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Expansion and Increased Function of NK Cells by Probiotic Bacteria in Cancer

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Author
Chiang, Jessica

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Expansion and Increased Function of NK Cells by Probiotic Bacteria in Cancer

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

by

Jessica Chiang

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ABSTRACT OF THE THESIS

Expansion and Increased Function of NK Cells by Probiotic Bacteria in Cancer

by

Jessica Chiang

Master of Science in Oral Biology
University of California, Los Angeles, 2017

Professor Anahid Jewett, Chair

Advances in understanding anti-tumor responses and cancer biology has revealed a dynamic interaction between immune effector cells like natural killer cells and cancer target cells. Natural killer cells are part of the innate immune system and targets cancer stem cells (CSCs) and healthy, non-transformed stem cells via secreted and membrane-bound IFN-\(\gamma\) and TNF-\(\alpha\). Probiotic bacteria has induced split-anergy in NK, thus having an increase in cytokine IFN-\(\gamma\) secretion leading to differentiation of CSCs. This innovative method of expanding large number of functional NK cells with the addition of probiotics is a breakthrough for adoptive NK immunotherapy. In this study, NK immunotherapy was studied in combination with supplementation of probiotic bacteria in pancreatic cancer patients and in a humanized mouse model. NK immunotherapy increased cytokine production in immune tissue of subjects, while probiotic supplementation further enhanced these effects, resulting in more differentiated tumors

*in vivo.*
The thesis of Jessica Chiang is approved.

Shen Hu

Nicholas A. Cacalano

Anahid Jewett, Committee Chair

University of California, Los Angeles

2017
DEDICATION

This thesis is first and foremost dedicated to my hard-working and loving family, KC, Meilan, and Ryan Chiang. You all have taught me the importance of hard work and I cannot have made it this far without the sacrifices you have made. Secondly, I would like to dedicate this thesis to my loving and supportive friends. I love you all so much and thank you for all your support.
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INTRODUCTION

Natural killer cells

Natural killer (NK) cells are immune cells that develop in the bone marrow and comprises 5-15% of the total lymphocytes in the peripheral blood [1]. NK cell effector functions are known to mediate direct natural cytotoxicity, antibody-dependent cellular cytotoxicity (ADCC), as well as inflammatory and cytokine secretion [2-3]. In addition, these key cytokines and chemokines produced by NK cells indirectly regulate the functions of other immune cells [3-4]. NK cells mediate cytotoxicity against transformed and healthy cells through the release of perforin and granzyme B, pre-formed granules of proteins. Of which, these proteins can induce apoptosis of target cells [4-6]. NK cells preferentially target and kills cancer stem cells (CSCs)/undifferentiated tumors, as well as healthy, non-transformed stem cells, which express low levels of major histocompatibility complex I (MHC-1), B7H1, CD54, but high levels of CD44 [7].

Human NK cells are identified by two distinct NK cell populations i.e. CD56 bright CD16 dim and CD56 dim CD16 bright, based on phenotypic and functional analyses [8,10]. The CD56 bright CD16 dim phenotype is a major subset in the peripheral blood that mediates cytotoxicity, whereas CD56 dim CD16 bright plays a role in secretion of cytokines [8,10].

Previous studies have shown that NK cells differentiate CSCs, otherwise known as undifferentiated or poorly differentiated tumors, via secreted and membrane-bound IFN-γ and TNF-α, which can lead to a slow-down in tumor growth and microenvironment transformation [9]. Both active receptors and co-receptors that recognize ligands expressed on tumors or virus-infected cells are responsible for NK cell activation [10,11]. In fact, the risk of cancer is significantly reduced when medium/high cytotoxic activity of lymphocytes are involved, and a
high level of NK infiltration within the tumor is linked to better prognosis [1, 11]. Therefore, low cytotoxic function of NK cells is associated with increased cancer risk [13]. Significant NK cell cytotoxic and cytokine secretion function were diminished in cancer patients [12-22].

**Split anergy in NK cells**

Our laboratory coined the term ‘split anergy’ to explain the relationship of reduced NK cell cytotoxicity in the presence of augmented secretion of IFN-γ [23] Split-anergized NK cells promotes differentiation of target cells via secreted and membrane-bound factors, increases tumor cell resistance to NK cell-mediated cytotoxicity, as well as inhibits inflammation due to the reduction of cytokine and chemokine production after tumor differentiation [24-29].

**Probiotic Bacteria**

Probiotics have been increasingly studied due to its relation to immune system improvement. In the early 20th century, Ellie Metchinkoff discovered certain strains of bacteria in the human gut that were beneficial to homeostasis of the gut; therefore, these beneficial bacteria were termed probiotics [30]. Probiotics are commonly used in foods and supplements to enhance the innate immune system, including NK cells activity, and maintain the digestive tract’s microbial balance [31-33]. Majority of probiotics are lactic acid-producing bacteria, which includes *lactobacilli*, *streptococci*, and *bifidobacteria*. Probiotics influence the production of immunoglobulin A [34-36], stimulation of macrophage activity [37], and are likely to lower the toxic side-effects of anti-cancer therapy [38]. Probiotics also help induce immature dendritic cells to differentiate into regulatory dendritic cells, induce the presence of regulatory T cells and increase NK cell activity, resulting in local intestinal defense [30-32]. Our lab has shown that
probiotic bacteria can also induce split-anergy in activated NK cells, leading to substantial induction of IFN-γ and TNF-α and induce significant expansion of NK cells [49]. Research into probiotics is increasingly growing, with promising results in treatment of various medical conditions such as mucosal pathologies, allergies, obesity, metabolic syndrome, heart disease, and cancer prevention or treatment, and so on [39-44]. However, though research on the benefits of probiotics has continued to grow and improve the lives of many, the underlying mechanisms of NK cell immunomodulation is not understood. This study will provide further information regarding the outcome of probiotics in combination with NK immunotherapy.

