hESC-derived NK Cells Expressing CD4/ζ CAR for Enhanced Anti-HIV-1 Cytotoxic Activity \textit{in vitro}

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ABSTRACT

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Cell-based approaches utilizing hematopoietic stem cells (HSC) and T cells with naturally occurring, or experimentally introduced, genetic modifications that confer either HIV resistance or enhanced anti-HIV properties have emerged recently as viable approaches to treating HIV infection. However, a number of technical issues, such as a lack of protocols to maintain and expand HSC in vitro, inefficient gene transfer technologies, and the induction of T cell exhaustion (due to extensive in vitro expansion prior to adoptive transfer) have impeded the progress in this area. Human embryonic stem cells (hESC), which can be used to derive any type of immune cells including natural killer (NK) cells, may be a superior source of genetically modified cells compared with primary NK cells or HSC. hESC can be maintained in culture in an undifferentiated state
indefinitely, and as such they can be extensively genetically manipulated, characterized for potential genotoxic events at the nucleotide level, and expanded to clinically relevant quantities. Given that these cells can be clonally expanded from a single cell, virtually all of the progeny of the genetically engineered hESC will carry the same modification at the same genomic position. Furthermore, as these cells can be effectively cryopreserved and differentiated into the desired lineage at different time points, the same cell populations can be used for preclinical studies and patient treatment. Importantly, in the case of lymphoid lineages, due to the extended length of their telomere ends, hESC-derived NK cells are less likely than their normal counterparts to undergo cellular senescence or immunological exhaustion upon expansion. We generated hESC lines that express an anti-HIV chimeric antigen receptor (CAR), which is a fusion molecule consisting of human CD4 with the signaling domain of the CD3 complex zeta-chain. CD4 binding to HIV-1 gp120 envelope on the surface of infected cells will trigger NK-cell recognition of infected cells and activation of their effector functions through zeta-chain signaling. Importantly, this vector also carries two anti-HIV shRNA sequences that confer protection from HIV-1 infection to the transduced cells. Here we present the phenotypic and in vitro functional characterization of the hESC-derived, anti-HIV CAR-expressing, NK cells.
Clinical data for HIV-1

The Centers for Disease Control and Prevention estimated that human immunodeficiency virus (HIV) currently infects more than 1.2 million people in the United States with a consistent rate of 50,000 new infections per year. Worldwide, there was an estimated 36.9 million people living with HIV (CDC.gov). The staggering statistical numbers demonstrate that HIV is a very infectious disease. HIV can be transmitted from an infected person to an uninfected person via bodily fluids such as blood, semen, vaginal secretions, and breast milk. These transmissions can come in the form of unprotected sex, needle sharing, during pregnancy or during childbirth, or receiving organ transplants from an HIV-infected donor. Transmission rates are high due to the long asymptomatic period during HIV infection, leading thousands unaware of their infection (Murray, Rosenthal et al. 2013).

The initial symptoms of HIV infections, which occurs within 2-4 weeks after infection, have flu-like symptoms including fever, chills, rash, night sweats, muscle aches, sore throat, fatigue, swollen lymph nodes, and mouth ulcers (Murray, Rosenthal et al. 2013). These symptoms are not exhibited by all who are infected with HIV and should not be used as a method for diagnosing HIV infection due to the symptoms being similar to flu symptoms. After this early stage of HIV infection, people infected with HIV enter a latency stage of chronic infection. At this chronic infection stage, viral replication is at low levels with some people exhibiting no HIV-related symptoms or mild symptoms. By
this stage, it is crucial for people infected with HIV to be on antiretroviral therapy to control HIV replication and maintain low levels of viral particles in their blood. If left untreated, HIV infection reaches a final stage of acquired immunodeficiency syndrome (AIDS.gov). At this final stage, viral replication has not been controlled and the virus will weaken the infected person’s immune system by killing their CD4+ helper T cells. This weakened state of the immune system will leave the infected person vulnerable to opportunistic infections such as pneumonia, lymphoma, and tuberculosis (AIDS.gov).

The infection and elimination of CD4+ helper T cells due to HIV infection disrupts the body’s normal immune response to infections. CD4+ helper T cells play a crucial role in mediating an immune response to an infection by releasing cytokines to recruit and activate B lymphocytes and cytotoxic T cells, in order to eliminate the pathogen infecting the body (Luckheeram, Zhou et al. 2012) Therefore, the loss of CD4+ helper T cells will break the chain in the immune response cascade due to the absence of CD4+ helper T cells, which are vital in recruiting and activating B lymphocytes and cytotoxic T cells to fight off the infection. When an HIV-infected person has reached the AIDS stage of HIV infection, their immune system lacks CD4+ helper T cells required to recruit B lymphocytes and cytotoxic T cells to mount an effective response against opportunistic infections (AIDS.gov; Luckheeram, Zhou et al. 2012).

Currently there are no cures for HIV infections, but there are measures that can be taken to prevent HIV infection and antiretroviral drug treatments available. Preventative measures include avoiding exposure to infected body fluids and pre-exposure prophylaxis (PrEP). The current approved pre-exposure prophylaxis is a combination of two HIV medicines, consisting of tenofovir and emtricitabine (both are non-nucleoside reverse
transcriptase inhibitors). This drug combination is sold under the name Truvada® (CDC.gov; FDA.gov).

Antiretroviral therapies (ART) help control the replication of the virus in the infected person. Controlling viral replication allows infected persons to rebuild their immune systems, helping to prevent serious infections from opportunistic infections.

There are five main antiretroviral drug classes. (1) Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs) interfere with viral reverse transcriptase’s ability to convert HIV RNA into HIV DNA (PrEP falls under this category)(FDA.gov). (2) Protease Inhibitors (PI) block the proteolytic activity of the viral protease and prevent viral particle assembly (FDA.gov). (3) Fusion Inhibitors prevent the entry of HIV into the host cell by blocking viral fusion to the cellular membrane (FDA.gov). (4) Entry Inhibitors interfere with the virus’ ability to interact with host CCR5 co-receptor and prevents viral infection of the cell (FDA.gov). (5) HIV integrase strand transfer inhibitors block HIV integrase, an enzyme required by the virus to integrate its genome into the host’s genome (FDA.gov).

These antiretroviral drugs block or inhibit crucial viral replication steps in order to keep HIV replication in an infected person at very low levels.

**HIV-1 replication cycle**

HIV-1 is a deadly virus because it replicates in and destroys CD4+ helper T cells. The coat protein gp120 found on HIV-1 recognizes and interacts with CD4 molecules found on CD4+ helper T cells (Murray, Rosenthal et al. 2013). In addition, CCR5 co-receptors expressed on CD4+ helper T cells take part in the CD4-gp120 interaction by
triggering the virus to begin fusion to the cellular membrane of the CD4+ helper T cell. It has been shown that HIV-1 requires both CD4 and CCR5 in order to begin fusion to the cellular membrane (Murray, Rosenthal et al. 2013). Some HIV-1 subtypes have the ability to utilize the CXCR4 co-receptor to trigger viral particle fusion to the host cellular membrane in the absence of CCR5 (Murray, Rosenthal et al. 2013). These two types of co-receptor requirements for HIV-1 fusion have given scientists a method to categorize the tropism of HIV-1 (Berger, Doms et al. 1998; Coakley, Petropoulos et al. 2005). R5-tropic HIV-1 requires the co-receptor CCR5 to initiate fusion while X4-tropic HIV-1 requires the co-receptor CXCR4 for fusion initiation (Coakley, Petropoulos et al. 2005). An individual infected with HIV-1 can have either R5- or X4- tropic HIV-1 or have both at one time (Coakley, Petropoulos et al. 2005). As previously mentioned, entry inhibitor antiretroviral drugs target CCR5 co-receptors to block HIV-1 interactions with the co-receptor and therefore could prevent the infection of host cells. There have also been studies showing the capabilities of shRNAs to target cellular CCR5 mRNA to mediate degradation of CCR5 mRNA (Hutter, Nowak et al. 2009; Ringpis, Shimizu et al. 2012; Kamata, Kim et al. 2015). Degradation of CCR5 mRNA would prevent the expression of CCR5 on the cell surface and protect it from HIV-1 infection (Hutter, Nowak et al. 2009; Ringpis, Shimizu et al. 2012).

