Novel Cell and Gene Therapies for HIV

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Highly active antiretroviral therapy dramatically improves survival in HIV-infected patients. However, persistence of HIV in reservoirs has necessitated lifelong treatment that can be complicated by cumulative toxicities, incomplete immune restoration, and the emergence of drug-resistant escape mutants. Cell and gene therapies offer the promise of preventing progressive HIV infection by interfering with HIV replication in the absence of chronic antiviral therapy. Individuals homozygous for a deletion in the CCR5 gene (CCR5 Δ 32) are largely resistant to infection from R5-tropic HIV-1 strains, which are most commonly transmitted. A recent report that an HIV-infected patient with relapsed acute myelogenous leukemia was effectively cured from HIV infection after transplantation of hematopoietic stem/progenitor cells (HSC) from a CCR5 Δ 32 homozygous donor has generated renewed interest in developing treatment strategies that target viral reservoirs and generate HIV resistance in a patient's own cells. Although the development of cell-based and gene transfer therapies has been slow, progress in a number of areas is evident. Advances in the fields of gene-targeting strategies, T-cell-based approaches, and HSCs have been encouraging, and a series of ongoing and planned trials to establish proof of concept for strategies that could lead to successful cell and gene therapies for HIV are under way. The eventual goal of these studies is to eliminate latent viral reservoirs and the need for lifelong antiretroviral therapy.

In the era of highly active antiretroviral therapy (HAART), HIV replication can be suppressed to low or undetectable levels (Kaufmann et al. 2000; Maartens et al. 2007; Kitahata et al. 2009) with a corresponding but variable increase in CD4 T-cell counts (Valdez et al. 2002; Moore et al. 2005; Geng and Deeks 2009). Survival has improved dramatically, and HAART has turned HIV infection into a chronic, manageable illness. However, survival

remains reduced by at least 10 years compared with the general population, and there are long-term complications (Lewden et al. 2005; Lohse et al. 2007) including accelerated cardiovascular disease, liver and renal failure, neurocognitive dysfunction, and HIV-associated malignancies (Cheung et al. 2005; Lekakis and Ikonomidis 2010; Nunez 2010; Xia et al. 2011). Ongoing immune activation and inflammation can occur and may contribute to

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increased morbidity and mortality (Giorgi et al. 1999; Rodger et al. 2009). Many patients, particularly those who began HAART in the setting of advanced immunodeficiency, do not achieve complete CD4 reconstitution (≥ 500 cells/mm³) (Valdez et al. 2002; Kelley et al. 2009), which has been linked to increased morbidity (Lewden et al. 2007; Baker et al. 2008). Moreover, even when viral replication is suppressed for many years, replicating HIV typically reappears in plasma once HAART is discontinued, indicating that patients are not cured and that long-lived viral reservoirs persist (Chun and Fauci 1999; Blankson et al. 2002). Given the limitations and the potential complications of HAART, there has been renewed interest in novel approaches to control or preferably cure HIV infection (Richman et al. 2009).

With advances in understanding the molecular basis for HIV replication and mechanisms for host control, a number of investigators are focusing on cell-based and gene therapy either as stand-alone approaches or as adjuvants to pharmacological drug regimens. There have been several excellent reviews of this topic (Poluri et al. 2003; Strayer et al. 2005; Edelstein et al. 2007; Rossi et al. 2007). These approaches have potential advantages compared to conventional drugs. In particular, the self-renewing properties of cell and genetic strategies at least in principal can obviate the need for lifelong antiretroviral medications. As will be discussed, cell-based approaches involving hematopoietic stem cell transplantation have the potential to be curative (Hütter et al. 2009; Allers et al. 2010; Deeks and McCune 2010). This review will consider novel therapies that focus on modifying host cells to resist HIV infection with approaches directed at viral or cellular targets. One general consideration is that although nucleic acid-based antivirals can be designed to have high specificity for HIV-1 targets at the gene, RNA, or protein level, as with pharmacologic approaches, viral escape is a major concern. In contrast, cellular targets are far less prone to mutational escape, although with the exception of CCR5, the side effects of down-regulating cellular targets for the long term are unknown.

HISTORY

Cell and gene therapy strategies have been proposed from the earliest days of the HIV epidemic. Friedmann and Anderson have reviewed the conception and early history of gene transfer therapy (Friedmann and Roblin 1972; Anderson 1984; Friedmann 1992). Baltimore first proposed a gene therapy approach for treating HIV/AIDS, in which a retrovirus could be engineered to antagonize HIV by expressing dominant negative RNA or protein inhibitors (Baltimore 1988).

Allogeneic and Xenogeneic Transplantation

Xenotransplantation was proposed as a cell-based therapy, with the rationale that HIV-1-infected humans could be reconstituted with nonhuman primate bone marrow, an approach that would take advantage of the intrinsic resistance of nonhuman species to infection with HIV-1. In 1995, a 38-yr-old patient infected with HIV for more than 15 years underwent a controversial experiment following approval by the U.S. Food and Drug Administration. His bone marrow was suppressed by sublethal doses of radiation and chemotherapeutic drugs, after which he received an infusion of bone marrow cells from a baboon. This experiment occurred before the advent of HAART therapy, at a time when the standard treatment approaches were failing to control HIV, and the AIDS activist community was increasingly convinced that radical approaches should be explored. The patient survived and improved for reasons that are unclear, as the baboon cells failed to engraft and were not detectable in his bone marrow beyond the first month posttransplant (Michaels et al. 2004).

