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Gene Therapy for HIV-1 Infection

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1. Introduction

The introduction of the highly active antiretroviral therapy (HAART) for the treatment of HIV-1-infection has dramatically improved the quality of life and the survival of HIV-infected patients. HAART can effectively suppress virus replication and thereby helps to preserve immune functions. However, as HIV-1 persists in latently infected reservoirs (Finzi et al., 1997), complete eradication of the virus by antiretroviral drugs has never been achieved and life-long treatment is required. Moreover, emerging viral resistance and drug toxicity restrict long-term therapeutic efficacy (Brinkman et al., 1999; Vigouroux et al., 1999). As a consequence, HAART has not had a major impact on the global prevalence of HIV-infection and there is no vaccine in sight that could prevent further virus spread.
In addition to HAART and vaccines, gene therapy approaches for HIV-1 infection have been under investigation for more than two decades. Gene therapy could theoretically overcome the limitations of standard antiretroviral drug therapy and facilitate sustained suppression of virus replication after only few treatment cycles. Moreover, the choice of adequate genes or combinations of genes and expression systems could greatly reduce toxicity and prevent the generation of resistant virus strains. Although gene therapy is an expensive and technically challenging therapy today, future developments could simplify the procedures involved and bring down costs. Two basic gene therapeutic strategies for immune reconstitution of AIDS patients have been developed and the safety and efficacy of different approaches have been examined in preclinical and clinical studies. The first strategy aims to specifically kill HIV-infected cells by enhancing the antiviral host immune responses. The second approach, termed 'intracellular immunization', is based on the expression of antiviral genes that prevent HIV-1 replication in its target cells. Furthermore, therapeutic or prophylactic vaccination strategies that aim to enhance anti-HIV immunity and use DNA or viral vectors to express the viral antigens can formally be classified as gene therapy approaches. However, such vaccination strategies are not in the scope of this review.

2. Enhancing HIV-specific immunity: Adoptive transfer of $CD8^+$ T cells

The striking ability of HIV-1 to evade control by the host immune system is a fundamental problem in AIDS pathogenesis. Although most patients develop natural anti-HIV immune responses, the virus does not possess the immunogenicity to mount long-lasting responses strong enough to entirely suppress replication and to allow complete virus eradication from the body. In fact, most affected individuals initially develop immunodominant $CD8^+$ T cell

(cytotoxic T lymphocyte, CTL) responses during the acute phase of HIV-1 infection, resulting in transient virus control and a decrease in plasma viremia (Borrow et al., 1994). The importance of CTLs was further confirmed in experiments with SIV-infected non-human primates, where acute infection could not be controlled in animals depleted of $CD8^+$ T lymphocytes (Schmitz et al., 1999). There is a strong negative correlation between emergence of virus-specific CTLs and the viral set point, as patients with strong early CTL responses show significantly lower viral set points and a slower disease progression (Streeck et al., 2009). However, later, during the chronic phase of HIV-1 infection generation of $CD8^+$ T cell responses seems to be impaired and dysfunctional CTLs fail to control virus replication in most patients. In contrast, long-term non-progressors – individuals which are HIV-1 seropositive, but do not progress to AIDS – retain high levels of virus-specific T cells, indicating that functional CTL responses are crucial for efficient virus control also in chronic infection (Rinaldo et al., 1995).

One reason for the failure of HIV-specific immunity during the chronic stage of infection may be the high variability of the virus resulting from a high replication rate and error-prone reverse transcription (Phillips et al., 1991). Moreover, HIV-1 attacks the immune system itself, the $CD4^+$ helper T cell being its major target cell. This results in the preferential infection and a massive loss of HIV-specific $CD4^+$ T cells already early upon infection, as the virus-specific helper cells migrate to the site of infection where they become activated thereby becoming more susceptible to HIV-1 infection (Demoustier et al., 2002; Douek et al., 2002). Yet, virus-specific $CD4^+$ T cells are urgently needed to help generating strong, durable immune responses and their absence impairs CTL activation and maturation (Kemball et al., 2007; Matloubian et al., 1994). In addition, progressive exhaustion of virus-specific $CD8^+$ T cells is a hallmark of the chronic immune activation during ongoing HIV-1 infection. Here, the constant antigen persistence prevents contraction of the effector T cell pool and development of long-lived memory cells. Instead, a stepwise exhaustion characterized by metabolic and transcriptional changes, reduced cytokine and chemokine secretion, loss of proliferation capacity and cytolytic activity is observed, resulting in impaired CTL effector functions and finally apoptosis (Shankar et al., 2000; Trimble & Lieberman, 1998).

Boosting of the natural CTL responses against HIV-1 by cell and gene therapeutic strategies may help to overcome these problems. For this purpose two different strategies have been developed; the adoptive transfer of autologous antigen-specific T cells after *ex vivo* selection and expansion, and the infusion of genetically armed $CD8^+$ T cells expressing HIV-specific T cell receptors (TCRs). A therapy combining these approaches with the protection of $CD4^+$ T cells to preserve important T-helper cell functions could potentially impact infection dynamics and ultimately facilitate clearance of the virus.

The adoptive transfer of autologous, antigen-specific $CD8^+$ T cell clones has been used successfully to treat persistent viral infections and cancer. The allogeneic organ transplantation from cytomegalovirus or Epstein-Barr virus seropositive donors, for instance, is associated with risks for the recipient, if untreated. Co-transplantation of virus-antigen-specific T cells isolated from the donor and expanded *in vitro*, enhanced T cell immunity to the viruses and prevented adverse effects in the immunosuppressed recipients (Heslop et al., 1996; Walter et al., 1995). For HIV-1, however, the transfer of *ex vivo* expanded virus-specific CTLs had only limited success in patients so far. In a study reported by Brodie et al. autologous Gag-specific CTLs were isolated and reinfused into HIV-positive individuals (Brodie et al., 1999). The CTLs engrafted in the patients and were found to migrate to the lymph nodes, which are the major sites of HIV-1 replication (Brodie et al.,

2000; Hufert et al., 1997). However, although the CTLs were capable of lysing HIV-1 infected cells *in vivo*, only a transient effect on virus replication was observed (Brodie et al., 1999; Brodie et al., 2000). A major problem of this therapeutic concept is that the CTLs isolated from patients with advanced disease are often exhausted and terminally differentiated and lack full effector functions. As *in vitro* expansion of these cells is accompanied with a further loss of function, the transferred cell numbers may be insufficient to induce long-term effects. Methods that allow generation of large numbers of fully functional CTLs therefore would be required to facilitate success of this promising therapy approach.

