

# Current Status of Gene Therapy Strategies to Treat HIV/AIDS

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Progress in developing effective gene transfer approaches to treat HIV-1 infection has been steady. Many different transgenes have been reported to inhibit HIV-1 *in vitro*. However, effective translation of such results to clinical practice, or even to animal models of AIDS, has been challenging. Among the reasons for this failure are uncertainty as to the most effective cell population(s) to target, the diffuseness of these target cells in the body, and ineffective or insufficiently durable gene delivery. Better understanding of the HIV-1 replicative cycle, host factors involved in HIV-1 infection, vector biology and application, transgene technology, animal models, and clinical study design have all contributed vastly to planning current and future strategies for application of gene therapeutic approaches to the treatment of AIDS. This review focuses on the newest developments in these areas and provides a strong basis for renewed optimism that gene therapy will have an important role to play in treating people infected with HIV-1.

**Key Words:** AIDS, cellular cofactors, gene therapy, transduction, viral vectors

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## INTRODUCTION

The success of highly active antiretroviral therapy (HAART) has brought into question the need for continued research in AIDS gene therapy. Why study expensive, risky, and potentially impractical treatments if safe pharmacologic approaches produce durable remissions? If HAART were curative, inexpensive, and without significant toxicity this argument would be cogent. However, HAART is not totally effective and has problematic side effects. Drug-resistant HIV-1 is increasingly frequent [1–5], even in lymph nodes of patients receiving HAART with undetectable HIV-1 in the blood [6]. Studies of structured treatment interruptions, designed to provide respite from HAART's burdensome toxicities, complexity, and cost, show that HAART cannot be effectively suspended, even briefly [7–11]. Investigation into additional therapeutic approaches should therefore continue. Gene delivery will probably not supplant pharmacotherapy, but may rather play an important supporting role.

The goals of anti-HIV-1 gene therapy are to deliver transgenes: (a) to hematopoietic progenitor cells (HSC) to protect their differentiated progeny from HIV-1; (b) directly to HIV-1-susceptible cells, to render them resistant to HIV-1 infection or inhibit HIV-1 replication in

them; (c) to immunize against HIV-1 antigens; and (d) to inhibit HIV-1 in discrete organ target sites (e.g., central nervous system). This discussion focuses on the first two of these issues. Gene delivery to immunize against HIV-1, either for treatment or prophylaxis, and solid organ-directed gene transfer are separate topics and are not addressed here.

To date, gene therapy targeting HIV-1 has largely failed in all of these tasks. There is nonetheless considerable reason to be optimistic about its future for HIV-1 therapeutics. Analysis of unsuccessful anti-HIV-1 gene therapy studies provides insights that are being applied to improve transgene effectiveness and to understand better what cell populations to target and how to target them. Recent work in rodent and primate models of HIV-1 infection documents the ability of gene delivery *ex vivo* and *in vivo* to inhibit *in vivo* simian immunodeficiency virus (SIV) or HIV-1 challenges [12–14].

Much remains to be done before any type of anti-HIV-1 gene therapy reaches routine clinical practice, but there is reason to believe that this goal is attainable. This review attempts to summarize anti-HIV-1 genetic therapies, emphasizing recent advances in transgene technology, vector development, animal models, and clinical studies.

## DEVELOPMENT OF POTENT ANTI-HIV-1 TRANSGENES

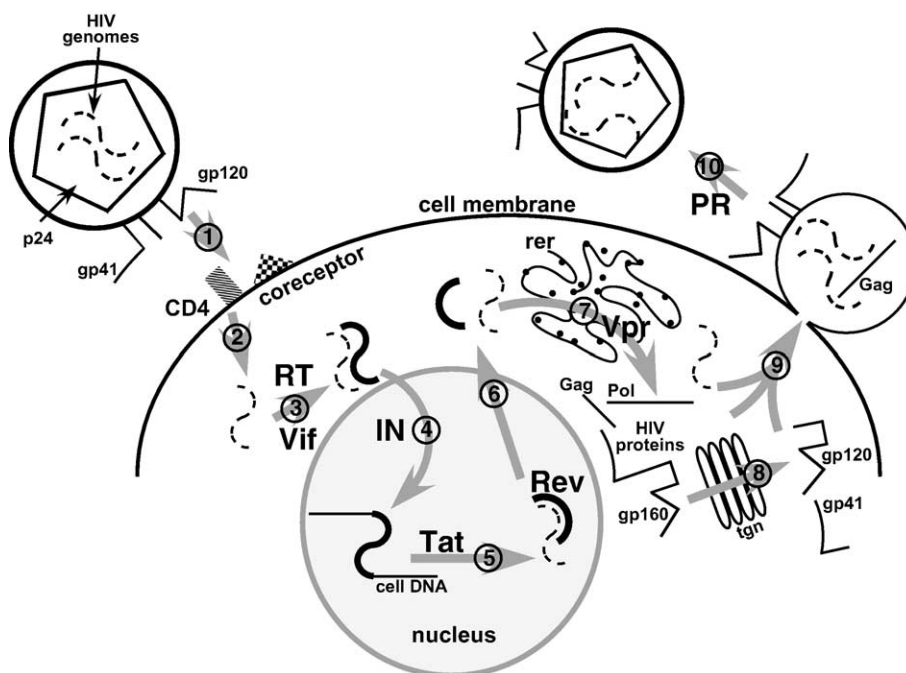
### Strategies to Inhibit HIV-1 by Gene Transfer

The principles of gene delivery to express a gene product and inhibit HIV-1 were enunciated in 1988 [15]. A target function, either an intrinsic HIV-1 function or a cellular function, must first be identified. The replicative cycle of HIV-1 is vulnerable to interruption at many different points. Fig. 1 illustrates some stages in the sequence of HIV-1 infection that have been targeted. Myriad transgenes, acting via diverse mechanisms and expressed with various constitutive and conditional promoters, have been devised. This section illustrates some of these possibilities (see below). A discussion of both standard and newer vectors used experimentally to treat HIV-1-susceptible cells and their derivatives is presented in a subsequent section.

### Protein Strategies

Protein-based anti-HIV-1 strategies have been the single largest area of anti-HIV-1 gene transfer trials in humans (reviewed in [16–18]). Briefly, protein structures developed to inhibit HIV-1 include transdominant negative mutants, intrakines, toxins, single-chain antibodies, and DNA-based vaccines. The first transdominant derivative of an HIV-1 protein that inhibited HIV-1 replication, a Gag mutant [19], stimulated design of other transdominant HIV-1 protein homologs. RevM10, which retains

two Rev functions—the ability to bind RRE and the ability to form Rev multimers—was the first transdominant protein tested in human trials [20,21]. Subsequent examples include *tat* [22] and a fusion of the *tat* and *tat* transdominant genes coding for a Tat/Rev fusion protein, Trev [23]. Intracellular toxins or conditionally toxic proteins, such as herpes simplex thymidine kinase [24], diphtheria toxin [25], and even modified lytic viruses, have been designed for anti-HIV-1 activity [26,27]. HIV-1 uses cellular CD4 as a receptor, and a chemokine-binding coreceptor, to infect cells. Thus, intracellular expression of SDF-1, the ligand for CXCR4, or RANTES or MIP-1 $\alpha$ , ligands for CCR5, may block or sequester receptors intracellularly and decrease membrane receptor molecules available for HIV-1 entry [28–30]. However, inhibition of HIV-1 by these intrakines may involve cytokine secretion and extracellular competition with HIV-1 for their shared receptor [31]. Indeed, very recent work suggests that overproduction of a CCR5 ligand may be a natural means of protection from HIV [32]. CD4 itself can also be used to inhibit HIV-1 infection when expressed intracellularly [33]. Intracellular HIV-1-specific single-chain Fv antibodies (SFv) can target and redirect essential HIV-1 proteins away from required subcellular compartments and block the function or processing of such essential proteins as HIV-1 gp120, Rev, Gag, reverse transcriptase (RT), and integrase (IN) [34–38].