After HIV-1 fuses to the cellular membrane, the packaged viral proteins including proteases, reverse transcriptase, and integrase, along with viral RNA, are released into the cellular cytoplasm. At this point, viral DNA is produced as a result of the reverse transcriptase that was packaged with the virus, converting viral RNA into viral DNA. There are non-nucleoside reverse transcriptase inhibitor antiretroviral drugs that inhibit
the activity of reverse transcriptase, blocking viral RNA from producing viral DNA. Apart from reverse transcriptase inhibitors, there have been studies showing shRNA that can target and mediate the degradation of HIV-1 RNA to prevent its conversion to DNA (Kamata, Kim et al. 2015). It is crucial for the virus to convert its genetic material from RNA to DNA during its replication cycle, because the successful production of viral DNA will then lead to its integration into the host’s genome (Liao, Marchand et al. 2010).

The integrase enzyme, packaged with the virus, will incorporate the viral DNA into the host’s genome. This will allow for the virus to take advantage of the host’s transcription and translation mechanisms to produce viral proteins and viral RNA. These viral products will be assembled to produce new viral particles to infect additional host cells. The integrase inhibitor antiretroviral drugs target viral integrase to prevent the integration of viral DNA into the host’s genome. After the production of viral proteins and RNA, these products are transported toward the cellular membrane of the infected cell for assembly of new viruses. The processed gp120 viral coat proteins (encoded by the env gene) will integrate into the cellular membrane to prepare for the budding of packaged viruses. Viral protease activity is required for correct cleavage of viral proteins for virus assembly. The protease inhibitor antiretroviral drugs target viral proteases to inhibit their proteolytic activity and prevent the assembly of new viruses (Liao, Marchand et al. 2010).

Once the assembly of new viruses is complete, the new viral particles will bud off from the host cell and take a portion of the cellular membrane to make a new viral coat. The continued production of new viral particles budding from one host cell will eventually stress the infected cell, compromising the host cell’s cellular membrane. This
will then lead to the lysis of the infected cell. These new viral particles are infectious and will travel through the body to find a new host cell. Apart from cell lysis, the infected person’s immune system will also respond to the infection by sending cytotoxic T cells and NK cells to attack the infected cells. This will begin to weaken the host’s immune system by depleting the person’s CD4⁺ helper T cells. Furthermore, CD4⁺ helper T cells play an integral part in activating CD8 cytotoxic T cells to combat infections (Murray, Rosenthal et al. 2013).

**Introduction of chimeric antigen receptors (CARs) to T cells**

Viral replication is well understood and there are many different types of antiretroviral drugs available to control the replication of HIV-1 in an infected individual. There are developments in the field of immunotherapy suggesting the use of immune cells as a way to target and destroy HIV-1 infected cells rather than trying to pharmacologically maintain low levels of HIV-1 particles in the blood (Ni, Knorr et al. 2011; Ni, Knorr et al. 2014). There have been recent developments in engineering T cells with chimeric antigen receptors (CARs), which are modified T cell receptors, to target specific cell surface proteins. These modifications redirect the target specificity of T cells expressing the chimeric antigen receptors, to target cells expressing surface proteins recognizable by the CAR. (Jackson, Rafiq et al. 2016).

The first-generation chimeric antigen receptors consist of an extracellular antigen-recognition domain linked to an intracellular signaling domain. The antigen-recognition domains are usually an antibody single-chain variable fragment, a peptide, or
a protein. The intracellular signaling domain is usually made of the CD3ζ (zeta) chain of the T cell receptor (Frigault and Maus 2016). These CARs allow the T cells to specifically target an antigen of interest, and the recognition of the protein signals the proliferation of the T cells, along with secretions of cytotoxins and cytokines, to kill the target cell expressing the protein of interest (Jackson, Rafiq et al. 2016).

**Success of CARs in medicine**

Recent studies have shown success in the use of T cells engineered with chimeric antigen receptors to target B-cell acute lymphoblastic leukemia (Jackson, Rafiq et al. 2016), chronic lymphocytic leukemia and non-Hodgkin’s lymphoma (Frigault and Maus 2016). These studies demonstrated the efficacy of T cells expressing CARs to specifically target cancer cells showing an overall response rate of 57% in patients with chronic lymphocytic leukemia. In addition, the functional CAR-T cells persisted in the patients beyond 4 years (Frigault and Maus 2016). The persistence of CAR-T cells adds to the benefit of using engineered T cells as a form of immunotherapy to reduce the need for continual dosage of anti-cancer drugs. The successes of these studies using engineered T cells expressing chimeric antigen receptors to target cancer suggests that chimeric antigen receptors can target other types of diseased cells such as those infected with HIV-1.
Natural killer cells characteristics and development

In an attempt to combat HIV-1 infections using CAR-expressing immune cells, we needed to find an appropriate white blood cell to attack HIV-infected cells. Prior studies have shown the efficacy of T cells engineered with cancer targeting CARs, but in the case of HIV, we needed a different cytotoxic white blood cell. The need for a different cytotoxic immune cell is due to HIV-1 targeting CD4+ helper T cells for infection and CD4+ helper T cells contributing to CD8+ cytotoxic T cell activation. We selected NK cells as the immune cells to express chimeric antigen receptors specific to HIV-1.

Natural killer cells have similar cytotoxic capabilities to that of CD8+ cytotoxic T cells, with differences in that they do not require prior antigen sensitization (Fauci, Mavilio et al. 2005; Caligiuri 2008; Iannello, Debbeche et al. 2008). NK cells are an important part of the innate immune system and are effective against cancer cells and virus-infected cells (Fauci, Mavilio et al. 2005; Caligiuri 2008; Iannello, Debbeche et al. 2008). Lymphoid progenitor cells give rise to lymphocytes including T cells, B cells, and NK cells. Although NK cells arise from the lymphoid lineage, they play a crucial role in the innate immune system compared to T and B cells being a part of the adaptive immune system (Sun and Lanier 2009). NK cells lack specific antigen recognition receptors (Sun and Lanier 2011) and therefore a single NK cell can recognize a wider range of cell types compared to the specific antigen receptors expressed on T and B cells.

