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USE OF INDUCED PLURIPOTENT STEM CELLS FOR HUMAN IMMUNODEFICIENCY VIRUS TREATMENT

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ABSTRACT

HIV is a retrovirus that mainly infects helper T lymphocytes. This pathogen causes the acquired immune deficiency syndrome (AIDS), affecting approximately 36.9 million people worldwide. AIDS is characterized by a quantitative and qualitative decrease in these cell counts. The pharmacological treatment is a combination of antiretrovirals that are administered for life. Due to the large number of people infected with this pathogen, finding a cure has become a public health goal. In 2007, the recovery of an HIV-positive patient was confirmed by transplanting hematopoietic stem cells with a mutation in the CCR5 coreceptor, providing infection resistance. Search for techniques that managed to inhibit or eliminate the CCR5 expression through gene silencing began. Induced pluripotent stem cells or iPSCs were produced *in vitro* after nuclear reprogramming of somatic cells by expressing transcription factors such as OCT3/4, SOX2, KLF4, and MYC, allow genetic modifications to be made so that HIV-resistant cells are obtained. Although this therapy is a promising alternative to treat and cure this illness, there are currently no clinical studies using genetically modified iPSCs to ensure their safety and effectiveness.

Keywords: human immunodeficiency virus, helper T lymphocytes, drug therapy, induced pluripotent stem cells.

INTRODUCTION

Approximately 36.9 million people around the world are living with the human immunodeficiency virus (HIV). 75 % know their condition, and most of them are undergoing treatment.[1] HIV is grouped into 1 and 2. The leading global agent of acquired immune deficiency syndrome (AIDS) is HIV-1. The other is restricted to some regions of West and Central Africa,[2] causing a similar but less aggressive disease. For its part, HIV-1 is acquired through infected body fluids, particularly blood and semen. The most ordinary ways of transmission are sexual, parenteral (recipients of blood or blood products, injection drug users, and occupational exposure to contaminated products), and vertical (mother to fetus).[3]

HIV-1 is a single-stranded RNA retrovirus of the lentivirus family. It invades cells that contain specific membrane receptors and incorporates a copy of its genetic material into the host genome.[3] Infections generally show a chronic course, with a long clinical latency, persistent viral replication, and central nervous system involvement.[2] The pathogen infects the immune system cells, mainly helper T lymphocytes (CD4+), macrophages, dendritic cells, and Langerhans cells.[4]

AIDS is characterized by an immunodeficiency secondary to a quantitative and qualitative decrease in CD4+ T cells. The expression of the CD4 molecule on its cell surface serves as the primary cellular receptor. Still, for the virus entrance, a coreceptor is required. The two main ones are the chemokines receptors CCR5 and CXCR4.[5]

When treatment for the virus is started early, the life expectancy of infected people approximates the non-infected ones. The main objective is to suppress its replication completely. Management that achieves an undetectable viral load produces a lasting therapeutic response. For this, a combination of no less than three drugs from at least two distinct classes is necessary. Medications can be grouped into five main classes: antiretroviral nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors

(PIs), entry inhibitors (fusion inhibitors), and integrase inhibitors.[6]

Its life-long administration and many infected people worldwide have made finding a cure a primary objective of public health. One advance is its complete eradication in two patients by transplantation of hematopoietic stem cells (HSCs) without CCR5 receptor. Due to this finding, the search for new techniques to inhibit or eliminate the expression of CCR5 by gene silencing, and thus, replace infected cells with a new population of cells resistant to HIV-1 is pursued.[7]

Against this background, this article's objective is to compile the available information on the utilization of pluripotent stem cells for future treatment against HIV.

PATHOPHYSIOLOGY AND IMMUNE RESPONSE TO INFECTION

In ancient times, chimpanzees and other primates' species were hunted and slaughtered. During these practices, direct human exposure to animal blood or mucosal secretions has provided the simplest explanation for the cross-species transmission of the simian immunodeficiency viruses (SIVs) to become HIVs.[8]

Viral and host factors determine the variability of HIV-1 infection and rates of disease progression in infected individuals. Cellular tropism that defines the viral phenotype and receptors-coreceptors (determine viral entry into various cell types) are the main factors that influence its pathogenesis.[9]

One mechanism for weakening the immune system is infecting and destroying CD4+ T cells, leading to immunodeficiency in the later disease stage. It binds to the CD4 protein on the cell surface to enter through the viral glycoproteins gp120 and gp41.[10, 11] However, its presence is not sufficient for the entrance into other cells. This situation led to the discovery of chemokine receptors. There are distinct types for several cells that HIV variants employ to infect them. The two main ones are CCR5 and CXCR4 (or fusin). They are structurally related receptors (seven-transmembrane G-protein coupled receptors or GPCRs). By binding to their respective ligands, they are activated and mediate various cellular functions, including leukocyte trafficking, angiogenesis, and immune response.[9, 12, 13, 14, 15]

Since the pathogen has a two-receptors model, the primary targets are CD4+ T lymphocytes and monocyte/macrophage lineage cells. Furthermore, the activation state of CD4+ has a significant impact on their ability for successful replication. After emerging from the thymus, they have not found their cognate antigen and are resting. At that time, they do not express CCR5 and are resistant to HIV. In contrast, the activated ones are transcribing DNA, promoting more significant viral replication.[16]

CCR5 is expressed at high levels in memory T cells, macrophages, and dendritic cells but not in naïve T lymphocytes. CXCR4 is expressed in both. In the early stages, the CCR5 receptor allows to infect cells, even though as immunodeficiency progresses, the virus evolves to enter through CXCR4. Although dendritic cells are difficult to infect, the virus capture promotes its transmission to neighboring helper T lymphocytes. Subsequently, the pathogen can bind to the follicular dendritic network within the lymph nodes' B cell follicles. Also, it occasioned lymphoid tissue fibrosis through various mechanisms, including T regulatory cell upregulation and the release of transforming growth factor-β (TGF-β). This molecule is a cytokine involved in cellular processes such as hematopoiesis, proliferation, angiogenesis, differentiation, migration, and apoptosis. Moreover, it has pleiotropic effects on adaptive immunity, primarily regulating effector and regulatory responses of CD4+ T lymphocytes.[17, 18, 19, 20, 21]

