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Review

Engineered DNA modifying enzymes: Components of a future strategy to cure HIV/AIDS

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ABSTRACT

Despite phenomenal advances in AIDS therapy transforming the disease into a chronic illness for most patients, a routine cure for HIV infections remains a distant goal. However, a recent example of HIV eradication in a patient who had received CCR5-negative bone marrow cells after full-body irradiation has fuelled new hopes for a cure for AIDS. Here, we review new HIV treatment strategies that use sophisticated genome engineering to target HIV infections. These approaches offer new ways to tackle the infection, and alone or in conjunction with already established treatments, promise to transform HIV into a curable disease.

Contents

1. Introduction

Combination antiretroviral therapy (ART), introduced into clinical practice in the mid-1990s, has profoundly reduced HIV-associated morbidity and mortality, changing a lethal disease into a chronic illness (Palella et al., 1998; Thompson et al., 2010). Although ART can suppress viral loads below the detection limit of standard clinical assays (<50 HIV-1 RNA copies/mL), it cannot eliminate HIV. This is due on the fact that ART targets virus entry or the viral enzymes, but not the integrated provirus. Therefore, ART requires lifelong treatment, potentially leading to problems of cost (Chen et al., 2006; Schackman et al., 2006), adherence (Mannheimer et al., 2002; Paterson et al., 2000), drug resistance (Little et al., 2002; Richman, 2006) and toxicity (Dybul et al., 2002). Particularly, long-term treatment frequently results in secondary complications, such as diabetes, hyperlipidemia, cardiovascular disease, osteoporosis, and chronic kidney disease (Calmy et al., 2009; Deeks and Phillips, 2009).

More importantly, patients successfully treated with ART for several years still do not fully recover their immune responses, and show increased levels of immune activation along with its harmful effects (Ostrowski, 2010; Plana et al., 1998). Consequently, low-level viral replication may persist along with an established pool of latently infected cells (Finzi et al., 1997, 1999; Palmer

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et al., 2008). When ART treatment is interrupted viral load can quickly rebound, even in patients who have suppressed plasma viremia to levels below detection limits for many years (Davey et al., 1999). Therefore, developing novel therapeutic strategies aiming to cure HIV infection must address the pool of cells that harbor the latent HIV reservoir (Chun and Fauci, 2012; Deeks et al., 2012; Richman et al., 2009).

In principle, two qualitatively different types of cure have been defined (Dieffenbach and Fauci, 2011; Lafeuillade, 2011; Lewin et al., 2011). In a "functional cure" the patient's immune defense fully controls HIV in the absence of ART. However, proviral DNA can still be found in the body. In contrast, a "sterilizing cure" eradicates HIV and no viral genes remain in the infected host. Clearly, a functional cure may be easier to achieve, but a sterilizing cure is considered to be the holy grail of HIV therapy.

2. Purging the reservoir

The long-lived resting cells that contain HIV reservoirs reside primarily in tissues, in other words sanctuary sites that may not be easily accessible (Lafeuillade, 2012; Palmer et al., 2011; Smith et al., 2012). The proviral DNA (i.e. the integrated replication-competent HIV genome) in these cells is transcriptionally silenced, mainly due to epigenetic modifications of the viral long terminal repeat (LTR) promoter region (Coiras et al., 2009; Geeraert et al., 2008; Richman et al., 2009). Hence the viral antigens are not expressed, and in consequence, these HIV-infected host cells evade immune surveillance. Importantly, the existence of these viral reservoirs is believed to be the main hurdle to quantitatively clearing the virus from an infected organism. So far, the main and also best characterized reservoir comprises latently infected resting memory $CD4^+$ T cells containing replication-competent but dormant proviral DNA (for recent reviews on HIV reservoirs see Chun and Fauci, 2012; Lafeuillade, 2012; Margolis, 2011a; Palmer et al., 2011; Smith et al., 2012).

An important mechanism for maintaining transcriptional quiescence of the provirus, and hence viral latency, relies on cellular chromatin remodeling enzymes, in particular histone deacetylases (HDACs) (Hakre et al., 2011; Margolis, 2011b). Therefore, a main strategy currently being investigated for eliminating HIV reservoirs is based on pharmacologically inhibiting HDACs, thereby specifically activating latent proviral genomes in resting $CD4^+$ T cells. Upon HIV antigen expression, it is expected that these cells will be eliminated through either direct cytophatic viral effects or immune responses of the host (e.g. cytotoxic T cells; CTL).