**FIG. 1.** Steps of the HIV-1 replicative cycle commonly targeted, and potentially targetable, for intervention by gene delivery. A diagrammatic representation of the phases of the HIV-1 replicative cycle that are commonly targeted by various gene therapeutic interventions, or that may be attractive targets, and some of the molecular species involved in those steps is shown. (1) HIV-1 binding to cell membrane. (2) HIV-1 entry into the cell. (3) Reverse transcription (RT, reverse transcriptase). Vif also appears to be involved in this step. (4) Transport of the HIV-1 proviral genome into the nucleus and integration into the cellular DNA (IN, integrase). (5) Transcription of the HIV-1 proviral genome to produce both spliced and unspliced HIV-1 RNAs. (Tat, trans-activator of transcription). (6) Binding of the HIV-1 protein, Rev, to some HIV-1 transcripts, to chaperone them through the nuclear membrane into the cytosol. (7) Production of HIV-1 proteins: Vpr, among other functions, enhances production of HIV-1 proteins. (8) Posttranslational modification of HIV-1 gene products, here gp160<sup>Env</sup> is cleaved by cellular enzymes in the trans-Golgi network (tgn) to gp120<sup>Env</sup> and gp41<sup>Env</sup>. (9) HIV-1 virion assembly and morphogenesis within the cell. (10) Maturation of the immature virion into a completely infectious particle. Shown here is the processing of p55<sup>Gag</sup> by HIV-1 protease (PR) to its components.

**RNA Strategies**

RNA approaches include antisense, ribozymes, RNA aptamers and decoys, and RNA interference (RNAi) [16]. Antisense molecules were shown by several groups to inhibit HIV-1 *in vitro* when targeted to such critical HIV-1 genes as *tat*, *rev*, and integrase [39–45]. RNA decoys are RNA homologues, such as TAR and RRE, that bind viral proteins and compete with native ligands necessary for replication [41,46,47]. A “second generation” of RNA-based antisense transgenes encoded ribozymes, RNA molecules that cleave RNA at specific sequences, and aptamers, both of which target HIV-1 at critical sites such as Tat, Rev, and Gag [48–55]. A recent advance in this area is the use of RNAi to inhibit HIV-1 [56–60]. However, RNAi strategies may also be circumvented by escape mutations in the targeted portion(s) of the viral genome, even without necessarily changing the encoded protein [61]. Some transgenes are more effective than others, but it is not easy to articulate guidelines comparing the usefulness of individual transgenes or classes of transgenes. Investigators have greeted discoveries of new types of transgenes enthusiastically, only for the ardor to wane with further study (e.g., RNAi, see above).

There have been so many transgenes tested, with so many promoters and delivery and challenge settings, that comparative efficacy is difficult to determine. Protection from HIV-1 *in vitro* usually depends on challenge dose: many transgenes effective vs low HIV-1 concentrations fail with stiffer challenges. Few studies compare the relative effects of transgenes targeted to specific targets (e.g., RT vs IN, Rev, etc.) (however, see [62]). Similarly, few reports vary the timing of challenges (e.g., effect on acute infection vs controlling chronic infection). Most importantly, it is unclear how protection from HIV *in vitro* would translate to *in vivo* settings.

Some general principles can be enunciated, however. Transgenes directed against HIV genes (e.g., ribozymes, RNAi) work best when directed at highly conserved HIV sequences, although they are prone to HIV escape mutation [63]. Combinations of transgenes targeting diverse parts of the HIV-1 replicative cycle are more likely to be effective than individual transgenes [62]. Finally, because cellular genes mutate much less readily than viral genes, cellular cofactors required for HIV infection are probably better targets than HIV genes or gene products [64]. It is more likely that combinatorial approaches will succeed for anti-HIV gene therapy, as for anti-HIV chemotherapy.

**TARGETING CELLULAR GENE PRODUCTS AND PROCESSES THAT ARE ESSENTIAL FOR HIV-1**

Every virus must use some host cell functions for its replication. For HIV-1 and related primate lentiviruses, several cellular proteins are thought to be essential for efficient viral replication and are now being considered as

**TABLE 1:** Gene therapy targeting cell cofactors utilized by HIV-1 and innate mediators of cellular resistance to HIV-1: potential targets new and old

Type of target	Specific structure	Transgene types used
Receptor	CXCR4	Single-chain antibody (SFv) Ribozyme, siRNA, intrakines
	CCR5	
	CD4	
PIC transport Integration cofactors	$\beta$ -Importin HMG Ini1, LEDGF <sup>a</sup>	I $\kappa$ B, siRNA Dominant negative, RNA-based transgenes
	Vif binding proteins Transcription factors Tat cofactors	
Rev cofactors Gag, morphogenesis and budding	CRM-1, exportin-1 TSG101 and others	siRNA
Proprotein processing	Enzymes in the <i>trans</i> -Golgi network (furin, PC6b, etc.)	$\alpha$ 1-Antitrypsin, antisense
Vpr cofactors	ATR, Rad-3 related protein	siRNA
General viral inhibitors	Interferons	HIV-1-responsive conditional expression of IFN- $\alpha$ 2
Early resistance effects	Ref1, cyclophilin	

<sup>a</sup> The roles of these species in integration, vs PIC transport, are not yet clear.

potential targets for antiviral therapy. Some of these are listed in Table 1. Currently identified cellular factors probably are the tip of the iceberg: many more will be emerging soon.

**HIV-1 Entry**

HIV-1 infects cells by binding to cell membrane structures, fusion of virus envelope and cell membrane, and uncoating, whereby HIV-1 introduces its genome into the cytoplasm. The main HIV-1 receptor, CD4, and one of several coreceptors, mainly CXCR4 and CCR5, are essential to initiate the entry process. Not surprisingly, gene therapy strategies aimed at downregulating CD4, CXCR4, or CCR5 via RNAi have been extremely successful in inhibiting viral entry *in vitro* and in severe combined immunodeficiency (SCID) mice bearing human cells [65–68]. The clinical applicability of receptor/coreceptor downregulation in the near future, however, is uncertain. Widespread or indiscriminate downregulation of CD4 or CXCR4 is likely to have deleterious consequences for immune function or cellular maturation and homing. In contrast, individuals who lack CCR5 have no apparent defects, immune or otherwise, and yet show decreased susceptibility to HIV-1 infection and delayed progression to AIDS (reviewed in

[69]). CCR5 thus is an attractive target for anti-HIV-1 therapeutics.

Approaches used to decrease receptor and coreceptor concentrations at the cell membranes include SFV antibodies and “intrakinases” (see above) [28,29,31,70–72], as well as a variety of RNA-based approaches, including antisense, ribozymes, and siRNAs [54,55,57–59,68,73].

In addition to promoting viral entry into permissive cells, CD4 inhibits the infectivity of released particles [74]. To overcome this effect HIV has evolved mechanisms to ensure removal of CD4 from the surface of infected cells (reviewed in [75]). Recent data suggest that CD4 downmodulation plays an important role in HIV pathogenesis and replication *in vivo*. Disease progression correlates with enhanced virus-induced CD4 downmodulation, and a subset of long-term nonprogressors is infected with viruses defective in this function [76–78]. These findings suggest that selectively inhibiting CD4 downregulation might constitute the basis for novel anti-HIV therapies. When cells were transduced with truncated CD4s that were resistant to downmodulation by Nef and Vpu, the infectivity of released progeny virions was reduced 1000-fold [79]. Furthermore, lentiviral vectors expressing truncated CD4 blocked HIV-1 replication in cell lines and in CD4-positive primary lymphocytes [79].

### Preintegration Complexes and Provirus Integration

Following entry, viral cores are partially disassembled to become preintegration complexes (PICs). Inside PICs, viral RT converts the RNA genome to a linear, double-stranded DNA intermediate, which constitutes the template for integration into cellular DNA. In nondividing cells, PIC for most retroviruses cannot enter the nucleus, because of size constraints at the nuclear pore. HIV-1 and related lentiviruses, however, utilize nuclear transport machinery to gain access actively to the nucleus of nondividing cells such as macrophages and microglia [80]. Gene therapy strategies that downregulate selected elements of this nuclear transport may prove effective in preventing establishment of productive infections in macrophages and microglial cells. Thus,  $\beta$ -importin, which binds to nuclear localization signals in the various proteins of HIV-1 PICs, is a potential target [81]. Recent studies using RNAi against importin 7 confirmed the feasibility of this approach [82]. However, targeting any native cellular protein risks adverse effects because that protein's normal functions may be disrupted.

A postentry event that has recently captured significant attention is viral restriction. This phenomenon is a collection of cellular activities that provide potent protection from viral infection. Restriction differs from conventional innate immune responses in that restriction is present constitutively and is not activated by viral infection (reviewed in [83]). These activities act at a postentry step, during or immediately before reverse transcription. A restriction factor called lentivirus suscept-

ibility factor-1 has recently been associated with the rhesus monkey gene encoding TRIM5 $\alpha$  ( $\alpha$  spliced variant of tripartite interaction motif 5) [84]. TRIM5 $\alpha$  restricts HIV-1 infection in rhesus macaque cells. The human homolog of TRIM5 $\alpha$  has Ref-1 activity [85–87]. Thus, gene delivery may be used to express restriction factors in heterologous species in which they appear to be most efficient: rhesus TRIM5 $\alpha$  strongly protects human cells from HIV-1 [84].

