ENGINEERING HUMAN PLURIPOTENT STEM CELLS FOR ENHANCED LYMPHOCYTE DEVELOPMENT AND FUNCTION

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THESIS ABSTRACT

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) provide accessible, genetically tractable and homogenous starting cell populations to efficiently study human blood cell development. These cell populations provide platforms to develop new cell-based therapies to treat both malignant and non-malignant hematological diseases. Our group has previously demonstrated the ability of hESC-derived hematopoietic precursors to produce functional natural killer (NK) cells. hESCs and iPSCs, which can be reliably engineered in vitro, provide an important model system to study human lymphocyte development and produce enhanced cell-based therapies with potential to serve as a "universal" source of anti-tumor lymphocytes for novel clinical therapies. My studies have focused on the generation of NK cells from hESCs and iPSCs, their function both *in vitro* and *in vivo* against a variety of different tumor types, and modification of these cells with genetic constructs to enhance their anti-tumor capabilities.

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CHAPTER 1

Background and significance

PLURIPOTENT STEM CELLS TO STUDY BLOOD DEVELOPMENT

For over 40 years, hematologists and oncologists have utilized transplantation of hematopoietic stem cells (HSC) to treat and cure hematologic malignancies[1]. HSCs continue to be the only routinely used stem cell population for clinical therapies, though other stem cell-based therapies have been used in clinical trials. Since the derivation of human embryonic stem cells (hESCs) over a decade ago[2], numerous groups have successfully differentiated this pluripotent source to fully mature and functional subsets of each germ layer. hESCs remain one of the most promising cell sources for regenerative medicine. Phase I clinical trials using hESC-derived cells have been approved by the United States Food and Drug Administration (FDA) but are in their infancy[3]. Most recently, investigators have successfully used hESC-derived retinal pigmented epithelial cells to treat patients with a form of macular degeneration[4]. Studies on derivation and differentiation of human induced pluripotent stem cells (iPSCs) are also rapidly advancing[5-8]. Therefore, the prospect to utilize hESC- and iPSC-derived hematopoietic products for diverse clinical therapies is not a distant prospect, but a reasonable expectation in the next few years[9].

Shortly after the original derivation of hESCs, Kaufman *et. al.* demonstrated hematopoietic cell development using an in vitro co-culture model and defined condition[10]. These studies utilized co-culture of hESCs on irradiated stromal cells (serving as a microenvironment) and showed that during differentiation, hESC-derived cells acquired typical hematopoietic genes and surface antigen expression. Since these initial studies, we and others have further defined the

factors mediating almost all human blood lineages from hESCs[9]. Hematopoietic cells can be consistently produced from hESCs using two separate methods: stromal cell co-culture and embryoid body formation[11,12]. These hESC-derived hematopoietic precursor/progenitor cells can produce erythroid, myeloid, and lymphoid lineage cells *in vitro*[13-15]. However, use of hESCs to derive HSCs capable of long-term, multilineage engraftment when transplanted using *in vivo* models (such as immunodeficient mice) has been limited[16-20]. Several groups have demonstrated similar hematopoietic development from human induced pluripotent stem cells (iPSCs)[21-23]. These studies provide the intriguing possibility that iPSC-derived hematopoietic progenitors could be derived on a patient-specific basis and serve as the definitive example of personalized medicine.

HUMAN INDUCED PLURIPOTENT STEM CELLS

Recently, the development of iPSCs has provided another platform to study human development. iPSCs can now be routinely derived from terminally differentiated somatic cells through expression of several transcription factors known to support the pluripotent state. These include various combinations of the following factors: octamer-binding transcription factor 4 (OCT4), SRY (sex determining region Y)-box 2 (SOX2), Krueppel-like factor 4 (KLF4), cellular myelocytomatosis (C-MYC), and LIN-28[24-26]. iPSCs derived from mice undergo tetraploid complementation and demonstrate germ line chimerism- the most stringent test of pluripotency[27]. Human iPSCs, similar to hESCs, are capable of differentiating into mature cell types of all three germ layers[8,21,24,26,28]. The

recent explosion of iPSC technology has led to successful derivation of iPSCs without integrating transgenes[29,30], a technology that may better enable clinical translation. iPSC technology also enables derivation of disease specific lines enabling in vitro study of diseases with natural genetic or biochemical defects[6,8,31-34]. Building on decades of studies done in mouse and human hematopoietic progenitors several groups have used hESCs, and now iPSCs, to study the mechanisms regulating blood cell development.

LYMPHOCYTES DERIVED FROM HUMAN PLURIPOTENT STEM CELLS

The interplay of transcription factors, cytokines, and tissue microenvironment in hematopoietic and, more specifically, lymphoid development has been well studied in mouse models. However, these successes have not been easily duplicated in human studies[35-37]. Within the adaptive immune system, our knowledge of B and T cells has advanced as one of the most well defined developmental paradigms. In contrast, many aspects of NK cell development and education, such as the underlying mechanisms driving newly-defined NK cell subsets to acquire effector function through a process of “licensing”, remain to be elucidated. Several reviews have focused on this important, growing area of research[38-41].

hESC-derived NK cells provide a genetically defined population to study NK cell development and overcome the high level of donor heterogeneity, such as when using peripheral blood NK (PB-NK) cells. Our group has demonstrated the ability of hESC-derived hematopoietic progenitor cells to produce functional NK cells *in vitro*[14,42]. hESC-derived NK cells express activating and inhibitory

receptors similar to peripheral blood NK (PB-NK) cells and are highly efficient at direct cell-mediated cytotoxicity, antibody dependent cell-mediated cytotoxicity, and cytokine (IFN-γ, TNF-α) production[42]. hESC-derived NK cells provide a reliable developmental model to study human NK cells in vitro. Our studies of hESC-derived NK cells have also demonstrated *in vivo* function[43]. Using a tumor xenograft model, Woll *et. al.* showed complete tumor clearance with hESC-derived NK compared to umbilical cord blood(UCB)-derived NK cell or sham injected controls[43]. For cancer immunotherapy, preclinical mouse models such as this have been the cornerstone to translate novel therapies into the clinic. To date, several factors critical to the efficacy of NK cell-based therapies, including licensing, trafficking, and homing are not well defined in humans. Therefore, hESC-derived hematopoietic progenitors provide both a novel system to study basic developmental processes, as well as an unlimited source of cells for cancer immunotherapy and could potentially serve as a universal source of lymphocytes for off-the-shelf therapy.

CURRENT STATUS OF NK CELLS FOR CLINICAL ADOPTIVE IMMUNOTHERAPY

For over two decades Rosenberg and colleagues at the NIH have been steadily advancing adoptive transfer immunotherapy trials for patients with metastatic melanoma, renal cell cancer, and other malignancies[44-46] A seminal study in 2002 by Ruggeri et. al. demonstrated a more specific function of NK cells for cancer therapy[47]. Here, NK cell-mediated alloreactivity could eliminate relapse, graft rejection, and protect against graft-vs-host disease (GVHD) for patients

with acute myelogenous leukemia (AML)[47]. Another study by Miller and colleagues evaluated the in vivo efficacy of expansion for haplo-identical NK cells with interleukin 2 (IL-2) in a non-transplant setting. In an evaluation of 43 patients with advanced cancer (melanoma, renal cell carcinoma, and poor prognosis AML), IL-2 and a high-dose immunosuppressive regimen was shown to promote expansion of donor derived NK cells[48]. This finding positively correlated with the serum concentrations of IL-15 (a key cytokine involved in NK cell development) induced by the high-dose immunosuppressive regimen. Intriguingly, 5 of 19 patients with poor-prognosis AML had a complete remission. Similar to the Ruggeri study, 4 out of the 5 patients in remission had an alloreactive NK cell receptor repertoire.

Recent studies identify several potential mechanisms attributing to both the successes and failures in NK cell based immunotherapy[49-52]. IL-2, used to expand T or NK cells *in vitro* and *in vivo*, also promotes the expansion of T regulatory cells (Tregs) which express the IL2 receptor alpha chain (CD25). T regulatory cells have been shown to directly suppress both T and NK cell responses[53,54]. Additionally, it has not been well defined why NK cell immunotherapy works in AML but not other hematological or solid malignancies. Here, the ability to genetically modify NK cells for more direct recognition of tumor targets could enhance their therapeutic potential against a broader range of cancers. To date, most of this work has focused on antigen-specific T cells, with less emphasis placed on engineering NK cells. However, this type of gene therapy is difficult achieve in primary peripheral blood (PB)-

NKs and T cells, whereas high efficiency genetic modification is routinely feasible in hESC-derived NK cells.

Based on this record of successful (though limited) NK cell-based therapies against cancer, NK cells derived from hESCs or iPSCs now provide an intriguing starting point to expand this immunotherapeutic approach. Strictly considering cell number, the ability to create enough hESC-derived NK cells for adoptive immunotherapy (~10^7- 10^8 NK cells per patient)[48] is far more reasonable than the possibly insurmountable number of cells needed to generate one unit of RBCs (10^{12} RBCs per unit). Additionally, since the hESC-derived NK cells are void of any contaminating T or B cells, they could be positively selected on the basis of NK cell surface markers. This is in contrast to current strategies using peripheral blood NK cells that involve treatment of the aphaeresis product with CD3 beads (to deplete T cells) and CD20 beads (to deplete B cells and prevent “passenger lymphocyte syndrome”).

hESC-derived NK cells also have their own barriers to translation. The reliable derivation of hematopoietic progenitors from hESCs is dependent on stromal cell co-culture models. The use of mouse stoma provides a barrier to clinical translation, though not insurmountable if a master cell bank of the stromal cells is produced. The traditional “feeder-free” system of embryoid body (EB)-mediated differentiation of hESCs/iPSCs may be utilized but is more variable in differentiation into hematopoietic progenitor cells. Alternative methods such as “spin-EBs”[55] have standardized this approach and have the potential to provide the large numbers of cells needed for clinical trials[56]. By aggregating

undifferentiated hESCs through centrifugation, spin EBs provide a uniform and reproducible method to produce hematopoietic progenitors in the absence of murine stroma[57]. More studies are needed to determine if this is an effective means to generate fully functional NK cells similar to those developed on stroma.

hESC-derived NK cells have been extensively characterized and have defined effector functions parallel to PB-NKs, which are now routinely used for clinical trials[43]. Compared to UCB-derived NK cells, hESC-derived NK cells have superior ability to kill tumor cells both *in vitro* and *in vivo*[58]. In fact, UCB-derived NK cells are less functionally mature, and their use in clinical trials has fallen out of favor. Our studies demonstrate hESC-derived NK cells are similar to PB-NKs in several ways. Like PB-NK cells, hESC-derived NK cells express a number of NK cell effector proteins on their surface (CD16 (FcRIII), NKG2D, NKp46, Fas ligand, TRAIL, and KIR family members). They also have potent anti-tumor effects both in vitro and in vivo as hESC-derived NK cells completely clear the CML tumor K562 in vivo[43]. Because the use of NK cells has shown encouraging results in patients with AML[43], new clinical trials have been initiated using NK cells to treat other hematologic and solid tumors.

With the seemingly effective clinical use of PB-NK cells[48,49], there is often a question of what advantage hESC or iPSC-derived NK cells could provide. To answer this, it is important to consider the relative ease to genetically-engineer hESCs (or iPSCs)[59-62]. In this manner, we have tested whether or not the expression antigen specific receptors in hESCs and iPSCs could provide an

enhanced cellular therapeutic capable of specifically recognizing and killing tumor cells. We hypothesize the genetically-modified hESC or iPSC-derived NK cells could dramatically increase the tumor cell populations amenable to this type of immunotherapy. Additionally, autologous NK cells can be derived from iPSCs. Presumably, these iPSC-derived cells would not require significant chemotherapy to be given to the patient, as is done in part to promote in vivo survival of the allogeneic NK cells that have been used in clinical trials. Third, it may also be possible to utilize hESC- or iPSC-derived NK cells to treat infectious diseases such as HIV where autologous peripheral blood lymphocytes would not be a good option due possible infection of donor cells. Indeed, several studies suggest NK cells play a role in anti-HIV immunity[62-64].

USE OF hESC AND iPSC-DERIVED NK CELLS FOR CANCER THERAPEUTICS

Perhaps the most promising direction for hESC-based immunotherapies is to engineer hESCs or iPSCs to express chimeric antigen receptors (CARs) capable of directing cytotoxic lymphocytes to tumor sites. CARs typically contain an extracellular domain, derived from the Fab (antigen-specific) portion of an antibody that has high specificity to recognize a tumor antigen[65]. The external domain is genetically linked to a transmembrane domain and an intracellular signaling domain[65]. Upon antigen binding, the CAR initiates a signaling cascade leading to release of cytotoxic granules. Several iterations of CARs have utilized distinct signaling and co-stimulatory domains in different combination to optimize function. Carpenito *et. al.* showed greater numbers of

co-stimulatory domains can increase the half-life of engineered lymphocytes *in vivo*. This advance lead to increased tumor clearance in a tumor xenograft model[66]. They have also advanced these studies to show that addition of chemokine receptors with CARs leads to enhance lymphocyte trafficking to tumor sites[67].

Decreased in vivo half-life of transferred cells is considered one of the major obstacles hindering successful adoptive transfer therapies. Restifo and colleagues have shown that using naïve, "younger" populations of T lymphocytes results in greater in vivo expansion and tumor clearance in mice. This finding correlates to longer telomeres in this cell population and fits the hypothesis that limited replicative potential causes rapid decline of infused cells. Dudley and Rosenberg have adopted this strategy to expanding tumor infiltrating lymphocytes (TILs) *ex vivo* for use in clinical trials[68]. They also found that younger TILs express high levels of co-stimulatory molecules and have longer telomeres. This work suggests that since hESCs and iPSCs have persistent telomerase activity[2,69], the hESC and/or iPSC-derived lymphocytes may survive better in vivo and provide enhanced anti-tumor activity. However, it is not currently known whether hESC-derived NK cells have longer or shorter telomeres than PB-NKs or how this would change throughout *in vitro* expansion. Although CARs provide antigen-specific recognition and proliferation of lymphocytes, their function in young or mature populations is also yet to be determined.

While CAR-based therapies against cancer (and potentially infectious disease) are intriguing, this system needs to be advanced with caution in light of recent reports of CAR-engineered lymphocytes leading to serious adverse events when infused into patients[70-72]. For example, when investigators infuse CAR-engineered T cells with powerful anti-tumor activity, robust recognition and killing of large tumor burdens can lead to tumor lysis syndrome or a cytokine storm. Both of these conditions can lead to end organ damage and ultimately failure. Second, in one study the infusion of HER-2/neu specific T cells lead to a serious adverse event in a patient with colon cancer. This was thought to be due to the low level of HER-2/neu expression in the lung[71]. Although these adverse events may dampen some of the enthusiasm for CARs, they reiterate the importance for in depth preclinical testing to define tissue specific expression of antigens recognized by CARs. CAR-specific T cells activated upon contact with antigen produce no inhibitory signal if they encounter the antigen on normal tissue. In a manner similar to decreased GVHD in NK cell adoptive transfer trials, one could hypothesize hESC-derived NK cells and PB-NKs would remain more tolerogenic than T cells, as the infused NK cells would still co-express inhibitory receptors recognizing “self”. Alternatively it may be difficult to inhibit this overriding activation signal, therefore second generation CARs that have fewer intracellular activation domains, could in fact be safer for clinical trials.

An alternative approach for hESC/iPSC-derived NK cells would be to express tumor-specific (T cell receptors) TCRs. NK cells express all of the components

needed to signal through the TCR[72], making them a suitable platform to utilize ectopic TCR expression. Studies by the Rosenberg group[43] provide direct evidence for the potential of adoptive immunotherapy as a successful treatment for lethal malignancies. Although their initial studies used tumor infiltrating lymphocytes from actual patient cancers, more recently they have used T cells engineered with T cell receptors (TCRs) specific for tumor associated antigens (TAA)[73]. While this group is able to modify T cells from individual patients, this expensive and cumbersome process is not feasible for multi-institutional study and routine clinical application. Another disadvantage to using TCRs is the restrictive nature of specific major histocompatibility (MHC) molecules. The use of HLA-A2 restricted TCRs covers roughly 30-40% of the Caucasian population but is not an optimal TCR-MHC combination for each individual or malignancy. The ideal cellular source would function independently of MHC haplotype but remain specific for tumor antigen(s). Engineering hESCs to express antigen specific receptors could provide an enhanced cellular therapeutic capable of specific recognition and killing of tumor cells. As NK cells express key CD3 chains (ζ and ε) required for TCR signaling and activation, engagement of TCR-expressing NK cells will lead to functional cytolytic activity[74].

One advantage to implementing tumor-specific TCRs in NK cells is reduced competition for TCR binding between endogenous and exogenous α/β subunits. A recent study found aberrant pairing of transgenic and endogenous α/β subunits can create a repertoire of self-reactive lymphocytes resulting in autoimmune pathology [75]. Another major advantage in using TCRs stems from

years of epitope-discovery and TCR generation. This provides an assortment of optimal tumor-specific TCRs suitable for preclinical testing. TCR-expressing hESC-derived NK cells would need to recognize tumor-specific peptide antigens in the context of the correct MHC, but could be designed (using different ES cell lines) to cover a larger repertoire of MHC molecules and used as a universal source of anti-tumor lymphocytes for HLA matched patients.

In a simpler model, unmodified hESC-derived NK cells could be used in combination with current practices. Some of the most successful immunotherapies in human cancer are monoclonal antibodies (mAbs)[76]. Over the past decade, identification and manufacturing of monoclonal antibodies (e.g. anti-CD20, anti-VEGF, anti-HER2, anti-CD52) has been a major focus of both the academic and private sectors. We now have a better understanding of their *in vivo* function and have tailored therapies to accommodate many specific disease mechanisms. Of the many modifications made to mAbs over the years, most important is decreasing their immunogenicity by making chimeric or humanized forms. Also, several groups identified the isotypes (typically IgG1) that are most stable, have greatest affinity for the FcγRIIIA receptor, and increased ability to fix complement. One of the major cell types needed for antibody dependent cellular cytotoxicity (ADCC) are NK cells. Our studies have shown that surface expression of the activating FcR, FcγRIII (CD16), on hESC-derived NK cells parallels that of PB-NKs and can also mediate ADCC[14]. Although this remains to be tested *in vivo*, we hypothesized that combination therapeutics with monoclonal antibodies that target tumors (or other desired cell

populations) and hESC-derived NK cells would add an additional mechanism to kill human tumors, without the potential risks of using CARS. Another approach has been the development of agonists of the apoptosis inducing proteins Fas ligand and TRAIL[77]. Because hESC-derived NK cells also express high levels of both these ligands, one can begin to imagine the synergy and application of utilizing hESC-derived NK cells in combination therapeutics to treat a wide variety of malignancies.

THESIS STATEMENT

The following body of work describes both translational and proof-of-principle experiments involving NK cell development from pluripotent stem cells. Initial work to characterize NK cell development from iPSCs demonstrated their similarities to hESC-derived NK cells, both in phenotype and function (Chapter 2). This broadly parallels what investigators have found in other fields where iPSC-derived cells genocopy and phenocopy the behavior of their hESC-derived counterparts. These are important findings in iPSC biology and could one day allow autologous, iPSC based therapies that are syngeneic to the patient.

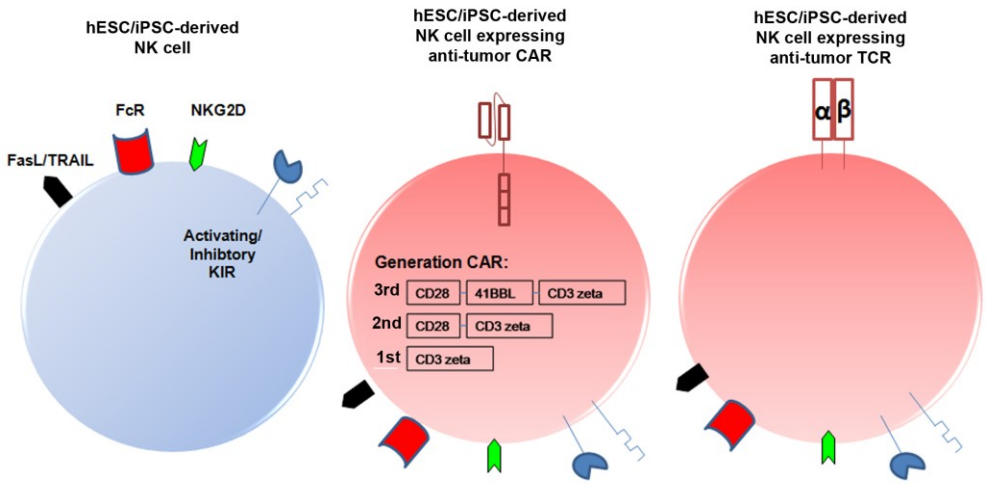
Before NK cell therapy can be translated into clinical trials, there are two major obstacles. First is the reliance on murine stromal cell co-cultures. These studies demonstrate a step-wise approach to move to completely defined conditions for the derivation of mature and functional NK cells. Second is the generation of enough NK cells to infuse into patients. This was accomplished through the use of more efficient differentiation protocols as well as expansion using artificial

antigen presenting cells. From less than one plate of hESCs we can now derive enough NK cells to treat patients with cell numbers similar to what is used from peripheral blood donors (Chapter 2).

Using hESC-derived NK cells in pre-clinical models we had previously shown the potent ability of these cells to kill leukemia cells *in vivo*. We have now utilized novel *in vivo* imaging models to monitor NK cell trafficking to tumor sites *in vivo* indicating the direct anti-tumor effect of hESC-derived NK cells (Chapter 3). Although the treatment of leukemia is a logical step in translating hESC-derived NK cells into clinical trials, there are several other malignancies that could benefit from hESC-derived NK cell immunotherapy. In particular, we have shown that hESC-derived NK cells can kill solid tumors at a similar or higher level than PB-NK cells. We tested our cells *in vivo* against ovarian cancer using a novel delivery system of direct intraperitoneal (IP) injection. We found this was a suitable and effective approach for NK cell based immunotherapy in ovarian cancer (Chapter 4). Although IP delivery of NK cells can effectively control ovarian cancer, we also sought to use hESCs and iPSCs as a novel platform for gene therapy by overexpressing tumor specific receptors.

Chimeric antigen receptors are antigen specific receptors with high avidity for tumor antigens. By expressing CARs in hESCs and iPSCs, we were able to demonstrate their stability, high expression, and efficient differentiation into NK cells containing the tumor specific receptors. We then demonstrated that CARs can redirect NK cells for specific killing of tumor targets (Chapter 5).

Figure 1.1. Models of proposed hESC/iPSC-derived NK cells suitable for clinical trials against cancer. Unmodified hESC or iPSC-derived NK cells express a variety of NK cell effector molecules at high levels, including: Fc receptor (CD16), Fas ligand (FasL), TRAIL, NKp46, NKp44, NKG2D, and killer immunoglobulin-like receptors (KIRS). hESC and iPSC-derived NK cells can be modified with chimeric antigen receptors (CARs) for specific tumor antigens. Schematic of important functional domains of CARs is illustrated. Each individual co-stimulatory domain combination is fused to an antigen-specific recognition motif on the surface of the cell (e.g. Fab' portion of an antibody). hESC and iPSC-derived NK cells can be modified with cloned T cell receptors (TCRs) for specific tumor antigens. As NK cells express the CD3 chains (ζ and ε) required for TCR signaling and activation, engagement of TCR-expressing NK cell will lead to functional cytolytic activity.



CHAPTER 2

Enhanced development of natural killer cells from human pluripotent stem cells

Adoptive transfer of anti-tumor lymphocytes has gained intense interest in the field of cancer therapeutics over the past two decades. Human natural killer (NK) cells are a promising source of lymphocytes for anti-cancer immunotherapy. NK cells are part of the innate immune system and exhibit potent anti-tumor activity without need for HLA matching and without prior antigen exposure. Moreover, the derivation of NK cells from pluripotent stem cells could provide an unlimited source of lymphocytes for “off-the-shelf” therapy. To date, most studies on hematopoietic cell development from human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSCs) have used incompletely defined conditions and on a limited scale. Here, we have utilized a two-stage culture system to efficiently produce NK cells from hESCs and iPSCs in the absence of cell sorting and without need for xenogeneic stromal cells. This novel combination of embryoid body formation using defined conditions and membrane-bound IL21-expressing artificial antigen presenting cells allows production of mature and functional NK cells from several different hESC and iPSC lines. While different hESC and iPSC lines had varying efficiencies in hematopoietic development, all cell lines tested could produce functional NK cells. These methods can be used to generate enough cytotoxic NK cells to treat a single patient from less than 250,000 input hESCs/iPSCs. Additionally, this strategy provides a genetically amenable platform to study normal NK cell development and education in vitro.

INTRODUCTION

Human natural killer (NK) cells provide an important arm of the innate immune system by producing cytokines and killing virally-infected and malignant cells. Whereas anti-tumor T cell responses require a priming phase and are HLA-restricted, mature NK cells effectively kill malignant cells without any prior exposure. Both T cell and NK cell-based adoptive immunotherapies have been utilized to treat patients with refractory malignancies[45,48,78]. A major hindrance to expanded use of these therapies is the need for cell processing and donor selection. Therefore, a standardized, off-the-shelf therapeutic with a potent anti-tumor response could be used to treat thousands of patients with refractory malignancies. Although T cell development from human pluripotent stem cells has been reported, it remains relatively inefficient[15,79]. In contrast, use of hESCs and iPSCs to generate NK cells with anti-tumor and anti-viral capacity is routine[14,58,80]. NK cells derived from hESCs and iPSCs possess a mature phenotype, secrete cytokines, and are cytotoxic against both hematologic and solid malignancies in vitro and in vivo[14,58,80]. However, previous work on NK cell generation from both hESCs and iPSCs has relied on the use of murine stromal cell layers and sorting of small numbers of hESC/iPSC-derived hematopoietic progenitor cells[14,58,80]. Although the use of murine stromal layers does not absolutely prohibit clinical translation (if a master cell banks is used), use of culture systems that eliminate xenogeneic cells provides more defined conditions for NK cell development. Elimination of murine stromal support also provides an improved developmental model to study receptor-ligand

interactions driving NK cell licensing. Here, we describe an efficient system for the development of functional NK cells from both hESCs and iPSCs as well as an improved method suitable for clinical translation.

MATERIALS AND METHODS

Maintenance and stromal cell-mediated differentiation of hESCs/iPSCs. hESCs (H9 and H1) and iPSCs (UCBiPS7, NHDF-iPS, BJ1-iPS) were maintained on mouse embryonic fibroblasts (MEF). Stromal cell-mediated differentiation of hematopoietic progenitors on M210-B4 (ATCC, Manassas, VA) cells was done as previously described[16,58,81]. Briefly, undifferentiated hESCs or iPSCs were placed on M210-B4 in medium containing RPMI 1640 (Invitrogen), 15% defined FBS (Hyclone), 2 mM L-glutamine (Cellgro), 1% non-essential amino acids (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 0.1 mM β-mercaptoethanol (Invitrogen). Media was changed three times per week and after 18-21 days cells were harvested for $CD34^+CD45^+$ progenitor cell enrichment [58]. Approximately 1 x 10^5 $CD34^+CD45^+$ cells were placed onto EL08-1D2 stroma with 1 mL NK cell initiating cytokines (IL-3, IL-7, IL-15, stem cell factor (SCF), fms-like tyrosine kinase receptor-3 ligand (FLT3L), all from Peprotech). NK cell cultures we refreshed with 0.5 mL of cytokine containing media every 4-5 days. Mature NK cells were measured at 28-35 days of culture on EL08-1D2.

