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# Mechanisms for Controlling HIV-1 Infection: A Gene Therapy Approach

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Additional information is available at the end of the chapter

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## Abstract

Current anti-retroviral treatment (ART) for HIV-1 is highly effectively at controlling the infection. However, during early infection the virus establishes a latent reservoir, which is not impacted by ART. Any treatment interruption rapidly results in virus rebound from the latent reservoir to pre-therapy levels and thus ART does not constitute an HIV-1 cure. Alternate treatments are currently being explored in the form of gene therapy, following the success of the Berlin patient who has had undetectable virus since 2007. This review will describe the contrasting cure approaches that are currently the focus of multiple studies to control HIV, then focus in on functional cure gene therapy strategies; specifically, epigenetic silencing of HIV-1 by various methods, including RNA-directed transcriptional gene silencing. The various delivery strategies for targeting cells of the latent reservoir will be reviewed and finally, the clinical status and current challenges for *ex vivo* versus *in vivo* gene therapy delivery approaches will be discussed.

**Keywords:** HIV, functional cure, “block and lock”, epigenetic silencing, si/shRNA, latent reservoir, gene therapy

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## 1. Introduction

HIV-1 was first identified in the 1980s and currently infects ~37 million people world-wide [1]. There has been a concerted global effort in strategic planning for HIV prevention, with the current goals in multiple countries for the end of 2020 being to significantly reduce or virtually eliminate new infections. This goal is based on the increased uptake and availability

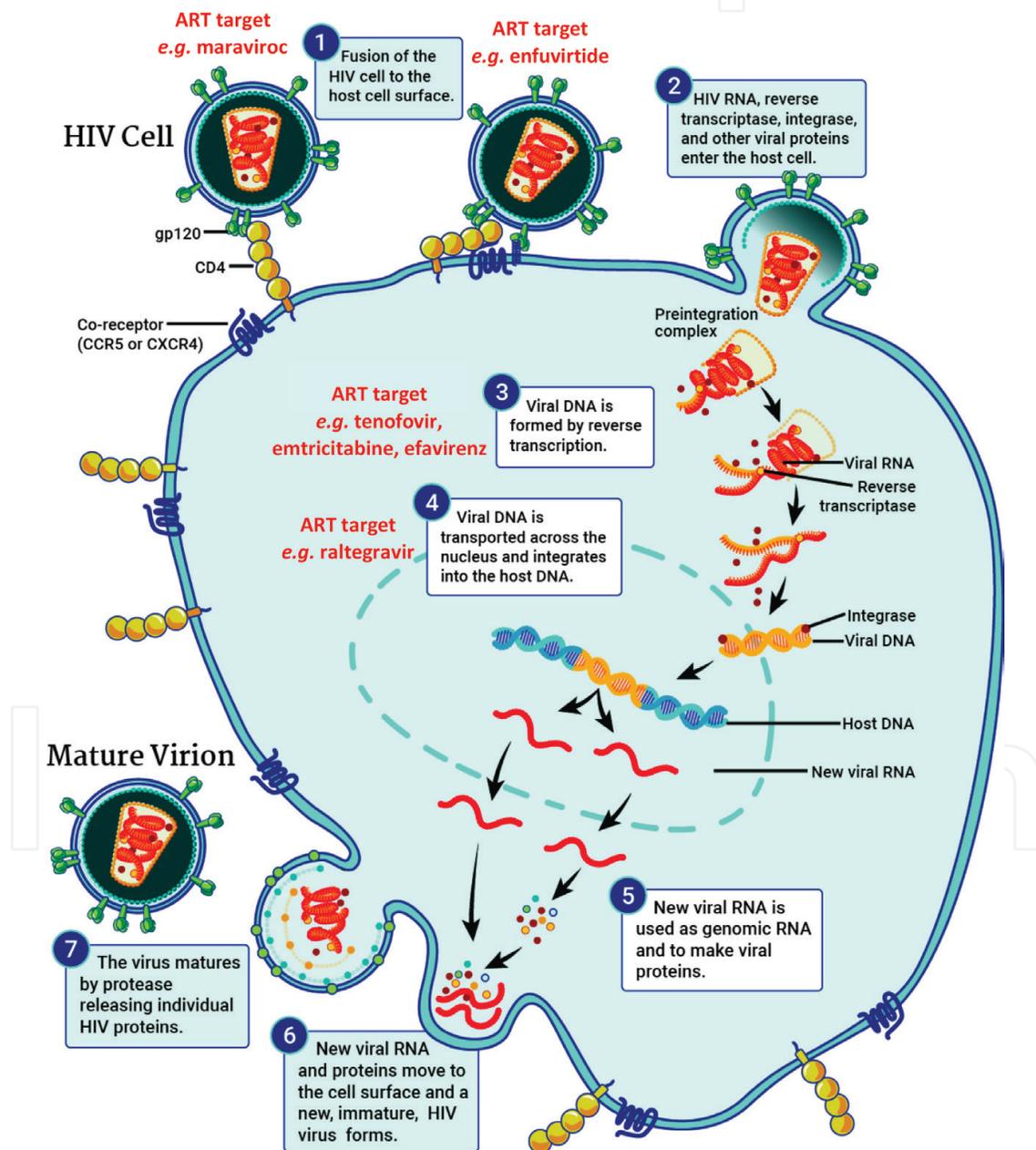
of anti-retroviral therapy (ART) and the more recent pre-exposure prophylaxis (PrEP), which has been embraced by many countries, particularly the US, UK and Australia [2], and demonstrated in landmark studies to prevent transmission by 99% [3, 4]. Additionally, the recent OPPOSITES ATTRACT study [5, 6] and PARTNER study [7] both showed that ART effectively reduces that rate of transmission to zero in homosexual male serodiscordant couples.

However, while ART is highly effectively at controlling HIV-1 infection, it does not impact the latent reservoir, which is established early during virus infection. This allows the latent virus to recrudescence following any treatment interruption and results in rapid virus rebound to pre-therapy levels. Therefore, ART does not constitute an HIV-1 cure. As is the case for many other diseases, gene therapy is being explored as an alternate HIV-1 treatment. There are, however, challenges specific to HIV, that may not arise in gene therapy approaches for other diseases. In the context of HIV, the latent reservoir represents a major barrier for developing an HIV cure, since ART has no effect on the integrated provirus and there is currently lack of a biomarker to identify cells that carry latent virus. Combined with the inability to identify latent cells and the rare frequency of latently-infected cells (~1 in  $10^6$  cells) these hurdles make the development of a gene delivery platform uniquely challenging, especially in terms of a systemic *in vivo* approach. Another difficulty is that not all integrated provirus is the same, with some being full-length intact genomes and others being defective genomes that carry large deletions and will not result in productive infection following reactivation [8]. These are the barriers that must be addressed to ensure a gene therapy approach to HIV translates into patient outcomes. The unique challenges facing HIV-1 gene therapy and current solutions are described in this chapter.

## 2. HIV-1 life cycle: challenges for an HIV-1 cure

HIV-1 can infect a wide range of cells, predominantly targeting CD4<sup>+</sup> T cells, dendritic cells, macrophages and other myeloid lineage cells [9]. This is achieved by binding of the viral envelope glycoprotein gp120 to the CD4 receptor, triggering a conformational change that allows for CCR5 or CXCR4 coreceptor binding. Further conformational changes in gp41 initiate a membrane fusion reaction that allows the viral capsid cytoplasmic entry [10]. Upon entry, the capsid disassembles to release viral RNA and proteins. To protect this genetic material, host restriction factors are subverted. With the aid of commandeered host cell machinery, a reverse transcription complex is formed and complementary viral DNA (cDNA) generated. The resulting pre-integration complex is transported via cytoskeletal manipulation to a nuclear pore complex where it is actively imported. The ability to traverse the nuclear membrane allows HIV to productively infect non-dividing cells. Viral integrase then facilitates the integration of viral DNA into the host genome to form the provirus [11]. Thus, HIV tethers its survival to the longevity of the cell and establishes the latent provirus reservoir, from which virus can reactivate. Using host replication machinery, viral RNA is then transcribed and exported to the cytoplasm. Proteins required for infectivity are synthesised and trafficked to the plasma membrane. Along with two RNA copies of the HIV genome, these proteins are assembled and packaged into immature virions in a process mediated by the viral Gag polyprotein. Once released from the plasma membrane, viral protease cleaves Gag into three structural proteins to create the mature infectious virion [12]. The HIV-1 life cycle is summarised in **Figure 1**.