**Osteoclasts for NK Expansion**

NK cells have also been identified within inflamed synovial fluid and express RANKL and M-CSF, which during their interaction with monocytes, can trigger the formation of osteoclasts in a process that is RANKL and M-CSF dependent [45]. Osteoclasts are a type of bone cell, derived from hematopoietic stem cells. In the bone, homeostasis is achieved when there is a balance between osteoblast bone formation and osteoclast bone resorption [46]. Osteoclasts can resorb bone tissues, which is critical for the maintenance, repair, and remodeling of bones, while osteoblasts can produce and secrete matrix proteins and transport mineral into the mix [47]. Overall, these osteoclasts are significant activators of NK cell expansion and function [45]. In this study, the rate of expanding functional NK cells from patient and healthy donors using osteoclasts as feeder cells and sAJ2 probiotic is studied.

**Humanized Mouse Model**
When using a NOD-\textit{scid} and NSG strain mice model, varying levels of NK cell impairment could explain the discrepancies in the ability of CSCs to give rise to human tumors in these different immunodeficient strains [48]. Many have attempted to create an animal model system comprised of a fully reconstituted human immune system, however many questions have been raised regarding specific immune subsets and their roles in controlling cancer growth and metastasis. Since it is difficult to assess the growth of human tumors using immunodeficient mouse strains, humanized mice with restored immune systems, offers to be the most fitting platform to implant such tumors [49].

Many studies have attempted to generate mice with a fully reconstituted human immune system. Since it is critical for the background strain to harbor severe immunodeficiency, NSG or NRG mice have typically been the strain of choice [50,51]. The methods of creating various humanized mouse models include variables such as age of mice, transplanted cell type, source or donor cell type, etc. Upon these variations, one of the simplest methods consists of injecting immunodeficient mice with human peripheral blood mononuclear cells (PBMCs), obtained from adult healthy donors/patients [52,53]. The downside is that these types of mice can only be used for short term experiments, since circulating mature immune cells in mice can initiate graft versus host disease (GvHD) against murine recipients [54].

The BLT humanized mouse (hu-BLT) mouse model stands to be the most advanced and complete humanized mouse model that displays mucosal immunity. The human engraftment protocol consists of surgically implanting pieces of human fetal liver and thymus tissue under the kidney capsule of NSG mice, followed by tail vein IV injection of same-donor CD34+ hematopoietic cells to support full reconstitution of the human bone marrow [49, 55]. Thus, developing T cells undergo positive and negative selection in human thymus. Consequently,
immature T cells become functional CD4+ helper and CD8+ cytotoxic T cell after human MHC class I and class II restriction [55,58]. These hematopoietic stem cells develop, at least to some extent, into human T cells, B cells, NK cells, monocytes, myeloid derived suppressor cells (MDSCs), macrophages, dendritic cells, erythrocytes, and platelets in the BLT’s tissues [60-63]. It is important to note that NSG-BLT mice (BLT mice developed from NSG background strain) exhibit substantially higher levels of human leukocyte reconstitution in their peripheral blood than NOD-\textit{scid}-BLT mice [64]. These characteristics demonstrate that hu-BLT, derived from a NSG background, is the best available model for studying human immunity, thus far.

**Purpose of Study**

The purpose of this study is to further investigate the role of probiotic bacteria as therapy with NK immunotherapy to increase the function and survival of NK cells and their ability to target pancreatic stem cells in hu-BLT mice, and understand the effects of probiotic bacteria in pancreatic cancer patients.
THESIS OUTLINE

**Specific Aim 1:** To investigate the role of probiotics in activation of NK cells and their role in induction of split anergy.

**Specific Aim 2:** To study NK cell function in pancreatic cancer and healthy patients before the use of probiotics.

**Specific Aim 3:** To investigate the function of probiotic bacteria AJ2 in activation of NK cells in humanized-BLT mice model and in pancreatic cancer patients.
MATERIALS AND METHODS

Cell lines, reagents, and antibodies

Human immune cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, CA). Oral squamous carcinoma stem cells (OSCSCs) were isolated from oral cancer patient tongue tumors at UCLA School of Medicine and cultured in RPMI 1640, supplemented 10% FBS (Gemini Bio-Products, CA), 1.4% antibiotic antimycotic, 1% sodium pyruvate, 1.4% MEM non-essential amino acids, 1% L-glutamine, 0.2% gentamicin (Gemini Bio-products, CA) and 0.15% sodium bicarbonate (Fisher Scientific, PA). MIA PaCa-2 (MP2) were cultured in DMEM with 10% FBS and 1% penicillin and streptomycin (Gemini Bio-Products, CA, USA).

Antibodies to CD16 were purchased from Biolegend (San Diego, CA, USA). Recombinant IL-2 was obtained from NIH-BRB. Antibodies against isotype control, MHC-I, CD45 (human), CD45 (mouse), CD3, CD16, CD56, CD8, B7H1, CD14, CD19, and CD11b were purchased from Biolegend (San Diego, CA). Human NK purification kits were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

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

Bacteria sonication

AJ2 is a combination of 8 different strains of gram positive probiotic bacteria (Streptococcus thermophiles, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium
*infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, and Lactobacillus bulgaricus* used to induce differentiation of stem cells (doi:10.3389/fimmu.2014.00269). AJ2 was weighed and resuspended in RPMI Medium 1640 containing 10% FBS at a concentration of 10mg per 1mL. The bacteria were thoroughly vortexed, then sonicated on ice for 15 seconds, set at a 60% amplitude. Sonicated samples were then incubated for 30 seconds on ice. After every five pulses, a sample was taken to observe under the microscope until at least 80 percent of cell walls were lysed. It was determined that approximated 20 rounds of sonication/incubation on ice, were conducted to achieve complete sonication. Finally, the sonicated samples (sAJ2) were aliquoted and stored in a -80 degrees Celsius freezer for future use.

