U.PORTO

MESTRADO INTEGRADO EM MEDICINA

CRISPR-Cas9 against HIV infection

Ricardo Filipe Soares Nogueira

M

2021

Artigo de Revisão Bibliográfica

CRISPR-Cas9 against HIV

Dissertação de candidatura ao grau de Mestre em Medicina, submetida ao Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto.

AUTOR: Ricardo Filipe Soares Nogueira

Estudante do 6º ano do Mestrado Integrado em Medicina

Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto

Endereço: Rua de Jorge Viterbo Ferreira nº 228, 4050-313 Porto

Nº de aluno: 201504791

Endereço eletrónico: heyricardonogueira@gmail.com

ORIENTADORA: Doutora Carolina Luísa Cardoso Lemos

Professora auxiliar no Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

Endereço: Largo Prof. Abel Salazar, 4099-001 Porto

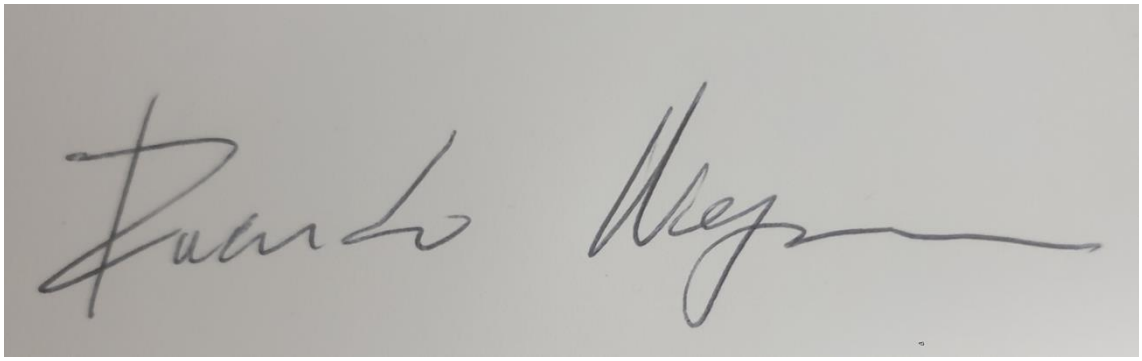
COORIENTADOR: Miguel Alves Ferreira

Investigador no i3S- Instituto de Investigação e Inovação em Saúde, Universidade do Porto

Endereço: i3S - Rua Alfredo Allen, 208; 4200-135 Porto

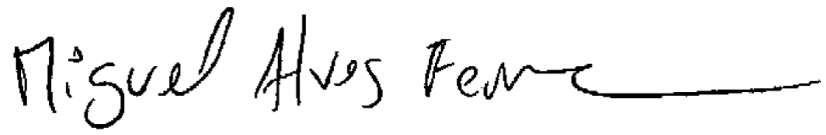
Porto, junho de 2021

Estudante:



Orientação:

Coorientação:



Agradecimentos

À Doutora Carolina Lemos pela sua dedicação, entusiamo e por ter aceite a orientação deste trabalho.

Ao Doutor Miguel Alves Ferreira, por toda a paciência e por toda a dedicação investida neste trabalho, assim como por ter aceite a coorientação do mesmo.

Aos meus pais, por me terem apoiado desde o início de tudo e por me terem permitido tirar este curso.

Aos meus irmãos, por todo o apoio incondicional desde sempre.

Aos meus amigos pelo apoio emocional e por todos os momentos vividos.

Resumo

O Vírus da Imunodeficiência Humana (VIH) está na base etiológica da Síndrome da Imunodeficiência Adquirida, condição essa que tem por base a diminuição do número dos linfócitos TCD4+ e, consequentemente, uma deterioração progressiva do sistema imunitário, que predispõem a várias infeções oportunistas e tumores.

Segundo dados da Organização das Nações Unidas, em 2019 havia aproximadamente 39 milhões de pessoas infetadas pelo vírus de imunodeficiência humana (VIH), sendo que quase uma terça parte das mesmas não tem acesso a tratamento por antirretrovirais. O VIH situava-se ainda, no ano de 2019, na lista dos 10 maiores problemas de saúde pública, segundo a Organização Mundial de Saúde (OMS).

A OMS e o Programa Conjunto das Nações Unidas sobre VIH/SIDA (UNAIDS) estimam que a patologia em questão tenha sido responsável pela morte de mais de 25 milhões de pessoas, desde a sua identificação em 1981, o que a torna numa das mais devastadoras pandemias de que há registo.

Têm sido levados a cabo vários estudos *ex-vivo* no sentido de combater a infeção por VIH, que acaba por se integrar no genoma do hospedeiro *ad eternum*. A fase de latência do vírus tem lugar nos linfócitos T de memória, não cedendo a nenhum dos fármacos antirretrovirais existentes.

Uma das inovadoras estratégias terapêuticas tem por base o redireccionamento da endonuclease Cas9 para a porção do ADN alvo que está integrado no genoma dos linfócitos T infetados. Um dos estudos redirecionou esta promissora ferramenta de terapia genética para a região LTR (*Long Terminal Repeats*) do genoma do VIH, em linfócitos T CD4+. Verificou-se posteriormente uma diminuição da carga viral, assim como a eliminação do vírus latente. Foi ainda registada a resistência a uma nova infeção pelo vírus em linfócitos T provenientes da diferenciação de iPSCs (*Induced pluripotent stem cells*) transduzidas com vetores contendo CRISPR/Cas9 contra a região LTR.

Tendo como alvo o material genético do HIV-1 surgiram diversos estudos no sentido de inibir a replicação, infeção e integração do vírus, assim como para eliminar o vírus de células infetadas de forma latente.

Outra estratégia passou pela modulação do recetor CCR5 (CC chemokine motif receptor type 5) que é crucial para que exista a infeção das células T. A inviabilização deste recetor tendo por base o redireccionamento da Cas9 mostrou, após xenotransplantação de células da linhagem

hematopoiética em ratinhos, efeitos *off-target* reduzidos. A inviabilização do gene *CCR5* motivou o nascimento dos primeiros bebés geneticamente modificados, as gémeas Lulu e Nana, no sentido de adquirirem a capacidade de não serem infetados pelo VIH. Outro recetor que foi alvo de pesquisa quer de forma singular, quer em conjunto com o CCR5, foi o CXCR4 (CXC chemokine receptor type 4).

O CRISPR-Cas9 foi simplificado de forma a constituir uma ferramenta de uso mais eficiente e prático, tendo sido por isso reduzida a dois componentes que agora são apanágio da sua utilização: sgRNA (single guide RNA) e Cas9.

SgRNA é uma forma híbrida do duplex CRISPR RNA e o crRNA de ativação em trans (crRNA-tracrRNA). O crRNA permitirá a ligação ao ADN alvo e o tracrRNA permitirá a ligação ao Cas9. Portanto, para modificar o ADN alvo, é apenas necessário modificar 20 nucleótidos do 5' terminal do sgRNA, que corresponde à região do protoespaçador do crRNA.

É importante reiterar que, o ADN alvo necessita de um motivo protoespaçador adjacente (PAM), uma vez que é necessário para o desenrolamento do alvo pela Cas9. Na ausência da sequência PAM, o ADN alvo não é reconhecido por Cas9, mesmo que a sequência de nucleótidos do sgRNA seja totalmente complementar. A interação da sequência PAM e Cas9 resulta na separação da cadeia dupla de ADN imediatamente após esta sequência, o que permitirá, posteriormente, a clivagem.

A principal desvantagem desta técnica são os efeitos *off-target,* ou seja, outras sequências de ADN não desejadas podem ser reconhecidas e clivadas. No entanto vários métodos, como o uso de *nickases* ou dCas9, foram e estão a ser desenvolvidos para aumentar a especificidade e eficiência. O uso de ortólogos poderá contribuir para uma maior abrangência terapêutica desta técnica.

Palavras-chave: HIV infection; CRISPR-Cas Systems; gene editing.

Abstract

The Human Immunodeficiency Virus (HIV) is at the etiological basis of the Acquired Immunodeficiency Syndrome, a condition that is based on the decrease in the number of TCD4 + lymphocytes and, consequently, a progressive deterioration of the immune system, which predispose to various opportunistic infections and tumors.

According to data from the United Nations, in 2019 there were approximately 39 million people infected with the human immunodeficiency virus (HIV), with almost a third of them having no access to antiretroviral treatment. HIV was still, in 2019, on the list of the 10 biggest public health problems, according to the World Health Organization (WHO).
WHO and the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimate that the disease in question has been responsible for the death of more than 25 million people since its identification in 1981, making it one of the most devastating registered pandemics.

Several ex-vivo studies have been carried out to combat HIV infection, which ends up integrating into the host genome ad eternum. The latency phase of the virus takes place in the memory T lymphocytes, not yielding to any of the existing antiretroviral drugs.

One of the innovative therapeutic strategies is based on the redirection of the Cas9 endonuclease to the portion of the target DNA that is integrated in the genome of the infected T lymphocytes. One of the studies redirected this promising gene therapy tool to the LTR (Long Terminal Repeats) region of the HIV genome, in CD4 + T lymphocytes. There was subsequently a decrease in viral load, as well as the elimination of the latent virus. Resistance to a new infection by the virus was also recorded in T lymphocytes from differentiation of iPSCs (Induced pluripotent stem cells) transduced with vectors containing CRISPR / Cas9 against the LTR region.
Several studies have emerged in order to inhibit the replication, infection and integration of the virus, as well as to eliminate the virus from latently infected cells, by targeting the genetic material of HIV-1.

Another strategy was the modulation of the CCR5 receptor (CC chemokine motif receptor type 5), which is crucial for T cell infection to exist. The impracticability of this receptor based on Cas9 redirection showed, after xenotransplantation of cells of the hematopoietic lineage in mice, reduced off-target effects. The unfeasibility of the CCR5 gene motivated the birth of the first

genetically modified babies, the twins Lulu and Nana, in the sense of acquiring the ability to not be infected with HIV.
Another receptor that was the target of research, either singularly or in conjunction with CCR5, was CXCR4 (CXC chemokine receptor type 4).

