Title

Super-charged NK cells increase immune infiltration in the tumor microenvironment and inhibit tumor growth in humanized-BLT mice

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Publication Date

2017

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Super-charged NK cells increase immune infiltration in the tumor microenvironment and inhibit tumor growth in humanized-BLT mice.

A dissertation submitted in partial satisfaction of the requirements for the

degree Doctor of Philosophy in Oral Biology

by

Kawaljit Kaur

2017

ABSTRACT OF DISSERTATION

Super-charged NK cells increase immune infiltration in the tumor microenvironment and inhibit tumor growth in humanized-BLT mice

By

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Doctor of Philosophy in Oral Biology

University of California, Los Angeles 2017

Professor Anahid Jewett, Co-chair

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NK cells are known to limit growth and expansion of cancer stem cells and oral tumors by providing key cytokines such as IFN-γ and TNF-α, which drive differentiation of stem-like/poorly differentiated cancer stem cells. The detailed understanding of how and why their function is affected by the cancer microenvironment is very important for outlining the most efficient NK cell-based immunotherapies for cancer patients. In this regard, we first characterized NK cells from the cancer patients for their IFN-γ secretion, cytotoxic function against the cancer stem cells, and their surface receptors. In previous studies, we demonstrated that IFN-γ plays a dominant role in up-regulation of B7H1, CD54 and MHC1 surface receptor expression and inhibition of tumor growth by differentiating the cancer stem cells, and via inflammatory cytokine release when they are in contact with cancer stem cells. We also showed that induction of NK cell-mediated

cytotoxicity resistance and differentiation in the stem cells correlated with the increased expression of CD54, B7H1, and MHCI, and mediated by the combination of membrane-bound or secreted IFN-γ. In this study, we demonstrated that NK media adjusted based cells from cancer patients secreted lower IFN-γ. When we used the NK cell condition media from the cancer patient and healthy NK cells to treat the cancer stem cells, the condition media from cancer patient NK cells was less capable of differentiating the cancer stem cells. The cancer stem cells treated with patient NK cells condition media, were more susceptible to NK cell-mediated cytotoxicity and displayed lower surface expression of CD54, MHC1, and B7H1 when compared to cancer stem cells treated with healthy IFN-γ.

We also demonstrated that among all the cells we characterized to design a novel strategy to expand highly potent NK cells, osteoclasts fit best based on the NK receptors ligand expression and secreted cytokines required for NK cells expansion and activation. In this regard, we tested the cancer patient osteoclasts (OCs) for their surface expression of MHC1 and MICA/B, both were showed lower expression levels on cancer patient OCs. So, not just the NK cells receptors required for tumor recognition are lower on cancer patient NK cells, but the ligands required to increase the expression of those receptors are also down-modulated in cancer patient OCs. Next, we cultured the cancer patient NK cells with healthy donor OCs, and we were able to increase the surface expression of NKG2D on patient NK cells but, again the cytotoxic activity and IFN-γ were lower in patient NK cells. We confirmed our finding by analyzing the OCs from KRAS mutated mice and hu-BLT mice injected with tumor.

Based on our data, we were not convinced in the use of autologous NK cells to treat the cancer patients, our strategy was to use expanded allogenic NK cells as cancer cell immunotherapy.

Next, we tested the expanded allogeneic NK cells in tumor implanted hu-BLT mice. With just one NK cell injection (1.5 million cells), tumor growth was inhibited in the mice. On analyzing the immune cell compartments, NK cell-mediated cytotoxicity and IFN-γ from immune cells was improved significantly. Tumors dissected from animals that received NK therapy showed differentiation profile, high MHC1, CD54 and B7H1 on their surface and lower growth in the culture when compared to tumor dissected from hu-BLT mice without NK cell immunotherapy. It has been known that mature alloreactive NK cells can be safely infused into patients with no increased incidence of graft versus host disease (GvDH). To avoid any kind of risk of GvHD, we can also consider isolating the contaminating T cells from the super-charged NK cells, and injecting the high purity NK cells.

The dissertation of Kawaljit Kaur is approved.

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2017

DEDICATION

This dissertation is dedicated to my husband Navroze for believing in me, I would not be what I am and where I am today without his unconditional love and support, and all the sacrifices he made for me.

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Acknowledgements

I owe my deepest gratitude to my advisors, Dr. Anahid Jewett and Dr. Ichiro Nishimura, for their excellent guidance, mentoring, support and encouragement from day I joined the lab. I was fortunate enough to work and develop under tutelage of two successful and well respected researchers in their own field. Dr. Jewett is patient, caring, and provides an excellent atmosphere for professional development in her lab. Her intellectual knowledge, thinking, enthusiasm and devotion to work have been and will always be a source of inspiration to me in both my professional and personal life. On other hand, Dr. Ichiro Nishimura nurtured me to be an independent thinker and provides different prospective to my project. His dedication to detail and passion for science always motivates me to give my best.

A special thanks to my committee members Dr. Nicholas Cacalano, Dr. Rueben Kim, and Dr. Gwack Yousang for their expertise and precious time. They were always there for their generous help and great advice.

Special thanks to my parents, parent-in-laws, brothers, brother-in-law and sister-in-laws.

Last, but definitely not least, my lab members and colleagues, especially Jessica Cook and Brett Ploussard, for being the are best students.

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INTRODUCTION

Natural Killer cells

Natural Killer (NK) cells are large granular lymphocytes that function at the interference of innate and adaptive immunity [1]. Discovered in the early 1970's by accident when investigators were studying specific cytotoxic effects of lymphocytes, it was not until the 1980's that they became generally accepted despite the accumulated evidence [2]. NK cells are a subset of cytotoxic lymphocytes able to recognize and lyse tumor cells and virally infected cells without prior sensitization [3]. Traditionally they have been classified as effectors of innate immunity due to the lack of antigen specific cell surface receptors [4]. NK cells derive from CD34+ hematopoietic stem cells (HSC's) found in the bone marrow. They can be found throughout the body in the spleen, liver, placenta, and peripheral blood [5]. Human NK cells are defined phenotypically by the surface expression of CD56 and CD16, and by their lack of CD3 surface expression [6]. CD56 is a human neural-cell adhesion molecule, but its function on human NK cells is yet to be understood. Although the function of CD56 is unknown, its expression correlates with the expression of other surface markers that confer important functional properties to NK cells [1]. Two subsets of NK cells have been identified based on surface expression of CD56 and CD16. The major subset of NK cells, about 90% of human NK cells, is defined by low expression of CD56 ($CD56^{dim}$) and high expression of CD16 ($CD16^{bright}$). The minor subset makes up approximately 10% of human NK cells and is defined by high expression of CD56 ($CD56^{bright}$) and low or lack of CD16 ($CD16^{dim}$) expression [1, 6]. The $CD56^{dim}$ $CD16^{bright}$ cells were found to be the more cytotoxic subset of human NK cells. On the other hand, $CD56^{bright}$ $CD16^{dim/-}$ NK cells were found to secrete more cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), TNF-β, granulocyte

macrophage-colony stimulating factor (GM-CSF), interleukin-10 (IL-10), and IL-13 after being stimulated with pro-inflammatory cytokines [1, 6, 7].

NK cells develop in the bone marrow and constitute about 5-10% of total lymphocytes in the peripheral circulation and secondary lymphoid organs [8]. Effector function of NK cells include direct natural cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), as well as secretion of inflammatory cytokines and chemokines that indirectly regulate the functions of other immune cells [9, 10]. NK cells mediate cytotoxicity against transformed tumor cells, as well as healthy cells, by releasing pre-formed granules of proteins, known as perforin and granzyme B, which can induce apoptosis, or programmed cell death in target cells [11-13]. NK cells have also been identified within inflamed synovial fluid and express RANKL and M-CSF which during their interaction with monocytes can trigger the formation of osteoclasts in a process that is RANKL and M-CSF dependent [14].

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, 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.

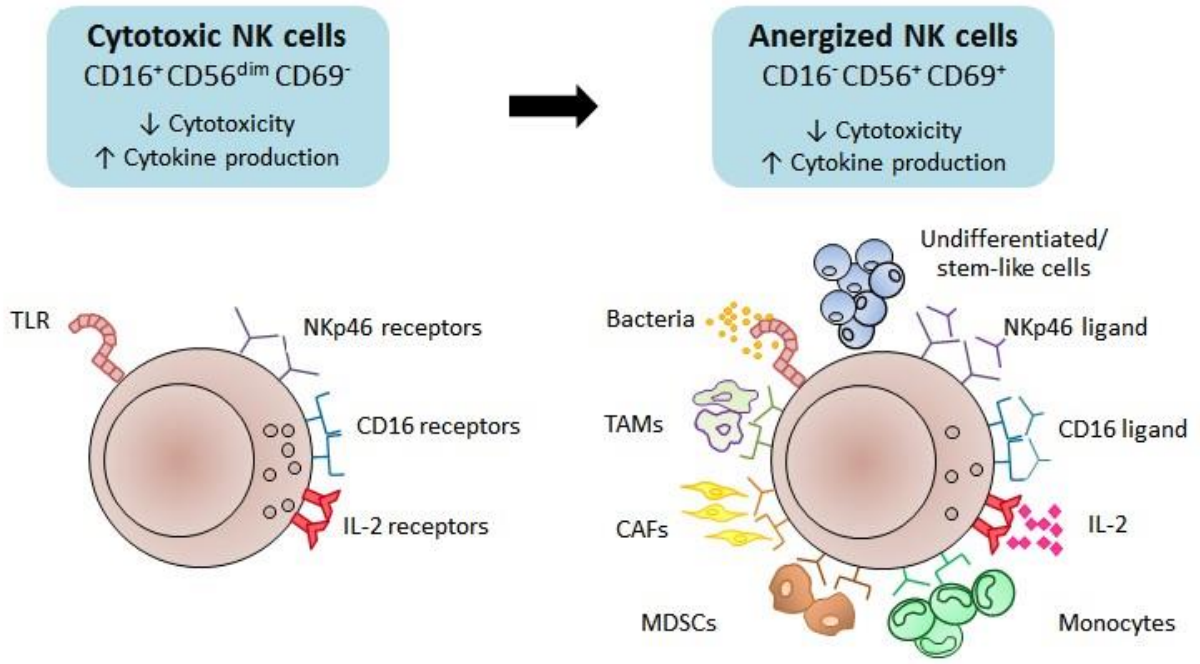

Figure 1: Diagram of split anergy

NK cells in Cancer Patients

Medium and high cytotoxic activity of peripheral-blood lymphocytes are associated with reduced cancer risk, and high NK-cell infiltration of the tumor is associated with better prognosis [18, 19], whereas low activity is associated with increased cancer risk [20]. Suppression of NK cells is mediated by downregulation of NK receptors in the tumor microenvironment [21-28]. NK cell function was previously shown to be significantly reduced in tumor patients [20, 21, 23-27, 29-31]. It has also been shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors, also NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [17, 20, 32-42].

Why cancer Immunotherapy?

Despite intense research and improvement in therapeutic schemes, the 5-year survival rate remained unimproved in all cancers, with an average of less than 5%. This is due to the fact that majority of cancers progresses rapidly, lacking early symptoms and most patients are diagnosed at locally advanced or metastatic stages that do not qualify them for a surgery. For such patients, options are limited to different chemotherapeutic regimes including FOLFIRINOX and nab-paclitaxel with gemcitabine [43-45]. However, this approach is limited by high toxicity and morbidity, and even so chemotherapy is rarely curative. The cellular and molecular factors that promote resistance of pancreatic carcinoma to conventional therapy are aims of intense investigation. Recently, cancer stem cells (CSCs), a population of poorly differentiated cells, capable of unlimited self-renewal in both primary tumor and metastases, have been proposed to contribute in cancer progression and chemotherapy resistance due to increased expression of multi-drug resistance and DNA mismatch repair genes. Indeed, CSCs have been found to enrich chemo-resistant populations in pancreatic cells [46-48]. After many years of disappointments, finally strategies targeting cancer have changed, and immunotherapy has been introduced and validated in the clinical setting.

Engineered TCR, CAR/T, TIL and NK therapies

Due to recent advances in immunotherapeutic strategies and the demonstrated effectiveness of such therapies in arresting or inhibiting disease progression, immunotherapy is now regarded as one of the main strategies for cancer treatment. T cells engineered to express tumor-specific TCRs with high affinity for tumor antigens are currently being used in clinical trials of patients with relapsed tumors. Chimeric antigen receptor T (CAR/T) cells which combine both

antibody-like recognition site with T cell activating function is also a novel therapeutic direction with promising results. Tumor infiltrating lymphocytes (TILs) have been isolated and expanded ex-vivo for infusion in cancer patients. NK cells can be expanded and delivered to patients to eliminate cancer stem cells, which seed the cancer, along with their use in promoting differentiation of remaining tumors in an effort to inhibit cancer metastasis.

Although impressive results were obtained by strategies indicated above in certain cancer patients it is still unclear whether such treatments will deliver long-lasting remission in patients. In addition, toxicities associated with these treatments have halted the effort for the continuous infusion of these cells for the complete elimination of the tumors. Future studies will be directed towards designing cellular therapies which will be specific to tumors and will have long term persistence of engineered cells in patients with low overall toxicities. Until then, cancer patients will continue receiving monoclonal antibodies to immune check points such as PD-1 and CTLA-4 which have shown efficacy in treating tumors in a selected group of patients. Future strategies will not only use the engineered and expanded T, CAR/T, TIL and NK cells but also they will combine such treatments with a number of immune checkpoint inhibitors.

Currently, adoptive NK cell therapy has been less efficient than T cell therapy in treating cancer patients due to a number of limitations. With existing strategies, fewer numbers of potent NK cells can be expanded and used for cancer immunotherapy compared to T cells. Cytokine and tumor-activated NK cells neither survive long enough nor maintain their cytotoxic function to effectively eliminate tumors, due to rapid NK cell inactivation by the suppressive microenvironment in cancer patients. To circumvent such limitations, we have recently established a strategy to expand large numbers of functionally potent NK cells using specific strains of sonicated probiotic bacteria in combination with osteoclasts optimized for potent expansion and

activation of NK cells [49]. This strategy has not only allowed for greater expansion of NK cells accompanied by much higher levels of cytotoxicity and cytokine secretion, but also prevented NK cells from undergoing cell death when compared to NK cells expanded by conventional strategies (Chapter 2). We have previously shown that human osteoclasts produce IL-15, IL-12, IL-18 and IFN-alpha, and display low expression of MHC class I and II, CD14, CD11b and CD54 [49].

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 [50]. 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 [51].

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 [52, 53]. 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 [54, 55]. 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 [56].

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 [51, 57].

The BLT humanized mouse (hu-BLT) represents the most advanced and complete humanized mouse model generated, to date [57]. 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 [51, 58, 59]. 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 [61]. 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 [62-65]. 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 [66,

67], 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 [61]. These features demonstrate that the hu-BLT model, developed from NSG background, is arguably the best available model for studying human immunity, thus far.

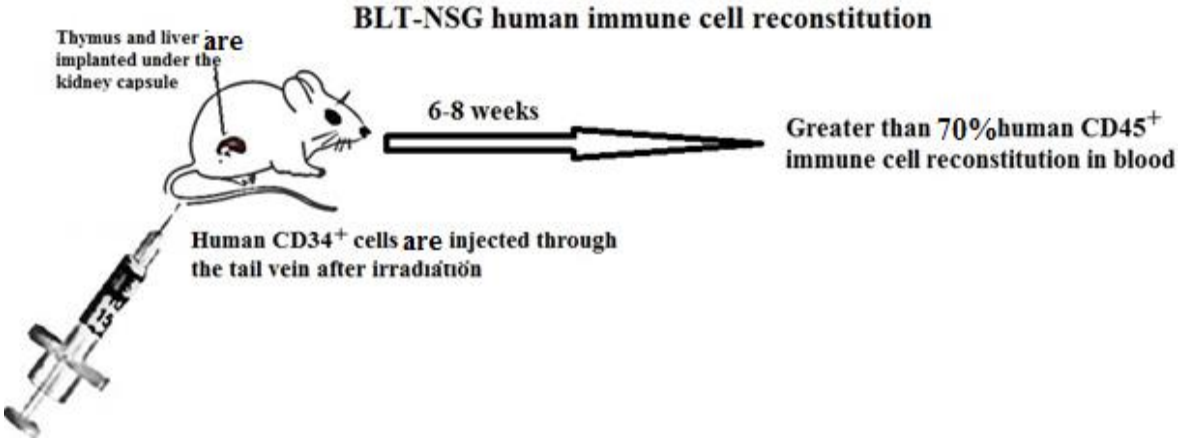

Figure 3: Generation of humanized BLT mice

Humanized-BLT (hu-BLT; human bone marrow/liver/thymus) mice were generated by surgical implantation of human fetal liver and thymus tissue under the renal capsule of 6-8 weeks old immunocompromised NOD.CB17-Prkdcscid/J and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice

Based on our recent studies and those of others, we suggest that the hu-BLT mouse model is the best available preclinical model to study novel immunotherapeutic approaches in the context of the reconstituted immune repertoire. Thus far, hu-BLT animals provide the best available model for determining the intricate human immune cell interactions with human tumors when delivering immune therapeutics, and studying the molecular and cellular mechanisms of tumor resistance

leading to therapy failure. Moreover, this model can further be refined by the delivery of *ex vivo* derived, osteoclast-expanded, functionally potent human NK cells in order to overcome the observed limitations in the numbers and function of autologous NK cells found in humanized mice.

Interaction of Osteoclasts with other immune cells

Bone and immune system are functionally interconnected. Immune and bone cells derive from the same progenitors in the bone marrow, they share a common microenvironment and are influenced by similar mediators; different immune cells such as macrophages, B lymphocytes, mast cells, natural killer cells (NK), etc. have been shown to influence bone cells, as well [68]. Immune cells and their products (cytokines) play an important role in the regulation of skeletal development and function, particularly with osteoclasts, implying that immune cell dysfunction may be involved in the pathogenesis of certain skeletal disorders [69]. IFN-γ, produced by both NK cells and Th1 lymphocytes, has been shown to inhibit osteoclastogenesis *in vitro* [70]. However, the *in vivo* effects of IFN-γ on bone tissue are less clear since they often provide a contrasting effect when compared to *in vitro* studies [71, 72]. Reduced functioning of osteoclast and NK cell function coexist in osteoporotic mutant rat [69]. OC progenitor activity is positively regulated by TNF-α and negatively regulated by IFN-γ [73]. IFN-γ binds to its receptor on osteoclasts, degrades RANKL signaling and thus inhibits the activation of osteoclasts and protects our bones from being resorbed. IFN-γ is produced predominantly by NK and natural killer T (NKT) cells involved in the innate immune response, and by $CD4^+$ Th1 and $CD8^+$ cytotoxic T lymphocyte (CTL) effector T cells, once antigen-specific immunity develops [74]. ITIM-bearing NK receptor, positively regulates osteoclast differentiation, and immunoreceptor tyrosine-based activation motif (ITAM)-mediated signaling is critical for osteoclast differentiation [75, 76].

Cross-talk between the skeletal system and T cells, is termed as osteoimmunology. RANKL expressed by CD4+ and CD8+ T-cells can induce osteoclastogenesis, providing a link between the immune and skeletal systems. Osteoclasts produce chemokines that recruit CD8+ T cells. Osteoclasts induced secretion of IL-2, IL-6, IFN-γ and induced proliferation of CD8 positive T cells. CD8 positive T cells activated by osteoclasts expressed FoxP3, CTLA4, and receptor activator of NF-kB ligand [77]. Anti-CD3/CD28-stimulated γδ T cells or CD4+ T cells inhibit human osteoclast formation and resorptive activity *in vitro*. Cytokine production by CD3/CD28-stimulated γδT cells and observed a lack of IL-17 production and producing abundant interferon (IFN)-γ. Neutralization of IFN-γ markedly restored the formation of osteoclasts from precursor cells and the resorptive activity of mature osteoclasts, suggesting that IFN-γ is the major factor responsible for the inhibitory role of activated γδT cells on osteoclastogenesis and the resorptive activity of mature osteoclasts [77].

Feng et al. [78] 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 [79]. It was previously shown that osteoclasts, in comparison to dendritic cells and monocytes, are significant activators of NK cell expansion and function [49]. Additionally, osteoclasts secrete significant amounts of IL-12, IL-15, IFN-γ and IL-18, which are known to activate NK cells; osteoclasts also express important NK-activating ligands.

Central Hypothesis

Due to severe immunosuppressive microenvironment in cancer patients, we have hypothesized that by changing the microenvironment to enhance NK activation and/or Supplying super-charged competent NK cells should provide complementary strategies to eliminate cancer stem-cells and differentiate the remaining tumors; thereby providing effective immunotherapeutic strategies to treat cancer patients.

Objective of the study

To delineate how NK cells, lose function in cancer patients and design strategies to reverse or restore NK cell numbers and function in patients to prevent cancer progression.

Specific Aims

Specific Aim 1: **To investigate immune cells cytotoxic activity and IFN-γ secretion in cancer patients, tumor-bearing hu-BLT mice and KRAS mutated mice.**

Sub-aim 1: Characterization and functional studies of healthy donor and cancer patient PBMCs, NK cells, T cells and osteoclasts.

Sub-aim 2: Functional studies of NK cells, monocytes and osteoclasts of healthy and tumor injected hu-BLT mice.

Sub-aim 3: Characterization and functional studies for NK cells in various tissue compartments of WT and KRAS mutated mice.

Specific Aim 2: **To investigate the underlying mechanism for the loss of NK function at the pre-neoplastic and neoplastic stage; reversal of NK cells function using osteoclast.**

Sub-aim 1: Significance of reduced surface expression of NKG2D receptor ligand, MICA/B on cancer patient and KRAS mutated mice osteoclasts and low level of ADCC by cancer patient NK cells.

Sub-aim 2: Role of IL-12 and IL-15 in osteoclasts mediated NK expansion.

Sub-aim 3: OCs preferentially expand NK cells and CD8+T cells, and not CD4+T cells; NK cells mediate cytotoxicity against CD4+T cells.

Specific Aim 3: **Osteoclast-expanded super-charged NK cells inhibit growth and progression of stem-like/poorly differentiated oral and pancreatic tumors in vivo in humanized BLT mice.**

Sub-aim 1: Methodology to expand super-charged NK cells using osteoclasts and sAJ2.

Sub-aim 2: Treatment strategy for cancer using super-charged NK cells in hu-BLT mice injected with oral and pancreatic tumors.

Sub-aim 3: Increased surface expression of CD8+T cells on the immune cells in various tissue compartments of hu-BLT mice injected with super-charged NK cells.

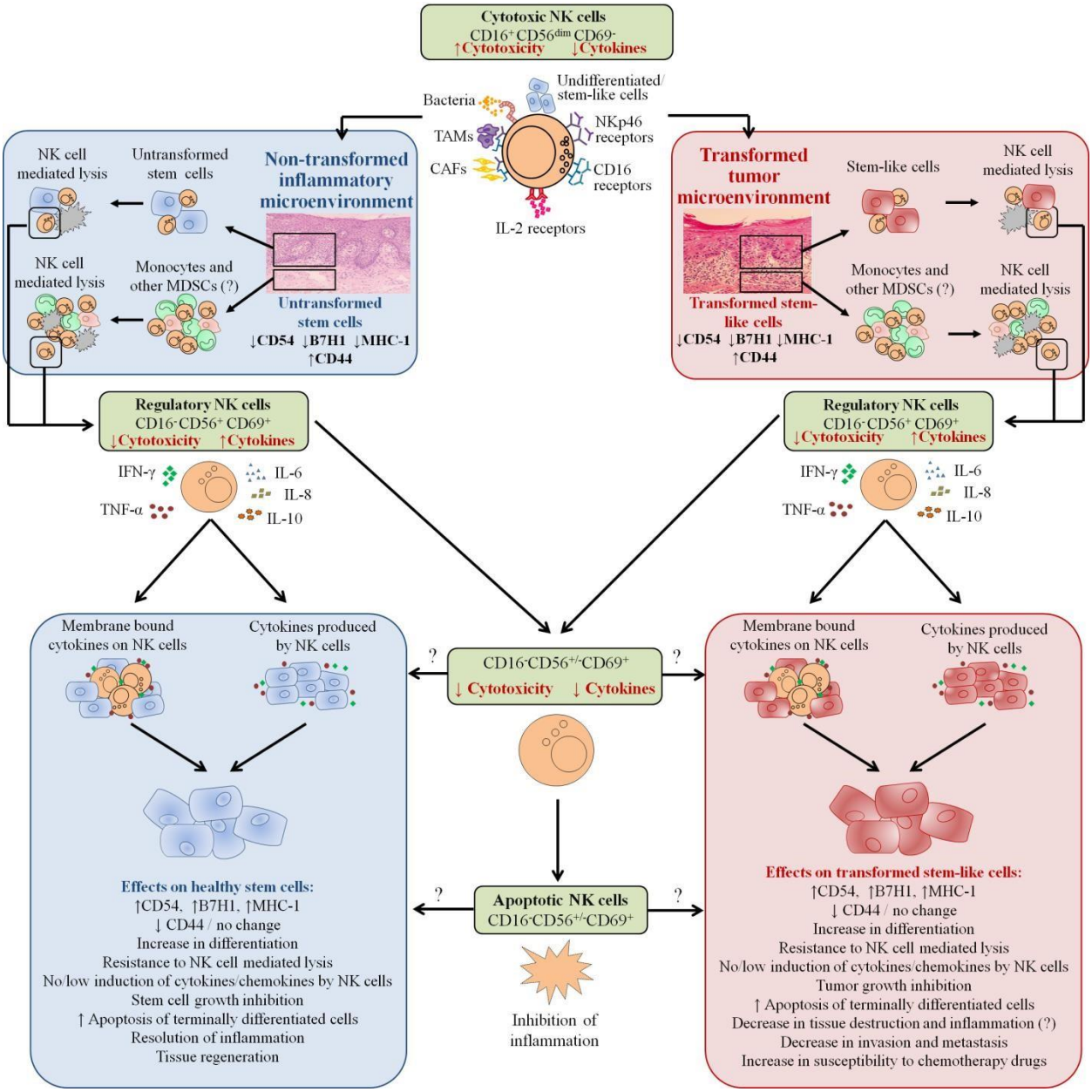

Figure 3: Hypothetical model of induction of regulatory NK cells by immune inflammatory cells and by the effectors of connective tissue to support differentiation of non-transformed stem cells and cancer stem cells

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MATERIALS AND METHODS

Cell lines, reagents, and antibodies

RPMI 1640 complete medium with 10% fetal bovine serum (FBS) (Gemini Bio-Product) was used for cell cultures. Oral squamous carcinoma cells (OSCCs) and oral squamous carcinoma stem cells (OSCSCs) were isolated from cancer patients with tongue tumors at UCLA [1-4]. Alpha-MEM (Life Technologies, CA) with 10% FBS was used for osteoclast (OC) and dendritic cell (DC) cultures. M-CSF was purchased from Biolegend (CA) and RANKL, GM-CSF and IL-4 were purchased from PeproTech (NJ), and rh-IL-2 was obtained from NIH-BRB. Human CD3/CD28 T cell activator was purchased from Stem Cell Technologies.

Antibodies for human MHC-I, KIR2, KIR3, CD44, CD54, B7H1, CD16, NKG2D, MICA/B, KLGR1, CD45, CD3/16/56, CD8, CD3, CD4, GL3, NKp40, NKp30, NKp44, NKp46 and CD94 were purchased from Biolegend (San Diego, CA). ULBP 1-6 antibodies were purchased from R&D Systems. Antibodies to mouse CD45, DX5, Ly49A and F4/80 were purchased from Biolegend (San Diego, CA). Propidium iodide (PI) was purchased from Sigma (St. Louis, MO). sAJ2 was prepared as described previously [5].

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 monocytes and generation of osteoclasts from hu-BLT mice and, from human PBMCs

The study, as well as the procedures, were approved by the UCLA Institutional Review Board (IRB), and all participants signed written informed consent in accordance with the Declaration of Helsinki. Human monocytes were purified as described previously [6]. Monocytes from hu-BLT mice were positively isolated from bone marrow using human the CD14 isolation kit (eBioscience, San Diego, CA). Greater than 95% purity was achieved for each subset based on flow cytometric analysis. Monocytes were differentiated to osteoclasts by with M-CSF (25 ng/mL) and RANKL (25 ng/mL) for 21 days.

Purification of NK cells and T cells from the human peripheral blood

Written informed consents, approved by UCLA Institutional Review Board (IRB), were obtained from healthy blood donors, and all procedures were approved by the UCLA-IRB. Peripheral blood was separated using Ficoll-Hypaque centrifugation, after which the white, cloudy layer, containing peripheral blood mononuclear cells (PBMC), was harvested, washed and 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 and T cells were negatively selected and isolated from PBLs using the EasySep® Human NK cell enrichment kit and T cells isolation kit, respectively purchased from Stem Cell Technologies (Vancouver, BC, Canada). Isolated NK cells and T cells were stained with anti-CD16 and anti-CD3 antibody, respectively, to measure the cell purity using flow cytometric analysis. Purified NK cells and T 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). T cells from hu-BLT splenocytes were positively purified using isolation kits from Stem Cell Technologies (Stem Cell Technologies, Vancouver, Canada).

Expansion of NK cells

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

NK cells and T cells supernatants used for stem cell differentiation

As described above, human NK cells and T cells were purified from PBMCs of healthy donors. NK cells were treated with a combination of anti-CD16mAb (3μg/mL) and IL-2 (1,000 U/mL) for 18 hours before supernatants were removed and used for differentiation experiments. NK cells were treated with a combination of anti-CD16mAb (3μg/mL) and IL-2 (1,000 U/mL) for 18 hours before they were cultured with osteoclasts and sAJ2, supernatants were harvested on day 6 after the culture and used for differentiation experiments. T cells were treated with a combination of anti-CD3/anti-CD28 (1 μg/mL) and IL-2 (100 U/mL) for 18 hours before supernatants were

removed and used for differentiation experiments. T cells were treated with a combination of anti-CD3/anti-CD28 (1 μg/mL) and IL-2 (100 U/mL) for 18 hours before they were cultured with osteoclasts and sAJ2, supernatants were harvested on day 6 after the culture and used for differentiation experiments. The amounts of IFN-γ produced by activated NK cells were assess with IFN-γ ELISA (Biolegend, CA, USA). OSCSCs were differentiated with gradual daily addition of increasing amounts of NK cell supernatants (of corresponding treatments). On average, to induce differentiation, a total of 3,500pg of IFN-γ containing supernatants were added for 4 days to induce differentiation and resistance of OSCSCSs to NK cell-mediated cytotoxicity. Afterwards, target cells were washed with 1xPBS, detached and used for experiments.

Analysis of human OSCSCs cell growth in immune-deficient 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 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 [7, 8].

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) and human

pancreatic tumor (MP2) 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 injected 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, and MP2 were injected 0.5 X 10^6 cells mixed with 10 ul HC Matrigel into the pancreas. Immediately prior to tumor cell injection, 5.0-mg/kg carprofen 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 super-charged NK cells via tail vein (IV) injection. Seven days following super-charged NK cells injections, anti-PD1 (50 µg/mice) was injected via tail vain injection.

Conditional KRAS^{G12D} mouse model

To study the effect of a high-fat, high caloric diet on immune function during pancreatic cancer development, the conditional KRAS^{G12D} model from Hingorani and colleagues was used [9]. *LSL-KRAS^{G12D}*, *p48-Cre*, (or *PDX-1-Cre)* mice were maintained as heterozygous lines. After weaning, offspring of *LSL-KRAS^{G12D}* and *p48-Cre* (*or PDX-1-Cre*) mice were fed either a high-fat, high calorie diet (HFCD) or a control diet (CD) for 4 to 6 months. The exact composition of the diets is described elsewhere [10]. In brief, compared to the CD the HFCD has increased caloric content (4,536 KRASal/kg vs. 3,726 KRASal/kg), which stems from an increase in corn oil-based fat content (1,800 KRASal/kg vs. 450 KRASal/kg). While ~12% of the total calories in the AIN-

76A CD come from fat, about 40% of total caloric intake in the HFCD stems from fat. After 4 to 6 months, animals were euthanized and the entire pancreas, visceral adipose tissues, and other organs were harvested. Formalin-fixed, paraffin-embedded tissues were sectioned (4 μm) and stained with H&E. Sections of pancreatic tissues were histologically evaluated by a gastrointestinal pathologist for the presence and stage of murine PanIN lesions (mPanIN) as described elsewhere [11]. Animal studies were approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Genotyping analysis

Before randomization to the diets the presence of the $KRAS^{G12D}$ and *Cre* allele were determined by PCR analysis of genomic DNA, as described elsewhere, obtained from tail biopsies [12]. Animals with both the $KRAS^{G12D}$ and *Cre* allele were designated as mutant ($KRAS^{+/G12D}$) and animals with neither the $KRAS^{G12D}$ nor the *Cre* allele were deemed wildtype ($KRAS^{+/+}$). At the end of the study at sacrifice the successful excision-recombination events were confirmed by PCR by the presence of a single LoxP site in the pancreas as described elsewhere [12].

Preparation of single cell suspension from WT and KRAS mice.

To prepare a single cell suspension of mouse tissues for subsequent analyses, animals at sacrifice underwent a cardiac perfusion, and organs (pancreas, spleen, visceral adipose tissue) were harvested. The pancreas and visceral adipose tissue were immediately cut into $1mm^3$ pieces and placed into a digestion buffer containing 1mg/ml collagenase IV (for pancreas) or collagenase II (for fat tissue), 10U/ml DNAse I, and 1% bovine serum albumin in DMEM and incubated for 20

minutes in a 37°C oven with a 150 rpm shaker. After digestion, the sample was filtered through a 40µm cell strainer and centrifuged at 1500 rpm for 10 minutes at 4°C. The pellet was re-suspended in DMEM and cells counted. The spleen was directly mashed and filtered through a 40µm cell strainer after harvest, and centrifuged for 5 minutes at 1500 rpm at 4°C. The splenic sample was re-suspended in 0.5ml ACK lysis buffer (150mM NH_4Cl, 1mM $KHCO_3$, 0.1mM EDTA) to eliminate red blood cells and incubated for 5 minutes at room temperature followed by centrifugation for 5 minutes at 1500 rpm at 4°C. The pellet was re-suspended in DMEM and cells counted.

Experimental diets

The diets were obtained from Dyets, Inc., Pennsylvania (Table 1). A slightly modified AIN-76A purified rodent diet served as a control diet. Compared to the control diet, our HFCD has increased caloric content (4,536 KRASal/kg vs. 3,726 KRASal/kg), which stems from an increase in corn oil-based fat content (1,800 KRASal/kg vs. 450 KRASal/kg). While ~12% of the total calories in the AIN-76A control diet come from fat, about 40% of total caloric intake in the HFCD stems from fat. The corn oil contains about 60% omega-6 polyunsaturated fatty acids (linoleic acid), saturated fatty acids (10.8% palmitic, 2.1% stearic), mono-unsaturated fatty acids (26.5% oleic), and small amounts of omega-3 polyunsaturated fatty acids (0.6% linolenic). Importantly, the amount of sucrose, salts, and vitamins are kept identical in both diets. To compensate for the increase in corn oil, we reduced the amount of cornstarch in the HFCD accordingly. The diets were handled under low light conditions, and stored at -20°C. The diets were replaced twice weekly. The stability of the fatty acids in the diets was regularly monitored by the UCLA Nutritional Biomarker and Phytochemistry Core.

Cell dissociation and cell culture from tissues of no tumor and 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. Peripheral blood mononuclear cells (PBMCs) were obtained using Ficoll-Hypaque centrifugation of heparinized blood specimens. The white, cloudy layer, containing 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.

Mouse NK cells culture with monocytes, or and osteoclasts

All animal work performed was based on the guidelines established and approved by UCLA Office of Animal Research Oversight. Single cell preparations of mouse splenocytes were used to negatively select for mouse NK cells using mouse NK isolation kit purchased from Stem Cell Technologies (Vancouver, Canada). The purity of mouse NK cell was greater than 90% based on staining with PE-conjugated CD49b antibodies. NK cells were treated with IL-2 ($1X10^4$ U/million NK cells). Bone marrow cells were isolated by flushing femurs with PBS supplemented with 2% heat-inactivated FBS. Murine monocytes were then purified from bone marrow cells using monocyte isolation kit obtained from Stem Cell Technologies (Vancouver, Canada). The purity of monocytes was between 86-96% based on staining with PE-conjugated F4/80 and ant-CD14 antibody. Monocytes were differentiated to osteoclasts by treatment with M-CSF (25 ng/mL) and RANKL (25 ng/mL) for 21 days. IL-2 treated NK cells were either treated with monocytes or osteoclasts, and the culture was treated with LPS (100 ng/ml).

Surface staining

$1x10^5$ cells from each condition were stained in 100ul of cold 1%PBS-BSA with pre-determined 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 micro-well 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.

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:

$$\%\ \text{cytotoxicity} = \frac{\text{experimental cell count} - \text{spontaneous cell count}}{\text{spontaneous cell count}} \times 100\%$$

LU $30/10^7$ 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 were purchased from Biolegend (San Diego, CA). ELISA was performed to detect the level of IFN-γ produced from cell cultures. The assay was conducted as described in the manufacturer's protocol. Briefly, 96-well EIA/RIA plates were coated with diluted capture antibody corresponding to target cytokine and incubated overnight at 4°C. After 16-18 hours of incubation, the plates were washed 4 times with wash 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 diluted 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).

Statistical analysis

An unpaired or paired, two-tailed student t-test was performed for the statistical analysis. ***(p value <0.001), **(p value 0.001-0.01), *(p value 0.01-0.05).

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Participating subjects

Healthy donors: n=24

Cancer patients: n=16

	Gender	**Cancer type**	**Cancer stage (TNM)**
Patient 1	M	Oral	Stage 4
Patient 2	M	Oral	Stage 4
Patient 3	M	Oral	Stage 4
Patient 4	F	Pancreatic	Stage 4
Patient 5	M	Pancreatic	Stage 4
Patient 6	F	Pancreatic	Stage 4
Patient 7	F	Pancreatic	Stage 4
Patient 8	F	Pancreatic	Stage 4
Patient 9	M	Pancreatic	Stage 4
Patient 10	M	Pancreatic	Stage 4
Patient 11	M	Pancreatic	Stage 4
Patient 12	M	Pancreatic	Stage 4
Patient 13	M	Colon	Stage 4
Patient 14	M	Colon	Stage 4
Patient 15	M	Colon	Stage 4
Patient 16	M	Prostate	Stage 4

CHAPTER 1

To investigate immune cells cytotoxic activity and IFN-γ secretion in cancer patients, tumor-bearing hu-BLT mice and KRAS mutated mice

Abstract

The cytotoxic function of NK cells is suppressed in the tumor microenvironment by a number of distinct effectors. Furthermore, decreased peripheral blood NK cell cytotoxicity has been documented in cancer patients. In this regard, we found the cytotoxic function and IFN-γ secretion by NK cells from cancer patients and, those from hu-BLT mice engrafted with tumor decreased when compared to healthy human donor and cancer-free hu-BLT mice respectively. When NK cells from cancer patients and hu-BLT mice injected with tumors were co-cultured with OC, there was more expansion of T cells, resulting in NK loss, providing an important mechanism of NK loss in cancer patients. We also tested T cells from healthy donor and cancer patients, cancer patient T cells showed high surface expression of effector- memory indicating those T cells are activated. The most interesting finding is the reduced NK cells cytotoxic function at the pre-neoplastic stage, NK cells were found to mediate significant cytotoxicity against primary tumor or cancer stem cells, and low NK cell cytotoxic activity provided a favorable micro-environment for tumors to grow.

Introduction

Advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells.

Effectors of the immune system are thought to shape the survival and maturation of tumor cells and to select for cancers with reduced immunogenicity. However, recent data from our laboratory indicted that the same effector mechanisms were likely responsible for shaping the survival and maturation of healthy stem cells for the ultimate goal of the regeneration of damaged tissues and the resolution of inflammation. Although, immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, neither their underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are completely understood.

NK cells arise from the bone marrow and constitute 5-15% of total lymphocytes in the peripheral blood. They are known to mediate direct natural cytotoxicity as well as antibody-dependent cellular cytotoxicity (ADCC). By producing key cytokines and chemokines, NK cells are known to regulate the functions of other immune cells [1, 2]. Conventional human NK cells are identified by the expression of CD16 and CD56, and by the lack of surface CD3 expression. NK cells mediate their function through a number of important activating and inhibitory cell receptors listed in Table 1 [3]. It is thought that the balance between activating and inhibitory signals which NK cells receive from their surface receptors determines their functional fate [3]. Many of the receptors listed in Table 1 including CD16, killer immunoglobulin like receptors (KIR), NKG2 family of receptors which form a heterodimer with CD94, NKG2D and natural cytotoxicity receptors (NCR) have all been the subject of many studies. Likewise, several key cytokines, chemokines and adhesion molecules are found to have significant roles in maturation, differentiation, and effector function of NK cells. Much less is known regarding the interaction of NK receptors, especially NKG2D with their ligands on the osteoclasts.

Although a lot is known about the inhibitory and activating receptors that modulate the function of NK cells, and many previous studies have indicated that NK cells may recognize and become activated by irradiated or stressed cells [2, 3], no previous studies have shown NK cells receptors playing an important role in NK cells expansion when they interact with osteoclasts. In this chapter, we provide data regarding the factors and mechanisms involved in shaping the function of NK cells in cancer as well as the interactions of NK cells from healthy donors and cancer patients with autologous and allogeneic osteoclasts. Furthermore, we discuss the emerging view that the NK cells function is affected way before the cancer is established. NK cells have less cytotoxic activity at the pre-neoplastic stage, and that could be the part of reason for cancer development.

In the early 20th century, Elie Metchnikoff discovered that certain strains of bacteria in the human gut were beneficial to gut homeostasis; these beneficial bacteria were named probiotics [4]. Probiotics are commonly used in foods and supplements in an effort to enhance the innate immune system, including NK cells activity, and maintain the digestive tract's microbial balance [5-7]. The majority of probiotics consist of lactic-acid producing bacteria, including *lactobacilli, streptococci,* and *bifidobacteria*. The wide range of benefits observed with the use of probiotic bacteria suggests their integral role in the modulation of local gut immunity, as well as systemic immunity [8-10]. These beneficial bacteria effect production of immunoglobulin A [10-12], stimulate macrophages activity [13] and may reduce the effects of toxicity in anti-cancer therapy [14]. 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 [4, 6]. Areas of research pertaining to probiotic bacteria are fast-growing, with a promising

outlook for treatment of medical conditions such as gut mucosal pathologies, allergies, obesity, metabolic syndrome, heart disease, and cancer prevention or treatment, and so on [15-18].

Table 1- List of NK cell Activating and Inhibitory surface receptors and their ligands

Receptors	Ligands
Activating/inhibitory Receptors	
FcγRIII (CD16)	Fc of antibodies
CD2	CD58 (LFA-3)
LFA-1	ICAM-1
2B4	CD48
CD69	Unknown
DNAM-1 (CD226)	CD112, CD155
NKp80	AICL
Tactile (CD96)	CD155, CD111
TIGIT	CD112,CD113,CD155
CRTAM	TSLC1
C-type Lectin receptors –Activating/Inhibitory	
CD94/NKG2A/B	HLA-E
NKG2D	MICA, MICB, ULBP-1, ULBP -2, ULBP -3, ULBP -4, ULBP -5, ULBP -6
CD94/NKG2C	HLA-E
CD94/NKG2E/H	HLA-E, Qa-1b
Natural cytotoxicity receptors (NCR)	
NKp46 (NCR1)	Viral Hemagglutinin
NKp44 (NCR2)	Viral Hemagglutinin
NKp30 (NCR3)	B7h6, HCMV-pp65

Although the benefits and role of probiotics in immune modulation has been well known, the underlying mechanisms of NK cell immunomodulation is not understood. This study will provide further information regarding the outcome of probiotics in regulating NK cell function. Probiotic bacteria induce significant split anergy in activated NK cells, leading to a significant induction of IFN-γ and TNF-α. In addition, probiotic bacteria induce significant expansion of NK cells [19] .

Pancreatic ductal adenocarcinoma (PDAC) is the most fatal form of pancreatic malignancy with a 5-year survival of less than 4% [20]. The genes mutated in pancreatic cancer include KRAS2, p16/CDKN2A, TP53, and SMAD4/DPC4, and these are accompanied by a substantial compendium of genomic and transcriptomic alterations that facilitate cell cycle deregulation, cell survival, invasion, and metastases [21, 22]. In most cases, pancreatic ductal adenocarcinoma is initiated by oncogenic mutant KRAS, which has been shown to drive pancreatic neoplasia [23]. Analysis showed that KRAS mutations were associated with reduced patient survival in both malignant exocrine and ductal adenocarcinomas (PDAC). Patients with PDACs that had KRAS mutations showed a median survival of 17 months compared to 30 months for those without mutations [24].

In this study, we demonstrated the role loss of NK cells function in the growth and metastasis of cancer. We show that PBMC and NK cells but not T cells in cancer patients, tumor-bearing hu-BLT mice and KRAS mutated mice secrete less cytokines required for differentiation of cancer stem cells, and they have less cytotoxic activity too. Based on our data, we propose that autologous or allogeneic NK cell immunotherapy might be a novel, promising strategy for patients with cancer.

Results

Sub aim 1: Characterization and functional studies of healthy donor and cancer patient PBMCs, NK cells, T cells and osteoclasts.

NK cells, but not T cells, from cancer patients have low cytotoxic activity and low IFN-γ secretion.

The same volume of peripheral blood from cancer patients gave significantly lower number of PBMCs compared to healthy donors (Fig. 1A). When we analyzed the proportions of immune cells in the PBMCs of both healthy donors and cancer patients, PBMCs from cancer patients showed increased numbers of NK cells, CD14 monocytes and CD11b cells, but reduced numbers of the CD3+T and CD19+ cells (Fig. 1B-1F). Similar number of cells from healthy donors and cancer patients were used to determine the cytotoxicity against the oral squamous cancer stem-like cells (OSCSCs). Cancer patients PBMCs have less cytotoxic activity against the OSCSCs (Fig. 1G), IFN-γ secretion of PBMCs from cancer patients was also lower (Fig. 1H). Next, we analyzed the proportions of CD4+T and CD8+T cells in the total T cells, the number of CD4+T cells are lower, and the numbers of CD8+T cells are higher in cancer patients (Fig. 2A), resulting in decreased ratio of CD4+T/CD8+T numbers in cancer patients when compared to healthy donors (Fig. 2B).

Purified NK cells of cancer patients had modulation for most of the NK surface receptors tested (Table 2), including low surface expression of NKG2D (Fig. 3A and B) and CD16 (Fig. 3C and D), both cytotoxic function (Fig. 3 E) and IFN-γ (Fig. 3F) secretion was low in cancer patient NK cells when compared to healthy donors. No significant differences or a slight inhibition of cytotoxicity (Fig. 4A) and IFN-γ (Fig. 4B) secretion could be seen in cancer patient T cells,

although T cells surface receptors were modulated and displayed the effector-memory T cell profile in cancer patients (Fig. 4C).

Decreased cytotoxicity and lower IFN-γ secretion by NK cells from patients coincides with increased expansion of T cells

When NK cells were cultured with OCs for expansion as showed in Fig. 5A, 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 11% of the remaining cells in the culture were NK cells (Fig. 5B), whereas the healthy donor NK cell culture had 95% NK cells and only 1-2% T cells (Fig. 5C). 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. 5E and 5H), and the levels of expanding T cells were significantly higher than NK cell expansion (Figs. 5F and 5G). 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 (Figs. 5F and 5G). Patient NK cells cultured with OCs lysed OSCSCs significantly less when compared with the healthy NK cells cultured with OCs (Fig. 5I). OC-expanded patient NK cells secreted significantly less IFN-γ when compared to healthy OC-expanded NK cells (Fig. 5J). The levels of NKG2D surface expression were similar on healthy as compared to patient NK cells expanded by the osteoclasts (Fig. 5K). The intensity of CD94 expression is higher on the surface of patient NK cells as compared to healthy control (Fig. 5K). KIR2, NKp30, NKp44 and NKp46 expressions were lower on the surface of OC-expanded patient NK cells when compared to healthy NK cells (Fig. 5K), whereas

KIR3 expression was either the same or lower on the surface of OC-expanded patient NK cells when compared to healthy NK cells (Fig. 5K).

Based on the data shown in Fig. 5K, surface expression of NKG2D on OC-expanded patient NK cells was similar to OC-expanded healthy NK cells, thus we expected to observe similar cytotoxic function and cytokine secretion. To determine the NK cells function, we isolated the NK cells using a NK isolation negative selection kit both from cancer patient and healthy donor OC-expanded NK cells culture, treated them with rh-IL-2 for 18-19 hours, and used them for cytotoxicity against the OSCSCs, the supernatant was used to determine the IFN-γ secretion. Both cytotoxic function (Fig. 5L) and IFN-γ secretion (Fig. 5M) were improved in cancer patients, but was still low compared to healthy donor. Expanded T cells in patient OC-expanded NK cells expressed T-effector memory surface profile (Fig. 5N). No significant differences or a slight inhibition of cancer patients, T cells expansion was seen when T cells were expanded without/with OC (Fig. 5O). Although OC-expanded T cells from patients secrete IFN-g less, the decrease is less than that observed by their NK cells (Fig. 5P and 5Q).

IFN-γ secreted from the NK cells of cancer patients induced less differentiation of oral cancer stem-like cells when compared to similar levels of to IFN-γ secreted from NK cells of the healthy donor.

OSCSCs were treated with equal volumes of pg/ml IFN-γ secreted by patient and healthy donors NK cells treated with IL-2+anti-CD16 (Fig. 6D and Fig. 6E) and IL-2+anti-CD16+ sAJ2 (Fig. 6F and 6G), and T cells treated with IL-2+anti-CD3/CD28 (Fig. 6H and 6J) and IL-2+anti-CD3/CD28+ sAJ2+OC (Fig. 6K and 6L) as described in Materials and Methods. Although the

same levels of IFN-γ was used, there was 6.5 fold increase in MHC-1, 1.4 fold increase in CD54 and 1.53 fold increased in B7H1 when the patient NK cells supernatants were used whereas there was 35 fold increase in MHC1, 35 fold increase in CD54 and 6 fold increase in B7H1 using the healthy NK cells supernatant (Fig. 6D). Increases in MHC1, CD54, B7H1 expression were also lower when using NK+OC supernatant from cancer patient when compared to NK+OC supernatant from healthy donor (Fig. 6F). No or slight difference in surface receptor modulation was seen when either T cells or T+OC supernatant from healthy donor and cancer patient was used to treat the cancer stem cells (Fig. 6H and 6K). When compared to patient NK cells, a large decrease in NK cell-mediated cytotoxicity for OSCSCs was observed when treated with either healthy NK (Fig. 6E) or NK+OC (Fig. 6G) supernatants, whereas no or slight difference was seen in decreased NK cell-mediated cytotoxicity with either T cells (Fig. 6J) or T+OC (Fig. 6K) supernatants was used to treat OSCSCs.

Osteoclasts from the cancer patients are less potent in expanding the NK cells, increasing their cytotoxic activity and IFN-γ secretion in NK cells when compared to healthy osteoclasts.

To determine whether the osteoclasts generated from healthy donor and cancer patient have different interaction profile with NK cells, we cultured the NK cells from healthy donor with allogeneic cancer patient and healthy donor's osteoclasts, osteoclasts from cancer patients expanded less NK cells (Fig. 7A), did not increase cytotoxic activity as much (Fig. 7B) and increased IFN-γ secretion by a lower amount (Fig. 7C).

