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Supercharged Natural Killer cells preferentially expand CD8+ Cytotoxic T cells while targeting CD4+ Helper T cells

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Supercharged Natural Killer cells preferentially expand $CD8^+$ Cytotoxic T cells while targeting $CD4^+$ Helper T cells

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

by

Narek Ohanian

2017

ABSTRACT OF THE THESIS

Supercharged Natural Killer cells preferentially expand $CD8^+$ Cytotoxic T cells while targeting $CD4^+$ Helper T cells

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Narek Ohanian

Master of Science in Oral Biology

University of California, Los Angeles, 2017

Professor Anahid Jewett, Chair

NK mediated expansion of T-cells preferentially expands CD8+ but not the CD4+ subsets in both healthy and cancer donors. This results in a lower CD4/CD8 ratio. While NK Cells target $CD4^+$ helper T-cells they do not target their $CD8^+$ cytotoxic counterparts. This study also reveals that Cancer patients NK cells have reduced cytotoxic ability and secrete much less IFN-γ than healthy donors and Osteoclast and sAJ2 expanded super-charged NKs intended for immunotherapeutic use from healthy donors expand more immune cells at a faster rate. NK immunotherapy was studied in combination with supplementation of probiotic bacteria in the humanized mouse model. NK immunotherapy increased CD8+ cytotoxic T cell population in immune tissue compartments and Increased immune infiltration in the tumor microenvironment, while probiotic supplementation further enhanced these effects, resulting in decreased tumor burden. This novel expansion technique can provide a non-invasive and highly effective treatment for the treatment of oral squamous cell carcinoma and other tumorigenic cancers.

The thesis of Narek Ohanian is approved.

Ichiro Nishimura

Nicholas A. Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2017

DEDICATION

I dedicate this thesis to the innocent people around the world that are lost to cancer every year.

May you rest in peace, as the world perseveres and struggles to find a cure in your honor.

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ACKNOWLEDGEMENTS

I would like to thank and acknowledge my coworkers and colleagues who have always been there for me throughout this journey. I could not have done it with out your help and support.

Thank you to Kawal for her guidance throughout this process and to Dr. Jewett for her unwavering support.

Finally, a heartfelt thanks goes out to my parents for their immense sacrifice. Thank you for always keeping nothing and giving everything.

Introduction

Natural Killer Cells

Natural killer cells (NK) make up ten to fifteen percent of the total circulating lymphocytes population and are key members of the immune response. Functioning and the interference of the innate and adaptive immunity, NK cells play a crucial role in the early response to pathology. Derived in the bone marrow, NK cells differentiate from $CD34^+$ hematopoietic stem cells. In the body they can be found in the peripheral blood, spleen, liver and even in the placenta [29]. We can identify NK cells by the surface of expression of CD16 and CD56, combined with the lack of expression of CD3.

Effector functions of NK cells include direct cytotoxicity against cancer stem cells, and virally infected cells as well as secretion of potent granzymes, perforin, interferon-g, tumor necrosis factor-a and others when activated by pro-inflammatory cytokine. NK cells kill their targets via direct contact by binding to the transformed cell and releasing pre-formed granules of proteins designed to penetrate the plasma membrane of the target, releasing its cytoplasm while also activating apoptotic pathways for good measure (15-29). In the past, our laboratory has shown, that following lysis, NK cells differentiate CSCs, otherwise known as undifferentiated or poorly differentiated tumors, via secreted and membrane-bound IFN-γ and TNF-α. This leads to tumor growth prevention and tumor microenvironment remodeling [4].

Split-anergy in NK cells

Our laboratory coined the term ‘split anergy,’ to indicate reduced NK cell cytotoxicity in the presence of significant secretion of cytokines [15-17]. Induction of split anergy in NK cells promotes differentiation of target cells via secreted and membrane-bound factors, increases key differentiation receptors on tumor cells [55], induces tumor cell resistance to NK cell-mediated cytotoxicity, and inhibits inflammation due to a decrease or shutdown of cytokine and chemokine production after tumor differentiation.

T-cells

Adaptive immunity creates immunological memory after an initial response to a specific pathogen, leading to a faster response to repeat stimulation in the future. Like the innate system, the adaptive system includes both humoral immunity components and cell-mediated immunity components [19]. T-cells are a crucial component of the adaptive immune system. Lower than normal cell counts of just one type of T-cell are all too evident in the poor prognosis seen in patients with HIV/AIDS [31]. Meanwhile the loss of function due to T-cell exhaustion is a hallmark of tumor metastasis and growth [24, 28]. There are several different kinds of T-cell; broadly speaking they can be divided into two different types, killer T-cells and helper T-cells. Helper T-cells are characterized phenotypically by their surface expression of CD4 co-stimulatory proteins whereas killer T-cells express CD8.

Helper T-cells are involved in the activation of the various immune responders, primarily B-cells [7]. As helper T-cells patrol the body, they are scanning the body for abnormal cells,

recognized by the display of antigens. If activated, they initiate an antibody mediated immunity pathway activating B-cell that start producing a flood of antibodies which can bind to the pathogen an initiate cytotoxic pathways, activate other immune cells, and contribute to ADCC [17]. Helper T-cells also play a crucial role in inflammatory response mitigation and macrophage activation as well through IFN-γ and Interleukin 2 (IL-2) secretion, IL-4, IL-5 and IL-10 [12, 13]. The expanding roles of CD4+ cells are yet to be understood [7].

Killer T-cells (CD8) or cytotoxic T-cells (CTL) patrol the body looking for cancer cells or virally infected cells [13]. When a T-cell encounters another cell, protrusions from the outer membrane scan its surface with their T-cell Receptor (TCR) searching for MHC-I and MHC-II proteins and antigens. If an MHC molecule is presenting a non-self or an intracellular stress-induced antigen is presented, cytotoxic granules travel to the interface between the CTL and target cell where they are released to deliver the most potent response [22, 24]. This activated stage also allows CTLs to secrete primarily tumor necrosis factor alpha (TNF-α) and IFN- γ. As a result, CTLs play an important role in tumor growth suppression by targeting and eliminating cancer cells. Unfortunately, due to mechanisms which are yet to be understood, T-cells often fall into a state of exhaustion in cancer patients. They lose their effector functions and no longer contribute to the fight against cancer, allowing tumors to grow rapidly and spread. This decrease in function and cell count may contribute to the poor prognosis of many Stage III and Stage IV cancer patients.

Oral Squamous Cell Carcinoma

With a 5-year survival rate of just 64.5%, oral squamous cell carcinoma (OSCC), is a cancer of the head, neck and throat that can have devastating effects on a patients quality of life, regardless of survival. Current therapies include surgical intervention followed by strict chemo- and radio-therapeutic regiments. The morbidities associated with this form of cancer and the invasive nature of the surgeries required has deforming effects on the patients' physiology and quality of life [25].

Transformation of the thin flat cells that constitute OSCC begins most commonly with the development of leukoplakia. Many forms of chewing tobacco, cigarettes and opioid drug abuse are associated with the development of leukoplakia. If not treated, leukoplakia can develop into OSCC. Oral squamous cancer stem cells (OSCSC) are characterized by their down regulation of the major histocompatibility complex (MHC-I) [52]. This helps OSCSCs avoid immune detection by circulating immune cells, however, NK cells are known to target such cells. The absence of MHC-I triggers NK cells to kill via direct cytotoxicity or via cross linking of the NK Fc receptor with the targets ligand, known as antibody dependent cellular cytotoxicity (ADCC) [55].

Humanized Mouse Model

Varying levels of NK cell impairment and/or deletion in nude, NOD-scid and NSG strains could explain discrepancies in the ability of CSCs to give rise to human tumors in these different

immune-deficient strains [3]. Many questions have been raised, based on previous studies performed on - animals, regarding specific immune subsets and their roles in controlling cancer initiation, growth, and metastasis. Since it is difficult to assess and compare the aggressiveness and metastatic potential of primitive CSCs using immune-deficient mouse strains, humanized mice, with restored human immune systems, offer the most suitable platform to implant such tumors.

There have been numerous attempts to generate mice that bear a fully reconstituted human immune system. There are also differences between human immune system reconstitution levels supported by specific mouse strains. Since it is critical for the background strain to harbor severe immunodeficiency, NSG or NRG mice have typically been the strain of choice [17,18]. There are many methods in creating various humanized mouse models, with differences in the age of mice, transplanted cell type, source or donor cell type, injection/implantation method, irradiation, etc. Of these variations, the simplest humanization method consists of injecting immune-deficient mice with human PBMCs, obtained from adult healthy donors/patients [32, 33]. PBMCs circulate in the blood, either dying or migrating to other tissues; the downside is that these mice can only be used for short term experiments, since circulating mature immune cells in mice initiate graft versus host disease (GvHD) against murine recipients [17].

Another method uses isolated $CD34^+$ progenitor cells originating from the peripheral blood, cord blood or fetal liver. $CD34^+$ cells are injected into either newly born or adult NSG mice. They stably engraft into the bone marrow and are capable of differentiating into all hematopoietic lineages of the human immune system. The CD34+ humanized mouse model's major limitation is that it lacks the presence of a human thymus; so instead, T cells undergo selection in the context of the mouse MHC [17].

The BLT humanized mouse (hu-BLT) represents the most advanced and complete humanized mouse model generated, to date [33]. The human immune engraftment protocol consists of surgically implanting pieces of human fetal liver and thymus tissue under the renal capsule of NSG mice, followed by tail vein IV injection of same-donor $CD34^+$ hematopoietic cells to support full reconstitution of the human bone marrow [3, 17, 33]. Thus, positive and negative selection of developing T cells occurs in the presence of human thymus. Consequently, immature T cells become functional $CD4^+$ helper and $CD8^+$ cytotoxic T cells after human MHC class I and class II restriction [57, 60]. The hu-BLT model is the only known humanized mouse model to displays mucosal immunity [15]. Hematopoietic stem cells (HSCs) develop, at least to some extent, into human T cells, B cells, NK cells, monocytes, myeloid derived suppressor cells (MDSCs), macrophages, dendritic cells, erythrocytes, and platelets in the BLT's tissues [42-45]. Long-term peripheral reconstitution of human $CD45^+$ immune cells is usually within the 30-80% range, as detected in the blood, spleen and bone marrow (manuscript in preparation). Human immune cells have been detected in the reproductive tract of females, intestines and rectum [45], as well as the gingiva (manuscript in prep). It is also worth noting that NSG-BLT mice (BLT mice developed from NSG background strain) exhibit substantially higher levels of human leukocyte reconstitution in their peripheral blood than NOD-scid-BLT mice [15]. These features demonstrate that the hu BLT model, developed from NSG background, is arguably the best available model for studying human immunity, thus far.

Osteoclasts

Osteoclasts are a type of bone cell, derived from hematopoietic stem cells. Their function,

resorbing bone tissue, is critical for the maintenance, repair, and remodeling of bones. Bone homeostasis is achieved when there is a balance between osteoblast bone formation and osteoclast bone resorption [49]. Osteoclasts mature through stimulation from osteoblasts expressing RANKL, and their interaction, mediated by firm adhesion via ICAM-1[8]. Feng et al.[8] showed that osteoclasts also express many ligands for receptors present on activated NK cells. They reported that osteoclasts express ULBP-1, ULBP-2/5/6 and ULBP-3, but little or no MIC-A, MIC-B, or MHC class I-like ligands for NKG2D, the activating receptor of NK cells [50].

