

Linqi Zhang · Sharon R. Lewin *Editors*

HIV Vaccines and Cure

The Path Towards Finding an Effective
Cure and Vaccine

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Editors

HIV Vaccines and Cure

The Path Towards Finding an Effective Cure
and Vaccine



Springer

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Preface

The success of antiretroviral therapy (ART) in the management of HIV infection has been one of the most spectacular successes in medicine in the last century. ART led to the transformation of HIV from a universal death sentence to a chronic manageable disease. In every part of the world, we have seen a dramatic reduction in HIV-related morbidity and mortality, and treatment is now available to 21 million people – over half the number of people living with HIV. In addition, ART eliminates sexual transmission of the virus meaning that treatment is also prevention. Multiple other strategies, in addition to ART for HIV-infected individuals, clean needles and condoms, can now prevent transmission of HIV including male circumcision and pre-exposure prophylaxis. However, despite these great advances over one million people die of HIV-related illnesses each year and there are 1.8 million new infections. Two profound scientific challenges remain that must be solved to truly see an end to HIV – finding a cure and an effective vaccine.

In this book, we have invited an impressive array of international experts to review the current science and future challenges in relation to HIV cure and vaccine research. The subject matter is written for the non-expert with plenty of figures and tables to summarise complex concepts. The chapters span discovery, translational and clinical research.

To find a cure for HIV or a safe way to stop lifelong treatment, we first need a detailed understanding of how and where the virus persists on ART. Understanding the molecular and cellular factors that allow for HIV persistence are critical to identifying new interventions to eliminate HIV persistence. Understanding how HIV latency is established, maintained and reversed needs robust *in vitro* and animal models. The advantages and disadvantages of each of these models needs to be fully understood, prior to embarking on any research. Our capacity to measure intact replication competent virus in blood and tissue in people living with HIV on suppressive ART has also advanced and better approaches are still needed to optimally assess any interventions aimed at cure. Finally, despite all the unknowns and all the unanswered questions in relation to HIV latency and persistence on ART, multiple clinical trials are underway. To date these trials have been small studies of a single intervention, but an immense amount has been learned already in relation to what

will be needed to eliminate or reverse latency and the requirement for concomitant immune-based interventions.

In the challenging areas of prophylactic vaccines, we are still facing challenges for both empirical and rational approaches. From the empirical aspect, the best case scenario came from the Phase 3 RV144 trial in Thailand, the only HIV-1 vaccine efficacy trial to show a moderate protective effect. By digging deeper into the potential immunologic correlates of protection in this trial, we will be able to better design the next generation of vaccines based on the pox prime and protein boost vaccine strategy. The correlates of protection could also serve as critical surrogate markers for more rational-based approach in vaccine design. From the rational aspect, the field has made significant progress in better understanding of antibody and T cell responses during natural infection. In particular, a handful of broadly neutralising antibodies have been identified with far more potent and broad activity against global HIV-1 panels compared to antibodies isolated during early studies. Structural and functional analysis of their epitopes have for the first time provided the precise targets for vaccine design.

However, translation of antigenicity into immunogenicity remains a big challenge in both theoretical and practical terms. Some progress has been made in the development of antibody ontogeny-based HIV-1 subunits, or trimeric Env immunogens with emphasis on triggering specific antibody germline ancestors. These immunogens, however, are only able to trigger B-cell receptors or stimulate affinity maturation in transgenic mice models, failing to simultaneously sustain both processes. Whether any of these approaches could ultimately be successful will largely rely on efficacy trials in humans.

We still lack an appropriate animal model that predicts vaccine efficacy in human trials. Nevertheless, rhesus macaques infected with simian immunodeficiency virus (SIV) and chimeric simian human immunodeficiency virus (SHIV) have provided valuable models. With more SHIVs became increasingly available, some envelope-based vaccine approaches can be more thoroughly investigated before moving into human trials. Finally, in the absence of a successful vaccine in the foreseeable future, other prevention strategies must be pursued and implemented. Apart from behavioural interventions, successful biomedical approaches should already be used. While small molecule drugs have already been approved for pre-exposure prophylaxis, long-acting agents including small molecules and antibodies may lead to far better uptake and sustainability. The field has high expectations for these agents currently being tested in human trials.

Professors Lewin and Zhang met as young post-doctoral fellows in New York at the Aaron Diamond AIDS Research Centre, The Rockefeller University over 20 years ago. At the time, they were working under the guidance of Professor David Ho, on multiple projects related to HIV cure and vaccine research. It was the very beginning of ART and there was much to learn about this extraordinary virus. Since returning to their home countries of Australia and China, they now both lead large multidisciplinary research groups and their passion to find a cure and a vaccine for HIV remain stronger than ever.

Because of the great successes in the HIV response, there are many who believe that the fight against HIV is over. This book highlights how untrue this is. We hope that many scientists and clinicians in low-, middle- and high-income countries are inspired to take up the great scientific challenge of finding a cure and a vaccine for HIV. The scientific discoveries are moving at lightning pace but the major victories are still to come.



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We also acknowledge the contribution of all people living with HIV and those who we have unfortunately lost as a result of HIV infection, for their advocacy, commitment and participation in research. The great advances in HIV medicine and science over the last 30 years would never have been possible without their contribution. We very much hope that this extraordinary partnership between the community and researchers working on HIV only further strengthens, as we all strive together to discover, develop and implement an effective HIV cure and vaccine globally.

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Part I

HIV Vaccines

Chapter 1

HIV Vaccine Efficacy Trials: RV144 and Beyond



Elizabeth Heger, Alexandra Schuetz, and Sandhya Vasan

Abstract Despite progress in antiretroviral therapy, pre-exposure prophylaxis, microbicides, and other preventive strategies, a vaccine to prevent HIV-1 infection remains desperately needed. Development of an effective vaccine is challenged by several immunologic features of HIV-1 evidenced by the failure of five of the six HIV-1 candidate vaccine efficacy trials to date. This chapter reviews these efficacy trials with a focus on the Phase 3 RV144 trial in Thailand, the only HIV-1 vaccine efficacy trial to show a moderate protective effect of 31% with respect to placebo administration. Although modest, this protection has allowed for the study of potential immunologic correlates of protection to improve development of future HIV-1 pox-protein and other vaccine strategies. Trials in Thailand and South Africa have built upon the RV144 framework to provide additional immunologic insights which enable current and future efficacy testing of related vaccine candidates.

Keywords HIV · Vaccine · Correlates · Immunogenicity · Efficacy

The views expressed are those of the authors and should not be construed to represent the positions of the US Army or US Department of Defense.

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1.1 Challenges of HIV-1 Vaccine Development

Despite decades of effort, development of a highly effective vaccine to prevent human immunodeficiency virus (HIV)-1 infection remains elusive, largely due to several immunologic features unique to retroviruses and particularly to HIV. During the HIV life cycle, HIV RNA must be reverse transcribed to DNA, a process with a high error rate, creating rapid and large sequence diversity. The sequence diversity of HIV envelopes (Env) within a single geographic clade can vary by up to 15% and more than 35% across all clades within Group M, the “major” group of viruses responsible for the global HIV epidemic (Gaschen et al. 2002; Hemelaar 2012). Structural elements of the outer HIV Env are conserved, which may allow the development of vaccine immunogens capable of eliciting immunity to a diverse array of HIV variants (Jiang et al. 2010; Almond et al. 2010). However, because there are no cases of natural clearance of the vaccine, it is unknown whether effective vaccines must utilize HIV gene sequences matched to the predominant circulating recombinant forms in the geographic region of the target population or if they can rely on cross clade immunity. Alternatively, vaccine sequences may utilize designer immunogens expressing ancestral, consensus, or mosaic HIV sequences (Liao et al. 2006; Gao et al. 2004; Barouch et al. 2010; Santra et al. 2010; Ndhlovu et al. 2011; Barouch et al. 2013; Nkolola et al. 2014).

In HIV-infected individuals, broadly neutralizing antibodies against HIV-1 develop only years after infection, as these antibodies require extensive somatic hypermutation to be formed (Deeks et al. 2006; Dhillon et al. 2007; Binley et al. 2008; Gray et al. 2009; Simek et al. 2009; Sather et al. 2009; Bonsignori et al. 2017; Kepler et al. 2014; Fera et al. 2014; Sok et al. 2013). These antibodies must be able to penetrate the “glycan shield” on the surface of the HIV Env, where antigenic proteins are masked by heavy glycosylation, leaving little protein exposure for antibody binding (Schieff et al. 2009; Wei et al. 2003; Stewart-Jones et al. 2016; Panico et al. 2016). In addition, these antibodies demonstrate features of autoantibodies and may bind to host proteins such as cardiolipin, phospholipids, and glycolipids (Alam et al. 2007; Liao et al. 2011; Haynes et al. 2005; Matyas et al. 2009). This phenomenon may be due to the low density of trimeric gp120 spikes on the surface of the HIV Env, necessitating heterodimerization with gp120 and the viral membrane for adequate binding and cross-linking (Mouquet et al. 2010, 2012).

Kinetics of the immune response are an additional hurdle. Because HIV is a retrovirus, it integrates quickly into host DNA, necessitating that transmitted virions be cleared early, prior to the establishment of reservoir sites throughout the body (Miller et al. 2005; Spira et al. 1996; Haase 2010; Liu et al. 2016). Thus, vaccines must not only elicit specific and effective immune responses but must do so relatively quickly after initial exposure. Finally, the longevity of antibody responses to HIV Env must be improved to elicit not only effective but also durable vaccine protection.

1.2 Challenges to HIV Efficacy Testing

Although nonhuman primate experiments allow modeling of the potential efficacy of HIV vaccines against simian-human immunodeficiency virus (SHIV) challenge, results can vary depending upon the timing, route, and strain of challenge virus being used. Ultimately, the efficacy of an HIV vaccine candidate can only truly be tested in the conduct of large Phase 3 efficacy trials in populations at risk for HIV. Conduct of a randomized, placebo-controlled HIV vaccine efficacy trial involves enrollment of HIV-uninfected volunteers who are at risk for HIV acquisition and randomizing them to receive either vaccine or placebo and following infection rates in both groups over time while providing continuous ongoing counseling on behavioral HIV risk reduction. Successful completion of such a trial relies on ensuring that (1) there is sufficient HIV incidence in the population being studied, (2) these at-risk populations are accessible and willing to enroll in a trial to receive an investigational HIV candidate vaccine, and (3) that a sufficiently high percentage of volunteers enrolling in the trial will attend all visits and continue to completion to achieve a statistically meaningful result.

Each of these requirements brings its own challenges. For example, determination of annual HIV incidence, defined as the number of new HIV infections in any given year, requires a formal prospective study in the population intended for participation in the subsequent efficacy trial. These incidence estimates are used to statistically model the sample size for the efficacy trial to ensure a statistically meaningful result where the 95% confidence interval for efficacy will fall within an intended scientific target. However, if there is too great an interval between the incidence calculation and the efficacy trial, there is a risk of the incidence changing during this interval due to changing demographics, risk factors, or novel interventions (e.g., pre-exposure antiretroviral prophylaxis, or PreP), which could affect the outcome of the trial. Thus, incidence estimates must not only be accurate but current. Incidence estimates must also factor in the phenomenon of behavioral risk reduction in trial participants, as regular access to HIV prevention counseling and condoms may further reduce incidence in trial participants (Robb et al. 2012; Janes et al. 2013). In addition, the populations at highest risk for HIV are also often those hardest to reach. Recruiters, community stakeholders, and researchers must not only be able to reach such populations but also be able to establish relationships and provide education to assure their rights regarding individual privacy and voluntary participation. Potential risks of participation must be carefully explained, including vaccine-induced seroreactivity, where antibodies to candidate HIV vaccines may cross-react on clinical HIV diagnostic tests, causing a false-positive HIV test that must be resolved by further nucleic acid testing. These false-positive results could engender potential social harms, such as loss of employment or visa status, inability to donate blood or organs, and loss of health insurance, so close coordination with study staff regarding HIV testing status during and after the trial is paramount (Enterprise et al. 2015). Study staff must also ensure volunteer understanding that

the vaccine candidate under study is experimental and should not be assumed to be protective to avoid potential behavioral risk enhancement (Newman et al. 2009, 2010; Young et al. 2014). Finally, high-risk populations may be the most difficult to retain throughout a study due to geographic mobility, economic instability, comorbidities, or incarceration. Methods to minimize loss to follow-up include establishment of trust with the study team at the onset of the study, reminders via phone or social media between visits, and obtaining permission from ethical committees and local authorities to follow participants during incarceration (de Bruyn et al. 2005; Etcheverry et al. 2013; Harrison et al. 1995; Thapinta et al. 2002). Implementation of such trials is therefore a herculean effort by many stakeholders including but not limited to trial sponsors, pharmaceutical partners, academic investigators, governmental and other relevant host nation agencies, nongovernmental partners, community leaders and stakeholders, and clinical care facilities.

1.3 Challenges to HIV Efficacy Testing

1.3.1 *VAX003 and VAX004 Efficacy Trials*

VAX003 and VAX004 were two of the first HIV Phase 2b/3 vaccine efficacy studies conducted. Both trials were sponsored and funded by VaxGen, Inc., and targeted populations with a high risk of acquiring HIV infection. However, the routes of exposure in each population differed, with VAX003 targeting injecting drug users and VAX004 targeting men who have sex with men (MSM), bisexual men, and women who were at high risk of acquiring HIV through sexual contact.

VAX003 was a randomized, double-blind placebo-controlled trial (Vanichseni et al. 2004). It was initiated in 1999, recruiting 2500 injecting drug users from 17 drug treatment clinics in Bangkok, Thailand. The vaccine tested was AIDSVAX B/E®, which consisted of 300 µg each of subtype B MN rgp120 plus subtype E (A244 CRF01 AE) rgp120 adjuvanted with 600 µg alum; participants were assigned 1:1 to active vs. placebo treatment arms. The primary vaccine series was administered at 0, 1, and 6 months, with boosts at 12, 18, 24, and 30 months.

VAX004 was sponsored and partially funded by VaxGen, and partially funded by the US Centers for Disease Control and Prevention, and the US National Institutes of Health, and initiated in June 1998. It was a randomized, double-blind, placebo-controlled trial in 5000 MSM and 300 women at risk for heterosexual acquisition of HIV (Harro et al. 2004). Healthy, HIV-uninfected volunteers were randomized 2:1 active to placebo arms. VAX004 tested AIDSVAX B/B®, containing two different subtype B gp120 proteins: 300 µg each of MN and GNE8 adjuvanted in 600 µg alum. The study was conducted at 61 sites located in the USA, Canada, and the Netherlands. The vaccination schedule was identical to that used in VAX003.

The efficacy results of both VAX003 and VAX004 were disappointingly similar. In VAX003, protective efficacy was 0.1% and in VAX004 was 6.0% (Pitisuttithum et al. 2006; Flynn et al. 2005). Neither vaccine had a significant effect on viral load, CD4⁺ T cell counts, rates of ART-initiation, or disease progression. Despite the lack of efficacy, both vaccines resulted in development of antibodies to the gp120 components. In VAX004, the peak antibody responses were significantly inversely correlated with the incidence of HIV (Gilbert et al. 2005). However, when antibody responses were compared between those who were low, medium, and high vaccine responders, there was no consistent pattern between antibody levels and rate of infection.

1.3.2 STEP and Phambili Efficacy Trials

The STEP and Phambili studies tested the same vaccine, MRKAd5 HIV-1 gag/pol/ nef subtype B, which was developed by Merck & Co., Inc. The trials were funded by the National Institute of Allergy and Infectious Diseases (NIAID) and Merck and executed by the HIV Vaccine Trials Network (HVTN). However, the studies were conducted in two distinct regions and in different populations. The STEP trial recruited 3000 HIV-uninfected individuals at high risk for acquiring HIV infection, including MSM, sex workers, and high-risk heterosexuals. Volunteers were assessed for risk based on self-reports of the frequency and type of intercourse reported, drug use, prostitution, or sexually transmitted disease diagnoses. Volunteers were screened for adenovirus type 5 (Ad5) antibody titer and grouped accordingly into those with titers less than 200 and those with titers greater than 200. The study, which initiated in December 2004, included sites distributed across North America (the USA and Canada), the Caribbean, South America, and Australia (Buchbinder et al. 2008). An interim analysis was designed to take place after 30 HIV infections were confirmed in the STEP study per protocol participants and was held by the study Data Safety Monitoring Board in September 2007. The study unexpectedly met futility criteria at this meeting, resulting in suspension of further vaccinations and the decision to unblind all participants.

The STEP study team defined efficacy as reduction in HIV acquisition rates or, in those who became infected, a decrease in HIV viral load set point by an average of two \log_{10} HIV RNA values approximately 3 months post-diagnosis. The results of the interim analysis revealed that the infection rate in per protocol male participants (those who received at least 2 injections) was nearly double than that of placebo recipients (4.00% per year vs. 2.12%), but no difference in viral load set point was detected in vaccine recipients who became HIV-infected (4.60 vs 4.57 \log_{10} copies/mL). Further analysis of HIV infections in male participants by baseline Ad5 titer revealed that Ad5 titer was associated with the number of HIV infections. STEP study volunteers who became HIV-infected were followed to assess the effect of the

vaccine on mean \log_{10} viral load and progression to AIDS (Fitzgerald et al. 2011). No significant effect of vaccine was found on viral load, on CD4 $^{+}$ T cell counts, or on time to initiation of ART in participants followed for up to 4 years.

Immunologic investigations immediately following suspension of the STEP trial were inconclusive as to a mechanism for enhanced infection in the male vaccine recipients. Many hypotheses were investigated in subsequent years, including the relationship between baseline Ad5 titer and expansion of Ad5-specific CD4 $^{+}$ T cells (Hutnick et al. 2009). The authors concluded that “Ad5-specific CD4 $^{+}$ T cells were unlikely to have had a role in the possible increased susceptibility to HIV infection observed in the STEP trial.” In a companion paper, O’Brien et al. (O’Brien et al. 2009) hypothesized that baseline Ad5-specific neutralizing antibodies are a surrogate for Ad5-specific T lymphocyte responses and that Ad5-specific CD4 $^{+}$ T cells in volunteers with positive baseline Ad5 titers provided a susceptible target for HIV infection. However, the authors found no correlation between baseline Ad5-specific interferon- γ (IFN- γ) ELISPOT responses and neutralizing antibody titers. They repeated, however, the finding of lower vaccine-induced responses (gag-specific antibody; IFN- γ $^{+}$ CD4 $^{+}$ and IFN- γ $^{+}$ CD8 $^{+}$ T cells) in participants with positive baseline Ad5 neutralizing antibodies than in those with no detectable baseline titer, suggesting that the vaccine vector was partially neutralized in the baseline Ad5 positive population (O’Brien et al. 2009).

A comprehensive in vitro investigation of the immunologic basis for the apparent enhancement of infection in the STEP trial was published in 2014, where the authors demonstrated that Ad5-specific CD4 $^{+}$ T cells are, as suggested by O’Brien et al. (2009), highly susceptible to HIV infection and are preferentially lost in HIV-infected individuals when compared with cytomegalovirus (CMV)-specific CD4 $^{+}$ T cells (Hu et al. 2014). Regardless of whether the Ad5-specific CD4 $^{+}$ T cells were induced by natural infection or by vaccination, they were absent or significantly reduced in the peripheral blood of HIV-infected volunteers (12%). In contrast, Ad5 hexon-specific CD4 $^{+}$ T cell responses were detectable in a majority of HIV-uninfected volunteers (75%). Hu et al. also found that the expression of $\alpha 4\beta 7$ in Ad5-specific CD4 $^{+}$ T cells of Ad5 HIV vaccine recipients was significantly higher (45%) than in CMV-specific CD4 $^{+}$ T cells (9.8%). The $\alpha 4\beta 7$ integrin has been shown to be responsible for migration of CD4 $^{+}$ T cells to gastrointestinal tissues (Arthos et al. 2008), where depletion during acute HIV infection occurs (Brenchley et al. 2004).

Phambili was conducted in the Republic of South Africa and enrolled 801 of the targeted 3000 sexually active population before the trial was halted at the time the STEP study interim analysis results were known (Gray et al. 2011). Because the study was discontinued early (active between January and September 2007), a formal analysis of results was not completed. However, in the participants who enrolled, a trend similar to that of the STEP trial was seen: there were 34 HIV-infected cases in the vaccine group (incidence of 4.54) and 28 cases in the placebo group (incidence of 3.70). Additionally, there was no significant difference in the mean viral load set point between arms.

1.3.3 HVTN 505 Efficacy Trial

HVTN 505 tested a DNA prime, rAd5 boost vaccine developed by the Vaccine Research Center at NIH. The study, conducted by the Division of AIDS and the HVTN between 2009 and 2013, enrolled 2500 high-risk participants (MSM, transgender women) across the USA (Hammer et al. 2013). As with STEP, the study was halted for futility at the first interim analysis. There was no effect of the vaccine on the acquisition of HIV nor was there an effect on the viral load set point post-infection. The vaccine was immunogenic, inducing 100% response of IgG to the three Env proteins encoded in the vaccine (subtypes A, B, C), to the Group M gp140 consensus sequence, and to the gp41 sequence. However, it did not induce robust binding antibody (bAB) responses to the first and second variable regions of HIV-1 envelope (V1 V2), which had been identified as a correlate of reduced risk of infection in the RV144 trial by the closure of the study (Haynes et al. 2012).

1.4 RV144 Trial Design, Conduct, and Outcome

RV144, which was the first efficacy trial of a prime-boost HIV vaccine strategy, was funded and conducted from 2003 to 2009 by the US Army, US NIH, the Thai Ministry of Public Health (MOPH), and the Mahidol University Vaccine Trial Center, enrolling over 16,000 Thai participants in a 1:1 randomization (Rerks-Ngarm et al. 2009). Vaccine recipients received ALVAC-HIV (vCP1521) prime at 0, 1, 3, and 6 months, followed by AIDSVAX B/E® protein boost during months 3 and 6. Sanofi Pasteur produced the ALVAC-HIV prime, which consisted of a canarypox vector with subtype B *gag/pro* inserts and a CRF-01 AE gp120 *env* insert (Nitayaphan et al. 2004). AIDSVAX B/E®, produced by VaxGen, contained two gp120 proteins: MN subtype B and A244 CRF01-AE; the same vaccine tested in VAX003 (Pitisuttithum et al. 2006).

Eight sites in the Chonburi and Rayong provinces in Thailand recruited participants in the surrounding communities; in contrast to the previous efficacy trials, the enrolled population was largely heterosexual and of low to moderate risk for infection with HIV. The primary endpoints were prevention of HIV infection and effect on early viral load after infection. Following the vaccination series, participants returned for HIV testing at 6-month intervals for 3 years and for infection risk-reduction counseling. Participants who were confirmed HIV-infected during the trial were referred to a companion protocol that followed progression of disease through plasma and seminal plasma viremia, CD4⁺ T cell counts, and initiation of ART.

Efficacy was assessed after the final visit in which volunteers received all the remaining vaccinations (3.5 years) and were confirmed to be HIV-negative upon trial entry (modified intention-to-treat analysis; MITT). The vaccine regimen resulted in a modest 31.2% reduction in HIV-1 (Fig. 1.1). However, there was no effect of the vaccine on early viral load. In a post hoc analysis of the trial results,

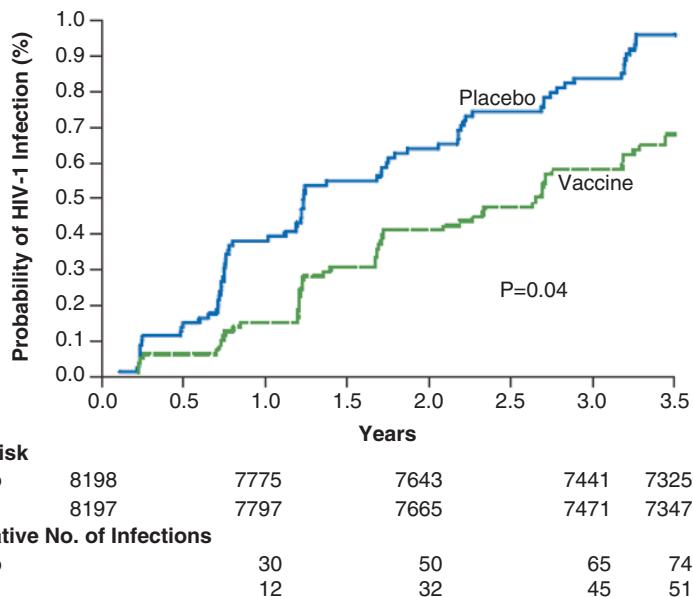


Fig. 1.1 Kaplan-Meier cumulative rates of HIV-1 infection from the RV144 Modified Intention-to-Treat Analysis. Adapted from Rerks-Ngarm et al. NEJM 2009

efficacy was assessed at 6-month intervals after all vaccinations were completed in the MITT population (Robb et al. 2012). In this analysis, the vaccine efficacy reached 59.9% high at 12 months postvaccination, waning over time to 30.4% at 36 months.

1.5 Immune-Correlates Analysis of RV144

Almost all currently licensed vaccines against viral pathogens protect through antibodies in serum or at mucosal sites that block viral infection. These correlates of protection are identified by studying immune responses in individuals who block or clear infection. Immune correlates of protection for HIV vaccines have remained elusive, due in part to the fact that no individuals have cleared HIV naturally. RV144 provided the first evidence that HIV vaccines could potentially provide protective efficacy against HIV acquisition (Rerks-Ngarm et al. 2009), thus affording the opportunity for identification of immune responses that were potentially predictive for the trial outcome and hypothesis generation as to which of those responses are responsible for protection (Qin et al. 2007). An initial pilot study involving an international team of collaborators evaluated 32 assays of 17 types to detect antibody, innate, and cellular immune responses. Based on reproducibility, nonredundancy, low false-positive rate, and large dynamic range, six primary assays were identified.

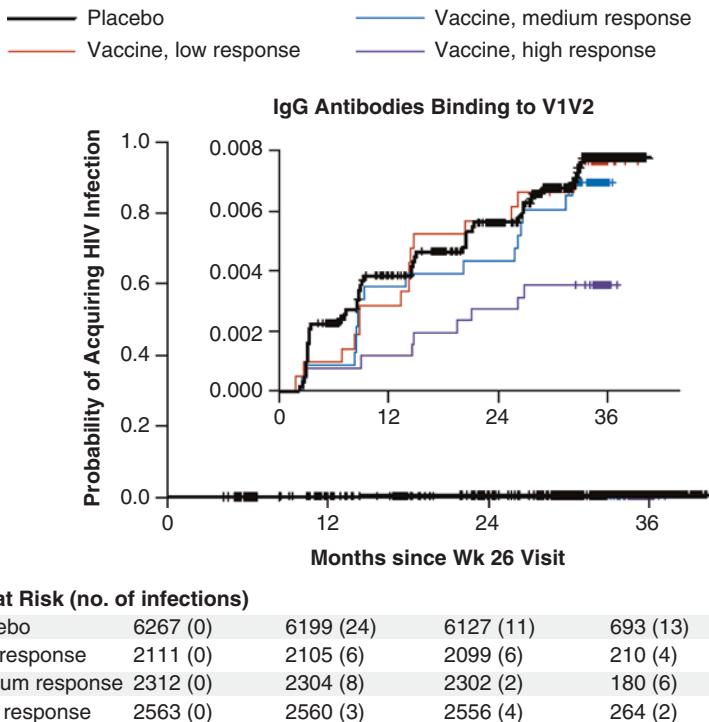


Fig. 1.2 Estimated Cumulative HIV-1 Incidence Curves for Participants with Plasma IgG Binding Antibodies to HIV V1V2. Adapted from Haynes et al. NEJM 2012

Samples from 41 HIV-infected, vaccinated cases, and 205 HIV-uninfected, vaccinated controls were analyzed at peak-immunogenicity and 2 weeks after the fourth vaccination to identify correlates of risk (CoR) (Haynes et al. 2012). CoR are measured immune responses from vaccine trials associated with the rate of a particular endpoint, such as HIV infection in RV144, and those are statistically relevant (Plotkin and Gilbert 2012; Plotkin 2010). Based on this case-control analysis, IgG avidity, antibody-dependent cell-mediated cytotoxicity (ADCC), neutralizing antibodies (Nab), and cellular immune responses did not correlate with the rate of HIV infection. However, two immune responses were identified that correlated significantly with HIV infection.

First, plasma IgG-binding antibodies to gp70 V1 V2 CaseA2 (subtype B) scaffold inversely correlated with HIV infection. Prior analyses had shown that RV144 samples at peak-immunogenicity tested against linear peptide arrays derived from the A244CRF01_AE strain of HIV exhibited a high-binding pattern to peptides in the V1 V2 region, a pattern that is considerably different from HIV-infected individuals (Gottardo et al. 2013; Karasavvas et al. 2012). Vaccines with the highest V1 V2 IgG-binding antibodies were more likely to be protected than those with low titers (Fig. 1.2). Follow-up analyses using a wide range of V1V2 antigens including

antigens derived from a variety of clade C isolates showed similar association between high binding to V1 V2 and reduced HIV acquisition (Zolla-Pazner et al. 2014a). It is of note that V1V2 antibody response rate and magnitude waned over time and was associated with waning efficacy (Corey et al. 2015). In addition, monoclonal antibodies from RV144 vaccine recipients appeared to bind to a region of V2 that partially overlaps with the binding sites of the broadly neutralizing antibodies (bNAbs) CH01 and PG9 (Bonsignori et al. 2012a; Doores and Burton 2010) and are directed at a linear epitope including a lysine residue at amino acid 169 on the HIV envelope. Though not broadly neutralizing, these V2-specific antibodies mediated ADCC responses, and further analyses showed that this ADCC response was directed primarily against the first conserved (C1) conformational region of gp120 Env (Bonsignori et al. 2012b). C1-specific IgG antibodies potentially act synergistically with V2-specific antibodies, possibly by inducing conformational changes that improve the exposure of V2 (Pollara et al. 2014).

Second, plasma Env-specific IgA-binding antibodies directly correlated with HIV infection. IgA-binding antibody levels did not enhance infection but rather abrogated the protective effect of vaccination. In the setting of low Env-specific IgA, a reduction in the risk of infection was seen for ADCC and NAb (Gottardo et al. 2013; Montefiori et al. 2012). One potential hypothesis is that IgA may block the action of IgG in the context of ADCC and phagocytosis (Griffiss 1975; Mathew et al. 1981). ADCC responses in RV144 were mainly directed against the C1 conformational region of HIV gp120, although other epitope specificities contributed to the overall response (Bonsignori et al. 2012b; Ferrari et al. 2011; Liao et al. 2013; Moody et al. 2012). Further studies showed that plasma IgA specific for the C1 conformational region could block C1-specific IgG binding and ADCC effector function due to their ability to bind to different Fc receptors (Tomaras et al. 2013). In addition, individuals with low levels of Env-specific IgA antibodies also had high levels of antibodies to V3 peptides, and vaccine efficiency was about 70% among the subgroup of vaccinees with low to no Env-specific serum IgA response and high V3 antibodies (Gottardo et al. 2013; Zolla-Pazner et al. 2014b).

1.6 RV144 Post Hoc Analyses

The initial RV144 correlates analyses in 2011 afforded a new understanding of HIV Env regions such as the V1 V2 loop and the importance of non-neutralizing antibody responses that provide new hypotheses and targets for the development of future vaccine concepts (Haynes et al. 2012). Since that time, international research collaborations have produced a number of scientific publications that have substantiated the hypothesis that antibody-mediated protection played a predominant role in the observed efficacy in RV144 but have also elucidated additional immunologic insights regarding the RV144 vaccine regimen.

The confluence of results from RV144 and improved HIV sequencing techniques have allowed the use of HIV genetics to test hypotheses regarding the impact of

vaccines on HIV breakthrough infections (Edlefsen et al. 2013). Characterization of viruses that evade a vaccine-induced host-immune response to establish HIV infection can provide key insights into vaccine-induced immune pressure on HIV to inform subsequent immunogen design. Genetic sieve analysis of viral isolates from individuals who became HIV-infected in the RV144 trial revealed that isolates from vaccinated individuals were less likely to possess a lysine at amino acid (aa) 169 of the Env V2 region than placebo recipients. Vaccine efficacy was significantly higher against HIV manifesting a lysine at aa 169 than against HIV with a different residue at position 169 (Rolland et al. 2012). Over 85% of the circulating HIV strains in Thailand have lysine at aa 169; however, it was present in only 66% of the RV144 HIV-infected vaccine recipients, suggesting vaccine-induced immune pressure altered this region of the virus (Corey et al. 2015). Researchers went on to perform a comprehensive sieve analysis of the entire HIV genome and identified 56 aa signature sites that differed between vaccine and placebo recipients. Of those, 19 sites were in the regions comprising the RV144 vaccine, and 9 sites in the gp120 Env were significantly enriched for known antibody-associated sites (Edlefsen et al. 2015). In particular, site aa 317 in the V3 loop was linked to antibody recognition, and site aa 369 and aa 424 were linked to CD4-binding site neutralization. The fact that signature sites significantly covaried with other sites across the genome more than did non-signature sites suggests functional and/or structural relevance of the signature sites.

Although the initial RV144 correlates analyses did not detect a strong cellular signal associated with risk of infection (Haynes et al. 2012), several subsequent observations highlighted the potential contribution of cellular immune responses. Advances in flow cytometry have enabled high-dimensional and high-throughput measurements of individual cells that allow interrogation of cell population heterogeneity. With the additional usage of Bayesian analysis using a new computational tool, COMPASS (combinatorial polyfunctionality analysis of antigen-specific T-cell subsets), researchers were able to model all observed functional cell subsets and select those most likely to exhibit antigen-specific responses (Larsen et al. 2012; Newton et al. 2004). Using the same intracellular cytokine staining (ICS) assay that did not show any correlate in the initial study (Haynes et al. 2012) and including COMPASS analysis of the polyfunctionality of antigen-specific cellular immune responses revealed an inverse relationship between polyfunctionality score and HIV infection (Lin et al. 2015). Vaccines with Env-specific CD4⁺ T cells expressing CD154, IL-2, IL-4, IFN- γ , and TNF- α showed the strongest correlation, with a reduced rate of infection compared to those who did not make such a polyfunctional response. Moreover, this response was an independent correlate of infection after accounting for the primary correlates of IgG binding to V2 and IgA binding to Env (Lin et al. 2015). In addition, a three-function subset of CD4⁺ T cells expressing CD154, IL-2, and IL-4 was also inversely related to infection (Kim et al. 2015). These data indicate the potential importance of initiating strong helper T-cell responses with vaccinations. This is also supported by a study conducting fine epitope mapping of CD4⁺ T-cell responses in RV144, showing that two CD4 epitopes are contained within the V2 loop, one encompassing the $\alpha 4\beta 7$ integrin-binding

site and the other nested between two previously described genetic sieve signature sites, aa 169 and aa 181 (Ratto-Kim et al. 2015).

Based on the findings that two antibody responses correlated with HIV acquisition (Haynes et al. 2012; Rerks-Ngarm et al. 2009; Robb et al. 2012), researchers tested whether human leukocyte antigen (HLA) class II genotypes affected HIV-specific antibody levels and HIV acquisition in RV144. HLA class II molecules (DR, DQ, and DP) are found on the surface of antigen-presenting cells presenting foreign extracellular peptides to CD4⁺ T cells, which then induce antibody production by B cells. The HLA class II genes that encode these molecules are highly polymorphic, and several HLA alleles have been shown to be associated with humoral responses mediated by vaccines (Caillat-Zucman et al. 1998; Ovsyannikova et al. 2007; Paris et al. 2012; Stephens et al. 1995). Therefore, it is possible that differences in vaccine-induced immune responses could partially be due to variation in HLA class II genes (Baldwin et al. 2015). HLA class II genotypes and antibody levels of vaccinated HIV-infected and matched vaccinated HIV-uninfected control subjects from RV144 were analyzed, and antibody response correlated with acquisition only in the presence of single host HLA class II alleles (Prentice et al. 2015). Env-specific IgA antibodies were associated with an increased risk of acquisition specifically in individuals with DQB1*06. IgG antibody responses to Env (120–204) were higher and associated with a decreased risk of acquisition (Haynes et al. 2012). Among individuals with high levels of IgG antibody responses, vaccine efficacy was 58% and increased to 71% in conjunction with DPB1*13. Prentice et al. (Prentice et al. 2015) also observed that the immune response induced by vaccination in individuals carrying DPB1*13 differed from those without DPB1*13, as vaccinated DPB1*13 positive individuals selected specific HIV variants (Klein and Sato 2000; Prentice et al. 2015). Overall, the underlying genetic findings indicate that interactions of certain HLA class II genes modulated the antibody responses in RV144, thus affecting HIV acquisition.

Analysis of case-control data and subsequent post hoc analyses from RV144 revealed complex compositions and interactions of immune responses that potentially underlie the observed vaccine efficacy. Even though antibody titers and NAbs are often correlates of protection against other viral pathogens, those mechanisms alone do not account for protective immunity in RV144 (Pulendran and Ahmed 2011; Ferrari et al. 2011). There is mounting evidence for the potentially critical role of non-neutralizing antibody functions such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent phagocytosis (ADCP), antibody-dependent complement deposition (ADCD), and antibody-dependent respiratory burst (ADRB) in protective immunity against HIV-1 (Barouch et al. 2015; Bournazos et al. 2014; Hessell et al. 2007). Based on this observation, a new “systems serology” approach is using a battery of modeling techniques that emphasize covariation among those measurements to reveal features of vaccine-induced “fingerprints” that potentially offer new insights in polyclonal antibody immune responses elicited by the vaccine (Chung et al. 2015). This approach was able to identify features that were reported in previous correlates analyses, such as the elevated IgG3 response in RV144 marking a distinct IgG1/IgG3 subclass selection profile (Chung et al. 2014; Yates et al. 2014)

and the vaccine-induced V1V2-specific IgG responses that inversely correlated with risk of HIV infection (Zolla-Pazner et al. 2014a). Moreover, this approach revealed indirect connections between V1 V2-specific IgG1 or IgG3 responses and antibody functions such as ADCC, ADCP, and ADCD. The fact that IgG1 responses were largely tethered to antibody function suggests that the IgG3 “protective” signature may either represent a surrogate of an effective antibody response or only contribute in combination with multiple other antibody features. This is supported by the observation that depletion of IgG3 antibodies from RV144 vaccinees resulted in a significant loss of ADCP and ADCC activity. However, the activity was not completely depleted, suggesting that IgG3 antibodies alone do not mediate the activation in polyclonal RV144 sera (Chung et al. 2014). ADCP was enhanced in vaccinees exhibiting a high V1 V2 IgG3/IgG1 response shown previously to be associated with protection in the nonhuman primate model (Barouch et al. 2013), thus raising the possibility that ADCP may represent a critical function within the polyfunctional antibody profile. This novel approach could help to dissect HIV vaccine regimen profiles in greater depth and provide a framework to better understand immune profiles that segregate with previously defined correlates of risk.

1.7 Post RV144 Trial Progress

1.7.1 *RV305 Clinical Trial in Thailand*

In follow-up to the partial success of the RV144 trial, two additional trials were conducted with the same products in hopes of improving the immunogenicity of this vaccine regimen, with a focus on the V1 V2 correlate described above. RV305 was a clinical trial conducted in Thailand and sponsored by the US Army in collaboration with the US NIH Thailand MOPH, Mahidol University, and the Royal Thai Army (Rerks-Ngarm et al. 2017). This trial was conducted from 2012 to 2017 in 162 volunteers from the original RV144 trial who had been randomized to receive active vaccines and had completed all vaccinations. RV305 volunteers were further randomized to receive two vaccinations 6 months apart, consisting of either AIDSVAX® B/E alone, ALVAC-HIV, the two in combination, or placebo in a double-blinded manner. Because the interval between the original priming RV144 vaccine series and the RV305 boosting series was 6–8 years, the purpose of the study was to evaluate the safety and immunogenicity of these late boost vaccinations, as well as to characterize the immune response in mucosal compartments, which was not performed in RV144 due to the trial magnitude.

Consistent with the RV144 priming series, additional late boosts in RV305 were generally safe and well tolerated. Quantification of plasma IgG bAb to full-length gp120 subtype E as well as the subtypes B and E V1 V2 scaffolds revealed a consistent pattern. All vaccine recipients of AIDSVAX B/E®, either alone or in combination with ALVAC-HIV, formed HIV-specific antibodies, whereas responses in

ALVAC-HIV alone or placebo recipients were low. There was no significant difference in response to AIDSVAX B/E® alone from AIDSVAX B/E® with ALVAC-HIV, indicating that ALVAC-HIV plays little role in boosting the humoral response. Interestingly, the magnitude of response to the initial vaccination was higher than the peak response to the initial RV144 vaccination series, but the IgG response to the second vaccine 6 months later was not as high and of similar magnitude to the peak RV144 response (Rerks-Ngarm et al. 2017). Unfortunately, the IgG response to all antigens waned rapidly, within 6 months of the last vaccination. Analysis of IgG subclasses revealed that Env IgG1 and IgG4 were boosted higher than the peak RV144 response after the initial boost, while Env/V1 V2 IgG3 was not boosted higher than the peak RV144 response and decreased with additional protein boosts (Williams et al. 2015; Akapirat et al. 2016c). Plasma IgA responses, which were a correlate of risk in RV144, followed a similar pattern (Rerks-Ngarm et al. 2017).

Weak neutralizing activity was detected prior to boosting, evidencing long-lived Env-specific B-cell responses generated by previous RV144 immunizations. Boosting immunizations enhanced the Tier 1 neutralization response against more sensitive viruses in a similar pattern to IgG and IgA antibody boosting, without generating strong neutralization against the less easily neutralized Tier 2 viruses (Rerks-Ngarm et al. 2017; Seaman et al. 2010). However, sorting of individual B cells from vaccine recipients revealed an expansion of a subdominant pool of B-cell clonal lineages producing antibodies with qualities of broadly neutralizing antibodies. Specifically, late vaccine boosts of AIDSVAX B/E® with or without ALVAC resulted in increased somatic hypermutation and lengthening of the heavy chain complementary determinant region 3 (HCDR3) in Env-reactive antibodies, both qualities associated with broadly neutralizing antibodies (Meffre et al. 2001; Wardemann et al. 2003; Haynes et al. 2012). Furthermore, the germline precursors and affinity matured B-cell clonal lineage members neutralized a Tier 2 primary viral isolate (Easterhoff et al. 2017), expanded prior ADCC-mediated antibodies induced in RV144, and generated new HIV-1 V2-specific ADCC-mediated antibodies (Pollara et al. 2017).

Cellular responses in blood consisted predominantly of CD4⁺ T-cell IFN- γ responses against HIV Env, with some CD4⁺ IL-2 responses against HIV Env in participants boosted with AIDSVAX B/E® with or without ALVAC but not ALVAC alone. CD4⁺ T-cell polyfunctionality scores to HIV Env increased in these same two groups post late boosting (Rerks-Ngarm et al. 2017). RV305 also afforded the opportunity to evaluate the response at mucosal sites at the point of initial HIV entry during sexual transmission. HIV-specific IgG antibodies were evident in a similar pattern to plasma IgG antibodies in semen and cervical and rectal secretions (Akapirat et al. 2014a; Akapirat et al. 2014b), although encouragingly, HIV-specific IgG was detectable in cervicovaginal secretions 1-year postvaccination (Akapirat et al. 2015). Based on these results, participants in the RV305 trial were then optionally enrolled to receive an additional AIDVAX B/E® boost 3–4 years after the RV305 series, in the hopes of further expanding this clonal lineage. Analyses of this additional boost are currently ongoing.

1.7.2 *RV306 Clinical Trial in Thailand*

In parallel, the RV306 clinical trial enrolled and randomized 360 healthy volunteers in Thailand to receive either placebo or the RV144 priming series over 6 months, followed by a boost with AIDSVAX B/E® alone or with ALVAC at month 12, or both vaccines at month 15 or 18, to utilize a more compressed vaccination schedule which incorporated a late boost (Pitisuttihum et al. 2014). In all vaccine groups, addition of the late boost increased HIV Env-specific and V1 V2-specific IgG plasma, mucosal binding antibody titers, and Tier 1 neutralization, with a strong correlation in response magnitudes across assays (Akapirat et al. 2016a; Akapirat et al. 2016b; Wieczorek et al. 2016). As in RV305, the predominant cellular responses in blood were CD4⁺ T cell expressing IFN- γ and/or IL-2 to HIV Env (Eller et al. 2017). Late boosting also increased both HIV-specific plasmablast and memory B-cell responses quantified by B-cell enzyme-linked immunospot (ELISpot) (Chuenarom et al. 2016). Interestingly, the magnitude of the humoral and peripheral cellular responses, including polyfunctionality, improved as the interval between the priming series and the booster vaccine increased. Furthermore, increasing the boosting interval improved the durability of HIV-specific neutralizing antibody responses (Wieczorek et al. 2017). These data are consistent with the RV305 results and demonstrate that a prolonged interval between priming and boosting may be immunologically beneficial for the anamnestic immune response. However, at a population level, this must be balanced with the need to provide continued protection from HIV during the vaccination series, as responses wane rapidly postvaccination, and a longer interval between priming and boosting could lead to a period of increased risk prior to boosting.

Interestingly, HIV-specific responses in the gut, as measured by flow cytometry on volunteers undergoing optional sigmoidoscopy, revealed a different pattern than in the periphery. While the RV144 priming regimen did not induce detectable HIV-specific responses in the gut, following late boost, responses to HIV Env were detected in both the CD4⁺ and CD8⁺ T-cell compartments but consisted primarily of T cells secreting TNF- α (Schuetz et al. 2017). Thus, the quality of the cellular response to HIV candidate vaccines may differ between anatomical sites. Increases in activated CD4⁺ CCR5⁺ target cells have been shown to correlate with increased risk of intrarectal Simian Immunodeficiency Virus (SIV) acquisition in rhesus macaques (Carnathan et al. 2015). CD4⁺ CCR5⁺ T cells in the gut mucosa of RV306 vaccinees showed no evidence of increased cellular activation postvaccination with respect to placebo recipients (Schuetz et al. 2016), which provides important reassurance before moving this vaccination regimen into efficacy trials in populations with increased risk of intrarectal transmission.

1.7.3 HVTN 097 and HVTN 100 Clinical Trials in South Africa

In parallel to the development efforts in Thailand, the AIDSVAX/ALVAC candidate vaccine regimen is moving forward in the Republic of South Africa under the leadership of the Pox-Protein Public-Private Partnership (P5), comprised of the NIAID, the Bill & Melinda Gates Foundation, the South African Medical Research Council, HVTN, Sanofi Pasteur, GlaxoSmithKline plc, and the US Military HIV Research Program. The initial trial in this effort, HVTN 097, evaluated the vaccines used in RV144 in the same vaccination schedule in a randomized, double-blinded placebo-controlled Phase 1b trial. In 68 participants, vaccines were safe and well tolerated, and overall response rates of plasma IgG and Env-specific CD4⁺ T cells expressing IFN- γ and/or IL-2 were similar to RV144 (Gray et al. 2014; Lazarus et al. 2014).

This trial was followed by HVTN 100, conducted in healthy volunteers in the Republic of South Africa to test a vaccine regimen similar to RV144 and RV306. Although the importance of matching HIV vaccine products to the predominant circulating HIV molecular subtype in the target population remains unknown, HVTN 100 utilized vaccines expressing genes from HIV subtype C predominant in Southern Africa. ALVAC-HIV vCP2438 expressing HIV subtype C gp120 *env* and subtype B gp41, *gag*, and protease was administered at months 0, 1, 3, 6, and 12, with bivalent subtype C gp120 administered at months 3, 6, and 12, mimicking the 12-month late boost arm in RV306. While the AIDSVAX B/E® bivalent protein used in RV144, RV305, and RV306 was adjuvanted with Alum (Vanichseni et al. 2004), the bivalent subtype C gp120 was adjuvanted with MF59®, a squalene-based adjuvant that has been used in the evaluation of candidate influenza and HIV vaccines (Nitayaphan et al. 2000; Durando et al. 2008; Churchyard et al. 2016). The goal of this clinical trial was to determine whether the HVTN 100 vaccine regimen matched or exceeded the immunogenicity of four prespecified “go/no-go” criteria, specifically Env IgG, V1 V2 IgG, Env CD4⁺ T cell, and antibody Fc effector function over similar responses in RV144.

1.8 Future HIV-1 Candidate Vaccine Efficacy Trials Building Upon RV144

In 2016, it was announced that all HVTN 100 prespecified immunogenicity criteria had been met, prompting the decision to proceed with a Phase 2b double-blinded, placebo-controlled efficacy trial of this subtype C regimen in South Africa (Tomaras 2016). This trial, HVTN 702, is enrolling 5400 men and women at risk for HIV. They are being randomized to receive the HVTN 100 regimen or placebo over 12 months and followed for 24 to 36 months while receiving HIV preventative counseling and referral to services for voluntary medical male circumcision and/or PrEP if desired. Results on HIV incidence in each group and vaccine efficacy are expected between 2019 and 2021.

In parallel, development efforts are underway to manufacture the next generation of bivalent B/E proteins for clinical development, with the goal of pursuing an efficacy trial in regions where subtypes B and E circulate. Thus, the immunologic insights of the RV144 trial and its successors continue to drive the pursuit of a licensed, effective vaccine to prevent HIV infection in multiple regions.

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Chapter 2

HIV T-Cell Vaccines



Beatrix Mothe and Christian Brander

Abstract Some of the strongest immune correlates of controlled HIV infection include markers related to antiviral T-cell responses, especially responses mediated by CD8+ cytotoxic T lymphocytes (CTL). These observations and lessons learned from other viral infections have motivated the development of T-cell vaccine candidates to HIV in the preventive and especially in the therapeutic setting. While none of the T-cell vaccine concepts tested to date have shown sufficient efficacy, the last few years have seen some advances indicating that strategies activating the cellular adaptive immune response to HIV may present a critical component of an effective therapeutic and possibly preventive HIV vaccine. Some of the important components that a successful T-cell vaccine may need to contain and additional considerations for the design and delivery of such vaccines are discussed in this chapter.

Keywords T-cell vaccine · HIV vaccine design

2.1 Support for the Relevance of T-Cell-Based HIV Vaccines

This chapter does not aim to provide an in-depth review of T-cell-related biomarkers and related host genetic factors associated with relative control of HIV in natural infection, as there is plenty of literature on the topic. Still, to appreciate the challenges of developing effective HIV T-cell vaccines, a few key aspects merit

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mentioning. These include especially studies addressing host immunity mediated by CD4+ and CD8+ T-cells that recognize viral antigen presented in the context of HLA class I and class II molecules. The first description of a cytotoxic T lymphocyte (CTL) immune responses to HIV dates back 30 years, when in 1987 Walker and Plata independently identified HIV-seropositive individuals with CD8+ T-cells that recognized viral antigen (Plata et al. 1987; Walker et al. 1987). Although subsequent studies started to provide an ever more detailed picture of the specificity and the high magnitude of these responses, a clear mechanistic link to in vivo HIV control was not defined (Koup et al. 1994). Ten years after their first description, the critical role of virus-specific CD8+ CTL responses was demonstrated by CD8+ T-cell depletion studies in SIV-infected macaques, providing at the time the most compelling evidence for direct involvement of CTL responses in the control of HIV infection (Schmitz et al. 1999). At the same time, studies in HIV-infected humans also began to differentiate responses based on their HLA restriction and identified an ever-growing number of HLA class I alleles that were associated with relatively slower progression of HIV disease (Kaslow et al. 1996). These studies further supported the notion that at least some HIV-specific T-cell responses may be capable to reduce plasma viral loads and control an established infection. In addition, early studies identifying virus-specific T-cell responses in highly exposed seronegative individuals (HESN) suggested that HLA class I-restricted CD8+ T-cell may even be able to prevent infection all together (Rowland-Jones et al. 1995). Some more recent data showing HESN to mount broad T-cell responses with unusual effector phenotypes may provide novel tools to better characterize these responses (Ruiz-Riol et al. 2015). However, although studies in HESN have regained considerable interest over the last years, it is still unclear whether these cells are indeed responsible for effective protection from (abortive) infection or whether they merely represent exposure to noninfectious viral particles. Similarly, the characterization of virus-specific T-cells in established and long-term chronic infection may also simply yield surrogate markers of otherwise controlled infection, and care needs to be taken not to confuse surrogate markers with functional correlates of a truly effective immune response (Mothe et al. 2009). In this regard, some strong evidence supporting an antiviral effect of virus-specific CTL responses stems from the analyses of viral evolution in response to CTL-mediated immune surveillance. Studies from a number of groups have shown extensive adaptation of HIV to HLA-restricted CTL activity and identified HLA genetics as one of the strongest factors driving global HIV variability (Bhattacharya et al. 2007). The fact that HIV replicative fitness can suffer tremendously by viral escape from these CTL responses strongly suggests these CTL are effective in vivo and directly affect viral replication (Frater et al. 2007; Murakoshi et al. 2017). In addition, frequent reversion of these mutations in the absence of immune pressure further documents the antiviral effect of these cells (Leslie et al. 2004). While such gradual adaptation may reduce viral pathogenicity (Payne et al. 2014), the variability of HIV greatly complicates the interpretation of some of these analyses and poses a major challenge for T-cell vaccine development.

