Stabilized CD16a Expression on Pluripotent Stem Cell-Derived Natural Killer Cells

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Abstract

Human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) have been shown to successfully generate functional hematopoietic cells of multiple lineages. One of the cells that these studies have shown is possible to generate are cytotoxic natural killer (NK) cells. These pluripotent stem cell-derived NK cells could potentially be used as a cell-based therapy to treat a wide range of cancers. A potent method through which NK cells kill their targets is antibody-dependent cell-mediated cytotoxicity (ADCC). During this process an Fc gamma receptor, CD16a, recognizes antibody coated target cells and leads to the stimulation of the NK cell's cytotoxic pathways. However, upon CD16a activation, a metalloprotease known as ADAM17 has been shown to cleave CD16a leading to a decrease in NK cell ADCC. This project seeks to generate genetically modified NK cells that have CD16a expression stabilized to maximize ADCC. One method to accomplish this is the generation of a non-cleavable version of CD16a. This cDNA construct has been transfected into iPSCs using *Sleeping Beauty* transposase to stably express CD16a. These cell's CD16a molecules are resistant to cleavage upon cell activation and are also highly expressed on the surface of the NK cells. Preliminary testing has shown that when combined with antibodies specific to their cognate target ligand, these cells have a greater cytolytic response than untransfected peripheral blood NK cells (PBNK). Also, CRISPR-Cas9 has been employed to knock-out ADAM17. Going forward, comparing the cytotoxicity of these two NK cells will show which is best to employ in the treatment of cancers that express ligands for therapeutic antibodies that have proven refractory to other therapies and adjuvants.

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Introduction

The derivation and generation of human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC) have helped to drastically advance the fields of developmental biology and regenerative medicine (Thomson et al., 1998; Takahashi et al., 2007). While these two cell populations are capable of generating mature cells from all three embryonic germ layers, they are obtained in two different methods. hESCs are isolated from the inner cell mass of human blastocysts. iPSCs on the other hand can be generated from mature cells such as fibroblasts. While there are many methods to do this, it is most commonly done by viral transduction of mature cells with vectors that drive expression of four transcription factors (Malik et al., 2013). These include octamer-binding transcription factor 4 (Oct4), SRY-box 2 (SOX2), Krueppel-like factor 4 (Klf4) and cellular myelocytomatosis (c-Myc). In the years since their derivation, scientists have taken advantage of the fact that these cells are capable of differentiating into mature cells of many lineages under the right conditions, facilitating the study of the *in vitro* development for various tissues and cells.

In the field of hematology, hESCs and iPSCs have proved a powerful tool for scientists looking to study the development of hematopoietic cells. For example, Kaufman et al. found that hESCs are capable of giving rise to multiple hematopoietic cell lineages when co-cultured with stromal cells (Kaufman et al., 2001). This study and others like it were modeled on knowledge gained from research into the mechanisms of mouse and human hematopoietic development. Human hematopoiesis starts two to three weeks into embryonic development as blood islands appear in the secondary yolk sac (Migliaccio et al., 1986). One of the hallmarks of this cellular population is the cell surface receptor FLK1 (VEGFR2) (Ema et al., 2003; Park et al., 2004). They are thought to arise from mesoderm cells, termed the hemangioblast, which are able to generate both hematopoietic and endothelial cells (Huber et al., 2004; Kennedy et al., 2007). The blood cells generated in these blood islands are both myeloid and erythroid while lymphoid generation is less well understood (Palis et al., 1999). Studies have suggested that the yolk sac contains cells at this time capable of giving rise to ancestors of the adult

hematopoietic system (Samokhvalov et al., 2007). The human embryo itself also has true HSCs during ontogenesis, which develop in the aorta-gonad-mesonephros (AGM) region, capable of rescuing hematopoiesis in lethally irradiated mice (Ivanovs et al., 2011). This cellular population is thought to arise from the hemogenic endothelium, endothelial cells capable of giving rise to hematopoietic cells (Jaffredo et al., 1998). Certain endothelial cells at this stage exhibit upregulation of hematopoietic markers, become rounded and eventually separate from the endothelium (Bertrand et al., 2010; Lam et al., 2010; Boisset et al., 2010). Later in ontogenesis definitive blood cells and hematopoietic stem cells can be found in the fetal liver (Uchida et al., 2001; Tavian et al., 2005). These HSCs eventually seed the bone marrow and continue to generate blood throughout adult life.

Since Kaufman et al. 2001, there have been advances in *in vitro* human hematopoietic development from hESCs and iPSCs. These have been partly stimulated by the difficulties posed by xenogeneic components to potential cell therapies. Culture systems that do not require xenogeneic stromal co-culture and have defined, humanized conditions have been shown to successfully generate human hematopoietic progenitors (Ng et al., 2005). When generating hematopoietic progenitor cells from ESCs and iPSCs, this system utilizes spin embryoid bodies (EB) which can be used to recapitulate the different niches that mesodermal and hemato-endothelial progenitors develop in during embryonic blood development (Ng et al., 2008). The spin EBs are cultured with media containing cytokines important to early hematopoietic development, such as VEGF which stimulates FLK1, a hallmark of the hemogenic endothelium. These spherical cystic structures contain hematopoietic, endothelial and stromal cells that can support the derivation and proliferation of hematopoietic progenitor cells capable of generating mature hematopoietic cells of both lymphoid and myeloid lineages (Knorr et al., 2013, Ran et al., 2013).

The realization that hESCs and iPSCs can give rise to lymphocytes has led to the establishment of techniques that allow for the derivation of natural killer (NK) cells for use in cell-based therapies to treat cancers (Woll et al., 2005; Knorr et al., 2013, Hermanson et al., 2015). These differentiation protocols involve the use of cytokines to

influence hematopoietic progenitor differentiation into NK cells and have been modeled on *in vivo* human NK cell development. Human NK cell development is a progressive process in which hematopoietic stem cells (HSC) housed in the bone marrow give rise to NK cells through a number of intermediate states (Yu et al., 2014). The first step occurs when HSCs divide into common lymphoid progenitors (CLP) (Kondo et al., 2001). These cells retain the ability to differentiate into all lymphoid cell populations however, over time some of their progeny acquire IL-15 receptor beta (CD122) which is an important step toward committed NK cell differentiation (Blom et al., 2006; Boos et al., 2008; Ikawa et al., 1999). IL-15 promotes NK cell differentiation, maturation and survival (Becknell et al., 2005). The interactions that HSCs and NK precursors have with stromal cells in the bone marrow are also important as they have been shown to provide cytokines such as IL-3, IL-7, FMS-like tyrosine kinase 3 ligand (Flt3L) and c-kit ligand (SCF) (Drexler et al., 2004; Gibson et al., 1995; Lyman et al., 1998; Pillai et al., 2004; Guimond et al., 2010). As these NK cell precursors mature, they acquire functional markers such as CD161, then CD56, CD94/NKG2A, NKG2D and finally KIRs and CD16 (Grzywacz et al., 2006; Perussia et al., 2005). The expression level of CD56 is used to determine the developmental stage of mature NK cells. Those $CD56^{bright}$ NK cells are shown to have lower cytotoxicity capabilities but high cytokine production in response to soluble factors (Cagliuri et al., 2008). As CD56 decreases in expression, the cell's inherent cytotoxicity increases and the ability of the cells to release cytokines in response to target cells predominates (Cagliuri et al., 2008).

In vitro NK cell development has been modeled off of *in vivo* NK cell maturation. These observations have driven the use of defined cytokines to stimulate cells and derive cytotoxic NK cells from hematopoietic progenitor cells. The studies that pioneered this experimental paradigm used purified mouse HSCs and have led to the notion that NK cell development proceeds through two major steps; lineage committed progenitor generation and development of mature cells capable of cytotoxic activity (Colucci et al., 2003). This method was later modified to differentiate human bone marrow HSCs into NK cells using cytokines including c-kit ligand (SCF), Flt3-Ligand

(Flt3L), interleukin-7 (IL-7), IL-3, IL-2 and mouse fetal stromal cells (AFT-024) (Miller et al., 1999). Since then, members of the Kaufman lab modified this protocol to differentiate NK cells from hESC-derived hematopoietic precursors (Woll et al., 2005). However, xenogeneic components within the aforementioned differentiation systems would prohibit effective clinical translation of any NK cell based therapies. It has been demonstrated that the addition of SCF, Flt3L, IL-7 and IL-3 can replace stromal cells and help to generate NK cells from hematopoietic precursors (Mrozek et al., 1996; Williams et al., 1997). Therefore, Knorr et al., 2013 and Hermanson et al., 2015 further modified the NK differentiation protocol to efficiently generate NK cells while excluding all xenogeneic components from hematopoietic and NK differentiation protocols. Once NK cells have been derived, they are exponentially expanded using IL-21 transfected K562 cells (Denman et al., 2012). This cytokine has been shown to promote NK cell proliferation and maturation in the bone marrow (Parrish-Novak et al., 2000).

NK cells are lymphocytes typified by their $CD56^{+}CD3^{-}$ surface expression and are integral members of the innate immune system (Herberman et al., 1981). This is due to the fact that they are capable of lysing transformed and virally infected cells without prior sensitization or genome rearrangement to produce antigen-specific receptors (Yokoyama et al., 2003). Therefore, they have a critical role in tumor surveillance. One of the ways that NK cells survey for tumors is by assessing the relative expression of MHC class I molecules (Karre et al., 1986). During development, these killer-cell immunoglobulin-like receptors (KIRs) ligate to their respective ligands and gain the ability to determine the identity of healthy self-cells (Ljunggren et al., 1990). When an inhibitory subset of KIRs recognizes appropriately expressed self-MHC I molecules, they transduce inhibitory signals through recruitment of protein tyrosine phosphatases preventing NK cell killing of target cells (Romagne et al., 2011; Narni-Mancinelli et al., 2013). Those cells that have non-self MHC I or no MHC I expression do not trigger inhibitory signals within the NK cell through these KIRs allowing for cytotoxicity to proceed (Long et al., 1999; Moretta et al., 2000; Colonna et al., 2000; Yokoyama et al., 1993; Lopez-Botet et al., 2000). NK cells can also use germ-line encoded activating

receptors (natural cytotoxicity receptors: NCRs) such as NKG2D, NKp46 and 2B4 to differentiate between normal and affected cells (Vivier et al., 2012). Each of these receptors recognize an antigen(s) on the surface of target cells that is either not produced by normal cells or is differentially expressed on affected cells (Girardi et al., 2001). It has been seen that cancerous cells, which express appropriate KIR ligands, can be targeted by NK cells due to the expression of these markers (Gras Navarro et al., 2014; Bauer et al., 1999). When activated, many receptors dimerize with transmembrane signaling domains stimulating tyrosine kinase-dependent cell activation (Krzewski et al., 2008). Finally, cytokines such as IL-15, IL-12 and IL-18 can lead to activation of the general NK cell population and increased anti-tumor activity (Cooper et al., 2009).

Within the NK cell, signals from receptors and cytokines are integrated leading to a potentially cytotoxic event (Vivier et al., 2004). However, in order for target cell lysis to occur the NK cell must strongly engage its target (Orange JS., 2008). This involves the formation of the immunological synapse; a tight NK cell to target cell association. After formation, excitatory receptor signals can lead to NK cell killing of the target (Orange JS., 2008). NK cells can kill target cells by a variety of mechanisms once they have generated an immunological synapse (Bromley et al., 2001). The most potent and common is through degranulation of cytolytic granules (Childs et al., 2015). These granules contain perforins and granzymes which work together to trigger target cell apoptosis (Froelich et al., 1996; Browne et al., 1999). While the exact mechanism by which perforins act is unknown, it is hypothesized that perforins destabilize target cell plasma membranes which assist in granzyme uptake by endosomal structures (Keefe et al., 2005). There are multiple granzymes with slightly different mechanisms to kill target cells (Trapani et al., 2002). In the case of granzyme B, uptake leads to direct and indirect caspase activation through the activity of BCL-2 family proteins (Sutton et al., 2000). This granzyme also has the ability to destabilize mitochondrion and facilitate release of cytochrome c into the cytoplasm leading to further induction of the pro-apoptotic program (Alimonti et al., 2001). Granzyme A alternatively induces apoptosis via cleavage of nuclear proteins leading to double strand breaks in the target cell's DNA

(Beresford et al., 1999). NK cells can also trigger their target's death receptor pathways through their expression of tumor necrosis factor related apoptosis-inducing ligand (TRAIL) and Fas ligand (FASL) (Trapani et al., 2002). These pathways trigger caspase activation and target cell death when ligands of the death receptors bind at immune synapses (Czabotar et al., 2014). After lytic granule release has finished, NK cells undergo a period of inactivity and eventually detach from the lysed target cell (Orange JS., 2008).

