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POTENTIAL NUCLEASE-BASED STRATEGIES FOR HIV GENE THERAPY

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1. ABSTRACT

Gene therapy for HIV (human immunodeficiency virus) involves the introduction of a therapeutic gene into the infected individual for the purposes of reducing viral load and ultimately reconstituting a healthy immune system. Clinical trials for HIV gene therapy have not yet reported therapeutic benefit. In addition to improving the efficiency of gene delivery and the maintenance of gene expression, better therapeutic genes must be designed

before this therapy becomes available to patients. A new class of therapeutic genes expressing nucleases may be designed. These nucleases may be classified into three categories based on their mode of action: (i) 'targeted nucleases' for specifically cleaving HIV RNA within the cell, (ii) 'colocalized nucleases' for cleaving HIV genomic DNA or RNA present within the cell or progeny virus, and (iii) 'cytotoxic nucleases' for conferring selective toxicity to HIV-infected cells. The focus of this review is on the design and application of these nucleases for HIV gene therapy.

2. INTRODUCTION

HIV infection leads to the slow and progressive destruction of the immune system. As a result, the infected individual becomes susceptible to opportunistic infections leading to the diseased state known as acquired immunodeficiency syndrome (AIDS). Drug therapies have improved the quality of life of many infected individuals; however, current drug cocktails cannot completely eradicate the virus from the body (1). In contrast, gene therapy for HIV-infected individuals offers a promising approach in the fight against AIDS (2). It involves the introduction of a therapeutic gene into the infected individual for the purposes of reducing viral load and ultimately reconstituting a healthy immune system. HIV gene therapy, however, has not been able to provide therapeutic benefit to date. In addition to improving the efficiency of gene delivery and the maintenance of gene expression, better therapeutic genes must be designed before this therapy becomes available to patients. A new class of therapeutic genes may be designed to encode nucleases that inhibit HIV replication. These nucleases may be classified into three categories based on their mode of action: (i) 'targeted nucleases' for specifically cleaving HIV RNA within the cell, (ii) 'colocalized nucleases' for cleaving HIV proviral DNA or genomic RNA present within the cell or the progeny virus, and (iii) 'cytotoxic nucleases' for conferring selective toxicity to HIV-infected cells. This review will focus on the design of anti-HIV nucleases and their application in HIV gene therapy. A brief review of HIV molecular biology is presented first.

3. HIV MOLECULAR BIOLOGY

3.1. HIV life cycle

HIV is a member of the lentivirus genus in the *Retroviridae* family (3). In addition to *gag*, *pol* and *env*, the virus encodes six other genes. Upon fusion with the host cell, the RNA genome is reverse transcribed and integrated into the host chromosome. Viral gene expression leads to the assembly, release and maturation of progeny virions, yielding infectious virus (figure 1).

3.1.1. Viral entry

Viral entry is determined by specific protein-protein interactions between the HIV envelope (Env) protein complex on the surface of the virion and cellular receptors (4). The HIV Env protein is initially produced in the host cell as the glycoprotein gp160 and subsequently cleaved by a cellular convertase (within the endoplasmic reticulum, ER) into the gp120 surface subunit and the gp41 transmembrane subunit (5). Both subunits remain noncovalently attached and are targeted to the host plasma membrane by vesicular transport (6).

The gp120 surface subunit binds to cellular CD4 and a coreceptor belonging to the chemokine receptor family (7, 8). The CD4 receptor is found primarily on the surface of T-helper lymphocytes, monocytes/macrophages, dendritic cells and brain microglial cells (9). The primary coreceptors utilized by the virus are either CCR5 or CXCR4 (10, 11). HIV variants that use the CCR5 coreceptor are termed macrophage-tropic, while variants that use the CXCR4 coreceptor are termed T cell-tropic (12). Upon initial binding of gp120 to the cellular receptors, conformational changes allow the N-terminus of the gp41 transmembrane subunit to mediate fusion between the viral membrane and the host cell membrane (13). The Env protein can also mediate fusion between infected cells and uninfected cells in a process called syncytium formation (14).

3.1.2. Reverse transcription

Initiation of reverse transcription of the viral RNA genome requires the formation of a ternary complex between HIV Reverse Transcriptase (RT), a cellular $tRNA_3^{Lys}$ which serves as a primer, and the viral RNA genome (15). RT has an RNase H domain and a polymerase domain (16). Initiation primarily occurs post-fusion within the cytoplasm of the newly infected cell where there is an abundance of dNTPs; although, proviral DNA has been detected in some rare virions (17, 18). The $tRNA_3^{Lys}$ anneals to complementary sequences within the 5' region of the viral genome, called the primer binding site (PBS) (19). RT then polymerizes negative strand strong-stop DNA to the 5' end of the genome (20). During polymerization, the RNase H domain cleaves the RNA in the RNA/DNA hybrid (21, 22). Sequence complementarity of negative strand DNA favours an intermolecular jump to the 3' end of the other genomic copy of HIV RNA (23); although, an intramolecular strand transfer has also been shown to occur suggesting that two genomic RNA copies may not be necessary for proviral DNA synthesis (24). Negative strand DNA synthesis then proceeds to the PBS, while the RNase H domain cleaves RNA within the RNA/DNA hybrid (25). RNase H also nicks the genomic RNA 3' to two PPTs (polypurine tracts) upstream of the 3' LTR, which then act as primers for positive strand DNA synthesis (26). Sequence complementarity to the PBS favours circularization of the template and allows for RT to complete synthesis of double stranded proviral DNA (27). Recombination may occur during reverse transcription by intermolecular jumping of RT between both genomic RNA copies (28). The diploid nature of HIV suggests that recombination serves a central function in virus evolution.

3.1.3. Nuclear localization

Since HIV can infect non-dividing cells (29), the pre-integration complex must translocate through the nuclear pore complex (NPC). It is this process that allows infection of monocytes/macrophages (30). Little is known of the intermediates involved during import of the

preintegration complex. However, nuclear import is mediated by independent pathways that use the Vpr (virion protein R), MA (matrix) or IN (integrase) protein. The Vpr protein uses two different import pathways (31) that are distinct from the classical NLS (nuclear localization signal)- and M9-mediated pathways. The Vpr protein does not seem to use cellular receptors involved in import, but rather appears to interact directly with the NPC (31, 32). Unlike the Vpr protein (33), the MA protein contains a classical NLS sequence (34) and binds to a member of the karyopherin-alpha family (35). In addition, tyrosine phosphorylation at the C-terminus of the MA protein is needed for import (36). The IN protein has an atypical bipartite NLS that utilizes the importin/karyopherin pathway (37).

3.1.4. Integration

The IN protein of HIV catalyzes the insertion of proviral DNA into the genome of the host. The integration event is not site specific but certain topological features of the chromosome may be more amenable to integration (38). The IN protein recognizes short inverted repeats at both ends of the proviral DNA and cleaves a dinucleotide at the 3' end, leaving an AT overhang at the 5' end (39). The IN protein also catalyzes the cleavage of the host genome and subsequently ligates the 5' overhang to the cellular genome creating a 5 bp repeat on either side (40).

3.1.5. Early and late gene expression

Transcriptional regulation of HIV gene expression is controlled by co-operative interactions between host-cell transcription factors and viral gene products. The 5' LTR contains binding sites for transcription factors such as AP-1, NF-kappa B, NF-AT, IRF, and Sp1 (41). The transcription initiation site is in the U3 region and transcription occurs under control of RNA polymerase II. The primary transcript remains either unspliced, singly spliced or multiply spliced, thereby permitting translation of nine open reading frames (42). Multiply spliced mRNAs code for the early gene products, *trans*-activator of transcription (Tat), regulator of viral proteins (Rev) and the negative factor (Nef). The viral gene product Tat positively regulates the production of all three types of HIV mRNAs, while Rev positively regulates the production of unspliced and singly spiced HIV mRNAs which code for the late gene products. Unspliced HIV mRNA serves as the viral genome and mRNA coding for the group antigen (Gag) and Gag/polymerase (Gag/Pol) precursors, while singly spliced HIV mRNAs code for the virion infectivity factor (Vif), Vpr, viral protein U (Vpu), and Env (42).

An essential transcriptional activator required for HIV gene expression is the viral Tat protein. Tat contains an NLS and nuclear export signal (NES) allowing it to cycle in and out of the nucleus (43, 44). Tat promotes transcriptional elongation by binding to the viral *trans*-activation response (TAR) element located immediately 3' to the transcriptional start site (45). The TAR element is a 59 nucleotide-long RNA stem loop structure having two important sequence elements - a three nucleotide bulge and a six nucleotide loop (46). Tat recruits cellular cofactors to the TAR element in a process that involves Tat binding to the bulge and cofactor binding to the loop (47). Tat acts to promote the processivity of RNA pol II by recruiting a CTD (C-terminal domain) kinase which allows the phosphorylation of the CTD of the RNA pol II enzyme (48). This results in the production of high levels of the primary transcript which is available to the spicing pathway. HIV has evolved the required mechanism for exporting newly synthesized unspliced and singly spliced HIV mRNAs, by using the Rev protein.

The HIV Rev protein regulates the production of viral gene products encoded by singly spliced and unspliced HIV mRNAs. The Rev protein contains an NLS and NES and thus shuttles back and forth between the nucleus and cytoplasm (43). Rev binds to a 234 nucleotide-long viral RNA multi-stem-loop structure, called the Rev-responsive element (RRE) (49). Rev initially binds to stem loop IIB within RRE (50). This then leads to the recruitment of other Rev molecules in a multimerization process (51). Nuclear export of the Rev/RNA complex is mediated by exportin Crm1 and Ran-GTP (52), while nuclear import of Rev is mediated by importin beta (53). Rev has also been shown to promote polysomal loading during translation of viral mRNAs (54).

