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Omar A. Garcia Martinez
Augustana College, Rock Island Illinois

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Omar Garcia Martinez

HIV Vaccines: Progress, Limitations and a CRISPR/Cas9 Vaccine

Introduction

The human immunodeficiency virus type 1 (HIV-1) pandemic is a global health problem that continues to affect millions of people worldwide. By the end of 2014, there were 36.9 million HIV-infected individuals living around the world (UNAIDS 2015). HIV-1 is notorious for its elusive nature towards the immune system, its high mutation rate and the lack of symptoms that occur after initial infection. The lack of symptoms causes people to unknowingly transmit the deadly virus most commonly during sexual intercourse. HIV-1 transmission primarily occurs through mucosal epithelial cells, which are found in genital and rectal mucosal surfaces (White et al. 2015). Statistics show that about 2 million people are infected annually with HIV (UNAIDS 2015). Once a person is infected with HIV-1 there is a 5-10 day window in which infected cells could become eradicated. After this small window, the disease becomes too widespread and diverse to eradicate completely (McMichael et al. 2009). Relatively recent advances in HIV treatment have given rise to highly active retroviral therapy (HAART). HAART has been shown to lower levels of viremia to almost undetectable levels by inhibiting HIV in various steps of its viral life cycle such as virus entry, reverse transcription, integration, or maturation. This therapy can stop the virus from infecting new cells, which in turn, will delay the onset of AIDS (Fig. 1). HAART, however, cannot eradicate HIV that is already integrated into the host genome (Marsden and Zack 2013). In addition, the rising numbers of people living with HIV, due to the advent of HAART therapy, increase the likelihood that the disease will be transmitted from a quantitative standpoint. Thus, there is a need for a prophylactic medication to

stop the HIV-1 virus from integrating into the host genome or one that is able to eradicate HIV-1 proviral DNA altogether. Ideally the best intervention in combatting the HIV-1 pandemic is by a preventative vaccine that is highly effective, reliable, cost-efficient, and with no serious side effects (Hemelaar 2011).

Difficulties in treating HIV

The HIV-1 virus has developed many different mechanisms to evade our immune system making it difficult to develop an HIV-1 vaccine. Part of understanding the reasons for the difficulties in treating HIV lies in its pathogenic viral life cycle (Fig. 2). First, HIV-1 will bind to the CD4 receptor protein as well as a coreceptor protein on the surface of a cell, such as a T cell or B cell. Next, the viral and the host cell membrane fuse together and the viral RNA is released into the inside of the cell. The enzyme, reverse transcriptase, converts RNA into double-stranded DNA. As stated before, the main characteristic of a retrovirus is that it can integrate its RNA into the host genome and will remain there for the rest of the cell's lifespan (Marsden and Zack 2013). Thus, HIV-infected cells that contain HIV-1 proviral DNA can persist for decades without expressing or transiently expressing the viral proteome. The problem with these latent reservoirs is that HIV expression is often re-initiated upon interruption of HAART treatment and viremia returns to pre-therapy levels. HAART can stop the virus from replicating but it has no effect on HIV that is integrated into the host genome. However, the main mechanism HIV-1 uses to evade the immune system is not the advent of latent reservoirs but rather its high escape mutation rates. Shortly after infection, HIV-1 begins establishing a latent reservoir that is abundantly filled with HIV-1 variants. It has been shown that mutations advantageous for continual HIV-1 infection of non-infected cells are selected for by natural selection. Often, these mutations occur in order to evade cytotoxic T lymphocytes (Siliciano and Greene 2011). These

escape mutations inhibit the recognition of infected cells by cytotoxic T lymphocytes. As a result, the latently affected cells will continue to survive and replicate further, which will only increase infection and levels of HIV latent cells. Additionally, the high mutation rates of HIV will also increase the likelihood that the virus becomes resistant to HAART therapy (Levy 2015). In order for HAART to continue to work, the drug combinations administered have to be changed since HIV can build a resistance. Finally, taking HAART for a lifetime can make HIV patients more susceptible to various diseases. Widespread use of HAART may be linked to the development of resistant isolates (Levy 2015). Thus, a prophylactic vaccine is needed since it will avoid the various mechanisms and mutations HIV-1 undergoes that make HIV-1 infection so difficult to treat.

Adaptive Immune Response

In general, vaccines work by providing active adaptive immunity to a particular disease. Vaccines can be both prophylactic and therapeutic. A killed or weakened form of a microbe or surface protein is typically used in vaccines. These microbes and proteins will induce an adaptive immune response upon introduction to the body. The adaptive immune response is antigen-specific and will develop memory B and T cells during first exposure to the antigen (Flajnik and Kasahara 2009). Antigens are molecules capable of inducing an immune response. Antigens can be parts of a virus or bacteria. For example, the outer envelope surrounding the HIV-1 virion and the flagella present on some bacteria can be antigens. The immunological memory established upon first exposure allows for a greater immune response to the previously encountered antigen (Flajnik and Kasahara 2009). There are two arms to the adaptive immunity. One arm, known as humoral immunity, typically uses B cells to defend the host from foreign antigens. The other

arm, known as cell-mediated immunity, involves mostly cytotoxic T cells that recognize and subsequently eradicate infected host cells.

Humoral

As stated previously, the humoral arm of the adaptive immune system responds to foreign antigens circulating in the body. The humoral immunity is the first response when a foreign antigen is introduced into the body. After initial infection, B lymphocytes (B cells), which are specific to certain antigens, will bind to their corresponding antigen and engulf the pathogen. The pathogen will be broken up and a peptide fragment of the pathogen will be presented on the surface of a B cell by MHC class II molecules (Flajnik and Kasahara 2009). After initial recognition, a helper T cell (CD4+ T cell) will recognize and bind to the MHC class II protein and peptide fragment, which will then activate the B cell. An activated B cell will replicate and differentiate into plasma and B memory cells specific to that specific antigen (Calame 2001). These B memory cells can live for decades waiting to be activated again by the reintroduction of the same antigen into to the host organism. The plasma made from proliferating B cells will produce antibodies that are specific for that same antigen (Calame 2001). These antibodies will recognize the antigen upon future infection and bind to it. Antibodies serve as a marker when bound to an antigen so non-specific phagocytes can more quickly recognize the invading pathogen and kill it (Calame 2001). In summary, vaccines work by presenting foreign microbes or proteins to the body in an attempt to trigger an adaptive response, which will better prepare the body upon future infection.

