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Expansion of Highly Cytotoxic Human Natural Killer Cells by Osteoclast for Cancer Immunotherapy

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

By

So-Hyun Park

2015

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2015

ABTRACT OF THE THESIS

Expansion of Highly Cytotoxic Human Natural Killer Cells by Osteoclasts to Enhance Expanded NK Cell Function for Cancer Immunotherapy

By

So-Hyun Park

Master of Science in Oral Biology

University of California, Los Angeles, 2015

Professor Anahid Jewett, Chair

Natural Killer (NK) cells have a crucial role in immune surveillance against a variety of infectious microorganisms and tumors. NK cells are known to mediate direct cytotoxicity as well as antibody dependent cellular cytotoxicity (ADCC) against a variety of tumor cells. Also, they are known to regulate the functions of other cells by producing key cytokines and chemokines. In the tumor microenvironment, cytotoxic function of NK cells is suppressed by a number of distinct effectors and their secreted factors. It has been shown that many cancer patients have decreased peripheral blood NK cell function, so NK cell-based immunotherapy

has been used as a treatment in order to enhance NK cell function. However, limited availability of NK cells and ability to expand has restricted development of NK cell immunotherapy. Overcoming NK cell tolerance against tumors by developing new ways of activating endogenous NK cells that increase the expression of ligands for activating NK cell receptors or that render them more sensitive to NK cell mediated killing is crucial.

In this study, we found the novel way to expand NK cells and the functionality of NK cells generated under this condition demonstrated enhanced expression of activating NK receptors. Also, significant cytotoxic killing potential after culture was discovered as well as augmented cytokine secretion. Therefore, these expanded NK cells are highly functional in comparison to primary NK cells. Through the help of cytokines, sAJ2 bacteria and osteoclast, NK cells can be expanded and activated in vitro and furthermore, these expanded NK cells can be used to target tumors in vivo. Expanded NK cells can be used in combination with other treatment modalities, potentially leading to synergistic antitumor activities.

The thesis of So-Hyun Park is approved by

Ichiro Nishimura

Nicholas A.Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2015

DEDICATION

This dissertation work is dedicated to my parents, Sun Park and Sun Hyeung Park who have been a constant source of support and encouragement during the challenges of graduate school and life. I cannot eloquently depict all that they have sacrificed and done for me. I wholeheartedly share this dissertation and degree with my parents because I would not be where I am and who I am today without their unconditional love and support.

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First and foremost, my deepest gratitude and thanks to my PI, Dr.Anahid Jewett, for her guidance, caring, patience, support and providing me with an excellent atmosphere throughout this study, and especially for her confidence in me. Also, I sincerely wish to thank my committee members, Dr. Nicholas Cacalano and Dr. Ichiro Nishimura for their expertise and precious time.

I would like to acknowledge my fellow researchers and friends at the Weintraub Center for Reconstructive Biotechnology. I truly enjoyed working with them who assisted, advised and supported my research and writing efforts over the years.

Last, but definitely not least, I would like to thank my parents, Sun Park and Sun-hyeung Park, who gave up their careers and decided to immigrate to the US solely for the future of my brother and I and for that I am forever grateful. I also would like to thank my parents for teaching me that anything great in life is earned with diligence and perseverance and Hyun-Jun Park for being my only brother that I love so much. Finally, I would like to thank my husband, Ji-Hoon Kyon, who was always there cheering me up and stood by me through the good times and bad.

"Trust in the Lord with all your heart, and lean not on your own understanding, in all you ways acknowledge Him, and He shall direct your paths." Proverbs 3:5-6

"I can do all things through Christ who gives me strength." Philippians 4:13

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INTRODUCTION

Natural Killer (NK) Cells

Natural killer (NK) cells are large granular lymphocytes that are important innate immune effector cells with a critical role in early host defense against invading pathogens while adaptive immune responses are being activated [1]. They are known as first line of defense that mediate direct cytotoxicity as well as antibody-dependent cellular cytotoxicity (ADCC) against a variety of tumor cells, virally infected cells and neoplasia [2]. Substantial evidence has demonstrated the importance of NK cell function in the immune system in reports of individuals lacking NK cells or with low activity of NK cells. One such patient was constantly afflicted with a variety of viral infections and recurrent cervical carcinoma [3, 4]. Therefore, NK cells are 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 migrate to inflamed tissues and organs of the body in response to pro-inflammatory stimuli. Typically, immune cells detect major histocompatibility complex (MHC) presented on the surface of infected cells, triggering cytokine secretion, causing lysis or apoptosis. However, NK cells kill tumor cells without recognizing tumor-specific antigen and MHC or prior sensitization, allowing for a much faster immune reaction.

NK cells are distinct from T and B cells and represent a unique subset of lymphocytes that contributes to host antimicrobial and antitumor defense. Comprising 10-15% of all peripheral blood lymphocytes, human NK cells can be divided into two functional subsets based on their surface expression of CD56 and the lack of expression of CD3; $CD56^{bright}$ immunoregulatory cells and $CD56^{dim}$ cytotoxic cells [1]. In addition, 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]. 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]. Several key cytokines, chemokines and adhesion molecules have significant roles in maturation, differentiation and effector function of NK cells.

It is that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of cancer patients have significantly reduced cytotoxic activity [8-11]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [12-14]. In cancer patients since the majority of NK cells have lost cytotoxic activity, they may contribute the progression of cancer. This will eventually lead to the growth and expansion of the pool of cancer stem cells. Knowledge of the distinct functional attributes of $CD56^{bright}$ and $CD56^{dim}$ human NK cell subsets and factor 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, the selective expansion of $CD56^{bright}$ NK cells and optimizing the efficacy of using 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.

Thesis Outline

Specific Aim 1: **To establish osteoclasts as key immune effectors capable of expanding NK cells and modulating the function of NK cells**

- Sub-aim 1A: Osteoclasts are potent inducers of NK cell expansion and function
- Sub-aim 1B: NK cells expanded by osteoclasts retain activating receptors on their surface
- Sub-aim 1C: Expanded NK cells target tumor cells and induce significant IFN-γ secretion
- Sub-aim 1D: The functionality of expanded NK cells are more potent than primary NK cells

Specific Aim 2: **To investigate expansion of NK cells by tumor and non-tumors and the capacity of osteoclast-expanded NK cells to target pancreatic tumor (MP2) in hu-BLT mice**

- Sub-aim 2A: Live tumors neither support the expansion of NK cells nor enhance their functions
- Sub-aim 2B: Irradiated tumors neither support the expansion of NK cells nor enhance their functions
- Sub-aim 2C: Irradiated PBMCs do not support the expansion of NK cells whereas osteoclasts support the expansion of NK cells for more than a month
- Sub-aim 2D: Single injection of expanded NK cells inhibited tumor growth in hu-BLT mice implanted with MP2 stem like/poorly differentiated tumors

Specific Aim 3: To investigate the role of IL-10 as a regulator of IFN-gamma secretion in NK cell interaction with monocytes or osteoclasts

- Sub-aim 3A: Monocytes induced significant secretion of IFN-γ in the presence of anti-IL-10mAb
- Sun-aim 3B: Monocytes induced significant secretion of IL-10 in the absence of anti-IL-10mAb
- Sub-aim 3C: Osteoclasts induced more IFN-γ secretion in the presence of anti-IL-10mAb than monocytes in the presence of anti-IL10mAb
- Sub-aim 3D: Osteoclasts inhibited NK cell cytotoxic function in the presence of anti-IL10mAb

Chapter 1

Specific Aim 1: To establish osteoclast as a key immune effector capable of expanding NK cells and modulating the function of NK cells.

INTRODUCTION

Expansion of Natural Killer (NK) Cells

Natural killer (NK) cells play an important role in immune surveillance against a variety of microbial pathogens. Infusions of NK cells are a promising tool for cancer cell therapy. However, immunotherapy with NK cells has been limited by the inability to obtain sufficient number of highly functional NK cells. Therefore, the development of clinically applicable methods to produce large numbers of highly functional NK cells is critical for cancer cell therapy.

Many cytokines have been studied in efforts to induce NK cell expansion as well as increase their cytotoxicity. Activated NK cells secrete interferon (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]. Also, IL-4, IL-7 and IL-12 induced 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-15Rα, it promoted NK cell survival and expansion [23]. Also, when feeder cell populations, such as Epstein-Barr virus-trans-formed 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]. IL-2 appears to be necessary but not the only source for preferential expansion of NK cells. Therefore, NK cells require costimulatory signals for optimal proliferation.

Interaction with other immune 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 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, approximate 25-fold expansion of NK cells was observed. Yu et al. also [28] 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 immuostimulatory cells by expressing high levels of MHC class II molecules, adhesion molecules and costimulatory receptors [30]. In order to enhance NK cell cytotoxicity and IFN-γ production, direct cell-to-cell contract 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 contact mature osteoclasts [31]. Suppression of bone erosion by the interaction between NK cells and osteoclasts had shown but 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 RANKL 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 preceptors 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].

In this aim 1, we found the novel method to expand NK cells by osteoclasts and enhanced sensitization of tumor target cells to NK cell-mediated apoptosis as well as

cytokine production. These recent discoveries in NK expansion to potentiate their functions against tumors will fuel translational research that will lead to clinical trials for future cancer therapy.

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% gentimicin 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. 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, NKp44, CXCR1, CXCR3, DNAM, and NKG2D 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% purify based on flow cytometric analysis of CD14 antibody stained monocyte cells.

Generation of Dendritic Cells (DCs) and Osteoclasts

Purified monocytes from health donor's PBMCs were differentiated into DCs and cultured in RPMI 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). Zolendronic acid (500nM) was purchased from UCLA Ronald Reagan Pharmacy and used to treat osteoclast.

Surface Staining

$1x10^5$ NK cells from each condition were stained in 50ul of cold 1%PBS-BSA with pre-determined optimal concentration of PE conjugated isotype control, CD16, NKp44, CXCR1, CXCR3, DNAM, and NKG2D and incubated at 4°C for 30 minutes. Then, the cells were washed and resuspended in 1%PBS-BSA. The Epics C (Coulter) flow cytometry 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 ($1x10^5$ 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 ($5x10^5$ OSCSCs/well) were labeled with 50μCi ^{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;

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

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

Enzyme-Linked Immunosorbent Assays (ELISAs)

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 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 200rmp 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 $H_2SO_4$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

Osteoclasts are potent inducers of NK cell expansion and function

Osteoclasts were used in order to investigate whether they can support expansion of NK cells. As shown in Fig.1, NK cells cultured with osteoclasts expanded NK cells whereas NK cells cultured without osteoclasts did not. Especially, IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts expanded NK cells the most (Fig.1). IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts induced higher IFN-γ secretion than NK cells cultured without osteoclasts (Fig.2). Moreover, IL-2+anti-CD16mAb+sAJ2 treated NK cells expanded by osteoclasts mediated the highest cytotoxicity against oral squamous cancer stem cells (OSCSC) (Fig.3).

Osteoclasts were compared with monocytes and dendritic cells (DCs) whether osteoclasts can support expansion of NK cells. As shown in Fig.4, both osteoclasts and DCs and much less monocytes supported the expansion of NK cells on day 6. Especially, DCs and osteoclasts that were cultured with IL-2+anti-CD16mAb+sAJ2 bacteria treated NK cells expanded NK cells the most. Indeed, 35.6 more NK cells were expanded by DCs and osteoclasts compared to NK cells activated with IL-2 and anti-CD16mAb. In order to determine whether the cells that were expanded by DCs and osteoclasts are activated NK cells, the levels of CD16 and NKp44 surface expression were determined on day 8. As shown in Fig. 5, the conditions that had the most expanded NK cells retained the surface expressions of CD16 and NKp44. The conditions that had the least number of NK cells expanded with

monocytes had low expressions of CD16 and NKp44 in comparison to NK cells expanded with either DCs or osteoclasts (Fig.5).

NK cells that were expanded with DCs, osteoclasts or monocytes were replenished with a combination of IL-2 and anti-CD16mAb had low level of CD16 receptors (Fig.5). Also, surface expression profiles of chemokine receptors and activation receptors on these cells were determined. As shown in Fig.6, there were no clear differences in surface expressions of CXCR1, CXCR3 and DNAM in the cultures of NK cells with DCs, osteoclasts or monocytes.

Fig 1.

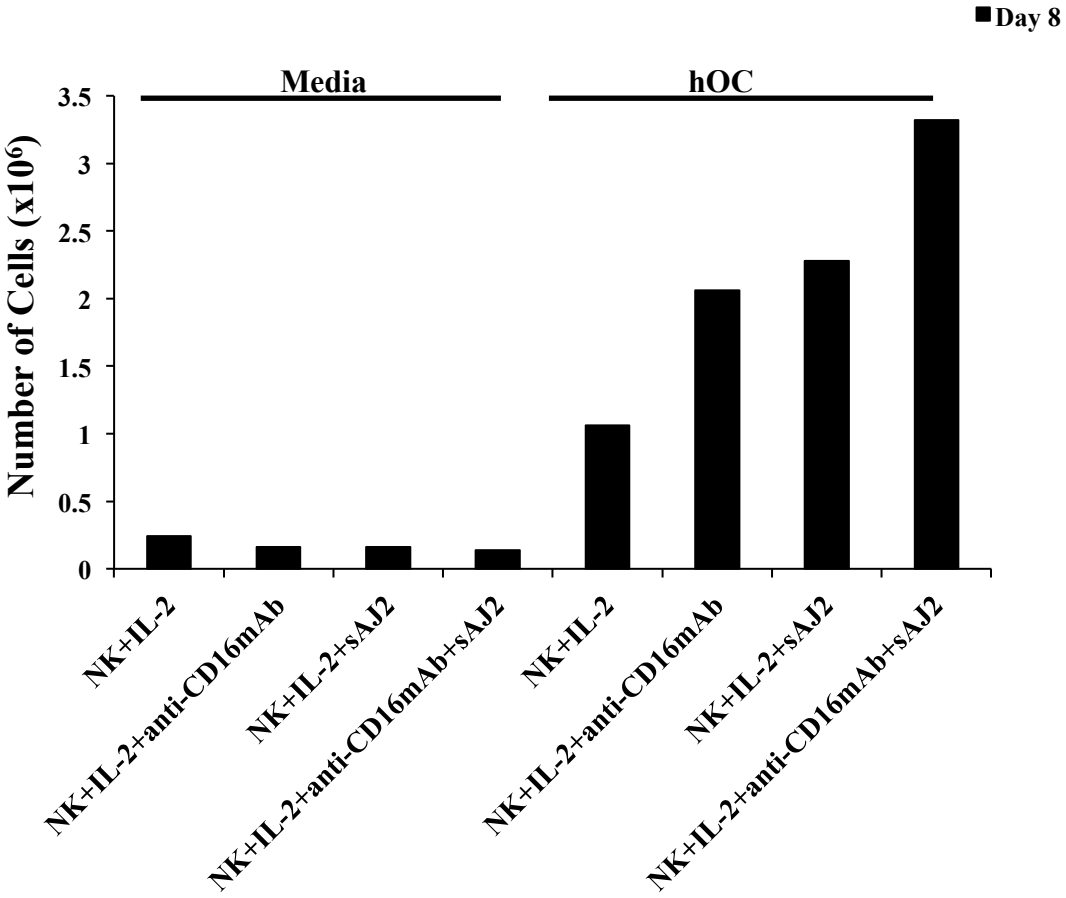


Figure 1. Number of NK cells on day 8 after co-culture with osteoclasts

Osteoclasts (hOCs) were prepared as described in Materials and Methods and seeded at $4x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. Then, they were cultured alone or with autologous hOCs at an effector to target ratio of 1:1. On the 8^{th} day of experimental period the number of cells was assessed by microscopic evaluation.

Fig. 2

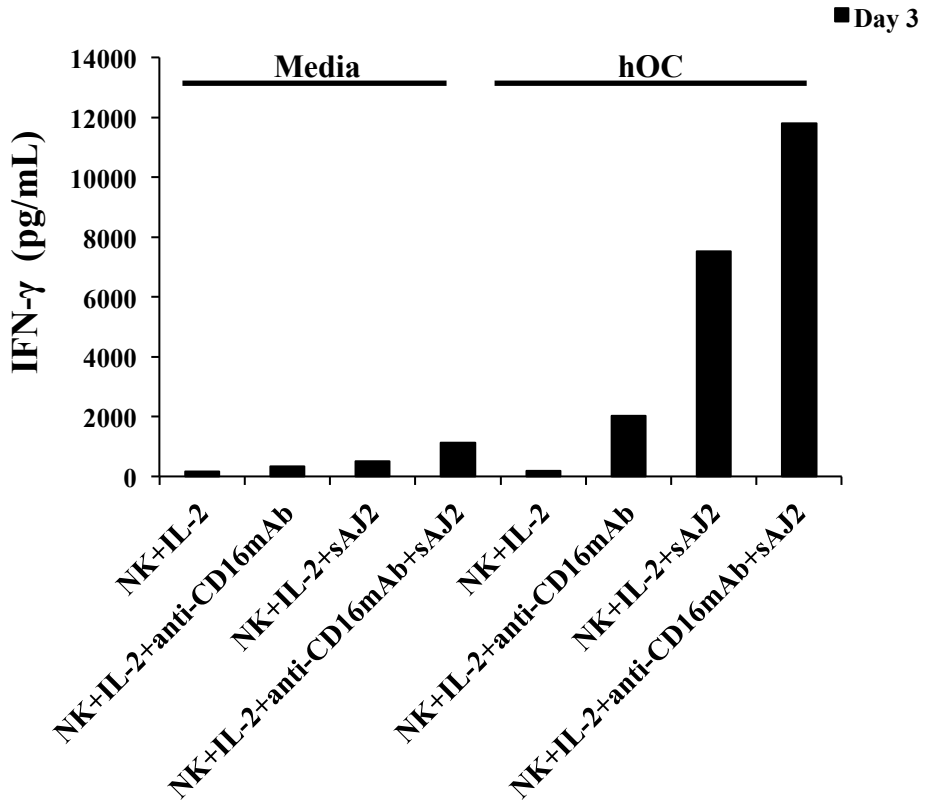


Figure 2. The levels of IFN-γ secreted in the cultures of NK cells with osteoclasts were higher than controls.

Osteoclasts (hOCs) were prepared as described in Materials and Methods and seeded at $4x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. Then, they were cultured alone or with autologous hOCs or at an effector to target ratio of 1:1.6. The supernatants from each culture were harvested on day 3. The level of IFN-γ produced by NK cells was measured with a specific Elisa.

Fig. 3

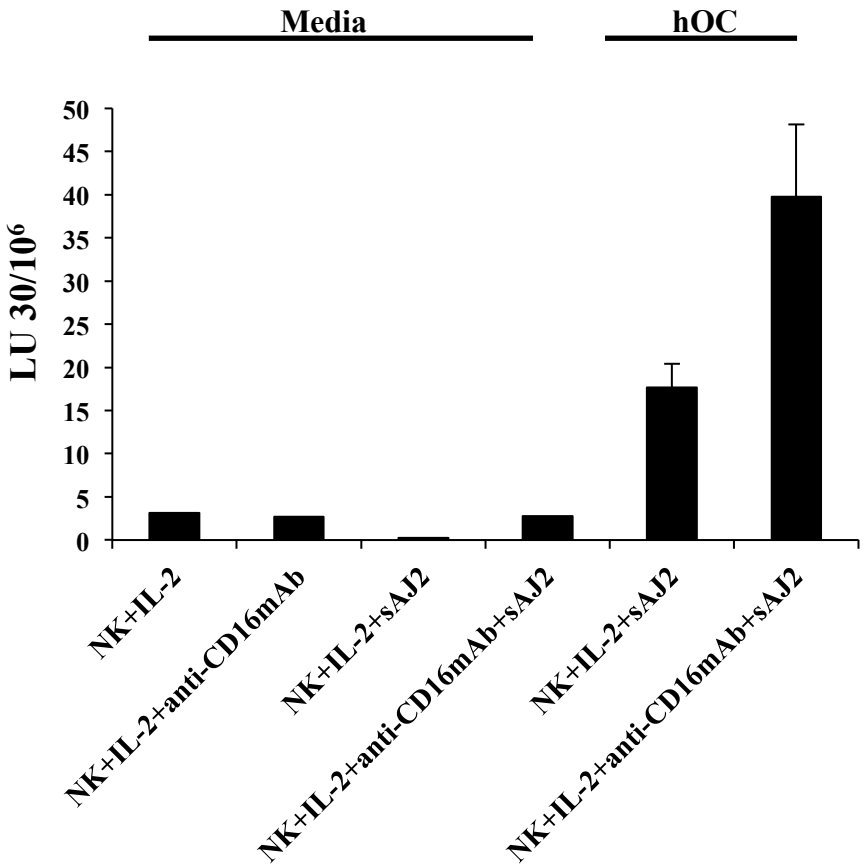


Figure 3. Expanded NK cells by osteoclasts mediated the highest cytotoxicity against OSCSCs in comparison to controls.

Osteoclasts (hOCs) were prepared as described in Materials and Methods and seeded at $2x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. Then, they were cultured alone or with autologous hOCs or at an effector to target ratio of 1:1. On day 8, the same number of NK cells from each culture was used in standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x100.

Fig. 4

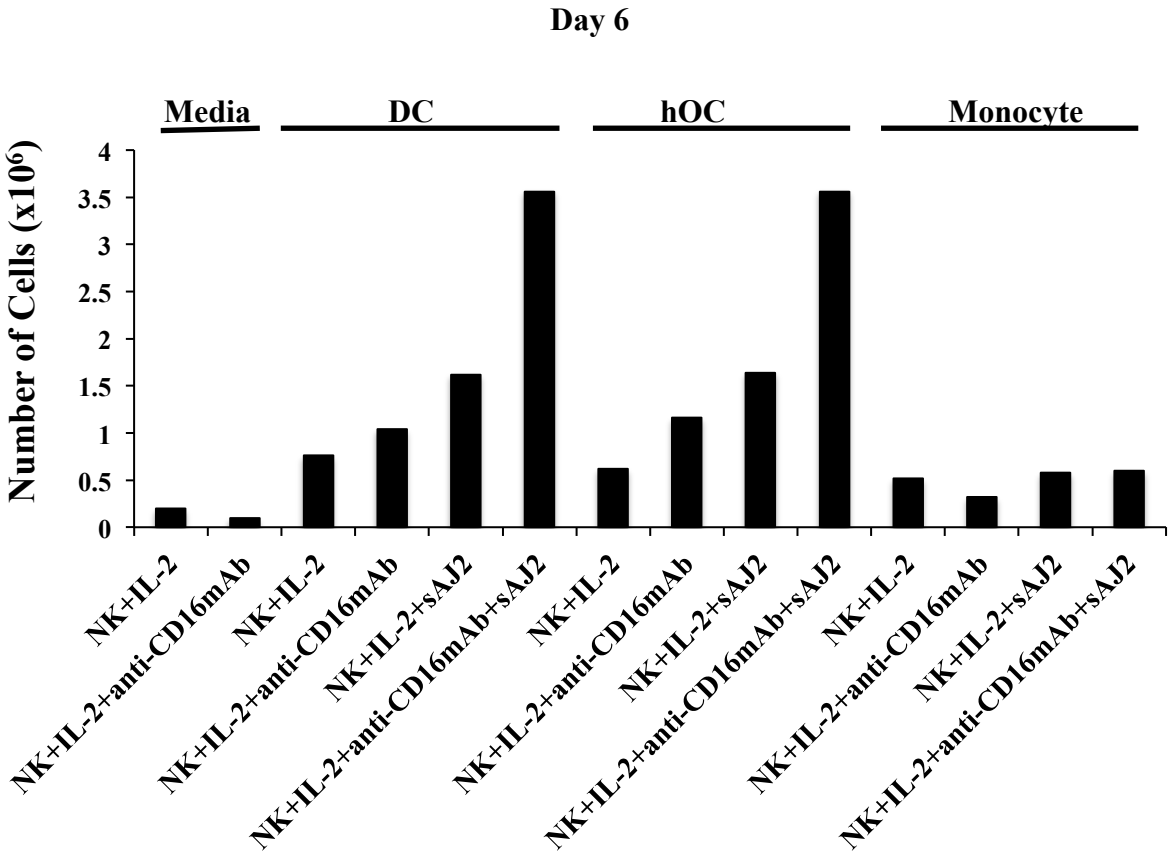
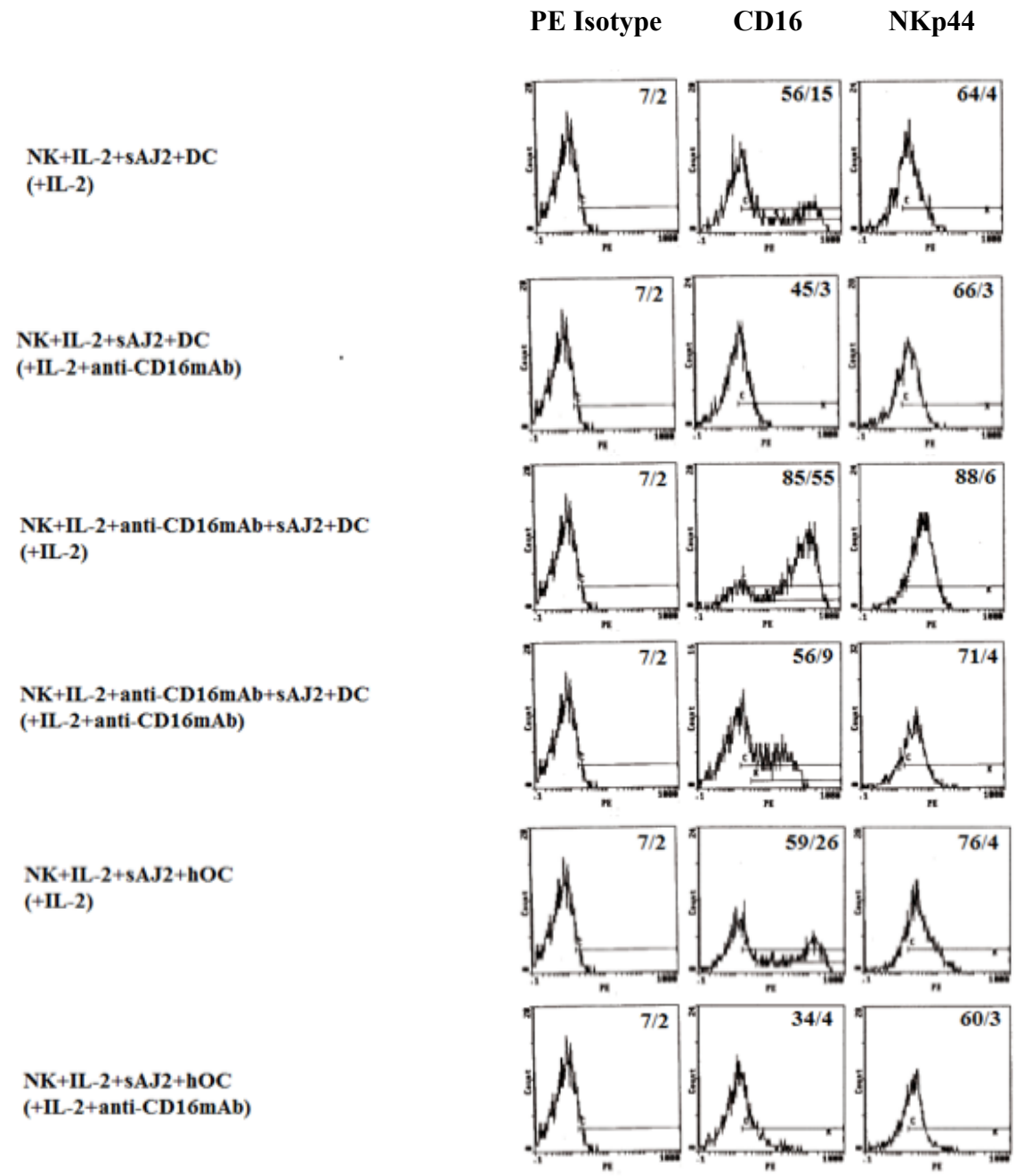


Figure 4. Number of NK cells on day 6 after co-culture with dendritic cells, osteoclasts or monocytes

Dendritic cells (DCs), osteoclasts (hOCs) and monocytes were prepared as described in Materials and Methods and seeded at $4x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. Then, they were cultured alone or with autologous DCs, hOCs or monocytes at an effector to target ratio of 1:1. On the 6^{th} day of the experimental period the number of cells was assessed by microscopic evaluation.