The third early gene product encoded by HIV is the Nef protein. The Nef protein downregulates cell surface CD4 and major histocompatibility 1 (MHC I) proteins (55). The N-terminus of the Nef protein is post-translationally modified by myristoylation (56). Myristoylation promotes targeting to the inner surface of the plasma membrane where Nef promotes binding of a clathrin adaptor complex with the cytoplasmic tails of CD4 or MHC I protein (57). This interaction promotes their internalization and transport to lysozomes for degradation *via* independent pathways (58, 59). Downregulation of cell surface CD4 protein may prevent complex formation between CD4 and Env proteins at the plasma membrane and facilitate release of HIV virions (60). Downregulation of MHC I inhibits cytotoxic T lymphocyte (CTL)-mediated lysis of HIV-infected cells (61). The Nef protein is also packaged into virions during viral budding and seems to enhance virion infectivity; HIV virions produced in the absence of Nef were shown to inefficiently reverse transcribe the RNA genome (62).

Rev-dependent accumulation of singly spliced and unspliced HIV mRNAs allow for the production of late gene products. The main function of the Vif protein is to enhance the infectivity of HIV virions (63); however, Vif may not be incorporated into virions during viral assembly and may therefore function in the cytoplasm to somehow modify HIV virions through a cellular factor (64). The Vpr protein is packaged into virions and allows eventual nuclear import of the preintegration complex (65). The Vpr protein is also involved in arresting HIV-infected cells in the G2 phase of the cell cycle (66). The HIV LTR was shown to be more active in G2-arrested cells, maximizing virus production (67). The Vpu protein is unique to HIV-1 and is absent in HIV-2. Vpu selectively targets CD4 to a degradation pathway in the ER (68). This permits the release of Env from the ER, which may be complexed with

CD4 (69). The Vpu protein also selectively enhances virion release (70). Vpu has been proposed to make an ion channel in the plasma membrane to facilitate the release of virions; however the mechanism by which Vpu promotes virion budding is unknown (71).

3.1.6. Viral assembly and maturation

The virus is assembled at the plasma membrane. Two copies of unspliced genomic RNA and cellular $tRNA_3^{Lys}$ are packaged into the budding viral particle along with virion structural and enzymatic proteins (72). Host cellular proteins are also packaged into virions (73). The pathway through which hundreds of subunits self-assemble into an infectious virus has been difficult to determine. However, all the information needed to form virus-like particles resides within the HIV Gag protein (74). Initially, the *gag* gene product is translated as a precursor from cytosolic ribosomes. Ribosomal frameshifting during translation of unspliced mRNA also allows for the production of the Gag/Pol polyprotein precursors (75). The Gag and Gag/Pol precursors are then post-translationally modified by removal of the N-terminal methionine and attachment of myristic acid to the second amino acid glycine (76). The hydrophobic interactions between the lipid bilayer and the myristate moiety along with the electrostatic contacts between flanking residues and the acidic phospholipids allow for efficient membrane targeting (77). Ordered Gag-Gag and Gag-Gag/Pol aggregation is achieved through multiple protein-protein interactions between Gag molecules at the plasma membrane (74).

Proteolytic cleavage by the Protease (Pro) domain of the Gag/Pol precursors allows for large conformational changes which may promote induction of curvature and virion release from the infected cell (78). HIV Gag precursors are processed at the membrane and in the virus into (from N- to C-terminus) the MA, capsid (CA), nucleocapsid (NC) and core-envelope-link (CEL) proteins (79). Upon protease cleavage, the MA protein forms a matrix under the viral envelope and the CA protein condenses to form a conical core surrounding the NC-coated HIV RNA genome. The CEL protein is thought to become associated with the Env protein. The Gag/Pol precursors are also processed into the above products, plus the HIV enzymatic proteins Pro, RT and IN. The association of Gag and Gag/Pol precursors with the Env protein is mediated by protein/protein interactions between the MA domain and gp41 protein (80).

All of the information needed for packaging two copies of genomic RNA is contained within the unspliced HIV RNA. The packaging signal (psi), adjacent to the 5' splice donor, is necessary for recognition by the NC domain of the Gag precursor during viral assembly (81). In addition, sequences within the *env*-coding region of HIV RNA have been shown to promote packaging (82). Dimerization of two copies of genomic RNA is mediated by palindromic sequences within the dimer linkage structure (DLS) (83). The DLS overlaps the psi element (83); however, dimerization may not necessarily be a prerequisite for packaging genomic RNA. Dimerization is catalyzed by the NC domain *in vitro* (84). Thus, the NC domain not only allows for recognition but also seems to allow for packaging two copies of genomic RNA. Cellular $tRNA_3^{Lys}$ is also packaged into virus by binding to the RT domain of the Gag/Pol precursor (85).

Correct folding and trafficking of proteins may be mediated by cellular factors. For example, virions lacking cyclophillin A are non-infectious (86). The central region of the CA domain binds to cyclophillin A; however, the function of cyclophillin A during virus assembly is largely unknown (87). Furthermore, this association with cyclophillin A has not been shown with other closely related retroviruses and seems to be unique to HIV (88).

3.2. HIV pathogenesis

HIV infection ultimately leads to the destruction of the cells within the immune system. Infected cells infiltrate the lymph nodes and multiple cell types within the thymus, brain and gut (89-91). The onset of AIDS occurs as the progressively deteriorating immune system becomes susceptible to opportunistic infections (92). The pathogenic mechanisms leading to AIDS are not well understood but involve both viral and host factors.

Viral replication causes disease and the host immune response reduces viral load. These two opposing forces on viral population may eventually fall out of equilibrium as the continuous and rapid turnover of virus leads to the emergence of variants that evade the immune response (93). As a result, infection leads to a massive depletion of cells within the immune system, particularly of the CD4+ T-lymphocytes.

Host factors may regulate the life cycle of HIV resulting in acute, chronic or latent infection. Acutely infected cells are short-lived, whereas chronically infected cells and resting cells harbouring latent HIV proviral DNA are relatively long-lived (1). Resting cells likely evade treatment and control by the immune response (94, 95).

The infected cells may die as a result of cytopathic effects such as syncytium formation or viral budding (96). Thus far, only *in vitro* results show that cell death results from direct infection of the cell (97, 98); however, this may not be the case *in vivo* (99, 100). Instead, cell death *in vivo* may also be caused by host factors.

Mechanisms other than syncytium formation and virally induced cytopathicity may contribute to the massive depletion of cells in the immune system. For example, studies have shown that a pathogenic CTL response may be induced in which uninfected CD4+ cells are destroyed (101). Additionally, other bystander effects such as T-cell anergy *in vivo* (102, 103) or apoptosis (104) may be mediated by superantigens, like HIV gp120. In addition, host genetic factors also play a role in pathogenesis. It is known that both non-MHC (105, 106) and MHC (107, 108) genes influence HIV replication and progression to AIDS.

3.3. Drug therapy

The World Health Organization has estimated that 33 million people are infected worldwide (109). The complete eradication of the virus in the infected individual has not been fully realized by drug therapy (1). Anti-viral

drugs have extended the lives of many individuals infected with HIV in the United States; however, they have not proven effective in the long term and have exhibited toxic side effects (110). Drug therapy has not been able to deal with the latent reservoir (111) and undetected viral compartments or sanctuary sites (112). Unfortunately, viral resistance to all classes of anti-HIV drugs has been described (113). In addition, the high cost of anti-viral drugs makes it impossible for developing countries, where 95% of all HIV-infected people live, to have access to the treatment. Therefore, a treatment must be sought that can also be cost-effective.

3.4. Gene therapy

HIV infection leads to the acquisition of viral genes by the host. Thus, AIDS can be considered as an acquired genetic disorder. Gene therapy would decrease viral load and protect susceptible cells, resulting in the eventual reconstitution of a healthy, HIV-resistant immune system. In addition, a single gene therapy treatment could manage the infection and prevent transmission for the life of the patient. Thus, gene therapy has the potential to be less costly without the complication of compliance associated with drug therapy.

Therapeutic genes can be designed to inhibit viral replication such that infected cells do not produce any infectious progeny. Therapeutic genes are designed to encode RNAs (*i.e.* antisense RNAs, sense RNAs, and ribozymes) and/or proteins (*i.e. trans*-dominant mutants, nucleases, single-chain antibodies, suicide proteins, HIV receptors, and HIV coreceptors and their ligands). The therapeutic gene products may inhibit one or multiple sites within the viral life cycle or cellular processes essential for viral replication by blocking initial infection, inhibiting viral replication, or rendering progeny virus non-infectious. The inhibitory strategy that will be most successful is the one that is able to completely block any given step in the viral life cycle. Alternatively, the therapeutic gene products may disable the infected cells, thereby eliminating the infected cells, so that viral spread would not occur. A significant decrease in the viral load will prevent new infections and also protect gene-modified and unmodified cells from bystander effects. Current clinical trials have focused on several anti-HIV genes, even though they do not confer complete resistance to HIV replication *in vitro*. Since the emergence of viable escape mutants has been the primary obstacle with the efficacy of current anti-HIV drugs, the isolation of therapeutic genes conferring 100% protection with zero probability of developing viable escape mutants is crucial for long term resistance. Thus, there is a clear need to explore better therapeutic genes that completely inhibit HIV replication while disallowing the emergence of a viable escape mutant.

