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The Resistance of Super Charged NK Cells to Immunosuppression in Tumor Microenvironment

Author
Chen, Po-Chun

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The Resistance of Super Charged NK Cells to Immunosuppression in Tumor Microenvironment

A thesis submitted in partial satisfaction of

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by

Po-Chun Chen

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ABSTRACT OF THE THESIS

The Resistance of Super charged NK Cells to Immunosuppression in Tumor Microenvironment

by

Po-Chun Chen

Master of Science in Oral Biology

University of California, Los Angeles, 2021

Professor Anahid Jewett, Chair

We have previously demonstrated that natural killer (NK) cells are the primary immune effectors with the ability to mediate selection and differentiation of several different cancer stem cells/undifferentiated tumors via lysis and secreted or membrane-bound IFN-γ and TNF-α, respectively, leading to growth inhibition and curtailment of tumor metastasis. Here, we present an overview of our recent findings on the biology and significance of NK cells in the selection/differentiation of stem-like tumors using in vitro and in vivo studies conducted in the NSG and humanized-BLT mice. To establish the phenotypic and functional differences between primary NK and super-charged NK cells in vitro and in vivo, we analyzed their surface receptors, cytotoxicity, and cytokine secretion in either NK cells culture, NK-tumor co-culture, and CD34+ humanized mouse model condition. In in vitro studies, super-charged NK cells were more polyfunctional. They expressed higher surface receptors, performed higher cytotoxicity, and secreted higher IFN-γ compared to primary NK cells in vitro. When cultured with tumor tissues,

super-charged NK cells were also more resistant to immunosuppression by the tumor tissues. Moreover, we observed similar inhibition of tumor growth by super-charged NK cells in the NSG-CD34+ humanized mouse model as we saw in hu-BLT mice. Therefore, due to their indispensable role in targeting cancer stem-like/undifferentiated tumors and various other vital functions of NK cells which are presented in this thesis, NK cells should be placed high in the armamentarium of tumor immunotherapy.

The thesis of Po-Chun Chen is approved.

Ichiro Nishimura

Nicholas A. Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2021

DEDICATION

I dedicate this thesis primarily to my parents,

Chen, Chung-Cheng

and

Huang, Jing-Hua

who have given me all the supports throughout this period of time and provided me endless educational opportunities, and to my brother and sister-in-law who have encouraged me and been there with me all this time.

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Last but not least, I would like to thank Dr. Sung for all his help with collecting blood sample

Introduction

Natural Killer Cells

Natural Killer (NK) cells are bone marrow-derived large granule lymphocytes which are recognized by the expression of CD16 and CD56 and the absence of surface CD3 expression. They compose 5 to 15% of all circulating lymphocytes, peripheral blood mononuclear cells (PBMCs). In humans, the role of NK cells is similar to cytotoxic T cells in the adaptive immune response. By recognizing the absence of self-markers of Major histocompatibility complex class I (MHC-I) and antibodies, NK cells can kill cells undergoing tumor formation or those under stressors such as viruses or intracellular pathogens through a faster immune reaction. NK cells' ability to destroy cells which are missing MHC-I markers is important because T lymphocytes are unable to detect and kill those cells. As immune system effectors, NK cells are thought to be able to influence tumor cell survival and development and contribute to cancer eradication. Also, NK cells mediate both direct killing and antibody-dependent cellular cytotoxicity (ADCC), and control the activities of other immune cells by generating important cytokines and chemokines, such as Interferon gamma (IFN-γ) and Tumor necrosis factor alpha (TNF-α). There are two subsets of NK cells: $CD56^{dim}CD16^{+}$NK cells and $CD56^{bright}CD16^{-}$NK cells. $CD56^{dim}CD16^{+}$NK cells mainly appear in the spleen and peripheral blood, and their primary function is killing.$CD56^{bright}CD16^{-}$NK cells are cytokine producers and are mostly found in lymph nodes and tonsils. NK cells' functional ability is determined by the balance between activating and inhibitory signals received by NK cells from their surface receptors, such as NKG2D, NKp46, NKp44, and CD54, which are activating receptors, and KIR2 which is inhibitory. NK cells have diverse functions against cancer. They are known as the first responder in the immune system.

They also possess various defenses against cancer cells, including the direct killing of stem-like tumors, tumor differentiation, activating CAT-NK, CAR-T, and T cells, killing of immune suppressor cells, includes tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs).

Stem-like/Poorly Differentiated and Well Differentiated Tumors

Stem-like/poorly differentiated tumors have higher CD44 expression and low CD54, MHC-I, and PD-L1 expression. They grow faster, making them susceptible to NK cell-mediated cytotoxicity, and they induce NK cells to secret IFN-γ. They are resistant to chemotherapeutic and radiotherapeutic treatments due in part to their ability to self-renew themselves and differentiate into heterogeneous lineages of cancer cells. On the other hand, highly differentiated tumors have lower CD44 expression and express high amounts of CD54, MHC class 1, and PDL1. They grow slower and expand less, and are therefore susceptible to NK-mediated cytotoxicity, and do not induce or induce less IFN-γ secretion by NK cells. They are also sensitive to chemotherapy and radiotherapy.

Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (OSCC) is one of the head and neck squamous cell carcinomas (HNSCCs), the most common malignancies that arise in the head and neck. HNSCC is the sixth most common cancer worldwide in 2018[1]. Among all of the malignant neoplasms of the oral cavity, OSCC is the most common malignancy, making up 80-90% of oral malignant neoplasms[2]. Risk factors for development of OSCC include tobacco and alcohol usage and Human Papilloma Virus (HPV). Current treatments for OSCC include surgery, radiotherapy, chemotherapy, immunotherapy, or combinatorial therapies, but none of these options have

provided satisfactory outcomes for patients. Even amongst the emergence of several new treatment strategies, many have not been able to produce a robust clinical response [3,4]. The survival rate of OSCC, which is about 40-50%, has not significantly changed over the past decades[5].

Pancreatic Ductal Adenocarcinoma

Pancreatic ductal adenocarcinoma (PDA) is the 14th most common cancer and the 7th highest cause of cancer mortality globally [6]. Despite current developments in cancer therapy, the mortality rate of PDA has only slightly decreased in recent years; most PDA patients do not respond to existing treatments, and the 5-year survival rate is 10.8%, the poorest prognosis rate of all the cancer types. Although numerous studies on cancer treatment have been conducted in recent decades, we still lack suitable therapies or cures for several aggressive cancers. As a result, there is an urgent need to find efficient medical treatments of cancer which have minimal side effects and high efficiency.

Immune and NK Cell Defects in Cancer Patients

Many studies have shown correlations between the rate of cancer with low cytotoxicity by the PBMCs [7]and cancer patients' NK cell function, which is determined by NK cell-mediated cytotoxicity and IFN- γ secretion is significantly lower when compared to healthy individuals' NK cells[8]. Reduced cytotoxicity of peripheral blood NK cells are correlated with poorer outcomes and advanced stage of several cancers, including prostate cancer, colorectal carcinoma, cervical cancer, malignant melanoma, and head and neck cancer[9]. Similar NK cell malfunction can be found in tumor-bearing humanized bone marrow-liver-thymus (hu-BLT) mice[10],even at

the pre-neoplastic stage of pancreatic cancer mice model which is induced by genetic and environmental factors[11].

AJ2 Probiotic Bacteria

AJ2 combines eight different strains of gram-positive probiotic bacteria, including *Streptococcus thermophiles*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus bulgaricus*. AJ2 can synergistically induce a balanced mix of pro and anti-inflammatory cytokines, chemokines, and growth factors secretion in NK cells. They induce NK cells to yield a closer ratio of IFN- γ to IL-10 to obtain the balance that when NK cells are activated with IL-2 and anti-CD16 mAb in the absence of bacteria. Since such treatment provides significant differentiation of cancer stem cells[12,13].

Osteoclast-expanded NK Cells (Super-charged NK)

Super-charged NK (sNK) cells, which are NK cells expanded by osteoclasts, have higher rates of proliferation, cytotoxicity, and IFN-γ secretion when compared to other myeloid cells such as monocytes and dendritic cells[14]. More importantly, although cord blood and iPSC-derived NK cells can expand large numbers of cells with the NK phenotype, they are not capable of lyse-targeting oral squamous carcinoma stem-like cells (OSCSCs)/poorly differentiated tumors. Futhermore, in comparison with primary NK (pNK) cells derived from peripheral blood or sNK cells, they cannot produce a sufficient amount of IFN-γ[15].

p53 Mutation

P53 is a transcription factor that senses cellular stress. It can trigger transient cell cycle arrest, permanent cell cycle arrest, and apoptosis in response to stressors, which are most often associated with tumor initiation. Emerging studies have shown that p53 also modulates other cellular processes, including cellular metabolism, stem cell maintenance, invasion, metastasis, and promoting communication within the tumor microenvironment (TME). The TP53 gene, which controls p53, is the most commonly mutated gene in human cancers. Tumors lacking p53 are more likely to have malignant characteristics such as a lack of cellular differentiation, genetic instability, and increased invasiveness metastatic potential[16]. The relationship of p53 and NK cells depends on the tumor types. P53 can either upregulate or downregulate the expression of UL16-binding protein 1 (ULBP1) and ULBP2, which are NKG2D ligands. Overexpression of wtp53 can upregulate ULBP1/2 on non-small cell lung carcinomas and activate NK cells. However, in contrast, overexpression of miR-34a/c, a microRNA which is targeted by p53, will downregulate ULBP1/2 on melanoma tumors and inactivate NK cells[17]. To date, the exact relationship between p53 mutations on tumors and NK cell functions are not known. In our research, we performed a tp53 targeted gene library prep and sequencing on the tumor tissue to elucidate more information.

Suppression of Anti-tumor Function and Change of NK cell Phenotype in Tumor Microenvironment

Tumor-infiltrating NK cells display significant phenotypic and functional alterations compared to circulating NK cells found in various cancers, including breast, pancreatic, lung, colorectal cancer, and gastrointestinal tumors. Immunosuppressive tumor microenvironments (TME)

weaken the anti-tumor activity of NK cells directly or indirectly by impairing the function of NK activating immune effectors. Tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and regulatory T cells (Tregs) are examples of immune effectors which have been shown to exert suppression of NK function within the TME. Several other immunosuppressive factors of TME have also been identified, such as cytokines, soluble receptors, metabolites, and healthy non-transformed stromal cells. Moreover, altered cellular metabolism in TME has also been known to induce suppression of NK function[18].