Cell-mediated

The cell-mediated response involves mostly T cells and responds to infected cells by destroying it, rather than acting on a free flowing antigen. Antigen presenting cells (APC) engulf the antigen and break it down to peptide fragments. This ability is similar to B cells, however, MHC class I molecules present the peptide fragment on the surface of an APC instead of MHC class II molecules. The difference being that MHC class I molecules bind to CD8+ T cells, which are also known as cytotoxic T lymphocytes (Flajnik and Kasahara 2009). These CD8+ T cells will activate upon recognition of the MHC class I molecule and the peptide fragment. Activated CD8+ T cells proliferate into more CD8+ T cells as well as memory T cells (McMichael and Rowland-Jones 2001). Memory T cells, like B memory cells, can live for decades in case this type of infection occurs in the future. Memory T cells allow for more efficient recognition and killing of infected cells. In addition, activated CD8+ T cells have the ability to secrete cytokines and chemokines that makes surrounding cells more prepared to fight the infection (McMichael and Rowland-Jones 2001).

HIV Vaccine Strategies

Currently, there are several strategies in making an HIV-1 vaccine. The advent of recombinant DNA technology has given rise to novel approaches in fighting HIV-1 infection. The strategies that show the most promise are attenuated or live vector vaccines, viral peptide vaccines, and recombinant DNA vaccines (Fig. 3; NIH 2012). In general, these vaccines attempt to induce an effective, long-lasting, broad antibody response. Acute HIV-1 infection results in perturbations to B cells so vaccines that prime the immune system to induce an effective secondary response are also needed (McMichael et al. 2009).

Live Vector Vaccines

To start with, attenuated or live vector vaccines use a weakened version of a virus or bacteria to transmit a recombinant DNA plasmid to the host cell so its contents can later be expressed. Plasmids are small circular double-stranded DNA molecules that can replicate independently outside the host genome. The plasmid usually contains genes encoding antigens that can stimulate a strong immune response. The weakened virus or bacteria is still able to infect host cells but it will not express the viral or bacterial genome since that may be toxic to the host organism. The transfected foreign DNA will then use the host cell's machinery to make HIV viral proteins. These proteins are later broken down by proteasomes in the cell and the viral fragments are either secreted or presented on the surface of the cell so it can be recognized by B and T cells. This will induce the creation of memory B and T cells as well as antibodies that will make the immune system more prepared upon subsequent infection. HIV-1 vaccines using viral vectors have shown promise and have been studied in clinical trials (NIH 2012).

This strategy of using attenuated, live vectors works well in eliciting a strong immune response since the introduction of viral or bacterial vectors into the organism mirrors a real infection. Additionally, viral vector vaccines are relatively facile to manufacture since they do not contain many genes, which also increases control of viral expression. However, these vaccines have significant limitations as they have the potential to be unsafe or toxic. The microbe used as a vector is technically still alive. A highly mutagenic virus like HIV should not be used as a vector, even if it is weakened, since certain mutations can change the virus back to its virulent form. Furthermore, patients with compromised immune systems are not able to take this type of vaccine since their immune system will not be able to create an immune response. Using an inactivated vaccine would reduce the potential danger of mutations but will most likely not induce a strong enough immune response.

Viral Peptide Vaccines

Another vaccine strategy used to combat HIV-1 infection is by using vaccines containing viral peptides or virus-like particles. Instead of inserting a DNA plasmid into a host cell so it can express antigens, peptide vaccines already contain the antigens. Injecting patients with viral antigens can stimulate their CD4+ T cells and elicit an immune response. Some prominent vaccines using virus-like particles contain envelope glycoproteins, which are normally expressed on the surface of HIV-1 (Pitisuttithum et al. 2006). The glycoproteins would ideally contain highly conserved epitopes making HIV easier for the immune system to recognize upon future infection. The faster antibodies are made the better since HIV-1 needs only 5-10 days upon initial infection to replicate, differentiate and insert itself into immune cells (McMichael et al. 2009).

Viral peptide vaccines are generally safer than live vector vaccines since they contain specific conserved antigens rather than a whole microbe. As was previously stated, microbes have the potential to mutate and revert back to its virulent form. Nonetheless, these vaccines may not induce a strong enough immune response. A better understanding of the specific antigens most recognized by the immune system is needed to develop an effective peptide or viral particle vaccine.

DNA Vaccines

Lastly, recombinant DNA vaccines are still in early development and have shown great potential in eliciting an effective immune response. DNA vaccines are similar to live vector vaccines. Both use recombinant DNA technology but DNA vaccines do not necessarily need a bacteria or virus to carry the plasmid into the host cell. Injecting naked DNA into the host

organism will cause the DNA to be taken up by some cells. These cells will either integrate the foreign plasmid DNA into its own genome or will keep it separated from the host chromosome (Wolff and Budker 2005). DNA vaccines use a recombinant DNA plasmid carrying different HIV related genes encoding for viral proteins. Similar to live vector vaccines, these proteins will be expressed and cleaved by enzymes and shuttled to the surface of the cell where it will be recognized by B cells, helper T cells and cytotoxic T cells.

Furthermore, DNA vaccines are promising because they have the ability to elicit a long-term immune response since the plasmid inside the host cell will continuously be expressed. If a person gets infected with HIV-1, the body will already have produced enough antibodies to effectively fight the infection. Further research is needed to identify the genes that code for antigens that elicit a strong immune response. Another upside is that DNA vaccines are relatively easy to manufacture due to the growing knowledge of recombinant DNA technology.

In contrast, naked DNA vaccines have a major limitation that hinders its effectiveness. Overall, relatively low expression levels are achieved when attempting to transfect DNA into host cells. The host cells that do manage to take up the naked DNA plasmid is highly variable. It has been shown that cells farther away from initial injection site will have lower levels of successfully transfected DNA (Wolff and Budker 2005). Transfection is the uptake of DNA into a eukaryotic cell. In order to counteract these low levels of transfection other measures to increase DNA uptake need to be administered along with the injection of naked DNA. Electroporation techniques have been shown to be effective in greatly increasing DNA uptake (Wolff and Budker 2005). Electroporation is a technique in which an electrical field is applied to cells to increase their membrane permeability. A previous study has shown that intramuscular injection of naked DNA into mice followed by subsequent electroporation increased the levels of

DNA taken up by the host cell by 6- to 34-fold, when compared to the dose that did not undergo electroporation (Wang et al. 2004). More techniques to increase DNA uptake are being developed year after year. As a result, DNA vaccines are now beginning to approach expression levels achieved by live viral vectors (Wolff and Budker 2005).