AJ2 Probiotic Bacteria

AJ2 is patented combination of 8 strains of gram-positive probiotic bacteria. These strains include, *Streptococcus thermophiles, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, and Lactobacillus bulgaricus.* The resulting combination of probiotic bacteria in the small and large intestine contribute to the activation of PBMC's [38]. This leads to a balanced release of pro- and anti-inflammatory cytokines. Our recent work has shown elevated levels of IFN-γ secretion, which promotes CSC differentiation. These beneficial bacteria also effect production of immunoglobulin A [11, 38], stimulate macrophages activity [55] and may reduce the effects of toxicity in anti-cancer therapy [10]. They also induce immature dendritic cells to differentiate into regulatory dendritic cells, induce the presence of regulatory T cells and increase NK cell activity, resulting in local intestinal defense. We have more bacterial cells colonizing our gut than we have cells in our body, thus other possible benefits and role of these microbes is still yet

to be understood.

Purpose of Study

The purpose of this study is to investigate and characterize the dynamics of expansion and function in immune cells of cancer patients while elucidating the roll of Natural Killer cells in the observed T-cell expansion.

Thesis Outline

Specific Aim 1: Characterization of healthy and cancer patient donor osteoclast induced expanded NK cells.

Specific Aim 2: Elucidate the mechanisms by which NK cells mediate expansion of CD8+ T-cells but not CD4+.

Specific Aim 3: Increased expansion of $CD8^+$ T-cells by supercharged NK cells in Hu-BLT Mice.

MATERIALS AND METHODS

Cell lines, reagents, and antibodies

Human immune cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, CA). Oral squamous carcinoma stem cells (OSCSCs) were isolated from oral cancer patient tongue tumors at UCLA School of Medicine and cultured in RPMI 1640, supplemented 10% FBS (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% MEM non-essential amino acids, 1% L-glutamine, 0.2% gentimicin (Gemini Bio-products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). Antibodies to CD16 were purchased from Biolegend (San Diego, CA, USA). Recombinant IL-2 was obtained from NIH-BRB. Antibodies against isotype control, MHC-I CD45 (human), CD45 (mouse), CD3, CD16, CD56, CD8, HLADR, and CD11b were purchased from Biolegend (San Diego, CA). Human NK purification kits were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

Human monocytes/osteoclasts were cultured in alpha-MEM medium (Life Technologies, CA), supplemented with 10% FBS, and penicillin-streptomycin (Gemini Bio-Products, CA). Human M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -80°C.

Bacteria sonication

AJ2 is a combination of 8 different strains of gram positive probiotic bacteria (Streptococcus thermophiles, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, and Lactobacillus bulgaricus) used to induce differentiation of stem cells (doi:10.3389/fimmu.2014.00269). AJ2 was weighed and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were thoroughly vortexed, then sonicated on ice for 15 seconds, at 6 to 8 amplitude. Sonicated samples were then incubated for 30 seconds on ice. After every five pulses, a sample was taken to observe under the microscope until at least 80 percent of cell walls were lysed. It was determined that approximated 20 rounds of sonication/incubation on ice, were conducted to achieve complete sonication. Finally, the sonicated samples (sAJ2) were aliquoted and stored in a -80 degrees Celsius freezer.

Purification of NK cells from the peripheral blood

Written informed consents, approved by UCLA Institutional Review Board (IRB), were obtained from healthy blood donors, and all procedures were approved by the UCLA-IRB. Peripheral blood was separated using Ficoll-Hypaque centrifugation, after which the white, cloudy layer, containing peripheral blood mononuclear cells (PBMC), was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS and plated on plastic tissue culture dishes. After 1-2 hours of incubation, non-adherent, human peripheral blood lymphocytes (PBL) were collected. NK cells were negatively selected

and isolated from PBLs using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, BC, Canada). Isolated NK cells were stained with anti-CD16 antibody, to measure NK cell purity using flow cytometric analysis. The isolated NK cell population was greater than 90% purity. Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% MEM non-essential amino acids (Invitrogen, Life Technologies, CA).

Purification of monocytes from the peripheral blood

Written informed consents, approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors, and all procedures were approved by the UCLA-IRB. Peripheral blood was separated using Ficoll-Hypaque centrifugation, after which the white, cloudy layer, containing peripheral blood mononuclear cells (PBMC), was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS and plated on plastic tissue culture dishes. After 1-2 hours of incubation, the adherent subpopulation of PBMCs was detached from the tissue culture plates. Monocytes were purified using the EasySep® Human monocyte cell enrichment kit obtained from Stem Cell Technologies (Vancouver, BC, Canada). Based on flow cytometric analysis of CD14 the antibody-stained, enriched monocyte cells, the monocyte population was found to have greater than a 95% purify.

Generation of osteoclasts

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

Medium was refreshed every 3 days with fresh alpha-MEM, containing M-CSF (25ng/mL) and RANKL (25ng/mL).

Analysis of human OSCSCs cell growth in immunodeficient and humanized mice

Animal research was performed under the written approval of the UCLA Animal Research Committee (ARC) in accordance to all federal, state, and local guidelines. Combined immunodeficient 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 [54].

Prior to tumor implantation, selected mice were fed $5x10^9$ AJ2 bacteria (the combination of 8 probiotic strains listed above) every other day for one week. This adjuvant therapy was continued every other day until the day of sacrifice. For each mouse, lyophilized AJ2 was resuspended in 200 µL of fat free milk, and fed to them via pipetting.

In vivo growth of human oral squamous carcinoma stem cells (OSCSCs) was determined by orthotopic cell implantation of tumor cells into hu-BLT mice. To establish orthotopic tumors, mice were first anesthetized using an isoflurane set up, and OSCSCs were then transferred by direct injection of $1x10^6$ cells mixed with 10 µl HC Matrigel (Corning, NY,USA) into the oral cavity, to the floor of the mouth. Immediately prior to tumor cells injection, 5.0-mg/kg carprofen 1 2 was injected subcutaneously, and this injection was repeated every 24 hours for 48 hours. Following injection of tumor cells, all mice were continuously monitored for disease progression

every other day. Mice were observed for overall signs of morbidity, such as loss of weight, ruffled fur, hunched posture, and immobility. Seven days after tumor implantation selected hu-BLT mice received $1.5x10_6$ human expanded NK cells via tail vein (IV) injection.

Cell dissociation and cell culture from tissues of tumor bearing hu-BLT and NSG mice

At the end of the experiment, mice were euthanized and oral tumor, liver, bone marrow, spleen and blood were obtained from hu-BLT or NSG mice. Single cell suspensions were obtained by digesting tissues using DMEM medium supplemented with collagenase II (1mg/mL) (oral tumor) (Invitrogen, CA) and DNAse (10u/mL) (Sigma-Aldrich, CA) and 1%BSA. The digested tissues were passed through 70 μM filters (Fisher Scientific, CA) to obtain single cell suspensions. Femurs and spleens were harvested from animals, and bone marrow cells and splenocytes were passed through 70 μM filters (Fisher Scientific, CA) to obtain single cell suspensions. Murine peripheral blood mononuclear cells (PBMCs) were obtained using Ficoll-Hypaque centrifugation of heparinized blood specimens. The white, cloudy layer, containing peripheral blood mononuclear cells (PBMCs), were harvested, washed and re-suspended in medium. Single cell suspensions of each tissue were cultured in the presence and/or absence of IL-2 (1000 units/mL) treatment, using RPMI 1640 media (Life Technologies, CA), supplemented with 10% FBS.

Purification of human T cells from hu-BLT mice

CD3+ T cells from hu-BLT mice were positively selected from splenocytes using isolation kits from Stem Cell Technologies (Vancouver, BC, Canada). Cells were cultured at

$1x10^6$ cells/mL in RMPI 1640 media (Life Technologies, CA), supplemented with 10% FBS, along with IL-2 (1000 units/mL) treatment. Flow-through cells (negative for CD3, following the positive selection for T cells) were also cultured in the same manner.

Surface staining

$1x10^5$ cells from each condition were stained in 100ul of cold 1%PBS-BSA with predetermined optimal concentration of PE conjugated antibodies, as detailed in the experiments, and incubated at 4°C for 30 minutes. Then, cells were washed and resuspended in 1%PBS-BSA. The Epics C (Coulter) flow cytometer was used for cellular surface analysis.

^{51}Cr release cytotoxicity assay

^{51}Cr was purchased from Perkin Elmer (Santa Clara, CA). Standard ^{51}Cr release cytotoxicity assays were used to determine NK cell cytotoxic function in the experimental cultures and the sensitivity of target cells to NK cell mediated lysis. The effector cells ($1x10^5$ NK cells/well) were aliquoted into 96-well round-bottom microwell plates (Fisher Scientific, Pittsburgh, PA) and titrated at four to six serial dilutions. The target cells ($5x10^5$ OSCSCs) were labeled with 50μCi ^{51}Cr (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour. Following incubation, target cells were washed twice to remove excess unbound ^{51}Cr. ^{51}Cr-labeled target

cells were aliquoted into the 96-well round bottom microwell plates containing effector cells at a concentration of $1x10^4$ cells/well at a top effector:target (E:T) ratio of 5:1. Plates were centrifuged and incubated for a period of 4 hours. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. Total (containing ^{51}Cr-labeled target cells) and spontaneous (supernatants of target cells alone) release values were measured and used to calculate the percentage specific cytotoxicity.

The percentage specific cytotoxicity was calculated using the following formula:

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

LU $30/10^6$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells x100.

Enzyme-Linked Immunosorbent Assays (ELISAs) and multiplex cytokine assay

Human ELISA kits for IFN-γ and IL-10 were purchased from Biolegend (San Diego, CA). ELISA was performed to detect the level of IFN-γ and IL-10 produced from cell cultures. The assay was conducted as described in the manufacturer's protocol. Briefly, 96-well EIA/RIA plates were coated with diluted capture antibody corresponding to target cytokine and incubated overnight at 4°C. After 16-18 hours of incubation, the plates were washed 4 times with wash

buffer (0.05% Tween in 1xPBS) and blocked with assay diluent (1%BSA in 1xPBS). The plates were incubated for 1 hour at room temperature, on a plate shaker at 200rpm; plates were washed 4 times following incubation. Then, 100uL of standards and samples collected from each culture were added to the wells and incubated for 2 hours at room temperature, on the plate shaker at 200rpm. After incubation, plates were washed 4 times, loaded with detection antibody, and incubated for 1 hour at room temperature, on the plate shaker at 200rpm. After 1 hour of incubation, the plates were washed 4 times; wells were loaded with Avidin-HRP solution and incubated for 30 minutes at room temperature, on the plate shaker at 200rpm. After washing the plates 5 times with wash buffer; 100uL of TMB substrate solution was added to the wells and plates were incubated in the dark until they developed a desired blue color (or up to 30 minutes). Then, 100uL of stop solution (2N H_2SO_4) was added per well to stop the reaction. Finally, plates were read in a microplate reader, at 450nm to obtain absorbance values (Biolegend, ELISA manual). The levels of cytokines and chemokines were examined by multiplex assay, which was conducted as described in the manufacturer's protocol for each specified kit. Analysis was performed using a Luminex multiplex instrument (MAGPIX, Millipore, Billerica, MA) and data was analyzed using the proprietary software (xPONENT 4.2, Millipore, Billerica, MA).