Fig. 5


PE Isotype
CD16
NKp44
NK+IL-2+sAJ2+DC
(+IL-2)
7/2
56/15
64/4
NK+IL-2+sAJ2+DC
(+IL-2+anti-CD16mAb)
7/2
45/3
66/3
NK+IL-2+anti-CD16mAb+sAJ2+DC
(+IL-2)
7/2
85/55
88/6
NK+IL-2+anti-CD16mAb+sAJ2+DC
(+IL-2+anti-CD16mAb)
7/2
56/9
71/4
NK+IL-2+sAJ2+hOC
(+IL-2)
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(+IL-2+anti-CD16mAb)
7/2
34/4
60/3

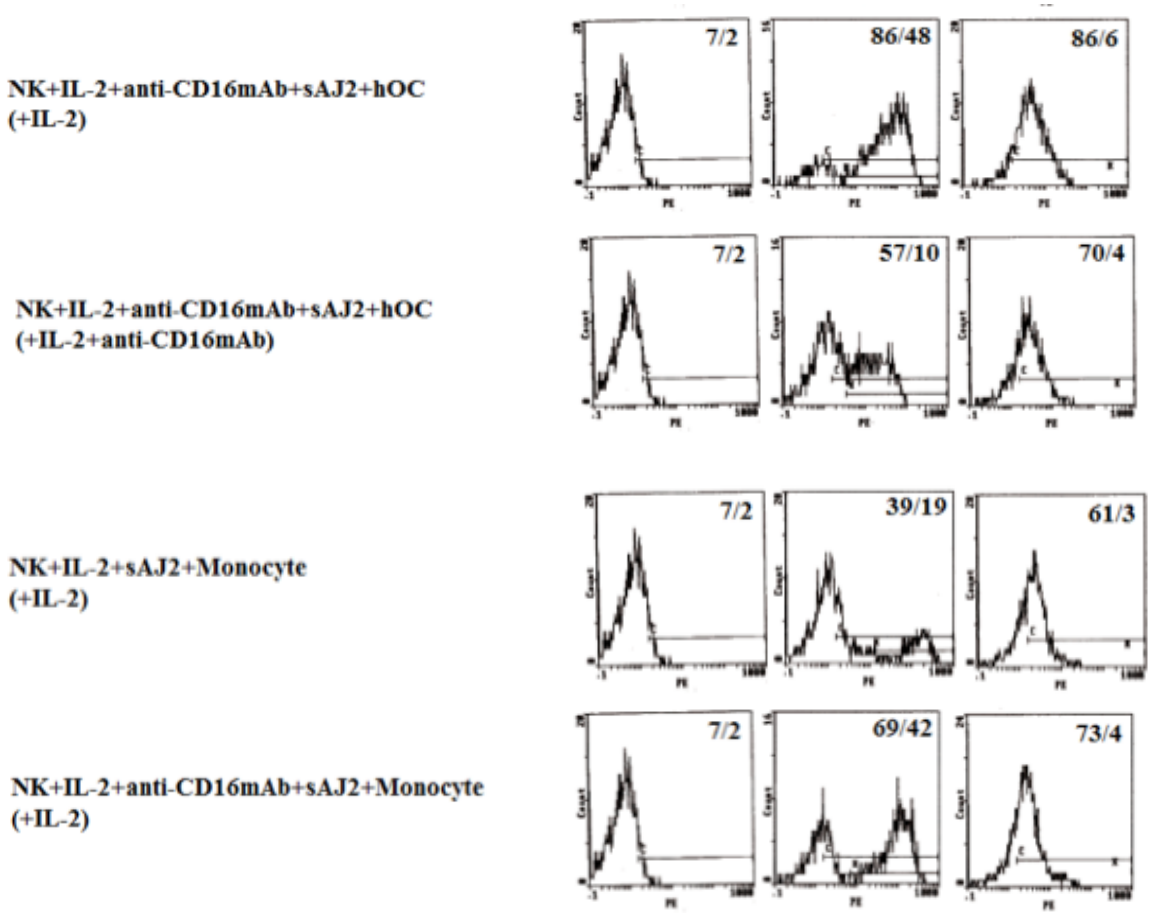
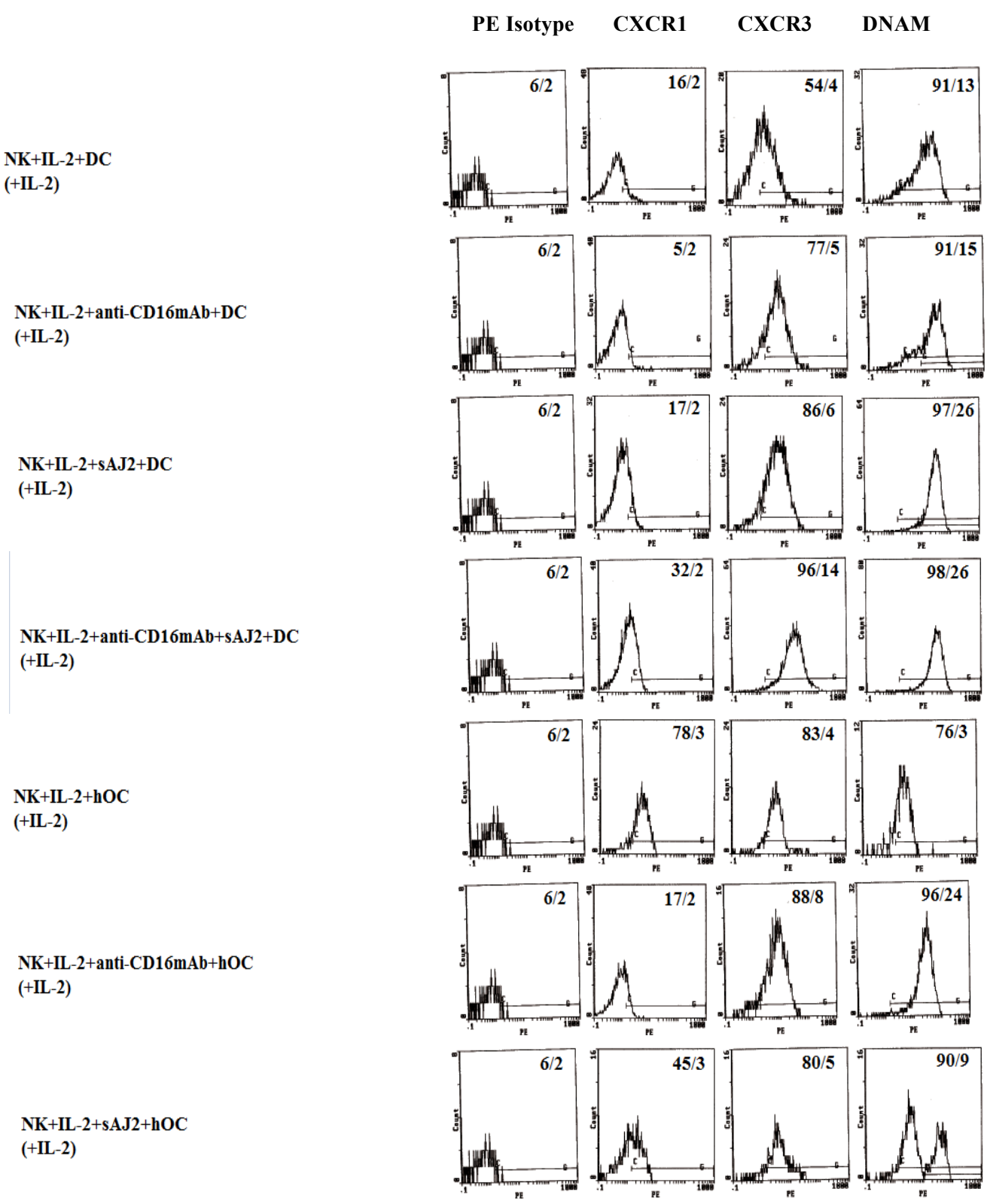


Figure 5. Surface expression profiles of expanded NK cells by dendritic cells, osteoclasts or monocytes

NK cells were co-cultured with dendritic cells (DCs), osteoclasts (hOCs) or monocytes for 6 days as described in Fig.4 and then treated with additional IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 48 hours. Surface expression of CD16 and NKp44 on expanded NK cells were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram.

Fig. 6


PE Isotype
CXCR1
CXCR3
DNAM
NK+IL-2+DC (+IL-2)
6/2
16/2
54/4
91/13
NK+IL-2+anti-CD16mAb+DC (+IL-2)
6/2
5/2
77/5
91/15
NK+IL-2+sAJ2+DC (+IL-2)
6/2
17/2
86/6
97/26
NK+IL-2+anti-CD16mAb+sAJ2+DC (+IL-2)
6/2
32/2
96/14
98/26
NK+IL-2+hOC (+IL-2)
6/2
78/3
83/4
76/3
NK+IL-2+anti-CD16mAb+hOC (+IL-2)
6/2
17/2
88/8
96/24
NK+IL-2+sAJ2+hOC (+IL-2)
6/2
45/3
80/5
90/9

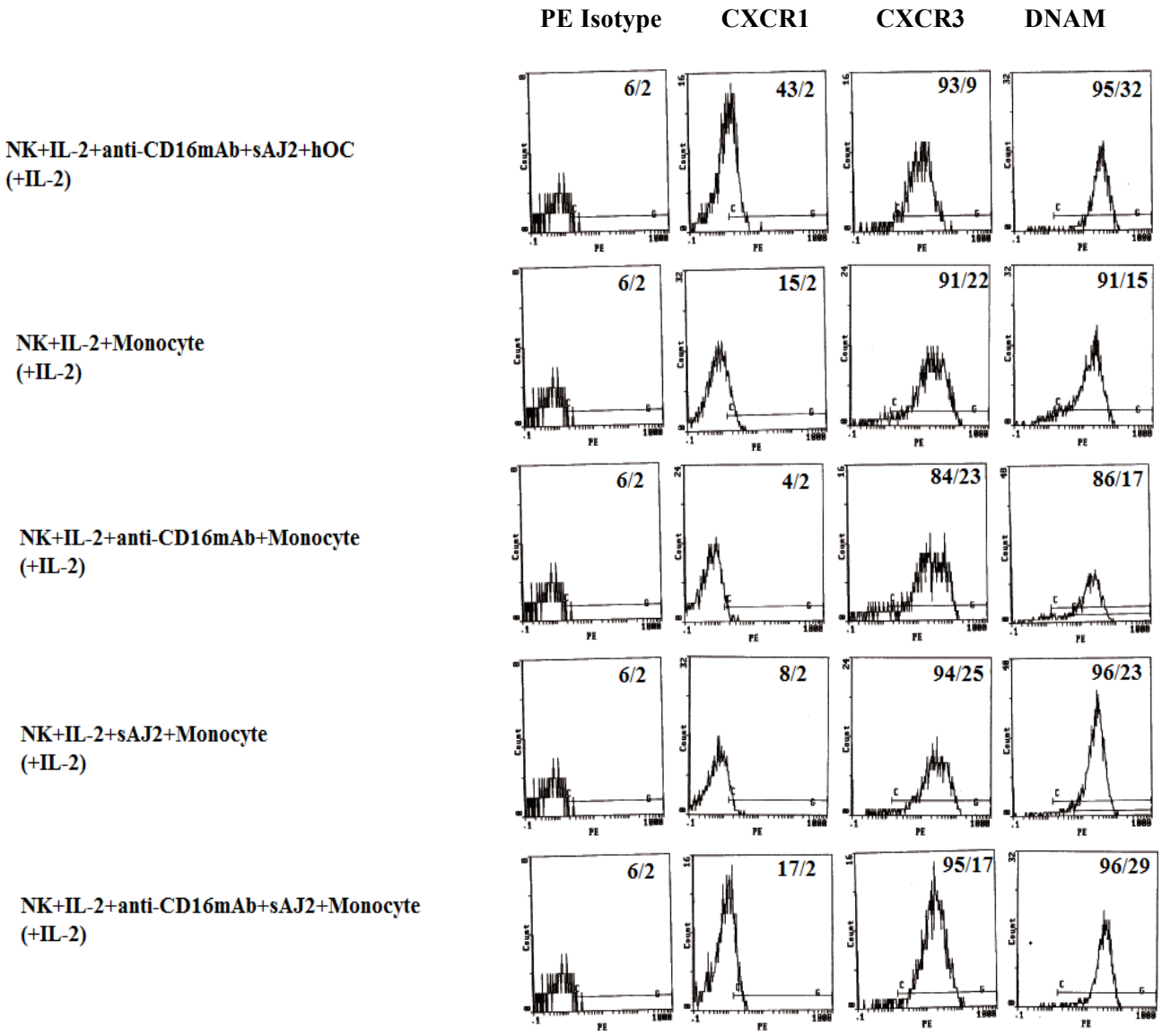


Figure 6. Surface expression profiles of chemokines and activation receptors on NK cells co-cultured with dendritic cells, osteoclasts and monocytes

NK cells were co-cultured with dendritic cells (DCs), osteoclasts (hOCs) and monocytes as described in Fig.5 and then surface expression profiles of CXCR1, CXCR3, and DNAM on NK cells were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram.

Expanded NK cells target tumor cells and induce significant IFN-γ secretion

NK cells expanded by dendritic cells (DCs), osteoclasts and monocytes were counted and equal numbers of NK cells from each expanded subset were used in cytotoxicity assay against OSCSCs, which is a very sensitive NK cell target. IL-2+anti-CD16+sAJ2 bacteria treated NK cells expanded by osteoclasts had the highest cytotoxicity, followed by those expanded by DCs and the least cytotoxicity was seen by IL-2 treated NK cells which were expanded by monocytes (Fig. 7). IL-2+anti-CD16mAb and IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts mediated higher cytotoxicity than IL-2+anti-CD16mAb and IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with monocytes ($p<0.05$) (Fig.7).

The levels of IFN-γ secretion by IL-2 and IL-2+anti-CD16mAb treated NK cells cultured either with DCs, osteoclasts or monocytes from day 0 to day 6 of cultures were compared. As shown in Fig. 8, the levels of IFN-γ secreted in the cultures of NK cells with osteoclasts rose significantly from day 0 to day 3 and remained high until day 6, and the secreted levels were much higher than those induced in the cultures of NK cells either with DCs or monocytes. On day 6, IL-2+anti-CD16mAb activated NK cells cultured with osteoclasts secreted less IFN-γ than IL-2+anti-CD16mAb activated NK cells cultured with DCs ($p<0.05$) (Fig. 8). However, the level of IFN-γ secreted by IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts was significantly higher than IL-2+anti-CD16mAb +sAJ2 treated NK cells cultured either with DCs or monocytes ($p<0.05$) (Fig.8).

Indeed, within 5 hours of cultures of NK cells secreted more IFN-γ when cultured with osteoclasts than DCs, and the levels were much less when cultured with monocytes (Fig. 9). On day 6 after 5 hours, IL-2+anti-CD16mAb treated NK cells cultured with DCs secreted more IFN-γ than IL-2+anti-CD16mAb treated NK cells cultured with osteoclasts ($p<0.05$) (Fig.9). However, IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts secreted 1.5 fold more of IFN-γ than IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with DCs ($p<0.05$) whereas IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts secreted 3.7 fold more of IFN-γ than IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with monocytes ($p<0.05$) (Fig.9).

After 6 days of cultures, $0.3x10^5$ cells from each condition were treated with an additional IL-2 for 48 hours and the levels of IFN-γ secretion were determined. Still, NK cells with osteoclasts were keeping the levels of cytokine secretion high for 8 days when compared to the cultures of NK cells either with DCs or monocytes (Fig. 10). On day 8 after replenishment with IL-2, IL-2+anti-CD16mAb treated NK cells cultured with osteoclasts secreted more IFN-γ than IL-2+anti-CD16mAb treated NK cells cultured either with DCs or monocytes ($p<0.05$) (Fig.10). Moreover, IL-2+anti-CD6mAb+sAJ2 treated NK cells cultured with osteoclasts secreted more IFN-γ than IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured either with DCs or monocytes ($p<0.05$) (Fig.10).

Expanded NK cells retain high expressions of CD16 and NKG2D on the surface in comparison to primary NK cells (Fig. 11). As shown in Fig.12, expanded NK cells by osteoclasts secreted more IFN-γ than primary NK cells. IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts with an additional IL-2 secreted 449 fold more of IFN-γ

than primary NK cells treated with IL-2 ($p<0.05$) (Fig 12). Moreover, expanded NK cells mediated the highest cytotoxicity against OSCSCs compared to primary NK cells (Fig. 13). IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts with an additional IL-2 had 2.6 fold more of cytotoxic activity than primary NK cells treated with IL-2 ($p<0.05$) (Fig.13).

Fig. 7

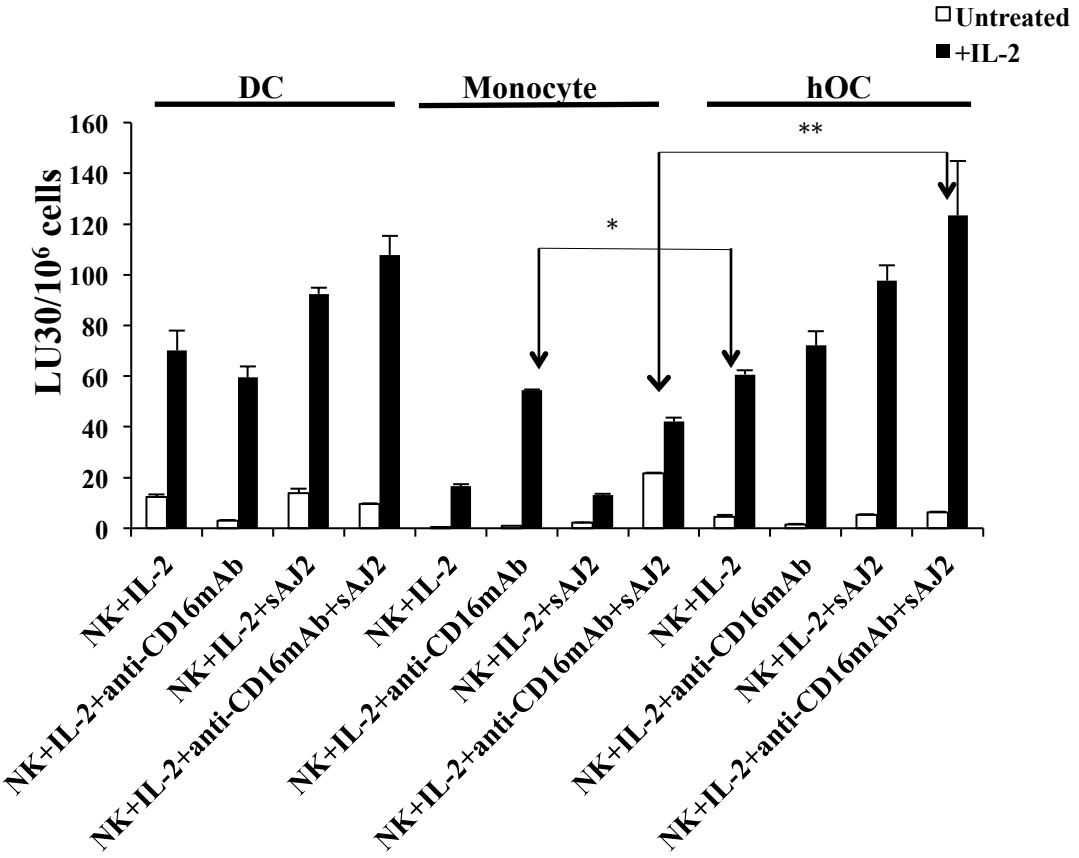


Figure 7. Expanded NK cells by osteoclasts mediated the highest cytotoxicity against OSCSCs.

Pre-activated NK cells, as described in Fig.4, were co-cultured with autologous dendritic cells (DCs), osteoclasts (hOCs) or monocytes for 6 days. Afterwards, the NK cells were divided into two groups, untreated or treated with an additional IL-2 (1000 units/mL), for 48 hours and used in a standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100. *The difference between NK+IL-2+anti-CD16mAb+monocyte and NK+IL-2+anti-CD16mAb+hOC is significant at $p<0.05$. **The difference between NK+IL-2+anti-CD16mAb+sAJ2+monocyte and NK+IL-2+anti-CD16mAb+sAJ2+hOC is significant at $p<0.05$.

Fig. 8

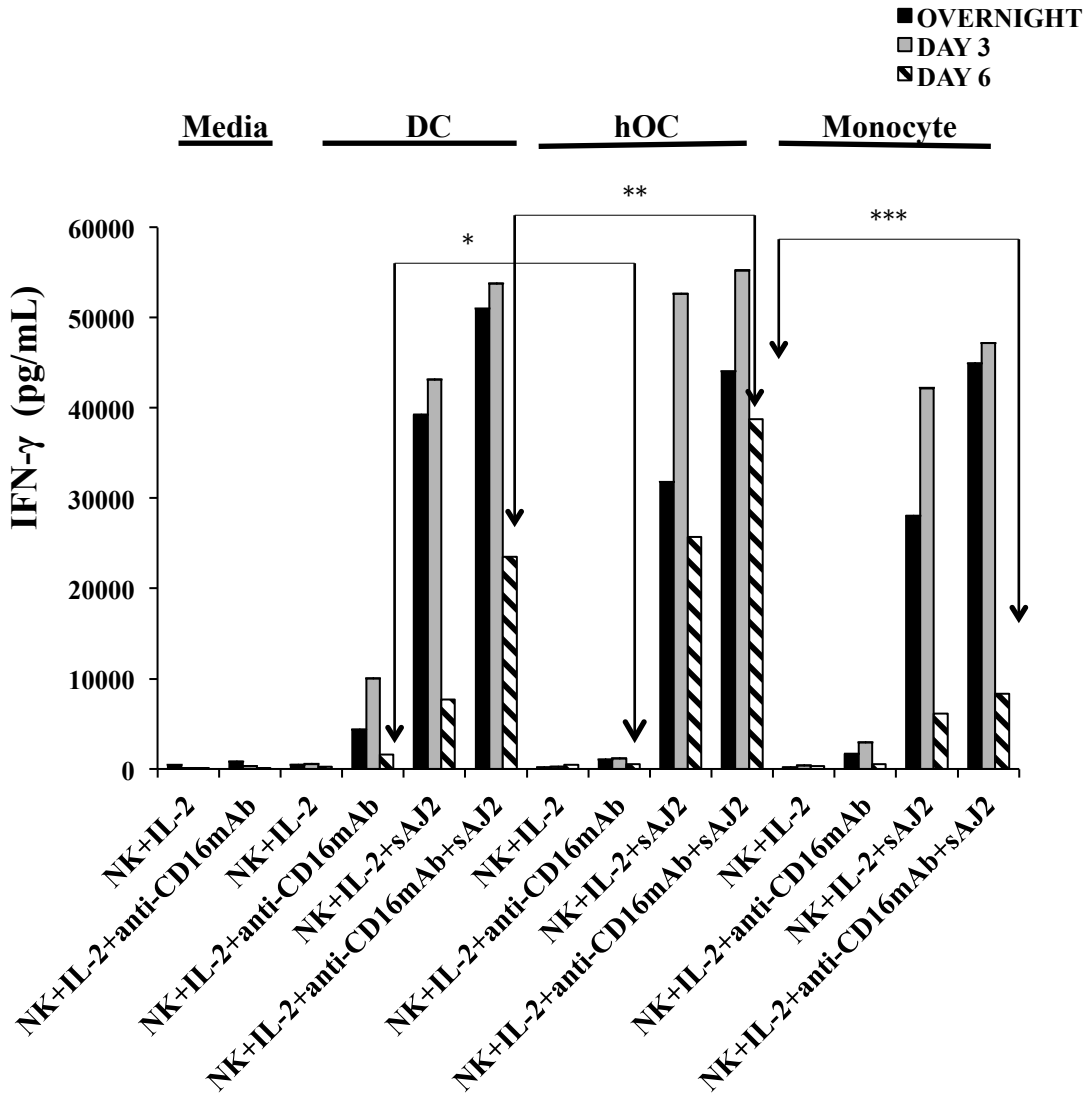


Figure 8. The levels of IFN-γ secreted in the cultures of NK cells with osteoclasts were higher than those induced in the cultures of NK cells either with dendritic cells or monocytes.

NK cells were cultured with dendritic cells (DCs), osteoclasts (hOCs) or monocytes as described in Fig.4. The supernatants from each culture were harvested overnight, day 3 and day 6. The level of IFN-γ produced by NK cells was measured with a specific Elisa. *The difference between NK+IL-2+anti-CD16mAb+DC and NK+IL-2+anti-CD16mAb+hOC is significant at $p<0.05$. **The difference between NK+IL-2+anti-CD16mAb+sAJ2+ DC (or ***monocyte) and NK+IL-2+anti-CD16mAb+ sAJ2+hOC is significant at $p<0.05$.