TGF-β secreted from tumors, healthy stromal cells, and immune cells, can increase the proliferation of suppressive immune cells, such as Tregs. In addition, TGF-β can directly limit cytotoxicity and secretion of IFN-γ in NK cells by downregulating the expression of activating receptors NKp30 and NKG2D and NKG2D's ligand MICA in cancer patients. TGF-β can also inhibit NK cell function by targeting mTOR, a crucial signaling integrator of pro- and anti-inflammatory cytokines, such as interleukin-15 (IL-15), in murine and human NK cells[19]. TGF-β is proposed to differentially induce chemokine repertoires, reducing the expression of those attracting $CD56^{dim}$ NK cells such as CXCL2, CX3CL1, CXCL1, and CXCL8, and increasing those which favor the recruitment of $CD56^{bright}$ NK cells such as CXCL9, CXCL10, CXCL19, and CCL5[20].

After a week of IL-2 and anti-TGF-β antibody treatment of tumor-bearing mice, the maturity of tumor-associated NK cells was increased; this either suggests that these cells possess the ability to mature and become activated or that the activation of NK cells was slowed down by the treatment in these mice, resulting in the maintenance of the higher NK cell surface receptors,

since over-activation of NK cells could result in the increased shedding of surface receptors and decreased function of NK cells[21].

The plasticity of NK cell responses is a double-edged sword. On the one hand, if we can determine the exact mechanisms by which NK cells are signaled to maintain their maturity and functional state for a more extended period, we could perhaps increase the effectiveness of such killers against tumors. Unfortunately, it also indicates that the tumors can easily convert activated NK cells to suppressed or even pro-tumoral effectors.

NK cell infiltrates in prostate tissues are mainly $CD56^{+}$ and are thought to display either an unexpected immature phenotype[22], or as indicated above, perhaps a mature phenotype with increased shedding of the surface receptors, since these cells are found to be of activated phenotype with low/no cytotoxic potential displaying reduced CD57, NKp46, NKG2D, ILT2, and CD107 expressions, and increased CD69 and NKp44 expressions when compared with peripheral blood and control tissue-infiltrating NK cells. The cytokine milieu in the prostate tissue environment impacts NK cell functions partly through TGF-β1. Coculture experiments of prostate cancer cells and NK cells revealed that prostate cancer cells induced the expression of the inhibitory receptor (ILT2/LILRB1) and decreased expression of activating receptors NKp46 (NCR1), NKG2D (KLRK1), and CD16 (FCGR3) by NK cells, thus preventing recognition of tumor cells by the NK cells. Taken together, immature or likely overly activated infiltrating NK cells with hyporesponsive function and imbalanced expression of activating and inhibitory NK cell receptors in TME may be part of the underlying mechanism of cancer progression[22].

Several characteristics of Hodgkin's Lymphomas (cHL) account for the impaired function of NK cells. Locally, cancer cells and other cells from the TME express ligands for inhibitory receptors on NK cells, including HLA-E, HLA-G, and programmed death-ligand 1 (PD-L1). The secretion of chemokines and cytokines, including soluble IL-2 receptor (sCD25), TGF-β, IL-10, CXCL9, and CXCL10, mediates systemic immunosuppression. Immunosuppressive cytokines, IL-10 and TGF-β, are actively secreted by cancer cells and TME cells, both of which favor Treg recruitment and expansion and reduce lymphocyte production of IFN-γ involved in attracting NK cells. TGF-β can also diminish the expression of NKG2D ligands (MICA, ULBP2, and ULBP4) and downregulate activating receptors NKG2D and NKp30 (NCR). TGF-β has been shown to directly mediate the transformation of NK cells into tumor-tolerant type 1 innate lymphoid cells in TME[23].

Tumor-associated Cells Shape the Function of NK Cells

Tumor-associated mesenchymal stem cells (MSCs) block the activity of NK cells within the lung tumor microenvironment. In the TME of lung cancers, NK cells primarily display a $CD56^{dim}$ phenotype and have low expression of multiple activating receptors and reduced function. MSCs can inhibit NK cell proliferation, cytotoxicity, and cytokine production by secreting several factors, such as IDO1, TGF-β, HLA-G, and PGE2. Squamous cell lung carcinoma-derived MSCs can also downregulate NK cell-activating receptors NKp44, NKp30, NKG2D, DNAM-I, and NKG2A. MSC-mediated inhibition of IFN-γ was predominantly seen in the $CD56^{bright}$ subpopulation of NK cells, which was expected because secretion of IFN-γ is the primary function of these cells.

In contrast, NK cell degranulation was inhibited by MSCs in the $CD56^{dim}$ subtype due to cytotoxicity being the primary function of these cells. Inhibition of both functions, particularly cytokine production, was primarily contact-dependent. IL-6 and particularly PGE2 are implicated in T-MSC-mediated inhibition of NK cell function. The observed shift towards the $CD56^{dim}$ NK cell phenotype after exposure to MSCs is consistent with low expression of CD56 by the NK cells in vivo and selective inhibition of the $CD56^{bright}$ subtype rather than expansion of the $CD56^{dim}$ subpopulation, indicating the lack of transitions of NK cells from $CD56^{dim}$ to $CD56^{bright}$ subpopulations[24].

Tumor-associated fibroblasts (TAFs), present in the TME, can exert immunosuppressive effects on NK cells, favoring tumor immune escape. Co-culture of NK cells with TAFs can prevent IL-2-mediated NKG2D upregulation. The ability of NK cells to kill autologous TAFs might represent a control mechanism to reduce the TAF-mediated immunosuppressive effect on NK cell function. These findings suggest that TAFs in the TMEs of cancer patients can downregulate the immune cell recognition of tumor cells by NK cells[24].

Due to their essential roles during cancer progression and ability to reduce the effects of immunotherapies, MDSCs and Tregs are attractive therapeutic targets in patients with cancer. Tregs suppress NK cell activity by producing high amounts of TGF-β or acting as a sink for IL-2. In the in vivo renal cell carcinoma model and an in vitro human RCC system, Tregs suppressed NK cell activity via the production of TGF-β. Therefore, another approach to overcoming the immunosuppressive mechanisms of the TME has been to engineer NK cells to silence or express dominant negative (DN) TGF-β receptors to decrease NK cell responsiveness to TGF-β [25].

In tumor-bearing mice, MDSCs were shown to suppress NK-cell activation and cytolytic capacity via membrane-bound TGF-β. In contrast, it has been proposed that MDSCs may also exert a stimulatory function on NK cells through ligation of the NKG2D receptor. In human cancers, it is less known how MDSCs interact with NK cells. A recent study has shown that suppression of NK cells by MDSCs was mediated through the ligand of NKp30 in patients with hepatocellular carcinoma. Tumor-derived prostaglandin-E2 (PGE2) plays an essential role in inducing MDSCs. Patient-derived MDSCs inhibited NK cell activity through the production of TGF-β. In vitro, the binding of PGE2 to EP2 and EP4 receptors on monocytes activated the p38MAPK/ERK pathway and resulted in elevated secretion of TGF-β. Similar to MDSCs, PGE2 treated monocytes were able to potently suppress NK-cell activity through the production of TGF-β. Furthermore, silencing COX-2 in murine breast cancer cell lines reduced the accumulation of $CD11b^{+}Gr1^{+}$ MDSCs in the spleen, resulting in simultaneous improvement in in vivo clearance of NK-cell sensitive lymphoma cells. Since NK cells are known to target immunosuppressive cells, including MDSCs, suppression of NK cell function could, in turn, increase the numbers of MDSC and increase the immunosuppressive potential of these cells on NK cells.

Impaired Cellular Metabolism within TME Leads to Dysfunctional NK Cells

Altered NK cell metabolism is likely to be an essential factor in NK cell dysfunction. TMEs are metabolically restrictive and can be deficient of nutrients such as glucose, the critical fuel for NK cells. Tumors can also have high concentrations of lactate and a low pH, both of which can impair NK cell anti-tumor functions. Indeed, a recent human study showed that lactate produced

by colorectal liver metastasis resulted in a decrease in the intracellular pH of tumor-infiltrating liver-resident NK cells, leading to mitochondrial dysfunction and apoptosis. Oxysterols 25-hydroxycholesterol and 27-hydroxycholesterol inhibit the activation of SREBP transcription factors, which are critical regulators of NK cell metabolism. The enzyme indoleamine 2,3-dioxygenase 1 (IDO1) is often highly expressed in tumor cells or tumor-associated cells such as tolerogenic dendritic cells. IDO1-mediated metabolism of tryptophan and the IDO1-derived metabolite l- kynurenine can inhibit human NK cell proliferation and cytotoxicity[26]. Adenosine, a purine metabolite present at high concentration in the TME, also acts by limiting the activity of protective immune infiltrates, including NK cells, and enhancing Tregs and MDSCs. The engagement of the A2A adenosine receptor (A2AR) acts as a checkpoint that limits the maturation of NK cells. Global and NK cell-specific conditional deletion of A2AR in mice model enhanced proportions of terminally mature NK cells ($CD11b^{+}CD27^{-}CD11b^{+}CD27^{-}$, $KLRG^{+}$, $Ly49C/I^{+}$) at homeostasis, following reconstitution, and in the tumor microenvironment. Notably, A2AR-deficient, terminally mature NK cells retained proliferative capacity and exhibited heightened reconstitution in competitive transfer assays. Moreover, targeting A2AR specifically on NK cells also improved melanoma tumor control and delayed tumor initiation[27].

When stimulated with cytokines, NK cells from obese mice or humans with obesity fail to engage a metabolic response. They have substantially reduced metabolic rates compared with NK cells from lean mice or humans. Indeed, we have recently shown that mice with a Kras mutation, when fed a high-fat calorie diet, demonstrated significantly less ability to expand NK cells, mediate cytotoxicity and secrete IFN-γ compared to mice fed with a lean diet. They

developed pancreatic tumors at a much faster rate[11,28]. In addition, when fed with a high-fat calorie diet, healthy mice also demonstrated decreased capabilities of NK cell expansion and function compared to those provided with a lean diet (Please see below and[11]). It is also shown that NK cells from obese mice and humans fail to kill tumor cells, in part because they fail to form a synapse with target cells correctly; the cytotoxic machinery does not traffic to the NK cell–tumor cell synapse[29]. Thus, obese mice and humans have greater risks of developing cancer due to their dysfunctional NK cells[11]. The metabolic dysfunction in NK cells has been linked to peroxisome proliferator-activated receptor (PPAR)-driven lipid accumulation in NK cells, leading to altered gene expression, the downregulation of MYC and mTORC1 signaling, and decreased rates of glycolysis and OXPHOS25.

Purpose of study

Based on the previous studies, we know that cancer patients' NK cell number and function are decreased when compared to healthy donors. Second, we know that osteoclast-expanded NK cells can decrease tumor growth and extend survival days in hu-BLT mice models. In this research, we have combined these two findings to study whether sNK cells are able to withstand suppression by the TME and not become inactivated.

Thesis Outline

Specific aim 1: To establish phenotypic and functional differences between activated pNK cells and sNK cells.

