

Review

Gene Therapy Approaches to Functional Cure and Protection of Hematopoietic Potential in HIV Infection

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Abstract: Although current antiretroviral drug therapy can suppress human immunodeficiency virus (HIV) replication, a lifelong prescription is necessary to avoid viral rebound. The problem of persistent and ineradicable viral reservoirs in HIV-infected people continues to be a global threat. In addition, some HIV-infected patients do not experience sufficient T-cell immune restoration despite being aviremic during treatment, and this is likely due to altered hematopoietic potential. To achieve global eradication of HIV disease, a cure is needed. To this end, tremendous efforts have been made in the field of anti-HIV gene therapy. This review will discuss the concepts of HIV cure and relative viral attenuation and provide an overview of various gene therapy approaches aimed at a complete or functional HIV cure and protection of hematopoietic functions.

Keywords: human immunodeficiency virus; acquired immunodeficiency syndrome; hematopoietic stem/progenitor cells; gene therapy

1. Introduction

Human immunodeficiency virus (HIV) infection causes acquired immunodeficiency syndrome (AIDS). The depletion of memory CD4⁺ T cells preceding AIDS manifestation may be mainly due to the infection of these cells. However, HIV may also reduce the production of naïve T cells by infecting CD4⁺ thymocytes. On the other hand, although the dynamics of hematopoietic stem/progenitor cells (HSPCs) in HIV-infected settings is still unclear, it is well established that HIV infection is associated with hematological changes such as anemia and pancytopenia [1]. These hematological changes are likely due to modified HSPCs and hematopoietic potential of the host. Therefore, a cure for HIV disease should consider not only the absence of newly HIV-infected CD4⁺ cells but also the normal production rates of CD4⁺ T cells and other hematopoietic cells (**Figure 1**).

2. The idea of intracellular immunization

In 1988, Baltimore presented his idea of intracellular immunization by gene therapy [2], and his concepts are still valid today. First, he suggested expressing inhibitory molecules against HIV in target cells. Second, he proposed using retroviral vectors to transduce cells, although lentiviral vectors are widely used today. Third, he conceived the use of gene-modified HSPCs to replace the hosts' immune system with an HIV-resistant one. These concepts may be summarized as intracellular artificial immune systems designed against HIV and working independently from HIV-specific CD4⁺ helper T cells, the most vulnerable HIV targets [3]. Since then, a number of candidate gene therapies have been proposed and tested and are described later in this article.

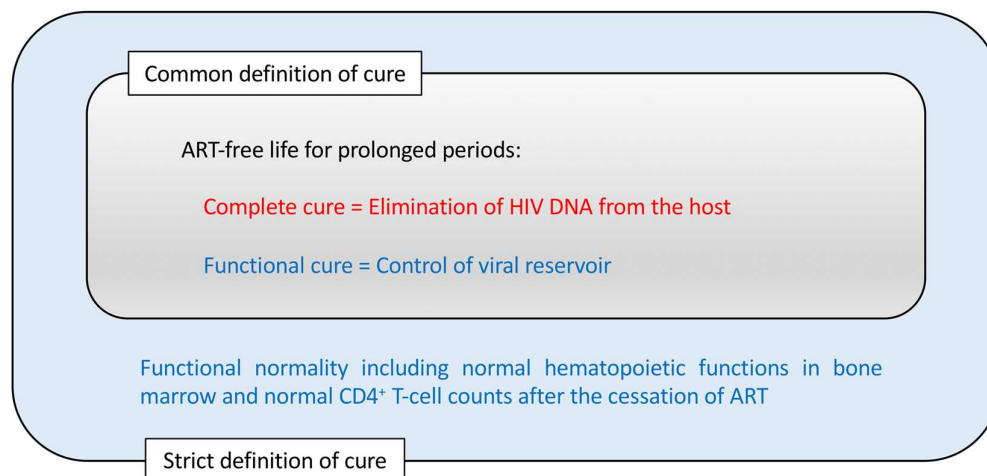


Figure 1. The concepts of human immunodeficiency virus (HIV) cure. A cure for the HIV disease is commonly interpreted as antiretroviral drug therapy (ART)-free life without viral rebound for prolonged periods. In addition, the cure of bone marrow dysfunctionalities observed in HIV-infected patients could be included in a stricter definition of HIV cure.

