UNIVERSITY OF CALIFORNIA

Los Angeles

Osteoclast-induced super-charged NK cells preferentially select and expand CD8+ T cells, differences with primary NK cells, and the role of CD16 receptors on NK activation

Meng-Wei Ko

2021

ABSTRACT OF THE DISSERTATION

Preferentially selection and expansion of CD8+ T cells
by osteoclast-induced, super-charged NK cells
and the role of CD16 receptors on NK activation

by

Meng-Wei Ko

Doctor of Philosophy in Oral Biology
University of California, Los Angeles, 2021
Professor Anahid Jewett, Chair

Natural killer (NK cells) are known to play crucial role in halting the progression of cancer. They have the abilities to selectively lyse and differentiate cancer stem cells (CSCs) *via* direct cytotoxicity and secreted IFN-γ secretion and membrane-bound TNF-α, respectively, which leads to slower tumor growth and limit tumor metastasis. However, lower numbers and functions of NK cells from cancer patients have been reported. This study provides evidences that CD16 receptor but not Toll-like receptor signaling pathway on both NK cells and monocytes from cancer patients is defective resulting the lack of activation in cancer patients, which resulting less differentiation ability of NK cells from cancer patients. In addition, for NK immunotherapy, a methodology to

expand and enhance the numbers and functions of NK cells was previously established. The expanded NK cells with potent functions are celled super-charged NK cells. This study investigated the differences of primary and super-charged NK cells and showed that super-charged NK cells have higher level of proliferation markers, more cytokine and chemokine secretion, and more polyfunctional population and are not inactivated after encountering tumor cells. Lastly, it was illustrated that super-charged NK cells preferentially expand CD8+ memory T cells by lysing CD4+ T cells during the expansion process. These compelling findings provides novel approaches for cancer immunotherapy encompassing the stabilization of CD16 receptor on both NK cells and monocytes of cancer patients as well as the possibilities of combination cell adoptive immunotherapy with allogeneic NK and autologous CD8+ T cells.

The dissertation of Meng-Wei Ko is approved.

Ichiro Nishimura

Nicholas A. Cacalano

Shen Hu

Anahid Jewett, Committee Chair

University of California, Los Angeles

2021

DEDICATION

To my mother and father

Table of Contents

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Anahid Jewett, for her continuous guidance and providing me the environment to grow as a scientist. I would like to thank the members of my dissertation committee, Dr. Ichiro Nishimura, Dr. Nicholas A. Cacalano and Dr. Shen Hu for all their exceptional advice.

I would like to acknowledge all the current and previous lab members of the Jewett Lab for good discussion as well as aiding me in personal growth. Also, I would like to thank all my friends and everyone I met during my PhD journey for their companion at both the most exciting and difficult times.

I wish to acknowledge the contributions of all to Chapter 1, 2 and 3 of this dissertation. They are as follow;

Chapter 1: Tahmineh Safaei, Christine Sutanto, Wuyang Chen and Paul Wong

Chapter 2: Emanuela Senjor, Lucy Wanrong Gao, Paul Wong, Po-Chun Chen, Whitaker Cohn and Dr. Julian P Whitelegge

Chapter 3: Dr. Kawaljit Kaur, Nick Ohanian and Jessica Cook

Finally, to my mother and father, for all their love, encouragement and support. I would never become who I am without them. I owe any success I have to them.

VITA

2009-2014	D.V.M., National Chung Hsing University, Taichung, Taiwan
2014-2016	M.V.M., National Chung Hsing University, Taichung, Taiwan
2017-2021	Doctoral Candidate, Oral Biology and Medicine, University of California, Los Angeles, California, USA

INTRODUCTION

Natural killer cells

Natural killer (NK) cells were first identified as anti-tumor T cell independent immune cells with natural cytotoxicity in 1970s [1]. They are defined as immune cells with CD16 and CD56 and the lack of CD3 expression on their cell surfaces [2]. They are a part of peripheral blood mononuclear cells (PBMCs) derived from hematopoietic stem cells and are known to mediate cytotoxicity against viral and bacterial infected cells or malignant cells without prior immunization as first line defender in the immune system as well as secret cytokines and chemokines to regulate the function of other immune cells [3, 4]. We have previously proposed a 4-stage of NK maturation hypothesis hinging on their surface expression and functionality. In the first stage, NK cells express $CD3^{-}CD16^{+}CD56^{dim}$ on the cell surfaces and possess high cytotoxicity and lower cytokine secretion. With surface receptor crosslinking with antibodies or ligands on other cells, NK cells would be induced into the second stage, called split anergy. Split anergized NK cells are $CD3^{-}CD16^{-}CD56^{bright}$ and have higher cytokine secretion ability but lower cytotoxicity. Without proper stimulation or activation signals, NK cells would enter stage 3, where they lose both of their cytotoxicity and cytokine secretion and become dysfunctional, which eventually triggers apoptosis and leads to the death of the cells as stage 4 [5].

The function of NK cells are dominated by their cell surface receptors which is regulated by the summation of inhibitory and activating signals receiving through their surface receptors upon immune synapse formation [6]. There is a panel of various activating receptors on NK cells,

such as NKG2D, NKp46 and DNAM-1 as well as inhibitory receptors, including KIR2, NKG2A and KLRG2 [7]. It is well established that infected cells would increase their MICA/B on the cell surfaces which are the ligands for NKG2D resulting in NK activation upon ligation [8]. On the other hand, healthy or well-differentiated cells would express MHC class I as self-identified molecule on the cell surfaces which binds to KIRs and inhibits NK cell function to prevent the cell lysis [9], whereas, poorly differentiated or stem like cells would lose the MHC class I expression and wouldn't be able to inhibit the cytotoxic function of NK cells leading to NK cell mediated cell lysis [10].

NK cell functions

One of the most distinct function of NK cells, especially for those are at stage 1, is mediating direct cytotoxicity against target cells. They perform the cytotoxic function through three main pathways. First, perforin/granzyme pathway, NK cells release the cytotoxic granule upon immune synapse formation, which contains perforin and granzyme B to permeate target cell membrane and induce apoptosis in the target cells, respectively [11]. In addition, NK cells induce target cell death through TNF-related apoptosis-inducing ligand (TRAIL) and Fas-FasL pathways to transduce apoptotic signals [12-14]. Other than direct cytotoxicity, NK cells are capable of mediating antibody-dependent cellular cytotoxicity (ADCC) through CD16, also known as FcγRIIIa, which binds to the Fc region of antibodies. Upon ligation, NK cells would release perforin- and granzyme-containing granules to lyse the antibody-coated target cells [15, 16].

In addition to cytotoxicity, as one of the main lymphocytes in innate immunity, NK cells are also known for their cytokine and chemokine secretion to enhance and regulate adaptive immune responses to influence other immune cells in the PBMCs. It has been shown that NK cells promote the proliferation of $CD4^+$ T cells through tumor necrosis factor (TNF) superfamilies and other costimulatory molecules [17]. Also, NK cells were reported in several studies to play important roles in T cell recruitment and activation after intracellular pathogen infection through the secretion of interferon (IFN)-γ [18-21]. Nevertheless, the IFN-γ production from NK cells has also reported to inhibit B cell transformation at the early stage of the viral infection and restrict the pathogen while allowing more time for adaptive immune system to acquire specific immunity and be equipped to defeat the pathogen [22]. Aside from regulatory effects on other immune cells, secreted IFN-γ and membrane-bound TNF-α from NK cells were shown to differentiate stem like cells, both normal and cancerous cells [10, 23-25].

NK cells in cancer development

NK cell has shown to possess great abilities to halt the progression of cancer development through selectively lysing and differentiating poorly differentiated cancer stem cells (CSCs) *via* direct cytotoxicity and secreted IFN-γ and membrane-bound TNF-α, respectively [23, 26-28]. Since these CSCs have lower levels of MHC class I expression on their cell surfaces, NK cells are able to identify the "missing-self" signal and further initiate the killing process through perforin-granzyme pathway leading to apoptosis of these CSCs [29]. Moreover, NK cells are also known to differentiate CSCs through IFN-γ and TNF-α. It was previously reported that CD44, a surface maker for stemness, is decreased on CSCs while CD54, PD-L1 and MHC class I, surface markers

established previously for differentiated cells, are all increased after treated with NK cell culture supernatants [26, 30]. As a result, these CSCs grow slower or eventually lose their self-renewal ability. Therefore, through directly targeting and differentiating CSCs, NK cells are capable of eliminate the seeds of cancer and prevent cancer from progression.

It has been unearthed by our laboratory and others that the numbers and functions of NK cells are significantly lower in cancer patients. And we have reported these observations of reduced functions of NK cells are associated with both genetic mutation and lifestyle prior to tumor is established. It was shown that mice with both KRAS mutation and fed with high fat calory diet had the lowest NK cell functions followed by either KRAS mutation or high fat calory diet mice, then control mice when pancreatic intraepithelial neoplasias (PanINs) are induced, yet pancreatic ductal adenocarcinoma (PDAC) was not formed, indicating that both genetics (KRAS mutation) and environmental factors (high fat calory diet) play crucial role in the loss of NK cell function at the pre-neoplastic stage of pancreatic cancer [31, 32]. The same observation is made with tumor-bearing humanized BLT mouse models and the peripheral blood obtained from cancer patients. The numbers and functions of NK cells are significantly lower than those from healthy individuals [31, 33, 34].

NK cell in cancer immunotherapy

Given the crucial role of NK cells in halting cancer progression, there have been numerous studies endeavor to rescue or supplement healthy functional NK cells as a novel approach to cancer immunotherapy. We have previously established a robust methodology to expand and enhanced

the number and functions of NK cells using osteoclast (OC) as feeder cells and a mixture of sonicated probiotic bacteria, sAJ2 [35]. And these expanded NK cells have shown to possess great functional abilities in mediating lysing and differentiating CSCs *in vitro* assays as well as *in vivo* studies with humanized-BLT mouse models of oral and pancreatic tumor [33, 34].

Central Hypothesis

Due to the defectiveness of the number and functions of NK function seen in cancer patients, we proposed that CD16 pathway is responsible for the lack of activation of NK cells not TLR signaling pathway in cancer patients and super-charged NK cells preferentially expand CD8+ T cells through lysing CD4+ T cells.

Objective of the study

To identify the pathway responsible for the lack of activation of NK cells in cancer patients; to understand the differences of primary and super-charged NK cells super-charged NK cells; to study the mechanism of NK cells super-charging process preferentially expand CD8+ T cells in order to provide more insight to improve current knowledge of NK immunotherapeutic strategies.

Specific Aims

Aim 1: Defects in CD16 receptor but not toll-like receptors signaling pathway in NK cells and monocytes in cancer patients.

Sub-aim 1: Differences of cell type composition of PBMCs and cytokine/chemokine profile in the sera between cancer patients and healthy individuals.

Sub-aim 2: Differences of NK cell functions in PBMCs from cancer patients and healthy individuals

Sub-aim 3: Monocytes from cancer patients increased IFN-γ secretion of NK cells when treated with IL-2 and sAJ2, but not with the presence of anti-CD16 monoclonal antibody

Aim 2: Phenotypic and functional differences between primary and super-charged NK cells

Sub-aim 1: Phenotypic differences between primary and super-charged NK cells

Sub-aim 2: Functional differences between primary and super-charged NK cells.

Sub-aim 3: Proteomics analysis of primary and super-charged NK cells

Sub-aim 4: Unlike primary NK cells, the cytotoxic function of super-charged NK cells is not suppressed after encountering tumor

Aim 3: Super-charged NK cells preferentially select and expand memory CD8+ T cells while eliminating CD4+ T cells during expansion

Sub-aim 1: Decreased in functions of NK cells and less activation ability in osteoclasts in cancer patients.

Sub-aim 2: Osteoclasts expanded NK cells and osteoclasts preferentially expand CD8+ T cells

Sub-aim 3: Osteoclasts expanded super-charged NK cells contain the growth of T cells and preferentially expand CD8+ T cells by selectively lysing CD4+ T cells

Sub-aim 4: Characteristics of super-charged NK cells expanded memory CD8+ T cells and their functionality

Reference

1. Greenberg, A.H., *The origins of the NK cell, or a Canadian in King Ivan's court.* Clin Invest Med, 1994. **17**(6): p. 626-31.
2. Moretta, A., et al., *A novel surface antigen expressed by a subset of human CD3- CD16+ natural killer cells. Role in cell activation and regulation of cytolytic function.* J Exp Med, 1990. **171**(3): p. 695-714.
3. van Meir, H., et al., *Impact of (chemo)radiotherapy on immune cell composition and function in cervical cancer patients.* Oncoimmunology, 2017. **6**(2): p. e1267095.
4. Bonavida, B., L.T. Lebow, and A. Jewett, *Natural killer cell subsets: maturation, differentiation and regulation.* Nat Immun, 1993. **12**(4-5): p. 194-208.
5. Jewett, A., et al., *Multiple Defects of Natural Killer Cells in Cancer Patients: Anarchy, Dysregulated Systemic Immunity, and Immunosuppression in Metastatic Cancer.* Crit Rev Immunol, 2020. **40**(2): p. 93-133.
6. Kumar, S., *Natural killer cell cytotoxicity and its regulation by inhibitory receptors.* Immunology, 2018. **154**(3): p. 383-393.
7. Pegram, H.J., et al., *Activating and inhibitory receptors of natural killer cells.* Immunol Cell Biol, 2011. **89**(2): p. 216-24.
8. Eleme, K., et al., *Cell surface organization of stress-inducible proteins ULBP and MICA that stimulate human NK cells and T cells via NKG2D.* J Exp Med, 2004. **199**(7): p. 1005-10.

9. Tomasello, E., et al., *Signaling pathways engaged by NK cell receptors: double concerto for activating receptors, inhibitory receptors and NK cells.* Semin Immunol, 2000. **12**(2): p. 139-47.

10. Jewett, A., Y.G. Man, and H.C. Tseng, *Dual functions of natural killer cells in selection and differentiation of stem cells; role in regulation of inflammation and regeneration of tissues.* J Cancer, 2013. **4**(1): p. 12-24.

11. Trapani, J.A., *Target cell apoptosis induced by cytotoxic T cells and natural killer cells involves synergy between the pore-forming protein, perforin, and the serine protease, granzyme B.* Aust N Z J Med, 1995. **25**(6): p. 793-9.

12. Arase, H., N. Arase, and T. Saito, *Fas-mediated cytotoxicity by freshly isolated natural killer cells.* J Exp Med, 1995. **181**(3): p. 1235-8.

13. Oshimi, Y., et al., *Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells.* J Immunol, 1996. **157**(7): p. 2909-15.

14. Zamai, L., et al., *Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells.* J Exp Med, 1998. **188**(12): p. 2375-80.

15. Hatjiharissi, E., et al., *Increased natural killer cell expression of CD16, augmented binding and ADCC activity to rituximab among individuals expressing the Fc{gamma}RIIIa-158 V/V and V/F polymorphism.* Blood, 2007. **110**(7): p. 2561-4.

16. Jewett, A., J.V. Giorgi, and B. Bonavida, *Antibody-dependent cellular cytotoxicity against HIV-coated target cells by peripheral blood monocytes from HIV seropositive asymptomatic patients.* J Immunol, 1990. **145**(12): p. 4065-71.

17. Zingoni, A., et al., *NK cell regulation of T cell-mediated responses.* Mol Immunol, 2005. **42**(4): p. 451-4.

18. Vankayalapati, R., et al., *NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen.* J Immunol, 2004. **172**(1): p. 130-7.

19. Combe, C.L., et al., *NK cells help to induce CD8(+)-T-cell immunity against Toxoplasma gondii in the absence of CD4(+) T cells.* Infect Immun, 2005. **73**(8): p. 4913-21.

20. Ge, M.Q., et al., *NK cells regulate CD8+ T cell priming and dendritic cell migration during influenza A infection by IFN-gamma and perforin-dependent mechanisms.* J Immunol, 2012. **189**(5): p. 2099-109.

21. Teoh, J.J., et al., *Acute Virus Control Mediated by Licensed NK Cells Sets Primary CD8+ T Cell Dependence on CD27 Costimulation.* J Immunol, 2016. **197**(11): p. 4360-4370.

22. Strowig, T., et al., *Tonsilar NK cells restrict B cell transformation by the Epstein-Barr virus via IFN-gamma.* PLoS Pathog, 2008. **4**(2): p. e27.

23. Jewett, A., et al., *Natural killer cells as effectors of selection and differentiation of stem cells: role in resolution of inflammation.* J Immunotoxicol, 2014. **11**(4): p. 297-307.

24. Tseng, H.C., et al., *Differential Targeting of Stem Cells and Differentiated Glioblastomas by NK Cells.* J Cancer, 2015. **6**(9): p. 866-76.

25. Tseng, H.C., N. Cacalano, and A. Jewett, *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): p. 8947-59.

26. Tseng, H.C., et al., *Induction of Split Anergy Conditions Natural Killer Cells to Promote Differentiation of Stem Cells through Cell-Cell Contact and Secreted Factors.* Front Immunol, 2014. **5**: p. 269.

27. Bui, V.T., et al., *Augmented IFN-gamma and TNF-alpha 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.* Front Immunol, 2015. **6**: p. 576.

28. Lanzon, C.J., *Strategies to target pancreatic cancer stem cells using natural killer cells and chemotherapeutic drugs*. 2015, University of California, Los Angeles,: Los Angeles. p. 1 online resource (vi, 56 p.

29. Bern, M.D., et al., *Inducible down-regulation of MHC class I results in natural killer cell tolerance.* J Exp Med, 2019. **216**(1): p. 99-116.

30. Tseng, H.C., et al., *Differential Cytotoxicity but Augmented IFN-gamma Secretion by NK Cells after Interaction with Monocytes from Humans, and Those from Wild Type and Myeloid-Specific COX-2 Knockout Mice.* Front Immunol, 2015. **6**: p. 259.

31. Kaur, K., et al., *Suppression of Gingival NK Cells in Precancerous and Cancerous Stages of Pancreatic Cancer in KC and BLT-Humanized Mice.* Front Immunol, 2017. **8**: p. 1606.

32. Jewett, A., et al., *Natural Killer Cells: Diverse Functions in Tumor Immunity and Defects in Pre-neoplastic and Neoplastic Stages of Tumorigenesis.* Mol Ther Oncolytics, 2020. **16**: p. 41-52.

33. Kaur, K., et al., *Super-charged NK cells inhibit growth and progression of stem-like/poorly differentiated oral tumors in vivo in humanized BLT mice; effect on tumor differentiation and response to chemotherapeutic drugs.* Oncoimmunology, 2018. **7**(5): p. e1426518.

34. Kaur, K., et al., *Probiotic-Treated Super-Charged NK Cells Efficiently Clear Poorly Differentiated Pancreatic Tumors in Hu-BLT Mice.* Cancers (Basel), 2019. **12**(1).

35. Kaur, K., et al., *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.* Front Immunol, 2017. **8**: p. 297.

MATERIAL AND METHOD

Cell lines, reagents and antibodies

RPMI 1650 (Gibco, Thermo Fisher Scientific, USA) complete medium with 10% fetal bovine serum (FBS)(Gemini Bio-Products, San Diego, USA) was used for immune cells and oral squamous carcinoma cells (OSCCs) and oral squamous carcinoma stem cells (OSCSCs) isolated from cancer patients with tongue tumor at UCLA [1, 2]. Alpha-MEM (Gibco, Thermo Fisher Scientific, USA) with 10% FBS was used for osteoclasts (OCs) cultures. RPMI 1650 complete medium with 10% human AB serum off the clot (Gemini Bio-Product, San Diego, USA) was used for super-charged NK cell culture. DMEM complete medium with 10% FBS was used for MIA PaCa-2 (MP2) and PL12, which are generous gifts from Dr. Guido Eibl (David Geffen School of Medicine, UCLA) and Dr. Nicholas Cacalano (Jonsson Comprehensive Cancer Center, UCLA), respectively [3]. RANKL and GM-CSF were purchased from PeproTech (NJ, USA), rh-IL-2 was obtained from NIH-BRB. Anti-CD16 monoclonal antibody and human CD3/28 T cell activator was purchased from StemCell Technologies. CDDP was purchased from Ronald Reagen UCLA Medical Center Pharmacy.

Antibodies for human used for flow cytometry were purchased from BioLegend (San Diego, USA), including CD45, CD3, CD16, CD56, CD4, CD8, CD19, CD14, CD44, CD54, B7H1 (PD-L1), MHC class I, NKG2D, KIR2, NKp44, NKp46, CD54, CD62L, CD45RA, CD45RO, CD28, CCR7, granzyme B and Ki-67. Propidium iodide (PI) was purfachsed from Sigma-Aldrich (St. Louis, MO, USA).

Microscope for expansion pictures

Images of NK cells were taken under 400x magnification using DMI6000 B inverted microscope and LAS X software (both Leica, Wetzlar, Germany).

Bacteria sonication

AJ2 is a combination of eight strains of Gram-positive probiotic bacteria (*Streptococcus thermophiles, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus pacasei and Lactobacillus bulgaricus*). AJ2 was resuspended in RPMI 1640 containing 10% FBS at 10 mg/mL. The bacterial suspension then was placed on ice and was sonicated at 8 amplitude for 30 seconds, rest for 15 seconds as a pulse. A sample of sonicated bacteria suspension would be taken and observed under the microscope. It was determined that the sonication was completed when greater than 80% of the cell had been lysed. The sonicated AJ2 (sAJ2) suspension were aliquoted and stored in -80°C freezer for further use.

Purification of NK cell, T cells and monocyte from human PBMC

The study, as well as the procedures, were approved by the UCLA Institutional Review Board (IRB)(IRB#11-000781), and all participants signed written informed consent in accordance with the Declaration of Helsinki. Peripheral blood was diluted with PBS. After Ficoll-Hypaque centrifugation, PBMC was harvested, washed resuspended with RPMI for experiment or Robosep in desirable concentration for negative selection using EasySep™ human NK cell enrichment kit, EasySep™ human CD4+ T cell isolation kit, EasySep™ human CD8+ T cell isolation kit and

EasySep™ human monocyte enrichment kit without CD16 depletion purchased from Stem Cell Technologies (Vancouver, BC, Canada). Purity of isolated NK cells and CD4+ T cells, CD8+ T cells and monocytes were analyzed by flow cytometric analysis. All cells were resuspended in RMPI 1640 complete medium.

Purification and generation of osteoclasts from human PBMC

PBMC were harvest from peripheral blood as describe above and resuspend into alpha-MEM with 1% autologous plasma. Fifty million cells were plated per tissue culture dish with 151.9 cm^2 culture area and incubated at 37°C for 30 min. After incubation, floating cells were removed from the culture dish. Fresh alpha-MEM with 1% autologous plasma was replenished and recombinant human macrophage colony-stimulated factor (M-CSF) (25 ng/mL) (BioLegend, SD, USA) was supplemented every three days and RANKL (25 ng/mL) was supplemented every three days after Day6 for 21 days.

Generation of super-charged NK cells

Nature killer cells were purified from PBMC as described above. Freshly isolated NK cells are treated with recombinant human IL-2 (rh-IL-2) (1,000 U/mL) and anti-CD16 monoclonal antibody (5 μg/mL) (Biolegend, San Diego, USA) at 37°C for 16-18 hours. After the incubation time, NK cells are co-cultured with osteoclasts and sAJ2 (NK: OC: sAJ2, 2: 1: 4) (preparation as describe above) at 37°C. The culture media was replenished every 3 days with rh-IL-2 [4].

Supernatants of NK cells used for induction of cell death and differentiation

Both primary and super-charged NK cells were treated with rh-IL-2 (1,000 U/mL) at 37°C for 16-18 hours in the concentration of 10×10^6 cells /mL. Supernatants of primary and super-charged NK cells were collected and used in the experiments for desire concentrations.

Surface staining, cell death assays and flow Cytometry Analysis

1×10^5 cells were used for each sample. All samples were stained with 100µL of 1% BSA-PBS (Gemini Bio-Products, CA) and pre-determined optimal concentration of desire fluorochrome (PE, FITC or PEcy5) conjugated antibodies and incubate at 4°C for 30 min. The sample then was washed and resuspend with 1% BSA-PBS. Intracellular staining was performed as described in manufacturer's protocol. The sample was fixed with fixation buffer for an hour in dark at room temperature. Two ml of perm buffer was added to wash the sample and the sample was centrifuged at 1400 rpm for 5 min. Repeated the washing step once and discarded the supernatants. Resuspend the sample in Perm buffer and added fluorochrome conjugated antibody and incubate in dark at room temperature for 30 minutes. Repeat the washing step with Perm buffer once and 1%BSA/PBS. Resuspend the sample with 1%BSA/PBS. Propidium iodide (PI)(100 µg/ml) was used for cell death assay. The Epics C XL flow cytometer (Beckman Coulter, Pasadena, CA) and Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA) were used. And FlowJo v10.4 (BD, Oregon, USA) was used for analysis.

^{51}Cr release cytotoxicity Assay

^{51}Cr was purchased from Perkin Elmer (Santa Clara, CA). Standard 4 hour ^{51}Cr release cytotoxicity assays were used to determine NK cytotoxic function in the experimental cultures and the sensitivities of target cells to NK mediated cell lysis. Effector cells (1-2.5×10^5 cells/well) were aliquoted into 96-well round-bottom micro-well plate (Fisher Scientific, Pittsburgh, PA) and titrated at 4-6 serial dilutions with RPMI 1640. Target cells were label with 50μCi ^{51}Cr (Perkin Elmer, Santa Clara, CA) for an hour and excessive ^{51}Cr then being moved by washing with medium twice. ^{51}Cr-labeled target cells (1×10^4 per well) were aliquoted into 96-well round-bottom micro-well plate containing effector cells at a top effector:target (E:T) ratio of 12.5-5:1. Plates were centrifuged and incubated for 4 hours. Following incubation, supernatant was collected from each well into glass tubes and was counted for release radioactivity using the gamma counter. Total release (^{51}Cr-labeled target cells) and spontaneous release (supernatant harvested from wells only contains ^{51}Cr-labeled target cells) values were measured to calculate the percentage specific cytotoxicity. The percentage specific cytotoxicity was calculated by the following formula:

$$\%\ \text{cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100\%$$

Lytic unit 30 per million cells (LU30/10^6 cells) was calculated using the inverse of the number of effector cells needed to lyse 30% of target cells × 100.

Target Visualization Assay (TVA)

Target cells were incubated with TVA^{TM} dye at 37 ˚C for 15 mins and then cultured with effector cells for 4 hours. Afterwards, the target cells were counted with ImmunoSpot® S6 universal analyzer/software (Cellular Technology Limited, OH, USA) at 525 nm emission wavelengths. The percentage specific cytotoxicity was calculated as follows:

$$\%\ \text{cytotoxicity} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Total cpm} - \text{Spontaneous cpm}} \times 100\%$$

LU30/10^7 cells was calculated using the inverse of the number of effector cells needed to lyse 30% of target cells × 1000.

Enzyme-Linked Immunoosorbent Assay (ELISA) and Luminex multiplex assays

Human ELISA kits for IFN-γ (BioLegend, San Diego, USA) and CD137 (Thermo Fisher Scientific, MA, USA) were used to detect the level of IFN-γ and CD137 produced from cell cultures, respectively. 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 washed 4 times with wash buffer (0.05% Tween20 in PBS), the coated plates were blocked with 1%BAS/PBS for an hour at room temperature on a shaker at 200 rpm. The plates then were washed again with wash buffer for 4 times and 100 µl of prepared standards or samples were added to the wells and incubated for 2 hours at room temperature on a plate shaker at 200 rpm. After incubation, the plates were washed 4 times with wash buffer and 100 µl of detection antibody was added to each well and the plates were incubated at room temperature for 1 hours on a shaker at 200 rpm. The plates were washed 4 times with wash buffer and were loaded with Avidin-HRP solution and incubated for 30 minutes at room

temperature on the plate shaker at 200 rpm. After washing the time for 5 times with wash buffer, 100 µl of TMB solution was added to each well and the plates were incubated in dark at room temperature until they developed a desired blue color (or up to 15 minutes). Then, 100 µl of stop solution (2N H_2SO_4) was added to each well to stop the reaction. Finally, the plates were read at 450 nm to obtain absorbance values.

The levels of cytokines and chemokines were examined by multiplex assay, which was conducted as described in manufacturer's protocol for each specific kit. The quantitate signal were detected using a Luminex multiplex instrument, MAGPIX (Millipore, Billerica, MA, USA) and data was analyze using the proprietary software, xPONENT 4.2 (Millipore, Billerica, MA, USA).

Enzyme-linked immunospot (ELISpot) assay

The ELISpot were conducted according to manufacturer's instructions. Briefly, the plate was coated with primary antibody overnight at 4˚C. After washing the plate with PBS twice, desirable cell number were added into each well (40,000 cells/well for PBMCs and NK and monocyte co-culture experiments; 50,000 cells/well for primary and super-charged NK cells and CD8+ T cells) and incubate at 37˚C for 16-18 hours. The plate was washed with PBS and wash buffer twice after the incubation period and detection antibody was added into each well and incubated at room temperature for 2 hours. After the incubation period, the plate was washed three times with wash buffer (0.05% Tween20/PBS). Tertiary solution was added into each well and the plate was incubated at room temperature in dark for 30 minutes. The plate was washed twice with wash buffer and twice with DI water before the blue development solution was added into each

well and was incubated for 15 minutes in dark at room temperature. The reaction was stopped by gently rinsing the pate with water for 3 times. And the plate was air-dried for 24 hours before being read. The number of IFN-γ secreting cells was analyzed using Human IFN-γ Single-Color Enzymatic ELISPOT Assay, ImmunoSpot® S6 UNIVERSAL analyzer and ImmunoSpot® SOFTWARE (all CTL Europe GmbH, Bohn, Germany).

OSCSC differentiation assay

OSCSCs were treated with 100 µl of supernatants collected from NK and monocyte crisscross coculture of cancer patients and healthy individuals on Day 0 and Day 1. On Day 2, OSCSCs were harvested and surface expression of CD44, CD54, B7H1 and MHC class I were assessed using flow cytometry to determine the differentiation status of OSCSCs by the coculture supernatants.

IsoPlexis single cell secretome analysis

Polyfunctional secretome of primary and super-charged NK cells was assessed using CodePlex single-cell cytokine profiling 32-plex (IsoPlexis, Branford, CT, USA). NK cells were activated for 24 hours and then added into a single-cell barcoded chip with 32-plex antibody array. After incubating for 16 hours, the signals for specific cytokines were measured and digitalized on single cell basis. Polyfunctionality of the cells is defined as more than one analytes tested found per cell. Polyfunctional strength index (PSI) is the percentage of polyfunctional cells in the total population multiplied by mean fluorescence intensity of specific analytes tested.

Proteomic analysis

Cell were then pelleted and reconstituted in buffer before being denatured by heat. Afterwards, samples were reduced, alkylated, trypsinized and incubated at 37°C overnight. Using Rappsilber's protocol [5], samples were desalted and a peptide bicinchoninic acid assay was performed. Samples were labeled using isobaric TMT tags and normalized to total protein before being pooled and desalted. Finally, the pooled sample was fractionated by high pH reverse phase and desalted before being injected into the Thermo Q Exactive Plus Orbitrap. Raw data was analyzed via Proteome Discoverer 2.2. Protein-protein associations were identified using STRING analysis.

Oral and pancreatic tumor implantation in humanized BLT mice model

Animal research was performed under the written approval of the UCLA Animal Research Committee (ARC)(protocol #2012-101-13) in accordance to all federal, state, and local guidelines. Combined immune- deficient NOD.CB17-Prkdcscid/J and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG mice lacking T, B, and natural killer cells) were purchased from Jackson Laboratory and maintained in the animal facilities at UCLA in accordance with protocols approved by the UCLA animal research committee. Humanized-BLT (hu-BLT; human bone marrow/liver/thymus) mice were prepared on NSG background as previously described [6].

OSCSCs or MP2 (0.5×10^6 cells) were injected with 10 7µl HC Matrigel (Corning, NY, USA) into the oral cavity (the floor of the mouth) or the pancreas. Following the tumor implantation, the condition of the mice was monitored every day. Mice are euthanized when they start to show the signs of morbidity, such as loss of weight, ruffled fur, hunched posture, and immobility [3, 7].

Cell dissociation, isolation and cell culture of tissues from hu-BLT mice

Cell dissociation and single cell suspension preparation were performed as described previously [3, 7]. Biefly, oral and pancreatic tumor cells were harvested from hu-BLT mice and cut into 1 mm^3 pieces and are used for co-culture experiments or are put with digestion buffer (1mg/ml collagenase II or IV, 10 U/ml DNAse I, and 1% bovine serum albumin (BAS) in DMEM medium for 20 min at 37°C for single cell suspension preparation. The samples were then filtered through a 40 mm cell strainer and centrifuged at 1500 rpm for 10 min at 4°C. To obtain single cell suspensions from bone marrow, femurs were flushed using medium and filtered through a 40 µm cell strainer. Spleens were harvested and single cell suspensions were prepared and filtered through a 40 µm cell strainer and centrifuged at 1500 rpm for 15 min at 4°C. The pellets were re-suspended in ACK buffer to remove the red blood cells. Blood were collected through heart puncher and peripheral blood mononuclear cells (PBMCs) were isolated using ficoll-hypaque centrifugation.

Single cells suspension from bone marrow, spleen, peripheral blood were used in the experiment or were used in isolation for specific cell types, such as human CD3+ selection kit and CD14 + isolation kits, followed manufacturer's protocol.

Statistical Analysis

A paired or unpaired, two-tailed Student t-test was performed for the statistical analysis using Prism-8 software (Graphpad Prism, CA, USA). The following symbols represent the levels of statistical significance within each analysis,*(p-value<0.01-0.05), **(p-value<0.01-0.001), ***(p-value<0.001).

Reference

1. Tseng, H.C., et al., *Induction of Split Anergy Conditions Natural Killer Cells to Promote Differentiation of Stem Cells through Cell-Cell Contact and Secreted Factors.* Front Immunol, 2014. **5**: p. 269.
2. Tseng, H.C., et al., *Increased lysis of stem cells but not their differentiated cells by natural killer cells; de-differentiation or reprogramming activates NK cells.* PLoS One, 2010. **5**(7): p. e11590.
3. Kaur, K., et al., *Probiotic-Treated Super-Charged NK Cells Efficiently Clear Poorly Differentiated Pancreatic Tumors in Hu-BLT Mice.* Cancers (Basel), 2019. **12**(1).
4. Kaur, K., et al., *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.* Front Immunol, 2017. **8**: p. 297.

5. Rappsilber, J., M. Mann, and Y. Ishihama, *Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips.* Nat Protoc, 2007. **2**(8): p. 1896-906.

6. Wege, A., et al., *Functional and phenotypic characterization of the humanized BLT mouse model*, in *Humanized Mice*. 2008, Springer. p. 149-165.

7. Kaur, K., et al., *Super-charged NK cells inhibit growth and progression of stem-like/poorly differentiated oral tumors in vivo in humanized BLT mice; effect on tumor differentiation and response to chemotherapeutic drugs.* Oncoimmunology, 2018. **7**(5): p. e1426518.

CHAPTER 1

Defects in CD16 receptor but not Toll-like receptors signaling pathway in NK cells and monocytes in cancer patients

Introduction

Natural Killer (NK) cells are known as the first-line of defense against infections and neoplasia. NK cells have been identified for their cytotoxic activity in the early 1970s [1]. NK cells participate in innate immune mechanisms, and constitute 10% to 15% of human peripheral blood lymphocytes [2]. NK cells express CD56 and CD16, and do not express CD3 on their surface. Based on the surface expressions, two distinct population of NK cells are $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$, having cytotoxic and immunoregulatory properties, respectively [3]. As one of key immune effectors, NK cells exhibit cytotoxic activity due to direct and indirect target recognition [4]. Our previous studies have demonstrated that in addition to their cytotoxic function, NK cells have a significantly influential role in promoting differentiation of cancer stem cells (CSCs) by providing critical signals via secreted cytokines, IFN-γ and TNF-α, as well as direct NK cell-stem cell interaction [5-13]. IFN-γ is an important cytokine produced by NK cells and, plays significant role in anti-tumor immunity [14]. Differentiation of tumors was previously shown directly correlate to the tumor's insensitivity to NK cell mediated cytotoxicity, sensitivity to chemotherapeutic drugs, and also growth inhibition of those tumors [15, 16].

Monocytes appear to be recruited in tumors, and found to play an important role in cancer prevention and metastatic spread of cancer [17-21]. Human monocytes express HLADR, CD11b, CD86, and in late 1980s monocytes have been classified is 3 subsets based on CD14 and CD16 surface expression; classical monocytes $CD14^{bright}$ $CD16^{dim}$, non-classical monocytes $CD14^{dim}$ $CD16^{bright}$, and intermediate monocytes $CD14^{bright}$ $CD16^{bright}$ [22, 23]. Non-classical monocytes have been demonstrated to play role in recruitment of NK cells to tumors [18, 24, 25]. Monocyte-derived IL-15 signaling found to be required for cytotoxic NK cells recruitment to tumors [24]. We also have previously demonstrated that monocytes synergize with NK cells in the presence of probiotic bacteria to induce CSCs differentiation [10].

In our previous study, we demonstrated that NK cells' function, and expansion were suppressed in mice at pre-neoplastic and neoplastic stage as well in cancer patients [26-32]. Cancer patients found to express lower PBMC proliferation and production of IFN-γ and TNF-a [33]. NK cells exhibit several important activating and inhibitory surface receptors, including CD16, and the balance between activating and inhibitory signals which NK cells receive through these surface receptors determines NK cells' functional fate [34-36]. CD16 plays role in activating antibody-dependent cellular cytotoxicity (ADCC) activity in NK cells, and is a major FcγR on NK cells [37-40]. In cancer patients, impairment of NK cells' CD16 surface expression and function was demonstrated [41-44]. Studies have also shown that NK cells associated with tumor microenvironment (TME) are unresponsive to CD16 receptor stimulation, resulting in diminished NK cell-mediated cytotoxicity against tumors [45]. In addition, NK cells' effector function of IFN-γ and TNF-a secretion as well NK cells' percentages were found to be impairment in association with tumor-associated monocytes/macrophages [46].

In this chapter, we investigated the activation of NK cells by monocytes of cancer patients and healthy individuals and demonstrated that there is defect in CD16 but not Toll-like receptor signaling pathway in cancer patient's NK cells and monocytes.

Result

Sub-aim 1: Differences of cell type composition of PBMC and cytokine/chemokine profile in the sera between cancer patients and healthy individuals

Higher the percentage of monocyte and lower of B cells in PBMC and decreased in IFN-γ and other cytokines and chemokines in sera from cancer patients when compared to those from healthy individuals.

To understand the composition differences of PBMCs between cancer patients and healthy individuals, flow cytometric analysis were performed to determine the proportion of each cell type. The average percentage of NK cells is higher in cancer patients but there is no statistical significance (**Fig. S1A**). There is no difference in total number of T cells (CD3+)(**Fig. 1B**) between cancer patients and healthy individuals. However, PBMCs obtained from cancer patients showed lower percentage of B cells (CD19+)(**Fig. S1C**) and higher percentage of monocytes (CD14+)(**Fig. S1D**) cells when compared to those from healthy individuals.

To evaluate cytokine and chemokine profile of sera in cancer patients, sera were collected from cancer patients and healthy individuals and multiplex Luminex assays were performed to determine the cytokine and chemokine profile. Among 21 cytokines and chemokines tested, the levels of IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-7, IL-12p70 and IL-13 are lower than those from healthy individuals (**Fig. S1E-S1J**).

Sub-aim 2: Suppression of NK cell functions in PBMCs from cancer patients when treated with the combination of IL-2 and anti-CD16 mAb

Suppression of NK cell mediated cytotoxicity, decreased in IFN-γ secreting cells and decreased in IFN-γ secretion in PBMCs from cancer patients when treated with the combination of IL-2 and anti-CD16 monoclonal antibody

Next, to investigate the functions of PBMCs, both PBMCs from cancer patients and healthy individuals were left untreated, treated with IL-2 alone or treated with IL-2 in combination of anti-CD16 mAb, anti-CD3/28 antibody or sonicated probiotic bacteria (sAJ2) overnight before being used in functional assays. To evaluate NK cell mediated cytotoxicity, 4-hour ^{51}Chromium release assay was conducted using oral squamous carcinoma stem cells (OSCSCs) as target cells. In one representative experiment, overall, PBMCs from cancer patients showed lower cytotoxicity against OSCSCs in all the treated groups (**Fig. 1A**). When compiled experiments, PBMCs from cancer patients treated with the combination of IL-2 and anti-CD16 mAb demonstrated less NK cell mediated cytotoxicity than those from healthy individuals' (**Fig. 1D**).

To assess IFN-γ secretion levels of PBMCs, both ELISpot and ELISA were used for single cell basis and total population manners, respectively. PBMCs from cancer patients had lower spot counts in all treatment groups when compared to those from healthy individuals except for untreated group (**Figs. 1B** and **1E**). In ELISA, PBMCs from cancer patients exhibited lower IFN-γ secretion in all the treated groups (**Fig. 1C**). Meanwhile, the compiled data revealed that PBMCs from cancer patients exhibited lower IFN-γ secretion when treated with IL-2 and the combination of IL-2 and anti-CD16 mAb (**Figs. 1D** and **1F**). However, there is no difference in IFN-γ secretion between PBMCs obtained from cancer patients and healthy individuals in the treatment of IL-2 and anti-CD3/28 antibody or sAJ2 (**Fig. 1F**). Together, these observations indicate that although PBMCs obtained from cancer patients contain more monocytes, their functions of activating the NK cell mediated cytotoxicity and IFN-γ secretion of PBMCs are less in the presence of the combination of IL-2 and the crosslink of CD16 receptor in comparison to those from healthy individuals.