- Sub-aim 1: Phenotypic differences between primary and sNK cells.
- Sub-aim 2: Functional differences between primary and sNK cells.
- Sub-aim 3: Differences between pNK and sNK cells assessed by proteomic analysis.

Specific aim 2: To determine the effect of oral and pancreatic tumors on the inactivation of activated pNK cells and sNK cells.

- Sub-aim 1: When cultured with human tumor chunks, the cytotoxicity and IFN-γ secretion of pNK cells decrease significantly compared to sNK cells.
- Sub-aim 2: The cytotoxicity of sNK cells is more resistant to suppression by tumors implanted in hu-BLT mice when compared to those embedded in NSG mice.
- Sub-aim 3: Induction of cell death as an essential mechanism for the inactivation of pNK cells when cultured with human tumor chunks.
- Sub-aim 4: Tumor induced mutations in p53 as a means for regulating the activation of NK cells.

Specific aim 3: To determine the in vivo significance of sNK cells in eliminating tumors in a $CD34^+$ humanized mouse model compared to those in hu-BLT mice.

- Sub-aim 1: Decrease tumor burden in NSG-$CD34^+$mice injected with sNK cells.
- Sub-aim 2: Systemic regulation of sNK cell function by AJ2 probiotic bacteria fed to tumor-bearing NSG-$CD34^+$ mice leads to decreases in tumor size.

Method and Materials

Method and Materials

Cell Lines, Mouse models, Reagents, and Antibodies

RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, CA, USA) was used for the cultures of NK cells. 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). PE-conjugated anti-CD54, anti-CD44, anti-B7H1 antibodies were obtained from BioLegend (San Diego, CA, USA). The human NK purification kits were obtained from Stem Cell Technologies (Vancouver, BC, Canada). Humanized BLT mice were generated from NOD/SCID mice from UCLA-DLAM, and NSG-CD34+ mice were from the Jackson laboratory.

Purification of NK Cells from Human Peripheral Blood

UCLA Institutional Review Board (IRB) approved written informed consent from healthy blood donors, and UCLA approved all procedures. Peripheral blood was separated by Ficoll Hypaque centrifugation. Then, the cloudy white buffy coat containing peripheral blood mononuclear cells (PBMC) was collected, washed, and resuspended in RPMI 1640 (Invitrogen by Life Technologies, CA) with 10% added. EasySep® Human NK Cell Enrichment Kit purchased from

Stem Cell Technologies (Vancouver, BC, Canada) was used to negatively select and isolate NK cells from PBMC. The isolated NK cells were stained with anti-CD45, anti-CD16, anti-CD56, and anti-CD3 antibodies to measure cell purity by flow cytometry analysis. Purified NK cells in RPMI 1640 medium supplemented with 10% FBS (Gemini BioProducts, CA), 1% antibiotics/antifungals, 1% sodium pyruvate, and 1% non-essential amino acid MEM (Invitrogen, Life Technologies, CA).

Sonicating AJ2

AJ2 was weighed and resuspended in RPMI 1640 medium supplemented with 10% FBS (Gemini Bio-Products) at a final concentration of 106/ml. After every five pulses, a sample was taken and observed under a microscope until at least 80% of the cell wall is dissolved. The sample was sonicated for 15 seconds while on ice, then incubated for 30 seconds on ice. This process was repeated 20 times to achieve complete sonication. Lastly, the sonicated samples were aliquoted and stored in a −80°C freezer.

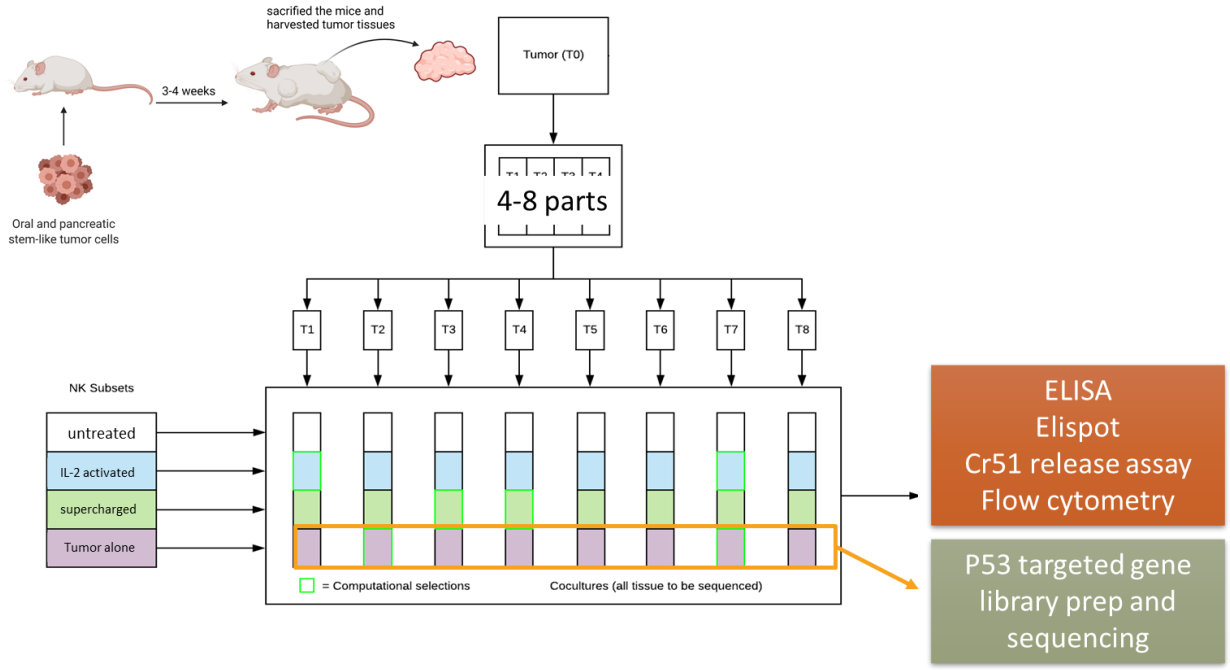
Generation of Osteoclasts and Expansion of NK Cells

The method was described in-depth in a previous paper. To briefly outline it, purified human NK cells were activated with rhIL-2 (1000 U/ml) and anti-CD16 mAb (3 ug/ml) for 18-20 hours and then co-cultured with osteoclasts and sAJ2 in an NK: osteoclast: sAJ2 as 2:1:4 ratio. The medium was renewed with rhIL-2 every three days[14].

Experiment Design of NK-tumor Co-culture

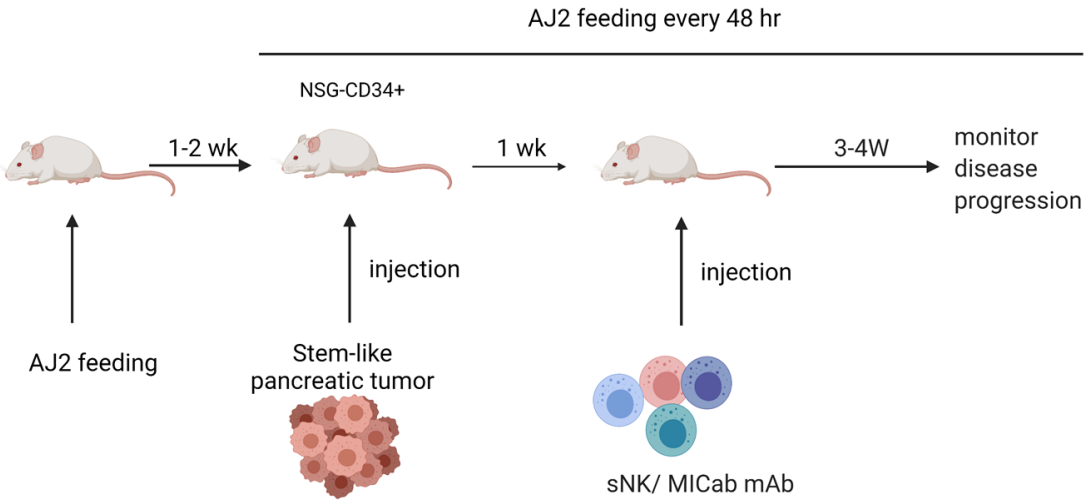
Animal research was performed under the written approval of the UCLA Animal Research Committee (ARC). Humanized-BLT (hu-BLT; human bone marrow/liver/thymus) mice were

prepared in our core facility as previously described[31,32]. Oral and pancreatic stem-like tumor cells were implanted in both humanized BLT and NSG mice. Mice were anesthetized using isoflurane, and tumor cells were then transferred by direct injection in the pancreas with 7µl HC Matrigel (Corning, NY, USA). Mice were euthanized when signs of morbidity were evident. Tumors were harvested after 3-4 weeks. Each tumor was cut into 4-8 small parts, and then each portion was cut into four small pieces. One piece was saved for p53 library prep and sequencing. The other pieces were cocultured with either untreated pNK or IL-2 (1000 U/ml) treated pNK or sNK cells within RPMI 1640 medium supplemented with 10% FBS (Gemini BioProducts, CA), 1% antibiotics/antifungals, 1% sodium pyruvate, and 1% non-essential amino acid MEM (Invitrogen, Life Technologies, CA) overnight. Then the supernatant was collected for ELISA, and the NK cells were used for Elispot, chromium release assay, and flow cytometry.



Analysis of Human Oral Cancer Cell Growth in CD34$^+$ Humanized Mouse Model

Animal research was performed under the written approval of the UCLA Animal Research Committee (ARC). CD34+ humanized mice were fed AJ2 (5 billion bacteria/dose), beginning one or two weeks before MP2 tumor cells implantation, and were fed every 48 hours throughout the experiment. sNK and/or anti-MICa/b mAb was injected through a tail vein or i.p. one week after implantation. Disease progression was monitored and mice were sacrificed when they showed tumor-bearing symptoms.



Dissociation and Culture of Cells from Tissues of CD34$^+$ Humanized Mice

To prepare a single cell suspension of mouse tissues for subsequent analyses, animals were sacrificed, and peripheral blood, BM, spleen, and pancreatic tumors were obtained. For future studies, the pancreatic tumors were immediately cut into one mm3 piece and froze with 50% of DMEM, 40% FBS, and 10% DMSO. Single-cell suspensions from BM and spleen were obtained by digesting tissues, as described previously[33]. PBMCs were obtained using ficoll-Hypaque

centrifugation of heparinized blood specimens. The buffy coat containing PBMCs was harvested, washed, and re-suspended in RPMI 1640 medium.