Generally, NK cell killing is determined by a summation of both positive and negative signals. No single NCR is capable of stimulating NK cells to kill a potential target without co-stimulation from another receptor and a lack of inhibitory signaling (Long et al., 2013). The Fcγ receptor CD16a however is able to activate NK cell cytotoxicity autonomously of these checkpoints (Long et al., 2013). As a member of the Fcγ receptor family, CD16a stimulates NK cell killing by complexing with the Fc portion of IgG molecules (Nimmerjahn et al., 2008). This causes intracellular immunoreceptor tyrosine-based inhibitory motif (ITAM) phosphorylation and eventual RAS-RAF-MAPK signaling resulting in cell activation and antibody-dependent cell-mediated cytotoxicity (ADCC) (Nimmerjahn et al., 2008). While there are a number of different Fcγ receptors expressed on different immune cells, NK cells only express CD16a (Nimmerjahn, 2008).

In recent years, clinical trials have been conducted using allogeneic peripheral blood derived NK (PBNK) cells to treat a number of cancers (Miller et al., 2005; Geller et al., 2011). These adoptive immune cell therapy trials have demonstrated the utility of NK cells in this setting as well as showing that allogeneic NK cell therapies do not generate prohibitive adverse side effects. While these treatments do run the risk of eliciting side effects such as anemia and neutropenia, Miller et al. 2005 demonstrated that haploidentical allogeneic NK cells could effectively combat acute myeloid leukemia (AML) without sparking GVHD (Miller et al., 2005). Clinical trials involving solid tumors have also been conducted showing the viability of haploidentical NK cells to attack solid tumors like ovarian cancer in patients (Geller et al., 2011). However, none of the patients went into a complete remission, showing that there is work that needs to be

done in order to augment current adoptive NK cell therapy options in the case of solid tumors.

While the PBNKs tested in the aforementioned trials are certainly effective at killing hematological cancers such as acute myeloid leukemia (AML), they did not performed so well when treating solid tumors (Miller et al., 2005; Geller et al., 2011). Therefore, one route to remedy this problem is using hESC and iPSC-derived NK cells. These cells have demonstrated potent *in vitro* and *in vivo* killing of solid tumors and express similar receptors to those seen on PBNKs (Woll et al., 2009). One reason to derive NK cells from hESCs and iPSCs is that they can be banked and validated based on alloreactivity. Unlike PBNK donor cell populations which are highly variable, these banked cells would be a uniform population. Another advantage over PBNK cells is that the hESC and iPSC populations used to derived NK cells are highly amenable to a number of genetic modification mechanisms (Wilber et al., 2007; Gropp et al., 2003). These can be used to modify and augment the functional NK cell's activity and increase their ability to kill cancers. For these reasons, we hypothesize that proceeding with pluripotent stem cell derived NK cells over PBNKs is preferable.

One potential genetic modification of relevance to NK cell clinical applications involves the Fcγ receptor CD16a. Because CD16a recognizes the Fc region of IgG1 and IgG3 isotype antibodies, it is thought that combining these cells with FDA approved monoclonal antibody therapies could help to treat solid tumor cancers (Nagarajan et al., 1995). Monoclonal antibodies have been used for the treatment of a range of cancers for over a decade and have revolutionized the field of cancer therapeutics (Scott et al., 2012). For example, Cetuximab, Trastuzumab and Rituximab recognize surface proteins expressed by the tumor cells that they target (Olive et al., 2010). The antibodies themselves can lead to target cell death in two ways. They can directly act upon target cancer cells to kill them by preventing the function of surface proteins vital to proliferation and survival (Ludwig et al., 2003). Also, these antibodies can kill tumor cells by directing NK cell ADCC through CD16a (Alderson et al., 2011). CD16a comes in two single nucleotide polymorphism (SNP) variants, the high Fc affinity FcγRIIIa^{158V}

and low affinity FcγRIIIa^{158F} (Nimmerjahn et al., 2008). It has been shown that lymphoma patients with the higher affinity allele exhibited improved clinical responses when treated with the therapeutic monoclonal antibody Rituximab (Cartron et al., 2002; Weng et al, 2004; Weng et al., 2003). In patients with ovarian cancer, CD16a expression has been shown to be decreased on NK cells (Lai et al., 1996; Jewett et al., 2011). This decrease in expression is due to the effects of a metalloprotease called ADAM17 (Romee et al., 2013). In response to activating signals from CD16a, ADAM17 cleaves CD16a from the cell surface (Romee et al., 2013; Jing et al., 2015). ADAM17 is a member of the ADAM family of metalloproteases and has a role in the cleavage of many substrates including some relevant to NK biology such as L-selectin and TNF-alpha (Scheller et al., 2011). Mice with ADAM17 knocked out had defects in their vasculature, lungs, eyes, hair and skin. These problems were responsible for their premature deaths between E17.5 and a few days after birth (Peschon et al., 1998). A condition in humans has also been studied which seems to be the result of a homozygous mutation in the ADAM17 gene. These patients developed skin lesions, diarrhea and cardiovascular issues. However, none of this data suggested that actual hematopoietic development was noticeably affected by ADAM17 mutations.

Since CD16a downregulation leads to a decrease in NK cell cytolytic activity, our lab aims to prevent its cleavage from the surface of NK cells. In order to preserve the effects of this receptor, our lab and collaborators have devised two methods to maintain its expression on the surface of NK cells. We have designed a non-cleavable version of CD16a that is not susceptible to cleavage by ADAM17 (Jing et al., 2015). The mutant CD16a has a single residue (197) mutated from a serine to proline. This has been stably integrated into iPSCs using the *Sleeping Beauty* transposase. Also, we have used CRISPR-Cas9 to knock-out ADAM17 in hESCs and iPSCs. We hypothesize that preventing downregulation of this molecule could increase the effectiveness of therapies treating solid tumors when combined with therapeutic antibodies.

Materials and Methods

hPSC Subculture Conditions

Maintaining human pluripotent stem cells (hPSCs) in an undifferentiated state over a long period of time allows researchers to keep a constant reservoir of functional cells that can be used for multiple iterations of an experiment. The hPSCs used for these experiments included H9 and UCBiPS7 cells. H9 cells were isolated from human blastocysts as described in Thomson et al. and were purchased from WiCell (Thomson et al., 1998). UCBiPS7 cells were derived by prior members of the Kaufman lab from umbilical cord blood cells positive for CD34. Once sorted, these CD34 positive cells were then transduced with lentivirus containing Oct4, Sox2, Klf4 and c-Myc which drove transcription of genes responsible for reprogramming these cells into pluripotent cells (Ye et al., 2013). In order to maintain the undifferentiated state of hPSCs, the following steps were executed. In culture, hPSCs were maintained on irradiated (3,000 rads) mouse embryonic fibroblast (iMEF) feeder cells plated on 0.1% gelatin at 100,000 cells/well in 6 well plates. These cells were stored in an incubator at 37^{o}C and 5% CO_2. hPSCs were fed daily with hPSC media consisting of Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Invitrogen) base media supplemented with 1mM L-Glutamine (Invitrogen), 0.5% PenStrep (Invitrogen), 1% non-essential amino acids (NEAA) (Invitrogen), 15% knock-out serum replacer (KOSR) (Invitrogen), 4 ng/mL basic Fibroblast Growth Factor (bFGF) (Addgene) and 0.1 mM 2-Mercaptoethanol (Thermo Fisher Scientific). When hPSCs reached a confluence of approximately 90% they were passaged onto a new plate of MEFs. These cells are TrypLE (Invitrogen) adapted which allows them to be passaged as a single cell suspension. To accomplish this, hPSCs were passaged approximately 10 times using TrypLE. At this point, the whole population should be cells that detach well under these conditions. In order to passage cells, TrypLE must be warmed to 37^{o}C and the cells should be incubated with TrypLE for approximately 3 minutes but no longer than 5. Using a pipette, the cells were washed off of the plate and diluted out in at least twice the volume of TrypLE using hPSC media to neutralize the TrypLE. Cells were then passed onto the new plate at the desired passage

ratio. In order to freeze down cells, individual wells of a 6 well plate were grown up to near confluence and then detached using TrypLE. Each well of cells was then put into a vial suspended in 1 mL of freezing media. This consists of 60% hESC media, 30% fetal bovine serum (FBS) (Invitrogen) and 10% dimethyl sulfoxide (DMSO; Sigma Aldrich).

Flow Cytometry

Flow cytometry was done using the LSR II H1160 at the University of Minnesota's core facility. Antibodies used included CD34-PE, CD45-PE, CD56-PE, CD33-PE, CD14-PE, CD16-PE, CD34-PE-Cy7, CD45-PE-Cy7, CD56-PE-Cy7, CD31-APC, CD43-APC, CD45-APC, CD73-APC, KDR-APC, CD117, CD144-APC, CD56-APC, CD158a/b/I-APC, CD94-APC, NKp44-APC, NKp46-APC, NKg2D-APC, Fas ligand-APC, Trail-APC, CD16-APC (all BD), ADAM17-primary (Abcam) and Alexafluor 594 secondary (Abcam). Cells were stained with antibody for 30 minutes following the producers' recommended quantities. The collected events were analyzed using Flowjo software.

Cancer Cell Culture

In order to test the inherent *in vitro* cytotoxicity of various NK cell populations, different cancer cell lines were grown and put into chromium release assays. These include; K562 human chronic myelogenous leukemia line, Raji human Burkitt's lymphoma cells, SK-OV-3 human ovarian carcinoma (HER2 high) and MA-148 human ovarian carcinoma (HER2 low). The first three have been bought from ATCC and were cultured per their recommendations. MA-148 cells were isolated at the University of Minnesota in the lab of Dr. Ramakrishnan. K562 cells were maintained in T-25 flasks with 10 mL of K562 media which included Roswell Park Memorial Institute (RPMI) media (Invitrogen) with 10% FBS, 1% PenStrep, 1% L-Glutamine and 1% NEAA. Cells were counted daily and when they reached $0.7\text{-}0.8*10^6$ cells/mL they were passed to $0.3*10^6$ cells/mL in order to keep them in log growth phase. Raji cells were also maintained in T25 flasks using K562 media. When they reached $2\text{-}3*10^6$ cells/mL they were passaged down to $.5\text{-}5*10^5$ cells/mL. MA-148 cells were maintained in 15 mL K562 media while attached to a T-75 flask. When cultures reached 95% confluence they were passaged using 0.05% Trypsin-

EDTA warmed to 37ºC for 5 minutes. This was neutralized with 10 mL K562 media and the cells were then passaged at a ratio of 1:3-6. SK-OV-3 cells were maintained in 15mL SK-OV-3 media affixed to a T75 culture flask. SK-OV-3 media includes McCoy's 5a media (Invitrogen), 10% FBS, 1% L-Glutamine, 1% NEAA and 1% PenStrep. The passaging was done the same as for the MA-148 cells.

Spin Embryoid Body (EB) Hematopoietic Differentiation

Hematopoietic progenitor cells that are $CD34^+/CD43^+$ and $CD34^+/CD45^+$ have been shown to be capable of generating NK cells *in vitro* (Vodyanik et al., 2006). Under the specified conditions, the spin embryoid body (EB) system can be used to recapitulate the hematopoietic niche *in vitro* and drive hPSCs to differentiate into hematopoietic progenitor cells (Ng et al., 2008). This system can therefore be used in the differentiation of NK cells from undifferentiated hPSCs. Before generating spin EBs it is necessary to synchronize the population of cells into logarithmic growth phase. 250,000-500,000 hPSCs (depends on rate of division) should be passaged one day before EB formation onto iMEFs at 100,000 cells/well. When these hPSCs reach a confluence of approximately 70% they are ready to be detached by TrypLE passaging. Only pure cell cultures without differentiating colonies were used. In order to remove clumps, the passaged cells were run through a 70 micron filter. This single cell suspension was added to Albumin Polyvinylalcohol Essential Lipids (APEL) media and cytokines. APEL is a defined media and contains no xenogeneic components and was made as previously described (Ng et al., 2008). For this stage of hematopoietic differentiation: VEGF (20 ng/mL), BMP4 (20 ng/mL) and SCF (40 ng/mL) (all R&D) were added to the APEL. The single cell suspensions with APEL and cytokines were pipetted into ultra-low attachment 96 well plates (NUNC) at 2,500 cells per well and spun at 1500 rpm for 5 minutes. These cells were then put into an incubator at 37ºC and 5% CO_2 and left for 2-3 days without being touched. On day 6, a half media change with doubled concentrations of VEGF and SCF were given to further promote hematopoietic progenitor cell development.

Maintenance of OP9 Stromal Cells

OP9 cells are mouse fibroblast-like cells that can be used to support the differentiation of NK cells from hematopoietic progenitors (Tabatabaei-Zavareh et al., 2007). OP9 cells (ATCC) have been transduced using *Sleeping Beauty* to constitutively express Delta-like ligand 1 (OP9-DL1) which acts through the Notch pathway to support NK differentiation (Carotta et al. 2006). Before use, these cells must be mitotically inactivated. To accomplish this, cells were treated with 300 uL of 500ug/mL Mitomycin C (Millipore) for 3 hours. These cells were seeded at 27,000 OP9 cells per well onto 0.1% gelatinized 24 well plates. Once EBs had matured for 11 days they were transferred onto these cells. OP9 media consists of alpha-minimum essential media (alpha-MEM) (Invitrogen) supplemented with 20% FBS, 1% L-Glutamine, 26 mM Sodium Bicarbonate and 1% PenStrep.

