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Antiretroviral Drug Studies in Nonhuman Primates: a Valid Animal Model for Innovative Drug Efficacy and Pathogenesis Experiments

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Abstract

Several nonhuman primate models are used in HIV and AIDS research. In contrast to HIV-1 infection of chimpanzees, infection of macaque species with simian immunodeficiency virus (SIV) isolates results in a disease (simian AIDS) that shares many similarities with HIV infection and AIDS in humans. Although each animal model has its limitations and can never completely mimic HIV infection of humans, a carefully designed study allows experimental approaches, such as the control of certain variables, that are not feasible in humans, but that are often the most direct way to gain better insights in disease pathogenesis and provide proof-of-concept for novel intervention strategies. In the early days of the HIV pandemic, nonhuman primate models played a relatively minor role in the anti-HIV drug development process. During the past decade, however, the development of better virologic and immunologic assays, a better understanding of disease pathogenesis, and the availability of better drugs have made these animal models more practical for drug studies. In particular, nonhuman primate models have played an important role in demonstrating: (i) preclinical efficacy of novel drugs such as tenofovir; (ii) the benefits of chemoprophylaxis, early treatment and immunotherapeutic strategies; (iii) the virulence and clinical significance of drug-resistant viral mutants; and (iv) the role of antiviral immune responses during drug therapy. Comparison of results obtained in primate models with those observed in human studies will lead to further validation and improvement of these animal models. Accordingly, well-designed drug studies in nonhuman primates can continue to provide a solid scientific basis to advance our scientific knowledge and to guide future clinical trials. **(AIDS Reviews 2005;7:67-83)**

Key words

Macaque. Monkey. Prophylaxis. Chemotherapy. Resistance.

Introduction: the need for an appropriate animal model

An increasing arsenal of anti-HIV drugs is currently being used, and many novel candidates are continuously being developed[1]. The main anti-HIV drugs that have been approved or are being developed target several key steps or enzymes in the viral replication cycle: attachment, fusion, reverse transcriptase (RT), integrase or protease. During recent years, combination therapy of these compounds, so-called highly active antiretroviral therapy (HAART), has led to major improvements in the clinical management of HIV-infected people[2]. Despite this considerable success, there is no reason for complacency as long-term administration of these drugs is associated with problems of cost, toxicity, compliance, and drug resistance. Accordingly, the quest for better antiviral drug regimens continues. The ideal antiviral drug regimen would be one that induces strong and persistent suppression of virus replication, gives prolonged immunologic and clinical benefits without toxicity, can be administered at infrequent dosage intervals, is affordable and easy to store, and can thus benefit the greatest number of HIV-infected people, including those in developing countries.

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The pipeline that new drug candidates need to cross between the first demonstration of *in vitro* antiviral effects and approval for clinical use is tedious, time-consuming, and very expensive. Most compounds that inhibit virus replication *in vitro* are never further developed (due to lack of resources), or they fail in preclinical testing or clinical trials due to unfavorable pharmacokinetics, toxicity, or insufficient antiviral efficacy.

A confounding obstacle in the drug development process is that many drugs have already been approved for HIV-infected patients. It is considered unethical to treat "control" groups with anything less than the currently available "gold standard" of combination therapy. Therefore, the efficacy of new drugs is now often evaluated by including the compound as part of a combination regimen, often in patients failing currently available HAART regimens, who may have existing drug-resistance mutations, low CD4+ cell counts, or poor adherence. Thus, the response in such "worst-case scenario" patients may underestimate the potency of the drug for treatment-naive patients. These dilemmas underscore the need for an evaluation of the role of animal models in the drug development process. Appropriate animal models that allow rapid evaluation of the efficacy and toxicity of antiviral compounds can assist in sorting out those drugs which are promising and deserve to enter human clinical trials first, from those drugs that should probably be discarded[3].

While murine and feline models are appropriate for initial screening, further testing is best done in nonhuman primate models that better resemble HIV infection of humans. Nonhuman primates are phylogenetically the closest to humans. The similarities in physiology (including drug metabolism, placentation, fetal and infant development, etc.) and immunology allow a more reliable extrapolation of results obtained in primate models to clinical applications for humans. While chimpanzees can be infected with HIV-1, this animal model is not practical due to the low availability, high price, low viral virulence, and ethical issues[4,5]. Many nonhuman primate species in Africa are naturally infected with simian immunodeficiency virus (SIV) strains; despite persistent high-level virus replication, these natural hosts do not develop disease, possibly because infection is associated with little immune activation[6,7]. In contrast however, infection of non-natural hosts, such as macaques, with virulent SIV isolates results in a disease which resembles human AIDS (including generalized immune activation, CD4+ T-cell depletion, opportunistic infections, weight loss and wasting), and the same laboratory markers can be used to monitor disease progression[8]. Compared to HIV infection of humans, infection of macaques with virulent SIV or simian-human immunodeficiency virus (SHIV) isolates results in an accelerated course, as most animals develop clinical disease within one to three years. Similar to observations in HIV-infected human infants, the disease course in newborn macaques following inoculation with virulent SIV strains is usually accelerated[9,10]. It is important, however, to remember that SIV or SHIV infection of macaques is not necessarily fatal, as there are many attenuated or nonpathogenic virus isolates which give transient or low-level viremia, and slow or no disease. This wide spectrum of infection outcomes makes this model suitable to assess how genetic changes in the virus (e.g. drug-resistance mutations) affect viral virulence.

Primate models are powerful tools in many areas of HIV research. In addition to allowing investigators to unravel virus-host interactions during disease pathogenesis and to test vaccines[8], macaques allow us to model the different aspects of antiviral drug treatment, including pharmacokinetics, toxicity, and antiviral efficacy. The balance among all these *in vivo* interactions (which is impossible to model accurately *in vitro*) determines the long-term clinical usefulness of the antiviral drug (Fig. 1).

Besides being a test system for preclinical screening of novel drug regimens, an animal model can also be used to test hypotheses that are difficult or impossible to explore in humans. By manipulating certain variables (e.g. the initiation of drug treatment relative to virus inoculation, duration of treatment, the age of the animals, the virulence and drug susceptibility of the virus inoculum, the status of the immune system), investigators can design studies to address very specific questions. As discussed further in this review, examples of this are studies focused on evaluating chemoprophylaxis, the *in vivo* virulence and clinical implications of drug-resistant viral mutants, and the role of antiviral immune responses on antiviral drug efficacy.