Fig. 9

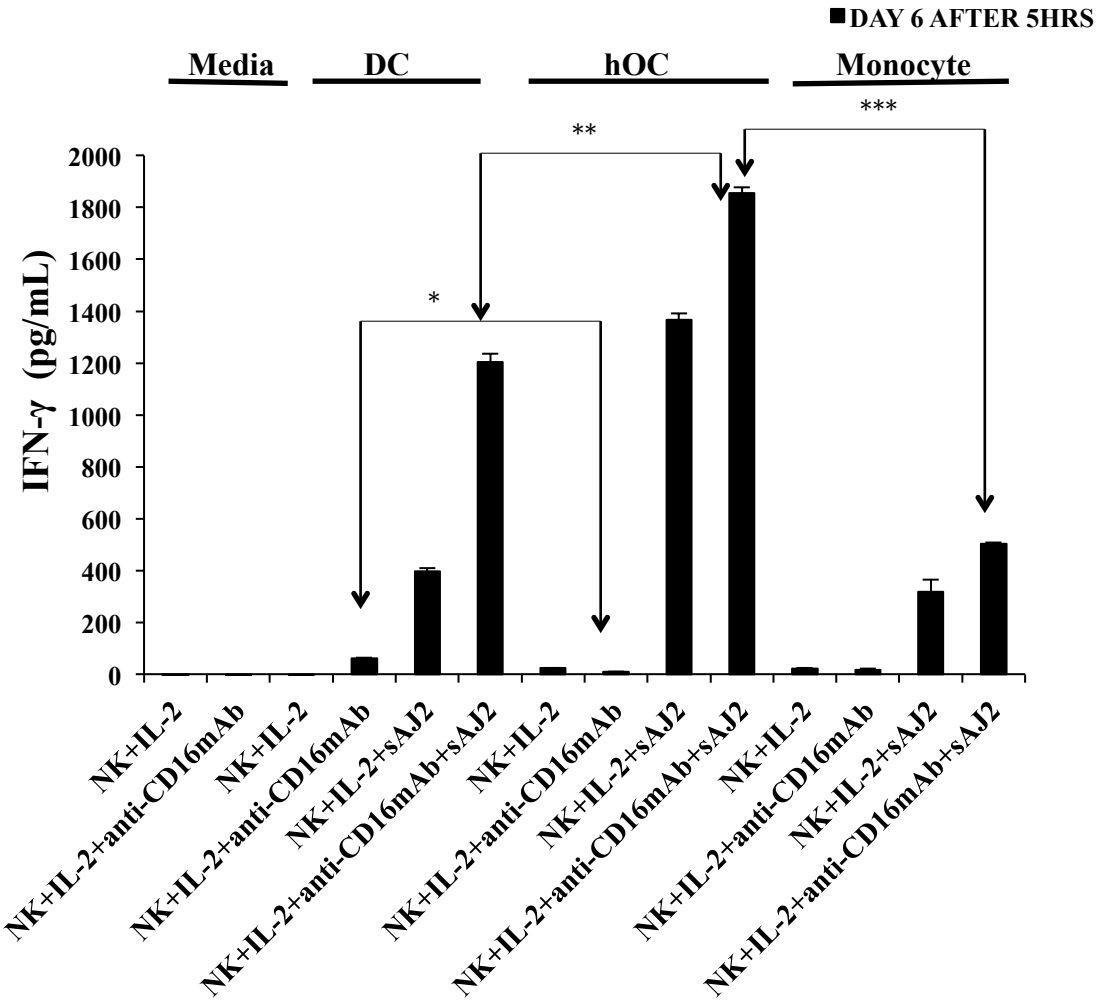


Figure 9. The level of IFN-γ secretion by NK cells on day 6 after 5 hours of supernatant collection

On the 6th day of experimental period, the culture medium was refreshed and cells were cultured for an additional 5 hours. The level of IFN-γ produced by NK cells was measured with a specific ELISA. *The difference between NK+IL-2+anti-CD16mAb+ DC and NK+IL-2+anti-CD16mAb+hOC is significant at $p<0.05$. **The difference between NK+IL-2+anti-CD16mAb+sAJ2+DC (or ***monocyte) and NK+IL-2+anti-CD16mAb+sAJ2 +hOC is significant at $p<0.05$.

Fig. 10

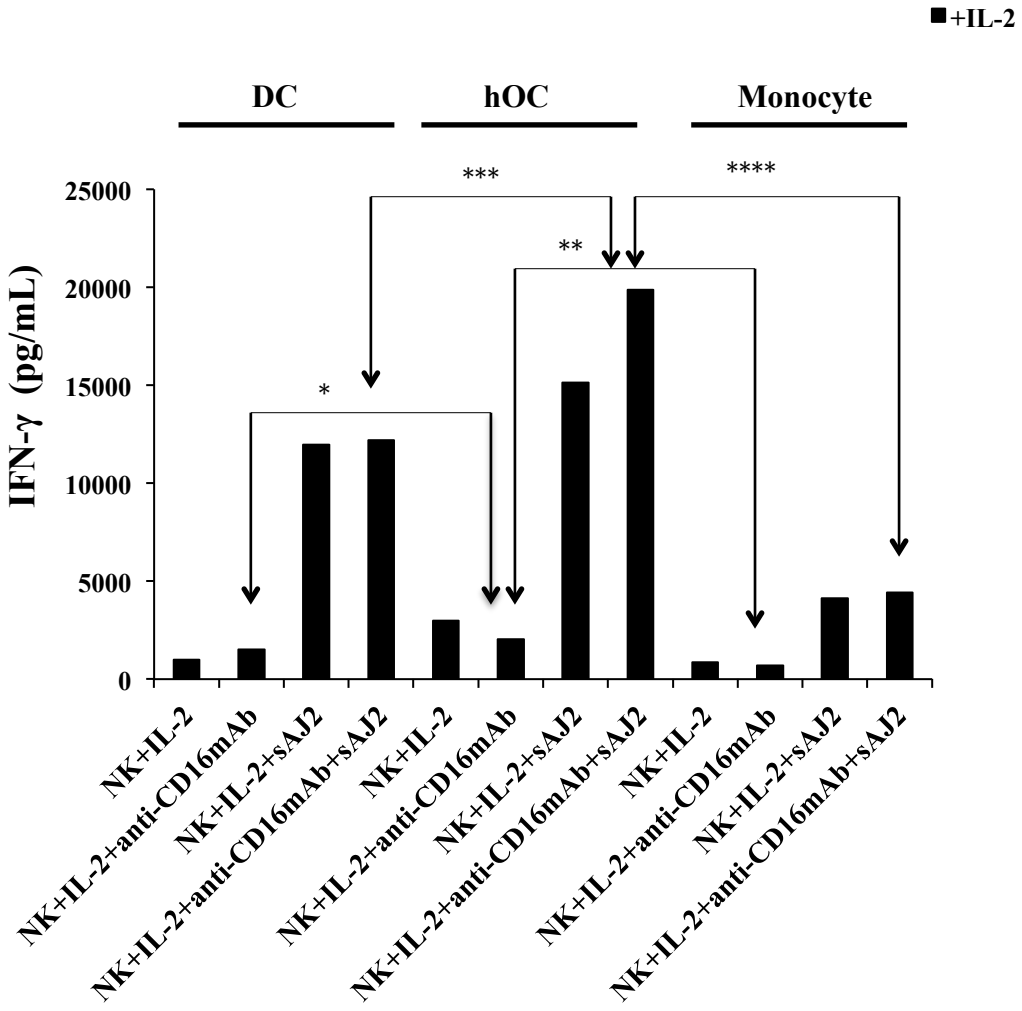


Figure 10. The level of IFN-γ secretion in the cultures of NK cells with dendritic cells, osteoclasts or monocytes on day 8

After 6 days of cultures, $0.3x10^5$ cells from each condition were treated with an additional IL-2 (1000 units/mL) for 48 hours and the supernatants were harvested. The level of IFN-γ produced by NK cells was measured with a specific Elisa. *The difference between NK+IL-2+anti-CD16mAb+DC (or **monocyte) and NK+IL-2+anti-CD16mAb+hOC is significant at $p<0.05$. ***The difference between NK+IL-2+ anti-CD16mAb+sAJ2+DC (or ****monocyte) and NK+IL-2+anti-CD16mAb+sAJ2+hOC is significant at $p<0.05$.

Fig. 11

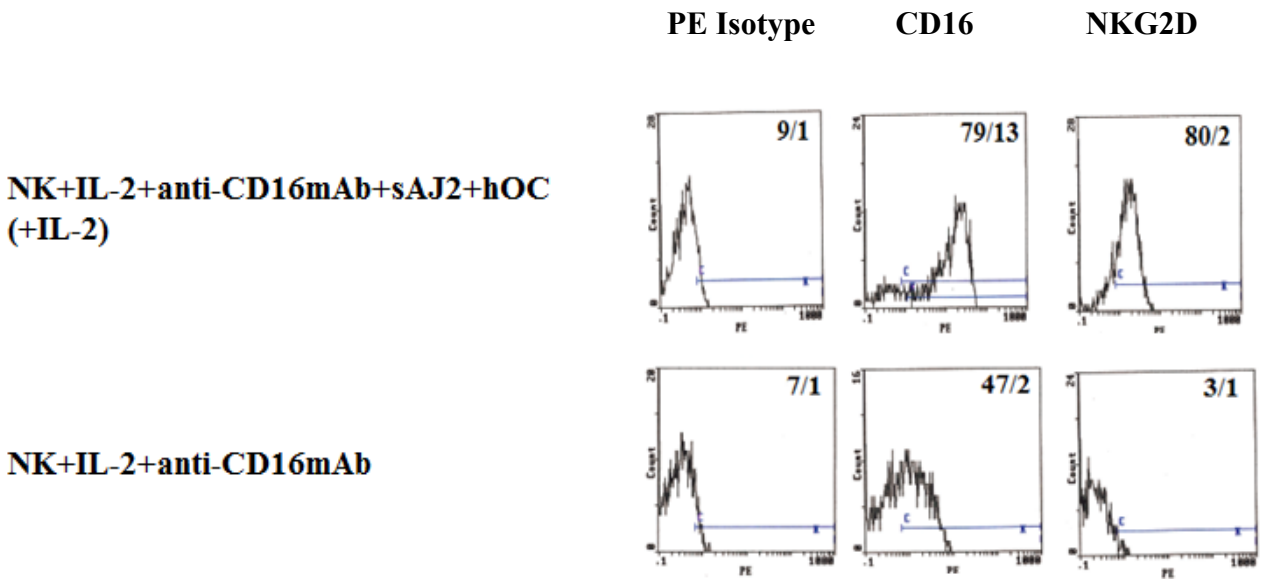


Figure 11. Expanded NK cells retain high expressions of CD16 and NKG2D on the surface compared to primary NK cells.

After 8 days of culture, IL-2 (1000 units/mL), anti-CD16mAb (3ug/mL) and sAJ2 bacteria treated NK cells co-cultured with osteoclasts (hOCs) were treated with an additional IL-2 (1000 units/mL) for overnight. Primary NK cells were purified and treated with a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for overnight. Surface expression profiles of CD16 and NKG2D were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and mean channel fluorescence intensities for each histogram.

Fig. 12

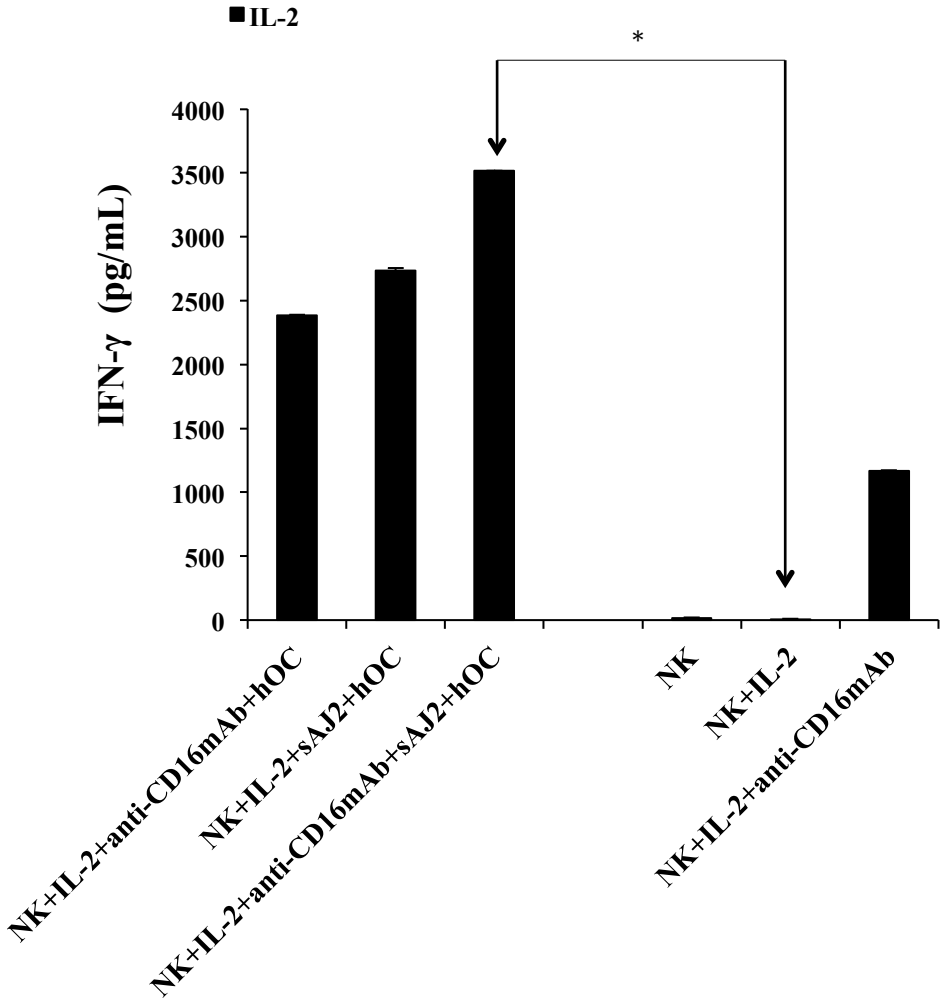


Figure 12. Expanded NK cells by osteoclasts secrete more IFN-γ than primary NK cells.

After 8 days of culture, NK cells from each condition were treated with an additional IL-2 (1000 units/mL) for overnight and primary NK cells were purified and treated with either IL-2 (1000 units/mL) or a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for overnight. The supernatants from each condition were collected and the level of IFN-γ produced by NK cells was measured with s specific Elisa. *The difference between NK+IL-2+anti-CD16mAb+hOC and NK+IL-2 is significant at $p<0.05$.

Fig 13.

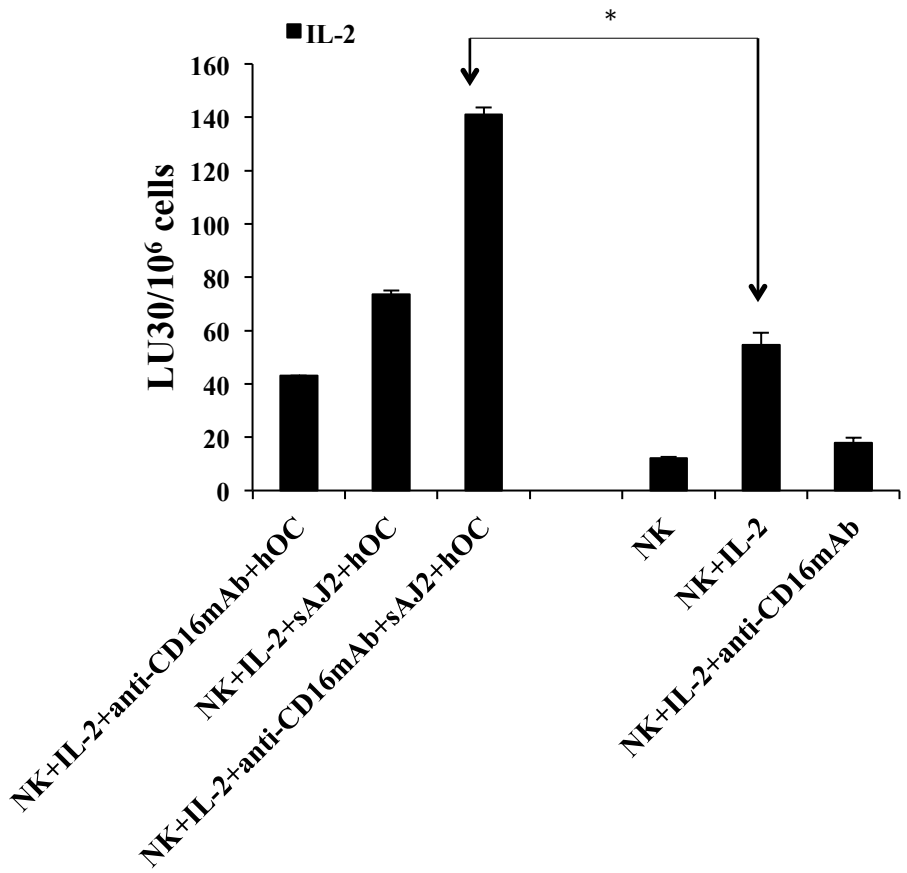


Figure 13. Expanded NK cells by osteoclasts mediated the highest cytotoxicity against OSCSCs compared to primary NK cells.

NK cells from each condition were cultured as described in Fig.11 and used in standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x100. *The difference between NK+IL-2+anti-CD16mAb+sAJ2+hOC and NK+IL-2 is significant at $p<0.05$.

DISCUSSION

In this study, we found the novel way to expand NK cells by osteoclasts with potent cytotoxic function as well as augmented cytokine secretion. There are several studies that reported about NK expansion with a combination of cytokines with other immune cells but no one has been shown NK expansion by osteoclasts. Osteoclasts are found to be the best targets for the expansion of fully functional NK cells under the optimized conditions of NK cell stimulation as shown in this study. Indeed, IL-2+anti-CD16 activated NK cells triggered by sAJ2 bacteria and osteoclasts expanded significant number of NK cells with a capacity of high susceptibility to NK cell mediated lysis and inducing high IFN-γ secretion. Previously, we showed that anergized NK cells contribute to the differentiation and resistance to NK cell mediated cytotoxicity of transformed stem cells by secreting key cytokines [36]. Also, we demonstrated that NK differentiated stem cells not only resist NK cell lysis but also they do not trigger secretion of cytokines or chemokines, potentially contributing to the inhibition of inflammation [36]. TNF-α and IFN-γ secreted by the NK cells synergistically augmented differentiation of cells resulting in an increase in MHC class I, CD54 and B7H1 and 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 in increase in MHC class I surface expression when either treated with the combination of TNF-α and IFN-γ or with activated NK supernatants, which are known to increase maximally MHC class I and II (manuscript submitted). When osteoclasts were compared to freshly isolated autologous monocytes, decreases in CD14, CD11b, CD44, MHC-class I and II and CD54 were more

profound on the surface of osteoclasts (manuscript submitted). This result suggested 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. This may be one reason why NK cells in peripheral blood remain relatively quiescent, even in the presence of competent cytotoxic machinery. Also, this may be one reason why osteoclasts are found to be the best target for the expansion of functionally potent NK cells. Perhaps, osteoclasts are sensitive to NK cell mediated cytotoxicity.

Osteoclast-expanded NK cells not only exhibited high cytotoxic capacity but also they mediated significant secretion of IFN-γ when compared to DCs- and monocyte-stimulated NK cells. Osteoclast-expanded NK cells responded to IL-2 activation and substantially increased IFN-γ secretion per cell basis when compared to DCs or monocytes. Moreover, osteoclast-expanded NK cells express high levels of CD16, NKp44 and NKG2D on the cell surface. NKp44 is a NK activating receptor and binds to viral heamagglutinin (HA) and HA-neuraminidase (HN) as well as tumor-associated ligands [37]. Another NK activiating receptor, NKG2D, is constitutively expressed on all NK cells [38]. NK ligation phophotylates 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]. Expanded NK cells secrete more IFN-γ than primary NK cells while mediating the highest cytotoxicity against OSCSCs in comparison to primary NK cells. These results suggested that osteoclast expanded NK cells are highly activated NK cells with potent cytotoxic function and augmented cytokine secretion. This observation is of outmost

importance since this strategy can overcome NK cell tolerance against tumors and may be used to expand NK cells for delivery to cancer patients.

CONCLUSION

This study demonstrated that osteoclasts are potent immune effectors capable of expanding NK cells and modulating the function of NK cells. Osteoclasts-expanded NK cells generated under this optimal condition demonstrated the highest cytotoxic activity and augmented secretion of IFN-γ. Also, osteoclast-expanded NK cells regained CD16 receptors and are fully activated by retaining activating receptors such as NKp44 and NKG2D. These expanded NK cells are highly functional compared to primary NK cells and they are potent targets of stem like/poorly differentiated tumor cells. Through the help of cytokines, sAJ2 bacteria and osteoclast, NK cells can be expanded and activated in vitro and furthermore, they can be used to target tumors in vivo. Therefore, osteoclast expanded NK cells are highly activated NK cells with potent cytotoxic function and augmented cytokine secretion. These recent discoveries in NK expansion to potentiate their functions against tumors will contribute to clinical trials for cancer therapy.

Chapter 2

Specific Aim 2: To investigate expansion of NK cells by tumors and non-tumors and its ability to target MP2 (pancreatic tumor) in BLT mice

INTRODUCTION

NK cells are innate lymphoid cells that can target for malignant and virally infected cells. They recognize targets without prior sensitization, which makes them excellent candidates to manipulate for cancer therapy. Most cytotoxic activity of NK cells is a result of direct killing, which is mediated by perforin and granzyme release. Perforin causes perforation of the target cell membrane by allowing apoptosis-inducing granules to enter the cell then, the caspase system is activated through the intrinsic pathway [40]. Granzymes trigger apoptosis in the target cell by activating an enzyme cascade. Another apoptotic mechanism is via interactions between death receptors expressed on target cells and the corresponding ligand expressed on NK cells. Fas-Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL)-TRAIL ligand induce apoptosis in the target cell through the extrinsic pathway [40, 41]. However, cytotoxic function of NK cells is suppressed in the tumor microenvironment by many distinct effectors and their secreted factors.

NK cells release pro-inflammatory cytokines so they can augment adaptive responses by promoting differentiation, activation and recruitment of accessory immune cells in the site of infection. Accumulated evidence from out laboratory indicated that NK cells are the effectors of selection, differentiation and resistance of undifferentiated or stem like cells. We showed that conditioned or anergized NK cells induce resistance of healthy stem cells and

transformed cancer stem cells through both secreted cytokines and direct cell-to-cell contact [2]. We believe that this mechanism is likely responsible for shaping the maturation of healthy stem cells for the regeneration of damaged tissues and the resolution of inflammation [2].

There is a complex dynamic interaction between the immune effectors and the immune cells. The tumor microenvironment can induce tumor escape from immune surveillance by suppressing the effector NK cell function and evading through selection of immunogenic tumor cells [42]. When NK cells are cultured with sensitive tumor target cells, the target binding NK cells undergo phenotypic and functional changes. NK cells inactivated by target cells express CD16-CD56dim/-CD69+ phenotype [43, 44]. Significant down-modulation of CD16 receptor expression and decreased NK cell cytotoxic function were observed in patients with HIV infection, oral and ovarian cancer [45-47]. We have previously shown that triggering CD16 on NK cells resulted in down-modulation of CD16 receptors and in a significant loss of cytotoxicity and augmented secretion of IFN- γ, which we coined the term, "split anergy"[43, 44, 48-50]. We have seen that K562 causes loss of NK cell cytotoxicity and induces cell death in a small 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]. Therefore, CD16 receptors play a critical role in target cell induced loss of NK cell cytotoxicity.

Expansion of highly cytotoxic NK cells by tumor cells for cancer cell therapy was also reported. Fujisaki et al [52] modified leukemia cell line K562 to express a membrane-bound form of IL-15 and 41BB ligand (K562-mb15-41BBL) to generate NK cells with enhanced cytotoxicity. They reported 21.6 fold expansion of $CD56^{+}CD3^{-}$ NK cells from

peripheral blood after they were co-cultured with irradiated K562-mb15-41BBL for 7 days and these expanded NK cells were more potent than those produced by stimulation with IL-2, IL-12, IL-15, and/or IL-21 [52]. Artificial antigen-presenting cells (aAPCs) produced by genetic modification of K562 expressing costimulatory molecules and membrane-bound cytokines were used to co-culture with unfractionated PBMC in order to expand NK cells [53]. We used K562 and irradiated PBMCs to expand NK cells with potent function in the same way we expanded NK cells with osteoclasts but we were unable to expand NK cells.

The results from our laboratory suggested important functions for NK cells. One potential function of NK cells is to eliminate the number of stem cells after their interaction with other immune inflammatory cells or effectors of connective tissue [2, 54]. Another potential function is to support differentiation of the stem cells since they will be conditioned to lose cytotoxicity and induce cytokine secretions. [2, 54]. Since cancer stem cells were found to be 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, combination therapy of repeated allogeneic NK cell transplantation and chemotherapeutic drugs should be more effective in the elimination of both cancer stem cells and differentiated tumors [2, 54]. In this regard, developing new ways of expanding NK cells is a crucial for adoptive therapy, which might be ideal in some situations. Not only expanding the number of NK cells but also sustaining the potent functions of NK cells, NK cell mediated cytotoxicity and augmented cytokine secretion, is very important in order to overcome NK cell tolerance against tumor.

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, DNAM, NKp44, and CD3/CD16^{+}CD56 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% purify based on flow cytometric analysis of CD14 antibody stained monocyte cells.

Generation of Osteoclasts

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

$1x10^5$ NK cells from each condition were stained in 50ul of cold 1%PBS-BSA with pre-determined optimal concentration of PE conjugated isotype control, CD16, CD45, CD56, CXCR1, CXCR3, DNAM, NKp44 and CD3/$CD16^+$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 cytometry 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 ($1x10^5$ 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 ($5x10^5$ OSCSCs/well) were labeled with 50μCi ^{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;

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

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

Enzyme-Linked Immunosorbent Assays (ELISAs)

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 200rmp 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 $H_2SO_4$to stop the reaction. The plates were read in a microplate reader at 450nm to obtain absorbance value (Biolegend, ELISA manual).

Analysis of human pancreatic cancer cell growth in immunodeficient and humanized mice

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

In vivo growth and metastatic behavior of human pancreatic cancer cell lines were determined by orthotopic cell implantation into 8-10 week-old NSG mice, as previously

described . To establish orthotopic tumors, mice were first anesthetized with ketamine (100 mg/kg) and xylzine (10 mg/kg), and then the pancreas was exposed through an abdominal incision (laparotomy). Tumor cells were then transferred by direct injection of a single cell suspension ($0.5X10^6$ or $1X10^6$ cells into NSG or hu-BLT mice, respectively) into the pancreas or transplantation of a subcutaneous tumor fragment ($2mm^3$) onto the pancreas, secured by a 7-0 Prolene suture. After tumor implantation, all mice were monitored at least twice weekly for disease progression by abdominal palpation and for overall signs of morbidity such as ruffled fur, hunched posture, and immobility. 7 days after the surgery selected hu-BLT mice received $1.5x10^6$ human expanded NK cells via teil vein injection. Moribund mice were euthanized by isofluorane or CO2 inhalation. For survival studies, mice were followed until death or euthanized when signs of morbidity were evident. Tumor size was measured with a caliper. Pancreatic tumors, livers, lungs, bone marrow, spleen and blood were harvested from mice at the end of the experiment following orthotropic tumor implantation or when tumor size reached 2cm diameter.

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

Live tumors neither support the expansion of NK cells nor enhance their functions

NK cells expanded by osteoclasts were compared with NK cells expanded by oral squamous cancer stem cells (OSCSCs) whether OSCSCs can also support expansion of NK cells. As shown in Fig.14, osteoclasts supported the expansion of NK cells whereas OSCSCs did not. Especially, osteoclasts that were cultured with IL-2+anti-CD16mAb+sAJ2 bacteria treated NK cells had the most expansion. NK cells cultured with either osteoclasts or OSCSCs were counted and equal numbers of NK cells from each subset were used in cytotoxicity assay against OSCSCs, which is a very sensitive NK cell target. IL-2+anti-CD16mAb treated hOC with an additional IL-2 had higher cytotoxicity than IL-2+anti-CD16mAb treated OSCSCs with an additional IL-2 ($p<0.05$) (Fig.12). However, IL-2+anti-CD16mAb+sAJ2 bacteria treated NK cells expanded by osteoclasts with an additional IL-2 treatment had the highest cytotoxicity whereas NK cells expanded by OSCSCs had no cytotoxicity ($p<0.05$) (Fig. 15).

Then, the levels of IFN-γ secretion by IL-2+sAJ2 bacteria or IL-2+anti-CD16mAb +sAJ2 bacteria treated NK cells cultured either with osteoclasts or OSCSCs were determined from day 0 to day 8. As shown in Fig.16, the levels of IFN-γ secreted in the cultures of NK cells with osteoclasts rose significantly from day 0 to day 6 and remained high until day 8, and the secreted levels were much higher than those induced in the cultures of NK cells with OSCSCs. NK cells cultured with OSCSCs induced high levels of IFN-γ until day 3 but then the levels of IFN-γ secretion decreased significantly since day 6 (Fig.16).