CRISPR-Cas9 has been simplified in order to constitute a tool for more efficient and practical use, having therefore been reduced to two components that are now the prerogative of its use: sgRNA (single guide RNA) and Cas9. SgRNA is a hybrid form of the CRISPR RNA duplex and the trans activation crRNA (crRNA-tracrRNA). The crRNA will allow binding to the target DNA and the tracrRNA will allow binding to Cas9. Therefore, to modify the target DNA, it is only necessary to modify 20 nucleotides of the 5 'terminal of the sgRNA, which corresponds to the protospacer region of the crRNA. It is important to reiterate that the target DNA needs an adjacent protospace motif (MAP), since it is necessary for the target to unfold by Cas9. In the absence of the PAM sequence, the target DNA is not recognized by Cas9, even if the nucleotide sequence of the sgRNA is completely complementary. The interaction of the PAM and Cas9 sequences results in the separation of the double strand of DNA immediately after this sequence, which will subsequently allow cleavage.

The main disadvantage of this technique is the off-target effects, that is, other unwanted DNA sequences can be recognized and cleaved. However, several methods, such as the use of nickases or dCas9, have been and are being developed to increase specificity and efficiency. The use of orthologists may contribute to a wider therapeutic range of this technique.

Keywords: HIV infection; CRISPR-Cas Systems; gene editing.

List of abbreviations

AAVs - adenovirus and adeno-associated viruses
AIDS - Acquired Immunodeficiency Syndrome
ART - Antiretroviral therapy
ATI - analytical treatment interruption
Cas - CRISPR associated proteins
CCR5 - CC chemokine motif receptor type 5
cDNA - complementary synthesized DNA
CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats
CXCR4 - CXC chemokine receptor type 4
DSB - double-strand break
GALT - lymphoid tissue associated with the intestine
HDR - homologous recombination
HIV - Human Immunodeficiency Virus
hPSC - human pluripotent stem cells
HSCT - hematopoietic stem-cell transplantation
iPSCs - induced pluripotent stem cells
LASER ART - long-acting slow-effective release antiviral therapy
LTR's - long terminal reapeat sequences
NHEJ - non-homologous recombination
PAM - protospacer adjacent motif
pre-crRNA - pre-CRISPR RNA
RNPs - Ribonucleoproteins
sgRNA - single-guide RN
SNVs - single-nucleotide variations
TALENs - transcription activator-like effectores
tracrRNA - transactivating CRISPR-RNA
WHO - World Health Organization
ZFNs - Zinc Finger Nuclease

Índex

Introduction

Living organisms have been fighting infectious and invasive agents, such as virus, since the beginning of their existence. We, human beings, fight these and other invaders daily, from HIV, through Influenza, to the current SARS-Cov2. This is a war that makes victims every day.

Thus, living beings that faced pathogenic invasions developed what we call the immune system, in order to defend themselves. The immune system is divided into two subtypes: innate immune system and adaptive immune system. The main difference between them is that the first is less specific, so it only recognizes general characteristics of pathogens, whereas the adaptive immune system recognizes specific characteristics of invaders, acquiring immune memory. Until the discovery of CRISPR, the scientific community believed that adaptive immune systems were exclusive to eukaryotes, which is not the case. [1]

Adaptive immunity is acquired using genetic material from the invader. In bacteria, small portions of that same genetic material will be integrated into regions of repetitive sequences called CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats. [2,3]

CRISPR can then be translated into small fragments of RNA that later associate with specific proteins called Cas (CRISPR associated proteins), thus forming the CRISPR-Cas complex that will degrade the invader's genetic material. [2,4]

The nuclease of the CRISPR system is guided by an RNA chain, which recognizes the target DNA by base complementary of Watson-Crick. [5]

There are many types of CRISPR-Cas systems, however, the most studied and used is CRISPR / Cas type II, originally from *Streptococcus pyogenes*, due to its less complexity.[5,6]

CRIPR-Cas9 have been repurposed in order to create a powerful technology allowing genome editing, epigenetic modulation and genome imaging. This system also provides the opportunity to precisely manipulate almost any genomic sequence, granting the understanding of the function of the gene under analysis and its relationship with certain pathology**.** This technology is also of crucial importance in preventing diseases with a genetic etiology, since it allows the correction of the mutations that cause it. It is also of particular importance in the fight against cancer, since it is possible to activate cancer suppressor genes, as well as inactivating oncongenes.[7]

Two main advantages of this genetic editing system are that we only need to build a given guide RNA to theoretically promote the hybridization or cleavage of any target gene; and the fact that this technology allows us to manipulate multiple genes simultaneously, thus it is possible to create more complex disease models in a relatively simple way.[8]

In addition to its efficiency, there are reports of less toxicity when compared to the use of ZFNs and TALENs. [9]

In the future, it will become possible to treat and even cure various genetic and oncological diseases. CRISPR also promises to facilitate the fight against infectious agents, such as viruses. CRISPR can make immunization, treatment and even the cure of viral infections like HIV possible.

The first tests for the use of this technology in the treatment and eradication of HIV have already been made. Above all, it is important to mention that the first babies immune to the HIV virus were born (ethical issues will not be discussed here). [10]

CRISPR-Cas will change medicine and the world forever, making therapies used nowadays obsolete. However, for this technology to increase its therapeutic potential, it is necessary to avoid / reduce off-target effects, that is, mutations in regions other than the desired one. [7]

1. CRISPR-Cas9

The CRISPR-Cas system is divided into two classes and six types. The classes were divided based on the number of effector proteins. Class 1, which contains types I, III and IV, requires a high number of effector proteins and class 2, which contains types II, V and VI, requires a single RNA-guided endonuclease to lead to double-strand cleavage of DNA. Of all the mentioned types, type II is the most studied, due to its properties that guarantee an easy, precise and efficient edition.[11-13]

1.1. Mechanism

A typical CRISPR locus, of a CRISPR-Cas type II system, consists of 3 essential parts:

1. CRISPR array: which consists of a set of repetitive sequences (repeats) interspersed with a set of non-repetitive sequences (spacers);
2. Cas operon: a set of CRISPR-associated genes;
3. Trans-activating CRISPR RNA (tracrRNA) gene: which has the function of encoding a unique noncoding RNA with homology to the repeat sequences.[7]

The mechanism of action of the CRISPR-Cas system (Fig.1) consists of three phases: adaptation, expression and interference. The first phase is called adaptation, in which there is exposure to the invader's genetic material and a small segment of target DNA or RNA (protospacer) is integrated into the CRISPR array, using the Cas operon machinery (Cas1, Cas2, and Csn2), forming immune memory.

When re-exposure occurs, the expression phase takes place as part of the immune response, in which there is transcription of the CRISPR locus, in a long pre-CRISPR RNA (pre-crRNA), together with the transactivating CRISPR-RNA (tracrRNA). The tracrRNA, paired with the repetitions of the pre-crRNA, and, later, RNase III, from the host, performs a cleavage, in order to produce the mature crRNAs. These crRNAs are then combined with the Cas endonucleases to form an active Cas-crRNA complex.[14-16]

The last phase will be the interference phase, where cleavage of the double strand of DNA occurs, by Cas9. However, because it is a type II system, it is necessary for the crRNA-tracrRNA-Cas9 complex to find the PAM (protospacer adjacent motif) sequence, immediately below the target site in the invading DNA, for cleavage to occur.[6,15,16] PAM is a small sequence of 3 nucleotides located immediately downstream from the recognition and cleavage region in the target genome and plays

an essential role in type II systems. Many type II systems have different types of PAM, however, the most common is the NGG sequence, where N can take any nucleotide and G the guanine nucleotide.

To facilitate the laboratory process, a hybrid form of crRNA and tracrRNA called sgRNA was created.[6] The cleavage made by Cas9 triggers the DNA damage repair pathways used as a strategy for genomic manipulation.[4] With this system, the nuclease activity of Cas9 can be directed to any DNA sequence of the type N 20 - Protospacer Sequence, it is only necessary to change the first 20 nucleotides of the sgRNA, which are complementary to the target sequence and guide Cas9.[8,17]

The binding of sgRNA by complementarity and the presence of PAM in the opposite strand allows double cleavage of the DNA strand (double-strand break - DSB) by Cas9.[18]

1.2. Cas9

Structurally, Cas9 contains 2 independent enzyme domains, HNH and RuvC. Each domain is responsible for the cleavage of a DNA strand. The HNH domain cleaves the complementary DNA strand, while the RuvC domain, structurally more complex, cleaves the non-complementary DNA strand.[4,6,19]

Recently, a structural analysis of the CRISPR / Cas9 complex made it possible to verify the existence of two lobes with different functions, one for recognition (REC) and one for nuclease (NUC). In REC, Cas9 interacts with RNA-DNA in several ways, depending on the conformation acquired, which allows us to infer that the Cas9 nuclease does not have any specificity in targeting, so the presence of crRNA-tracrRNA is crucial to be able to exercise its activity.[20,21] However, even with the crRNA-tracrRNA duplex form, the cleavage process by Cas9 is not entirely specific, and off-target cleavages may occur.[22]

If one of the two domains (HNH and RuvC) of the Cas9 nuclease is inactivated through small mutations, variants of Cas9 are produced with only one catalytic domain, called nickases which proved to be useful in genetic engineering since they catalyze only one cut in the double strand of DNA. To increase the specificity of the DSB in the target, a complex with two nickases was developed, following the analogy of the ZFN and TALEN dimers and thus increasing the number of recognized bases. This complex mimics the double cutting of DNA made by a single nuclease, increasing the efficiency and correction in the repair. On the other hand, any off-target cleavage is precisely repaired, which makes this complex of nickases a promising strategy to increase the specificity of Cas9.[4]

If the two enzymatic domains of Cas9 are inactivated, it is catalytically dead - dead Cas9 (dCas9), and when it binds to DNA it can repress transcription by stereochemical impedance of RNA polymerase.[4]

A more robust expression in the regulation of gene expression can be found by targeting multiple sgRNAs to multiple targets in the same gene. However, in screening studies, regulation by only a few sgRNAs is preferred, due to the size of the sgRNA libraries.[18]

1.3. DNA repair

The DSB will be processed by 2 DNA repair mechanisms (Fig.2): homologous recombination (HDR) and non-homologous recombination (NHEJ). In general, NHEJ has a more predominant activity than HDR, due to the fact that it does not require any DNA template and the mechanism is used preferentially during the S and G2 phases of the cell cycle.[18,23] However, it is more prone to errors through small insertions or genetic deletions. These *indels* alterations can lead to a change in the function of the target gene. HDR is more accurate since it requires the presence of a corrective synthetic mold sequence to proceed with the repair, reducing the error.[23,24]

1.4. Off-target effects

The entire CRISPR / Cas9 system has undergone major developments in order to increase specificity and efficiency for the target sequence and reduce the off-target effects.[18]

The 18-20 nucleotide region corresponding to the protospacer in the gRNA is responsible for a large part of the off-target and on-target effects. In theory, a single nucleotide sequence should correspond to a single target sequence that has a characteristic adjacent PAM region, however it has been found that there is more than one PAM sequence that can activate the same nuclease, although with different affinities.[19,25] In the case of Cas9 from the *S. pyogenes* bacterium, the “NGG” sequence is recognized as the optimal sequence, however a “NAG” sequence can also be recognized by the same enzyme, although less frequently. Progress has been made towards the development of increasingly rigid recognition sites in order to optimize the specificity of the nuclease for a given PAM region.[19]

It is assumed that a decrease in the concentration of Cas9 protein and sgRNA may be associated with a decrease in off-target effects. Other factors, such as accessibility of chromatin at the target site and the capacity for cellular response to injuries induced by CRISPR, are also determinants in the success of the CRISPR-Cas9 system.[19,26]

Another proposal to increase specificity is the development of double complexes of nickases, in which the activity of one depends strictly on the activity of another, increasing the specificity of recognition.[8]

All of these developments can be combined and developed in order to further optimize the CRISPR--Cas9 system.