In parallel to studies addressing the specificity and antiviral activity of CD8+ T-cells, CD4+ T-cell responses to HIV have been studied in detail, although not to the extent as CD8+ T-cell immunity. The first comprehensive assessment of virus-specific CD4+ T-cell responses has been conducted by Kaufmann and colleagues in 2004 and identified the regions, particularly in Gag and Nef that are most frequently targeted by CD4+ T-cells (Kaufmann et al. 2004) (Buggert et al. 2012). More recently, virus-specific CD4+ T-cell responses have been linked to HIV disease control, with different protein specificities being associated with variable effects on viral load (Laher et al. 2017). These findings are further supported by the statistical associations between HLA class II alleles and HIV control, which have been reported for several cohorts and have been linked to specific CD4+ T-cell epitopes presented by class II molecules (Tang et al. 2002; Ferre et al. 2010; Ranasinghe et al. 2013) (Oriol-Tordera et al. 2017). Since these CD4+ T-cells can have a direct effect on the activity of CD8+ CTL through various mechanisms (Chevalier et al. 2011), vaccine efforts, preventive and therapeutic alike, may thus profit from incorporating strong CD4 targets as well. In many cases, immunogen design does not focus specifically on this potentially critical requirement, but especially in full protein approaches, CD4+ T-cell targets are automatically represented as well. However, as not all CD4+ T-cell responses will contribute equally to viral control and since the required effector phenotype that can drive a most effective CD8+ T-cell response is not well defined, further studies in this regard are urgently needed. As highlighted in the further sections, studies in acute and chronic HIV infection, longitudinal analysis of the coevolution between virus and T-cell responses, study of the interaction between the CD4 and CD8 arm of the cellular adaptive immunity, and animal studies employing potent vaccine vectors could be especially informative for T-cell immunogen design, guide vaccine vector development, and help select optimal adjuvants and route of vaccination for effective, preventive, and therapeutic HIV vaccines.

2.2 T-Cell Immunogen Design for HIV

The field of T-cell vaccines against HIV has seen a large number of different approaches for immunogen design of which several have made it to clinical testing, either in HIV-uninfected or HIV-infected individuals. In general, these approaches are either purely HIV sequence-informed strategies or based on immune data (total protein screens, optimal CTL epitopes), or they combine sequence information with immune data. Purely sequence-based strategies have been used to build immunogen sequences that contain certain regions of the viral proteome, often selected based on their specific conservation scores or viral gene expression kinetics or both. Some examples of such approaches are provided in the following. In cases where immune data have lent some guidance for immunogen design, information on CTL and CD4+ T-helper cell responses, their specificity, HLA restriction, and target conservation have generally be considered for inclusion in the immunogen sequence.

Thereby, clearly more attention has been given to targets of the CD8+ T-cell response than CD4+ cells since CD8+ CTL are considered the effector cells acting directly on the infected cells. However, as eluded above, the importance of CD4+ T-cell responses is likely not fully appreciated to date in the vaccine field, and more studies that assess CD4+ and CD8+ T-cell responses in parallel may help to refine immunogen design in the future (Swain et al. 2012). Importantly however, and regardless of whether the activities of CD4+ or CD8+ T-cells are being assessed separately, it is critical to note that natural HIV infection induces very robust and broad CD4+ and CD8+ T-cell responses across all protein subunits of the virus in the vast majority of infected individuals (Frahm et al. 2004). For CD8+ T-cell-mediated immunity, these responses generally exceed the magnitude of CTL responses to most other pathogens several folds (Bihl et al. 2005) and oftentimes appear to be driven by viral antigen availability (i.e., viral load), although this may not be generalized to all specificities (Gasser et al. 2011). Evidently though, these responses are in the majority of HIV infections unable to control viral replication. Therefore, the assessment of total HIV-specific immunity by high-throughput assays such as INF γ ELISpot assays clearly misses some critical attributes of an effective CTL response against HIV and/or measures, in addition to relevant responses, also responses that are not exerting any substantial antiviral effect. It can therefore be quite reasonably concluded that a vaccine that would contain all or most of the viral proteins would simply induce “more of the same” immune responses as natural infection does and likely not provide any advantage for viral control. Thus, the ability to discriminate beneficial from non-beneficial or even harmful, if any, CTL responses would greatly help to guide rational T-cell immunogen design. Indeed, a number of studies in the early 2000 have dissected specific T-cell response patterns to the HIV proteome by their specificity and identified CTL responses targeting the HIV Gag protein as being associated with relative control of viral load in chronic, untreated HIV infection (Zuñiga et al. 2006). In contrast, HIV envelope-specific T-cells were dominating the total virus-specific responses in individuals with elevated viral loads (Kiepiela et al. 2007), indicating that immunogen design for HIV should likely be restrictive rather than all-inclusive and that less may be more as the inclusion of additional, non-beneficial targets into a T-cell immunogen sequence may dilute the effects of T-cells targeting relevant targets. While many of the currently developed T-cell immunogen designs indeed attempt to avoid such “decoy” targets (Rolland et al. 2007), this may not always be possible. This is especially the case where the vaccine is also expected to induce B-cell immunity to viral surface proteins and the immunogen thus includes at least portion of the envelope protein. The potential drawbacks of such combined strategies need to be evaluated carefully and may require sequential immunization schedules for the induction of T- and B-cell immunity, respectively (McMichael and Koff 2014).

One example of an immunogen design that is driven by functional T-cell immune data is the HIVACAT T-cell immunogen (HTI) developed by the HIVACAT consortium in Barcelona (Mothe et al. 2011). This sequence comprises those and only those region of the viral proteome that are deemed “beneficial” since they are overproportionally frequently targeted in HIV controllers and subjects with low

viral loads when compared to individuals with elevated in vivo viral replication. These regions were identified by large-scale immune screenings of more than 1000 chronically HIV-infected, treatment-naïve subjects across 3 different continents. Of note, beneficial regions predominantly locate to Gag and Pol protein sequences but also include Vif and a short fragment of Nef (Mothe et al. 2011). At the same time, no beneficial regions were identified in HIV Env, with the potential caveat that a proportion of responses to this more variable protein sequence may have been missed by the consensus-based peptide test set used (Altfeld et al. 2003). Still, these findings are in line with a potentially detrimental effect of mounting T-cell responses to envelope, be it due to the virus being able to escape them rapidly and at little fitness costs or due to Env-specific responses preventing the induction of T-cell response to other more vulnerable regions of the virus (Altfeld and Allen 2006). Given the high proportion of beneficial regions in Gag and Pol, and although the immunogen design was not guided by sequence alignment information (such as level of conservation across clade or group M-specific sequences), the identified segments turn out to be significantly more conserved than the rest of the viral genome. These segments were also shown to induce T-cell responses of higher functional avidity, a criteria that may be a decisive characteristic of effective T-cell responses against highly variable pathogens (Mothe et al. 2012) (Yerly et al. 2008). Since the sequence design is based on screening of a large number of HIV-infected individuals and comprises segments between 13 and 78 amino acids in length, it is largely HLA independent and does not represent a “CTL epitope-string” immunogen, which often suffers limitations in regards to immunogenic content and coverage of host HLA diversity (Mothe et al. 2015). The immunogen candidate has been tested preclinically in mice and macaques (Mothe et al. 2015) and has entered human clinical trials in Europe as a therapeutic vaccine candidate.

Other immunogen designs informed by immune data often focus on strategies that use individual, well-defined CTL epitopes known to be presented by specific HLA class I molecules. In order to achieve broad human population coverage (i.e., including at least one or few epitopes for a specific HLA allele), these approaches include by necessity epitopes presented by common HLA class I alleles. As recognized more than 20 years and shown in several newer and more extensive analyses, the problem with this approach can be that dominant epitopes presented by most frequent HLA class I alleles in a given population may have mutated away and the escape mutations have become fixed in the circulating viral strains (Shankar et al. 1996) (Brander et al. 2003). This has, for instance, been described for specific epitopes presented by HLA-A24 and B51, two alleles prevalent in the Japanese population, where the respective dominant epitopes have largely been deleted from currently circulating viral isolates (Akahoshi et al. 2012; Koga et al. 2010). The observations are likely inferable to global viral evolution as HLA genetics have been found to be a strong driver of viral evolution (Kawashima et al. 2009). Thus caution needs to be taken when designing such epitope-string immunogens, and epitope-based approaches may need to prioritize the inclusion of subdominant epitopes and/or epitopes generally not targeted in natural infection (Frahm et al. 2006). Alternatively, epitope-string-based vaccines may benefit from the inclusion of epitopes presented

by multiple different HLA class I molecules (Frahm et al. 2007). In these HLA supertype-based designs, epitopes can be identified that have clear binding preferences to closely related class I alleles but which differ in the T-cell receptor usage and consequently show different CTL escape pathways depending on the presenting HLA molecule (Sidney et al. 2008) (Kloverpris et al. 2015). There are by now plenty of examples of such promiscuously binding CTL epitopes in the Los Alamos HIV immunology database (Llano et al. 2013), and their escape pathways have in many cases been well explored (Karakas et al. 2015; Carlson et al. 2015). This may open up the opportunity for novel CTL epitope-based immunogen designs that are not limited by the aforementioned restriction and could induce responses in a large proportion of HLA diverse individuals (Roman et al. 2013).

Aside from immune data-guided immunogen design, several vaccine concepts that are based entirely or predominantly on HIV sequence alignment have reached or are about to reach clinical testing. Two prominent examples of such sequence-guided immunogen designs are the conserved elements (CE) approach pioneered by Mullins and colleagues and the conserved regions approach developed by Hanke et al. (Rolland et al. 2007; Letourneau et al. 2007). In the former, focus was given on regions of HIV with highest conservation scores, and only short sequences of Gag p24 that show highest levels of sequence conservation (>98% of group M sequences) are included. This gave rise to 7 conserved elements between 12 and 25 amino acids in length (Rolland et al. 2007; Mothe et al. 2012; Kulkarni et al. 2012) that contained a multitude of HLA class I- and class II-restricted T-cell epitopes. Of note, some of these CE overlapped with defined CTL epitopes presented by HLA class I alleles associated with delayed HIV disease progression. Interestingly, a comparative analysis in HIV controllers and non-controllers that excluded subjects with these beneficial HLA alleles still showed preferential targeting of at least some of these CE in the controllers arm. These data suggest that targeting these highly conserved regions could be effective, even in the context of non-protective HLA alleles, and provides strong rational to explore the immunogenicity of the CE design in human clinical trials. In addition, the CE design has, given its focus on highly conserved segments, the great advantage that it provides broadest coverage of viral diversity globally and thus has the potential to induce T-cell immunity reactive with most viral isolates.

The second example of a HIV sequence-informed immunogen design, referred to as HIVConsV, has been developed at the University of Oxford and is based on the inclusion of 14 conserved regions in the HIV Gag, Pol, Env, and Vif protein sequences (Letourneau et al. 2007). The sequence comprises a total of close to 900 amino acids and, with regard to the aforementioned caveat regarding inclusion of Env sequences, also covers a component of envelope. It also includes additional parts of the viral proteome for which other studies have not seen a favorable association between CTL reactivity and viral loads (Mothe et al. 2011). The immunogen has been tested in several clinical trials including HIV-infected and HIV-uninfected individuals (Borthwick et al. 2017; Mutua et al. 2016) (Mothe 2016) and using different, potent vaccine vectors. Interestingly, a parallel analysis of this sequence with the above-described HTI and CE immunogen designs by Hancock et al. in

individuals that became infected after being vaccinated with the STEP vaccine raises the concern that the additional, non-beneficial regions included in HIVConsV may indeed divert some T-cell activity toward decoy targets (Hancock Plos Path 2015). In fact, individuals with broad T-cell responses to HTI showed a lower viral load and stronger in vitro viral inhibition activity than subjects with dominant responses to HIVConsV or individuals with responses to all seven CE. Interestingly, among the CE responders, the ones who targeted the segments containing the described beneficial epitopes also showed reduced in vivo viral loads and greater in vitro inhibition of viral replication (Hancock et al. 2015).

A newer version of the HIVConsV vaccine concept developed by the same group, HIVConsVx, has recently been presented and may overcome some of the concerns related to the HIVConsV (Ondondo et al. 2016). HIVConsVx aims to combine the conserved regions/elements approaches with the inclusion of functional immune data that were the bases for the HTI immunogen design. As a result, the HIVConsVx sequence is close to 900 amino acids in length and only contains regions in Gag and Pol, avoiding potential issues of envelope targets while covering many of the segments contained in the HTI sequence. Still, compared to HTI, almost half its length was not found to contain beneficial regions in the earlier studies by Mothe and could thus contain decoy targets (Mothe et al. 2011). Alternatively, these additional regions may indeed contain desirable targets, but their presence may have gone unnoticed by Mothe et al. due to reduced statistical power to identify beneficial, but subdominant, T-cell response.

In addition to these examples of sequence- or combined sequence/immune data-driven designs, several other immunogen sequences have been proposed, which cannot be all reviewed here. One of them, a short conserved region immunogen candidate by Yang and colleagues (Yang et al. 2015), also contains regions located in Gag but also in Env and Nef. It will be interesting to assess whether these Env- and Nef-derived segments represent potential decoy targets or whether they cover additional truly beneficial T-cell targets that have not been identified thus far. This may be especially interesting for segments in Nef, for which responses have generally been related to uncontrolled HIV infection. Yet, some short segments of Nef have been identified and included in the HTI sequence as well and may be present in other designs, too, providing a potentially useful stimulus. However, their identification is complicated by the strong immunodominance of Nef in natural HIV infection and the overall elevated sequence variability of this protein. In addition, Nef may not harbor many highly effective T-cell targets at all, given that Nef is dispensable for HIV infection and replication and escape from these responses may readily occur (Learmont et al. 1999).

Other immunogen concepts, many of which have been designed a number of years back and have thus already been tested in human clinical trials, include full protein sequences. While some of these approaches are limited to Gag and Pol many also include Env and Nef, raising again concerns as to the antiviral activity of some of the induced responses. While DNA/MVA or MVA-only-based delivery of some of these approaches was safe but did not induce very robust T-cell responses in vivo (Thompson et al. 2016; Gomez et al. 2011), the PENNVAX-GP immunogen,

consisting of Gag, Pol, and Env, delivered by four electroporated DNA plasmids together with a IL12 plasmid, was recently reported to be highly immunogenic in close to 100% of HIV-negative trial participants.

Finally, autologous approaches for the therapeutic vaccine setting have been developed and partly been tested in clinical trials as well. These strategies take either actual virus or viral sequence information from the infected individual to build a patient-tailored immunogen and deliver it either loaded on autologous dendritic cells or by viral and non-viral vaccine vectors. The former approach of entire viral particles has been tested in phase I and II clinical trials and has shown delayed viral rebound and lower viral load set points in individuals vaccinated with their autologous virus compared to the placebo controls (Gil et al. 2011; Garcia et al. 2013). A similar approach, where RNA-encoding autologous virus Gag, Rev, Vpr, and Nef genes were loaded onto dendritic cells, however, failed to show an antiviral effect (Jacobson et al. 2016). Additional autologous virus-based immunogen designs are currently under development, of which at least two are guided by information on the subjects HLA genotype and viral sequence polymorphisms found in the viral populations in either the peripheral blood and/or the integrated latent viral reservoir ((Papuchon et al. 2014) www.HIVACAR.org). Evidently, aside from what regions of the viral genome should be covered for these design (for instance, Gag/Pol/Rev/Tat for the HIVACAR immunogen), the success of these approaches may also hinge critically on the time point in the infection from which viral sequence information is being obtained. Since it remains unclear from what cellular source and compartment the rebounding virus originates, once cART treatment is stopped, the preference of either plasma-, tissue-, or cell-integrated viral sequences over any of the other sources is problematic, and it may be necessary to cover multiple potential virus sources. Also, while it is well accepted that the viral reservoir is seeded early in acute infection, ongoing viral evolution and longitudinal changes in immune targets may further complicate the definition of the most relevant time point for sampling and sequence analyses (Deng et al. 2015). Detailed studies in early clinical trials, using such sequence designs and employing vectors that induce a potent enough response to act on the recrudescing virus, may hopefully shed some light on these questions.

Ideally, different T-cell vaccine concepts would be tested in comparative studies so that they can inform each other and accelerate their respective refinement. However, this would increase the number of study arms needed and complicate trials significantly. Thus, clinical trials to date have been conducted in variable settings that differ in many potential cofounders, in particular, also the inclusion criteria of trial participants. Especially for therapeutic vaccine studies, patient selection may strongly impact the outcome as it can be expected that individuals diagnosed and treated during early HIV infection may respond differently to vaccination than subject treated only after prolonged chronic infection. In addition, to assess whether one or the other immunogen sequence is per se superior in inducing more effective T-cell responses would require the application of identical or at least comparable trial endpoints and immune measurements. Furthermore, immunogens should be delivered by the same vaccination route and using same vector backbones to allow objective

comparisons. Animal models may help in comparing different vaccination approaches while controlling for some of these parameters, but different immunogen designs may not translate equally from animal models to human testing, if they, for instance, include some strongly dominant CTL epitopes for the given animal species. Thus, while animal models can possibly help to eliminate the most poorly performing vaccine candidates and delivery regimens from further human trials, it is unlikely that the respective value of different vaccine strategies can be precisely evaluated before entering human clinical trial testing. As this implies later failure of some candidates and wasted investments, the one hope remains that the field will learn at least some lessons from these early-stage failures so that more promising candidates can be improved. An important analysis tool in this context will likely be the “sieve effect” analyses for T-cell immune selection pressure which may still yield a signal even if the main endpoint of the study is missed (Rolland et al. 2012). These analyses use sequence signatures in the breakthrough or rebounding virus to identify potential immune selection pressure mounted by preventive or therapeutic vaccination. Especially in the setting of therapeutic vaccination, where preexisting viral population at treatment starts and before vaccination can be compared to rebounding viral quasi-species, such analyses may provide important guidance to refine and improve the content of T-cell immunogens for HIV. Parallel functional studies may also help identify functional characteristics of vaccine-induced T-cell responses that are associated with viral control or failure of protection, further informing immunogen design and vector choice and application route.

2.3 Vaccine Vectors for T-Cell Vaccines

Aside from adequate immunogen design, one of the most important aspects of vaccination for T-cell responses is possibly the choice of a suitable vaccine vector or of multiple vectors for prime-boost regimens. Thereby, a number of vector-specific characteristics may be critical to induce responses with the desired functional profile that may be reflective of an effective T-cell response. Aside from antigen specificity, which will be defined by the immunogen insert, this may include the induction of broad T-cell responses (i.e., targeting numerous epitopes), the magnitude of these individual responses (i.e., the frequency of epitope-specific CTL), homing profiles of vaccine-induced T-cells, specific effector function profiles, the cytotoxic capacity, and possibly other parameters that have been related to relative control of established HIV infection or have gone unnoticed thus far. Likely, many of these desired attributes will only be defined empirically through clinical trials with hard immunological and virological endpoints (see below) and in which at least partially positive signals can be observed (i.e., some fraction of participants responding to vaccination with the intended outcome). On the other hand, some approximations of optimal vector design may be possible based on existing data and knowledge of antigen processing, T-cell receptor use and homing markers that could guide vector design, and their use in either preventive or therapeutic vaccination.

For instance, broad(er) CD8+ CTL responses or CD8+ T-cell responses of greater magnitude may be achieved by targeting the adequate antigen-processing pathways and antigen-presenting cells. Efficient antigen processing has been to some degree achieved with adding specific targeting sequences to the actual immunogen sequence so that endosomal or cytosolic processing machineries can be accessed. For the induction of CD8+ T-cell responses, *Escherichia coli* heat-labile toxin (EtxB) or anthrax toxin's lethal factor (LF) subunit (De Haan et al. 2002; Goletz et al. 1997) may represent such a suitable sequence, while HLA class II antigen presentation may be augmented by targeting the intracellularly expressed antigen preceded by the lysosomal-associated membrane protein 1 (LAMP1) targeting sequence (Su et al. 2002). However, if antigen presentation occurs via cross presentation on dendritic cells and other professional antigen-presenting cells (pAPC) after uptake of immunogen released by the cells containing the vaccine vector, then secretion signals such as GMCSF leader sequences may be more effective (Mothe et al. 2015) as these promote release from immunogen-expressing cells. However, although presentation of exogenous antigen is not limited to HLA class II molecules on pAPC but can occur for HLA class I epitopes as well (Brander et al. 1993; Kovacsics-Bankowski et al. 1993) (Goodridge et al. 2013), such cross presentation has been found to be suboptimal for the induction of strong CD8 T-cell responses (Sei et al. 2015). To overcome this drawback, the development of vectors with specific tropism for the intended pAPC may need to be engineered. However, targeting different APC cell types can also result in differential antigen processing and presentation of distinct set of epitopes (Dinter et al. 2015), which could profoundly impact response patterns to the immunogen and result in unfavorable immunodominance patterns and reduced antiviral efficacy.

But even if antigen processing would be largely independent of the processing preferences of the targeted APC, the vector may exert its own specific effects on further antigen processing and presentation. This has been most dramatically illustrated by the data obtained with CMV-based vaccine vectors for SIV, where Picker and colleagues used a rhesus cytomegalovirus (RhCMV) vector expressing SIV proteins and observed the induction of broad CD8+ T-cell responses restricted by MHC class II molecules (Hansen et al. 2013). This nonclassical MHC restriction was attributed to the absence of a set of specific RhCMV genes and the effect of the RhCMV homolog of the viral US11 protein, which is known to inhibit antigen presentation by HLA class I. On the other hand, a fibroblast-adapted CMV vector induced predominantly CD8+ T-cell responses of a classical MHC class I restriction (Murray et al. 2017). This example highlights the possibilities that genetic manipulation of vaccine vectors offers to steer the vaccine-induced T-cell response into the desired direction – although oftentimes the target profile of such responses still are far from being well defined. Similar modification of viral vectors has been reported for modified vaccinia virus Ankara (MVA) where the removal of specific viral antigens can change the patterns of vector and insert-specific immune responses (Sanchez-Sampedro et al. 2013; Sanchez-Sampedro et al. 2012). Furthermore, with

the advent of effective nanoparticle delivery technologies, specific targeting – and protection from degradation – can be achieved, for instance, for DNA plasmids vaccine vectors, ensuring that suitable professional antigen-presenting cells could induce a robust T-cell response (Farris et al. 2016). Thus, while some of the observed vaccine effects may depend on the immunogen insert; vector choice may have a profound impact. However, examples where the same insert has been used in human clinical trials employing different viral vaccine vectors are likely too scarce to draw consistent conclusions in this regard (Garcia-Arriaza et al. 2015).

On the other hand, it is also clear that some significant differences exist in the immune response induced by viral and non-viral vectors and between different delivery routes, even if the same immunogen is being used. These differences are generally assessed as the magnitude of responses and, less frequently, as the number of induced specificities (breadth of responses). While this is informative to assess the “immunogenicity” of the vaccine, just maximizing the sheer magnitude of the vaccine-induced response may not be the most suitable guiding principle for designing optimal vaccine regimens and prime/boost combinations that achieve maximal in vivo efficacy. In particular, it has been shown that low-dose immunization can lead to T-cell responses of markedly higher functional avidity compared to vaccine approaches that maximize antigen dose (Bullock et al. 2003; Bullock et al. 2001; Billeskov et al. 2017; Alexander-Miller et al. 1996). This may be especially relevant for the induction of potent antiviral T-cells to highly variable pathogens, such as HIV but also HCV, where viral escape from T-cell surveillance is a major challenge and where, for both infections, the functional avidity of virus-specific T-cells has been associated with superior recognition of viral sequence variants and viral control (Yerly et al. 2008; Berger et al. 2011; Mothe et al. 2012). It will therefore be interesting to see whether prime/boost regimens with a relatively weakly immunogenic prime vaccine vector will lead to responses of higher functional avidity compared to regimens where the most immunogenic vectors are already being used in the prime vaccination. While these parameters can be assessed to some degree in animal models, testing such a concept for vaccine efficacy in therapeutic HIV vaccination will require complex and costly clinical trial design with multiple arms.

An additional, critical component of any vaccine approach is vector-specific immunity and its impact on vaccine take and immunogenicity. The maybe most prominent example for the effect that preexisting vector-specific immunity can have on vaccination outcome is the STEP trial, a preventive HIV vaccine trial in HIV-uninfected individuals that used a serotype 5 human adenovirus (Ad5) vector and which was halted prematurely in 2007 due to a higher number of HIV infections in the vaccine group compared to the placebo arm (Buchbinder et al. 2008). The increased risk for HIV acquisition was especially pronounced in individuals with highest antibody titers against Ad5, raising concerns regarding the use of human adenoviruses with a high seroprevalence in the general population (D’Souza and Yang 2015). To overcome the issue of preexisting immunity to viral vaccine vectors, several groups have identified alternative human adenovirus vectors with lower

seroprevalence and have moved some of these candidates into preclinical and clinical testing (Baden et al. 2016). Still, some levels of preexisting immunity in the human population may hinder their wider application. As an alternative, several groups have explored the potential use of adenovirus vaccine vectors from nonhuman primate species, especially chimpanzees (Tatsis and Ertl 2004; Dicks et al. 2012; Colloca et al. 2012). Extensive testing of more than 1000 different chimpanzee adenovirus (ChAd) strains for existing immunity in different human populations has identified a number of potential candidate ChAd vectors, with several of them having reached human clinical trial (Colloca et al. 2012). Of these, ChAd63 encoding a conserved HIV sequence immunogen has shown great immunogenicity in chronic and early HIV-infected individuals, especially when combined with a MVA-based booster vaccination (Mutua et al. 2016) (Mothe 2016). These ChAd vectors can be produced with relative ease in well-established cell lines and at high titers so that large-scale production is feasible. What remains however poorly defined is the cross-reactivity of vaccine-induced responses to different, often closely related, ChAd vectors and how this could limit the broader application of the ChAd vaccine platform to different vaccine fields. In the case that broad cross-reactivity would be observed between different ChAd vectors, it will be important to assess the longevity of vector-specific immunity and how this could potentially interfere with the ideal interval of booster vaccinations for different pathogens. The need for such “booster” or “maintenance” vaccination will depend on the specific pathogen and clinical setting, but it is clear that in a situation where repeated vaccination over a short period of time is required, boosting of vector-specific immunity could pose a considerable problem. Keeping this issue to a minimum may possibly be achieved by the use of “maintenance vaccines” with vectors that do not induce adaptive memory immune responses all, such as DNA or RNA vectors.

These concerns surrounding vector-specific immunity and occasional concerns regarding the safety of viral vectors have strongly motivated the further development of non-viral vaccine vectors. Aside from DNA plasmid vaccines, the use of RNA-based delivery of HIV-based T-cell immunogens has made some progress. Compared to DNA plasmids, RNA vectors have the additional advantage that they cannot integrate into the host genome, further reducing safety concerns. Furthermore, their stability and immunogenicity can be dramatically increased by specific formulation and inclusion of self-replicating mechanisms, which can prolong immunogen persistence and lead to markedly stronger vaccine-specific immune responses (Ljungberg and Liljestrom 2015; Lundstrom 2016). The combination with additional RNAs to stimulate innate immune responses has been shown to further increase the magnitude of responses (Guardo et al. 2017). Such combinations of multiple RNA sequences may also help overcome potential limitations in immunogen cargo size, which can be limiting for DNA and RNA vectors and which can also negatively impact the genetic stability of viral vectors (Bett et al. 1993).

2.4 Clinical and Immune Endpoints for T-Cell Vaccines in HIV Infection

Vaccine outcome in humans for existing preventive vaccines is generally measured by determining antibody titers to the vaccine insert. This is driven by the thought that antibodies induced by these vaccines are the main effector mechanism by which these vaccines exert their protective effect. Although it is not clear that these antibody responses are in all cases the true functional correlate of immune protection, determining immunogen-specific antibody titers appears to be a sufficiently accurate measure of vaccine efficacy. The situation for T-cell vaccines in general and HIV vaccines in particular is somewhat more complicated. Especially in the therapeutic setting, where a T-cell-based vaccine may be most effective, simply measuring T-cell responses by current assays systems such as the INF γ ELISpot assay may not provide a reliable marker of vaccine efficacy. As outlined above, effector functions different from INF γ release may contribute more directly to viral control, and adequate ex vivo analyses of vaccine-induced responses should ideally capture these effector functions. A possibly more suitable test than ELISpot or the traditional intracellular cytokine analyses by flow cytometry is the measurement of vaccine-induced T-cell's ability to inhibit viral replication in vitro. This viral inhibition assay (VIA) measures virus production in autologous CD4+ T-cells in the presence or absence of virus-specific CD8+ T-cells and is currently considered a gold standard assay for measuring HIV vaccine-induced CTL responses. Although the assay may show limited sensitivity and dynamic range, it is possibly the one assay that currently measures the most relevant effector function of T-cell responses to HIV (i.e., inhibition of viral replication) in a more or less physiological manner and is thus being broadly applied to clinical trial immune monitoring.

Another, more informative endpoint for therapeutic HIV vaccination is stopping antiretroviral treatment and measuring time to viral rebound. There exists extensive literature on the most responsible and informative manner to conduct such treatment interruption (Graziani and Angel 2015), and the field has moved from more or less unsupervised analytical treatment interruptions (ATI) to closely monitored antiretroviral pauses (MAP). While the latter is clearly safer for the patient since treatment is being reinitiated upon first signs of rebounding virus, it does not allow to monitor the natural course of the rebounding viral kinetics and the possible achievement of a reduced viral set point. Still, since rebounding virus can be isolated at first sign of rebound, the MAP approach also offers to conduct detailed sieve effect analyses on the recrudescing viral population and can thus inform vaccine refinement. However, to validate any of the routine immune assays and to establish a relationship between HIV vaccination and sieve effect, clinical trials with at least some individuals responding positively to vaccination are needed. For the first time, this may have been achieved partially in the recently reported BCN-02 trial (NCT#02616874) where individuals who were treated within 6 months of their HIV infection were vaccinated with a ChAd/MVA prime-boost regimen and treated with the latency reactivating drug romidepsin (Mothe et al. 2017). The vaccinated subjects showed a

remarkable capacity to redirect the immune focus of their existing HIV-specific T-cell response toward epitopes contained in the HIVConsV immunogen sequence, effectively shifting immunodominance to potentially more effective targets (Mothe 2016). Remarkably, data presented at the 2017 annual Conference on Retroviruses and Opportunistic Infections (CROI) showed that when stopping treatment, about 35% of the individuals were able to control viral replication to levels of below 2000 copies/ml for up to 8 months (Mothe et al. 2017). Certainly, more studies like the BCN-02 trial will be needed for an iterative improvement of T-cell immunogen design, vector choice, and vaccination regimen before an effective therapeutic T-cell vaccine will be at hand. If, however, this endeavor should have a successful outcome, its use as a preventive T-cell vaccine or at least as a component of combined preventive vaccine strategies certainly deserves consideration as well.

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Chapter 3

HIV Broadly Neutralizing Antibodies: VRC01 and Beyond



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Abstract Developing an effective prophylaxis HIV-1 vaccine is likely to require the elicitation of broadly neutralizing antibodies (bnAbs). As the HIV-1 envelope (Env) glycoprotein – the sole target of bnAbs – has evolved multiple mechanisms to evade antibody neutralization, the processes for bnAb generation are highly selective and time-consuming. Benefiting from antibody isolation technologies of single B cell culturing and direct single B cell sorting and cloning, a new generation of monoclonal bnAbs has been isolated since 2009, exhibiting remarkable breadths and potencies, thus breaking through a nearly 20-year-long limit of four monoclonal bnAbs with moderate breadth and potency. The discovery of a long list of monoclonal bnAbs has provided in-depth understanding of the sites of vulnerability on the HIV-1 Env and the complexity of human B cell immunology to generate such responses, thus presenting both guidance and challenges to move the Env immunogen design effort forward.

Keywords HIV-1 · VRC01 · Monoclonal antibody · Neutralization

3.1 Introduction

The development of HIV-1 broadly neutralizing antibodies (bnAbs) in a subset of infected individuals has demonstrated the ability of the human immune system to mount effective antibody responses against the virus (Simek et al. 2009; Doria-Rose et al. 2010). As the HIV-1 envelope (Env) glycoprotein is the only viral protein expressed on the surface of the virion, bnAbs target the HIV-1 Env to block viral infection. Because HIV-1 Env has evolved a number of mechanisms to evade antibody neutralization (Wei et al. 2003; Moore et al. 2009), the processes of bnAb generation are proven highly selective and time-consuming. Nonetheless, HIV-1 bnAbs with 50% breadth are developed in half of HIV-1 chronically infected individuals (Hraber et al. 2014), supporting the feasibility of inducing a similar

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spectrum of antibody responses with optimal Env immunogens. To inform Env immunogen design and improve our understanding of human B cell immunology to mount an effective antibody response, the isolation of monoclonal bnAbs has become critical.

Though high in demand, there were only four monoclonal HIV-1 bnAbs isolated before 2009, largely due to limitations in methods for monoclonal antibody (mAb) isolation and lack of knowledge for HIV-1 bnAbs at that time. Since 2009, improved methods of single B cell culturing and direct single B cell cloning were introduced and applied to HIV-1-infected samples, leading to a major breakthrough in identifying a new generation of bnAbs with remarkable breadths and potencies. The new era of HIV-1 monoclonal bnAb discovery and understanding of these antibodies opened up new avenues for basic research in immunology, structural biology, and vaccinology, as well as in translational and clinical research for HIV-1 infection. As we contributed to the isolation of the first HIV-1 monoclonal bnAb VRC01 by single B cell sorting and cloning, I review the critical path and scientific thoughts that drove the progress of the project and eventually led to the discovery of this exciting antibody.

3.2 HIV-1 Neutralization by Polyclonal Sera

Sera or plasma profiling, as a straightforward analysis, usually takes place first by directly sampling the polyclonal antibodies in donor sera or plasma for binding specificities to antigens and for antibody functions against pathogens. Since the identification of HIV as the cause of AIDS in the early 1980s (Barre-Sinoussi et al. 1983; Gallo et al. 1983, 1984), polyclonal sera and plasma samples from HIV-infected individuals have been rigorously tested for binding specificities to various viral antigens and for activity to neutralize the virus. Initial neutralization studies used lab-adapted strains such as IIIB, MN, HXB2, and NL4-3 and found that sera and plasmas from vaccinated or infected donors potently neutralized lab-adapted strains (Mascola et al. 1994; Kovacs et al. 1993; Schwartz et al. 1993). However, subsequent studies using HIV-1 primary isolates showed high levels of resistance to sera and plasma neutralization from vaccinated and infected donors (Moore et al. 1995; Parren et al. 1998). This difference in neutralization sensitivity indicated that the Envs presented on the surface of lab-adapted strains were different from those presented on primary isolates. To categorize the neutralization sensitivity among lab-adapted strains and primary isolates, a “tiered” system (Seaman et al. 2010) was introduced with tier 1A representing the easy-to-neutralize lab-adapted strains, followed by tier 1B, then tier 2 representing the typical primary isolates, and finally tier 3 representing the most difficult-to-neutralize strains.

Tested against various panels of tier 2 strains, many chronically infected polyclonal sera and plasma samples have been ranked based on their neutralization breadths and potencies. Though most samples lacked activities to neutralize genetically diverse tier 2 strains, small fractions (10–20%) were identified with such activities (Simek et al. 2009; Doria-Rose et al. 2010). These donors have

been referred to as “broad neutralizers” and “elite neutralizers” if ranked in the top 1%. The identification of broad and elite neutralizers was exciting because they demonstrated that the human immune system can mount effective antibody responses against the virus, thus providing a scientific basis for Env immunogen design to elicit similar antibody responses to block infection. The first step to implement this strategy was to understand the antibody specificities that mediated the observed neutralization breadths, i.e., where did the antibodies bind on the HIV Env to block viral entry? As the plasma neutralization test itself did not inform antibody specificities or targets, the analyses followed were sera mapping (Walker et al. 2010; Binley et al. 2008; Gray et al. 2009). The caveat was that, because sera and plasmas are mixes of polyclonal antibodies, it has been extremely difficult to dissect complex antibody specificities and precisely map the epitopes and targets mediating neutralization. Before the isolation of monoclonal bnAbs, there had been an extensive debate on whether polyclonal antibodies or mAbs mediated neutralization breadth. To address this question and to precisely map the epitopes targeted by bnAbs, the solution was to isolate monoclonal bnAbs. For this purpose, samples from the identified broad and elite neutralizers received the highest priority and were selected for the isolation of mAbs that could account for donor’s serological activities.

3.3 mAb Isolation and the First Generation of Monoclonal bnAbs

Before 2009 only four mAbs, b12, 2G12, 2F5, and 4E10, all isolated in the early 1990s (Table 3.1), demonstrated superior neutralization spectrums over the other mAbs available at that time. For nearly 20 years, these four mAbs had been used for almost all bnAb-related basic, pre-clinical and clinical research, including epitope mapping, Env structural analysis, Env immunogen design, passive immunization in nonhuman primates (Mascola et al. 1999, 2000), and a clinical trial to treat HIV-infected individuals (Trkola et al. 2005). Because of their wide uses for such a prolonged period, the four mAbs became well known for their role in defining HIV-1 monoclonal bnAbs. Given the fact that a mAb only binds to a single epitope on the Env antigen, a single mAb may not be expected to neutralize a broad range of circulating strains. Based on a conservative expectation, the relatively narrow breadths (30–60%) and weak potencies (mean or median IC₅₀s >2 µg/ml) of the four mAbs (Walker et al. 2009) were accepted by the research community as the limit of neutralization for a single mAb. Meanwhile, the breadths and potencies displayed by a number of well characterized broadly neutralizing plasmas from HIV-1 chronically infected donors (Li et al. 2007), including the NIH donor 45 from whom VRC01 was later isolated, greatly exceeded those displayed by the four mAbs. Thus, there was a discrepancy in the observed neutralization breadths between the polyclonal plasmas and the four known monoclonal bnAbs. Because of this discrepancy, HIV neutralization breadth was thought to be mediated by polyclonal antibodies and not

Table 3.1 Summary of unique HIV-1 monoclonal bnAbs

#	mAb ID	Donor (viral clade)	Env target, B cell probe	V-genes (hypermutation)	CDR3 length (amino acids)	References
<i>First generation of bnAbs</i>						
1	b12	(B)	CD4bs ^a	VH1-3 (13%), VK3-20 (14%)	H3: 18, L3: 9	Burton et al. (1994)
2	2G12	(B)	gp120 glycan cluster	VH3-21 (21%), VK1-5 (14%)	H3: 14, L3: 9	Trkola et al. (1995)
3	2F5	(B)	MPER ^a	VH2-5 (14%), VK1-13 (12%)	H3: 22, L3: 9	Muster et al. (1993)
4	4E10	(B)	MPER	VH1-69 (14%), VK3-20 (7%)	H3: 18, L3: 9	Buchacher et al. (1994)
<i>New generation of bnAbs isolated by HIV-1 Env probes</i>						
1	VRC01	NIH45 (B)	CD4bs, RSC3	VH1-2 (32%), VK3-20 (18%)	H3: 12, L3: 5	Wu et al. (2010)
2	3BNC117	RU3 (B)	CD4bs, gp120 core	VH1-2 (26%), VK1-33 (16%)	H3: 10, L3: 5	Scheid et al. (2011)
3	12A12	IAVI57	CD4bs, gp120 core	VH1-2 (23%), VK1-33 (19%)	H3: 13, L3: 5	Scheid et al. (2011)
4	1B2530	RU1 (B)	CD4bs, gp120 core	VH1-46 (28%), VL1-47 (18%)	H3: 16, L3: 11	Scheid et al. (2011)
5	8ANC131	RU8 (B)	CD4bs, gp120 core	VH1-46 (26%), VK3-20 (19%)	H3: 16, L3: 9	Scheid et al. (2011)
6	8ANC195	RU8 (B)	gp120-gp41, gp120 core	VH1-3 (28%), VK1-5 (16%)	H3: 20, L3: 9	Scheid et al. (2011)
7	VRC-PG04	IAVI74 (AD)	CD4bs, RSC3	VH1-2 (30%), VK3-20 (19%)	H3: 14, L3: 5	Wu et al. (2011)
8	VRC-CH31	CH0219 (A)	CD4bs, RSC3	VH1-2 (24%), VK1-33 (15%)	H3: 13, L3: 5	Wu et al. (2011)
9	3 BC176	RU3 (B)	trimer, cell BaL gp140	VH1-2 (24%), VL2-23 (15%)	H3: 19, L3: 10	Klein et al. (2012)
10	VRC-PG19	IAVI23	CD4bs, RSC3	VH1-2 (23%), VL2-14 (14%)	H3: 11, L3: 5	Zhou et al. (2013)
11	VRC23	NIH-127/C (B)	CD4bs, RSC3	VH1-2 (22%), VK3-15 (15%)	H3: 12, L3: 5	Georgiev et al. (2013)
12	CH103	CH505 (C)	CD4bs, RSC3	VH4-61 (17%), VL3-1 (11%)	H3: 13, L3: 10	Liao et al. (2013)
13	VRC13	NIH44 (B)	CD4bs, RSC3	VH1-69 (34%), VL2-14 (24%)	H3: 21, L3: 6	Zhou et al. (2015)
14	VRC16	NIH-C38 (B)	CD4bs, RSC3	VH3-23 (18%), VK1-39 (19%)	H3: 20, L3: 9	Zhou et al. (2015)
15	VRC18	NIH-C38 (B)	CD4bs, RSC3	VH1-2 (27%), VK3-20 (18%)	H3: 10, L3: 5	Zhou et al. (2015)
16	VRC27	NIH-Z258 (B)	CD4bs, RSC3	VH1-2 (30%), VK1-33 (27%)	H3: 13, L3: 5	Zhou et al. (2015)
17	179NC75	EB179 (B)	CD4bs, gp120 core	VH3-21 (28%), VL3-1 (22%)	H3: 24, L3: 10	Freund et al. (2015)

(continued)

Table 3.1 (continued)

#	mAb ID	Donor (viral clade)	Env target, B cell probe	V-genes (hypermutation)	CDR3 length (amino acids)	References
18	DRVIA7	DRVI01 (B')	CD4bs, RSC3	VH1-2 (19%), VK1-5 (17%)	H3: 11, L3: 5	Kong et al. (2016a)
19	N123-VRC34	NIH-N123 (B)	gp120-gp41/FP ^a , SOSIP	VH1-2 (15%), VK1-9 (10%)	H3: 13; L3: 9	Kong et al. (2016b)
20	ACS202	D12950 (B)	gp120-gp41/FP, SOSIP	VH3-30 (16%), VK1-33 (16%)	H3: 22; L3: 9	van Gils et al. (2016)
21	PCDN-33A	IAVI-PC76 (C)	N332 supersite, gp120	VH4-34 (12%), VK3-20 (11%)	H3: 20; L3: 8	MacLeod et al. (2016)
22	N90-VRC38.01	NIH-N90 (B)	V1V2 apex, VLP ^a	VH3-13 (18%), VK2-28 (9%)	H3: 16; L3: 10	Cale et al. (2017)
<i>New generation of bnAbs isolated by B cell culture and micro-neutralization</i>						
23	PG9	IAVI24 (A)	V1V2 apex	VH3-33 (13%), VL2-14 (6%)	H3: 28, L3: 11	Walker et al. (2009)
24	CH01	CH0219 (A)	V1V2 apex	VH3-20 (13%), VK3-20 (10%)	H3: 24, L3: 9	Bonsignori et al. (2011)
25	PGT121	IAVI17 (A)	N332 supersite	VH4-59 (17%), VL3-21 (18%)	H3: 24, L3: 12	Walker et al. (2011a)
26	PGT128	IAVI36 (AG)	N332 supersite	VH4-39 (19%), VL2-8 (9%)	H3: 19, L3: 10	Walker et al. (2011a)
27	PGT135	IAVI39 (C)	N332 supersite	VH4-39 (17%), VK3-15 (16%)	H3: 18, L3: 9	Walker et al. (2011a)
28	PGT145	IAVI84 (A or D)	V1V2 apex	VH1-8 (18%), VK2-28 (16%)	H3: 31, L3: 9	Walker et al. (2011a)
29	10E8	NIH-N152 (B)	MPER	VH3-15 (21%), VL3-19 (14%)	H3: 20, L3: 12	Huang et al. (2012)
30	VRC24	NIH-N27 (B)	N332 supersite	VH4-4 (23%), VL1-15 (18%)	H3: 24, L3: 9	Georgiev et al. (2013)
31	CAP256-VRC26	CAP256 (C)	V1V2 apex	VH3-30 (14%), VL1-51 (10%)	H3: 37, L3: 12	Doria-Rose et al. (2014)
32	PGT151	IAVI31 (C)	gp120-gp41/FP	VH3-30 (20%), VK2-29 (12%)	H3: 26, L3: 9	Falkowska et al. (2014)
33	35O22	NIH-N152 (B)	gp120-gp41	VH1-28 (35%), VL2-14 (24%)	H3: 14, L3: 10	Huang et al. (2014)
34	CH235	CH505 (C)	CD4bs	VH1-46 (8%), VK3-15 (5%)	H3: 13, L3: 8	Gao et al. (2014)
35	N6	NIH-Z258 (B)	CD4bs	VH1-2 (31%), VK1-33 (25%)	H3: 13, L3: 5	Huang et al. (2016)
36	CAP248-2B	CAP248 (C)	gp120-gp41/MPER	VH4-31 (22%), VL2-14 (14%)	H3: 15, L3: 19	Wibmer et al. (2017)
<i>New generation of bnAbs isolated by other methods</i>						
37	HJ16	242315 (B)	CD4bs	VH3-30 (29%), VK4-1 (20%)	H3: 19, L3: 8	Corti et al. (2010)

^aCD4bs, CD4-binding site; MPER, membrane proximal external region; FP, fusion peptide; VLP, virus-like particle

by mAbs. This view would disfavor vaccine development strategies to target a conserved site or site of vulnerability on the HIV Env. To address the discrepancy between broadly neutralizing plasmas and few known monoclonal bnAbs, it was necessary to identify additional monoclonal bnAbs, should they exist.

Historically mAbs were isolated by phage display, Epstein-Barr virus (EBV) transformation, electrofusion, and hybridomas. Despite limitations and weaknesses, these technologies dominated the field since their discoveries in the 1970s to 1990s. Before 2009, HIV-1-specific mAbs had been isolated by a few labs that specialize in mAb isolation using these technologies. Disappointingly these technologies suffered from low efficiency when applied to HIV-1-infected samples. Another limiting factor was the mAb screening process that was not based on virus neutralization but instead was based on binding affinities to gp120 monomers, gp120 peptides, or gp41 peptides, thus further reducing the number of bnAbs yielded from these efforts. Despite numbering only four, the discoveries of monoclonal bnAbs still provided tremendous knowledge about HIV-1 neutralization. For example, epitope mapping indicated that b12 targets the CD4-binding site (CD4bs) on gp120, that 2G12 targets a cluster of glycans on gp120, and that 2F5 and 4E10 each binds to a peptide in the membrane-proximal external region (MPER) of gp41. Therefore, we learned that the CD4bs, a cluster of gp120 glycans, and MPER are sites of vulnerability on the HIV-1 Env. Additionally, passive immunizations using a single or combinations among the four mAbs demonstrated protection in nonhuman primate models (Mascola et al. 1999, 2000), thus providing a basis for developing Env immunogens aiming to elicit similar bnAbs. The four monoclonal bnAbs also provided antibody sequences that hinted at common genetic features of HIV-1 bnAbs such as high levels of somatic hypermutation and relatively long heavy chain CDR3 lengths. Nonetheless, after intensive research on the four mAbs for such a long period of time, there was an increasing urgency to identify additional monoclonal bnAbs to verify and address critical questions such as: (1) Were the four monoclonal bnAbs generalizable to other infected donors? If so, could similar mAbs be identified from those donors? (2) Were there mAbs that account for neutralization breadths and potencies observed in donor plasmas? (3) Was HIV-1 neutralization breadth mediated by polyclonal antibodies or mAbs?

3.4 Sera Mapping and the CD4-Binding Site (CD4bs)

If HIV-1 neutralization breadth were mediated by polyclonal antibodies and not by mAbs, searches for monoclonal bnAbs would likely fail. Though the chance of success was small, there had been sera mapping data strongly supporting the presence of monoclonal bnAbs in some chronically infected donors. Using antibody adsorption and elution from gp120 proteins with and without a point mutation D368R that knocks out CD4 binding, Li et al. mapped the neutralizing specificities of two broadly reactive sera, the NIH donors 1 and 45, to the CD4bs (Li et al. 2007). The fractionated plasmas were tested for neutralization against four HIV-1 strains, with

similar results obtained. Therefore, antibodies bound to the D368 site neutralized at least four genetically distant strains, supporting an antibody specificity that targeted the conserved D368 residue and mediated neutralization against different strains.

The rationale for sera mapping studies focusing on the CD4bs was based on the fact that HIV depends on CD4 to initiate viral entry and infection; thus the CD4bs must be functionally conserved. Structurally the CD4bs has been defined at the atomic level in a liganded complex of a gp120 core bound with a 2-domain soluble CD4, along with a mAb 17b antigen-binding fragment (Fab) directed at the co-receptor binding site (Kwong et al. 1998). The crystal structure revealed three domains of gp120 core, an inner domain, an outer domain, and a bridging sheet that connects the two. The gp120 residues in contact with CD4 were discontinuous and spread out in all three domains but were more concentrated in the outer domain. Notably the spans of 365–371, later termed the CD4-binding loop, and 425–430 at β 20–21, as part of the bridging sheet, contributed 57% of the total CD4 contact area on gp120 (Kwong et al. 1998). The residue D368 in the middle of the CD4-binding loop was identified as one of the key contact residues for CD4 interaction, and the D368R mutation specifically knocked out CD4 binding. Sera mapping using gp120 proteins with and without the D368R point mutation indicated that novel antibodies to this site were elicited in some HIV-1-infected individuals, and exposure of this conserved site to memory B cells in these individuals might probe the B cells expressing such antibodies. The CD4bs-targeting monoclonal bnAb b12 (Burton et al. 1994) also supported the CD4bs as a site of vulnerability.

3.5 b12

The mAb b12 was isolated in the year 1994 by phage display using PBMCs from an HIV-1 clade B-infected individual (Burton et al. 1994). The phage display library lost antibody heavy and light chain pairing information and produced Fabs with randomly paired heavy and light chains, thus capturing antibodies not “naturally” produced in donor plasmas. This had raised questions and criticism in the field. Moreover, panning of phage display libraries relied on monomeric gp120 binding. Hence, the phage display effort largely yielded mAbs that were capable of binding to monomeric gp120 but not necessarily neutralizing the virus. Nonetheless, mAb b12 exhibited an overall 35% neutralization breadth against cross-clade HIV strains and a preferential 58% breadth against clade B strains (Walker et al. 2009). Competition ELISA with CD4-Ig indicated that b12 efficiently competed with CD4 to bind to gp120, suggesting that the b12 epitope overlaps with the CD4bs (Moore and Sodroski 1996).

The b12 Fab atomic level crystal structure in complex with HXB2 core was solved in 2007, revealing that only the b12 heavy chain interacted with gp120 and that the vast majority of b12 epitope was on the outer domain (Zhou et al. 2007). The b12 heavy chain CDR1, CDR2, and CDR3 “grasped” all around the CD4-binding loop, making direct contact with each of the 10 consecutive residues from 364 to 373. As

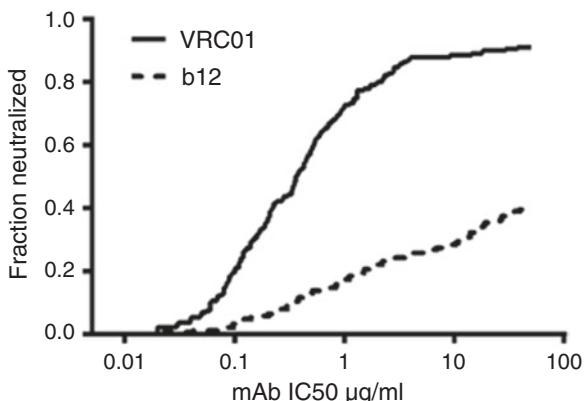
expected, the D368R mutation in the CD4-binding loop knocked out b12 binding. Though both primarily targeted the CD4-binding loop, CD4 only formed contacts with one side (six residues), yet b12 grasped on both sides (all ten residues) of the CD4-binding loop (Zhou et al. 2007). Therefore, b12-escape mutations commonly arose on the four residues dispensable for CD4 interaction in the CD4-binding loop (Wu et al. 2009). When the footprints of b12 and CD4 were superimposed on gp120 core, they overlapped primarily on the outer domain, defining an appealing target that was functionally conserved, structurally stable, and antibody accessible. Therefore, new designs of gp120 core proteins to specifically present this region of gp120 to memory B cells in chronically infected donors might probe the B cells expressing antibodies targeting this region. With a powerful single B cell sorting and cloning platform introduced to the field (Tiller et al. 2008; Scheid et al. 2009), new opportunities for functional mAb discovery appeared on the horizon particularly suited to the quest of identifying novel monoclonal bnAbs against HIV.

3.6 VRC01 and Its Class

The platform of single B cell sorting and cloning was first used to recover influenza-specific mAbs from the plasmablasts of vaccinated individuals (Wrammert et al. 2008) and had not been applied to HIV-infected samples before 2009. With PBMCs from broad neutralizers and a Yu2 gp140 foldon trimer protein readily available, the single B cell sorting platform was first applied and attempted to isolate novel monoclonal bnAbs in 2009. Using the Yu2 gp140 as bait, Scheid et al. processed PBMC samples from four broad neutralizers, including the NIH donor 45, and recovered more than 500 gp140-binding mAbs (Scheid et al. 2009). However, none of the isolated mAbs was broadly neutralizing against HIV-1. It was unclear at that time why the recovered gp140-binding mAbs were not broadly neutralizing, and this result would have supported the view that polyclonal antibodies but not mAbs mediate HIV-1 neutralization breadth. Though inexperienced, our group decided to test the single B cell sorting and cloning platform in our hands using a modified gp120 core as bait.