**Purification of NK cells from the peripheral blood**

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

**NK cell supernatants used for stem cell differentiation**

As described above, human NK cells were purified from PBMCs of healthy donors. NK cells were left untreated, treated with sAJ2 at 1:3 (NK:sAJ2), and/or a combination of anti-CD16mAb (3μg/mL) and IL-2 (1,000 U/mL) for 18 hours before supernatants were removed and used for differentiation experiments. The amounts of IFN-γ produced by activated NK cells were assessed with IFN-γ ELISA (Biolegend, CA, USA). OSCSCs/MP2s were differentiated with gradual daily addition of increasing amounts of NK cell supernatants (of corresponding treatments). On average, to induce differentiation, a total of 4,500pg of IFN-γ containing supernatants, obtained from IL-2+anti-CD16mAb+sAJ2-treated NK cells, was added for 4 days to induce differentiation and resistance of OSCSCSs to NK cell-mediated cytotoxicity. Afterwards, target cells were washed with 1xPBS, detached and used for experiments.

**Purification of monocytes from the peripheral blood**

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

**Generation of osteoclasts**

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

**Expansion of NK cells**

Human purified and hu-BLT enriched NK cells were activated with rh-IL-2 (1000 U/ml) and anti-CD16mAb (3ug/ml) for 18-20 hours before they were co-cultured with feeder cells and SAJ2. The culture media was refreshed with rh-IL-2 every three days.

**Analysis of MP2 tumor cell growth in immunodeficient and humanized mice**

Animal research was performed under the written approval of the UCLA Animal Research Committee (ARC) in accordance to all federal, state, and local guidelines. Combined immunodeficient NOD.CB17-Prkdcsid/J and NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ (NSG mice lacking T, B, and natural killer cells) were purchased from Jackson Laboratory and maintained in the animal facilities at UCLA in accordance with protocols approved by the UCLA animal research committee. Humanized-BLT (hu-BLT; human bone marrow/liver/thymus) mice were prepared on NSG background as previously described [63, 64].
Prior to tumor implantation, selected mice were fed 5x10^9 AJ2 bacteria (the combination of 8 probiotic strains listed above) every other day for one week. This adjuvant therapy was continued every other day until the day of sacrifice. For each mouse, lyophilized AJ2 was resuspended in 200 μL of fat free milk, and fed to them via pipetting.

*In vivo* growth of pancreatic stem-like MP2 cells was determined by surgical implantation of tumor cells into hu-BLT mice. To establish pancreatic tumors, mice were first anesthetized using an isoflurane set up, and MP2s were then transferred by direct injection of 1x10^6 cells mixed with 10 μl HC Matrigel (Corning, NY, USA) into the pancreas. Immediately prior to tumor cells injection, 5.0-mg/kg carprofen was injected subcutaneously, and this injection was repeated every 24 hours for 48 hours.

Following injection of tumor cells, all mice were continuously monitored for disease progression every other day. Mice were observed for overall signs of morbidity, such as loss of weight, ruffled fur, hunched posture, and immobility. Seven days after tumor implantation selected hu-BLT mice received 1.5x10^6 human expanded NK cells via tail vein (IV) injection.

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

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

**Purification of human T cells from the peripheral blood**

CD3+ T cells from patient and healthy donors were positively selected from peripheral blood using isolation kits from Stem Cell Technologies (Vancouver, BC, Canada). Cells were cultured at 1x10^6 cells/mL in RPMI 1640 media (Life Technologies, CA), supplemented with 10% FBS, along with IL-2 (1000 units/mL) treatment.

**Surface staining**

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

**51Cr release cytotoxicity assay**

51Cr was purchased from Perkin Elmer (Santa Clara, CA). Standard 51Cr release cytotoxicity assays were used to determine NK cell cytotoxic function in the experimental cultures and the sensitivity of target cells to NK cell mediated lysis. The effector cells (1x10^5 NK cells/well)
were aliquoted into 96-well round-bottom microwell plates (Fisher Scientific, Pittsburgh, PA) and titrated at four to six serial dilutions. The target cells (5 x 10^5 OSCSCs) were labeled with 50μCi ^{51}\text{Cr} (Perkin Elmer, Santa Clara, CA) and chromated for 1 hour. Following incubation, target cells were washed twice to remove excess unbound ^{51}\text{Cr}. ^{51}\text{Cr}-labeled target cells were aliquoted into the 96-well round bottom microwell plates containing effector cells at a concentration of 1 x 10^4 cells/well at a top effector:target (E:T) ratio of 5:1. Plates were centrifuged and incubated for a period of 4 hours. After a 4-hour incubation period, the supernatants were harvested from each sample and counted for released radioactivity using the gamma counter. Total (containing ^{51}\text{Cr}-labeled target cells) and spontaneous (supernatants of target cells alone) release values were measured and used to calculate the percentage specific cytotoxicity. The percentage specific cytotoxicity was calculated using the following formula:

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

LU 30/10^6 is calculated by using the inverse of the number of effector cells needed to lyse 30% of target cells x 100.