Integration is essential for all retroviruses, and strategies to block HIV-1 integration inhibit replication effectively. Although HIV-1 IN has been targeted [38,88], the cellular cofactors involved in HIV-1 integration have not yet received much attention as targets for therapies using genetic approaches. These cofactors include the integrase cofactor, high mobility group A1 protein (HMG) [89]. In the past 2 years, several reports have established lens epithelium-derived growth factor/transcriptional coactivator p75 (LEDGF/p75) as a key cellular protein with roles in nuclear and chromatin targeting of HIV-1 IN [90–92], as well protection from proteasomal degradation [93]. RNAi-mediated downregulation of LEDGF/p75 inhibited nuclear localization of IN and also decreased its intracellular half-life dramatically. Therefore, LEDGF/p75 is emerging as a potential antiviral target for RNAi.

Another IN-interacting protein whose role in HIV replication is less well characterized is integrase-interactor-1 (Ini1/hSNF5). Recent studies indicate that Ini1/hSNF5 binds to HIV-1 IN specifically [94]. A fragment of Ini1/hSNF5 spanning the minimal IN interaction domain demonstrated transdominant activity, inhibited HIV-1 production by  $\geq 5$  orders of magnitude, and protected T cells from HIV-1 [94]. Therefore, strategies to deliver the transdominant mutant of Ini1/hSNF5 to hematopoietic stem cells could constitute a novel approach to inhibiting HIV-1. Since the Ini1/hSNF5 transdominant mutant acts by inhibiting particle assembly and production, heterologous vectors, rather than those derived from HIV-1, would be most appropriate.

### HIV-1 Gene Expression

Several postintegration cellular cofactor sites may be therapeutic targets. For example, cellular transcription factors are exploited during the HIV-1 life cycle. The best studied of these is nuclear factor- $\kappa$ B (NF- $\kappa$ B) and its natural inhibitor, I $\kappa$ B. RNAi vs NF- $\kappa$ B, or overexpression of I $\kappa$ B, is being explored in this light [95–97].

HIV-1 gene expression involves the viral transactivators Tat and Rev, both of which require cellular functions to be active. Since both transactivators are essential for HIV-1 infectivity, interfering with the cellular processes that support Tat function should provide avenues for potent viral inhibition. Early studies showed that TAR RNA “decoy” sequences can act as competitive inhibitors of Tat recognition of TAR and inhibit viral replication [98–100]. Additional potential targets include Tat cofac-

tors cyclin T and CDK9 [101]. The absolute requirement for human cyclin T makes its inhibition an important objective, whether via transdominant negative mutants or RNA-based approaches [102].

HIV-1 Rev, the viral regulatory protein that allows nucleus-to-cytoplasm transport of unspliced viral RNA in infected cells, requires interactions with the cellular transport moiety CRM-1. Thus, inhibition of CRM-1 may also alter HIV-1 replication, leading to a Rev-negative phenotype [103]. Since CRM-1 participates in many nuclear export mechanisms, its inhibition may involve problematic toxicities. Cellular cofactors that participate in Rev:Rev-response element (RRE) interactions may be important targets for inhibiting HIV-1 [104]. Rev cofactors  $\beta$ -importin and exportin-1 [105], and several general transcription factors [101], are also potential sites for therapeutic manipulation.

The lentiviral accessory protein Vif has generated considerable excitement lately. When Vif is mutated a “dead” viral phenotype results in certain infected cells, which are considered “nonpermissive.” Recently, it was shown that Vif inhibits a cellular protein, initially named CEM15 and now called APOBEC3G. APOBEC3G causes hypermutation of the viral DNA and then likely inactivation of the provirus. Vif targets APOBEC3G for ubiquitination and then trafficking to proteasomes for degradation [106–112]. Gene therapeutic approaches to augmenting APOBEC3G may allow inhibition of HIV-1 replication.

The accessory protein Vpr induces cell cycle arrest in G<sub>2</sub> phase. This cell cycle manipulation moderately, but significantly, augments HIV-1 replication *in vitro*: activity of the viral LTR increases during G<sub>2</sub> [113,114], as does translational efficiency of viral mRNAs [115]. The cellular pathway that mediates these effects of Vpr is controlled by ATR (ATM- and Rad3-related protein) [116,117]. ATR normally detects certain forms of DNA damage or replicative stress. HIV-1 mutants deleted in *vpr* replicate slower in culture. Importantly, there is a frequent mutation in *vpr* in long-term nonprogressors [118]. This finding suggests that inhibition of Vpr function may retard HIV-1 replication *in vivo*. In support of this idea, RNAi-mediated inhibition of ATR abrogated transactivation and cell cycle arrest by Vpr [116,117]. Importantly, downregulation of ATR, or its downstream effector, the growth arrest and DNA damage-associated protein, GADD45, dramatically relieved Vpr-induced apoptosis [119]. Thus, a gene delivery strategy to interfere with Vpr function could impede HIV-1 replication and protect infected cells from the multiple cytopathic effects of Vpr.

#### HIV-1 Proprotein Processing, Virus Assembly, and Budding

Processing of HIV-1 gp160 envelope proprotein requires participation of one or more serine proteases in the cellular *trans*-Golgi network (tgn), to cleave gp160 into

its active substituents, gp120 and gp41. There is disagreement as to which tgn enzyme(s) mediates this catalysis [120–123], but the requirement for a cellular serine protease at this critical step early in producing HIV-1 structural proteins suggests that serine protease inhibitors (serpins) could be effective anti-HIV-1 transgenes. One such native serpin,  $\alpha$ 1-anti-trypsin ( $\alpha$ 1AT), was used very effectively *in vitro* for this purpose and is an extraordinarily potent anti-HIV-1 moiety [124,125].  $\alpha$ 1AT has additional—unanticipated—antiretroviral activity: via a different active site, it also blocks HIV-1 protease (PR).  $\alpha$ 1AT thus also inhibits PR processing of HIV-1 Gag [126].

Assembly of HIV-1 includes an association between the capsid protein (CA) and a cellular enzyme, cyclophilin A, CypA [127]. How and why the binding between CypA and CA is necessary for HIV-1 infectivity has been obscure until very recently [128]. Binding to CypA precludes recognition of CA by a cellular restriction factor known as Ref-1, presumably by steric hindrance. Thus, therapeutic strategies aimed at disrupting CypA or its binding to CA may be successful in blocking productive infection by HIV-1.

HIV-1 virions exit cells by membrane budding, which typically occurs at the plasma membrane but may also occur into multivesicular bodies (MVBs). The steps leading to virus budding have been studied intensively (reviewed in [129]). The vacuolar protein sorting machinery is required for virus budding and membrane fission. Budding is initiated by interaction of the p6 subunit of Gag with Tsg101, a cell protein that links p6 with the vacuolar protein sorting machinery. siRNAs vs Tsg101 profoundly inhibit [130] release of virus by HIV-1-infected cells.

Many cellular factors are involved in Gag morphogenesis and viral budding, including—but not limited to—Tsg101. Other cellular factors, such as intracellular sorting proteins Tsg101 and ESCRT-1, interact early in budding of HIV-1, while others, e.g., CHMP4 and ESCRT-3, interact late in this pathway. As well, the cellular protein AIP1 appears to link the early and late complexes. These new findings suggest numerous sites at which these various cellular cofactors that are critical in initial viral budding into MVBs, can be targeted utilizing gene therapeutics [131].

#### Innate Cellular Resistance Mechanisms

Finally, there are cell- and species-specific innate resistance pathways against HIV-1 and other nonhuman primate lentiviruses. One of the best described forms of early innate immunity or resistance in human cells against HIV-1 is the Ref-1 restriction factor. Cyclophilin A, a host cell protein that binds to the HIV-1 p24 capsid protein, is critical for this effect. It appears that cyclophilin A protects HIV-1 from the Ref-1 restriction factor [128]. Molecular therapeutic approaches that target cyclophilin A or disrupt its interaction with the HIV-1 Gag protein would be a promising area of research.