As mentioned above, the overlapping footprint of b12 and CD4 on gp120 core revealed the portion of CD4bs on the outer domain to be functionally conserved, structurally stable, and antibody accessible; thus new designs of gp120 core proteins to specifically present this region might specifically probe the B cells expressing antibodies targeting this region. With designs from William Schief at the Scripps Institute, Yang and colleagues at the Vaccine Research Center expressed and tested a series of proteins that preserved the part of CD4bs on the outer domain but altered the rest of the protein surface to non-HIV-1 (Wu et al. 2010). These modified gp120 core proteins were termed resurfaced stabilized core (RSC) proteins. Among the expressed RSC proteins, RSC3 performed the best in retaining b12 binding but reducing binding to other non-neutralizing mAbs. Because the inner domain and bridging sheet were altered, RSC3 lost stable binding to CD4, thus abrogating CD4

Fig. 3.1 Neutralization breadth (y-axis) is plotted at the corresponding potency (x-axis) for VRC01 and b12 using the IC50 data from Wu et al. 2010 against a total of 190 HIV-1 Env-pseudotyped viruses representing strains circulating globally



interference. Using RSC3 along with a negative control Δ RSC3, which deleted a single residue I371 in the CD4-binding loop, we stained a PBMC sample from the NIH donor 45 and sorted individual IgG-expressing B cells that stained RSC3 + Δ RSC3-. From the sorted individual B cells, we recovered three monoclonal bnAbs, VRC01, VRC02, and VRC03, which belonged to the same B cell lineage and same class of bnAbs – the VRC01-class – and recapitulated the RSC3 + Δ RSC3-binding profile (Wu et al. 2010).

Because the prototype mAb for RSC3 design was b12, we compared the gp120 binding and viral neutralization profiles of VRC01 to b12 (Wu et al. 2010). VRC01 clearly targeted the CD4bs, displaying all known binding characteristics of mAbs directed to the CD4bs, including efficient competition with CD4-Ig and b12 and reduced binding with the D368R mutation. Importantly, VRC01 exhibited superior neutralization breadth and potency than b12 (Fig. 3.1), reaching an overall breadth of 90% with a geometric mean IC50 of 0.33 µg/ml against global circulating strains. Furthermore, VRC01 accounted for a major fraction of the neutralization activities measured in the donor plasma (Wu et al. 2010). Therefore the isolation of VRC01 supported the presence of highly conserved sites of vulnerability on the HIV-1 Env and that neutralization breadths observed in the donor plasma were in a large part mediated by mAbs. Crystal structure analysis of the VRC01 Fab in complex with gp120 core revealed that the VRC01 epitope overlapped with CD4 on the outer domain (Zhou et al. 2010), precisely targeting the site that RSC3 was designed to expose to B cells. Therefore, the RSC3 bait and VRC01 isolation demonstrated an example of successful protein design and engineering. The atomic structure also indicated that the heavy chain CDR2 of VRC01 partially mimicked the CD4 interaction with gp120, forming contacts with only one side of the CD4-binding loop, thus avoiding escape mutations arising from the other side of the CD4-binding loop, a strategy exploited by the virus to escape b12. This mimicry of CD4 by VRC01 partially explained its broader neutralization spectrum than that of b12. Later studies determined that the virus mainly mutates gp120 loop D and V5 to escape VRC01 neutralization (Li et al. 2011).

Following the successful isolation of VRC01 by single B cell sorting and cloning with a specific Env bait, more HIV-1 monoclonal bnAbs have been isolated using similar methods. From the B cells sorted with Yu2 gp140, re-PCRs with improved primers yielded VRC01-class of monoclonal bnAbs (Scheid et al. 2011), indicating that the PCR primers used in the first study missed these highly mutated bnAbs because the original primers did not account for possible mutations at the start of the mAb-coding region. To date the VRC01-class of bnAbs has been isolated from more than 10 donors (Table 3.1), rendering it a category of antibody response generalizable across individuals.

In addition to the VRC01-class, six other classes of bnAbs have been identified that target the CD4bs as exemplified by mAbs HJ16, 8ANC131, CH103, VRC13, VRC16, and 179NC75 (Table 3.1). A collection of crystal structures of the CD4bs-targeting bnAbs in complex with gp120 cores revealed substantially overlapping epitopes and different modes of gp120 recognition (Zhou et al. 2013; Zhou et al. 2015), with the VRC01-class and 8ANC131-class partially mimicking the CD4 interaction with gp120. Though the CD4bs itself is not glycosylated, it is surrounded by glycans, and the bnAbs targeting this site all avoided or accommodated glycans to reach their epitopes. Also, because the CD4bs of gp120 is always readily available to interact with CD4, the Env trimer packaging and conformational changes have minimal impact on bnAbs that target this site. However, this is not the case for non-neutralizing mAbs. Since current vaccines induced non-neutralizing mAbs to the CD4bs, it will be important to consider and address how to modify and adjust the mode or angle of antibody binding to gp120 in Env immunogen design.

3.7 Antibody Genetic Composition and Next-Generation Sequencing (NGS)

Because each unique B cell receptor has its own VDJ recombined composition, bnAb nucleotide sequences are essential to establish the corresponding B cell lineages. For mAbs identified by single B cell cloning, their variable region nucleotide sequences are obtained for paired heavy and light chains. As illustrated in Fig. 3.2 using heavy chain as an example, mAbs are usually characterized by their V-gene usage, somatic hypermutation, and CDR3 length. While the antibody's primary antigen interacting regions CDR1 and CDR2 are coded within the V-gene, its CDR3 is composed of VD junction, D gene, DJ junction, and part of the J gene. Thus, CDR3 is the most diverse region of the antibody. The VRC01-class of bnAbs from multiple donors shared a sequence signature of IGHV1-2 gene usage, high levels of somatic hypermutation, and a short light chain CDR3 length of five amino acids (Zhou et al. 2013; West et al. 2012). This distinct sequence signature has been used to select naïve B cells with the potential to evolve and mature to become the VRC01-class of bnAbs (Jardine et al. 2016).

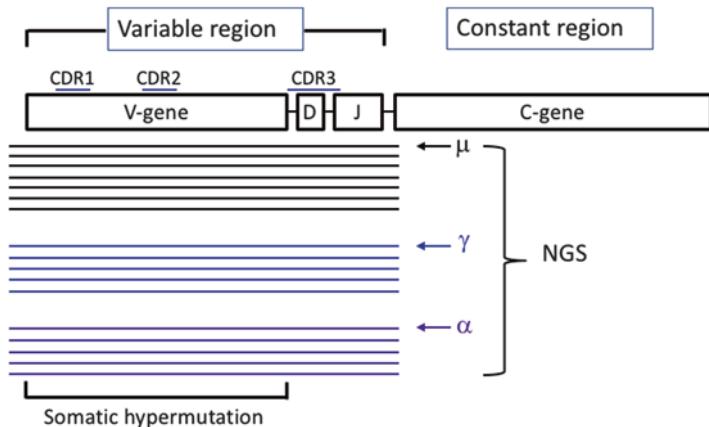


Fig. 3.2 A schematic of the gene structure for antibody heavy chain showing characteristic elements of V-gene usage, somatic hypermutation, and CDR3. For repertoire studies using next-generation sequencing (NGS), reverse primers annealing to the start of the constant region distinguish between μ chain for IgM, γ chain for IgG, and α chain for IgA

As bnAbs are part of the donor antibody repertoire, the availability of bnAb sequences has inspired and stimulated antibody repertoire analyses by next-generation sequencing (NGS) including IgM, IgG, and IgA (Fig. 3.2) in HIV-1-infected and HIV-1-uninfected donors (Chen et al. 2012; Xiao et al. 2013; Yin et al. 2013; Zhang et al. 2013; He et al. 2014). The NGS data from longitudinal samples of the same donor also allowed for tracking bnAb lineages (Wu et al. 2015; Doria-Rose et al. 2014; Bonsignori et al. 2016; van Gils et al. 2016; MacLeod et al. 2016). However, a caveat to the currently available sequencing platforms is the loss of paired heavy and light chains, and thus antibodies cannot be reconstituted with naturally paired heavy and light chains based on the NGS data alone. Though not yet readily available, new sequencing platforms are being developed to address this issue (DeKosky et al. 2013, 2015). As of today, NGS combining with single B cell sorting and sequencing, which maintains the heavy and light chain pairing information, is still the most practical and comprehensive system to study B cell lineages of interest.

3.8 Other HIV-1 bnAbs

Pioneered by the Burton group using individual B cell cultures with micro-neutralization screening to isolate monoclonal bnAbs PG9 and PG16 (Walker et al. 2009), a similar system has been applied by other laboratories to isolate many more HIV-1 bnAbs targeting Env regions outside of the CD4bs. Along with single B cell sorting using soluble or cellular gp140 trimer and virus-like particle (VLP) baits (Table 3.1), HIV-1 bnAbs targeting Env regions outside of CD4bs have

also been isolated by the single B cell sorting and cloning platform. HIV-1 bnAbs have been extensively reviewed in the literature (Mascola and Haynes 2013; Kwong and Mascola 2012; Kwong et al. 2013; Wu and Kong 2016). Briefly, there are currently seven known categories of bnAbs targeting different regions of the HIV-1 Env. From the Env trimer apex to gp41, known sites of vulnerability include (1) the V1 V2 apex targeted by PG9, CAP256-VRC26, PGT145, N90-VRC38, and others; (2) a gp120 glycan cluster targeted by 2G12; (3) the V3 base glycans targeted by PGT121, PGT128, and others; (4) the CD4bs targeted by VRC01 and others; (5) the gp120 and gp41 interface targeted by 35O22 and others; (6) the fusion peptide targeted by PGT151, VRC34, and ACS202; and (7) the MPER targeted by 10E8, 2F5, and 4E10. These bnAbs play a central role in informing and guiding HIV-1 Env immunogen designs, including defining conserved sites of vulnerability on HIV-1 Env and validating Env immunogens for proper presentation of intact bnAb epitopes. Although still a work in progress, sequential Env variants that evolved during the natural course of infection and stimulated bnAb responses are being identified and cloned (Gao et al. 2014; Bhiman et al. 2015; MacLeod et al. 2016), and the identification of these Envs would rely on the identification of both autologous and heterologous neutralizing antibodies. Additional studies have focused on longitudinal analyses to track bnAb lineages and delineate their maturation pathways (Doria-Rose et al. 2014; Bonsignori et al. 2016; van Gils et al. 2016; MacLeod et al. 2016). As single B cell sorting and cloning has been increasingly included in post-immune analyses (Sundling et al. 2012, 2014; Navis et al. 2014), a relatively complete list of bnAb genetic compositions has been highly informative regarding whether similar bnAb precursors have been engaged by the tested immunizations. The broadest and most potent bnAbs have also been high in demand for research in passive immunization and in HIV-1 treatment and cure.

3.9 Conclusion

With 41 unique bnAbs isolated to date (Table 3.1) that define 7 general categories of effective targets on the HIV-1 Env, current research has moved on to unveil the mechanisms of human B cell responses that produce bnAbs and to investigate the entire bnAb maturation pathway starting from naïve B cell engagement. The pathways for bnAb generation remain mysterious because current knowledge of basic B cell immunology has been gained primarily from mouse studies, yet mouse may not possess the proper B cell repertoire to generate similar highly functional antibodies against HIV (Hu et al. 2015). Since SHIV-infected nonhuman primates have demonstrated the ability to produce HIV-1 bnAbs (Jia et al. 2016; Walker et al. 2011b; Shingai et al. 2012), emphasis on nonhuman primate models may hold promise to elucidate details of HIV-1 bnAb production *in vivo* and fill gaps of knowledge in this regard.

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Chapter 4

Structural Features of Broadly Neutralizing Antibodies and Rational Design of Vaccine



Tongqing Zhou and Kai Xu

Abstract Despite sequence diversity, glycosylation, and conformational flexibility of the human immunodeficiency virus type 1 (HIV-1) envelope (Env), antibodies that neutralize diverse HIV-1 strains develop in selected HIV-1-infected individuals. The application of single B cell-based approaches has identified many broad and potent human antibodies from infected donors. Structural studies on antibody recognition of HIV Env have revealed that these broadly reactive antibodies target epitopes covering entire exposed and glycosylated surface on the viral spike; several classes of antibodies recognize the viral spike with converged modes. Critical structural features, such as antibody mimicry of cellular receptors, enable effective HIV-1 neutralization. However, other structural and genetic features, such as long CDR H3, fixed length of CDR L3, restricted germline usage, and high rate of somatic hypermutation, may explain the difficulties in eliciting these antibodies by vaccination. Accumulating information on antibody recognition of HIV-1 Env and ontogenesis suggests distinct pathways for generating effective HIV-1 vaccine based on specific antibody ontogeny or specific target site.

Keywords HIV-1 · Antibody · Structure · Neutralization · Vaccine · Lineage-based design · Epitope-based design

4.1 Introduction

The envelope spike (Env) on the surface of the HIV-1 virion is composed of trimers of heterodimers formed by two subunits, gp120 and gp41, and is the sole target of neutralizing antibodies. The infection process of HIV-1 starts when gp120 binds to the receptor CD4 and co-receptors CCR5 or CXCR4 on host cell; the conformational change induced by gp120 interaction with receptors triggers the

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gp41-mediated membrane fusion between the virus and host cell. Natural HIV-1 infection elicits robust antibody responses to both gp120 and gp41. These Env-directed responses do not contribute significantly to viral control and are predominantly non-neutralizing or strain-specific antibodies. However, the selection pressure imposed by these antibodies does promote mutagenesis and hence diversity in the HIV-1 Env that outpaces the developing antibody-mediated immune response (Bar et al. 2012; Gray et al. 2007; Mikell et al. 2011; Wei et al. 2003).

Aside from sequence diversity, several other mechanisms, especially extensive glycosylation and conformational masking of epitopes, enable HIV-1 to evade the humoral responses. Structural definition of the glycans on the surface of HIV-1 Env reveals the glycan shield protrudes 20-Å above the protein surface and covers most of the Env surface, including the membrane-proximal gp41, the site of CD4 binding, and the variable loops (Stewart-Jones et al. 2016). Molecular dynamics and glycan array analysis indicated that glycan shield impedes antibody access to protein epitopes (Stewart-Jones et al. 2016). Moreover, mutagenesis can cause addition, deletion, and shift of potential N-linked glycosylation sites that alter the glycan shield (Sagar et al. 2006; Wei et al. 2003; Zhou et al. 2018). The “self” feature of glycans on the Env glycoproteins also acts as camouflage to hide the HIV-1 Env from human immune system.

The trimeric HIV-1 Env is metastable and adopts different conformational states. Structural definition of the HIV-1 trimeric Env in its pre-fusion state by crystallographic and cryo-electron microscopy (cryo-EM) (Julien et al. 2013a; Lyumkis et al. 2013; Pancera et al. 2014; Bartesaghi et al. 2013) revealed how gp120 and gp41 interact and a quaternary arrangement of the V1V2 and V3 loops at the apex of the trimer that is different from conformation in the CD4-bound state (Kwong et al. 1998; Ozorowski et al. 2017). Certain functionally conserved sites on HIV-1 Env are conformationally masked (Kwong et al. 2002). For example, upon binding of receptor CD4 to the initial CD4-binding site on the outer domain of gp120, the bridging sheet is formed by antiparallel β -strands from the base of V1V2 loop and β 20-21 which uncovers the co-receptor-binding site that is otherwise absent in the pre-fusion closed state. Therefore, HIV-1 utilizes conformational masking to disguise important sites from antibody recognition.

Despite multiple ways of immune evasion, high titers of cross-reactive neutralizing activity arise in some individuals after 2 to 4 years of HIV-1 infection (Doria-Rose et al. 2009; Gray et al. 2011; Mikell et al. 2011; Sather et al. 2009; Moore et al. 2009). Mapping analysis demonstrates that these broadly reactive neutralizing sera contained antibodies targeting several regions of the HIV-1 Env, including the CD4-binding site on gp120, the glycan-containing regions on the surface of gp120, and the membrane-proximal external region of gp41 (Walker et al. 2010; Binley et al. 2008; Li et al. 2007, 2009; Tomaras et al. 2011; Gray et al. 2009). Although there were only four isolated monoclonal antibodies, including b12 to the CD4-binding site, 2F5 and 4E10 to the membrane-proximal external region (MPER), and 2G12 to a high-mannose glycan cluster, that neutralized diverse primary strains of HIV-1

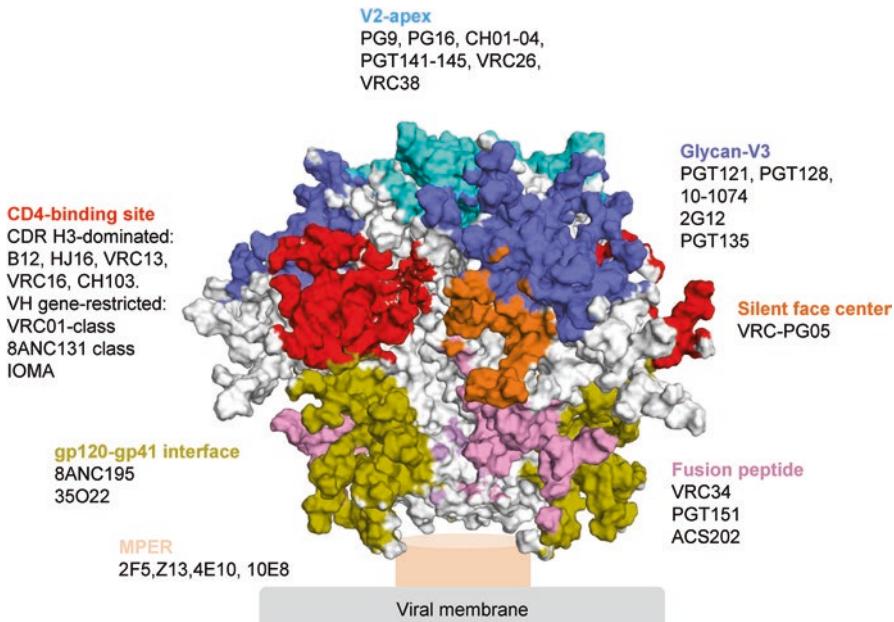


Fig. 4.1 Major sites of vulnerability to neutralizing antibodies on the pre-fusion closed HIV-1 Env trimer. The target sites for CD4-binding site antibodies, the V1V2 apex antibodies, the glycan V3 antibodies, the fusion peptide-targeting antibodies, the gp120-gp41 interface antibodies, and the MPER antibodies are colored on the trimeric HIV-1 Env in its pre-fusion state. The epitope for VRC-PG05 targeting the center of the silent face is also shown in orange

before 2009 (Burton et al. 1994; Muster et al. 1994; Stiegler et al. 2001; Trkola et al. 1996), it proved in principle that human immune system can generate potent neutralizing antibodies against the HIV-1 Env.

Several methodological advances brought the breakthrough in isolation of broadly neutralizing HIV-1 mAbs. Among them, single cell antibody cloning techniques, which amplify genes of antibody variable regions from single B cells, allowed recovery and expression of antigen-specific mAbs (Tiller et al. 2008; Wardemann et al. 2003). Development of high-throughput microculture method enabled direct neutralization screening with secreted IgG from small numbers of B cells; antibody heavy and light chains from positive cells could then be recovered by single B cell cloning (Walker et al. 2009). Structure-based design of engineered protein probes made it possible to identify and sort epitope-specific B cells (Wu et al. 2010). Starting with the identification of mAbs PG9 and PG16 by the microculture method and the isolation of VRC01 by the probe-based method, dozens of broad and potent monoclonal antibodies have been cloned from infected individuals. The neutralizing antibodies can be categorized into five major groups based on their dominant target sites: (1) the CD4-binding site, (2) V1V2 apex, (3) glycan V3, (4) fusion peptide (FP), (5) gp120-gp41 interface, and (6) MPER (Fig. 4.1).

4.2 Antibodies Targeting the CD4-Binding Site

The CD4-binding site on HIV-1 gp120, which mediates the initial step of virus-host interaction, is functionally conserved for efficient association with CD4 receptor and is vulnerable to neutralizing antibodies to HIV-1 (Zhou et al. 2007; Chen et al. 2009). Even though the CD4-binding site on gp120 is recessed and optimized to bind the single-headed Ig-like CD4 (Kwong et al. 1998), the human immune system nonetheless found ways to target this site with the dual-headed immunoglobulin with paired heavy and light chains. One class of these antibodies, prototyped by VRC01 and derived from the VH1-2 germline, was isolated from ten donors (Scheid et al. 2011; Wu et al. 2010, 2011; Zhou et al. 2010; Georgiev et al. 2013; Huang et al. 2016; Zhou et al. 2015; Wu et al. 2015). A second class of these antibodies, prototyped by 8ANC131 and derived from the VH1-46 germline, was isolated from three donors (Bonsignori et al. 2016; Scheid et al. 2011). Additional CD4-binding site antibodies were isolated from multiple donors, however, each with a germline origin that is different from VH1-2 or VH1-46.

Structural studies indicated that VRC01-class antibodies bind gp120 with VH domains mimicking domain 1 of CD4, with a ~40° rotation and 6-Å shift from the CD4-defined orientation to accommodate the pairing light chain. The interaction is dominated by the heavy-chain second complementarity-determining region (CDR H2) by providing half of the paratope surface. Both VRC01-class antibodies and CD4 use their C' strands to engage the CD4-binding loop on gp120. Moreover, the detailed chemistry of key interaction between CD4 and gp120 was also mirrored: The salt bridges between Asp368 in the CD4-binding loop of gp120 and Arg59 of CD4 were mimicked by Arg71 of VRC01-class antibodies. In some of the VRC01-class antibodies, such as VRC03, VRC-PG20, 12A21, VRC23, VRC27, and N6, a Phe, His, Arg, or Tyr residue at position 54 was used to contact with the gp120 bridging sheet region in a manner analogous to a critical Phe43 of CD4 (Zhou et al. 2015). Mutation of the Gly54 in NIH45–46 and VRC07 to a Trp or His could enhance the antibody potency (Diskin et al. 2011). Even though the light chains of VRC01-class antibodies were of different origins, all VRC01-class antibodies have 5-amino acid-long CDR L3 with a conserved Glu/Gln at position 96 (West et al. 2012; Zhou et al. 2010, 2015) (Table 4.1). The shorter than usual length is possibly restricted by the size of the void formed by CDR H3, CDR L1, N-term of the light chain, and gp120 loop D and V5, where CDR L3 is located. In addition, CDR L1 of VRC01-class antibodies is relatively short or containing glycine residues to avoid clashes with gp120 (Zhou et al. 2013; Diskin et al. 2011). Antibodyomics data from multiple donors indicated that the 5-amino acid CDR L3 were selected from specific V-J recombination, while the deletion or glycine mutations were from affinity maturation process (Zhou et al. 2013).

Crystal structures of 8ANC131, 8ANC134, 1B2530, and CH235.12 in complex with HIV-1 gp120 also indicated CD4 mimicry for these VH1-46-derived antibodies (Wu et al. 2015; Bonsignori et al. 2016). Similar to VRC01-class antibodies, CDR H2 of these 8ANC131-class antibodies dominate the interaction with gp120

Table 4.1 Structural features of antibodies targeting major sites on the HIV-1 pre-fusion closed Env

Target site	Class	Antibody features	Antigen features
CD4BS	VRC01	Germline HV1-2*02	Epitope on multiple gp120 elements, not dependent on glycan
		Heavy chain mimics CD4 domain 1	
		5-amino acid CDR L3	
		Indel or glycine in CDR L1	
	8ANC131	Germline HV1-46*02	Epitope on multiple gp120 elements, not dependent on glycan
		Heavy chain mimics CD4 domain 1	
	IOMA	Germline HV1-2*02, heavy chain mimics CD4 domain 1, normal CDR L3, no indel in CDR L1	Epitope on multiple gp120 elements
	HJ16 VRC13 VRC16	Different germlines, CDR H3-dominated interaction, key resides mimic CD4	Epitope on multiple gp120 elements
V1V2 apex	PGT145 PGDM1400 BG1 PG9 PG16	Protruding long electronegative CDR H3, each HIV Env binds one antibody	Electropositive sink and glycan N160 ring on quaternary epitope
	VRC38	Normal CDR H3, use side chain-side chain contacts, varied stoichiometry	Required quaternary epitope
V3-glycan	PGT121 PGT128 PGT135	Use two protruding CDR loops to interact with glycan and peptide	N332 and nearby glycan cluster, V3 loop is part of epitope
Fusion peptide	VRC34	CDRs form shallow hydrophobic groove and CDRs L1 and H3 form salt bridge with N-term amine of fusion peptide	Conformationally flexible 8 N-term residues of fusion peptide, need full cleavage of gp160, and involves glycan
gp120-gp41 interface	8ANC195 35O22 3 BC176 3 BC315	Normal length CDRs without protruding loops	Epitope at gp120-gp41 interface, involves glycans N88, N230, N241, N276 with different dependency
MPER	10E8 4E10	Trp at the tip of CDRH3 extends into the viral lipid membrane	Linear peptide in helix form, special orientation relative to viral membrane, epitope may involve membrane

(Table 4.1). Even though the binding angles of VH1-46-derived antibodies relative to that of CD4 are different from the VH1-2-derived antibodies due to the longer CDR L3 (9–11 amino acids), the salt bridges with gp120 Asp368 are conserved by Arg71, and in antibody 1B2530, Arg54 engages the gp120 in a manner similar to Phe43 of CD4 and VRC01-class antibody VRC23.

Like the VH1-46-derived antibodies, VH1-2 germline can also be paired with light chain with conventional CDR L3 in length. IOMA, a new VH1-2-derived antibody, was isolated containing normal 8-amino acid CDR L3 with 50% breadth(Gristick et al. 2016). Crystal structure in complex with BG505 SOSIP indicated that IOMA binds to gp120 in an orientation slightly different from the orientations of both VH1-2/VRC01-class and VH1-46/8ANC131-class bNAbs; however, key CD4-mimetic features, such as the Arg71 salt bridges with gp120 Asp368 and CDRH2 mimicking the C" strand of CD4, are retained. In addition, W100F in IOMA CDR H3 preserved the interactions with gp120 Asn279 and Asn280 observed for VRC01-class CDR H3 W100B. The longer CDRL3 of IOMA is accommodated by a shift in the gp120 V5 loop to allow IOMA's CDR L3 to penetrate the groove between the D and V5 loops, with Asp93 interacting Asn280 and Arg456 from both D and V5 loops (Table 4.1).

Unlike VH1-2- and VH1-46-derived CD4-binding site antibodies which use CDR H2-dominated interactions to mimic domain 1 of CD4, structures of antibodies from germlines other than VH1-2 or VH1-46 (CH103, VRC13, VRC16, and HJ16) revealed antibody interfaces dominated by canonical CDR H3 (Liao et al. 2013b; Zhou et al. 2015), which explains the low preference for specific germline V gene usage. Using long CDRs to reach into the recessed CD4-binding site, antigen-binding fragments of these antibodies mimic the overall approaching angle to gp120 of CD4 domains 1 and 2. Furthermore, the salt bridges to gp120 Asp368 were conserved through Arg96 for VRC13, His53 for VRC16, and Arg97 for CH103 (Zhou et al. 2015). Tyr100E of VRC16 is oriented almost identically to that of CD4 Phe43 (Zhou et al. 2015). Thus, the CDR H3 dominated antibodies, with known breadth over 80%, also mimic the mode of CD4-gp120 interaction in a way that is different from those derived from VH1-2 and VH1-46 germlines (Table 4.1).

4.3 The V1V2 Apex Antibodies

The V1V2 site is another major site of vulnerability to neutralizing antibodies. Structures of HIV-1 Env in the pre-fusion closed state showed that V1, V2, and V3 from three protomers form the trimer apex at the membrane-distal portion of the spike (Julien et al. 2013a; Pancera et al. 2014). Structure of fully glycosylated HIV-1 Env trimers revealed glycans form a canopy 20 Å above protein surface and the apex N160 glycan branches interact and assemble into an interprotomeric oligomannose ring around the threefold axis. V1V2 undergoes significant conformational changes upon binding CD4 (Ozorowski et al. 2017). Many antibodies targeting this conformation dependent site, for example, PG9 and PG16 (Walker et al. 2009), CH01-CH04 (Bonsignori et al. 2011), PGT141-PGT145 (Walker et al. 2011), PGDM1400 (Sok et al. 2014), CAP256-VRC26.01–12 (Doria-Rose et al. 2014), PCT64 (Landais et al. 2017), BG1 (Wang et al. 2017), and VRC38 (Cale et al. 2017), were isolated from multiple donors and showed dependence on or preference for quaternary epitopes. Of these antibodies, PGDM1400 showed exceptional

breadth (83%) and potency ($IC_{50} = 0.003 \mu\text{g/ml}$). Crystal structures revealed both PG9 and CH03 use hammerhead-like CDR H3 loops to interact with glycans N160 and N156 and a peptide of the V2 domain (McLellan et al. 2011; Gorman et al. 2016). EM structure showed PG9, CAP256-VRC26.09, PGT145, PGDM1400, and PCT64 bind the trimer with a stoichiometry of 1:1. PG9, CH03, and PCG64 bind the apex adjacent to the threefold axis at a slightly off-vertical angle and contact two gp120 monomers of the HIV-1 trimer (Julien et al. 2013b; Doria-Rose et al. 2014; Landais et al. 2017; Lee et al. 2017), whereas PGT145 and PGDM1400 bind along the threefold axis with the long electronegative CDR H3 loops penetrating the glycan ring and the electropositive sink at the trimer apex. One striking feature for most V1V2-targeting antibodies is the unusually long protruding loops, with the longest being the 37-residue CDRH3 of VRC26.09 (Doria-Rose et al. 2014). However, antibody with shorter CDRH3 loop can also infiltrate HIV's Env apex. VRC38.01, which has a non-protruding 16-residue HCDR3, recognizes the V2 apex by making side chain-side chain contacts (Cale et al. 2017). EM 2D class averages of VRC38.01 Fab-trimer complexes indicated variable stoichiometry with one or two Fabs per trimer and a footprint away from the threefold axis (Cale et al. 2017) (Table 4.1).

4.4 The Glycan V3 Antibodies

The region around the highly conserved glycan N332 on gp120 forms a third site of vulnerability to neutralizing antibodies. This site includes the base of the gp120 V3 loop, glycan N332, and a cluster of nearby glycans on the outer domain of gp120. Antibodies targeting the glycan V3 site include different families derived from different germline lineages, mainly the PGT121-123, PGT133-134, and 10–1074 lineage from the IGHV4-59*07 germline and the PGT125-128, PGT130-131, and PGT135-137 lineages from the IGHV4-39*01 germline (Julien et al. 2013c; Kong et al. 2013; Mouquet et al. 2012; Pejchal et al. 2011; Walker et al. 2011). Crystal structures of PGT128 in complex with gp120 outer domain containing “mini-V3” showed its CDR H2 and CDR H3 penetrate the glycan shield and recognize two conserved glycans at N332 and N301 as well as a short β -strand segment of the gp120 V3 loop that contains the GDIR motif (Pejchal et al. 2011). Crystal structure of PGT135 in complex with gp120 core revealed that PGT135 interacts with three glycans at Asn332, Asn392, and Asn386, using two long CDR loops (H1 and H3) to penetrate the glycan shield and access the gp120 protein surface (Kong et al. 2013). The binding mode for the PGT121/10–1074 clonal family was provided by the structure of clonal variant PGT122 in complex with BG505 SOSIP.664 gp140 trimer (Julien et al. 2013a; Julien et al. 2013c; Pancera et al. 2014). PGT122 interacted with glycan N322 and a different set of glycans at Asn137, Asn156, Asn301 and protein components of the V1 and V3 loops. Both CDRH3 and CDR L2 of PGT122 contact glycan N332 which sits at the junction of heavy and light chains. The cleft between CDR H3 and H2 interact with glycan N137; in addition, light-chain

framework region 3 contacted glycan N301 that has a significant effect on PGT122 neutralization. While most of the glycan V3-directed broadly neutralizing antibodies recognize glycan N332 and surrounding glycans and protein components, antibody 2G12 recognizes only N-linked glycans at N295, N332, and N395 in a unique domain-swapped architecture that combines both Fab arms into a single, large antigen-binding (Fab)2 unit (Calarese et al. 2003; Doores et al. 2010). Derived from different germline lineages, the glycan V3 bNabs use protruding CDR loops to penetrate the glycan shield to interact with glycan and protein components surrounding the N332 glycan, however, in diverse ways (Table 4.1).

4.5 Fusion Peptide Targeting Antibodies

The fusion peptide (FP) is thought to be sequestered. However, the isolation and structural solution of antibody VRC34 have defined the FP as a site of vulnerability to neutralizing antibodies (Kong et al. 2016). VRC34.01 has a medium somatic hypermutation frequency (15% VH and 10% VK on nucleotide) and ordinary length of CDR H3 (13 residues). The epitope of VRC34 comprises the linear first 8 residues of FP, locating on the N-terminus of gp41 subunit, and the adjacent glycan N88, locating on the gp120 subunit. The hydrophobic 8 residues on FP, adopted in an extended conformation, were embedded in a shallow hydrophobic groove spanning most of the complementarity-determining regions of VRC34, contributing significantly to the binding energy. It is worth noting that the free amine group on the N-terminus of FP forms salt bridges with two negatively charged residues on CDRs L1 and H3, stabilizing the interaction. Most of the rest of the binding energy is contributed by the recognition of glycan N88. The recognition of glycan N88 is mediated by the interaction between the base N-acetylglucosamine moieties and the two tyrosine residues located on CDRs L1, L2, and H3 of VRC34.

Other antibodies that target the FP include PGT151 (Blattner et al. 2014), which has a slightly higher somatic hypermutation frequency (20% VH and 12% VK on nucleotide) and much longer CDR H3 (28 residues) than VRC34.01. PGT151 forms extensive contact network with the FP, the surrounding residues, and the glycans. The long CDR H3 were able to extend deeply into the FP area and contact with FP residues spanning from 512 to 522. The N-terminal 4 residues of FP form antiparallel β -sheet with CDR H3 of PGT151. The fucosylation in the glycan core of N611 and N637 has been reported to be the essential part of PGT151 epitope. It is not clear whether two other glycans in close proximity, N240 and N488, contribute positively to PGT151 binding.

4.6 gp120-gp41 Interface-Targeting Antibodies

Antibodies from different donors have been isolated that target the interface of gp120 and gp41 on the pre-fusion closed HIV-1 Env (Table 4.1). One of these is 8ANC195 which neutralizes 66% of viruses in a diverse viral panel. Initial mapping of its epitope indicated it targets a site that is distinct from any known supersites (Scheid et al. 2011). Crystal structure in complex with gp120 indicated that 8ANC195 recognizes gp120 exclusively with its heavy chain, and the epitope involves two glycans N234 and N276 and protein components including the gp120 inner domain, loop D, and loop V5 with glycans providing two thirds of the interface (Scharf et al. 2014). Binding to glycan N234 is provided by CDRH2 and somatically mutated framework residues. It is of note that a somatic two-residue deletion at the CDR H2–framework 3 boundary is needed to permit these interactions which would otherwise clash with inner domain residue Asn234 and its neighbors. Binding to glycan N276 involves framework residues and the N-terminal portion of CDRH1 which forms an interface involving only the core pentasaccharide common to both high-mannose and complex-type N-glycans, allowing 8ANC195 to recognize both complex-type and high-mannose glycans at position 276. Further study using EM suggested that light-chain CDRL1 and CDRL2 of 8ANC195 may contact gp41 (Scharf et al. 2014).

Derived from IGHV-1-18*02- and IGLV-2-14*02 germline genes, antibody 35O22, isolated from the same donor N152 of antibody MPER 10E8, is highly somatically mutated in variable genes of both heavy chain (35%) and λ light chain (24%) with 62% breadth (Huang et al. 2014). N88A, N230A, N241A, and N625A mutations diminish neutralization potency, suggesting the requirement of glycans and elements of both gp120 and gp41 for 35O22 recognition of the HIV-1 Env. Crystal structure of 35O22 in complex with BG505.SOSIP.664 revealed that heavy-chain CDRs of 35O22 form extensive contacts with the N-linked glycan N88, while the heavy-chain framework 3 region interacts with strand β 1 of the 7-stranded inner domain sandwich of gp120. Heavy-chain CDR3 and light-chain framework 3 of 35O22 also interact with the α 9 helix and glycan N618 of gp41 (Pancera et al. 2014).

Additional interface antibodies, 3BC176 and 3BC315, were isolated from the same donor of the VRC01-class antibody 3BNC117. Crystal structures of 3BC315 and 3BC176 Fabs and Cryo-EM structures in complex with BG505 SOSIP.664 reveal that the 3BC315 and 3BC176 epitopes are very similar and situated at the interface between two gp41 subunits (Lee et al. 2015). Unlike 35O22, 3BC315 and 3BC176 recognition of trimer does not require glycan N88 on gp120.

4.7 The MPER Antibodies

The gp41 MPER is a critical component of the viral entry process (Buzon et al. 2010; Weissenhorn et al. 1997). Several antibodies recognizing this region have been isolated (Huang et al. 2012; Zhu et al. 2011; Zwick et al. 2005; Nelson et al. 2007). Among them, antibodies 4E10 and 10E8, which were identified by the microculture approach, can neutralize 90% and 98% of tested viruses, respectively. Crystal structures of MPER antibody complexes indicated that antibodies 2F5, Z13e1, 4E10, or 10E8 recognize different conformations of the MPER. 2F5 and Z13e1 recognize non-helical conformations of the MPER (Ofek et al. 2004; Pejchal et al. 2009). In contrast, 4E10 and 10E8 recognize residues 672–683 of MPER folded into an α -helix right before the transmembrane-spanning region (starting at residue 684) (Cardoso et al. 2005; Huang et al. 2012). Crystal structures of Fab in complex with phospholipid headgroups (Irimia et al. 2016; Irimia et al. 2017) indicate antibodies 10E8 and 4E10 to recognize both MPER and membrane with the indole ring of Trp100c at the tip of CDRH3 in 10E8 extending into the viral lipid membrane. Several light-chain basic residues are in the same plane with the lipid headgroup (Irimia et al. 2016, 2017). Consistently, a number of other HIV-1 broadly neutralizing antibodies have been proposed to interact with viral membrane, including antibodies 2F5 (Ofek et al. 2014), CAP248-2B (Wibmer et al. 2017), and DH511 (Williams et al. 2017). A Trp-substitution at the tip of CDR H3 at position 100c in 10E8v4 and at position 100a in 4E10 showed increased level of binding to MPER over that of 10E8v4 and 4E10 in the membrane context (Kwon et al. 2018). Modelling indicated that the MPER assumes a perpendicular orientation to the virion membrane during 10E8 neutralization of HIV-1 (Irimia et al. 2016). Recent cryo-EM of 10E8 in complex with JR-FL Δ CT Env suggested that non-MPER sites on the HIV Env may also contribute to binding of 10E8 (Lee et al. 2016) (Table 4.1).

4.8 Implication for Vaccine Development

Antibodies that target and neutralize diverse HIV-1 isolates have been isolated in naturally infected HIV donors. Information on modes of antibody recognition and pathways of antibody development can inform vaccine design that aims to re-elicit antibody responses. Structural and biochemical studies have revealed that the epitopes of these antibodies essentially cover all exposed surfaces on the pre-fusion closed Env trimer which nonetheless cluster into several major “supersites” of vulnerability (Zhou et al. 2018). Moreover, antibody lineages repeatedly elicited in multiple donors have been identified. To re-elicit the antibodies by vaccination, vaccine designs can either focus on common sites that are targeted by diverse antibodies, namely, epitope-based design, or focus on certain antibody lineages that repeatedly elicited in multiple donors, namely, lineage-based design. Special structural features for different class of antibodies on their recognition of the HIV Env

implies special considerations should be taken for vaccine design (Table 4.1). Regardless of the design approaches, the process starts by designing immunogens to increase immunogenicity of target sites or to properly trigger the putative germline, followed by rational boosting to guide the maturation of antibodies, and finishing with immunogens that closely mimic native viral spike to guide the development of antibodies for neutralization of diverse HIV-1 strains (Fig. 4.2).

Multi-donor analysis revealed several classes of antibodies isolated from different donors share the same germline genes, for example, the VH1-2-derived VRC01-class antibodies, the VH1-46-derived 8ANC131-class antibodies, and the VH4-59-derived PGT121-class antibodies (Table 4.2), suggesting the elicitation of these antibodies is reproducible. To re-elicit the same antibody responses by immunization, lineage-based designs by germline-targeting is one potential strategy (Fig. 4.2). This approach needs to develop specifically designed molecules with sufficient affinities to germline versions of the target antibodies. For the VRC01-class antibodies, an engineered germline-targeting gp120 domain (eOD-GT8 60mer) can bind and activate VRC01-class precursors in a germline-reverted VRC01 H-chain knock-in mouse model (Jardine et al. 2015). For PGT121-class antibodies targeting the glycan V3 supersite, wild-type Env glycoproteins also lack detectable affinity-related germline antibodies. A structure-guided directed evolution approach using mammalian cell surface display was developed to design stabilized Env trimers with gradient affinity for germline-reverted and mature versions of PGT121-class antibodies. PGT121-like responses were induced in PGT121 inferred-germline knock-in mice (Escolano et al. 2016).

However, lineage-based designs can be complicated by many other unusual characteristics of broadly neutralizing antibodies, such as the 5-amino acid CDR L3 for VRC01-class antibodies and the long and rare CDR H3 for the V2 apex antibodies, that require special V-D-J or V-J recombination. An alternative approach is the epitope-based designs which focus on the super epitopes of antibodies and implement designs that increase the immunogenicity of target site to elicit antibodies regardless of germline origin (Fig. 4.2). For example, the CD4-binding site is functionally conserved for receptor binding and is a target not only for antibodies derived from VH1-2 or VH1-46 germlines but also for other antibodies using conventional CDR H3 recognition, such as VRC13 and CH103. Molecular dynamics analysis indicates glycans impede antibody accessibility to the CD4-binding site that is evolutionarily optimize for access by CD4 with single Ig-like domain. Epitope-focusing by strategical removal of glycans around the CD4-binding site exponentially increased the antibody titers to the CD4-binding site (Zhou et al. 2017).

Fusion peptide (FP), a site of HIV vulnerability illustrated by two bNAbs epitope and the essential part of the viral entry machinery, represents a unique target for vaccine development that may benefit from the epitope-based approach (Kong et al. 2016; van Gils et al. 2016). The linear fusion peptide can adopt multiple conformations, allowing recognition by antibodies in different modes, which potentially increase the chance of antibody elicitation. For the epitope-focusing strategy, multimerization by conjugating the fusion peptide onto carriers such as KLH may be beneficial for focusing the antibody response to fusion peptide in the initial

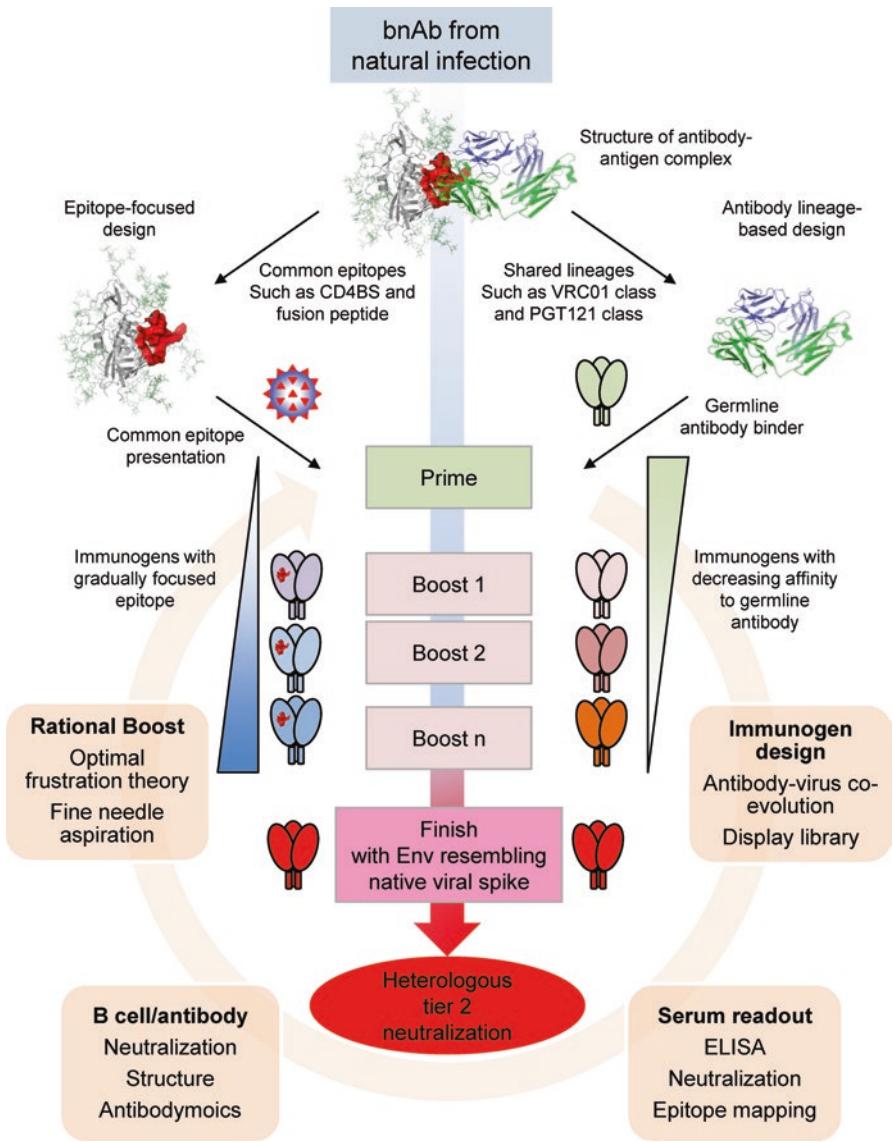


Fig. 4.2 Scheme of structure-based vaccine design. The process starts with understanding of antibody responses from natural infection. Information on modes of antibody recognition and pathways of antibody development inform vaccine design that aims to re-elicit antibody responses. Designs can either focus on certain antibody lineages that repeatedly elicited in multiple donors or focus on common sites that targeted by diverse antibodies. Serial immunization steps involving prime, boot, and finish with designed immunogens are required to achieve neutralization of heterologous tier viruses. A feedback system (peach color) that informs rational boosting for guiding the maturation of neutralizing antibodies is critical. Studies on antibody-virus coevolution can provide clues on the special features that enable the maturation of natural antibodies. Library-based selection of immunogens can accurately control the affinity of boosting immunogens to the BCR at different stages of immunization. Theory-based immunogen selection, which balances the selection and mutation pressure with optimal frustration on B cells to be boosted, can aid the development breadth. In combination, techniques for B cell readout, such as fine-needle aspiration, antibody isolation, and structural and genomic characterization of developing antibodies, provide essential feedback on iterative design process

Table 4.2 Features of classes of antibodies that share common germline genes

Target/class	Representative lineage	Common germline	Special features
CD4BS VH1-2-derived VRC01 class	VRC01	HV1-2*02	High somatic hypermutation rate 5-amino acid CDR L3 In CDR L3, third position hydrophobic, fourth position E/Q CDR L1 deletion or polyglycine mutation
	VRC-PG04		
	VRC-CH31		
	VRC-PG20		
	3BNC117		
	12A12		
	VRC23		
	VRC18b		
	N6		
CD4BS VH1-46-derived	1B2530	HV1-46*02	High somatic hypermutation rate Variable CDR L3
	8ANC131		
	CH235.12		
Glycan V3	PGT121-123	HV4-59*07	High somatic hypermutation rate Glycan dependent
	10-1074		
	PGT133-134		
	PGT125-128	HV4-39*01	High somatic hypermutation rate Glycan dependent
	PGT130-131		
	PGT135-137		

immunization. However, due to the limited surface area of the short peptide, the interaction between antibody and fusion peptide may not be tight enough to prevent the Env protein from conformational change. Therefore, additional engagement of antibody to the fusion peptide-surrounding region in Env protein would likely render tighter binding and higher neutralization potency. The FP surrounding region comprises a mixture of conserved and strain-specific components. To elicit bNAbs, immunogens and immunization strategies need to be carefully designed to minimally presenting the strain-specific components. On the other hand, the conserved glycans, such as glycan N88, N611, and N637, in a proper format, can be included into the immunogens. The immunogen design can use two strategies, either ontogeny-based or site-focusing. For the ontogeny-based strategy, it is more convenient to follow VRC34.01 development, which undergoes less SHM and gene editing. The experimental or bioinformatics analysis of VRC34.01 developmental pathway hence is important to carry out the ontogeny-based vaccine design. For the site-focusing strategy, sequentially immunization of FP immunogens in various formats may be beneficial for focusing the antibody response to FP and its surrounding conserved areas. FP immunogens in a multiple-valency format would be necessary in the initial immunization to enhance its immunogenicity, while FP immunogens in the native trimer context would be suitable for the later boost to guide the antibody maturation toward the native conformation on virus.

Most of the broadly neutralizing HIV-1 antibodies have high somatic mutation rates resulting from multiple encounters with Env variants during natural infection, implying the need of rational boosts in the immunization setup after priming.

Boost immunogens with gradient affinity for germline-reverted and mature antibodies can be selected from display libraries expressing stabilized Env trimers (Steichen et al. 2016). Studies of antibody-virus coevolution can also inform the selection of boost immunogens by following the virus strains at critical points along the pathway to bNAbs from an infected individual (Liao et al. 2013a; Bonsignori et al. 2016; Doria-Rose et al. 2014; Bonsignori et al. 2017). It is also important to incorporate the principle of optimal frustration into design and selection of boost immunogens, which balance the selection and mutation pressure on BCR and may lead to maximal breadth (Wang et al. 2015; Shaffer et al. 2016). The finishing step involves boosts with HIV-1 Env that closely resemble the native state on the virion (Fig. 4.2). However, it is yet to be determined if new state other than the state represented by BG505.SOSIP exists and if it will be more appropriate for boosting certain types of antibody response.

Lastly, an evaluation system with feedback for iterative design is critical (Fig. 4.2). For example, vaccine-relevant animal models are also critical in developing bNAb-based vaccines with iterative optimization processes. Antibody lineage knock-in and humanized mouse models have been shown to provide informative feedbacks on critical parameters, such as germline precursor frequency, affinity of immunogens, and adjuvants and prime-boost strategies (Abbott et al. 2018; Dosenovic et al. 2015; Escolano et al. 2016; Sok et al. 2016; McGuire et al. 2013; Tian et al. 2016). Protocols combining conventional readouts, such as serum ELISA or neutralizing titers with new techniques, such as fine-needle aspiration (Pauthner et al. 2017), B cell immortalization, monoclonal antibody isolation, next generation sequencing of B cell repertoire, and structural characterization of elicited antibodies, will be important contributors to iterative vaccine design process.

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Chapter 5

Monkey Models and HIV Vaccine Research



Zhiwei Chen

Abstract Since the discovery of acquired immunodeficiency syndrome (AIDS) in 1981, it has been extremely difficult to develop an effective vaccine or a therapeutic cure despite over 36 years of global efforts. One of the major reasons is due to the lack of an immune-competent animal model that supports live human immunodeficiency virus (HIV) infection and disease progression such that vaccine-induced correlates of protection and efficacy can be determined clearly before human trials. Nevertheless, rhesus macaques infected with simian immunodeficiency virus (SIV) and chimeric simian human immunodeficiency virus (SHIV) have served as invaluable models not only for understanding AIDS pathogenesis but also for studying HIV vaccine and cure. In this chapter, therefore, we summarize major scientific evidence generated in these models since the beginning of the AIDS pandemic. Hopefully, the accumulated knowledge and lessons contributed by thousands of scientists will be useful in promoting the search of an ultimate solution to end HIV/AIDS.

Keywords SIV · HIV · SHIV · AIDS · Vaccine · Model · Nonhuman primate

5.1 Introduction

Since the discovery of the acquired immunodeficiency syndrome (AIDS) in 1981 and its causative agent human immunodeficiency virus (HIV) in 1983, an effective AIDS vaccine remains to be discovered. Due to the lack of a preventive vaccine, HIV/AIDS pandemic continues to grow and has resulted in about 40 million deaths due to AIDS-related illness, while another 36.7 million people are currently living with HIV (PLHIV) according to the recent global statistics from the UNAIDS in 2016. In the past 36 years, the search for an effective AIDS vaccine has always

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been a top priority among the HIV/AIDS research community. Besides issues related to HIV genetic diversity, unknown immune correlates of protection, and limited resources for vaccine clinical trials, the lack of a proper HIV-infected animal model has also seriously halted AIDS vaccine discovery. Although HIV originated probably from the simian immunodeficiency virus (SIV) found among West-Central African nonhuman primates through cross-species (zoonotic) transmissions (Sharp and Hahn 2011), HIV does not establish persistent infection and AIDS among any nonhuman primate (NHP) species tested except for chimpanzees (*Pan troglodytes troglodytes*). Unfortunately, as an endangered animal species, chimpanzees are not readily available for AIDS vaccine research. Moreover, chimpanzees chronically infected with HIV often show undetectable viral loads in plasma and poor disease progression to AIDS (Letvin 1998), making them unsuitable for vaccine evaluation.

Despite genetic differences between HIV and SIV, NHP models infected with SIV have made tremendous contributions to understanding HIV/AIDS pathogenesis, transmission, and therapeutic interventions. SIV first isolated from the Asian rhesus macaques (*Macaca mulatta*) with lymphoma showed similar morphology, growth characteristics, and antigenic properties to HIV (Daniel et al. 1985). Because macaques experimentally infected with purified SIV displayed AIDS-like symptoms such as body weight loss, opportunistic infections, and CD4 cell count drop, this model provided not only scientific evidence that the virus indeed causes AIDS but also acted as a useful tool for the study of vaccines (Letvin et al. 1985). Interestingly, since immune sera induced by HIV envelope glycoprotein gp120 in goat, horse, and rhesus monkeys neutralized the viral infectivity in vitro, the gp120 subunit AIDS vaccine was suggested to be theoretically possible (Robey et al. 1986). Moreover, rhesus monkeys immunized with purified, disrupted, noninfectious SIV generated weak neutralizing antibodies yet allowed complete protection among two of six vaccinated monkeys (Desrosiers et al. 1989). Meantime, immunization with a formalin-inactivated whole SIV vaccine potentiated with an adjuvant resulted in the protection of eight out of nine rhesus monkeys (Murphrey-Corb et al. 1989). These results provided the initial hope that vaccine-induced immunity against HIV infection might be possible. Obviously, this hope has yet to become a reality due to complex lentiviral diversity, host genetics, and HIV transmissibility. In this chapter, we focus on lessons learnt on NHP models for AIDS vaccine development so that the knowledge can be used to promote the discovery of an effective HIV vaccine.