Indeed, the HDAC inhibitor (HDACi) suberoylanilide hydroxamic acid (SAHA; Vorinostat), an FDA-approved drug for treating cutaneous T cell lymphoma, did specifically reactivate HIV from latency in chronically infected cell lines and primary cells (Archin et al., 2009; Contreras et al., 2009; Edelstein et al., 2009). More recently, SAHA has been administered to ART-treated HIV-positive patients with fully suppressed viremia (Archin et al., 2012). In a majority of these patients, SAHA not only affected cellular acetylation but also upregulated HIV-specific RNA expression in their resting $CD4^+$ T cells. Clearly, this increase in cell-associated HIV RNA does not necessarily imply that the respective cells could produce viral progenies. Nevertheless, reactivation of latent HIV expression by applying chromatin remodeling drugs, such as HDAC inhibitors, may be an essential mechanism to trigger HIV eradication *in vivo* (Durand et al., 2012). Doubtless, such a strategy will be applied in combination with ART to avoid *de novo* infection during activation of the latent virus reservoir.

As mentioned above, HDACi-induced (i.e. SAHA-induced) activation of latent HIV was generally expected to result in cell death due to either cytopathic viral effects or CTL action. Unfortunately, in another recent study it was shown that neither is the case, even when autologous CTLs from ART-treated patients were present (Shan et al., 2012). Instead, after virus reactivation $CD4^+$ T cells were only killed by CTLs when the cytotoxic T cells were pre-stimulated with HIV-1 Gag peptides.

These data demonstrate that HDAC inhibitor-induced activation of latent HIV will presumably not suffice to eradicate the long-term viral reservoirs by clearing the pool of latently infected cells. It has therefore been suggested that some form of therapeutic vaccination and/or additional interventions may be required for successful purging/eradication attempts (Archin et al., 2012; Shan et al., 2012). These may include gene therapy strategies (Kiem et al., 2012; van Lunzen et al., 2011). This notion is also supported by a more recent study in which various HDAC inhibitors (HDACis), including SAHA, were analyzed with respect to HIV production (Blazkova et al., 2012). It was demonstrated that, in aviremic individuals, HDACis only stimulate HIV expression in a small fraction of latently infected resting $CD4^+$ T cells and may therefore not be able at eliminating these viral reservoirs. The authors therefore suggested that alternative therapeutic strategies that incorporate HIV-specific targeting and/or immune activation approaches will be necessary to clear latent HIV (Blazkova et al., 2012).

3. A singular case of an HIV cure

In 2009, publication of the so-called "Berlin patient" case report revived the notion that a cure for HIV infection might be feasible (Hütter et al., 2009). This HIV-infected patient suffered from acute myeloid leukemia (AML). After failure of chemotherapy, the patient received hematopoietic stem cells (HSCs) from an HLA-identical donor selected for CCR5Δ32 homozygosity. This very rare mutation in Caucasians (~1% occurrence) inactivates the *CCR5* gene which encodes a critical HIV co-receptor (Liu et al., 1996). The patient received fully ablative and potentially lethal conditioning regimes in combination with two successive HSC transplantations. This procedure led to a complete remission of the AML (Hütter et al., 2009). Importantly, however, prior to transplantation the patient discontinued ART and for more than five years now shows no signs of HIV infection (Allers et al., 2011; Hütter and Thiel, 2011).

This is of particular interest, since before treatment a minor population (2.9%) of CCR5-independent virus variants (i.e. CXCR4-tropic or dual-tropic viruses) was also detected in the patient. Why these viruses did not rebound after ceasing ART, particularly in light of the fact that a high proportion of potential target cells (e.g. activated memory $CD4^+$ T cells) were recovered after transplantation, is unclear at the moment (Hütter and Ganepola, 2011). Nonetheless, it is conceivable that the harsh myeoablative conditioning of the patient or other immune reactions may have been responsible for this fortunate outcome.