4. DESIGNING ANTI-HIV NUCLEASES

Nucleases are enzymes capable of hydrolyzing the phosphodiester bonds present in nucleic acids. Nucleases which cleave DNA are called deoxyribonucleases (DNases), while nucleases which cleave RNA are called ribonucleases (RNases). DNases may cleave single stranded DNA or double stranded DNA. Also, DNases known as restriction endonucleases can recognize and cleave DNA at specific sequences. RNases may cleave single stranded RNA, double stranded RNA or RNA present in a DNA/RNA hybrid. Until now no RNase has been found that exhibits restriction endonuclease-like specificity. However, the specific nature of RNA processing in mammalian cells suggests that RNases do recognize specific sequence motifs. Furthermore, nature has used nucleases as a defense mechanism against viral infections. For example, RNase(s) with anti-HIV activity are associated with human chorionic gonadotropin which when produced at high levels prevent transmission of HIV from mother to fetus (114).

Nucleases have a unique advantage as therapeutic agents because of their reaction specificity and catalytic efficiency. Therapuetic genes expressing nucleases have been designed for use in HIV gene therapy (115-117). HIV gene therapy strategies that require stoichiometric binding of interfering molecules to HIV RNA/protein molecules require an excess of the interfering molecules to shift the equilibrium towards the bound form. Since, the HIV RNA/protein is not consumed in the reaction, a slight shift in the equilibrium may permit HIV replication to proceed beyond the inhibitory step, allowing virus to propagate. In contrast, anti-HIV nuclease-based strategies may be preferred since they would result in a permanent loss of HIV DNA/RNA function. Furthermore, since nucleases act in a catalytic manner, low concentrations would be required for them to be effective. By designing anti-HIV nucleases whose function depends on several essential viral functions, the overall probability of the emergence of escape mutants would be reduced to the multiple of their individual probabilities. Thus, HIV will have little latitude for the emergence of escape mutants since any such mutation will be deleterious and confer a replicative disadvantage to the virus. In addition, nucleases may be designed to be effective against various subtypes of both HIV-1 and/or HIV-2.

Genetic engineering may allow for the construction of three types of anti-HIV nucleases: (i) 'targeted nucleases' can be designed to specifically cleave HIV RNA within the cell, (ii) 'colocalized nucleases' can be designed to cleave HIV proviral DNA or virion RNA present within the cell or viral progeny, and (iii) 'cytotoxic nucleases' can be designed to confer selective toxicity to the HIV-infected cells (figure 1, table 1).

4.1. Targeted nucleases

Definition: A targeted nuclease is defined as a nuclease which would specifically bind to and cleave its target RNA. Inhibition of HIV replication would involve the intracellular production of a targeted nuclease in infected cells so that specific cleavage of HIV RNA would prevent subsequent steps within the viral life cycle. The nuclease should be designed to inhibit HIV replication without causing any cytotoxicity.

Design: Most existing nucleases that cleave RNA, cleave all single stranded RNAs at a specific ribonucleotide or all double stranded RNAs at any

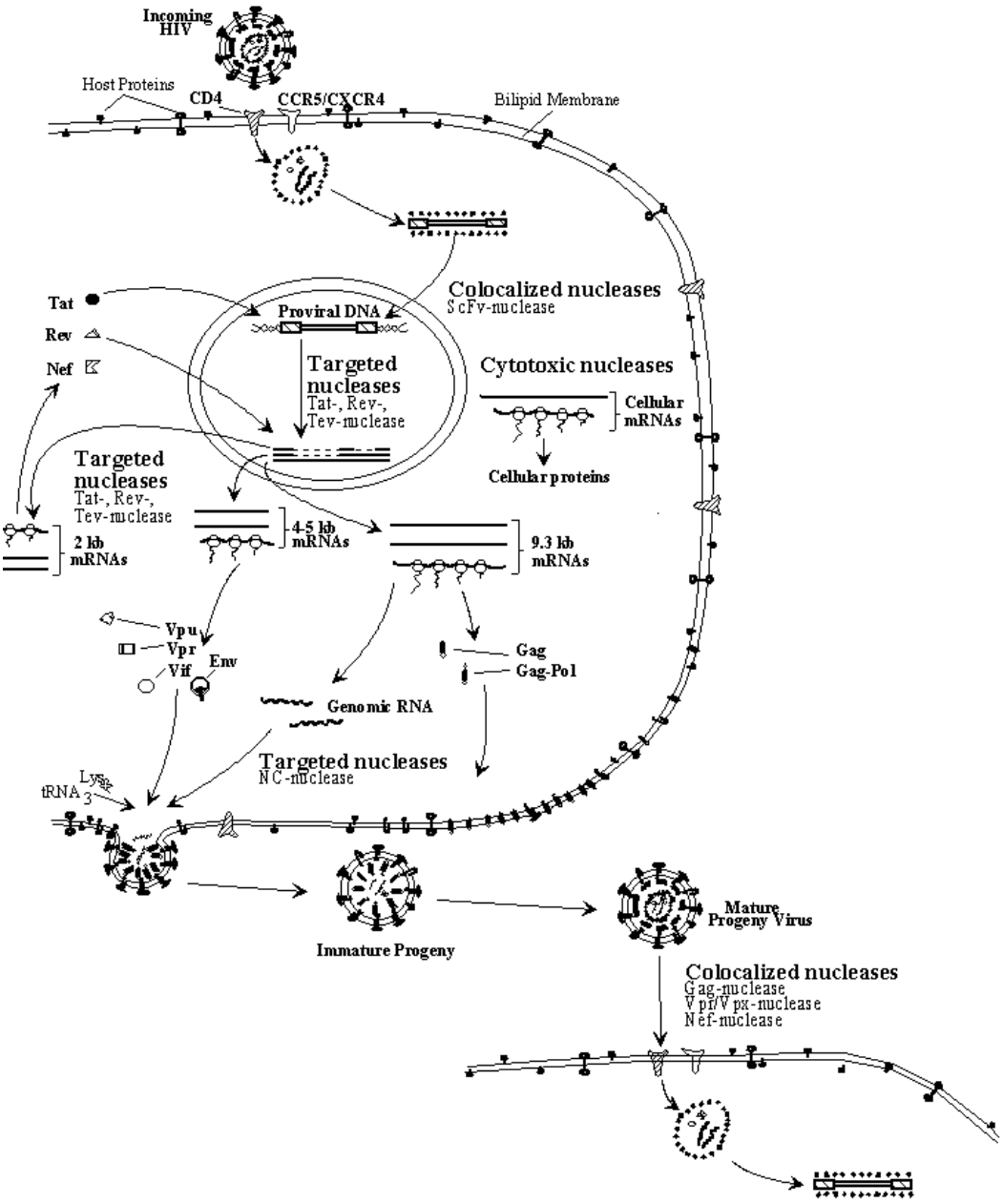

Figure 1. HIV-1 life cycle and various steps that can be blocked *via* nuclease-based gene therapy strategies. Following entry inside the cell, HIV-1 RNA reverse transcribes and integrates within the cellular genome. Upon transcription, the full length 9.3 kb viral RNA is produced which is differentially spliced to give rise to various HIV-1 mRNAs. The 2 kb RNAs then give rise to Tat which enhances gene expression and Rev which allows export of 4-5 and 9.3 kb HIV-1 RNAs. Translation of these RNAs then gives rise to various structural and maturation proteins. Virus assembly then takes place and recruits 2 copies of full length HIV RNA and cellular $tRNA_3^{Lys}$. Some of the steps taking place during the subsequent round of infection are also shown. colocalized scFv-nucleases would cleave the incoming virion RNA/DNA and thereby block provirus DNA integration. Targeted nucleases, including Tat-, Rev-, and Tev-nucleases would cleave HIV transcripts both within the nucleus and the cytoplasm. Targeted NC-nucleases would cleave the HIV-1 genomic RNA before virion assembly takes place, such that the particles produced will be non-infectious as they will lack virion RNA. Colocalized packageable nucleases including Gag-, Vpr/Vpx-, and Nef-nucleases would cleave virion RNA during or after assembly, such that the virions produced will be non-infectious and subsequent rounds of infection will not take place. Intracellular cytotoxic nucleases would cause cell death once expressed inside the HIV-infected cells, and secreted cytotoxic nucleases would cause cell death upon selective uptake by the HIV-infected cells.

Table 1. Nuclease-based strategies in HIV gene therapy

Nuclease used		Localization required for activity	Target RNA	Interference site	Fate of the HIV-infected cell
Targeted nucleases	Tat-nuclease	Nuclear/cytoplasmic	2, 4-5, and 9.3 kb HIV RNAs	Early/late gene expression	Protected
	Rev-nuclease	Nuclear/cytoplasmic	4-5 and 9.3 kb HIV RNAs	Late gene expression	Protected
	Tev-nuclease	Nuclear/cytoplasmic	2, 4-5, and 9.3 kb HIV RNAs	Early/late gene expression	Protected
	NC-nuclease	cytoplasmic	9.3 kb HIV genomic RNA before virion assembly	Infectious virus production	Cell death
Colocalized nucleases	ScFv-nuclease	cytoplasmic/nuclear	incoming virion RNA/ provirus DNA	Provirus DNA integration	Protected (no HIV provirus)
	Gag-nuclease	Virion	Virion RNA in the progeny virus	Infectious virus production	Cell death and virus production
	Vpr/Vpx-nuclease	Virion	Virion RNA in the progeny virus	Infectious virus production	Cell death and virus production
	Nef-nuclease	Virion	Virion RNA in the progeny virus	Infectious virus production	Cell death and virus production
Cytotoxic nucleases	Intracellular cytotoxic nuclease	Nuclear/cytoplasmic	Cellular RNA	Virus production	Cell death
	Secreted cytotoxic nuclease	Cytoplasmic	Cellular RNA	Virus production	Cell death

ribonucleotide (118), but lack target RNA specificity. The ribonucleotide specificity of a nuclease resides within the ribonucleotide binding motif. Target RNA specificity may be provided by modifying a nuclease to contain an HIV RNA binding motif to direct the active site to the HIV target RNA. This may be accomplished by fusing a gene encoding an HIV RNA binding domain to a gene encoding a nuclease. HIV RNA binding motifs that have been well characterized include the RRE binding domain of the HIV Rev protein (49, 119), the TAR RNA binding domain of the HIV Tat protein (120), and the psi binding domain of the HIV NC protein (44). Also, the HIV-1 Tev protein (produced from a gene containing the first coding exon of *tat*, a portion of *env*, and the second coding exon of *rev*) may be used as it contains both the TAR- and RRE-binding domains (121).