Fig. 1

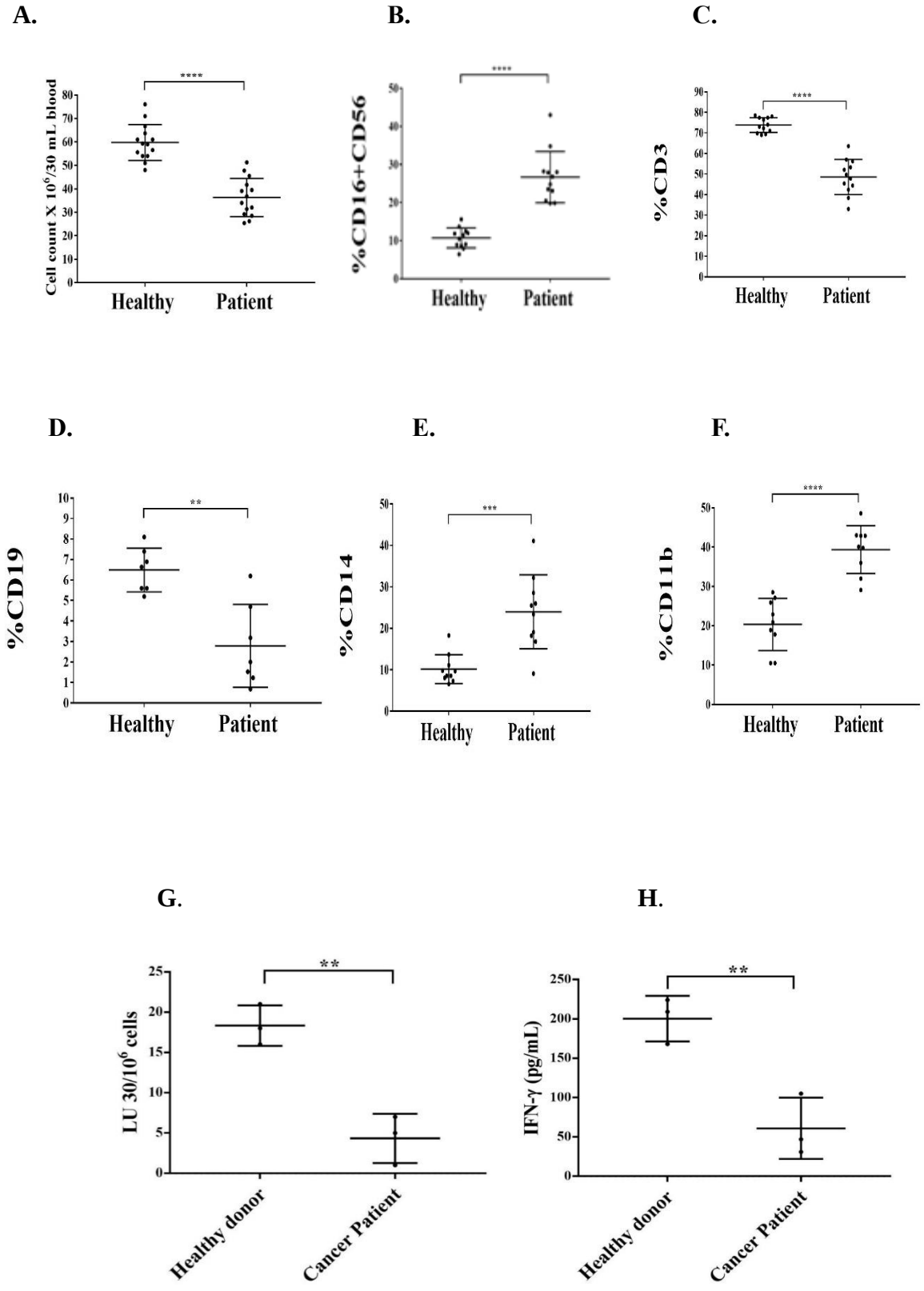
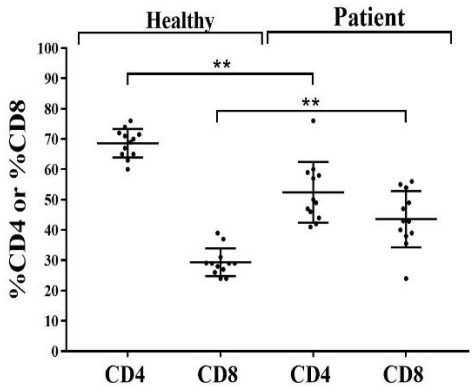

A.
B.
C.
Cell count X 10^6/30 mL blood
%CD16+CD56
%CD3
Healthy
Patient
D.
E.
F.
%CD19
%CD14
%CD11b
G.
H.
LU 30/10^6 cells
IFN-γ (pg/mL)
Healthy donor
Cancer Patient

Figure 1: Characterization of healthy donor and cancer patient's PBMC

Human PBMCs were isolated from 30 ml of peripheral blood of the healthy donors and cancer patients and number of cells were assessed by microscope using hematocytometer (n=14) **(A)**. Human PBMCs were isolated from peripheral blood of healthy donors and cancer patients, surface expression of CD3 (n=12), CD16 (n=12), CD56 (n=12), CD19 (n=9), CD14 (n=10) and CD11b (n=9) were analyzed within CD45+ immune cells using flow cytometry **(B-F)**. Freshly isolated PBMCs from healthy donors and cancer patients were treated with IL-2 (1000 units/mL) for 18 hours before the cells were used as effector cells in ^{51}Cr release assay against tumor cells cultured with the PBMCs. The lytic units $30/10^6$ cells were determined using inverse number of PBMCs required to lyse 30% of the target cells X100 (n=3) **(G)**. Freshly isolated PBMCs from healthy donors and cancer patients were treated with IL-2 (1000 units/mL) for 18 hours before the supernatants were removed from the co-cultures and the levels of IFN-γ secretions were determined using specific ELISAs (n=3) **(H)**.

Fig. 2

A.



B.

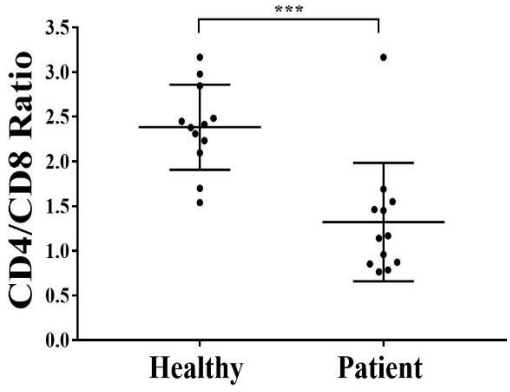


Figure 2: Decreased percentages of CD4 T cells and an increased percentage of CD8 T cells within cancer patients' PBMCs as compared to healthy PBMCs

Human PBMCs were isolated from peripheral blood of the healthy donors and cancer patients, surface expression of CD4 and CD8 were analyzed within CD3+ immune cells using flow cytometry (n=12) **(A)**, and ratio of CD4 to CD8 was determined (n=12) **(B)**.

Table 2

	Healthy NK	Patient NK
CD16	++++	++
Nkp30	+	++
Nkp44	+	+/-
Nkp46	+	+++
CD94	++	+++
NKG2D	+++	+
KIR2	+	+/-
KIR3	+	+/-

Table 2: NK receptors in freshly isolated healthy donor's and cancer patient's NK cells

Surface expression on the surface of freshly isolated healthy donors' and cancer patients' NK cells using anti-human antibodies for Nkp30, Nkp44, Nkp46, CD94, NKG2D, KIR2 and KIR3, one from total 7 representative experiments is shown in the table.

Fig. 3

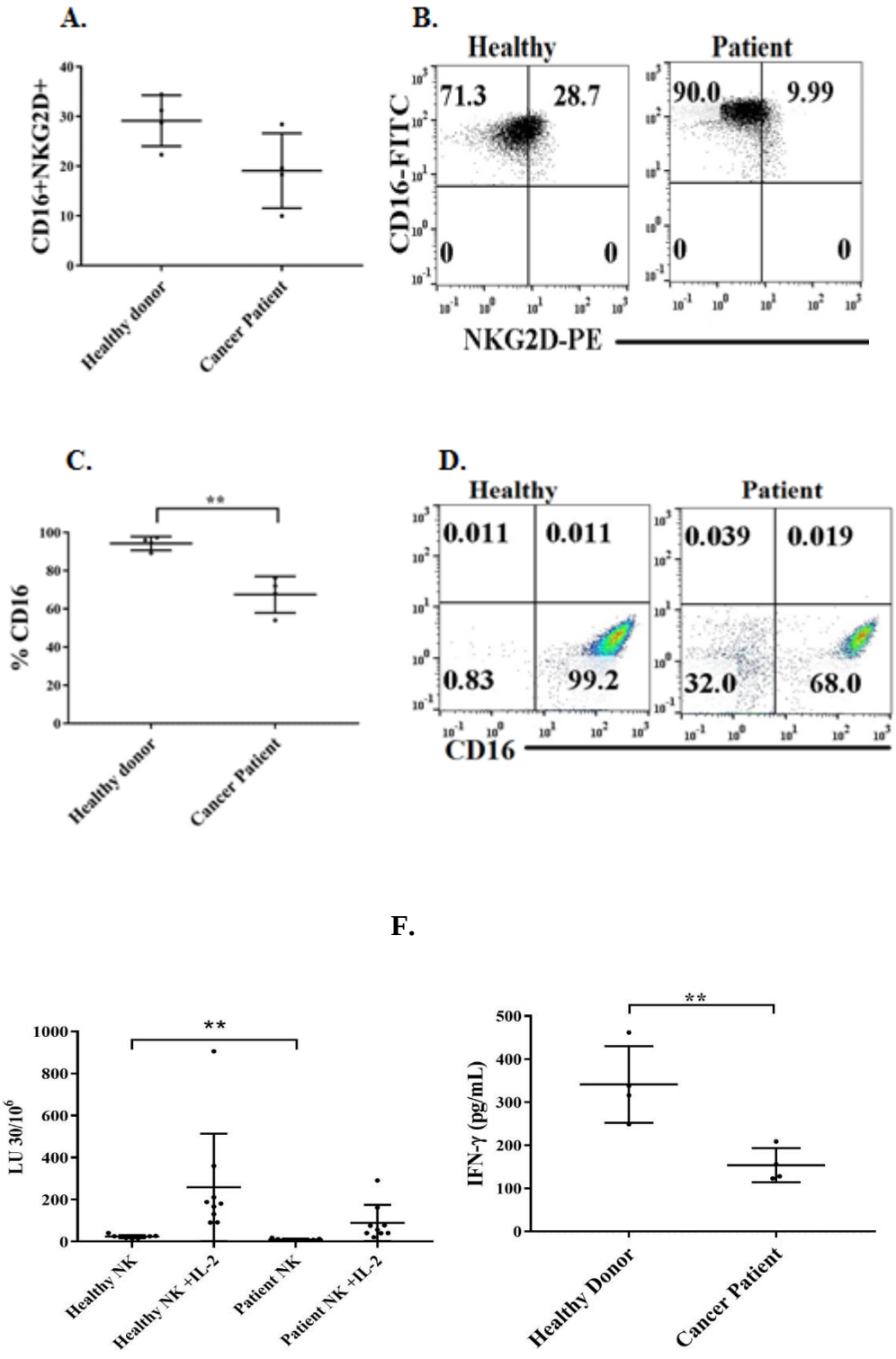

A.
CD16+NKG2D+
Healthy donor
Cancer Patient
B.
Healthy
Patient
71.3
28.7
90.0
9.99
0
0
0
0
CD16-FITC
NKG2D-PE
C.
**
% CD16
Healthy donor
Cancer Patient
D.
Healthy
Patient
0.011
0.011
0.039
0.019
0.83
99.2
32.0
68.0
CD16
E.
**
LU 30/10^6
Healthy NK
Healthy NK +IL-2
Patient NK
Patient NK +IL-2
F.
**
IFN-γ (pg/mL)
Healthy Donor
Cancer Patient

Figure 3: Low surface expression of NKG2D and CD16, and decreased NK cell cytotoxicity and IFN-γ secretion by patient NK cells as compared to healthy NKs.

Human NK cells were isolated from healthy and cancer patient's PBMC as described in Materials and Methods. Surface expression on the surface of freshly isolated healthy donors' and cancer patients' NK cells using anti-human antibodies for (n=4) NKG2D **(A and B)**, and CD16 (n=4) **(C and D).** Purified NK cells ($1x10^6$ cells/ml) were treated with IL-2 (1000 U/ml) for 18 hours before they were added to ^{51}Cr labeled oral squamous cell carcinoma stem cells (OSCSCs) at various effector to target ratios as described in the Materials and Methods. NK cell-mediated cytotoxicity for healthy donors and cancer patients (n=9) were determined using a standard 4-hour ^{51}Cr release assay against the oral squamous cell carcinoma stem cell line (OSCSCs) **(E)**. Freshly isolated NK cells from healthy donors and cancer patients were treated with IL-2 (1000 units/mL) for 18 hours before the supernatants were removed from the co-cultures and the levels of IFN-γ secretions were determined using specific ELISAs (n=4) **(H)**.

Fig. 4

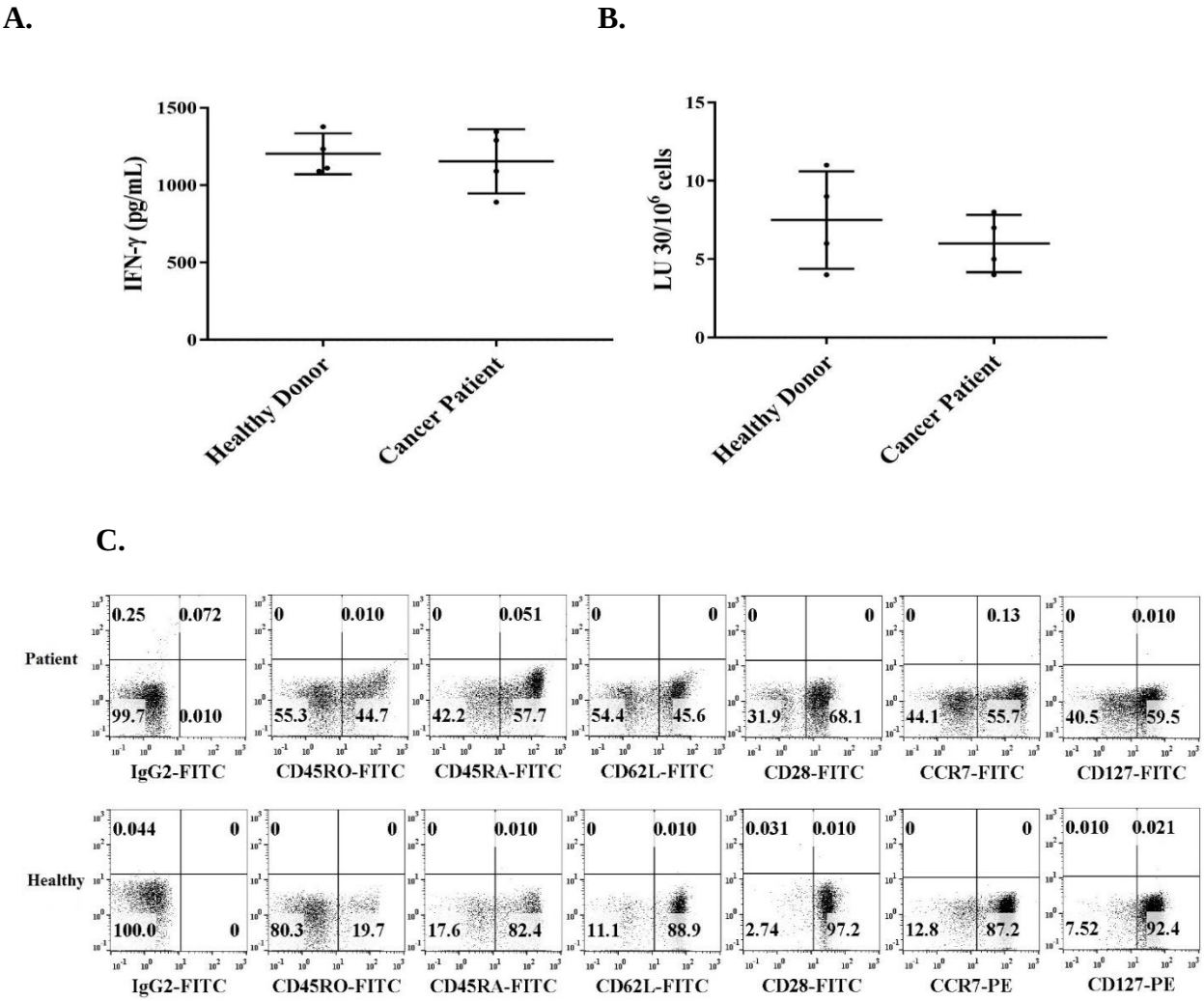
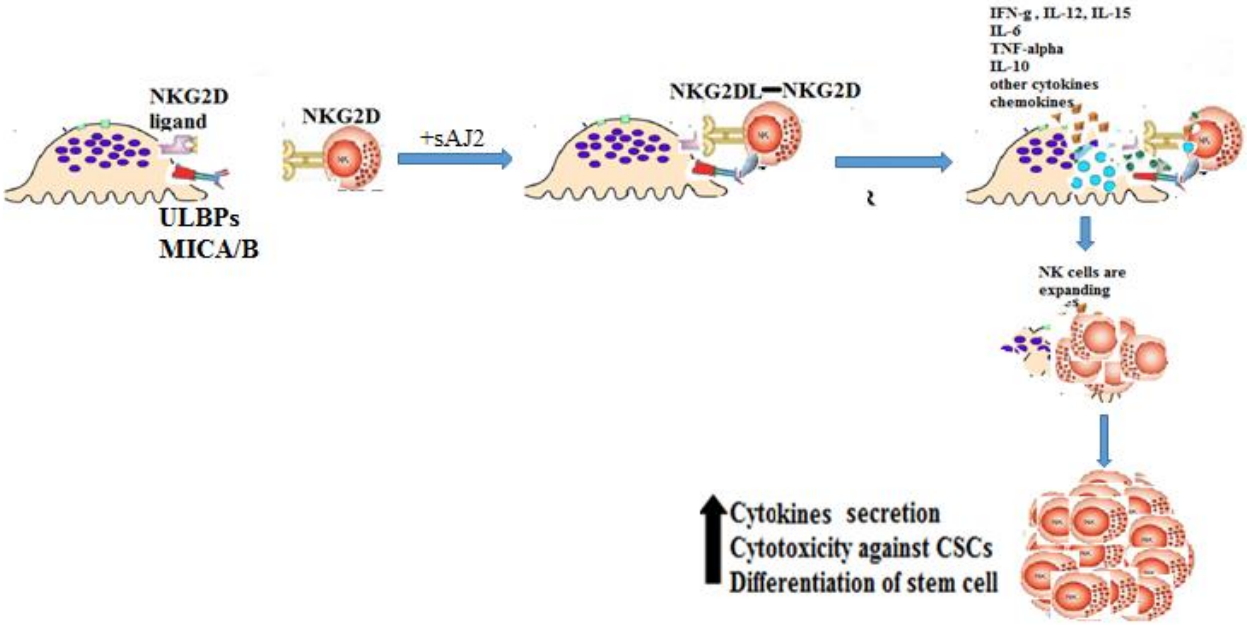


Figure 4: Patient T cells have T-effector memory surface profile.

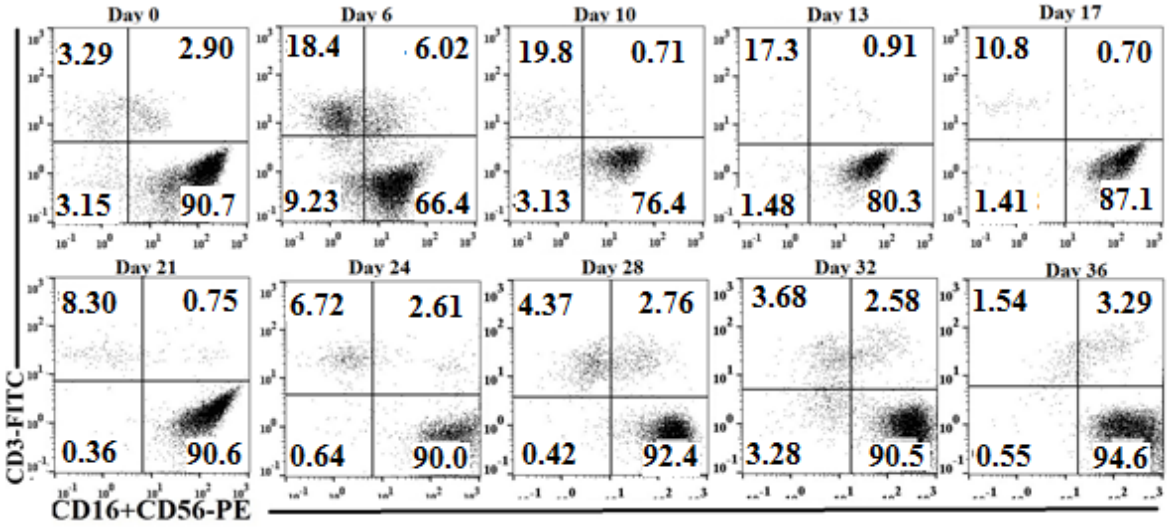
Freshly isolated T cells from healthy donors and cancer patients were treated with IL-2 (100 units/mL) for 18 hours before the supernatants were removed from the co-cultures and the levels of IFN-γ secretions were determined using specific ELISAs (n=4) **(A)**.Purified T cells ($1x10^6$ cells/ml) were treated with IL-2 (100 U/ml) for 18 hours before they were added to ^{51}Cr labeled oral squamous cell carcinoma stem cells (OSCSCs) at various effector to target ratios as described in the Materials and Methods. Cytotoxicity for healthy donors and cancer patients (n=4) were determined using a standard 4-hour ^{51}Cr release assay against the oral squamous cell carcinoma stem cell line (OSCSCs) **(B)**. Freshly isolated T cells from healthy donors and cancer patients were analyzed for surface expressions of CD45RO, CD45RA, CD62L, CD28, CCR7, and C127 after they were stained with anti-human antibodies and were ran on flow cytometry, one of the 4 representative experiment is shown in the Figure **(C)**.

Fig. 5

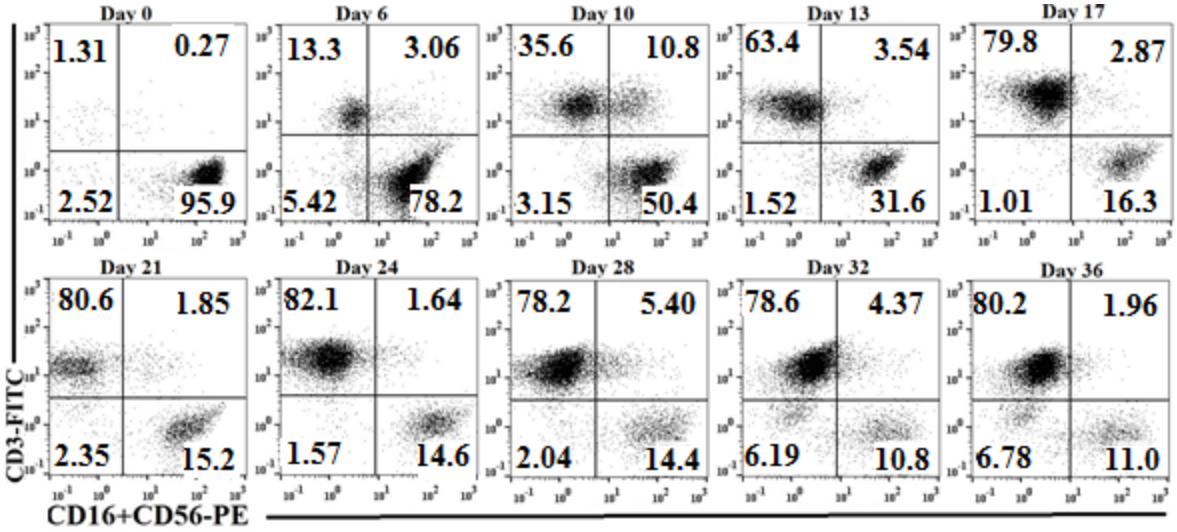
A.


NKG2D ligand
ULBPs
MICA/B
NKG2D
+sAJ2
NKG2DL—NKG2D
IFN-g , IL-12, IL-15
IL-6
TNF-alpha
IL-10
other cytokines
chemokines
NK cells are expanding
Cytokines secretion
Cytotoxicity against CSCs
Differentiation of stem cell

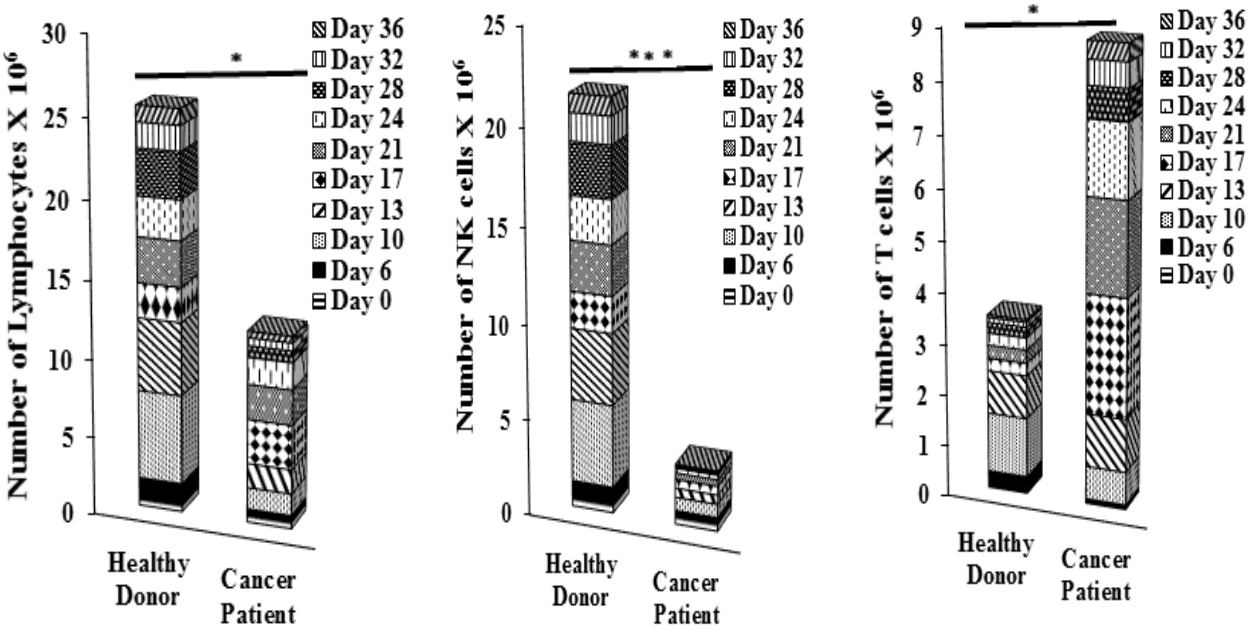
B.


Day 0
3.29
2.90
3.15
90.7
Day 6
18.4
6.02
9.23
66.4
Day 10
19.8
0.71
3.13
76.4
Day 13
17.3
0.91
1.48
80.3
Day 17
10.8
0.70
1.41
87.1
Day 21
8.30
0.75
0.36
90.6
Day 24
6.72
2.61
0.64
90.0
Day 28
4.37
2.76
0.42
92.4
Day 32
3.68
2.58
3.28
90.5
Day 36
1.54
3.29
0.55
94.6
CD3-FITC
CD16+CD56-PE

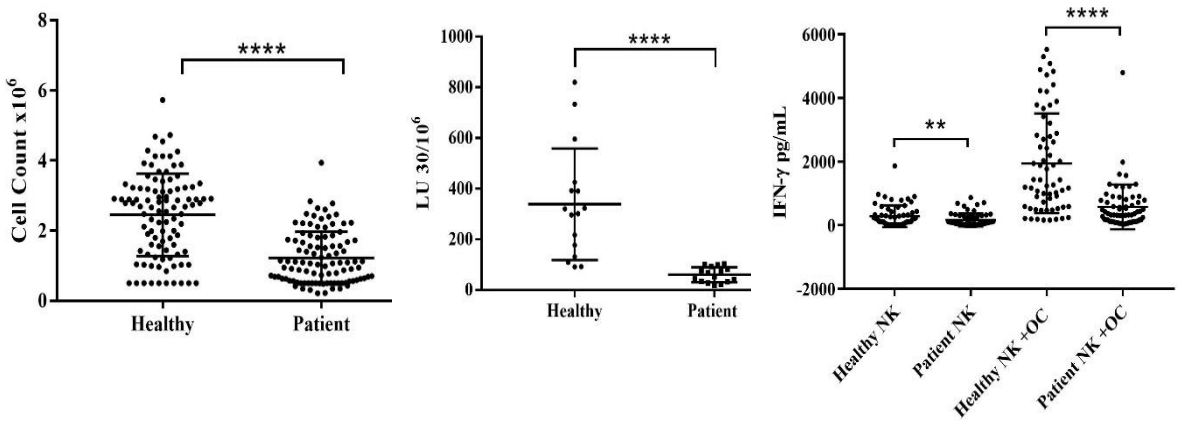
C.


Day 0
1.31
0.27
2.52
95.9
Day 6
13.3
3.06
5.42
78.2
Day 10
35.6
10.8
3.15
50.4
Day 13
63.4
3.54
1.52
31.6
Day 17
79.8
2.87
1.01
16.3
Day 21
80.6
1.85
2.35
15.2
Day 24
82.1
1.64
1.57
14.6
Day 28
78.2
5.40
2.04
14.4
Day 32
78.6
4.37
6.19
10.8
Day 36
80.2
1.96
6.78
11.0
CD3-FITC
CD16+CD56-PE

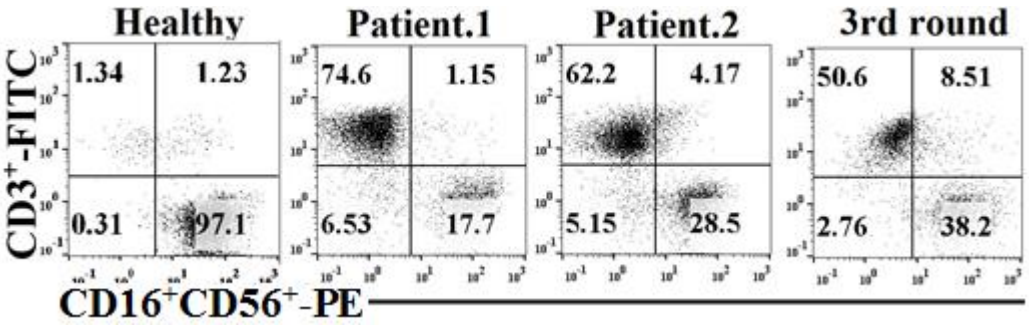
D. **E.** **F.**


Number of Lymphocytes X 10^6
Number of NK cells X 10^6
Number of T cells X 10^6
*
* * *
Day 36
Day 32
Day 28
Day 24
Day 21
Day 17
Day 13
Day 10
Day 6
Day 0
Healthy Donor
Cancer Patient

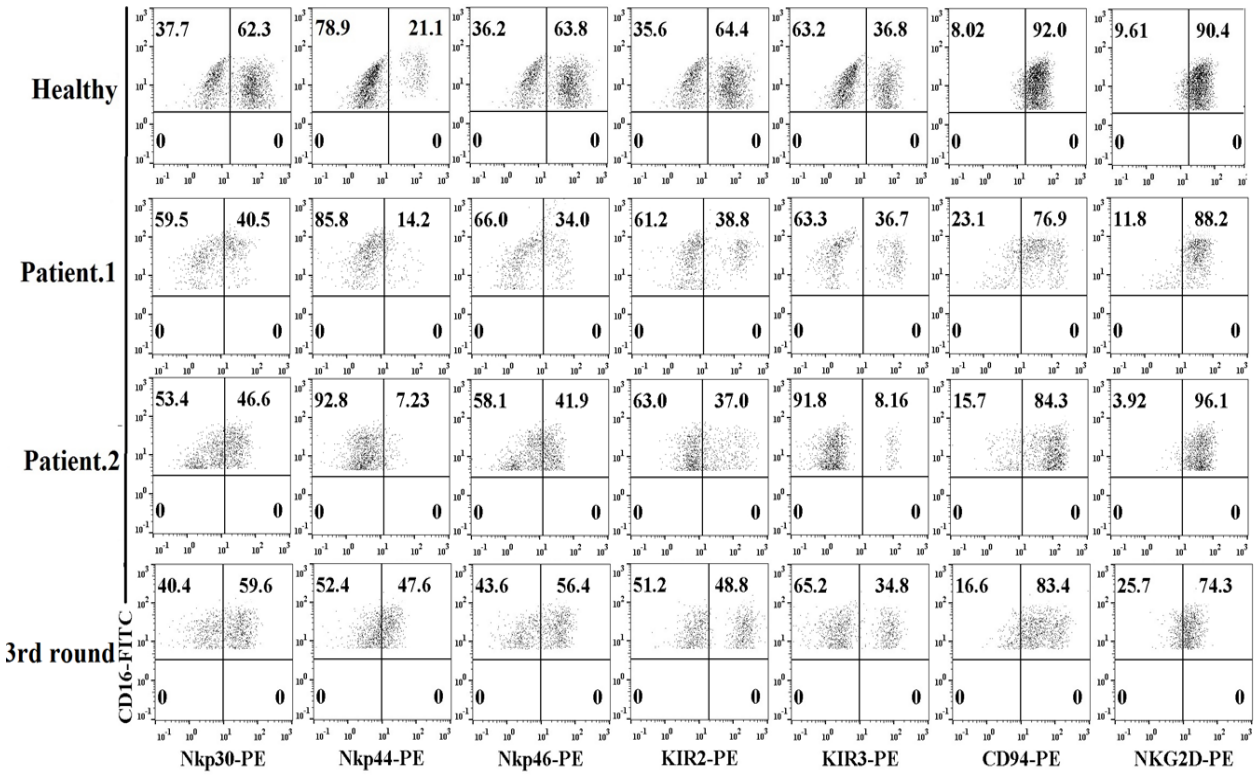
G. **H.** **I.**


Cell Count x10^6
****
Healthy
Patient
LU 30/10^6
****
Healthy
Patient
IFN-γ pg/mL
**
****
Healthy NK
Patient NK
Healthy NK +OC
Patient NK +OC

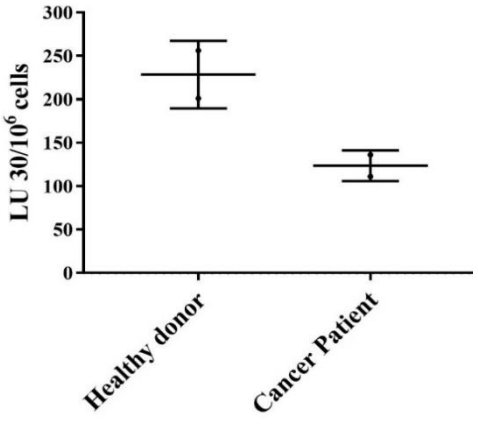
J.


Healthy
Patient.1
Patient.2
3rd round
CD3+-FITC
CD16+CD56+-PE
1.34
1.23
0.31
97.1
74.6
1.15
6.53
17.7
62.2
4.17
5.15
28.5
50.6
8.51
2.76
38.2

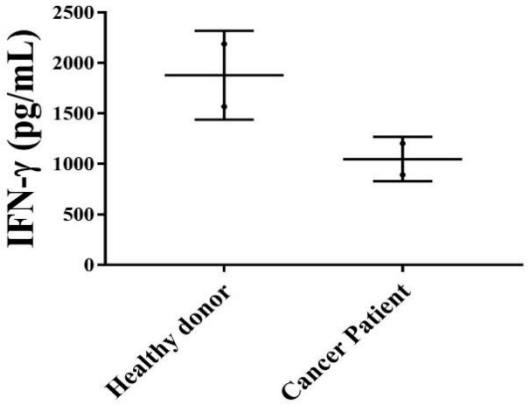
K.


Healthy
Patient.1
Patient.2
3rd round
CD16-FITC
Nkp30-PE
Nkp44-PE
Nkp46-PE
KIR2-PE
KIR3-PE
CD94-PE
NKG2D-PE
37.7 62.3 0 0
78.9 21.1 0 0
36.2 63.8 0 0
35.6 64.4 0 0
63.2 36.8 0 0
8.02 92.0 0 0
9.61 90.4 0 0
59.5 40.5 0 0
85.8 14.2 0 0
66.0 34.0 0 0
61.2 38.8 0 0
63.3 36.7 0 0
23.1 76.9 0 0
11.8 88.2 0 0
53.4 46.6 0 0
92.8 7.23 0 0
58.1 41.9 0 0
63.0 37.0 0 0
91.8 8.16 0 0
15.7 84.3 0 0
3.92 96.1 0 0
40.4 59.6 0 0
52.4 47.6 0 0
43.6 56.4 0 0
51.2 48.8 0 0
65.2 34.8 0 0
16.6 83.4 0 0
25.7 74.3 0 0

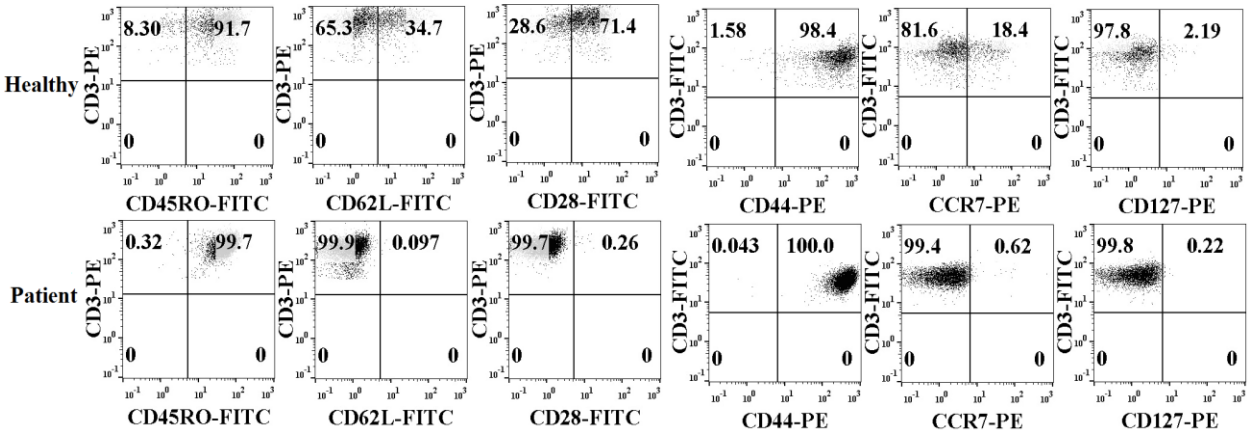
L.


LU 30/10^6 cells
300
250
200
150
100
50
0
Healthy donor
Cancer Patient

M.


IFN-γ (pg/mL)
2500
2000
1500
1000
500
0
Healthy donor
Cancer Patient

N.


Healthy
Patient
CD3-PE
CD45RO-FITC
CD62L-FITC
CD28-FITC
CD3-FITC
CD44-PE
CCR7-PE
CD127-PE
8.30
91.7
65.3
34.7
28.6
71.4
1.58
98.4
81.6
18.4
97.8
2.19
0.32
99.7
99.9
0.097
99.7
0.26
0.043
100.0
99.4
0.62
99.8
0.22
0

O. **P.** **Q.**

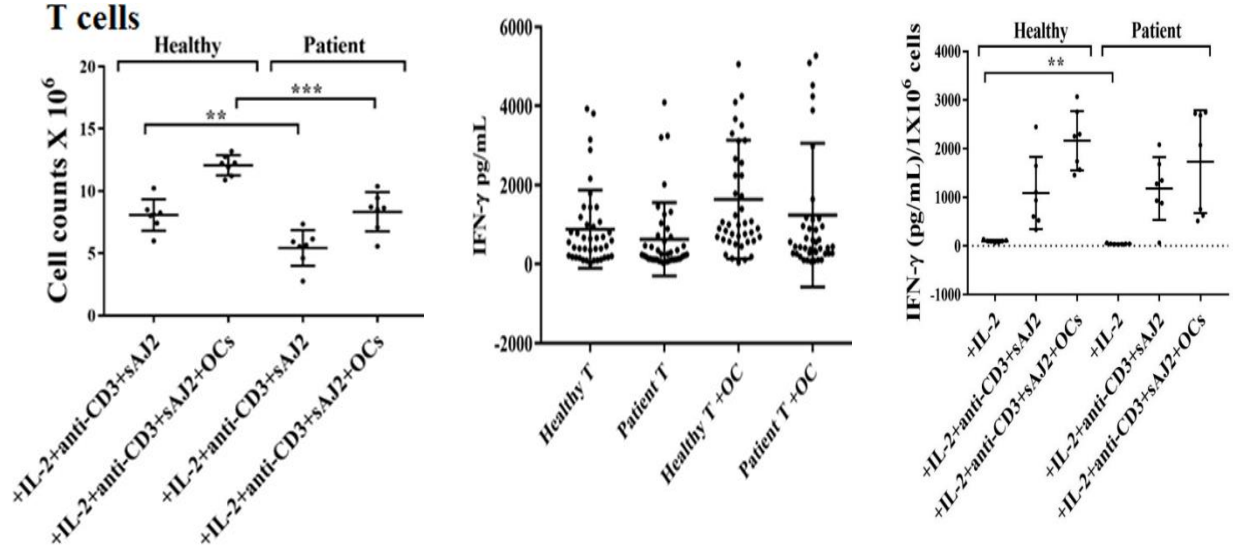
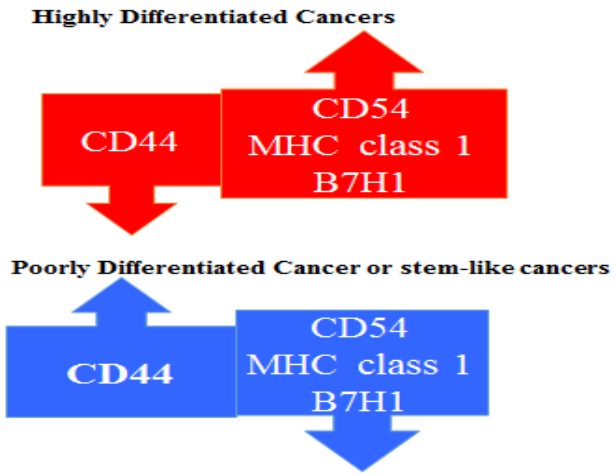

T cells
Healthy
Patient
Cell counts X 10^6
**
***
+IL-2+anti-CD3+sAJ2
+IL-2+anti-CD3+sAJ2+OCs
IFN-γ pg/mL
Healthy T
Patient T
Healthy T +OC
Patient T +OC
IFN-γ (pg/mL)/1X10^6 cells
+IL-2

Figure 5: Purified NK cells cultured with OCs from cancer patients expand more T cells than NK cells, mediate much lower cytotoxicity, and cytokine secretion compared to those expanded from healthy donors.

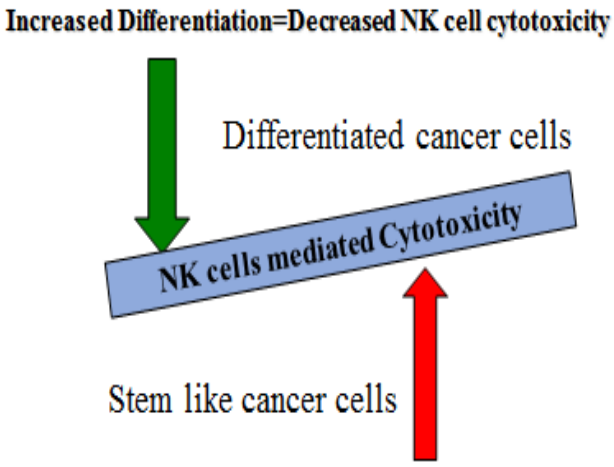
Freshly purified NK cells from healthy donor and cancer patient were treated and co-cultured with monocyte-derived OCs **(A)**, surface expression of CD3, CD16 and CD56 was analyzed on expanding cells at days 6, 9, 12, 15, 18, 21, 24, 27, and 31 of healthy donor (**B**) and cancer patient (**C**) using antibody staining followed by flow cytometric analysis**.** After 6, 9, 12, 15, 18, 21, 24, 27, and 31 days of co-culture, expanded lymphocytes were manually counted using microscopy (n=70) **(D and G).** The numbers of NK cells **(E)** and T/NKT **(F)** cells were determined using the percentages of NK and T/NKT cells within the total expanding cells in Fig. B and C**.** Cytotoxicity of lymphocytes was determined on days 12 and 15 using standard 4-hour ^{51}Cr release assay against OSCSCs (n=18) **(H)**. The supernatants were harvested from the overnight, day 6, 9, 12, 15, 18, 21, 24, and 27 co-cultures and IFN-γ secretion was determined using single ELISAs (n=63) **(I)**. Freshly purified NK cells from healthy donors, cancer patient with tonsillar carcinoma (patient.1) and pancreatic cancer (patient.2) were treated and co-cultured with OCs as described in Fig. 5A. Surface expressions of CD3, CD16, and CD56 were analyzed on lymphocytes from co-cultures of day 21 for healthy donor and patient NKs, and day 87 (3rd stimulation) of healthy donors (**J**), and surface expression of Nkp30, Nkp44, Nkp46, KIR2, KIR3, CD94 and NKG2D was analyzed within CD16 positive cells (**K**). IgG2 isotype control antibodies were used as control **(K)**. The NK cells were isolated form NK+OC culture from healthy donor and cancer patients on day 21, treated isolated NK cells with IL-2 (1000 U/ml) for 18-20 hours before they were used to determine cytotoxicity against untreated and NK supernatant-differentiated OSCSCs in 4-hour ^{51}Cr release assay **(L)**, supernatant from the culture was harvested and IFN-γ secretion was determined using single ELISAs **(M)**. Freshly purified NK cells from healthy donors and cancer patient were treated and co-cultured with OCs as described in Fig. 5A. Surface expressions of CD3, CD45RO, CD62L, CD28, CD44, CCR7, and CD127 were analyzed on lymphocytes from co-cultures of day 21 for healthy donor and patient T cells in OC expanded NK cells (**N**). Purified T cells (1x10^6 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 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 (n=7) **(O)** and the supernatants were harvested from the day 6, 9, 12 and 15 co-cultures, IFN-γ secretion was determined using single ELISAs (n=45) **(P)** and normalized per million lymphocytes counts (n=7) **(Q)**.

Fig. 6

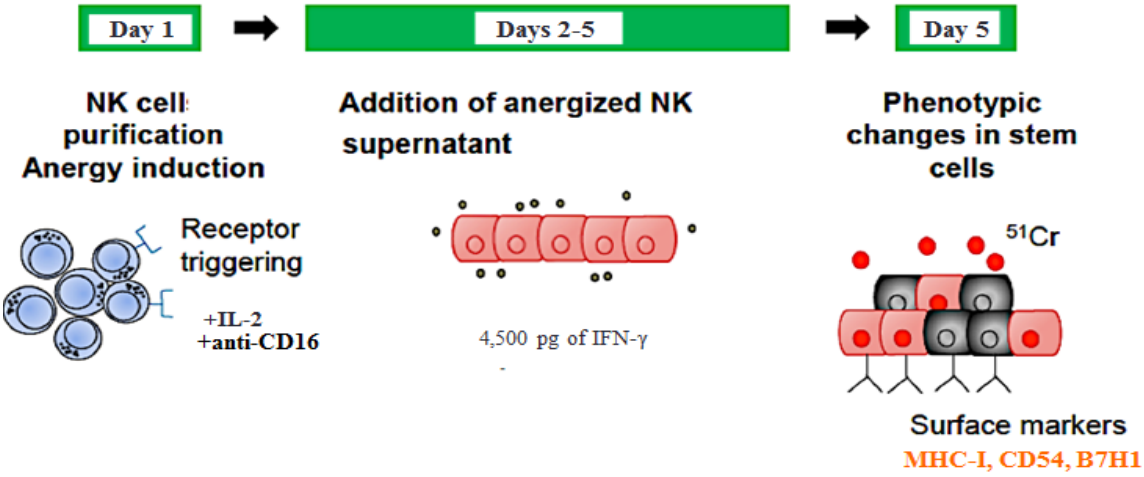
A.

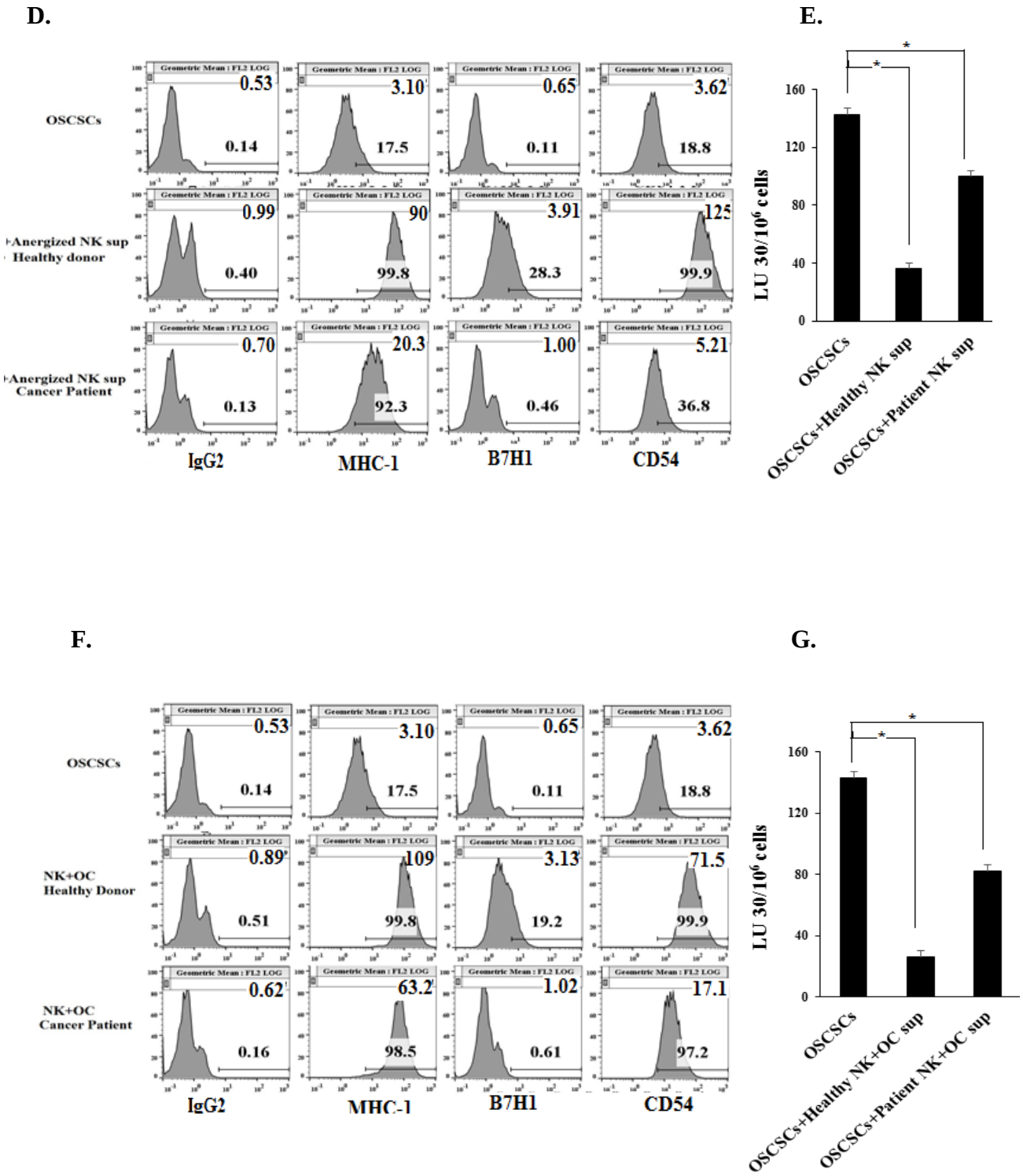

Highly Differentiated Cancers
CD44
CD54
MHC class 1
B7H1
Poorly Differentiated Cancer or stem-like cancers
CD44
CD54
MHC class 1
B7H1

B.