Obviously, this approach cannot be applied to larger HIV patient cohorts for various reasons. For example, HLA-matched CCR5Δ32 homozygous donors are extremely rare, which in fact has so far prevented the treatment of another patient (Hütter and Thiel, 2011). Also equally prohibiting is the relatively high rate of mortality (~26%) connected with the procedure of allogeneic HSC transplantation (Gooley et al., 2010). Nevertheless, this unique case of the "Berlin patient" obviously jump-started the field of HIV eradication and latency research by demonstrating that an HIV cure is possible under certain, although extremely rare conditions. This case may also suggest that the genetic alteration of host cells, rendering them resistant to HIV, may be an important component of future eradication strategies.

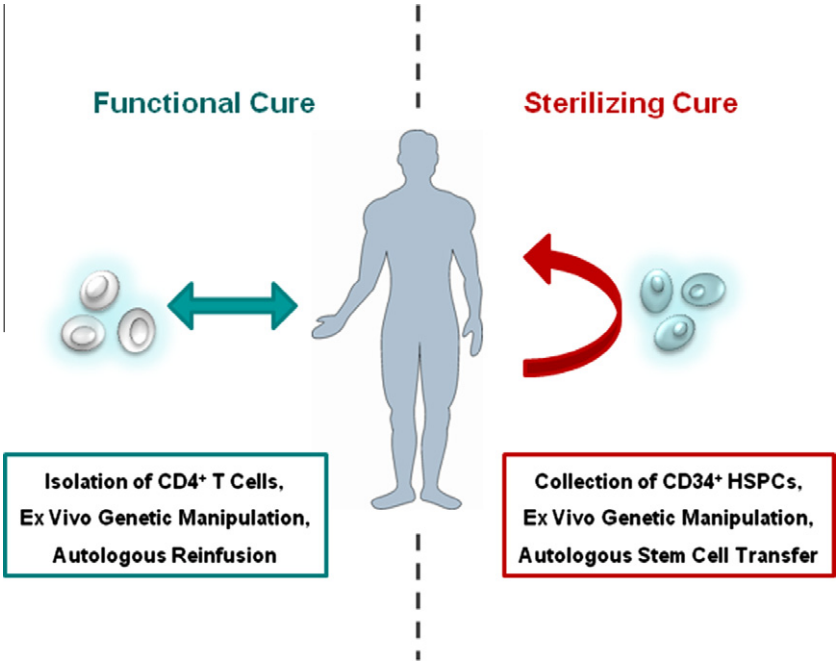

Fig. 1. Genetic therapies against HIV. Either the patient's peripheral $CD4^+$ T cells or the patient's $CD34^+$ HSPCs are usually genetically modified *ex vivo* using gene transfer vectors. Autologous re-infusion of enriched modified T cells is expected to result in antiviral, although transient, effects. This technical approach will probably find its major application in the context of a functional cure. Bone marrow-derived $CD34^+$ stem cells are commonly collected upon G-CSF mobilization by leukapheresis. Following genetic *ex vivo* manipulation, autologous transplantation and engraftment of the patient's bone marrow with these cells is expected to perpetually repopulate the patient's peripheral blood and lymphoid tissues with HIV-1 resistant cells. Therefore, stem cell-based gene therapies will most likely be applied in the context of a sterilizing cure. Clearly, both approaches may also be combined, either with each other, or for example, with vaccination strategies or novel pharmacological approaches.

4. CCR5 inactivation by gene therapy

In principle, genetic therapies against HIV either modify the patient's peripheral blood $CD4^+$ T cells or patient-derived $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) (Kiem et al., 2012; Rossi et al., 2007; Scherer and Rossi, 2011) (Fig. 1). T cell modification most likely results in transient effects, and may therefore be the strategy applied in a functional cure. In contrast, the genetic alteration of HSPCs allows the perpetual repopulation of the patient's hematopoietic system with genetically modified cells of all lineages, including the most relevant HIV host cells (e.g. lymphocytes, and monocytes). These HIV-resistant cells are expected to be selected *in vivo* (Baltimore, 1988), an assumption that clearly remains to be proven in a clinical setting. In theory, the patient's immune system should be functionally reconstituted, which is considered to be an important precondition for elimination of virus reservoirs (i.e. virus eradication). Therefore, stem cell gene therapy will most likely be the method of choice when a sterilizing cure is pursued.