Allogeneic and syngeneic (twin) hematopoietic stem cell transplantation (HSCT) were proposed as a treatment for AIDS, based on the positive experiences with allogeneic HSCT in children with severe combined immunodeficiency (Cavazzana-Calvo and Fischer 2007). Patients with HIV/AIDS-associated malignancies for whom HSCT was standard therapy provided an opportunity to determine the regenerative potential of bone marrow and

mature lymphocytes in the setting of HIV infection. The first report of allogeneic HSCT described two men with AIDS and Kaposi's sarcoma who were given infusions of partially compatible bone marrow from uninfected family donors (Hassett et al. 1983). No clinical benefit occurred, and engraftment of the donor bone marrow was brief, probably because no conditioning regimen was given to the patients. In retrospect, it was not realized at that time that even though AIDS patients have severe immunosuppression, they are still able to mount potent rejection responses to organ and bone marrow grafts. Lane and colleagues conducted the first study using infusion of bone marrow from a healthy identical twin to a patient with AIDS. The patient was given a single infusion of bone marrow without conditioning, followed by repeated infusions of syngeneic peripheral blood lymphocytes from the donor (Lane et al. 1984). Transient clinical benefit was observed with increased CD4 counts that peaked three months after stem cell infusion. These early experiments were conducted in the pre-HAART days in the presence of high levels HIV viremia, and the results may have been more durable with long-term immune reconstitution had effective antiretroviral therapy been available. Consistent with this notion, Holland and coworkers reported the case of a patient with HIV and lymphoma who received an allogeneic HSCT with concomitant zidovudine therapy. The patient engrafted with donor marrow, but died from progressive lymphoma 47 days after HSCT. An autopsy revealed no evidence for residual HIV-1 by culture or polymerase chain reaction (PCR) of numerous tissues, suggesting that myeloablative chemoradiotherapy combined with antiretroviral therapy had a substantial impact on latent viral reservoirs in host cells (Holland et al. 1989).

PROTEIN-BASED INHIBITORS

A number of criteria have been proposed for developing genetic inhibitors of HIV for human clinical trials, including (1) potency of inhibition, (2) lack of immunogenicity, (3) lack of toxicity, (4) the stage in the viral life cycle that

they target, and (5) their potential for selecting resistant viruses (Dropulic and June 2006). Mathematical modeling has predicted that postintegration inhibitors lead to the persistence of cells carrying an integrated provirus, resulting in an accumulation of HIV-1-infected cells that could ultimately counteract their antiviral effect. In contrast, inhibitors that act before integration, even those with lower potency, are predicted to exert a systemic antiviral effect with the expansion of transduced cells capable of resisting HIV infection (von Laer et al. 2006).

Protein-based inhibitors can be directed toward cellular or viral targets and have included dominant negative inhibitors, intrabodies, intrakines, fusion inhibitors, and zinc finger nucleases (ZFNs). Each type of protein inhibitor is described in detail below. These inhibitors are typically expressed from a viral vector long terminal repeat (LTR), but in several instances are produced from strong constitutive promoters inserted within the bodies of the viral vectors. Strictly speaking, ZFNs are protein-based inhibitors that have a fundamentally different mechanism than the other inhibitors in this class, and so are discussed in a separate section.

Dominant Negative Inhibitory Proteins

The first protein used in a gene therapy trial for treating HIV infection was a mutant form of the HIV Rev protein called M10 (Woffendin et al. 1996). Rev M10 is believed to work by blocking the export of singly spliced and unspliced HIV RNA from the nucleus to the cytoplasm thereby preventing viral assembly and subsequent transmission (Malim et al. 1992). This mutant protein remains one of the most potent inhibitors of HIV replication and has been evaluated in human clinical trials (Ranga et al. 1998; Morgan et al. 2005; Podsakoff et al. 2005). Although there was no long-term benefit, a modest survival advantage of M10-expressing CD4 cells was observed.

Intrabodies and Intrakines

The expression of intracellular antibodies (termed "intrabodies"), typically as single-chain Fv fragments that target viral or cellular proteins,

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and chemokines (termed “intrakines”) have also proven to be very potent inhibitors of HIV replication in vitro (Yang et al. 1997; Schroers et al. 2002; Poluri et al. 2003; Lobato and Rabbitts 2004; Lo and Marasco 2008; Zhang et al. 2009). These proteins bind to viral or cellular target proteins leading to their intracellular degradation. Notable cellular targets for this approach have included the HIV coreceptors CCR5 and CXCR4 (Yang et al. 1997; Schroers et al. 2002; Zhang et al. 2009).

Fusion Inhibitors

von Laer and colleagues developed a novel protein-based fusion inhibitor, termed C46, which binds to HIV gp41 at the cell surface and blocks viral entry (Hildinger et al. 2001; Egelhofer et al. 2004; Perez et al. 2005). C46 is comprised of amino acids from the second heptad repeat of gp41. As with other protein-based inhibitors, it can be expressed constitutively by retroviral vectors and applied to a gene therapy setting. A membrane-anchored form of C46 (maC46) was shown to confer HIV resistance to hematopoietic stem cells in nonhuman primates (Trobridge et al. 2009). In a comparative study, the maC46 fusion inhibitor was compared to an HIV-1 tat/rev-specific small hairpin (sh) RNA (Lee et al. 2002) and an RNA antisense gene specific for the HIV-1 envelope glycoprotein (Dropulic et al. 1996). Notably, maC46 proved to be the most potent in conferring a selection advantage to transduced cells following HIV-1 inoculation in vitro and in humanized mice in vivo (Kimpel et al. 2010). The maC46 transgene was tested in a phase I trial in 10 patients with late-stage HIV infection (van Lunzen et al. 2007). The infusions of transduced T cells were well tolerated, and a significant albeit transient increase of CD4 counts was observed after infusion.

Inhibitors of Viral Restriction Factor, TRIM5 α

An early event in the viral life cycle that is well suited to protein-based inhibition is uncoating of the viral capsid following entry, which is highly restricted for HIV-1 in rhesus macaques

by the host protein tripartite motif (TRIM)5- α (Stremlau et al. 2004; Nisole et al. 2005; Malin and Bieniasz 2011). Under permissive conditions the HIV and SIV capsid proteins have evolved to escape binding by TRIM5- α , which otherwise binds and leads to degradation of the capsid and a failure of reverse transcription. It has been shown in vitro that in human cells expressing either TRIM5- α from a nonpermissive primate species or a human TRIM5- α that was rendered competent for binding to the HIV capsid, these proteins act in a dominant negative manner to inhibit replication (Yap et al. 2005; Stremlau et al. 2006). These approaches represent promising interventions for future protein-based gene therapy.

