Treating Diffuse Large B Cell Lymphoma Using HLA Class I Molecule Deficient Anti CD19 CAR-NK Cells

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Gene Editing and CRISPR Technology

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2/2/2022

Diffuse Large B Cell Lymphoma (DLBCL), a sub-species of Non-Hodgkin's Lymphoma remains as the most common diagnosis of Lymphoma in western countries. At present, the de facto pathway of treatment is R-CHOP chemotherapy,and in the case of relapsed or refractory DLBCL, a second line of chemotherapy is used, along with high dose therapy and autologous stem cell rescue(HDT/ASCR). Although these treatment pathways serve to be a potential cure for a good fraction of patients, there still remains a large percentage of patients with whom such treatments are not applicable. A novel treatment currently used is CAR therapy, where Chimeric Antigen Receptors(CAR) are attached to lymphocytes to program their targeting specificity and cytotoxicity. Recent therapies all use autologous T-Cells, however this is a very tedious and expensive process since the therapy is specialized to the patient(allogeneic T-Cells are very prone to HLA mismatch, which results in Graft versus. Host Disease(GvHD). Alternatively, by creating HLA Class I-deficient anti CD19 CAR-NK cells through CRISPR/Cas9 gene editing technology and using allogeneic NK cells derived from Induced Pluripotent Stem Cells(IPSCs) as a source, it would be possible to develop an "off-the shelf" CAR-based immunotherapy to target DLBCL in patients. In this paper, I propose such a CAR-based immunotherapy and predict that it could potentially be used in cancer therapeutics, as it would bypass the HLA mismatch restriction, lower risk of cytokine release syndrome, and also minimize the risk of GvHD in patients, all while eliminating malignant B-cells.

Introduction

The immune system is the human body's primary defense system against infections categorized into 4 groups: Viruses, Intracellular bacteria, Extracellular bacteria, and Parasitic Worms. Immune cells make distinctions between healthy and unhealthy cells by identifying stress-induced ligands on the cells. It distinguishes between normal, healthy cells and unhealthy cells by recognizing a variety of "danger" cues called danger-associated molecular patterns (DAMPs). Viruses and Bacteria, on the other hand, release pathogen-associated molecular patterns (PAMPs), which are also recognized by the Immune System. The Immune System can be divided into two major branches of preventative measures - Adaptive Immunity and Innate Immunity(1,2).

Innate immunity is one of the two preventative measures that the human body can activate in order to eliminate a desired pathogen. Unlike the adaptive response, it is a first line defense against infection and malignancies . The benefit of this is that it eliminates the wait-time between activation of the adaptive immune system and effectiveness of the cytotoxic measures applied when a new foreign pathogen is encountered. The cells that are part of the innate immune system are not specific to particular pathogens like in the Adaptive system. Innate immunity depends on a set of proteins and phagocytic cells that recognize highly conserved features in pathogens, which are then quickly activated to eliminate invading cells. Three types of main innate immune system cells that respond to invading - granulocytes, natural killer cells, and macrophages(1,2,3). The Innate Immune system is also responsible for the activation of T cells and coordinating the Adaptive Immune response, through the production of cytokines, and antigen presentation. Cytokine proteins emitted by innate cells activate nearby immune cells and recruit them to clear the pathogen or stressed cells. Macrophages and dendritic cells can

then process and present antigens, a piece of a foreign substance used to instigate an immune response against that substance, to adaptive cells after the system detects danger(1, 2, 3, 4).

The second preventative measure used by the human body is the adaptive immune system, which mainly consists of T cell and B cell lymphocytes(1, 2). Adaptive immune cells provide antigen based immunity that is tailored specifically to the pathogen presented at the time of infection. The adaptive immune system also serves as a life-long protective measure from infections that have already been experienced by a patient. The activation of the adaptive immune response is dependent on the process of antigen presentation. Antigen presentation is the process by which cells of the innate immune system, including dendritic cells and macrophages, present antigens to adaptive immune cells resulting in the activation of these antigen specific cells. Only cells which can recognize the antigen via a T-cell receptor or B cell receptor are selectively activated and undergo clonal expansion. The activation of adaptive immune cells is fortified by cytokine proteins that are secreted by innate immune cells during an immune response(5). The adaptive immune system is split into two broad classes of immune responses - humoral responses and cell-mediated immune responses. Both classes of immune responses play critical roles in the overall immune response of the human body, and are antigen dependent immune responses which provide immunological memory for specific pathogens(1, 2, 8, 12).

Immune cells

All lymphocytes including T cells, B cells, and NK cells share a common progenitor that is abundant in the bone marrow(6, 7). Lymphocytes are a type of white blood cell utilized by the immune system. Adaptive lymphocytes, as previously mentioned, are generally classified into

two broad types of cells: T cells and B cells(1,2, 5, 6, 8). On the other hand, NK cells are classified as cytotoxic effector lymphocytes which means that while they are fully capable of eliminating foreign pathogens within the human body. NK cells are also capable of presenting antigens and releasing cytokines to recruit other nearby lymphocytic cells such as T cells or B cells to induce an antigen specific response(9, 10).

B cells are predominantly responsible for carrying out antibody responses during an immune response. Activated B cells secrete antibodies or immunoglobulins, which drift through the human body, and can bind to specific antigens, and inactivating the antigen. After binding to the antigen, the immunoglobulins are also capable of being secreted and marking antigens for destruction, in order to aid the phagocytic cells of the innate immune response(2, 8).

T cells are cytotoxic lymphocytes which are responsible for cell mediated immunity during an immune response. There are two prominent types of T cells: CD8-positive cytotoxic T cells and CD4-positive helper T cells. Helper T cells rely on antigen-presenting cells that express MHC Class I proteins on their membrane such as dendritic cells to identify target antigens. Killer T cells are then activated by Helper T cells. When Helper T cells identify an antigen presenting cell, they produce cytokines, which in turn activate Killer T cells.The difference between an antibody-mediated response and a cell mediated response as with T cells is that T cells interact directly with the stressed cell to conduct cell lysis(2, 6, 8, 9, 12). Helper and Killer T cells are distinguished through an important factor known as the Cluster of Differentiation. Each unique group of molecules and other markers on the surface of a cell is designated a different CD number, allowing for cells to be phenotyped by their expression of these surface markers and functional markers. CD antigens are the ones that react in response to recognition of some level of expression of the Cluster of Differentiation it was numbered for(11). Helper T cells have been designated as CD4+ T cells, whereas killer T cells have been designated as CD8 T cells.