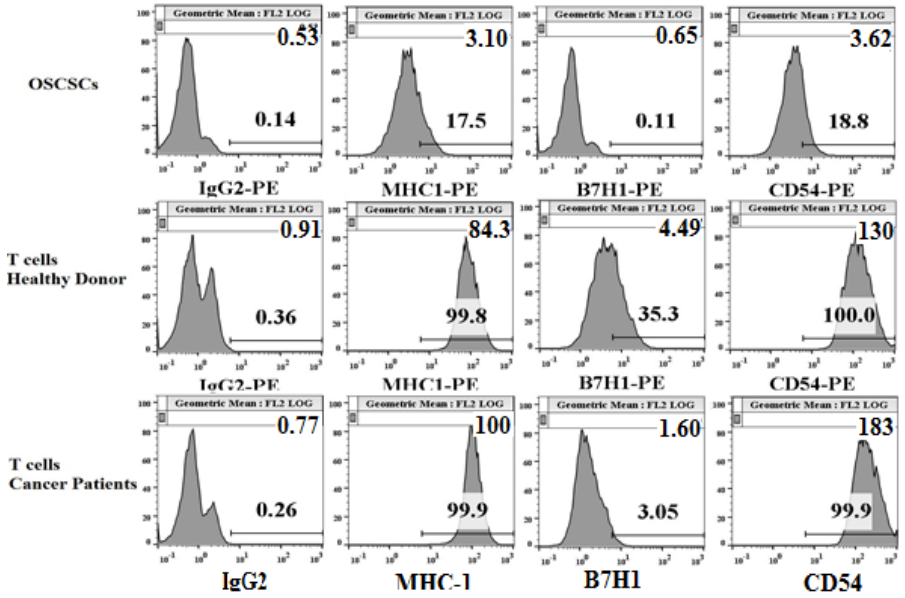

Increased Differentiation=Decreased NK cell cytotoxicity
Differentiated cancer cells
NK cells mediated Cytotoxicity
Stem like cancer cells

C.

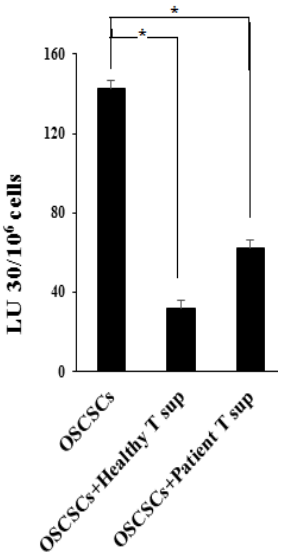

Day 1
Days 2-5
Day 5
NK cell purification
Anergy induction
Receptor triggering
+IL-2
+anti-CD16
Addition of anergized NK supernatant
4,500 pg of IFN-γ
Phenotypic changes in stem cells
51Cr
Surface markers
MHC-I, CD54, B7H1


D.
E.
OSCSCs
Anergized NK sup
Healthy donor
Anergized NK sup
Cancer Patient
0.53
0.14
3.10
17.5
0.65
0.11
3.62
18.8
0.99
0.40
90
99.8
3.91
28.3
125
99.9
0.70
0.13
20.3
92.3
1.00
0.46
5.21
36.8
IgG2
MHC-1
B7H1
CD54
LU 30/10^6 cells
160
120
80
40
0
OSCSCs
OSCSCs+Healthy NK sup
OSCSCs+Patient NK sup
F.
G.
OSCSCs
NK+OC
Healthy Donor
NK+OC
Cancer Patient
0.53
0.14
3.10
17.5
0.65
0.11
3.62
18.8
0.89
0.51
109
99.8
3.13
19.2
71.5
99.9
0.62
0.16
63.2
98.5
1.02
0.61
17.1
97.2
IgG2
MHC-1
B7H1
CD54
LU 30/10^6 cells
160
120
80
40
0
OSCSCs
OSCSCs+Healthy NK+OC sup
OSCSCs+Patient NK+OC sup

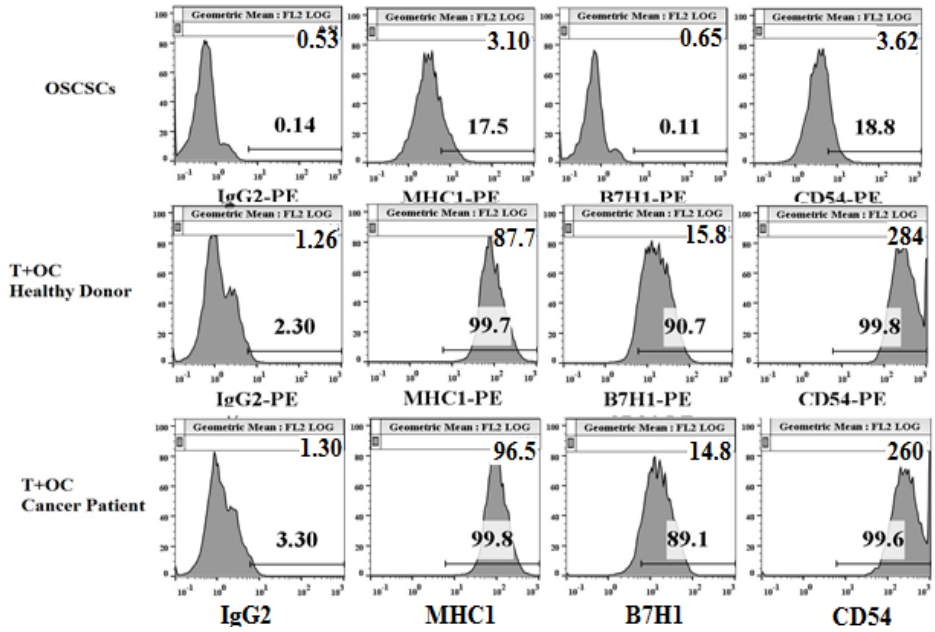
H.


OSCSCs
T cells Healthy Donor
T cells Cancer Patients
Geometric Mean : FL2 LOG
0.53
0.14
3.10
17.5
0.65
0.11
3.62
18.8
IgG2-PE
MHC1-PE
B7H1-PE
CD54-PE
0.91
0.36
84.3
99.8
4.49
35.3
130
100.0
0.77
0.26
100
99.9
1.60
3.05
183
99.9
IgG2
MHC-1
B7H1
CD54

I.


LU 30/10⁶ cells
160
120
80
40
0
*
*
OSCSCs
OSCSCs+Healthy T sup
OSCSCs+Patient T sup

J.


OSCSCs
T+OC Healthy Donor
T+OC Cancer Patient
Geometric Mean : FL2 LOG
0.53
0.14
3.10
17.5
0.65
0.11
3.62
18.8
IgG2-PE
MHC1-PE
B7H1-PE
CD54-PE
1.26
2.30
87.7
99.7
15.8
90.7
284
99.8
1.30
3.30
96.5
99.8
14.8
89.1
260
99.6
IgG2
MHC1
B7H1
CD54

K.

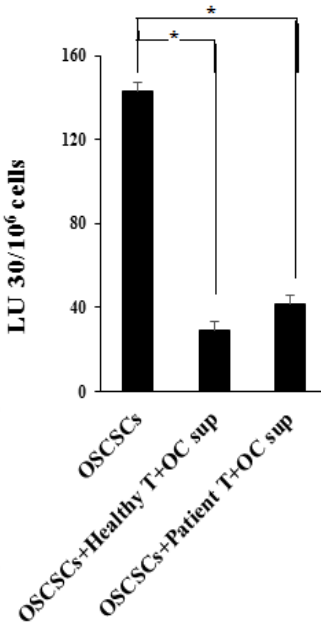

LU 30/10⁶ cells
160
120
80
40
0
*
*
OSCSCs
OSCSCs+Healthy T+OC sup
OSCSCs+Patient T+OC sup

Figure 6: Similar level of IFN-γ secreted by cancer patient NK cells induced lesser differentiation of oral cancer stem-like cells when compared to IFN-γ secreted from healthy donors NK cells.

Highly purified NK cells from healthy donor and cancer patient were treated with IL-2 (1000 units/mL) and anti-CD16mAb (3μg/mL) with for 18 hours before the supernatants were harvested and added to OSCSCs for 4 days. Afterwards untreated OSCSCs and those treated with different NK cell supernatants indicated in the figure, were detached from the tissue culture plates, extensively washed with 1X PBS, surface expression of MHC1, B7H1 and CD54 were analyzed on the surface of OSCSCs **(D)** and labeled with ^{51}Cr. Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/mL) for 24 hours before the cells were used as effector cells in ^{51}Cr release assay against OSCSCs treated with NK supernatants. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 **(E)**. Highly purified NK cells from healthy donor and cancer patient were treated with IL-2 (1000 units/mL) and anti-CD16mAb (3μg/mL) with for 18 hours before they were cultured with OC and sAJ2, the supernatants were harvested on day 6 after the culture, and added to OSCSCs for 4 days. Afterwards untreated OSCSCs and those treated with different NK cell supernatants indicated in the figure, were detached from the tissue culture plates, extensively washed with 1X PBS, surface expression of MHC1, B7H1 and CD54 were analyzed on the surface of OSCSCs **(F)** and labeled with ^{51}Cr. Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/mL) for 24 hours before the cells were used as effector cells in ^{51}Cr release assay against OSCSCs treated with NK supernatants. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 **(G)**. Highly purified T cells from healthy donor and cancer patient were treated with IL-2 (100 units/mL) and anti-CD3/CD28 (1μg/mL) with for 18 hours before the supernatants were harvested and added to OSCSCs for 4 days. Afterwards untreated OSCSCs and those treated with different NK cell supernatants indicated in the figure, were detached from the tissue culture plates, extensively washed with 1X PBS, surface expression of MHC1, B7H1 and CD54 were analyzed on the surface of OSCSCs **(H)** and labeled with ^{51}Cr. Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/mL) for 24 hours before the cells were used as effector cells in ^{51}Cr release assay against OSCSCs treated with NK supernatants. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 **(I)**. Highly purified T cells from healthy donor and cancer patient were treated with IL-2 (100 units/mL) and anti-CD3/CD28 (1μg/mL) with for 18 hours before they were cultured with OC and sAJ2, the supernatants were harvested on day 6 after the culture, and added to OSCSCs for 4 days. Afterwards untreated OSCSCs and those treated with different NK cell supernatants indicated in the figure, were detached from the tissue culture plates, extensively washed with 1X PBS, surface expression of MHC1, B7H1 and CD54 were analyzed on the surface of OSCSCs **(J)** and labeled with ^{51}Cr. Freshly isolated NK cells were left untreated or treated with IL-2 (1000 units/mL) for 24 hours before the cells were used as effector cells in ^{51}Cr release assay against OSCSCs treated with NK supernatants. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 **(K)**.

Fig. 7

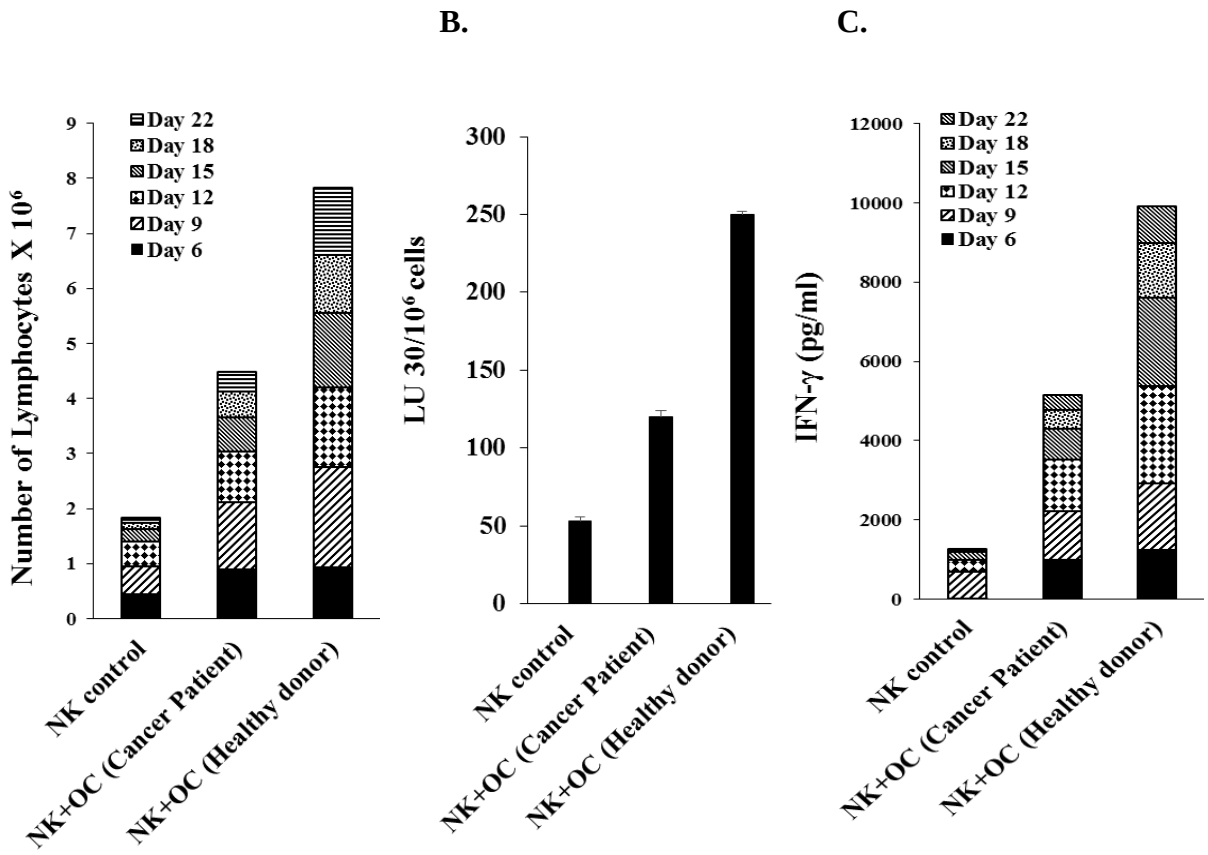


Figure 6: Osteoclasts from the cancer patients are less potent to expand the NK cells, to increase the cytotoxic activity and IFN-γ secretion in NK cells when compared to healthy osteoclasts.

Freshly purified NK cells from healthy donor were treated with IL-2 and anti-CD16mAb and co-cultured with monocyte-derived OCs from healthy donor and cancer patients, after 6, 9, 12, 15, 18, and 22 days of co-culture, expanded lymphocytes were manually counted using microscopy **(A).** Cytotoxicity of lymphocytes was determined on days 12 and 15 using standard 4-hour ^{51}Cr release assay against OSCSCs **(B)**. The supernatants were harvested from the overnight, day 6, 9, 12, 15, 18, and 22 after the co-cultures and IFN-γ secretion was determined using single ELISAs **(C)**.

Sub aim 2: Functional studies of NK, monocytes and osteoclasts of healthy and tumor injected hu-BLT mice.

PBMCs from tumor-bearing hu-BLT mice have low cytotoxicity activity against CSCs and secrete less IFN-γ.

Humanized BLT mice had human immune cells in various tissue compartments (Fig. 1B), and the number of T cells, CD4+T cells, CD8+T cells and monocytes in hu-BLT PBMCs were very similar to human PBMCs, except the NK cells which are low in hu-BLT mice (Fig. 1C-1F). There was significantly low cytotoxic activity and low IFN-γ secretion from the PBMCs of OSCSCs injected hu-BLT mice (Fig. 2C and 2D), similar results were seen in MP2 injected hu-BLT mice (Fig. 2E and 2F).

Oral tumors in humanized mice preferentially expand T cells resulting in the loss of NK cytotoxicity while retaining IFN-γ secretion.

Humanized-BLT mice were implanted with oral tumors, mice were sacrificed 4 weeks after tumor implantation. The spleens from hu-BLT mice were harvested, and T cells were sorted out. The flow-through cells containing B cells were then treated with IL-2 and anti-CD16mAb for 18-20 hours before they were cultured with BLT-OCs. 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. 3B). 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 (Fig. 3B). The total numbers of expanded lymphocytes were lower in tumor-bearing mice when compared to those without tumors (Fig. 3C), with the majority being T cells and not NK cells (Fig. 3D and E).

NK enriched cells from tumor-bearing mice, when co-cultured with OCs, were able to lyse OSCSCs significantly less than those of control mice without tumor (Fig. 3F). In addition, when cytotoxicity was assessed per NK cell basis, less cytotoxicity was seen with NK cells from tumor-bearing mice as compared to NK cells from control mice without tumor (Fig. 3G). NK enriched cells from tumor-bearing mice secreted significantly higher IFN-γ (Fig. 3H), lower IL-10 (Fig. 3I), and slightly lower IL-6 (Fig. 3J) when compared to the control mice without tumor. Sera from peripheral blood of tumor bearing hu-BLT mice exhibited increased secretion of IFN-γ, IL-10, and IL-6 as compared to the control mice with no tumor (Fig. 3K).

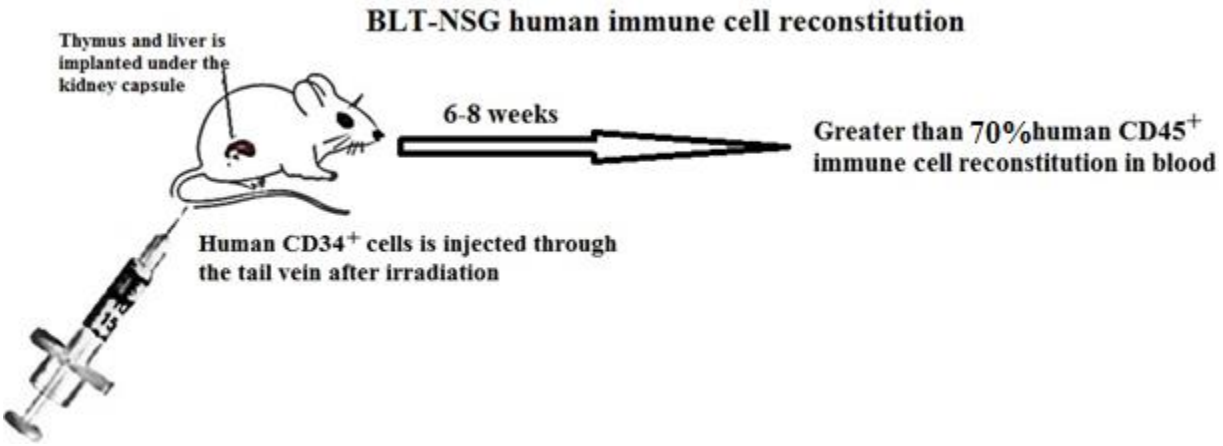
Monocytes and OC from the hu-BLT mice with tumor also have inhibited function.

Next, we studied the effect of monocytes on the autologous NK cells in the tumor environment. We purified NK cells from the spleen and monocytes from the bone marrow cell. NK cells were left untreated and treated with IL-2. Further IL-2 treated cells were cultured with/without autologous monocytes, for 12 days and then cells were used for cytotoxicity against the OSCSCs. NK cells from the BLT animal with tumor only mediated low cytotoxicity against OSCSCs when compared to NK cells from hu-BLT injected with tumor in combination with NK

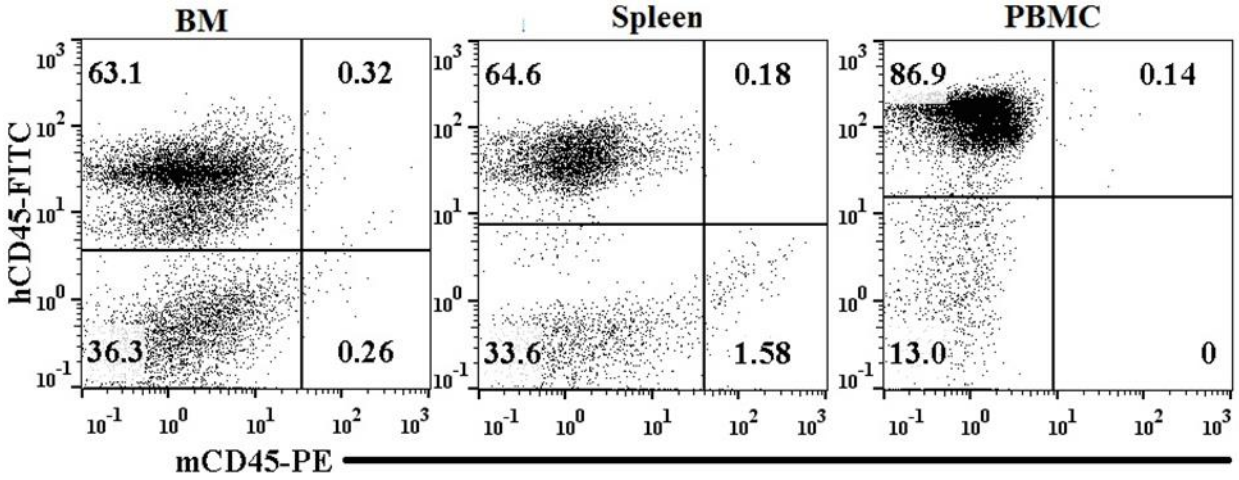
cells or those injected with the differentiated tumor (Fig. 4A). From a different set of tumors injected hu-BLT animals, we purified NK cells from the spleen and monocytes from bone marrow cells. NK cells were left untreated and treated with IL-2. Further IL-2 treated cells were cultured with/without autologous monocytes for 12 days, and IFN-γ secretion was determined per million cells in the culture, NK cells from the BLT animal with tumor secreted low IFN-γ, when compared to NK cells from hu-BLT injected with tumor in combination with NK cells or those injected with differentiated tumor, or those injected with differentiated tumor in the presence of antibodies to TNF-α and IFN-γ (Fig. 4B). Similar results were seen when NK and monocytes were cultured from MP2 injected hu-BLT mice (Fig. 4C). We then cultured the monocytes from the BLT animals in alpha-MEM media supplemented with M-CSF and RANKL for 21 days to generate the osteoclasts. Highly purified fresh human NK cells were treated with IL-2 in combination of anti-CD16mAb for 18 hours, and co-cultured with BLT-OC with the addition of probiotic bacteria (sAJ2). Human NK cells cultured with hu-BLT-OC were counted using microscopic assessment, OC-induced NK expansion was low by the osteoclasts generated from MP2 injected hu-BLT mice when compared to NK cells from hu-BLT injected with tumor and received NK cells therapy or those injected with differentiated tumor (Fig. 4D). NK cells secreted less IFN-γ (Fig. 4E), and mediated low NK cells-mediated cytotoxicity against OSCSCs (Fig. 4F) by the osteoclasts generated from MP2 injected hu-BLT mice when compared to NK cells from hu-BLT injected with MP2 and received NK cells therapy or those injected with differentiated tumor (Fig. 4E and Fig. 4F).

Fig. 1

A.


BLT-NSG human immune cell reconstitution
Thymus and liver is implanted under the kidney capsule
6-8 weeks
Greater than 70%human CD45+ immune cell reconstitution in blood
Human CD34+ cells is injected through the tail vein after irradiation

B.


BM
Spleen
PBMC
hCD45-FITC
mCD45-PE
63.1
0.32
36.3
0.26
64.6
0.18
33.6
1.58
86.9
0.14
13.0
0

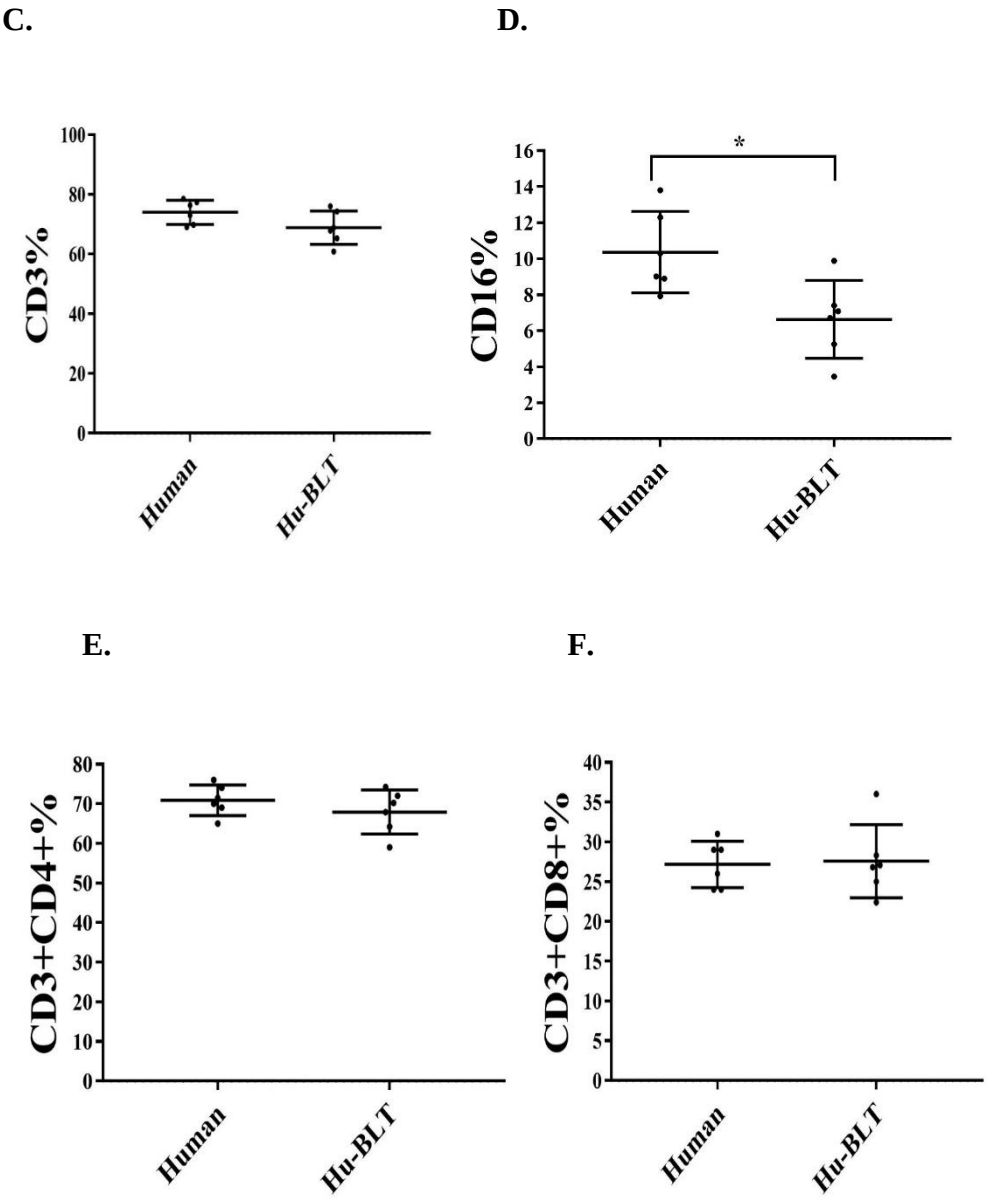
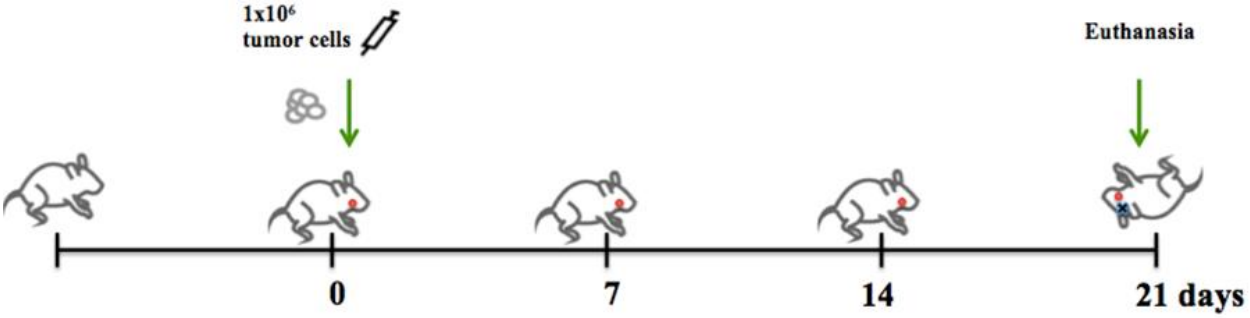


Figure 1: Hu-BLT mice generation and human immune cell proportion in various tissues.

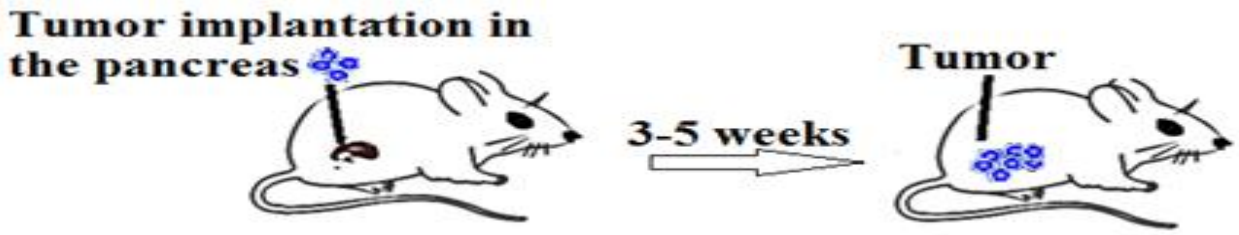
Humanized-BLT (hu-BLT; human bone marrow/liver/thymus) mice were generated by surgical implantation of human fetal liver and thymus tissue under the renal capsule of 6-8 weeks old immunocompromised NOD.CB17-Prkdcscid/J and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice **(A)** [25]. 4-6 weeks post tissue transplant, mice were sub-lethally irradiated and intravenously injected with $CD34^+$ cells isolated from fetal liver to support full reconstitution of human bone marrow. 8-12 weeks after injection with $CD34^+$ cells, reconstitution of human immune system was analyzed using blood, BM and spleen **(B)**. PBMCs were stained for human CD45, CD4, CD8, CD14, CD16, CD19, and CD56 expression and analyzed using flow cytometry **(C-F)**.

Fig. 2

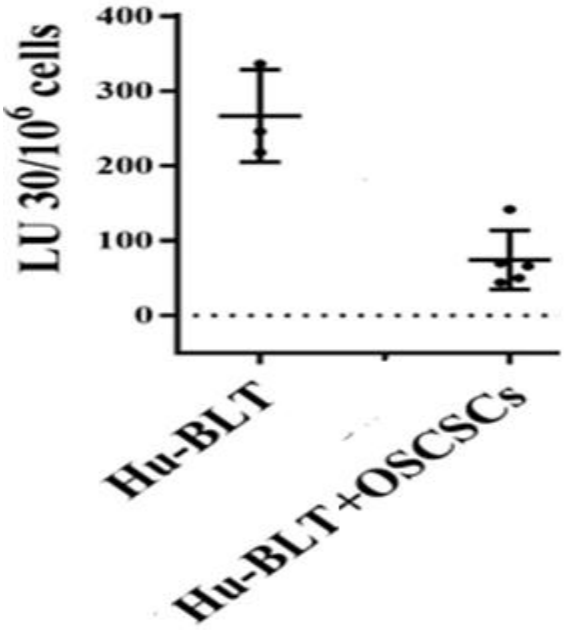
A.


1x10⁶
tumor cells
Euthanasia
0
7
14
21 days

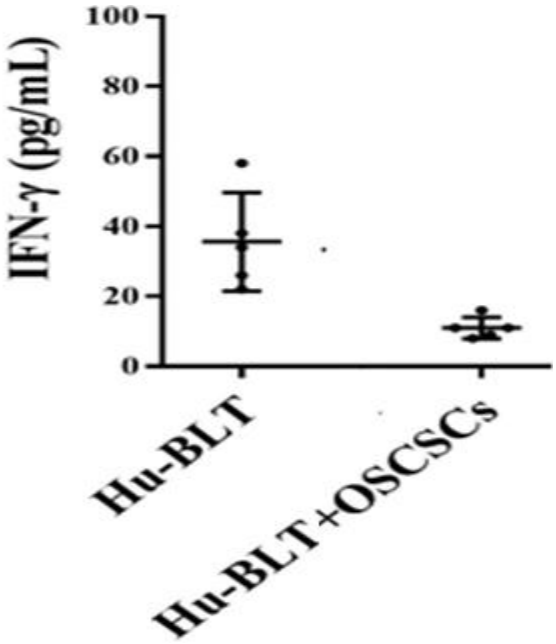
B.


Tumor implantation in
the pancreas
3-5 weeks
Tumor

C.


LU 30/10⁶ cells
400
300
200
100
0
Hu-BLT
Hu-BLT+OSCSCs

D.


IFN-γ (pg/mL)
100
80
60
40
20
0
Hu-BLT
Hu-BLT+OSCSCs

E. **F.**

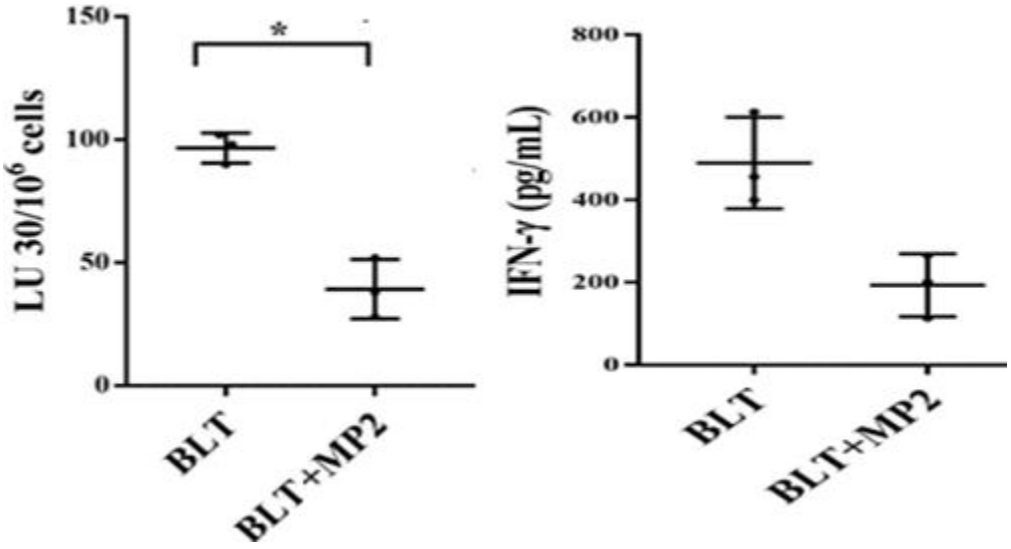
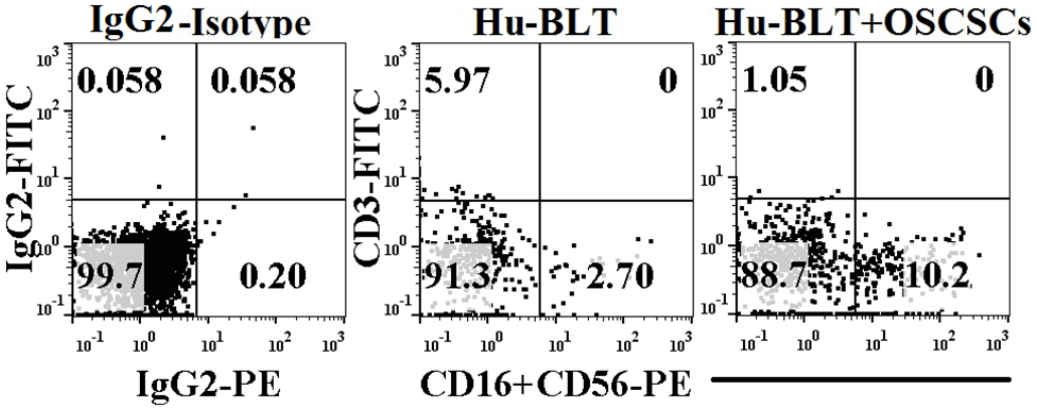


Figure 2: PBMCs from tumor-bearing hu-BLT mice mediated low cytotoxicity against cancer stem cells and secreted less IFN-γ when compared to no tumor hu-BLT mice.

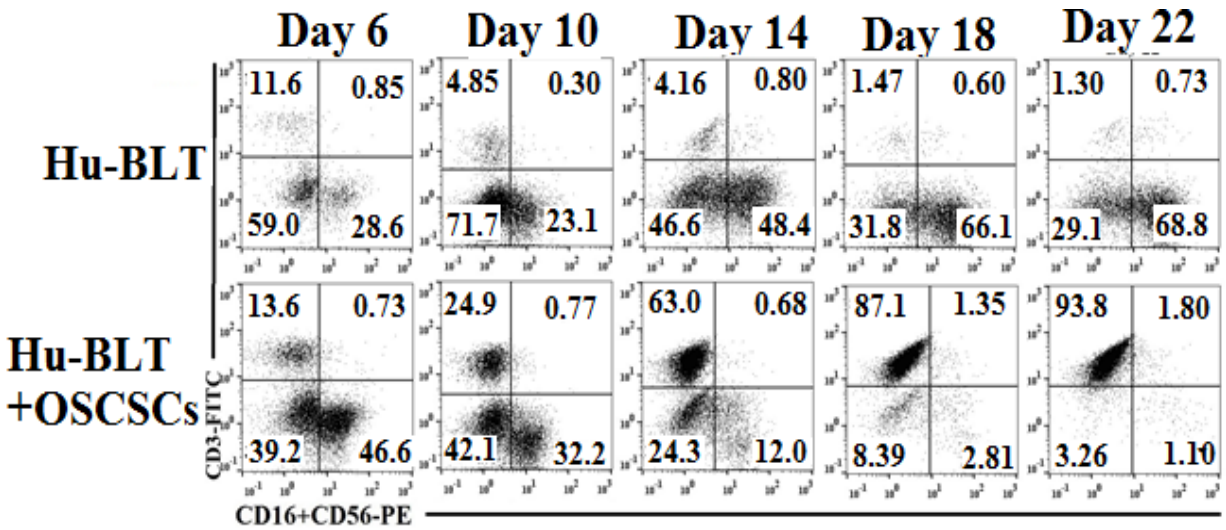
Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) mice were orthotopically injected with $1x10^6$ of human OSCSCs into the flora of the mouth, monitored for 3-4 weeks before the animals were euthanasized **(A)**. Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) mice were orthotopically injected with $0.5x10^6$ of human MP2 in the pancreas, monitored for 3-4 weeks before the animals were euthanasized **(B)**. At the end of this experiment peripheral blood was collected, single cell suspension was obtained from each tissue as described in Materials and Methods. PBMC were treated with or without IL-2 (1000 units/ml) for 7 days, cytotoxicity assay was performed was determined using standard 4-hour ^{51}Cr release assay against OSCSCs and the lytic units $30/10^6$ cells were determined using inverse number of PBMCs **(C and E)**. PBMC were treated with or without IL-2 (1000 units/ml) for 7 days, the supernatants were harvested and IFN-γ secretion was determined using single ELISAs **(D and F)**.

Fig. 3

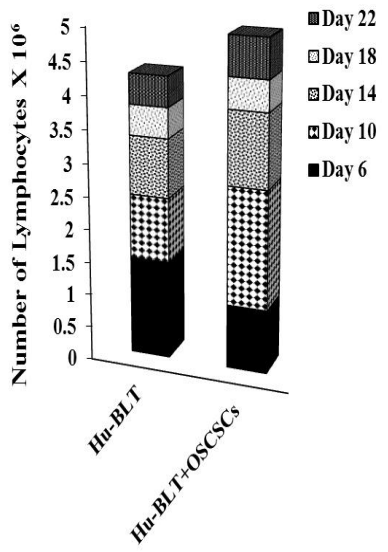
A.


IgG2-Isotype
Hu-BLT
Hu-BLT+OSCSCs
IgG2-FITC
CD3-FITC
0.058
0.058
99.7
0.20
5.97
0
91.3
2.70
1.05
0
88.7
10.2
IgG2-PE
CD16+CD56-PE

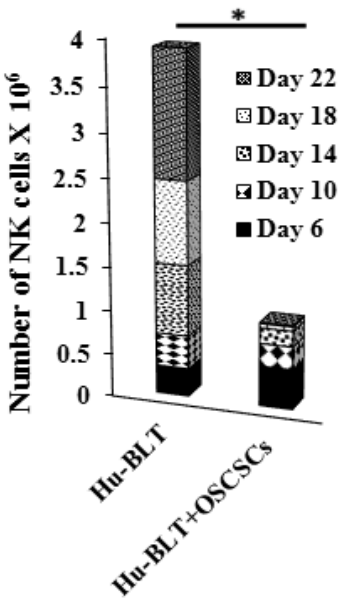
B.


Day 6
Day 10
Day 14
Day 18
Day 22
Hu-BLT
Hu-BLT +OSCSCs
CD3-FITC
11.6
0.85
59.0
28.6
4.85
0.30
71.7
23.1
4.16
0.80
46.6
48.4
1.47
0.60
31.8
66.1
1.30
0.73
29.1
68.8
13.6
0.73
39.2
46.6
24.9
0.77
42.1
32.2
63.0
0.68
24.3
12.0
87.1
1.35
8.39
2.81
93.8
1.80
3.26
1.10
CD16+CD56-PE

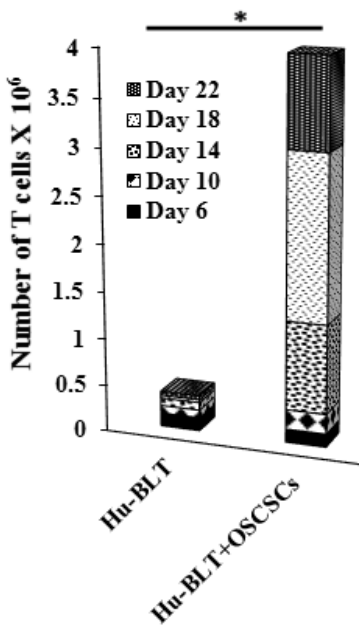
C.


Number of Lymphocytes X 10⁶
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 22
Day 18
Day 14
Day 10
Day 6
Hu-BLT
Hu-BLT+OSCSCs

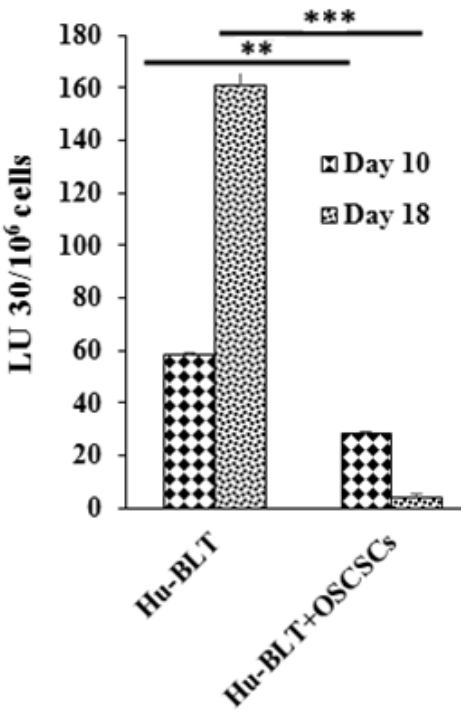
D.


*
Number of NK cells X 10⁶
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 22
Day 18
Day 14
Day 10
Day 6
Hu-BLT
Hu-BLT+OSCSCs

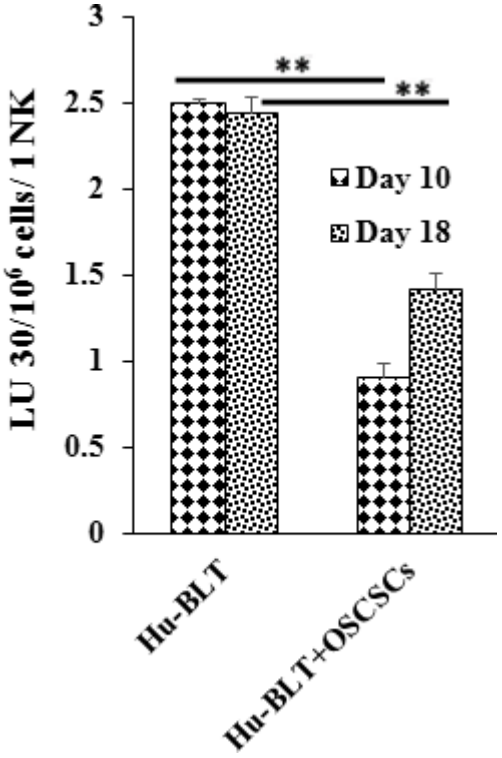
E.


*
Number of T cells X 10⁶
4
3.5
3
2.5
2
1.5
1
0.5
0
Day 22
Day 18
Day 14
Day 10
Day 6
Hu-BLT
Hu-BLT+OSCSCs

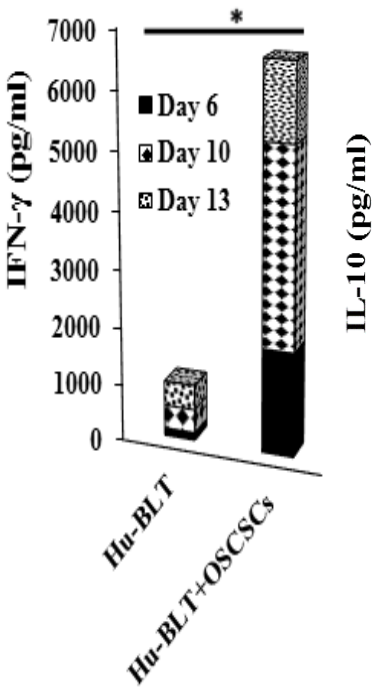
F.


***
**
LU 30/10⁶ cells
180
160
140
120
100
80
60
40
20
0
Day 10
Day 18
Hu-BLT
Hu-BLT+OSCSCs

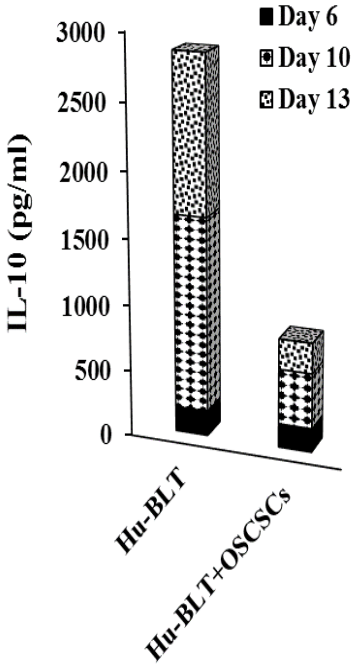
G.


**
**
LU 30/10⁶ cells/ 1 NK
3
2.5
2
1.5
1
0.5
0
Day 10
Day 18
Hu-BLT
Hu-BLT+OSCSCs

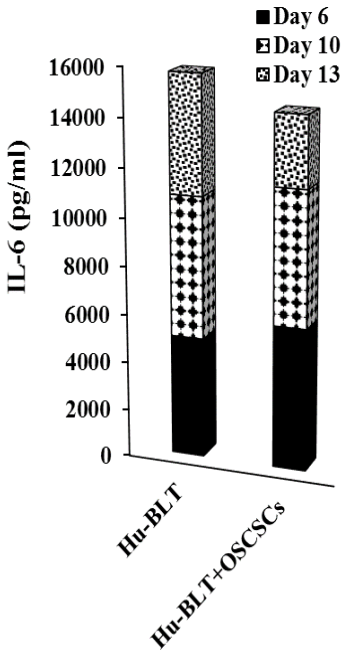
H.


*
7000
6000
5000
4000
3000
2000
1000
0
IFN-γ (pg/ml)
Day 6
Day 10
Day 13
Hu-BLT
Hu-BLT+OSCSCs

I.


3000
2500
2000
1500
1000
500
0
IL-10 (pg/ml)
Day 6
Day 10
Day 13
Hu-BLT
Hu-BLT+OSCSCs

J.


16000
14000
12000
10000
8000
6000
4000
2000
0
IL-6 (pg/ml)
Day 6
Day 10
Day 13
Hu-BLT
Hu-BLT+OSCSCs

K.

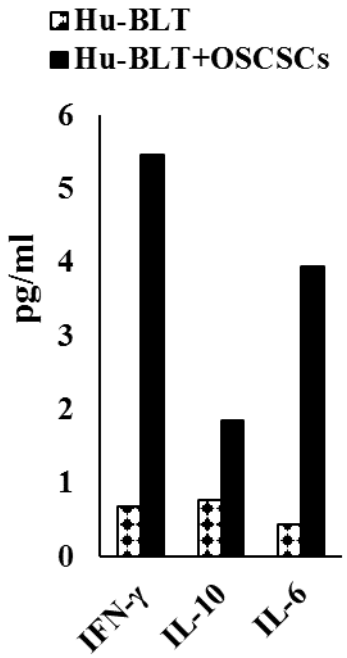
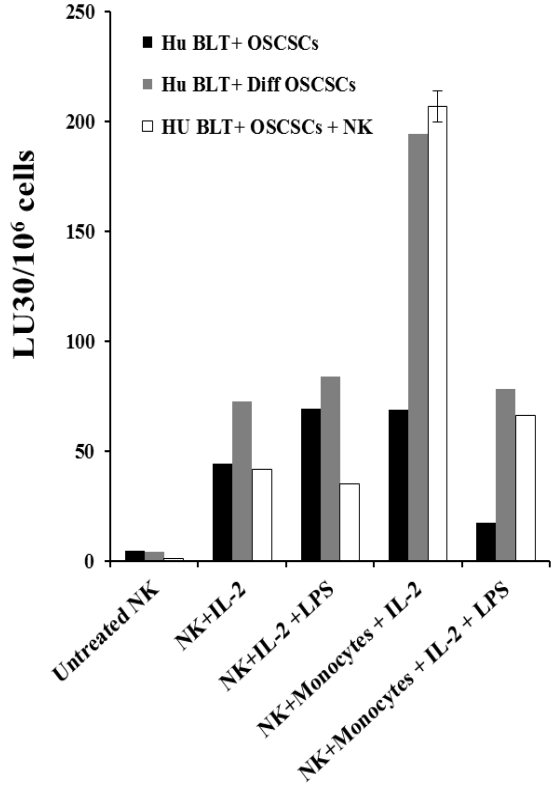

Hu-BLT
Hu-BLT+OSCSCs
6
5
4
3
2
1
0
pg/ml
IFN-γ
IL-10
IL-6

Figure 3: Spleen NK cells depleted of T cells from tumor bearing humanized BLT mice cultured with OCs expand small fraction of contaminating T cells faster than NK cells obtained from healthy hu-BLT mice cultured with OCs.