Although protein-based approaches can have potent antiviral effects in vitro, a principal limitation is not only the need to maintain sufficient levels of expression from the vector, but to avoid immunogenicity in vivo, as immune responses could confound persisting antiviral activity. Thus, for protein inhibitors that are advanced to clinical trials both their potency and immunogenicity will need to be assessed.

NUCLEIC ACID-BASED INHIBITORS

A number of RNA-based approaches, including antisense RNAs, aptamers, decoys, ribozymes, and si/shRNAs have been described. An advantage of these inhibitors is that in contrast to protein-based inhibitors, they do not elicit adaptive T-cell responses and are unlikely to be immunogenic. However, RNA approaches can potentially have off-target toxicity because of activation of innate immune responses and competition with endogenous RNA functions (Lares et al. 2010).

Antisense RNA

Short and long antisense RNA transgenes that pair with HIV transcripts to form duplexes that are nonfunctional have been effective in blocking HIV replication in hematopoietic cells. The first demonstration of this principle came from studies using adeno-associated virus vectors to deliver a short anti-U5 region antisense

RNA (Chatterjee et al. 1992). More recently, Levine and coworkers infused patients with autologous CD4 T cells that were genetically modified by a conditionally replicating lentiviral vector expressing a long antisense RNA to the HIV envelope mRNA (Levine et al. 2006). In a follow-up study (clinicaltrials.gov NCT00295477), Tebas and coworkers showed a decrease in viral load in the majority (88% [7/8]) of these patients after antiretroviral therapy was discontinued, with one patient maintaining undetectable HIV RNA for 104 days (Tebas et al. 2010). Although the mechanism by which these antisense transcripts inhibited HIV replication is not clear, it could have triggered extensive adenosine deamination of the HIV/antisense duplex, resulting in nuclear retention of transcripts or the generation of multiple viral disabling mutations (Lu et al. 2004). A further analysis of transduced cells from patients in this trial using 454 pyrosequencing showed no evidence for abnormal expansion of cells because of vector-mediated insertional activation of proto-oncogenes, suggesting that no adverse events were apparent at the molecular level (Wang et al. 2009).

Aptamers

RNA aptamers are RNA molecules with structural features that facilitate high-affinity interactions with targeted ligands. They are amenable to rapid selection in vitro and can be designed to bind to virtually any protein of choice (Nimjee et al. 2005). There are now a number of highly effective aptamers available for testing in gene therapy settings (Symensma et al. 1996; Joshi et al. 2003; Held et al. 2006, 2007; Kissel et al. 2007), although to date none have been tested in clinical trials for HIV infection.

RNA Decoys

The HIV Tat protein binds to a structure on the 5' UTR of mRNAs termed the TAR motif, and is required for efficient transcription (Arya et al. 1985; Sodroski et al. 1985). Short RNA oligonucleotides corresponding to the TAR sequence, termed TAR decoys, bind to Tat and block its

interaction with the authentic TAR region to inhibit HIV gene expression and replication (Lisiewicz et al. 1993; Bohjanen et al. 1997). RNA decoys have also been developed to prevent Rev from binding to the Rev response element (RRE), which is required to transport unspliced and singly spliced mRNAs from the nucleus (Lee et al. 1994). A clinical trial to evaluate the safety and feasibility of an RRE decoy was conducted using bone marrow from four HIV-1-infected pediatric subjects (Kohn et al. 1999). The approach was safe; however, expression of the RNA decoy was likely insufficient for antiviral effects to be seen.

Ribozymes

Ribozymes are antisense RNAs that not only bind but enzymatically cleave targeted mRNAs. The first demonstration that ribozymes were effective in inhibiting HIV replication was published in 1990 (Sarver et al. 1990). Since then, many ribozyme-based antivirals have been developed, and some have been evaluated in clinical trials including those targeting HIV genes *tat* and *rev*, and the U5 region of the viral LTR. Ribozymes in these trials were either expressed from the retroviral LTRs as long, capped, polyadenylated transcripts from the retroviral LTR promoter (Bauer et al. 1997; Ngok et al. 2004), or as a discrete, chimeric Pol III tRNA-ribozyme transcript (Leavitt et al. 1994; Li et al. 2005). Four of these trials involved retroviral vector delivery of decoy or ribozyme genes into autologous hematopoietic progenitor cells isolated from HIV-1-infected individuals (Kohn et al. 1999; Amado et al. 2004; Mitsuyasu et al. 2009; DiGiusto et al. 2010). Following retroviral transduction, cells were reinfused either in the absence of bone marrow conditioning, or in one case with bone marrow conditioning to treat an AIDS-related lymphoma (DiGiusto et al. 2010). Although these trials showed that ribozymes and decoys could be safely introduced into mobilized stem cells or peripheral blood mononuclear cell cultures (PBMCs) and reinfused in patients, no significant anti-HIV effects were observed. However, in a recent phase II clinical

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trial, Mitsuyasu et al. (2009) showed that an anti-tat ribozyme could be safely introduced in autologous stem cells from a gammaretroviral vector. This vector (termed OZ1) expressed a hammerhead ribozyme that targeted the overlapping reading frames of the viral *vpr* and *tat* genes in unspliced and spliced viral transcripts, respectively. Although no significant differences were observed in the viral load between OZ1-treated and placebo groups at the time of study's primary end point at 12 months, they observed a significantly lower viral load at later time points, and OZ1-treated patients had higher CD4 cell numbers at all time points. This study is notable because it is the first randomized, double-blinded phase II clinical trial for HIV-1 infected patients to test gene-modified HSC, and showed the feasibility of conducting complex cell and gene transfer trials that will likely be used in future protocols to eradicate HIV infection.