Macaque species and virus isolates used in antiviral drug studies

Anti-HIV drug studies in macaques generally used rhesus macaques *(Macaca mulatta)* or cynomolgus macaques *(M. Fascicularis)*[11]. The SIV isolates usually belonged to a few groups, in particular SIVmac, SIVsmm and SIVmne. Because the polymerase region of these SIV isolates has about 60% and 85% amino acid homology to HIV-1 and HIV-2, respectively, SIV is susceptible to many of the same nucleoside RT inhibitors (NRTI; e.g. zidovudine), nucleotide RT inhibitors (tenofovir, adefovir), integrase and protease in-

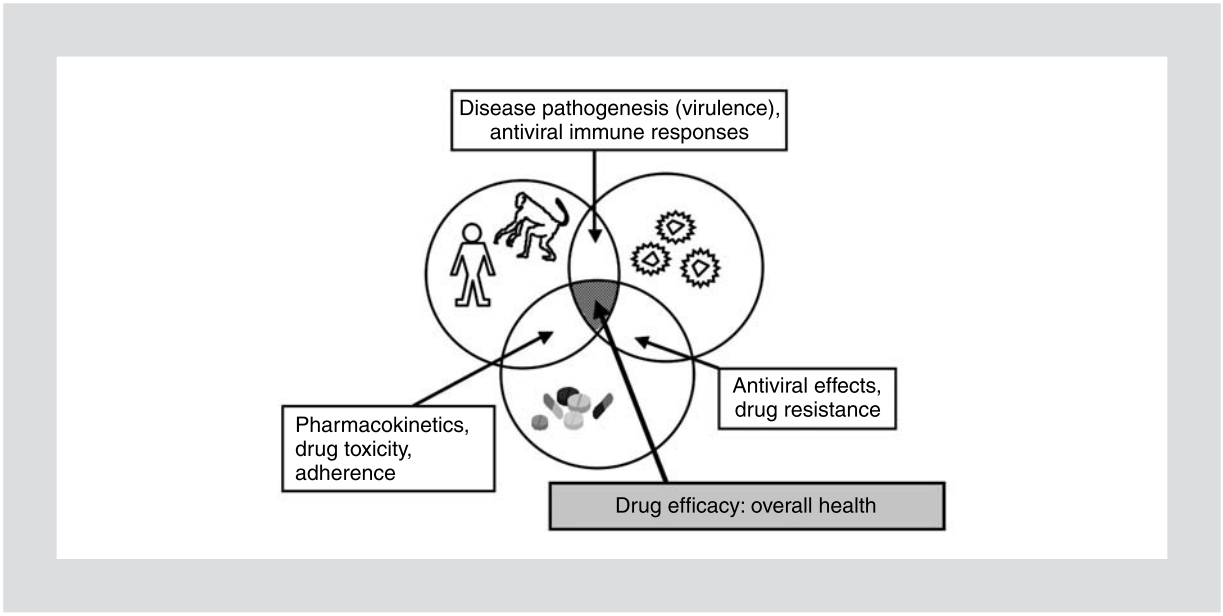

Figure 1. *Overall outcome of antiviral drug treatment. The ultimate goal of drug treatment is to improve the overall health of the host and indefinitely delay disease progression. This outcome is determined by many interactions between the virus, the host, and the antiviral drugs, most of which cannot be mimicked appropriately by in vitro studies. Animal models allow us to control and manipulate certain variables through experimental approaches that are not feasible in humans (such as experimental inoculation of animals with drug-resistant mutants, or in vivo depletion of certain immune cells), but that are often the most direct way to address certain questions regarding antiviral drug treatment.*

hibitors[12-16]. Due to their CCR5 chemokine coreceptor usage, SIV isolates are also susceptible to CCR5-targeting entry inhibitors[17]. Some compounds, however, including nonnucleoside RT inhibitors (NNRTI) such as nevirapine and efavirenz, are active only against HIV-1 and not against HIV-2 or SIV[18]. The construction of infectious SIV/HIV-1 chimeric viruses, in which the RT gene of SIV was replaced by its counterpart of HIV-1 (so called RT-SHIV), has been proven useful to evaluate NNRTI in primate models[19-23]. Other SHIV have been constructed and contain the envelope region (so called env-SHIV) or other genes of HIV-1. Many env-SHIV are attenuated. Most pathogenic env-SHIV such as SHIV-89.6P, while useful to address specific questions, have the limitation that their disease pathogenesis (including CXCR4 coreceptor usage and very rapid CD4+ cell depletion) is different from the typical course seen with HIV and SIV infection[24]. Currently available CCR5-using env-SHIV (such as SHIV-SF162P)[25] have the limitation that, after the initial peak of viremia, many untreated animals are able to suppress viremia to undetectable levels; while these isolates are useful to test prophylactic or early post-infection interventions, this large variability in chronic viremia set-point and disease outcome makes them less practical for testing antiviral drug efficacy during chronic infection, especially with limited animal availability. Accordingly, SIV is in general a more appropriate and practical model to test anti-HIV strategies[26,27].

Development of primate models: from initial obstacles to validation

During the first decade of the HIV pandemic, the role of nonhuman primate models in testing anti-HIV drugs was rather limited. Although SIV is susceptible to many anti-HIV drugs *in vitro*, many initial drug studies in macaques were not very successful in demonstrating *in vivo* efficacy[3,28]. Several factors are responsible for these observations. Most drugs that were available at that time had complicated dosage regimens (e.g. a short half-life necessitating frequent administration) or problems of toxicity and were thus not suitable for long-term administration. The time course of SIV disease progression in juvenile and adult macaques is highly variable as the asymptomatic period can range from months to years; it was therefore hard to determine whether a small difference in clinical outcome was due to host factors or to the drug treatment, especially with only relatively small numbers of animals and short-term treatment regimens[29]. In retrospect, another important reason for the poor efficacy results of the initial drug studies was that at that time the role of antiviral immune responses in determining antiviral drug efficacy was not

recognized. Untreated macaques infected with virulent isolates such as SIVmac251 have higher viremia, lower cell-mediated antiviral immune responses, and a more rapid disease course than HIV-infected humans[30]. As discussed further in this review, an antiretroviral drug becomes less effective in suppressing viremia without the assistance of effective antiviral immune responses. As the drugs available at that time were not very potent in suppressing viremia in HIV-infected humans, it is now no surprise that they were even less effective in suppressing viremia in immunodeficient SIV-infected macaques. Finally, sensitive assays to accurately quantitate viremia were not available at that time.

Many of these problems have been solved in the past decade. Sensitive assays, similar to those used to monitor HIV infection of humans, have been developed to monitor virus replication in SIV-infected macaques, including quantitative viral RNA assays[31-33]. The development of a pediatric SIV model has also been very useful, as the more uniformly rapid disease course (~ 3 to 4 months) observed in infant macaques infected with virulent SIV isolates permits evaluation of drug efficacy, including viremia and disease-free survival, in a relatively short time[29,34,35]. Infant macaques are also easier to handle for drug administration and require less drug, which is useful especially for compounds that are initially very expensive to produce in test quantities. The first report on the RT inhibitor tenofovir (9-[2-(R)-(phosphonomethoxy)propyl]adenine; PMPA) in 1995 was a milestone in validating this animal model because it was the first compound found to be highly effective against SIV infection[34,36]. The strong therapeutic benefits observed with tenofovir in the monkey studies have been predictive of tenofovir's efficacy in HIV-infected humans, and have contributed to its clinical development[37-39]. Altogether, these developments over the past decade have sparked further interest in using nonhuman primate models for antiretroviral drug studies.