3. Evidence of modified CD34⁺ cell dynamics and functions in HIV infection

HIV-1 may cause the loss of primitive hematopoietic progenitors without directly infecting these cells [4]. However, HIV infection does not cause the complete loss of CD34⁺ stem cells, and it is, therefore, possible to harvest stem cells from HIV-infected patients suffering from lymphoma [5], albeit with reduced efficiencies in relation to the reduction of peripheral CD4⁺ T-cell counts [6] or reduced *in vitro* lymphopoiesis capacities [7]. The recovery of CD4⁺ T-cell counts after successful antiretroviral drug therapy treatment may depend on the recovery of CD34⁺ cell counts [8].

A number of potential mechanisms involved in the changes of CD34⁺ cells in the presence of HIV have been suggested, such as reduced expression of the proto-oncogene *c-mpl* on CD34⁺ cells [9] and elevated plasma stromal cell-derived factor 1 (SDF-1) levels [10]. HIV-1 infection causes upregulation of inflammatory cytokine production that may affect the dynamics and functions [11] or induces Fas-mediated apoptosis [12] of bone marrow CD34⁺ cells. On the other hand, HSPCs themselves may contribute to inflammation and allergies [13]. This may be partly because inflammatory signals are involved in HSPC development [14]. Recent evidence has suggested that CD34⁺CD226(DNAM-1)^{bright}CXCR4⁺ cells may represent a subset of common lymphoid progenitors associated with chronic HIV infection and inflammation, reflecting the altered dynamics of natural killer cells and α/β T cells [15].

Humanized mouse models are useful for analyzing bone marrow CD34⁺ loss or changes following HIV-1 challenge. In studies with humanized mice infected with CXCR4-tropic HIV-1_{NL4-3}, CD34⁺ hematopoietic progenitor cells were depleted and showed impaired *ex vivo* myeloid/erythroid colony forming capacities after challenge [16,17]. Reduction of bone marrow CD34⁺ cell counts after CCR5-tropic HIV-1 infection was also detected in another study [18]. Interestingly, depletion of bone marrow CD34⁺ cells following CCR5-tropic HIV infection has been reported to depend on plasmacytoid dendritic cells [19] or to be associated with the expression of CXCR4 [20]. The latter implicates a potential role of the SDF-1/CXCR4 axis in the loss of CD34⁺ cells. Another recent *in vitro* study suggested that CD34⁺CD7⁺CXCR4⁺ lymphoid progenitor cells may be depleted in the presence of CXCR4-tropic HIV-1 in the coculture of HIV-infected cord-derived CD34⁺ cells with OP9-DL1 [21].

4. The protection of bone marrow CD34⁺ cells by an anti-HIV gene therapy demonstrated *in vivo*

There have been few reports testing the protection of CD34⁺ cells after HIV infection by gene therapy. This may be because viral suppression and CD4⁺ counts have been widely accepted as measures for the effect of gene therapies against HIV. However, the true goal for any gene therapy

against HIV should be the protection of hematopoietic potential because this is another arm of the definition of AIDS, i.e., the loss of cellular immunity (**Figure 1**). We have recently reported that the transcriptional gene silencing (TGS) approach using a short hairpin (sh) RNA, called shPromA, resulted in limited CXCR4-associated depletion of bone marrow CD34⁺ cells following CCR5-tropic HIV infection in humanized mice, suggesting that anti-HIV gene therapy can support the preservation of hosts' hematopoietic potential [20].