Natural killer cells develop in multiple sites in the human body such as the thymus, lymph nodes, liver, and spleen, but the main site of development is the bone
marrow (Huntington, Vosshenrich et al. 2007). During development NK cells express specific surface receptors including killer cell immunoglobulin-like receptors (KIRs) and killer activation receptors (KARs). The killer cell inhibitory receptors are required for NK cells to establish efficient missing-self recognition. As described below, these receptors interact with MHC class I molecules leading to the generation of functional NK cells in the peripheral blood (Karre, Ljunggren et al. 1986; Ljunggren and Karre 1990; Sun and Lanier 2011). The responsiveness of NK cells depends on the number of interactions it encountered during development between its KIRs and MHC class I molecules. If the NK cells had no interaction, they become anergic or nonresponsive (Karre, Ljunggren et al. 1986; Ljunggren and Karre 1990). If the NK cells encountered two or more interactions, they will become highly responsive. These interactions prepare mature NK cells to be able to patrol the periphery of the body and recognize cancer cells or virally infected cells that have down-regulated the expression of MHC class I molecules (Karre, Ljunggren et al. 1986; Ljunggren and Karre 1990). These characteristics suggest NK cells may be a suitable tool for combating HIV-1 infections.

Using natural killer cells as an immunotherapy for HIV-1 infection

It has been shown that activation of NK cells is associated with protection against HIV-1 infection and the inhibition of HIV-1 replication both in vitro and in vivo (Alter, Martin et al. 2007; Iannello, Debbeche et al. 2008). Therefore, we have engineered NK cells to express anti-HIV-1 chimeric antigen receptors to specifically target HIV-1 infected cells in hopes that it would prove to be more effective at killing HIV-1 infected
cells. We used human embryonic stem cells (hESCs) as the starting material to generate NK cells, using an adapted *in vitro* differentiation method (Woll, Martin et al. 2005; Ni, Knorr et al. 2011).

hESCs have the pluripotent capabilities of differentiating into any cell type of the human body and being able to self-renew indefinitely to provide a large reservoir of cells with which to work (Thomson, Itskovitz-Eldor et al. 1998). Studies have confirmed the expansion of stem cells in culture using mouse embryonic fibroblasts as feeder cells and efficient, viable cryopreservation in liquid nitrogen (Galic, Kitchen et al. 2006; Galic, Kitchen et al. 2009). It has also been shown that hESCs can be differentiated in a controlled manner to give rise to human blood cells including HSCs, T cells, and NK cells (Woll, Martin et al. 2005; Galic, Kitchen et al. 2006; Galic, Kitchen et al. 2009). These tools allowed us to produce clinically relevant numbers of NK cells to test the efficacy of the addition of anti-HIV-1 chimeric antigen receptors. Additionally, the transduction of hESCs enabled us to successfully isolate transduced hESCs and to expand the cell line to give us a genetically homogenous population of cells compared to transducing patient-extracted NK cells. My results suggest that the generation of NK cells using hESCs is a potential tool for HIV-1 immunotherapy.
MATERIALS AND METHODS

Human embryonic stem cell culturing media

Human embryonic stem cell (HES) media was used during the culturing and expansion of hESCs on mouse embryonic fibroblasts (MEFs) feeder layer. Media in the cultures were exchanged for fresh media on a daily basis. HES media was made with D-MEM/F12 containing 20% knockout serum, 2 mM concentration of L-glutamine, 8 ng/ml of basic fibroblast growth factor (bFGF), 100 µM of each nonessential amino acids (NEAA), and 0.1 µM of β-mercaptoethanol (2-ME) (Galic, Kitchen et al. 2006).

Mouse embryonic fibroblast culturing media and feeder layer plating

Mouse embryonic fibroblasts (MEFs) were plated onto 6-well plates coated with 0.1% gelatin a day prior to the passaging of hESCs. MEFs were plated at a cell density of 3x10^5 per well in a 6-well plate. MEF media was made with D-MEM containing 10% fetal bovine serum (FBS), 2 mM of L-glutamine, and 100 µM of NEAA.

Human embryonic stem cell expansion method

Human embryonic stem cells were cultured in 6-well plates and were passaged weekly. HES media was removed from the cultures and collagenase (1 mg/ml in D-MEM/F12) was added and incubated at 37°C for approximately 5 minutes or until the edges started to curl up. At that point, 1-2 ml of HES media was added and colonies were broken up
into small fragments with a glass pipet. Cells were transferred to a test tube and centrifuged at 1,000 rpm for 5 minutes. The supernatant was removed and HES media was added for another centrifugation at the same speed and duration. The supernatant was removed and cells were resuspended with enough HES media to plate 2 ml per well in a 6-well plate. A 40 µm nylon cell strainer was set inside of a different test tube and the resuspended cells were passed through the cell strainer. Cell fragments that passed through the cell strainer were then equally split into wells of 6-well plates that contained a feeder layer of MEFs (Galic, Kitchen et al. 2009). All work with human embryonic stem cells was approved by the UCLA Embryonic Stem Cell Research Oversight Committee.

**Embryoid bodies formation for hematopoietic stem cell differentiation method**

Human embryonic stem cells were differentiated into HSCs by forming embryoid bodies (EBs) with dissociated hESCs. These embryoid bodies were then cultured with cytokines for 15 days to obtain HSCs. Embryoid bodies were formed by first removing HES media from hESC cultures and 1 ml of dispase (0.5 mg/ml in D-MEM/F12) was added to each well of a 6-well plate. These plates were then incubated for approximately 10 minutes. Colonies of hESCs were then dissociated gently with a glass pipet and collected into 50 ml test tubes for centrifugation at 1.0 x 1,000 rpm for 2 minutes. The supernatant was removed and EB media was added to wash and break up large cell aggregates. Cells were then centrifuged once more at 1.0 x 1,000 rpm for 90 seconds. The supernatant was removed and EB media was added to resuspend the cells. The resuspension was split into new 6-well low-attachment plates with a final media volume of 3 ml. EB media was
made with Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 15% non-heat-inactivated defined fetal bovine serum, 1% NEAA, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 units/ml penicillin, and 100 µg/ml streptomycin. EB media was exchanged every 2 days and starting on day 4, EB media was supplemented with 10 ng/ml bone morphogenetic protein-4 (BMP-4), 300 ng/ml stem cell factor (SCF), and 20 ng/ml Flt-3 ligand. Starting on day 12, EB media was no longer supplemented with BMP-4 (Galic, Kitchen et al. 2009).

**Sorting of CD34+ hematopoietic stem cells**

Following differentiation with EB media supplemented with cytokines, cells were sorted for CD34+ HSCs using anti-CD34 multisort microbeads (Galic, Kitchen et al. 2009).

**AFT-024 culturing media and plating for co-culture with hematopoietic stem cells**

AFT-024 fetal liver-derived stromal cells were plated as a feeder layer for NK cell differentiation a day prior to purification of CD34+ HSCs. AFT-024 cells were plated at a cell density of 5.0 x 10^5 cells per well in a 6-well plate with 2 ml of AFT-024 media.

AFT-024 media was made with D-MEM consisting of 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.05 µM β-mercaptoethanol (Woll, Martin et al. 2005).
Natural killer cells differentiation media and method

CD34+ HSCs were sorted and co-cultured with AFT-024 stromal cells that had been plated prior. Hematopoietic stem cells were plated at a cell density of 1.0 x 10^5 cells per well in a 6-well plate. NK cell differentiation media was exchanged every 3-4 days. NK cell differentiation media was made with DMEM supplemented with 28.3% F-12 (HAM), 15% heat-inactivated (56°C for 1 hour) Human AB serum, 1 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 25 μM β-mercaptoethanol, 2.5 μg sodium selenite, 50 μM ethanolamine, 28.95 μ/ml L-ascorbic acid, 20 ng/ml SCF, 20 ng/ml IL-7, 10 ng/ml IL-15, 20 ng/ml Flt-3 ligand, and 5 ng/ml IL-3. IL-3 was only supplemented in the NK cell differentiation media for the first 7 days (Woll, Martin et al. 2005).