Being a retrovirus, it can integrate its DNA into the host's genome. This mechanism makes it difficult to eradicate with current therapies. After entering a cell, single-strand RNA is reverse transcribed into DNA, and through viral integrase, becomes part of the host's DNA. Then, proteins are produced and cleaved, releasing mature virions, with the complete viral DNA attached. The viral protease divides this genetic material into segments outside the host cells, obtaining fully functional particles to infect others.[17, 22]

During early infection, chemokine induction can bring more target cells to the infection and inflammation site, spreading the virus. Replication ultimately causes CD4+ T lymphocyte dysfunction and cell death (severe cellular immunodeficiency).[12, 23, 24]

The disease progression is correlated with an increase in viral load and elevated apoptosis levels. HIV-1 has various strategies to survive in infected individuals. HIV-1 Nef protein is a small molecule essential for pathogen replication and AIDS progression. It is abundantly expressed during the early infection stages. Its functions imply changing cell signaling pathways, increasing viral infectivity, and downregulating surface antigens on infected cells.[13]

Interferon-α (IFN-α), a family of specific proteins with diverse biological functions (antiviral, antiproliferative, and immunomodulatory activities), is a crucial pathogenesis marker. Although its expression in the initial stages may restrict viral replication, its virus-mediated overstimulation during chronic infection favors disease progression.[25, 26]

Regarding the host's immune response, the human leukocyte antigen-B*5701 (HLA-B*5701) is associated with the infection control in the absence of therapy, modulating the adaptive and innate immune response.[17, 27] The approximate 100-fold decrease in viral load as the

acute infection resolves is attributable to HIV-specific cytotoxic T lymphocytes (CD8+) development. With few exceptions, this response cannot lastingly control the virus, and with chronic exposure, the cells become dysfunctional. As a complement, the virus generates mutations, leading to a reservoir of viral strains impervious to the host's primary clearance mechanisms.[17]

Natural Killer (NK) cells also contribute through viral recognition by killer-immunoglobulin receptors (KIR), exerting immunological pressure. The virus evades such responses by selecting sequence polymorphisms in KIR.[27]

Immune activation includes several components, such as the homeostatic response to CD4+ T cell depletion and immune hyperactivation that induces amplified cell division in memory T cells and augmenting their self-renewal capacity. Thus, a higher cell count is generated. Nonetheless, activated helper T lymphocytes have a short life span and are lost relatively quickly due to activation-induced cell death or apoptosis. Plus, an inflammatory response occurs because of an increase in circulating pro-inflammatory cytokines and chemokines. Persistent immune activation prevents the establishment of IL-2 producing memory CD4+ and CD8+ T cells, generating deleterious effects on the immunity of specific CD4+ T lymphocytes against HIV. This interleukin promotes proliferation types and downregulates immune responses to prevent autoimmunity.[13, 28, 29]

CLINICAL PICTURE

Virus detection in the blood (usually measured as viral RNA levels) is often associated with a brief symptomatic phase marked by fever, generalized lymphadenopathy, nonspecific rash, myalgia, and/or malaise. More severe complications involving meningitis can occur, but many people are asymptomatic. During this time, plasma RNA levels are typically at their peak (approximately 106 to 107 copies per ml). The symptoms' severity is strongly correlated with peak viral load. Once the immune response develops, they drop approximately 100-fold to a viral set point.[17]

This level is correlated with the clinical outcome. Individuals with a high viral load generally progress to AIDS and death more rapidly than those with lower ones.[30] The few patients with low scores are elite controllers.[31]

A recently infected person may present within one to three weeks symptoms of a viral illness (high plasma viremia, headache, retroorbital pain, muscle aches, sore throat, anorexia, low or high fever, swollen lymph nodes, and non-pruritic macular erythematous rash involving the trunk and the extremities). In some cases, oral candidiasis and ulcerations happen in the esophagus or anal canal, and central nervous system disorders (encephalitis) may be seen. In other people with acute infection, pneumonia, diarrhea, and other gastrointestinal complaints have been reported. These symptoms generally last one to three weeks, although lymphadenopathy, lethargy, and general malaise persist for many months. In general, the primary infection is followed by an asymptomatic period of many months to years.[32, 33]

Subsequently, an inactive (or latent) infection is established within the memory CD4+ T lymphocytes.[34] These cells are maintained indefinitely through homeostatic proliferation. Some have the self-renewal capacity of stem cells.[35] Once HIV DNA integrates into the host chromatin, it can restart replication rounds if the cell persists.[17]

DIAGNOSIS

An antigen/antibody test is recommended based on the simultaneous detection of anti-HIV antibodies and the HIV-1 p24 antigen.[36] Positive results must be confirmed with an antibody assay capable of distinguishing between HIV-1 and HIV-2 infections. Detection of acute infection prevents its transmission.[17]

The laboratory tests employed for its diagnosis can be classified as direct and indirect. Direct procedures allow detecting the virus's presence or its constituents (proteins and nucleic acids), even before an antibody response has been generated. Their drawback is that they are costly.[37] Some assays are p24 antigenemia (p24 antigen is a protein of the viral core; concentrations can be detected in the people blood around 11 to 13 days after infection by enzyme-linked immunosorbent assay or ELISA),[38, 39] quantitative detection of its viral genome in plasma or viral load testing (prognostic marker to control antiretroviral therapy and to estimate the infection potential),[40] and polymerase chain reaction (PCR). The latter allows the quantification of HIV DNA and RNA in cells, detection during the window period (before specific antibodies generation) and in newborns and determining HIV-1 and HIV-2 infections.[41]

Indirect analyses show the immune response generated based on the antibodies' detection in the serum. They are classified in:[37]

- Screening test (ELISA or micro-ELISA serology): requires an antigenic base that captures the specific antibodies in the sample.[42] Antigens are coated onto a plastic surface of the ELISA plate and incubated with a patient's serum. The binding of autoantibodies to antigens resembles the *in vivo* situation.[43]
- Confirmatory test (Western Blot serology): the distinct viral proteins are separated by electrophoresis according to their molecular weight and transferred to a nitrocellulose membrane on which the patient's serum is added and incubated. ELISA detects antigen-antibody binding. If the serum contains antibodies against the protein, a colored band is produced.[42]