Although live K562 expanded NK cells in some extent, they were not able to expand NK cells to the same extent as osteoclasts (Fig.17). NK cells expanded by osteoclasts and K562 were counted and equal numbers of NK cells from each expanded subset were used in cytotoxicity assay against OSCSCs. IL-2+anti-CD16mAb+sAJ2 bacteria treated NK cells expanded by osteoclasts had the highest cytotoxicity whereas there was no cytotoxicity mediated by IL-2+anti-CD16mAb+sAJ2 treated NK cells co-cultured with K562 ($p<0.05$)(Fig.18). The levels of IFN-γ secretion by IL-2+sAJ2 bacteria and IL-2+anti-CD16mAb+ sAJ2 bacteria treated NK cells cultured either with osteoclasts or K562 from day 0 to day 8 of cultures were compared. As shown in Fig.19A, the levels of IFN- γ secreted in the cultures of NK cells with osteoclasts rose significantly from day 0 to day 3 and remained high until day 6, and the secreted levels were much higher than those induced in the culture of NK cells with live K562. On day 3, IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts secreted 5.7 fold more of IFN-γ than IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with K562 ($p<0.05$) (Fig.19A). On day 6, even though the amount of IFN-γ secreted by IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts decreased in comparison to day 3, the levels of IFN-γ secretion was still higher than IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with K562 ($p<0.05$) (Fig.19A). Indeed, within 5 hours of cultures IL-2+anti-CD16mAb+sAJ2 treated NK cells secreted 5.5 fold more IFN-γ when cultured with osteoclasts than K562 ($p<0.05$) (Fig.19B).

Surface expression profiles of expanded NK cell either by osteoclasts or K562 were compared at different time points. On day 3 of culture, IL-2+anti-CD16mAb +sAJ2 treated NK cells expanded by osteoclasts had low expression of $CD16^{+}/CD56^{+}$ (Fig. 20A). Also as

you can see in Fig. 17A, there was no clear difference in the surface expression of CXCR1 among NK cells co-cultured with osteoclasts or K562 and CXCR3 and DNAM were up-regulated in these conditions. On day 6, IL-2+anti-CD16mAb+sAJ2 treated NK cells expanded by osteoclasts retained $CD16^{+}/CD56^{+}$ receptors on its surface and had relatively higher surface expression of $CD16^{+}/CD56^{+}$ in comparison to NK cells expanded by K562 (Fig. 20B). As shown in Fig. 20C, CD16 and CD56 receptors were down-modulated on day 8 compared to day 6 but the expanded condition by osteoclasts still presented more CD16 and CD56 receptors than any other conditions. There was no clear difference in the surface expression of DNAM (Fig. 20C).

Fig. 14

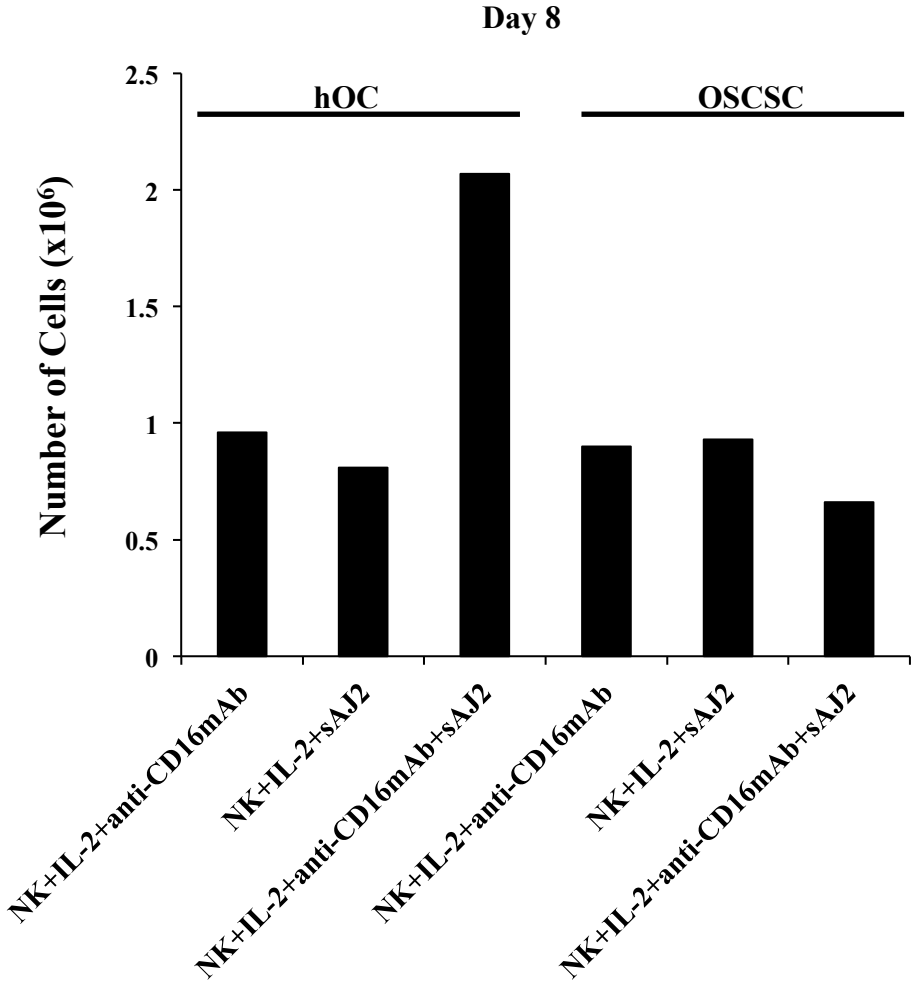


Figure 14. Number of NK cells on day 8 after co-culture either with osteoclasts or OSCSCs

Osteoclasts (hOCs) were prepared as described in Materials and Methods and seeded at $3x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. These NK cells were cultured either with autologous hOCs or OSCSCs at an effector to target ratio of 1:1 for 8 days. On the 8^{th} day of the experimental period the number of cells was assessed by microscopic evaluation.

Fig. 15

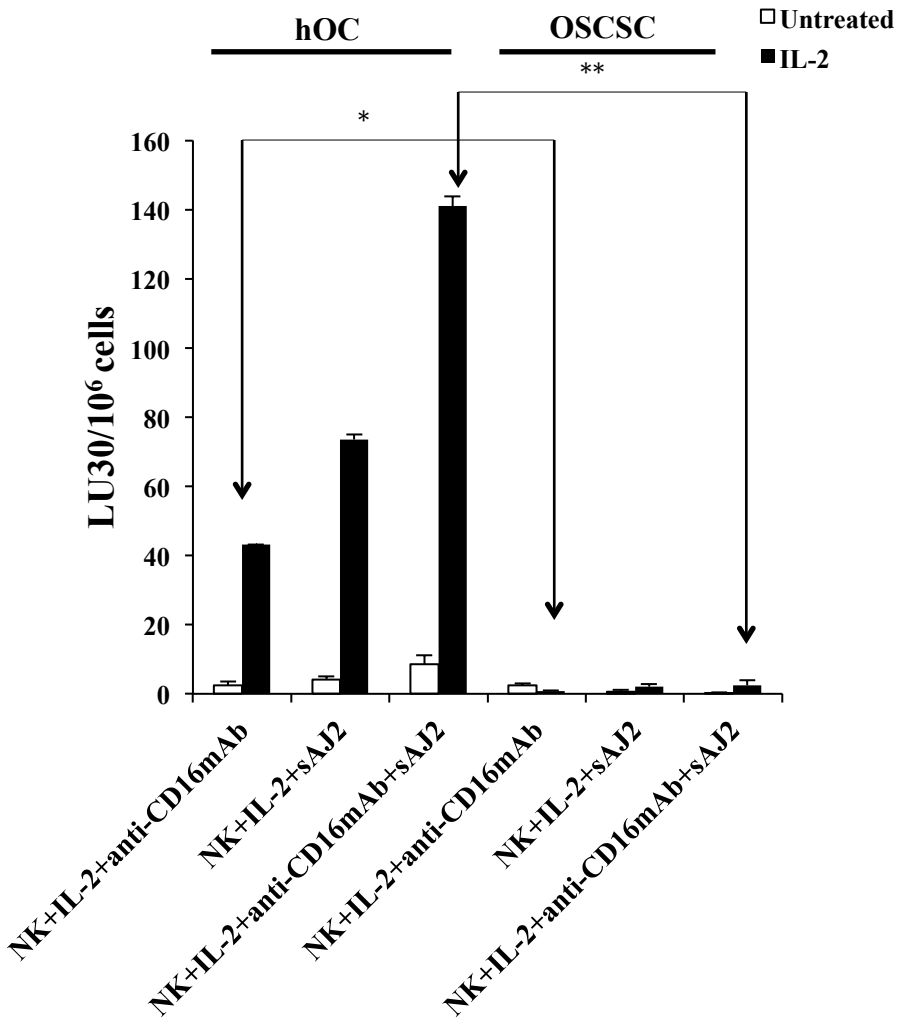


Figure 15. Expanded NK cells by osteoclasts mediated the highest cytotoxicity against OSCSCs compared to NK cells co-cultured with OSCSCs.

Osteoclasts (hOCs) were prepared as described in Materials and Methods and pre-activated NK cells were cultured either with hOCs or OSCSCs as described in Fig.14. On day 8, NK cells were divided into two groups, untreated or treated with an additional IL-2 (1000 units/mL), for 24 hours and used in a standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100. *The difference between NK+IL-2+anti-CD16mAb+hOC and NK+IL-2+anti-CD16mAb+OSCSC is significant at $p<0.05$. **The difference between NK+IL-2+anti-CD16mAb+sAJ2+hOC and NK+IL-2+anti-CD16mAb+sAJ2+ OSCSC is significant at $p<0.05$.

Fig. 16

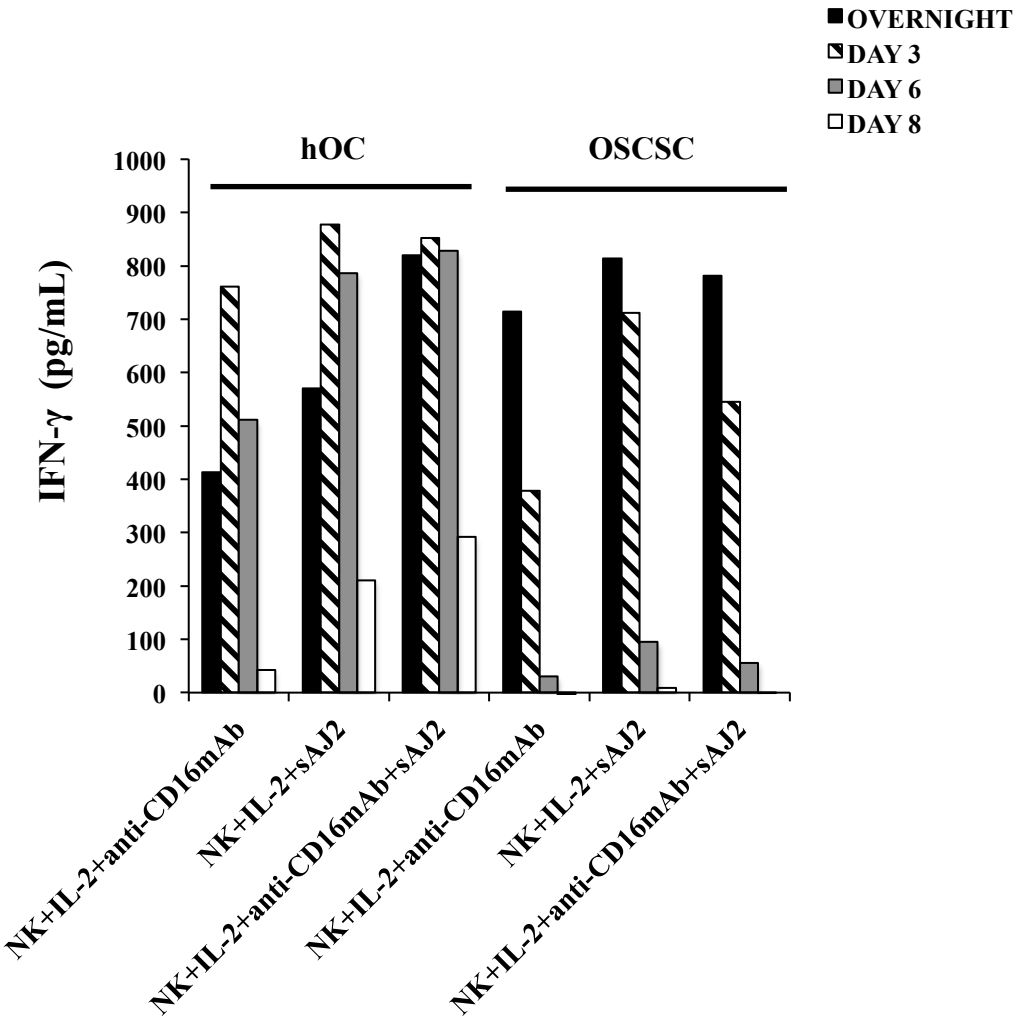


Figure 16. The levels of IFN-γ secreted in the cultures of NK cells with osteoclasts were higher than those induced in the cultures of NK cells with OSCSCs.

NK cells were cultured with osteoclasts (hOCs) or OSCSCs as described in Fig.14. The supernatants from each culture were harvested overnight, day 3, day 6, and day 8. The level of IFN-γ produced by NK cells was measured with a specific Elisa.

Fig 17.

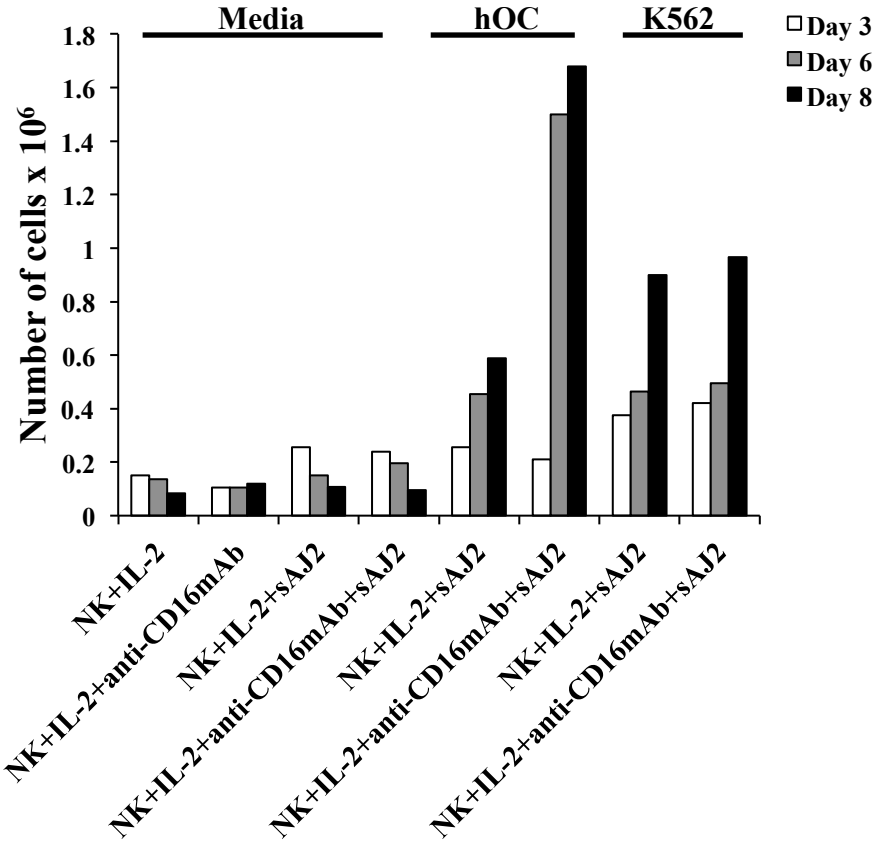


Figure 17. Number of NK cells expanded either by osteoclasts or K562 at different time points

Osteoclasts (hOCs) were prepared as described in Materials and Methods and seeded at $2x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. These NK cells were cultured either with autologous hOCs or K562 at an effector to target ratio of 1:1. On the 3rd, 6th, and 8th day of the experimental period the number of cells was assessed by microscopic evaluation.

Fig. 18

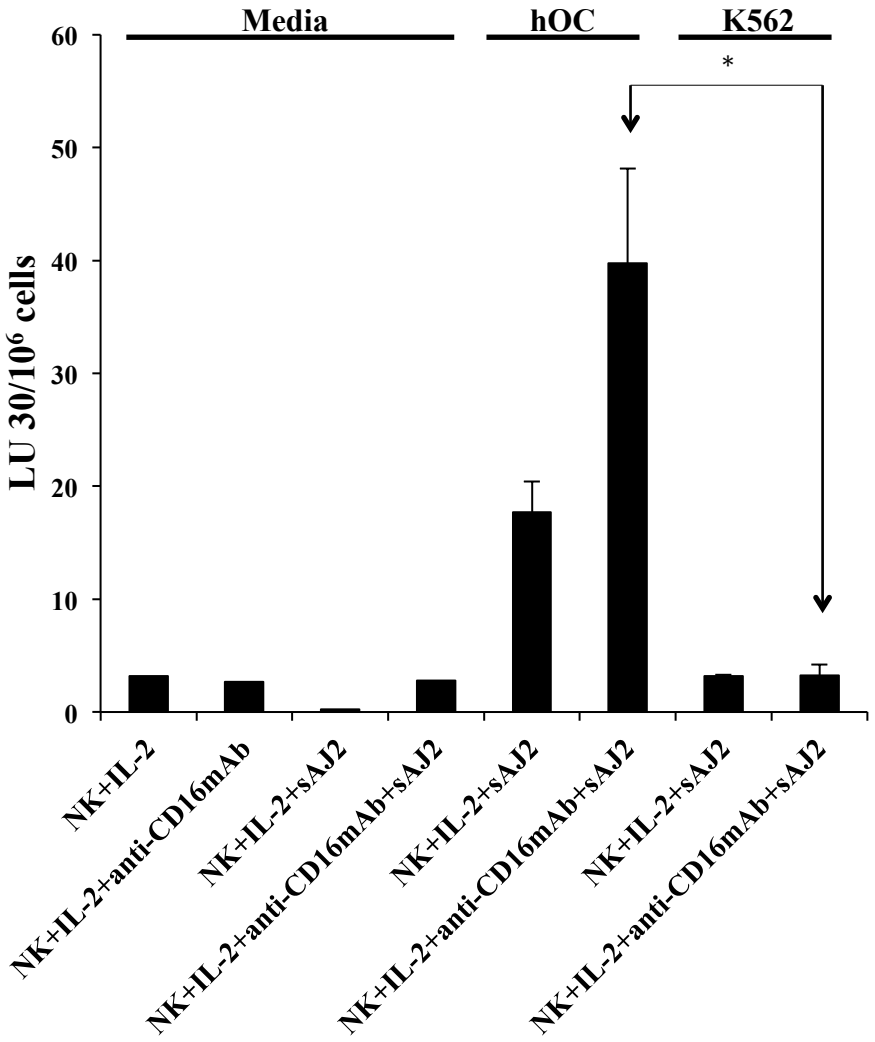


Figure 18. Expanded NK cells by osteoclasts mediated the highest cytotoxicity against OSCSCs compared to expanded NK cells by K562.

Osteoclasts (hOCs) were prepared as described in Materials and Methods and pre-activated NK cells were cultured either with hOCs or K562 as described in Fig.17. On day 8, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100. *The difference between NK+IL-2+anti-CD16mAb+sAJ2+hOC and NK+IL-2+anti-CD16mAb+sAJ2 +K562 is significant at $p<0.05$.

Fig. 19

A

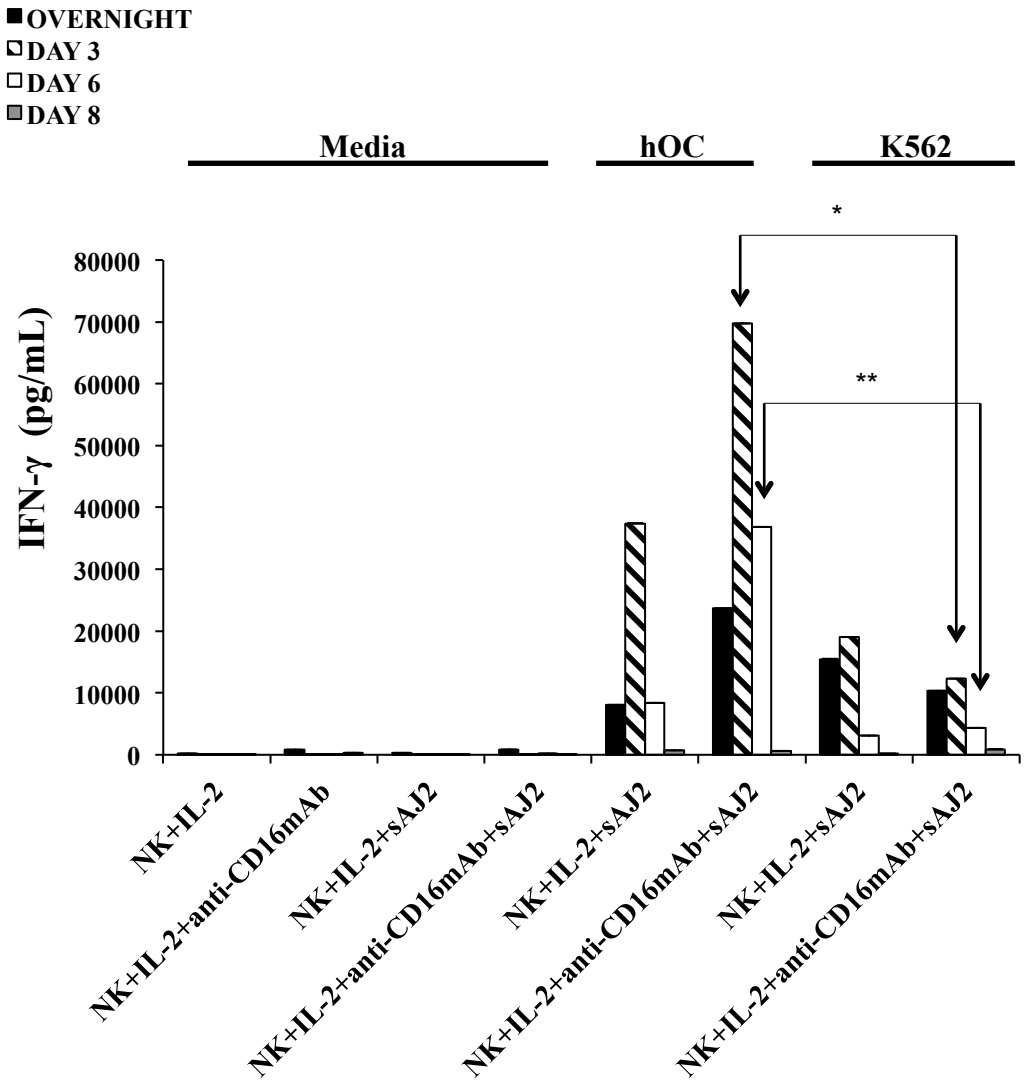


Figure 19A. The levels of IFN-γ secreted in the cultures of NK cells with osteoclasts were higher than those induced in the cultures of NK cells with K562.

NK cells were cultured with osteoclasts (hOCs) and K562 as described in Fig.17. The supernatants from each culture were harvested overnight, day 3, day 6, and day 8. The level of IFN-γ produced by NK cells was measured with a specific Elisa. *The difference between NK+IL-2+anti-CD16mAb+sAJ2+hOC and NK+IL-2+anti-CD16mAb+sAJ2+ K562 on day 3 (or **day 6) is significant at $p<0.05$.

B

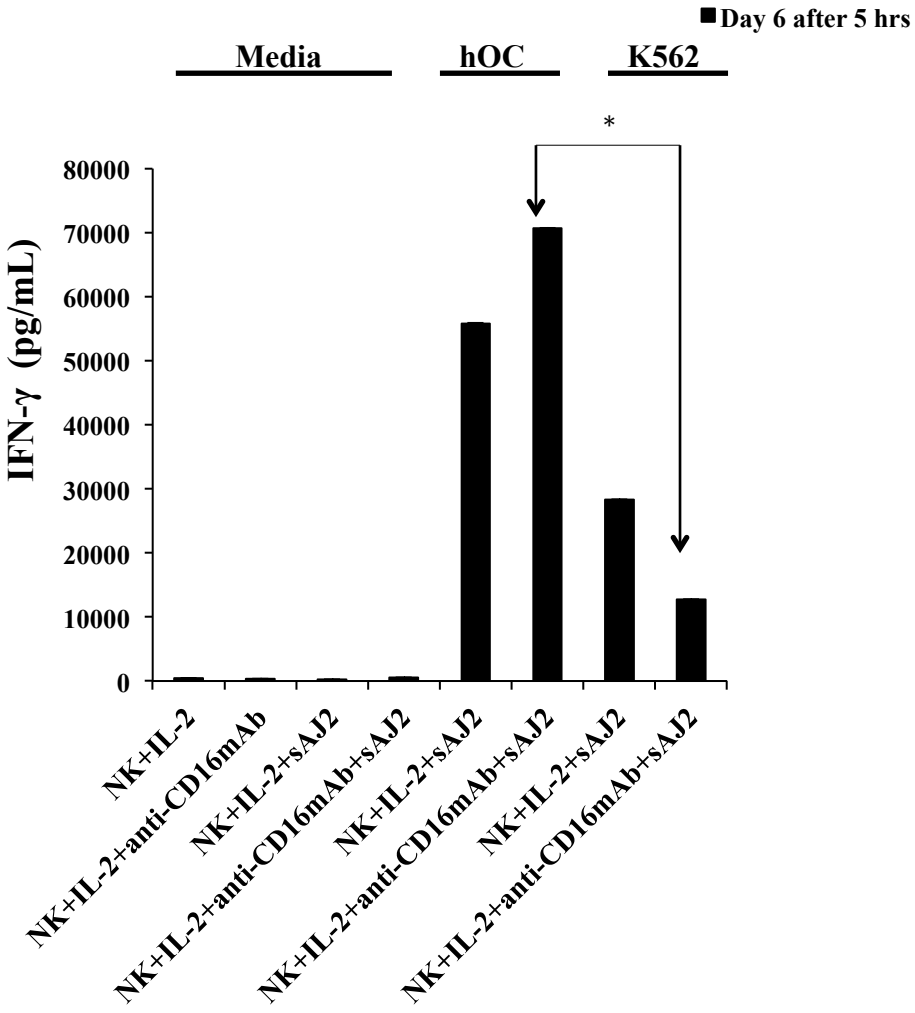


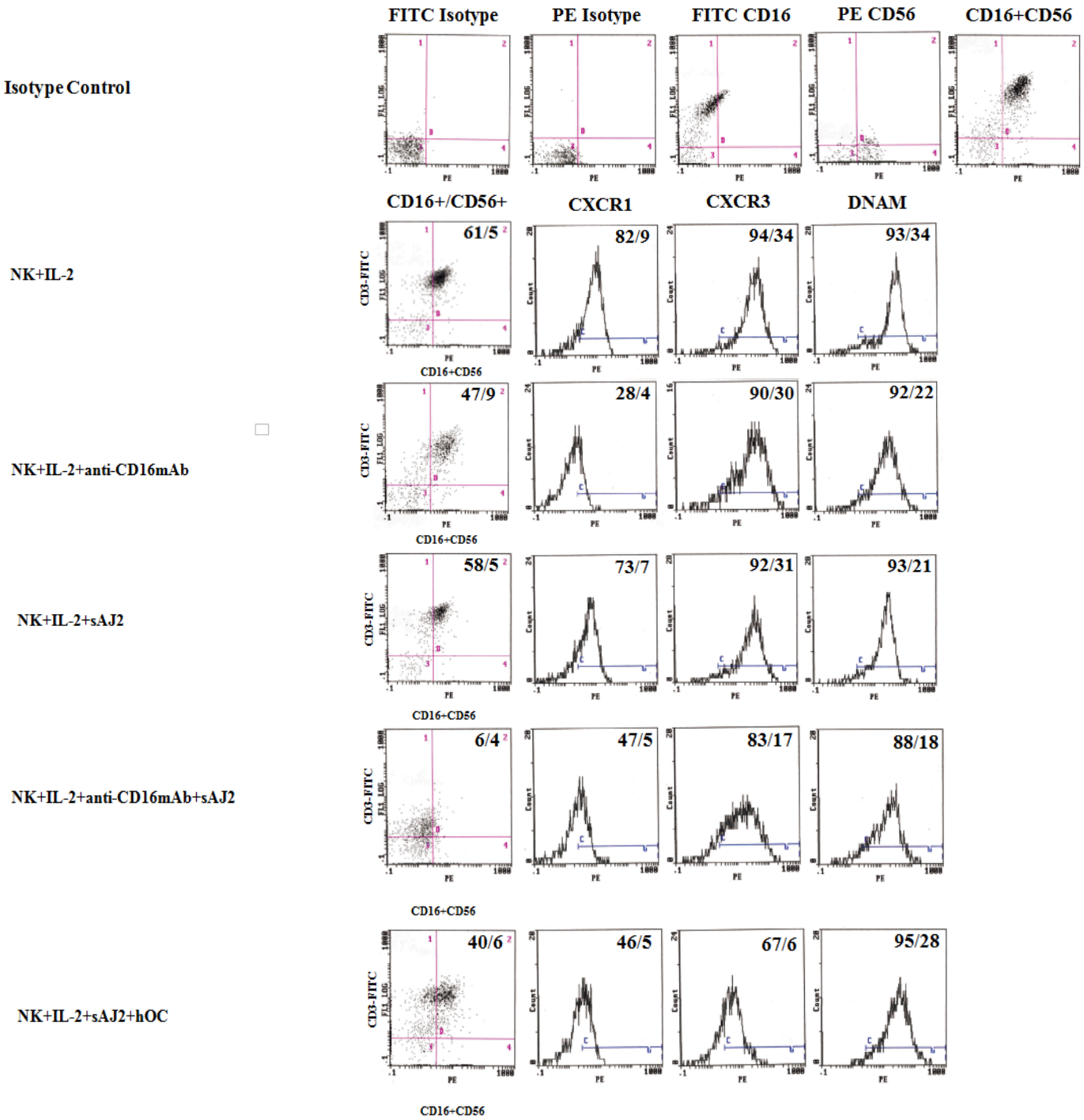
Figure 19B. The level of IFN-γ secretion by NK cells on day 6 after 5 hours of supernatant collection

On the 6th day of experimental period, the culture medium was refreshed and cells were cultured for an additional 5 hours. The level of IFN-γ produced by NK cells was measured with a specific ELISA. *The difference between NK+IL-2+anti-CD16mAb+ sAJ2+hOC and NK+IL-2+anti-CD16mAb+sAJ2+K562 is significant at $p<0.05$.

