Development of Cytotoxic Natural Killer Cells for Ovarian Cancer Treatment

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Veethika Pandey
University of Central Florida

DEVELOPMENT OF CYTOTOXIC NATURAL KILLER CELLS FOR OVARIAN CANCER TREATMENT

by

VEETHIKA PANDEY
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Major Professor: Deborah Altomare

ABSTRACT

Ovarian cancer is a leading cause of gynecological malignancy. Cytoreductive surgery and frontline platinum/taxane-based chemotherapy provides good initial efficacy in the treatment, but poor long-term patient survival. This is mainly caused by tumor relapse due to intraperitoneal spreading and ineffective alternate therapies to treat these resistant tumors. The challenge in the field is to develop strategies that would prove effective in these patients and extend overall survival.

Over the years, various treatments have been developed for the treatment of cancer amongst which, adoptive cell immunotherapy has shown promising results. But despite the efficacy seen in the clinic, there are concerns with the complexity of treatment and associated side effects. Therefore, there is still a need for better understanding of how different components of the immune system react to the presence of tumor. In this study, healthy human peripheral blood mononuclear cells (PBMCs) were used to examine the immune response in a mouse model with residual human ovarian tumor, where natural killer (NK) cells were found to be the effector cells that elicited an anti-tumor response. Presence of tumor was found to stimulate NK cell expansion and cytotoxicity in mice treated intraperitoneally (IP) with PBMCs+Interleukin-2 (IL-2). Intravenous (IV) adoptive transfer of isolated NK cells has been attempted in ovarian cancer patients before, but showed lack of persistence in patients resulting in lack of anti-tumor efficacy. Experiments in this study highlight the significance of NK cell-cytotoxic response to tumor, which may be attributed to interacting immune cell types in the PBMC population (when treated IP), as opposed to clinically used isolated NK cells showing lack of anti-tumor efficacy in ovarian cancer patients (when treated IV).

NK cell immunotherapy is mainly limited by insufficient numbers generated for adoptive transfer, limited *in vivo* life span after adoptive transfer, lack of cytotoxicity and some logistical concerns that impede its widespread implementation. Therefore there is a need to develop methods of NK cell expansion that provide stimulation similar to other immune cell types in the PBMC population. The second part of this study utilizes a method of *in vivo* NK cell expansion using a particle-based approach in which plasma membranes of K562-MB21-41BBL cells (K562 cells expressing membrane-bound IL-21 and 41BB ligand) are used for specific NK cell expansion from PBMCs. NK cells expanded with this method were cytotoxic, showed *in vivo* persistence and biodistribution in different organs.

Collectively, these studies show that NK cells are a major innate immune component that can recognize and kill the tumor. Their cytotoxic ability, using particle-based stimulation, can be enhanced for a second-line treatment of relapsed tumors such as in ovarian cancer as well as other cancer types.

Dedicated to
my parents, who always encouraged me to take on
adventures including this one, and without the support and
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LIST OF ABBREVIATIONS

HGS	high-grade serous
LGS	low-grade serous
EMT	epithelial- to-mesenchymal
IV	intravenous
IP	*intraperitoneal*
HIPEC	hyperthermic IP chemotherapy
CIK	cytokine induced killer
ACT	adoptive cell therapy
mAb	monoclonal antibody
VEGF	vascular endothelial growth factor
TIL	tumor infiltrating lymphocytes
GVHD	graft-vs-host disease
DC	dendritic cell
NK	natural killer
CAR	chimeric antigen receptor
PBMC	peripheral blood mononuclear cells
IFN	interferon
IL	interleukin
NSG	NOD.Cg-Prkdc^{scid} Il2rγ^{tm1Wjl}/SzJ
GFP	green fluorescent protein
IHC	immunohistochemistry

FITC	fluorescein isothiocyanate
CTLA-4	cytotoxic T lymphocyte antigen-4
PD-1	programmed cell death receptor-1
HLA	human leukocyte antigen
KIR	killer immunoglobulin receptor
Treg	regulatory T cell
TBI	total body irradiation
Hi-Cy/Flu	high dose cyclophosphamide/fludarabine
cGMP	current good manufacturing practices
PM21	plasma membrane bound IL-21
MDSC	myeloid derived suppressor cell
AAALAC	association for assessment and accreditation of laboratory animal care
IACUC	institutional animal care and use committee
Luc	luciferase gene
ANOVA	analysis of variance
LN	lymph node
IDO	indolamine 2,3 dioxygenase
AML	acute myelogenous leukemia
CML	chronic myelogenous leukemia
PB	peripheral blood
4-1BBL	4-1BB ligand
CTViolet	cell trace violet
AW	abdominal wash

ADCC	antibody dependent cell mediated cytotoxicity
BiKE	bispecific killer cell engager

CHAPTER 1: BACKGROUND

Ovarian cancer: The disease

Ovarian cancer is the most lethal amongst gyenecological malignancies. Due to an asymptomatic early phase and lack of reliable early detection methods, patients are diagnosed at an advanced stage of the disease. About 21,000 women are diagnosed each year and 14,000 die from the disease. This poor prognosis is attributed to metastasis, leading to 5-year survival rates of 70% and 30% for local and distant metastases respectively (American Cancer Society. Cancer Facts and Figures. New York: American Cancer Society; 2015).

Ovarian tumors can arise from different locations in the ovary like epithelial cells, stromal cells or germ cells, but majority (>90%) of the tumors are of epithelial origin. Based on different histotypes, cancers of epithelial origin are further characterized as high-grade serous (HGS), low-grade serous (LGS), mucinous, endometroid and clear cell. Amongst these, HGS is the most prevalent form and the most deadly because of late stage diagnosis when the tumor has already metastasized. Ovarian cancer is unique in the way it metastasizes, compared to other solid tumors. Tumor cells shed from the primary tumor site and spread through the peritoneal cavity, as opposed to following the classical mechanism of intravasation and extravasation to and from the blood or lymphatic system [1]. After they undergo an epithelial to mesenchymal transition (EMT), cells from the bulk of the tumor are distributed in the peritoneal cavity, mainly in the form of spheroids through the ascites fluid that builds up in the cavity as the disease advances [2]. This is why, despite the advances in the techniques for surgical debulking of the visible primary tumor, most patients succumb to disease relapse caused by free-floating single cells or spheroids that cannot be adequately removed by surgery.

Ovarian Cancer: Therapies

Chemotherapeutics

One of the first agents used intravenously (IV) for the treatment of ovarian cancer, Cisplatin, was discovered in the 1970s. Single agent response rate with Cisplatin was in the range of 13-30% [3]. Then, in the early 1990s, second-generation platinum based agent Carboplatin was introduced. Use of these agents predominantly in combination with Cyclophosphamide or Anthracyclin showed patient survival rates similar to that with Cisplatin [4]. These drugs were used as a standard of care for a few years before taxanes were introduced in the late 1990s. Since then, Carboplatin and Paclitaxel have been used in combination (3-week cycle) as a standard chemotherapeutic treatment for ovarian cancer [5].

Attempts were made to further improve the efficacy of the platinum/taxane-based approaches by utilizing more than two agents, together or sequentially but these strategies failed to show any further improved outcome in the overall patient survival [6]. Despite the successful outcomes of IV administered platinum/taxane-based therapies, tumor relapse rates remain high and 5-year survival rates are poor, depending on the site and extent of metastases.

A clinical trial was designed to compare the effects of IV and intraperitoneal (IP) administration of Cisplatin in previously untreated stage III epithelial ovarian cancer patients. This study showed a significant improvement in the overall survival of patients receiving Cisplatin IP (49 months) as compared to patients receiving IV treatment only (41 months) [7]. Several other studies since then, with better combinatorial regimes that were carried out after this trial also showed better patient survival in favor of IP therapy [8]. Despite the extension in overall ovarian cancer patient survival offered by IP chemotherapy, there were various

complications associated with it, such as increased hematologic and gastrointestinal cytotoxicity amongst other side effects [9].

Recently, a modified form of IP therapy has emerged for the treatment of ovarian cancer patients, called as hyperthermic IP chemotherapy (HIPEC), which offers some advantages [10]. HIPEC is applied at the time of cytoreductive surgery, without any interval between surgery and chemotherapy. Also, high volumes of chemotherapy can be delivered while the patient is under general anesthesia that ensures a homogenous distribution of the drug, which is otherwise difficult because of abdominal distention and pain. Most importantly, hyperthermia offers pharmacokinetic benefits and has been shown to improve tumor penetration of Cisplatin and DNA crosslinking [11, 12].

Even after obtaining data over several years, the utility of HIPEC is not accepted universally. Techniques, drugs and dosages differ and toxicity related issues with chemotherapy still remain unaddressed even after implementation of this technique. Even though the treatment of primary tumors has been improved with advances in chemotherapy regimens, tumor relapse rates in ovarian cancer patients remain high (70% for advanced stage and 20%-25% for stage I and II) [13] and there's a lack of credible second-line options for treating chemotherapy resistant relapsed tumors. Therefore, the challenge is to develop novel strategies that can be effective in these patients to prevent/treat relapse, extend progression free and overall survival, improving quality of life.

Immunotherapy

Overview

One of the strategies that has gained momentum in the past few years for cancer treatment is immunotherapy [14]. Under the umbrella of immunotherapy, there are various types of therapies that hold promise and are under development such as checkpoint inhibition, antibody-based therapies, cytokine-induced killer (CIK) cell-based therapies, adoptive cell therapies (ACT) etc.

Checkpoint inhibition: A main challenge that faces immunotherapy in ovarian cancer is the highly immunosuppressive tumor microenvironment. One of the most promising approaches towards overcoming this suppression has been the use of immune checkpoint inhibitors. The rationale behind checkpoint blockade strategy is to block immunomodulatory signals that dampen T cell responses. Main target molecules are CTLA-4 and PD-1 and PD-Ligand1 (PD-L1). Some of the ongoing clinical trials in patients with tumor types such as melanoma, renal cell carcinoma, pancreatic cancer etc. are showing promising results (NCT01666353, NCT01295827). For ovarian cancer treatment, there are several clinical trials using anti-CTLA-4 (Ipilimumab, Tremelimumab), anti-PD-1 (Nivolumab, Pembrolizumab) and anti-PD-L1 (BMS-936559, MEDI4736) antibodies that are underway whose results are awaited in terms of increasing overall survival of patients [15].

Antibody-based therapies: Some other antibody-based therapies have become well established in the past fifteen years for several tumor types and many such antibodies are being used in ovarian cancer treatment as well. For example, Bevacizumab, which is a monoclonal antibody (mAb) for vascular endothelial growth factor (VEGF), is one of the promising candidates for ovarian cancer as VEGF gene expression in ovarian tumor tissues correlates with

poor prognosis [16]. Use of this antibody in combination with chemotherapy showed an increase in the overall response rate from 11% to 27% [17]. Other antibodies that have shown promising results in the treatment of ovarian cancer are Cetuximab, Panitumumab, Catumaxomab and some antibodies targeting tumor-associated macrophages [18].

CIK cell-based therapies: CIK cells are a heterogeneous population of immune cells stimulated by multiple cytokines and are known to show anti-tumor activity [19] and $CD3^+$ $CD56^+$ NKT cells have been shown to be main effectors within this population [20]. This strategy is promising with respect to ovarian cancer because second–line treatment with CIK cells in advanced epithelial ovarian cancer patients has showed an improved progression-free survival (with some side-effects) [21]. However, its effect on overall survival of ovarian cancer patients is still unclear.

Adoptive Cell Therapy: This is a technique that uses anti-tumor lymphocytes for adoptive transfer into patients, in an autologous or allogeneic setting to induce tumor cytotoxicity. A seminal study published in 2003 showed clinical evidence that the presence of tumor infiltrating lymphocytes (TILs) in ovarian cancer patients was associated with favorable prognosis [22]. The 5-year overall survival rate of patients with tumors lacking TILs and the ones that contained TILs was 4.5% and 38% respectively. This study suggested that ovarian tumors may be susceptible to attack by immune cells and provided rationale for therapeutic applications for ovarian cancer that aim at bolstering immune cell function. Initial clinical trials utilizing ACT for the treatment of ovarian cancer used *ex vivo* activated autologous T cells from peripheral blood that were retargeted to the folate receptor by a bispecific monoclonal antibody [23]. Results showed an encouraging overall response rate of 27% without any major side effects. This study established that autologous T cells, if specifically redirected, could result into tumor regression

with minimal off-target effects. Alternatively, another study utilized endogenous TILs for T cell transfer instead of peripheral T cells. Patients were treated with TILs following surgical debulking of the tumors and chemotherapy. There was a 100% 3-year progression-free survival rate observed in these patients as compared to 67.5% in the group that received chemotherapy only [24]. Despite the promising anti-tumor effects observed in patients with different tumor types with ACT of autologous or allogeneic lymphocytes, many types of adverse effects have been reported. Some of them include cytokine release syndrome, hypothyroidism, vitiligo, autoimmune thyroiditis etc. [25]. ACT in patients with lymphomas have also been shown to suffer from respiratory obstruction, perhaps due to T cell induced inflammatory responses [26]. Apart from these side effects, graft vs host disease (GVHD) has also been one of the major side effects of ACT that can limit its beneficial effects [27].

T cell based adoptive cell therapy

Development of chimeric antigen receptor (CAR) T cell technology has given a strong boost to T cell based adoptive cell therapy in several types of cancers, including ovarian cancer. Studies with the ID8 model of ovarian cancer indicate development of anti-tumor immunity in mice and tumor free survival with no relapse. This study utilizes engineered T cells that express an NKG2D CAR that imparts specificity to them. After regression of the tumors, mice were also shown to develop T cell memory, as a result of which 90% of the mice rejected tumor re-challenge [28]. Following promising results in mouse models, some other ovarian tumor specific antigens are now being utilized for generation of CAR-T cells and their efficacy is being tested in patients. For example, a phase I clinical trial is using autologous adoptive transfer of CAR-Ts with specificity towards the folate receptor found on ovarian tumor cells [29] [30]. Such studies

confirm that large amounts of modified T cells can be safely administered into patients, but there are some major concerns with off-target effects and cytokine release syndromes, GVHD etc. [31]. Even though T cell infusions are well tolerated in patients, it failed to show anti-tumor responses in ovarian cancer patients because of poor trafficking and complications with *in vivo* persistence and function [30].

NK cell based adoptive cell therapy

In contrast to T cells, NK cells are lymphocytes of the innate immune system that target virally infected or transformed tumor cells. The genes coding for receptors on NK cells do not undergo any rearrangements to give them specificity against particular target antigens, but are germline encoded and their specificities to certain antigens is pre-determined. The unique feature of NK cells is that they have diverse inhibitory and activating receptors. Engagement of these receptors with their corresponding ligands on target cells determines activity of NK cells. Absence of an inhibitory ligand on the target cell can trigger the NK cell to eliminate the target. For example, in the case of tumor cells that lack HLA-I on their surface, which is a ligand for killer immunoglobulin receptor (KIR) on NK cells, NK cells get activated and degranulate to release granzymes stored in their cytoplasmic granules and kill the target cell. This is also called as the 'missing self hypothesis' of NK cell activation [32]. Because of this unique ability of NK cells to recognize tumor targets that downregulate class-I MHC molecules, perhaps to evade T cell mediated killing, they are a very important avenue for developing tumor-targeting strategies.

Autologous NK cell adoptive therapy: NK cells have been exploited for cancer treatment for a long time using various techniques to activate their cytotoxic ability. One of the strategies was with cytokines such as IL-2, IL-15, IL-21, IL-18, IL-12 and type-I IFNs administered *in vivo*

(systemically)[33]. But systemic administration of cytokines was associated with severe side effects in patients such as vascular leak syndrome, in addition to Treg cell expansion that directly inhibits NK cell function [34, 35]. Instead of systemic administration of cytokines, adoptive transfer of NK cells expanded *ex vivo* was attempted and showed improved responses in breast cancer patients [36] without severe side effects. However, this strategy also showed marginal *in vivo* anti-tumor responses because autologous NK cells inherently, can be insufficiently cytotoxic towards tumors [37].

Allogeneic NK cell adoptive therapy: Unlike autologous NK cell transfer, allogeneic transfer offers benefits because of mismatch between KIRs on donor NKs and KIR ligands in the host. There was greater anti-tumor activity demonstrated by allogeneic NK cells adoptively transferred into patients as compared to their own stimulated NK cells. Donors for these cells are selected based on established KIR mismatch criteria [38]. This strategy has been successful in solid tumors and leukemia with relatively minimal side effects [39-41]. A very important factor for the success of this regimen was lymphodepleting the patients before NK cell infusion, either by total body irradiation (TBI) or by using chemotherapy. Efficacy of this procedure was defined in a trial with metastatic melanoma and renal carcinoma patients treated with high-dose cyclophosphamide and fludarabine (Hi-Cy/Flu) chemotherapeutics (lymphodepleting) before adoptive transfer of NK cells [39]. Efficacy of allogeneic NK cell transfer has also been tested in solid tumors such as breast and ovarian cancer [42]. In this study, patients did not show optimal NK cell expansion *in vivo* following chemotherapy, so they were subjected to TBI in order to improve expansion. Unfortunately, there was no benefit seen in terms of NK cell expansion. Also, patient Treg population was found to be increased at 14 days after NK cell infusion, which may have further limited NK cell expansion. Another drawback with this strategy is that due to

KIR mismatch in the allogeneic NK cells, they eventually get rejected by the host's effector T cells, suppressed by myeloid suppressor cells and are negatively effected by a myriad of other host factors [43]. Therefore, even after incremental advances in the field of adoptive transfer of NK cells, we still need to address the problems of optimal *in vivo* persistence, expansion and cytotoxicity.

NK cell expansion for clinical applications

For obtaining sufficient numbers of functional NK cells, several methods have been developed in recent years [44]. NK cells can be expanded from bone marrow, cord blood, embryonic stem cells and peripheral blood. Irradiated autologous feeder cells, Epstein-Barr virus-transformed lymphoblastoid cell lines and modified K562 cells have been used for NK cell expansion in the past [45]. Campana *et al* showed that large-scale expansion and activation of NK cells was possible using modified K562 cells that express membrane-bound IL-15 and 4-1BB ligand (K562-mb15-41BBL) [46]. NK cells expanded with this method, expand several hundred fold for up to two weeks but then experience senescence [47]. Moreover, this methodology using feeder cell cultures for NK cell expansion is not completely safe. Firstly, with co-cultures of NK cells with feeder cells, there is always a possibility of feeder cells or their genetic material being injected into patients. Secondly, co-culture systems are complex, variable and require cGMP facilities that make the process less feasible for widespread application. Thirdly, these is a need for a final check process to ensure that there are no feeder cells present in the product being infused into patients. In order to overcome these impediments, a novel NK cell expansion method was developed that did not require live feeder cells but instead used the plasma membranes obtained from K562-MB15-41BBL cells. Since the membranes of these cells

express NK stimulating molecules, using just the plasma membranes would be capable of stimulating NK cells without the drawbacks associated with live feeder cells [48]. Apart from membrane bound IL-15, K562-MB21-41BBL cells have also been used for expansion of NK cells showing sustained *ex vivo* proliferation [49]. Here in this study, we used plasma membranes derived from K562-MB21-41BBL cells (PM21) and tested the *in vivo* expansion of NK cells activated with PM21 particles.

Functional properties of NK cells make them applicable for clinical use in the treatment of cancers, but there has been limited success in harnessing the complete potential of this cell type, specially in fighting solid tumor types. In order to develop NK cell therapy to its full potential, there is a need to fully understand NK cell biology and how it reacts to the presence of tumor and also device methods for achieving optimal *in vivo* NK cell expansion, persistence and tumor cytotoxicity.

CHAPTER 2: ANTI-OVARIAN TUMOR RESPONSE OF DONOR PERIPHERAL BLOOD MONONUCLEAR CELLS IS DUE TO INFILTRATING CYTOTOXIC NK CELLS

Introduction

Ovarian cancer is the second leading gynecological cancer [50]. Although current chemotherapy using carboplatin-paclitaxel is effective as a first-line therapy, 70% of patients with advanced disease succumb to tumor relapse within less than five years, despite initial response to chemotherapy [51]. Thus, there is an urgency to develop strategies for prevention of tumor relapse and for increasing overall patient survival. The immune system is protective against ovarian cancer and thus, adjuvant immunotherapy post-surgery and with chemotherapeutics could be effective for preventing relapse and extending survival [22, 52]. Studies show the link between cancer stem cells and ovarian tumor recurrence, and that treatment with chemotherapeutics selects resistant tumor populations causing increased risk of tumor relapse [53, 54]. Such resistant tumor cell populations are susceptible to lymphocyte-mediated lysis, particularly by NK cells [55]. Therefore, NK cell-based adjuvant immunotherapy could have significant impact on sustaining remission and improving overall treatment efficacy.

Immunotherapy has impacted treatment of multiple cancers. Monoclonal antibody-based approaches are beneficial for long-term effects, but a challenge is potential escape by tumors [56]. Adoptive cell therapy (ACT) with different immune cell types, T cells, dendritic cells (DCs) or NK cells in clinical trials hold promise. A landmark publication in 2003 showed that the infiltration of T cells into ovarian tumors improved survival [22]. Since tumor infiltrating immune cells influence the severity and overall disease outcome, attempts have been made to adoptively transfer TILs after lymphodepletion with successful results in metastatic melanoma

patients [57, 58]. DCs can activate the adaptive immune system for a robust response against infection or against transformed cells, and trials using DCs are ongoing (Clinicaltrials.gov identifier: NCT01875653). DCs function as antigen presenting cells for naïve CD4 and CD8 T cells [59]. To circumvent the need for T cell "education", chimeric antigen receptor (CAR)-T methods can genetically modify patient T cells to express a targeting receptor for a tumor specific antigen. In contrast to naïve $CD4^+$ and $CD8^+$ T cells that need to be "educated" or engineered *ex vivo*, innate NK cells are naturally cytotoxic towards malignant cells [60]. *In vitro* studies show that resting NK cells from healthy donors target isolated tumor cells from the peritoneal ascites of ovarian carcinoma patients [61]. In this respect, ACT using cytolytic NK cells for cancer treatment is more advantageous since NK cells do not require prior sensitization with an antigen and are not limited to targeting only tumors that have a specific marker as in CAR-T methods [62].

Clinical studies for ovarian and breast cancer using IV delivered NK cells enriched by CD3 depletion of PBMCs from haploidentical donors failed to show *in vivo* NK cell expansion perhaps due to suppression by host Treg cells or myeloid-derived suppressor cells (MDSCs) [42]. Therefore, there is still an insufficient understanding about factors required for NK cell expansion and *in vivo* persistence for successful clinical outcome. A previous pre-clinical study showed that IP delivered enriched NK cells could have anti-tumor response against ovarian cancer, and that NK cell cytotoxicity may be affected by the mode of delivery that could bypass hurdles of NK cell homing to the tumor location [63].

The quality of immune response to ovarian cancer has a significant impact on disease prognosis [64-66]. In the context of a complete immune system, innate NK cells can have direct cytotoxicity towards transformed cells as well as interact with DCs to induce interferon IFN-γ

production which primes Th1 cells [67] and further enhances cytotoxic T cell responses [68]. NK cells are indispensable for effective DC-based immunotherapy, as loss of NK cells has shown to result in defective tumor immunity [69]. Such studies highlight the importance of NK cell interactions with both, innate and adaptive immune cell types, to affect adaptive immunity for effective anti-tumor response.

