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**Proliferation of Highly Functional Human Expanded Natural Killer Cells by
Osteoclasts for Cancer Immunotherapy Against OSCSCs**

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

By

Christian Jesus Mendoza

2016

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2016

ABSTRACT OF THE THESIS

**Proliferation of Highly Functional Human Expanded Natural Killer Cells by
Osteoclasts for Cancer Immunotherapy Against OSCSCs**

By

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Master of Science in Oral Biology

University of California, Los Angeles, 2016

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2016

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INTRODUCTION

Cancer Today

Cancer is a complex heterogeneous disease in which abnormal cells divide without control, able to invade other tissues and metastasize to distant sites. With about 8.2 million cancer deaths in 2012 and around 13.2 cancer deaths projected to occur worldwide by 2030, cancer is clearly one of the most alarming health problems our society faces today [71]. The standard therapeutic procedures currently in practice for all types of cancers include surgery, radiation therapy and chemotherapy. Although these therapies have proven to be highly effective in eradicating the primary tumor, advanced metastatic disease cannot be cured by these measures [71]. Other concerns, such as increasing chemotherapeutic drug resistance, relapse of disease after achieving certain remission, ineffectiveness against cancer stem cells, are other limitations associated with these therapies. Therefore, new and more effective methods of treating these patients are urgently needed.

Natural Killer (NK) Cells

Natural Killer (NK) cells are innate immune effectors that are primarily involved in immunosurveillance to spontaneously eliminate malignantly transformed and virally infected cells without prior sensitization [71]. NK cells trigger targeted attack through release of cytotoxic granules, and secrete various cytokines and chemokines to promote subsequent adaptive immune responses [71]. NK cells represent the first line of defense that mediates direct cytotoxicity as well as antibody-dependent cellular cytotoxicity (ADCC) against a variety of tumor cells, virally-infected cells and neoplasia [2]. Extensive evidence gathered in clinical trials illustrates the importance of NK cell function in the immune system. In a study, a patient lacking active NK

cells was constantly afflicted with a variety of viral infections and recurrent cervical carcinoma [3, 4]. As a result from this and many other examples, numerous studies regard NK cells as a vital component of the immune response that constantly protects an individual from life-threatening infections.

NK cells develop, differentiate and mature in the bone marrow, lymph node, spleen, tonsils and thymus where they then enter into the circulation [5]. These NK cells then have the ability to migrate to inflamed tissues and organs of the body in the direction of proinflammatory stimuli. Normally, effector immune cells detect and target infected cells in the context of major histocompatibility complex (MHC), triggering cytokine secretion, or causing lysis or apoptosis. However, NK cells kill tumor cells without recognizing tumor-specific antigen and MHC, allowing for a much faster innate immune reaction.

NK cells represent a unique subset of lymphocytes, distinct from T and B cells. Human NK cells, which comprise 10-15% of all peripheral blood lymphocytes (PBL), can be divided into two functional subsets based on the amount of CD56 and the lack of CD3 expressed on their surface: CD56^{bright} immunoregulatory cells and CD56^{dim} cytotoxic cells [1]. NK cells are a crucial source of immunoregulatory cytokines and interact with other immune cells to trigger an adaptive, or antigen-specific, immune response [6]. Several key cytokines, chemokines and adhesion molecules have significant roles in maturation, differentiation and effector function of NK cells. The CD56^{bright} NK cells are the primary population of NK cells that produces immunoregulatory cytokines, including interferon (IFN)- γ , tumor necrosis factor (TNF)- α , TNF- β , granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-10, and IL-13 [7].

Interestingly however, the freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [12-14]. As expected, NK cells obtained from the peripheral blood of cancer patients have significantly reduced cytotoxic activity [8-11]. Since in cancer patients the majority of NK cells have lost cytotoxic activity against OSCSCs, they may contribute the progression of cancer by expanding the pool of cancer stem cells. Knowledge of the distinct functional attributes of CD56^{bright} and CD56^{dim} human NK cell subsets and other cofactors involved in their expansion may enable us to design strategies that preferentially activate the subset with the greatest therapeutic potential for a particular disease. Therefore, optimizing the expansion of CD56^{bright} NK cells and enhancing the potency of these expanded NK cells are crucial for the immunotherapy of cancer. In this study, we have shown the novel way to expand NK cells with enhanced cytotoxic function and cytokine secretion. Moreover, the functionality of NK cells generated under this condition demonstrated enhanced expression of activating NK receptors, as demonstrated in previous studies conducted in our lab.

Thesis Outline

Specific Aim 1: To investigate the effects of K562, OSCSCs, and monocytes in NK cell expansion and function in comparison to osteoclasts.

- Sub-aim 1A: To reconfirm that osteoclast-induced NK cell expansion induces higher cytotoxicity against OSCSCs and higher IFN- γ secretion than their precursor cells, monocytes.
- Sub-aim 1B: To establish that osteoclast-induced NK cell expansion induces higher cytotoxicity against OSCSCs and higher IFN- γ secretion in comparison to NKs co-cultured with OSCSCs
- Sub-aim 1C: To establish that irradiated tumors secrete higher IFN- γ than live tumors; however, irradiated osteoclasts are still more potent inducers of NK cells function than irradiated tumors.
- Sub-aim 1D: To establish that irradiated osteoclasts are more potent inducers of NK cell expansion than irradiated K562.

Specific Aim 2: To investigate the role of cytokines IL-12 and IL-15 in NK cells co-cultured with osteoclasts.

- Sub-aim 2A: To understand how IFN- γ and cytotoxicity is affected with anti-IL-12 and anti-IL-15 at two different concentrations: 100 ng/mL and 1 μ g/mL.
- Sub-aim 2B: To understand the dynamics of CD16⁺/CD56⁺ expression on NK cells co-cultured with osteoclasts at two different concentrations of anti-IL-12 and anti-IL-15 mAbs: 100 ng/mL and 1 μ g/mL.

- Sub-aim 2C: To understand how blocking IL-12, IL-15, or a combination of both at two different concentrations affects NK cell expansion rates.

Specific Aim 3: To investigate the differences between dendritic cell- and osteoclast-induced NK cell expansion and function.

- Sub-aim 3A: To investigate the dynamics of lymphocyte cell proliferation and NK cell expansion induced by different myeloid cell lines (osteoclasts, monocytes, and dendritic cells) in long-term co-cultures.
- Sub-aim 3B: To understand how osteoclasts, monocytes, and dendritic cells influence NK to T/NKT cell proportions in long-term co-culture with NK cells.
- Sub-aim 3C: To investigate how NK to T/NKT cell proportions affect cytotoxicity and IFN- γ secretion induced by each myeloid cell line.

Chapter 1

Specific Aim 1: To investigate the effects of K562, OSCSCs, and monocytes in NK cell expansion and function in comparison to osteoclasts.

INTRODUCTION

Expansion of Natural Killer (NK) Cells

Natural killer (NK) cells play a crucial role in immune surveillance against a variety of microbial pathogens, viruses, and cancerous cells. Infusions of NK cells are a promising tool for cancer immunotherapy. Therefore, the development of clinically applicable methods to produce large numbers of highly potent NK cells is critical for cancer cell therapy.

Many cytokines have been studied in efforts to induce NK cell expansion while maintaining their split energy capabilities of inducing pro-inflammatory cytokines and inducing cytotoxicity against oral squamous carcinoma stem cells (OSCSCs) for a longer period of time. Activated NK cells secrete (IFN)- γ , tumor necrosis factor (TNF) and granulocyte/macrophage colony-stimulating factor (GM-CSF)[15]. NK cells can be activated by interleukin (IL)-2 to mediate potent cytotoxicity against a variety of tumor target cells and virally infected cells. IL-2 is known to increase NK cell cytotoxicity and it can also stimulate their proliferation but only a minority of NK cells can maintain proliferation after the initial response [16-18]. IL-4, IL-7, and IL-12 also induce some proliferation of NK cells, but are less potent than IL-2 [19]. NK cell proliferation in response to optimal concentrations of IL-2 was at least 10-fold more than proliferation to optimal concentrations of IL-4, IL-7, or IL-12 [19]. IL-15 is known to promote NK cell maturation and survival [20-21]. IL-15 alone or in combination with IL-2 or other

growth factors did not induce significant expansion of NK cells [22]. However, when IL-15 was presented to NK cells in trans as a membrane bound complex with IL-15R α , it promoted NK cell survival and expansion [23]. Furthermore, when feeder cell populations, such as Epstein Barr virus-transformed lymphoblastoid cells (EBV-LCL), or gene-modified K562 cells expressing NK-stimulatory molecules, such as 4-1BB ligand and IL-15 to NK cells, NK cell expansion was dramatically enhanced [22]. Thus, IL-2 appears to be necessary but is not the only source for preferential expansion of NK cells. Therefore, NK cells require costimulatory signals for optimal proliferation.

Interaction with other immune myeloid cells such as monocytes [24] or B-lymphoblastoid cells [17, 25, 26] is required in order to sustain proliferation of NK cells. Monocytes play critical roles in immune defense in response to inflammation signals. Monocytes can migrate quickly to sites of infection in the tissues and differentiate into dendritic cells to elicit immune response. Miller et al. [24] reported an approximate 30-fold expansion of NK cells after they were co-cultured with IL-2 (1000 IU/mL) and monocytes for 18 days. Perussia et al. [27] found that after the interaction of NK cells with irradiated B-lymphoblastoid cells for 2 weeks, an approximate 25-fold expansion of NK cells was observed. Yu et al. [28] also reported that the co-culture of NK cells with dendritic cells (DCs) resulted in enhancement of NK cell cytotoxicity and IFN- γ production. DCs, which are derived from hematopoietic bone marrow progenitor cells, are the most potent antigen-presenting cells (APCs) with a critical role in initiation of primary immune response [29]. Following maturation, DCs decrease their antigen processing capacity and become immunostimulatory cells by expressing high levels of MHC class II molecules, adhesion molecules and costimulatory receptors [30]. In order to enhance NK cells cytotoxicity and IFN- γ production, direct cell-to-cell contact between DCs

and NK cells is necessary with combinations of different cytokines and costimulatory molecules [28].

NK cells are found proximate to the surface of bone, where they may directly mature osteoclasts [31]. Suppression of bone erosion by the interaction between NK cells and osteoclasts had been shown; however, the expansion of NK cells by osteoclasts has never been studied. Osteoclasts, derived from hematopoietic stem cells, are a type of bone cells that resorbs bone tissue. This function is critical in the maintenance, repair, and remodeling of bones. Bone homeostasis is achieved by a balance between bone formation by osteoclasts and bone resorption by osteoclasts [32]. Osteoclasts mature by stimulating RANK-L expressed on osteoblasts and the cognate interaction is mediated by firm adhesion via ICAM-1 [33]. Feng et al. [34] showed that osteoclasts 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 or MIC-B, all MHC class I-like ligands for NKG2D, the activating receptor of NK cells [35]. Moreover, they also showed that osteoclasts express CD155 (poliovirus receptor, PVR), but not CD112 (PVR2 or Nectin-2), which are ligands for the activating NK receptor DNAX accessory molecule-1 (DNAM-1) [34]. IL-15 activated NK cells triggered osteoclast apoptosis, resulting in drastically decreased bone erosion [34].

There is a complex dynamic interaction between immune effector cells and the tumor microenvironment (TME). The TME has the capability of inducing a tumor to escape from the immune surveillance system by suppressing the effector NK cell function and evading through selection of immunogenic tumor cells [42]. When NK cells interact with sensitive tumor target cells *in vitro*, the target binding NK cells undergo phenotypic and functional changes. These dormant NK cells inactivated by target cells express CD16⁻CD56^{dim}/CD⁺ phenotype [43,44].

Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were observed in patients diagnosed with HIV, oral, or ovarian cancer [45-47]. In previous studies, we have shown that by triggering CD16 on NK cells, there is a down-modulation of CD16 receptors, resulting in a significant loss of cytotoxicity and augment secretion of IFN- γ , a phenomenon we coined as “split anergy” [43, 33, 48-50]. We have seen that K562, which is a modified leukemia cell line as described by Fujisaki et al. [52], causes loss of NK cell cytotoxicity and induces cell death in a subset of NK cells [43,44]. Down-modulation of CD16 surface receptors causes a major decrease in NK cell cytotoxicity against K562 tumor cells [51]. Thus, CD16 receptors play a critical role in the loss of NK cell cytotoxicity induced by target cells.

The expansion of highly cytotoxic NK cells by tumor cells for cancer immunotherapy has been reported. The modified K562 leukemia cell line expresses a membrane-bound form of IL-15 and 41BB ligand (K562-mb15-41BBL) to generate NK cells with enhanced cytotoxicity. Some studies have reported as much as 21.6 fold expansion of CD56⁺CD3⁻ NK cells from peripheral blood after they were co-cultured with irradiated K562 for 7 days. Furthermore, these expanded NK cells were reported to be more potent in function than those produced by stimulation of IL-2, IL-15, and/or IL-21 [52]. We used irradiated K562, along with irradiated OSCSCs as a control, to attempt to expand NK cells and enhance their function, and subsequently compare these results alongside with osteoclast-induced expanded NK cells.

Extensive results originating from our lab have underscored the importance of Natural Killer cells in eliminating the number of stem cells after their interaction with other immune inflammatory cells or effectors of connective tissue [2, 54]. A second, but equally important, function of NK cells is their potential to support differentiation of stem cells through the

induction of cytokine secretion [2, 54]. It is a well-established fact that oral cancer stem cells are significantly more sensitive to NK cell mediated killing but resistant to certain chemotherapeutic drugs, whereas differentiated oral tumors were more sensitive to chemotherapeutic drugs but resistant to NK cell mediated killing. Therefore, a combinational therapy of repeated autologous NK cell transplantation and chemotherapeutic drugs should come to the forefront of cancer immunotherapy, as they will be more effective in eliminating both cancer stem cells and differentiated tumors [2, 54]. Using this rationale, it is crucial to find innovative ways of not only expanding NK cells, but also of enhancing their potency in both NK cell mediated cytotoxicity and augmented cytokine secretion. These new lineage of potent NK cells will have the capability of overcoming NK cell tolerance against tumors.

For aim 1, we found a novel method to expand NK cells with osteoclasts, which enhanced sensitization of tumor target cells to NK cell-mediated cytotoxicity and increased cytokine induction. These recent *in vitro* discoveries in regards to increased NK cell expansion and potency against tumors warrant future translational research that will lead to clinical trials for more efficient cancer immunotherapy treatments.

MATERIALS AND METHODS

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA) was used for the cultures of human immune cells. Oral Squamous Cancer Stem Cells (OSCSCs) were isolated from oral cancer patient tongue tumors at UCLA School of Medicine and cultured in RPMI 1640 supplemented 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic (100x), 1% sodium pyruvate (100nM), 1.4% MEM non-essential amino acids, 1% L-Glutamine, 0.2% gentamicin sulfate (Gemini Bio-products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). Alpha-MEM medium (Life Technologies, CA) supplemented with 10% FBS and penicillin-streptomycin (Gemini Bio-Products, CA) was used to culture human osteoclasts. M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -80°C. PE conjugated isotype control, CD16, NKp44, CXCR1, CXCR3 and DNAM were purchased from Biolegend (San Diego, CA).

Bacteria Sonication

sAJ2 is a combination of 8 different strains of sonicated gram positive probiotic bacteria selected by Dr. Anahid Jewett for their superior ability to induce optimal secretion of both pro-inflammatory and anti-inflammatory cytokines in NK cells (manuscript in prep). The whole bacteria was weighed and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were vortexed thoroughly and then sonicated for 15 seconds on ice at an amplitude from 6 to 8. After that, the samples were incubated for 30 seconds on ice. After every 5 pulses, a sample was taken to observe under the microscope every

time until we obtained at least 80 percent of cell walls lysed. Then, the sonicated samples were aliquoted and stored in -20 to -80 degrees Celsius for long-term studies.

Purification of NK cells

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. The Ficoll-Hypaque technique was used to fractionate the blood, and the white cloudy layer called peripheral blood mononuclear cells (PBMC) was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes (PBL) were obtained after 1-2 hours incubation at 37°C on a plastic tissue culture dish. NK cells were negatively selected and isolated using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, Canada). The NK cell population was found to have greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained NK cells. Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% MEM non-essential amino acids (Invitrogen, Life Technologies, CA).

Human Peripheral Blood Monocyte Purification

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll-hypaque centrifugation. PBMCs were cultured onto the tissue culture plate for an hour to two hours and the adherent

subpopulation of PBMCs was detached from the tissue culture plate. Monocytes were purified using the EasySep® Human monocyte cell enrichment kit obtained from Stem Cell Technologies (Vancouver, Canada). The monocyte population was found to have greater than 95% purity based on flow cytometric analysis of CD14 antibody stained monocyte cells.

Generation of Osteoclasts (hOCs)

Osteoclasts were generated from monocytes and cultured in alpha-MEM medium containing M-CSF (25ng/mL) and Rank Ligand (RANKL) (25ng/mL) for 21 days, or otherwise specified. Medium was refreshed every 3 days with fresh alpha-MEM containing M-CSF (25ng/mL) and RANKL (25ng/mL). Zoledronic acid (500nM) was purchased from UCLA Ronald Reagan Pharmacy and used to treat osteoclasts.

Surface Staining

1×10^5 NK cells from each condition were stained in 50ul of cold 1%PBS-BSA with pre-determined optimal concentration of PE and FITC conjugated isotype control, FITC CD3 conjugated with PE CD16+CD56, and incubated at 4°C for 30 minutes. Then, the cells were washed and resuspended in 1%PBS-BSA. The Epics C (Coulter) flow cytometer was used for surface analysis.

⁵¹Cr release cytotoxicity assay

⁵¹Cr was purchased from Perkin Elmer (Santa Clara, CA). NK cell cytotoxic function in the experimental cultures and the sensitivity of target cells to NK cell mediated lysis were determined using standard ⁵¹Cr release cytotoxicity assays. The effector cells (1×10^5 NK cells/well) were aliquoted into 96-well round-bottom microwell plates (Fisher Scientific,

Pittsburgh, PA) and titrated to four serial dilutions. The target cells (5×10^5 OSCSCs/well) were labeled with $50 \mu\text{Ci } ^{51}\text{Cr}$ (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour. The cells were washed twice to remove excess unbound ^{51}Cr . The ^{51}Cr -labeled target cells were incubated with the effector cells for 4 hours. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

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

LU $30/10^6$ is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X100.

Enzyme-Linked Immunosorbent Assays (ELISAs)

Human IFN- γ Elisa kit was purchased from Biologend (San Diego, CA). ELISA was performed to detect the level of IFN- γ produced from NK cells co-cultured with different cancer or immune cells. The 96-well EIA/RIA plates were coated with 100ul of diluted capture antibody (1:200) corresponding to target cytokine and incubated overnight at 4°C . After 16 hours incubation, the plates were washed 4 times with wash buffer (0.05% Tween in 1xPBS) and blocked with 200ul of assay diluent (1%BSA in 1xPBS). The plates were incubated on a plate shaker at 200rpm for 1 hour followed by washing 4 times with wash buffer. 100ul of standards and samples collected from each culture were added to the wells and incubated on the plate shaker at 200rpm for 2 hours at room temperature. Then, the plates were washed 4 times with wash buffer and 100ul of diluted detection antibody (1:200) were added to the wells and incubated for 1 hour on the plate shaker at 200rpm at room temperature. After 1 hour of

incubation, the plates were washed 4 times with wash buffer and 100ul of diluted Avidin-HRP solution (1:1000) were added to the wells and incubated on the plate shaker at 200rpm for 30minutes. After washing the plates 5 times with wash buffer, 100ul of TMB substrate solution were added to the wells and incubated in the dark until the wells developed a desired blue color before adding 100ul of stop solution (2N H₂SO₄) to stop the reaction. The plates were read in a microplate reader at 450nm to obtain absorbance values (Biolegend, ELISA manual).

Statistical analysis

An unpaired, two-tailed student t-test was performed for the statistical analysis. One-way ANOVA with a Bonferroni post-test was used to compare the different groups.