2. CRISPR-Cas9: therapeutic approach

2.1. Transport of the CRISPR-Cas9 to the cell

The transport of the CRISPR-Cas9 system remains a major challenge for all genetic engineering, because CRISPR-Cas9 system is still difficult to introduce only in the desired cells.[27,28] We highlight three main methods for transporting the CRISPR-Cas9 system into cells: plasmids, viral vectors and ribonucleoproteins.

2.1.1. Plasmids

This is the most common method of transporting the CRISPR-Cas9 system into cells. In this method cells are transfected simultaneously with plasmids, which encode Cas9, crRNA and tracrRNA, by electroporation methods.[27]

In order to simplify this method a plasmid coding for sgRNA was developed, and more recently a vector coding for Cas9 and sgRNA in the same plasmid, reducing the plasmids to be delivered from three to only one. This technology permits the rearrangement of plasmids in order to code for multiple sgRNA, allowing multiple corrections in the locus.[17]

This method has the advantages of easy in vitro production and has some disadvantages such as the fact that it can only be applied ex vivo.[17,27]

In vivo studies demonstrate some limitations of this method due to the epigenetic silencing as well as the low delivery efficiency.[29] It was found that in a proportion of transfected cells, there was random integration of the genetic material into the host genome, which, on the one hand, allows for continuous production of Cas9 and sgRNA, but on the other hand, it may lead to off-target effects and mutations by insertion. If this feature is not desired, the plasmid is lost after a few cell cycles.[19,30]

2.1.2. Viral vectors

Viral vectors are widely used as a tool for introducing an exogenous DNA fragment into primary cells or cells refractory to plasmid transfection.[19]

There are two main viral vectors: adenovirus and adeno-associated viruses (AAVs), being non-integrative viruses, whose viral DNA disappears in a few hours.[27,31]

The advantages of adenovirus are that it is easy to produce and clone, as well as having a good correlation between packaging efficiency and size. However, it has the disadvantage that it can lead to an immune response from the host that can culminate in the destruction of cells.[32]

Compared to adenovirus, AAVs have the advantage that they induce a mild immune response and can provide long-term expression in cells that are not dividing. Their disadvantage is their small size, which would lead to packaging difficulties. However, this problem already has a solution, which involves replacing Cas9 from *staphylococcus aureus* with a Cas9 from *S. pyogenes*, the latter being more appropriate.[32]

2.1.3. Ribonucleoproteins (RNPs)

Unlike plasmids and viral vectors that encounter prolonged expression of Cas9 and sgRNA in cells and therefore with a higher rate of off-target effects, RNPs cause a specific change in the target site immediately after being delivered to the cell, being degraded shortly thereafter.[19,33]

RNPs are traditionally delivered by direct microinjection, but cationic liposomes or electroporation technique can also be used.[34]

The delivery of RNPs in a cell culture synchronized in a specific phase of the cell cycle allows to increase the success rate of the technique.[19]

The construction of positively charged nanoparticles can also be used, as a result of the conjugation of Cas9, sgRNA and cell penetrating proteins that have the ability to deliver Cas9 and sgRNA to the nucleus of target human cells efficiently, while maintaining cell viability.[35] RNPs have the advantage of fast and easy in vitro production.[30]

2.1. Therapeutic approach

CRISPR / Cas9 can be used in gene therapy, which can be ex vivo, through the modification of human cells in culture, being subsequently transplanted into humans, or in vivo, through direct editing of cells in the target organism.[24]

2.1.1. In vivo genetic editing

It consists of the direct injection of components of the CRISPR-Cas9 system through viral or non-viral vectors.[24]

2.1.2. Genetic editing in embryos

The introduction of CRISPR / Cas9 components (Cas9, sgRNA and template nucleotide chain for HDR) in a zygote or in an early embryonic phase allows modifying the genome in all cells of the organism, including germline cells. This type of approach allows for permanent changes that can be passed on to subsequent generations, which makes it possible to eliminate inherited genetic diseases.[36]

There are very few studies on human embryos because it is a controversial and delicate topic that raises ethical concerns in the scientific community and in the general public.[24]

2.1.3. Gene editing in somatic cell

In this case, the genes are edited externally in somatic cells or induced pluripotent stem cells (iPSCs) that are reprogrammed, then undergoing a selection process where only recombinant clones that have correction in the target allele and without off-target mutations will be chosen to transplant back into the human organism.

IPSCs can be developed from human fibroblasts, propagate infinitely and can be differentiated in any cell in the body, which represents an immeasurable potential in gene therapy.

One of the great advantages of ex vivo gene therapy is that it allows the selection and analysis of cells before transplantation.[24]

3.0. CRISPR-Cas9 in the potential treatment of HIV

3.1. HIV

3.1.1. The pandemic

The Human Immunodeficiency Virus (HIV) is at the etiological basis of the Acquired Immunodeficiency Syndrome (AIDS), a condition that is based on the decrease in the number of TCD4 + lymphocytes and, consequently, a progressive deterioration of the immune system, which predispose to various opportunistic infections and tumors.

According to data from the World Health Organization (WHO), at the end of 2019 there were approximately 39 million people infected by the HIV in the world, with almost a third of them having no access to antiretroviral treatment. In the same year, approximately 1.7 million people were re-infected and approximately 690,000 died from HIV-related causes.

HIV was also, in 2019, on the list of the 10 major public health problems, according to the WHO.

WHO estimate that AIDS has been responsible for the death of more than 25 million people since its identification in 1981, making it one of the most devastating registered pandemics. [37]

3.1.2. The biology and the life cycle

HIV is a retrovirus and is part of the lentivirus family. It is an encapsulated virus formed by two single-stranded RNA molecules, surrounded by a capsid. It has an RNA genome in the order of 9.8kb, which is composed of nine genes called env, gag, nef, pol, rev, tat, vpr, vpu and vif limited by two long terminal repeat sequences (LTR's), encoding 10 viral proteins.[38-40]

There are two types of HIV virus: HIV-1 and HIV-2. HIV-1 is the main focus of the scientific community, because it is largely more frequent, more transmissible and more pathogenic, although HIV-2, which is extremely rare, can also lead to AIDS.[41]

The virus is transmitted in three main ways: sex, intravenous injections and vertical transmission.[42]

The HIV virus infects cells that express CD4 glycoproteins, the main receptor for HIV-1 and HIV-2, such as CD4 T lymphocytes, regulatory T cells, monocytes, macrophages, and dendritic cells. After binding to CD4, interaction with a co-receptor is necessary for fusion of the viral membrane to the host cell membrane.[43]

It is important to analyze the life cycle of HIV-1 since this way we will be able to understand possible targets for the CRISPR-Cas9 technology.

The life cycle consists of 5 stages:

1. Binding and entry: HIV enters in the host cells, which correspond mainly to T cells, macrophages, microglial cells, astrocytes and macrophages of the central nervous system.

The virus starts this process by linking its glycoprotein gp120 to the surface of the CD4 receptor on the surface of the cell, then it also binds to a coreceptor, which may be CCCR5 or CXCR4. This connection culminates in the fusion of the virus with the cell membrane, allowing the virus to enter the cell.

2. Reverse transcription: HIV-1 RNA is transcribed into double-stranded DNA by reverse transcriptase.

3. Integration: viral DNA enters the nucleus and is integrated into the nucleus by integrase.

4.Replication and assembly: the proviral DNA generated can then be used to produce viral proteins. These proteins then associate with viral RNA and move to the cell surface to form immature viral particles.