Here, we examine NK and T cells' response to tumor as part of an unbiased whole PBMC population as opposed to treating with selectively enriched NK or T cell populations. The study examines the kinetics of effector subtypes involved in the acute anti-tumor response of innate and adaptive components of PBMCs and identifies NK cells as the main effector cell of PBMCs' response, acting as a first line of anti-tumor defense. It also highlights the importance and points to the need for further studies to delineate other interacting immune cell types to strategically employ them as an adjuvant regimen for a safe and effective NK cell-based immunotherapeutic approach.

Materials & Methods

Animals

Animal care and use was at the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited University of Central Florida (UCF) vivarium. Protocols were approved by the UCF Institutional Animal Care and Use Committee (IACUC) and were compliant with NIH guidelines. NOD.Cg-*Prkdc*^{scid} *Il2r*γ^{tm1Wjl}/SzJ (NSG) mice were obtained through an institutional material transfer agreement with Jackson Laboratory, Bar Harbor, Maine, USA.

Human Ovarian Tumor Cells and Engraftment in Mice

Human ovarian tumor cell line SKOV-3/GFP-Luc was obtained from Cell BioLabs, Inc. (San Diego, CA, USA). The cell line was passaged in an NSG mouse once, then sorted for green fluorescent protein (GFP) expressing tumor cells using a cell sorter (FACSAria), BD Biosciences, Franklin Lakes, NJ, USA). NSG mice were IP injected with $1x10^6$ SKOV-3-GFP/Luc cells and tumors seeded for 7-10 days. Tumor growth was monitored by *in vivo* bioluminescent imaging.

In vivo Imaging

SKOV-3/GFP-Luc tumor formation and growth was monitored using the Xenogen *In vivo* Imaging System (IVIS, Caliper Life Sciences, Hopkinton, MA, USA). Luciferin (Gold Biotechnology, Inc., St. Louis, MO, USA,) was IP injected at a dose of 0.15mg/g body weight fifteen minutes prior to imaging. Maximum/minimum of the luminescence signal intensity was adjusted to be the same throughout the experiment (Living Image software, Perkin Elmer, Waltham, MA, USA).

Human PBMCs

Human PBMCs were obtained from healthy human donor source leukocytes (OneBlood, Orlando, FL, USA) by standard Ficoll gradient. Isolated PBMCs were tested for viability using Annexin V staining and NK cell percentages by flow cytometry using a BD Accuri C6, BD Biosciences, San Jose, CA, USA) following staining by CD45, CD3 and CD56 antibodies. The initial NK cell percentages for PBMCs used in the experiments were 8-11% of total $CD45^+$ cells. After tumor engraftment (7-10 days after inoculation), 1 x 10^6 viable PBMCs and 1,000U human IL-2 (Preprotech, Rocky Hill, NJ, USA) were IP injected. 1,000U IL-2 injections were given to mice weekly on Monday, Wednesday and Friday throughout the experiments.

Flow Cytometry

Fifteen minutes after subcutaneous saline injections to hydrate the animals, <100 μL of peripheral mouse blood was collected by submandibular bleeds once a week. 50 μL of blood was treated with red blood cell lysis buffer and stained with fluorophore conjugated antibodies against hCD45 (eBiosciences, San Diego, CA, USA), hCD56 (Miltenyi Biotec, Bergisch Gladbach, Germany) and hCD3 (Beckman Coulter, Brea, CA, USA). Peritoneal washes obtained by flushing ~2mL PBS in the peritoneal cavity were also processed similarly. Analysis of the data was done using the software Flowlogic (Inivai Technologies, Victoria, Australia). All NK cells ($CD56^+$, $CD3^-$) were first gated on human $CD45^+$ cells.

Histology

Tumors were fixed in 10% neutral-buffered formalin (Surgipath, Leica, Buffalo Grove, IL). Tissue was processed, embedded in paraffin blocks and 5 μm sections were stained. Immunohistochemistry (IHC) was done using Leica's BondMax automated immunostainer.

Antibodies included NKG2D (GeneTex, Inc., Irvine, CA, USA). Granzyme B (Spring Bioscience, Pleasanton, CA, USA), Cleaved Caspase 3 (Cell Signaling Technology, Inc., Danvers, MA, USA), CD3 (Abcam Cambridge, MA, USA).

Granzyme Assay

From the tumors of mice treated for 14 days with PBMCs, cells were perfused using 18 gauge needles. They were washed and incubated with SKOV-3/GFP-Luc cells (50,000/well) on a 96-well plate for 1 hour. Cells were stained with anti-CD45, -CD3 and -CD56 antibodies in a granzyme substrate (PanToxiLux kit; Oncolmmunin, Gaithersburg, MD, USA). After 30min incubation with antibodies, samples were analyzed by flow cytometry (CantoII, BD Biosciences) and analyzed using Flowlogic software. To analyze the granzyme substrate signal (Fluorescein isothiocyanate, FITC) in the background fluorescence from the endogenous GFP of SKOV-3/GFP-Luc cells present in the NK cell (or T cell)-SKOV-3/GFP-Luc conjugates, a sample without granzyme substrate was used to define the fluorescent intensity for events correlating to cells that lack granzyme activity (Figure 4b).

Cytotoxicity Assay

SKOV-3/GFP-Luc cells were plated on a 96-well plate at 10,000 cells/well and incubated overnight at 37°C. NK cells (EasySep human NK cell enrichment kit, Stem Cell Technologies Inc., Vancouver, BC, Canada) were stimulated with 100 U IL-2 overnight and then incubated with plated tumor cells at different Effector (NK cells) : Target (tumor cells) ratios (E:T) for 48 hours. Cytotoxicity was measured using the CytotoxGlo assay (Promega Corp., Madison, WI, USA) as per manufacturer's instructions.

Results

Treatment with unselected healthy PBMCs clears human ovarian tumors engrafted in mice.

The interplay among multiple immune cell types in response to the presence of a tumor is complex and is still poorly understood. To address the therapeutic effectiveness of unselected immune cells from normal donor PBMCs in response to the presence of tumor, NSG mice that were IP inoculated with 1 x 10^6 SKOV-3/GFP-Luc cells were monitored for engraftment. Mice that showed engraftment 7 days post inoculation were then treated with PBS as a control, low dose IL-2 only (1,000 U thrice weekly), or with human PBMC+IL-2. Another group of non-tumor bearing mice was injected with PBMC+IL-2 as a control. Treatment effectiveness was assessed by monitoring tumor size and overall health for 7 weeks after starting treatments. Serial imaging (Figure 1a) shows significant differences in tumor progression between the mice injected with PBS or IL-2 only ('untreated' group) and the 'PBMC+IL-2 treated' group. Untreated mice succumbed to disease in ~3 weeks, whereas tumor-engrafted mice treated with PBMC+IL-2 showed reducing tumor burden. Figure 1a shows reduction of tumor size in the treated group and total luminescence flux from the peritoneal tumor images acquired after PBMC injections (Figure 1b) also demonstrates the effect of treatment compared with no treatment ($p=0.0003$, two-way ANOVA). A clear survival difference was observed in PBMC-treated compared to untreated mice ($p=0.001$, Log-rank Test) (Figure 1c). Untreated mice were euthanized upon health deterioration including abdominal bloating due to ascites and hunched posture. Overall, tumor-engrafted mice treated IP with whole PBMCs showed tumor regression within 3 weeks of PBMC treatment as compared to the untreated mice that succumbed to the disease within the same time frame.

Expansion of NK cells in peripheral blood and peritoneal cavity of PBMC treated tumor-engrafted mice

To determine if the cytolytic effector components in the anti-tumor response with the PBMC+IL-2 treatment is due to a single or synergistic lymphocyte populations in the allografted PBMCs, levels of immune cells were serially tracked in mouse peripheral blood for up to 7 weeks after PBMC injection. Delivering unselected healthy donor PBMCs along with low-dose IL-2, the expansion of NK cells was observed in the blood of only mice seeded with tumor. Also, mice that were injected only with PBMCs+IL-2 but without any tumor, showed no engraftment of NK cells (Figure 2). In a subsequent experiment, sets of mice treated with PBMC+IL-2 were collected on days 7, 14 and 21 post-treatment to examine the human lymphocyte population and their interaction with the tumor in the intraperitoneal cavity (Figure 3). Similar to the previous experiment, treatment of tumor-seeded mice with PBMC and IL-2 delivered IP resulted in reduced tumor and complete clearance by 21 days after initiation of treatment. For each of the mice sacrificed on days 7, 14 and 21, cells were collected from the intraperitoneal cavity and were analyzed by flow cytometry. As seen previously in the analysis of peripheral blood, NK cell expansion occurred only in the presence of tumor, and despite IL-2 stimulation, mice without any tumor did not show NK cell engraftment, expansion or persistence. NK cell number peaked at day 14, when tumor signal intensity was also at its peak (Figure 1b) and then started to decline as the tumor regressed. Data from the blood analyzed during this experiment is shown in Supplemental Figure 1 with a similar trend as seen in the blood analysis of the survival experiment (Figure 2). These results show that the presence of tumor elicits specific expansion of NK cells from an unselected PBMC population, in the presence of low dose IL-2.

NK cells infiltrate tumor and show markers of cytotoxicity

To corroborate that NK cell expansion in the presence of tumor *in vivo* and subsequent tumor remission was due to NK cell mediated cytotoxicity, shrinking tumors were collected from 14 day treated mice and from untreated mice for analysis by histology. NKG2D was used as an NK cell marker because it is one of the main activating receptors on NK cells that recognizes various stress induced ligands on tumor cells (22). Staining of a tumor nodule section (Day 14 after PBMC inoculation) from the PBMC+IL-2 treated group with anti-NKG2D showed positive NKG2D staining (Figure 4), which is indicative of intratumoral infiltration of NK cells. Granzyme release was observed in the same region of the tumor. This is consistent with the presence of granzyme containing cytotoxic granules released from NK cells upon tumor cell recognition to mediate apoptosis by caspase dependent pathways [70]. Detection of cleaved caspase 3 by staining in the same region as that of granzyme release confirms NK cell induced apoptosis of tumor cells (Figure 4).

Since T cell expansion was observed in the peripheral blood (Supplemental Figure 2) and the peritoneal cavity (Supplemental Figure 3), their presence was also examined by staining with anti-CD3. CD3 staining was minimal (last panel in Figure 4) as compared to NKG2D staining in the same region of the tumor or as compared to CD3 staining in a human lymph node used as a positive control, thus confirming that the cytotoxicity markers were attributable to infiltrating NK cells killing tumors cells and not T cells. The NKG2D staining appears to be dim in the region shown, perhaps because of actively dying cells in that area due to granzyme release. A different region of the tumor shows clearer NKG2D staining (Supplemental Figure 4). These results indicate that NK cells expand in the vicinity of the tumors (peritoneal cavity) and

infiltrate into the small tumor nodules to cause tumor regression observed in the PBMC+IL-2 treated mice.

NK cells show direct cytotoxicity towards tumor cells ex vivo

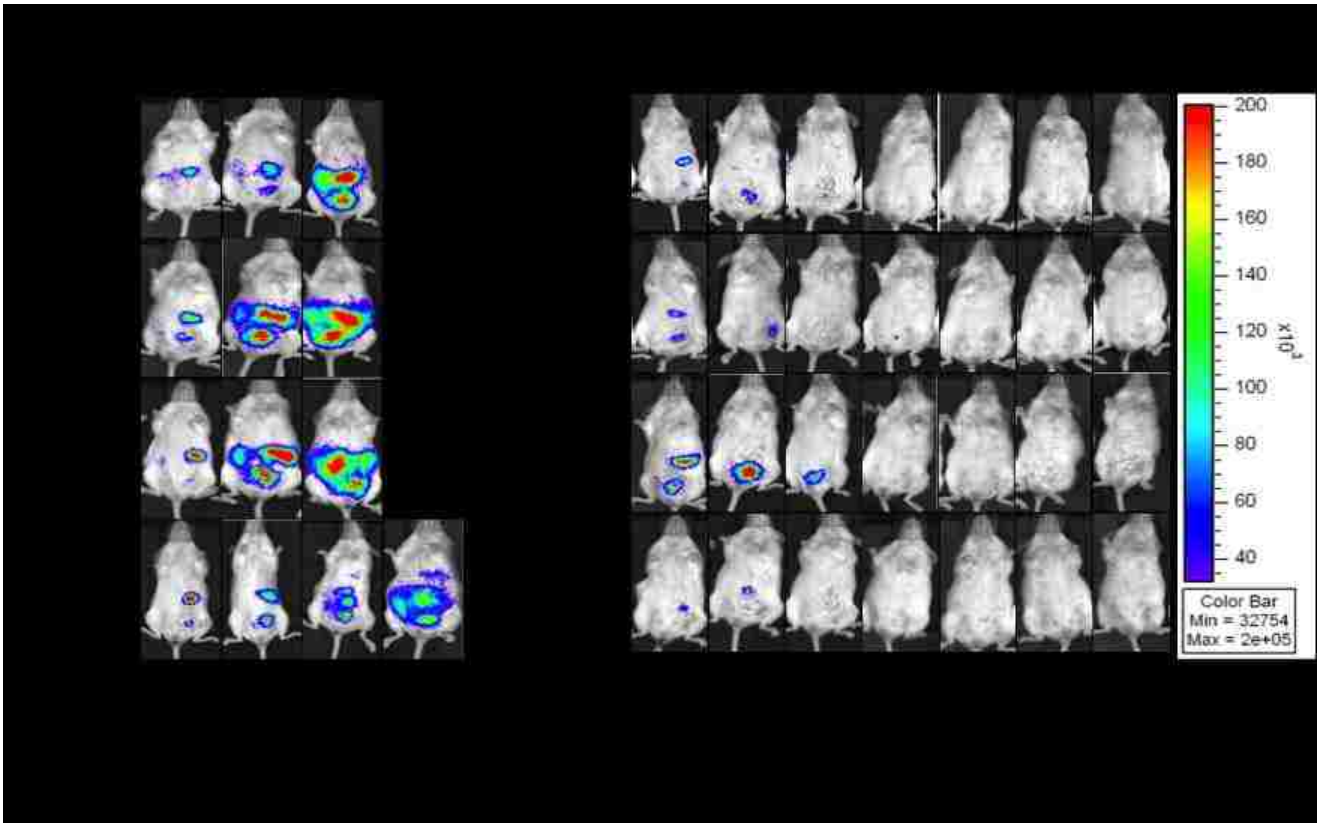
A cytotoxicity assay (CytotoxGlo) was done with enriched NK cells from the same PBMC source as used in the experiment shown in figure 3 to show that NK cells present in the PBMC source were cytotoxic towards the tumor cells. Reducing cytotoxicity of tumor cells was seen with decreasing E:T ratios (figure 6). To confirm that the intratumoral NK cells were also cytotoxic and not anergic, intratumoral perfusions were isolated from two tumor bearing mice being treated with PBMCs+IL-2 for 14 days and were tested for granzyme activity as discussed in the methods section. Flow cytometric analysis of granzyme activity in singlet vs. doublet tumor-lymphocyte conjugate populations was performed as previously described [71], to determine the cytotoxic response of intratumoral NK and T cells against fresh SKOV-3/GFP-Luc cells (Figure 13). Differences in scatter properties (forward scatter height vs area plots) and CD45 intensity were used to determine the conjugates (tumor cells and immune cells) and singlet cell populations. Populations were further gated for $CD56^{+}$, $CD3^{-}$ NK cells and $CD3^{+}$ T cells, and for signal from granzyme activity. Figure 5 represents the events positive for granzyme activity seen in singlet and conjugate cells. Overall, significant difference ($p=0.0002$, two-way ANOVA) was observed in the number of granzyme-positive NK cells as compared to T cells with majority of the NK cells forming conjugates with tumor cells. These results show that isolated intratumoral NK cells are not anergic and highly cytotoxic towards SKOV-3/GFP-Luc tumor cells, and gives strong evidence that the tumor shrinkage effect observed *in vivo* is due to cytolytic activity from NK cells and not T cells.

Whole PBMC treatment results in longer survival as compared to treatment with PBMCs depleted of one or more cell types

In order to show that the cytotoxicity against tumor and overall survival of tumor bearing animals is optimal when they are treated with NK cells as a part of the whole PBMC population, an experiment was set up in which mice inoculated with the tumor cells for a week were treated with isolated NK cells, NK cell depleted PBMCs and T cell depleted PBMCs. All mice received 1000 U of IL-2 IP, thrice a week for the entire length of the study. Figure 7a shows *in vivo* imaging of all the animals over 7 weeks after the start of treatment (Day 0). Untreated mice started to decline in health in the third week after the start of treatments. 'Isolated NK cell treated' group as well as the 'T cell depleted PBMCs' group also started to decline in health because of growing tumors and no expansion of lymphocytes, as seen in figure 8. This was perhaps due to the lack of sustained IL-2 supply form T cells necessary for NK cell to thrive, in addition to low-dose IL-2 provided exogenously. On the other hand, mice in the whole 'PBMC treated' group and the 'NK cell depleted PBMCs treated' group showed extended survival, as shown in figure 7b. Even though, mice in both of these groups showed tumor remission, mice in the NK cell depleted PBMCs treatment group showed signs of GVHD. Mice appeared to be suffering from diarrhea and eventually became lethargic and weak before they died or were euthanized because of severely declining health condition. Mice in the whole PBMC treated group however, survived longer but ultimately succumbed to GVHD phenotypes a week later. Figure 8 shows data obtained from weekly blood analysis done using flow cytometry. Mice in the PBMC treated group show high NK cell numbers on day 14, which is consistent with the earlier experiments that showed NK cells to be the cytotoxic population. Mice treated with NK cell depleted PBMCs do not show any NK cells (as expected), but show expansion of NKT cells

and T cells. Perhaps, the tumor clearance in this group of animals despite the absence of NK cells could be attributed to these cell types. However, after tumor clearance, high number of T cells in this group (day 28) contributed to GVHD-like symptoms and ultimately caused death whereas PBMC treated animals who had relatively low number of T cells survived for another week. In conclusion, overall health with respect to the tumor and GVHD symptoms, was found to be best in PBMC treated mice amongst the conditions tested here.

Figures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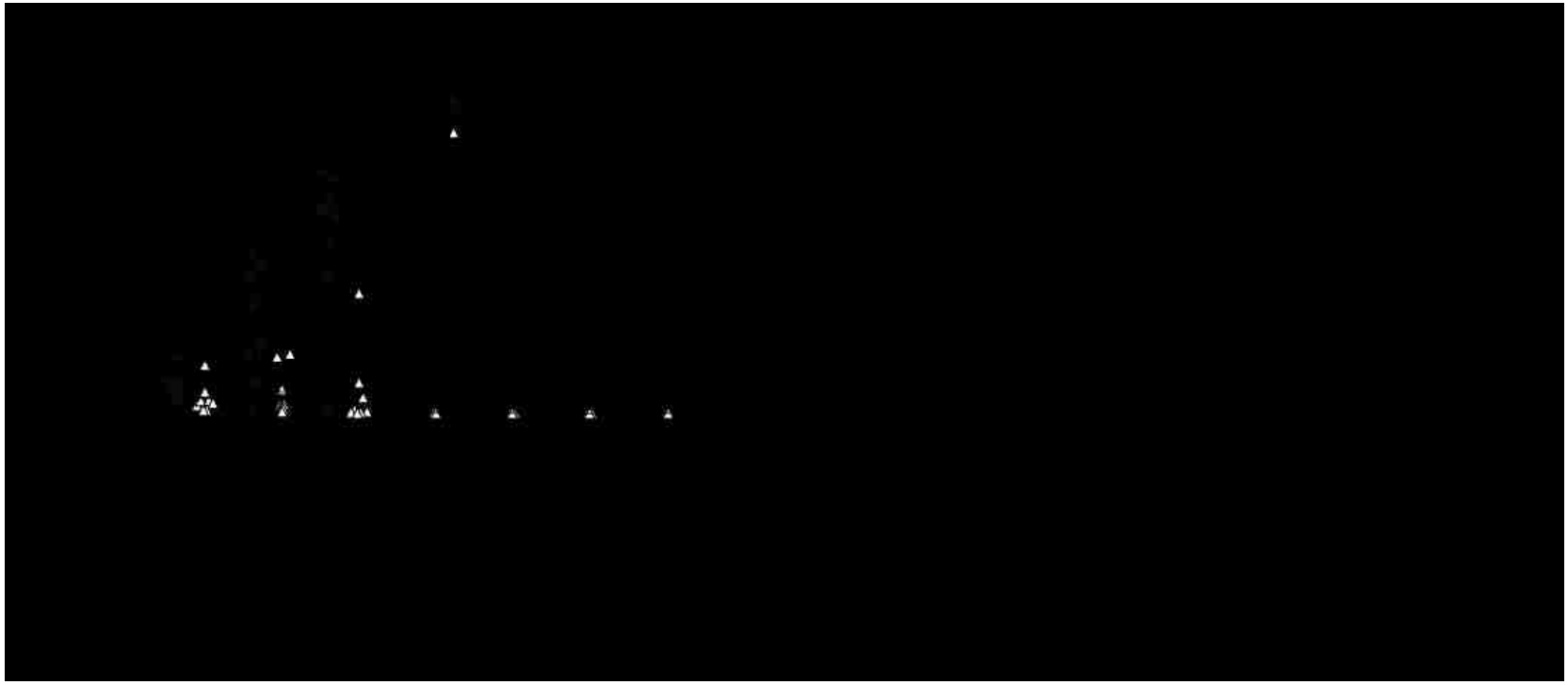


Figure 1: SKOV-3/GFP-Luc tumor bearing mice show complete remission of tumor upon healthy human PBMC and IL-2 treatment.

a) Bioluminescence of representative mice showing SKOV-3/GFP-Luc tumor burden over 7 weeks. Mice shown in the left panel were untreated (PBS or IL-2 only), whereas the ones in the right panel were treated with PBMCs+IL-2 and monitored for tumor growth. b) Quantification of the tumor signal (total flux) obtained from imaging. P-value was <0.001 for the first three time-points (two-way ANOVA, GraphPad Prism). c) Curve showing the difference in overall survival of the untreated and PBMC treated mice from the day of tumor cell inoculation. p= 0.001 (Log-rank test, GraphPad Prism).