5.2 Evolutional Relationship Between HIV and SIV

AIDS in humans is caused by two types of lentiviruses, namely, HIV-1 and HIV-2. While HIV-1 has already resulted in the AIDS pandemic with estimated 80 million infections, HIV-2 remains primarily endemic in West African countries. Based on the phylogenetic analysis, both HIV-1 and HIV-2 are likely the consequences of

multiple cross-species transmissions of SIVs in naturally infected African NHP species (Sharp and Hahn 2011). Apparently, the primate reservoir of HIV-2 was identified as the African sooty mangabey (*Cercocebus torquatus atys*) because closely related SIVsm strains were isolated from this monkey species not only in captivity in the United States but also among household pets and wild animals in West African countries including Liberia and Sierra Leone (Fultz et al. 1989; Marx et al. 1991; Chen et al. 1996). In particular, one SIV in a sooty mangabey and one HIV-2 in a human subject were found from the same village in West Africa (Chen et al. 1996). These findings, therefore, support the hypothesis that HIV-2 subtypes in West Africans probably originated from widely divergent SIVsm strains, transmitted by independent cross-species events in the same geographic and endemic locations.

Similarly, the first chimpanzee SIV was isolated from wild-born animals found in Gabon with positive antibody responses to HIV-1 antigens (Peeters et al. 1989). In a search for the primate reservoir of HIV-1, additional SIVcpz strains were found in chimpanzee subspecies in Africa, the central *Pan troglodytes troglodytes* (*P. t. troglodytes*) and the eastern *P. t. schweinfurthii* (Gao et al. 1999). Since the natural range of *P. t. troglodytes* coincides with the endemic areas of HIV-1 groups M, N, and O, this animal species was likely the primary reservoir for HIV-1. However, the virus most closely related to HIV-1 at that time was a SIV identified in members of the chimpanzee subspecies *P. t. troglodytes* held in American captivity. Interestingly, this missing gap was subsequently filled in after SIVcpz nucleic acids were recovered in fecal samples from free-ranging *P. t. troglodytes* in southern Cameroon, which establishes distinct, geographically isolated chimpanzee subspecies as the possible origins of pandemic (group M) and nonpandemic (group N) HIV-1 (Keele et al. 2006).

The discovery of HIV-1 origins enhances the investigation of immune responses in natural host species, which provides useful information for AIDS vaccine development. To date, more than 40 different NHP species have been naturally infected with genetically distinct SIV strains. Since these entire NHP species were found in Africa but not in Asia, it was possible that SIV emerged after the physical separation of African and Asian Old World monkeys around 6–10 million years ago (Sharp and Hahn 2011). After the introduction of SIV into African monkeys and apes, the virus has evidently undergone a tremendous evolution. As a result of the virus and host coevolution, SIV genetic diversity has reached over 40% and does not commonly cause AIDS in their natural hosts despite active viral replication. For example, the majority of SIV-infected sooty mangabeys remain disease free for more than 24 years. Thus far, only one reported case developed classic AIDS after an 18-year incubation of a natural SIVsm infection (Ling et al. 2004). Most infected sooty mangabeys had relatively high set-point viral loads but lacked aberrant immune activation and AIDS during chronic SIV infection (Pandrea et al. 2009). Sooty mangabeys showed substantially reduced levels of innate immune activation because their plasmacytoid dendritic cells (pDCs) produced markedly less interferon-alpha in response to SIV infection (Mandl et al. 2008). Furthermore, low CC-chemokine receptor 5 (CCR5) expression on sooty mangabey CD4⁺ T cells

might promote an AIDS-free status by protecting central memory CD4⁺ T cells from direct SIV infection (Paiardini et al. 2011). The low immune activation despite high viremia and low CCR5 expression on long-lived central memory CD4⁺ T cells as a possible nonpathogenetic mechanism evolved over thousands of years in sooty mangabeys naturally infected with SIV, which may model HIV-infected nonprogressor cohorts (Muenchhoff et al. 2016). SIV infection in African natural hosts such as sooty mangabeys, African green monkeys, and mandrills, therefore, has become a critical NHP model for the investigation of immune correlates of protection useful for immunological interventions against HIV infection (Sodora et al. 2009).

5.3 SIV-Infected Asian Macaques as Models for HIV/AIDS

Unlike African natural hosts, Asian macaques infected with SIV or molecularly cloned SIV commonly develop simian AIDS with clinical signs similar to AIDS in humans (Letvin et al. 1983; Kestler et al. 1990). Since Asian macaques have been regularly bred in captivity without issues of endangerment, they have been extensively used for modeling HIV/AIDS and, therefore, have greatly improved our understanding of viral transmission, pathogenesis, latency, antiviral therapy, and vaccine development. Thus far, three species of Asian macaques have been used for HIV vaccine research, including rhesus macaques (*Macaca mulatta*), pig-tailed macaques (*Macaca nemestrina*) and cynomolgus macaques (*Macaca fascicularis*) (Baroncelli et al. 2008). We indicated previously that SIV strains isolated from these Asian macaques, namely, SIVmac and SIVmne, originated from SIVsm through cross-species transmission similar to the situation of HIV-2 (Chen et al. 1996). Recently, by tracking historical records and stored specimens, both rhesus and pig-tailed macaque models of HIV/AIDS are the results of the accidental transmission of SIV from sooty mangabey carriers to naive Asian macaques during the course of human kuru experimental transmission studies at the University of California, Davis, during the 1960s (Gardner 2016). These studies led to the discovery of infectious prions but inadvertently transmitted SIVmac and SIVmne, unknown at that time, from African sooty mangabeys to two Asian macaque species.

SIV infection in Asia-derived rhesus macaques not only causes simian AIDS experimentally but also shares many biological properties with HIV. First, we reported that primary SIV strains mainly use CCR5 as the co-receptor for entry into CD4⁺ T cells (Marx and Chen 1998; Chen et al. 1997). Second, SIV infection in NHP models is characterized by massive and rapid depletion of memory CD4⁺ T cells in mucosa-associated lymphoid tissues (Veazey et al. 1998). Third, SIV infects not only activated and proliferating T cells but also resting T cells to establish similar latent reservoir (Reece et al. 2014). Like HIV, plasma set-point viral load after the acute SIV infection predicts the progression of simian AIDS. Fourth, acute SIV infection in NHP models resolves with the onset of antigen-specific immune responses. However, SIV utilizes similar evasion mechanisms to escape from host

immune surveillance (Roederer et al. 2014). Fifth, using single-genome amplification and a model of random virus evolution, early plasma viral sequences coalesce to transmitted/founder viruses during SIVmac251 or SIVsmE660 infections (Keele et al. 2009). In addition, SIV integration has similar preference to HIV-1 for actively transcribed regions in the genome but not for the CpG islands or transcription start sites, which may impact on viral life cycle and latency establishment (Crise et al. 2005). These findings validate the SIV/macaque models for HIV-1 vaccine and immunotherapy research.

SIV infection has been mainly established in two subspecies of Asian *Macaca mulatta* as NHP models for HIV/AIDS research. One is rhesus macaque of Indian origin (IRM). The other is rhesus macaque of Chinese origin (CRM). Since IRMs were readily available at American primate centers when SIVmac was isolated, SIV/IRM has been the most extensively studied NHP model. However, after the supply of IRMs became limited, more studies have used SIV/CRM as an alternative NHP model since 2001. The differences between IRM and CRM in physiology, viral fitness, immunological responses, and genetic backgrounds have improved our knowledge to model HIV infection in diverse human populations. Interestingly, when compared with IRM, SIVmac pathogenesis in CRM was closer to HIV-1 infections in untreated adult humans, suggesting some advantages over the rapid and highly pathogenic SIV/IRM model (Ling et al. 2002). After in vivo passage and adaptation of SIVmac in CRM, however, rapid progressors to simian AIDS were also found among infected CRMs (Burdo et al. 2005; Wei et al. 2016). Besides viral adaptation, host factors might affect SIVmac pathogenesis in rhesus macaques, which affects vaccine studies.

5.4 Host Factors in Governing Immune Responses in SIV-Infected Asian Macaques

Rhesus MHC expression influences the susceptibility and disease progression of Asian macaques to SIV infection. Similar to human leukocyte antigen (HLA) genes (human MHC), rhesus MHC genes express multiple predominant *Mamu-A* and *Mamu-B*, corresponding to *HLA-A* and *HLA-B*, respectively, but not to *HLA-C*. Normally, one *Mamu-A* and three B locus genes are highly expressed, which are different in gene combination and copy number compared with human HLA (Otting et al. 2005). IRM and CRM share 70% *Mamu-A* and *Mamu-B* haplotypes in terms of MHC-I diversity (Karl et al. 2013). The presence of *Mamu-A*01* allele mainly in IRM is closely related with lower viral load, enhanced restriction of viral replication, and preservation of CD4⁺ T cells during SIVmac infection (Pal et al. 2002; Mothe et al. 2003). *Mamu-B*08* and *Mamu-B*17* alleles are significantly over-presented among elite controllers, macaques that are able to control SIV viral load below 1000 RNA copies per ml without antiviral treatment (Yant et al. 2006; Loffredo et al. 2007). In particular, *Mamu-B*17* is associated with reduced plasma viral load by 26-fold in an analysis of 181 SIVmac-infected rhesus macaques (Yant et al. 2006).

Vaccine-induced three *Mamu-B*08*-restricted CD8⁺ T-cell epitopes, a macaque model for *HLA-B*27*-mediated elite controller, can control replication of pathogenic clonal SIV_{mac239} in IRMs, which is associated with high frequencies of CD8⁺ T cells against Vif and Nef epitopes in the blood, lymph nodes, and colon (Mudd et al. 2012). In pigtail macaques, animals with *Mane-A*10* have been shown to have lower set-point SIV levels than non-*Mane-A*10* animals (Smith et al. 2005). The protective effects of macaque MHC in SIV/macaque models need to be taken into consideration in vaccine efficacy studies.

Besides rhesus MHC, other host factors that may also affect SIV infection, disease progression, and vaccine testing are CD4, CCR5, tripartite-motif-containing protein 5 (TRIM5), apolipoprotein B-editing catalytic subunit-like 3 (APOBEC3), tetherin (also known as BST2 or CD317), and SAM domain- and HD-domain-containing protein 1 (SAMHD1). CD4 is an indispensable primary receptor for the pathogenic and neutralizing-resistant molecularly cloned SIV_{mac239}, yet the non-pathogenic molecularly cloned SIV_{mac1A11} infects rhesus CCR5⁺ T cells in a CD4-independent way (Chen et al. 1997). We found that SIV_{mac1A11} is highly sensitive to neutralization by CD4-induced (CD4i) antibodies that are abundantly induced by SIV and various vaccines encoding SIV gp160 (Tang et al. 2017). Similar antibodies to HIV-1 CD4i epitopes are induced by vaccination and may play a role in controlling SHIV_{162P3} infection (DeVico et al. 2007). We discovered that SIVrcm isolated from red-capped mangabeys uses CCR2 instead of CCR5 as an entry co-receptor due to homozygous delta24 deletion in host CCR5 genes (Chen et al. 1998). Due to this co-receptor difference, SIVrcm does not cause simian AIDS in IRM after serial in vivo passages but attenuates pathogenic SIVmac challenges (Ling et al. 2008). Rhesus monkeys that express two TRIM5 alleles that restrict SIV replication are more likely to be protected from infection than monkeys that expressed at least one permissive TRIM5 allele (Letvin et al. 2011). APOBEC3G (A3G)-mediated innate immunity may also impact adjuvant-assisted vaccine against SIV_{mac251} infection (Sui et al. 2010). These findings highlight the need to analyze both immune and genetic correlates of protection in trials of vaccine strategies. Meantime, overcoming host restriction may result in HIV-1/NHP models in the future (Hatzioannou and Bieniasz 2011).

5.5 SIV-Infected Macaques as Models for Evaluation of AIDS Vaccine Strategies

An effective AIDS vaccine needs to prevent viral transmission and, if infection occurs, control and eliminate viral replication without latency formation. After SIV/macaque model was established, multiple vaccine strategies were tested in 1980s–1990s with positive results obtained. Vaccination with a formalin-inactivated whole SIV vaccine resulted in eight out of nine rhesus monkeys being protected from challenge with ten animal-infectious doses of pathogenic virus (Murphey-Corb et al. 1989). Rhesus monkeys immunized by multiple inoculations with

purified, disrupted, noninfectious SIV protected two of six vaccinated monkeys following the live virus challenge (Desrosiers et al. 1989). A live recombinant virus, vaccinia-based vaccination elicited a SIVmac Gag-specific CD8⁺ CTL response in rhesus monkeys (Shen et al. 1991). Oral administration of a recombinant SIV antigen induced mucosal immune responses in macaques (Lehner et al. 1992). Moreover, the intramuscular plus oral inoculation of formalin-treated SIV in biodegradable microspheres conferred protection among five of six macaques against vaginal challenge (Marx et al. 1993). The seemingly protective vaccines in these studies, however, did not result in any effective HIV vaccine due to various technical limitations. Some vaccines did not induce potent neutralizing antibodies and CD8⁺ CTL responses. Some protected against SIV intravenous challenges but did not protect against a heterosexual route of infection. Some protected against SIV mucosal challenges yet due to responses against host components instead of viral antigens. Apparently, primary SIVmac strains propagated in monkey PBMC were difficult to protect (Hulskotte et al. 1995). One important lesson learnt is that vaccine efficacy studies using cell-line-derived SIV challenge stocks are misleading and unacceptable.

The most effective and reproducible AIDS vaccine in 1990s was probably the live attenuated and nonpathogenic SIV_{mac239deltanef} (deletion in nef). Rhesus monkeys vaccinated with SIV_{mac239deltanef} were protected against challenge by intravenous inoculation of live, pathogenic SIVmac (Daniel et al. 1992). Eight cynomolgus macaques vaccinated with the attenuated SIV were all protected against both cell-free and cell-associated SIV (Almond et al. 1995). African green monkeys vaccinated with SIV_{agm3deltanef} also demonstrated a strong protection against live SIVagm (Beer et al. 1997). Moreover, eight Indian macaques vaccinated with SIV_{mac239deltanef} displayed a significant reduction in acquisition of the pathogenic heterologous swarm isolate SIV_{smE660} against ten repeated low doses of intrarectal challenges (Reynolds et al. 2010). Although the live attenuated SIV has proven highly effective in protecting macaques against challenge with pathogenic SIV strains, the immune correlates of protection remain incompletely understood. By examining immune responses in animals vaccinated with SIV_{mac239deltanef} or SIV_{mac239delta 3} (deletions in nef, vpr, and upstream sequences in U3), vigorous CTL responses against the SIV Gag and Env proteins were detected for more than 6 years after a single inoculation (Johnson et al. 1997). However, although attenuated SIV_{mac239deltanef} elicited specific CTL precursor cells (CTLp), no correlation was observed between breadth or strength of CTLp responses to SIV structural proteins Env, Gag, or Pol and protection against infection (Nixon et al. 2000). By an ex vivo viral suppression assay, lung CTL in SIV_{mac239deltanef}-immunized animals, unlike blood CD8 T cells, effectively suppress virus replication by up to 80%, suggesting that functional immunity at mucosal sites might contribute to protection (Harris et al. 2010).

Since there was a trend toward increased protection with the length of time of vaccination and the solid vaccine protection was achieved by 79 weeks with the highly attenuated SIV_{mac239delta3}, the titer of SIV_{mac251}-neutralizing activity in serum on the day of challenge was associated with protection (Wyand et al. 1996). This finding might be relevant because SIV_{mac251} infection induced neutralizing antibodies

at 20 to 32 weeks after infection (Yeh et al. 2010). Interestingly, SIV_{mac239deltanef} vaccination recruits plasma cells and induces ectopic lymphoid follicle formation beneath the mucosal epithelium in rhesus female reproductive tracts, producing antibodies reactive with viral gp41 trimers, and these antibodies are concentrated on the path of virus entry by the neonatal FcR in cervical reserve epithelium and in vaginal epithelium for protection (Li et al. 2014). Besides neutralizing antibodies, SIV_{mas239deltanef} induced Env-specific antibody-dependent cell-mediated cytotoxicity (ADCC) 3 weeks after inoculation and increased progressively over time (Alpert et al. 2012). Recently, the protection of SIV_{mac239deltanef} vaccination against vaginal challenge was correlated with the absence of an epithelium-innate immune cell-CD4⁺ T-cell axis response in the cervical mucosa (Shang et al. 2017). Multiple mechanisms, therefore, may contribute to SIV_{mac239deltanef}-induced protection in rhesus macaques. Although replication-competent SIV_{mac239deltanef} and SIV_{mac239del3} vaccines have provided protection in rhesus monkeys, the long-term safety of such vaccines in human populations prevents them from clinical development especially when pathogenic Nef reversion does occur during prolonged SIV/HIV infections, leading to subsequent disease progression (Chakrabarti et al. 2003; Learmont et al. 1999).

Since conventionally killed and live attenuated vaccines were not successful, vector-based vaccines have been explored since the late 1990s in SIV/macaque models. In particular, the findings of the resolution of acute HIV/SIV infections and control of the subsequent persistent infection by antiviral cellular immune responses promoted T-cell vaccine discovery substantially (Jin et al. 1999; Schmitz et al. 1999). The modified vaccinia virus Ankara (MVA) was explored as a vector, with SIV Gag-specific CD8⁺ T lymphocytes detected in lymph nodes of the immunized monkeys by both functional and tetramer-binding assays (Seth et al. 1998). A DNA prime-MVA boost vaccination regimen elicited high levels of CTLs specific for a single SIV gag-derived epitope in rhesus macaques despite the lack of protection against intrarectal challenge with pathogenic SIV_{mac251} (Hanke et al. 1999). Improved DNA vaccines using either an IL-2/Ig fusion protein or a plasmid expressing IL-2/Ig substantially augment T-cell immune responses in rhesus monkeys (Barouch et al. 2000). A codon-optimized SIV gag DNA vaccine elicits SIV-specific CTL responses in rhesus monkeys, which facilitates the control of viral replication following a pathogenic SIV_{smE660} challenge (Egan et al. 2000). Rhesus macaques received a DNA plus MVA regimen against nearly all SIV proteins had significantly reduced peak viremia (Horton et al. 2002). Against 12 weekly rectal challenges with the heterologous SIV_{smE660}, the DNA plus MVA regimen and the MVA alone expressing SIVmac239 Gag, PR, RT, and Env sequences elicited similar 61–64% reductions in the per challenge risk of viral transmission (Lai et al. 2012). A DNA vaccine with the CCR10L adjuvant results in significant protection against an intra-vaginal SIV_{smE660} challenge in rhesus macaques (Kutzler et al. 2016).

When DNA-, MVA-, and a replication-incompetent adenovirus type 5 (Ad5) vector-based SIV vaccine were compared in rhesus monkeys, the most effective responses were elicited by the Ad5 vector, used either alone or as a booster inoculation after priming with a DNA vector (Shiver et al. 2002). This finding laid

the foundation for the subsequent development of the MRKAd5 HIV-1 *gag/pol/nef* vaccine in the STEP human phase 2b efficacy trial, which directly assessed the efficacy of a T-cell vaccine to protect against HIV-1 infection or control of early plasma HIV-1 levels. Unfortunately, despite being highly immunogenic for inducing HIV-specific CD8⁺ T cells, this T-cell vaccine did not prevent HIV-1 infection or reduce early viral levels, and HIV-1 incidence was higher in vaccine-treated than in placebo-treated Ad5-seropositive uncircumcised men (Buchbinder et al. 2008; McElrath et al. 2008). Since baseline Ad5-specific neutralizing antibodies were not correlated with Ad5-specific T-cell responses and Ad5-seropositive subjects did not develop higher vector-specific cellular immune responses, one could not conclude that activated Ad5-specific T lymphocytes were the cause of the potential enhanced HIV-1 susceptibility in the STEP study (O'Brien et al. 2009). More importantly, the efficacy results were predicted previously and later recapitulated in the SIV/macaque model, further enhancing the role of this system in helping facilitate the prioritization of candidate HIV-1 vaccines (Qureshi et al. 2012; Watkins et al. 2008).

After the disappointing STEP study, much attention was placed on vaccine strategies using a heterologous prime and boost regimen. A DNA prime-adenovirus boost strategy demonstrated that vaccine-induced cellular immunity in the absence of any Env-specific antibodies could control viral replication following multiple low-dose challenges with the highly pathogenic SIV_{mac239} (Wilson et al. 2006). A similar regimen failed to protect against SIV_{mac251} infection, yet 50% of vaccinated monkeys were protected from infection with SIV_{smE660} (Letvin et al. 2011). An Ad26 prime-Env gp140 boost regimen demonstrated complete protection in 50% of vaccinated animals against a series of repeated, heterologous, intrarectal SIV_{mac251} challenges (Hel et al. 2000). A rAd26 prime and rAd5 boost regimen showed a 1.4 log reduction of peak and a 2.4 log reduction of set-point viral loads as well as decreased AIDS-related mortality with durable immune control of a pathogenic SIV challenge for more than 500 days (Liu et al. 2009). We demonstrated that a modified replicating vaccinia virus Tiantan strain mucosal prime plus an intramuscular Ad5 boost regimen elicited robust cellular immune responses that reduced SIV_{mac239} set-point viral loads and delayed simian AIDS for years (Sun et al. 2013). Interestingly, a participant-level meta-analysis of randomized trials indicated an increased risk of HIV-1 infection by MRKAd5 over all follow-up time but does not support increased risk of HIV-1 infection by the DNA/rAd5 regimen in the HVTN505 trial (Huang et al. 2015). Therefore, the HVTN505 trial was stopped because the vaccine was ineffective in preventing HIV-1 infections and lowering viral load among those participants who had become infected with HIV-1 (Hammer et al. 2013). This outcome was in line with data generated in the SIV_{mac251}/macaque model but not in the SIV_{smE660}/macaque one (Letvin et al. 2011). When human adenovirus-5 (AdHu5), chimpanzee adenovirus-6 (AdC6) or adenovirus-7 (AdC7), vaccinia virus (VV), and DNA given by electroporation (DNA/EP), all expressing SIV_{mac239}Gag/Tat, were compared in five regimen groups of DNA/EP-AdC6-AdC7, VV-AdC6-AdC7, DNA-/EP-VV-AdC6, DNA/EP-VV-AdC7, and AdHu5-AdHu5-AdHu5, these immunization regimens did not protect against SIV_{mac239} acquisition via repeatedly low-dose challenges but did result in an approximately 1.6-log decline in set-point

viremia, and the levels of activated CD4⁺CCR5⁺ T cells in the rectal mucosa likely predict the risk of SIV acquisition in vaccinated macaques (Carnathan et al. 2015). More prime and boost vaccine strategies should be carefully evaluated in future human studies for improving vaccine efficacy while avoiding vaccine-induced risk of HIV-1 infections.

One possible reason underlying the failure of STEP and HVTN505 trials is probably due to insufficient vaccine-induced protective T-cell immunity at the entry site of viral infection. Since it is difficult to study this hypothesis in humans, studies in SIV/macaque models open a paradigm. Rhesus cytomegalovirus (RhCMV) was developed as a live vaccine vector to express SIV Gag, Rev-Tat-Nef, and Env (Hansen et al. 2009). This RhCMV-based vaccine persistently infected rhesus macaques and induced robust, SIV-specific CD4⁺ and CD8⁺ effector memory T-cell (TEM) cell responses, which conferred resistance to acquisition of progressive SIV_{mac239} infection upon repeated limiting-dose intrarectal challenge (Hansen et al. 2013a). Impressively, about 50% of vaccinated animals manifest durable, aviremic control of SIV_{mac239} infection. Over time, these protected macaques lost signs of SIV infection because ultrasensitive quantitative assays did not detect SIV RNA or DNA sequences or replication-competent SIV even after adoptive transfer of 60 million hematolymphoid cells to naive monkeys (Hansen et al. 2013a). These data provide evidence that vaccine-induced TEM responses did not prevent SIV_{mac239} infection but conferred continuous immune surveillance for progressive latency clearance. Mechanistically, genetically programmed RhCMV-based vaccine elicited SIV-specific CD8⁺ T cells that recognize unusual, diverse, and highly promiscuous epitopes, including dominant responses to epitopes restricted by class II major histocompatibility complex (MHC) molecules (Hansen et al. 2013b). These studies, therefore, shed lights for the development of both preventive and therapeutic T-cell vaccines.

5.6 SHIV-Infected NHP Models for Evaluation of AIDS Vaccine Strategies

In order to analyze HIV-1 *env* gene functions and to evaluate vaccines based on HIV-1 Env antigens in a nonhuman primate model, infectious recombinant viruses between the pathogenic molecular clone of SIV_{mac239} and molecular clones of HIV-1 subtype B were generated to establish the SHIV/macaque model (Luciw et al. 1995). Even since, the SHIV/macaque model has become useful for HIV-1 pathogenesis and vaccine studies because SHIV-infected Asian macaques developed AIDS-like diseases (Reimann et al. 1996; Joag et al. 1996). Despite comparable levels of viral replication, rhesus macaques infected with the R5-tropic SHIV_{SF162P} and X4-tropic SHIV_{SF33A} displayed distinct pathogenic outcomes, providing a useful *in vivo* model for preclinical examination of HIV-1 vaccines and therapeutic agents in the context of the HIV-1 envelopes (Harouse et al. 1999). Meanwhile, X4-tropic SHIV_{DH12R} and SHIV_{HXBc2}, dual R5/X4-tropic SHIV_{89,6P} and SHIV_{DH12}, as

well as R5-tropic SHIV_{KU-1} and SHIV_{BaL} were also generated using *env* genes derived from HIV-1 subtype B strains (Shibata et al. 1997; Letvin et al. 1997; Joag et al. 1998; Cafaro et al. 1999). Along with other researchers, we have further constructed R5-tropic SHIV strains bearing HIV-1 subtype AE, C and B' envelopes, which have made it possible to conduct cross-subtype vaccine efficacy studies (Klinger et al. 1998; Chen et al. 2000; Wang et al. 2010; Song et al. 2006). Since pathogenic SHIV strains target different subsets of CD4⁺ T cells in vivo, R5-tropic SHIV_{SF162P3}/macaque is one of the most widely used models for developing an effective prophylactic vaccine by mimicking HIV-1 primary infection via mucosal transmission.

Rhesus macaques previously infected with a live attenuated SIV were protected from subsequent infection with SHIV, but the subsequent SHIV exposure in cases with low CD4⁺ T-cell numbers revealed possible risk of inducing disease progression (Bogers et al. 1995). Rhesus macaques immunized with SIV_{mac239delta3} or SIV_{mac239delta2} achieved strong long-term protection against pathogenic SHIV_{DH12} (Shibata et al. 1997). However, live attenuated SHIV vaccines conferred partial protection against pathogenic SHIV_{KU-1} via the intravaginal route (Joag et al. 1998). Immunization with a DNA prime and DNA plus protein boost regimen completely protects monkeys from infection after intravenous challenge with a neutralizing-sensitive SHIV_{HXBc2} strain (Letvin et al. 1997). Subsequently, vaccination of cynomolgus monkeys with HIV-1 Tat protein reduced infection of the highly pathogenic SHIV_{89.6P} to undetectable levels and prevented CD4 T-cell loss (Cafaro et al. 1999). Alum-adjuvanted gp120 did not improve a DNA/MVA regimen against SHIV_{89.6P} infection probably due to the lack of neutralizing antibodies in immunized macaques (Buge et al. 2003). An optimized IL-15 immune adjuvant delivered with a DNA vaccine can impact the cellular immune profile in rhesus macaques and lead to enhance suppression of SHIV_{89.6P} replication (Boyer et al. 2007). An adenovirus serotype 26 (Ad26) expressing SIV_{smE543} Env, Gag, and Pol and AS01B-adjuvanted SIV_{mac32H} Env gp140 boost demonstrated complete protection in 50% of vaccinated animals against acquisition of neutralization-resistant intrarectal SIV_{mac251} or tier-2 SHIV_{SF162P3} challenges in rhesus monkeys (Barouch et al. 2015). While diverse vaccine strategies have been tested in SHIV/macaques models, only a few entered human trials.

Up until now, only one preventive HIV vaccine decreased 31% risk of HIV-1 acquisition in the Thai RV144 vaccine trial, which involved a recombinant vaccine containing Aventis Pasteur's canarypox vector (ALVAC)-HIV and gp120 alum regimen (Haynes et al. 2012). Subsequent analysis indicated that vaccine-induced antibody responses were associated with two signatures in V2 at amino acid positions 169 and 181 and vaccine-induced V2 responses likely played a role in the protection observed in the RV144 trial (Rolland et al. 2012). Env-specific IgA antibodies from RV144 vaccines, however, blocked the ability of natural killer cells to kill HIV-1-infected CD4⁺ T cells coated with RV144-induced IgG antibodies (Tomaras et al. 2013). Four V2 monoclonal antibodies from RV144 vaccines recognize residue 169, neutralize laboratory-adapted HIV-1, and mediate killing of field-isolate HIV-1-infected CD4⁺ T cells (Liao et al. 2013). In the SIV/macaque models, we reported

that the MVTTgpe prime and AD5gpe regimen induced a predominant antibody response to V2, which correlated with delayed simian AIDS against pathogenic SIV_{mac239} infection (Sun et al. 2013). The anti-V2 antibodies, however, were not boosted naturally upon SIV_{mac239} challenge and diminished in infected macaques at the stage of simian AIDS (Guo et al. 2015). An RV144 equivalent ALVAC-SIV and gp120 alum vaccine, but not an ALVAC-SIV plus gp120 MF59 vaccine, was efficacious in delaying the onset of SIV_{mac251} infection in rhesus macaques (Vaccari et al. 2016). Interestingly, we recently reported that a highly conserved N-linked glycosylation site N277 in SIV C2 region governs the induction of anti-V2 antibodies (Tang et al. 2017). Future studies should investigate mechanisms underlying protective anti-V2 antibodies and ways to elicit such antibodies for effective AIDS vaccines.

5.7 Passive Immunization as Prevention in NHP Models

Because of tremendous difficulties in vaccine design for inducing broadly neutralizing antibodies (bNAbs), it is logical to develop passive immunization as prevention and immunotherapy against HIV/AIDS. Early studies suggested that heat-inactivated convalescent sera protected five out of seven macaques at a dose of 9 ml kg⁻¹ (Putkonen et al. 1991). Purified IgG from chimpanzees infected with several different HIV-1 isolates could confer complete protection in some SHIV-challenged macaques (Shibata et al. 1999). Although there were only a few bNAbs available before 2009, some experiments have demonstrated the critical role of bNAbs against HIV/AIDS in nonhuman primate models. While individual HIVIG, 2F5, or 2G12 was not effective, 2F5/2G12 and HIVIG/2F5/2G12 combinations showed compete protection in some macaques against pathogenic SHIV_{89.6PD}(Mascola et al. 1999). Similarly, macaques which received a triple combination of F105, 2G12, and 2F5 were protected against intravenous SHIV_{IIIB} challenges (Baba et al. 2000). Vaginal administration of a single bNAb b12 can protect some macaques from tier-1 SHIV_{SF162P4} challenge through the same route (Veazey et al. 2003). The interaction between bNAb Fc-receptor-bearing effector cells and bNAb-complexed infected cells is essential in reducing virus yield from infected cells (Hessell et al. 2007). Moreover, by adeno-associated virus (AAV)-vectored transfer of antibody gene in monkeys, long-lasting neutralizing antibodies in serum conferred complete protection against intravenous challenge with pathogenic SIV (Johnson et al. 2009). With many novel and potent bNAbs discovered in recent years, sterilizing immunity was achieved in all rhesus monkeys pre-administered with 5 mg/kg and 1 mg/kg bNAb PGT121 against a single high dose of intravaginal challenge of SHIV_{SF162P3} (Moldt et al. 2012). VRC01-LS, a modified version of bNAb VRC01 with enhanced FcRn binding, has increased gut mucosal tissue localization and mediated protection superior to that of VRC01 against intrarectal SHIV infection (Ko et al. 2014). AAV-delivered eCD4-Ig that neutralized HIV-1, HIV-2, and SIV was able to express in rhesus macaques for more than 40 weeks, preventing

several SHIV_{AD8} challenges (Gardner et al. 2015). Although lower amounts of antibody than previously thought may provide protection for typical human exposure to HIV-1 (Hessell et al. 2009), long-lasting HIV-1 prevention using bNAbs either alone or in combination remains technically challenge for practical application.

5.8 SIV Latency and T-Cell Immunotherapeutic Cure in NHP Models

Th17-lineage CCR6⁺ CD4⁺ T cells are primary targets of SIV_{mac239} infection during vaginal transmission (Stieh et al. 2016). By immunoPET (antibody-targeted positron emission tomography), qRT-PCR, and immunohistochemistry, SIV was detected primarily in foci in the small bowel, some lymphoid areas, and the male reproductive tract of elite controllers (Santangelo et al. 2015). Similar to HIV-1 infection, the treatment of SIV infection with combination antiretroviral therapy (cART) suppresses viral load below the limit of detection. Infected resting CD4⁺ T cells, however, are the major reservoir of latent SIV with slow turnover in chronically infected macaques of low viral loads, which leads to viral load rebound after stopping cART (Reece et al. 2014). In SIV-infected macaques, cART led to a biphasic decay in viremia, and the frequencies of resting CD4⁺ T cells harboring replication-competent virus were reduced but not eradicated in lymph nodes, spleen, and peripheral blood (Dinoso et al. 2009). The turnover of resting CD4⁺ T-cell SIV proviral DNA was higher for escape during early infection than for escape later in infection. The median level of integrated SIV proviral DNA is 1660 copies and 866 copies per million rhesus PBMC during untreated acute and chronic SHIV infection, respectively (Mavigner et al. 2016). Integrated SIV proviral DNA, however, was readily detected in lymph nodes and spleen of cART-treated macaques with total 3319 copies per million PBMC and integrated 3160 copies per million cells after a median of 404 days of treatment. Although resting CD4⁺ T cells are the major latent reservoir, infected macaques with CD4⁺ lymphocyte depletion showed a similar peak of viremia and faster disease progression likely due to the lack of post-peak decline of virus replication (Ortiz et al. 2011). These results indicated that infected macaques are a relevant model for HIV-1 cure research.

To eliminate latently infected cells, besides cART, SIV-specific immune responses expanded by therapeutic NYVAC-SIV immunization control pathogenic SIV_{mac251} replication in 6/8 vaccinated macaques (Hel et al. 2000). Three vaccinations with chemically inactivated SIV-pulsed dendritic cells led to a 50-fold decrease of SIV DNA and a 1000-fold decrease of viral loads in peripheral blood of SIV_{mac251} infected macaques (Lu et al. 2003). Macaques immunized by a DNA prime and Ad5 boost regimen showed prolonged survival with preserved central memory CD4⁺ T lymphocytes (Letvin et al. 2006). Chinese macaques vaccinated by a vaccinia MVTT prime and Ad5 boost regimen showed a similar prolonged survival and viral replication control (Sun et al. 2013). By targeting at host factors, *in vivo* PD-1

blockade improved virus-specific CD8⁺ T cells for SIV control and reduced type I IFN signaling in the blood and mucosal tissues of rhesus macaques (Dyavar Shetty et al. 2012). Critically, conventional CD8-depletion experiments in SIV-infected macaques increase latent cells more than viremia (Rouzine et al. 2015). In addition, a recent study shows that CD8⁺ T cells play a critical role in controlling viral production even during cART in SIV-infected rhesus macaques (Cartwright et al. 2016). Therefore, there is no doubt that vaccine-induced CD8⁺ T cells are useful to control and to eliminate infected cells, which is useful to HIV-1 cure efforts.

In recent years, how to induce latent provirus especially latently infected cells for elimination or even eradication has been a key focus for HIV-1 cure studies. One strategy called “shock and kill” aims to induce expression of latent proviruses resulting in elimination of the affected cells through viral cytolysis or immune clearance or combined mechanisms. For example, the ability of the histone deacetylase inhibitor (HDACi) romidepsin (RMD) has recently been tested to reactivate latent SIV in infected rhesus macaques after receiving cART for 9 months. RMD treatment resulted in a rapid and massive surge in T-cell activation and significant viral load rebounds but did not alter the ability of SIV-specific CD8⁺ T cells to control the reactivated virus (Policicchio et al. 2016). It remains elusive, however, if HDACi treatment alone can induce all latent proviruses, which will rely on careful investigation of viral reservoirs and various cure strategies continuously. The co-stimulatory molecule CD2 and its ligand CD58 signals can induce latent SIV without the co-engagement of the T-cell receptor (Shen et al. 2007). Interestingly, in vivo Treg depletion results in both reactivation of the latent virus and a significant boost of SIV-specific CD8⁺ T-cell frequency with faster clearance of reactivated virus (He et al. 2016). Combining Ad26/MVA vaccination and Toll-like receptor 7 (TLR7) stimulation decreased proviral DNA in lymph nodes and peripheral blood and delayed viral load rebound following discontinuation of cART (Borducchi et al. 2016). In addition to SIV-specific T cells, antibody-mediated latency removal is another critical strategy to cure HIV-1 infection.

5.9 Passive Immunization for Immunotherapeutic Cure in NHP Models

Recently, many studies have investigated the role of bNAbs for immunotherapeutic cure in SHIV-infected rhesus macaques. During the acute phase of infection, rapid passive immunization may eliminate early viral foci and thereby prevent the establishment of viral reservoirs. When administered together to recently infected macaques, two bNAbs 3BNC117 and 10-1074 that target CD4-binding site and the V3 region rapidly suppressed plasma viremia for 3–5 weeks and successfully controlled virus rebound in some SHIV_{AD8}-infected macaques (Shingai et al. 2013). A combination of VRC07-523 and PGT121 suppressed acute SHIV_{SF162P3} plasma viremia and limit CD4⁺ T-cell-associated viral DNA (Bolton et al. 2015). Infant

macaques subcutaneously injected with bNAbs on days 1, 4, 7, and 10 were all free of virus in blood and tissues at 6 months after oral SHIV_{SF162P3} exposure (Hessell et al. 2016). Importantly, early administration of bNAbs 3BNC117 and 10-1074 leads to the establishment of potent CD8⁺ T-cell immunity and resultant long-term control against SHIV_{AD8} infection (Nishimura et al. 2017). During the chronic phase of SHIV_{SF162P3} infection, a cocktail of bNAbs, as well as the single PGT121, resulted in a rapid and 3-log decline of plasma viremia in rhesus macaques, while a subset of animals maintained long-term viral load control after the antibody infusions (Barouch et al. 2013). Moreover, a fully protective dose of the bNAb PGT121 was able to mediate protection against the intravaginal pathogenic SHIV_{SF162P3} challenge and clearance of infectious virus in distal tissues (Liu et al. 2016). These exciting results have indicated the critical role of passive immunization in elimination of latently infected cells for sustained long-term viral suppression.

5.10 Conclusion

Tremendous amount of work generated in NHP models has greatly enriched our knowledge on HIV/AIDS pathogenesis and vaccine development. Many factors, however, may potentially affect vaccine experimental results in NHP models such as model selection, dose and route of vaccination, dose and route of viral challenge, vaccination regimen, combination of vaccination with cART, bNAb, PD-1 blockade or other immune interventions, and so on. With the exciting and increasing knowledge obtained daily, there are still many unanswered scientific questions. First, is it true that productive SIV infection restricted to CD4⁺ follicular helper T cells in germinal center (GC) B cell follicles is difficult to be eliminated by antiviral CD8⁺ T-cell responses (Fukazawa et al. 2015)? A recent study demonstrated that the increase in CXCR5⁺ CD8⁺ T cells, a unique subset of antiviral CD8 T cells, was associated with the presence of higher frequencies of SIV-specific CD8 T cells in the GC that might play a role in the control of pathogenic SIV infection (Mylvaganam et al. 2017). Then, how about similar situation that may apply for other immune privileged organs such as brain and cells such as CD34⁺ stem cells in bone marrow (Wu et al. 2016)? Second, is transient but total CD4⁺ T-cell depletion necessary for eliminating latently infected cells? CD4⁺ T cell-depleted rhesus macaques showed a similar peak of viral load but did not manifest any post-peak decline of virus replication (Ortiz et al. 2011). If so, how about transient CD4⁺ T cell-depletion in combination with cART, bNAb, and other immune interventions? Third, why did RhCMV-based vaccine achieve only 50% of durable, aviremic control of SIV_{mac239} infection in vaccinated macaques? It remains unknown if similar complex but protective CD8⁺ T cells can be induced by human CMV-based vaccine in clinical trials. Fourth, SIV-infected macaques treated with cART and an anti-alpha4beta7 integrin antibody maintained low to undetectable viral loads and normal CD4⁺ T-cell counts for more than 9 months (Byrareddy et al. 2016). The protection was not associated with neutralizing antibody or conventional cell-mediated immune responses but

with reduced damage to mucosal T lymphocytes, yet can similar findings be made in humans? We do not have clear answers to many of these questions but experimental evidence generated in NHP models has paved the road that may lead to the ultimate success of HIV vaccine or immunotherapeutic cure.

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Chapter 6

Treatment of HIV for the Prevention of Transmission in Discordant Couples and at the Population Level



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Abstract The scientific breakthrough proving that antiretroviral therapy (ART) can halt heterosexual HIV transmission came in the form of a landmark clinical trial conducted among serodiscordant couples. Study findings immediately informed global recommendations for the use of treatment as prevention in serodiscordant couples. The extent to which these findings are generalizable to other key populations or to groups exposed to HIV through nonsexual transmission routes (i.e., anal intercourse or unsafe injection of drugs) has since driven a large body of research. This review explores the history of HIV research in serodiscordant couples, the implications for management of couples, subsequent research on treatment as prevention in other key populations, and challenges in community implementation of these strategies.

Keywords HIV transmission · Treatment as prevention · Serodiscordant couples

6.1 Introduction

Now, 37 years after the first reported case of HIV, we have made considerable progress on prevention of infection (El-sadr et al. 2013), short of a preventive vaccine. A focus on “combination prevention” embraces both behavioral and biological tools that reduce the risk of an HIV transmission event.

The evolution of the HIV pandemic has made clear that sexual transmission of the virus is of greatest importance and that HIV incidence is bounded by the prevalence of HIV in a community, mobility of sexual partners, and risk-taking behaviors

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(Grabowski et al. 2014). The number of partners, the probability of exposure to an infected partner, and the precise sexual behaviors define the risk of HIV acquisition (Baggaley et al. 2006, 2010; Powers et al. 2008; Boily et al. 2009). The details of sexual transmission of HIV have been carefully described (Cohen et al. 2007; Joseph et al. 2015).

In a landmark study of couples, Quinn et al. demonstrated that the concentration of HIV in the blood of untreated HIV-infected individuals could be directly correlated with the risk of HIV transmission (Quinn et al. 2000). An infected person with less than 1500 copies of HIV/ml blood plasma demonstrated no transmission risk, while those with a viral load greater than 50,000 copies/ml had a transmission rate of 23.0 per 100 person-years (PY). Importantly, these results were repeated with a focus on the concentration of HIV in the genital tract, which is more closely tied to the sexual HIV transmission event (Baeten et al. 2011). These and other studies over many years support the idea that reduction of the concentration of HIV in the genital tract can be expected to reduce HIV transmission. Before antiretroviral agents (ARVs) were available, treatment of STDs – which increase the concentration of HIV in the genital tract (Cohen et al. 1997, 2007) – was a mainstay of HIV prevention. With the development of ARVs, the idea that treatment with these agents could also dually serve as prevention gradually evolved into a central hypothesis (Cohen et al. 2007). In this chapter, we will explore “treatment as prevention” (TasP) as it relates to the management of HIV discordant couples and in the control of the spread of HIV at the population level.

6.2 Treatment as Prevention in Discordant Couples

6.2.1 *Historical Perspective*

All HIV transmission occurs between an infected individual and a susceptible individual. In the context of HIV, either both partners are HIV negative (concordant negative) or positive (concordant positive) or the couple is discordant (one negative and one positive). Heterosexual discordant couples – i.e., two individuals engaged in a “stable” relationship – represent a distinct population. In clinical trials designed to study HIV transmission, an eligible discordant couple is typically defined by cohabitation or marriage and/or relationship duration (a minimum 3–6 months), an intention to stay in the partnership, and recent unprotected sex. However, discordant couples do not allow entirely reliable assessment of HIV transmission: up to 30% of HIV incidence among HIV-uninfected partners originates from external partnerships (Celum et al. 2010; Gray et al. 2011; Cohen et al. 2011), and HIV discordant relationships are at an increased risk of dissolution relative to partnerships unaffected by HIV (Porter et al. 2004; Mackelprang et al. 2014).

Early in the epidemic, studies of HIV discordant couples were initiated to better understand the dynamics of HIV transmission (Harris et al. 1983; Laurian et al. 1989; de Vincenzi and HIV 1994). Subsequent studies in Africa, particularly in the

Rakai Study cohort in Uganda (Gray et al. 2000, 2001; Wawer et al. 2005), led to a better sense of the risk of HIV transmission and to strategies to prevent transmission. As this area of research grew, it was widely assumed by both healthcare providers and those living with HIV that a partner in a stable relationship would likely be HIV-infected. However, in a series of studies, it was found that only about half of such couples were HIV concordant (Chamaletty et al. 2012), although risk of HIV transmission persisted at a steady and alarming rate (Carpenter et al. 1999; Ahmed et al. 2001).

Studies of HIV discordant couples led to several important considerations. First, the overall contribution of transmission within couples to HIV incidence and prevalence has been debated; the role of defined, stable partnerships to population-level spread of HIV varies greatly. Second, failure of HIV transmission within a discordant couple led to consideration of a population of exposed uninfected (EU) (Lederman et al. 2010), i.e., people who might have unique resistance to HIV infection. While studies of EU have mostly focused on female sex workers (Fowke et al. 1996), they have also extended to discordant couples and found evidence that innate genetic protection against HIV exists (Dunkle et al. 2008). Third, reduction of transmission in discordant couples through counseling and/or biomedical interventions has been a cornerstone of HIV prevention policies worldwide (Allen et al. 1992, 2003).

The prevalence of discordant partnerships varies substantially across Africa, based on geography and the type of epidemic. In a summary of studies by Curran et al., approximately 2–8% of all stable couples in Africa are discordant, though this varies dramatically based on setting (Curran et al. 2012). During recruitment for one clinical trial taking place among discordant couples, an estimated 51,900 couples of unknown serostatus were tested across sub-Saharan Africa, of whom 6543 (13%, range 8–31%) tested as discordant (Lingappa et al. 2009). A mathematical modeling study based on demographic and health survey (DHS) data estimated that stable discordant couples make up 0–17% of all stable partnerships and that 36–88% of HIV-infected individuals in stable partnerships in Africa have an HIV-uninfected partner (Chamaletty et al. 2012). In generalized epidemics, a greater portion of couples are HIV concordant, but serodiscordant relationships also account for a greater proportion of all partnerships than in concentrated epidemics.

Today, heterosexual discordant couples likely play an important role in sustaining the global epidemic, though their contribution to overall incidence is only partially understood. Modeling studies estimate that in hyperendemic sub-Saharan Africa a substantial proportion of infections originated prior to couple formation or from external partners. One study estimated that 29% (range 10–52%) of overall transmission originates from within-couple transmission (Chamaletty et al. 2013), while another estimated that extra-couple transmission accounted for 27–61% and 21–51% of new infections among men and women in cohabiting couples, respectively (Bellan et al. 2013). As individuals in sub-Saharan Africa are frequently unaware of their own HIV status (ICF International 2012) or their partner's status (Matthews et al. 2014), it is also likely that many more serodiscordant couples exist that are not captured through existing public health channels and continue to contribute to HIV incidence.

6.2.2 Identification of Discordant Couples in Africa

Randomized controlled trials (RCTs) have screened and recruited couples through ART clinics (via partner testing), antenatal care (ANC) and prevention of mother-to-child transmission (PMTCT) clinics (via male partner testing), home-based counselling and testing (HBCT), referral of partners of known HIV-infected individuals engaged in the health system, and community promotion of joint couples' counselling and testing (Lingappa et al. 2009; Mujugira et al. 2011). Some couples were also reached indirectly (i.e., through joint voluntary counselling and testing, or VCT) via community programs, including peer outreach, radio, and street theater (Lingappa et al. 2008). In sub-Saharan Africa, women are equally as likely to be the HIV-infected partner in a couple, and ANC clinics may be an effective source for male partner testing (Eyawo et al. 2010). Of Kenyan women presenting at ANC clinics, 40 out of 148 (27%) had partners that were tested for HIV (Roxby et al. 2013), which could be indicative of the coverage of serodiscordant couples available through ANC recruitment. Community-based HIV testing results in higher rates of testing and greater acceptability than do facility-based approaches (Suthar et al. 2013).

Barriers to HIV testing include low perception of risk, stigma, economic costs of accessing care (e.g., difficulty of transportation or childcare), and gender inequality (Musheke et al. 2011; Lankowski et al. 2014). Men, in particular, also engage with health services far less than do women (Skovdal et al. 2011); for example, in rural Zimbabwe, 80% of individuals tested through clinic-based VCT were female, and men were more likely to consider testing only after becoming ill (Chirawu et al. 2010).

A fraction of serodiscordant couples may present themselves to the health system through these pre-existing channels; however, those couples that present may already be at a lower risk of transmission, potentially as a result of having a greater awareness of existing HIV prevention strategies, or the partnership may have remained discordant over time due to a lower set-point viral load in the partner or some other factor.

6.2.3 Risk of HIV Transmission in a Discordant Couple

Few observed estimates of HIV-1 incidence are available among couples unaware of their serodiscordant status. The Rakai Study in Uganda retrospectively identified stable serodiscordant couples in which HIV transmission had occurred within the partnership and found that the probability of HIV transmission varied between 0.0007 and 0.0082 per sex act, depending on the stage of HIV infection (Wawer et al. 2005). However, only 235 couples were observed, and estimates may also

suffer from selection bias; those couples in the community with the highest propensity to transmit are more likely to have seroconverted prior to the study period. The Partners in Prevention HSV/HIV Study, in which enrolled couples were aware of their serodiscordant status, estimated that male-to-female and female-to-male transmission probabilities per sex act were 0.0019 and 0.0010, respectively (Hughes et al. 2012). Systematic reviews of the literature show that per act transmission risk varies greatly by factors such as presence of other STIs or stage of HIV infection (Powers et al. 2008; Boily et al. 2009). In addition, the risk of transmission among MSM via receptive anal intercourse is estimated to be an order of magnitude higher than heterosexual sex, at 0.0138 per sex act (Patel et al. 2014). The aggregated yearly risk of HIV acquisition in a negative partner, considering exposure to HIV both within and outside the partnership, may be as high as 12% with limited counseling and less than 2% per year with couple counseling (Quinn et al. 2000; Celum et al. 2010; Cohen et al. 2011).

Modifiable risk factors can increase the basic transmission probability substantially (Powers et al. 2008). Early- (acute) and late-stage HIV infection are associated with a 26- and 7-fold increase in transmission respectively (Hollingsworth et al. 2008), though the relative infectiousness of acute infection may be lower than previously thought (Bellan et al. 2015). The presence of other sexually transmitted infections (STIs) increases both the risk of transmission and acquisition of HIV (Wasserheit 1992); genital herpes in particular has played an important role in the epidemic's spread within sub-Saharan Africa (Abu-Raddad et al. 2008). For women, the composition of the vaginal microbiome can also strongly influence susceptibility to HIV; an increased presence of certain bacteria is associated with nearly a 13-fold increased risk of infection (Passmore et al. 2016).

Hormonal contraception and pregnancy are both associated with an approximately twofold increase in the relative risk of both HIV acquisition and transmission (Mugo et al. 2011; Heffron et al. 2012). Inadequate measurement or adjustment for confounding by condom use is thought, however, to overestimate the association between the former and risk of HIV acquisition (Schwartz et al. 2013), a topic of ongoing investigation in the Evidence for Contraceptive Options and HIV Outcomes (ECHO) Study (Colvin et al. 2015). Anal sex and forcible sex further increase the risk of transmission within a partnership (Baggaley et al. 2010; Dunkle et al. 2013).

Characteristics that are associated with a greater risk of transmission among stable serodiscordant couples are younger age, cohabitation, having fewer children, high plasma viral load, and recent occurrence of unprotected sex (Kahle et al. 2013). Data from the Partners in Prevention HSV/HIV Study were used to develop a risk score algorithm, which used these factors to identify couples with a high risk of HIV transmission within the partnership (Kahle et al. 2013). Discordant couples with a risk score ≥ 5 (from a maximum of 12) were associated with annual incidence of 3/100 PY, and those couples with a score ≥ 6 accounted for 67% of all HIV transmissions, despite making up only 28% of the population (Kahle et al. 2013).

6.2.4 HIV Prevention Options for Serodiscordant Couples

Rates of HIV transmission among discordant couples can be lowered substantially through behavioral and biomedical interventions, including voluntary counselling and testing (VCT), condom promotion, and male circumcision (Jiwatram-Negrón and El-Bassel 2014). Joint couples' VCT resulted in condom use improving from <3% to >80% in a cohort of couples in Zambia (Allen et al. 2003), and awareness of HIV serodiscordance led to increased and consistent condom use for couples in South Africa (Rosenberg et al. 2013). With regular counselling, testing, and the provision of free condoms, incidence in the placebo arms of RCTs among discordant couples was reduced to 2/100 PY without any biomedical interventions (Celum et al. 2010; Cohen et al. 2011). Nevertheless, without such an intervention, condom use among stable couples is generally low (Maharaj and Cleland 2005; Ngure et al. 2011), and couples frequently express a desire to conceive (Ngure et al. 2014). Male circumcision also has the potential to reduce transmission in couples; there is an approximately 60% reduction in risk for circumcised HIV-uninfected men (Auvert et al. 2005; Bailey et al. 2007; Gray et al. 2007).