Generation of spin EBs

In order to generate spin EBs amenable to aggregation, hESCs and iPSCs were passaged in TrypLE Select (Invitrogen) on low density mouse embryonic fibroblasts (MEFs, 90,000 cells/well) for a minimum of 10 passages. For the spin EB studies, we used an H9 line modified with a GFP/firefly luciferease construct[16] for future in vivo experiments. For iPSCs, we tested the UCBiP7 line derived from UCB $CD34^+$ hematopoietic progenitors (Supplementary Figure 1). To generate TypLE adapted hESCs or iPSCs, cultures around 60-70% confluency were dissociated and filtered through a 70 micron sterile filter. Only pure cultures of hESCs lacking any signs of differentiation were used. Cells were passaged 1:1 on low density MEFs in regular hESC media until cellular proliferation allowed passing at more dilute ratios, typically occurring around passage 10. To set up TrypLE passaged hESCs into Stage I spin EBs, adapted cells around 70% confluency were dissociated with TrypLE and filtered through a 70 micron filter to remove any clumps. Cells were then counted and placed at a concentration of 3000 cells per well (100 µl volume) of a round-bottom 96-well plate in BPEL media containing stem cell factor (SCF, 40 ng/ml), vascular endothelial growth factor (VEGF, 20 ng/ml), and bone morphogenic protein 4 (BMP4, 20 ng/ml). BPEL media was made in 200 mL volumes and contained Iscove's Modified Dulbecco's Medium (IMDM, 86 mL, Invitrogen), F12 Nutrient Mixture with Glutamx I (86 mL, Invitrogen), 10% deionized Bovine Serum Albumin (BSA, 5 mL, Sigma), 5% Polyvinyl alcohol (10 mL, Sigma), Linoleic acid (20uL of 1 mg/mL solution, Sigma), Linolenic acid (20uL of 1 mg/mL

solution, Sigma), Synthecol 500X solution (Sigma), α-monothioglyceral (Sigma), Protein-free hybridoma mix II (Invitrogen), ascorbic acid (5 mg/mL, Sigma), Glutamax I (Invitrogen), Insulin-transferrin-selenium 100x solution (Invitrogen), and Penicillin/streptomycin (Invitrogen). The outer wells of the plate were filled with sterile water to prevent any evaporation of the media. Plates were then spin aggregated at 1,500 RPMs for 5 minutes at room temperature and placed undisturbed in a 37°C incubator with 5% CO_2. Cells were not removed for at least 3 days to ensure formation of spin EBs in the plates.

NK cell differentiation from spin EBs

At day 11 of spin-EB differentiation, 6 wells of a 96 well plate were directly transferred to one well of a 24-well plate in NK cell initiating cytokines, as above. Initially, the 24-well plates contained 100,000 irradiated (3000 Rads) EL08-1D2 cells per well. For completely defined conditions, 6 wells of spin EBs were directly transferred to uncoated 24-well plates. On the day of analysis each well was collected, filtered, and washed.

Flow cytometry

The following antibodies were used: CD34-APC, CD45-PE, CD31-PE, CD31-APC, CD-73PE, CD43-PE, NKp46-PE, NKp44-PE, CD56-APC, NKG2D-PE, TRAIL-PE, FAS ligand-PE, CD16-PercpCy5.5, CD117-PercpCy5.5, all from Becton Dickson. CD158a/h-PE, CD158j-PE, CD158i-PE, CD158e1/e2, CD159a-PE and CD159a-APC were obtained from Beckman Coulter.

CD107aPercpCy5.5 and INF-γ PacBlue were obtained from eBioscince. Flow cytometry was done on a BD FACS Calibur or LSRII and data analyzed using FlowJo (Treestar).

In vitro cytotoxicity

Tumor targets (K562, SVP10, S2013, OPM2, RPMI 8226, U266) were incubated with ^{51}Chromium for 2 hours at 37°C, washed three times and co-cultured with NK cells at indicated effector to target (E:T) ratios. After a period of 4 hours, cells were harvested and analyzed. Specific ^{51}Cr lysis was calculated using the equation: % specific lysis =

100 x (test release - spontaneous release) / (maximal release - spontaneous release).

CD107a and IFN-γ assays

NK cells were incubated with or without K562 targets at 1:1 effector to target ratios. CD107a-PerCPCy5.5 antibody was added to each well and allowed to incubate for one hour. GolgiStop and GolgiPlug (BD Biosciences) were then added to each well and incubated for another 4 hours. At the completion of incubation, cells were washed, stained with CD56APC, and fixed with 2% paraformaldehyde for 10 minutes on ice. Cells were then permeablized with 1% saponin for 20 minutes at 4°C, washed, and stained for IFN-γ.

Stromal cell functional assays

To assess the endothelial and mesenchymal stromal cell (MSC) characteristics of the stroma derived in NK cell cultures, non-adherent cells were first washed away and then the adherent layer was trypsinized and washed. Cells were stained with endothelial (CD31) and MSC (CD73) markers, as well as the hematopoietic (CD45), monocyte/macrophage (CD14, CD15), and dendritic cell (CD11c) markers. To assess the ability of each of the stromal cell layers (EL08-1D2, HUVECs, Feeder free stroma) to support NK cell development, each was plated at 100,000 cells per well of a 24-well plate and irradiated (3,000 Rads). HUVECs were grown as previously described[81]. Each well was then seeded with 500 $CD34^+$ cells from umbilical cord blood. No stroma conditions contained UCB $CD34^+$ cells and media alone. Cells were then cultured in standard NK cell conditions as above.

Artificial antigen presenting (aAPC) cell expansion.

To expand hESC-derived NK cells from EL08-1D2 or feeder free conditions, each was placed in culture with Clone 9.mbIL-21 aAPCs [82]. For the first week of culture, aAPCs were irradiated with 10,000 rads and added to NK cells at a ratio of 2:1 (day 0). The following stimulations with aAPCs (every 7 days) were carried out at a 1:1 ratio. Cultures were fed three times per week (RPMI 1640, 15%FBS, 1% penicillin/streptomycin, 50U/mL interleukin-2), maintaining cell counts at 250,000 cells/mL for optimal expansion.

Statistical analysis.

Differences between groups were compared using a Student's t-test or ANOVA using Prism 4 (GraphPad Software, San Diego, CA). Results were considered significant at *P* values of 0.05 or less.

RESULTS

hESC- and iPSC-derived Hematopoietic Progenitor Cells Can Develop Into NK Cells

Initial studies used a stromal cell co-culture method[14,58,80] to compare hematopoietic and NK cell developmental potential of two different hESC lines (H1 and H9) and three different iPSC lines (BJ1-iPS12, UCBiPS7, and DRiPS16). UCBiPS7 and DRiPS16 were derived and characterized in our lab (Supplementary Figure 2.1). For this method, hESCs or iPSCs were cultured on M210-B4 stromal cells in media containing only FBS. Over a period of 3 weeks, all hESC and iPSC lines generated hematopoietic progenitor cells co-expressing CD34 and CD45 (Figure 2.1). The H9 cells gave rise to the highest percentage of hematopoietic progenitor cells expressing CD34 and CD45 (6.46 ± 1.75%), other hESC and iPSC lines yielded consistently lower numbers. Specifically, 1.45 ± 0.18% for H1 hESCs, 2.46 ± 1.71% for UCBiPS7, 0.92 ± 0.14% for DRiPS16, and 1.43 ± 0.35% for the BJ1-iPS line (Figure 2.1B). These numbers were similar to what we and others have previously shown, where the efficiency of hematopoietic development using the stromal cell based system were relatively limited[7,43]. After demonstrating that different iPSC lines gave rise to varying numbers of hematopoietic progenitor cells, we generated NK cells from each of the hESC/iPSC-derived $CD34^+CD45^+$ cell populations. Here, $CD34^+CD45^+$ cells are sorted and cultured in conditions known to support human NK cell development, including the murine stromal cell line EL08-1D2 and cytokines (SCF, FLT3L, IL15, IL7, IL3)[58] for four weeks. Although distinct

lines of hESCs or iPSCs gave rise to varying frequencies of hematopoietic progenitor cells, each cell line was able to produce phenotypically mature and functional NK cells. Both hESC- and iPSC-derived NK cells consisted of a homogenous population of cells expressing CD56, killer immunoglobulin-like receptors (KIRs), CD16, NKp44, NKp46, and NKG2D (Figure 2.1C). Also, NK cells from all five hESC/iPSC populations were able to kill tumor cells similar to NK cells isolated from peripheral blood (PB-NK) (Supplementary Figure 2.2). These data demonstrated that although individual hESCs and iPSCs have reproducible differences in their ability to derive hematopoietic progenitor cells, each was capable of making mature, cytolytically active NK cells.

Enhanced Generation of Progenitor Cells Eliminates Cell Sorting in the Derivation of hPSC-derived NK Cells

In an effort to better understand specific stimuli required to mediate derivation of NK cells from hESCs or iPSCs and to improve culture efficiency, we took a step-wise approach to translate these methods to completely feeder-free and serum-free culture systems. First, undifferentiated hESCs and iPSCs were supported to produce hematopoietic progenitor cells using a “spin-EB” method[56,83]. Here, defined numbers (3,000 cells) of undifferentiated hESCs (H9) or iPSCs (UCBiPS7) were spin aggregated in serum-free media in a 96 well format (Supplementary Figure 2.3A). Over a period of 11 days these cultures demonstrated hematopoietic cell development and proliferation (Supplementary Figure 2.3B). Not only does this method remove the need for M210-B4 murine

stroma, we find it allows higher frequency and more consistent generation of hematopoietic progenitor cells from both hESCs and iPSCs. For hESCs we found a majority of the hematopoietic cells expressed CD34 (55.9 ± 6.4%) with many coexpressing CD43 (41.8 ± 9.51%) or CD45 (26.2 ± 6.6%) (Figure 2.2A and 2.2B). iPSCs also generated $CD34^+$ cells (12.06 ± 5.40%) and $CD45^+$ cells (3.20 ± 1.43%), though typically less than hESCs. This method provides significant improvement over the M210-B4 stromal based system (Figure 2.1) for both hESCs and iPSCs.

We next tested the ability of the hematopoietic progenitor cells generated in the spin EB system to derive NK cells. To do this we directly transferred spin EBs, without dissociation or sorting, into NK cell initiating conditions containing cytokines and EL08-1D2 stroma. As previously shown, this stage II culture system provides reliable development of NK cells over a period of 4 weeks[58,80]. Similar to stromal cell co-culture derived progenitor cells, spin-EB derived hematopoietic progenitors acquired NK cell surface markers culminating in a mature NK cell phenotype (Figure 2.2C). Indeed, we found that spin-EB-derived cells differentiated into a homogenous population of $CD56^+$ NK cells expressing CD94 in the absence of CD117[58]. Additionally, these cells expressed high levels of KIR, CD16, NKG2D, NKp46 and TRAIL. Spin-EB derived NK cells (from both hESCs and iPSCs) killed K562 tumor cells at a similar level to PB-NK cells, and were more cytotoxic than UCB-derived NK cells (Figure 2.2D). These data demonstrate the spin EB approach not only provides a feeder free system to generate high numbers of hematopoietic progenitor cells, but these

progenitors do not require sorting to differentiate into phenotypically mature NK cells with cytotoxic function similar to activated PB-NK cells.

hPSC-derived Stroma Support NK cell Development From Hematopoietic Progenitor Cells

To more completely define the conditions required for NK cell development from hESCs and iPSCs, we next tested spin EB-derived cells in a feeder-free and serum-free stage II system containing NK cell promoting cytokines (IL3, IL7, IL15, SCF, FLT3L) without EL08-1D2 or other exogenous stromal cells (Supplementary Figure 2.3A). Within the first two weeks following transfer, there was proliferation of non-adherent hematopoietic cells from the spin EBs at a similar level to what is seen with EL08-1D2 stroma (Figure 2.3A). Additionally, we found these cells started to produce their own adherent cells in culture (Figure 2.3B). We have previously demonstrated development of endothelial cells (ECs) and mesenchymal stromal cells (MSCs) from hESCs[81,84]. Here, we demonstrated both of these cell types ($CD34^+CD31^+$ ECs and $CD34^+CD73^+$ MSCs)(Supplementary Figure 2.4) are routinely produced in the spin EB cultures. As non-hematopoietic cells such as ECs and MSCs that reside in the bone marrow are known to support NK cells in vivo, we hypothesized that these adherent cells could efficiently support growth of NK cells from hESCs in vitro[85,86]. Notably, we found the spin EB stromal cell layers expressed high levels of CD31 and CD73 (Figure 2.3C) in addition to MHC class I molecules (HLA-A,B,C and HLA-E) known to be important for NK cell development and

acquisition of killer immunoglobulin-like receptors (KIRs)[87]. Additionally, these spin-EB-derived stromal cells support the development of NK cells from UCB $CD34^+$ cells similar to EL08-1D2 stroma and more efficiently than human umbilical vein endothelial cells or cytokines alone (Figure 2.2D).

Using these defined conditions with no exogenous stromal cells, NK cells developed in similar numbers and phenotype compared to stage II conditions utilizing the EL08-1D2 stromal cells (Figure 2.1C and 2.2C). These cells expressed a mature NK cell phenotype and were comparable to their stromal-derived counterparts in cytotoxicity assays, indicating proper NK cell education and acquisition of effector function. Spin EB-derived NK cells degranulate, make IFN-γ, and also have activity against diverse tumor targets including pancreatic cancer and multiple myeloma (Figure 2.4). These data demonstrate for the first time, successful in vitro derivation of functional, cytotoxic lymphocytes in the absence of any sorting or murine stromal cell support. Avoiding xenogeneic feeder layers provides a novel, genetically amenable system to study human NK cell education, as well as a defined human source for adoptive immunotherapy.

Clinical Scale Expansion of hESC-derived NK Cells for Anti-tumor Immunotherapy

Although this EB-based approach shows marked expansion and clinical feasibility, we next aimed to further enhance the number of NK cells generated through another clinically amenable method. Recently, several groups have

utilized artificial antigen presenting cells (aAPCs) to expand T cells or NK cells for adoptive immunotherapy[88]. One major hindrance of this approach is that high levels of in vitro expansion lead to shortening of telomeres and cellular senescence. Lee et. al. have generated an aAPC line expressing membrane bound IL-21 (Clone 9.mbIL-21) leading to marked expansion of PB-NK cells while maintaining telomere length and in vitro activity[82]. We tested whether these aAPCs could lead to further expansion of hESC-derived NK cells and found that Clone 9.mbIL-21 aAPCs mediated an additional 2-3 log expansion of both the EL08-1D2 and feeder-free derived NK cells (Figure 2.5A). The aAPC-expanded cells maintained their NK cell phenotype as well as in vitro activity (Figure 2.5B and 2.5C). Additionally, these hESC-derived NK cells could be maintained and continually expanded in culture for more than 2 months. Combined, the spin EB method and aAPC expansion provide a straight forward, translatable approach to generate enough human NK cells from hPSCs for cancer immunotherapy.

Discussion

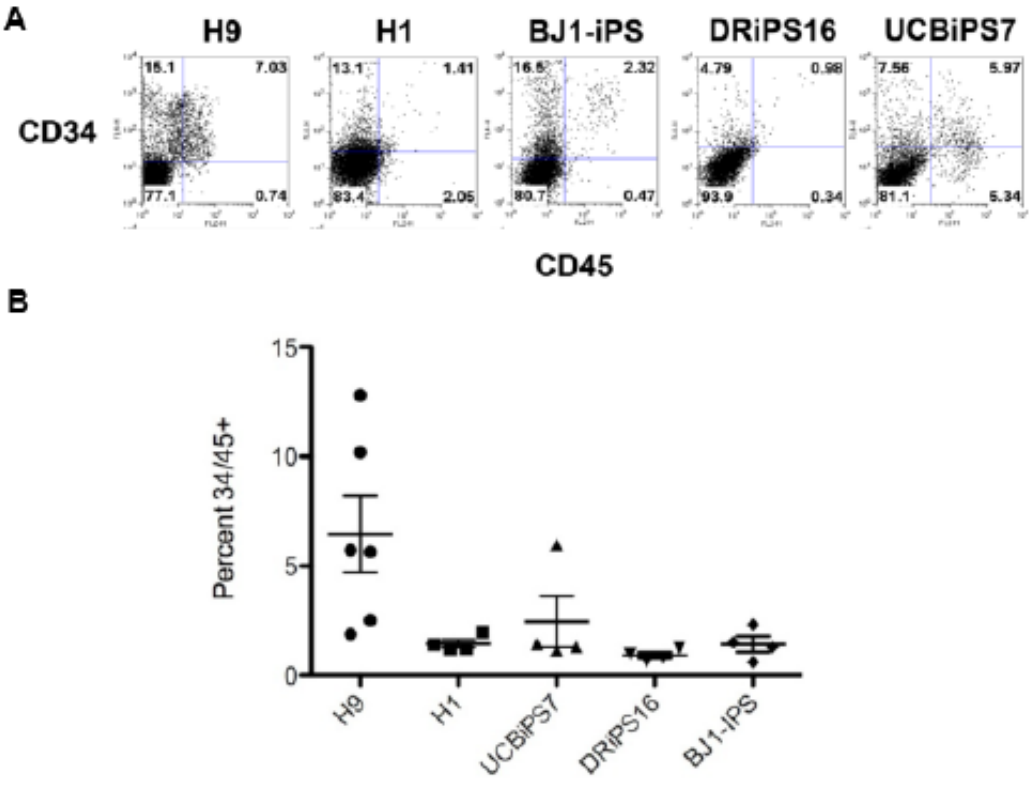
With the improved efficiency and defined components of this system, clinical translation of hESC/iPSC-derived cells becomes feasible. Current adoptive NK cell-based immunotherapy uses an NK cell containing clinical product (typically comprising approximately 50% NK cells) consisting of about 2 x 10^7 cells per kilogram[48]. Our methods without the aAPCs would provide this number of NK cells from about 13 x 10^6 undifferentiated hESCs or iPSCs (~1-6 well plate).

Using the aAPCs would mean that fewer than 10^6 undifferentiated hESCs/iPSCs would be required per patient at current NK cell doses. This process can be utilized to produce substantially more NK cells starting from a single, homogenous and well-characterized starting cell population than can be done with individual apheresis donors used for peripheral blood NK (PB-NK) cells. Additionally, these methods decrease the amount of cell processing compared to that of peripheral blood, which requires depletion with anti-CD3 antibodies against T cells to prevent GVHD and anti-CD20 antibodies against B cells to prevent passenger lymphocyte syndrome. Neither T cells nor B cells are present in our cultures[9]. Utilizing the expanding knowledge of KIR receptors and allo-reactivity, NK cells from diverse genetic backgrounds could be generated to create the optimal NK cell "super donor", a concept recently established in a large cohort of subjects indicating that particular KIR haplotypes (Centromeric B/B) are optimal in clearing residual leukemia in patients undergoing allogeneic hematopoietic stem cell transplantation[89]. Improved treatment of patients with other tumors may also be feasible with these hESC- and iPSC-derived cells that have cytolyic activity against ovarian, pancreatic, breast cancer, prostate cancer and myeloma cells[58] (Figure 2.4 and data not shown) Treatment of HIV or other chronic viral infections may also be possible[80]. Additionally, engineering hESCs and iPSCs with anti-tumor and anti-viral chimeric antigen receptors (CARs) would provide an "off-the-shelf" product of targeted lymphocytes for immunotherapies[90-93].

Clinical translation of hESC- and iPSC-derived cells continues to be steadily advancing. Indeed, investigators have shown the delivery of retinal pigment epithelial cells derived from hESCs are safe, and may be effective, in patients with a form of macular degeneration[4]. Clinical use of hESC/iPSC-derived hematopoietic cells has been of keen interest for over a decade[9]. Strictly considering cell number, the ability to create enough hESC-derived NK cells for therapy is more feasible than the number of cells needed to generate one unit of RBCs (10^{12} RBCs per unit). Studies on more efficient derivation of human induced pluripotent stem cells (iPSCs) using non-integrating methods more suitable for clinical translation are also advancing[94]. Therefore, our ability to now produce large numbers of cytotoxic NK cells means the prospect hESC- and iPSC-derived hematopoietic products for diverse clinical therapies can be realized in the not too-distant future.

Our data demonstrate an improved method to develop NK cells from human pluripotent stem cells. Using a step-wise approach, we were able to transition to a completely defined system amenable to clinical translation. We also demonstrated that hESC-derived progenitors produce their own adherent stromal cells supporting NK cell development. This system not only provides a system for clinical scale expansion of anti-tumor lymphocytes but a genetically amenable platform to study human NK cell development.

Figure 2.1. Derivation of functional NK cells from hESCs and iPSCs. A) $CD34^+CD45^+$ progenitors derived from hESCs (H1, H9) or iPSCs (BJ1-iPS, DRiPS16, UCBiPS7) following 21 days on M210-B4 stroma. **B)** Differentiation efficiencies of hESCs and iPSCs, at least 4 separate experiments for each line. **C)** NK cells derived from hESCs, iPSCs (BJ1-iPS, NHDF-iPS, UCB-iPS), UCB 34^+ progenitors, or isolated from adult peripheral blood (PB-NK). Histogram plots are gated on $CD56^+$ events. KIR plots utilized a cocktail of KIR antibodies (CD158a/h, CD158e1/e2, and CD158i). Similar to PB-NKs, hESC- and iPSC-derived NK cells express markers of functionally mature NK cells (CD16, NKG2D, NKp44, NKp46, CD161). Histograms are representative of at least 3 individual experiments.



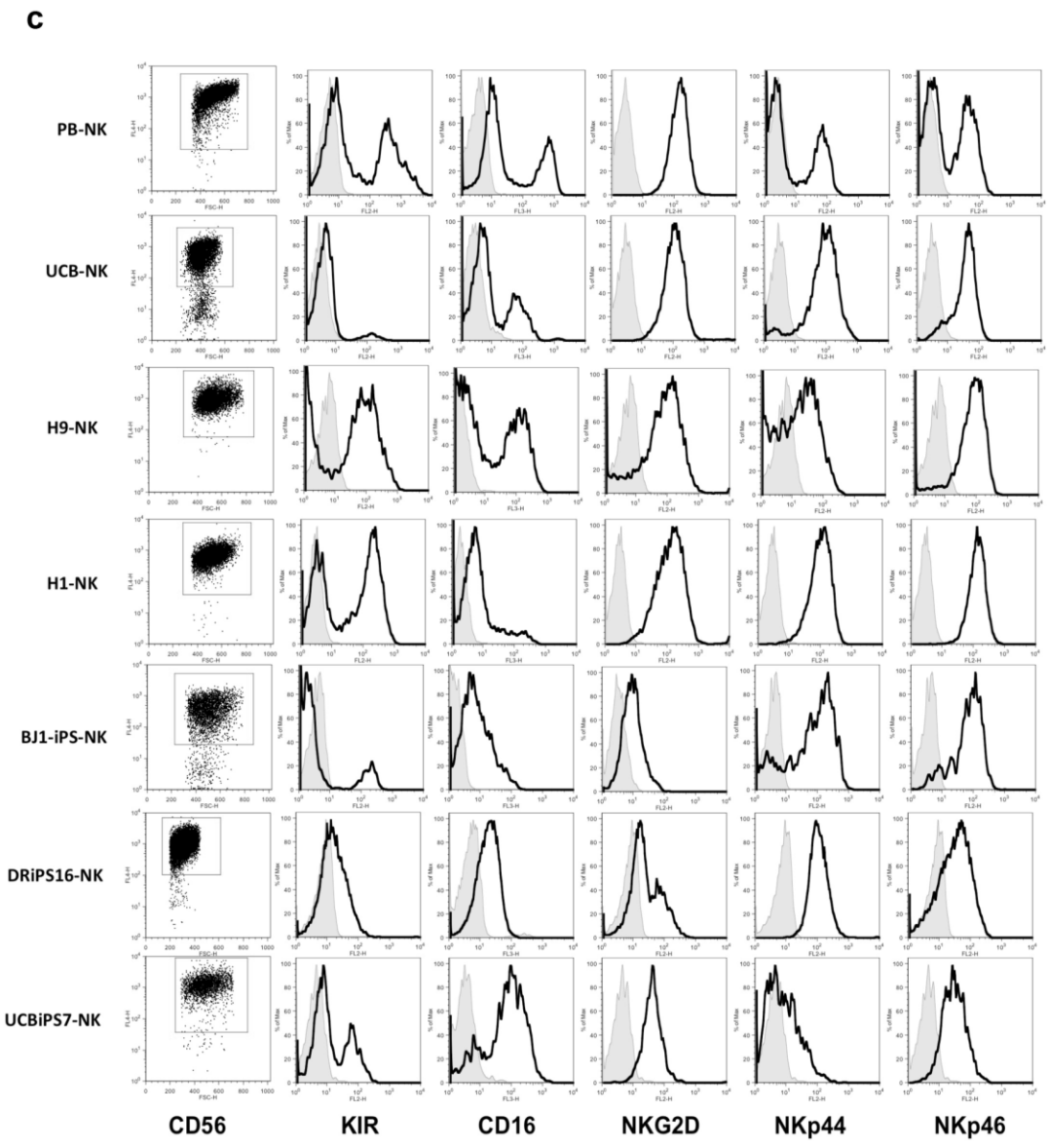

c
PB-NK
UCB-NK
H9-NK
H1-NK
BJ1-iPS-NK
DRiPS16-NK
UCBiPS7-NK
CD56
KIR
CD16
NKG2D
NKp44
NKp46

Figure 2.2. Derivation of functional NK cells in feeder free conditions. A) Spin EBs generate higher frequencies of hematopoietic progenitors cells from hESCs and iPSCs than co-culture on murine stromal cells. At day 11 of spin EB culture, individual EBs were collected, dissociated and stained with antibodies against CD34, CD43, and CD45. **B)** Quantification of the high level of blood progenitors produced in the spin EB system. Percentages of cells expressing CD34 alone or in combination with CD45, CD43, CD31, and CD73 are shown. Each dot represents results from a separate experiment. Lines indicate mean $\pm$ SEM. **C)** $CD56^+$ NK cells derived from hESCs (with or without EL08-1D2 feeders), PB-NKs, or iPSCs (UCBiPS7). hESC- and iPSC-derived NK cells form a $CD117^-94^+$ homogenous, mature population similar to activated PB-NKs. Each also expresses various effectors molecules, including KIR, CD16, NKG2D, NKp46, and the apoptosis inducing ligand TRAIL. Histograms representative of at least 3 independent experiments. **D)** Cytotoxicity assay against the leukemic cell line K562 (N=4 per cell type). hESC- and iPSC-derived NK cells kill K562 cells similar to activated PB-NK cells and significantly better than UCB-NK cells (p value= 0.0054). Data are represented as mean +/- SEM.