^{51}Cr Release Cytotoxicity Assay

^{51}Cr was purchased from Perkin Elmer (Santa Clara, California). A standard ^{51}Cr release cytotoxicity assay was used to determine the cytotoxic function of NK cells in experimental cultures. Aliquots of the effector cells ($1x10^5$ cells/well) were placed into 96-well round-bottom microplates (Fisher Scientific, Pittsburgh, PA) and titrated with 4 to 8 serial dilutions. Target cells ($1*10^6$) were labeled with 100 µCi ^{51}Cr (Perkin Elmer, Santa Clara, CA) and stained for 1 hour. After incubation, the target cells were washed twice to remove excess unbound ^{51}Cr. The^{51}Cr-labeled target cells were aliquoted into 96-well round-bottom microtiter plates containing effector cells at a concentration of $1*10^4$ cells/well, and the effector cell: target (E:T) ratio was 5:1. The plate was centrifuged and incubated for 4 hours. After the 4-hour incubation period, the supernatant of each sample was collected, and the released radioactivity was counted using a gamma counter. The total (containing ^{51}Cr-labeled target cells) and spontaneous (containing target cell supernatant only) release values were measured and used to calculate the percentage of specific cytotoxicity. The following formula calculates the percentage of particular cytotoxicity:

$$\%\ Cytotoxicity = \frac{Experimantal\ cpm - spontaneous\ cpm}{total\ cpm - spontaneous\ cpm}$$

Lu30/10^6 was calculated using the inverse of the number of effector cells needed to lyse 30% of target cells times 100.

Enzyme-Linked Immunosorbent Assays (ELISAs)

The IFN-γ ELISA kit was purchased from BioLegend (San Diego, CA). An ELISA was performed to detect the level of IFN-γ produced from the cell culture. The determination was carried out as described in the manufacturer's protocol. Briefly, a 96-well EIA/RIA plate was coated with a diluted capture antibody corresponding to the target cytokine and incubated overnight at 4°C. After 16-18 hours of incubation, the plate was washed three times with Wash 18 Buffer (0.05% Tween in 1x PBS) and blocked with Assay Diluent (1% BSA in 1x PBS). The plate was incubated on a plate shaker at 500 rpm for 1 hour at room temperature and then washed three times after incubation. Then, 100 µl of standards and samples collected from each culture were added to the wells and incubated on a plate shaker at 500 rpm for 2 hours at room temperature. After incubation, plates were washed three times, loaded with detection antibody, and incubated on a plate shaker at 500 rpm for 1 hour at room temperature. After 1 hour incubation, plates were washed three times, Avidin-HRP solution was added to the wells, and plates were set on a plate shaker at 500 rpm for 30 minutes at room temperature. After washing the plates three times with wash buffer, 100 µL of TMB Substrate Solution was added to the wells and the plates were incubated in the dark for up to 30 minutes, or until they appear blue as desired. Then 50 µl of Stop Solution (2N H2SO4) was added to each well to stop the reaction. Finally, plates were read in a microplate reader at 450 nm to obtain absorbance values (BioLegend, ELISA manual).

Elispot

Elispot kits were purchased from ImmunoSpot (Cleveland, OH). The procedure was carried out as described in the manufacturer's protocol. Briefly, an ELISPOT plate with a PVDF membrane was coated with an analyte-specific antibody overnight. Afterward, the plate was washed with a buffer and then plated with freshly isolated or cultured cells with the antigens of interest

overnight to allow for the activation of NK cells and the induction of their cytokine secretion. The cells are then washed away and IFN-γ antibody was added. The plates were incubated at room temperature for two hours and then washed. Streptavidin was added and set for 30 minutes. Last, chromogenic substrate was added to develop an enzyme-catalyzed spot.

Surface Staining and Cell Death Assays

$30*10^3$ cells from each condition were stained in 100 μL of cold 1% BSA-PBS with a 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. For the cell death assay, 30*103 cells in 100 μl of cold 1% BSA-PBS were stained with 8 mg/ml propidium iodide and brought to 200 μl with BSA-PBS. The Attune NxT flow cytometry (Waltham, MA) and Flowjo (Ashland, OR) software was used for data analysis.

TP53 Target Gene Library Preparation and Sequencing

Genome DNA (gDNA) was extracted from 44 tumor tissue samples from either hu-BLT or NSG mice using the DNeasy® Blood & Tissue kit purchased from Qiagen (Germantown, MD) and purified with PureLink® PCR Purification Kit from Invitrogen (Waltham, MA). gDNA was extracted and purified following the protocols from the kits. Briefly, tumor tissues were cut and lysed overnight by the lysis buffer and proteinase K at 56 degrees Celsius. After sample lysis, the binding buffer was added, and sample gDNA was bonded to the column membrane. Wash buffer 1 and 2 were used to wash out the proteins and salts from the tissues and previous reagents and centrifuged one more time to ensure the membranes were dry and without any wash buffer remained. Then water or elution buffer was used to elute gDNA from the column membrane. After gDNA extraction, gDNA was used to prepare the p53 targeted gene library prep. TP53

region targeting probes were designed by Illumina experts, and the DNA Prep with Enrichment kit was purchased from Illumina. All the preparation followed the provided protocols. Briefly, specific beads were first used to cleave and tag gDNA, then the indexes were added, and PCR was performed. After indexing, the samples were pooled together. Then, custom tp53 targeted probes were added to catch the fragments which included the regions of interest, and magnet beads were used to enrich targeted fragments for sequencing.

Statistical analysis

An unpaired, two-tailed student t-test was performed for the statistical analysis. One-way ANOVA with a Bonferroni post-test was used to compare different groups. (n) denotes the number of mice used for the experiment. For cytotoxicity and cytokine analysis, either duplicate or triplicate samples were used for assessment. The following symbols represent the levels of statistical significance within each analysis, ***(p-value <0.001), **(p-value 0.001-0.01), *(p-value 0.01–0.05).

Results

Chapter 1: Establish the phenotypic and functional differences between primary activated NK cells and super-charged NK cells.

SNK (sNK) cells are larger and polymorphic in morphology and express more activating receptors and cytotoxicity protein.

Primary NK cells are rounded and small, whereas super charged NK cells exhibit polymorphic morphology and are larger. Once activated, pNK cells were plated with osteoclasts, and the pNK cells immediately attached to them. On day five of the NK cells-osteoclast co-culture, most of the osteoclasts were gone, and the NK cells were elongated and became polymorphic and clustered. On day seven and after, sNK cells grew in clumps. They were either floating or attached to the plate(Manuscript in preparation). In addition, flow cytometry analysis demonstrated an elevation pf both percent and mean channel fluorescence intensity (MFI) of sNK cells exhibiting inhibitory, KIR2s, and activating receptors, including NKG2D and NKp44 NKp46, CD54 compared to those in pNK cells.

On the other hand, CD62L, which is known to be downregulated in activated lymphocytes, was lower in sNK cells when compared to pNK cells from healthy donors (Manuscript in preparation). When the NK cell receptors, CD16 and CD56, were tested, CD56 was higher in sNK cells while CD16 expression was diminished, similar to the profile of cytokine producer NK cells in lymph nodes and tonsils. However, intracellular staining showed that granzyme B

was also higher in sNK cells. Moreover, the level of Ki-67, a proliferation marker, showed that sNK cells have a higher level in both percentage and MFI, which is consistent with the increased expansion rate as we have previously published[14] (Manuscript in preparation). Overall, these results indicated that sNK cells demonstrated the phenotype of activated cells, exhibited activation markers, and showed higher amounts of cytotoxic granules and proliferation markers.

sNK cells mediate higher direct cytotoxicity and secret more IFN-γ than primary NK cells, and their cytotoxicity remains higher after being treated with rhIL-2 and anti-CD16 mAb.

Super-charged and primary NK cells were treated with IL-2 or a combination of IL-2 and anti-CD16 mAb overnight and used in the ^{51}Chromium release assay. sNK cells showed higher cytotoxicity against OSCSCs than pNK cells in rhIL-2 alone (Figure 1. A). As previously reported, the cytotoxicity of NK cells decreases because of the induction of split Anergy by the combination of IL-2 and anti-CD16 mAb. The decrease in cytotoxicity of super-charged NK cells is not as severe as it is in primary NK cells. (Data not shown). Besides mediating cytotoxicity against tumor cells, another crucial function of NK cells is stopping cancer progression through cytokine secretion, especially IFN-γ. We have previously shown the significance of IFN-γ in differentiating cancer stem cells, leading to decreased tumor growth and sensitization of cancer cells to chemotherapeutic drugs. For total population evaluation, the level of IFN-γ was tested in the supernatant of both pNK and sNK cells by ELISA. A greater level of IFN-γ was detected in the supernatant from sNK cells than those from pNK cells (Figure 1. B). Elispot was also used to evaluate the IFN-γ secretion level of these two cell types in a single-cell manner. The number of IFN-γ secreting cells of sNK cells was substantially higher than pNK

cells. Overall, sNK cells showed more IFN-γ secretion than pNK cells in total population and single-cell level (Figure 1. C).

Then, we assessed the polyfunctionality of sNK cells on a single-cell basis by using isoplexis. Polyfunctionality is defined as secreting more than one cytokine/chemokine/functional protein per cell, meaning that one cell can secrete two or more cytokines or chemokines. Within the same treatment, sNK cells exhibited more secretions and were more polyfunctional cell subsets than pNK cells (Manuscript in preparation). Donors' profiles were compiled and made into a bar graph (Manuscript in preparation). sNK cells continuously demonstrated more polyfunctionality, and interestingly, the treatment of sAJ2 also induced polyfunctionality in pNK cells. Next, the power of polyfunctionality was presented as polyfunctionality strength index, which was calculated using the percentage of polyfunctional cells multiplied by the intensities of tested cytokines and chemokines (Manuscript in preparation). sNK cells also showed higher levels of polyfunctional strength index than pNK cells, especially when compared within IL-2 and anti-CD16 mAb treated groups. Collectively, these results suggest that sNK cells possess a greater capacity for secretion.

sNK cells showed higher protein abundances in cell cycle, cellular metabolism, immune system process, and higher-level of proteins and transcription factors related to proliferation, cytotoxicity, and IFN-γ secretion in proteome.

We investigated the differences between super-charged NK cells and those of primary NK cells at the proteomic level. The comparison of pNK and sNK cells within the same donors showed

more differences in protein abundance than comparisons between different donors (Manuscript in preparation). When compared within the same donors, there were increases in the abundance of 271 proteins and decreases in 172 proteins in super-charged NK cells (Manuscript in preparation). We took the increased proteins in both scenarios and analyzed them by STRING analysis (Manuscript in preparation) to measure the protein-protein functional interaction. Of all 43 proteins raised in sNK cells, 7 are involved in the cell cycle process, 29 are associated with cellular metabolic function, and 14 are involved in the immune system process. For cell proliferation, the amount of minichromosome maintenance protein complex (MCM), which is essential for genomic DNA replication, was higher in super-charged NK cells, but not in primary NK cells (Manuscript in preparation). Ki-67 expression was also increased in super-charged NK cells (Manuscript in preparation). Parallel to the increased cytotoxic ability of super-charged NK cells, proteins that are known to be essential in the cytotoxicity were also analyzed, and it was found that granzyme A, granzyme B, and granulysin, which are known as effector molecules in mediating cytotoxicity in granule exocytosis, were more abundant in super-charged NK cells (Manuscript in preparation). Signal transducer and activator of transcription (STAT) were also analyzed using proteomics. In super-charged NK cells, expression of STAT1 and STAT4, which are known to be crucial regulators of IFN-γ production and NK cell-mediated cytotoxicity, were elevated (Manuscript in preparation).