An alternative to the isolation and expansion of existing antigen-specific T cells from a patient, is the genetic modification of cells resulting in the expression of recombinant HIV-specific receptors. The cell types used for this genetic 'redirection' can be peripheral blood T cells, but also hematopoietic stem cells (HSC), which can afterwards differentiate into immune cells targeting HIV-1. As the isolation and manipulation of T cells is currently easier to perform compared to stem-cell modification, all studies that have been conducted so far used peripheral blood T cells. The receptors used to redirect the immune cells to target HIV-1 can either be natural T cell receptors or artificial chimeric antigen receptors (CARs). For the natural TCRs, CTL clones with high avidity TCRs specific for the target antigens are selected *in vitro.* The alpha and beta chains of these TCRs are then cloned and used to transduce patient T cells. This approach has been used successfully in the clinic to treat patients with melanoma, where in some cases tumor regression has been observed (Morgan et al., 2006). The isolation and cloning of high-avidity HIV-specific TCRs is also feasible. Joseph and colleagues constructed a lentiviral vector expressing a TCR specific for the HIV-1 Gag epitope SL9. Transduction of human primary T cells led to the conversion of peripheral blood $CD8^+$ T cells into HIV-specific CTLs (Joseph et al., 2008). These CTLs exerted anti-HIV activity *in vitro* and *in vivo* in a humanized mouse model. In a similar study, an SL9-specific TCR with enhanced affinity was shown to efficiently mediate control of HIV-1 *in vitro* (Varela-Rohena et al., 2008). Currently this approach is evaluated in a phase I clinical trial (www.ClinicalTrials.gov; identifier: NCT00991224). The use of natural TCRs to generate virus-specific CTLs is limited by the major histocompatibility complex (MHC)-restriction of the TCR. TCRs recognize peptides only if they are presented on a specific type of MHC. Therefore, T cells expressing a given TCR can only be used for treatment of MHC-matched patients. The clinical trial described above, for instance, can only include patients with the HLA-type A*02. For a broader application of this therapy concept a set of TCRs would be required that allows treatment of patients with various haplotypes. Besides, in gene modified CTLs, the genetically transferred TCR can mispair with the endogenous TCR, which may affect its function and lead to autoimmunity. Another drawback of the approach is the downregulation of MHC molecules in HIV-infected cells, which impedes the presentation of viral peptides and recognition by the CTLs (Sommermeyer et al., 2006).

CARs are chimeric antigen receptors composed of an extracellular antigen binding motive connected to an intracellular signal transduction domain via a flexible linker and a transmembrane domain. The antigen binding motive usually is an antigen-specific single-chain variable fragment derived from a monoclonal antibody, while the signal transduction part comes from the CD3 ζ-chain. CARs trigger an MHC-independent antigen recognition (Eshhar et al., 2001). Two CARs with HIV-1 specificity have been developed by Roberts and colleagues (Roberts et al., 1994). The antigen-binding part consists either of the gp120 binding domain from human CD4 or an antibody against gp41. T cells transduced with either of the receptors specifically recognized and killed HIV-infected cells *in vitro.* The

receptor containing CD4 has been tested in three clinical trials. In these studies local effects on virus replication were observed, but unfortunately there was no overall change in viral load (Deeks et al., 2002; Mitsuyasu et al., 2000; Walker et al., 2000).

3. Intracellular immunization: Protection of $CD4^+$ T cells

The second gene therapeutic strategy for HIV-1 infection was termed 'intracellular immunization' (Baltimore, 1988) and involves the expression of an antiviral gene in cells susceptible to HIV-1 infection. The target cells for intracellular immunization strategies therefore are mainly peripheral T cells or hematopoietic stem cells. The gene product can either be a protein or an RNA that inhibits HIV-1 replication by interfering with crucial steps of the viral life cycle or by targeting a cellular factor required for virus replication. Efficient genetic protection of the HIV-1 target cell population, i.e. mainly $CD4^+$ T-helper cells, will deprive the virus of the possibility to produce progeny and is therefore expected to result in a drop of viral load and a regeneration of T cell counts. An additional antiviral effect can be achieved, if sufficient T-helper cell clones specific for HIV-1 antigens are protected against viral infection. These gene-protected $CD4^+$ T cells could support the immunologic control of viral replication, without risking infection facilitated by HIV-1 antigen activation. As mentioned above, previous studies have shown that HIV-specific T-helper cell clones, which are crucial for an effective immune control of HIV-1 replication, are preferentially infected by HIV and lost during the course of the disease (Douek et al., 2002).

3.1 Antiviral proteins

Various types of anti-HIV proteins have been developed over the past years. Dominant-negative forms of both, viral proteins and cellular proteins required for virus replication have been described. These dominant-negative mutant proteins antagonize the activity of their corresponding wild-type proteins and thus prevent viral replication. A transdominant form of the HIV-1 Rev protein, RevM10, has been extensively studied *in vitro* and *in vivo*. RevM10 prevents the export of genomic viral RNA from the nucleus and as a result inhibits production of progeny virus (Malim et al., 1989). In clinical trials genetic modification of $CD4^+$ T cells and $CD34^+$ hematopoietic stem cells with RevM10 has been shown to be safe and provide some selective survival advantage. However, no sustained absolute accumulation of gene-modified cells and accordingly no antiviral effect was observed (Morgan et al., 2005; Podsakoff et al., 2005; Ranga et al., 1998; Woffendin et al., 1996). Furthermore, transdominant mutants of HIV-1 Tat that prevent Tat transactiviation have been developed (Fraisier et al., 1998; Pearson et al., 1990), but were never tested in clinical trials. The same is true for transdominant forms of the HIV-1 proteins Gag (Trono et al., 1989) and Vif (Morgan et al., 1990; Vallanti et al., 2005).