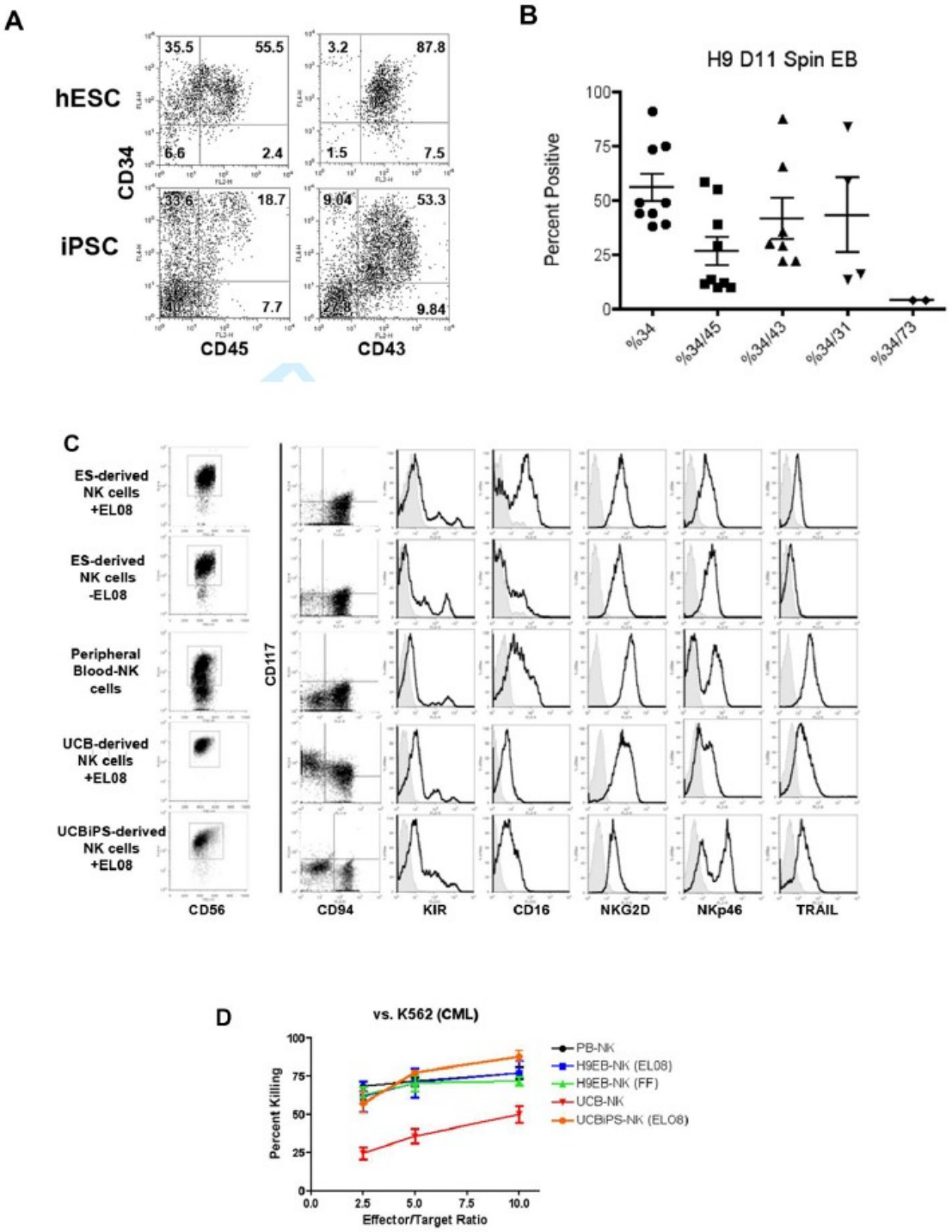

A
hESC
iPSC
CD34
CD45
CD43
35.5
55.5
6.6
2.4
3.2
87.8
1.5
7.5
33.6
18.7
7.7
9.04
53.3
9.84
B
H9 D11 Spin EB
Percent Positive
%34
%34/45
%34/43
%34/31
%34/73
C
ES-derived NK cells +EL08
ES-derived NK cells -EL08
Peripheral Blood-NK cells
UCB-derived NK cells +EL08
UCBiPS-derived NK cells +EL08
CD56
CD117
CD94
KIR
CD16
NKG2D
NKp46
TRAIL
D
vs. K562 (CML)
Percent Killing
Effector/Target Ratio
PB-NK
H9EB-NK (EL08)
H9EB-NK (FF)
UCB-NK
UCBiPS-NK (EL08)

Figure 2.3. hPSC-derived stroma support development of mature NK cells. **A)** hESC-derived NK cells proliferate in the presence or absence of murine EL08-1D2 stromal cells. At 4 weeks of culture with or without EL08 cells, the stromal cells provided 56.8-fold expansion and feeder free cells expanded 40.4-fold. N=4 for each condition. **B)** hESC-derived stroma following 2 weeks of NK cell culture. Cells were imaged at 40x magnification. The hematopoietic cells were then washed away and cells were re-imaged to evaluate only the stromal layer. The stromal cells also express GFP, indicating their hESC origin (the parent H9 line used is GFP^+). **C)** Stroma from feeder free conditions express surface antigens typical of both endothelial (CD31) and mesenchymal stromal cells (MSCs), but do not express the pan-hematopoietic marker CD45 or myeloid markers (CD14, CD15, CD11c). **D)** Stroma derived from feeder free conditions support the development of NK cells from UCB $CD34^+$ HSCs. Each stromal layer (EL08-1D2, HUVEC, feeder free stroma) was evaluated at D28 for the presence of NK cells (CD56). A no stroma, cytokine only condition is also shown. $CD56^+$ events were then evaluated for the expression of CD117, CD94, NKG2A, and KIR (N=2).

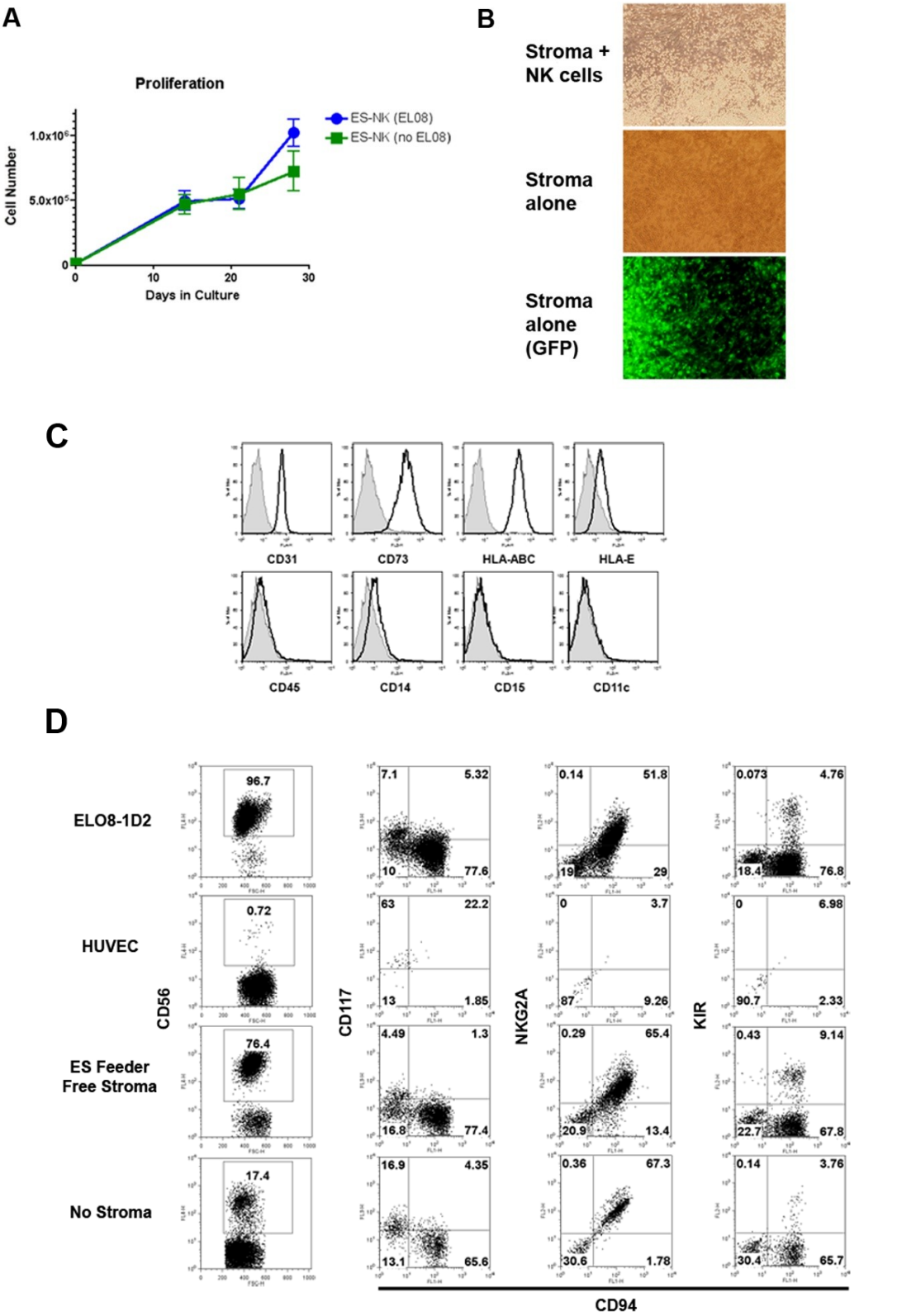
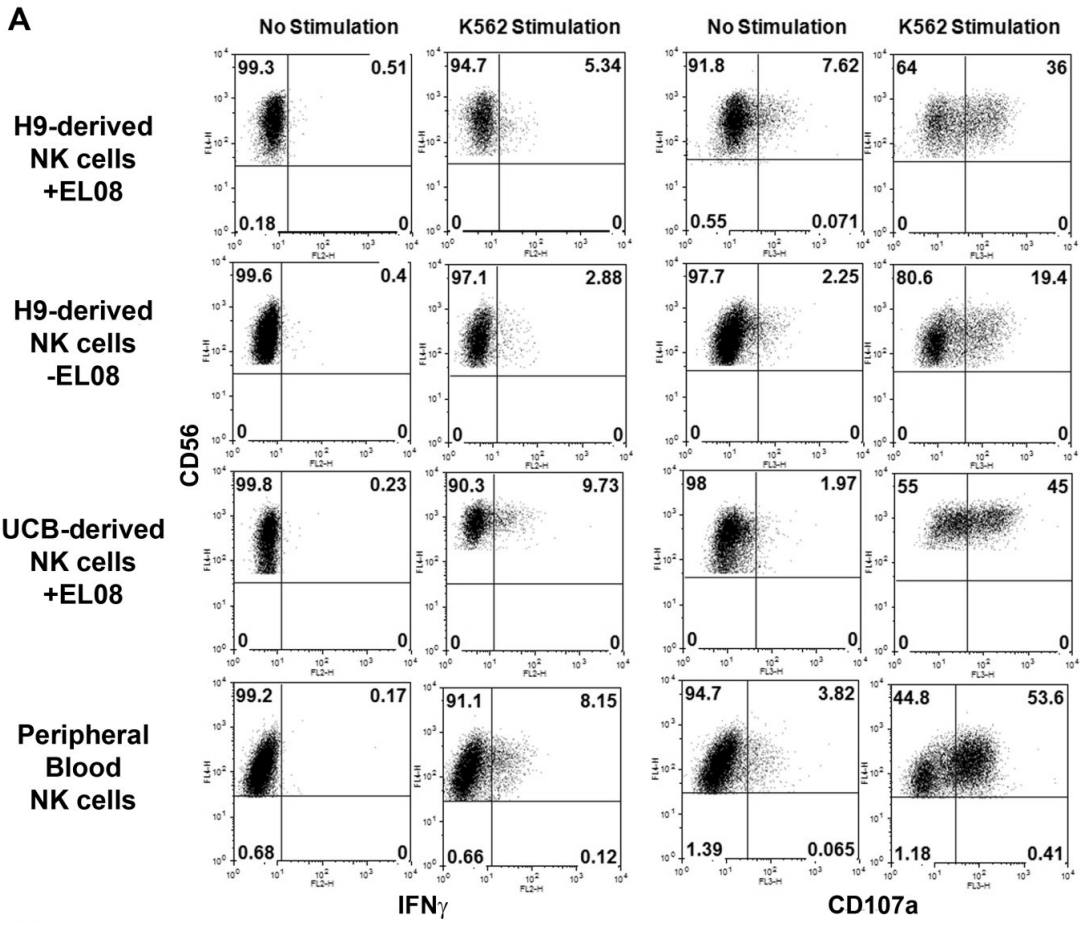

A
Proliferation
ES-NK (EL08)
ES-NK (no EL08)
Cell Number
1.0x10^6
5.0x10^5
0
0
10
20
30
Days in Culture
B
Stroma + NK cells
Stroma alone
Stroma alone (GFP)
C
CD31
CD73
HLA-ABC
HLA-E
CD45
CD14
CD15
CD11c
D
ELO8-1D2
HUVEC
ES Feeder Free Stroma
No Stroma
CD56
CD117
NKG2A
KIR
CD94
96.7
7.1
5.32
10
77.6
0.14
51.8
19
29
0.073
4.76
18.4
76.8
0.72
63
22.2
13
1.85
0
3.7
87
9.26
0
6.98
90.7
2.33
76.4
4.49
1.3
16.8
77.4
0.29
65.4
20.9
13.4
0.43
9.14
22.7
67.8
17.4
16.9
4.35
13.1
65.6
0.36
67.3
30.6
1.78
0.14
3.76
30.4
65.7

Figure 2.4. **Spin-EB derived NK cells are functional against a variety of targets. A)** hESC-derived NK cells in feeder or feeder free conditions, UCB-derived NK cells, and PB-NK cells were tested against K562 targets for IFN-γ secretion and CD107a expression. Effectors were incubated with targets for 5 hours and analyzed by flow cytometry. **B)** Each effector population was also tested against myeloma (RPMI 8226, U266, OPM-2) and pancreatic cancer (S2013, S2VP10) targets using a standard ^{51}Chromium release assay. Data are represented as mean +/- SEM.



B

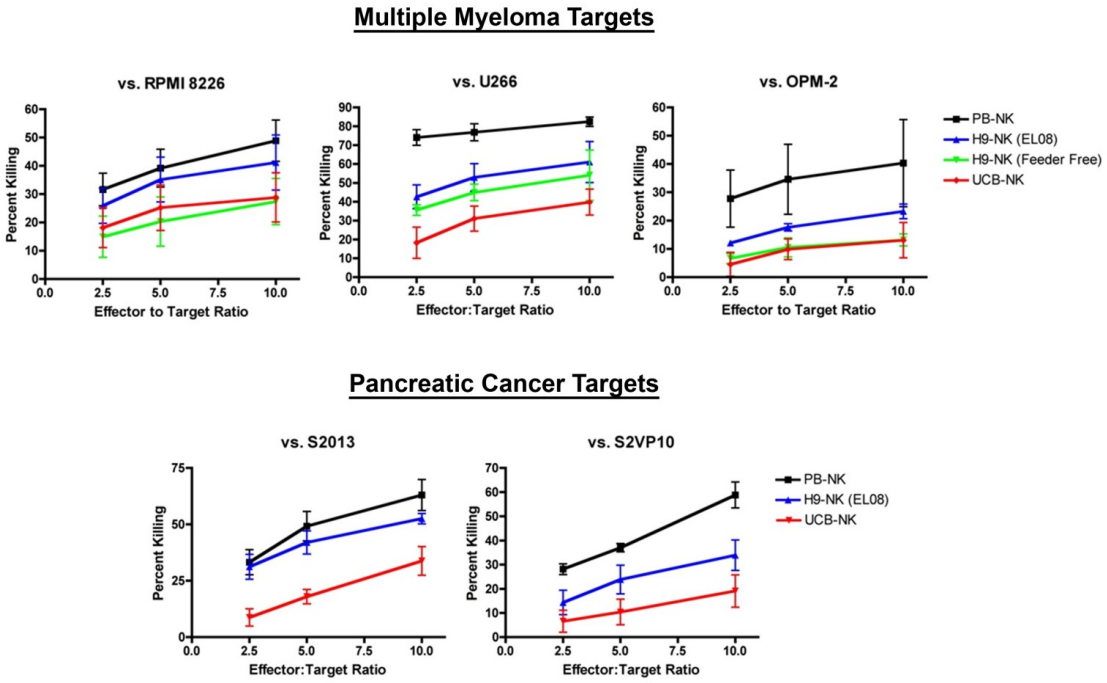
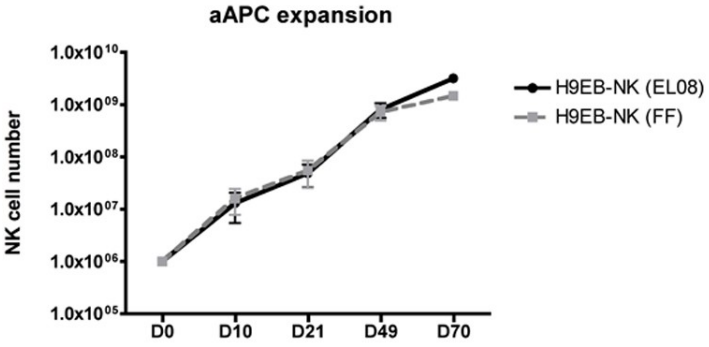
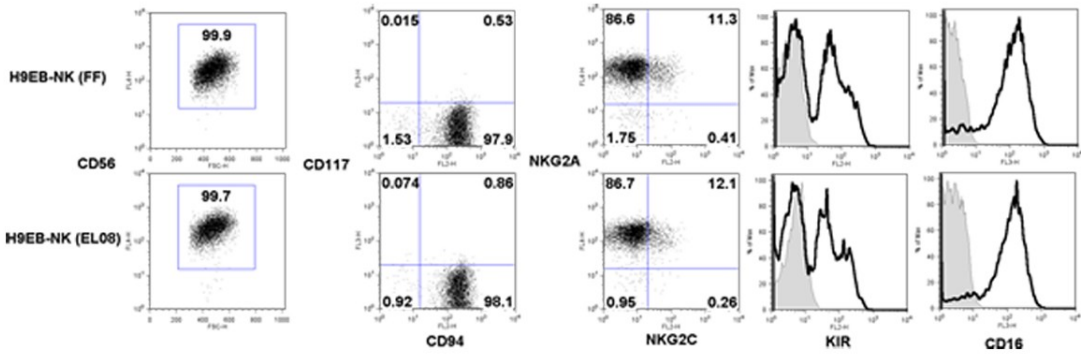

Multiple Myeloma Targets
vs. RPMI 8226
vs. U266
vs. OPM-2
Percent Killing
Effector to Target Ratio
Effector:Target Ratio
PB-NK
H9-NK (EL08)
H9-NK (Feeder Free)
UCB-NK
Pancreatic Cancer Targets
vs. S2013
vs. S2VP10
Percent Killing
Effector:Target Ratio
PB-NK
H9-NK (EL08)
UCB-NK

Figure 2.5. NK cell expansion with artificial antigen presenting cells (aAPCs). A) Cultures containing aAPCs and $1x10^6$ hESC-derived NK cells (EL08-1D2 or feeder free) were evaluated for NK cell expansion at days 10, 21, 49 and 70 (D10 and 21 n=4, D49 and 70 n=2). **B)** Following 3 weeks of expansion both the EL08-1D2 and feeder free NK cell cultures maintain pre-expansion phenotype and are similar to expanded PB-NK cells. Each contains pure cultures of $CD56^+$ NK cells that remain $CD94^+CD117^-$. Each expresses high levels of KIR, CD16 and NKG2A with a small percentage of the cells expressing NKG2C (n=3). **C)** Expanded NK cells maintain their in vitro function. Each was tested in a standard ^{51}Chromium release cytotoxicity assay against K562 targets (n=3 for each). Data are represented as mean +/- SEM.

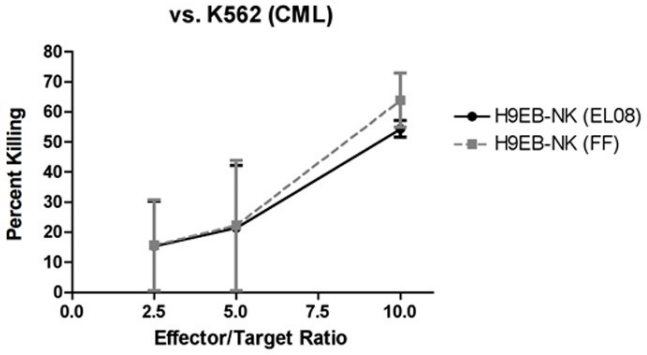
A


aAPC expansion
NK cell number
1.0x10^10
1.0x10^09
1.0x10^08
1.0x10^07
1.0x10^06
1.0x10^05
D0
D10
D21
D49
D70
H9EB-NK (EL08)
H9EB-NK (FF)

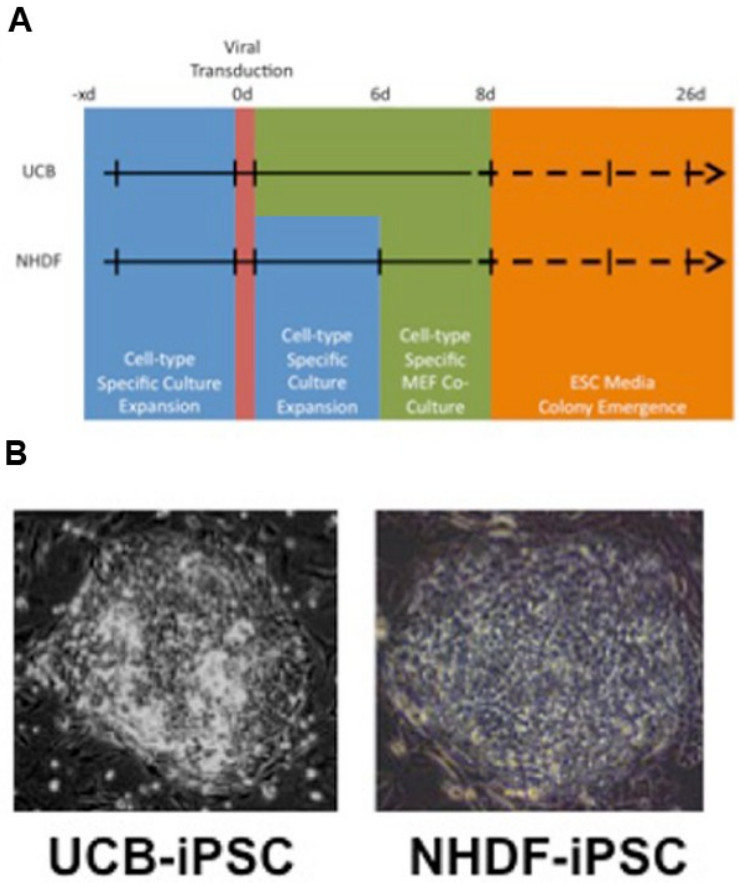
B

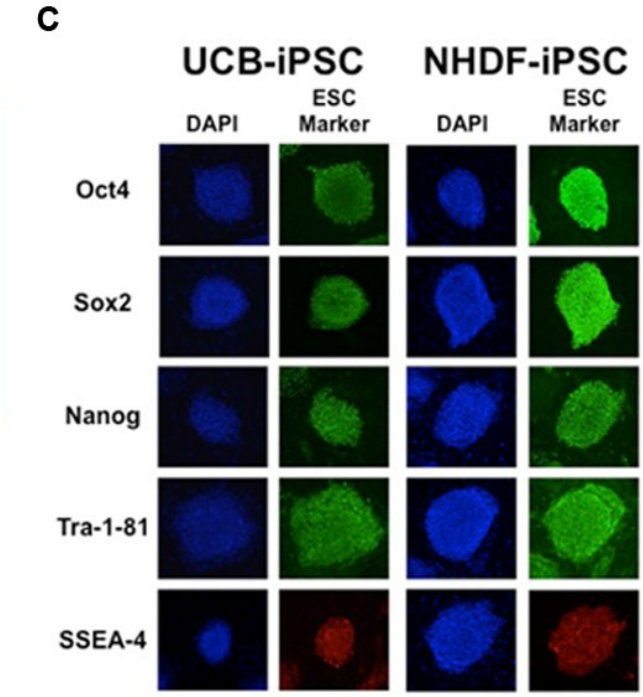

H9EB-NK (FF)
H9EB-NK (EL08)
CD56
CD117
CD94
NKG2A
NKG2C
KIR
CD16
99.9
99.7
0.015
0.53
1.53
97.9
0.074
0.86
0.92
98.1
86.6
11.3
1.75
0.41
86.7
12.1
0.95
0.26

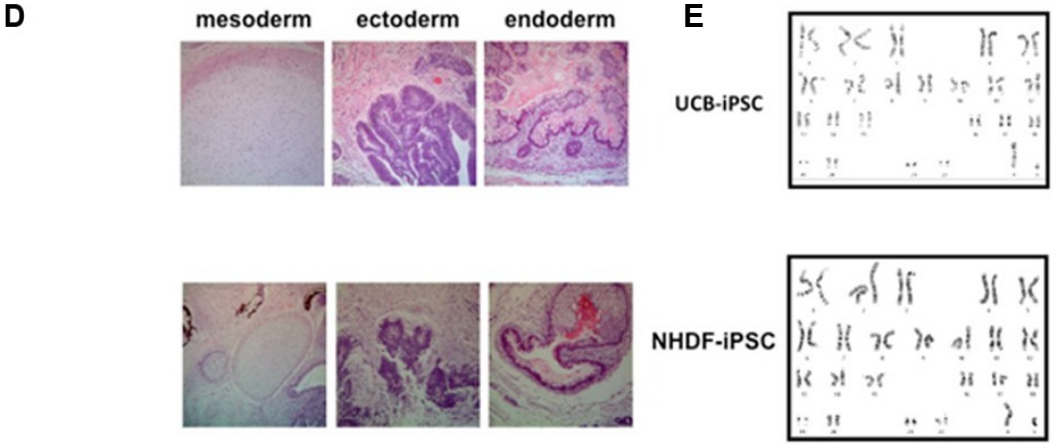
C


vs. K562 (CML)
Percent Killing
80
70
60
50
40
30
20
10
0
0.0
2.5
5.0
7.5
10.0
Effector/Target Ratio
H9EB-NK (EL08)
H9EB-NK (FF)

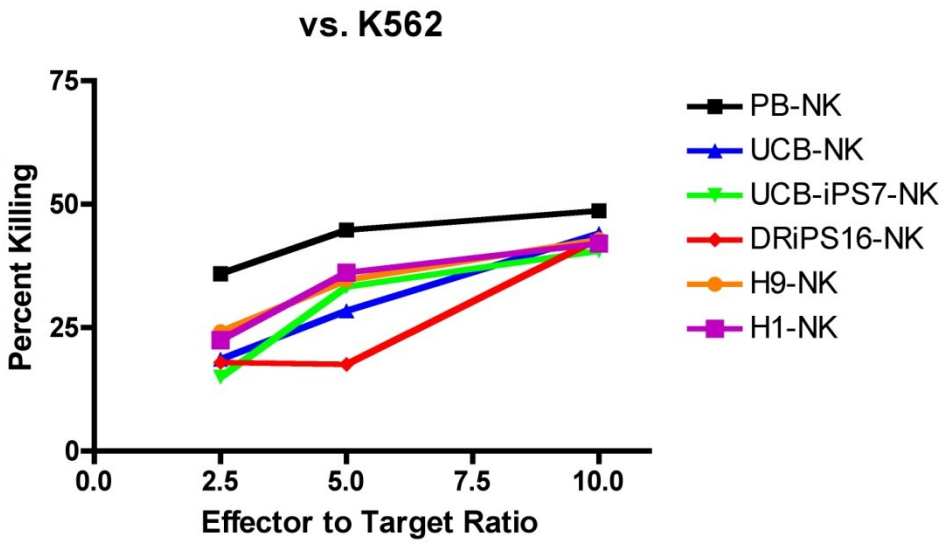
Supplementary Figure 2.1. Reprogramming strategy and characterization of iPSCs. A) Reprogramming scheme illustrating the strategy to reprogram each cell line from UCB 34^+ progenitor cells (UCBiPS7) or neonatal dermal human fibroblasts (NHDF, DRiPS16). **B)** Phase images of colonies between Day 11 and 20 post infection. **C)** Immunostaining of pluripotent markers Oct3/4, Sox2, Nanog, SSEA-4, and Tra-1-81. Blue for DAPI staining, green for Alexa Fluor® 488 secondary antibody, and red for primary PE-conjugated antibody. **D)** Teratoma formation showing examples of all three germ layers and **E)** normal karyotype.



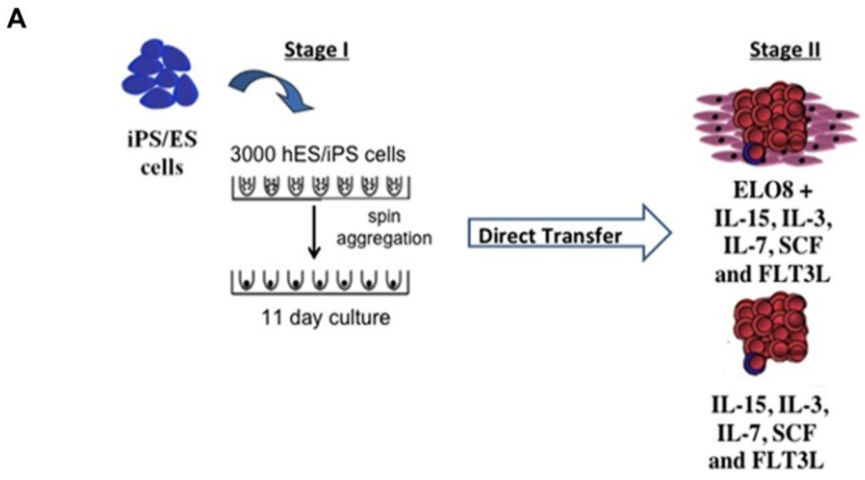

C
UCB-iPSC
NHDF-iPSC
DAPI
ESC Marker
DAPI
ESC Marker
Oct4
Sox2
Nanog
Tra-1-81
SSEA-4

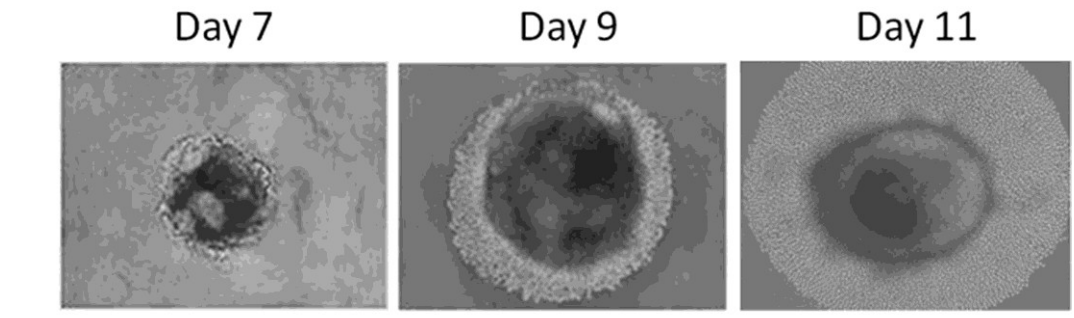

D
mesoderm
ectoderm
endoderm
E
UCB-iPSC
NHDF-iPSC

Supplementary Figure 2.2. Cytotoxicity of iPSC-derived NK cells. Cytotoxicity assay against K562 tumor cells. hESC and iPSC-derived NK cells kill CML targets cells at a significantly higher level than UCB-derived NK cells. Means of cytotox, n=3 for each except H1 n=2, BJ1iPS-derived NK cell antitumor activity has previously been demonstrated[80].