Rapid HIV tests with blood from a finger-stick or oral fluid collection can provide test data for HIV-1 infection in 30 minutes. They are beneficial in settings where patient care is challenging. However, most have limited sensitivity to detect acute infection. If possible, more sensitive assays should be added when risk factors suggest it. The reduced sensitivity on oral fluid secretions rises approximately six weeks to detection in blood samples. As a complement, immunoassays can become non-reactive after long-term suppressive therapy in some infected individuals, so they should not be performed in treated people already confirmed as positive.[17, 44]

TRADITIONAL TREATMENT

Its objective is to reduce the virus replication in the body, diminishing transmission and preventing AIDS progression. Medications are not curative, but they raise the patient's functionality and life expectancy. The treatment consists of a three-drug combination to be consumed for the rest of life.[45, 46] A more significant benefit has been observed by starting treatment immediately in asymptomatic patients before the CD4+ count falls below 350 cells/mm^3.[47, 48]

There are six drug types, classified into five groups, as shown in **Table 1**. They block or inhibit the HIV's life cycle, preventing the viral particles from replication.

Table 1: Drugs used in traditional antiretroviral therapy according to their site of action and pharmacological group.[45, 46, 49]

Site of action	Pharmacological group	Mechanism of action	Examples
Initial fixation	CCR5 antagonist	Binds to the CCR5 receptor to block the viral gp120 binding.	Maraviroc
Membranes' fusion	Fusion inhibitor	Inhibits viral fusion by binding gp41 and CD4+ T lymphocyte.	Enfuvirtide
Reverse transcription of virus DNA	Non-nucleoside reverse transcription inhibitors	Bind to a protein site other than the active one of the reverse transcriptase, causing a conformational change.	Delavirdine, efavirenz, etravirine, nevirapine, rilpivirine
	Nucleoside reverse transcription inhibitors	Introduce simultaneously as the reverse transcriptase, generating competition with the nucleosides themselves and ending the process elongation due to the lack of a 3-hydroxyl group.	Abacavir, didanosine, emtricitabine, lamivudine, stavudine, tenofovir alafenamide, tenofovir disoproxil fumarate, zidovudine
	Integrase inhibitors	Interfere the integrase cations, preventing the formation of covalent bonds between the host DNA and the virus.	Dolutegravir, elvitegravir, raltegravir
Budding of new viral particles	Protease inhibitors	Competitive inhibitors of viral protease, generally binding between the phenylalanine and proline amino acids.	Fosamprenavir, atazanavir, darunavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, tipranavir

Nowadays, treatments with biotechnological processes have grown in the global pharmaceutical industry.[50, 51] This situation does not embrace HIV treatment because of the pathophysiology complexity. Despite this, research on biological therapies continues to be studied.

A significant group is monoclonal antibodies. They are homogeneous immunoglobulins produced by a single clone of B lymphocytes with the same antigenic specificity. They are widely utilized in pathologies with a molecular component such as autoimmune diseases and cancer, biomedical research (analytical techniques for biochemical and imaging diagnosis), labeling products, and therapeutic proteins purification. By being produced through efficient platform-based approaches, they are generally well-tolerated and precise. Besides, the risk of unexpected safety problems in clinical trials is lower than other therapeutic products.[52, 53, 54, 55] One drug already approved is the monoclonal antibody ibalizumab, a recombinant humanized immunoglobulin G4 (IgG4).

Its mechanism of action inhibits the pathogen entry into the CD4+ T lymphocytes.[56]

Nonetheless, the investigation of these molecules against the disease has encountered barriers, preventing the selection of broadly neutralizing antibodies. For example, not all those produced by the immune response serve to counteract each virus strain. VRC01 partially mimics the CD4+ T lymphocyte interaction with gp120, but a slight change allows it to bypass the receptor. It is in phase IIb clinical trials. Additionally, monoclonal antibodies 3BNC117 and 10-1074 were administered together and directed to this same lymphocyte's binding site with the HIV-1 envelope spike. Although the sample is small, benefits have been seen from its employment.[57, 58, 59, 60, 61]

Preventive and therapeutic vaccination has also been examined. Therapeutic vaccines seek to prevent AIDS development, replace the daily oral treatment, and reduce transmission. To date, there is no approved vaccine. Only immunization with RV144 has reduced the infection risk within clinical studies, producing IgG1Ab and IgG3 specific for gp120. The demonstrated efficacy was 60 % at 12 months, decreasing to 31 % at 3.5 years.[62, 63]

Likewise, strategies have been established to induce various immune responses. The most promising regimen is HVTN 702, like that of RV144 in a South African population. Still, the lack of people who recovered from HIV naturally, the variation in immune responses induced by the virus, and the several strains limit the production of a global vaccine. As a result, there is the alternative of creating mosaic immunogens, selecting a consensus sequence close to the viruses' natural or ancestral. In this way, better coverage of a population would be provided.[64, 65]

The above reflects that the search for a cure has expanded. Nevertheless, no molecule acts when HIV is already inserted into the DNA of CD4+ T cells. Other things to consider are that most T lymphocytes die after infection and that the virus latency hinders finding a cure. Besides, current medications' costs and accessibility, chronic adherence to treatment, and adverse effects encourage the search for new methods.[66, 67] Against this background, two cases of patients who were treated with stem cells are interesting. These examples are described below.

PATIENTS HEALED WITH STEM CELLS

In 2008, a 40-year-old male patient with acute myeloid leukemia and HIV underwent an allogeneic transplant to treat leukemia in Berlin. His treating doctor looked for a donor with a CCR5 Delta 32 (CCR5Δ32) mutation in CD4+ T cells.[68, 69]

The homozygous CCR5Δ32 deletion, observed in approximately 1 % of the white population, offers a natural resistance to HIV acquisition. After transplantation, treatment was stopped, and 20 months later, no active, replicating virus was detected. This observation was remarkable because this homozygosity is associated with high, but not complete, resistance to HIV-1. Its blood absence indicated the immune stimulus elimination of the virus. Antibodies against its envelope antigens remained detectable at continuously decreasing levels. It was considered that sustained secretion could be caused by long-lived plasma cells resistant to standard immunosuppressive therapies.[69]