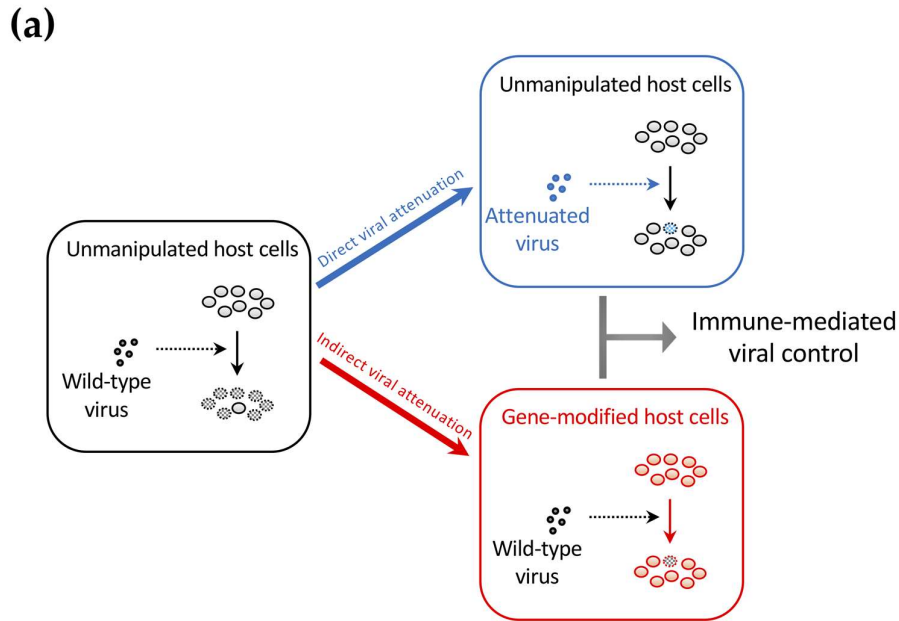
5. Complete cure vs. functional cure of HIV infection

Strategies for the treatment of HIV infection include eliminating all the HIV DNA copies within the host, termed a complete cure (**Figure 1**). In pursuing the feasibility of this goal, tremendous efforts have been made to (1) find a method to detect all the latently infected HIV DNAs in viral reservoirs, and to (2) eliminate all the detected HIV DNAs so that the host would become sterile in terms of HIV infection [22]. Among the methods to achieve this, the so-called "shock and kill" method, in which reactivation of the viral reservoir is attempted with a shock-inducing agent followed by the immune-mediated killing of the reactivated cells, has been widely investigated [23-27]. These efforts have been partly successful [22,28]. However, the difficulty of viral eradication *in vivo* is not limited to HIV; other viruses such as herpes simplex viruses, varicella-zoster virus, cytomegalovirus, and Epstein-Barr virus induce long-lasting latent infection, making them ineradicable [29]. HIV may differ from other latently infecting viruses, as viral replication from the latent reservoir can resume quickly, even if the host is not considered immunocompromized [30]. Moreover, even in the case of the so-called Berlin patient who exhibited no sign of HIV existence following allogeneic transplantation with CCR5-Δ32/Δ32 hematopoietic stem cells, a complete cure was assumed rather than being fully demonstrated [31,32].

Alternatively, some potential gene therapy methods aim at a functional cure that is evidenced by the control of HIV replication below the limit of detection and the immune system being functionally normal despite residual cells harboring HIV proviral DNAs in the host (**Figure 1**) [28,33]. This approach might be more practical than the complete cure approach, given that many successful vaccines for chronic viral infections so far exert a functional cure rather than elimination of the targeted viruses [34]. In light of this, it could be stated that for those pathogens where an effective vaccine has not been developed to date, researchers could, instead, develop gene therapies aiming at a functional cure. In this way, there is an overlap between the concept of functional-cure gene therapy and the concept of vaccines against chronic pathogens [35].