Target cell Visualization Assay (TVA)

Target cells were incubated with TVA™ Dye at 37°C for 15 minutes. After a 4-hour incubation period with effector cells, cells were harvested from each sample and the target cells are counted with ImmunoSpot® at 525nm emmission wavelengths. Cytotoxicity percentage were calculated as followed:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cell count - Spontaneous cell count x100}}{\text{Spontaneous cell count}}$$

LU $30/10_7$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells x100.

Statistical analysis

An unpaired, two-tailed student t-test was performed for the statistical analysis of two groups. One-way ANOVA with a Bonferroni post-test was used to compare more than two groups. ***(p value <0.001), **(p value 0.001-0.01), *(p value 0.01-0.05).

Chapter 1

Specific aim 1: Characterization of healthy and cancer patient donor osteoclast induced expanded NK cells.

Results

Primary NK cells from patients peripheral blood have decreased cytotoxicity against OSCSCs and diminished IFN-γ secretion when cultured overnight.

To investigate the functional differences in Natural killer cells from cancer patients, we first negatively selected NK cells from the peripheral blood of both healthy donors and cancer patients. Purity was assessed by flow cyotometery, staining for the presence of CD3 and CD16 +CD56. Healthy donor has a smaller population of cells that have down-regulated or lost $CD3^+$ and $CD16/CD56^+$ Receptors as compared to cancer patient (Fig 1). As seen in Figure 1, both the healthy and cancer patient have pure NK populations with no infiltrating CD3+ cells. The cells were then incubated in media overnight as untreated control, and activated with IL-2 or IL-2+anti-CD16mAb. Following overnight culture, the various conditions of activated NKs were assayed for their ability for NK cell-mediated lysis using the 51Cr release assay (Fig 2). IL-2 activated NK cells from both healthy and cancer patients were the most cytotoxic towards OSCSCs, however patient NK cells were much less cytotoxic as compared to healthy in all three conditions (Fig 2). When the supernatants of the overnight cultures were analyzed for IFN-γ concentration, NK cells activated by IL-2+anti-CD16mAb, induced split-anergy caused by the crosslinking of the Fc receptors, secreted the largest amounts of IFN-γ as seen in Figure 3. IL-2

activation also increased the IFN-γ production of the NK cells as compared to control.

Osteoclast and sAJ2 expanded super-charged NKs from cancer patients expand less functional immune cells at a slower rate while losing NK population.

Decreased cytotoxicity and lower IFN-γ secretion by NK cells from cancer patients coincides with increased expansion of T cells. When NK cells were cultured with osteoclast (OCs) for expansion as showed in Fig. 4, purified NK cells from cancer patients were unable to maintain the expansion of NK cells and indeed, by day 12, greater than half of the expanding cells were T cells. Moreover, by day 31, only 9.6% of the remaining cells in the culture were NK cells (Fig. 7B), whereas the healthy donor NK cell culture had 95% NK cells and only 1-2% T cells (Fig. 7A). In addition, when total numbers of expanded NK and T cells were determined within 31-36 days of expansion in cancer patients, there were less expanding cells from cancer patients when compared to healthy controls (Fig. 4), and the levels of expanding T cells were significantly higher than NK cell expansion (Figs. 7B). In contrast, NK cells isolated from healthy donors maintained the expansion of NK cells and the levels of NK expansion were significantly higher than T cells. Patient NK cells cultured with OCs lysed OSCSCs significantly less when compared with the healthy NK cells cultured with OCs (Fig. 5). OC-expanded patient NK cells secreted significantly less IFN-γ when compared to healthy OC-expanded NK cells (Fig.6).

In addition, when total numbers of expanded NK and T cells were determined within 31-36 days of expansion in repeat experiments cancer patients displayed less expanding cells when compared to healthy controls (Fig. 8B). In contrast, NK cells isolated from healthy donors

maintained the significantly higher expansion of NK cells (Fig 8B). Patient NK cells cultured with OCs lysed OSCSCs significantly less when compared with the healthy NK cells cultured with OCs (Fig. 8A) and also secreted significantly less IFN-γ (Fig 8C).

Figure 1

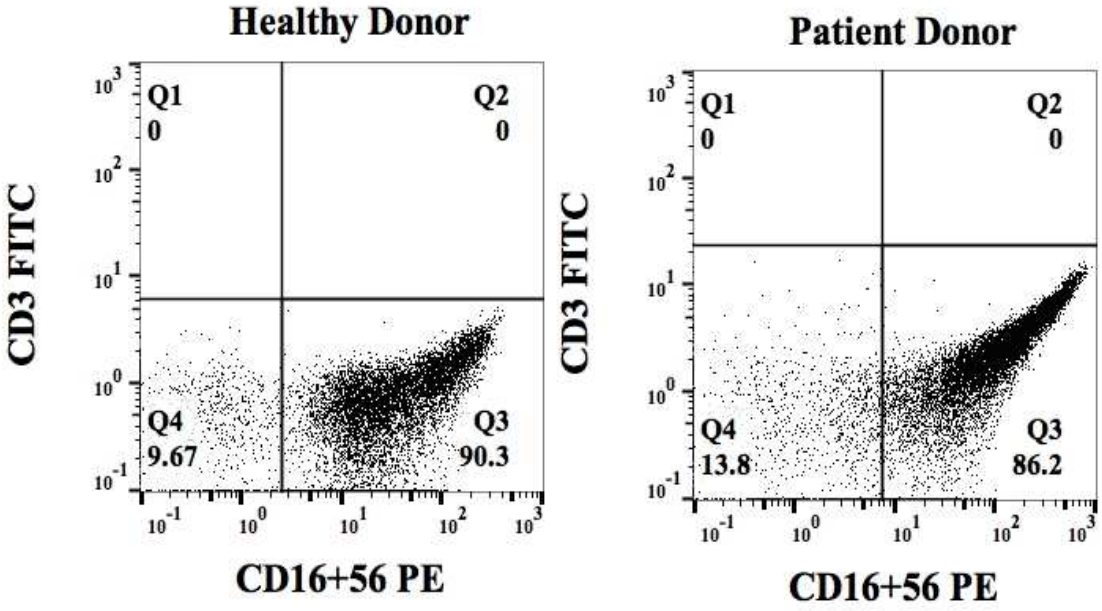


Figure 1: Flow cytometery of healthy and patient donor negatively selected NK cells.
Human PBMCs were isolated from 60 mL of peripheral blood of the healthy donors and cancer patients and number of cells were assessed by microscope using hematocytometer. Equal numbers of PBMCs from both donors were used for negative selection and $1.0x10^5$ purified cells were stained with CD3 FITC and CD16+56PE antibodies for flow cytometery.

Figure 2

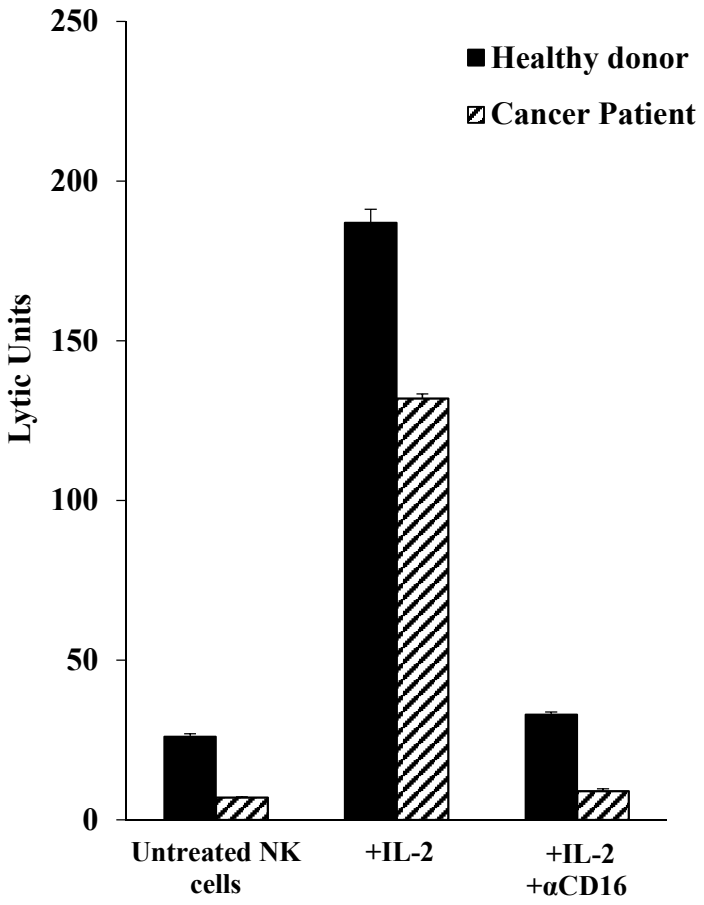


Figure 2: Primary NK cells from healthy and patient donors are activated and incubated overnight then assayed for cytoxicity against OSCSCs.

Purified NK cells (1x10$_6$/mL) were left untreated, IL-2 (1000 units/mL) treated, or anti
CD16mAb (3 μg/mL) and IL-2 (1000 units/mL) treated and incubated for 12-18 hours. Following overnight incubation, they were added to ^{51}Cr labeled OSCSC cells (target cells). NK cell cytotoxicity was determined by conducting a standard 4-hour ^{51}Cr release assay. A gamma counter was used to measure the radioactivity released into the supernatants. Levels of NK cell-mediated cytotoxicity against radioactively labeled OSCSCs were determined using lytic units (LU 30/10$_6$).