Sub-aim 3: Monocytes from cancer patients does not increase IFN-γ secretion of NK cells when treated with IL-2 and anti-CD16 mAb, however, they do activate and increase IFN-γ secretion fo NK cells with IL-2 and the presence of sonicated AJ2

Increased of cytotoxicity of NK cells from healthy individuals by autologous but not cancer patients' monocytes with IL-2 and CD16 receptor crosslinking

Our findings then prompted us to investigate the interaction of the only two cell types in the PBMC that express CD16 on their cell surfaces, NK cells and monocytes. To explore the

relationship of NK cells and monocytes, they were isolated from both cancer patients and healthy individuals' PBMCs and treated with IL-2 alone or IL-2 in combination of anti-CD16 mAb or sAJ2. NK cells were cultured with either autologous or allogeneic monocytes at 1:1 ratio overnight before NK cell functions were measured. One representative experiment is shown in **Fig. 2A-2C** and compiled experiments are shown in **Fig. 2D-2E**. In general, NK cells from healthy individuals have higher cytotoxicity than those from cancer patients and the cytotoxicity is increased when co-cultured with monocytes from healthy individuals while the same observation was not seen when co-cultured with monocytes from cancer patients (**Fig. 2A-2F**).

When treated with IL-2, cytotoxicity of NK cells from both cancer patient and healthy individual was decreased when co-cultured with monocytes obtained from cancer patient. There is significant higher cytotoxicity of NK cells from healthy individual than those from cancer patient when co-cultured with healthy monocytes than when co-cultured with patient's monocytes. Even when co-culture with autologous monocytes, patient's NK cells still has lower cytotoxicity than NK cells from healthy individuals (**Fig. 2A**). n the compiled data, there is no statistical significance of cytotoxicity between NK cells from cancer patients and healthy individuals in all culture condition (**Fig. 2D**).

A significant higher cytotoxicity of NK cells from healthy individuals was observed across all culture groups within the treatment of IL-2 and anti-CD16 mAb. Notably, when co-cultured with autologous monocytes, the cytotoxicity of NK cells from healthy individual is higher when compared to those from cancer patients (**Fig. 2B**). When the experiments are compiled, the only difference was seen when healthy individuals' NK cells were co-cultured with autologous

monocytes (**Fig. 2E**). These data suggested that with the treatment of anti-CD16 mAb, overall cytotoxicity of NK cells from cancer patients was decreased and their monocytes also failed to activate the cytotoxicity of either autologous or allogeneic NK cells.

However, when treated with the combination of IL-2 and sAJ2, the cytotoxicity function of both cancer patients' and healthy individuals' NK cells was ameliorated, the only differences were seen when comparing NK cell with autologous monocytes and when both are co-cultured with monocytes from healthy individual. In either condition, cytotoxicity was increased when co-cultured with monocytes from healthy individual in the representative experiment (**Fig. 2C**). In the compiled data, no differences were observed across all culture groups (**Fig. 2F**). Taken together, these results demonstrate that the cytotoxic function of NK cells of healthy individuals is only enhanced by autologous monocytes but not by those from cancer patients with the presence of anti-CD16 mAb. And the same observation was not seen in NK cells from cancer patients. Meanwhile, NK cell mediated cytotoxicity of both cancer patients and healthy individuals were increased when treated with the combination of IL-2 and sAJ2.

Increased IFN-γ secretion of healthy individuals' NK cells when co-cultured with either autologous or allogeneic monocytes treated with the combination of IL-2 and/or anti-CD16 mAb

Other than cytotoxicity, one of the most critical functions of NK cells is IFN-γ secretion, especially in cancer immunotherapy, since IFN-γ had previously shown to be responsible for differentiate cancer stem cells (CSCs) and limiting the tumor growth as well as sensitizing CSCs

to chemotherapeutic therapies [30]. ELISpot assays were performed to determine the IFN-γ secretion in single cell producing manner. In **Figures 3A** and **3B**, NK cells and monocytes from both cancer patients and healthy individuals were isolated, co-cultured and treated with IL-2. Monocytes from healthy individuals showed significantly increased number of IFN-γ producing NK cells from cancer patients when compared to those cultured alone or co-cultured with monocytes from cancer patients, whereas, monocytes from cancer patients did not increase the IFN-γ producing cell numbers of cancer patient NK cells. On the other hand, for NK cells obtained from heathy donors, the numbers of IFN-γ producing NK cells were both increased either co-cultured with autologous or allogeneic monocytes (**Figs. 3A** and **3B**). Notably, when NK cells were co-cultured with autologous monocytes, healthy individuals exhibited significant higher number of IFN-γ producing cells (**Fig. 3B**).

When treated with the combination of IL-2 and anti-CD16 mAb, the number of IFN-γ producing cells of NK cells from cancer patients only increased by monocytes from healthy individuals but not by those from cancer patients (**Figs. 3A** and **3C**). However, the IFN-γ producing cell number of NK cells obtained from healthy individuals were increased by both monocytes from cancer patients and healthy individuals (**Figs. 3A** and **3C**). Lastly, when treated with the combination of IL-2 and sAJ2, there is no differences among all culture groups (**Figs. 3A** and **3D**), which might be due to the profound effect of sAJ2 on activating NK cells' IFN-γ production in general as shown in **Figure 3E**. These data indicate that when treated with the combination of IL-2 and anti-CD16 mAb, cancer patients' monocytes do not promote the secretion of NK cells from either cancer patients or healthy individuals as healthy monocytes do. Moreover, when treated with IL-2 and sAJ2, there is no difference seen all the treated groups which is possibly attributed to the

substantial effect of IL-2 and sAJ2 on increasing the IFN-γ secretion in all co-cultured groups (**Fig. 3E**).

We utilized ELISA and multiplex Luminex assay to evaluate IFN-γ secretion for total population. In ELISA, the level of IFN-γ secretion was measured and presented in the representative experiments (**Figs. 4**). A crisscross co-culture experiments of NK cells and monocytes with three different treatments were conducted as described above. NK cells from healthy individuals showed higher IFN-γ secretion than those from cancer patients when cultured alone or co-cultured with monocytes from healthy individuals, whereas, monocytes from cancer patients did not increase the IFN-γ secretion of NK cells from healthy individuals (**Figs. 4A**). In the combination of IL-2 and anti-CD16 mAb treated groups, there is a general increase in IFN-γ secretion when NK cells are co-cultured with monocytes from healthy individuals, whereas, monocytes from cancer patient did not mediate the same effect (**Figs. 4B**). Also, autologous co-culture of healthy individuals significantly increased IFN-γ secretion when compared to those from cancer patients (**Fig. 4B**). Monocytes obtained from healthy individuals were able to improve the IFN-γ secretion of cancer patients' NK cells more than monocytes from cancer patients to NK cells from healthy individuals (**Fig. 4B**). In brief, monocytes from cancer patients were not able to increase the IFN-γ secretion of NK cells from both cancer patient and healthy individuals with the presence of anti-CD16 mAb.

When the culture is treated with the combination of IL-2 and sAJ2, the IFN-γ secretion was increased in both cancer patient and healthy individual (**Figs. 4C)**. As seen in IL-2 and sAJ2 treated groups, the IFN-γ secretion of NK cells from cancer patient was increased when co-cultured with

monocytes from healthy NK cells when compared to those from cancer patients (**Fig. 4C**). Notably, there is no difference of the IFN-γ secretion when healthy individual's NK cells are co-cultured with monocytes from either cancer patient or healthy individual (**Fig. 4C**). These observations were seen when using multiplex Luminex assay (**Table 1**).

Collectively, these results demonstrated that NK cells from cancer patients showed lower IFN-γ secretion in general and their monocytes are lack of the ability to activate the IFN-γ secretion of NK cells from both cancer patients and healthy individuals in the presence of anti-CD16 mAb. However, with the treatment of IL-2 and sAJ2, monocytes from cancer patients were able to restore their function of activating the IFN-γ secretion level of NK cells from both cancer patients and healthy individuals.

Supernatant from NK cell and monocyte co-culture of cancer patients showed less differentiation effect on tumor cells

As previously established, one of the crucial function of NK cells is to differentiate CSCs leading to slower tumor growth and therefore abrogate tumor metastasis [30, 47]. Here, we performed cancer stem cells (CSCs) differentiation experiment with the supernatants harvested from the NK cell and monocyte co-culture to investigate the differentiation ability of the secreted factors of NK cells from cancer patients and healthy individuals with the activation of monocytes. CD54 and MHC class I have been identified as surface markers for differentiation status. The increase of CD54 and MHC class I correlates to the differentiation status of tumor cells [16, 48].

Overall, supernatants from NK cells of healthy individual had higher induction of CD54 (**Fig. 5A**) and MHC class I (**Fig. 5B**). In IL-2 and anti-CD16 mAb treated group, it is obvious that the supernatants from patient autologous coculture had the least induction of CD54 and MHC class I and those from healthy individual autologous had the highest level of induction. In IL-2 and sonicated AJ2 treatment groups, the supernatants from both cancer patients and healthy individuals were able to induce high levels of CD54 and MHC class I expression when compared to IL-2 treated groups (**Figure 5A** and **5B**). Together with ELISA and ELISpot results, the limited in differentiation effect on CSCs by cancer patients' NK cells, especially when co-cultured with autologous monocytes with the presence of anti-CD16 mAb, is likely the corollary of the lack of IFN-γ secretion.

Down-regulation of CD16 expression on monocytes obtained from cancer patients

To further understand the mechanism contributes to the observation mentioned above, receptors on surface of monocytes were measured using flow cytometry. Monocytes freshly isolated from peripheral blood of cancer patients and healthy individuals and the expression of CD16 were analyzed within CD14+ population (**Fig. 6A**). There is no difference in the percentage of CD16 positive monocytes from cancer patients and healthy individuals (**Fig. 6B**), however, monocytes from cancer patients showed significantly lower level of mean channel fluorescence (MFI) of CD16 when compared to those from healthy individuals (**Fig. 6C**), which indicates that the expression of CD16 is lower on the surfaces of monocytes from cancer patients.

Discussion

Previous studies from our laboratory and those of others have shown defects in NK function in cancer patients, however, the underlying mechanisms for such defects has not been clearly delineated, nor is it known whether other immune effectors that are known to activate NK cells are also defective in cancer patients. Therefore, in this paper, we show that NK cell cytotoxicity and induction of IFN-γ in PBMCs of cancer patients are significantly defective when compared to those obtained from healthy individuals. Although the defect can be seen in most activation signals including those treated with IL-2 only, the combination of IL-2 with anti-CD16 mAb, IL-2 with anti-CD3/28 antibody or IL-2 with sAJ2, the most significant effect was seen in those treated with IL-2 and anti-CD16 mAb. The fewest differences could be seen in those treated with IL-2 and sAJ2. Due to the fact that we had previously seen down-modulation of CD16 on patient NK cells [49] and significant differences in the function of PBMCs when they were activated with IL-2 and anti-CD16 mAb, we undertook studies to understand the underlying mechanisms of the insufficient NK activation. We purified peripheral blood NK cells and studied their functions following co-cultures with autologous and allogeneic monocytes obtained from healthy individuals in the presence of IL-2 and anti-CD16 mAb activation, and compared the effect to those activated with IL-2 and sAJ2. As presented in the results section, the levels of cytotoxicity were decreased when patient or healthy NK cells were cultured in the presence of patient monocytes, as compared to healthy monocytes in a representative experiment. However, when we compiled the data from several donors, the differences became less significant, but the pattern was maintained in the presence of IL-2 treatment. This could be due to the variability seen between the donors and experimental procedures. Interestingly, treatment with sAJ2, unlike anti-CD16 mAb, maintained higher NK cell cytotoxicity in the patient and healthy NK cell cultures with both patient

and healthy monocytes. Healthy and patient NK cells in the presence of healthy monocytes generally had higher cytotoxicity, when compared to those cultured with patient monocytes and treated with IL-2 or IL-2 with anti-CD16 mAb. However, it is important to note that the experimental conditions were optimized to observe the differences between NK cultures with monocytes and not NK cells alone. Treatment with anti-CD16 mAb, but not sAJ2, induced split anergy in NK cells, leading to decreased cytotoxicity in the presence of increased IFN-γ secretion. Furthermore, sAJ2 treatment maintained or increased cytotoxicity, especially in patients' NK cells with autologous and allogeneic monocytes. This could be due to internalization or shedding of CD16 receptors, when compared to TLR receptors. We have previously hypothesized and shown that decreases in CD16 receptor and inhibition of NK cell cytotoxicity could be a physiological programming for the selection and differentiation of the cells with which NK cells interact [5]. Such physiological conditioning of NK cells are not only important for defense against infections, trauma and other insults, but also after the transformation of NK cells. By increasing differentiation of tumors, this transformation of NK cells can lead to low numbers of tumor cells, and also may induce vulnerability of these NK differentiated tumors to a number of other therapeutic strategies, such as chemotherapy, radiotherapy, checkpoint inhibitors and T cell mediated effects. The negative aspect of such NK conditioning is the potential lack of tumor cell lysing from NK cells.

When cultured with monocytes, NK cells also either decrease, maintain the same level, or in very few cases slightly increase the lysis of tumor cells, however, they always substantially increase the levels of induction and secretion of IFN-γ. Indeed, the only time an increase in NK cytotoxicity by monocytes was seen was when the co-cultures were treated with sAJ2, however

the differences were not statistically significant. Therefore, monocytes are also important effectors in inducing split anergy in NK cells, as a mechanism to drive differentiation in tumor cells.

Our results demonstrate that patient monocytes induced lower levels of IFN-γ spots, as well as secretion, when cultured with autologous and allogeneic NK cells in ELISpot assays. Monocytes from healthy individuals induced higher levels of IFN-γ spots in cultures with both autologous and allogeneic NK cells from patients. Therefore, monocytes obtained from healthy individuals were capable of increasing and restoring the IFN-γ induction of patient NK cells in IL-2 or IL-2 and anti-CD16 mAb treated groups. Therefore, infusing allogeneic monocytes from healthy individuals to cancer patients could pose another strategy to increase patient NK cell function. Interestingly, treatment with sAJ2 was also able to increase and restore IFN-γ induction in patient NK cells cultured with autologous monocytes. On average, the differences between patient NK cells cultured with either healthy or patient monocytes and healthy NK cells cultured with patient and healthy monocytes when treated with sAJ2 were very slight when assessed using the ELISpot assay.

We have previously established that differentiated OSCSC express higher levels of CD54, MHC class I, and B7H1, but lower levels of CD44 [47]. We have also shown that equivalent amounts of IFN-γ secreted from patients have much lower ability to increase differentiation of tumor cells, when compared to those obtained from healthy individuals. Thus, we sought to determine the functional ability of IFN-γ produced by NK cells in the presence and absence of autologous and allogeneic monocytes. Similar to the profiles obtained by ELISpot and ELISA, we saw significantly decreased levels in induction of CD54 and MHC class I by supernatants obtained

from patients, when compared to those from the healthy individuals. The addition of monocytes from healthy individuals to patient NK cells increased the levels of IFN-γ secretion and augmented the levels of differentiation in the OSCSCs. Patient monocytes, when added to healthy NK cells, decreased the levels of differentiation in the tumor cells, when compared to healthy NK cells cultured with autologous monocytes. Therefore, the decreased capacity to differentiate tumors should be regarded as one of the major causes of survival and expansion of poorly differentiated tumors in cancer patients. Furthermore, the restoration of IFN-γ function in patients is going to be indispensable in developing successful treatments for cancer patients. It should be noted that the loss of differentiation is much more severe when co-cultures of NK and monocytes were treated with IL-2 and anti-CD16 mAb, when compared to those treated with IL-2 with sAJ2. Thus, treatment with sAJ2 may partly serve to restore the loss in ability for patient IFN-γ to differentiate tumors. Whether the secreted IFN-γ in patients is complicated by its shed receptors or whether the IFN-γ is bound to an inhibitor to prevent their differentiation function requires future investigation.

We next determined the levels of CD16 receptors on monocytes, and it was found that CD16 receptor expression was significantly decreased on the surface of patient monocytes, when compared to those of healthy individuals. Down-modulation and/or shedding of CD16 receptors from NK cells [49] compounded with the same effects in monocytes may present one of the underlying mechanisms for dysfunction in NK cells. Currently, we are in the process of delineating the role of such down-modulation in the functions of NK cells.

We have previously found that monocytes are one of the major cell types in imparting resistance towards cell death in tumor cells [12], and therefore, increases in monocytes in the

absence of NK or CD8+ T cells in the tumor microenvironment may allow for monocytes to provide resistance to tumor cells as a product of their interaction. Since monocytes also provide increased survival for NK cells and increases in IFN-γ secretion from NK cells, the role of monocytes becomes very complex within the tumor microenvironment. If the number of monocytes rise in patients in the presence of decreased lymphocytes, this may be a troubling sign since the monocytes may end up aiding the tumors in survival instead of helping NK or CD8+ T cells to increase their respective functions. There should be further studies investigating the interaction of these immune cells in the tumor microenvironment to predict clinical outcome.

Reference:

1. Pross, H.F. and M. Jondal, *Cytotoxic lymphocytes from normal donors. A functional marker of human non-T lymphocytes.* Clin Exp Immunol, 1975. **21**(2): p. 226-35.
2. Moretta, L., et al., *Human natural killer cells: origin, receptors, function, and clinical applications.* Int Arch Allergy Immunol, 2014. **164**(4): p. 253-64.
3. Campbell, K.S. and J. Hasegawa, *Natural killer cell biology: an update and future directions.* J Allergy Clin Immunol, 2013. **132**(3): p. 536-544.
4. Vivier, E., et al., *Functions of natural killer cells.* Nat Immunol, 2008. **9**(5): p. 503-10.
5. Tseng, H.-C., et al., *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**: p. 269.
6. Jewett, A., et al., *Natural killer cells as effectors of selection and differentiation of stem cells: Role in resolution of inflammation.* J Immunotoxicol, 2014. **11**(4): p. 297-307.
7. Jewett, A., Y.G. Man, and H.C. Tseng, *Dual functions of natural killer cells in selection and differentiation of stem cells; role in regulation of inflammation and regeneration of tissues.* J Cancer, 2013. **4**(1): p. 12-24.
8. Jewett, A., et al., *Natural killer cells preferentially target cancer stem cells; role of monocytes in protection against NK cell mediated lysis of cancer stem cells.* Curr Drug Deliv, 2012. **9**(1): p. 5-16.

9. Jewett, A. and H.C. Tseng, *Potential rescue, survival and differentiation of cancer stem cells and primary non-transformed stem cells by monocyte-induced split anergy in natural killer cells.* Cancer immunology, immunotherapy : CII, 2012. **61**(2): p. 265-74.

10. Bui, V.T., et al., *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.* Front Immunol, 2015. **6**: p. 576.

11. Jewett, A., Tseng H.C., *Tumor Microenvironment may Shape the Function and Phenotype of NK Cells Through the Induction of Split Anergy and Generation of Regulatory NK Cells*, in *The Tumor Immunoenvironment*, M. Shurin, Viktor Umansky and Anatoli Malyguine, Editor. 2013, Springer. p. 361-384

12. Jewett, A., et al., *Strategies to rescue mesenchymal stem cells (MSCs) and dental pulp stem cells (DPSCs) from NK cell mediated cytotoxicity.* PloS one, 2010. **5**(3): p. e9874.

13. Jewett, A. and H.C. Tseng, *Potential rescue, survival and differentiation of cancer stem cells and primary non-transformed stem cells by monocyte-induced split anergy in natural killer cells.* Cancer Immunol Immunother, 2012. **61**(2): p. 265-74.

14. Ikeda, H., L.J. Old, and R.D. Schreiber, *The roles of IFN gamma in protection against tumor development and cancer immunoediting.* Cytokine Growth Factor Rev, 2002. **13**(2): p. 95-109.

15. Tseng, H.C., et al., *Increased lysis of stem cells but not their differentiated cells by natural killer cells; de-differentiation or reprogramming activates NK cells.* PloS one, 2010. **5**(7): p. e11590.

16. Kozlowska, A.K., et al., *Differentiation by NK cells is a prerequisite for effective targeting of cancer stem cells/poorly differentiated tumors by chemopreventive and chemotherapeutic drugs.* J Cancer, 2017. **8**(4): p. 537-554.

17. Qian, B.Z., et al., *CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis.* Nature, 2011. **475**(7355): p. 222-5.

18. Hanna, R.N., et al., *Patrolling monocytes control tumor metastasis to the lung.* Science, 2015. **350**(6263): p. 985-90.

19. Franklin, R.A., et al., *The cellular and molecular origin of tumor-associated macrophages.* Science, 2014. **344**(6186): p. 921-5.

20. Movahedi, K., et al., *Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes.* Cancer Res, 2010. **70**(14): p. 5728-39.

21. Kitamura, T., et al., *Monocytes Differentiate to Immune Suppressive Precursors of Metastasis-Associated Macrophages in Mouse Models of Metastatic Breast Cancer.* Front Immunol, 2017. **8**: p. 2004.

22. Ziegler-Heitbrock, L., et al., *Nomenclature of monocytes and dendritic cells in blood.* Blood, 2010. **116**(16): p. e74-80.

23. Passlick, B., D. Flieger, and H.W. Ziegler-Heitbrock, *Identification and characterization of a novel monocyte subpopulation in human peripheral blood.* Blood, 1989. **74**(7): p. 2527-34.

24. Kubo, H., et al., *Primary Tumors Limit Metastasis Formation through Induction of IL15-Mediated Cross-Talk between Patrolling Monocytes and NK Cells.* Cancer Immunol Res, 2017. **5**(9): p. 812-820.

25. Plebanek, M.P., et al., *Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche.* Nat Commun, 2017. **8**(1): p. 1319.

26. Jewett, A., et al., *Natural Killer Cells: Diverse Functions in Tumor Immunity and Defects in Pre-neoplastic and Neoplastic Stages of Tumorigenesis.* Mol Ther Oncolytics, 2020. **16**: p. 41-52.

27. Kaur, K., et al., *Probiotic-Treated Super-Charged NK Cells Efficiently Clear Poorly Differentiated Pancreatic Tumors in Hu-BLT Mice.* Cancers (Basel), 2019. **12**(1).

28. Kaur, K., et al., *Suppression of Gingival NK Cells in Precancerous and Cancerous Stages of Pancreatic Cancer in KC and BLT-Humanized Mice.* Front Immunol, 2017. **8**: p. 1606.

29. Kaur, K., et al., *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.* Front Immunol, 2017. **8**: p. 297.

30. Kaur, K., et al., *Super-charged NK cells inhibit growth and progression of stem-like/poorly differentiated oral tumors in vivo in humanized BLT mice; effect on tumor differentiation and response to chemotherapeutic drugs.* Oncoimmunology, 2018. **7**(5): p. e1426518.

31. Kaur, K., et al., *Deficiencies in Natural Killer Cell Numbers, Expansion, and Function at the Pre-Neoplastic Stage of Pancreatic Cancer by KRAS Mutation in the Pancreas of Obese Mice.* Front Immunol, 2018. **9**: p. 1229.

32. Kaur, K., et al., *Osteoclast-expanded super-charged NK-cells preferentially select and expand CD8+ T cells.* Sci Rep, 2020. **10**(1): p. 20363.

33. Evans, C.F., et al., *The effect of colorectal cancer upon host peripheral immune cell function.* Colorectal Dis, 2010. **12**(6): p. 561-9.

34. Pegram, H.J., et al., *Activating and inhibitory receptors of natural killer cells.* Immunol Cell Biol, 2011. **89**(2): p. 216-24.

35. Gogali, F., et al., *CD3(-)CD16(-)CD56(bright) immunoregulatory NK cells are increased in the tumor microenvironment and inversely correlate with advanced stages in patients with papillary thyroid cancer.* Thyroid, 2013. **23**(12): p. 1561-8.

36. Lopez-Cobo, S., et al., *Impaired NK cell recognition of vemurafenib-treated melanoma cells is overcome by simultaneous application of histone deacetylase inhibitors.* Oncoimmunology, 2018. **7**(2): p. e1392426.

37. Yeap, W.H., et al., *CD16 is indispensable for antibody-dependent cellular cytotoxicity by human monocytes.* Scientific Reports, 2016. **6**(1): p. 34310.

38. Bhatnagar, N., et al., *FcgammaRIII (CD16)-mediated ADCC by NK cells is regulated by monocytes and FcgammaRII (CD32).* Eur J Immunol, 2014. **44**(11): p. 3368-79.

39. Oboshi, W., et al., *The influence of NK cell-mediated ADCC: Structure and expression of the CD16 molecule differ among FcgammaRIIIa-V158F genotypes in healthy Japanese subjects.* Hum Immunol, 2016. **77**(2): p. 165-71.

40. Nimmerjahn, F. and J.V. Ravetch, *Fcgamma receptors as regulators of immune responses.* Nat Rev Immunol, 2008. **8**(1): p. 34-47.

41. Kono, K., et al., *Impaired antibody-dependent cellular cytotoxicity mediated by herceptin in patients with gastric cancer.* Cancer Res, 2002. **62**(20): p. 5813-7.

42. Kawaguchi, Y., et al., *Cetuximab induce antibody-dependent cellular cytotoxicity against EGFR-expressing esophageal squamous cell carcinoma.* Int J Cancer, 2007. **120**(4): p. 781-7.

43. Watanabe, M., et al., *NK cell dysfunction with down-regulated CD16 and up-regulated CD56 molecules in patients with esophageal squamous cell carcinoma.* Dis Esophagus, 2010. **23**(8): p. 675-81.

44. Nakajima, T., et al., *Augmentation of antibody-dependent cellular cytotoxicity with defucosylated monoclonal antibodies in patients with GI-tract cancer.* Oncology letters, 2018. **15**(2): p. 2604-2610.

45. .

46. Peng, L.-s., et al., *Tumor-Associated Monocytes/Macrophages Impair NK-Cell Function via TGFβ1 in Human Gastric Cancer.* Cancer Immunology Research, 2017. **5**(3): p. 248-256.

47. Tseng, H.C., N. Cacalano, and A. Jewett, *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): p. 8947-59.

48. Tseng, H.C., et al., *Differential Cytotoxicity but Augmented IFN-gamma Secretion by NK Cells after Interaction with Monocytes from Humans, and Those from Wild Type and Myeloid-Specific COX-2 Knockout Mice.* Front Immunol, 2015. **6**: p. 259.

49. Kaur, K., et al., *ADCC against MICA/B Is Mediated against Differentiated Oral and Pancreatic and Not Stem-Like/Poorly Differentiated Tumors by the NK Cells; Loss in Cancer Patients due to Down-Modulation of CD16 Receptor.* Cancers (Basel), 2021. **13**(2).

Figure legend

Figure 1, PBMCs obtained from cancer patients showed lower NK cell mediated cytotoxicity, less spot counts of IFN-γ and lower IFN-γ secretion.

PBMCs were isolated as described in Materials and Methods from cancer patients and healthy individuals and were left untreated or treated with a combination of IL-2 (1000 U/ml) and anti-CD16 mAb (3 μg/ml), anti-CD3/28 antibody (25 μl/ml) or sAJ2 (PBMC:sAJ2, 1:20) for 18 hours before used in functional assays. NK cell mediated cytotoxicity of PBMCs was determined using standard 4-hour ^{51}Cr release assay against OSCSCs. The lytic units (LU) $30/10^6$ cells were determined using the inverse number of NK cells required to lyse 30% of OSCSCs ×100 (**A** and **D**). The number of cells secreting IFN-γ in the PBMCs were determined as spot counts using ELISpot assay (**B** and **E**). A value of 900 spot counts was assigned when it exceeds the limit of detection as too numerous to count (TNTC) as shown in red dots. Supernatants were harvested from PBMCs to determine IFN-γ secretion using single ELISA (**C** and **F**). A representative experiment is shown in (**A-C**) and data are presented as Mean±SD. Compile data is shown in (**D-E**) and data are presented as Mean±SEM. Student t tests were performed.

Figure 2, Co-cultured with autologous monocytes, but not monocytes from cancer patients, increased the cytotoxicity of NK cells from healthy individuals when treated with the combination of IL-2 and anti-CD16 mAb.

NK cells and monocytes were isolated as described in Material and Methods from cancer patients and healthy individuals. NK cells and monocytes were treated with IL-2 (1000 U/ml) alone (**A** and **D**) or in combination with anti-CD16 mAb (3 μg/ml)(**B** and **E**) or sAJ2 (cells: sAJ2, 1:20)(**C** and **F**). A crisscross NK cells and monocyte experiment was performed. NK cell mediated cytotoxicity

were measured after 18 hours of co-culture using standard 4-hour ^{51}Cr release assay against OSCSCs. The lytic units (LU) $30/10^6$ cells were determined using inverse number of NK cells needed to lyse 30% of target cells OSCSCs ×100. A representative experiment is shown in **(A-C)** and data are presented as Mean±SD. Compile data is shown in **(D-E)** and data are presented as Mean±SEM. Student t tests were performed.

Figure 3, Increased number of IFN-γ secreting NK cells from both cancer patient and healthy individuals when co-cultured with monocytes obtained from healthy individuals when treated IL-2 alone or in combination with anti-CD16 mAb.

NK cells and monocytes were isolated as described in Material and Methods from cancer patients and healthy individuals. NK cells and monocytes were treated with IL-2 (1000 U/ml) alone (**A** and **B**) or in combination with anti-CD16 mAb (3 μg/ml)(**A** and **C**) or sAJ2 (cells: sAJ2, 1:20)(**A** and **D**). A crisscross NK cells and monocyte experiment was performed. After 18 hours of coculture, the number of IFN-γ secreting cells were determined using ELISpot assay as spot counts. The numbers of IFN-γ secreting cells of NK alone cultures across three treatments are re-graphed as shown in (**E**). A representative experiment is shown in **(A)**. Compile data is shown in **(B-E)** and data are presented as Mean±SEM. Student t tests were performed (*, $p<0.05$; **, $P<0.01$; ***, $P<0.001$).

Figure 4, Lower IFN-γ secretion in NK cells from cancer patients when co-cultured with autologous monocytes.

NK cells and monocytes were isolated as described in Material and Methods from cancer patients and healthy individuals. NK cells and monocytes were treated with IL-2 (1000 U/ml) alone (**A**) or

in combination with anti-CD16 mAb (3 μg/ml)(**B**) or sAJ2 (cells: sAJ2, 1:20)(**C**). A crisscross NK cells and monocyte experiment was performed. After 18 hours of coculture, supernatants were harvested and used in single ELISA to measure IFN-γ secretion. A representative experiment is shown and data are presented as Mean±SD.

Figure 5, Supernatants harvested from autologous cancer patient coculture showed less differentiation effect on OSCSCs when compared to those from healthy individuals when treated with IL-2 and anti-CD16 mAb

OSCSC differentiation assay were conducted as described in Material and Method using the supernatants collected from NK cell and monocyte crisscross coculture experiment followed by assessing the expression of CD54 **(A)** and MHC class I **(B)** using flow cytometry to evaluate the induction of differentiation of OSCSCs.

Figure 6, Monocytes obtained from cancer patients exhibited lower level of CD16 on the cell surfaces.

Monocytes were isolated as described in Material and Method from cancer patients and healthy individuals and the surface expression of CD16 within CD14+ population were determined using flow cytometry. One representative experiment is shown in **(A)**. Compiled experiments of % percentage and MFI are as shown in **(B)** and **(C)**, respectively and data are presented as Mean±SD. Student t tests were performed.

Figures

Fig.

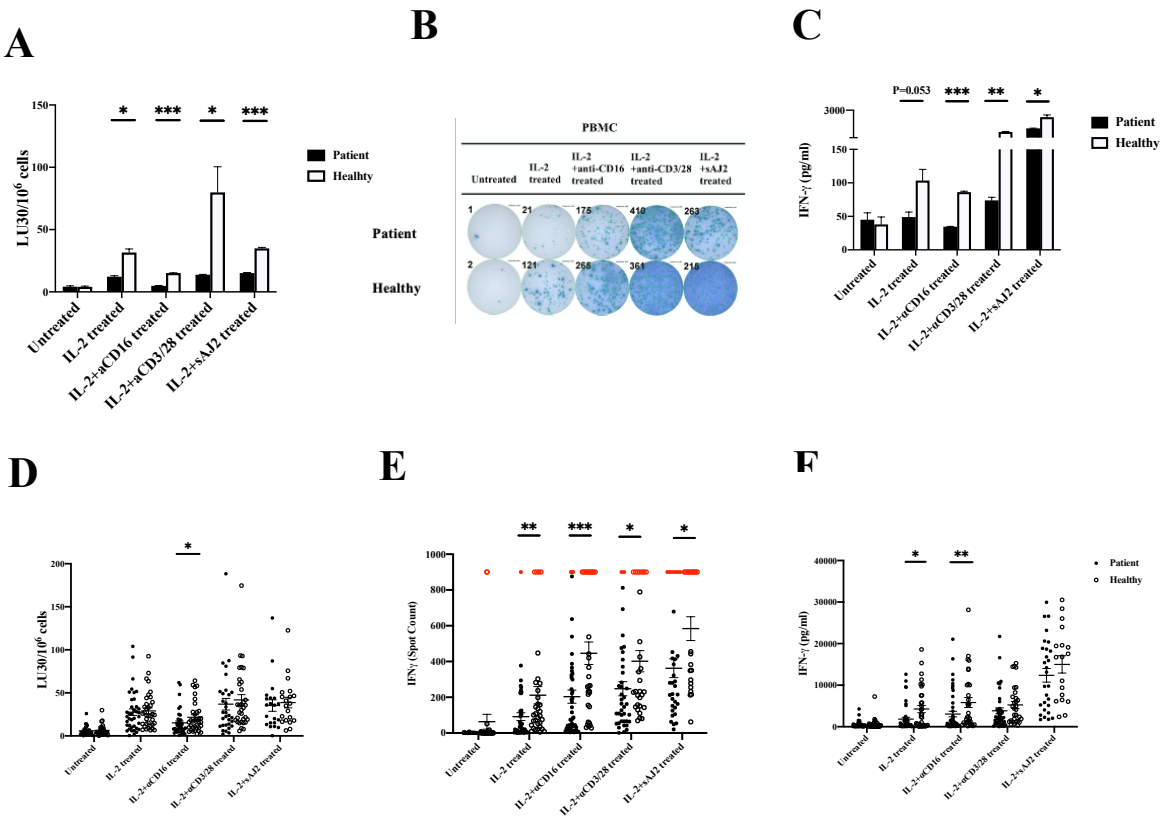

A
B
C
D
E
F
LU30/10^6 cells
Patient
Healthy
Untreated
IL-2 treated
IL-2+αCD16 treated
IL-2+αCD3/28 treated
IL-2+sAJ2 treated
PBMC
IL-2 +anti-CD16 treated
IL-2 +anti-CD3/28 treated
IL-2 +sAJ2 treated
IFN-γ (pg/ml)
P=0.053
IFNγ (Spot Count)

Fig. 2

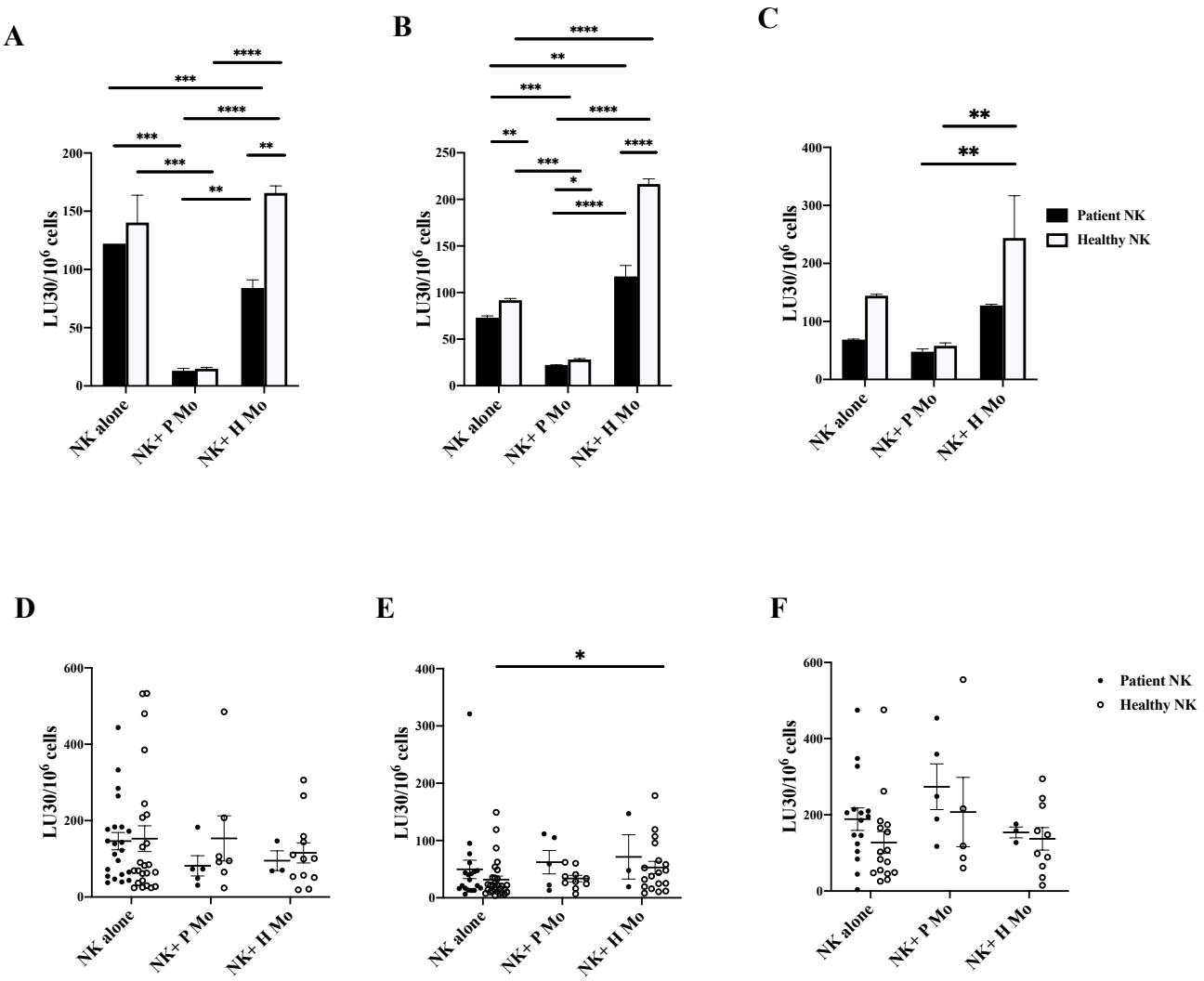

A
B
C
D
E
F
LU30/10^6 cells
NK alone
NK+ P Mo
NK+ H Mo
Patient NK
Healthy NK

Fig. 3

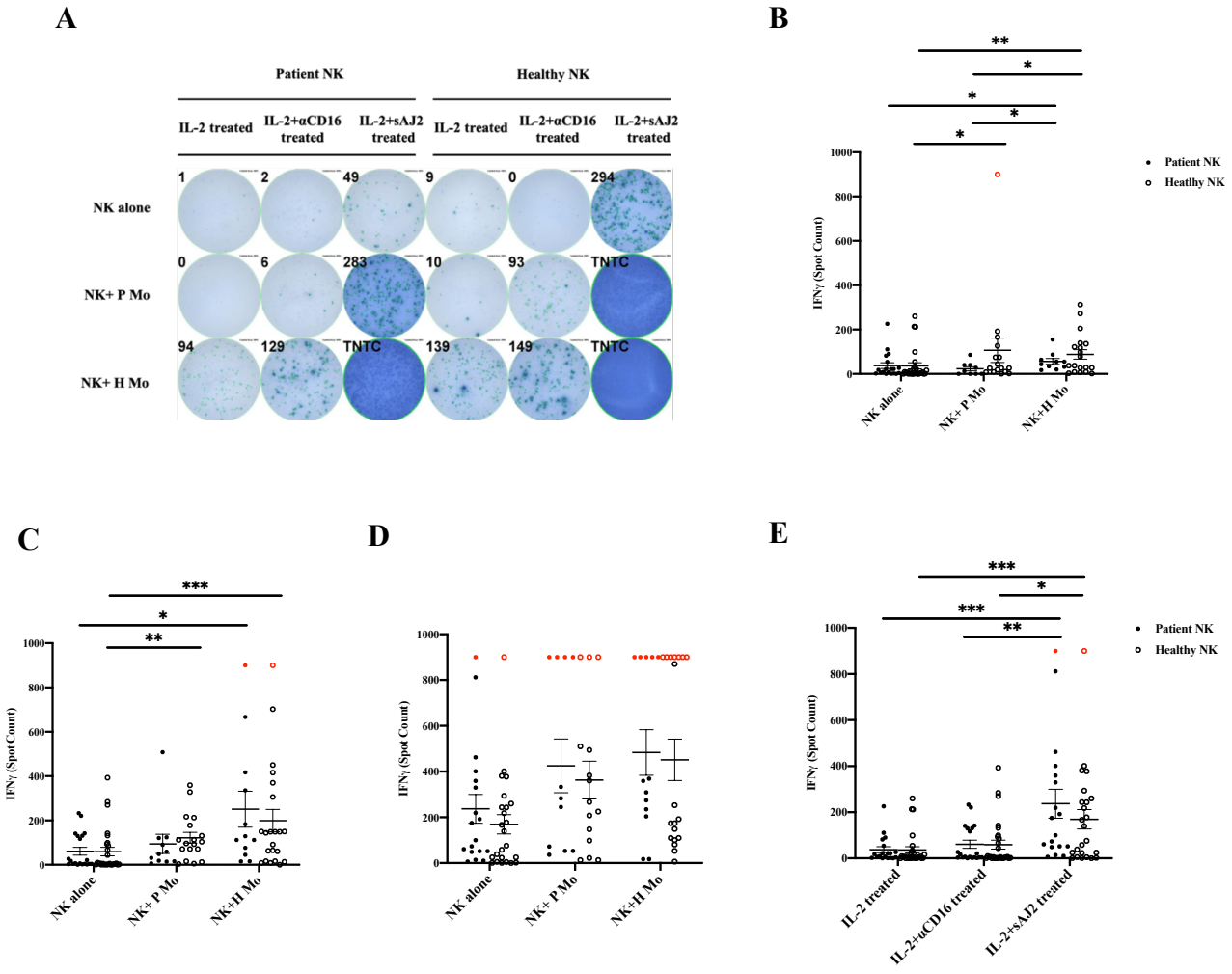
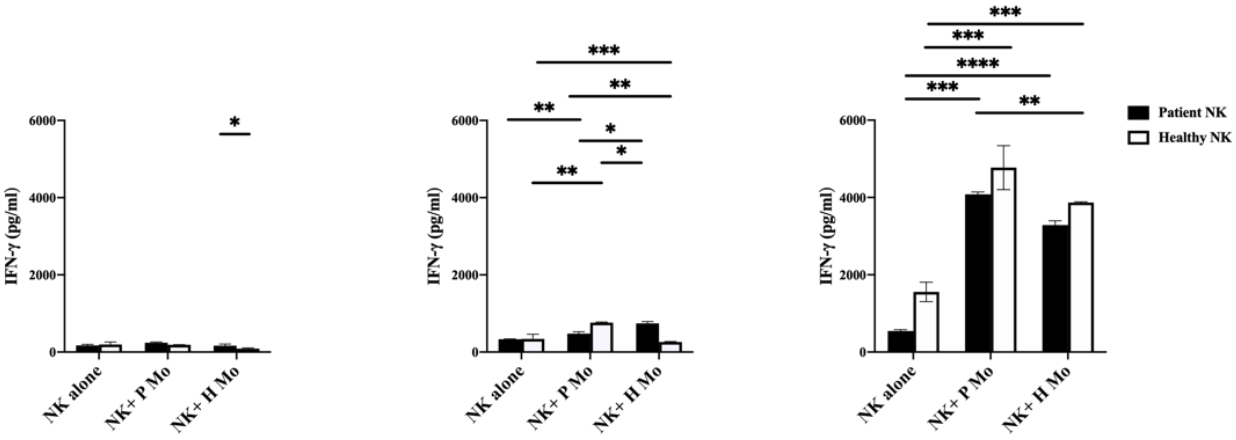
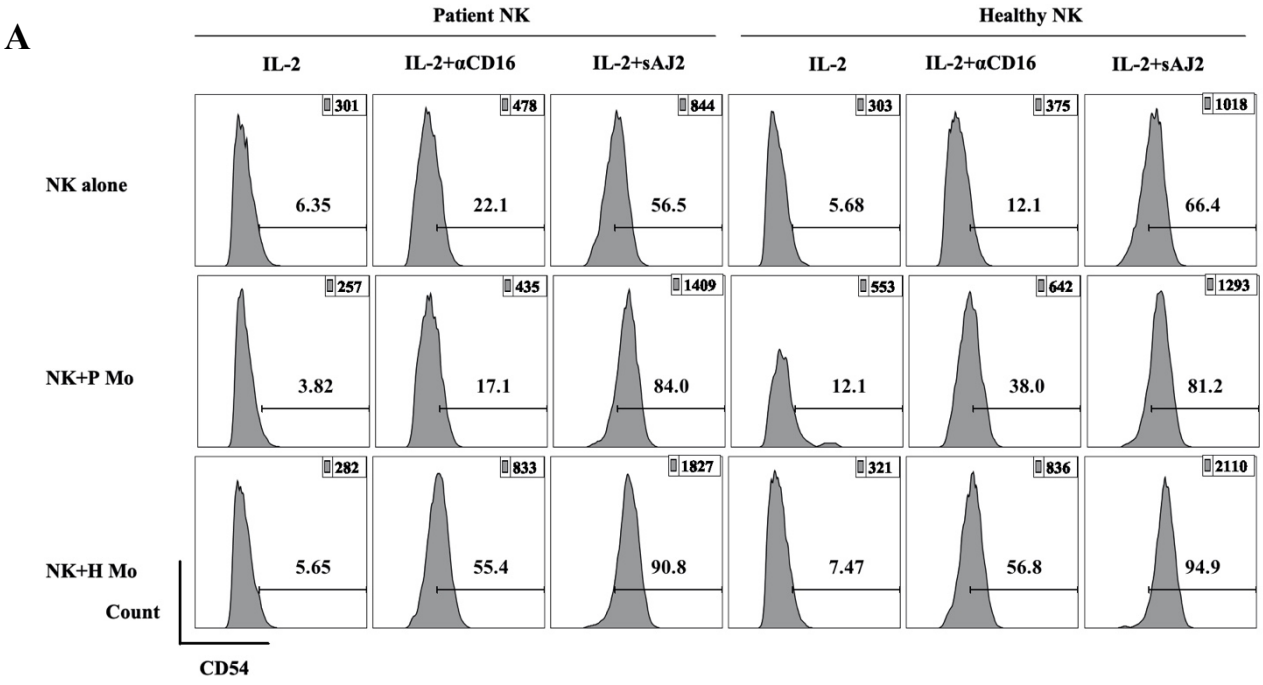