Among host mechanisms that may restrict replication of HIV-1 and other viruses are cytokines such as interferons (IFN) [132–134]. There are multiple mechanisms by which IFNs may impede HIV-1 replication, all varying with the timing, amounts, and species of IFN; the cell types; the strain of HIV-1 tested; etc. Recombinant IFN proteins, particularly IFN- $\alpha$ 2, inhibit HIV-1 replication in human cells *in vitro* and in hu-PBL-SCID mice *in vivo* [135–142], but the magnitude of inhibition by rIFN- $\alpha$ 2 is moderate and virus replication returns when treatment is stopped [143–145]. However, gene delivery of IFN- $\alpha$ 2, driven by the HIV-1 LTR as a promoter that is activated in the presence of HIV-1 but is otherwise quiescent, provides HIV-1-responsive IFN- $\alpha$ 2 expression and powerful inhibition of HIV-1 *in vitro* [146,147]. Such conditional expression of IFNs and, perhaps, other antiviral proteins may inhibit virus strongly in susceptible cells, with limited treatment side effects.

Very soon, we expect to see enormous growth in our understanding of how cellular functions contribute to lentivirus replication. Effectively exploiting this knowledge to design new therapies requires (a) specifically targeting HIV-1-infected cells while sparing uninfected ones and/or (b) identifying processes whose inhibition is not harmful to uninfected cells.

## VIRAL VECTORS FOR GENE THERAPY OF HIV/AIDS

### Retroviruses as Gene Delivery Vectors

Among the *Retroviridae* family, animal retroviruses, lentiviruses of animals and human, and simian and bovine spumaviruses have all been used for gene delivery. As described above, these RNA viruses “reverse” their gene sequences in DNA and to integrate this DNA into the host genome. The first viral vector-based gene therapy studies were done in cancer patients using murine leukemia virus (MLV)-derived vectors (reviewed in [148,149]). Despite the development of several virus-based gene vectors, MLV vectors continue to be among the most reliable means of clinical gene transfer research (reviewed in [150]). MLV-based studies in AIDS patients are described below. As also noted above, a key disadvantage of these vectors is that migration of the preintegration complex from cytoplasm to nucleus requires cell division for the PICs to access the cellular genome for integration [151]. Thus, quiescent cells cannot be readily transduced with MLV. In addition, MLV integration and transgene expression are susceptible to alteration or silencing by flanking host chromatin and to other position-related influences on gene expression [152–154]. It should be noted that MLV vectors integrate throughout the genome but do not do so randomly: they preferentially integrate near transcriptional start sites [155]. Therefore, other members of the *Retroviridae* family are being evaluated as vectors.

### Lentivirus Vector-based Gene Delivery

**Lentiviral vectors for gene therapy for HIV/AIDS.** Since gene therapy for HIV-1-infected patients is not likely to eliminate HIV-1 from the body, persistence of a therapeutic anti-HIV-1 gene is important for long-term treatment solutions. Lentivirus-based retroviral gene delivery vectors are of particular interest since they can genetically modify nondividing cells [156,157], although truly quiescent G0 cells are not well transduced by these vectors [158,159], and deliver genes into dividing cells at high efficiencies [160–162]. Their adaptation to anti-HIV gene therapy required many modifications, including rendering the vectors resistant to the antilentic viral transgenes to be delivered [163]. HIV-1-based vectors expressing various anti-HIV-1 genes have been previously described for delivery into lymphocytes, monocytes, stem cells, and cells in the central nervous system [58,164–170].

**Safety considerations.** The two key safety concerns regarding lentivirus vectors are insertional mutagenesis and development of replication-competent lentivirus (RCL). Lentivirus-based vectors may actually offer increased safety in terms of insertional oncogenesis. Unlike oncoretroviruses [171], lentiviruses are not associated with tumor development. HIV-1-infected individuals have, on average, proviral DNA in 1/55 to 1/2800 cells [172], of which  $\geq 30\%$  is replication defective [173,174]. Thus, approximately 1/165 to 1/8400 cells in HIV-1-infected individuals harbor provirus that does not replicate to kill the cell, suggesting that the risk of HIV-1-induced insertional oncogenesis in T cells is small. As well, leukemia is not a recognized side effect of HIV-1 infection, although memory T lymphocytes are known to harbor virus for years [175]. It should be noted that HIV-derived vectors, although their integration sites are widely distributed in the genome, prefer to integrate in regions where expressed genes are concentrated [155]. Thus, continued animal and human studies with lentivirus vectors are needed to ensure that they are not oncogenic.

Today, the primary safety concern with lentiviral vectors is recombination to yield RCL—for which there is no FDA-approved assay—that may be pathogenic or transmissible. This is of particular concern in HIV-1-naïve individuals or in anyone if the RCL were to contain a non-HIV-1 envelope protein, such as the glycoprotein (G) of vesicular stomatitis virus (VSV), with broader cellular tropism. Although nonhuman lentivirus-based vectors could circumvent this problem, as lentivirus infection is species-specific [176,177], recombination between vectors and endogenous retroviral sequences could theoretically generate new human pathogens.

**Engineering safety into lentivirus vectors.** There has been a gradual evolution of lentivirus vectors, from first-generation vectors to those improved vectors having less

chance for recombination events. In an effort to minimize or eliminate completely the possibility of generating RCL during vector production, a variety of modifications have been made in the vector production system. First-generation HIV-1 vectors were generated by transient transfection of human 293T cells with plasmid containing the desired transgene inserted in an HIV-1 vector, a packaging plasmid for VSV-G [178]. Because VSV-G receptors are widely expressed among cell types [179], adding VSV-G expanded the potential population of transducible cells. However, copackaging of RNAs derived from the packaging plasmid within the same virion with the vector genome yielded RCL during subsequent reverse transcription in transduced cells. Second-generation HIV-1 vectors were generated without Vif, Vpr, Vpu, and Nef, through mutation or deletion of these genes from the packaging plasmid [180–182]. Accumulating evidence suggests that these accessory proteins are crucial for HIV-1 virulence [183]. These vectors were further improved by replacing the U3 region of the 5' LTR with the strong immediate early cytomegalovirus enhancer [18,184,185]. Such third-generation lentiviral vectors were not Tat-dependent: the absence of *tat* during vector production further reduced the possibility of generating replication-competent HIV-1. Enhancer and promoter sequences in the U3 region of the 3' LTR were also removed to yield so-called self-inactivating (SIN) vectors [185,186]. Since the 3' LTR U3 region is the template for the U3 regions of both LTRs, neither LTR in a SIN vector should be transcriptionally active. Transgene expression should thus depend totally on an inserted cis-regulatory sequence. Inactivation of LTRs should also render replication-competent HIV-1 incapable of rescuing a SIN vector and spreading it from transduced cells to other nontransduced cells [186]. Recent work, however, suggests that SIN vectors may not be so transcriptionally crippled as originally supposed: they retain promoter activity within transcribed sequences outside the 5' LTR [187].

With these modifications, only three HIV-1-encoded proteins, Gag, Pol, and Rev, are required for vector production. Further modifications in the packaging plasmid aim to eliminate the requirement for Rev in vector production [188]. Alteration of codon usage in *gag-pol* removed sequences thought to be responsible for nuclear retention and instability of HIV-1 messages, yielding a vector with Rev-independent Gag and Pol expression [188]. The need for only *gag* and *pol* in HIV-1 vector production makes recombination to generate RCL even less likely. Such modifications should make RCL production improbable via either recombination between packaging plasmid and vector genome or copackaging of RNAs from the packaging construct and vector in the same virion, although recombination between a Rev-independent Gag/Pol and a patient's HIV-1 could be problematic.

Since lentiviral vectors can be designed to introduce no new genetic sequences into the study subject, HIV-1 infection is an appropriate disease to test the safety of these vectors. Patients can be monitored for nonnative HIV-1 envelope sequences as a measure of a putative RCL generation (i.e., recombination between packaging constructs and the vector genome in producer cells) during vector production. Furthermore, gene transfer can be evaluated first as “salvage therapy” in patients who do not tolerate HAART or who carry drug-resistant HIV-1.

HIV-1-based vectors have an additional advantage over other vectors for HIV/AIDS treatment. An anti-HIV-1 effect may be a combination of the effect of the transgene and competition with wild-type (wt) HIV-1 by *cis*-acting elements within the vector (e.g., TAR and RRE decoys), thus enhancing efficacy.

Lentiviral vectors thus offer both considerable promise and cause for caution. Their utility is currently being tested in a phase I clinical trial (see below).