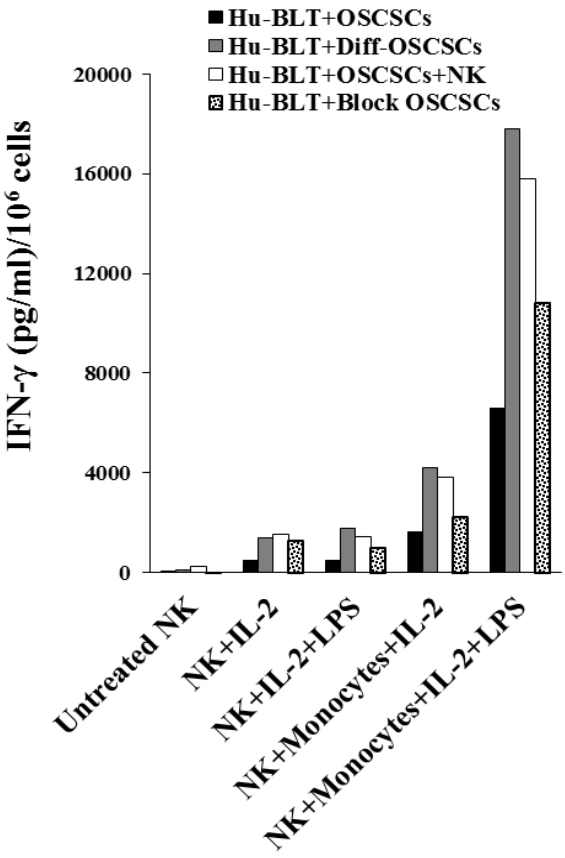
Reconstituted BLT mice were orthotopically injected with $1x10^6$ of human OSCSCs into the floor of the mouth. Disease progression and weight loss was monitored for another 4-5 weeks. Mice were sacrificed, the spleens were harvested, and single cell suspensions were obtained as described in supplementary materials and methods. CD3+ T cells were sorted out using positive selection kit and the flow through cells were analyzed for surface expression of human CD3/CD16/CD56 after staining with the respective antibodies. Isotype control antibodies were used as a control **(A)**. CD3-negative cells ($1x10^6$ cells/ml) from hu-BLT mice were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were cultured with OCs in the presence of sAJ2 at 1:2:4 ratios (OC:NK:sAJ2). Surface expression of CD3, CD16, and CD56 was analyzed on days 6, 10, 14, 18, and 22 using flow cytometric analysis **(B).** After 6, 10, 18, and 22 days of co-culture, expanded lymphocytes were manually counted using microscopy **(C).** The numbers of NK cells **(D)** and T/NKT **(E)** cells were determined using the percentages of NK and T/NKT cells within the total expanding cells. Cytotoxicity of NK cells co-cultured for 10 and 18 days was determined using standard 4-hour ^{51}Cr release assay against OSCSCs and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100 **(F).** Lytic units were normalized and adjusted per NK cell lysis against OSCSCs **(G)**. The supernatants were harvested from the co-culture on day 6, 10, and 13, and IFN-γ **(H)**, IL-10 **(I)**, and IL-6 **(J)** secretion was determined using single ELISAs. Peripheral blood was collected post-mortem by cardiac puncture from hu-BLT mice and serum samples were harvested and analyzed for IFN-γ, IL-10, and IL-6 secretion using multiplex arrays **(K).**

Fig. 4

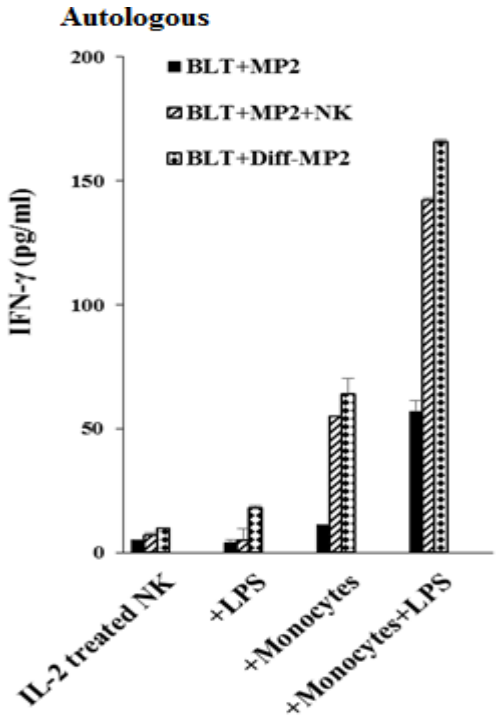
A.


Hu BLT+ OSCSCs
Hu BLT+ Diff OSCSCs
HU BLT+ OSCSCs + NK
LU30/10⁶ cells
250
200
150
100
50
0
Untreated NK
NK+IL-2
NK+IL-2 +LPS
NK+Monocytes + IL-2
NK+Monocytes + IL-2 + LPS

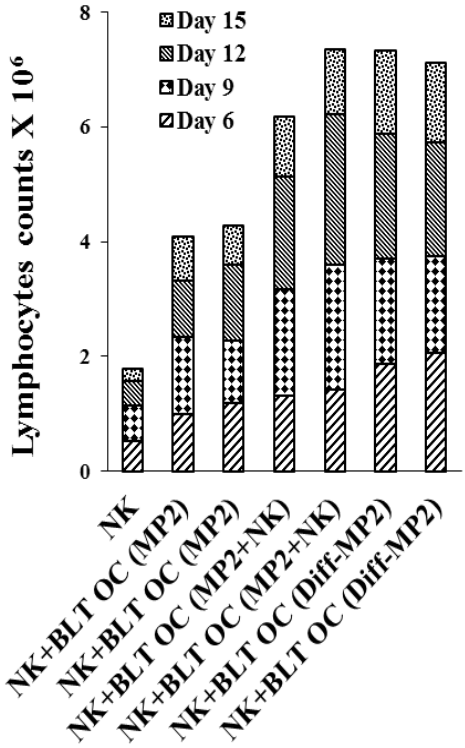
B.


Hu-BLT+OSCSCs
Hu-BLT+Diff-OSCSCs
Hu-BLT+OSCSCs+NK
Hu-BLT+Block OSCSCs
IFN-γ (pg/ml)/10⁶ cells
20000
16000
12000
8000
4000
0
Untreated NK
NK+IL-2
NK+IL-2+LPS
NK+Monocytes+IL-2
NK+Monocytes+IL-2+LPS

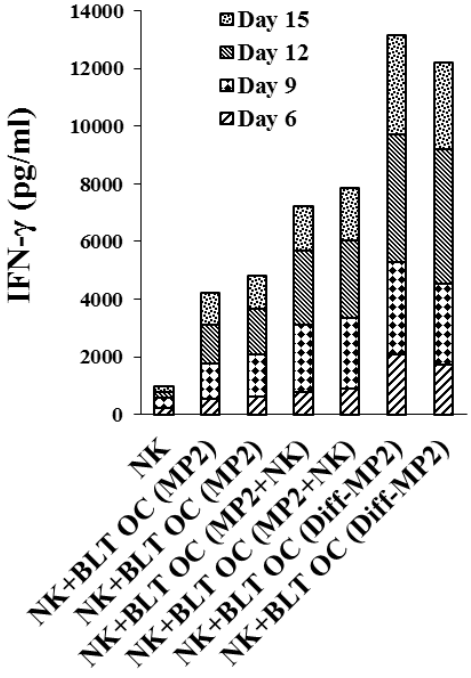
C.


Autologous
BLT+MP2
BLT+MP2+NK
BLT+Diff-MP2
IFN-γ (pg/ml)
200
150
100
50
0
IL-2 treated NK
+LPS
+Monocytes
+Monocytes+LPS

D.


Day 15
Day 12
Day 9
Day 6
Lymphocytes counts X 10⁶
8
6
4
2
0
NK
NK+BLT OC (MP2)
NK+BLT OC (MP2)
NK+BLT OC (MP2+NK)
NK+BLT OC (MP2+NK)
NK+BLT OC (Diff-MP2)
NK+BLT OC (Diff-MP2)

E.


Day 15
Day 12
Day 9
Day 6
IFN-γ (pg/ml)
14000
12000
10000
8000
6000
4000
2000
0
NK
NK+BLT OC (MP2)
NK+BLT OC (MP2)
NK+BLT OC (MP2+NK)
NK+BLT OC (MP2+NK)
NK+BLT OC (Diff-MP2)
NK+BLT OC (Diff-MP2)

F.

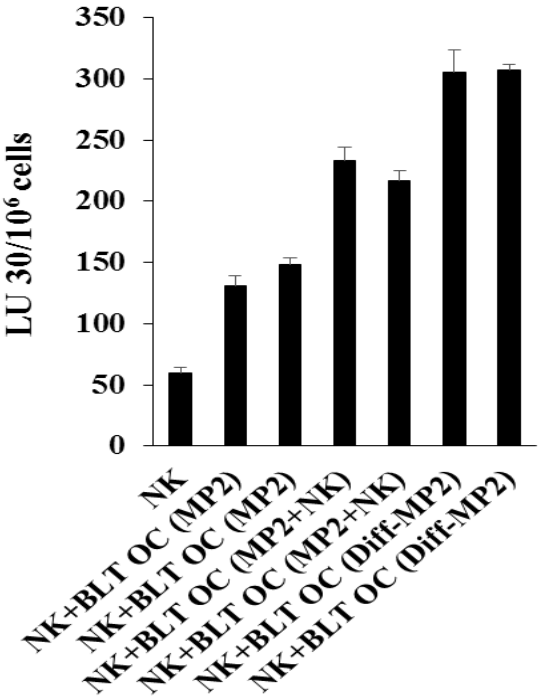

LU 30/10⁶ cells
350
300
250
200
150
100
50
0
NK
NK+BLT OC (MP2)
NK+BLT OC (MP2)
NK+BLT OC (MP2+NK)
NK+BLT OC (MP2+NK)
NK+BLT OC (Diff-MP2)
NK+BLT OC (Diff-MP2)

Figure 4: Monocytes and OC from the hu-BLT mice with tumor also have inhibited function.

Spleen and bone marrow was harvested from human OSCSCs, diff-OSCSCs, OSCSCs in combination with NK, or monoclonal antibody blocked OSCSCs injected hu-BLT mice, splenocytes and bone marrow was processed to obtain single cell suspension as described in Materials and Methods. Human CD56+ NK were positively selected from splenocytes. Human CD14+ monocytes were positively selected from bone marrow. NK and monocytes cultures were treated with IL-2 alone or IL-2 in combination with LPS (100 ng/ml). Day 7 after the co-culture, NK cells were used as effector cells in a standard 4 hour ^{51}Chromium release assay against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the target cells X100 **(A)**. Supernatants were harvested from cultured and IFN-γ secretion was determined using single ELISA **(B)**. Spleen and bone marrow was harvested fromm human MP2, Diff-MP2 and MP2 in combination with NK injected hu-BLT mice, splenocytes and bone marrow was processed to obtain single cell suspension as described in Materials and Methods. Human CD56+ NK were positively selected from splenocytes. Human CD14+ monocytes were positively selected from bone marrow. NK and monocytes cultures were treated with IL-2 alone or IL-2 in combination with LPS (100 ng/ml). Day 7 after the co-culture, supernatants were harvested from cultured and IFN-γ secretion was determined using single ELISA **(C)**. Osteoclasts (OC) were generated from the hu-BLT monocytes as described in Material and Methods. Purified NK cells from healthy human donors were pre-treated with IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then cultured alone or with hu-BLT-OC at effector to target ratio of 2:1 and probiotic bacteria (sAJ2) were added at 2:1 NK cells:bacteria ratio. Number of lymphocytes in the culture were manually counted using microscope **(D)**, the supernatants were harvested from the culture on day 6. day 9, day 12 and day 15, the level of IFN-γ was determined using single ELISA **(E).** Cytotoxicity of NK cells co-cultured on day 15 was determined using standard 4-hour ^{51}Cr release assay against OSCSCs and the lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100**.** Lytic units were normalized and adjusted per NK cell lysis against OSCSCs **(F)**.

Fig. 5

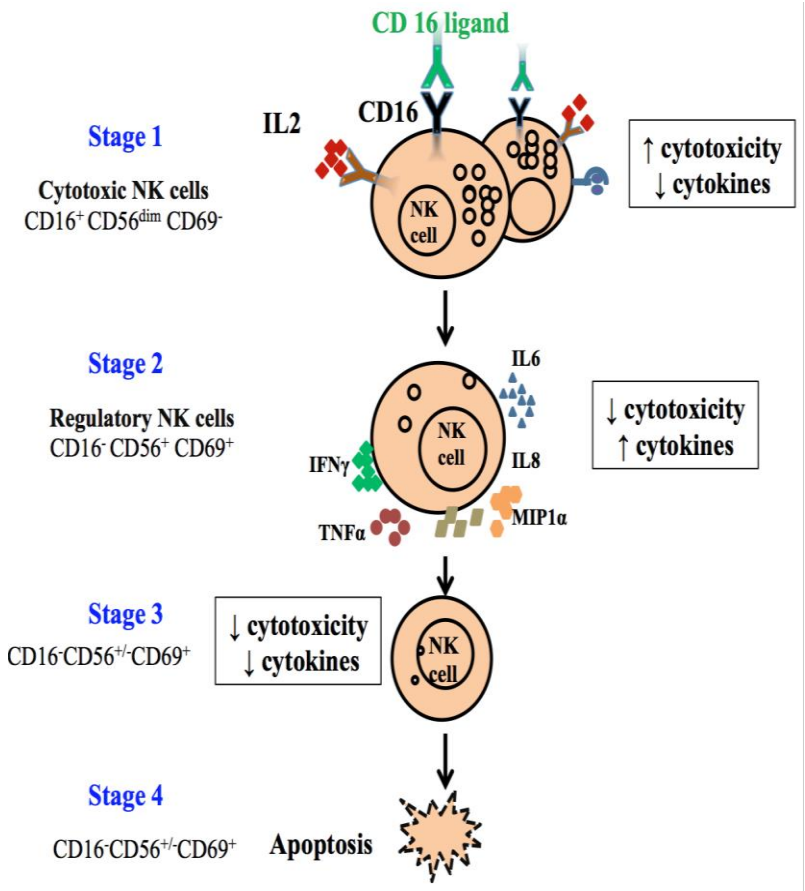


Figure 5: NK cells from cancer patients and tumor bearing hu-BLT mice showed stage 3 feature for NK regulation.

Sub-aim 3: Characterization and functional studies for NK cells in various tissue compartments of WT and KRAS mutated mice.

PanIN lesions with high-fat calorie diet in WT and KRAS^{G12D} mice model.

KRAS mice fed the HFCD for 3 months showed significantly more advanced PanIN-2 and -3 lesions when compared to KRAS mice on the CD (Table 1). Importantly, no invasive PDAC was found in KRAS mice fed either diet at 3 months (Table1). No pancreatic neoplastic lesions were found in WT mice fed either the CD or HFCD. In addition, KRAS mice fed the HFCD had significantly more enhanced pancreatic inflammation (Table 1).

Severe decrease in number of NK cells with high-fat calorie diet and KRAS mutation alone and more so with the combination KRAS mutation and high-fat calorie diet.

Primary tissues from the WT and KRAS animals either on control diet or high fat calorie diet had no or slight change in the number of NK cells (Fig. 2A), but when we cultured the various tissue compartments from the WT and KRAS^{G12D} mice on control diet and high-fat diet with IL-2 activation and tested the cells on day 7 for the NK cells surface marker DX5, NK cell numbers were decreased in all tissue compartments with high-fat and KRAS mutation, and effect was more severe with the combination of high-fat and KRAS mutation (Fig. 2B), the survival rate of NK cells were better in WT mice on control diet (Fig. 2B).

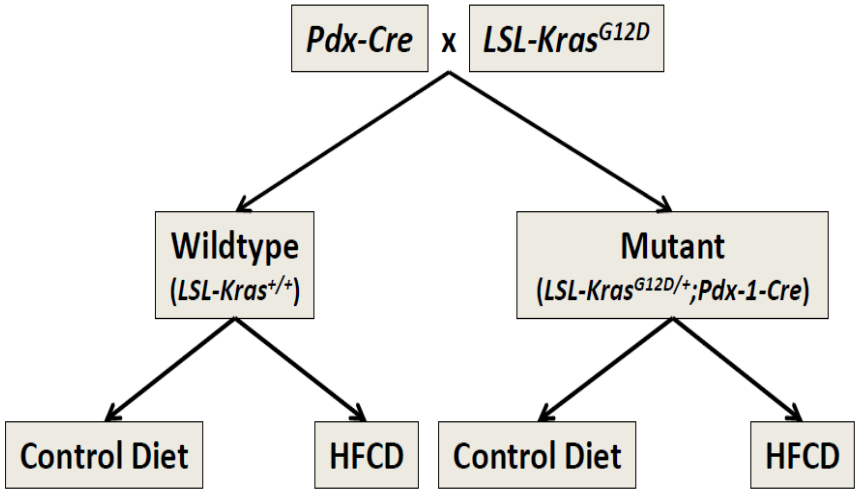
Decreased NK cell mediated cytotoxicity but augmented secretion of IFN-γ by the immune cells cultured from spleen, pancreas, PBMCs and per-pancreatic tissues of WT and

KRAS^{G12D} mice fed with high-fat diet; contrasting results with Bone Marrow derived immune cells

To determine whether similar effects of KRAS mutation and high-fat calorie diet on NK cytotoxicity and secretion of IFN-γ by the immune cells is observed in different tissue compartments, we determined both functions by the dissociated cells prepared from spleen, pancreas, peri-pancreatic fat, peripheral blood, gingiva, bone marrow, and compared those with the tissues obtained WT animals on control diet. Single cell preparations from spleen, pancreas, peri-pancreatic fat, PBMCs recovered from peripheral blood, bone marrow and oral gingiva cells were obtained and left untreated or treated with IL-2. On day 7 after the cultures, cells were used to determine NK cell cytotoxicity, and the supernatants were harvested and assayed for the released IFN-γ. Interestingly, in the most assessments for spleen, pancreas, peripancreatic fat and PBMCs the following pattern of cytotoxicity could be obtained from high to low for WT and KRAS^{G12D} mice fed with either control or high-fat diet (WT/CD>WT/HFCD> KRAS^{G12D}/CD> KRAS^{G12D}/HFCD) (Fig. 3A). The complete opposite was obtained for the secretion of cytokines IFN-γ from low to high as follows (WT/CD<WT/HFCD< KRAS^{G12D}/CD< KRAS^{G12D}/HFCD) (Fig. 3B). A similar pattern was seen in oral gingiva isolated cells (Fig. 3C). In contrast, bone marrow exhibited the opposite of spleen, pancreas and adipose in which increased cytotoxicity from low to high was observed as follows (WT/CD<WT/HFCD< KRAS^{G12D}/CD< KRAS^{G12D}/HFCD) (Fig. 3D), whereas secretion of IFN-g was the complete opposite as follows (WT/CD>WT/HFCD>KRAS^{G12D}/CD> KRAS^{G12D}/HFCD) (Fig. 3E).

Fig. 1

A.



B.

	AIN-76A			HFCD		
	kcal/gm	grams/kg	kcal/kg	kcal/gm	grams/kg	kcal/kg
Casein	3.58	200	716	3.58	200	716
Sucrose	4	380.8	1,523.2	4	380.8	1,523.2
Cornstarch	3.6	269.2	969.12	3.6	119.2	429.12
Cellulose	0	50	0	0	50	0
Corn Oil	9	50	450	9	200	1,800
DL-Methionine	4	3	12	4	3	12
Salt Mix	0.47	35	16.45	0.47	35	16.45
Vitamin Mix	3.92	10	39.2	3.92	10	39.2
Choline Bitartrate	0	2	0	0	2	0
		1000	**3,725.97**		**1000**	**4,535.97**

Figure 1. Conditional KRASG12D mouse model.

To study the effect of diet-induced obesity on NK cell function during pancreatic cancer development, the conditional KRASG12D model from Hingorani and colleagues was used [26, 27]. Offspring of LSL-KRASG12D and PDX-1-Cre mice were fed either a HFCD or a control diet (CD) for 3 months. Before randomization to the diets, the presence of the KRASG12D and Cre alleles were determined by PCR analysis of genomic DNA, as described previously [27]. Animals with both the KRASG12D and Cre allele were designated as mutant, and animals with neither the KRASG12D nor the Cre allele were deemed wildtype. At sacrifice, the successful recombination events were confirmed by PCR by the presence of a single LoxP site in the pancreas as described previously [27]. Individually tagged mice had free access to diet and water. Food intake and body weight of each animal were measured weekly. After 3 months, animals were euthanized, and the pancreas and other organs were harvested. Animal studies were approved by the Chancellor's Animal Research Committee of the University of California, Los Angeles in accordance with the NIH Guide for the Care and Use of Laboratory Animals **(A)**. The diets were obtained from Dyets, Inc. A modified AIN-76A purified rodent diet served as a control diet (CD). Compared with the CD, the high-fat, high calorie diet (HFCD) had increased caloric content (4,536 KRASal/kg vs. 3,726 KRASal/kg), resulting from an increase in corn oil-based fat content (1,800 KRASal/kg vs. 450 KRASal/kg). Approximately 12% of the total calories in the CD come from fat, while about 40% of total caloric intake in the HFCD stems from fat. Details about the diets was mentioned earlier [27]. The diets were handled under low-light conditions, and stored at -20°C. The diets were replaced twice weekly **(B)**.

Table 1

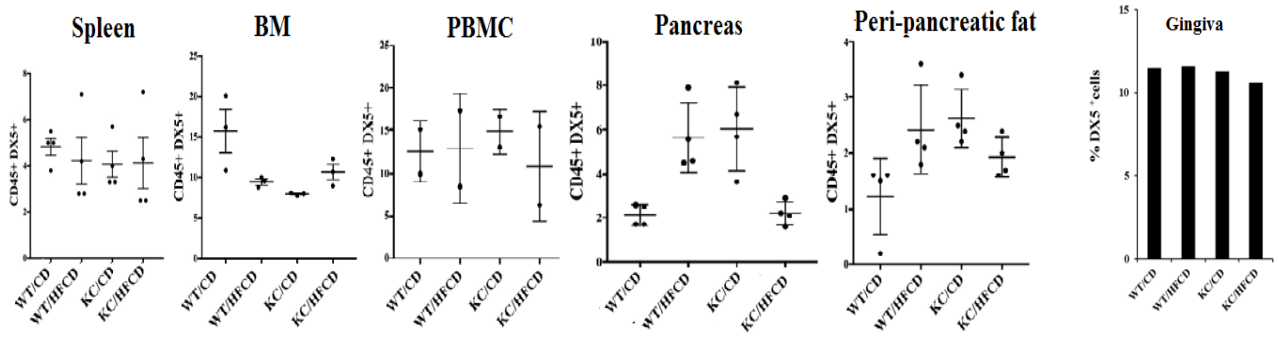
	PanIN1a	PanIN1b	PanIN2	PanIN3	ND	Total	Acinar Cell Loss	Inflammation	Fibrosis	Pancreatitis Score
WT/CD	0	0	0	0	100	100	0	0	0	0
WT/HF	0	0	0	0	100	100	0	1	0	1
KC/CD	15	8	2	0	75	100	1	1	1	3
KC/HF	30	20	6	4	40	100	2	2	1	5

Table 1: Histological quantification of pancreas.

Formalin-fixed, paraffin-embedded tissues were sectioned (4 mm) and stained with H&E. Six to eight sections (100 µm apart) of pancreatic tissues were histologically evaluated by a gastrointestinal pathologist blinded to the experimental groups. Murine PanIN lesions (mPanIN) were classified according to histopathologic criteria as recommended elsewhere [28]. To quantify PanIN lesions, the total number of ductal lesions and their grade were determined. About 100 pancreatic ducts were analyzed for each animal. The relative proportion of each mPanIN lesion to the overall number of analyzed ducts was recorded for each animal. For the assessment of pancreatic inflammation, full histologic cross-sections of each pancreas were graded using a semi-quantitative scoring system, as previously described [27]. Pancreatic inflammation was given an index score (0–12) reflecting the sum of scores for acinar loss, lobular inflammation, and fibrosis. Histologic quantification of PanIN lesions and normal ducts (ND) in wildtype mice (WT) on control diet (CD) or high-fat, high calorie diet (HFCD) in percentage of total ducts analyzed. Pancreatic inflammation was quantified histologically by grading (from 0-4) acinar cell loss, inflammatory cell infiltration, and extent of fibrosis, resulting in a cumulative pancreatic inflammation score (0-12). Details about the histologic grading was described previously [27].

Fig. 2

A.



B.

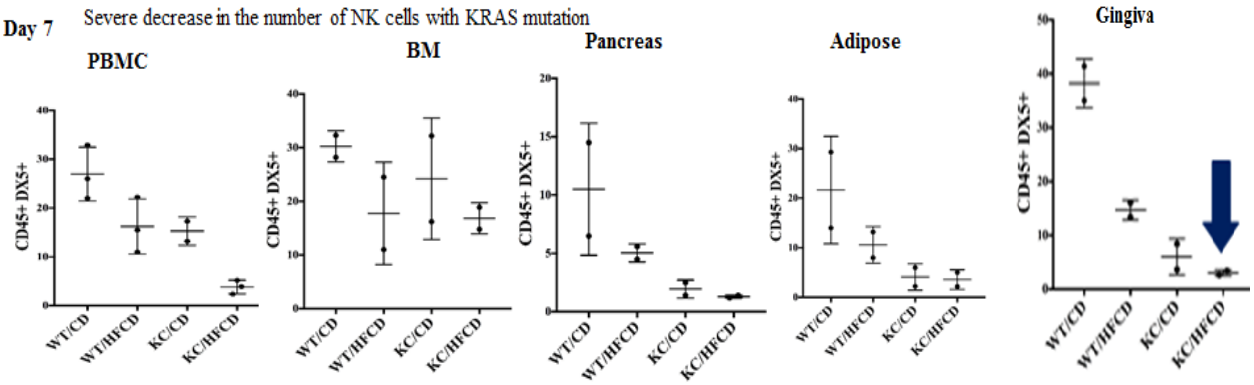
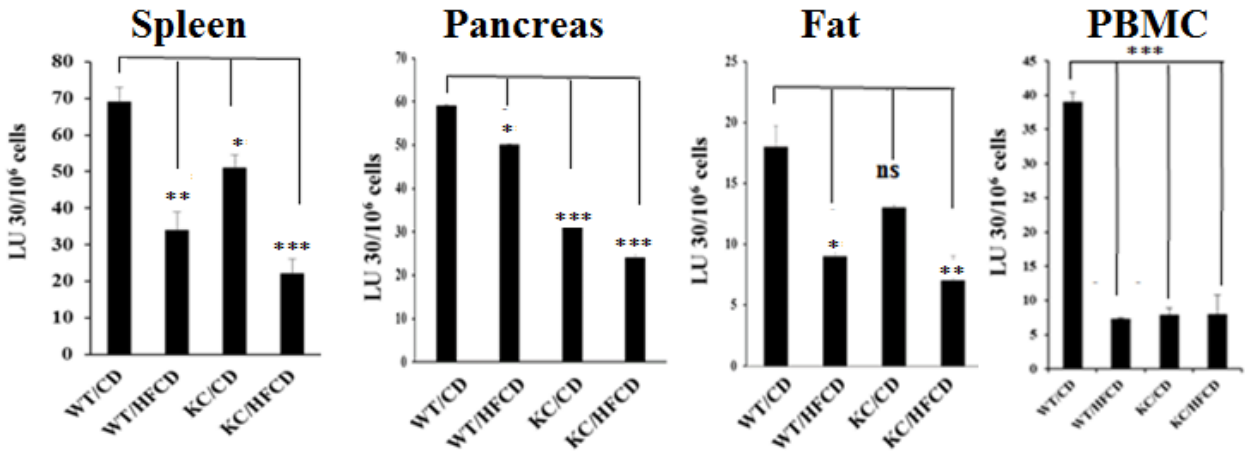


Figure 2: Severe decrease in the number of NK cells with high-fat calorie diet and KRAS mutation alone and more so with the combination KRAS mutation and high-fat calorie diet.

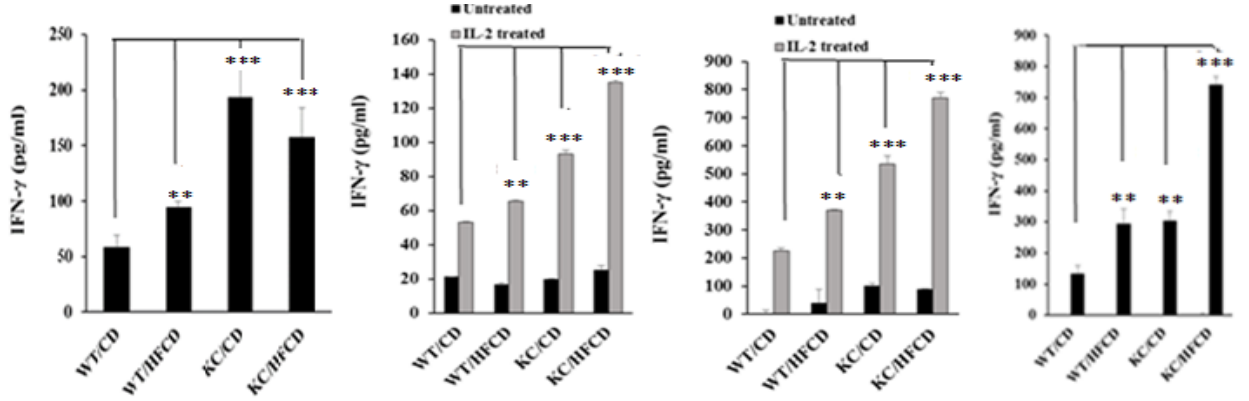
WT and $KRAS^{G12D}$ mice were fed with control diet and high-fat calorie diet as described in Material and Methods. Spleen, pancreas, peri-pancreatic fat, peripheral blood, bone marrow and oral gingiva were harvested after the animals were sacrificed and single cell suspension from each tissue was obtained as described in Material and Methods. Surface expression DX5 in CD45+ immune cells (n=4) were determined using flow cytometric analysis after staining with the respective PE-conjugated and FITC-conjugated antibodies. Isotype control antibodies were used as control **(A).** All the tissues were treated with IL-2 (10,000 U/ml) and were cultured for 7 days, on day 7 surface expression of DX5 was determined with CD45+ immune cells on the pancreas cells surface **(B)**.

Fig. 3

A.


Spleen
Pancreas
Fat
PBMC
LU 30/10⁶ cells
WT/CD
WT/HFCD
KC/CD
KC/HFCD
ns

B.


IFN-γ (pg/ml)
Untreated
IL-2 treated
WT/CD
WT/HFCD
KC/CD
KC/HFCD

C. **D.**

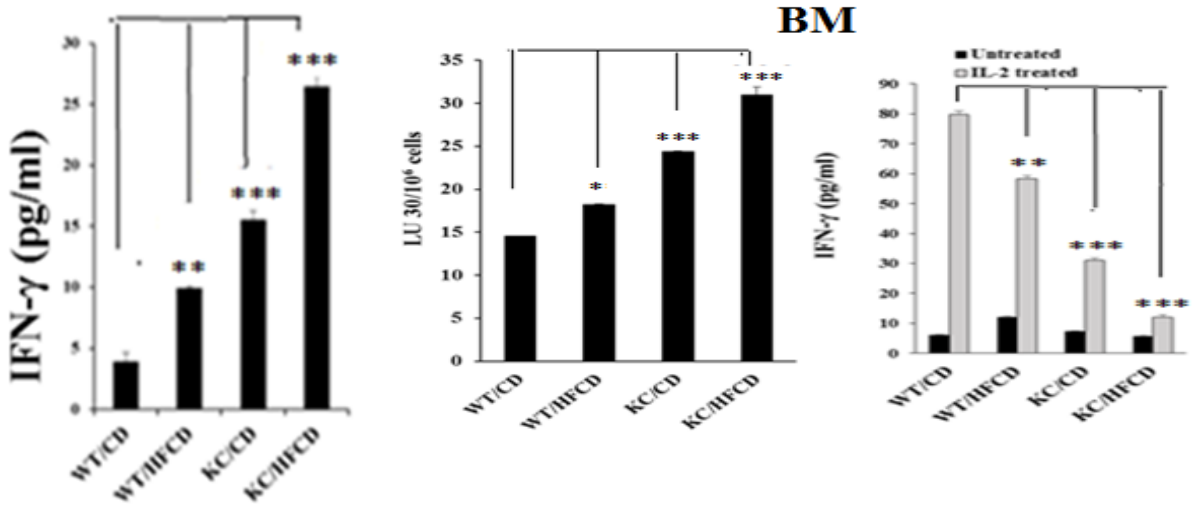


Figure 3: Effect of KRAS mutation and high fat calorie diet on various tissue compartments of mice.

WT and $KRAS^{G12D}$ model were fed with control diet and high-fat calorie diet as described in Material and Methods. Spleen, pancreas, peri-pancreatic fat, peripheral blood, bone marrow and oral gingival tissue were harvested from these animals after sacrifice, and were digested to obtain single cell suspensions as described in Materials and Method. Spleen, pancreas, peri-pancreatic fat and PBMCs, were cultured in the presence of IL-2 (10,000 U/ml). 7 days after the culture cells were used as effectors against ^{51}Cr labeled ST63 cells at various effector to target ratios in a standard 4 hour ^{51}Cr release assay. The lytic units $30/10^6$ cells were determined using an inverse number of effector cells required to lyse 30% of the ST63 cells X100 **(A), s**upernatants were harvested and the levels of IFN-γ **(B)** was measured with specific ELISA. Cells isolated form oral gingiva tissue were cultured in the presence of IL-2 (10,000 U/ml). 7 days after the culture, **s**upernatants were harvested and the levels of IFN-γ **(C)** was measured with specific ELISA. Bone marrow flow-through cells were cultured in the presence of IL-2 (10,000 U/ml). 7 days after the culture cells were used as effectors against ^{51}Cr labeled ST63 cells at various effector to target ratios in a standard 4 hour ^{51}Cr release assay. The lytic units $30/10^6$ cells were determined using an inverse number of effector cells required to lyse 30% of the ST63 cells X100 **(D), s**upernatants were harvested and the levels of IFN-γ **(D)** was measured with specific ELISA.

Fig. 4

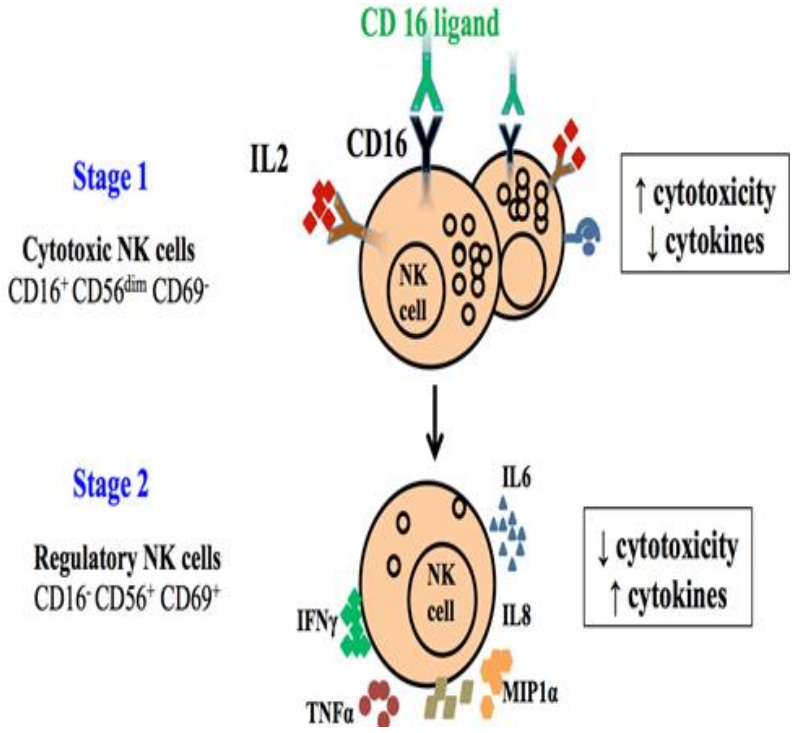


Figure 4: NK cells from bone marrow in KRAS mutated animals have stage 1 feature, while other tissues examined have stage 2 features.

Discussion of chapter 1

Previous studies attributed significant detrimental effects on immune cell function in the presence of cancer, especially NK cells, however, the underlying mechanisms for the low activity of NK cells in cancer patients have not been elucidated. Here we demonstrate that NK surface receptors, and their ligands on cells responsible for expanding and activating them, especially osteoclasts, are down-modulated in cancer patients, resulting in substantial decrease in NK cell-mediated cytotoxic activity and IFN-γ secretion of NK cells. NK cell cytotoxic activity is required to target cancer stem cells, and cytokines, especially IFN-γ, play a very important role in the differentiation of cells and the cessation of inflammation. NK cell-mediated cytotoxic function and IFN-γ secretion are inhibited in cancer patients, a less extreme effect was observed on T cell IFN-gamma secretion. When OCs was used to expand NK cells from cancer patients a very distinct profile was observed. Cancer patients' OCs also expanded T cells early in the culture, with decline in expansion of NK cells at different days. When assessing the function of patient NK cells after OC cultures, a significant loss of NK cell cytotoxicity and a decrease in IFN-γ secretion could be observed per NK cell. This observation is important, since it indicates that faster expansion of the small fraction of T cells in purified NK cultures in cancer patients correlates with the loss of cytotoxic function by NK cells. Whether loss of NK cytotoxicity in cancer patients is responsible for allowing the expansion of T cells, and/or expanding T cells have an inhibitory effect on the expansion of patient NK cells requires further investigation. It is also possible that T cells have, in general, greater survival advantage compared to NK cells and are able to proliferate faster than NK cells. These possibilities are under investigation presently in our laboratory. However, it is likely that the loss of NK cells may also provide the fertile ground for the continued growth and eventual metastasis of cancer stem cells.

A major decrease in NK cell killing activity has been observed by exposure to NK sensitive target cells- K562 and it was associated with the downregulation of CD16 surface receptors [29, 30]. Loss of CD16 was associated with increased production of IFN-γ and up-regulation of CD107a a marker of degranulation [30]. Significant downregulation in CD16 receptor expression and loss of NK cell cytotoxic function were seen in oral and ovarian cancer patients [31, 32] and also when NK cells were co-cultured with ovarian tumors. Triggering of CD16 receptor on NK cells by anti-CD16 antibody, which mimics the ligand binding effect, was also been found to result in down-modulation of CD16 receptors, a great loss of cytotoxicity, and gain in cytokine secretion. For this phenomenon, observed in NK cells upon interaction with target cells, we previously coined the term "split anergy" [33-39]. Besides split anergized cells, a small subpopulation of NK cells can undergo cell death similar to that seen during the interaction of NK cells with sensitive tumors [37, 38].

To test whether OCs obtained from humanized mice implanted with tumors, similar to cancer patients OCs, expand the contaminating fraction of T cells within purified NK cultures faster than their non-tumor bearing counter parts, we implanted OSCSCs in the floor of the mouth. After five weeks, mice were euthanized and splenic T cells were depleted before the remaining cells were cultured with autologous and allogeneic OCs, and the rates of NK cell expansion was determined. Similar, to healthy donor NK cells, NK cells from hu-BLT mice without tumor expanded NK cells for a longer period of time, whereas those from tumor bearing mice expanded the small fraction of contaminating T cells within the NK cultures faster, favoring the expansion of T cells. Interestingly, similar to the loss of NK cell cytotoxicity observed in cancer patients, we also observed significant loss of NK cell cytotoxicity in hu-BLT mice implanted with tumors, which as indicated above may be the underlying mechanism for the expansion of T cells. However,

OC-expanded NK cells from the tumor bearing mice secreted higher levels of IFN-γ when compared to those without tumors, suggesting the potential induction of split anergy in NK cells to drive differentiation of cancer stem cells. This was found to be the case since single cell preparation of tumors in NK-injected tumor bearing hu-BLT mice demonstrated higher differentiation antigens, including MHC class I and were resistant to NK cell-mediated cytotoxicity (data not shown).

Rapid expansion of T cells and decreased NK cell numbers in peripheral blood/tissues of cancer patients and the humanized mice could be detrimental for targeting MHC class I low targets including cancer stem cells/undifferentiated tumors by NK cells in order to minimize the tumor load. In addition, NK cells also provide large amounts of IFN-γ to promote optimal differentiation of cancer stem cells to increase MHC class I expression, thereby paving the road for targeting of NK differentiated tumors by CD8+ T cells. When the same amount of IFN-γ secreted from healthy and cancer patients was used to treat the OSCSCs, cancer patient's IFN-γ was less capable at inducing differentiation of OSCSCs. This data provides evidence that not just the secretion of IFN-γ, but also the potential of that secreted IFN-γ to differentiate CSCs is not working in the cancer microenvironment, which could be one of very important reasons for cancer growth in cancer patients.

Although it is known that NK cells are functionally inactivated in many cancer patients, it has yet to be shown whether inactivation of NK cell function is the consequence of establishment of cancer or precedes the establishment of cancer, during the pre-neoplastic stage, in which case their functional loss may be responsible for the establishment of cancer. In this study, we demonstrated that the loss of NK cell function occurs at the pre-neoplastic stage and is correlated with the increased levels and the intensity of pre-neoplastic lesions, increased inflammation,

fibrosis, pancreatitis score and loss of normal ducts. In addition, we have also shown that both environmental and genetic factors contribute to the loss of NK cell function, since their synergistic effect is responsible for the greater loss of NK cell cytotoxic function and augmented inflammatory cytokines and chemokines. Indeed, almost equal degrees of the loss of NK cell cytotoxicity and augmented secretion of cytokines can be seen by WT mice fed with HFCD and KRAS mice fed with CD in a number of experiments indicating equal contribution of factors to the loss of NK cell function.

Furthermore, receptors and ligands required for NK cells function and expansion were found to be down-modulated in cancer patients and in pre-neoplastic stage, providing evidence that this is a complex mechanism and multiple factors are involved in either affecting the NK cell activity or tumor progression in cancer patients. We also tested the effect of high fat, and it synergized with the KRAS mutation in the pancreas in significantly impairing NK cell number and function. Both NK and NK-activating immune cells, notably osteoclasts and monocytes, within the microenvironment are defective in expanding NK cells and activating their function, NK cells are impaired at or before the establishment of pre-neoplastic stage. Defect in NK cell function may result in the loss of tumor differentiation, increased survival of cancer stem cells, and progression from the pre-neoplastic to neoplastic stage, leading to invasion and metastasis of tumors. Therefore, NK cells are prime candidates for use in designing an immunotherapeutic strategies.

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CHAPTER 2

To investigate the underlying mechanism for the loss of NK function at the pre-neoplastic and neoplastic stages; reversal of NK cell function using osteoclasts.

Abstract

Natural killer (NK) cells are known to target cancer stem cells and undifferentiated tumors. In this study, we provide a novel strategy for expanding large numbers of super-charged NK cells with significant potential to lyse and differentiate cancer stem cells, and demonstrate the differences in the dynamics of NK cell expansion between healthy and cancer patients. Expansion and functional activation of super-charged NK cells by osteoclasts is dependent on NK receptors, their ligands on other immune cells, and secreted cytokines such as IL-12 and IL-15. NK cells from cancer patients have decreased surface receptor expression, especially NKG2D and CD16, and osteoclasts from cancer patients have reduced expression of NK receptor ligands ULBPs and MICA/B. Thus, in this report we not only provide a novel strategy to expand super-charged NK cells, but also demonstrate that rapid and sustained expansion of residual CD8+T cells within the purified NK cells during expansion with osteoclasts could be a potential mechanism by which the numbers and function of NK cells decline in cancer patients and in BLT humanized mice. Thus, restoration of NK cell numbers and function in cancer patients will be important to establish effective tumor control.

Introduction

Natural killer (NK) cells lyse and differentiate cancer stem cells/undifferentiated tumors with lower expression of MHC class I, CD54 and B7H1 and higher expression of CD44 [1, 2]. Medium and high cytotoxic activity of peripheral-blood lymphocytes are associated with reduced cancer risk, and high NK-cell infiltration of the tumor is associated with a better prognosis [3, 4], whereas low activity is associated with increased cancer risk [5]. Suppression of NK cells is mediated by downregulation of NK receptors in the tumor microenvironment [6-13]. Function of NK cells was shown previously to be significantly reduced in tumor patients [5, 6, 8-12, 14-16]. Effector function of NK cells includes direct natural cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), as well as secretion of inflammatory cytokines and chemokines that indirectly regulate the functions of other immune cells [17, 18]. Antibodies are linked to immune effector functions by the Fc fragment, which is capable of initiating ADCC when binding to Fc receptors, specifically FcγRIII (CD16) on NK cells, which initiates a sequence of cellular events culminating in the release of cytotoxic granzyme-containing granules and IFN-γ secretion [19]. NK cell effector functions can be exploited for the treatment of some tumors through their ability to mediate ADCC [20]. Tumor-associated NK cells are refractory to CD16 receptor stimulation, resulting in diminished ADCC against autologous tumor cells [21]

Several *in vitro* NK expansion techniques have been developed to allow for a higher therapeutic cell dose [22-30]. The stimulation of peripheral blood mononuclear cells (PBMCs) or a purified population of NK cells with feeder cells such as K562 cells expressing interleukin (IL)-15 and 41BB ligand, EBV-TM-LCL, Wilm's tumor or irradiated PBMCs have resulted in greater numbers of NK cells with adequate function [28, 31, 32]. The generated NK cells expressed higher levels of NKG2D, natural cytotoxicity receptors, DNAM-1, and ICAM-1 [30]. Thus, various

methods to obtain *ex vivo*-expanded, activated, and $CD3^+$ T cell-depleted NK cells have been established for clinical use [33]. In addition, Miller and colleagues established the safety and efficacy of adoptive cellular transfer of HLA-haploidentical NK cells in patients with advanced cancer [34]. Additionally, clinical trials have shown that allogeneic NK cells play a therapeutic role in solid tumors, and are safe for transfer into patients [35-37].

Immunotherapy with NK cell has been limited due to inability to obtain sufficient numbers of highly functional NK cells. In this study, we provide a novel strategy to expand highly functional NK cells using osteoclasts as feeder cells, at levels which are significantly potent. In addition, expansion of patient NK cells unlike NK cells from healthy individuals is significantly limited due to the expansion of a small fraction of contaminating T cells which could potentially crowd out or inhibit NK cell expansion by their faster proliferating capability. This trend was also observed in NK cells from tumor-bearing humanized mice.

Results

Sub-aim 1: Significance of reduced surface expression of NKG2D receptor ligand, MICA/B on cancer patient and KRAS-mutated mice osteoclasts, and low level of ADCC by cancer patient NK cells.

Important ligands and cytokines involved in NK cells activation, osteoclasts as a potential candidate to activate NK cells.

Freshly isolated monocytes were compared with mature DCs and OCs for expression of key surface receptors. CD54 was upregulated on DCs and OCs, whereas MHC-I was decreased on DCs and OCs, when compared to monocytes (Table 1). NK receptor ligands, ULBPs and MICA/B surface expression were higher on the surface of osteoclasts when compared to OSCSCs and K562 (Table 1). Killer cell immunoglobulin-like receptors *(*KIR*)*, KLRG1, and MICA/B were higher on OCs, intermediate on monocytes, and very low on DCs (Fig. 1A and Table 1). ULBP1-6 was high on monocytes, intermediate on OCs and low on DCs (Fig. 1A). When we cultured the NK cells with OSCSCs, K562, monocytes, DCs or OCs, and compared the cytokine secretions and cytotoxic function of NK cells from these cultures, pattern was like this OCs>DCs>monocytes/OSCSCs/K562 (Table 1). NK cell receptors including CD94 and NKG2D were higher on OC-expanded NK cells (Fig. 1B) as compared to untreated primary NK cells (Fig. 1B). KIR2 and KIR3 expression were intermediate on expanded NK cells (Fig. 1B).

ADCC is low from the cancer patient's NK cells when compared to healthy donor's NK cells.

Next, we analyzed the NK target cells, cancer stem cells and their differentiated counterparts for the presence of MICA/B on their surface. We wanted to block the MICA/B expression on the surface of target cells to reduce the NK cells mediated cytotoxicity, through the mechanism of interrupting the interaction of NK cells through NKG2D surface receptor and their ligands on the target cells. We selected OSCSCs, MP2, differentiated OSCSCs and MP2 using the supernatants from the NK+IL-2+anti-CD16mAb treated NK cells as described in Materials and Methods. We also analyzed OSCC and PL12, established differentiated cell lines. OSCSCs and MP2 differentiated with NK supernatants, OSCC, and PL12 displayed higher MICA/B marker expression on their surface when compared to their stem-like counterparts, OSCSCs (Fig. 2A) and MP2 (Fig. 2E).

As shown previously [2, 38, 39], NK cells-mediated much higher cytotoxicity against oral squamous carcinoma stem-like cells (OSCSCs) when compared to their differentiated counterpart, oral squamous carcinoma cells (OSCCs), and differentiated cells have higher surface expression of MICA/B. When we treated the NK and target cells culture with anti-MICA/B during the cytotoxicity activity, NK cell-mediated cytotoxicity by healthy NK cells and cancer patients NK cells was not significantly different against OSCSCs and MP2 (Fig. 2B and 2F). On the other hand, when NK and target culture treated with anti-MICA/B where we had diff-OSCSCs (Fig. 2C), diff-MP2 (Fig. 2G), OSCCs (Fig. 2D) and PL12 (Fig. 2H), NK cell-mediated cytotoxicity increased with anti-MICA/B through ADCC with healthy donor NK cells, but less or no effect was seen with cancer patient NK cells (Figs. 2C, 2D, 2G and 2H).

We also down-modulated the CD16 surface expression on NK cells from healthy donor and cancer patients, freshly isolated NK cells were left untreated, treated with IL-2 and treated with

IL-2+antiCD16mAb. As mentioned earlier NK cells when are activated with IL-2, they mediate more cytotoxicity against the stem-like cells, whereas the treatment with IL-2+anti-CD16mAb block the cytotoxicity activity of NK cells, but secrete more IFN-γ. So, when NK cells were used for cytotoxicity assay, IL-2 treated NK cells from healthy donor, the increase in cytotoxicity activity was 7.2 fold, whereas in NK cells from cancer patients fold increase was 4 fold (Fig. 2I), similar effect was seen for IFN-γ secretion (Fig. 2J).

Decreased surface expression of NK receptors ligand, MICA/B and RAE-1 delta on the surface of osteoclasts from cancer patients and KRAS mutated mice respectively.

Now we know that NK cells down-modulate the surface expression of two very important surface receptors, NKG2D and CD16, through their interaction with other immune cells such as osteoclasts and ADCC respectively. Next question was if cancer or pre-neoplastic lesions have any effect on the NK receptors ligands also, and answer was yes, they do. Osteoclasts from cancer patients displayed lower marker expression of MHC1, CD54, KIR2, KIR3, KLRG1, and MICA/B, reduced expression of MICA/B on the surface of cancer patient OCs (Fig. 3A), indicate that both NK receptors and their ligands on the osteoclasts are affected by cancer microenvironment, providing the mechanism for why cancer patient NK cells are less capable of expansion and survival. Similar results were seen on the osteoclasts from KRAS mutated mice. MHC1 and RAE1-delta, mouse NKG2D ligand, were decreased on the surface of osteoclasts from KRAS mutated mice (Fig. 3B).