Drug studies in nonhuman primates: overview and lessons learned

Pharmacokinetics and toxicity

Macaques, which are similar in physiology and metabolism to humans, have been very useful for studying the toxicity and pharmacokinetics of antiviral drugs, including the effects of pregnancy and drug transfer across the placenta and into breast milk[40-46]. While most studies used short-term drug administration (in the order of days to weeks), studies with tenofovir have also assessed the safety of prolonged treatment (> 1 to 10 years), starting at birth and continuing throughout adulthood, including pregnancy[47]. These studies found that prolonged daily treatment with a high dose of tenofovir resulted in a Fanconi-like syndrome (proximal renal tubular disorder) with bone pathology, while short-term administration of relatively high doses and prolonged low-dose regimens were safe[47]. Such long-term studies in primates are very relevant as they mimic life-long treatment of HIV-infected humans.

Prophylaxis: prevention of infection

Many studies in nonhuman primates have focused on investigating whether drug administration starting near the time of virus inoculation could prevent infection. Prevention of infection is traditionally considered as the complete absence of any viral or immunologic evidence of infection; however, the development of more sensitive techniques (including DNA PCR, viral RNA quantitation) has sometimes resulted in transient detection of low-level signs of infection, usually within the first months after virus inoculation[48,49]. Accordingly, for the purposes of this review, prophylaxis is defined as "protection against persistent infection", with persistent infection being defined as "persistent viremia or persistently detectable virus-specific immune responses".

A few studies in macaque models have evaluated the efficacy of antiviral compounds as topical microbicides against mucosal infection; topical high-dose administration of a number of compounds protected adult macaques against intravaginal or intrarectal SIV or SHIV infection at varying rates of efficacy[50-56].

Most studies have used systemic drug administration to try to prevent infection. Early studies, which mostly used zidovudine (AZT), were not very effective in preventing infection, but a likely reason for this was the high dose of virus used in these experiments[57-61]. In subsequent studies, when a lower dose of virus was used to inoculate animals, administration of several drugs (including zidovudine, adefovir (PMEA), tenofovir (PMPA) and 3'-fluorothymidine) starting prior to or at the time of virus inoculation was able to prevent virus infection[48,49,62-69]. Very few compounds have been shown to prevent infection when treatment was started after virus inoculation: i.e. tenofovir, BEA-005, and GW420867. A combination of the timing and duration of drug administration was found to determine their success rate[21,36,63,70-72]. Of these three compounds, tenofovir was effective following virus inoculation by different routes (intravenous, oral, intravaginal, intrarectal), and is currently the only one ap-

proved for therapeutic use in humans; BEA-005 and GW420867 are no longer in clinical development.

The demonstration that antiviral drugs can prevent infection in macaques has provided a solid scientific rationale to administer anti-HIV drugs to humans following exposure to HIV in several clinical settings. Antiviral drugs are now recommended, usually as combination regimens, to prevent HIV infection following occupational exposure (e.g. needlestick accidents of health care workers) and non-occupational exposure (e.g. sex or injection-drug use)[73,74]. Similarly to the animal studies, transient viremia has been described in some humans receiving postexposure prophylaxis[75].

Because an efficacious HIV vaccine has so far not been identified, tenofovir's prophylactic success in the macaque models has sparked clinical trials to investigate whether uninfected adult persons who engage in high-risk behavior will have a lower infection rate by taking tenofovir once daily. The ethical controversies surrounding these trials, which are being held at several international sites and target different high-risk populations, are reviewed elsewhere[76].

Antiviral drugs, especially zidovudine and nevirapine, have played a very important role in the prevention of mother-to-infant transmission of HIV, including in developing countries[77-79]. To counteract potential problems of drug-resistance mutations that are induced by the nevirapine regimen in women in developing countries[80], the promising data of a two-dose tenofovir regimen in the newborn macaque model[49,64] have spurred interest to test the feasibility of a two-dose tenofovir regimen to reduce perinatal HIV transmission (PACTG-394 and HPTN-057).

Therapy: treatment of infection

Many studies in the macaque model have demonstrated that, even when infection was not prevented, early drug treatment delayed or reduced the peak of acute viremia that occurs during the first weeks of infection, enhanced antiviral immune responses, and delayed disease progression[16,19,21,29,57,59,60,66,81-94]. These same benefits of early treatment have now been confirmed in human studies[95-100].

When macaques were started on short-term drug regimens during the stage of acute viremia, the outcome once treatment was withdrawn depended on the virus isolate. With pathogenic env-SHIV isolates, short-term suppression of acute viremia was usually effective to induce strong antiviral immune responses that controlled virus replication and delayed disease for an extended time in the absence of drug treatment[16,90,101]. In contrast, with highly virulent SIV isolates (such as SIVmac251), viremia usually increased again once short-term drug treatment was stopped, similarly to what is observed in most HIV-infected humans[26,27,94,102-105].

Macaque studies have also investigated the effects of antiviral therapy on established, chronic SIV infection (i.e. after the acute viremia stage), and the often disappointing results have puzzled researchers for a long time. Initial studies with zidovudine were not very successful in reducing viremia once SIV infection was established[29,62,106]. As selection for zidovudine-resistant viral mutants was slow[107], these data are consistent with the relative weakness of zidovudine monotherapy compared to newer compounds. Lamivudine (3TC) and emtricitabine ((-)-FTC) treatment of SIVmac251-infected infant macaques also had little effect on viremia and disease progression. However, there was rapid emergence of drug-resistant mutants with the M184V mutation in RT, suggesting that drug levels were sufficient to inhibit replication of wild-type virus[108]. The CCR5 inhibitor CMPD 167 reduced viremia fourfold to 200-fold in chronically SIV-infected macaques, but in some animals this effect was transient[17]. Similarly, efavirenz treatment led to reduced viremia in RT-SHIV infected animals, and selection for drug-resistant mutants led in some animals to viral rebound[23]. The integrase inhibitor L-870812 reduced viremia in SHIV-89.6P-infected macaques if initiated during early infection (before CD4+ cell depletion)[16]. In most studies, tenofovir has been highly effective to reduce established viremia[34,109-112]. During prolonged tenofovir therapy, the emergence of viral mutants with reduced *in vitro* susceptibility did not always lead to a rebound in viremia as some animals maintained low viremia[34,113]. However, there have been reports where tenofovir therapy was not effective in suppressing viremia despite the presence of drug-susceptible virus at the onset of treatment[35,101,109,112,114], suggesting that antiviral drug therapy is more complex than just a matter of having sufficient drug levels and susceptible virus. As discussed below, a growing body of evidence obtained from monkey studies creates a picture of drug therapy in which the efficacy of a drug regimen to reduce viremia is the combined result of several factors: (i) direct inhibitory activity of the drug(s) against the virus, determined by pharmacokinetic and pharmacodynamic factors; (ii) drug resistance (including likelihood of emergence, level of reduced susceptibility, effect of mutations on viral replication fitness and virulence); and (iii) the status of the host immune system (including antiviral immune responses). Primate studies

have provided valuable insights into these interactions.