Figure 3

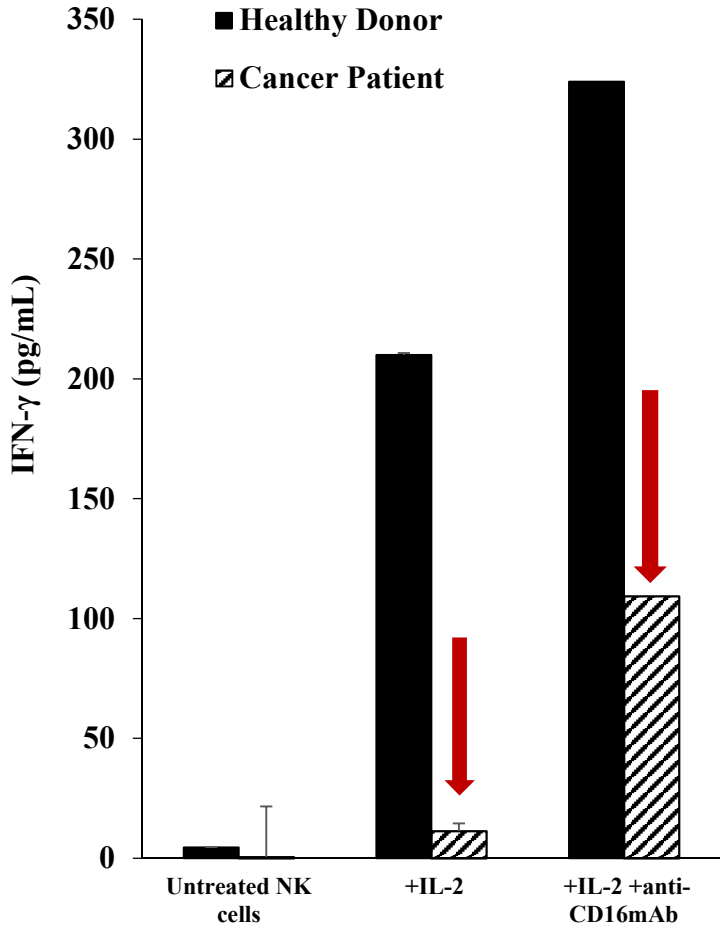


Figure 3: NK cells from patient donors produce less IFN-γ than healthy donors.
Purified NK cells (1x10$_6$/mL) were left untreated, treated with IL-2 (1000 units/mL), or anti-CD16mAb (3 μg/mL) and IL-2 (1000 units/mL) and incubated overnight. Supernatants of cultures were harvested and used for ELISA analysis. Above are the levels of secretion for IFN-γ.

Figure 4

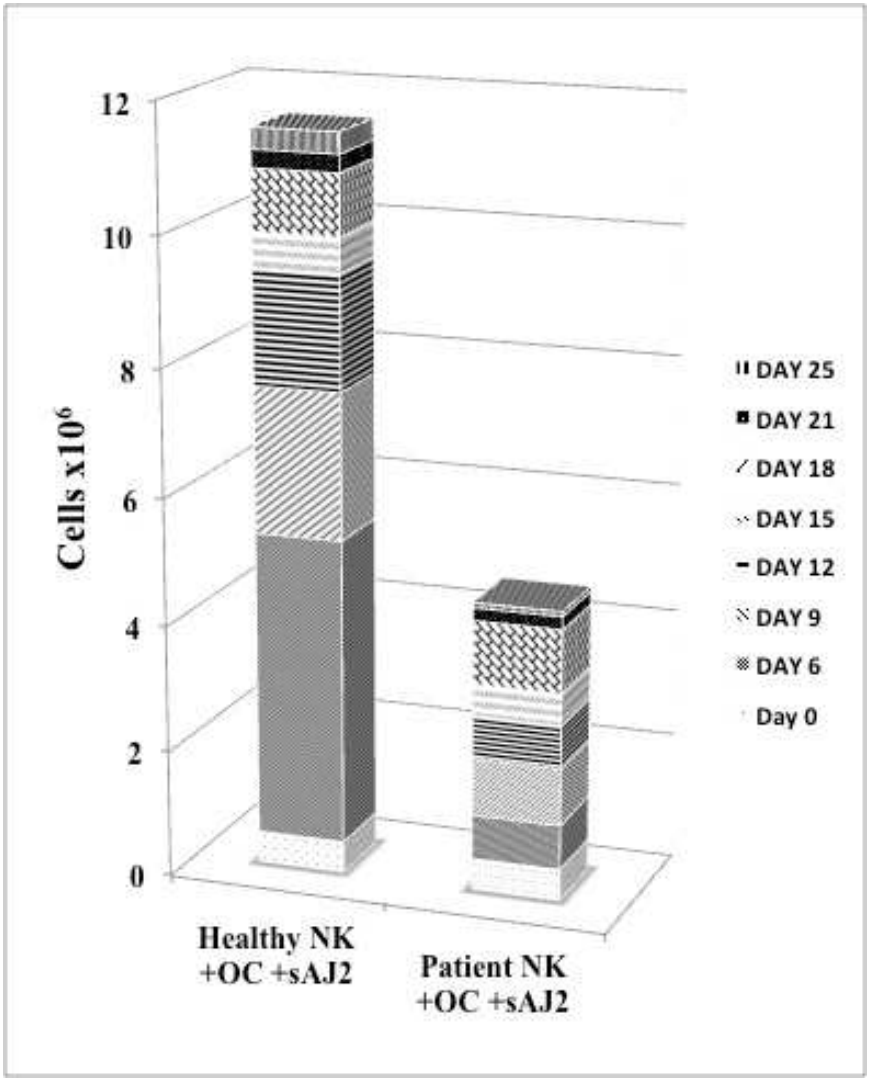


Figure 4: Osteoclast and sAJ2 expanded super-charged NKs from healthy donors expand more immune cells at a faster rate. Freshly purified NK cells from healthy donor and cancer patient were treated and co-cultured with monocyte-derived OCs. After 6, 9, 12, 15, 18, 21, and 25 days of co-culture, expanded lymphocytes were manually counted using microscopy and a hematocytometer.

Figure 5

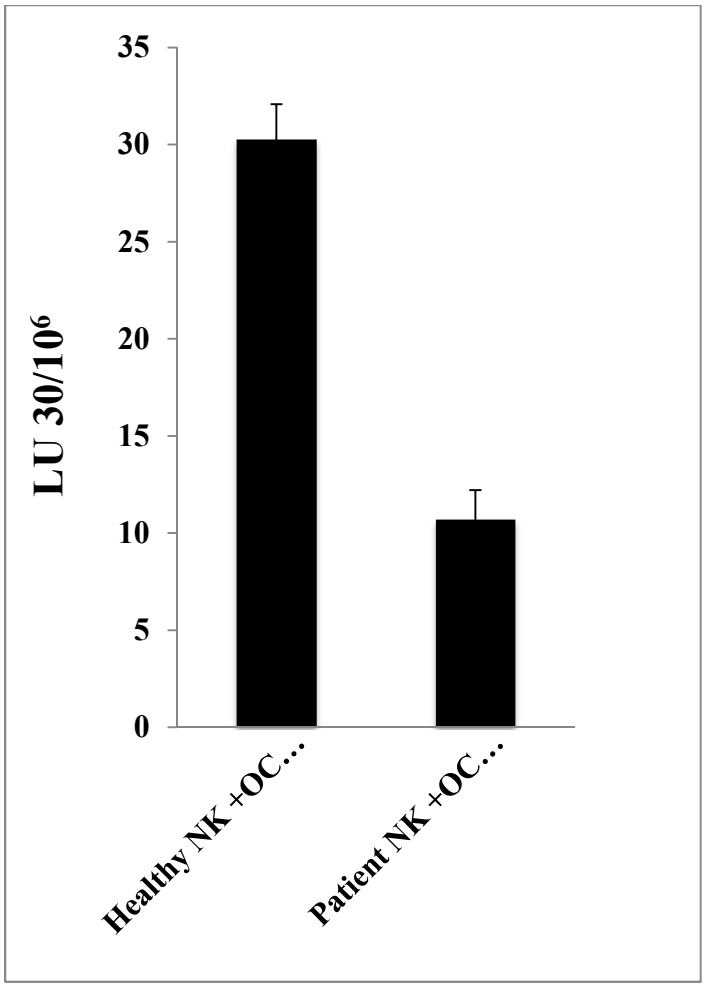


Figure 5: Osteoclast and sAJ2 expanded NK cells from cancer patients demonstrate low cytotoxic ability against OSCSCs.
Freshly purified NK cells from healthy donor and cancer patient were treated and co-cultured with monocyte-derived OCs. After 14days of co-culture, expanded lymphocytes were manually counted using microscopy and a hemocytometer and cytotoxicity of lymphocytes was determined using standard 4-hour ^{51}Cr release assay against OSCSCs. A gamma counter was used to measure the radioactivity released into the supernatants. Levels of NK cell-mediated cytotoxicity against radioactively labeled OSCSCs were determined using lytic units (LU $30/10^6$).

Figure 6

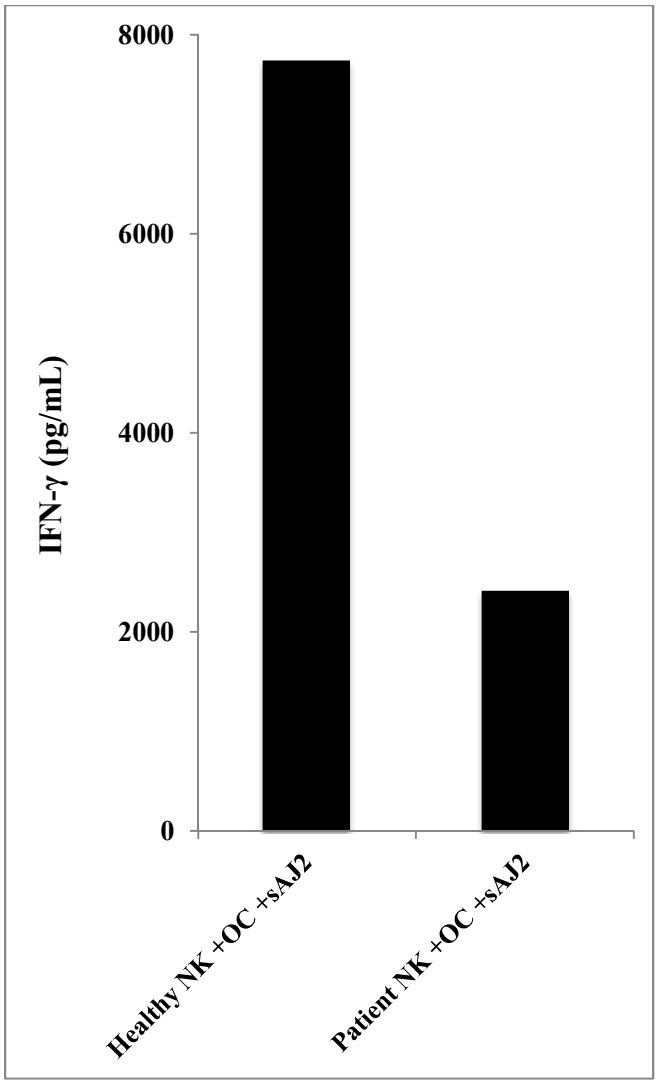


Figure 6: Osteoclast and sAJ2 expanded NK cells from cancer patients demonstrate decreased IFN-γ secretion.
Freshly purified NK cells from healthy donor and cancer patient were treated and co-cultured with monocyte-derived OCs. After 14 days of co-culture, expanded lymphocytes were manually counted using microscopy and a hemocytometer and supernatants of cultures were harvested and used for ELISA analysis. Above are the levels of secretion for IFN-γ.