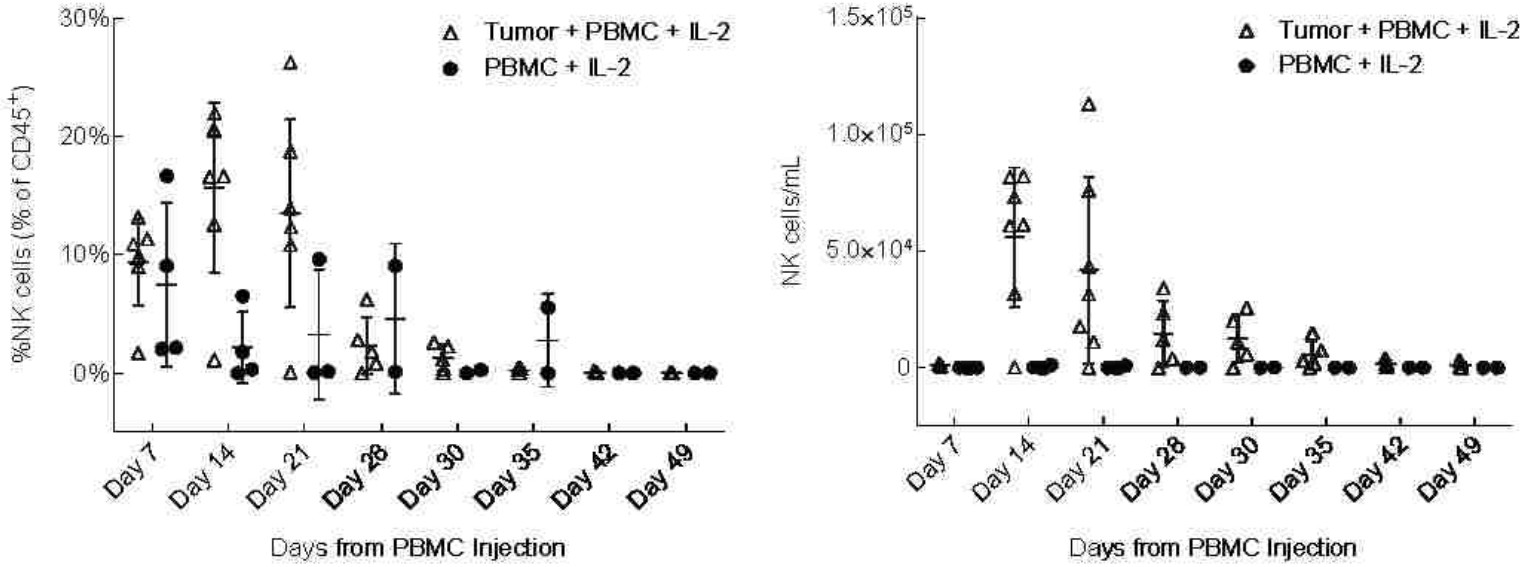
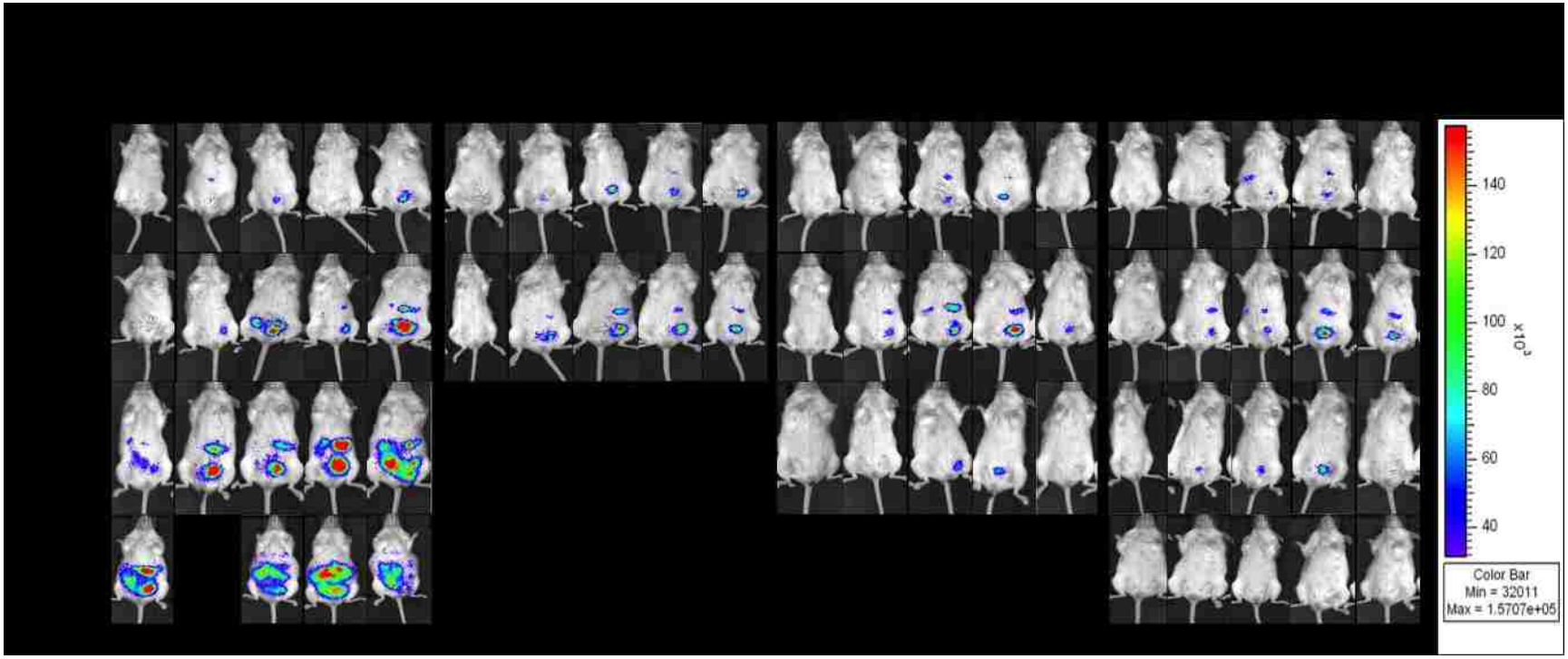


Figure 2: NK cells increase in peripheral blood in response to the presence of engrafted tumor.

Plots show the fraction of human lymphocytes (left) and concentration (right) of human $CD45^+$, $CD56^+$, $CD3^-$ NK cells in mouse peripheral blood at the indicated number of days after initiation of treatment. p=0.005 (left); p= 0.009 (right) (two-way ANOVA, GraphPad Prism).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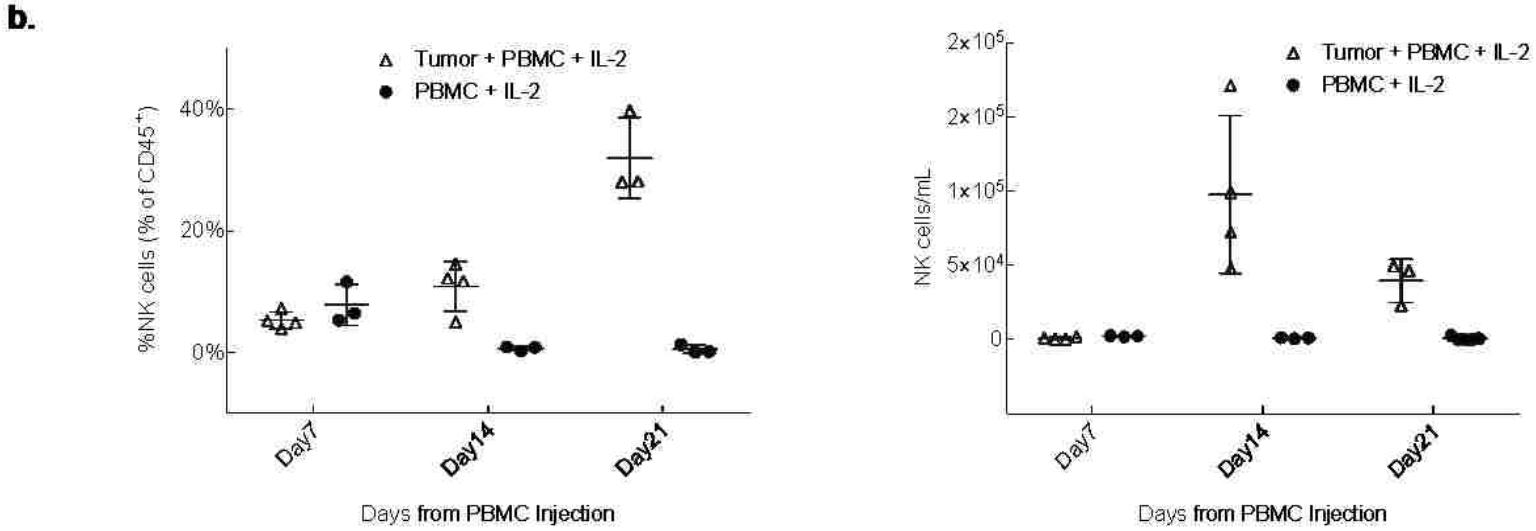


Figure 3: NK cells from whole PBMCs expand in the presence of tumor within the peritoneal cavity.

a) After 10 days of SKOV-3/GFP-Luc engraftment, 3 groups were treated with $1x10^6$ human PBMC+IL-2. Five mice were euthanized every week for 3 weeks for the analysis of peritoneal cavity wash. Another 3 groups of mice (not shown here) with no tumor and injected only with PBMCs+IL-2 were also collected along with the tumor bearing mice, as controls. b) Plots show the trend of expansion and reduction of NK cells in the peritoneal cavity expressed as fraction of human lymphocytes (left) and as concentration (right) over a time period of 3 weeks. p=0.0001(left); p=0.0052 (right) (two-way ANOVA, GraphPad Prism).

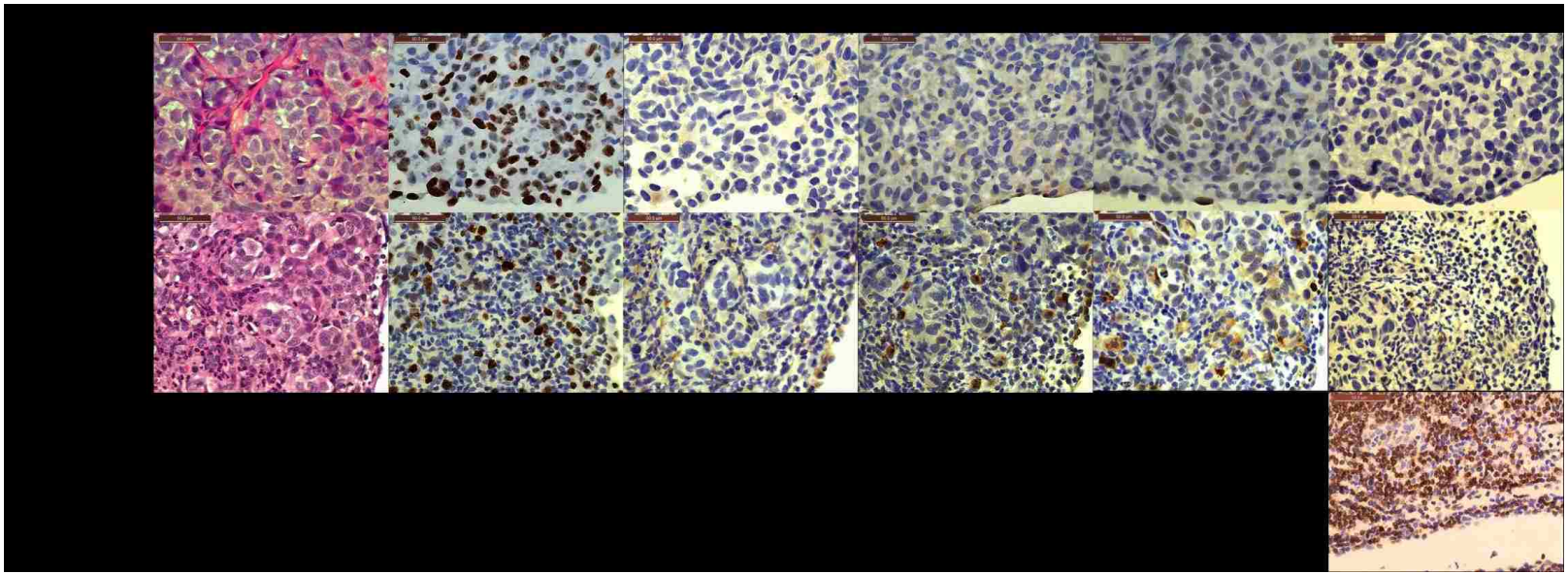


Figure 4: *In vivo* expanded NK cells infiltrate tumors and mediate tumor killing.

The left panel shows representative images of a tumor nodule from the 'untreated' group of animals; the right panel shows that of 'PBMC+IL-2 treated' ones. The H&E image in the top panel shows infiltrating immune cells compared to the untreated tumor. Tumor cells have Ki67 staining indicating proliferation. NKG2D staining shows NK cells within the tumor of the PBMC+IL-2 treated group. Granzyme B staining shows NK cell granules containing granzyme which mediate target cell lysis. Cleaved Caspase 3 staining indicates apoptosis in the tumor cells. The last panel shows the lack of CD3 staining in the same region of the tumor showing apoptosis. A human lymph node (LN) is shown as a positive control for the human CD3 antibody. All images were acquired using a 40X objective and scale bars represent a length of 50µm.

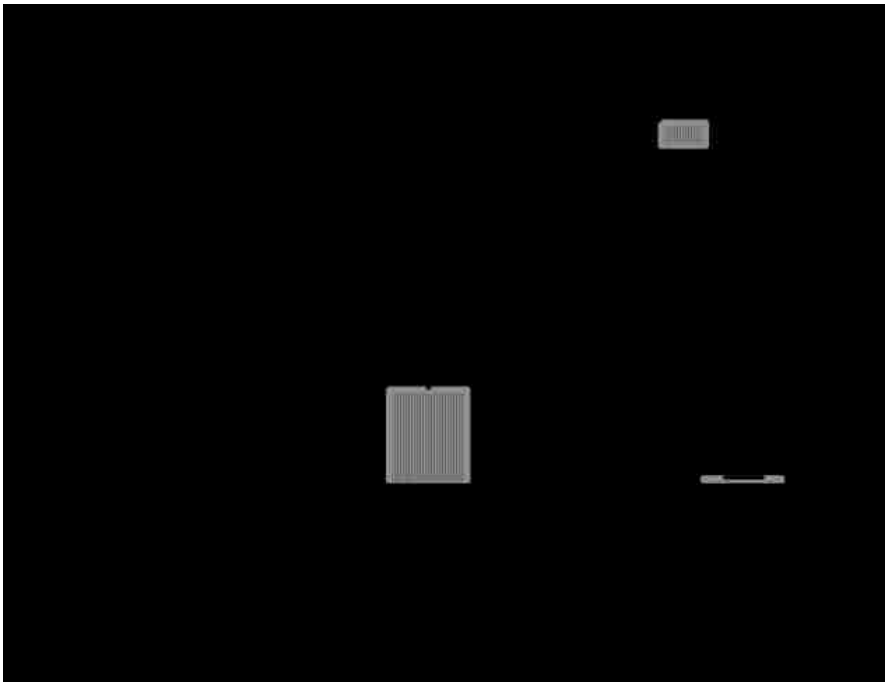


Figure 5: Granzyme assay showing actively cytotoxic NK cells perfused from tumors.

Graph representing granzyme positive events of NK and T cells present in $CD45^+$ singlet or conjugated populations upon perfusion from tumors. p=0.0002 (two-way ANOVA).

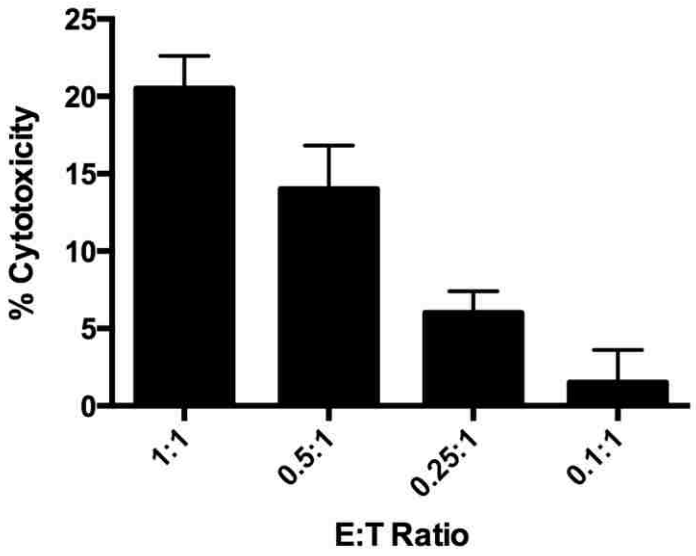
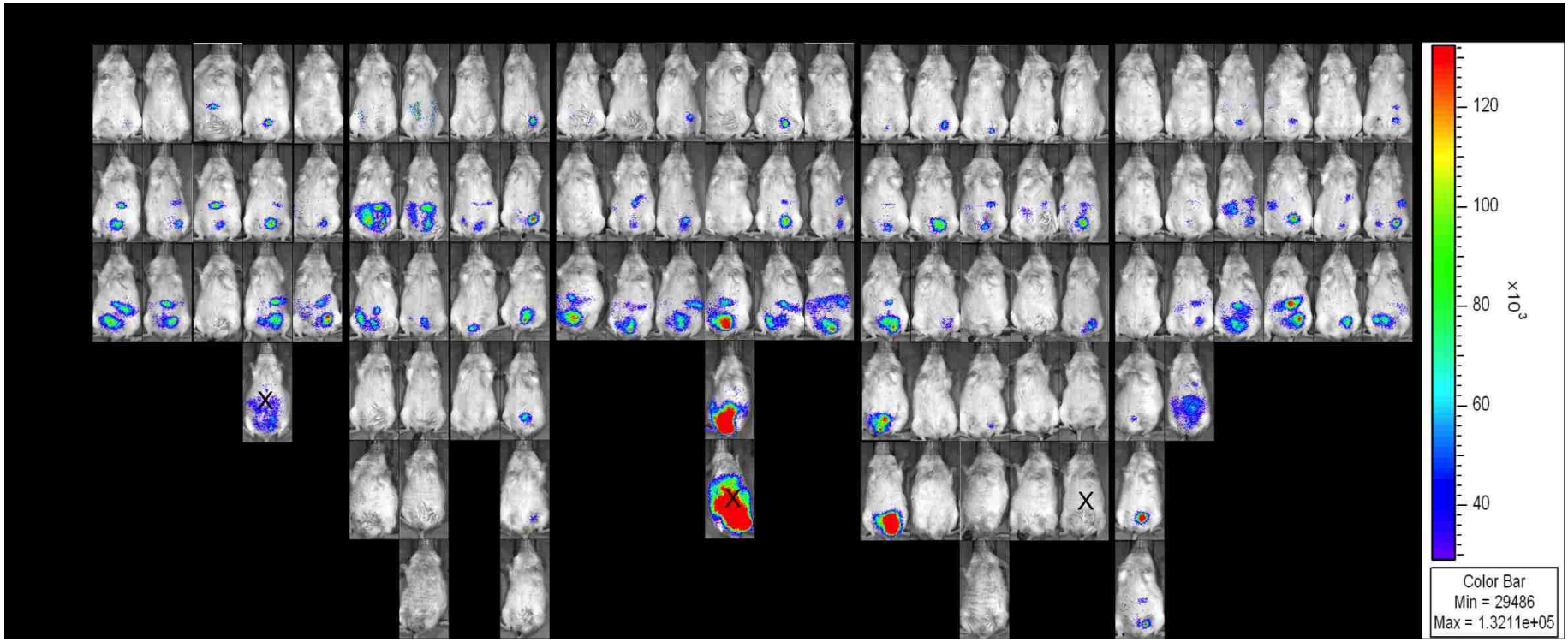


Figure 6: Cytotoxicity against SKOV-3/GFP-Luc is NK cell dependent.

Graph showing %cytotoxicity by isolated NK cells (effector) in SKOV-3/GFP-Luc (target) cells at different effector : target (E:T) ratios. Decreasing number of NK cells against the same number of tumor cells shows a decreasing trend in cytotoxicity.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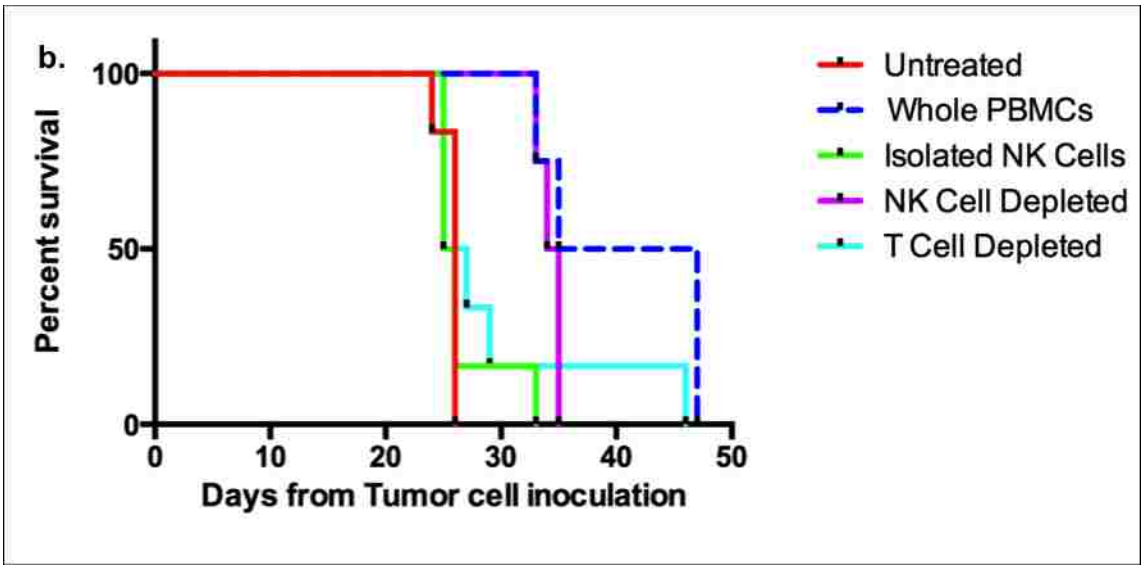


Figure 7: Survival difference between the untreated group and groups treated with PBMCs or PBMCs depleted with different cell types.

a) Bioluminescence images of mice showing SKOV-3/GFP-Luc tumor burden (seeded for 7 days) over 5 weeks from the start of treatment. Mice shown in the panels from left were, untreated, treated with whole PBMCs, treated with isolated NK cells, treated with PBMCs depleted of NK cells and treated with PBMCs depleted of T cells. All mice were injected with 1000 U IL-2 thrice a week. b) Survival curve showing the difference in overall survival between different groups from the day of tumor cell inoculation. p= 0.002 (Log-rank test, GraphPad Prism).