The demonstration of tenofovir's antiviral efficacy in SIV-infected macaques has sparked many other drug studies in this animal model. Tenofovir-containing regimens have been used to gain a better understanding of disease pathogenesis and drug therapy, and to test additional intervention strategies. While SIV infection leads to rapid depletion of CD4+ T-cells from gut-associated lymphoid tissue (GALT) and gastrointestinal dysfunction[115-117], early tenofovir therapy was found to decrease mucosal virus levels and restore the CD4+ T-cell population in GALT; this was associated with up-regulation of growth factors and genes involved in repair and regeneration of the mucosal epithelium[118,119]. Combination treatment of SIV-infected macaques with tenofovir and two protease inhibitors (indinavir and nelfinavir) was found to improve immune responses against other organisms such as mycobacterium[120]. The macaque model has also been used to investigate the viral reservoirs during drug treatment: SIV-infected pigtailed macaques treated with tenofovir and emtricitabine were found to have viral reservoirs in resting CD4+ T-lymphocytes[121]. Similar to observations in humans, a combination of tenofovir, lamivudine, and Efavirenz was also found to be very effective to suppress viremia in RT-SHIV infected macaques, with no detectable emergence of drug-resistant mutants during treatment[122].

A number of studies have combined antiviral drug treatment with other strategies aimed at enhancing antiviral immune responses, so that when drug treatment was stopped, viremia was controlled better. These immunotherapeutic strategies include structured treatment interruption, the combination of antiviral therapy with active immunization with or without cytokine administration, and immune reconstitution via administration of autologous CD4+ T-cells collected prior to SIV infection[123-130]. A caveat in interpreting the data of several of these studies, however, is that the combination of a high dose of tenofovir, didanosine, and hydroxyurea in macaques is plagued by problems of pancreatic toxicity (probably due to didanosine), which sometimes results in life-threatening diabetes (including after drug withdrawal); the published reports do not discuss whether drug-related toxicity may have contributed to the mortality observed in some of these studies.

The value of primate models in studying drug resistance

Many individuals do not show the desired strong and persistent suppression of viral replication during HAART. Although other factors, such as compliance and individual variability in pharmacokinetics, also contribute to reduced efficacy of HAART, a major limiting factor is the emergence of viral mutants with reduced *in vitro* susceptibility to antiviral drugs (so called "drug-resistant mutants")[131]. Due to the high mutation rate of the virus, incomplete suppression of replication selects for viral variants with mutations that allow better replication in the presence of drugs. The relationship between drug adherence and the emergence of drug-resistant mutants is complex and seems to depend on the drug class[132].

While the correlation between specific mutations in the viral genome and *in vitro* reduced susceptibility has been well documented for most antiviral compounds, many unanswered questions remain regarding the exact clinical implications of these drug-resistant variants *in vivo*, and how to use this information to make treatment decisions. If drug resistance means that the drug is no longer effective, then it can just as well be withdrawn; but if there is still a partial response, then it will be counterproductive to discontinue drug administration unless better alternatives can be offered[133-135]. Many studies, including those utilizing drug interruptions, have demonstrated that HAART can still have therapeutic virologic and/or immunologic benefits even in the presence of drug-resistant virus, and this may be due to some residual drug activity and/or the altered pathogenesis of drug-resistant variants[136-146]. Thus, it is important to note that the terms "drug resistance" and "reduced susceptibility" are *in vitro* measures, and "drug resistance" does not necessarily imply that drug efficacy is completely abolished *in vivo*.

An important question about mutants with reduced *in vitro* susceptibility to drugs concerns the replicative fitness and virulence of such mutants in comparison to wild-type virus. Because the mutations that reduce susceptibility are at very low or undetectable frequency in the absence of drug treatment, these mutations are expected to reduce the ability of the virus to replicate. However, primary drug-resistance mutations are often followed by compensatory mutations to improve replicative fitness. So what is the final result? Are drug-resistant mutants attenuated in virulence (i.e. their ability to cause disease) to such extent that the purpose of continuing drug therapy could be to prevent reversion to the more virulent wild-type form?

Studies measuring *in vitro* replication kinetics of drug-resistant HIV mutants can never completely predict their *in vivo* virulence. *In vivo* virulence is determined by complex pharmacologic, viral and host factors (including many tissue- and cell-specific factors)

that are difficult to mimic *in vitro*, such as drug pharmacokinetics, primary and compensatory mutations (and their impact on replication fitness, but also on immunogenicity), cell tropism, and the complex role of the immune system (which supports virus replication, but at the same time also tries to contain it). Studies in the SIV-macaque model have demonstrated repeatedly that the correlation between *in vitro* markers (viral replication fitness, cell tropism, and cytopathogenicity) and *in vivo* measures (replication fitness, cell tropism, and virulence) is often weak as virus isolates that replicate well and are very cytopathogenic *in vitro* can be severely attenuated or have a different cell tropism following inoculation in macaques[147-149]. Thus, the extrapolation of results from *in vitro* growth kinetic studies to decisions affecting clinical management of HIV-infected patients should be performed with caution. Similarly, it has been difficult to correlate data of *in vitro* drug susceptibility assays (which can demonstrate small to large changes in susceptibility) with changes in antiviral efficacy *in vivo*[150].

Some information regarding the relative replication fitness and stability of drug-resistant HIV mutants *in vivo* can be gathered from case reports, such as those documenting primary infection with drug-resistant HIV-1, as well as those monitoring the reversion of drug-resistant virus to wild-type following discontinuation of drug treatment[144,151,152]. An animal model, however, allows approaches which are impossible in humans, but which are the most direct ways to study the clinical implications of drug-resistant virus: animals can be inoculated with drug-resistant viral mutants or their wild-type counterparts, and their replication fitness and virulence can be compared in drug-treated versus untreated animals.

Drug-resistance studies in the macaque model

Several methods have been used to generate drug-resistant SIV variants *in vitro*, including selection through serial passage as well as site-directed mutagenesis of molecular clones[23,153,154]. Only a few studies have evaluated the emergence of drug-resistant viral mutants in treated macaques. Treatment of RT-SHIV infected macaques with nevirapine or efavirenz gave rise to the emergence of mutations at codon 103 and 181 in RT, similar to observations in treated HIV-1 infected patients[22,23].

A zidovudine-treated SIVmac251-infected macaque developed a glutamine-to-methionine substitution at codon 151 of RT (Q151M), associated with high-level (> 100-fold) *in vitro* resistance to zidovudine[29,107]. Inoculation of the Q151M SIVmac isolate into naive newborn macaques demonstrated that this mutation did not significantly reduce viral replication and viral virulence; the Q151M mutation (which is the result of two base changes) was also very stable in the absence of zidovudine treatment[107]. This Q151M mutation has not been found in HIV-1 infected patients receiving zidovudine monotherapy, but has been found in HIV-1 infected patients receiving sequential or combination therapy with dideoxynucleoside analogues[155,156]. However, the Q151M mutation is found frequently in HIV-2 infected patients receiving NRTI therapy[157,158]. This latter observation indicates that, due to much sequence homology, HIV-2 and SIV use similar mutational pathways that are sometimes distinct from those of HIV-1.