RNA Interference

RNA interference (RNAi) is a regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules as triggers to direct homology-dependent control of gene activity (Hannon and Rossi 2004). Known as small interfering RNAs (siRNA) these ~21- to 22-bp-long dsRNA molecules have characteristic two-nucleotide 3' overhangs that allows them to be recognized by the enzymatic machinery of RNAi leading to homology-dependent degradation of the target mRNA. To date, preclinical studies indicate that this is the most potent RNA-based inhibitory mechanism available for therapeutic application. Virtually all of the HIV-encoded mRNAs have been shown to be susceptible to RNAi down-regulation in cell lines, including *tat*, *rev*, *gag*, *pol*, *nef*, *vif*, *env*, *vpr*, and the LTR (Rossi et al. 2007).

A substantial challenge for clinical applications of RNAi inhibitors is the high mutation rate of HIV, which readily generates escape mutants. Ideally, conserved sequences on transcripts encoding gene products required for critical functions in the viral life cycle and that cannot tolerate mutations will need to be

targeted (Zhang et al. 2007). An alternative approach to relying solely on RNAi is to combine a single shRNA with other antiviral genes, thereby providing synergistic inhibition. This approach has been successfully performed by coexpressing an anti-tat/rev shRNA, a nucleolar localizing TAR decoy, and an anti-CCR5 ribozyme in a single vector backbone (Li et al. 2005). A pilot trial testing this triple combination lentiviral vector transduced into stem cells from patients with HIV and non-Hodgkin's lymphoma was recently reported (DiGiusto et al. 2010). Engraftment occurred by 11 d, with low but persisting levels of gene marking observed in several cell lineages for up to 24 mo. In preclinical studies, this triple combination vector was compared to the protein-based maC46 fusion inhibitor and to a long RNA antisense vector, and was found to be less efficient than C46 in providing a selective advantage in HIV-1-infected cultures of primary T cells (Kimpel et al. 2010).

CCR5 AS AN ANTI-HIV TARGET AND ERADICATION STRATEGIES

The identification of CCR5 as the major coreceptor for transmitted HIV-1 isolates provided an explanation for the previously noted resistance of individuals who were frequently exposed to HIV, but remained uninfected, and were found to be homozygous for the CCR5 Δ 32 allele (Samson et al. 1996). The mutation is caused by a 32-bp deletion resulting in a frameshift mutation that truncates CCR5 and prevents its expression on the cell surface (Huang et al. 1996; Samson et al. 1996; Zimmerman et al. 1997). Given that individuals lacking CCR5 are entirely healthy, this observation provided a strong impetus for the development of drugs that target the virus-CCR5 interaction, one of which is now FDA approved (Gulick et al. 2008).

The "Berlin Patient"

Although CCR5 small molecule inhibitors are proving to be clinically useful (Gulick et al. 2008), their activity did not predict the remarkable outcome of a patient who has apparently

been cured of HIV infection following an allogeneic HSC transplant from a homozygous CCR5 Δ 32 donor in an extraordinary experiment conducted by Gero Hütter and colleagues in Berlin (Hütter et al. 2009). The patient, infected with HIV for 10 years and well controlled on antiretroviral therapy (ART), developed acute myelogenous leukemia unrelated to his HIV. When ART was interrupted during antileukemia chemotherapy, HIV plasma RNA not surprisingly increased to a high level, and was again controlled when ART was restarted. Despite achieving a remission, the patient's leukemia relapsed, requiring that an allogeneic HSC transplant be performed as lifesaving therapy. In the German database for unrelated donors, one individual was identified who was MHC compatible and also homozygous for the CCR5 Δ 32 mutation in CCR5. The patient received a HSC transplant from this donor, with ART being discontinued at the time of stem cell infusion. Strikingly, 3½ years post-transplant and in the continued absence of antiretroviral therapy, no HIV has been detected using the most sensitive molecular assays for viral RNA or DNA in plasma, lymphoid tissues, and peripheral blood CD4 cells (Allers et al. 2010; Hütter and Thiel 2011). For a variety of reasons, including the low frequency of CCR5 Δ 32 homozygotes in the general population and the logistics and feasibility of identifying suitable donors, similar transplants in HIV-infected patients are unlikely to be performed anytime soon, leaving open many questions (discussed further below) as to how HIV was eradicated in this patient (Deeks and McCune 2010). However, given this proof of concept that HIV infection can be apparently cured, the challenge to the field is to develop more generally applicable approaches that (1) do not require intensive myeloablative chemotherapy and (2) can be performed using autologous rather than allogeneic cells.

HIV Latency

The establishment of HIV latency and the failure of antiviral therapies to eradicate persisting, long-lived viral reservoirs is a central issue in the

field (discussed by Siliciano and Greene 2011). Whereas the clinical result from the Berlin patient suggests that donor CCR5-negative HSCs and engraftment of HIV-resistant T cells can ultimately suppress or prevent HIV replication and eradicate HIV reservoirs, other mechanisms likely contributed (Deeks and McCune 2010; Cannon and June 2011). It is possible or even likely that the myeloablative therapy could have provided a synergistic effect. However, there are numerous reports of HIV-infected individuals undergoing allogeneic HSC or bone marrow transplants from donors not selected to be CCR5-negative, demonstrating that HIV-1 was not eliminated by the particular chemotherapy regimens used (Hassett et al. 1983; Angelucci et al. 1990; Bardini et al. 1991; Schlegel et al. 2000; Kang et al. 2002; Avettand-Fenoel et al. 2007; Wolf et al. 2007; Woolfrey et al. 2008; Kamp et al. 2010; Polizzotto et al. 2010), with one possible exception (Holland et al. 1989). Of note, the Berlin patient's myeloablative therapy consisted of amsacrine, fludarabine, cytarabine, and cyclophosphamide, a regimen that has not previously been reported in patients with HIV infection. He was also treated with total body irradiation (TBI), both to enhance the antileukemic chemotherapy and for immunosuppression to facilitate engraftment; although historically, TBI used in conjunction with myeloablative therapy has not eradicated HIV (Giri et al. 1992; Tomonari et al. 2005; Polizzotto et al. 2010). Of potential importance, the Berlin patient was also treated with several agents to prevent graft versus host disease, including antithymocyte globulin (ATG), cyclosporine, and mycophenolate mofetil, all of which have potent cytolytic or suppressive effects on T cells. Given the role of CD4/CCR5 memory T cells as a long-lived reservoir for HIV (Han et al. 2007), it is likely that these agents directly targeted this reservoir. ATG, in particular, is not only a potent immunosuppressive drug, but contains polyclonal antibodies directed against all known lymphocyte subsets (Rebellato et al. 1994). The effects of ATG in the setting are largely unknown, because this potent immunosuppressive agent has only rarely been given to