Natural Killer Cell Differentiation from Spin EBs

In order to influence hematopoietic progenitor cells to differentiate into NK cells, EBs can be placed into media containing cytokines important for NK cell development (Knorr et al., 2013). On day 11 of the hematopoietic differentiation, spin EBs were transferred from their 96 well plates to 24 well plates. Before transfer, approximately half the APEL is removed using a multichannel pipette. The EBs are then transferred to a 24 well plate with Natural Killer differentiation media (NKDM) and cytokines. This was made as described in previous publications and supplemented with Flt3L (10 ng/mL), IL-7 (20 ng/mL), IL-15 (10 ng/mL), SCF (20 ng/mL) and IL-3 (5 ng/mL only on the day of the transfer) which were all purchased from R&D systems Minneapolis, MN (Knorr et al., 2013). These were passed onto 24 well plates, either coated with gelatin then seeded with OP9-DL1 cells, or bare plastic. 6-8 EBs were transferred into each well. Every week, half of the media was changed. After 4 weeks one well of cells was harvested to determine the quantity of CD56-expressing cells in culture using flow cytometry. Briefly, cells were harvested and filtered to remove debris and EBs. These cells were then stained with CD56-APC, CD33-PE and CD14-PE to determine which cells were present in these cultures.

Artificial Antigen Presenting Cell NK Cell Stimulation

Stimulation of NK cells was done using IL-21 transduced K562 myelogenous leukemia cells. IL-21 is a gamma chain cytokine that synergizes *in vivo* with Flt3L, IL-2 and IL-15 to generate NK cells from the bone marrow (Denman et al., 2012). IL-21 may also help to upregulate cytolytic activity by upregulating NCRs, perforins and granzymes (Denman et al., 2012). It also helps in the proliferation and maturation of NK cells in culture (Denman et al., 2012). Therefore, after 4-5 weeks in NKDM, cultures that had CD56 cells (as determined by flow cytometry) were transferred into NK expansion conditions. First, non-adherent cells were harvested from 24 well plates and filtered through a 70 micron filter to remove the EBs. This cell suspension was counted using a standard hemocytometer. These cells were then put into artificial antigen presenting cell (aAPC) co-culture at an initial ratio of 2 IL-21 transduced K562: 1 NK cell. The cells were incubated in NK expansion media which consists of RPMI with 10% FBS, 1% PenStrep, 1% L-Glutamine, 1% non-essential amino acids and IL-2 (R&D). 4 days after the cells were put into culture the media was changed. Every 7 days after initiation the K562 cells were replenished at a 1:1 ratio. This was done weekly until 2 months after transfer into NKEM conditions. Periodically over the course of the two months, cells were frozen down and stocked. The freezing media consists of 50% FBS, 40% NKEM without IL-2 and 10% DMSO.

Validation of Hematopoietic Differentiation

In order to determine the identity of cells within the differentiating EBs, flow cytometry was run on disaggregated EBs. Briefly, 48-96 EBs were collected from 96 well plates, washed with DPBS and incubated with 1 mg/mL collagenase IV (Thermofisher Scientific) for 2 minutes. This was then neutralized with double the volume of I10 (10% FBS in Iscove's Modified Dulbecco's Medium (IMDM)) (Invitrogen). After washing again with DPBS, cells were incubated with 0.05% Trypsin + EDTA (Invitrogen) + .02% chick serum for 8-10 minutes. This was also neutralized with double the volume of I10. The cells were then put through a strainer and flow cytometry was run. To determine the quantity of hematopoietic progenitor cells, the expression of CD34-PE was compared to CD31-APC, CD43-APC, CD45-APC and CD73-APC.

NK Cell Immunophenotyping

Receptors such as killer-cell immunoglobulin-like receptors (KIRs), natural cytotoxicity receptors (NCRs) and CD16a are the primary mediators of NK cell cytotoxicity. Their presence or absence could have profound implications on the success or failure of *in vitro* cytotoxicity experiments. In order to determine the complement of receptors present on the surface of NK cells after NK expansion, flow cytometry was used. This was also used to quantify the relative expression of CD16a on different cell lines such as the knock-in CD16a wild-type, mutant and un-transfected cells. The expression of CD56-PE was compared to receptors such as CD158a/b/h-APC, CD94-APC, NKp44-APC, NKp46-APC, NKg2D-APC, Fas ligand-APC, Trail-APC and CD16-APC.

In Vitro Cytotoxicity

In order to compare the inherent cytotoxicity of NK cells transduced with mutant CD16a, with ADAM17 knocked out, isolated from peripheral blood (PBNK) and untransfected UCBiPS7-derived NK cells, chromium release assays were used. This assay works by incubating target tumor cells with Chromium-51 containing CrO_4^{2-} (Perkin Elmer). These labeled cells can then be incubated with different quantities of effector (NK) cells. The relative release of Chromium into the supernatant can be used to determine the efficacy of the NK cells. Briefly, tumor cells (K562, MA-148, SK-OV-3 and Raji) were incubated for 1 hour at 37^{o}C with 0.2 millicuries of radioactive CrO_4^{2-}. These cells were then washed 3 times with I5 (IMDM+5% FBS) and co-cultured with NK cells at the specified effector to target (E:T) ratios in I20. To test the effect that adding antibodies would have on the NK cell's cytotoxicity (ADCC), Rituximab (targeting Raji cells at 10 ug/mL; Genentech) and Herceptin (targeting HER2-expressing cells at 100ug/mL; Genentech) were incubated with the effector cells for 30 minutes before adding the target cells. After 4 hours, the plates were spun down and 100 uL of the media was collected (without pellet) for gamma scintillation measurements. Specific lysis was calculated as follows: Percentage of specific lysis = 100*(Test Release – Spontaneous Release) / (Maximal Release – Spontaneous Release) (Knorr et al., 2013).

Confirmation of ADAM17 KO

During previous Kaufman lab studies, UCBiPS7 and H9 cells were transfected with CRISPR-Cas9 targeting exon 1 of ADAM17. Flow cytometric analysis on these cells showed which clones had a knock-out of ADAM17 as determined by a lack of surface expression. The sgRNA was designed by our collaborator Jimmy Wu and had the sequence 5'-ccg CCG AAG CCC GGG TCA TCC GG-3'. First, cells were grown in zeocin containing hPSC media to select out for non-transfected cells. The survivors were then sorted by isolating GFP positive cells (the CRISPR-Cas9 vector constitutively expresses GFP). These cells were then grown and plated at low density. Homogenous clones were picked and grown in a 12 well plate on 30,000 iMEFs/well with normal hPSC media. When confluent, they were detached using TrypLE and flow cytometry was run as described using an ADAM17 primary antibody and an Alexafluor 594 secondary antibody. Those clones whose flow plots matched that of their isotype were selected as potential knock-out clones. To determine if these non-expressing cells were truly ADAM17$^{-/-}$, the clones' DNA flanking the exon 1 cut site were isolated for sequencing. First, genomic DNA was isolated using a Qiagen DNeasy blood and tissue kit. This was then used as a template to derive a PCR product using primers procured from Invitrogen and a high fidelity *Taq* polymerase (Thermofisher Scientific). The sequence of the upstream primer was 5'GGGTGGAGTTGGGACTCATAC3' and of the downstream primer was 5'ACACCCCCTTCTACACTGAAAA3'. This PCR product was then run on a 1 % agarose gel to see if the band localized to the appropriate region of the gel (655 bp).

Statistical Analysis

Differences between groups were compared using two way ANOVA testing via Prism 4 (GraphPad Software, San Diego, CA). Results with a p-value less than .05 were considered significant.

Results

Transfection of hES and iPS Cells with Crispr-Cas9 Plasmids Targeting ADAM17 Leads to its Knock-out

ADAM17 is expressed on many different cells within the human body (Scheller et al., 2011). As demonstrated by previous studies in the Kaufman lab, ADAM17 is specifically expressed on hES cells, PBNK and iPSC-derived NK cells (Fig. 1A). In order to generate ADAM17$^{-/-}$ hES and iPS cells, H9 and UCBiPS7 cells were transfected with a vector that expresses a sgRNA sequence targeting exon one of the ADAM17 transcript (Fig. 1B). This vector constitutively expresses GFP and confers recipient cells with antibiotic resistance. These features were used to fluorescence-activated cell sort (FACS) out cells that had been successfully transfected with the vector after having been cultured for 72 hours. After these cells were isolated, they were grown up in bulk and passaged at a low enough confluence to isolate clones that had arisen from one cell. These clones were then stained for ADAM17 and surface expression was determined by flow cytometry analysis. Previous Kaufman lab analysis on the H9 cells showed 7 clones whose surface expression of ADAM17 matched that of the isotype. Flow cytometric analysis on the iPS cells found 4 clones whose surface expression of ADAM17 matched that of the isotype (Fig. 1C). These cells were then expanded and frozen down in the liquid nitrogen.

To confirm the knock-out genetically, the DNA flanking exon one of the ADAM17 gene was isolated using primers targeting sequences upstream and downstream of the cut site. Genomic DNA from the clones thought to be ADAM17$^{-/-}$ was isolated and purified. The DNA flanking the exon 1 cut site of the clones was then amplified via PCR reactions. The DNA products of these reactions were then run on a 1% agarose gel to determine if the primers in fact generated products of the appropriate 655 nucleotide length (Fig. 1D). Unlike the band produced from iPSC clone 10, most of the products generated a strong signal and were of the appropriate length. DNA from this clone will be re-isolated in order to generate a more pure PCR product which can in the future be sequenced along with DNA from the other clones. Initial isolation of iPSC clone 9 was

not effective and therefore DNA from another batch of the cells was isolated. Both of the products were run and while the second band from the right did not produce a band, the other iPSC clone 9 was able to generate a product (Fig. 1D).

hESC Derived Hematopoietic Progenitors Failed to Produce NK Cells

Upon the derivation of these ADAM17 knock-out H9 cells, the undifferentiated pluripotent stem cells were put into spin EBs to foster hematopoietic differentiation. For these trials, an H9 untransfected control, H9 CRISPR-Cas9 transfected control (expresses ADAM17) and two knock-out clones (5 and 10) were used. Over the 11 day time course, EBs grew and their progression can be seen before the day 6 half media change as well as before the day 11 transfer to NK differentiation conditions in Figures 2A and 2B. These EBs developed cystic structures and often showed an appreciable accumulation of round hematopoietic cells surrounding the EB. After 11 days in culture, the EBs were disaggregated to analyze the relative populations of primitive and mature hematopoietic, endothelial and stromal cells within the EB by flow cytometry (Fig. 2C). These EBs can produce immature cells ($CD34^+$) and immature endothelial cells ($CD34^+CD31^+$) with the ability to give rise to blood cells. Critically they are also capable of generating cells both $CD34^+CD43^+$ and $CD34^+CD45^+$ which have been shown previously to give rise to NK cells *in vitro* (Tien et al., 2009; Vodyanik et al., 2005). One concern regarding this project was that the knock-out of ADAM17 may inhibit hematopoietic differentiation. It has been shown that ADAM17 is responsible for cleaving many proteins from the surface of cells that contribute to hematopoiesis such as Flt3L (Scheller et al., 2011; Gilliland et al., 2002). Importantly, the hematopoietic differentiations were not determined to be significantly affected by the knock-out of ADAM17 (Fig.2D). The percentages of cells $CD34^+CD31^+$, $CD34^+CD43^+$, $CD34^+CD45^+$ and $CD34^+CD73^+$ did not differ significantly between iPSCNK transfected ADAM17 expressing and the two iPSCNK ADAM17 KO cell lines (Fig. 2D).

While these studies did demonstrate production of hematopoietic progenitor cells based on phenotype, the progenitor cells derived from the EBs did not generate H9-derived NK cells when placed into NK differentiation media (NKDM). Approximately 2 weeks after

transfer, the cultures proliferated heavily almost covering the whole well (Fig. 2E). By day 30 in NKDM however, these cultures which previously proliferated strongly were devoid of hematopoietic cells and all that could be seen were stromal cells (Fig. 2E). When analyzed by flow cytometry, these cultures uniformly gave rise to $CD33^{+}CD56^{-}$ myeloid cells (Fig. 2F). Even repeated passaging on OP9-DL1 stromal cells has not permitted generation of CD56 positive cells. Potentially, the ADAM17 knock-out could have somehow adversely affected NK cell differentiation independent of hematopoietic progenitor differentiation. It has been shown that Notch, a target of ADAM17, can help to contribute to NK cell generation (Beck et al., 2009). However, even H9 control cells that do express ADAM17 normally were not, up to this point, capable of making NK cells (Fig. 2F). Likewise, $ADAM17^{-/-}$ iPSC-derived NK cells have been generated, further detracting from the theory that this developmental deficit is due to ADAM17's knock-out (Fig. 3C).