#### Using Recombinant SV40-Derived Vectors to Deliver Anti-HIV-1 Genetic Therapy

As gene delivery vehicles, rSV40 vectors are both old-timers and newcomers. Among the first reported studies of experimental gene transfer with viral vectors were those that employed rSV40 vectors [189]. Nonetheless, they are being studied, developed, and applied to anti-HIV-1 gene therapy by a small number of investigators. rSV40s have been tested extensively both in tissue culture and, more recently, in rodent models of HIV-1 infection but are not yet in clinical trials.

**Properties of recombinant SV40-derived vectors.** These vectors are usually made by deleting large T antigen, plus a variable amount of the remainder of the SV40 genome from cloned wt SV40 genomes (reviewed in [190]). All SV40 coding sequences may be deleted, leaving only the origin of replication and packaging signal, to accommodate transgenes of up to 5 kb. These vectors are made at very high titers, often  $>10^{11}$  infectious units/ml, and are extremely hardy: they can be lyophilized and stored in that form at room temperature with minimal loss of infectivity. More importantly, these vectors infect almost all cell types that are currently targeted in HIV gene therapy, including HSC, lymphocytes, macrophages, and dendritic cells, without selection, whether cells are resting or cycling, and with efficiencies approaching 100%. Vector DNA integrates into cellular DNA, yielding permanent gene delivery. Uniquely, rSV40s are not antigenic: not only do transduced cells elicit no cell-mediated cytotoxic responses, but the vectors themselves induce no detectable neutralizing antibody. They thus can be administered repeatedly without loss of transduction efficiency [191–193]. Levels of protein production delivered by these vectors are lower than with, e.g., adenovirus, but once a level of expression is reached, it endures indefinitely [193].

**Applications of rSV40S to gene delivery to inhibit HIV-1.** Their high transduction efficiency allows for sequential delivery of different anti-HIV-1 transgenes, to improve inhibition of HIV-1 and minimize escape mutants. rSV40 gene transfer to resting CD34<sup>+</sup> cells [194] yields long-lasting transgene expression for gene delivery to both HIV-1-susceptible cells [195] and their progenitors *ex vivo* or *in vivo*. Strong inhibition of HIV-1 is seen *in vitro* in rSV40-transduced cell lines and primary cells. *In vivo* studies are described below.

**Transgenes used to inhibit HIV-1.** Many anti-HIV-1 transgenes have been incorporated into rSV40 vectors, including single-chain Fv antibodies, ribozymes, antisense, and siRNAs, targeting both HIV-1 functions and cellular collaborators. In general, rSV40 gene delivery of a particular promoter plus anti-HIV-1 transgene construct to *unselected* cells is as effective in inhibiting HIV-1 as is retroviral delivery of the same expression cassette to *selected* cells [196].

**Combination genetic therapeutics to inhibit HIV-1.** Combination strategies require highly efficient delivery and multiple administrations and so are well suited to SV40-derived vectors [62]. Combinations of two or three individual rSV40s, each carrying a different anti-HIV transgene, were administered sequentially. Over 98% of unselected cells simultaneously expressed multiple transgenes. Most combinations of two transgenes gave synergistic protection vs HIV-1: combinations of three anti-HIV-1 transgenes provided even greater protection. The same principle, using multiple transgenes to downregulate CCR5, has also been shown to protect monocytes, microglia, and macrophages from R5-tropic HIV-1 [197].

**Protection from HIV-1 challenge *in vivo*.** The effectiveness of *in vivo* rSV40 gene delivery in protecting from *in vivo* HIV-1 challenge was tested using a thy/liv SCID-hu model (for details of this model system, see below). Human thymic xenografts in SCID mice were injected with SV(Aw), carrying SFv against HIV-1 IN. It was noted that 80–90% of the human thymic implant cells expressed the anti-IN SFv. The grafts were then challenged by direct injection of an R5-tropic clinical isolate of HIV-1. HIV-1 replication, assayed using a quantitative coculture assay, was inhibited approximately 90%, compared to mock-transduced and control rSV40-transduced thymic implants [12]. A similar approach to *in vivo* gene delivery, targeting CCR5 and protecting from R5-tropic HIV, has recently been reported in abstract form [14].

**Transduction of CD34<sup>+</sup> cells.** The utility of these vectors in delivering genes to cultured CD34<sup>+</sup> cells has been reported [194]. *In vitro* transduction of CD34<sup>+</sup> cells with rSV40s occurs whether cells are resting or dividing, and gene expression in differentiated progeny has been

documented. Finally, following a single injection of a marker rSV40 vector directly into the femoral bone marrow, expression of the marker protein was detected in between 2 and 10% (average, 5%) of peripheral blood nucleated cells >1 year later [198].

Thus, SV40-derived gene delivery vectors are amenable to HIV-1-inhibitory strategies that may be difficult to approach using other gene delivery systems. These vectors have not yet been tested in clinical trials, so their safety and efficacy in humans remain to be determined.

## ANIMAL MODELS USED IN HIV-1 GENE THERAPY

Understanding the likely therapeutic efficacy of anti-HIV-1 gene therapy requires use of animal model systems. No perfect animal model of human HIV-1 infection exists. Investigators have used both rodents and primates for this purpose.

### Murine Models for HIV-1 Gene Therapy

**Characteristics of murine models of HIV-1 infection.** For *in vivo* experiments with HIV-1, innovative approaches utilized immunodeficient mice (SCID mice) for transplantation with human cells and tissue that are susceptible to viral infection [199,200]. Two xenograft mouse models are currently used for HIV-1 pathogenesis and drug evaluation. The hu-PBL-SCID mouse model is created by reconstituting with adult human PBLs, whereas the SCID-hu mouse thy/liv model (SCID-hu) is created by transplanting human fetal liver and thymus tissue under the kidney capsule [199,200]. There is more general experience with the latter, so this discussion focuses on the thy/liv SCID-hu model. In that system, the thymus and liver are sources of stem cells as well as a stromal microenvironment in which hematopoietic progenitor cells undergo lineage-specific differentiation. Within months postimplantation, a conjoint organ (thy/liv) that resembles human thymus develops by vascularization and fusion of fetal liver and thymic tissue. These grafts sustain lymphopoiesis as a predominant feature for as long as 1 year. Human T cells detected in the organ are phenotypically and functionally normal and can be identified in the blood around 2 months after implantation. Infection with HIV-1 leads to high viral load. X4-tropic strains cause severe thymocyte depletion, thus mimicking key aspects of viral pathogenesis [199,201,202].

Of these two chimeric mouse models, the SCID-hu mouse model was further developed to permit exogenous hematopoietic stem cell transfer and reconstitution [203]. Purified human CD34<sup>+</sup> hematopoietic progenitor cells obtained from different sources such as fetal liver, bone marrow, and umbilical cord blood, when introduced into thy/liv grafts, develop normally into normal T lymphocytes. Additionally, transduction with retroviral vectors into CD34<sup>+</sup> cells prior to their reconstitution into the grafts yields cells which retain and express

vector sequences [203,204]. Although this system does not mimic all aspects of hematopoiesis and HIV-1 pathogenesis perfectly, the combination of HIV-induced cell depletion and gene-transduced CD34<sup>+</sup> cell reconstitution occurring within a relatively short time facilitates study of many critical issues relevant for the success of gene therapy approaches [204]. At present the SCID-hu system is the only *in vivo* system to evaluate accurately the thymopoietic potential and HIV-1 resistance of vector-transduced hematopoietic progenitor cells. It also allows evaluation of whether T cell differentiation is altered by, or itself alters, transgene delivery and expression.

Other notable advantages of using the SCID-hu system are: (1) Vector-transduced cells can be injected directly into the grafts, providing direct access to the thymic stroma and thus expediting the thymic homing step for the progenitor cells. (2) Most injected cells remain in the graft and can develop into T cells, thus simplifying cell biopsy collection for further analysis. (3) Thymic grafts can be serially biopsied up to three times as needed, thus providing a rich source of thymocytes at various stages of differentiation. (4) Adequate numbers of differentiated T cells containing the vector can be sorted from the graft based on donor cell HLA markers for *in vitro* evaluation. (5) Clonality of differentiated cells can easily be assessed. (6) Human CD4<sup>+</sup> lymphocytes in grafts can be challenged directly with HIV-1 to test protection of transgene-containing cells *in vivo*. (7) Finally, efficacy data can be obtained in a short time, within 3 months.