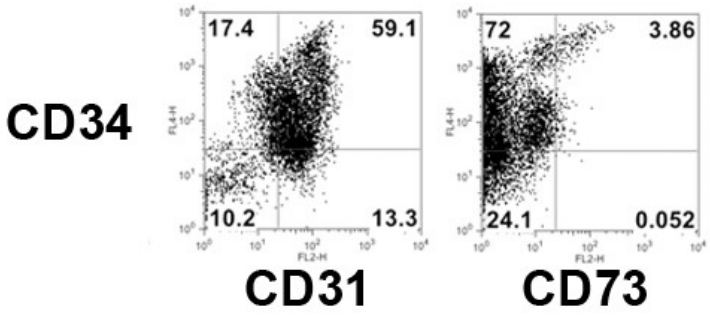


Supplementary Figure 2.3. Hematopoietic differentiation using spin-EB cultures. A) Schematic of hematopoietic and NK cell development from undifferentiated hESCs/iPSCs using the spin-EB method. **B)** Aggregated hESCs or iPSCs form discrete spin-EBs in each individual well and proliferate over a period of 11 days. Pictures were taken at 4x magnification at days 7, 9, and 11. At day 11 there are a large number of hematopoietic appearing cells surrounding the EBs.





Supplementary Figure 2.4. Spin EBs contain progenitors for both endothelial cells and mesenchymal stromal cell. Day 11 (day of transfer to NK cell conditions) spin EBs were analyzed for the presence of the endothelial (CD31) or mesenchymal stromal cell (CD73) markers. A large portion of the $CD34^+$ cells co-express CD31. Flow cytometry plots are representative of at least 3 independent experiments.



CHAPTER 3

hESC-derived natural killer cells to study cell trafficking and immunotherapy in vivo

Human embryonic stem cell (hESC) derived NK cells are a promising source of anti-tumor lymphocytes for off-the-shelf immunotherapeutics. They also provide a genetically tractable platform uniquely suited for study of anti-tumor immunotherapies in pre-clinical models. We have previously demonstrated the potency of hESC-derived NK cells *in vivo*. We have used both bioluminescent and fluorescent imaging to demonstrate trafficking of hESC-derived NK cells to tumors in vivo. Our dual-imaging approached allowed us to more specifically define and study the kinetics of NK cell trafficking to tumor sites. NK cell persistence and trafficking were further evaluated by flow cytometry and immunohistochecmistry. This integrated approach provides an improved system to study the kinetics and biodistribution of adoptively transferred lymphocytes which is broadly applicable to the field of immunotherapy.

INTRODUCTION

Natural killer (NK) cells are lymphocytes of the innate immune system with powerful anti-tumor activity and can be derived from both hESCs and iPSCs[14,38,80,92]. Previous experiments by our group used hESC-derived NK cells in a mouse xenograft model to show complete tumor clearance in mice engrafted with human K562 leukemia cells[58]. However, whether or not NK cells trafficked to and were responsible for the tumor regression was unclear. The kinetics of NK cell trafficking to tumors *in vivo* has not been previously explored, but is crucial to gain a basic understanding of the activity of these cells. Human pluripotent stem cells provide a unique platform to study cell-based therapies.

Their ability to be routinely and stably genetically modified allows for enhanced monitoring of cell survival and migration both *in vitro* and *in vivo*[59,95]. For therapeutic purposes, the ability to derive almost any blood cell from hESCs could provide banks of cells for off-the-shelf therapeutics[9]. Alternatively, given the relative ease of manufacturing iPSCs and technological advances in this process, the thought of using patients' autologous cells for therapy is also reasonable[96]

Previous studies by our group demonstrated that hESC and iPSC-derived NK cells have potent ability to kill diverse tumor cells both *in vitro* and *in vivo*. However, a key question of these studies is how directly (or indirectly) do the NK cells interact with tumor cells? Our studies have focused on using hESC-derived NK cells as a system to test lymphocyte engraftment and trafficking *in vivo*. To test this, we administered immunodeficient NOD/SCID/$\gamma C^{-/-}$ (NSG) mice intraperitoneal (IP) or intravenous (IV) injections of hESC-derived NK cells and followed them *in vivo*. Interestingly, we found a persistence of the cells for more than 25 days *in vivo* by bioluminescent imaging and flow cytometric analysis. We next engineered the leukemia cell line K562 to express a membrane bound *Gaussia* luciferase (mbGluc) construct, which is distinct from the Firefly luciferase (FLuc) constitutively expressed in our hESC-derived NK cells. These two luciferase reporters have been used in combination before to demonstrate trafficking and serve as a genetic reporter in the same cell type[97], but to our knowledge this is the first to utilize both in an anti-tumor model. This allowed parallel monitoring of both the tumors and NK cells *in vivo*, non-

invasively over time. Although this approach allows dual-bioluminescense imaging, it is technically difficult and limiting because of the need to deliver the mbGluc substrate intravenously to each mouse. To overcome this, we subsequently modified K562-tumor cells with the fluorescent protein turboFP650. TurboFP650 is a far-right shifted fluorescent reporter which allows optimal *in vivo* imaging because of its reduced background compared to other fluorescent reporters *in vivo*. By doing this, we were able to successfully recapitulate our findings from the dual-luciferase model by showing NK cell trafficking with similar kinetics. As a third measure of NK cell trafficking we evaluated invasion of hESC-derived NK cells into tumor sites and were able to show NK cell trafficking by immunohistochemistry.

These results confirm the ability of hESC-derived NK cells to persist and traffic to the site of tumor in a xenograft model. The data also validate hESC-derived blood cells as a model system to study *in vivo* trafficking and are broadly applicable across a variety of models.

MATERIALS AND METHODS

hESC maintenance and NK cell differentiation

hESCs were maintained on low-density (90,000 cells/well of a 6 well plate) mouse embryonic fibroblasts (MEF). Generation of hematopoietic progenitor cells from hESCs was accomplished using an established method[83]. For NK cell differentiations, six wells of spin EBs from a 96-well round bottom plate were transferred to one well of an uncoated 24-well plate in media and cytokines we have previously described[58]. Following 4 weeks of NK cell culture cells were phenotype further expanded using artificial antigen presenting cells (aAPCs)[82]. aAPCs were kindly provided by Dr. Dean A. Lee.

Cell lines

K562 cells were obtained from ATCC. K562 cells expressing mbGLuc were generated as follows. First, the mbGluc portion was PCR amplified using the primers: 5'-CATACAGAATTCATGGCTCTCCCAGTGACTGCCCTACTGCTT and 5'- CATACAGAATTCGGATCCCTATTATTGAATCCGCCTGTGGTT-3'. EcoRI sites are underlined. The mbGluc fragment was then digested and subcloned in to an EcoR1 digested pKT2-mCAGs-IRES-GFP:zeo construct containing an EcoRI splice junction between the mCAGs promoter and the internal ribosomal entry site (IRES). Orientation was confirmed by restriction enzyme digest at sites within the distal ends of the subcloned mbGluc sequences. To generate tuboFP650 expressing K562 cells, we PCR amplified the sequence containing the TurboFP650 (Evrogen) as above, using the

primers: 5'-CATACAATCGATATGGGAGAGGATAGCGA-3' and 5'-CATACAAGATCTATCAGTTATCTAGATCCGGT-3'. ClaI and BglII sites are underlined, respectively. The PCR fragment was then digested with ClaI and BglII and ligated into the pKT2-mbGluc-IRES-GFP;zeo construct in place of the GFP:zeo fusion protein. Confirmed constructs were then nucleofected into K562 cells using a Lonza 4D-nucleofector device. TurboFP650 expressing K562 cells were sorted on a FACsAria cell sorter (BD Biosciences).

***In Vivo* Fluorescent and Bioluminescent Imaging to Follow Trafficking of hESC-derived NK Cells**

At 24 hours before tumor inoculation, 6- to 8-week old nonobese diabetic/severe combined immunodeficiency with gamma-chain knockout (NOD/SCID/ $\gamma C^{-/-}$) were given a sublethal dose of irradiation (225-250 cGy). A total of 1 x 10^6 mbGluc$^+$ or mbGluc$^+$/turboFP650$^+$ K562 cells were resuspended in 200 µl Iscoves modified Dulbecco medium (IMDM) (HyClone Laboratories) supplemented with 20% FBS (Gibco). Cells were then injected subcutaneously into the upper left thorax of the mice. The tumors were allowed to engraft for 4 (mbGluc$^+$) or 7 days (turboFP650$^+$). Mice were then given an intraperitoneal (IP) injection of 10 x 10^6 hESC-NK cells resuspended in 300 µl IMDM supplemented with 20% FBS. For all experiments, mice receiving no NK-cell infusion were included as a negative control and tumor-only mice were included as a positive control for tumor engraftment. All mice received IP injections of IL-2 (1 x 10^4 U/mouse) and IL-15 (10 ng/mouse) every day for the first 7 days after NK-cell

injection followed by IL-2 only every 2 to 3 days until mice were sacrificed. All mice were housed, treated, and handled in accordance with the guidelines set forth by the University of Minnesota Institutional Animal Care and Use Committee and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

To follow tumor progression and NK cell trafficking simultaneously, we utilized two dual-imaging schemes. To track the mbGluc^{+} K562 cells and Fluc^{+} hESC-NK cells, bioluminescent imaging was performed using a Xenogen IVIS Spectrum imaging system (Caliper Life Science, Hopkinton, MA). Prior to imaging, mice were anesthetized with isoflurane. A bioluminescent image was acquired using a 1 minute exposure 10-15 sec following intravenous (IV) injection of coelenterazine (320 µg, Nanolight Technology) or 10 minutes after IP injection of D-luciferin (150 mg/kg, GOLD Bio Technology). Mice were imaged individually following injection of coelenterazine and allowed to recover prior to injection of D-luciferin. Optical images were analyzed with Living Image software version 4.2 (Caliper Life Science, Hopkinton, MA).

To track turboFP650^{+} K562 cells and Fluc^{+} hESC-NK cells, fluorescent and bioluminescent imaging was performed using a Xenogen IVIS Spectrum imaging system. Prior to imaging, mice were anesthetized as described. A bioluminescent image was acquired for a total 1 minute exposure 10 minutes after IP injection of D-luciferin. Immediately following, a fluorescent imaging sequence was acquired by performing an emission scan for turboFP650 (Excitation: 605, Emission: 660-720) and background signal (Excitation: 570,

Emission: 640-720) using autoexposure settings. To separate the tumor and background signal, fluorescent imaging sequences were spectrally unmixed and set to a standard scale using Living Image software version 4.2 (Caliper Life Science, Hopkinton, MA).

Immunohistochemistry

Tumor tissue collected at the time of sacrifice was fixed in 10% formalin for 24-36 hours and embedded in paraffin. Four micron sections were cut using a microtome, mounted onto uncharged slides, and rehydrated according to standard protocols. Slides were pretreated with a citrate buffer, 6.0 pH, in a Oster steamer for 30 minutes, and allowed to cool for 15 minutes. Primary antibodies were used at the following concentrations: Human NKp46 (R&D Systems, AF1850, 1:100), IgG1 kappa isotype control (eBioscience, Cat # 14-4714-82, 1:50). Antibody detection was by horseradish peroxidase-labeled streptavidin and DAB chromagen (Covance). Tissue sections were counterstained in hematoxylin. In every experiment, human tonsil tissue was stained as a positive control and tumor tissue from mice receiving no NK cell injection as a negative control. Images were taken at 10x, 40x, and 63x magnifications.

RESULTS

Generation of NK cells from firefly luciferase expressing hESCs for *in vivo* tracking

In order to study trafficking in this mouse model, we used a well characterized differentiation protocol to derive natural killer cells from hESCs[14,58,80]. We have previously demonstrated the derivation and function of NK from both hESCs and iPSCs[58,80]. hESC-derived NK cells have potent anti-tumor activity both *in vitro* and *in vivo*, however, our initial studies were unable to directly implicate tumor clearance as a direct result of trafficking NK cells. Using hESCs that we have modified to express firefly luciferase and GFP[16], we first demonstrated their ability to differentiate into hematopoietic progenitor cells and subsequently NK cells (Figure 3.1). We have previously shown these cells kill a variety of targets *in vitro* as well as K562 erythroleukemia cells *in vivo*[58]. To further explore the function of hESC-derived NK cells *in vivo*, we developed a model to monitor NK cell persistence and trafficking as well as tumor burden. We compared the survival of hESC-derived NK cells upon transfer into immunodeficient (NSG) mice via the intravenous (IV) or intraperitoneal (IP) route. Traditionally, effector cells for adoptive immunotherapy have been administered IV, but this may not be an optimal deliver system for all malignancies, such as ovarian cancer. Additionally, injection of NK cells into a non-hematopoietic or non-lymphoid compartment using an IP approach would provide a more rigorous test of trafficking. Mice also received injections of IL-2 (10,000 units/mouse) and IL-15 (10 ng/mouse) for the first 7 days followed by

injections of IL-2 10,000 units/mouse) every other day until the end of the study. Mice receiving IP delivery of NK cells had prolonged persistence compared to those injected IV (Figure 3.2). IV delivery of NK cells first trafficked to the lungs of the mice but were absent by day 4, whereas IP delivery of NK cells lead to persistence for greater than 4 weeks. At day 19, we sacrificed mice and looked for engraftment within the peripheral blood, spleen, bone marrow, and peritoneum. Both routes of NK cell delivery had low levels of engraftment within the peripheral blood, bone marrow, and spleen as measured by GFP^+ and $CD56^+CD45^+$ cell surface antigens. However, there was a high level of engrafting NK cells in the peritoneum of IP injected mice compared to IV injected mice or controls (Figure 3.2). This corresponds with the bioluminescent imaging data and led us to conclude that IP delivery of NK cells allows enhanced persistence of NK cells *in vivo* and would be optimal for our trafficking studies.

NK cells persist and co-localize with tumors *in vivo*

Over the past 10 years there have been several advances in small animal imaging that have allowed investigators to monitor biologic processes non-invasively over time. One of the most common is the family of luciferase proteins[98,99]. Here we took advantage of firefly luciferase within our hESC-derived NK cells as a well-characterized reporter of lymphocyte persistence *in vivo*[97,99]. Our previous studies demonstrating the powerful anti-tumor activity of hESC-derived NK cells in vivo used luciferase positive tumor cells; however, we

were unable to follow NK cells concurrently. To image both NK cells and tumors in the same mouse, we adopted another luciferase reporter of the *Gaussia* luciferase family. Using a recombinant form of the *Gaussia* protein that has been modified to be tethered to the membrane (membrane-bound Gaussia lucifearse, mbGluc, kindly provided by Dr. Renier Brentjens)[100], we were able to utilize two different substrates to image both tumors and NK cells in the same mouse. We initially subcloned the mbGluc gene into a *Sleeping Beauty* backbone driven by an mCags promoter (Figure 3.3). We were able to stably transduce K562 tumor cells and select for cells with luciferase activity in response to the substrate coelenterazine but not luciferin.

To use both reporters *in vivo*, we took advantage of the fact that mbGLuc, which utilizes coelenterazine as its substrate and is rapidly degraded *in vivo* [100]. Firefly luciferase (expressed in the hESC-derived NK cells) is reciprocally stable *in vivo*[100,101] and was delivered second. Using these two reporters, we were able to initially image tumor mbGluc^{+} cells, and then image the hESC-derived NK cells in the same mouse. We replicated our initial model by allowing K562 tumor cells to engraft in sublethally irradiated mice for 4 days prior to NK cell injection[58] . At day 0, NK cells were given IP and mice were treated with cytokines. Mice were evaluated for both tumor size and NK cell trafficking at days 0, 4, 7, 9 to 12. As we previously hypothesized, NK cells were capable of trafficking to tumor sites (Figure 3.4). This typically occurred between day 9 and 12 but was variable among mice. Additionally, not every mouse demonstrated trafficking by bioluminescence. We conclude from this that hESC-derived NK

cells can be followed for trafficking in this dual-bioluminscent system. Although not every mouse demonstrated trafficking, this could be due to the absolute number of luciferase^{+} cells needed to demonstrate bioluminescent signal and the negative mice could be below the limit of detection. Additionally, we found that increasing the tumor burden of the mice (1 x10^{6} cells vs. 200,000 cells) was necessary to allow enough NK cells to accumulate and give a bioluminescent signal over background.

Improved dual reporter imaging with firefly luciferase and the fluorescent protein turboFP650

The use of mbGluc in conjunction with firefly luciferase provided a reliable, yet technically challenging, model to study NK cell trafficking. This is primarily because the substrate for Gaussia luciferase, coelenterazine, needs to be delivered intravenously in order to be effective. This was prohibitive for several reasons. First, due to the decay kinetics of the substrate, a limited amount of mice could be imaged simultaneously[100]. Also, repeated injection of the coelenterazine substrate lead to destruction of the tail vein over time, making this delivery site suboptimal for later injections. To overcome this, we took advantage of a more recently described fluorescent reporter that can be imaged *in vivo*[102,103]. TurboFP650 is a red-shifted fluorescent reporter (excitation 592 nm, emission 650 nm) giving it good tissue penetrance for optimal *in vivo* imaging. It also doesn't require delivery of a second substrate. We used a similar cloning approach to express the turboFP650 protein in K562 cells using

Sleeping Beauty (see materials and methods). Stable expression of TurboFP650 was determined by flow cytometry (BD Fortessa) using the 561 nm laser and 660/40 filter set. Following confirmation of stable transduction for more than 1 week, cells were sorted using the same parameter as above (BD FACS Aria). Sorted cells maintained expression of TurboFP650 protein and were used for further *in vivo* studies (Figure 3.5).

Using the same in vivo model as above, mice were engrafted with 1 million TurboFP650^{+} K562 tumor cells and allowed to engraft for 7 days. At day 0, mice were then given firefly luciferase expressing NK cells intraperitoneally and followed for trafficking. Similar to our dual-luciferase studies, we found that NK cells were able to track to turboFP650^{+} tumor cells in four of the five mice, which occurred within 9-12 days post-NK cell injection (Figure 3.5). These data support the use of turboFP650 as a reporter compared to mbGluc. By overcoming the technical limitations of using coelenterazine based reporters these studies provide enhanced *in vivo* system to monitor two cell populations over time.

Trafficking of hESC-derived NK cells to tumor confirmed by immunohistochemistry

To definitively show NK cells at the tumor site, we paraffin embedded tumors taken from mice at the time of sacrifice. Compared to the tumor only (no NK cell injection) group and isotype controls, mice with demonstrated NK cell trafficking by bioluminescent imaging had human NK cells present as demonstrated by NKp46 staining on IHC (Figure 3.6). Human tonsil tissue was used as a positive

control. The use of NKp46 is a more specific marker of human NK cells, as CD56 can also marker other tissue types. The NK cells positive by IHC staining were uniformly dispersed throughout each tumor tissue section stained. These data further support the trafficking of NK cells to the tumor site and that bioluminescent imaging using firefly luciferase is an effective model to study lymphocyte trafficking *in vivo*.

Discussion

The use of hESCs and iPSCs to study blood and lymphocyte development has several advantages. First, each line provides an unlimited number of cells and serves as an individual donor allowing a homogenous starting source. Our most recent studies have improved on the method in generating NK cells from hESCs and iPSCs, allowing the generation of enough cells to treat a single patient from 250,000 starting cells. Second, hESCs and iPSCs are genetically amenable platforms to allow stable transgene expression as well as the potential for gain- and loss-of-function studies. Here, we have utilized hESCs that constitutively express firefly luciferase, although there are other reporters that can be used. Additionally, having a genetically tractable system allows modification of the input cells. Some recent studies have used transfer of certain chemokine receptors into effector cells to get enhanced homing to the tumor site[104]. This could be tailored on a patient, disease specific basis if a bank of hESC-derived effector cells was used. Finally, there are numerous well defined protocols leading to the development of almost all hematopoietic

lineages, not just NK cells[9,13,105-107]. Studies have aimed at genetic correction of erythroid progenitors in hopes of delivering red blood cells to patients with hemoglobinopathies[9,13,105-107]. The major challenge of hESCs and iPSCs is the continued inability to generate and isolate functional HSCs capable of long-term, multilineage engraftment[17,20]. Until a protocol to generate definitive HSCs is found, delivery of more differentiated blood types (such as lymphocytes or erythrod cells) is the best available option of hESC- and iPSC-based hematopoietic cell therapies.

By using hESC-derived NK cells to monitor trafficking, we found that delivery of cells intraperitoneally lead to enhanced persistence of NK cells, not surprisingly within the peritoneum. Enhanced persistence of IP delivered NK cells could be due to several reasons. First, although firefly luciferase is sensitive *in vivo* reporter, signal is most prevalent when there are collections of cells. IV delivery of NK cells could lead to enhanced distribution as the cells traffic through the circulation, thereby minimizing the luciferase signal. Alternatively, IP delivered NK cells persist for longer periods of time because they have more direct access to injected cytokines, which are also delivered IP.

Importantly, these studies were able to demonstrate trafficking of hESC-derived NK cells to the site of tumor. Without the utility of a dual-bioluminescent imaging model, trafficking would have been much more difficult to discern. Not all mice (50-75%) demonstrated trafficking by bioluminescent imaging and signs of trafficking ranged over 9-12 days. It is possible that some NK cells traffic and are below our limits of detection, or that NK cells can indeed traffic to tumor but

don't receive the correct signals to stay within the tumor environment. In this case, one could modify the NK cells with tumor specific receptors to enhance intratumoral persistence and activity[104]. This has been recently accomplished using chimeric antigen receptors (CARs) in human T cells[66,90]. hESCs and iPSCs provide an optimal platform for such a modification. The studies also confirm our initial findings that unmodified hESC-derived NK cells traffic to tumor sites to clear disease.

Immunohistochemistry was performed to confirm the above evidence and validate the ability of this dual reporter system. As NSG mice are deficient of all lymphocytes, and the NKp46 marker is specific to human NK cells, we can conclude definitive trafficking of NK cells to the tumor site. By having effector cells labeled with firefly luciferase we were able to monitor whole body trafficking whereas other methods are limited to longitudinal analysis of engraftment in the peripheral blood. Although we didn't see high levels of engraftment in other organs such as spleen or bone marrow by bioluminescent imaging, the NSG mouse may not be the best host to support the hESC-derived NK cell persistence at these sites. This does not completely explain the deficiency of hESC-derived NK cells trafficking to these sites as PB-NK cells can be found here. It remains important to more closely define the different receptors between these two cell types dictating this phenomenon.

Together, these data provide a model system to follow two different populations of human cells in mice and should not be limited to anti-tumor therapies. The

use of two diverse reporter systems combined with hESC/iPSC-derived cells has broad applicability to a number of biological systems.

For therapeutic purposes, the ability to derive almost any blood cell from hESCs could provide banks of cells for off-the-shelf therapeutics[9]. Alternatively, given the relative ease of manufacturing iPSCs and technological advances in this process, the thought of using patients autologous cells for therapy is also reasonable[96]. hPSCs also provide a platform to take gain- and loss-of-function approaches in studying lymphocyte development and trafficking. Constitutive or conditional knock down of particular molecules affecting these processes would be beneficial and a major advantage over using cells isolated from primary sources (HSCs or peripheral blood), which are intrinsically resistant to genetic modification.

Figure 3.1. Derivation of NK cells from hESCs expressing firefly luciferase. A) Schematic for the derivation of hESC-derived NK cells. GFP-luciferase^{+} hESCs were dissociated and plated in spin EB conditions for 11 days. Cells were then transferred to conditions supporting NK cell development. **B)** After 11 days in spin EB culture, cells were dissociated and stained for the progenitor markers CD34, CD45, and CD43 for FACs analysis. Spin EB derived progenitor cells expressed high levels of CD34, CD43, and CD45. **C)** Following 4 weeks in NK cell culture, cells were harvested and stained for FACs analysis. hESCs expressing GFP and luciferase differentiate into pure populations of NK cells expressing CD56, CD16, NKp46, and NKG2A. Each flow plot is representative of at least 5 independent experiments.

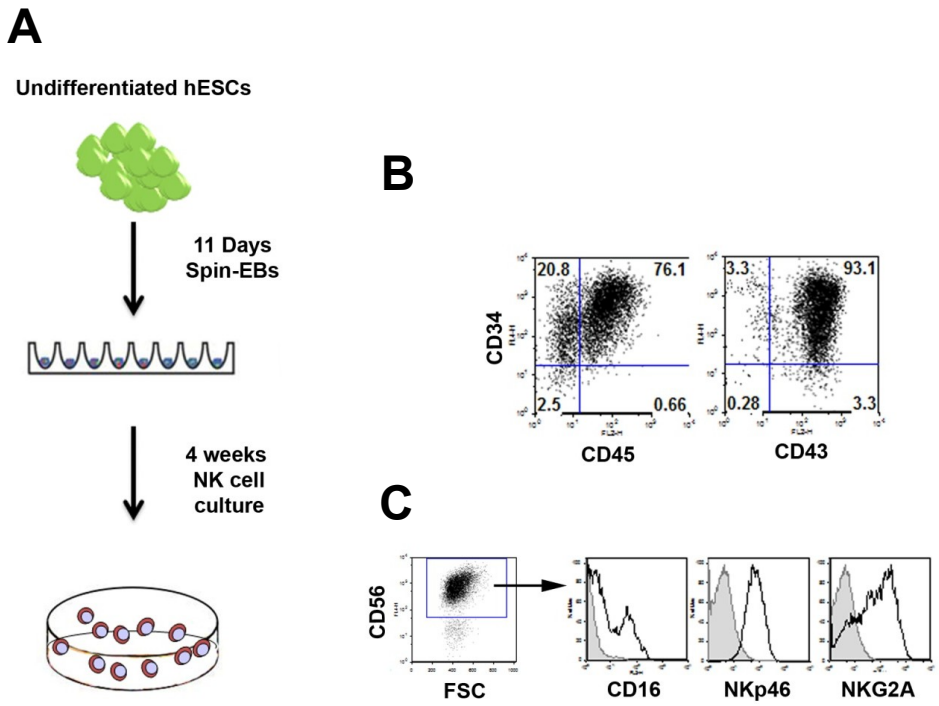
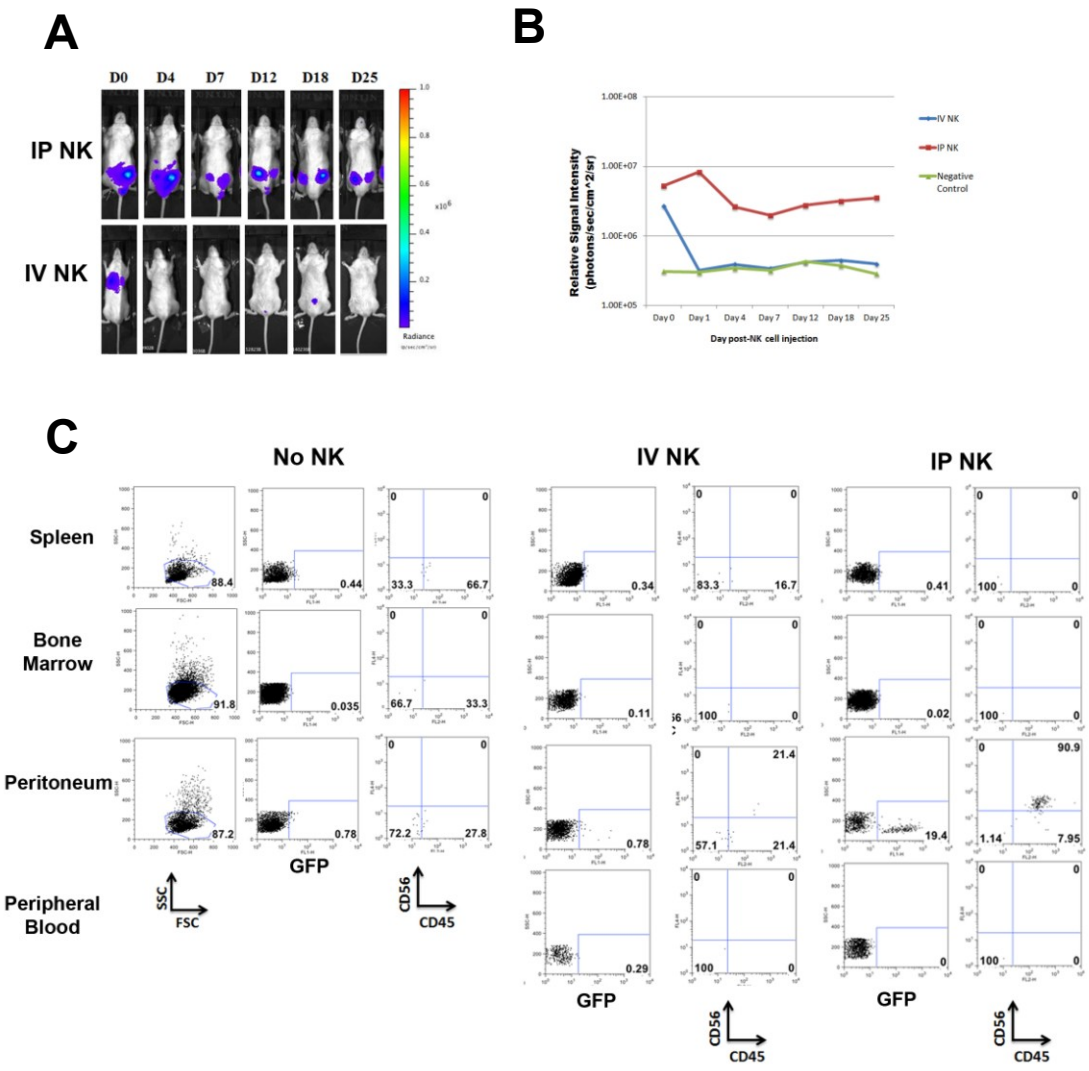


Figure 3.2. Persistence of hESC-derived NK cells. A) Persistence of hESC-derived NK cells injected IV vs. IP was monitored by bioluminescent imaging at the indicated timepoints (D= days) following NK cell injection. **B)** Quantification of hESC-derived NK cell luciferase signal in mice receiving cells IV vs. IP and compared to non-injected controls. IP injected NK cells persisted for the entire 25 days whereas IV injected cells were undetectable by day 4. **C)** Analysis of some mice at day 19 to assess engraftment of IV and IP injected NK cells within the spleen, bone marrow, peritoneum, and peripheral blood compared to non-injected controls. hESC-derived NK cells were analyzed for their expression of GFP or staining of CD56 and CD45 surface antigens. Plots are representative of 4 IP mice and 3 IV mice. For non-injected control group, two mice were analyzed.