6. Connection between functional-cure gene therapies and live-attenuated vaccine approaches

Live-attenuated vaccines have been tested in macaque AIDS models using simian immunodeficiency virus (SIV) strains [36]. After the infection of a host with a live-attenuated SIV or simian-human immunodeficiency virus (SHIV), the vaccine strain is controlled by T-cell response but remains slowly replicating in the infected host. This results in further immunization of the host to prepare for subsequent superinfections of wild-type SIV or SHIV. Therefore, even if live-attenuated vaccines are powerful, they provide a functional but not a complete cure. This means that there is a scientific connection between live-attenuated vaccines and gene therapy approaches for a functional cure (**Figure 2**). In other words, anti-HIV gene therapies make CD4⁺ cells more resistant to HIV infection, and this can be interpreted as the relative attenuation of the infected virus to the gene-modified host cells (**Figure 2**). Relative viral attenuation might, thus, help host immunity to control the virus [37].



(b)

Given the following parameters:

v_0 ... Virulence of the wild type virus

v_1 ... Virulence of the live-attenuated virus

r_0 ... Viral resistance of the unmanipulated host cells

r_1 ... Viral resistance of the gene-modified host cells

“Relative viral attenuation $f(t_1)$ ” can be defined as follows, using the direct viral attenuation index (v_0/v_1) and the indirect viral attenuation index (r_1/r_0).

$$f(t_1) = \frac{v_0}{v_1} \times \frac{r_1}{r_0}$$

Direct attenuation of the virus to make a vaccine strain
Gene therapy-mediated increase in viral resistance of the host cells

Figure 2. The concept of relative viral attenuation. (a) A schema describing direct and indirect viral attenuation. HIV usually infects host CD4⁺ cells efficiently and replicates rapidly. As a result, the host immune system fails to control viral replication (left). However, accumulating evidence in macaque AIDS models suggests that a live-attenuated virus, which infects and replicates slowly because of partial defects in the viral genome, can be controlled by the immune system and help further immunization for potential superinfection with immunodeficiency virus strains that are homologous to the vaccine strain (upper right). The live attenuation method cannot be directly applied to HIV infection in humans because of safety concerns. However, indirect viral attenuation can be achieved by rendering the host cells HIV-resistant by an “intracellular immunization” gene therapy (lower right). (b) The definition of relative viral attenuation. This idea can connect live-attenuated vaccine studies and gene therapy approaches to achieve a functional cure.

7. Gene therapy strategies against HIV

The so-called Berlin patient, an HIV-positive male United States citizen who was diagnosed with HIV while attending university in Berlin and later suffered from acute myelogenous leukemia, received a transplantation of allogeneic hematopoietic stem cells homozygous for CCR5- Δ 32. This resulted in a subsequent functional HIV cure [31,32]. Because CCR5 is critical in HIV infection and transmission, as observed with CCR5- Δ 32 homozygous cells resistant to HIV infection [38], the manipulation of CCR5 expression on HIV target cells has been intensively investigated and is considered effective [39-61]. CCR5 can be targeted by zinc finger nucleases [62,63], ribozymes [64], CRISPR/Cas9 methods [65], transcription activator-like effector nucleases [65], and shRNAs [42,66,67]. Among these, several gene therapy methods, including one using lentiviral vector LVsh5/C46, expressing shRNA against CCR5, and HIV-1 entry inhibitor C46 [68] have been tested in clinical trials [51]. While CCR5 is involved in numerous pathologic states including inflammatory and infectious diseases [69], a complete knockout of CCR5 can be related to an increased sensitivity to some viral infections [70,71]. Therefore, CCR5 gene editing should only be considered for HIV cure [69].