Most recently, antiretroviral treatment of HIV has evolved into the backbone of a combination prevention strategy for discordant couples. A large series of observational studies (with rare exception (Wang et al. 2010; Birungi et al. 2015)) demonstrated that ART provided to HIV-infected partner reduced or eliminated HIV transmission (Cohen and Gay 2010; Smith et al. 2011; Cohen et al. 2013). To better quantify both the magnitude and durability of treatment for prevention, a randomized clinical trial – HIV Prevention Trials Network 052 (HPTN 052) – was conducted among 1763 heterosexual couples in 9 countries. This study reported 96% protection against HIV acquisition for the HIV-uninfected partner over 18 months (Cohen et al. 2011), with durable 93% protection over the course of more than 10,000 person-years of follow-up (Cohen et al. 2016). Importantly, the only HIV transmission events observed in the face of treatment were noted very early after initiation of therapy or when treatment failed. More recently, an observational study of MSM who engaged in condomless sex demonstrated prevention of HIV transmission of HIV ascribed to ART (Rodger et al. 2016). In the PARTNER Study, there were no cases of within-couple HIV transmission if the HIV-positive partner was taking ART.

An alternative use of ART in a discordant couple is pre-exposure prophylaxis (PrEP). Combination tenofovir/emtricitabine (TDF/FTC) PrEP, known by the brand name Truvada, has proven efficacious for a number of key populations, including serodiscordant couples (Baeten et al. 2012), MSM (Vargas et al. 2010), single heterosexuals (Thigpen et al. 2012), and people who inject drugs (Choopanya et al. 2013). The Partners PrEP Study demonstrated 67–75% protection against HIV acquisition (Baeten et al. 2012), with the potential for greater protection (>90%) associated with detectable levels of tenofovir in blood plasma samples (Donnell et al. 2014). In addition, serodiscordant couples had high adherence to PrEP [median 97%, IQR 91–100%] by electronic monitoring] in the context of regular clinical

follow-up and with the support of an adherence intervention (Haberer et al. 2013; Ware et al. 2015). PrEP can provide protection from HIV if the infected partner fails treatment or from another HIV-infected partner not taking ART.

The best way to offer PrEP in a discordant relationship is currently unknown. The maximal benefit might be realized if early ART is offered to an infected partner and PrEP is offered to an uninfected partner for up to 6 months, until the infected partner achieves viral suppression (Hallett et al. 2011; Baeten et al. 2016). PrEP has also been used as additional protection during planned conception, when the infected person (generally the male partner) is treated and the woman receives PrEP. However, given the near-perfect protection afforded by treatment of an infected person, long-term PrEP for a discordant HIV-negative partner in a monogamous relationship would be of limited additional benefit (and see modeling discussion below).

6.2.5 Mathematical Modeling of the Cost-Effectiveness of Prevention Strategies

For stable discordant couples, immediate ART for the HIV-infected partner is likely to be cost-effective or potentially cost-saving (Hallett et al. 2011; Walensky et al. 2013). However, despite high efficacy, TasP will likely not reduce all HIV acquisition within discordant couples, particularly given that in some settings, an observed 20–30% of HIV incidence originates from external partnerships (Celum et al. 2010). In addition, achieving viral suppression on ART may take up to 6 months for most HIV-infected individuals (Cohen et al. 2011), leaving a window of vulnerability for HIV-uninfected partners. As noted above, one potentially feasible and effective opportunity for oral PrEP use in couples is as a “bridge” to ART initiation; the combination of prevention strategies reduced incidence among high-risk couples by an estimated 96% in the Partners Demonstration Project, based on a counterfactual simulation of expected infections in the absence of universal ART and PrEP (Baeten et al. 2016). However, for low-risk couples (i.e., incidence of 2/100 PY), oral PrEP may only be cost-effective if HIV-infected partners initiate ART immediately and universally, as PrEP is, in general, most effective and cost-effective when used for a short period of time during a temporary period of increased risk (Jewell et al. [forthcoming-a, b](#)). When targeted to high-risk couples, using PrEP as a bridge to ART initiation may be cost-saving under certain circumstances (Jewell et al. [forthcoming-a, b](#); Ying et al. 2015). However, the absolute number of infections averted by PrEP in even the most optimistic scenarios is small compared to those averted by ART over a long time horizon (Jewell et al. [forthcoming-a, b](#)).

Cost-effectiveness modeling studies are dependent on assumptions surrounding the epidemiology of HIV infection, the sexual behavior of couples, and the economic costs of ART, PrEP, and identifying couples for prospective interventions. For example, the ratio of the cost of PrEP relative to the cost of ART is highly

influential for determining the cost-effectiveness of providing PrEP to discordant couples (Ying et al. 2015). If clinic costs are reduced through task-shifting, antiretroviral costs are negotiated to be based on local prices rather than ceiling costs, or if monitoring costs shift to being point-of-care rather than clinic-based, cost-effectiveness calculations could also change substantially. In addition, potentially major barriers to cost-effectiveness (e.g., costs of identifying high-risk couples) are not included in most modeling studies. While antiretroviral-based interventions may still be cost-effective when paying a premium to find high-risk couples, outreach costs could ultimately reduce the cost-effectiveness of an intervention (Jewell et al. *forthcoming-a, b*). Furthermore, delays in identifying couples could severely decrease the impact of TasP or PrEP, as a proportion of high-risk couples would have seroconverted by the time they were identified by the health system (Jewell et al. *forthcoming-a, b*).

These studies underscore that approaches to managing stable serodiscordant couples will need to be flexible and specialized. Not all strategies will be applicable for all types of couples at all points in time. As the epidemic changes, the recommendations for the management of stable serodiscordant couples will also need to be dynamic and responsive to changes with the partnerships themselves, as well as the development of new technologies and methods of antiretroviral administration.

6.2.6 Contraceptive Needs of Discordant Couples

In addition to management of HIV prevention, discordant couples may also have a concurrent need for contraceptive methods (Heffron et al. 2012). In sub-Saharan Africa, hormonal contraception is popular among women; an estimated 46% of women in 2012 were using injectables or implants, with a further 21% using oral contraceptives (Darroch and Singh 2013). Hormonal contraceptives – in particular, injectable depot-medroxyprogesterone acetate (DMPA) – have been linked to an increased risk of HIV transmission, which could have important implications for HIV prevention (Morrison et al. 2009; Heffron et al. 2012; Polis and Curtis 2013). However, results across studies have been mixed, and there have been concerns about bias in some cases (e.g., confounding, lack of follow-up, and small sample sizes) (Schwartz et al. 2013). If injectable hormonal contraception does increase the risk of HIV transmission, continuing use could lead to excess new HIV infections among women, though discontinuation of hormonal contraception could also lead to unintended pregnancies and additional maternal or neonatal mortality (Butler et al. 2013). Women's preferences for contraceptive methods should also be accommodated for when optimizing HIV prevention strategies, and safe and effective contraception alternatives should be made available.

6.2.7 Future Antiretroviral-Based Options for Discordant Couples

Long-acting injectable ART and PrEP may be particularly attractive to discordant couples by minimizing the impact of adherence, reducing the frequency of clinic visits, and potentially improving cost-effectiveness (Walensky et al. 2016b). Several candidates for injectable ART and PrEP have shown promise in early studies. A 2016 study of combination injectable cabotegravir and rilpivirine for ART-naïve patients showed that 94–95% of individuals achieved viral suppression after 32 weeks, compared to 91% of those on oral therapy (Margolis et al. 2017). Cabotegravir and rilpivirine have also shown promise as candidates for quarterly PrEP dosing (Andrews and Heneine 2015; Jackson and McGowan 2015) and are being developed for testing in phase III trials. If an effective long-acting form of injectable PrEP is developed, a few injections of PrEP might be used for the HIV-uninfected partner in a couple as a bridge to viral suppression for the HIV-infected partner initiating ART.

6.3 Treatment for Prevention at the Population Level

The realization that effective ART reduces sexual HIV transmission risk in serodiscordant couples led to early and persistent calls to use HIV treatment as prevention. These were manifested in editorial form (Montaner et al. 2006), influential mathematical models (Blower et al. 2000; Abbas et al. 2006; Granich et al. 2009), and a policy recommendation (“The Swiss Statement”) (Vernazza et al. 2008). But broader scale-up of treatment as prevention remained controversial for several reasons: (1) lack of compelling empirical data (prior to the HPTN 052 study) and (2) questions surrounding the risks and safety about early ART initiation (Walensky et al. 2009). The “when to start” question remained unresolved until the completion of HPTN 052 and, more recently, the INSIGHT START (INSIGHT START Study Group and Lundgren 2015) and TEMPRANO ANRS 12136 (Group 2015) studies.

CD4 count served as the threshold for initiation of ART until 2016. When ART was first developed, it was believed that the cost and toxicity of the treatment exceeded the health benefits until falling CD4+ T-cell count indicated more immediate risk of infection or neoplasm. However, randomized trials demonstrated risk much earlier in HIV infection and irrevocable damage to the immune system without treatment. In 2016 guidelines, the US Department of Health and Human Service (Council 2016), the USA Panel of the International Antiviral Society (Society-USA 2016), and the World Health Organization (WHO) (World Health Organization 2016) all called for universal and immediate ART with detection of infection, regardless of stage of infection or CD4+ T-cell count (Organization 2013).

The combination of accepted health benefits and public health advantages of ART led to the 90-90-90 targets issued by UNAIDS in 2014. This program is based on the concept of a “care cascade”(Gardner et al. 2011) which breaks down by stage the process of linkage into care, from HIV testing to viral suppression. The 90-90-90 targets call for 90% of all people living with HIV know their status, 90% of people with diagnosed HIV to be on ART, and 90% of treated individuals to achieve viral suppression by 2020. The targets aim for a viral suppression rate in all HIV-infected people of 73%, the threshold believed necessary to reduce the basic reproductive number to below 1.0 – thereby stopping further spread of HIV – by 2030 (UNAIDS 2014).

6.3.1 Combination Prevention

The biological plausibility of ART for HIV prevention has generated renewed interest in the potential impact of universal test-and-treat (UTT) interventions. By shifting the focus of prevention from targeting “at-risk populations” of uninfected people to those already infected with the virus, treatment as prevention represents a paradigm shift in HIV prevention (Hayes et al. 2014). But the magnitude of potential benefit from this program remains controversial, as will be discussed in the modeling section below.

In addition, no single prevention method is an adequate means of stopping the HIV epidemic. Studies of UTT scale-up have been developed to incorporate individual prevention strategies into large combination prevention “packages” that include other proven HIV preventive methods. The simultaneous use of several partially protective interventions is thought to provide a more effective alternative, as it can impede HIV transmission at multiple points in the “transmission cycle” (Fig. 6.1). It can also leverage potentially additive or synergistic effects of multiple interventions. This approach, known as “combination prevention,” has been defined by UNAIDS as:

The strategic, simultaneous use of different classes of prevention activities (biomedical, behavioral, social/ structural) that operate on multiple levels (individual, relationship, community, societal), to respond to the specific needs of particular audiences and modes of HIV transmission, and to make efficient use of resources through prioritizing, partnership, and engagement of affected communities. ((UNAIDS) 2009)

Combination strategies commonly include voluntary HIV counselling and testing, safer sex counselling (including condom distribution), screening and treatment for sexually transmitted infections, and ART for prevention (Kurth et al. 2011), in addition to many other possible interventions. An advantage of combination approaches is the flexibility to tailor packages to address the specific transmission dynamics of a particular setting.

A second challenge is in the design of prevention packages to address transmission risk at multiple levels that also account for local epidemiological features. For

$R_0 = \beta c D + \beta c \Gamma$ <p> β: transmission probability per contact c: rate of partner change D: mean duration of infectiousness Γ: mean partnership duration </p> <p>Individual-level interventions</p> <ul style="list-style-type: none"> • Reduce susceptibility: vaccine, broadly neutralizing antibodies, medical male circumcision, STI treatment, pre-exposure prophylaxis • Reduce transmissibility: STI treatment, suppressive ART • Reduce effective contacts: delay sexual debut, condom use, reduce number of sexual/drug using partners, reduce frequency of equipment sharing <p>Dyad/couple-level interventions</p> <ul style="list-style-type: none"> • Reduce age-mixing/intergenerational sex/drug use partnerships, reduce partner concurrency <p>Structural-level interventions</p> <ul style="list-style-type: none"> • Address gender-based income differentials: conditional cash transfers • Laws & Policies criminalizing homosexuality, commercial sex, drug use and possession of drug paraphernalia • Improving healthcare access, particularly in marginalized populations
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Fig. 6.1 Levels of HIV prevention interventions to consider for combination prevention

one, evidence of an effect in one setting is no guarantee that it will readily transfer to other epidemiological or cultural circumstances (Geffen et al. 2014). Findings from studies of HIV combination prevention effectiveness (many launched by the HIV Prevention Trials Network [HPTN] or the Methods for Prevention Package Program [MP3]) can also be difficult to interpret given wide variation in package composition and study settings. Modeling exercises have attempted to extract lessons to inform package assembly by disentangling the effects of individual tools within a package (Cummings and Mehta 2016) or by comparing the hypothetical effectiveness of various package compositions in a single setting (Anderson et al. 2014). As with all mathematical models, findings must be interpreted in light of the necessary trade-offs between model complexity and real-world relevance, in particular with the assumption that each particular prevention tool prevents HIV as effectively when implemented in combination as it does in isolation.

In addition, it is hoped that an HIV vaccine will become part of a combination prevention package. A randomized controlled trial (RV 144) appeared to demonstrate a vaccine with 1 year of 37% protection (Rerks-Ngarm et al. 2009). A repeat trial designed to reproduce or improve upon these findings (HVTN 702) has started (HIV Vaccine Trials Network 2016). Recent modeling exercises suggest a vaccine 50% effective with between 40 and 80% coverage would have a bigger effect than the 90-90-90 program. However, in the absence of such a vaccine, combination prevention must proceed as planned (Dimitrov et al. 2015).

In light of these challenges, assembly of an efficacious HIV prevention package that is also safe, acceptable, and sustainable will likely rely not only on scientific evidence but also on pragmatism, judgment (Piot et al. 2008), and on what Jones and colleagues refer to as “the art of public health” (Jones et al. 2014).

6.3.2 Community Randomized Controlled Trials to Inform TasP

Three ongoing (HIV Prevention Trials Network; of 2013; Chan et al. 2015) and one completed (Inserm-ANRS) community randomized controlled trials (RCTs) are expected to provide critical insight into the effectiveness of large-scale UTT interventions (Tables 6.1 and 6.2). Trial outcomes will be valuable both to provide evidence of UTT efficacy and to offer critical insight into implementation requirements and to inform best practices about testing and linkage to care. At the least, results are hoped to help determine the minimum threshold of ART uptake and care retention needed to impact population-level HIV incidence.

Investigators in KwaZulu-Natal have previously reported a strong correlation between provision of ART to a community and reduced incidence of HIV (Tanser et al. 2013). Results from this observational study inspired ANRS 12249 Treatment as Prevention Trial in the same province (Iwuji et al. 2013). The study was completed in 2016, and no detectable difference in HIV incidence between communities randomly assigned to immediate treatment and those treated according to the then-current South African standard of care was observed (Hayes et al. 2017). However, logistical issues including uneven recruitment across age and sex (men and younger individuals were systematically undersampled), low retention, and equivalent ART uptake across study arms all compromised the ability of the study to properly address the treatment as prevention hypothesis. In addition, sexual mixing with partners from beyond the study area is also thought to have diluted the protective effects of ART in intervention communities.

HPTN071 (“PopART”) (Hayes et al. 2014) is being implemented across 21 communities in Zambia and South Africa comprising an estimated 1.2 million residents. The three study arms compare 3-year HIV incidence to assess the effect of two prevention packages, one of which administers immediate ART and the other that treats patients according to national guidelines. HIV incidence is monitored in a smaller “incidence cohort” making up about 20% of randomly sampled households in each study village. HPTN071 study objectives also include operations research question assessing the feasibility, acceptability, cost-effectiveness, and sustainability of the proposed intervention and the use of viral molecular phylogeny to better interpret results (see below) (Hayes et al. 2014, 2017).

The Sustainable East Africa Research in Community Health (SEARCH) trial (of 2013) in Kenya and Uganda features a “Collapse the Cascade” model of streamlined HIV care that uses a model of community health – including multi-disease care, a patient-centered to HIV, text message technology, and individualized adher-

Table 6.1 Full-scale community randomized trials of universal test and treat

Trial name, duration	Sponsors	Site	Study design	Intervention (combination components)	Exposure assessment	Study outcome
HPTN 071 (PopART); 2012–2017	HIV Prevention Trials Network	Zambia, South Africa (21 communities; total study population size estimate = 1.2 million)	Triplet-matched, 3-armed community randomized trial	VCT (home-based testing); “active” linkage to care; referral to ART initiation in HIV-infected individuals (Arm A, immediate referral (regardless of immunological or clinical staging); Arm B, referral based on national guidelines); medical male circumcision referrals; behavioral counselling and condom promotion; TB screening; PMTCT referral services. [Control arm: existing prevention and testing services; ART eligibility according to national guidelines]	HIV viral loads and self-reported ART adherence among the <i>population cohort</i> , a random sample of 2500 adults (one from each household) from each study community	HIV incidence as inferred from HIV prevalence assessed on an annual basis for 3 years in the <i>population cohort</i> , a random sample of 2500 adults (one from each household) from each study community

(continued)

Table 6.1 (continued)

Trial name, duration	Sponsors	Site	Study design	Intervention (combination components)	Exposure assessment	Study outcome
Sustainable East Africa Research in Community Health (SEARCH); 2013–2018	Led by University of California, San Francisco; funded by the US President's Emergency Plan for AIDS Relief (US CDC)	Kenya, Uganda (32 communities; total study population size estimate = 300,000)	Pair-matched, 2-phased, 2-armed community randomized controlled trial	Targeted PrEP, targeted VCT (hybrid mobile testing and home-based testing), targeted care model (immediate ART regardless of immunological or clinical staging; point-of-care CD4 testing; streamlined care delivery); and annual community health campaigns (multi-disease screening, hypertension and diabetes screening, malaria rapid diagnostic testing for participants with fever, male condom distribution, referral for medical male circumcision, family planning services, and cervical cancer screening for women). [Active comparator arm: community-based VCT; immediate ART regardless of immunological or clinical staging; streamlined care delivery model; baseline multi-disease testing]	ART patients followed up for medical assessments monthly for the first 3 months and then quarterly thereafter. Viral load was assessed at enrollment and at 6 and 12 months	Cumulative 3-year HIV incidence in men and women ages ≥15 years, assessed by finger-prick blood-based HIV antibody testing

<p>Botswana Combination Prevention Project (BCPP); 2013–2017</p>	<p>Led by the Harvard AIDS Initiative; funded by the US President's Emergency Plan for AIDS Relief (US CDC)</p> <p>Botswana (30 communities; total study population size estimate = 180,000)</p>	<p>Pair-matched, 2-armed community randomized controlled trial</p>	<p>VCT (hybrid mobile testing and home-based testing); point-of-care CD4 testing; linkage to care support; expanded ART (for CD4 counts of ≥ 551 cells/μL or higher, viral load of $\geq 10,000$ copies/mL, as well as local criteria); retention/adherence counseling; enhanced male circumcision services. [Active comparator arm: “enhanced” care model: receive guidance and improved technical support for quality management and data systems]</p>	<p>Coverage parameters (intervention uptake) measured in 20% of the sample taken from the household and end of study surveys and program monitoring data</p>	<p>Cumulative 3-year HIV incidence measured via longitudinal follow-up of community-based cohort of ~9000 adults enrolled from a random (~20%) sample of community households. Incident infections evaluated by phylogenetic linkage to assess likelihood of infection acquisition from inside an intervention village</p>	<p>HIV incidence measured at 24 months through repeat longitudinal HIV testing of blood samples collected on DBS during the home visits</p>
<p>ANRS 12249</p>	<p>Led by the Africa Centre for Health and Population Studies; funded by the French National Agency for Aids and Viral Hepatitis Research (ANRS) and Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ)</p>	<p>KwaZulu-Natal province of South Africa; total study population size estimate = 34,000</p>	<p>2-phased, 2-armed community randomized controlled trial</p>	<p>VCT (home-based testing), immediate ART regardless of immunological or clinical staging; pre-ART care and positive prevention services for HIV-positive individuals not on ART; care and treatment for HIV-related morbidities; linkage to care support; adherence counseling; TB/STI screening. [Control arm: ART eligibility per the 2011 SA and 2010 WHO guidelines]</p>	<p>ART initiation is tracked at the individual level, and patients receiving treatment are followed on a monthly basis</p>	<p>HIV incidence measured at 24 months through repeat longitudinal HIV testing of blood samples collected on DBS during the home visits</p>

Table 6.2 Insights from community randomized controlled trials (RCTs) on the effectiveness of large-scale UTT interventions

Trial	Time point of assessment	First 90: knowledge of HIV status (among HIV+ people) (%)	Second 90: receiving ART (%)	Third 90: virologically suppressed (%)
HPTN 071 (or “PopART”) ((PEPFAR) et al. 2017)	Data up to 08/2016	86.0	60.0	90.0
BCCP(Gaolathe et al. 2016)	2 years post intervention	83.3	87.4	96.5
SEARCH (Petersen et al. 2016)	2 years post intervention	97.0	94.0	90.0
ANSR 12249 (Iwuji et al. 2017)	End of study (4 years)	88.6	34.9	92.8

ence counseling – to improve population suppression. The trial is divided into two phases, the first to compare the streamlined UTT strategy with the standard of care and the second to examine the impact of additional prevention package components such as targeted PrEP and targeted viral suppression interventions at annual health fairs. A central focus of the SEARCH trial is to investigate not only health effects but also the economic and educational impacts of these interventions. Preliminary results from SEARCH suggest an exceptional level of coverage, reaching 82% viral suppression after the first 2 years of the trial (Petersen et al. 2016).

Last among the UTT trials, the Botswana Combination Prevention Program (BCCP) (Control) covers 30 rural and peri-urban sites across the country. In response to shifting national treatment guidelines, the study protocol has already been amended once to offer immediate treatment to all HIV patients, rather than according to CD4+ T-cell count. Plans by the Botswana Ministry of Health to provide universal ART in 2016 are expected to soon affect non-intervention communities as well. The same “incidence cohort” technique used by PopART is applied to assess HIV transmission rates throughout the study period; however, incident infections are also evaluated using viral genetic linkage to assess the proportion that are likely to have originated from an intervention site, rather than from outside the communities (see below).

6.3.3 Use of Viral Genetic Information: Phylogenetics and Phylodynamics

Viral genetic information can be used to evaluate the time of acquisition of HIV and the relationship between a person with HIV infection and their partners (Volz and Frost 2013). When sequences are sufficiently similar, it is possible to identify a

linked transmission (Eshleman et al. 2011), although it has been difficult to prove “directionality” and to exclude the possibility of a third partner who may be responsible for transmission.

The use of viral phylogeny is potentially important in assessing the benefits and limits of TasP. If TasP is working well, dominant sequences from infected people may be reduced in incident cases of infection. Conversely, if migration is common, new sequences might be introduced from people living outside a treated community. Grabowski et al. noted that people living in fishing villages who receive less attention were responsible for fewer infections than previously assumed in other distant communities (Grabowski et al. 2014). Working in Botswana, Novitsky et al. have used phylogenetics to track the number of circulating lineages of HIV-1C, which causes the most severe disease, in order to guide balancing of different components of HIV combination packages (Novitsky et al. 2017).

6.3.4 *Challenges of UTT*

Practical lessons from the cumulative implementation experience amassed from the UTT trials and the global shift toward reaching the UNAIDS 90-90-90 targets have brought to light several of the following novel challenges.

6.3.4.1 *Acceptability of Repeat Testing*

Success of UTT strategies will hinge on universal and routine HIV testing by a sufficient proportion of the at-risk population. Consistent testing is particularly critical for curtailing onward transmissions from individuals with early HIV infection, who may as a group be responsible for up to a third of new infections in some settings (Powers et al. 2011). Novel approaches to increase testing uptake, such as home-based testing (Organization et al. 2012), testing from mobile units (Morin et al. 2006; van Schaik et al. 2010), and self-testing (Organization 2016), among others, have all been shown to improve HIV testing in key populations. What is yet to be seen is the acceptability and uptake of HIV testing over the course of repeated campaigns, in some cases as often as twice a year. If testing fatigue degrades uptake rates over time, or if rates of testing attrition vary across groups – e.g., if higher risk individuals become more reluctant than others to sustain routine testing – a treatment-based prevention method cannot be expected to deliver hypothesized results over the long term. The existing UTT paradigm also generally proposes annual screenings, though findings from a mathematical model set in KwaZulu-Natal, South Africa, suggest that a province-wide testing campaign conducted every 5 years (along with treatment uptake consistent with the 2013 WHO eligibility guidelines) would cost-effectively halve new infections within 10 years (Ying et al. 2016). The ANSR 12249 trial is exploring the social impact of such strategies by tracking perceptions and attitudes toward repeat testing in trial participants over the course of the study (Orne-Gliemann et al. 2015).

6.3.4.2 ART for Healthy Individuals

As more countries adopt WHO treatment guidelines or pursue UTT strategies to achieve the 90-90-90 targets, a larger proportion of individuals with higher CD4+ T-cell counts are expected to initiate ART. These patients are often younger, healthier, and generally asymptomatic, raising concerns that they will have lower motivation to practice strong medication adherence and retention in care (Brown et al. 2016; Bigna et al. 2016). A prospective study of treatment outcomes in HIV patients with CD4+ T-cell counts over 350 in rural Uganda (the Early Antiretroviral Therapy in Resource Limited Settings in Patients with High CD4+ Cell Counts Study) reported high patient retention, self-reported adherence, and viral suppression after 2 years of follow-up. The results are encouraging but must be considered together with the relatively modest sample size ($N = 197$) and specialized features of the care delivery model that was used, which included a streamlined, nurse-driven system that provided patients with unprecedented access to clinicians (participants were given his/her clinician's mobile phone numbers) (Jain et al. 2014). Data from a second analysis of the same study population further suggests that earlier ART may also yield economic benefits by keeping treated adults in the workforce longer and minimizing costly morbidities (Thirumurthy et al. 2013).

6.3.4.3 Missing Men

Interim results from the HPTN 071 and BCCP trials both report lower uptake of testing in men (Gaolathe et al. 2016; Hayes et al. 2017). Trial procedures are partly responsible for this sampling bias, as community-based outreach generally calls on homes during the day when working-age men are less likely to be reachable. However lower rates of male engagement in HIV care have long been observed in African settings (Mills et al. 2011; Johnson et al. 2013; Nsanzimana et al. 2015) and are attributed to factors such as higher migratory needs in men, differential patterns in health-seeking behavior (Courtenay 2000; Jewkes and Morrell 2010; Mills et al. 2012), and lack of reliable life events such as pregnancy during which most women undergo HIV testing (Gunn et al. 2016). Improved engagement of men into HIV care is not only needed for more valid measurement of UTT effects in trials but will also be fundamental to stopping HIV transmission in epidemics sustained by heterosexual sex.

6.3.4.4 Mobility and Mixing

One reason cited by the ANSR 12249 study for lack of detected effect is due to individuals acquiring HIV from those outside of their study region. Whether this may be due to high in- and out-migration of people from the trial area or preferential sexual mixing of trial participants with those in non-intervention sites is yet to be determined. Results from an observational study in Rakai noted the importance of

HIV-infected people living in fishing villages with higher mobility and who are less likely to be treated (Grabowski et al. 2014). However, interim results from the BCCP study suggest that sexual mixing across communities in other settings in sub-Saharan Africa may not be common enough to undermine TasP efforts (Novitsky et al. 2017).

6.3.4.5 Financing and Staffing Considerations

The resource intensity of UTT strategies is twofold: first, expanded access to ART dramatically increases the number of people eligible for care and treatment; second, effective treatment will considerably extend survival among treated individuals. Beyond the direct costs of antiretroviral drugs are the demands the TasP strategy places on human and physical resources (e.g., routine viral load testing for larger patient pools) (Scott Braithwaite et al. 2014; Baernighausen et al. 2016). Human resource constraints are particularly difficult in settings plagued by structural issues such as the lack of higher quality medical schools in low- and middle-income countries (Labor 2009) or high rates of emigration among qualified healthcare providers (i.e., “brain drain”) (Tankwanchi et al. 2013; World Health Organization Assembly 2013). Task-shifting has demonstrated success in many settings to reduce the level of training and support required, though success of these programs has been shown to depend on regular supervision from experienced ART providers (Bedelu et al. 2007).

Several models have examined the economic costs and benefits of treatment as prevention strategies, all of which demonstrate the cost-effectiveness of this approach in the long run (Granich et al. 2012; Eaton et al. 2014; Jain et al. 2015; Walensky et al. 2016a). The optimism of such findings stands in stark contrast to the reality of plateauing HIV/AIDS funding since 2008 and the lack of political consensus on the considerable funds that would need to be mobilized to close the treatment gap and the optimal way to apply such funds (Dutta et al. 2015; Atun et al. 2016). In place of traditional aid-based approaches, some teams have proposed alternatives models, such as fee-based programs where patients bear some of the cost of care (Kakaire et al. 2016) or the use of a HIV allocative efficiency model to guide program prioritization and funding (Shattock et al. 2016).

6.3.4.6 Community Perceptions of Test and Treat

Rollout of community UTT trials and strategies has also afforded insights into the lived experience of treatment as prevention at the community level. One of the earliest groups poised to benefit from the scientific discovery that ART stops sexual HIV transmission has been discordant couples, to whom results of the HPTN 052 study were most transferable. In reality, however, the first step to preventive treatment requires status disclosure to one’s partner, for which participants in the SEARCH trial described many barriers, including risk of violence, abandonment, or couple

dissolution (Maeri et al. 2016). Beyond this, HIV and the use of treatment to prevent HIV appear to be concepts couched within the broader framework of personal concerns about personal health (Bond et al. 2016), love, and moral behavior (Viljoen et al. 2016). These conceptualizations of ART and treatment as prevention as lived by individuals in the high prevalence regions may provide critical insight into cognitively acceptable and culturally appropriate messaging of public health interventions.

6.3.5 Mathematical Modeling

In the formative years of research on ART for HIV prevention, several transmission models were greatly influential in pushing the TasP research agenda (Cohen and Gay 2010). Results of the HPTN 052 study have solidified consensus on the centrality of suppressive ART in future HIV control efforts. Yet questions persist regarding the feasibility of achieving the necessary program coverage or practical considerations of program implementation given limited resources. To address such questions, a new wave of mathematical models dedicated to exploring key aspects of program implementation have emerged (Table 6.3).

Overall functions of these models fall into four key areas. First, models can help inform country decisions regarding assembly of optimal and locally adaptable prevention packages by disentangling effects individual prevention tools comprising a combination package (Charlebois et al. 2011; Marshall et al. 2012; Brookmeyer et al. 2014; Abuelezam et al. 2016). Multiple simulations can also predict the potential effects of incomplete coverage of a given intervention. Some models also compare the relative impact of key parameters on model outputs in order to identify modifiable process indicators expected to disproportionately impact program success. A model by Hallett and Eaton, for example, identifies multiple pathways to care among HIV patients, suggesting that a more nuanced version of the “cascade of care” would allow program managers to target alternative channels to care to improve future health benefits of ART (Hallett and Eaton 2013). Related models have explored the relative importance of other processes such as care retention (Bendavid et al. 2009), behavior change in response to treatment (Cori et al. 2014), and development of drug resistance (Duwal et al. 2015).

Third, as continually broader treatment recommendations are released by the WHO, countries will have to confront difficult decisions regarding allocation of treatment efforts in the face of finite resources. Models comparing treatment effects in various risk groups (Charlebois et al. 2011; Marshall et al. 2012; Kato et al. 2013; De Vos et al. 2014) (including discordant couples and pregnant women) (Stover et al. 2012) provide policy makers with much needed insight into the trade-offs of various targeted strategies.

An argument for more focused testing has recently put forth. In a modeling exercise focused on HIV prevention in Lesotho, Coburn et al. argue that maximal benefit

Table 6.3 Characteristic of mathematical models assessing the impact of ART on population HIV incidence, published since the landmark HPTN 052 study in 2011

	Authors (year)	Setting (population)	Goals	Model structure	Findings	Limitations/acknowledged assumptions
1	Abuelezam et al. (2016)	South Africa (general)	To assess the potential for HIV elimination when preventative ART is used together with non-treatment-focused programs (condoms, male circumcision)	Agent-based, simulation	HIV elimination is possible in 50 if all available interventions are perfected and combined. A test-and-treat model alone cannot achieve elimination due to transmissions occurring during acute infection	No accounting for role of adolescents who acquired HIV through maternal transmission at birth; rates of intervention uptake stay constant over time (no accounting for participant fatigue); UTT rates of testing and treatment uptake set at idealized levels
2	Bendavid (2010)	South Africa (general)	To assess the extent to which UTT scale-up strategies will be hindered by potentially suboptimal linkage to care or loss to follow-up	Stochastic, transmission	UTT scale-up without changes in linkage to care is sufficient to substantially reduce HIV-related mortality and new HIV infections, but measures to address linkage to care and loss of follow-up can nearly double the benefits of UTT	Lower HIV transmission risk in infected individuals on ART; no behavioral risk modification with decreasing disease burden; stable fertility rate over course of assessment period
3	Brookmeyer et al. (2014)	South Africa (general)	To assess the potential efficacy of a TasP-based combination strategy targeting MSM	Agent-based, simulation	Of the various combinations of each of four package components (universal HIV testing, ART for infected individuals with CD4 < 350, behavioral interventions to reduce unprotected anal intercourse, and PrEP) those that included both behavioral and biomedical components were the most effective at reducing HIV incidence	Forecast limited to 5 years; regular and main partners did not change over the 5 years of the simulation; no accounting for transmission within and across other risk groups; infectiousness remained fixed over the course of disease; closed population

(continued)

Table 6.3 (continued)

	Authors (year)	Setting (population)	Goals	Model structure	Findings	Limitations/acknowledged assumptions
4	Charlebois et al. (2011)	San Francisco (MSM)	To measure the impact of strategies to treat all HIV patients already in care, a strategy that would require no additional investment in outreach or expanded HIV testing	Deterministic, simulation	Expanding reduced ART to all HIV-infected persons could reduce new HIV infections by 59% in 5 years. If HIV test expansion was added, new infections would fall by 76% in the same amount of time	Acutely infected persons are not explicitly modeled; effects of drug resistance on infectiousness are not accounted for in the treated population
5	Cori et al. (2014)	Zambia and South Africa (general)	To predict the impact of the intervention package to be delivered during the HPTN 071 (PopART) trial, with extensive uncertainty and sensitivity analysis to quantify the influence of process variables	Deterministic, compartmental	Trial interventions could reduce HIV population-level incidence by 60% over 3 years, but wide variability across uncertainty analyses supports the need for a trial	Model does not account for age structure and non-heterosexual sex and assumes independence between risk group and the propensity to have sexual contacts outside of the community. The model also does not include selection and transmission of drug-resistant strains of virus
6	de Vos et al. (2014)	Amsterdam (PWID)	To evaluate the impact of existing ART coverage in observed and hypothetical PWID populations	Agent-based	ART has only led to 2% decrease in new infections, though a TasP strategy could substantially lower new infections, especially in a younger epidemic. Treatment leads to far greater reductions in incidence compared to syringe exchange	Uptake of services not stratified by risk group; role of arrest or incarceration not modeled

7	Duwal et al. (2015)	South Africa (general)	To assess the impact of ART on HIV transmission under two different treatment paradigms (a “diagnostic-guided” and a “proactive” treatment strategy)	Coarse-grained stochastic model of within-host viral dynamics	Both treatment paradigms perform better than the current clinical protocols in reducing new HIV transmissions, and the diagnostic-guided strategy performs nearly as well as the proactive approach	Relative estimates of secondary transmissions did not require explicit modeling of underlying transmission dynamics nor risk behaviors such as sexual contacts
8	Eaton J et al. (2012)	South Africa (general)	To compare a common set of outputs across 12 independent models that assessed the potential effects of a standard set of UTT scenarios	8 deterministic (HIV portfolio, STI-HIV Interaction, BBH, CD4 HIV/ART, Eaton, Granich, Goals, Fraser) and 4 microsimulation models (EMOD, STDSIM, synthesis transition, Bendavid)	All 12 models broadly agreed on short-term epidemiologic impacts of UTT scale-up, but diverged in terms of longer-term projections. Differences between model predictions could not be explained by differences in model structure or parameterization that were hypothesized to affect intervention impact	All models assumed high efficacy of ART to reduce transmission; only one model explicitly accounted for the role of drug resistance on compromised treatment efficacy
9	Goldman et al. (2014)	USA	To estimate the effects of early ART initiation observed in the US from 1996–2009 on HIV incidence during the same time period	Deterministic, compartmental	Early ART (initiating at CD4 cell count above 350) is responsible for 188,000 averted infections, among which “very early” ART (initiating above 500) accounted for 80% of averted infections	Model does not account for transmission through same-sex partnerships or injection drug use; sexual partnerships are assumed to form at random

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Table 6.3 (continued)

Authors (year)	Setting (population)	Goals	Model structure	Findings	Limitations/acknowledged assumptions
10 Goodreau et al. (2012)	USA and Peru (MSM)	To estimate the proportions of transmissions occurring in main vs. casual partnerships, as well as by sexual role, stage of infection, and testing and treatment history of the infected partner	Network, simulation (2 parallel models that vary by assumptions about stage-specific infectiousness)	Regardless of country setting (USA or Peru), a minority of HIV transmission results from contacts with acute-stage partners, far more stem from contact with chronically infected partners. Roughly two thirds of infections are attributed to casual partnerships	Temporal variability in treatment effectiveness assumed to be fixed; all causal partnerships modeled as “one-offs” without any repeated contact; contact with female sex partners not modeled; male sex work and transgender identity also not modeled
11 Heaton et al. (2015)	Africa (general)	To estimate the impact to date to PEPFAR’s support for ART, PMTCT, and male circumcision in Africa on the number of HIV infections averted over 10 years	Spectrum model	\$42 billion in cumulative funding helped avert 2.9 million HIV infections and helped avert the orphaning of almost 9 million children	No counterfactual modeled; non-biomedical interventions such as behavioral and structural interventions were not included as part of the evaluation
12 Hontelez et al. (2013)	South Africa (general)	To compare 9 structurally different mathematical models to test the robustness of a previously reported finding that a UTT strategy could eliminate HIV in South Africa in 7 years	Models range in complexity and realism, ranging from deterministic to stochastic microsimulation approaches	All models predict eventual elimination of HIV, but the more complex among them indicate elimination will occur at a far later date (20–25 years from intervention start)	Development and transmission of drug-resistant strains not modeled; model only sought functional elimination (incidence below 1/1000 person-years) instead of actual elimination
13 Jansson et al. (2014)	Australia (general)	To estimate the impact of a potential TasP strategy in Australia	Agent-based, simulation	Treatment of all HIV-infected people with CD4 < 500 will reduce new infections by 30.9%. Overall program performance depends on its effectiveness in MSM and on levels of risk compensation	Non-biomedical interventions not assessed; effects of population-level drug resistance not accounted for in the model

14	Kato et al. (2013)	Vietnam (various)	To assess the impact of a UTT strategy on HIV incidence with and without targeting subpopulations (MSM, PWID, FSW, clients of FSW, and general population) with condom promotion and methadone maintenance	Deterministic, compartmental	A UTT approach that targets each population will achieve an 81% reduction in new HIV infections and lead to HIV elimination in 14 years	Model did not account for clean needle exchange; information on ART efficacy for reducing parenteral HIV transmission risk still uncertain
15	Marshall et al. (2014)	New York Metropolitan Area (PWID)	To assess which interventions should be included when considering combination strategies for TasP in PWID (whole population includes PWID, those who take drugs through non-injection modes (pills, smoking, snorting, etc.), and those who do not use drugs)	Agent-based, stochastic	A “high-impact” combination strategy (UTT in addition to improved access to substance abuse treatment and increased use of needle and syringe programs) may reduce HIV incidence by 62% as compared to the status quo	Stable rates of engagement in risk behavior as HIV progressed; no accounting for sex work, the types of drugs used, and comorbidities such as infection with hepatitis C virus or STIs; no accounting for interventions that might affect those who do not use drugs (e.g., STI screening, UTT, etc.)
16	Sood et al. (2013)	Los Angeles, USA (MSM)	To assess the potential impact of an expanded UTT strategy in MSM	Deterministic, compartmental	Expansion of an UTT in MSM will lead to a 34% reduction in new infections over 10 years. A near doubling in the prevalence of multidrug-resistant HIV is also thought of	No stratification by risk, ethnicity, nor age; model does not consider potential effects of PrEP. Risk behavior among ART initiators assumed constant

(continued)

Table 6.3 (continued)

Authors (year)	Setting (population)	Goals	Model structure	Findings	Limitations/acknowledged assumptions
17 Stover et al. (2014)	25 countries ranging in endemicity	To investigate the effects of scaling up to WHO guidelines, TasP, PrEP, and an HIV vaccine	Deterministic, compartmental	Immediate implementation of the 2013 WHO antiretroviral therapy guidelines could reduce new HIV infections by 80%. Further reductions may be achieved by moving to a “test-and-treat” approach, PrEP, and an HIV vaccine	No consideration of age structure or drug resistance
18 Tao et al. (2017)	Chaoyang District, Beijing (general)	To investigate the effects of ART coverage scale-up on HIV incidence. Risk groups include MSM, male and female sex workers, PWID, transgender individuals, and members of the general population	Deterministic, compartmental	Overall ART coverage of 50% leads to a decline in new infections by 1.6% while 90% coverage leads to a 15.0% decline	Risk behaviors remain fixed irrespective of ART initiation; retention in care is assumed perfect; drug resistance not considered; study area restricted to a single district of a large metropolitan city

of TasP can only be realized if spatial dispersion of infected people is considered and that an infection “density” criterion is used to direct resources (Coburn et al. 2013).

Lastly, models developed in conjunction with ongoing programmatic efforts are useful in guiding the collection of data that can provide a fuller understanding of the key mechanisms driving treatment as prevention (Hallett and Eaton 2013).

6.3.6 Natural Experiments in Treatment as Prevention

Either by circumstance or due to shifts in national guidelines seeking to achieve the 90-90-90 targets, a large amount of observational data has become available for assessing the effectiveness of UTT-like situations occurring in non-trial settings. Here, we review several of the most notable observational studies that have furthered our understanding of the prevention benefit of ART in the real world, as well as several common limitations of such approaches.

A well-known investigation of treatment as prevention in the real world involves population-level effects observed over a 7-year period in KwaZulu-Natal, South Africa (Tanser et al. 2013). The study found that communities in which ART coverage reached at least 30–40% were associated with reduced rates of HIV infection as compared to communities where <10% of HIV-infected individuals were being treated with ART. However, there remains the risk of residual confounding, through either direct effects (i.e., communities with high ART coverage may also have benefited simultaneously from the greatest risk reduction) or indirect effects (i.e., epidemic dynamics may be such that in communities where the epidemic is naturally declining, ART coverage is higher due to a greater proportion of individuals having late-stage disease).

In British Columbia, Canada, a prospective cohort study of people who inject drugs (PWID) cited evidence of a strong preventive effect of ART based on associations detected between HIV incidence and median viral loads measured among PWID with HIV (a widely used and potentially problematic measure referred to as community viral load (Miller et al. 2013). Beyond the concerns of mis-measurement common to many ecological studies (Smith et al. 2012), some have questioned whether the declines in incidence observed in PWID in British Columbia were not at least partially due to behavioral interventions to reduce injection-related HIV risk (Grulich and Wilson 2010). Indeed, findings from a mathematical model comparing HIV and HCV trends among British Columbia PWID suggest that most of the declines in HIV incidence in the same time period can be attributed to intensive harm reduction, with only marginal reductions brought about by ART (Fraser et al. 2016).

Two examples of observational studies of treatment as prevention in MSM come from Denmark and Australia. An analysis of data from the Danish HIV Cohort Study used CD4 back-calculation methods to infer annual incidence rates, which was found to be strongly correlated with estimated proportions of MSM on treat-

ment over time (Okano et al. 2016). The study from Australia, the ongoing Australian “TAIPAN” study (Treatment with Antiretrovirals and their Impact on Positive And Negative men), consists of large (>10,000) HIV-positive and HIV-negative cohorts of MSM who will be tracked to assess temporal associations between community prevalence of viremia and the incidence of HIV infection (Callander et al. 2016). As with the Wood et al. study, both analyses evaluate TasP effectiveness by examining statistical associations between treatment coverage and HIV incidence, an approach that conflates cause for association and can overlook the potential effects of other secular trends (Smith et al. 2012).

As part of their national HIV control efforts, the country of Sweden claims to have already achieved their 90-90-90 targets (Gisslen et al. 2016). Though results from this higher-income setting may not be directly generalizable to more high priority settings such as sub-Saharan Africa, lessons from the Sweden case study underscore the importance of consolidated treatment and monitoring guidelines and the utility of the care cascade (Gardner et al. 2011) as a framework for monitoring the epidemic.

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Part II

HIV Cure

Chapter 7

HIV Persistence on Antiretroviral Therapy and Barriers to a Cure



Julia Marsh Sung and David M. Margolis

Abstract HIV persists within the body despite successful suppression of virus replication with antiretroviral therapy (ART). HIV lurks in latent and active reservoirs, leading to rebound of virus spread if ART is interrupted. The latent HIV reservoir is a natural consequence of the life cycle of HIV, with integration of HIV into the genomes of cells that are or later enter the resting state, resulting in transcriptionally quiescent provirus. Resting CD4 T cells comprise the majority of the latent reservoir, although new evidence points to additional, smaller cellular reservoirs of latent HIV. An alternate, so-called active reservoir of HIV also exists within cells such as those found in the B cell follicle of lymph nodes, where expression of HIV RNA can be found, again despite the full suppression of viremia and viral replication. Multiple factors such as the degree of virus exposure, timing of ART, and host factors can influence the size and characteristics of the HIV reservoir. Constructing effective strategies for HIV eradication and measuring their impact will require a sophisticated knowledge of the HIV reservoir.

Keywords Latent reservoir · Persistent infection · Central memory resting CD4 T cell

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7.1 Introduction

The discovery of highly active antiretroviral therapy (ART) in the mid-1990s revolutionized the treatment of HIV, turning a once fatal disease into a manageable chronic condition. Although highly potent antiretroviral therapy is capable of completely suppressing viral replication in plasma below the level of detection, thus halting spread of the virus, it does not cure HIV. In patients who are successfully suppressed by ART, cessation of ART leads to a predictable pattern of rebound of plasma viremia within weeks in most individuals (Chun et al. 2010; Chun et al. 1997b).

7.1.1 *Consequences of a Persistent HIV Reservoir*

The persistence of HIV despite ART has clinically relevant consequences. ART is associated with a significant cost (estimated at \$326,500 over the lifetime of an HIV-infected individual (Schackman et al. 2015; Schackman et al. 2006)), side effects, and ongoing stigma. ART is imperfectly deployed, and so does not completely prevent person-to-person spread of infection, and in the treated individual does not fully restore normal immune function. This persistent immune dysfunction, characterized by low-level inflammation, is currently associated with increased risks of comorbidities such as cardiovascular and neurologic disease and malignancy (Psomas et al. 2016; Sinha et al. 2016; Feinstein et al. 2016; Hunt et al. 2016; Muyanya et al. 2016; Siedner et al. 2016a, b).

7.1.2 *The Life Cycle of HIV*

The life cycle of HIV yields important clues into the mechanisms of HIV persistence despite complete suppression of replication and the major cellular sources of persistence. HIV preferentially infects activated CD4 T cells through engagement of the HIV-1 envelope with the CD4 receptor and a chemokine co-receptor (most commonly, CCR5, and less frequently CXCR4), leading to fusion of the virus and host cell membranes and entry of viral RNA and proteins into the cell. HIV RNA is rapidly reverse transcribed into double-stranded proviral DNA, relying on prepackaged viral proteins carried within the virion into the host cell and upon the use of host machinery. This complex of proviral DNA with virus and host cell proteins forms the pre-integration complex (PIC). The PIC is transported into the nucleus and integrated into the host genome. Integration results in on average one copy of DNA per infected cell. Integration sites are random, but influenced by the state of the chromatin, with HIV more likely to integrate into chromatin that is in a more “open” and heavily transcribed state, due in part to interactions with host factors such as LEDGF (Kvaratskhelia et al. 2014). In activated T cells with high levels of active transcription of the genome, viral mRNA is transcribed from the proviral

genome, translated into protein and packaged into viral particles ready to burst from the host cell membrane, leading to cell death via viral cytopathic effects, beginning a new round of infection. During active replication, at least in ex vivo culture, this process is exponential, with one infected cell capable of producing anywhere from an estimated 1,000 to 50,000 virions (Chen et al. 2007; Hockett et al. 1999). Unchecked HIV infection leads to a vicious cycle of activation and infection, accelerated by widespread activation and cell death of bystander, uninfected CD4 T cells (Doitsh et al. 2014; Ho et al. 1993). The average life span of an actively infected CD4 T cell is short, with a half-life of approximately 0.7 days (Ho et al. 1995).

ART halts virus replication and spread by targeting HIV at different stages of the life cycle. With modern ART regimens that include integrase inhibitors, complete virus suppression to below the limit of detection in the peripheral blood can be rapidly achieved within a matter of weeks. However, the cure that would be predicted given the half-life of 0.7 days of productively infected cells is not observed. Viral rebound occurs almost uniformly in those who stop ART. This is because a small but non-negligible fraction of CD4 T cells harboring proviral DNA are in a resting rather than activated state. These infected resting CD4 memory T cells still contain HIV provirus integrated into the host genome, but because the cell environment is transcriptionally repressive, the HIV provirus is largely transcriptionally silent. In this state, HIV viral proteins cannot be detected, and the infected cells appear unrecognized by the host immune system. However, transcription of HIV provirus from these latently infected resting CD4 T cells occurs infrequently and perhaps transiently, due either to stochastic events or following the encounter of a latently infected cell with cognate antigen or stimulatory cytokines or chemokines (Chun et al. 1997b). In the absence of ART, such events can be amplified and lead to rebound of plasma viremia.

The latent reservoir within the resting CD4 T cell population is long-lived (see Table 7.1 for a list of key terms and definitions). Two rigorous studies examining the decay of the replication-competent latent reservoir independently identified the half-life of infected resting CD4 T cells as 43 and 44 months, respectively (Crooks et al. 2015; Siliciano et al. 2003). Based on this stable half-life, eradication of HIV virus from the body would take over 70 years on ART alone. HIV cure efforts have therefore centered on two possible strategies:

1. Decreasing the size of the latent reservoir, through:
 - (a) Early treatment.
 - (b) Purging the latent reservoir from the body through strategies that rely on a latency-reversing agent to induce viral expression, thereby exposing the infected cell, and strategies that augment the clearance of infected cells. This two-step approach has been termed “kick and kill.”
 - (c) Excising the latent reservoir through gene-editing techniques such as CRISPR/Cas, a technology still in its infancy.
2. Inducing a state of “super latency,” through a so-called “block and lock” strategy aimed not at eradicating virus from the body, but rather at preventing the existing virus from reactivating from the latent state

Table 7.1 List of key definitions

Term	Definition
Anatomic sanctuary	A site that may provide a sanctuary for the active reservoir, putatively due to either poor ART penetrance or poor access by cytotoxic immune effector cells
Active reservoir	Persistently infected cells that continue to transcribe HIV RNA, presenting HIV antigen
Homeostatic proliferation	Proliferation of cells under the regulation of cytokines such as IL-7 and IL-15 that allows for the self-renewal and expansion of a small population of cells
Latent reservoir	Intact HIV provirus that is transcriptionally silent, but capable of being induced to produce replication-competent viral particles
Latency-reversing agent	An intervention that reverses latency by inducing latently infected cells to produce detectable viral particles
Productive infection	HIV infection with active transcription and production of viral particles
Replication-competent virus	The majority of HIV DNA that is measured represents defective virus of unclear relevance to cure efforts and thus overestimates the size of the latent reservoir. Replication-competent, inducible virus, in contrast, represents integrated HIV provirus that is capable of producing virions that will propagate further spread of infection in the absence of ART. The current gold standard to measure replication-competent virus is the quantitative viral outgrowth assay (see below and Chap. 11), but this assay underrepresents the true size of the latent reservoir

These strategies will be covered in more depth in Chap. 12 “Clinical Interventions in HIV Cure.”

7.2 The Latent Reservoir

7.2.1 Cellular Reservoirs: Resting CD4 T Cells

HIV most efficiently infects activated CD4 T cells that have the transcriptional machinery necessary to promulgate the HIV life cycle, but subsequently die relatively quickly due to viral cytopathic effects. The latent reservoir, however, resides in long-lived resting CD4 T cells. The latent reservoir may likely be established when a small but non-negligible fraction of activated, infected CD4 T cells transition back to a resting state, after integration of HIV into the genome but prior to the production of sufficient viral particles to cause cell death (Siliciano et al. 2003). Recent in vitro studies employing a dual-labeled HIV-1 reporter virus, however, suggest that direct infection of resting CD4 T cells is possible and can lead to latent but inducible infection upon subsequent stimulation (Calvanese et al. 2013; Chavez et al. 2015). These studies confirmed that activated CD4 T cells were more permissive for HIV infection than resting CD4 T cells, leading to a higher number of both active productively infected and latently infected cells, but also showed that cells that were infected in the resting state were more likely to lead to latent infection

(Chavez et al. 2015). To what extent this phenomenon may occur in vivo is still unknown, although some have argued that the presence of integrated HIV DNA in naïve CD4 as well as memory CD4 T cells suggests direct infection of resting CD4 T cells as a contributing mechanism to the establishment of latency (Archin et al. 2014; Ostrowski et al. 1999; Josefsson et al. 2013). However, other studies have found HIV DNA but failed to recover significant levels of replication-competent latent HIV virus from naïve CD4 T cells (Soriano-Sarabia et al. 2014).