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patients with chronic HIV-1 infection (Wolf et al. 2007). Finally, it is possible that innate or acquired immunity delivered by the donor immune system may have contributed to the elimination of residual HIV reservoirs. The Berlin patient developed graft versus host disease, and an allogeneic immune response directed against host lymphocytes could have had a purging effect on latent HIV reservoirs. Allogeneic immune effects are among the most powerful known immune responses, and are capable of eradicating host lymphocytes and stem cells as well as malignant hematopoietic cells in the recipient (Barrett and Malkovska 1996; McSweeney et al. 2001). In summary, although an allogeneic transplant from a normal donor cannot in itself explain the remarkable clinical outcome reported by Hütter and colleagues, it is possible that transplantation with HIV-resistant cells, myeloablative therapy, anti-T-cell therapy, and alloimmune responses could all have contributed to the observed long-term control of HIV (Hütter et al. 2009; Allers et al. 2010; Hutter and Thiel 2011).

GENE THERAPY STRATEGIES TO REDUCE CCR5 EXPRESSION

As noted previously, there are a number of gene therapy approaches to inhibit CCR5 expression with a goal of mimicking the CCR5 null phenotype of a *ccr5Δ32* homozygote (Nazari and Joshi 2008). These can act at the RNA level with RNA interference (Anderson et al. 2003; Qin et al. 2003; Anderson and Akkina 2005) or ribozymes (Bai et al. 2000, 2001; Cordelier et al. 2004), or at the protein level with CCR5-targeted intrabodies (Cordelier et al. 2004; Swan et al. 2006) or intrakines (Yang et al. 1997; Schroers et al. 2002). Improvements in humanized mouse models that support the generation of human T cells in vivo (Denton and Garcia 2009) are permitting analyses of their relative efficacies. Recent reports have highlighted the potential of RNA interference to down-regulate CCR5 expression, including the possibility of exploiting in vivo delivery of siRNAs through the use of T-cell-targeted

nanoparticles (Kumar et al. 2008; Kim et al. 2009). Alternatively, RNA interference can be achieved through the stable expression of shRNAs targeting CCR5 from lentiviral vectors. Transduction of such vectors into human CD34⁺ HSC allowed HIV resistance to be conferred on both macrophages derived in vitro from the transduced cells (Liang et al. 2010) as well as T-cell progeny that differentiated in vivo in a bone marrow/liver/thymus (BLT) mouse model (Shimizu et al. 2010). A targeted strategy to deliver lentiviral vectors expressing an anti-CCR5 shRNA specifically to CCR5⁺ cells in vivo was also shown using a PBMC transplanted mouse (Anderson et al. 2009) and in nonhuman primates after stem cell transplants (An et al. 2007).

Aside from these encouraging results in humanized mouse and nonhuman primate models, the utility of genetic approaches to treat HIV infection will ultimately require evaluation in patients. Given the chronic but typically manageable nature of HIV infection with ART, the clinical and ethical criteria for patient selection in gene therapy trials will need to be carefully considered. Notably, patients who develop AIDS-related malignancies, particularly lymphomas, a subset of which will require autologous HSC transplants, represent a unique cohort in which gene therapy approaches can be evaluated, because their HSCs are mobilized and harvested before chemotherapy. This provides both an opportunity to engineer HSCs to be resistant to HIV infection and to increase the chances of engraftment of the modified cells following a myeloablative conditioning regimen (DiGiusto et al. 2010). Moreover, as noted above, anti-lymphoma chemotherapy in this setting may have the added benefit of targeting HIV reservoirs.

GENE-EDITING STRATEGIES TO DISRUPT CCR5

All of the strategies described above have the challenge of achieving and maintaining sufficient levels of anti-CCR5 activity to decrease CCR5 expression to levels that are insufficient for HIV infection. In addition, in the case of

lentiviral vector-based approaches, there remains the theoretical risk of these integrating vectors causing insertional mutagenesis in host DNA. More recently, gene-editing approaches to achieve permanent CCR5 gene disruption have been described using ZFNs that do not require continuous expression of a therapeutic gene.

ZFNs are engineered fusion proteins that contain two linked domains: a DNA-binding zinc finger protein and the endonuclease domain of a type-I restriction enzyme (Fig. 1) (Urnov et al. 2010). ZFNs have been designed to target many genes, including CCR5 and CXCR4, and are currently being evaluated in preclinical and clinical settings as HIV therapeutics (Lombardo et al. 2007; Perez et al. 2008; Holt et al. 2010; Wilen et al. 2011). The zinc finger protein domain is an artificial array of zinc finger peptides that confer sequence-specific

DNA-binding properties. This occurs because three to four residues toward the tip of each zinc finger peptide make contact with three or four base pairs of DNA. Altering the contact residues changes the specificity of the zinc finger for a DNA sequence. Moreover, linking multiple zinc fingers extends the length of the DNA sequence that is recognized, and because ZFNs are dimerized via their endonuclease domain, this further extends the length and the specificity of a targeted sequence. In this way, a ZFN pair engineered to bind to the sense and the antisense regions of a gene can target a sequence that is theoretically unique in the human genome, although the potential for off-target effects is always a consideration.