Fluorescence activated cell sorting for natural killer cell surface markers

During NK cell differentiation, cells were sampled from 6-well plates and were antibody labeled for NK cell surface markers. Cells were labeled with monoclonal antibodies for CD4, CD16, CD45, CD56, CD158b, CD314, CD335, and CD336. Monoclonal antibodies were purchased from Beckman Coulter, eBioscience, and BioLegend. Samples were analyzed by flow cytometry using a BD LSRFortessa (BD BioSciences) and phenotypes determined using FlowJo software (Woll, Martin et al. 2005).
Immunohistochemistry for pluripotent stem cell markers

CD4/ζ CAR-transduced hESCs were characterized for pluripotent stem cell markers as previously described (Galic, Kitchen et al. 2006).

GFP-expressed hESCs-derived natural killer cells cytotoxic function killing assay

Natural killer cells derived from EGFP-transduced hESCs were tested for their cytotoxic functions by culturing with target K562 cells. K562 cells are human chronic myelogenous leukemia cells and are commonly used in NK cell cytotoxic assays. The killing assays were performed as described previously (So, Sallin et al. 2013), with changes in antibody labeling and live/dead staining. NK cells and target K562 cells were co-cultured at effector to target ratios of 0, 0.1, 1, and 10. A BD LSRFortessa (BD BioSciences) flow cytometer was used to analyze cleaved-caspase 3 in target K562 cells with cleave-caspase 3 antibodies along with live/dead stain performed with Zombie Yellow Fixable Viability Kit (BioLegend) (Woll, Martin et al. 2005; Galic, Kitchen et al. 2006). Statistical analyses performed using one way ANOVA with Bonferroni’s multiple comparison post test.
CD4/ζ CAR expressed hESCs-derived natural killer cells cytotoxic function killing assay

Natural killer cells generated from hESCs transduced with CD4/ζ CAR vector were tested for their cytotoxic functions and specificity for targeting HIV-1 latently infected U1 cells. Cytotoxic assays were performed as described previously (So, Sallin et al. 2013) with changes in antibody labeling and live/dead staining. U1 HIV-1-infected cells were pretreated with the latency reversing agent prostratin overnight prior to co-culture and washed thoroughly. CD4/ζ CAR expressed NK cells were co-cultured with target U1 cells at varying effector to target cell ratios of 0, 0.31, 0.63, 1.25, 2.5, and 5. Gag protein expression in U1 cells were labeled using anti-HIV-1 core antigen, clone: KC57 antibodies (Fisher Scientific) along with live/dead stain performed with GHOST Red 780 (TONBO biosciences). Data points represent means with standard deviation represented by y-error bars. Statistical analyses performed using two way ANOVA with Bonferroni’s multiple comparison post test.
RESULTS

Fluorescence-activated cell sorting (FACS) analysis of differentiated H1 hESC-derived NK Cells for specific NK cell surface markers

In order to reach our goal of using genetically modified NK cells to specifically kill HIV-infected cells, we began by generating NK cells from H1 hESCs. This H1 hESCs cell line had not been genetically modified to target HIV infected cells, but was transduced with a vector containing green fluorescent protein (GFP) to allow us to identify cells that have been successfully transduced. This step was taken to show the differentiation process was successful in generating NK cells from hESCs. We adapted a developed protocol to generate NK cells from hESCs as seen in the differentiation schematic in Figure 1. The differentiation process began with the expansion of hESCs on mouse embryonic fibroblasts. Then the cells were differentiated to HSCs using an embryoid bodies culture method. Hematopoietic stem cells were co-cultured with AFT-024 (mouse fetal liver-derived stromal cells) and further differentiated and developed into mature NK cells. Towards the end of the differentiation process, cells from the co-culture in step 4, Figure 1, were harvested and labeled with antibodies for specific NK cell surface markers. FACS analysis was performed to characterize the antibody-bound cells displayed in Figure 2. The cell populations of interest were first gated by their forward and side scatter profiles and were subsequently gated based on GFP expression. In the cell population of interest, 93 percent of the gated cells expressed GFP and CD45+ markers. This told us that we differentiated our hESCs, which expressed GFP, into lymphoid lineage cells. Further analysis of GFP positive cells revealed that 61 percent of GFP positive cells
expressed both CD45\(^+\) and CD56\(^+\) NK cell markers. Additional NK cell surface markers were examined including: CD158b, CD314, CD335, and CD336. In the CD45\(^+\)/CD56\(^+\) population, 52 percent of the cells expressed both CD158b and CD336 markers, in addition, 82 percent of the population expressed both CD314 and CD335 markers. The positive detection of the previously listed surface markers confirmed the presence of mature NK cells (So, Sallin et al. 2013; Ni, Knorr et al. 2014). This analysis was performed in collaboration with lab member Hongying Chen at UCLA.
Figure 1. Flow chart of natural killer (NK) cell differentiation starting from human embryonic stem cells (hESCs). EGFP-transduced hESCs were first expanded on mouse embryonic fibroblasts (MEF) and then embryoid bodies of hESCs were formed. Embryoid bodies were cultured with media containing BMP-4, SCF, and Flt3-L for 15 days. Embryoid bodies were then dissociated into single cells and purified with magnetic beads for CD34+ cells. Purified CD34+ HSCs were plated onto AFT-024 feeder layers for NK differentiation. NK differentiation media contains cytokines such as IL-3 (for the first 7 days of culture), IL-7, IL-15, Flt3-L, and SCF. A list of cytokines and additional key ingredients is to the right of the culturing step. These methods were adapted from Dan Kaufman’s lab (Woll, Martin et al. 2005; Ni, Knorr et al. 2011).
Figure 2. Fluorescence-activated cell sorting (FACS) analysis of differentiated H1 hESC-derived NK Cells for specific NK cell surface markers. Hematopoietic stem cells co-cultured with AFT-024 and NK differentiation medium were stained for NK cell surface markers. Cells were first gated by their forward and side scatter profiles and were subsequently gated based on green fluorescent protein (GFP) expression. Cells expressing both CD45 and CD56 were gated to examine for additional NK surface markers including: CD336, CD158b, CD335, and CD314. FACS analyses were performed in collaboration with lab member Hongying Chen at UCLA.
Measuring cleaved caspase 3 and using live/dead staining to examine cytotoxic functions of H1 hESC-derived natural killer cells

Once mature NK cells derived from hESCs were confirmed with the detection of NK cell surface markers, the cells were tested for cytotoxic functions. H1 hESC-derived NK cells were co-cultured with CellTrace Violet labeled K562 target cells at various effector:target (E:T) ratios. K562 cells are myelogenous leukemia cells that are sensitive to NK cells, allowing us to observe and measure the killing capabilities of the NK cells that were generated. Cells were then stained with Zombie Yellow, which stains dead cells, along with cleaved caspase 3 antibodies, which detects cells actively undergoing apoptosis. The results showed that there was an increase in cleaved caspase 3 and dead cells as the ratio between NK cells and K562 in the co-culture increased. Representative flow cytometry histograms and dot plots represent cleaved caspase 3 and Zombie Yellow stained dead target cells seen in Figure 3. These data were obtained by lab member Emily Lowe at UCLA. Further statistical analysis was performed to quantitatively analyze the cytotoxic functions of the generated NK cells.
Figure 3. Measuring cleaved Caspase 3 and using live/dead staining to examine cytotoxic functions of H1 hESC-derived natural killer cells. H1 hESC-derived NK cells were co-cultured with CellTrace Violet labeled K562 target cells at various effector:target (E:T) ratios. Cells were then stained with Zombie Yellow, which stains dead cells, along with cleaved caspase 3 antibodies, which detects cells actively undergoing apoptosis. Representative flow cytometry histograms and dot plots are of cleaved caspase 3 and Zombie Yellow stained dead target cells. These data were obtained by lab member Emily Lowe at UCLA.
Statistical analysis of a cleaved caspase 3-based killing assay using H1 hESC-derived natural killer cells