Fig. 20

A

Day 3


FITC Isotype
PE Isotype
FITC CD16
PE CD56
CD16+CD56
Isotype Control
CD16+/CD56+
CXCR1
CXCR3
DNAM
NK+IL-2
61/5
82/9
94/34
93/34
CD3-FITC
CD16+CD56
NK+IL-2+anti-CD16mAb
47/9
28/4
90/30
92/22
NK+IL-2+sAJ2
58/5
73/7
92/31
93/21
NK+IL-2+anti-CD16mAb+sAJ2
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40/6
46/5
67/6
95/28

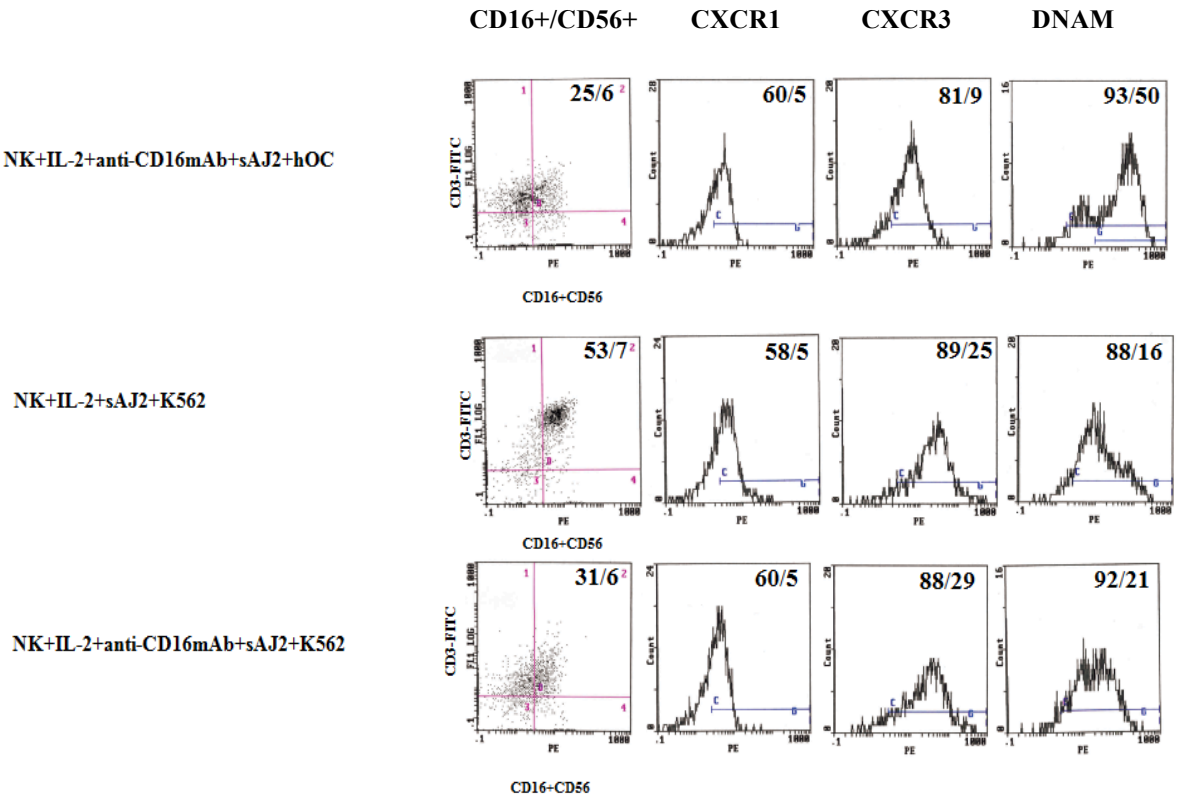
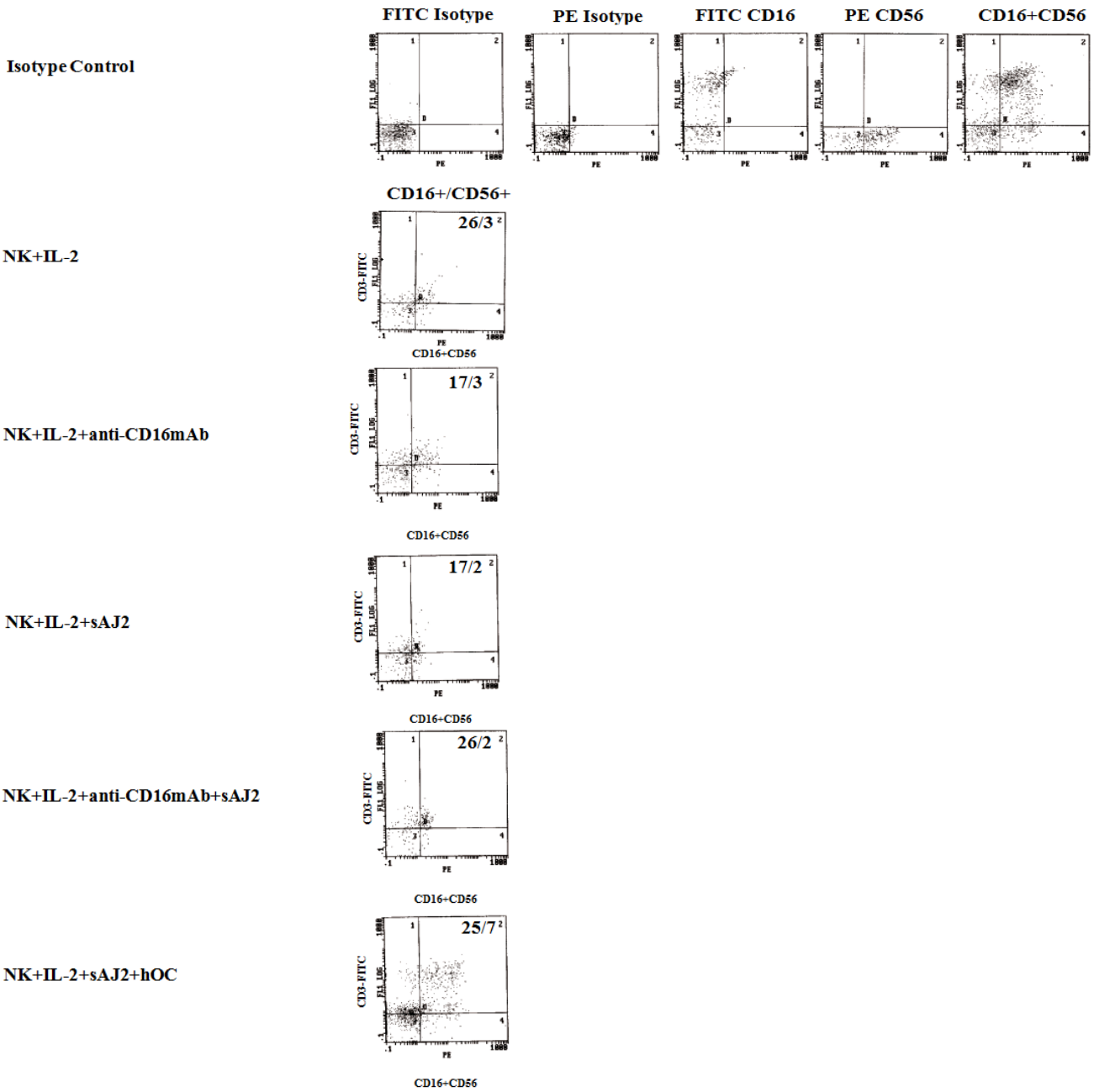


Figure 20A. Surface expression profiles of NK cells expanded either by osteoclasts or K562 on day 3

Osteoclasts (hOCs) and K562 were co-cultured as described in Fig.17. Surface expression profiles of $CD16^{+}/CD56^{+}$, CXCR1, CXCR3 and DNAM were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and mean channel fluorescence intensities for each histogram.

B

Day 6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PE Isotype
FITC CD16
PE CD56
CD16+CD56
Isotype Control
CD16+/CD56+
NK+IL-2
26/3
NK+IL-2+anti-CD16mAb
17/3
NK+IL-2+sAJ2
17/2
NK+IL-2+anti-CD16mAb+sAJ2
26/2
NK+IL-2+sAJ2+hOC
25/7
CD3-FITC
CD16+CD56
PE

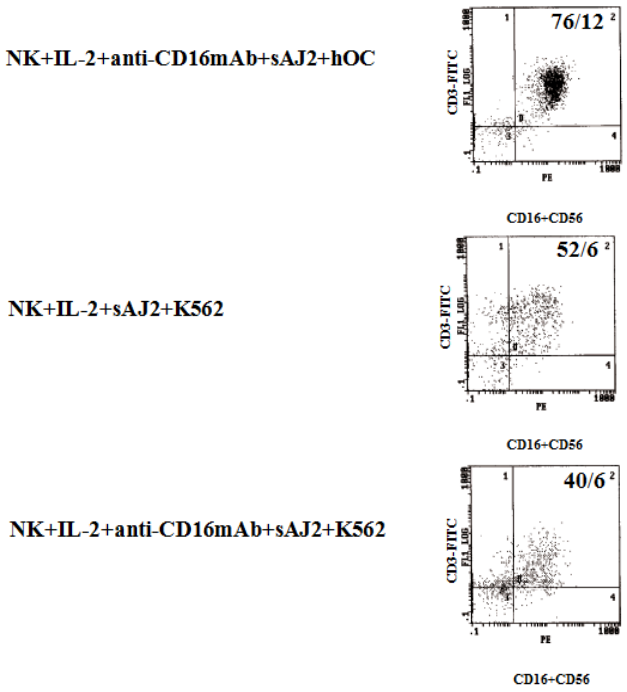


Figure 20B. Surface expression profiles of NK cells expanded either by osteoclasts or K562 on day 6

Osteoclasts (hOCs) and K562 were cultured as described in Fig. 17. Surface expression profiles of $CD16^{+}/CD56^{+}$ were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and mean channel fluorescence intensities for each histogram.

C **Day 8**

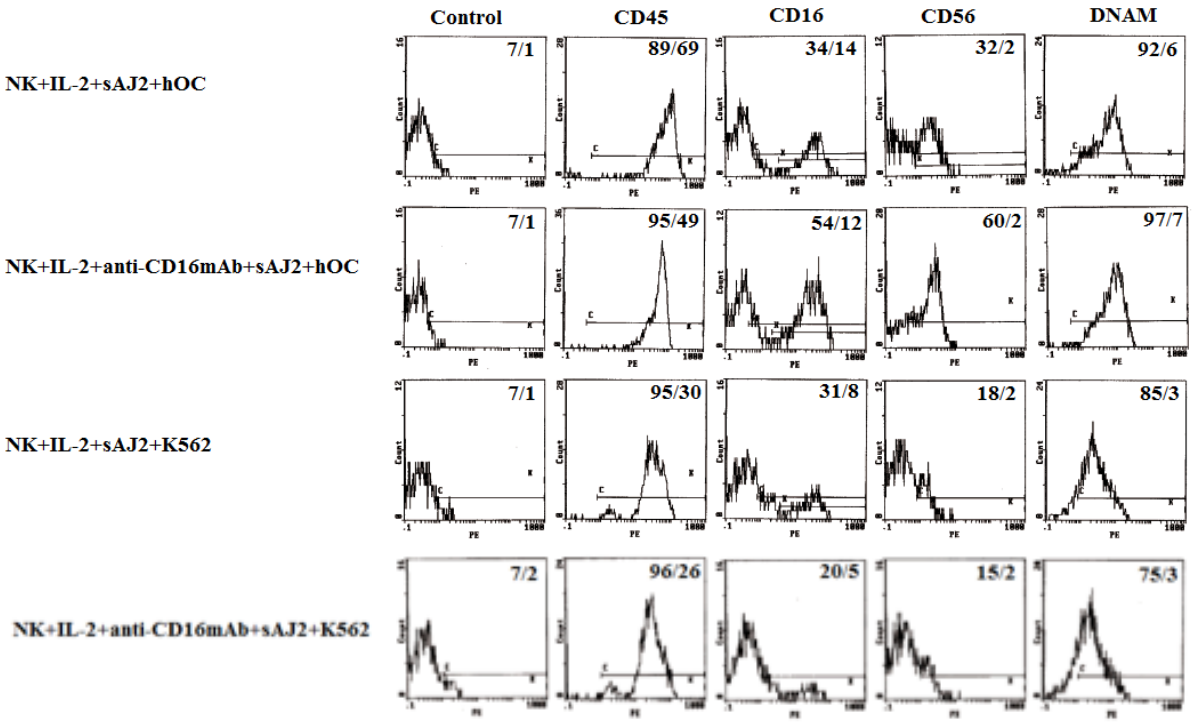


Figure 20C. Surface expression profiles of NK cells expanded either by osteoclasts or K562 on day 8

Osteoclasts (hOCs) and K562 were cultured as described in Fig.17. Surface expression profiles of CD45, CD16, CD56 and DNAM were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and mean channel fluorescence intensities for each histogram.

Irradiated tumors neither support the expansion of NK cells nor enhance their functions

The function of NK cells expanded by live tumors or irradiated tumors were studied. The levels of IFN-γ secretion by NK cells co-cultured with live tumors and irradiated tumors were determined from day 0 to day 8. As shown in Fig.21, the levels of IFN-γ secreted in the cultures of NK cells with live tumors and irradiated tumors decreased significantly since day 3. Overall, the secreted levels in the cultures of NK cells with irradiated tumors especially K562 were much higher than those induced in the cultures of NK cells with live tumors (Fig.21).

NK cells expanded by live K562 and irradiated K562 were compared with NK cells expanded by live osteoclasts and irradiated osteoclasts. As shown in Fig. 22, on day 6 live osteoclasts and irradiated osteoclasts supported the expansion of NK cells whereas live K562 and irradiated K562 did not. NK cells cultured either with live osteoclasts and irradiated osteoclasts secreted more IFN-γ than NK cells cultured either with live K562 or irradiated K562 (Fig. 23). NK cells cultured with live osteoclasts, irradiated osteoclasts, live K562 and irradiated K562 were counted and equal numbers of NK cells from each subset were used in cytotoxicity assay against OSCSCs, which is a very sensitive NK cell target. As shown in Fig.24, NK cells expanded by live osteoclasts with an additional IL-2 treatment had higher cytotoxicity than NK cells expanded by live K562. Also, irradiated osteoclasts and irradiated K562 showed the same trend. After cytotoxicity assay, equal number of expanded NK cells from each condition was cultured at the same concentration with an additional IL-2 (1000 units/mL) treatment. As shown in Fig. 25, the levels of IFN-γ secretion by expanded NK cells

cultured with osteoclasts gradually decreased on day 10 and day 13 since day 7 but still remained high when compared to the levels of IFN-γ secretion by expanded NK cells cultured with live K562 and irradiated K562.

Osteoclasts are more differentiated cells than monocytes and dendritic cells (DCs) so whether more differentiated tumors can support the expansion of NK cells was determined. Oral squamous cell carcinoma (OSCC), OSCSC, pancreatic cancer cell line 12 or PANC 10.05 (PL-12), and MIA Paca-2 (MP-2) were irradiated and used to expand NK cells. As shown in Fig.26, none of irradiated stem-like or more differentiated tumors were able to expand NK cells except osteoclasts. Also, the levels of IFN-γ secreted in the cultures of NK cells with osteoclasts remained high until day 8, and the secreted levels were much higher than those induced in the cultures of NK cells with stem like or more differentiated tumors (Fig. 27).

Fig. 21

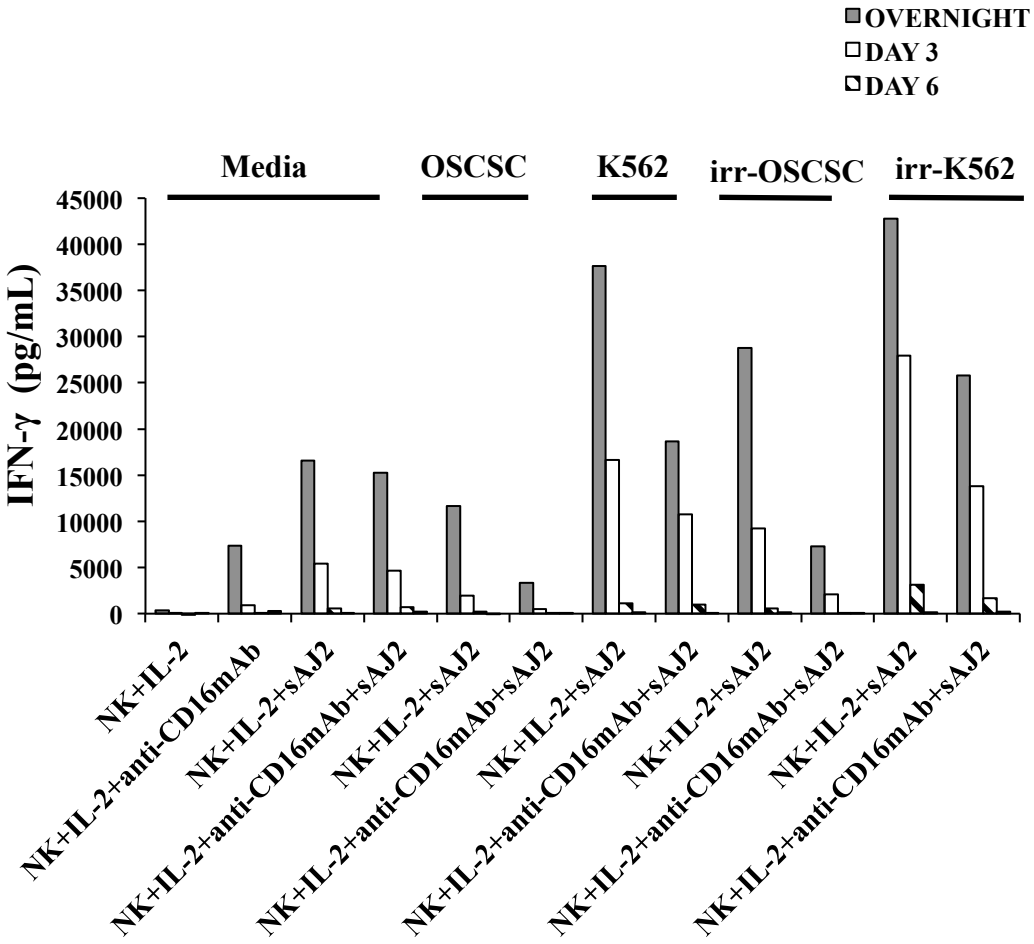


Figure 21. The levels of IFN-γ secreted in the cultures of NK cells with irradiated tumors were higher than those induced in the cultures of NK cells with live tumors

OSCSCs and K562 were irradiated at 40 Gray. Live OSCSCs, live K562, irr-OSCSCs and irr-K562 were seeded at$1x10^6$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to garget ratio of 1:2. These NK cells were cultured with live tumors or irradiated tumors at an effector to target ratio of 1:1 for 8 days. The supernatants from each culture were harvested overnight, day 3, day 6 and day 8. The level of IFN-γ produced by NK cells was measured with a specific Elisa.

Fig. 22

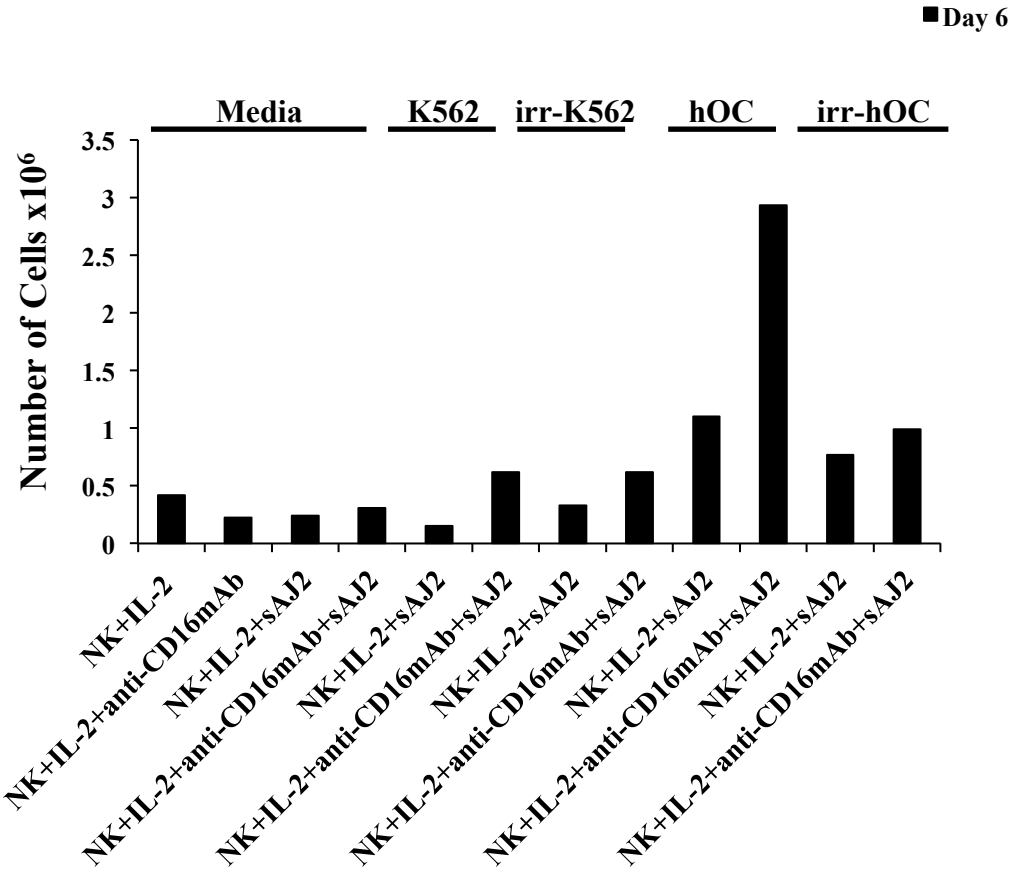


Figure 22. Number of expanded NK cells by live K562, irradiated K562, live osteoclasts and irradiated osteoclasts on day 6

Osteoclasts (hOCs) were prepared as described in Materials and Methods. K562 was irradiated at 40 Gray and hOCs were irradiated at 20 Gray. They were seeded at $3x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. These NK cells were cultured with live K562, irr-K562, live hOCs or irr-hOCs at an effector to target ratio of 1:1 for 7 days. On the 6^{th} day of the experimental period the number of cells was assessed by microscopic evaluation.