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

Human ELISA kits for IFN-γ and IL-10 were purchased from Biolegend (San Diego, CA). ELISA was performed to detect the level of IFN-γ and IL-10 produced from cell cultures. The assay was conducted as described in the manufacturer’s protocol. Briefly, 96-well EIA/RIA plates were coated with diluted capture antibody corresponding to target cytokine and incubated overnight at 4°C. After 16-18 hours of incubation, the plates were washed 4 times with wash buffer (0.05% Tween in 1xPBS) and blocked with assay diluent (1% BSA in 1xPBS). The plates were
incubated for 1 hour at room temperature, on a plate shaker at 200rpm; plates were washed 4 times following incubation. Then, 100uL of standards and samples collected from each culture were added to the wells and incubated for 2 hours at room temperature, on the plate shaker at 200rpm. After incubation, plates were washed 4 times, loaded with detection antibody, and incubated for 1 hour at room temperature, on the plate shaker at 200rpm. After 1 hour of incubation, the plates were washed 4 times; wells were loaded with Avidin-HRP solution and incubated for 30 minutes at room temperature, on the plate shaker at 200rpm. After washing the plates 5 times with wash buffer; 100uL of TMB substrate solution was added to the wells and plates were incubated in the dark until they developed a desired blue color (or up to 30 minutes). Then, 100uL of stop solution (2N H₂SO₄) was added per well to stop the reaction. Finally, plates were read in a microplate reader, at 450nm to obtain absorbance values (Biolegend, ELISA manual).

The levels of cytokines and chemokines were examined by multiplex assay, which was conducted as described in the manufacturer’s protocol for each specified kit. Analysis was performed using a Luminex multiplex instrument (MAGPIX, Millipore, Billerica, MA) and data was analyzed using the proprietary software (xPONENT 4.2, Millipore, Billerica, MA).

**Statistical analysis**

An unpaired, two-tailed student t-test was performed for the statistical analysis of two groups. ***p value <0.001), **(p value 0.001-0.01), *(p value 0.01-0.05).
CHAPTER 1

Specific Aim 1: To investigate the role of probiotics in activation of NK cells and their role in induction of split anergy.

RESULTS

Probiotic bacteria induce cytokine secretion, but does not elevate cytotoxicity

To study the effect of probiotic bacteria on NK cytotoxic function, NK cells were purified from healthy donors. NK cells were left untreated, treated with IL-2, or the combination of anti-CD16mAb and IL-2 in the presence or absence of individual probiotic bacteria strains and a $^{51}$Cr release assay was conducted. Neither untreated or activated NK cells showed significant differences in cytotoxic activity of NK cells in their ability to target OSCSCs (Fig. 1). Thus, NK cell treatment with probiotic bacteria did not elevate cytotoxic function.

To study the effects of probiotics on NK cell function, NK cells were cultured either with or without probiotic strains under different activation conditions. Supernatants were collected and was used for multiple array analysis. Activated NK cells with IL-2 or IL-2+anti-CD16mAb, treated with individual probiotic strains induced higher levels of IFN-γ (Fig. 2). NK cells activated with IL-2 or the combination with IL-2+anti-CD16mAb induced slight secretion of IL-10 (Fig. 3). Thus, probiotic bacteria does enhance NK cytokine secretion function, while maintaining the same level of cytotoxicity.

Supernatants of sAJ2 bacteria treated with IL-2+anti-CD16mAb+sAJ2 NK cells induce differentiation and resistance of OSCSCs to NK cell-mediated cytotoxicity
Next, we determined whether resistance to NK cell-mediated cytotoxicity correlates with a change in cytokine and chemokine secretion by NK cells in cultures with differentiated MP2s (Fig. 4,6) and OSCSCs (Fig. 5). The supernatants from NK cells activated with IL-2+anti-CD16mAb+sAJ2 were harvested to treat stem-like MP2 pancreatic tumor cell line for 4 days. Untreated tumors and tumors treated with NK cell supernatants were washed and labeled with $^{51}$Cr to determine the cytotoxic function of NK cells, as described previously. Notable difference was seen between untreated MP2 and tumors treated with NK from IL-2+anti-CD16mAb+sAJ2 supernatant in the absence or presence of anti-TNF-α or anti-IFN-γ were significant at a p value of <0.05 (*). No significant differences were seen between untreated MP2 and tumors treated with supernatants from NK cells treated with IL-2+anti-CD16mAb+sAJ2 with a combination of anti-TNF-α and anti-IFN-γ antibody (Fig. 4). OSCSCs were treated as described in Figure 4. OSCSCs treated with IL-2+anti-CD16mAb-activated NK cell supernatants triggered less IFN-γ secretion by NK cells; however, the extent of inhibition was far less than OSCSCs treated with IL-2+anti-CD16mAb+sAJ2- treated NK cell supernatants (P < 0.05) (Fig. 5). The addition of anti-IFN-γ alone, but not anti-TNF-α alone, to IL-2+anti-CD16mAb+sAJ2 was able to restore the IFN-γ secretion back to the levels observed with untreated OSCSCs (Fig. 5).

To determine whether supernatants obtained from probiotic bacteria and IL-2 + anti-CD16mAb-treated NK cells are capable of inducing differentiation and resistance in MP2 stem-like pancreatic tumors, MP2 tumors were stained for surface expression of B7H1 and MHC-I (Fig. 6). PE isotype control antibody were used as controls. The numbers on the top, right hand corner are percentages and the mean channel fluorescence intensities for each histogram (Fig. 6). Indeed, the expression of B7H1 and MHC-I on IL-2+anti-CD16mAb+sAJ2 treated NK cells was higher than in the presence of MP2s treated with IL-2+anti-CD16mAb+sAJ2 and anti-IFN-γ,
anti-TNF-α, or the combination of both (Fig. 6). Thus, NK cells treated with probiotic bacteria supernatants further differentiated MP2 tumor cell line and delineate that tumors treated with the supernatants from treated/activated NK cells are resistant to NK-mediated cell killing.
Figure 1: Probiotic bacteria does not elevate NK cell-mediated cytotoxicity against OSCSCs.