One way ANOVA with Bonferroni’s multiple comparison post-test statistical analysis of the killing assays were performed to test for significant differences between varying effector:target cell ratios. Specific killing is defined as the percent of cells with cleaved caspase 3 at a specified E:T ratio minus the percent cleaved caspase 3 in target cells alone. It can be seen in Figure 4 that with increased numbers of NK cell to K562 cell ratios, there was a higher mean percentage of cleaved caspase 3, indicating K562 cells were undergoing apoptosis. In addition, it was also shown that increased numbers of K562 cells underwent apoptosis due to the specific targeting and cytotoxic function of the NK cells. One way ANOVA with Bonferroni’s multiple comparison post test statistical analyses were performed by Emily Lowe of UCLA. Up to this point, we have shown that we were able to generate NK cells from hESCs using a protocol that we have adapted and have shown that these NK cells have cytotoxic functions. Our next step was to create a genetically modified H1 hESCs cell line that specifically targets HIV-infected cells.
Figure 4. Statistical analysis of a cleaved caspase 3-based killing assay using H1 hESC-derived natural killer cells. (Left) Bar graph shows the mean percent of cells with cleaved caspase 3 from three independent experiments. The y-error bars indicate standard deviation. (Right) Specific killing is defined as percent of cells with cleaved caspase 3 at specified E:T ratio minus the percent cleaved caspase 3 in target cells alone. Statistical analyses were performed using one way ANOVA with Bonferroni’s multiple comparison post test. *$P<0.05$, ***$P<0.0001$. Statistical analyses were performed by Emily Lowe of UCLA.
Anti-HIV-1 chimeric antigen receptor (CAR) in the triple CD4/ζ chain CAR lentiviral vector

H1 hESCs were transduced with a triple CD4/ζ CAR lentiviral vector to generate NK cells expressing CD4/ζ chimeric antigen receptors. The schematic of a portion of the vector can be seen in Figure 5. The expressions of the enhanced green fluorescent protein (EGFP) and CAR genes were controlled by the constitutive ubiquitin C promoter. EGFP expression, which is a protein that fluoresces green, allowed for the identification of successfully transduced cells. The chimeric antigen receptor consists of the extracellular and transmembrane domains of human CD4 fused to the cytoplasmic domain of the CD3 T-cell receptor zeta chain. The CD4 portion of the receptor allows for the recognition of HIV-1 gp120 viral coat proteins, while the CD3 portion signals NK cells to release cytotoxic granzyme and perforin to induce apoptosis in HIV-infected cells. The gp120 viral coat proteins are present on the surface of HIV-1 infected cells that are actively producing viral particles. To protect the transduced cells from HIV-1 (R5-tropic HIV-1) infection and (both R5- and X4-tropic strains of HIV-1) replication, the vector also contains CCR5 shRNA and shRNA516. CCR5 shRNA and shRNA516 mediate degradation of cellular CCR5 mRNA and HIV RNA respectively. CCR5 co-receptor and CD4 on T cells are needed for the fusion of HIV to the T cell. The control vector only expresses EGFP and excludes the gene for the CD4/ζ chain CAR. The vector and vector schematic were generated and kindly provided by Drs. Irvin Chen and Masakazu Kamata of UCLA. This vector was used to transduce H1 hESCs and to differentiate the transduced hESCs into NK cells following the protocol in Figure 1, ultimately generating NK cells expressing CD4/ζ CARs to specifically target HIV infected cells.
Figure 5. Anti-HIV-1 chimeric antigen receptor (CAR) in the triple CD4/CD3 zeta chain CAR lentiviral vector. Constitutive expressions of the EGFP and CAR genes were controlled by the ubiquitin C promoter. EGFP expression allowed for the identification of successfully transduced cells. The CAR consists of the extracellular and transmembrane domains of human CD4 fused to the cytoplasmic domain of the CD3 T-cell receptor zeta chain. To protect the transduced cells from HIV-1 (R5-tropic HIV-1) infection and (both R5- and X4-tropic strains of HIV-1) replication, the vector also contains CCR5 shRNA and shRNA516 respectively. CCR5 shRNA and shRNA516 mediate degradation of cellular CCR5 mRNA and HIV RNA respectively. The control vector only expressed EGFP and excludes the gene for the CD4/CD3 zeta chain CAR. The vector and vector schematic were generated and kindly provided by Drs. Irvin Chen and Masakazu Kamata of UCLA.
Karyotyping of hESCs that have been transduced with either control or CD4/ζ CAR vectors

Post transduction of hESCs with the CD4/ζ CAR vector, hESCs were expanded and examined for EGFP expression. The presence of green fluorescence indicated the success of the transduction. These successfully transduced hESCs were then characterized by karyotyping to ensure cells had not developed any damages, losses, or additions of chromosomes (Bates 2011). The karyotype results, as seen in Figure 6, indicated that the insert of the vector into the transduced hESC did not produce gross abnormalities or defects in their chromosomes. Immunohistochemistry and teratoma assays were next performed after it was determined that these transduced hESCs did not accrue genetic damages.
Figure 6. Karyotyping of hESCs that have been transduced with either control or CD4/zeta CAR vectors. The karyotype results indicated the insert of the vector into the hESC did not produce gross abnormalities in their chromosomes. This figure was obtained from Drs. Irvin Chen and Masakazu Kamata at UCLA.
**Immunohistochemistry characterizing pluripotent state of control and CAR-transduced H1 human embryonic stem cells (hESCs)**

Further characterizations of successfully transduced hESCs were required to confirm the pluripotent state of the hESCs. This step was important because we had to ensure that the hESCs were still pluripotent and would allow us to differentiate them into NK cells. Immunohistochemistry was performed to analyze transduced hESCs for the pluripotent stem cell markers OCT4, SSEA-4, TRA-1-60, and TRA-1-81 (Thomson, Itskovitz-Eldor et al. 1998; Sundberg, Andersson et al. 2011; Christensen, Calder et al. 2015). As shown in Figure 7, the detection of these surface markers is indicated by green fluorescence seen throughout the cells. These data were obtained by Drs. Irvin Chen and Masakazu Kamata of UCLA. This test confirmed that our transduced hESCs were still pluripotent due to the expression of known pluripotent cell proteins.
Figure 7. Immunohistochemistry characterizing pluripotent state of control and CAR-transduced H1 human embryonic stem cells (hESCs). The pluripotency of control and CAR-transduced H1 hESCs were analyzed using immunohistochemistry for stem cell markers OCT4, SSEA-4, TRA-1-60, and TRA-1-81. Positive detection of stem cell markers is shown in green fluorescence along with DNA staining with DAPI, shown in blue (in the top right corner for each sample respectively). These data were obtained by Drs. Irvin Chen and Masakazu Kamata of UCLA.
Teratoma assay of control and CAR-transduced H1 human embryonic stem cells to test pluripotent capabilities