5. Finally, the mature virus is formed through the release of immature viral particles from the cell and the production of proteases by those that then break down a long protein chain.[44]

3.1.3. The formation of reservoirs

HIV, after the process of reverse transcription of its RNA into DNA, integrates it into the genome of the target cell, forming a pro-virus. After the initial infection, there is a set of cells that remain lately infected, which is one of the major obstacles to the complete cure of HIV. It should be noted that, as soon as the provirus is activated, the host cell in question produces viral particles and dies. When the T lymphocytes decrease to low levels, we reach the stage of acquired immunodeficiency syndrome (AIDS), in which the individual is highly susceptible to infections or malignant transformations.[45]

CD4+ memory lymphocytes, macrophages astrocytes, microglia and intestinal lymphoid cells are described in the literature, as examples of reservoir cells.[46]

Reservoir cells usually integrate tissues where there is poor penetration by antiretroviral therapy, such as the central nervous system or lymphoid tissue associated with the intestine (GALT).[47]

These cells are latently infected with transcriptionally inactive HIV-1 pro-viruses, but competent for replication. However, this reservoir remains due to the clonal expansion of latently infected T cells. The integration of the HIV pro-virus is found predominantly in genes associated with cell cycle regulation or cancer, which does not mean that it occurs predominantly in these loci, it only means that these loci confer a selective proliferative advantage, thus allowing for greater expansion and persistence of these cells.[48-50]

The molecular mechanisms that allow HIV latency are not yet fully understood, however, it is thought to have to do with the formation of repressive chromatin around the proviral integration site.[51]

Thus, the eradication of reservoirs with antiretroviral therapy is unlikely.[52]

It has only been proven that an early start of antiretroviral therapy would lead to a very small size of the reservoirs. However, all patients experienced rebound after stopping antiretroviral drugs.[53]

Other methods have been tried to eradicate the reservoirs, such as the allogeneic transplantation of hematopoietic cells to treat malignant hematological neoplasia and there was still rebound after a few months. However, it has thus been shown that the allogeneic hematopoietic stem-cell transplantation (HSCT) can reduce HIV reservoirs for a short period of time.[54]

Another strategy called “shock and kill” was also aimed at reducing HIV reservoirs. The name itself explains the method that aims to reactivate all cells in the reservoirs, something extremely unlikely, since it is difficult for all proviruses to be activated and transcribed at the same time, followed by “killing”, that is, the death of all these cells “Awake”. This study was also unsuccessful in reducing reservoirs.[55]

3.1.4. Clinical Aspects

Acute HIV-1 infection is based on three pillars: high levels of viral replication, decreased CD4 T lymphocytes in peripheral blood and the establishment of a latent infected CD4 T cell reservoir.[56]

In the context of acute HIV-1 infection, individuals may present with a mononucleosis-like condition, approximately 2 to 6 weeks after infection, however approximately 60% of individuals do not present symptoms at this stage of the disease, which is often overlooked.[37,56]

After acute infection, patients enter often an asymptomatic period of chronic HIV infection with relative stable viremia and progressive decline in CD4 T lymphocyte count. After approximately 8 to 10 years after HIV infection, patients have a profoundly immunodeficient state with CD4 T lymphocyte counts below 200 cells / L, putting them at risk for opportunistic infections and life-threatening neoplasms.[57]

3.1.5. Current therapy

Antiretroviral therapy (ART) can suppress the replication of the virus, reducing the risk of progression to AIDS and allowing the life expectancy of people living with HIV who respond to ART to be similar to that of the general population. It is also important to note that ART fails in eradicating HIV, and the interruption of ART is followed by viral rebound within weeks in most patients, so it needs to be administered for life. This is because, current therapy is ineffective in eradicating the reservoir virus, making the disease in question a chronic disease.[52,58]

Importantly, this therapy has high costs and side effects, such as bone or renal toxicity, dyslipidemia, insulin resistance and cardiovascular disease.[59]

Therefore, there is a need to create new therapies. Genetic editing, using CRISPR-Cas9, for example, is one of the options that appear, since it allows disruption of the HIV life cycle at different stages. With this technique entry into the cell can be prevented, through the ablation of co-receptors, or inhibit the infection, replication and integration of the virus. The great challenge, however, remains the elimination of the virus that lately infects certain cells in the host's organism.

3.1.6. The cure

First, it is important to understand what "cure" is. Two types of cure are described:

1. Sterilizing cure: complete eradication of HIV-1

2. Functional cure: long-term control of HIV-1 replication with preservation of CD4 T lymphocyte count in the absence of antiretroviral therapy.

Although no curative treatment has been found so far, there are two documented cases of HIV patients, the Berlin patient and the London patient, who were cured of HIV infection after allogeneic HSC transplants.[60,61]

In both cases, the donor had a homozygotic deletion of 32 base pairs in the CCR5 gene (CCR5Δ32), which was previously known to impair HIV-1 T-cell infection, and both patients had malignant haematological neoplasms, thus indicating an allogeneic hematopoietic stem cell transplant. Patients were then monitored and sterilizing cures were found in both cases.[60-62]

Both patients had graft-versus-host disease, which may have mediated depletion of the "autologous HIV reservoir". Therefore, it is likely that intense conditioning regimens have facilitated the eradication of HIV reservoirs and the reconstitution of a CCR5D32 resistant to the HIV immune system, thus enabling the cure of these patients.[62,63]

The study by Kordelas et al. highlights the importance of screening such patients for the absence of viruses with tropism for X4. After the allogeneic HSCT with D32 graft, one patient had an HIV rebound with tropism to X4 during analytical treatment interruption (ATI).[64]

It remains to be noted, the limited availability of CCR5D32 donors and the side effects of allogeneic HSCT prevent a wider use of this approach. Even if in the future, conditioning regimens without chemotherapy and radiation may be available to circumvent some of the serious adverse effects, these new approaches need to be combined with effective depletion of the HIV reservoir and genetically modified autologous HSCs to make the immune system resistant to HIV.[65,66]

3.2. Genome edition

3.2.1. Ablation of co-receptors

The coreceptors that are relevant to this subject are just two: CC chemokine motif receptor type 5 (CCR5) and CXC chemokine receptor type 4 (CXCR4), because they are closely related to the tropism of HIV. [67]

There are two major types of HIV viruses, subdivided because of their natural tropism: macrophage (M) -tropic strain virus (CCR5dependent, also called R5-tropic) and a T lymphocyte (T) -tropic strain virus (CXCR4-dependent, also called X4-tropic). The former is predominant in earlier stages of infection and is responsible for transmission, while the latter is prevalent in the later stages and is responsible for the rapid depletion of CD4 T lymphocytes and consequently disease

progression. It is important to mention that, in the final infection process, the strains of R5 tropism can transform in order to obtain tropism for both co-receptors.[68,69]

In general, the main disadvantages of coreceptor ablation are the efficiency of ex vivo editing, as well as the proliferation of cells edited after ex vivo transfusion. It is also important to note that this therapy may not be able to block the infection in most of the latent reservoirs and ablation does not treat previously infected cells. Thus, this therapy may represent only an addition to the current therapy, with no effects of toxicity or off-target.[70]

An alternative is to interrupt the HIV genome itself, and since it is not an endogenous sequence it is less susceptible to off-target effects, however, it has the disadvantage of mutational escape. But it is important to mention that there is a solution for viral escape, this can be reduced if a therapy is targeted at several targets.[71]

3.2.1.1. CCR5

Physiologically, CCR5 plays an relevant role as a co-stimulating molecule in immune synapses, however what matters is to understand its correlation with HIV infection.[72]

There are individuals who are naturally more resistant to HIV infection but are not completely immune. What makes them so unique is a 32-nucleotide long deletion (D32) in the CCR5 locus, making them resistant to R5-tropic HIV-1 strains. Importantly, genetic association studies have shown that homozygous individuals show resistance to the infection by the virus with the tropism under analysis and heterozygous individuals show a slower progression of the infection when compared to individuals without this mutation.[62,73]

This mutation is extremely rare, being more common in North-Eastern Europe, where it can reach 10%.[74]

CCR5 is the principal coreceptor for the entry of R5 tropic HIV into cells, thus playing an important role in the onset of viral infection. It was also found that the levels of this coreceptor are related to the degree of infection. Therefore, some authors have started to try to induce changes in this cell surface protein.[67,75]

As the modified T cells would have a limited life span, it is not clear how long they could protect the individual against HIV, so the scientific community has focused, predominantly, on hematopoietic stem cell (HSCs) research, since these have the capacity for self-renewal to remain present as stem cells and can originate any cell of the hematopoietic lineage, for example CD4 + myeloid cells.[76]

Therefore, the interruption of the CCR5 coreceptor represents one of the fronts of CRISPR-Cas9 in this battle against HIV.

One of the approaches to eliminate the latent reservoir of HIV-1 is the ex-vivo ablation of the CCR5 gene, attempting to recreate the CCR5Δ 32 mutation that led to the cure for HIV-1 infection in two previously mentioned cases, patients of Berlin and London. In this approach, HSCs are extracted from patients, after which the gene in question is ablated by CRISPR-Cas9 and are then reinfused. Treated cells become more resistant to infection and, if proliferation is successful, resistant cells supplant cells susceptible to HIV-1. It should be noted that HIV viruses with another type of tropism and cell-to-cell transmission are not treated. Equally important, in order for viral reproduction to be harmed efficiently, a biallelic knockout is necessary.[70]

Wang et al. used TZM.bl cells as the object of study, and these cells express the CCR5 and CXCR4 receptors. The targets of the CRISPR-Cas9 system were three regions of the CCR5 gene (CR1, CR2 and CR3). Seven days after treatment, the expression of CCR5 in the treated cells was analyzed and what was found was that only CR2 had a percentage of cells negative for CCR5 expression above 50% (63.8%). Li et al. did a similar study but directed the gRNAs to eight regions of the CCR5 gene (sgR5-3 and sgR5-10) and obtained better results. sgRNA-5 obtained the highest percentage of this study of mutations in the CCR5 gene allele (74.1%), followed by sgRNA-8 with 63.8%.[77,78]

Both authors infected the cells under study with HIV-1 and found resistance to viruses with R5 tropism. However, there was no resistance to viruses with tropism for R4.[77,78]

In order to verify the impact of editing, primary CD4 + T cells were evaluated and it was found that susceptibility to HIV-1 infection decreased considerably.[78]

Based on these previous studies, it is noticeable that the ablation of CCR5 has an enormous clinical benefit.

To check the in vivo efficacy, Xu et al. resorted to the study on NPG rats. In this study, the authors transplanted modified cells, hematopoietic and progenitor stem cells (HSPCs) containing CCR5 or unedited ablation, to animal models. Subsequently, after being infected with HIV with R5 tropism, a reduction in the viral RNA in the peripheral blood was verified two weeks after the beginning of the study. Twelve weeks after the procedure, edited cells were detected, after hematopoietic reconstitution. This study allowed us to validate the approach of transplanting cells edited in the CCR5 gene as a possible cure tool in the fight against HIV.[79]

In order to mimic what occurs naturally, some authors began to induce deletions of 32 base pairs in the CCR5 gene (CCR5Δ32), generating a stop codon, leading to the absence of the

coreceptor and making the patient resistant to HIV-1 with R5 tropism or making possible a slower progression of the pathology.[80,81]

Ye et al. induced this biallelic or monoallelic CCR5Δ32 mutation in hematopoietic stem cells, through Cas9 and gRNA directed to the CCR5 gene. The cells differentiate into macrophages and monocytes. After being infected with HIV-1 R5-tropic, the authors reported a reduction of viral replication when compared to unaffected cells.[82]

QI et al. induced the same change with Cas9 and two gRNAs using CD4 + T cells and primary Jurkat cells from peripheral blood. A lentiviral vector was used and the Δ32 deletion was found in 20% of CD4 + T cells and in 60% of primary Jurkat cells. [80]

It is now important to mention two cases of in vivo results.