While the above cycle is the most common model of HIV-1 infection, it is not exclusive. Numerous alternate pathways at varying stages of infection have been observed. For example, rather than the release of free HIV-1 virions, cell-to-cell transmission via infectious synapses can occur [13]. This modification is a more efficient means of infectivity [14]. Additionally, the above model does not account for infection of the central nervous system [15], in particular cells such as astrocytes, which lack the CD4 receptor [16]. While some of the proposed pathways remain controversial, HIV-1 is undeniably a versatile virus capable of hijacking diverse systems. The infectious route it takes may depend on the cell type, its available resources and activation status.



**Figure 1.** HIV-1 life cycle and stages targeted by antiretroviral therapy (ART). ART drugs target various stages of the HIV life cycle, with some common drugs shown. Credit: National Institute of Allergy and Infectious Diseases (NIAID).

## 2.1. The Life cycle and ART treatment

Due to the inherent sequence variability of HIV-1 and the ability for virus resistance to arise, multiple stages in the virus life cycle need to be targeted by ART to control the infection. As illustrated in **Figure 1**, stages that are targeted can include binding, fusion, reverse transcription and integration, among others, with either one or more ART drugs from each stage being utilised to provide sufficient HIV-1 control. An example of one such drug combination is TRUVADA<sup>®</sup>, which combines two drugs targeting the reverse transcription stage, emtricitabine and tenofovir and has been widely embraced for PrEP treatment.

## 2.2. The latent reservoir

Although the versatility of HIV-1 presents a challenge for the development of therapeutics, by far, the latent cellular reservoirs are the greatest barrier to developing a cure. The difficulties in controlling these virus reservoirs arise when the infection becomes latent. While the exact mechanisms of HIV latency are still being precisely defined, studies have demonstrated epigenetic regulation is involved in suppressing virus transcription, with the presence of classic epigenetic repressive marks, including methylation (*i.e.* histone 3 lysine 27 trimethylation (H3K27me3)) and deacetylation (*i.e.* Histone deacetylase 1 (HDAC1)) on N-terminal histone tails inducing specific epigenetic chromatin compaction, termed heterochromatin [17].

The latent reservoir resides in resting memory CD4<sup>+</sup> T cells [18, 19], such as central memory, effector memory and transitional memory cells [8], T follicular helper cells [20], macrophages and other myeloid cells, as well as in immune privileged sanctuary sites, such as the gut [21], lymph nodes and associated germinal centers and the brain. Physiologically, in their resting states CD4<sup>+</sup> T cells have low endocytic and metabolic rates that are sufficient for maintenance of housekeeping functions [22]. As such, they are not impacted by ART. To retain dormancy, they negatively regulate gene activation via inhibition of cellular transcription factors, such as NF- $\kappa$ B and NFAT [23]: host factors essential for initiating active HIV-1 virus production [24–26]. Consequently, the integrated provirus is reversibly silenced by epigenetic repression and evades host immune detection. This presents a clinically daunting prospect. As memory cells are long lived, with ART controlling the infection, not only would eradication of the reservoir take over 70 years [18], but theoretically, one infected cell could sufficiently sustain a life-long infection. Further, upon reactivation of the infected cell/s by any stimuli, virus recrudescence rapidly occurs and thus while ART can effectively control HIV-1 infection, it does not represent a cure.

## 3. HIV-1 cure strategies

Various approaches to overcome the viral reservoir barrier are being pursued. Primarily, they can be separated into two main categories: sterilising and functional. Both approaches aim for an undetectable viral load without the need for ART, with the sterilising approach being defined as complete eradication of the virus, and conversely, the functional approach is defined as controlling the virus reservoir without its eradication.

### 3.1. Shock and kill

The most studied sterilising approach is aptly named “shock and kill”. This concept explores the use of *latency reversing agents* (LRAs) to “shock” the virus into reactivation, whereby it is detected and “killed” by either its own cytotoxicity or the host immune system. To date, this purging strategy has been largely unsuccessful. Proposed agents either induce global immune reactivation, leading to increased pro-inflammatory cytokines and severe side effects, or *in vitro* efficacy has simply failed to translate *in vivo* [27–32]. Extensive descriptions of LRAs and the next step of optimising the “kill” step is reviewed by Kim et al., [33] and are not the focus of this chapter. However, one recent study has demonstrated a specific kill response mediated by ricin A, which is initially encapsulated in a novel nanocapsule polymer shell and then activated and released via the ricin A peptide crosslinkers being cleaved by HIV-1 protease [34]. Some of the LRAs under investigation are summarised in **Table 1** and a schematic of the process is shown in **Figure 2**.

### 3.2. Block and lock

The limited success of sterilising cures has shifted the priority to identifying a functional cure; which is now seen as a more realistic approach to controlling the viral reservoir, without ART. The functional cure approach is termed “block and lock”. This concept exploits the use of *latency inducing agents* (LIAs) to “block” virus gene transcription at the promoter via epigenetic mechanisms and “lock” the integrated virus genome in a permanent super-latent state, which resembles the natural latent reservoir. We and others are pursuing this approach to provide a sustained virus remission, without ART. Some of the LIAs under investigation are summarised in **Table 1** and a schematic of the process is shown in **Figure 2**.

#### 3.2.1. Tat-inhibitor didehydro-cortistatin A (dCA)

In 2012, the Valente group reported a novel small molecule inhibitor of HIV transcription, termed Didehydro-cortistatin A (dCA) [35]. Derived from a marine sponge, dCA inhibits Tat-mediated transactivation of integrated HIV provirus via disrupting binding of the transactivation response (TAR) element to Tat through direct binding competition with the RNA hairpin TAR-binding domain of Tat. Effectively disrupting the Tat/TAR complex, dCA induced prolonged suppression of virus transcription in HeLa-CD4 cells infected with HIV-1<sub>NL4.3</sub> for 2 months with constant treatment and out to a further 27 days following cessation of treatment, as determined by measuring viral RNA levels. The inhibitory effect of dCA on HIV replication *ex vivo* in primary CD4+ T cells isolated from eight HIV-1 infected individuals on suppressive ART was ~60% compared to no treatment, while ART treatment alone reduced viral production to ~40% and dCA treatment alone reduced virus production to ~25% [35]. The study reported there were no apparent cytotoxic effects in cell culture models or when assessed in C57BI-6 mice at the concentrations used. Further optimisation of dCA will be required if the treatment is to be effective in the absence of ART, as desired for a functional HIV cure, and to increase the longevity of the suppressive effect.