Figure 7

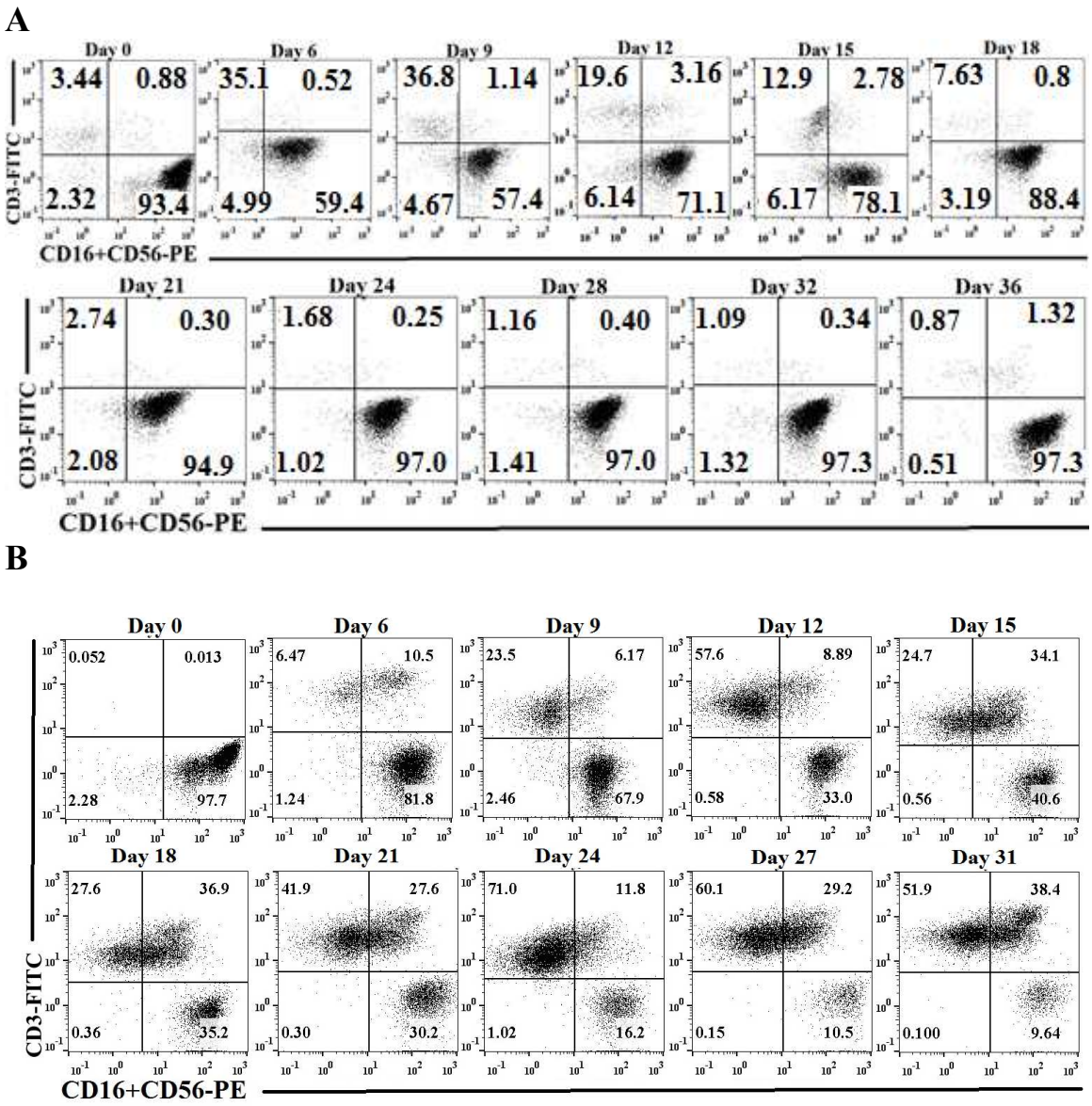
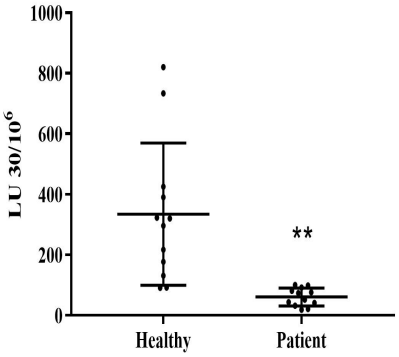


Figure 7: Healthy donors continually expand NK cells whereas cancer patients expand a larger percentage of $CD3^+$ cells

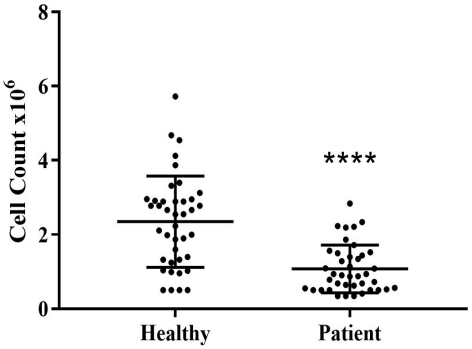
Freshly purified NK cells from healthy donor and cancer patient were treated and co-cultured with monocyte-derived OCs, surface expression of CD3, CD16 and CD56 was analyzed on expanding cells at days 6, 9, 12, 15, 18, 21, 24, 27, and 31 of healthy donor (A) and cancer patient (B) using antibody staining followed by flow cytometric analysis.

Figure 8

A


1000
800
600
400
200
0
LU 30/10^6
**
Healthy
Patient

B


8
6
4
2
0
Cell Count x10^6
****
Healthy
Patient

C

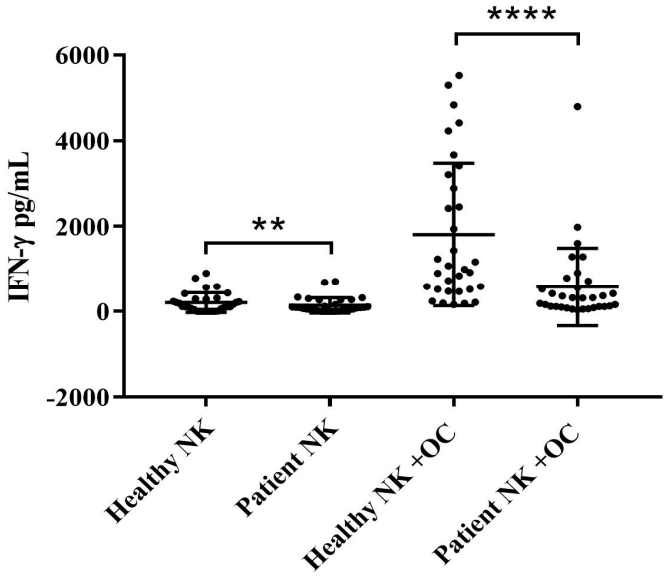

6000
4000
2000
0
-2000
IFN-γ pg/mL
**
****
Healthy NK
Patient NK
Healthy NK +OC
Patient NK +OC

Figure 8: Osteoclast expanded NK cells from cancer patients have significantly reduced ability to kill OSCSCs, secrete IFN-g, and expand immune cells.

Freshly purified NK cells from healthy donor and cancer patient were treated and co-cultured with monocyte-derived OCs. After 14 days of co-culture, expanded lymphocytes were manually counted using microscopy and a hematocytometer and cytotoxicity of lymphocytes was determined using standard 4-hour 51Cr release assay against OSCSCs. A gamma counter was used to measure the radioactivity released into the supernatants. Levels of NK cell-mediated cytotoxicity against radioactively labeled OSCSCs were determined using lytic units (LU $30/10^6$)(**A)** and supernatants of cultures were harvested and used for ELISA analysis(**C**). After 6, 9, 12, 15, 18, 21, and 25 days of co-culture, expanded lymphocytes were manually counted using microscopy and a hematocytometer (**B**).

Chapter 2

Specific aim 1: Osteoclasts preferentially expand and activate NK and $CD8^+$ cells but not $CD4^+$

Results

NK Cells target $CD4^+$ helper T-cells but not their $CD8^+$ cytotoxic counterparts.

Increased expansion of CD8+T cells in the OC-expanded NK cells cultures seen in both healthy and cancer patients. Majority of contaminated T cells in the osteoclasts expanded NK cell culture both in healthy donor and cancer patient were CD8+T cells (Fig. 10A and 10B). When T cells were purified from PBMC and cultured with OCs, CD8+T cells numbers increased and not the CD4+T cells (Fig. 10A).

Next, we purified the NK cells from healthy donors, and used those NK cells to run the cytotoxicity assay against the CD4+T cells and CD8+T cells. Results indicate that NK cells target and lyse the CD4+T cells, but not CD8+T cells (Fig. 10C).

Osteoclasts preferentially expand and activate NK cells and CD8+T cells more than they do CD4+T.

Next, we purified the CD4+T cells and CD8+T cells, and cultured them with OCs to track the expansion profile. When expanded cells were tracked for 15 days, cumulative cell count showed increased expansion of CD8+ cells as compared to CD4+. Based on this data, we know OCs expand CD8+T cells more than CD4+T cells, next we compared all three type of immune cells, NK, CD4+T cells and CD8+T cells under the same conditions (Fig. 9A). Results showed that

NK cells and CD8+T cells had similar fold expansion of cells with OCs, whereas CD4+T cells expanded less (Fig. 9a), when IFN-γ was adjusted based on 1 million cells, NK cells secreted higher IFN-γ, CD8+T cells second higher and CD4+T cells secreted lower IFN-γ when they interacted with OCs (Fig. 9B).