A promising gene therapy approach that somehow mimics the case report of the "Berlin patient" is disrupting the *CCR5* gene by expressing an engineered zinc finger nuclease (ZFN). ZFNs are modular, designer DNA editing enzymes that comprise an array of zinc finger domains (commonly three to six) each recognizing a specific DNA triplet (Porteus and Carroll, 2005; Schiffer et al., 2012; Urnov et al., 2010). This substrate binding domain is fused to an unspecific nuclease domain commonly derived from the restriction endonuclease FokI. Since ZFNs act as dimers, appropriate positioning of two ZFN monomers, binding to the opposite strands on either site of a spacer region, results in DNA double-strand breaks (DSBs) at the spacer region (Fig. 2). DSBs are then frequently "repaired" by the cell's error-prone, non-homologous end joining (NHEJ) pathway, a process that often results in localized sequence deletions or the addition of unrelated bases (Naldini, 2011; Porteus and Carroll, 2005). Thus, specifically directing ZFNs to the *CCR5* locus can disrupt the cellular CCR5 receptor, conferring resistance to *de novo* infection by CCR5-tropic HIV-1.

In experiments, adenovirus (Ad) vector-mediated transient expression of CCR5-specific ZFNs specifically disrupted ~50% of *CCR5* alleles in a pool of primary human $CD4^+$ T cells; furthermore, CCR5-tropic HIV-1 infected mice engrafted with these transduced T cells displayed lower viral loads than animals engrafted with ZFN-untreated $CD4^+$ T cells (Perez et al., 2008). A subsequent study extended this T cell-based strategy to mice that were engrafted with human $CD34^+$ HSPCs. Prior to transplantation, transfection of the HSPCs with ZFN-expressing plasmid vectors resulted in CCR5 disruption (5–7% of $CCR5^{-/-}$ cells in the transfected population) and *in vivo* selection of ZFN-modified cells in the hematopoietic multi-lineage progeny. Again, analysis of viral loads and $CD4^+$ T cell counts demonstrated that ZFN-treated animals controlled HIV-1 replication more efficiently than mice that received ZFN non-transfected HSPCs (Holt et al., 2010).

Taken together these studies suggest that ZFN-mediated inactivation of CCR5, by modifying either peripheral $CD4^+$ T cells or $CD34^+$ HSPCs, is a highly promising approach to conferring resistance to HIV-1 *in vivo*. Evidently, this approach closely resembles the treatment strategy applied in the case of the "Berlin patient" to facilitate virus eradication (Deeks and McCune, 2010; Durand et al., 2012; Schiffer et al., 2012). It should be noted that a clinical trial is currently underway to analyze the potential of CCR5-specific ZFN in the context of a functional cure. In this trial peripheral $CD4^+$ T cells are isolated from HIV-infected patients, genetically modified *ex vivo* using an Ad-vector, and returned by autologous re-infusion (Tebas et al., 2011).

As outlined, ZFNs are valuable tools for site-directed genome engineering (Urnov et al., 2010), particularly for disrupting the *CCR5* gene as part of clinical HIV eradication studies. However, various undesired toxic effects may be connected with this