Table 1

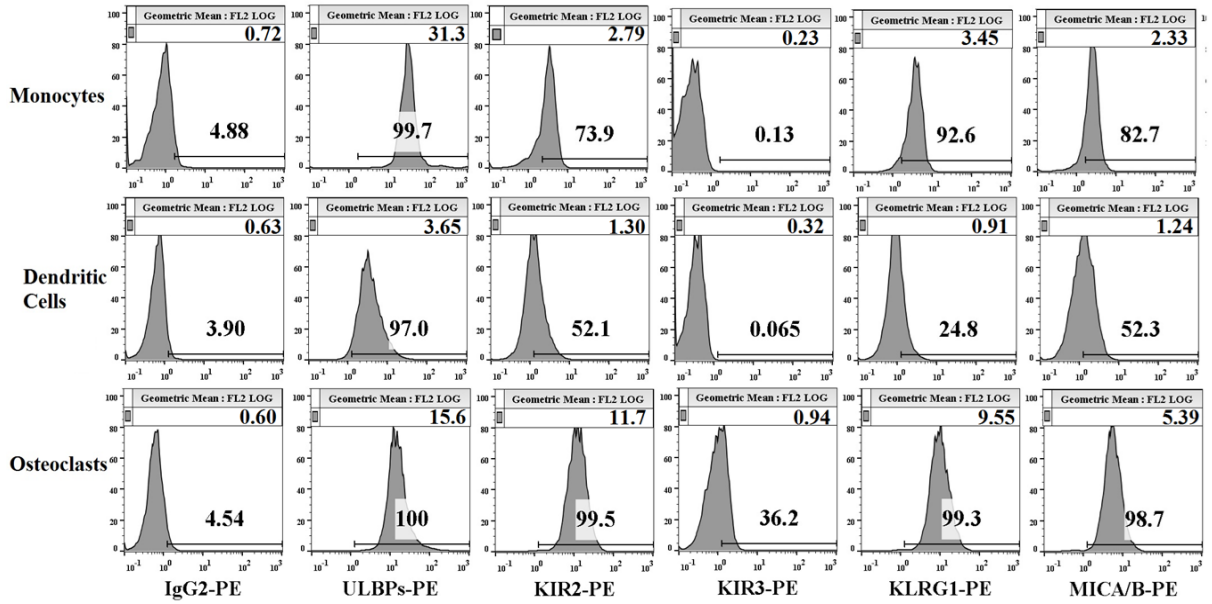
	OSCSCs	K562	Monocytes	Dendritic cells	Osteoclasts
ULBPs	NA	NA	32 ± 2.1	3.7 ± 1.8	18.2 ± 3.4
KLRG1	0.6 ± 0.45	0.7 ± 2	5.3 ± 3.6	0.9 ± 1.1	12 ± 1.7
MICA/B	0.36 ± 1.9	1.5 ± 1	3.5 ± 0.98	0.8 ± 1.36	6.8 ± 2.9
KIR2	1.9 ± 0.87	0.7 ± 0.3	5.3 ± 2.6	0.9 ± 0.4	10 ± 3.2
KIR3	0.91 ± 0.46	0.6 ± 0.7	0.23 ± 0.89	1 ± 0.6	0.8 ± 1.5
CD54	5 ± 1.9	15.2 ± 3	7.39 ± 1.8	30 ± 4.3	34 ± 7.6
MHC1	2.34 ± 1.3	0.5 ± 0.7	36 ± 9	23 ± 5.8	12 ± 3.8
B7H1	0.78 ± 0.4	1.02 ± 0.5	2.8 ± 1.6	22 ± 7.2	1.6 ± 1.2
IFN-g	+	+	+	++	++++
IL-6	+	+	+	++	+++
IL-10	+	+	+	++	+++
Effect of NKs cytotoxic function	+	+	+	++	++++

Table 1: Higher expression of NK activating ligands and cytokines required for NK cells activation by Osteoclasts

Monocytes were isolated, dendritic cells and osteoclasts were generated as described in Materials and Methods, OSCSCs and K562 tumor cell lines were cultured as described in Materials and Methods, $1x10^4$ cells were used to analyze ULBPs, KLRG1, MICA/B, CD54, MHC1, B7H1, KIR2 and KIR3 surface expressions, employing PE-conjugated antibodies and flow cytometry. IgG2 isotype was used as a control. NK cells were activated with IL-2+anti-CD16mAb and cultured with OSCSCs, K562, monocytes, dendritic cells and osteoclasts respectively, 24 hours after the cultures, the supernatant were harvested and single ELISAs for IFN-γ, IL-6, and IL-10 was used to determine the secretion level, and NK cells were used to assess the cytotoxic activity against OSCSCs.

Fig. 1

A.


Monocytes
Dendritic Cells
Osteoclasts
Geometric Mean : FL2 LOG
0.72
4.88
31.3
99.7
2.79
73.9
0.23
0.13
3.45
92.6
2.33
82.7
0.63
3.90
3.65
97.0
1.30
52.1
0.32
0.065
0.91
24.8
1.24
52.3
0.60
4.54
15.6
100
11.7
99.5
0.94
36.2
9.55
99.3
5.39
98.7
IgG2-PE
ULBPs-PE
KIR2-PE
KIR3-PE
KLRG1-PE
MICA/B-PE

B.

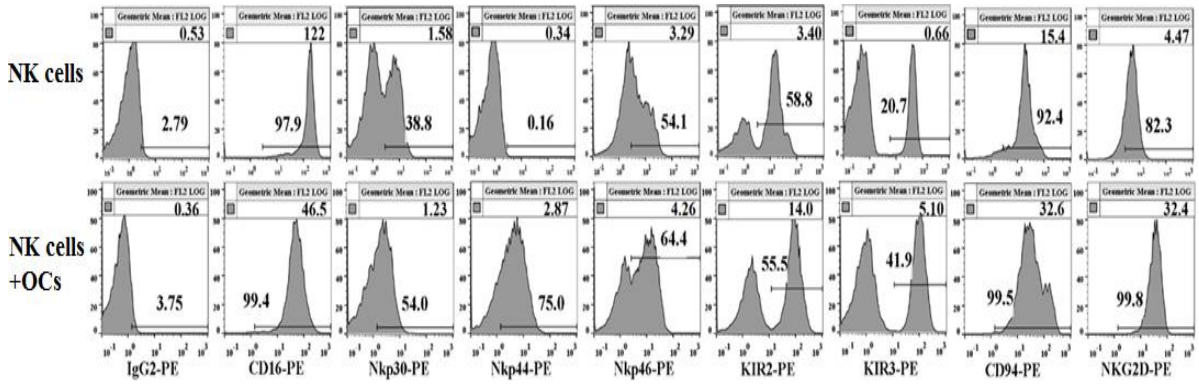
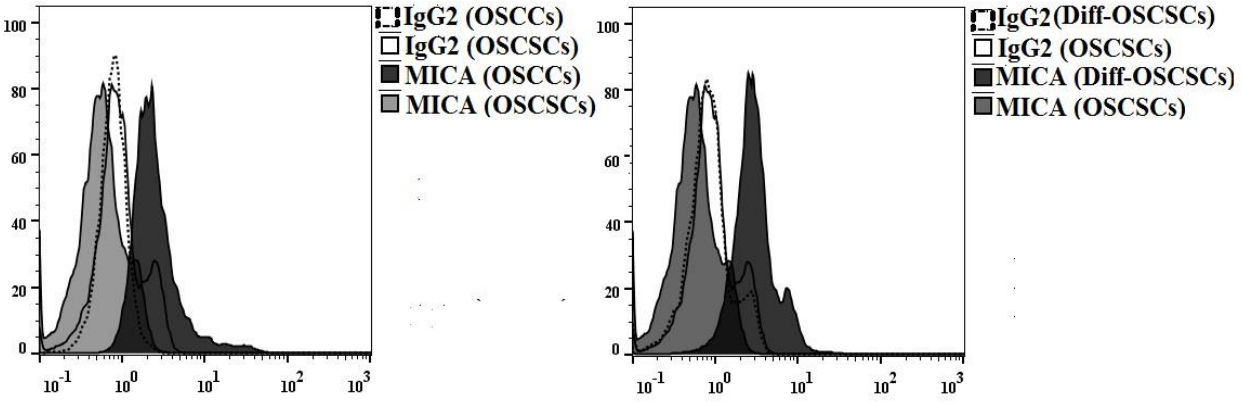

NK cells
NK cells +OCs
0.53
2.79
122
97.9
1.58
38.8
0.34
0.16
3.29
54.1
3.40
58.8
0.66
20.7
15.4
92.4
4.47
82.3
0.36
3.75
46.5
99.4
1.23
54.0
2.87
75.0
4.26
64.4
14.0
55.5
5.10
41.9
32.6
99.5
32.4
99.8
IgG2-PE
CD16-PE
Nkp30-PE
Nkp44-PE
Nkp46-PE
KIR2-PE
KIR3-PE
CD94-PE
NKG2D-PE

Figure 1: Higher expression of NK activating ligands by Osteoclasts

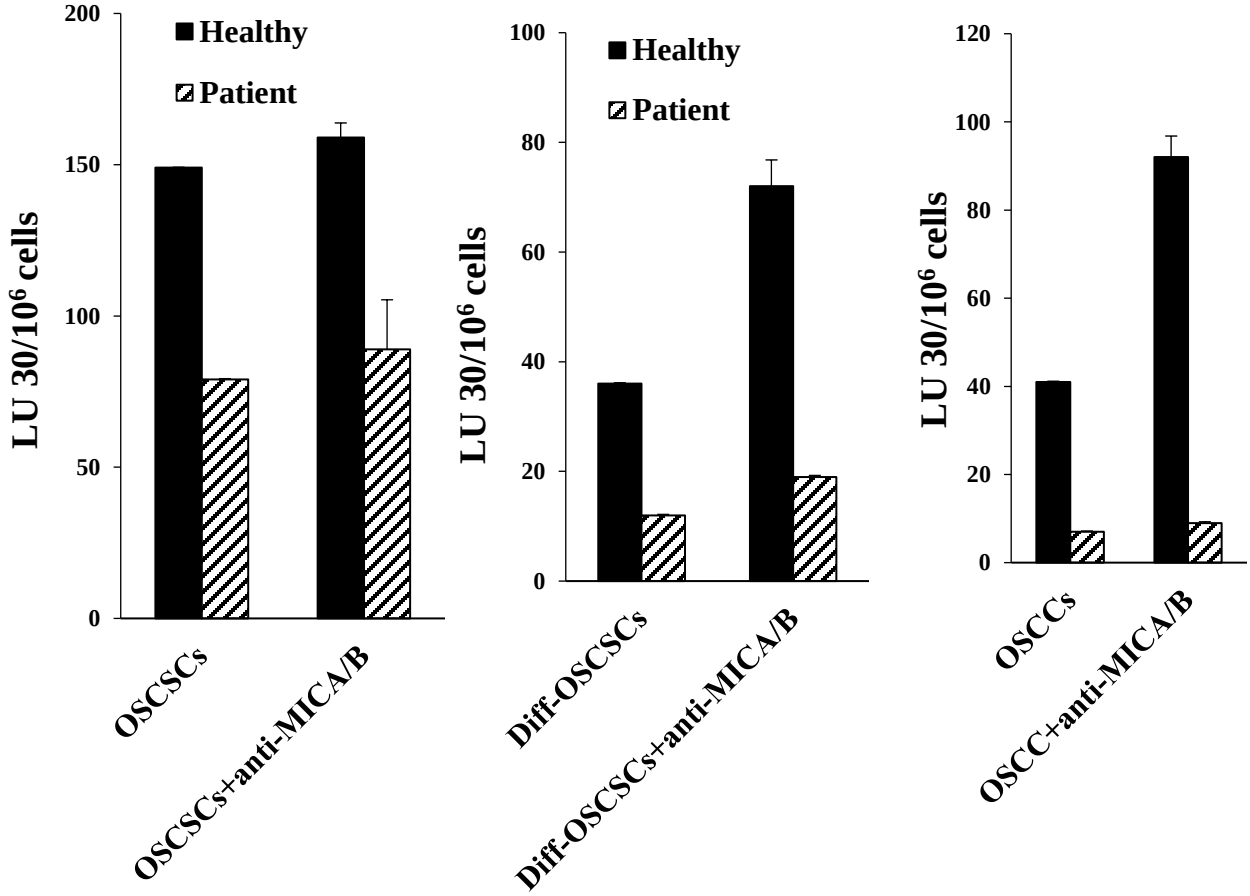
Monocytes were isolated, dendritic cells and osteoclasts were generated as described in Materials and Methods, $1x10^4$ cells were used to analyze ULBPs, KIR2, KIR3, KLRG1 and MICA/B surface expressions, employing PE-conjugated antibodies and flow cytometry. IgG2 isotype was used as a control **(A)**. Freshly isolated NK cells (upper row) and NK cells co-cultured with autologous osteoclasts and expanded as described in Materials and Methods (lower row) were used to analyze CD16, Nkp30, Nkp44, Nkp46, KIR2, KIR3, CD94, and NKG2D surface expression using, PE-conjugated antibodies. IgG2 isotype control antibody was used as control **(B)**.

Fig. 2

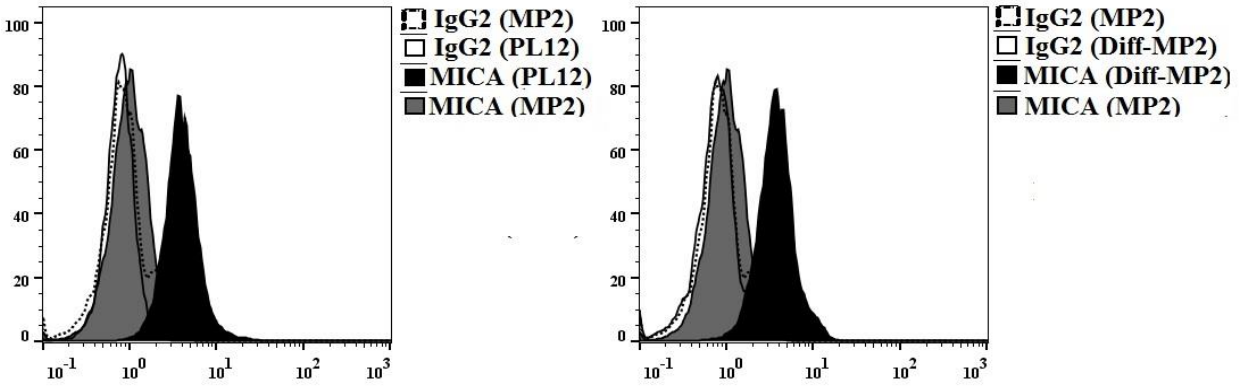
A.


IgG2 (OSCCs)
IgG2 (OSCSCs)
MICA (OSCCs)
MICA (OSCSCs)
IgG2(Diff-OSCSCs)
IgG2 (OSCSCs)
MICA (Diff-OSCSCs)
MICA (OSCSCs)

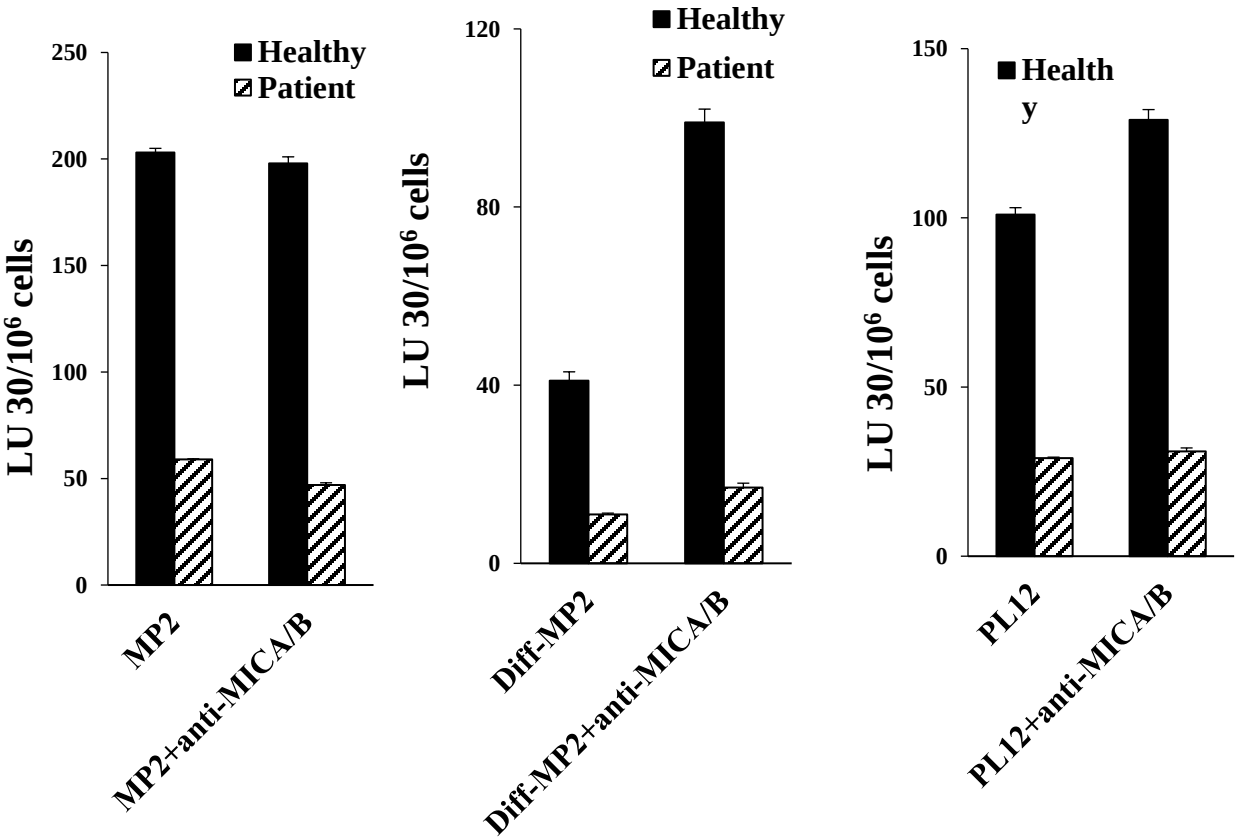
B. **C.** **D.**


Healthy
Patient
LU 30/10^6 cells
OSCSCs
OSCSCs+anti-MICA/B
Diff-OSCSCs
Diff-OSCSCs+anti-MICA/B
OSCCs
OSCC+anti-MICA/B

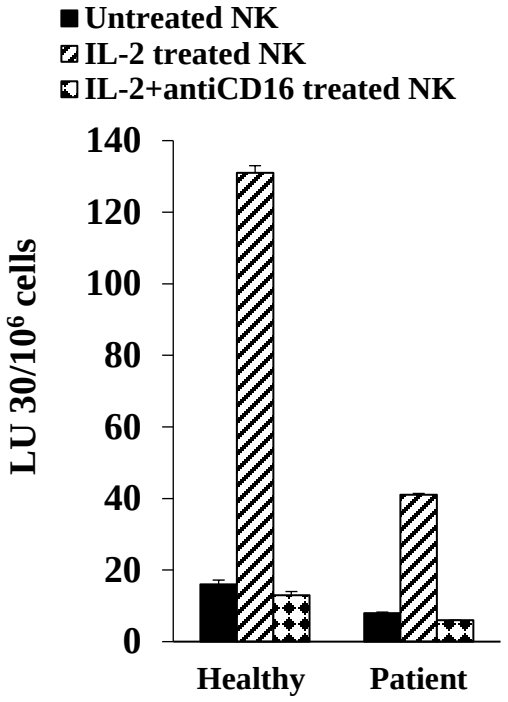
E.


IgG2 (MP2)
IgG2 (PL12)
MICA (PL12)
MICA (MP2)
IgG2 (MP2)
IgG2 (Diff-MP2)
MICA (Diff-MP2)
MICA (MP2)

F. **G.** **H.**


Healthy
Patient
LU 30/10^6 cells
MP2
MP2+anti-MICA/B
Diff-MP2
Diff-MP2+anti-MICA/B
Healthy
PL12
PL12+anti-MICA/B

I.



J.

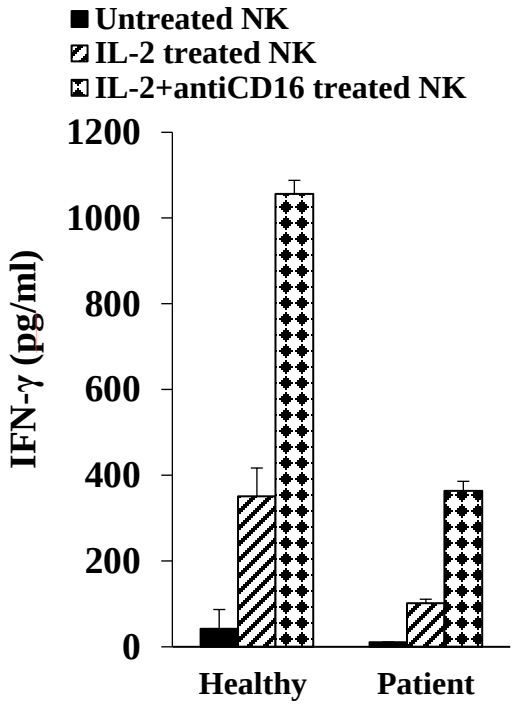
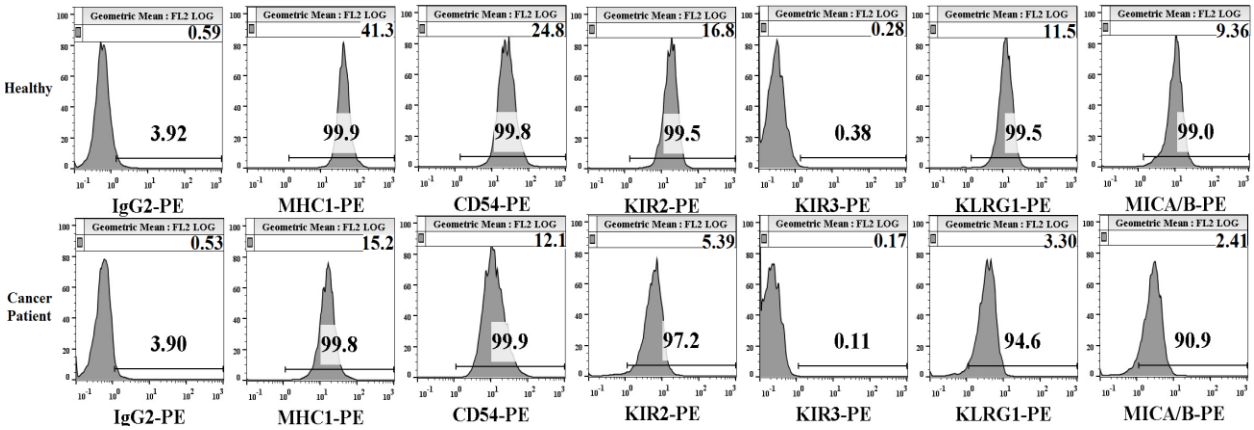


Figure 2. NK cells from cancer patients have low level of NK cells mediated ADCC.

The surface expression of MICA/B on OSCSCs, Diff-OSCSCs and OSCCs was assessed using flow cytometric analysis after staining with respective PE-conjugated antibodies. Isotype control antibodies were used as controls **(A)**. The cytotoxicity against untreated OSCSCs, untreated Diff-OSCSCs, untreated OSCCs, and all three tumor cell types treated with anti-MICA/B during the 4 hours incubation time with NK cell was determined using healthy donor and cancer patient NK cells **(B-D)**. The surface expression of MICA/B on MP2, Diff-MP2 and PL12 was assessed using flow cytometric analysis after staining with respective PE-conjugated antibodies. Isotype control antibodies were used as controls **(E)**. The cytotoxicity against untreated MP2, untreated Diff-MP2, untreated PL12, and all three tumor cell types treated with anti-MICA/B during the 4 hours incubation time with NK cell was determined using healthy donor and cancer patient NK cells **(F-H)**. NK cells ($1X10^6$/ml) from healthy donor and cancer patients were left untreated or treated with IL-2 (1000 units/ml), or a combination of IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 12-24 hours before they were added to ^{51}Cr labeled OSCSCs. NK cell cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay, and the lytic units $30/10^6$ were determined using inverse number of NK cells required to lyse 30% of OSCSCs X 100 **(I)**. NK cells were treated as described above, after an overnight culture, supernatants were removed from the cocultures and the levels of IFN-γ secretion were determined using specific ELISAs **(J)**. One of a minimum thirteen representative experiments for each stem cell population is shown in this figure

Fig. 3

A.


Healthy
Cancer Patient
Geometric Mean : FL2 LOG
0.59
3.92
41.3
99.9
24.8
99.8
16.8
99.5
0.28
0.38
11.5
99.5
9.36
99.0
0.53
3.90
15.2
99.8
12.1
99.9
5.39
97.2
0.17
0.11
3.30
94.6
2.41
90.9
IgG2-PE
MHC1-PE
CD54-PE
KIR2-PE
KIR3-PE
KLRG1-PE
MICA/B-PE

B.

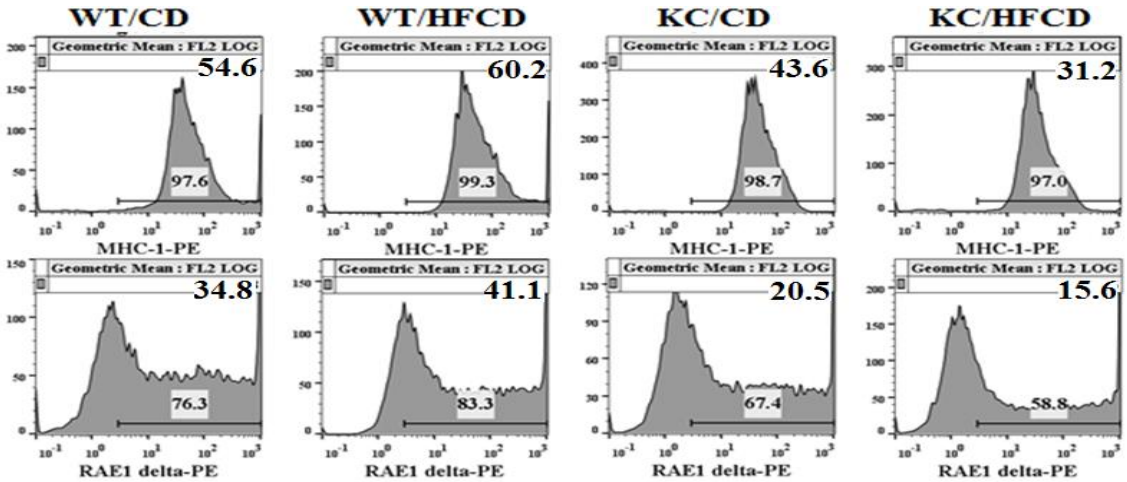

WT/CD
WT/HFCD
KC/CD
KC/HFCD
Geometric Mean : FL2 LOG
54.6
97.6
60.2
99.3
43.6
98.7
31.2
97.0
MHC-1-PE
34.8
76.3
41.1
83.3
20.5
67.4
15.6
58.8
RAE1 delta-PE

Figure 3: Low surface expression of NK activating ligands by cancer patient osteoclasts

Monocytes were isolated from healthy donor and cancer patient, and osteoclasts were generated as described in Materials and Methods, $1x10^4$ cells were used to analyze MHC1, CD54, KIR2, KIR3, KLRG1 and MICA/B surface expressions, employing PE-conjugated antibodies and flow cytometry. IgG2 isotype was used as a control **(A)**. Monocytes were isolated from bone marrow flow-through cells of WT and KRAS mice on control diet and high fat calorie diet, and osteoclasts were generated as described in Materials and Methods, $1x10^4$ cells were used to analyze MHC1, and RAE1 delta surface expressions, employing PE-conjugated antibodies and flow cytometry. IgG2 isotype was used as a control **(B)**.

Sub-aim 2: Role of IL-12 and IL-15 in osteoclasts mediated NK expansion.

IL-15, in part, mediates expansion of NK cells by osteoclasts, whereas IL-12 is responsible for increased IFN-γ secretion by NK cells

We determined the levels of cytokines, chemokines, growth factors, and ligands secreted by primary NK cells and day 6 OC-expanded NK cells (Fig 1A). The majority of secreted cytokines, chemokines, growth factors, and ligands were higher by OC-expanded NK cells when compared to those secreted by the primary NK cells activated with IL-2 and anti-CD16mAb (Fig. 1A). 50-60 fold higher induction of IL-12 and 20-26 fold higher IL-15 secretion were seen by OC-expanded NK cells as compared to primary NK cells (Fig. 1A). Addition of anti-IL-12 and/or anti-IL-15 mAbs significantly reduced cell expansion with 1 ug/ml of anti-IL-15 having the largest effect (Fig. 1B). The treatment with anti-IL-12 and/or anti-IL-15 did not affect the cytotoxic function of NK cells at day 9 (Fig. 1C), but it was inhibited significantly at day 15 (Fig. 1C). The levels of IFN-γ secretion by OC-expanded NK cells were reduced more by anti-IL-12 when compared to anti-IL-15 treatment (Fig. 1D).

Osteoclasts from KRAS mutated mice fed with HFCD are defective in promoting expansion and cytotoxicity of allogeneic wild type NK cells, whereas they trigger IFN-g secretion

We next purified monocytes from the bone marrow, differentiated them into osteoclasts and used them in expansion of NK cells. As shown in Fig. 2A osteoclasts from KRAS mice fed with HFCD had the lowest ability to expand NK cells when compared to WT mice fed with CD. There was a decrease in the expansion of NK cells by osteoclasts from WT mice fed with HFCD

as well as KRAS mice fed with CD (Fig. 2A). NK cells expanded by the osteoclasts from WT mice fed with HFCD and KRAS fed with HFCD mediated much less cytotoxicity when compared to WT or KRAS mice fed with CD (Fig. 2B). In contrast, NK cells expanded with osteoclasts from KRAS mice fed with HFCD secreted the highest levels of IFN-g when compared to those from WT mice fed with CD or HFCD or KRAS mice fed with CD (Fig. 2C). NK cells expanded by osteoclasts from both WT mice with HFCD or KRAS mice with CD secreted higher levels of IFN-g when compared to those obtained from WT mice fed with CD (Fig. 2C).

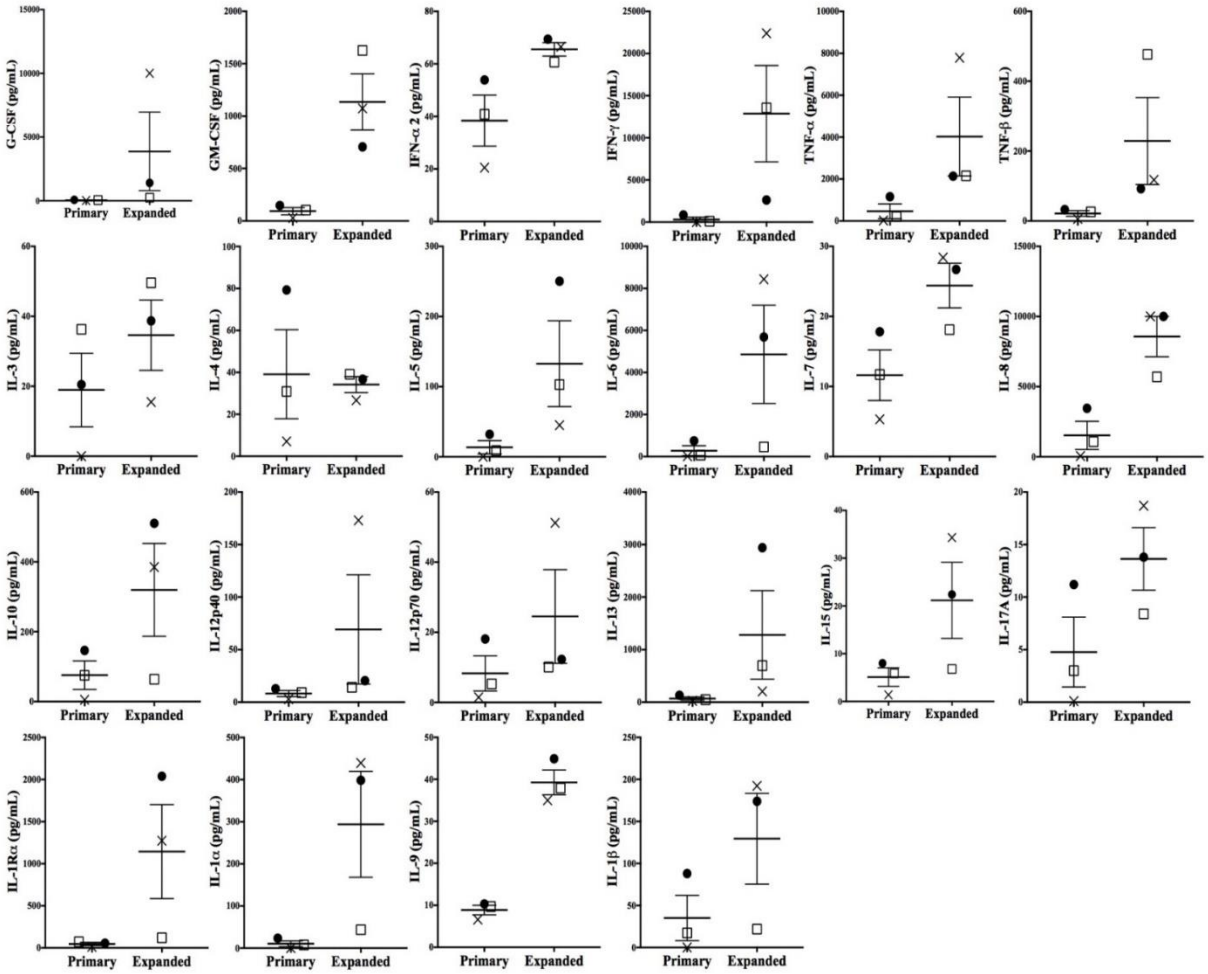
Decreased NK cell cytotoxicity and lower secretion of IFN-g by purified NK cells from spleen tissues of WT and Kras^{G12D} mice fed with high fat diet

NK cells were purified from the spleens of WT and Kras^{G12D} mice fed with control diet or high fat calorie diet and the levels of NK cell mediated cytotoxicity and IFN-g were determined. Similar to spleen, pancreas and adipose tissue dissociated cells, purified activated NK cells demonstrated the following pattern of cytotoxicity from high to low for WT and Kras^{G12D} mice fed with either control or high fat diet (WT/CD>WT/HFCD> Kras^{G12D}/CD> Kras^{G12D}/HFCD (Fig. 3A). Cytotoxicity of IL-2 treated NK cells cultured with and without autologous monocytes also followed similar trend as indicated above except the levels of cytotoxicity by NK cells sorted from WT mice fed with HFCD and KRAS fed with CD were similar, and both were high than those obtained from KRAS mice fed with HFCD (Fig. 3A). Treatment of NK cells with a combination of IL-2+LPS in the absence of monocytes lowered cytotoxicity substantially when compared with IL-2 treated NK cells, keeping the same trend within the animal groups for the levels of cytotoxicity; however, in the presence of monocytes, IL-2+LPS treated NK cell cytotoxicity was decreased substantially in all animal groups (Fig. 3A). In contrast to secretion of IFN-g by

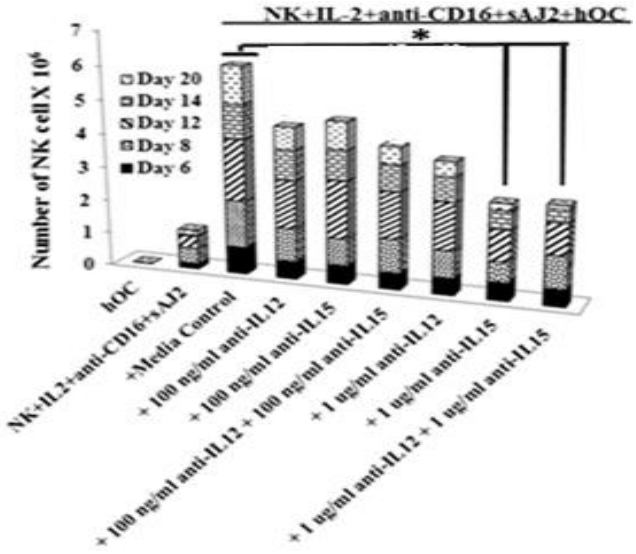
splenocytes, PBMCs, pancreas and adipose in which an upregulation of cytokine secretion could be seen in mice fed with high fat calorie diet, NK cells from $Kras^{G12D}$ fed with HFCD had the least secretion of IFN-g followed by $Kras^{G12D}$ fed with control diet (Fig. 3B and C) and WT/HFCD fed mice had slightly higher than WT/CD fed mice (Fig. 3B and C). When synergistic induction of IFN-g by the purified NK cells was assessed between the 4 groups of mice activated with IL-2+LPS and/or monocytes similar profiles of IFN-g secretion as seen with IL-2 activated NK cells could also be observed (Fig. 3B and C).

Fig. 1

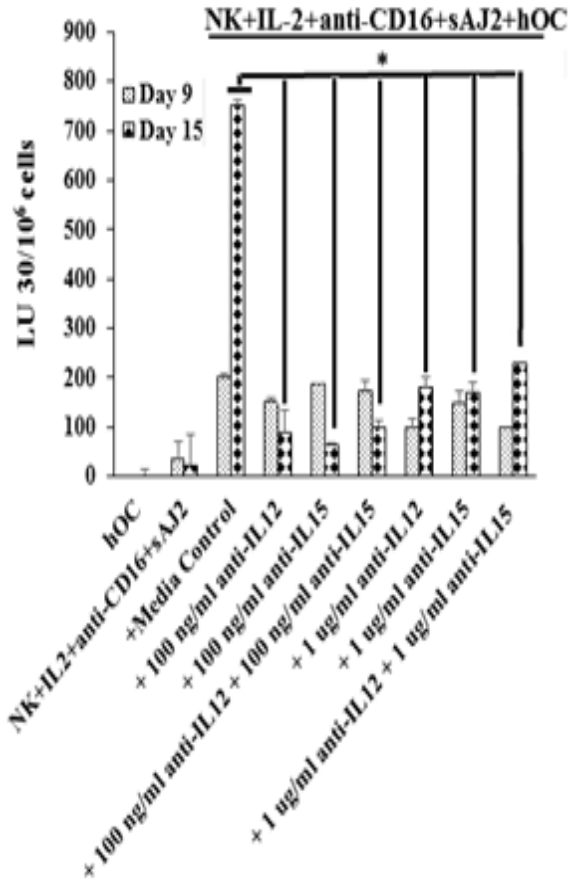
A.


G-CSF (pg/mL)
GM-CSF (pg/mL)
IFN-α 2 (pg/mL)
IFN-γ (pg/mL)
TNF-α (pg/mL)
TNF-β (pg/mL)
IL-3 (pg/mL)
IL-4 (pg/mL)
IL-5 (pg/mL)
IL-6 (pg/mL)
IL-7 (pg/mL)
IL-8 (pg/mL)
IL-10 (pg/mL)
IL-12p40 (pg/mL)
IL-12p70 (pg/mL)
IL-13 (pg/mL)
IL-15 (pg/mL)
IL-17A (pg/mL)
IL-1RA (pg/mL)
IL-1α (pg/mL)
IL-9 (pg/mL)
IL-1β (pg/mL)
Primary
Expanded

B.


NK+IL-2+anti-CD16+sAJ2+hOC
*
Number of NK cell X 10^6
Day 20
Day 14
Day 12
Day 8
Day 6
hOC
NK+IL2+anti-CD16+sAJ2
+Media Control
+ 100 ng/ml anti-IL12
+ 100 ng/ml anti-IL15
+ 100 ng/ml anti-IL12 + 100 ng/ml anti-IL15
+ 1 ug/ml anti-IL12
+ 1 ug/ml anti-IL15
+ 1 ug/ml anti-IL12 + 1 ug/ml anti-IL15

C.


NK+IL-2+anti-CD16+sAJ2+hOC
*
LU 30/10^6 cells
Day 9
Day 15
hOC
NK+IL2+anti-CD16+sAJ2
+Media Control
+ 100 ng/ml anti-IL12
+ 100 ng/ml anti-IL15
+ 100 ng/ml anti-IL12 + 100 ng/ml anti-IL15
+ 1 ug/ml anti-IL12
+ 1 ug/ml anti-IL15
+ 1 ug/ml anti-IL12 + 1 ug/ml anti-IL15

D.

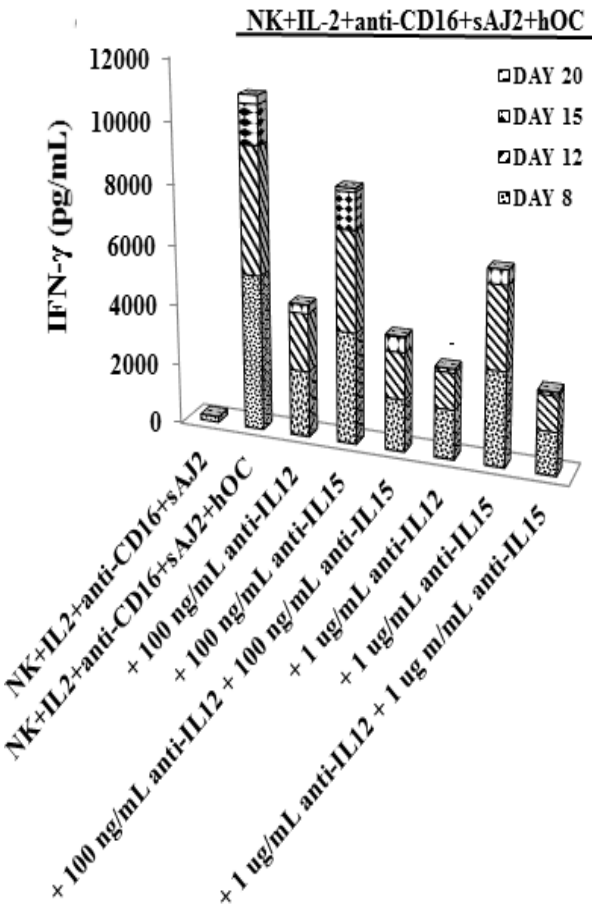

NK+IL-2+anti-CD16+sAJ2+hOC
IFN-γ (pg/mL)
DAY 20
DAY 15
DAY 12
DAY 8
NK+IL2+anti-CD16+sAJ2
NK+IL2+anti-CD16+sAJ2+hOC
+ 100 ng/mL anti-IL12
+ 100 ng/mL anti-IL15
+ 100 ng/mL anti-IL12 + 100 ng/mL anti-IL15
+ 1 ug/mL anti-IL12
+ 1 ug/mL anti-IL15
+ 1 ug/mL anti-IL12 + 1 ug m/mL anti-IL15

Figure 1: **Blocking IL-12, IL-15, or a combination of both resulted in reduced NK cell expansion, NK cell mediated cytotoxicity and cytokine secretion.**

Highly purified NK cells and monocytes were obtained from peripheral blood mononuclear cells (PBMCs) of healthy donors and NK cells were treated ($1x10^6$ cells/ml) with IL-2 (1000 U/ml) for 18 hours before the supernatant was harvested. To generate osteoclasts, monocytes were cultured in alpha-MEM media containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 21 days. For expansion, purified NK cells ($1x10^6$ cells/ml) 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 autologous osteoclasts in the presence of sAJ2 bacteria at 1:2:4 ratios (OC:NK:sAJ2), respectively. The supernatant was harvested after 6 days of co-culture and multiplex assay was used to determine cytokines **(A)** levels secreted by the primary and expanded NK cells. Freshly purified NK cells from a healthy donor were treated and co-cultured with autologous osteoclasts as described in Fig. 1A in the presence and absence of anti-IL12, -IL-15, or a combination of anti-IL-12 and –IL-15 mAbs at 100 ng/ml and 1 μg/ml respectively. Co-cultures were replenished with IL-2 (1000 units/mL) every 2 days. NK cells were counted using microscopy on days 6, 8, 12, 14, and 20 **(B)**. On day 9 and day 15, $1x10^5$ NK cells from each sample were used in standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100 **(C)**. The supernatants were harvested from the co-cultures on day 8, 12, 15 and 20, and IFN-γ secretion was determined using single ELISA **(D)**.

Fig. 2

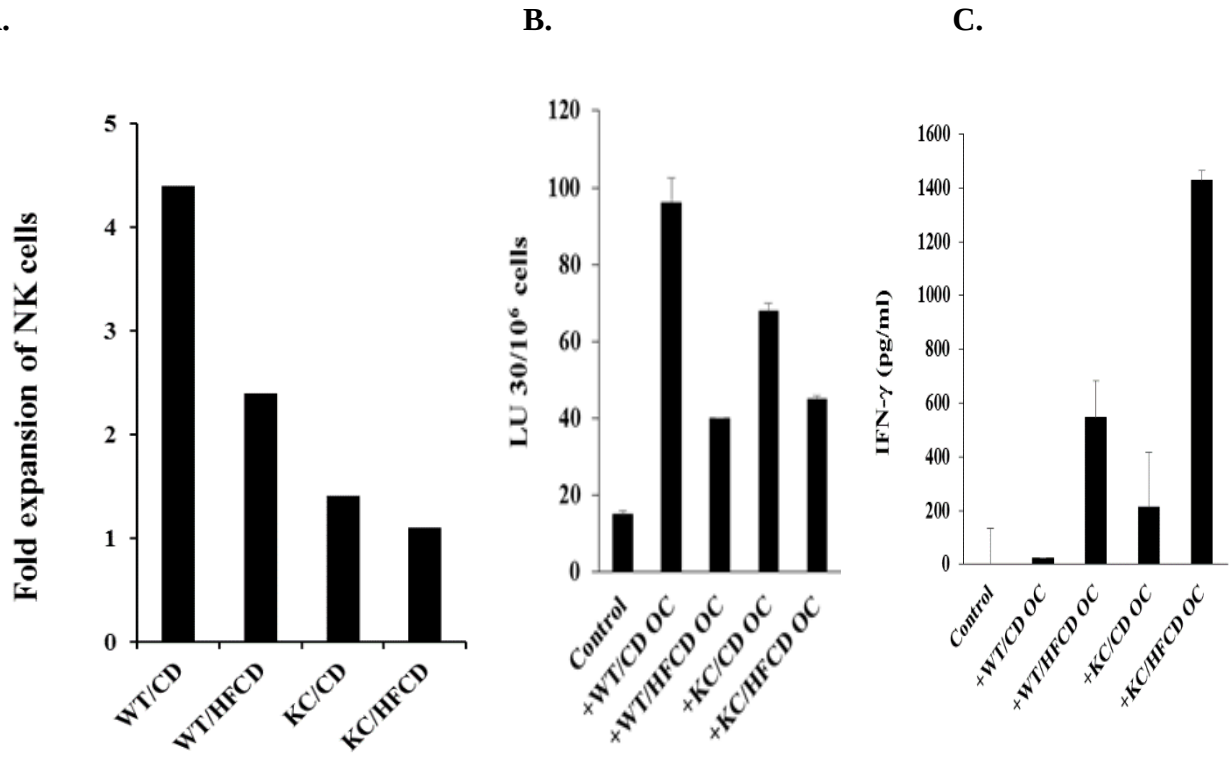


Figure 2: Osteoclasts from high-fat calorie diet animals induce more IFN-γ secretion as compared to osteoclasts from control diet animals on interaction with NK cells.

WT and $KRAS^{G12D}$ model were fed with control diet and high-fat calorie diet as described in material and methods. The femur was harvested after sacrificing the animals and bone marrow cells were obtained as described in Material and Methods, monocytes were purified from bone marrow cells using negative selection. Mouse osteoclasts (OCs) were generated as described in Materials and Methods. Purified NK cells from the spleen of WT animal were left untreated and pre-treated with IL-2 (10,000 units/mL) for 18 hours and then cultured alone or with mouse OCs (NK:OC, 2:1) and culture was treated with sAJ2 bacteria (1:2, 1 million NK:2 million sAJ2). The lymphocytes were counted manually using microscope on day 7 after culture, and fold expansion was calculated as a ratio of day 7: day 0 NK cells counts **(A).** The lymphocytes were used from the culture on day 7 as effector cells in a standard 4 hour ^{51}Chromium release assay against St63 cells. The lytic units $30/10^6$ cells were determined using an inverse number of NK cells required to lyse 30% of the target cells X100 **(B)**, and the supernatant was harvested to determine the level of IFN-γ secretion **(C)**.

Fig. 3

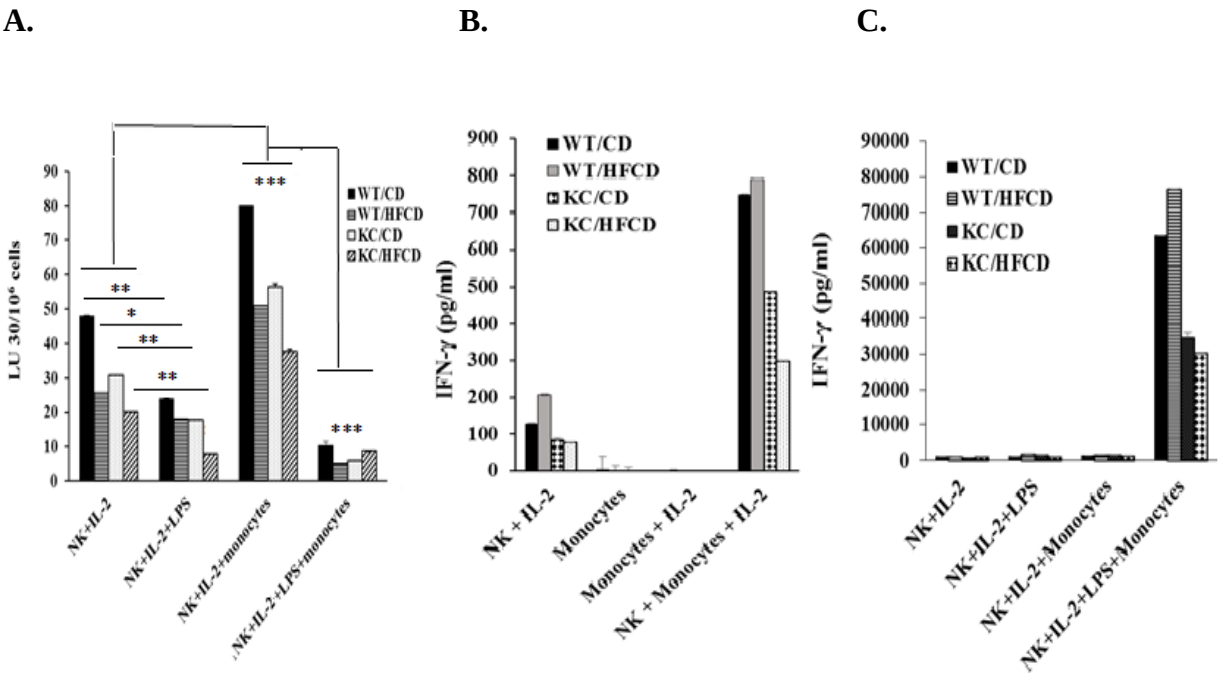


Figure 3: The decrease in cytotoxicity and IFN-γ secretion when NK cells from high-fat calorie diet wild-type and KRAS^{G12D} model were cultured with autologous monocytes.

WT and KRAS^{G12D} model were fed with control diet and high-fat calorie diet as described in material and methods. Spleen and bone marrow were harvested after sacrificing the animals. NK cells were purified using NK negative selection kit from the splenocytes, purity of NK cells was determined by staining them with anti-mouse DX5 antibody and were analyzed by flow cytometry. Bone marrow flow-through cells were obtained as described in Material and Methods, monocytes were purified from bone marrow cells using negative selection. Purity of monocytes was determined using F4/80 mouse antibody**.** NK cells obtained from wild-type and KRAS^{G12D} animals were either treated with IL-2 treated (10,000 U/ml) alone and in combination of IL-2 (10,000 U/ml) with LPS (100 ng/ml), and were cultured with autologous monocytes for 7 days. On day 7, NK cells were used as effector cells in a standard 4 hour ^{51}Chromium release assay against St63 cells. The lytic units 30/10^6 cells were determined using an inverse number of NK cells required to lyse 30% of the target cells X100 **(A)**. The supernatant was harvested from the NK and monocytes co-culture as described in Fig. A and the levels of IFN-γ secretion were determined using specific ELISAs **(B and C)**.

Sub-aim 3: NK cells preferentially expand NK cells and CD8+T cells, and not CD4+T cells; NK cells mediate cytotoxicity against CD4+T cells.

Increased expansion of CD8+T cells in the OC-expanded NK cells culture both in 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. 1A and 1B). When T cells were purified from PBMC and cultured with OCs, CD8+T cells numbers increased and not the CD4+T cells (Fig. 1A and 1B). Similar, to primary T cells from the cancer patients, T cells expanded with the OCs also expressed the effector memory surface profile (Fig. 1 C). Next, we purified the NK cells from healthy donor, 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 the CD4+T cells, but not CD8+T cells (Fig. 1D).