Cellular proteins required for virus replication have also been targeted by dominant-negative mutants. Membrane expression of chemokine receptor 5 (CCR5), which acts as a co-receptor for HIV-1, has been blocked by transdominant negative CCR5 variants upon retroviral expression in human T cells (Luis Abad et al., 2003). Even though inhibition of virus replication was observed in the gene-modified cells, this concept was not pursued further. A truncated soluble form of the cell surface receptor CD4 (sCD4) has been described to protect T cells from entry of laboratory-adapted strains of HIV-1, however, inhibition was less efficient for primary virus isolates (Daar & Ho, 1991; Morgan et al., 1994; Morgan et al., 1990). In a clinical phase I study recombinant soluble CD4 was administered by continuous

intravenous infusion to paediatric AIDS patients. Although the therapy was well tolerated, evidence of *in vivo* antiviral activity was not observed and consequently, sCD4 has never been investigated in gene therapy clinical trials (Husson et al., 1992).

Nevertheless, gene therapeutic strategies targeting early steps in the viral life cycle are expected to be the most promising therapeutics for HIV/AIDS, as discussed below. Protein-based inhibitors targeting the virus entry process are thought to be especially powerful tools as they can prevent infection of the cell. Our group has previously developed a membrane-anchored gp41-derived HIV-1 fusion inhibitor, maC46. This protein is expressed on the surface of T cells after transduction with retroviral or lentiviral vectors (Egelhofer et al., 2004; Hermann et al., 2009b; Perez et al., 2005). The protein binds to the HIV-1 gp41 heptad repeat 1 region thereby interfering with six-helix bundle formation during the viral and cellular membrane fusion process. MaC46 expressing T cells are almost completely protected from HIV-1 entry and have a strong selective survival advantage compared to unmodified cells *in vitro* and in mouse models of HIV-1 infection (Egelhofer et al., 2004; Hermann et al., 2009a; Kimpel et al., 2010). Likewise, maC46 has been shown to be one of the most potent anti-HIV gene products currently available (Kimpel et al., 2010). However, in a clinical trial with 10 HIV-1-infected patients with advanced disease and HAART failure, infusion of autologous $CD4^+$ T cells genetically modified to express maC46 did not achieve sustained success. Although a significant rise in overall $CD4^+$ T cell counts was observed in this study, the gene-protected cells did not accumulate over time and consequently, viral loads were not affected (van Lunzen et al., 2007). Recently, we described a secreted version of the fusion inhibitory C46 molecule. This '*in vivo* secreted antiviral entry inhibitor' (iSAVE) showed promising anti-HIV activity *in vitro* and has the potential to confer an overall antiviral effect *in vivo* despite low levels of gene marking (Egerer et al., 2011).

A different protein-based approach for HIV-1 gene therapy uses antibodies that bind and inactivate proteins and enzymes required for virus replication. Antibodies can be expressed within gene-modified cells as single-chain fragments (scFv), so-called intrabodies, or they can be secreted into the supernatant as neutralizing antibodies. Various intrabodies against HIV-1 proteins including Tat, Vif, Reverse Transcriptase and Integrase have been shown to inhibit virus replication in gene-modified cells *in vitro* (Goncalves et al., 2002; Kitamura et al., 1999; Levy-Mintz et al., 1996; Mhashilkar et al., 1995; Shaheen et al., 1996). Moreover, intrabodies against the viral co-receptors CXCR4 and CCR5 have been designed that retain these proteins in the ER (BouHamdan et al., 2001; Cordelier et al., 2004; Swan et al., 2006). Although these approaches efficiently inhibited HIV-1 replication in cell culture systems, intrabody techniques have not been further evaluated *in vivo*. The secreted version of the broadly neutralizing anti-gp41 monoclonal antibody 2F5 has been analyzed in a humanized mouse model of HIV-1 infection (Sanhadji et al., 2000). In this study, gene-modified cell lines expressing the antibody were implanted as neo-organs into immunodeficient mice repopulated with human $CD4^+$ T cells. The neo-organs engrafted in the peritoneum and permitted continuous secretion of the antibody. Upon infection of the mice with HIV-1, viral loads were greatly reduced compared to control animals. However, due to safety problems, the implantation of neo-organs is not an option for treatment of patients. Recently, Joseph and colleagues reported the secretion of therapeutic concentrations of the broadly neutralizing anti-gp120 antibody 2G12 in humanized mice. Here, immunodeficient mice were transplanted with human hematopoietic stem cells that had previously been modified with a lentiviral vector encoding 2G12. The transduced stem cells differentiated into human