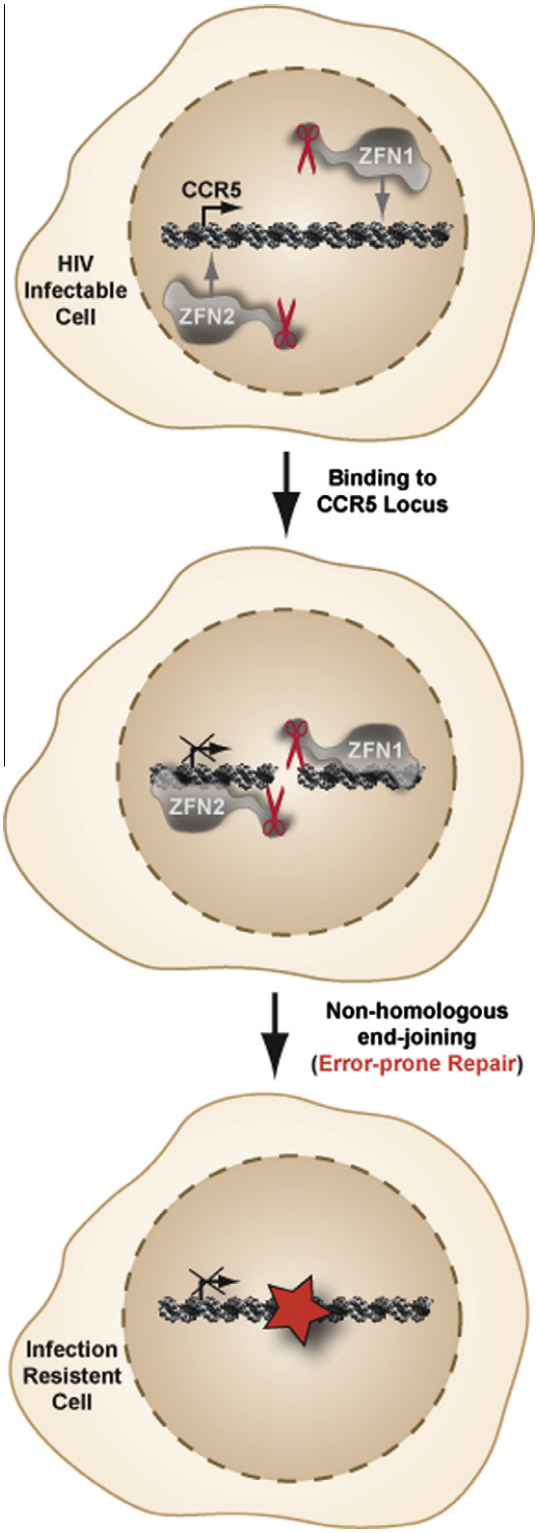

Fig. 2. Mode of action of CCR5-specific ZFNs. A dimer of *in vitro* engineered ZFNs specifically recognizes the *CCR5* locus via its zinc finger domains. Subsequently, the unspecific ZFN nuclease domains (scissors) introduce DNA double-strand breaks (DSBs) that are "repaired" by the cellular error-prone non-homologous end-joining (NHEJ) pathway. This process frequently results in disruption of the *CCR5* locus (asterisk), rendering the cell resistant to infection with CCR5-tropic HIV.

technology. ZFNs may recognize unrelated genomic sequences that share some degree of homology with the intended target sequence. For example, it has already been established that CCR5-specific ZFNs also target the *CCR2* locus to a significant extent (Perez et al., 2008). Two recent independent studies reported CCR5-specific ZFN cleavage of additional (≥13) human gene sequences (Gabriel et al., 2011; Pattanayak et al., 2011). Clearly, these off-target effects may be particularly troubling when stem cell (HSPC)-based gene therapies using CCR5-specific ZFNs are considered for clinical use.

The problem of genotoxicity due to unspecific cleavage may be avoided by using transcription activator-like effector nucleases (TALENs). Like ZFNs, TALENs are modular, structured designer nucleases that commonly combine an extended DNA targeting motif containing a variable number of tandem 34 amino acid repeats that each recognize a single nucleotide, plus the FokI endonuclease domain (Bogdanove and Voytas, 2011; Li et al., 2011). Since TALENs are engineered to recognize longer target sequences, binding specificity is greatly improved, thereby minimizing off-target effects. Supporting this notion, a CCR5-specific TALEN recently compared side-by-side with the corresponding ZFN demonstrated similar gene disruption activities, but clearly reduced nuclease-associated cytotoxicities (Mussolino et al., 2011).

Another drawback of ZFN- as well as TALEN-mediated CCR5 knockout may derive from the fact that the cleavage (and hence disruption) of the *CCR5* locus results in DSBs that activate the cellular error-prone NHEJ pathway. Unfortunately, DSBs represent one of the most dangerous lesions for a cell, and can potentially result in oncogenic catastrophe (Hiom, 2010; Porteus and Carroll, 2005).

Finally, it should also be noted that disrupting the CCR5 molecule is not an effective strategy to block infection or outgrowth of CCR5-independent viruses, such as CXCR4-tropic or dual-tropic HIV-1.

5. Targeting the HIV provirus

Specific endonucleases, such as ZFNs, TALENs, or homing endonucleases (HEs) (Stoddard, 2011), may also be used to directly target integrated HIV proviral DNA. Here, a DSB is induced in an essential region within the provirus, again followed by host cell-mediated error-prone NHEJ. Indeed, it has been recently demonstrated that a lentiviral vector-derived artificial GFP reporter construct, that was engineered to contain a single HE recognition site, was inactivated by HE expression (Aubert et al., 2011). So far, however, no HE being capable of recognizing a native HIV target sequence has been reported, which would be prerequisite to an application in future HIV eradication strategies.