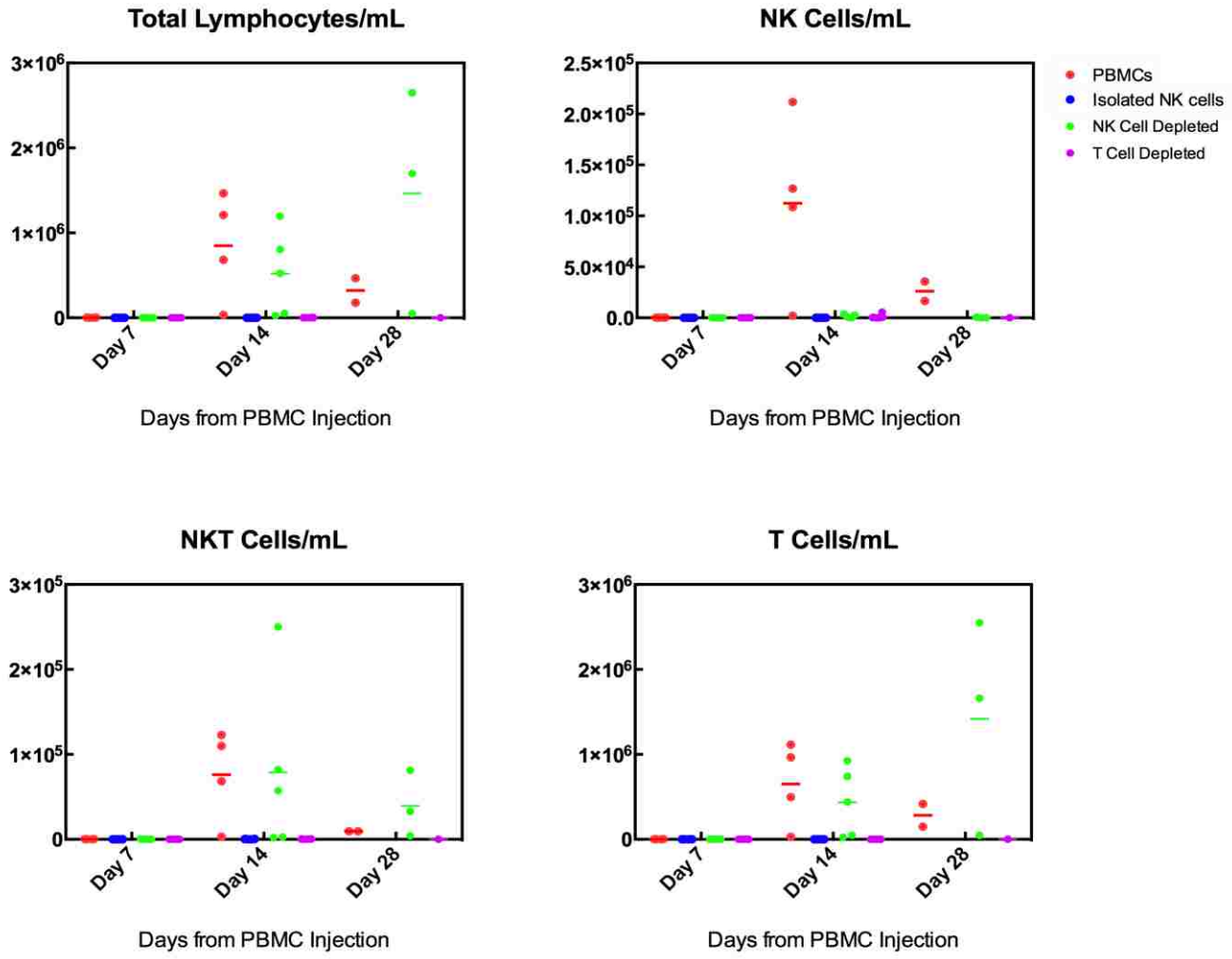


Figure 8: Expansion of peripheral blood cell types in different groups.

Plots showing total lymphocytes, NK cells, NKT cells and T cells per mL in mouse peripheral blood at the indicated number of days after initiation of treatment in different groups.

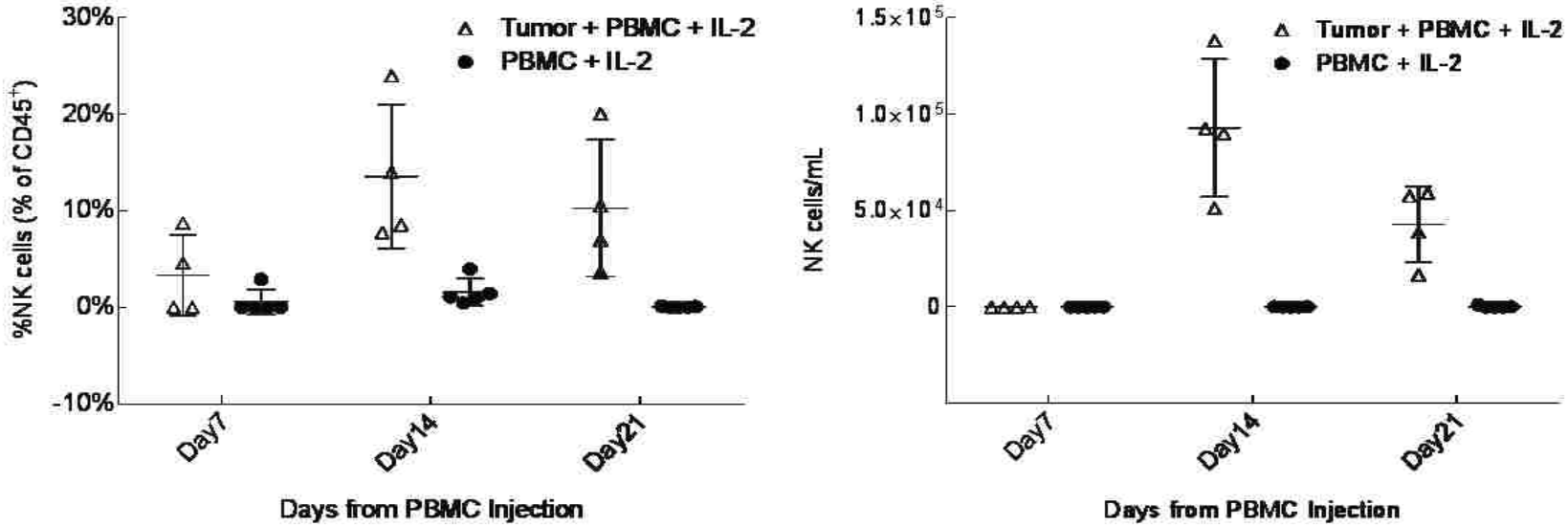


Figure 9: Supplemental Figure 1.

Plots showing NK cell expansion and reduction expressed as a fraction (left) and as concentration (right), in peripheral blood. These were analyzed parallel to the data shown in Figure 3. p=0.07 (left); p=0.0001 (right) (two-way ANOVA).

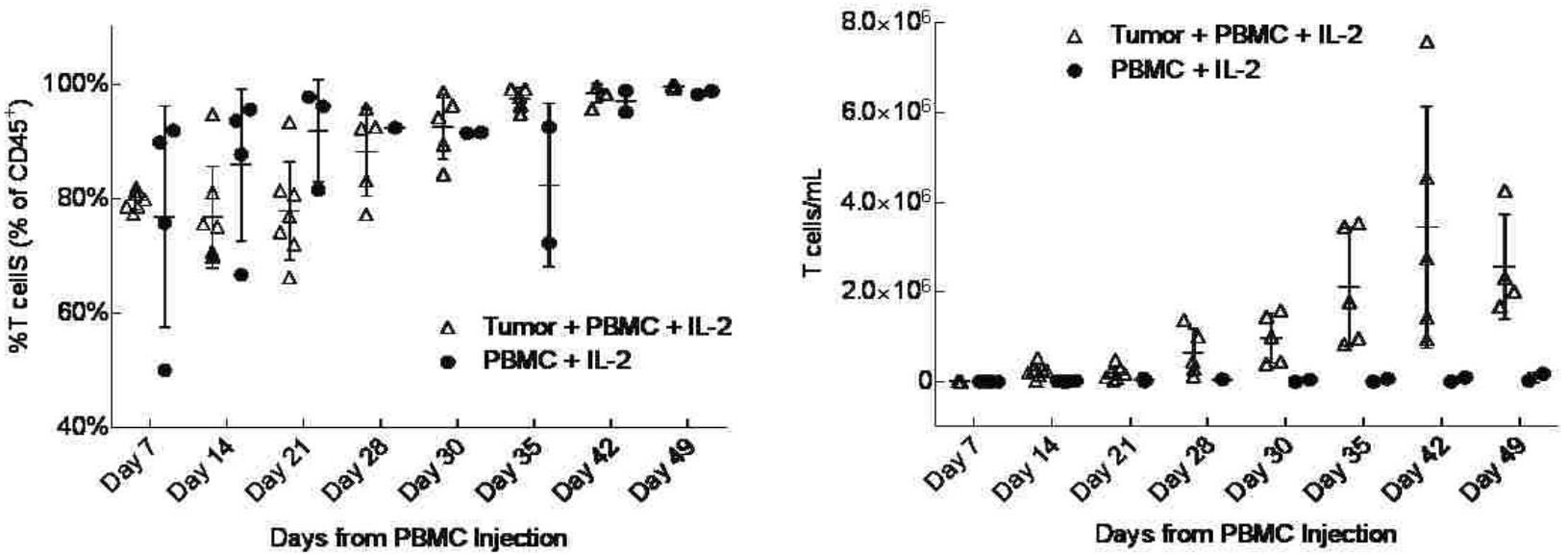


Figure 10: Supplemental Figure 2.

Plots showing T cells in the peripheral blood over time in tumor bearing and non-tumor bearing mice treated with PBMC+IL-2, as a fraction (left) and as concentration (right). This data is from the survival experiment shown in Figure 1. p=0.01 (left); p=0.06 (right) (two-way ANOVA).

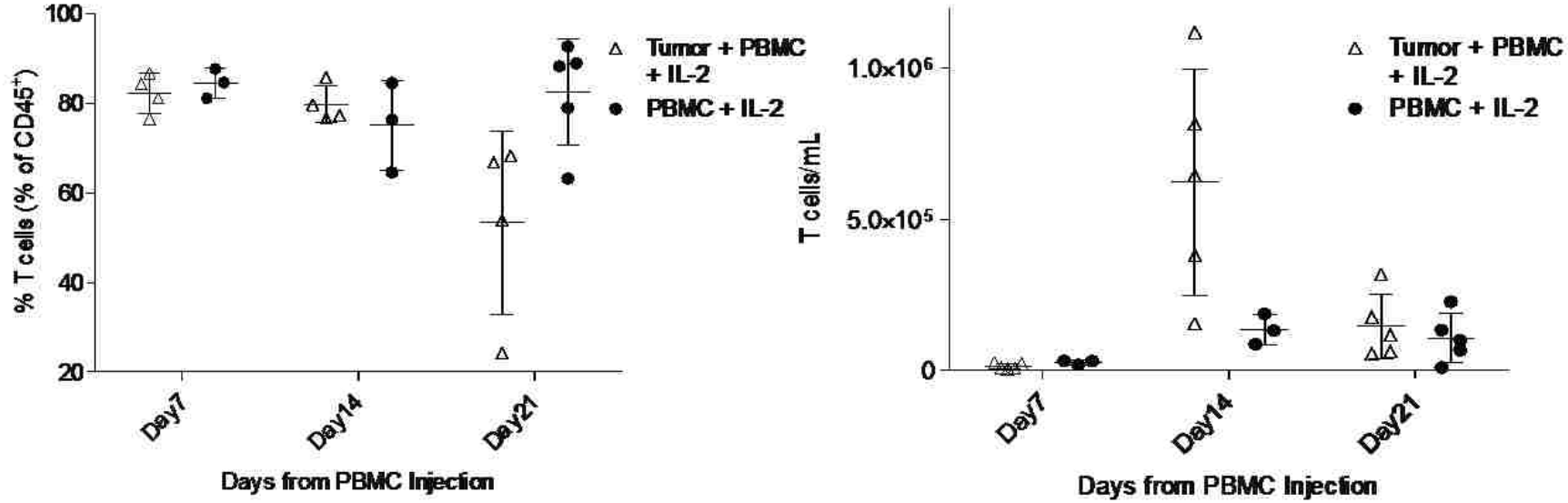


Figure 11: Supplemental Figure 3.

Plots showing T cells in the peritoneal wash over time in tumor bearing and non-tumor bearing mice treated with PBMC+IL-2. This data is from the experiment shown in Figure 3. p=0.02 (left); p=0.01 (right) (two-way ANOVA).

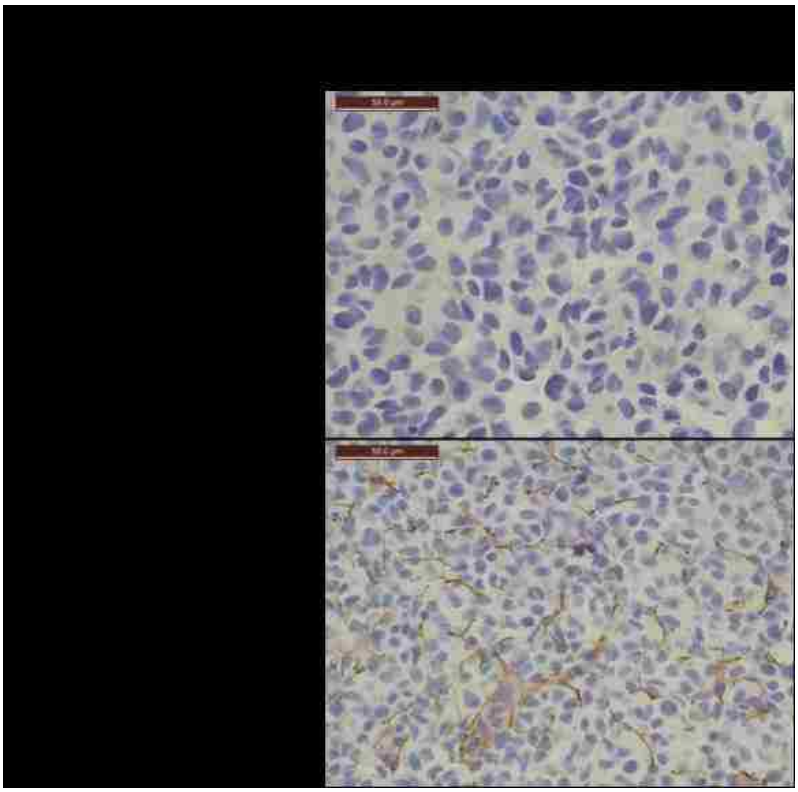
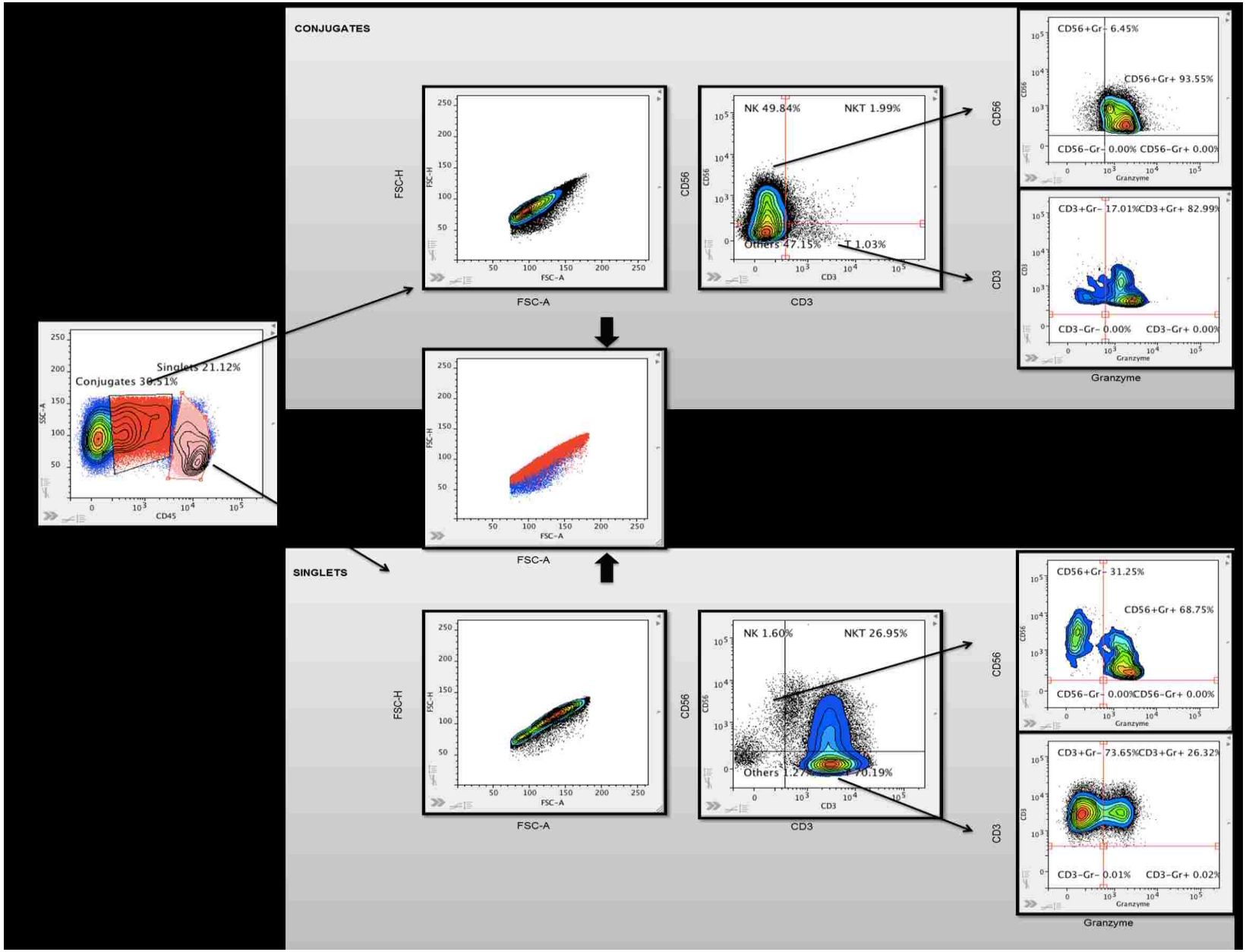


Figure 12: Supplemental Figure 4.

IHC images showing NKG2D staining in untreated and PBMC +IL-2 treated tumor tissue, indicating infiltration of NK cells into the tumors.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Conjugates 30.51%
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FSC-A
NK 49.84%
NKT 1.99%
Others 47.15%
T 1.03%
CD56
CD3
CD56+Gr- 6.45%
CD56+Gr+ 93.55%
CD56-Gr- 0.00%
CD56-Gr+ 0.00%
Granzyme
CD3+Gr- 17.01%
CD3+Gr+ 82.99%
CD3-Gr- 0.00%
CD3-Gr+ 0.00%
SINGLETS
NK 1.60%
NKT 26.95%
Others 1.27%
T 0.19%
CD56+Gr- 31.25%
CD56+Gr+ 68.75%
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Figure 13: Supplemental Figure 5.

Schematic of the gating strategy for the granzyme activity assay shown in Figure 5. First panel (left) shows the two distinct $CD45^{+}$ populations, conjugates and singlets. In the second panel, FSC-H vs FSC-A plots for both populations are represented as overlays show conjugate formation. Third panel shows gating of the two populations for CD56 and CD3. The last panel shows granzyme activity positive events gated on the NK cell population.

Discussion

In this study, complete tumor remission and increased survival of tumor bearing NSG mice was observed when mice were treated with healthy donor derived PBMCs, as compared to the untreated ones. From the whole PBMC population injected, NK cell expansion was observed in the peritoneal cavity as well as in the peripheral blood of the tumor bearing mice, whereas no immune cell expansion or NK cell engraftment was seen in the control mice without any tumor. There was also a correlation between tumor shrinkage and decreasing number of NK cells in the treated mice. Importantly, flow cytometry of granzyme activity of immune cells isolated from tumors showed that 1) there were more NK cells than T cells, 2) the majority of tumor conjugates were that of tumor-NK cells and 3) these tumor-NK cell conjugates had granzyme activity. These data collectively led us to conclude that NK cells were the effector cells responsible for the tumor regression and progression-free survival of the mice.

Previous studies with either allogeneic or autologous NK cell adoptive therapy have been inconsistent in producing successful clinical outcomes due to various reasons, most importantly the inability of NK cells to expand and persist *in vivo* [44]. A phase II study with IV delivered haploidentical NK cells in ovarian and breast cancer patients with recurrent disease following lymphodepleting chemotherapy showed sub-optimal NK cell expansion [42]. Another study with adoptively transferred autologous NK cells showed proliferation and persistence *in vivo*, but the NK cells failed to exhibit cytotoxicity due to immunosuppression without any overall benefit to the health of the patients [72]. A study in a xenograft mouse model showed progress with *in vivo* persistence of NK cells by changing the mode of delivery from IV to IP, but this study required very high starting dose of *in vitro* IL-2 pre-activated NK cells ($20x10^6$), enriched by T and B cell depletions of PBMCs, and high dose IL-2 (75,000 U/day IP daily for 3 weeks) in order to allow

reduction in tumor burden [63]. In contrast, only 1 x 10^6 whole PBMCs (8-11% NK cells) were injected IP along with very low dose of IL-2 (1,000 U thrice weekly) to induce complete tumor remission in this study. This suggests that a supportive or synergistic role of other cell types in the whole PBMC population is crucial for optimal NK cell cytotoxic activity against tumors.

Cell types that are known to augment NK cell proliferation and maintenance are monocytes, DCs and T cells. Monocytes have been shown to aid in NK cell expansion mediated by some soluble factors [73]. Results from *in vitro* and clinical studies indicate that cytokines such as IL-2, 1L-12 and IL-21 enhance NK cell proliferation and effector functions such as IFN-γ secretion [74, 75]. DCs also play a role in NK cell effector functions like IFN-γ production and cytolytic activity *in vitro* [76, 77]. Mouse and human studies show that they mediate their support mainly through secretion of IL-12 [78, 79] and trans-presentation of membrane bound IL-15 on mature DCs [76, 80-82]. In addition to triggering NK cell expansion and activation through a direct cell-to-cell contact, DCs can also influence NK cells by releasing exosomes [83, 84]. A clinical study showed that vaccination with dendritic cell derived exosomes harboring NKG2D ligands and functional IL-15Rα, synergize for NK cell proliferation and activation *in vitro* and in patients [85]. In addition to monocytes and DCs, NK cells are also dependent on activated $CD4^+$ T cells for the availability of IL-2. In our study, the presence of T cells in the peritoneal cavity (Figure 11) may have provided IL-2 for NK cell expansion in addition to low-dose exogenous IL-2. Data from figures 7 and 8 supports this and clearly shows the dependence of NK cells on T cells because, treatment with isolated NK cells did not lead to expansion *in vivo*. Secretion of IL-2 by these cells also causes Treg cell expansion as a negative feedback to prevent overstimulation of $CD4^+$ T cells. Therefore, Tregs restrict the availability of IL-2 for NK cells causing suppression of NK cell expansion and activity [86, 87], thus validating the effect of

Treg depletion to enhance tumor suppression by NK cells [88, 89]. In our study, the presence of T cells in the peritoneal cavity (Figure 11) may have provided IL-2 for NK cell expansion in addition to low-dose exogenous IL-2. Apart from indirect inhibition of NK cells, Tregs can also directly suppress NK cells in a tumor microenvironment by secreting TGF-β, which is known to suppress NKG2D expression on NK cells and mitigate tumor cell lysis [34]. From these examples, it is clear that cross talk of NK cells with different components of the PBMCs plays a critical role in the regulation of NK cell proliferation and function.

In addition to being regulated by different components of PBMCs, NK cells are also involved in maintaining fine balances in immune responses, specially contributing towards prevention of GVHD. Secretion of IL-2 by T cells can also cause an abundance of T cells that may lead to GVHD. NK cells are known to inhibit T cells in such a case and reduce GVHD by increasing apoptosis in T cells [90, 91]. The experiment shown in figure 7 and 8 supports this and shows that in the group treated with NK depleted PBMCs, animals succumbed to GVHD symptoms, whereas the PBMC treated group showed a delay in GVHD symptoms, perhaps after NK cell numbers waned down after day 14.