progeny cells that secreted the functional antibody into the serum. This genetic immunization clearly reduced viral burden upon HIV-1 infection (Joseph et al., 2010).
Another strategy described by Sarkar et al. involves the expression of a modified version of the Cre recombinase (termed Tre) in HIV-1 infected cells. This recombinase has been engineered in a directed evolution approach to recombine a sequence present in the HIV-1 LTRs resulting in site-specific excision of the integrated provirus from an HIV-1 infected host cell genome (Sarkar et al., 2007). Even though proof-of-concept was provided *in vitro*, this approach is far from clinical application as the Tre recombinase is specific for one exclusive LTR sequence and does not recognize the LTRs of other virus strains.
Finally, zinc finger nucleases (ZFNs) are a novel tool in protein-based HIV-1 gene therapy. ZFNs are artificial fusion proteins composed of a DNA-binding and a DNA-cleavage domain. They can be engineered to bind any desired genome sequence and induce double-strand breaks in the targeted DNA. Repair of the damaged DNA is associated with the introduction of high-frequency deletions and insertions at the site of cleavage. Individuals with a naturally occurring 32 bp deletion mutant of the CCR5 receptor (CCR5Δ32) are perfectly healthy, but resistant to infection with R5-tropic strains of HIV-1 (Huang et al., 1996; Samson et al., 1996). Consequently, disruption of the CCR5 locus using ZFNs is not expected to alter immune functions, making it an ideal target for ZFN-based gene therapy. CCR5-specific ZFNs have been studied *in vitro* and in animal models and were shown to render the treated cells resistant to HIV-1 infection (Holt et al., 2010; Perez et al., 2008). Currently three clinical trials are recruiting patients to test this promising approach *in vivo* (www.ClinicalTrials.gov; identifier: NCT01044654, NCT01252641 and NCT00842634).

3.2 Antiviral RNAs

Antiviral RNAs for intracellular immunization can be grouped into four major categories: RNA interference (RNAi), ribozymes, anti-sense RNAs and RNA decoys. Several RNAi-based gene therapy regimens for treatment of HIV-1 infection have proven to be effective in blocking viral replication by selective degradation of either viral RNAs or mRNAs of host factors that are essential for HIV-1 replication. Basically all HIV-1 RNAs have been successfully downregulated by RNAi *in vitro* (Chang et al., 2005; Coburn & Cullen, 2002; Jacque et al., 2002; N. S. Lee et al., 2002; Novina et al., 2002). However, systemic delivery of siRNAs to the relevant cell types *in vivo* is difficult. Kumar and colleagues administered antiviral siRNAs conjugated to a T cell-specific single-chain antibody that undergoes internalization upon binding to T cell surface receptors to humanized mice. This approach allowed targeted delivery of the siRNAs to T cells, which resulted in effective virus inhibition and preserved $CD4^+$ T cell numbers (Kumar et al., 2008). An alternative to the regular injection of exogenous siRNAs is the expression of shRNAs directly in the HIV-1 target cells, but achieving stable transgene expression in the gene-modified cells is a challenge. Yet, constant endogenous expression would be required to obtain efficient suppression of HIV-1 replication and prevent viral escape mutants. At the same time, expression levels have to be tightly regulated in order to avoid cellular toxicity, as saturation of the cellular small RNA-processing pathway due to overexpression of shRNAs can lead to downregulation of cellular microRNAs (miRNAs) and cause severe toxicity (Grimm et al., 2006). Insertion of shRNAs into a natural miRNA backbone has been shown to reduce such toxic effects (McBride et al., 2008).
The high mutation rate of HIV-1 is an additional challenge in developing RNAi-based therapeutics, as a single point mutation within the targeted HIV-1 RNA sequence can

abolish function of small RNAs and escape mutants emerge rapidly (Boden et al., 2003; Das et al., 2004; Sabariegos et al., 2006). This problem can be partly overcome by using a combination of small RNAs targeting several conserved regions of the viral genome and ideally expressed from a single therapeutic vector (ter Brake et al., 2006). Alternatively, cellular genes required for virus replication can be targeted, including CD4, CXCR4, CCR5, nuclear factor κB, or LEDGF/p75, which all have been shown to be susceptible to RNAi silencing, thereby blocking viral entry or replication (Anderson & Akkina, 2005; Cordelier et al., 2003; Novina et al., 2002; Surabhi & Gaynor, 2002; Vandekerckhove et al., 2006). The CCR5 receptor is a particularly promising target, as disruption of the CCR5 gene does not alter immune functions (Huang et al., 1996; Samson et al., 1996). A highly potent and non-cytotoxic siRNA directed against CCR5 has been developed by the group of Irvin Chen. Stable long-term expression of the siRNA and silencing of the CCR5 gene was observed after transplantation of gene-modified $CD34^+$ hematopoietic progenitor cells in non-human primates. Gene-modified cells isolated from the animals were resistant to simian immunodeficiency virus infection *ex vivo* (An et al., 2007; Liang et al., 2010). The only RNAi approach that has been examined in patients so far is a tat/rev specific short hairpin RNA, which was tested in combination with a ribozyme targeting CCR5 and a TAR decoy in four patients receiving $CD34^+$ hematopoietic progenitor cell transplantation due to AIDS-related lymphoma. In this recently reported clinical trial, stable, but low-level expression of the antiviral RNAs from gene-modified cells was observed for up to 24 months; however, there were no major effects on viral load (DiGiusto et al., 2010).

Ribozymes are anti-sense RNA molecules with enzymatic activity that have been designed to target and site-specifically cleave essential viral RNAs or cellular mRNAs leading to gene silencing. Many ribozyme-based strategies for treatment of HIV-1 infection have been developed and show promising antiviral activity *in vitro* (Hotchkiss et al., 2004; Sarver et al., 1990; Zhou et al., 1994). Three ribozymes directed against HIV-1 tat/vrp (Amado et al., 2004; Macpherson et al., 2005; Mitsuyasu et al., 2009), HIV-1 rev/tat (Michienzi et al., 2003) and the viral U5 leader region (Wong-Staal et al., 1998) have already been tested in separate clinical trials. The gene transfer was proven to be safe in all studies, but none showed significant antiviral efficacy. As mentioned above, a CCR5-specific ribozyme has recently been analyzed in the clinic in combination with two other types of anti-HIV RNAs, but did not have a major influence on HIV-1 replication (DiGiusto et al., 2010).

Antisense RNAs are short or long single-stranded RNA molecules binding to complementary HIV-1 mRNAs resulting in the formation of non-functional duplexes. Antisense molecules directed against the HIV-1 trans-activation response element (TAR) and the viral envelope RNA have been developed (Humeau et al., 2004; Lu et al., 2004; Vickers et al., 1991). The conditionally replicating lentiviral vector VRX496™ encodes a long antisense gene against the HIV-1 envelope. In clinical trials patients received autologous $CD4^+$ T cells transduced with VRX496™. A stabilization of viral load and slightly increased $CD4^+$ T cell counts were observed, the significance of these results, however, remains unclear (Levine et al., 2006).