The emergence of HIV mutants preventing HIV RNA recognition by the targeted nuclease would also prevent recognition by wild type Rev, Tat, Tev or NC protein. Viable escape mutants would be rare since simultaneous mutations in both the HIV protein and HIV target RNA would be required for recognition and retention of function by wild type proteins and not the targeted RNase.

4.1.1. Tat-nuclease

Tat binds the TAR RNA element within HIV RNA. Since TAR is present at the 5' and 3' ends of all HIV RNAs, a Tat-nuclease will recognize and cleave all HIV RNA transcripts and inhibit subsequent steps leading to viral production. The cleavage of HIV RNA will take place either in the nucleus or in the cytoplasm since HIV Tat cycles in and out of the nucleus (44).

Results obtained in HIV gene therapy: Tat-RNase H was engineered in our laboratory by fusing the HIV-1 TAR RNA binding domain of HIV-1 Tat with the RNase H domain of HIV-1 RT (116). RNase H cleaves RNA in RNA/DNA hybrids and can cleave RNA in RNA/RNA hybrids at a lower efficiency (122). Unlike most RNases, the RNA binding activity and catalytic activity of HIV RNase H are separable (*i.e.* the RNA binding motif is within the polymerase domain). Thus, specificity of Tat-RNase H can be mediated by the TAR RNA binding domain of Tat. Tat-RNase H was shown to specifically recognize and cleave HIV-1 TAR RNA *in vitro* (116). However, Tat-RNase H did not inhibit HIV-1 replication in MT4 cells (a human T-lymphoid cell line) (our unpublished results). Tat-RNase H may have failed to cleave HIV-1 RNA *in vivo*. Lack of inhibition of HIV-1 replication suggests that HIV-1 RNA/DNA hybrids may not have been available for cleavage by Tat-RNase H during transcription. It is also possible that the fusion protein has a specific ion requirement for cleavage of RNA/RNA hybrids that may be missing *in vivo.* Alternative RNase domains may be sought to improve the Tat-nuclease strategy.

4.1.2. Rev-nuclease

Rev binds to the RRE within HIV RNA. A Rev-nuclease will contain the RRE binding domain and thus specifically cleave RRE-containing HIV RNAs. Since Rev cycles in and out of the nucleus (43), a Rev-nuclease is expected to cleave HIV RNA within the nucleus as well as in the cytoplasm. RRE-containing RNAs that would be cleaved include the unspliced and singly spliced HIV mRNAs (123). Cleavage of primary HIV transcripts, before they enter the splicing pathway, will inhibit the production of all early and late gene products. Cleavage of unspliced and singly spliced viral transcripts, after they leave the splicing pathway, will inhibit the production of all late viral gene products.

Results obtained in HIV gene therapy: Rev-nucleases have not yet been analyzed for their ability to inhibit HIV replication.

4.1.3. Tev-nuclease

Tev contains the RNA binding domains of both Tat and Rev and can thus target both TAR and RRE within

HIV RNA. Tev contains an NLS and is predicted to act primarily within the nucleus. A Tev-nuclease should target all HIV transcripts and inhibit the production of both early and late viral gene products. Furthermore, HIV-1 Tat and Rev proteins also bind HIV-2 TAR and RRE (124, 125). A Tev-nuclease is therefore expected to confer protection against both HIV-1 and HIV-2.

Results obtained in HIV gene therapy: We designed the fusion protein Tev-RNase T1 which contains the HIV-1 Tev domain and the *Aspergillus oryzae* RNase T1 domain (117). RNase T1 specifically recognizes and cleaves guanylate residues within single stranded RNAs. The catalytic activity and RNA binding activity of RNase T1 cannot be uncoupled (126) and were both present within the fusion protein. Since the RNase T1 domain still retains its own RNA binding activity, Tev-RNase T1 specificity *in vivo* is expected to be dependent on the initial binding event of HIV-1 Tev to TAR or RRE which occurs with up to a 10^4 fold higher affinity than the binding of RNase T1 to RNA (49, 127, 128). Therefore, Tev-RNase T1 is predicted to cleave HIV-1 RNA at available guanylate residues within and outside the TAR and RRE regions. Tev-RNase T1 was shown to be enzymatically functional and intracellular production of Tev-RNase T1 in MT4 cells and human PBLs resulted in a significant delay in replication of a laboratory strain and a clinical isolate of HIV-1, respectively (117). Also, cells producing Tev-RNase T1 were viable and showed no signs of toxicity suggesting that Tev-RNase T1 did not cleave host cellular RNA. The absence of cytotoxicity suggests that nuclear host RNAs are not available for cleavage by Tev-RNase T1. The compartmentalization of nuclear bodies within the nucleus during RNA metabolism (129) may prevent any association of nuclear host RNA/protein complexes with Tev-RNase T1. Host nuclear RNA exclusion in combination with the specific affinity and compartmentalization of Tev-RNase T1 with HIV RNA may thus be important factors contributing to the lack of toxicity and specificity of Tev-RNase T1. Tev-RNase T1 was shown to interfere with the HIV-1 life cycle post-integration (117). Our results suggest that Tev-RNase T1 may not cleave incoming HIV genomic RNA. Rather, Tev-RNase T1 seems to act by specifically decreasing HIV-1 transcript levels, resulting in the inhibition of the subsequent steps within the viral life cycle. In addition to acting as a targeted RNase, our results cannot exclude the possibility that Tev-RNase T1 also acts as a *trans*-dominant negative mutant since this would also result in a reduction of HIV RNA levels.

4.1.4. NC-nuclease

The HIV NC protein binds specifically to the psi element, which is retained in unspliced HIV RNA (130). It is also important in the dimerization of the HIV genomic RNA and allows specific annealing of $tRNA_3^{Lys}$ to the PBS during HIV RNA reverse transcription (131). The NC domain within the HIV Gag precursor is essential for packaging HIV genomic RNA into virus (132). The functional properties of the NC domain are dependent on the maintenance of two highly conserved cysteine-histidine boxes separated by a basic linker (133). Thus, an NC-nuclease should allow for specific cleavage of unspliced HIV RNAs containing the psi element. The cleavage will take place in the cytoplasm where the NC-nuclease is expected to bind to and cleave unspliced HIV RNAs. This in turn should inhibit the production of HIV Gag and Gag/Pol precursors and the availability of HIV genomic RNA for packaging. The HIV-1 NC protein also binds to the psi element of HIV-2 RNA (130). An NC-nuclease fusion protein should therefore inhibit replication of both HIV-1 and HIV-2.

Results obtained in HIV gene therapy: NC-RNase T1 is currently being developed in our laboratory.

4.1.5. Improved targeted nucleases

Complete inhibition of HIV-1 replication was not observed *in vitro* using Tev-RNase T1 (117). Protein design and selection strategies can be used to build improved targeted nucleases. An effective targeted nuclease strategy would depend on both cleavage efficiency and specificity. The nuclease domain may be evolved with a higher catalytic activity under varied conditions. One study described a general method to evolve protein catalysts *in vitro* by using staphylococcal nuclease (SN) as a model enzyme in a phage display library (134). SN was displayed as a pIII fusion protein on phage and substrate DNA was covalently attached to the phage to allow intramolecular cleavage. The modified phage was attached to a solid support by the substrate. Reaction conditions favourable for cleavage allowed for the release and enrichment of phage having enzymatically functional SN variants. Similar strategies can be used to develop targeted nucleases and selection can be done under shorter reaction times, varying temperatures, solvent conditions and pH for optimal activity *in vivo.*

Improved HIV RNA binding domains may be developed by selecting or evolving variants of known RNA binding structural motifs. Various families of RNA binding domains like the RNP motif, the arginine-rich motif, the ds-RNA binding motif and the zinc finger motif have all been well characterized (135). Combinatorial libraries of peptides based on sequences from known RNA binding motifs can be used to isolate specific HIV RNA binding peptides that bind as tightly or better than HIV Tat, Rev, Tev or NC protein. *In vivo* selection of HIV RNA binding peptides based on the arginine-rich RNA binding motif has been demonstrated (136, 137). Peptides were isolated with high affinity for RRE (136, 137). To this end, the 19 amino acid N-terminal RNA binding domain of the bacteriophage lambda antitermination protein N was replaced with a library of arginine-rich peptides, while the box B hairpin was replaced by HIV RRE. Anti-termination was observed with only specific peptide-RRE interactions *in vivo* (136, 137). In another study, a zinc finger library was displayed on bacteriophage and peptides that had a high affinity for stem loop IIB within HIV RRE were selected (138). The novel zinc fingers selected in the assay were shown to bind to RRE with affinities that were comparable or better than that of monomeric Rev. The selected zinc finger peptides were also shown to bind to RRE stem loop IIB *in vivo* in an HIV-1 LTR-CAT reporter gene expression system in which the RNA binding domain of Tat was replaced with the zinc

finger peptide and the TAR element by the RRE-IIB element.