**Studies applying these models.** A number of studies had exploited this model to evaluate the efficacy of different anti-HIV-1 constructs. In protein-based gene therapy approaches, CD34<sup>+</sup> cells transduced with a transdominant Rev-M10 protein-coding gene, when introduced into SCID-hu grafts, differentiated normally into CD4<sup>+</sup> cells that were resistant to *in vitro* HIV-1 challenge [205]. Similarly, efficacy of an HIV-1-inducible diphtheria toxin A gene was demonstrated in this model [206]. Later studies evaluated many RNA-based anti-HIV-1 constructs. Ribozymes targeted to HIV-1 Tat, Rev, and Env, as well as Rev aptamer constructs, were tested for efficacy in differentiated cells [204,207,208]. Potential toxic effects of these constructs on the lineage-specific differentiation of vector-transduced stem cells into thymocytes were also evaluated. In addition to confirming their efficacy, results of these studies showed no apparent adverse effects of the vector or the transgene on the T cell progeny. A ribozyme targeted to the cellular HIV-1 coreceptor CCR5 was also tested in this system [207]. Conventional retroviral vectors based on MLV were used in the above studies. In a more recent work, a novel SV40-derived vector was used to deliver single-chain antibodies against HIV-1 integrase. Vector-transduced thymocytes in SCID-hu grafts resisted direct HIV-1 challenge [12].

**New trends in the use of murine models to study HIV-1 gene therapy.** A recent trend in HIV-1 gene therapy studies is to take advantage of the improved lentiviral vectors for transducing anti-HIV-1 genes [209]. This is based on their capacity to transduce the normally quiescent hematopoietic progenitors and their relative resistance to gene silencing during cell differentiation. This, together with the discovery that the RNAi mechanism could be harnessed to suppress HIV-1 replication, has added new thrust and excitement to the HIV-1 gene therapy field [204,210,211]. Ongoing work with RNAi/siRNAs continues to yield encouraging results. However, their ultimate therapeutic use depends on translating these findings to *in vivo* applications.

Since siRNAs are new tools, many important questions remain such as whether they have any deleterious effects on stem cell differentiation or possible toxicity. In a recent study addressing some of these issues, anti-*tat/rev* siRNA-transduced CD34<sup>+</sup> cells were introduced into the SCID-hu thy/liv grafts to determine if T cell maturation and vector expression can occur [209]. The transduced progenitors matured into T lymphocytes following the normal thymopoietic pathway. The immunophenotypes of the differentiated cells were normal and they proliferated in response to stimulation with PHA and IL-2. When the transgenic thymocytes were challenged *in vitro* with T-cell-tropic HIV-1 NL4-3, they displayed remarkable antiviral resistance. These studies established for the first time the potential utility of siRNAs in a stem cell setting for HIV-1 gene therapy. However, given the propensity of HIV-1 to mutate and generate viral escape variants, combinatorial constructs incorporating multiple inhibitory RNA effector motifs are currently under development to overcome such resistance [212]. In this regard, the SCID-hu mouse model is expected to play a major role in their preclinical testing.

### Nonhuman Primates in HIV-1 Gene Therapy

#### **Advantages of the nonhuman primate animal model.**

Nonhuman primates, specifically rhesus macaques, are key to the goal of developing gene therapy and vaccines against HIV-1. It has been shown from genetic, morphologic, physiologic, hematopoietic, immunologic, and developmental perspectives that monkeys are most similar to humans and are, therefore, an essential and appropriate animal model of human development and disease. Nonhuman primates respond to the administration of human cytokines for bone marrow stem cell mobilization for bone marrow transplantation and the animals can effectively undergo apheresis procedures to collect these cells [213–215]. In addition, many antibodies to human protein effectively cross-react with nonhuman primate tissues and cells, such that procedures like fluorescence-activated cell sorting, immune selection, immunohistochemistry, and Western blotting

can effectively be performed without the necessity of the development of species-specific reagents.

The rhesus macaque model provides a unique opportunity to assess the relative effectiveness and gene transfer efficiency of various vector systems. The close phylogenetic relationship between humans and nonhuman primates supports the concept of the use of nonhuman primates in preclinical (safety and toxicity) studies for gene therapy.

**SIV infection and disease pathogenesis as a model for HIV-1.** In the 1990s, several research groups developed models in which nonhuman primates can be naturally infected or challenged experimentally with primate lentiviruses [216]. In these models, rhesus macaques develop simian AIDS after being experimentally infected with various SIV strains of differing virulence and course of disease progression that for the most part mirrors that of human disease [217]. In addition, hybrid SIV and HIV-1 viruses (simian-human immunodeficiency viruses, SHIVs) have been developed for use in nonhuman primates. These SHIVs, which are typically composed of SIV Gag and Pol and HIV-1 Env, have advantages over SIV for vaccination development strategies [218].

The acute phase of infection is characterized by high levels of viremia and transient leukopenia and is often accentuated by fever, rash, diarrhea, and lymphadenopathy [219]. Plasma virus levels during acute and chronic infection of macaques are highly variable and mirror patterns observed in humans [220]. SIV-specific antibodies and cytotoxic T lymphocytes (CTL) develop and the plasma viremia is resolved, and infected animals enter an asymptomatic phase. During this phase the animals appear to be in a healthy state; however, most exhibit a progressive decline in the absolute number of CD4<sup>+</sup> T cells to levels below 100 CD4<sup>+</sup> T cells/mm<sup>3</sup>. As the CD4 counts begin to bottom out animals are susceptible to opportunistic infections [221–223].

As observed in HIV-1-infected humans, the immune response to SIV during primary and chronic infection differs [224–226]. SIV escape from immune elimination, has been demonstrated in the rhesus system and is similar to virus escape in humans [227,228]. The central role of CD8<sup>+</sup> T cells in containing the infection has been shown by depletion studies [229–231].

The significance of the rhesus macaque/SIV model has been markedly enhanced by the recent characterization of major histocompatibility complex class I molecules in Indian rhesus macaques [232] and the identification of SIV peptides presented by these molecules [227]. These results led to the development and application of tetramer technology for monitoring the levels and specificity of CD8<sup>+</sup> T cells [232–234]. In addition, accurate assays to measure neutralizing antibodies to primary and laboratory-adapted viruses have been developed in the SIV/rhesus model [235].

**Gene transfer and gene therapy studies.** Studies analyzing the effectiveness of HIV-1 gene therapy strategies have been performed in the rhesus model system. Rosenzweig and colleagues have performed studies focused on the delivery of anti-HIV-1 gene therapy strategies into rhesus CD34<sup>+</sup> lymphohematopoietic stem/progenitor cells [236,237]. In their first study, they delivered an anti-HIV-1 *tat* gene to rhesus CD34<sup>+</sup> cells with an oncoretrovirus vector. Challenge studies done on the *in vitro*-derived mature CD4<sup>+</sup> T cells and macrophage-like cells showed them to be highly resistant to SIV infection [236]. This group has also performed studies in the CD34<sup>+</sup> stem/progenitor cells analyzing the effectiveness of an SIV-specific ribozyme in rhesus CD34<sup>+</sup> cells. After delivery of the ribozyme, CD34<sup>+</sup> cells were again differentiated *in vitro* into CD4<sup>+</sup> T and macrophage-like cells, which were greatly protected from SIV [237].

Other anti-HIV-1 studies performed in the rhesus model have focused on targeting the delivery of the antiviral elements to mature peripheral blood mononuclear cells (PBMCs). In one of the only demonstrations of the potential *in vivo* efficacy of antiviral gene therapy strategies, retroviral vectors expressing an antisense molecule to HIV-1 *tat/rev* were delivered to mature CD4-enriched populations of rhesus lymphocytes. Populations of autologous lymphocytes expressing the antisense construct were transplanted in rhesus macaques, which were subsequently challenged with SIV<sub>mac239</sub>. The results of this study revealed that although the animals were productively infected, those receiving PBMCs expressing the antisense construct showed marked reductions in viral burden in both peripheral blood and lymph nodes and sustained total numbers of CD4<sup>+</sup> cells, compared to control animals. Subsequent studies have focused on methods to enhance *in vitro* expansion of rhesus CD4-enriched populations of PBMCs for similar studies in the future [13]. These studies suggest that mature CD4-enriched PBMCs and CD34<sup>+</sup> stem cell populations of PBMCs may be viable candidates for anti-HIV-1 gene therapy.

## CLINICAL STUDIES

### Strategies Used for Genetic Therapy of HIV-1 Infection: Cell-Based Gene Therapy

There have been two main strategies for anti-HIV-1 gene therapy to date—transduction of T lymphocytes with infusion of *ex vivo* expanded cells and transplantation of hematopoietic stem cells (HSCT) after *ex vivo* genetic modification.