In our study, NK cells infiltrated into small diffuse tumor nodules in the peritoneal cavity and were able to degranulate to induce cytotoxicity. This models residual tumor burden or diffuse persistent ovarian carcinoma where tumor sizes are small and perhaps not as immunosuppressive as compared to an advanced tumor. For the treatment of advanced tumors, NK cell therapy in combination with other treatment modalities may be required to achieve maximal clinical benefit. Various types of combination immunotherapies have been carried out in ovarian carcinoma patients, with promising results [18]. A new clinical trial is recruiting patients with recurrent ovarian and fallopian tube cancer along with other primary peritoneal

carcinomas, for a phase1 study looking at the effect of *in vivo* suppression of T cells by using an indoleamine 2,3-dioxygenase (IDO) inhibitor, along with IP delivery of haploidentical NK cells (Clinicaltrials.gov identifier: NCT02118285). IDO is prevalent in ~56% of ovarian carcinomas and is associated with an immunosuppressive tumor microenvironment [92]. Also, in the ID8-VEGF ovarian carcinoma mouse model, decreased proliferation and the inability of the tumor infiltrating lymphocytes to produce cytokines was attributed to the expression of CTLA-4 and PD-1 makers [93]. Therefore, a combination of NK cell therapy with other strategies such as checkpoint inhibition may result in better clinical responses. For HLA expressing tumors that are resistant to NK cell lysis, combining NK cell therapy with an anti-KIR receptor antibody may be useful [94]. Because of the heterogeneous nature of tumors and a myriad of escape mechanisms used by tumors to evade the immune system, combining different approaches that inhibit mechanisms for immune escape in eclectic combinations is a rational approach.

NK cell function is regulated by a balance between various combinations of activating and inhibitory signals that allow the diversity of NK cell repertoire in an individual, but the link between NK cell diversity and NK cell function is only starting to be understood. In an analysis of peripheral blood NK cells, a study showed that there are 6-30,000 phenotypic variants of NK cells in an individual [95]. While inhibitory receptor expression is largely genetically governed, activation receptors are influenced by environmental factors in an individual [95]. The whole PBMC approach in our study rather than using enriched, *in vitro* IL-2 pre-activated NK cells, likely resulted in the conservation of the diversity of the activated NK cell repertoire due intercellular stimulation of NK cells with other components of the healthy donor PBMC population and tumor. This may have contributed to effectiveness of tumor induced NK cell expansion *in vivo* from low starting percentages (8-11%) to ~20% NK cells in the total

lymphocyte population, and stimulation of anti-tumor efficacy. In adoptive transfer of isolated, pre-activated NK cells, the lack of intercellular stimulation from other lymphocyte types and potentially skewing of NK cells during *in vitro* pre-activation may possibly account for the lack of NK cell expansion and anti-tumor efficacy in ovarian carcinoma patients. For an effective anti-tumor response, all components are likely to have a role, but the absence of NK cells has shown to impair tumor rejection [96].

Results from these studies suggest that the cross talk of NK cells with other cell types in the PBMC population may be responsible for NK cell proliferation, infiltration into small and diffuse tumors and anti-tumor cytotoxicity. This may be an important consideration for utilizing NK cell-based immunotherapy as an adjuvant after surgical tumor resection and frontline chemotherapy, especially in case of tumors where the elimination of residual tumor could be very significant to prevent relapse, such as in ovarian cancer treatment. Therefore, this study provides a proof-of-principle for the therapeutic potential of NK cells in the anti-tumor response and highlights the importance of further investigating cell-to-cell interactions within PBMC population, based on which, different combination approaches could be designed to significantly attenuate tumor evasion.

CHAPTER 3: NK CELLS STIMULATED WITH PM21 PARTICLES EXPAND AND BIODISTRIBUTE *IN VIVO*: CLINICAL IMPLICATIONS FOR CANCER TREATMENT

Introduction

Natural killer (NK) cells are a component of the innate immune system, identified by being $CD56^{+}CD3^{-}$, and can naturally recognize and lyse cells that are virally compromised or are malignant. Cell therapy with NK cells is promising as a cancer treatment and multiple clinical trials have been conducted and are currently underway for treatment of various cancers (AML, lymphoma, non-Hodgkin lymphoma, breast, ovarian, neuroblastoma, non-small cell lung carcinomas). For effective anti-cancer therapy with NK cells, three general aspects must be considered: 1) a large enough dose of NK cells must be delivered; 2) NK cells must be highly cytotoxic; and 3) NK cells must reach, possibly localize at the site of disease, persist and specifically target tumor cells [97].

For clinical efficacy in an AML setting, Miller and co-workers have recommended attaining a dose that would provide at least 100 NK cells per µL of peripheral blood (PB) at two weeks post infusion [98]. In some examples where treatment with adoptive NK cell therapy was efficacious, over 1,000 NK cells per µL of PB were observed. These observations highlight the importance of proficient NK cell expansion methods for delivery of a sufficient dose for overall treatment efficacy.

Currently, there are broadly three different clinically used strategies for NK cell expansion for adoptive cell therapy. First, *in vivo* expansion with cytokines such as IL-15 and IL-2, combined with host lymphodepletion/irradiation, may stimulate *in vivo* expansion from the relatively low amount of injected donor NK cells [39, 99, 100]. Second, *ex vivo* methods with

cytokines, mainly using IL-2 and IL-15 [101], can activate NK cells, although expansion is relatively low and variable. Also, NK cells activated *ex vivo* with cytokines undergo cytokine withdrawal after infusion and the NK cells undergo apoptosis [102, 103]. Third, feeder cell methods for *ex vivo* NK cell expansion use co-cultures with other cells that are stimulatory. Feeder cell methods for NK cell stimulation include Epstein-Barr virus-Lymphoblastoid cell lines [45, 104], or engineered tumor cells. Co-culture with K562 leukemia cells expressing membrane bound IL-15 (mb15) and 4-1BB ligand (41BBL) (K562-mb15-41BBL) are able to expand NK cells several hundred fold in about two weeks, but the NK cells expanded by this method experience senescence [47, 49]. In addition, NK cells activated with IL-15 lose surface CD16 by proteolytic activity of ADAM17 [105]. Rather K562 cells expressing mb21 (focus of this study), instead of mb15, significantly improves NK cell expansion while avoiding telomere shortening and consequent NK cell senescence [49, 106]. Expansion of NK cells with the K562-mb21-41BBL is very efficient and a mean 48,000-fold expansion with >85% enrichment is typically achieved in three weeks [49]. All of these methods are actively being investigated in clinical trials.

While NK cell expansion methods have improved, there are still disadvantages and challenges. To date, highly toxic doses of IL-2 are required regardless of expansion method for survival of the infused NK cells, and the persistence of the NK cells is still limited. While *ex vivo* methods with feeder cells have been effective for expansion to generate large amounts of NK cells, concerns have been raised that long term *ex vivo* culturing of NK cells causes loss of ability to home to the site of disease such as bone marrow [107]. Thus, there has been a debate about the overall benefits of *in vivo* vs. *ex vivo* expansion [108]. An optimal NK cell expansion procedure

would be a method which has the proliferation capability of an *ex vivo* feeder cell based method, but could be performed either *ex vivo* or *in vivo*.

Recently, a novel particle based method for rapid and selective expansion of cytotoxic NK cells starting with PBMCs was reported [48]. In this report, the particles corresponding to closed plasma membrane vesicles were prepared from plasma membrane of K562-mb15-41BBL cells (PM15-particles) and allowed selective NK cell expansion of 250-fold in 14 days and 1,265-fold after 17 days, which is comparable to the expansion efficiency using K562-mb15-41BBL feeder cells in co-culture. PM15-particle activated NK cells, similar to feeder cell expanded NK cells, were highly cytotoxic towards leukemia cells *ex vivo*. Importantly, these particles offer many advantages over the currently used feeder cell methods. First, they can be prepared in advance, tested and stored for more than a year and can be used as an "off-the-shelf reagent" to simplify the clinical logistics. Second, use of the PM-particles, instead of feeder cells to stimulate NK cells, eliminates steps needed for safety measures when using tumor-derived feeder cell such as feeder irradiation and testing their presence and proliferation in the final product. Third, tumor-derived feeder cells cannot be injected as an adjuvant therapy whereas the PM-particles are injectable to stimulate *in vivo* expansion of NK cells. The advantages offered by the PM-particle based method for NK cell expansion would likely allow for significant clinical benefits.

Here in this work, we test the efficacy of PM-particles prepared from K562-mb21-41BBL for *in vivo* expansion of adoptively transferred NK cells, pre-activated with a relatively short and simple procedure that could be easily implemented in a clinical setting. The method overcomes the shortcomings of previous studies with IV infusion of adoptive NK cells that only allowed very minimal *in vivo* NK cell expansion and had limited persistence. For the current

study, efficacy is shown for PM21-particle stimulated *ex vivo* and *in vivo* expansion of NK cells from unselected PBMCs injected into the peritoneal cavity, which is intended to serve as site for incubation and stimulation by PM21-particles. This method is expected to be useful for the *in vivo* expansion of NK cells at therapeutically relevant amounts and presents means to make NK cell-mediated immunotherapy more widely accessible to patients.

Materials & Methods

Human Samples

Leukocyte source (One Blood, Orlando, FL) or fresh blood collected from healthy volunteers who signed and IRB approved informed consent were used as healthy samples. Primary leukemia blasts were obtained from patients, who signed an IRB-approved informed consent, during active disease and comparable PB was collected from these patients during remission. PBMCs were isolated using Ficoll-Paque (GE Healthcare, Pittsburgh, PA) as previously described [48]. All samples were de-identified and viably cryopreserved.

Reagents and Cell Lines

K562 cell line was obtained from ATCC (Manassas, VA). K562-mb15-41BBL was kindly provided by Dr. Dario Campana (St. Jude Children's Research Hospital, Memphis, TN). Annexin-V FITC kit for cytotoxicity assays and Enumeration Flow-Count beads purchased from Beckman Coulter (Miami, FL). The following dye conjugated antibodies were used for phenotyping: CD16-FITC, NKG2A-PE, NKp46-PE, CD3-APC (Beckman Coulter); CD4-APC-Cy7, CD8-PE, CD56-BV421, CD94-APC (BD Biosciences); CD3-Alexa488, NKG2D-APC, CD62L-PE-Cy7, CD45-eFluor450, CD45-APC (eBiosciences); CD56-PE, KIR2D-APC (Miltenyi); NKG2C-PE NKp44-APC, TRAIL-PE (R&D Systems).

Preparation And Characterization Of Plasma Membrane Particles

PM-particles were prepared from K562-mb15-41BBL or K562-mb21-41BBL cells as previously described [48]. PM-particle preparations were quantified by protein concentration by BCA assay and specified as μg of membrane protein/mL. Presence of IL-21 and 41BBL on PM-particles was confirmed by ELISA and Western Blot.

Ex Vivo NK Cell Expansion From PBMCs

NK cells from PBMCs were expanded using PM21-particles as previously described [48]. Briefly, PBMCs were seeded at 0.1 x 10^6 NK cells/mL in SCGM (CellGenix, Portsmouth, NH) supplemented with 10% FBS, 2 mM Glutamax, 100 U/mL IL-2 (Peprotech, Rocky Hill, NJ) and 200 µg/mL PM21-particles. Media with supplements was replaced routinely every 2-3 days after day 5.

In Vivo Expansion of NK Cells in NSG Mice

PBMCs, either freshly thawed or pre-activated for two days with 200 µg/mL PM21 and 100 U/mL IL-2, were washed twice and resuspended in phenol red-free RPMI media. 1 x 10^5 NK cells in a whole PBMC cell suspension were injected IP into NSG mice. PM21-particles (amounts specified in figure legends, twice weekly) and IL-2 (1,000 U, thrice weekly) were also injected IP and PB was collected by cheek bleeds or cardiac puncture. Organs were collected at necropsy were perfused to obtain single cell suspensions for analysis.

In Vivo Proliferation Analysis

PBMCs, freshly thawed or pre-activated with PM21-particles and 100 U/mL IL-2 for two days (PM21-PBMCs) were labeled with Cell Trace (CT) Violet (Invitrogen, Carlsbad, CA). 2x10^6 non pre-activated PBMCs and PM21-PBMCs were injected IP to NSG mice. Mice in all groups received 1,000 U IL-2, IP, thrice weekly. Two of the groups of mice were also injected with 400 µg of PM21-particles, IP, twice weekly. Mice from each group were euthanized on day 6 and the peritoneal wash was analyzed by flow cytometry for CT violet fluorescence of $CD45^+$, $CD3^-$, $CD56^+$ NK cells. Peritoneal wash was collected by making a small incision in the

peritoneum immediately after euthanizing the mice and flushing PBS in the cavity a few times using a pasteur pipette.

In Vivo PM21 Titration

PBMCs were pre-activated *ex vivo* with PM21-particles and 100 U/mL IL-2 for 2 days. PM21-PBMCs in the amount containing 0.2 x 10^6 of viable NK cells were injected IP to NSG mice. Mice in all groups received 1,000 U of IL-2, IP thrice weekly. Mice were also injected with 0, 400, 800, 1,600 µg of PM21-particles, IP twice weekly. Peripheral blood was analyzed by flow cytometry for $CD45^+$ lymphocytes twice weekly starting on day 5 and NK, T and B cell amounts were determined based on staining for CD3, CD56, CD19.

NK Cell Biodistribution Analysis

0.2 x 10^6 cells NK cells as part PBMCs, were pre-activated *ex vivo* with PM21-particles and 100 U/mL IL-2 for 2 days and were injected IP to NSG mice. Mice in all groups received 1,000 U of IL-2, IP, thrice weekly. Mice were also injected with 0 or 800 µg of PM21-particles, IP, twice weekly. Mice were sacrificed 16 days after initial injection IP of PM21-PBMCs. On day of euthanasia, bone marrow (femur), spleen, lung, brain and liver were collected, organs were perfused while femur was washed to recover cells. Cells were stained with antibodies against CD3, CD45, CD56, CD19 for flow cytometry analysis.

NK Cell Expansion from Different Donor Sources

PBMCs from different sources were pre-activated *ex vivo* for 2 days with PM21-particles and 100 U/mL IL-2 for 2 days (PM21-PBMCs) and were injected IP to NSG mice. Mice in all groups received 1,000 U of IL-2, IP, thrice weekly. Peripheral blood was analyzed by flow

cytometry for $CD45^+$ lymphocytes twice weekly starting on day 5 and NK, T and B cell amounts were determine d based on staining for CD3, CD56, CD19.

Phenotype Analysis

PM21-PBMCs, activated *ex vivo* with PM21-particles for two days, were split and either injected IP into NSG mice or further cultured *ex vivo* for 14 days. Mice were given 800 μg per injection, IP twice weekly or *ex vivo* culture included 200 μg/mL of PM21-particles. NK cells were obtained from *ex vivo* culture, from peripheral blood, or from wash of the abdominal cavity. Cells were stained with respective fluorescent conjugated antibodies and were analyzed by flow cytometry. Expression levels of the various cell surface proteins on NK cells were assessed with fluorescence intensities of events gated by $CD45^+$, $CD56^-$, $CD3^-$ were inspected. Gates for the positive populations were set based on FMO (fluorescence minus one)-isotype control samples.

Confocal Imaging

NK cells were expanded from PBMCs using PM21 particles for 12 days when NK cells were at >80%. Cells were centrifuges and resuspended in 700uL SCGM + 10% FBS + 2mM Glutamax + 1000U/mL IL2.

For the isolated NK cell setup, PBMC vials were thawed, cells were transferred to 40mL RPMI + 10% FBS, centrifuged and resuspended in 1mL DPBS + 0.5%BSA + 2mM EDTA (Isolation buffer; room temp). NK cell isolation was done using EasySep human NK cell enrichment kit (Stem Cell Technologies Inc., Vancouver, BC, Canada). Cell count after isolation showed 95.9% NK cells. 1mL SCGM + 10%FBS + 2mM Glutamax + 1000U/mL IL2 was added to the cell suspension and transferred to a 6-well plate, placed in 37C, 5% CO_2 incubator overnight. SKOV-3/GFP-Luc cells plated in a 24-well glass bottom plate at ~60% confluency

and allowed to adhere to the surface for 3-4 hours. Media was replaced by the NK cell suspensions from both conditions mentioned above. Ultraview live cell imaging system was used for imaging. GFP and DIC (differential interference contrast) channels were captures over 18 hours at 10X magnification

Leukemia cell lines Cytotoxicity Assay

NK cells were cultured for 14 days with PM21-particles. Target cells were labeled with TFL4 dye (OncoImmunin, Gaithersburg, MD) prior to the assay. Expanded PM21-NK cells were co-incubated with target cells at varying ratios for 4 hours, placed on ice and stained for 20 minutes with Annexin V-Alexa488 (BD Biosciences) and then analyzed by flow cytometry. The amount of spontaneous target cell death was determined using a "Target Alone" control. Percent cytotoxicity was determined by subtracting spontaneous cell death from target cell death and then dividing by total target cells.

Autologous Patient NK Cell Cytotoxicity Assas

Cytotoxicity assays of patient derived NK cells against autologous AML tumor cells was assayed with Annexin V (BD Bioscience). NK cells expanded for 18-20 days were stained with TFL4 dye. Target tumor cells were co-cultured at 0.5 x 10^6 $CD34^+$ cells/mL with NK cells at E:T ratios of 1:1, 2:1, 5:1, and 10:1 for 2 hours in 37 °C, 5% CO_2 atmosphere. The cells were then centrifuged and resuspended in Annexin V labelling buffer containing Annexin V-FITC, anti-CD34-PE, and anti-CD56-PC7 and incubated for 15 minutes at 4 °C. The labeled cells were diluted to 250 µL and analyzed by flow cytometry on an Accuri instrument (BD Bioscience).

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Results

PM21 particles show better expansion of NK cells as compared to PM15 particles

It was recently reported that NK cells can be expanded using particles derived from plasma membranes of K562-mb15-41BBL feeder cells (denoted PM15) [48]. The PM15-particles perform very similar to K562-mb15-41BBL feeder cells to induce similar levels of NK cell expansion, and the expanding NK cells also have the same characteristics of senescing after ~3 weeks of expansion. Since K562 cells engineered to express mb21, have been reported to have better efficiency for NK cell expansion without senescence [49], PM-particles were prepared from K562-mb21-41BBL cells, denoted PM21-particles, and tested for their NK cell expansion capabilities.

PBMCs were cultured side-by-side with either PM15- or PM21-particles (200 μg/mL) for 28 days. NK cells stimulated with PM21-particles expanded more rapidly as compared to the ones stimulated with PM15-particles, and the content of NK cells reached >90% by day 14 in PM21-particle stimulated NK cell cultures compared to 21 days in PM15-particle stimulated cultures (Figure 14 A,B). Cumulative analysis of NK cell expansions, at day 14±1 of culture, showed that PM21-particles (mean 825 fold expansion, range 163-2,216, n=13) are significantly (p=0.021) more effective as compared to PM15-particles (mean 424 fold, range 290-570, n=30) (Figure 14 C). Furthermore, NK cells stimulated with PM21-particles expanded exponentially during the period of 28 days reaching over 100,000 fold expansion, in contrast to the NK cell expansion with PM15-particles which stalled by day 22 of culture due to senescence. Thus, PM21-particles have improved NK cell expansion proficiency over the PM15-particles and comparable to the K562-mb21-41BBL feeder cells from which they were derived [49].

PM21 particle show expansion of NK cells from patient blood

The NK cell expansion capabilities of PM21-particles were further tested with PBMCs from leukemia patients in remission. PM21-particles induced NK cell expansion relatively efficiently from all three patient derived samples in 14 days of culture (113±7 fold for F021, 810±81 fold for M038, and 352±86 fold for M050, Figure 14 D). The expansion was specific for NK cells where the percentage of NK cells of total $CD45^+$ cells rose preferentially (Figure 14 E).

PM21 particle-pre-activated PBMCs show NK cell expansion in vivo which can be improved by additional in vivo dosing of PM21

An unprecedented capability of the PM-particles would be as an injectable to spur *in vivo* expansion. To test if PM21-particles stimulate *in vivo* NK cell expansion and to determine if *ex vivo* pre-activation is required, NSG mice were injected IP with 0.1×10^6 NK cells as part of either non pre-activated PBMCs or PM21-particle pre-activated PBMCs (PM21-PBMCs). Mice injected with non pre-activated PBMCs had low amounts of NK cells in PB and only T cells increased as a percentage of total $CD45^+$ cells over 15 days post injection. In significant contrast, PB of mice injected with PM21-PBMCs were found to have elevated amounts of NK cells that peaked 12 days post IP injection (Figure 15). The NK cell content enriched to 53±8 % of $CD45^+$ cells. In the same experiment, the efficacy was tested for *in vivo* IP application of PM21-particles to promote better *in vivo* NK cell expansion. For mice injected with non pre-activated PBMCs, additional *in vivo* PM21-particles did not stimulate NK cell expansion. However, applying PM21-particles *in vivo* to mice grafted with PM21-PBMCs had an effect where NK cell amounts were higher compared to the mice with PM21-PBMCs that did not receive *in vivo* PM21-particles (Figure 15).

To provide evidence that the PM21-particles induce *in vivo* NK cell proliferation, analysis was performed with CTViolet labeled NK cells expanding *in vivo* at 6 days post IP inoculation. The cells from mice injected with non pre-activated PBMCs showed none or very little decrease in the CTViolet fluorescence, indicating that there was none or very few cell divisions of NK cells (Figure 16, top panel). The NK cells from mice injected with PM21-PBMCs showed significant diminishment of the CTViolet fluorescence intensities (Figure 16, bottom panel). Fitting of the fluorescence intensities showed that the intensity decrease correlates with the major population, dividing 7 cell divisions *in vivo* within 6 days. For the NK cells obtained from mice that received IP injections of PM21-particles, one more division can be observed. This additional doubling with administration of the *in vivo* PM21-particles correlates with the higher NK cell amounts observed in PB with *in vivo* PM21-particles.

Enhanced NK cell expansion due to in vivo delivery of PM21 is dose-dependent

To further verify if *in vivo* delivered PM21-particles enhance *in vivo* NK cell expansion, a dose dependence of *in vivo* PM21-particles was studied (Figure 17). A dose dependent increase in NK cells in PB was observed from 0 to 800 μg of PM21-particles per injection. At a dose of 800 μg, 470±40 NK cells per μL of PB was observed at 12 days after IP injection of the PM21-PBMCs. This NK cell concentration in PB was 5 fold higher than the concentration that is generally thought to be therapeutically efficacious [98]. The dose dependent effect for *in vivo* expansion was specific for NK cells where T cell amounts did not increase significantly (Figure 17 b, c). At a higher amount of 1,600 μg per injection, PB NK cell amounts diminished, similar to the effect observed *ex vivo* where ~200-400 μg/mL is optimal for PM21-particles (data not shown) or PM15-particles and higher amounts attenuated NK cell expansion [48].

In vivo expanded NK cells home to different organs and homing increases with in vivo dosing of PM21

The observation of significant amounts of NK cells in PB shows that NK cells expand in the peritoneal cavity along with IP injection of PM21-PBMCs, can migrate out from the cavity to the PB. To verify that the adoptively transferred NK cells can migrate to potential sites of disease, NK cells in various organs were quantified (Figure 18). Human NK cells were found in every organ inspected, and higher amounts of NK cells were found in organs from mice treated *in vivo* with 800 µg of PM21-particles, all significantly higher (p<0.05) except in livers. Furthermore, the organs from the mice treated with 800 µg of PM21-particles had higher percentage of NK cells as a fraction of total $CD45^+$ cells.