A
Patient NK
Healthy NK
IL-2 treated
IL-2+αCD16 treated
IL-2+sAJ2 treated
IL-2 treated
IL-2+αCD16 treated
IL-2+sAJ2 treated
NK alone
NK+ P Mo
NK+ H Mo
1
2
49
9
0
294
0
6
283
10
93
TNTC
94
129
TNTC
139
149
TNTC
B
IFNγ (Spot Count)
Patient NK
Heatlhy NK
NK alone
NK+ P Mo
NK+H Mo
C
IFNγ (Spot Count)
NK alone
NK+ P Mo
NK+H Mo
D
IFNγ (Spot Count)
NK alone
NK+ P Mo
NK+H Mo
E
IFNγ (Spot Count)
Patient NK
Healthy NK
IL-2 treated
IL-2+αCD16 treated
IL-2+sAJ2 treated

Fig. 4



	Cancer Patient			Healthy donor		
	IL-2 treated	IL-2+αCD16 treated	IL-2+sAJ2 treated	IL-2 treated	IL-2+αCD16 treated	IL-2+sAJ2 treated
NK alone	16.88	18.19	18.19	14.25	17.91	154.23
NK+P monocyte	16.42	19.79	300.06	18.49	23.01	1658
NK+H monocyte	19.44	29.88	1255	23.01	39.11	2516
monocyte alone	6.25	15.12	42.82	17.91	17.37	254.51

Fig. 5


A
Patient NK
Healthy NK
IL-2
IL-2+αCD16
IL-2+sAJ2
IL-2
IL-2+αCD16
IL-2+sAJ2
NK alone
NK+P Mo
NK+H Mo
Count
CD54
301
6.35
478
22.1
844
56.5
303
5.68
375
12.1
1018
66.4
257
3.82
435
17.1
1409
84.0
553
12.1
642
38.0
1293
81.2
282
5.65
833
55.4
1827
90.8
321
7.47
836
56.8
2110
94.9

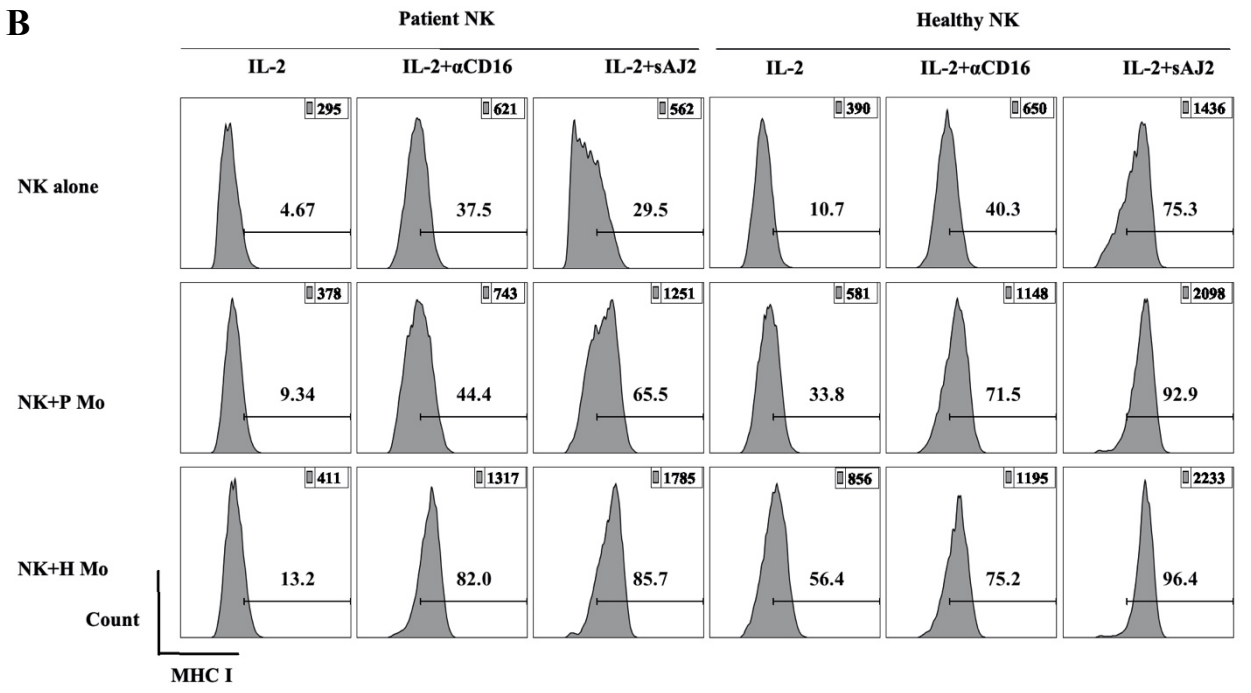
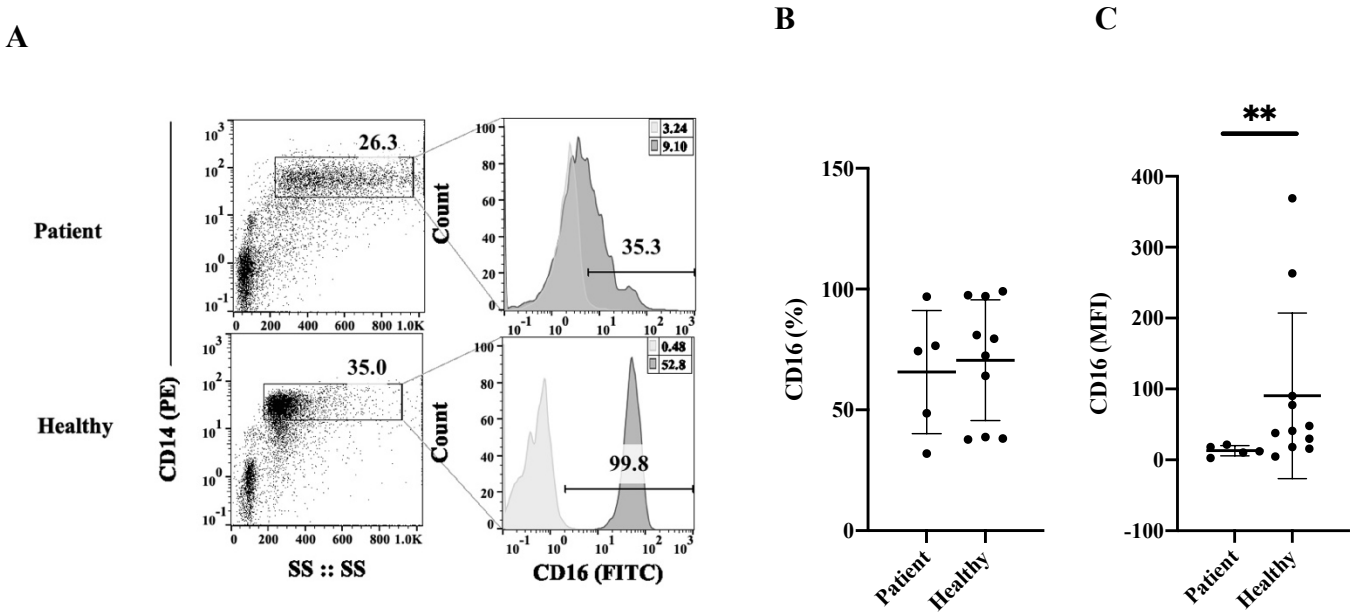

B
Patient NK
Healthy NK
IL-2
IL-2+αCD16
IL-2+sAJ2
IL-2
IL-2+αCD16
IL-2+sAJ2
NK alone
NK+P Mo
NK+H Mo
Count
MHC I
295
4.67
621
37.5
562
29.5
390
10.7
650
40.3
1436
75.3
378
9.34
743
44.4
1251
65.5
581
33.8
1148
71.5
2098
92.9
411
13.2
1317
82.0
1785
85.7
856
56.4
1195
75.2
2233
96.4

Fig. 6


A
B
C
Patient
Healthy
CD14 (PE)
SS :: SS
26.3
35.0
Count
35.3
99.8
3.24
9.10
0.48
52.8
CD16 (FITC)
CD16 (%)
CD16 (MFI)
**
Patient
Healthy

CHAPTER 2

Phenotypic and Functional Differences between Primary and Super-Charged NK cells

Introduction

Natural killer (NK) cells are one of the most essential effector cells in the immune system and are known for their cytotoxicity and regulation of both innate and adaptive immune functions through the release of a variety of pro- and anti-inflammatory cytokines and chemokines [1]. NK cells mediate direct cell lysis in response to the sum of activating and inhibitory signals received upon cell-cell interaction [2]. NK cells also form immune synapses to determine the status of the encountered cells. The loss of the self-identification molecule (MHC class I) on the cell surfaces triggers the NK cell mediated cytotoxic function and ultimately leads to the release of cytotoxic granules containing perforin and granzyme B, resulting in the induction of cell death and lysis of the target cells [3]. In addition to its cytotoxic functions, NK cells release cytokines, such as IFN-γ and IFN-α, which serve to differentiate stem-like cells [4]. Our laboratory has previously identified various surface markers to determine the differentiation status of cells: CD44, CD54, B7H1 (PD-L1) and MHC class I. When stem-like cells are treated with the culture supernatant of NK cells, they are shown to exhibit a more differentiated profile, which features lower levels of CD44, higher levels of CD54, B7H1 and MHC class I [5].

With regards to their cytotoxic and cytokine secretion abilities, NK cells are considered to be essential in shaping the tumor microenvironment and in halting the progression of tumor. They

eliminate cancer stem cells through direct lysis, as well as differentiating malignant cells through cytokine secretion, leading to the curtailment of tumor [6]. Especially for their differentiation effect on tumor cells, which was reported to enhance the sensitive of tumor cells to chemotherapeutic drugs [7]. However, it was previously demonstrated that the functions of NK cells are ineffective in cancer patients as well as during pre-neoplastic and neoplastic stages in mice [8, 9]. NK cells in these circumstances have less cytotoxicity and do not secrete sufficient level of cytokines in the microenvironment. As a whole, these dysfunctions in NK cells are associated with increased cancer risk, higher changes of tumor establishment and metastasis, and poor prognosis for cancer patients.

Due to the defectiveness of NK cell function, immunotherapy that is engineered to improve the function of NK cells in cancer patients is urgently needed. We have previously established a novel strategy to expand NK cells with potent abilities, deeming them “super-charged” NK cells. In this expansion process, primary NK cells are purified from the peripheral blood and treated with IL-2 and anti-CD16 monoclonal antibody (mAb) overnight before their co-culture with the feeder cells, osteoclasts and sonicated bacteria, sAJ2. Upon receiving the signals from cell-cell interaction, surface receptor crosslinking and cytokine stimulation, NK cells were shown to exhibit high proliferation rate, high cytotoxicity, and high cytokine secretion. Furthermore, these cells demonstrated great therapeutic potential in cancer immunotherapy though their ability to limit tumor growth and improve the immune system and efficacy of immunotherapy in oral and pancreatic tumor-bearing humanized BLT mice models [10].

In this chapter, we discuss the phenotypic differences between primary and super-charged NK cells from various aspects, ranging from surface markers to functionalities. To further illustrate the characteristics of super-charged NK cells, proteomics was applied for a broader and more profound understanding that verified the observations seen in the functional assays. Lastly, we investigated the functions of NK cells after they encounter tumor cells to assess how the immune cells sustain their functions in the tumor microenvironment.

Result

Sub-aim 1: Phenotypic differences between primary and super-charged NK cells. Super-charged NK cells

Super-charged NK cells are bigger in size and more polymorphic under the microscope than primary NK cells.

The most noticeable difference between culturing primary and super-charged NK cells is the change in morphology. Primary NK cells are usually rounded, but occasionally, one can see limited amount of irregular elongated cells when activated with IL-2 (**Fig. 1A**). At the beginning of the super-charging process, NK cells are observed to attach to the feeder cells, mature osteoclasts, layering at the bottom of cell culture plate and start to form clumps (**Fig. 1B**). After 5 to 7 days of culturing, there are no feeder cells left in the culture and NK cells are elongated, with these cultured NK cells being more polymorphic and notably bigger in size than primary NK cells (**Fig. 1C**) under the microscope. From then on, super-charged NK cells start to expand in clumps either attach to the plate or float in the culture medium (**Fig. 1D**).

Super-charged NK cells express higher level of proliferation marker, activating and inhibitory receptors, activation markers and granzyme B than primary NK cells, except for CD16 and CD62L

In addition to morphologic changes, super-charged NK cells exhibit a faster proliferation rate than primary NK cells, in terms of cell count fold expansion. A known proliferation marker, Ki-67, was measured through flow cytometry to assess the proliferation rate of primary and super-charged NK cells. It was shown that the percentage of Ki-67 positive population and mean channel fluorescence (MFI) were both significantly higher in super-charged than primary NK cells when compared within the same donors (**Fig. 2A**) or among several donors **(Fig. 2B**).

It is well known that NK cells behave in response to the sum of signals from the activating and inhibitory receptors on its cell surfaces. To further understand the difference between super-charged and primary NK cells, several surface markers were measured using flow cytometry (**Figs. 2C-2J)** and **Table 1**). Either percent positive population or mean channel fluorescence (MFI) showed that inhibitory and activating receptors, including NKG2D, KIR2, NKp44, NKp46, CD54 were found higher in super-charged NK cells than those in primary NK cells (**Figs. 2C-2G**). On the other hand, CD62L was found to be higher in primary than super-charged NK cells (**Figs. 2H**). It is worth noting that when the NK cell identifying receptors, CD56 and CD16, were tested, CD56 was higher in super-charged NK cells while CD16 expression was lower (**Figs. 2I** and **2J**). Intracellular staining of granzyme B, which is one of the most important elements for NK cell in mediating cytotoxicity, also showed that it is more prominent in super-charged NK cells (**Fig. 2K**).

This data showed that, in general, super-charged NK cells have higher surface marker expression and are also in a more active status than primary NK cells.

Sub-aim 2: Functional differences between primary and super-charged NK cells. Super-charged NK cells possess higher cytotoxicity and more prominent cytokine and chemokine secretion ability than primary NK cells.

Super-charged NK cells possess higher direct cytotoxicity than primary NK cells and their cytotoxicity does not decrease after treated with IL-2 and anti-CD16 mAb as primary NK cells.

Super-charged and primary NK cells were treated with IL-2 or a combination of IL-2 and anti-CD16 mAb overnight before use in the ^{51}Chromium release assay for cytotoxicity assessment. Super-charged NK cells exhibited higher cytotoxicity against oral squamous cancer stem cells than primary NK cells in either IL-2 alone (**Fig. 3A**) or IL-2 in combination with anti-CD16 mAb treatment (**Fig. 3B**). As previously reported, the cytotoxicity of NK cells decreases as a result of split anergy induced from the combination of IL-2 and anti-CD16 mAb. The fold decrease in cytotoxicity was calculated to evaluate whether split anergy effects would also be induced in super-charged NK cells as it has been seen in the primary NK cells. As shown in **Fig. 3C**, the decrease in cytotoxicity of super-charged NK is not as prominent as it is in primary NK cells. Taken together, super-charged NK cells mediate greater cytotoxicity against cancer stem cells without a reduced functional proficiency that is seen in primary NK cells after treated with IL-2 and anti-CD16 mAb.

Super-charged NK cells mediate lower levels of antibody dependent cellular cytotoxicity than primary NK cells

We have shown that super-charged NK cells expressed less CD16 on their cell surfaces and the treatment of IL-2 and anti-CD16 mAb does not reduce the direct cytotoxicity of super-charged NK cell. Here, we investigate one crucial function of CD16, antibody dependent cellular cytotoxicity (ADCC) of super-charged NK cells. Both primary and super-charged NK cells used were obtained from the same donor. Oral squamous cancer cells (OSCCs) were treated with MICA/MICB antibodies, cetaximab or left untreated before using in ^{51}Chromium release assay with untreated, IL-2 treated and IL-2 and anti-CD16 mAb treated primary and super-charged NK cells. In untreated and IL-2 treated groups, primary NK cells mediated more cell death in antibody treated OSCCs than untreated tumor cells, whereas the same observation was not seen in IL-2 and anit-CD16 mAb treated group which is because the CD16 receptors on NK cells' surface were blocked before they were co-cultured with tumor cells (**Figs. 4A-4D**). However, the same observation was not seen in super-charged NK cells. Among all three treatment groups, super-charged NK cells did not mediate higher cytotoxicity to antibody-treated tumor cells (**Figs. 4A-4D**). When ADCC-induced fold increases in Lu30/10^6 were calculated in cells, primary NK cells exhibited greater increase than super-charged NK cells (**Fig. 4E**). In sum, super-charged NK cells did not mediate ADCC against anti-MICA/MICB or cetaximab treated OSCCs.

Super-charged NK cells have higher IFN-γ secretion than primary NK cells in both total population and single cell manner.

Besides mediating cytotoxicity against tumor cells, another crucial function for NK cells to halt the progression of cancer is through cytokine secretion. We have previously shown the importance of IFN-γ in differentiating cancer stem cells which can lead to slower tumor growth and sensitization of cancer cells to chemotherapeutic drugs. Therefore, it is essential to assess the IFN-γ secretion of primary and super-charged NK cells. For total population evaluation, the level of IFN-γ were tested in the supernatant of both primary and super-charged NK cells. A greater level of IFN-γ was detected in the supernatant from super-charged NK cells than those from primary NK cells (**Figs. 5A** and **5B**). Then, Elispot was exploited to evaluate the IFN-γ secretion level in single cell manner of these two cell types. The number of IFN-γ secreting cells was substantially higher than primary NK cells in all treatment groups (**Fig. 5C**). Overall, super-charged NK cells showed higher IFN-γ secretion than primary NK cells in total population and single cell manner.

Super-charged NK cells secrete more cytokine and chemokine as well as are more polyfunctional than primary NK cells

Other than IFN-γ, other cytokines and chemokines were determined between primary and super-charged NK cells. CD137 (4-1BB), a co-stimulatory receptor on NK cells was shown to be more abundant in the super-charged NK cell culture supernatant than in that of primary NK cells using ELISA (**Fig. 6**). In addition, we exploited Luminex multiplex to determine the secretion profile of primary and super-charged NK cells. Super-charged NK cells exhibited higher levels of cytokine and chemokine secretion when compared to either IL-2 or IL-2 and anit-CD16 mAb treated primary NK cells. This trend was seen in 13 out of 15 cytokines and 4 out of 5 chemokines

tested, including GM-CSF, IFN-γ TNF-α, Franktalkine, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-21, IL-23, ITAC, MIP-1α and MIP-3α (**Figs. 7A-7Q**, **Table 2**). IL-2 and anti-CD16 mAb treated primary NK cells exhibited higher secretion of IL-1β, IL-6 and MIP-1β than IL-2 treated primary NK cells and super-charged NK cells (**Figs. 7R-7T**).

Other than the cytokine and chemokine secretion profiles in total population, we also investigated the cytokine and chemokine secretion on a single cell basis, using Isoplexis platform to further understand super-charged NK cells' polyfunctionality in secretion, which is defined as the ability for cells to secrete more than one cytokine/chemokine/effector tested. Within the same treatment, super-charged NK cells exhibited more diverse secretions and were shown to be more polyfunctional cell subsets than primary NK cells (**Fig. 8A**). Also, there are higher percentages of the polyfunctional population in super-charged NK cells than in primary NK cells with the treatment of IL-2 and anti-CD16 mAb or sAJ2 (**Fig. 8B**). When polyfunctionality strength index (PSI) was calculated, super-charged NK cells also showed higher levels than primary NK cells, especially, when treated with IL-2 and anti-CD16 mAb (**Fig. 8C**). Collectively, these results suggest that super-charged NK cells possess a more comprehensive capacity in secreting cytokines, chemokines and other functional proteins.

Supernatants obtained from super-charged NK cells induced higher levels of cell death and differentiation in tumor cells than those from primary NK cells.

We have previously shown that the supernatant from primary NK cells induce cell death in cancer stem cells. Thus, our findings led us to investigate the effects of the supernatant from

super-charged NK cells on both oral squamous cancer cells (OSCCs) and their poorly differentiated counterpart, oral squamous cancer stem cells (OSCSCs). Supernatants were collected from cell cultures of both primary and super-charged NK cells overnight after treatment with IL-2 (1000 U/ml). Oral cancer cells were treated with the supernatant at concentrations equivalent to 1×10^6 or 2.5×10^6 cells /ml of NK cells. Cell death and differentiation markers were subsequently evaluated using flow cytometry after 24 or 48 hours of treatment. Supernatants from both primary and super-charged NK cells showed induction of cell death in OSCCs and OSCSCs, however, the effect was shown to be more profound in OSCCS than OSCSCs after 24h (**Fig. 9A**) and 48 hours of treatment (**Fig. 9B**). Moreover, 2.5×10^6 cells/ml of super-charged NK cells culture supernatant induced the highest cell death, and there was a decreasing trend in cell death induction starting from the treatment of 2.5×10^6 cells/ml of super-charged NK cell culture supernatant, followed by 1×10^6 cells/ml of super-charged NK cell culture supernatant, 2.5×10^6 cells/ml and 1×10^6 cells/ml of primary NK culture supernatant. (**Figs. 9A** and **9B**)

In addition to oral tumors, the effects of supernatant from primary and super-charged NK cells on PL12, well differentiated pancreatic cancer cells, and its counterpart, poorly differentiated pancreatic cancer stem cells, MP2, were also tested. Supernatants from NK cells induced cell death in PL12 and MP2; but unlike oral tumors, the cell death induction was more remarkable in MP2 than PL12 cells (**Figs. 9C** and **9D**). Also, the decreasing trend was only seen after 48 hours of treatment (**Figs. 9C** and **9D**). These findings indicate that the supernatant from super-charged NK cells are richer in cytokines/chemokines, along with other functional proteins, which results in a greater induction of cell death in both oral and pancreatic cancer cells than supernatant from primary NK cells.

Apart from cell death induction, differentiation induced by NK cell culture supernatants were assessed in both oral tumor cell lines after 24 and 48 hours of treatment. Levels of CD44, CD54, B7H1 (PD-L1) and MHC class I on the tumor cell surfaces were measured using flow cytometry. After treatment with NK cell supernatant, there was an overall decrease in CD44 (**Fig. 10A**, whereas CD54 (**Fig. 10B**), B7H1 (**Fig. 10C**) and MHC class I (**Fig, 10D**) showed increases in OSCCs and OSCSCs after treatment with either NK cells' supernatants at all concentrations and at both time points. There were greater effects seen in the expression of CD54 and B7H1 in OSCSCs than OSCCs. The same findings were observed in the pancreatic tumor cell lines, PL12 and MP2. In both tumor cell line types, 2.5×10^6 cell/ml of super-charged NK cells' supernatant had the most prominent effect on differentiating tumor cells, most notably on cancer stem cells followed by supernatants from 1×10^6 cell/ml of super-charged NK cells, 2.5×10^6 cell/ml of primary NK and 1×10^6 cell/ml of primary NK cells (**Fig. 11**). Collectively, these data demonstrate that the high abundance of cytokines, chemokines and functional proteins secreted by super-charged NK cells are in concordance with their substantial effects in cell death induction and tumor cell differentiation.

Although OC supernatant with or without sonicated OC mediated NK expansion showed comparable expansion rate and NK purity as standard OC mediated NK expansion, the functionalities of those are not as potent as standard OC-expanded NK cells.

To understand the mechanism which bestows the potent functionalities on NK cells, we modified the protocol for super-charging NK cells by using the sonicated OCs and the supernatant

of osteoclasts cell culture to render supporting molecules and cytokines and chemokines from osteoclasts, respectively. As shown in **Figure 12A** and **12B**, the expansion rate and NK purity of these three culture conditions are comparable with one another. However, the functions of NK cells, both cytotoxicity and IFN-γ secretion, are not as enhanced when co-cultured with sonicated osteoclasts and/or the supernatant of osteoclasts cell culture (**Fig. 12C-12E**). These results indicate that albeit it might be sufficient to expand NK cells with sonicated OCs and/or the cytokines and chemokines secreted by OCs, to reach the greatest optimization of NK cell functions, osteoclasts are required as feeder cells.

Sub-aim 3: Proteomics Analysis of primary and super-charged NK cells.

Alteration of protein abundances in primary and super-charged NK cells and protein-protein association using string analysis

To grasp a deeper understanding of how super-charged NK cells are superior to primary NK cells, we took a step further and investigated their respective changes in protein expression levels. Super-charged NK cells are generated as described in the Materials and Methods. Primary NK cells were purified freshly from peripheral blood of healthy donors. After treatment with IL-2 (1000 U/ml), protein abundance was measured in primary and super-charged NK cells. Fold changes in the protein abundance of super-charged to primary NK cells across donors or within the same donors were calculated for measured proteins. When compared across different donors, there were 90 proteins that were higher and 21 that were lower in super-charged NK cells (**Fig.**

13A). On the other hand, 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 (**Fig. 13B**).

Only proteins that either increased or decreased in expression within super-charged NK cells from both cross donors and same donor datasets were selected for further analysis. Protein-protein interactions between these proteins were analyzed using STRING analysis. Of all 43 increased proteins (n=43), these were mostly involved in cell cycle processes (7/43), cellular metabolic processes (29/43) and immune system processes (14/43) (**Fig. 14A**). On the other hand, proteins whose expression decreased in super-charged NK cells (n=11) were relevant to regulated exocytosis (7/11), regulation of immune system processes (6/11) and actin cytoskeletons (6/11) (**Fig. 14B**).

Changes in protein abundance correspond to the phenotypic and functional differences observed in primary and super-charged NK cells

Here, proteins with abundance alterations between primary and super-charged NK cells within the same donors were selected and categorized accordingly to their biological function and further analyzed. For cell proliferation, minichromosome maintenance protein complex (MCM) is known to be essential for genomic DNA replication, which was found mostly higher in super-charged NK cells, but not in primary NK cells (**Fig. 15A**). Ki-67 expression was also increased in super-charged NK cells (**Fig. 15A**), which corresponds to the flow cytometric analysis described earlier (**Figs. 2A and 2B**). These results verified the observation seen in the cell culture process

that super-charged NK cells possess a higher proliferation rate than primary NK cells which ultimately leads to profound expansion in cell numbers.

Twelve cell surface clusters of differentiation (CDs) were also detected in the proteomics analysis. Seven of them are more abundant in super-charged NK cells than primary NK cells, and these CDs are known to be important in a variety of functions such as: host defense and immune response, co-stimulatory molecules (CD2 and CD48), cell activation, growth and motility (CD53 and CD59), and in migration, adhesion and cell-cell interactions (CD44, CD63, CD97). CD36 was the only protein detected with higher abundance in primary cells rather than in super-charged NK cells (**Fig. 15B**). Although the exact activation makers were not measured using flow cytometry, these data still support the concept of the higher activation status of super-charged NK cells.

In virtue of the substantial cytotoxic ability of super-charged NK cells, proteins known to be essential in the cytotoxicity were analyzed. Granzyme A, granzyme B, and granulysin, which are known as effector molecules in mediating cytotoxicity in granule exocytosis, were found to be more abundant in super-charged NK cells (**Fig. 15C**). Unexpectedly, cathepsin C, a key enzyme to activate granzyme B was found in lesser quantities in super-charged NK cells. Meanwhile, cystatin F (CST7), a cathepsin C-directed protease inhibitor, was also found to be elevated in super-charged NK cells (**Fig. 15D**). To address this inconsistent observation, protein kinetic assay of granzyme B was performed, in which the results showed that super-charged NK cells exhibited greater enzyme activity (data not shown). More studies still are needed to evaluate the biological activity or localization of cathepsin C and cystatin F for further clarification.

Given the evidence of high activation status in super-charged NK cells through activation makers, cytotoxicity and cytokine/chemokine profile as described above, important cytokine receptor downstream signaling pathways, specifically signal transducer and activator of transcription (STAT), were analyzed. In super-charged NK cells, there was shown to be elevated expression of STAT1 and STAT4, which are known to be crucial regulators of IFN-γ production and NK cell mediated cytotoxicity (**Fig. 15C**). In conclusion, proteomics analysis strongly supports the findings of phenotypic and functional differences between super-charged and primary NK cells.

Sub-aim 4: Unlike primary NK cells, the functions of super-charged NK cells are not suppressed after encountering tumor.

Super-charged NK cells showed less inactivation in cytotoxicity after co-cultured with tumor tissue than primary NK cells.

The cytotoxicity of untreated and IL-2 treated primary NK cells and super-charged NK cells were measured after co-cultured with oral tumor derived from humanized BLT mice or single cells of OSCSCs using for ^{51}Chromium release assay. Percent killing to effector-target ratio (E:T ratio), lytic unit 30/10^6 cells (Lu30/10^6 cells) were presented. In general, super-charged NK cells showed higher cytotoxicity when compared to either untreated or IL-2 treated primary NK cells (**Figs. 16A-16F**). Untreated primary NK cells showed diminished cytotoxicity after co-cultured with tumor tissue and OSCSCs (**Figs. 16A** and **16D**). For IL-2 treated primary NK cells, less cytotoxicity was seen when co-cultured with both tumor tissue and OSCSCs. Moreover, the

percent killing descended more drastically along with E:T ratio when co-cultured with tumor tissue than with OSCSCs (**Figs. 16B** and **16E**). Whereas, there is no difference in cytotoxicity of super-charged NK cells regardless of co-culture conditions (**Fig. 16C** and **16F**). Percent decreased in $Lu30/10^6$ cells were calculated. In both tumor tissue and OSCSC co-culture condition, untreated primary NK cells exhibited the highest in % decrease in lytic unit 30, followed by IL-2 treated primary NK cells and super-charged NK cells (**Fig. 16G** and **16H**). In brief, super-charged NK cells possess the highest cytotoxicity among the three groups of NK cells tested and their cytotoxicity is not inactivated after encountering tumor tissue or single cell culture as seen in primary NK cells.

Increased IFN-γ secretion in IL-2 treated primary and super-charged NK cells after co-cultured with tumor but not in untreated primary NK cells

In addition to cytotoxicity, IFN-γ secretion of NK cells is also evaluated after co-cultured with tumor tissue and OSCSCs using Elispot and ELISA. Untreated primary NK cells had the least IFN-γ secreting cells among all three types of NK cells with or without tumor co-culture (**Figs. 17A** and **17B**). There is an increase in IFN-γ secreting cells of IL-2 treated primary NK cells when co-cultured with OSCSCs when compared to those co-cultured with tumor tissue or without co-cultured (**Figs. 17A** and **17C**). Meanwhile, super-charged NK cells had the most IFN-γ secreting cells without any co-culture condition when compared with untreated or IL-2 treated primary NK cells. Also, numbers of IFN-γ secreting cells increased after co-cultured with tumor tissue or OSCSCs (**Figs. 17A** and **17D**).

Collectively, these results showed that primary NK cells lose their cytotoxicity and increase their IFN-γ secretion after encountering tumor tissue or OSCSCs, which supports the concept of split anergy which was successfully demonstrated in vitro using the treatment of IL-2 and anti-CD16 mAb. More importantly, it is noteworthy that super-charged NK cells not only did not lose their cytotoxicity but also exhibited greater IFN-γ secretion after co-cultured with tumor. Taken together, these data demonstrated that super-charged NK cells are not inactivated through tumor cells as primary NK cells.

Discussion

The primary function of the cytoskeleton is to give the cell its shape, mechanical resistance to deformation and are important in cell physiology. The alternation of cytoskeleton abundance in cells changes cell morphology, motility and deformability. In this chapter, proteomics analysis revealed that there was a decrease of several cytoskeleton associated proteins in the super-charged NK cells, specifically vinculin (VCL), filamin A (FLNA), zyxin (ZYX). It has been shown that the decrease of vinculin (VCL) changed the cell morphology and enhanced cell spreading and growth in clumps [11, 12]. Gelsolin (GSN) and filamin A (FLNA) are known to be associated with the deformability of the cells [13, 14]. Zyxin-depleted cells showed spindle shape-like morphology [15]. Moreover, an increase in other cytoskeleton associated proteins, like stathmin (STMN1), which is a protein that prevents and promotes disassembly of microtubules, was also observed [16]. The variation of these proteins in super-charged NK cells from primary NK cells could be expected to alter cell morphology and behavior, which coincide with the elongation and irregular shape of super-charged NK cells and aggregation growth pattern observed in the culture. Aside from morphology and growth patterns, the modulation of cytoskeleton proteins also contributes to cell deformability, an essential process for cell proliferation. For example, overexpression of stathmin has been shown to facilitate cell proliferation in tumor cells [16], and filamin A is known to negatively regulate the cell proliferation in lung tumor cells [17]. The increase in stathmin and decrease in filamin A are in agreement with the fast proliferation rate seen in super-charged NK cells. Along with the increase of proteins associated with deformability contributing by the down-modulation of cytoskeleton, there are other standard markers for cell proliferation were also elevated, such as proliferating cell nuclear antigen (PCNA), Ki-67 and minichromosome

maintenance (MCM). Of which, the increase of Ki-67 was confirmed with flow cytometric analysis. These observations tally with the fast proliferation rate of super-charged NK cells reported previously [10] and the capability of rapid expansion bestow large scale production of super-charged NK cells for cancer immunotherapy.

There is evidence supporting that super-charged NK cells are more active in general than primary NK cells. First, NK cells functions are governed by the interplay between activating and inhibitory receptors [18]. Both inhibitory and activating receptors are found elevated in super-charged NK cells, including NKG2D, KIR2, NKp44, NKp46 and CD54 (ICAM-1) along with the decrease of CD62L, which is commonly observed in activated immune cells [19, 20]. Secondly, in the proteomic analysis, super-charged NK cells showed more abundance of proteins involved in cell metabolism. What's more, signal transducer and activator of transcription 4 (STAT4) was also shown higher in the super-charged NK cells, which is known to increase the cytotoxicity through the binding to the perforin gene promoter as well as cytokine secretion ability in response to IL-12 activation [21-23]. Lastly, it has been previously established that CD16 and CD56 are two surface receptors to distinguish subsets of NK cells: $CD16^{+}CD56^{dim}$ as cytotoxic NK cells and $CD16^{-}CD56^{bright}$ as regulatory NK cells [24, 25]. It is worth noting that super-charged NK cells exhibited higher CD56 and lower CD16 when compared to primary NK cells, which is similar to the tissue-associated NK cell profile or split anergy induced with the combination of IL-2 and anti-CD16 mAb *in vitro*, which have higher cytokine secretion ability and less cytotoxic function [1, 24].

Even though super-charged NK cells showed $CD56^{bright}CD16^{dim}$ profile, which is supposed to behave similarly to split anergy NK cells, both flow cytometry and proteomic analysis revealed that super-charged NK cells contained higher level of granzyme B. Indeed, in chromium release assay, when activated with IL-2, super-charged NK cells exhibited much more profound cytotoxicity against stem-like oral tumor cells (oral squamous cancer stem cells, OSCSCs) when compared to primary NK cells. Interestingly, when attempting to induce split anergy in both primary and super-charged NK cells with the combination of IL-2 and anti-CD16 mAb, only primary NK cells showed the decrease in the cytotoxicity. Super-charged NK cells, on the other hand, still possess significant cytotoxic function against OSCSCs. Yet, proteomic analysis revealed that aside from granzyme B, cystatin F is also elevated in super-charged NK cells. Cystatin F is known to be indispensable in regulating NK cell mediated direct killing via perforin/granzyme B pathway through inhibiting the activity of cathepsin C, a convertase for granzyme B [26]. Unlike our findings here with elevated cystatin F in the super-charged NK cells, it was reported that increased cystatin F in the split anergized NK cells is responsible for the decrease of cytotoxic function [27]. It is possible that the detected cystatin F are not being transported into nucleus, where the regulatory function takes place, due to its glycosylation status [28]. However, due to the sample processing, it is hard to determine if the cystatin F seen in the super-charged NK cells is monomer or dimer and its glycosylation status. Further study on the both structure and activity of cystatin F needs to be explored by western blot or other kinetics assays to have a deeper understanding of the mechanisms behind the potent cytotoxic function of super-charged NK cells.