NK cells are classified as innate cytotoxic effector lymphocytes, which means that NK cells are both capable of eliminating foreign pathogens within the human body, as well as presenting antigens and releasing cytokines to recruit local lymphatic cells like T cells or B cells to induce antigen-specific immune responses. The main qualities of NK cells as compared to T cells and B cells is that they do not need to be activated by dendritic cells, or other antigen presenting cells to carry out their primary function(1, 2,3, 4, 10). NK cell cytotoxicity is regulated by the operation of Activating and Inhibitory receptors. Activating receptors detect the presence of “stressor” ligands on the surface of a cell in distress. Stress-induced ligands that are produced by the cell mark the cell as abnormal to the immune system, and these ligands are consequently recognized by NKG2D, as well as other NK Activating cell Receptors. Activating Receptors are also capable of recognizing other alert molecules such as infectious allogeneic ligands like the cytomegalovirus encoded m157 which is recognized by Ly49-H(in the mouse)(13). Another receptor that is seen to have expression on NK cell surfaces is TLR4 (Toll-Like Receptor 4)which is part of the family of Toll-Like Receptors, which is part of the pattern recognition receptor family. TLR4 has an important function of recognizing lipopolysaccharide (LPS), a component that is present in the majority of gram-negative bacteria. The receptor is also capable of recognizing other ligands and molecules such as polysaccharide and Palmitic acid, as well as various other viral proteins and endogenous proteins. In essence, Activating receptors induce cell apoptosis as a result of recognizable stress ligands on the surface of the cell in question. Once the stress ligand binds with its corresponding receptor, the NK cell secretes cytotoxic molecules such as granzymes and perforin, which eliminate the cell(3,13, 14).

Inhibitory receptors, on the other hand, are tasked with preventing the unnecessary killing of healthy and autologous cells. This is managed through the recognition of low concentrations of

MHC (Major Histocompatibility Complex) Class I molecules that are present on every cell in the body. MHC proteins are fundamentally different for all humans, so inhibitory receptors are only capable of recognizing autologous MHC molecules. When MHC Class I is not presented to the inhibitory receptors, the inhibitory cell receptor is inactivated, allowing for the activating cell receptor to behave at full capability. When the activating cell receptor recognizes a stress ligand in the absence of an inhibitory signal, it will initiate the process of producing cytokines and killing cells. Rather, when autologous MHC Class I is recognized by the inhibitory receptor, this leads to an increased inhibitory signal being present which inactivates the NK cell(2, 3, 4,10, 14).

MHC

The Major Histocompatibility Complex is a fundamental part of all human beings, and is unique to the individual. It can also be referred to as the HLA - Human Leukocyte Antigens. MHC is a gene locus composed of genes that encode for proteins expressed on cell surfaces. The surface proteins are correspondingly referred to as MHC molecules. The function of these molecules is to bind peptide fragments (antigens) which are seen to be expressed in pathogens to the surface of the cell, and display it to the appropriate T cell for elimination. The uniqueness of the MHC can be connected back to two important characteristics - the MHC is polygenic, and is also polymorphic. Because it is polygenic, there exists a wide range of class I and class II gene loci that all encode for the same protein(15, 16, 17). As a result of the MHC being polymorphic, there also exists multiple alleles of each loci. Since there are so many permutations and combinations possible, it is highly unlikely for two people to have the same set of MHC molecules. The result of the MHC being unique to each person is that NK cells are only capable of recognizing autologous MHC molecules(15, 16).

Non-hodgkin’s Lymphoma

Malignant Lymphomas have always been a source of disagreement in regards to subcategories and classifications, and diagnosis, ever since the original report written by Thomas Hodgkin in 1832. It was only recently in 2001, when the World Health Organization updated and refined the 1994 classification Revised European-American Classification(REAL), finally defining 27 distinct lymphomas, under which Non-hodgkin's Lymphoma is classified(18, 19). The classification of major Lymphomas is generally determined by the type of cell malignantly affected - NK cell, T cell, or B cell. Non Hodgkin's Lymphoma mainly arises from B cell lymphocytes, and its sub-species Diffuse Large B-cell Lymphoma(DLBCL) is the most commonly diagnosed cancer in Western countries, comprising 30% of new diagnoses(18). Non-Hodgkin's Lymphoma is distinguished from Hodgkin's Lymphoma through the absence of certain malignant B-cells identified as Reed-Sternberg cells(20). DLBCL is clinically aggressive, and is composed of various similarly aggressive B-cell Lymphomas. Identification and categorization of DLBCL, as defined by WHO, was determined through three distinct origin cell types - germinal center B-cell(GCB), activated B cell(ABC), and primary mediastinal B-cell (PMBL). GLB DLBCL is the result of malignant B-cells with the germinal center B-cells undergoing somatic hypermutations and ongoing CD10 expression. ABC DLBCL, which is associated with a poorer prognosis when treated with standard chemoimmunotherapy, is characterized by the activation of the NF-κB pathway(21, 22, 23, 24). Finally, PMBL results from a thymic B-cell molecule, and has similar traits to nodular sclerosing Hodgkin lymphoma (NSHL) including utility of the NF-κB and JAK-STAT signaling pathways, as well as other genetic modifications that help immune evasion. There also remains another approximately 20% of DLBCL cases that are uncategorized(21).

Current Treatments and Cures

DLBCL does not have any guaranteed cure, however patients are treated with the intention to fully cure, and many patients are also able to achieve a long-term disease free status(18). The current preferred regimen of treatment is a type of chemotherapy. Chemotherapy is conducted through the injection of extremely cytotoxic chemicals into the region of the tumor. The most commonly utilized chemotherapy is a cyclical treatment process consisting of four drugs - cyclophosphamide, doxorubicin, vincristine, and prednisone(CHOP), along with a CD20 monoclonal antibody rituximab(R-CHOP) - that is administered every 3 weeks(21, 24).

However, there still remains the problem of refractory and relapsing DLBCL. In order to combat this, a second line of chemotherapy is applied, along with administration of High Dose Therapy and Autologous Stem cell Rescue(stem cell mobilization)(HDT/ASCR), or autologous bone marrow transplantation(18, 21). This pathway of treatment offers a possible long-term cure for a fraction of patients. The final fraction of patients, to whom such treatments are not applicable for various reasons, have a very poor percentage of positive outcomes. To this extent, a novel cure must be developed in order to guarantee a long-term disease-free outcome.

A novel treatment comes in the form of CAR-T cell Therapy(Chimeric Antigen Receptor). CAR Constructs are programmable antigen receptors that allow one to manipulate a T-cell's targeting specificity, cytotoxicity, as well as other functions such as inhibition and activation. By programming autologous T-cells extracted from the patient's body to direct them towards malignant B-cells, you are producing a sot of "living drug", where even after the tumor cells have been eliminated, the CAR-T cells will continue to persist in the body, allowing for your adaptive immune system to automatically protect against relapsed and refractory DLBCL for a few years after the initial injection.

