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Abstract

Once infected with HIV-1, the host immune system is incapable of ridding itself of the virus. HIV-1 uses latent viral reservoirs (LR) within CD4+ T cells to replicate. Within these reservoirs, HIV-1 is able to go into a latent state where it cannot be detected by the host's immune system or current HIV-1 treatments. By utilizing viewing assays and CRISPR-Cas9, there may be a possibility to identify, isolate, and then cut out HIV-1 from an infected cell. Two possible viewing assays have been proposed and studied in recent research. PCR assays are quicker and easier to administer while viral outgrowth assays (VOA) can measure the activation of the resting memory CD4+ T cells and the release of the HIV-1 from latency. Although research has shown that similar results may be able to be found from PCR, VOA is still the primary assay for HIV-1 LR studies. Current limitations of these assays include the PCR tending to overestimate the size of the LR and VOA underestimates the size, which, in turn, gives a possible false HIV-1 negative reading. Being able to view these LR at a precise reading is vital for the success of CRISPR-Cas9. Several strategies for the use of CRISPR-Cas9 have been proposed to aid in the excision of the HIV-1 LR from the genome of a cell, including saCas9 and spCas9. saCas9 would create a double-stranded break within the HIV-1 genetic code and would fully terminate the virus. Current research is focused on finding a viral transporter for the CRISPR-Cas9 that is big enough to fit the genetic coding needed to ensure the correct cleavage around the HIV-1 yet small enough to be successful. spCas9 is researched as a genome-editing tool that can cleave a eukaryotic double helix due to its double-stranded binding site. Along with dCas9, this technique can be used to potentially ‘cut out' the HIV-1 LRs and catalyze an HIV-1 reactivation which will cause virus-producing cell death. This ability causes both strands of the studied eukaryotic cell (in this case HIV-1) to be inactive and can thus repress the expression of the targeted genes. A method of delivery is needed to be studied in order for this strategy to be feasible.

Keywords: HIV-1, CRISPR-Cas system, HIV-1 latency, spCas9, saCas9

Table of Contents

Introduction

According to the US Centers for Disease Control and Prevention (CDC), in 2017 an estimated 36.9 million people were living with HIV-1 in the world. In 2006, the CDC reported that the average treatment regimen for one person living with HIV-1 to prevent the progression to AIDS would have an estimated annual cost of $19,912. This means annually, 36.9 million people have to decide their life is worth $19,912. Even with current medicine, for many people who are HIV-1 positive, there is not a possible way to fully stop the progression. In this review, the knowledge known prior to and necessary to the primary research will be looked at and will be discussed. This review will go through the most common viewing assays proposed to measure latent reservoirs as well as two of the proposed Cas9 strategies that are being studied.

Background

CRISPR/Cas9

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a gene editing technique that was first discovered in archaea (Broad Institute, 2018). Francisco Mojica, who discovered CRISPR, hypothesized that it was used as a defense mechanism for archaea from viruses (Broad Institute, 2018). This theory was then confirmed by researchers in 2007 (Broad Institute, 2018). CRISPR has been largely used to genetically modify plants to fight off fungi or other antigens; however, in 2013 the first method of using the technique in mice was introduced (Broad Institute, 2018). CRISPR and one of its enzymes (in this paper it will be specifically

Cas9) bind to a target section of DNA and cuts it (Broad Institute, 2018). This review will be specifically concerned about the CRISPR-Cas9 system. Cas9 is a protein that is derived from bacteria (Yin *et al*, 2017). Many different variations of Cas9 can be used depending on the size needed or other factors that may be important to the specific research. This paper will look specifically at Cas9 from *Staphylococcus aureus* and *Streptococcus pyogenes*. This combination of CRISPR and Cas9 has been used in science since 2005 (Ishino, 2018).