Fig. 23

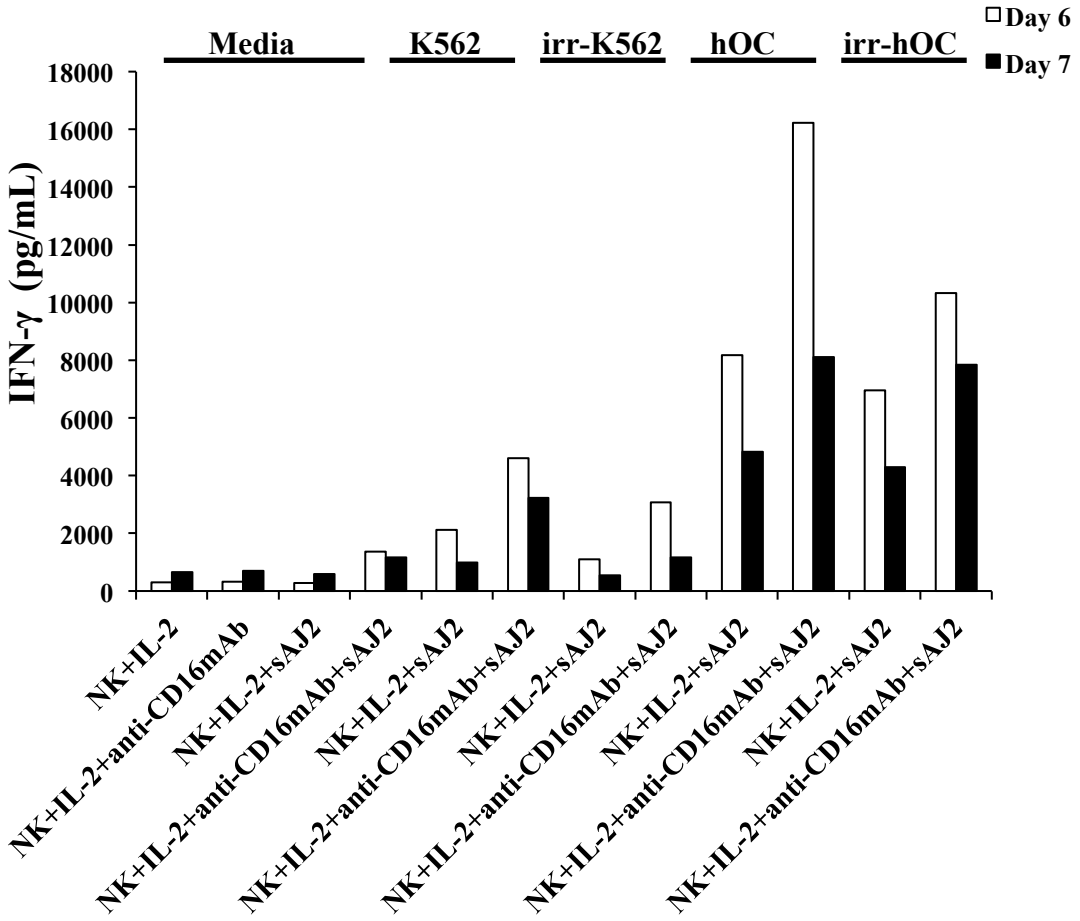
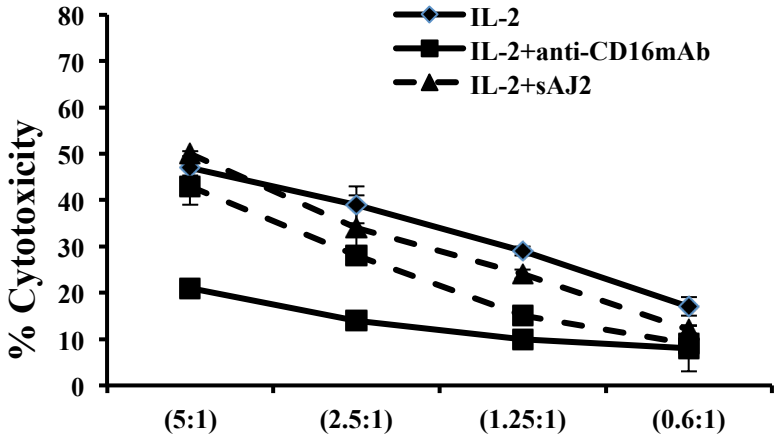


Figure 23. The levels of IFN-γ secreted in the cultures of NK cells with live K562, irradiated K562, live osteoclasts and irradiated osteoclasts

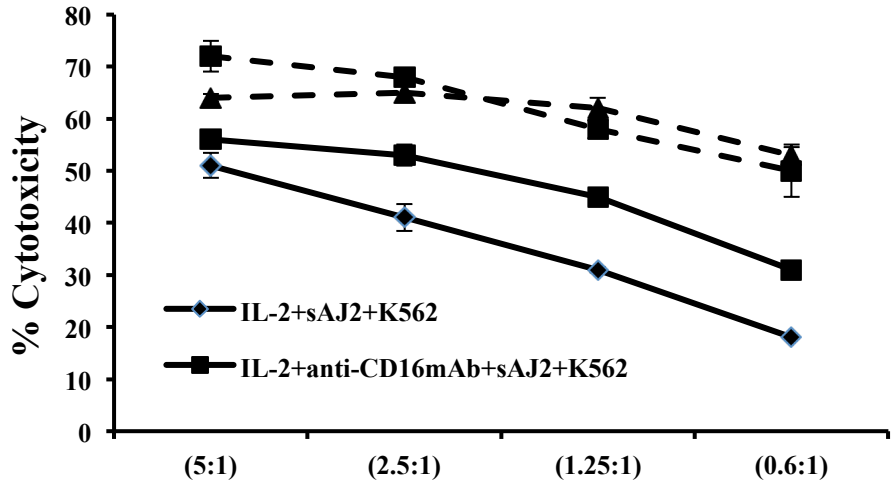
Live K562, irradiated K562, live osteoclasts (hOCs) and irradiated hOCs were cultured with activated NK cells as described in Fig.22**.** The supernatants from each culture were harvested on day 6 and day 7. When harvesting the supernatants, an additional IL-2 (1000 units/mL) was added to each condition. The level of IFN-γ produced by NK cells was measured with a specific Elisa.

Fig. 24

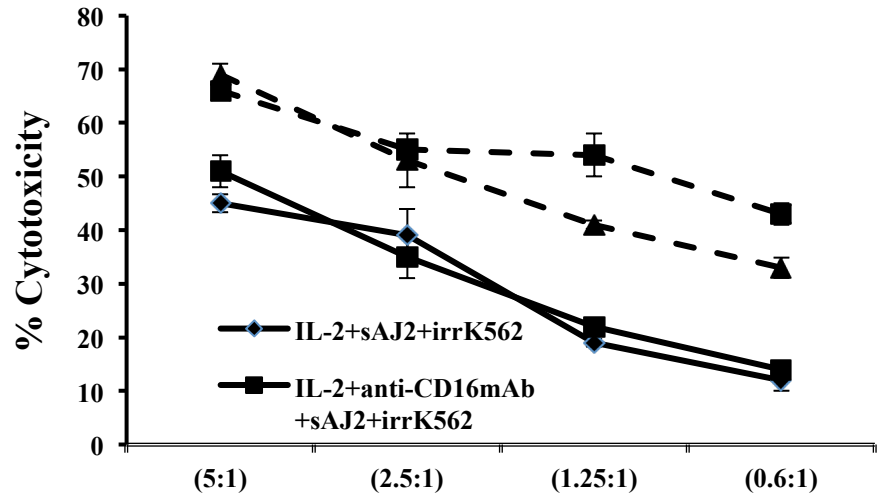
A. Control



B. K562 and hOCs



C. irr-K562 and irr-hOCs



D

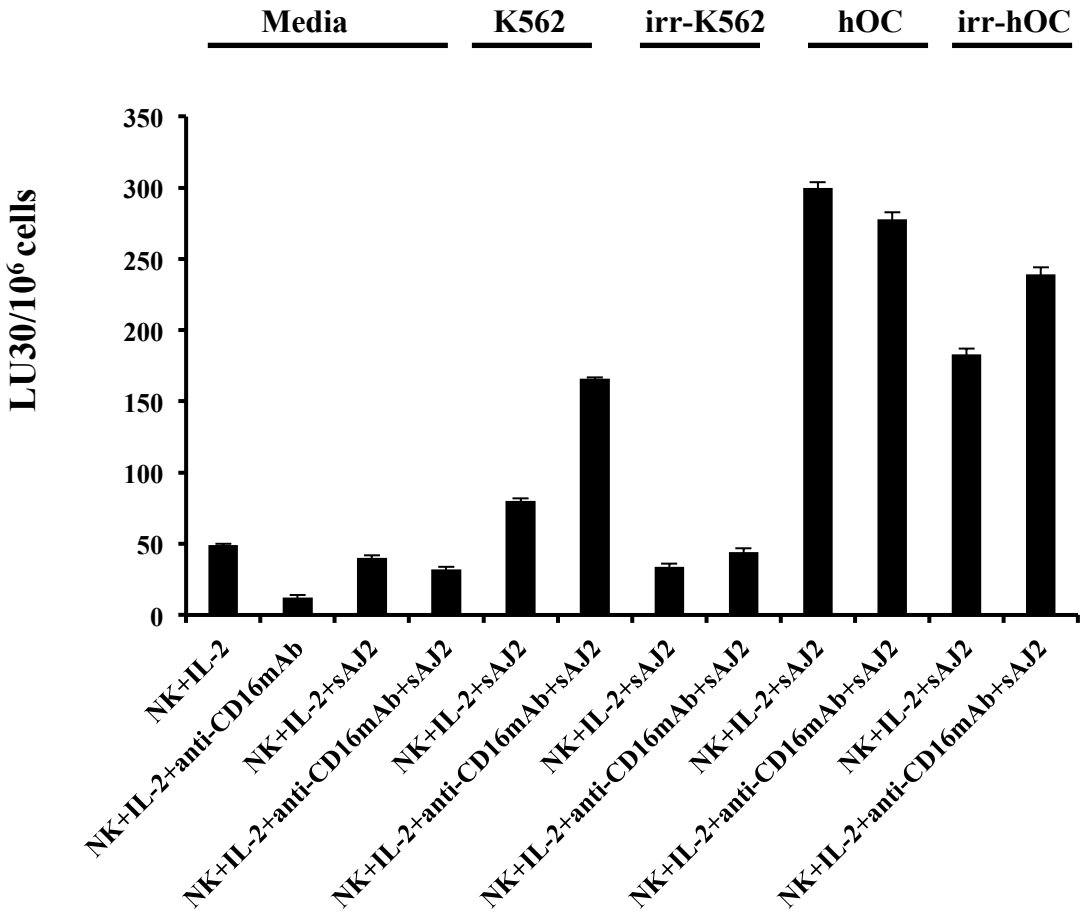


Figure 24. Expanded NK cells by osteoclasts mediated the highest cytotoxicity against OSCSCs compared to expanded NK cells by live or irradiated tumors.

Live osteoclasts (hOCs) irradiated hOCs, live K562 and irradiated K562 were cultured with activated NK cells as described in Fig.22. On day 7, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100.

Fig. 25

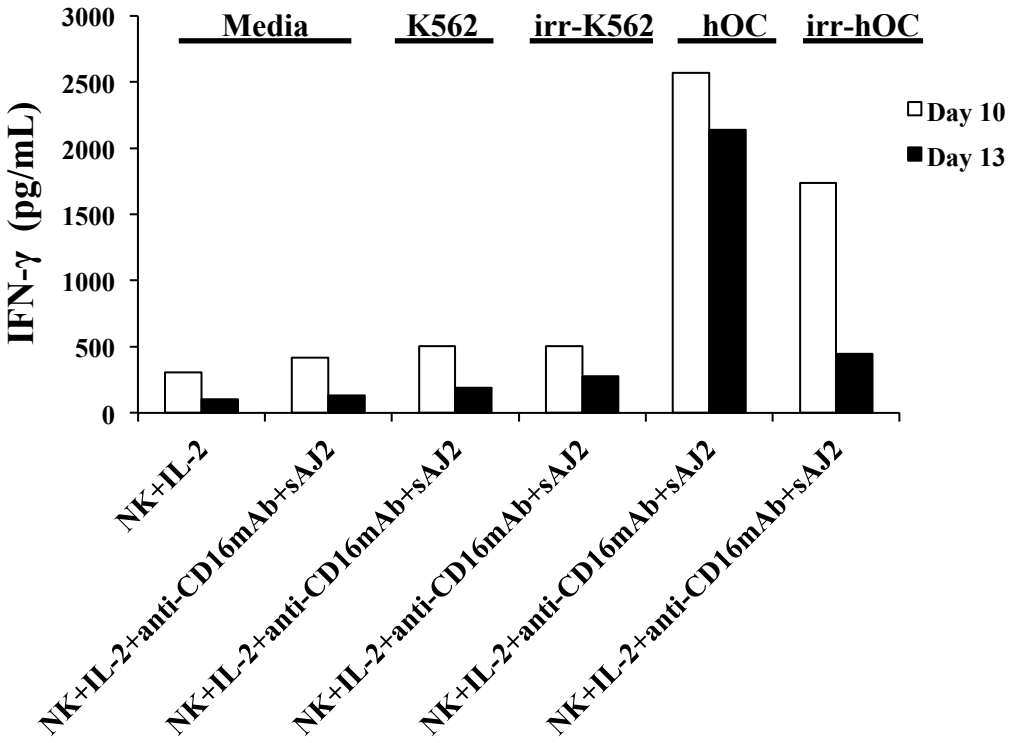


Figure 25. Expanded NK cells by osteoclasts induced the highest secretion of IFN-γ for until day 13.

Live K562, irradiated K562, live osteoclasts (hOCs) and irradiated hOCs were cultured with activated NK cells as described in Fig.22**.** $4x10^5$ of expanded NK cells from each condition were cultured in snap cap tubes in 1mL of media with an additional IL-2 (1000 units/mL). The supernatants from each culture were harvested on day 10 and day 13. When harvesting the supernatants, an additional IL-2 (1000 units/mL) was added to each condition. The level of IFN-γ produced by NK cells was measured with a specific Elisa.

Fig. 26

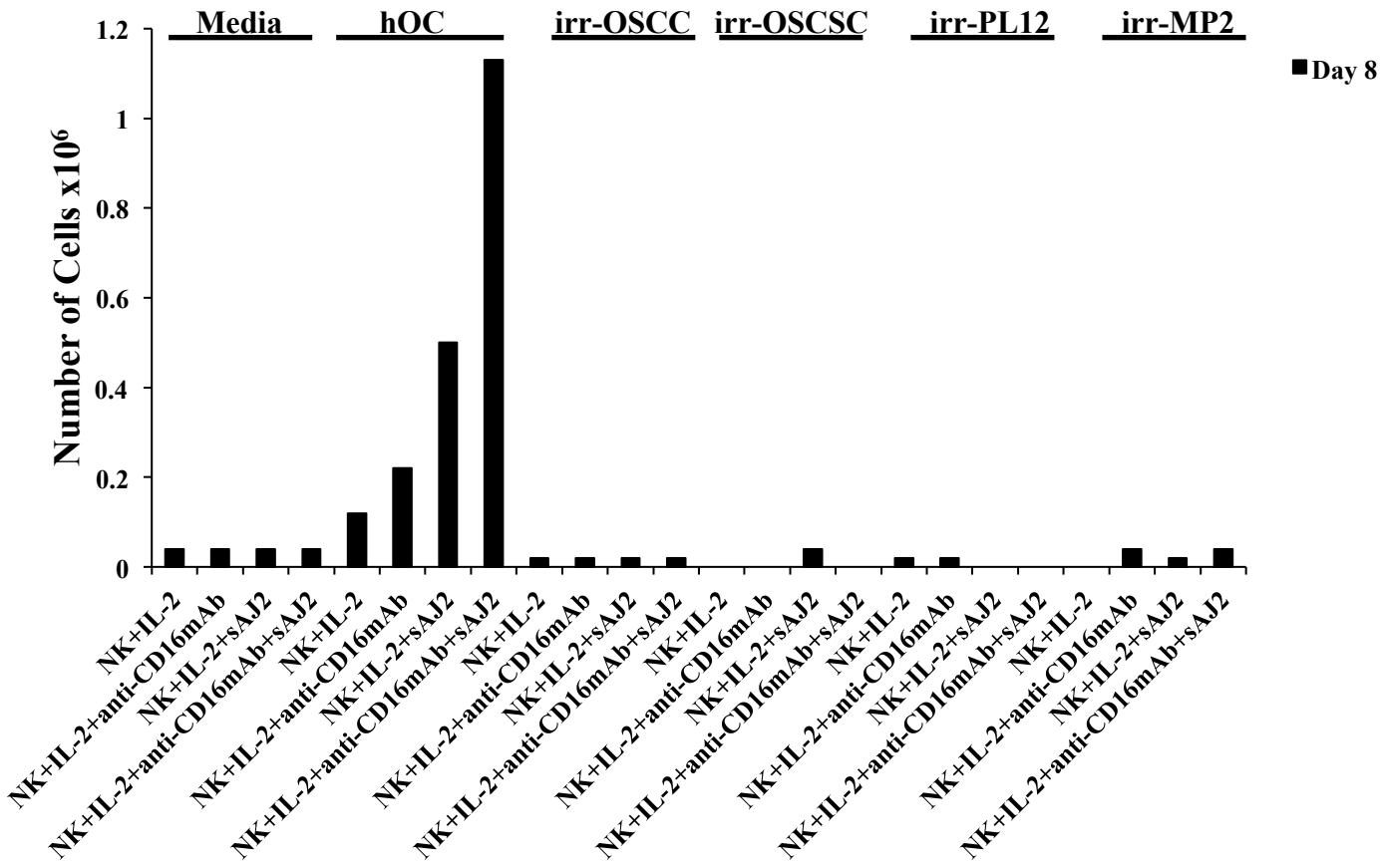


Figure 26. Number of expanded NK cells by osteoclasts, stem-like tumors and differentiated tumors on day 8

Osteoclasts (hOCs) were prepared as described in Materials and Methods. OSCCs, OSCSCs, PL-12 and MP2 were irradiated at 75 Gray. They were seeded at $3x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. These NK cells were cultured with hOCs, irr-OSCCs, irr-OSCSCs, irr-PL12, and irr-MP2 at an effector to target ratio of 5:1 for 8 days. On the 8^{th} day of the experimental period the number of expanded NK cells was assessed by microscopic evaluation.

Fig. 27

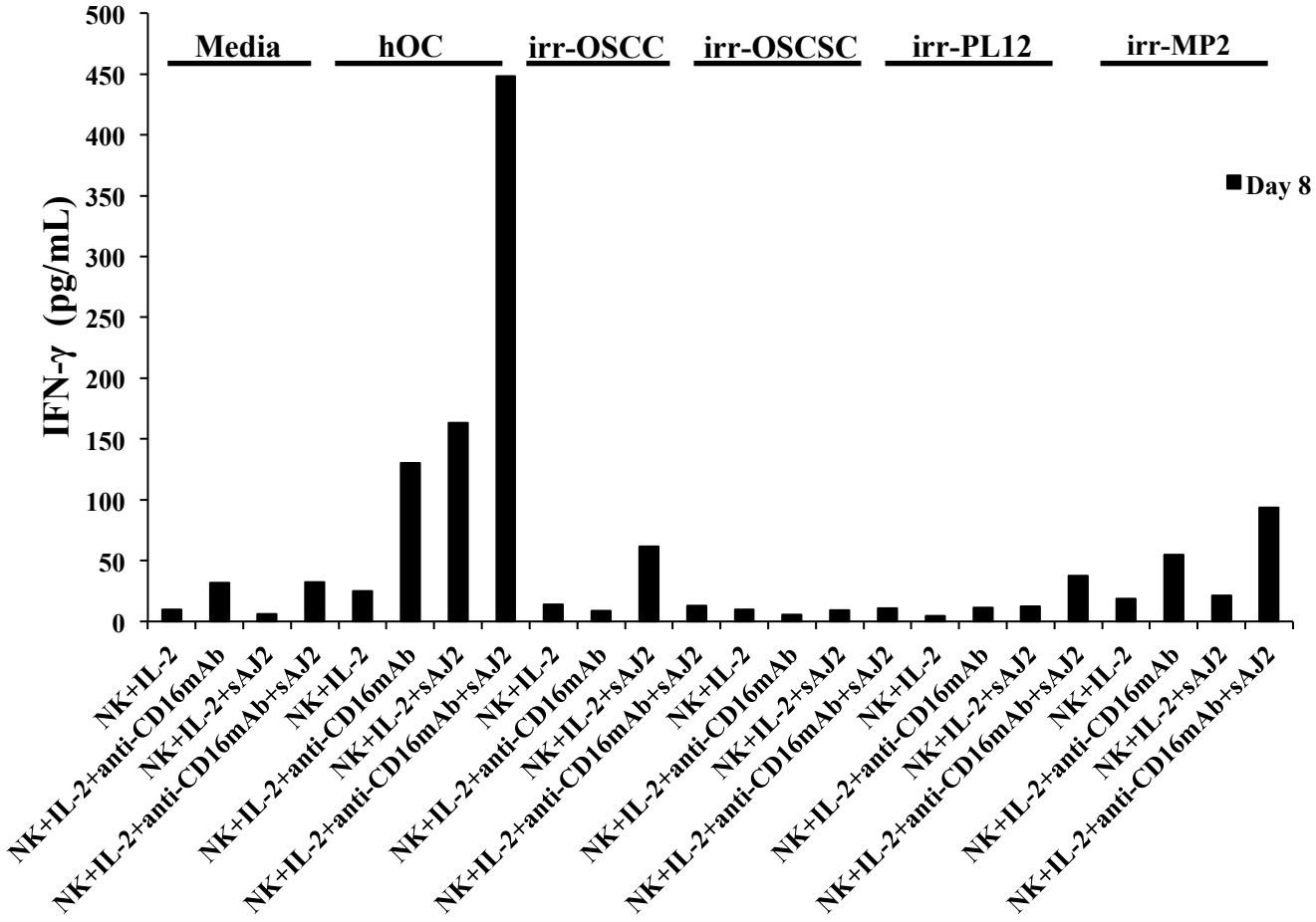


Figure 27. The levels of IFN-γ secreted in the cultures of NK cells with osteoclasts were higher than those induced in the cultures of NK cells with stem-like tumors and differentiated tumors.

Osteoclasts (hOCs), irr-OSCCs, irr-OSCSCs, irr-PL12 and irr-MP2 were cultured with activated NK cells as described in Fig. 26. The supernatants from each culture were harvested on day 6 and day 8. When harvesting the supernatants, an additional IL-2 (1000 units/mL) was added to each condition. The level of IFN-γ produced by NK cells was measured with a specific Elisa.

Irradiated PBMCs do not support the expansion of NK cells whereas osteoclasts support the expansion of NK cells for long period of time

NK cells expanded by osteoclasts were compared with NK cells expanded by irradiated PBMCs whether PBMCs can also support the expansion of NK cells. On day 33 of experimental period, osteoclasts were able to support the expansion of NK cells whereas irradiated PBMCs and monocytes could not (Fig.28) Surface analysis was performed at different time points in order to determine whether cells expanded by osteoclasts for 33 days were still activated NK cells. Surface expression profiles of expanded NK cells by osteoclasts showed that about 88% were $CD3^-/CD16^+CD56^+$ for 33 days (Fig.29).

NK cells cultured with osteoclasts, monocytes and irradiated PBMCs were counted and equal numbers of expanded NK cells from each subset were used in cytotoxicity assay against OSCSCs, which is a very sensitive NK cell target. IL-2+anti-CD16mAb+sAJ2 bacteria treated NK cells expanded by osteoclasts with an additional IL-2 treatment had the highest cytotoxicity whereas IL-2+anti-CD16mAb+sAJ2 treated NK cells expanded by monocytes or irr-PBMC had low cytotoxicity ($p<0.05$) (Fig. 30). Then, the levels of IFN-γ secretion by IL-2+ anti-CD16mAb+sAJ2 bacteria treated NK cells cultured with osteoclasts, monocytes or irr-PBMCs from day 0 to day 15 of cultures were determined. As shown in Fig.31, the levels of IFN-γ secreted in the cultures of NK cells with osteoclasts rose significantly and remained high until day 15, and the secreted levels were much higher than those induced in the cultures of NK cells with monocytes and irr-PBMCs.

Fig. 28

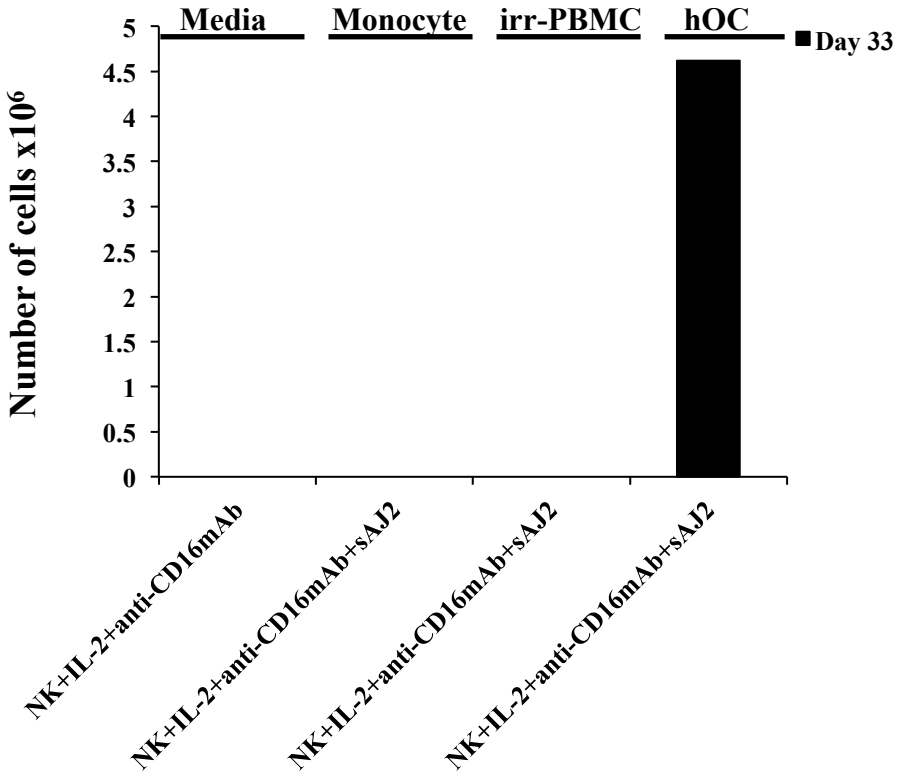


Figure 28. Expanded NK cells by osteoclasts, monocytes, and irradiated PBMCs on day 33

Osteoclasts (hOCs) were prepared as described in Materials and Methods. PBMCs were irradiated at 75 Gray. hOCs, monocytes and irr-PBMCs were seeded at $5x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. These NK cells were cultured with hOCs, monocytes and irr-PBMCs at an effector to target ratio of 2:1 for 33 days. Number of expanded cells was assed by microscopic evaluation.

Fig. 29

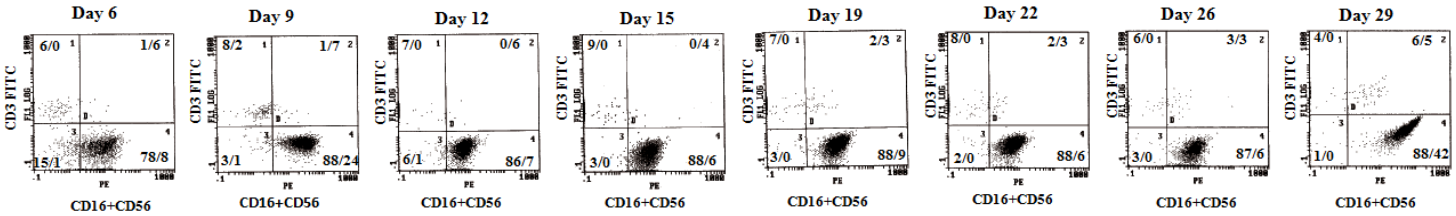


Figure 29. Surface expression profiles of NK cells expanded by osteoclasts at different time points

Osteoclasts (hOCs) were cultured with activated NK cells as described in Fig.28. Surface expression profiles of CD3/CD16^{+}CD56^{+} were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and mean channel fluorescence intensities for each histogram.