HIV DNA can be found largely in the central and transitional memory CD4 T cell subsets (Chomont et al. 2009; Josefsson et al. 2013). However, replication-competent, inducible HIV virus is largely restricted to the central memory compartment (Soriano-Sarabia et al. 2014). An examination of different resting CD4 T cell subsets, including naïve, central memory (T_{CM}), and transitional memory (T_{TM}), revealed that the majority of the replication-competent, inducible reservoir resides in the central memory CD4 T cell population (Soriano-Sarabia et al. 2014). In fact, latent infection was so enriched in this population; in two individuals in whom no virus was recovered on culturing of total resting CD4 T cells because of low reservoir size, culturing of isolated central memory T cells was able to yield virus. In contrast, replication-competent virus decayed relatively rapidly in the transitional memory compartment within months of ART initiation, although HIV DNA continued to be detected, suggesting the accumulation of predominantly defective HIV provirus within this compartment (Soriano-Sarabia et al. 2014).

Less well-differentiated CD4 T cell subsets and novel, long-lived T cell subsets have also been identified as potential contributors to the latent reservoir. CD4 memory stem cells (T_{SCM}), a very long-lived and poorly differentiated CD4 T cell that is thought to represent the earliest identified stage of memory T cell development, have also been found to harbor integrated HIV DNA within their genome (Buzon et al. 2014). These long-lived CD4 T_{SCM} were susceptible to infection by HIV, but less vulnerable to killing by viral cytopathic effects, creating what might be ideal conditions for the establishment of a latent reservoir. HIV-1 DNA was found in the CD4 T_{SCM} subsets in ART-suppressed HIV-infected individuals, accounting for approximately a median of 8% of the total viral reservoir in CD4 T cells as measured by DNA. This contribution was increased in those with smaller T_{CM} reservoirs and also increased over time on suppressive ART. Whether the HIV DNA present in the CD4 T_{SCM} subset is, in part at least, replication-competent has not yet been clearly demonstrated.

7.2.2 *Beyond CD4 T Cells: Other Cell Populations*

Because CD4 T cells are the primary target of infection by HIV, resting CD4 T cells are the best characterized latently infected cell and comprise the majority of the latent reservoir. However, expression of surface receptors necessary for HIV infection, such as CD4 and CCR5, can be transiently expressed on other cell types. Induced expression of CD4 under the right circumstances could enable HIV infection of cell types beyond the canonical CD4 T cell. In order for the infected cell to be

considered a latent reservoir, it must also be a long-lived cell without detectable HIV expression prior to induction but able to spread infection following induction. Identification of latent replication virus in cell types beyond resting CD4 T cells may have significant implications for cure strategies, as the different cell populations may respond differently to latency-reversing agents, and require different clearance strategies.

As proof of this, a recent rigorous study revealed the presence of an inducible, replication-competent reservoir within peripheral $\gamma\delta$ T cells (Soriano-Sarabia et al. 2015). In contrast to conventional $\alpha\beta$ T cells, such as CD4 T cells, $\gamma\delta$ T cells do not traditionally express CD4 on the cell surface and represent only a minor fraction of T cell subsets. In the presence of an acute viral infection such as HIV, however, $\gamma\delta$ T cells expand rapidly as part of the innate immune response, upregulate CD4 upon activation, and can subsequently revert to a long-lived memory subset, much like resting memory CD4 T cells. The frequency of replication-competent, latent virus was relatively high in this cell subset, reaching as high as 1 in just 5000 $\gamma\delta$ T cells in some ART-suppressed, HIV-infected individuals (Soriano-Sarabia et al. 2015), indicating that although $\gamma\delta$ T cells make up only a small percentage of the total T cells, they harbor a relevant proportion of the latent reservoir.

Macrophages and monocytes have long been hypothesized to harbor a latent HIV reservoir. Newer studies have shown that macrophages are capable of self-renewal and revalidated productive HIV infection of macrophages, albeit at lower levels than CD4 T cells (Sieweke and Allen 2013). This productive infection of macrophages persists despite ART (Arainga et al. 2017). However, whether such cells harbor transcriptionally silent integrated virus that is capable of reactivation—a latent reservoir of similar characteristics to the memory CD4 cell—is still unknown. Developing *in vitro* assays to investigate the possible presence of an inducible, quiescent latent reservoir in macrophages, similar to what has been performed for CD4 T cells, has been technically challenging (Archin et al. 2014). Animal models may provide more definitive evidence regarding the possible role of persistent infection of macrophages on ART (Archin et al. 2014; Honeycutt et al. 2016; Arainga et al. 2017). The “MOM” (myeloid-only mouse) model, a variation of the bone-marrow-liver-thymus (BLT) mouse that, as its name implies, only has myeloid but not T cells, is being used in current ongoing studies to address this question (Honeycutt et al. 2016).

7.3 Maintenance of a Stable Latent Reservoir

Just how the latent reservoir sustains itself over decades with such a slow decay is a fascinating problem that many groups are attempting to unravel. The presence of low-level, residual viremia in the plasma of some patients despite ART sparked the hypothesis that the latent reservoir could be replenished through ongoing low-level replication despite ART. Very sensitive assays are capable of detecting low-level viremia in the plasma, below the limit of detection of commercial assays (Palmer et al. 2008). This low-level residual viremia most likely stems from cells infected

prior to initiation of ART. Sequences from CD4 T_{CM} and T_{SCM} show phylogenetic similarities to sequences of plasma virus obtained from individuals on suppressive ART therapy (Buzon et al. 2014). Because HIV replication is error prone, active replication of HIV is associated with dramatically rapid mutation rates. Rapid mutation with viral diversification is seen when replication is unchecked by ART. In contrast, viral sequences do not show evidence of evolution once ART has been initiated (Josefsson et al. 2013; Kearney et al. 2014; Kieffer et al. 2004), suggesting there is no ongoing low-level replication. Furthermore, ART intensification studies have on the whole not shown a reduction in this low-level replication (Gandhi et al. 2012; Tiraboschi et al. 2017; Dinoso et al. 2009; Gandhi et al. 2010; Hatano et al. 2011).

Proliferation of latently infected cells, driven either by natural, homeostatic proliferation or due to dysfunctional proliferation triggered by the disruptive presence of an HIV provirus within the gene body of a cell cycle control gene, has also been invoked as a mechanism of sustaining the long-lived, stable reservoir (Chomont et al. 2009; Bailey et al. 2006; Wagner et al. 2014; Maldarelli et al. 2014). Homeostatic proliferation, driven by cytokines such as IL-7 and IL-15, can induce proliferation of T cells without reactivation of virus (Bosque et al. 2011), as can exposure to mitogens in a fraction of cells (Hosmane et al. 2017; Ho et al. 2013), making this explanation plausible.

Clonal proliferation of T cells harboring HIV genomes integrated in or near host genes associated with cell cycle control could also lead to maintenance of the reservoir. Consistent with this, residual viremia is often dominated by clonal sequences (Tobin et al. 2005; Bailey et al. 2006), and sequencing of proviral DNA also reveals clonal expansion of identical viral sequences (Bailey et al. 2006; Bruner et al. 2016). The contribution of a clonally expanded population to the inducible, replication-competent latent reservoir is not yet clear. While some studies have indicated the majority of HIV DNA detected in clonally expanded populations represents the accumulation of defective provirus incapable of making viral particles (Bruner et al. 2016), other studies have clearly shown in at least some instances, latently infected cells with replication-competent HIV DNA can clonally expand, producing progeny cells capable of reactivating HIV virus (Hosmane et al. 2017; Simonetti et al. 2016). Both observations may be true, as it appears that even rare proliferation of replication-competent provirus, superimposed on the durability of memory cells, may be enough to maintain the longevity of the viral reservoir.

7.4 Correlates of Size of the Latent Reservoir

Various factors have been associated with the size of the latent reservoir. This may have implications for cure strategies for two reasons: 1) if a smaller reservoir is founded in early infection prior to the implementation of ART, that HIV-infected individual is much closer to eradication; and 2) designing strategies aimed at further reducing the size of the reservoir may be informed by the factors that we already know can restrict the size of the reservoir, if these factors can be intentionally replicated.

Initiation of ART early in the course of HIV infection has been associated with a smaller reservoir size when examining multiple metrics of reservoir size, including measurement of DNA by PCR and of replication-competent inducible latent virus by the quantitative virus outgrowth assay (Soriano-Sarabia et al. 2014; Siliciano et al. 2003; Chun et al. 1997a; Ananworanich et al. 2016a; Robb and Ananworanich 2016; Jain et al. 2013; Archin et al. 2012). This is related to the amount of virus exposure, or the area under the curve, composed of both the magnitude of the viral load (peak and set point) and the duration of viremia prior to suppression with ART (Archin et al. 2012). More recent studies based in countries with relatively high rates of HIV incidence have been able to push the boundaries of early treatment, with twice weekly monitoring for HIV viral load in high-risk HIV-negative individuals allowing for the identification of individuals infected with HIV as early as prior to peak viral load being attained (Crowell et al. 2016; Ananworanich et al. 2016b; Ndhlovu et al. 2015).

Other factors that have been found to be associated with the reservoir size include the CD4 nadir (Boulassel et al. 2012) and initiation of treatment during infancy in perinatal-acquired infection (Upadhyay et al. 2017; Rainwater-Lovett et al. 2017). Levels of immune activation have also been correlated with the size of the reservoir, although causality is difficult to tease out in this situation.

7.5 Viral Diversity Within the Latent Reservoir

A potential major obstacle to immune-mediated clearance of the latent reservoir aside from impaired effector cell phenotype is the remarkable ability of HIV to rapidly mutate to evade T cell responses (Goonetilleke et al. 2009; Liu et al. 2013; Deng et al. 2015; Papuchon et al. 2013). As cells infected with this diverse array of HIV variants enter into latency, the index, “wild-type” sequence, and variants that have escaped from existing T cell responses (escape variants) are archived in the latent reservoir, ready to evade immune responses that are not equipped to handle the escape variants. To what extent this will need to be taken into account when formulating strategies for an HIV cure is an area of intense investigation.

7.6 Other Sources of HIV Persistence: The Active Reservoir

In contrast to the latent reservoir, consisting of latently infected long-lived cells that are transcriptionally silent, the active reservoir may represent a population of cells that continue to support varying degrees of productive virus transcription despite ART. Because of their potential to contribute to virus rebound on cessation of ART, understanding sites and characteristics of active reservoirs is important for HIV cure efforts.

The best described active reservoir that fits this definition is the T follicular helper CD4 cell (T_{FH}). T_{FH} are specialized T helper cells localized to the B cell follicle of the lymph node and characterized by coexpression of CXCR5 and PD-1. T_{FH} cells are highly susceptible to HIV infection and replication due to both cell intrinsic factors as well as colocalization within the B cell follicle with follicular dendritic cells, which can facilitate cell-to-cell transmission of virus (Heesters et al. 2015). A recent rigorous study identified outgrowth of replication-competent virus from T_{FH} cells isolated from the lymph nodes of ART-suppressed HIV-infected individuals (Banga et al. 2016).

How productive infection is sustained in the face of ART is a widely debated topic. Some have pointed to certain anatomic sites, such as the lymph node, CNS, and gut as providing anatomic sanctuaries for ongoing HIV replication due to hypothesized poor penetration of ART into these tissues. However, measuring the active form of ART—which are often intracellular metabolites—can be challenging and hinders accurate analysis of this problem. Further, if there is ongoing replication in drug-poor areas, it would be expected that drug resistance would randomly evolve during replication and then be selected when this virus was later exposed to ART in another anatomic locale. The lack of the widespread emergence of drug resistance, therefore, suggests that if ongoing replication does occur, it is vanishingly rare. As detailed above, there is little to no evidence of ongoing active viral replication in the presence of ART. It is possible, instead, that productive infection may be ongoing and maintained in long-lived cells in the absence of virus spread and replication and without inducing viral cytopathic-mediated cell death. Anatomic location in immune-privileged sites such as the B cell follicle may allow these productively and persistently infected cells to evade detection by the immune system (Paiardini and Licherfeld 2016). Examination of other putative sites of anatomic sanctuary, such as the CNS, for rigorous evidence of the existence of an active reservoir, has been technically challenging (Hellmuth et al. 2015).

The active reservoir is an evolving concept in the HIV field. Understanding sources of persistence are important when designing trials evaluating immunotherapeutic and therapeutic vaccines for HIV cure. Therapeutics aimed at enhancing the immune response to HIV must be able to impact these sanctuaries—directly or indirectly. However, as active reservoirs already maintain productive, transcriptionally active infection, their relevance in trials aimed at reversing latency lies only in how an impact of latency-reversing agents on degree of productive infection in these cells could confound interpretation of the results of this study. For example, if a latency-reversing agent is found to increase plasma HIV RNA levels, is the source of this RNA truly from previously transcriptionally silent latently infected cells or due to an increase in transcription in the already productive active reservoir? Untangling these effects will be a difficult but important task of HIV cure studies going forward and can be aided by in-depth, sophisticated sequencing approaches (Barton et al. 2016).

7.7 The Latent HIV Reservoir in Special Populations

The establishment and maintenance of the latent reservoir are subject to influence from complex biologic systems. Beyond biology, complex social and behavioral factors also influence transmission of HIV and access to and engagement in care (and hence duration of viremia) (Johnston and Heitzeg 2015). How different demographics (age, gender, race) may influence characteristics of the latent reservoir or response to latency-reversing agents (Johnston and Heitzeg 2015) is still an evolving field of research. If an HIV cure will be scaled up to the general, heterogeneous, population, it will be imperative to understand these differences.

7.7.1 Infants/Children

Out of all of the special populations outlined below, the dynamics and characteristics of the latent HIV reservoir in infants are perhaps the most well-characterized and have generated the most interest in the research community. Because of vastly different immune landscapes between infants and adults, with relatively small populations of memory T cells in the blood in neonates, the kinetics of HIV infection and clinical outcomes are also different (Martinez-Bonet et al. 2015b). For example, peak plasma viral load is sustained over a longer time period when HIV infection is acquired prenatally and is associated with a more rapid progression to AIDS (Mofenson et al. 1997).

The “Mississippi child,” who received ART as early as 30 hours of age and experienced control of HIV infection for 27 months after ART was stopped (Persaud et al. 2013; Luzuriaga et al. 2015), has provided many valuable insights into the reservoir in infants. Although initial excitement over the potential cure was tempered by her rebound 27 months after ART cessation, this case has galvanized research into the area of cure for perinatal-acquired HIV infection.

One of the lessons of the Mississippi child case that has been born out in studies in animal models is how early the latent reservoir is established. Treatment as early as 30 hours in the Mississippi child may have been sufficient to restrict the size of the latent reservoir to the point where a transient remission was attainable, but was still not early enough to prevent establishment of the latent reservoir that can serve as a source of rebound virus following ART cessation. Early treatment initiated in the more realistic realm of 1–3 months of age is still associated with a smaller reservoir size, with an average infectious units per million resting CD4 T cells of just 0.32 (Rainwater-Lovett et al. 2016; Rainwater-Lovett et al. 2017; Persaud et al. 2012), but still with rebound of virus upon cessation of ART (Martinez-Bonet et al. 2015b). Many infants treated early in the course of infection also lack HIV-specific T cell and antibody responses, presumably as a consequence of early control of viral replication (Shah et al. 2014; Martinez-Bonet et al. 2015a). This knowledge has sparked current efforts to investigate combining early and prolonged treatment of perinatal infection with therapeutic vaccine strategies.

7.7.2 Aging

The prevalence of HIV infection in those over 50 years of age is rapidly increasing (Luther and Wilkin 2007). Interestingly, although historically older individuals are less likely to participate in HIV clinical trials in general (Manfredi 2002), the median age of HIV-infected individuals participating in complex and demanding phase I cure studies at our center tends to be higher than that of the general population of HIV-infected individuals. How older age may influence measurements of the latent reservoir, and response to therapy, is still unclear. Age is known to play a role in mediating clinical outcomes in HIV infection and response to treatment with ART (High et al. 2012). Older age is associated with a higher risk of progression to AIDS or death upon ART initiation, less robust recovery in CD4 count following ART initiation, a faster viral load rebound on cessation of ART, and increased measures of immune activation (Egger et al. 2002; Kalayjian et al. 2013; Sabin et al. 2009; Luther and Wilkin 2007; Kolte et al. 2002; Goodkin et al. 2004). Some studies have linked this to a smaller thymus size with reduced capacity for thymopoiesis (Kalayjian et al. 2013; Kolte et al. 2002; Rickabaugh et al. 2011), as well as social and behavioral factors that lead to presentation for care at lower CD4 counts and more advanced disease (Luther and Wilkin 2007; Althoff et al. 2010a, b). On the other hand, once diagnosed with HIV infection, older patients are more likely to achieve durable viral suppression (Johnston and Heitzeg 2015).

With a different immunologic and virologic starting point at the time of ART initiation, aging may impact the size of the latent reservoir and the response of older participants to immunotherapeutic approaches in curative strategies. Whether this is true is currently unstudied and unknown, as the majority of HIV cure-related studies do not examine age as an independent variable (Johnston and Heitzeg 2015).

7.7.3 Women

Although women comprise half of HIV-infected individuals worldwide, they are vastly underrepresented in HIV cure studies (Curno et al. 2016). This is not a unique problem to HIV cure research. It is an increasingly recognized problem that despite the fact that disease phenotype and treatment response differ between men and women in many areas, women are underrepresented in biomedical research across many fields (Zakiniaeiz et al. 2016; Raz and Miller 2012). In a recent systematic review examining the representation of women in publications of 132 cure-related studies between 1995 and 2012, of the 79% reporting the sex of participants, 29% did not include any women, and only 21% of participants overall were women (Curno et al. 2016). This number did not increase over time.

Over the past 20 years, there has been a push to more rigorously identify and address sex bias in biomedical research at every stage of research, as early as the preclinical stage (Zakiniaeiz et al. 2016; Clayton and Collins 2014). This includes the more transparent identification of sex in cell and animal models. Progress

remains slow however; as recently as 2009, 1 study found that 80% of published rodent studies spanning 10 fields of biomedical research included the use of only male animals, unchanged from numbers 20 years earlier (Beery and Zucker 2011).

Sex-based differences exist in acquisition, progression, severity, and response to treatment of HIV infection. Routes of transmission differ: women are more likely to experience transmission through the lower genital tract (LGT) via penile-vaginal transmission, rather than via other mucous membrane surfaces (anal, oral). This may have implications for characteristics of transmitted virus and reservoir establishment in the LGT and upper genital tract. Persistent shedding of virus from the LGT has been observed in women despite suppressive ART (Neely et al. 2007). Women also have lower viral loads and higher CD4 counts during active viremia, but, unlike what would be predicted based on natural history from studies of men, they have faster disease progression than men at similar viral loads. Women also have faster recovery of CD4 counts and lower mortality rates on treatment. There are also differences in pharmacokinetics and pharmacodynamics in women taking ART, leading to increased and different adverse event profiles and likely contributing to differences in treatment adherence.

Extending this to the HIV cure field specifically, new data has emerged suggesting that we may also encounter sex-based differences in reservoir characteristics and response to treatment. Viral load area under the curve is associated with reservoir size in individuals treated during acute infection (Archin et al. 2012). Whether and how the lower viral load observed in women prior to treatment may influence the size of the latent reservoir are not yet known. Estrogen has known impacts on HIV replication and spread, and so differences in estrogen levels may impact HIV pathogenesis, reservoir establishment and maintenance, and response to latency-reversing agents. Women produce higher and more variable levels of estrogen compared to men. While estrogen levels in men range from 10 to 50 pg/ml, levels in women oscillate during the menstrual cycle between lows of 25–80 pg/ml to peaks of 80–300 pg/ml (Alonso and Rosenfield 2002). Estrogen can increase the expression of CCR5 in CD4+ T cells, thus facilitating HIV infection of CD4+ cells (Mo et al. 2005), while at the same time, it can also reduce the cytotoxicity of NK cells (Hao et al. 2007), limiting the innate immune response's ability to contain infection. Estrogen can also have direct effects on HIV transcription through the formation of a repressor complex between β -catenin and estrogen receptor- α at the HIV promoter (Szotek et al. 2013). HIV viral load in untreated women can vary depending on the timing of the menstrual cycle, with lower plasma viral loads measured during the follicular phase of the cycle when estrogen levels are at their peak (Greenblatt et al. 2000). Early in vitro studies also suggest that estrogen may decrease and impair the efficacy of some latency-reversing agents (Karn 2015), although further work is necessary to confirm these findings.

Many barriers impede the study of women in the HIV cure field, including social and behavioral factors (Johnston and Heitzeg 2015) as well as obstacles inherent to the experimental nature of HIV cure efforts. For example, the predominance of small, experimental phase I trials in examination of interventions at this stage in HIV

research likely contributes to the lower proportion of women enrolled, especially women of childbearing age: the tolerance for risk is lower in this population, and some of the most promising latency-reversing agents cannot be used in this group due to possible genotoxicity seen in some animal models. It is clear from existing work, however, that understanding the latent reservoir and how to purge it in women cannot be directly derived from studies of men. Identifying gender as a variable in HIV cure studies will be critical as the field moves closer to a cure.

7.7.4 *Race*

Race can modulate many factors related to HIV care and treatment, both from a biologic perspective as well as due to complex socioeconomic factors. Race and ethnicity, including as seen in African-Americans and Hispanic/Latinos, are associated with differences in rates of CD4 decline and viral load at time of ART initiation, later diagnosis and engagement in care, and lower rates of viral suppression (Smith et al. 2003; CDC 2012).

HLA alleles, which can cluster variably according to race and ethnicity, can play a large role in influencing the immune response to natural infection and vaccines as well as viral escape patterns (Gartland et al. 2014). HLA type can predict either spontaneous viremic control, in so-called elite controllers, as is seen with HLA-B*27 or HLA-B*57 alleles, or faster disease progression, such as is seen with HLA-B*35. HLA-B*5701, for example, is less prevalent in those of African descent than in white individuals (Saag et al. 2008).

Similar to biomedical research in general, nonwhite individuals are underrepresented in current HIV cure-related studies. In the United States, 67% of HIV-infected individuals are nonwhite, with African-Americans and Hispanics/Latinos experiencing a disproportionate burden of disease (CDC 2016). In a systematic review of 151 publications through 2013, only 30% included information on race, and of those, only 23.6% of participants overall were nonwhite (Johnston and Heitzeg 2015).

7.8 Conclusion

Over the past two decades, a wealth of evidence has been brought to light about the latent HIV reservoir. Although resting CD4 T cells provide the greatest contribution to the latent reservoir, emerging evidence points to an increasingly complex composition of the latent reservoir, with minor but significant contributions from multiple distinct cell subtypes and tissues. Additionally, HIV-infected individuals represent a heterogeneous population, and how different demographics such as age, gender, and

race may impact the latent reservoir is an evolving area of research. Host-related differences, and within a host, cell and tissue reservoir differences, may influence responses to treatments aimed at eradicating HIV virus, so fully understanding the landscape of the latent reservoir will be critical to achieving an HIV cure.

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Chapter 8

The Molecular Biology of HIV Latency



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Abstract HIV remains incurable due to the existence of a reservoir of cells that harbor intact integrated genomes of the virus in the absence of viral replication. This population of infected cells remains invisible to the immune system and is not targeted by the drugs used in the current antiretroviral therapies (cART). Reversal of latency by the use of inhibitors of chromatin-remodeling enzymes has been studied extensively in an attempt to purge this reservoir of latent HIV but has thus far not shown any success in clinical trials. The full complexity of latent HIV infection has still not been appreciated, and the gaps in knowledge prevent development of adequate small-molecule compounds that can effectively perturb this reservoir. In this review, we will examine the role of epigenetic silencing of HIV transcription, posttranscriptional regulation, and mRNA processing in promoting HIV-1 latency.

Keywords Latency · Transcriptional interference · mRNA processing · Tat-P-TEFb

8.1 Introduction

As all viruses, the HIV-1 depends on host cells to complete its replication life cycle. After entry via specific cell surface receptors, the viral RNA genome is reverse transcribed, thanks to the viral reverse transcriptase and cellular elements, into a complementary DNA. During this step, a duplication of sequences at both ends of the proviral DNA [called 5' and 3' long terminal repeats (LTRs, Fig. 8.1)] takes

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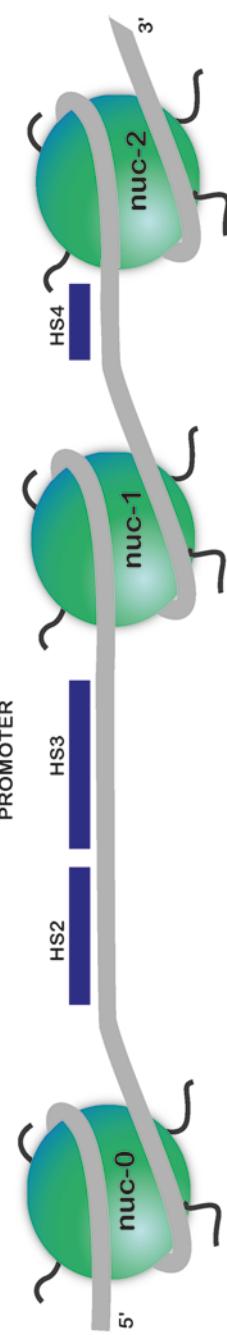
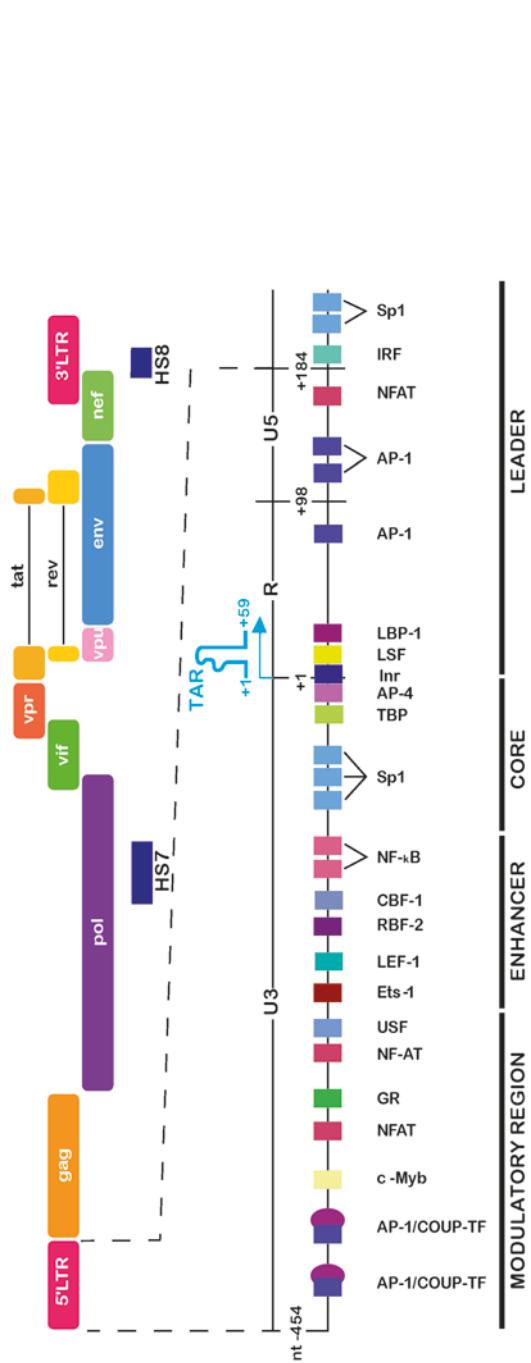


Fig. 8.1 Schematic representation of the HIV-1 5'LTR. The HIV-1 genome is represented at the top of the figure. Major hypersensitive sites (HS, blue boxes) located through the viral provirus and nucleosomal organization of the HIV-1 genome 5'LTR region are indicated. The U3, R, U5, and leader regions are indicated. Nucleotide 1 (nt +1) is the transcription start site of HIV-1. The four functional domains involved in transcriptional regulation as well as the transcription factor-binding sites identified in these domains are shown. See text for details (This figure is adapted from Colin and Van Lint, Retrovirology 2009, with permission from BioMed Central)

place. Despite their similar sequences, the two LTRs play different roles: the 5'LTR functions as the viral promoter and the 3'LTR as the termination site during transcription. HIV-1 is a complex retrovirus encoding, in addition to the structural genes common to all retroviruses (*gag*, *pol*, and *env*), several so-called “regulatory” and “accessory” genes (*vif*, *vpr*, *tat*, *rev*, *vpu*, *nef*) (Fig. 8.1). After integration into the host cell genome, HIV-1 viruses replicate using the cellular transcriptional and translational machineries to synthesize viral messenger RNAs (mRNAs) and proteins, respectively. In contrast to simple retroviruses, HIV-1 generates, in addition to unspliced and singly spliced mRNAs, multiply spliced mRNAs. New HIV particles are then assembled at the cell surface and released from infected cells. Viral particle maturation mediated by the viral protease is next necessary for the formation of infectious virions able to target and infect new uninfected cells.

Once integrated into the host genome, the HIV-1 provirus is organized into chromatin as all cellular genes. Although most latent HIV-1 proviruses are found integrated into actively transcribed genes (Han et al. 2008), HIV-1 proviruses present a transcriptional repression, leading to establishment and maintenance of post-integration latency and of viral reservoirs. HIV-1 post-integration latency is a multifactorial phenomenon still incompletely understood and controlled by mechanisms operating at the transcriptional but also at posttranscriptional levels.

In this chapter, we consider our present understanding of the molecular mechanisms involved in HIV-1 latency, after a brief description of the organization of the HIV-1 promoter, a necessary prerequisite for the proper understanding of those mechanisms.

8.2 Organization of the HIV-1 Promoter

The molecular regulation of HIV-1 latency is a multifaceted process dictated in part by the abundance of cellular transcription factors (TFs) that induce or repress HIV-1 promoter activity. Indeed, HIV-1 transcription is initiated by the viral promoter present at the 5'LTR region, which contains numerous binding sites for cellular TFs. These factors help to control the rate of RNA polymerase II (RNA Pol II) transcription initiation of the provirus, and their abundance in different cell types or at different times likely determines whether a provirus is actively transcribed or not. In addition to the presence or absence of these cellular TFs, nucleosome positioning in the 5'LTR appears to be specific and not static and so implicated in transcriptional latency and activation from latency. Consequently, characterization of the HIV-1 promoter structural organization is important for the comprehension of the molecular biology of latency.

The 5'LTR has been divided in three regions [U3 (unique in 3'), R (repeated), and U5 (unique in 5')], in reference to their respective origin in the viral RNA genome] and in four functional domains (from the 5'end to the 3'end: the modulatory region, the enhancer composed of a distal region and a proximal region, the core promoter, and the leader region) (Fig. 8.1). Binding sites for several TFs have

been identified in each of these domains using in vitro footprinting and electrophoretic mobility shift assays (Fig. 8.1). In addition, the latter region encodes the trans-activating response (TAR) element (nucleotide (nt) 1-59, where nt +1 represents the transcription start site) whose RNA forms a stable stem-loop structure.

Under basal conditions, two major DNase I-hypersensitive sites (short chromatin regions detected by their hypersensitivity to cleavage by DNase I and other nucleases) are present in the 5'LTR: HS2 (nt -234→-132) and HS3 (nt -67→-7), which map to the HIV-1 promoter in the U3 region (Fig. 8.1). Site HS4 (nt 201→265) is located immediately downstream of the 5'LTR in a region overlapping the primer-binding site (Fig. 8.1). Of note, the 3'LTR, containing the polyadenylation signal for viral transcripts, exhibits a pattern of DNase I-hypersensitivity that is different from that of the 5'LTR, with only a single major hypersensitive site (HS8) mapping to nt 8867→9034 (Fig. 8.1). Besides the presence of DNase I-hypersensitive sites in the LTRs, a single major hypersensitive site (named HS7) has been identified in the part of the *pol* gene coding for the integrase (centered on nt 4035→4311, Fig. 8.1). Several ubiquitous and cell-specific TFs have been shown to be recruited to the HS7 region and to be important for viral transcription and replication (Colin et al. 2011b; Goffin et al. 2005).

Chromatin analyses with micrococcal nuclease (which cuts DNA in nucleosome-free regions and in linker regions separating nucleosomes) and with restriction enzymes allowed the mapping of nucleosome positioning in the 5'LTR. Two nucleosomes (called nuc-0 and nuc-1) are positioned at the viral 5'LTR promoter DNA at precise locations with respect to regulatory elements (Fig. 8.1). Nuc-0 is positioned immediately upstream of the modulatory region and nuc-1 immediately downstream of the viral transcription start site. These nucleosomes define two open regions of chromatin corresponding to the modulatory region plus the enhancer/core promoter region (HS2 + HS3) and a regulatory domain in the leader region downstream of the transcription start site (HS4), respectively. Interestingly, nucleosomes within the LTR are not deposited according to their thermodynamically most favorable positions (Mahmoudi 2012). Indeed, the BAF subcomplex of ATP-dependent SWI/SNF chromatin remodeling complex specially functions to counteract intrinsic histone-DNA sequence preferences at the LTR and to move a preferred nucleosome from HS2 and HS3 regions to position nuc-1 over sub-optimal sequences immediately downstream of the transcriptional start site. Moreover, nucleosome positioning in the 5'LTR appears to be an intrinsic property of the LTR, as the same positions were observed independently of the different integration sites in cell lines. In addition, during HIV transcriptional activation, the organization of nuc-1 is specifically disrupted but not the organization of the other nucleosomes present on the HIV-1 genome (Verdin et al. 1993).

8.3 Regulation of HIV-1 Transcription

Transcription inhibition is critical to the establishment and maintenance of HIV latency, and the processes which cause this inhibition are of interest given their potential to be manipulated to activate HIV-1 expression and hopefully allow latently infected cells to die or, in contrast, to permanently silence HIV transcription (Darcis et al. 2017). Since there is no viral transcription inhibitor, HIV-1 latency results from a complex and variable combination of multiple cellular elements acting at the initiation and elongation phases of viral transcription (Fig. 8.2). This heterogeneous combination of cellular mechanisms represses expression of the HIV-1 transactivating factor Tat and consequently impedes Tat accumulation to a critical level needed to radically activate HIV transcription. In the following sections, we will focus on these cellular factors implicated in HIV-1 transcriptional regulation.

8.3.1 Viral Integration Site

After reverse transcription in the cytoplasm, both viral and cellular proteins associate with the double-stranded viral DNA in the so-called pre-integration complex (PIC). The PIC migrates to the nucleus where the linear viral DNA is incorporated nonrandomly into cellular DNA. Cellular lens epithelium-derived growth factor (LEDGF/p75) binds both chromosomal DNA and HIV integrase and directs integration by a tethering interaction in active genes and more specifically in introns of actively transcribed genes. Furthermore, integration site sequencing has identified peculiar genes that represent hot spots of viral integration (Wagner et al. 2014; Maldarelli et al. 2014).

In cells depleted for LEDGF/p75, integration is still not a random process but is (1) less frequent in transcription units, (2) less frequent in genes regulated by LEDGF/p75, and (3) more frequent in GC-rich DNA (Ciuffi et al. 2005). Indeed, in the absence of LEDGF/p75, residual replication is predominantly mediated by the hepatoma-derived growth factor-related protein 2 (HRP-2), the only cellular protein besides LEDGF/p75 that contains an integrase-binding domain (Schrijvers et al. 2012). Knockdown of HRP-2 upon LEDGF/p75 depletion results in a more random HIV-1 integration pattern (Schrijvers et al. 2012), although gene targeting remains significantly enriched. Indeed, other host factors likely contribute to gene-tropic integration. Sowd et al. recently showed that the cleavage/polyadenylation specificity factor 6 (CPSF6) knockout decreased integration into transcriptionally active genes, spliced genes, and regions of chromatin enriched in genes and activated histone modifications (Sowd et al. 2016). Disruption of CPSF6 expression by RNA interference or CRISPR-mediated gene knockout resulted in a redistribution of HIV-1 integration sites away from gene bodies and gene dense chromosomal regions (Sowd et al. 2016).

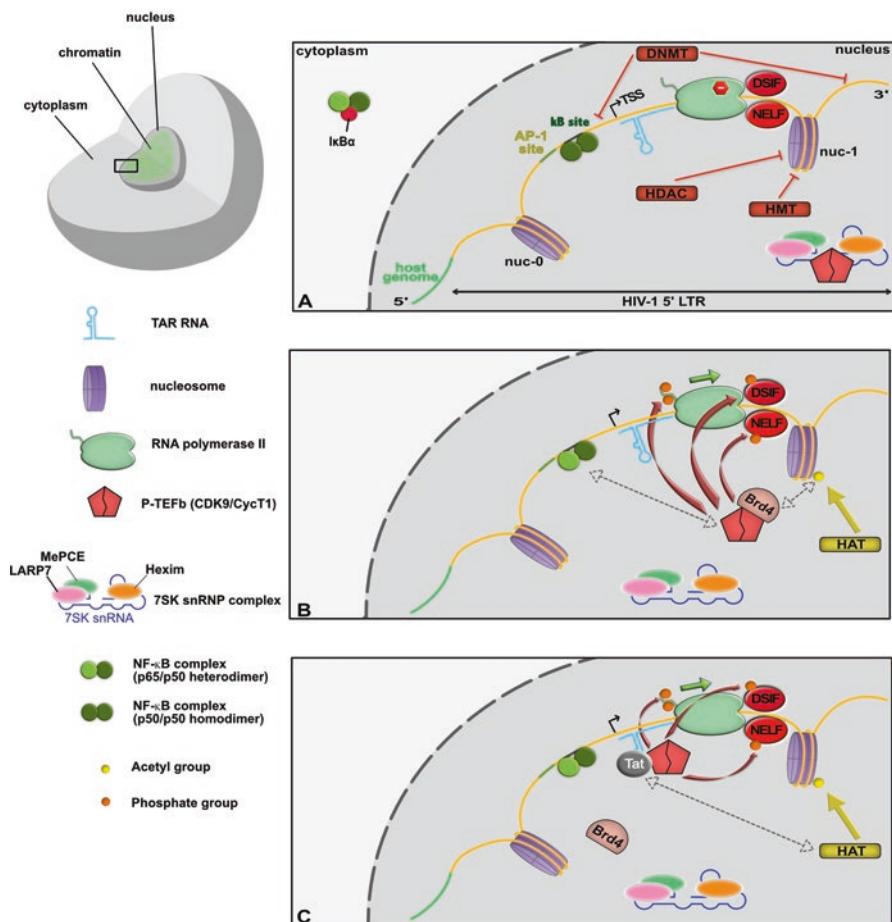


Fig. 8.2 Regulations of HIV-1 transcription initiation and elongation

Upon nuclear entry, the HIV-1 genome integrates in the periphery of the nucleus and adopts a chromatin structure. During latency (a), HIV-1 expression is repressed by the presence of nuc-1, a repressive epigenetic environment (DNA methylation and histone deacetylation and methylation), and the sequestration of the NF-κB in the cytoplasm and of P-TEFb in the inactive 7SK snRNP complex. Upon activation and in the absence of the Tat transactivator (b), the epigenetic environment of the HIV-1 LTR becomes permissive for transcription, and active NF-κB and P-TEFb complexes are recruited to the HIV-1 LTR through direct binding to DNA and interaction with Brd4, respectively. When Tat is present (c), it competes with Brd4 and recruits active P-TEFb complex and histone acetyltransferases (HATs) to allow efficient transcription elongation of the viral genes

Transcriptional interference has been proposed to explain HIV-1 promoter repression when integrated into introns of highly expressed genes. It refers to several mechanisms impeding promoter activity including enhancer trapping, promoter occlusion, and steric hindrance (Van Lint et al. 2013; Shan et al. 2011) (Fig. 8.3).

Enhancer trapping is a phenomenon which occurs when the enhancer located in the HIV-1 5'LTR is placed near the promoter of a cellular gene and acts on the transcriptional activity of this cellular promoter, thereby preventing the enhancer action on the viral promoter (Fig. 8.3). When the provirus integrates in the opposite orientation compared to the host gene, this may lead to collisions between the RNA polymerase complexes elongating from the viral and cellular promoters, resulting in a premature termination of transcription from the weaker or from both promoters, a process named promoter occlusion (Fig. 8.3). Steric hindrance occurs when the provirus integrates downstream and in the same transcriptional orientation as the cellular host gene. The “read-through” RNA polymerase transcription from the upstream cellular promoter displaces key transcription factors from the HIV-1 promoter and prevents assembly of the pre-initiation complex on the viral promoter (Fig. 8.3).

Latently infected transformed cell lines give a good example of the influence of HIV integration site on basal transcriptional rate. J-Lat cell lines, derived from Jurkat cells, were developed with an HIV-1-based vector containing an intact Tat/TAR axis (Jordan et al. 2003). These cells carry a unique provirus with the coding sequence for the green fluorescent protein (GFP) replacing the *nef* gene (Jordan et al. 2003). There is a 75-fold difference in expression level between the highest and lowest expressing clones. Those differences in expression levels are due to the integration site and are not controlled by DNA methylation or histone acetylation (Jordan et al. 2001). Lewinski et al. have infected Jurkat T cells with an HIV-based vector transducing GFP and shown that low-level GFP expression correlates with integration in (1) gene deserts, (2) centromeric heterochromatin, and (3) very highly expressed cellular genes (Lewinski et al. 2005). These latter data suggest that viral integration site, along with cellular environment, influences the balance between latency and HIV-1 proviral expression.

Sherrill-Mix et al. have compared the latency status of HIV-1 proviruses in five cellular model systems with the genomic features surrounding their integration site such as histone posttranslational modifications or chromatin accessibility (Sherrill-Mix et al. 2013). They have observed that proviruses from the same cellular model integrated in nearby positions share the same latency status much more often than predicted by chance, indicating the existence of local features influencing latency. However, these were not consistent among models, reflecting the multiplicity and the heterogeneity of the mechanisms leading to the establishment and maintenance of latency (Sherrill-Mix et al. 2013).

If the site of integration plays a role on HIV-1 transcriptional rate, it may also influence the survival of infected cells and consequently favor viral persistence. Indeed, HIV integration into genes associated with cancer or cell cycle regulation confers a survival advantage that allows these cells to persist during cART, with cell proliferation appearing to serve as an important mechanism of HIV persistence

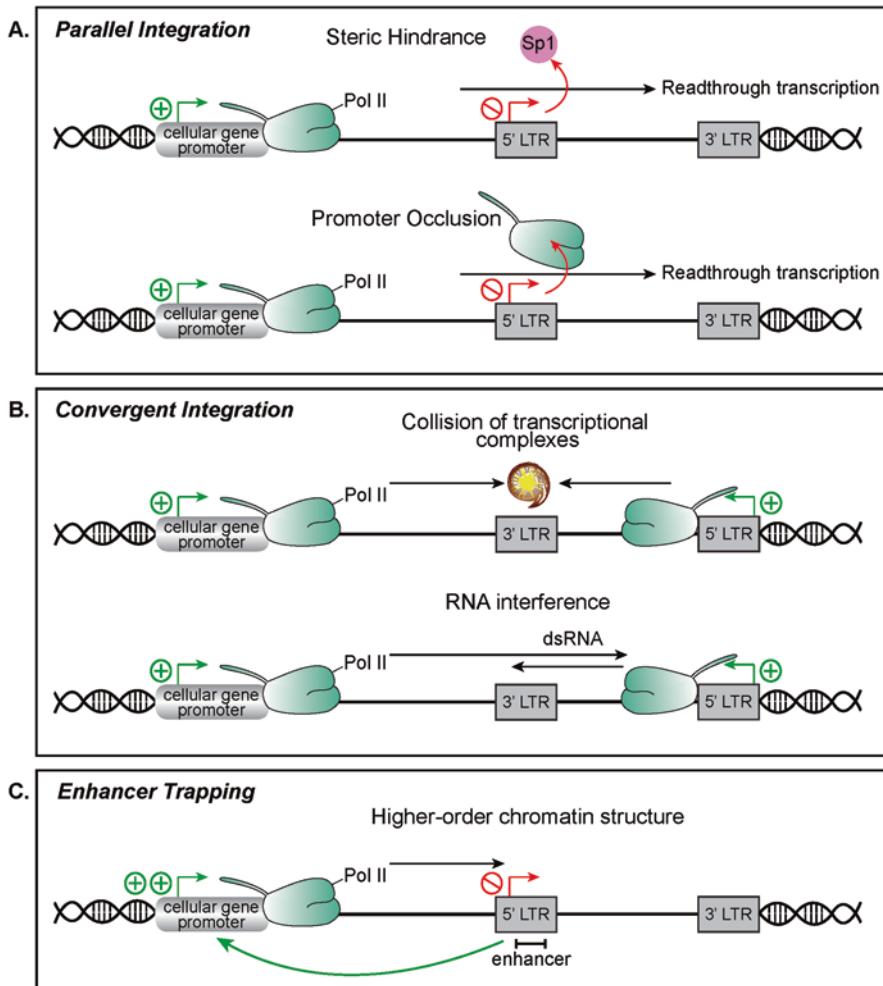


Fig. 8.3 Transcriptional interference preventing HIV-1 transcription

The site of integration could impact HIV-1 transcription. (a) Parallel integration might prevent association of transcription factors such as SPI with the HIV promoter (5'LTR) or displacement of the RNA polymerase II (Pol II) complex from the HIV LTR due to Pol II initiating transcription at the upstream cellular gene promoter. (b) Convergent integration is not permissive for HIV transcription as either the collision of converging Pol II complexes results in termination of transcription or the generation of RNA from both strands of the HIV genome results in triggering of RNA interference pathways. (c) Enhancer trapping is the ability of HIV 5'LTR to enhance an upstream weak cell promoter, thus preventing its activity on the 5'LTR

(Wagner et al. 2014; Maldarelli et al. 2014). The sites of HIV integration play an important role in the persistence of infected cells. Indeed, some cells with HIV integration sites in specific genes such as BACH2 are strongly selected because these integrations promote the survival and expansion of the infected cells (Maldarelli et al. 2014). However, Cohn et al. have highlighted that these clonally expanded infected cells may not or only poorly contribute to the inducible viral reservoir since they mainly contain defective proviruses (Cohn et al. 2015). More recently, Simonetti et al. have reported conflicting results showing that clonally expanded CD4⁺ T cells carry replication-competent proviruses and could be a reservoir of infectious HIV-1 (Simonetti et al. 2016). Consequently, it seems that at least some cells carrying replication-competent HIV-1 can undergo clonal expansion. This observation implies that reductions in the latent reservoir could not be stable since some of the remaining infected cells may be able to proliferate and thus replenish the HIV-1 reservoir (Kim and Siliciano 2016).

8.3.2 *Nuclear Topography*

A fundamental property of genomes is their topological organization in three-dimensional space in the intact cell nucleus. The spatial organization of the chromatin fiber and the genome as a whole dramatically affects the function of DNA (Misteli 2007). Besides the arrangement of the genetic information itself, the cellular factors implicated in transcription, replication, and genomic architecture are organized in sophisticated patterns within the cell nucleus (Lamond and Sleeman 2003). Many transcription factors, chromatin proteins, and RNA-processing factors are localized in specific nuclear domains corresponding to distinct nuclear functions. Processes such as transcription and replication occur at spatially defined locations in the nucleus (Misteli 2007). Therefore, the nuclear topography of HIV-1 integration may radically impact its transcriptional level. Importantly, HIV-1 pre-integration complex preferentially targets those areas of open chromatin that are proximal to the nuclear pore, while excluding the internal regions in the nucleus as well as the peripheral regions associated with the nuclear lamina (Marini et al. 2015). This integration into active chromatin close to the nuclear pore complex (NPC) corresponds to the first open chromatin regions the virus meets along its route into the nucleus (Marini et al. 2015). Marini et al. have established the role of nucleoporins in HIV-1 transcriptional regulation since they have observed a reduction in proviral transcription following Tpr and Nup153 silencing in latent J-Lat cells (Marini et al. 2015). Consequently, the nuclear pore complex may provide a chromatin topology and transcriptional environment encouraging HIV-1 replication. Indeed, the interaction between NPCs and specific chromosomal regions, called nucleoporins-associated regions, indicates that the NPC could have a role in organizing the three-dimensional nuclear architecture (Capelson et al. 2010). Lelek et al. have provided direct evidence for the exclusive involvement of Nup153 and Tpr in distinct and complementary steps of the HIV-1 life cycle (Lelek et al. 2015).

They have demonstrated that the flexible C-terminus domain of Nup153 is required for HIV-1 nuclear import. They have also observed that Tpr depletion specifically reduces HIV-1 expression and proposed a model in which HIV-1 integration preferentially occurs in chromatin regions proximal to NPC and where Tpr remodels these regions in an active state favorable for HIV-1 replication (Lelek et al. 2015). In this way, some NPC components are critical for normal integration of the HIV genome. Indeed, following nucleoporin knockdown, the structure of the chromatin is altered, leading to integration into regions less optimal for efficient viral gene expression (Wong et al. 2015).

Nuclear topology and active gene movement can also mediate HIV-1 transcriptional regulation and have implications for controlling HIV-1 latency (Lusic et al. 2013). Silenced but transcriptionally competent HIV-1 proviruses reside in close proximity to PML (promyelocytic leukemia) NBs (nuclear bodies). This association inhibits HIV-1 gene expression and coincides with transcriptionally inactive facultative heterochromatic marks, notably G9a-mediated H3K9me2, at the viral genome (Lusic et al. 2013). During transcriptional reactivation following 12-O-tetradecanoylphorbol-13-acetate (TPA), HIV-1 repositions its genome away from PML NBs through an active movement requiring nuclear actin polymerization that coincides with loss of facultative heterochromatin marks from the proviral DNA (Lusic et al. 2013). Notably, other studies have suggested that PMLs can interact with the histone-acetyltransferase p300/CBP and sequester cyclin T1 (sub-unit of the cellular transcription elongation factor p-TEFb) (Marcello et al. 2003; Doucas et al. 1999). The site of integration and the nuclear topography of HIV-1 are consequently intricate with other mechanisms involved in HIV-1 transcriptional regulation.

8.3.3 Regulation of HIV Transcription by Tat/P-TEFb

HIV mRNA transcription is controlled by the cellular RNAP II. RNAP II executes a series of distinct steps: it binds to promoters, initiates RNA synthesis, and then pauses in early transcriptional elongation. The paused RNAP II remains stably associated with the nascent RNA and is capable of quickly resuming elongation. However, further signals are needed to elicit the transition from the paused RNAP II to a productive elongation complex (Adelman and Lis 2012). Like for cellular promoters, RNAP II accumulates around position nt + 30 with respect to the HIV transcription start site but also shows additional pausing at nt + 90, which is immediately downstream of the TAR element and other distal sites in the HIV LTR (Jadlowsky et al. 2014). Latent HIV proviruses are primed for rapid transcriptional responses due to promoter proximal pausing of RNAP II. The switch from promoter proximal pausing to productive elongation is mediated by the viral transactivator Tat and the cellular P-TEFb active complex, an essential elongation transcription factor constituted of cyclin T1 (CycT1), CDK9, and the bromodomain-containing protein 4 (BRD4). Resting CD4⁺ T cells are characterized by an extremely low level

of cyclin T1 due to actions of specific miRNAs and of the cellular factor NF-90 that blocks translation of CycT1 mRNA (Budhiraja et al. 2013; Chiang and Rice 2012; Chiang et al. 2012). The sequestration of P-TEFb within the 7SK small nuclear ribonucleoprotein (snRNP) repressive complex including the 7SK snRNA, the hexamethylene bisacetamide-inducible protein 1 (HEXIM1), the 5' methylphosphate capping enzyme (MePCE), and the La-related protein (LARP7), as well as the combined inhibition by the negative elongation factor (NELF) and the 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor (DSIF), reinforces the block of transcriptional elongation in the absence of Tat.

Activation of several signaling pathways results in posttranslational modifications of P-TEFb subunits and HEXIM-1 which can increase the level of P-TEFb subunits and prevent the sequestration of P-TEFb in the 7SK snRNP complex (Mbonye et al. 2015). Therefore, following cellular activation and before the production of Tat, P-TEFb is released from the 7SK snRNP complex and associates with BRD4 to form the active P-TEFb complex (Fig. 8.2). This complex is recruited to the HIV promoter through interactions of the BRD4 bromodomains with acetylated histones. Once Tat has been synthesized, on the one hand, Tat competes with BRD4 for binding to P-TEFb, but on the other hand, Tat is also able to disrupt the inactive P-TEFb complex by displacing HEXIM1 and forming a stable complex with P-TEFb. Together with P-TEFb, Tat binds the TAR stem-loop RNA structure, allowing CDK9 to phosphorylate the RNAP II carboxy-terminal domain (CTD) and the negative transcription elongation factors NELF and DSIF, allowing efficient elongation by RNAP II (Karn and Stoltzfus 2012).

Tat can also recruit, in addition to P-TEFb, other elongation factors such as ELL2 (elongation factor for RNA polymerase II), AFF4 (AF4/FMR2 family member 4), ENL (eleven-nineteen-leukemia protein), and AF9 (ALL1-fused gene from chromosome 9 protein), thereby forming the superelongation complex (Sobhian et al. 2010; Cherrier et al. 2013), reviewed in Cary et al. (2016). Importantly, Besnard et al. have recently demonstrated a role of mTOR (mechanistic target of rapamycin) in HIV-1 latency reversal. They have shown that mTOR inhibitors suppressed the reactivation of latent virus through the suppression of Tat-independent and Tat-dependent transactivation of the HIV promoter and the reduction of the global CDK9 phosphorylation induced by TCR co-stimulation in CD4+ T cells (Besnard et al. 2016). These results suggest that mTOR pathway might be used to lock the latent reservoir.