The basic human immune system

A healthy immune system works to rid the body of any antigens that may have entered through bodily fluids or through other external sources. According to the Institute for Quality and Efficiency in Health Care (IQWiG), there are two different parts to the immune system: innate and adaptive (InformedHealth.org, 2010). The innate immune system is in charge of going around the body and looking for antigens (most commonly bacteria) which it will eliminate. In the adaptive immune system, the body has already been introduced to the antigen and has stored the "memory" for a later time to be recalled (InformedHealth.org, 2010). A main component of this adaptive system are cells named CD4+ T cells. These cells help B cells produce the antibodies that are used to protect the body (Zhu et. al., 2008). These CD4+ T cells are a key target of HIV-1.

These two aspects of the immune system work together to keep the body healthy and in working condition.

HIV-1 infected immunity

HIV-1 has been found to be a particularly tough virus to work with because it is very easy to unknowingly transfer to others due to its ability to cross the blood-tissue barrier, it is very aggressive, and it can "hide" within the infected cells (Darcis *et al*, 2018). The virus attacks CD4+ T memory cells and then enters a state of latency so it cannot be detected by the body as an antigen (Darcis *et al*, 2018). While in these CD4+ T cells, the virus will replicate until the original CD4+ T cell becomes so full of the virus that it explodes and each viral cell finds a new CD4+ T cell to replicate itself within the cell. This virus is also very susceptible to creating a drug-resistant mutant due to its very high rate of transcription error (Gang *et al,* 2018).

Another aspect as to why HIV-1 is nearly impossible to rid the body of once infected is due to the latent viral reservoirs (LR). It is within these reservoirs in which the virus goes into its latent stage and replicates in the CD4+ T cell (Darcis *et al*, 2018). LRs can be formed with partial or full infection of HIV-1 in the host (Bruner *et al,* 2015). This makes even early intervention too late for the full eradication of the virus. These reservoirs also have a half-life of 44 months which can be credited for why treatment must be ongoing and lifelong.

HIV-1 itself can be split into two stages: acute HIV-1 and chronic HIV-1. Acute HIV-1 is defined as the 2-4 week window after the initial infection (AIDSInfo B, 2018). It is within this time that levels of HIV-1 is highest in the blood and the chance of transmission is extremely high. In this stage, the host may feel symptoms similar to that of the flu. The chronic HIV-1 stage is also referred to as asymptomatic HIV-1 infection or clinical latency (AIDSInfo B, 2018). It is at this stage where the virus may still be passed on through blood, semen, or mucous linings,

but the infected host may not experience any symptoms of HIV-1. Even without signs or symptoms, the virus is still multiplying within the host cells at a slow rate. Without treatment, HIV-1 will progress to Acquired immunodeficiency syndrome (AIDS). The average progression is roughly 10 years without medicine (AIDSInfo B, 2018). An HIV-1 infected patient may be diagnosed with AIDS if they have a CD4 count of fewer than 200 cells/mm^3 (AIDSInfo B, 2018). AIDS is the most severe stage due to the already severely damaged immune system left by HIV-1. Due to this, a patient whose diagnosis has progressed to AIDS almost always dies of a common infection that the weakened immune system cannot fight off. The life expectancy of someone who has reached the diagnosis of AIDS is three years (AIDSInfo B, 2018).

Current Available Treatments

HIV-1 cannot be cured, however, there are many drugs to help slow down the progression of HIV-1 to AIDS (Mayo Clinic). Current treatments are available to maintain low HIV-1 titres and maintain T cell levels. Having more accessible treatment regimens that are less aggressive to one's lifestyle has helped with patient compliance rates. There are many drugs that go into the general treatment regimen for HIV-1. These drugs are referred to as antiretroviral therapy (ART). It is recommended that ART is taken in combination from at least two of the seven different drug classes (AIDSInfo A, 2018). This drug combination consists of at least three pills and is then called combined antiretroviral therapy (cART) (AIDSInfo A, 2018). This is because each of the pills taken targets a different point in the virus's life cycle. There currently is

not one pill to target the virus throughout its entire cycle. ART is able to block the passing on of the virus but is unable to target the latent viral reservoirs.