The final characterization test for pluripotency is the teratoma assay. This assay tests the capabilities of the transduced hESCs to differentiate into the three different germ layers (Gropp, Shilo et al. 2012). The three germ layers are the ectoderm, endoderm, and mesoderm. These three germ layers give rise to distinct types of cells in the human body during development. The ectoderm gives rise to cells of the nervous system, cornea, and epidermis. The endoderm gives rise to cells of the digestive tube, respiratory structures, and accessory digestive organs. The mesoderm gives rise to cells that make up muscles, bones, vascular system, and urogenital organs (Alberts, Johnson et al. 2008). As seen in Figure 8, both control and CAR-transduced hESCs were shown to be capable of producing cells of the ectoderm, endoderm and mesoderm germ layers. This figure is courtesy of Drs. Irvin Chen and Masakazu Kamata at UCLA. Extensive characterization of our transduced hESCs confirmed that our hESCs have been successfully transduced with the CD4/ζ vector and that our hESCs are pluripotent with the capability to differentiate into any cell type.
Figure 8. Teratoma assay of control and CAR-transduced H1 human embryonic stem cells to test pluripotent capabilities. These cell lines were tested for their ability to differentiate into the three different germ layers. Both control and CAR-transduced hESCs were shown to be capable of producing cells of the ectoderm, endoderm and mesoderm germ layers. This figure is courtesy of Drs. Irvin Chen and Masakazu Kamata at UCLA.
FACS analysis of differentiated CD4/ζ CAR-transduced NK cells for specific NK cell surface markers

After characterization and confirmation of pluripotency, transduced hESCs were differentiated to make NK cells following the differentiation protocol described previously in Figure 1. CD4/ζ CAR-transduced NK cells co-cultured with AFT-024 in NK differentiation media were stained with fluorescent antibodies specific to NK cell surface markers (CD56, CD158b, and CD335) (So, Sallin et al. 2013; Ni, Knorr et al. 2014) and analyzed with FACS as shown in Figure 9. Initial gating was based on the forward and side scatter profile typical for NK cells. The cells were subsequently gated for cells expressing GFP due to the expression of EGFP gene found on the vector. Eighty-nine percent of cells expressed GFP from the initial forward and side scatter gating. The GFP positive cells were examined for the presence of NK cell surface markers, along with CD4, which are found on cells expressing CD4/ζ CARs. The dot graphs indicate that more than 90 percent of the GFP positive cells were expressing CD4 and mature NK cell surface markers (CD56, CD158b, and CD335). These FACs analyses confirm mature NK cells have been derived from transduced hESCs using the differentiation protocol. FACs analyses were performed in collaboration with Hongying Chen at UCLA.
Figure 9. FACS analysis of differentiated CD4/zeta CAR-transduced NK cells for specific NK cell surface markers. CD4/zeta CAR-transduced NK cells co-cultured with AFT-024 in NK differentiation media were stained with fluorescent antibodies specific to NK cell surface markers and analyzed with FACs. Initial gating was based on the forward and side scatter of cells. The cells were subsequently gated for cells expressing GFP. The sorted cells were examined for the presence of NK cell surface markers. Positive detection for CD56, CD4, CD158b, and CD335 can be seen on the bottom portion of the figure. FACS analyses were performed in collaboration with Hongying Chen at UCLA.
**CD4/ζ CAR-transduced natural killer cells killing assays**

Following the success of generating NK cells expressing CD4/ζ CARs confirmed by FACS analysis in Figure 9, transduced mature NK cells were collected and tested for their cytotoxic functions. NK cells containing CD4/ζ or CD19 CAR were used in a killing assay with U1 (latently infected with HIV-1) target cells at various E:T ratios to test specific killing. CD19 CAR recognizes CD19 receptors expressed on B cells and B cell lymphomas, which in this case was used as a control. In Figure 10, NK cells expressing CD4/ζ CAR displayed a decreased mean percentage of intracellular Gag expression in U1 target cells. Gag expression is indicative of viral structural protein production and was labeled using anti-HIV-1 core antigen, clone: KC57 antibodies, and measured using flow cytometry. The decrease in Gag expression in U1 target cells implied that the U1 cells were not producing viral particles. Live/dead staining, using GHOST Red 780, revealed that CD4/ζ CAR NK cells co-cultured with KC57+ cells have more dead KC57+ cells compared to CD19 CAR NK cells co-cultured with KC57+ cells. This suggested that CD4/ζ CAR NK cells targeted HIV-1 infected cells with more specificity compared to CD19 CAR NK cells and were able to induce apoptosis in U1 cells to halt the production of viral particles. The killing assays and two way ANOVA with Bonferroni’s multiple comparison post test were performed by Emily Lowe at UCLA.
Figure 10. CD4/zeta CAR-transduced natural killer cells killing assays. Natural killer cells containing CD4/zeta or CD19 CAR were used in a killing assay with U1 (latently infected with HIV-1) target cells at various E:T ratios to test specific killing. CD19 CAR recognizes CD19 receptors expressed on B cells and B cell lymphomas, which in this case was used as a control. (Left) NK cells expressing CD4/zeta-CAR displayed a decreased percentage of intracellular Gag expression in U1 target cells. Gag expression is indicative of viral structural protein production and was measured using flow cytometry with clone KC57 antibody. (Right) Live/dead staining revealed that CD4/zeta co-cultured with KC57+ cells have more dead KC57+ cells compared to CD19 CAR co-cultured with KC57+ cells. Live/dead staining was done with GHOST Red 780. All data points represent mean with standard deviation. Statistical analyses performed using two way ANOVA with Bonferroni’s multiple comparison post test. *P<0.05, **P<0.01, ***P<0.001. This figure was produced by Emily Lowe at UCLA.
DISCUSSION

Summary and significance of results

The pluripotent characteristics of hESCs enabled scientists to use hESCs to generate, in a controlled manner, any cell type of the human body including blood cells, HSCs, NK cells, and T cells) (Woll, Martin et al. 2005; Galic, Kitchen et al. 2006; Galic, Kitchen et al. 2009). hESCs also have the ability to self-renew indefinitely, enabling the expansion of hESCs using mouse embryonic fibroblasts as feeder cells (Thomson, Itskovitz-Eldor et al. 1998). Due to the undifferentiated state of hESCs maintained in culture, they can undergo extensive genetic manipulation and genotypic characterizations. hESCs can be clonally expanded from a single cell and therefore all of the progeny of the genetically modified hESCs will carry the same modification at the same genomic position compared to transducing patient-extracted peripheral blood mononuclear cells (PBMCs), in which case the modification position may differ from one cell to the other. Furthermore, viable cryopreservation methods of hESCs using liquid nitrogen have been demonstrated as an efficient method to maintain a stable source of hESCs (Galic, Kitchen et al. 2006; Galic, Kitchen et al. 2009). The ability to indefinitely expand and cryopreserve hESCs provides a large reservoir of cells to work with compared to using PBMCs extracted from a donor. These advantages of using hESCs compared to using PBMCs, would enable us to produce clinically relevant numbers of NK cells to test the efficacy of the addition of anti-HIV-1 chimeric antigen receptors.
Our results demonstrated that we were able to generate NK cells using H1 hESCs following an adapted NK cell differentiation protocol (Woll, Martin et al. 2005). EGFP-transduced hESCs were expanded on mouse embryonic fibroblasts (MEFs) and embryoid bodies (EBs) were formed to differentiate the hESCs into HSCs (Galic, Kitchen et al. 2009). These cells were then purified for CD34+ HSCs and were co-cultured with AFT-024 fetal mouse liver-derived stromal cells to further differentiate and develop into NK cells. We have also shown that the NK cells that we generated expressed mature NK cell surface markers including CD56, CD158b, CD335, and CD336 (Woll, Martin et al. 2005). In addition, we have confirmed the cytotoxic capabilities of our generated NK cells by performing in vitro killing assays using K562 human myelogenous leukemia cells as the targets for our NK cells.