Proposed Vaccines and Limitations

Unfortunately, all proposed vaccines have shown little to no efficacy during vaccine efficacy trials (McMicheal et al. 2009). Recent, notable efficacy trials include the AIDSVAX trial, the STEP study, and the Thai trial (Pitisuttithum et al. 2006; Buchbinder et al. 2008; Rerks-Ngarm et al. 2009). The vaccine combination tested in the Thai trial is the only one that showed some protective effect towards HIV-1 infection, although statistical analysis showed these results to be insignificant. More research is needed on the earliest responses of the immune system in order to find ways to safely induce neutralizing antibodies with broad-specificity (McMicheal et al. 2009). Vaccines have been the most effective means for fighting deadly infectious diseases like small pox or polio (NIH 2008). More and more people are living with HIV-1 due to HAART therapy so more individuals are capable of further transmitting the disease even if their viremia levels are low. We must continue research so we can find an effective prophylactic vaccine and put an end to the HIV-1 pandemic.

The AIDSVAX Trial

To begin with, the first efficacy trial in Asia was started in the mid-90s and completed in 2006 for the virus-like particle vaccine known as AIDSVAX B/E. Phase I and phase II trials showed the vaccine was safe to use in humans so the study moved on to phase III trials. The virus-like particle vaccine consisted of envelope recombinant gp120 glycoproteins derived from

HIV-1 subtypes B and E (Pitisuttithum et al. 2006). The envelope gp120 glycoprotein is normally found on the surface of the HIV-1 envelope, which is the protective covering surrounding the HIV-1 virion. A recombinant gp120 glycoprotein was used so it would elicit antibodies specific for two subtypes of HIV-1. In this case recombinant gp120 glycoproteins from HIV-1 group M subtypes B and E were used for the vaccine because these HIV-1 strains are among the most abundant in the United States and Thailand, respectively. The vaccine works by inducing an antibody response that subsequently incapacitates HIV-1. More specifically, the antibodies produced by proliferating B cells, specific for gp120, can bind to the gp120 protein on the surface of HIV-1 and remove the receptor off the surface. If HIV-1 is not able to bind to cells then that would essentially make HIV-1 incapable of infecting any host cells (Pitisuttithum et al. 2006). About 2500 at-risk individuals from Thailand were tested with AIDSVAX. These individuals were all volunteers and were at-risk for contracting HIV-1 because of their injection drug use history.

The AIDSVAX trial proved to be unsuccessful after failing to prevent or delay disease progression even though the vaccine did manage to induce antibodies to gp120 (Pitisuttithum et al. 2006). The cause for the failure seemed to be because the vaccine did not produce a broad enough immune response making the individual susceptible to HIV-1 isolates. The vaccine induced an entirely humoral response so infected cells were capable of proliferating since there was a lack of cell-mediated response. This study identified the need for a vaccine that would stimulate both arms of the adaptive immune system instead of only the humoral response. Even though the vaccine was shown to have low efficacy, its longitudinal data regarding disease progression and at-risk behavior gives useful information for future studies. Additionally, this study showed that it is possible to recruit a high number of injection drug users and these users

were found to be compliant and most benefited from the counseling they received as part of the study (Pitisuttithum et al. 2006).

The STEP Study

Secondly, a newer efficacy trial, known as the STEP study, was conducted for an adenoviral vector vaccine. This study followed the AIDSVAX trial and was the second efficacy trial ever conducted (McElrath et al. 2008). Instead of testing the humoral response, the STEP study was focused on stimulating the second arm of the adaptive immune response, the cell-mediated response. The researcher's ultimate question was to find if the STEP vaccine induced cytotoxic T lymphocytes specific for HIV-1 (Buchbinder et al. 2008). The researchers attempted to induce the creation of long-term memory CD8+ T cells that recognize conserved viral epitopes. The three vaccines used were viral vectors using replication incompetent adenovirus serotype 5. The plasmid carried genes encoding for HIV-1 gag, pol, and nef proteins (Buchbinder et al. 2008). The vaccines worked by expressing intracellular HIV proteins in the host cells, which the researchers hoped would induce the production of memory cytotoxic T lymphocytes. Around 3000 individuals were tested and were administered the three gag, pol, nef vaccines or a placebo (McElrath et al. 2008).

Vaccination in the STEP study was discontinued before it was scheduled to finish as it was found to have increased HIV-1 infection in a subgroup of at-risk individuals. The protocol for the study predicted that there would be equal or fewer HIV-infected individuals among those who received the vaccine compared to participants who only received the placebo. However, the data indicated a much different result. Out of the 914 male vaccine recipients, 49 became infected with HIV-1 while out of the 922 male placebo recipients, 33 were found to be HIV-1

positive (Buchbinder et al. 2008). The fact that there were more vaccine recipients affected with HIV-1 than placebo recipients forced the study to be discontinued since the vaccine was found to be ineffective. However, there is no definitive data that shows that the vaccine increased the likelihood of contracting HIV-1. It can be argued that cytotoxic T lymphocytes are better for reducing viral load after infection than actually preventing HIV-1 infection. The absence of genes encoding HIV-1 envelope proteins may also decrease the likelihood that antibodies will sense HIV-1 infection since only gag, pol, and nef regions of HIV-1 were expressed. Vaccines that induce both the humoral and cell-mediated response should be more effective against HIV-1 infection than using a vaccine that only affects one arm of the adaptive immune system.

The Thai Trial

Third of all, the ALVAC/AIDSVAX vaccine was tested for efficacy in the Thai trial, which began in October 2003 and ended in July 2006. Similar to the AIDSVAX trials, they tested the vaccine on at-risk individuals living in Thailand. The vaccine was a prime-boost combination of two vaccines consisting of the failed AIDSVAX vaccine, described previously, and ALVAC. The ALVAC vaccine contained a dual ability pox virus that was able to induce cell-mediated responses as well as prime the immune system for the creation of antibodies. The ALVAC vaccine used a harmless recombinant canary pox vector containing genes that express HIV-1 gag and pol proteins as well as gp120 (Rerks-Ngarm et al. 2009). The AIDSVAX vaccine was used to boost the immune system. As previously mentioned, the AIDSVAX vaccine contains a recombinant gp120 glycoprotein from HIV-1 subtypes B and E. The prime-boost vaccination was tested on 16,402 HIV-negative participants making it the largest efficacy trial ever conducted.

Lastly, the Thai trial tested the efficacy of the ALVAC/AIDSVAX vaccine. The Thai trial is the only efficacy trial that has given any positive clinical results in preventing HIV-1 infection. Out of the 16, 402 total participants, 125 of them were found to have contracted HIV. A total of 51 people were infected with HIV-1 in the vaccine group compared to 74 from the placebo group (Rerks-Ngarm et al. 2009). This time instead of the vaccine group having a higher number of HIV-infected individuals, the vaccine group showed a reduction in HIV-1 infected individuals when compared to recipients from the placebo group. The initial reports indicated that there was a 31.2% reduction in HIV-1 infection among vaccine recipients compared to placebo recipients (Rerks-Ngarm et al. 2009). However, there was no reduction in viremia levels in HIV-1 infected participants from the vaccine group compared to HIV-1 infected participants in the placebo group. Statistical analysis of the study found the reduction in HIV-infected participants to be not significant. The findings albeit promising, should be taken with a grain of salt since there still is no definitive data suggesting we have found a successful prophylactic HIV-1 vaccine.