CAR-T cell Therapy has ultimately been an incredible breakthrough of a treatment for cancer-based illnesses, but even then there are still problems that remain. Perhaps the most important problem is that CAR-T cells are extremely expensive due to the exclusivity of the treatment. Each patient must have CAR-T cells extracted from their own body, and only these CAR-T cells can be modified. This is due to the recognition system of the immune system - The immune system recognizes healthy cells if they express low concentrations of MHC molecules on the surface of their cell membrane. If such molecules are not sensed on the surface of a cell, the immune system will be activated to eliminate these allogeneic cells, causing the patient to eliminate the very treatment applied, which can also result in extreme Graft versus. Host Disease(GvHD), and or Cytokine Release Syndrome(CRS). As a result, doctors must extract T-cells from the patient, and modify those specific autologous T-cells(26, 27). Such exclusivity only invites extreme expenses, as well as major resources, and time being spent on a singular patient's treatment. Currently, there are two existing CAR-T cell therapies which are being used commercially: axicabtagene ciloleucel and tisagenlecleucel, both which target CD19, a Cluster of Differentiation protein that is expressed heavily on the membranes of malignant B cells(30, 31). In a clinical study, approximately 50 to 70% of patients were still alive after 12 months of treatment, however the fact that multiple other patients succumbed to adverse side-effects such as CRS and or GvHD should not be ignored(28, 30). Still, studies have shown that CAR-T cell therapy can be successful for patients on whom R-CHOP chemotherapy failed as such its shortcomings must be further understood to fully exploit the power of CAR-T cell therapy in the clinic (24).

Generally speaking, while CAR-T cell therapy has been proven to be a successful treatment, its feasibility as a treatment option for the general public is quite low, with only a small number of people being able to afford it, as well as the immense risk of adverse side-effects.

CRISPR/Cas 9 Gene Editing Technology

In recent years, many breakthroughs in gene editing technologies have been made through a method known as CRISPR/CAS9, which stands for Clustered regularly interspaced palindromic repeats, and Cas9 is recognized to be a CRISPR associated protein. Originally, CRISPR and Cas9 served as a mechanism of immunity in bacteria. When a bacterium detects the presence of viral DNA within the cell, the bacteria produces two strands of RNA. One of which is the corresponding RNA strand to the viral DNA strand. The two RNA strands form a complex with the CRISPR associated protein, known as Cas proteins(32, 33, 34, 35, 36). The Clustered regularly interspaced palindromic repeats are the second important piece to gene editing technology. The matter of interest however, is not these palindromic repeats, but rather the DNA sequences in between each of these repeats, referred to as spacers(32-36, 37). These spacers were discovered to be highly conserved DNA strands in different pathogens, as well as other mobile genetic elements. Experimental evidence towards this conclusion came in the form of trials run by Horvath, depicting that after being challenged by a phage genome, the bacteria Streptococcus thermophilus had in fact incorporated new spacer sequences which had been derived from the bacteriophage's genetic material(32, 34). These spacers specify to the CRISPR associated protein complex what the target of elimination is. Although the CRISPR-Cas9 system is the one discussed commonly due to its applications in the fields of gene editing, there are many more systems of CRISPR-Cas associated proteins that exist. CRISPR-Cas9 is a Type II system, and considered to be one of the more simple systems of adaptive immunity that is utilized by a bacterium(33, 35, 38).

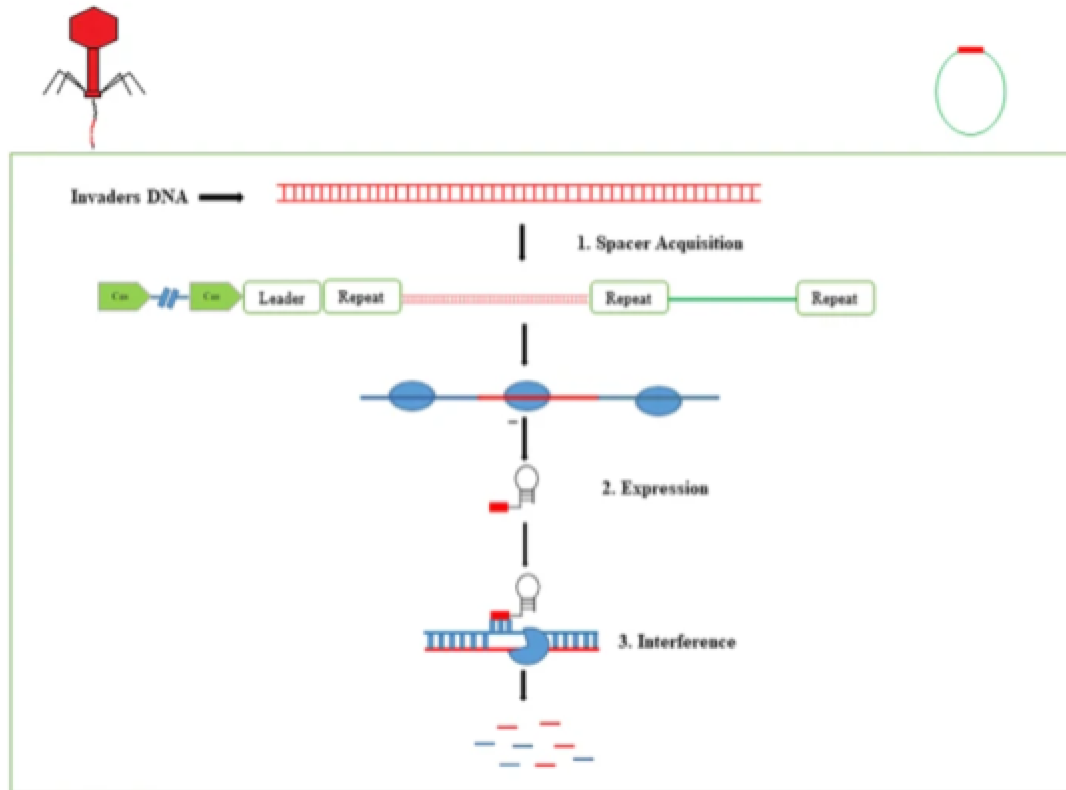
There are two different scenarios under which CRISPR-Cas9 is capable of operating on. The first is the more common occurrence when the viral DNA inserted by the bacteriophage is already conserved within a spacer sequence. In this scenario, bacterial microbes capture

fragments of the viral DNA which was inserted into the bacterium, and process it through all the spacer sequences until it reaches a spacer sequence which conserves some of that specific viral phage's DNA. Transcription of the CRISPR array produces a piece of RNA known as pre-crRNA which is processed into individual crRNAs with unique specificity. crRNA serves as a guide for Cas9 protein to a complementary sequence of viral DNA. Cas9 proteins are produced from an array of genes known as Cas genes. The Cas genes are located upstream of the Crispr. Cas proteins are helicases which target specific cutting regions to make double stranded DNA cuts(33, 36).