Purpose of the Research

The current cost of the therapy required for those living with HIV-1 is far too high. Even with a copay or government assistance, the fact that people have to spend so much of their money on the choice of staying alive is absurd. For many, it isn't even an option. The current medicine only maintains the levels of HIV-1 already in the body. This means that without the medicine, the individual will get worse but they have to continue to purchase the drugs until they pass away. In 2009, the CDC figured that a lifetime of HIV-1 treatment costs was roughly $367,134. If science was able to find a way to eradicate HIV-1 from the body indefinitely then there would be a significantly lower cost of treatment as well as fewer check-ups and time taken out of the diagnosed patients life to maintain the diagnosis.

Findings of Research

Techniques Proposed For Viewing Latent Reservoirs.

Being able to visualize the infected host on a cellular level is vital for fully understanding what is happening with the interaction between the host cell and the virus. There are few viewing assays available now that make viewing and modifying HIV-1 by CRISPR-Cas9 a possibility. The two possible viewing assays that have been proposed and studied within recent research are Viral Outgrowth Assays (VOA) and Real-Time PCR (Bruner *et al,* 2015).

Viral Outgrowth Assays (VOA)

Viral Outgrowth Assays, also known as VOA, were first used to successfully view and measure the size of LRs within a host cell (Bruner *et al,* 2015). Although research has shown that similar results may be able to be found from PCR, VOA is still the primary assay for HIV-1 LR studies. VOA can measure viruses that replicate along with T cell activation (Bruner *et al,* 2015). The proposed use for VOA to help view HIV-1 DNA within recently infected cells is to measure the activation of the resting memory CD4+ T cells and the release of the HIV-1 from latency. VOA is used by purifying CD4+T cells from infected blood samples of patients who are on ART. These cells then get diluted into Phytohemagglutinin (PHA) and get added to activate the latent virus (Bruner *et al,* 2015). CD4+T cells from HIV-1 negative patients are then added to the isolated HIV-1 latently infected CD+T cell sample to promote viral growth (Finzi *et al*, 1997). The virus is then grown in cultures at a high enough level to be examined using an ELISA assay (Finzi *et al*, 1997). Advantages to VOA is that it can quantify the amount of "induced replication- competent" proviruses without having to detect the defective proviruses as well (Bruner *et al,* 2015). There are different categories of proviruses: Induced provirus and noninduced proviruses (Bruner *et al,* 2015). Noninduced proviruses are defective and pose no issue with the eradication of HIV-1. VOA does not detect these so it sorts out the proviruses that do not pose a threat. The induced proviruses have the ability to produce HIV-1 within the assay (Bruner *et al,* 2015). These have a possibility of reinitiating infection, which poses an issue with finding a cure (Bruner *et al,* 2015). One limitation of VOA is that it would only be useful to

measure the latent reservoirs in patients that have been using ART for a prolonged period to suppress the replication of HIV-1 due to the required levels for detection (Bruner et al, 2015). Other limitations include the cost, time, and high blood volume tied to the assay. VOA requires 120-180 mL of blood and a 2-3 weeks tissue culture in a specialized lab (Bruner et al, 2015). There also may be unknown proviruses that could potentially limit the probability of success of a cure that cannot be detected by VOA. There may be unknown proviruses have the ability to become replication-competent (Bruner et al, 2015). Lastly, VOA cannot get a precise enough reading and underestimates the size of the LR, which, in turn, gives a possible false HIV-1 negative reading. In order for the eradication of HIV-1, all transcribable information must be extracted. Therefore having a near exact reading is very important.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is another common way to measure HIV-1 LR. PCR works similarly to VOA. The DNA of the sample must be isolated and denatured to have two single strands of the sample DNA. An enzyme, Taq polymerase, is added to build two new strands of replicated DNA (NIH, 2015). This new DNA can then be amplified and read through gel electrophoresis. Similar to VOA, PCR measures replication-competent provirus DNA, however, it can also detect free-floating HIV-1 DNA as well as defective proviruses (Bruner *et al*, 2015). The main exclusion of detection from PCR is the detection of proviruses that have had a substantial amount of deletion within its DNA (Bruner *et al*, 2015). Unlike VOA, PCR is not over consuming with the amount of time spent in labs or excessive in the volume of required materials such as blood. A current restriction of PCR is the tendency to overestimate the size of