In contrast to the antiviral RNA molecules described above, RNA decoys do not attack the HIV-1 RNA. Instead, these small RNA fragments, which are derived from cis-acting elements in the viral genome, competitively bind and sequester viral proteins, thereby interfering with HIV-1 replication. Anti-HIV decoys are mainly based on the HIV-1 regulatory sequences TAR and Rev-responsive-element (RRE), which are bound by the two HIV-1 regulatory proteins Tat and Rev, respectively. The TAR element is a sequence

contained in the 5′ region of all HIV-1 mRNAs, which forms a stable stem-loop structure (Baudin et al., 1993; Muesing et al., 1987). Binding of the HIV-1 Tat (trans-activator) protein to the TAR element mediates increased viral gene expression (Keen et al., 1996); moreover, the TAR region is required for initiation of reverse transcription (Harrich et al., 1996). Disruption of the Tat/TAR interaction by TAR decoy RNA was shown to effectively prevent HIV-1 replication *in vitro* (Sullenger et al., 1990, 1991). A combination of three antiviral RNAs including a TAR decoy was successfully tested in humanized mouse models of HIV-1 infection (Anderson et al., 2007) and, as mentioned above, was shown to be safe in a clinical trial, although only minor effects on HIV-1 infection could be observed (DiGiusto et al., 2010). Interaction of the viral Rev protein with the RRE is critically required for transport of the unspliced genomic viral mRNA to the cytoplasm (Olsen et al., 1990). RRE decoys provide strong inhibition of HIV-1 replication by blocking the nuclear export of genomic HIV-1 RNA (T. C. Lee et al., 1992; Michienzi et al., 2006). Genetic modification of $CD34^+$ hematopoietic stem cells by retroviral transfer of an RRE decoy gene followed by infusion of the gene-modified cells, was shown to be safe in a clinical trial with paediatric AIDS patients. However, transduction and engraftment levels were very low and no antiviral effect was observed (Kohn et al., 1999).

3.3 The mode of action: Classification of antiviral genes

The impact of gene-modified cells on systemic HIV-1 kinetics depends critically on the stage of the viral replication cycle at which the inhibition occurs. The antiviral genes can accordingly be grouped into three classes, depending on their effect on the viral life cycle (von Laer et al., 2006). Class I genes inhibit the first steps of the replication cycle prior to integration of the proviral DNA into the host cell genome and thus prevent infection of the cell. Hence, class I includes genes encoding entry inhibitors, as well as inhibitors of reverse transcription and integration. Class II genes have no effect on early steps of the viral replication cycle, but prevent the expression of viral RNA or proteins. Thus, they inhibit the production of infectious virus progeny and the viral cytopathic effect, however, integration of the proviral genome into the host cell chromosomes is not hindered. Cells expressing a class II gene and infected with HIV-1 resemble latently infected cells and according to computer simulations accumulate with time, counteracting the antiviral effect (von Laer et al., 2006). Furthermore, reverse transcription, which can give rise to resistant virus variants, is not inhibited by class II genes. Class III genes interfere with late steps in the viral life cycle such as virion assembly and budding. Consequently, they neither protect the infected cell from recognition by the immune system, as viral protein production is not inhibited, nor from the viral cytopathic effect. Therefore, class III genes alone are not expected to have an overall antiviral effect unless high percentages of cells are genetically modified.

Mathematical modelling predicts that only genes inhibiting early steps in the viral replication cycle provide a selective advantage strong enough to allow for the selection and accumulation of the gene modified cells (Lund et al., 1997; von Laer et al., 2006). Consequently, class I genes are the most promising candidates for successful intracellular immunization strategies. The group of Warner Greene recently found that in *ex vivo* cultures of human tonsils infected with HIV-1 the vast majority of $CD4^+$ T cells died due to non-productive abortive infection. In these non-permissive cells DNA reverse transcription intermediates elicited proapoptotic responses resulting in release of proinflammatory cytokines and caspase-mediated cell death (Doitsh et al., 2010). Inhibition of HIV-1 entry or early steps of reverse transcription could protect the cells from these fatal effects, indicating

that very early inhibitors are even more favorable, as they can prevent the massive "bystander" killing observed in HIV-1 infection. However, combination of several antiviral genes, targeting different steps in the viral life cycle might synergize most efficiently and could be the only way to achieve sustained suppression of HIV-1 replication.

3.4 Selective survival advantage and bystander effect

The major drawback of intracellular immunization approaches is the huge number of HIV-1 target cells in the human body (> 10^{11}) that cannot be genetically modified with the available technologies. The frequencies of gene-modified $CD4^+$ T cells achieved *in vivo*, whether by T cell or stem cell targeting, have been disappointingly low, in the range of 0,01% to 1%, or less (Amado et al., 2004; Levine et al., 2006; Macpherson et al., 2005; Morgan et al., 2005; van Lunzen et al., 2007). A significant impact of these few genetically HIV-resistant cells is neither expected on overall HIV-1 infection dynamics nor on immune reconstitution. However, if the gene-protected cells are able to proliferate and preferentially survive compared with unmodified cells, they could accumulate with time and progressively repopulate the immune system (Lund et al., 1997; von Laer et al., 2006). A number of gene products that have been developed could theoretically mediate such a selective survival advantage of the transduced cells as they have been shown to efficiently suppress virus replication and protect the cells from the viral cytopathic effect *in vitro*. However, selective accumulation of gene-protected cells has never been observed in clinical trials so far. In a comparative study, we recently evaluated three intracellular immunization strategies that had previously been used in the clinic, with respect to antiviral activity and survival advantage (Kimpel et al., 2010): (1) the viral entry inhibitor maC46 (class I gene); (2) an HIV-1 tat/rev-specific small hairpin (sh) RNA (class II gene); and (3) an RNA antisense gene specific for the HIV-1 envelope (class II gene). We found robust inhibition of HIV-1 replication with the fusion inhibitor maC46 and the antisense envelope inhibitor. Interestingly, and importantly, a survival advantage was merely demonstrated for cells expressing the maC46 fusion inhibitor both *in vitro* and *in vivo* in a humanized xenotransplant mouse model (Kimpel et al., 2010). This finding confirms *in silico* predictions stating that only class I genes can confer a sufficient selective advantage to allow preferential survival and accumulation of gene-protected, non-infected cells *in vivo* (von Laer et al., 2006). However, even this highly active fusion inhibitor failed to show a clear accumulation of gene-protected cells to therapeutic levels in a previous clinical trial in 10 AIDS patients (van Lunzen et al., 2007). These data show that efficient engraftment and proliferation of the gene-protected cells remain a major challenge in gene therapy for HIV-1 infection.