NK cell expansion with PM21 is independent of the PBMC source

The mice based studies described here showed that the procedure combining *ex vivo* short pre-activation with PM21-particles and *in vivo* administration of PM21-particles induces significant *in vivo* NK cell expansion, potentially in the therapeutically relevant range. To show consistency, necessary for clinical use, the procedure was applied to leukocyte sources from three different donors (different from those used in other experiments) (Figure 19). The average amount of NK cells in both PB and abdominal wash was relatively consistent between leukocyte sources (p=0.8). The percentage of NK, T cells and other $CD45^+$ cells was also very consistent for mice within the group injected with the PM21-PBMCs from a particular leukocyte source (n=3) and also between leukocyte sources L8 and L10.

Phenotype of NK cells expanded with PM21-particles

The anti-tumor cytolytic activity of NK cells is determined by the balance of stimuli from activating and inhibitory signals. Here, a detailed comparative inspection was performed for the

PM21-particle stimulated NK cells 1) expanded *ex vivo* with PM21 for 12 days, 2) expanded *in vivo* and isolated from PB, and 3) expanded *in vivo* and isolated from the abdominal wash (AW). These comparisons were made using cells from a single donor in all of the settings and performed in parallel (Figure 20).

Presence of CD16, the Fcγ receptor, on NK cells is required for effective antibody dependent cytotoxicity (ADCC). Nearly all NK cells from *in vivo* expansion show expression of CD16 (97% and 87% for PB and AW, respectively). CD94 is a surface receptor that forms heterodimeric complexes with NKG2C or NKG2A. About half of the NK cells expanded *ex vivo* have CD94 expression. For NK cells expanded *in vivo*, cells from the AW (64±9%) have higher expression than NK cells from PB (38±13%). Receptors of the NKG2 family both bind to CD94, inclusive of NKG2C as an activating receptor and NKG2A as an inhibitory receptor. The *ex vivo* expanded NK cells had relatively low expression of NKG2C, but NK cells from the AW were higher (53±8%) and even more increased in PB (61±2%). The fraction of NK cells that express NKG2A were higher in the AW (82±8%) than PB (67±12%) and those from *ex vivo* (74%). NKG2D is another important activating receptor found on NK cells and its expression was found on 61±6% of AW NK cells, 26±3% from PB and about 75% of NK cells expanded *ex vivo*. The expression of CD62L, known to be correlated with bone marrow homing, was higher for NK cells in PB (63±10%) and lower for that in the AW (39±14), which is consistent with the expression being higher on cells that were mobilized. NKp44 and NKp46 are members of the natural cytotoxicity receptor family and play a role in NK cell mediated cytolysis. NKp46 was expressed on NK cells from both PB (76±9%) and AW (89±5%). NKp44 was relatively poorly expressed in these NK cells from all the sources. On the other hand, NKp46 was well expressed from both PB (89±5) and AW (76±9). TRAIL is a ligand on NK cells that induces apoptosis of

targets via the death receptor pathway. TRAIL was expressed on 36±6% of NK cells from AW, 20±4% of PB and 26% of *ex vivo*-expanded cells. KIR2D is the killer immunoglobulin-like receptor (KIR) 2D subtype and a minority (about 1/3) of the NK cells *in vivo* or *ex vivo* expressed KIR2D. The proportion of $CD8^+$ and $CD4^+$ T cells were analyzed and it was found that $CD8^+$ T cells were more abundant than CD4 T cells from the *in vivo* samples. We also tested for presence of NK suppressive Treg cells and very few (<0.1% of all $CD3^+$ cells) were observed in *in vivo* samples.

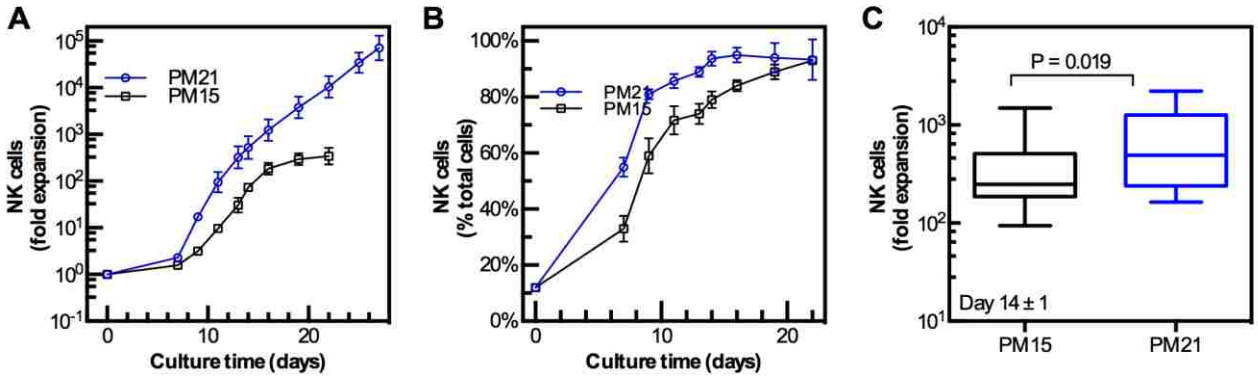
PM21-stimulated NK cells are cytotoxic against tumor cells in vitro

In order to test the cytotoxic capacity of PM21-expanded NK cells in comparison to fresh, non pre-activated NK cells, SKOV-3/GFP-Luc cells were co-incubated with them and imaged over time. Figure 21 shows PM21-expanded NK cells attacking attached SKOV-3/GFP-Luc cells causing them to lift, aggregate and die (top panel). Whereas, isolated NK cells, stimulated with 10,000 U IL-2 overnight show close minimal activity against attached tumor cells.

Apart from ovarian tumor cells, PM21-activated NK cells also showed cytotoxicity against different leukemia cell lines such as K562 (Chronic myelogenous leukemia or CML), HL-60 (Acute promyelocytic leukemia or APML) and KG-1 (Acute myelogenous leukemia or AML). Also, PM21-expanded NK cells from PBMCs of one of the leukemia patients (F021) in remission (shown in figure 14 D,E) were tested for cytotoxicity in an autologous setting against tumor blasts obtained from the same patient during active disease. At a relatively low effector to target ratio (E:T) of 1:1, 78±3% of tumor cells were apoptotic. Thus, this method of NK cell

stimulation could potentially be used for the treatment of various types of cancers and could possibly prove to be successful in an autologous transplant setting.

Figures


A
NK cells (fold expansion)
PM21
PM15
Culture time (days)
B
NK cells (% total cells)
PM21
PM15
Culture time (days)
C
NK cells (fold expansion)
P = 0.019
Day 14 ± 1
PM15
PM21

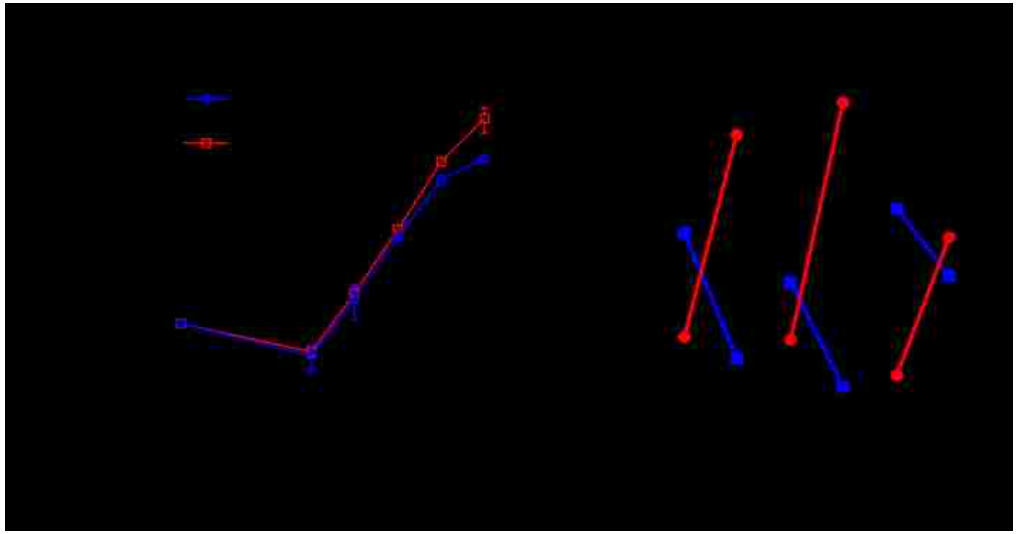


Figure 14: PM-21 particles expand cytotoxic NK cells efficiently and selectively.

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte source and seeded at 0.1 x 10^6 NK cells/mL in SCGM supplemented with 10% FBS, 2 mM Glutamax and 50 U/mL IL-2. PBMCs were stimulated with PM15 (□, black) or PM21 (○, blue) particles at 200 µg/mL for 27 days, the cell content was tested every 2-3 days and shown are relative fold of NK cell expansion **A**) and the percentage of suspension cells **B**). PM21-particles (825 ± 188 fold, N=13, 4 donors) (blue) are more efficient for NK cell expansion compared to PM15-particles (425 ± 71, N=35, 9 donors) (black) based on cumulative analysis of day 14 data for NK cell expansion **C**). PBMCs isolated from three AML patients in remission were cultured for 14 days with PM21-particles (200 µg/mL), seeded at 0.5 x 10^6 NK cells/mL in SCGM with 10% FBS, 2 mM Glutamax, 50 U/mL IL-2. Shown is fold of NK cell expansion from the primary PBMCs **D**) and lymphocyte content **E**) ($CD56^+CD3^-$ NK cells (●, red), $CD56^-CD3^+$ T cells (■, blue) and $CD56^+CD3^+$ NKT cells (▲, black)). PBMCs from patient F021 were cultured for 14 days as previously above and autologous cytotoxicity toward AML tumors from the same patient was analyzed (Experiment courtesy of Jeremiah L. Oyer, Copik Lab).

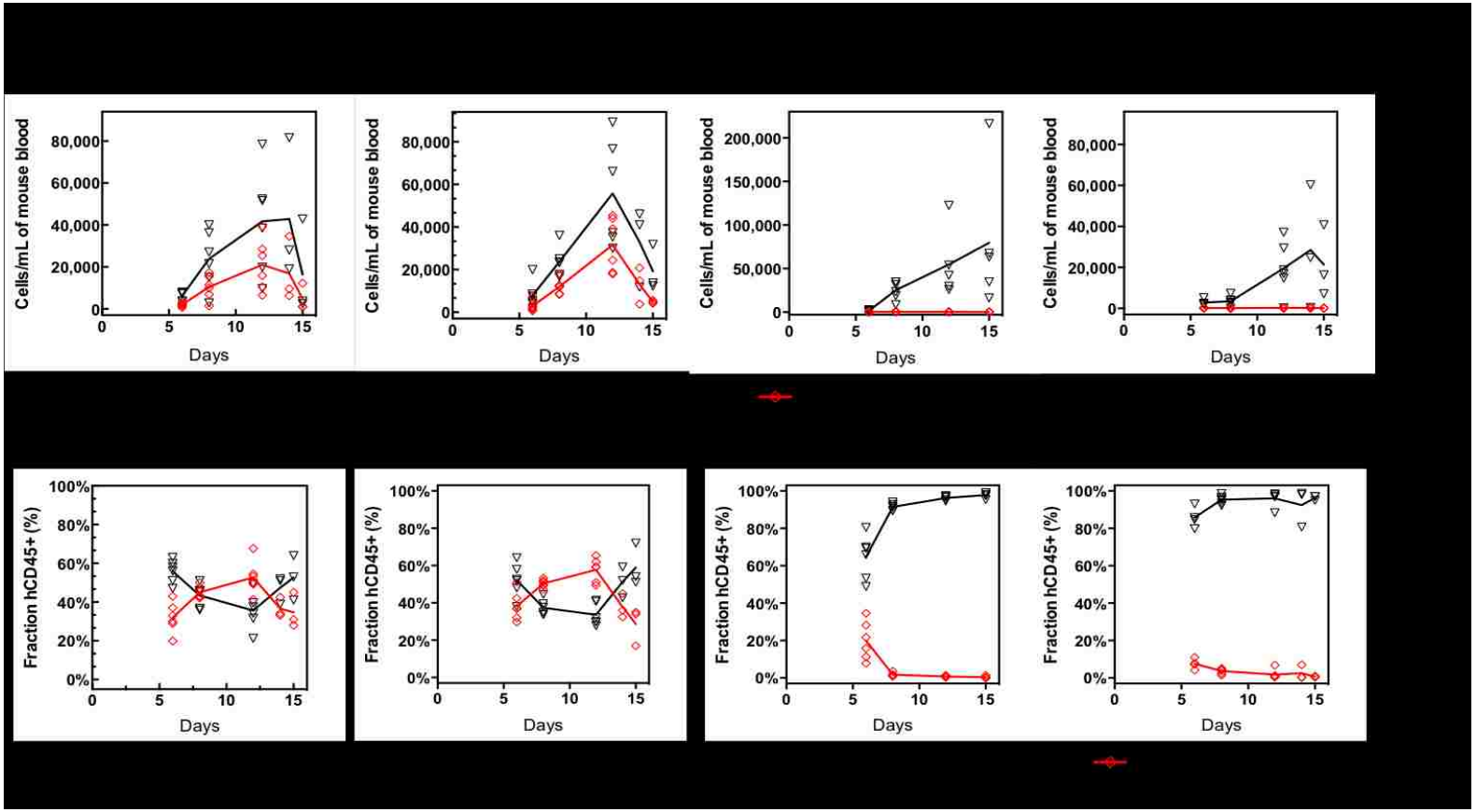


Figure 15: Pre-activation of unselected PBMCs with PM21-particles induces *in vivo* NK cell expansion.

NSG mice were injected IP with $2x10^6$ cells of either pre-activated PBMCs *ex vivo* with PM21-particles (left) or non pre-activated PBMCs and 100 U/mL IL-2 for two days (PM21-PBMCs). Mice in all groups received 1,000 U of IL-2, IP thrice weekly. Groups of mice were also injected with 400 µg of PM21-particles, IP twice weekly. Peripheral blood was drawn by sequential cheek bleeds and analyzed by flow cytometry for $CD45^+$ human lymphocytes twice weekly starting on day 6. Cell amounts were determined based on staining for CD3, CD56, CD19. The top plots in each experimental group show concentration of hNK cells per µL of PB. The bottom plots show the percentage of NK cells (○, red) and T cells (▽, black) as fraction of total $CD45^+$ cells.

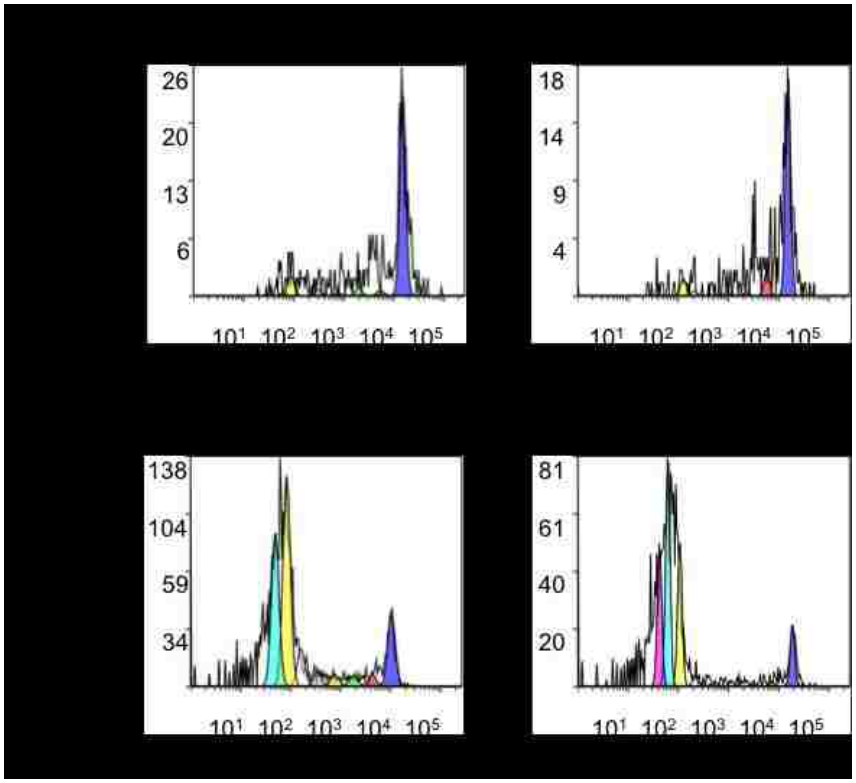
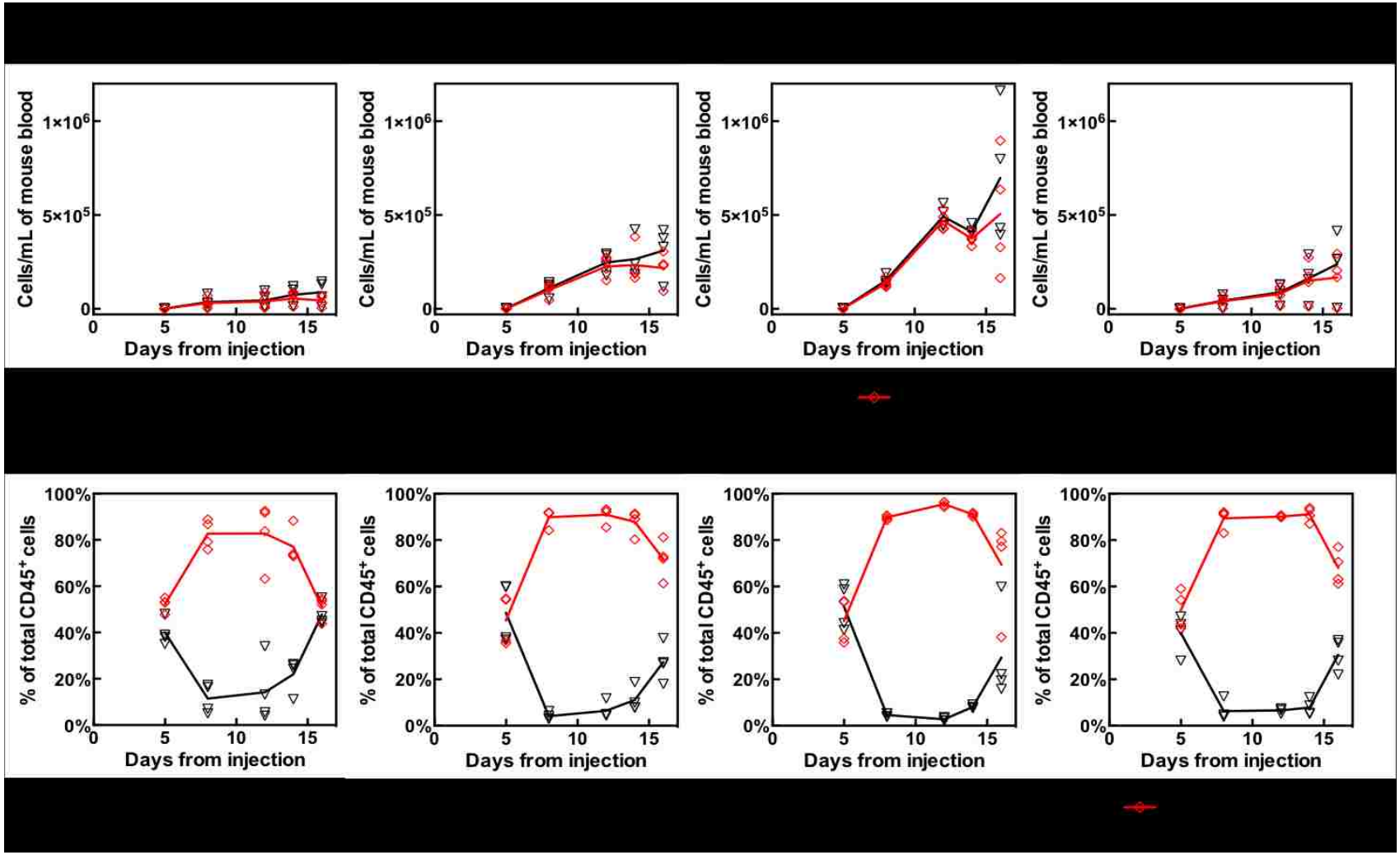


Figure 16: Proliferation analysis evidences *in vivo* NK cell expansion from PM21-PBMCs.

A, **B**) Non pre-activated PBMCs **C**, **D**) PM21-PBMCs. **B**, **D**) 2 groups were injected with 400 µg of PM21-particles. Histograms of the CT Violet fluorescence was analyzed through curve fitting using the Proliferation analysis suite within FlowLogic.


Cells/mL of mouse blood
1×10^6
5×10^5
0
0
5
10
15
Days from injection
% of total CD45+ cells
100%
80%
60%
40%
20%
0%

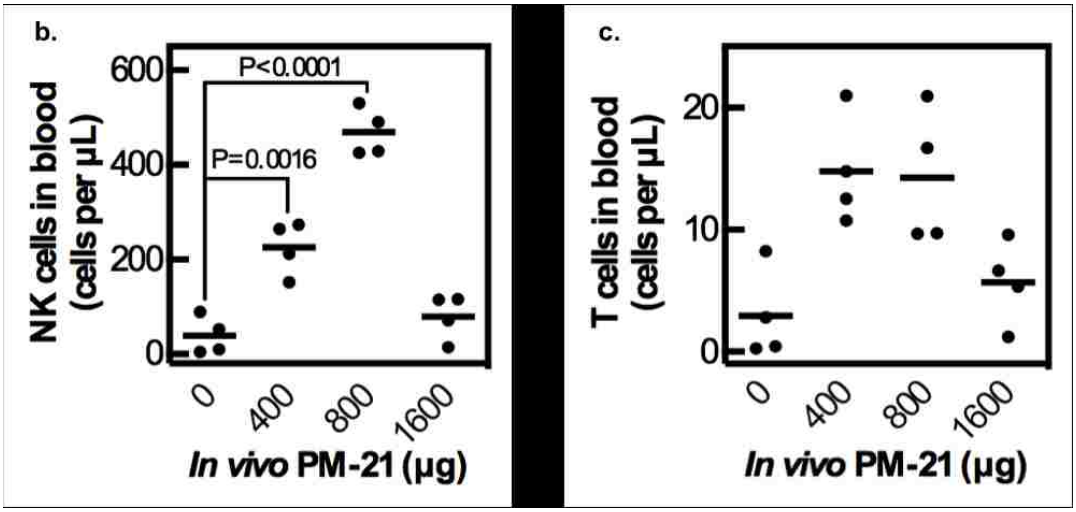


Figure 17: *In vivo* application of PM21 allows increase of NK cells in peripheral blood.

a) The top plots in each experimental group shows concentration of hNK cells per µL of PB. The bottom plots shows the percentage of NK cells (○, red) and T cells (▽, black) as fraction of total $CD45^+$ cells. b) Analysis of PB samples from day 12 after initial injection IP of PM21-PBMCs shows a dose dependent increase of hNK cells with respect to *in vivo* PM21-particle dose; c) while no significant dose dependent increase in total $CD3^+$ T cells was observed.