A
D0
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IP NK
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B
Relative Signal Intensity (photons/sec/cm^2/sr)
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Day 1
Day 4
Day 7
Day 12
Day 18
Day 25
Day post-NK cell injection
IV NK
IP NK
Negative Control
C
No NK
IV NK
IP NK
Spleen
Bone Marrow
Peritoneum
Peripheral Blood
GFP
SSC
FSC
CD56
CD45

Figure 3.3. Generation of K562 tumor cells expressing membrane bound Gaussian luciferase. A) Schematic of the *Sleeping Beauty* constructs used to modify the tumor cells. **B)** K562 cells modified with either control or mbGluc constructs were selected for using zeocin and monitored by flow cytometry until 90% were GFP^+.

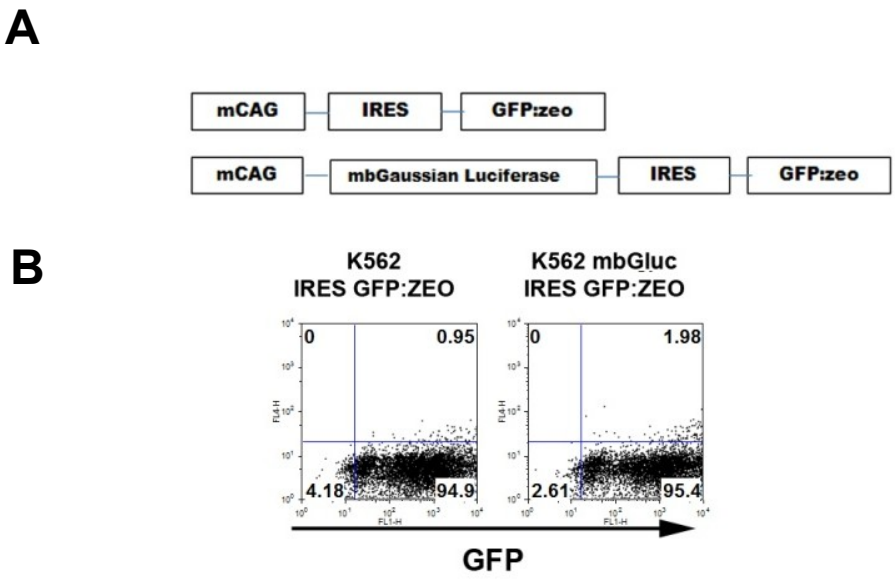


Figure 3.4. Dual-bioluminescent imaging to monitor hESC-derived NK cell trafficking *in vivo*. Monitoring of a single mouse over a period of 9 days for presence of both tumor cells (mbGluc^{+}, top row) and NK cells (firefly luciferase+, Fluc, bottom row). NK cell trafficking to the tumor site can be seen on day 9 in this particular mouse.

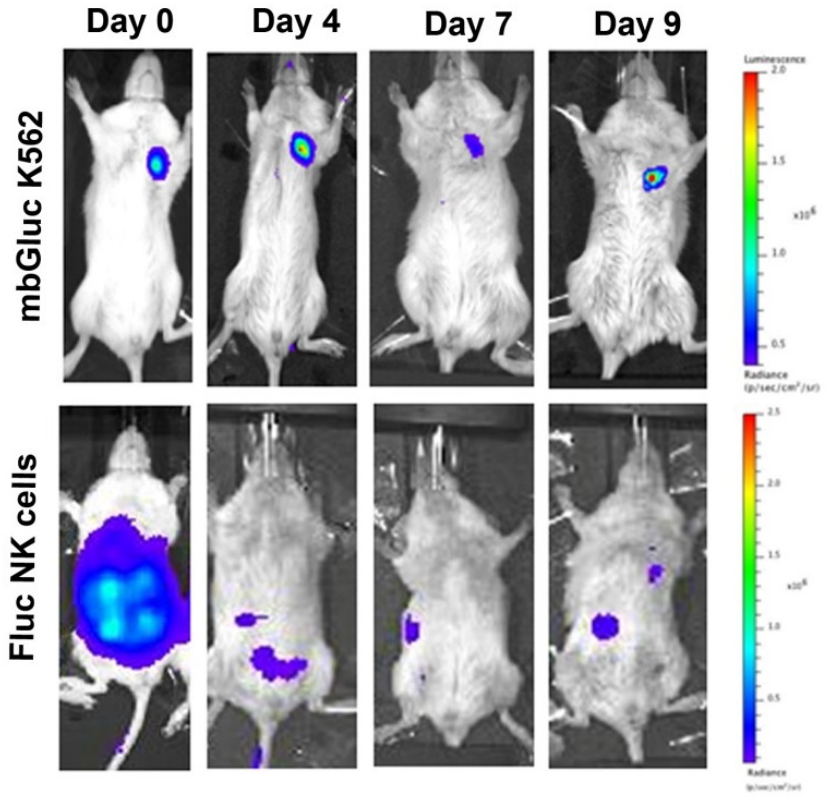
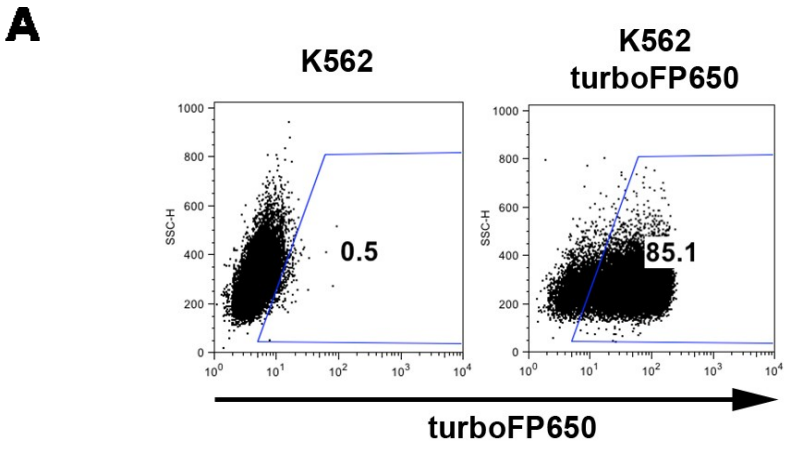


Figure 3.5. Enhanced dual-reporter imaging utilizing TurboFP650 expressing K652 cells. A) K562 cells modified with a TurboFP650 reporter were analyzed and sorted by FACs. **B)** 5 mice were injected with both TurboFP650^{+} K562 tumor cells and 10 x 10^{6} NK cells and followed for two weeks. The images demonstrate trafficking of hESC-derived NK cells expressing firefly luciferase at **B)** day 9 and **C)** day 12.



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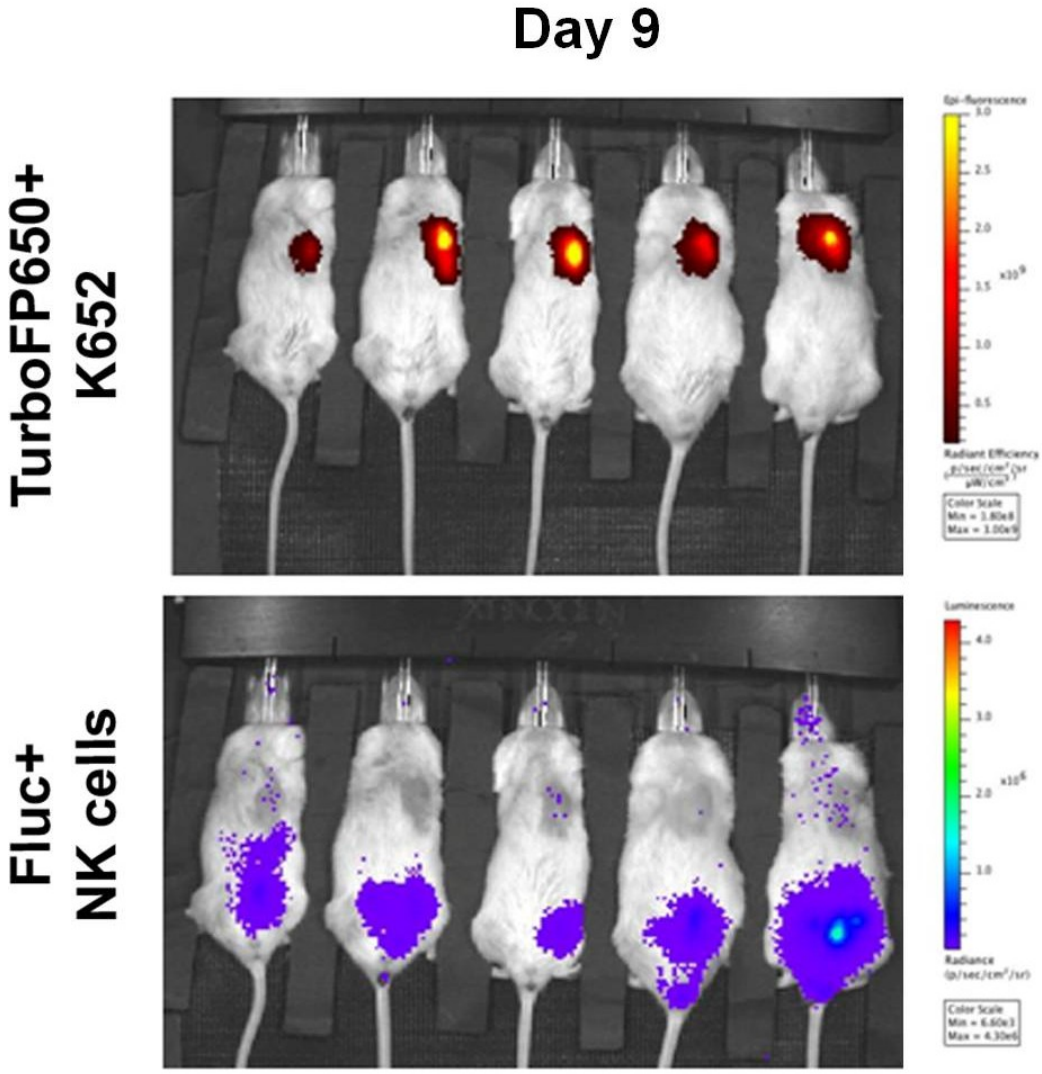

Day 9
TurboFP650+
K652
Fluc+
NK cells
Epi-fluorescence
Radiant Efficiency
Color Scale
Luminescence
Radiance
Color Scale

C

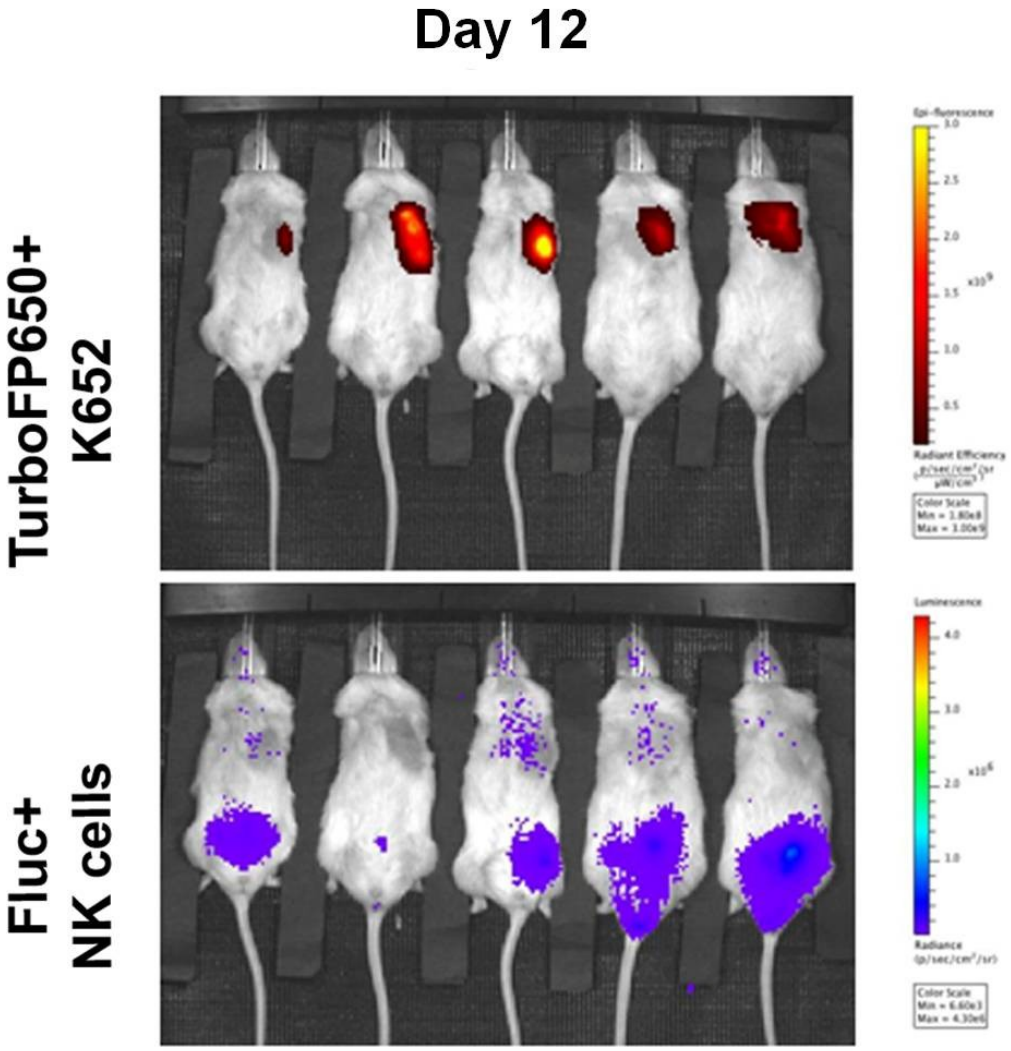
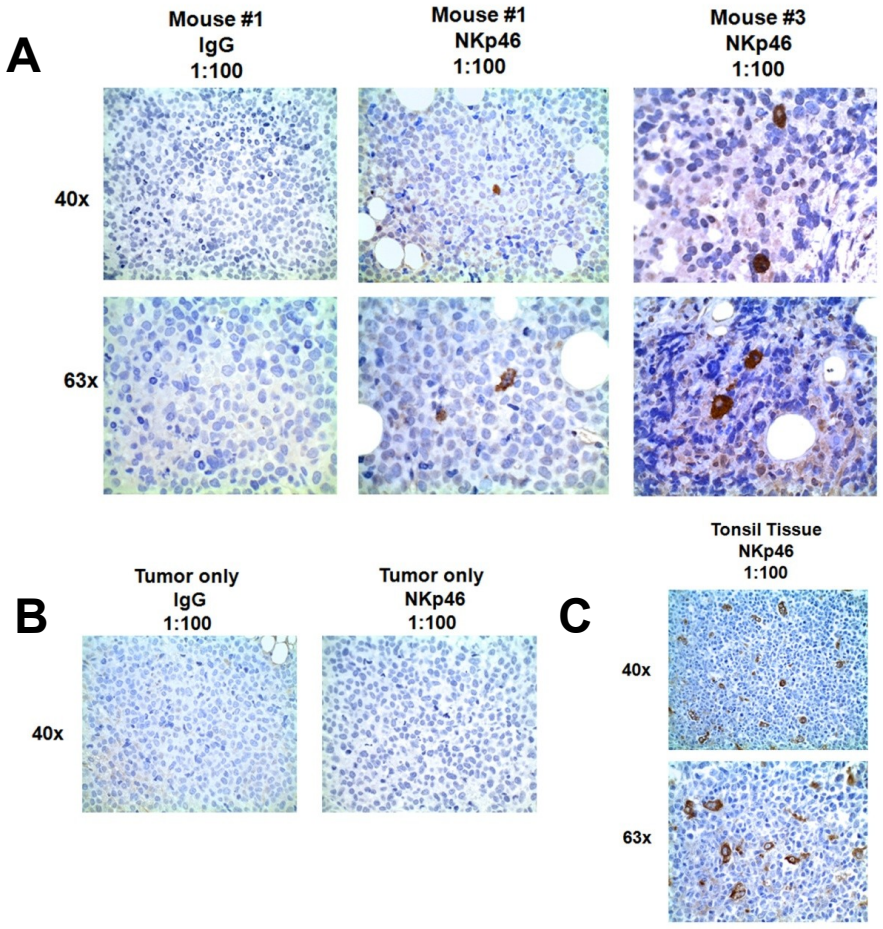

Day 12
TurboFP650+ K652
Fluc+ NK cells
Epi-fluorescence
Radiant Efficiency
Color Scale
Min = 1.80e8
Max = 3.00e9
Luminescence
Radiance
Color Scale
Min = 6.60e3
Max = 4.30e6

Figure 3.6. Immunohistochemical (IHC) confirmation of *in vivo* NK cell trafficking. A) Tumor tissue from mice with positive trafficking by bioluminescence imaging was formalin-fixed and paraffin embedded prior to IHC. Sections were then stained with isotype (IgG1) or NKp46 antibodies at the indicated concentrations. Isotype and NKp46 antibodies were performed on serial sections. **B)** Tumor only mice demonstrated no staining for NK cells whereas the positive control, human tonsil tissue (**C)**, had numerous NK cells present.



CHAPTER 4

Intraperitoneal delivery of human NK cells for treatment of refractory ovarian cancer

There is an urgent need for novel therapeutic strategies, as most women with relapsed ovarian cancer will die of progressive disease. Dramatic anti-tumor effects have been observed with IL-2 activated NK cells that are adoptively transferred into patients with other cancers. Persistence and *in vivo* expansion of intravenously delivered NK cells in ovarian cancer patients has not been successful to date. We investigated *in vivo* expansion and persistence of intraperitoneally (IP) delivered NK cells in an ovarian cancer xenograft model to determine if delivery mode can affect tumor cell killing and circumvent lack of NK cell expansion. By using an ovarian cancer xenograft mouse model, we were able to demonstrate that direct delivery of NK cells to the peritoneum resulted in similar levels of circulating NK cells as seen with intravenous (IV) delivery. IP delivery of NK cells was effective in clearing intraperitoneal tumor burden in mice receiving IL-2, IL-15 or in combination, and that NK cells remained within the peritoneal cavity 54 days following injection. The NK cells that persisted had markers of maturation and proved to be functional. These NK cells were able to kill ovarian cancer targets either at a rate similar to or higher than their pre-infusion levels supporting that *in vivo* functionality of human NK cells can be maintained following IP infusion. By demonstrating that IP delivery of NK cells leads to stable engraftment, expansion, and antitumor response in an ovarian cancer xenograft model, these data support further pre-clinical and clinical evaluation of IP delivery of allogeneic NK cells in ovarian cancer.

INTRODUCTION

Harnessing immune cells to treat malignancy has been a major goal over the past decades. Current standard therapies for recurrent ovarian cancer provide a unacceptably low (<20%) response rate[108]. There is an urgent need for effective therapeutic strategies, as most women with relapsed ovarian cancer will die of progressive disease. Natural Killer (NK) cells are a key part of the innate immune system with the ability to recognize and kill diverse types of tumor cells, including ovarian cancer[109]. Human T and NK cells can be applied to treat cancer [5,6]. T cell-based cellular therapy has produced objective responses in metastatic melanoma, however, recurrent disease after T-cell therapy is common[7-10]. In contrast to T cells, NK cells (defined by the $CD3^-/CD56^+$ phenotype) are capable of lysing virally infected cells or tumor cells without prior sensitization and are not restricted by variations in human leukocyte antigens (HLA). In clinical trials at the University of Minnesota, dramatic anti-tumor effects have been seen with IL-2 activated NK cells that are adoptively transferred into patients with refractory leukemia[48]. Importantly, clinical efficacy in these patients correlated with *in vivo* NK cell persistence and expansion. However, there has been less of a response in a Phase II trial of NK cell infusions in ovarian cancer patients[110]. Although the approach is promising, limitations have been identified. Unlike treatment of leukemia, there was limited persistence and no *in vivo* expansion of intravenously delivered NK cells in ovarian cancer patients. Here, we investigated the hypothesis that the mode of NK cell delivery contributes to the lack of persistence and expansion

experienced clinically when allogeneic NK cells were delivered IV. We developed a mouse ovarian cancer xenograft model to determine if the route of NK cell delivery may be a major obstacle in obtaining clinical responses in ovarian cancer. We found that IP delivery of NK cells lead to stable engraftment, expansion, and antitumor response. These data provide novel evidence for the ability of IP-delivered NK cells to not only inhibit tumor growth but to persist *in vivo* and traffic to the periphery and secondary lymphoid organs. The present findings are expected to stimulate further preclinical studies leading ultimately to clinical validation of NK cell immunotherapy in trials with the potential to impact clinical treatment in ovarian cancer.

Materials and Methods

Generation of firefly luciferase/GFP$^+$ ovarian cancer cells

K562 and OVCAR-3 cells were obtained from American Type Culture Collection (ATCC). The ovarian cancer cell line MA-148 cells were kindly provided by Sundaram Ramakrishnan (University of Minnesota). Luciferase and GFP expressing MA-148 cells were generated using a bicistronic pKT2 *Sleeping Beauty* cassette[16]. Approximately 5 x 10^5 MA-148 cells were nucleofected with 1 μg of pKT2 plasmid containing a GFP:zeocin fusion protein and firefly luciferase as well as 1 μg of SB100X transposase using the 4D-Nucleofector^{TM} system (Lonza). Cells were passaged in zeocin-containing media and sorted using a FACSAria (BD Biosciences).

Cells and Mice

Peripheral blood mononuclear cells (PBMCs) were isolated from 3-5 hour lymphapheresis products drawn from normal donors on the day prior to cell infusion. Mononuclear cells were first isolated from apheresis products through density gradient centrifugation. NK cells were enriched by depleting CD3$^+$ and CD19$^+$ using magnetic beads (Miltenyi Biotec, Auburn, CA). Use of PBMCs from donors was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota. Following CD3/CD19 depletion, cells were activated overnight with 100 Units/ml interleukin 2 (IL-2, Chiron). Cells were then harvested and injected intraperitoneally into mice (Day 0). Five days prior (Day -5) to NK cell injection, NOD/SCID/γc$^{-/-}$ (NSG) mice were sub-lethally

irradiated (225 cGy) and xenografted with firefly luciferase expressing MA-148 tumor cells (Day -4). After allowing the tumors to engraft for four days, mice were given 20 x 10^6 NK cells. Mice then received IP injections of IL-15 and IL-2 as indicated. Prior to NK cell injection (day -1) and on days 7, 14, 21, 40 and 53, mice were analyzed for presence of tumor cells by BLI using the Xenogen IVIS Imaging system (Caliper Life Science, Hopkinton, MA).

Antibodies and flow cytometry. The following antibodies were used: PE-conjugated CD11a, CXCR4, CD62L, CCR5, CXCR3, and CCR7 were obtained from Biolegend. CD45-PE, CD56-APC, CD16-PercpCy5.5, CD117-PercpCy5.5, from Becton Dickson. CD158a/h-PE, CD158j-PE, CD158i-PE, CD158e1/e2, CD159a-PE and -APC were obtained from Beckman Coulter. Flow cytometry was done on a BD FACS Calibur or LSRII and data analyzed using FlowJo (Treestar).

In vitro cytotoxicity. Tumor targets (K562, OVCAR3, and MA-148) were incubated with ^{51}Chromium for 1 hour at 37°C, washed three times and co-cultured with NK cells at indicated effector to target (E:T) ratios. Total lysis (maximal release) was achieved using 5% Triton-X 100. After a period of 4 hours, cells were harvested and analyzed. Specific ^{51}Cr lysis was calculated using the equation: % specific lysis =

100 x (test release - spontaneous release) / (maximal release - spontaneous release).

Engraftment analysis

On days 7, 14, and 21 mice were retro-orbitally bled for evaluation of NK cell engraftment. 100 µl of blood was collected, lysed, blocked with 0.5% human AB serum (Valley Biomedical), and stained for the presence of surface antigens present on human NK cells. At day 54 post-NK cell injection mice were sacrificed to evaluate intraperitoneal tumor burden as well as NK cell engraftment in the spleen and peritoneum. As above, cells were blocked and stained for the presence of various surface markers present on NK cells. Peritoneal washes were also evaluated for the presence of MA-148 tumor cells, which express GFP.

***Ex vivo* expansion of engrafted splenic NK cells**

At the time of sacrifice, each spleen was split in half; one half was analyzed by flow cytometry and the other half was expanded *ex vivo* using artificial antigen presenting cells. The engraftment of human NK cells within the spleen was determined by flow cytometry gating on $CD56^{+}CD45^{+}$ cells within a lymphocyte gate. This percentage was then applied to the absolute count of cells recovered from the spleen sample for expansion. Each individual sample was then stimulated with aAPCs expressing membrane bound IL-21 at a ratio of 2:1 (2 aAPCs:1 NK cell), which is known to markedly expand human NK cells ex vivo[82]. Cells were fed every 3 days as previously described and re-stimulated

with aAPCs every 7 days. Following 2 weeks of expansion cells were tested against K562 and MA-148 targets in a standard chromium release assay.

Statistical analysis. Differences between groups were compared using a paired Student's t-test analysis in Prism 4 (GraphPad Software, San Diego, CA). Results were considered significant at *P* values of 0.05 or less.