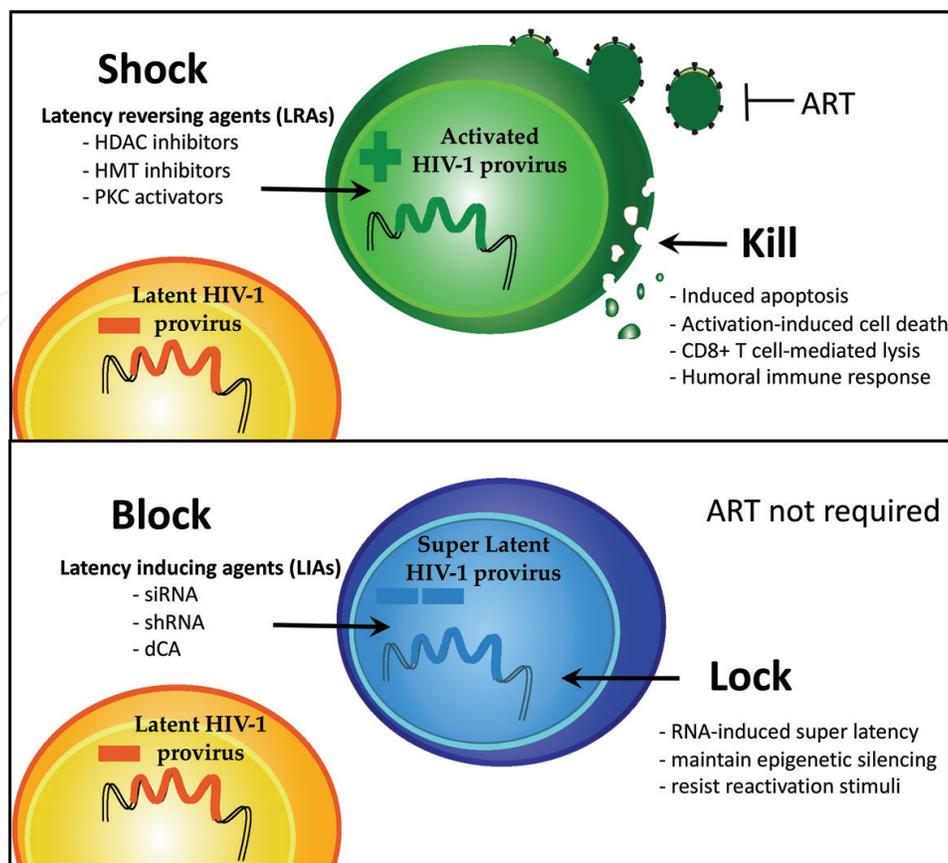
Latency reversing agents (LRAs)	
Class	Agent
Epigenetic Modifiers	Histone deacetylase inhibitors (HDACs)
	Methylation inhibitors
	Methyl-transferase inhibitors
	Bromodomain inhibitors
	P-TEFb activators
Protein Kinase C Agonists	Prostratin/Bryostatins
	Ingelol B/PEP0005
PI3K/Akt pathway	Disulfiram
	STAT5 signalling benzotriazole
	mTOR complex Rapamycin
TCR Activators	Immune checkpoint blockers
Cytokines	IL-15
TLR Agonists	TLR7
	TLR9
Latency inducing agents (LIAs)	
Class	Agent
Epigenetic Modifiers	Didehydro-cortistatin A (dCA)
	siRNA
	shRNA
	Ubiquitin-like, containing PHD and RING finger domains 1 protein (UHRF1)
	Nullbasic
HIV Integrase inhibitors	LEDGINs

P-TEFb, positive transcription elongation factor b; TLR, Toll-like receptor; mTOR, mechanistic target of rapamycin; STAT5, signal transducer and activator of transcription 5; IL-15, interleukin-15; dCA, Didehydro-cortistatin A; UHRF1, Ubiquitin-like, containing PHD and RING finger domains 1 protein.

**Table 1.** Agents that modulate HIV latency.

### 3.2.2. RNA silencing

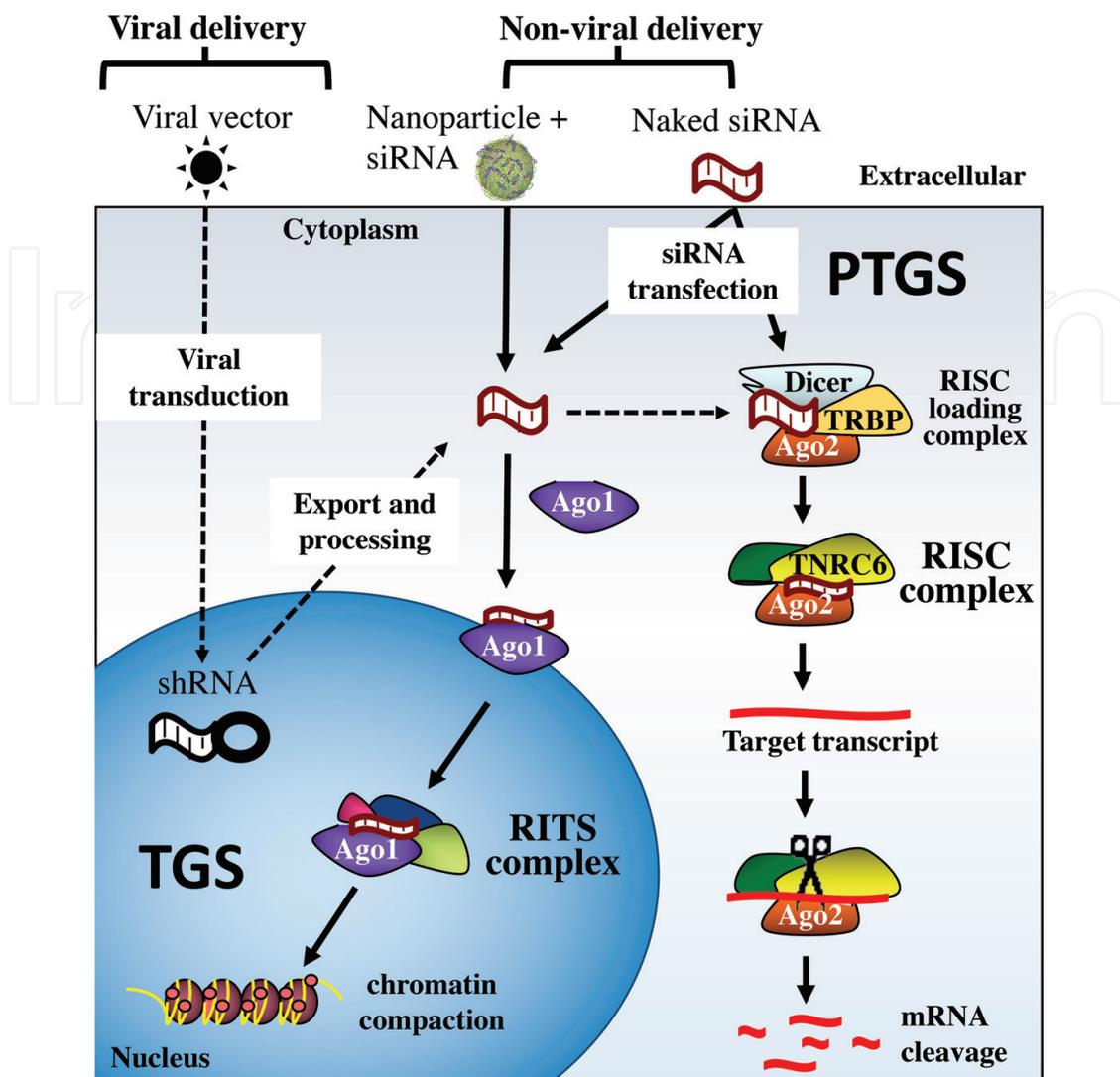
RNA interference (RNAi) is a fundamentally conserved process crucial for viral defence and the regulation of normal gene expression. Since its initial discovery in transgenic tobacco plants [36], the RNAi field has erupted with literature exploring the depth of its possibilities. From a tool to study basic gene functions, to a remedy for previously untreatable conditions, RNAi has the potential to revolutionise research and medicine. As a result, it has been extensively studied and characterised in a wide array of organisms, particularly plants (*Arabidopsis thaliana* [37, 38]), the nematode *Caenorhabditis elegans* [39] and fission yeast *Schizosaccharomyces pombe* (*S. pombe*) [40]. Two distinct pathways have since emerged: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS); also termed epigenetic silencing (refer to **Figure 3** for an overview).



**Figure 2.** “Shock and kill” versus “Block and Lock” HIV cure strategies. Latency reversing agents (LRAs) activate or “shock” the HIV-1 provirus awake with the aim that the infected cell will then undergo “kill” via cytotoxic or host immune mechanisms. Latency inducing agents (LIAs) mediate the opposite effect, by “blocking” virus replication and “locking” the virus genome in an induced “super-latency” that is refractory to reactivation. HDAC, histone deacetylase; HMT, histone methyl transferase; PKC, protein kinase C; dCA, didehydro-cortistatin A; ART, antiretroviral therapy.