Treatment of SIV-infected infant macaques with lamivudine (3TC) or emtricitabine ((-)-FTC) gave rise to the emergence of viral mutants with the expected M184V mutation in RT within five weeks of treatment[108]. The clinical implication of the M184V mutation was subsequently investigated by inoculating juvenile macaques with SIVmac239 clones having either wild-type sequence or the M184V mutation in RT (SIVmac239-184V). In comparison to wild-type virus, SIVmac239-184V was replication-impaired, based on virus levels one week after inoculation, and on the reversion of SIVmac239-184V to wild-type sequence in untreated animals. However, this reduced replication fitness was not sufficient to affect viral virulence, as animals inoculated with SIVmac239-184V and treated with emtricitabine (to prevent reversion) had similar viremia from two weeks after infection onwards, and the disease course and survival was indistinguishable from that of animals infected with wild-type virus[108]. In a different study, the M184V mutation did not revert in macaques inoculated with SIVmac239 containing both the M184V and E89G mutations; however, the M184V mutation in that study was engineered with two base changes in codon 184 (instead of the single base change that is normally seen during *in vitro* or *in vivo* selections)[159].

Long-term treatment of SIVmac251-infected macaques with tenofovir resulted in the emergence of virus with fivefold reduced *in vitro* susceptibility to tenofovir, associated with a lysine-to-arginine substitution at codon 65 (K65R) of RT[34,114]. Tenofovir also selects for the K65R mutation in HIV-1 RT[160-162]. The emergence of K65R in SIV was followed by additional RT mutations, which were likely to be compensatory mutations[34]. The emergence and distribution of K65R mutants is a complex process, with considerable variability among animals and among tissues[114]. The SIV macaque model has provided impor-

tant information regarding the clinical implications of K65R viral mutants during tenofovir treatment. Although some SIVmac251-infected animals show an increase in viremia following the emergence of K65R viral mutants, other animals continue to suppress viremia to low or undetectable levels for years (> 3 to 9 years)[34,113,163]. This success in persistently suppressing replication of the highly virulent SIVmac251 isolate with tenofovir monotherapy is unprecedented in this animal model[26,27]. To investigate whether this observation of suppressed viremia in some animals despite K65R virus was caused by an attenuating effect of the K65R mutation on viral replication fitness and virulence, two K65R SIV isolates were inoculated into new animals. In the absence of tenofovir treatment, the K65R SIV isolates were as fit and virulent as wild-type SIVmac251, based on their ability to induce high viremia and rapid disease (≤ 4 months) in newborn macaques[163]. However, in the presence of prolonged tenofovir treatment, the disease course was changed and two scenarios were possible: (i) K65R viremia was reduced and could become undetectable with prolonged disease-free survival (> 9 years)[113,163]; (ii) viremia remained high (> 10^6 to 10^7 RNA copies/mL plasma), but with continued tenofovir treatment, survival was increased significantly more than predicted based on viral RNA levels and CD4+ T-cell counts[35,113,163]. Such findings have not been observed with other antiviral drugs in the SIV-macaque model, which suggests that tenofovir treatment may have rather unusual interactions with the immune system. These observations instigated further *in vivo* experiments that identified a major role of the immune system in determining the efficacy of antiviral drug therapy to reduce viremia.

The role of the immune system on the efficacy of drug therapy

Viral kinetics during drug therapy depend on viral replication fitness, drug susceptibility of the virus, and drug potency[164-166]. When virus levels in plasma are reduced rapidly following the onset of drug therapy, the antiviral drugs are lauded for their potency, while the role of antiviral immune responses during drug therapy is less clear[166]. In this context, one is inclined to consider antiviral immune responses mostly as a backup plan to try to contain viremia whenever drug treatment is withdrawn or if drug-resistant virus would emerge[103]. Recently, however, a growing body of evidence from human and primate studies suggests that antiviral immune responses play a previously unrecognized role during drug therapy, which merits proper credit[16,35,113,143,167]. Drug studies in macaques have demonstrated the concept that the efficacy of antiviral drug therapy in reducing viremia is not only determined by the intrinsic potency of the drug in directly inhibiting virus replication, but is also strongly dependent on the status of the immune system[16,35,113]. In other words, antiviral drugs require the assistance of immune responses to reach full effectiveness in reducing viremia, both at the onset of treatment when the virus has wild-type susceptibility, as well as during prolonged treatment in the presence of drug-resistant mutants[113].

Several key studies using experimental depletion of CD8+ cells *in vivo* (through administration of anti-CD8 monoclonal antibody) are summarized in figure 2, and support the model shown in figure 3. When tenofovir treatment was started during acute viremia with wild-type SIVmac251, the efficacy of tenofovir to suppress acute viremia with wild-type SIVmac251 was significantly reduced in the absence of CD8+ cells[113]. These observations indicate that the otherwise rapid decline of productively infected cells (with half-life of ~ 1 to 2 days) after the onset of drug therapy is due to CD8+ cell-mediated killing or inhibition, rather than the natural death rate (as determined by the cytopathogenicity of the virus)[113]. In this model of drug therapy (Fig. 3), CD8+ cell-mediated antiviral immune responses contribute significantly to the antiviral effects of anti-HIV drugs, presumably by reducing the burst of virus replication in productively infected cells via cytolytic or noncytolytic pathways. In the absence of CD8+ cells, productively infected cells had a long half-life, suggesting that virulent SIV, during concomitant tenofovir treatment, is not as cytopathic as expected[113].

Even after the emergence of K65R SIV mutants, some tenofovir-treated animals were able to reach undetectable viremia[34,113]. A tempting explanation for this surprising observation, especially if seen in tenofovir-treated humans, would be to ascribe it to (i) a severe reduction in replication fitness caused by the K65R mutation (which, as discussed earlier, is not the case for K65R SIV isolates)[163], and/or (ii) sufficient residual inhibitory effect of tenofovir against these viral mutants (with ~ 5-fold reduced *in vitro* susceptibility). However, CD8+ cell-depletion experiments, which are not feasible in humans, revealed that the suppressed viremia of K65R SIV mutants during prolonged tenofovir treatment of macaques was largely due to strong CD8+ cell-mediated antiviral immune responses because, in the absence of CD8+ cells, (i) K65R viral mutants were very replication-competent, and (ii) tenofovir treatment alone was not sufficient to inhibit K65R SIV replication *in vivo* (Fig. 2)[113].