8.3.4 Epigenetic Silencing of HIV Transcription

In eukaryotic cells, DNA is wrapped around a nucleosome composed of a histone octamer. The histone tails are subject to multiple posttranslational modifications including acetylation, phosphorylation, sumoylation, ubiquitination, and methylation. These epigenetic marks are heritable modifications, which alter the way genes are expressed without altering the underlying DNA sequence. These histone

modifications as well as DNA methylation are reversible epigenetic marks that are catalyzed by chromatin-modifying enzymes. These marks affect chromatin condensation, a critical process for regulation of gene expression since it determines the accessibility of DNA to regulatory transcription factors. A constellation of epigenetic modifications contribute to the silencing of latent HIV-1 proviruses (reviewed in Van Lint et al. (2013)).

The chromatin structure and the epigenetic control of the HIV-1 promoter (5' LTR) are important mechanisms leading to transcriptional repression and thus post-integration latency. As stated earlier in this chapter, two nucleosomes named nuc-0 and nuc-1 are localized in the HIV-1 5' LTR in latently infected cell lines (Verdin et al. 1993). Importantly, nuc-1 is located immediately downstream of the transcription start site (TSS) and takes part in the blockage of transcriptional elongation. Nucleosomes on the 5' LTR of latent proviruses typically harbor epigenetic marks associated with transcriptional repression. However, stress signals have the potential to modify these epigenetic marks, thereby inducing nuc-1 remodeling that favors transcriptional elongation (Van Lint et al. 1996).

Histone acetyltransferases (HATs) and histone deacetylases (HDACs) influence transcription by selectively acetylating or deacetylylating the ε-amino group of lysine residues in histone tails. Deacetylation by HDACs generally produces a repressive heterochromatin environment and reduces the accessibility of DNA to transcription factors, whereas chromatin acetylation by HATs promotes chromatin opening (Yang et al. 2007). HDAC1, HDAC2, and HDAC3 are recruited by transcriptional repressors to the HIV-1 5' LTR that typically displays deacetylated histones. Deacetylation of the HIV-1 promoter chromatin by these enzymes plays a role in the establishment and maintenance of HIV-1 latency (Colin and Van Lint 2009). Indeed, the repressive nucleosome nuc-1, located downstream of the transcription start site, is specifically remodeled following treatment of infected cells with HDAC inhibitors or TNF alpha, concomitant with activation of HIV-1 gene expression (Verdin et al. 1993; Van Lint et al. 1996).

While histone hypoacetylation is a pattern associated with transcriptional repression, histone methylation can be linked to either transcriptional repression or activation. Trimethylation (such as H3K9 trimethylation (H3K9me3) as well as H3K27 trimethylation (H3K27me3)) is a pattern associated with transcriptional repression and has been shown to be associated with HIV-1 transcriptional silencing in different post-integration latency models (Imai et al. 2010; Friedman et al. 2011; Marban et al. 2007; du Chene et al. 2007). In microglial cells, the corepressor COUP-TF-interacting protein 2 (CTIP2) recruits HDAC1 and HDAC2 to promote local histone H3 deacetylation at the HIV-1 promoter region. In addition, DNA-bound CTIP2 also associates with the histone methyltransferase SUV39H1, which increases local histone H3K9 trimethylation (Marban et al. 2007).

The histone methyltransferase (HMT) enhancer of zeste homologue 2 (EZH2) is required for histone lysine 27 trimethylation (H3K27me3). This HMT is present at high levels in the LTR region of silenced HIV proviruses and is rapidly displaced following proviral reactivation (Friedman et al. 2011). EZH2 seems to be of particular importance since the knockdown of this enzyme strongly induced HIV-1 expres-

sion compared to the knockdown of SUV39H1, another HMT required for H3K9me3. Importantly, EZH2 interacts—within the context of the polycomb repressive complexes 2 and 3 (PRC2/3)—with DNA methyltransferases (DNMTs) in vivo, highlighting a direct connection between two key epigenetic repression systems (Viré et al. 2006). In addition to EZH2 and SUV39H1, the HMTs G9a and G9a-like protein (GLP), which are involved in H3K9me2, may also play a role in the maintenance of HIV-1 silencing (Imai et al. 2010; Ding et al. 2013).

DNA methylation at CpG dinucleotides is generally associated with gene silencing, either by directly blocking the binding of transcription factors to their recognition sequences due to steric hindrance or indirectly through the recruitment of methyl-CpG-binding domain (MBD) proteins, which in turn interact with HMTs and HDACs, leading to a repressive chromatin structure (Suzuki and Bird 2008). This link between DNA methylation and histone epigenetic marks is important for our understanding of the establishment of a latent HIV-1 infection. Indeed, DNA hypermethylation is present during latency at the two CpG islands surrounding the viral transcription start site and has also been associated with silencing of the HIV 5'LTR promoter in J-Lat cells and primary CD4+ T cells (Blazkova et al. 2009; Kauder et al. 2009). In CD4 T cells from HIV-infected individuals, DNA methylation of the HIV-1 promoter increases progressively during ART treatment, suggesting that this epigenetic contributes more to viral persistence than to latency establishment (Trejbalova et al. 2016).

8.3.5 *The Absence of Inducible Host Transcription Factors*

As explained above, the HIV-1 5'LTR contains several DNA-binding sites for various cellular transcription factors, including Sp1- and NF-κB-binding sites, essential for HIV-1 replication, whereas other sites, such as NFAT-, LEF-1-, COUP-TF-, Ets1-, USF-, and AP-1-binding sites, enhance transcription without being indispensable (Colin and Van Lint 2009). HIV-1 transcription is consequently coupled with the cellular activation status. Indeed, for example, NF-κB is sequestered in the cytoplasm of quiescent T cells in an inactive form through its interaction with an inhibitory protein from the family of inhibitors of NF-κB (IKB) (Fig. 8.1). Following T-cell activation, the phosphorylation of IKB by IKK (IKB kinase) leads to its dissociation from NF-κB, allowing translocation of NF-κB into the nucleus and transcriptional transactivation of NF-κB-dependent genes. Additionally, NF-κB stimulates transcriptional elongation by interacting with P-TEFb and directs the recruitment of a co-activator complex of HATs to the HIV LTR (Barboric et al. 2001; Perkins et al. 1997).

Binding sites for other transcription factors downstream of the transcription start site have been identified: three AP-1 sites, a motif binding the T-cell-specific factor NF-AT, a downstream interferon-responsive factor (IRF)-binding site, and juxtaposed Sp1 sites (Van Lint et al. 1997). The primary ability of HIV-1 to establish latent infection is controlled by a four-nucleotide AP-1 element just upstream of the

NF-κB element in the viral promoter (Duverger et al. 2013). Deletion of this AP-1 site mostly deprived HIV-1 of the ability to establish latent HIV-1 infection, while extension of this site to a 7-nucleotide AP-1 sequence massively promoted latency establishment, supporting the notion that HIV-1 latency is a consequence of transcription factor restriction (Duverger et al. 2013).

Moreover, analysis of the chromatin organization of integrated HIV-1 proviruses allowed the identification of a transcriptional cis-regulatory element associated with a nuclease-hypersensitive site in the HIV-1 *pol* gene (Van Lint et al. 1994; Goffin et al. 2005). Three AP-1-binding sites have been characterized in this important intragenic region in the HIV-1 genome (Van Lint et al. 1991), and mutations of these intragenic AP-1 sites individually or in combination altered HIV-1 replication (Colin et al. 2011a).

8.4 Posttranscriptional Regulation of HIV-1 Gene Expression

It is important to understand that all the mechanisms of transcriptional regulation, described in the previous section, act together and are influenced by nuclear processes now considered co-transcriptional in nature, such as pre-mRNA capping, splicing, and polyadenylation. Our understanding of the significance of these mechanisms has greatly increased during recent years.

Splicing, capping, and polyadenylation processes occur mostly co-transcriptionally (Karn and Stoltzfus 2012). First, pre-mRNAs are capped at their 5' end by capping enzymes: RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-N7) methyltransferase. This step is facilitated by RNAP II pausing. The capping reaction is also stimulated by Tat via TAR-dependent phosphorylation of the CTD (C-terminal domain of Pol II) by P-TEFb. Further processing of viral pre-mRNA by the host splicing machinery gives rise to a variety of transcripts and, consequently, many proteins due to alternative splicing. Indeed, nascent transcripts include several 5' splice donors (5), 3' splice acceptors (9), and branch point sites in order to result in over 40 differently spliced mRNAs (Purcell and Martin 1993).

Generated mRNAs are divided into three classes: (1) unspliced RNA (9 kb) serving as genomic RNA or to produce the Gag and Gag-Pol precursors; (2) singly spliced (4 kb) RNA encoding Tat exon1, Vif, Vpu-Env, and Vpr; or (3) fully spliced (2 kb) RNA expressing Tat exon1 + 2, Rev, and Nef (Fig. 8.4). Transport of unspliced and partially spliced mRNAs from the nucleus to the cytoplasm is mediated by the viral Rev protein, which interacts with the rev-responsive element (RRE) in the viral RNA and the cellular export factor Crm1 to connect these viral RNAs to the export machinery. Before the transport to the cytoplasm, the final step in the processing of the nascent transcript is polyadenylation. The 3' processing and polyadenylation of pre-mRNAs involve recognition of the upstream 5'AAUAAA3' and downstream 5'GU-rich3' nucleotide motifs surrounding the cleavage and poly(A)

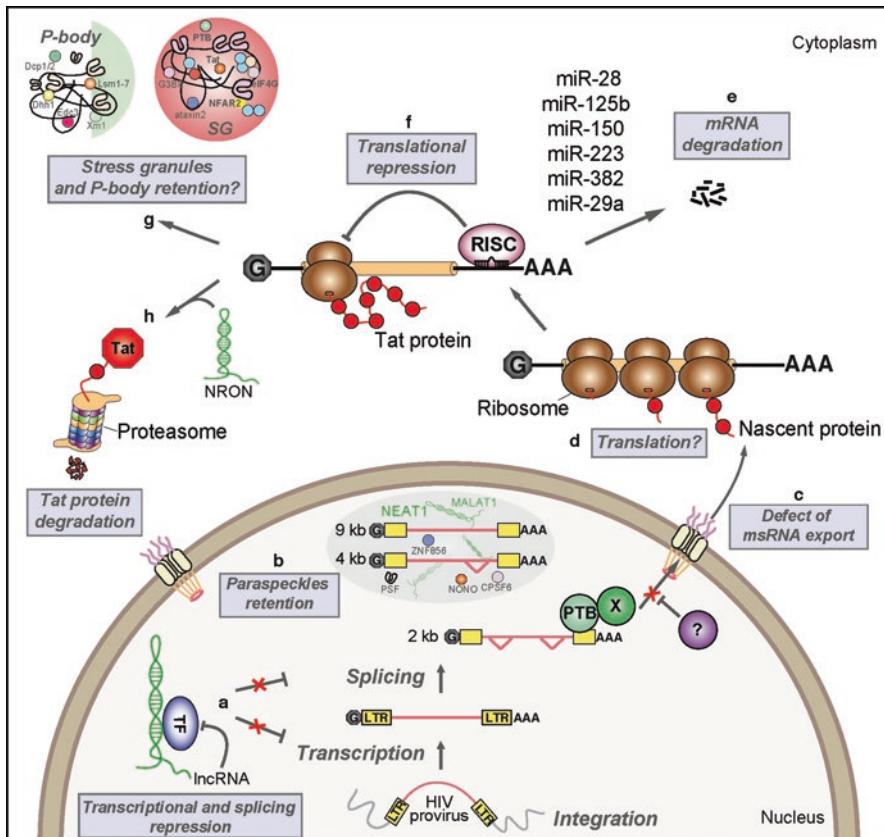


Fig. 8.4 Posttranscriptional regulations of HIV-1 gene expression in latently infected cells

Several factors and pathways have been implicated in the establishment and maintenance of latency:

- (a) Sequestration of essential transcription factors (TFs) and/or splicing factors by long noncoding RNA (lncRNA) in the nucleus of resting cells is an impediment for efficient transcription splicing of HIV mRNAs.
- (b) Paraspeckles' retention of unspliced (9 kb) and singly spliced (4 kb) mRNAs as well as several transcription (PSF, ZNF856), splicing (NONO), and cleavage/polyadenylation specificity factors (CPSF6) induced by lncRNAs NEAT1 (nuclear-enriched abundant transcript 1) and MALAT1 (metastasis-associated lung adenocarcinoma transcript 1).
- (c) Defect in viral RNA export due to insufficient levels of either Rev or HIV RNA-binding factors such as PTB (polypyrimidine tract-binding protein).
- (d) Aberrant localization of msRNA in the nucleus imposes a block on successful translation of all viral components required for productive infection.
- (e) Translation of cyclin T1 and PCAF is reduced as a result of inhibition by miRNA-mediated pathways that could lead to mRNA degradation.
- (f) Targeting the 3' untranslated region of HIV mRNAs, miRNAs could also result into translational repression.
- (g) The only viral factor implicated in regulation of latency is the absence of Tat or impaired Tat function that would favor latent infection. Because of its central role in transcriptional transactivation and production of full-length viral mRNAs, small stochastic changes in Tat expression could induce latency. For example, Tat retention in stress granules (SG) with several factors essential for transcription (NFAR2), splicing/export (PTB), and translation (eIF4G) could help in maintaining the cells latently infected.
- (h) Finally, it has been shown that HIV latency arises when Tat levels fall below a threshold induced by NRON (noncoding repressor of NFAT—nuclear factor of activated T cells), a lncRNA that promotes Tat degradation and therefore induces latency in resting CD4+ T cells.

addition site. For this, host cellular proteins such as the cleavage/polyadenylation specificity factor (CPSF), CstF, CF1m, CF2m, and poly(A) polymerase are required for endonucleolytic cleavage and polyadenylation of viral pre-mRNA. HIV provirus contains two identical LTR, and each of them has a poly(A) site. However, the poly(A) site in the 5'LTR has to be ignored for HIV transcription.

Gene expression regulations for both cellular and HIV-1 genome are not discrete or independent events and may occur simultaneously as they are very closely related both temporally and spatially. Several factors that are involved in a specific pathway often appear in another. However, these pathways do not all exert their effects in every cell and at every gene promoter, creating a multifaceted picture of gene expression regulation dependent on cellular environment and cell type.

Key players that appear to have starring roles in several aspects of the molecular control of HIV-1 latency are most notably Tat, HDACs, P-TEFb and its cofactors, and NF- κ B (see above). First, for Tat, p-TEFb, and NF- κ B, many levels of control exist to regulate the function of these factors, due to the delicate balance in protein level and protein function required for their roles in gene expression in preference to their involvement in other aspects of cell survival or the immune response. Second, Tat, HDACs, and NF- κ B exert effects in multiple pathways of gene expression regulation and are implicated in several aspects of HIV-1 latency establishment and/or maintenance. The transcriptional regulation of these factors has been widely studied and extensively discussed. In contrast, the posttranscriptional control of these proteins is less well studied, and their relative significance in HIV-1 latency is not well known.

8.4.1 HIV mRNA Processing Altered During Latency

Alternative splicing is a highly dynamic process, allowing a single mRNA template to give rise to several distinct protein-coding transcripts. Deep sequencing analysis revealed the existence of more than 109 species of mRNAs generated from the HIV-1_{89.6} genome (Ocieja et al. 2012), but the significance of this complexity in HIV-1 gene expression regulation is poorly understood. Splicing occurs co-transcriptionally, which facilitates its association with cellular factors that are involved in the earlier stages of transcription, including P-TEFb and HDAC enzymes. P-TEFb was shown to be important for linking the co-transcriptional processes of pre-mRNA capping and alternative splicing to transcriptional elongation (Lenasi et al. 2011), thereby facilitating efficient generation and processing of protein-coding mRNA.

In addition to their role in remodeling chromatin architecture, HDAC enzymes can affect splice site selection (Hnilicova et al. 2011), which may impact upon both Tat and Rev expression, both functions of which, as discussed previously, are imperative for productive infection of the cell. Within the context of HIV-1 latency, the levels of splice factors that promote the usage of splice sites for generation of msRNA have not been studied and may represent an additional block to the successful production of Tat and Rev in these cells.

The use of latency-reversing agents (LRAs) poses many impediments to HIV-1 gene expression and alternative splicing, including the bromodomain inhibitor JQ1, which was shown to detrimentally affect the levels of hnRNP proteins (Khoury et al. 2018). Nonspecific effects of LRAs, such as vorinostat, result in the upregulation of transcription from the upstream gene promoter at the integration site of HIV-1 (Bullen et al. 2014), thereby increasing the production of read-through transcripts in lieu of promoting activation of the HIV-1 5'LTR. Intermittent reactivation of latent HIV results in rapid viral rebound once individuals interrupt their ART regimen; however, the underlying triggers for the spontaneous release of virus from the reservoir are not known. A study by Imamichi et al. (2016) has shown the presence of aberrant transcription and splice products from defective proviruses in the latent reservoir that potentially allow the production of low levels of functional peptides, a putative pathway for stochastic reactivation of latently infected cells in the rare situations where Tat and Rev are produced (Imamichi et al. 2016). Other studies have proposed that conditions of cell stress such as fever, starvation, or drug stimulation may be linked to random reactivation of latent proviruses through the effects of the active HSF1 protein and its interaction with the HIV-1 LTR and cofactors of proviral transcription, p300 and P-TEFb (Pan et al. 2016; Khan et al. 2015).

In addition to the implication of transcriptional mechanisms in HIV-1 latency, posttranscriptional regulations such as inhibition of nuclear viral RNA export or translation are also implicated in HIV-1 latency. Export of msRNA from the nucleus is impacted upon by the levels of the polypyrimidine tract-binding protein (PTB), which is differentially regulated in resting and activated CD4+ T cells (Lassen et al. 2006). An aberrant accumulation of msRNA was observed in the nucleus of resting cells, which is reversed by the overexpression of PTB. However, the exact mechanisms governing PTB's involvement in the switch from nuclear to cytoplasmic localization of msRNA have not been elucidated. Export of the partially or unspliced variants of HIV mRNA is facilitated by the viral protein, Rev, and its association with several cellular factors and the RRE or instability elements in these mRNA species (Zolotukhin et al. 2003; Yedavalli and Jeang 2011; Kula et al. 2012; Kula et al. 2011). Blocks to export of these mRNAs and the involvement of these cellular factors, including Matrin 3 and PSF in HIV-1 latency, have not been described but may have a role due to their importance for successful expression of HIV-1 gene products.

8.4.2 Cross Talk Between Noncoding mRNAs and HIV Latency

Noncoding mRNAs have been studied for their involvement in HIV-1 latency, and examples of both miRNAs and long noncoding RNAs (lncRNAs) have been implicated in regulation of nonproductive HIV-1 infection.

The level of specific mRNA translation can be modulated by microRNAs (miRNAs). miRNAs are short single-stranded noncoding RNAs of 19–25 nucleotides

that mediate posttranscriptional gene silencing. Following RNAP II transcription, pri-miRNA transcripts are sequentially processed via the nuclear RNases III Drossha and Dicer to generate mature miRNAs which interact with a complementary sequence in the 3' untranslated region of target mRNAs by partial sequence matching, resulting in degradation of the mRNA and/or translational repression. Interestingly, modifications of the miRNA profile of HIV-1 infected individuals have been observed in different cohorts (Houzet et al. 2008; Witwer et al. 2012; Bignami et al. 2012). Mechanistically, Tat and Vpr are known to function as RNA silencing suppressors by modulating miRNA expression levels in infected cells (Qian et al. 2009; Hayes et al. 2011; Coley et al. 2010).

miRNAs can be of either cellular or viral origin and, in turn, can target either cellular or virally expressed mRNAs. HIV-1 infection can alter the expression levels of some cellular miRNAs, and these have been studied for their potential roles in regulating HIV-1 gene expression. For example, PCAF (p300/CBP-associated factor), a histone acetyltransferase that is involved in chromatin remodeling and modulation of Tat function, is targeted posttranscriptionally by miR-17/92 and miR-20a, both of which are downregulated in HIV-1 infection (Triboulet et al. 2007; Hayes et al. 2011). The P-TEFb component, Cyclin T1, is repressed by different miRNAs in monocytes (miR-198) and resting CD4 T cells (miR-27b, miR-29b, miR-150, miR-223) (Sung and Rice 2009; Chiang et al. 2012), which have implications for attempting to reverse HIV-1 latency in the different cell types that harbor latent proviruses. Additionally, cellular miRNAs, miR-28, miR-125b, miR-150, miR-223, and miR-382, upregulated in resting CD4 T cells, recognize the 3' end of HIV-1 mRNAs (Huang et al. 2007) and participate in repressing HIV-1 gene expression. Moreover, the HIV-1 nef gene contains a miR-29a-targeted site that interferes with HIV-1 replication (Ahluwalia et al. 2008; Nathans et al. 2009). Several HIV-1 derived miRNAs, called virus-derived vmiRNAs, have also been identified by *in silico* analysis, deep sequencing of infected cells, and miRNA expression profiling (Bennasser et al. 2004; Yeung et al. 2009; Schopman et al. 2012). These include TAR-derived miRNA-TAR5p/3p (Klase et al. 2007; Ouellet et al. 2008) and the nef-derived miR-N367 (Omoto et al. 2004). TAR-derived miRNA may have an anti-apoptotic effect by targeting apoptotic genes (Klase et al. 2009) or HIV-1 transcription as it has been recently proposed (Wagschal et al. 2012).

On the other hand, the HIV-1 proviral expression can be facilitated by miR-217 and miR-34a targeting SIRT1 (Zhang et al. 2012a, b), a class III HDAC involved in both the regulation of NF- κ B and Tat, where the deacetylation of either factor negatively impacts their function. While its levels in resting CD4+ T cells are low, upon activation TRIM32 levels increase (Ruelas et al. 2015). However, miR-155, a counter-regulator of TRIM32, is also upregulated upon activation and serves to antagonize the role of TRIM32 in NF- κ B activation and hence HIV-1 reactivation (Ruelas et al. 2015). The existence of several different pathways that lead to the NF- κ B activation designates a need for careful regulation of any of these arms due to the roles of this transcription factor beyond gene expression regulation. This implies that different methods that have been trialed for reactivation of HIV-1 latency may also be inadvertently activating counter-regulatory pathways that serve

to dampen the effects of these T-cell activation signals. This would lead to a limitation in the magnitude of reactivation achieved by these LRAs and would severely reduce the efficacy of the “shock” part of the “shock and kill” (Ruelas et al. 2015).

LncRNAs have only recently been appreciated for their role in gene expression regulation, with their influence extending from early transcription initiation through to protein translation and degradation. A lncRNA that has been previously discussed in this chapter is the 7SK RNA, and although it has a significant role in the regulation of active P-TEFb levels within the nucleus, its expression in resting CD4+ T cells is low and consequently does not appear to have an important involvement in regulating HIV-1 latency (Haaland et al. 2003; Sung and Rice 2006). Nuclear-enriched abundant transcript 1 (NEAT1) is a lncRNA associated with the pathway of HIV mRNA export dependent on Rev and other cellular cofactors (Zhang et al. 2013). Nuclear topography and, specifically, the association of HIV-1 mRNAs with paraspeckles appear to regulate the accumulation of partially or unspliced mRNAs in the nucleus (Zhang et al. 2013; Zolotukhin et al. 2003; Yedavalli and Jeang 2011), and while no clear links to HIV latency have been shown, NEAT1 is an example of a lncRNA that is involved in HIV gene expression. Expression levels of noncoding repressor of NFAT—nuclear factor of activated T cells—or NRON, a lncRNA which involvement in HIV-1 latency was observed to be inversely correlated with levels of HIV mRNA in resting CD4+ T cells (Li et al. 2016). Consequently, it was shown that NRON promotes HIV-1 latency by targeting Tat for degradation and that its knockdown results in rescue of proviral transcription in latently infected cells.

Alternative splicing can also be influenced by lncRNAs, and one studied example is MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), also known as NEAT2 (nuclear-enriched abundant transcript 2), which interacts with SR proteins (Tripathi et al. 2010), splicing factors that are extensively involved in HIV-1 alternative splicing. MALAT1 has not been studied in the context of HIV-1 gene expression, but given their appearance in so many aspects of gene expression regulation, the extent of ncRNA involvement in HIV-1 latency is most likely far greater than previously realized.

At the level of protein expression, regulation of mature mRNA translation allows rapid production of factors required for different aspects of cell function circumventing the need for de novo mRNA synthesis. In the cell, sets of genes that are functionally related may all be translationally modulated within the same regulatory circuit. CD4 T-cell subsets differ markedly in their functions, and this is reflected in their translational signatures. Proliferation of CD4 T-cell subsets is controlled at the level of translation, where eIF4E has been implicated in the control of mRNAs that encode genes associated with cell cycle and proliferation (Bjur et al. 2013). HIV-1 latency has been described in several subsets of CD4 T cells, where the proliferative profiles vary from viral subtype to viral subtype. As discussed here, it is believed that integration of HIV-1 into specific genes promotes proliferation of specific clones and facilitates persistence of particular populations of latently infected cells. The interplay between eIF4E and other translation regulatory factors may have a role in regulation of the proliferation of T-cell clones carrying latent HIV-1, and this could be occurring independently of the HIV-1 integration site.

8.5 Conclusion

Despite numerous different transcriptional and posttranscriptional molecular mechanisms identified, HIV-1 post-integration latency is still incompletely understood. Intensive fundamental research is still necessary in order to extend our understanding of HIV-1 latency. On other hand, since most of the studies on HIV-1 latency have been performed using blood T cells and since these circulating cells account for 2%, at most, of the CD4+ T cells in the body, it remains essential to study HIV-1 latency in the lymph nodes, bone marrow, gut, and other tissues. The contribution of other latent cellular reservoirs such as those from monocyte/macrophage lineage or from anatomical sanctuaries has been neglected. Despite the fact that the existence of proviruses in a similar latent state in macrophages is subject of intense debate, many of the factors that are involved in regulating latency in T cells are differentially expressed in macrophages, suggesting that latency mechanisms might differ between T and non-T target cells. In addition to the potential variations between cellular types, it is currently unknown whether the mechanisms leading to the establishment and persistence of HIV latency are uniform or vary from one cell to another or from one anatomical compartment to the other. Consequently, it seems probably impossible to reach HIV-1 remission without considering all these issues. Further understanding of HIV latency remains essential to accelerate the generation of new basic research insights and thereby the design of novel therapeutic approaches to decrease the size of the latent HIV reservoir.

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Chapter 9

Cellular Determinants of HIV Persistence on Antiretroviral Therapy



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Abstract The era of antiretroviral therapy has made HIV-1 infection a manageable chronic disease for those with access to treatment. Despite treatment, virus persists in tissue reservoirs seeded with long-lived infected cells that are resistant to cell death and immune recognition. Which cells contribute to this reservoir and which factors determine their persistence are central questions that need to be answered to achieve viral eradication. In this chapter, we describe how cell susceptibility to infection, resistance to cell death, and immune-mediated killing as well as natural cell life span and turnover potential are central components that allow persistence of different lymphoid and myeloid cell subsets that were recently identified as key players in harboring latent and actively replicating virus. The relative contribution of these subsets to persistence of viral reservoir is described, and the open questions are highlighted.

Keywords HIV reservoirs · CD4+ T-cell subsets · Macrophages · Dendritic cells · HIV susceptibility · Cell survival · Turnover potential

9.1 Introduction

Antiretroviral therapy (ART) has achieved impressive success in preventing progression of HIV-1 infection. However, ART is lifelong and is not a cure. HIV-1 persists in viral reservoirs (Barton et al. 2016) that lead to viral rebound if ART is discontinued. A better understanding of HIV reservoirs is fundamental in the search for an HIV cure (Deeks et al. 2016; Passaes and Saez-Cirion 2014). HIV-1 persistence under ART may be due to (1) low-level viral replication, in particular in tissues where concentration of antiretrovirals (ARVs) may not always reach optimal levels (Fletcher et al. 2014; Lorenzo-Redondo et al. 2016; Tobin et al. 2005), and (2)

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the long half-life and self-renewal of latently infected cells (Chomont et al. 2009). Although it is likely that HIV-1 persistence is a consequence of the combination of these two processes, we will focus here on intrinsic cell properties that determine the maintenance of infected cells.

HIV-1 infects cells from the myeloid and T-cell lineages, but not all cell populations contribute equally to HIV persistence (Barton et al. 2016). HIV persistence on ART is largely secondary to infected CD4+ T cells (Chomont et al. 2009; Chun et al. 1998). Macrophages and dendritic cells play a critical role as early targets for HIV infection and as vehicles for HIV-1 dissemination throughout the body, but their role in long-term persistence is still unclear (Abbas et al. 2015; Coleman and Wu 2009). There are multiple subsets of CD4+ T cells that follow a gradient of maturation stages including naïve (TNA), stem cell memory (TSCM), central memory (TCM), transitional memory (TTM), effector memory (TEM), and terminally differentiated (TTD) cells (Mahnke et al. 2013). In HIV-infected individuals on ART, HIV DNA is most frequently detected in memory CD4+ T cells and in particular TCM and TTM (Chomont et al. 2009). However, the relative contribution of these subpopulations to the HIV reservoir may vary depending on whether the treatment was initiated in primary or chronic infection, and after long periods of ART, there is a greater contribution of very long-lived infected cells such as TSCM (Ananworanich et al. 2015; Buzon et al. 2014; Jaafoura et al. 2014; Cheret et al. 2015).

The establishment and maintenance of HIV-1 reservoirs is a multifaceted process that depends (1) on the relative cell susceptibility to HIV infection, (2) the capacity of the infected cell to resist HIV-induced apoptosis and escape immune surveillance, and (3) the infected cell's life span and turnover potential (Fig. 9.1). All these processes are determined by each cell type's program and regulated by tissue location, activation, and differentiation state of the cells in response to environmental conditions and stress signals. This chapter analyzes each of these processes with respect to how they contribute to HIV persistence on ART.

9.2 Being a Good or a Bad Host to the Virus

HIV-1 cell tropism is determined by the expression on the cell surface of the main HIV-1 receptor CD4 and at least one additional co-receptor, mainly CCR5 or CXCR4 (Wilen et al. 2012). CD4+ T cells, monocytes/macrophages, and dendritic cells are the major targets of HIV-1 (Kandathil et al. 2016; Kumar et al. 2014). HIV-1 replicates preferentially in activated CD4+ T cells and less efficiently in macrophages and immature dendritic cells, while resting CD4+ T cells, monocytes, and mature dendritic cells are relatively resistant to HIV-1 infection (Steinman et al. 2003; Wu and KewalRamani 2006; Izquierdo-Useros et al. 2010; Descours et al. 2012; Diamond et al. 2004). These differences are mostly explained by the relative abundance of cellular factors that participate in the virus life cycle, either facilitating or interrupting viral replication. Several studies have identified hundreds of cellular

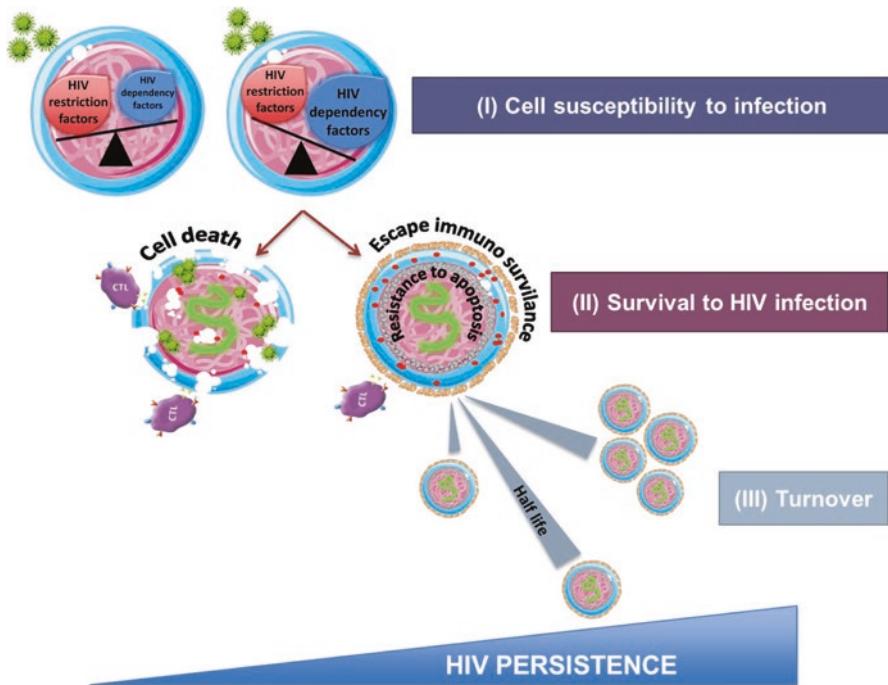


Fig. 9.1 Cellular determinants for the establishment and persistence of HIV on ART. HIV persistence is first determined by the susceptibility of different cells to infection (*i*), which is regulated by the balance of HIV host dependency factors and viral restriction factors present in the cells. In order to persist, infected cells need to resist apoptotic signals induced by viral infection and avoid immune surveillance (*ii*). These resistant infected cells will persist for variable periods of time depending on their specific life span and capacity to proliferate without enhancing HIV-dependent cell death signals (*iii*)

factors potentially required for HIV-1 to complete each step of its replication cycle, called HIV dependency factors (HDF) (Brass et al. 2008; König et al. 2008; Zhou et al. 2008; Chinn et al. 2010; Cleret-Buhot et al. 2015).

The expression of chemokine receptors varies with T-cell differentiation, impacting the susceptibility of the cells to HIV-1 (Sallusto et al. 1998; Veazey et al. 2000). Among CD4+ T helper lineages, Th2 cells are relatively resistant to HIV-1 infection, in particular to CCR5-using (R5) viruses due to low CCR5 expression (Gosselin et al. 2010; Sun et al. 2015) although these cells might be targeted by CXCR4-using (X4) viruses late in infection (Maggi et al. 1994). Th1 cells are susceptible to both R5 and X4 viruses but to a lower extent than Th1/Th17 or Th17 cells that express CCR6 (El Hed et al. 2010; Gosselin et al. 2010; Sun et al. 2015). The enhanced susceptibility of CCR6+ CD4+ T cells to HIV-1 infection was linked to an enhanced expression of HDF in these cells (Cleret-Buhot et al. 2015).

In addition to HIV entry receptors, some of the best known HDF include cyclophilin, which binds to HIV-1 capsid and facilitates decapsidation/reverse

transcription through an unknown mechanism (De Iaco and Luban 2014); cytoskeleton, which is required for intracellular trafficking of the virus (Menager and Littman 2016; Stolp and Fackler 2011); lens epithelium-derived growth factor (EDGF)/p75, which interacts with integrase and is responsible for the tethering and selective integration of HIV into active transcription units of the chromatin and possibly also in the regulation of HIV latency (Gerard et al. 2015; Engelman and Cherepanov 2008); positive transcription elongation factor (P-TEFb, composed of cyclin-dependent kinase 9 (CDK9) and of cyclin T1 or T2 (CycT1/T2)) and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF κ b) and nuclear factor of activated T cells (NAFT) transcription factors, which are required for HIV-1 transcription (Karn and Stoltzfus 2012); and endosomal sorting complex required for transport (ESCRT), which participates in HIV-1 budding (Usami et al. 2009).

While relying on numerous cellular factors, HIV-1 needs to overcome restriction factors that have evolved as part of our innate immune response to counteract infections (Arhel and Kirchhoff 2010). Although several cellular factors have been suggested to potentially inhibit HIV-1 infection (Liu et al. 2011), to date only a handful of restriction factors have been clearly validated. Serine incorporators 3 and 5 (SERINC3 and SERINC5) interfere with the delivery of viral particles to target cells (Rosa et al. 2015; Usami et al. 2015); apolipoprotein B mRNA editing enzyme catalytic subunit 3G (APOBEC3G), tripartite motif containing 5 (TRIM5alpha), and SAM domain- and HD domain-containing protein 1 (SAMHD1) impair reverse transcription (Santa-Marta et al. 2013; Simon et al. 2015); MX dynamin-like GTPase 2 (Mx2) hinders nuclear accumulation and integration of proviral DNA into the host chromatin (Goujon et al. 2013); bone marrow stromal cell antigen 2 (BST-2) retains newly produced viral particles at the surface of infected cells (Perez-Caballero et al. 2009). The strong expression of HDF present in immune cells suggests that HIV-1 needed to evolve to replicate in these cells, which contain critical factors for its replication cycle, and adapted to circumvent the action of restriction factors (Brass et al. 2008; Cleret-Buhot et al. 2015). Thus, the susceptibility of host cells to HIV-1 infection is largely dictated by the availability of HDF rather than the expression of restriction factors, with the exception of SAMHD1.

SAMHD1 possesses dNTPase and nuclease activities and can potentially interfere with HIV-1 reverse transcription by reducing the pool of intracellular deoxy-nucleoside triphosphate (dNTP) and by degrading incoming viral nucleic acids (Beloglazova et al. 2013; Laguette et al. 2011; Lahouassa et al. 2012), although the relative contribution of each of these activities is still unclear. This may vary as a function of cell type and cell cycle. SAMHD1 efficiently blocks HIV-1 infection in quiescent CD4+ T cells and monocytes and strongly decreases HIV-1 dynamics in differentiated myeloid cells, but it is inefficient in cycling CD4+ T cells (Laguette et al. 2011; Cribier et al. 2013).

The differences in the antiviral activity of SAMHD1 between these cell types are not related to its relative expression, but SAMHD1 antiviral activity is ablated by the phosphorylation of its threonine 592 (T592) residue (Cribier et al. 2013; White et al. 2013). Phosphorylation of SAMHD1 is regulated by cyclin-dependent kinases (CDK) 1/2, which coordinate T-cell division and differentiation in response to

antigen recognition (Wells and Morawski 2014). A recent report shows that tissue resident macrophages susceptible to HIV-1 infection such as microglial cells, which are responsible for HIV-1 persistence and compartmentalization in the brain (Schnell et al. 2011), are in a G1-like status and express high levels of phosphorylated (inactive) SAMHD1 (Mlcochova et al. 2017).

The influence of the cell cycle on HIV-1 replication is also well known (Goh et al. 1998). P21^{cip1/waf1}, a CDK inhibitor which regulates cell cycle arrest and is involved in monocyte differentiation (Asada et al. 1999; Xiong et al. 1993), is a potent inhibitor of HIV-1 infection (Bergamaschi et al. 2009). On the one hand, p21 controls the de novo synthesis of dNTPs by regulating the expression of the main enzymes involved in this process (Allouch et al. 2013; Valle-Casuso et al. 2017). Through its CDK inhibitor activity, p21 also controls the phosphorylation state of SAMHD1 (Allouch et al. 2014; Pauls et al. 2014b). p21 has also been shown to interfere with HIV-1 replication in hematopoietic stem cells (Zhang et al. 2007) and with CDK9-dependent transcription of HIV-1 in CD4+ T cells (Chen et al. 2011).

The differentiation state of the cells also influences the relative capacity of HIV-1 to replicate. HIV-1 replicates less well in naïve CD4+ T cells and monocytes than in memory CD4+ T cells and macrophages, and this is not only related to differential expression of HIV-1 co-receptors. Successful HIV infection requires stable integration of viral cDNA into the host cell genome. HIV integration occurs preferentially within transcription units of transcriptionally active genes (Han et al. 2004; Schroder et al. 2002). However, several mechanisms including epigenetic gene silencing, transcription gene silencing, and posttranscriptional gene silencing have been described to explain the establishment and maintenance of latency in target cells (reviewed in Van Lint et al. (2013)). Although these mechanisms are described in detail elsewhere in this book, it is interesting to note here that HIV gene expression is heavily dependent on the presence of several transcription factors, such as NFAT and NFκB. These factors are critical regulators of T-cell activation and differentiation, and their expression is necessary for rapid production of cytokines or effector molecules (Macian 2005; Oh and Ghosh 2013). Accordingly, NFAT and NFκB are expressed at very low levels in naïve and resting T cells and strongly expressed in activated and differentiated T cells.

In vitro studies suggest that latency can be established in both resting and activated CD4+ T cells (Chavez et al. 2015). However, it is reasonable to think that HIV-1 latency per integration event may be achieved more frequently in less-differentiated CD4+ T cells subsets than in effector cells, but this remains to be proven. The mammal target of rapamycin (mTOR) is a pivotal regulator of cell differentiation, cell cycle, proliferation, and survival (Chi 2012). mTOR directly regulates many HDF (e.g., cytoskeleton, NFAT) and it has been shown to control HIV-1 latency (Besnard et al. 2016). mTOR also regulates the metabolic activity of the cells (Powell and Delgoffe 2010), and expression of the glucose transporter Glut1 is required for HIV-1 replication (Loisel-Meyer et al. 2012). Thus, it is likely that mTOR has an important part in the regulation of HIV-1 infection in different cell subsets.

Much less evidence is available about the establishment of HIV latency in infected macrophages. As in CD4+ T cells, in macrophages HIV preferentially integrates into the transcriptionally active region of the chromatin (Kumar et al. 2014). However, it is not clear whether the mechanisms driving latency in CD4+ T cells are similar in macrophages. Latency can be established in macrophages in vitro (Brown et al. 2006), and HIV transcription is regulated in response to external signals and macrophage activation (Saez-Cirion et al. 2006). Moreover, the transcription factor B-cell CLL/lymphoma 11B (BCL11B/CTIP2), which is involved in multiple cellular processes including cell proliferation and survival, has been shown to repress HIV gene transcription in microglial cells by inhibiting the elongation factor P-TEFb and by inducing a compact, transcriptionally inactive, heterochromatic environment at the HIV promoter (Le Douce et al. 2010).

Although HIV-1 has evolved mechanisms to avoid the action of restriction factors such as APOBEC3G, this factor is expressed at very high levels in the cells that are more susceptible to HIV-1 infection, and this may come at a cost for the virus in terms of replication capacity in more differentiated cell subsets (Vetter et al. 2009). Finally, some cells, such as dendritic cells, are poorly or not susceptible to HIV-1 replication but can internalize free virions in non-cytolytic vesicles and transfer them at high concentrations to CD4 T cells upon interaction (Manches et al. 2014). Along these lines, follicular DCs have been shown to trap and retain infective HIV-1 particles for extended periods of time (Heesters et al. 2015).

9.3 Dodging Cell Death Upon Infection

9.3.1 Apoptosis

Although apoptosis is a major mechanism of defense against infection, viruses have evolved means to influence the balance of death and survival of the host cell in order to promote efficient virus replication and persistence of infection. Progressive CD4+ T-cell loss is a defining characteristic of uncontrolled HIV-1 infection. HIV-1 can provoke direct cytotoxicity on target cells. However, it is now well accepted that decline of CD4+ T cells in vivo is not solely due to direct viral cytotoxicity but to a multifactorial process that also includes apoptosis of “bystander” cells (Doitsh et al. 2010; Doitsh et al. 2014; Finkel et al. 1995) and killing of productively infected cells by immune effectors. Yet persistence of HIV-1 infected cells for long periods of time requires avoiding all these forms of cell death. Studies performed in vitro and ex vivo have shown the contribution of many different apoptotic molecules in CD4+ T cells and other HIV-1 susceptible cell subsets. The exact molecular mechanisms of HIV-1-induced cytotoxic or anti-apoptotic effects on infected cells that lead to long-lived HIV persistence are still not well understood.

Viral proteins such as tat (Li et al. 1995; Westendorp et al. 1995), env (Cicala et al. 2000), vpr (Muthumani et al. 2002), and nef (Muthumani et al. 2005; Xu et al. 1999) have been shown to have a pro-apoptotic effect in vitro. However, the action

of these proteins at physiological concentrations and in a complex immunological setting remains unclear. Moreover, the regulation of apoptosis depends on the interaction of viral factors with cell pathways, and the equilibrium between pro-apoptotic and anti-apoptotic signals may vary as a function of the stage of viral replication, nature, and state of the target/bystander cell and external signals. Nef has been shown to prevent apoptosis in productively infected cells upon Fas and tumor necrosis factor alpha (TNFalpha) ligation (Ohnimus et al. 1997) by inhibiting Fas signaling (Gelezunas et al. 2001). Nef was also shown to inactivate pro-apoptotic BCL2-associated agonist of cell death (Bad) protein, hence rendering infected T cells more resistant to apoptosis (Wolf et al. 2001).

On the other hand, myeloid lineage cells including monocytes and macrophages appear less sensitive to the cytopathic effect of HIV replication than T cells, suggesting that intrinsic properties of myeloid cells may render them selectively resistant to HIV-induced apoptosis (Kumar et al. 2014; Le Douce et al. 2010). Along these lines, telomerase activity increases in macrophages upon infection, rendering them more resistant to DNA damage or oxidative stress (Ojeda et al. 2014). Macrophages were observed to be more apoptosis resistant at least in part due to env (Swingler et al. 2007)- and nef (Olivetta and Federico 2006)-dependent alterations in apoptotic pathways.

Following ART, the contribution of direct viral cytotoxicity to cell death is likely minimal. Latently infected cells remain largely ignored by HIV-specific T cells due to the lack of expression of viral proteins, which favors persistence. However, infected CD4+ T cells have been reported to undergo integration-dependent cell death, linked to the recruitment of DNA-dependent protein kinase (DNA-PK) (Cooper et al. 2013). It is unlikely that latency is an “all or nothing” phenomenon, and it is possible that episodes of viral reactivation occur episodically in vivo even in the presence of effective cART (Nettles et al. 2005; Graf et al. 2013a). Recent studies have shown that reactivation of latently infected cells in ART-suppressed HIV-infected individuals with histone deacetylases (HDAC) inhibitors or other latency reversal agents was insufficient to promote cell death ex vivo or to decrease the frequency of integrated viral DNA or infectious units in vivo (Lehrman et al. 2005; Rasmussen and Lewin 2016; Rasmussen et al. 2014; Routy et al. 2012; Shan et al. 2012). Moreover, cells carrying latent replication competent viruses might be particularly resilient to CD8+ T cell-mediated killing even after viral reactivation (Huang and Jones 2017). Therefore, long-term HIV-1 persistence may be caused by cells that are particularly resistant to cell death, either due to their intrinsic properties or because of an anti-apoptotic state, supported by HIV factors.

Different studies using in vitro models of HIV-1 latency have described that establishment of latent HIV-1 infection is accompanied by the induction of anti-apoptotic proteins (e.g., B-cell CLL/lymphoma 2 (BCL2), cellular FLICE-like inhibitory protein (cFLIP), myeloid cell leukemia sequence 1 (Mcl-1) (Aillet et al. 1998; Berro et al. 2007; Tan et al. 2013) or the downregulation of pro-apoptotic proteins (e.g., BCL2-associated X protein (BAX), Fas-associating death domain-containing protein (FADD)) (Wang et al. 2011; Badley et al. 2013). Interestingly, in vivo, cells that support virus persistence such as TCM (Olvera-García et al. 2016)

and monocytes (Giri et al. 2009) had an anti-apoptotic gene signature in HIV-infected individuals compared to noninfected controls.

Death of nonproductively infected nonactivated T cells that do not express viral antigen has been reported to result from the detection of viral reverse transcription products by DNA sensor interferon gamma inducible protein 16 (IFI16), which leads to caspase-1 activation, thereby triggering pyroptosis (Doitsh et al. 2010; Monroe et al. 2014). However, the contribution of this form of cell death is probably limited once ART is initiated (Cai et al. 2016).

Apoptosis of bystander cells has also been linked to persistent immune activation seen in chronic infection via signaling by TNF family members (TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL), and TNF α) (Herbeauval et al. 2005, 2006; Katsikis et al. 1995; Sloand et al. 1997). Death signals delivered via Fas ligation (Katsikis et al. 1995; Sloand et al. 1997) were shown to have an important contribution to bystander cell depletion in HIV-infected individuals (Badley et al. 1997; Badley et al. 1996, 1998). However, these signals were observed to be counteracted, at least in part, by viral proteins expressed by infected cells. For example, Env was observed to induce resistance to TRAIL-induced apoptosis in macrophages (Swingler et al. 2007).

Overall, it seems that the activation status of cells plays a role in the susceptibility to cell death during HIV infection, which is also reflected by differential loss of cell populations from different tissues, where more or less activated cell phenotypes are found. For example, in the lymph nodes of HIV-infected individuals, the degree of apoptosis has been correlated with virus- and microbial-driven immune activation observed in infection and not the viral load (Muro-Cacho et al. 1995). It has been further shown that highly activated effector memory CD4+ T cells are depleted faster and first from gut mucosal sites (Grossman et al. 2006) and naïve T cells displaying a resting phenotype are resistant to depletion in lymphoid tissues (Veazey et al. 2000). Additionally, blood-derived CD4+ T cells that display a deeper resting state than lymphoid tissue-derived cells are more resistant to pyroptosis despite carrying viral genetic material (Muñoz-Arias et al. 2015). Thus, infected naïve, central memory, and stem cell-like memory T cells displaying a less activated phenotype could be less prone to these mechanisms of induced cell death than highly activated effector memory T cells. These differences could contribute to shaping where HIV persists, even before treatment initiation.

The relevance of these mechanisms during ART is unknown. ART undoubtedly halts loss of CD4+ T cells, but some level of abnormal chronic inflammation persists (Paiardini and Muller-Trutwin 2013), and it is likely that this may contribute to selective elimination during treatment of cells that have the highest susceptibility to apoptosis. The susceptibility of infected cells to cell death may also vary in tissues depending on the cytokine milieu. For example, interleukin (IL)-7 protected resting CD4+ T cells from death during in vitro HIV infection (Trinité et al. 2016), whereas IL-12 protected while IL-10 augmented Fas-mediated cell death of CD4 T cells from HIV-infected individuals (Estaquier et al. 1995).

9.3.2 Immune Clearance

HIV-1-specific cytotoxic CD8+ T cells and natural killer (NK) cells are able to eliminate infected cells, and these responses have been linked to protection against HIV transmission or natural control of infection (Betts et al. 2006; Graf et al. 2013b; Jennes et al. 2006; Martin et al. 2002; Ravet et al. 2007; Sáez-Cirián et al. 2007; Sáez-Cirián et al. 2009). However, during progressive infection, selection *in vivo* of viral variants that escape this immune pressure occurs progressively diminishing the capacity of these cells to counteract infection (Alter et al. 2011; Borrow et al. 1997; Deng et al. 2015; Mailliard et al. 2013). In elite controllers, HIV-1 persists for many years even in the presence of highly efficient CD8+ T-cell responses (Noel et al. 2016), suggesting that persistent infected cells are able to avoid immune surveillance. Latently infected resting CD4+ T cells that do not actively express viral epitopes escape immune surveillance although transient expression of viral antigens may trigger their killing (Graf et al. 2013a). Viral proteins such as Nef (Cohen et al. 1999) and Vpu (Apps et al. 2016) downregulate major histocompatibility complex (MHC) class I and may contribute to protect infected CD4+ cells from the CD8+ T-cell response (Collins et al. 1998; Xu et al. 1997) (although this could make these targets susceptible to NK mediated killing (Cohen et al. 1999)).

Infected macrophages might be more resistant than CD4+ T cells to killing by CD8+ T cells *in vitro*, independently of nef (Rainho et al. 2015; Vojnov et al. 2012). Infected macrophage can be eliminated by HIV-specific cytotoxic CD4+ T cells (Sacha et al. 2009), which have been found to increase during acute infection (Soghoian et al. 2012) and in elite controllers (Johnson et al. 2015). In addition, in macrophages, HIV-1 particle assembly in intracellular virus-containing compartments (VCCs) (Jouve et al. 2007; Welsch et al. 2011) may provide protection from immune recognition (Tan and Sattentau 2013).

In addition to escape mutations and latency, effective immune responses are also curtailed by the physical separation of effector cells from their targets residing in tissue sanctuaries. The central nervous system (CNS) has long been considered an “immune-privileged” site where infected macrophages, astrocytes, and microglial cells are relatively inaccessible to antiviral immune responses and variably accessible to ART (Letendre et al. 2008; Joseph et al. 2015), thus constituting an important viral reservoir.

Cerebrospinal fluid (CSF) from noninfected individuals contains CD4+ T cells with an activated central memory phenotype (Kivisäkk et al. 2003), which are a preferential target of HIV-1. Compared to the blood, the ratio of CD8/CD4 T cells is much lower in the CNS, and it has been suggested that antigen-specific CD8+ T cells found in the CNS do not provide durable immune surveillance in the absence of antigen (Young et al. 2011). Resident memory CD8+ T cells can be found in the CNS in the context of viral infection (Wakim et al. 2010), and it is now recognized that functional HIV-1-specific CD8+ T cells infiltrate CNS during acute (Kessing et al. 2017) and chronic (Ganesh et al. 2016) HIV infection. Remarkably, CD8+ T-cell responses in the CNS are detected in elite controllers with undetectable viral

load in the CSF and blood (Sadagopal et al. 2008) and during ART (Lescure et al. 2013; Miller et al. 2004) and contribute to the control of infection in the CNS (Marcondes et al. 2015).

Several reports have shown accumulation of virus bearing Tfh cells in germinal centers of lymphoid follicles (Banga et al. 2016; Perreau et al. 2013) where CD8+ T cells are found in low frequencies as compared to T-cell zones (Connick et al. 2007; Folkvord et al. 2005; Fukazawa et al. 2015; Hong et al. 2012). However, recently identified follicular cytotoxic T cells (Tfc) were shown to enter B-cell follicles of HIV-infected individuals and to have cytotoxic potential (Leong et al. 2016; Petrovas et al. 2017). Cytotoxic CD8+ T cells displaying viral target lysis are also detected in the lamina propria (Murphy-Corb et al. 1999) as well as vaginal epithelium and submucosa (Lohman et al. 1995) of SIV-infected rhesus macaques. Activated CD8+ T cells with cytotoxic potential were also located in adipose tissue (which carried infected CD4+ T cells and macrophages) of SIV-infected monkeys (Damouche et al. 2015). It is, therefore, probable that HIV-specific CD8+ T-cell responses in tissue contribute to the elimination of infected cells in nonlymphoid tissues and even the CNS, although more studies will be needed to define their characteristics.