PTGS was the first distinct pathway to be discovered in 1998 by Fire and Mello [39]. It can be considered the predominantly cytoplasmic arm of RNAi. Briefly, short interfering RNAs (siRNAs) 19–23 base pairs long are processed by the Dicer endonuclease and loaded onto the Argonaute 2 (Ago2) protein. Several of other proteins are recruited to form the RNA-induced silencing complex (RISC). This complex uses the siRNA as complementary anti-sense guides to identify target mRNA in the cytoplasm that are subsequently cleaved and degraded by the catalytic activity of Ago2 [41]. As a result, target gene expression is transiently downregulated prior to translation. Exhibiting enormous potential, PTGS has since been developed as a tool to selectively inhibit the expression of critical HIV-1 viral proteins *in vitro*, leading to significant reductions in viral replication [42–46]. The sequence specific nature of the PTGS process suggests there are minimal off-target effects [47], although these can still occur. Some reports have shown that introducing exogenous siRNA less than 24 base pairs in length does not trigger an interferon mediated immune response or defence mechanism to the synthetic material [48].

While these are all highly desirable traits for future gene therapeutics, PTGS has several limitations, particularly in the context of HIV-1, which leave it unsuitable for gene therapy. A consequence of its high specificity is that a nucleotide mismatches between the siRNA and its target could be sufficient to abrogate silencing [49]. Hence, in the context of a highly diverse virus with multiple subtypes, a conserved target sequence must be carefully selected. Due



**Figure 3.** Gene silencing pathways mediated by siRNA. siRNA duplexes induce posttranscriptional gene silencing (PTGS) predominately in the cytoplasm through RISC machinery initiating mRNA degradation, and transcriptional gene silencing (TGS) in the nucleus through the RITS complex inducing chromatin compaction to silence gene expression. Ago1, Argonaute 1; Ago2, Argonaute 2; shRNA, short hairpin RNA; RISC, RNA induced silencing complex; RITS, RNA-induced transcriptional silencing complex; TRBP, trans-activating response RNA-binding protein; TNRC6, trinucleotide repeat containing six proteins.

to the enormous diversity of the genome, a multiplexed approach would be necessary to provide adequate coverage across HIV subtypes and strains. Additionally, as its silencing effects are only transient, PTGS would require either (1) frequent siRNA administration to an infected individual, thus rendering it a recurring treatment and not a cure, or (2) provision of a self-sustaining system. This would involve viral vector delivery of a plasmid that can constitutively express target siRNAs. This may be in the form of short hairpin (sh)RNAs that are exported to the cytoplasm and processed into the desired siRNA. The greatest pitfall however, is that PTGS predominately functions in the cytoplasm, targeting mRNA from an actively transcribing provirus. As such, it cannot overcome the selective pressures driving HIV-1 mutation. Rather, this process allows the virus to rapidly transcribe escape variants [50]. From point mutations and deletions that disrupt target specificity, to the generation of alternative secondary structures to prevent RISC accessibility [51], HIV-1 can rapidly

circumvent a single PTGS therapeutic. Similar to ART, current PTGS therapeutics are having to be combined, to simultaneously target multiple HIV proteins and/or to target host targets, such as CCR5, to overcome the generation of resistance mutations [52].

### 3.2.2.1. RNA-directed latency inducing agents

In comparison, TGS can be considered the nucleic arm of RNAi. It offers the highly specific targeting of the integrated HIV provirus. Still controversial in mammals, this pathway begins in the cytoplasm where siRNA associate with Argonaute 1 protein (Ago1). These two components are trafficked to the nucleus [53] and recruit other proteins to form the RNA-induced transcriptional silencing (RITS) complex. The RITS protein components have been identified in *S. pombe* yeast and include Ago1, the GW protein, Tas3, and chromodomain protein 1 (Chp1) [54], however while Ago1 is present in mammalian cells, Tas3 and Chp1 homologues have not yet been identified. Although this complex can be considered as the equivalent of RISC from PTGS, it is not identical, due to the different functional requirements and distinct protein components. Via siRNA sequence complementarity, RITS identifies the target locus and induces chromatin compaction through epigenetic modifications, such as histone methylation [55]. By rendering it structurally inaccessible to transcriptional machinery, TGS can lock the virus in a latent state.

Like PTGS, TGS is capable of significantly suppressing HIV-1 production and is highly sequence specific, with minimal off-target effects and interferon mediated immune responses or defence mechanisms, dependent on the specific sequence targeted [56]. A single nucleotide mismatch between the siRNA and its target could be sufficient to disrupt silencing. Hence, conserved regions of the provirus must be carefully selected and a multiplexed approach may be necessary. TGS also offers several advantages over its cytoplasmic counterpart. By preventing the provirus from actively transcribing, it can silence HIV-1 prior to the generation of escape variants. Additionally, due to the heritable nature of the heterochromatin marks, daughter cells exhibit the same suppressive phenotype [57].

Our laboratory has described two siRNA sequences capable of inducing potent TGS in HIV-infected cells. The first siRNA, termed siPromA, was identified in 2005 and has been extensively characterised as inducing highly-sequence specific TGS via epigenetic repressive mechanisms [53, 55, 56, 58–60]. The siPromA sequence targets NF- $\kappa$ B tandem repeat motif, which is unique to the virus and is not homologous to any host cell NF- $\kappa$ B motifs. This is important as NF- $\kappa$ B is an important transcription factor for multiple cell signalling pathways. A second siRNA, termed si143, has recently been shown to also induce TGS and targets the COUP-TF and AP-1 transcription factor sites upstream of the siPromA target sequence [61]. When combined, these two siRNAs provide enhanced suppression and enforcement of latency through multiple epigenetic modifications. We have shown up to 1000-fold suppression of virus transcription following a single transfection of siPromA or si143 for up to 15 days in various HIV-infected cell cultures, including HeLa T4+ cells and Hut78 cells. Further, in MOLT-4 cells carrying shRNA expressing siPromA, we reported virus transcription was suppressed for over 1 year [60]. Specific epigenetic modifications in HIV cultures suppressed by siPromA or si143 have been investigated using ChIP assays and included enrichment of histone methylation on the N-terminal histone tail (such as H3K27me3, H3K9me2) and decreased acetylation (such as H3K9) [61]. We have also demonstrated effective *in vivo* virus suppression using a humanised mouse model of acute HIV in (NOD)/SCID/Janus kinase 3 (NOJ) knockout mice infected with

HIV-1<sub>JR-FL</sub>. In human PBMCs that were stably transduced with shPromA delivered by a lentivirus vector and transplanted into the NOJ mice, followed by immune reconstitution, mice were protected from HIV-1 challenge, with significantly decreased plasma viral loads and normal CD4:CD8 T cell ratios, compared to control group treated with cells transduced with an inactive siRNA sequence carrying three mismatches (shPromA-M2) [58]. We anticipate, much like combined ART, that a multiplexed approach of combining TGS-inducing siRNAs will be necessary to provide sufficient control across a wide range of HIV subtypes and strains.

The Chattopadhyay laboratory has also reported a TGS-inducing siRNA sequence specifically targeting the HIV-1 subtype C NF- $\kappa$ B triple repeat motif, termed S4-siRNA. They demonstrated S4-siRNA induced TGS in a TZM-bl cell line and *ex vivo* human PBMCs transfected with S4-siRNA and infected with various subtype C viruses, as determined by measuring viral RNA levels [62]. Further, CHIP assay confirmed the enrichment of epigenetic repressive marks using histone methylation markers, H3K27me3 and H3K9me2. This siRNA may have potential as an RNA therapeutic, since HIV-1 subtype C is prevalent in approximately half of the people living with HIV globally.

#### 3.2.2.2. RNA-aptamer silencing

The Morris laboratory has also described a TGS-inducing siRNA, termed, LTR362, which also targets the NF- $\kappa$ B tandem repeat motif [63] and overlaps with 8 bp of the siPromA sequence. This RNA therapeutic has recently been further developed with the addition of a delivery aptamer designed to the HIV-1 glycoprotein termed gp120 A-1 and multiplexing with PTGS-inducing siRNAs targeting Tat and Rev. [64], designed by the Rossi laboratory. They showed the LTR362 RNA localised to the nucleus of an HIV-infected T lymphoblastoid CEM cell line and primary human CD4<sup>+</sup> T cells. Virus suppression showed a 10-fold reduction of viral p24 levels compared to control cultures at 12 days post-infection. This potential dual therapeutic was assessed *in vivo* using an HIV-1 infected humanised NOD/SCID/IL2  $\gamma^{\text{null}}$  mouse model and demonstrated suppressed virus infection and protected CD4<sup>+</sup> T cell levels in viremic mice. However, the mechanism of virus suppression was determined to be PTGS, due to the lack of the CpG methylation, an epigenetic silencing mark, at the 5'LTR. Investigation of histone methylation may prove some involvement of TGS, however the study currently indicates that while cell-type specific aptamer delivery of TGS-inducing siRNA functions *in vitro*, the *in vivo* silencing effect will require significant optimising to achieve robust epigenetic modifications [64].