Following the success of our NK cell differentiation, we transduced our H1 hESCs with a vector containing EGFP, CD4/ζ chimeric antigen receptor (CAR), shRNAs to mediate degradation of cellular CCR5 mRNA and HIV-1 RNA. This vector was designed for NK cells expressing CD4/ζ CARs to enhance its specificity in targeting and killing HIV-1 infected cells, and at the same time, protecting the NK cells from potential HIV-1 infection (Hutter, Nowak et al. 2009; Ringpis, Shimizu et al. 2012; Kamata, Kim et al. 2015). We followed the same NK cell differentiation protocol to generate and confirm mature NK cells expressing either CD19 CAR or CD4/ζ CAR. The advantage in using NK cells expressing CD19 CARs, which were created to target and kill B cell lymphoma cells, would be that CD19 CARs does not have a preference in targeting HIV-infected cells. In addition, we were also able to demonstrate that the expression of CARs alone does not enhance the NK cells’ ability to target HIV-infected cells. On the other
hand, the CD4/ζ CARs were designed to recognize gp120 viral coat proteins and therefore hypothesized to show a difference in targeting HIV-infected cells compared to the CD19 CARs. We then performed cytotoxic assays of our NK cells expressing CD4/ζ CARs to test their ability to specifically target and kill HIV-1 latently infected promonocytic U1 cells \textit{in vitro}. Compared to NK cells expressing CD19 CARs, the NK cells expressing CD4/ζ CARs displayed a significant decrease in viral Gag protein expression in U1 HIV-1 infected cells at effector:target ratios between 0.63–2.5. In addition, live/dead staining also indicated a significant increase in dead cells for the killing assays using NK cells expressing CD4/ζ CARs between effector:target ratios of 1.25–5. The results from the cytotoxic assays of our NK cells do not directly confirm the immunity of HIV infection provided by the CD4/ζ CAR vector, although published work utilizing shRNA 1005, which down regulates CCR5 and shRNA 516, which targets the long terminal repeat R region of HIV-1, did inhibit HIV-1 replication in the cells expressing these shRNAs (Hutter, Nowak et al. 2009; Ringpis, Shimizu et al. 2012; Kamata, Kim et al. 2015). Ultimately, it can be concluded that the expression of CD4/ζ CARs enhances NK cells’ ability to kill HIV-1 infected cells.

NK cells are crucial in the recognition and elimination of virally infected cells and cancer cells (Fauci, Mavilio et al. 2005; Caligiuri 2008; Iannello, Debbeche et al. 2008). Our \textit{in vitro} experiments confirmed that NK cells killed HIV-infected cells, which was significantly improved by the expression of CD4/ζ CARs in NK cells. Other groups published similar results, demonstrating the ability of unmodified NK cells and NK cells expressing CD4/ζ CAR derived from H9 hESCs were successful in suppressing HIV-1 replication (Alter and Altfeld 2009; Ni, Knorr et al. 2011). The difference between our
generated CD4/ζ NK cells in comparison to previous studies is due to the fact that our NK cells were derived from H1 hESCs and the CD4/ζ vector also carries shRNAs to provide our CD4/ζ NK cells with immunity to HIV-1 infection. In using different hESC cell lines, there may be potential differences in our ability to generate functional NK cells purely due to the cell lines coming from different embryos and having different genetic backgrounds (Allegrucci and Young 2007). In the end, our data were similar to recent results and showed that there were significant differences in the specificity of CD4/ζ CAR NK cells in targeting and killing HIV-1 infected cells when CD4/ζ CAR NK cells and HIV-1 infected cells were co-cultured together. The results of our in vitro killing assays using CD4/ζ CAR NK cells against U1 HIV-1 infected cells had expected similar results compared to the in vitro results from another lab, where they found a significant increase in the killing ability of NK cells expressing CD4/ζ CAR derived from H9 hESCs compared to its unmodified counterpart (Ni, Knorr et al. 2014). These results validated the effectiveness of CARs as a method to direct NK cells to recognize and kill HIV-infected cells with more specificity than NK cells without CD4/ζ CAR.

Moreover, the motivation for using genetically modified NK cells to specifically target aberrant cells in the human body stems from the innate abilities of NK cells to target virally infected cells and cancer cells (Fauci, Mavilio et al. 2005; Caligiuri 2008; Iannello, Debbeche et al. 2008). NK cells are able to identify these abnormal cells due to their loss expression of major histocompatibility complex class I (Karre 2002). NK cells derived from hESCs have been shown to possess antitumor capabilities when tested with various cancerous cell lines: K562 (erythroleukemia), MCF7 (breast cancer), NTERA2 (testicular embryonal carcinoma), PC3 (prostate cancer), and U87 (glioma) (Woll,
Grzywacz et al. 2009). It was observed that hESC-derived NK cells demonstrated a significant increased cytolytic activity against erythroleukemia, breast cancer, and prostate cancer cell lines compared with NK cells derived from umbilical cord blood derived NK cells (Woll, Grzywacz et al. 2009). These results establish that hESC-derived NK cells have the same, if not better, cytotoxic capabilities as that of umbilical cord blood-derived NK cells. The innate abilities of NK cells to generally target abnormal cells along with the addition of CARs to a specific antigen would greatly enhance its targeting specificity and killing abilities. HIV-infected patients, whose immune system is lacking CD4+ helper T cells to activate B lymphocyte and CD8+ cytotoxic T cells are at an increased risk for cancer including Kaposi sarcoma, non-Hodgkin lymphoma, and cervical cancer (cancer.gov). The advantage in using NK cells is due to NK cells being a part of the innate immune system and do not require the activation of CD4+ helper T cells. From here, we can potentially increase the capabilities of hESC-derived NK cells to target and eliminate these cancerous cells with more specificity by genetically modifying them to express CARs to recognize surface molecules present on cancerous cells.

After seeing the potential of NK cells expressing CD4/ζ CARs against HIV-infected cells, I differentiated CD19 CAR expressing NK cells for Zoran Galic’s lab to explore their ability to target B cell lymphomas. B lymphocytes of the immune system are part of the adaptive immune system and play an important role in producing antibodies to specific antigens (LeBien and Tedder 2008). In order to create NK cells expressing CARs specific to B cell lymphomas, the CARs required a surface protein to recognize. There are numerous different surface molecules found on B cells including: CD19 (Ig superfamily), CD40 (TNF receptor), and CD72 (C-type lectin) (LeBien and
Tedder 2008). The CAR-expressing NK cells that we have generated were designed to target the CD19 surface molecule found on B cells and B cell lymphomas. Further testing is required before we can conclude the efficacy of CD19 CARs expressed on NK cells, but this demonstrates the versatility of CARs as a method to direct NK cells toward specific aberrant cells in the body.