On binding, ZFNs act as designer restriction enzymes, cutting both strands of DNA at the bound target sequence. Following cleavage, the double-stranded break is repaired in

Figure 1. Zinc finger nucleases. (A) ZFNs bind CCR5 target sequence (underlined), and create a five-base-pair double-stranded staggered cut. (B) The double-stranded break is repaired by the error-prone nonhomologous end-joining (NHEJ) repair pathway. (C) The NHEJ results in a variety of insertions or deletions that disrupt the open reading frame (ORF). In the case of the CCR5 ZFNs, the most common repair leads to a 5-pb duplication (boxed). This insertion introduces two adjacent stop codons in the ORF that result in premature termination. (Figure adapted from Cannon and June 2011; reprinted, with permission, from the author.)

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mammalian cells, predominantly by the error-prone nonhomologous end-joining (NHEJ) pathway, which typically generates a series of small deletions or additions at the break site with disruption of the open reading frame (ORF). ZFNs that target the coding sequence for the CCR5 amino terminus have been described (Perez et al. 2008) in which a 5-bp duplication at the target site is commonly observed (Perez et al. 2008; Holt et al. 2010). This insertion changes the translational reading frame and so introduces two adjacent stop codons that result in premature termination (Fig. 1).

A key feature of ZFN gene editing is that the ZFNs are only required to be expressed during a short time window; once the double-stranded break is created, the host NHEJ repair pathway creates a permanent gene knockout that is propagated with cell division. ZFNs can be delivered to a wide variety of human cells using standard gene delivery techniques. Notably, vectors that transiently express ZFNs are effective and may even be preferable because they eliminate the risk of immunogenicity that is inherent in protein-based gene therapy approaches. Vectors currently in use include adenoviral and nonintegrating lentiviral vectors. Nucleofection of plasmid DNA or in vitro-transcribed mRNA has also been used successfully, albeit with less efficiency (Lombardo et al. 2007; Perez et al. 2008; Holt et al. 2010).

ZFNs can be directed to the mature target cells that HIV-1 infects, or HSCs that give rise to these cells. Delivery of ZFNs to peripheral T cells has been especially effective using adenovirus vectors. In preclinical studies, Perez and colleagues (Perez et al. 2008) reported that adenovirus vector delivery of a CCR5-targeted ZFN pair led to disruption of ~50% of CCR5 alleles in populations of primary human CD4⁺ T cells. They further showed that the ZFN treatment generated HIV-resistant primary CD4⁺ T cells that expanded stably in HIV-infected cultures for several weeks, resulting in enrichment of ZFN-generated CCR5-modified cells in the population on long-term exposure to virus (>50 d). In addition, when the cells were transplanted into an

immunodeficient NOD/LtSz-scid IL2R λ null (NSG) mouse and followed by infection with a CCR5-tropic strain of HIV-1, the ZFN-modified T cells preferentially expanded, so that the proportion of modified cells present at the end of the experiment was greater than two- to threefold higher in the HIV-infected mice. Thus, a major finding from this work was that HIV infection itself can play an important role in selecting CCR5-negative cells. Even when present at low frequency, these cells are able to replenish and stabilize T-cell populations, whereas nonedited CCR5⁺ cells are destroyed by HIV.

Disrupting the CCR5 gene in HSCs could create a more potent antiviral effect than in peripheral T cells, given the added advantage that CCR5-negative cells would be generated in all hematopoietic lineages that HIV-1 infects, including macrophages as well as T cells. However, manipulating HSCs is technically challenging, as these cells are difficult to maintain in culture without losing viability or undergoing differentiation. An additional problem is that CCR5 is not expressed in HSCs, and therefore it is not possible to positively select for CCR5-disrupted cells. An early report described using nonintegrating lentiviral vectors to deliver ZFNs to CD34⁺ HSCs, but the efficiency was poor (Lombardo et al. 2007). However, Holt and colleagues recently showed that nucleofection of human HSCs with ZFN plasmid DNA was particularly effective with an average disruption rate of 17% of CCR5 alleles (Holt et al. 2010). Importantly, these ZFN-modified cells retained their “stemness” and were subsequently able to engraft NSG mice with the same efficiency as were nonmodified HSCs. In addition, the frequency of CCR5-disrupted genes in HSCs before engraftment was maintained in mature cells following differentiation in vivo and persisted through a secondary transplantation, clearly indicating that in SCID mice the ZFNs were able to modify HSCs and that these cells retained the capacity to regenerate all hematopoietic lineages. Moreover, similar to the study of Perez and colleagues, when mice transplanted with ZFN-modified HSCs were challenged with a

CCR5-tropic strain of HIV-1, CD4 cells that persisted in gut mucosa showed a potent selection for CCR5-negative, ZFN-modified cells (Holt et al. 2010).

CLINICAL TRIALS TESTING CELL AND GENE TRANSFER APPROACHES FOR CHRONIC HIV INFECTION

Cell-delivered gene therapy for HIV/AIDS has the potential to generate an immune system that is resistant to HIV that could theoretically contribute to the eradication of HIV reservoirs. As described in earlier sections, anti-HIV gene(s) can be introduced into HSC and/or T lymphocytes to provide a population of cells that is protected. Both HSC- and T-cell-directed approaches have shown promise. Although the potential benefits of HSC are substantial, there are two major challenges. First, the efficiencies of gene transfer with HSCs have been lower than with T-cell-directed therapies (Rossi et al. 2007; June et al. 2009), particularly regarding the ability to genetically modify long-term repopulating cells. Second, HSC appear to be more susceptible than mature T cells to genotoxicity from integrating viral vectors (Newrzela et al. 2008; Montini et al. 2009; Matrai et al. 2010). In contrast to the challenges in evaluating efficacy of anti-HIV gene therapy in HSCs, an attractive feature of gene therapy in T cells is that it is straightforward to determine therapeutic effects. Supervised treatment interruptions, if carefully performed, are safe and can provide definitive information on the antiviral efficacy of the vector by measuring changes in viral load or CD4 cell counts after the interruption. Recently, a subpopulation of postthymic T cells with extensive self-renewal capacity has been described (Zhang et al. 2005; Stemmerger et al. 2007; Turtle et al. 2009), and it is possible that the generation of populations of autologous, HIV-resistant HSCs and T cells will be an efficient approach to replicate the encouraging findings with allogeneic HSC using CCR5Δ32 donor cells.