The use of targeted nucleases in HIV gene therapy also raises the issue of potential immunogenicity. Protein engineering can be used to select for targeted nucleases with minimal functional requirements while leaving out immunodominant epitopes. Also targeted nucleases may be 'humanized' to contain TAR and RRE binding domains as well as nuclease domains of human origin. For example, the human TAR RNA binding protein (TRBP) interacts with the TAR RNA during HIV infection. Mutational analysis has identified a 15 amino acid stretch of TRBP protein that is necessary and sufficient for binding to TAR RNA (139). Two human proteins, with molecular masses of 120 kDa and 62 kDa, were shown to bind HIV-1 RRE RNA (140). Nucleases of human origin that may be used to develop humanized nucleases include pancreatic RNase, angiogenin, EDN (eonisophil derived nuerotoxin) and ECP (eonisophil cationic protein).

4.2. Colocalized nucleases

Definition: A colocalized nuclease is defined as a nuclease which will cleave HIV genomic RNA or proviral DNA by compartmentalizing with them during the early or late stages of the viral life cycle. A colocalized nuclease may act by compartmentalizing with the preintegration complex early in the viral life cycle and cleaving proviral DNA or by copackaging with progeny virus late in the viral life cycle and cleaving virion genomic RNA. Compartmentalization of a colocalized nuclease with the preintegration complex will prevent the acquisition of proviral DNA, while copackaging of the colocalized nuclease will degrade virion genomic RNA making the viral progeny non-infectious.

Design: A colocalized nuclease for HIV gene therapy can be designed by genetically fusing a gene encoding a protein that colocalizes with the preintegration complex or virion to a gene encoding a nuclease.

Single chain antibodies (scFvs) can be used to colocalize nucleases with the preintegration complex to inactivate proviral DNA. Such nucleases will be referred to as scFv-nucleases. An scFv-nuclease can be designed by fusing a gene encoding an scFv to a gene encoding a nuclease.

HIV proteins can be used to incorporate nucleases into viral particles. Such nucleases will be referred to as packageable nucleases. Well-characterized HIV proteins that can package nucleases into viral progeny include Gag, Vpr, Vpx, and Nef. Although Vif was originally believed to be packaged into virions, a recent study suggests otherwise (64). A packageable nuclease can be designed by fusing a gene encoding a virion protein to a gene encoding a nuclease. This strategy was first demonstrated in the inhibition of transposition of the yeast Ty1 element, whose replication resembles that of a retrovirus (141). Ty1 CA-nuclease fusion proteins were shown to be targeted to Ty1-virus-like particles and to degrade nucleic acid *in vitro*.

4.2.1. ScFv-nuclease

The colocalized scFv-nuclease may be designed to become compartmentalized with a nucleoprotein complex by specific protein/protein interactions; subsequently leading to the inactivation of HIV proviral DNA. Such a strategy may allow inactivation of the incoming viral genome prior to integration. Colocalization may be achieved by designing nuclease fusions with scFvs specific for HIV DNA binding proteins, like HIV IN. The scFv of an antibody is the smallest structural domain which retains the complete specificity and binding site capability of the parent antibody (142). One can derive scFvs from monoclonal antibodies produced by hybridomas (143). ScFvs may be isolated that bind to different domains of the IN protein. The application of scFvs would involve a bifunctional task in which binding of an scFv to a target antigen would allow colocalization of the nuclease to the site of cleavage. Since reverse transcription can already start in the virus, the preintegration complex may be partly made up of DNA during viral entry (17). Therefore, an scFv fusion with a nuclease having both DNase and RNase activities, such as SN, may be ideal. One study found that the intracellular production of anti-IN scFvs in PBLs specifically neutralizes IN activity (144). This study demonstrated that functional, non-cytotoxic scFvs can be produced. Furthermore, scFvs localized to the nucleus (*via* fusion to an NLS) also inhibited HIV replication, indicating scFv-nucleases can be designed to cleave HIV RNA/DNA within the cytoplasm and the nucleus (144). An scFv specific for the IN protein can be used to target a nuclease to a preintegrated HIV nucleoprotein complex and allow subsequent cleavage, preventing the host cell from acquiring the proviral DNA. This strategy should thus be effective in newly infected cells as well as in resting cells which may harbour extrachromosomal proviral DNA (145) that is normally invisible to the immune system and anti-HIV drugs.

Results obtained in HIV gene therapy: ScFv-nucleases have not yet been analyzed for their ability to inhibit HIV replication.

4.2.2. Gag-nuclease

The HIV Gag protein precursors are self-associating molecules specifically targeted to the site of virion assembly (74). The HIV Gag protein is able to form virus-like particles in the absence of other viral gene products.

In one study, the *gag* gene from the Murine Leukemia Virus (MLV) was genetically fused to the gene encoding SN. The Gag-SN fusion protein was shown to become incorporated into MLV virions and reduce their infectivity by degrading virion RNA (146). A minor steric effect also resulted in a loss in virion infectivity, suggesting that an aberrant structural phenotype of the heterochimeric viral progeny can prevent the next round of infection**.** This strategy was further extended by designing a Gag-RNase H1 fusion protein with a protease cleavage site between the MLV Gag and *E. coli* RNase H1 domains (147). Cleavage at this site was shown in MLV particles, demonstrating the feasibility of designing a packageable nuclease in which the

activation of the RNase is controlled by intravirion protease cleavage so that enzyme activity would be limited to within the virus and not within cells (147).

The Gag-GFP fusion protein containing the HIV-1 Gag domain and the 238 amino acid GFP (green fluorescent protein) domain was shown to associate with HIV-1 Gag suggesting that cellular routing of HIV-1 Gag was not affected by fusion to GFP (148). The HIV-1 Gag-beta-galactosidase fusion protein was shown to assemble with native HIV-1 Gag precursors into viral particles (149), demonstrating that foreign proteins could also be packaged by HIV. Thus, an HIV Gag-nuclease fusion protein should be co-packaged with HIV and inhibit subsequent rounds of infection. To take advantage of the HIV RNA binding properties of NC, HIV Gag-nucleases should be designed by genetically fusing sequences encoding the nuclease domain downstream of sequences encoding the NC domain of the Gag precursor. This should allow for efficient cleavage of virion genomic RNA.

Since the HIV Gag protein has multiple essential functions (74), it is unlikely that HIV will come up with a viable escape mutant against Gag-nucleases. The HIV-1 Gag also coassembles with HIV-2 Gag (150). Thus, a Gag-nuclease is expected to confer protection against a broad range of HIV-1 and HIV-2 subtypes. The existence of infectious proviral DNA within the virion has been established (17, 18). Therefore, the use of a nuclease such as SN with both RNase and DNase activities should be more effective.

Results obtained in HIV gene therapy: Gag-RNase T1 expressed from MGIN vector was shown to produce virus like particles in transient transfection experiments (Lamothe, Singwi and Joshi; unpublished results). This strategy is currently being tested for inhibition of HIV replication.

4.2.3. Vpr/Vpx-nuclease

HIV-1 Vpr and HIV-2 Vpx proteins are incorporated in virions (151). Thus, Vpr and Vpx proteins can also be used to package nucleases into HIV particles.

Results obtained in HIV gene therapy: Vpr-SN and Vpx-SN fusion proteins were genetically engineered and tested for their enzymatic activity and ability to copackage. Vpr-SN was shown to incorporate into virus-like particles *via* association with HIV-1 Gag, while Vpx-SN was shown to incorporate into virus-like particles *via* association with HIV-2 Gag. Furthermore, it was shown that Vpr-SN and Vpx-SN could compete with their wild type counterparts for incorporation into virus-like particles. Virus-like particles containing either Vpr-SN or Vpx-SN were also shown to possess nuclease activity as they could cleave lambda-phage DNA *in vitro*. In addition to testing the efficacy of the fusion protein in virus-like particles, Vpr-SN and Vpx-SN were also tested for their ability to copackage with HIV. When produced intracellularly, Vpr-SN and Vpx-SN were shown to be incorporated into HIV-1 and HIV-2 particles, respectively. However, nuclease activity was not retained since HIV Pro inactivated the SN moiety. In contrast, virion incorporated Vpr-SN and Vpx-SN were shown to be enzymatically active in the presence of a protease inhibitor. Thus, packageable nucleases should be designed using nucleases resistant to cleavage by HIV Pro. Susceptibility of the fusion nucleases to cleavage may be dependent on the colocalization of Vpr and Vpx with HIV Pro during and after viral assembly. Vpx can be packaged by HIV-1 (151). Vpx-nucleases may, therefore, be used to inhibit both HIV-1 and HIV-2 replication. Since Vpr has been shown to be packaged into HIV-1 and not HIV-2 (115), this strategy may only be used for inhibiting HIV-1 replication. Also, Vpr and Vpx proteins only enhance infectivity and are not essential for HIV replication. Thus, escape mutants of HIV may emerge that prevent packaging of Vpr or Vpx protein, and thus copackaging of a Vpr- or Vpx-nuclease.

4.2.4. Nef-nuclease

The Nef protein is incorporated into viral particles and could thus be used to package a nuclease. Virion associated Nef was shown to be cleaved by the viral Pro between amino acids 57 and 58 (152). Mutational analyses of Nef revealed that both myristoylation and an N-terminal cluster of basic amino acids were required for plasma membrane targeting and virion incorporation of Nef (153). The virion association of Nef is strongly enhanced by myristoylation and does not seem to require other HIV proteins as Nef could be efficiently incorporated into and cleaved inside MLV particles (152). Proteolytic cleavage of the Nef protein results in the liberation of the C-terminal core domain from the membrane-associated N-terminal domain (152). Therefore, Nef-nucleases may be designed by genetically fusing sequences encoding the nuclease domain at the 3'-terminus of the *nef* coding region. The intravirion localization of the C-terminal domain of the Nef protein is not known. This information is critical in predicting the usefulness of this strategy since the nuclease will need access to the genomic RNA in the virion core. Since the Nef protein only enhances infectivity *in vitro* (154), HIV escape mutants may emerge that prevent packaging of Nef and thus copackaging of a Nef-nuclease.