In 2019, Gupta et al. described the reduction of viral RNA to undetectable levels in HIV-1 infected patients after an allogeneic HSCT from a donor carrying the CCR5Δ32 biallelic mutation, in the context of a Hodgkin's lymphoma. The patient was monitored and CD4 + and CD8 + T lymphocytes were collected without CCR5 expression, and antiretroviral treatments were suspended 510 days after transplantation. This study proved that the Berlin patient was not an anomaly, that the remission of HIV-1 can be achieved with a lower drug intensity and without total irradiation from the body, with a single transplant.[62]

Hütter et al. showed similar results in a patient infected with HIV-1 and with acute myeloid leukemia.[60]

As a curiosity, the first babies genetically edited with the CRISPR-Cas9 system were born. The twins, Nana and Lulu, were born in China in November 2018, as a result of in vitro fertilization, using the gametes of the HIV positive father and the HIV negative mother, in combination with CRISPR-Cas9 genome editing which altered their CCR5 gene. This first genetic manipulation in humans, with the aim of inactivating the CCR5 gene to confer resistance to the HIV virus, results in the birth of a child in which both copies of the gene are mutated and a second child carrying a mutated and a normal copy. The most important point is that there is a potential risk of off-target effects, which are still unknown and difficult to predict the consequences of a genetic edition in the germline edition.[22]

3.2.1.2. CXCR4

Other studies have chosen to focus on CXCR4 ablation, using CRISPR-Cas9. As expected, resistance to HIV-1 in vitro has also been demonstrated here.[83]

Hou et al., in a study in Jurkat T cells, osteosarcoma-derived cells and infected primary human CD4 + T cells, used a lentivirus expressing Cas9 and 10gRNAs targeting conserved CXCR4 gene sites. The results showed that there was a decrease in the cells expressing CXCR4 to 20-30%.[84]

Schumann et al. carried out a study with primary human CD4 + T cells isolated from healthy donors, having induced indels in the CXCR4 gene, through the use of Cas9. They also introduced an exogenous model for HDR. It was found that 60% of the cells showed a reduction in the expression of this coreceptor on the surface.[85]

CXCR4 receptors are also closely related to the retention of hematopoietic stem cells in the bone marrow, and it is extremely important to understand their effect on hematopoietic cells. In this sense, Liu et al. developed a study in TZM.bl cells where they induced a mutation in the CXCR4 P191A gene, through CRISPR-Cas9 and the piggyBac transposon system, where it was found that the expression of CXCR4 was decreased from 99.8% to 18.4% and 12,0% after treatment with two different sgRNAs, without any negative effect on physiology, with prevention and even a decrease in HIV-1 infection. There was a high specificity of this method, with negligible off-target effects, with preservation of immune functions.[86]

3.2.1.3. CCR5 and CXCR4

The simultaneous ablation of both CCR5 and CXCR4 co-receptors using CRISPR / Cas9 was achieved in vitro, as well as the respective resistance to viruses with both tropisms, even if simultaneous.[87]

Liu et al. used this strategy in a study conducted on Jurkat T, TZM.bl and primary CD4 + T cells. The results demonstrated an indelible mutation rate of 40.5% for CXCR4 and 32.9% for CCR5. The results indicated that in TZM.bl cells, the indel mutation rate was up to 40.5% for CXCR4 and up to 32.9% for CCR5. In the TCD4 + lymphocytes there was a reduction in the co-receptors, with resistance to viruses with tropism R5 and / or X4 and without toxic effects registered.[88]

3.1.2. Inhibition of viral infection

One of the strategies would be to prevent viral infection, creating a prophylactic immune system against HIV infection, through the constitutive expression of a CRISPR-Cas9 system.

Several studies were directed at the most different cells.

Liao et al. conducted a study with human T cells, derived from lymphoblasts. The gRNA was targeted at several regions of the HIV-1 genome, leading to a persistent reduction in viral expression. The best results were found mainly for the U3 and R regions of the LTR.[89]

These results were corroborated by other studies on CD4 + T lymphocytes.[89]

The same strategy applied to other hematopoietic lines. Several lines of anti-HIV human pluripotent stem cells (hPSC) have been produced showing a well-established expression of Cas9**.** The acquisition of resistance to HIV-1 M-trophic virus was verified in cells derived from the previous ones and that would originate monocytes or macrophages.[89] There were no reported toxic effects or off-target.[89]

Kaminski et al. conducted a study in which the gene encoding Cas9 was placed under the control of a viral Tat-activated promoter. Tat stands for "Transcription Transactivator", playing an important role in the transcription of HIV-1 They found that the production of nucleases constitutively was stimulated by the cellular release of viral Tat in the course of infection.[90]

The cells under study were TZM.bl cells. These cells were treated with a vector that contained specific regions of the HIV-1 promoter and sequence for Cas9. Then, this cells received multiplexed gRNAs directed to the LTR region. After being infected with HIV-1, the cleavage of the viral genetic material was verified, which will conclude that there was Tat production during the infectious process. This strategy allows the ablation of HIV-1 at an early stage or reactivation of the latent virus.[90]

Thus, it was found that the use of CRISPR-Cas systems as a vaccine is extremely viable and, considering that the target is viral genomic sequences, this strategy is functional, regardless of tropism or type of virus.

3.1.3. Inhibition of viral replication

The inhibition of viral replication is another strategy to be taken into account, as it is based on the redirection of gRNAs to different regions of the virus genome, such as LTR, gag, pol, tat and ver, and it was found that the results were higher, in terms of efficiency, when the target was the LTR region.[91]

It is important to mention that it is possible to modify the Cas9 endonuclease, which does not have the nuclear localization signal, so that it remains in the cytoplasm, thus allowing it to reach

the viral DNA earlier. On the other hand, Cas9-NLS would allow HIV to reach both the cytoplasm and the nucleus.[92]

A study was carried out with TCD4 + cells extracted from healthy individuals, later infected with HIV-1 and with the lentivirus vector that distributes Cas9 and gRNAs directed to the LTR region. There has been a decrease in the number of HIV-1 copies in the cells in question. [90]

The decrease in the number of copies of HIV-1 was also observed in peripheral blood mononuclear cells of HIV-positive patients.[90]

The number of reduced copies is due to indels and single-nucleotide variations (SNVs) in the target regions. However, it is important that multiple indels are generated, in order to increase the effectiveness of the attack on the virus, because if they are ineffective, the virus can create adaptive improvements. It is therefore important to use multiple gRNAs for different HIV-1 targets to prevent viral escape. It is noteworthy that, the reduced viral replication led to a decrease in reverse transcriptase activity.[93-95]

3.1.4. Prevent viral integration

Another strategy aims to prevent viral integration by cleaving and degrading the HIV-1 genome in the cytoplasm, before it is integrated into the host's DNA.

Liao et al. infected, in vitro, cells with complementary synthesized DNA (cDNA) of HIV-1, to understand whether it would be degraded by Cas9, in order to prevent viral integration and infection. The result was a significant decrease in HIV-1 positive cells.[89]

Liao et al. demonstrated that the resistance to HIV-1 caused by this edition could be induced in human pluripotent stem cells and be maintained after cell differentiation. Thus, allowing us to think of this tool as not only a treatment option, but also as a preventive one.[89]

Yin et al. conducted an in vitro study with 293T cells treated with cas9-NLS and gRNA directed to the R and U5 regions of LTR and infected with HIV-1. They found significant reductions in integrated and pre-integrated viral DNA, but no significant reduction in cytoplasmic DNA. All of this can be explained because Cas9 had a nuclear location signal. The authors found that only the nuclear cas9 could root out the latent provirus.[92]

Thus, it was possible to verify that the CRISPR-Cas9 system is effective in inactivating the integrated HIV-1 genetic material, through indels, and in inactivating it before it is integrated, through the degradation of the genetic material of the pro-viruses.[92]

3.1.5. Latently Infected Cells

Regarding latently infected cells we have to take into account two strategies.

The first strategy targets LTR and it results in the simultaneous cleavage in two different regions of the genetic material of the provirus, leading to the elimination of the genetic material between these two points of DNA in the host genome.

Several studies have been carried out on cells (293T, HeLa and HEK293T cells) infected with HIV-1, using Cas9 associated with multiple gRNAs targeting predominantly LTR regions. The cells were infected with different HIV-1 strains expressing GFP. They found a decrease in GFP (and p24, when used), translating an effective response. The authors also noted the presence of indels in the LTR region.[39,89,96]

It was considered that a reduced dose of this treatment would result in low cellular toxicity and could culminate in the elimination of the provirus in the long term.[89]

The possibility of disruption of the provirus genome is one of the techniques most tested by several scientific groups, since it would prevent possible reactivation, which occurs in HIV-1 patients who attempt to discontinue pharmacological treatment.

Wang et al. confirmed this in Jurkat cells using lentivirus as a vector of the CRISPR-Cas9 system. Very low viral reactivation (4.5%) was observed compared to the control (25.9%). Thus, the importance of this strategy was verified in the chronic treatment of HIV infection.[39]

In addition, other types of cells that are relevant reservoirs, such as astrocytes and myeloid cells, can also be changed effectively by CRISPR-Cas9.[97]

A study in particular carried out by Hu et al. is important as they became the first to prove the eradication of the latent HIV-1 provirus through the CRISPR-Cas9 system. In this study, a latent HIV-1 infected myeloid cell model was used, which was later treated with Cas9 and four types of gRNA targeting four distinct regions of the U3 region of the LTR. A TSA histone deacetylase inhibitor was then administered to activate the transcription of the integrated pro-viruses. There was an important reduction in GFP expression in treated cells. This strategy culminated in the creation of several indels that led to a complete inhibition of HIV-1 replication or reactivation.[98]

Also, Kunze et al. found that this could also be achieved in HNSC.100 cells, an astrocyte cell model containing latent pro-virus. No effects on the cell genome or cytotoxicity have been reported.[97]

The second strategy acts on viral genes, thus allowing to change HIV, both its characteristics and its infectivity.