CRISPR/Cas9 System

Recent advances in engineered nuclease systems, like the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system, have given rise to novel gene editing treatment options for HIV-1 (Doudna and Charpentier 2014; Dance 2015). A recent proof-of-concept study showed that the CRISPR/Cas9 system could be used to prevent HIV-1 infection in myeloid cells, as well as having therapeutic effects *in vitro* (Hu et al. 2014). CRISPR are segments of prokaryotic DNA that contain short segments of DNA acquired from previous exposure to viruses or other pathogens. The CRISPR/Cas9 system is derived from a natural bacterial adaptive immune system that gives resistance to foreign genetic material, such as

retroviruses (Barrangou and Marrafini 2014). The system has the ability to efficiently cleave DNA by using Cas9, an endonuclease enzyme, coupled with a guide RNA (Fig. 4; Cas9/gRNA). The guide RNA directs the Cas9 enzyme to the corresponding base pair sequence on DNA (Doudna and Charpentier 2014). The Cas9 enzyme will then bluntly cut the DNA on both strands. A PAM (protospacer adjacent motif) sequence must immediately follow the target DNA cleavage site in order for Cas9 cleavage to occur. The PAM sequence is usually a 2-3 nucleotide guanine-rich sequence (Barrangou and Marrafini 2014). The need for a PAM sequence for Cas9 activity to occur has been developed in bacteria to distinguish bacterial self DNA from foreign DNA. Cas9/gRNA activity has the ability to cleave DNA integrated in the host genome as well as extrachromosomal DNA (Hu et al. 2014). Thus, Cas9/gRNA activity has the potential to immunize cells from HIV-1 infection since the HIV-1 DNA will be cleaved before it integrates itself into the host genome. This bacterial immune system shows great promise for treating or curing numerous genetic diseases. One day this bacterial adaptive immune system may be part of our own adaptive immune system.

Conclusion

In conclusion, the lack of effective vaccines sheds light on the unique ways HIV-1 evades the immune system. Only one vaccine combination, the Thai trial, has shown any promise. The findings from the AIDSVAX clinical trial, STEP study, and the Thai trial suggest that preventing HIV-1 infection with a prophylactic vaccine may be an all or nothing endeavor. The first battle with HIV-1 primarily takes place in the mucosal surface of epithelial cells. The vaccine will most likely only be successful if it is able to block or kill the HIV-1 virus before it enters the bloodstream. If a prophylactic vaccine cannot prevent infection, then the high escape mutation rates may make the HIV-1 infection too diverse and abundant in its latent reservoir to counteract

with a therapeutic vaccine (Siliciano and Greene 2011; Deng et al. 2015). This hypothesis is supported by the fact that viremia levels were no different in HIV-infected participants from the vaccine and placebo groups during the ALVAC/AIDSVAX clinical trials (Rerks-Ngarm et al. 2009). Thus, the main goal for a preventative vaccine should be in destroying the HIV virus at mucosal surfaces so it does not have a chance to differentiate and build its latent reservoir. More research is needed in finding novel vaccine strategies that are able to prevent HIV-1 infection. The positive findings from the Thai trial as well as recent advances in engineered nucleases like the CRISPR/Cas9 system suggest that we may be closer than ever to finding a prophylactic vaccine for HIV-1.

Study Proposal

Introduction

The proposed vaccines tested in phase III clinical trials have all been unsuccessful (Pitisuttithum et al. 2006; Buchbinder et al. 2008; Rerks-Ngarm et al. 2009). The Thai trial was the only efficacy trial that showed some positive results. The Thai trial tested the efficacy of the ALVAC/AIDVAX vaccine combination. A 31.2% reduction in HIV was seen when comparing participants from the vaccine group and placebo group (Rerks-Ngarm et al. 2009). Although this was not statistically significant, the ALVAC/AIDSVAX combination was a step in the right direction but there is still a need for novel ways in combatting HIV-1. Ideally an effective prophylactic HIV vaccine would have a 99.9% prevention rate.

Recent advances in engineered nucleases have given attention to gene editing techniques, like the CRISPR/Cas9 system and zinc finger nucleases. The former has been shown to effectively cut out integrated HIV-1 proviral DNA from latently infected human reservoir cell

types *in vitro* (Hu et al. 2014). This system has been shown to be successful *in vitro*; however, it has never been attempted *in vivo*. Additionally, never before has the CRISPR/Cas9 system been coupled with the ALVAC vaccine (CRISPR/Cas9/ALVAC). The objectives of this proof-of-concept study are to (1) evaluate if the CRISPR/Cas9 system in combination with the ALVAC vaccine can immunize host epithelial cells in the cervix of humanized mice; (2) determine whether HIV-1 proviral DNA is successfully excised from host epithelial cells; (3) and determine whether there are off-target effects due to Cas9/gRNA activity. HIV-1 infects people primarily through mucosal surfaces so this vaccine will target the cervix of female humanized mice since this area is a major target for HIV-1 (Fig. 5; White et al. 2015; Levy 2015). The vaccine will be successful if it is able to prevent epithelial cells from being infected with HIV-1. Not allowing HIV-1 to travel further into the organism will help us evade the problem of high escape mutation rates and latent reservoirs. If the virus manages to travel past epithelial cells then the hope is that the ALVAC vaccine will have primed the immune system enough to kill any remaining HIV-1 virions.

Materials & Methods

Plasmid Preparation

For the plasmid, a Cas9 expression vector will be used alongside 2 guide RNA (gRNA) sequences. The guide RNAs will form a complex with the Cas9 endonuclease enzyme and will be used to guide the enzyme to the promoter region found in the LTR sequence in HIV-1 DNA, regardless of whether it is proviral or extrachromosomal DNA (Hu et al. 2014). The target LTR sequence is followed by a PAM sequence, which is essential in order for Cas9 cleavage to occur (Barrangou and Marrafini 2014). The guide RNA base pairs with the corresponding sequence in

HIV-1 DNA and the Cas9 enzyme will cleave the DNA backbone immediately before the PAM sequence.