Results obtained in HIV gene therapy: Nef-nucleases have not yet been analyzed for their ability to inhibit HIV replication.

4.2.5. Improved colocalized nucleases

Since scFvs derived from hybridomas are murine in origin, they may be immunogenic in humans. Combinatorial phage libraries may be used to select for high affinity human scFvs. A human scFv gene library may be constructed by heavy and light chain shuffling followed by selection of phage that bind to antigens immobilized on a solid support (155, 156). Protein design can also be used to build better packageable nucleases. A phage-based method could be used to select for proteins with improved stability in a virion environment. For example, stabilized variants of RNase T1 were selected as follows: variants of RNase T1 were inserted into the gene-3-protein (g3p) of the phage head. Since a tight association between domains of g3p are required for infectivity, several cycles of *in vitro* proteolysis, infection and phage propagation were

performed to enrich for phage resistant to proteolysis and thus hosting the most stable variants of RNase T1. Three of ten RNase T1 variants were found to be significantly more stable than the wild type. This strategy can be used to select for packageable nucleases that are resistant to HIV Pro. The temperature, pH, solvent conditions and duration of proteolysis may be varied for selection of nucleases that would be active in the cytosol and virion.

HIV Gag- or Nef-based packageable nucleases may be designed with the minimum sequences required for myristoylation and plasma membrane targeting to minimize immunodominant epitopes. Alternatively, humanized packageable nucleases may be developed by designing therapeutic genes encoding fusion proteins with both the packaging and the nuclease domains of human origin. Cyclophillin A (157) and MAPK (mitogen activated protein kinase) (158) are two cellular proteins that are incorporated into virions. Also the human RNases, EDN and ECP, were shown to have antiviral activity against enveloped single stranded RNA virions *in vitro* (159, 160) could thus be used to construct packageable nucleases.

4.3. Cytotoxic nucleases

Definition: A cytotoxic nuclease is defined as a nuclease which will kill an HIV-infected cell so that viral spread will not occur. Cytotoxic nucleases can be designed to be produced intracellularly in infected cells. Alternatively, they could be designed to be secreted and taken up by infected cells *via* specific cell targeting.

Design: Cytotoxic nucleases are designed to cleave essential host RNAs in HIV-infected cells. Thus, intracellular cytotoxic nucleases must be produced inside infected cells, while secreted cytotoxic nucleases must be secreted from a producer cell and targeted to HIV-infected cells. The gene encoding the intracellular cytotoxic nuclease must be designed to be switched on if the genetically modified cell becomes infected. On the otherhand, the secreted cytotoxic nuclease must be genetically engineered to contain a protein domain which allows specific targeting and internalization into HIV-infected cells. In both cases, the cytotoxic nuclease may inhibit translation by cleaving host RNA in infected cells, thereby conferring specific cell death.

4.3.1. Intracellular cytotoxic nuclease

Nucleases have been shown to exhibit cytotoxic effects in the cell and can, therefore, confer cytotoxicity to HIV-infected cells. An intracellular cytotoxic nuclease should be produced and be cytotoxic in cells that become infected, thus destroying potential reservoirs of viral production. The feasibility of this approach has previously been demonstrated in a 'suicide' gene therapy strategy in which intracellular production of diphtheria toxin selectively killed HIV-1 infected cells (161). Production of this gene product was regulated by HIV-1 Tat and Rev proteins and thus restricted to infected cells only (161).

Various RNases have been also shown to be toxic to human cells and may be used to develop intracellular cytotoxic nucleases. Onconase from Rana pipens oocytes, which is homologous to RNase A, conferred cytotoxicity to glioma cells *via* degradation of 28S and 18S rRNA. RNase activity appears to be essential for cytotoxicity since a non-active form was 100 fold less cytotoxic (162). RNase A is less cytotoxic than Onconase. This may be due to the binding of PRI (placental RNase inhibitor), a 50 kDa RNase inhibitor (163). On the otherhand, Onconase is resistant to PRI and another RNase inhibitor, Inhibit-Ace. Angiogenin, BS-RNase and eosinophil-derived neurotoxin have low K_i values for PRI (164, 165). These nucleases may therefore prove to be better than those which can be inactivated in the cell.

Results obtained in HIV gene therapy: Intracellular cytotoxic nucleases have not yet been analyzed for their ability to inhibit HIV replication.

4.3.2. Secreted cytotoxic nuclease

The object of using a secreted cytotoxic nuclease is to eliminate HIV-infected cells. Unlike intracellular cytotoxic nucleases that employ a self-destructive mechanism, secreted cytotoxic nucleases are designed to destroy the HIV-infected target cell and not the cell they are produced in. A cytotoxic nuclease, secreted from a producer cell, would function by binding specifically to the infected cell, becoming internalized and causing cell death. Thus, the fusion protein would contain a cell targeting domain and a nuclease domain. Cell targeting may be achieved by genetically engineering a secreted cytotoxic nuclease to contain an scFv domain specific for surface antigens present on HIV-infected cells.

The feasibility of secreted cytotoxic nucleases was demonstrated in cancer therapy using recombinant RNase fusion proteins exhibiting cell-type specific cytotoxicity. For example, the gene for an scFv that recognizes the human transferrin receptor was fused in frame to the gene encoding angiogenin, a human homologue of pancreatic RNase. The chimeric protein was shown to bind to the transferrin receptor, to retain RNase activity and to selectively kill human tumor cells containing high levels of the transferrin receptor (166, 167).

Secreted cytotoxic nucleases and immunotoxins may fall under a larger class of cytotoxic molecules designed to specifically kill HIV-infected cells. The design of immunotoxins is based on a principle similar to the one used to develop secreted cytotoxic nucleases. The toxic moieties of immunotoxins are toxins derived from bacteria and plants. Toxins used to date include ricin, pseudomonas exotoxin A and diptheria toxin (168). The immunotoxin CD4-PE40, containing the CD4 domain and the toxic domain of pseudomonas exotoxin A, selectively killed HIV-infected cells *in vitro*; however, it failed to exhibit the same specificity when injected in patients. It also caused toxic side effects even at very low doses (169). Furthermore, the duration of treatment was limited by an immune response (169). Since nucleases can be of mammalian origin, a secreted cytotoxic nuclease should be less immunogenic. Furthermore, systemic delivery *via* gene therapy should allow for stable, continuous and sufficient production of the therapeutic gene product *in vivo*, obviating the need for patient compliance.

Amongst HIV antigens, only the HIV Env protein is a specific cell surface marker of infected cells. As the viral core enters the cell, gp41 remains intact on the cellular membrane, thereby marking the infected cell during viral entry, while gp120 is readily shed (170). Thus, gp41 alone is a marker of infected cells at both early and late stages of viral replication.

Interestingly, when certain RNases of the RNase A superfamily are added to *in vitro* cultures of HIV-infected cells, some viral inhibition is observed (171). BS-RNase and Onconase were shown to modestly inhibit HIV replication in infected cells while remaining non-toxic to uninfected cells (172). In the absence of a targeting domain, it appears that HIV particles may have carried the RNase into the target cell where it can efficiently degrade viral and/or cellular RNA. For a secreted cytotoxic nuclease to cause cell death, it must be internalized and delivered to the cytosol of the infected cell. This was demonstrated by targeting pokeweed antiviral protein (PAP) to CD4+ cells by conjugating it to monoclonal antibodies reactive with CD5, CD7 or CD4 protein on the cell surface. The anti-HIV potency of the conjugated PAP protein was increased by up to 1,000-fold in HIV-infected CD4+ cells (173).

The ability for an immunotoxin such as CD4-PE to kill a cell suggests that the gp120/gp41 complex allows internalization and access to the cytosol. However, the gp41 transmembrane protein may be a better target for a secreted cytotoxic nuclease since soluble gp120 shed from the virus and the infected cells may inhibit the binding of nucleases targeted to gp120. Studies have found that the cytoplasmic domain of HIV-1 gp41 has an internalization motif (174). This motif is similar to those found in the cytoplasmic domains of certain cell surface proteins, such as the transferrin receptor that undergoes rapid constitutive endocytosis in clathrin-coated pits (175). A highly conserved internalization motif is also found within the cytoplasmic domain of HIV-2 gp41 (176). Mutagenesis of specific tyrosine residues results in significantly reduced rates of endocytosis of the HIV-1 Env protein (176). Since the cytoplasmic domain of gp41 alone can confer the ability to undergo internalization in the absence of other HIV proteins, a secreted cytotoxic nuclease targeting gp41 should be sequestered into the infected cells early during the viral life cycle. This strategy should also be effective against latently infected cells harbouring the proviral DNA. Thus, reservoirs of infected cells may be killed before subsequent rounds of infection are initiated.

Results obtained in HIV gene therapy: Secreted cytotoxic nucleases have not yet been analyzed for their ability to inhibit HIV replication.