Also important were two studies, whose gRNA directions were common (env, gag, pol, ver, vif, and LTR). [39,89]

The first study was carried out by Liao et. al in HEK293T cells infected with HIV-1 and the results were a significant reduction in GFP.[89]

The second study carried out by Wang et al., redirected the gRNAs to 43 different HIV-1 genome sites and obtained similar results. It was found that there are several regions of the genome that allow the eradication of HIV-1, however the gRNAs targeting the LTR region were the most effective, when compared with the rest.[39]

Tests were carried out on animal models to better understand the treatment of HIV. Kaminski et al. used Tg26 transgenic mice, which contained samples of the HIV-1 virus integrated into their genome, thus mimicking HIV-1 infection. The target regions were the LTR and the gag. The vector used was AAV9 and the injections were in the caudal vein, which occurred twice with a 5-day spacing between both. On the fifteenth day of the experiment, the animals were euthanized, and DNA extracted from the heart, lung, spleen, kidney, liver, brain and blood lymphocytes was analyzed. Thus, for the first time, the eradication of HIV-1 in vivo was verified in several tissues. Kaminski et al. also analyzed the effects of excision on the thirty-second day, observing circulating lymphocytes and verified the proviral eradication. [99]

Yin et al., based on the same target and analysis method as the previous authors, proved that the eradication of the HIV-1 provirus was possible in other animals, such as nude mouse NCr, without thymus and with lymphocytopenia and infested with EcoHIV-eLuc. They used the AAV-DJ / 8 adenoviruses as a vector and used the gRNA targeting the same target regions as the previous authors, but in a multiplexed manner. On the 19th day, viral expression was significantly reduced, and it was verified that Cas9 and gRNA reached the various organs and tissues, having local effectiveness. This efficiency was also verified in animals with greater clinical interest, humanized bone marrow / liver / thymus (BLT) mice, which are immunodeficient animals formed with fragments of the human liver, thymus, and bone marrow.[100,101]

It remains to mention the study Bella et al. who used PBMC cells obtained from HIV-1 infected patients and on antiretroviral therapy and injected those same cells into NRG rats. In this study, the target regions of Cas9 and multiplexed gRNA were two regions, A and B, of the LTR. Two weeks later the results showed a reduction of more than 90% of the viral DNA and the elimination

of the viral fragment present between the target sites. This study showed, once again, the importance of multiplexed gRNAs in the fight against HIV and even opened the possibility of studies in humans in the future.[102]

Finally, it is important to focus in one of the greatest achievements in the fight against HIV, carried out by Kaminski et al. in 2019, in which it was possible, through long-acting slow-effective release antiviral therapy (LASER ART) and CRISPR / Cas9 treatments, the elimination, without viral rebound, of HIV-1 infection in a subset of humanized mice. The gRNAS were directed to the LTR and Gag regions and delivered in vivo by AAV. In the transfer of immunocytes from animals subject to treatment to animals without infection, the virus did not spread. And in animals subjected to treatment, the virus was not detected by ddPCR in the blood, bone marrow, lymphoid tissue and brain. Thus, it is concluded that, combining CRISPR-Cas9 with LASER ART, a cure is possible. It is important to highlight the effectiveness and viability of AAV as a vector. [103]

3.1.6. Off-target effects

Specificity remains a major concern in therapy based on CRISPR-Cas9. This is because, breaking the DNA in areas not corresponding to the desired one can lead to physiological and structural damage, which can compromise the viability of the cell. In order to increase specificity, several strategies have been developed.

The first group of strategies to talk about is the use of modified nucleases.

Kleinstiver et al. and Kulcsár et al. produced high-fidelity endonucleases that proved to be effective in reducing off-target effects.[104,105]

Vakulskas et al. induced a p.R691A mutation, which also allowed for a reduction in off-target effects, but a conservation of activity on the target.[106]

The second group of strategies is based on the chemical modification of the gRNA, which proved to be very useful.[33,107]

A third group of strategies is based on the use of software to assist in the selection of target sequences and optimization of the gRNA project.

Hsu et al. resorted to bioinformatics screening to score potential target genomic locations.[108]

Listgarten et al. have developed a technology capable of predicting the potential best gRNA sequence.[109]

Haeussler et al. developed an algorithm called CRISPOR that uses a database to identify off-target sites caused by a gRNA sequence.[110]

Another strategy was suggested by Senturk et al. who proved that a shorter cell exposure time to the CRISPR-Cas9 system led to a decrease in off-target effects.[111]

The use of ribonucleoproteins would be a good option, since the cell would degrade the components as soon as the DNA breaks down.[85]

A last strategy, mentioned previously, is the use of nickases Cas9.

Taking into account the studies published to date, no off-target effect was found after using CRISPR-Cas9 as a tool to combat HIV-1. However, prior to clinical studies in humans, it is necessary to ensure specificity parameters and reproducibility. With regard to HIV patients, the best method of gRNA design would be to take into account the analysis of their genomic variation and the virus sequence, in order to minimize the possible off-target effects.[112]

4. Conclusion

This work aimed to carry out a bibliographic review in order to achieve a better understanding of this promising new tool, the CRISPR-Cas9 system, but also of its applicability in HIV.

HIV is one of the major pandemics ever and one of the biggest challenges for the scientific community. The reality is that, today, it is possible for an individual who is infected and medicated correctly to live a life overlapping with that of a so-called normal individual, but never attains complete cure. The major obstacle to complete cure is the latent infection of some tissues and organs. Several strategies were tried, but always without success.

Genetic editing, namely through CRISPR-Cas systems, seems to be an important tool to achieve not only the cure, but also the prevention.

There are several studies that prove a certain effectiveness in the CRISPR-Cas9 fight against HIV. The ablation of the CCR5 coreceptor has gained tremendous strength, since it is based on the attempt to replicate the cure of patients of London and Berlin, the only cases of complete and successful cure. This technique gained even more prominence, since it motivated the birth of the first genetically modified children in history.

Other studies were based on reaching the HIV genetic material itself, preventing infection, replication or integration of the virus. The major obstacle continues, as mentioned, is the elimination of the virus from latently infected cells. However, all these study fronts present promising results, as we have reported throughout this work. Here emerges one of the biggest obstacles to the use of CRISPR-Cas9: ethics. This subject was not addressed in this work.

It remains for me to mention the growing importance of this technique of genetic editing in the scientific environment. The CRISPR-Cas system was awarded the Nobel Prize for Chemistry in 2020 and is one of the future great medical and scientific fronts in the fight against infectious, degenerative and neoplastic pathologies, hence the importance of a document like this that allows first a generalized understanding of the technique and finally its applicability in combating a disease as devastating as HIV.

Appendix

List of figures

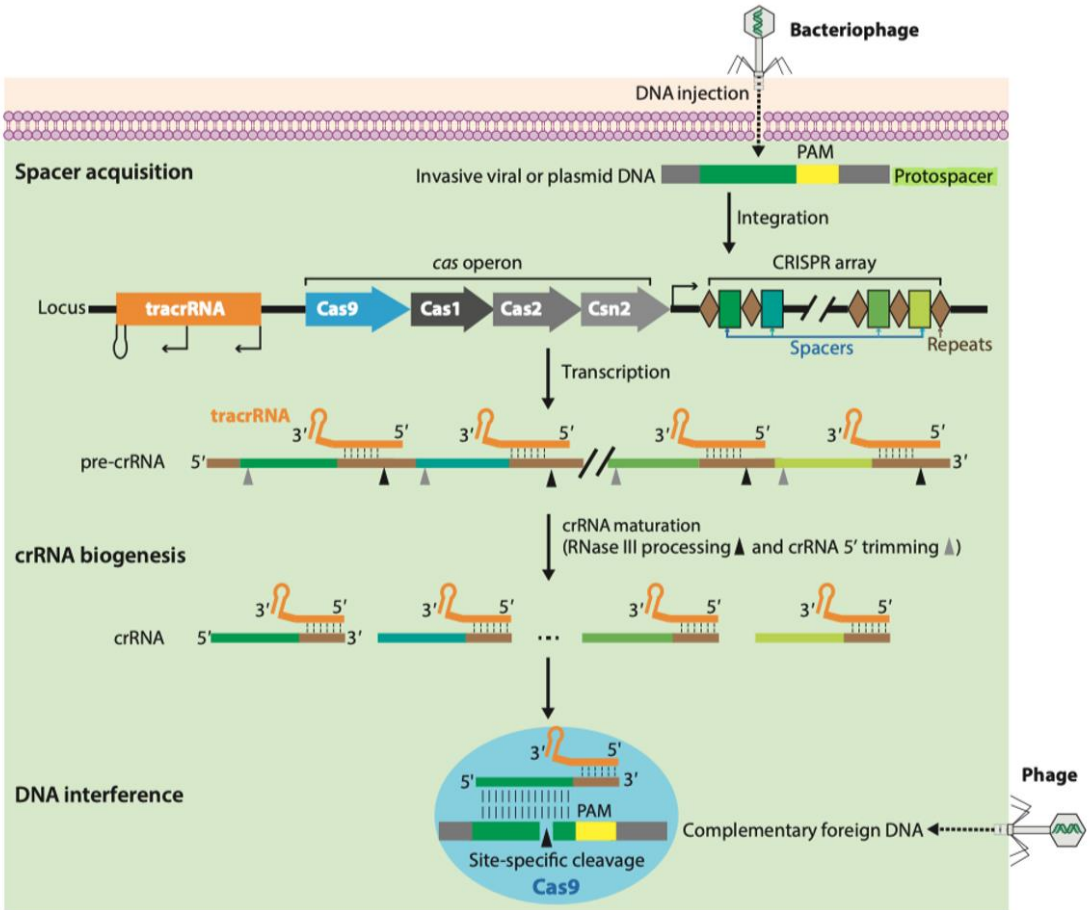


Figure 1 CRISPR–Cas9-mediated DNA interference in bacterial adaptive immunity- ***Jiang et al., 2017.***[7]