## 4. Successful gene therapy treatment of HIV-1: the “Berlin patient”

The first person cured of HIV-1 was Timothy Ray Brown, also known as the ‘Berlin patient’, who still today remains to be the only person to be cured of HIV-1. Diagnosed with HIV at the age of 29, the patient commenced ART [65], but then presented with acute myeloid leukaemia at the age of 40. At that stage the patient’s HIV was controlled with ART and classified as stage A2; asymptomatic with a CD4<sup>+</sup> T-cell count of 415 cells/ $\mu$ L [66]. ART interruption during the first initial treatment showed viral rebound, therefore ART was resumed and no further treatment was required until an acute myeloid leukaemia relapse 7 month after initial treatment [66]. The patient then received an allogeneic haematopoietic stem-cell transplantation (HSCT)

[65, 66]. HSCT was already shown to be feasible in HIV positive patients, but it was also known that HSCT alone was insufficient to eliminate HIV [67]. For many patients finding an HLA-matched stem-cell donor is a significant challenge, however a suitable match was identified for the Berlin patient and subsequent screens for possible donors with the homozygous CCR5-delta32 (CCR5 $\Delta$ 32/ $\Delta$ 32) allele were performed [65, 66]. High resistance against HIV infection has been reported for individuals who are homozygous for the CCR5-delta32 deletion [68, 69]. HIV requires CD4 and typically either CCR5 (or CXCR4) for cell entry, making it a promising candidate for intervention [69]. Unlike CD4 and CXCR4, the absence of CCR5 is not obviously deleterious for modified cells [69]. Therefore the approach to use CCR5-delta32 stem-cells for HSCT of HIV infected patients was pursued, as earlier described by Chow et al., in 2001 [70]. Using this treatment approach an HLA-matched stem-cell donor with the homozygous CCR5-delta32 allele was identified [66].

The patient ceased ART medication on the day prior to the HSCT procedure, which was successful with complete chimerism achieved and only grade I graft-versus-host disease (GvHD) as serious complications [66]. HIV infection was analysed by RNA and DNA-PCR and remained undetectable in peripheral blood and bone marrow, as well as in the rectal mucosa [66]. Analysis of macrophages in the intestinal mucosa found they were still expressing CCR5, indicating that 159 days post-HSCT these long-lasting cells were not yet replaced by the new immune system [66]. The CD4<sup>+</sup> T-cell count in peripheral blood stayed at a low level of less than 300 cells/ $\mu$ L after the first HSCT until leukaemia relapsed on day 332 after HSCT [66, 71]. Following a total body irradiation, the patient received a second HSCT from the same CCR5 $\Delta$ 32/ $\Delta$ 32 donor [66, 71]. Fortunately, after the second transplantation the HIV load remained undetectable for the following years in peripheral blood, bone marrow and tissue biopsies, including gut and brain [66, 71]. CCR5-expressing macrophages in the gut became undetectable over the years and the peripheral CD4<sup>+</sup> T-cell count increased greatly within the first 6 months after the second HSCT, to over 400 cells/ $\mu$ L [71]. While the treatment was successful in inducing remission from the acute myeloid leukaemia, recovery from the second HSCT was slow, with a long period of infections, GvHD reactions in the liver and a period of fever, dizziness and delirium [65, 71]. The patient experienced loss of short-term memory, was almost paralysed and had to learn to walk again [65, 71].

As a milestone in HIV cure research, there is the question if this is a one-time wonder cure or if it is reproducible? In 2014, Hutter et al. assessed six more cases of patients with HIV-1 receiving an allogenic CCR5 $\Delta$ 32/ $\Delta$ 32 HSCT [72]. Five of those patients died within the first 4 months due to relapse, GvHD or infection [72]. The only patient surviving for 12 months experienced a rebound of CXCR4-tropic HIV-1 rapidly after the transplantation and died from a relapse of cancer [72]. This shows the difficulties of HSCT in HIV infected patients and the importance for careful selection of donor to recipient, as well as considering the continuance of ART to prevent CXCR4-tropic HIV-1 from rebounding until the new immune system has become more established [72]. In light of these attempts to replicate the successful treatment of Timothy Ray Brown, it should be noted that he was in fact  $\Delta$ 32 heterozygous prior to his HSCT, which likely provided him an advantage in relation to providing protection via  $\Delta$ 32 expression after transplantation.

The mechanisms of Berlin patient HIV cure are currently being investigated and pose an interesting question-is it a functional or sterilising cure? To start to answer this question, we will likely only be able to use the information currently available, as ongoing updates on

this case may be limited due to the patient recently commencing pre-exposure prophylaxis (PrEP) in order to prevent contracting HIV a second time. Firstly, the patient had ceased taking ART for >4 years without experiencing viral rebound, secondly, the viral DNA level was below detection limit in the periphery and in tissue biopsies, and thirdly, the patient showed a decrease in anti-HIV antibodies, all indicating a lack of virus replication, which makes it possible to conclude that the patient is functionally cured of HIV [66, 71]. The principle of a sterilising cure is the complete eradication of a pathogen out of the human body. This would therefore mean that every single cell previously infected and therefore carrying the HIV-1 genome would need to be replaced by new donor-derived cells to completely eradicate HIV from the body. All tests for proviral DNA until now, showed no detectable HIV-DNA and Timothy Ray Brown remained without viral rebound for 4 years, indicating the possibility that even the long-lasting memory immune cells were replaced by cells derived from the donor. This could lead to the interpretation that it was in fact a sterilising cure. That being said it is important to take into account the current limits of detection and the fact every single cell in his body cannot be analysed. Further similar results were found in two other patients who did eventually rebound. Therefore, one cannot be definitive in whether the cure is functional or completely sterilising. Regardless of whether the final conclusion is potentially a sterilising cure, it was derived from a functional cure approach.

## 5. Genome editing

The ability to engineer specific changes in the genome of an organism has developed rapidly over the last 10 years. The technology of gene editing relies on nucleases, scissor-like enzymes, with the ability to cut genomic DNA in a highly specific manner. This process results in additions, deletions or alterations at the targeted site of the genome. There are two pathways that can achieve double stranded breaks (DSB) in DNA; (i) nonhomologous end joining (NHEJ) repair pathway, where deletions or insertions in the target gene result in gene disruption *e.g.* CCR5, or (ii) the homologous recombination or homology-directed repair (HDR) pathway, in which DNA sequences are introduced into the genome using a homologous DNA template. The HDR pathway is more precise, with limited off-target genome effects, due to more control over the integration site, copy number and expression of the DNA sequence [73]. Some examples of gene editing technologies include zinc finger nuclease (ZFN), transcription activator-like nucleases (TALENs), clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR/Cas9). Examples of specific gene editing technologies that have been explored in the context of HIV treatment are described below.

### 5.1. Zinc finger nucleases (ZFN)

ZFNs are a combination of zinc-finger proteins, which have DNA recognition specificity, and the nuclease activity of the cleavage domain of restriction enzyme, *FokI*. The most advanced ZFN pair targets the CCR5 host gene and HIV-1 co-receptor [74]. Preclinical studies in a mouse model, where mice received CD3/CD28-activated primary CD4<sup>+</sup> T cells treated with the ZFN delivered in a chimeric adenoviral vector, Ad5/F35, showed anti-HIV efficacy,

with disruption of 40–60% of all CCR5 alleles and 33% disruption of both CCR5 alleles [74]. Following the successful pre-clinical data, the ZFN progressed to phase I clinical studies and the first-in-human gene editing HIV treatment trial (#NCT00842634) commenced in 2009.