RESULTS

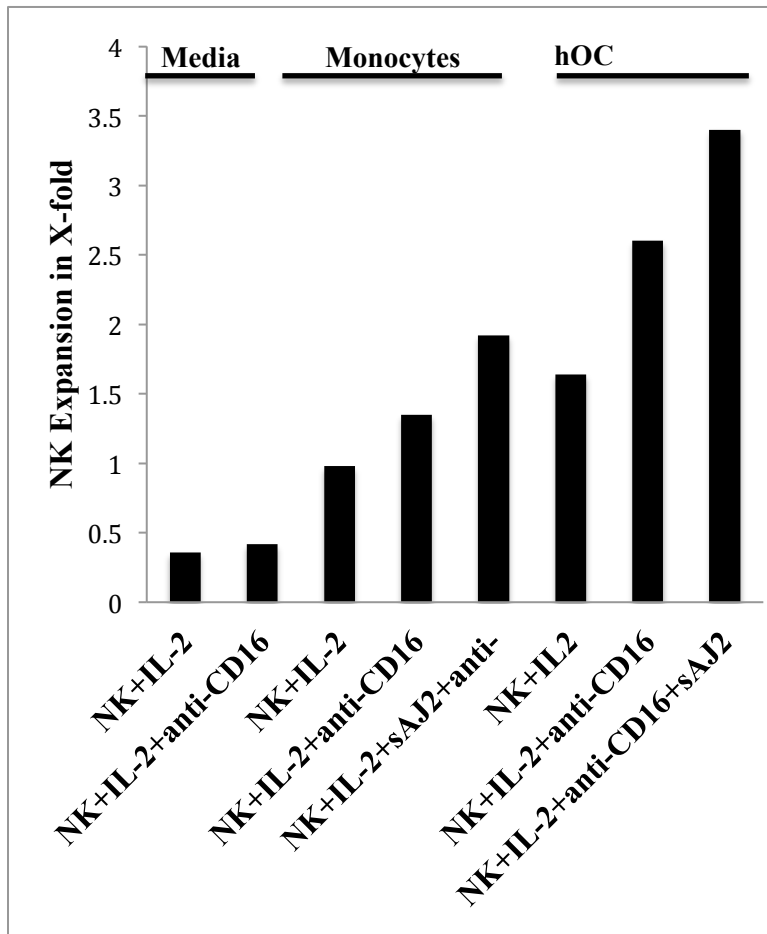


Figure 1: Activated NK cells co-cultured with osteoclasts and sAJ2 bacteria yielded the highest NK-cell fold expansion compared to the monocyte-induced NK cells.

Monocytes and osteoclasts were differentiated as described in the Materials and Methods. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL) and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. On day 12, NK cell counts were taken using a cell counter and microscope.

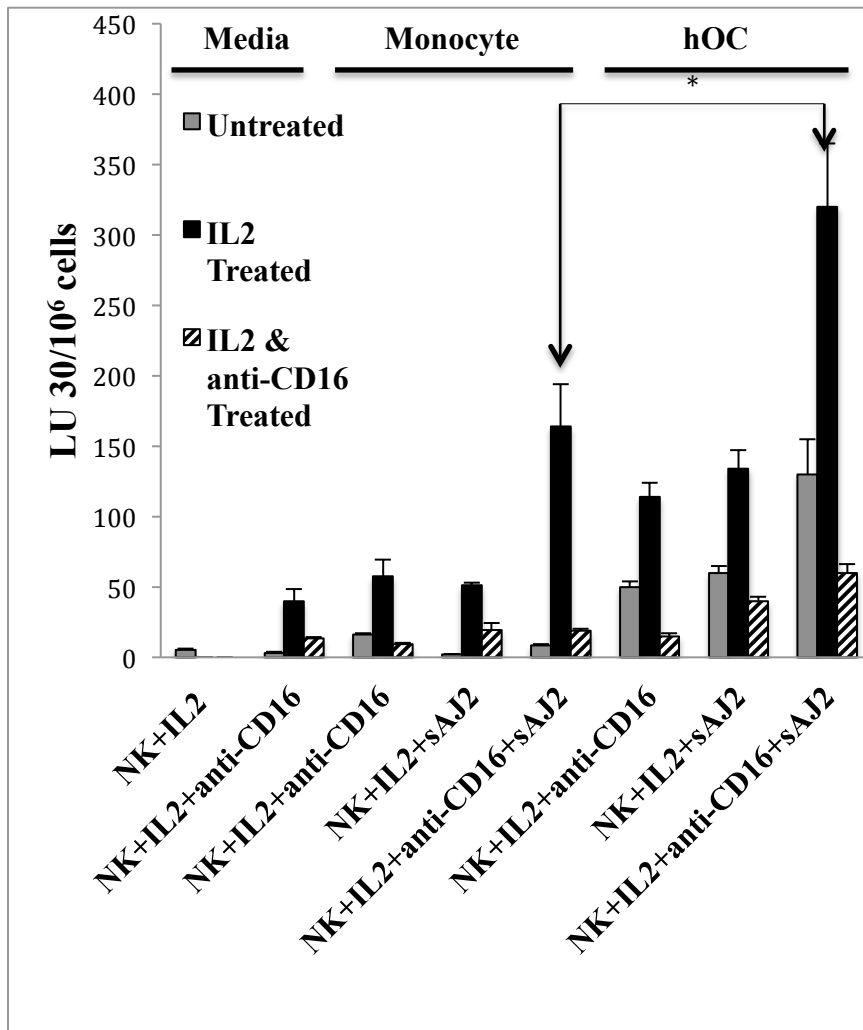


Figure 2. Expanded NK cells co-cultured with osteoclasts induced higher levels of cytotoxicity against OSCSCs than NKs co-cultured with monocytes.

Monocytes and osteoclasts were differentiated as described in the Materials and Methods. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL) and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was left untreated, treated with IL-2 (1000 units/mL), or re-activated with IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL) every 2 days. On day 10, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ⁵¹Cr release against OSCSCs. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

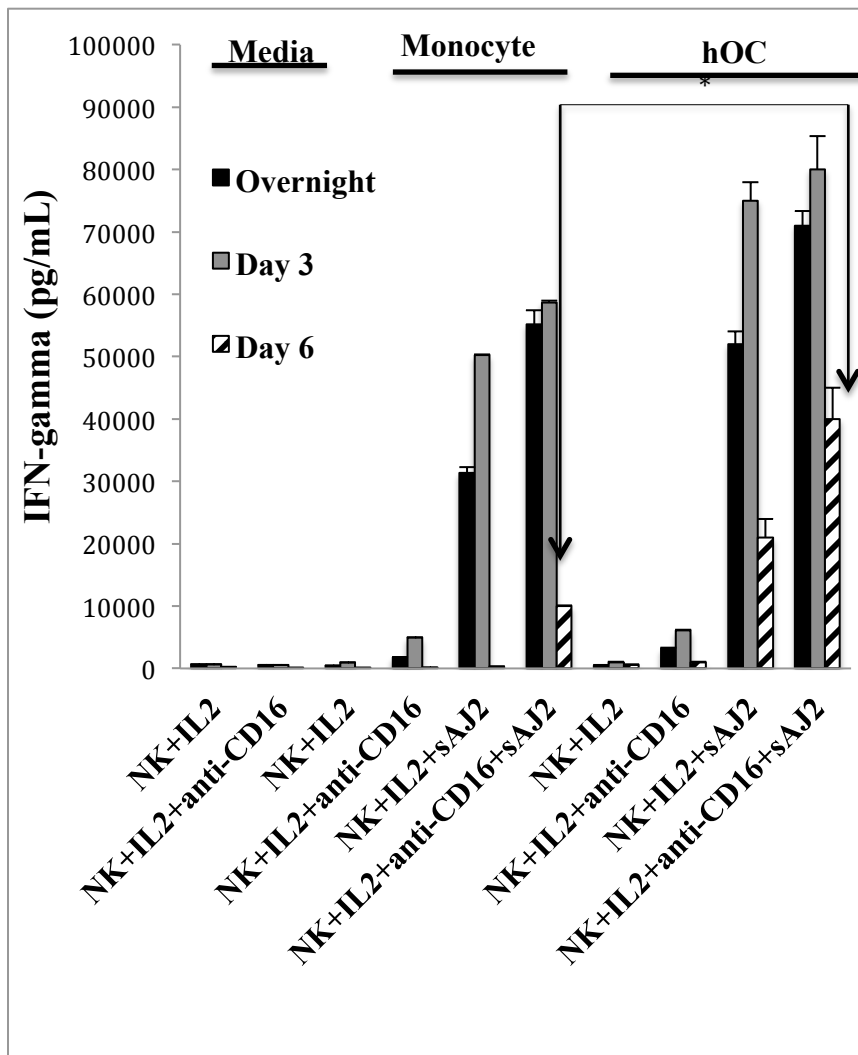


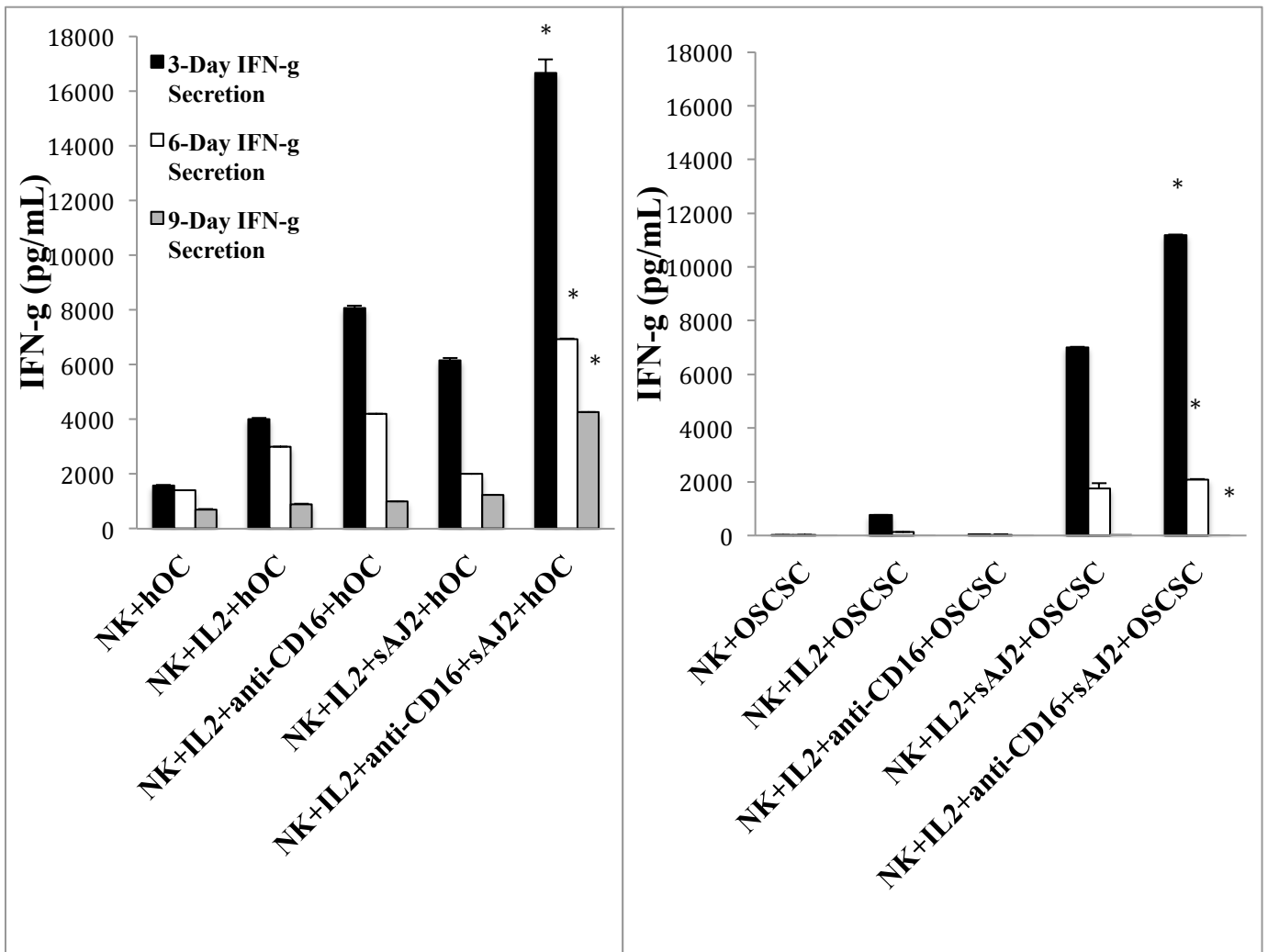
Figure 3. Expanded NK cells co-cultured with osteoclasts induced higher levels of IFN- γ than NK cells co-cultured with monocytes.

Monocytes and osteoclasts were differentiated as described in the Materials and Methods. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 $\mu\text{g}/\text{mL}$) and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Supernatants from each co-culture were collected overnight, on day 3, and day 6. The levels of IFN- γ were measured using a specific ELISA.

Irradiated tumors neither proliferate nor increase the potency of expanded NK cell function to the extent of osteoclast-induced expanded NK cells

Other scientists have used other methods of proliferating NK cells, including using irradiated K562 in co-culture with NK cells. We wanted to test this tumor cell line, as well as OSCSCs, to understand how irradiating tumors modulate the function of expanded NK cells using our model of NK cell expansion. Breast and Head and Neck Cancer radiation dosages usually range between 40 to 60 Gray. A Gray (Gy) is the measure of the amount of radiation energy absorbed by 1 kilogram of human tissue. To understand the effects of irradiated tumors on expanded NK cells, we had a controlled batch of live tumors and the rest were irradiated at 40 Gy. After irradiation, these irr-tumors and live tumors were cultured *in vitro* overnight before adding purified, activated NK cells. There is no dispute that irradiated-K562 and irradiated-OSCSCs induced higher IFN- γ than their live tumor counterparts (Figs. 8 & 9). However, by day 12 of the co-culture, the expanded NK cells that were generated insignificant levels of cytotoxicity against OSCSCs (Fig. 7). Irradiated K562 did induce NK cell expansion, whereas irradiated-OSCSCs did not. To control for this extra variable, radiation, we proceeded to irradiating osteoclasts at 40 Gy and compare their influence in NK cell expansion and function against irradiated K562. As expected, we found that irradiated osteoclasts yielded significantly higher numbers of expanded NK cells compared to irradiated K562 over the course of 20 days (Fig. 6). In addition, irradiated osteoclasts higher NK cell mediated killing against OSCSCs (Fig.11). It is important to note that although irradiated K562 induced higher IFN- γ by NK cells than irradiated osteoclasts overnight, by day 6, irr-K562-induced expanded NK cells secreted very little IFN- γ compared to irr-hOC-induced expanded NK cells (Fig. 10). There is a universal general trend that we observe in IFN- γ secreted by NK cells co-cultured with tumor

cells: the high induction of cytokines overnight but rapid decline thereafter. Therefore, osteoclasts remain the gold standard in expanding NK cells and retaining their cytotoxic and secretory functions for longer period of times, allowing for a more potent immunotherapy treatment against oral cancer stem cells.



Figures 5a & 5b: NK cells expanded with osteoclasts yield significantly higher levels of IFN- γ secretion than NK cells expanded with OSCSCs.

Osteoclasts were differentiated as described in the Materials and Methods. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL), or left untreated and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. OSCSCs were seeded at 0.25×10^6 cells/well. Supernatants from each co-culture were collected on day 3, 6, and 9. The levels of IFN- γ were measured using a specific ELISA.

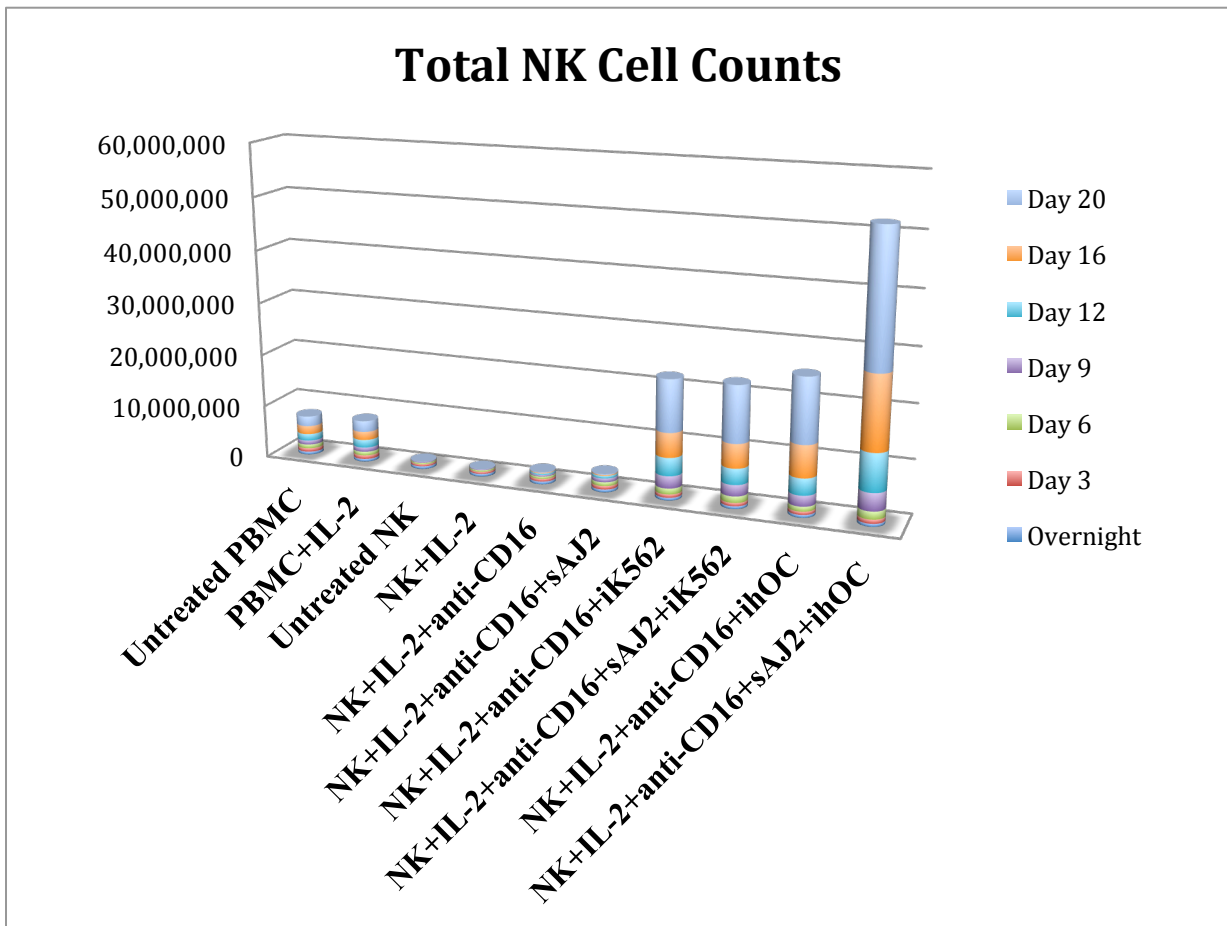


Figure 6: Irradiated osteoclasts expanded NK cells at a significantly higher rate than irradiated K562 when supplemented with probiotic bacteria.

Autologous osteoclasts were differentiated and irradiated at 40 gray (Gy) as described in the Materials and Methods. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Irradiated K562 cells were seeded at 0.25×10^6 cells/well. Purified NK cells were activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL), or left untreated and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures of NK to target cell ratios of 2:1. Each condition was treated with IL-2 (1000 units/mL) every 2 days. Using a cell counter and microscope, NK cell counts were taken every 3 or 4 days from day 1 to day 20 of the co-cultures.