**T cell strategy for gene therapy.** CD4<sup>+</sup> lymphocytes were first genetically modified to express the transdominant negative protein Rev-M10 and reinfused into patients in two experiments. The first used DNA-transfected T cells, and this showed that the Rev-M10-expressing cells

survived somewhat longer than the control cells [20]. Following infusion of murine retrovirus-transduced lymphocytes, the Rev-M10-expressing cells were detectable for an average of 6 months compared to approximately 3 weeks in the controls [21].

CD8<sup>+</sup> T lymphocytes were first genetically modified and reinfused into patients using a murine retrovirus encoding a marker gene. In this trial autologous HIV-1 *gag*-specific CTL clones retrovirally transduced with a chimeric suicide gene safely transferred CTL-based lytic function *in vivo* to AIDS patients [238]. These CD8<sup>+</sup> cells accumulated adjacent to HIV-1-infected cells in lymph nodes [239] but persistence of the infused cells was poor because of cellular immunity vs the marker gene [239]. McMichael and colleagues also found poor engraftment of adoptively transferred *gag*-specific CTLs, which were rapidly eliminated within hours of infusion [240]. Thus, autologous adoptive immunotherapy with antigen-specific T cells is possible in HIV-1 infection, but different cell expansion strategies allowing longer term function are needed.

CD8<sup>+</sup> T lymphocytes have been genetically modified to express an extracellular domain of human CD4 that binds HIV-1 Env and which was linked to the  $\zeta$  chain of the T cell receptor (CD4 $\zeta$ ) needed for T cell activation. Thus, syngeneic HIV<sup>+</sup> twins received genetically modified cells from their non-HIV<sup>+</sup> twin sibling, and detectable lymphocytes were present at 1 year after infusion at 0.1–1% of peripheral blood cells [241]. In addition, marked cells were detected in lymph nodes. In another study [242], T cells were expanded by CD3/CD28 costimulation and 2–3  $\times 10^{10}$  cells were safely infused into 24 subjects, who then received either IL-2 or no IL-2. CD4 $\zeta$  marker was detected in 1–3% of blood mononuclear cells at 8 weeks and 0.1% at 1 year after infusion. IL-2 did not enhance cell survival [243]. These studies show that genetic modification of T cells using retroviral vectors is feasible, infusion of up to 3  $\times 10^{10}$  cells is safe, and cells survive for months. It is possible that improved methods, with or without cytokine support, could lead to effective support of immune function in persons with HIV-1 infection. In any event, gene therapy approaches that target mature lymphoid populations have become a method of choice for initial evaluation of new gene therapy strategies.

The practical limitations of such immunotherapy are the requisite *ex vivo* T cell expansion. June and colleagues have demonstrated the growth-promoting effects of costimulatory signaling of T cell receptors [243]. Using CD3 and CD28 costimulation, large-scale enrichment and expansion of CD4 cells was possible. The anti-CD3 plus anti-CD28, immobilized on polystyrene beads, resulted in exponential growth of CD4 cells for more than 60 days with 10<sup>9</sup>- to 10<sup>11</sup>-fold expansion of cells. This was a polyclonal expansion as indicated by analysis of the T cell repertoire of TCR usage. The

cytokine pattern produced by these cells was that of a Th1 response. Using PBMCs from HIV-1-infected donors, this method has been shown to expand CD4 cells *in vitro* and to decrease the HIV-1 infection in these cells [244]. The decrease in HIV-1 infection is due to downregulation of CCR5, and this is gradually lost as CCR5 recovers [245,246]. These expanded and activated CD4 cells have been shown to be safe when up to 3  $\times 10^{10}$  are infused [247]. In that study, PBMCs were obtained by apheresis, and CD4<sup>+</sup> T cells were purified by negative selection and expanded in tissue culture for 14 days with anti-CD3/28 beads. To demonstrate safety, a phase I dose-escalation study started with an initial infusion of 3  $\times 10^9$  cells, followed by a second infusion of 1  $\times 10^{10}$ , and then a third infusion of 3  $\times 10^{10}$  cells at 6-week intervals. The infused cells were >95% CD4<sup>+</sup>, did not contain detectable replication-competent HIV-1, and had a Th1-like cytokine profile. Eight patients received 51 infusions of costimulated CD4 cells in this protocol. All showed increased CD4<sup>+</sup> cell counts and decreased CCR5 on circulating CD4 cells.

These studies indicate that genetic modification of T cells using retroviral vectors is feasible and that infusion of up to 3  $\times 10^{10}$  cells is safe. In addition, the early results indicate that cells survive for months. It is possible that with improved methods, with or without cytokine support, this could lead to effective support of immune function in persons with HIV-1 infection. Recently, this method of immunotherapy using costimulatory expansion of cells has been combined with lentivirus transduction to express antisense targeted to HIV-1 *env*. This is the first test of lentivirus vectors in humans and demonstrates the versatility of T cell approaches in phase I vector development (see below).

**Hematopoietic stem cell studies.** It is likely that if long-term T cell or macrophage protection is to be accomplished, gene modification and transfer to the HSC must become possible. HSCs are cells of the bone marrow (and umbilical cord blood of newborns) from which all the hematopoietic and lymphoid cells are derived. In a person with HIV-1 infection, all of the cells that are infected (CD4<sup>+</sup> cells, monocytes, macrophages, dendritic cells, microglial cells) are derived from HSCs. The fact that HIV-1 infection of the HSC is minimal to nil in the uncommitted progenitor further suggests that an efficient method of gene transfer and HSC transplantation could be effective in HIV-1. Thus, gene transfer via HSC transplantation (HSCT) is the obvious goal of such a strategy [39]. Gene transfer methods to HSCs to treat HIV/AIDS are currently limited by several factors: (1) most vectors used for HSC gene delivery to date require *ex vivo* transduction with reimplantation (transplantation), (2) safe and effective methods of cell transplantation are needed, (3) low levels of transduction efficiency and

transient transgene expression make it important to select for transferred stem cells, and (4) expression systems need to be devised that are not silenced by natural means after transfer.

**Requirement for *ex vivo* transduction.** Gene transfer to HSCs employs retroviral vectors (usually Moloney murine leukemia virus (MuLV) derived) or, more recently, lentiviral vectors (mostly derived from HIV). Unlike most other gene delivery methods (e.g., to liver or lungs), HSC transduction has been done virtually exclusively *ex vivo*. The reasons for this include the diffuse nature of the target organ (bone marrow), the relative infrequency of hematopoietic stem cells amidst the total bone marrow cell pool, the facts that HSCs are largely a nondividing population of cells and achievable titers of vectors used for HSC transduction are generally insufficient to transduce HSC in unfractionated cell populations, and a preference (e.g., lentiviral vectors) or requirement (MuLV vectors) for HSC to be actively dividing. The need for *ex vivo* transduction produces a string of ancillary problems (noted below).

Many of these problems could be circumvented if delivery vehicles could be produced in high titer and transduce resting HSCs effectively. However, such commonly used high-titer vectors as adenovirus and adeno-associated virus are either (respectively) not particularly useful for gene delivery to HSCs or the subject of considerable debate as to their utility as potential vehicles for HSC gene therapy [248–250]. SV40 vectors are made at high titers and transduce resting HSCs permanently and effectively [194,198] without selection. However, these vectors have been used only by a small number of investigators, so accumulated experience with them in HSCs is still limited [194,198,251].

**Method of hematopoietic cell transplantation for gene therapy.** Most approaches to HSC-directed gene delivery to date have focused on autologous HSCT. HSCT-directed gene transfer was first attempted using gene marking studies to elucidate the source of relapse during autologous HSCT for treatment of neuroblastoma or leukemia (reviewed in [252]). These studies showed that although low-level marking was possible, it never produced more than a small percentage of genetically marked peripheral blood cells, which were detected for only a few months. The main unanswered question is what is the optimal conditioning regimen for preparing the recipient for the HSCT? Engraftment of retrovirus-transduced autologous stem cells in HIV/AIDS patients was tested, either without conditioning or with complete marrow ablation [253–255]. Both groups received  $1\text{--}2 \times 10^6$  CD34<sup>+</sup> peripheral blood progenitor cells/kg. The nonconditioned group consisted of healthy HIV<sup>+</sup> volunteers, and the complete ablation group contained patients with AIDS lymphoma receiving high-dose chemotherapy [256]. Without con-

ditioning, almost no marking was detected in recipient peripheral blood cells. With complete conditioning (ablation), there was good engraftment of genetically modified stem cells, but it persisted only 3–6 months. Maximum peripheral blood cell positivity for the transgene was  $\sim 10^4$  cells/ml [254]. Thus, gene-modified HSCT is safe, and it does not lead to loss of control of HIV-1 infection even after complete marrow ablation. But successful engraftment requires pretreatment with marrow-toxic agents. In a study of retrovirus-transduced CD34<sup>+</sup> marrow-derived cells in children with AIDS using a vector carrying a RRE decoy transgene [255], at most 0.01–0.03% peripheral blood cells were marked cells. This level dropped to even lower percentages after 6 months. Finally, a completed phase I study of ribozyme gene transfer using retrovirus-transduced autologous HSCT in healthy HIV<sup>+</sup> patients showed late persistence of marked cells but at low levels similar to those seen with other retrovirus vectors [257]. Persistence of transduced cells after HSCT remains a challenge.