Blood, cerebrospinal fluid, lymph node, and gut samples were analyzed. In no case was HIV DNA or RNA detected, and no competent replication virus could be grown from peripheral blood mononuclear cells. Though, RNA was detected in plasma and DNA in the rectum at levels considerably lower than expected in patients with suppressed antiretroviral therapy. The replication absence after five years without therapy, the failure to isolate infectious viruses, and the decrease in specific immune responses indicated his cure.[70]

Moreover, there is evidence of a patient in London who in 2019 underwent an allogeneic transplant with stem cells that did not express CCR5 for the treatment of Hodgkin's lymphoma. He had a reported remission 18 months after retroviral treatment discontinuation. For the detection, ultrasensitive viral load assays of plasma, semen, and cerebrospinal fluid were performed, cerebrospinal fluid samples were taken, and peripheral CD4+ T lymphocyte sampling was done. In addition, droplet digital PCR, a technique where rapid and quantitative detection is possible based on amplifying a target DNA molecule in a plurality of separate droplets, allowing the exact quantification of DNA copy numbers, was made. With this method, HIV-1 DNA targeted long terminal repeat regions, glycosaminoglycans, and integrase.[71, 72]

After 30 months, the plasma HIV viral load remained undetectable using a limit of detection test of one copy per ml. Also, the CD4+ cell count was 430 per µl at 28 months, the viral load in semen was undetectable at 21 months, and the cerebrospinal fluid remained in normal parameters at 25 months with viral RNA below the detection limit. Besides, the result was negative in the rectum, caecum, and sigmoid colon and terminal ileum at 22 months and a very low positive viral DNA signal was detected in peripheral CD4+ memory T cells at 28 months.[72]

Like the Berlin patient, some highly sensitive tests showed shallow levels of HIV-1 DNA in tissue samples. Residual DNA in axillary lymph node tissue could represent a defective clone expanded during hyperplasia within the lymph node sampled. After performing a DNA sequence analysis of HIV-1 in lymph nodes, it was not successful. This procedure is based on DNA polymerase's ability to extend a primer until a nucleotide that terminated the chain is incorporated, and the resulting series of fragments are separated by

polyacrylamide gel electrophoresis. The assay failure could be attributed to the low number of copies. Therefore, it was impossible to completely rule out the possibility that minor levels of intact HIV provirus persisted. It could also be attributed to contamination.[72, 73]

With this, it was appreciated that bone marrow transplantation is effective but presents certain difficulties and specific situations such as that they were cancer patients and were willing to undergo operations with a high complication degree. Such a scenario is not an option for those who only have HIV. Although, these cases have been the basis for stem cell-based gene therapy. The idea is to reconstitute the patient's immune system by transplanting genetically engineered hematopoietic stem cells (induced pluripotent stem cells or iPSCs) with anti-HIV genes. In theory, they could continuously provide immune cells resistant to the virus throughout the patient's life.[74]

STEM CELLS

The term was mentioned at the beginning of the 20th century by the German scientist Ernst Haeckel. Stem cells are characterized by dividing indefinitely and differentiate into distinct types of specialized cells through mitotic division. The division can be symmetric (two identical cells that have the same potential as the parental one) and asymmetric (two cells where the first one preserves the properties of the stem cell, while the second initiates the differentiation process to another cell lineage). Regarding their differentiation potential, they can be classified into five types (from highest to lowest differentiation potential): totipotent, pluripotent, multipotent, oligopotent, and unipotent.[75, 76, 77, 78]

They can come from two sources: the internal cell mass of the blastocyst (embryonic) and various human tissues such as bone marrow, brain, liver, dental marrow, intestine, and skin (adult).[76, 79]

Embryonic stem cells (ESCs) represent a source for cell replacement therapy in type I diabetes and cardiovascular, Parkinson's, blood cell, and liver diseases. Though its application as a developmental biology model has been limited, cell replacement therapy is still under study. One of the biggest questions is the maturation stage in which they should be transplanted. Moreover, there is a concern regarding the danger associated with graft contamination produced by residual undifferentiated stem cells, leading to teratomas.[80]

For adult stem cells, a heterogeneous population such as hematopoietic, endothelial, stromal/mesenchymal, multipotent adult progenitor, and oval ones have been identified in the bone marrow. Similar populations have been appreciated in peripheral blood but at a lower concentration.[81]

Multiple punctures are performed for the bone marrow aspiration in the iliac crests of patients under anesthesia. Once obtained, they must be subjected to Ficoll density gradient centrifugation (cell volume decreases, while concentration and purity increase), based on the diverse densities between cells, allowing them to be separated after centrifugation.[81]

For achieving a fine aspiration in peripheral blood, strategies with stimulating factors are used (granulocyte colony-stimulating factor or G-CSF), releasing them from their natural habitat in the bone marrow and mobilizing them towards the blood. Once optimal levels have been reached, they are generally collected with a cell separator.[81]

INDUCED PLURIPOTENT STEM CELLS

The stem cell theory that existed at the beginning of the 20th century was corroborated in 1961, with the evidence presented by James Till and Ernest McCulloch demonstrating HSCs existence. Years later, in 1981, ESCs were isolated and cultured for the first time. With this, the differentiation process from embryonic to somatic cells was studied. Subsequently, in 1993 a live mouse was generated from ESCs, and in 1998, James Thomson described the possibility of isolating human ESCs, raising the expectation that pluripotent stem cells could become the source of new organs.[78]

However, investigations were limited, as there were questions about the techniques utilized and the embryos' destruction during their collection. Thus, the scientific community focused on searching for alternatives, ideally those present in the patient.[78, 82]

Pluripotency in ESCs is maintained briefly during mammals' embryonic development, in cells of the blastocyst's inner cell mass and the epiblast of the pre-gastrulation embryo. Their loss and differentiation to specific cell lines lead to changes in gene expression patterns, including the silencing of genes encoding key transcription factors for their maintenance, the expression of differentiation-inducing genes, and epigenetic changes in chromatin.[82]

In 2006, Takahashi and Yamanaka determined that somatic cells could revert to a pluripotent-like form by re-expressing essential embryonic genes suppressed in the somatic state.[78, 82] This discovery leads to a new concept called iPSCs. They are produced *in vitro* after somatic cells reprogramming through the forced expression of the following transcription factors:[83, 84]

1. OCT3/4: also known as the OCT3, OCT4, OTF3, or OTF4. Its official name in humans is POU5F1. It is located on chromosome 6p21.31 and encodes for a critical transcription factor in the pluripotency of embryonic cells. Likewise, it is associated with tumorigenesis in the case of an aberrant expression.