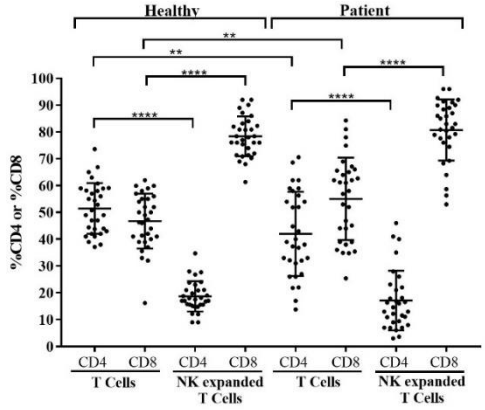
Osteoclasts preferentially expand and activate NK cells and CD8+T cells more than they do CD4+T cells.

Next, we purified the CD4+T cells and CD8+T cells, and cultured them without and with OCs to track the expansion profile, when expanded cells were tracked for 15 days, cumulative cell count had no or slight difference for CD4+T and CD8+T cells without OCs (Fig. 2A), but when both CD4+T and CD8+T cells were with OCs, CD8+T cells had significantly higher expansion when compared to CD4+T cells with OCs (Fig. 2A). Now, we assessed the fold expansion of

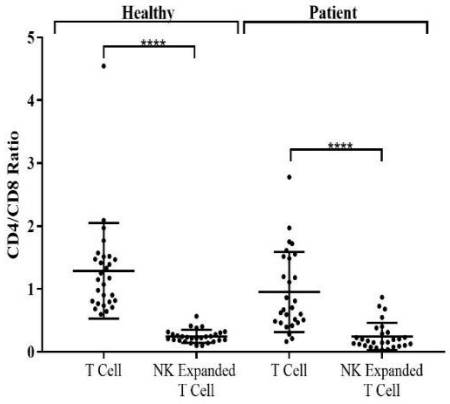
CD4+T cells and CD8+T cells with no OC or with OCs, without OCs there was slight different profile of expansion but both CD4+T and CD8+T cells reduced the expansion almost at same time point (Fig. 2B), but with OC, CD8+T cells continue expanding for a longer time-period, whereas CD4+T cells terminated the expansion earlier than the CD8+T cells (Fig. 2C). 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. Results showed that NK cells and CD8+T cells had similar fold expansion of cells with OCs, whereas CD4+T cells expanded less (Fig. 2D), 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. 2E).

Fig. 1

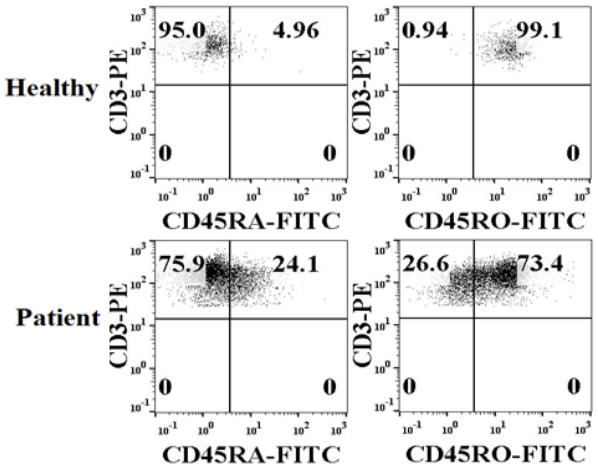
A.


Healthy
Patient
%CD4 or %CD8
CD4
CD8
T Cells
NK expanded T Cells

B.


Healthy
Patient
CD4/CD8 Ratio
T Cell
NK Expanded T Cell

C.


Healthy
Patient
CD3-PE
CD45RA-FITC
CD45RO-FITC
95.0
4.96
0.94
99.1
75.9
24.1
26.6
73.4

D.

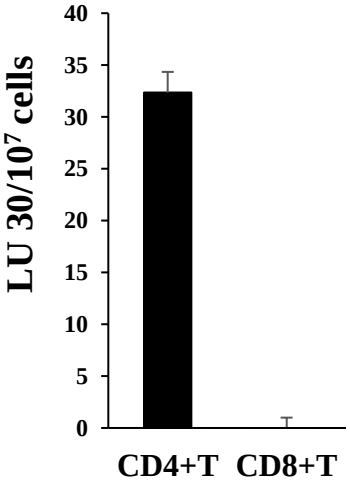
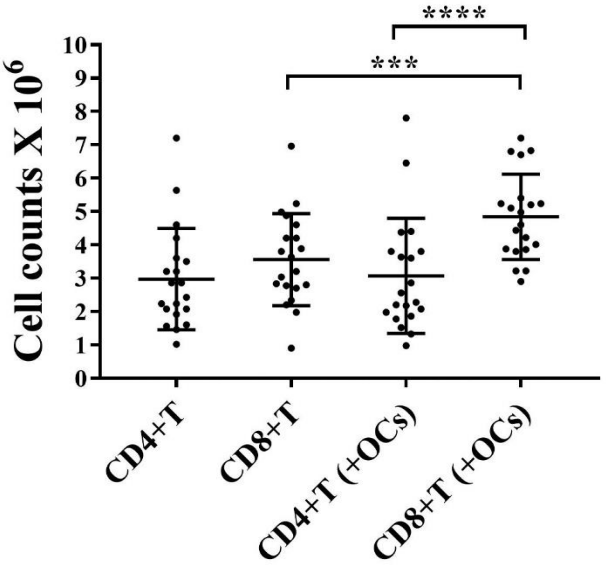

LU 30/10^7 cells
CD4+T
CD8+T

Figure 1: Osteoclast expanded NK cells promote expansion of CD8 T cells and not CD4 T cells, resulting in a substantial decrease in the ratios of CD4 T cells to CD8 T cells

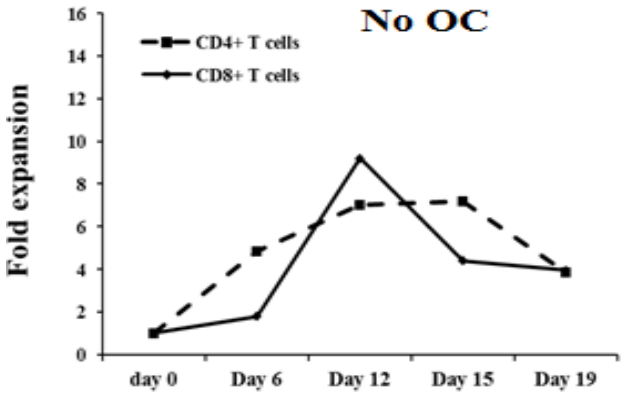
Purified NK cells ($1x10^6$ cells/ml) from the healthy donors and cancer patients were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with OCs in the presence of sAJ2 at 1:2:4 ratios (OCs:NK:sAJ2). Purified T cells ($1x10^6$ cells/ml) 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 co-cultured 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)**. T cells expanded in OC-induced NK cells culture were stained with CD45RO and CD45RA human anti-bodies, and were analyzed on flow cytometry **(C)**. 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 $30/10^7$ cells were determined using inverse number of NK cells required to lyse 30% of CD4+T or CD8+T x 100 **(D).**

Fig. 2

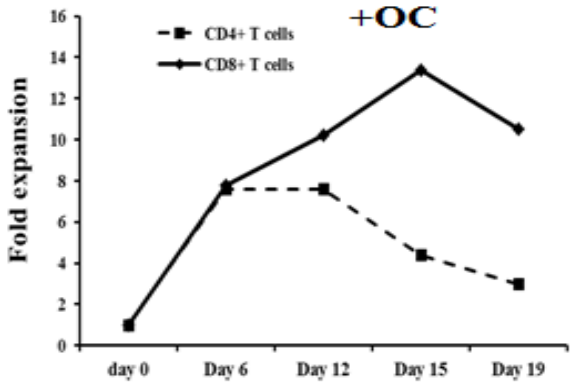
A.


Cell counts X 10^6
****
***
CD4+T
CD8+T
CD4+T (+OCs)
CD8+T (+OCs)

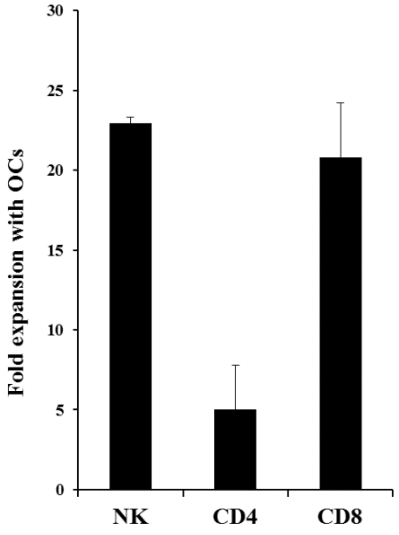
B.


No OC
CD4+ T cells
CD8+ T cells
Fold expansion
day 0
Day 6
Day 12
Day 15
Day 19

C.


+OC
CD4+ T cells
CD8+ T cells
Fold expansion
day 0
Day 6
Day 12
Day 15
Day 19

D.



E.

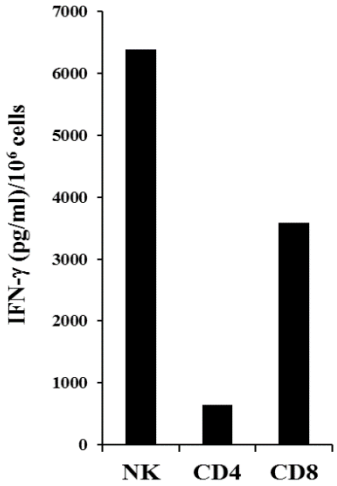


Figure 2: The numbers of CD8 T cells increase with their culture with OCs whereas the number of CD4s decreases substantially indicating that OCs will primarily support the expansion of CD8 T cells and not CD4 T cells.

Purified CD8T cells and CD4T cells ($1x10^6$ cells/ml) from the healthy 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). After 6, 12, 15 and 19 days of co-culture, lymphocytes were manually counted using microscopy, fold expansion for each time points are shown as indicated in figure **(A)**. Purified CD8T cells and CD4T cells ($1x10^6$ cells/ml) from the healthy 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 co-cultured with OCs in the presence of sAJ2 at 1:2:4 ratios (OCs:T:sAJ2). After 6, 12, 15 and 19 days of co-culture, lymphocytes were manually counted using microscopy, fold expansion for each time points are shown as indicated in figure **(B and C)**. Purified CD4T cells and CD8T ($1x10^6$ 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 **(D)**. 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 **(E)**.

Discussion of chapter 2

We have previously shown that osteoclasts are major activators of NK cells [40]. To our knowledge, using OCs as feeder cells is the best strategy to expand large numbers of NK cells compared to those previously described. Although cytotoxic function of expanded NK cells across studies is difficult to compare due to different types of targets used, our strategy provides large numbers of NK cells with significant capabilities to target and lyse cancer stem cells/undifferentiated tumors and secrete IFN-γ. In contrast to OCs, DCs stimulated preferential expansion of small numbers of contaminating T cells in purified NK cells, and the levels of T cells continued to rise for a month thereby decreasing NK expansion. Accordingly, cytotoxicity was lost against OSCSCs. Interestingly, when the cytotoxicity of NK cells was assessed at an earlier time point in which similar proportion of NK cells was observed with OC and DC cultures, a rise in NK cytotoxicity could be observed by the NK cells cultured with OCs (data not shown). Similarly, there was an increase in IFN-γ secretion by NK cells cultured with OCs when compared to those cultured with DCs, indicating that on a per cell basis, NK cells secreted higher IFN-γ when cultured with OCs as compared to those cultured with DCs (data not shown). OCs in comparison to DCs and other potential candidates for NK cell expansion, displayed higher expression of activating NK ligands, whereas they both demonstrated similar levels of differentiation antigen CD54. Interestingly, they both demonstrated lower levels of MHC class I expression when compared to monocytes. In addition, IL-15 secretion appeared to be important for the proliferation of NK cells, whereas IL-12 was important for the secretion of IFN-γ. Both cytokines were important in cytotoxic function of NK cells when assessed at the later day as compared to earlier time point in expansion.

NK cells expanded by OCs demonstrated much higher levels of activating receptor expression including NKG2D, NKp46, NKp44, NKp30, CD94 and increased inhibitory receptors KIR2 and KIR3 with much lower expression of CD16 receptor when compared to primary NK cells. Osteoclasts from cancer patients and KRAS mutated mice lacked higher expression of NK cell receptor ligands which could interfere in NK cell activation and expansion in the cancer microenvironment. Osteoclasts from KRAS mice fed with HFCD expressed much lower levels of MHC class I and Rae1-delta, suggesting that both inhibitory and activating ligands are decreased. The levels of decrease were much higher on KRAS mice fed with HFCD as compared to KRAS mice fed with CD and the highest expression was seen on the surface of WT mice fed with HFCD or CD. The decreased levels of MHC class I and Rae1-delta correlates with the generation of PanINs in KRAS mice fed with CD or HFCD, indicating that the loss of surface receptors of osteoclasts in combination with decreased expansion and function of NK cells is a better indicator of PanIN formation. Inhibition of NK cells in WT mice fed with HFCD may be responsible for the increased inflammation and pancreatitis score as compared to WT mice with CD, without any mutation in the pancreas may have less ability to drive downmodulation of important surface receptors on cells within the tumor microenvironment which are crucial for the activation of NK cells. Indeed, down modulation of both inhibitory and activating ligands for NK cells indicates that NK cells, although are receiving activating signals due to downmodulation of the ligand, are also not able to become activated in the presence of decreased inhibitory ligands. Thus, the defect may be both from the NK cells, as well as the cells which support NK activation. In this regard when NK cells from WT mice fed with CD were cultured with osteoclasts from different mouse groups, osteoclasts from KRAS mice fed with HFCD triggered larger secretion of IFN- γ in the presence of decreased NK cytotoxicity; however, when NK cells from KRAS mice fed with HFCD

were cultured with autologous monocytes both cytotoxicity and IFN- γ secretion were substantially decreased.

When CD4+T cell and CD8+T cell interactions with NK cells were compared, OCs preferentially expand CD8+T cells. NK cells mediate cytotoxicity against CD4+T cells but not the CD8+T cells. Reduced surface expression of NK cell receptors on the NK cells and their ligands on cancer patient OCs, explains why there is decline in NK cells. This decline in the numbers and function of NK cells in cancer patients may not only prevent control of cancer stem cell/undifferentiated tumors but it may also decrease expansion of CD8+ T cells, leading to an increase in tumor growth. Thus, depending on the tumor microenvironment and the levels of defect in immune cells and in those supporting their activation, different functions of immune cells may be compromised; restoration of NK cell numbers and function in cancer patients will be important to establish effective tumor control.

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CHAPTER 3

Osteoclast-expanded, super-charged NK cells inhibit growth and progression of stem-like/poorly differentiated oral and pancreatic tumors *in vivo* in humanized BLT mice.

Abstract

NK cells, through secreted factors and direct cell–cell contact, induce differentiation of poorly differentiated/stem-like oral and pancreatic tumors cells resulting in their resistance to NK cell-mediated cytotoxicity. Induction of NK cell resistance and differentiation of cancer stem is cells correlated with the increased expression of CD54, B7H1, and MHC class I, and mediated by membrane-bound and secreted IFN-γ and TNF-α from the NK cells. In addition, antibodies to both TNF-α and IFN-γ were required to prevent NK-mediated inhibition of tumor cell growth, and restored the numbers of cancer stem cells to the levels obtained when stem cells were cultured in the absence of NK cells. NK differentiated oral and pancreatic tumors did not trigger secretion of cytokines or chemokines by activated NK cells, however, this effect was tumor type dependent. Continuous presence of NK cells was required for the maintenance of cell differentiation since removal of NK cell secreted cytokines revert the phenotype and function of differentiated cells to their stem-like characteristics. Differentiated oral and pancreatic tumors, both hu-BLT mice derived and NK cell induced did not grow or metastasize to vital organs and were less susceptible to NK cell-mediated cytotoxicity, whereas their stem-like tumors grew rapidly, metastasized and were susceptible to NK cell-mediated cytotoxicity. Removal of NK cells from differentiated tumors reverted the tumors to their stem-like stage and induced metastasis. Poorly differentiated oral and pancreatic tumors grew in hu-BLT mice and the intravenous injection of highly potent NK cells prevented tumor formation. NK differentiated tumors were unable to grow in hu-BLT

mice. Tumors obtained from NK injected hu-BLT mice exhibited the differentiated phenotype, grew very slowly, did not expand and were resistant to NK cell mediated cytotoxicity, whereas tumors obtained from non-NK injected mice grew faster, expanded at a higher rate and remained highly susceptible to NK cell-mediated cytotoxicity. Based on our data, we propose immunotherapy with NK cells for the elimination of oral and pancreatic cancer stem cells.

Introduction

Oral squamous cell carcinoma (OSCC) is a significant subset of head and neck cancers, which are the sixth most common cancer worldwide [1]. Despite advances in current therapeutic strategies including radiotherapy, chemotherapy, or concurrent chemo/radiotherapy, most patients, relapse within months and around 50% of patients die of the disease within 5 years [2, 3]. Epithelial tumors including oral carcinoma are known to be phenotypically, etiologically, and biologically heterogeneous, partially due to accumulating mutations [4, 5], but recently there has been accumulating evidence that solid tumors are driven by a small population of cancer cells with stem cell properties, namely cancer stem cells (CSCs) [6]. CSCs are poorly differentiated, can self-renew and give rise to more differentiated progeny that constitute the tumor bulk. This theory has been demonstrated first in leukemia, and then in a variety of solid tumors including oral tumors [6-9] It has been shown that CSCs are resistant to chemo/radiotherapy and enrichment of CSCs after such therapies results in the relapse, metastasis and further resistance of tumors to available treatment [10-12]. CSCs of oral squamous carcinoma can be characterized by overexpression of CD44, a molecule that interacts with hyaluronan (HA), a glycosaminoglycan component of the extracellular matrix (ECM) [8, 13]. HA/CD44 signaling has been demonstrated to mediate solid tumor progression, invasion, metastasis, and resistance to multiple chemotherapeutic agents used

in head and neck cancer including cisplatin [14, 15]. The importance of CD44 was further inferred in studies, in which CD44+ head and neck tumors had greater tumorigenicity in nude mice compared with CD44- cancer cells [8].

Pancreatic adenocarcinoma is the fourth most lethal malignancy in the United States, with about 48,960 new diagnoses and 40,560 deaths expected in 2015 [2]. Despite intense research and improvement in therapeutic schemes, the 5-year survival rate continues to be the lowest among all cancers, with an average of less than 5%. This is because pancreatic cancer progresses rapidly, lacking early symptoms and most patients are diagnosed at locally advanced or metastatic stages that do not qualify them for surgery. For such patients, options are limited to different chemotherapeutic regimes including FOLFIRINOX and nab-paclitaxel with gemcitabine [16-18]. However, this approach is limited by high toxicity and morbidity, and even so chemotherapy is rarely curative. The cellular and molecular factors that promote resistance of pancreatic carcinoma to conventional therapy are aims of intense investigation. Recently, cancer stem cells (CSCs), a population of poorly differentiated cells, capable of unlimited self-renewal in both primary tumor and metastases, have been proposed to contribute to cancer progression and chemotherapy resistance due to increased expression of multi-drug resistance and DNA mismatch repair genes. Indeed, CSCs have been found to enrich chemo-resistant populations in pancreatic cells [19-21]. Li et al. confirmed that CD44+CD24+ESA+ pancreatic CSCs have an increased tumorigenic potential, and are responsible for the aggressiveness of pancreatic cancer [22]. $CD44^{high}$/CD133+/EpCAM+ triple positive phenotype of human PC MiaPaCa-2 and L3.6pl cells has been also found to correlate with stem-like features, including increased cell growth, migration, clonogenicity, and self-renewal capacity [23].

It has previously been shown that downregulation of MHC class I is a hallmark of poorly differentiated cells including mesenchymal stem cells, hematopoietic stem cells, embryonic stem cells, induced pluripotent stem cells, and CSCs in various types of tumors [24, 25]. Low MHC class I expression might favor survival of CSCs and explain limited effectiveness and success of T-cell based immunotherapies in cancer patients. MHC class I loss has been found to be frequently reduced in pancreatic carcinoma specimens and inversely correlated with cytotoxic T-cell infiltration [26, 27]. On the other hand, pancreatic malignant cells should become the targets of NK cells since NK cells are known to kill cells expressing decreased MHC class I levels. Indeed, NK cells in pancreatic cancer have been reported to mediate tumor cell lysis, and overall higher levels of circulating NK cells are associated with better prognosis [28, 29]. However, NK cell cytotoxic activity in the peripheral blood of patients is reduced and expression of NK cell activating receptors is diminished even at early stages and further decreased in advanced disease [30-32]. It is known that the cytotoxic function of NK cells is suppressed after their interaction with stem cells [33-35].

Based on the established immune-privilege and active immunoregulation of normal stem cells [24, 25], it has been proposed that CSCs may evade the host immune-surveillance, partially through absence or downregulation of MHC class I molecules [36]. It is known that MHC class I molecules are regulated not only on stem cells or immune cells but also on cancer cells, and that lower levels of MHC class I in certain types of cancers, including head and neck cancer, correlates positively with disease progression and therapeutic unresponsiveness [24, 37]. Therefore, cancer cells with diminished MHC class I expression may be able to avoid CTL lysis and can be an explanation for limited success of T cell based immunotherapies, but such cells should remain a good NK cell target [37-39]. However, it has been known that freshly isolated tumor infiltrating

NK cells are not cytotoxic to autologous tumors and NK cell cytotoxic activity and numbers in peripheral blood of head and neck patients are reduced and they correlate with clinical stage of the disease [40-42]. Türkseven and Oygür, demonstrated that the number of NK cells was significantly lower in oral tumor specimen graded as a poor prognostic group compared to the cases with a good prognosis [40].

The hypothesis of CSCs interactions with NK cells as well as with other immune subsets, and mechanisms related to cancer cell survival could not be tested in human cancers until now, simply because mice that accept xenografts are immune-deficient, whereas immunocompetent mice mount a powerful xenogeneic immune response against human cells. Discrepancies in the ability of cancer stem cells to give rise to human tumors in immune-deficient mice were reflected by different levels of immunodeficiency achieved in NOD/Shi-SCID and NOD/Shi-SCID IL-2Rγ^{null} (NSG) mice [43]. Recently, several humanized mouse models have been developed, with the humanized BLT (hu-BLT, human bone marrow, liver and spleen) mouse model being one of the best to mimic the full repertoire of interactions that occur between tumor, its environment and immune system, inside the human body [44, 45].

In this study, we demonstrate that primitive cancer stem cells isolated from oral and pancreatic tumor patient (OSCSCs and MP2) can give rise to solid tumors in hu-BLT mice, in the presence of the human immune system. We show how these oral and pancreatic CSCs can be targeted *in vivo* by human NK cells, as a result of split-anergized NK cell-mediated differentiation. Based on our data, we suggest that adoptive NK cell transfer does not mediate unwanted immune responses and can be used as a novel cellular immunotherapeutic strategy for oral and pancreatic tumor patients.

Results

Sub-aim 1: Methodology to expand super-charged NK cells using osteoclasts and sAJ2.

NK cells expansion using OCs and sAJ2.

We compared the activating effect of OCs, monocytes and DCs on NK cell expansion and function. NK cells were activated with IL-2 and anti-CD16mAb 18-20 hours before their co-culture with OCs and/or sAJ2. The combination of OCs and sAJ2 preferentially expanded NK cells while maintaining a low proportion of T cells (Fig 1A and 1B). The rate of expansion and the levels of contaminating T cells in NK cultures were then compared between the co-cultures with OCs, DCs, and monocytes treated with sAJ2. NK cells co-cultured with OCs preferentially expanded NK cells and the rate of contaminating T cells remained very low throughout the first one to two months of the culture (Figs. 1C-1F). In contrast, DCs preferentially expanded T cells and the proportion of NK cells remained low. Although monocytes were also able to expand T cells, T cell expansion remained lower than those of NK cells. There was an initial increase in the proportion of T cells in all three cultures, however, in subsequent cultures, the rate of T cell expansion decreased in the co-cultures with OCs, whereas T cells continued expanding, and substantially increased in cultures with DCs. A steady state of T cell expansion can also be seen in co-cultures with monocytes (Fig. 1C and 1F).

NK cells co-cultured with OCs were able to lyse OSCSCs significantly more than NK cells co-cultured with monocytes or DCs, and there was a significant increase from day 9 to day 15, co-relating with the higher numbers of NK cells in co-culture with OCs on day 15 (Fig. 1G). IL-2 and

anti-CD16mAb activated NK cells cultured with OCs secreted significantly higher amounts of IFN-γ, compared to NK cells co-cultured with monocytes or DCs (Fig. 1H). Upon analysis of NK cell expansion rate and population doubling (defined by the log of the ratio of the final count to the baseline count divided by the log of 2) it was found that OCs expanded NK cells 21,000-132,000 fold at day 20 and 300,000-5,100,000 fold on day 31, with 17-21 population doublings within 4 weeks (Fig. 1I-J).

Expansion of NK cells with osteoclasts remained high in the first month, gradually reduced in the second month and decreased substantially in the third month

Natural killer cells cultured with OCs expanded for 31–36 days while the percentages of contaminating T cells remained low (Fig. 2A). Day 36 expanded NK cells were re-cultured with OCs for a second round of expansion and the NK expansion was continued for 27 days (Figure 2B). Similarly, the percentages of T cell expansion remained very low in the second round of NK cell expansion with OCs (Fig.2B). Day 67 expanded NK cells were re-cultured with OCs for the third round of expansion; however, NK cells were gradually lost due to T cell expansion (Fig. 2C). When the total numbers of NK and T cells were determined during expansion, the numbers of NK cells remained high in the first and second month of expansion (Figs. 2D–I) and substantially decreased in the third month (Figs. 2J–L). No or slight levels of cell death could be observed in the expanding NK cells in the three rounds of expansion with OCs (Fig. 2M). The ability of NK cells to lyse cancer stem cells and secrete IFN-γ was gradually decreased from the first to second round of expansion, and in the third round, during which greater percentages of T cells were expanding, these functions became minimal (Fig. 2N–S). Interestingly, even though large numbers of NK cells were still expanding during the second round of stimulation with OCs

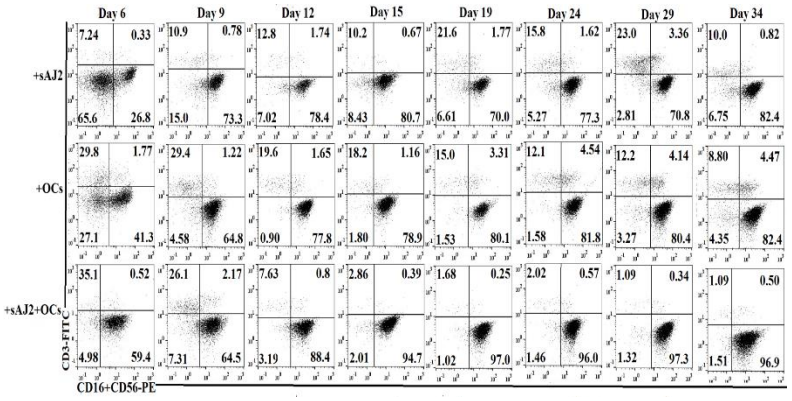
from days 40–63, the levels of NK cell cytotoxicity and secretion of IFN-g were substantially lower than those obtained from expanding NK cells in the first round of stimulation on days 0–34 (Fig. 2).

Osteoclasts, but not K562 or OSCSCs, expand NK cells and increase NK cell function.

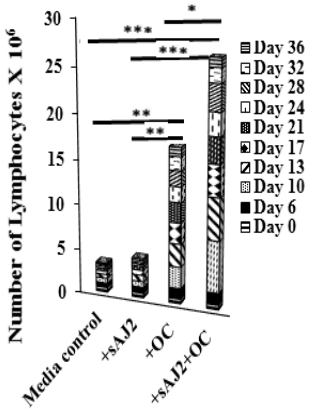
Activated NK cells were cultured with OSCSCs, K562, OCs, irradiated K562, or irradiated OCs in the presence of sAJ2 and the levels of NK expansion and their function were determined (Fig. 3A- 3H). NK cell expansion and function (cytotoxicity and IFN-γ secretion) induced by either non-irradiated, irradiated K562 or OSCSCs was significantly lower than those induced by non-irradiated or irradiated OCs (Fig. 3).

Fig. 1

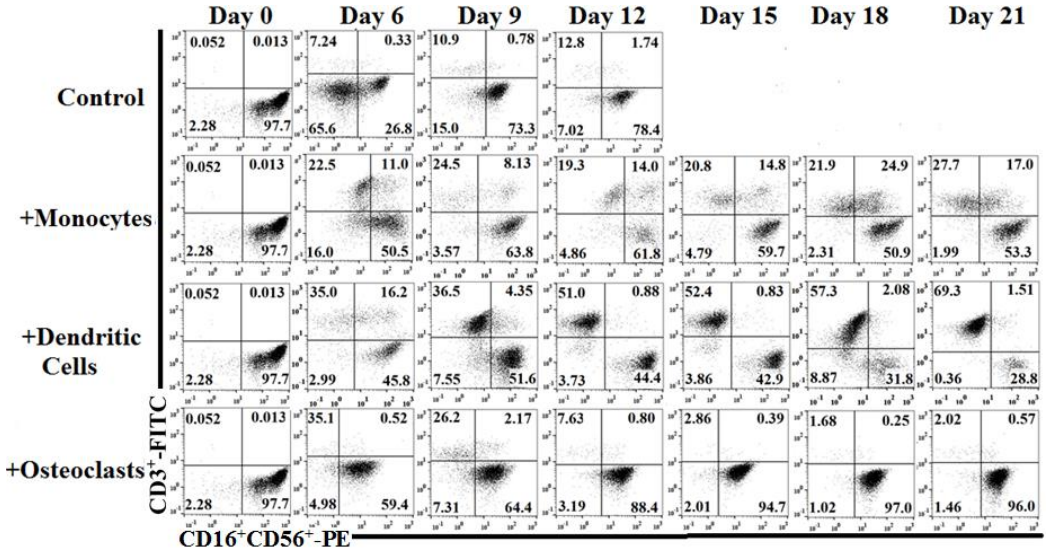
A.


Day 6
Day 9
Day 12
Day 15
Day 19
Day 24
Day 29
Day 34
+sAJ2
+OCs
+sAJ2+OCs
CD3-FITC
CD16+CD56-PE

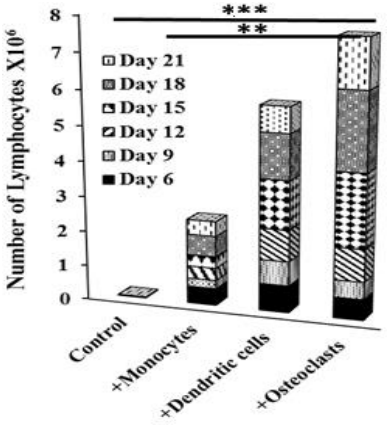
B.


Number of Lymphocytes X 10⁶
Media control
+sAJ2
+OC
+sAJ2+OC
Day 36
Day 32
Day 28
Day 24
Day 21
Day 17
Day 13
Day 10
Day 6
Day 0

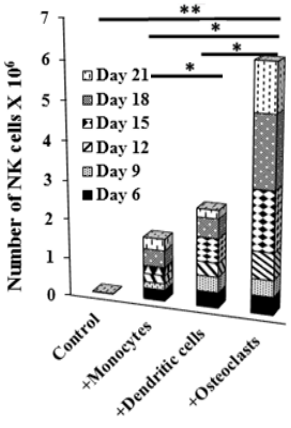
C.


Day 0
Day 6
Day 9
Day 12
Day 15
Day 18
Day 21
Control
+Monocytes
+Dendritic Cells
+Osteoclasts
CD3⁺-FITC
CD16⁺CD56⁺-PE

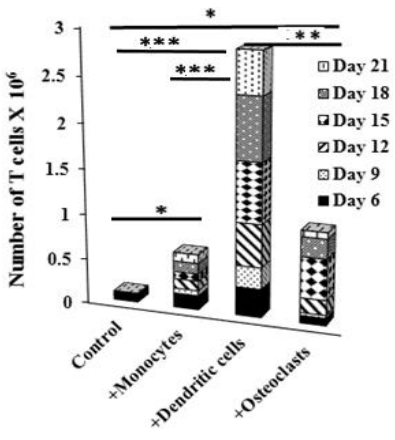
D.


Number of Lymphocytes X10^6
Day 21
Day 18
Day 15
Day 12
Day 9
Day 6
Control
+Monocytes
+Dendritic cells
+Osteoclasts

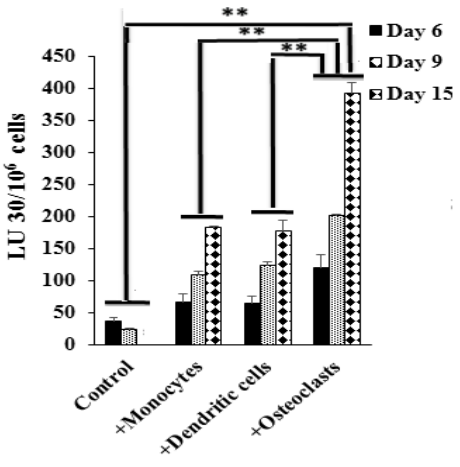
E.


Number of NK cells X 10^6
Day 21
Day 18
Day 15
Day 12
Day 9
Day 6
Control
+Monocytes
+Dendritic cells
+Osteoclasts

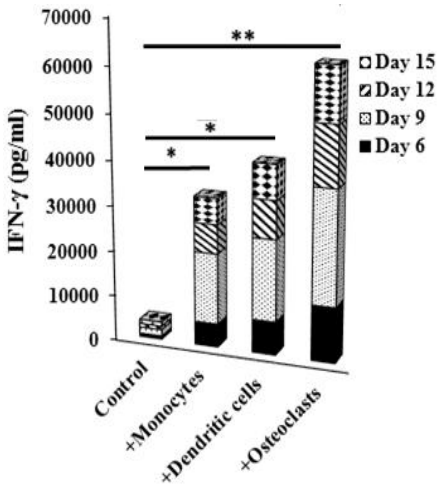
F.


Number of T cells X 10^6
Day 21
Day 18
Day 15
Day 12
Day 9
Day 6
Control
+Monocytes
+Dendritic cells
+Osteoclasts

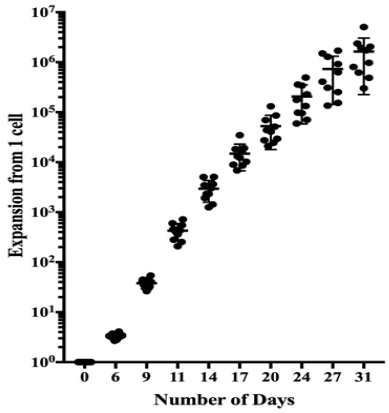
G.


LU 30/10^6 cells
Day 6
Day 9
Day 15
Control
+Monocytes
+Dendritic cells
+Osteoclasts

H.


IFN-γ (pg/ml)
Day 15
Day 12
Day 9
Day 6
Control
+Monocytes
+Dendritic cells
+Osteoclasts

I.


Expansion from 1 cell
Number of Days

J.

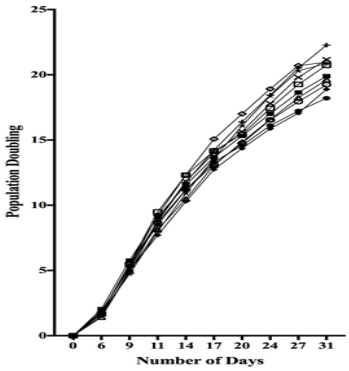
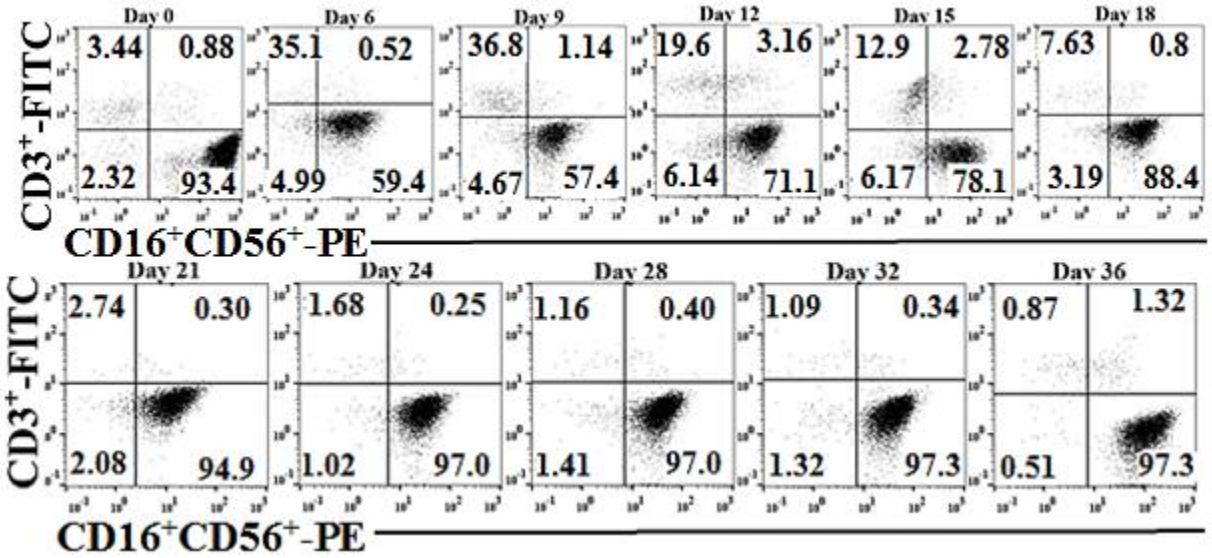

Population Doubling
Number of Days

Figure 1. Expansion of NK cells by osteoclasts and T cells by dendritic cells.

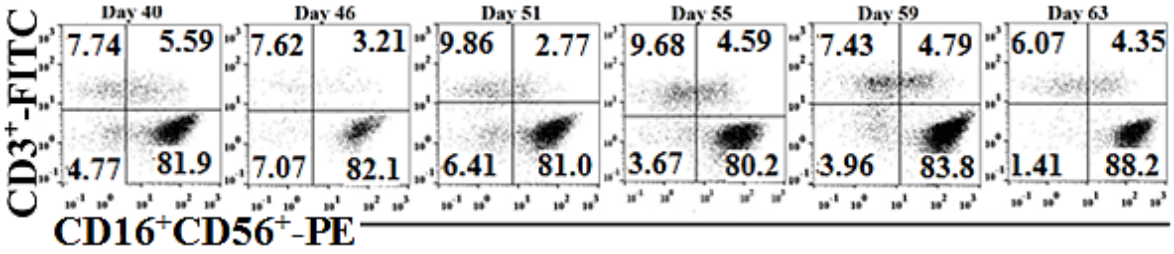
To generate osteoclasts (OCs), monocytes were cultured in medium containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 21 days. Highly purified NK cells ($1x10^6$ cells/ml) were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18 hours before they were co-cultured with autologous OCs in the presence or absence of sAJ2 bacteria at 1:2:4 ratios (OCs:NK:sAJ2), respectively. Surface expression of CD3, CD16, and CD56 was analyzed in $1x10^4$ lymphocyte samples from co-cultures at days 6, 9, 12, 15, 19, 24, 29, and 34 using flow cytometry, and culture medium was refreshed and supplemented with rh-IL-2 (1000 U/ml) **(A).** Cells were co-cultured as described in Fig. 1A and expanded lymphocytes were counted manually using microscope **(B).** Monocytes were purified from human PBMCs and were cultured with GM-CSF (150 ng/ml) and IL-4 (50 ng/ml) for 8 days to generate DCs. To generate osteoclasts, monocytes were cultured in alpha-MEM media containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 21 days For expansion, purified NK cells ($1x10^6$ cells/ml) were treated with the combination of IL-2 (1000 U/ml) and anti-CD16mAb (3□g/ml) for 18 hours before they were co-cultured with autologous monocytes, DCs or OCs in the presence of sAJ2 at 1:2:4 ratios (monocytes, DCs or OCs:NK:sAJ2). Surface expression of CD3, CD16, and CD56 was analyzed at days indicated in the figure using flow cytometry, and culture medium was refreshed and supplemented with rh-IL-2 (1000 U/ml) **(C).** Cells were co-cultured as described in Fig. 1A and the numbers of expanded lymphocytes were assessed using microscopic determination **(D).** The numbers of NK cells (**E**) and T/NKT (**F**) cells were determined using the percentages of NK and T/NKT cells (Fig. 1C) within the total expanding cells in Fig. 1D**.** Cells were co-cultured as described in Fig. 1A and cytotoxicity were determined on the days shown in the figure using a standard 4-hour ^{51}Cr release assay against the oral squamous cell carcinoma stem cell line (OSCSCs). The lytic units $30/10^6$ cells were determined using the inverse number of lymphocytes required to lyse 30% of OSCSCs x 100 **(G).** Supernatants were harvested from the co-culture of NK with OCs as described in Fig. 1A on days 6, 9, 12 and 15, and IFN-γ secretion was determined using single ELISA **(H).** NK cells were co-cultured with autologous osteoclasts and expanded from 10 healthy donors as described in Fig. 1A. Cumulative fold expansion of NK cells was calculated for each donor for 31 days **(I)**, and population doubling was calculated based on the log of the ratio of the final count to the baseline count divided by the log of 2 **(J)**.

Fig. 2

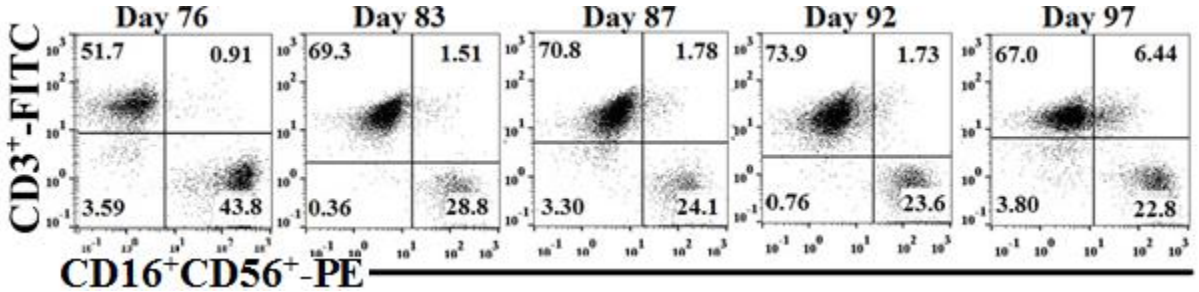
A.


Day 0
Day 6
Day 9
Day 12
Day 15
Day 18
3.44
0.88
2.32
93.4
35.1
0.52
4.99
59.4
36.8
1.14
4.67
57.4
19.6
3.16
6.14
71.1
12.9
2.78
6.17
78.1
7.63
0.8
3.19
88.4
CD3+-FITC
CD16+CD56+-PE
Day 21
Day 24
Day 28
Day 32
Day 36
2.74
0.30
2.08
94.9
1.68
0.25
1.02
97.0
1.16
0.40
1.41
97.0
1.09
0.34
1.32
97.3
0.87
1.32
0.51
97.3
CD3+-FITC
CD16+CD56+-PE

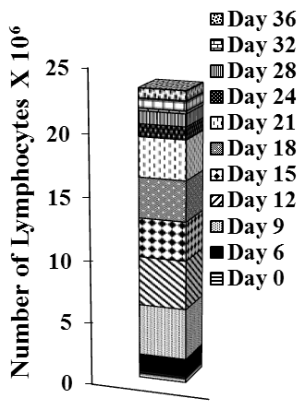
B.


Day 40
Day 46
Day 51
Day 55
Day 59
Day 63
7.74
5.59
4.77
81.9
7.62
3.21
7.07
82.1
9.86
2.77
6.41
81.0
9.68
4.59
3.67
80.2
7.43
4.79
3.96
83.8
6.07
4.35
1.41
88.2
CD3+-FITC
CD16+CD56+-PE

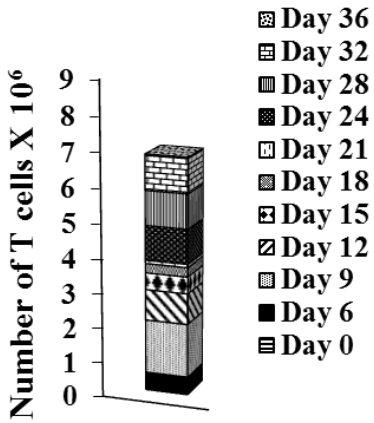
C.


Day 76
Day 83
Day 87
Day 92
Day 97
51.7
0.91
3.59
43.8
69.3
1.51
0.36
28.8
70.8
1.78
3.30
24.1
73.9
1.73
0.76
23.6
67.0
6.44
3.80
22.8
CD3+-FITC
CD16+CD56+-PE

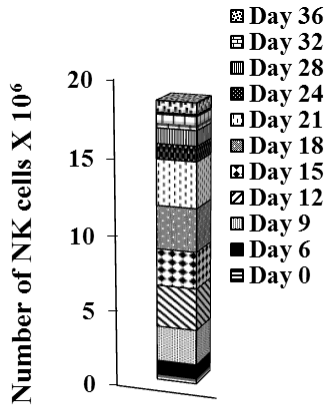
D.


Number of Lymphocytes X 10⁶
0
5
10
15
20
25
Day 36
Day 32
Day 28
Day 24
Day 21
Day 18
Day 15
Day 12
Day 9
Day 6
Day 0

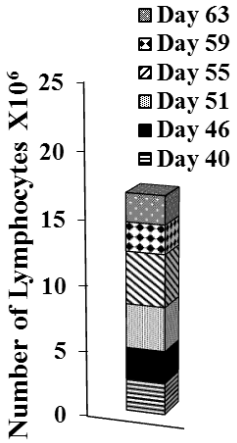
E.


Number of T cells X 10⁶
0
1
2
3
4
5
6
7
8
9
Day 36
Day 32
Day 28
Day 24
Day 21
Day 18
Day 15
Day 12
Day 9
Day 6
Day 0

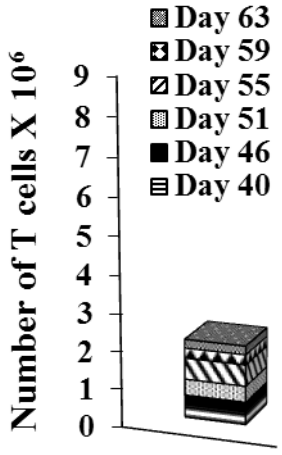
F.


Number of NK cells X 10⁶
0
5
10
15
20
Day 36
Day 32
Day 28
Day 24
Day 21
Day 18
Day 15
Day 12
Day 9
Day 6
Day 0

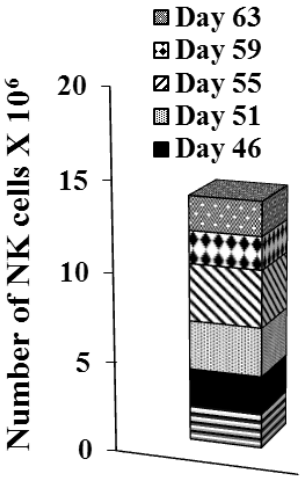
G.


Number of Lymphocytes X10⁶
0
5
10
15
20
25
Day 63
Day 59
Day 55
Day 51
Day 46
Day 40

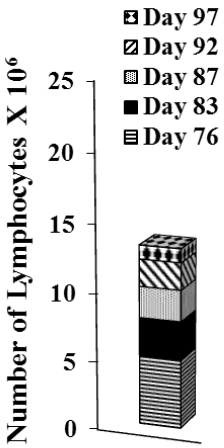
H.


Number of T cells X 10⁶
0
1
2
3
4
5
6
7
8
9
Day 63
Day 59
Day 55
Day 51
Day 46
Day 40

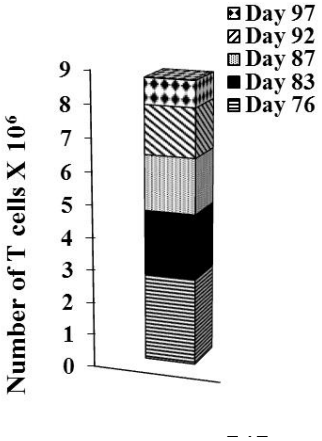
I.


Number of NK cells X 10⁶
0
5
10
15
20
Day 63
Day 59
Day 55
Day 51
Day 46

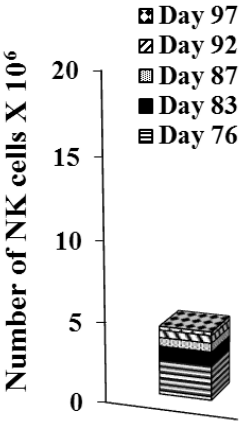
J.


Number of Lymphocytes X 10⁶
0
5
10
15
20
25
Day 97
Day 92
Day 87
Day 83
Day 76

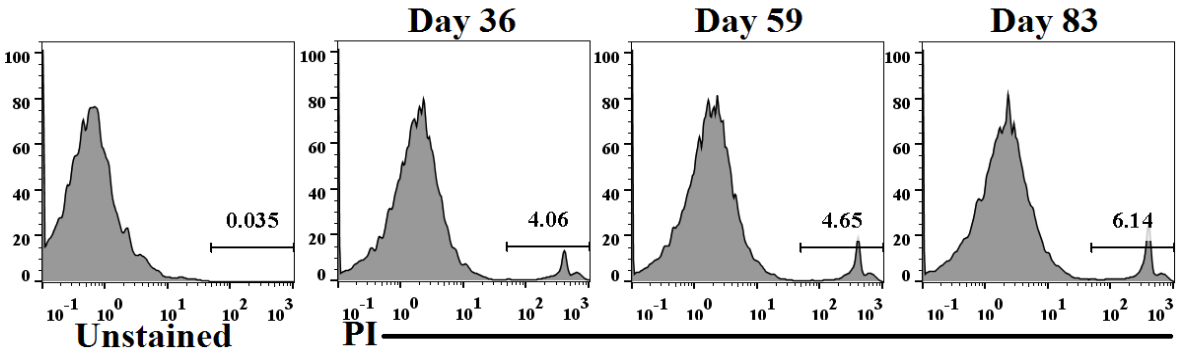
K.


Number of T cells X 10⁶
0
1
2
3
4
5
6
7
8
9
Day 97
Day 92
Day 87
Day 83
Day 76

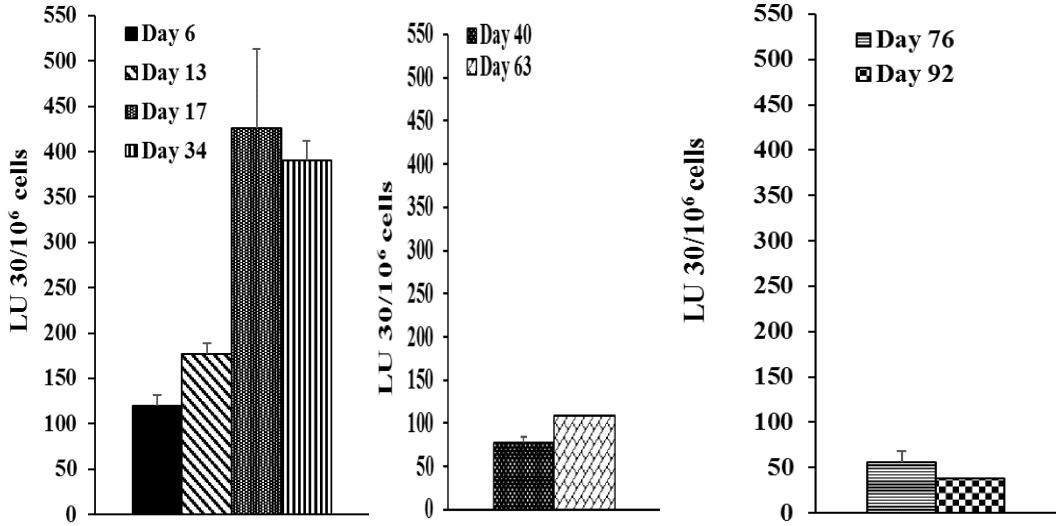
L.