Figure 9

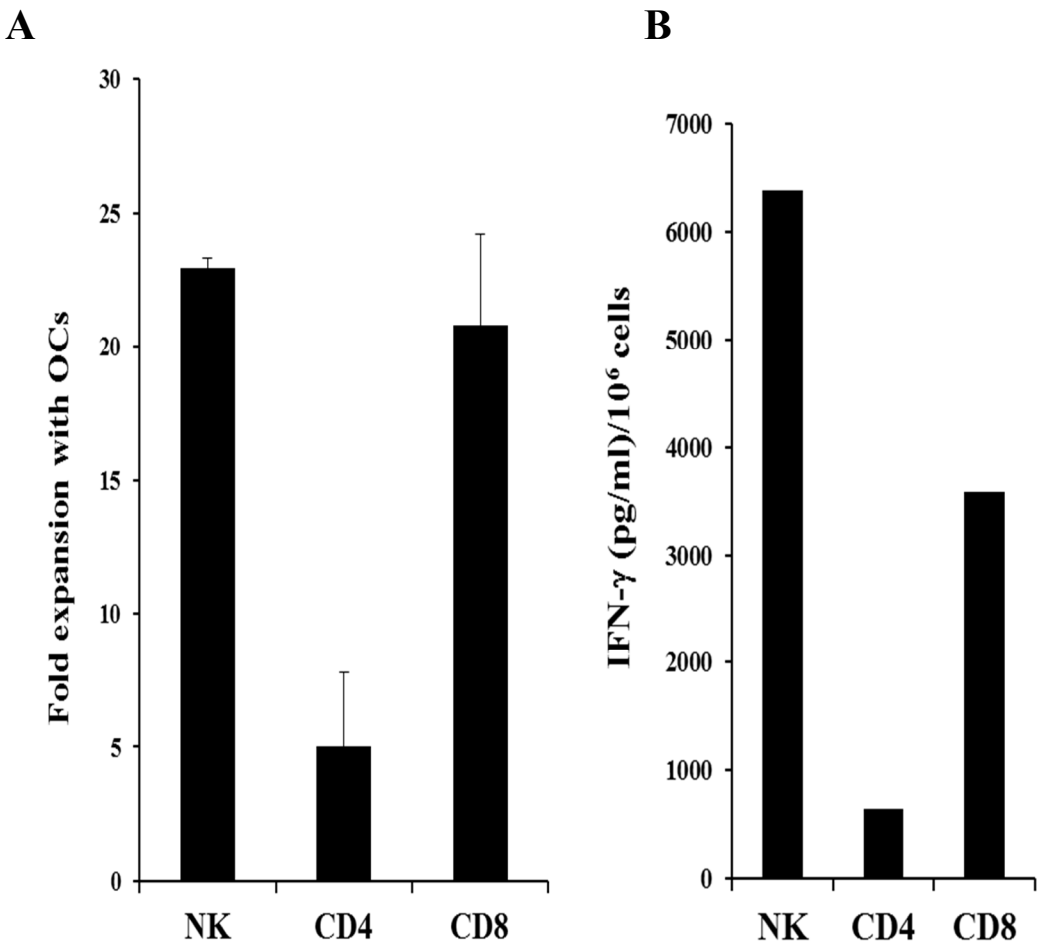
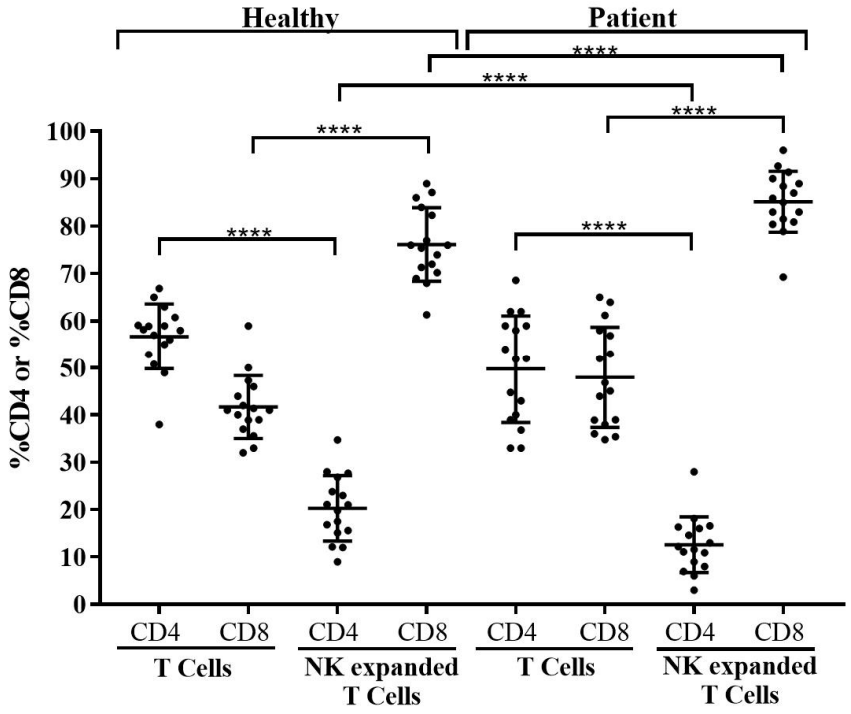


Figure 9: Osteoclasts preferentially expand and activate NK and CD8$^+$ cells but not CD4$^+$

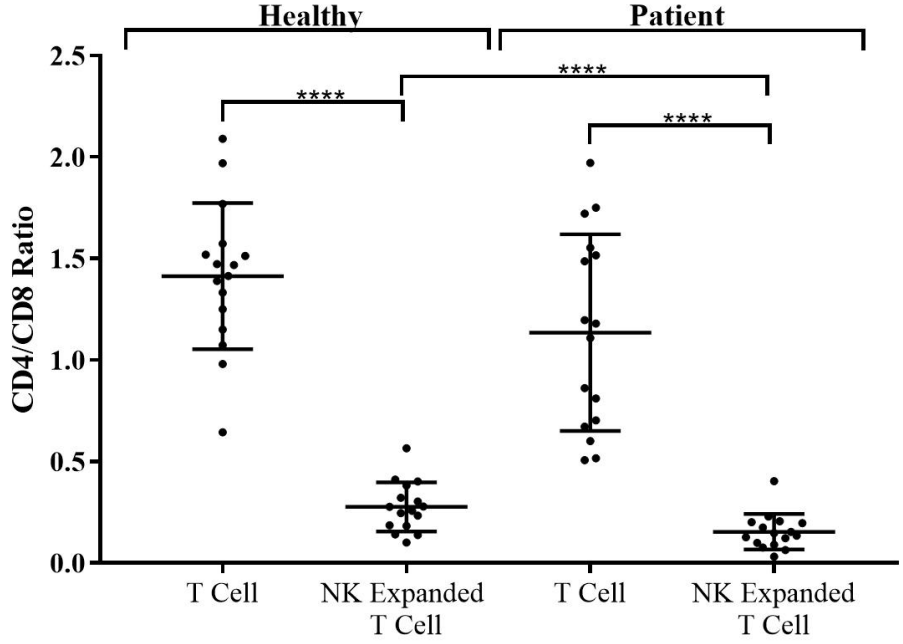
Purified CD4T cells and CD8T (1x106 cells/ml) from the healthy donors were treated with the combination of IL-2 (100 U/ml) and anti-CD3 (1 μg/ml)/CD28mAb (3 g/ml) for 18 hours before they were co-cultured with sAJ2 (T: sAJ2; 1:2) and OCs in the presence of sAJ2 at 1:2:4 ratios (OCs:T:sAJ2). After 6, 9, 12 and 15 days of co-culture, lymphocytes were manually counted using microscopy, the numbers of OC expanded NK, CD4 and CD8 cells were subtracted from the number of non-OC expanded control cells, and fold expansion of the cells were determined by dividing it to the initial input cells (**A**). The supernatants were harvested from the day 6, 9, 12 and 15 co-cultures, IFN-γ secretion was determined using single ELISAs and adjusted per 1 million of lymphocytes (**B**).

Figure 10

A


Healthy
Patient
****
****
****
****
****
****
%CD4 or %CD8
100
90
80
70
60
50
40
30
20
10
0
CD4
CD8
CD4
CD8
CD4
CD8
CD4
CD8
T Cells
NK expanded T Cells
T Cells
NK expanded T Cells

B


Healthy
Patient
****
****
****
CD4/CD8 Ratio
2.5
2.0
1.5
1.0
0.5
0.0
T Cell
NK Expanded T Cell
T Cell
NK Expanded T Cell

C

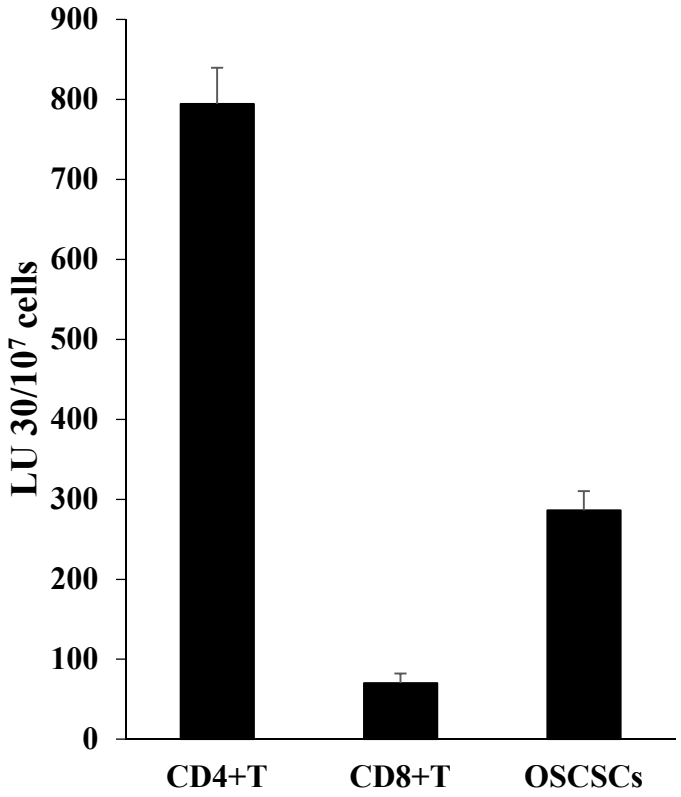


Figure 10: NK mediated expansion of CD8 T-cells could partly be due to lysis of CD4 T-cells by the NKs

Purified NK cells (1x106 cells/ml) from the healthy donors and cancer patients were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with OCs in the presence of sAJ2 at 1:2:4 ratios (OCs:NK:sAJ2). Purified T cells (1x106 cells/ml) from the healthy donors and cancer patients were treated with the combination of IL-2 (100 U/ml) and anti-CD3 (1 µg/ml)/CD28mAb (3 ug/ml) for 18 hours before they were cocultured with OCs in the presence of sAJ2 at 1:2:4 ratios (OCs:T:sAJ2). After 6, 9, 12 and 15 days of co-culture, surface expression of CD4 and CD8 were analyzed within CD3+ immune cells using flow cytometry (n=28) (**A**). Proportion of CD4+T cells were compared with the proportion of CD8+T cells and ratio is shown in figure (n=24) (**B**). Cytotoxicity of NK cells against CD4+T and CD8+T cells was determined suing
NK cells from the OC-expanded NK cells, using standard 4-hour TVA assay against CD4+T and CD8+T cells,the lytic units 30/107 cells were determined using inverse number of NK cells required to lyse 30% of CD4+T or CD8+T x 100(**C**).

Chapter 3

Specific aim 3: Increased expansion of $CD8^+$ T-cells by supercharged NK cells in Hu-BLT Mice.

Results:

Single Injection of Supercharged NK cells as immunotherapy resulted in reduced tumor weight post-excision, and decreased tumor growth when cultured *ex-vivo*.

Mice were fed orally with AJ2 throughout the experiment time period, a week later the first dose of AJ2, mice were implanted surgically with OSCSCs tumors and after one week of tumor growth, a single injection of 1.5 $X10_6$ purified NK cells with potent cytotoxic and cytokine secretion capabilities were performed (Figs. 12A). NK cells were purified from healthy donors and expanded in the presence of the treatment with IL-2+anti-CD16mAb and sonicated bacteria and osteoclasts as described in the Materials and Methods section and previous section of this chapter. Mice implanted with OSCSC tumors and injected with NK cells alone grew significantly smaller tumors when compared to those implanted with OSCSC tumors in the absence of NK injection (Fig 12B). Similar results were seen in MP2 injected mice (Data not shown).

Oral consumption of AJ2 in combination with NK immunotherapy further reduced tumor mass and increased immune infiltration in the tumor microenvironment.

Tumors excised from the mice were weighed then dissociated and suspended in single cell cultures and were allowed to incubate. Animals that received NK injection and NK injection in

combination with AJ2 diet supplementation had the smallest tumors and weighed the least out of all conditions (Fig 13A). When cultured for 22 days, NK injected and NK/AJ2 fed animals had drastically lower tumor cell proliferation. Not only was the overall cell count the low but so was the rate of expansion between each day (Fig. 13B). Flow cytometery analysis of the tumors immediately after excision and dissociation reveals that tumors from animals with supercharged NK injection showed higher numbers of infiltrating human immune cells as compared to non injected mice (Fig. 13C). Interestingly, animals that received NK injection and were also fed a supplemental AJ2 diet had 25% human immune cell infiltration in the tumor mass (Fig 13C).