Furthermore, an INV promoter and enhanced green fluorescent protein (EGFP) reporter gene sequence will be inserted into the CRISPR/Cas9 plasmid so plasmid expression can be verified (White et al. 2015). The INV promoter will add specificity and reduce possible unwanted cleavage of the host genome in non-epithelial cells since this promoter is only expressed in epithelial cells, the area that HIV-1 first comes in contact with during sexual transmission (White et al. 2015; Levy 2015). The EGFP will be used to determine whether the Cas9 enzyme is being expressed. Fluorescence microscopy will be used to generate a microscopic image of epithelial cells, which will show the presence or absence of the EFGP protein on the surface of the epithelial cells (Bierhuizen et al. 1999; Hu et al. 2014). The Cas9 expression vector will be incubated with appropriate lipids to make a CRISPR/Cas9/liposome complex. The liposome will facilitate the transfection of the CRISPR/Cas9 plasmid into host epithelial cells (Relloso and Esponda 2000).

Humanized Mice

A total of 30 humanized mice will be used for the experiment. Out of the 30 humanized mice, 10 will be used for the control group, 10 will be used to test immunization capability, and 10 to test therapeutic ability of CRISPR/Cas9/ALVAC vaccine.

Currently, humanized mice models are being used in preclinical efficacy studies of HIV-1 prevention and HIV-1 therapeutic interventions (Denton and Garcia et al. 2011). Additionally, humanized mice models are useful for this study since they can be used to study the prevention of rectal and vaginal HIV-1 transmission. Female humanized mice will be tested with the

CRISPR/Cas9 vaccine in combination with ALVAC since they are suitable models for the human immune system (Deng et al. 2015). Humanized mice will be developed by extracting CD34+ hematopoietic progenitor cells from healthy bone marrow and transplanting them into newborn immunodeficient mice (Fig. 6; Deng et al. 2015). Hematopoietic progenitor cells have the ability to differentiate into specific types of immune cells and will eventually develop into a functional immune system inside the mice (Denton and Garcia et al. 2011). Peripheral blood from the humanized mice can be tested to measure viral load, CD4+ T cell depletion, and immune activation. Overall, humanized mice models can be used to study virtually all aspects of HIV-1 biology and pathogenesis.

In-vivo gene transfer

Control Group

The 10 mice in the control group will be injected with known HIV-1 isolates intravenously using a thin syringe. The HIV-1 isolates will be purified and sequenced prior to injection. The control group will be used to determine if the mice are expressing HIV-1 viruses in their cervical epithelial cells 2 weeks after infection since that is when viral load levels start to peak (White et al. 2015). A Southern blot will be used to identify the presence of integrated HIV-1 proviral DNA.

Immunization Group

First, the CRISPR/Cas9/liposome complex, containing the CRISPR/Cas9 plasmid, will be injected into the cervix of three HIV-infected humanized mice using a thin microcapillary tube (Relloso and Esponda 2000). During injection of the first vaccine, electroporation will be used to enhance transfection of the plasmid/liposome complex into host cervix epithelial cells

(Wolff and Budker 2005). Immediately after injection is finished, the ALVAC vaccine will be injected intramuscularly into the gluteus to prime and also boost the immune system (Rerks-Ngarm et al. 2009). The ALVAC vaccine will not be introduced into the mice with electroporation. Finally, after 6 hours, HIV-1 isolates will be injected intravenously. The 6 hour time difference between the ALVAC and HIV-1 isolate injection is done so the ALVAC vaccine has enough time to induce antibody production.

Therapeutic Group

First, sequenced HIV-1 isolates will be injected into 10 humanized mice intravenously using a thin syringe. An incubation period of 3 weeks will then take place to give time for the viral loads to peak (McMichael et al. 2009). Next, peripheral blood will be extracted intravenously and biopsies from cervical epithelial cells will be analyzed to determine if HIV-1 specific antibodies and HIV-1 DNA levels are present using an ELISA assay and Southern blot, respectively. Identifying the presence of HIV-1 antibodies using an ELISA test will confirm the progression of HIV-1 infection. Next, the CRISPR/Cas9 vaccine will be injected into the cervix of 10 HIV-infected humanized mice using a thin microcapillary tube. Electroporation techniques will be used to enhance transfection of the plasmid/liposome complex into host cervical epithelial cells (Wolff and Budker 2005). The ALVAC vaccine will be injected intramuscularly into the gluteus, which is used to prime and also boost the immune system.

Data Collection

An ELISA test will be used on the mice in the control group, immunization group and therapeutic group to determine the presence of HIV-1 infection (Table 1). The 10 humanized mice in the control will be sacrificed 2 weeks after the HIV-1 isolate injection. The 10 mice in

the immunization group and the therapeutic group will be sacrificed 2 weeks after vaccination. Next, a biopsy of cervical epithelial tissue from all groups will be taken and their DNA will be analyzed using a Southern blot (Fig. 7). DNA sequencing will confirm whether HIV-1 is integrated into the host genome. A Southern blot will help determine if the HIV-1 LTR was successfully excised in the therapeutic group and if the cervical epithelial tissue was immunized. Fluorescence microscopy will indicate if the Cas9 expression vector containing the EGFP reporter gene was expressed. If the Cas9 expression vector is expressed, fluorescence micrographs will show bright green EFGP proteins on the surface of epithelial cells (Fig. 8). A one-way analysis of variance (ANOVA) will be used to statistically analyze the ELISA results. The one-way ANOVA will be used to determine whether there are significant differences between the means of the control, immunization, and therapeutic groups.

Expectations & Limitations

My hypothesis is that the CRISPR/Cas9 plasmid will be successfully expressed inside most, if not all, cervical epithelial cells (either extrachromosomally or while integrated into host genome) and the subsequent Cas9/gRNA activity will cleave all HIV-1 DNA encountered. By eliciting an antibody response in combination with gene editing techniques, we may be able to create an effective vaccine combination that will have both therapeutic and immunological functions. This vaccine combination will remove the HIV genome from the host genome, elicit an antibody response and will immunize host epithelial cells by way of memory cell response as well as Cas9/gRNA cleavage of viral DNA before integration into the host genome. The tissue specificity that the INV promoter gives is beneficial in order to reduce off-target effects by the Cas9/gRNA complex.

In contrast, inducing the uptake of the DNA plasmid into all cells *in-vivo* is difficult. DNA uptake into all epithelial cells would be ideal. Thus, eliciting an antibody response will hopefully assist in tagging any remaining HIV infected cells that were not transfected with the CRISPR/Cas9 plasmid. The subunit of HIV found in ALVAC will act as a sort of "sidekick" in fighting HIV-1 infection, while the Cas9/gRNA complex serves as the main line of treatment and defense. This vaccine may not be able to survive long-term inside a human. Instead of the vaccine being used for long-term prevention, it may be used as a topical microbicide that can be applied before sexual intercourse. The topical microbicide could be in the form of a spray that contains the Cas9 enzyme and several guide RNAs that correspond to conserved HIV-1 LTR sequences. However, it would be rather difficult to prevent the enzyme and guide RNAs from denaturing inside the container over time. Keeping the microbicide refrigerated or cooled may be sufficient in counteracting denaturation. A technique that would maintain the biochemistry of the enzyme and RNA molecule could be developed to make a viable Cas9/gRNA topical microbicide. The tissue specificity is a beneficial aspect for preventing HIV-1 infection but may not be advantageous in treating chronically infected individuals since they may have latent reservoirs in different anatomical regions of the body.