Figure 1.

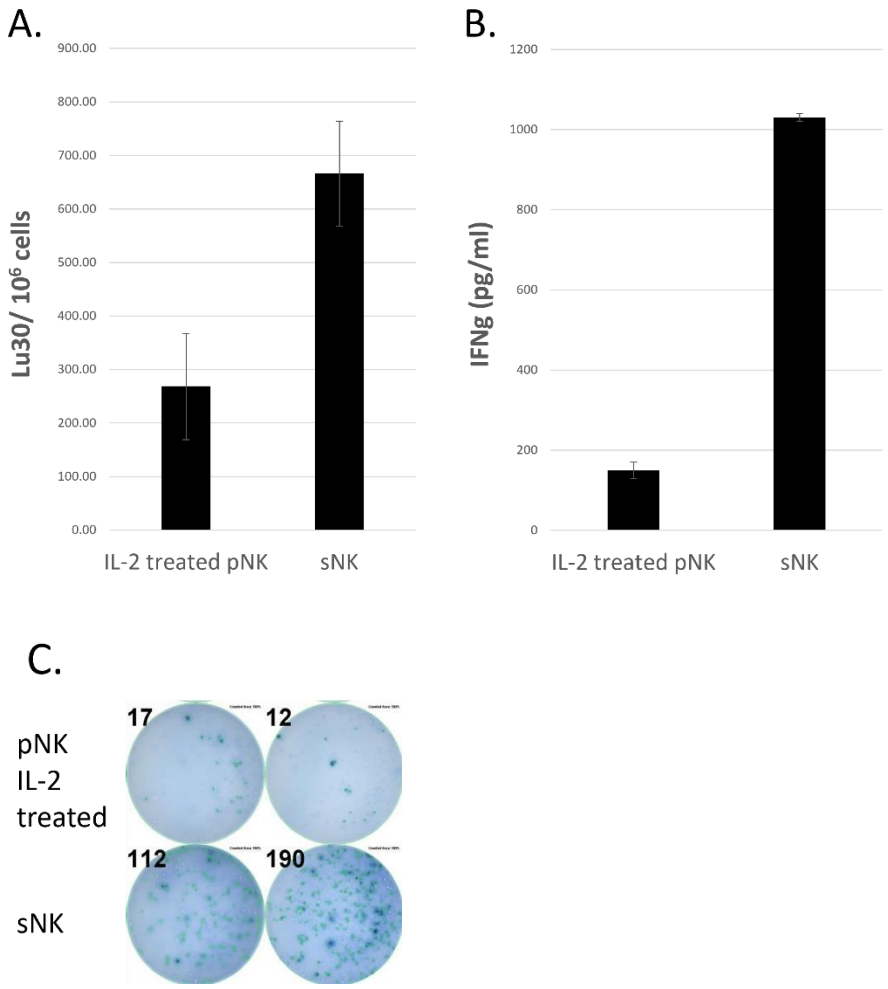


Figure 1. *sNK showed a greater ability to kill stem-like tumor cells and higher secretion of IFN-γ in comparison to pNK cells.* pNK cells were treated with IL-2 overnight, and measure the cytotoxicity was by a ^{51}Cr release assay (A). Supernatant from either pNK or sNK were collected and measured the IFN-γ by ELISA (B), and Elispot was used to evaluate the IFN-γ secretion level of these two cell types in a single-cell manner (C). The following symbols represent the levels of statistical significance within each analysis, ***(p-value <0.001), **(p-value 0.001-0.01), *(p-value 0.01–0.05).

Chapter 2: To determine the effect of oral and pancreatic tumors on the inactivation of primary activated NK cells and sNK cells.

Results

When cultured with human tumor chunks, the cytotoxicity and IFN-γ secretion of pNK cells decrease significantly compared to sNK cells.

The cytotoxicity of untreated and IL-2 treated primary NK cells, and super-charged NK cells was measured after their co-culture with oral tumor tissues derived from humanized BLT mice or single cells of OSCSCs using 51Chromium release assay. Super-charged NK cells exhibited higher cytotoxicity when compared to both untreated or IL-2 treated primary NK cells (Figure 2. A). Untreated primary NK cells showed significantly lower cytotoxicity after co-culture with tumor tissue and OSCSCs (Figure 2. B). Using the percent decrease analysis, untreated pNK cells showed the highest reduction, followed by IL-2 treated pNK cells. There was barely any decrease in sNK cells in both forms of tumor co-culture (Figure 2. C). The same result was observed when NK cells were co-cultured with pancreatic tumor tissues: there was a decrease in untreated and IL-2 treated pNK cells and higher cytotoxicity and less suppression of sNK cells (Figure 3. A-C).

In addition to cytotoxicity, IFN-γ secretion of NK cells was also evaluated after co-culture with tumor tissue and OSCSCs using Elispot and ELISA. The IFN-γ secretion in the single-cell level was measured by using Elispot. sNK cells had the highest cell count when co-cultured with

either oral or pancreatic tumor tissues (Figure 4. A, B). We also found that the percent decrease of cell count of rhIL-2 activated pNK cells cultured with either oral or pancreatic tumor tissues had a higher decrease level compared to sNK cells. On the other hand, the percent decrease of sNK remained low and even increased secretions compared to NK cells alone (Figure 4. C).

Next, to measure the IFN-γ secretion of NK cells in the total population, the supernatant from the NK-tumor co-culture was measured using ELISA. There was no drop in the IFN-γ secretion of the total population in both rhIL-2 activated pNK and sNK cells (Figure 5. A). But sNK cells still maintained the highest secretion compared to rhIL-2 activated pNK cells when cultured with tumor tissue (Figure 5. B-C).

The cytotoxicity and secretion of IFN- γ by sNK cells are more resistant to suppression by tumors implanted in BLT mice when compared to those embedded in NSG mice.

In NK cells cultured with oral tumors, there was higher suppression of cytotoxicity in rhIL-2 treated pNK cells cultured with tumors from NSG mice compared to those from BLT mice. However, sNK had the least amount of suppression when cultured with tumors from both BLT and NSG mice (Figure 6. A-B). There was also a higher suppression of IFN-γ secretion in both IL2 treated pNK cells and sNK cultured with tumors from NSG mice. On the other hand, NK cells cultured with tumors from BLT mice had less suppression or even increased IFN-γ secretion in IL2 treated and supercharged NK cells. To conclude, immune cells in TME are crucial to rescue NK cells from suppression (Figure 6. C-D).

Induction of cell death is an important mechanism for the inactivation of pNK cells when cultured with human tumor chunks.

NK cells with viability staining were analyzed using flow cytometry (Figure 7. A) and the percent decrease of live cells was calculated and shown in Table 1. We found that sNK has the slightest percent decrease of live cells (Table 1.), which means that the induction of cell death might be the reason for the inactivation of pNK cells when co-cultured with tumor chunks which correlate to the percent of cell death (Figure 7. B).

Tumor induced mutations in p53 as the means for regulating the activation of NK cells

To determine the role of p53 in the modulation of NK cells, we used three different conditions of the NK cells: untreated, treated with IL-2, and super-charged NK cells, and cultured them with tumor tissues derived from oral or pancreatic tumors grown in BLT mice. The results were compared to tumors grown in NSG mice. We used 132 tumor pieces and cultured them with the three NK conditions to assess NK activation and function. However, we also used 44 tumor pieces without the NK cells to extract the genomic DNA, followed by the RNA library prep to determine the specific sequences of the p53 gene. We were able to obtain good quality samples and performed sequencing and obtained sequencing data. We are currently analyzing the sequencing data, which will be correlated with the NK functional status, to determine associations between p53 gene mutations and NK function in the future.

Figure 2.

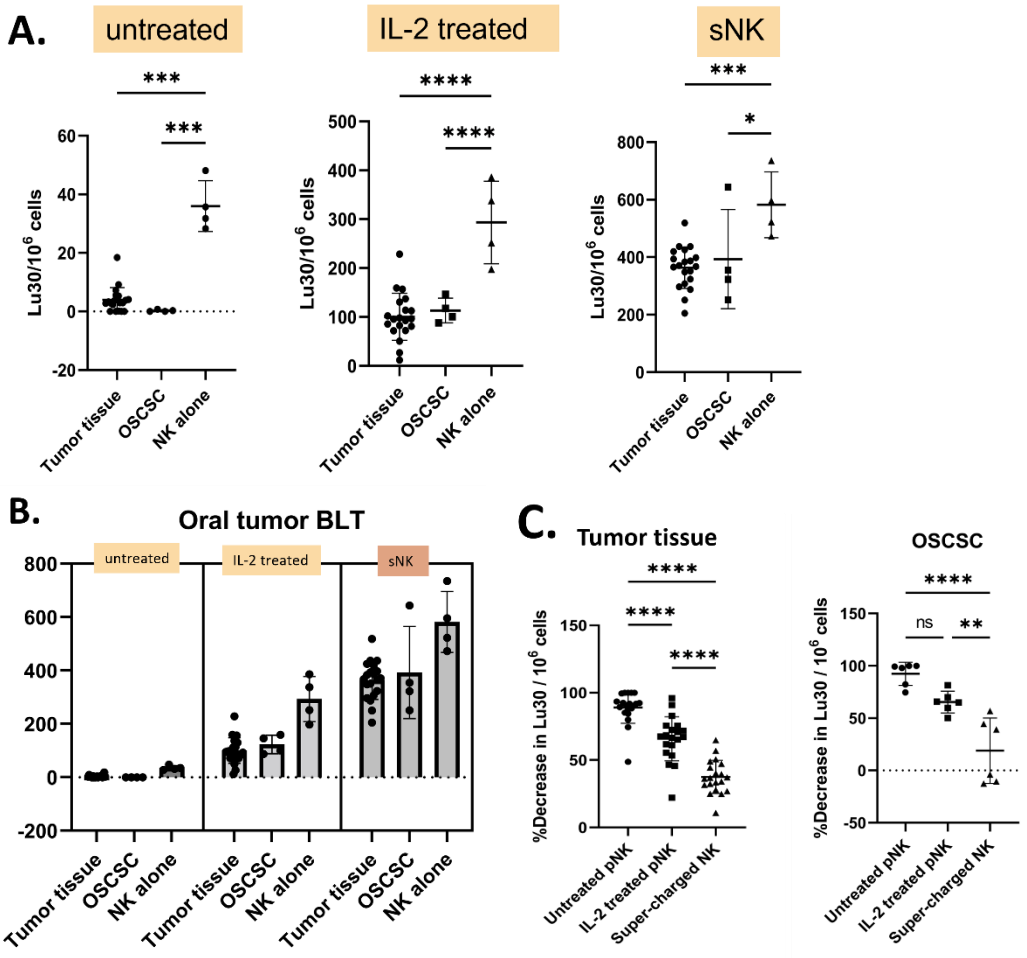


Figure 2. *Higher cytotoxicity of sNK cells than rhIL-2 treated pNK cells when cultured with OSCSC tumor tissues.* Oral stem-like tumor cells were implanted in both humanized BLT and harvested the tumors after 3-4 weeks. The tumor tissues were cut into 4-8 small parts and then each portion was cut into four small pieces. One of the pieces was saved for p53 library preparation and sequencing. The other pieces were co-cultured with either untreated pNK or IL-2 treated pNK or sNK for overnight. Then the cytotoxicity of NK cells was measured by chromium release assay. % decrease was calculated by the number of lytic unit 30 of control minus each sample and divided by the control (C). The following symbols represent the levels of statistical significance within each analysis, ***(p-value <0.001), **(p-value 0.001-0.01), *(p-value 0.01–0.05).