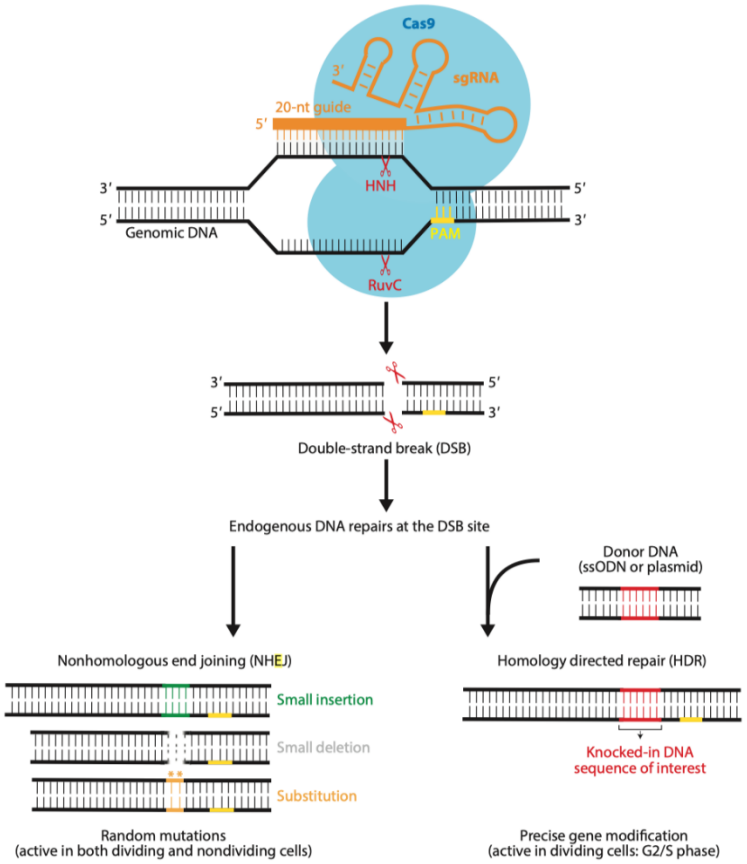


Figure 2 Mechanism of CRISPR–Cas9–mediated genome engineering - ***Jiang et al., 2017****.*[7]

Bibliography

1. Amitai G, Sorek R. CRISPR-Cas adaptation: insights into the mechanism of action. *Nat Rev Microbiol.* 2016;14(2):67-76.
2. Richter C, Chang JT, Fineran PC. Function and regulation of clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR associated (Cas) systems. *Viruses.* 2012;4(10):2291-2311.
3. Marraffini LA, Sontheimer EJ. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet.* 2010;11(3):181-190.
4. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell.* 2014;157(6):1262-1278.
5. Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science.* 2014;346(6213):1258096.
6. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science.* 2012;337(6096):816-821.
7. Jiang F, Doudna JA. CRISPR-Cas9 Structures and Mechanisms. *Annu Rev Biophys.* 2017;46:505-529.
8. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol.* 2014;32(4):347-355.
9. Doetschman T, Georgieva T. Gene Editing With CRISPR/Cas9 RNA-Directed Nuclease. *Circ Res.* 2017;120(5):876-894.
10. Greely HT. CRISPR'd babies: human germline genome editing in the 'He Jiankui affair'*. *Journal of Law and the Biosciences.* 2019;6(1):111-183.
11. Lo A, Qi L. Genetic and epigenetic control of gene expression by CRISPR-Cas systems. *F1000Res.* 2017;6.
12. Koonin EV, Makarova KS, Zhang F. Diversity, classification and evolution of CRISPR-Cas systems. *Curr Opin Microbiol.* 2017;37:67-78.
13. Makarova KS, Koonin EV. Annotation and Classification of CRISPR-Cas Systems. *Methods Mol Biol.* 2015;1311:47-75.
14. Jiang F, Doudna JA. The structural biology of CRISPR-Cas systems. *Curr Opin Struct Biol.* 2015;30:100-111.
15. Lee HB, Sundberg BN, Sigafoos AN, Clark KJ. Genome Engineering with TALE and CRISPR Systems in Neuroscience. *Front Genet.* 2016;7:47.
16. Wright AV, Nuñez JK, Doudna JA. Biology and Applications of CRISPR Systems: Harnessing Nature's Toolbox for Genome Engineering. *Cell.* 2016;164(1-2):29-44.
17. Mali P, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. *Nat Methods.* 2013;10(10):957-963.
18. Xue HY, Ji LJ, Gao AM, Liu P, He JD, Lu XJ. CRISPR-Cas9 for medical genetic screens: applications and future perspectives. *J Med Genet.* 2016;53(2):91-97.
19. Shui B, Hernandez Matias L, Guo Y, Peng Y. The Rise of CRISPR/Cas for Genome Editing in Stem Cells. *Stem Cells Int.* 2016;2016:8140168.
20. Jinek M, Jiang F, Taylor DW, et al. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science.* 2014;343(6176):1247997.
21. Nishimasu H, Cong L, Yan WX, et al. Crystal Structure of Staphylococcus aureus Cas9. *Cell.* 2015;162(5):1113-1126.
22. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* 2013;339(6121):819-823.
23. Heyer WD, Ehmsen KT, Liu J. Regulation of homologous recombination in eukaryotes. *Annu Rev Genet.* 2010;44:113-139.

24. Savić N, Schwank G. Advances in therapeutic CRISPR/Cas9 genome editing. *Transl Res.* 2016;168:15-21.
25. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature.* 2014;513(7519):569-573.
26. Sternberg SH, Redding S, Jinek M, Greene EC, Doudna JA. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature.* 2014;507(7490):62-67.
27. Jinek M, East A, Cheng A, Lin S, Ma E, Doudna J. RNA-programmed genome editing in human cells. *Elife.* 2013;2:e00471.
28. Khan FA, Pandupuspitasari NS, Chun-Jie H, et al. CRISPR/Cas9 therapeutics: a cure for cancer and other genetic diseases. *Oncotarget.* 2016;7(32):52541-52552.
29. Chen ZY, He CY, Ehrhardt A, Kay MA. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol Ther.* 2003;8(3):495-500.
30. Kim S, Kim D, Cho SW, Kim J, Kim JS. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res.* 2014;24(6):1012-1019.
31. Maggio I, Holkers M, Liu J, Janssen JM, Chen X, Gonçalves MA. Adenoviral vector delivery of RNA-guided CRISPR/Cas9 nuclease complexes induces targeted mutagenesis in a diverse array of human cells. *Sci Rep.* 2014;4:5105.
32. Ran FA, Cong L, Yan WX, et al. In vivo genome editing using Staphylococcus aureus Cas9. *Nature.* 2015;520(7546):186-191.
33. Hendel A, Bak RO, Clark JT, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol.* 2015;33(9):985-989.
34. Zuris JA, Thompson DB, Shu Y, et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. *Nat Biotechnol.* 2015;33(1):73-80.
35. Ramakrishna S, Kwaku Dad AB, Beloor J, Gopalappa R, Lee SK, Kim H. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res.* 2014;24(6):1020-1027.
36. Niu Y, Shen B, Cui Y, et al. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell.* 2014;156(4):836-843.
37. Organization WH. HIV/AIDS. November, 2020; https://www.who.int/health-topics/hiv-aids/#tab=tab_1. Accessed May, 12, 2021.
38. Human Immunodeficiency Virus (HIV). *Transfus Med Hemother.* 2016;43(3):203-222.
39. Wang Q, Liu S, Liu Z, et al. Genome scale screening identification of SaCas9/gRNAs for targeting HIV-1 provirus and suppression of HIV-1 infection. *Virus Res.* 2018;250:21-30.
40. Cassan E, Arigon-Chifolleau AM, Mesnard JM, Gross A, Gascuel O. Concomitant emergence of the antisense protein gene of HIV-1 and of the pandemic. *Proc Natl Acad Sci U S A.* 2016;113(41):11537-11542.
41. Nyamweya S, Hegedus A, Jaye A, Rowland-Jones S, Flanagan KL, Macallan DC. Comparing HIV-1 and HIV-2 infection: Lessons for viral immunopathogenesis. *Rev Med Virol.* 2013;23(4):221-240.
42. Shaw GM, Hunter E. HIV transmission. *Cold Spring Harb Perspect Med.* 2012;2(11).

43. Deng H, Liu R, Ellmeier W, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature.* 1996;381(6584):661-666.
44. Xiao Q, Guo D, Chen S. Application of CRISPR/Cas9-Based Gene Editing in HIV-1/AIDS Therapy. *Front Cell Infect Microbiol.* 2019;9:69.
45. Mbonye U, Karn J. The Molecular Basis for Human Immunodeficiency Virus Latency. *Annu Rev Virol.* 2017;4(1):261-285.
46. Maartens G, Celum C, Lewin SR. HIV infection: epidemiology, pathogenesis, treatment, and prevention. *Lancet.* 2014;384(9939):258-271.
47. Churchill MJ, Deeks SG, Margolis DM, Siliciano RF, Swanstrom R. HIV reservoirs: what, where and how to target them. *Nat Rev Microbiol.* 2016;14(1):55-60.
48. Kwon KJ, Siliciano RF. HIV persistence: clonal expansion of cells in the latent reservoir. *J Clin Invest.* 2017;127(7):2536-2538.
49. Wagner TA, McLaughlin S, Garg K, et al. HIV latency. Proliferation of cells with HIV integrated into cancer genes contributes to persistent infection. *Science.* 2014;345(6196):570-573.
50. Maldarelli F, Wu X, Su L, et al. HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. *Science.* 2014;345(6193):179-183.
51. Cary DC, Fujinaga K, Peterlin BM. Molecular mechanisms of HIV latency. *J Clin Invest.* 2016;126(2):448-454.
52. Siliciano JD, Kajdas J, Finzi D, et al. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat Med.* 2003;9(6):727-728.
53. Sáez-Cirión A, Bacchus C, Hocqueloux L, et al. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS Pathog.* 2013;9(3):e1003211.
54. Cummins NW, Rizza S, Litzow MR, et al. Extensive virologic and immunologic characterization in an HIV-infected individual following allogeneic stem cell transplant and analytic cessation of antiretroviral therapy: A case study. *PLoS Med.* 2017;14(11):e1002461.
55. Abner E, Jordan A. HIV "shock and kill" therapy: In need of revision. *Antiviral Res.* 2019;166:19-34.
56. Niu MT, Stein DS, Schnittman SM. Primary human immunodeficiency virus type 1 infection: review of pathogenesis and early treatment intervention in humans and animal retrovirus infections. *J Infect Dis.* 1993;168(6):1490-1501.
57. Vlahov D, Graham N, Hoover D, et al. Prognostic indicators for AIDS and infectious disease death in HIV-infected injection drug users: plasma viral load and CD4+ cell count. *Jama.* 1998;279(1):35-40.
58. Samji H, Cescon A, Hogg RS, et al. Closing the gap: increases in life expectancy among treated HIV-positive individuals in the United States and Canada. *PLoS One.* 2013;8(12):e81355.
59. Chun TW, Justement JS, Murray D, et al. Rebound of plasma viremia following cessation of antiretroviral therapy despite profoundly low levels of HIV reservoir: implications for eradication. *Aids.* 2010;24(18):2803-2808.
60. Hütter G, Nowak D, Mossner M, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med.* 2009;360(7):692-698.
61. Wu L, Gerard NP, Wyatt R, et al. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature.* 1996;384(6605):179-183.