In addition to cytotoxicity, NK cells shape tumor microenvironment through cytokine and chemokine secretion in order to communicate with other immune cells as well as mediate differentiation of cancer stem cells, especially through secreted and membrane-bounded IFN-γ and TNF-α, respectively [1, 29-31]. Most importantly, as previously established, differentiation of cancer cells enhances the sensitivity of these cells to chemo- and radiotherapy [7, 32]. In both bulk and single cell analysis, super-charged NK cells exhibited greater IFN-γ secretion than primary NK cells. Moreover, multiplex analysis discovered that super-charged NK cells possess a more diverse secretion profile and single cell profiling demonstrated that super-charged NK cells are more polyfunctional than primary NK cells. Positively, in the functional assays, with the same number of NK cells to tumor cells, supernatants from super-charged NK cells were able to induce higher level of differentiation to both pancreatic and oral stem-like cancer cells, which indicates that the application of super-charged NK cells in the cancer immunotherapy is essential to lay the foundation for the success of chemo- and radiotherapy. Furthermore, supernatants from super-charged NK cells induced higher levels of cell death in both pancreatic and oral tumor cells, which might result from the high levels of IFN-γ, TNF-α , or soluble FasL, which are known as mechanisms that NK cells mediate cell lysis [33, 34].

In regard to the superb functions of super-charged NK cells, they were used as immunotherapy in tumor bearing humanized-BLT mice. In both oral and pancreatic tumor model, tumor derived from mice received super-charged NK cells showed limited growth *ex vivo*. Additionally, the treatment of super-charged NK cell injection enhances the frequency and the functions of CD8+ T cells as well as immune function of the mice in general, which illustrated the promising potential of super-charged NK cells in cancer immunotherapy [35, 36]. However, it has

been suggested that the functions of NK cells are inactivated after encountering tumor cells [37, 38]. Therefore, functionality of super-charged NK cells after they come across target cells was evaluated. Interestingly, the cytotoxic function of super-charged NK cells remained the same despite co-cultured with tumor tissue or tumor cell culture while primary NK cells exhibited a major decrease in both scenarios. As mentioned earlier, the inactivation in cytotoxicity might be as a consequence of split anergy induction where NK cells lose their cytotoxic function but gain great secretion ability [29]. Due to the induction of split anergy is mediated by IL-2 and anti-CD16 mAb, it is possible that super-charged NK cells are unable to become split anergized as their CD16 was decreased on the cell surfaces, consequently no decrease in cytotoxicity after meeting target cells. Collectively, the abilities of super-charged NK cells, such as great expansion, superior cytotoxic function and cytokine secretion ability and not being inactivated by tumor cells bestow these cells great potential in immune cancer therapy.

Reference:

1. Jewett, A., Y.G. Man, and H.C. Tseng, *Dual functions of natural killer cells in selection and differentiation of stem cells; role in regulation of inflammation and regeneration of tissues.* J Cancer, 2013. **4**(1): p. 12-24.
2. Kumar, S., *Natural killer cell cytotoxicity and its regulation by inhibitory receptors.* Immunology, 2018. **154**(3): p. 383-393.
3. Clement, M.V., et al., *Involvement of granzyme B and perforin gene expression in the lytic potential of human natural killer cells.* Nouv Rev Fr Hematol, 1990. **32**(5): p. 349-52.
4. Wang, H., et al., *CD56(bright)CD16(-) natural killer cells are shifted toward an IFN-gamma-promoting phenotype with reduced regulatory capacity in osteoarthritis.* Hum Immunol, 2019.
5. Tseng, H.C., et al., *Differential Cytotoxicity but Augmented IFN-gamma Secretion by NK Cells after Interaction with Monocytes from Humans, and Those from Wild Type and Myeloid-Specific COX-2 Knockout Mice.* Front Immunol, 2015. **6**: p. 259.
6. Kaur, K., et al., *Natural killer cells target and differentiate cancer stem-like cells/undifferentiated tumors: strategies to optimize their growth and expansion for effective cancer immunotherapy.* Curr Opin Immunol, 2018. **51**: p. 170-180.
7. Kozlowska, A.K., et al., *Differentiation by NK cells is a prerequisite for effective targeting of cancer stem cells/poorly differentiated tumors by chemopreventive and chemotherapeutic drugs.* J Cancer, 2017. **8**(4): p. 537-554.
8. Kaur, K., et al., *Suppression of Gingival NK Cells in Precancerous and Cancerous Stages of Pancreatic Cancer in KC and BLT-Humanized Mice.* Front Immunol, 2017. **8**: p. 1606.

9. Jewett, A., et al., *Natural Killer Cells: Diverse Functions in Tumor Immunity and Defects in Pre-neoplastic and Neoplastic Stages of Tumorigenesis.* Mol Ther Oncolytics, 2020. **16**: p. 41-52.

10. Kaur, K., et al., *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.* Front Immunol, 2017. **8**: p. 297.

11. Coll, J.L., et al., *Targeted disruption of vinculin genes in F9 and embryonic stem cells changes cell morphology, adhesion, and locomotion.* Proc Natl Acad Sci U S A, 1995. **92**(20): p. 9161-5.

12. Rodriguez Fernandez, J.L., et al., *Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells.* J Cell Biol, 1993. **122**(6): p. 1285-94.

13. Kwon, H.J., et al., *Suppression of morphological transformation by radicicol is accompanied by enhanced gelsolin expression.* Oncogene, 1997. **15**(21): p. 2625-31.

14. Cunningham, C.C., et al., *Actin-binding protein requirement for cortical stability and efficient locomotion.* Science, 1992. **255**(5042): p. 325-7.

15. Mise, N., et al., *Zyxin is a transforming growth factor-beta (TGF-beta)/Smad3 target gene that regulates lung cancer cell motility via integrin alpha5beta1.* J Biol Chem, 2012. **287**(37): p. 31393-405.

16. Cassimeris, L., *The oncoprotein 18/stathmin family of microtubule destabilizers.* Curr Opin Cell Biol, 2002. **14**(1): p. 18-24.

17. Zhang, Y., et al., *FLNa negatively regulated proliferation and metastasis in lung adenocarcinoma A549 cells via suppression of EGFR.* Acta Biochim Biophys Sin (Shanghai), 2018. **50**(2): p. 164-170.

18. Pegram, H.J., et al., *Activating and inhibitory receptors of natural killer cells.* Immunol Cell Biol, 2011. **89**(2): p. 216-24.

19. Lin, S.J., et al., *Decreased intercellular adhesion molecule-1 (CD54) and L-selectin (CD62L) expression on peripheral blood natural killer cells in asthmatic children with acute exacerbation.* Allergy, 2003. **58**(1): p. 67-71.

20. Yang, S., et al., *The shedding of CD62L (L-selectin) regulates the acquisition of lytic activity in human tumor reactive T lymphocytes.* PLoS One, 2011. **6**(7): p. e22560.

21. Gotthardt, D. and V. Sexl, *STATs in NK-Cells: The Good, the Bad, and the Ugly.* Front Immunol, 2016. **7**: p. 694.

22. Thierfelder, W.E., et al., *Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells.* Nature, 1996. **382**(6587): p. 171-4.

23. Yamamoto, K., et al., *The human perforin gene is a direct target of STAT4 activated by IL-12 in NK cells.* Biochem Biophys Res Commun, 2002. **297**(5): p. 1245-52.

24. Vitale, M., et al., *The small subset of CD56brightCD16- natural killer cells is selectively responsible for both cell proliferation and interferon-gamma production upon interaction with dendritic cells.* Eur J Immunol, 2004. **34**(6): p. 1715-22.

25. Poli, A., et al., *CD56bright natural killer (NK) cells: an important NK cell subset.* Immunology, 2009. **126**(4): p. 458-65.

26. Perisic Nanut, M., et al., *Cysteine cathepsins as regulators of the cytotoxicity of NK and T cells.* Front Immunol, 2014. **5**: p. 616.

27. Magister, S., et al., *Regulation of split anergy in natural killer cells by inhibition of cathepsins C and H and cystatin F.* Oncotarget, 2015. **6**(26): p. 22310-27.

28. Colbert, J.D., A. Plechanovova, and C. Watts, *Glycosylation directs targeting and activation of cystatin f from intracellular and extracellular sources.* Traffic, 2009. **10**(4): p. 425-37.

29. Tseng, H.C., et al., *Induction of Split Anergy Conditions Natural Killer Cells to Promote Differentiation of Stem Cells through Cell-Cell Contact and Secreted Factors.* Front Immunol, 2014. **5**: p. 269.

30. Jewett, A., et al., *Natural killer cells as effectors of selection and differentiation of stem cells: role in resolution of inflammation.* J Immunotoxicol, 2014. **11**(4): p. 297-307.

31. Tseng, H.C., N. Cacalano, and A. Jewett, *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): p. 8947-59.

32. Lanzon, C.J., *Strategies to target pancreatic cancer stem cells using natural killer cells and chemotherapeutic drugs*. 2015, University of California, Los Angeles,: Los Angeles. p. 1 online resource (vi, 56 p.

33. Arase, H., N. Arase, and T. Saito, *Fas-mediated cytotoxicity by freshly isolated natural killer cells.* J Exp Med, 1995. **181**(3): p. 1235-8.

34. Wu, A.J., et al., *Interferon-gamma induced cell death in a cultured human salivary gland cell line.* J Cell Physiol, 1996. **167**(2): p. 297-304.

35. Kaur, K., et al., *Super-charged NK cells inhibit growth and progression of stem-like/poorly differentiated oral tumors in vivo in humanized BLT mice; effect on tumor differentiation and response to chemotherapeutic drugs.* Oncoimmunology, 2018. **7**(5): p. e1426518.

36. Kaur, K., et al., *Probiotic-Treated Super-Charged NK Cells Efficiently Clear Poorly Differentiated Pancreatic Tumors in Hu-BLT Mice.* Cancers (Basel), 2019. **12**(1).

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

38. Jewett, A. and H.C. Tseng, *Tumor induced inactivation of natural killer cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells.* J Cancer, 2011. **2**: p. 443-57.

Table 1. Surface receptor expression of super-charged NK cells compared to those of primary NK cells.

Receptors	% Percentage	MFI	Functionality
KIR2	↑	↑	Inhibitory receptor
NKG2D	↑	ns	Activating receptor
NKp44	ns	↑	Activating receptor
NKp46	↑	ns	Activating receptor
CD54	↑	↑	Migration
CD62L	↓	↓	Homing
CD16	↓	↓	Fc receptor
CD56	↑	↑	
Granzyme B	↑	ns	Cytotoxic function

↑: super-charged NK cells significantly higher than primary NK cells.
↓: super-charged NK cells significantly lower than primary NK cells.
ns: no significant difference between super-charged and primary NK cells.

Table 2, Cytokine and chemokine secretion profile of primary and super-charged NK cells using multiplex Luminex

	sNK > pNK		**IL-2+αCD16 mAb pNK > sNK**
Cytokine	GMCSF	IL-10	IL-1β
	IFN-γ	IL-12p70	IL-6
	TNF-α	IL-13	
	Franktalkine	IL-17A	
	IL-4	IL-21	
	IL-5	IL-23	
	IL-7		
Chemokine	IL-8		MIP-1β
	ITAC		
	MIP-1α		
	MIP-3α		

pNK: primary NK cells; sNK: super-charged NK cells

Figure Legend

Figure 1. Morphology of NK cells are changed after the process of super-charging by osteoclasts.

Primary NK cells were freshly isolated from PBMC **(A)**. Multinucleated osteoclasts (OCs) were differentiated from peripheral blood derived monocytes for 21 days with M-CSF and RANK-L. IL-2+anti-CD16 mAb treated primary NK cells are attached to the osteoclasts after overnight co-cultured with OCs **(B)**. After 5 days of co-cultured, OCs can't be seen in the culture and NK cells become bigger, elongated and irregular shaped as well as start to form clumps **(C)**. At the prime expansion period (Day7 and after) for super-charged NK cells, they proliferate in both floating and attaching clumps in the cell culture **(D)**.

Figure 2. Super-charged NK cells exhibited higher proliferation marker, activating and inhibitory receptor and granzyme B than primary NK cells.

Human NK cells were isolated from peripheral blood of healthy donors as described in Material and Methods. Ki-67 expression and other markers and protein of primary and super-charged were measured after 7 days treated with IL-2 (1000 U/ml) using flow cytometry analysis. Comparison of primary and super-charged NK from the same donor **(A)**. Compiled data are shown in (**B**). Other surface markers and protein were also measured on both primary and super-charged NK cells. NKG2D **(C)**, KIR2 **(D)**, NKp44 **(E)**, NKp46 **(F)**, CD54 **(G)**, CD62L **(H)**, CD16 **(I)**, CD56 **(J)**, Granzyme B (GZMB) **(J)**.

Figure 3, Super-charged NK cells showed superior cytotoxicity and less decrease in cytotoxicity when treated with IL-2+αCD16 mAb than primary NK cells. Primary NK cells

were freshly purified from PBMCs. Primary NK cells and super-charged NK cells were treated with IL-2 (**A**) and the combination of IL-2 and αCD16 mAb (**B**) overnight before the cytotoxicity was measured. 4-hour ^{51}Cr release assay was performed to assess the cytotoxicity of both primary and super-charged NK cells. Fold decrease in decrease cells were calculated using $Lu30/10^6$ as shown in (**C**) (n=3)

Figure 4, Primary NK cells mediate higher level of ADCC than expanded NK cells.

NK cells were purified from healthy donors' blood and expanded. After day 15 of expansion, primary NK cells were purified from the same donor (1×10^6 cells/mL) and were left untreated, treated with IL-2 (1,000 U.mL), and the combination of IL-2 (1,000 U/mL) and anit-CD16 mAb (3 μg/mL) for 18 hours. Super-charged NK cells (1×10^6 cells/mL) were also left untreated, treated with IL-2 (1,000 U.mL), and the combination of IL-2 (1,000 U/mL) and anit-CD16 mAb (3 μg/mL) for 18 hours. OSCCs were labeled with ^{51}Cr, and 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 **(B)** and combination of IL-2 and anti-CD16 mAb **(C)** of primary and super-charged NK cells against OSCCs untreated or treated with the antibody against MICA/MICB antibody or Cetaximab was determined using the stander 4 hours ^{51}Cr release assay. (Fig. A-C are representative experiments). The percentage of cytotoxicity for the same experiment is shown in **(D)**. ADCC induced fold increase in IL-2 treated primary and super-charged NK cells were calculated **(E)**.

Figure 5, Super-charged NK cells showed superior of IFN-γ secretion than primary NK cells in total population and single cell manner.

Primary NK cells were freshly purified from PBMCs. Primary NK cells and super-charged NK cells were treated with IL-2 for 48 hours before supernatant was collected. ELISA was used to determine the level of IFN-γ. Bar graph **(A)**, scatter plot **(B)** (n=7). Elispot was performed to determine the relative number of cell secreting IFN-γ shown as spot counts **(C)**. TNTC: too numerous to count

Figure 6, Super-charged NK cells showed superior of soluble CD137 (4-1BB) secretion than primary NK cells.

Primary NK cells were freshly purified from PBMCs. Primary NK cells were treated with IL-2, IL-2 and αCD16 mAb or IL-2 and sAJ2 or being left untreated overnight. Super-charged NK cells were treated with IL-2 overnight. The supernatants were collected and ELISA was used to determine the level of soluble CD137. (n=2)

Figure 7, Super-charged NK cells showed superior cytokines and chemokines secretion than primary NK cells.

Primary NK cells were freshly purified from PBMCs and treated with IL-2 or the combination of IL-2 and anti-CD16 mAb overnight before the supernatants were collected. Supernatant of super-charged NK cells were collected after being treated with IL-2 GM-CSF **(A)**, IFN-γ **(B)**, TNF-α **(C)**, Franktalkine **(D)**, IL-4 **(E)**, IL-5 **(F)**, IL-7 **(G)**, IL-8 **(H),** IL-10 **(I)**, IL-12p70 **(J)**, IL-13 **(K)**, IL-17A **(L)**, IL-21 **(M)** and IL-23 **(N),** ITAC **(O)**, MIP-1α **(P)**, MIP-3α **(Q)**, IL-1β **(R)**, IL-6 **(S)** and MIP-1β **(T)**.

Figure 8, Super-charged NK cells are more polyfunctional than primary NK cells.

Both primary and super-charged NK were treated with IL-2 and anti-CD16 mAb or sAJ2 and PMA before using in the commercial IsoCode Chip to measure the cytokine and chemokine secretion profile in per cell basis. The polyfunctionality of NK cells are defined as 2 or more cytokines or chemokines secreted per cell. The overall profile of %NK cytokine secreting profile is shown as heatmap in **(A)** and as bar graph in **(B)**. Polyfunctional strength index (PSI) is calculated as % polyfunctional cells multiplied by the intensity of the intensities of the secreted cytokines/chemokine **(C)**.

Figure 9, Supernatants from super-charged NK cells induced higher cell death in both oral and pancreatic tumor cells.

Supernatants of both primary and super-charged NK cells (5×10^6 cells/mL) were collected after treated with IL-2 (1000 U/mL) overnight. Both oral **(A, B)** and pancreatic **(C, D)** tumor cells were treated with the supernatants at the quantity equals to 1×10^6 cells/mL or 2.5×10^6 cells/mL for 24 hours **(A, C)** or 48 hours **(B, D)**.

Figure 10, Supernatants from super-charged NK cells induced higher differentiation oral tumor cells.

Supernatants of both primary and super-charged NK cells (5×10^6 cells/mL) were collected after treated with IL-2 (1000 U/mL) overnight. Both OSCCs and OSCSCs were treated with the supernatants at the quantity equals to 1×10^6 cells/mL or 2.5×10^6 cells/mL for 24 hours. CD44 (A), CD54 (B), B7H1 (C) and MHC I (D) on the cell surfaces were measured to evaluate the differentiation of tumor cells using flow cytometry. One representative of three independent experiments is as shown.

Figure 11, Supernatants from super-charged NK cells induced higher differentiation pancreatic tumor cells.

Supernatants of both primary and super-charged NK cells (5×10^6 cells/mL) were collected after treated with IL-2 (1000 U/mL) overnight. Both PL12 and MP2 were treated with the supernatants at the quantity equals to 1×10^6 cells/mL or 2.5×10^6 cells/mL for 24 hours. CD44 (A), CD54 (B), B7H1 (C) and MHC I (D) on the cell surfaces were measured to evaluate the differentiation of tumor cells using flow cytometry. One representative of three independent experiments is as shown.

Figure 12, Super-charged NK cells expanded by OCs as feeder cells bestowed the most prominent functionalities of super-charged NK cells when compared to those expanded by sonicated osteoclasts (sOC) and/or supernatant from OC culture.

NK cells are expanded with OCs, sOC or the combination of sOC with OC cell culture supernatant. The number of cells **(A)** and the NK cell purity **(B)** are monitored during the culture period using microscopy and flow cytometric analysis, respectively. Cytotoxicity **(C)** was measured using 4-hour chromium release assay and percent decrease cytotoxicity compared to OC expanded NK cells were calculated **(D)**. IFN-γ secretion of day6, 10 and 13 was measured using single ELISA **(E).**

Figure 13, Protein abundance difference in primary and super-charged NK cells in volcano plot.

2×10^6 cells of primary and super-charged NK cells were used for proteomic analysis. Red dots the fold change of protein abundance are of super-charged NK cells/primary NK cells is greater than 2 and with statistic differences(n=90**(A)**, 271**(B)**, $p<0.05$) and blue dots indicate the fold change

of protein abundance are of super-charged NK cells/primary NK cells less than 0.5 (n=21 **(A)**, 172 **(B)**, $p<0.05$) with statistically significance ($p<0.05$). The comparison of primary and super-charged NK cells obtained from different donors is as shown in **(A)** and from same donors is as shown in **(B).**

Figure 14, STRING analysis of protein-protein interaction of those are higher or lower in super-charged than primary NK cells.

Proteins with two or more fold higher **(A)** or lower **(B)** in both inter- and intra- donors were selected. (n=43) STRING analysis were applied to analyze the protein-protein interaction and their functional network.

Figure 15, Changes in protein abundance correspond to the phenotypic and functional differences observed in primary and super-charged NK cells.

Proteins with abundance alterations between primary and super-charged NK cells within the same donors were selected and categorized accordingly to their biological function. Paired t test was performed to determine the statistical differences (n=4, *$p<0.05$, **$p<0.01$). Proteins associated with cell proliferation **(A)**, cluster of differentiations (CDs) **(B)**, cytotoxicity **(C)** and signaling molecule **(D)** are as shown.

Figure 16, Super-charged NK cells showed less inactivation in cytotoxicity after co-culturing with tumor than primary NK cells.

Primary NK were freshly isolated from PBMCs and were left untreated or treated with IL-2 (1000 U/ml) overnight before co-culturing with tumor tissue or OSCSCs. ^{51}Chromium release assay were

conducted to determine the cytotoxicity of NK cells. Percent killing to effector-target ratio of untreated **(A)** and IL-2 treated **(B)** primary NK cells and super-charged NK cells **(C)** are as shown. Lytic unit 30 per million cells were calculated to compare the cytotoxicity of untreated **(D)** and IL-2 treated **(E)** primary NK cells and super-charged NK cells **(F)**. Percent decreased in lytic unit 30 were calculated for tumor tissue and OSCSCs co-cultures to evaluate the inactivation effect of tumor tissue **(G)** and OSCSCs **(H)** co-culture on NK cells.

Figure 17, Increased IFN-γ secretion in IL-2 treated primary and super-charged NK cells after co-cultured with tumor but not in untreated primary NK cells.

Primary NK were freshly isolated from PBMCs and were left untreated or treated with IL-2 (1000 U/ml) overnight before co-culturing with tumor tissue or OSCSCs. Elispot and ELISA were conducted to determine the IFN-γ secretion. One set of representative Elispot result is shown in **(A)** and the compiled analysis of untreated **(B)** and IL-2 treated **(C)** primary NK cells and super-charged NK cells **(D)** are as shown.

Figure

Fig. 1

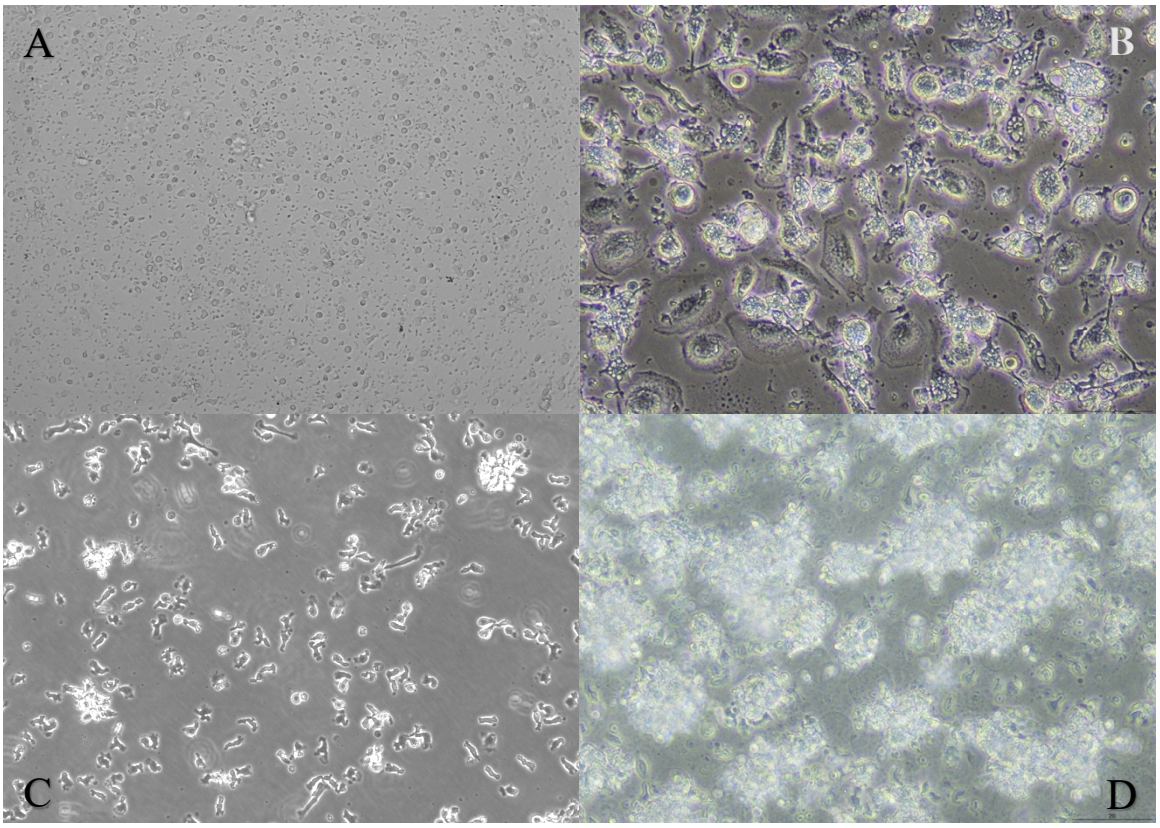


Fig. 2

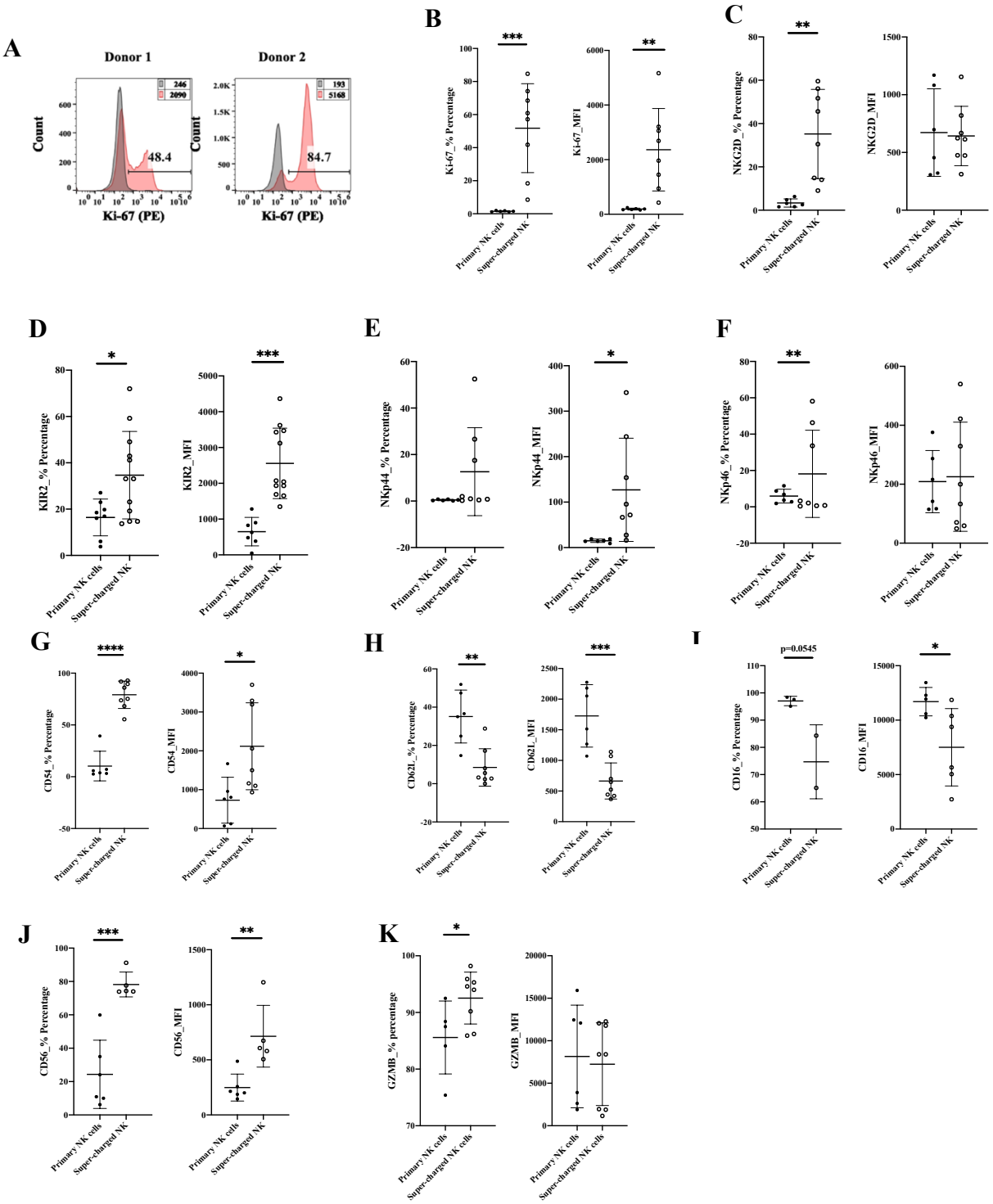

A
Donor 1
Donor 2
Count
Ki-67 (PE)
48.4
84.7
B
Ki-67_% Percentage
Ki-67_MFI
C
NKG2D_% Percentage
NKG2D_MFI
D
KIR2_% Percentage
KIR2_MFI
E
NKp44_% Percentage
NKp44_MFI
F
NKp46_% Percentage
NKp46_MFI
G
CD54_% Percentage
CD54_MFI
H
CD62L_% Percentage
CD62L_MFI
I
p=0.0545
CD16_% Percentage
CD16_MFI
J
CD56_% Percentage
CD56_MFI
K
GZMB_% percentage
GZMB_MFI
Primary NK cells
Super-charged NK
Super-charged NK cells

Fig. 3

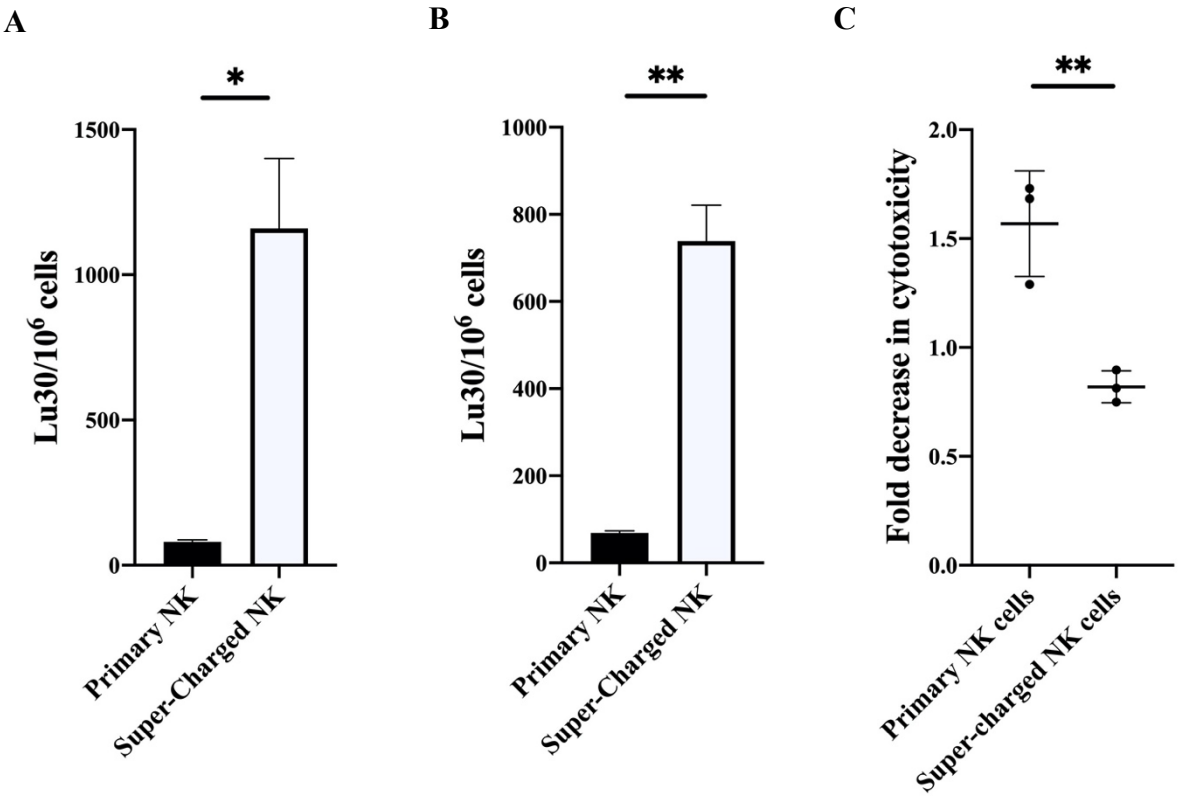

A
*
1500
1000
500
0
Lu30/10^6 cells
Primary NK
Super-Charged NK
B
**
1000
800
600
400
200
0
Lu30/10^6 cells
Primary NK
Super-Charged NK
C
**
2.0
1.5
1.0
0.5
0.0
Fold decrease in cytotoxicity
Primary NK cells
Super-charged NK cells

Fig. 4

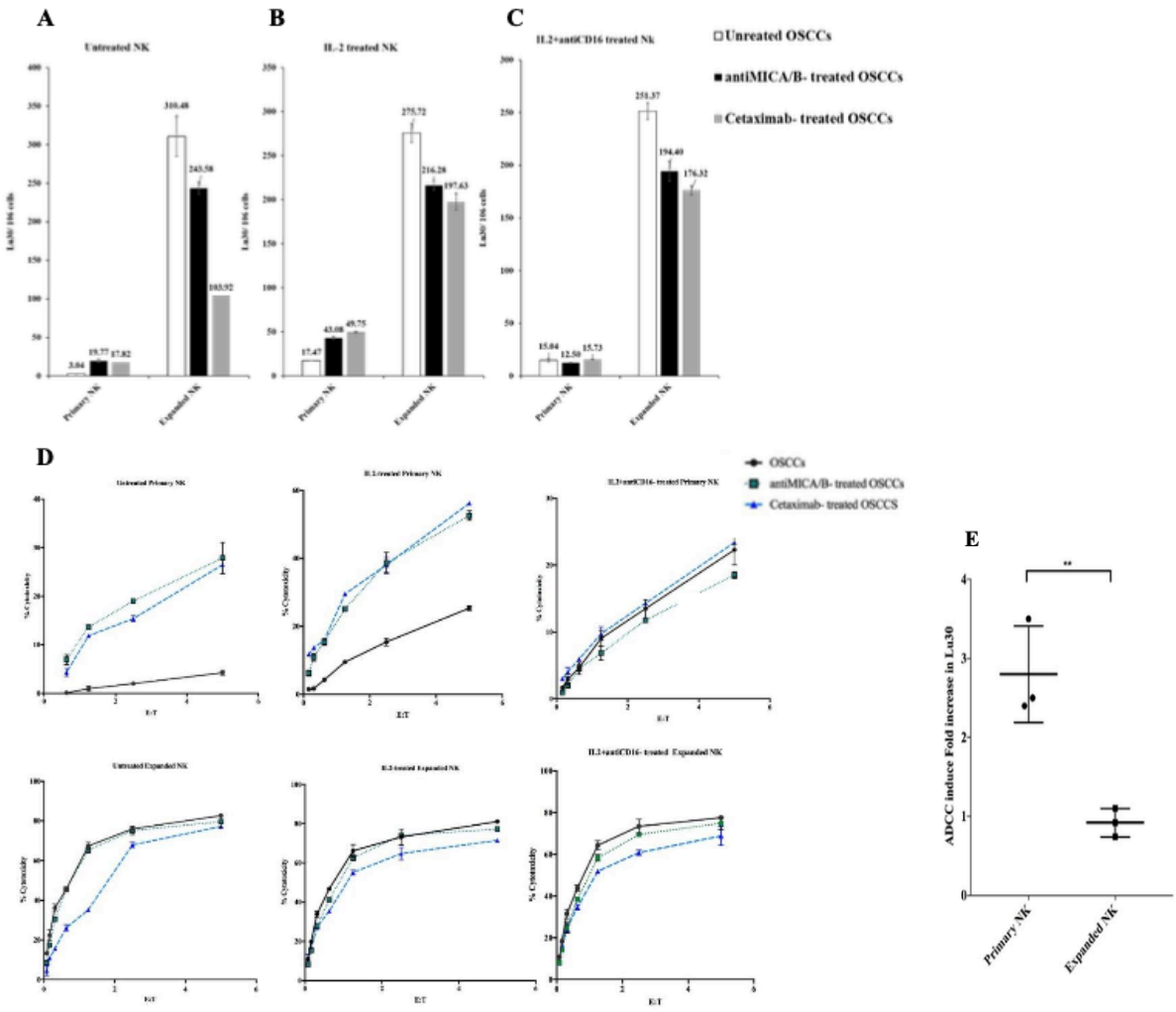

A
Untreated NK
B
IL-2 treated NK
C
IL2+antiCD16 treated Nk
Unreated OSCCs
antiMICA/B- treated OSCCs
Cetaximab- treated OSCCs
Primary NK
Expanded NK
D
OSCCs
antiMICA/B- treated OSCCs
Cetaximab- treated OSCCS
E
ADCC induce Fold increase in Lu30
Primary NK
Expanded NK

Fig. 5

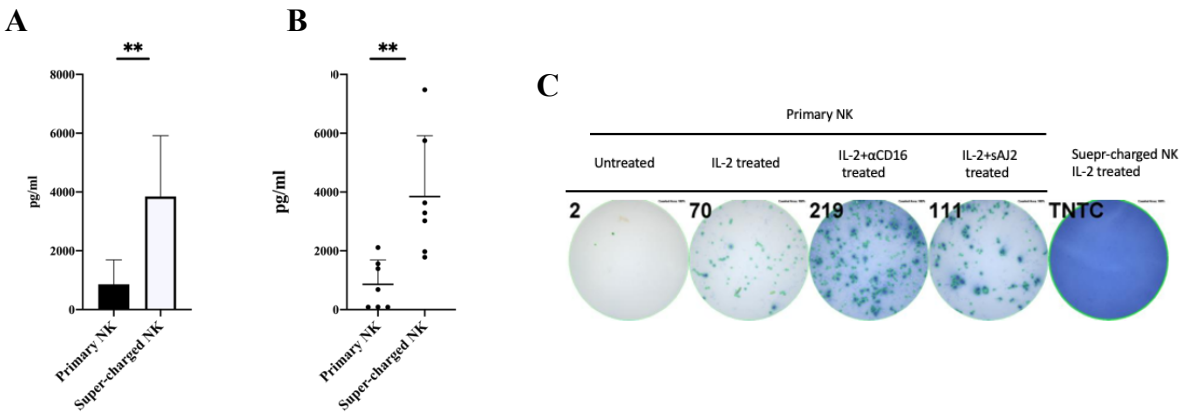

A
**
8000
6000
4000
2000
0
pg/ml
Primary NK
Super-charged NK
B
**
6000
4000
2000
0
pg/ml
Primary NK
Super-charged NK
C
Primary NK
Untreated
IL-2 treated
IL-2+αCD16 treated
IL-2+sAJ2 treated
Suepr-charged NK IL-2 treated
2
70
219
111
TNTC