The targeting of HIV RNA sequences by ribozymes or RNAs [58,72-83] and HIV DNA sequences by the CRISPR/Cas9 system [84] have been investigated and are also considered major strategies [59,85]. The latter method has recently been of great interest especially, primarily because of its potential for targeting and disrupting integrated HIV DNA sequences to achieve a complete cure. A recent study targeted and inactivated the HIV-1 long terminal repeat (LTR) U3 region *in vitro* by Cas9 and guide RNAs (gRNAs), and no off-target gene editing to the host cells was detected [86]. Another study also successfully targeted the HIV-1 LTR U3 region using the CRISPR/Cas9 system. However, this study also detected the emergence of escape variant viruses mediated by the host cells' error-prone non-homologous end joining (NHEJ) DNA repair following the CRISPR/Cas9 targeting [87]. The mutagenesis problem with CRISPR/Cas9 has also been observed in treatments of other diseases [88] but can be a serious problem when targeting the HIV DNAs because the strategy might require sustained expression of Cas9 and gRNA in the potential HIV target cells, meaning a sustained risk of mutagenesis [89]. Therefore, an improved method for disrupting HIV DNA while prohibiting the emergence of replication-competent escape variants caused by the NHEJ repair system might be necessary. Nevertheless, radical approaches can still be tested in cultured cells and animals. For example, a recent study demonstrated that *in vivo* gene delivery of multiplex single-gRNAs plus *Staphylococcus aureus* Cas9 to transgenic mice bearing HIV DNA using an adeno-associated virus (AAV) vector resulted in an efficient excision of HIV DNA in various tissues and organs [90]. If safety concerns are met, such a gene-delivery method can be a powerful tool to achieve systemic elimination of latent viral reservoirs in hematopoietic cells as well as nonhematopoietic cells such as astrocytes [91]. Wang et al. (2018) have written a thorough review of topics regarding the targeting of HIV DNA by CRISPR/Cas9 [84].

There is yet another strategy: the silencing approach, which aims to reduce the production rate of HIV viral particles per integrated HIV DNA copy [28]. Lentiviral gene delivery enables RNA-based gene silencing, including the previously characterized small interfering RNA (siRNA) called PromA [92,93]. PromA is a short RNA sequence specific for the two NF- κ B binding sites in the HIV LTR U3 region. While specific mRNA cleavage by post-transcriptional gene silencing is the best-known mechanism for siRNAs, PromA triggers TGS mediated by epigenetic changes such as DNA methylation and heterochromatin formation [76,94]. In fact, PromA has been shown to induce chromatin compaction in the HIV-1 promoter region [94]. This means that, in contrast to methods attempting to eradicate HIV DNA, PromA locks and stabilizes latently infecting HIV provirus and prevents viral reservoirs from reactivation induced by stimuli such as tissue necrosis factor (**Figure 3A**) [95-97]. The efficacy of PromA in suppressing HIV-1 replication *in vivo* was first demonstrated by an HIV challenge study using humanized NOD/SCID/JAK3^{null} (NOJ) mice transplanted with human peripheral mononuclear cells expressing shPromA [96]. Our recent study to extend the results using NOJ mice engrafted with shPromA-transduced CD34⁺ cells and their derivatives

further demonstrated that PromA could be an effective gene therapy for protecting bone marrow CD34⁺ cells and the hematopoietic potential of the host from HIV infection (**Figure 3B**) [20,98].

Other potential gene therapy methods include the secretion of soluble HIV entry inhibitors [99]; rescue of hematopoiesis including myelopoiesis, erythropoiesis, and megakaryopoiesis using *c-mpl* [100]; expression of a chimeric human-simian TRIM5 α [101]; expression of p68 kinase [102]; and expression of HIV Gag mutants [103].