NK cells were purified from peripheral blood and were left untreated, IL-2 (1000 units/mL), or combination of IL-2 and anti-CD16mAb (3μg/mL) in the presence or absence of probiotic bacteria sAJ2 at 1:5 ratio (NK: sAJ2) for 18 hours. NK cells were then used as effector cells against $^{51}$Cr labeled OSCSCs. NK cell mediated cytotoxicity was determined using a standard 4 hour $^{51}$Cr release assay and the lytic units 30/10^6 cells were determined using inverse number of NK cells required to lyse 30% of OSCSCs X100.
Figure 2: Treatment of NK cells with probiotic bacteria induces higher secretion of IFN-γ

NK cells were purified from peripheral blood and were left untreated, IL-2 (1000 units/mL), or combination of IL-2 and anti-CD16mAb (3μg/mL) in the presence or absence of probiotic bacteria at 1:5 ratio (NK: sAJ2) for 18 hours. Supernatants of cultures were harvested and used for multiplex array analysis. The levels of secretion for IFN-γ from each condition is detailed in the figure.
Figure 3: Activated NK cells with probiotic bacteria induces slight secretion of IL-10

Purified NK cells were treated as described in Figure 2. Supernatants of cultures were harvested and used for multiplex array analysis. The levels of secretion for IL-10 from each condition is detailed in the figure.
Figure 4: Induction of differentiation and resistance to NK cell mediated lysis of MP2 tumors treated with IL-2+anti-CD16mAb+sAJ2 NK cell supernatants is mediated by the combination of NK cell induced IFN-γ and TNF-α and not each cytokine alone

Purified NK cells were treated as described in Figure 3. Afterwards, supernatants from each NK sample were harvested and used to treat/differentiate MP2s for 4 days. MP2s were detached from tissue culture plates, washed, and NK cell-mediated lysis was evaluated using a standard 4-hour $^{51}$Cr release assay. Levels of NK cell-mediated cytotoxicity against radioactively labeled MP2s conditions were determined using lytic units (LU 30/10⁶).
Figure 5: The addition of anti-IFN-γ alone, but not anti-TNF-α alone, to IL-2 + anti-CD16mAb + sAJ2 was able to restore the IFN-γ secretion back to the levels observed with untreated OSCSCs.

Freshly isolated NK cells were treated with IL-2 (1000 U/mL) for 18 hours. Afterwards, NK cells were added to untreated OSCSCs and those differentiated with NK cell supernatants. After an overnight incubation, the supernatants were removed from the co-cultures, and the levels of IFN-γ secretions were determined using specific ELISAs.
Figure 6: Induction of differentiation of MP2 tumors treated with IL-2+anti-CD16mAb+sAJ2 NK cell supernatants is mediated by the combination of NK cell induced IFN-γ and TNF-α, but not each cytokine alone.

Purified NK cells were left untreated, treated with sAJ2 alone at 1:3 (NK: sAJ2) ratio, or in combination with IL-2 (1000 units/mL) and anti-CD16mAb (3μg/mL) for 18 hours. Afterwards, the supernatants from each NK sample was collected and used to treat/differentiate MP2 tumors for 4 days. Anti-IFN-γ (1:100) and anti-TNF-α (1:100) antibodies were added before the start of NK sup treatments. MP2s were detached from tissue culture plates, and cells from each treatment were used to measure surface expression of surface markers via flow cytometry. PE conjugated antibodies against isotype control, MHC-1, and B7H1 were used to stain untreated MP2s or those treated with NK cell supernatants, as detailed in the figure. Isotype control antibodies were used as controls. The numbers on the right hand corner are the percentages and the mean channel fluorescence intensities for each histogram.
CHAPTER 2

Specific Aim 2: To study NK cell function in pancreatic cancer and healthy patients before the use of probiotics.

RESULTS

Isolated PBMCs obtained from patients exhibit lower cell count, decreased percentages of T and B cells, and increased percentages of NK cells, CD14 monocytes, and CD11b.

To investigate the effects of probiotic bacteria AJ2 in cancer patients, an analysis into the immune profile between healthy and cancer donors before taking probiotics was identified. Purified PBMCs were isolated and obtained from the peripheral blood of patient and healthy donors, as described in the Materials and Methods section. PBMCs were isolated, washed, and counted. Significant differences were seen between the PBMC counts when comparing healthy and cancer patients. Cancer patients exhibit fewer number of PBMCs when compared to healthy individuals (Fig. 7). The remaining PBMCs from healthy and cancer patient donors were used to measure the proportion of certain immune cell subsets via flow cytometry (Fig. 8). Interestingly, pancreatic cancer patients were found to have increased percentages of NK, CD14 monocytes, and CD11b, regulator of leukocyte adhesion (Fig. 8A, D, E), and significant decreases of T and B cells (Fig. 8B, C).

Patient NK cell-mediated lysis significantly decreased when compared to the functionality of healthy NKs.
To study the cytotoxic function of NK cells isolated from patient and healthy donors, a baseline study was conducted to create a comparison between patient and healthy NK cytotoxicity. NK cells were left either untreated or with IL-2 for 18-20 hours before they were used in a $^{51}$Cr release assay. Upon analysis, patient NK cells exhibited decreased NK cell cytotoxicity compared to healthy individuals (Fig. 9).

Osteoclast-expanded NK cells from patients have less capability to expand, exhibit less NK cell-mediated lysis, and cytokine secretion.