Figure 3.

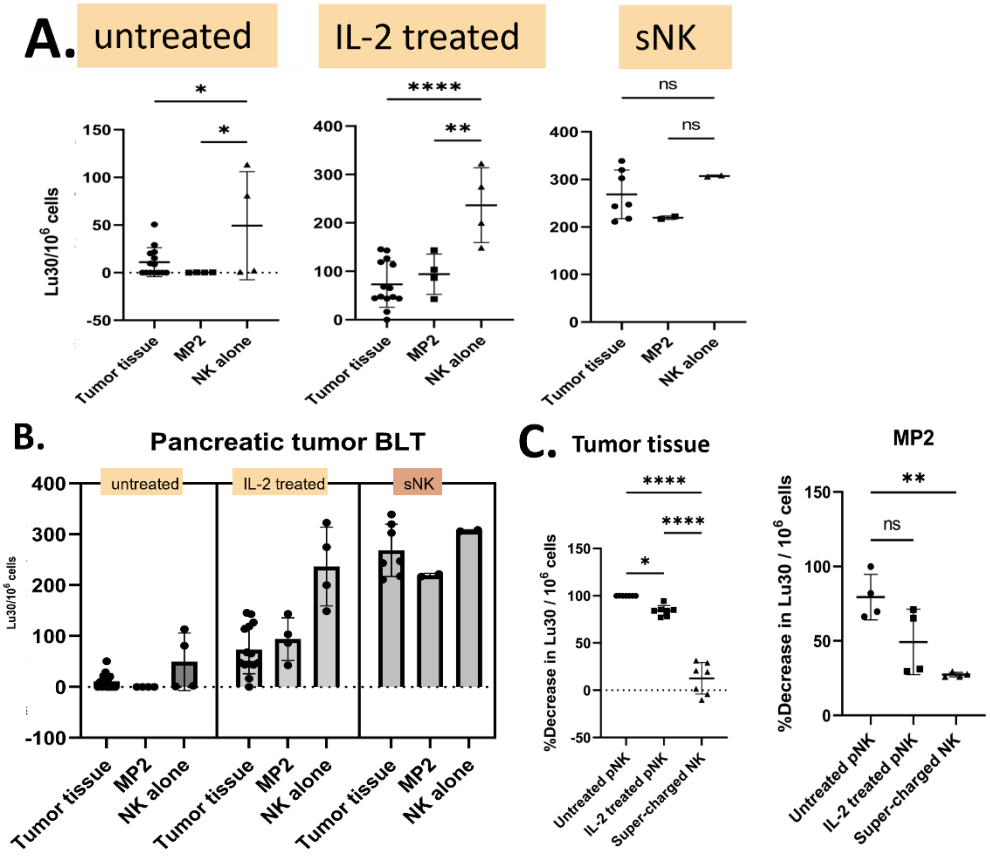
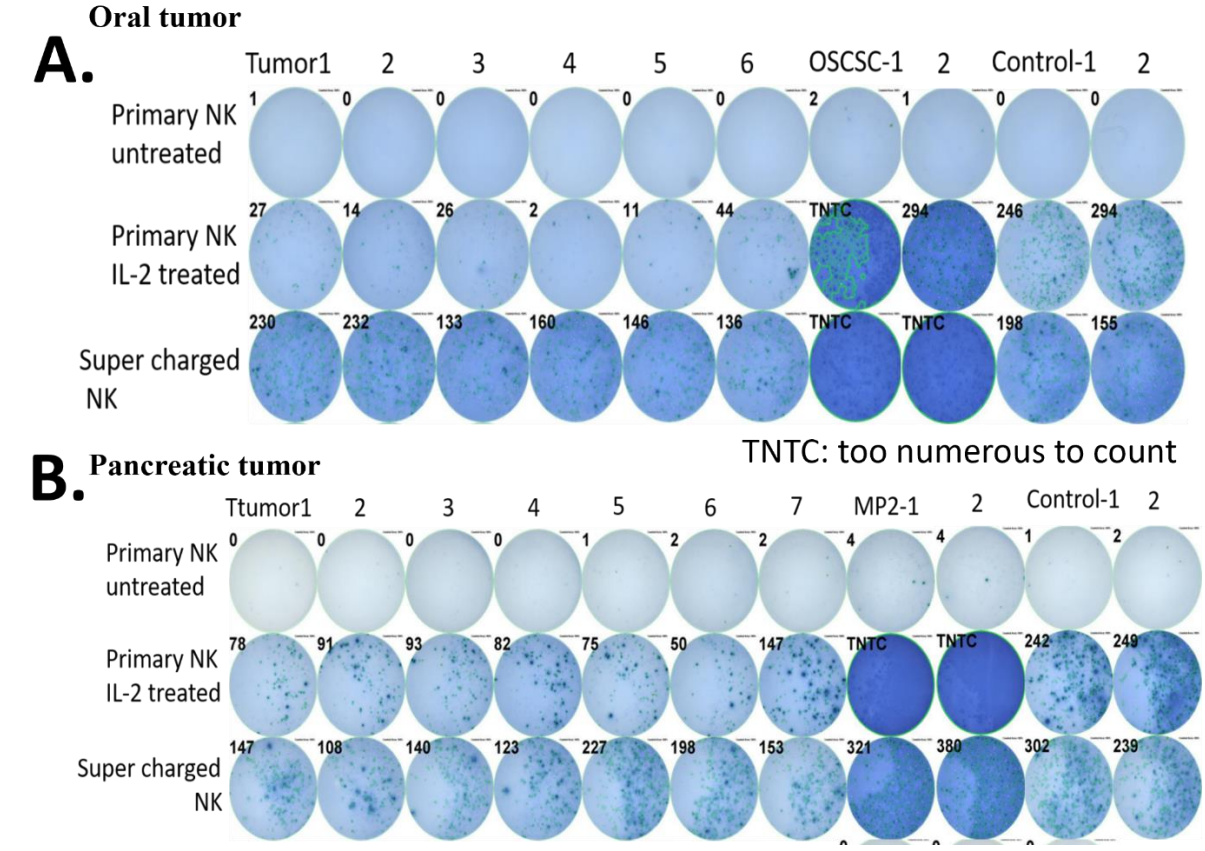


Figure 3. *Higher cytotoxicity of sNK cells than rhIL-2 treated pNK cells when cultured with MP2 tumor tissues.* Pancreatic stem-like tumor cells were implanted in both humanized BLT and harvested the tumors after 3-4 weeks. The tumor tissues were cut into 4-8 small parts and each portion was then cut into four small pieces. One of the pieces was saved for p53 library prep and sequencing. The other pieces were co-cultured with either untreated pNK or IL-2 treated pNK or sNK for overnight. Then the cytotoxicity of NK cells was measured using a chromium release assay. Percent decrease was calculated by the number of lytic unit 30 of control minus each sample and divided by the control (C). The following symbols represent the levels of statistical significance within each analysis, ***(p-value <0.001), **(p-value 0.001-0.01), *(p-value 0.01–0.05).

Figure 4.


Oral tumor
A.
Tumor1 2 3 4 5 6 OSCSC-1 2 Control-1 2
Primary NK untreated
1 0 0 0 0 0 2 1 0 0
Primary NK IL-2 treated
27 14 26 2 11 44 TNTC 294 246 294
Super charged NK
230 232 133 160 146 136 TNTC TNTC 198 155
TNTC: too numerous to count
B.
Pancreatic tumor
Ttumor1 2 3 4 5 6 7 MP2-1 2 Control-1 2
Primary NK untreated
0 0 0 0 1 2 2 4 4 1 2
Primary NK IL-2 treated
78 91 93 82 75 50 147 TNTC TNTC 242 249
Super charged NK
147 108 140 123 227 198 153 321 380 302 239

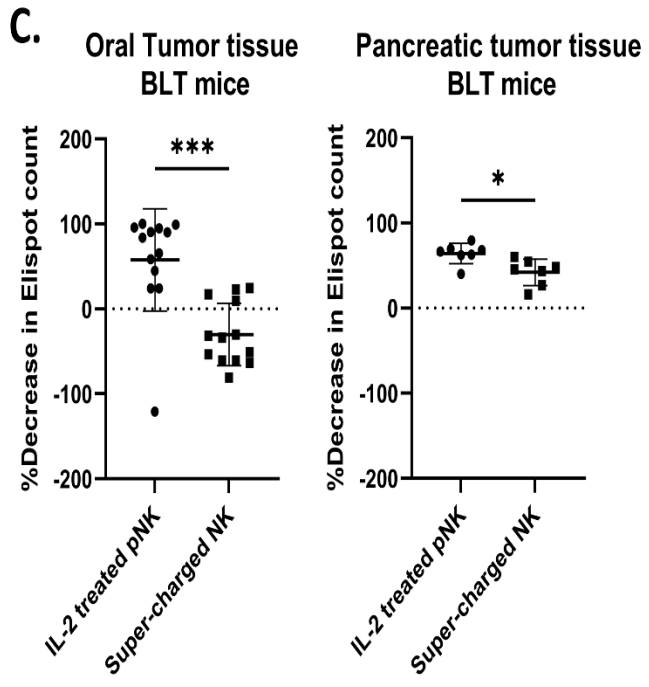

C.
Oral Tumor tissue BLT mice
Pancreatic tumor tissue BLT mice
%Decrease in Elispot count
200
100
0
-100
-200
***
*
IL-2 treated pNK
Super-charged NK

Figure 4. *Higher secretion of IFN-γ of sNK cells at the single-cell level.* As previously described, NK cells were co-cultured with either OSCSC or MP2 tumor tissues and Elispot was performed on the collected samples (A, B). The % decrease was calculated using the average number of controls minus the number of each sample and divided by the average of control (C). The following symbols represent the levels of statistical significance within each analysis, ***(p-value <0.001), **(p-value 0.001-0.01), *(p-value 0.01–0.05).