The second scenario is when the bacterium is challenged by an unrecognized bacteriophage. In this scenario as well, microbes of the bacterium will capture fragments of the viral DNA, and process it through the spacer sequences. The fragment of viral DNA will then be integrated into a new spacer sequence. The rest of the process is the same from the previous scenario, with the bacterium producing a pre-crRNA through transcription, and consequently forming a complex with a Cas protein to guide it to the source of infection(35, 36).

Once the pre-cRNA has been produced by RNA polymerase, it is then cleaved into small crRNA by specific endonucleases. Although crRNA have a variety of functions, one of their most highly recognized ones is that of a guide RNA. crRNA cleaved from the pre-crRNA is then hybridized with a tracrRNA, a type of noncoding trans-activating crRNA which is complementary to the crRNA to form a tracrRna:crRNA complex - a dual guide RNA(35, 36). This allows for bonding specific to Cas9 nucleases. The complex formed by the dgRNA with the Cas9 helicase is what allows for the Cas9 helicase to be able to target the viral DNA that infiltrated the bacterium. This is where the final stage of elimination/interference occurs(37). Directed by the dgRNA complex, the Cas9 endonuclease traverses the genomic material, scanning for the correct PAM(proto-spacer adjacent motif sequence) sequence which is located opposite to the target

strand(33, 36). Once the PAM site is correctly identified, the double stranded DNA is unwinded, and the Cas9-sgRNA complex binds to the target single strand DNA, and ultimately induces a blunt double stranded break(40).



(Reference: 35)
Figure 1: The following are the steps for CRISPR Cas9 Type II system based immunity. Once viral DNA has been inserted, spacer acquisition will occur, where the cell integrates a fragment of the viral DNA into a spacer sequence. Following this, the stage of expression occurs, where the cell transcribes the viral DNA, and a complex with Cas9 protein is formed. The final stage of interference occurs when the guide RNA complex has finally directed the Cas9 protein to the viral DNA, where the Cas9 protein induces a double-stranded break in the DNA.

The importance of this biological system is that scientists were successfully able to implement Cas9 endonuclease activity within human settings. It was discovered that by fusing the 3' end of the crRNA to the 5' end of the tracrRNA, it would be possible to form a single chimeric guide

RNA(sgRNA), which would be capable of forming a complex with Cas9 to direct it towards viral target DNA(33, 36, 39) . Such a discovery permitted researchers to be able to modify the specificity and location of the DNA cleavage that is to be conducted by Cas9. After Cas9 induces the double stranded break, the cell either undergoes NHEJ(non-homologous end joining), or homologous recombination. Both pathways have their advantages and disadvantages. In the case of Crispr/Cas9 technology, NHEJ tends to be the more common pathway of DNA preparation. In this pathway, the DNA repairs itself at the location of the double stranded break using endogenous repair machinery. This repair mechanism is very error prone, and oftentimes can result in the addition of small nucleotide insertions or deletions, which consequently result in various mutations including insertions, deletions, and frameshift mutations. On the other hand, HEJ(homologous end-joining) is a more precise pathway of reparation, which allows for researchers to incorporate new DNA sequences into the area where the DS break occurred. In order for the DNA to undergo this method of preparation, a template strand of the DNA sequence must be supplied to the cell(33, 41, 42). For the purpose of this paper, we will induce HEJ in the cell.

CAR Design

In recent years, there have been many breakthroughs in methods of modifying T cells and NK cells to target specific tumor cells. This was done through the addition of a Chimeric Antigen Receptor (CAR) to the NK cell. Integration of a CAR in a NK cell allows for an increase in overall cytotoxicity of the cell, as well as programmable targeting specificity for a desired antigen. CAR Nk cell history can be summarized over four generations of CAR NK cell. The fourth generation is the currently used, and most effective type of CAR Nk cell. A fourth generation CAR designed for integration with an NK cell typically consists of three main parts - the ectodomain, the transmembrane region, and the endodomain(42).

The ectodomain is made up of a signal peptide, and an extracellular antigen identification domain - typically a single chain variable antibody fragment (scFv). The CAR sequence begins with a short signal peptide, which are short sequences that are located on the N terminus of proteins, and contain data regarding the protein's post translational roles and destination. There is an immense number of variant Signal peptides, which makes its selection process quite difficult. Currently, CD8a SP is the most commonly used signal peptide, however it has not actually been scientifically determined as to what the optimal signal peptide is(42, 43).

The single chain fragment variant is a fusion protein composed of the variable regions in the heavy and light chains of an antibody. It is also known as the tumor antigen binding domain. An scFv is typically generated to contain an antigen binding site by cloning the heavy and light chain variable regions of an antibody(Vh and VL). The two chains are consequently linked to a flexible polypeptide linker, which is normally a multimer of the polypeptide glycine-serine. The scFv is an artificially created chain, so the orientation has also been artificially determined(44). At present, most scFvs have been constructed using a VH-VL orientation, as compared to a VL-VH orientation. In actuality, it has been proven by Fujiwara et. al in a paper regarding the construction of scFvs that orientation of heavy and light chains, as well as differences in the linker do not correlate to an alteration in CAR efficacy and function(42, 45). On the other hand, integrating heavy and light chains from multiple different antibodies can sometimes lead to increased CAR affinity(42, 46). Finally, there occurs a large number of different scFv chains, and it is possible for multiple variants of an scFv to attach onto different epitopes of the same protein. As a result, the scFv determines the targeting specificity of the CAR NK cell, as well its function. Determining the most appropriate scFv is a difficult process, however, as a large quantity of tumor-associated antigens are also expressed at lower levels in healthy cells, which

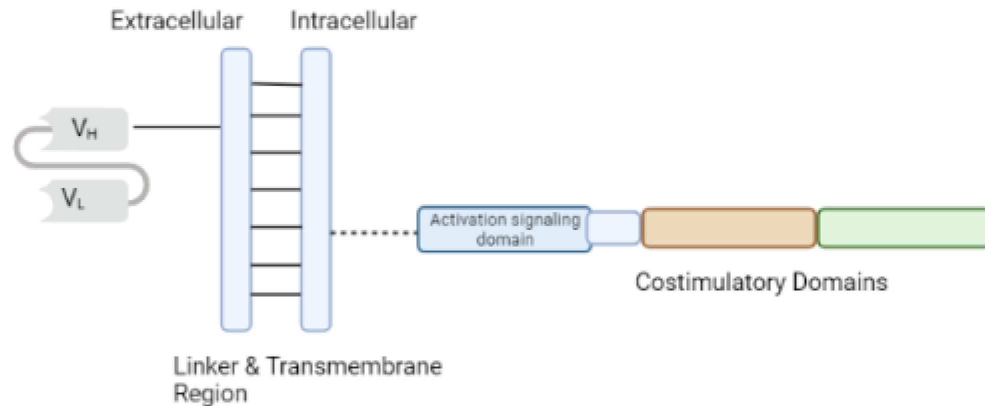
can possibly lead to self compromisation of healthy cells(CAR NK cells binding to normal/healthy tissue)(42).