To investigate the expansion rate and functionality of patient and healthy individual’s NK cells, NK cells were co-cultured with osteoclasts and sAJ2. To generate osteoclasts, monocytes were cultured in alpha-MEM media containing M-CSF and RANKL for 21 days. For expansion, purified NK cells were treated with the combination of IL-2 and anti-CD16mAb for 18 hours; and there was a significant decrease in cell count, cytotoxic function, and cytokine secretion from patient expanded NK cells compared to healthy donors. Patient NK significantly lacked in cell count, indicating poor expansion rates (Fig. 10A) Additionally, these poorly expanded patient NK cells showed significant decrease in cytotoxicity (Fig. 10B) and lower IFN-γ secretion from NK cells (Fig. 10C).
Figure 7: Significantly decreased number of PBMCs from the peripheral blood of pancreatic cancer patients

Peripheral blood was separated using Ficoll-Hypaque centrifugation, after which the white, cloudy layer, containing peripheral blood mononuclear cells (PBMC), was harvested, washed and re-suspended in RPMI 1640, and supplemented with 10% FBS. PBMCs were counted on day 0.
Figure 8: Increased percentages of NK cells, CD14 monocytes and CD11b, but significant decreases of T and B cells in pancreatic cancer patients’ PBMCs

Purified PBMCs were isolated and obtained from the peripheral blood of patient and healthy donors, as described in the Materials and Methods section. PBMCs were isolated, washed, and counted. Isotype control antibodies were used as controls. PBMCs were stained for CD16+CD56, CD3, CD19, CD14, and CD11b expression and analyzed using flow cytometry (A-E).
Baseline cytotoxicity  n=9

Figure 9: Purified NK cells from pancreatic cancer patients demonstrated significant decreases in NK cell cytotoxicity compared to healthy donors’ NK cells.

NK cells and monocytes were purified and isolated from the patient and healthy donor’s PBMCs. NK cells were left either untreated or with IL-2 (1000 units/mL) for 18-20 hours before they were used in a $^{51}$Cr release assay against OSCSCs. Levels of NK cell-mediated cytotoxicity of these NK cell conditions, against radioactively labeled OSCSCs (target cells) were determined using lytic units (LU 30/10$^6$).
Figure 10: Osteoclast-expanded NK cells from patients have less capability to expand, exhibit less NK cell-mediated lysis, and cytokine secretion.

Purified NK and monocytes from PBMCs were cultured and treated as described in Figure 7. To generate osteoclasts, monocytes were cultured in alpha-MEM media containing M-CSF (25 ng/mL) and RANKL (25 ng/mL) for 21 days. For expansion, purified NK cells (1x10^6 cells/mL) were treated with the combination of IL-2 (1000 units/mL) and anti-CD16mAb (3 μg/mL) for 18 hours; then they were co-cultured with autologous osteoclasts in the presence of sAJ2 at a 1:2:4 (OC:NK:sAJ2) ratio. NK cells were counted on day 0 (A). At day 7, NK cell-mediated cytotoxic function of these expanded NK cells were measured using a standard 4-hour ^51^Cr release assay. Levels of NK cell-mediated cytotoxicity of these NK cell conditions, against radioactively labeled OSCSCs (target cells) were determined using lytic units (LU 30/10^6) (B). Supernatants of expanding NK cells were harvested and levels IFN-γ were measured using human IFN-γ ELISA. Cells were replenished with fresh culture medium and re-supplemented with IL-2 (1000 units/mL) at respective days (C).
CHAPTER 3

Specific Aim 3: To investigate the function of probiotic bacteria AJ2 in activation of NK cells in humanized-BLT mice model and in pancreatic cancer patients.

RESULTS

The hu-BLT mouse model was used to investigate probiotic supplementation in combination with the use of expanded NK cells to target cancer stem-like cells. Hu-BLT mice generation and detailed description of the experimental design is outlined in Figure 11-13.

NK immunotherapy resulted in increased NK cell-mediated cytotoxicity; AJ2 probiotic supplementation further increased this effect

To investigate the cytotoxic function of NK cells, $^{51}$Cr release assay was conducted using immune cells from harvested tissues. PBMCs were cultured as described in Figure 7, and on day 7 were used in a $^{51}$Cr release assay to measure their cytotoxic function. PBMCs from healthy control were highly cytotoxic, and mice receiving NK immunotherapy had similar levels of cytotoxicity. Mice that were supplemented with AJ2 augmented their cytotoxic function, while tumor-bearing mice showed very low cytotoxicity function (Fig. 14). Similar to hu-BLT, pancreatic cancer patient NK cells supplemented with probiotic AJ2 improved cytotoxic function compared to pancreatic cancer patient NK cells without AJ2 supplementation (Fig. 18). Based on our results, tumor-bearing mice and pancreatic patients exhibited significant decrease in cytotoxic function compared to subjects who received NK immunotherapy, both alone or in combination with AJ2 supplementation.
NK immunotherapy resulted in increased NK cytokine secretion function; AJ2 probiotic supplementation further increased this effect

To understand the effect of NK immunotherapy and probiotic supplementation on cytokine secretion by NK cells, PBMCs were harvested from hu-BLT and the supernatants were analyzed for cytokine secretion levels. Mice that received NK immunotherapy, either alone or in combination with AJ2 supplementation, induced a high level of IFN-γ secretion (Fig. 15). This significant augment in cytokine secretion seen in hu-BLT was also seen in pancreatic cancer patients who took probiotic supplementation (Fig. 19). In the presence of probiotic supplementation and NK immunotherapy, cytokine secreting function was not only restored, but significantly improved.