Figure 5.

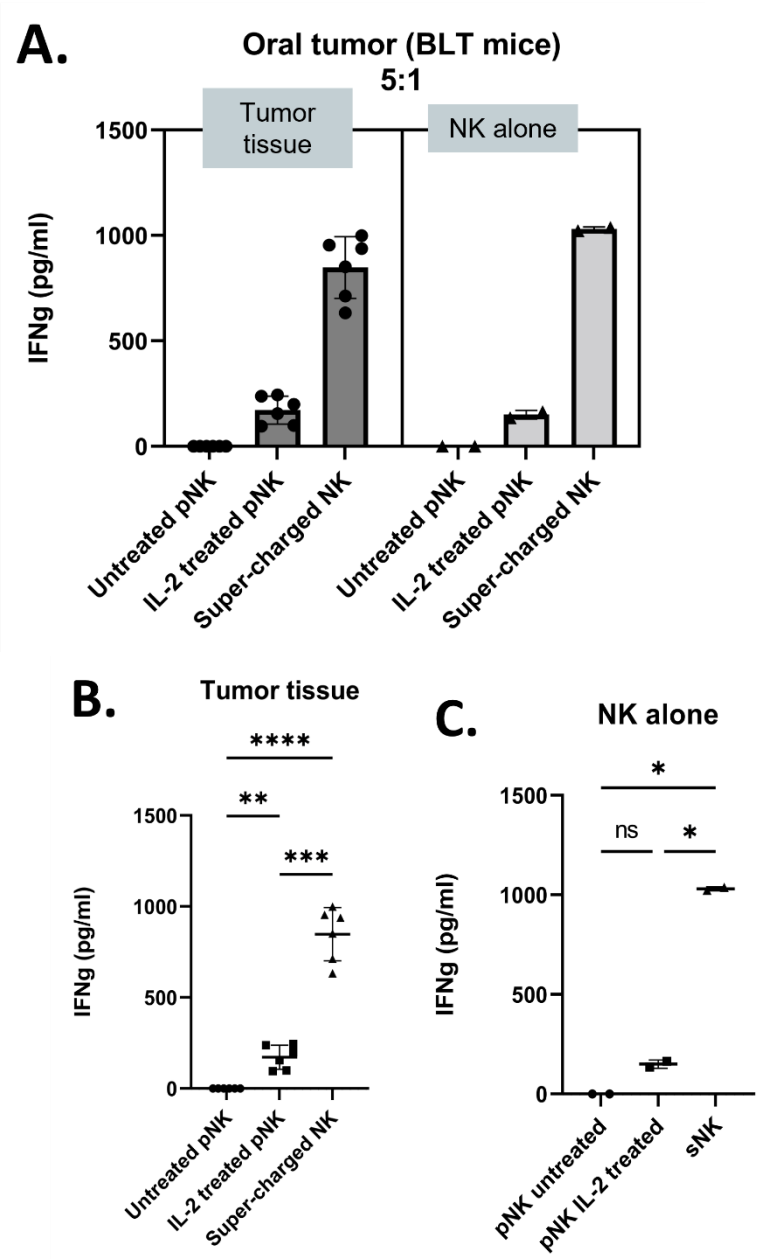


Figure 5. *The highest IFN-γ secretion in sNK cells when compared to pNK cells*. The supernatant from NK cells and tumor co-cultured was measured with an ELISA kit. The supernatant was diluted by a ratio of 1:5 when measured the IFN-γ secretion (A). The following symbols represent the levels of statistical significance within each analysis, ***(p-value <0.001), **(p-value 0.001-0.01), *(p-value 0.01–0.05).

Figure 6.

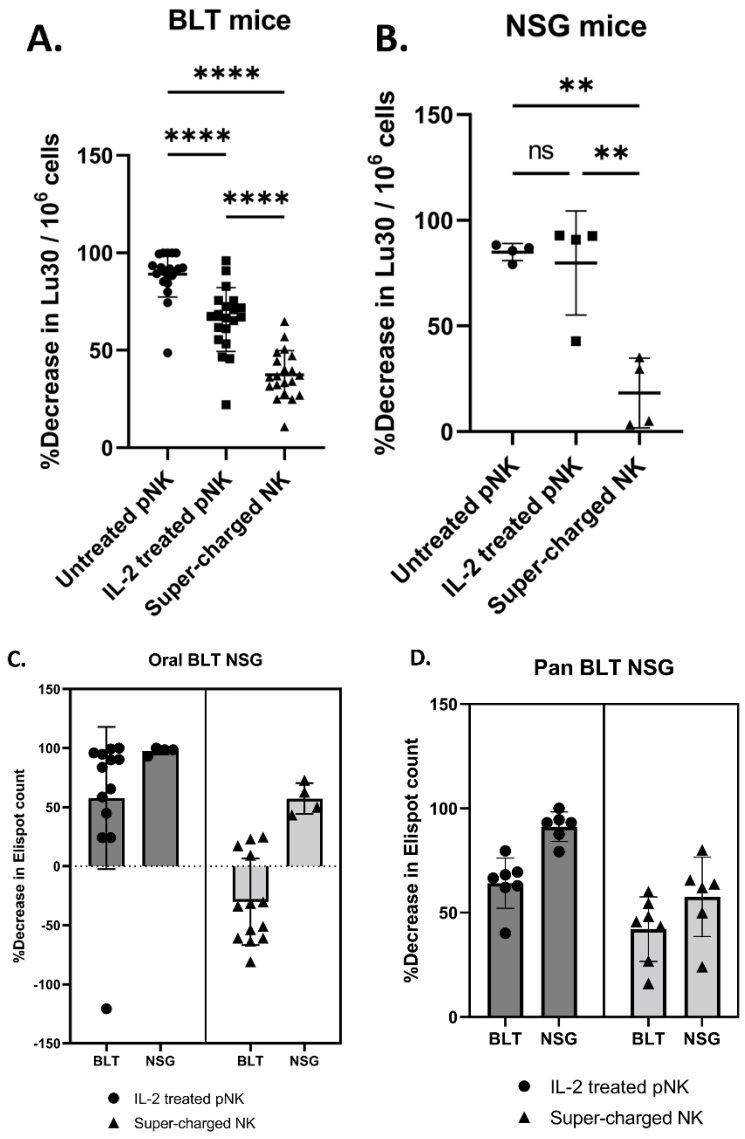

A.
BLT mice
****
****
****
%Decrease in Lu30 / 10^6 cells
150
100
50
0
Untreated pNK
IL-2 treated pNK
Super-charged NK
B.
NSG mice
**
ns
**
%Decrease in Lu30 / 10^6 cells
150
100
50
0
Untreated pNK
IL-2 treated pNK
Super-charged NK
C.
Oral BLT NSG
%Decrease in Elispot count
150
100
50
0
-50
-100
-150
BLT
NSG
BLT
NSG
IL-2 treated pNK
Super-charged NK
D.
Pan BLT NSG
%Decrease in Elispot count
150
100
50
0
BLT
NSG
BLT
NSG
IL-2 treated pNK
Super-charged NK

Figure 6. *The cytotoxicity and the secretion of IFN-γ of both rhIL-2 activated pNK and sNK cells were suppressed less when NK cells were cultured with tumors from hu-BLT mice than those from NSG mice.* Tumor implantation, NK cells and tumor tissues co-culture, chromium release assay, and Elispot were performed in NSG mice and hu-BLT mice. The percentage decrease of lytic unit 30 of NK cells cultured with oral stem-like tumor tissues was calculated by the average Lu30 of controls minus the Lu30 of each sample and divided by the average of control (A-B). The percent decrease of Elispot count of NK cells either cultured with oral or pancreatic tumor tissues was calculated by the average number of controls minus the number of each sample and divided by the average of control (C-D). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 7.

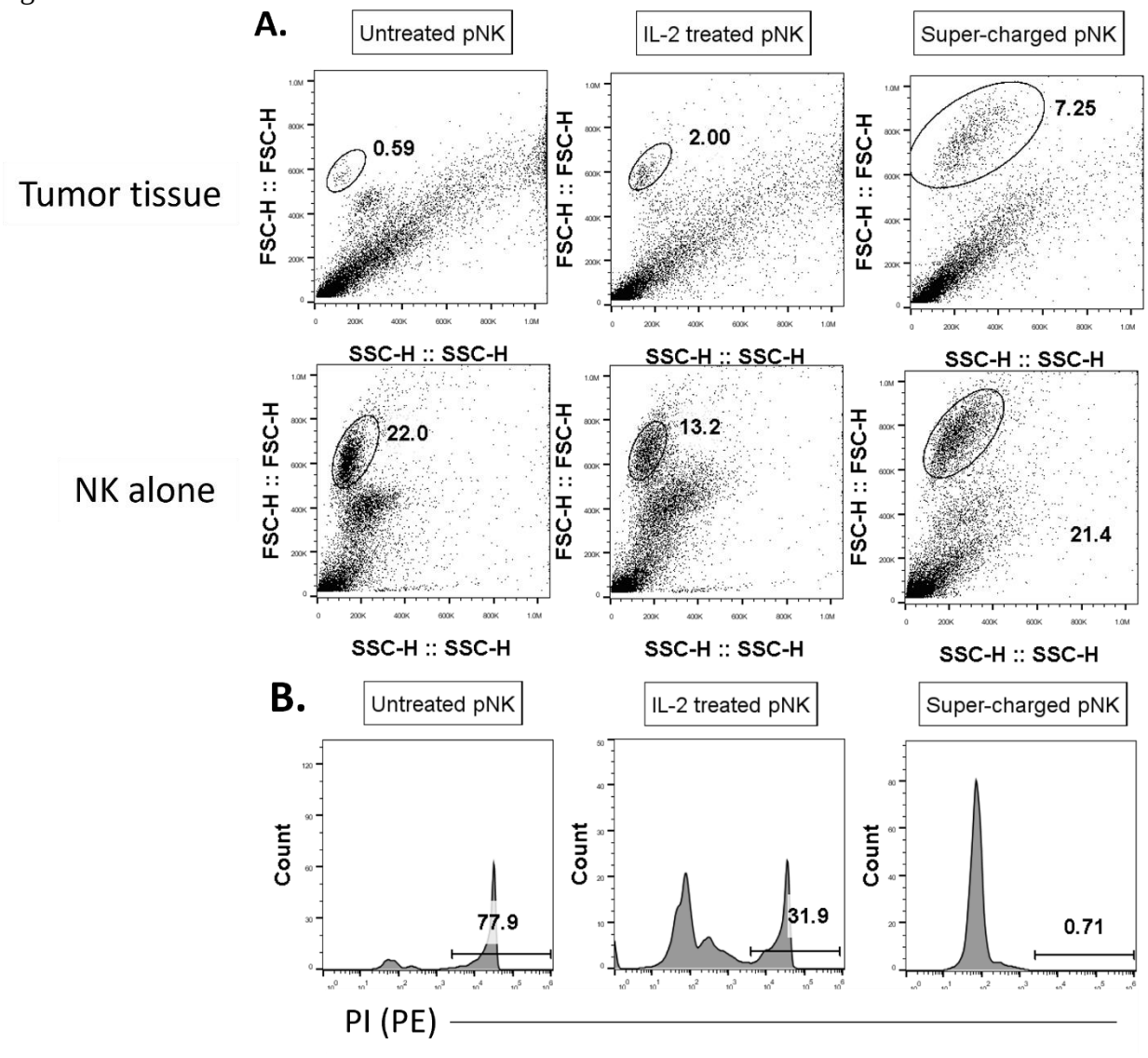


Figure 7. *Live cell gating and PI staining on NK cells from NK-tumor coculture showed that sNK cells had higher percent of live cells and lower percent of dead cells, indicating that cell death is an important mechanism for the inactivation of pNK cells when cultured with human tumor chunks.* NK cells from NK-tumor co-culture were stained with PE PI. Unstained cells were used as controls. The numbers showed on each graph are percentages of gated cells

Table 1.