2. SOX2: encodes a member of the family of transcription factors involved in regulating embryonic development and definition of the development pathway. It is required for the stem cells' maintenance in the central nervous system and regulates gene expression in the stomach. It is located on chromosome 3q26.3-q27, and its mutation can be associated with optic nerve hypoplasia and many ophthalmic malformations.
3. KLF4: encodes a transcription factor that regulates the expression of genes associated with maintaining pluripotency and preventing differentiation (activator or repressor of multiple genes). Besides, it regulates the p53/TP53 transcription, and it is located on chromosome 9q31.
4. c-MYC: located on chromosome 8q24.21. It encodes for a multifunctional nuclear phosphoprotein that participates in cell transformation and apoptosis and regulates the transcription of multiple genes related to cell growth. Its mutations are associated with hematopoietic tumors, leukemias, and lymphomas.

This advancement involved the unlimited production of functional human cell types without ethical controversy. Hence, they could be applied to discover new drugs, implement *in vitro* disease modeling, drug safety tests, cell-based therapies, and regenerative medicine.[82, 85]

REPROGRAMMING METHODS FOR THE IPSCs GENERATION

Since the discovery of iPSCs, genomic integrating and non-integrating vectors are the procedures to generate them. In the first instance, the possibility of reprogramming by somatic cell nuclear transfer (SCNT) was studied. It consists of introducing the nucleus of a somatic cell into an enucleated oocyte, subsequently becoming an embryo. These investigations led to the birth of Dolly, the lamb, in 1997.[82, 86] It was shown that changes during cell differentiation and deleted genes could be reversible processes, owing to epigenetic variations (changes in the chemical structure of DNA that do not alter its sequence coding) and not permanent changes.[82, 87]

Another method previously studied was the cytoplasmic or nuclear extracts of undifferentiated cells such as ESCs, inducing reprogramming in somatic cells and giving them the pluripotent capacity. Additionally, the fusion of cells (one somatic and one human ESC) allowed reaching the former's pluripotent state. Later, with Takahashi and Yamanaka's discovery, genetic integrating vectors were valuable to introduce the four transcription factors required for obtaining a cell in a pluripotent state.[82, 86]

Still, its use has certain disadvantages. They are generally retroviruses, and there is a possibility of mutations in the viruses' insertion sites and risk of permanent genome modification of iPSCs. For these reasons, effective alternatives have been sought. Some options are non-integrating adenoviruses, transient transfection with plasmids, transposons (PiggyBac transposition system), and Sendai virus. Other alternatives include small molecules, synthetic messenger RNA (mRNA), episomal vectors, minicircle vectors, recombinant proteins, and micro RNAs (miRNAs).[82, 86] These reprogramming techniques are explained below.

Genomic integrating methods

Retrovirus

Retroviral gene transfer to hematopoietic cells is a new therapy for various severe or life-threatening diseases. Despite the low gene-transfer efficiency, it remains the standard technology for clinical studies on genomic integration. Early retroviral transduction techniques required the co-culture of hematopoietic target cells with stromal cells, such as primary bone marrow fibroblasts or retrovirus producer cell lines. Notwithstanding a satisfactory gene-transfer result, safety, and logistic problems minimized their viability in clinical practice. Currently, hematopoietic cell transduction is performed with cell-free retrovirus-containing supernatant. Polycations (protamine or polybrene) are added to facilitate the initial interaction between the cell and the virus, and hematopoietic growth factors are incorporated to stimulate the cycle and expression of the retroviral receptor of the hematopoietic target cells. The incubation in the virus presence is done for 48 hours, and the transduction efficiency is analyzed by determining the colonies' percentage with resistance to 1.5 mg/ml of an aminoglycoside antibiotic.[88]

Another method is mouse fibroblasts. For this, retroviruses with high transduction efficiency are required. First, a green fluorescent protein is introduced into human dermal fibroblasts with an amphotropic virus, and as a control, the same fluorescent protein is introduced with an ecotropic virus in mouse embryonic fibroblasts. More than 80 % of the cells expressed green fluorescence, while less than 20 % expressed it with less intensity. For optimizing the transduction process, the mouse receptor for retrovirus Slc7a1 was introduced into human dermal fibroblasts. The fluorescent protein was then incorporated into these cells, obtaining a transduction efficiency of 60 % with a similar intensity to that of mouse fibroblasts.[89]

For the generation of iPSCs, retroviruses containing the human transcription factors OCT3/4, SOX2, KLF4, and c-MYC are introduced into human dermal fibroblasts Slc7a1 receptor. A few days after transduction, trypsinized cells are harvested and placed in SNL feeder cells treated with mitomycin C. The next day, the medium is replaced by one for culturing primate ESCs. Approximately one month later, colonies similar to ESCs are observed, which are disaggregated into small groups without enzymatic digestion. In previous investigations with Western Blot, it was possible to determine that OCT3/4 and SOX2 proteins' levels were like those of

human iPSCs. In addition, with a DNA microarray (DNA hybridization, where the probes are immobilized onto a solid surface applying robotic technology), the gene expression patterns were similar but not identical between the ESCs obtained and the human iPSCs.[89, 90]

One limitation is the low efficiency (between 0.001 and 0.1 %, depending on the method). Also, it has been reported that a significant proportion of the pluripotent cell population initially obtained after retroviral transduction is partially reprogrammed, and sustained expression of transgenes is required to acquire self-renewal capacity. The low efficiency could be obtained by the low rate of infectivity and retroviral integration, the heterogeneity in the retroviruses integration, and intrinsic differences of the diverse cell types to be reprogrammed.[82]

Lentivirus

Lentiviral vectors show the importance of monogenic gene therapy by integrating into the host genome and allow long-lasting gene expression. They have been used in human diseases, proving to be an efficient process for overexpression of proteins after intrathecal administration in animal models.[91] Unlike conventional retroviruses, they can efficiently infect cells that are not dividing.[92]