Number of NK cells X 10⁶
0
5
10
15
20
Day 97
Day 92
Day 87
Day 83
Day 76

M.


Day 36
Day 59
Day 83
0.035
4.06
4.65
6.14
Unstained
PI

N. **O.** **P.**


Day 6
Day 13
Day 17
Day 34
Day 40
Day 63
Day 76
Day 92
LU 30/10⁶ cells

Q. **R.** **S.**

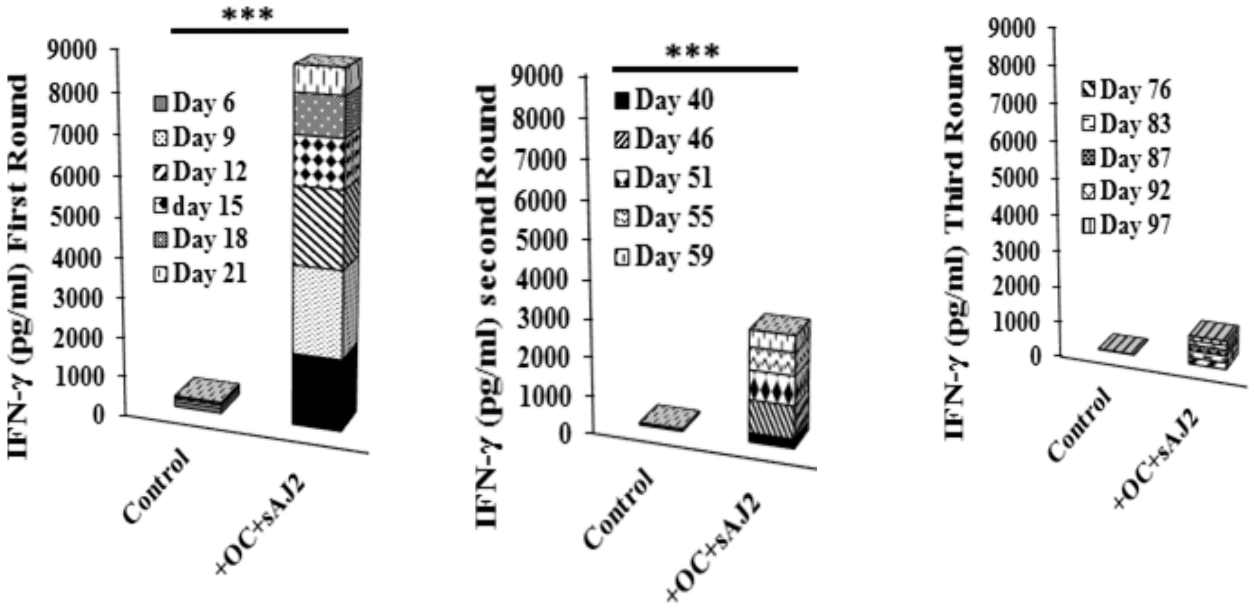
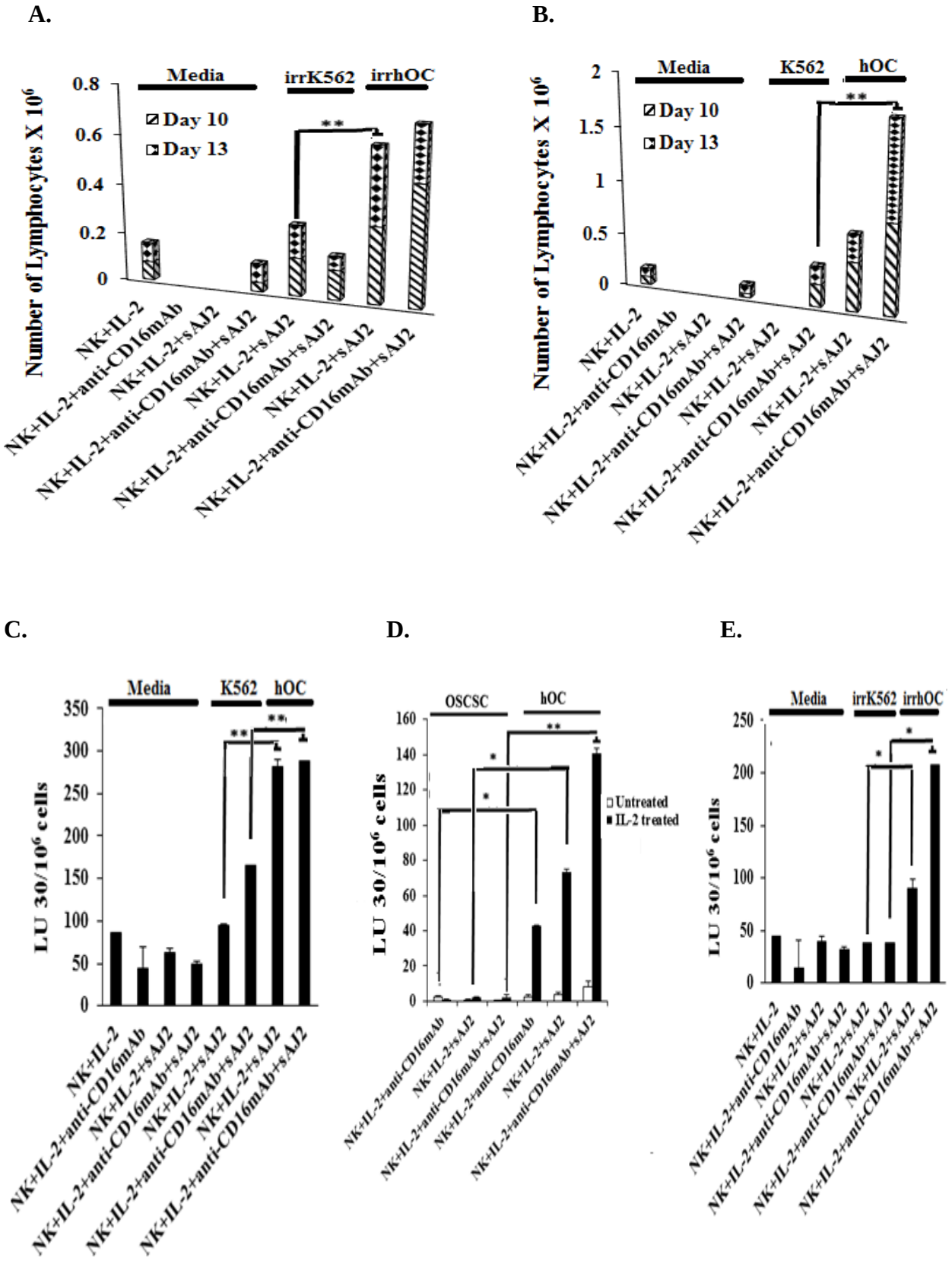


Figure 2. Reduced proportions of NK cells, NK cell-mediated cytotoxicity, and IFN-γ secretion with each successive monthly re-stimulation of NK cell cultures with osteoclasts and sAJ2 bacteria

Freshly purified NK cells were treated and co-cultured with monocyte-derived autologous osteoclasts as described in Fig. 1A. Surface expression of CD3, CD16 and CD56 was analyzed in $1x10^4$ lymphocyte from co-cultures at days indicated in the figure using flow cytometric analysis **(A)**. After 36 days, when NK cells ceased to expand, they were re-cultured with fresh autologous osteoclasts as described in Fig. 1A. Surface expression of CD3, CD16, and CD56 was analyzed on the days indicated in the Figure using antibody staining and flow cytometric analysis **(B)**. On day 63, when cells ceased to expand, they were re-cultured with OCs as described and surface expression of CD3, CD16 and CD56 was analyzed on days shown in the figure **(C)**. The numbers of expanded lymphocytes were assessed using microscopic determination (**D**, **G, J**) and the numbers of NK cells **(F, I, L)** and T/NKT **(E, H, K)** cells were determined using the percentages of NK and T/NKT cells within the total expanding cells **(D)**. Cell death was determined in lymphocytes at days 36, 59 and 83 using propodium iodide staining and flow cytometric analysis **(M)**. Freshly purified NK cells were treated and co-cultured with autologous osteoclasts as described in Fig. 1A. Lymphocytes were then tested for cytotoxicity using a standard 4-hour ^{51}Cr release assay against the OSCSCs after 6, 17, and 34 days of the co-culture **(N)**, 40 and 63 days of the co-culture **(O)** or 76 and 92 days of the co-culture (**P**). The lytic units $30/10^6$ cells were determined using the method described in Fig. 1E. The supernatants were harvested and IFN-γ secretion was determined using single ELISA using supernatants from days 6, 9, 12, 15, 18 and 21, **(Q);** days 40, 46, 51, 55, and 59 **(R),** and days 76, 83, 92, and 97 **(S)**.

Fig. 3


A.
Media
irrK562
irrhOC
Day 10
Day 13
Number of Lymphocytes X 10^6
0.8
0.6
0.4
0.2
0
**
NK+IL-2
NK+IL-2+anti-CD16mAb
NK+IL-2+sAJ2
NK+IL-2+anti-CD16mAb+sAJ2
B.
Media
K562
hOC
Day 10
Day 13
Number of Lymphocytes X 10^6
2
1.5
1
0.5
0
**
C.
Media
K562
hOC
LU 30/10^6 cells
350
300
250
200
150
100
50
0
**
D.
OSCSC
hOC
LU 30/10^6 cells
160
140
120
100
80
60
40
20
0
*
**
Untreated
IL-2 treated
E.
Media
irrK562
irrhOC
LU 30/10^6 cells
250
200
150
100
50
0
*

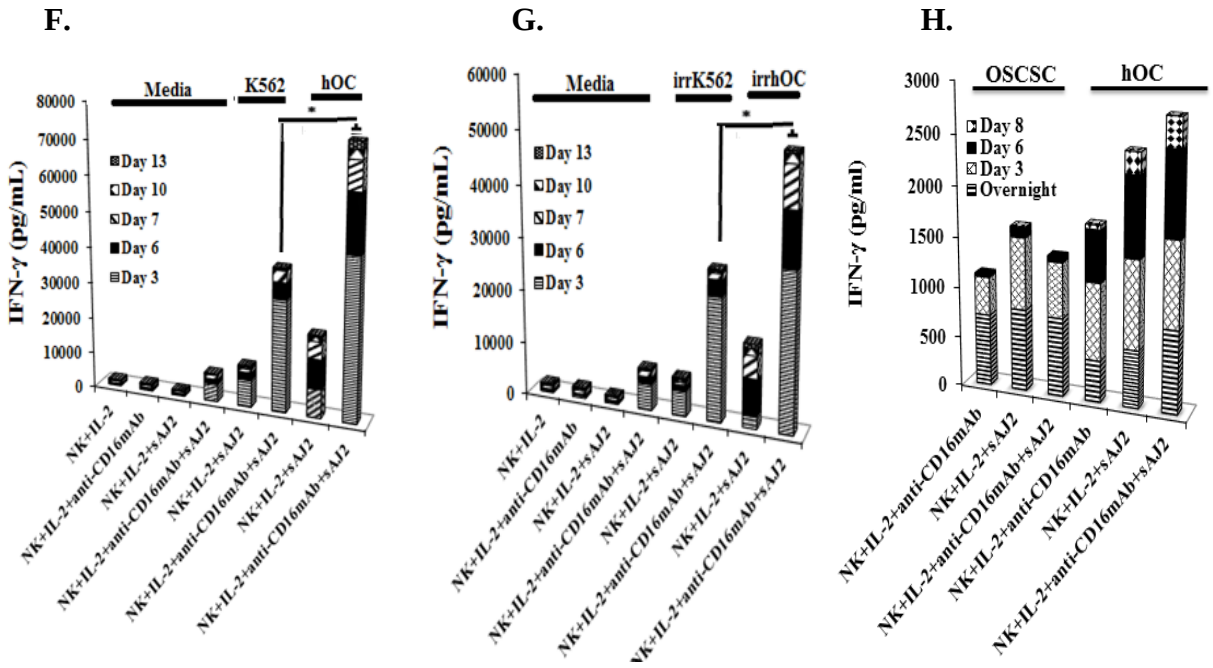


Figure 3: Osteoclasts, but not K562 or OSCSCs, expand NK cells and increase NK cell function substantially.

To generate osteoclasts, monocytes were cultured in medium containing M-CSF (25 ng/ml) and RANKL (25 ng/ml) for 21 days, K562 tumor cell lines were cultured as described in Materials and Methods. Highly purified NK cells ($1x10^6$ cells/ml) 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 K562 and autologous OCs in the presence of sAJ2 bacteria at 1:2:4 ratios (OCs:NK:sAJ2), respectively. Cells from the cultured were counted manually using microscope on day 6, 10 and 13 **(A)**. The osteoclasts generated as described in Materials and Methods, and K562 tumor cells lines were irradiated at 40 grays (Gy) as described in the Materials and Methods. NK cells were purified and treated as described in Materials and Methods, before they were co-cultured with irradiated K562 and irradiated autologous OCs in the presence of sAJ2 bacteria at 1:2:4 ratios (OCs:NK:sAJ2), respectively. Cells from the cultured were counted manually using microscope on day 6, 10 and 13 **(B)**. NK cells were purified and cultured with OCs and K562 as described in Materials and Methods, cytotoxicity of lymphocytes co-cultured for 6 days was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs **(C)**. NK cells were purified and cultured with OCs and K562 as described in Fig. S3B, cytotoxicity of lymphocytes co-cultured for 6 days was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs **(D)**. NK cells were purified and cultured with OCs and OSCSCs as described in Fig. S3A, cytotoxicity of lymphocytes co-cultured for 6 days was determined using a standard 4-hour ^{51}Cr release assay against OSCSCs **(E)**. NK cells were purified and cultured with OCs and K562, supernatant was harvested on day 3, 6, 7, 10 and 13, and IFN-γ secretion was determined using single ELISA **(F)**. NK cells were purified and cultured with irradiated OCs and irradiated K562, supernatant was harvested on day 3, 6, 7, 10 and 13, and IFN-γ secretion was determined using single ELISA **(G)**. NK cells were purified and cultured with OCs and OSCSCs, supernatant was harvested on day 1, 3, 6 and 8, and IFN-γ secretion was determined using single ELISA **(H)**.

Table 1: NK cells expansion methodologies

NK Source	Feeder cells	Expansion	References
Negative selection for NKs from PBMCs	Autologous and allogeneic osteoclasts	**21,000-132,000 fold** expansion at 20 days, 0.3-5.1 million at 31 days; **17-21 population doublings** (avg. 19) at 4 weeks	**Our methodology**
Whole PBMCs or purified NKs	Irradiated PBMCs, Wilms tumor cell line, K562-mb15-41BBL, irradiated EBV-TM-LCL, or none	**4-5,712 fold** at 14-21 days	Deng, X., et al. (2012), Luhm, J., et al. (2002), Parkhurst, M.R., et al. (2011), Harada, H., et al. (2002), Voskens, C.J., et al. (2010), Fujisaki, H., et al. (2009), Yang, H., et al. (2015), Berg, M., et al. (2009), Chang, Y.H., et al. (2013)
Whole PBMCs used initially, residual T-cells removed after 7 days using anti-CD3 Dynabeads, genetically modified with TERT for immortalization	K562-mb15-41BBL (continued stimulation)	TERT transformed: (**130-227 population doublings** over 1000 days) Non-transformed: (**11-20 population doublings** at 8-15 weeks)	Fujisaki, H., et al. (2009)
Cord Blood (CB), CD34+ selection	None	**2,000-15,000 fold** at 5 weeks	Spanholtz, J., et al. (2010, 2011)
NK-92 (cell line)	None	**218- 250 fold** at 15-17 days	Tam, Y.K., et al. (2003)

Sub-aim 2: Treatment strategy for cancer using super-charged NK cells in hu-BLT mice injected with oral and pancreatic tumors.

Lack of tumor growth and metastasis and long term survival of NSG mice after orthotopic injection of IL-2+anti-CD16mAb stimulated NK cell supernatant differentiated MP2 tumors in pancreas

To determine whether IL-2+anti-CD16 mAb stimulated NK cell supernatant differentiated MP2 loses the ability to grow significantly and establish in the pancreas of NOD/SCID; IL-2Rγ-/- mice we first injected mice (n=9) with MP2 stem-like tumors and compared the growth rate and metastatic ability to PL12 well differentiated tumors (n=9). As shown in Fig. 2A, MP2 stem-like tumors grew within 4 weeks and metastasized to both liver and lungs and killed the animals whereas mice injected with PL12 generated very small tumors within 12 weeks and did not metastasize nor kill the animals (Figs. 1A and 1B). Injection of IL-2+anti-CD16mAb stimulated NK cell supernatant differentiated MP2s to pancreas (n=6) did not exhibit growth nor metastasized to liver and lung and all mice survived at 12 weeks when the experiment was terminated (Fig. 1A). Since all of the IL-2+anti-CD16mAb stimulated NK cell supernatant differentiated MP2s reverted to their stem-like phenotype after 12 days of incubation when NK supernatants were removed from the cells and replaced by control media, as evidenced by gradual decrease in expression of MHC class I (data not shown) and CD54 and B7H1 and a gain in susceptibility to NK cell mediated cytotoxicity (data not shown), we injected the reverted MP2 cells (Diff-MP2-R) in the pancreas (n=3) and observed their growth and metastatic potential. Although Diff-MP2-R grew to a smaller size than the untreated MP2s (Fig. 1B), it retained its metastatic potential into the liver (Fig. 1B).

Differentiated MP2 tumors were unable to grow and form tumors in hu-BLTmice

Stem-like/poorly differentiated MP2 tumors were differentiated by supernatants from IL-2+anti-CD16mAb treated NK cells and $1X10^6$ tumors were implanted surgically in the pancreas of hu-BLT mice, and the growth dynamics and overall effect on the mice were compared to MP2 tumor implanted mice in the absence of NK differentiation. MP2 tumors grew rapidly and formed palpable tumors within 4 weeks and became visible by naked eye as they grew more within the pancreas (Fig. 2B). No tumors were palpable on touch or became visible at any point and the mice were active and did not exhibit any signs of morbidity up until the day of the sacrifice when NK supernatant differentiated MP2 cells were implanted in mice (Fig. 2B). In contrast, mice injected with MP2 were inactive and exhibited signs of morbidity at week 6. Animals were sacrificed at week 7 and MP2-implanted mice exhibited tumors which span the entire abdomen and had enveloped spleen, stomach and a portion of intestines (Fig. 2B). Pancreas which were implanted with NK differentiated MP2 tumors exhibited normal size and shape and no tumors could be seen (Fig. 2B). Furthermore, mice retained their weight and no signs of morbidity could be seen (Fig. 2B). Moreover, there was 9.37% CD45+ immune cells with 64% being CD3+ T cells in NK differentiated MP2 implanted pancreas, whereas only 3.37% CD45+ immune cells with 80% of the cells being CD3+ T cells from MP2 bearing pancreas (Fig. 2B).

Single injection of NK cells inhibited tumor growth in mice implanted with MP2 and OSCSCs stem-like/poorly differentiated tumors

Mice were implanted surgically with either MP2 or 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. 2A and 4). 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 MP2 tumors and injected with NK cells grew significantly smaller tumor when compared to those implanted with MP2 tumors in the absence of NK injection (Fig. 2B). Similar results were seen in OSCSCs injected hu-BLT mice (Fig. 4B and 4C). The tumor was localized and no involvement of other organs could be seen in mice injected with MP2 tumors and received activated NK cells, mice were much active and healthy (Fig. 2B). Similar results in OSCSCs tumor injected mice (Fig. 4A). When determining the percentages of CD45 immune cells between the two tumor types, significantly higher percentages of CD45+ tumor infiltrating tumor cells could be seen in MP2 tumors which received the injection of NK cells and the immune cells expressed CD56 bright, CD94, NKG2D and DNAM-1 surface receptors (Fig. 2B and data not shown). Moreover, in agreement with our in vitro experiments MP2 tumors from NK injected mice exhibited substantially decreased susceptibility to NK cell mediated killing whereas those obtained from no NK injected mice remained significantly more susceptible to NK cell mediated cytotoxicity (Fig. 2C). Similar results were seen is OSCSCs injected hu-BLT mice (Fig. 4I). Tumors dissected from OSCSCs + NK injected mice expressed significantly higher MHC1 on their surface, providing evidence that NK injections differentiated tumors in-vitro (Fig. 4D). When tumors dissected form OSCSCs + NK injected mice and compare them when OSCSCs alone injected mice, OSCSCs + NK tumors didn't grow in culture (Fig. 4E and 4G), OSCSCs + NK injected tumors secreted more IFN-γ (Fig. 4H), and expressed low Epcam on their surface (Fig. 4F), when compared with the tumors dissected from the mice injected with only OSCSCs (Figs.

4E-4H). Tumors dissected from OSCSCs + NK injected hu-BLT mice expressed high MHC-1 and CD54, and increased hu-CD45 immune cells infiltration during the culture (Table 1).

Single injection of NK cells with the combination of probiotics AJ2, inhibited tumor growth in mice implanted with MP2 and OSCSCs stem-like/poorly differentiated tumors.

Mice were fed orally with AJ2 throughout the experiment time period, a week later the first dose of AJ2, mice were implanted surgically with MP2 or 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. 2D and 5A). 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 MP2 tumors and injected with NK cells alone, or the combination of NK cells injection and fed with AJ2 grew significantly smaller tumor when compared to those implanted with MP2 tumors in the absence of NK injection (Fig. 2E and 2F). Similar results were seen in OSCSCs injected mice (Fig. 5B). When tumors were dissociated and equal numbers of tumors from each mouse were cultured for 3 days no cell attachment could be observed from tumors obtained from MP2+NK injected and MP2+NK+AJ2 animals, whereas significant attachment of tumors from MP2 implanted and no NK injected or NK+AJ2 mice were visible even at day 1 and the levels increased substantially by day 3-7 and covered the entire plate (Fig. 2H). When unattached tumors from MP2 implanted and no NK injected tumors were transferred to new plates, similarly a rapid kinetics of attachment and increased growth could be seen (data not shown). Therefore, when total numbers of tumors were counted in days 7, 10 and 14, a three fold increase in tumor growth can be seen in tumors taken from MP2 implanted and

no NK injected mice as compared to those resected from MP2 implanted + NK injected mice and MP2 implanted + NK injected AJ2 fed mice (Fig. 2G). Similar results were seen in OSCSCs injected mice (Fig. 5C). When IL-2 was added to the tumors, the number of tumors decreased substantially in both tumor types, however, MP2 implanted and NK injected alone or in combination with AJ2 tumors had only a few tumors growing whereas those from MP2 implanted and no NK injected mice grew to almost to the levels of MP2 implanted with NK injected without IL-2 addition (data not shown). Indeed, the levels of B7H1, MHC class I and CD54 were significantly higher on MP2 or OSCSCs tumors from NK injected alone or on combination with AJ2 mice when compared to no NK injected mice (data not shown). The supernatants harvested from the pancreas (tumor site) culture of MP2+NK secreted high, and MP2+NK+AJ2 mice secreted significantly higher IFN-γ when compared to MP2 alone injected mice (Fig. 2I). Again, in agreement with our in vitro experiments OSCSCs tumors from NK injected or AJ2 fed along with NK injected mice exhibited substantially decreased susceptibility to NK cell mediated killing whereas those obtained from no NK injected mice remained significantly more susceptible to NK cell mediated cytotoxicity (Fig. 5D). There was increase hu-CD45 immune cells infiltration in the tumor dissected for OSCSCs + NK animals and more so from OSCSCs + NK + AJ2 whereas those obtained from no NK injected mice remained had very low hu-CD45 immune cells (Fig. 5E).

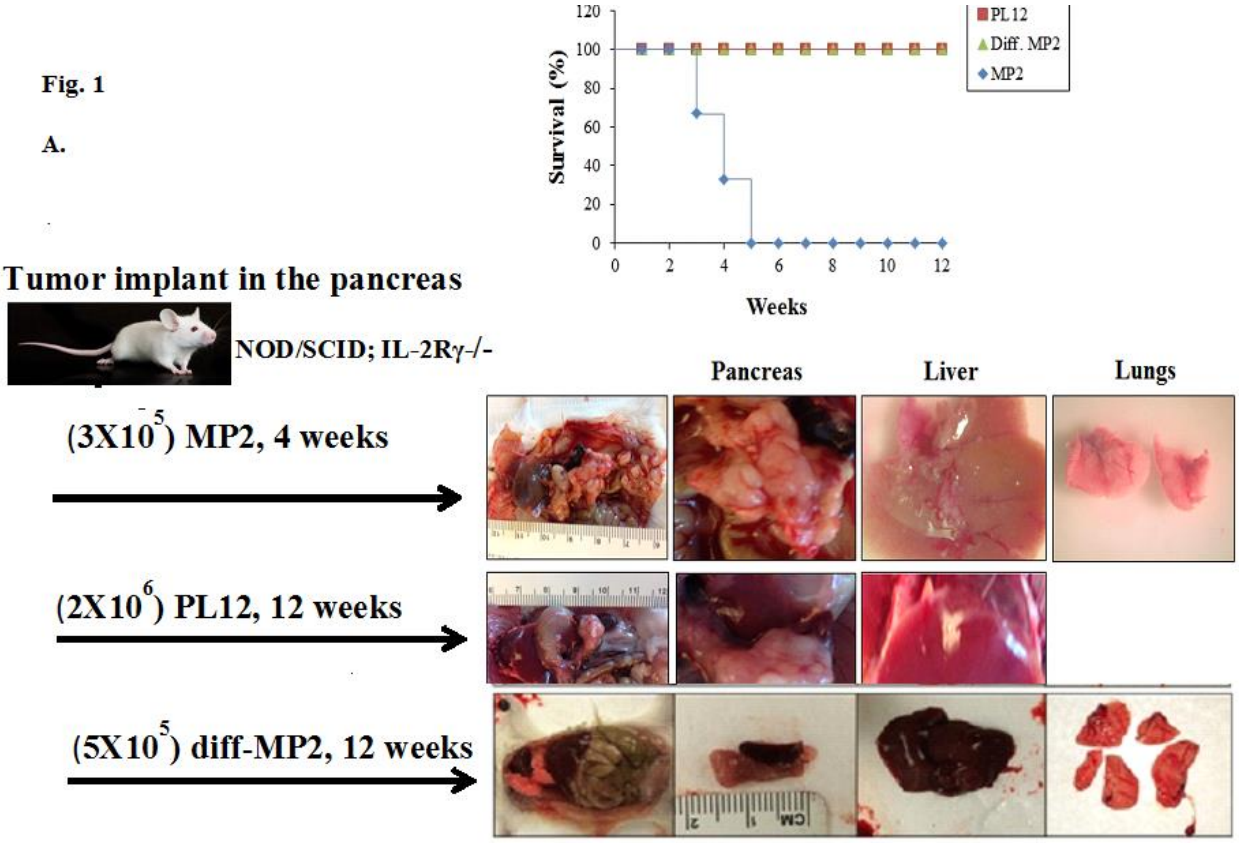
NK cells injection alone and more so in combination with AJ2 and anti-PD1 improved the IFN-γ secretion and cytotoxic function of immune cells in various tissue compartment of the hu-BLT mice.

To determine the effect of tumor on peripheral blood, spleen, BM and oral gingiva, tissues were collected at the end of the experiment when animals were euthanized, and single cell

suspension was harvested from each tissue as described in Materials and Methods, each tissue were cultured with IL-2 activation for 7 days before the NK cell mediated cytotoxicity and IFN-g secretion were determined. PBMCs, spleen, BM and oral gingiva tissue from MP2 and OSCSCs implanted tumors were severely suppressed both in terms of cytotoxicity and, also IFN-g secretion (Fig. 3A and 3B), whereas all these tissues from MP2+NK injected mice exhibited significant high levels of cytotoxicity and activation of IFN-g by IL-2 treatment (Fig. 3A and 3B). Similar results were seen is OSCSCs injected hu-BLT mice (Fig. 5F and 5G). Tissues harvested from the mice fed with AJ2 on top of NK injection, increased IFN-γ secretion and NK cells mediated cytotoxicity is PBMCs, spleen, and gingiva (Fig. 3A and 3B), whereas in BM secreted IFN-γ was not much improved with AJ2 addition although NK cell-mediated cytotoxicity was increased (Fig. 3A and 3B). The combination of AJ2 and NK cells increased the IFN-γ secretion and NK cells mediated cytotoxicity is PBMCs, spleen and BM for OSCSCs injected hu-BLT mice (Fig. 5F and 5G). Next, we combined the anti-PD1 injections along with NK and AJ2, when tissues from MP2+anti-PD1+NK injected mice were analyzed to determine the NK cell-mediated cytotoxicity and IFN-γ secretion, IFN-γ secretion was improved compared to MP2 alone or MP2+NK mice, but NK cells-mediated cytotoxicity was not improved further (Fig. 3C and 3D). The combination of anti-PD1 with MP2+NK+AJ2 was when compared with MP2+NK+AJ2 mice, the level of IFN-γ and NK cells mediated was decreased in PBMCs, spleen and oral gingiva (Fig. 3C and 3D), in BM although NK cell-mediated cytotoxicity was not improved but IFN-γ was increased when compare to MP2+NK+AJ2 mice (Fig. 3C and 3D). The combination of anti-PD1 with OSCSCs+NK+AJ2 was when compared with OSCSCs+NK+AJ2 mice, the level of IFN-γ secretion was decreased in PBMCs, spleen, not much changed on BM (Fig. 5F), cytotoxicity on the other hand was increased in PBMCs, spleen and BM (Fig. 5G).

Fig. 1

A.


Survival (%)
120
100
80
60
40
20
0
0 2 4 6 8 10 12
Weeks
PL12
Diff. MP2
MP2
Tumor implant in the pancreas
NOD/SCID; IL-2Rγ-/-
(3X10^5) MP2, 4 weeks
(2X10^6) PL12, 12 weeks
(5X10^5) diff-MP2, 12 weeks
Pancreas
Liver
Lungs

B.

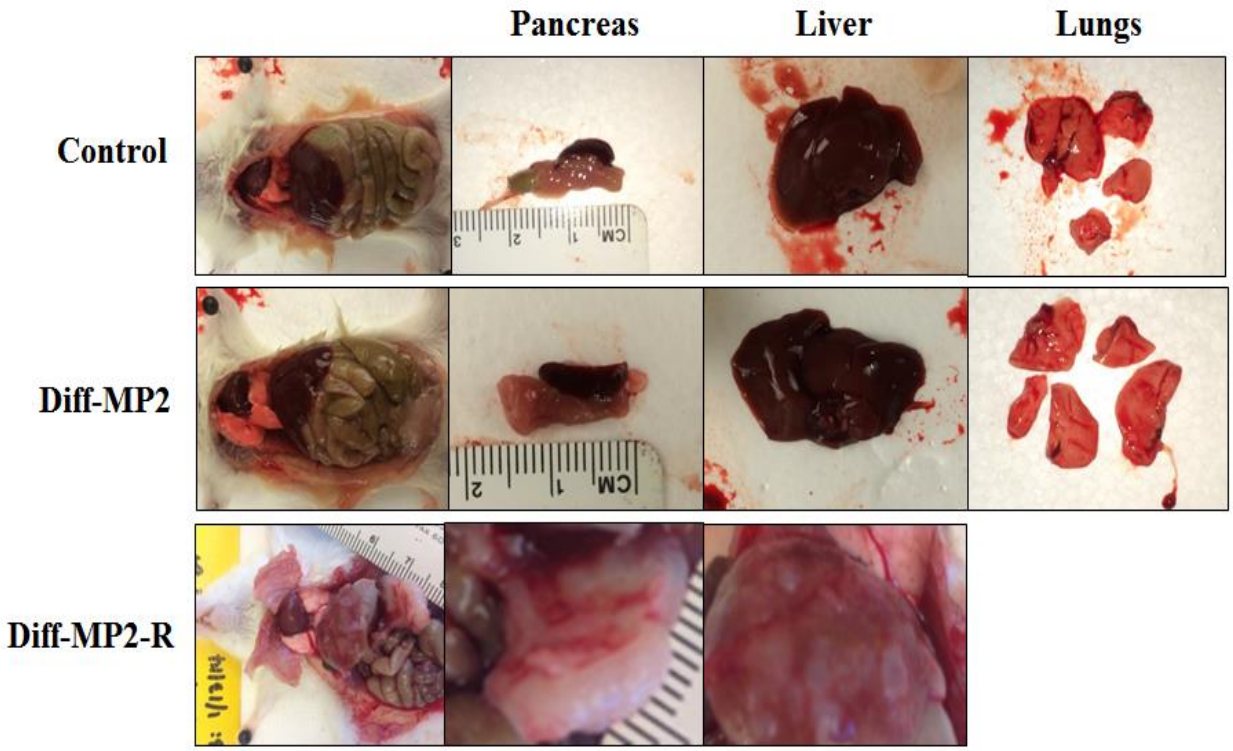


Figure 1: Lack of tumor growth and metastasis and long term survival of NSG mice after orthotopic injection of IL-2+anti-CD16mAb stimulated NK cell supernatant differentiated MP2 tumors in pancreas

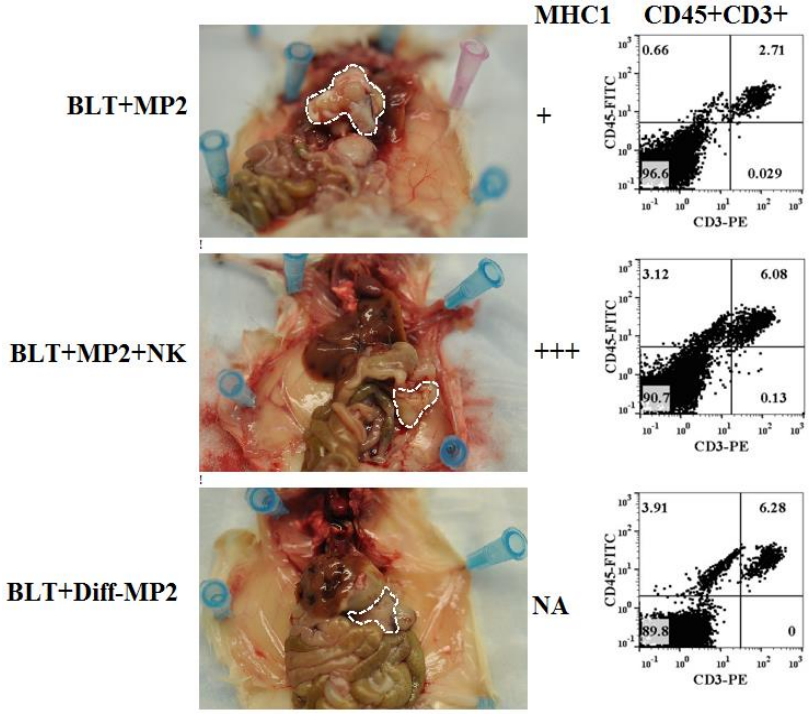
Successful orthotopic cell implantation of MP2 and PL12 (5 X 10^5 cells) into 8-10 weeks old NOD/SCID mice is shown. Method of implantation is described as in Materials and Methods. After 4 weeks (MP2) and 12 weeks (PL12) post implantation, the animals were euthanized by isoflurane or CO_2 inhalation and the pancreas, liver and lungs were analyzed for primary tumor growth and metastases **(A).** Highly purified NK cells were treated with IL-2 (1000 units/ml) and anti-CD16mAb (3ug/ml) for 24 hours before the supernatant was collected and added to MP2 cells for 5 days (Diff-MP2). The rate of survival between animals injected with PL12, MP2 and Diff-MP2 (5 X 10^5 cells) were tracked for 12 weeks **(A)**. MP2 cells were differentiated as described in figure 8B. Another set of MP2 cells were differentiated as described and then the cells were replenished with fresh medium without supernatant from NK cells for 2 weeks (Diff-MP2-R). Afterwards, the tumor cells were orthotopically implanted into NOD/SCID mice (5 X 10^5 cells). Mice with injected Diff-MP2 tumor cells were sacrificed at 10 weeks and mice with Diff-MP2-R tumor cells were sacrificed at 8 weeks. After the animals were euthanized, images of the pancreas, liver and lungs were taken **(B)**.

Fig. 2

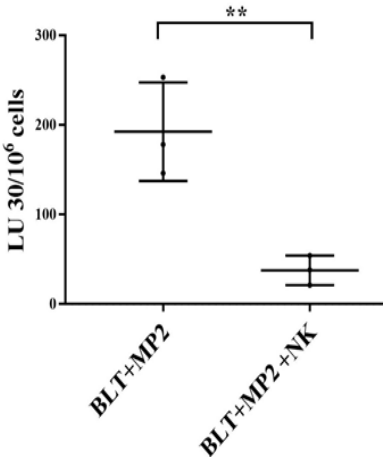
A.


Tumor implantation in the pancreas
Tail vein injection of super-charged NK cells
Euthanasia/Tissues harvesting/ single cell preparation of tissues
0 days
7 days
14 days
21 days
28 days
35 days

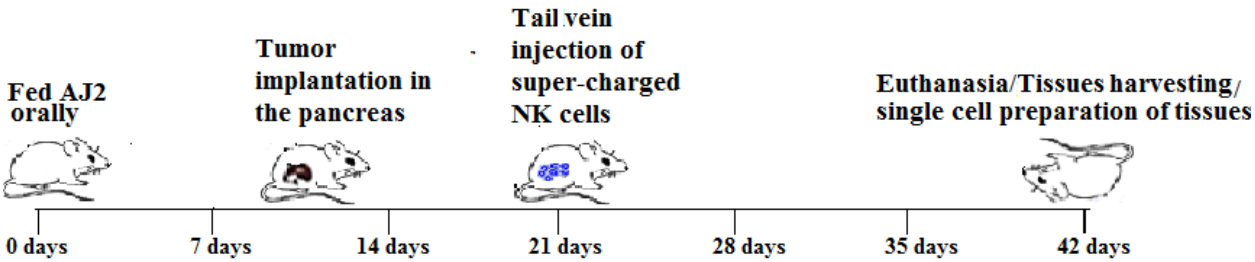
B.


MHC1
CD45+CD3+
BLT+MP2
+
BLT+MP2+NK
+++
BLT+Diff-MP2
NA
0.66
2.71
96.6
0.029
3.12
6.08
90.7
0.13
3.91
6.28
89.8
0
CD45-FITC
CD3-PE

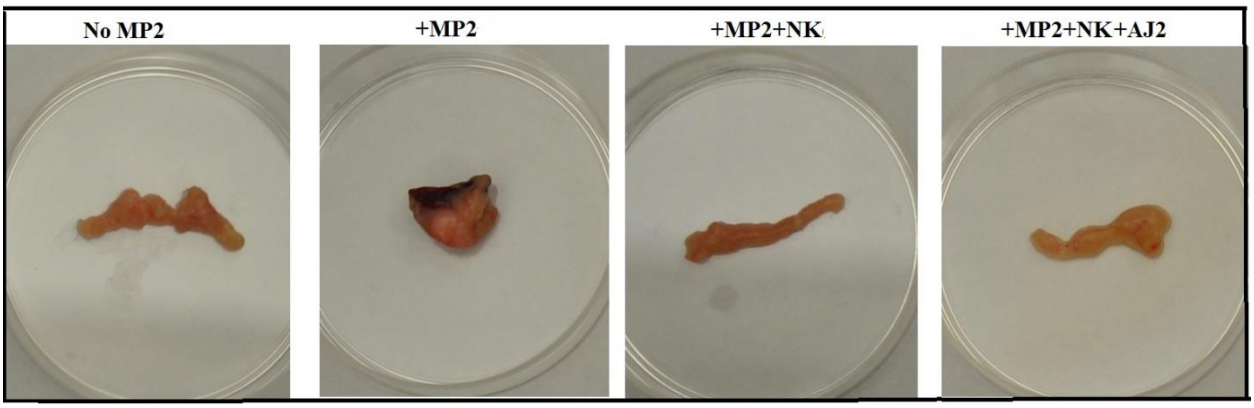
C.


**
LU 30/10^6 cells
300
200
100
0
BLT+MP2
BLT+MP2+NK

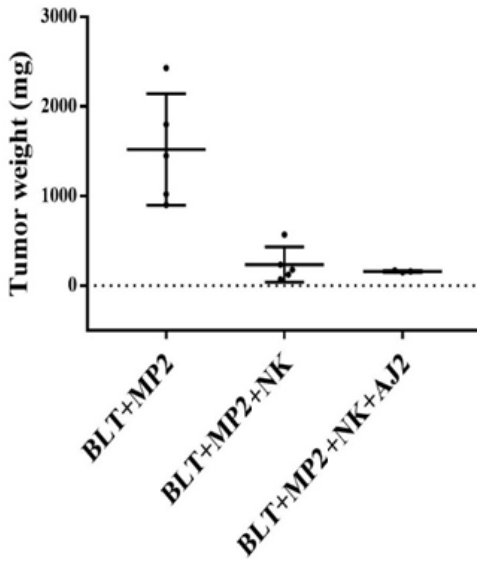
D.


Fed AJ2 orally
Tumor implantation in the pancreas
Tail vein injection of super-charged NK cells
Euthanasia/Tissues harvesting/ single cell preparation of tissues
0 days
7 days
14 days
21 days
28 days
35 days
42 days

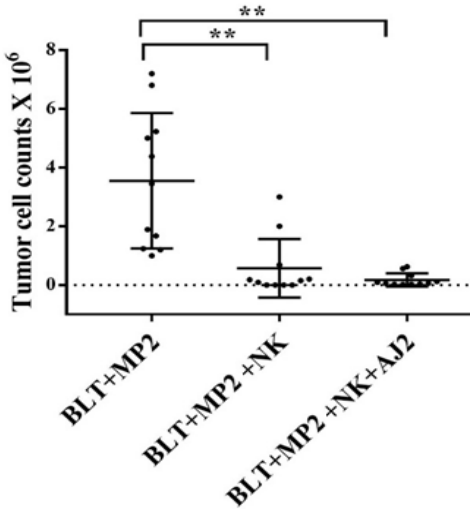
E.


No MP2
+MP2
+MP2+NK
+MP2+NK+AJ2

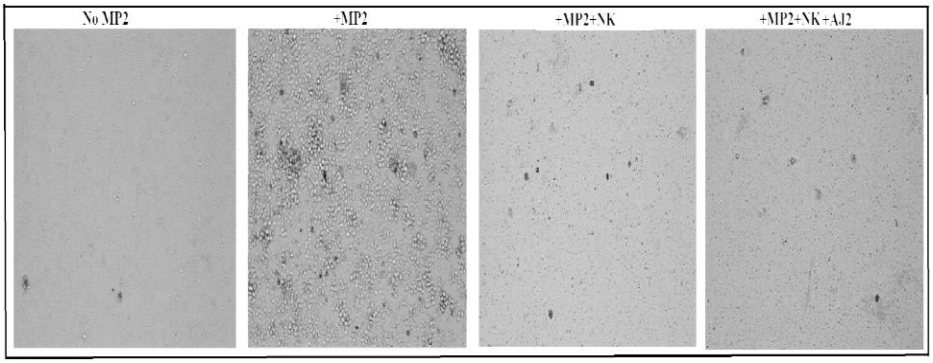
F.


Tumor weight (mg)
3000
2000
1000
0
BLT+MP2
BLT+MP2+NK
BLT+MP2+NK+AJ2

G.


Tumor cell counts X 10^6
8
6
4
2
0
**
**
BLT+MP2
BLT+MP2+NK
BLT+MP2+NK+AJ2

H.



I.

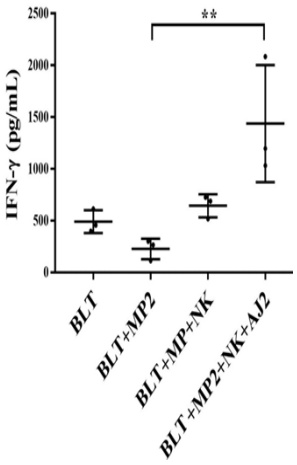
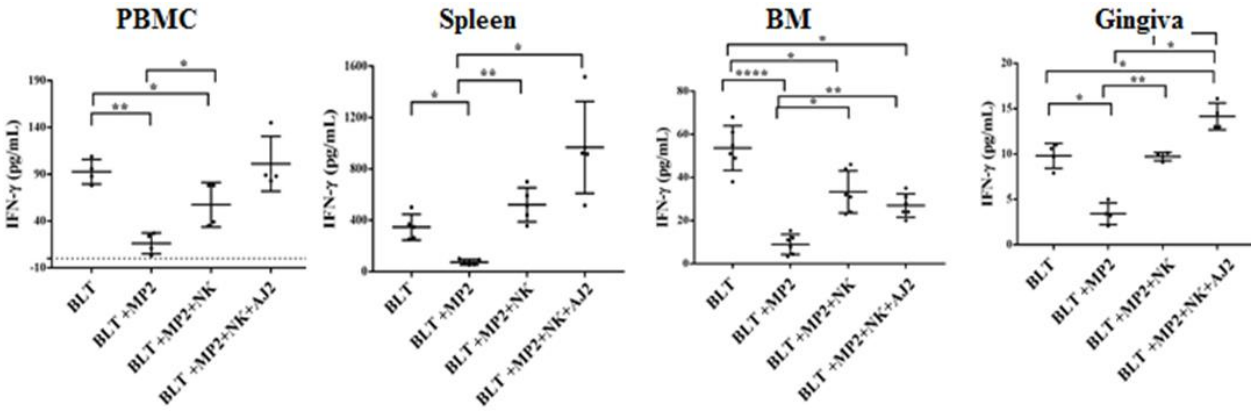


Figure 2: Single injection of expanded hu-NK cells inhibited the growth of poorly differentiated MP2 cancer stem cells in BLT mice and was further inhibited with combination of the probiotics.

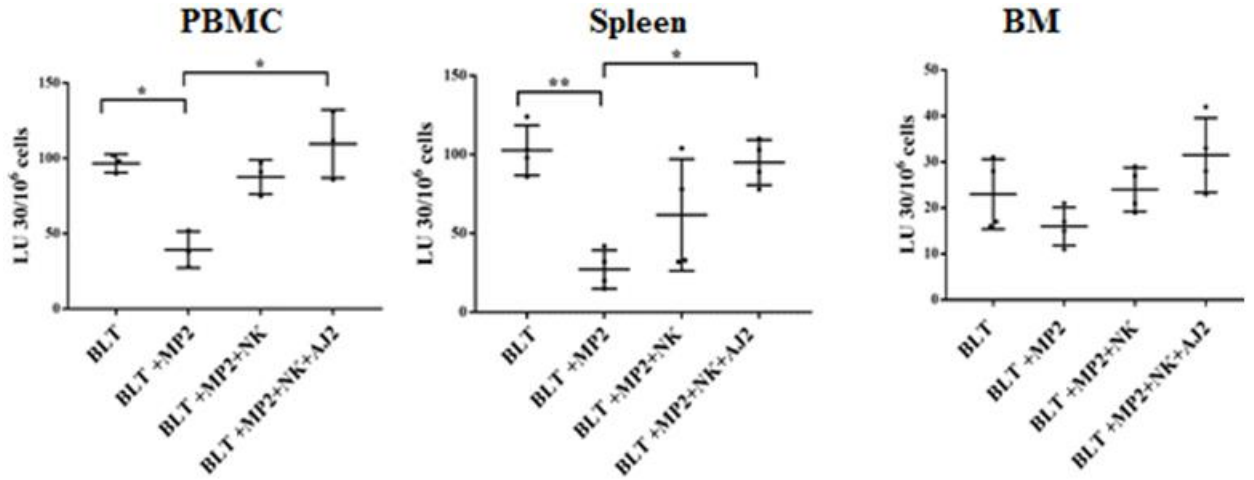
Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) were orthotopically injected with $1x10^6$ of human MP2 or diff-MP2 into the pancreas. One week after tumor implantation selected hu-BLT mice received 1.5 X 10^6human expanded NK cells via tail vein injection. Disease progression and weight loss was monitored for another 3-4 weeks **(A)**. Animals were sacrificed and tumor pictures were taken post mortem **(B)**, surface expression of human MHC1, CD45 and CD3 was analyzed on the surface of freshly dissociated tumor cells **(B)**. Purified NK cells ($1X10^6$ cells/ml) from the peripheral blood of the healthy donor were left untreated or treated with IL-2 (1000 units/ml) for 18 hours before they were added to ^{51}Cr labeled MP2 obtained from the BLT animals at various effector to target ratios. NK cell mediated cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the MP2 X100 **(C)**. Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) were orthotopically injected with $1x10^6$ of human MP2 into the pancreas. 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 **(D)**, 4-5 weeks later animals were sacrificed and tumor pictures were taken post mortem **(E)**, and tumor mass was weight **(F)**. Tumor cells from hu-BLT were treated with IL-2 (1000 units/ml), cumulative tumor cell counts from day 0 to day 20 are shown in fig. **(G),** on day 7 after the culture representative images were taken using Nikon TMS inverted microscope **(H)**. Tumor cells from hu-BLT were treated with IL-2 (1000 units/ml), the levels of IFN-γ was determined on day 3 and day 7 harvested supernatants using specific ELISAs **(I)**.

Fig. 3

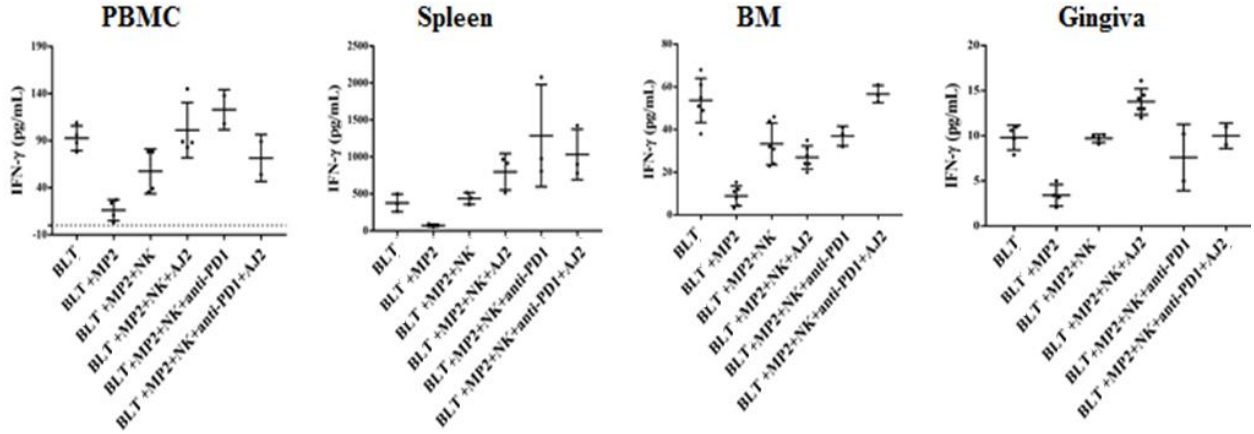
A.


PBMC
Spleen
BM
Gingiva
IFN-γ (pg/mL)
BLT
BLT +MP2
BLT +MP2+NK
BLT +MP2+NK+AJ2

B.


PBMC
Spleen
BM
LU 30/10^6 cells
BLT
BLT +MP2
BLT +MP2+NK
BLT +MP2+NK+AJ2

C.


PBMC
Spleen
BM
Gingiva
IFN-γ (pg/mL)
BLT
BLT +MP2
BLT +MP2+NK
BLT +MP2+NK+AJ2
BLT +MP2+NK+anti-PD1
BLT +MP2+NK+anti-PD1+AJ2

D.