Rapid expansion of $CD3^+$ T-cells coincides with the loss of $CD16^+CD56^+$NK cells in tumor implanted Hu-BLT mice.

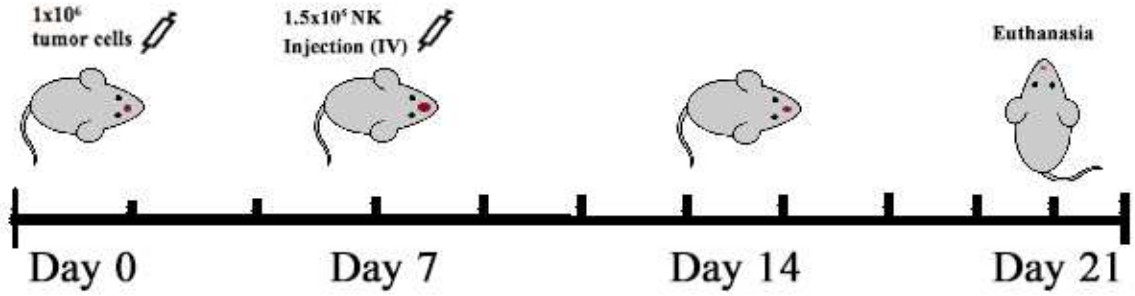
Even though tumor bearing hu-BLT mice contained larger percentages of NK cells (Fig. 3A), the expansion resulted in gradual and significant T cell expansion starting on day 6 and continuing until day 22, at which point 96% of the cells were T cells and only 1.1% were NK cells. In contrast, flow-through cells from hu-BLT mice with no tumor, which contained less NK cells initially, expanded NK cells, and the levels rose from 28.6% NK cells at day 6 to 69% NK cells at day 22 (Fig. 14) The levels of NK cells when cultured with autologous OCs were increased in both animals from the initial day of culture, although tumor free mice had a 10.59 fold increase from day 0 to day 6, whereas the mice with tumors had a 4.56 fold increase (Data not shown). The total numbers of expanded lymphocytes were lower in tumor-bearing mice when compared to those without tumors with the majority being T cells and not NK cells (Data now shown)

Increased surface expression of CD8$^+$ T cells in various immune tissue compartments of Hu-BLT mice implanted with tumors and received single dose of super-charged NK cells as immunotherapy.

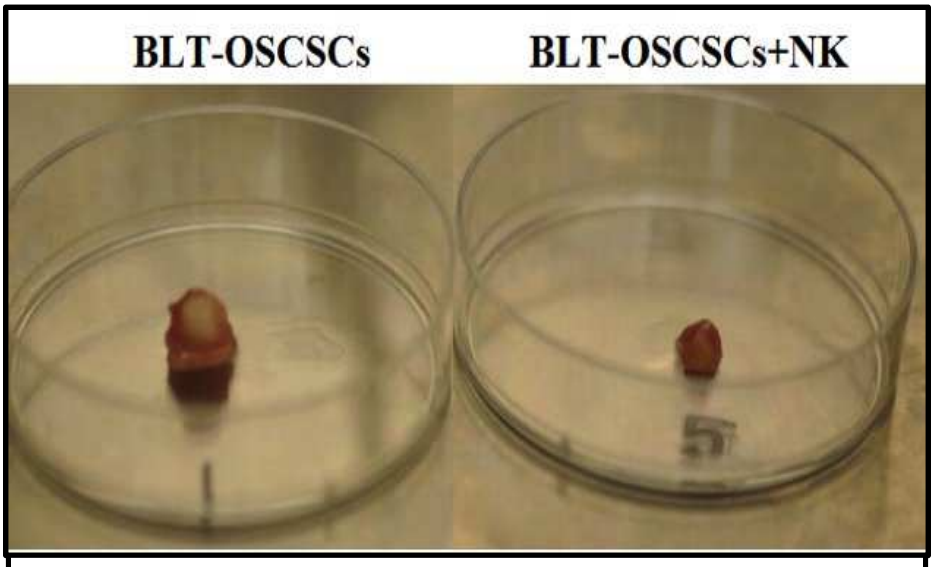
To investigate the immunomodulatory effect osteoclasts expanded NK cells and probiotic in the hu-BLT mice, various tissue compartments were harvested and analyzed for immune cell markers. When we analyzed spleen, bone marrow and blood, there was increased proportions of CD8+ cells in the animals injected with NK cells, and were further increased when AJ2 was combined with NK cells (Table 1, Table 2 and Fig. 1). The healthy control animal when compared to the OSCSC injectd animals display a larger proportion of circulating CD8+ in the PBMCs. Animals that were supplemented with AJ2 in combination with NK immunotherapy had significantly larger percentages of CD8+ immune cells in the dissociated spleen. CD8+ cells in the BM were highest in the AJ2 fed animals and also the NK injected mice (Fig. 15).

Figure 12

A



B



Tumors of OSCSCs-implanted BLTs, with and without Super-charged NK

Figure 12: Single injection of expanded hu-NK cells inhibited the growth of primitive oral cancer stem cells in BLT mice and was further inhibited with combination of the probiotics.

Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) and NSG mice were orthotopically injected with 1x106 of human OSCSCs into the flora of the mouth. One week after tumor implantation selected hu-BLT mice received 1.5 X 10^6 human expanded NK cells via tail vein injection. Disease progression and weight loss was monitored for another 3-4 weeks (**A**). Animals were sacrificed and tumor pictures were taken post mortem (**B**).

Figure 13

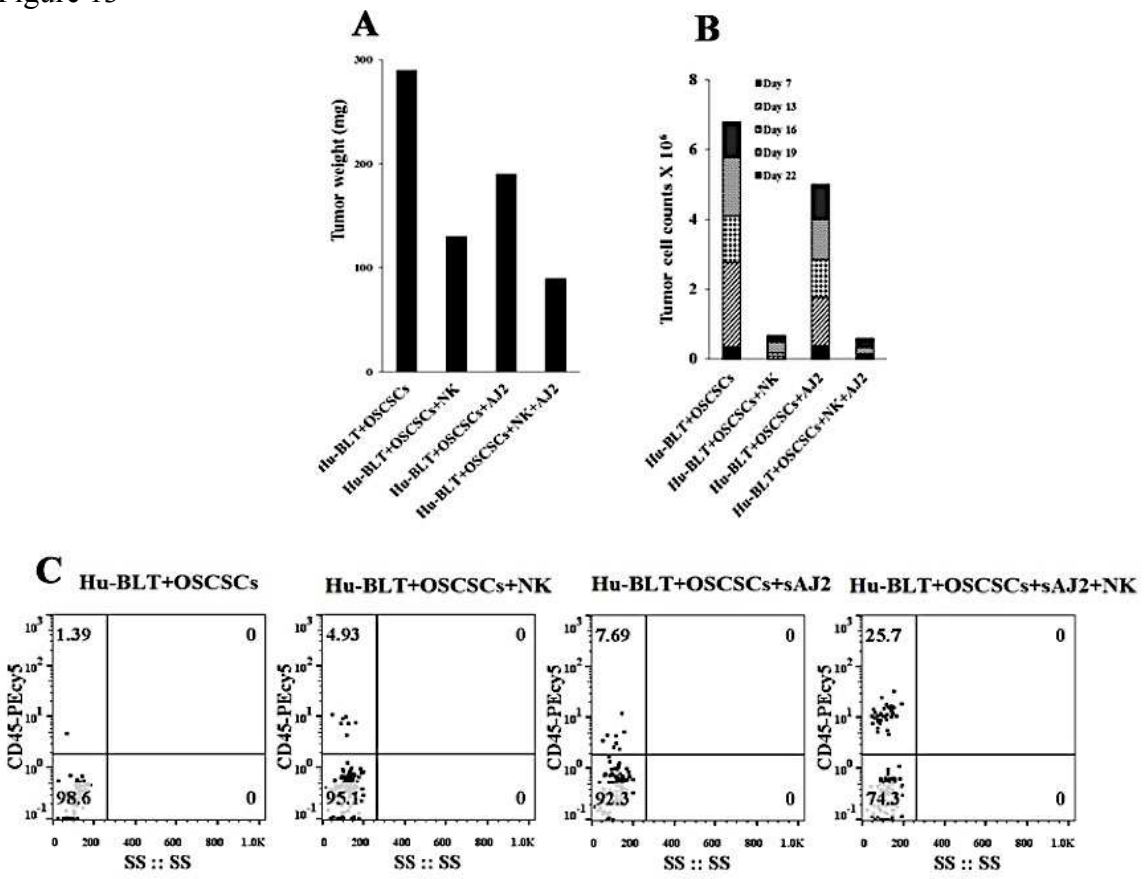


Figure 13: Oral consumption of AJ2 in combination with NK immunotherapy further reduced tumor mass and increased immune infiltration in the tumor microenvironment. Disease progression and weight loss was monitored for another 3-4 weeks, animals were sacrificed and tumor mass was recorded (**A**). Oral tumors from the animals were cultured in FBS media and the numbers of attached tumor cells were measured on day 7, day 13, day 16, day 19 and day 22 (**B**). Surface expression of human CD45 on the oral tumors from hu-BLT mice was determined using flow cytometeric analysis. Isotype control antibodies were used as control (**C**).

Figure 14

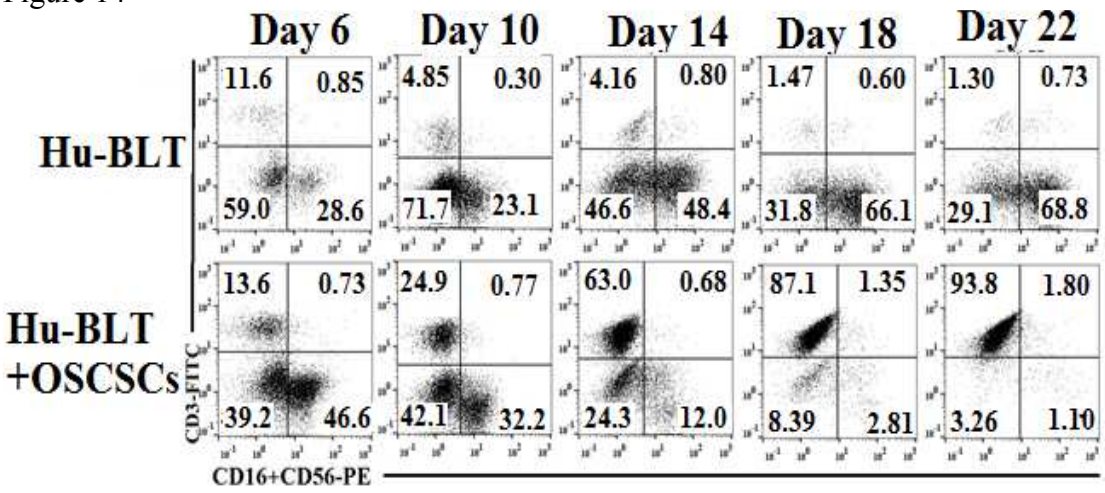


Figure 14: Rapid expansion of $CD3^+$ T-cells coincides with the loss of $CD16^+CD56^+$NK cells in tumor implanted Hu-BLT mice.