Infecting iPSCs with lentiviral inserts is a highly efficient process. Stem cells proliferate and remain undifferentiated under specific conditions.[91] Nevertheless, a limitation is the modifications of retroviral expression because of epigenetic phenomena, triggering the virus's possible silencing.[93]

Non-integrating methods

Viral vectors

Adenoviral vectors

Adenoviruses are non-enveloped viruses. Its genetic material is double-stranded DNA contained in an icosahedral nucleocapsid.[94, 95] They belong to the family *Adenoviridae* and can remain in the epichromosomal form in all cell types, except egg cells[95] (lower risk of insertional mutations).[96] Its characteristics include high throughput and infection efficiency in cells, whether replicating or not. Its replication deficiency allows transgenes to be delivered and expressed without genomic integration.[95]

Adeno-associated viral vectors

Adeno-associated viruses (AAV) belong to the family *Parvoviridae*. They are composed of an icosahedral capsid. Their genome is single-stranded DNA and are not pathogenic for humans. To replicate, they need a helper virus such as adenoviruses. In its absence, the genome stays episomal.[95, 97] The vectors can integrate into a genomic locus of human cells known as AAVS1. Recombinant AAVs (rAAVs), in which the viral coding sequences of the rep gene are removed, are available to avoid such a situation. rAAVs offer advantages such as reduced immunogenicity and cytotoxicity and improved packaging capacity.[97]

Sendai virus vectors

Viruses of the family *Paramyxoviridae* with non-segmented negative-stranded RNA can replicate exclusively in the cytoplasm, reducing the possibility of host cell genome modification. They can infect cells independently of the cell cycle. Moreover, it is neither pathogenic nor carcinogenic in humans.[95, 98, 99]

The disadvantage is their difficult elimination at the process end. Nonetheless, modifications have been done to make them temperature sensitive. Heat treatment is applied at 38 °C for three days, obtaining 0 % positive Sendai cells.[100]

To determine the most optimal reprogramming method, specific characteristics or properties of these vectors must be considered, including the number of transductions required to obtain iPSCs. A greater quantity of transductions implies higher viral titers, which would eventually be a factor of genomic integration. Polycistronic systems (contain several structural genes) consist of a single vector with multiple genes connected by the 2A peptide.[95, 97, 101]

Immunogenicity is another factor to consider. In some cases, viral protein-coding sequences are removed to decrease the response, like rAAVs.[97]

As for reprogramming efficiency, it is generally determined by the percentage or count of reprogrammed cells compared to all those that underwent the process. It is done by staining with alkaline phosphatase. Next, the count is performed, and the percentage is calculated.[97, 102]

Tropism is another relevant characteristic. It is influenced by the cells' receptors and the virus capsid proteins (virus affinity for specific receptors or tissues to later infected them).[97, 103]

The characteristics previously indicated for the three types of non-integrating viral vectors are shown in **Table 2**.

Table 2: Comparison of the properties of non-integrating viral methods.[95]

Characteristic	Adenovirus	AAV	Sendai virus
Number of transductions to reach the iPSCs	Multiple	Multiple	One
Ability to accommodate essential transgenes in a polycistronic manner	Yes	No (low transgene-carrying capacity)	Yes
Immunogenicity	Immunogenic	Very low (rAAV reduces viral coding sequences)	Immunogenic
Genomic integration capacity	There is a possibility due to the need for high viral titers	Minimum	There is a possibility
Stable and prolonged expression of transgenes over time	No, the expression is transitory, hindering the pluripotent state	Yes	Yes
Ease of vector removal	Easy	Easy	Complicated, because multiple passages are required, or a mutation is necessary to make it sensitive to temperature
Reprogramming efficiency	Low (0.0001 to 0.001 %)	Low	High (0.01 to 4 %)
Tropism	Yes	Yes	Yes
Ability of infecting replicating and non-replicating cells	Yes	Yes	Yes

DNA-based non-viral vectors

Plasmids

They are extrachromosomal circular DNA, not vulnerable to exonucleases with respect to linear DNA. Compared to viral vectors, they are simpler to produce, can be employed multiple times, and have low immunogenicity. However, they are inefficient in their transfections, requiring physical forces, vehicles, or special modifiers for their location in the nucleus. In addition, they are extensive and require multiple transfections. Polycistronic plasmids improve reprogramming performance.[95, 104]

Minicircle vectors

They are double-stranded DNA with a supercoiled organization and a reduced size, giving them better transfection efficiency relative to plasmids. They are transferred by electroporation and show the expression of transcription factors for a longer time. Its immunogenicity is low.[86, 95] Nevertheless, they have low reprogramming efficiency, requiring multiple transductions.[105]

Episomal vectors

They are derived from the Epstein-Barr virus without viral packaging,[86] having the capacity to replicate themselves, are poorly immunogenic, and require a single transfection (compared to plasmids and minicircular vectors).[95] Nonetheless, its reprogramming efficiency concerning viral vectors is lower, and the administration of transcription factors occurs individually by nucleofection, causing uptake differences and highly varied gene expression levels.[106]

Transposons

Transposons are mobile DNA capable of changing positions in the genome. It is believed that they could be

viruses incorporated into the human genome over time.[107]

As a technique, they work by a cut-and-paste mechanism, and their essential element is the transposase enzyme, which recognizes a segment of DNA from a plasmid, cuts it and integrates itself at a chromosomal locus.[108] The most relevant are PiggyBac (PB) and Sleeping Beauty (SB).[109, 110, 111]

They have better transfection efficiency, low immunogenicity, are inexpensive, easy to purify, simple to manufacture,[95] and combine properties of viral vectors and naked DNA because transgenes are integrated potentially life-long. Another element is that they are versatile vehicles and can deliver more oversized genetic cargoes than viruses.[108]

The problem is, as well as other non-viral vectors, that they show lower reprogramming efficiency. Also, transposons require an extra step (excision) with a transposase to eliminate the transgenes, implying the possibility that it jumps to a new place and reintegration occurs.[95, 108]

Liposomal magnetofection

It consists of liposomal complexes formed by cationic lipids. Being positively charged, they form complexes with negatively charged nucleic acids by phosphate in their structure.[112] The combination of self-assembling nucleic acids and enhancers in amalgamation with magnetic nanoparticles is concentrated at the cells' surface. This method only requires one transfection and has little immunogenicity.[95]