The hinge region, also known as a spacer, is what connects the scFv to the transmembrane domain.This region serves the purpose of enabling flexibility throughout the chain, easy access to the target antigen, and ensures CAR stability. Most CAR-NK constructs used in research have been variants of the CD8a, and CD28, but recent breakthroughs have noted that CD28 has an increased likeliness of promoting dimerization of CAR molecules, which results in a higher chance of cytokine release syndrome being induced during CAR-NK cell therapy. As a result, it was also noted that a hybrid version of the CD8a region was capable of increasing the safety of CAR-NK cell therapy(42, 47, 48) .

The transmembrane(TM) domain of the CAR protein docks the receptor to the NK cell membrane and connects the ectodomain to the intracellular activation signaling domains which allows for engagement of signaling cascades that leads to cytotoxic effector function. It has been proven that choice and modification of the TM domain can result in changes in NK cell killing capacity(42). Le et al showed that the incorporation of a NKG2D transmembrane domain into the chimeric antigen receptor resulted in increased cytotoxicity, whereas using another costimulatory transmembrane domain decreased the rate of cytotoxic granule release(49). As is evident from this study, it is important to select a transmembrane domain that will be capable of enhancing CAR NK cell functionality.

The transmembrane has a second function of connecting to the endodomain, which encompasses the intracellular activating signals(42, 43). As previously mentioned, there are four generations of CAR constructs, and the generation of a Construct is determined by the number of intracellular activating signal domains a Construct is composed of. Activating signals

are what are responsible for the activation of the NK cell upon recognition of the target antigen. NK cells use a variety of activating receptors, including various different cytokine receptors that each play fundamental roles in an NK cell's growth and development. An important factor to be considered is that a large number of receptors have common adaptor molecules and signal pathways. First generation CAR Nk cells consisted of only the CD3ζ signal molecule, which had a singular purpose of activating the NK cell to induce cytotoxic functions upon the target antigen. The second and third generations included the addition of one and two costimulatory molecules respectively(43). Costimulatory molecules have various functions, and are generally selected to modify the cell in ways such as improving safety profile, increasing cytotoxic functionality. Some of the more commonly used costimulatory molecules are derived from various groups such as the CD28 family, the tumor necrosis factor receptor, and, or the signaling lymphocytic activation molecule (SLAM)-related receptor family(42). Strong activation signals are important to induce a strong anti-tumor effect, however this can also result in rapid consumption of the CAR Nk cells. For this reason, different permutations and combinations of costimulatory molecules are utilized in order to program the desired immune response.
4-1BB/CD3ζ signals, for example, induce sustained antitumor activity in vivo, as well as memory associated genes(50).



(Reference: 72)
Figure 2
A 4th generation CAR construct. The single chain Fragment variable, consisting of a fusion of the heavy and light variable chains of an antigen, are connected to the transmembrane region via a Linker region. The transmembrane region docks the CAR onto the cell, as well as connecting to the intracellular signaling domains. In a fourth generation CAR construct, there are 4 signaling domains(42, 43).

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Safety mechanisms can also be installed onto these activating domains, allowing for rapid elimination of CAR modified Nk cells, to prevent adverse reactions such as Cytokine Release Syndrome. Incorporation of the suicide gene iCasp9, for example, has been shown to be an effective way to lyse all CAR carrying lymphocytes in the patient should the CAR Cells begin to mutate or behave abnormally(51).

To this extent, the fourth generation of CAR Nk cells have been further developed to express strong antitumor functionality, and have also been derived from TRUCKs(T cells redirected for antigen unrestricted cytokine initiated killing) (52). In fact, a large majority of the research conducted on CAR-Nk cells comes from the field of CAR-T cells. This can be attributed to various reasons, however one important one is that many signaling pathways and activating

molecules are shared by both Nk cells and T cells. As a result, various discoveries in the CAR-T cell field can be applied to CAR-Nk cells.(42, 43)

These three parts, along with various other variable regions, allow for the efficacy, cytotoxicity, and specificity of a CAR-NK cell to be modified to a researcher's advantage. Expression of a CAR molecule in a NK cell requires the insertion of a plasmid vector that encodes for a specific CAR gene. A typical vector backbone generally contains an origin of replication, a selection marker gene and/or an antibiotic resistance gene, a custom promoter for the desired gene, and transcriptional regulation fragments. Most commonly, viral vectors such as retroviruses and lentiviruses are used to introduce plasmids into eukaryotic cells (42). Another technique for inserting plasmids into human cells is electroporation, which involves placing the cells under an electrical current causing ion channels to open and inadvertently the uptake of plasmid vectors.

NK cell Sources

One of the problems that arises with an NK cell based treatment is the sourcing of functional NK cells. It is not possible to extract an adequate amount of NK cells from the patient's body, since Nk cells only comprise 10% of all lymphocytes, and a decent majority of Nk cells tend to be dysfunctional(52). Hence, the only alternative is to extract cells from a healthy donor, and create a sort of "off-the-shelf Nk cell source". One commonly used source is the NK-92 cell line. This Nk-cell Line is the only one out of six other recognized Nk cell lines that has consistently displayed antitumor functionality and cytotoxicity. Indeed, it is also seen to be the easiest cell to be able to genetically modify and program cell characteristics, such as targeting specificity, cytotoxicity, as well as other various roles that an Nk cell may be responsible for(52). Nk-92 cell Lines have previously been produced by immortalizing clonal Nk cell lines from patients affected by Nk-cell Lymphoma. The dilemma here is that there are a small number of patients that are