the LR. This can give a false reading for how much of the virus is left within the cells (Bruner *et al*, 2015). Another downfall of PCR is that it detects many proviruses that will have little to no value to the research (Bruner *et al*, 2015).

CRISPR CAS9 strategies proposed for excision

Currently, Cas9 and CRISPR are used in many different settings for editing a genetic code. Researchers have proposed that this gene-editing method would possibly serve as a base point to be used to edit the Latent Reservoirs (LRs) and cut out the HIV-1 from the genome of the infected host cell. Several approaches are being taken using the CRISPR-Cas9 system to aid in the excision of HIV-1 from the cell genome. Currently, all research has either been done in vitro or in vivo with mice. This review will be focused on the two variations of Cas9: saCas9 and spCas9, and the processes found to work successfully alongside them.

saCas9 (Thakore et al, 2018)

One of the proposed combinations of using CRISPR-Cas9 is using it alongside saCas9. SaCas9 is a version of the Cas9 enzyme isolated from *Staphylococcus aureus,* a common bacteria strain. A large advantage of using saCas9, compared to other versions of the enzyme, is that it would create a double-stranded cleavage within the HIV-1 genetic code and would render the virus useless. To find the value of this process, researchers looked at three different single-guide RNA (sgRNA) combinations that targeted the control center for gene expression which, in HIV-1 is the long terminal repeats (LTRs) (Yin *et al*, 2017). The three sgRNAs that the researchers looked at were labeled LTR-1, LTR-2, and LTR-3. Because HIV-1 is a virus that

only infects humans and a few closely related primates, to ethically perform in vivo research, a strain of HIV-1 was constructed to be able to infect mice cells. This strain is EcoHIV (Potash *et al*, 2005). Researchers then added Firefly luciferase to the strain to make the infected areas glow (Rabinovich et al, 2008). Using PCR viewing assay, the results show that all combinations of the three sgRNAs and saCas9 were reported between 95% and 99% elimination of EcoHIV-eLuc activity (Yin *et al*, 2017). The proposed delivery method to get the RNA/Cas9 combination into the host is through using adeno-associated virus (AAV) vectors (Yin *et al*, 2017). These vectors have found to be the most advantageous delivery method due to their ability to deliver to various tissue types and that it can be administered using the specific method that benefits the research, rather than be constrained to a single type of use (Thakore *et al*, 2018). The researchers wanted to find if they could make their results even closer to 100% by targeting the saCas9/sgRNA to the LTRs and the viral structural genes (Gag or Pol). The results show that all combinations resulted in a great reduction of EcoHIV-eLuc activity in the targeted cells except the pairings of GagB, GagC, and PolB (Yin *et al*, 2017). The most effective combination was when paired with GagD, however, it was also found that the pairing of more than one combination resulted in even higher rates of reduction of EcoHIV-eLuc activity and easier PCR genotyping, therefore following research carried on with the combination of LTR-1, LTR-3, GagD, and PolB. After looking at PCR results, it was confirmed that this combination is the most effective at eradicating the entirety of the HIV-1 genome (Yin *et al*, 2017).

spCas9 (Gang et al, 2018)