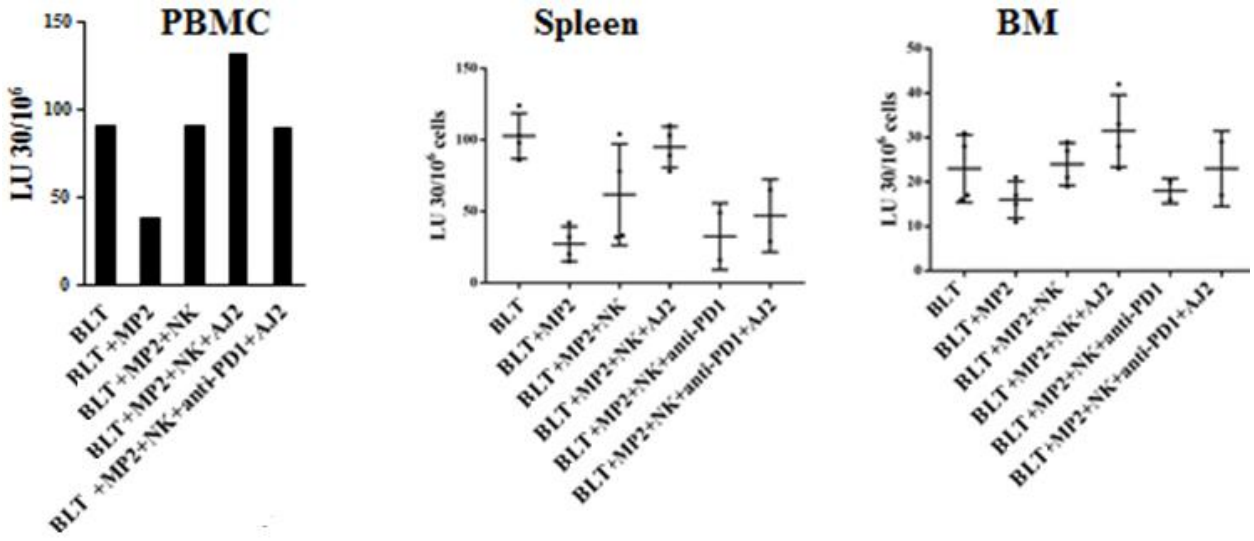
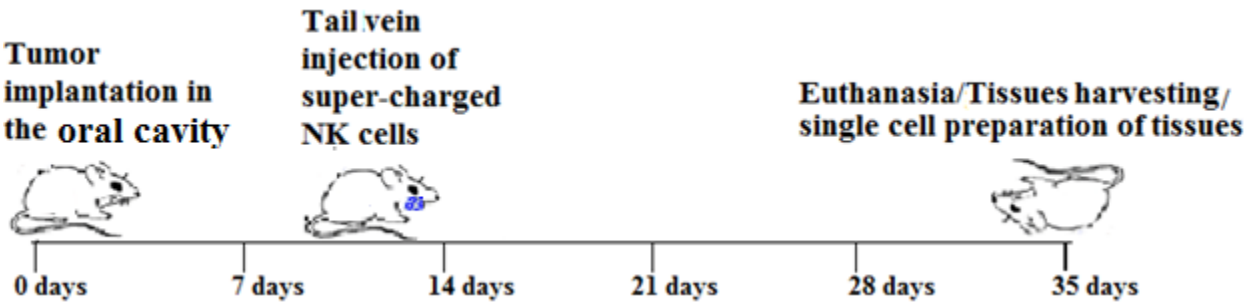
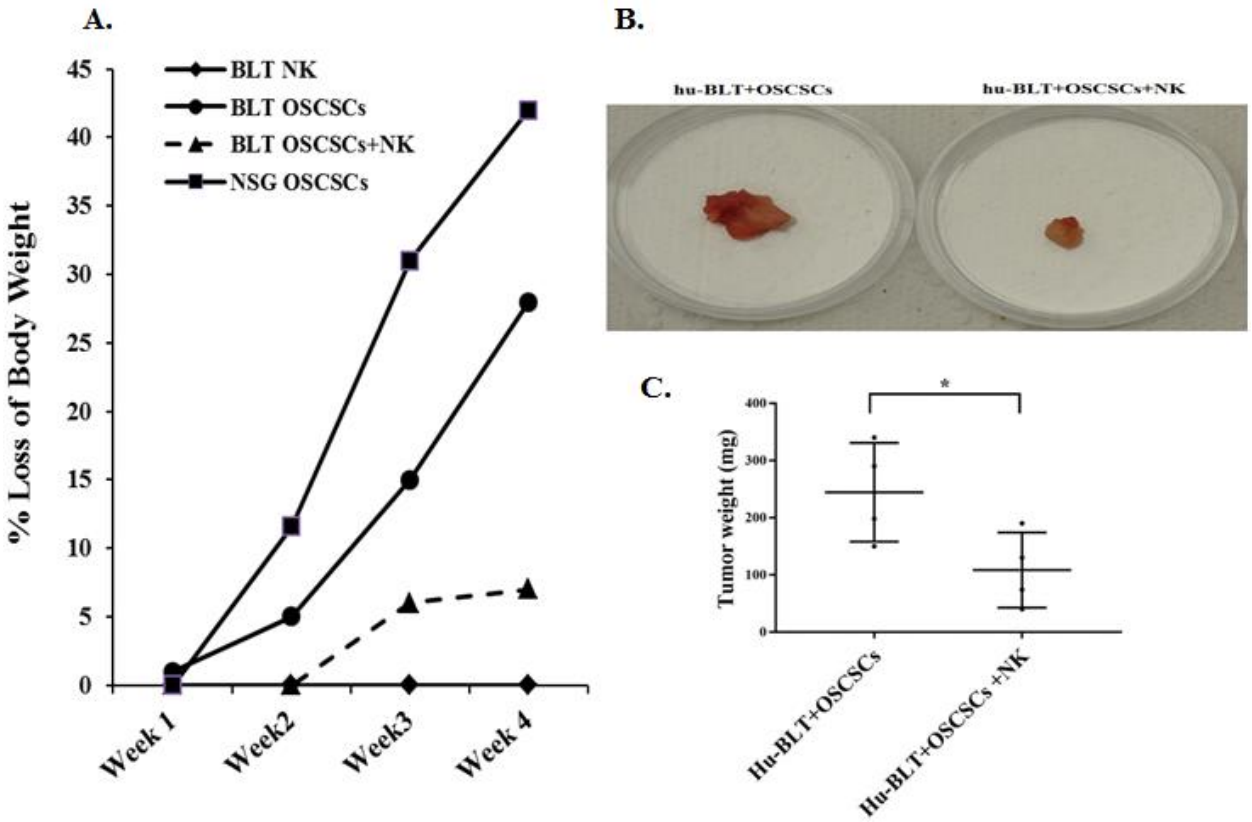


Figure 3: NK cells injection alone and more so in combination with AJ2 and anti-PD1 improved the IFN-γ secretion and cytotoxic function of immune cells in various tissue compartment of the hu-BLT mice.

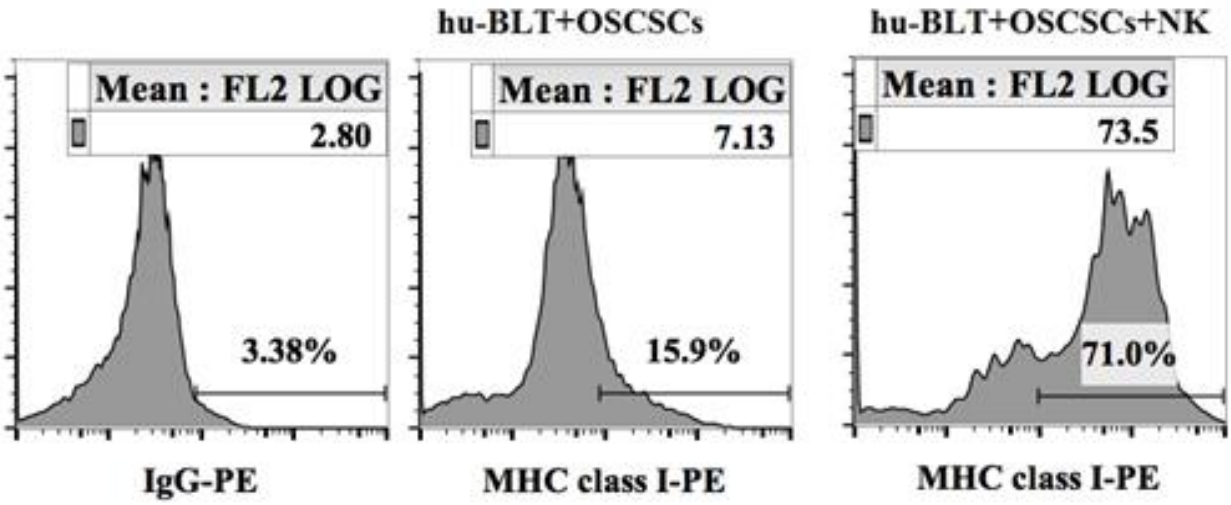
Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) were orthotopically injected with $1x10^6$ of human MP2 into the pancreas. One week after tumor implantation selected hu-BLT mice received 1.5 X 10^6 human expanded NK cells via tail vein injection, animals were orally fed with AJ2 (5 billion) every 48 hours throughout the experiment period **(2D)**. At the end of this experiment spleen, bone marrow, peripheral blood and oral gingiva tissues were collected, single cell suspension was obtained from each tissue as described in Materials and Methods, tissues were cultured with IL-2 activation, the supernatants were harvested on day 7 after the culture, and IFN-γ was determined using single ELISAs **(A)**, and cytotoxicity assay was performed was determined using standard 4-hour ^{51}Cr release assay against OSCSCs and the lytic units $30/10^6$ cells were determined using inverse number of splenocyte, bone marrow and PBMCs **(B)** required to lyse 30% of OSCSCs x 100 **(B)**. Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) 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, a week later the NK cells injections animals were injected with 50 ug/mice anti-PD1 via tail vein, animals were orally fed with AJ2 (5 billion) every 48 hours throughout the experiment period. At the end of this experiment spleen, bone marrow, peripheral blood and oral gingiva tissues were collected, single cell suspension was obtained from each tissue as described in Materials and Methods, tissues were cultured with IL-2 activation, the supernatants were harvested on day 7 after the culture, and IFN-γ was determined using single ELISAs **(C)**, and cytotoxicity assay was performed was determined using standard 4-hour ^{51}Cr release assay against OSCSCs and the lytic units $30/10^6$ cells were determined using inverse number of splenocyte, bone marrow and PBMCs **(D)** required to lyse 30% of OSCSCs x 100 **(D)**.

Fig. 4

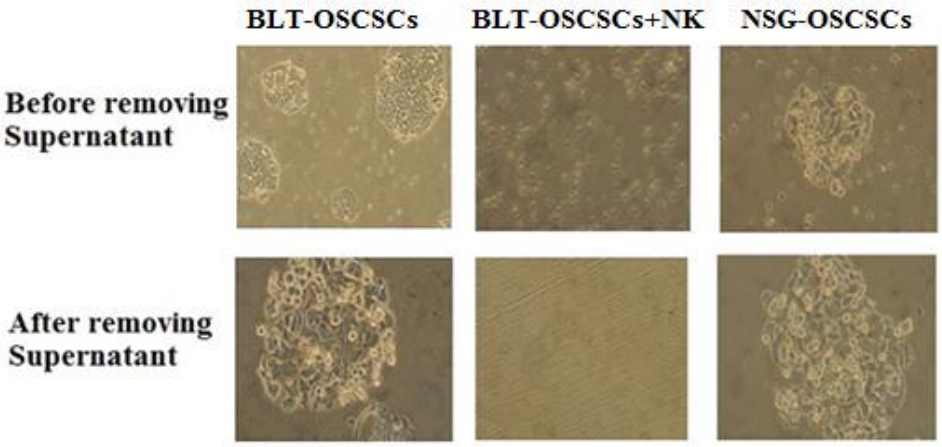

Tumor implantation in the oral cavity
Tail vein injection of super-charged NK cells
Euthanasia/Tissues harvesting/ single cell preparation of tissues
0 days
7 days
14 days
21 days
28 days
35 days


A.
B.
C.
BLT NK
BLT OSCSCs
BLT OSCSCs+NK
NSG OSCSCs
% Loss of Body Weight
Week 1
Week2
Week3
Week 4
hu-BLT+OSCSCs
hu-BLT+OSCSCs+NK
Tumor weight (mg)
Hu-BLT+OSCSCs
Hu-BLT+OSCSCs +NK

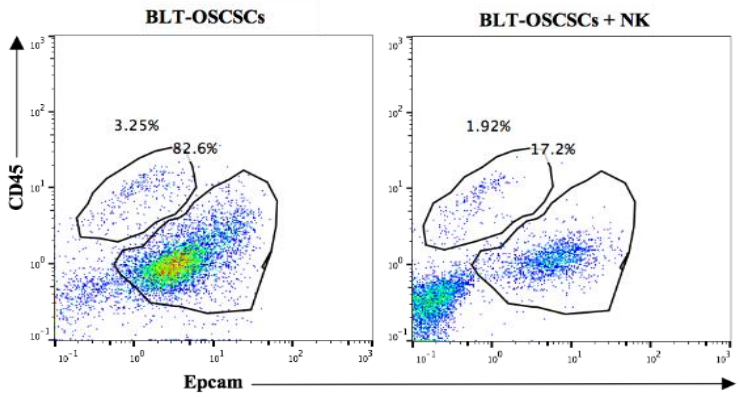
D.


hu-BLT+OSCSCs
hu-BLT+OSCSCs+NK
Mean : FL2 LOG
2.80
3.38%
IgG-PE
Mean : FL2 LOG
7.13
15.9%
MHC class I-PE
Mean : FL2 LOG
73.5
71.0%
MHC class I-PE

E.


BLT-OSCSCs
BLT-OSCSCs+NK
NSG-OSCSCs
Before removing Supernatant
After removing Supernatant

F.


BLT-OSCSCs
BLT-OSCSCs + NK
3.25%
82.6%
1.92%
17.2%
CD45
Epcam

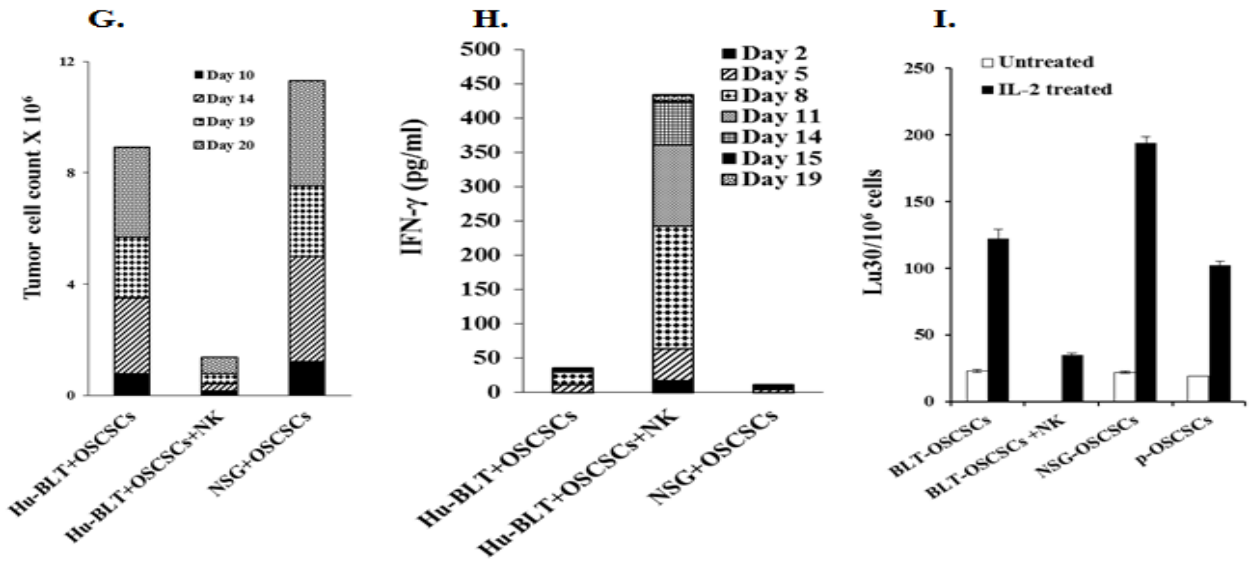


Figure 4: Single injection of expanded hu-NK cells inhibited the growth of primitive oral cancer stem cells in BLT mice and was further inhibited with combination of the probiotics.

Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) and NSG mice were orthotopically injected with $1x10^6$ of human OSCSCs into the flora of the mouth. One week after tumor implantation selected hu-BLT mice received 1.5 X 10^6human 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)**, and tumor mass was weight **(C)**. At the end of this experiment oral tumors were harvested and single cells suspensions were prepared as described in Materials and Methods. Surface expression of human MHC1 was determines using flow cytometry (**D)**. At the end of this experiment oral tumors were harvested and single cells suspensions were prepared as described in Materials and Methods. Oral tumors from the animals were cultured, on day 7 after the culture representative images were taken using Nikon TMS inverted microscope **(E)**. Surface expression of human CD45 and Epcam on the surface of tumors from the hu-BLT mice was determined using flow cytometric analysis after staining with the FITC-conjugated and PE-conjugated antibodies. Isotype control antibodies were used as control **(F)**. Oral tumors cells from hu-BLT and NSG mice were treated with IL-2 (1000 units/ml), and the supernatants were harvested after day 2, day 5, day 8, day 11, day 14, day 15 and day 19, cells were re-suspended in fresh medium and supplemented with additional IL-2 (1000 units/ml) every day supernatant was harvested and the cells were counted, cumulative tumor cell counts from day 0 to day 20 are shown in fig. **(G)**, the levels of IFN-γ was determined on harvested supernatants using specific ELISAs **(H)**. Purified NK cells ($1X10^6$ cells/ml) from the peripheral blood of the healthy donor were left untreated or treated with IL-2 (1000 units/ml) for 18 hours before they were added to ^{51}Cr labeled OSCSCs obtained from the BLT animals at various effector to target ratios. NK cell mediated cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the OSCSCs X100 **(I)**.

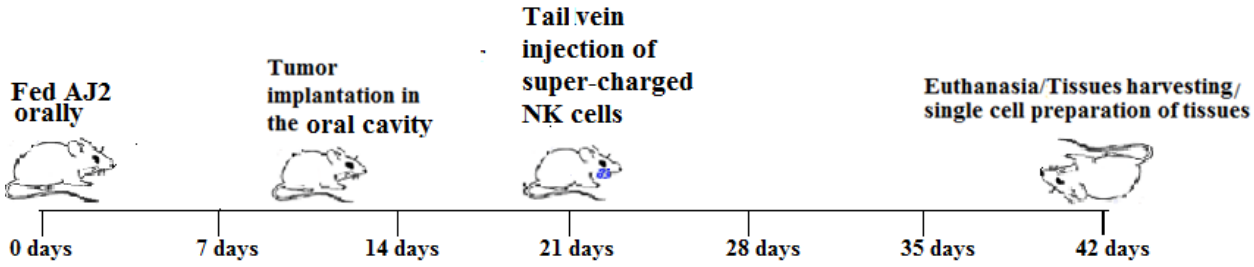
Table 1

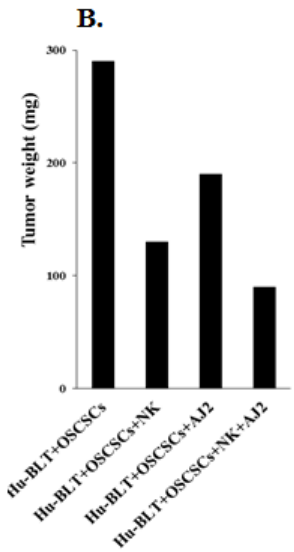
Day 14 culture	Hu-BLT+OSCSCs	Hu-BLT+OSCSCs +NK	NSG +OSCSCs
MHC1	5.3 ± 0.98	101 ± 8.9	12.2 ± 3.4
CD54	23 ± 2.09	48 ± 1.8	20 ± 1.1
hu-CD45	1.42 %	14 %	0.3 %

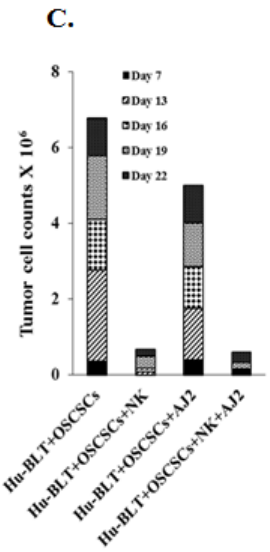
Table 1: Surface analysis of tumor cells from hu-BLT mice

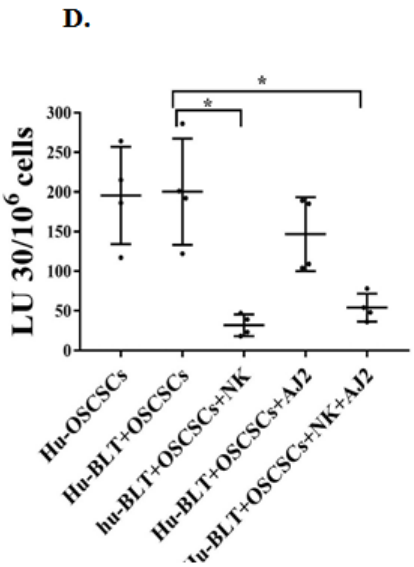
Oral tumors cells from hu-BLT and NSG mice were cultured, day 14 after the culture, tumor cells were detached, and the surface expression of CD54, CD45 and MHC-1 on oral tumors were assesed after staining with the PE-conjugated antibodies. Isotype control antibodies were used as control.

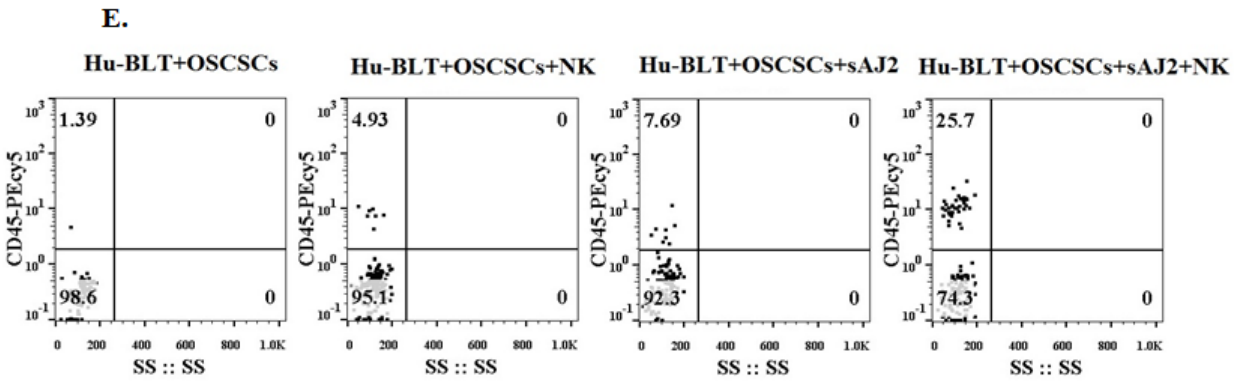
Fig. 5


A.
Fed AJ2 orally
Tumor implantation in the oral cavity
Tail vein injection of super-charged NK cells
Euthanasia/Tissues harvesting/ single cell preparation of tissues
0 days
7 days
14 days
21 days
28 days
35 days
42 days

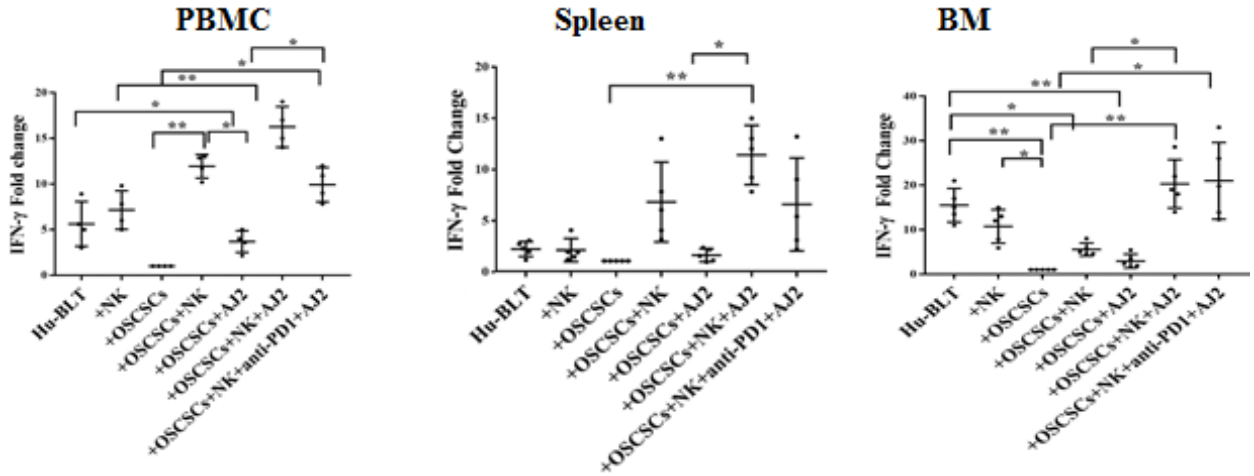

B.
Tumor weight (mg)
Hu-BLT+OSCSCs
Hu-BLT+OSCSCs+NK
Hu-BLT+OSCSCs+AJ2
Hu-BLT+OSCSCs+NK+AJ2


C.
Tumor cell counts X 10⁶
Day 7
Day 13
Day 16
Day 19
Day 22
Hu-BLT+OSCSCs
Hu-BLT+OSCSCs+NK
Hu-BLT+OSCSCs+AJ2
Hu-BLT+OSCSCs+NK+AJ2


D.
LU 30/10⁶ cells
Hu-OSCSCs
Hu-BLT+OSCSCs
hu-BLT+OSCSCs+NK
Hu-BLT+OSCSCs+AJ2
Hu-BLT+OSCSCs+NK+AJ2


E.
Hu-BLT+OSCSCs
Hu-BLT+OSCSCs+NK
Hu-BLT+OSCSCs+sAJ2
Hu-BLT+OSCSCs+sAJ2+NK
CD45-PEcy5
SS :: SS
1.39
98.6
4.93
95.1
7.69
92.3
25.7
74.3

F.


PBMC
Spleen
BM
IFN-γ Fold change
Hu-BLT
+NK
+OSCSCs
+OSCSCs+NK
+OSCSCs+AJ2
+OSCSCs+NK+AJ2
+OSCSCs+NK+anti-PD1+AJ2

G.

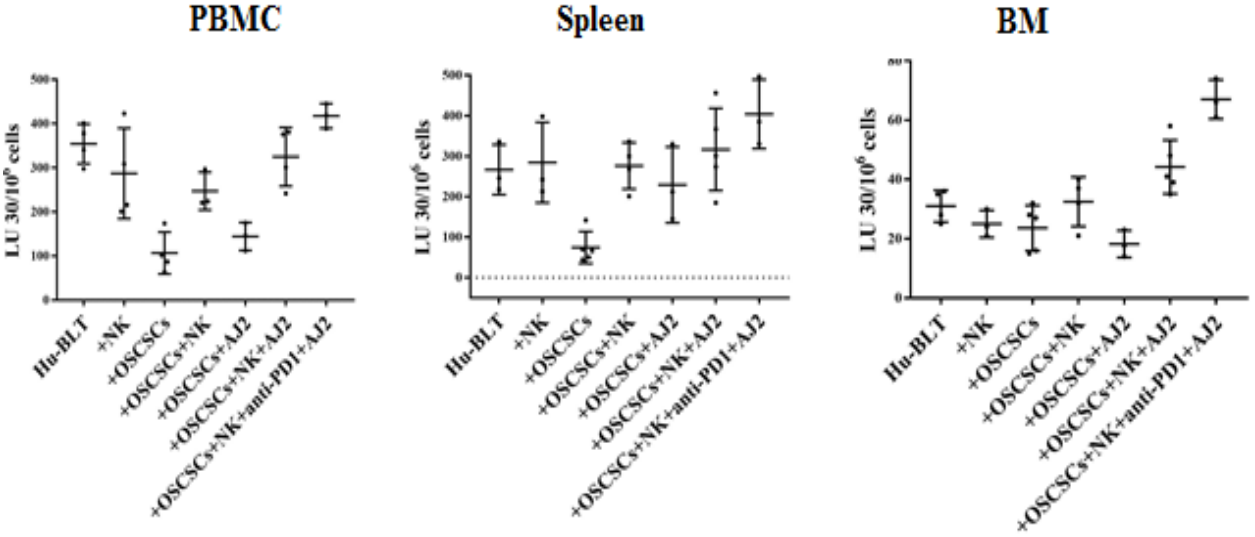

PBMC
Spleen
BM
LU 30/10^6 cells
Hu-BLT
+NK
+OSCSCs
+OSCSCs+NK
+OSCSCs+AJ2
+OSCSCs+NK+AJ2
+OSCSCs+NK+anti-PD1+AJ2

Figure 5: NK cells injection alone and more so in combination with AJ2 and anti-PD1 improved the IFN-γ secretion and cytotoxic function of immune cells in various tissue compartment of the hu-BLT mice.

Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) 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 **(A)**.Disease progression and weight loss was monitored for another 3-4 weeks, animals were sacrificed and tumor mass was weight **(B)**. Oral tumors from the animals were cultured, the number of attached tumor cells were measured on day 7, day 13, day 16, day 19 and day 22 **(C)**. Purified NK cells ($1X10^6$ cells/ml) from the peripheral blood of the healthy donor were left untreated or treated with IL-2 (1000 units/ml) for 18 hours before they were added to ^{51}Cr labeled OSCSCs obtained from the BLT animals at various effector to target ratios. NK cell mediated cytotoxicity was determined using a standard 4 hour ^{51}Cr release assay. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of the OSCSCs X100 **(D)**. Surface expression of human CD45 on the oral tumors from hu-BLT mice was determined using flow cytometric analysis. Isotype control antibodies were used as control **(E)**. Successfully reconstituted BLT (levels and lineages of T cells comparable to healthy donors) 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, a week later the NK cells injections animals were injected with 50 ug/mice anti-PD1 via tail vein, 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, tissues were cultured with IL-2 activation, the supernatants were harvested on day 7 after the culture, and IFN-γ was determined using single ELISAs **(F)**, and cytotoxicity assay was performed was determined using standard 4-hour ^{51}Cr release assay against OSCSCs and the lytic units $30/10^6$ cells were determined using inverse number of splenocyte, bone marrow and PBMCs **(G)** required to lyse 30% of OSCSCs x 100 **(G)**.

Sub-aim 3: Increased surface expression of CD8+T cells on the immune cells in various tissue compartments of hu-BLT mice injected with super-charged NK cells.

Increased proportions of CD3+T cells and CD8+T cells in various tissue compartments when hu-BLT mice were injected with NK cells or the combination of NK cells injections and AJ2 feeding.

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 CD3+ 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), there was increased HLADR+CD11b+ cells in bone marrow flow-through cells in the animals injected with NK cells and more so with the combination of NK cells and AJ2 (Table 1).

Table 1

PBMCs Day 0 CD45+	CD16+56	CD3
AJ2 control	9.67 (3.41)	66.0
OSCSCs	28.2 (8.97)	44.6
OSCSCs+NK	12.4 (4.46)	67.6
OSCSCs+AJ2	20.4 (7.57)	50.2
OSCSCs+NK+AJ2	10.0 (4.64)	72.8

Spleen Day 0 CD45+	CD16+56	CD3
AJ2 control	0.98	55.8
OSCSCs	2.86	38.2
OSCSCs+NK	0.81	64.1
OSCSCs+AJ2	3.66	52.1
OSCSCs+NK+AJ2	1.50	69.9

BM Day 0 CD45+	CD16+56	CD3	HLADR+CD11b
AJ2 control	0.91	2.14	10.2
OSCSCs	1.54	2.12	11.1
OSCSCs+NK	1.45	2.36	15.7
OSCSCs+AJ2	1.83	1.79	11.2
OSCSCs+NK+AJ2	2.51	5.98	16.1

Table 1: Single injection of super-charged NK cells increased the proportions on T cells in various tissues of hu-BLT mice and these proportions were increased further with the combination of AJ2.

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 anti-human CD3, CD16, and CD56 antibodies, bone marrow cells were also stained with anti-human HLADR and CD11b, and analyzed by flow cytometry. Isotype control antibodies were used as a control.

Table 2

BM Day 11 CD45+	CD16+56	CD3+	CD3+ CD8+
AJ2 control	67.4	8.32	4.70
OSCSCs	63.1	6.31	4.43
OSCSCs+NK	54.5	15.22	8.70
OSCSCs+AJ2	57.5	5.22	3.30
OSCSCs+NK+AJ2	48.5	37.02	15.8

Table 2: Single injection of super-charged NK cells increased the proportions on T cells, especially CD8+T cells in BM of hu-BLT mice and these proportions were increased further with the combination of AJ2.

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 bone marrow flow-through cells were collected, single cell suspension was obtained as described in Materials and Methods. Bone marrow flow-through cells were cultured with IL-2, on day 11 after the culture, bone marrow flow-through cells with anti-human CD3, CD8, CD16, and CD56 antibodies, bone marrow cells were also stained, and analyzed by flow cytometry. Isotype control antibodies were used as a control.

Fig. 1

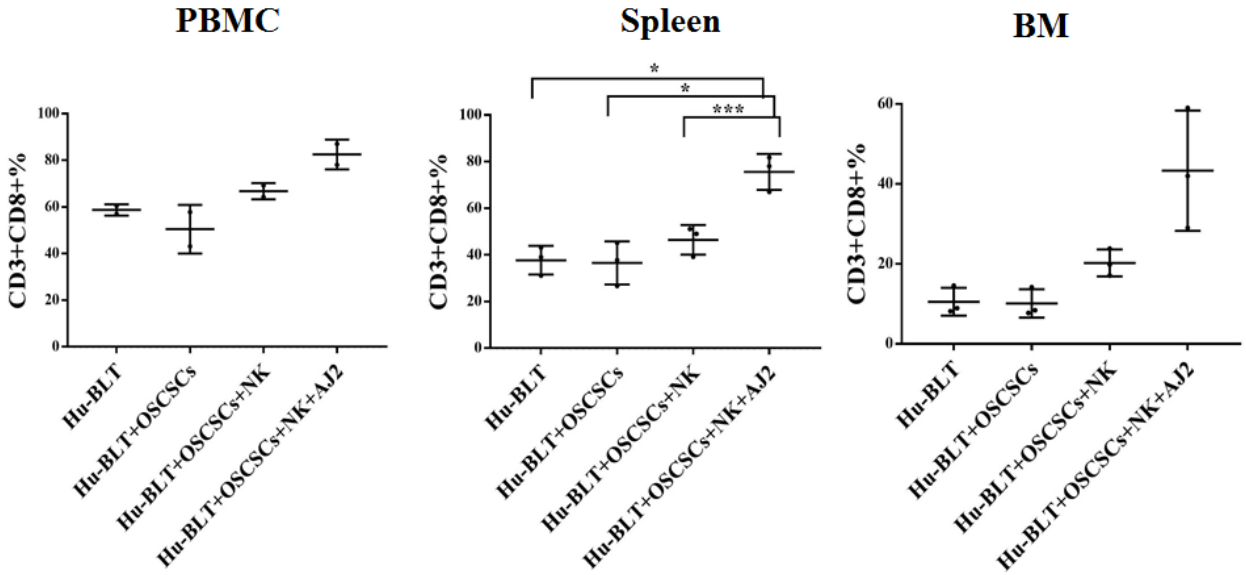


Figure 1: Single injection of super-charged NK cells increased the proportions on CD8+T cells in various tissues of hu-BLT mice and these proportions were increased further with the combination of AJ2.

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 anti-human CD3 and CD8 antibodies and analyzed by flow cytometry. Isotype control antibodies were used as a control.

Discussion of chapter 3

Osteoclast-expanded NK cells exhibit higher cytotoxic and cytokine secretion activity, both required to either target and differentiate the cancer stem cells. Potential mechanisms which govern pancreatic tumor cell survival and differentiation has not been fully understood and is the subject of this report. We demonstrated that NK cells play a significant role in limiting growth and expansion of pancreatic and oral tumors in vitro and in vivo. Stem-like/poorly differentiated pancreatic and oral tumors are lysed by the NK cells whereas their differentiated counterparts are more resistant to NK cell mediated lysis. In addition, NK cells are able to limit growth and expansion of pancreatic and oral tumors by providing key cytokines such as IFN-γ and TNF-α which drive differentiation of stem-like/poorly differentiated pancreatic and oral tumors. In this regard, secreted or membrane bound IFN-γ and TNF-α [46] are required for the induction of tumor resistance to NK cell mediated cytotoxicity, whereas IFN-γ plays a dominant role in up-regulation of surface receptor expression and inhibition of both tumor growth and inflammatory cytokine release, since the addition of antibody to IFN-γ and TNF-α abrogated resistance whereas IFN-γ antibody alone in the absence of TNF-α antibody was able to inhibit cell surface receptor expression while restoring tumor growth and release of inflammatory mediators. Therefore, tumor differentiation by the NK cells not only limit cell growth but also provides the means to inhibit inflammatory cytokine release which could fuel tumor growth. Indeed, as shown in this study, poorly differentiated pancreatic tumors formed large tumors and metastasized to liver and lungs in NSG mice whereas their NK differentiated tumors were unable to form tumors in the pancreas. In addition, well-differentiated pancreatic tumors when implanted in the pancreas formed much smaller tumors and the mice survived for a long period of time, whereas those implanted with stem-like/poorly differentiated tumors all died a few weeks after implantation and tumor growth.

Continuous presence of IL-2+anti-CD16mab treated NK supernatants were required for the maintenance of the tumors in differentiated state. In a previous study. we have shown when NK supernatants were removed from the differentiated oral tumors, they reverted to their stem-like stage within 2 weeks after removal of supernatants [46]. Although all the differentiated tumors expressed higher levels of MHC class I, B7H1 and CD54 upon differentiation, after the removal of the supernatants the levels decreased gradually from day 0 to day 2 and then days 6 to12 and by day 12 they were almost identical to the stem-like tumors. The reverted tumors became highly susceptible to NK cell-mediated cytotoxicity, grew rapidly and induced significant secretion of cytokines [46]. Moreover, when they were injected to NSG animals they formed large tumors and metastasized to the liver. Therefore, to maintain the tumor cells in a differentiated state the continued presence of competent NK cells is required for the prevention of metastasis and the progression of cancer.

It is interesting to note that stem-like/poorly differentiated MP2 tumors as well as stem-like oral tumors were easily grown in mice reconstituted with competent human immune cells in hu-BLT mice. These tumors grew within 3-5 weeks in the presence of competent T and B cells. Although the mice have NK cells, the average numbers are significantly lower in the peripheral blood when compared to humans. Thus, such reconstituted animals are great models to study not only the effect of NK cells on tumors but also their effect on stimulating T cells for effective eradication of tumor cells. Similar, to NSG mice, NK supernatant differentiated MP2 tumors did not grow or form tumors in hu-BLT mice. In addition, PBMCs obtained from BLT mice implanted with NK differentiated tumors, even though contained lower percentages of NK cells, mediated significant cytotoxicity against NK sensitive target cells, whereas higher percentages of NK cells from blood of MP2 implanted tumors did not mediate cytotoxicity. These results are similar, to

those which are seen in cancer patients. In addition, no loss of CD3+ T cells in the blood of NK supernatant differentiated tumors could be observed, whereas significant loss of CD3+ T cells could be observed in the blood of MP2 implanted tumors.

Single injection of expanded potent NK cells was able to prevent tumor growth and mediated significant differentiation of the tumors as evidenced by increased surface receptor expression of B7H1, CD54 and MHC class I, and resistance of tumor to NK cell mediated cytotoxicity. Collectively, these results are in great agreement with our *in vitro* studies and indicate that differentiation is a very important step in the inhibition of tumor progression and cessation of inflammation. It is likely that continued supplementation with NK cells may be required for the complete eradication of the tumors since the removal leads to reversion of the tumor growth [46]. Injection of expanded NK cells also prevented loss of CD3+ T cells from the peripheral blood and restored the cytotoxic function and IFN-γ secretion of peripheral blood NK cells, although the levels were still less when compared to that obtained from differentiated MP2 implanted tumors in mice. Both MP2 and MP2+NK implanted mice exhibited higher numbers of CD16+ cells in the peripheral blood, which were higher than that seen in the peripheral blood of NK differentiated MP2 implanted mice. The increase may be due to different mechanisms, since in MP2 implanted mice it could be due to the loss of CD3+ T cells, leading to increased populations of other immune cells including NK cells, whereas in MP2+NK implanted mice it could be the maintenance of the increased NK percentages since no loss of CD3+ T cells were observed. In addition, BLT mice injected with NK with no tumors contain a large number of CD3- cells.

NK cells injected in MP2 implanted tumors may also be able to provide necessary stimuli to activate other effector cells such as NKT cells, CD4+ and CD8+ T cells and gamma-delta T cells. Indeed, when expansion of NK cells was carried out for much longer time points (20-30

days), a subpopulation of CD3+CD56+ and CD3+CD56- T cells was observed (chapter 1). When the growth dynamics of MP2 and MP2+NK dissociated, tumors were followed, a greater potential for the growth was seen in tumors from MP2 implanted mice, resulting in a sustained elevation of tumor growth as compared to that of MP2+NK dissociated tumors. Indeed, it required much longer time for tumors to attach and grow from MP2+NK mice. Addition of IL-2 to tumor cells further reduced tumor numbers for MP2+NK tumors, however, at the beginning IL-2 stimulated tumor growth in MP2 tumors, especially the number of unattached tumors resembling spheres rapidly grew. After two to three transfers, IL-2 stimulations started reducing the tumor numbers in MP2 tumors indicating perhaps an activation event for infiltrating immune cells might have been underway during the transfers. Indeed, when the total number of tumors (both attached and floating) were counted at days 7, 10 and 14 IL-2 supplementations was able to reduce the number of tumors from the tumors of MP2 implanted mice to the levels obtained from non-IL-2 supplemented MP2+NK tumor. In addition, B7H1 expression from IL-2 supplemented MP2 tumors reached levels comparable to non-IL-2 supplemented MP2+NK tumors. These results indicate that NK injection provides the activation step to result in the differentiation of tumors in MP2+NK implanted mice, whereas in mice with MP2 tumors many tumors remained at the stem-like/poorly differentiated state.

Single delivery of potent NK cells after injection of oral tumors decreased the size of the tumors in mice and prevented weight loss substantially when compared to either NSG mice or BLT-NSG mice injected with tumors in the absence of NK cell injection. The percentages of T cells and NK cells remained similar between BLT-NSG mice injected only with NK cells as compared to those injected with both oral tumors and NK cells in blood, spleen and liver, whereas the levels of T cells were decreased and B cells increased in oral tumor injected in the absence of

NK cells. Decreased levels of NK cells were observed in oral tumor injected mice. Secretion of IFN-γ remained high by the cells of blood and spleen, as well as NK cells enriched from the spleen, whereas levels of IL-8 and IL-6 secretion were variable, exhibiting higher secretion by cells obtained from oral tumor and NK injected mice (data not shown). In contrast liver-derived cells from oral tumor and NK injected mice demonstrated lower secretion of IFN-γ, IL-8 and IL-6 when compared to oral tumor injected and no NK cell (data not shown). These results demonstrated that NK injected mice retained the ability to produce higher secretion of IFN- γ.

Oral tumors obtained from NK injected mice remained very small and contained approximately 3-9 fold more CD45+ cells and produced higher amounts of IFN-γ in all tested time points when compared to oral tumor injected mice in the absence of NK cells. When tumors were dissociated and similar numbers of cells were cultured in each well no tumors were able to grow from oral tumor and NK injected mice, whereas large numbers of tumors were obtained from mice injected with oral tumors in the absence of NK injection and from tumor-injected NSG mice. When enough tumor cells could be obtained from oral tumor and NK injected mice, the same number of tumors from each mouse were cultured, and only tumors from oral tumor injected BLT and NSG mice could grow and proliferate, whereas those obtained from oral tumor/NK did not grow significantly. Interestingly, tumors obtained from oral tumor injected BLT and NSG mice exhibited higher cell death when compared to oral tumor/NK injected mice. This is similar to the results seen in our *in vitro* culture of tumors differentiated with NK cell supernatants in which decreased growth and expansion was seen when compared to stem-like tumors [46]. Because NK differentiated tumors exhibited resistance to NK cell-mediated cytotoxicity in the *in vitro* differentiation experiments, we used tumor cells obtained from the three sets of mice to assess cytotoxicity. Oral tumors from NK injected mice exhibited significant resistance whereas those

from oral tumor injected BLT and NSG were highly susceptible to NK cell mediated cytotoxicity, reflecting the *in vitro* differentiation studies. Increased resistance to NK cell mediated cytotoxicity by oral tumor/NK injected tumors correlated with increased expression of MHC class I, CD54 and B7H1 as established previously in *in vitro* analyses. Reversion of differentiated phenotype, i.e., decrease in MHC class I, CD54 and B7H1 after a month of culture, of tumors from oral tumor/NK injected tumors coincided with the gain of their susceptibility to NK mediated cytotoxicity (data not shown). These results indicate that continuous secretion of IFN-γ by NK cells or NK activated CD3+ T cells is crucial for maintenance of tumor differentiation for the inhibition of tumor growth.

Hu-BLT mice contain lower frequencies of NK cells (1% to 3%) and it appears that the reason why stem like/ poorly differentiated oral tumors are established in these mice is due to low numbers of NK cells, since the tail-vein delivery of NK cells to mice differentiate the tumors injected in the oral mucosa, resulting in their growth inhibition and decreased proliferation. In addition, NK injected tumor-bearing mice contain higher percentages of CD3+CD8+ T cells, indicating the significance of NK cells in the recruitment of T cells to the tumor microenvironment. However, the percentages of tumor infiltrating NK cells remain very low, which could be due to increased induction of activation-induced cell death of NK cells within the tumor microenvironment.

When tumors were dissociated, and grown for more than 30 days, tumors of NK injected mice reverted to their stem-like stage and demonstrated decreases in differentiation antigens, increased susceptibility to NK cell-mediated cytotoxicity and increased growth and proliferation. In addition, osteoclasts from NK injected oral tumor and *in vitro* differentiated oral tumor recipients increased NK cell expansion obtained from human donors more than osteoclasts from mice injected with oral tumors in the absence of NK cells (Chapter 1). In addition, when NK cells

were isolated from these tumor-bearing mice and cultured in the presence of autologous monocytes, NK cells from NK injected oral tumor and *in vitro* differentiated oral tumor recipients retained their cytotoxic function when compared to NK cells from mice either injected with tumors alone or those injected with NK differentiated oral tumors in the presence of antibodies to TNF-α and IFN-γ (Chapter 1).

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Summary

NK cells are known to limit growth and expansion of pancreatic and oral tumors by providing key cytokines such as IFN-γ and TNF-α, which drive differentiation of stem-like/poorly differentiated pancreatic and oral tumors. The detailed understanding of how and why their function is affected by the cancer microenvironment is very important for outlining the most efficient NK cell-based immunotherapies for cancer patients. In this regard, we first characterized NK cells from the cancer patients by the IFN-γ secretion, cytotoxic function against the cancer stem cells, and their surface receptors. In previous studies, we demonstrated that IFN-γ plays a dominant role in up-regulation of B7H1, CD54 and MHC1 surface receptor expression and inhibition of tumor growth by differentiating the cancer stem cells, and via inflammatory cytokine release when they are in contact with cancer stem cells. We also showed that induction of NK cell-mediated cytotoxicity resistance and differentiation in the stem cells correlated with the increased expression of CD54, B7H1, and MHC class I, and mediated by the combination of membrane-bound or secreted IFN-γ [1]. In this study, we demonstrated that NK cells from cancer patients secreted lower IFN-γ secretion. When we used the NK cells condition media adjusted based same amount of secreted IFN-γ from a healthy donor and a cancer patient, condition media from the cancer patient NK cells was less capable of differentiating the cancer stem cells. The cancer stem cells treated with patient NK cells condition media, were more susceptible to NK cell-mediated cytotoxicity and displayed lower surface expression of CD54, MHC1, and B7H1 when compared to cancer stem cells treated with healthy IFN-γ.

NK cells express CD16, the low affinity Fcγ receptorfor immunoglobulins (FcγRIIIa) and mediate antibody-dependent cell mediated cytotoxicity (ADCC), which is considered an important

effector mechanism of many therapeutic antibodies [2-5]. NK cells anti-tumor response is regulated by the balance between the numerous activating and inhibitory receptors, among those we focus on NKG2D and CD16, these two play an important role in NK cell anti-tumor response through antigen recognition [6]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in oral and ovarian cancer patients [7, 8]. In addition, down-regulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [9]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [9]. Triggering of CD16 on NK cells by anti-CD16 antibody, which mimics the ligand binding effect, was found to down-modulate CD16 receptors. This causes a great loss of cytotoxicity and gain in cytokine secretion in NK cells which we have previously coined as "split anergy" [1, 9-15]. In this study, we demonstrated that NKG2D and CD16 surface expression was lower in cancer patients. NK cell-mediated ADCC was lower in cancer patients when compared to healthy donors. When CD16 receptor on NK cells was down-modulated, fold increase in IFN-γ secretion and fold decrease in cytotoxicity (split anergy) was lower in cancer patient NK cells when compared to healthy NK cells.

We also demonstrated that among all the cells we characterized to design a novel strategy to expand highly potent NK cells, osteoclasts (OCs) fit best based on the NK receptors ligand expression and secreted cytokines required for NK cells expansion and activation. In this regard, we tested the cancer patient osteoclasts (OCs) for their surface expression of MHC1 and MICA/B, both were displayed lower on cancer patient OCs. So, not just the NK cells receptors required for tumor recognition are lower on cancer patient NK cells, but the ligands required to increase the expression of those receptors are also down-modulated to cancer patient OCs. Next, we cultured

the cancer patient NK cells with healthy donor OCs, we were able to increase the surface expression of NKG2D on patient NK cells but, again the cytotoxic activity and IFN-γ were lower in patient NK cells. We confirmed our finding by analyzing the OCs from KRAS mutated mice and hu-BLT mice injected with tumor.

Based on our data, we were not convinced in the use of autologous NK cells to treat the cancer patients, so the based strategy for using expanded NK cells as cancer cell-immunotherapy would be allogeneic NK cells. Next, we tested the expanded allogeneic NK cells in tumor implanted hu-BLT mice. With just one NK cell injection (1.5 million cells), tumor growth was inhibited in the mice. On analyzing the immune cell compartments, NK cell-mediated cytotoxicity and IFN-γ from immune cells was improved significantly. Tumors dissected from the NK therapy received animals showed differentiation profile, high MHC1, CD54 and B7H1 on their surface and lower growth in the culture when compare to tumor dissected from hu-BLT mice without NK cell immunotherapy. 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 super-charged NK cells, and inject the high purity NK cells.

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Future direction

Future direction for the study would be to investigate the underlying mechanism of osteoclasts and NK cells, or other immune cells *in-vivo* and *ex-vivo* under both physiological and pathological conditions especially skeletal disorders and cancer metastasis to the bone. One of the major aspect need to be investigate, the signaling pathways involved in the osteoclasts and lymphocytes interaction, both under physiological and pathological conditions. All these studies could provide us new strategies to generate the equipment required to deal with the major health issues. While this study, we generated preliminary data showing that T cells expanded by dendritic cells (DCs) maintained higher numbers of CD4+T, and as we discussed in this study OCs expanded T cells maintained higher number of CD8+T cells. This need to be investigated further why OCs expand more CD8+T cells while DCs expand more CD4+T cells. In cancer patients PBMCs, CD8+T numbers were higher than CD4+T cells. This provide us clue that cancer patients DCs need to be screened, it is possible that DCs in cancer patients are also disable to activate the T cells, there may be down-modulation of T cell co-stimulatory ligands or factors required to maintain the balance to immune cells. Another cancer immunotherapy strategy can be investigated in *in-vivo* studies using hu-BLT mice is to inject them with the condition media from the OC-expanded NK cells.