The utilization of liposomal systems has specific transfection difficulties in some cells. However, the magnetic field optimizes this procedure.[113]

Small molecules

To improve the limitations of other methods, such as low reprogramming efficiency and oncogenic characteristics of transcription factors, the small molecules that regulate the reprogramming controlled by these factors have been studied. This method implies epigenetic modification through histone deacetylase inhibitors (a type of enzyme that removes acetyl groups from lysine residues in histones).[114] Since histones are proteins of the chromosome structure, they are positively charged by their amino groups, favoring phosphate groups' interaction. Histone acetylation generates a positive charge to be lost, decreasing the DNA affinity and allowing gene transcription.[115] For this reason, these molecules favor the genetic reprogramming process. Some of them are valproic acid, trichostatin A sodium butyrate, suberoylanilide hydroxamic acid, and vitamin C.[86, 116]

IPSCs MODIFIED TO BE HIV RESISTANT

Once the iPSCs are obtained, modifications are made to introduce mutations that allow cells to be HIV resistant. What happened to the Berlin (he died on September 29, 2020)[117] and London patients was a precedent. The possibility of performing allogeneic transplants is latent but not entirely feasible since the mutation frequency in the population is low (there would not be a good source of these cells), and the HLA of the donor and the patient must be compatible.[110]

As a complement, the genetic modification of HSCs has been considered. Still, it would be inefficient, as it cannot compete with unmodified endogenous cells.[110]

With this, iPSCs present the convenience of being an optimal source of any patient-specific cells. They are induced from the patient's somatic cells, can be expanded for long periods, be genetically modified, and differentiated to almost any type of cell in the body.[109]

Methods have been described to genetically modify iPSCs and gather the disruption of the CCR5 gene or a modification resulting in a CCR5Δ32 mutated gene. The procedures are zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs,) and clustered regularly interspaced short palindromic repeats (CRISPR).[118]

These processes cut at specific places in the DNA. The cells' characteristics' efficiency recognizes double-strand breaks (DSBs) as damage,[118] activating two repair mechanisms:

- Homologous recombination (HR): repair system that occurs during the S phase of the cell cycle. Activation of the mutated ataxia-telangiectasia gene causes the MRN complex (consisting of the MRE11, RAD50, and NBS1 genes) to bind to the DNA. Its 5' to 3' exonuclease activity processes the damaged ends, generating a single chain with free 3' ends, which invade homologous sequences (template to restore what has been damaged).[77] The DNA polymerase extends the ends. Subsequently, the extended end is removed from the homologous sequence and is paired with the single strand that exposes a 3' end of the original break. Finally, DNA polymerase and DNA ligase exert their respective action, recovering the double-strand integrity.[118]
- Non-homologous end joining (NHEJ): requires a DNA-dependent protein kinase system. It has three subunits in charge of recognizing the cuts. The ARTEMIS/DNA-PKcs complex, with nuclease activity, and the XRCC4/ligase IV complex, responsible for linking the ends, are involved. This repair process can result in losing nucleotides, as only the cutting ends are joined.[77]

ZFNs were the first specific gene-editing technique developed. It is composed of two elements for double-strand cuts: the restriction enzyme FokI (found in the

bacterium *Flavobacterium okeanokoites*) and the zinc finger proteins.[119]

For its part, the TALEN method consists of four proteins that specifically bind to each of the DNA nitrogenous bases (guanine, adenine, cytosine, and thymine), allowing the construction of a TALE that recognizes specific sites. It also requires the enzyme FokI.[119]

Nonetheless, they are various mechanisms. TALEN recognizes single-nucleotide and ZFN trinucleotides. Furthermore, the former shows greater binding efficiency and precision because, through the other procedure, its interactions are more complicated.[120]

In contrast, CRISPR or CRISPR/Cas is based on a natural protection system for bacteria and archaea against viruses and phages. When viral genetic material is introduced, the Cas9 protein functions as a nuclease and processes the invading foreign DNA into small fragments incorporated into the CRISPR locus of host genomes as spacers. Then, in response to viruses and phage infections, the spacers participate as transcriptional templates to produce RNA that guides the Cas protein to cut target DNA sequences.[121]

Its discovery began to be utilized for cutting any DNA sequence, changing the RNA single guide.[120] Repair options are next, inserting, deleting, or substituting a DNA sequence.[121]

The technology has been used extensively for its greater efficiency over TALENs and ZFNs. Nevertheless, it has certain limitations, such as unexpected mutations in other sites and dependence on a protospacer adjacent motif (PAM). This situation is because the CRISPR/Cas9 system's cleavage specificity requires a DNA sequence protospacer that matches the RNA and the PAM. Therefore, it cannot be applied to any DNA fragment since it requires a PAM sequence of two to five nucleotides downstream immediately after the target sequence.[121]

The homozygous CCR5Δ32 mutation is known to occur naturally in less than 1 % of European-derived populations. Additionally, it is less common in other racial groups and has not been harmful clinical effects. Current studies focus on this type of genetic modification instead of inducing complete gene disruption, as it could generate adverse effects that have not yet been discovered.[110, 122]

A study demonstrated how its generation from iPSCs by PiggyBac technology combined with CRISPR/Cas9 presented better efficiency than TALENs. Furthermore, macrophages resistant to HIV were obtained from the iPSCs.[110]

ANALYTICAL METHODS TO VERIFY EFFICIENCY AND SAFETY

There is a problem in defining reliable criteria as to whether a cell has successfully reverted to pluripotency or not. Studies on the iPSC isolation are based on morphological criteria and markers.[82] These markers include:

- Nanog gene: a specific gene in charge of encoding a transcription factor that carries a homeodomain, required to maintain the undifferentiated state of stem cells.[123]
- SSEA-3 and SSEA-4: present in human ESCs and human embryonal carcinoma cells. They are downregulated as they differentiate. Both are epitopes on related glycosphingolipids (GSL) called GL-5 and GL-7. GSLs consist of a carbohydrate moiety or chain attached to ceramide.[124]
- TRA-1-60: stem cell surface marker.[125]

One way to identify iPSCs is by labeling the Fbx15 protein, expressed in large amounts by immature cells and in small quantities by mature ones. Its presence is linked to pluripotency. In addition, the Nanog protein, found in high concentrations in undifferentiated cells, has been employed. When they mature, its concentration decreases, facilitating the differentiation of pluripotency to a greater extent than the Fbx15 labeling.[84]