**Future directions**

In order to test the efficacy of CD4/ζ CAR NK cells as a potential anti-HIV-1 treatment, further testing is required to test their ability to target HIV-1 infected cells in vivo with a mouse model. It has been reported that suppression of HIV-1 in peripheral blood lymphocyte-NOD SCID gamma (PBL-NSG) mice was successful with the use of H9 hESC and iPSC-derived NK cells expressing either CD4/ζ or its unmodified counterpart (Ni, Knorr et al. 2014). This suggested that H9 hESC and iPSC-derived NK cells can potentially be a source for HIV-1 suppression treatment, but the addition of CD4/ζ CAR did not enhance the NK cell’s ability to target HIV-1 cells in mice (Ni, Knorr et al. 2014). The next step for us would involve testing our H1 hESC-derived CD4/ζ CAR NK cells in vivo using HIV-1 infected mice to see if using H1 hESCs make a difference. This would enable us to test the efficacy of the addition of our CD4/ζ CARs on NK cells in targeting and killing HIV-1 infected cells in a living animal. The in vitro experiments demonstrated the cytotoxic abilities of CD4/ζ CAR NK cells in killing HIV-1 infected cells, however the U1 HIV-1 infected cells were the only foreign cells to the NK cells in the co-culture. The in vivo experiments involving the injection of CD4/ζ CAR
NK cells into mice will test the NK cells’ abilities to specifically target HIV-1 infected cells instead of other cells in the mice.

Although we have been able to demonstrate success in the generation of NK cells expressing CD4/ζ CARs to kill HIV-infected cells, there are additional experiments that may be performed to optimize the differentiation method to increase the yield of CD4/ζ CAR NK cells to make a clinical application more feasible. It has been observed that the cytokine IL-21 induces the differentiation and proliferation of B cells, NK cells, cytotoxic T cells, and macrophages (ncbi.nlm.nih.gov). Others have taken this a step further and created a K562 (myelogenous leukemia) cell line co-expressing 4-1BBL and MICA combined with soluble IL-21 to induce proliferation of NK cells in vitro (Jiang, Wu et al. 2014). The CD137L (4-1BBL) ligand/receptor plays a role in both the antigen presenting process and generation of cytotoxic T cells (SinoBiological.com). MICA is a stress molecule, often secreted by cancer cells, that stimulates the cytotoxic response of both lymphocytes and NK cells (Weiss-Steider, Soto-Cruz et al. 2011). In using a cell line such as K562, that co-expresses 4-1BBL and MICA, we could potentially expand mature NK cells generated from hESC to yield clinically relevant numbers.

In addition to increasing the yield of NK cells by co-culturing K562 cells co-expressing 4-1BBL and MICA, along with soluble IL-21, a change in the EB formation protocol can be tested for an increased yield of HSCs, ultimately leading to an increased yield in NK cells. Apart from creating EBs manually through scraping, there have been other methods of forming uniform EBs that can potentially provide us with better quality and yields. The spin-EB method is used to create uniform shapes and sizes of cell aggregates using a defined number of cells in the special AggreWell™ plates (Antonchuk...
This new method, which creates better quality EBs with more uniformity, may provide a better method for creating HSCs. Furthermore, with the ability to control the number of cells used in this method, one would be able to test the optimal EB size to produce the best yield in HSCs. It has been observed that the spin-EB method is a possible alternative to producing EBs to create HSCs and ultimately produce NK cells from the HSCs (Ni, Knorr et al. 2014). There should be additional studies trying to perfect the EB formation method to optimize HSC differentiation to yield greater numbers of NK cells. By doing this, we would be able to decrease the massive number of hESCs that are currently used and still yield clinically relevant numbers of NK cells. Optimizing the NK differentiation protocol would allow us to develop a more feasible method of generating NK cells in a clinical setting in which the cost of creating NK cells to treat patients does not outweigh the benefits of the treatment.

There are potential modifications that can be made to enhance CAR-expressing NK cells to better target HIV-1 infected cells. It has been reported that second- and third-generation CARs, which have additional signaling domains, can boost effector functions of T cells expressing the CARs. Second generation CARs have been used in T-cells to demonstrate their efficacy in treating B-cell acute lymphoblastic leukemia (B-ALL). The second generation chimeric antigen receptor used to treat B-ALL consisted of 3 parts, a CD19 antigen-recognition domain linked with either CD28 or CD137 co-stimulatory domain and a CD3ζ signaling domain (Jackson, Rafiq et al. 2016). CD28 in T cells is responsible for optimal T cell activation, proliferation, and survival (Weng, Akbar et al. 2009). CD137 is part of the tumor necrosis factor (TNF) receptor family and is responsible for the inhibition of T lymphocyte proliferation (Langstein, Michel et al.)
A third generation chimeric antigen receptor has been designed as a treatment for B-ALL and tested for its effectiveness compared to the second generation chimeric antigen receptor. The third generation chimeric antigen receptor consists of a CD19 antigen recognition domain, with both CD137 and CD28 co-stimulatory domains and a CD3ζ signaling domain (Jackson, Rafiq et al. 2016). Preclinical studies have shown that T cells expressing the third generation chimeric antigen receptor displayed a superior antitumor efficacy compared to T cells expressing the second generation chimeric antigen receptors (Jackson, Rafiq et al. 2016). The promising results of the third generation chimeric antigen receptors in T cells can be a key in enhancing the current CD4/ζ CARs expressed in our NK cells. Similar modifications to the current CD4/ζ CARs may also help boost the effector functions of the NK cells expressing the next-generation CARs (Frigault and Maus 2016; Jackson, Rafiq et al. 2016).

In order to make this cell-based immunotherapy effective for all patients, induced pluripotent stem cells (iPSCs)-derived NK cells may be an invaluable source of cells for immunotherapy in HIV-1 infected patients. Groups are currently trying to produce iPSCs-derived NK cells expressing CD4/ζ CAR to treat HIV-1 infections (Ni, Knorr et al. 2014). Utilizing iPSCs (differentiated cells that have been reprogrammed to be pluripotent) generated from patient tissues will allow for the bypass of donor-recipient incompatibility. In addition, iPSCs just like hESCs, are able to differentiate into any cell type of the human body. It has been shown that NK cells can be derived from iPSCs. This powerful tool would enable scientists to engineer iPSC-derived NK cells expressing anti-HIV CARs to treat HIV-infection for patient-specific treatments (Ni, Knorr et al. 2014). Therefore, it would beneficial for us to continue along this path and create an iPSCs cell
line to carry the CD4/ζ vector currently used in our H1 hESC. With this iPSCs cell line, we should test their ability to generate NK cells using the current NK cell differentiation protocol and further test their ability to eliminate HIV-infected cells. Along this line, we can compare the cytotoxic capabilities of the iPSCs-derived NK cells with the hESCs-derived NK cells to observe their cytotoxicity towards HIV-infected cells. Based on published work (Ni, Knorr et al. 2014), I hypothesize that NK cells derived from either iPSCs or hESCs should exhibit similar cytotoxicity towards HIV-infected cells.

The success of creating CAR-expressing NK cells that have enhanced targeting and killing abilities of HIV-1 infected cells in vivo may lead to viable cell-based immunotherapy for HIV-infected patients to suppress HIV replication and to maintain an undetectable viral load. CAR-expressing NK cells may not necessarily replace current antiretroviral drug therapies, but may potentially be used simultaneously with antiretroviral drugs or reduce the current daily dosage of antiretroviral drugs (FDA.gov). Ultimately, promising results from NK cells expressing anti-HIV CARs may potentially lead to the generation of NK cells expressing different types of CARs specific to other cancers and viral infections (Jackson, Rafiq et al. 2016).
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