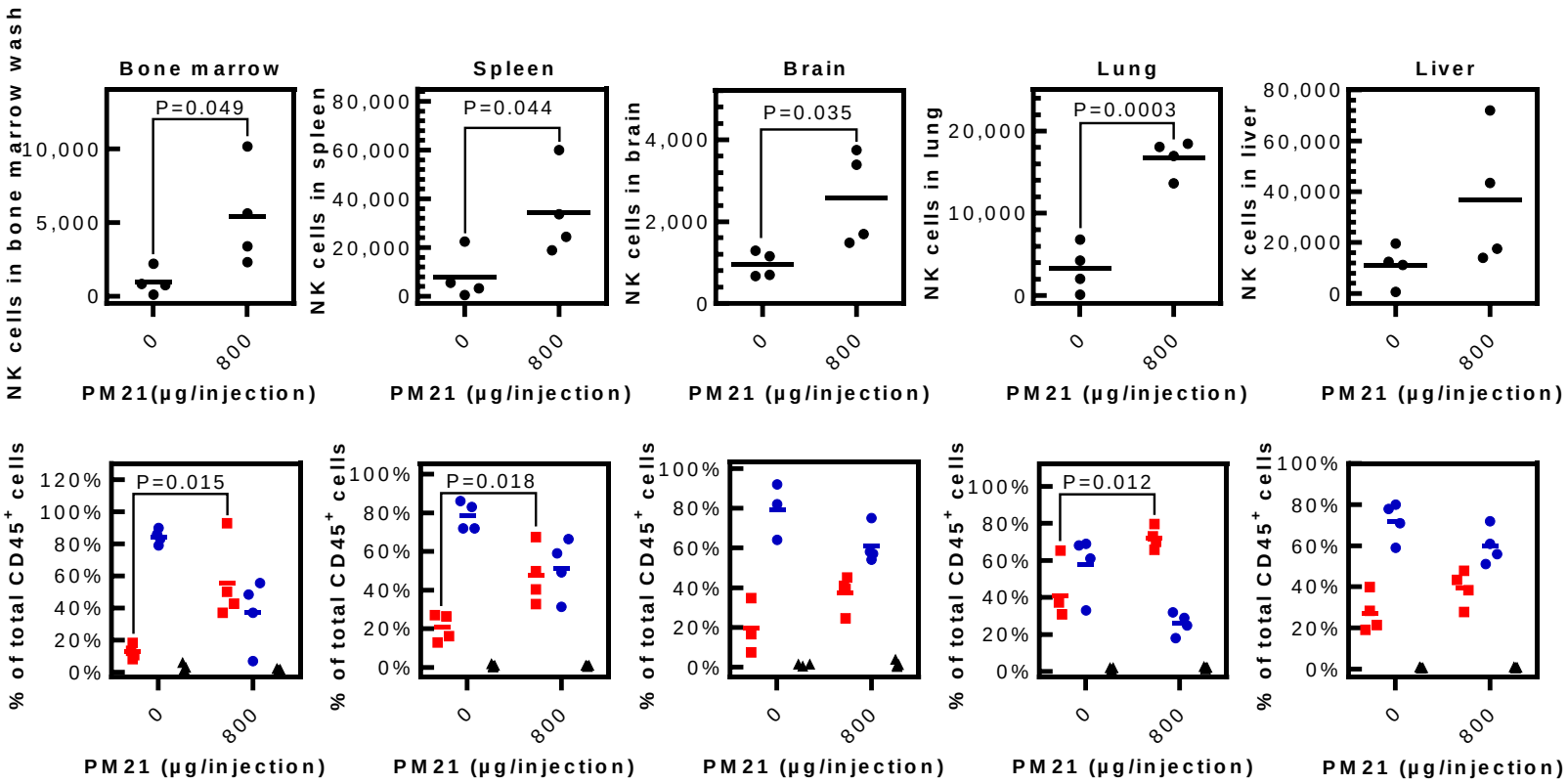


Figure 18: *In vivo* expanded NK cells bio-distribute to key physiological sites and the NK cell bio distribution is increased with *in vivo* application of PM21-particles.

Data for bone marrow, spleen, brain, lung and liver (left to right) are shown with the amount of $CD45^+CD56^+CD3^-$ NK cells (top plots for each organ) and percentages for $CD45^+CD56^+CD3^-$ NK cells (○, red), $CD45^+CD3^+$ T cells (□, blue) and $CD45^+$, $CD56^-CD3^-$ other lymphocytes (△, black) are shown (bottom plots for each organ). The thick bar for each represents the mean.

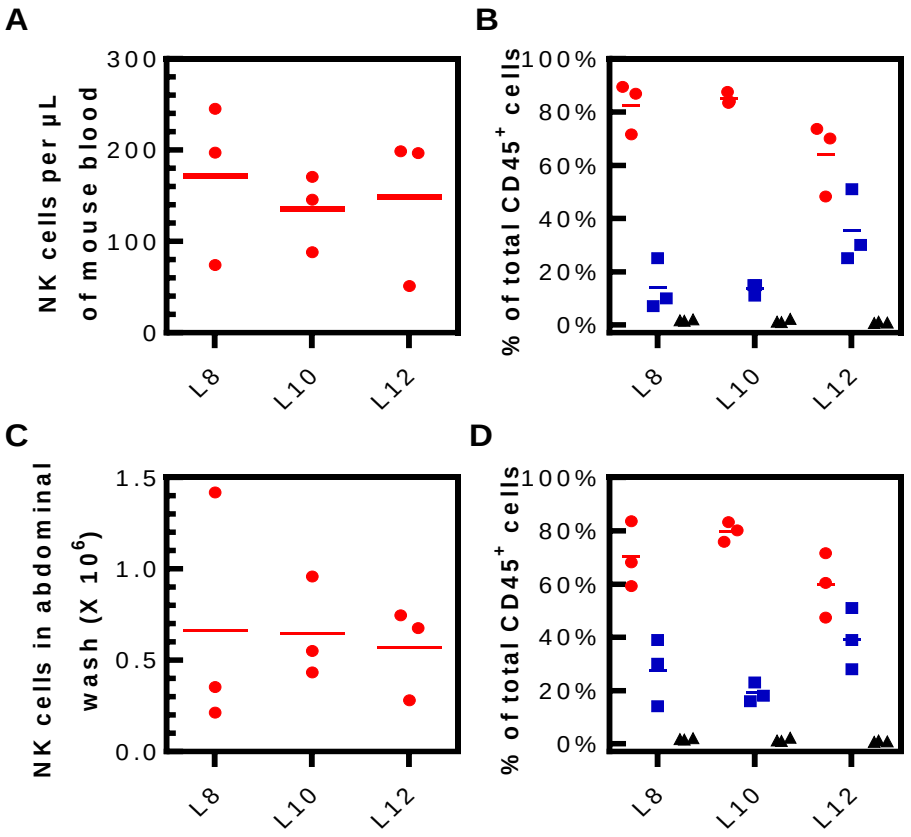
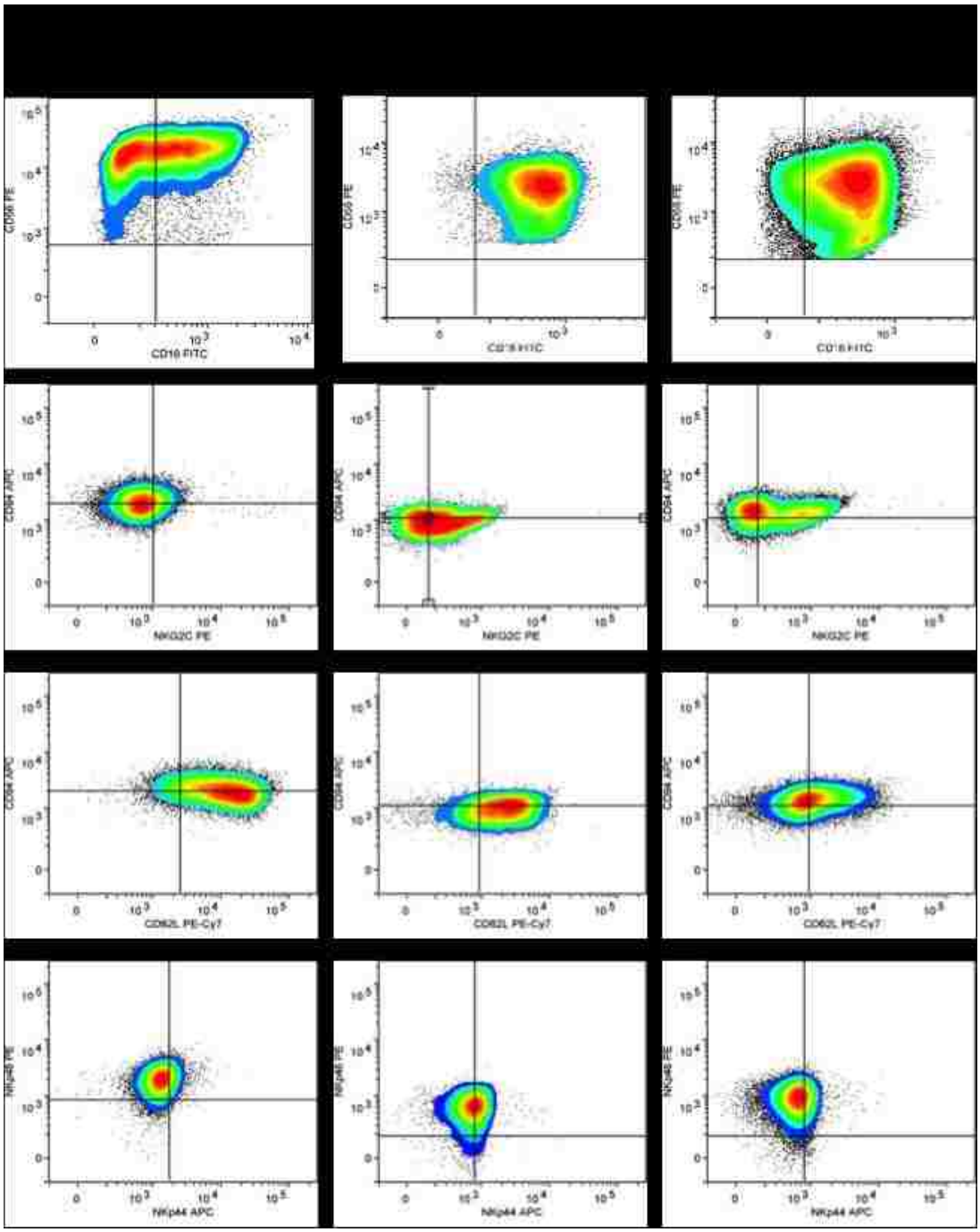


Figure 19: *In vivo* NK cells expansions from different donor sources are consistent.

A) Concentration of NK cells in blood 12 days after IP PBMC injection and C) the amount of NK cells collected in a wash of the abdominal cavity 14 days after IP PBMC injection were similar between the different groups injected with different NK cell sources (p=0.84 for PB and p=0.69). B, D) The corresponding cell content of NK cells (○, red), T cells (□, blue) and other $CD45^+$ cells (△, black) were also consistent between the groups injected with different PBMC sources in the peripheral blood and in the abdomen. The thick bar represents the mean.


CD56 PE
CD16 FITC
CD94 APC
NKG2C PE
CD62L PE-Cy7
NKp46 PE
NKp44 APC

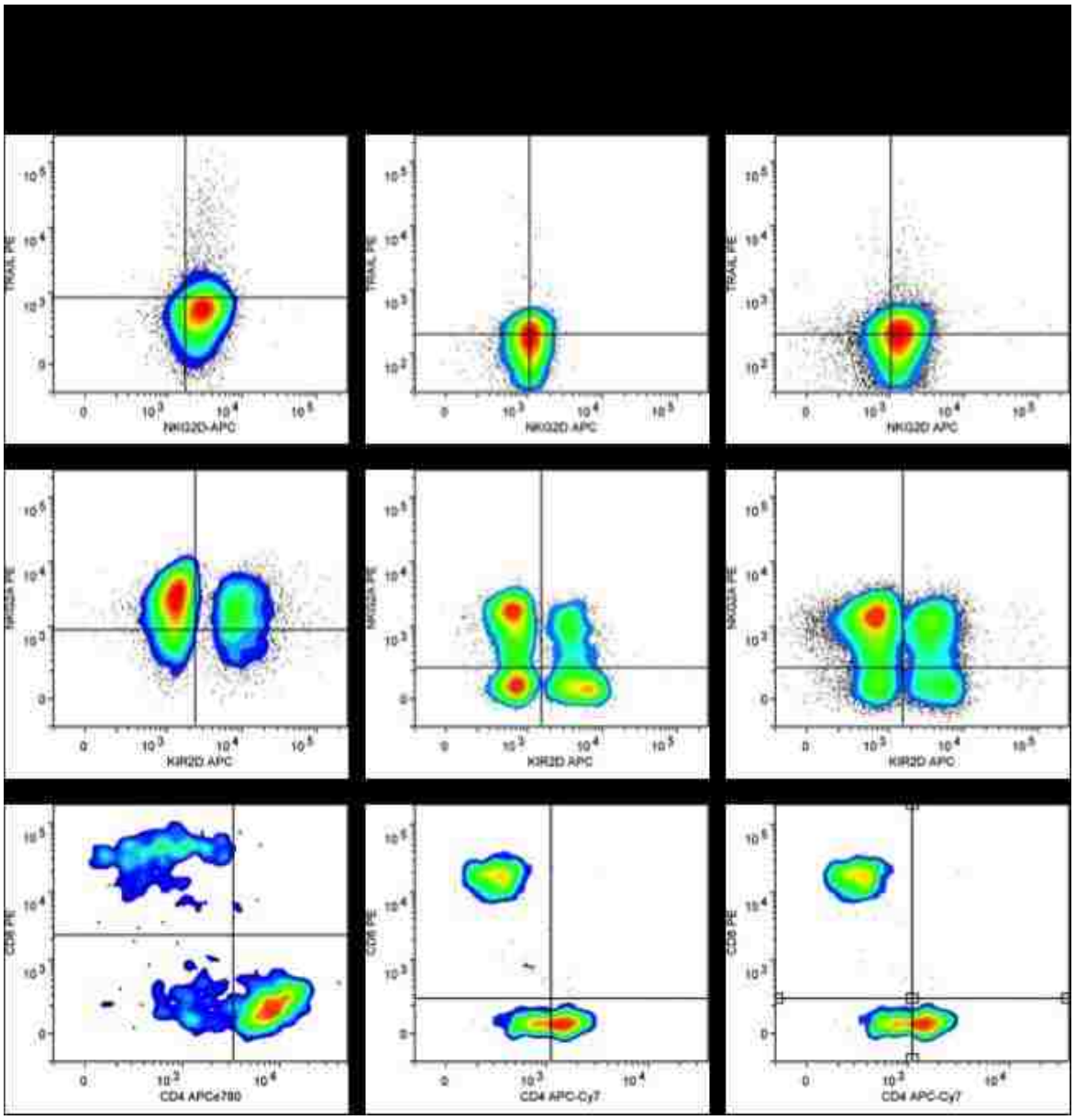


Figure 20: Comparative phenotype analysis of PM21-NK cells expanded from *in vivo* and *ex vivo* sources.

NK cells were obtained from *ex vivo* culture (left), from peripheral blood (PB, middle), or from wash of the abdominal cavity (AW, right). Plots show the expression of important activating and inhibitory receptors and markers of NK cells involved in different cytotoxicity pathways such as ADCC and death receptor pathways.

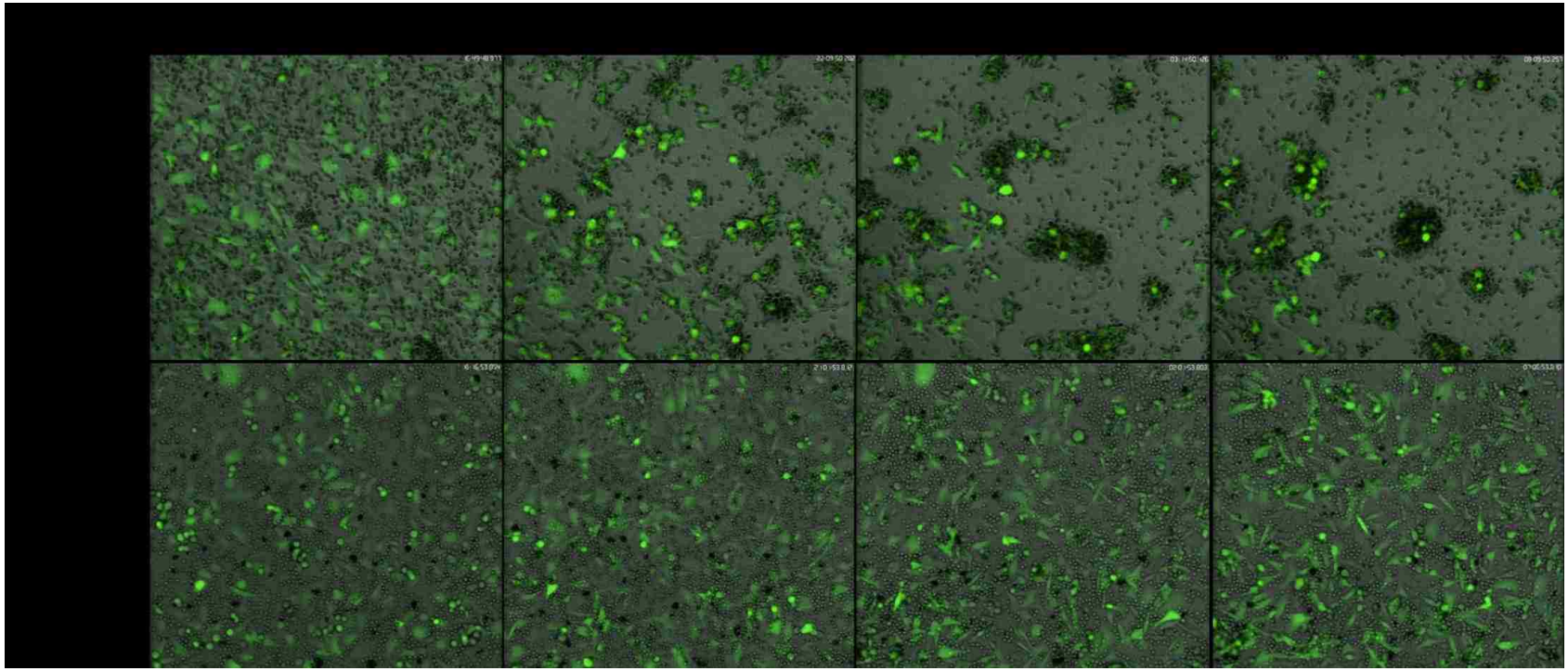


Figure 21: PM21 particle expanded NK cells kill SKOV-3/GFP-Luc cells *in vitro*.

Top panel of images shows PM21-expanded NK cells attacking adhered SKOV-3/GFP-Luc cells over time and the bottom panel shows isolated NK cells, not pre-activated with PM21 showing minimal activity against adhered SKOV-3/GFP-Luc cells, leaving the tumor cells attached to the plate.

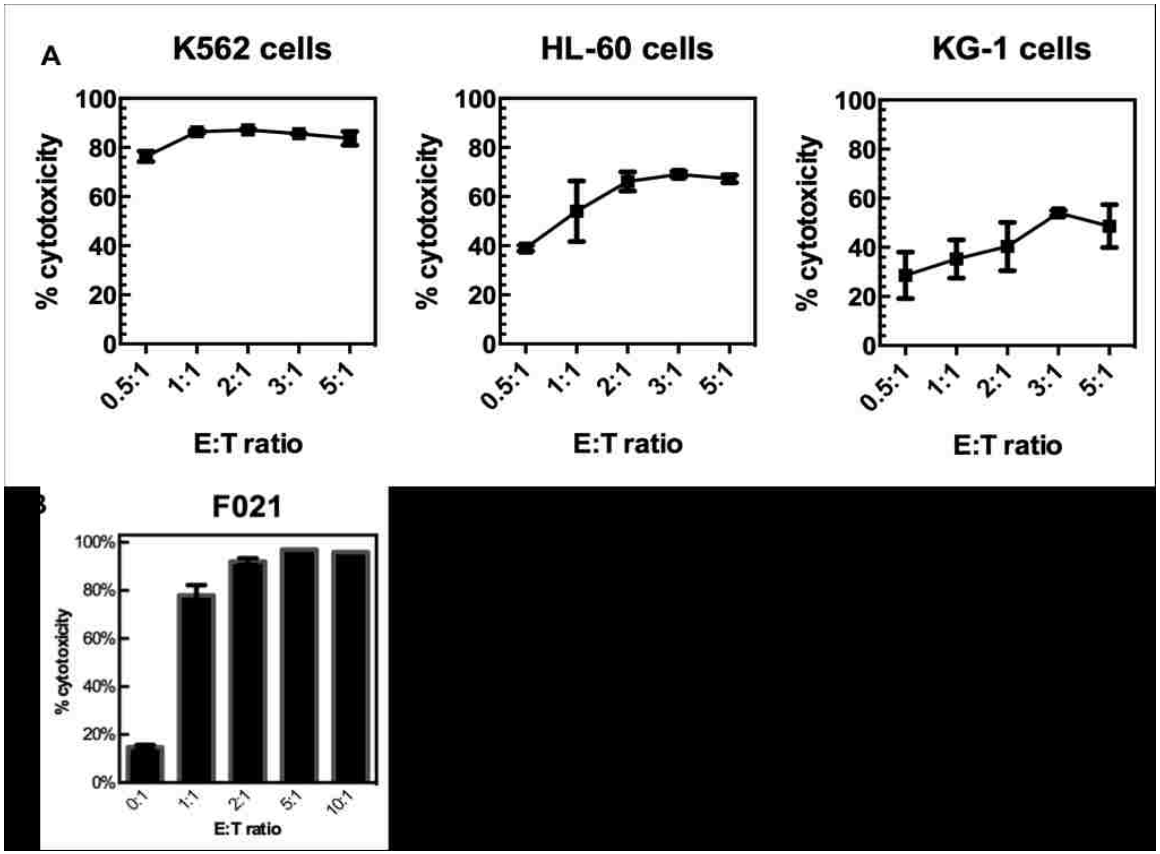


Figure 22: PM21-particle expanded NK cells kill leukemia cell lines and patient tumor in an autologous setting.

A) Percentage cytotoxicity of PM21 expanded NK cells against K562 (CML), HL-60 (APML) and KG-1 (AML) cell lines. Plots depict one representative cytotox assay and each data point was determined in duplicate with the error bars depicting the standard deviation. B) PM21-NK cells (from F021 patient PBMC) labeled with TFL4, co-incubated (2 hours) at indicated E:T ratios with AML cells from the same patient during active disease, and analyzed by flow cytometry. The amount of spontaneous dead target cells was determined using a "Target Alone" control. Each data point was determined in duplicate (Experiment courtesy of Jeremiah L. Oyer, Copik Lab).

Discussion

PM21-particles facilitate ex vivo and in vivo NK cell expansion to therapeutically relevant amounts.

Adoptive NK cell therapy holds high promise as a cancer therapy for initial treatment and remission maintenance of various tumors. A requirement for therapeutic use of NK cells is a method for rapid and selective NK cell expansion that is safe, simple, and overall therapeutically effective. Several cytokine and feeder cell based methods are currently being clinically investigated and the methodology using K562-mb21-41BBL cell line is among the most effective for *ex vivo* NK cell expansion. While feeder cell methods are effective for providing a high initial dose and can allow for multiple dosing, the ability of the *ex vivo* expanded NK cells for homing to the bone marrow may be affected in leukemia treatment and *in vivo* persistence of the infused NK cells may not be optimal. The combined *ex* and *in* vivo PM21-particle based NK cell expansion method described here could significantly enhance the efficacy of NK cell adoptive therapy.