NK immunotherapy, both alone or in combination with AJ2 supplementation, prevented tumor growth in vivo

Following the sacrifice of hu-BLT animals, pancreatic MP2 tumors were resected as outlined in Figure 13. When comparing sizes and weights side-by-side, it was notably shown that mice receiving NK immunotherapy had smaller tumors compared to mice with no NK immunotherapy. The size of the tumor diminished even more in mice that received NK immunotherapy and probiotic supplementation (Fig. 16). 150,000 pancreatic tumor cells from each respective hu-BLT condition were treated with IL-2 (1000 units/mL) and cultured. Supernatants were harvested after day 7, day 11, day 14, and day 17. This information was validated through tumor cells counts following dissociation of the harvested tumors on the day the mice were euthanized (Fig. 17).
Humanized-BLT mice were generated by surgical implantation of human fetal liver and thymus tissue under the renal capsule of 6-8 weeks old immunocompromised NOD.CB17-Prkdcscid/J and NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice.
Figure 12: Greater than 90% of immune cells within different tissue compartments of BLT hu-mice are of human CD45+ immune cells.

Humanized-BLT mice were generated as described in Figure 10. 4-6 weeks post tissue transplant, mice were sub-lethally irradiated and intravenously injected with CD34+ cells isolated from fetal liver to support the full reconstitution of human bone marrow. 8-12 weeks after injection with CD34+ cells, reconstitution of human immune system was analyzed using blood, bone marrow, and spleen.
A week before tumor implantation, mice were fed with $5 \times 10^9$ AJ2 bacteria (the combination of 8 probiotic strains listed above) every other day. This probiotic supplementation was continued every other day until day of sacrifice. Lyophilized AJ2 was resuspended in 200 μL of fat free milk, and fed to them via pipetting. MP2 tumors were injected into the pancreas of hu-BLT mice. Following injection of tumor cells, mice were monitored for disease progression every other day. Seven days after tumor implantation selected hu-BLT mice received $1.5 \times 10^6$ human, osteoclast-expanded NK cells via tail vein (IV) injection. Mice were observed for overall signs of morbidity, such as loss of weight, ruffled fur, hunched posture, and immobility. Mice were then sacrificed 3 weeks from initial tumor implantation.
Figure 14: Restoration of NK cell mediated cytotoxicity of IL-2 activated PBMC from hu-BLT mice.

Successfully reconstituted BLT were orthotopically injected with 1x10⁶ of human MP2 into the pancreas. Following injection of tumor cells, selected hu-BLT mice received 1.5 X 10⁶ human expanded NK cells via tail vein injection, and was orally fed with AJ2 (5 billion) every other day through pipetting. At the end of this experiment peripheral blood was collected and PBMCs were isolated and cultured with IL-2 activation. Cytotoxicity assay was performed and determined using standard 4-hour ⁵¹Cr release assay against OSCSCs and the lytic units 30/10⁶ cells were determined.
Figure 15: NK injection improved IFN-γ secretion of immune cells in peripheral blood of hu-BLT mice.

Successfully reconstituted BLT were orthotopically injected with $1\times10^6$ of human MP2 into the pancreas. Following injection of tumor cells, selected hu-BLT mice received $1.5 \times 10^6$ human expanded NK cells via tail vein injection, and was orally fed with AJ2 (5 billion) every other day through pipetting. At the end of this experiment peripheral blood was collected and PBMCs were isolated and cultured with IL-2 activation. The supernatants were harvested on day 7 after the culture, and IFN-γ was determined using single ELISAs.
Figure 16: Significant decreases in tumor size with single injection super-charged NK cells in the presence and absence of feeding with AJ2 in tumor-bearing BLT mice

Successfully reconstituted BLT were orthotopically injected with $1 \times 10^6$ of human MP2 into the pancreas. Following injection of tumor cells, selected hu-BLT mice received $1.5 \times 10^6$ human expanded NK cells via tail vein injection, and was orally fed with AJ2 (5 billion) every other day through pipetting. On day of mice sacrifice (day 0), tumors were extracted from hu-BLT and were individually weighed.
Figure 17: Significant decreases in tumor growth with single injection supercharged NK cells in the presence and absence of feeding with AJ2 in tumor-bearing BLT mice

Successfully reconstituted BLT were orthotopically injected with 1x10^6 of human MP2 into the pancreas. Following injection of tumor cells, selected hu-BLT mice received 1.5 X 10^6 human expanded NK cells via tail vein injection, and was orally fed with AJ2 (5 billion) every other day through pipetting. On day of mice sacrifice, tumors were extracted from hu-BLT and dissociated. 150,000 pancreatic tumor cells from each hu-BLT condition were treated with IL-2 (1000 units/mL) and cultured. Supernatants were harvested after day 7, day 11, day 14, and day 17. Cells were re-suspended in fresh medium and supplemented with additional IL-2 (1000 units/mL) every day supernatant was harvested and cells were counted. This figure illustrates the cumulative tumor cell counts from day 0 to day 17.
Figure 18: Significant increase in NK cell mediated cytotoxicity after intake of AJ2 by pancreatic cancer patients

NK cells and monocytes were purified and isolated from the patient and healthy donor’s PBMCs. NK cells were treated with IL-2 (1000 units/mL) for 18-20 hours before they were used as effector cells in a $^{51}$Cr release assay against OSCSCs. Levels of NK cell-mediated cytotoxicity of these NK cell conditions, against radioactively labeled OSCSCs (target cells) were determined using lytic units (LU 30/10⁶).
Figure 19: Significant increase in IFN-γ after intake of AJ2 by pancreatic cancer patients.