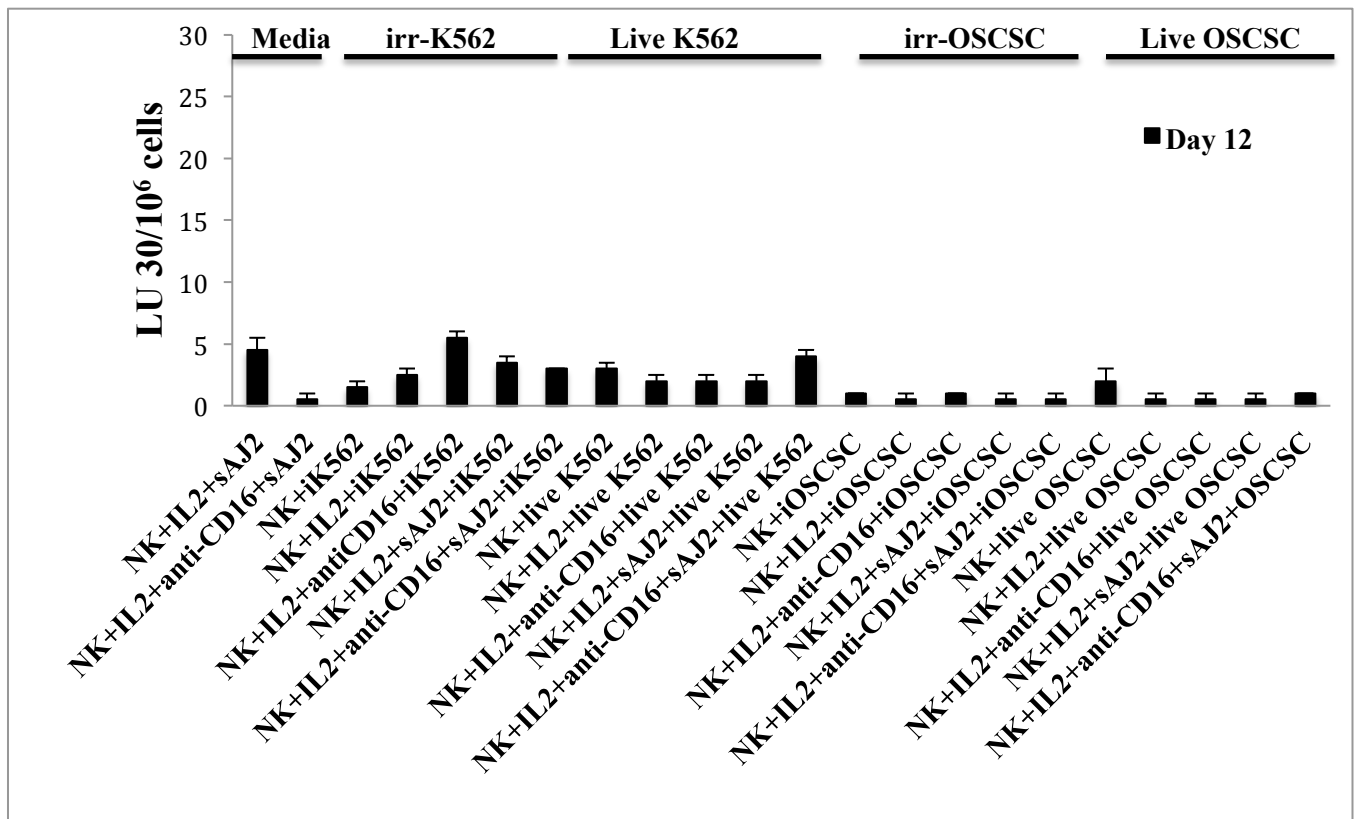


Figure 7: Neither live tumor cell lines K562 and OSCSCs nor the irradiated-K562 and irradiated-OSCSC induced significant cytotoxicity against OSCSCs by day 12 of NK cell co-cultures.

K562 and OSCSCs were either left live (untreated) or irradiated at 40 gray (Gy). These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were left untreated or activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL) and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. On day 12, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ⁵¹Cr release against OSCSCs. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

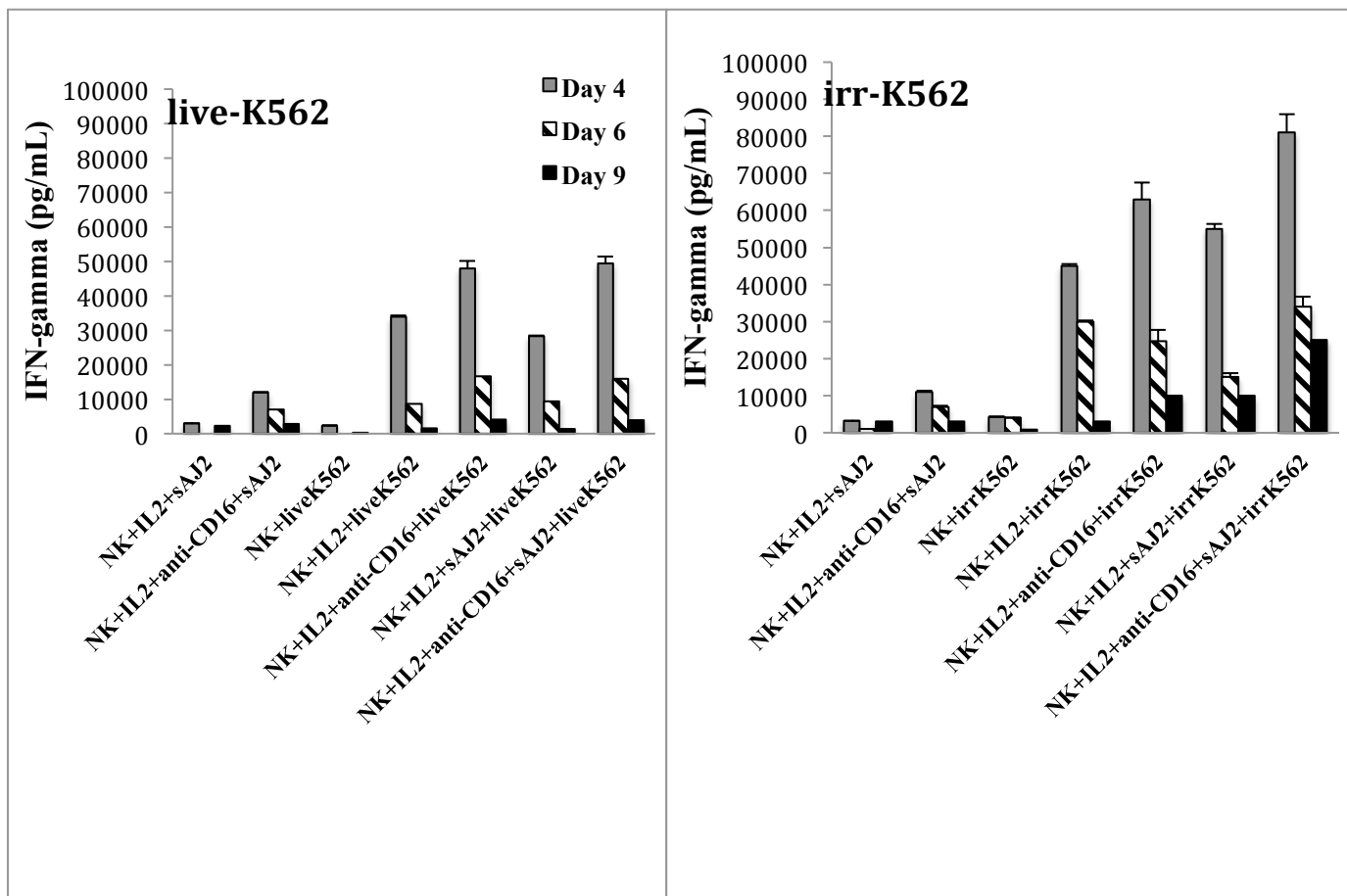


Figure 8: NK cells expanded with irradiated-K562 induced higher IFN- γ secretion than NK cells expanded with live-K562 cancer cells.

K562 cells were left untreated or irradiated at 40 Gy. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were left untreated or activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. Supernatants from each co-culture were collected on days 4, 6, and 9. The levels of IFN- γ were measured using a specific ELISA.

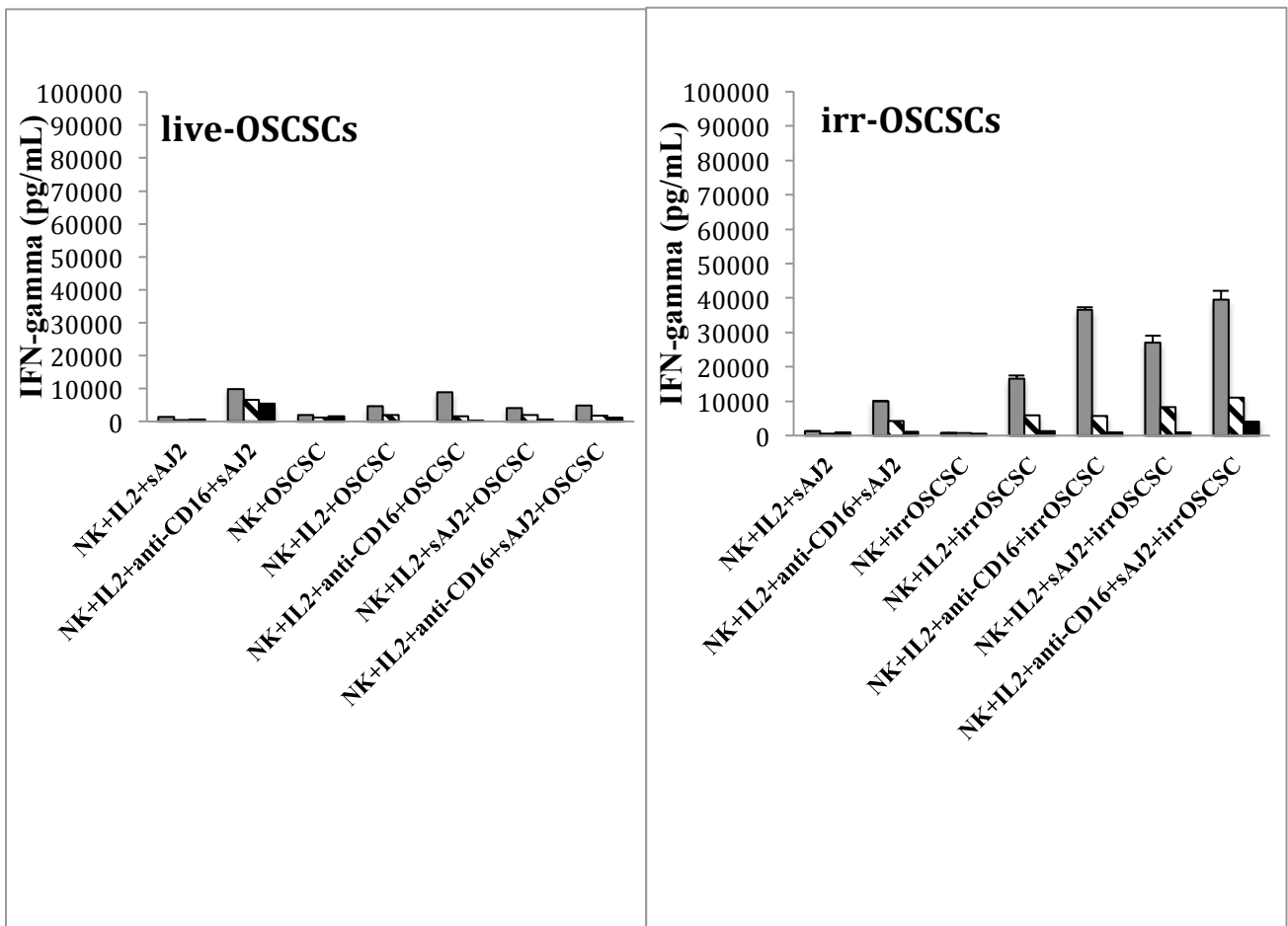


Figure 9: NK cells expanded with irradiated-OSCSC induced higher IFN- γ secretion than NK cells expanded with live-OSCSC cancer cells.

OSCSCs were left untreated or irradiated at 40 Gy. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were left untreated or activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 $\mu\text{g}/\text{mL}$), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. Supernatants from each co-culture were collected on days 4, 6, and 9. The levels of IFN- γ were measured using a specific ELISA.

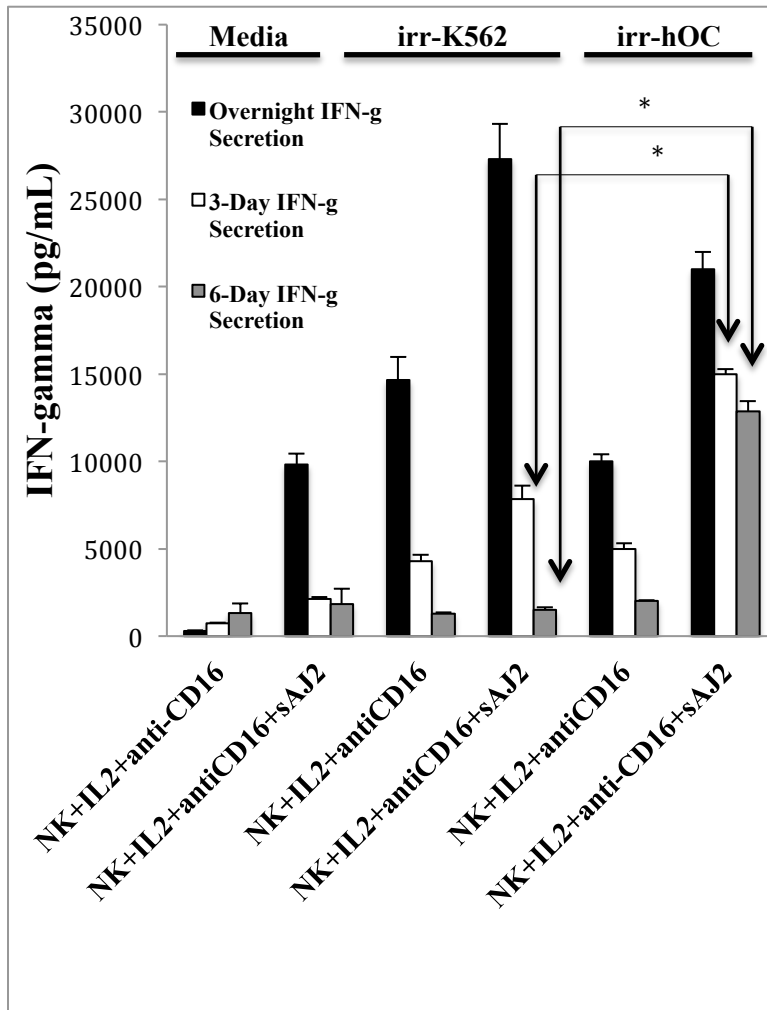


Figure 10: NK cells expanded with irradiated-osteoclasts induced significantly higher IFN- γ secretion levels than NK cells expanded with irradiated-K562 over a 6-day period.

Osteoclasts were differentiated as described in Materials and Methods. Osteoclasts and K562 cells were irradiated at 40 Gy. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 $\mu\text{g}/\text{mL}$), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. Supernatants from each co-culture were collected overnight and on days 3, and 6. The levels of IFN- γ were measured using a specific ELISA.

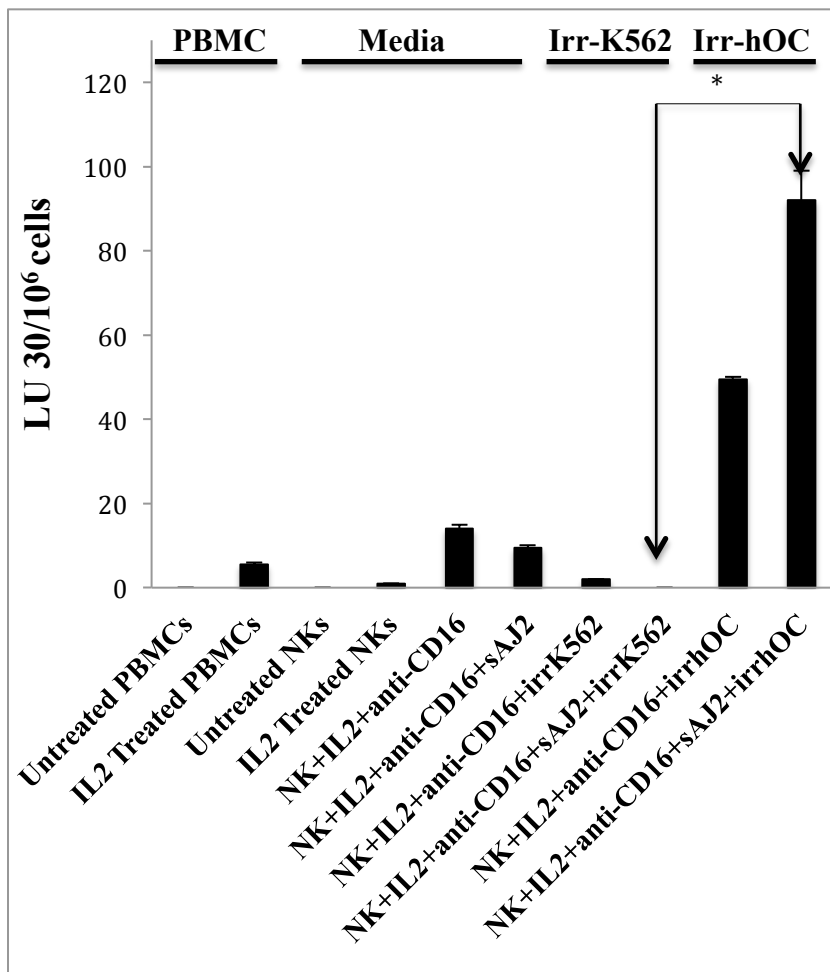


Figure 11: On day 12 of the co-cultures, NK cells expanded with irradiated osteoclasts yielded higher cytotoxicity levels than NK cells expanded with irradiated-K562.

K562 and OSCSCs were irradiated at 40 gray (Gy). PBMCs were purified from blood to use as a control group. These cells were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were left untreated or activated using either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL) and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. On day 16, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ⁵¹Cr release against OSCSCs. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

DISCUSSION

In our laboratory, we found a novel way to expand osteoclast-induced NK cells with potent cytotoxic function as well as augmented cytokine secretion. There are several studies that have shown NK expansion with a combination of cytokines with a variety of other feeder cells, most notably with K562 cells and dendritic cells. However, no study has been able to show NK expansion with the aid of human osteoclasts. Osteoclasts had been perceived only to play a crucial role in bone remodeling but for long have been overlooked as important immune effector cells for cancer immunotherapy until now. Osteoclasts have shown to be the best targets for the expansion of functional NK cells under the optimized conditions of NK cell stimulation as shown in this study. In fact, IL-2 + anti-CD16 activated NK cells triggered by sonicated probiotic (sAJ2) bacteria and osteoclasts expanded high numbers of NK cells with a high potency of NK cell-mediated lysis against OSCSCs and a high induction of IFN- γ secretion. Previously, we demonstrated that anergized NK cells contribute to the differentiation and resistance of transformed stem cells to NK cell-mediated cytotoxicity by secreting key cytokines [36]. In addition, we not only demonstrated that cells differentiated by NK cells resist NK cell-mediated lysis, but also that they do not trigger secretion of cytokines or chemokines, potentially contributing to the inhibition of inflammation [36]. TNF- α and INF- γ secreted by the NK cells synergistically augmented differentiation of stem cells, resulting in an increase of MHC class I, CD54, and B7H1, and an increase in their resistance to NK cell-mediated cytotoxicity and decrease in cytokine and chemokine secretion by the NK cells cultured with differentiated cells [36].

Osteoclasts express lower levels of MHC class I and II and resist an increase in MHC class I surface expression when either treated with the combination of TNF- α and INF- γ or with activated NK supernatants, which are all known to increase MHC class I and II significantly (manuscript submitted). When osteoclasts were compared to freshly isolated autologous monocytes, a decrease in all CD14, CD11b, CD44, MHC-class I and II, and CD54 was more profound on the surface of osteoclasts (manuscript submitted). These results suggest that monocytes in the periphery may be less activating for NK cells since they retain higher expression of MHC class I, whereas once they move to the tissues and down-modulate their surface receptors, they may become more activating to NK cells. This theory may be one reason why NK cells in peripheral blood remain relatively quiescent, even in the presence of competent cytotoxic machinery. It may also be another reason why osteoclasts are found to be the best targets for the expansion of functionally potent NK cells.