The current problem is how to study safe conditioning regimens for HIV/AIDS patients ethically. Marrow ablation cannot be used to evaluate new HSCT-based gene transfer methods on a broad basis. This is true both for HIV-1-infected patients and for persons with inborn genetic diseases. Use of reduced toxicity and nonmyeloablative regimens remains to be tested for HIV/AIDS. AIDS lymphoma treatment programs, however, are well suited to gene transfer studies. The outcome of poor-prognosis non-Hodgkin lymphoma has greatly improved using dose-intense chemotherapy (carmustine (BCNU), etoposide (VP16), and cyclophosphamide) with HSCT in non-HIV-1 settings [258]. Importantly, this approach provides potentially improved outcome for high-risk AIDS-related lymphoma and is a unique setting for evaluation of gene-modified HSCT in the setting of HIV-1 infection. Despite risks associated with HSCT-based gene transfer, this model provides an ethical approach to studying improved vector design that is unique in this patient population. In the future, initial studies of new vectors can be made in this setting, in which marrow ablation is therapeutically appropriate. While this is an extreme situation, if a vector cannot deliver its transgene in this setting, it is likely to fail in other HSC settings. Thus, the importance of this model is that it allows quick testing of a new vector's potential utility.

**Selection of gene-modified cells.** It is apparent when considering HSC-based gene transfer that untransduced stem cells will virtually always be present to compete with the transduced cells. To eliminate this possibility, one would have to have 100% transduction efficiency of donor HSC and complete marrow ablation of the recipient, an unlikely prospect. Yet, the use of gene-modified HSCs has produced the first “cure” of a disease using gene transfer in the patients with SCID [259,260].

In addition to other lessons learned from this trial, it is clear that, with sufficiently strong selective pressure, transduced cells can predominate. In this situation, the selection was linked to a T cell growth factor effect of the transgene, and in some patients this growth selection resulted in the occurrence of a T cell leukemia. Thus, the retrovirus-based gene therapy has potential for success, but must be approached with caution. A safe method of selection of such modified cells will be important if the protected cells are to become predominant in the setting of HIV-1 infection. Although there is a hope that HIV-1 itself would produce the pressure for such selection, a more reliable method of *in vivo* cell selection is needed.

By its nature, selection implies the use of a toxic effect and in the case of marrow toxicity, the goal is to develop a method with minimal toxicity. Methyl guanine methyl transferase (MGMT) can be used for selection of transduced HSC when used with BCNU and benzyl guanine (BG) [261]. However, BCNU plus BG is very toxic and unlikely to be readily applied to an HIV-1-infected patient after gene transfer therapy. The ability of a less toxic selection agent, temozolamide, to induce selection with MGMT will be tested, to determine the applicability to humans of an approach reported to be successful in a murine model [262]. Temozolamide is an oral agent and has been used for prolonged periods of therapy without serious adverse events [263].

Toward the development of a less toxic selection system that could have application to T cell selection, M. Jensen and colleagues have developed a method using type II inosine monophosphate dehydrogenase (IMPDH2) and mycophenylate mofetil (MMF) [264]. MMF acts as an immunosuppressive agent by inhibition of IMPDH2, which is essential for purine nucleoside production [265]. Like other IMPDH inhibitors, MMF has the ability to synergize with anti-HIV-1 agents, and it has been used in anti-HIV-1 pilot studies with minimal toxicity [266,267]. Thus, MMF potentiates the anti-HIV-1 effects of some antiretroviral chemotherapy regimens and is well tolerated in the AIDS patients [266]. The IMPDH-system was used to select T cells expressing shRNA and showed protection from HIV-1 infection *in vitro* [268]. It remains to be shown whether MMF could be used in conjunction with anti-HIV-1 gene therapy to support selection of cells protected from HIV-1 infection.

#### A Clinical Study Using Lentivirus Vectors

**Rationale for the study.** The first phase I clinical trial of a lentivirus-derived vector ([269]; [www.fda.gov/ohrms/dockets/ac/01/briefing/3794b3.htm](http://www.fda.gov/ohrms/dockets/ac/01/briefing/3794b3.htm)) is under way. VRX496 is a fully gutted HIV-1<sub>NL4-3</sub>-based second-generation lentivirus vector [270] with the native HIV-1 LTR, a cPPT/CTS sequence, and a 937-base antisense payload directed vs the HIV-1 envelope gene. In *in vitro* studies, VRX496 transduced >95% of CD4<sup>+</sup> T lymphocytes from naïve and HIV-1-infected subjects [271]. Modified lym-

phocytes strongly inhibited HIV-1 in naïve cells and in cells from HIV-1-infected people over 36 days. Treated cells had a selective advantage, compared to unmodified cells, and escape mutants were not seen [271,272].

**Study design.** Five late-stage HIV-1-infected patients who have failed two regimens of HAART (due to either intolerance or the evolution of drug-resistant strains of HIV-1) and who have a viral load above 5000 copies/ml, a CD4 count between 200 and 500, and no opportunistic infections were enrolled. Autologous T cells will be transduced and expanded and then readministered. Such approaches have been used safely as anti-HIV-1 therapies [21,242,247].

This trial aims to: (1) establish clinical safety parameters of such *ex vivo* lentiviral gene delivery; (2) reduce numbers of HIV-1-susceptible cells, thus lowering patients' viral loads; and (3) provide immunocompetent HIV-resistant cells to delay progression to AIDS. Transduced cells should have a selective advantage over unmodified cells and thus preferentially repopulate patients' immune systems over time, similar to modified cells in gene delivery trials for adenosine deaminase deficiency [256].

Since the primary goal of the study is safety, the success criteria focus on absence of adverse events: 0.5 log<sub>10</sub> increase in viral load or decrease in CD4<sup>+</sup> T cells within 3 weeks of dosing. Study subjects will receive 1 × 10<sup>10</sup> modified lymphocytes once and then be monitored at 24, 48, and 72 h; 1, 2, and 3 weeks; and 3 and 6 months later. Monitoring will include physical examination and measuring CD4 counts, viral load, numbers of circulating modified cells, blood chemistry, VSV-G DNA and RNA, TCR Vβ repertoire, biological RCL assay, and ELISPOT analysis. To date, safety has been demonstrated in two patients, although specific data are not yet released.

#### CONCLUSIONS

AIDS is a global scourge that has killed millions, has brought heartache and suffering to millions more, and is likely to continue its grim lethal crescendo for the foreseeable future. The success of HAART in controlling HIV-1 offers hope, but the difficulties of this therapy and the ability of HIV-1 to mutate into drug-resistant variants necessitate continuous development of new therapies. The sobering results of clinical gene therapy trials for AIDS are cause, not for despair, but for reflection. Gene delivery may yet have a role to play in the fight against AIDS: continuing work in tissue culture and animal models encourages cautious optimism.

Efforts to study HIV genetic therapy have been fragmented and reflect differing priorities among many investigative groups and organizations. It may be helpful to establish a coordinated multi-investigator and multi-

institutional effort to identify the most effective and promising transgenes, expression constructs, and delivery approaches to HIV gene therapy.

## NOTE ADDED IN PROOF

Two recent publications describe and corroborate reference #212, the utility of lentiviral vector delivered combinatorial RNA-based anti-HIV constructs. Banerjee, A., Li, M.-J., Remling, L., Rossi, J., and Akkina, R. (2004). Lentiviral vector transduction of HIV-1 Tar Decoy and CCR5 ribozyme into CD34+ progenitor cells and derivation of virus resistant T cells and macrophages. *AIDS Research and Therapy*, **1**: 2–13 and Anderson, J., and Akkina, R. (2005). HIV-1 resistance conferred by siRNA co-suppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. *AIDS Research and Therapy*, **2**: 1–12.

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