62. Gupta RK, Abdul-Jawad S, McCoy LE, et al. HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature.* 2019;568(7751):244-248.
63. Salgado M, Kwon M, Gálvez C, et al. Mechanisms That Contribute to a Profound Reduction of the HIV-1 Reservoir After Allogeneic Stem Cell Transplant. *Ann Intern Med.* 2018;169(10):674-683.
64. Kordelas L, Verheyen J, Beelen DW, et al. Shift of HIV tropism in stem-cell transplantation with CCR5 Delta32 mutation. *N Engl J Med.* 2014;371(9):880-882.
65. George BM, Kao KS, Kwon HS, et al. Antibody Conditioning Enables MHC-Mismatched Hematopoietic Stem Cell Transplants and Organ Graft Tolerance. *Cell Stem Cell.* 2019;25(2):185-192.e183.
66. Czechowicz A, Palchaudhuri R, Scheck A, et al. Selective hematopoietic stem cell ablation using CD117-antibody-drug-conjugates enables safe and effective transplantation with immunity preservation. *Nat Commun.* 2019;10(1):617.
67. Doms RW. Chemokine receptors and HIV entry. *Aids.* 2001;15 Suppl 1:S34-35.
68. Moore JP. Coreceptors: implications for HIV pathogenesis and therapy. *Science.* 1997;276(5309):51-52.
69. Verhofstede C, Nijhuis M, Vandekerckhove L. Correlation of coreceptor usage and disease progression. *Curr Opin HIV AIDS.* 2012;7(5):432-439.
70. Allen AG, Chung CH, Atkins A, et al. Gene Editing of HIV-1 Co-receptors to Prevent and/or Cure Virus Infection. *Front Microbiol.* 2018;9:2940.
71. Murray AJ, Kwon KJ, Farber DL, Siliciano RF. The Latent Reservoir for HIV-1: How Immunologic Memory and Clonal Expansion Contribute to HIV-1 Persistence. *J Immunol.* 2016;197(2):407-417.
72. Lopalco L. CCR5: From Natural Resistance to a New Anti-HIV Strategy. *Viruses.* 2010;2(2):574-600.
73. Ioannidis JP, Rosenberg PS, Goedert JJ, et al. Effects of CCR5-Delta32, CCR2-64I, and SDF-1 3'A alleles on HIV-1 disease progression: An international meta-analysis of individual-patient data. *Ann Intern Med.* 2001;135(9):782-795.
74. Solloch UV, Lang K, Lange V, Böhme I, Schmidt AH, Sauter J. Frequencies of gene variant CCR5-Δ32 in 87 countries based on next-generation sequencing of 1.3 million individuals sampled from 3 national DKMS donor centers. *Hum Immunol.* 2017;78(11-12):710-717.
75. O'Brien SJ, Moore JP. The effect of genetic variation in chemokines and their receptors on HIV transmission and progression to AIDS. *Immunol Rev.* 2000;177:99-111.
76. Wang CX, Cannon PM. Clinical Applications of Genome Editing to HIV Cure. *AIDS Patient Care STDS.* 2016;30(12):539-544.
77. Wang W, Ye C, Liu J, Zhang D, Kimata JT, Zhou P. CCR5 gene disruption via lentiviral vectors expressing Cas9 and single guided RNA renders cells resistant to HIV-1 infection. *PLoS One.* 2014;9(12):e115987.
78. Li C, Guan X, Du T, et al. Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9. *J Gen Virol.* 2015;96(8):2381-2393.
79. Xu L, Yang H, Gao Y, et al. CRISPR/Cas9-Mediated CCR5 Ablation in Human Hematopoietic Stem/Progenitor Cells Confers HIV-1 Resistance In Vivo. *Mol Ther.* 2017;25(8):1782-1789.
80. Qi C, Li D, Jiang X, et al. Inducing CCR5Δ32/Δ32 Homozygotes in the Human Jurkat CD4+ Cell Line and Primary CD4+ Cells by CRISPR-Cas9 Genome-Editing Technology. *Mol Ther Nucleic Acids.* 2018;12:267-274.

81. Liu R, Paxton WA, Choe S, et al. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell.* 1996;86(3):367-377.
82. Ye L, Wang J, Beyer AI, et al. Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Δ32 mutation confers resistance to HIV infection. *Proc Natl Acad Sci U S A.* 2014;111(26):9591-9596.
83. Wang Q, Chen S, Xiao Q, et al. Genome modification of CXCR4 by Staphylococcus aureus Cas9 renders cells resistance to HIV-1 infection. *Retrovirology.* 2017;14(1):51.
84. Hou P, Chen S, Wang S, et al. Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection. *Sci Rep.* 2015;5:15577.
85. Schumann K, Lin S, Boyer E, et al. Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *Proc Natl Acad Sci U S A.* 2015;112(33):10437-10442.
86. Liu S, Wang Q, Yu X, et al. HIV-1 inhibition in cells with CXCR4 mutant genome created by CRISPR-Cas9 and piggyBac recombinant technologies. *Sci Rep.* 2018;8(1):8573.
87. Yu S, Yao Y, Xiao H, et al. Simultaneous Knockout of CXCR4 and CCR5 Genes in CD4+ T Cells via CRISPR/Cas9 Confers Resistance to Both X4- and R5-Tropic Human Immunodeficiency Virus Type 1 Infection. *Hum Gene Ther.* 2018;29(1):51-67.
88. Liu Z, Chen S, Jin X, et al. Genome editing of the HIV co-receptors CCR5 and CXCR4 by CRISPR-Cas9 protects CD4(+) T cells from HIV-1 infection. *Cell Biosci.* 2017;7:47.
89. Liao HK, Gu Y, Diaz A, et al. Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. *Nat Commun.* 2015;6:6413.
90. Kaminski R, Chen Y, Salkind J, et al. Negative Feedback Regulation of HIV-1 by Gene Editing Strategy. *Sci Rep.* 2016;6:31527.
91. Yin C, Zhang T, Li F, et al. Functional screening of guide RNAs targeting the regulatory and structural HIV-1 viral genome for a cure of AIDS. *Aids.* 2016;30(8):1163-1174.
92. Yin L, Hu S, Mei S, et al. CRISPR/Cas9 Inhibits Multiple Steps of HIV-1 Infection. *Hum Gene Ther.* 2018;29(11):1264-1276.
93. Wang G, Zhao N, Berkhout B, Das AT. CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape. *Mol Ther.* 2016;24(3):522-526.
94. Lebbink RJ, de Jong DC, Wolters F, et al. A combinational CRISPR/Cas9 gene-editing approach can halt HIV replication and prevent viral escape. *Sci Rep.* 2017;7:41968.
95. Wang Z, Pan Q, Gendron P, et al. CRISPR/Cas9-Derived Mutations Both Inhibit HIV-1 Replication and Accelerate Viral Escape. *Cell Rep.* 2016;15(3):481-489.
96. Ebina H, Misawa N, Kanemura Y, Koyanagi Y. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. *Sci Rep.* 2013;3:2510.
97. Kunze C, Börner K, Kienle E, et al. Synthetic AAV/CRISPR vectors for blocking HIV-1 expression in persistently infected astrocytes. *Glia.* 2018;66(2):413-427.
98. Hu W, Kaminski R, Yang F, et al. RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. *Proc Natl Acad Sci U S A.* 2014;111(31):11461-11466.
99. Kaminski R, Bella R, Yin C, et al. Excision of HIV-1 DNA by gene editing: a proof-of-concept in vivo study. *Gene Ther.* 2016;23(8-9):690-695.

100. Yin C, Zhang T, Qu X, et al. In Vivo Excision of HIV-1 Provirus by saCas9 and Multiplex Single-Guide RNAs in Animal Models. *Mol Ther.* 2017;25(5):1168-1186.
101. Karpel ME, Boutwell CL, Allen TM. BLT humanized mice as a small animal model of HIV infection. *Curr Opin Virol.* 2015;13:75-80.
102. Bella R, Kaminski R, Mancuso P, et al. Removal of HIV DNA by CRISPR from Patient Blood Engrafts in Humanized Mice. *Mol Ther Nucleic Acids.* 2018;12:275-282.
103. Dash PK, Kaminski R, Bella R, et al. Sequential LASER ART and CRISPR Treatments Eliminate HIV-1 in a Subset of Infected Humanized Mice. *Nat Commun.* 2019;10(1):2753.
104. Kleinstiver BP, Pattanayak V, Prew MS, et al. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature.* 2016;529(7587):490-495.
105. Kulcsár PI, Tálas A, Huszár K, et al. Crossing enhanced and high fidelity SpCas9 nucleases to optimize specificity and cleavage. *Genome Biol.* 2017;18(1):190.
106. Vakulskas CA, Dever DP, Rettig GR, et al. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nat Med.* 2018;24(8):1216-1224.
107. Basila M, Kelley ML, Smith AVB. Minimal 2'-O-methyl phosphorothioate linkage modification pattern of synthetic guide RNAs for increased stability and efficient CRISPR-Cas9 gene editing avoiding cellular toxicity. *PLoS One.* 2017;12(11):e0188593.
108. Hsu PD, Scott DA, Weinstein JA, et al. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol.* 2013;31(9):827-832.
109. Listgarten J, Weinstein M, Kleinstiver BP, et al. Prediction of off-target activities for the end-to-end design of CRISPR guide RNAs. *Nat Biomed Eng.* 2018;2(1):38-47.
110. Haeussler M, Schönig K, Eckert H, et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biol.* 2016;17(1):148.
111. Senturk S, Shirole NH, Nowak DG, et al. Rapid and tunable method to temporally control gene editing based on conditional Cas9 stabilization. *Nat Commun.* 2017;8:14370.
112. Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol Ther Nucleic Acids.* 2015;4(11):e264.