The primary outcome of this study investigated the safety of ZFN modification of autologous CD4<sup>+</sup> T cells being delivered to HIV positive individuals [75]. A secondary outcome measured immune reconstitution and HIV resistance. Twelve ART-treated patients with undetectable viral loads were enrolled in two cohorts dependent on CD4<sup>+</sup> T cell count; cohort 1 included patients with CD4<sup>+</sup> T cell count >450/mm<sup>3</sup>, the median being 662/mm<sup>3</sup>, and cohort 2 was patients with lower CD4<sup>+</sup> T cells counts between 200 and 500/mm<sup>3</sup>, the median being 272/mm<sup>3</sup> [75]. Patients received one infusion containing 5×10<sup>7</sup> autologous CD4<sup>+</sup> T cells that were ZFN-modified. The infusion of ZFN-treated cells was deemed safe, with one serious adverse event reported that was infusion-related. All patients demonstrated engraftment, with the ZFN-modified cells being present for up to ≥42 months following infusion and showed expected characteristics. At 4 weeks post-infusion, cohort 1 ceased taking ART in a 12 week analytical treatment interruption (ATI), resulting in four out of six patients with detectable viral loads at 2–4 weeks post-ART cessation [75]. One of the six patients experienced a delayed increase in viral load at week 6, but was still below the viral set point [75]. This patient was later determined to be heterozygous for CCR5Δ32, suggesting that this genotype enhanced the ZFN treatment effect.

The successful modification of CD34<sup>+</sup> HPSCs was also shown using the same ZFN pair [76]. This has been further optimised to achieve HDR-induced gene modifications using an adeno-associated virus vector (AAV) serotype 6 and electroporation to deliver nuclease mRNA to both primary CD4<sup>+</sup> T cells and HPSCs [77], achieving between 8 and 60% and 15–40% CCR5 editing, respectively. Currently, there is no *in vivo* method that can effectively deliver nucleases to cells infected with HIV, and this will require further characterisation of the HIV sanctuary sites and identification of latent cell markers to allow specific targeting of cells that comprise the virus reservoir.

The generation of off-target genome modification is also a concern for the clinical application of ZFNs. This is exemplified by the off-target effect reported for the highly related CCR2 gene which was disrupted in 5.39% of ZFN-modified CD4<sup>+</sup> T cells that were targeting CCR5 and decreased CCR5 expression by 36% [74]. Optimisation of the CCR5 ZFN would be required if this off-target effect was determined to be deleterious.

## 5.2. CRISPR/Cas9

The development of therapeutics using CRISPR/Cas9 technology has rapidly intensified over the last decade. The system is based on a short guide RNA (gRNA) that targets a specific DNA sequence and the Cas9 endonuclease, which then cleaves the double stranded DNA. Mutations, either deletions or insertions, are introduced into the target sequence following DNA repair by the NHEJ repair pathway. Similar to ART, HIV has been shown to develop virus escape mutations when only a single gRNA is utilised and requires multiple gRNAs to prevent the emergences of virus resistance [78, 79]. Multiple studies have reported HIV-1 inactivation using this gene editing platform with dual gRNAs, via mutation at either target sites or complete excision of the virus sequence between the two target sites [80–83].

Although CRISPR technology has proven successful in inactivating HIV-1, like the ZFN system, successful delivery to all cells of the latent reservoir will be challenging without a known latency marker.

The CRISPR/Cas9 platform has recently been adapted to activate HIV transcription, akin to the “shock and kill” approach. This involves a mutation in the Cas9 catalytic domain which results in deactivated Cas9 (dCas9). The dCas9 can then be coupled to a strong transcription activation domain (AD) and targeted to the HIV-1 LTR can induce transcriptional activation via recruitment of transcription and chromatin modifying factors. One example of the dCas9-AD system is dCas9-VP64, which contains multiple copies of the herpes simplex virus (HSV) VP16-driven minimal AD and has been shown to activate HIV-1 promoter-driven gene expression. Interestingly, the most promising gRNA target in the HIV-1 5’LTR is single guide (sg)362F [84] and similar to the siPromA sequence described in the “block and lock” approach, targets the NF- $\kappa$ B binding motif.

## 6. Delivery

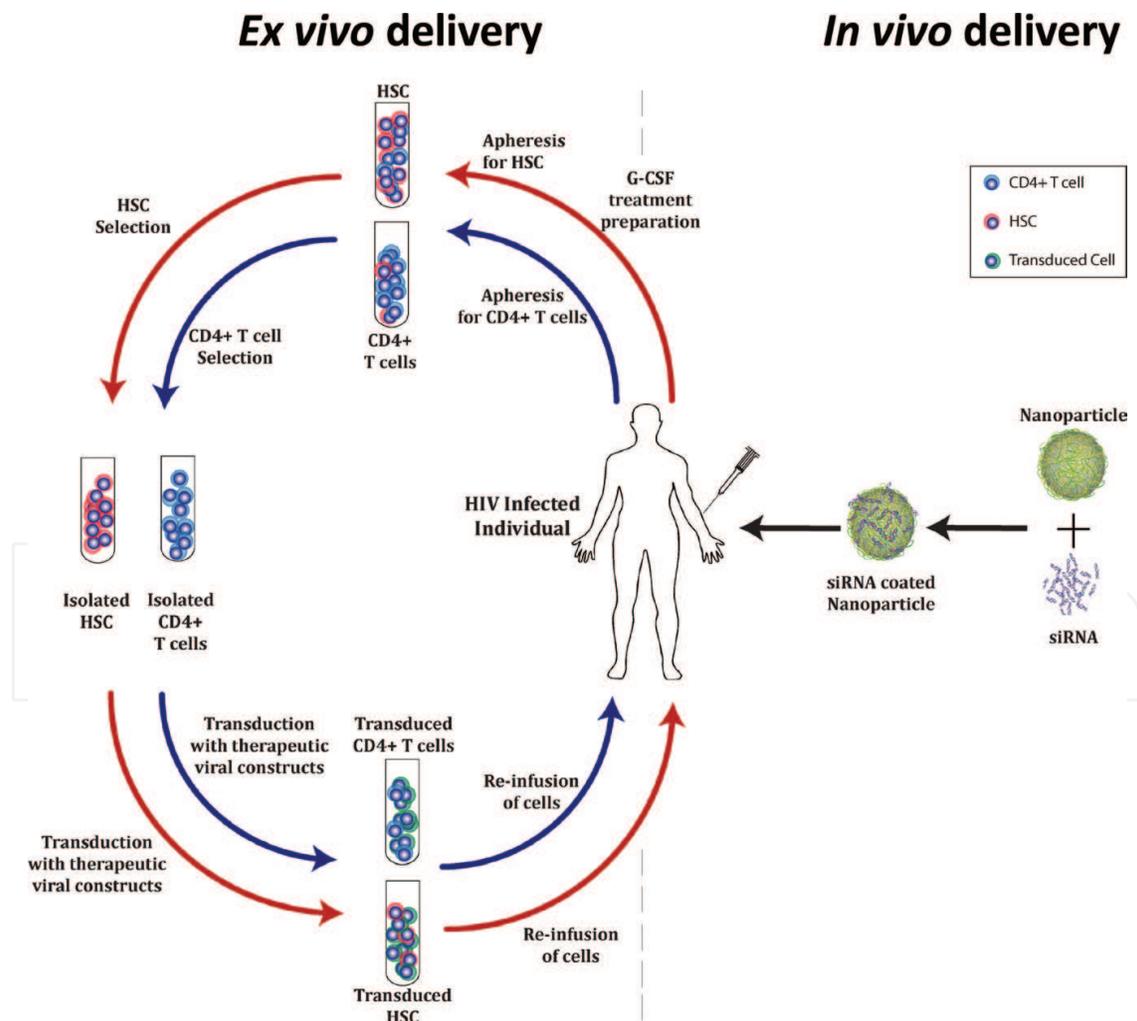
### 6.1. *Ex vivo* delivery by lentiviral vectors

Delivery of gene therapy to a specific target cell is another current challenge for an HIV cure. Viral vectors have become a regular method by which to deliver therapeutic genes and constructs [85]. There are multiple viable types of viral vectors that have been proven to be safe, relatively easy to construct and modify, and in the case of lentiviral vectors, these have the potential to transduce cells in a non-proliferative state [86]. Although the latter feature does not extend to non-proliferative leukocytes, due the presence of lentiviral restriction factors at and below the membrane.