CRISPR along with Cas9, derived from *Streptococcus pyogenes* (spCas9), is researched as a genome-editing tool that, due to its double-stranded binding sites, can cleave a eukaryote's double helix similar to saCas9 (Gang *et al,* 2018). spCas9 is currently used in the studies of editing many different eukaryotic cells (Hus *et al*, 2014). The process of cleaving the double helix then causes both strands of the spCas9 to be inactive. This is a form of Cas9 also known as dead Cas9 (dCas9). In this form, dCas9 is able to interfere with the transcriptional abilities of the DNA, yet still, bind with guide RNA, and can thus repress the expression of the targeted genes (Gang *et al,* 2018). SpCas9 differs from saCas9 in its highly selective nature of DNA targeting. This comes from its ability to recognize a protospacer adjacent motif (PAM) of NRG nucleotides (Yin *et al*, 2017). In order to ensure the Cas9 cleaves the intended point of the DNA, guide RNA (gRNA) is used to direct the Cas9 protein to its target (Gang *et al,* 2018). With the use of gRNA, the cleavage site is accurate within the intended 3' and 5' end of a nucleotide chain. Because of this and spCas9 already selective nature, using gRNA can drastically lower the potential for off-target modifications (Gang *et al,* 2018). Researchers have used this cleavage technique to study the effects both before HIV-1 infection or after infection. In an already infected genome, CRISPR-spCas9 is used to attack an established HIV-1 LR. This editing technique can also be introduced to a healthy cell and act as an extra line of defense for the cell (Gang *et al,* 2018). During the same techniques used for saCas9, it was found that pre-treatment of cells with the spCas9 and LTR sgRNAs, while successful at a much lesser rate of eliminating the EcoHIV-eLuc from the mice cells, left them to be resistant to new HIV-1 infection in vitro (Yin

et al, 2017). For either of these methods to be successful in vivo, there must be a delivery method that works with spCas9 such as AAV. The size of the AAV genome is roughly 4.7 kilobases and spCas9 would require 4.2 kilobases (Thakore *et al*, 2018). This has proven to be too big to be a successful delivery method.

Conclusions

The CRISPR-Cas9 system has proven to be a very promising direction to continue research pertaining to HIV-1/AIDS research. There are several other techniques that also use CRISPR-Cas9 to explore the possibilities of eradicating HIV-1 alongside other incurable diseases that find themselves in a gene sequence. This is a very important point of research because if one system doesn't work, there are many other combinations for research. Both spCas9 and saCas9 have proven to be successful in eliminating HIV-1 from the infected genome, however, with the current limitations of delivery size, saCas9 has proven to be the preferred route of study. This shows that CRISPR-Cas9 can be used to permanently inactivate HIV-1 latency. CRISPR-Cas9 based gene therapies are feasible strategies against HIV-1.

Future Directions

As of now, major research issues fall within not being able to narrow down the size restraints for viewing latent reservoirs. If researchers are able to find a viewing assay that can show the exact size of the latent reservoirs, then being able to detect how much of the cell needs to be targeted could be used to successfully rid it of the virus without overshooting and killing

the cell. Another issue that has surrounded HIV-1 research is the number of different types of research that are being conducted. For example, many studies show the theoretical possibility of the CRISPR-Cas9 system working to cut HIV-1 from the cell genome, however, more studies need to be conducted in order to reflect the true possibilities of what CRISPR-Cas9 processes may be the most efficient and/or feasible. There is also some discrepancy on the possibility of a successful delivery into the host. If the CRISPR-Cas9 system were able to work, there would have to be a big enough virus to house all of the gene editing tools within it. There are current studies finding close matches but further research must proceed in order for any of the proposed methods to be successful. Researchers are also looking into the possibility of CRISPR being used to modify host cells to be no longer vulnerable to HIV-1 infection (Gang *et al,* 2018). A wild type of genetic mutation has been found with the deletion of 32nt in the CCR5 gene. People who have been studied and have this have been known to be more resistant to HIV-1. There are many different directions that are proving sustainable options for the cure and resistance to HIV-1. The continuation of HIV-1 treatment and curative research must continue in all promising aspects in order to ensure the quickest and most effective findings.

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