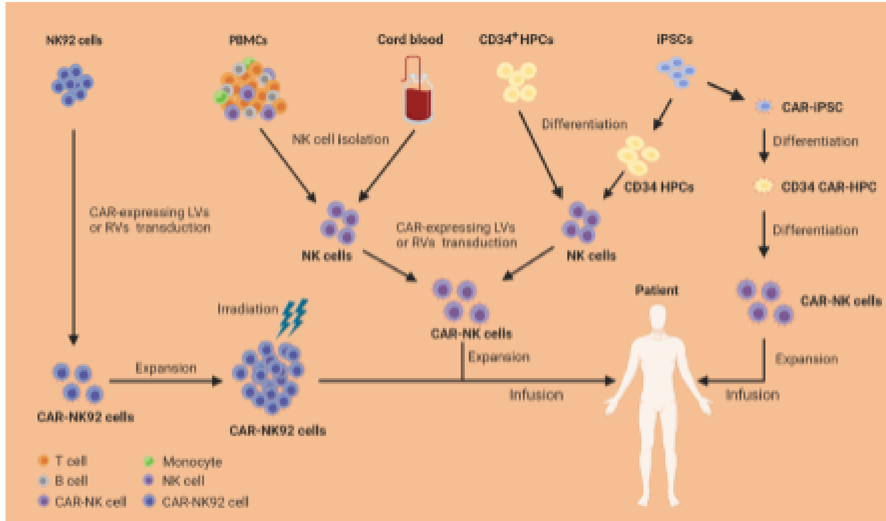
affected by Nk-cell Lymphoma, and furthermore, the chance of a clonal outgrowth of a cell-line is also incredibly low, resulting in only a handful of developed clonal Nk-92 cell Lines. On the other hand, once a cell line has been developed, they are quite easy to maintain and expand: the doubling time of a culture is 2-4 days, allowing for treatments to be administered on a flexible schedule. As a result, Nk-92 cell lines are so commonly used because they are the easiest to maintain, and are also inexpensive(42, 52, 54).

Although they seem to be very advantageous, they also have numerous flaws. Nk cells are developed from a malignant patient, so it raises the question as to whether it is safe to insert these cells for treatment purposes. As it turns out, in order to inject these Nk-cells into the patient, they must be irradiated first in order to prevent uncontrolled proliferation. On the other hand, by irradiating the cells, you are also reducing the capability of the Nk cell to reproduce in vivo should there be the need to eliminate more malignant cells, as well as their lifespan(52). The Nk-92 cell line has also been recognized to contain the Epstein-Barr virus, as well as being aneuploidy(42, 55).

Another attractive source of NK cells that has gained some traction over the past few years is deriving NK cells from human induced pluripotent stem cells(IPSCs). By reprogramming adult somatic cells through inducing genes and other factors that express the necessary properties of an embryonic stem cell, it is possible to produce a cell that retains an embryonic stem-cell like state. In order to derive Nk cells from IPSCs, the IPSCs must first be differentiated into CD34+ hematopoietic stem cells. Additional gene expression can be induced in order to further modify functionalities to resemble that of an NK cell.

Research has shown that NK cells derived from IPSCs have cytotoxic functionality, and are also capable of targeting tumor cells both in vitro and in vivo - experiments run by Ni et al. show

data that IPSC-derived NK cells were capable of inhibiting HIV-1 NL4-3 infection of CEM-GFP cells, and were also able to control/suppress HIV-1 infection CD4+ T-cells through three functionalities: direct lysis, production of cytokines, and/or antigen dependent cellular cytotoxicity(42, 55, 56). Furthermore, in another study conducted by Li et al.(2018), CAR expressing IPSC-derived NK cells displayed increased levels of antitumor functionality as compared to T-CAR-expressing iPSC-derived NK cells (T-CAR-iPSC-NK cells) and non-CAR-expressing cells when all three were directed towards the tumor-associated antigen mesothelin(49). IPSC-based NK cell lines are also advantageous over other primary stem cell sources as they have a comparably better safety profile than sources such as human embryos and bone marrow biopsies(42). Additionally, IPSCs are also feasible for clinical applications - as supported by results from Knorr et al. - they developed a novel method for producing cytotoxic(expressing CD56, killer immunoglobulin-like receptors (KIRs), CD16, NKp44, NKp46, and NKG2D) NK cells which would only need fewer than 250,000 input IPSCs to treat a single patient(55). There are, however, still some potential challenges with IPSCs. IPSC-derived cells always have the potential to malignantly transform and retain immunogenicity, which would cause an inflamed cytokine release storm. Such problems have not been seen in studies, however the problem is undeniably there(42). A possible solution includes attaching a costimulatory molecule to the CAR receptor that behaves as a “safety switch” in the chances that the cell starts behaving abnormally. Although IPSC-derived cells do have some minor flaws, they are apparently more advantageous to use a cell source than NK-92 cells.



(Reference: 73)
Figure 3: This figure depicts different potential NK cell sources. On the far-left is the currently most commonly used cell source: the NK-92 cell line. The NK-92 cell line simply undergoes introduction to the CAR vector to successfully integrate the CAR into the NK-92 cells, and those cells are used for treatment. Alternatively, on the far right, a possible cell source is IPSCs, which could go through two different pathways to differentiate into a CAR-NK cell. The pathway used for my experimental method is where the CAR vector is only added to the iPSC after it has gone hematopoietic differentiation, and further genetic modification to convert it into an NK cell.

Cell Design & Experimental Method

With the steadily growing popularity of CAR-NK cell based treatments, here I propose a hypothetical cell design, as well as the experimental method required to create it, that would be capable of treating DLBCL in patients.

As it is evident from previous studies and papers that the most advantageous cell source to be used is human IPSCs, from which we will derive our NK cells from this source. To begin, we must select a process for derivation, as well as a source from which to produce IPSCs. IPSCs are induced from differentiated adult somatic cells, so hypothetically any cell could be selected,

but some procedures tend to be more invasive than others(fibroblasts require skin biopsies as an example), so it would be preferable to select a cell source that does not intrude on the donor as much as possible. Such a source exists in the form of keratinocytes from human-plucked hair. Even though there are different types of hair follicles, for establishing a source of keratinocytes, either fine vellus or thick terminal hair follicles will suffice(57). Once the hair is plucked, it can be sustained in a DMEM media for multiple days. Such a long time implies more convenience on all parties involved: donors will not even have to leave their home, and researchers will not have to worry about being on a time crunch to preserve the hair follicles before they lose their ability to proliferate(57). After successfully preparing a culture of keratinocytes, we must induce an embryonic stem-cell state in the cells. This can be done through the overexpression of four transcription factors: OCT4, SOX2, cMYC, and KLF4(60, 61, 62). Additionally, a clinical study which has been performed showed that as compared to fibroblasts, keratinocytes have a 100-fold higher efficiency, as well as being two-fold faster to reprogram(57, 60). Following the completion of reprogramming, it is crucial to maintain the state of pluripotency, which can be done by using compounds that inhibit glycogen synthase kinase 3, lysine-specific demethylase 1, or G9a(60).