Fig. 6

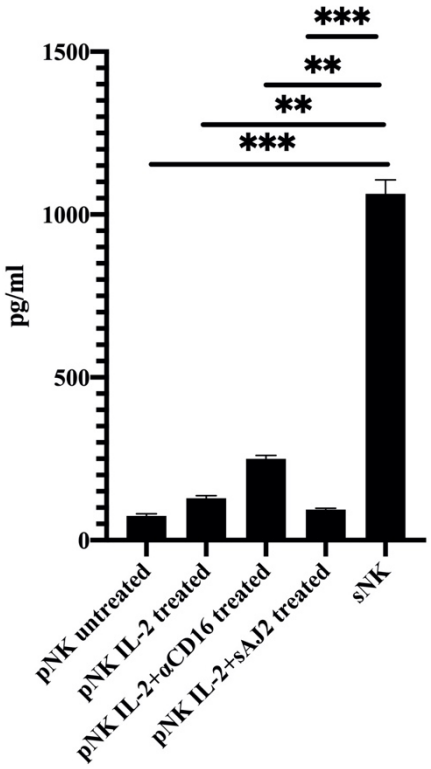
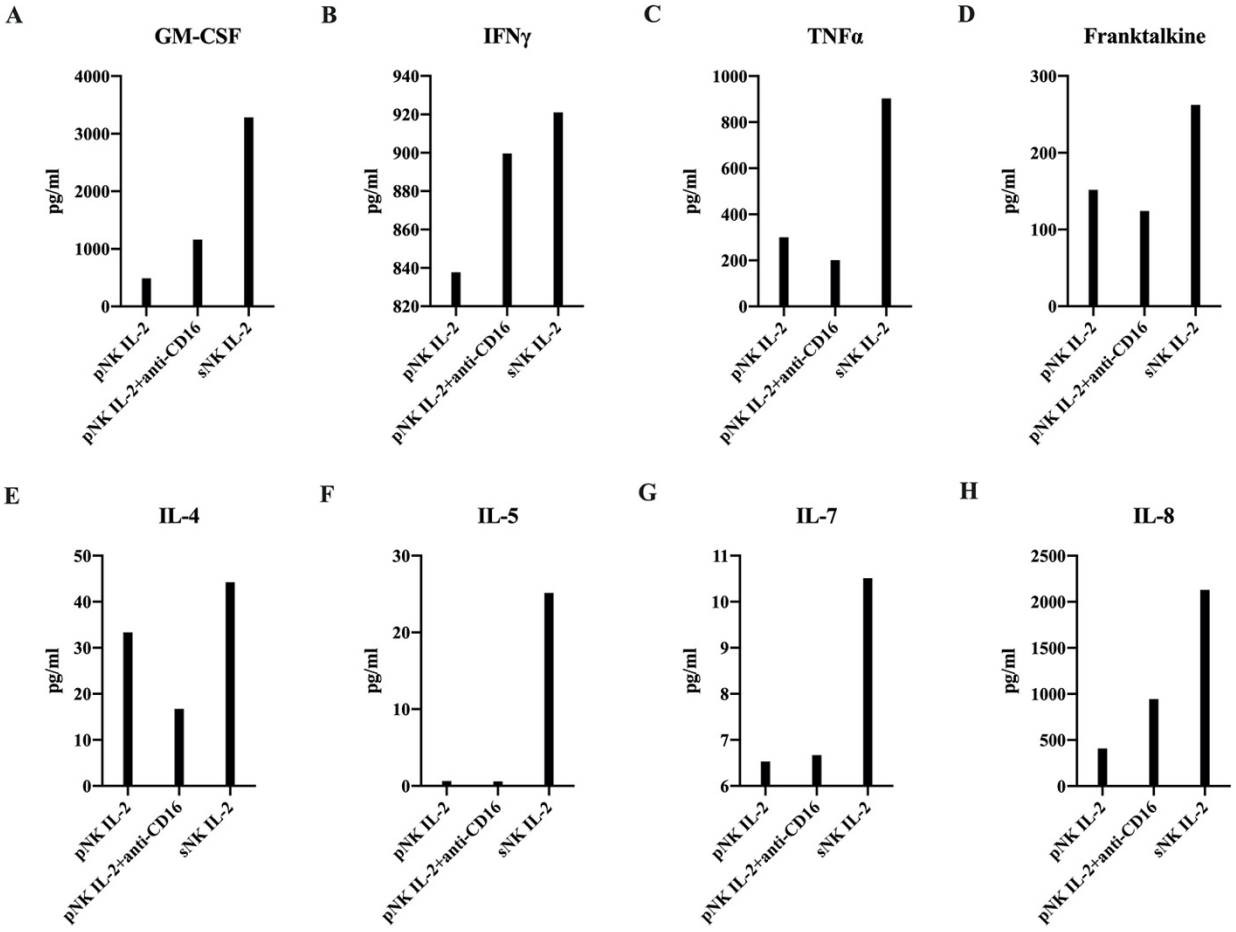

***
**
**
***
1500
1000
500
0
pg/ml
pNK untreated
pNK IL-2 treated
pNK IL-2+αCD16 treated
pNK IL-2+sAJ2 treated
sNK

Fig. 7


A
GM-CSF
pg/ml
4000
3000
2000
1000
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
B
IFNγ
pg/ml
940
920
900
880
860
840
820
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
C
TNFα
pg/ml
1000
800
600
400
200
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
D
Franktalkine
pg/ml
300
200
100
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
E
IL-4
pg/ml
50
40
30
20
10
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
F
IL-5
pg/ml
30
20
10
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
G
IL-7
pg/ml
11
10
9
8
7
6
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
H
IL-8
pg/ml
2500
2000
1500
1000
500
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2

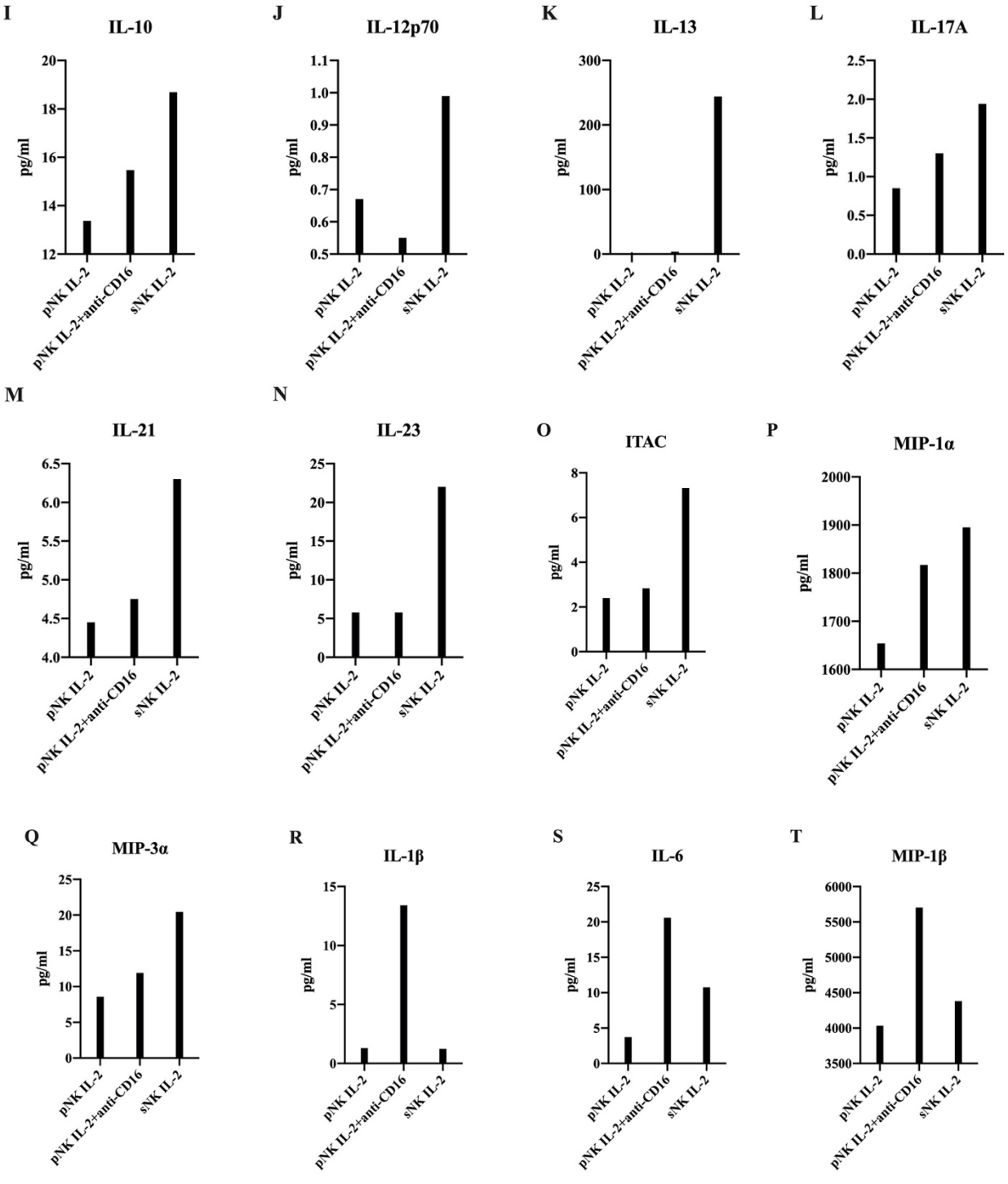

I
IL-10
pg/ml
20
18
16
14
12
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
J
IL-12p70
pg/ml
1.1
1.0
0.9
0.8
0.7
0.6
0.5
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
K
IL-13
pg/ml
300
200
100
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
L
IL-17A
pg/ml
2.5
2.0
1.5
1.0
0.5
0.0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
M
IL-21
pg/ml
6.5
6.0
5.5
5.0
4.5
4.0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
N
IL-23
pg/ml
25
20
15
10
5
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
O
ITAC
pg/ml
8
6
4
2
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
P
MIP-1α
pg/ml
2000
1900
1800
1700
1600
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
Q
MIP-3α
pg/ml
25
20
15
10
5
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
R
IL-1β
pg/ml
15
10
5
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
S
IL-6
pg/ml
25
20
15
10
5
0
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2
T
MIP-1β
pg/ml
6000
5500
5000
4500
4000
3500
pNK IL-2
pNK IL-2+anti-CD16
sNK IL-2

Fig. 8

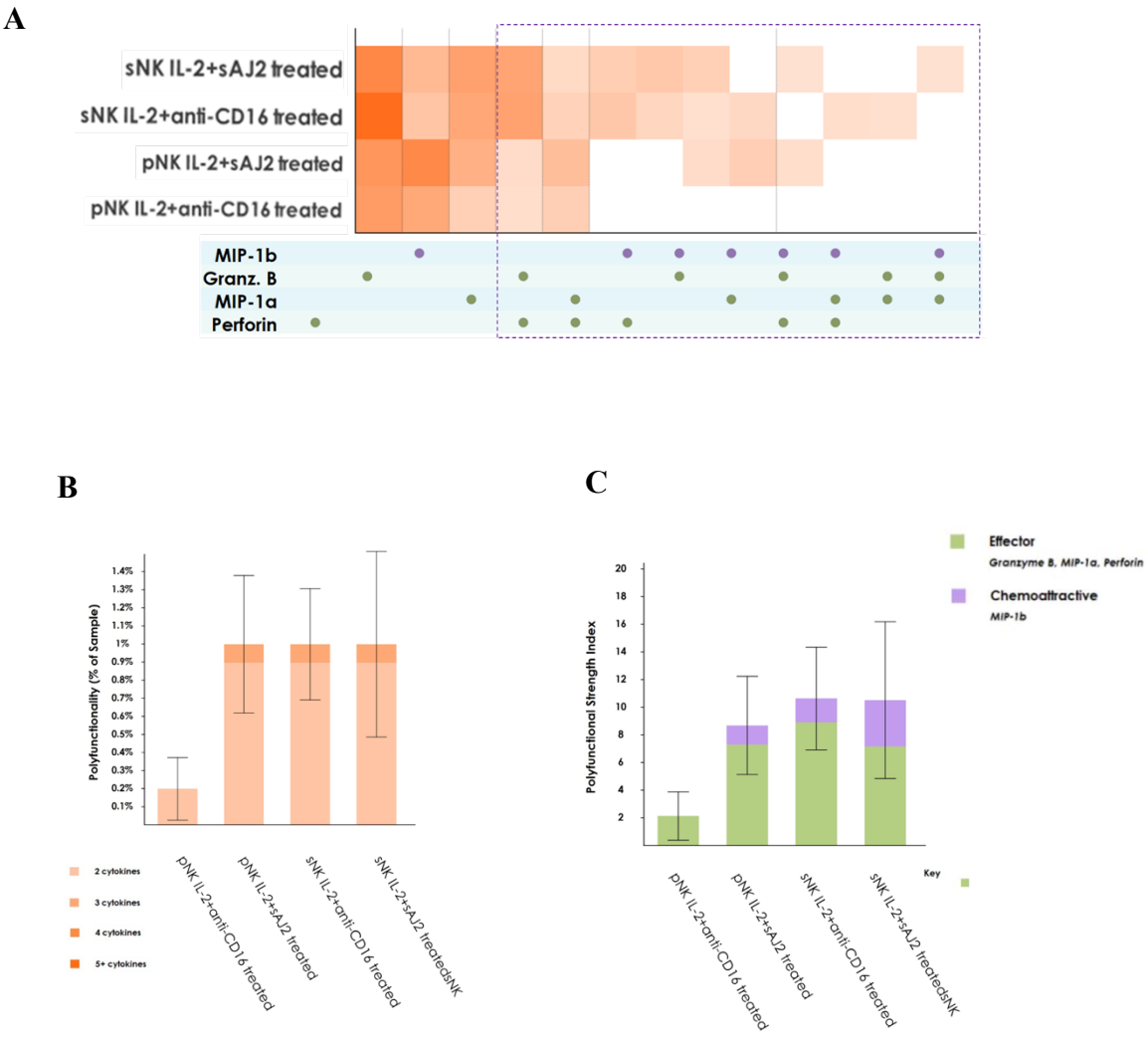

A
sNK IL-2+sAJ2 treated
sNK IL-2+anti-CD16 treated
pNK IL-2+sAJ2 treated
pNK IL-2+anti-CD16 treated
MIP-1b
Granz. B
MIP-1a
Perforin
B
Polyfunctionality (% of Sample)
1.4%
1.3%
1.2%
1.1%
1%
0.9%
0.8%
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
2 cytokines
3 cytokines
4 cytokines
5+ cytokines
pNK IL-2+anti-CD16 treated
pNK IL-2+sAJ2 treated
sNK IL-2+anti-CD16 treated
sNK IL-2+sAJ2 treatedsNK
C
Polyfunctional Strength Index
20
18
16
14
12
10
8
6
4
2
Effector
Granzyme B, MIP-1a, Perforin
Chemoattractive
MIP-1b
Key
pNK IL-2+anti-CD16 treated
pNK IL-2+sAJ2 treated
sNK IL-2+anti-CD16 treated
sNK IL-2+sAJ2 treatedsNK

Fig. 9

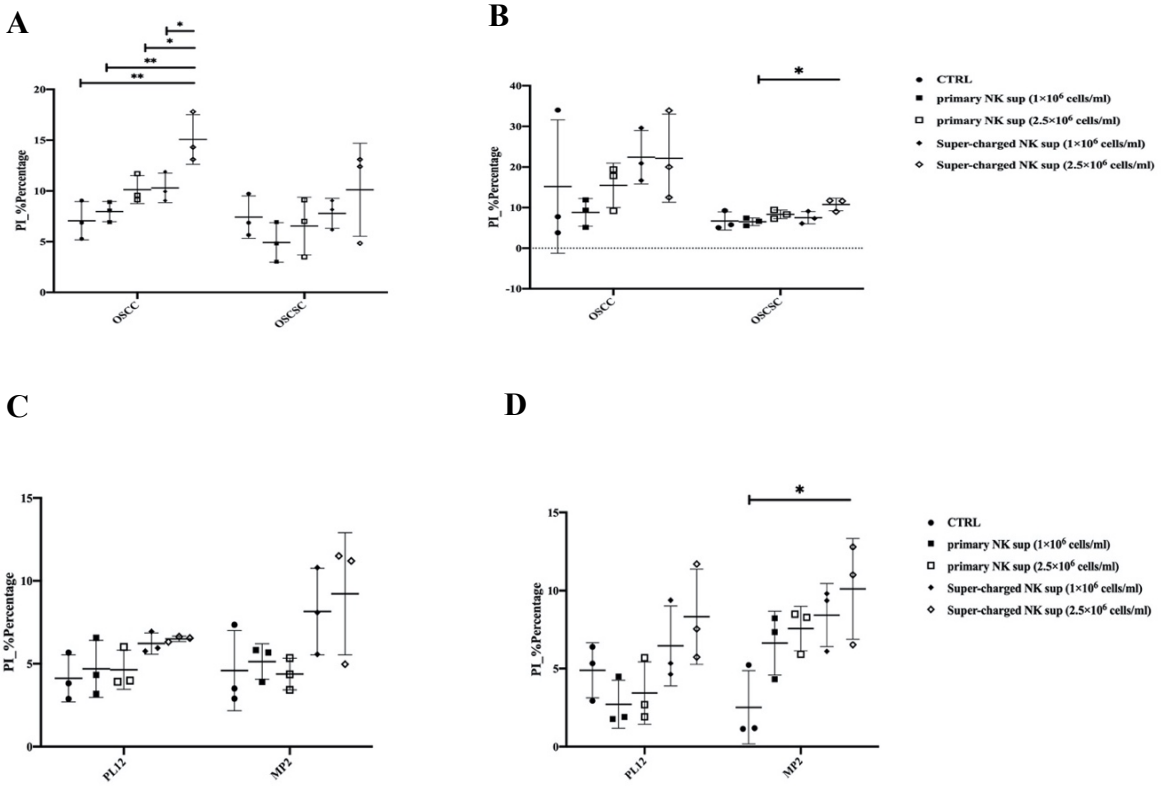

A
B
C
D
PI_%Percentage
OSCC
OSCSC
PL12
MP2
CTRL
primary NK sup (1×10⁶ cells/ml)
primary NK sup (2.5×10⁶ cells/ml)
Super-charged NK sup (1×10⁶ cells/ml)
Super-charged NK sup (2.5×10⁶ cells/ml)

Fig. 10

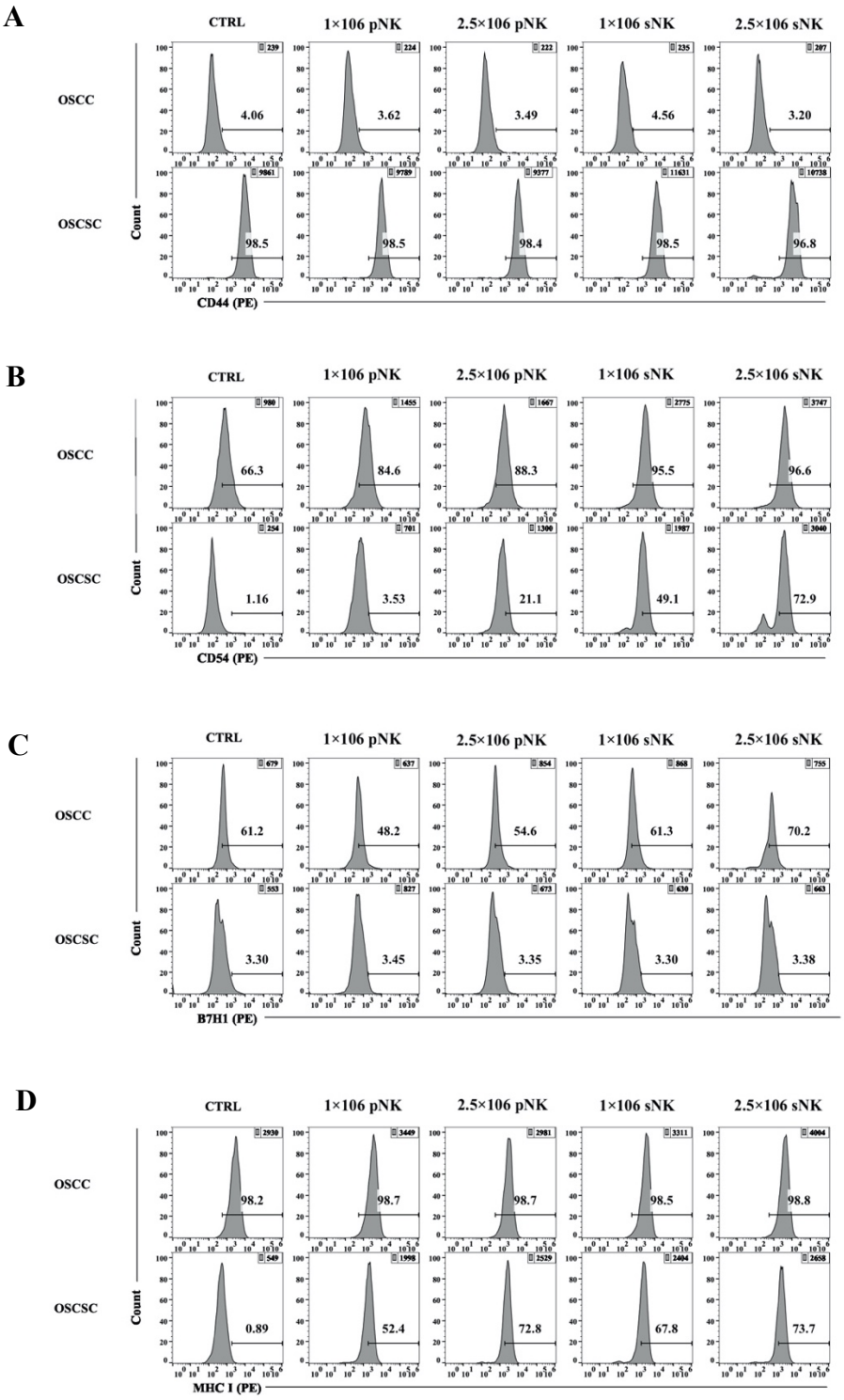

A
CTRL
1×106 pNK
2.5×106 pNK
1×106 sNK
2.5×106 sNK
OSCC
OSCSC
Count
4.06
3.62
3.49
4.56
3.20
98.5
98.5
98.4
98.5
96.8
CD44 (PE)
B
66.3
84.6
88.3
95.5
96.6
1.16
3.53
21.1
49.1
72.9
CD54 (PE)
C
61.2
48.2
54.6
61.3
70.2
3.30
3.45
3.35
3.30
3.38
B7H1 (PE)
D
98.2
98.7
98.7
98.5
98.8
0.89
52.4
72.8
67.8
73.7
MHC I (PE)

Fig. 11

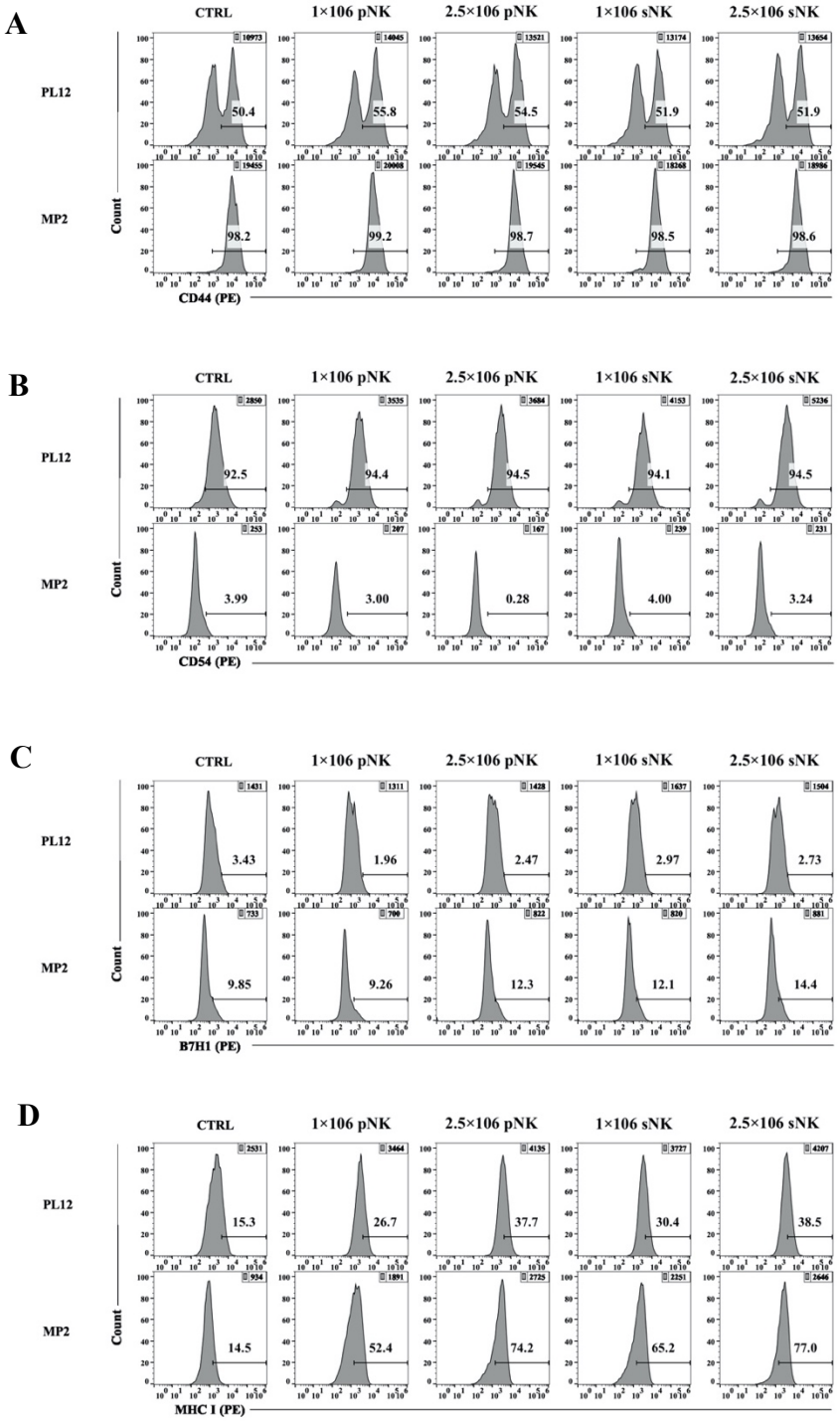

A
CTRL
1×106 pNK
2.5×106 pNK
1×106 sNK
2.5×106 sNK
PL12
MP2
Count
50.4
55.8
54.5
51.9
51.9
98.2
99.2
98.7
98.5
98.6
CD44 (PE)
B
92.5
94.4
94.5
94.1
94.5
3.99
3.00
0.28
4.00
3.24
CD54 (PE)
C
3.43
1.96
2.47
2.97
2.73
9.85
9.26
12.3
12.1
14.4
B7H1 (PE)
D
15.3
26.7
37.7
30.4
38.5
14.5
52.4
74.2
65.2
77.0
MHC I (PE)

Fig. 12

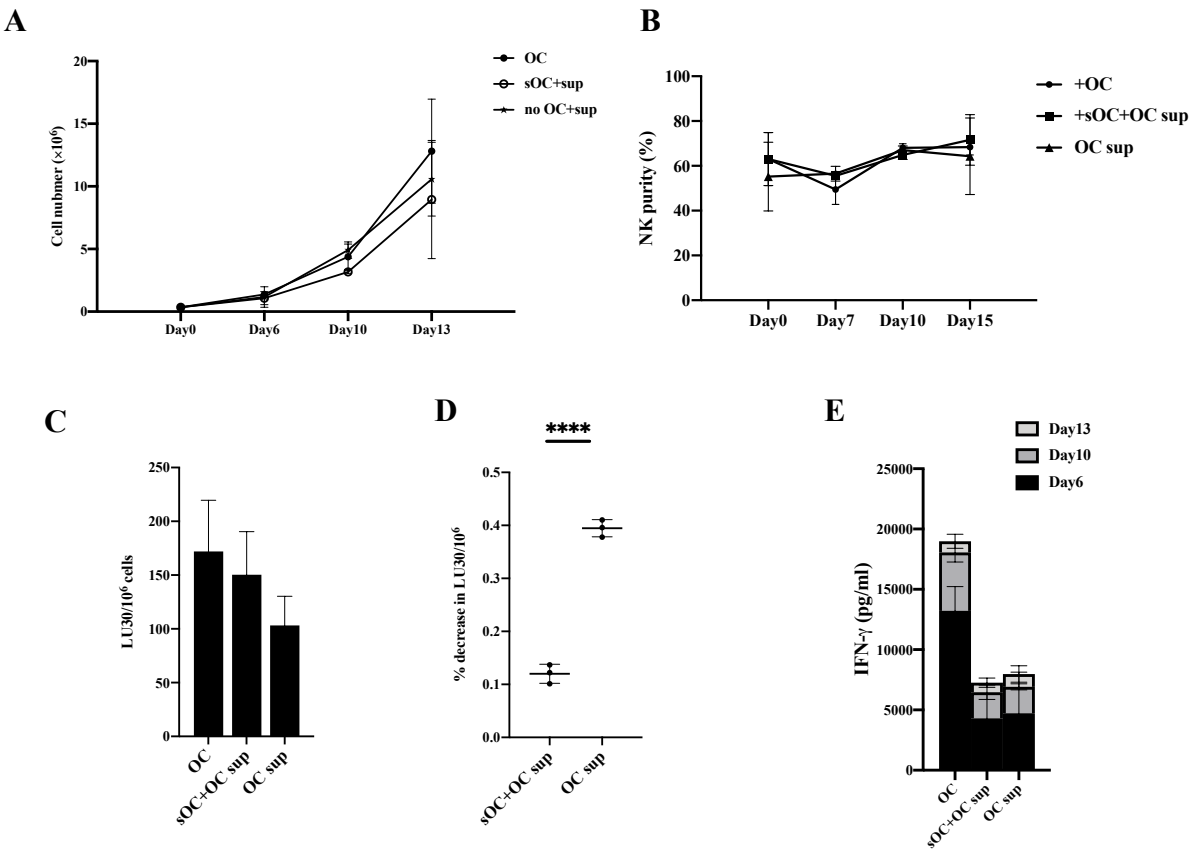
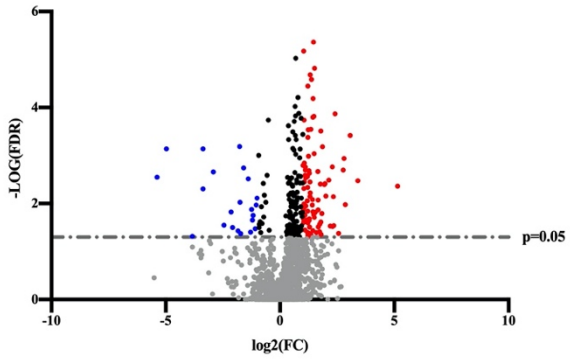

A
Cell nubmer (×10⁶)
OC
sOC+sup
no OC+sup
Day0
Day6
Day10
Day13
B
NK purity (%)
+OC
+sOC+OC sup
OC sup
Day0
Day7
Day10
Day15
C
LU30/10⁶ cells
OC
sOC+OC sup
OC sup
D
****
% decrease in LU30/10⁶
sOC+OC sup
OC sup
E
IFN-γ (pg/ml)
Day13
Day10
Day6
OC
sOC+OC sup
OC sup

Fig. 13

A


-LOG(FDR)
log2(FC)
p=0.05
6
4
2
0
-10
-5
0
5
10

B

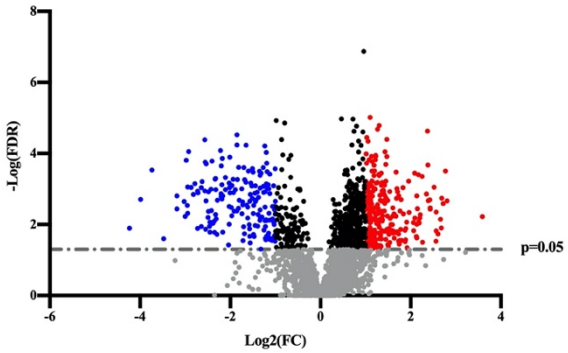
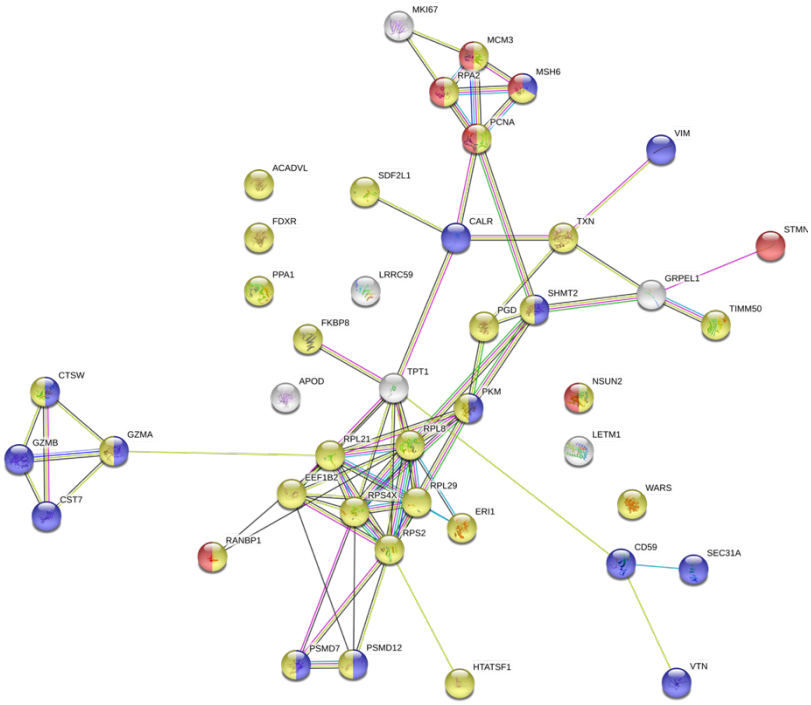
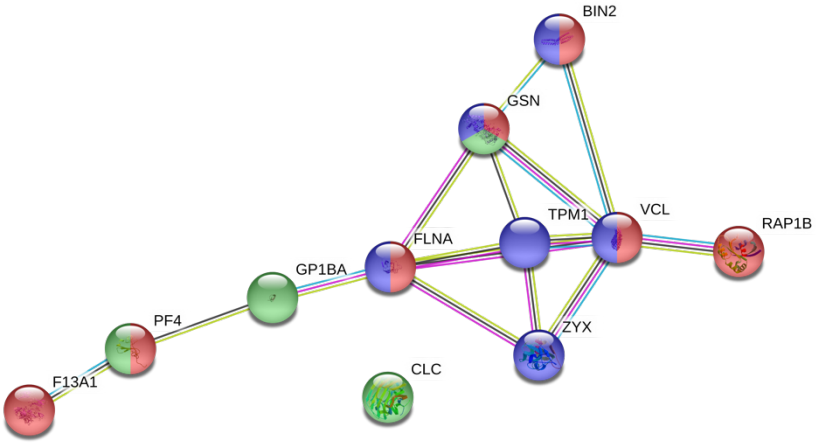

-Log(FDR)
Log2(FC)
p=0.05
8
6
4
2
0
-6
-4
-2
0
2
4

Fig. 14A



	GO-Term	Description	False Discovery Rate
	GO:0022402	Cell cycle process (7)	0.0467
	GO:0044237	Cellular metabolic process (29)	0.0413
	GO:0002376	Immune system process (14)	0.0111

Fig. 14B



	GO-Term	Description	False Discovery Rate
	GO:0045055	Regulated exocytosis (7)	1.85×10^{-5}
	GO:0002682	Regulation of immune system process (6)	0.0466
	GO:0015629	Actin cytoskeleton (6)	8.61×10^{-6}

Fig. 15

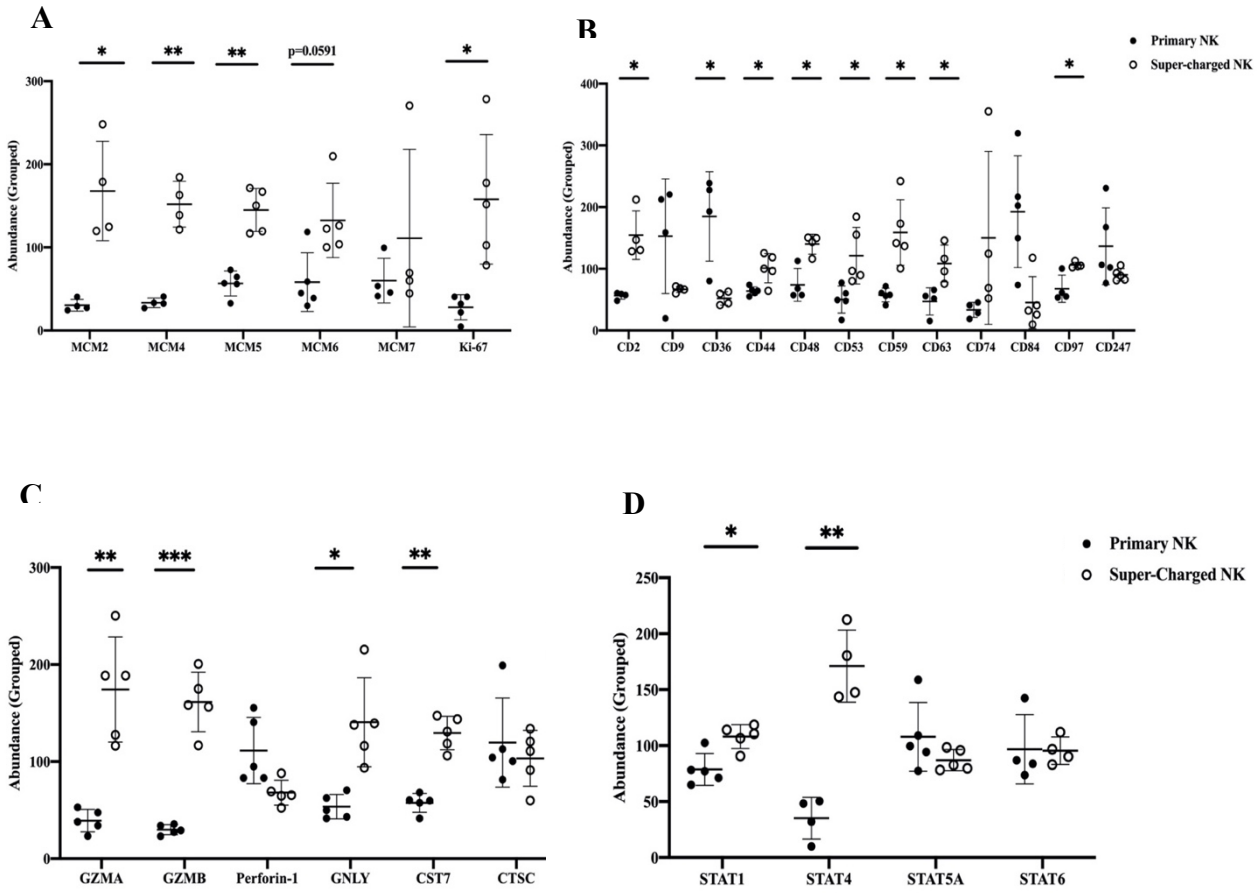

A
B
C
D
Abundance (Grouped)
MCM2
MCM4
MCM5
MCM6
MCM7
Ki-67
p=0.0591
CD2
CD9
CD36
CD44
CD48
CD53
CD59
CD63
CD74
CD84
CD97
CD247
Primary NK
Super-charged NK
GZMA
GZMB
Perforin-1
GNLY
CST7
CTSC
Primary NK
Super-Charged NK
STAT1
STAT4
STAT5A
STAT6

Fig. 16

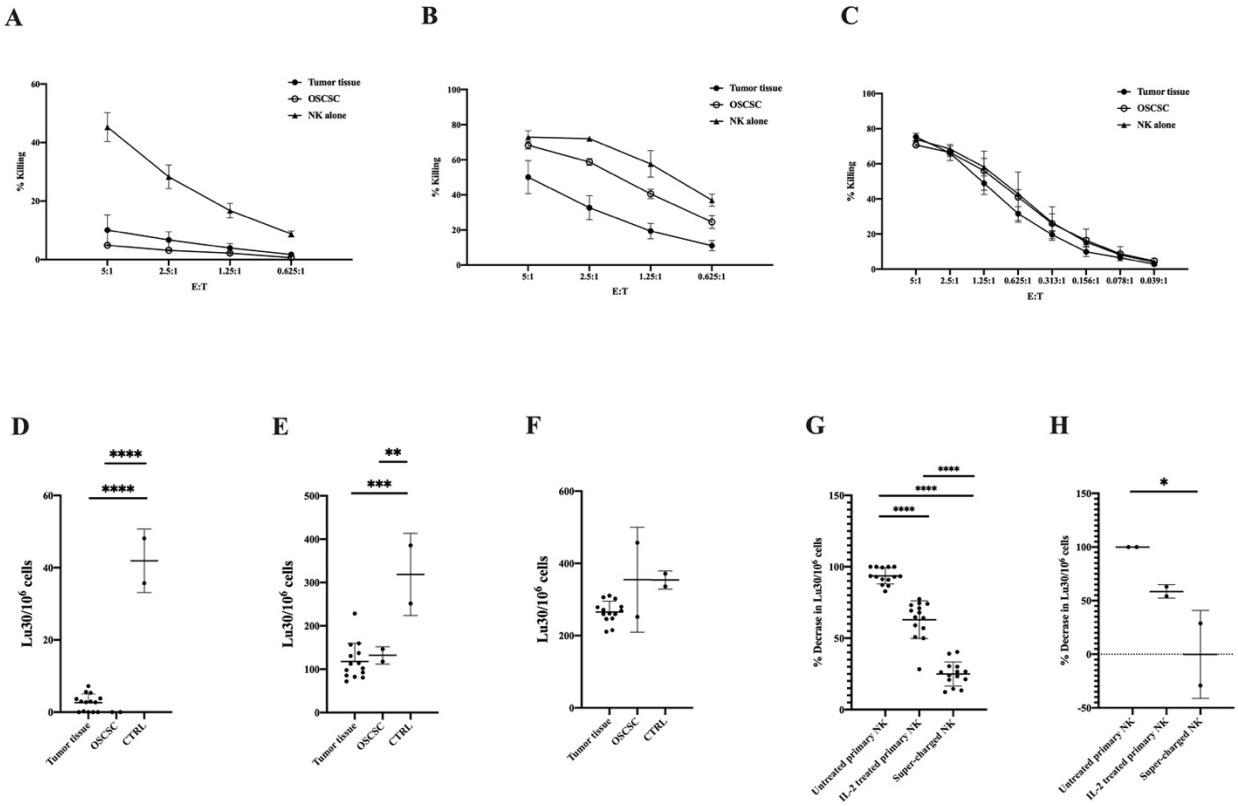
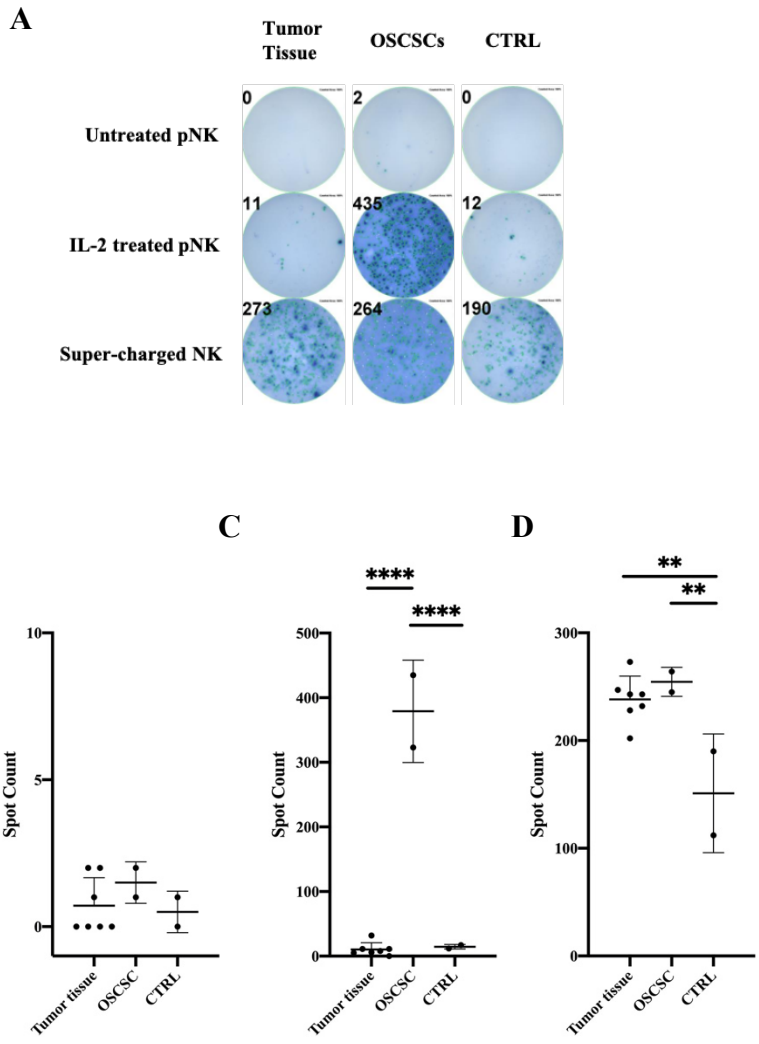

A
B
C
Tumor tissue
OSCSC
NK alone
% Killing
E:T
5:1
2.5:1
1.25:1
0.625:1
0.313:1
0.156:1
0.078:1
0.039:1
D
E
F
G
H
Lu30/10^6 cells
Tumor tissue
OSCSC
CTRL
% Decrase in Lu30/10^6 cells
Untreated primary NK
IL-2 treated primary NK
Super-charged NK

Fig. 17


A
Tumor Tissue
OSCSCs
CTRL
Untreated pNK
IL-2 treated pNK
Super-charged NK
0
2
0
11
435
12
273
264
190
B
C
D
Spot Count
Tumor tissue
OSCSC
CTRL
****
****
**
**

CHAPTER 3

Osteoclast-induced super-charged NK cells preferentially select and expand CD8+T cells; studies in humanized-BLT mice and cancer patients

Introduction

Tumor cells are known to decrease the numbers of NK and CD8+ T cells as well as suppressing their function [1-5]. Similar profiles of decrease in NK and CD8+ T cells have been seen in patients suffering from COVID-19 [6, 7]. The interconnectedness of these two important cytotoxic cells has not been clearly delineated previously even though correlations were seen in increased percentages of NK and CD8+ T cells in peripheral blood and in bone marrow (BM) of cancer patients in the presence of decrease in CD4+ T cells [8-12]. It is of particular interest to note that such profile is largely seen in the BM of multiple myeloma (MM) patients where osteoclasts (OCs) are found. OCs are largely known for their influence on the turnover rate of the bone. However, in recent years the role of OCs in the modulation of different immune effectors, in particular NK cells has received great attention [13-15]. Therefore, even though the classical function of OCs were long regarded as influencing the architecture of the bone, recent reports from our laboratory and those of the others have bestowed a well-deserved status to OCs as immunomodulatory effectors which their function go beyond their well-known role in bone remodeling [13-17].