NK Cell Development from $ADAM17^{-/-}$ iPSC-Derived Hematopoietic Progenitor Cells

After ADAM17 was knocked out in iPS cells, the clones were differentiated into hematopoietic progenitors. For these trials, an iPSC untransfected control, iPSC CRISPR-Cas9 transfected control (expresses ADAM17) and two knock-out clones (9 and 10) were used. After 11 days in culture, the EBs were disaggregated to analyze the relative populations of primitive and mature hematopoietic, endothelial and stromal cells within the EB by flow cytometry (Fig. 3A). These EBs can produce immature cells ($CD34^{+}$) and immature endothelial cells ($CD34^{+}CD31^{+}$) with the ability to give rise to blood cells. Critically they are also capable of generating cells both $CD34^{+}CD43^{+}$ and $CD34^{+}CD45^{+}$ which have been shown previously to give rise to NK cells *in vitro* (Tien et al., 2009; Vodyanik et al., 2005). Similar to what was seen in the H9 ADAM17 knock-out cultures; there was no significant difference between the hematopoietic differentiations seen in the control cell populations compared with the knock-out cells (Fig. 3B). When these cells were placed into NKDM they managed to proliferate into $CD56^{+}$ NK cells as shown from plots derived from clone 9 (Fig. 3C).

Non-cleavable CD16a knock-in iPSC Derived Hematopoietic Progenitors Can Give Rise to NK Cells

iPS cells transduced with *Sleeping Beauty* transposase to integrate a vector which constitutively expresses both a non-cleavable and wild-type CD16a protein (along with GFP) were also put into hematopoietic differentiation conditions to generate $CD34^+CD43^+$ and $CD34^+CD45^+$ hematopoietic progenitor cells (Jing et al., 2015 and Fig. 4A). After 11 days in culture, the EBs were disaggregated to analyze the relative population of primitive and mature hematopoietic, endothelial, and stromal cells within the EB by flow cytometry (Fig. 4B). Much like the H9 EBs, these EBs contained cells that were $CD34^+$, $CD34^+CD31^+$, $CD34^+CD43^+$ and $CD34^+CD45^+$ and the two different cell lines had no significant difference in expression of these markers (Fig. 4C). Once placed into NKDM conditions, these hematopoietic progenitors were capable of giving rise $CD56^+$ NK cells (Fig. 4D). These cells appear to be immature due to their expression of c-kit receptor (CD117) and have some cytolytic capabilities due to the expression of the NCR NKp44.

After aAPC Co-culture iPSC-derived NK Cells Acquire Receptors Necessary for Cytotoxicity

After deriving $CD56^+$ NK cells from iPSC CD16a knock-in-derived hematopoietic progenitors, these cells were placed into aAPC co-culture with K562 cells that stably express membrane-bound IL-21 (Denman et al., 2012). These cells help to drive the proliferation of NK cells while also stimulating the maturation of these cells in combination with the IL-2 added to the media (Denman et al., 2012). After 2-4 weeks in culture, there were enough NK cells to perform experiments. One of which was an immnunophenotyping panel using flow cytometry to stain the cells for various receptors integral to cytotoxicity. In the case of iPSCNK untransfected cells, the aAPC expansion yielded a highly pure $CD56^+$ population of cells (Fig. 5A). These cells expressed the death receptor ligand Trail as well as the NCRs NKp46 and NKG2D (Fig. 5A). Interestingly, this cellular population highly expressed CD16a and almost 90% of the cells were positive for this receptor (Fig. 5A). Immunophenotyping was also done on

iPSCNK CD16a mutant NK cells (Fig. 5A). These cells were almost 100% $CD56^+$ (Fig. 5A). They expressed the death receptor ligand Trail along with the NCRs NKp46 and NKG2D (Fig. 5A). Over 98% of these cells expressed CD16a helping to confirm the hypothesis that CD16a mutant knock-in cells would maintain expression of CD16a to a much greater extent than untransfected control cells (Fig. 5A). It is worth noting that these cells were not stimulated in a method that would trigger ADAM17 activation and CD16a cleavage. We hypothesize that if the cells were stimulated with either PMA or IL-12 and IL-18 the disparity between the iPSCNK cells and iPSCNK CD16a mutant cells would be far more striking.

These cells were also tested to determine their *in vitro* cytotoxic capacity. This was performed using the chromium release assay. NK cells were co-cultured with both K562 and SKOV3 cells in the presence or absence of Herceptin to determine whether non-cleavable CD16a overexpression confers an advantage in killing targets that overexpress the antibodies' target (epidermal growth factor receptor 2: HER2) in the presence of the antibody. When PBNK, iPSCNK and iPSCNK CD16a mutant cells were incubated with K562 cells, the effector NK cells killed their targets at approximately the same level regardless of whether Herceptin was added (Fig. 6A). This is to be expected as K562 cells do not express HER2. Also, PBNK cells performed better in this trial than iPSCNK CD16a mutant NK cells. When these same effector cells were co-cultured with HER2-expressing SKOV3 cells however, the addition of Herceptin augmented the killing of the effector cells (Fig. 6B). In fact, the iPSCNK CD16a mutant cell's killing was improved by the addition of Herceptin such that it was able to kill SKOV3 cells more effectively than PBNK cells in the presence of Herceptin. While these results are promising, they are only comprised of one biological replicate. In order to solidify these conclusions, more replicates are needed.

Discussion

Thus far the project has yielded positive results. Preliminary results have shown consistent generation of NK cells from iPS cells. Likewise, testing of iPSC-derived NK cells has reinforced the hypothesis that non-cleavable CD16a molecules can help to augment ADCC. The next step for this project is to obtain H9-derived NK cells for cytotoxicity testing. Despite not generating any H9-derived NK cells, the differentiation of iPSC-derived NK cells was successful. This makes identifying the problem(s) within the H9 differentiation difficult as these cells were often cultured in parallel with the same cytokines and media that successfully generated iPSC-derived NK cells. One possibility is that the actual H9 cells transfected with the CRISPR-Cas9 sgRNA targeting ADAM17 were not capable of generating NK cells. The control cell lines that were run in parallel with the ADAM17 knock-out clones also did not generate NK cells. These H9 untransfected cells were also from the same stock that had been used for the ADAM17 knock-out transfection. To help determine whether the starting cell population was in some way defective, a new control is being tested (H9GFP:Luc) in parallel with H9 ADAM17 knock-out cells. If this control yields NK cells but the knock-out still doesn't, the result would suggest that the starting cell population is deficient.

It is also possible that the H9 cells which have been differentiated to obtain these results were in some way damaged during the course of cell culture. One possibility is that a process called culture adaptation occurred. It has been shown that culture adapted hES cells exhibit altered or skewed differentiation capacities (Baker et al., 2007). This can manifest through an abnormal karyotype as well as changes to the chromatin of hES cells (Baker et al., 2007). However, other cell lines cultured in parallel with these cells and using the same MEFs generated NK cells. Nonetheless, the next step in trouble shooting this possibility is to try and differentiate clones from a stock that was frozen down before these experiments were undertaken. If these cells do generate NK cells then it is possible that the cells which have previously been subjected to differentiation acquired some issues and should be thrown away in favor of this more primitive population. However, if these primitive cells do not generate NK cells then it may be

necessary to re-transfect another H9 cell line with the CRISPR-Cas9 targeting exon 1 of ADAM17. This could be done using cells that have been proven in previous publications to efficiently generate NK cells.

Having derived $CD56^{+}$ cells from the $ADAM17^{-/-}$ iPS cells, the next step is to expand these cells in aAPC and then conduct the same chromium cytotoxicity experiments as was done for the iPSC CD16a mutant cells. This will allow for a direct comparison between the two cell line's inherent cytotoxicity and help to determine which would be a more effective cell-based therapy.

Preliminary cytotoxicity results from the iPSC-derived CD16a mutant NK cells have been consistent with the overall experimental hypothesis. These effector cells became activated in the presence of antibodies and elicit an improved cytotoxic response when compared to conditions without the antibody (Fig. 6B). Likewise, this phenomenon was only observed when the effector cells were co-cultured with cancerous cells that express the antibody's target (Fig. 6A). The next step for these experiments is to replicate the assay while also adding on different cancer cell lines. While SKOV3 cells highly express HER2, another ovarian epithelial cancer cell line; MA-148, does not express HER2 at such high levels as determined by previous Kaufman lab experiments (Fig. 6C). Comparing the results garnered from assays in which the non-cleavable CD16a transfected NK cells target both SKOV3 and MA-148 in the presence of Herceptin would help to show the true strength of these effector cells. Likewise, this therapy could be employed against other cancers besides ovarian carcinomas. Raji cells, a Burkitt's lymphoma cell line that express CD20, can be targeted using Rituximab and are an example of another cell line that could be tested to determine the versatility of these effector cells.

Another important aspect to this project is the acquisition all three genotypic variants derived from the phenylalanine (F) and valine (V) SNPs at residue 158 of CD16a. It has been shown that patients homozygous for the high Fc-affinity 158V allele have a better prognosis when treated for B-cell lymphoma with Rituximab than the heterozygous (FV) and homozygous (FF) patients (Cartron et al., 2002; Weng et al, 2004;

Weng et al., 2003). The vector that overexpresses the non-cleavable CD16a within iPS cells contains DNA coding for the high affinity allele. The iPSC-derived NK cells being compared to the iPSC CD16a mutant-derived NK cells are heterozygous for this SNP. However, generating control iPSC-derived NK cells from populations homozygous for both SNPs would allow for a complete comparison showing the iPSC CD16a mutant-derived NK (158V) relative to different iPSC-derived NK cells resembling the heterogeneity of the human populace. Currently, our collaborators are working to determine the genotypes of four different iPSC lines and hopefully more can be acquired.

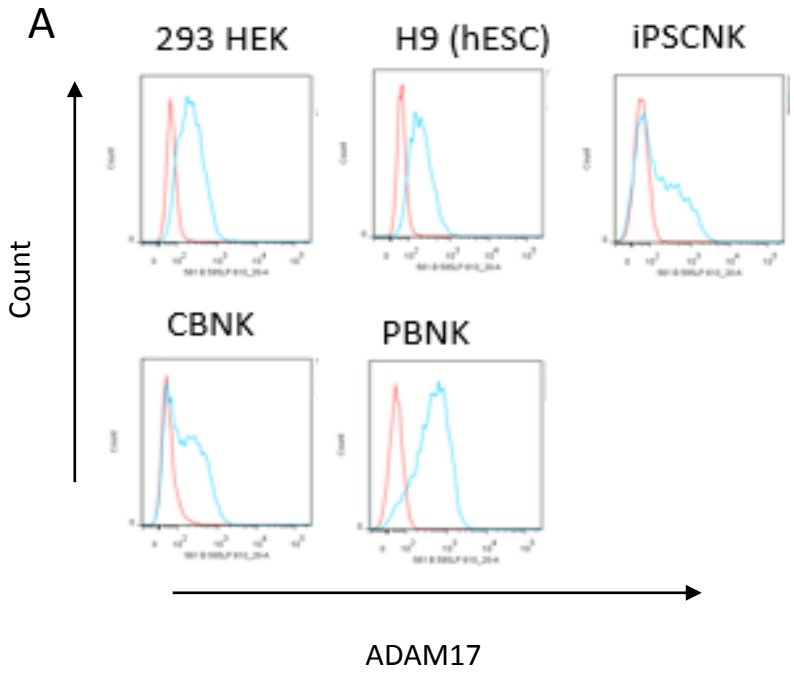
Finally, it still remains to be conclusively determined whether or not the putative ADAM17$^{-/-}$ clones are true knock-out cells. While the flow cytometry data shows that these clones lack ADAM17 expression, it is necessary to confirm that an indel mutation has occurred in both of the alleles. Initial stages in this process have been completed. PCR products corresponding to the region flanking the exon 1 cut site have been amplified from genomic DNA isolated from individual clones. It has also been shown the primers themselves are capable of generating a PCR product that corresponds with the nucleotide length of the theoretical product (Fig. 1D). Going forward, this PCR product should be sequenced to look for indels of one or two nucleotides that would suggest a frameshift mutation that very likely would give rise to a non-sense mutation. Clones homozygous for these mutations would then be selected going forward for further differentiation.

In recent years, pioneering work on adoptive NK cell immunotherapy has seen interest in the field expand greatly. These studies employed PBNK cells and were often highly effective at treating hematological malignancies (Miller et al., 2005). Likewise, trials involving T cell chimeric antigen receptor (CAR) adoptive therapies have also shown great promise in the treatment of hematologic malignancies (Grupp et al., 2013). However, when applied to solid tumors, NK cells did not elicit such positive responses (Geller et al., 2011). This has led to a search for methods to improve upon previous results so solid tumors refractory to current therapies and adjuvants can be better treated. One avenue to augment previous trials is to use hES and iPS cell-derived NK cells. The

discovery that allogeneic PBNK cells could safely be used to treat various malignancies has led to the possibility of using allogeneic hES and iPS cell-derived NK cells (Miller et al., 2005). These cells, both the pluripotent stem cells and NK cells, can be banked leading to a uniform therapeutic unlike donor-derived PBNK cells. Also, these pluripotent stem cells can critically be genetically manipulated in order to accentuate different immunoregulatory and cytolytic pathways (Wilber et al., 2007; Gropp et al., 2003). It is through these alterations that hES and iPS cell-derived NK cells can gain an advantage over PBNK cells. Stabilizing the expression of CD16a is one way to increase the anti-tumor effects of these cells. Knocking-out ADAM17 or transfecting cells with a non-cleavable version of CD16a should confer an advantage over other unmodified cells when attacking cancer cells through ADCC. Under these conditions, the patient would concurrently be given a therapeutic antibody that targets their specific type of cancer. Therapeutic antibody treatments have been used for quite some time and are ever evolving (Slamon et al., 2011; Wilken et al., 2010). Due to the wide range of antibodies available today and with more on the horizon, this strategy has the potential to be applied to any cancer that can be targeted effectively with therapeutic antibodies.