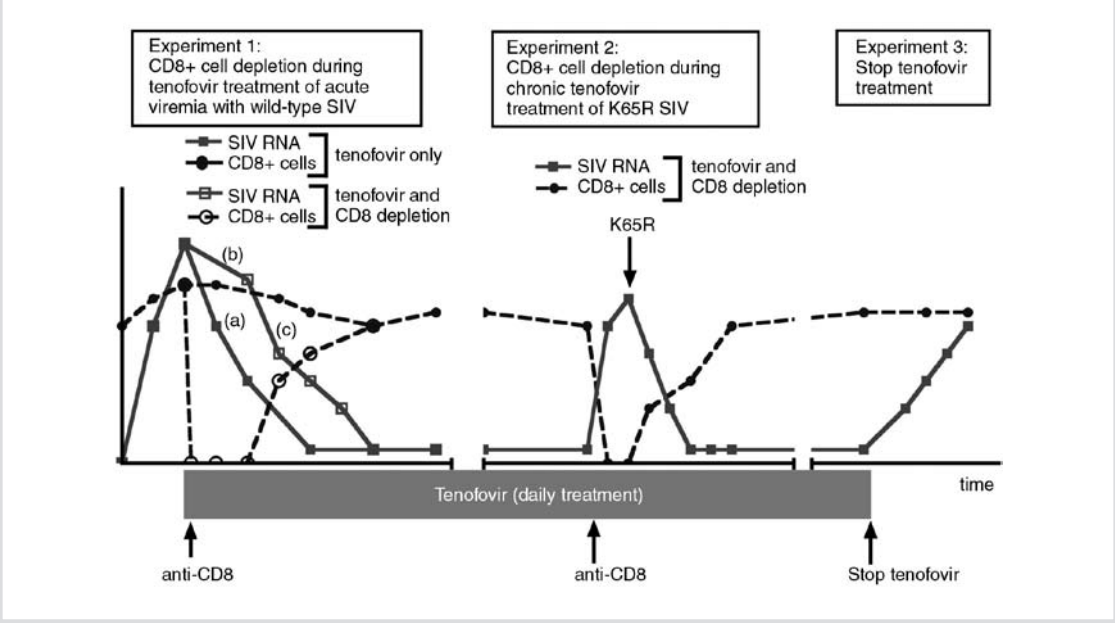

Figure 2. *Importance of CD8+ cells for the efficacy of tenofovir treatment: summary of CD8+ cell-depletion experiments. A schematic simplification of previously published data is presented[113]. In Experiment 1, animals were inoculated with wild-type virulent SIVmac251 and started on tenofovir therapy two weeks later. While untreated animals had persistently high viremia (not shown), animals started on tenofovir treatment (closed square) showed a rapid reduction of viremia* ***(A)****, with estimated half-life of productively infected cells of 1 to 2 days in the presence of CD8+ cells. At the onset of tenofovir treatment, one group (open square and circle) was also depleted of CD8+ cells via administration of the anti-CD8 monoclonal antibody (cM-T807); in the absence of CD8+ cells, tenofovir-treated animals had little reduction in viremia* ***(B)****, suggesting a half-life of productively infected cells of 4 to 6 days. When CD8+ cells became detectable, viremia was reduced rapidly with a half-life of 1 to 2 days* ***(C)****. Despite the emergence of K65R mutants (with fivefold reduced* in vitro *susceptibility to tenofovir), some animals were able to reach undetectable viremia after prolonged tenofovir treatment[113]. In Experiment 2, when such chronically treated animals were depleted of CD8+ cells, viremia of K65R virus increased transiently and returned to baseline values upon return of CD8+ cells. Thus, tenofovir treatment alone was not sufficient to control viremia of K65R mutants in the absence of CD8+ cells. In Experiment 3, when prolonged tenofovir treatment was withdrawn, viremia of K65R virus increased slowly, demonstrating that CD8+ cell-mediated immune responses alone were not sufficient to maintain maximal suppression of viremia. Thus, both tenofovir and CD8+ cells were required for optimal suppression of viremia, both at the onset of therapy (when virus was still wild-type) as well as during prolonged therapy (when virus had reduced* in vitro *susceptibility and the K65R mutation in RT)[113].*

Further experiments demonstrated that continued tenofovir treatment was required to maintain suppression of K65R SIV replication because tenofovir withdrawal led to a slow increase in viremia (Fig. 2)[113]. Thus, both tenofovir and effective CD8+ cells were required to maximally suppress replication of virulent virus in this animal model. Because the anti-CD8 antibody depletes both CD8+CD3+ T-lymphocytes and CD8+CD3- natural killer (NK) cells, the relative contribution of these two cell populations and their antiviral effector mechanisms could not be identified in these experiments[113]. These observations of reduced viremia of K65R SIV mutants associated with improved antiviral immune responses in tenofovir-treated macaques are consistent with clinical observations of strong antiviral immune responses in HAART-treated HIV-1-infected people who have low-level viremia with drug-resistant virus[143,168]. Temporal variability in the strength of such immune responses may also be the direct cause of transient blips of viremia that are observed in many HAART-treated individuals[169,170]. Antiviral immune responses may thus also play a role in determining viral reservoirs in HAART-treated patients[171].

As mentioned previously, tenofovir treatment initiated during early stages of SIV infection was usually very effective in reducing viremia. In contrast, several studies documented that tenofovir therapy was not very effective in rapidly suppressing viremia, despite the presence of drug-susceptible virus at the onset of treatment, especially when tenofovir therapy was started later in infection, with more virulent isolates, and in animals with high viremia and immunodeficiency[35,101,109,112,114]. However, the rapid emergence of K65R virus that has been described in some of these studies is a reflection of strong selection pressure, and

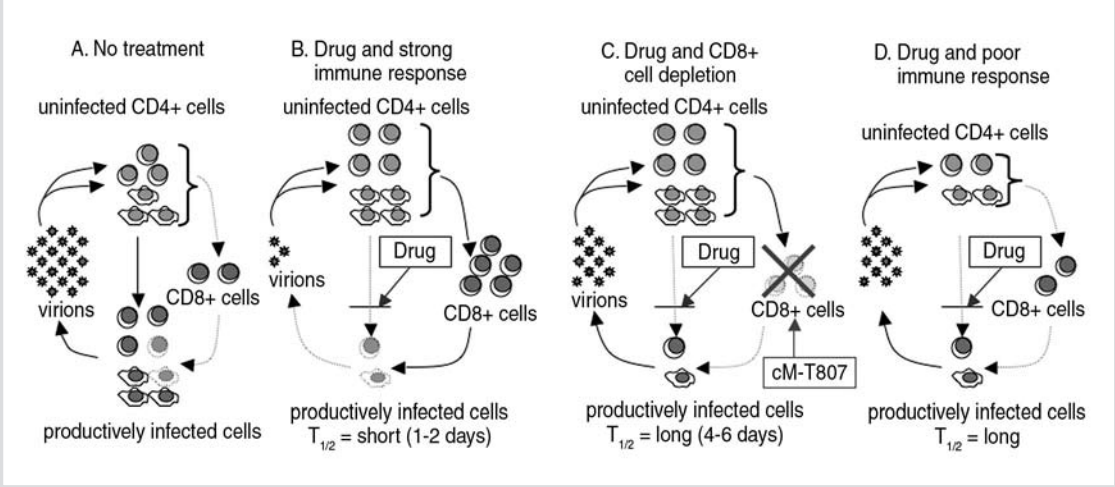

Figure 3. *Proposed model of drug and immune-mediated effects on virus replication.* ***A:*** *Without drug treatment, virulent virus can replicate to high titers because of high infection rates of CD4+ T-helper cells and antigen-presenting cells which are unable to provide sufficient assistance to CD8+ cell-mediated immune responses to contain virus replication.* ***B:*** *A potent drug regimen reduces the number of CD4+ T-helper cells and antigen-presenting cells that become newly infected. Potent CD8+ cell-mediated immune responses reduce the half-life, and thus the burst size of viral progeny, for those cells that already became infected. The combined antiviral activities of drug and antiviral CD8+ cells are efficient to induce and maintain low viremia, even after the emergence of drug-resistant viral mutants (as shown for tenofovir in the macaque model[113]).* ***C:*** *During artificial CD8+ cell depletion, productively infected cells survive longer and produce more progeny virus, resulting in higher viremia (see also Fig. 2)[113].* ***D:*** *During immunodeficiency, the reduced function of antigen presenting cells and CD4+ T-helper cells results in insufficient assistance to antiviral CD8+ cells to remain active, especially at lower levels of viremia. Even when infection of new cells is reduced by an efficient drug regimen, the half-life of the productively infected cells is long, resulting in a slower decrease of viremia. Without sufficient immune restoration, the emergence of drug-resistant mutants is likely to lead to a rebound in viremia[16,35].*
Modified from reference 113.