NK cells and monocytes were purified and isolated from the patient and healthy donor’s PBMCs. NK cells were treated with IL-2 (1000 units/mL) for 18-20 hours before the supernatants were collected and levels of IFN-γ secretions were determined using specific ELISAs.
DISCUSSION

Our lab has previously shown that cytokines that are secreted by NK cells, primarily IFN-γ and TNF-α, are responsible for the differentiation of cancer stem cells (CSCs) and result in the increase in differentiation antigens such as MHC class I, CD54, and B7H1 and decrease in CD44 [11]. This study illustrates the profound capability probiotic bacteria has on NK cells to induce significant increase in cytokine secretion, known as split anergy.

sAJ2 is a combination of 8 strains of probiotic bacteria for their ability to induce significant secretion of IFN-γ when added to IL-2 or IL-2+anti-CD16mAb treated NK cells [11]. The ratio of bacteria added to create sAJ2 was adjusted to yield a ratio of IFN-γ to IL-10 for when cells are activated with IL-2 or IL-2+anti-CD16mAb without bacteria. This ratio was established to obtain a similar ratio when NK cells are activated with IL-2+anti-CD16mAb without bacteria, since this NK treatment provided increased differentiation of stem cells. IL-10, an anti-inflammatory cytokine, was taken into consideration to balance the significant amount of IFN-γ secreted by cells during the process of differentiation. This combination of bacterial strains was selected due to its optimal induction of pro- and anti-inflammatory cytokine and growth factors by the NK cells. Injecting expanded potent NK cells with probiotic bacteria could prevent tumor growth and metastasis and mediate significant differentiation of tumors evidenced by increased surface receptor expression of MHC class I and B7H1 (Fig. 6).

There was no significant difference observed in the NK cell cytotoxic levels of AJ2 treatment, however augmented cytokine secretion was observed in NK cells treated with probiotic bacteria, suggesting that NK cells exhibit a split anergy profile (Fig. 1-3). sAJ2 bacteria treated NK cells and split anergized NK cell supernatants induced greater differentiation and resistance of OSCSCs to NK cell mediated cytotoxicity (Fig. 4). The differentiation of stem cells
induced by split anergized NK cells treated with sAJ2 is mediated through cytokine secretion of both IFN-γ and/or TNF-α (Fig. 4-6). Thus, sAJ2 probiotic treatment of NK cells induces augmented cytokine secretion, resulting in further tumor differentiation, and reduced tumor growth.

Previous studies have attributed the presence of cancer with dampened immune responses, however the underlying mechanisms as to why immune activity in cancer patients have not been elucidated [13]. Here, we demonstrate that fewer PBMCs were collected from cancer patients compared to healthy donors (Fig. 7). In fact, the proportion of immune cells in PBMCs from cancer patients exhibited increased number of NK cells, CD14 monocytes and CD11b cells, but reduced proportions of CD3+ and CD19+ cells (Fig. 8A-E). In pancreatic cancer patients, it was found that NK surface receptors, especially osteoclasts, are down-modulated in cancer patients leading to the substantial decrease in NK cell-mediated cytotoxicity activity of NK cells. When osteoclasts (OCs) was used to expand NK cells from cancer patients, a significant loss of NK cell numbers, cell-mediated cytotoxicity, and decrease in IFN-γ secretion was observed (Fig. 10A-C). These results clearly show that pancreatic cancer patients have tremendous proportions of NK cells, but their NK cells have lost all cell function, and provides a substantial ground for the continued growth of cancer stem cells.

Single injection of potent NK cells increased cytotoxic activity and cytokine function from PBMCs in hu-BLT, and was further enhanced with probiotic bacteria. By injecting NK cells, tumor growth was inhibited as exhibited by decreased tumor cell counts and initial tumor weight (Fig. 16, 17). Injection of expanded NK cells inhibited tumor growth of MP2s through differentiation by the high levels of NK cell secretion of IFN-γ in humanized mice. Similar to our hu-BLT findings, pancreatic cancer patients who took or did not take probiotics exhibited
similar profiles as found in hu-BLT. Isolated NK cells from cancer patients with AJ2 substantially increased cytokine and cytotoxic function. These results clearly indicate that NK cell-induced tumor differentiation is important in the limitation of tumor growth and aggressiveness. It also showed that probiotic bacteria, combined with NK activated cytokines can provide a condition for NK cells to promote tumor differentiation.

The use of NK immunotherapy supplemented with AJ2 enhanced the function of NK cells in both hu-BLT and pancreatic cancer patients. PBMCs collected from the peripheral blood of hu-BLT mice secreted high levels of IFN-γ. Meanwhile, with NK immunotherapy in combination with AJ2 supplementation, there was a significant increase in cytokine secretion level compared to NK immunotherapy alone (Fig. 15). Mice receiving NK immunotherapy treatment significantly improved cytotoxic function compared to tumor bearing mice. Similar to tumor bearing mice, NK cells of pancreatic cancer patients exhibited lack of function in terms of cytotoxicity and cytokine secretion (Fig. 18,19). Meanwhile, mice that received NK immunotherapy following tumor injection demonstrate both cytotoxic as well as cytokine secretory functions (Fig. 14,15). This study collectively demonstrates the continued in vivo potency of ex vivo expanded NK cells with probiotic supplementation can differentiate cancer stem-like tumor cells in the BLT humanized mouse model and in cancer patients.
CONCLUSION

In conclusion, expanded NK cells can select and differentiate CSCs in \textit{in vitro} and \textit{in vivo} systems. The use of NK immunotherapy supplemented with AJ2 diminished tumor weight and tumor cell counts, enhance NK cell function, especially their cytokine secreting ability. It was also discovered that NK immunotherapy could select and differentiate pancreatic tumors in the presence of probiotic bacteria, and is a substantial foundation in immunology research. From our study, we now understand the significant effects of our NK immunotherapy in humanized mice, notably in the ability to differentiate tumors and prevent aggressive tumor growth. In future direction, applying our method of NK immunotherapy and probiotic supplementation can be brought into the clinical setting. It would be of interest to study the effects of probiotic supplementation combined with chemotherapy or immunotherapy. NK cells, and their capability of differentiating tumors may be the key breakthrough to preventing cancer relapse in patients. Thus, it is important to further conduct research that primarily focuses on the field of NK cells in cancer immunology.
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