NK and T cells comprise a large portion of the lymphocyte population in peripheral blood mononuclear cells (PBMCs). NK cells are mainly known as the effectors of innate immunity due to their lack of antigen-specificity; however, this notion has been challenged in recent years [18-

20]. Decreased peripheral blood lymphocyte infiltration and lower cytotoxic activity, particularly in NK cells, result in poor prognosis in cancer patients [21-26]. However, cancer-mediated immune suppression remains a complex and multifactorial problem. Various studies have shown that factors in the tumor microenvironment suppress NK cells by downregulating NK cell surface receptors [27-34]. Studies have shown that NK cells can activate and induce the proliferation of T cells through direct cell-cell contact [35, 36]; IL-2–activated NK cells can also directly induce the maturation of dendritic cells (DCs) and enhance their ability to stimulate allogeneic naive $CD4^+$ T cells [37]. Several other studies have demonstrated the immunoregulatory roles which NK cells play in killing chronically activated leukocytes [38-41] and eliminating activated autologous CD4+ T cells [41-43].

CD4+ and CD8+ T cells mediate the adaptive cellular immunity, which closely collaborate with the innate immune system [44, 45]. CD8+ T cells spearhead the cellular immune responses that protect against tumors [46]. High levels of tumor-infiltrating CD8+ T cells are associated with complete responses to standard chemotherapeutic regimens [47], and the presence of CD8+ memory T cells is associated with patient survival [48, 49]. Increased T regulatory (Treg) cell infiltration into solid tumors is negatively associated with overall survival in cancer patients [50].

NK and CD8+ T cell-based immunotherapies are among the leading standards in cancer therapeutics [51, 52]. NK immunotherapies have been limited due to the low numbers of these cells in the peripheral blood and our inability to expand large numbers of functional NK cells with extended survival in patients. To overcome this problem, we previously introduced a novel NK expansion methodology using a combination of OCs and sonicated probiotic bacteria (sAJ2) to generate activated super-charged NK cells with the potential to kill and differentiate cancer stem

cells (CSCs) [15]. Later in the expansion process of NK cells by OCs we have also observed that this methodology led to the expansion of a very small population of contaminating T cells which were initially not detectable [15]. However, after many rounds of expansion a small population of CD8+ T cells was expanding in NK cultures which coincided with the lower ability of NK cells to lyse tumors [15]. Previous studies have also shown that NK cells can accelerate CD8+ T cell responses against viral infections, such as those caused by cytomegaloviruses [53, 54]. We have also uncovered the differences in the dynamics of NK cell expansion in healthy individuals and cancer patients [15].

In this study we sought to determine how NK and T cells differ in their numbers and responses in cancer patients and in healthy controls using our novel methodology to expand NK cells. In addition, we observed that OC-activated super-charged NK cells specifically expand CD8+ T cells, whereas DC-activated NK cells demonstrate a preference for the CD4+ T cell expansion. Our *in-vitro* study revealed how supercharged NK cells might affect the balance of T cell subsets, cytokine secretions, and cytotoxic activity of immune cells in various tissue compartments of the healthy and cancer-bearing hu-BLT mice. Finally, we demonstrated that super-charged NK cells lyse activated CD4+ T and not CD8+ T cells, thus selecting and preferentially expanding mostly effector memory CD8+ T cells through IFN-γ secretion.

Result

Sub-aim 1: Decrease in functions of NK cells and less activation ability in osteoclasts in cancer patients.

Allogeneic OC-mediated expansion, and augmented function of NK cells from cancer patients is greatly suppressed when compared to those of healthy individuals

To determine the extent of NK and T cell expansion and function, we expanded NK and T cells of cancer patients and healthy individuals using our expansion strategy as previously described [15]. Cancer patients' NK cells showed significantly decreased levels of expansion (**Fig. 1A** and **2E**), and expanded NK cells exhibited significantly lower cytotoxicity (**Fig. 1B** and **2F**), and IFN-γ secretion (**Fig. 1C, 1D** and **2G**) when compared to those of healthy individuals. Cancer patients' T cells exhibited similar decreases in expansion rate (**Fig. 1E**) and IFN-γ secretion (**Fig. 1F-1G**).

To assess whether NK and T cells exhibit distinct expansion profiles, we cultured NK and T cells of healthy individuals in the presence and absence of OCs and found that T cells expanded faster than NK cells in the absence of OCs (**Fig. 1H**). However, OCs induced 2.6 to 4.5-fold and 1.2 to 1.6-fold expansion in NK and T cells, respectively, when compared to those cultured in the absence of OCs (**Fig. 1H**). These results indicated that OCs induce higher expansion of NK cells when compared to T cells. T cells secreted significantly higher IFN-γ secretion in the absence of OCs while IFN-γ secretion in both NK and T cells exhibited comparable increases in the presence of OCs (**Fig. 1I**).

Cancer patients' OCs induced lower cell expansion, IFN-γ secretion and cytotoxicity in NK cells when compared to healthy individuals' OCs

To investigate the function of patients' OCs, we cultured the healthy individuals' NK cells with either autologous OCs or with patients' OCs (allogeneic). Patients' OCs were less capable of inducing NK cell expansion (**Fig. 2A** and **2E**), cytotoxicity (**Fig. 2D** and **2F**) and IFN-γ secretion (**Fig. 2C, 2D** and **2G**). We next assessed the function of patient NK cells in the context of autologous OCs. Severe decreases in NK cell expansion, IFN-γ secretion, and cytotoxicity were observed when patients' NK cells were cultured with autologous OCs (**Fig. 2E-2G**). These results indicated severe functional defects in both NK cells and OCs of cancer patients.

Sub-aim 2: Osteoclast expanded NK cells preferentially expands CD8+ memory T cells while selectively lyse CD4+ T cells

OC-induced T cell mediated expansion increased CD8+ T cells moderately when compared to OC-induced NK cell mediated expansion of CD8+ T cells

We next analyzed the surface phenotype of memory and naïve subpopulations of T cells, and observed increase in CD45RO+ cells (activated T cells) and decrease in CD45RA+ cells (naïve T cells) on cancer patients' T cells (**Fig. 3A**). We also noted reduced surface expressions of CD62L, CD28, CCR7, and CD127 on cancer patients' T cells (**Fig. 3A**). Moreover, percentages of CD4+ T cells were decreased with corresponding increase in the percentages of CD8+ T cells in cancer patients' PBMCs (**Fig. 3B**). Accordingly, we observed decreased CD4+/CD8+ T cell ratios in

cancer patients' PBMCs, suggesting an overall increase in the CD8+ T cell subset in cancer patients when compared to healthy individuals (**Fig. 3C**).

We next cultured NK cells in the presence of healthy allogeneic OCs and determined the fractions of expanded CD4+ and CD8+ T cells within the expanded NK cells. No detectable T cells could be seen initially after NK cell purifications, however, after several rounds of NK cell expansions we were able to detect T cell expansion within the NK cells. The expanded T cells were primarily CD8+ T cells with no or very low levels of CD4+ T cells in cultures of expanded NK cells from both healthy individuals and cancer patients (**Fig. 3D**), and the relative CD4+/CD8+ T cell ratios remained similar between cancer patients and healthy individuals (**Fig. 3E**). However, it should be noted that the patients have higher percentages of CD8+ T cells than CD4+ T cells in their PBMCs when compared to those of healthy individuals (**Fig. 3B-3E**).

When purified T cells were cultured with allogeneic healthy OCs, cancer patients but not healthy individuals exhibited higher percentages of CD8+ T cells with lower CD4+/CD8+ T cell ratios since the levels of CD8+ T cells were constitutively higher in cancer patients PBMCs in the absence of expansion (**Fig. 3D-3E**). Also, it should be noted that for the sake of comparison we chose to activate T cells by IL-2 and anti-CD3/CD28 signaling since NK cells were activated by IL-2 and anti-CD16 mAbs before they were cultured with OCs. Thus, NK and T cells were pre-activated before their culture with OCs. We observed much higher percentages and numbers of CD8+ T cell expansion with no or very low remaining CD4+ T cells in OC-expanded NK cells, whereas OC-induced T cells expanded CD8+ T cells moderately with significant percentage of CD4+ T cells still remaining in the culture (**Fig. 3D-3E**).

Increased NK numbers and NK-mediated cytotoxicity by OC-expansion in comparison to DC-expansion; OCs preferentially expand CD8+ T cells whereas DCs preferentially expand CD4+ T cells in NK cells cultures

To assess whether the activation of NK cells by OCs vs. DCs differentially affects expansion profile and function, we cultured NK cells from healthy individuals either alone, with OCs, or with DCs. Significantly higher cell counts were observed in NK cells cultured with OCs in comparison to those cultured alone or with DCs (**Fig. 4A**). Next, we determined the subpopulations of CD16, and CD3 expressing cells within the NK cells cultured alone, or with OCs, or with DCs and counted the numbers of NK and T cells within total lymphocytes. Significantly higher NK cell counts (**Fig. 4B**) and lower T cell counts (**Fig. 4C**) were observed in the presence of OCs versus DCs. OC-expanded NK cells displayed significantly higher levels of cytotoxicity against oral squamous cancer stem-like cells (OSCSCs) (**Fig. 4D-4E**). Additionally, NK cells cultured with OCs secreted significantly higher levels of IFN-γ than those cultured with DCs (**Fig. 4F**).

In addition, we characterized the subpopulations of T cells expanded within the NK cell cultures with OCs or DCs and found that DCs preferentially expanded CD4+ T cells (**Fig. 4G, 4I-4J and 4M**) whereas OCs favored the expansion of CD8+ T cells (**Fig. 4H-4J and 4M**). T cells expanded in NK cell cultures with OCs similar to those expanded by DCs did not express either killer cell lectin-like receptor G1 (KLRG1) or T cell immunoglobulin/mucin domain-containing protein 3 (TIM3), whereas they had similar levels of PD-1 (**Fig. 4J**). Thus, no significant levels of these check point inhibitors could be seen on T cells expanded by either OC- or DC-expanded NK cells (**Fig. 4J**). We also noticed slight differences in the expression levels of CD4, CD8, KLRG1, TIM3, and PD-1 in purified T cells cultured with OCs versus DCs in the absence of NK cells (**Fig.**

4K). T cells in NK+OC co-cultures expressed higher levels of CD45RO; lower levels of CD62L, CD28, CCR7, and CD127; and similar levels of CD44 when compared to NK+DCs co-cultures (**Fig. 4L-4M**). When purified T cells were expanded in the presence of OCs, we observed slightly higher levels of CD45RO and CD28; lower levels of CD62L and CCR7; and similar levels of CD127 and CD44 when compared to those expanded in the presence of DCs (**Fig. 4L-4M**).

Correspondingly, higher levels of cytokines and chemokine secretions were seen in NK cells in comparison to T cells when both were cultured with OCs (**Fig. 4N-4Q**). NK cells secreted higher levels MIP-1α, MIP-1β, sCD137, FasL, GM-CSF, IFN-γ, sFas, and perforin when compared to T cells (**Fig. 4N-4P**). CD8+ T cells sorted out from OC-expanded NK cells culture secreted higher levels of GM-CSF, sCD137, IFN-γ, FasL, IL-10, and TNF-α when compared to OC-expanded CD8+ T cells in the absence of NK cells (**Fig. 4Q**).

OCs induce higher cell expansion and IFN-γ secretion in CD8+ T cells than in CD4+ T cells

OCs were found to induce higher expansion of CD8+ T cells in NK cultures when compared to those with purified T cells (**Fig. 5A**) or purified CD8+T cells (**Fig. 5B**). No significant differences in the degree of expansion could be seen when purified CD4+ and CD8+ T cells were treated with anti-CD3/CD28+IL-2 and cultured in the absence of OCs (**Fig. 5C-5D**). However, in contrast to CD4+ T cells, a continuous rise in the fold expansion of CD8+ T cells could be seen when the cells were treated with anti-CD3/CD28+IL-2 and cultured with OCs (**Fig. 5C and 5E**). Under the same experimental condition CD4+ T cell counts increase initially, but plateaued soon after, and then declined after day 12 of culture (**Fig. 5E**). We then compared the expansion and secretion of IFN-γ by the NK, CD4+ T, and CD8+ T cells after they were treated as described

above and cultured with OCs. Higher cell expansion (**Fig. 5F**) and IFN-γ secretion (**Fig. 5G**) could be observed in NK and CD8+ T cells as compared to CD4+ T cells.

Increased CD8+ T cells, IFN-γ secretion, and cytotoxicity in various tissue compartments of oral tumor-bearing hu-BLT mice in response to NK cell immunotherapy

Hu-BLT mice were implanted with OSCSCs in the oral cavity and injected with OC-expanded NK cells with potent cytotoxic and cytokine secretion capabilities. After 4-5 weeks, the mice were sacrificed and tissues were harvested and dissociated in order to obtain single-cell suspensions for analysis (**Fig. 6A**). We observed increased proportions of CD3+CD8+ T cells in the bone marrow (BM) (**Fig. 6B**), spleen (**Fig. 6E**), and peripheral blood (**Fig. 6H**) of tumor-bearing mice injected with OC-expanded NK cells when compared to tumor-bearing mice injected with vehicle only or healthy non-tumor bearing mice. NK cell immunotherapy also augmented the IFN-γ secretion and NK cell-mediated cytotoxicity in BM (**Fig. 6C and 6D**), spleen (**Fig. 6F and 6G**), and peripheral blood (**Fig. 6I and 6J**) in tumor-bearing mice. Increased secretion of IFN-γ (**Fig. 6K**), IL-6 (**Fig. 6L**), and ITAC (**Fig. 6M**) and decreased secretion of IL-8 (**Fig. 6N**) and GM-CSF (**Fig. 6O**) were also seen in sera harvested from the peripheral blood of tumor-bearing mice injected with OC-expanded NK cells versus those injected with vehicle alone or non-tumor bearing mice injected with OC-expanded NK cells (**Fig. 6K-6L**).

Sub-aim 3: OC expanded Super-charged NK cells contain the growth of T cells and preferentially expand CD8+ T cells while lysing CD4+ T cells.

NK expansion with additional autologous CD4+ and CD8+ T cells showed the same expansion rate and NK cell functions while preferentially expand CD8+ T cells

Primary NK cells were freshly isolated from healthy donor's PBMCs and treated overnight with IL-2 and anti-CD16 mAb before co-cultures with feeder cells, osteoclasts, and sonicated probiotics, sAJ2, as previously reported. Additional CD4+ and CD8+ T cells were added into the NK expansion culture. Fresh medium and IL-2 were replenished every three days after the first 7 days of culture. NK expansions with or without additional autologous CD4+ and CD8+ T cells showed comparable level of expansion, NK cell mediated cytotoxicity and IFN-γ secretion (**Fig 7A-7D**). The purity of NK cells in the expansion was determined using flow cytometric analysis in the period of expansion. NK purity decreased drastically during the first 7-10 days in culture, and as the NK cells started to expand and increase in cell count, NK purity subsequently began to increase and returned to the original or higher purity after 10-15 days of culturing. The same observation was seen in either standard NK expansion or the expansion with additional autologous T cells (**Fig. 7E**).

The percentages of CD4+ and CD8+ T cells were measured using flow cytometric analysis during the expansion period, and the ratios of CD4+ or CD8+ T cells to total T cells were calculated. In the beginning stages of the expansion, there were more CD4+ T cells than CD8+ T cells (2:1), in the groups with additional autologous T cells. However, after the first 7 days of expansion, the percentage of CD8+ T cells was higher than CD4+ T cells in the culture, with or without additional autologous T cells (**Fig. 7F**). After 10-15 days of expansion, the levels of CD4+ T cells were almost extinguished in the culture, and CD3+ T cells were shown to be mostly of the CD8+ subset

(**Fig. 7F**). These findings indicate that NK cells retain their purity and preferentially expand CD8+ T cells in the expansion process, even with additional autologous T cells in the culture.

No difference in sensitivity to cell death between CD4+ and CD8+ T cells.

Our findings prompted us to investigate the sensitivity to cell death of CD4+ and CD8+ T cells. Both CD4+ and CD8+ T cells were purified from PBMCs and treated with IL-2, the combination of IL-2 and anit-CD3/28 antibody, or were left untreated overnight before use. CDDP was used as a treatment to provide extra cellular stress to either subset of T cells to assess their sensitivity to cell death. No treatment or CDDP (0.5 μM) treated groups were conducted, and the cell numbers were examined on Days 1, 3 and 6 to quantify cell death in CD4+ and CD8+ T cells. There was no difference in cell number or cell number descending trend between CD4+ and CD8+ T cells across the experiment period with or without the presence of CDDP (**Fig. 8**).

The same experiments were conducted using propidium iodide (PI) staining to detect cell death of CD4+ and CD8+ T cells using flow cytometric analysis. There was a slight increase in % cell death in CD8+ T cells in Day 3 and Day 6 in both no treatment and CDDP treated groups, but a statistically significant difference was not seen after statistical analysis (**Fig. 9** and **10**). These data suggest that neither CD4+, nor CD8+ T cells, are more vulnerable to cell death with or without the addition of CDDP.

Super-charged NK cells preferentially target CD4+ T cells but not CD8+ T cells.

To further understand the mechanism in which super-charged NK cells expand CD8+, but not CD4+ T cells, untreated and IL-2 treated primary NK cells and super-charged NK cells were used to determine whether NK cells lyse either subset of T cells through using target visualization assay (TVA). Both CD4+ and CD8+ T cells were isolated and treated overnight as previously described before used. Both untreated and IL-2 treated primary NK cells showed no or limited cytotoxicity against CD4+ and CD8+ T cells (**Fig. 11A** and **11B**). Unlike primary NK cells, super-charged NK cells exhibited notable cytotoxicity against IL-2 and anti-CD3/28 antibody-treated CD4+ T cells, but demonstrated no cytotoxicity against CD8+ T cells regardless of treatments (**Fig. 11C** and **11D**). Whereby, super-charged NK cells, but not primary NK cells, preferentially lyse activated CD4+ T cells.

Together, NK cells demonstrate greater expansion capabilities with the inclusion of additional autologous T cells, while retaining their purity. In addition, super-charged NK cells preferentially expand CD8+ T cells through selectively lyse activated CD4+ T cells.

Sub-aim 4: Characteristics of super-charged NK expanded CD8+ T cells

Super-charged NK cell expanded CD8+ T cells are shown to be memory T cells.

Given the distinguished functions and promising potential of super-charged NK cells in cancer immunotherapy, it is crucial to understand the characteristics of super-charged NK cell-expanded CD8+ T cells. Several surface markers were measured on the surfaces of primary and super-charged NK-expanded CD8+ T cells using flow cytometric analysis. CD45RA+ was in higher levels in primary CD8+ T cells, (**Fig. 12A**) whereas CD45RO+ was higher in super-charged

NK-expanded CD8+ T cells (**Fig. 12B**), indicating that the majority of super-charged NK cell-expanded CD8+ T cells are memory T cells.

More surface markers were tested to further categorize super-charged NK cell-expanded CD8+ T cells, including CD28, CD62L and PD-1. Both CD28 and CD62L were expressed at higher levels in primary CD8+ T cells than in super-charged NK cell-expanded CD8+ T cells (**Fig. 12C** and **12D**). On the other hand, PD-1 and CCR7 were greater expressed on the surface of super-charged NK cell-expanded CD8+ T cells than in their primary counterparts (**Fig. 12E** and **12F**). As illustrated in **Fig. 13**, central memory T cells are CD45RO+CCR7+CD28+CD62L+PD-1+CD44+ and effector memory T cells are CD45RO+CCR7-CD28-CD62L-PD-1+CD44+++. Therefore, we propose that super-charged NK cell-expanded CD8+ T cells are mostly effector memory T cells, with a portion of them being central memory T cells.

Cytokine secretion profile of super-charged NK cell expanded CD8+ T cells

In addition to phenotypes of super-charged NK cell expanded CD8+ T cells, we investigated the cytokines and chemokines secretion of super-charged NK cellex expanded, osteoclasts (OCs) expanded and primary CD8+ T cells using Luminex multiplex assay. Overall, primary NK cells exhibited the lowest secretion of all the cytokines and chemokines tested among all three cell types. Super-charged NK cells showed the highest levels of soluble FasL, granzyme B, TNF-α, GM-CSF, soluble CD137, IFN-γ and IL-10, whereas OCs expanded CD8 exhibited the highest levels of MIP-1α, IL-13, granzyme A, IL-4 and perforin (**Table 1**). These indicated that either super-charged NK cell expanded or OCs expanded CD8+ T cells have more superior cytokine/chemokine secretion ability than primary CD8+ T cells.

Discussion

The dynamics of NK cell mediated regulation and activation of CD4+ and CD8+ T cells have not been explored fully and are the subjects of this chapter. We have previously shown that NK functional inactivation and loss of numbers occurs at both the pre-neoplastic and neoplastic stages of pancreatic cancer due to the effects of both the KRAS mutation and high fat calorie diet [55-57]. In this chapter we also demonstrate that patients with pancreatic cancer as well as a few other cancers have severely suppressed NK functions. Both cytotoxicity and the ability to secrete IFN-γ are suppressed in patient NK cells. In addition, we also demonstrate that the percentages of NK, monocyte, and CD11b+ immune cells are increased in cancer patients, even though the total numbers of PBMCs are severely decreased. In addition, the percentages of CD3+ T cells and B cells are substantially decreased. Thus, although the percentages of NK cells are elevated in cancer patients, the function of NK cells are severely depressed, indicating a profound immunosuppression of NK cells from cancer patients. Even when NK cells were purified and expanded and super-charged by the use of OCs [15, 56, 57], the cells from cancer patients had much lower ability to expand and mediate cytotoxicity and secrete IFN-γ when compared to those expanded from healthy individuals. Thus, lower recovery of PBMCs from cancer patients could partly be due to the inability of different lymphocyte subsets such as NK cells to proliferate and expand when compared to those expanded from healthy individuals. Both primary and OC-expanded NK cells from cancer patients are defective in their function, therefore, the defects observed in patients' primary NK cells are dominant and are only moderately improved when these cells are expanded in the presence of allogeneic OCs [15]. Expansion of patients' T cells as well as IFN-γ secretion from OC-expanded T cells are also decreased under different activation

conditions (Fig. 1E-1G). Cancer patients' immune effectors demonstrated higher percentages of CD45RO and decreased percentages of CD62L surface expressions indicating the increased status of immune activation *in vivo*. This is also evident from the increased percentage of CD8+ T cells and decreased ratios of CD4/CD8 in patients (Fig. 3B-3C).

Increased percentages of NK cells in cancer patients can be one reason why we see preferential increase in CD8+ T cells and lower ratios of CD4/CD8 T cells. Our studies indicate that NK cells are very important in the preferential expansion of CD8+ T cells. In particular, OCs are important in the expansion of NK cells as reported previously [15, 56, 57]. The majority of T cells expanded by the NK cells are CD8+ T cells, and similar profile of CD8+ T cell expansion by the NK cells is seen when NK cells are obtained from both healthy individuals and cancer patients indicating that NK cells are indispensable for the expansion of CD8+ T cells. Although OCs have some effect on the decreased ratios of CD4+ to CD8+ T cells in both healthy individuals' and cancer patients' T cells, the ratios are substantially decreased in the presence of NK cells indicating higher selection and expansion of CD8+ T cells and loss of CD4+ T cells by the expanded NK cells (Fig. 3E).

Interestingly, significant differences are observed between DC-induced expansion of NK cells and OC-induced NK cell expansion. Whereas OC-induced expansion of NK cells increased CD8+ T cell expansion, DC-induced expansion of NK cells resulted in expansion of CD4+ T cells. At the moment, the mechanisms governing the differential expansion of CD4+ vs. CD8+ T cells by DC- vs. OC-expanded NK cells respectively are not fully understood. However, there is larger increases in percentages of CD45RO and a higher decrease in percentages of CD62L surface expressions in T cells expanded by OC-expanded NK cells than DC-expanded NK cells indicating increased activation of T cells by the NK cells (Fig. 4L).

It is possible that the higher activation signals by the OC-expanded NK cells are necessary for greater expansion of CD8+ T cells than CD4+ T cells. Indeed, OC-induced expansion of CD8+ T cells when total CD3+ T cells were used for expansion resulted in moderate increase in the expansion of CD8+ T cells and in the slight decline of CD4+ T cells (Fig. 3D and 5A). Therefore, signals from both OCs and NK cells appear to be important in expansion and activation of CD8+ T cells, although the effect of NK cells appears to be more dominant than OCs. In addition, NK cells expanded by OCs have greater cytotoxic activity than those expanded by the DCs potentially providing the mechanism for targeting of CD4+ T cells and sparing CD8+ T cells. In support of this observation we have also observed that NK cells differentially targeted activated CD4+ and CD8+ T cells (Fig 11). The OC-expanded NK cells as well as IL-2+anti-CD16mAb treated NK cells but less primary IL-2 activated NK cells were able to target activated CD4+ T cells (Fig. 11). Indeed, it has previously been shown that NK cells inhibit proliferation of CD4+ T cells under chronic antigen stimulation in the model of GVHD through Fas receptor and not perforin mediated killing, and that the lysis was mediated through the NKG2D ligand expression [43]. In agreement, $CD56^{bright}$ NK cells in comparison to $CD56^{dim}$ subset were found to have higher degranulation and lysis of activated CD4+ T cells [58]. $CD56^{bright}$ NK cells were previously shown by many laboratories to have higher secretion of cytokines in the presence of no or lower cytotoxicity similar to those found with IL-2+anti-CD16mAb treated NK cells which we have previously coined as split anergized NK cells [59, 60]. Therefore, it is possible that the underlying mechanisms of CD4+ T cell lysis is through their death receptors triggered by Fas ligand, TNF-a and TRAIL on NK cells [61]. Indeed, both split anergized NK cells [60] and OC-expanded NK cells have very high induction of Fas ligand and TNF-α (Table 1). However, since super-charged NK cells have also significantly higher granule content with potent cytotoxic function (manuscript

in prep), the granule mediated lysis of CD4+ T cells can't be ruled out in our system at present. Furthermore, it was also shown that upon stimulation with antigen and co-stimulatory signals CD4+ T cells undergo activation induced cell death through Fas receptors whereas CD8+ T cells are rendered non-responsive but gain function when IL-2 is provided [61]. Therefore, there are clear differences between CD4+ and CD8+ T cell subsets in their susceptibility to cell death and mode of expansion. Thus, greater expansion of CD8+ T cells by both OCs and NK cells suggests increased selection as well as expansion of CD8+ T cells since NK cells select and also trigger expansion of CD8+ T cells. On the other hand, it appears that OCs will only aid moderately in expansion since these cells were not shown to have cytotoxic capability. Whether there are differences between the CD4 and CD8 T cells in the magnitude of expansion also requires further studies.

Although OCs were able to expand CD8+ T cells somewhat, the expansion of these cells were significantly accelerated in the presence of OC-expanded NK cells (Fig. 3D-3E and 5A-5B). Therefore, there could be potentially two different mechanisms of CD8+ T cell expansion by the OC-expanded NK cells. One mechanism is likely contributed by the OCs in the initial phases of expansion where there still remain some OCs in the NK cultures which could be approximately up until day 6 or maximum 9 of expansion with fewer or minor expansion of CD8+ T cells. By day 9 no OCs are remaining in the culture of NK cells and therefore, there are only expanding super-charged NK cells with more rapidly expanding CD8+ T cells. Thus, the second mechanism is contributed by the super-charged NK cells which are likely through targeting of remaining CD4+ T cells and selection of CD8+ T cells and activation of CD8+ T cells. Although there are significant numbers of CD4+ T cells remaining after OC-mediated expansion of T cells, in the presence of OC-expanded NK cells the majority if not all are primarily CD8+ T cells (Fig. 3D). At the moment

it is not clear how OCs contribute to the preferential expansion of CD8+ T cells. As for OC-expanded NK mediated expansion of CD8+ T cells, a significantly higher activation of CD8+ T cells in terms of increased percentage of cells expressing CD45RO and lower percentage of cells expressing CD62L is seen with OC-expanded NK cells when compared to just OC activated T cells (Fig. 4L-4M), therefore, it is possible that either NK cells differentially target and kill activated CD4 cells and/or that activation induced cell death is higher in CD4+ T cells than it is in CD8+ T cells. The higher activation of CD8+ T cells by the NK cells in comparison to OC-induced CD8+ T cells is also seen when different cytokine levels were assessed (Fig. 4N-4Q).

The patients are found to have on average higher percentages of CD8+ T cells in their peripheral blood when compared to those obtained from healthy individuals (Fig. 3B-3C). When OC-derived from healthy individuals were used to expand both healthy and patient T cells, OC-expanded T cells also demonstrated higher percentages of CD8+ T cell expansion from both healthy and patient derived T cells, which were higher than those seen from those obtained initially from the peripheral blood (Fig. 3B-3E). Thus, the higher expansion seen with patients' OC-expanded T cells is likely due to the higher frequencies of CD8+ T cells in the patients when compared to healthy individuals. Indeed, on average 1 percent primary CD8+ T cells when expanded by healthy OCs will give rise to 1.2 percent expanded CD8+ T cells by patient T cells, but that average is at 1.6 percent with T cells from healthy individuals, which is higher. Thus, anti-CD3/CD28 activation and signaling through T cells augments the percentages of CD8+ T cells moderately, and the levels of expansion are less by patients' CD8+ T cells when compared to CD8+ T cells expanded from healthy individuals (Fig. 3D-3E). In addition, OC-expanded NK cells expanded 2.73 percent CD8+ T cells from 1 percent of CD8+ T cells from healthy individuals, whereas from patients those percentages remained lower at 1.75 percent which on average is an

almost one percentage point difference (Fig. 3B-3E). Thus, the extent of CD8+ T cell selection and expansion by OC-expanded NK cells is much higher in healthy individuals than those obtained from patients. Based on these results, it is evident that the selection and expansion of CD8+ T cells from both OC-expanded T and OC-expanded NK cells is significantly inferior by the patient cells when compared to those of healthy individuals.

In a second series of experiments we determined the rate of OC mediated CD8+ T cell expansion from both healthy and cancer patients using autologous OCs. Patients' OCs had lower ability to expand autologous CD8+ T cells from OC-expanded T and NK cells and the percentages of expansion were much less when compared to CD8+ expansion from OC expanded T and NK cells from healthy donors in an autologous system. In addition, when the numbers of expanded NK cells and the levels of cytotoxicity and secretion of IFN-γ were assessed in an autologous system much lower levels of expansion, cytotoxicity and IFN-γ secretion could be observed from patient OC-expanded NK cells when compared to OC-expanded NK cells from healthy individuals (Fig. 2E-2G). As expected, using patient OCs for the expansion of NK cells from healthy individuals or healthy OCs with patient NK cells, we observed much lower expansion and function when compared to those obtained from OC-expanded NK cells in healthy individuals in an autologous system (Fig. 2). The lowest levels of expansion and function compared to the three groups mentioned above were seen when patient OCs were used to expand autologous NK cells, indicating that there are functional deficiencies in both NK and OCs from cancer patients, when compared to the function of both cell types from healthy individuals. Thus, these factors should be considered when designing immunotherapeutic strategies using autologous and allogeneic NK cells since even when one provides the best expansion and the highest quality of allogeneic NK cells to patients, the expansion and function of those NK cells will be short lived since the

supporting cells are also defective and will likely not provide the adequate signals for the continued expansion of NK cells. Indeed, when comparing the surface receptor expression between patient and healthy OCs, there is significant down-modulation of activating receptors which could be one reason why the patient OCs may not support the expansion of allogeneic or autologous NK and CD8+ T cells [56]. However, we also see a substantial decrease in MHC-class I inhibitory signals which should provide an activating signal due to decrease binding and inhibition of NK cells through MHC-class I inhibitory receptors. It appears that most receptors are down-modulated on the surface of patient OCs irrespective of whether they are activating or inhibitory ligands for the NK cells [56]. In this scenario it is possible that lack of activating ligands supersedes the effect of lack of inhibitory ligands since OCs from patients are not able to activate autologous NK cells.

In agreement with our *in vitro* studies, injection of OC-expanded NK cells to tumor-bearing hu-BLT mice increased the numbers of CD8+ T cells in BM, spleen, and peripheral blood resulting in the increased levels of NK cell-mediated cytotoxicity as well as increased secretion of IFN-γ (Fig. 6B-6J). Increased levels of IFN-γ, IL-6, ITAC were also observed in the sera of tumor-bearing hu-BLT mice injected with OC-expanded NK cells (Fig. 6K-6O).

Potential relevance of our observations could be seen in the studies reported with multiple myeloma (MM) patients. Indeed, these patients have multifocal neoplastic proliferation of monoclonal plasma cells in the bone marrow where significant numbers of OCs reside. It was shown that these patients had higher levels of NK and CD8+ T cells in both peripheral blood and bone marrow aspirates when compared to heathy controls [12]. It was also found that the ratio of CD4/CD8 was decreased in the patients and this decrease was co-related with an increase in human leukocyte antigen (HLA)-DR expression by CD8+ but not CD4+ T cells [62]. Moreover, it was noted that patients with long-term disease control exhibited an expansion of cytotoxic CD8+ T

cells and natural killer cells [9]. T cell expansions in MM patients have a phenotype of cytotoxic T cells, with expanded V-beta TCR populations having predominantly CD8+, CD57+, CD28- and perforin+ phenotype [11]. Although our observations are likely relevant to MM patients since they exhibit significant BM pathology, it is also likely that the mechanisms discussed in this chapter can also occur in patients who may sustain bone metastasis or have primary tumors inflicting bone. Prostate, breast, and lung cancers have predilection to metastasize to bone [63-66], although pancreatic and colon cancers can also metastasize to bone [67-70]. Whether these mechanisms are operational in osteoporosis or during non-metastatic bone pathologies should await future studies.

Reference:

1. Vela-Ojeda, J., et al., *Post-treatment improvement of NK cell numbers predicts better survival in myeloma patients treated with thalidomide-based regimens.* Int J Hematol, 2019. **110**(3): p. 306-312.
2. Tang, Y.-p., et al., *Prognostic value of peripheral blood natural killer cells in colorectal cancer.* BMC Gastroenterology, 2020. **20**(1): p. 31.
3. Levy, E.M., M.P. Roberti, and J. Mordoh, *Natural killer cells in human cancer: from biological functions to clinical applications.* Journal of biomedicine & biotechnology, 2011. **2011**: p. 676198-676198.
4. Kuss, I., et al., *Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck.* Clin Cancer Res, 2004. **10**(11): p. 3755-62.
5. Kim, J.W., et al., *Expression of pro- and antiapoptotic proteins in circulating CD8+ T cells of patients with squamous cell carcinoma of the head and neck.* Clin Cancer Res, 2004. **10**(15): p. 5101-10.
6. Qin, C., et al., *Dysregulation of immune response in patients with COVID-19 in Wuhan, China.* Clin Infect Dis, 2020.
7. Zheng, M., et al., *Functional exhaustion of antiviral lymphocytes in COVID-19 patients.* Cell Mol Immunol, 2020.
8. Krejcik, J., et al., *Daratumumab depletes CD38+ immune regulatory cells, promotes T-cell expansion, and skews T-cell repertoire in multiple myeloma.* Blood, 2016. **128**(3): p. 384-94.
9. Pessoa de Magalhaes, R.J., et al., *Analysis of the immune system of multiple myeloma patients achieving long-term disease control by multidimensional flow cytometry.* Haematologica, 2013. **98**(1): p. 79-86.
10. San Miguel, J.F., et al., *Lymphoid subsets and prognostic factors in multiple myeloma. Cooperative Group for the Study of Monoclonal Gammopathies.* Br J Haematol, 1992. **80**(3): p. 305-9.
11. Raitakari, M., et al., *T-cell expansions in patients with multiple myeloma have a phenotype of cytotoxic T cells.* Br J Haematol, 2000. **110**(1): p. 203-9.
12. Pasiarski, M., et al., *Assessment of peripheral blood and bone marrow T, NK, NKT and dendritic cells in patients with multiple myeloma.* Postepy Hig Med Dosw (Online), 2015. **69**: p. 1435-42.
13. Tseng, H.C., et al., *Bisphosphonate-induced differential modulation of immune cell function in gingiva and bone marrow in vivo: role in osteoclast-mediated NK cell activation.* Oncotarget, 2015. **6**(24): p. 20002-25.
14. Kozlowska, A.K., et al., *Adoptive transfer of osteoclast-expanded natural killer cells for immunotherapy targeting cancer stem-like cells in humanized mice.* Cancer Immunol Immunother, 2016. **65**(7): p. 835-45.
15. Kaur, K., et al., *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.* Front Immunol, 2017. **8**: p. 297.