Fig. 30

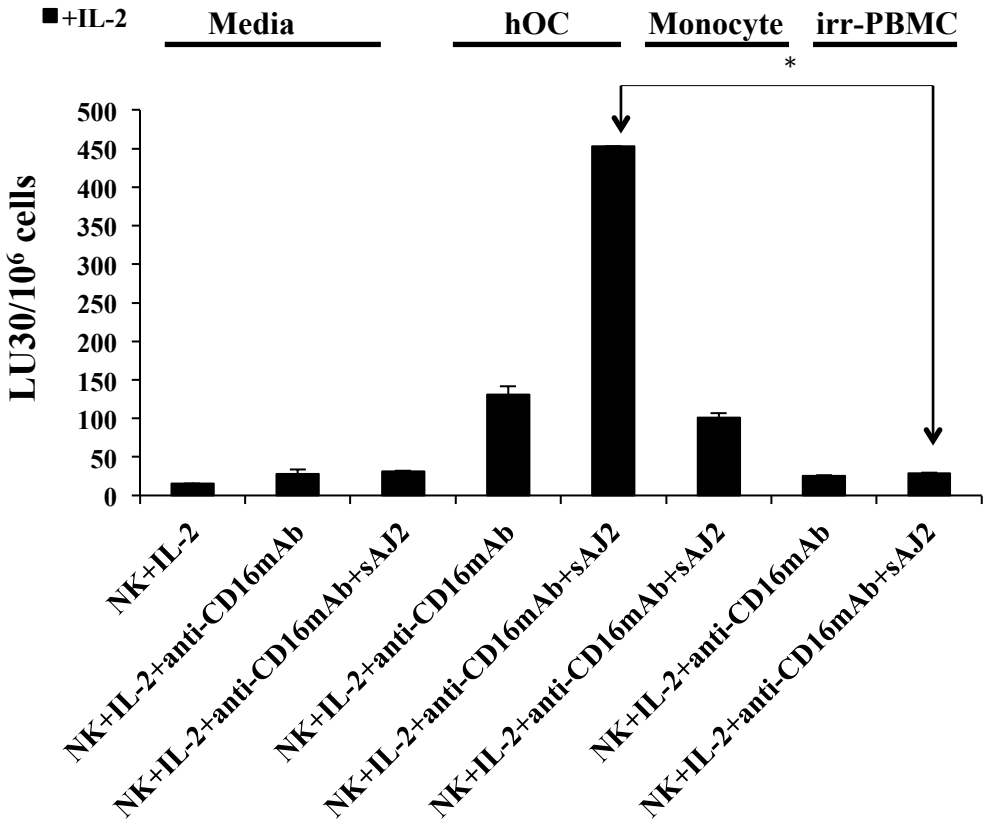


Figure 30. Expanded NK cells by osteoclasts mediated the highest cytotoxicity against OSCSCs compared to expanded NK cells by irradiated PBMCs

Osteoclasts (hOCs), monocytes and irr-PBMCs were cultured with activated NK cells as described in Fig. 28. On day 3 and 6, supernatants were collected and the culture medium was refreshed with an additional IL-2 (1000 units/mL) treatment. On day 9, equal numbers of NK cells from each expanded subset were used in a standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100. *The difference between NK+IL-2+anti-CD16mAb +sAJ2+hOC and NK+IL-2+anti-CD16mAb+sAJ2+irr-PBMC is significant at $p<0.05$.

Fig. 31

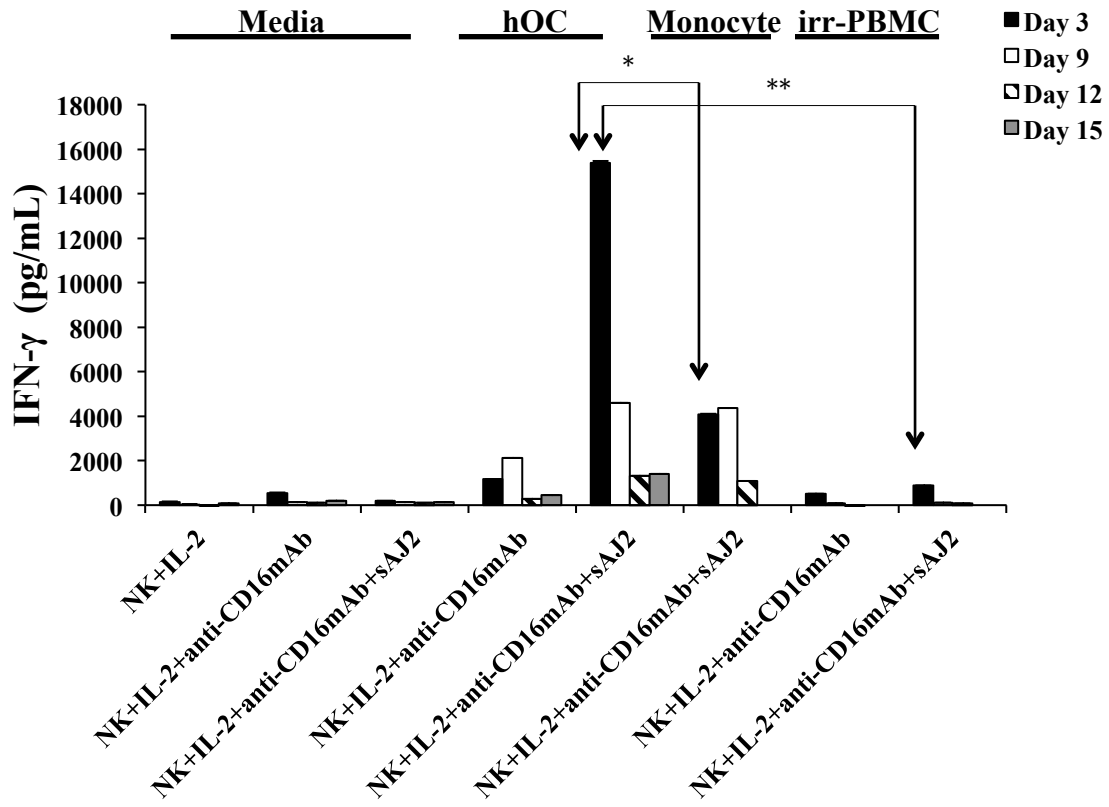


Figure 31. The levels of IFN-γ secreted in the cultures of NK cells with osteoclasts were higher than those induced in the cultures of NK cells either with monocytes or irradiated PBMCs.

Co-culture was done as described in Fig.28. The supernatants from each culture were harvested on day 3, 6, 9, and 15. When collecting the supernatants, the culture medium was refreshed and an additional IL-2 (1000 units/mL) was added to each condition. The level of IFN-γ produced by NK cells was measured with a specific Elisa. *The difference between NK+IL-2+anti-CD16mAb+sAJ2+hOC and NK+IL-2+anti-CD16mAb+sAJ2+monocyte (or **irr-PBMC) is significant at $p<0.05$.

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

Hu-BLT mice were implanted surgically with MP2 tumors and after one week of tumor growth, a single injection of 1.5 $X10^6$ purified NK cells with potent cytotoxic and cytokine secretion capabilities were performed. NK cells were purified from healthy donors and expanded in the presence of the treatment with IL-2+anti-CD16mAb and sAJ2 bacteria and osteoclasts as described in the materials and methods section and (manuscript in prep). In order to determine whether the cells that were expanded by osteoclasts are activated NK cells with potent cytotoxic and cytokine secretion capabilities, the levels of CD16 and NKp44 surface expression were determined on day 8. As shown in Fig. 32, the expanded NK cells had up-regulation of CD16 and NKp44 surface expressions.

As shown in Fig. 33, mice implanted with MP2 tumors and injected with expanded NK cells grew significantly smaller tumor when compared to those implanted with MP2 tumors in the absence of expanded NK injection. The tumor was localized and no involvement of other organs could be seen in mice injected with MP2 tumors and received activated expanded NK cells (Fig.33).

Fig. 32

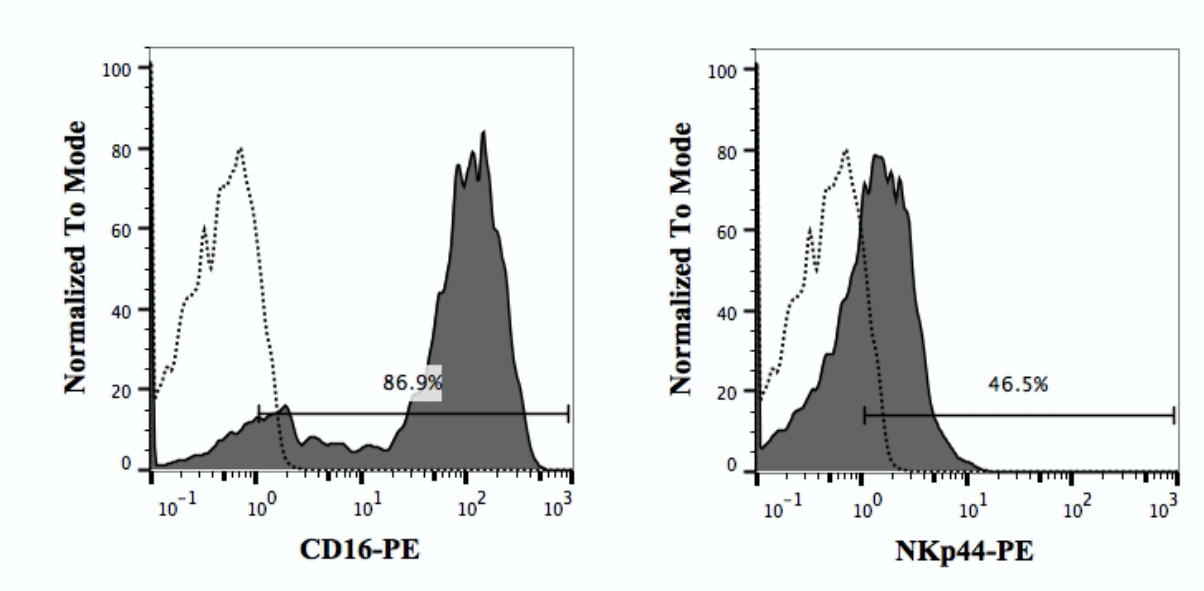
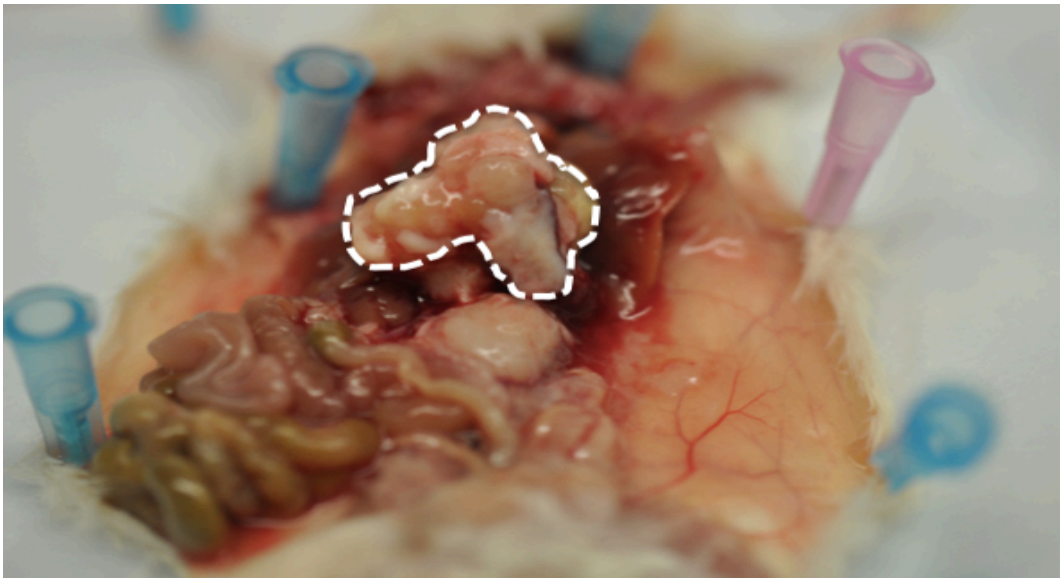


Figure 32. Surface expression profiles of expanded NK cells by osteoclasts

Osteoclasts (hOCs) were prepared as described in Materials and Methods and seeded at $3x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2. These NK cells were cultured with autologous hOCs at an effector to target ratio of 1:1 for 6 days. On day 3 and 6, the culture medium was refreshed with an additional IL-2 (1000 units/mL) treatment. On day 8, surface expression of CD16 and NKp44 on expanded NK cells were assessed with flow cytometric analysis after staining with the respective PE-conjugated antibodies. Isotype control antibodies were used as control. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram.

Fig. 33

A. MP2 alone



B. MP2 + Expanded NKs

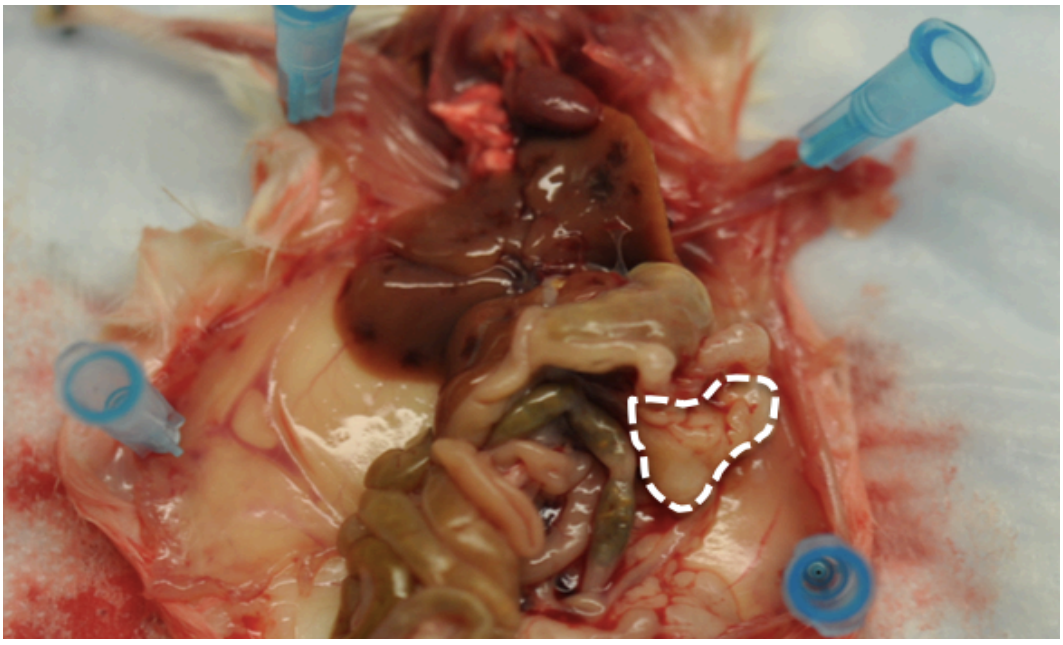


Figure 33. Size of tumor growth in hu-BLT mice after MP2 tumor implantation with or without expanded NK injection

Mice were implanted with MP2 tumors alone or injected with expanded NK cells as described in Materials and Methods. The size of tumor growth in both subsets was observed.

DISCUSSION

Many studies have shown that modified K562 can expand NK cells with enhanced cytotoxicity so in this study, we investigated whether K562 can also expand NK cells to the same extent as osteoclasts under the same optimal condition. Even though K562 expanded NK cells in some extent, they were not able to expand NK cells to the same extent as osteoclasts. NK cells expanded by K562 exhibited low cytotoxic activity and mediated low level of IFN-γ secretion. It was reported that K562, which was modified to express membrane-bound form of IL-15 and 41BB ligand, expanded NK cells with potent cytotoxic activity [52]. This result demonstrated that K562 alone is not sufficient to expand NK cells and co-stimulatory of IL-15, which is known o be one of NK activating cytokine, is needed to fully activate NK cells. Osteoclast expanded NK cells retained $CD16^+/CD56^+$ on the surface and had relatively higher surface expression of $CD16^+/CD56^+$ compared to NK cells expanded by K562. We have previously shown that down-modulation of CD16 receptors causes a great loss of cytotoxicity. This may be the reason why NK cells expanded by K562 lost their cytotoxic activity due to down-modulation of CD16 receptors after their interaction whereas osteoclast expanded NK cells are highly sensitive to NK mediated cytotoxicity because they retain CD16 receptors.

The same phenomenon was observed when NK cells were co-cultured with other tumors such as OSCCs, OSCSCs, PL12 and MP2 in efforts to expand NK cells. Considering the fact that osteoclasts are more differentiated tumors, we also investigated whether more differentiated tumors can support the expansion of NK cells better than stem-like tumors. Whether tumors were stem-like cells or more differentiated cells, the differentiation status

was not a main factor that contributes to expansion of NK cells. NK cells have critical roles in host immunity against cancer and have powerful cytotoxic activity, but their activity may be eluded by the tumor microenvironment. We believe that signals received from these tumors alter the phenotype of NK cells and cause NK cells to lose cytotoxicity. Furthermore, these tumor cells can interfere with NK cell activation pathways or the complex receptor array that regulate NK cell activation and antitumor activity.

Irradiated osteoclasts expanded more NK cells than K562. Moreover, NK cells expanded by irradiated osteoclasts mediated higher cytotoxicity and produced more IFN-γ secretion than NK cells expanded by K562. However, irradiated osteoclasts were less effective than non-irradiated osteoclasts in terms of their functionality and capacity to expand NK cells. Moreover, osteoclasts-expanded NK cells retained $CD16^{+}/CD56^{+}$receptors and proliferate for more than a month with IL-2 supplement, which demonstrated that they remain as activated NK cells for long term.

Irradiated osteoclasts do not secret much of cytokine when compared to non-irradiated osteoclasts. IL-12, IL-15, which are known to be NK activating cytokines, and IL-18 are produced by NK cells upon the interaction of NK cells with osteoclasts. Therefore, we believe that a combination of these cytokines are required to fully activate NK cells but when osteoclasts are irradiated, receptors between NK cells and osteoclasts are still remained so that these receptors can be recognized by NK cells.

As NK cells lyse target cells without prior sensitization and provide immune regulatory cytokines, infusion of NK cells have been proposed as a means to target various

cancers. Single injection of expanded NK cells with potent cytotoxic and cytokine secretion capabilities to the mice implanted with MP2 tumors significantly grew smaller tumor when compared to those implanted with MP2 tumors in the absence of NK injection. We have shown in previous studies that osteoclast expanded NK cells are significantly more susceptible to NK mediated cytotoxicity than primary NK cells. Also, expanded NK cells secrete significantly more IFN-γ than primary NK cells. As tumors acquire resistance to lysis by cellular effectors, high susceptibility to NK cell lysis is crucial to target tumors. We have shown that cytokines secreted by NK cells make stem-like cells to be more differentiated. Since cancer stem cells were found to be 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 mediated killing, differentiated tumors by cytokines secreted by NK cells can be eliminated by chemotherapeutic drugs. Since osteoclasts expanded NK cells are highly sensitive to NK cell mediated cytotoxicity, they can target cancer stem cells selectively while some cells can be differentiated by cytokines so that they can be either eliminated by chemotherapeutic drugs or surgically. In this regard, we believe that osteoclasts expanded NK cells with potent cytotoxic activity and augmented cytokine secretion have an important role in future therapies against certain cancers in elimination of cancer stem cells as well as differentiated tumors.

CONCLUSION

This study demonstrated that tumors are not potent immune effectors capable of expanding NK cells and modulating the function of NK cells. Irradiated osteoclasts expand more NK cells than K562 and have relatively high cytotoxicity and augmented secretion of IFN-γ compared to K562−expanded NK cells but the capacity to expand NK cells and the functionality of expanded NK cells to target tumor is the greatest with osteoclast. Also, osteoclasts-expanded NK cells are good candidates for long-term therapy since they remain as activating NK cells by retaining $CD16^{+}/CD56^{+}$ receptors and IFN-γ secretion high for long term. Osteoclasts are more differentiated than monocytes or dendritic cells but the differentiation status of tumors did not affect the expansion of NK cells. NK cells produce IL-12, IL-15 and IL-18 when they interact with osteoclasts and a combination of these cytokines are required to fully activate NK cells with potent cytotoxic activity and augmented cytokine secretion. Dual functions of NK cells, high cytotoxicity and augmented cytokine secretion, are crucial in elimination of cancer stem cells and differentiated tumors for cancer immunotherapy.

Chapter 3

Specific Aim 3: To investigate the role of IL-10 as a regulator of IFN-gamma secretion in NK cell interaction with monocytes or osteoclasts

Introduction

Pro-inflammatory cytokines are activated in order to initiate host defense against microbial invasion. Anti-inflammatory mechanisms maintain or restore tissue homeostasis by regulating the production of pro-inflammatory molecules. Interleukin (IL)-10 is a potent anti-inflammatory cytokine, which has a crucial role in preventing inflammatory and autoimmune disease. B cells, T helper cells, natural killer (NK) cells, monocytes, dendritic cells and macrophages primarily produce IL-10. IL-10 modulates inflammatory response by suppressing the production of pro-inflammatory cytokines such as IL-12 and IL-18 from macrophages [55]. IL-12 is known to be a main activator of NK cells and increase their cytotoxicity capacity. IL-18 is another important pro-inflammatory cytokine produced by macrophages and shown to be suppressed by IL-10 [56]. Inflammatory response to microbial challenge can be enhanced by deficiency or aberrant expression of IL-10. However, this can cause development of inflammatory bowel disease and a number of autoimmune diseases [57-59].

NK cells produce the pro-inflammatory cytokine IFN-γ in particular during infection [60]. Scott et al [55] showed that IL-10 has an inhibitory effect on NK cell activation by blocking IL-10 in mice with a neutralizing antibody. Also, it was shown that IFN-γ

dependent mechanisms of host resistance could be evaded by pathogens following IL10 stimulation [61-64]. IL-10 selectively suppressed NK cell IFN-γ production [65] and inhibits the differentiation of dendritic cells [66]. We have previously shown that the interaction of monocytes with human mesenchymal stem cells (hMSC) significantly protected the hMSC from NK cell mediated cytotoxicity while substantially increasing the production of IFN-γ [67]. Also, we reported that the blocking of IL-10 on NK cells triggered by sAJ2 bacteria and monocytes significantly augmented the production of IFN-γ and increased resistance against NK cell mediated cytotoxicity (manuscript in prep). IL-10 has a capacity to down-modulate MHC class II expression and to inhibit the production of pro-inflammatory cytokines by monocytes [68]. Therefore, IL-10 has important regulatory effects on immunological and inflammatory responses.

The role of IL-10 in regulating the production IFN-γ during viral infection has well established by many studies. However, the role of IL-10 as a regulator of IFN-γ secretion in NK cell interaction with osteoclasts has not been studied. The cause and effect relationship between IL-10 and IFN-γ secretion in the interaction of sAJ2 triggered NK cells with either monocytes or osteoclasts will be also investigated in this study. We hypothesize that blocking IL-10 on IL-2+anti-CD16+sAJ2 bacteria treated NK cells cultured with osteoclasts significantly up-regulates the production of IFN-γ and increases resistance against NK cell mediated cytotoxicity.

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% gentimicin 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. Human M-CSF (Biolegend, CA) and soluble RANKL (PeproTech, NJ) were dissolved in alpha-MEM and stored at -80°C.

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% purify based on flow cytometric analysis of CD14 antibody stained monocyte cells.

Generation of Osteoclasts

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).

^{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 ($1x10^5$ 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 ($5x10^5$ OSCSCs/well) were labeled with 50μCi ^{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;

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

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

Enzyme-Linked Immunosorbent Assays (ELISAs)

Human IFN-γ and IL-10 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 200rmp 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 $H_2SO_4$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

IL-10 is a regulator of IFN-gamma secretion in NK cell interaction with monocytes or osteoclasts

NK cells were co-cultured either with monocytes or osteoclasts in the presence or absence of anti-IL10 in order to investigate the role of IL-10 as a regulator of IFN-gamma secretion. sAJ2 bacteria treated NK cells cultured with monocytes in the presence of anti-IL10mAb (5ug/mL) induced more IFN-γ secretion than those cultured with monocytes in the absence of anti-IL10mAb ($p<0.05$) (Fig. 34). However, when anti-IL10mAb was added, it induced IFN-γ secretion and suppressed IL-10 secretion as seen in Fig. 35. sAJ2 treated NK cells cultured with monocyte induced significant amount of IFN-γ in comparison to sAJ2 treated NK cells cultured with monocytes in the presence of anti-IL10mAb ($p<0.05$) (Fig.35).

The role of IL-10 was also investigated in the cultures of NK cells with osteoclasts. NK cells cultured with osteoclasts in the presence or absence of anti-IL10mAb also showed the same trend as monocytes but NK cells cultured with osteoclasts induced more IFN-γ secretion than those cultured with monocytes (Fig. 36). IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with monocytes in the presence of anti-IL10mAb secreted more IFN-γ than those without anti-IL10-mAb ($p<0.05$) (Fig.36). The same trend was seen in cultures of osteoclasts. IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts in the presence of anti-IL-10mAb secreted more IFN-γ than those cultured without anti-IL10mAb ($p<0.05$) (Fig.36). However, IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts in the presence of anti-IL10mAb secreted 1.5 fold more of IFN-γ than those

cultured with monocytes ($p<0.05$) (Fig.36). IL-2+anti-CD16mAb+sAJ2 treated NK cells cultured with osteoclasts in the presence of anti-IL10mAb had reduced cytotoxic function compared to those cultured without anti-IL10mAb ($p<0.05$) (Fig.37).

Fig. 34

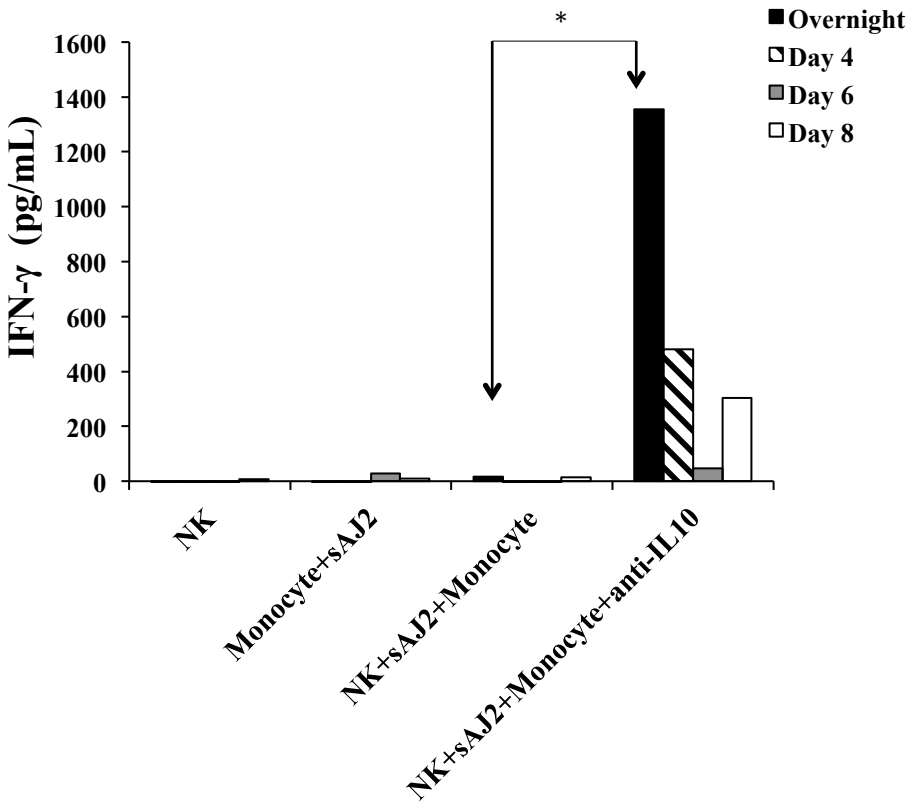


Figure 34. More IFN-γ secreted in the cultures of sAJ2 bacteria treated NK cells with monocytes in the presence of anti-IL10mAb

Monocytes were prepared as described in Materials and Methods and seeded at $3x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were treated with sAJ2 bacteria at an effector to target ratio of 1:2 and anti-IL10mAb (5ug/mL). Then, they were cultured with or without monocytes at an effector to target ratio of 1:1. The supernatants from each culture were harvested on overnight, day 4, day 6 and day 8. When collecting the supernatants, the culture medium was refreshed. The level of IFN-γ produced by NK cells was measured with a specific Elisa. *The difference between NK+ sAJ2+monocyte and NK+sAJ2+monocyte+anti-IL10 is significant at $p<0.05$.