Figures

Figure 1: Transfection and validation of both H9 and iPSC ADAM17 knock-out. A) Flow cytometry analysis from previous Kaufman lab studies showing surface expression of ADAM17 on various cell lines. Starting from the top left corner and going clockwise: 293 HEK cells, H9 ES cells, iPSC NK cells, PB NK cells and CB NK cells. For flow cytometry histograms, the red plot is the isotype and the blue is the expression corresponding to each cell line. B) pD1321-AD plasmid containing ADAM17 exon 1 sgRNA and Cas9 nuclease used to knock out ADAM17 in both H9 and iPS cells. C) Flow cytometry showing the relative expression of ADAM17 on H9 and ctrl iPS cells in both isotype controls (top two plots on left) and stained cells (bottom two plots on left). The grid of 12 plots on the right contains cells from the iPSC GFP positive cell population (transient expression) that were single clone selected and tested for ADAM17 expression. Those in the red boxes were isolated for further analysis and testing. D) Using primers designed to anneal to the regions both upstream and downstream of the exon 1 cut site, a PCR product was generated from clones that were negative for ADAM17 expression as assessed by flow cytometry. This product was expected to be 655 nucletides long and the bands fall just below the 700 nucleotide band on the ladder (6^{th} from top). From left to right: H9 clone 1, H9 clone 2, H9 clone 5, H9 clone 7, H9 clone 8, H9 clone 10, iPSC clone 9, iPSC clone 9 and iPSC clone 10.



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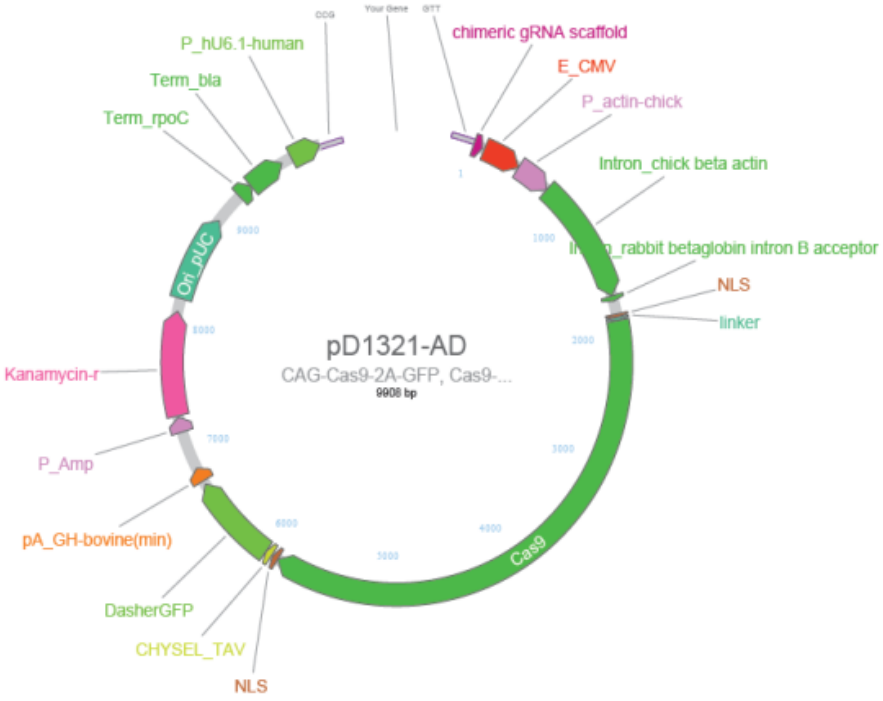
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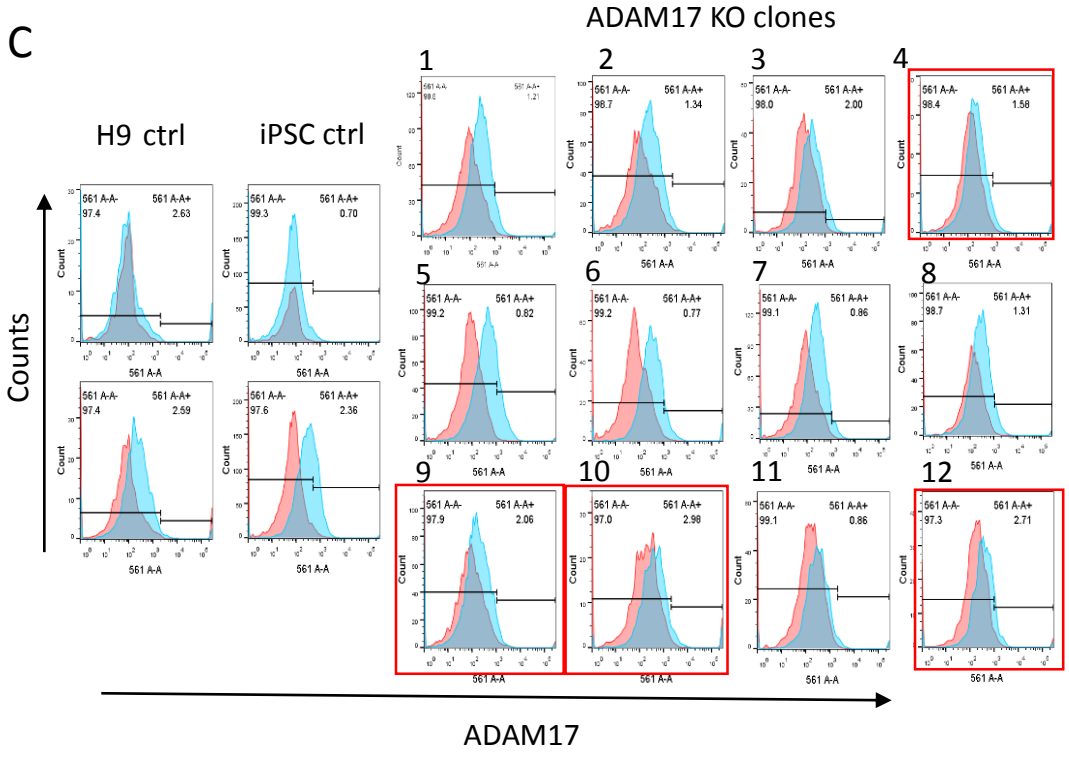
ADAM17 KO clones
H9 ctrl
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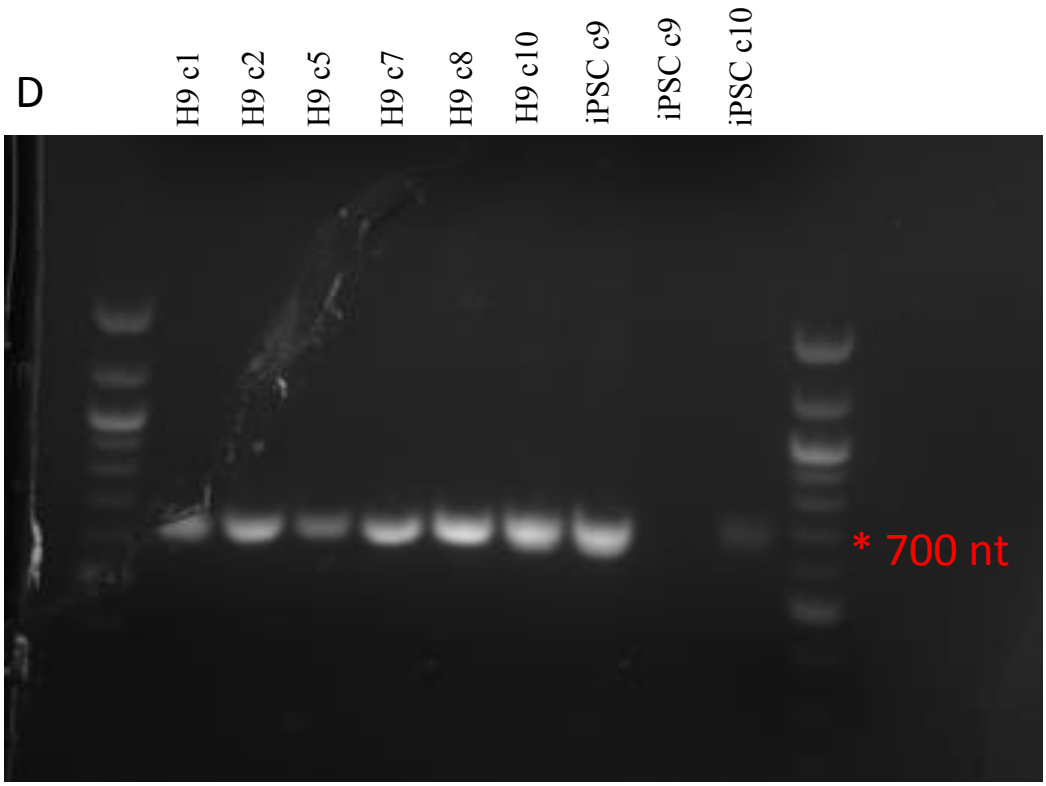
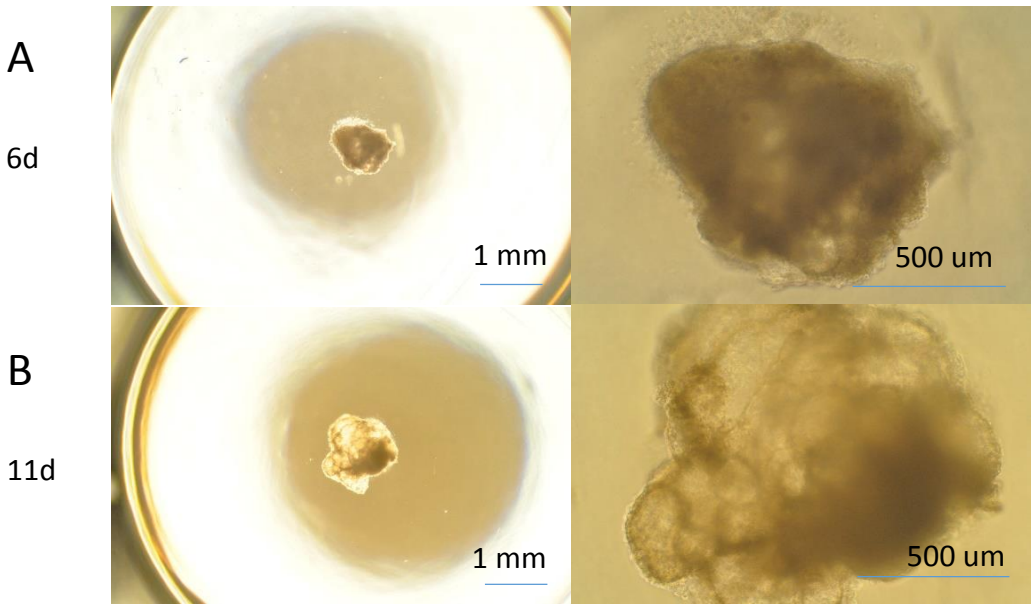


Figure 2: Differentiation of H9 ADAM17 knock-out clones and controls yields hematopoietic progenitors but no NK cells. A and B) Representative phase contrast images showing H9 derived EBs during hematopoietic differentiation. The pictures in A show the EBs after six days in culture while the pictures in B show them after 11 days. Scales bars are for 1 mm on the left and 500 um on the right). C) Representative flow cytometry analysis from day 11 spin EB differentiation of (clockwise from top left) H9 transfected control clone 2, H9 ADAM17 KO clone 5 and H9 ADAM17 KO clone 10 cells. D) Statistical analysis on the populations of H9 derived cells $CD34^+$, $CD34^+CD31^+$, $CD34^+CD43^+$ and $CD34^+CD45^+$ after 11 days in stage one conditions (n=3 for H9 ctrl c2, KO c5 and KO c10) showed no significant difference in the differentiation of any of these hematopoiesis related cell types. E) Phase contrast images showing H9 derived cultures at D16 (on the left) in NKDM and (on the right) at D30. These differentiations included OP9-DL1 cells. The D16 cultures contain many hematopoietic cells while the D30 cultures only possess OP9-DL1 cells coating. The top two pictures correspond with H9 ADAM17 expressing clone 2 derived cells, the middle two correspond to H9 ADAM17 KO clone 5 derived cells and finally the bottom two correspond to H9 ADAM17 KO clone 10 derived cells. Scale bars are 1 mm. F) Flow cytometry analysis of the three aforementioned cell lines (from Fig. 2C). These cells uniformly express CD33 (myeloid marker) but neither CD56 nor CD14 (macrophage) after 21 days in NK differentiation conditions.