indicates efficient inhibition of wild-type virus replication by the tenofovir regimen[35]. An integrase inhibitor was also found to be less effective in reducing viremia when initiated during late infection[16]. These data provide further support for this model in which antiviral immune responses assist anti-HIV drugs in reducing viremia. In the absence of effective antiviral immune responses, antiviral drugs face a more daunting task to control viremia as already infected cells survive longer and produce more viral progeny (Fig. 3D)[35,113]. Because virulent SIV isolates induce immune dysfunction at many stages of the immune response (including antigen presentation and CD4+ T-helper cell function[172,173]), CD8+ cell-mediated immune responses become inactive at lower levels of antigen, and thus it is less likely that viremia can be suppressed to low or undetectable levels, especially once drug-resistant mutants emerge[174-176]. This model in which both drugs and antiviral immune responses play a role in reducing viremia helps to explain the different patterns of viremia that are seen in drug-treated SIV-infected macaques and HIV-infected infants and adults[177,178]. Several main scenarios of models of viremia during drug therapy are presented in figure 4. Note, however, that an individual's pattern may shift to another one based on changes in drug regimen, the potential of immune restoration (including increased potency of antiviral immune responses), and the acquisition of additional drug resistance mutations (which can affect virulence and replication fitness). Even in an individual host, patterns of viral kinetics and turnover may vary among different tissues, based on tissue-specific differences in target cells, drug levels, and antiviral immune-effector mechanisms; this could explain observations of highly uneven distribution of SIV mutants in drug-treated macaques[114]. Such mechanisms of immune-mediated clearance of virus during drug therapy are probably not unique to lentiviruses, as a similar correlation has been described between the status of the immune system and clearance of hepatitis B virus following lamivudine treatment in patients with dual HIV and hepatitis B infection[179]. Despite this recent progress in better appreciating the role of antiviral immune responses during drug therapy, we need to acknowledge the big gaps that still remain in our knowledge of these antiviral immune responses. Direct *in vivo* ma-