4.3.3. Improved cytotoxic nucleases

Protein design and selection strategies can be used to build better cytotoxic nucleases. For intracellular cytotoxic nucleases, residues can be mutated to diminish an interaction with a putative cellular protein such as PRI (165). Mammalian cytotoxic nucleases should be sought to lower the immunogenic potential. Libraries of phage displaying peptides can also be screened for secreted cytotoxic nucleases having better binding affinities to a receptor. As already discussed, combinatorial phage libraries can be used to select for high affinity human scFvs (155, 156). In one study, an scFv was obtained with a five to six fold higher affinity compared to antibodies produced from mouse hybridoma cell lines generated with the same antigen (155). Such high affinity scFvs will have lower dissociation constants and thus be present on the cell surface for longer periods of time. With regards to secreted cytotoxic nucleases, the use of human scFvs with lower dissociation constants should allow for higher internalization efficiencies. The best scFv will be the one directed against a well conserved, neutralizing, immunorecessive epitope. This would allow protection against a broad variety of subtypes of HIV-1 and HIV-2, and minimize any competition for the receptor binding site with antibodies produced by the host immune response. Humanization of a cytotoxic nuclease can be achieved by humanizing the antibody, as stated above, and also using a cytotoxic nuclease of human origin (166).

5. GENE DELIVERY AND EXPRESSION

Since blood tissue is amenable to manipulations *ex vivo*, gene therapy for HIV can be carried out *ex vivo*. This involves isolation of autologous or allogeneic cells followed by their genetic modification. Genetically modified cells are then transplanted into the patient. Retroviral vectors can allow for stable gene delivery, cell targeting, and long term gene expression. These vectors are, therefore, widely used to deliver anti-HIV genes. Several retroviral vectors, including MLV-based vectors, can allow for therapeutic gene delivery into PBLs (177, 178); however human HSCs seem to be difficult to transduce because they are difficult to isolate and are quiescent (179). *Ex vivo* delivery of the therapeutic gene into pluripotent HSCs *via* a retroviral vector requires that the stem cell be active, dividing, and not differentiating (179). The right environment and factors needed to foster these events *ex vivo* must be further elucidated for efficient gene delivery into pluripotent HSCs and not intermediate progenitor cells. Gene marking protocols in human HSCs result in less than 1% differentiated, gene-marked progeny cells in the peripheral blood (180, 181).

Stem cells have a low level of amphotropic receptors, which is part of the reason why they are poorly transduced by MLV-based vectors (182-184). Specific targeting with high efficiency has been achieved using pseudotyped vectors. The vesicular stomatitis virus-G protein (185, 186) and gibbon ape leukemia virus Env protein (187) have been shown to improve transduction efficiencies of HSCs. However, since the receptors for these Env proteins are widespread, gene delivery was not cell type-specific. Targeted gene delivery may be achieved by changing the extracellular SU domain of Env with a ligand or an scFv that recognizes a specific cell surface receptor (188). Further advances in cell targeting should be significant since specific cell targeting will be a requirement for *in vivo* gene delivery into HSCs.

Alternatively, lentiviral vectors have been shown to transduce non-dividing cells, obviating the need to find the right conditions for cell division. HIV-based vectors were shown to transduce activated CD34+ cells in G_0/G_1 phase, while MLV-based vectors failed to do so (189). Most fascinating, was the ability of HIV-based vectors to mediate stable *in vivo* gene transfer into terminally differentiated neurons (190). *In vivo* gene therapy should provide a less invasive and cost effective treatment for HIV. Specific *in vivo* delivery of the therapeutic gene to all cell types susceptible to HIV infection may be achieved by using HIV-based replication-incompetent retroviral vector particles, since they would have the same tropism as HIV. HIV-based replication-competent, non-cytopathic vectors can be designed to achieve higher transduction efficiencies *in vivo*. However, since HIV does not infect all target cells, transduction efficiencies may not be very high. Furthermore, HIV-based vectors may be subject to inactivation by preexisting neutralizing antibodies in infected individuals. Strategies which circumvent these obstacles should be explored.

Once the gene has been delivered gene expression must be maintained throughout the treatment at adequate levels in appropriate cell types. The proviral DNA carrying the therapeutic gene may be genetically or epigenetically inactivated, once inserted into the host cell chromosome. Genetic phenomena include proviral DNA rearrangements or deletions, while epigenetic phenomena include suppression of therapeutic gene expression resulting from differential utilization of identical DNA sequences. Differential utilization will be dependent on the chromosomal environment of the therapeutic gene. Euchromatin contains transcriptionally active genes, while heterochromatin is more condensed and therefore is transcriptionally inactive (191). Maintenance of stable gene expression may require the design of vectors capable of targeting the therapeutic gene to specific regions of the chromosome that remain transcriptionally active during cell differentiation and proliferation. Further advances in the area of developing site specific integrases will be needed (192-194). Also, the discovery of locus control regions which insulate promoters from position effects can allow for position-independent gene expression (195, 196).

The retroviral vector can be designed to be HIV Tat- and/or Rev-inducible so that the therapeutic gene product is only manufactured if the cell becomes infected. This can be accomplished by designing, within the retroviral vector, elements that allow HIV Tat- and/or Rev-inducible production of the therapeutic gene product. Tat-inducible gene expression can be achieved by using the HIV LTR promoter or heterochimeric promoters containing the HIV TAR element (197, 198). Rev-inducible production of the therapeutic gene product may be achieved by designing, within the coding region of the therapeutic gene, instability elements (INS) and the RRE (199). INS sequences decrease mRNA stability, whereas the binding of HIV Rev to the RRE overrides this negative effect and increases the half-life of the transcripts produced, making the gene product Rev-inducible (199). MLV-based retroviral vectors allowing Tat- and Rev-inducible gene expression have been constructed (200, 201). HIV-based retroviral vectors were also designed to allow Tat- and Rev-inducible production of the therapeutic gene product (202). Tat- and Rev-inducible gene expression reduces basal expression and limits the therapeutic gene expression to HIV-infected cells. Tightly controlled production also prevented cytotoxic effects by the protective proteins (202). Thus, inducible production of intracellular cytotoxic nucleases should allow the cell to evade cytotoxic effects, if any, prior to HIV infection. Alternatively, the therapeutic genes may be expressed constitutively depending on the nuclease-based strategy used.

5.1. Expression of genes encoding targeted nucleases

Genes encoding targeted nucleases, which are designed to downregulate the HIV gene product post-integration, will have to be expressed once the cell is infected. 100% downregulation is an overwhelming task. Hypothetically, an infinite loop of competition may occur between the therapeutic gene product's ability to downregulate the HIV gene product and the HIV gene product's ability to overcome the downregulation since viral transcription will still continue.

Targeted nucleases designed to cleave HIV TAR, RRE or the psi signal must be maintained at sufficient inhibitory levels to prevent gene expression and/or production of infectious viral progeny. Targeted nucleases designed to cleave the TAR element may be more effective, since TAR RNA interaction with HIV Tat protein is required for *trans*-activation of HIV gene expression (203). A reduction in HIV Tat mRNA level should also decrease the amount of Tat protein available for *trans*-activation. In contrast, targeted nucleases designed to cleave RRE and psi signal may have to be produced at higher levels since viral transcription will continue.

Since a Tat-nuclease is designed to cleave TAR RNA and prevent the production of early gene products like Rev, it cannot be produced in a Rev-inducible manner. A Tat-inducible gene encoding a Tat-nuclease would also be ineffective since it would be self-inhibitory. A gene encoding a Rev-nuclease cannot be Tat-inducible since it should be able to cleave the primary HIV transcripts before they enter the splicing pathway, thus inhibiting the production of early gene products like Tat. Rev-inducible production of a gene encoding a Rev-nuclease would also be ineffective since it would be self-inhibitory. Thus, genes encoding a Tat-nuclease or Rev-nuclease cannot be designed to manufacture the therapeutic gene product in a Tat- and/or Rev-inducible manner and must, therefore, be expressed in a constitutive manner. On the otherhand, the production of an NC-nuclease can be either Tat- and/or Rev-inducible since it is designed to cleave HIV genomic RNA while allowing the production of early gene products, like HIV Tat and Rev.

5.2. Expression of genes encoding colocalized nucleases

The mechanism of inhibition of colocalized nucleases is not very demanding in contrast to targeted nucleases which must act perpetually to downregulate HIV gene products at a post-transcriptional level. An scFv-nuclease

designed to cleave HIV proviral DNA within the pre-integration complex should, therefore, be effective at completely inhibiting virus production. The number of integration events are likely to be finite, since only a limited number of viral particles can enter a cell and get reverse transcribed. Thus, the scFV-nucleases need only inactivate a finite number of preintegration complexes for 100% inhibition of viral replication, until the cell encounters another virus. Colocalized nucleases designed to cleave incoming proviral DNA should, therefore, be produced constitutively so that the therapeutic gene product would be present at the time of viral entry.

Packageable nucleases are designed to produce non-infectious viral progeny. The number of viral progeny produced per infected cell should also be a finite, since the amount of cellular plasma membrane available for viral budding may be limited. Thus, packageable nucleases need only be incorporated and be active in all budding viral particles for 100% inhibition of subsequent rounds of infection. This strategy can potentially disarm all viral progeny produced from the infected cells and, therefore, contribute significantly to decreasing viral loads. The packageable nucleases should not be produced in uninfected cells. Thus, the therapeutic gene product should be manufactured in a Tat- and/or Rev-inducible manner. This can be achieved with Gag-nuclease fusion proteins since the Rev-response is normally required for stability of the HIV Gag transcript (204, 205). Thus, production of the Gag-nuclease fusion gene can be induced in the presence of HIV Rev and synchronized with wild type production of HIV Gag for efficient heterochimeric assembly and virion inactivation. Therapeutic genes designed to produce packagable nucleases based on Vpu, Vpr or Nef should also be produced in a Tat- and/or Rev-inducible manner.