Once a source of IPSCs have been produced, begin the second step of processing the IPSCs through hematopoietic differentiation in order to produce a source of CD34+ Hematopoietic Stem cells. Hematopoietic differentiation can be achieved through a process introduced by Knorr et al. Differentiation is mediated by stromal cells, and the IPSCs are initially briefly placed in an RPMI 1640 medium promoting preservation and NK Cell differentiation. After replacing medium every 3 days for 18-21 days, the cells were placed onto EL08-1D2 stroma with 1 ml of NK cell differentiation cytokines (IL3, IL15, IL7, Stem cell factor and FLT3L). Mature NK cells will then arise after 28-35 days of coculture(63).

Once an NK cell source has successfully been developed, we can begin genetic modification through CRISPR/Cas9 technologies to modify the NK cell to modify targeting specificity, as well as some other functions.

One of the main problems currently arising with CAR-T cell therapies, as well as novel CAR-NK cell therapies is HLA mismatch. As previously mentioned, if autologous HLA molecules are not recognized on the surface of a cell, the cell will be marked for elimination. In the case of NK cells, there are slight concentrations of HLA Class I molecules on the cell membrane(1, 2, 3). These molecules interact with various inhibiting receptors of the NK cell to indicate that the cell is healthy. Since there are multiple receptors which interact with Class I HLA molecules, it would be more efficient to simply prevent surface-level expression of HLA Class I molecules. This is possible through inducing a double stranded break in the B2M gene, which has shown to have correlation with the rate of surface-level HLA Class I molecule expression on cell membrane(64). Additionally, by coexpressing a single chain HLA Class E trimer which is capable of inhibiting NK cell-dependent lysis, we can successfully prevent NK cell fratricide, which is one of the main dilemmas surrounding CAR-based therapy(65).

To induce a double-stranded break in the B2M gene, a lentiviral CRISPR/Cas9 vector pLE38-Cas9-sgB2M/gNKG2A must be used. The target sequences for the B2M gene can be created using an sgRNA designer, and then cloned into the Esp3I sites of the vector using annealed oligonucleotides(64). Instead of allowing the DNA to repair itself through the Non-homologous end joining pathway, we will induce homology directed recombination of DNA by introducing a piece of template DNA into the NK cell. The purpose of this will be to perform a gene knock-in, where we substitute the CAR gene into the location of double-stranded break.

As of now, the most commonly utilized CAR vector to combat lymphomas has been the anti CD19 vector, because of its high expression on the surface of malignant B cells.. To this extent, I will be modeling this hypothetical CAR receptor to be capable of targeting CD19 in malignant B cells. The components of the Chimeric Antigen Receptor will be: a single chain fragment variable FMC63, which has been proven to exhibit potent targeting ability towards CD19, and has effectively lysed CD19-positive cell lines when cocultured in vitro with them(the scFv was incorporated into a basic CAR construct backbone)(66). The hinge region attached to the scFv will be a CD8a region, which multiple studies have shown to be the most optimal hinge region for CARs given its high safety profile and reduced chance of inducing cytokine release storms(42, 47). A transmembrane domain of NKG2D and a costimulatory domain of 4-1BB will be conjoined onto the other end of the hinge region. These two domains specifically have been identified to increase anti-tumor activity, as well as promoting antigen-induced NK cell-mediated cytotoxicity(49, 67),. Finally, a suicide gene known as iCasp9 will be added as a costimulatory molecule to the CAR vector. The iCasp9 will serve as a safety switch to effectively lyse all CAR expressing cells from the patient in the case of unexpected mutations or other problems(51). This CAR gene will be transduced into a retroviral vector, as retroviruses allow for the CAR to be permanently integrated into the host cell's genome(68). There is also much precedent for retroviral-based CAR vectors - they are safe and have FDA approved protocols(68). The vector will additionally include an ampicillin resistance gene, as well as the fluorescent protein marker AmCyan1. Both of these genes will be important for functional assays at the end of cell development.

An additional plasmid vector utilizing an Streptomycin antibiotic resistance gene, mCherry fluorescent protein marker gene, and also containing the single-chain HLA Class E molecule will be made. The HLA single-chain class E molecule is a non-polymorphic polypeptide which

inhibits the capability of the cell to interact with receptors NKG2A and CD94, which prevents NK cells from initiating cell-dependent lysis, hence successfully preventing NK cell fratricide(65).

The process will begin by utilizing a CRISPR lentiviral vector to target the PD-1 receptor gene in the NK cell in order to induce a double stranded break. PD-1 is an inhibiting receptor that studies have shown to have a high correlation with preventing NK cells from eliminating malignant and unhealthy B cells that express PD-L1(70, 71). By inducing a double stranded break here, we are preventing PD-1 from playing its role as an inhibiting receptor, and instead will introduce the broken DNA to the single-chain HLA Class E molecule expressing vector to induce homologous recombination in the DNA.. This will protect the CAR-NK cells from cell fratricide.

After this process is conducted, the secondary process of breaking the B2M gene in the IPSC-derived NK cells will be initiated. Similar to the first process, we will use a lentiviral CRISPR vector pLE38-Cas9-sgB2M/gNKG2A in order to target the B2M gene. Targeting specificity will be determined by the sgRNA, which can be created with an sgRNA designer. Once the double stranded break has been induced, we will coculture the NK cells with our CAR vectors. Integration of the CAR gene can be checked after a period of time. In order to verify that both of these genetic modifications were made, we will be conducting a series of functional assays.

Functional Assays

Before beginning to test whether the cell acquired the desired genetic modifications, it is important to determine a negative control, which will allow us to be able to determine what genetic modifications the cell successfully integrated into its own DNA. My negative controls

would include stained and unstained primary peripheral blood (HLA-positive/CAR-negative) NK cells and unmodified IPSC derived NK cells. Positive controls would include a known CAR-NK cell line and a known HLA deficient human cell line.There will also be 2 other negative controls in the case of only one vector being successfully integrated into the NK cell. The permutations are hereby described in the table below.