Yet, such a strong selection and accumulation may not be required for a secreted antiviral gene product. Secreted antiviral proteins or peptides are expected to produce a bystander effect on unmodified neighboring cells, thereby suppressing virus replication and protecting the overall T cell pool even at low levels of gene modification. Such a bystander effect can only be conferred by antiviral proteins, but not by RNAs, as secretion is limited to proteins and peptides. However, the number of reports on secreted antiviral proteins in HIV-1 gene therapy is still very limited. Examples are neutralizing antibodies (Sanhadji et al., 2000), truncated soluble CD4 (Morgan et al., 1994; Morgan et al., 1990) and interferon β (Gay et al., 2004). We have recently reported the generation of an *in vivo* secreted antiviral entry inhibitor (iSAVE), which exerted a strong bystander effect in cell culture (Egerer et al., 2011). Lymphatic tissue is the major site of HIV-1 replication and thus T or B cells could be an ideal target cell for gene therapy approaches based on secreted gene products. Secretion of

antiviral proteins directly in the lymphoid tissues is likely to lead to high and stable local peptide concentrations and to substantially suppress virus replication. On the other hand, secreted antiviral gene products no longer depend on expression in HIV-1 target cells, but instead several other cell types could serve as producer cells in the body. This facilitates the development of direct *in vivo* gene transfer approaches (e.g. by the use of AAV-vectors), making gene therapy less complex and practicable also for treatment of patients in the developing world. Furthermore, such strategies have the potential for application as a gene transfer vaccine in a prophylactic setting. In this regard, a secreted antiviral gene product could for instance be used as a genetic topical microbicide that aims at the prevention of HIV-1 mucosal transmission. High-level secretion of the antiviral molecules from gene-modified target cells in the vagina or rectum has the potential to confer local sterilizing immunity, thus preventing HIV-1 genital transmission.

3.5 Immunogenicity of the antiviral gene product

Gene therapeutic strategies based on the expression of antiviral proteins are limited by the potential immunogenicity of the antiviral gene product, which can severely impair survival of the transduced cells. Antiviral RNAs have an advantage here, as they generally lack immunogenicity. Also, natural or only slightly altered variants of human proteins are not expected to mount significant immune responses. However, many antiviral proteins are non-self and bear the risk of eliciting a cellular immune response. Thus, to prevent selective deletion of the gene-modified cells by transgene-specific CTLs, it is necessary to minimize or eliminate immunogenicity of the antiviral gene product. The fusion of a Glycine-Alanine repeat derived from the Epstein-Barr virus nuclear antigen 1 (EBNA-1) to immunogenic proteins, such as transdominant HIV-1 Gag, has been shown to significantly reduce immunogenicity and prolong survival of transduced cells *in vivo* (Hammer et al., 2008). The Glycine-Alanine repeat protects the fusion protein from proteasomal degradation and prevents subsequent presentation of potentially immunogenic peptides on MHC class I molecules (Levitskaya et al., 1997). This frustrates the induction of CTLs directed against the transgene product and conceals it from CTL-mediated immune attack.

Another possibility to facilitate immune-evasion, which is feasible for small antiviral peptides only, is the generation of peptides which are devoid of MHC class I epitopes. Our group recently developed antiviral C peptides with potentially reduced immunogenicity, by mutating *in silico*-predicted immunodominant CTL epitopes within the peptide sequence. The mutated peptides retained excellent anti-HIV activity, while no immune responses could be detected in ELIspot assays (unpublished data).

3.6 The target cell for intracellular immunization strategies

Target cells for intracellular immunization are usually cells that can become infected with HIV-1 (mainly $CD4^+$ T cells) or their progenitors (hematopoietic stem cells). For gene therapeutic approaches based on secreted antiviral molecules, the modification of non-HIV-target cells is also feasible. So far, mature T cells and hematopoietic stem cells have been used in clinical trials. Advantages and disadvantages of both cell types are summarized in Table 1. Both have in common that they are relatively easy to obtain, and there are protocols for efficient *ex vivo* cultivation and transduction available. Gene modification of HSC has greater therapeutic potential, as it could restore a normal T cell repertoire, allow regeneration of HIV-specific T-helper cells and also protect monocytes/macrophages. However, current stem-cell based therapies are associated with greater risks and toxicity.

Target cell	T cell	HSC
Easy to obtain	++	+
Conditioning required	-	+
Cell dose	>10^{10}	10^8-10^9
Regeneration of T cell repertoire	-	+
Protection of all HIV-1 target cells	-	+
Insertional mutagenesis	Limited	+

Table 1. The target cell: T cells versus hematopoietic stem cells (HSC).

4. Vector systems for gene transfer

The choice of the vector system may have a major impact on the efficacy of HIV-1 gene therapy approaches. There is no ideal vector suitable for all purposes, but the pros and cons have to be balanced for each application. Table 2 summarizes advantages and disadvantages of several vector systems commonly used for gene transfer.