Osteoclast-induced expanded NK cells not only exhibited higher cytotoxic capacity but they also mediated significantly higher secretion levels of IFN- γ when compared to monocyte-, OSCSC- and K562-stimulated expanded NK cells. Osteoclast-expanded NK cells responded to IL-2 activation and substantially increased IFN- γ secretion per cell basis when compared to monocytes, OSCSC or K562 cells. Moreover, osteoclast-expanded NK cells express high levels of CD16, NKp44, and NKG2D on the cell surface (So-Hyun Park, 2015). NKp44 is a NK activating receptor and binds to viral hemagglutinin (HA) and HA-neuraminidase (HN) as well as tumor-associated ligands [37]. Another NK activating receptor, NKG2D, is constitutively expressed on all NK cells [38]. NK ligation phosphorylates YINM motifs on DAP10, which allows recruitment and activation of growth factor receptor bound protein (GRB2) and p85 subunit of phosphatidylinositol-3-kinase (PI3K) in order to trigger NK cytotoxicity [39]. In

addition, expanded NK cells secrete more IFN- γ than primary NK cells while mediating the highest cytotoxicity against OSCSCs when compared to primary NK cells. These results suggest that osteoclast-induced expanded NK cells are highly activated in terms of potent cytotoxic function and augmented cytokine secretion. These observations are of utmost importance since this strategy can overcome NK cell tolerance against tumors. As a result, there is potential of delivering functionally potent NK cells that had been expanded *ex vivo*.

CONCLUSION

This study demonstrated that human osteoclasts are potent immune effectors capable of expanding NK cells, but more importantly enhancing their function against OSCSCs. Osteoclast-induced expanded NK cells generated under optimal condition demonstrated the highest cytotoxic activity and augmented secretion of IFN- γ . In addition, osteoclast-induced expanded NK cells exhibited high potency, as they regained CD16 receptors and retained activating receptors such as NKp44 and NKG2D. These expanded NK cells were highly functional compared to primary NK cells, as they were potent effector cells for stem like/poorly differentiated tumor cells. Through the synergistic effects of cytokines, sAJ2 bacteria and osteoclasts, activated NK cells can optimally expand while retaining their high potency against OSCSCs and other poorly differentiated tumor cells, and high cytokine secretion induction *in vitro*. These expanded NK cells can then be used to target tumors *in vivo*. It is a fact now that osteoclast-induced expanded NK cells are highly activated NK cells with potent cytotoxic function and augmented cytokine secretion capabilities. This recent discovery in how we expand NK cells while retaining their functions is imperative for the advancement of cancer immunotherapies. The next two aims are intended to investigate through which mechanisms osteoclasts induce NK cell expansion while retaining their function against poorly differentiated tumors, and to compare our method of expanding NK cells with another gold standard involving the use of dendritic cells as immune effectors for NK cell expansion.

Chapter 2

Specific Aim 2: To investigate the role of cytokines IL-12 and IL-15 in NK cell expansion and function when co-cultured with osteoclasts.

INTRODUCTION

Although utilizing cytokines to enhance NK cell functionality is a crucial component in passive immunotherapy, it is only one part of a comprehensive approach to enhance NK cell antitumor activity. Other approaches include the blockade of inhibitory signals/cells, and enhancement of NK cell recognition of tumor target cells [72]. NK cells are further defined by their functional attributes, including proliferation, production of cytokines/chemokines, natural killing lymphokine-activated killing, and antibody-dependent cellular cytotoxicity (ADCC) via CD16/Fc γ RIIIa [72]. NK cells are of immense clinical importance as demonstrated in patients that are selectively deficient in NK cells, who develop often fatal viral infections. Moreover, a large epidemiological study showed that low NK cell cytotoxicity predicted an increased risk of developing cancer [72].

Cytokines help maintain the homeostasis of NK cells. But more importantly, several of these cytokines have been implicated in promoting different stages of NK cell differentiation and survival [72]. Moreover, cytokines may also be influencing the activation state of NK cells by providing microenvironment as a cue to determine whether it should raise or lower the threshold for triggering NK cells through surface receptors [72].

IL-2 and IL-15 are the two best understood cytokines that yield a number of positive

functional effects on NK cells to increase antitumor responses. When signaling occurs downstream of the IL-2/15R, the Jak1/3 and STAT3/5 are activated, leading to the activation of PI3K and MAPK pathways, and ultimately the NF- κ B. All of these signaling pathways are crucial in NK cell development and homeostasis, proliferation, cytokine production, and enhanced cytotoxicity [cytokines review]. . IL-2 and IL-15 share the IL-2/15R β and γ_c as the primary signaling subunits and interact with the heterodimer with intermediate affinity (IA). CD56^{bright} NK cells always express CD25/IL-2R α , which forms a high affinity heterotrimeric IL-2R $\alpha\beta\gamma$. Now, the IL-2R $\alpha\beta\gamma$ gets activated with picomolar concentrations of IL-2. CD25 and the high affinity (HA) receptor are induced on both CD56^{bright} and CD56^{dim} NK cells after combined cytokine activation with IL-12+IL-15+IL-18. Much less activation is induced following IL-2 or IL-15 activation alone.

Recombinant human (rh)IL-2 was one of the first cytokines that were used clinically to enhance antitumor immunity. For example, a high dose of IL-2 induces remissions in some patients with renal cell carcinoma (RCC) and metastatic melanoma. Unfortunately, the mechanism by which this is achieved is still unclear. IL-2 therapy is essential in activating NK cells *in vivo*; however, low dose IL-2 therapy also helps expands regulatory T cells, which are known to limit NK- and effector T-cell responses.

IL-12, also known as “NK cell stimulatory factor (NKSF),” is a cytokine composed of p35 and p40 subunits (IL-2 α and β chains). According to previous studies, IL-12 has shown the ability to enhance NK cytotoxicity [72]. Activated dendritic cells and macrophages release IL-12, which binds to its cognate receptor expressed on activated T and NK cells. Binding to the IL-12 receptor transduces signals through JAK2, TYK2, STAT3, 4, and 5. Upon interaction of NK cells with IL-12, significant amounts of IFN- γ and TNF- α are produced. IL-12R is expressed on

resting NK cells, which allows for a fast immune response prior to activation. Many preclinical studies have highlighted IL-12's potential for antitumor activity, including INF- γ dependent antitumor responses against melanoma and RCC in mice.

Thus, the following experiments are aimed to determine to see if IL-12 and IL-15 also play an important role in osteoclast-induced exp-NK cell proliferation and functions. In fact, we do see that these two cytokines play an important role in exp-NK cell proliferation and IFN- γ induction, but not so much in NK-cell mediated cytotoxicity against OSCSCs.

MATERIALS AND METHODS

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA) was used for the cultures of human immune cells. Oral Squamous Carcinoma Cells (OSCCs) and Oral Squamous Cancer Stem Cells (OSCSCs) were isolated from oral cancer patient tongue tumors at UCLA School of Medicine and cultured in RPMI 1640 supplemented 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic (100x), 1% sodium pyruvate (100nM), 1.4% MEM non-essential amino acids, 1% L-Glutamine, 0.2% gentimicin sulfate (Gemini Bio-products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). Human erythroleukemia cell line (K562) was derived from a patient with chronic myeloid leukemia (CML) and they were cultured in the same RPMI 1640 medium.

Dr. Nicholas Cacalano (UCLA Johnson Comprehensive Cancer Center) provided human pancreatic cancer cell lines, MIA PaCa-2 (MP2) and PL12. MP2 was cultured in DMEM in supplement with 10% FBS and 2% Penicillin-Streptomycin (Gemini Bio-Products, CA) and PL12 was cultured in RPMI 1640 medium supplemented with 10% FBS and 2% Penicillin-Streptomycin.

Alpha-MEM medium (Life Technologies, CA) supplemented with 10% FBS and penicillin-streptomycin (Gemini Bio-Products, CA) was used to culture human osteoclasts. Human M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -80°C. PE conjugated isotype control, CD16, CD45, CD56, CXCR1, CXCR3 and DNAM were purchased from Biolegend (San Diego, CA).

Bacteria Sonication

sAJ2 is a combination of 8 different strains of sonicated gram positive probiotic bacteria selected by Dr. Anahid Jewett for their superior ability to induce optimal secretion of both pro-inflammatory and anti-inflammatory cytokines in NK cells (manuscript in prep). The whole bacteria was weighted and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were vortexed thoroughly and then sonicated for 15 seconds on ice and the amplitude was set from 6 to 8. After that, the samples are incubated for 30 seconds on ice. After every 5 pulses, a small sample was taken to observe under the microscope to obtain at least 80 percentages of cell walls to be lysed. Then, the sonicated samples were aliquoted and stored in minus 20 to 80 degrees for long term studies.

Purification of NK cells

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. The Ficoll-Hypaque technique was used to fractionate the blood, and the buffy layer called peripheral blood mononuclear cells (PBMC) was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes (PBL) were obtained after 1-2 hours incubation at 37°C on a plastic tissue culture dish. NK cells were negatively selected and isolated using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, Canada). The NK cell population was found to have greater than 90% purity based on flow cytometric

analysis of anti-CD16 antibody stained NK cells. Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% MEM non-essential amino acids (Invitrogen, Life Technologies, CA).

Human Peripheral Blood Monocytes Purification

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll-hypaque centrifugation. PBMCs were cultured onto the tissue culture plate for an hour and the adherent subpopulation of PBMCs was detached from the tissue culture plate. Monocytes were purified using the EasySep® Human monocyte cell enrichment kit obtained from Stem Cell Technologies (Vancouver, Canada). The monocyte population was found to have greater than 95% purity based on flow cytometric analysis of CD14 antibody stained monocyte cells.

Generation of Osteoclasts (hOCs)

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

Surface Staining

1×10^5 NK cells from each condition were stained in 50ul of cold 1%PBS-BSA with pre-determined optimal concentration of PE and FITC conjugated isotype control, FITC CD3 conjugated with PE CD16+CD56, and incubated at 4°C for 30 minutes. Then, the cells were washed and resuspended in 1%PBS-BSA. The Epics C (Coulter) flow cytometer was used for surface analysis.

^{51}Cr release cytotoxicity assay

^{51}Cr was purchased from Perkin Elmer (Santa Clara, CA). NK cell cytotoxic function in the experimental cultures and the sensitivity of target cells to NK cell mediated lysis were determined using standard ^{51}Cr release cytotoxicity assay. The effector cells (1×10^5 NK cells/well) were aliquoted into 96-well round-bottom microwell plates (Fisher Scientific, Pittsburgh, PA) and titrated to four serial dilutions. The target cells (5×10^5 OSCSCs/well) were labeled with 50 μCi ^{51}Cr (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour. The cells were washed twice to remove excess unbound ^{51}Cr . The ^{51}Cr -labeled target cells were incubated with the effector cells for 4 hours. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

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

LU 30/ 10^6 is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells X100.

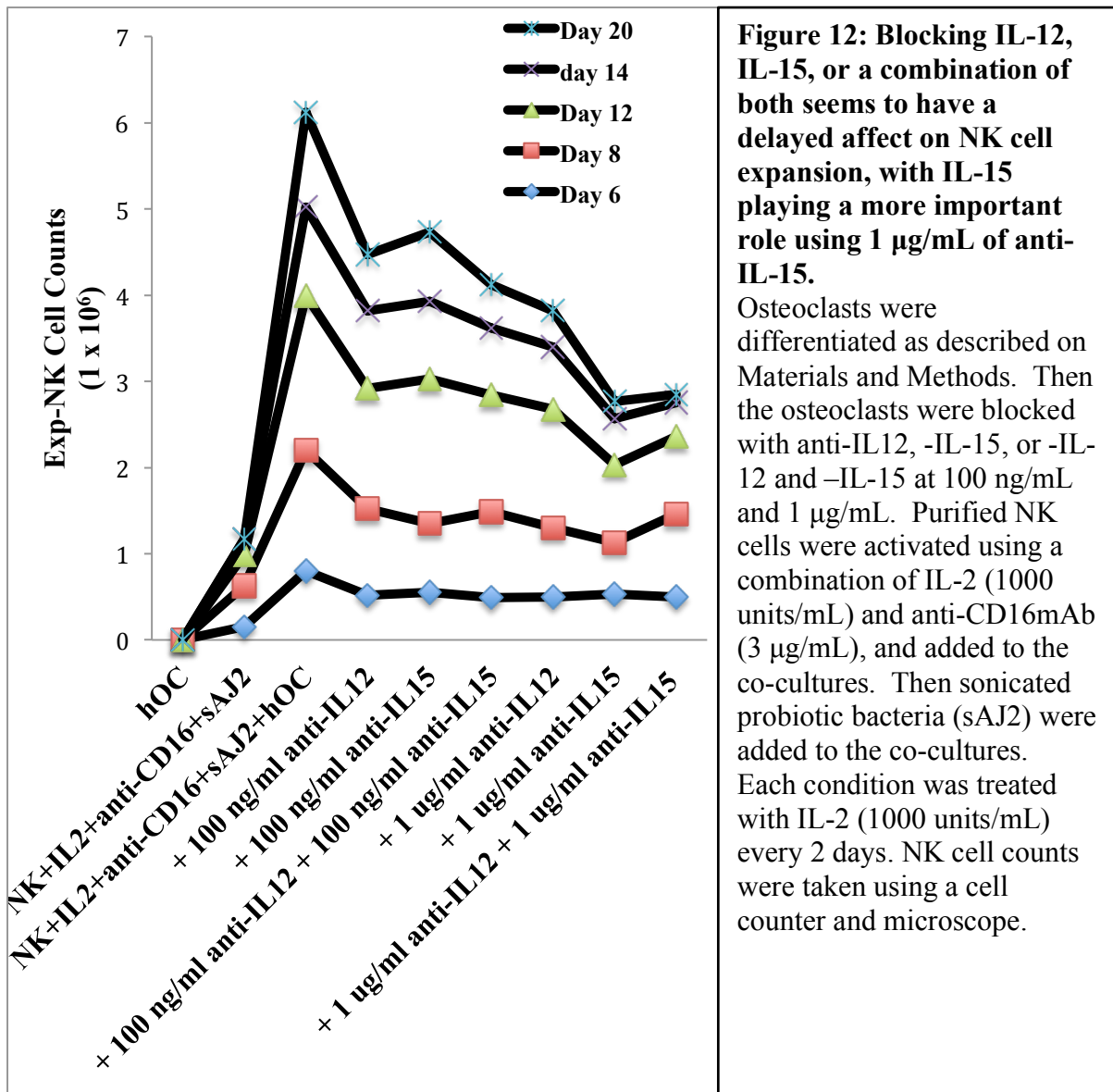
Enzyme-Linked Immunosorbent Assays (ELISAs)

Human IFN- γ and IL-8 Elisa kits were purchased from Biolegend (San Diego, CA). Elisa was performed to detect the level of IFN- γ produced from NK cells co-cultured with different cells. The 96-well EIA/RIA plates were coated with 100ul of diluted capture antibody (1:200) corresponding to target cytokine and incubated overnight at 4°C. After 16 hours incubation, the plates were washed 4 times with wash buffer (0.05% Tween in 1xPBS) and blocked with 200ul of assay diluent (1%BSA in 1xPBS). The plates were incubated on a plate shaker at 200rpm for 1 hour followed by washing 4 times with wash buffer. 100ul of standards and samples collected from each culture were added to the wells and incubated on the plate shaker at 200rpm for 2 hours at room temperature. Then, the plates were washed 4 times with wash buffer and 100ul of diluted detection antibody (1:200) were added to the wells and incubated for 1 hour on the plate shaker at 200rpm at room temperature. After 1 hour of incubation, the plates were washed 4 times with wash buffer and 100ul of diluted Avidin-HRP solution (1:1000) was added to the wells and incubated on the plate shaker at 200rpm for 30minutes followed by washing the plates 5 times with wash buffer. The, 100ul of TMB substrate solution was added to the wells and incubated in dark until the wells developed a desired blue color before adding 100ul of stop solution (2N H₂SO₄ to stop the reaction. The plates were read in a microplate reader at 450nm to obtain absorbance value (Biolegend, ELISA manual).

Statistical analysis

An unpaired, two-tailed student t- test was performed for the statistical analysis. One-way ANOVA with a Bonferroni post-test was used to compare the different groups.

RESULTS



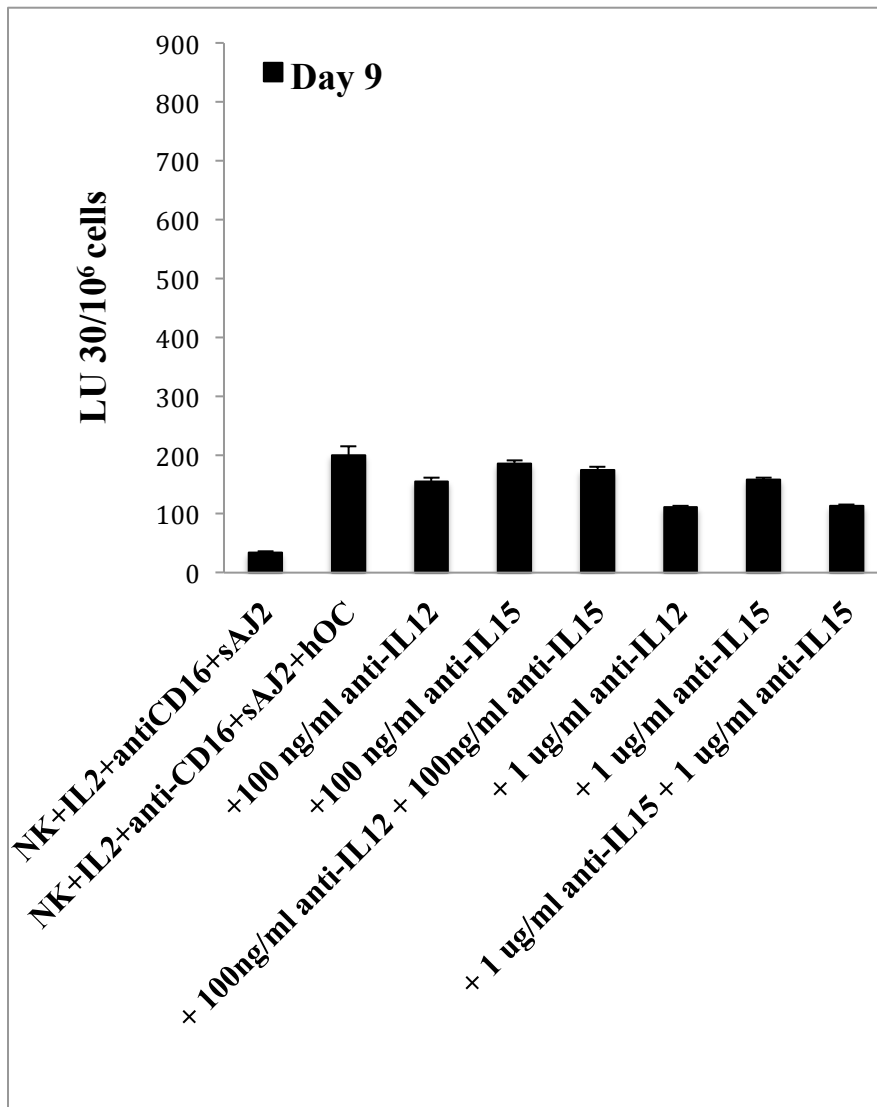


Figure 13: On day 9, blocking IL-12 and IL-15 had no significant impact on NK cell mediated lysis against OSCSCs.