Conclusion

This study provides a novel way to combat HIV-1 infection using a CRISPR/Cas9/ALVAC vaccine *in vivo.* An HIV-1 vaccine is needed since vaccines have historically been the most effective method for fighting and protecting people from deadly infectious diseases (NIH 2008). This vaccine not only has the potential to immunize individuals but can also have therapeutic effects. Mucosal HIV-1 transmission is the predominant transmission pathway for new HIV-1 infections (Denton and Garcia et al. 2011). Consequently,

the main goal for a preventative vaccine should be in destroying the HIV-1 virus at mucosal surfaces so it does not have a chance to differentiate and build up its latent reservoir. More research is needed in finding efficient novel vaccine strategies that are able to safely prevent and target HIV-1 infection specifically at mucosal sites.

Appendix

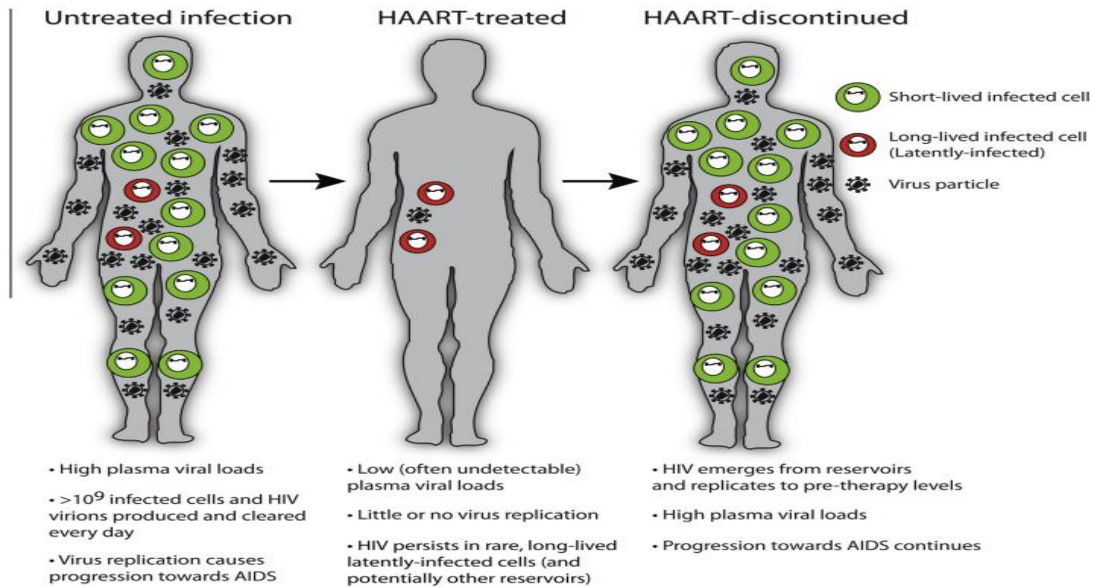

Fig. 1. HAART treatment and viral rebound upon HAART treatment discontinuation. Plasma viral loads are high in untreated HIV-infected individuals. HAART treatment greatly reduces viral loads but latent reservoirs can emerge after HAART discontinuation (Marsden and Zack 2013).

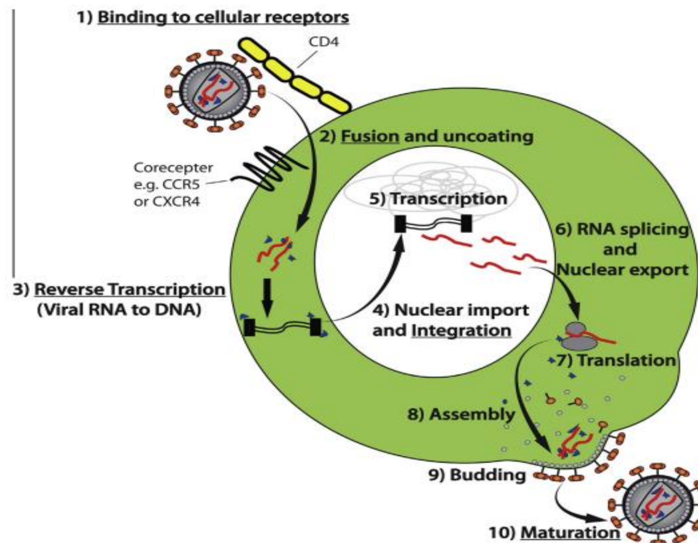

Fig. 2. Essential steps in the viral life cycle of HIV-1 (Marsden and Zack 2013).

HIV Vaccine Designs and Strategies

Vaccine Design	How Does This Vaccine Work?	Issues for HIV Vaccine
DNA	1. A few HIV genes are inserted into a backbone of DNA known as plasmid 2. The vaccine is injected into muscle of the recipient where the HIV genes are expressed into proteins. 3. The viral proteins are degraded into small peptide fragments, which are then presented by molecules on the cell surface. T cells recognizing these molecules generate an immune response.	No DNA vaccines have yet been approved for use in humans by FDA. FDA guidance on DNA vaccines can be found on FDA's Considerations for Plasmid DNA Vaccines for Infectious Disease Indications
Live Vectors: (Viral and Bacterial)	1. The HIV or SIV genes are inserted into the genomes of live, infectious, but non-disease-causing forms of viruses (e.g., adenovirus) or bacteria (e.g., Bacille Calmette-Guerin (BCG). 2. These vectors shuttle "foreign" genes along with their own into cells. 3. HIV proteins generated from these recombinant genes inside the cell are either secreted or displayed on the cell surface and presented to the immune system.	1. The development of viral vectors has been robust, with a few entering Phase III trials. 2. Only a few bacterial vectors are under development in small and large animal models, and some Phase I trials. The complex nature of bacteria hampers the development of bacterial vector systems.
Viral Proteins or Viral Peptides	Chemically synthesized pieces of HIV peptides or proteins that elicit strong T and B cell responses.	Peptide-based preparations require the addition of an adjuvant to enhance immunogenicity. At present, alum is the only adjuvant authorized by FDA for general medical use, however many products are being tested, and some are in clinical trials.
Virus-like Particles (VLPs)	1. Empty, non-infectious shells of the HIV envelope protein; they mimic the outer coat of the virus but lack a genome inside and cannot reproduce. 2. Because VLPs resemble the virus, they can induce high titers of neutralizing antibodies to protect against viral challenge.	VLPs represent an exciting new strategy for HIV vaccines but it has been difficult to make them reproducibly.