Cell type	amCyan1	mCherry	Streptomycin resistance	Ampicillin resistance
HLA-, CAR+	+	+	+	+
HLA+, CAR+	+	-	-	+
HLA-, CAR-	-	+	+	-
HLA+, CAR-	-	-	-	-

An initial functional assay will be performed to verify that the process of converting keratinocytes into IPSCs, and the consecutive process of IPSCs undergoing hematopoietic differentiation was successful, and that a population of adult, mature, healthy NK cells has been produced. To immunophenotype the IPSC populations by flow cytometry, cells will be incubated with fluorescently labeled antibodies that are specific to keratinocytes, IPSCs, mature and immature NK cells, and hematopoietic stem cells. Through this method, we will know that an adult, mature population of NK cells has been developed if the cell only is detected to have fluorescent antibody that is specific to a receptor expressed by mature NK cells. There are a few possibilities for different receptors that you could select: NK cells express CD56, and CD16 receptors, while they do not express CD3, CD14, CD19(these are T-cell and B-cell receptors. Once we have determined that hematopoietic differentiation has successfully occurred, and a mature, adult, healthy population of NK cells has been developed, we can proceed to the next set of functional assays.

The first functional assay for the genetic modifications performed will be through fluorescent microscopy. We will use fluorescent microscopy to determine whether the exists expression of the two fluorescent protein marker genes in our NK cell culture. The cell where both cell modifications successfully happened should express a cherry colored fluorescent marker(mCherry), as well as cyan colored fluorescent marker(amCyan1).

A secondary functional assay can be performed in vitro, taking the 4 different cell populations, and exposing them to a Streptomycin-based culture, checking for the deaths of any populations, and then consequently placing the remaining populations in an Ampicillin -based culture. By this method, after the duration in both the Streptomycin cultures and Ampicillin cultures, the only population that should be remaining is the HLA Class I deficient CAR-NK cell one.

Once we have determined that an HLA Class I deficient CAR-NK cell population has been successfully created, we must use another functional assay to determine the cytotoxic functionality of the cell, as well as its targeting specificity. This can be analyzed through conducting a Chromium release assay with malignant B-cells sourced from a patient of DLBCL being marked with Chromium-51. If the CAR-NK cells successfully recognize the overexpression of CD-19 on the cells, it will attempt to eliminate these cells through cytotoxic function such as the release of perforin and granzyme molecules. Once the malignant cells have been eliminated, only the Chromium-51 marker will remain, which through a centrifuge machine, can be used to determine the effective cytotoxic potential of the HLA Class I deficient CAR-NK cells as compared to the other 3 negative control populations we developed. I predict that there will be a comparably larger concentration of Chromium-51 in the HLA Class I deficient CAR-NK cell population, as opposed to the other three.

Another functional assay must be done to determine whether the expression of the single-chain HLA class E molecule successfully prevents NK cell fratricide in vitro. This can simply be done through coculturation of the NK cells with HLA Class I deficient CAR-NK cells for a period of time, and then conducting another round of fluorescence microscopy to determine what populations of cells are still alive in the culture. My prediction for this assay is that the cells with no surface-expression of HLA Class I molecules, and coexpression of a single-chain HLA Class E molecule will be able to successfully bypass the HLA mismatch restriction, and no NK cell fratricide will occur. Opposing this, the cell populations that do express HLA Class I molecules on their surface will be eliminated by the “autologous” NK cells.

The final functional assay to be conducted will be to determine the successful lysing of the CAR-NK cell in vitro through the inducible suicide gene iCasp9. A population of experimental cells and regular NK cells will be placed together, and then the small molecule dimerizer drug AP1903 will be injected into the culture. The injection of AP1903 into a culture with cells expressing the suicide gene should immediately induce apoptotic function, causing rapid lysis of any cells expressing iCasp9.

Finally, once these functional assays have been conducted to determine the effectiveness and functionality of HLA Class I deficient CAR-NK cells in vitro, we must determine their success in vivo. Prior clinical trials with CAR-NK cells and CAR-T cells have been conducted using a mouse model, so for the purpose of this experiment, we will use one as well. A culture of malignant B-cells with overexpression of CD-19 and PD-L1 will be fostered in vitro, and then injected into a mouse, to simulate the formation of a tumor. Once this has been completed, we will inject our population of experimental cells, and record the cells’ cytotoxic functionality, as well as targeting specificity and efficiency in vivo, and compare to the other three negative control populations. Seeing how very few clinical trials have been performed with CAR-NK cells,

it is a little tough to predict what the outcome may be, but since there exists data on CAR-T cells, and both of these cells utilize CAR constructs that are very related, I can extrapolate from data about cytotoxicity of CAR-T cells to determine that the HLA Class I deficient CAR-NK cells will be capable of successfully eliminating the malignant B-cells, and at a faster and higher success rate with less adverse side-effects when compared to other CAR-T cell and CAR-NK cell constructs.

Conclusion

Until now, the prognosis of patients with DLBCL resistant to conventional therapies has been incredibly poor with little to now hope in becoming better. However, with the innovations of CRISPR Cas9 gene editing technology, as well as Chimeric Antigen Receptors, alternative treatments for DLBCL, as well as other lymphomas can only get better. With the introduction of CAR-T cell therapies into commercial medical-care one might believe that an optimal alternative treatment has been found, but this is not the case. The CAR-T cells currently used still have many flaws, such as requirement of autologous T-cells for sourcing to prevent HLA mismatch, and adverse side-effects of Cytokine Release Syndrome, and Graft versus. Host Disease. With my proposed cell design of allogeneic IPSC-derived HLA Class I molecule deficient anti CD19 CAR-NK cells, I predict that these constraints that pose problems for the applications of CAR-T cells in the medical field will be bypassed by my cell treatment. By inhibiting HLA Class I molecule surface expression, as well as coexpressing a single-chain HLA Class E molecule, the cell will be able to bypass the NK cell inhibiting receptors that interact with HLA Class I molecules, and instead recognize the expression of the single-chain HLA Class E molecule, which will inhibit the NK cell’s ability to induce cell-dependent cytotoxicity. Furthermore, with the addition of an anti CD19 CAR that also contains a transmembrane domain and costimulatory domain of NKG2A and 4-1BB respectively as well as the breaking of the PD-1 receptor, the

cell's cytotoxic functionality and targeting of CD19 on malignant B-cells will marginally increase. In the case of mutation of abnormal behavior, the inducible suicide gene iCasp9 can be activated, causing near-immediate lysis of all the cells contains CARs. Finally, by using an induced pluripotent stem cell line as a source of NK cells, we are afforded much more freedom and flexibility with regards to the availability of my treatment commercially.

To summarize, this experimental cell I have proposed here has many prospective applications in the medical field. It will also possibly serve as a jumping point to advance the current research there is available on CAR-NK cells. At the current rate technology and science is advancing, I predict that there will be immense innovation in these two fields, and that the prognosis of lymphoma patients will increase exponentially.

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