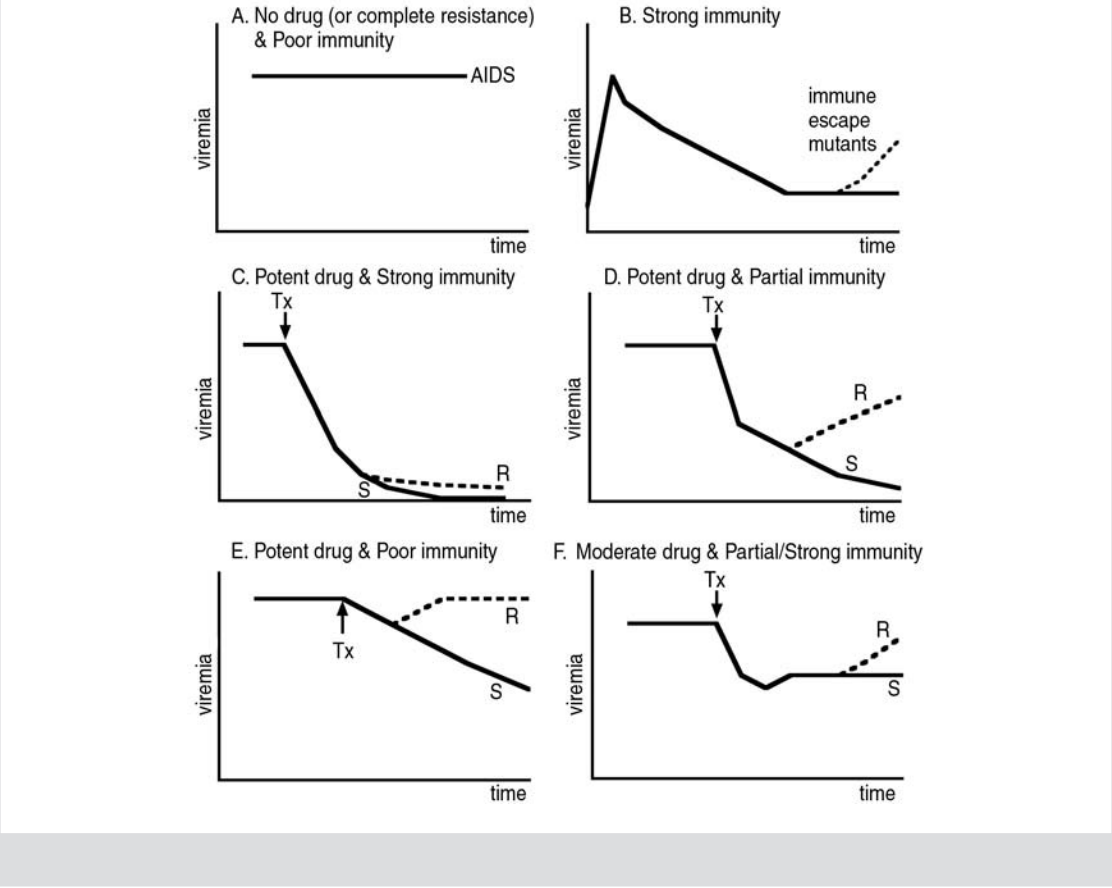

Figure 4. *Models of viremia during antiviral drug therapy: interaction of drugs and antiviral immune responses. Several scenarios are presented using different combinations of variables, including the strength of antiviral immune responses, the potency of the antiviral drug regimen against the virus, and the virulence and replication fitness of the virus. Tx indicates the start of drug treatment; R indicates the emergence of drug-resistant mutants with sufficient replication fitness, while S indicates viremia of wild-type virus (and/or drug-resistant mutants with severely reduced replication fitness). Intermediate levels of viral fitness are possible (not shown). "Potent drug" indicates a highly effective (single or combination) drug regimen that would completely prevent infection of new cells.* ***A:*** *Without effective antiviral immune responses and antiviral drugs (or in the presence of totally ineffective therapy due to complete drug resistance), viremia remains persistently high and leads to rapid disease.* ***B:*** *In the absence of anti-HIV drug therapy, some individuals are able to mount strong antiviral immune responses that initially control viremia, but usually are lost (due to progressive immune dysfunction and/or the emergence of immune escape mutants).* ***C:*** *Starting a potent drug regimen at a time of strong antiviral immune responses (e.g. during acute viremia) leads to rapid reduction of viremia; viremia can become and remain undetectable, even after the emergence of replication-fit drug-resistant virus (as observed in tenofovir-treated SIV-infected macaques[113]; see Fig. 2).* ***D:*** *Starting drug treatment at a moment of partial immunity (e.g. most HIV-infected patients with chronic infection) leads to a first phase of rapid decline in viremia, followed by phases of slower decline. These phases, generally believed to reflect distinct populations of infected cells[164], may alternatively also reflect antiviral immune responses that, without sufficient assistance of antigen-presenting cells or T-helper cells, become less active at lower levels of antigen[196]. In the absence of sufficient immune restoration, the emergence of drug-resistant virus or withdrawal of drug treatment is likely to lead to increased viremia.* ***E:*** *Without effective antiviral immune responses (e.g. SIV- or SHIV-infected macaques with severe immunodeficiency)[16,35,108], treatment with an otherwise highly potent drug does not result in rapid reduction in viremia, despite the presence of wild-type virus. Viremia can only continue to decrease if the drug is 100% effective in preventing infection of new cells and there is no emergence of drug-resistant mutants.* ***F:*** *With a partially effective drug regimen (or suboptimal levels of a potent drug), the reduction in viremia is limited because the relative increase in CD4+ cells provides more target cells for virus replication; as a result, viremia can stabilize at a lower level. Because wild-type virus can still replicate (albeit at reduced levels), the detection of drug-resistant mutants is delayed (e.g. zidovudine[29,107]).*

nipulations of the immune system (such as experimental depletions), which are often the best way to get a better understanding of *in vivo* antiviral immune mechanisms, can be performed in animal models, but are usually not feasible in humans. Instead, the need to rely on *in vitro* and *ex vivo* immune assays has the limitation that the currently available assays, especially when performed on peripheral blood, are not able to accurately grasp the variety, breadth, and strength of antiviral immune-effector mechanisms that control virus replication *in vivo*, especially in the lymphoid tissues and at mucosal sites[143,180-184].

It is important to note that the effects of antiviral immune responses during drug therapy are not mutually exclusive of the effects of reduced replication fitness of mutant virus and/or residual drug activity. In particular, even a relatively minor decrease in replication fitness, or a partial inhibition of virus replication by the drug regimen, can have a major impact on viremia if it provides more opportunity for effective antiviral immune responses to kill productively infected cells prior to the major viral burst. In contrast, in the absence of effective antiviral immune responses (such as during late-stage disease), a small difference in replication fitness may not translate into any significant difference in viremia and clinical outcome[108,113,185].

As mentioned previously, a surprising observation was that tenofovir-treated animals that maintained high viremia of K65R virus had prolonged disease-free survival, significantly more than predicted based on viral RNA levels and CD4+ T-cell counts[35,163]. This improved survival despite high viremia was only observed in the presence of tenofovir treatment, and has so far not been described for any other drugs in this animal model[107,108]. This prolonged survival despite high viremia in tenofovir-treated macaques is reminiscent of "discordant" or "paradoxical" results that have been described in HAART-treated HIV-infected adults and children, especially with regimens containing protease inhibitors. In such discordant patients, there is immunologic benefit (as measured by improved CD4+ T-lymphocyte counts and/or antigen-specific immune responses) and clinical benefits despite virologic failure[140-142,144,177,186-188]. The available data suggest that a combination of factors plays a role in such discordant results, including a decreased replicative fitness and T-cell activating ability of the drug-resistant mutants[136,138,144,146], an anti-apoptotic effect of protease inhibitors that preserves CD4+ T-cells[189], improved virus-specific cellular immunity[190], and direct antimicrobial properties of protease inhibitors[191,192]. Our study with tenofovir-treated SIV-infected macaques had the surprising finding that improved survival despite high viremia was even observed in animals in the absence of a significant immunologic response (based on standard immunologic parameters such as CD4+ T-cell counts and antibody responses to SIV and test antigens)[35,163]. Such clinical benefits would be difficult to detect in human studies as it requires years of follow-up, and without a good virologic and immunologic response, drug regimens would probably be changed in the meantime. As discussed elsewhere, it is unclear whether this phenomenon of prolonged disease-free survival in tenofovir-treated macaques with high viremia is due to residual antiviral activity of tenofovir against K65R virus in particular cell types (for example, antigen-presenting cells), potentially leading to relative preservation of innate immunity, or due to immunomodulatory effects that are independent of its antiviral effects, but that may partially protect the immune system against the deleterious effects of persistent virus replication and/or immune activation[35]. Tenofovir, which has many immunomodulatory effects in murine models[193], primed rhesus macaque cells for increased interleukin-12 secretion *in vitro*[194].

Such observations further highlight our relatively poor understanding of disease pathogenesis, and the need for further research to unravel the complex interactions between viral, host, and pharmacologic factors that determine (i) control of virus replication, and (ii) overall clinical outcome. The data of these macaque studies also suggest that the criteria for changing treatment regimens that were established with older drug regimens (based on correlations between viral RNA levels, CD4+ cell counts and disease progression) may have to be modified for regimens that include newer drugs (such as tenofovir). Please note, however, that tenofovir-treated animals with high viremia, despite having improved survival, eventually still develop disease. Thus, the ultimate goal of antiviral therapy remains to inhibit virus replication maximally and restore the immune system, using regimens that are feasible with regard to safety, cost, and adherence.

Studies in SIV-infected macaques have shown that improvement of immunologic control of viremia is possible with adoptive transfer of autologous antigen-presenting cells, CD4+ T-helper cells, or other immunization strategies[124-130,195]. The studies with tenofovir in macaques have proven the concept that the combination of a potent drug regimen and good antiviral immune responses is able to induce long-term suppression of viremia and prolonged disease-free survival (> 3 to 9 years), even in the presence of mutants with reduced drug susceptibility[113]. Accordingly, these primate studies provide a strong scientific rationale to explore other strategies to boost or restore antiviral immune responses during antiviral therapy. The demonstration in SIV-infected macaques that antiviral immune responses already contribute significantly to rapidly reducing viremia immediately after the onset of drug therapy (Fig. 2) provides the scientific impetus to also explore the feasibility of starting immunotherapeutic strategies near to or simultaneously with the onset of antiviral drug therapy, instead of waiting until viremia has reached lower levels.

Conclusions

The development of better reagents and more sensitive virologic and immunologic assays, the discovery of more potent drugs, and a better understanding of disease pathogenesis have made nonhuman primate models a more practical and adaptable system (i) to rapidly evaluate novel prophylactic and therapeutic drug strategies, and (ii) to test hypotheses that cannot be mimicked appropriately by *in vitro* experiments and are difficult to explore in humans. The comparison and correlation of results obtained in monkey and human studies is leading to a growing validation and recognition of the relevance of this animal model. Although each animal model has its limitations, carefully designed drug studies in nonhuman primates can continue to advance our scientific knowledge and guide future clinical trials.

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