RESULTS

Intraperitoneal delivery of allogeneic peripheral blood NK cells for treatment of ovarian cancer

Peripheral blood NK cells can be easily enriched from allogeneic donors for adoptive immunotherapy. This has been successfully been utilized for therapy in refractory AML[48], however, NK cell immunotherapy has been less effective in solid tumors[78,111]. We hypothesized that deficiencies of adoptively transferred NK cells *in vivo* was not due to their inability to kill ovarian cancer cells, but instead their inability to effectively traffic from the circulation to the tumor site. We first confirmed the ability of activated donor allogeneic PB-NK cells to kill ovarian cancer cells *in vitro*. Donor PB-NK cells were isolated and prepared as described (Materials and Methods) (Figure 4.1). We found that both resting and activated PB-NK cells were able to kill the NK cell sensitive target K562 at similar levels, however, IL-2 activated PB-NK cells performed significantly better against the ovarian cancer targets MA-148 and OVCAR3 (Figure 4.1). This is most likely due to upregulation of several surface effector molecules (CD16, NKp46, NKG2A/CD94, KIR, NKG2D, perforin) following cytokine stimulation, as has been demonstrated in other studies[112-114]. Since the NK cells are capable of recognizing and killing ovarian tumor targets *in vitro*, we hypothesized that delivery of anti-tumor lymphocytes directly to the site of tumor origin would overcome the defects in NK cell trafficking into the peritoneum. Ovarian cancer provides an optimal model to study this as we can position both the tumor targets and effector NK cells in murine recipients similar to the metastatic

disease found in humans. Additionally, although ovarian cancer is a lethal disease with a poor prognosis, its distribution is usually limited to the peritoneal cavity even at disease recurrence as the primary route of spread is direct seeding throughout the peritoneal cavity. Even though IP delivery of NK cells directly to the peritoneum appeared as the logical next step in treatment of this disease, it has never been demonstrated that direct delivery of NK cells to the peritoneum would be capable of engraftment and expansion at this site.

Intraperitoneal delivery of NK cells leads to stable engraftment, expansion, and antitumor response

We next tested whether or not IP delivery of PB-NK cells had any benefit over IV delivery. Our main focus was to measure levels of engraftment within the periphery to verify that IP delivery of NK cells would provide circulating anti-tumor effectors. Interestingly, we found that cells given IP had similar frequencies of circulating NK cells as those delivered IV (Supplementary Figure 4.1). We also found high frequencies of NK cells in the spleen and bone marrow weeks following delivery (Supplementary Figure 4.1). This remains important as although ovarian cancer is most commonly limited to the peritoneal cavity, IP delivered NK cells capable of circulating to the periphery and secondary lymphoid organs could be capable of controlling metastatic disease.

Although IP delivery of human NK cells leads to stable engraftment, we aimed to demonstrate that this was an effective means to clear intraperitoneal tumor burden. To test this in an *in vivo* model, we stably modified MA-148 ovarian

tumor cells with a *Sleeping Beauty* cassette carrying both GFP and firefly luciferase reporter genes (Supplementary Figure 4.2). We then devised a model based on our previous clinical experience for adoptive transfer of NK cells to patients with ovarian cancer. Following surgical debridement patients usually undergo paclitaxel and carboplatin chemotherapy[108]. NK cells are then given to clear residual disease. Initially, we engrafted NOD/SCID/$\gamma c^{-/-}$ (NSG) immunodeficient mice IP with 200,000 luciferase^{+} MA-148 cells and allowed them to engraft for 4 days. Mice then received 20 million PB-NK cells IP, as above. Tumor burden was followed using bioluminescence imaging. Two weeks post-NK cell delivery we could see marked tumor reduction in mice receiving IP NK cells compared to tumor only controls (Figure 4.2). Each mouse inoculated with tumor but not receiving NK cells exhibited tumor growth whereas those mice receiving NK cells with IL-2 had significantly decreased tumor burden. Given these data, we conclude that IP delivery of NK cells is an effective method for anti-tumor therapy leading to both stable engraftment and disease regression.

IP delivery of NK cells persist in vivo while maintaining a mature phenotype

We followed the mice for disease progression and peripheral engraftment for approximately 8 weeks. At day 54 we sacrificed the mice to evaluate NK cell engraftment within the peritoneum and organs. After mice were sacrificed, we performed peritoneal washings using sterile PBS to recover any free-floating

cells (lymphocytes and/or tumor cells). In mice receiving NK cells there were high numbers of NK cells within the peritoneal cavity at time of sacrifice with no notable differences between groups receiving IL-2 or IL-2/IL-15 combination. However, the mice receiving IL-15 alone had lower frequencies of NK cells (Figure 4.3). This finding is most likely due to the fact that the dose of IL-15 used is sub therapeutic for long-term persistence and expansion. In these experiments we used a dose of 100 ng/mouse, however, more recent studies are using between 2 to 5 µg/mouse to show successful *in vivo* expansion[115]. Although sub-therapeutic for expansion, the injection of IL-15 with or without IL-2 led to maintenance of CD56 expression on cells in both the peritoneum and the spleen, where IL-2 only mice had lower CD56 expression (Figure 4.3). It is likely that IL-2 induced hyperactivation of NK cells leads to down-regulation of CD56, however, the functional consequence of this down-regulation is not known[116,117]. Studies have previously shown *in vitro* that IL-15 is required for maintenance of CD56 expression[118]. Reciprocally, mice receiving IL-2 treatment vs. IL-15 only treatment had higher levels of KIR and NKG2A, indicating a more functional subset of NK cells with anti-tumor activity. As we demonstrate later, lack of CD56 expression can be restored following ex vivo expansion. While we still observed high numbers of NK cells in the mice there remained signs of localized tumor burden on bioluminescence imaging and at necropsy (Supplementary Figure 4.3). Bioluminescent imaging of each group prior to sacrifice (day 53) demonstrated significantly reduced tumor burden in the mice receiving PB-NK cells with IL-2 or IL-15 alone. The combination of IL-2/15

showed no significant differences to the tumor only group (Supplementary Figure 4.3). At necropsy, we found that IP tumor burden in mice receiving NK cells and IL-2 was significantly reduced to the level of the negative control. Whereas the tumor only mice had high levels of peritoneal tumor burden (34.3% ± 8.43 GFP^+ MA-148 tumor cells), NK cell treated mice had markedly reduced levels, with the IL-2 only group having 0.375% ± 0.144, the IL-2/IL-15 combination group having 3.33% ± 1.59, and the IL-15 only group having 19.11% ± 9.17, likely due to a lower frequency of NK cells (Figure 4.4). The poor response in mice receiving a combination of cytokines could result in competition of IL-2 and IL-15 for similar receptors on the surface of the NK cell. These data strongly support the hypothesis that IP delivery of NK cells is effective at clearing ovarian cancer. It remains possible that IP injected NK cells are fully capable of clearing ovarian cancer cells but cannot traffic to extra-peritoneal sites because of their activated phenotype following IP injection of cytokines.

Persisting NK cells remain functional and have markers of maturation

Although the NK cells present had markers indicating their functionality (KIR, NKG2A), we could not rule out the possibility that persisting NK cells were hypo-responsive. To test this, we aimed to test the function of NK cells *ex vivo*. First, we had to overcome the low yield and absolute numerical differences between mice in order to test function *ex vivo*. Recently, several groups have utilized artificial antigen present cells (aAPCs) to expand both NK cells and T

cells[82]. By calculating the percentage of human NK cells in each mouse spleen, samples were stimulated with aAPCs at a 2:1 ratio as previously demonstrated[82]. We found that this is an effective method to expand NK cells for further *in vitro* testing. Samples from each mouse, although varied in their initial starting cell number, expanded at an equal rate to levels far exceeding our expectations (Figure 4.5). On average, each sample expanded approximately 100-fold. This allowed for further phenotypic and functional testing. Although the input cells varied in their phenotype, we found that expansion with aAPCs restored NK cells to baseline level demonstrating high levels of CD56 expression as well as KIR and CD16. These data strongly support the *in vivo* phenotype and functionality of the persisting NK cells. We proceeded to test these cultures against both K562 and MA-148 tumor targets using a standard chromium cytotoxicity assay and found that each sample was effective at killing both targets similar to, or higher, than their pre-infusion levels (Figure 4.5). These findings further support our hypothesis that *in vivo* functionality of human NK cells can be maintained and they do not become exhausted, yet are only effective “on-site” and are limited in their ability to traffic when activated. These results have profound clinical implications as current therapies using NK cells in the adoptive transfer setting are currently given IV with intermittent IL-2 administration to maintain and expand the cells in vivo[48,78,110,119].

Given the long-term persistence of functional NK cells, we tested the expanded cells for markers of maturation. After 2 weeks of expansion, we found about one

third of the NK cells expressed the activating receptor NKG2C with a smaller proportion expressing the terminal maturation marker CD57 and CD158b (Figure 4.5). Nearly all of the NK cells expressed the inhibitory receptor NKG2A. The above markers are characteristic of mature NK cells and in conjunction with our functional data support the idea that NK cells, commonly thought of as short-lived, innate lymphocytes, could serve as a promising anti-ovarian cancer treatment when delivered IP.

Discussion

Current standard therapies for recurrent ovarian cancer provide a dismal response rate[108]. Thus there remains a critical need for novel therapeutic strategies, as most women with relapsed ovarian cancer will die of progressive disease. Our study focuses on using an immunotherapeutic strategy, specifically harnessing the ability of natural killer cells, to kill ovarian cancer cells[110,119]. Although NK cells are active against ovarian tumors *in vitro*, their activity *in vivo* has been limited[110]. Lack of *in vivo* activity can be correlated to several clinical findings. First, to date, clinical trials have been unable to achieve successful NK cell expansion *in vivo* in an ovarian cancer population[110,119]. Using doses of IL-2 sufficient to expand adoptively transferred NK cells in AML fails to expand NK cells in ovarian cancer patients. The reasons for lack of expansion may be multifactorial. One hurdle is that the preparative regimen used in the ovarian cancer trials does not allow for sufficient “cleared space” for successful expansion of adoptively transferred

lymphocytes. Alternatively, following lymphodepleting chemotherapy there is a high level of expansion of T regulatory cells (Tregs). The Tregs are of recipient origin and further support the insufficient level of immunosuppression preceding adoptive transfer. Tregs are capable of outcompeting NK cells for the exogenous IL-2 through their constitutive expression of the high affinity IL-2 receptor. It is also known that immunosuppressive cytokines such as TGF-β are high in ovarian cancer patients, which may further lead to the development and expansion of Tregs[120-122]. TGF-β also blocks the antitumor activity of NK cells in ovarian cancer[122].

It is unknown whether the traditional IV route of delivery of adoptively transferred NK cells is optimal. There is little known about the natural biology of NK cell trafficking and it is unlikely that NK cells administered IV receive the proper signals to traffic into the peritoneum of ovarian cancer patients. We know from previous clinical experience that IV administered adoptively transferred NK cells has not been effective to date[110]. Because NK cells are highly effective at killing ovarian cancer cells *in vitro*[109], we therefore reasoned that NK cells maintain the intrinsic capacity to kill ovarian cancer cells *in vivo*, but face limitations such as poor expansion and trafficking to the tumor site. Our studies sought to overcome these limitations by testing the ability of IP injected NK cells to treat ovarian cancer. In comparing the differences between IV vs IP PB-NK cells, we were surprised to find that delivery of lymphocytes IP led to similar levels of engraftment in all organs tested, including peritoneum, peripheral blood, spleen, and bone marrow. This indicates that adoptively transferred NK

cells are not only capable of expansion *in vivo,* but also maintain the ability to traffic to extraperitoneal sites. Once cells are in the lungs it is possible they don't have the proper receptors to traffic across the venous endothelium to the arterial circulation. NK cell delivered IP could have direct access the lymphatic system allowing entrance into circulation. By using a xenograft model of ovarian cancer, we were able to show the activity of IP transferred NK cells. NK cells injected directly into the peritoneum of mice with previously established ovarian tumors were able to limit disease compared to non-injected controls (Figures 4.2 and 4.4). Most surprisingly, at time of necropsy, mice receiving IP delivered NK cells in combination with IL-2 had no peritoneal disease, indicating potent and sustained activity of IP delivered NK cells. Mice not receiving NK cells had marked ascites and tumor burden as indicated by necropsy and bioluminescent imaging.

Lack of complete tumor clearance did not appear to be due to intrinsic inability of the NK cells to recognize and kill tumor targets. Following *ex vivo* expansion, adoptively transferred NK cells recovered high levels of CD56 expression as well as the effector molecules CD16 and KIR. These are the first studies to demonstrate this type of persistence for adoptively transferred NK cells within an *in vivo* tumor model. We believe that in opposition to the current dogma, NK cells can be long lived as recent studies in mice, and humans, have recently suggested[123-125]. We tested whether NK cells in this system had markers of persistence and found that they indeed expressed markers of terminally differentiated NK cells[126]. These include expression of CD57, NKG2C, and

CD158b. Although these data do not functionally support the potential for NK cell memory in this model, it would be interesting to see if NK cells could be manipulated to gain this function in patients to treat diseases with high rates of recurrence, such as ovarian cancer. It is also possible that an *ex vivo* expansion model could work in patients in which NK cells could be recovered and expanded *ex vivo* for re-infusion, thereby increasing the effector to target ratio until a patient is cleared of their disease.

Collectively, these data provide evidence for advancing clinical trials in women with ovarian cancer. We believe that IP delivery of NK cells provides an optimal platform of immunotherapy in this disease. As patients today often are treated with IP chemotherapy, this seems the next logical step. By overcoming the inability to expand adoptively transferred lymphocytes in this setting, direct delivery of effector cells to the tumor site has the potential to provide more rapid clinical responses. Additionally, we have shown the ability of NK cells to traffic to extraperitoneal sites, here demonstrating the similarities in engraftment between IV and IP administration of NK cells. Although ovarian cancer is a lethal disease with poor prognosis, its distribution is usually limited to the boundaries of the peritoneal cavity even in later disease stages. Our findings beg the question of whether patients would also benefit from IP delivery of exogenous cytokines such as IL-2. The recent advancement of IL-15 also holds intense promise for such an approach. Particularly in a disease such as ovarian cancer where there is massive expansion of host Tregs following adoptive transfer of allogeneic NK cells, IL-15 would be solely utilized by adoptively

transferred NK cells while not expanding Tregs[127,128]. Other strategies that influence NK cell trafficking may be used as well. Although we have shown that IP delivery of NK cells still allows for peripheral engraftment, blocking lymphocyte egress from the peritoneum would theoretically allow NK cells to persist intraperitoneally in higher percentages, optimizing anti-tumor response. Clinical trials have shown reagents such as sphingosine-1-phosphate receptor modulators could be a promising avenue to this approach[129].

These data provide evidence in support of a readily translatable approach to immunotherapy with much promise. The above studies provide the basis for the development of further strategies to manipulate the NK cell product, host and targets with the ultimate goal of enhancing the therapeutic benefit of NK cell based immunotherapy while minimizing the risks and toxicities for women with ovarian cancer.

Figure 4.1. Activity of allogeneic NK cells against ovarian cancer targets. **A)** Representative example of the post-CD19 and CD3 depletion product for infusion. Following depletion with CD3 and CD19 magnetic beads, there are minimal $CD3^+$ T cells or $CD19^+$ B cells with high percentages of $CD56^+$ NK cells and $CD14^+$ monocytes. **B)** Allogeneic PB-NK cells are active following CD3/19 depletion. Overnight resting (B0, squares with solid lines) and activated (B1000, triangles with dashed lines) NK cells were tested for their cytotoxicity against the leukemia target K562 or the ovarian cancer cell lines MA-148 and OVCAR3.

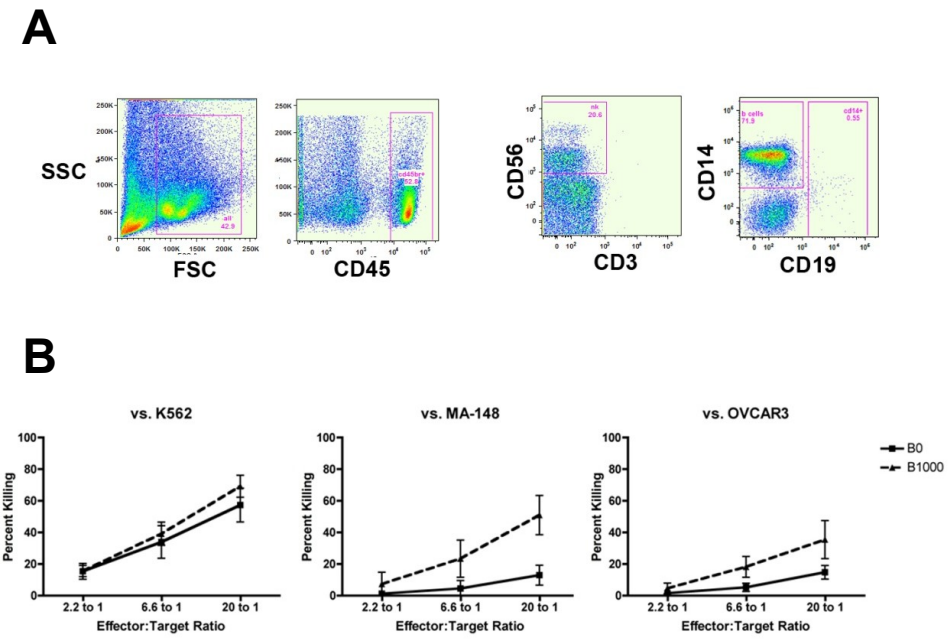
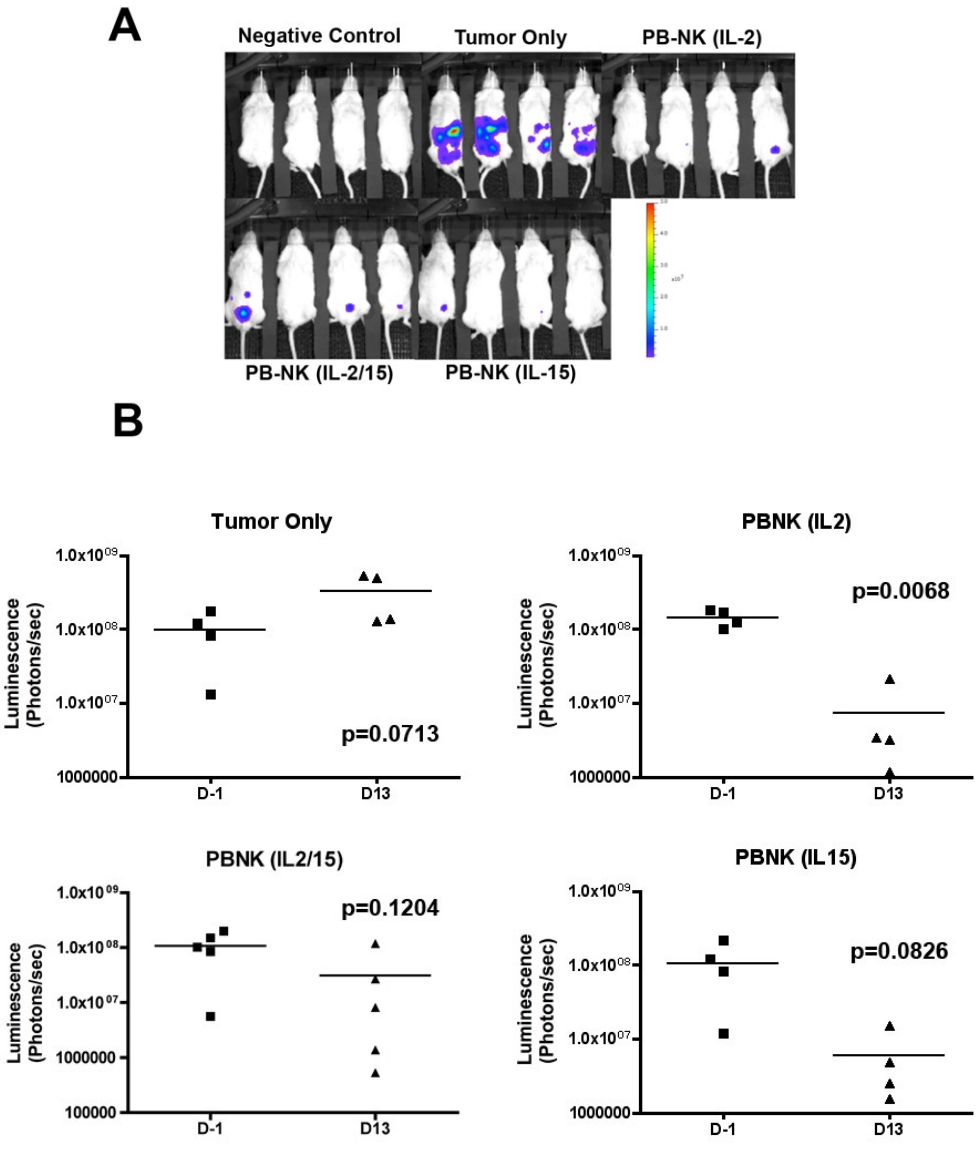


Figure 4.2. Intraperitoneal delivery of NK cells leads to decreases in tumor burden. A) Tumor burden was measured by bioluminescent imaging (BLI) 13 days following infusion of NK cells. **B)** Quantification of BLI signals demonstrates that the **Tumor Only** group experienced an increase in tumor burden. Only the **PB-NK (IL-2)** treatment group demonstrated a significant reduction in tumor signal ($p= 0.0068$) at day 13 vs day -1. The reductions in signal in the **PB-NK (IL-15)** and **PB-NK (IL-2/15)** groups were non-significant ($p= 0.0826$ and $p= 0.1204$, respectively). Differences within groups were determined using a paired Student's t-test.


A
Negative Control
Tumor Only
PB-NK (IL-2)
PB-NK (IL-2/15)
PB-NK (IL-15)
B
Tumor Only
PBNK (IL2)
PBNK (IL2/15)
PBNK (IL15)
Luminescence (Photons/sec)
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1.0x10^08
1.0x10^07
1000000
100000
D-1
D13
p=0.0713
p=0.0068
p=0.1204
p=0.0826

Figure 4.3. Engraftment analysis at day 54 post-NK cell injection. At time of sacrifice, mice were analyzed for engraftment of NK cells within the peritoneal cavity and spleen. Human NK cells were identified by their expression of CD56 and CD45. $CD56^{+}$ cells were further analyzed for the expression of killer immunoglobulin like receptors (KIR) and NKG2A.

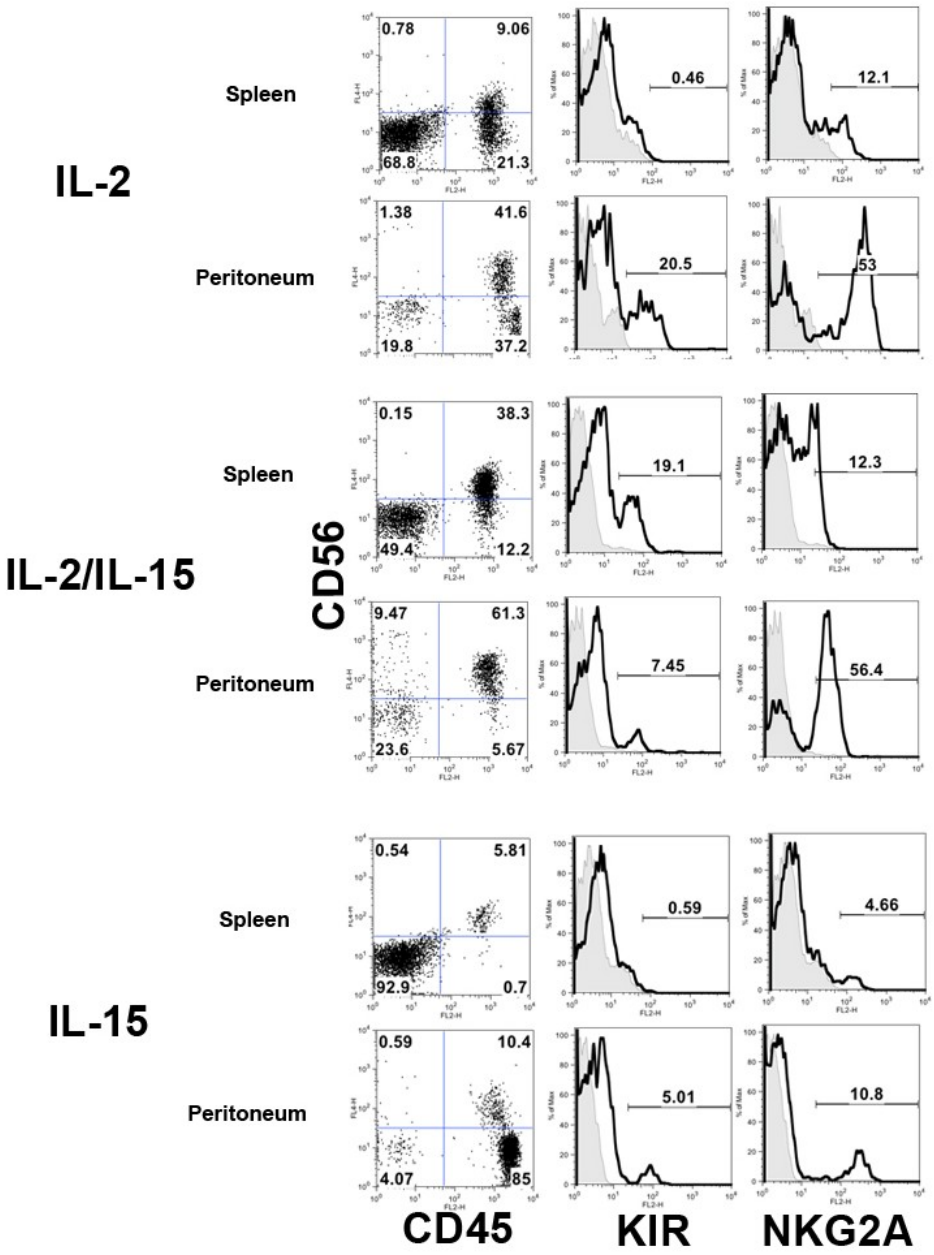

IL-2
Spleen
0.78
9.06
68.8
21.3
0.46
12.1
Peritoneum
1.38
41.6
19.8
37.2
20.5
53
IL-2/IL-15
Spleen
0.15
38.3
49.4
12.2
19.1
12.3
Peritoneum
9.47
61.3
23.6
5.67
7.45
56.4
CD56
IL-15
Spleen
0.54
5.81
92.9
0.7
0.59
4.66
Peritoneum
0.59
10.4
4.07
85
5.01
10.8
CD45
KIR
NKG2A

Figure 4.4. Peritoneal tumor burden at day 54 post-NK cell injection. A) At time of sacrifice, mice were analyzed for tumor burden within the peritoneal cavity. Peritoneal washes were performed using sterile PBS. Cells were then analyzed by flow cytometry for presence of GFP^+ MA-148 tumor cells. Representative plots of each treatment group shown with quantification in **B).** Differences between groups were analyzed using a student's t-test.