Importantly, PM21-particles can be used for *in vivo* stimulation to promote *in vivo* expansion and persistence. The methodology developed here used a short 2 day *ex vivo* pre-activation, followed by *in vivo* administration of PM21-particles. *In vivo* application of the PM21-particles induces higher *in vivo* NK cell expansion, which is dose dependent on the *in vivo* applied PM21-particles. With our current optimized procedure, PB NK cells were increased by an average of 360-fold between days 5 and 12 after IP injection of PM21-PBMCs, with perhaps even greater fold of expansion in the intraperitoneal cavity. For comparison, it was shown in a recent study [109] that following IV infusion of 1-2 x 10^6 NK cells only about 5 to 17 NK cells

per µL of blood were observable on day 14 after infusion. In contrast, PM21 particles resulted in >400 NK cells/µL of blood on day 12 after IP infusion of 2.0 x 10^6 PM21-PBMCs (11%, *i.e.* 0.2 x 10^6 NK cells). Moreover, the former study used 5 µg (~50,000 U) of either IL-2 or IL-15 thrice weekly, whereas a relatively low dose of IL-2 (1,000 U/injection, thrice weekly) was used in our study. In a different study [47], 30 x 10^6 NK cells were preferentially expanded *ex vivo* with K562-mb15-41BBL feeder cells and then were injected IV followed by tracking the injected human lymphocytes using anti-CD45 antibody (not by a combination of anti-CD56 and anti-CD3). However, the deficiencies in this method were that high dose of IP injected IL-2 (25,000 U/daily) was required for lymphocyte persistence and NK cell concentrations were implied but not determined. Therefore, in comparison to these previous studies, the resultant expansion of NK cell numbers *in vivo* from the PM21 particle method is unprecedented and a unique capability of the PM21 particles.

Here, the route of delivery of the PM21-PBMCs to NSG mice was by the IP injection, similar to previous pre-clinical studies [47, 63]. In comparison, the PM21 particle based method has several advantages. First, the combination of *ex vivo* pre-activation and *in vivo* stimulation with PM21-particles enables the use significantly fewer unselected PBMCs compared to cytokine activation of isolated NK cells [63], which are obtained from blood apheresis followed by extensive laboratory processing for NK cell enrichment. Second, the PM21-particle based method only requires a short 2 day pre-activation, instead of two week culture based expansion [47], and therefore may allow for better preservation of physiologically relevant functionality. Third, the current method allows for significantly greater *in vivo* expansion of NK cells and *in vivo* persistence without the use of high dose IL-2, which has been associated with clinical toxicity. For intraperitoneal tumors such as ovarian cancer, the advantages of the currently

described method may significantly enhance the overall anti-tumor effect. In the absence of intraperitoneal tumors, the intraperitoneal cavity still may provide an environment for PM21-particle to facilitate *in vivo* expansion, as shown clearly shown by proliferation analysis with CT Violet, so that the NK cells can migrate out at significant amounts to the PB and circulate to other organs. NK cells were not only observed in PB, but were found in organs and also were more abundant with *in vivo* application of PM21-particles. The NK cell amounts measured in bone marrow is comparable to those in a study [110] using NK cells generated from $CD34^+$ umbilical cord blood stem cells, indicating that these NK cells are competent for marrow homing.

Phenotyping of NK cells expanded in parallel *ex vivo* or *in vivo* (Figure 20) indicated that the resulting cells were similar, irrespective of the approach. Interesting differences were observed with respect to expansion of $NKG2A^-$ and $NKG2C^+$ subpopulations that were mostly observed with NK cells expanded *in vivo* but not in *ex vivo* [111]. $NKG2C^+$ NK cell populations have been observed during viral reactivation, associated with "memory-like" response and were recently shown to be dependent on monocytes for production of IL-12 [111-113]. Presence of $NKG2C^+$ NK cells in patients with CMV reactivation after stem cell transplantation for AML was also associated with better outcomes and less relapse [114]. Also, significant population of $NKG2A^-$ NK cells that should be resistant to HLA-E induced inhibition may be important in treatment of multiple myeloma patients where cells downregulate HLA class I but express HLA-E to evade NK cells response [115]. Approaches aimed at downregulation of NKG2A have been proposed as a means to improve NK cell cytotoxicity and thus their therapeutic potential [116]. Since *ex vivo* expanded cells were mostly $NKG2A^+$, shortening the time of *ex vivo* culture with

subsequent *in vivo* expansion may provide additional benefit in generation of NK cells with greater phenotypic diversity and potentially good cytotoxicity against targets.

Potential clinical utility of PM21-particles. The capabilities of PM21-particles for NK cell expansion may allow wider use of adoptive NK cell therapy for cancer treatment and potentially for other maladies as well. The PM21-particles can easily be substituted for the feeder cells currently used in clinical trials to ease logistics and mitigate risks. For regulatory jurisdictions where the use of tumor derived feeder cells are prohibited or approval is difficult to obtain, the PM21-particles are a ready solution of *ex vivo* expansion and activation. Moreover, *in vivo* administration of the PM21-particles can further expand the NK cells *in vivo*, an unprecedented capability, and possibly diminish T cell expansion to mitigate GVHD. For treatment of peritoneal cancer and other intraperitoneal tumors such as in persistent ovarian epithelial cancer or desmoplastic small-round-cell tumor, this NK cell expansion method could potentially be clinically translated. Anti-tumor efficacy experiments for elimination of intraperitoneal ovarian tumor are currently underway.

Importantly, the NK cells expanded by this method biodistribute out from the abdominal cavity to peripheral blood and multiple organs that are potential sites of various other cancers. While the IP route of injection is unconventional for treatment of hematological malignancies, delivery of NK cells by this IP path results in PB concentration of NK cells that would be relevant for AML treatment. Therefore, future exploration and testing may be worthwhile to consider for IP delivery of NK cells and PM21-particles for treatment of non IP cancers such as AML.

The particle based approach for NK cell specific signaling could be a platform to include other signaling molecules or even a vehicle for packaged delivery of agents for further targeted

stimulation of NK cells to enhance homing, anti-tumor cytotoxicity and persistence. The PM21-particles can be highly complementary with all the innovative NK cell specific immunotherapy methods (check point inhibitors [117], CARs [118-121], bispecific killer cell engagers (BiKE) [122], Diptheria toxin fused IL-2 for Treg depletion [109], etc.) being developed and with the effects of these being beneficially compounded upon *in vivo* expansion of NK cells with PM21-stimulation. Even as a pre-clinical utility, the currently described method can allow an unprecedented method to study such combination methods. While of course there are murine models, there are no other methods to study human NK cells that can be present *in vivo* for a significant duration.

To summarize, this procedure with PM21-particles allows *in vivo* preferential NK cell expansion at levels typically only achieved with *ex vivo* expansion with feeder cells, but without the need of cell culture with feeder cells or high cytokine doses that are toxic. Furthermore, PM21-PBMCs with *in vivo* delivery of PM21-particles could be used in autologous settings, to take advantage of beneficial synergistic effect of other immune cells on NK cell function [123-125] and further combined with other strategies such as anti-KIR antibodies or BiKEs to maximize NK cell cytotoxicity [94, 122, 126]. Thus this method meets the criteria for generation of NK cells for potential therapeutic efficacy while being simple, more amenable for clinical translation, and can be impactful for treatment of cancer or other maladies.

CHAPTER 4: CONCLUSION

Evidence over the past several years has shown that ovarian cancers are immunogenic in nature. As discussed before, there are credible chemotherapeutic drugs and procedures used in addition to cytoreductive surgery for the treatment of primary tumors. The challenge is to develop new feasible strategies that offer maximum clinical benefit to treat relapsed tumors. Studies suggest that platinum based chemotherapeutics can trigger the immune system by causing cell death, leading to anti-tumor immune response [127, 128]. Since platinum based drugs are the main agents used in front-line ovarian cancer treatment and are involved in enhancement of immunogenic cell death of tumor cells, there's a strong rationale for development of immunotherapeutic strategies as adjuvant therapy against ovarian tumors. Chapter 2 delineates the importance of innate NK cells as a main effector population in the PBMCs that act as the first-line of defense against the tumor. The study implies that there is cooperation or synergy within the PBMC population that NK cells require for their activity. This could explain the lack of NK cell responsiveness seen in the clinic because they are usually expanded and administered as an isolated population. The study underscores the importance of further development of existing NK cell based therapeutic strategies, mainly adoptive cell transfer, specially focusing on overcoming the side effects. Adoptive cell therapy using NK cells has shown promising results against some cancers such as leukemia and melanoma, but there is still a large scope for improvement in the understanding of NK cell function especially with respect to solid tumors such as ovarian cancer.

Miller and co-workers have defined a guideline for clinically efficient NK cell expansion as achieving 100 NK cells/μL of blood 14 days following infusion [98]. The NK cell expansion

strategies including *in vivo* and *ex vivo* expansion using IL-15/IL-2 stimulation has marginal benefits and several side-effects. There has been improvement in *ex vivo* expansion using feeder cell co-cultures with modified K562 cell line expressing membrane bound cytokines, mainly IL-15 and IL-21. Although these feeder cell-based methods offer improvement in NK cell expansion, there are other challenges pertaining to long *ex vivo* culturing time periods. Expansion with K562-mb15-41BBL may results in, first, senescence of NK cells and second, loss of their CD16 receptor leading to decrease in the capacity to mediate one of the major effector functions through ADCC. Expansion with the membrane bound IL-21 variant of the cell line overcomes the problem of senescence but issues with exhaustion, *in vivo* persistence and the ability to home to different organs remain. Besides this, the logistics of using feeder cells for expansion limit feasibility of their use in clinic.

Taking into consideration, all these drawbacks and challenges, Copik *et al* devised the method to expand NK cells using plasma membranes obtained from K562-mb15-41BBL cells and showed that NK cell expansion was comparable to that from the feeder cells the membranes were derived from and that the cells expanded *ex vivo* using this method were cytotoxic [48]. This study showed that the use of plasma membrane bound cytokines including but not limited to IL-15 could perhaps be a strong alternative to the existing feeder cell co-culture methods, without associated drawbacks that have been discussed above.

In the light of advantages seen from K562-mb21-41BBL expanded NK cells over that of K562-mb15-41BBL, such as better expansion and lack of senescence, same strategy was applied to obtain membrane bound IL-21 and 41BBL, which is the focus of the study presented in chapter 3. IL-21 is a cytokine secreted mainly by $CD4^{+}$T cells and belongs to the γ-chain-dependent family of cytokines such as IL-2 and IL-15 [129]. Besides benefitting NK cells in

terms of activating and promoting their survival, IL-21 is also shown to increase the expression of co-stimulatory molecules and NK cell effector molecules such as perforins and granzyme [130]. These findings in addition to advantages of K562-mb21-41BBL expanded NK cells as discussed before, strengthen the rationale for using this interleukin for stimulating NK cell expansion and cytotoxicity. Experiments discussed in chapter 3 clearly show that PM21 particles show better *in vitro* expansion of NK cells as compared to that from PM15 particles. It also shows that NK cells pre-activated with PM21 can expand *in vivo* and that this expansion can be augmented by further administration of PM21 *in vivo*. NK cells expand in the peritoneal cavity, migrate to the peripheral blood and home to different organs. Besides this, these particles were able to expand NK cells from patients in remission and they were shown to be cytotoxic against the same individual's tumor cells, which gives an indication that this strategy might be helpful in autologous NK cell therapy as well. Most importantly, PM21 pre-activated NK cells showed cytotoxicity against SKOV-3/GFP-luc cells *in vitro* along with some leukemia cell lines. During *in vivo* expansion, not only did NK cells expand to 360 fold in 5 to 12 days (>400 NK cells/μL of blood on day 12, which is way more that the set benchmark), but they constituted high percentage of total $CD45^+$ cells in mouse blood, indicating that the expansion was NK cell specific with low T cell numbers.

Current studies in our group include, determining *in vivo* cytotoxicity of PM21 stimulated NK cells in tumor models such as the ovarian cancer model discussed in chapter 2. To further the clinical relevance, efficacy could also be tested using patient derived xenograft mouse models of ovarian cancer, in terms of overall survival. Future studies will involve the study of *in vivo* cytotoxicity against leukemia cell lines. Considering hindrances because of lack of appropriate models for the disease, perhaps an orthotopic model will be developed for leukemia and then the

efficacy of PM21 stimulated NK cells would be studied in terms of homing to the bone marrow in the presence of tumor and cytotoxicity against that tumor. If and when tumor clearance *in vivo* is established, another interesting avenue would be to study NK cell memory possibly by re-challenging the mice with tumors. Another relatively unknown aspect of this study is the trafficking of the plasma membrane particles *in vivo* and how it may affect NK cell expansion. There are indications of PM21 particles being taken up by some other immune cell types in the injected PBMC population (data not shown). Such clarifications will shed light on the involvement and importance of other immune cell types in augmenting or diminishing NK cell expansion, persistence and cytotoxic efficacy. By dissecting the involvement of different signaling components essential for NK cell activation, plasma membranes could possibly be used to express involved molecules, for targeted stimulation of NK cells to enhance various aspects important for NK cell based immunotherapy. For example, currently experiments are underway to develop membrane bound IL-2 in order to make NK cells independent of exogenous or T cell-provided IL-2 for proliferation.

Overall, the first part of this study shows the relevance of an innate immune cell type in tumor cell recognition and mounting a first-line defense that resulted in complete clearance of residual ovarian tumors, which is relatively under-appreciated and unexploited in the field of immunotherapy for cancer. The second part of the study shows a novel methodology of NK cell expansion that has tremendous potential in advancing the field of NK cell based immunotherapy for ovarian and other cancer types. This strategy could perhaps also be combined with other immunotherapies that are currently being tested in the clinic and that have great potential for ovarian and other types of cancers, such as checkpoint blockade, mAb therapies, vaccines etc. Tumor cells can adopt various escape mechanisms simultaneously in order to evade immune

recognition. Thus, it is rational to combine immunotherapies in order to target tumor-escape mechanisms at various levels, possibly resulting in cooperation or synergy amongst different strategies and achieving maximum benefit, hopefully curing cancer.

APPENDIX A: IACUC PERMISSIONS

University of Central Florida

Office of Research & Commercialization

September 9, 2013

Dr. Deborah Altomare
Burnett School of Biomedical Sciences
6900 Lake Nona Blvd.
Orlando, FL 32827

Subject: Institutional Animal Care Use Committee (IACUC) Addendum Submission.

Dear Dr. Altomare,

This letter is to inform you that the following addendum submitted was approved by the IACUC.

Animal Project:	11-35
Title:	Strategies to Improve Immune Reconstitution and Anti-Cancer Responsiveness of Natural Killers (NK) Cell Immunotherapy. (Addendum #2)
Approval Date:	9/7/2013

Please see the attached copy of the approved addendum and please keep a copy for your records. Should you have any questions, please do not hesitate to call me at (407) 822-1164.

Sincerely,

Cristina Caamaño
Assistant Director, Research Program Services
Office of Research & Commercialization

12201 Research Parkway • Suite 501 • Orlando, FL 32826-3246 • 407-823-3778 • Fax 407-823-3299

An Equal Opportunity and Affirmative Action Institution

University of Central Florida

Office of Research & Commercialization

9/26/2014

Dr Deborah Altomare
Burnett School of Biomedical Sciences
Lake Nona
6900 Lake Nona Blvd
Orlando, FL 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr Deborah Altomare:

This letter is to inform you that your following animal protocol was re-approved by the IACUC. The IACUC Animal Use Renewal Form is attached for your records.

Animal Project #:	13-32
Title:	Establishing particle-activated natural killer cell therapy for treatment of AML in preclinical NSG mouse model
First Approval Date:	11/13/2013

Please be advised that IACUC approvals are limited to one year maximum. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur. Furthermore, should there be a need to extend this protocol, a renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval. If the protocol is over three years old, it must be rewritten and submitted for IACUC review.

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 882-1164.

Please accept our best wishes for the success of your endeavors.

Best Regards,

Cristina Caamaño
Associate Director, Research Program Services

Copies: Facility Manager (when applicable.)

12201 Research Parkway • Suite 501 • Orlando, FL 32826-3246 • 407-823-3778 • Fax 407-823-3299

UNIVERSITY OF CENTRAL FLORIDA
RESEARCH & COMMERCIALIZATION

6/26/2015

Dr Deborah Altomare
Burnett School of Biomedical Sciences
Lake Nona
6900 Lake Nona Blvd
Orlando, FL 32827

Subject: Institutional Animal Care and Use Committee (IACUC) Protocol Submission

Dear Dr Deborah Altomare:

This letter is to inform you that your following animal protocol was re-approved by the IACUC. The IACUC Animal Use Renewal Form is attached for your records.

Animal Project #:	14-28
Title:	Preclinical efficacy of adoptive cell therapy against ovarian cancer
First Approval Date:	7/10/2014

Please be advised that IACUC approvals are limited to one year maximum. Should there be any technical or administrative changes to the approved protocol, they must be submitted in writing to the IACUC for approval. Changes should not be initiated until written IACUC approval is received. Adverse events should be reported to the IACUC as they occur. Furthermore, should there be a need to extend this protocol, a renewal must be submitted for approval at least three months prior to the anniversary date of the most recent approval. If the protocol is over three years old, it must be rewritten and submitted for IACUC review.

Should you have any questions, please do not hesitate to call the office of Animal Welfare at (407) 882-116

Please accept our best wishes for the success of your endeavors.

Best Regards,

Cristina Casmaño
Associate Director, Research Program Services

Copies: Facility Manager (when applicable.)

12201 Research Parkway • Suite 501 • Orlando, FL 32826-3246 • 407-823-3778 • Fax 823-3299

APPENDIX B: IRB APPROVAL

Florida Hospital
Institutional Review Board
212 E. Winter Park Street
Orlando, FL 32804
Telephone: (407) 303-5581
Fax: (407) 303-3638
FWA: 00002060
IRB Registration #: 00000842

DATE:	June 21, 2012
TO:	Alicja Copik, PhD
FROM:	Florida Hospital Institutional Review Board (IRB)
PROJECT TITLE:	[238177-4] Generation of Highly Cytotoxic Natural Killer Cells for Cellular Therapy of Cancers Using Novel Microparticle Approach
SPONSOR:	None
REFERENCE #:	2586-2990
SUBMISSION TYPE:	Other
ACTION:	APPROVED
APPROVAL DATE:	June 21, 2012
EXPIRATION DATE:	September 11, 2012
REVIEW TYPE:	Expedited Review

Note: If this is an expedited or exempt action, the IRB members will be made aware at the next convened meeting.

Thank you for your submission of Other materials for this project. The Florida Hospital IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on the applicable federal regulations. Material reviewed for this submission includes:

- Consent Form - volunteer Informed consent (UPDATED: 06/19/2012)
- Consent Form - Patient Informed consent (UPDATED: 06/19/2012)
- Cover Sheet - Change request - title change, extension of study duration, and other minor changes (UPDATED: 06/19/2012)
- Protocol - Updated study protocol (UPDATED: 06/19/2012)

Please remember that informed consent is a process beginning with a description of the study and assurance of participant understanding followed by a FHIRB approved signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require that each participant receives a copy of the consent document.

Please note that any revision to previously approved materials must be approved by the FHIRB prior to initiation. Please use the appropriate revision forms for this procedure.

Florida Hospital
Institutional Review Board
212 E. Winter Park Street
Orlando, FL 32804
Telephone: (407) 303-5581
Fax: (407) 303-3638
FWA: 00003060

DATE:	September 4, 2012
TO:	Alicja Copik, PhD
FROM:	Florida Hospital Institutional Review Board (IRB)
PROJECT TITLE:	[238177-6] Generation of Highly Cytotoxic Natural Killer Cells for Cellular Therapy of Cancers Using Novel Microparticle Approach
SPONSOR:	None
REFERENCE #:	2586-2990
SUBMISSION TYPE:	Response/Follow-Up
ACTION:	APPROVED
APPROVAL DATE:	September 4, 2012
EXPIRATION DATE:	August 27, 2013
REVIEW TYPE:	Expedited Review

Note: If this is an expedited or exempt action, the IRB members will be made aware via published meeting minutes.

Thank you for your submission of Response/Follow-Up materials for this project. The Florida Hospital IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Expedited Review based on the applicable federal regulations. Material reviewed for this submission includes:

- Consent Form - Revised Volunteer informed consent (UPDATED: 08/31/2012)
- Consent Form - Revised Patient informed consent (UPDATED: 08/31/2012)
- Other - Note to file- response to p.3 of Modification Required Letter (UPDATED: 09/4/2012)
- Protocol - Revised protocol (UPDATED: 08/31/2012)

Other documents reviewed and approved by the convened IRB on 8/28/12 included:

- Continuing Review/Progress Report - Progress report (UPDATED: 08/20/2012)
- Cover Sheet - change request form (UPDATED: 08/20/2012)
- Other - Reviews Preparatory to Research Elizabeth Griffith (UPDATED: 08/21/2012)
- Other - Reviews Preparatory to Research Jeremiah Oyer (UPDATED: 08/21/2012)
- Other - delegation responsibility log (UPDATED: 08/20/2012)

Please remember that informed consent is a process beginning with a description of the study and assurance of participant understanding followed by a FHIRB approved signed consent form. Informed

University of Central Florida Institutional Review Board
Office of Research & Commercialization
12201 Research Parkway, Suite 501
Orlando, Florida 32826-3246
Telephone: 407-823-2901, 407-882-2901 or 407-882-2276
www.research.ucf.edu/compliance/irb.html

From : **UCF Institutional Review Board**
FWA00000351, IRB00001138

To : **Alicja Copik**

Date : **February 19, 2013**

IRB Number: **SBE-13-09129**

Study Title: **Generation of highly cytotoxic natural killer cells for cellular therapy of cancers using novel microparticle approach**

Dear Researcher:

The research protocol noted above was reviewed by the University of Central Florida IRB Designated reviewer on 2/19/2013. **The UCF IRB accepts Florida Hospital's Institutional Review Board review and approval of this study for the protection of human subjects in research. The expiration date (8/27/2013) will be the date assigned by the Florida Hospital's Institutional Review Board and the consent process will be the process approved by that IRB.**

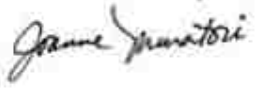
This project may move forward as described in the protocol. It is understood that the Florida Hospital IRB is the IRB of Record for this study, but local issues involving the UCF population should be brought to the attention of the UCF IRB as well for local oversight, if needed.

All data, including signed consent forms if applicable, must be retained in a locked file cabinet for a minimum of three years (six if HIPAA applies) past the completion of this research. Any links to the identification of participants should be maintained on a password-protected computer if electronic information is used. Additional requirements may be imposed by your funding agency, your department, or other entities. Access to data is limited to authorized individuals listed as key study personnel.

Failure to provide a continuing review report for renewal of the study to the Florida Hospital IRB could lead to study suspension, a loss of funding and/or publication possibilities, or a report of noncompliance to sponsors or funding agencies. If this study is funded by any branch of the Department of Health and Human Services (DHHS), an Office for Human Research Protections (OHRP) IRB Authorization form must be signed by the signatory officials of both institutions, and a copy of the form must be kept on file at the IRB office of both institutions.

On behalf of Sophia Dziegielewski, Ph.D., L.C.S.W., UCF IRB Chair, this letter is signed by:

Signature applied by Joanne Muratori on 02/19/2013 10:58:40 AM EST



IRB Coordinator

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