One significant obstacle to the effective delivery of sufficient quantities viral vector is the ability to transduce sufficient quantities of target cells. To overcome this, apheresis is performed in order to concentrate the desired cells. Currently, gene therapy protocols for HIV first require the isolation of the desired cells to be modified, typically following apheresis [85]. Apheresis is the process of removing mononuclear cells from blood and returning neutrophils, platelets, plasma and red blood cells to the donor. This process is performed in order to collect more of the desired cells of the blood than could be separated from a unit of whole blood of ~550 mL. While CD4+ T cells are the main target for HIV infection, other cells such as dendritic cells, macrophages, monocytes and to a lesser extent, haematopoietic stem cells (HSC) have been found to be susceptible to HIV infection [87–89]. It is known that if HSC are transduced, or modified in any way, then a wide range of subsequent immune cells including macrophages, dendritic cells, CD4+ T cells and NK cells will carry that modification [90]. While transduction of CD4+ T cells will result in only CD4+ T cells being modified, the approach of transducing HSC provides protection from HIV to a broader range of cell-types, making it a highly desirable target for treatment/modification. Once a large volume of cells has been collected over several hours, they can then be transduced with the desired viral vector and reintroduced to the individual where the cells will migrate back to peripheral blood, lymph nodes, and bone marrow. This delivery method has been used in dozens of clinical trials and has become a

widely accepted method for delivering viral vectors to large numbers of cells, in particular to HSC in the bone marrow [85]. The *ex vivo* gene therapy process is depicted in **Figure 4**.

As HSC predominantly reside in the bone marrow, in order to increase the quantity of HSC in peripheral blood, it is common to use granulocyte colony stimulating factor (G-CSF) as a mobilising agent to encourage recirculation of HSC. This causes cells to migrate from the bone marrow and lymph tissue into the peripheral blood. The use of G-CSF or other stimulating factors is essential when HSC are to be transduced with the therapeutic gene/vector, with various trials showing that HSC cell counts in peripheral blood increase 20–50-fold over the course of G-CSF administration [91–93]. To aid with re-engraftment of HSC back into the bone marrow after transduction, a technique known as myeloablation has been utilised in some clinical trials prior to the reintroduction of HSC via infusion, in order to provide an immunological niche and improve engraftment of the gene-containing cells [94]. This procedure involves the eradication of resident HSC, thereby reducing the population of non-transduced cells, and creating more space for the transduced cell population to reconstitute the bone marrow. A delay of the presence of newly ‘protected CD4+ T cell’ population would



**Figure 4.** Gene therapy delivery strategies; *ex vivo* lentivirus transduction of isolated patient haematopoietic stem cells (HSC) and/or CD4+ T cells to deliver the gene modification versus systemic, *in vivo* delivery of the gene therapy directly to the patient, which requires a cell specific moiety to ensure targeted cell delivery.

be expected due to the required production of cells, thus delaying the effect of the therapeutic gene(s). Production of new CD4<sup>+</sup> T lymphocytes from the thymus has been predicted to be at a rate of approximately 1.65 cells/ $\mu$ L of blood/day due to thymic function [95]. The resulting production of a stable population of protected cells would be expected to gradually create a positive impact on CD4<sup>+</sup> T cell number and help suppress viral load.

While the modification of HSC has the benefit of long-lasting and broad-spectrum protection via the differentiation of stem cells, this approach still lacks the immediate benefit of targeting the existing CD4<sup>+</sup> T cells population. The use of CD4<sup>+</sup> T cells as a target for HIV gene therapy has been explored and assessed in several studies. Isolation and modification of CD4<sup>+</sup> T cells is relatively simple, as they largely populate and consistently traffic through peripheral blood. Accordingly, no stimulatory factors (such as G-CSF) are required to mobilise them prior to collection. This method has the benefits of providing an immediate benefit via the reintroduction of a protected population of the primary target cells for HIV infection [96]. This has been performed and shown to be both safe in treatment, and effective in delivery of the therapeutic gene [96, 97].

Lentiviral vectors are being increasingly used in clinical trials to treat a variety of diseases ranging from cancers, to genetic diseases such as haemophilia and sickle cell anaemia, as well as several trials treating HIV. The largest such trial in HIV gene therapy demonstrating the safety of lentiviral vectors was the Phase II trial whereby a Tat/Vpr specific anti-HIV ribozyme (OZ1) or placebo was delivered in autologous CD34<sup>+</sup> haematopoietic progenitor cells. The trial involved 74 patients where there were no adverse events related to the vectors or infusion process [98].

As outlined above, present gene therapy efforts to target HIV are primarily defensive in approach, as they encode future HIV resistance and may not influence the HIV reservoir in the short-term. Given the success of CAR T cell therapy in various cancer trials, many investigators are now multiplexing HIV resistance alongside a CAR construct that can target HIV. As cellular markers for the HIV reservoir are often shared in various leukocyte niches, the equivalent to the anti-CD19 approach used in B cell leukaemia has yet to be determined. Rather, investigators have now turned to potent broadly neutralising antibodies, which have been screened and cloned from various HIV positive patients and target HIV envelope. In this setting, several pre-clinical studies are underway in non-human primates in the laboratories of Kiem and Jerome, where resistance afforded by C46 and shRNA is complexed with one of several CAR modules that incorporates the single variable change of well characterised broadly neutralising antibodies [99].

In contrast, work led by the Berger laboratory has taken a similar but different approach to CAR T cell development. Rather than incorporating a neutralising antibody, they have complexed the first Ig-Like domains of CD4 with the serum mannose binding lectin [100]. This approach enables global recognition of HIV envelope, as it engages the CD4 binding site and also the abundant glycosylation sites that decorate the antigenic silent face of HIV Env. In the CAR T cell context, CD8<sup>+</sup> T cells are generated alongside HIV resistant CD4<sup>+</sup> T cells to mediate attack on the HIV reservoir. The only problem with the latter approach is that the major HIV reservoir *in vivo* resides in the germinal centers of secondary lymphoid tissue and actively excludes CD8<sup>+</sup> T cells, given they lack the germinal homing receptor CXCR5. Therefore, whilst CD4<sup>+</sup> T cells may transverse the germinal center, CD8<sup>+</sup> T cells will not.

To overcome this lack of secondary lymphoid targeting of CAR CD8<sup>+</sup> T cells, the Skinner laboratory has recently developed a hybrid CAR construct that encompasses not only HIV targeting, but also the CXCR5 receptor [101]. In theory, this enables CD8<sup>+</sup> T cells not only to target the HIV reservoir, but also transverse the site where the reservoir is located.

#### 6.1.1. *Cal-1 lentiviral vector*

One vector of note that has been extensively studied is the Cal-1 vector, which uses both the maC46 fusion inhibitor and shRNA-CCR5. This construct has been extensively studied, consistently showing therapeutic benefits *in vitro*. Additionally, this construct has also shown its enhanced efficacy when compared against individual genes, as the effect is induced by the use of two therapeutic targets [102]. This has not only led to stronger protection from HIV infection, but also is likely to result in reduced risk of mutation resistance [102]. This has been examined in mouse studies and non-human primates, where it has shown safety, high levels of engraftment (including in CD34<sup>+</sup> cells), and a selective growth advantage [102–105].

The Cal-1 therapeutic construct is currently undergoing Phase I/II clinical trials [106]. The study involves 12 HIV positive patients, which have undergone transduction of both HSC and CD4<sup>+</sup> T cells with a lentiviral vector carrying both the shRNA-CCR5 and C46 fusion inhibitor. The patients were divided into 3 equal groups, group 1 received no busulfan pre-conditioning, group 2 received 4 mg/kg busulfan, and group 3 received 2 doses to a total of 6 mg/kg busulfan conditioning. This study is currently ongoing but will provide important data on the optimised conditioning treatment to guide future treatment studies.