Fig. 3. HIV vaccine designs and strategies. The most prominent vaccines use DNA, live vector, viral proteins or virus-like particles (NIH 2012)

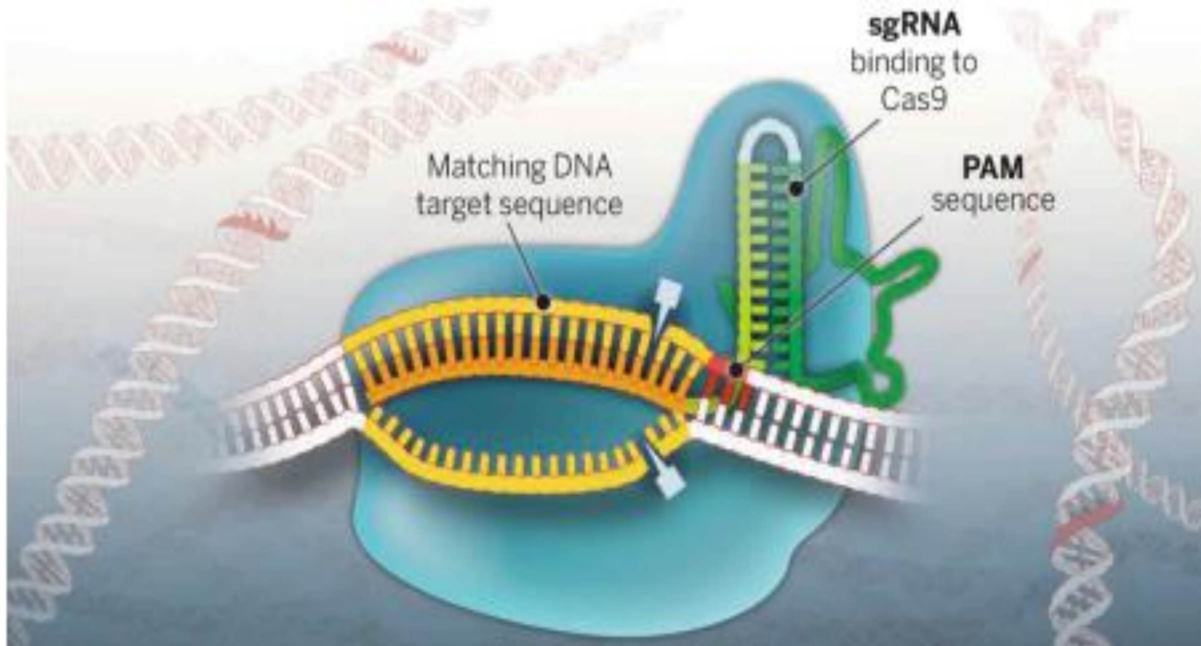

Fig. 4. Cas9/gRNA complex. The guide RNA stabilizes the enzyme on the corresponding DNA sequence. Cas9/gRNA activity causes a double-stranded break immediately before the PAM sequence (Doudna and Charpentier 2014).

Study Proposal Appendix

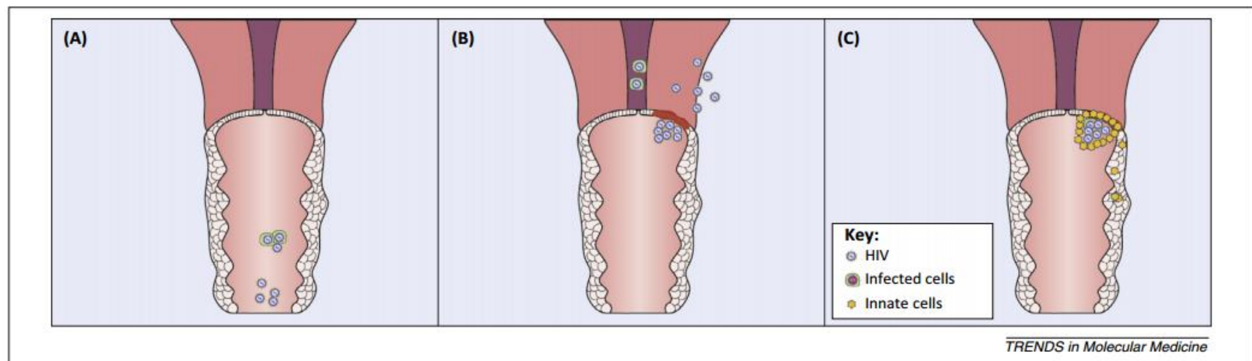

Fig. 5. Early events in vaginal HIV transmission. (A) Introduction of HIV virus into vaginal tract. (B) Innate cells respond to HIV presence on cervix. (C) Innate cells attempt to block viruses from entering further through the cervix (Levy 2015).

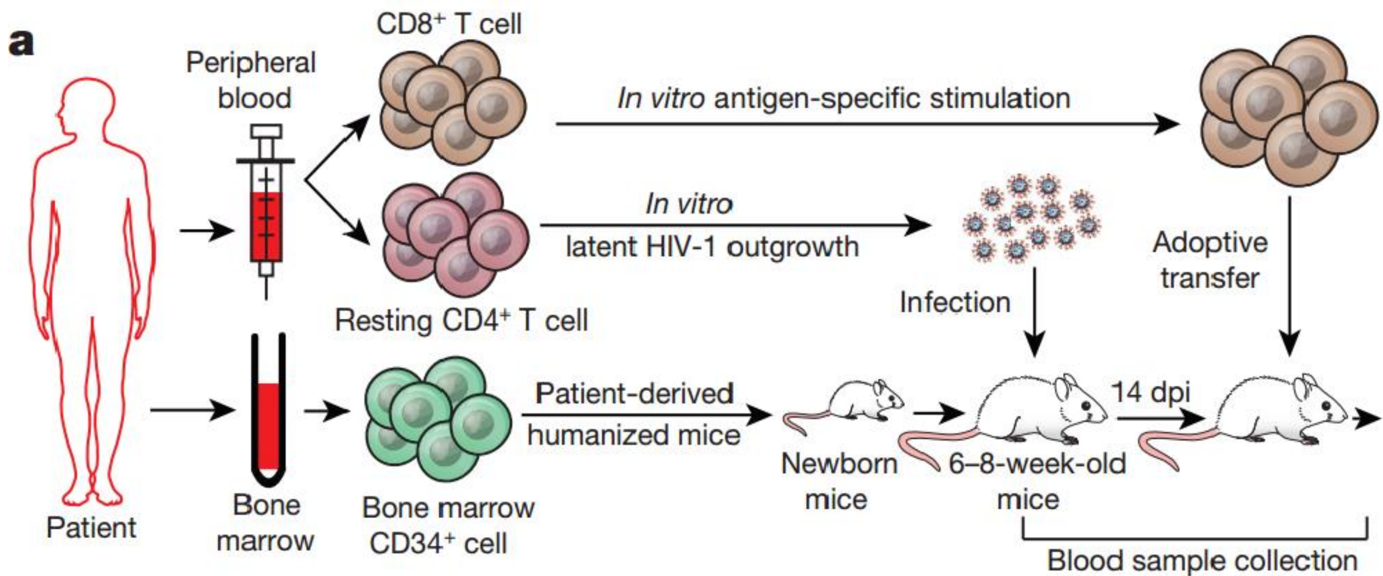

Fig. 6. The development and procedure of humanized mice models. Bone marrow hematopoietic cells are inserted into newborn mice, which will make a functional immune system inside the mice (Deng et al. 2015).