A variety of the anti-HIV strategies discussed above has been tested in tissue culture and in animal models, and some have progressed to

clinical trials (Table 1). The first direct test of the safety and feasibility of CCR5 knockout T cells is currently being evaluated in a phase I clinical trial sponsored by Sangamo Biosciences (California), in conjunction with investigators at the University of Pennsylvania. Details of the protocol design can be found at clinicaltrials.gov NCT00842634. An overview of the clinical trial is shown in Figure 2.

CONCLUDING REMARKS: TOWARD HIV ERADICATION

Combination ART has dramatically improved the survival of patients with HIV infection and reduced the frequency of opportunistic infections and cancers. However, its use can be associated with significant long-term toxicities, and persisting inflammation and immune activation even in the face ART can impact clinical outcomes and survival (Butler et al. 2011). In addition, even with improvements in pharmacologic formulations of anti-HIV drugs, HIV infection continues to represent a significant economic burden, with the discounted, lifetime medical care cost of HIV-1 infection in the United States estimated at \$303,100 (2005 dollars) per person with 73% of this cost attributed to ARV drugs (Schackman et al. 2006). These realities provide an additional rationale for developing eradication therapies with the goal of long-term, drug-free control of HIV-1, a concept that before the Berlin patient, was unthinkable. As described in this review, the toolbox of strategies to render hematopoietic and lymphoid systems resistant to HIV infection continues to expand and to improve. Key challenges facing this emerging field are numerous and include the development of robust assays to measure the impact of therapies on latent HIV-1 reservoirs. Another issue is whether pluripotent stem cells or progenitor cells constitute a potential reservoir, as suggested by a recent study (Carter et al. 2010). If confirmed, this study has major implications for therapies that contemplate the use of gene-modified HSCs. Another barrier to implementing gene modification strategies is a possible requirement for myeloablative therapy to

Table 1. Completed and ongoing cell and gene transfer trials for HIV

Protocol description	Phase	Status	Payload	Cellular vehicle	Transfer vector	Reference(s)
T cells						
A randomized study of HIV-specific T-cell gene therapy in subjects with undetectable plasma viremia on combination antiretroviral therapy	2	Completed	CD4 receptor coupled with the CD3 signaling chain ζ	Autologous CD4 ⁺ and CD8 ⁺ T cells; (3) repeat doses	Murine retrovirus	Deeks et al. 2002
Evaluation of safety, tolerability, and persistence of escalating and repeat doses of genetically modified syngeneic CD8 ⁺ or CD4 ⁺ /CD8 ⁺ cells	1	Completed	CD4 receptor coupled with the CD3 signaling chain ζ	Syngeneic CD8 ⁺ or CD4 ⁺ /CD8 ⁺ cells; single or multiple doses	Murine retrovirus	Walker et al. 2000
Evaluation of safety, tolerability, and tissue trafficking of a single dose of genetically modified autologous CD4 ⁺ and CD8 ⁺ cells	1	Completed	CD4 receptor coupled with the CD3 signaling chain ζ	Autologous CD4 ⁺ and CD8 ⁺ cells; single dose	Murine retrovirus	Mitsuyasu et al. 2000
A clinical trial of CD4 ζ gene-modified T-cell infusion with and without IL-2 in HIV-infected participants	1/2	Completed	CD4 receptor coupled with the CD3 signaling chain ζ	Autologous CD4 ζ cells, randomized comparison to cells only or IL-2 only	Murine retrovirus	Aronson et al. 2008
Evaluation of safety and tolerability of a single infusion of autologous CD4 ⁺ T cells modified with a dominant negative anti-HIV gene	1	Completed	Rev M10	Autologous CD4 ⁺ cells; single dose	Gold particles	Woffendin et al. 1996
Evaluation of safety and tolerability of a single infusion of autologous retrovirally modified CD4 ⁺ T cells to express a dominant negative anti-HIV gene	1	Completed	Rev M10	Autologous CD4 ⁺ cells; single dose	Murine retrovirus	Ranga et al. 1998
A marker study of therapeutically transduced CD4 ⁺ peripheral blood lymphocytes in HIV discordant identical twins	1	Completed	Anti-HIV-1 tat ribozyme (Rz2)	Syngeneic CD4 ⁺ cells; single dose	Murine retrovirus	Macpherson et al. 2005