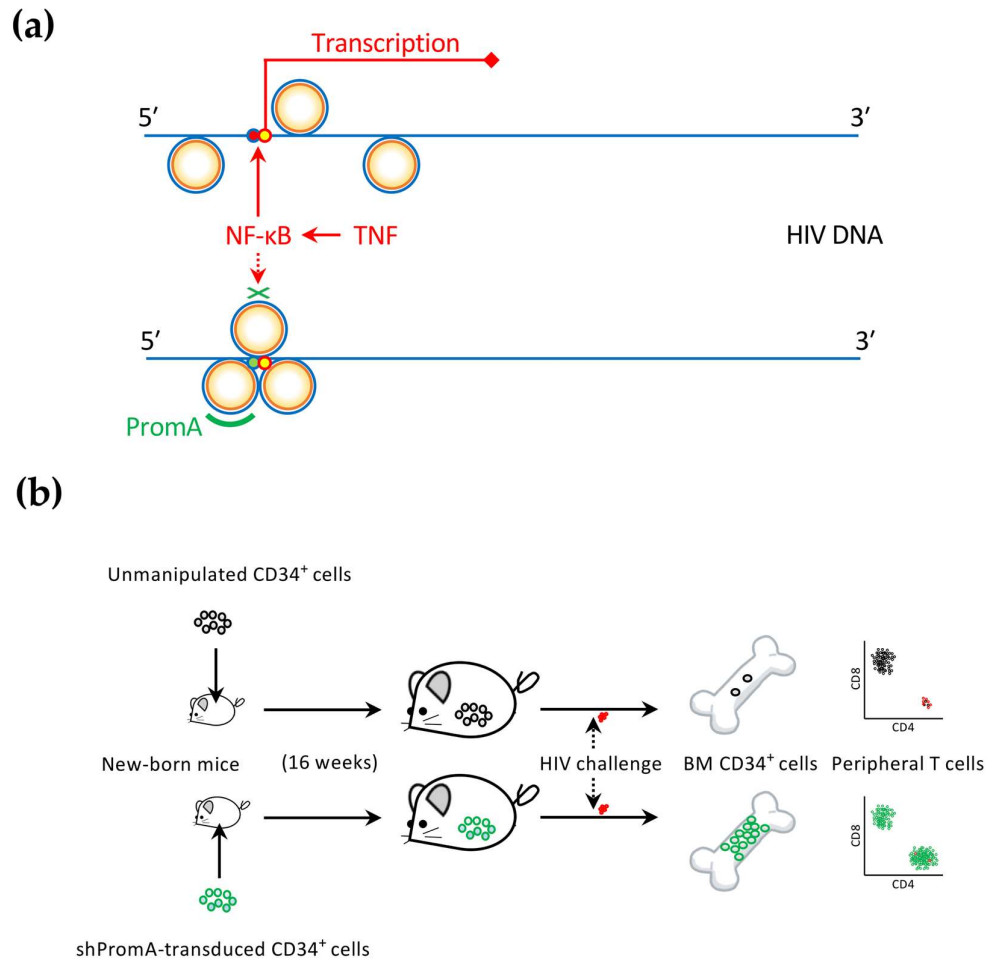


Figure 3. A schematic overview of PromA. (a) PromA induces chromatin compaction in the human immunodeficiency virus (HIV)-1 promoter. This prevents HIV-1 DNA from reactivation such as NF- κ B-mediated reactivation by tissue necrosis factor (TNF). For details on the molecular mechanisms involved in transcriptional gene silencing induced by PromA, see Klemm et al., 2016 [92] and Mendez et al., 2018 [97]. (b) Summary of the humanized mouse study to test the efficacy of shRNA PromA (shPromA) [20,98]. Newborn NOD/SCID/Jak3^{null} mice were intrahepatically transfused with unmanipulated cord-derived CD34⁺ cells or CD34⁺ cells lentivirally transduced with shPromA. Those mice showing engraftment of human cells were challenged with CCR5-tropic HIV-1_{JRFL}. Two weeks after challenge, the mice were sacrificed, and their bone marrow (BM) CD34⁺ cells and peripheral T cells were analyzed. Interestingly, mice transplanted with unmanipulated CD34⁺ cells showed unexpectedly low BM CD34⁺ cell counts 2 weeks after HIV infection, with concomitant depletion of peripheral CD4⁺ T-cells. On the other hand, mice engrafted with shPromA-expressing CD34⁺ cells showed preserved BM CD34⁺ cell and peripheral CD4⁺ T-cell populations at 2-weeks post challenge.