NK cells from healthy control and orthotopically injected humanized mice were purified and cultured in the presence of OC and sAJ2. Cells were stained and analyzed by flow cytometeric analysis on day 6, 10, 14, 18, and 22.

Figure 15

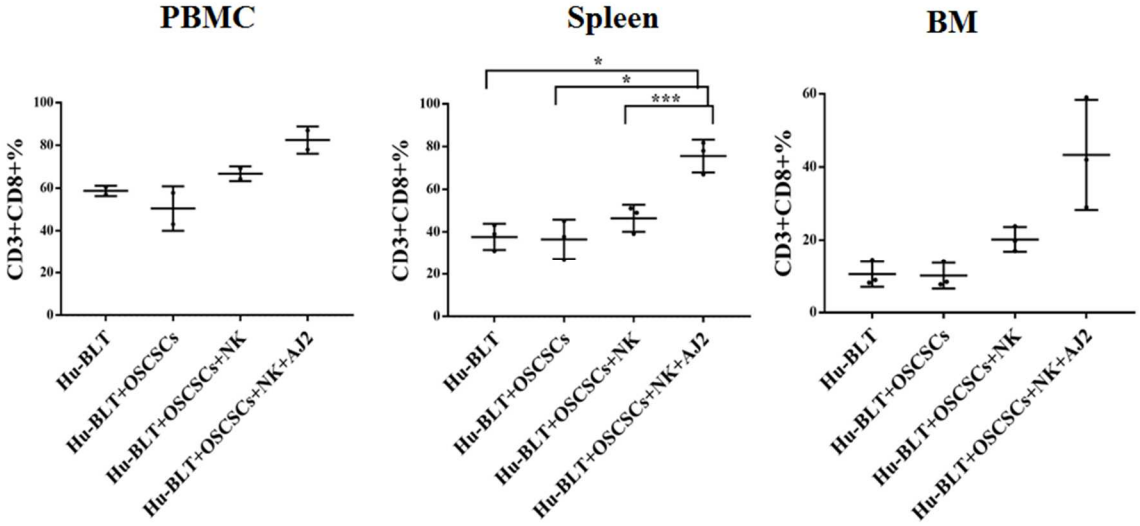


Figure 15: Increased surface expression of $CD8^+$ T cells in various immune tissue compartments of Hu-BLT mice implanted with tumors and received single dose of super-charged NK cells as immunotherapy.
Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) mice were orthotopically injected with $1x10_6$ of human OSCSCs into the flora of the mouth. One week after tumor implantation selected hu-BLT mice received 1.5 X 10_6 human expanded NK cells via tail vein injection, animals were orally fed with AJ2 (5 billion) every 48 hours throughout the experiment period. At the end of this experiment spleen, bone marrow and peripheral blood was collected, single cell suspension was obtained from each tissue as described in Materials and Methods. PBMCs from peripheral blood, spleen and bone marrow flow-through cells with antihuman CD3 and CD8 antibodies and analyzed by flow cytometry. Isotype antibodies were used as a control.

Discussion

In an effort to understand the behavior of primary NK cells purified from the peripheral blood, we examined the cytotoxicity and IFN-γ secreting ability of both healthy donors and cancer patients prior to our patented immunotherapeutic osteoclast and sAJ2 activation and expansion process. The results reveal that cancer patient NK cells are less capable of eliciting direct cytotoxicity against oral squamous cancer stem cells under each of the various activating conditions (Fig. 2). Once can assume a host of underlying factors triggering this loss of functional and direct cytotoxicity. The literature reports that the down regulation of activating receptors such as NKG2D and other suppressive phenomena such as checkpoint inhibitors like PD-1 and PD-L1 in the tumor microenvironment could be contributing factors to loss of function [5]. However our study was not focused on the mechanistic aspects of NK or T-cell exhaustion but on the comparative differences amongst the NK cells of healthy donors and cancer patients that may be responsible for the persistence of tumor growth.

Although direct cytotoxicity of NK cells against cancer stem cells could slow if not halt tumor progression, the secretion of IFN-γ has been shown to be responsible for the differentiation of cancer stem cells into target cells with increased expression of various differentiation markers, most importantly MHC-I. It is understood that activated CD8+ T cells are unable to kill cancer stem cells however they do target and kill transformed cells displaying non-self or stress induced antigens on MHC-I, such as those from malignant cancer cells that have been differentiated. Thus when trying to interpret the effects of the overwhelming decrease in IFN-γ secretion of NK cells from cancer patients in comparison to healthy donors, we can assume that the tumor mass of the these patients do not receive the necessary differentiating

signals that would be provided by healthy NK cells (Fig. 3). With that in mind, we can infer that if the function of NK cells was restored to, at least, that of healthy NK cell levels, patients may have a better prognosis.

Given our findings, we wanted to examine the functional and expansion response of cancer patient NK cells to our patented osteoclast and sAJ2 activation/expansion process. We have recently shown in *in vivo* and *in vitro* studies that osteoclasts are major activators of NK cells [43]. Our osteoclast induced and sAJ2 expansion process was able to maintain expansion of super-charged NK cells for over two months from healthy donors. Accordingly, the cytotoxic function of NK cells remained significantly higher in healthy donors but was severely reduced in cancer patients. The same purified primary NK cells from both donors were expanded and as seen in figure 4, patient NK cells were less capable of expanding functional immune cells and after day 25, healthy patients had more than double the amount of expanding immune cells. The largest difference in cell expansion can be seen on day 6, where healthy cells expanded nearly 6 times more that patient NK cells. This could likely be due to a decrease in cancer patients cells ability to respond to stimulating ligands or cytokines. Interestingly during the entire duration of the expansion process, health donors continually expanded CD16+CD56+ subsets of immune cells. Most notably increasing their proportion of such cells on day 31 to 97%. It is important to note that the expanding immune cells of cancer patients, although initially pure of contaminating T-cells, were eventually unable to sustain CD16+CD56+ expansion, losing 90% of the starting NK population and instead resulting in greater than 88% contaminating CD3+ T-cells (Fig. 7B, 7A). When we examined the cytotoxic ability of these expanding cells against OSCSCs on day 14, there was a drastic reduction in the killing ability of immune cells from patients as compared to healthy donors (Fig. 5). This also correlates with their ability to secrete differentiation-

inducing IFN-γ (Fig. 6). In an effort to further validate our findings, repeat experiments of NK isolation, expansion, cytotoxic assays and IFN-γ secretion profiling were done to 11 more cancer patients and compared to new healthy donors. The compiled results significant differences in the loss of NK cytotoxicity, IFN-γ secretion and expansion (Fig. 7).

In an effort to determine why the immune profile of expanding cells in our patented expansion process varies between healthy and cancer patients, we purified the CD4+T cells and CD8+T cells, and cultured them with OCs to track their expansion profile as compared to that of NK cells. We were able to uncover that Osteoclasts preferentially expand and activate NK cells and CD8+T cells more than they do CD4+T. Fold expansion of NK and CD8+ cells was much greater than that of CD4+, which concludes that there may be various activating or inhibitory ligands expressed on osteoclast that preferentially expand the CD8+ subset over the CD4+. IFN-γ secretion by from each of the expanding populations closely mimics the expansion profile, where NK and CD8+ cells secrete much higher amounts of IFN-γ as compared to CD4+ (Fig. 8). An even more interesting finding is that NK mediated expansion of CD8 T-cells could partly be due to lysis of CD4 T-cells by the NKs. As seen in figure 10A and 10B, when T-cell were expanded in the presence of NK cells, there is skewed ration of the CD4+ to CD8+ population. This preferential expansion of CD8+ cells is not seen in the absence of NK cells in both healthy and cancer patients T-cells. The differences in molecular and gene expression causing this difference in the presence of NK cells will require much more research to uncover, but a simple cytotoxicity assay against CD4+ and CD8+ T-cell as targets and supercharged expanded NKs as effector cells revealed a very important finding. We can conclude, that by through either direct or indirect forms of cytotoxicity, NK cells can target and kill helper T-cell and not CTLs. Even more surprising is that when compared to the control OSCSCs, which NKs are known to be

highly cytotoxic against, CD4+ T-cells were eliminated to a much greater extent (Fig. 10C).

Status quo, increased PBMC numbers in the peripheral blood as well as increased infiltrating immune cells in the tumor microenvironment provide physicians with greater options for immune-check point inhibition treatments and provide patients with an overall better prognosis for cancer treatment [2, 15, 30]. Based on our data, the use of autologous NK cells is not be a viable option for immunotherapeutic treatment of tumorigenic cancers, instead our strategy focuses on the use of supercharged expanded NK cells of allogeneic origin. We tested our expanded supercharged allogeneic NK cells in hu-BLT mice inoculated with oral tumors. With just one dose of NK cell injection (1.5 million cells), animals bearing tumors had a significant decrease in tumor size, ex-vivo tumor cell proliferation (Fig. 13A and 13B). Combinatorial treatment with probiotic AJ2 supplementation revealed a synergistic increase in tumor infiltrating immune cells (Fig. 13C). When analyzing immune cell compartments, NK cell-mediated cytotoxicity and IFN-γ from immune cells was improved significantly and an Increased surface expression of $CD8^+$ T cells in various immune tissue compartments of Hu-BLT mice implanted with tumors and received single dose of super-charged NK cells as immunotherapy. This verifies that allogeneic supercharged NK cells used as for immunotherapy not only increase and expand CD8+ cytotoxic T-cells in vitro, but also in a tumor bearing in-vivo model. Such a result could provide patients with a much less invasive treatment option with unprecedented results. It has been shown that mature alloreactive NK cells can be safely infused into patients with no increased incidence of graft versus host disease (GvDH) [16]. To avoid any kind of risk of GvHD, we can also consider isolating the contaminating T cells from the supercharged NK cells, and inject the high purity NK cells.

Conclusion

Our work has shown the importance of the supercharged Natural Killers cells for use as immunotherapy and demonstrated their ability to target and kill cancer stem cells, which I believe to be the main contributors to the pathogenicity of the malignancies. More importantly, our allogeneic supercharged NK cells may kill cancer cells that spawn more transformed daughter cells, but their ability to secrete larger amounts of IFN-γ helps differentiate these cells into a less proliferate cell cycle. This allows for the CD8+ cytotoxic T-cells, which have increased in number as a result our immunotherapy treatment, to now recognize, target, and kill these differentiated cancer cells leading to decreased tumor burden and a potentially better prognosis. To conclude, our work reveals that there is still much more research to be done on the various intracellular changes that result in the findings of our work. Mechanistic studies of the interaction between immune cells involved in the remodeling and elimination of the tumor mass and its inflammatory effects, although very challenging, remain to be the key to understanding the bodies response to malignancies. I firmly believe that the human body, with guidance, can infiltrate the inflammatory tumor mass, reducing its burden, eliminating it from the body more efficiently and less invasively as compared to the current surgical and chemotherapeutic treatment options.

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