Evaluation of safety and tolerability of multiple infusions of syngeneic CD4 ⁺ lymphocytes modified with anti-HIV genes	1	Completed	<i>Trans</i> -dominant rev and/or <i>trans</i> -dominant rev with TAR antisense	Syngeneic CD4 ⁺ cells; two doses	Murine retrovirus	Morgan et al. 2005
Evaluation of safety and tolerability of ribozyme gene therapy of HIV-1 infection	1	Completed	Anti-HIV-1 ribozyme to the U5 leader sequence and pol	Autologous CD4 ⁺ cells; single dose	Murine retrovirus	Wong-Staal et al. 1998
Evaluation of safety and tolerability of a single dose of autologous T cells transduced with VRX496 in HIV-positive patient subjects	1	Completed	Anti-HIV-1 antisense against the envelope gene	Autologous CD4 ⁺ T cells; single dose	HIV-derived lentivirus, conditionally replicating	Levine et al. 2006
Evaluation of safety, tolerability, and antiviral effects of autologous CD4 ⁺ T cells expressing the HIV fusion inhibitor M87	1	Completed	gp41 fusion peptide inhibitor	Autologous CD4 ⁺ T cells	Murine retrovirus	van Lunzen et al. 2007
An open-label, multicenter study to evaluate the safety, tolerability, and biological activity of repeated doses of autologous T cells transduced with VRX496 in HIV-positive subjects	2	Ongoing	Anti-HIV-1 antisense against the envelope gene (VRX496)	Autologous CD4 ⁺ T cells; (4 or 8) repeat doses	HIV-derived lentivirus, conditionally replicating	ClinicalTrials.gov NCT00131560
An open-label, single center study to evaluate the tolerability, trafficking, and therapeutic effects of repeated doses of autologous T cells transduced with VRX496 in HIV-infected subjects	1/2	Ongoing	Anti-HIV-1 antisense against the envelope gene (VRX496)	Autologous CD4 ⁺ T cells; (6) repeat doses	HIV-derived lentivirus, conditionally replicating	Collman et al. 2009; ClinicalTrials.gov NCT00295477
Autologous T cells genetically modified at the CCR5 gene by zinc finger nucleases SB-728 for HIV (zinc-finger)	1	Ongoing	CCR5 modified T cells (SB 728 T)	Autologous CD4 ⁺ T cells	Ad5/35 ZFN	ClinicalTrials.gov NCT00842634
Study of autologous T cells genetically modified at the CCR5 gene by zinc finger nucleases	1/2	Ongoing	CCR5-modified T cells (SB 728 T)	Autologous CD4 ⁺ T cells	Ad5/35 ZFN	ClinicalTrials.gov NCT01252641
Phase 1 dose escalation study of autologous T cells genetically modified at the CCR5 gene by zinc finger nucleases in HIV-infected patients	1	Ongoing	CCR5-modified T cells (SB 728 T)	Autologous CD4 ⁺ T cells	Ad5/35 ZFN	ClinicalTrials.gov NCT01044654

Continued

Table 1. Continued

Protocol description	Phase	Status	Payload	Cellular vehicle	Transfer vector	Reference(s)
Redirected high-affinity gag-specific autologous T cells for HIV gene therapy	1	Ongoing	HLA-A2 gag-specific CTLs (wild-type and high-affinity TCR)	Autologous CD8 ⁺ T cells	HIV-derived SIN lentivirus	ClinicalTrials.gov NCT00991224
HSCs						
Nonmyeloablative conditioning followed by transplantation of genetically modified HLA-matched peripheral blood progenitor cells for hematologic malignancies in patients with AIDS	1	Completed	Trans-dominant Rev	Autologous CD34 ⁺ cells isolated from mobilized peripheral blood	Murine retrovirus	Kang et al. 2002; Hayakawa et al. 2009
Evaluation of retroviral-mediated transfer of a rev-responsive element decoy gene into CD34 ⁺ cells from the bone marrow of HIV-1-infected children	1	Completed	RRE-decoy	Autologous CD34 ⁺ bone marrow cells	Murine retrovirus	Kohn et al. 1999
Evaluation of safety, tolerability, and persistence of transplantation with autologous bone marrow transduced with a retroviral vector expressing dominant negative Rev or a control gene	1	Completed	Dominant negative RevM10	Autologous CD34 ⁺ bone marrow cells	Murine retrovirus	Podsakoff et al. 2005

Tat and <i>tat/rev</i> ribozyme in autologous CD34 ⁺ cells and control vector in patients with and without ablation	1	Completed	Rev/ <i>tat</i> ribozyme	Autologous CD34 ⁺ bone marrow cells	Murine retrovirus	Michienzi et al. 2003; Look for Rossi
Evaluation of safety and tolerability of autologous CD34 ⁺ hematopoietic progenitor cells transduced with an anti-HIV ribozyme	1	Completed	Anti-HIV-1 <i>tat</i> ribozyme (Rz2)	Autologous CD34 ⁺ cells isolated from mobilized peripheral blood	Murine retrovirus	Amado et al. 1999, 2004
A randomized, double-blind, controlled trial to evaluate the safety and efficacy of autologous CD34 ⁺ hematopoietic progenitor cells transduced with placebo or an anti-HIV-1 ribozyme (OZ1) in patients with HIV-1 infection	2	Completed	Anti-HIV ribozyme OZ-1	Autologous CD34 ⁺ cells isolated from mobilized peripheral blood	Murine retrovirus	Mitsuyasu et al. 2009
A pilot study of safety and feasibility of stem cell therapy for AIDS lymphoma using stem cells treated with a lentivirus vector encoding multiple anti-HIV RNAs	1	Completed	Triple combination vector coexpressing an anti- <i>tat/rev</i> shRNA, a nucleolar localizing TAR decoy, and an anti-CCR5 ribozyme in a single vector backbone	Autologous CD34 ⁺ HSCs	HIV-derived lentivirus	DiGiusto et al. 2010

CCR5 knockout in autologous T cells can be achieved by infection with an Ad5/35 vector (SB-728) expressing left and right ZFNs, linked by a self-cleaving protease 2A sequence. In an ongoing phase I clinical trial, the CCR5-modified T cells are expanded ex vivo using antibodies to CD3 and CD28 for ~10 days and adoptively transferred to the patient. The study end points are listed.

TAR, *trans*-activation response; TCR, T-cell receptor; RRE, Rev response element.

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Figure 2. First clinical application of CCR5 ZFNs (protocol NCT00842634; see text).

facilitate engraftment of gene-modified HSCs. However, there is progress in the area of inducible selection systems for HSCs, for example, using modified methylguanine methyltransferase (Milsom and Williams 2007; Trobridge et al. 2009; Beard et al. 2010), and if proven safe, these approaches may circumvent this issue. Given the remarkable proof of concept that HIV infection can be eradicated (Hütter et al. 2009; Allers et al. 2010; Hutter and Thiel 2011), this field will be an exciting one for renewed research efforts.

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