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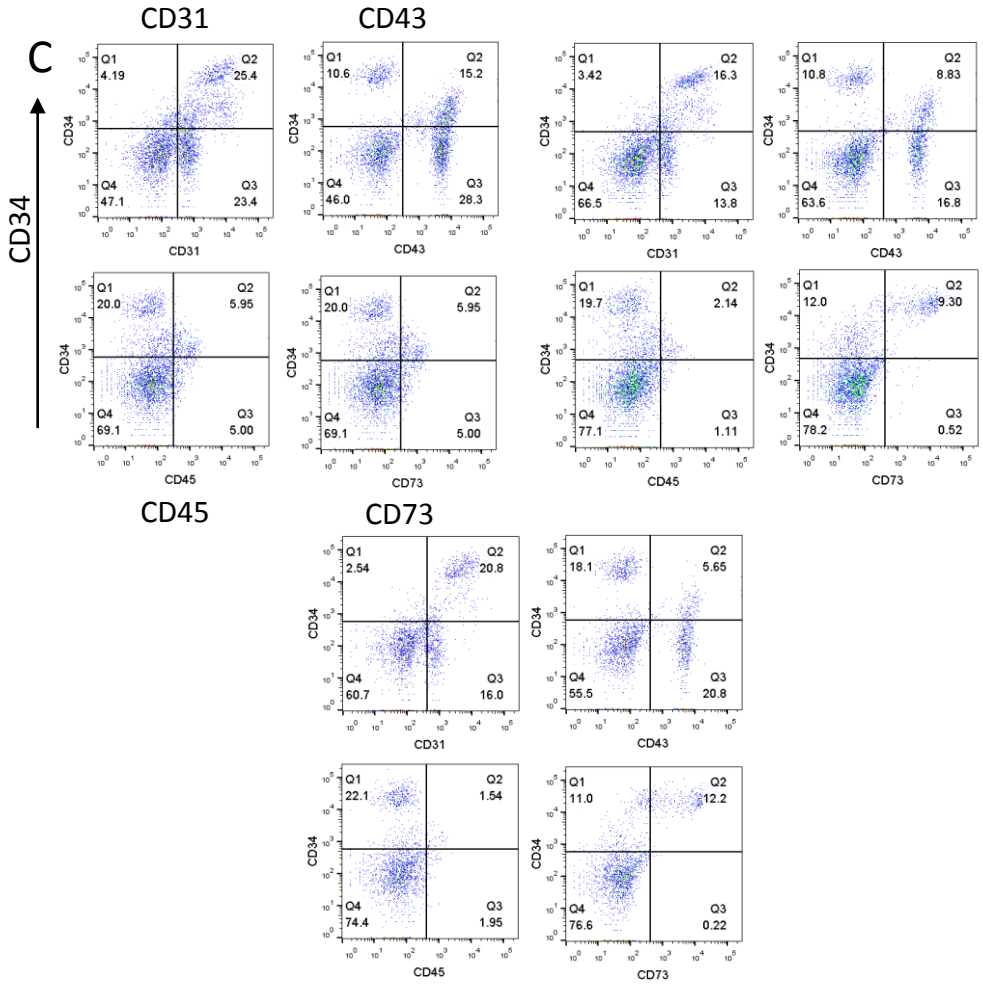
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Q1 3.42
Q2 16.3
Q4 66.5
Q3 13.8
Q1 10.8
Q2 8.83
Q4 63.6
Q3 16.8
Q1 20.0
Q2 5.95
Q4 69.1
Q3 5.00
Q1 20.0
Q2 5.95
Q4 69.1
Q3 5.00
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Q4 55.5
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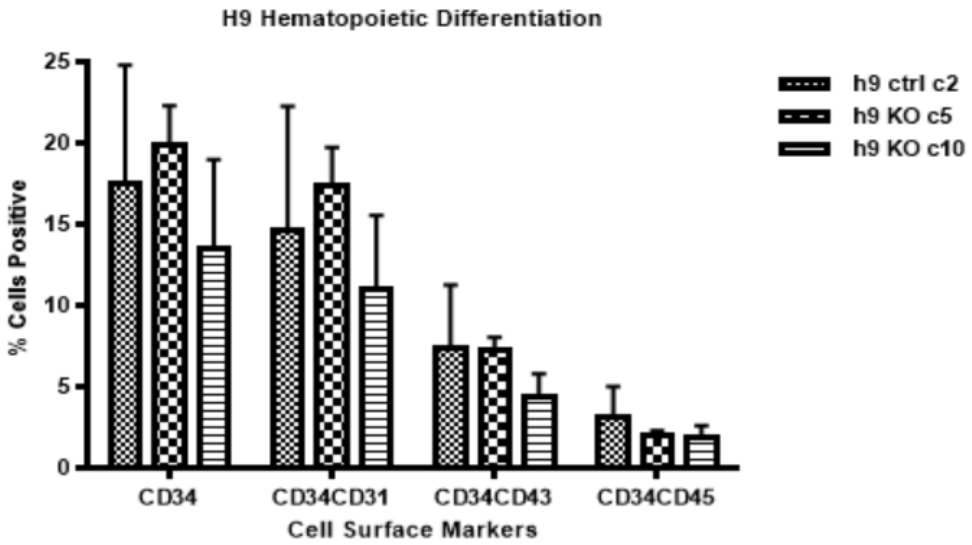
H9 Hematopoietic Differentiation
% Cells Positive
25
20
15
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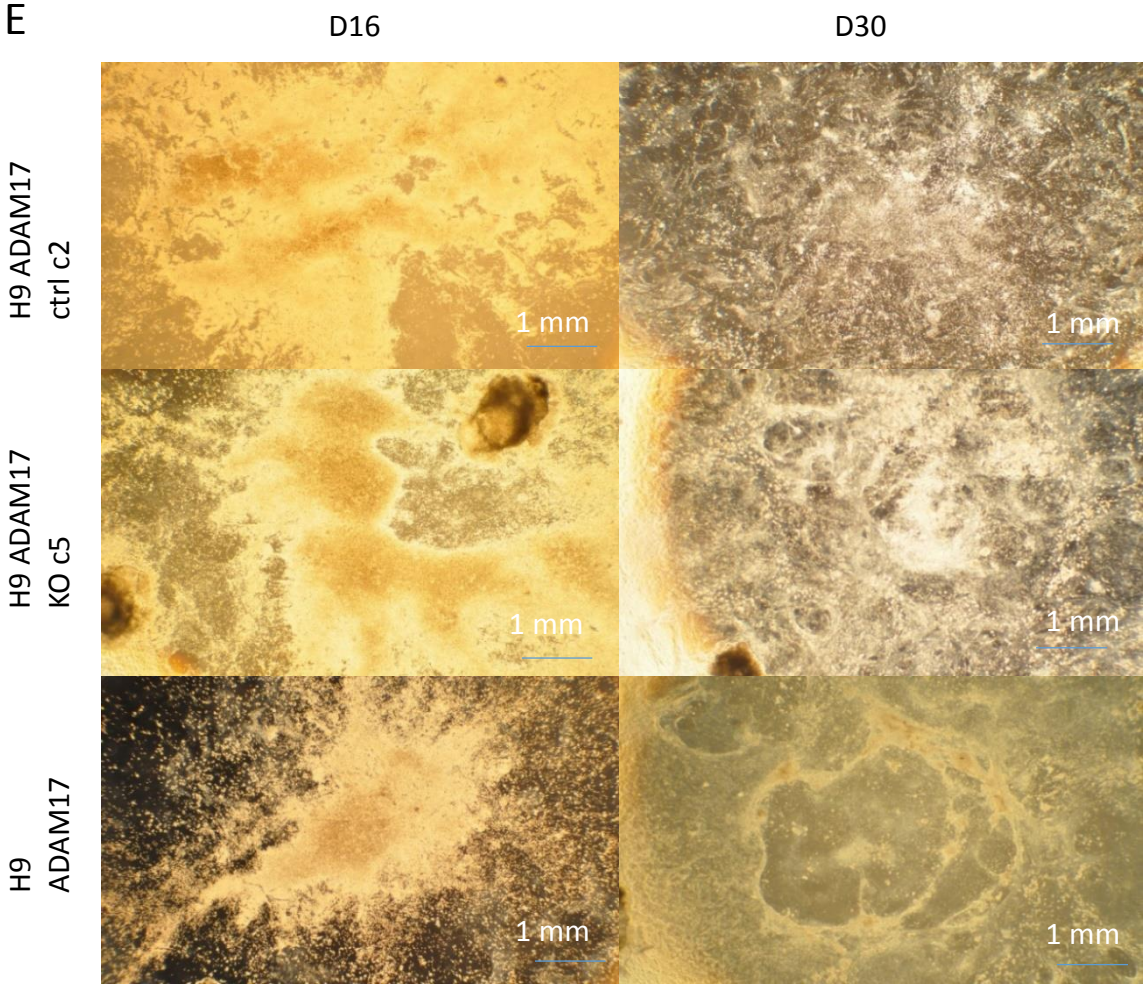
D16
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1 mm

F

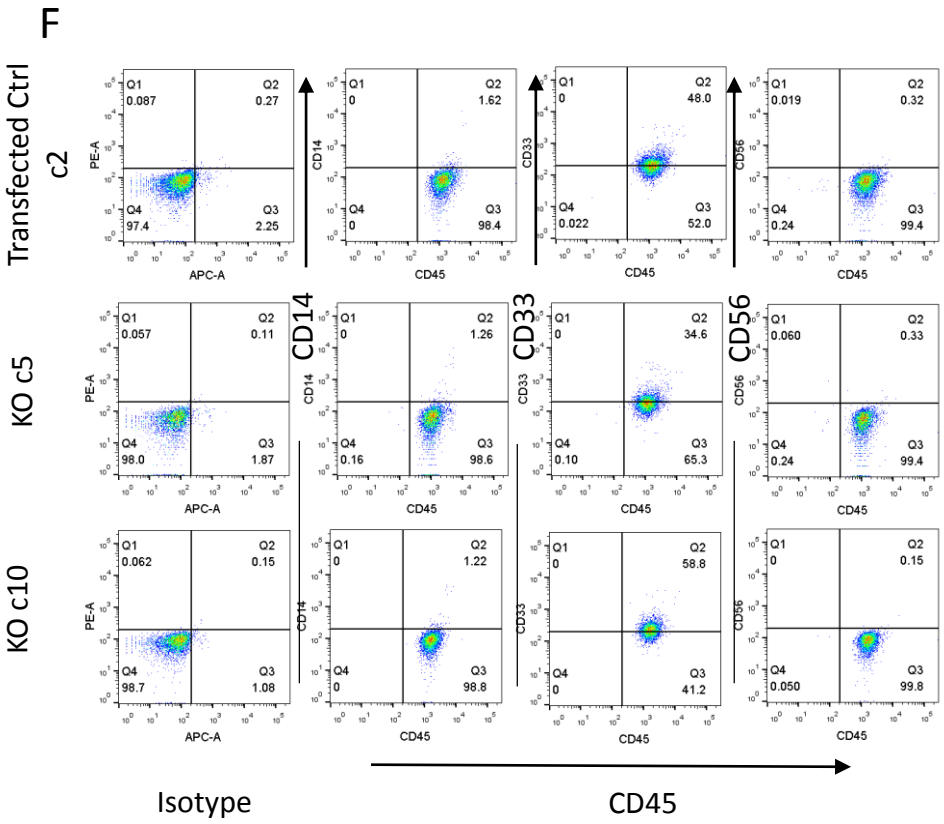
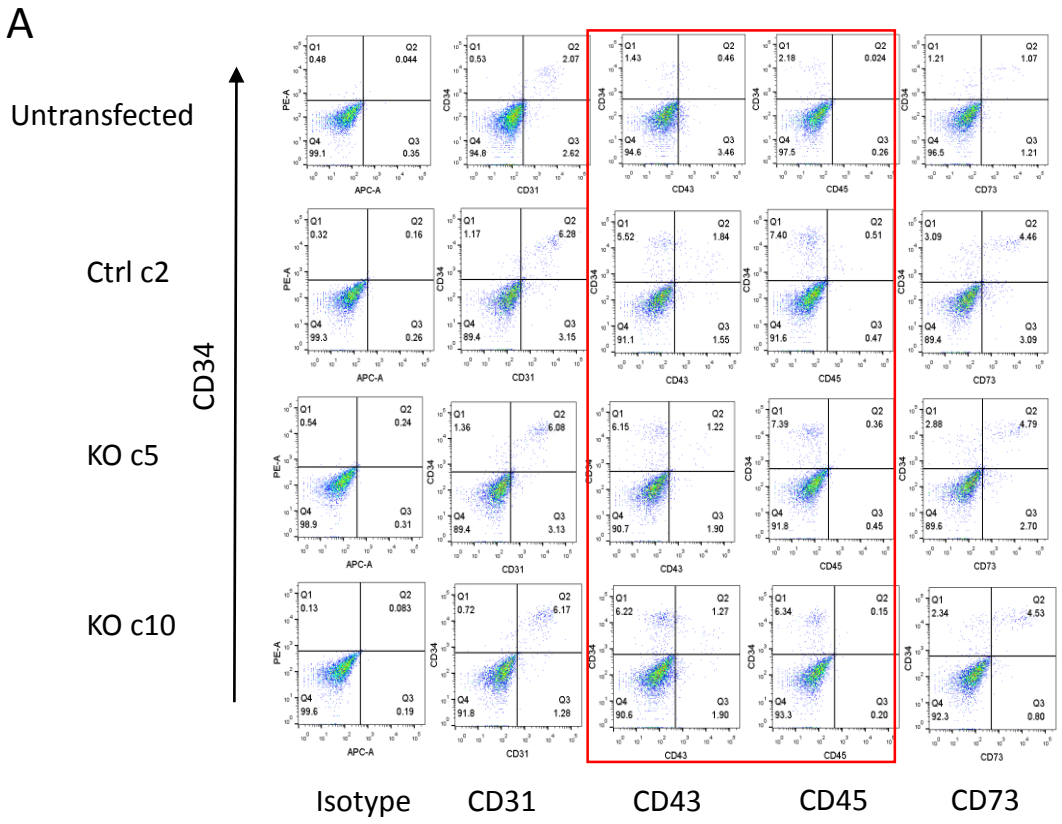
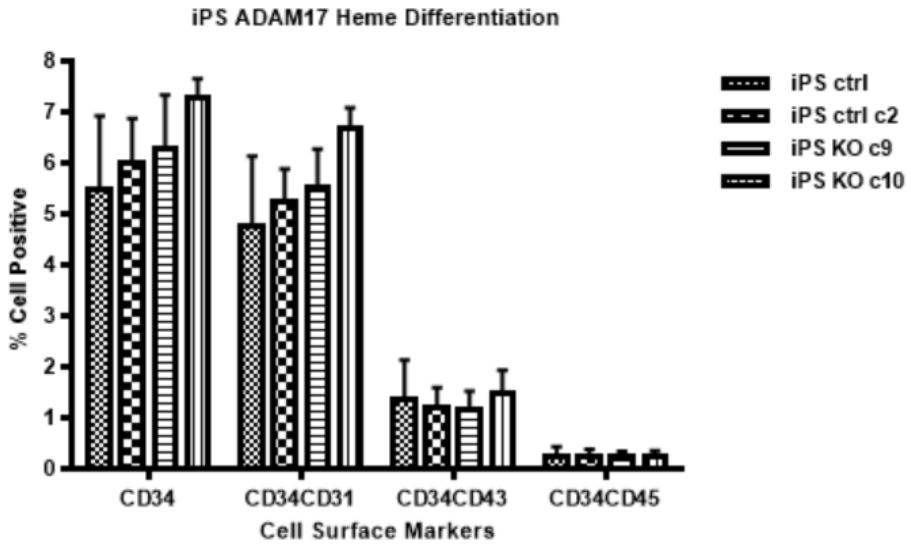