Osteoclasts were differentiated as described on Materials and Methods. Then the osteoclasts were blocked with anti-IL12, -IL-15, or -IL-12 and -IL-15 at 100 ng/mL and 1 μ g/mL. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. On day 9, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ⁵¹Cr release against OSCSCs. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

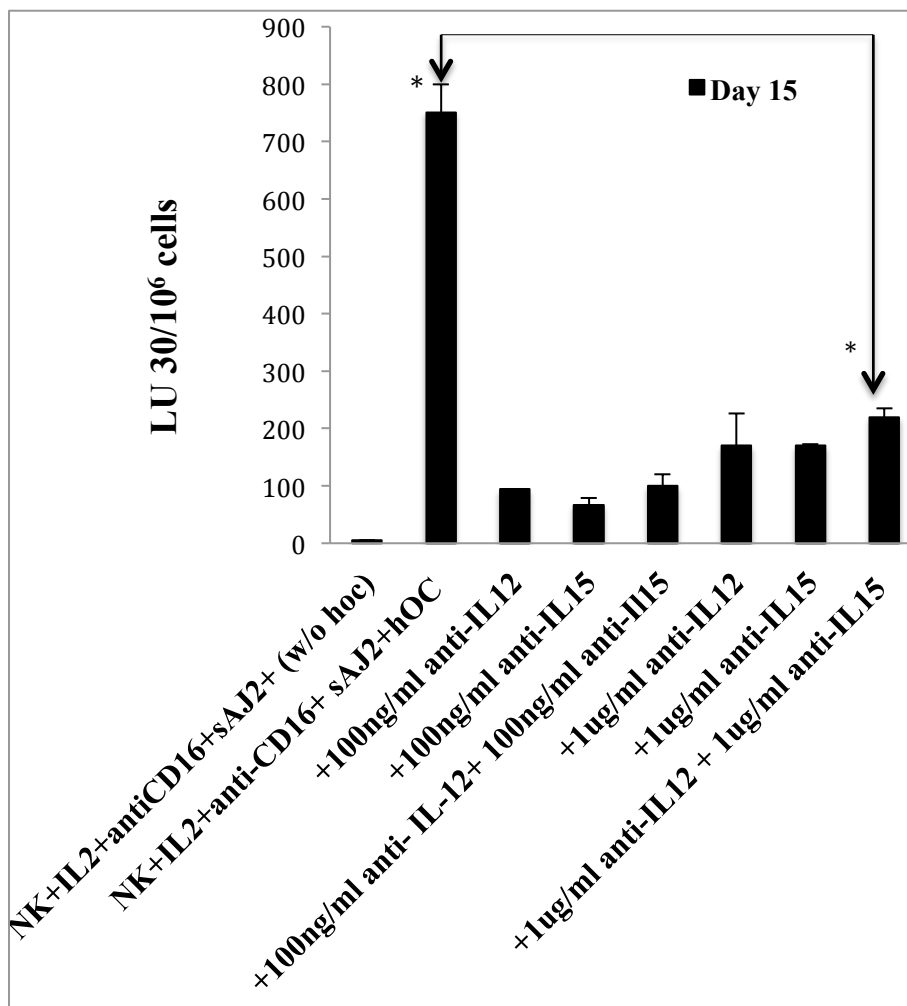


Figure 14: Both cytokines, IL-12 and IL-15 seem to play an equally important role in the amount cytotoxicity expanded-NK cells induce against OSCSCs when co-cultured with osteoclasts. The slightly higher cytotoxicity levels when increasing the concentration from 100 ng/mL to 1 µg/mL may be due to NK cell priming/apoptosis.

Osteoclasts were differentiated as described on Materials and Methods. Then the osteoclasts were blocked with anti-IL12, -IL-15, or -IL-12 and -IL-15 at 100 ng/mL and 1 µg/mL. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 µg/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. On day 15, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ⁵¹Cr release against OSCSCs. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

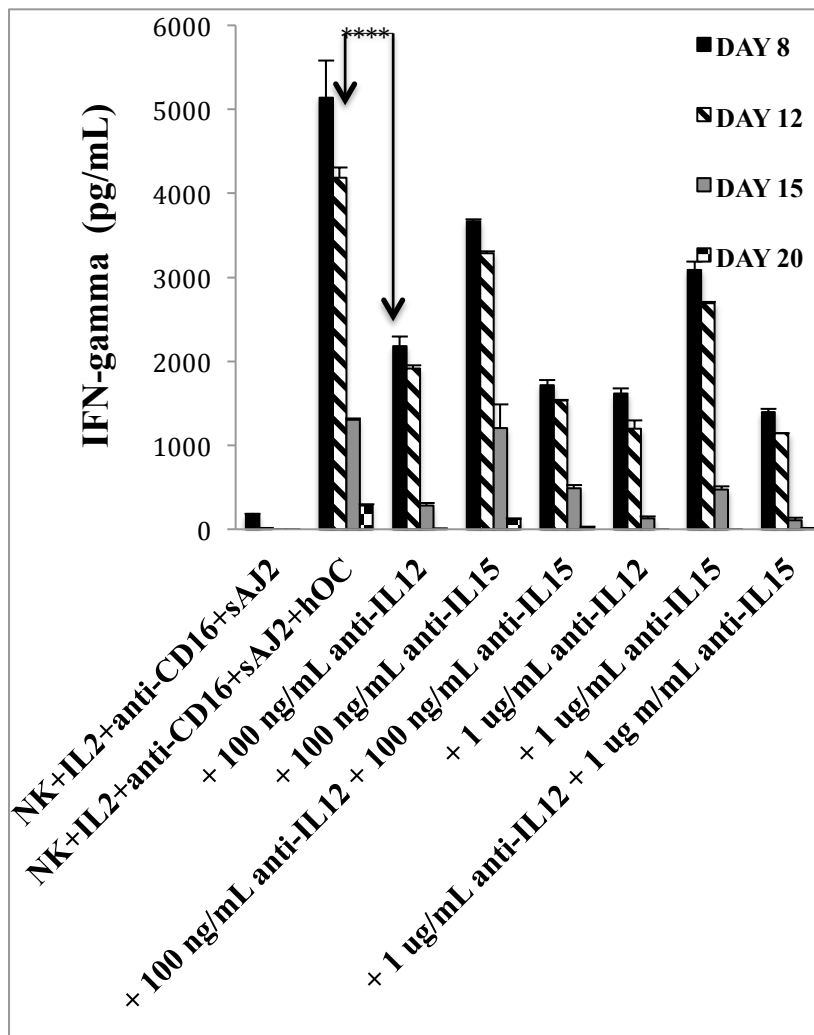
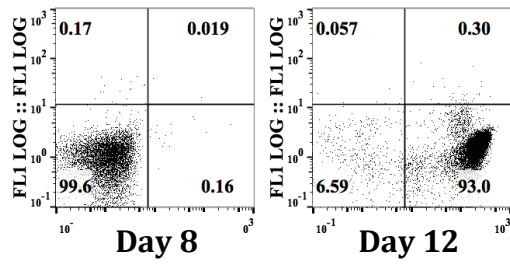


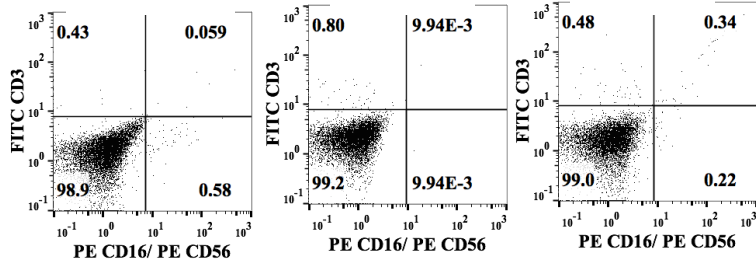
Figure 15: Both cytokines, in particular IL-12, plays a crucial role in the secretion of IFN- γ by osteoclast-induced expanded NK cells. Blocking IL-12 with 1 μ g/mL of respective antibody.

Osteoclasts were differentiated as described on Materials and Methods. Then the osteoclasts were blocked with anti-IL12, -IL-15, or -IL-12 and -IL-15 at 100 ng/mL and 1 μ g/mL. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. Supernatants from each co-culture were collected on days 8, 12, 15, and 20. The levels of IFN- γ were measured using a specific ELISA.

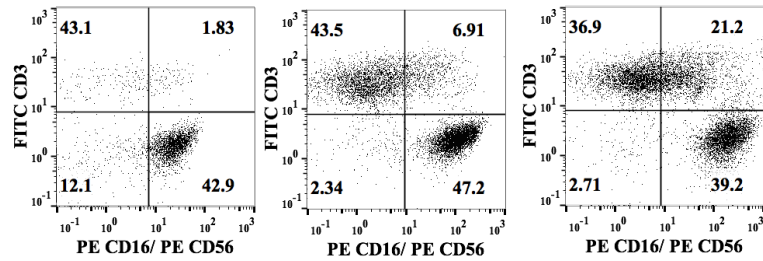
IgG Isotype Control



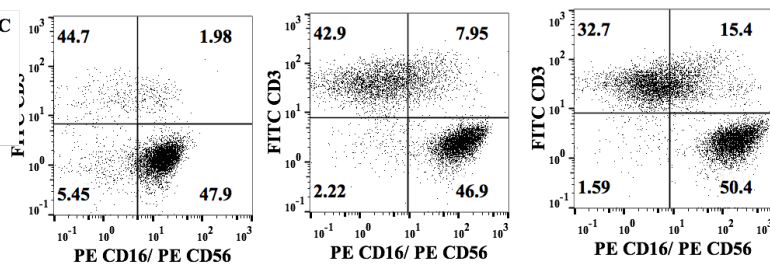
NK+IL2+anti-CD16+sAJ2



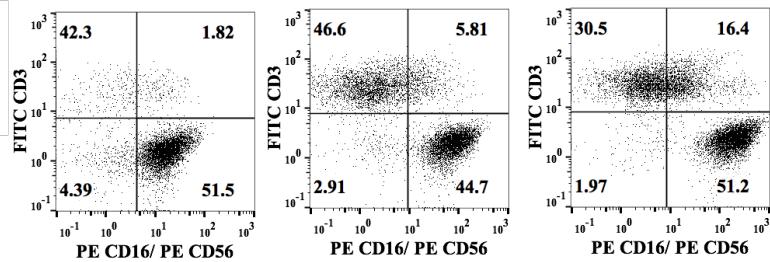
NK+IL2+anti-CD16+sAJ2+hOC



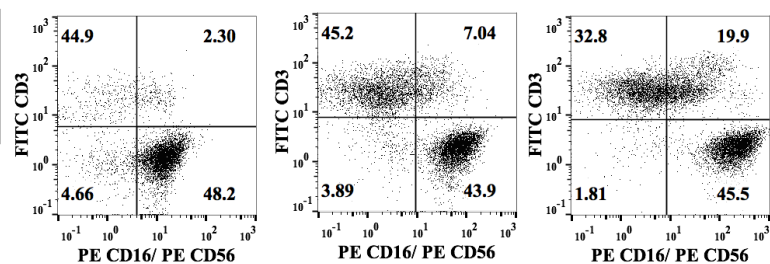
NK+IL2+anti-CD16+sAJ2+hOC
+100 ng/ml anti-IL12



NK+IL2+anti-CD16+sAJ2+hOC
+100 ng/ml anti-IL15



NK+IL2+anti-CD16+sAJ2+hOC
+1 ug/ml anti-IL12



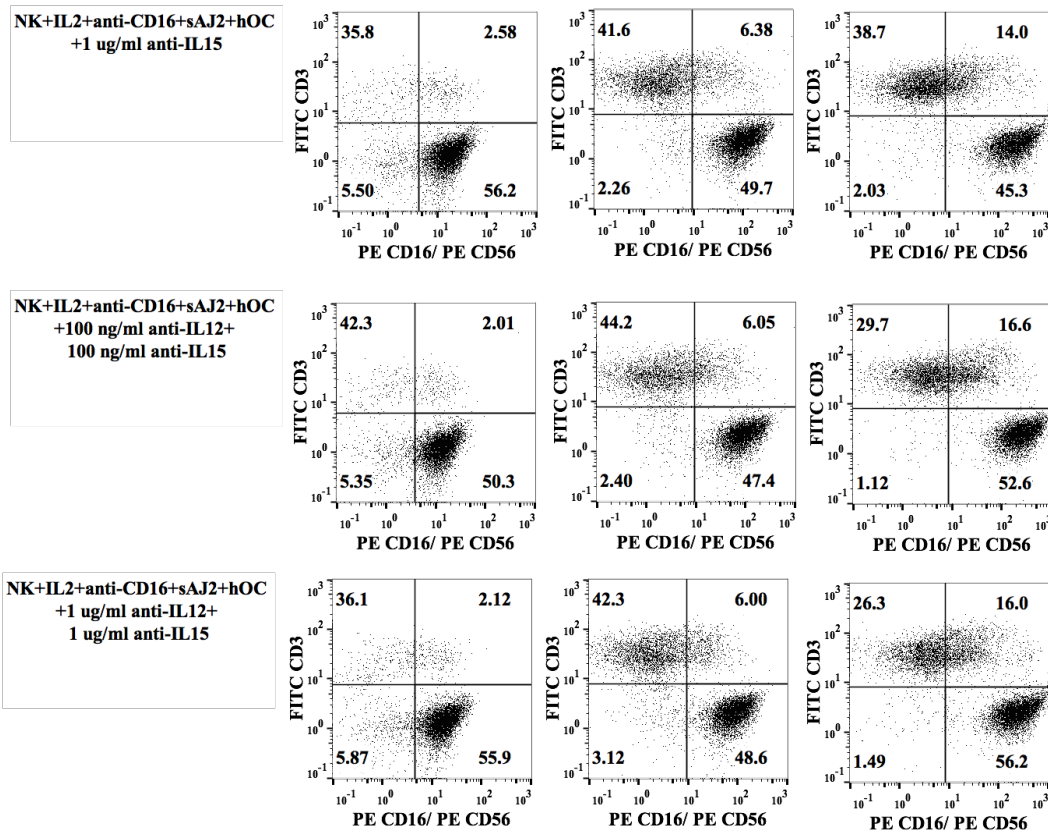


Figure 16: IL-12 and IL-15 seem to have not only an important impact on CD16⁺/CD56⁺ expression on NK cells, but also influence CD3⁺ expression on T cells. Blocking IL-12 and IL-15 seems to prevent the induction of T cells, allowing for the larger population to proliferate to T cells.

Osteoclasts were differentiated as described on Materials and Methods. Then the osteoclasts were blocked with anti-IL12, -IL-15, or -IL-12 and -IL-15 at 100 ng/mL and 1 µg/mL. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 µg/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. Samples of at least 50,000 cells from each conditions were collected, washed, and stained with a two color antibody, FITC CD3/ PE (CD16/CD56) on days 8, 12, and 15 of the co-cultures, and ran with Flow Cytometry. Flow Jo software was used for further analyses.

DISCUSSION

It is not only important to know that osteoclasts are the best candidate myeloid cells in expanding and increasing the potency Natural Killer cells, but it is also as equally important to understand which cytokines are contributing to the dynamics of osteoclast-induced Natural Killer cell functions.

IL-12 and IL-15 are two cytokines that have been extensively studied in the roles they play in NK cell activation, proliferation, and function, but never in the context of osteoclast-induced Natural Killer cells. If IL-12 and IL-15 contribute to the increase in cytotoxicity and IFN- γ secretion of osteoclast-induced expanded NK cells, then blocking these cytokines would hinder their function. To observe and determine the roles IL-12 and IL-15 play in osteoclast-induced expanded NK cells, primary NK cells were activated with anti-CD16 and IL-2 overnight, supplemented with sAJ2, and then co-cultured with human osteoclasts at a NK cell to osteoclast ratio of 2:1 (500,000: 250,000). Similar studies had been conducted where different concentrations of anti-IL12 or IL-15 were used to assess the affect on solely NK cell activation and proliferation, With a controlled condition (activated NK cells+ hOC), the remaining conditions were given anti-IL-12, anti-IL15, or a combination of both at concentrations of 100ng/ml and 1 μ g/ml. These co-cultures were incubated undisturbed for at least 6 days before functional assays were conducted.

In this study, we were able to determine that blocking IL-12, IL-15, or both does not have an immediate impact on NK cell expansion. Its effect is delayed as we still see relatively similar NK cell expansion between the controlled condition (NK+IL-2+anti-CD16+sAJ2+hOC) and the experimental blocking conditions on day 6 of the co-culture. It is until day 8, when we start observing significant inhibition of NK cell proliferation, particularly when osteoclasts are

blocked with either anti-IL-12 or anti-IL-15 at 1 $\mu\text{g}/\text{mL}$ (Fig. 12). Thus, these two cytokines are not solely responsible for NK cell expansion, although blocking these two cytokines does decrease the rate of NK cell expansion over a 20-day period *in vitro*.

Based on chromium⁵¹ release assays taken on day 9 of co-cultures, blocking IL-12 and IL-15 with antibodies at either concentration of 100 ng/ml or 1 $\mu\text{g}/\text{mL}$ did not affect the cytotoxicity levels of osteoclast-induced expanded Natural Killer cells against OSCSCs (Fig. 13). However, by day 15, blocking either cytokine begins to induce a significant decrease in NK cell-mediated lysis against OSCSCs. There does not seem to be a synergistic affect when blocking both cytokines. The increase in cytotoxicity levels as a result of increasing the antibody from 100 ng/mL to 1 $\mu\text{g}/\text{mL}$ may be due to self-induced cell death/apoptosis (Fig. 14). Conclusively, it is plausible to suggest that IL-12 and IL-15 do not have an immediate influence over the expanded NK's lysis potential against OSCSCs.

Understanding these cytokines' involvement in long-term IFN- γ secretion is as crucial as understanding the cytotoxicity mechanisms. When comparing both IL-12 and IL-15, there is a clear distinction in terms of which cytokine has more influence in inducing higher IFN- γ secretion by osteoclast-induced Natural Killer cells. As illustrated on figure 15, blocking IL-12 at 100 ng/mL yields a significantly lower IFN- γ levels compared to the condition where we block IL-15 at 100 ng/mL. Increasing the antibody concentration from 100 ng/mL to 1 $\mu\text{g}/\text{mL}$ decreases the amount of IFN- γ secretion induction with statistical significance ($p < 0.05$). However, there are synergistic reductions in IFN- γ secretion when both antibodies for IL-12 and IL-15 are not statistically significant when compared to blocking of only IL-12 (Fig. 15). As a result of observing a significant decline in IFN- γ secretion levels by day 8, we can conclude that

IL-12 and IL-15 play a more important role in differentiating tumor cells than their involved in inducing NK-cell mediated lysis against OSCSCs.

Although IL-12 and IL-15 are crucial in modulating NK cell-mediated cytotoxicity against OSCSCS, inducing IFN- γ secretion, and maintaining NK cell expansion, these cytokines do not seem show a specificity of expanding NK cells over T cells. In other words, these cytokines seem to maintain relatively equal proportions of NK cells (CD16⁺CD56⁺) and T/NKT (CD3⁺CD16⁻CD56⁻/CD3⁺CD16⁺CD56⁺) cells (Fig. 16). In fact, blocking IL-12 and Il-15 at 1 μ g/mL seems to slightly increase the proportions of NK cells over T/NKT cells, suggesting that there's an overlap in these cytokines' functions. In other words, IL-12 and IL-15 are not only important in maintain NK cell expansion, but they are equally important in expanding CD3⁺ cells. These observations suggest that both of these cytokines are as equally important for NK cell activation and development as they are for T cell activation and development.

CONCLUSION

Beginning to understand which cytokines are involved in osteoclast-primary NK cells interactions to produce potent expanded NK cells is imperative to move forward from *in vitro* to *in vivo* preclinical trial experiments. Based on functional assays both IL-12 and IL-15 equally contribute to expanded NK cells' cytotoxicity potency against OSCSCs. Increasing the concentration of anti-IL-12/anti-IL15 from 100 ng/ml to 1 µg/ml, however, does not increase cytotoxicity. This indicates that supplementing expanded-NK cells with a stronger dose of either IL-12 or IL-15 will not increase the cytotoxic capabilities of Natural Killer cells on a significant level. In addition, according to our studies, blocking IL-12 and/or IL-15 has a delayed affect on the decrease of cytotoxicity levels, indicating that these cytokines are not the main contributors of NK cell-mediated lysis against OSCSCs.

In terms of IFN- γ secretion, IL-12 plays a more important role than IL-15 as shown in figure 15. But unlike in NK cell-mediated lysis, these two cytokines exhibit a synergistic affect on IFN- γ induction and more important to note, their impact is immediate. Figure 14 illustrates how IL-12 and IL-15 have a delayed but effective affect on NK cell proliferation. However, these same cytokines are shown to be neutral in proliferating NK cells over T cells or vice versa.

Chapter 3

Specific Aim 3: To investigate the differences between dendritic cell- and osteoclast-induced NK cell expansion and function.