8. Target cells for anti-HIV gene therapies

Recent studies indicate that ideal anti-HIV gene therapy targets should be hematopoietic stem cells rather than more differentiated cells such as peripheral CD4⁺ T cells because the transduced cells could engraft the host bone marrow and act as a lifelong source of HIV-resistant CD4⁺ cells [104,105]. Potential gene therapies using CD34⁺ cells have been investigated *in vitro* using cell culture experiments or *in vivo* using humanized mice. Furthermore, transplantation of macaques with gene-modified autologous CD34⁺ cells followed by infection with SIV has also been tested [106,107], although strategies may differ between gene therapies [105]. Based on such basic study results, clinical trials using transplantation of retrovirally or lentivirally gene-modified CD34⁺ cells in HIV-positive patients have been carried out [73,108,109]. Gene therapies of CD34⁺ cells have been considered as a cure for monogenic immune diseases. For example, patients with adenosine deaminase deficiency [110], Wiskott–Aldrich syndrome (WAS) [111], and X-linked severe combined immunodeficiency [112,113] were successfully treated in clinical trials by transplantation of autologous CD34⁺ cells retrovirally or lentivirally transduced with the wild-type gene. Lentiviral vectors may be more efficient in gene transfer into resting stem cells at the G0/G1 phase compared with murine retroviral vectors [114]. Both retroviral and lentiviral vectors, if applied to gene therapy of HSPCs, could have adverse effects including deregulation of gene expression [115] and triggering p53 [116]. However, lentiviral vectors may be safer than retroviral vectors because the latter may occasionally cause insertional mutagenesis near active start regions of genes, possibly leading to oncogenesis and cancers such as leukemias [112]. Self-inactivating retroviral or lentiviral vectors lacking the U3 region of 3' LTRs have further safety advantages [117]. Moreover, recent evidence has shown that transplantation of WAS patients with autologous CD34⁺ cells transduced with lentiviral vectors encoding WAS protein results in long-term survival of genetically engineered hematopoietic stem cells and lymphoid-committed progenitors [118], giving hope for lifelong protection from HIV.

Induced pluripotent stem cells (iPSCs) may also be candidates for anti-HIV gene transfer. iPSCs can be generated from the patients' somatic cells, differentiate to any cells *in vitro*, and are expected to be utilized for the treatment of a broad range of genetic diseases [119-122]. While CD34⁺ cells engraft in the bone marrow following transplantation and differentiate to hematopoietic cells *in vivo*, iPSCs may be more convenient for *in vitro* hematopoiesis than CD34⁺ cells because of their ease of culture [123]. Interestingly, the impact of shPromA-transduced iPSCs on the suppression of viral replication *in vitro* has recently been demonstrated, suggesting that the large-scale production of gene-modified monocytes or lymphocytes *in vitro* for adoptive therapy could be a future option [124]. Additionally, generation of iPSCs from HIV epitope-specific CD8⁺ cytotoxic T cells followed by their re-differentiation to the identical epitope-specific CD8⁺ T cells for adoptive transfer could be an effective immunotherapy [125].

9. Application of gene therapy methods to immunotherapies

Immunotherapy approaches based on gene therapy methods have been extensively investigated. Chimeric antigen receptor (CAR) T-cells are engineered T cells expressing CARs for the recognition and killing of target cells [126]. Most typical CARs are engineered to recognize an antigen with a monoclonal antibody-derived extracellular domain conjugated to T-cell receptor-derived transmembrane and intracellular domains. Therefore, despite the use of T-cell signaling pathways, such CAR T-cell therapies might be regarded as an enhancement of antibody-based therapies [127,128]. To date, most successful CAR T-cell therapies have been against cancers [129]. For example, the high efficacy of the adoptive transfer of CAR T-cells recognizing CD19 has been demonstrated for the treatment of patients with B-cell acute lymphoblastic leukemia [130] and diffuse large B-cell lymphoma [131]. In contrast, CAR T-cell therapies may require manufacturing autologous CAR T cells for each patient and, thus, are not yet widely available [131]. Several broadly neutralizing antibodies have been considered for generating CAR T cells against HIV infection. Despite the shared concern of escape mutations with antibody-based therapies, CAR T cells are MHC-independent and more potent than neutralizing antibody administration, so better