Transfected Ctrl c2
KO c5
KO c10
CD14
CD33
CD56
PE-A
APC-A
CD45
Q1 0.087
Q2 0.27
Q4 97.4
Q3 2.25
Q1 0
Q2 1.62
Q4 0
Q3 98.4
Q1 0
Q2 48.0
Q4 0.022
Q3 52.0
Q1 0.019
Q2 0.32
Q4 0.24
Q3 99.4
Q1 0.057
Q2 0.11
Q4 98.0
Q3 1.87
Q1 0
Q2 1.26
Q4 0.16
Q3 98.6
Q1 0
Q2 34.6
Q4 0.10
Q3 65.3
Q1 0.060
Q2 0.33
Q4 0.24
Q3 99.4
Q1 0.062
Q2 0.15
Q4 98.7
Q3 1.08
Q1 0
Q2 1.22
Q4 0
Q3 98.8
Q1 0
Q2 58.8
Q4 0
Q3 41.2
Q1 0
Q2 0.15
Q4 0.050
Q3 99.8
Isotype
CD45

Figure 3: Differentiation of iPSC ADAM17 knock-out clones yield hematopoietic progenitors that give rise to NK cells. A) Representative flow cytometry data from day 11 spin EB differentiation showing iPSC untransfected control cells (top), Crispr-Cas9 transfected ADAM17 expressing clone 2 (second from top), ADAM17 knock-out clone 9 (third from top) and ADAM17 KO clone 10 cells (bottom). B) Statistical analysis on the populations of iPSC untransfected ctrl, transfected ctrl c2, ADAM17 KO c9 and KO c10 derived cells $CD34^+$, $CD34^+CD31^+$, $CD34^+CD43^+$ and $CD34^+CD45^+$ after 11 days in stage one conditions (n=3) show that there is no significant difference in the differentiation of any of these hematopoiesis related cell types. C) Flow cytometry data showing differentiation of iPSC ADAM17 knock out clone 9 cells after 28 days in NK differentiation conditions. Isotype is on the left.



B


iPS ADAM17 Heme Differentiation
% Cell Positive
0
1
2
3
4
5
6
7
8
CD34
CD34CD31
CD34CD43
CD34CD45
Cell Surface Markers
iPS ctrl
iPS ctrl c2
iPS KO c9
iPS KO c10

C

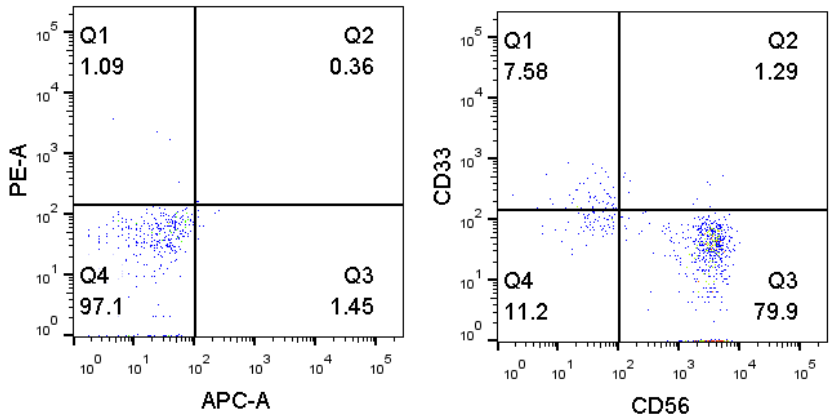
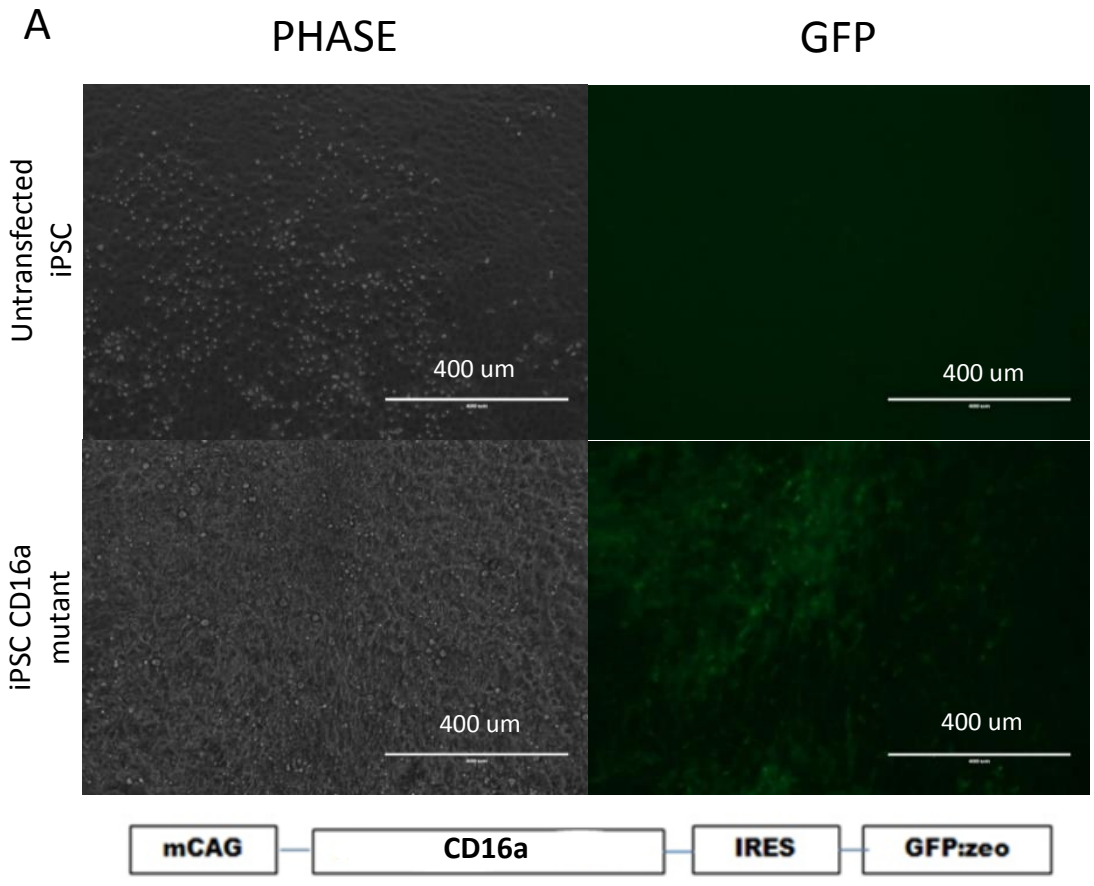
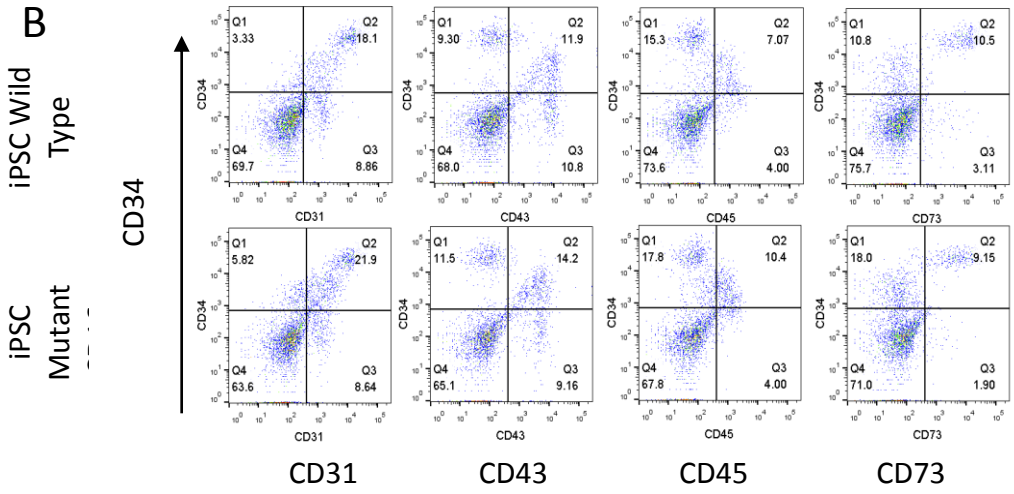

Q1
1.09
Q2
0.36
Q4
97.1
Q3
1.45
PE-A
APC-A
Isotype
Q1
7.58
Q2
1.29
Q4
11.2
Q3
79.9
CD33
CD56

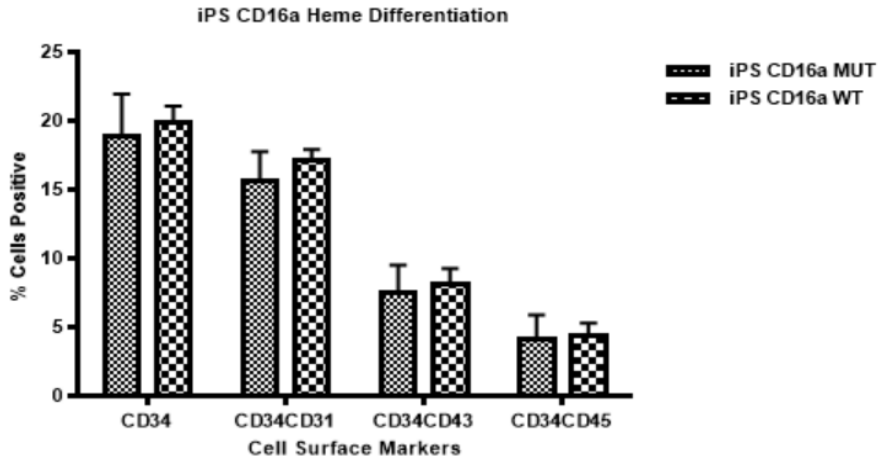
Figure 4: Differentiation of iPSC CD16a mutant and wild-type clones yield hematopoietic progenitors that give rise to NK cells. A) Phase contrast and fluorescent images showing GFP expression in NK differentiation cultures positive for CD56 cells. The top two photos correspond to untransfected iPS cells that do not express GFP. The bottom two correspond to iPSC CD16a mutant derived cells that are transfected with a pKT2-mCAG-IRES-GFP transposon that constitutively expresses GFP. Scale bars are 400 um. B) Representative flow cytometry data from day 11 spin EB differentiation showing iPSC CD16a wild-type transfected (top) and mutant transfected (bottom) cells. C) Statistical analysis on the populations of iPSC CD16a mutant and wild-type derived cells $CD34^+$, $CD34^+CD31^+$, $CD34^+CD43^+$ and $CD34^+CD45^+$ after 11 days in stage one conditions (n=5) show that there is no significant difference in the differentiation of any of these hematopoiesis related cell types. D) Flow cytometry data showing differentiation of iPSC CD16a mutant NK cells after 18 days in NK differentiation conditions.