Fig. 35

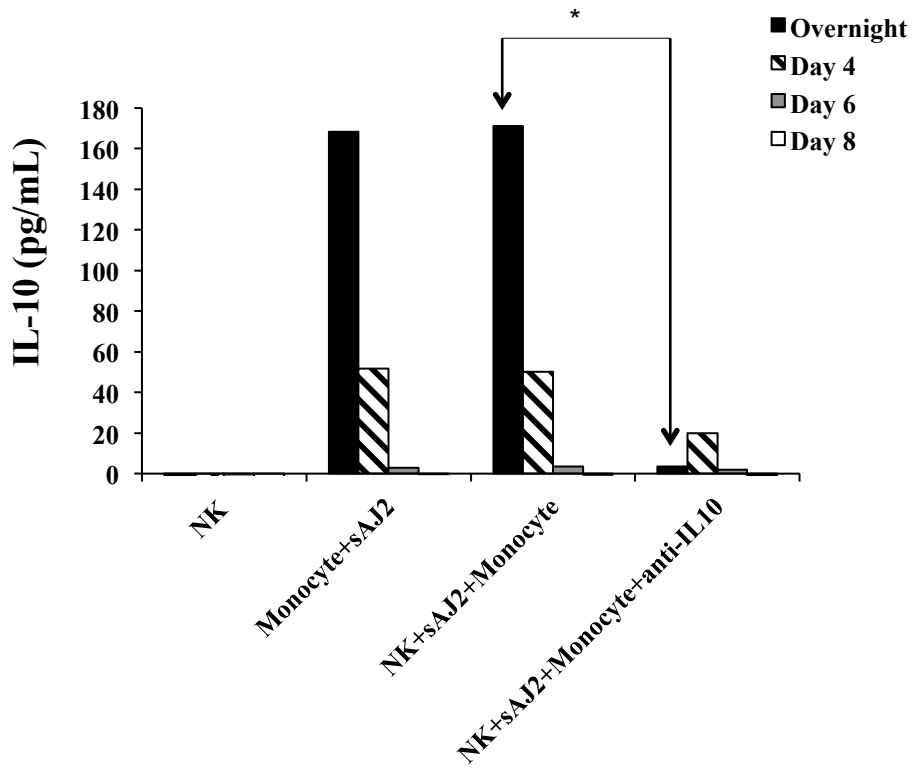


Figure 35. High level of IL-10 secreted in the cultures of sAJ2bacteria treated NK cells with monocytes in the absence of anti-IL10mAb

Monocytes were prepared as described in Materials and Methods and co-culture was performed as described in Fig. 34. The supernatants from each culture were harvested on overnight, day 4, day 6 and day 8. When collecting the supernatants, the culture medium was refreshed. The level of IL-10 produced by NK cells was measured with a specific Elisa. *The difference between NK+sAJ2+monocyte and NK+sAJ2+monocyte+anti-IL10 is significant at $p<0.05$.

Fig. 36

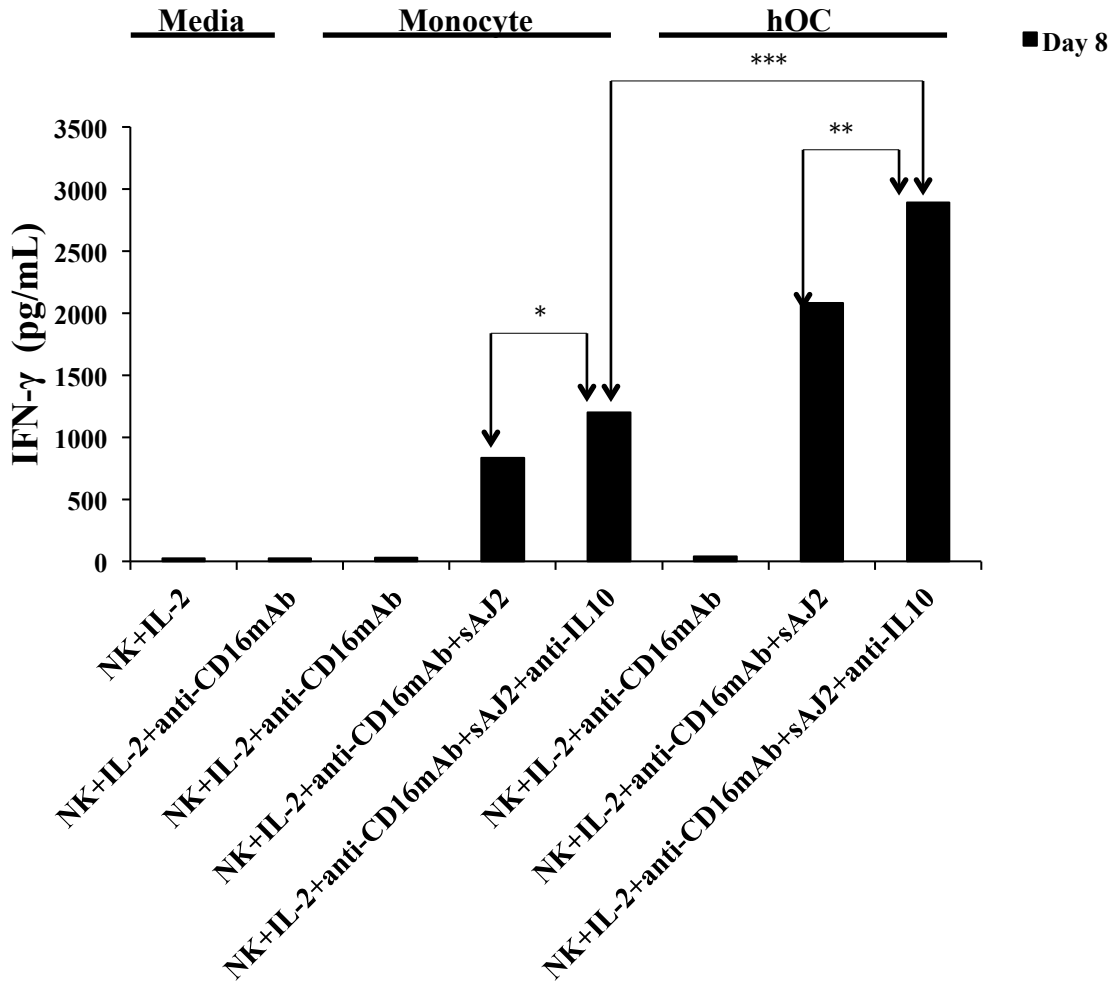


Figure 36. More IFN-γ secreted in the cultures of IL-2+anti-CD16mb+sAJ2 bacteria treated NK cells with osteoclasts in the presence of anti-IL10mAb than those with monocytes

Monocytes and osteoclasts (hOCs) were prepared as described in Materials and Methods and seeded at $6x10^5$ cells/well in 12 well plates and incubated overnight. Purified NK cells were pre-treated with IL-2 (1000 units/mL) and a combination of IL-2 (1000 units/mL) and anti-CD16mAb (3ug/mL) for 18 hours and then treated with sAJ2 bacteria at an effector to target ratio of 1:2 and anti-IL10mAb (5ug/mL). Then, they were cultured either with monocytes or hOCs at an effector to target ratio of 1:1. The supernatants from each culture were harvested on day 8. When collecting the supernatants, the culture medium was refreshed. The level of IFN-γ produced by NK cells was measured with a specific Elisa. *The difference between NK+IL-2 +anti-CD16mAb+sAJ2+monocyte (or **hOC) and NK+IL-2+anti-CD16mAb+sAJ2+ monocyte (or **hOC)+anti-IL10mAb is significant at $p<0.05$. ***The difference between NK +IL-2+anti-CD16mAb+sAJ2+monocyte+anti-IL10mAb and NK+IL-2+anti-CD16mAb+ sAJ2+hOC+anti-IL10mAb is significant at $p<0.05$.

Fig. 37

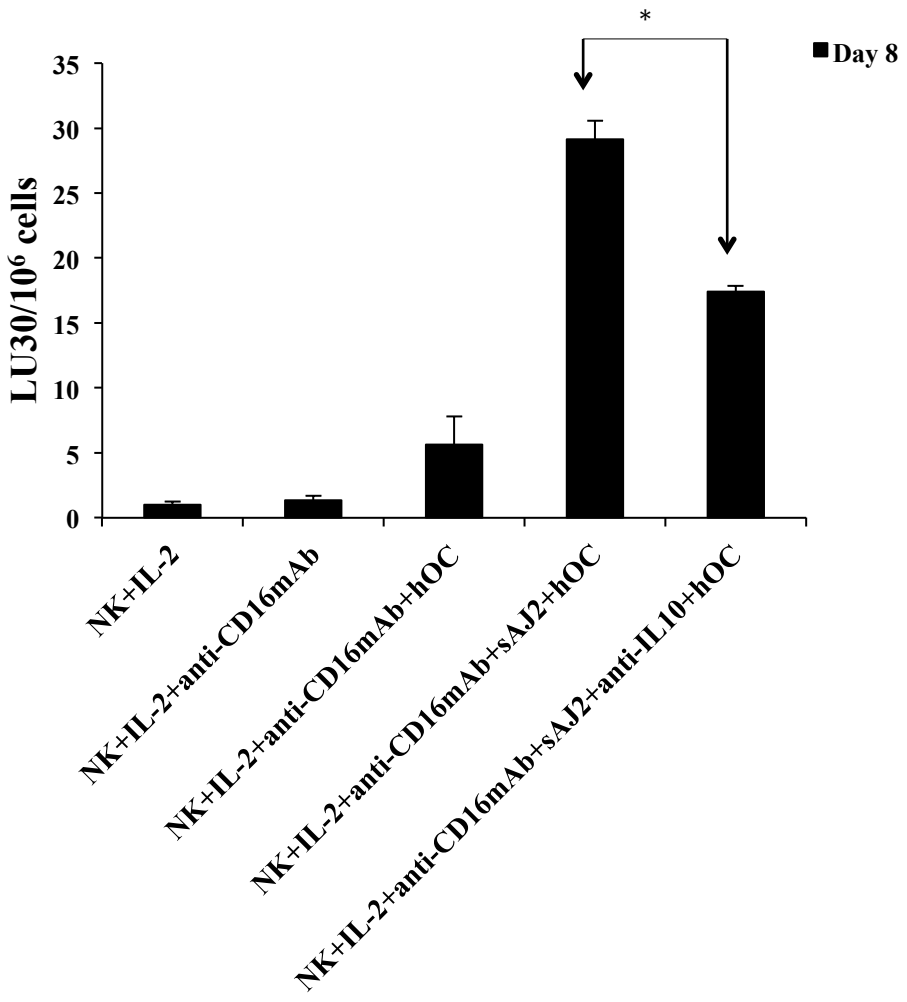


Figure 37. Osteoclasts inhibited NK cell cytotoxic function in the presence of anti-IL10mAb

NK cells cultured with osteoclasts (hOCs) in the presence or absence of anti-IL10mAb (5ug/mL) were used in a standard 4-hour ^{51}Cr release against OSCSCs. The lytic units $30/10^6$ cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs x 100. *The difference between NK+IL-2+anti-CD16mAb +sAJ2+hOC and NK+IL-2+anti-CD16mAb+sAJ2+ hOC+anti-IL10mAb is significant at $p<0.05$.

DISCUSSION

In this study we demonstrated that human IFN-γ and IL-10 are produced in relatively large quantities by monocytes following activation. Low level of IL-10 was detected after activation of NK cells with monocytes and sAJ2 bacteria in the presence of anti-IL10mAb. However, the addition of anti-IL-10mAb blocked the production of IL-10 on the NK cells while inducing significant amount of IFN-γ. IL-2+anti-CD16mAb+sAJ2 bacteria treated NK cells cultured with osteoclasts in the presence of anti-IL10mAb caused more induction of IFN-γ than those cultured under the same condition with monocytes. We have previously shown that monocytes inhibited the function of NK cell mediated cytotoxicity. When osteoclasts were cultured with IL-2+anti-CD16mAb+sAJ2 bacteria triggered NK cells in the presence of anti-IL10mAb, the cytotoxic activity against OSCSC was greatly reduced. The more induction of IFN-γ, the more resistance of the stem cells to NK cell mediated cytotoxicity.

IL-10 is an anti-inflammatory cytokine with a crucial role in preventing inflammatory and autoimmune disease. Sellon R K et al [57] reported that IL-10 deficient mice caused more inflammation. Since IL-10 has an inhibitory effect on the synthesis of IFN-γ by human NK cells [69, 70], it blocks differentiation of stem cells by inhibiting the secretion of IFN-γ on NK cells. Based on our accumulated data, IL-10 plays a significant role in regulating tumor progression and differentiation of stem cells.

CONCLUSION

This study demonstrated that IL-10 is a regulator of IFN-γ secretion in NK cell interaction with either monocytes or osteoclasts. The role of IL-10 in regulating the production of IFN-γ following the interaction of NK cells with monocytes has been well established by many studies. It was shown that monocytes are great inducers of IL-10. The significance of this study is that the role of IL-10 as a regulator of IFN-γ secretion in NK cells interaction with osteoclasts has been shown. IL-2+anti-CD16mAb+sAJ2 bacteria treated NK cell interaction with osteoclasts in the presence of anti-IL-10mAb produced significant amount of IFN-γ but they were resistant to NK cell mediated cytotoxicity. Taken together, IL-10 is a strong regulator of IFN-γ in NK cell interaction with osteoclasts. Furthermore, IL-10 is important in balancing or slowing differentiation by blocking IFN-γ, which will suppress immune and inflammatory responses.

FINAL CONCLUSION

Tumor-promoting inflammation and escape from immune surveillance are major factors responsible for the establishment and progression of cancer. Increased cancer incidence is associated with sustained immunosuppression and immune deficiency. The tumor microenvironment challenges to the efficacy of NK cells because it hinders both infiltration and activation of NK cells at tumor nests while inducing chronic immune suppressive signals that select for tumor cells. Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between immune effectors and the tumor cells. Decreased in NK cell activity in cancer patients implicates the role of NK cells in tumor surveillance. Applicability of NK cells for cancer immunotherapy has been evoked by improved methodologies for NK expansion.

In this study, we found the novel way to expand NK cells by osteoclast. When osteoclasts interacted with IL-2+anti-CD16mAb+sAJ2 treated NK cells, they expanded the NK cells the most and osteoclast-expanded NK cells generated under this condition remain as activated NK cells for longer period of time by retaining CD16, NKp44 and NKG2D receptors on the surface. Also, osteoclast-expanded NK cells were found to be highly functional compared to primary NK cells due to their high cytotoxic activity and augmented IFN-γ secretion. Considering that NK cells may reside primarily in the immune rich compartments, it is likely that other immune effector may shape the phenotype and function of NK cells. In this regard, osteoclasts as potent inducers of NK cells shape the phenotype and functional of NK cells so that they become highly activated and functional. We believe that a combination of IL-12, IL-15 and IL-18 is required to fully activate NK cells as NK

cells secrete these cytokines when they interact with osteoclasts. The underlying mechanism of how osteoclasts interact with NK cells for expansion is not completely understood but this recent discovery is of outmost importance since NK expansion to potentiate their functions against tumors will contribute to clinical trials for cancer therapy.

REFERENCES

1. Farag, S.S. and M.A. Caligiuri, *Human natural killer cell development and biology.* Blood Reviews, 2006. **20**(3): p. 123-137.

2. Jewett, A., Y.G. Man, and H.C. Tseng, *Dual Functions of Natural Killer Cells in Selection and Differentiation of Stem Cells; Role in Regulation of Inflammation and Regeneration of Tissues.* J Cancer, 2013. **4**(1): p. 12-24.

3. Biron, C.A., K.S. Byron, and J.L. Sullivan, *Severe Herpesvirus Infections in an Adolescent without Natural Killer Cells.* New England Journal of Medicine, 1989. **320**(26): p. 1731-1735.

4. Ballas, Z.K., et al., *A patient with simultaneous absence of "classical" natural killer cells (CD3-, CD16+, and NKH1+) and expansion of CD3+, CD4-, CD8-, NKH1+ subset.* J Allergy Clin Immunol, 1990. **85**(2): p. 453-9.

5. Iannello, A., et al., *Antiviral NK cell responses in HIV infection: I. NK cell receptor genes as determinants of HIV resistance and progression to AIDS.* J Leukoc Biol, 2008. **84**(1): p. 1-26.

6. Trinchieri, G., *Biology of Natural Killer Cells*, in *Advances in Immunology*, J.D. Frank, Editor. 1989, Academic Press. p. 187-376.

7. Cooper, M.A., et al., *Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset.* Blood, 2001. **97**(10): p. 3146-51.

8. Tsuboi, I., et al., *Nonsteroidal anti-inflammatory drugs differentially regulate cytokine production in human lymphocytes : Up-regulation of TNF, IFN-γ and IL-2, in contrast to down-regulation of IL-6 production.* Cytokine, 1995. **7**(4): p. 372-379.

9. Miescher, S., et al., *Preferential clonogenic deficit of CD8-positive T-lymphocytes infiltrating human solid tumors.* Cancer Res, 1988. **48**(24 Pt 1): p. 6992-8.

10. Qin, J., B. Han, and J. Pang, *[The relationship between TIL from human primary hepatic carcinoma and prognosis].* Zhonghua Yi Xue Za Zhi, 1997. **77**(3): p. 167-70.

11. Han, X., et al., *Tumor lymphocytes in patients with advanced ovarian cancer: changes during in vitro culture and implications for immunotherapy.* Gynecol Oncol, 1997. **65**(3): p. 391-8.

12. Aggarwal, S. and M.F. Pittenger, *Human mesenchymal stem cells modulate allogeneic immune cell responses.* Blood, 2005. **105**(4): p. 1815-22.

13. Selmani, Z., et al., *Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells.* Stem Cells, 2008. **26**(1): p. 212-22.

14. Spaggiari, G.M., et al., *Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2.* Blood, 2008. **111**(3): p. 1327-33.

15. Ljunggren, H.G. and K.J. Malmberg, *Prospects for the use of NK cells in immunotherapy of human cancer.* Nat Rev Immunol, 2007. **7**(5): p. 329-39.

16. Trinchieri, G., et al., *Response of resting human peripheral blood natural killer cells to interleukin 2.* J Exp Med, 1984. **160**(4): p. 1147-69.

17. London, L., B. Perussia, and G. Trinchieri, *Induction of proliferation in vitro of resting human natural killer cells: IL 2 induces into cell cycle most peripheral blood NK cells, but only a minor subset of low density T cells.* J Immunol, 1986. **137**(12): p. 3845-54.

18. Lanier, L.L., et al., *Interleukin 2 activation of natural killer cells rapidly induces the expression and phosphorylation of the Leu-23 activation antigen.* J Exp Med, 1988. **167**(5): p. 1572-85.

19. Robertson, M.J., et al., *Costimulatory signals are required for optimal proliferation of human natural killer cells.* J Immunol, 1993. **150**(5): p. 1705-14.

20. Carson, W.E., et al., *A potential role for interleukin-15 in the regulation of human natural killer cell survival.* J Clin Invest, 1997. **99**(5): p. 937-43.

21. Cooper, M.A., et al., *In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells.* Blood, 2002. **100**(10): p. 3633-8.

22. Srivastava, S., A. Lundqvist, and R.W. Childs, *Natural killer cell immunotherapy for cancer: a new hope.* Cytotherapy, 2008. **10**(8): p. 775-83.

23. Gras Navarro, A., A. Björklund, and M. Chekenya, *Therapeutic potential and challenges of Natural killer cells in treatment of solid tumors.* Frontiers in Immunology, 2015. **6**.

24. Miller, J.S., et al., *Role of monocytes in the expansion of human activated natural killer cells.* Blood, 1992. **80**(9): p. 2221-9.

25. Rabinowich, H., et al., *Increased proliferation, lytic activity, and purity of human natural killer cells cocultured with mitogen-activated feeder cells.* Cell Immunol, 1991. **135**(2): p. 454-70.

26. Igarashi, T., et al., *Enhanced cytotoxicity of allogeneic NK cells with killer immunoglobulin-like receptor ligand incompatibility against melanoma and renal cell carcinoma cells.* Blood, 2004. **104**(1): p. 170-7.

27. Perussia, B., et al., *Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines.* Nat Immun Cell Growth Regul, 1987. **6**(4): p. 171-88.

28. Yu, Y., et al., *Enhancement of human cord blood CD34+ cell-derived NK cell cytotoxicity by dendritic cells.* J Immunol, 2001. **166**(3): p. 1590-600.

29. Steinman, R.M., *The dendritic cell system and its role in immunogenicity.* Annu Rev Immunol, 1991. **9**: p. 271-96.

30. Romani, N., et al., *Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells.* J Exp Med, 1989. **169**(3): p. 1169-78.
31. Sˆderstrˆm, K., et al., *Natural killer cells trigger osteoclastogenesis and bone destruction in arthritis.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(29): p. 13028-33.
32. SUDA, T., N. TAKAHASHI, and T.J. MARTIN, *Modulation of Osteoclast Differentiation.* Endocrine Reviews, 1992. **13**(1): p. 66-80.
33. Tanaka, Y., S. Nakayamada, and Y. Okada, *Osteoblasts and osteoclasts in bone remodeling and inflammation.* Curr Drug Targets Inflamm Allergy, 2005. **4**(3): p. 325-8.
34. Feng, S., et al., *IL-15-activated NK cells kill autologous osteoclasts via LFA-1, DNAM-1 and TRAIL, and inhibit osteoclast-mediated bone erosion in vitro.* Immunology, 2015.
35. Lanier, L.L., *NK cell receptors.* Annu Rev Immunol, 1998. **16**: p. 359-93.
36. Tseng, H.C., N. Cacalano, and A. Jewett, *Split anergized Natural Killer cells halt inflammation by inducing stem cell differentiation, resistance to NK cell cytotoxicity and prevention of cytokine and chemokine secretion.* Oncotarget, 2015. **6**(11): p. 8947-59.
37. Brandt, C.S., et al., *The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans.* J Exp Med, 2009. **206**(7): p. 1495-503.
38. Raulet, D.H., *Roles of the NKG2D immunoreceptor and its ligands.* Nat Rev Immunol, 2003. **3**(10): p. 781-90.
39. Wu, J., et al., *An activating immunoreceptor complex formed by NKG2D and DAP10.* Science, 1999. **285**(5428): p. 730-2.
40. Chavez-Galan, L., et al., *Cell death mechanisms induced by cytotoxic lymphocytes.* Cell Mol Immunol, 2009. **6**(1): p. 15-25.
41. Pan, G., et al., *The receptor for the cytotoxic ligand TRAIL.* Science, 1997. **276**(5309): p. 111-3.
42. Vesely, M.D., et al., *Natural innate and adaptive immunity to cancer.* Annu Rev Immunol, 2011. **29**: p. 235-71.
43. Jewett, A. and B. Bonavida, *Target-induced inactivation and cell death by apoptosis in a subset of human NK cells.* J Immunol, 1996. **156**(3): p. 907-15.
44. Jewett, A. and B. Bonavida, *Target-induced anergy of natural killer cytotoxic function is restricted to the NK-target conjugate subset.* Cell Immunol, 1995. **160**(1): p. 91-7.
45. Hu, P.F., et al., *Natural killer cell immunodeficiency in HIV disease is manifest by profoundly decreased numbers of CD16+CD56+ cells and expansion of a population of CD16dimCD56- cells with low lytic activity.* J Acquir Immune Defic Syndr Hum Retrovirol, 1995. **10**(3): p. 331-40.

46. Kuss, I., et al., *Clinical significance of decreased zeta chain expression in peripheral blood lymphocytes of patients with head and neck cancer.* Clin Cancer Res, 1999. **5**(2): p. 329-34.

47. Lai, P., et al., *Alterations in expression and function of signal-transducing proteins in tumor-associated T and natural killer cells in patients with ovarian carcinoma.* Clin Cancer Res, 1996. **2**(1): p. 161-73.

48. Jewett, A., et al., *Differential secretion of TNF-alpha and IFN-gamma by human peripheral blood-derived NK subsets and association with functional maturation.* J Clin Immunol, 1996. **16**(1): p. 46-54.

49. Jewett, A., et al., *Rapid and potent induction of cell death and loss of NK cell cytotoxicity against oral tumors by F(ab')2 fragment of anti-CD16 antibody.* Cancer Immunol Immunother, 2008. **57**(7): p. 1053-66.

50. Jewett, A., M. Cavalcanti, and B. Bonavida, *Pivotal role of endogenous TNF-alpha in the induction of functional inactivation and apoptosis in NK cells.* J Immunol, 1997. **159**(10): p. 4815-22.

51. Patankar, M.S., et al., *Potent suppression of natural killer cell response mediated by the ovarian tumor marker CA125.* Gynecol Oncol, 2005. **99**(3): p. 704-13.

52. Fujisaki, H., et al., *Expansion of highly cytotoxic human natural killer cells for cancer cell therapy.* Cancer Res, 2009. **69**(9): p. 4010-7.

53. Denman, C.J., et al., *Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells.* PLoS One, 2012. **7**(1): p. e30264.

54. Tseng, H.-C., et al., *Induction of split anergy conditions Natural Killer cells to promote differentiation of stem cells through cell-cell contact and secreted factors.* Frontiers in Immunology, 2014. **5**.

55. Scott, M.J., et al., *Interleukin-10 suppresses natural killer cell but not natural killer T cell activation during bacterial infection.* Cytokine, 2006. **33**(2): p. 79-86.

56. Oberholzer, A., C. Oberholzer, and L.L. Moldawer, *Interleukin-10: a complex role in the pathogenesis of sepsis syndromes and its potential as an anti-inflammatory drug.* Crit Care Med, 2002. **30**(1 Suppl): p. S58-63.

57. Sellon, R.K., et al., *Resident Enteric Bacteria Are Necessary for Development of Spontaneous Colitis and Immune System Activation in Interleukin-10-Deficient Mice.* Infection and Immunity, 1998. **66**(11): p. 5224-5231.

58. O'Garra, A., et al., *Strategies for use of IL-10 or its antagonists in human disease.* Immunol Rev, 2008. **223**: p. 114-31.

59. Gazzinelli, R.T., et al., *In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha.* J Immunol, 1996. **157**(2): p. 798-805.

60. Seki, S., et al., *Role of liver NK cells and peritoneal macrophages in gamma interferon and interleukin-10 production in experimental bacterial peritonitis in mice.* Infect Immun, 1998. **66**(11): p. 5286-94.

61. Silva, J.S., et al., *Interleukin 10 and interferon gamma regulation of experimental Trypanosoma cruzi infection.* J Exp Med, 1992. **175**(1): p. 169-74.

62. Denis, M. and E. Ghadirian, *IL-10 neutralization augments mouse resistance to systemic Mycobacterium avium infections.* J Immunol, 1993. **151**(10): p. 5425-30.

63. Khan, I.A., T. Matsuura, and L.H. Kasper, *IL-10 mediates immunosuppression following primary infection with Toxoplasma gondii in mice.* Parasite Immunol, 1995. **17**(4): p. 185-95.

64. Finkelman, F.D., et al., *Regulation and biological function of helminth-induced cytokine responses.* Immunol Today, 1991. **12**(3): p. A62-6.

65. Peppa, D., et al., *Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection.* PLoS Pathog, 2010. **6**(12): p. e1001227.

66. Buelens, C., et al., *Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/macrophage-colony-stimulating factor.* Eur J Immunol, 1997. **27**(3): p. 756-62.

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

68. de Waal Malefyt, R., et al., *Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.* J Exp Med, 1991. **174**(5): p. 1209-20.

69. Hsu, D.H., K.W. Moore, and H. Spits, *Differential effects of IL-4 and IL-10 on IL-2-induced IFN-gamma synthesis and lymphokine-activated killer activity.* Int Immunol, 1992. **4**(5): p. 563-9.

70. Marchant, A., et al., *Interleukin-10 controls interferon-gamma and tumor necrosis factor production during experimental endotoxemia.* Eur J Immunol, 1994. **24**(5): p. 1167-71.