% Decrease in live cells	Untreated pNK	IL-2 treated pNK	SNK
Tumor tissue	97.3%	84.8%	66.1%

Chapter 3: To determine the in vivo significance of sNK cells in eliminating tumors in CD34+ humanized mouse model in comparison to those in BLT mice.

Results

In our previous work, we have demonstrated that tumors were unable to grow or grew smaller tumors in BLT mice injected with super-charged NK cells, and they were susceptible to chemotherapy drugs[34]. Furthermore, NK cells were able to differentiate tumors through the secretion of IFN-γ, resulting in decreased tumor growth and expansion, upregulation of MHC class I, CD54, and PD-L1, and lower expression of CD44 surface receptors in vivo. We found out that mice implanted with NK-differentiated pancreatic tumor cells had smaller tumors. The tumor cells were unable to grow in the BLT mice and were susceptible to chemotherapy. However, when the implanted tumor cells were treated with anti-IFN-γ and TNF-α antibodies, the tumors grew faster and bigger in BLT mice and were resistant to chemotherapy. Also, we found that sNK cells injection decreased stem-like pancreatic tumor growth, and the tumor cells dissociated from those tumors expressed higher differentiation antigens such as PD-L1, MHC class I, and CD54. More importantly, we observed that the mice given the sNK injection and fed with AJ2 had increased immune cell infiltration in tumors and tumor-induced decreases in NK cytotoxicity and IFN-γ secretion were restored/increased within PBMCs, splenocytes, and bone marrow cells. Lastly, we found IFN-γ secretion was increased, whereas the secretion of IL-6 was decreased in tumor dissociated cells from those mice.

To conclude, sNK cells prevent pancreatic tumors through tumor lysis and differentiation, curtailing stem-like tumors' growth and metastatic potential. In comparison with BLT mice, we also used NSG mice reconstituted with CD34+ bone marrow cells and observed similar trends. The experiment design was mentioned under the Methods and Materials section. Briefly, 8 to 12-week-old NSG mice were irradiated and injected intravenously with CD34+ hematopoietic stem cells (HSCs) derived from umbilical cord blood. After 12 weeks of $CD34^+$ cell injection, over 25% of the cells in the peripheral blood were human CD45+ cells. The mice were fed AJ2 starting one week before MP2 tumor implantation and fed every two days after implantation until they were sacrificed. sNK and anti-MICa/b were given one week after tumor implantation.

Decreased tumor burden in NSG-$CD34^+$mice injected with sNK cells

The results of our preliminary study demonstrated that there was a significant decrease in tumor weights from tumor-bearing CD34+ humanized mice treated with sNK combined with anti-MICa/b mAb compared to controls (Figure 8. A and B).

The Systemic regulation of sNK cell function by AJ2 probiotic bacteria fed to tumor-bearing NSG-CD34+ mice lead to decrease in tumor size.

The tumor weight from mice which received AJ2 also had a significant decrease compared to the tumor from mice which had not received AJ2 (Figure 8. A and B).

Figure 8.

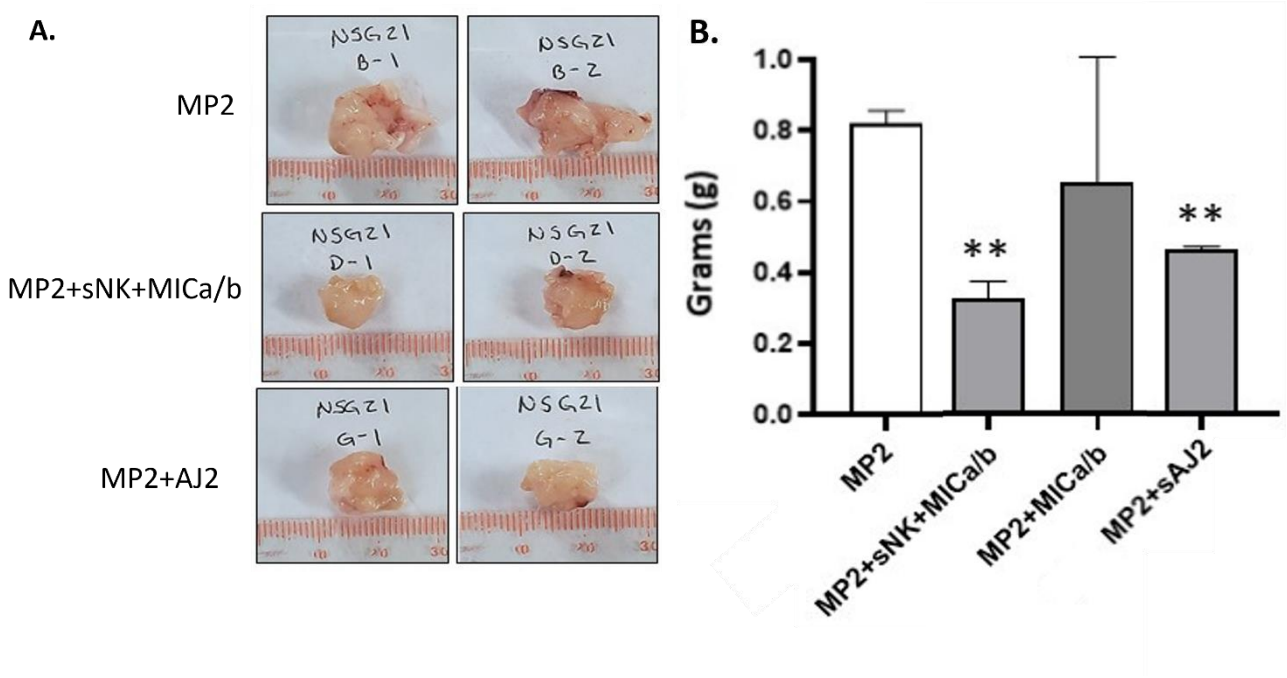


Figure 8. *Tumor weights decreased in both sNK cells treated, and AJ2 fed CD34+ humanized mice.* The humanized CD34+ mice were fed with AJ2 one or two weeks before stem-like pancreatic tumor implantation and then were fed every two days until they were sacrificed. sNK and/or MICa/b antibodies were injected through a tail vein or i.p. one week after implantation. Disease progression was monitored mice were sacrificed when they demonstrated tumor-bearing symptoms. The results of chromium release assay, Elispot, and are still being analyzed. The following symbols represent the levels of statistical significance within each analysis, ***(p-value <0.001), **(p-value 0.001-0.01), *(p-value 0.01–0.05).

Conclusion

sNK cells have higher expression of activating receptors, includes NKG2D, NKp44, NKp46 and granzyme B, higher cytotoxicity and IFN-γ secretion, and are more polyfunctional compared to pNK cells. In proteomic analysis, sNK cells have a higher abundance of proteins associated with the cell cycle, cellular metabolism, and immune system markers, and higher levels of proteins related to proliferation, cytotoxicity, and IFN- γ secretion compared to those in pNK cells. These differences can help us understand how sNK cells are resistant and maintain higher cytotoxicity when co-cultured with tumor tissues from tumor-bearing BLT mice. In tumor tissue co-culture experiments, we also found that the infiltrating immune cells in TME decrease suppression of the function of the primary and sNK cells. However, sNK cells are less functionally inactivated as compared to untreated or IL-2 treated primary NK cells both in BLT and NSG implanted tumors. Lastly, we observed the inhibition of tumor growth by sNK cells in NSG-$CD34^+$ mice, which was consistent with our previous studies in hu-BLT mice.

Discussion

We have previously shown that cytotoxicity of NK cells becomes largely inactivated after their interaction with tumor cells. Indeed, when assessing the NK function after the use of different treatment strategies, the highest inactivation of NK function after their interaction with the tumor mass was seen in the untreated primary NK cells, followed by rhIL-2 treated primary NK cells, as shown above. When the level of inactivation of cytotoxicity after interaction with the tumor mass or tumor cells of super-charged NK cells was compared to either untreated or rhIL-2 treated primary NK cells, super-charged NK cells only lost approximately 10%-30% of their cytotoxicity when compared to rhIL-2 treated primary NK cells, which lost around 60%-80% and untreated NK cells (about 90%-100%). Super-charged NK cells retain their cytotoxic function and mediate cytotoxicity for the second round of killing. Thus, these cells may be capable of serial killing the tumor cells and are inactivated much less by the tumor mass. Indeed, multiple super-charged NK cells were found to bind and target glioblastoma stem cells (GSCs) in a spheroid tumor model as assessed by the confocal microscopy (manuscript submitted). It is likely that super-charged NK cells withstand the suppressive signals from the TME and thus remain activated during their interaction with the tumor cells. In addition, it is possible that sNK cells are able to lyse the suppressive cells within the TME such as Tregs, MDSCs, MSCs and TAMs. Due to lysis of these suppressive cells less inhibitory signals are delivered to sNK cells.

We have also shown that sNK cells have the most minor decrease in the live population of NK cells and are not signaled to undergo cell death to the extent we see with activated primary NK cells. These results indicate that these cells have higher anti-apoptotic proteins such as BCL-2

and lower pro-apoptotic proteins such as Bax. These studies are on the way and will be conducted in the future.

Overall, the ability of sNK cells to withstand suppression by the TME is an essential area of investigation and should provide important clues regarding their ability to eliminate tumors.

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