Vector type	Application	Titer	Packaging capacity	Inte-gration	Immuno-genicity	Clinical trials
Adenoviral	*ex vivo* + *in vivo*	10^{13} VP/ml	up to 36 kb	-	++	+
AAV	*in vivo*	10^{13} VP/ml	3-5 kb	only with Rep	+	+
Gamma-retroviral	*ex vivo*	10^5-10^7 TU/ml	8-10 kb	+	-	+
Lentiviral	*ex vivo*	10^7 TU/ml (10^9-10^{10} TU/ml)*	up to 10 kb	+	-	+
SV-40	*ex vivo*	10^{12} VP/ml	up to 5 kb	+	-	-
Foamyviral	*ex vivo*	10^5-10^6 TU/ml (10^7 TU/ml)*	>9 kb	+	-	-

Table 2. Vector systems for gene transfer in HIV-1 gene therapy.
AAV, Adeno-Associated Virus; kb, kilo bases; SV-40, Simian Virus-40; TU, transducing units; VP, vector particles; * after concentration.

Basic questions to be asked are, whether *ex vivo* or *in vivo* gene transfer is preferred and if long-term expression of the gene product is required. To our knowledge, a vector system that allows efficient direct *in vivo* gene transfer specifically into $CD4^+$ T cells or HSC has not been developed so far. Therefore, approaches based on modification of these cell types (arming of T cells with TCRs or CARs, most intracellular immunizations) always rely on *ex vivo* gene transfer. In contrast, genes encoding secreted antiviral molecules may also be delivered to distinct production sites (e.g. liver, muscle) directly *in vivo* using adenovirus (Ad) or adeno-associated virus (AAV)-derived vectors. The second basic question deals with the long-term-expression of the transferred antiviral gene. Strategies involving arming of T

cells with antigen-specific receptors and intracellular immunization require stable and long-lasting production of the antiviral molecules in proliferating cells (T cells or HSC). Consequently, for such approaches, integrating vectors are favorable. These include SV-40 vectors and vectors derived from retroviruses. Stable expression of secreted gene products from slowly dividing cells like liver or muscle cells may also be achieved using non-integrating vectors like adenoviral or AAV vectors.

4.1 Non-integrating viral vectors

The only vectors systems that currently allow direct *in vivo* gene transfer are non-integrating viral vectors. As their genome is not stably incorporated into the host cell chromosomes, these vectors have an improved safety profile compared to integrating vectors. For a direct *in vivo* application of integrating vectors, efficient systems for targeted vector delivery would be required, which are not yet available for use in man. Despite the lack of integration, non-integrating vector systems still allow sustained long-term transgene expression, if cells or tissues with a slow turnover are targeted, where the vector genome can stably persist. Moreover, non-integrating vectors can also be used to deliver zinc finger nucleases, which require only transient expression, to diving cells. A number of non-integrating viral vectors have been evaluated for gene transfer. Currently, adenoviral vectors and vectors derived from the adeno-associated virus are in the focus of interest. Accordingly, Ad vectors are currently used in the above mentioned clinical trials to deliver CCR5-specific zinc finger nucleases to T cells *ex vivo*.

Recombinant adenoviral vectors have been utilized as a gene transfer and vaccine platform for a long time. Ad vectors provide a huge packaging capacity (36 kb), allowing the transfer also of multiple therapeutic genes. Moreover, high-titer production is possible, facilitating direct *in vivo* application with high transduction efficacies. The major obstacle of adenoviral vectors is pre-existing immunity in the general human population. Vector-mediated immune responses cause rapid clearance of Ad vectors, moreover, severe side effects have been observed. This can be partly overcome by using engineered adenovirus serotypes (Dharmapuri et al., 2009). Moreover, production of Ad vectors is prone to contamination with replication competent adenovirus, which complicates clinical grade vector production.

AAV is a non-pathogenic virus that belongs to the family of Parvoviridae. AAV-derived vectors have recently gained particular interest as gene transfer vehicles due to their apathogenicity and very low immunogenicity. Moreover, they can be used for direct *in vivo* gene delivery to both dividing and non-dividing cells. AAV can infect a variety of cell types *in vivo* and different serotypes of AAV have been shown to have varying preferences in their target cell type of choice (Chao et al., 2000; Halbert et al., 2000). However, AAV variants with a preference for T cells or hematopoietic stem cells have not been described. In the absence of the viral Rep protein, AAV vectors do not integrate into the host cell genome, but are maintained in episomal form in the nucleus. This allows very stable transgene expression without causing genotoxicity. The major disadvantage of AAV vectors is their small packaging capacity. In addition, vector production used to be laborious in the past and large-scale manufacturing for clinical trials was complicated. However, novel production systems facilitate faster and simpler high-titer production of AAV vectors in scaleable processes (Clement et al., 2009; Lock et al., 2010).

4.2 Integrating viral vectors

Vectors derived from gamma-retroviruses (mostly murine leukaemia virus) and lentiviruses (HIV-1) have been used in numerous clinical trials, including *ex vivo* gene transfer trials for HIV-1 infection (DiGiusto et al., 2010; van Lunzen et al., 2007). Replication incompetent viral vectors are made from these viruses by deletion of all genes encoding enzymes and structural proteins (Gag, Pol, Env) from the viral genome. These genes have to be added *in trans* to produce infectious, but replication incompetent, vector particles. The tropism of the vector particles can be altered by modification of the envelope glycoprotein or by pseudotyping with the envelope protein from a different virus (Frecha et al., 2008; Funke et al., 2008). A major difference between gamma-retroviral and lentiviral vectors is that lentiviruses can infect dividing as well as non-dividing cells. In contrast, gamma-retroviruses can only transduce dividing cells, as they rely on the collapse of the nuclear membrane during mitosis to enter the nucleus (Roe et al., 1993). Lentiviral transduction protocols therefore usually require a shorter period of pre-activation of the cells. As prolonged *in vitro* culture is associated with differentiation and a loss of *in vivo* repopulation potential, especially for HSC, lentiviral vectors have an advantage here. However, large-scale production of lentiviral vectors is more difficult than gamma-retroviral vector production due to a lack of stable packaging cell lines.