INTRODUCTION

Natural Killer (NK) Cell Expansion vs. T Cell Expansion

Numerous experiments have induced NK expansion by enriching peripheral blood mononuclear cells (PBMCs) by T cell and B cell depletion of the PBMCs followed by a positive selection for NK cells using anti-CD56 conjugated magnetic beads and depletion of CD3⁺ cells. In our lab, however, we use a negative selection to purify out NK cells from PBMCs. These same experiments have shown a significant improvement in the NK cells' potency with the addition of IL-2 and IL-15. This potency is enhanced even further when activated NK cells are supplemented with sAJ2 and monocytes or its derivatives, being dendritic cells (DCs) or human osteoclasts (hOC).

Two and three days after monocytes were stimulated with IL-4 and GM-CSF, these cells begin to manifest themselves as enlarged, spindle-shaped structures or small branch-shaped structures. By day 5, the morphological characteristics become more apparent towards DCs: star-like, dendritic, or radiant-shaped cells are observed. However, these DCs are immature, as illustrated by flow cytometry, showing these cells to positive for DC1a (marker for DCs), but negative for CD83 (a marker for maturation) [73]. DCs have a complex interaction with NK cells. DCs have been used as feeder cells in NK cultures. Scientists have claimed that DCs are potent inducers of NK cell proliferation and enhancers of NK cell function [73]. DCs have also shown to help NK cell secrete IFN- γ via IL-12 and stimulate NK cell proliferation via IL-15

[73]. A previous study showed that NK cells cultured for 15 days in the presence of autologous, immature DCs yielded the highest expansion rate when the ratio of NK cells: DCs was 2:1 [73]. In addition, others have shown that DCs not only increase NK cell proliferation but also increase NK cell cytotoxicity [73]. The highest NK cell purity and the highest rates of NK cell proliferation were obtained when the ratio of NK cells to DCs was 2:1 [73]. Moreover, NK cell-mediated cytotoxicity peaked after 15 days in culture when the NK cell to target ratio was 10:1.

Previous studies have suggested antitumor immunity is likely limited by Treg expansion, which is a concern for NK cell adoptive transfer that requires rhIL-2 post infusion to support NK cell vitality and function. Dendritic cells have been used extensively as feeder cells to proliferate Natural Killer cells to be used *ex vivo* in immunotherapy treatments against various cancers. However, my studies show DCs are not the best group of cells to be used as feeder cells if we want to optimize Natural Killer cells' potency in killing against OSCSCs and differentiating tumor cells. DCs in fact have shown a preference towards T-cell proliferation over NK-cell proliferation.

MATERIALS AND METHODS

Cell Lines, Reagents, and Antibodies

RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA) was used for the cultures of human immune cells. Oral Squamous Cancer Stem Cells (OSCSCs) were isolated from oral cancer patient tongue tumors at UCLA School of Medicine and cultured in RPMI 1640 supplemented 10% Fetal Bovine Serum (FBS) (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic (100x), 1% sodium pyruvate (100nM), 1.4% MEM non-essential amino acids, 1% L-Glutamine, 0.2% gentamicin sulfate (Gemini Bio-products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). Alpha-MEM medium (Life Technologies, CA) supplemented with 10% FBS and penicillin-streptomycin (Gemini Bio-Products, CA) was used to culture human osteoclasts. M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -80°C. PE conjugated isotype control, CD16, NKp44, CXCR1, CXCR3 and DNAM were purchased from Biolegend (San Diego, CA).

Bacteria Sonication

sAJ2 is a combination of 8 different strains of sonicated gram positive probiotic bacteria selected by Dr. Anahid Jewett for their superior ability to induce optimal secretion of both pro-inflammatory and anti-inflammatory cytokines in NK cells (manuscript in prep). The whole bacteria was weighed and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were vortexed thoroughly and then sonicated for 15 seconds on ice at an amplitude from 6 to 8. After that, the samples were incubated for 30 seconds on ice. After every 5 pulses, a sample was taken to observe under the microscope every

time until we obtained at least 80 percent of cell walls lysed. Then, the sonicated samples were aliquoted and stored in -20 to -80 degrees Celsius for long-term studies.

Purification of NK cells

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. The Ficoll-Hypaque technique was used to fractionate the blood, and the white cloudy layer called peripheral blood mononuclear cells (PBMC) was harvested, washed and re-suspended in RPMI 1640 (Invitrogen by Life Technologies, CA) supplemented with 10% FBS. Non-adherent human peripheral blood lymphocytes (PBL) were obtained after 1-2 hours incubation at 37°C on a plastic tissue culture dish. NK cells were negatively selected and isolated using the EasySep® Human NK cell enrichment kit purchased from Stem Cell Technologies (Vancouver, Canada). The NK cell population was found to have greater than 90% purity based on flow cytometric analysis of anti-CD16 antibody stained NK cells. Purified NK cells were cultured in RPMI Medium 1640 supplemented with 10% FBS (Gemini Bio-Products, CA), 1% antibiotic antimycotic, 1% sodium pyruvate, and 1% MEM non-essential amino acids (Invitrogen, Life Technologies, CA).

Human Peripheral Blood Monocyte Purification

Written informed consents approved by UCLA Institutional Review Board (IRB) were obtained from healthy blood donors and all the procedures were approved by the UCLA-IRB. Peripheral blood mononuclear cells (PBMCs) were obtained after Ficoll-hypaque centrifugation. PBMCs were cultured onto the tissue culture plate for an hour to two hours and the adherent

subpopulation of PBMCs was detached from the tissue culture plate. Monocytes were purified using the EasySep® Human monocyte cell enrichment kit obtained from Stem Cell Technologies (Vancouver, Canada). The monocyte population was found to have greater than 95% purity based on flow cytometric analysis of CD14 antibody stained monocyte cells.

Generation of Dendritic Cells (DCs) and Osteoclasts (hOCs)

Purified monocytes from healthy donors' PBMCs were differentiated into dendritic cells by culturing them in alpha-MEM medium containing GM-CSF (150ng/mL) and IL-4 (50ng/mL) for 7 days. Osteoclasts were generated from monocytes and cultured in alpha-MEM medium containing M-CSF (25ng/mL) and Rank Ligand (RANKL) (25ng/mL) for 21 days, or otherwise specified. Medium was refreshed every 3 days with fresh alpha-MEM containing M-CSF (25ng/mL) and RANKL (25ng/mL).

Surface Staining

1×10^5 NK cells from each condition were stained in 50ul of cold 1%PBS-BSA with pre-determined optimal concentration of PE and FITC conjugated isotype control, FITC CD3 conjugated with PE CD16+CD56, and incubated at 4°C for 30 minutes. Then, the cells were washed and resuspended in 1%PBS-BSA. The Epics C (Coulter) flow cytometer was used for surface analysis.

⁵¹Cr release cytotoxicity assay

⁵¹Cr was purchased from Perkin Elmer (Santa Clara, CA). NK cell cytotoxic function in the experimental cultures and the sensitivity of target cells to NK cell mediated lysis were determined using standard ⁵¹Cr release cytotoxicity assays. The effector cells (1x10⁵ NK cells/well) were aliquoted into 96-well round-bottom microwell plates (Fisher Scientific, Pittsburgh, PA) and titrated to four serial dilutions. The target cells (5x10⁵ OSCSCs/well) were labeled with 50μCi ⁵¹Cr (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour. The cells were washed twice to remove excess unbound ⁵¹Cr. The ⁵¹Cr-labeled target cells were incubated with the effector cells for 4 hours. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. The percentage specific cytotoxicity was calculated as follows;

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

LU 30/10⁶ 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)

Human IFN-γ Elisa kit was purchased from Biolegend (San Diego, CA). ELISA was performed to detect the level of IFN-γ produced from NK cells co-cultured with different cancer or immune cells. The 96-well EIA/RIA plates were coated with 100ul of diluted capture antibody (1:200) corresponding to target cytokine and incubated overnight at 4°C. After 16 hours incubation, the plates were washed 4 times with wash buffer (0.05% Tween in 1xPBS) and blocked with 200ul of assay diluent (1%BSA in 1xPBS). The plates were incubated on a plate shaker at 200rpm for 1 hour followed by washing 4 times with wash buffer. 100ul of standards

and samples collected from each culture were added to the wells and incubated on the plate shaker at 200rpm for 2 hours at room temperature. Then, the plates were washed 4 times with wash buffer and 100ul of diluted detection antibody (1:200) were added to the wells and incubated for 1 hour on the plate shaker at 200rpm at room temperature. After 1 hour of incubation, the plates were washed 4 times with wash buffer and 100ul of diluted Avidin-HRP solution (1:1000) were added to the wells and incubated on the plate shaker at 200rpm for 30minutes. After washing the plates 5 times with wash buffer, 100ul of TMB substrate solution were added to the wells and incubated in the dark until the wells developed a desired blue color before adding 100ul of stop solution (2N H₂SO₄) to stop the reaction. The plates were read in a microplate reader at 450nm to obtain absorbance values (Biolegend, ELISA manual).

Statistical analysis

An unpaired, two-tailed student t-test was performed for the statistical analysis. One-way ANOVA with a Bonferroni post-test was used to compare the different groups.

RESULTS

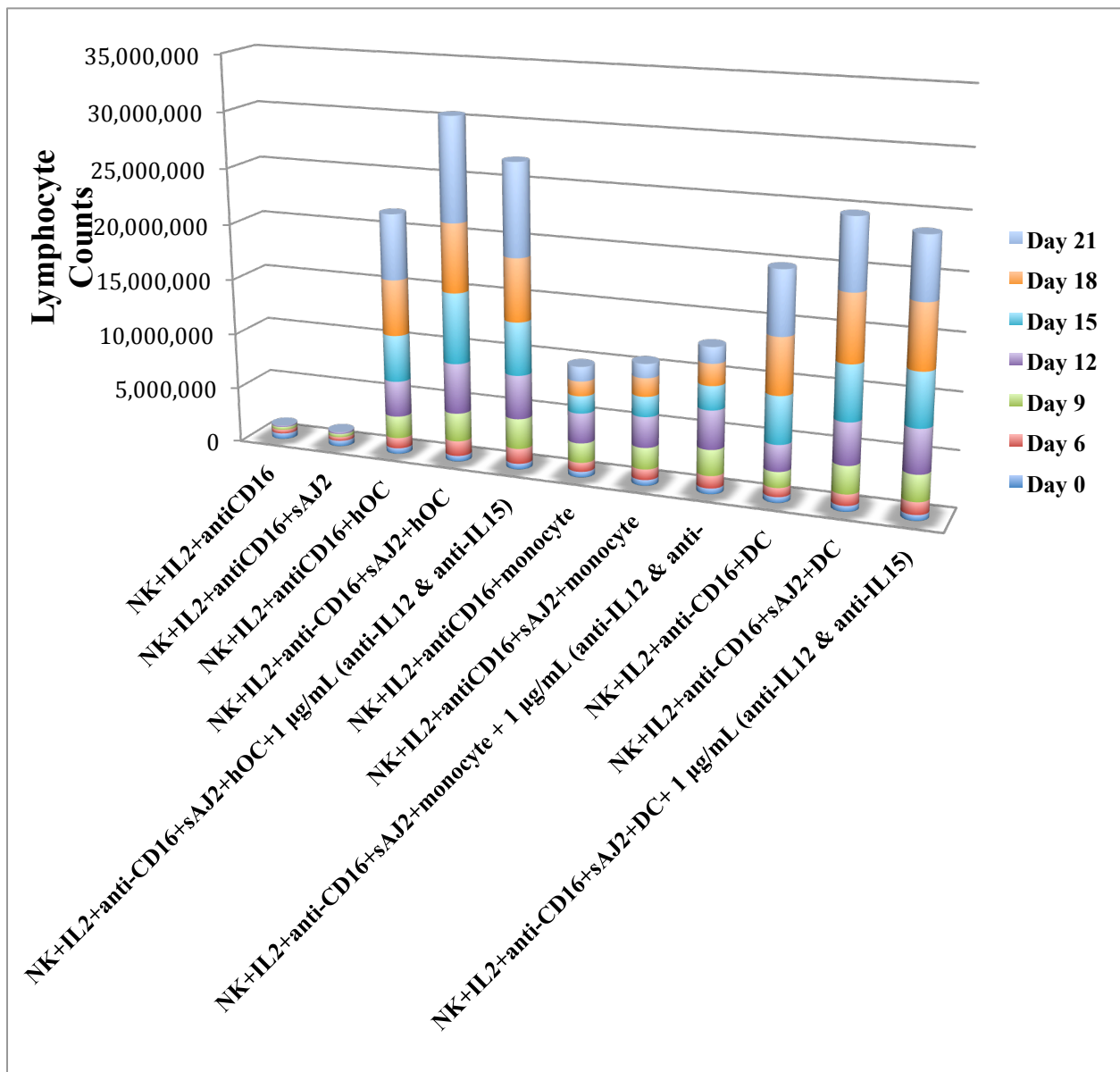
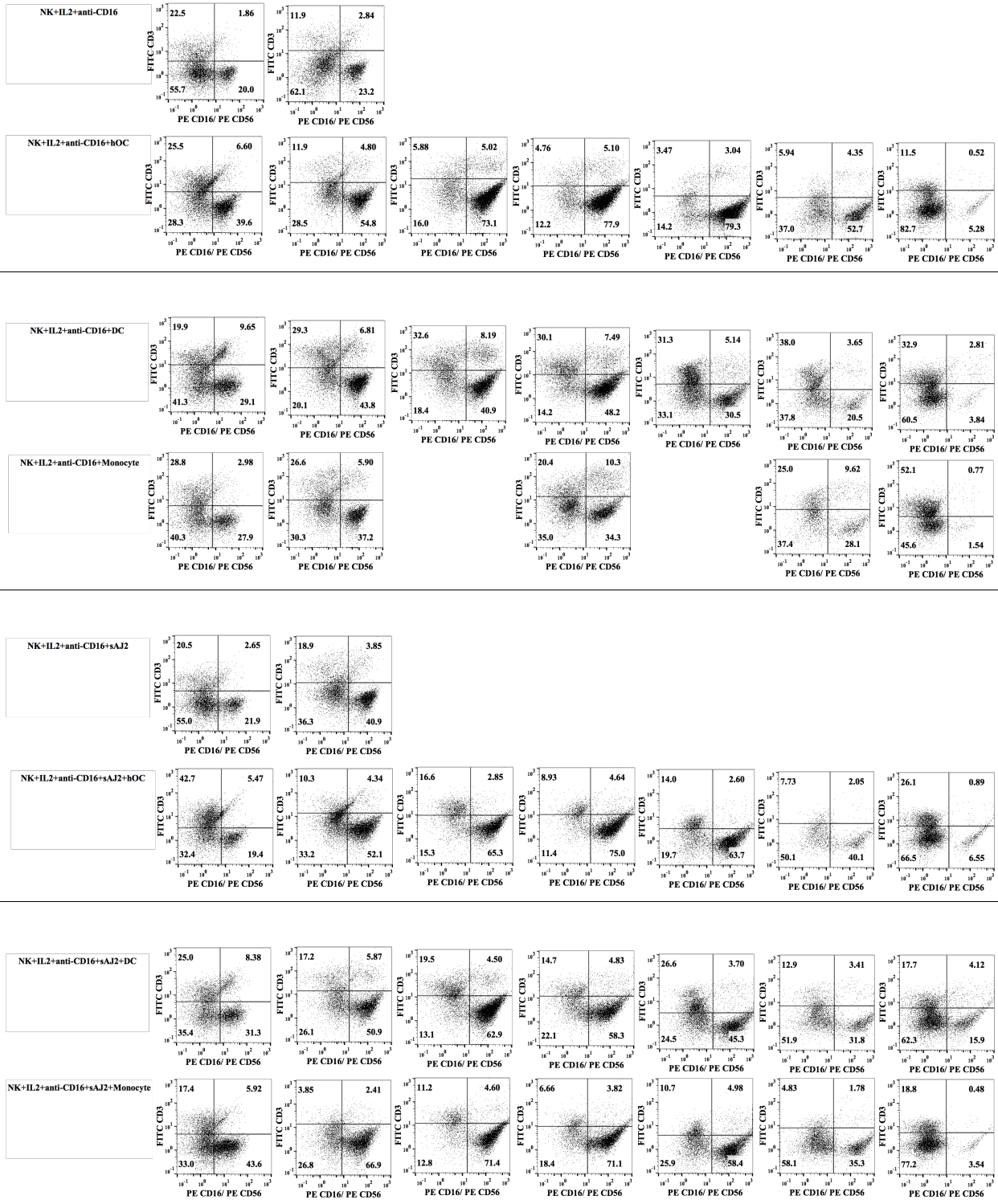


Figure 17: When comparing expanded NK cells co-cultured with osteoclasts, monocytes, or dendritic cells, we can observe that DCs induce the highest numbers of $CD3^+$ T cells and $CD3^+CD16^+CD56^+$ NKT cells over a 21-day period.

Osteoclasts and dendritic cells were differentiated as described on Materials and Methods. Monocytes, osteoclasts, and dendritic cells were seeded at 0.25×10^6 cells/well, one of each blocked with 1 $\mu\text{g/mL}$ of anti-IL-12 and anti-IL-15 for two hours. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 $\mu\text{g/mL}$), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. All cells (lymphocytes) were counted from day 0 to day 21 of the co-cultures using a cell counter and microscope.



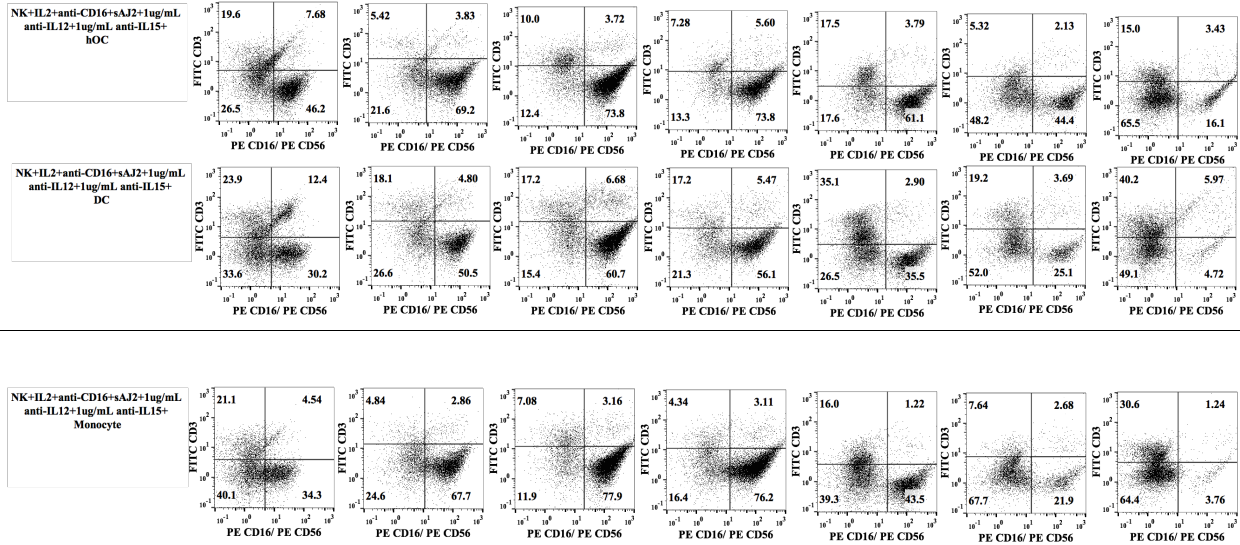


Figure 18: On average, there is a steady increase in CD16⁺CD56⁺ expression on OC-induced expanded NK cells. On the other hand, there is a steady increase of CD3⁺ and CD3⁺CD16⁺CD56⁺ expression on DC-induced expanded NK cells over the course of a 21-day co-culture. sAJ2 proves to be crucial in increasing the ratio of NK cells to T and NKT cells *in vitro*.

Osteoclasts and dendritic cells were differentiated as described on Materials and Methods. Monocytes, osteoclasts, and dendritic cells were seeded at 0.25×10^6 cells/well, one of each blocked with 1 $\mu\text{g}/\text{mL}$ of anti-IL-12 and anti-IL-15 for two hours. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 $\mu\text{g}/\text{mL}$), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. NK cell and T/NKT cell proportions were tracked over a 25-day period using a two color antibody FITC CD3/ PE (CD16/CD56), and subsequently run with Flow Cytometry. Flow Jo was software was used for further analyses.