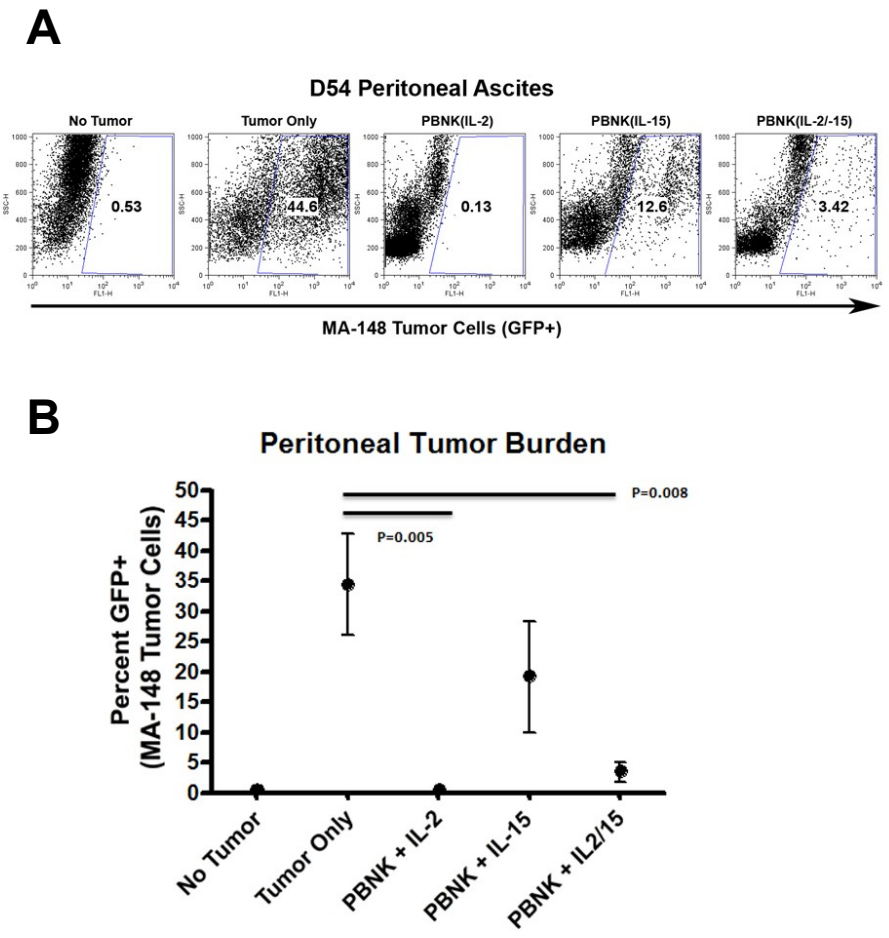
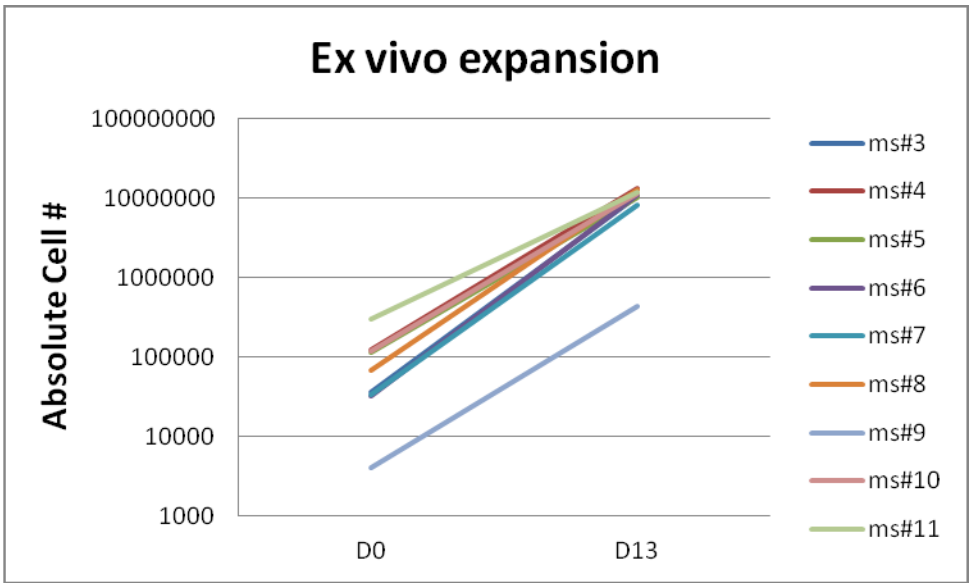


Figure 4.5. *Ex vivo* phenotype, expansion and function of splenic NK cells. **A)** Following analysis of splenic NK cell engraftment, cells were stimulated with aAPCs expressing mbIL-21. After 13 days of expansion (2 stimulations) each mouse sample was quantified and analyzed. **B)** Representative flow cytometry plots from two individual mice are shown. Following 13 days of expansion there were numerous $CD56^+$ NK cells. Gating on $CD56^+$ cells demonstrates high co-expression of CD16 and KIR without any human $CD3^+$ T cells. Dark black lines in histogram represent the individual stains compared to an unstained control (shaded gray). **C)** Each expanded line was then tested for their ability to kill K562 or MA-148 cells in a chromium release cytotoxicity assay.

A


Ex vivo expansion
Absolute Cell #
100000000
10000000
1000000
100000
10000
1000
D0
D13
ms#3
ms#4
ms#5
ms#6
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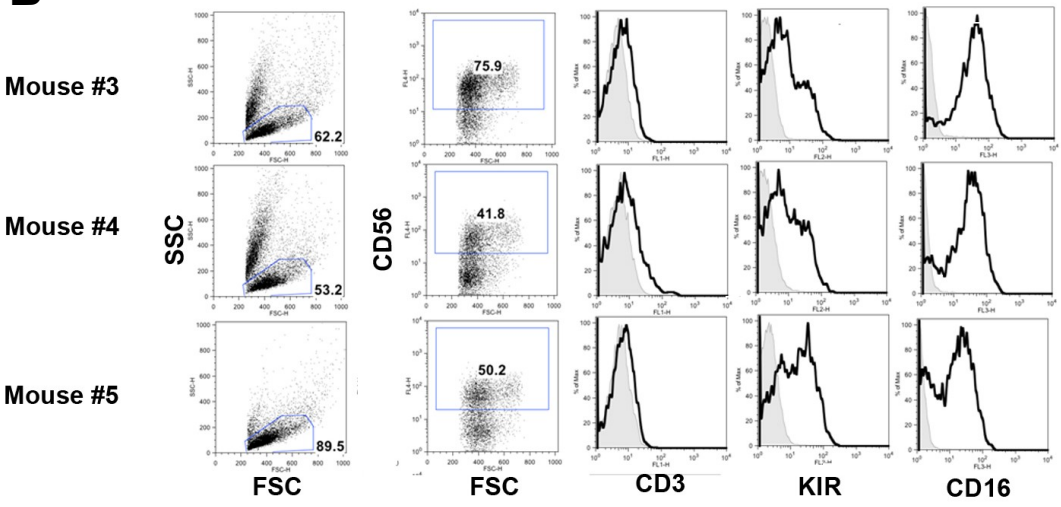
ms#9
ms#10
ms#11

B


Mouse #3
Mouse #4
Mouse #5
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53.2
89.5
CD56
FSC
75.9
41.8
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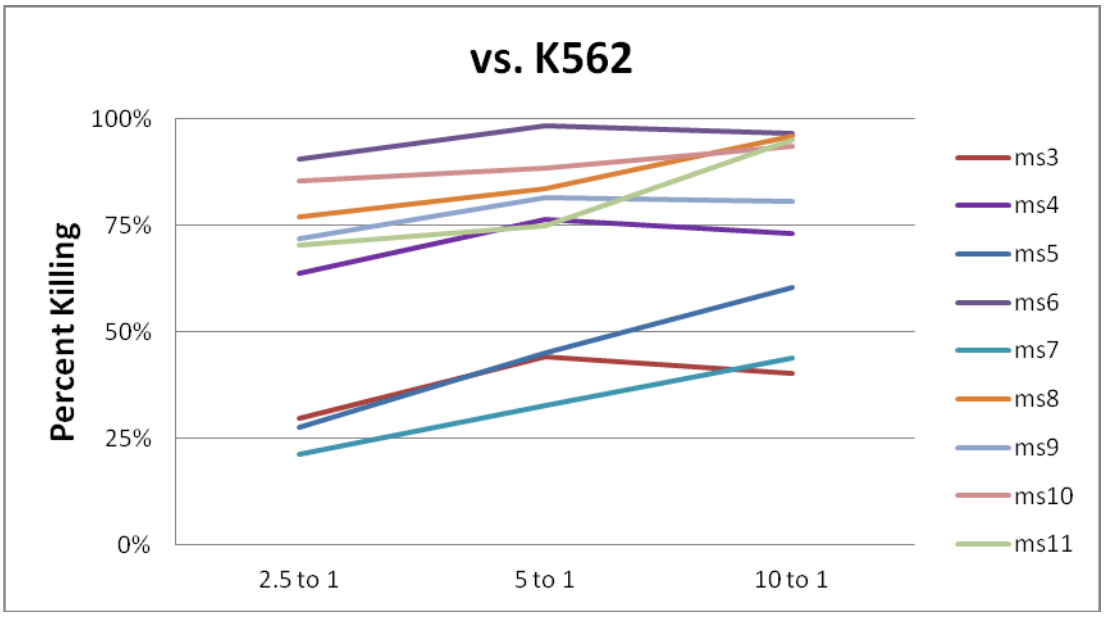
KIR
CD16

C


vs. K562
Percent Killing
100%
75%
50%
25%
0%
2.5 to 1
5 to 1
10 to 1
ms3
ms4
ms5
ms6
ms7
ms8
ms9
ms10
ms11

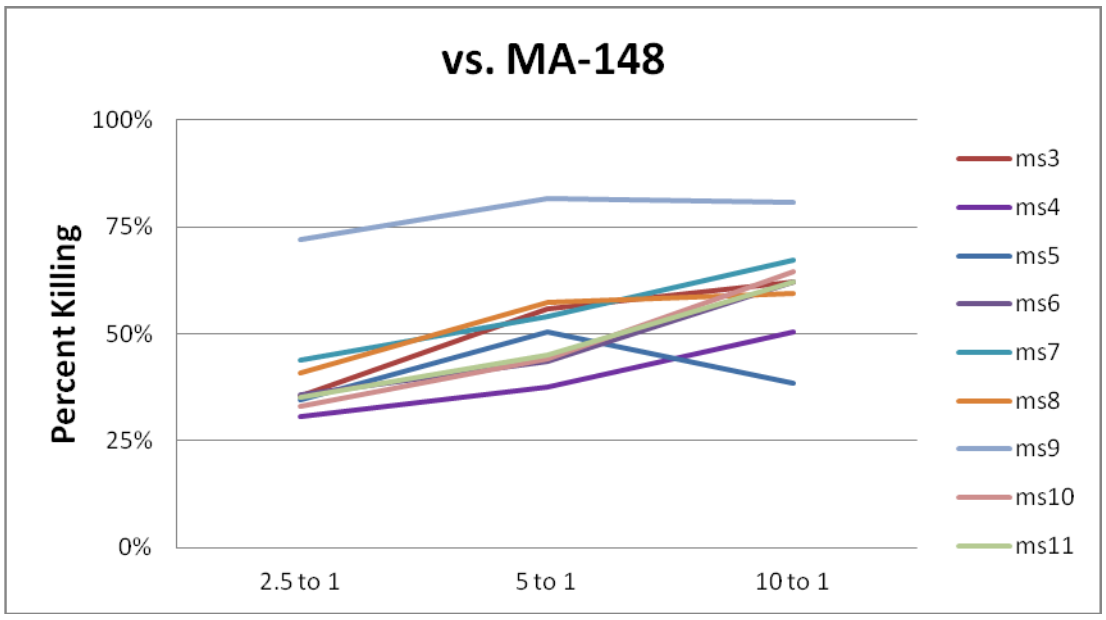
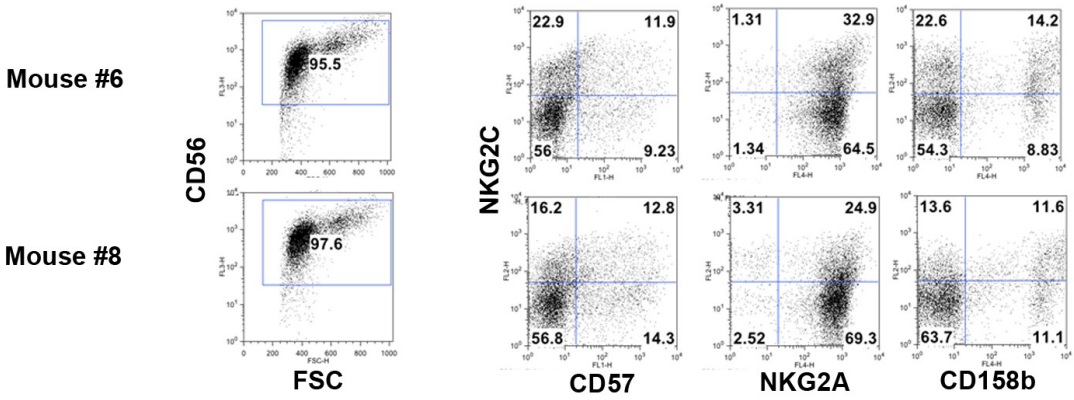
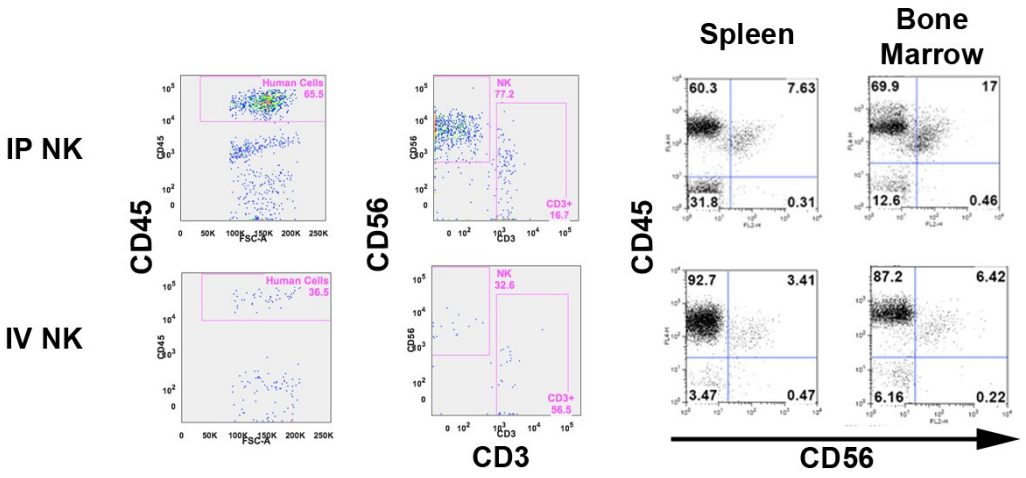

vs. MA-148
Percent Killing
100%
75%
50%
25%
0%
2.5 to 1
5 to 1
10 to 1
ms3
ms4
ms5
ms6
ms7
ms8
ms9
ms10
ms11

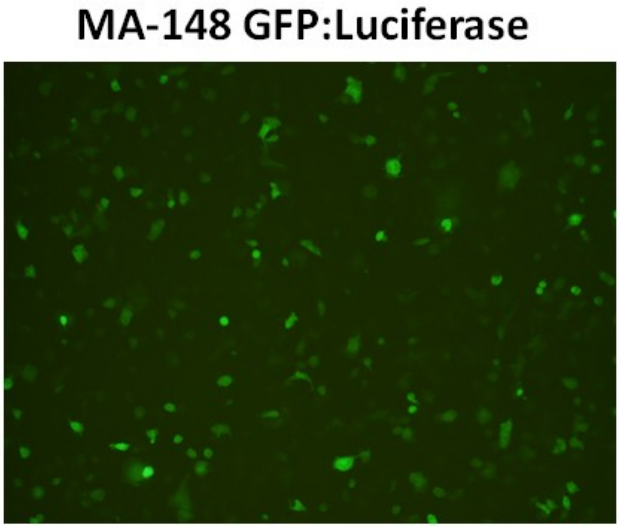
Figure 4.6. Long lived, persistent NK cells have markers of maturity. Following expansion, NK cells recovered from the spleen were measured for markers of maturity. Representative mice demonstrate that each group demonstrates high levels of mature NK cells markers. $CD56^{+}$ NK cells were analyzed for co-expression of the markers NKG2C, CD57, NKG2A, and CD158b.



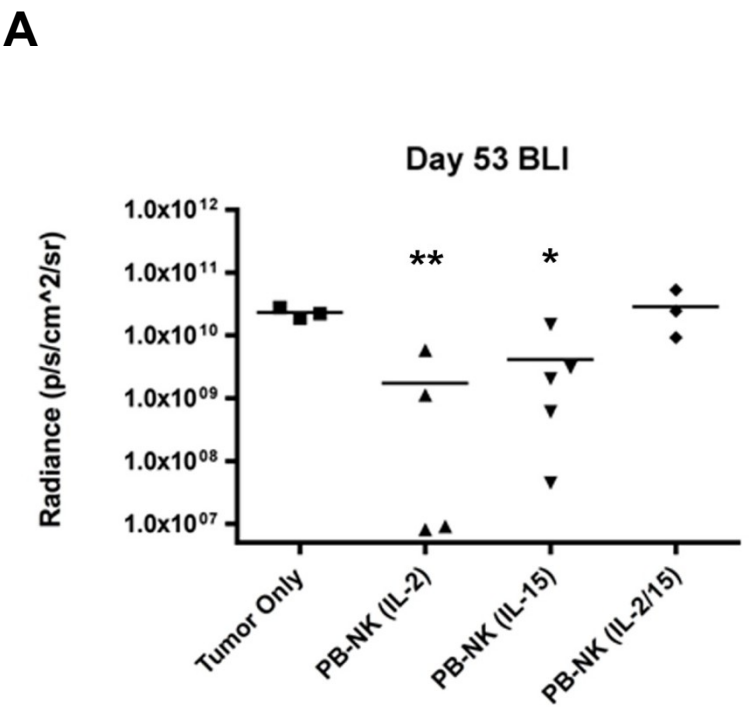
Supplementary Figure 4.1. Comparison of the engraftment between IP and IV delivered NK cells in peripheral blood, spleen, and bone marrow. At D17, a separate group of tumor bearing mice were sacrificed in order to quantify the difference in engraftment between IP and IV injected cells. IP and IV injected cells demonstrated similar frequencies of engraftment in the peripheral blood, spleen, and bone marrow.



Supplementary Figure 4.2. MA-148 tumor cells modified with a *Sleeping Beauty* GFP:Luciferase reporter. Stably modified MA-148 cells examined for expression of GFP by fluorescence microscopy.



Supplementary Figure 4.3. Residual tumor burden analyzed by bioluminescent imaging correlates with necropsy photos at time of sacrifice. A) Bioluminescent imaging quantified at D53 shows significant reduction of tumor burden in mice treated with NK cells compared to tumor only mice. PB-NK (IL-2) group p=0.0006 (**), PB-NK (IL-15) p=0.0038 (*), PB-NK (IL-2/15) p=0.6841. **B)** Bioluminescent imaging at day 53 demonstrates residual tumor in each group of mice. Necropsy photos indicated most of the tumor burden is within subcutaneous, circumscribed tumors. **C)** Correlation with bioluminescence imaging with necropsy findings of a liver metastasis is demonstrated from a mouse in the PB-NK (IL-2/15) group.



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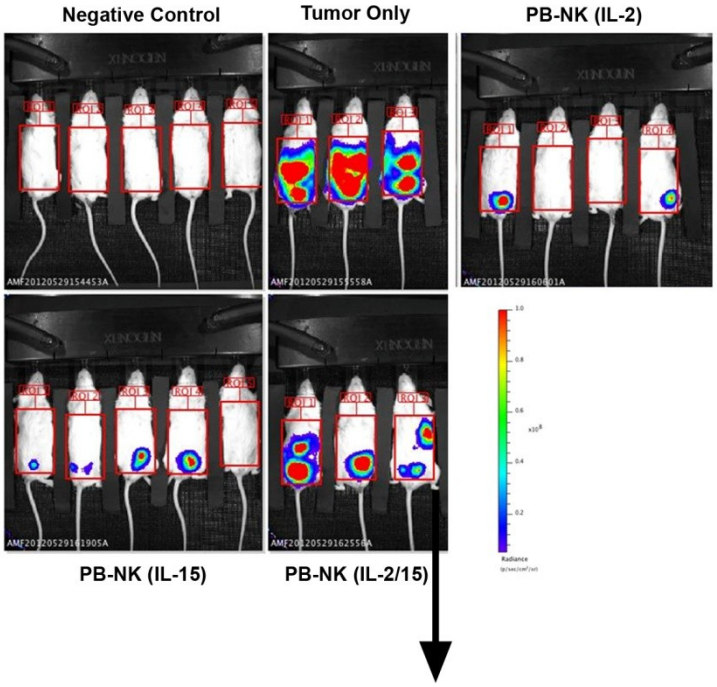

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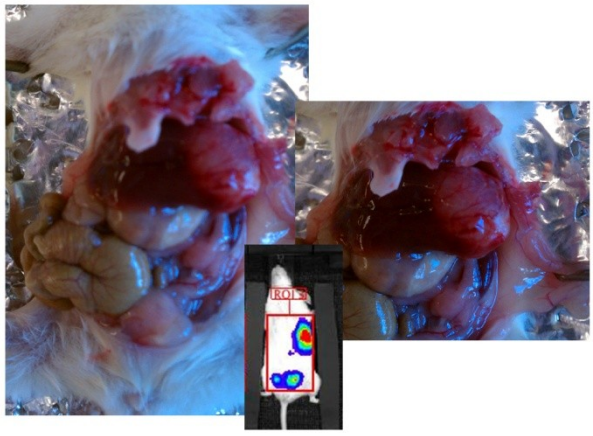


CHAPTER 5

Chimeric antigen receptor modified human pluripotent stem cells as a platform for directed, universal adoptive immunotherapy

The use of chimeric antigen receptors (CARs) to redirect anti-tumor lymphocytes for refractory malignancies has gained increased interest following recent promising clinical reports. However, few groups have focused on making this therapy more feasible to clinical centers without large cell processing facilities. Having a cellular product that doesn't require production on a patient specific basis would be ideal. To overcome this, we have used hESCs and iPSCs as a platform for CAR based immunotherapeutics. Here we demonstrate the feasibility of expressing CARs in both hESCs and iPSCs to derive pure populations of cells that homogeneously express the receptor of interest. Expression of CARs in hESCs and iPSCs did not affect their pluripotent state or their ability to generate high frequencies of hematopoietic progenitor cells. By differentiating these cells into mature natural killer cells, we demonstrate non-altered development of lymphocytes with anti-tumor activity. CAR-expressing NK cells were able to recognize and kill tumors in an antigen specific manner both in vitro and in vivo. These data support the use of human pluripotent stem cells for CAR based therapies, providing a characterized and universal source of anti-tumor lymphocytes for adoptive transfer immunotherapy.

INTRODUCTION

Human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), provide immense promise for regenerative medicine[2,9,28,130]. hPSCs are capable of differentiating into all three germ layers, including mesoderm and subsequent blood cell development[10]. Although little advancement has been made on the generation of an engraftable hematopoietic stem cell (HSC) from hPSCs[17,43,131], much progress has been made on the development of more differentiated blood lineages[7,9,15,132-136]. We have previously demonstrated efficient development of mature natural killer (NK) cells from both hESCs and iPSCs[58,80]. Natural killer cells are lymphocytes of the innate immune system with potent anti-tumor function, and as opposed to T cells, act in an MHC-independent fashion. The derivation of NK cells from hPSCs in sufficient numbers for clinical translation is one of the most prominent examples of the potential of using these cells in clinical trials. However, the current use of NK cells for adoptive immunotherapy using peripheral blood NK (PB-NK) cells has only been successful in the setting of AML[48,78]. Considering NK cell therapies can be used across a wider range of donors (because of their HLA-independent function) it is of interest to broaden this therapy to more tumor types. Because little is known about the natural biology driving NK cell activity against different tumor types, it is not yet possible to maximize NK cell activity based on endogenous receptor-ligand interactions. We performed proof-of-principle experiments aimed at redirecting hESC-derived natural killer cells against ovarian cancer using CARs.

Chimeric antigen receptors are based on recombinant technology and the fusion of two natural technologies of B and T cells within the adaptive immune system[93,137,138]. By fusing the epitope specific portion of an antibody, the single-chain variable fragment (scFv), to the intracellular signaling domains of a T cell receptor, Esshar and colleagues[137] first demonstrated the feasibility of this approach. Although CAR-redirected T cells showed enhanced function *in vitro*, they had limited *in vivo* clinical activity[139]. Following this, Sadelain and colleagues hypothesized that given the requirement for additional signals needed to activate T cells optimally *in vivo*, fusing more T cell signaling domains on to the intracellular portion would enhance activity *in vivo*[140,141]. These modifications have led to impressive clinical trials in CLL in which patients' autologous T cells modified with anti-CD19 specific receptors led to significant decreases in tumor burden as well as persistence and expansion in vivo without need for prior conditioning[90,142].

Although the derivation of CAR-modified T cells on a patient specific basis may be ideal, it remains a prohibitive therapy until technologies in lentiviral production and gene transfer become more practical and available. While it seems that retroviral or lentiviral constructs, which integrate into the host genome, are not as promiscuous as was found with HSC gene therapy[143-145], there is always concern for insertional mutagenesis. Additionally, limiting this modification to effector cells such as T cells may not be optimal either, as presence of the endogenous T cell receptor (TCR) in these cells could lead to

lethal side effects or autoimmunity. Recently, Cooper and colleagues have overcome this approach in hope to making this a more universal approach[91].

We aimed at using CAR based therapies from hESCs and iPSCs for several reasons. First, hPSCs provide a genetically tractable platform in which 100% of the cells express the transgene of interest. Although successful in clinical trials, there is a very low level of transgene expression (~5%) in human primary T cells due to poor transduction[90]. Improving this could increase the therapeutic effect and allow a much smaller number of cells to be used. Next, the integration of CAR constructs into bulk CD3+ T cells, including CD4 and CD8 subsets, is random and the function of each of these not well characterized. Using hPSCs, in which targeted integration is readily available, would allow intense characterization and safely validation prior to infusing into patients.

The following experiments demonstrate the feasibility of modifying human pluripotent stem cells with CARs for adoptive immunotherapy. CAR-modified cells were able to maintain their pluripotent state, differentiated in to their desired effectors cells, and were functional in an antigen-specific manner.

Materials and Methods

Human pluripotent stem cell culture maintenance and modification with chimeric antigen receptors

TypLE adapted hESCs were maintained on low-density (90,000 cells/well of a 6 well plate) mouse embryonic fibroblasts (MEF)[83]. Both the anti-mesothelin[66] and anti-CD19[146] CARs were PCR amplified from previously published lentiviral constructs on the pELNS backbone. The anti-mesothelin CAR was PCR amplified using the following primers: 5'-CATACAGAATTCATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCC-3', and 5'-CATACAGAATTCTTAGCGAGGGGGCAGGGCCTGCATGTGAAGGGCGTCGTA-3'. The anti-CD19 CAR was amplified with the following primers: 5'-CATACAGAATTCATGGCCTTACCAGTGACCGCCTTGCT-3', and 5'- CATACAGAATTCTTAGCGAGGGGGCAGGGCCTGCAT-3'. EcoRI sites are underlined. Each was then digested and subcloned in to an EcoR1 digested pKT2-mCAGs-IRES-GFP:zeo or pKT2-mCAGs-IRES-puro construct containing an EcoRI splice junction between the mCAGs promoter and the internal ribosomal entry site (IRES). Orientation was confirmed by restriction enzyme digest of sites within the distal ends of the subcloned CAR sequences. Functional constructs were then nucleofected into NK-92 cells or hESCs using a Lonza 4D-nucleofector device.

Differentiation of hPSCs into hematopoietic progenitor cells and NK cells

Generation of hematopoietic progenitor cells expressing CARs was performed as previously described[83]. Adapted hESC lines were set up as spin EBs weekly to maintain continuous cultures. For analysis, spin EBs were collected in a 15 mL conical tube (BD Falcon) following 11 days of differentiation in media contain stem cell factor (SCF), vascular endothelial growth factor (VEGF), and BMP4 (bone morphogenic protein 4). Spin EBs were allowed to fall to the bottom of the tube and the supernatant was set aside. Spin EBs were then trypsinized (0.5% Trypsin, Gibco) to remove remaining hematopoietic cells from inside the EB. Trypsinization was stopped as soon as the spin EBs started to flatten out and was never allowed to progress for more than 5 minutes duration. For NK cell differentiation, whole spin EBs were transferred to uncoated 24-well plates in media and cytokines we have previously described[58]. Following 4 weeks of NK cell culture, cells were phenotyped by flow cytometry and tested in vitro or in vivo. For in vivo studies, NK cells were further expanded using artificial antigen presenting cells (aAPCs, kindly provided by Dr. Dean A. Lee)[82].

Cell lines

K562, Raji, Daudi, and OVCAR-3 cells were obtained from ATCC. K562.meso cells were kindly provided by Dr. Carmine Carpenito. TurboFP650 expressing MA-148 cells were generated using a pKT2 *Sleeping Beauty* cassette previously developed in our lab[58,59]. Cells were sorted by FACS (FACSAria, BD Biosciences).

Cytotoxicity assays

Tumor targets (K562, K562.meso, Raji, Daudi, OVCAR3, and MA-148) were incubated with ^{51}Chromium for 1 hour at 37°C, washed three times and co-cultured with NK cells at indicated effector to target (E:T) ratios. After a period of 4 hours, cells were harvested and analyzed. Specific ^{51}Cr lysis was calculated using the equation: % specific lysis =100 x (test release - spontaneous release) / (maximal release - spontaneous release).