Both, gamma-retroviral and lentiviral vectors integrate randomly into the host cell genome. While gamma-retroviruses usually integrate near transcriptional start sites, lentiviruses have a preference for transcribed regions (Mitchell et al., 2004). As a consequence, transduction with these vectors is always associated with a risk of transformation due to insertional mutagenesis. Indeed, severe side effects caused by vector integration have been reported in gene therapy clinical trials (Hacein-Bey-Abina et al., 2008; Howe et al., 2008). Experiments in animal models showed that vector genotoxicity is higher for transduction of hematopoietic stem cells than for mature T cells (Newrzela et al., 2008) and lower for self inactivating (SIN) vectors compared to conventional long terminal repeat (LTR)-driven vectors (Modlich et al., 2009). SIN vectors have deletions in the promotor and enhancer elements of the 3'LTR, thereby reducing the genotoxic risks, as transactivation of neighboring protooncogenes is less likely. In these vectors, expression of the transgene cassette is driven from an internal promoter.

Foamyviruses also belong to the family of retroviruses. Foamyviral vectors are generated by deleting enzymes and structural genes from the viral genome and adding these *in trans* during vector production (Rethwilm, 2007). Foamyvirus-derived vectors efficiently transduce resting cells, which makes them an ideal tool to transduce hematopoietic stem cells *ex vivo* (Hirata et al., 1996; Leurs et al., 2003). However, just like SV-40 vectors, foamyviral vectors have not yet been tested in clinical trials.

Simian virus-40 (SV-40) belongs to the family of Polyomaviridae. It has been one of the first viruses used as a gene transfer vehicle (Gething & Sambrook, 1981). For the construction of gene transfer vectors, all coding sequences except the origin of replication and the packaging signal can be deleted from wild type SV-40 (Strayer et al., 2002). The resulting vectors efficiently transduce hematopoietic stem cells and lymphocytes *in vitro*, but have never been tested in clinical trials (Strayer et al., 2005).

4.3 Targeted integration

Past clinical trials have shown that random integration of a transgene delivered by an integrating vector bears the risk of severe side effects due to insertional mutagenesis.

Targeted integration of transgenes into the host cell genome is therefore expected to massively increase safety. The CCR5 locus is considered to be a safe harbor for transgene integration, as a naturally occurring deletion of 32 bp in the coding sequence for CCR5 causes no clinical symptoms. Moreover, this deletion is associated with a reduced susceptibility for HIV-1 infection (Huang et al., 1996; Samson et al., 1996). Therefore, targeted integration of an anti-HIV transgene into the CCR5 locus could even provide an additional antiviral effect, due to disruption of the CCR5 gene. Zinc finger nucleases binding to CCR5 have been used *in vitro* and in animal models to destroy the CCR5 locus, rendering the treated cells resistant to HIV-1 infection (Holt et al., 2010; Perez et al., 2008). A combination of this approach with targeted integration of antiviral genes holds especially great promise. For such a strategy a donor DNA encoding the desired antiviral gene and containing sequences homologous to the target site has to be present in the cells during the repair of ZFN-induced double-strand breaks by cellular enzymes. This results in the incorporation of the foreign DNA into the targeted region of the host genome by non-homologous end joining mediated by the cellular DNA repair machinery (Cathomen & Joung, 2008). Such approaches require only transient expression of the zinc finger nuclease and the transgene to achieve stable integration into the host cell genome, which allows use of non-integrating vector systems for gene transfer.

As an alternative to zinc finger nucleases, AAV vectors that contain the viral Rep protein in *cis* or in *trans* can also be used for targeted integration, as in the presence of Rep, AAV vectors target their genome preferentially to a locus on the human chromosome 19, termed AAVS1, without causing any apparent adverse effects (Surosky et al., 1997). As the CCR5 locus, AAVS1 is thus considered a safe harbor for vector integration.

5. Conclusions

Gene therapeutic approaches for the treatment and possibly prevention of HIV-1 infection hold considerable promise. Although the final breakthrough has not yet been achieved in clinical trials, there has been substantial progress over the last years and future developments might leverage this technology. The major reason for the limited efficacy seen in all HIV-1 gene therapy clinical trials up to now has been the insufficient level of gene modification. It will therefore be particularly important to develop optimized therapeutic regimen and gene transfer technologies that allow therapeutically effective engraftment levels of functional, gene modified cells. Efficient protection of $CD4^+$ T cells could possibly be achieved by using a combination of antiviral genes targeting different steps of the viral life cycle, conferring a substantial *in vivo* selective survival advantage and ideally also a therapeutic bystander effect on unmodified cells. This review describes the potent gene therapeutic tools that have been developed in the past years and it will be exciting to see if these can be integrated into an effective treatment regimen in the near future.

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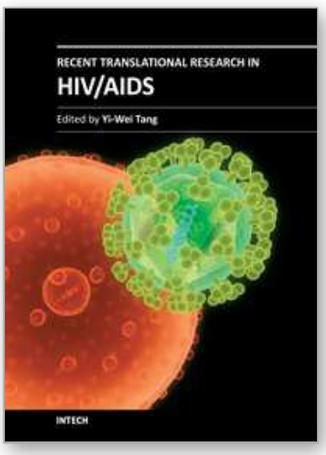

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The collective efforts of HIV/AIDS research scientists from over 16 countries in the world are included in the book. This 27-chapter Open Access book well covers HIV/AIDS translational researches on pathogenesis, diagnosis, treatment, prevention, and also those beyond conventional fields. These are by no means inclusive, but they do offer a good foundation for the development of clinical patient care. The translational model forms the basis for progressing HIV/AIDS clinical research. When linked to the care of the patients, translational researches should result in a direct benefit for HIV/AIDS patients.

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