NK+IL-2+anti-CD16

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
*T cells/NKT	54.9%	38.8%	x	x	x	x	x
**NK cells	45.1%	61.2%	x	x	x	x	x

NK+IL-2+anti-CD16+hOC

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	44.8%	23.4%	13.0%	11.3%	7.6%	16.3%	69.5%
NK cells	55.2%	76.6%	87.0%	88.7%	92.4%	83.7%	30.5%

NK+IL-2+anti-CD16+DC

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	50.4%	45.2%	49.9%	43.8%	54.4%	67.0%	90.3%
NK cells	49.6%	54.8%	50.1%	56.2%	45.6%	33.0%	9.7%

NK+IL-2+anti-CD16+Monocyte

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	53.3%	46.6%	x	47.2%	x	55.1%	97.2%
NK cells	46.7%	53.4%	x	52.8%	x	44.9%	2.8%

NK+IL-2+anti-CD16+sAJ2

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	51.3%	35.8%	x	x	x	x	x
NK cells	48.7%	64.2%	x	x	x	x	x

NK+IL-2+anti-CD16+sAJ2+hOC

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	44.5%	22.0%	22.9%	15.3%	20.7%	19.7%	80.4%
NK cells	55.5%	78.0%	77.1%	84.7%	79.3%	80.3%	19.6%

NK+IL-2+anti-CD16+sAJ2+DC

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	51.5%	31.1%	27.6%	25.2%	40.0%	33.9%	57.8%
NK cells	48.5%	68.9%	72.4%	74.8%	60.0%	66.1%	42.2%

NK+IL-2+anti-CD16+sAJ2+Monocyte

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	34.9%	8.6%	18.1%	12.9%	21.2%	15.8%	84.5%
NK cells	65.1%	91.4%	81.9%	87.1%	78.8%	84.2%	15.5%

NK+IL-2+anti-CD16+sAJ2+1ug/ml anti-IL-12+ 1ug/ml anti-IL-15+hOC

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25

T cells/NKT	37.1%	11.7%	15.8%	14.9%	25.8%	14.3%	53.3%
NK cells	62.9%	88.3%	84.2%	85.1%	74.2%	85.7%	46.7%

NK+IL-2+anti-CD16+sAJ2+1ug/ml anti-IL-12+ 1ug/ml anti-IL-15+DC

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	54.5%	31.2%	28.3%	28.7%	51.7%	47.7%	90.7%
NK cells	45.5%	68.8%	71.7%	71.3%	48.3%	52.3%	9.3%

NK+IL-2+anti-CD16+sAJ2+1ug/ml anti-IL-12+ 1ug/ml anti-IL-15+Monocyte

	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 25
T cells/NKT	42.7%	10.2%	11.6%	8.9%	28.3%	32.2%	89.4%
NK cells	57.3%	89.8%	88.4%	91.1%	71.7%	67.8%	10.6%

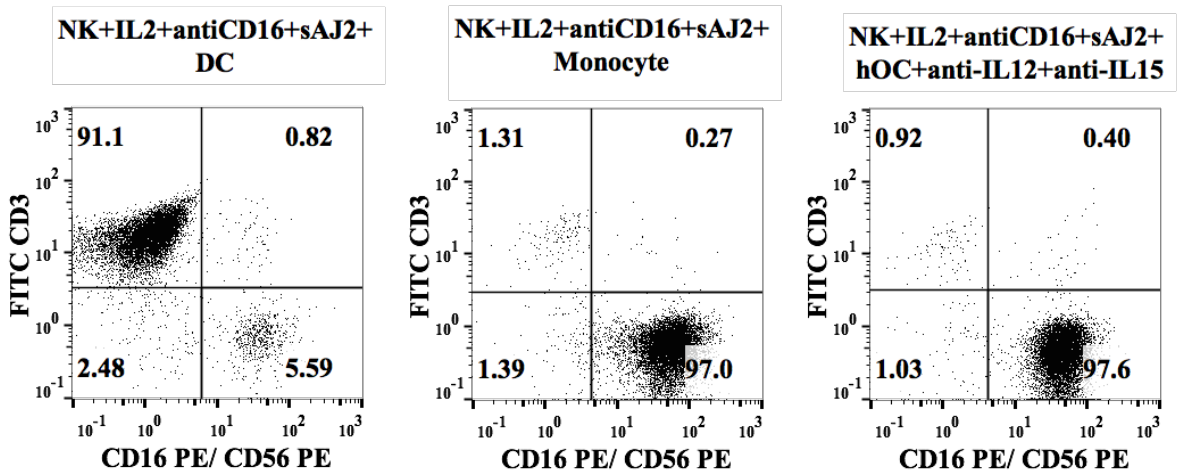
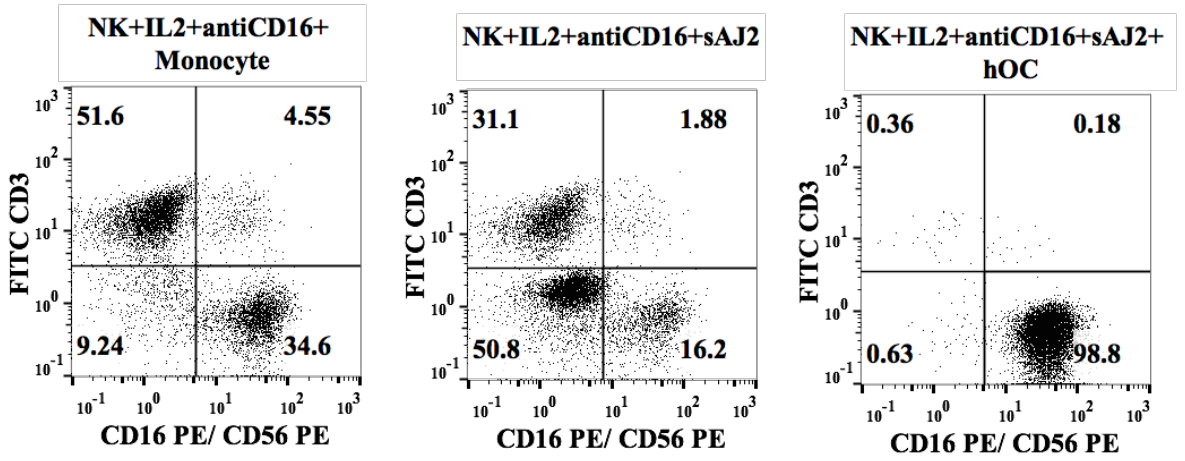
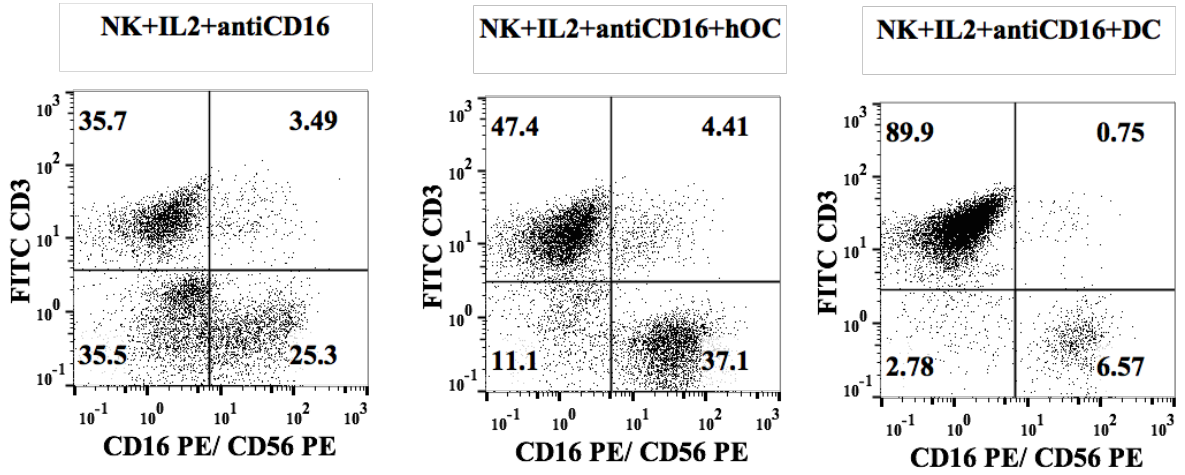
*T cells: (CD3⁺CD16⁻CD56⁻)

*NKT cells: (CD3⁺CD16⁺CD56⁺)

**NK cells: (CD3⁻CD16⁺CD56⁺)

Figure 19: On average, there is a steady increase in CD16⁺CD56⁺ expression on OC-induced expanded NK cells. On the other hand, there is a steady increase of CD3⁺ and CD3⁺CD16⁺CD56⁺ expression on DC-induced expanded NK cells over the course of a 21-day co-culture. sAJ2 proves to be crucial in increasing the ratio of NK cells to T and NKT cells *in vitro*. However, the ratio flips towards a higher T/NKT cell ratio over NK cells when the majority of the cell population undergoes apoptosis.

Osteoclasts and dendritic cells were differentiated as described on Materials and Methods. Monocytes, osteoclasts, and dendritic cells were seeded at 0.25 x 10⁶ cells/well, one of each blocked with 1 ug/mL of anti-IL-12 and anti-IL-15 for two hours. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 µg/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. NK cell and T/NKT cell proportions were tracked over a 25-day period using a two color antibody FITC CD3/ PE (CD16/CD56), and subsequently run with Flow Cytometry. Flow Jo was software was used for further analyses. In addition, dead/unstained cells were ignored to get live cell proportions of NK cells vs. T cell/NKT cells.



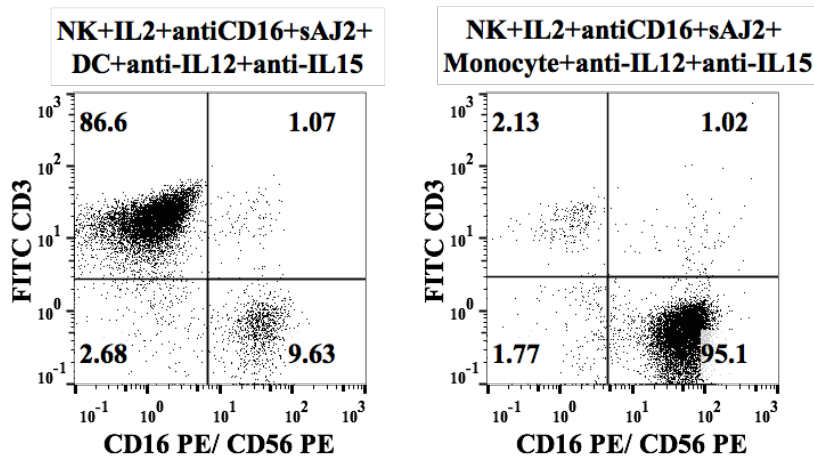


Figure 20: Dendritic cells (DCs) show to be potent inducers of T cell proliferation, and not NK cell proliferation from one case study.

At day 12 of *in vitro* co-cultures of NK cells with osteoclasts, DCs, and monocytes, samples of each condition were ran in flow cytometry using a two-color antibody [FITC CD3⁺/ PE (CD16⁺/CD56⁺) to determine NK cell to T cell proportions. In this extreme case study, DCs proliferated T cells extensively from 5% at day 0 to over 90% by day 12. This same trend stayed unchanged until day 30 when most cells underwent apoptosis. In this figure, we can observe the importance sAJ2 plays in NK cell expansion. Without sAJ2, monocytes and osteoclasts are limited in expanding NK cells, comprising less than 50% of the entire lymphocyte population. However, with the induction of sAJ2, NK cell expansion increases dramatically well up to over 90% of the entire lymphocyte population, staying unchanged during the course of 30 days.

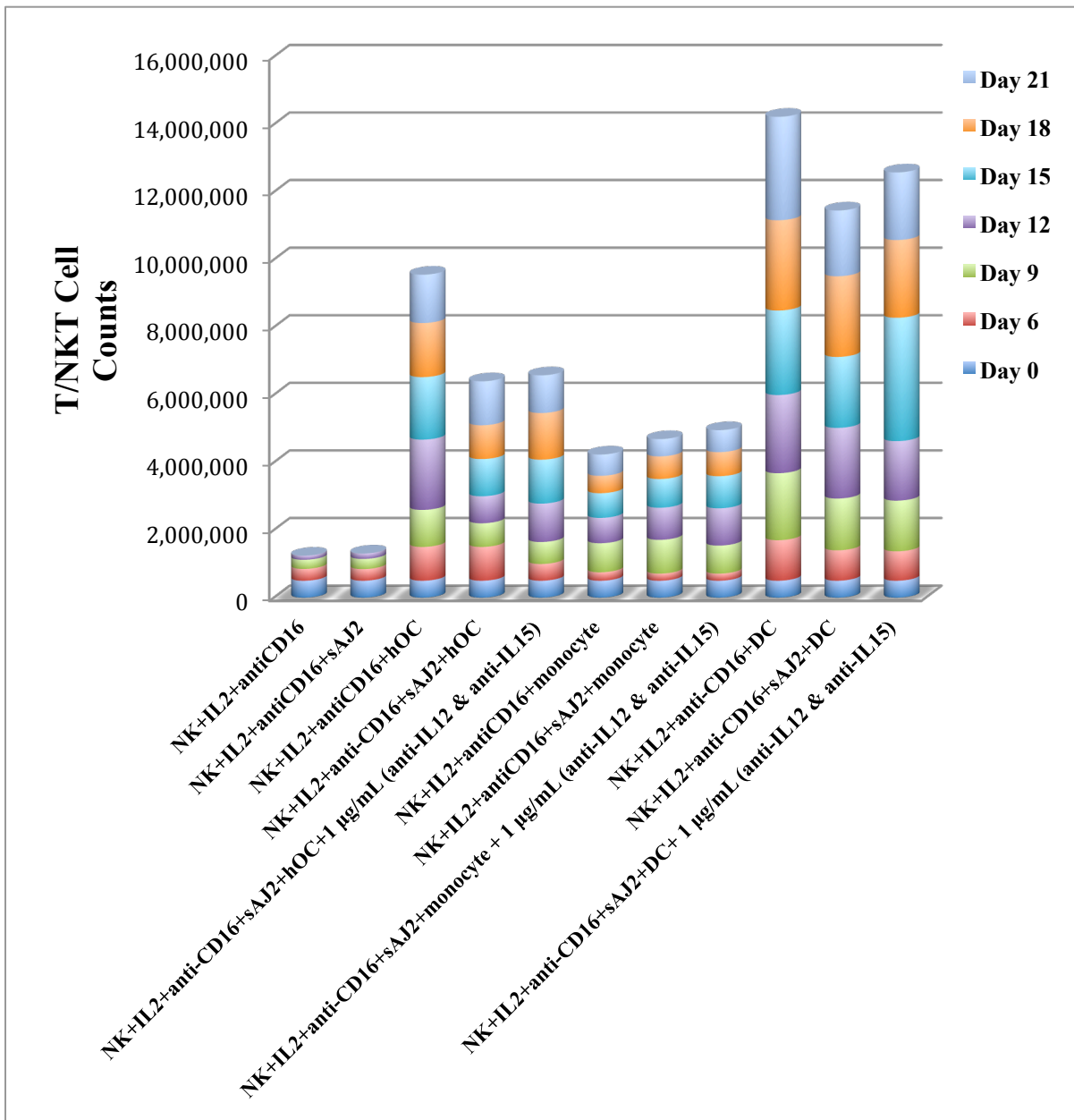


Figure 21: Dendritic Cells (DCs) expand T/NKT cells at a higher rate than monocytes, and/or osteoclasts.

Osteoclasts and dendritic cells were differentiated as described on Materials and Methods. Monocytes, osteoclasts, and dendritic cells were seeded at 0.25×10^6 cells/well, one of each blocked with 1 $\mu\text{g}/\text{mL}$ of anti-IL-12 and anti-IL-15 for two hours. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 $\mu\text{g}/\text{mL}$), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. All cells (lymphocytes) were counted from day 0 to day 21 of the co-cultures using a cell counter and microscope. T cells and NKT cells were calculated using flow cytometry analyses after staining with FITC CD3/PE (CD16-CD56) antibody.

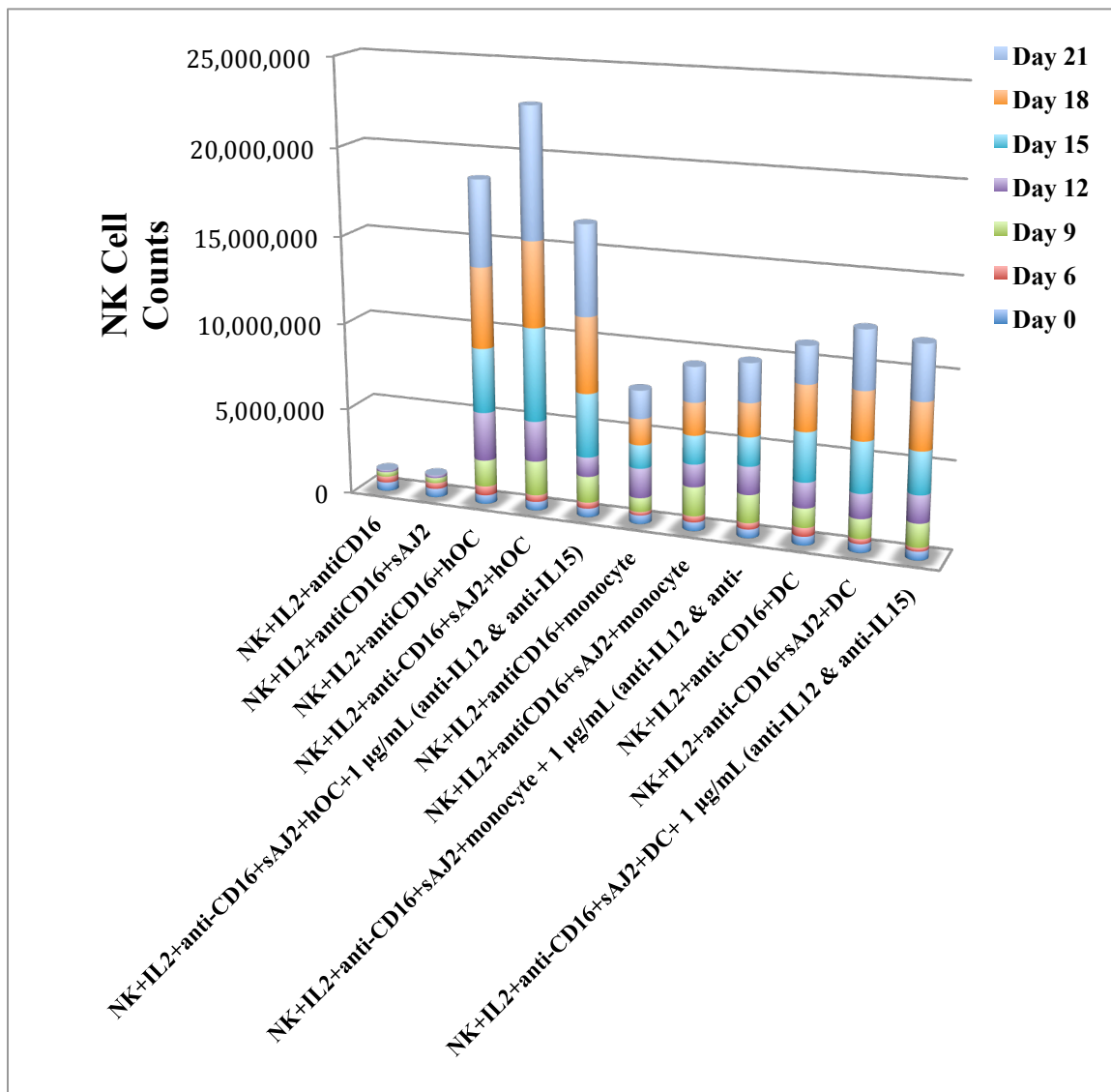


Figure 22: Osteoclasts expanded NK cells at a higher rate than monocytes, and significantly at a higher rate than DCs.