outcomes can be expected. A further improvement of HIV CAR gene therapy has been tested to make CAR T cells HIV-resistant by insertion of the HIV CAR gene expression cassette into the CCR5 locus, resulting in the disruption of CCR5 [132]. Finally, an adoptive transfer therapy using autologous CD34⁺ cells transduced with lentivirus expressing a CD4-based CAR able to bind the HIV envelope protein has been tested in pigtail macaques infected with a SHIV [133].

Another exciting gene transfer-based immunotherapy is programming the production of specific anti-HIV antibodies [134,135]. Compared with vaccination, passive immunization using a set of broadly neutralizing antibodies is customizable, MHC-independent, and provides instant and reliable protection against HIV [136,137]. However, neutralizing antibodies need repeated administration to provide prolonged protection [137]. Thus, the concept of antibody gene transfer is to overcome the limitation of passive immunization, which is only transiently effective [138]. It was demonstrated in a humanized mouse model that antibody gene transfer by intramuscular inoculation of an AAV vector encoding full-length antibody was able to induce production of the antibody by muscle cells and confer protection against intravenous HIV-1 challenge [139]. In another study, an adenovirus serotype 5 (Ad5) vector encoding an HIV-1-specific broadly neutralizing antibody PGT121 (Ad5.PGT121) afforded a more rapid and robust antibody response than an AAV encoding PGT121 (AAV1.PGT121) in HIV-1-infected bone marrow-liver-thymus humanized mice [140].

10. Biosafety and bioethics concerns regarding the application of anti-HIV gene therapies to human germline cells for pregnancy

In late 2018, it was reported that a Chinese researcher gave birth to two children bearing CRISPR/Cas9-mediated CCR5 double knockouts to confer HIV resistance [141]. However, inheritable gene modification of human germline cells culminating in human pregnancy is currently unacceptable [142]. CRISPR/Cas9, if applied to germline cells, could cause additional inheritable mutations to the host genome DNA [88,143-145], and the influence of this is not entirely predictable at this moment. Therefore, such investigation on human germline cells should be limited to nonclinical (i.e., *in vitro*) studies. Regarding the targeted gene, it should be emphasized that CCR5 knockout has not been proven safe. Even if a small population of people, mostly of Caucasian origin, is living without functional CCR5 alleles, this does not mean that the loss of CCR5 is universally harmless. This is partly because CCR5 has been reported to play important roles in some viral infections [70,71]. Moreover, there is an unexcluded possibility that the lack of CCR5 function among those carrying the CCR5-Δ32/Δ32 double mutations could be compensated by accompanying genetic variations that do not exist in the majority of human populations having the wild-type CCR5 alleles. We refer to the statement published in 2017 by an American Society of Human Genetics workgroup regarding human germline genome editing [146].

11. Conclusions

Evidence suggests that HIV infection alters the bone marrow hematopoietic potential of the host. This can lead to impaired CD4⁺ T-cell generation and contributes to the loss of peripheral CD4⁺ T cells and the manifestation of AIDS. Further investigations on the topics discussed in this review will collectively enhance our understanding of the important role that HIV gene therapy can contribute toward an HIV cure. Intracellular immunization gene therapies, including silencing approaches, are expected to confer relative viral attenuation without interfering with the HIV genome and assist cellular immunity to kill HIV-infected cells, leading to better viral control and a functional cure. In light of this, long-term preservation of bone marrow CD34⁺ cells and hematopoietic potential, in addition to aviremic states and restored peripheral CD4⁺ T-cell counts, may be an appropriate endpoint of future anti-HIV gene therapies. Furthermore, recent advances in gene therapy-based immunotherapy approaches against HIV have also been described in this review.

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