Antibodies and flow cytometry.

The following antibodies were used: CD34-APC, CD45-PE, CD43-PE, NKp46-PE, CD56-APC, NKG2D-PE, CD16-PercpCy5.5, all from Becton Dickson. CD158a/h-PE, CD158j-PE, CD158i-PE, CD158e1/e2, CD159a-PE and -APC were obtained from Beckman Coulter. Flow cytometry was done on a BD FACS Calibur and data analyzed using FlowJo (Treestar).

***In vivo* xenograft model**

NOD/SCID/$\gamma c^{-/-}$ (NSG) mice were sub-lethally irradiated (225 cGy) and xenografted with TurboFP650 expressing MA-148 tumor cells (Day -4). After allowing the tumors to engraft for four days, mice were given 10 x 10^6 NK cells intraperitoneally (IP). Mice then received IP injections of IL-15 and IL-2 as indicated. Prior to NK cell injection (day -1) and on days 7, 14, 21, 40 and 54, mice were analyzed for presence of tumor cells by BLI using the Xenogen IVIS Imaging system (Caliper Life Science, Hopkinton, MA). On days 7, 14, and 21

mice bled by facial vein for evaluation of NK cell engraftment. 100 µl of blood was collected, lysed, blocked with human AB serum, and stained for human CD56 and CD45 antigens.

RESULTS

Modification of NK cells with chimeric antigen receptors (CARs) enhances their cytotoxicity

Our first aim was to test the function of anti-mesothelin and anti-CD19 CARs in NK cells. A few reports have demonstrated the ability of CARs to function and redirect NK cells[147-149], but given the unknown function of the costimulatory domains with our CARs (CD28, 4-1BB, and CD3ζ) within NK cells, we first tested their function in the NK cell line NK-92. NK-92 cells are a malignant large granular lymphocyte cell line derived from a patient and have several similarities with NK cells[150]. They also function to kill tumor cells and have been utilized as a cellular therapeutic in clinical trials. We modified NK-92 cells with our *Sleeping Beauty* constructs containing either anti-CD19 or anti-mesothelin CARs. The constructs also contain a GFP;zeocin fusion protein which allows drug selection as well as monitoring of CAR expression by flow cytometry. NK-92 cells can be readily modified with both the anti-CD19 and anti-mesothelin CARs (Figure 5.1). Addition of CARs to NK-92 cells dramatically enhanced their ability to kill tumor targets in an antigen specific manner. Normally, NK-92 cells have a low level of cytotoxicity against the NK cell sensitive target K562 and no killing against the B cell targets Raji or Daudi. Addition of the anti-CD19 or anti-

mesothelin CARs did not affect the ability of NK-92 cells to kill K562 targets. However, when K562 cells overexpressing the antigen mesothelin were tested, the anti-mesothelin CAR-expressing NK-92 cells showed significantly higher killing (Figure 5.1). Vice versa, when tested against the B cell targets which express CD19, neither the native nor anti-mesothelin CAR-expressing NK-92 cells were able to kill, whereas the anti-CD19 CAR-expressing NK-92 cells showed significantly enhanced levels of killing. These results suggest that CARs containing the intracellular signaling domains CD28, 4-1BB, and CD3ζ function in NK cells and we therefore hypothesized they would function in hESC-derived NK cells.

Generation of hESCs and iPSCs stably expressing CARs

hESCs and iPSCs are widely amenable to a variety of overexpressed transgenes without affecting their pluripotent state[59,151]. We hypothesized that because neither of the antigens recognized by our CARs (CD19 or mesothelin) are expressed on hPSCs, there would be no inadvertent activation of the stem cells. We have relied on the *Sleeping Beauty* system to reliably and easily modify hPSCs[59]. As opposed to retro- or lentiviral methods, use of a transposon-based system is more clinically feasible[152]. As we have previously shown with other transgenes[59], hESCs and iPSCs can be stably modified to express CARs against CD19 or mesothelin while maintaining their pluripotent state, here measured by expression of SSEA-4 (Figure 5.2). Next, we tested whether or not CAR-modified hESCs and iPSCs could differentiate into

hematopoietic progenitor cells using a spin-EB approach[83]. Briefly, hPSCs (+/- CARs) were dissociated and plated at 3000 cells/well of a 96-well plate containing the cytokines stem cell factor (SCF), vascular endothelial growth factor (VEGF), and bone morphogenic protein 4 (BMP-4). These conditions reliably drive mesoderm and subsequent hematopoietic cell development from both hESCs and iPSCs[56]. As we have previously shown, this method gives rise to high frequencies of progenitor cells expressing the progenitor marker CD34 as well as CD43 and CD45. The presence of either the anti-CD19 or anti-mesothelin CAR during development did not affect the differentiation process, as all progenitor cells demonstrated high levels of CD34, CD43, and CD45 expression (Figure 5.2).

Generation of hESC-derived, antigen specific NK cells directed against mesothelin$^+$ tumors

We then transferred the cells from the stage I spin EBs into conditions supporting NK cell development. This is a culture system containing only the NK cell promoting cytokines IL-15, IL-3, IL-7, SCF, and FLT3L, which we have shown is an effective method to derive functional NK cells from hESCs and iPSCs without the use of any murine feeder layers. Transfer of hematopoietic progenitor cells expressing the anti-CD19 or anti-mesothelin CARs to NK cell conditions gave rise to phenotypically mature NK cells after four weeks culture (Figure 5.3). Surprisingly, the presence of the CARs during development did not affect their ability to generate mature, educated NK cells, given there was no

differences in the expression of killer immunoglobulin-like receptors (KIR), NKp46, or NKG2A (Figure 5.3). Also, 100% of the NK cells maintained expression of the CARs. As we have previously demonstrated, this two-stage, feeder-free method gives rise to high numbers of NK cells that can also be further expanded using reagents such as aAPCs[82]. Therefore, production of functional NK cells modified with CARs from hESCs and iPSCs could provide an off-the-shelf therapeutic ready for clinical translation in the not too distant future.

hESC- and iPSC-derived NK cells expressing CARs function in an antigen specific manner

Although the CAR-expressing NK cells had a normal phenotype by flow cytometry ($CD56^+/KIR^+/CD16^+$), it was unclear whether or not the presence of CAR could adversely affect the function of NK cells. NK cell recognition and killing of target cells is normally driven through the interaction of receptors expressed on the surface of NK cells and respective ligands on tumor cells[38]. As we have shown in the NK-92 cells, hESC-derived NK cells expressing chimeric antigen receptors can be redirected to kill tumor cells in an antigen specific manner. Specifically, whereas both hESC-derived NK cells and hESC-derived NK cells expressing the anti-mesothelin CAR where capable of killing the NK cell sensitive target K562, addition of the mesothelin antigen to the K562 targets increase their susceptibility to killing by hESC-derived NK cells expressing the anti-mesothelin CAR (Figure 5.3).

DISCUSSION

Following promising clinical trials there has been intense interest in using CAR-based therapies to treat a variety of malignancies[90]. Although the optimal platform for this type of therapy has not been established, several groups are testing different effector populations. There are also clinical trials being conducted to test the method of gene delivery (retroviral versus lentiviral vectors). It also remains unclear whether or not CAR-based therapies will be effective across a wide variety of malignancies[153]. Cancers overexpressing CD19 as the target antigen are most likely optimal because of the high level of CD19 expression as well as the limited tissue distribution. The hypogammaglobulinemia caused by depletion of $CD19^+$ B cells can easily be treated. NK cells, which act through a complement of germline encoded activating and inhibitory receptors, maintain activity even in the absence of CAR expression (Figure 5.3). As we and others have demonstrated, CARs can function in NK cells. Even though the signaling domains used for a majority of CARs have been optimized for T cells, it is likely that the similarities of NK cells with CD8 T cells allows these domains to function in NK cells[154]. Other possibilities include the addition of cytokines to enhance survival (IL-2, IL-15) or chemokine receptors to enhance trafficking to tumor sites.

Having a platform for universal delivery of CAR-based therapies would be optimal. One group has done this in T cells by also knocking down the endogenous T cell receptor using zinc finger nucleases (ZFNs). This would

prevent any aberrant activity of the TCR should it recognize normal host tissues leading to graft-vs-host disease. However, this type of modification is still done on a patient-specific basis and in order to select enough transduced T cells containing the CAR the cells need to be expanded *ex vivo*. *Ex vivo* expansion of lymphocytes leads to exhaustion, thereby limiting their *in vivo* efficacy[45]. Although newer strategies of *in vitro* expansion have aimed to circumvent some of these concerns[82], it is not known how this affects effector cell survival in patients. Stable modification of hESCs or iPSCs would provide an HLA-typed, and characterized, source of cells for immunotherapy. Also, the level of transgene expression that can be achieved using this method is far superior to that achieved by transducing primary T cells or NK cells.

One concern of modifying hESCs and iPSCs with CARs was the effect it may have on pluripotency (cells capable of differentiating into all three germ layers). As we have previously found, overexpression of a wide variety of transgenes in hESCs and iPSCs does not appreciably affect pluripotency[59]. Here, we found that hESCs could be stably modified with CARs and maintained a pluripotent state as measured by their expression of the surface marker SSEA-4 (Figure 5.2). Although this is not a true test for pluripotency, it is sufficient for our proof-of-principle experiments. Although we were confident that we could overexpress CARs in hESCs, it was unclear what effect the CARs would have on either the hematopoietic progenitor cell or NK cell differentiations. We hypothesized that since the target antigens (mesothelin or CD19) are not expressed through the differentiation process[155], CAR expression would have no effect on

hematopoietic or NK cell development. We found that presence of CARs did not affect the ability of hESCs to differentiate into blood progenitor cells or NK cells (Figure 5.2). Although we did not formally test whether or not crosslinking of the CAR through addition of exogenous antigen would adversely alter the differentiation, we would propose this would have the most effect on NK cell differentiation as this process is highly influence by the activating and inhibitory signals received throughout development[154]. This would be important if one was to consider the use of other CARs that have antigens that come up in the NK cell developmental process, such as CD33 or other markers of progenitors, including CD117. If the presence of antigens did negatively affect development, one could consider the use of inducible vectors that would only allow the drug to be expressed at specific cellular developmental stages[151]. This may also be useful *in vivo* as it would allow the clinician to alter the level of CAR expression based on the clinical response while reducing the adverse side effects of CAR expression as is seen in the studies using the CD19 CAR which lead to long term B cell aplasia and hypogammaglobulinemia[90].

Following stable expression of CARs in hESC-derived NK cells, we tested whether or not they could be redirected against refractory tumor targets in an antigen-specific manner. In vitro, hESC-derived NK cells were more cytotoxic against tumor cells overexpressing the tumor antigen of interest, while the non-CAR expressing NK cells still maintain a certain level of cytotoxicity (Figure 3). As opposed to T cells, hESC-derived NK cells also express high levels of the

effector molecules KIR and CD16. KIR could have both positive and negative effects on NK cells expressing CARs. In conjunction with the activating receptors, CARs could potentiate the activity of NK cells by synergizing with activating receptors recognizing stress ligands in parallel. Alternatively, it is possible that the presence of inhibitory KIR on the NK cells could negatively affect intracellular signaling domains of the CAR through the phosphatase activity of the inhibitory KIR receptors. This could be avoided clinically through concurrent use of antibodies directed against inhibitory KIR, which are currently being tested clinically. If using hESC- or iPSC-derived NK cells, one could also engineer the NK cells with zinc finger nucleases (ZFNs) or TAL-effector nucleases (TALENs) to knockout the KIR locus following development, as has been accomplished with the TCR in combination with CAR expression[91]. As with T cells, this would have to be done following the development of mature NK cells, which rely on KIR receptors for proper acquisition of effector functions. Overall, these studies provide an additional platform for the generation of tumor specific lymphocytes for adoptive immunotherapy. Modification of hESCs and iPSCs with CARs could provide a universal platform for cellular therapeutics.

Figure 5.1. Modification of NK cells with CARs generates tumor specific NK cells. A) Stable expression of anti-CD19 and anti-mesothelin CARs in the NK cell line NK-92. Cells were stably modified with a *Sleeping Beuaty* construct containing the anti-CD19 or anti-mesothelin CARs and a GFP:zeocin fusion protein. Stable integration was accomplished by co-injection of the plasmid containing the transposase SB100x. **B)** Expression of the anti-mesothelin or anti-CD19 CARs in the NK cell line NK-92 demonstrates enhanced tumor-specific activity of NK cells containing the CAR. Cells were tested against various $CD19^{+}$ (Raji, Daudi) or $mesothelin^{+}$ (K562.meso) targets in a standard 4hr cytotoxicity assay.

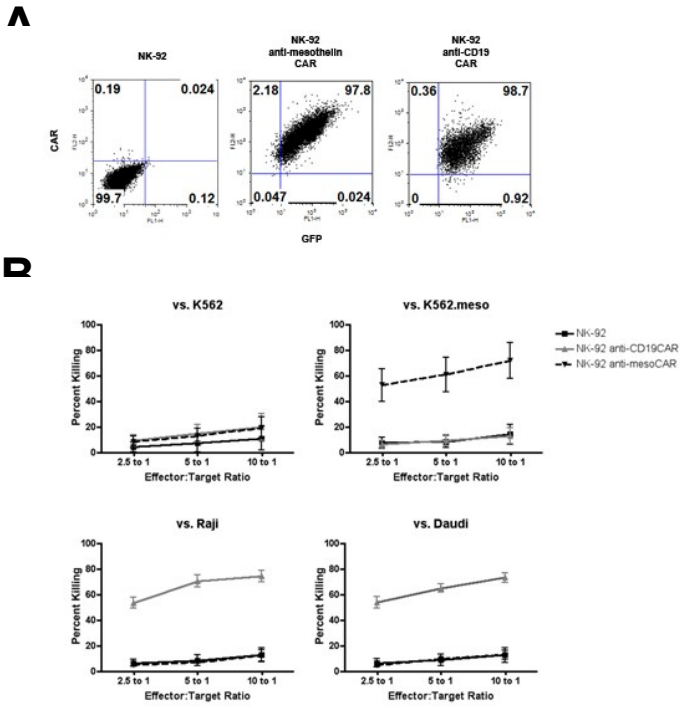
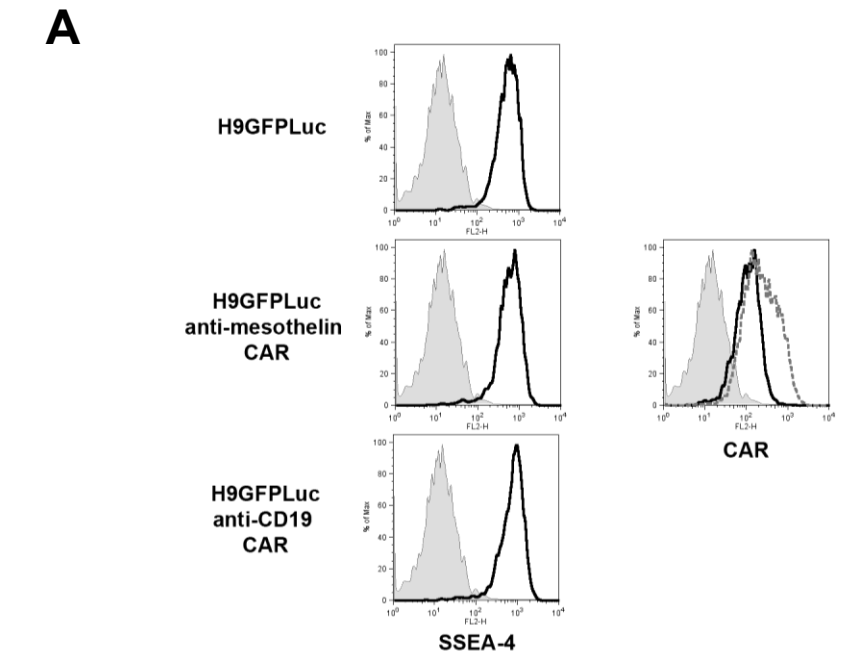
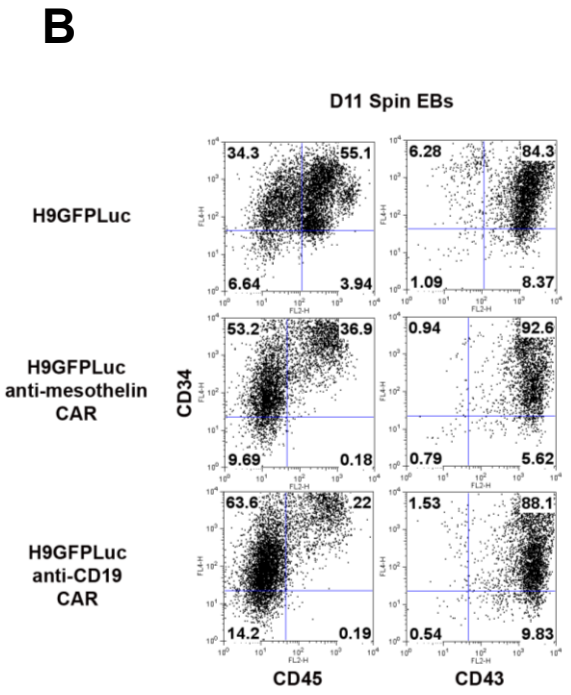


Figure 5.2. Stable expression of CARs in hESCs. A) Stable expression of CARs in hESCs. The H9GPFluc^{+} line was modified with anti-CD19 or anti-mesothelin CARs. Stable integration and expression of both CARs did not affect the pluripotent state of these cells, as each line maintained high levels of SSEA-4 expression. Staining for the anti-CD19 CAR (gray dashed line) and anti-mesothelin CAR (solid black line) is shown compared to an isotype control. **B)** hESCs stably expressing CARs were able to differentiate into high percentages of hematopoietic progenitor cells, similar to controls. They expressed high levels of CD34, CD45, and CD43. **C)** Following 11 days of hematopoietic differentiation in spin EB culture, CAR modified hESCs also downregulated SSEA-4, further indicating their ability to differentiate from the pluripotent state.


A
H9GFPLuc
H9GFPLuc anti-mesothelin CAR
H9GFPLuc anti-CD19 CAR
SSEA-4
CAR


B
D11 Spin EBs
H9GFPLuc
H9GFPLuc anti-mesothelin CAR
H9GFPLuc anti-CD19 CAR
CD34
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34.3
55.1
6.64
3.94
6.28
84.3
1.09
8.37
53.2
36.9
9.69
0.18
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92.6
0.79
5.62
63.6
14.2
0.19
1.53
88.1
0.54
9.83

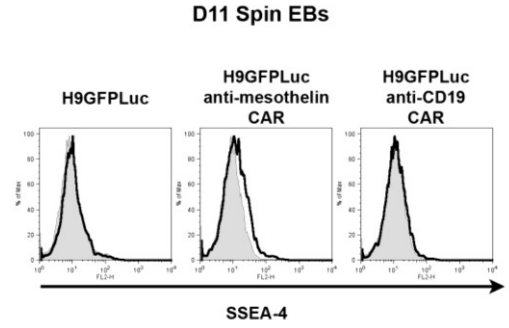
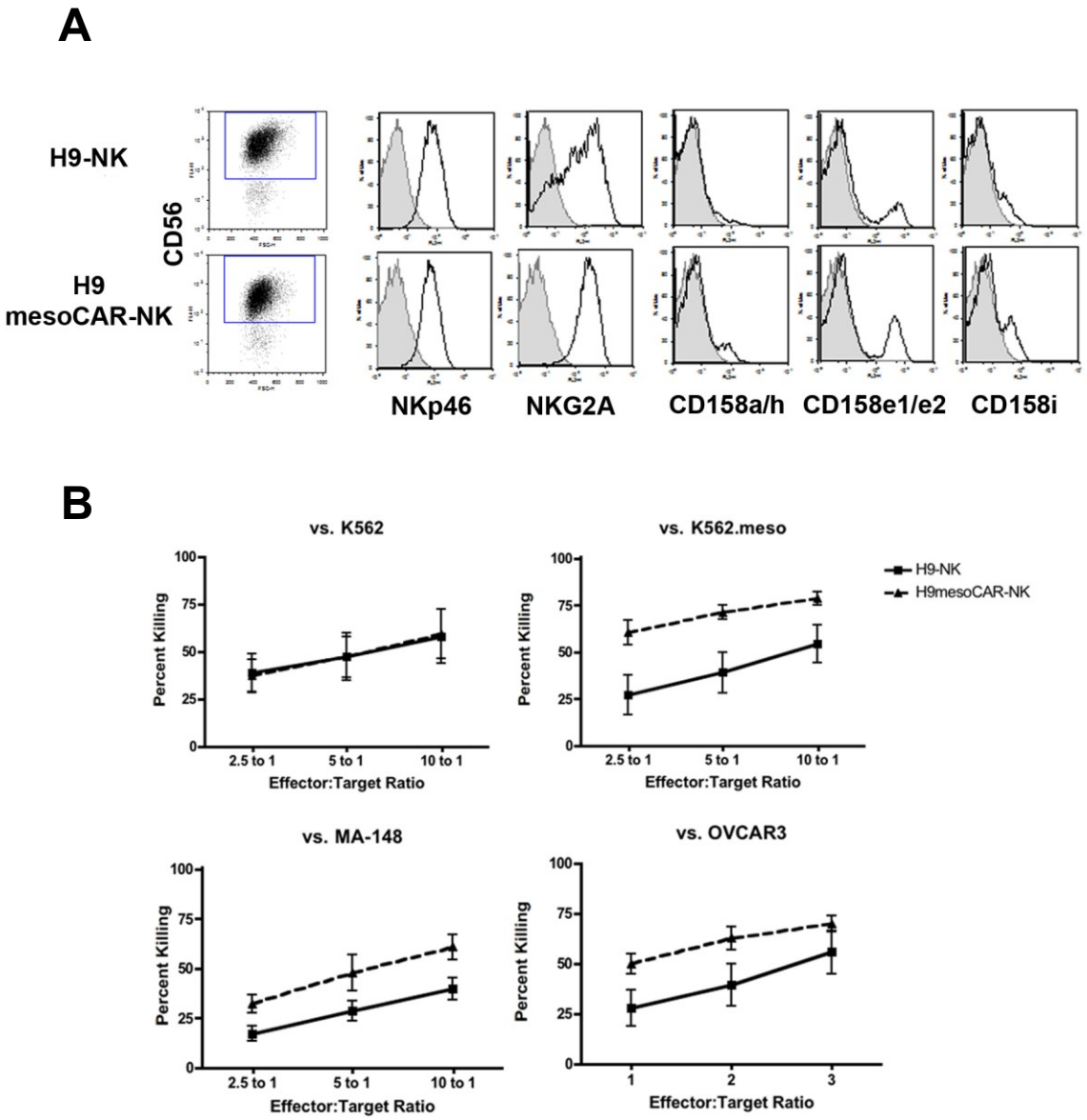

C
D11 Spin EBs
H9GFPLuc
H9GFPLuc anti-mesothelin CAR
H9GFPLuc anti-CD19 CAR
SSEA-4

Figure 5.3. CAR-expressing NK cells have a mature phenotype and demonstrate antigen specific activity. A) CAR^+ hematopoietic progenitor cells differentiate into mature NK cells following 4 weeks culture. Similar to CAR^- control cells, NK cells are generated from both the anti-CD19 and anti-mesothelin CAR lines. They expressed high levels of CD56, NKp46, NKG2A, and KIR. **B)** hESC-derived NK cells containing tumor specific CARs demonstrated enhanced cytotoxicity. Against the NK cell sensitive target K562, both H9-NK cells, and H9-mesoCAR NK cells kill to a similar level. Exposure of both effector populations against the mesothlin expressing tumors K562.meso, OVCAR3, or MA-148 demonstrates the H9-mesoCAR NK cells display enhanced killing.


A
H9-NK
H9
mesoCAR-NK
CD56
NKp46
NKG2A
CD158a/h
CD158e1/e2
CD158i
B
vs. K562
vs. K562.meso
vs. MA-148
vs. OVCAR3
Percent Killing
Effector:Target Ratio
2.5 to 1
5 to 1
10 to 1
H9-NK
H9mesoCAR-NK

CHAPTER 6

Conclusions

Human pluripotent stem cells hold tremendous potential for basic biological studies as well as regenerative medicine. However, a better understanding of how we manipulate these cells to safely get to the desired endpoint remains. These questions face several biological and technical hurdles. For example, recapitulating the normal ontogeny of blood cells is no trivial task. During blood development there are two developmental waves, and much work still needs to be done on describing blood cell development from hPSCs. Notwithstanding, there are several protocols for the derivation of different types of blood cells from hPSCs. Although these types of cells don't provide long-term engraftment they could be used as transient therapies, such as RBCs, platelets, or other cell therapies are utilized from blood banks today. When using such differentiated cells one must consider the regulatory concerns of translating these therapies into patients. Using hESC- and iPSC-derived NK cells as a model system for cancer therapy, we aimed to enhance the development and expansion of these cells for potential clinical use. We first described the similarities of NK cell development and function between hESCs and iPSCs. Although hESCs and iPSCs are similar in several aspects there is a growing body of literature demonstrating differences at both the genetic and epigenetic levels. By demonstrating that iPSC-derived NK cells are similar to hESC-derived NK cells in phenotype and function (Chapter 2), one can think about cellular therapies on a patient specific basis. For the NK cell based therapies described in Chapters 2-6, there is a distinct advantage of using NK cells from allogeneic donors because of how they recognize and kill tumor cells. Using both hESCs and

iPSCs, we have demonstrated the capability to generate enough NK cells to treat a patient from less than a single plate of hPSCs.

Although hPSCs contain promise in regenerative medicine and potentially anti-cancer therapeutics, they are often overlooked for basic biological studies. This is most likely due to the cost and technical details involved in performing hPSC research. hPSCs have the intrinsic capacity for unlimited self renewal as well as multilineage differentiation. This means each line can provide an unlimited source of cells for study from any germ layer. Studies in Chapter 3 used hESC-derived NK cells as a model system to study lymphocyte trafficking in a model of immunotherapy. Because hPSCs provide a tractable system for genetic modification, we modified our cells with a bioluminescent reporter to follow the expansion and biodistribution of hESC-derived NK cells in immunodeficient mice. Because we can also label the tumor cells with a fluorescent reporter, we were able to measure both NK cells and tumor cells in the same mouse. This platform allowed us to discern the optimal kinetics of NK cell trafficking to the tumor site and also further explored a direct role for hESC-derived NK cells in the clearance of human leukemia cells *in vivo*. This model was also able to demonstrate that IP injection of NK cells was optimal for *in vivo* persistence.

We then set to apply the concept of IP adoptive therapy of lymphocytes for cancer. To do this we focused on ovarian cancer (Chapter 4). By delivery human NK cells IP to immunodecificient mice with previously established ovarian cancer we were able to demonstrate tumor reduction. We were also able to demonstrate that IP delivery of NK cells leads to engraftment in the

spleen and peripheral blood at a similar level to IV injected NK cells. Intraperitoneally delivered NK cells were also able to expand *in vivo* in response to cytokines. Importantly, those mice with successful expansion following treatment with IL-2 saw significant reductions in peritoneal tumor burden at 8 weeks. These studies will help direct upcoming clinical trials in which patients with refractory ovarian cancer will receive injections of allogeneic cells directly to the peritoneum, the major site of tumor burden.

Although we feel that IP delivery of NK cells to ovarian cancer patients will lead to objective clinical responses, solid tumors are generally refractory to cell based therapies. To overcome this, we are testing hPSCs as a novel platform for modifying NK cells with tumor specific receptors (Chapter 5). This has several advantages, including targeted genetic modification, non-viral integration, homogenous transgene expression, and ability for extensive preclinical testing. By modifying hESCs with chimeric antigen receptors we demonstrated stable expression without any adverse affects on pluripotency or differentiation into blood progenitor cells and NK cells. Importantly, by modifying hESC-derived NK cells with CARs we were able to show this provides enhanced recognition and killing of refractory targets in an antigen specific manner.

The collective studies described above address major points in the study of natural killer cell development and function from hPSCs. First, the ability to generate therapeutic numbers of mature and cytotoxic lymphocytes from iPSCs is an achievable goal. Next, the ability to generate enough NK cells from hPSCs

in a completely defined system readies this type of therapy for use in clinical trials in the not too distant future. Finally, by providing a genetically amenable system, hPSCs are uniquely suited for the creation of targeted therapies for cancer in which large banks of cells can be made and tailored on a patient specific basis by the clinician.

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