16. Boyce, B.F., E.M. Schwarz, and L. Xing, *Osteoclast precursors: cytokine-stimulated immunomodulators of inflammatory bone disease.* Curr Opin Rheumatol, 2006. **18**(4): p. 427-32.
17. Mansour, A., A. Wakkach, and C. Blin-Wakkach, *Emerging Roles of Osteoclasts in the Modulation of Bone Microenvironment and Immune Suppression in Multiple Myeloma.* Frontiers in immunology, 2017. **8**: p. 954-954.
18. Vivier, E., et al., *Innate or adaptive immunity? The example of natural killer cells.* Science, 2011. **331**(6013): p. 44-9.
19. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends Immunol, 2001. **22**(11): p. 633-40.
20. Sun, J.C. and L.L. Lanier, *Is There Natural Killer Cell Memory and Can It Be Harnessed by Vaccination? NK Cell Memory and Immunization Strategies against Infectious Diseases and Cancer.* Cold Spring Harb Perspect Biol, 2018. **10**(10).
21. Burke, S., et al., *New views on natural killer cell-based immunotherapy for melanoma treatment.* Trends Immunol, 2010. **31**(9): p. 339-45.
22. Larsen, S.K., Y. Gao, and P.H. Basse, *NK cells in the tumor microenvironment.* Crit Rev Oncog, 2014. **19**(1-2): p. 91-105.
23. Nolibe, D. and M.F. Poupon, *Enhancement of pulmonary metastases induced by decreased lung natural killer cell activity.* J Natl Cancer Inst, 1986. **77**(1): p. 99-103.
24. Imai, K., et al., *Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population.* Lancet, 2000. **356**(9244): p. 1795-9.
25. Harning, R., G.C. Koo, and J. Szalay, *Regulation of the metastasis of murine ocular melanoma by natural killer cells.* Invest Ophthalmol Vis Sci, 1989. **30**(9): p. 1909-15.
26. Coca, S., et al., *The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma.* Cancer, 1997. **79**(12): p. 2320-8.
27. Bruno, A., et al., *A think tank of TINK/TANKs: tumor-infiltrating/tumor-associated natural killer cells in tumor progression and angiogenesis.* J Natl Cancer Inst, 2014. **106**(8): p. dju200.
28. Gross, E., J.B. Sunwoo, and J.D. Bui, *Cancer immunosurveillance and immunoediting by natural killer cells.* Cancer J, 2013. **19**(6): p. 483-9.
29. Mirjacic Martinovic, K.M., et al., *Decreased expression of NKG2D, NKp46, DNAM-1 receptors, and intracellular perforin and STAT-1 effector molecules in NK cells and their dim and bright subsets in metastatic melanoma patients.* Melanoma Res, 2014. **24**(4): p. 295-304.
30. Gubbels, J.A., et al., *MUC16 provides immune protection by inhibiting synapse formation between NK and ovarian tumor cells.* Mol Cancer, 2010. **9**: p. 11.
31. Balsamo, M., et al., *Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity.* Proc Natl Acad Sci U S A, 2009. **106**(49): p. 20847-52.
32. Castriconi, R., et al., *Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4120-5.

33. Pietra, G., et al., *Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity.* Cancer Res, 2012. **72**(6): p. 1407-15.
34. Krockenberger, M., et al., *Macrophage migration inhibitory factor contributes to the immune escape of ovarian cancer by down-regulating NKG2D.* J Immunol, 2008. **180**(11): p. 7338-48.
35. Assarsson, E., et al., *NK cells stimulate proliferation of T and NK cells through 2B4/CD48 interactions.* J Immunol, 2004. **173**(1): p. 174-80.
36. Zingoni, A., et al., *Cross-talk between activated human NK cells and CD4+ T cells via OX40-OX40 ligand interactions.* J Immunol, 2004. **173**(6): p. 3716-24.
37. Gerosa, F., et al., *Reciprocal activating interaction between natural killer cells and dendritic cells.* J Exp Med, 2002. **195**(3): p. 327-33.
38. Crouse, J., et al., *NK cells regulating T cell responses: mechanisms and outcome.* Trends Immunol, 2015. **36**(1): p. 49-58.
39. Crome, S.Q., et al., *Natural killer cells regulate diverse T cell responses.* Trends Immunol, 2013. **34**(7): p. 342-9.
40. Zingoni, A., et al., *NKG2D and DNAM-1 activating receptors and their ligands in NK-T cell interactions: role in the NK cell-mediated negative regulation of T cell responses.* Front Immunol, 2012. **3**: p. 408.
41. Pallmer, K. and A. Oxenius, *Recognition and Regulation of T Cells by NK Cells.* Front Immunol, 2016. **7**: p. 251.
42. Nielsen, N., et al., *Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A.* PloS one, 2012. **7**(2): p. e31959-e31959.
43. Noval Rivas, M., et al., *NK cell regulation of CD4 T cell-mediated graft-versus-host disease.* J Immunol, 2010. **184**(12): p. 6790-8.
44. Shaw, S.Y., et al., *Selective modulation of autophagy, innate immunity, and adaptive immunity by small molecules.* ACS Chem Biol, 2013. **8**(12): p. 2724-2733.
45. Pant, H., et al., *Accumulation of effector memory CD8+ T cells in nasal polyps.* Am J Rhinol Allergy, 2013. **27**(5): p. e117-26.
46. Zanetti, M., *Tapping CD4 T cells for cancer immunotherapy: the choice of personalized genomics.* J Immunol, 2015. **194**(5): p. 2049-56.
47. Seo, A.N., et al., *Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer.* Br J Cancer, 2013. **109**(10): p. 2705-13.
48. Hadrup, S., M. Donia, and P. Thor Straten, *Effector CD4 and CD8 T cells and their role in the tumor microenvironment.* Cancer Microenviron, 2013. **6**(2): p. 123-33.
49. Kim, S.T., et al., *Tumor-infiltrating lymphocytes, tumor characteristics, and recurrence in patients with early breast cancer.* Am J Clin Oncol, 2013. **36**(3): p. 224-31.
50. Shah, W., et al., *A reversed CD4/CD8 ratio of tumor-infiltrating lymphocytes and a high percentage of CD4(+)FOXP3(+) regulatory T cells are significantly associated with clinical outcome in squamous cell carcinoma of the cervix.* Cell Mol Immunol, 2011. **8**(1): p. 59-66.

51. Tomala, J., et al., *In vivo expansion of activated naive CD8+ T cells and NK cells driven by complexes of IL-2 and anti-IL-2 monoclonal antibody as novel approach of cancer immunotherapy.* J Immunol, 2009. **183**(8): p. 4904-12.
52. Tanaka, J., et al., *Differential expression of natural killer cell receptors (CD94/NKG2A) on T cells by the stimulation of G-CSF-mobilized peripheral blood mononuclear cells with anti-CD3 monoclonal antibody and cytokines: a study in stem cell donors.* Transplant Proc, 2004. **36**(8): p. 2511-2.
53. Robbins, S.H., et al., *Natural killer cells promote early CD8 T cell responses against cytomegalovirus.* PLoS Pathog, 2007. **3**(8): p. e123.
54. Wodarz, D., S. Sierro, and P. Klenerman, *Dynamics of killer T cell inflation in viral infections.* J R Soc Interface, 2007. **4**(14): p. 533-43.

Table 1. Cytokines and chemokines secretion profile of super-charged NK cell expanded, osteoclast expanded and primary CD8+ T cells.

	sFasL	Granzyme B	TNF-α	GM-CSF	sCD137	IFNγ	IL-10
CD8 sorted from expanding NK	3500	294000	6000	5500	1000	1796500	1000
OC Expanded CD8	1500	292000	3000	3500	500	1304000	500
primary CD8	1000	56500	2000	500	0	359500	0

Unit: pg/mL

Table 1 continued, Cytokines and chemokines profile of super-charged NK cell expanded, osteoclast expanded and primary CD8+ T cells

	MIP-1α	IL-13	Granzyme A	IL-4	Perforin
CD8 sorted from expanding NK	31500	1500	1450500	4500	17000
OC Expanded CD8	66000	2000	2747000	9000	25000
primary CD8	4000	0	167000	0	3000

Unit: pg/mL

Table 1 continued, Cytokines and chemokines profile of super-charged NK cell expanded, osteoclast expanded and primary CD8+ T cells

	MIP-1b	sFas	IL-5	IL-6
CD8 sorted from expanding NK	135000	21000	500	0
OC Expanded CD8	138500	21000	500	0
primary CD8	38500	15500	0	0

Unit: pg/mL

Figure Legend

Figure 1: OC-expanded NK cells from cancer patients have much lower capacity to expand, mediate cytotoxicity, and secrete IFN-γ.

NK cells from cancer patients and healthy individuals were expanded by OCs as described in Materials and Methods. the numbers of lymphocytes were counted using microscopy (n=70) **(A)**. And the cytotoxicity of day 15 cultured OC-expanded NK cells was determined using standard 4-hour ^{51}Cr release assay against OSCSCs (n=16) **(B)**. Supernatants of the culture were collected on days 6, 9, 12 and 15 to determine IFN-γ secretion using single ELISA (n=63) **(C)**. The amounts of IFN-γ secretion were analyzed based on 1×10^6 cells as shown in **(D)**(n=63). T cells (1×10^6 cells/ml) from healthy individuals and cancer patients were treated with a combination of IL-2 (100 U/ml) and anti-CD3 (1 μg/ml)/CD28mAb (3 μg/ml) for 18 hours before they were co-cultured with healthy individuals' OCs and sAJ2 at a ratio of 1:2:4 (OCs:T:sAJ2). On days 6, 9, 12, and 15, the OC-expanded T cells were counted using microscopy; the cumulative cell counts from day 0 to day 15 are displayed in the figure (n=7) **(E)**. The supernatants were harvested on days 6, 9, 12, and 15, and the levels of IFN-γ secretion were determined using single ELISA (n=42) **(F)**. Amounts of IFN-γ secretion shown in Fig. 1F were adjusted based on 1×10^6 cells (n=42) **(G)**. NK cells and T cells were treated and cultured as described in Fig. 1A and Fig. 1E respectively. The cells were counted using microscopy on days 6, 9, 12, and 15; the cumulative cell counts from day 0 to day 15 are displayed in the figure (n=10) **(H)**. The supernatants of OC-expanded NK and T cells cultures were then harvested on days 6, 9, 12, and 15 and the amounts of IFN-γ secretion were determined using single ELISA; the cumulative levels of IFN-γ secretion from day 0 to day 15 are displayed in the figure (n=10) **(I)**.

Figure 2: Unlike those from healthy individuals, OCs from Cancer patients induced decreased cell expansion, IFN-γ secretion and cytotoxicity in allogeneic NK cells obtained from healthy individuals.

NK cells from healthy individuals were expanded by OCs from either cancer patients or healthy individuals. On days 6, 9, 12, 15, 18 and 22 of co-culture, the numbers of NK cells were counted using microscopy (n=12) **(A)**. The culture supernatants were harvested on days 6, 9, 12, 15, 18 and 22 to determine IFN-γ secretion using single ELISA (n=12) **(B)** and adjusted based on 1×10^6 cells (n=12) **(C)**. Cytotoxicity of days 9 and 15 OC-expanded NK cells were determined using a standard 4-hour ^{51}Cr release assay against OSCSCs (n=4) **(D)**. NK cells (1×10^6 cells/ml) from healthy individuals and cancer patients were cultured alone or expanded with autologous OCs. On days 6, 9, 12, and 16 of co-culture, the numbers of NK cells were counted using microscopy **(E)**. Cytotoxicity of days 9 and 15 cultured NK cells with or without autologous OCs were determined using a standard 4-hour ^{51}Cr release assay against OSCSCs (n=4) **(F)**. The supernatants were collected on days 6 and 12 to determine IFN-γ secretion using single ELISA **(G)**.

Figure 3: T cells from Cancer patients exhibit lower CD4+/CD8+ T cell ratio both in peripheral blood and after expansion.

T cells purified from PBMCs of healthy individuals and cancer patients were analyzed for the surface expression of CD45RO, CD45RA, CD62L, CD28, CCR7, and CD127 using flow cytometry. IgG isotype control was used to assess non-specific binding. One of 12 representative experiments is shown in the figure **(A)**. PBMCs isolated from the peripheral blood of healthy individuals and cancer patients were used to determine the surface expression of CD4 and CD8 using flow cytometry, and the percentages of CD4+ and CD8+ T cells were determined within

CD3+ populations (n=12) **(B)**, The ratio of CD4:CD8 is shown in the figure (n=12) **(C)**. NK and T cells of healthy individuals and cancer patients were treated and co-cultured as described in Fig. 1A and Fig. 1E respectively. On days 6, 9, 12, and 15 of co-culture, the surface expression of CD4 and CD8 were analyzed by flow cytometry to obtain the percentages of CD4+ and CD8+ T cells within CD3+ populations (n=28) **(D)**. The ratio of CD4:CD8 is shown in the figure (n=28) **(E)**.

Figure 4A-M: OC-expanded NK cells induced CD8+ T cell expansion whereas DC-expanded NK cells promote CD4+ T cell expansion.

OCs and DCs were generated as described in Materials and Methods. NK cells from healthy individuals (1×10^6 cells/ml) were treated with a combination of IL-2 (1000 U/ml) and anti-CD16mAb (3 μg/ml) for 18 hours before they were co-cultured with autologous DCs or OCs in the presence of sAJ2 at 1:2:4 ratios (DCs or OCs:NK:sAJ2). The expanding cells were counted on days 8, 11, 15, and 18 using microscopy (n=30) **(A).** NK cells were co-cultured with OCs or DCs as described in Fig. 4A, and the surface expressions of CD3, CD16, and CD56 were analyzed on days 8, 11, 15, and 18 using flow cytometry. The numbers of NK cells and T cells were determined using the percentages of CD16+ and CD3+ cells, respectively, within the total cells in Fig. 4A (n=30) **(B, C)**. NK cells were co-cultured with OCs or DCs as described in Fig. 4A and cytotoxicity of day 15 expanded cells was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs. LU 30/10^6 cells were determined using the method described in Fig. 1B (n=12) **(D)**. The surface expressions of CD16 were analyzed on day 15 using flow cytometry. The levels of the cytotoxicity was determined based on 1% of CD16+NK cells (n=12) **(E)**. NK cells were co-cultured with OCs or DCs as described in Fig. 4A; the supernatants were harvested on days 8, 11, 15, and 18 of the co-cultures, and the amounts of IFN-γ secretion were determined using single

ELISA (n=12) **(F)**. On days 8, 11, 15, 18, 22 and 25 of the co-cultures the surface expressions of CD3+CD4+ and CD3+CD8+ T cells were determined using flow cytometry, and the percentages were used to determine the total numbers of CD3+CD4+ and CD3+CD8+ cells within the total cells (n=12) **(G, H).** the surface expressions of CD3, CD4, and CD8 were analyzed on days 8, 11, 15, and 18 using flow cytometry. Percentages of CD4+ and CD8+ T cells within the CD3+ populations are shown in this figure (n=12) **(I)**. The surface expressions of CD4, CD8, KLRG1, TIM3, and PD-1 were analyzed within CD3+ cells on day 27 of the co-cultures using flow cytometry (n=8) **(J).** T cells (1×10^6 cells/ml) from healthy individuals were treated with a combination of IL-2 (100 U/ml) and anti-CD3 (1 μg/ml)/CD28mAb (3 μg/ml) for 18 hours before they were co-cultured with autologous DCs or OCs in the presence of sAJ2 at 1:2:4 ratios (DCs or OCs:T:sAJ2). Surface expressions of CD4, CD8, KLRG1, TIM3, and PD-1 were analyzed within CD3+ cells on day 27 of the co-culture using flow cytometry (n=8) **(K)**. Surface expressions of CD45RO, CD62L, CD28, CD44, CCR7, and CD127 were analyzed within CD3+ cells on day 12 of the co-culture using flow cytometry (n=8) **(L)**. The surface expressions of CD3, CD16, CD56, CD4, CD8, CD28, and CD62L were analyzed on day 12 of the co-culture using flow cytometry (n=8) **(M)**.

Figure 4N-Q: OC-mediated activation induced higher secretion of cytokines and chemokines from NK cells when compared to T cells.

Freshly purified NK cells from the healthy individuals were treated and co-cultured. On day 12 of the co-culture; NK and NK-expanded CD8+ T cells were isolated from the expanded NK cells using the corresponding isolation kits. NK cells were treated with a combination of IL-2 (1000 U/ml) and anti-CD16 mAb (3 μg/ml) and, CD8+ T cells were treated with IL-2 (100 U/ml) and

anti-CD3 (1 μg/ml)/CD28 mAb (3 μg/ml) for 18 hours. The supernatants were then harvested and were used to determine the levels of cytokines, chemokines, and growth factors using multiplex array kits. The amounts of all tested factors were adjusted based on 1 million cell counts and, the ratios of secretion between NK and CD8+ T cells (NK/CD8+ T cells) were determined and fold increase in the secreted levels for NK cells were determined **(N)**. The supernatants were then harvested on day 6 of co-culture, and the levels of cytokines, chemokines, and growth factors were measured using multiplex array kits. Ratios of secretion between NK and T cells (NK/ T cells) were determined and fold increase in the secreted levels for NK cells were determined. **(O)**. The secreted levels shown in Fig. S4B were adjusted based on 1 million cell counts and, the ratios of secretion between NK and T cells (NK/ T cells) were determined and fold increase in the secreted levels for NK cells were determined **(P)**. In a separate culture, freshly isolated CD8+ T cells purified from healthy individuals were treated with IL-2 (100 U/ml) and anti-CD3 (1 μg/ml)/CD28 mAb (3 mg/ml) for 18 hours before they were cultured with OCs at a ratio of 1:2:4 (OCs:CD8+T:sAJ2). On day 12, CD8+ T cells were isolated from OC-expanded NK cells. CD8+ T cells isolated from OC-expanded NK cells and those from OC-expanded CD8+ T cells were further treated with IL-2 (100 U/ml) and anti-CD3 (1 μg/ml)/CD28 mAb (3 mg/ml) and after 18 hours of incubation, the supernatants were harvested from both CD8+ T cells cultures, and the levels of cytokines, chemokines, and growth factors were measured using multiplex array kits. Ratios of secretion between CD8+ T cells isolated from OC-expanded NK cells and OC-expanded CD8+ T cells were determined and fold increase in the secreted levels for CD8+ T cells isolated from OC-expanded NK cells were determined **(Q)**. One of three representative experiments is shown here.

Figure 5: OC-induced activation increases CD8+ T cells.

NK cells and T cells were purified from healthy individuals' PBMCs and the surface expressions of CD3, CD16, and CD56 on freshly isolated NK cells (left panel) and of CD3, CD4 and CD8 on freshly isolated T cells (upper right panel) were determined using flow cytometry. NK cells were treated and co-cultured with OCs and sAJ2 as described in Fig. 1A (middle right panel), and T cells were treated and co-cultured with OCs and sAJ2 as described in Fig. 1E (lower right panel). Surface expressions of CD3, CD4, and CD8 were analyzed on day 12 of the co-culture using flow cytometry **(A)**. NK cells were treated and co-cultured with OCs and sAJ2 as described in Fig. 1A (left bar). Freshly purified CD8+ T cells (1×10^6 cells/ml) from healthy individuals were treated with a combination of IL-2 (100 U/ml) and anti-CD3 (1 µg/ml)/CD28mAb (3 µg/ml) for 18 hours before they were co-cultured with OCs and sAJ2 at 1:2:4 ratios (OCs:CD4T or CD8T:sAJ2) (right bar). On days 6, 9, 12 and 15 of the co-cultures, the surface expressions of CD3+CD8+ T cells were determined using flow cytometry, and the percentages were used to determine the numbers of CD8+ T cells within the total cells. Fold expansion for each time point is shown in the figure (n=4) **(B)**. Freshly purified CD8+ T cells and CD4+ T cells (1×10^6 cells/ml) from healthy individuals were treated with a combination of IL-2 (100 U/ml) and anti-CD3 (1 µg/ml)/CD28mAb (3 µg/ml) for 18 hours before they were co-cultured with sAJ2 (T:sAJ2; 1:2) or with OCs and sAJ2 at 1:2:4 ratios (OCs:CD4T or CD8T:sAJ2). On days 6, 12, 15, and 19 of co-culture, the expanded cells were counted using microscopy (n=20) **(C)**. Purified CD8+ T cells and CD4+ T cells were treated and co-cultured with sAJ2 as described in Fig. 5C. On days 6, 12, 15 and 19 of co-culture, the expanded cells were counted using microscopy. Fold expansion for each time point is shown in the figure **(D)**. Freshly purified CD8+ T cells and CD4+ T cells were treated and co-cultured with sAJ2 and OCs as described in Fig. 5C. On days 6, 12, 15 and 19 of co-culture,

the expanded cells were counted using microscopy. Fold expansion for each time point is shown in the figure **(E)**. NK cells were treated and co-cultured with OCs as described in Fig. 1A**.** CD8+ T cells and CD4+ T cells were treated as described in Fig. 5C. On days 6, 9, 12 and 15 of co-culture, the expanded cells were counted using microscopy. The numbers of OC-expanded NK, OC-expanded CD4+ T and OC-expanded CD8+ T cells were subtracted from the number of non-OC-expanded control cells and the fold expansions were determined by dividing the resulting value by the initial input cells (n=6) **(F)**. NK cells were treated and co-cultured with OCs as described in Fig. 1A. CD8+ T cells and CD4+ T cells were treated as described in Fig. 5C. The supernatants were harvested from the co-cultures on days 6, 9, 12, and 15 and the secretions of IFN-γ were determined using single ELISA, and the values were adjusted based on a million lymphocytes (n=3) **(G)**.

Figure 6: OC-expanded NK cell immunotherapy increased CD8+ T cells, IFN-γ secretion, and NK cell-mediated cytotoxicity in BM, spleen, and peripheral blood of hu-BLT mice.

Hu-BLT mice were orthotopically injected with 1×10^6 human OSCSCs into the floor of the mouth. One to two weeks after the tumor implantation, mice received OC-expanded NK cells via tail-vein injection. The disease progression and weight loss were monitored for another 3-4 weeks (n=3) **(A)**. Hu-BLT mice were implanted with OSCSC tumors and were injected with NK cells as depicted in Fig. 6A. At the end of experiment, hu-BLT mice were sacrificed; the spleens, BM, and peripheral blood were harvested; and single cell suspensions were obtained and cultured as described in the Materials and Methods. Surface expressions of CD3 and CD8 were analyzed on day 7 of BM cultures (n=3) **(B)**, spleen cultures (n=3) **(E)**, and PBMC cultures (n=2) **(H)** using flow cytometry. The supernatants were harvested from the cultures on day 7 of BM culture (n=3)

(C), spleen culture (n=3) **(F)**, and PBMC culture (n=2) **(I),** and the secretions of IFN-γ were determined using single ELISA. Cytotoxicity of day 7 cultured BMs (n=3) **(D)**, spleens (n=3) **(G)**, and PBMCs (n=2) **(J)** were determined against OSCSCs using standard 4-hour ^{51}Cr release assay. LU 30/10^6 cells were determined using the method described in Fig. 1B. Reconstituted hu-BLT mice were injected with 1×10^6 human OSCSCs into the floor of the mouth. One to two weeks later, hu-BLT mice were intravenously injected with 1.5×10^6 OC-expanded NK cells. Disease progression was monitored for another 3-4 weeks, after which mice were sacrificed. Peripheral blood was collected in heparin-free vials post-mortem by cardiac puncture, and serum samples were harvested as described in Supplementary Materials and Methods, and analyzed for IFN-γ **(K)**, IL-6 **(L)**, ITAC **(M)**, IL-8 **(N),** and GM-CSF **(O)** secretions using multiplex arrays. One of the three representative experiments is shown in here.

Figure 7, NK expansion with additional autologous CD4+ and CD8+ T cells showed the same expansion rate as well as comparable NK functions. NK cells were freshly isolated from healthy donor's PBMCs and treated with IL-2 (1000 U/ml) and anti-CD16 mAb (3 μg/ml) overnight before co-culturing with feeder cells, osteoclasts, and sonicated probiotic bacteria, sAJ2. Autologous CD4+ and CD8+ T cells were isolated and were added into the purified NK cells for co-culture. Cell number were counted on days 6, 10, 13 and 15 using microscopy **(A)**. Cytotoxicity of expanded NK cells was assessed using chromium release assay **(B)** IFN-γ secretion of expanded NK cell culture was determined on day6, 10 and 13 **(C)**. NK purity was determined using flow cytometric analysis during the culture period **(D)**. CD4+ and CD8+ T cells are measured and analyzed with CD3+ population (total T cell population) as shown in **(**without additional CD3 **(E)**; with additional CD3 **(F)**. (n=3)

Figure 8, There is no difference in cell number between CD4+ and CD8+ T cells among all treatment groups. CD4+ and CD8+ T cells are isolated from PBMCs and treated with IL-2, the combination of IL-2 and anit-CD3/28 antibody or being left untreated overnight before using in the experiment. No treatment **(A)** and CDDP (0.5 μM)**(B)** were conducted to evaluate the cell death of CD4+ and CD8+ T cells. Cell number were counted for 6 days to determine the viable cell number in the culture.

Figure 9, No difference in % cell death between CD4+ and CD8+ T cells. CD4+ and CD8+ T cells are isolated from PBMCs and being left untreated **(A, B)**, treated with IL-2 **(C, D)**, the combination of IL-2 and anit-CD3/28 antibody **(E, F)** overnight before using in the experiments. Propidium iodide staining was used to evaluate the % cell death using flow cytometric analysis. One representative experiment is as shown in (**A**, **C**, and **E**) and compiled experiments are shown in (**B, D,** and **F**)

Figure 10, There is no difference of cell death between CD4+ and CD8+ T cells when treated with CDDP. CD4+ and CD8+ T cells are isolated from PBMCs and being left untreated **(A, B)**, treated with IL-2 **(C, D)**, the combination of IL-2 and anit-CD3/28 antibody **(E, F)** overnight before CDDP (0.5μM) was added to the cell culture. Propidium iodide staining was used to evaluate the % cell death using flow cytometric analysis. One representative experiment is as shown in (**A**, **C**, and **E**) and compiled experiments are shown in (**B, D,** and **F**)

Figure 11, Primary NK cells show no or limited cytotoxicity against both CD4+ and CD8+ T cells among all treated groups. CD4+ and CD8+ cells were isolated from PBMCs and were left

untreated, treated with IL-2 or treated with IL-2 and anti-CD3/28 antibody overnight before using in TVA. Primary NK cells were isolated from PBMCs and both primary and super-charged NK cells were left untreated or treated with IL-2 overnight before using in the TVA as effector cells. Lytic unit $30/10^6$ cells or Lytic unit $30/10^7$ were calculated to evaluate the cytotoxicity of NK cells to CD4+ or CD8+ T cells. Compiled data for untreated **(A)** and IL-2 treated **(B)** primary NK cells are as shown. One representative experiment of super-charged nK cells is as shown in **(C)**. A scatter plot of lytic unit $30/10^7$ cells of three independent experiments is shown in **(D)**

Figure 12, Super-charged NK cell expanded CD8 exhibited central memory T cell profile. Primary CD8+ T cells are freshly isolated from PBMC of healthy donors and super-charged NK cell expanded CD8 were obtained from super-charged NK cell culture. Both cells were treated with IL-2 (1000 U/ml) overnight before using for flow cytometry. CD45RA **(A)** CD45RO **(B)**, CD28 (**C**), CD62L (**D**), PD-1 (**E**) and CCR7 (**F**)

Figure 13. Scheme of surface markers expression on T cells at different stage. Changes of surface markers on the surface of T cells across different stage are obtained from three publications and as illustrated.

Figure

Fig. 1

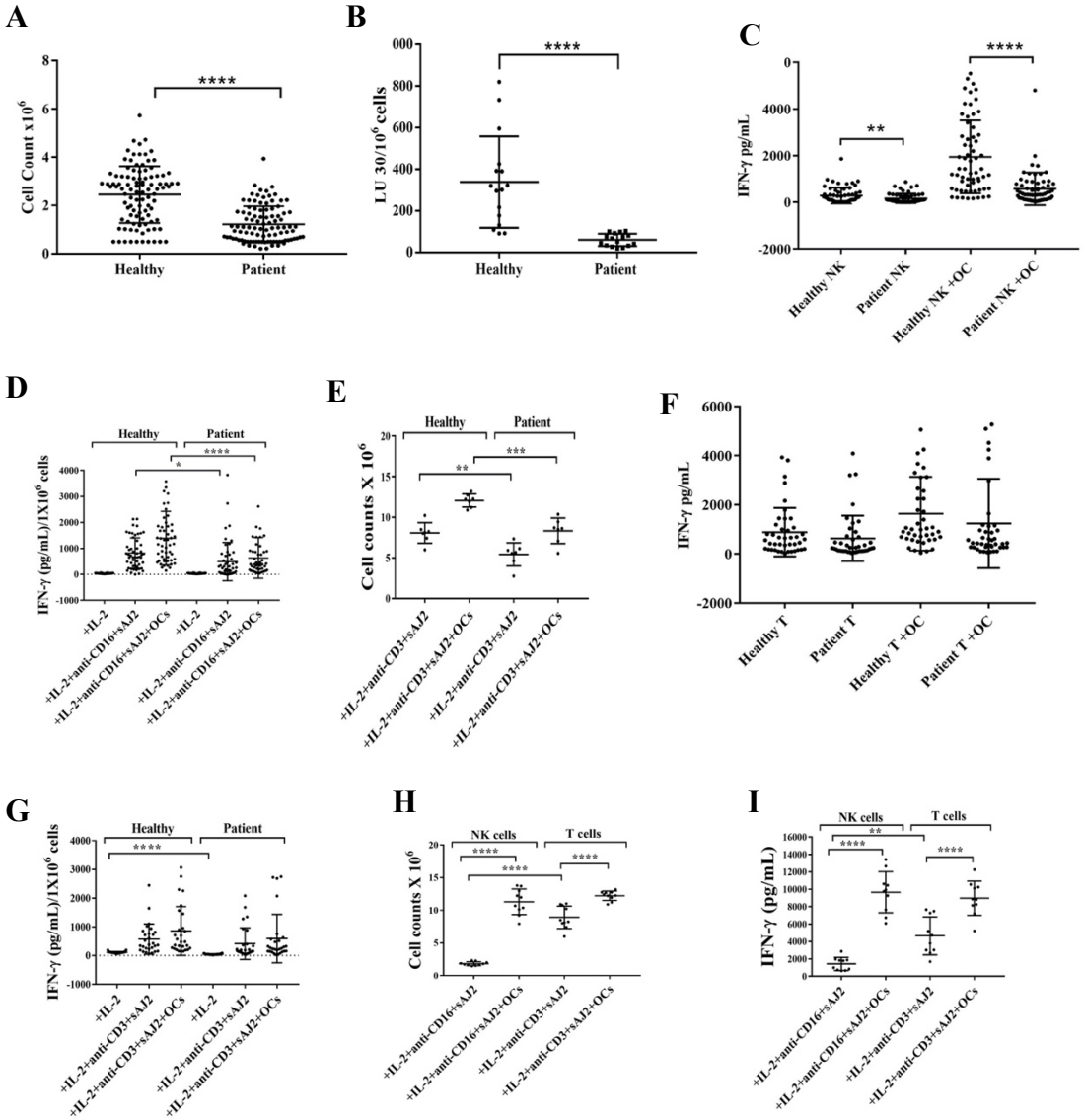

A
Cell Count x10^6
****
Healthy
Patient
B
LU 30/10^6 cells
****
Healthy
Patient
C
IFN-γ pg/mL
**
****
Healthy NK
Patient NK
Healthy NK +OC
Patient NK +OC
D
IFN-γ (pg/mL)/1X10^6 cells
Healthy
Patient
+IL-2
+IL-2+anti-CD16+sAJ2
+IL-2+anti-CD16+sAJ2+OCs
E
Cell counts X 10^6
Healthy
Patient
+IL-2+anti-CD3+sAJ2
+IL-2+anti-CD3+sAJ2+OCs
F
IFN-γ pg/mL
Healthy T
Patient T
Healthy T +OC
Patient T +OC
G
IFN-γ (pg/mL)/1X10^6 cells
Healthy
Patient
H
Cell counts X 10^6
NK cells
T cells
I
IFN-γ (pg/mL)
NK cells
T cells

Figure 2

Fig. 2A

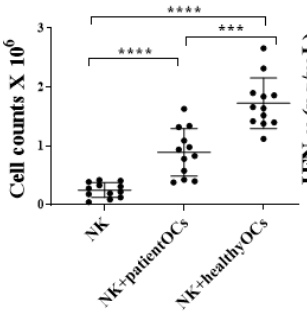


Fig. 2B

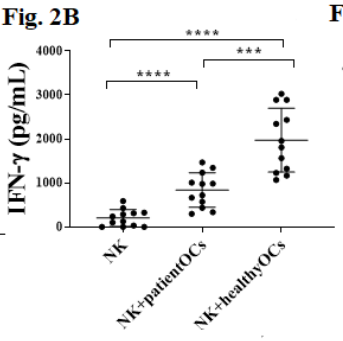


Fig. 2C

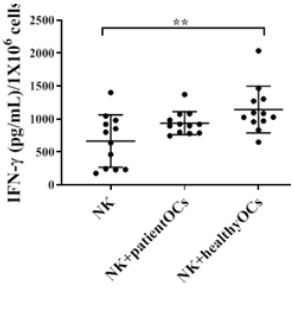


Fig. 2D

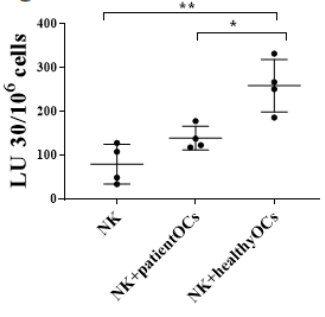


Fig. 2E

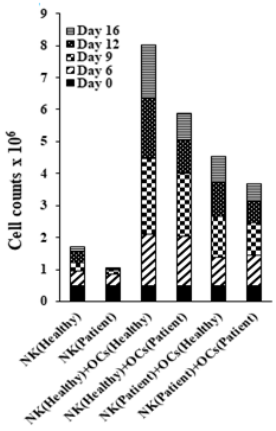


Fig. 2F

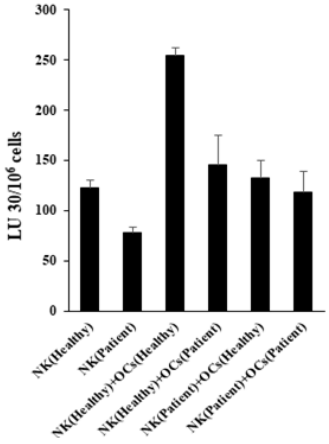


Fig. 2G

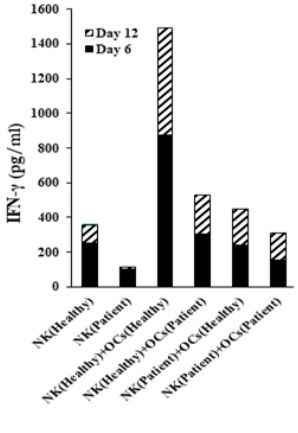


Figure 3

Fig. 3A

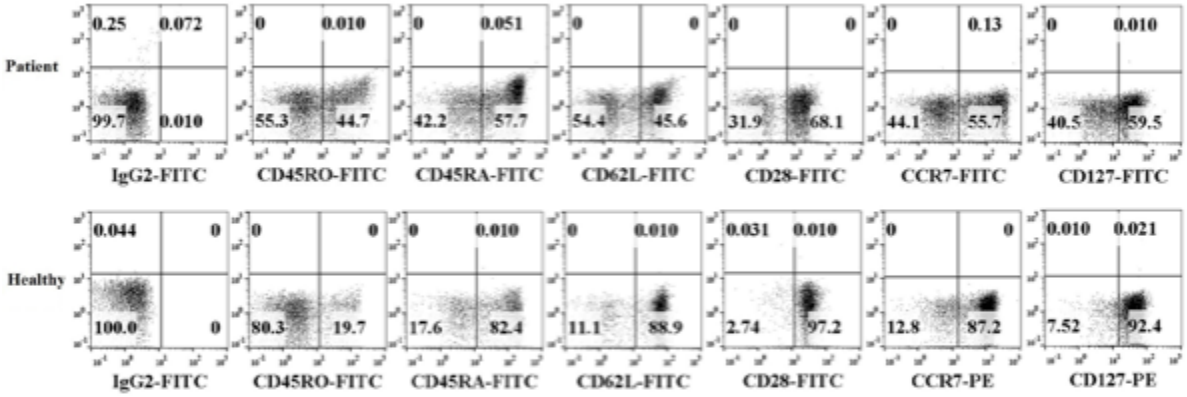


Fig. 3B

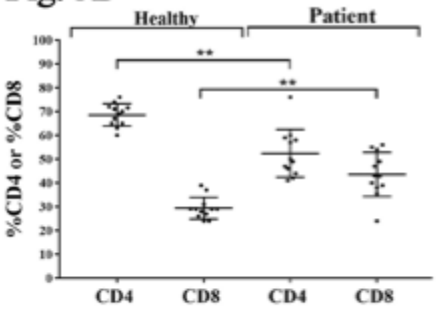


Fig. 3C

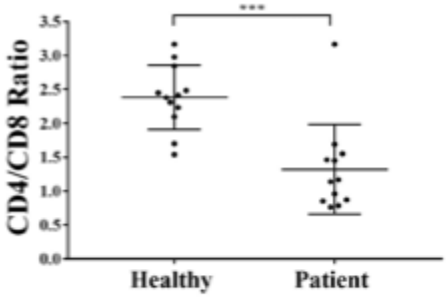


Fig. 3D

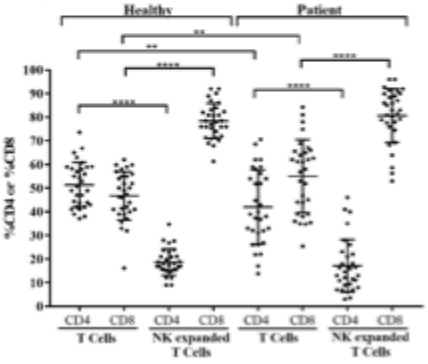


Fig. 3E

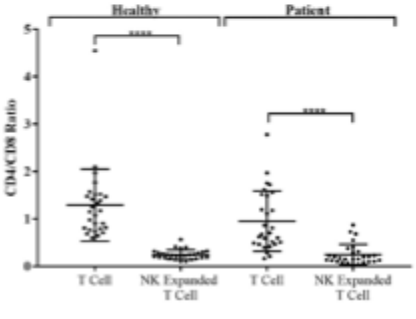


Figure 4

Fig. 4A

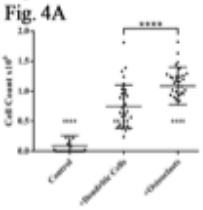


Fig. 4B

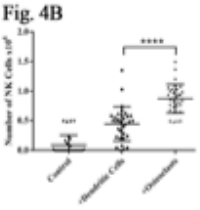


Fig. 4C

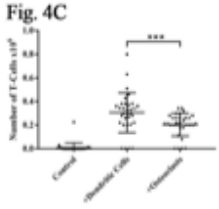


Fig. 4D

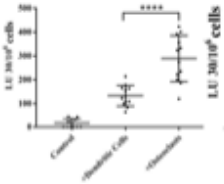


Fig. 4E

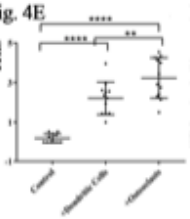


Fig. 4F

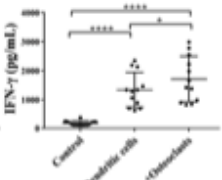


Fig. 4G

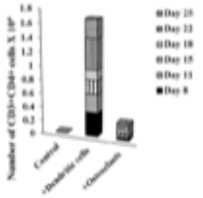


Fig. 4H

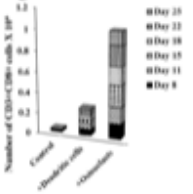


Fig. 4I

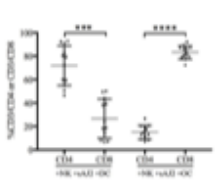


Fig. 4L

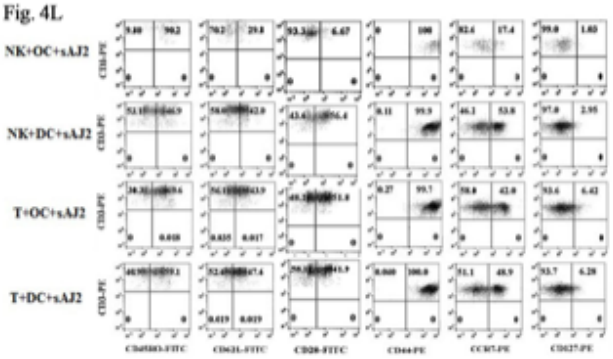


Fig. 4J

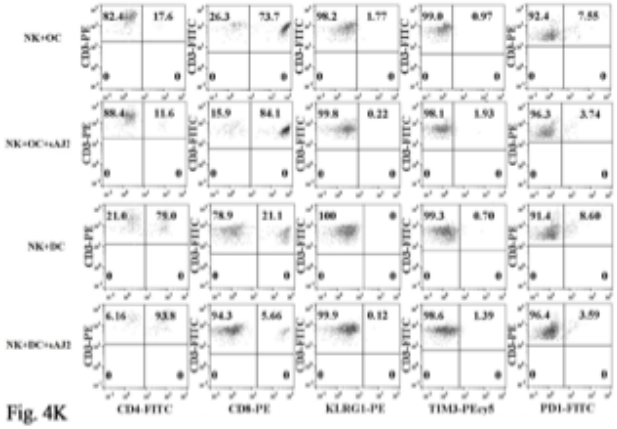


Fig. 4K

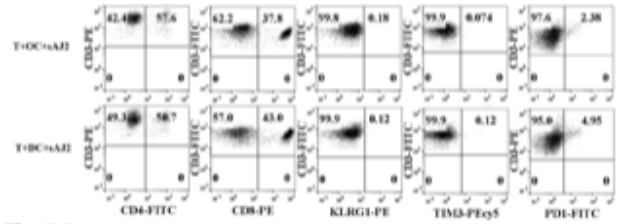


Fig. 4M

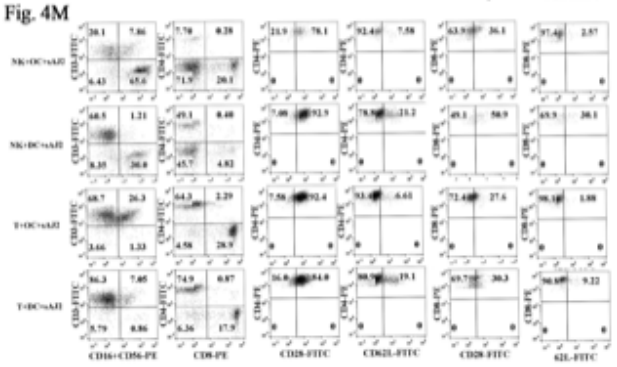


Fig. 4N

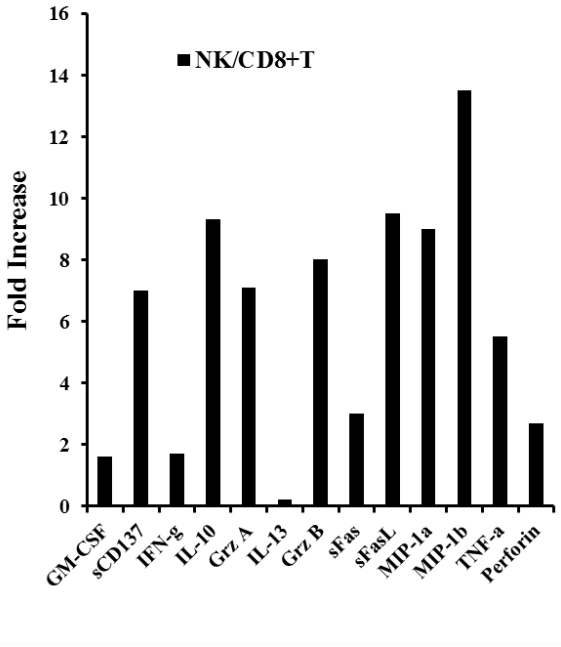


Fig. 4O

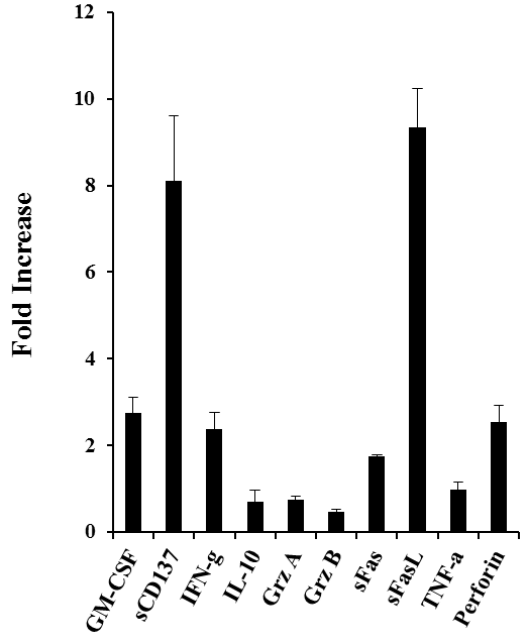


Fig. 4P

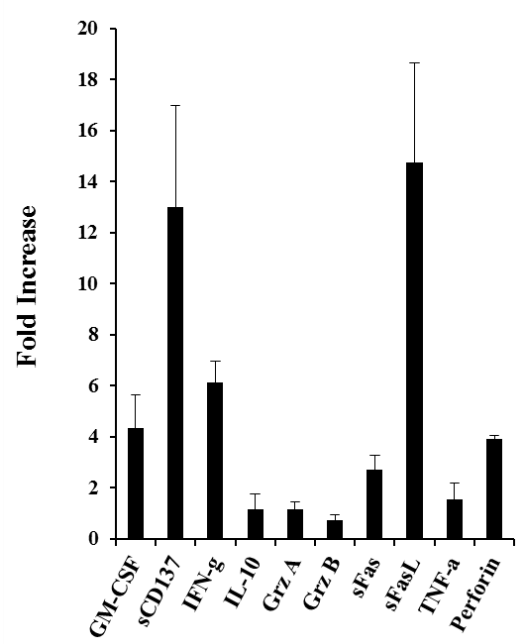
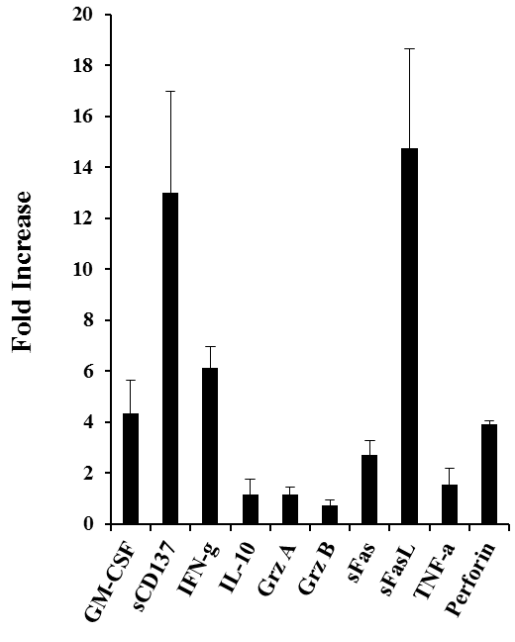