5.3. Expression of genes encoding cytotoxic nucleases

An intracellular cytotoxic nuclease is designed to kill the cell once it is infected and therefore must be produced in a Tat- and Rev-inducible manner. The production of the intracellular cytotoxic nuclease can be achieved such that cell death would occur before viral production ensues, thus inhibiting subsequent rounds of replication. There will have to be stringent requirements on the regulation of gene expression since any basal level expression may kill the cell prior to infection. During vector particle production, strategies must also be designed to avoid any cytotoxicity to the packaging cell lines.

A secreted cytotoxic nuclease can be constitutively secreted, while the infection persists. Secreted cytotoxic nucleases must contain a signal sequence for targeting into the endoplasmic reticulum so that these nucleases can be routed to the cell surface *via* the Golgi complex (206). Secreted cytotoxic nucleases must be delivered at appropriate physiological concentrations to achieve an inhibitory effect. This can be modulated by the number of transduced producer cells that are transplanted.

6. TARGET CELLS FOR INTRACELLULAR AND SYSTEMIC DELIVERY

The therapeutic gene should not only allow inhibition of HIV replication but also the eventual reconstitution of a healthy immune system. Thus, all cell types susceptible to infection should be protected by the therapeutic gene product. These cell types are primarily derived from the HSCs and circulate within the peripheral blood and lymphatic system. The population of cells that need to be genetically modified will depend on the type of nuclease-based strategy employed.

6.1. Intracellular delivery *via* PBLs and HSCs

When the therapeutic gene is designed to protect the cell it is expressed in, all cell types susceptible to infection must be transduced to completely inhibit the spread of the virus. *Ex vivo* transduction of human PBLs will mainly allow protection of a subpopulation of T-lymphocytes and macrophages/monocytes. However, HIV infection also results in the destruction of other cell types, like the dendritic cells and brain microglial cells (207-209). In contrast, human HSCs do give rise to all cell types susceptible to HIV infection. Their pluripotential, differentiative capacity and ability for self-renewal make them an ideal target for therapeutic gene transfer (210). Delivery of the therapeutic gene into this single population of cells should, upon differentiation and proliferation, give rise to a population of mature progeny cells that have acquired the therapeutic gene. The primary source of HSCs include bone marrow, peripheral blood and umbilical cord blood (211-213). Autologous cells may be obtained in the early stages of infection when the percentage of infected cells is low. Allogeneic cells may also be used from healthy, uninfected donors; however, strategies must be developed to minimize the host immune response to a foreign cell source. Alternatively, cell lines of HSCs may be used. Human embryonic stem cell lines could be proliferated *in vitro* for up to five months and still give rise to gut epithelium, cartilage, bone, smooth muscle, striated muscle, neural epithelium, embryonic ganglia, and stratified squamous epithelium (214). Pluripotent stem cell lines will offer a significant advance in HSC gene therapy.

6.2. Systemic delivery *via* producer cells

Gene therapy for HIV may also be implemented by allowing *in vivo* spread of the therapeutic gene product to the infected cells. This may be achieved by systemic delivery of the therapeutic gene product to all sites of infection from a small population of implanted, autologous producer cells. Producer cells may be derived from myoblasts and fibroblasts, since they are not susceptible to infection and stimulate little or no immune response.

Genetically modified myoblasts implanted in the forelimb of a mouse were shown to allow the systemic delivery of scFvs for several months (215). The same study demonstrated that other cell types that are amenable to autologous transplantation, like skin fibroblasts and hepatocytes, are also capable of secreting scFvs *in vitro* (215). The secreted scFvs were able to bind efficiently to their antigen, suggesting that the proper folding mechanism is available in cells other than plasmocytes. This study suggests that it may also be possible for scFv fusion proteins to be secreted by non-B cells. Exocrine organs such as the pancreas and liver can also be used to produce and secrete the therapeutic gene product into the blood. The

therapeutic gene product, if small enough, will diffuse into the lymphatic system and home into the lymph nodes (216). Also, since the Langerhan cells can migrate from the skin through the lymph vessels and to the lymph node, genetic modification of Langerhan cells in the skin can allow for localization of the therapeutic gene product to the lymph nodes (217).

7. RECONSTITUTION OF A HEALTHY IMMUNE SYSTEM

In the case of PBL gene therapy, the therapeutic gene is delivered into a subpopulation of mature progeny cells. Since these cells are not self-renewing and have a finite life span, PBL gene therapy will require repeated cycles of transduction and transfusion until viral load is sufficiently diminished and the immune system is reconstituted with healthy cells. In contrast, since HSCs can self-renew, the delivery of the therapeutic genes into these cells should not require repeated cycles of transduction and transfusion.

Unless all cells susceptible to infection can be transduced, the eventual reconstitution of a healthy immune system will require that genetically modified cells have a selective survival advantage over untransduced cells. Given that HIV will kill the untransduced cells, natural selection of transduced cells containing genes conferring resistance will happen *in vivo*. Once the viral load begins to decrease, repopulation of uninfected transduced and untransduced cells would result in eventual reconstitution of a healthy immune system. In contrast, for genes conferring negative selection, the immune system would have to be populated with a higher proportion of transduced cells. These cells could serve to reduce viral load over time, resulting in the eventual reconstitution of a healthy immune system.

Since gene therapy may likely be applied to patients already on anti-HIV drug therapy, a low viral load (maintained by anti-HIV drugs) may not constitute enough of a selection pressure on transduced cells. Thus, for gene therapy strategies conferring positive and especially negative selection, the proportion of transduced cells will have to be increased by additional selection *in vivo* (218). A recent study demonstrated that upon transduction of murine stem cells with the antifolate resistant DHFR gene and *in vivo* drug selection, transplanted mice were repopulated with a significantly increased percentage of vector expressing peripheral blood erythrocytes, platelets, granulocytes, and T and B lymphocytes (219). Furthermore, transduced cells were detected in the bone marrow, spleen, lymph nodes and thymus (219).

Systemic delivery of the therapeutic gene product is achieved from producer cells such as myoblasts or fibroblasts that are not susceptible to infection. Thus, selection criteria are not required for such strategies which do not require *in vivo* spread of the genetically modified cells, but rather *in vivo* spread of the therapeutic gene product.

7.1. Reconstitution of a healthy immune system using targeted nucleases

A targeted nuclease–based strategy should confer protection to the genetically modified cells while allowing maintenance of normal cellular function. Thus, positive selection of protected, functional cells will take place until viral load is sufficiently diminished. Maintenance of normal cellular functions will depend on the step(s) in the viral life cycle the therapeutic gene is designed to inhibit. The level of cytotoxicity will be directly proportional to the passaging of the viral life cycle that is allowed in the transduced cells, and most pronounced if viral gene products appear and viral progeny begins to assemble. A targeted nuclease-based strategy may be used in PBL or HSC gene therapy. Tat and Rev-nucleases will allow selective destruction of viral RNA early in the viral life cycle. An NC-nuclease, on the other hand, will still allow production of early and some late gene products which may be toxic to the cell. Thus, a therapeutic gene designed to inhibit early stages of the viral life cycle may be preferred over those designed to inhibit the later stages of the viral life cycle.

7.2. Reconstitution of a healthy immune system using colocalized nucleases

An scFv-nuclease, designed to inhibit HIV replication before integration, is ideal since it will prevent the acquisition of proviral DNA by the host cell and allow the maintenance of normal cellular functions. Thus, an scFv-nuclease can be used in PBL or HSC gene therapy. Consequently, the immune system will be reconstituted with a population of transduced cells lacking proviral DNA until viral loads are sufficiently diminished for reconstitution by healthy cells.

A packageable nuclease can also be used in PBL or HSC gene therapy. This strategy involves interference at the late phase of the viral life cycle, rendering the progeny virus produced from the genetically modified cells non-infectious. Packageable nucleases can potentially inhibit viral replication very effectively, especially in chronically infected cells. However, the genetically modified cells that do become infected may not function normally because production of viral proteins is still allowed. This strategy may, thus, confer negative selection to the infected cells by inhibiting normal cellular functions. Thus, PBL gene therapy will require repeated cycles of transduction and transfusion to decrease the viral load incrementally. Reconstitution of the immune system with healthy cells will take place once viral loads are sufficiently diminished. HSC gene therapy will require a significant proportion of total HSCs to be transduced. Only then will the immune system be continuously repopulated with a sufficient amount of genetically modified cells to reduce viral load.

7.3. Reconstitution of a healthy immune system using cytotoxic nucleases

Since intracellular cytotoxic nucleases are designed to catalyze the self-destruction of infected cells, this strategy clearly results in negative selection of the genetically modified cells. PBL gene therapy may be considered for this strategy, but will require repeated cycles of transduction and transfusion to decrease viral load

incrementally. Like in the case of a packageable nuclease strategy, HSC gene therapy using intracellular cytotoxic nucleases will also require a significant proportion of the total HSCs to be transduced to allow reconstitution of the immune system with mainly genetically modified cells.

In contrast to the above strategies which rely on *in vivo* spread of the genetically modified cells, the secreted cytotoxic nuclease strategy relies on *in vivo* spread of the fusion protein to infected cells. The continuous and regulated *in vivo* production of secreted cytotoxic nucleases will require only a limited number of transduced producer cells. Reconstitution of the immune system will take place once the viral load is sufficiently diminished. The transplanted producer cells may then be removed, if required.

8. CONCLUSIONS

Many obstacles must be overcome for HIV gene therapy to realize its true potential. The resolution of these obstacles will depend on the convergence of many subdisciplines including therapeutic gene design, gene expression, gene delivery, transplantation, and immunology. It is also conceivable that drug therapy and gene therapy will complement each other. Anti-HIV nucleases offer a novel approach that should be further exploited for effective inhibition of HIV replication. Furthermore, nuclease-based strategies may also be applicable for the treatment of other genetic disorders.

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