Another approach that likely depends on gene therapy directly targets the integrated proviral DNA using a tailored long terminal repeat (LTR)-specific recombinase (Tre-recombinase) (Buchholz and Hauber, 2011; Sarkar et al., 2007). The Tre enzyme specifically recognizes and recombines a 34 bp sequence, called loxLTR that is located in the proviral LTRs. This results in excising the intermediary sequences from the genome of the host cell, including all viral genes (Sarkar et al., 2007). A single LTR remains at the chromosomal integration site, while the circular integration-deficient excision product is eventually degraded by cellular nucleases (Fig. 3). Thus, Tre-recombinase can reverse an already established infection by removing integrated HIV-1 from infected host cells. Fortunately, this process is independent of virus tropism, i.e. CCR5- and CXCR4-tropic viruses are removed equally well.

Recapitulating the gene therapy scenarios discussed above, a Tre-based eradication strategy may include lentiviral vector (LV)-mediated Tre delivery into either the patient's peripheral $CD4^+$ T cells or $CD34^+$ HSPCs. Moreover, the fact that Tre is only required in HIV-1 infected cells permits conditional expression of Tre either by placing the *tre* gene under the control of a

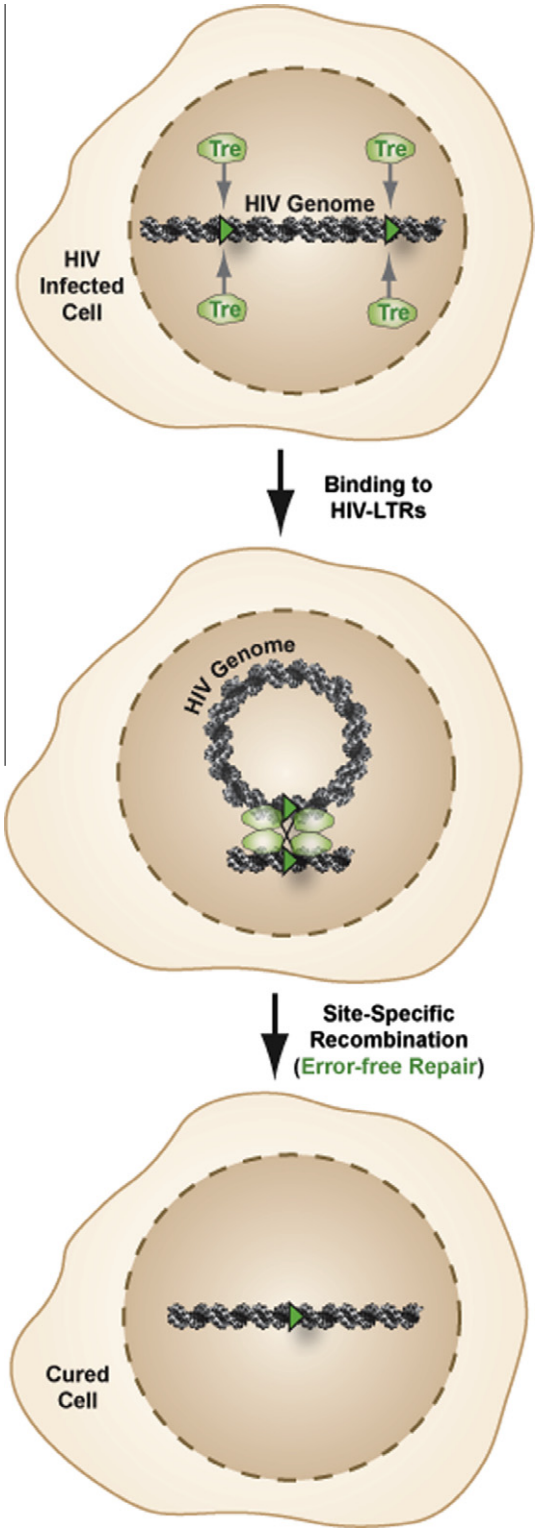

Fig. 3. Mode of action of LTR-specific Tre-recombinase. Tre-recombinase is a Cre-derived enzyme obtained *in vitro* by molecular evolution. Tre acts error-free as a tetramer recognizing and recombining a sequence located in the proviral LTRs (triangle). This process results in excision of the proviral genome from the host cell chromosome. The excision product is degraded and a single LTR remains at the integration site.