Table 1. Enzyme Linked Immunosorbent Assay (ELISA):

Results in Control, Immunization and Therapeutic Groups

Control Group	**HIV-1 Positive** n (%)	**HIV-1 Negative** n (%)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
Immunization Group	**HIV-1 Positive** n (%)	**HIV-1 Negative** n (%)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
Therapeutic Group	**HIV-1 Positive** n (%)	**HIV-1 Negative** n (%)
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

Southern blot: Expected Results

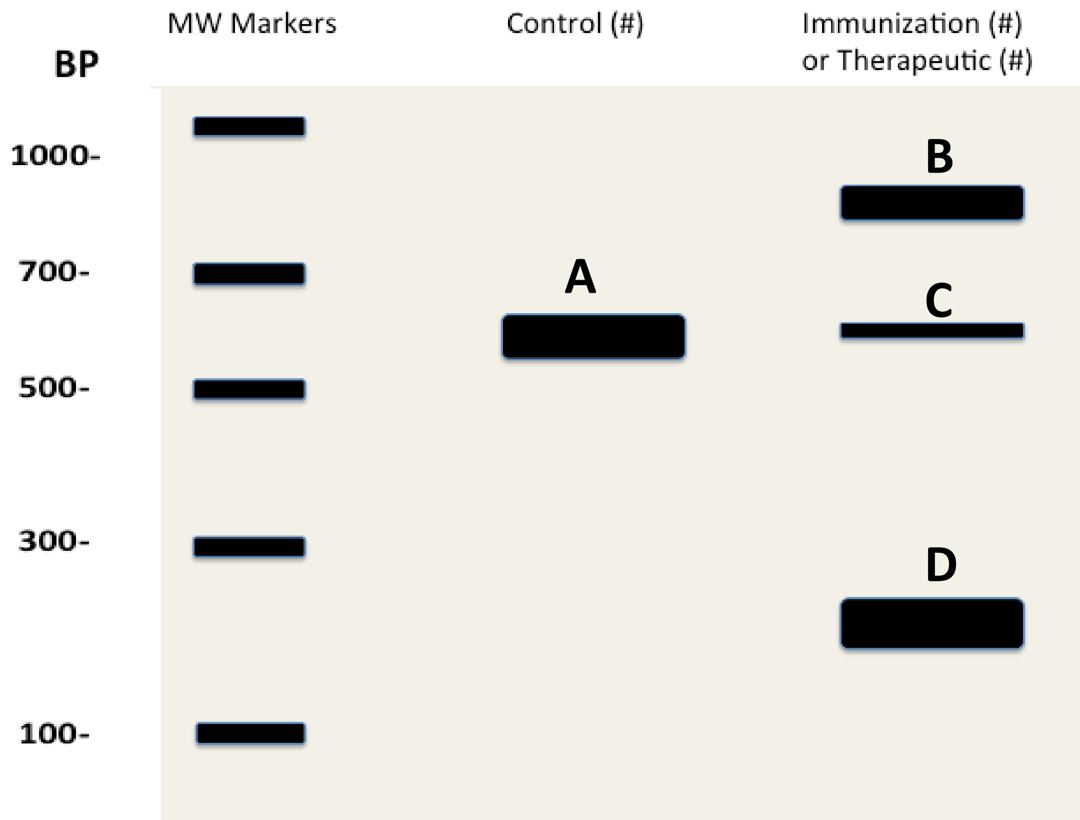

Fig. 7. Theoretical Southern blot for cervical epithelial cell DNA. Humanized mice from each group were numbered (#). Band A will be present, as shown, if HIV-1 is successfully integrated into host epithelial cells. The size of band A would roughly correspond to the size of the HIV-1 genome. Band B will be seen if an insertion occurs after HIV-1 DNA is excised. Band D will occur if host DNA is ligated after cleavage of HIV-1 DNA. Finally, band C will be present if HIV-DNA was not successfully cleaved (Hu et al. 2014).

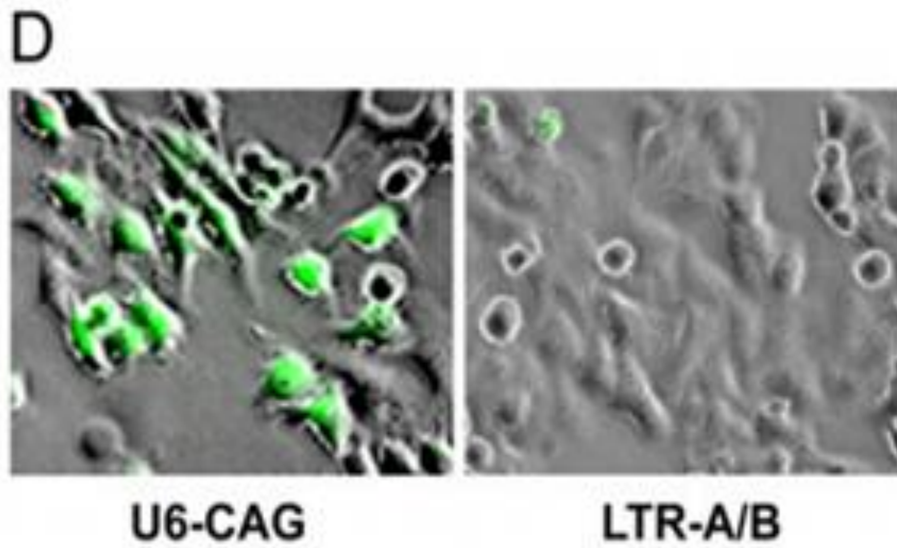

Fig. 8. Presence and absence of EGFP reporter protein on surface of latently infected HIV-1 cells. (Left) Shows presence of EGFP protein. (Right) Shows absence of EGFP protein (Hu et al. 2014).

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