Regarding the analyzes, it must be shown that mutations did not occur at unexpected sites. To do this, the site's prediction where such mutations could occur is made using software such as Cas-OFFinder®. Then, some of the sites indicated by the program are evaluated with a nuclease assay[109] (technology to discover and map known and unknown mutations).[126] Another strategy is complete genome sequencing. In cell differentiation, flow cytometry can be considered to verify or analyze the collection of cells (macrophages) through antibodies against markers such as CD4, CD45, HLA-DR, and CD14.[109]

Referring to official methodologies, the United States Food and Drug Administration (FDA) published a guide in 2008 that applied for human somatic cell therapy. Manufacturing aspects are detailed, as well as the quality tests that must be executed, like microbiological (sterility, mycoplasma, adventitious agents), identity, purity (residual contaminants, bacterial pyrogenicity/endotoxins), potency, and others (safety, cell viability, cell number/dose).[127] For their procedures, the United States Pharmacopeia (USP) information can be taken as a reference.[128]

ADDITIONAL STUDIES WITH STEM CELLS FOR HIV TREATMENT

The first clinical trial of stem cell gene therapy for HIV was conducted in 1996. A tat/rev ribozyme (virus protein that regulates virus gene expression) was transduced into peripheral blood stem cells of five infected adults who had not progressed to AIDS. A control vector

(transferred resistance to neomycin) and a therapeutic vector (transferred anti-HIV ribozyme and resistance to neomycin) were tested. This procedure was done to see a selective survival advantage of cells transduced with the anti-HIV gene against a viral load.[129]

The results indicated that the transduction efficiency and gene marking in peripheral blood were low, and the number of transduced CD34+ cells infused into the individuals was small compared to the number in the bone marrow. Plus, there were no differences in cells labeled with the control gene versus those labeled with the anti-HIV gene.[129]

In another investigation, around 2006, the impact of allogeneic HSCs transplant on the peripheral blood viral reservoir was evaluated in two infected patients. Patient A was a man with perinatally acquired HIV-1 infection and diagnosed with stage IV, nodular sclerosing Hodgkin's lymphoma. Patient B was a man with sexually acquired HIV-1, with slow disease progression within 20 years of being diagnosed.[130]

Both underwent HSCs transplants during combination antiretroviral therapy. There was a significant reduction in the peripheral blood reservoir of HIV-1 after complete donor chimerism (all detected cells are from the donor) and recovery of CD4+ T lymphocyte counts. After complete chimerism, the loss of detectable HIV-1 suggested that latently infected host cells were replaced by donor cells protected against infection.[130]

Subsequently, beginning in 2006, a multicenter, phase II, randomized, double-blind, placebo-controlled clinical trial for HIV was conducted, in which participants were followed for at least 15 years. The safety and efficacy of OZ1 (specific anti-HIV ribozyme) were evaluated. The enzyme-transduced CD34+ hematopoietic progenitor cells were grafted, divided, and differentiated *in vivo* to produce a group of mature myeloid and lymphoid cells protected from productive HIV-1 replication. Seventy-four infected participants were randomized and received OZ1-transduced CD34+ cells or placebo (autologous CD34+ hematopoietic progenitor cells). The protocol included two interruptions of antiretroviral therapy to provide positive selective pressure for OZ1-protected cells.[131]

The molecule's impact on plasma viral load was assessed at the end of the second treatment interruption (primary endpoint). Besides, secondary endpoints such as the quantitative marking (presence of the gene) and the expression (active RNA form of OZ1) of the gene transfer product, the time-weighted area under the curve for viral load, the absolute number and percentage of CD4+ lymphocyte count, and the presence of proviral DNA were analyzed up to week 100. Although the primary endpoint's efficacy was not achieved, HIV-1 viral loads were consistently lower in the OZ1 group for all analyses. Furthermore, there were no adverse effects related to the therapy. This study provided the first indication that cell-delivered gene transfer is safe and biologically active in HIV patients and could be developed as a conventional therapeutic product.[131]

Finally, a clinical trial was conducted in five patients with AIDS-related lymphoma to evaluate autologous stem cell transplantation's safety and feasibility. The CD34+ HSCs and progenitor cells from the individuals were genetically modified with a lentiviral vector.[132]

Four of them achieved the preparation of gene-modified hematopoietic stem and progenitor cells (HIV-1 resistant) products for infusion. Besides, all transplant patients had detectable gene marking and expression for two years. However, the initial level of genetic marking of the more primitive HSCs was low. This research demonstrated the safety of the therapy, long-term engraftment, and sustained expression of these antiviral products, but not enough virus-resistant blood cells were found to observe clinical benefit.[132]

CONCLUSIONS

The use of iPSCs is promising against HIV. It has the advantage of being an autologous transplant therapy, unlike allogeneic ones, where the compatibility between patient and donor must be verified.

There are multiple methods for reprogramming these cells. Those of genomic integration have a higher reprogramming efficiency. Though, vectors like retrovirus have great concern of viral components reactivation in the host, inducing mutations in the insertion sites of the retrovirus, and risk of permanent modification of the iPSCs genome. In contrast, in the non-integrating ones, these hazards are reduced. Nevertheless, its reprogramming efficiency is low, and it is necessary to improve these mechanisms.

Regarding the genetic modification of iPSCs to obtain cells resistant to HIV, the studies indicate three main techniques: ZFNs, TALENs, and CRISPR/Cas9. They work by cutting DNA in certain places. Then, the DNA repair mechanisms (HR or NHEJ) commonly utilized by cells take place.

It is preferable to generate the CCR5Δ32 mutation regarding gene therapy because people have it naturally, and no harmful effects have been observed. On the contrary, total gene disruption could lead to unknown clinical effects.

Although this therapy could be an excellent opportunity to lower viral load in patients, it has its dangers. Currently, no clinical studies are employing genetically modified iPSCs as a treatment for HIV. The *in vitro* investigations show that modified iPSCs are resistant to HIV. Still, there is a possibility that the virus may infect cells with tropism to CXCR4 receptors. In this way, these investigations should continue to develop a

curative therapy that will be effective and safe against HIV in the medium term.

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