drug-inducible (e.g. doxycycline-inducible) promoter element (Lachmann et al., 2012), or by employing a promoter responsive to the HIV-1 Tat transcriptional *trans*-activator. Particularly, the latter strategy is expected to be combined with and to benefit from the concomitant administration of viral reservoir purging drugs (e.g. SAHA). Clearly, such a Tre expression strategy could minimize potential transgene-related (i.e. Tre-related) toxicities. A recent analysis of Tat-dependent Tre expression in HIV-1-infected humanized mice indeed demonstrated pronounced antiviral effects of Tre-recombinase in the absence of cellular toxicities, irrespective of whether the animals were engrafted with either Tre vector-transduced human $CD4^+$ T cells or Tre-transduced human $CD34^+$ HSPCs (Buchholz & Hauber, unpublished). These studies suggest that Tre-recombinase may indeed become an important tool in therapies that aim to overcome the obstacle of virus clearance.

Tre is a site-specific recombinase derived from Cre recombinase engineered *in vitro* by molecular evolution to act on a native HIV-1 target site (Sarkar et al., 2007). Cre recombinase is widely used in mouse genetics and has been intensively studied (Glaser et al., 2005; Van Duyne, 2001). Particularly in clinical applications, it seems to be advantageous that such recombinases, including Tre, neither produce DSBs nor require additional host factors such as the NHEJ pathway. As a result, the recombination process is very precise and usually error-free (Glaser et al., 2005; Van Duyne, 2001).

Nevertheless, prior to clinical application various potential problems connected with the Tre technology have to be resolved. For example, current Tre-recombinase was raised against a primary HIV-1 subtype A isolate (Blackard et al., 1999). It is therefore expected that for broader applications a Tre-recombinase also recognizing a majority of HIV-1 subtypes must be developed. Likewise, Tre treatment may select for outgrowth of resistant viruses resulting from target (loxLTR) site mutation. Both aspects may be addressed by identifying Tre target sequences that are highly conserved in the LTRs of a vast majority of HIV-1 isolates. The recent development of a novel "locus of recombination site" search tool and the description of a collection of conserved sequences covering a maximum of HIV-1 variants will certainly be helpful in achieving this goal (McIntyre et al., 2009; Surendranath et al., 2010).

Even if it turns out that a sterilizing cure cannot be achieved, Tre technology may also be applicable in a functional cure for *ex vivo* treatment of PBMCs. For this, Tre-recombinase could be expressed as a fusion with a cell-penetrating protein transduction domain (PTD) or membrane translocation motif (TLM) (Fonseca et al., 2009). As reported recently, directly adding recombinant PTD/TLM-Tre fusion protein to a productively infected T cell culture resulted in efficient protein translocation and excision of the full-length HIV-1 proviral DNAs from their chromosomal integration sites (Mariyanna et al., 2012).

6. Conclusions and future perspectives

The growing recognition that a cure for HIV infection is not only needed but also feasible is based on significant advances in basic, translational, and clinical research (Deeks et al., 2012). The remarkable case of the "Berlin patient" particularly revived the idea of gene therapy strategies to eradicate HIV (Kiem et al., 2012; van Lunzen et al., 2011). Indeed, expression of *in vitro* engineered enzymes disrupting the CCR5 surface receptor and/or excising the HIV-1 proviral DNA may become critical components of future therapies aiming at virus eradication.

It is generally expected that, if achievable at all, no single approach will lead to a sterilizing cure. Rather, a clever combination of drug treatments, therapeutic vaccination strategies, possibly in combination with antiviral gene therapy, likely offers the highest hope for defeating HIV. Gene therapies expressing engineered enzymes generally modify cells of the hematolymphoid system, with

the goal of conferring HIV resistance. In turn, this should contribute to improving the patient's immune responses, enabling the clearance of latently infected cells. Obviously, direct targeting of these resting cells with any antiviral vector will not be possible in the very near future. However, a Tre-based approach in combination with chromatin remodeling drugs specifically activating the HIV LTR promoter of latent proviruses, as well as the Tre-expressing vector, may be conceivable as part of a future strategy to eradicate latent infection. In more general terms it is envisaged that stem cell-based gene therapies, employing designer enzymes, will provide the groundwork for adding various additional antiviral strategies to achieve a cure for HIV infection.

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