Osteoclasts and dendritic cells were differentiated as described on Materials and Methods. Monocytes, osteoclasts, and dendritic cells were seeded at 0.25×10^6 cells/well, one of each blocked with 1 µg/mL of anti-IL-12 and anti-IL-15 for two hours. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 µg/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. All cells (lymphocytes) were counted from day 0 to day 21 of the co-cultures using a cell counter and microscope. NK cells were calculated using flow cytometry analyses after staining with FITC CD3/PE (CD16-CD56) antibody.

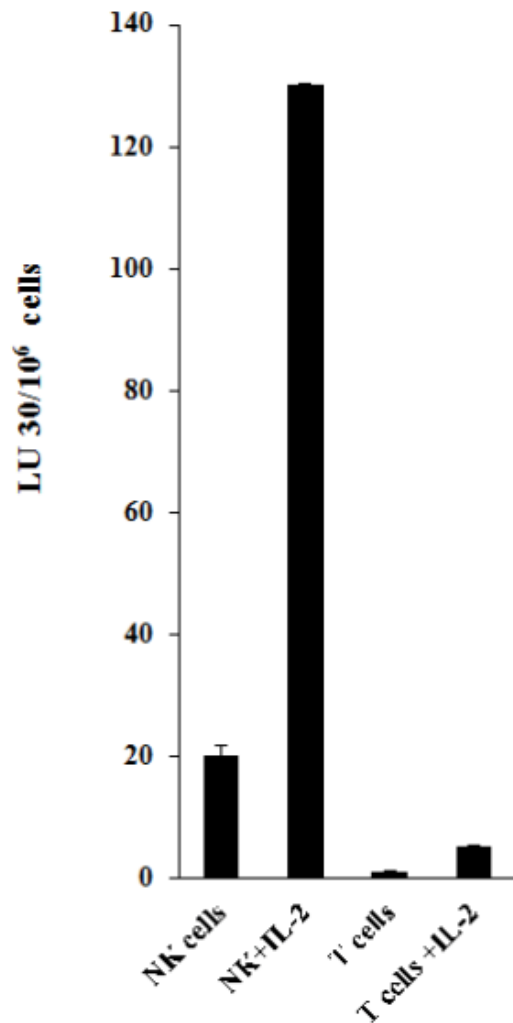


Figure 23. Primary NKs treated with IL-2 induced significantly higher cytotoxicity levels against OSCSCs than primary T cells treated with IL-2. T cells and NK cells were purified from PBMCS as described in the Materials and Methods section. These purified NK cells and T cells were left untreated or activated with IL-2 (1000 units/mL) and added to the co-cultures. On day 1, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ⁵¹Cr release against OSCSCs. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

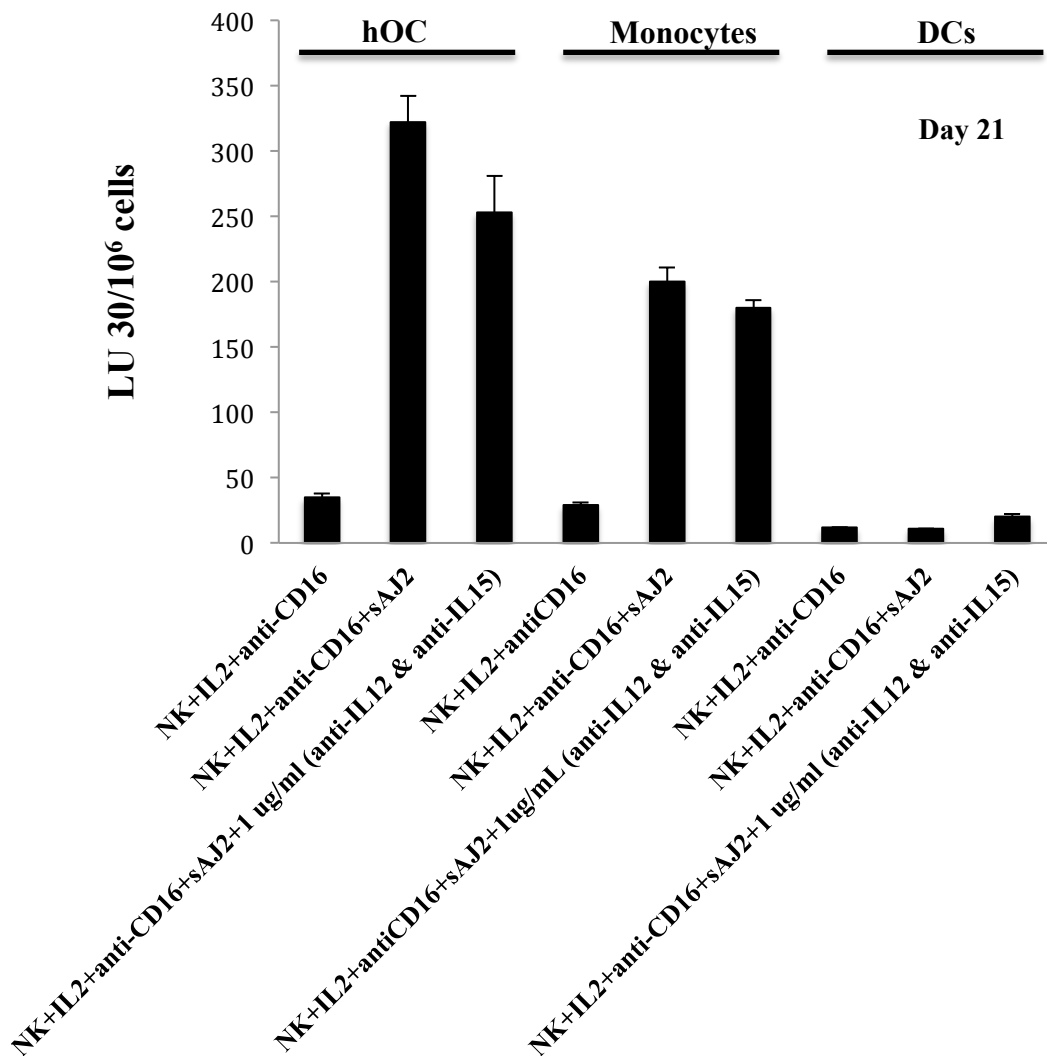


Figure 24: By day 21, when DCs have expanded significant numbers of CD3⁺ cells, these cells exhibit significantly lower levels of cytotoxicity against OSCSCs compared to osteoclast- and/or monocyte-induced expanded NK cells.

Osteoclasts and DCs were differentiated as described on Materials and Methods. Then the osteoclasts, monocytes, and DCs were blocked with anti-IL12 and anti-IL-15 at 1 μ g/mL in certain conditions. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to the co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2-3 days. On day 21, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ⁵¹Cr release against OSCSCs. The lytic units 30/10⁶ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

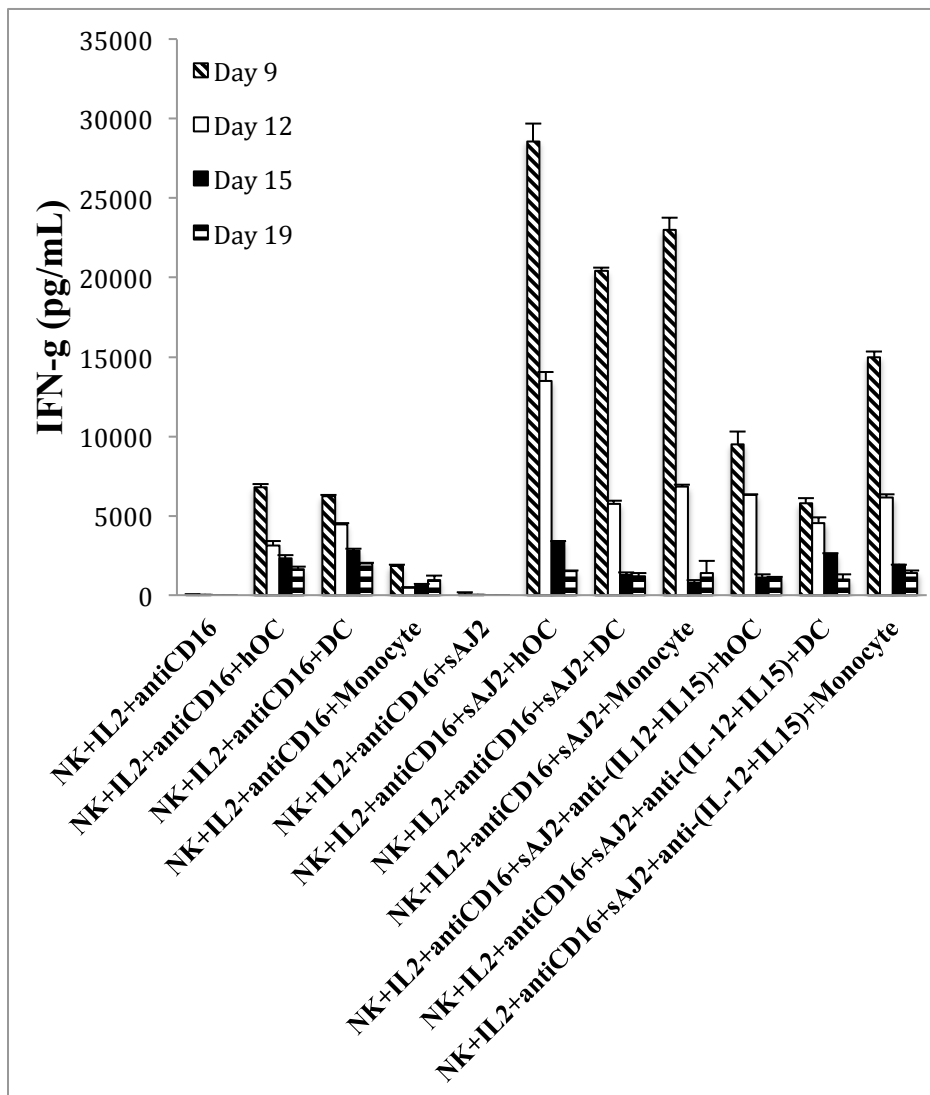


Figure 25: DC-induced expanded NK cells do not secrete IFN- γ to the same higher levels as osteoclast-induced expanded NK cells during the first 12 days *in vitro* co-cultures.

Osteoclasts and DCs were differentiated as described in Materials and Methods. Osteoclasts, monocytes, and DCs were seeded at 0.25×10^6 cells/well in a 6-well plate overnight. Purified NK cells were activated using a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μ g/mL), and added to the co-cultures. Then sonicated probiotic bacteria (sAJ2) were added to appropriate co-cultures. Each condition was treated with IL-2 (1000 units/mL) every 2 days. Supernatants from each co-culture were collected on days 9, 12, 15, and 19. The levels of IFN- γ were measured using a specific ELISA.

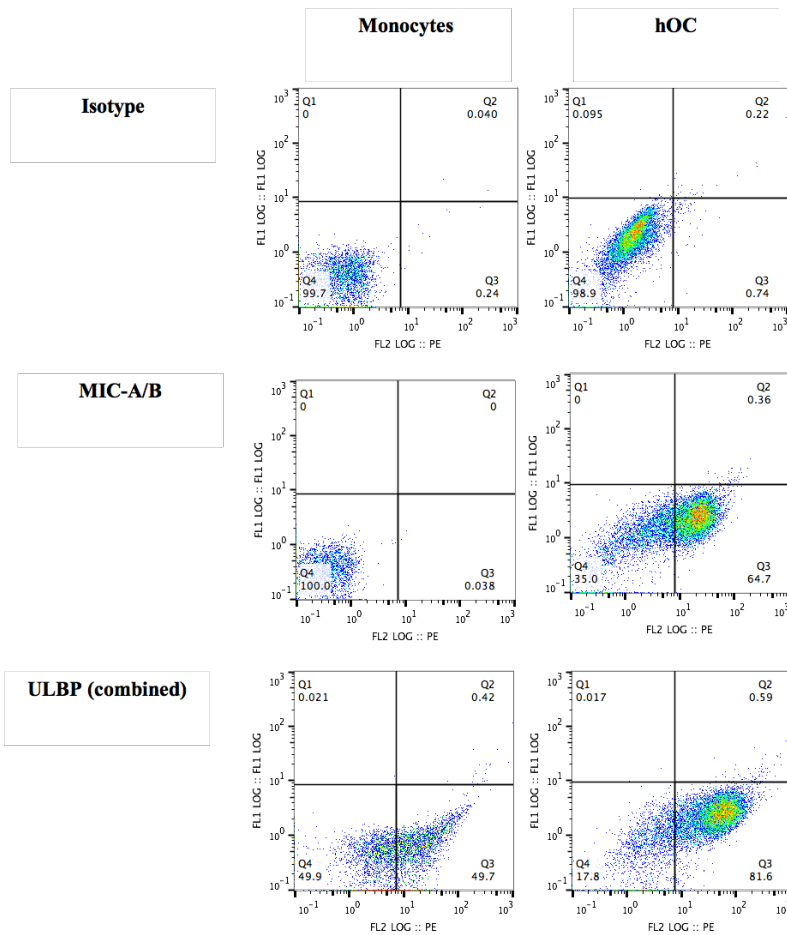


Figure 26: Mature osteoclasts (day 21) express MIC-A/B, whereas autologous monocytes do not. In addition, a higher percentage of osteoclasts express ULBPs than autologous monocytes.

Osteoclasts were differentiated as described in the Materials and Methods section. Monocytes and osteoclasts were stained with either IgG as a control, PE-conjugated MIC-A/B or with PE-conjugated ULBP (combined). Samples were run with a flow cytometer and analyzed using quadrant analysis in FlowJo software.

DISCUSSION

Some studies have emphasized the importance of DCs in stimulating NK cell cytotoxicity and IFN- γ via an IL-12 mediated pathway [73]. Data has shown an increase in the expression of IFN- γ after NK cell expansion. IFN- γ plays an important role in regulation of GVHD in patients receiving allogeneic hematopoietic stem cell transplantation [73]. This pro-inflammatory cytokine has also been reported to facilitate GVL, while inhibiting GVHD in these patients. Although autologous immature DCs could be used for *in vitro* expansion of NK cells, no one has conducted experiments matching these DCs against osteoclasts, or any other subset of myeloid cells in long-term co-cultures with Natural Killer cells.

NK cells with a purity of 90% or higher were activated with anti-CD16 and IL-2 at 10×10^6 cells/mL before being incubated overnight at a concentration of 1×10^6 cells per mL. Then, the activated NK cells were added to 6-well plates containing monocytes, osteoclasts, or dendritic cells at a ratio of NK cells to myeloid cells of 2:1, which has been shown to induce the highest rates of NK cell proliferation [73]. These co-cultures were stimulated further with a mixture of gram-positive probiotic sonicated bacteria strains (sAJ2) to help modulate the vitality of expanded NK cells by slowing down the process of NK-cell mediated apoptosis. These co-cultures were incubated in optimal conditions (37°C, 5% CO₂) for 6-7 days untouched to allow for successful NK cell proliferation.

As shown in previous studies, osteoclasts induce cell expansion at a significantly higher rate than monocytes, but it also induces a higher cell proliferation rate than DCs (Fig. 17). But more importantly, it is important to highlight that DCs expand T cells at a higher rate than

osteoclasts and/or monocytes (Fig. 21). Equally as important is the fact that osteoclasts expand NK cells at a higher rate than monocytes and/or monocytes (Fig. 22).

On day 6 of the co-culture, we begin observing significant differences in NK cell versus T cell proportions. Staining samples with a two-color antibody [FITC CD3/PE (CD16+CD56)], we were able to observe T/NKT cells and NK cell percentages in culture clearly using flow cytometry. Typically, Natural Killer cells co-cultured with DCs showed a preferential expansion of T cells over NK cells. There is a clearly steady increase in the T/NKT cells being expanded in the total cell populations when NK cells are being induced with DCs (Figs. 18 & 19). The preferential of T cell expansion with DCs becomes more evident when there is no sAJ2 present in the co-cultures, underscoring its importance in stimulating NK cell expansion (Figs. 18 & 19). However, if the source of contamination from both NK cell and monocyte purifications ranged between 4-5%, T cells this phenomenon is exacerbated, inducing T-cell proliferation up to 90% of the entire cell population over a period of 21 as observed in one patient (Fig. 20). However, on co-cultures of NK cells with either monocytes or osteoclasts, we observe a considerably lower rate of T cell expansion being induced (Figs. 18 & 19). Expanding T cells is an undesirable outcome if we want to treat oral cancer because primary T cells treated with IL-2 exhibit very low cytotoxicity against Oral Squamous Carcinoma Stem Cells (OSCSCs) in comparison to primary NK cells treated with IL-2 (Fig.23). Therefore, as the proportion of T/NKT cells to NK cells increases in vitro, the DC-induced expanded NK cells lose more their cytotoxic function against OSCSCs (Fig. 24). Moreover, DCs decrease the amount of IFN- γ being secreted by expanded NK cells at a higher rate than monocytes or osteoclasts (Fig. 25). This has huge negative implications if we are interested in using a combinational immunotherapy treatment

plan entailing expanded-NK cell injections with chemotherapy. Secreting less IFN- γ and TNF- α will differentiate OSCSCs at a lower rate, making them less susceptible to chemotherapy.

We would expect DC-induced and hOC-induced expanded NK cells to secrete similar levels of IFN- γ since T cells and NKT cells are known to secrete high levels of IFN- γ . However, these CD3⁺/CD3⁺CD16⁺CD56⁺ cells may be regulatory T cells, which are known to inhibit NK cell function, or may be dormant. In other words, may not be activated at this point, and thus, not inducing as much IFN- γ secretion levels as expected. Therefore, we need to investigate what kind of T cells are present in the cell populations being induced by these two myeloid cell subset types. Lastly, DCs induce expanded NK cells to go through apoptosis at a faster rate than monocytes or osteoclasts (Fig. 18). These differences in the dynamics of how NK cells proliferate and anergize *in vitro* are imperative in understanding how we translate our method of using expanded NK cells for cancer immunotherapy *in vivo*.

CONCLUSION

As of today, many *in vitro* studies have successfully demonstrated NK cell expansion with enhanced function with the co-culture of DCs; however, there are no studies that highlight the differences in NK cell potency when co-cultured with various potent effector immune cells. Comparing DCs to other effector immune cells, such as monocytes and osteoclasts, is imperative in finding the most effective mechanism from which NK cell potency can be maximized to its fullest potential for cancer immunotherapy.

Monocytes and osteoclasts maintained NK to T cell proportions significantly higher than DCs, suggesting that DCs play a key role in T cell maturation and expansion. This phenomenon becomes more apparent in an extreme case where DCs expanded T cells from 5% to over 90% of the entire lymphocyte population; the mechanisms that underlie this clear-cut observation are still unclear. However, these observations, along with other functional assays, gives us clues as to a primitive ranking system of effector immune cells that can proliferate and enhance the functions of expanded NK cells.

FINAL CONCLUSIONS

In addition to osteoclast to NK cell contact interactions, other chemokines/cytokines must be involved in optimizing the functions of expanded NK cells. However, highly purified NK cells must be purified in order to use them as a highly effective anti-tumor therapeutic tool. If other lymphocytes are present at a critical, unknown percentage, in the co-culture, such as T cells, the proportions of NK cells over T cells do not increase over time. Previous studies have suggested Tregs as a key factor in limiting antitumor activity, suggesting that inhibiting T cell proliferation is as equally important as stimulating NK cell expansion. As a result, to maximize the purity of expanded NK cells, it is imperative to increase the number of times we purify our lymphocytes to get rid of any T/NKT cells that may have expanded in co-cultures. Doing so would improve the results we observe *in vivo* when we inject BLT humanized mice with expanded NK cells.

When we analyze the cell surface expression of MIC-A/B, which belongs to the CD94/NKG2 family and ULPBs that are known to serve as a ligand for NKG2D, we can easily observe that osteoclasts express these surface markers at a significantly higher percentage than its autologous monocytes (Fig. 26). These observations serve as further clues as to why osteoclasts are much more potent immune effector cells for NK cell expansion compared to other myeloid cell subsets and tumor cells, such as K562 and OSCSCs.

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