Fig. 4Q



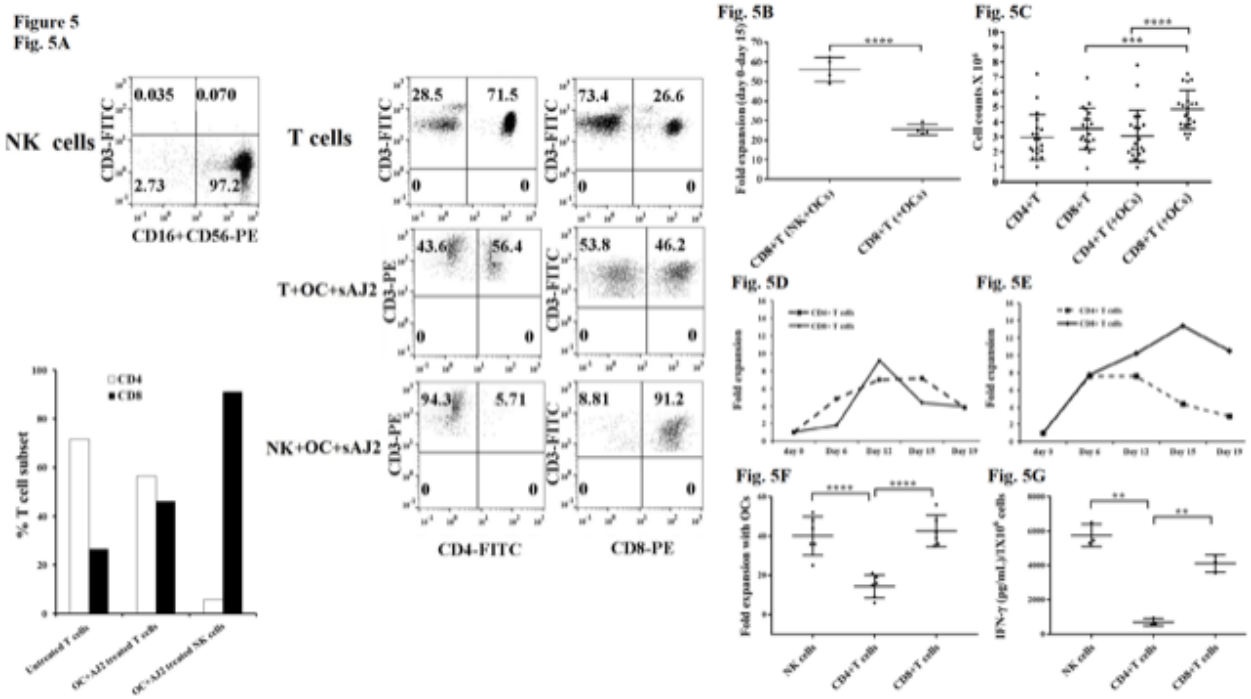


Figure 5
Fig. 5A
NK cells
0.035
0.070
2.73
97.2
CD3-FITC
CD16+CD56-PE
T cells
28.5
71.5
0
0
73.4
26.6
T+OC+sAJ2
43.6
56.4
53.8
46.2
NK+OC+sAJ2
94.3
5.71
8.81
91.2
CD3-PE
CD4-FITC
CD8-PE
CD4
CD8
% T cell subset
Untreated T cells
OC+AJ2 treated T cells
OC+AJ2 treated NK cells
Fig. 5B
Fold expansion (day 0-day 15)
CD8+T (NK+OCs)
CD8+T (+OCs)
Fig. 5C
Cell counts X 10⁵
CD4+T
CD8+T
CD4+T (+OCs)
CD8+T (+OCs)
Fig. 5D
Fold expansion
Fig. 5E
Fig. 5F
Fold expansion with OCs
NK cells
CD4+T cells
CD8+T cells
Fig. 5G
IFN-γ (pg/mL)/1X10⁶ cells

Figure 6

Fig. 6A

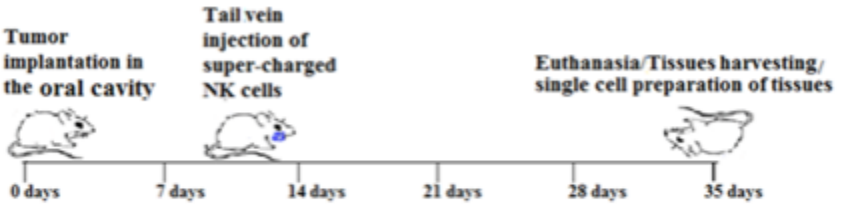


Fig. 6B

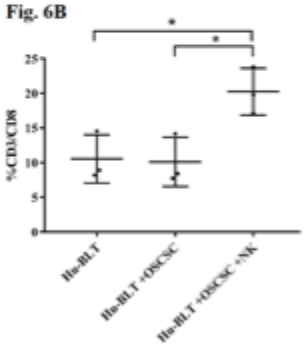


Fig. 6C

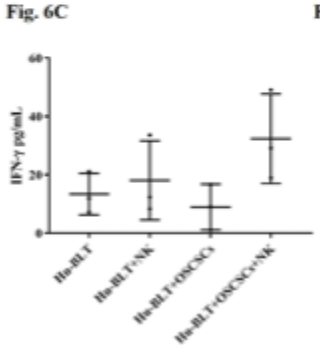


Fig. 6D

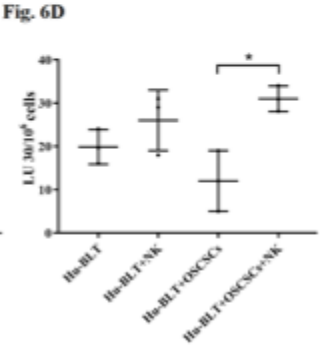


Fig. 6E

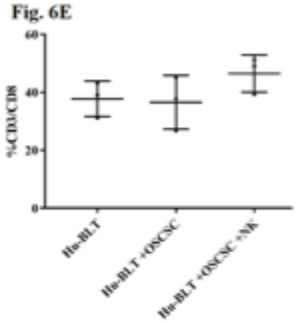


Fig. 6F

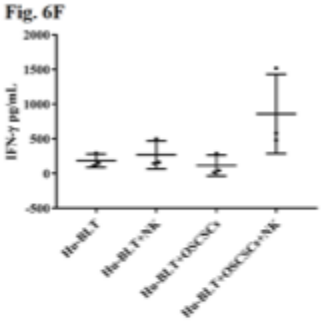


Fig. 6G

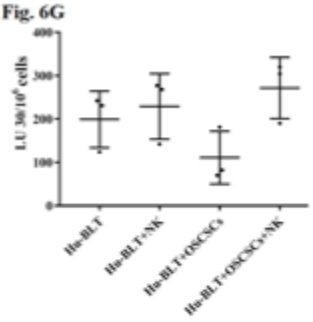


Fig. 6H

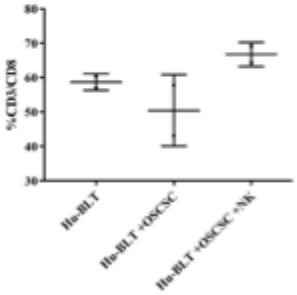


Fig. 6I

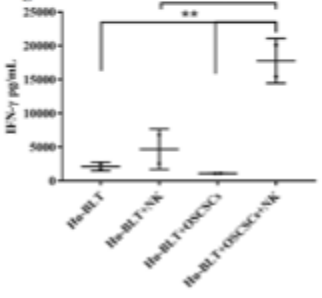


Fig. 6J

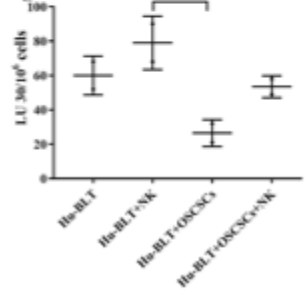


Fig. 6K

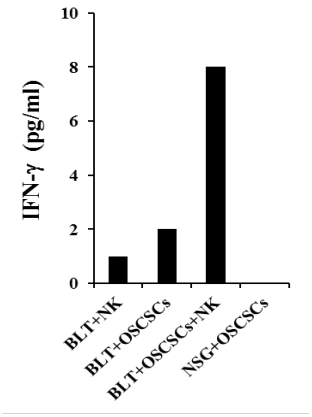


Fig. 6L

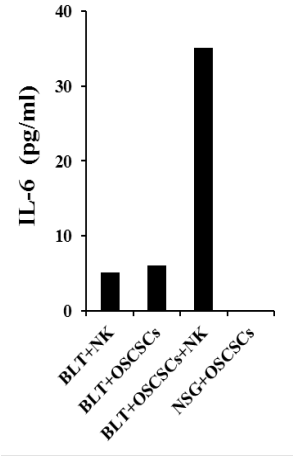


Fig. 6M

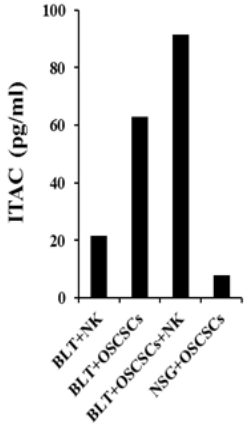


Fig. 6N

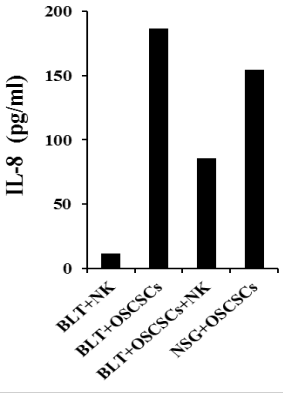


Fig. 6O

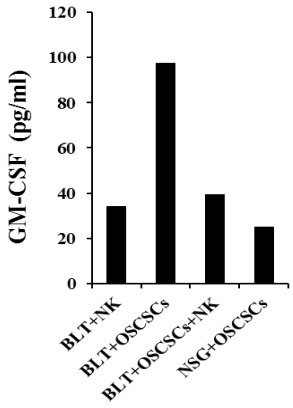


Fig.

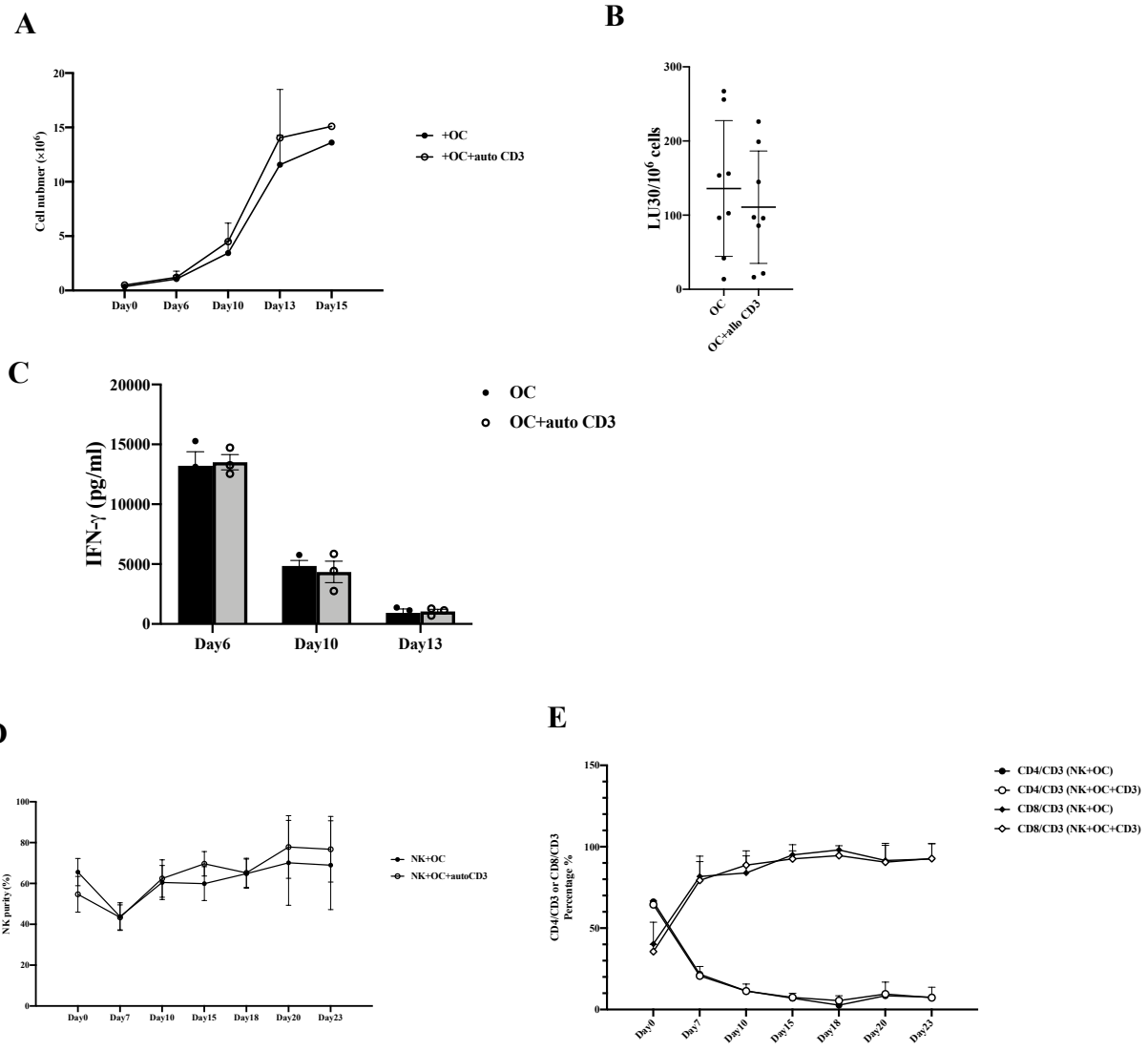

A
Cell nubmer (×10⁶)
+OC
+OC+auto CD3
Day0
Day6
Day10
Day13
Day15
B
LU30/10⁶ cells
OC
OC+allo CD3
C
IFN-γ (pg/ml)
OC
OC+auto CD3
Day6
Day10
Day13
D
NK parity (%)
NK+OC
NK+OC+autoCD3
Day0
Day7
Day10
Day15
Day18
Day20
Day23
E
CD4/CD3 or CD8/CD3 Percentage %
CD4/CD3 (NK+OC)
CD4/CD3 (NK+OC+CD3)
CD8/CD3 (NK+OC)
CD8/CD3 (NK+OC+CD3)

Fig. 8

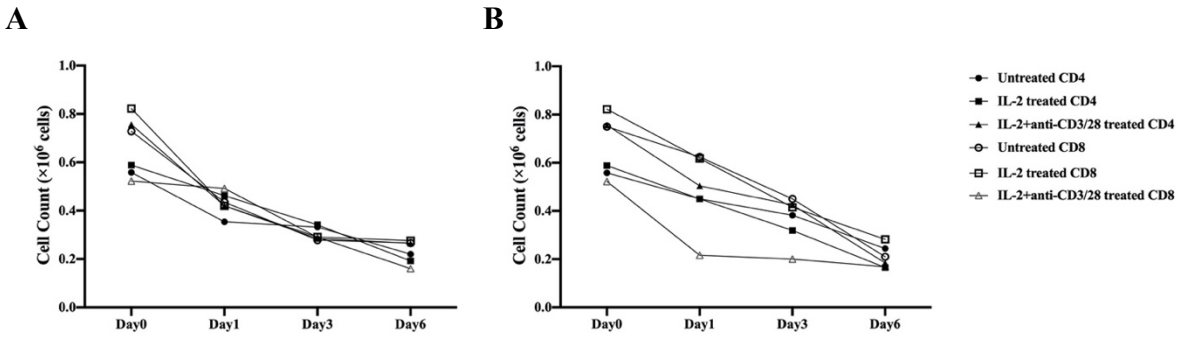

A
B
Cell Count (×10^6 cells)
1.0
0.8
0.6
0.4
0.2
0.0
Day0
Day1
Day3
Day6
Untreated CD4
IL-2 treated CD4
IL-2+anti-CD3/28 treated CD4
Untreated CD8
IL-2 treated CD8
IL-2+anti-CD3/28 treated CD8

Fig. 9

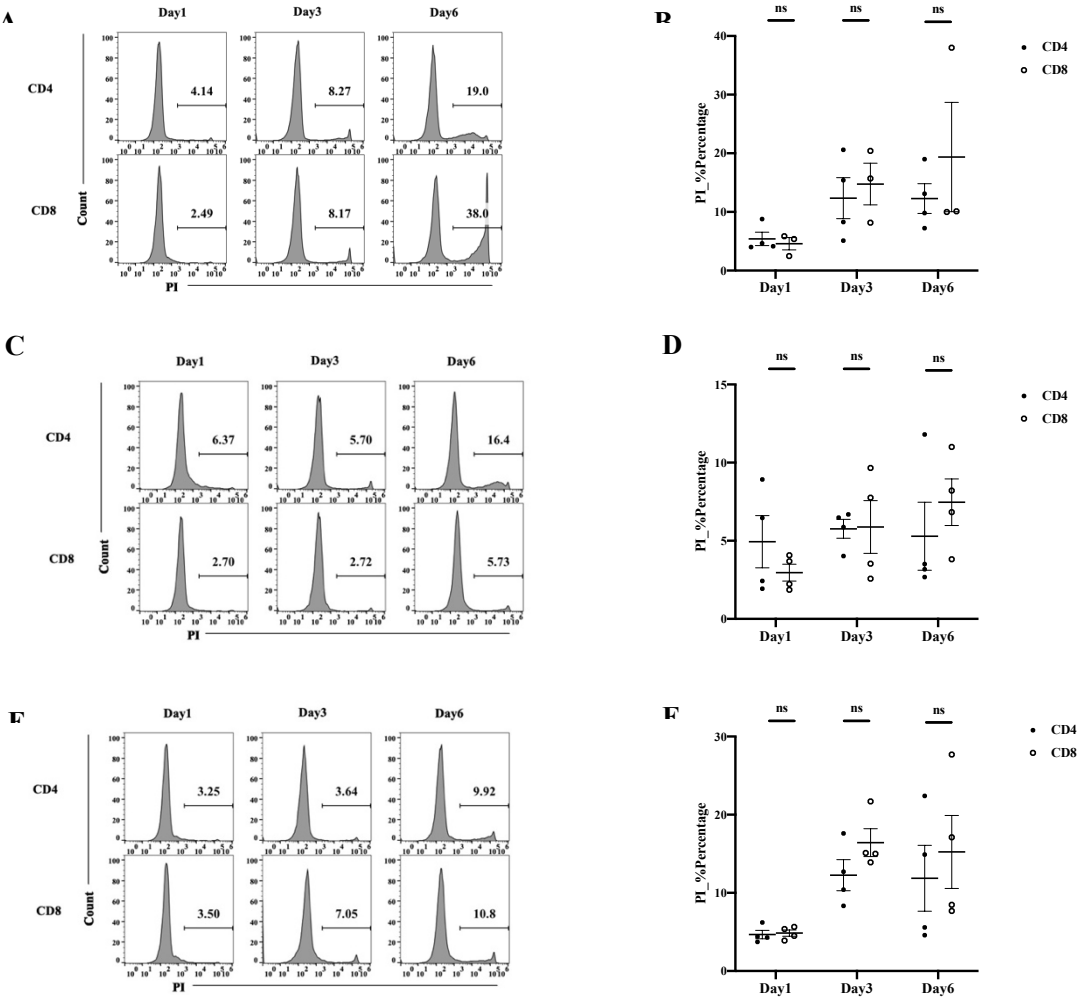

A
Day1
Day3
Day6
CD4
CD8
Count
PI
4.14
8.27
19.0
2.49
8.17
38.0
B
ns
PI_%Percentage
CD4
CD8
Day1
Day3
Day6
C
6.37
5.70
16.4
2.70
2.72
5.73
D
E
3.25
3.64
9.92
3.50
7.05
10.8
F

Fig. 10

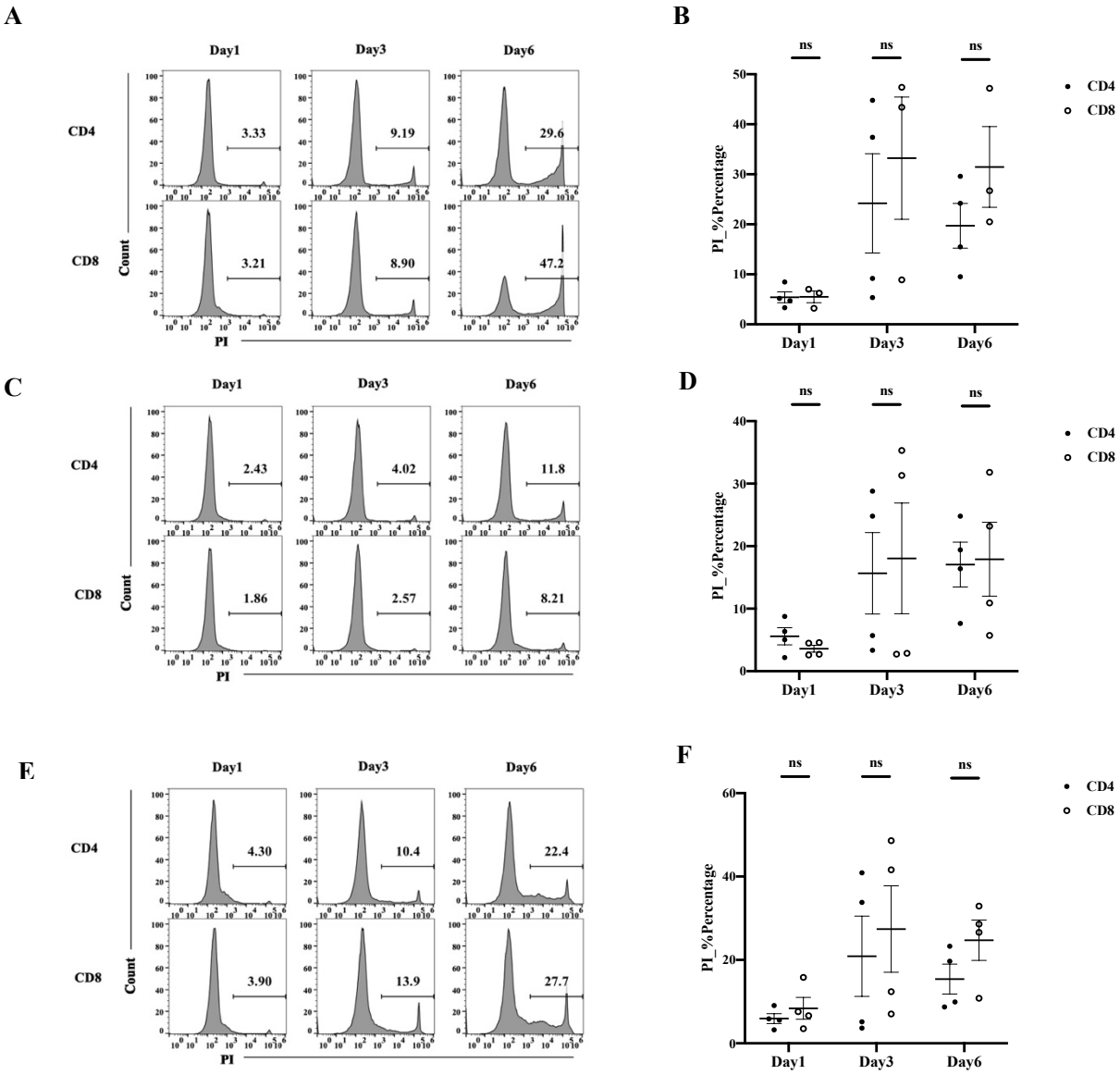

A
Day1
Day3
Day6
CD4
CD8
Count
3.33
9.19
29.6
3.21
8.90
47.2
PI
B
ns
PI_%Percentage
CD4
CD8
Day1
Day3
Day6
C
2.43
4.02
11.8
1.86
2.57
8.21
D
E
4.30
10.4
22.4
3.90
13.9
27.7
F

Fig. 11

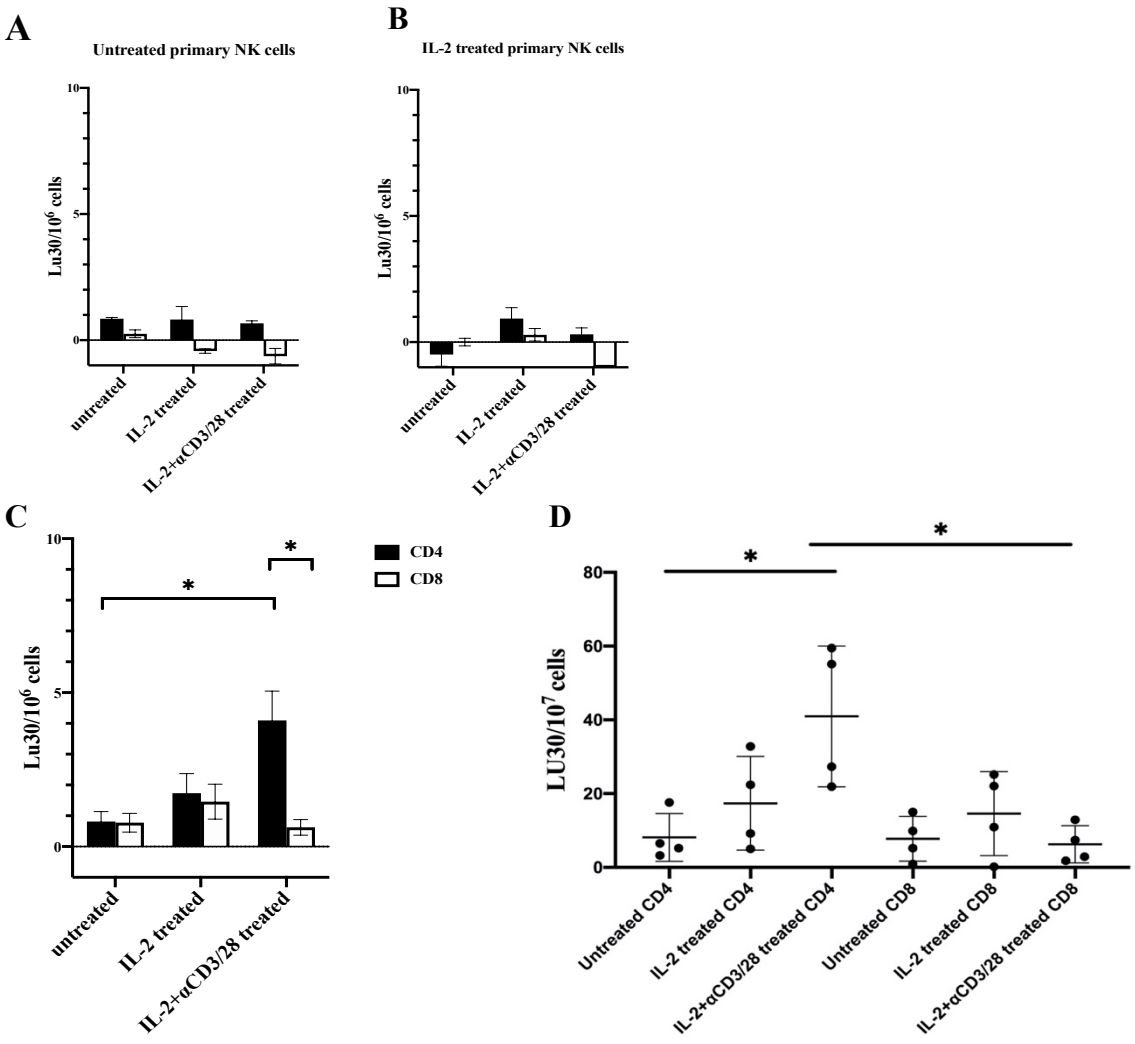

A
Untreated primary NK cells
Lu30/10^6 cells
untreated
IL-2 treated
IL-2+αCD3/28 treated
B
IL-2 treated primary NK cells
Lu30/10^6 cells
untreated
IL-2 treated
IL-2+αCD3/28 treated
C
CD4
CD8
Lu30/10^6 cells
untreated
IL-2 treated
IL-2+αCD3/28 treated
D
LU30/10^7 cells
Untreated CD4
IL-2 treated CD4
IL-2+αCD3/28 treated CD4
Untreated CD8
IL-2 treated CD8
IL-2+αCD3/28 treated CD8

Fig. 12

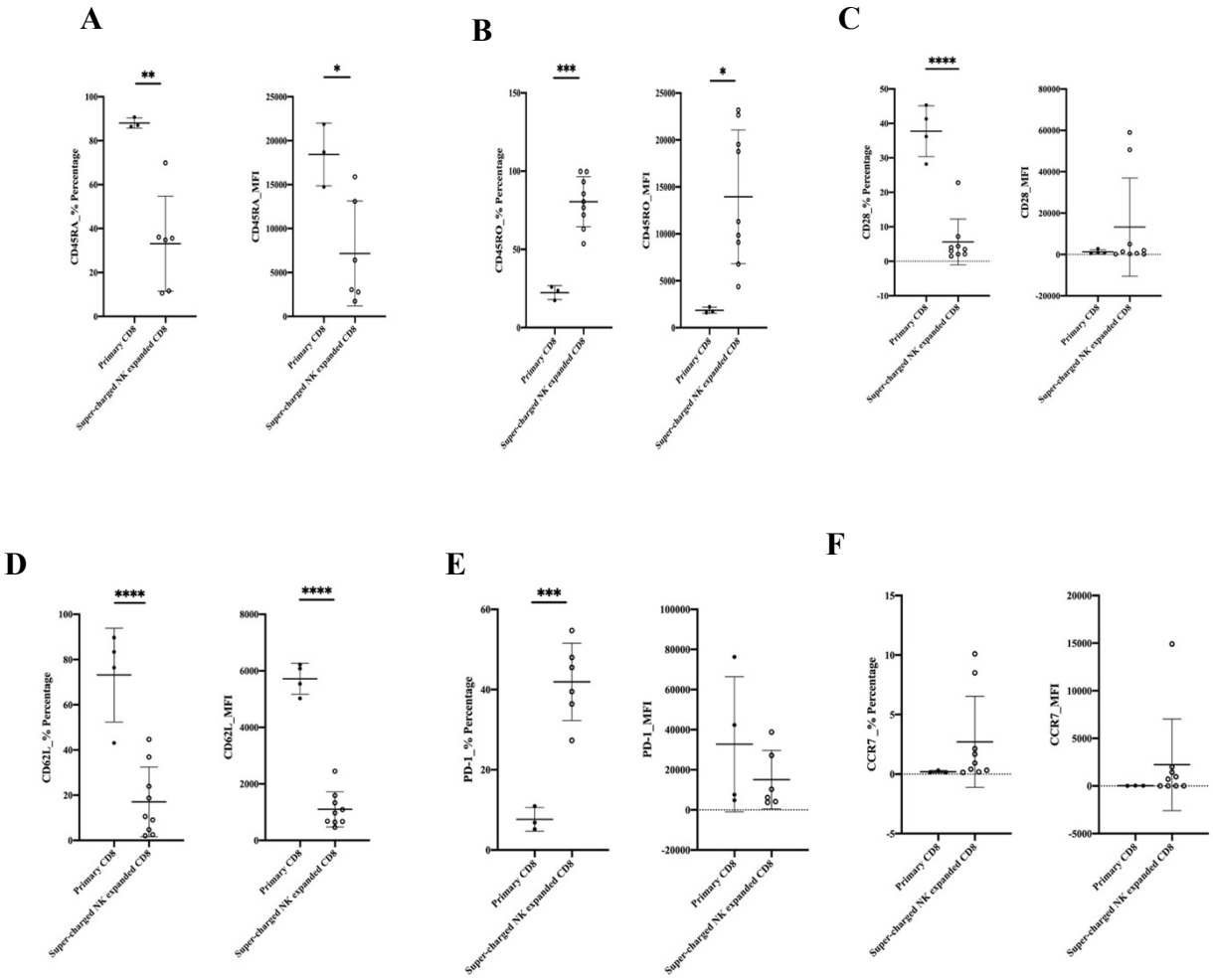
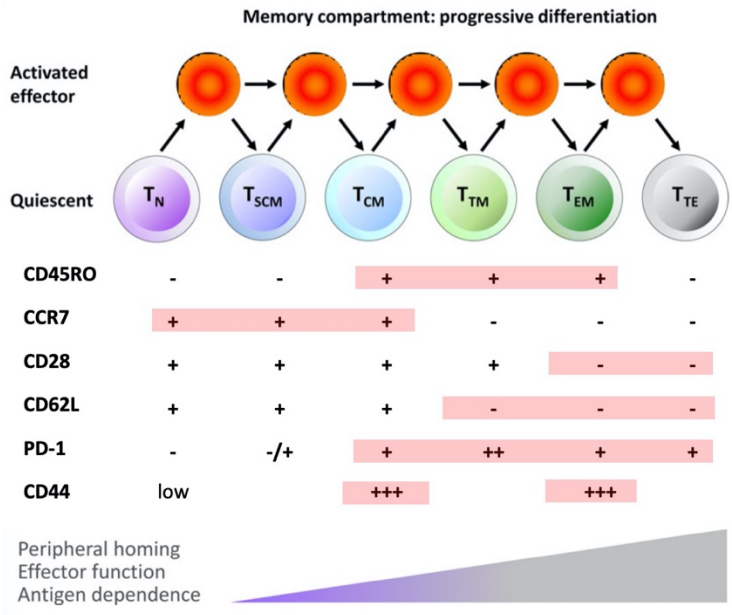

A
B
C
D
E
F
CD45RA_% Percentage
CD45RA_MFI
CD45RO_% Percentage
CD45RO_MFI
CD28_% Percentage
CD28_MFI
CD62L_% Percentage
CD62L_MFI
PD-1_% Percentage
PD-1_MFI
CCR7_% Percentage
CCR7_MFI
Primary CD8
Super-charged NK expanded CD8

Fig. 13



T_N: naïve T Cells
T_{SCM}: stem cell memory T cells
T_{CM}: central memory T cell
T_{TM}: transitional memory T cell
T_{EM}: effector memory T cell
T_{TE}: terminal effector T cell

(Mahnke, Y. D., 2013)

(Bono, MR. , et al, 2015; Mueller, S.N., et al, 2012)

(Mahnke, Y. D., 2013)

CONCLUSION

In this study, we identified that CD16 as a potential target for cancer immunotherapy. Also, we demonstrated how NK cells regulate inflammation by their secretion capability and presented the evidence of super-charged NK cells activating CD8+ T cell by preferentially expanding and enhancing their cytokine and chemokine secretion ability in the process of NK expansion. Furthermore, it showed that super-charged NK cells mediate greater tumor differentiation by their supernatants and are capable of targeting CD4+ T cells to possibly limit Treg population and eliminating suppressive monocytes. Lastly, we provided the evidence of super-charged are superior than primary NK cells and are not inactivated after encountering tumor cells.

FUTURE DIRECTION

It is of pivotal importance to explore the strategies to stabilize the level of CD16 on the cell surfaces of NK cells and monocytes either through the increase expression of CD16 receptor on the cell surfaces using viral vectors or prevent the shedding of CD16 from the cell surfaces with pharmaceutical treatments, such as ADAM17 inhibitor. Moreover, as elucidated, PD-1 is up-regulated in super-charged NK expanded CD8+ T cells, it would be essential to measure the level of CTLA-4, which is also a known immune checkpoint, on their cell surfaces as another potential treatments in combination with NK immunotherapy. Lastly, it would be crucial to expand the study of super-charging NK cells and investigate the mechanism of which super-charged NK cells preferentially expand CD8+ T cells by using IFN-γ antibody to determine the role of IFN-γ in maintenance of CD8+ T cells by super-charged NK cells.