#### 6.1.2. *Limitations of lentiviral vector delivery*

Whilst we now have therapeutic approaches that can focus our efforts on a HIV cure, delivery of these components still presents a barrier. Lentiviral vectors have proven to be extremely useful in providing delivery of therapeutic genes, although there are still limitations. As mentioned, cells can only be modified *ex vivo*, thus requiring apheresis. Additionally, in the case of HIV, as ART will prevent uptake of the lentiviral vector, patients must first stop ART, thus raising various health concerns and ethical obstacles. Furthermore, current approved lentiviral platforms can only transduce T cells that are activated, as this over comes lentiviral restrictions at the membrane and underneath the membrane. The sum of these problems significantly increases the cost of the clinical approach. In the setting of CAR-T cells the estimates for treatment of refractory B cell leukaemia is approximately \$US400,000. Given this substantial cost, the accessibility of this type of therapeutic intervention is low. Thus, efforts are underway that will improve the process of this gene delivery pipeline. For instance, lentiviral vectors could be developed to target fresh leukocyte populations *ex vivo*, obviating the need for large scale apheresis. Additionally, lentiviral vectors could be modified to target leukocyte subsets, so the cells with the greatest stem-like attributes are re-infused and not diluted with cells that may not proceed down the differentiation pathway. This could potentially include a sub-population of resting T cells (*e.g.* Stem T cells) being isolated, genetically modified and re-infused in a manner that may not require apheresis. However, whether re-infusion of a smaller population of stem T cell would result in the same outcome that maybe achieved with a large population of bulk T cells obtained by leukopheresis needs to be thoroughly investigated.

## 6.2. *In vivo* delivery by nanoparticles

The *ex vivo* delivery of an HIV gene therapy treatment will only be achievable in developed countries with the appropriate resources to facilitate the approach and this will not be feasible in countries which currently have the largest burden of HIV, such as sub-Saharan Africa. We and others are working on an alternate and highly relevant systemic, *in vivo* approach, which may ultimately be accessible to all. This approach utilises nanotechnology to deliver the HIV therapeutic to target cells, ideally those of the latent reservoir. According to the Recommendation of the European Commission in 2011 the currently accepted definition of a nanoparticle (NP) is a particle where one or more external dimensions is in the size range of 1 to 100 nm [107]. However, larger particles with sizes up 1000 and 2000 nanometres are commonly referred to as 'nano', especially since for medical purposes the size range of  $\leq 100$  nm is not always practical, as a larger surface can carry more drug on a single particle [108, 109]. However, to be able to be used in the human body, NPs must be biocompatible and without cytotoxic side effects [108, 109].

Concentrating on HIV drug delivery, NPs have the unique feature of being able to absorb and carry other compounds on their relatively large functional surface [109]. Using NPs as delivery agents has the potential advantages of highly specific and controlled drug delivery to a targeted tissue or cell, such as those of the latent reservoir, keeping non-target organs and cells free of the drug, thereby reducing toxicity. Further, by releasing the drug in a controlled manner at a predetermined rate, achieved through changes in the physiological environment like pH, temperature or enzymatic activity, the resulting therapeutic efficacy can be increased [108–110]. Importantly, nano-based delivery systems have been shown to transport therapeutics across the blood-brain barrier, which is highly relevant for treating neuro-degenerative diseases and specifically the HIV reservoir in the central nervous system [111]. Prior to use of NPs in humans, the following basic prerequisites need to be known: drug incorporation and release, formulation stability and shelf life, biocompatibility, biodistribution and targeting, potential toxicities as well as functionality [109]. Another consideration are the possible adverse effects of residual material after drug delivery, therefore biodegradable NPs with a limited life span are optimal [109].

There are many types of NPs reported as delivery vehicles for HIV therapeutics, such as liposomes, micelles, polymer capsules, inorganic gold particles and dendrimers [112]. The number of different formulations of NPs being explored for HIV and other diseases is steadily increasing and a focused review on nanoparticle systems is provided by Pelaz et al. [112]. An example of the *in vivo* gene therapy process is depicted in **Figure 4**.

In the case of HIV, NPs have been used to deliver antiretroviral drugs or anti-HIV therapeutics, such as siRNAs. Inorganic gold particles delivering antiretrovirals have progressed through to *in vivo* delivery in mouse models, as have poly(amidoamine) PAMAM dendrimers and RNA-aptamer conjugates (as previously describes in Section 3.2.2.2), that deliver a combination of anti-HIV siRNAs. The gold particles and PAMAM dendrimer nano-platforms will be discussed below to highlight the challenges of targeting the HIV latent reservoir.

The NP platforms delivering an antiretroviral drug were comprised of inorganic gold nanoparticles (AuNPs) ~2–10 nanometers in diameter and were conjugated with an HIV integrase inhibitor, raltegravir [113]. Modification of raltegravir was necessary to link the inhibitor

to the gold nanoparticle and involved incorporation of a thiol group to generate thiolated raltegravir. Cellular uptake and toxicity of AuNPs was assessed in three different cell types; PBMCs, macrophages and HBMECs and confocal microscopy showed AuNPs inside all three cell types 24 hours post-delivery [113]. No toxicity was observed between 24 and 72 hours post-delivery. Importantly, the study investigated *in vivo* delivery of AuNPs and reported the presence of AuNPs in multiple sites, with the highest to lowest levels observed in spleen, liver, kidney, tail, heart, blood, lungs, muscle and brain of BALB mice 24 hours post-delivery [113]. Accumulation of AuNPs in the spleen and liver was attributed to reticuloendothelial system clearance, which is the body's first line of defence for any *in vivo* delivered therapy. The lack of a specific marker for the latent reservoir is an ongoing challenge for targeting cells which harbour integrated HIV DNA and have the potential to reactivate and produce productive virus. Although this approach does successfully penetrate some cells of the latent reservoir, *i.e.* lymphocytes and macrophages, and to a modest degree cells in the brain, it is not a targeted approach and will most likely need further development of functional groups to penetrate the majority of cells of the latent reservoir. Due to the rarity of cells harbouring latent provirus, which is estimated to be 1 in every  $10^6$  cells, targeting these cells is the current challenge for an HIV cure.

The cationic PAMAM dendrimer NP system is comprised of highly branched, chemical polymers with cationic primary amine groups on a spherical surface that form stable, uniform nanoscale complexes. The PAMAM dendrimer interacts electrostatically with negatively charged dicer substrate siRNAs (dsRNAs). The combination of anti-HIV siRNAs in this study included *tat/rev*, as well as the siRNAs targeting the CD4 and TNPO3 genes [114]. In this study, humanised Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice were generated and infected with HIV-1<sub>NL4-3</sub> 12 weeks following engraftment, then dendrimer-siRNA complexes were delivered via *i.v.* injection using equal amounts of all three siRNAs. Injections were continued weekly for 4 weeks. A significant decrease in HIV viral load by 3 logs relative to controls was reported and persisted up to 3 weeks post-treatment, however virus rebound was observed in the majority of animals after this time point [114], as is the standard response in patients following ART cessation. The study then investigated redosing of the dendrimer-siRNA complexes 3 months following the last administration and observed a further virus suppression which persisted for 3 weeks past the additional treatment. Assessment of mRNA levels of the three targeted genes (HIV *tat/rev*, CD4 and TNPO3) showed reductions in mRNA levels relative to the controls corresponded to the dosing schedule and confirmed sequence-specific and efficient gene silencing [114]. The main challenge with this approach is the need for continual treatment, or alternately the further development of a sustained-release approach. Further, whether this approach will be able to target very rare cells harbouring the latent reservoir remains to be investigated.

## 7. Conclusion

The field of HIV gene therapy is rapidly evolving, with development of both novel anti-HIV therapeutics and delivery systems to ensure cell specific targeting. While an *ex vivo* gene therapy approach for HIV is well on the path to patient translation, further targeting of the latent reservoir will be necessary to achieve a systemic, *in vivo* gene therapy approach. This will require identification of biomarker/s for latently-infected cells and novel ways to incorporate them into viral vectors and/or nanoparticle platforms. Once achieved, the next challenge

will be the cost of treatment, which is becoming a driving factor in the context of HIV, as the global burden of HIV is predominantly in sub-Saharan Africa. The cost of *ex vivo* gene therapy approaches is prohibitive in developing countries and *in vivo* nanoparticle approaches, whilst more cost effective, do not yet achieve sustained virus remission. Further optimisation and refinement of current delivery systems is required to enable wide scale application of a functional HIV cure.

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## Conflict of interest

The authors declare no conflict of interest. GS, AK and CA hold patents for si/shRNA protection against HIV-1 and GS is an employee of Calimmune Inc./CSL.

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