B


iPSC Wild Type
iPSC Mutant
CD34
Q1 3.33
Q2 18.1
Q4 69.7
Q3 8.86
CD31
Q1 9.30
Q2 11.9
Q4 68.0
Q3 10.8
CD43
Q1 15.3
Q2 7.07
Q4 73.6
Q3 4.00
CD45
Q1 10.8
Q2 10.5
Q4 75.7
Q3 3.11
CD73
Q1 5.82
Q2 21.9
Q4 63.6
Q3 8.64
CD31
Q1 11.5
Q2 14.2
Q4 65.1
Q3 9.16
CD43
Q1 17.8
Q2 10.4
Q4 67.8
Q3 4.00
CD45
Q1 18.0
Q2 9.15
Q4 71.0
Q3 1.90
CD73
CD31
CD43
CD45
CD73

C


iPS CD16a Heme Differentiation
iPS CD16a MUT
iPS CD16a WT
% Cells Positive
25
20
15
10
5
0
CD34
CD34CD31
CD34CD43
CD34CD45
Cell Surface Markers

D

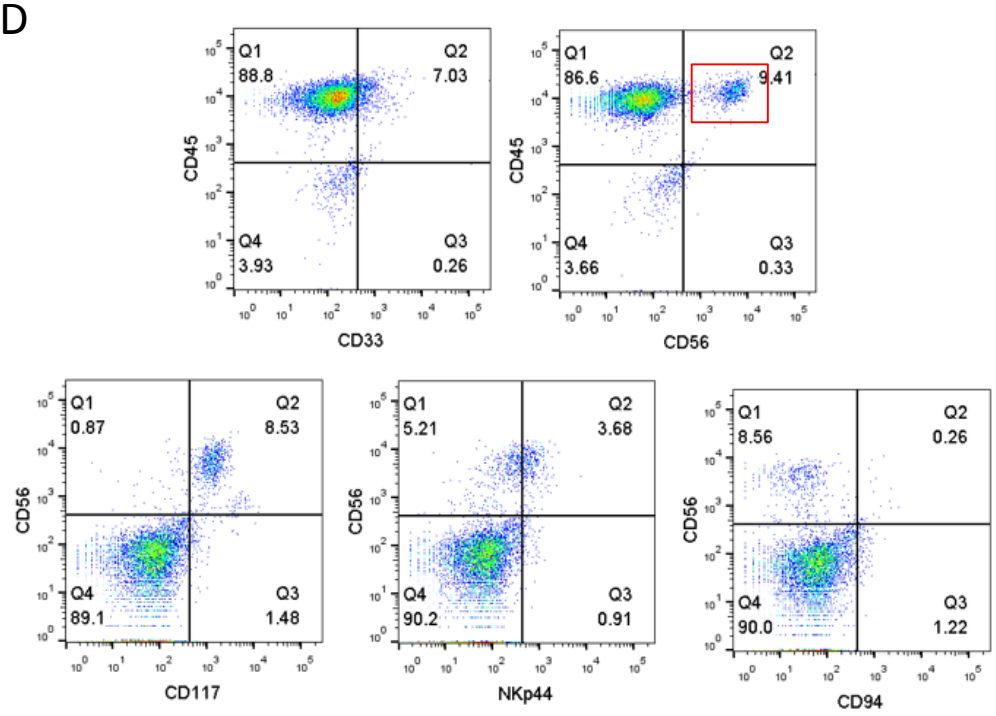

Q1
88.8
Q2
7.03
Q4
3.93
Q3
0.26
CD45
CD33
Q1
86.6
Q2
9.41
Q4
3.66
Q3
0.33
CD45
CD56
Q1
0.87
Q2
8.53
Q4
89.1
Q3
1.48
CD56
CD117
Q1
5.21
Q2
3.68
Q4
90.2
Q3
0.91
CD56
NKp44
Q1
8.56
Q2
0.26
Q4
90.0
Q3
1.22
CD56
CD94

Figure 5: Expanded NK cells have cytolytic immunophenotype. Flow cytometry analysis of iPSCNK and iPSCNK CD16a mutant cell's immunophenotype. These cells express TRAIL, a cell death receptor ligand as well as NKp46 and NKG2D which are NCRs. Finally, both of these expanded cells highly express CD16a.

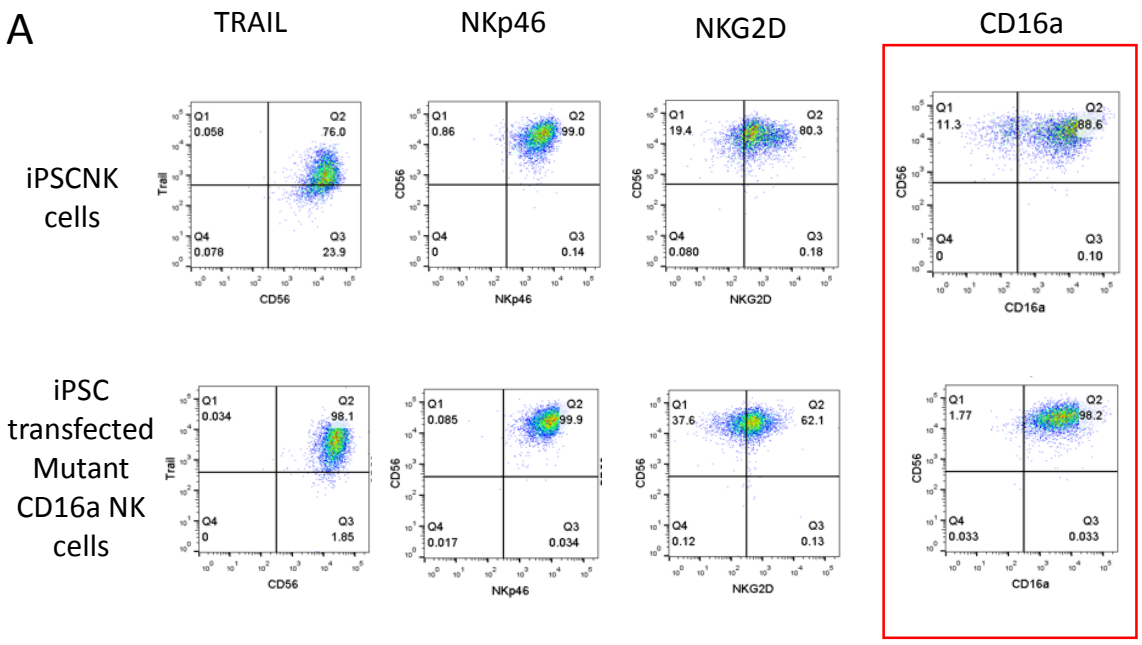
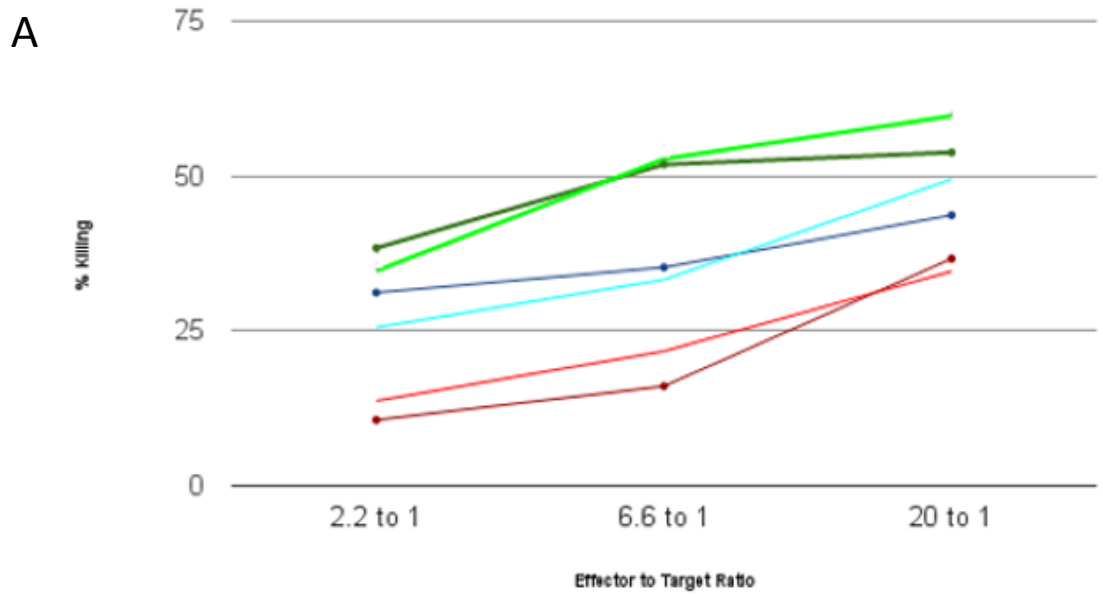
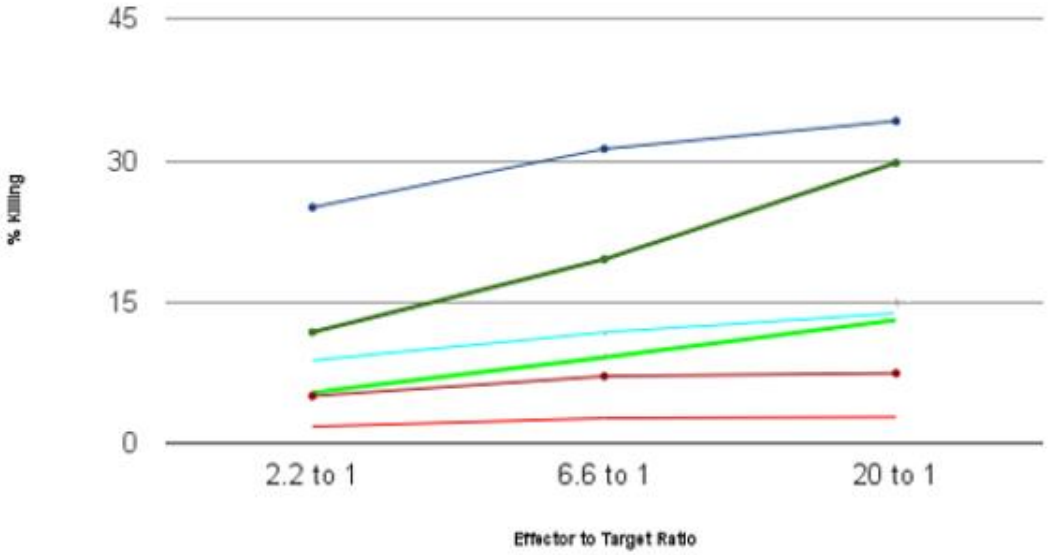


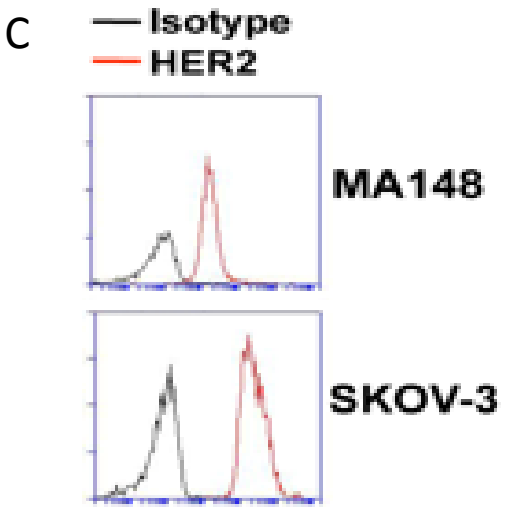
Figure 6: Chromium release assays show the effect that Herceptin has in killing HER2 overexpressing cancers when combined with NK cells. A) Standard chromium release assay showing relative killing of PBNK (green), iPSCNK (red) and iPSCNK CD16a mutant cells (blue) against K562 cells in the presence and absence of Herceptin. The darker curves with dots represent the conditions with antibody. (n=1) B) Standard chromium release assay showing relative killing of PBNK (green), iPSCNK (red) and iPSCNK CD16a mutant cells (blue) against SKOV3 cells in the presence and absence of Herceptin. The darker curves with dots represent the conditions with antibody. (n=1) C) Previous lab flow cytometric analysis showing the relative expression of HER2 on the surface of MA-148 ovarian carcinoma and SKOV3 ovarian carcinoma. The SKOV3 cells more highly express HER2.



B


45
30
15
0
% Killing
2.2 to 1
6.6 to 1
20 to 1
Effector to Target Ratio

C


Isotype
HER2
MA148
SKOV-3

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