9.4 Endurance and Renovation

The number of cells containing HIV DNA decreases sharply during the first months following initiation of ART (Finzi and Siliciano 1998; Avettand-Fenoel et al. 2016). A steady state appears to be reached by 2 years on ART, although DNA decay continues for longer periods of time when ART is initiated during primary infection (Avettand-Fenoel et al. 2016). Modeling the dynamics of decline in HIV DNA showed that this decay occurs in several phases that have been attributed to the sequential loss of infected cells of varying half-life (Finzi and Siliciano 1998; Perelson 2002). In the context of ART efficiently blocking systemic HIV replication, cells with active viral replication are expected to be eliminated within a few days due to cytopathic effects or immune clearance (see above). However, some cells like macrophages can produce infectious viral particles for long periods of time without being killed and resting CD4+ T cells carrying latent provirus persist despite multiple decades of treatment.

The maintenance of infected CD4+ T cells under ART is driven by survival of long-lived cells and homeostatic proliferation (Chomont et al. 2009). The life span of quiescent CD4+ T cell progressively decreases with differentiation. Naïve CD4+ T cells are much longer lived than memory cells, and early differentiated memory CD4+ T cells have a longer half-life than terminally differentiated cells. TNA, TSCM, and TCM upregulate genes associated with survival and are less prone to undergo apoptosis, at least in vitro (Lugli et al. 2013; Mahnke et al. 2013). It has been estimated that one TNA has a half-life of 1 to several years, a TCM a few months to 1 year, while a TEM would only be a few weeks (Macallan et al. 2004;

Vrisekoop et al. 2008). TSCM have the highest survival capacity among memory T cells in the absence of cognate antigen (Lugli et al. 2013; Gattinoni et al. 2011). However, these estimations, largely based on the *in vivo* analysis of incorporation of deuterated glucose or water on CD4+ T cells, are limited by the lack of resolution on cells that migrate to the tissues. The recently described resident memory T cells are programmed to persist locally and not recirculate even in the absence of antigen (Clark 2015). The role of these cells in the context of HIV-1 infection has not been clarified yet but their potential contribution to the persistence of HIV on ART deserves analysis.

The contribution of infected macrophages to HIV persistence on ART is debated (DiNapoli et al. 2017). Resting CD4+ T cells constitute the bulk of persisting infected cells, but the potential implication of infected macrophages as source of rebounding virus if treatment is interrupted should not be overlooked (Crowe et al. 2003). Although it is accepted that viral decay in monocytes/macrophages is slower than in activated CD4+ T cells, it is often assumed that the half-life of infected monocytes/macrophages is shorter than that of quiescent CD4+ T cells (Finzi and Siliciano 1998; Stevenson 2003; Van Lint et al. 2013). However, the life span of macrophages, as for CD4+ T cells, also varies greatly. Depending on their tissue location, macrophages can live from a few months to several years. Alveolar macrophages, which can be infected by HIV (Jambo et al. 2014), have been found to persist for over 3 years in analyses performed after lung transplant (Eguiluz-Gracia et al. 2016; Nayak et al. 2016), and microglial cells persist for years in the CNS (Tay et al. 2017). Moreover, macrophages may be better prepared than CD4+ T cells to resist apoptosis under conditions of metabolic stress (Carter and Ehrlich 2008; Jones and Power 2006; McNelis and Olefsky 2014).

It was previously assumed that activation of HIV-infected CD4+ T cells driving them to proliferation would reverse viral latency and decrease the half-life of cells carrying replication competent virus. However, recent phylogenetic studies using ultra deep whole genome sequencing have shown the presence of proviruses with identical sequences in clonally expanded infected CD4+ T cells (Chomont et al. 2009; Cohn et al. 2015; Maldarelli et al. 2014; Simonetti et al. 2016; Wagner et al. 2014; Boritz et al. 2016). Moreover, it is now clear that these expanded infected CD4+ T cells can also harbor intact proviruses able to spread infection (Hosmane et al. 2017; Simonetti et al. 2016). Several reports have shown that proliferation of infected CD4+ T cells could be at least partially driven by the selective integration of HIV-1 into genes that have been associated with cell growth, division, and cancer (Maldarelli et al. 2014; Wagner et al. 2014).

In addition, CD4+ T cells can divide in response to antigenic stimulation or to homeostatic signaling to balance cell numbers although capacity of self-renewal is lost with progressive differentiation of memory CD4+ T cells (Berard and Tough 2002; Mahnke et al. 2013). Antigenic stimulation through the T-cell receptor entails the activation of the cell and triggers cell differentiation. However, naïve and early differentiated cells require a higher signaling threshold and prolonged contact with antigen-presenting cells, and they also depend more on co-stimulatory signals than more differentiated cells to respond to antigens. Thus, low levels of antigen during

treated infection might provide a suboptimal signal allowing some degree of activation of these cells. It is however unknown whether, *in vivo*, some transiently activated cells might escape cell death despite some degree of viral production to later regain a quiescent state. In contrast, *in vitro* studies have confirmed that infected CD4+ T cells can undergo homeostatic proliferation without significant viral production or cell death (Bosque et al. 2011).

Homeostatic proliferation is governed by members of the common gamma chain family of cytokines in the absence of antigenic stimulation (Boyman et al. 2007; Seddon et al. 2003; Surh and Sprent 2008). In particular, IL-7 plays a central role in CD4+ T-cell homeostasis and survival. In the case of naïve CD4+ T cells, IL-7 signaling and contact with self-MHC-peptides complexes promotes cell survival without inducing proliferation. In contrast, IL-7 signaling can promote proliferation of memory CD4+ T cells independently of TCR activation. Responsiveness to IL-7 is not equal among all CD4+ T-cell memory subsets. TSCM and TCM express high levels of the IL-7 receptor (CD127) and have strong proliferative potential, while TEM express lower levels of CD127 and have a limited proliferative potential (Mahnke et al. 2013). Other common gamma chain cytokines (such as IL-2 or IL-15) also influence the survival and turnover of T cells in their inflammatory environment (Pennock et al. 2013). Overall, once established, memory CD4+ T cells can persist for decades in the absence of antigen (Hammarlund et al. 2003).

Macrophages and dendritic cells are terminally differentiated cell populations that cannot be propagated *in vitro*. However, macrophage subsets that are susceptible to HIV-1 infection are not in a quiescent state and share some characteristics of cycling cells (Badia et al. 2016; Mlcochova et al. 2017; Pauls et al. 2014a). Although infected macrophages did not show evidence of division *in vitro*, it is now clear that *in vivo* some macrophages can proliferate locally in tissues in response to inflammatory signals (Jenkins et al. 2011; Robbins et al. 2013; Zamarron et al. 2016). Thus, the possible persistence of some infected macrophages through cell division cannot be discarded.

9.5 Conclusion

The persistence of HIV-1-infected cells on ART depends on a combination of cell intrinsic characteristics including the susceptibility of cells to infection and their capacity to survive and proliferate (Fig. 9.2). However, it is also influenced by the responsiveness of these cells to external signals (such as inflammatory cytokines or contact with antigen-presenting cells) or their localization and capacity to circulate. For instance, HIV-specific CD4+ T cells are preferentially infected by HIV during treatment interruption when compared to other antigen-specific memory CD4+ T cells, and this is likely due to the selective localization of activated HIV-specific cells to the sites of viral replication (Douek et al. 2002). HIV-1 can thus reside in multiple cell subsets in multiple tissues, while the main mechanisms for persistence are diverse. It is of the outmost importance to define which specific infected cell

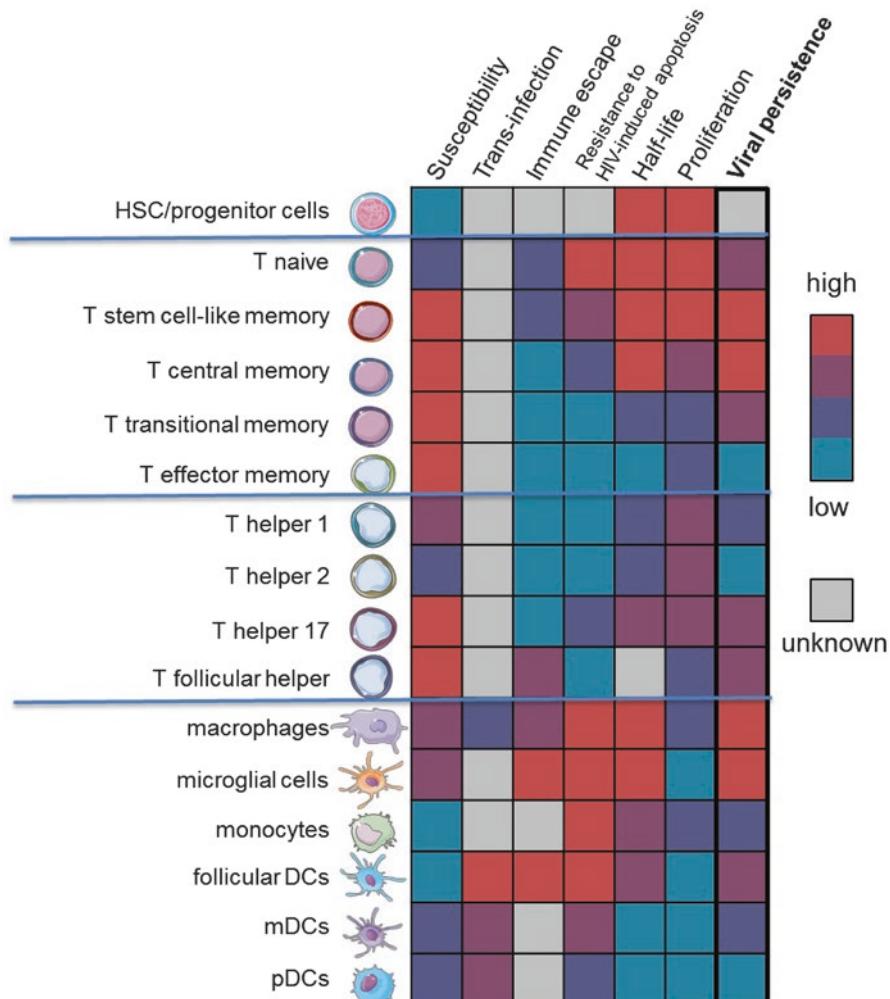


Fig. 9.2 Heatmap showing multiple features of different cell subsets that lead to HIV persistence on ART. Color codes were inferred on the basis of the literature discussed in this chapter. Among T-cell subsets, naïve, central memory, and stem cell-like memory T cells have a major contribution to long-term HIV persistence on ART, while effector memory and terminally differentiated helper T-cell subsets are depleted first during infection and have limited proliferative potential. Myeloid cells, despite their relatively low susceptibility to infection, are now increasingly recognized as important contributors to HIV persistence on ART in both lymphoid and non-lymphoid tissues due to their long half-life and resistance to apoptosis and immune-mediated killing. Although not shown here, tissue localization and activation status influence various parameters of persistence, with actively infected cells being more susceptible to cell death, thus reducing their contribution to long-term persistence.

subset leads to viral rebound off ART and to determine if some of these subsets should be preferentially targeted. Irrespective, tackling HIV persistence will require multiple diverse strategies that target these difference mechanisms leading to cell survival.

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Chapter 10

In Vitro and In Vivo Models of HIV Latency



James B. Whitney and R. Brad Jones

Abstract Latently infected cells are very infrequent in CD4+ T cells from antiretroviral (ARV) treated individuals, with only approximately one in a million infected CD4+ T cells in blood. Given the low frequency of infected cells in vivo, multiple in vitro latency models have been developed to facilitate investigations into mechanisms of HIV latency, as well as to enable the evaluation of pharmacological and immunological interventions aimed at depleting latently infected cells. These in vitro models include clones of transformed cell lines with integrated HIV proviruses or primary CD4+ T cells from uninfected donors that have been infected with HIV in particular conditions. This chapter presents a description of these various in vitro models, along with an overview of their advantages and limitations.

Prec clinical animal models represent a critical bridge between in vitro studies and human clinical trials. Simian immunodeficiency virus (SIV) infection of Indian origin rhesus macaques has been well established as an informative model of HIV infection. Recent years have seen breakthroughs in ARVs that permit the potent suppression of SIV replication, enabling studies of latency and putative curative interventions in this model. Small animal models of HIV infection can be generated by engrafting immunodeficient mice with human immune cells. These “humanized mice” have provided valuable insights into HIV pathogenesis and are under development as models for studying HIV latency. We summarize both the promise of these models and outstanding challenges that remain to be overcome to realize their potential to inform efforts to cure HIV infection.

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10.1 Introduction

In individuals on long-term antiretroviral therapy (ART), latently HIV-infected cells comprise an exceedingly infrequent proportion of total CD4⁺ T cells. Typically, on the order of 1 in 1000 CD4⁺ T cells contains proviral DNA; however, this is primarily comprised of defective proviruses (Bruner et al. 2016; Ho et al. 2013). Only a median of ~1 in 10^6 CD4⁺ T cells harbors an intact HIV provirus that can be activated following a single round of maximal in vitro stimulation (Siliciano et al. 2003; Crooks et al. 2015). This low frequency of infected cells poses a substantial challenge to studying HIV latency in samples from people living with HIV. For example, $5-10 \times 10^6$ CD4⁺ T cells are needed to determine whether a single compound is capable of reversing HIV latency. Since the yield of CD4⁺ T cells from peripheral blood mononuclear cells (PBMCs) is typically 10–20%, $\sim 50 \times 10^6$ PBMCs are needed to test a single replicate of a given compound. A relatively large volume blood draw of 150 mL thus allows one to test a single compound along with a negative and positive control.

Higher cell numbers, on the order of 10^{10} from a single participant, can be obtained by a procedure called leukapheresis, which separates leukocytes from blood and retains other blood constituents to the circulation. While leukapheresis samples are invaluable for cure research, the cell numbers obtained are still limiting for many applications. There is also a limited ability to manipulate latently infected cells in order to probe mechanisms of latency. These and other considerations have motivated the development of a diverse set of in vitro models of HIV latency that have yielded valuable insights. In addition to in vitro models of latency, recent years have seen significant breakthroughs in the development of animal models of HIV latency that can support the preclinical development of therapeutic strategies. In the current chapter, we aim to provide an overview of the current state of the art in terms of in vitro and in vivo models of HIV latency, framed by a historical perspective to provide context.

10.2 Cell Line Models of Latency

The first investigations of HIV latency relied on the use of in vitro immortalized T cell or monocytic cell lines such as ACH2 or U1 (Folks et al. 1986; Folks et al. 1987; Duh et al. 1989; Folks et al. 1988). Although cell lines have been used with success in delineating mechanisms of viral transcriptional suppression (Poli et al. 1991), much of the early focus was on nuclear factor kappa B (NFkB) regulation of gene expression in these systems (Duh et al. 1989; Devadas et al. 2004; Poli et al. 1990;

Nabel and Baltimore 1987). Perhaps the most important findings using these immortalized cell systems were in the screening and determination that a number of repurposed drugs, such as histone deacetylase inhibitors (HDACi) (Van Lint et al. 1996a, b), could function, albeit at varying levels, to reverse HIV latency (Doyon et al. 2013; O'Brien et al. 1995; Rasmussen et al. 2013). More recent analysis has relied on these lines for the screening of anti-apoptotic agents (Moren et al. 2017).

Subsequent studies have indicated that many cell lines harbor virus that are not truly latent per se but rather have impaired capacity to upregulate HIV transcription activity in response to external stimulation. Several investigators have noted some of these same cell lines contained proviral mutations that contributed to or enhanced the latent state. For example, U1 cells were shown to encode a truncated *tat* gene, resulting in a virus that was transcriptionally impaired (Emiliani et al. 1996; Emiliani et al. 1998). Emiliani et al. showed that ACH2 T cells were shown to have provirus harboring a mutation in the TAR region that impaired responsiveness to Tat (Emiliani et al. 1998). Recent work using integration site analyses has shown that latently infected cell lines with intact virus frequently have low levels of ongoing virus replication (Symons et al. 2017).

Some investigators had concluded that that latently infected cells *in vivo* may harbor large mutation which will favor a latent state (Yukl et al. 2009). Further investigation of viral mutations regulating latency indicated that this is not an important mechanism (Chun et al. 1995). In fact, latency in many cell lines is similar to latently HIV-infected primary T cells, with the majority of HIV integration events occurring in actively transcribed host genes (Siliciano et al. 2007; Siliciano and Siliciano 2005). Several reports indicate that the integration site of virus in latently infected cell lines is found primarily within actively expressed host cell genes similar to latently infected cells from HIV-infected individuals on ART (Han et al. 2004; Han et al. 2008). Despite this, there is evidence for some HIV-infected immortalized cells in which provirus is not integrated into actively transcribed DNA domains (Winslow et al. 1993).

10.2.1 Reporter Cell Lines for Drug Screening HIV LRAs

The use of surrogate markers to track active HIV expression from latency was first addressed through the generation of latently infected cell lines that contain a reporter virus that expresses green fluorescent protein (GFP) (Jordan et al. 2003). Many of these latently infected reporter cell lines harbor virus integration in active host cell genes similar to that observed in latently infected primary T cells (Jordan et al. 2003; Han et al. 2004; Lassen et al. 2004; Shan et al. 2012; Shan et al. 2011).

Latently HIV-infected reporter cell lines have been generated using numerous approaches but generally there is an assumption that latency persists in cells not expressing GFP, as an “off” phenotype. These selected clones, presumed latent, without expressed GFP, are then confirmed functional, represented by GFP “on” after TNF- α or PMA stimulation (Pearson et al. 2008). Interestingly, these cell lines

revert to a latent state following HIV reactivation with TNF- α or PMA, suggesting a common mechanism controlling latency in these systems (Han et al. 2004; Lassen et al. 2004).

Reporter cell lines have provided significant advantages over immortalized lines such as U1 and ACH2, as these systems remain cumbersome for advanced high throughput drug screening approaches (reviewed in Planelles et al. 2011). HIV-infected reporter cell lines designed for drug screening have included an LTR-Tat construct that regulates the expression of GFP in conjunction with secreted alkaline phosphatase (SEAP) (Micheva-Viteva et al. 2011; Edelstein et al. 2009; Micheva-Viteva et al. 2005). While GFP expression was used to select latently infected cell clones, SEAP production was used to detect and measure levels of HIV transcriptional activity. A similar system (LWI-6) was specifically designed for use in a high-throughput screen (Jones et al. 2007) and also employs a GFP-based readout for viral transcription.

10.3 Primary Cell Models of Latency

10.3.1 Introduction

The factors that regulate whether an HIV-infected cell establishes a state of latency can be divided into (1) virus-intrinsic, (2) integration site or epigenetic, and (3) host cell-intrinsic. As is reviewed below, immortalized cell line models of latency have proven invaluable in probing the first two of these factors. Their utility is ultimately limited, however, by the disparity between the functional states of these highly activated cell lines – often T lymphoblast lines – and the quiescent resting memory CD4 $^{+}$ T cells that engender latency in individuals on long-term ART (Chun et al. 1995; Chun et al. 1997; Finzi et al. 1997; Wong et al. 1997). As a result, relatively few of the candidate latency reversal agents (LRAs) that have been identified in cell line model screens have proven effective in reactivating virus from using CD4 $^{+}$ T cells from HIV-infected individuals on ART.

The activation state of a host cell plays a critical role in the establishment and maintenance of latency. This is evident both in the observation that *in vivo*, latently infected cells are predominately limited to resting memory CD4 $^{+}$ T cells and in the ability of diverse T-cell activating agents, such as mitogens and antigens, to reverse latency. This can be attributed specifically to deficiencies of factors required for HIV transcription, such as P-TEFb, in resting CD4 $^{+}$ T cells, and more generally to differences in metabolic or biosynthetic pathways between resting and activated CD4 $^{+}$ T cells (reviewed in MacIver et al. 2013). Based on such reasoning, considerable effort has been directed toward developing models of HIV latency in primary (i.e., non-transformed) resting CD4 $^{+}$ T cells.

10.3.2 Direct Infection Latency Models

One approach to establishing latency in vitro is direct infection of resting CD4⁺ T cells (Fig. 10.1). This approach is limited by the fact that resting CD4⁺ T cells are resistant to infection, relative to activated CD4⁺ T cells, due to inefficient reverse transcription, nuclear import, and integration (Spina et al. 1995; Zack et al. 1990; Zack et al. 1992; Sun et al. 1997; Stevenson et al. 1990). Despite these barriers, with the use of strategies to enhance levels of viral attachment, such as centrifugation to bring virus into contact with cells (“spinoculation”), viral integration can be achieved at low levels in resting cells (Swiggard et al. 2005; Agosto et al. 2007). In earlier studies, activation with IL-7 or anti-CD3/anti-CD28 was shown to induce HIV expression that could be detected by flow cytometry, establishing the viability of pre-activation models (Swiggard et al. 2005). To follow convention in the field, we term this approach the “O’Doherty model.” Improvements have since been made to this schema:

Inspired by the relative ease with which resting CD4⁺ T cells can be infected in lymphoid organ cultures, the *Lewin model* pretreats resting CD4⁺ T cells with the chemokines CCL19 and CCL21 to substantially enhance their susceptibility to direct infection. Mechanistically, this has been linked to chemokine-induced changes in the actin cytoskeleton of resting cells (Cameron et al. 2010). This model has been utilized to screen novel latency-reversing agents (Wightman et al. 2013), to probe mechanisms of latency establishment or reversal (Venkatachari et al. 2015);

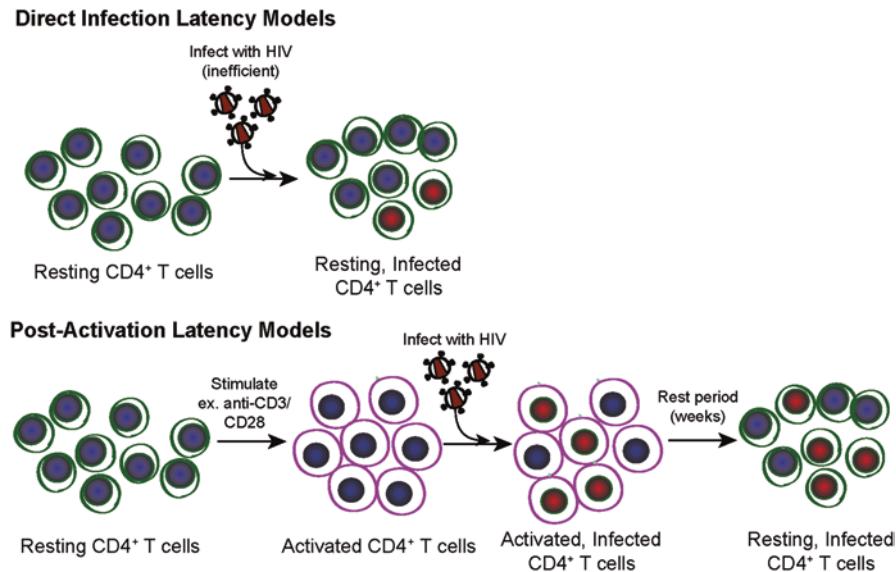


Fig. 10.1 General schematics of primary cell latency models

Jamaluddin et al. 2016), to determine which LRAs effectively drive antigen presentation to autologous CD8⁺ T cells (Jones et al. 2016), and to screen for biomarkers of latently infected cells (Raposo et al. 2017).

The *Greene model* utilizes a dual-reporter HIV that expresses mCherry and luciferase to directly infect resting CD4⁺ T cells. The spinoculation method used to directly infect resting CD4⁺ T cells results in a high level of attachment of virus to cells, with relatively little viral integration. This poses a challenge to distinguishing bona fide de novo viral protein expression, from viral proteins that are simply associated with cells in the form of bound virus. The Greene model solves this challenge through the use of a reporter mCherry-T2A-Luciferase cassette upstream of *nef* (separated by an internal ribosome entry site (IRES)). As the resulting mCherry is not appreciably packaged into virions, the expression of this fluorescent protein can be reliably used to assess frequencies of infected cells expressing de novo viral products. The luciferase reporter expressed in tandem with mCherry further provides the ability to evaluate average quantities of expression on a per-cell basis, allowing assessment of this additional dimension of latency reversal. Insight gained from the use of this model includes the description that different cell subsets respond differently to different LRAs (Lassen et al. 2012) and the demonstration that stimulation of innate immune receptors can enhance the elimination of reactivated cells (Li et al. 2016).

10.3.3 Post-activation Latency Models

An alternative method to generating a pool of latently infected cells is to activate CD4⁺ T cells in vitro, infect them in this activated state, and then allow them to return to resting over prolonged in vitro culture (Fig. 10.1). These strategies are collectively referred to as post-activation latency models. A key challenge in establishing these models is that the large majority of HIV-infected activated CD4⁺ T cells die before returning to a resting state, likely due to a combination of viral cytopathic and non-viral effects, such as activation-induced cell death (Green et al. 2003; Sprent and Surh 2003). A number of strategies have been developed which successfully mitigate this issue, allowing the generation of substantial numbers of latently infected resting CD4⁺ T cells to survive.

The *Cloyd model* was based on the observation that the co-culture of activated CD4⁺ T cells on a brain tumor-derived attached cell line (H80) facilitated their transition to a quiescent state and subsequent survival for many months (Sahu et al. 2006). Intriguingly, a subset of the HIV-infected cells that were cultured in this manner exhibited persistent low-level virus production, including those that had the phenotype of resting CD4⁺ T cells (CD69⁻HLA⁻DR⁻CD25⁻). Stimulation with prostratin was shown to result in a ~2-fold increase in the frequencies of HIV-Gag-expressing cells. While this model represented a significant and innovative advance, the ongoing expression of HIV limited its utility as a latency model.

The *Karn model* builds upon the Cloyd model, using the same H80 feeder cell co-culture strategy to enhance CD4⁺ T-cell survival (Tyagi et al. 2010). Cells are

infected with viral clones in which *nef* has been replaced with a fluorescent reporter (GFP or mCherry). While in an activated state, reporter-expressing cells are sorted by flow cytometry to enrich for infected cells. These cells are then expanded for 4–6 weeks with agonistic anti-CD3 and anti-CD28 antibodies (anti-CD3/28) and then plated on H80 feeder cells in the presence of IL-2. Over the course of 6 weeks, cells enter into a quiescent state, as evidenced by a reduction in cell size, loss of HIV reporter expression, loss of the CD25 activation marker, and reduced DNA synthesis. Remarkably, these cells can be maintained in co-culture with H80 cells for more than 6 months without noticeable losses in HIV viability or reactivation capability. During this time they maintain a resting central memory phenotype, consistent with the population that harbors the majority of the latent HIV reservoir *in vivo* (Chomont et al. 2009).

The *Siliciano model* improves the survival of HIV-infected CD4⁺ T cells by transducing the cells with the anti-apoptotic protein B cell lymphoma gene (*Bcl2*) prior to HIV infection (Yang et al. 2009; Kim et al. 2014). The method comprises activating resting CD4⁺ T cells with anti-CD3/CD28 for 3 days, transduction with a *Bcl2*-encoding lentivirus, and then a 3-week culture period to select for *Bcl2*-expressing cells (on the basis of improved survival). Transduced cells are then expanded for 10 days with IL-2, reactivated with anti-CD3/CD28 and infected with a GFP reporter virus. Cells are then cultured for 4–6 weeks to allow for the reestablishment of a latent state and sorted by flow cytometry to deplete productively infected cells (GFP⁺). The result is a resting CD4⁺ T-cell population of which typically 1–3% of cells are latently infected, as demonstrated by induction to express GFP with a strong LRA. This model has been used to screen for LRAs that are active in latently infected primary CD4⁺ T cells (Xing et al. 2012; Xing et al. 2011; Spivak et al. 2014), as well as an assessment of death of latently infected cells *in vitro* (Shan et al. 2012).

The *Bosque/Planelles model*, also known as the “cultured T_{CM} model” begins with the isolation of purified naïve CD4⁺ T cells from peripheral blood which, upon stimulation with anti-CD3/CD28 in the presence of transforming growth factor (TGF)- β and blocking antibodies against IL-4 and IL-12, mature into non-polarized (NP) memory CD4⁺ T cells (Bosque and Planelles 2011). These NP memory CD4⁺ T cells have many features in common with central memory CD4⁺ T cells (T_{CM}), which represent a significant portion of the latent reservoir *in vivo* (Chomont et al. 2009). As with T_{CM}, NP memory CD4⁺ T cells do not produce IFN- γ or IL-4 upon stimulation but can produce IL-2 and be driven to differentiate into either T_{H1} or T_{H2} cells (Messi et al. 2003; Iezzi et al. 2001) and exhibit high capacities for survival and proliferation (Sallusto et al. 1999; Bosque and Planelles 2011). The latter two factors also make these cells particularly well suited to the generation of post-activation latently infected cells. In the latest iteration of this model, naïve CD4⁺ T cells are (1) stimulated for 3 days in the conditions indicated above, (2) allowed to proliferate for an additional 4 days, (3) infected with replication-competent HIV for 6 days, and (3) allowed to return to a resting state in the presence of ARVs for at least 4 days. Cells with persistent HIV expression are then depleted based on their CD4 surface expression. The result is a resting CD4⁺ T-cell population of which typically 1–3% of cells

are latently infected, as defined by induction to express HIV-Gag with a strong LRA (Martins et al. 2016). The insights that have arisen from this model include identification of a TLR-1/2 agonist as a novel LRA (Novis et al. 2013) and benzotriazoles as agents that act in synergy with IL-2 to reverse latency (Bosque et al. 2017), insight into the role for p53 in the establishment of latency (White et al. 2016), and investigations into the relationship between proviral integration site placement and latency (Sherrill-Mix et al. 2013). Of note, clonal expansion of latently HIV-infected cells occurs in this model, recapitulating an important feature of latently infected cells in HIV-infected individuals on ART (Sunshine et al. 2016).

10.3.4 Comparisons Across Models

A study by Spina et al. compared the activity of putative LRAs in the Greene, Lewin, Planelles/Bosque, Siliciano, and Spina primary cell latency models, as well as against CD4⁺ T cells from HIV-infected individuals on ART (Spina et al. 2013). Each of the 12 putative LRAs tested exhibited activity in at least one model; however, only protein kinase C (PKC) agonists and mitogens (PHA/PMA) showed uniform activity in all models. The two post-activation models, Planelles/Bosque and Siliciano, were in fairly good agreement with each other, indicating potent LRA activity with mitogens and PKC agonists and little to no activity with the HDAC inhibitor vorinostat. The pre-activation Greene and Lewin models exhibited differences, however, with the profile of the Greene model fitting closely with that of the Planelles and Siliciano models, while the Lewin model additionally showed LRA activity by the HDAC inhibitors vorinostat, MRK-1, and MRK-11. Using a hierarchical analysis, the results using CD4⁺ T cells from HIV-infected individuals on ART clustered most closely with the Lewin model. Whereas most studies with ex vivo CD4⁺ cells have shown weak or no latency reversal with vorinostat as a single agent (in line with the Planelles, Siliciano, and Greene models), activity as an LRA increased when used in combination with the PKC agonist bryostatin (Laird et al. 2015). There is thus value in performing investigations in multiple latency models in parallel and in carefully validating any novel insights using CD4⁺ T cells from HIV-infected individuals on ART (Table 10.1).

10.4 Humanized Mouse Models of HIV Latency

10.4.1 Introduction

Murine cells do not support HIV infection or replication due to the absence of entry receptors, as well as multiple postentry blocks (Bieniasz and Cullen 2000; Browning et al. 1997). This has led to the development of “humanized mice,” comprising

Table 10.1 Comparisons of primary cell latency models

Name	Type	Cell phenotype	Time to generate latency	Yields of latency	Virus	Readout of reactivation
O'Doherty	Direct infection	HLA-DR ⁻ , CD25 ⁻ , CD69 ⁻	2–3 days	Low	III-B, NL4-3, YU-2	Intracellular Gag
Lewin	Direct infection	CCR7 ⁺ , CD45RA ⁺ , HLA-DR ⁻ , CD25 ⁻ , CD69 ⁻	3–4 days	Low	NL4-3 (wt)	Intracellular Gag, reverse transcriptase assay
Greene	Direct infection	T _{CM} , T _{TM}	3–4 days	Low	NL4-3 (mCherry:Luc)	Fluorescent reporter, luciferase
Cloyd	Post-activation	CD25 ⁻ , HLA-DR ⁻ , CD69 ⁺	4–6 weeks	High		Intracellular Gag
Karn	Post-activation	CD45RO ⁺ , CCR7 ⁺ , CD25 [±] , CD38 ⁺	4–6 weeks	High		Fluorescent reporter
Siliciano	Post-activation	CD45RO ⁺ , CD62L ⁺ , CCR7 ⁻	12 weeks	High	NL4-3 Δ6-drEGFP	Fluorescent reporter
Planelles/Bosque	Post-activation	CCR7 ⁺ , CD27 ⁺ , CD45RO ⁺ , CD25 [±] , CD69 ⁻	2–4 weeks	Medium	NL4-3 (wt) (newest assay)	Intracellular Gag

immunodeficient mouse strains xenografted with human cells and/or tissues, and thus supports HIV replication in human CD4⁺ T cells and/or myeloid cells (reviewed in Marsden and Zack 2015). Humanized mouse models have proven useful in studying aspects of HIV pathogenesis and transmission. More recent efforts have focused on adapting these models for the study of HIV latency.

hu-PBL-SCID mice: In the earliest iteration of this approach, human PBMCs were injected into the intraperitoneal cavity of CB17-severe combined immunodeficiency (SCID) mice (Mosier et al. 1988). These mice supported robust HIV infection for up to 16 weeks after infection but were limited somewhat in these early experiments by relatively poor and inconsistent levels of engraftment, driven by the rejection of human cells by the murine natural killer (NK) cells that remain in CB17-*scid* mice. Engraftment of human PBMCs is dramatically enhanced in the more recently developed NOD SCID IL2ry^{null} (NSG) mouse (Spranger et al. 2012) though, to our knowledge, studies of HIV have not been revisited in this setting. Graft-versus-host disease (GVHD) also develops relatively quickly (apparent within 3–4 weeks) in hu-PBL-SCID mice, limiting the duration of experiments.

SCID-hu (Thy/Liv) mice: Another approach to “humanizing” mice involves the implantation of portions of human fetal thymus tissue under the kidney capsule of SCID mice to generate a thymic organoid that supports the thymopoiesis of human

T cells (McCune et al. 1988; Namikawa et al. 1990). An important limitation of these mice is that human cells remain largely confined to the thymic organoid, though animals can support HIV replication following injection of virus directly into this site (Namikawa et al. 1988). An additional limitation is the complexity involved in acquiring and the surgical implantation of human fetal thymic tissues to generate these mice. GVHD develops in SCID-hu mice within 14–20 weeks (Lockridge et al. 2013).

Hu-HSC mice: In a third approach, mice are sublethally irradiated to open up bone marrow niches and then injected with human hematopoietic stem cells (HSCs), derived from either fetal liver, cord blood, or bone marrow (Lapidot et al. 1992). These mice become reconstituted with multiple myeloid and lymphoid lineages, including T cells, and this reconstitution is particularly robust in newborn NSG mice (Brehm et al. 2010). GVHD develops in Hu-HSC mice within 14–20 weeks (Lockridge et al. 2013).

Bone marrow-liver-thymus (BLT) mice: The BLT mice is a combination of the SCID-hu (Thy/Liv) and Hu-HSC mice, where animals are implanted with fetal liver and thymus tissue and then sublethally irradiated and injected with HSCs (Lan et al. 2006; Melkus et al. 2006). This model displays robust reconstitution of multiple organs with lymphocytes, including excellent CD4⁺ T-cell reconstitution, and thus supports sustained and high-level HIV replication (Denton et al. 2008).

Considerable enthusiasm was generated by the observation that these mice elicit virus-specific T-cell responses, including some level of HIV-specific T-cell responses capable of selecting viral escape mutants in vivo (Dudek et al. 2012). Substantial improvement is still needed, however, before this potential could be realized (Akkina 2013). While these efforts are ongoing in multiple laboratories, at present this model system is limited by the absence of vigorous autologous immune effectors and thus is not currently suited to testing “shock-and-kill” HIV eradication strategies. As with the other models presented above, GVHD also constitutes a major limitation of the BLT mouse, in particular for studying HIV latency/persistence or eradication studies that generally require long timelines. BLT mice reach a level of reconstitution considered suitable for most studies at 11 weeks post-engraftment and begin to succumb to GVHD within 13 weeks. Although there is some variability from batch to batch, it is typical for half of the mice to succumb from GVHD within ~28 weeks (Greenblatt et al. 2012). There is thus less than a 15-week window for experiments between reconstitution and severe GVHD.

T-cell-only mice (ToM) and myeloid-only mice (MoM): Innovative recent work has developed two variants of the above models that hold promise for dissecting the relative roles of T cells and macrophages in HIV persistence. ToM are a variant of the thy/liv model that are generated in NSG, rather than NOD/SCID, and show systemic reconstitution exclusively with human T cells (Honeycutt et al. 2013). In contrast, MoM are generated by transplanting CD34⁺ hematopoietic stem cells into NOD/SCID mice, resulting in engraftment with myeloid and B cells but not T cells (Honeycutt et al. 2016). Studies in the latter model have demonstrated both that HIV can replicate in macrophages in this immunodeficient in vivo environment

(Honeycutt et al. 2016) and that in HIV-infected Mo mice on suppressive ART, these macrophages can seed viral rebound once ART is ceased (Honeycutt et al. 2017). Unfortunately, the development of spontaneous thymic lymphomas in NOD/SCID mice imposes limitations on the timelines of experiments in MoM. This precludes studies involving more than several weeks of ART and thus the evaluation of long-term persistence.

10.4.2 Contributions of Humanized Mice to HIV Research

Humanized mouse models have yielded important breakthroughs in the areas of HIV pathogenesis, therapeutics suppression of HIV viremia, HIV transmission/prophylaxis, and others (reviewed in (Marsden and Zack 2015)). Some recent examples include the demonstration of in vivo replication competence of HIV strains isolated from HIV-infected elite controllers (Salgado et al. 2014), the observation of in vivo HIV infection of human hematopoietic progenitor cells (suggesting a mechanism for hematopoietic abnormalities in patients) (Nixon et al. 2013), the demonstration of in vivo activity of novel antiviral compounds (Liu et al. 2013; Kumar et al. 2008; Neff et al. 2011) and of novel long-acting nanoformulations of ARVs (Dash et al. 2012), and the successful interruption of HIV transmission across mucosal surfaces by a number of prophylactic strategies (Halper-Stromberg et al. 2014; Stoddart et al. 2011; Denton et al. 2008; Denton et al. 2010; Denton and Garcia 2012). Thus, despite the limitations noted above, HIV research in humanized mice has yielded many valuable insights that would have otherwise been not possible or would have been prohibitively expensive to address in nonhuman primate models.

10.4.3 Humanized Mouse Models of HIV Latency

In recent years, both the BLT and Hu-HSC humanized mice, described above, have been explored as potential models of HIV latency. In both models, viral replication can be suppressed by ARV regimens, and the virus can be reactivated from resting human CD4⁺ T cells isolated from pooled tissues (Denton et al. 2012; Choudhary et al. 2012; Marsden et al. 2012). In BLT mice, the administration of an anti-gp120 immunotoxin was shown to suppress viral expression that persisted in multiple tissues of ARV-treated animals, demonstrating the utility of this model for testing strategies to target residual virus expression in the setting of ART (Denton et al. 2014). In Hu-HSC mice, a combination of broadly neutralizing anti-HIV antibodies and a cocktail of the latency-reversing agents, vorinostat, I-BET, and anti-CTLA-4, were shown to reduce the viral reservoir as measured by time to viral rebound upon ART cessation (Halper-Stromberg et al. 2014).

10.4.3.1 Graft-Versus-Host Disease as a Limitation of Current Humanized Mouse Models

Despite this promise, both of these models are substantially limited by GVHD in three ways: First, GVHD imposes limitations on the timelines of experimental protocols to 11–15 weeks (post-reconstitution), after which point mice rapidly succumb to GVHD (Greenblatt et al. 2012). Given that infection must be first established and then suppressed prior to testing an intervention and that an additional period is required if one wishes to measure time to viral rebound, this leaves only a very short window in which to test interventions. Related to this, these models are limited to viral reservoirs that are generated by very short-term ART and cannot replicate many aspects of HIV persistence that mature over years to decades in people living with HIV. Thirdly, GVHD creates an inflammatory environment, including spontaneous production of IFN- γ (Greenblatt et al. 2012), which may directly activate virus expression and limit the pool of truly quiescent latently infected cells.

While an artificial reservoir generated by short-term (weeks) ARV suppression of mice may share some features with the natural reservoir present in people living with HIV who have been on ART for years/decades, there are many limitations. For example, the clonal expansion of cells harboring proviruses with specific integration sites that has recently been described in HIV-infected individuals on ART is unlikely to appreciably occur in these mice (Wagner et al. 2014; Maldarelli et al. 2014). The natural reservoir is also highly complex, involving intact and defective proviruses, intact proviruses that are induced by a single round of activation, and intact proviruses that, for poorly understood reasons, are not induced by a single round of activation. The latter are likely to represent a critical barrier to HIV eradication and may not be represented in these artificial reservoirs.

The stability of the HIV reservoir in HIV-infected individuals on ART has recently been associated in cells associated with anergy (Seu et al. 2015). This and other features of host cells may contribute to the phenomenon of intact non-induced proviruses or to the variability within and across patients in responsiveness to different LRAs. Existing humanized mouse models do not allow time for the selection of such diverse cells to occur.

Longer-term experiments may be greatly facilitated by the recently developed T cell-only mouse (ToM, see above). As these mice do not develop GVHD, some of the limitations raised above are mitigated. Additional study is needed to determine whether these animals will support a long-term reservoir (preliminary data show rebound after 8 weeks of ART) and whether these reservoirs will recapitulate features of HIV persistence in HIV-infected individuals on ART.

10.4.3.2 Paucity of Autologous HIV-Specific Immune Effectors into Humanized Mouse Models

Shock-and-kill HIV eradication strategies combine the use of LRAs to induce virus expression with immune effectors to eliminate these reactivated target cells. While immunotoxins can be used in humanized mice to achieve the “kill,” all existing

humanized mouse models are severely limited in terms of testing other immune effectors, such as cytotoxic T cells (CTL). As ToM lack cells of the myeloid lineage, including dendritic cells, HIV-specific CTL responses neither naturally occur nor can be elicited by vaccination. One could contemplate adoptive transfer experiments with CTL from HIV-infected subjects; however, this is logically challenging given the need to match the MHC-I restriction of the clone to the fetal tissue used to generate a given batch of mice. Allogeneic reactions would also be a consideration in this scenario, manifesting either as rejection of CTL and/or as inflammation that could disrupt the reservoir.

Hu-HSC and SCID-hu (thy/liv) mice also fail to generate detectable endogenous HIV-specific T-cell responses and thus have similar limitations. BLT mice have more promise in this regard, as HIV-specific T-cell responses have been reported to emerge in natural infection and to apply selective pressure on virus (Dudek et al. 2012). There is, at present, limited ability to manipulate these responses. To our knowledge, the only solution for effectively combining CTL responses with one of the humanized mouse models described above was presented in a study that obtained HSCs from the bone marrow of HIV-infected individuals. This group was thus able to generate Hu-HSC mice that were autologous to HIV-specific CTL clones from HIV-infected subjects and to show *in vivo* antiviral efficacy of adoptively transferred CTL (Deng et al. 2015). While this was an impressive feat, the need to obtain bone marrow from HIV-infected patients to generate mice severely limits the utility of this model.

10.4.4 Conclusions

Humanized mouse models hold the tantalizing potential to transform the field of HIV latency and to greatly accelerate the development of potentially curative therapeutics. While important insights have been generated with current models, several key limitations stand in the way of realizing this full potential. These primarily stem from limitations imposed on experimental timelines by the onset of GVHD and spontaneous lymphomas. Efforts to overcome these limitations comprise a very active area of ongoing research.

10.5 Nonhuman Primate Models of HIV Latency

The Simian immunodeficiency virus (SIV) infection of Indian origin rhesus macaques has long served as a model for HIV infection in humans (Letvin et al. 1983; Desrosiers and Letvin 1987). The rhesus macaque model is well suited for the study of HIV latency, particularly the early events of seeding and development of the reservoir (Whitney et al. 2014). But, it is important to note that this model utilizes SIV, which may differ from HIV in the regulation of viral latency, and thus may have subtle differences as compared to latent HIV.

Nevertheless, it is well described that the host immunologic response and viral dynamics during SIV infection closely mimic those responses observed in HIV-infected patients. Importantly, this model, through its clinical similarities with HIV-infected individuals and the ease of obtaining longitudinal biopsies, greatly facilitates the study of latency in the anatomic tissues that harbor the viral reservoir. Moreover, macaque models allow the study of latency in relevant and anatomically restricted compartments such as the central nervous system (CNS) (Zink et al. 2010; Clements et al. 2005). Detailed analysis of the reservoir generated and maintained in an “*in vivo*” context is critical to a complete understanding of viral latency and to the development of methods to perturb or clear the persistent reservoir. Thus, this model has also proven invaluable for the preclinical development of candidate HIV cure therapeutics.

Early models studied by Shen and colleagues (Shen et al. 2003) used SIV-infected pigtailed macaques that were treated with two reverse transcriptase (RT) inhibitors. They detected SIV provirus in quiescent CD4⁺ T cells from the peripheral blood, spleen, and lymph node (LN). Importantly, after cellular activation, the presence of replication-competent SIV was shown to exist in this population. This early investigation was unfortunately limited due to the limited degree of SIV suppression offered by only using two ARVs.

The historic lack of potent ART and durable virus suppression in these models has been a major drawback in the use of this model system. Advances in ART in NHP models have now allowed for the evaluation of latency (Dinoso et al. 2009; North et al. 2010). Dinoso et al. found that the number of resting cells harboring replication-competent SIV was greatly diminished in ART-treated macaques and found that the overall frequency of these cells was comparable between the blood and anatomic tissues, highlighting a process of “viral homeostasis” as a possible means of initial reservoir spread.

Similarly, early studies by North et al. used ART in rhesus macaques infected with a reverse transcriptase (RT)-SHIV virus (SIV virus that expresses the HIV envelope) making the chimeric virus susceptible to nonnucleoside RT inhibitors (Deere et al. 2010). In this study, viral DNA and RNA levels were measured in treated animals in nearly all tissues including the LN, gut, and CNS tissues. Additional studies by other investigators have shown that simply increasing the number of ARVs can increase viral suppression but also highlight significant issues using drugs formulated for humans into macaques (Shytaj et al. 2012).

The somewhat recent development of easily dosed, highly potent regimens with stable long-term pharmacokinetics will allow the field to advance, importantly using a common means by which to suppress SIV (or SHIV) in macaques to the levels observed in patients treated with long-term ART (Whitney et al. 2014). Using this regimen, typically including tenofovir, lamivudine, and dolutegravir, seeding of the reservoir and decay characteristics on ART have been clearly defined offering a means to study the genesis and maintenance of the SIV reservoir (Del Prete et al. 2016; Whitney et al. 2014).

These SIV-infected ART treated macaque models have also highlighted the importance of the lymphoid viral reservoir as a major obstacle to long-term

HIV remission (Whitney et al. 2014) and will offer continued advances to the understanding of latency in anatomic sites (Deleage et al. 2016). With advances in ART delivery and effective virus suppression, the ART-treated macaque model now offers an ideal system to evaluate therapeutics designed to reactivate and clear the viral reservoir. Whether the same results achieved with a macaque model are eventually achieved in HIV-infected individuals on ART remains to be determined.

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Chapter 11

Measuring HIV Persistence on Antiretroviral Therapy



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Abstract In order to determine if an eradication strategy for HIV is effective, it will be important to measure persistent replication-competent virus, the current barrier to a cure. Various assays are available that measure persistent virus, each with advantages and disadvantages that must be balanced in order to select the best assay for the experimental aim. Assays of free virus do not measure the latent form of the virus but can be utilised in conjunction with other assays in order to better understand HIV persistence on ART. The quantitative viral outgrowth assay (QVOA) is the gold standard assay for measuring persistent replication-competent virus, but it, along with assays that vary the classical QVOA method, underestimates the frequency of latently infected cells in blood due to the presence of non-induced yet intact and replication-competent proviruses. Assays that **quantify** or sequence specific genomic regions of HIV overestimate the size of the reservoir as they are unable to distinguish between intact and defective virus. As an alternative, sequencing the full-length integrated genome can better distinguish replication-competent provirus, but these methods may be expensive and time-consuming. Novel assays, and the application of these assays to novel questions, will be key to the development of future curative therapies for HIV.

Keywords HIV reservoir · Replication-competent virus · QVOA · HIV genome sequencing

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11.1 Introduction

The development of effective antiretroviral therapy (ART) has been key in reducing the morbidity and mortality associated with HIV infection (Palella et al. 1998). With continued ART, people living with HIV can achieve sustained viral suppression, defined as clinically undetectable levels of virus in the blood (< 20 RNA copies/mL plasma). Despite the apparent lack of HIV in the bloodstream, long-term follow-up studies of HIV-infected individuals have demonstrated that replication-competent HIV can still be found in the long-lived memory CD4⁺ T cells from participants on ART (Chun et al. 1997; Finzi et al. 1997; Wong et al. 1997; Finzi et al. 1999). It is this persistent, replication-competent virus that is currently the main barrier to finding a cure for HIV.

Assaying the frequency of cells that harbour replication-competent virus on ART is relatively challenging as these cells are rare. Traditional assays fall into two main categories, being either cell culture- or polymerase chain reaction (PCR)-based procedures. Each assay has advantages and disadvantages that must be weighed by the researcher when determining the best way of assaying the reservoir for each individual experiment. This review will examine these considerations, before delving into how different forms of the virus can be quantified.

11.2 Considerations When Choosing an Assay

The choice of assay when examining HIV persistence on ART relies on finding a balance between the information needed for addressing a specific research question and the limitations of each individual assay. Four questions can be asked during experimental design that will assist with the selection of the best assay for each unique experiment.

11.2.1 *What Compartment of the Body Is to Be Examined?*

The anatomical location being studied will determine which assays can be used to measure viral persistence. Cell-free virus may be easily detected in the plasma and cerebrospinal fluid (CSF), but this cell-free virus is not representative of latently infected cells. Peripheral blood cells can be collected from a participant relatively painlessly and quickly following a blood draw or leukapheresis; therefore participants are more willing to provide these samples. Additionally, the high proportion of target cells – most commonly CD4⁺ T cells – makes it easy to perform in-depth studies of this compartment.

Cells may also be collected from other anatomical compartments; however access to sufficient numbers of cells from these areas is more difficult. It is known

Table 11.1 General characteristics of the assays used to measure persistent HIV

Assay	Traditional sample input	Cell input requirements (relative)	Does the assay indicate replication competency?	Does the assay over- or underestimate reservoir size?
<i>Assays of free virus</i>				
Sampling of free virus	Plasma, cerebrospinal fluid	–	Yes	–
<i>Assays of cell-associated HIV</i>				
DNA	CD4 ⁺ T cells	Low	No	Over
RNA	CD4 ⁺ T cells	Low	No	–
<i>Assays of the inducible provirus</i>				
QVOA	Resting (HLA-DR [–]) peripheral blood mononuclear cells or CD4 ⁺ T cells	High	Yes	Under
MVOA	Resting (HLA-DR [–]) peripheral blood mononuclear cells or CD4 ⁺ T cells	High	Yes	Under
<i>Assays of translation-competent provirus</i>				
TILDA	CD4 ⁺ T cells	High	Indicates translational competency	Under
Single cell analysis	CD4 ⁺ T cells	Low	Indicates translational competency	Under
<i>Sequencing of the virus</i>				
Single-region sequencing assays	CD4 ⁺ T cells	Low	No	Over
Full genome sequencing	CD4 ⁺ T cells	Depends on methodology	Yes	Unknown; likely a small overestimate

QVOA quantitative viral outgrowth assay, *MVOA* murine viral outgrowth assay, *TILDA* Tat-/Rev-induced limiting dilution assay

that the gut-associated lymphoid tissues – as well as peripheral lymphoid tissues – are sites of early viral infection and dissemination (Pantaleo and Fauci 1995; Brenchley et al. 2004; Mehandru et al. 2004) and hence may be important sites in terms of harbouring latently infected cells. Biopsies, however, are relatively invasive and therefore of greater inconvenience, and are more expensive to perform. Tissue biopsy results in a smaller number of cells being harvested, and only a small part of the tissue is accessed. Due to these limitations latently infected cells in these anatomical sites are not as well characterised compared to the peripheral blood. The smaller number of cells also limits the assays that can be performed: assays such as the quantitative viral outgrowth assay (QVOA), for example, which requires a high cell input (Table 11.1), may be difficult to perform with the cells collected from a tissue biopsy.

11.2.2 What Type of Cell Is to Be Examined?

Traditionally, memory CD4⁺ T cells are considered the predominant cell infected with latent HIV. Early studies indicated that latency on ART could be found in resting CD4⁺ T cells (Chun et al. 1997; Finzi et al. 1997; Wong et al. 1997; Finzi et al. 1999). However, memory CD4⁺ T cells are a highly heterogeneous cell population, and the contribution of each cell subset to the latent HIV reservoir is very different. Central memory cells constitute the largest proportion of memory CD4⁺ T cells, comprising around 60–70% of the total memory CD4⁺ T-cell population (Fritsch et al. 2005), and a lower frequency of latently infected central memory cells has been associated with posttreatment control when ART is ceased (Sáez-Cirión et al. 2013). Recent work, however, has questioned the relative contribution of central memory cells in the peripheral blood to the latent HIV reservoir (Hiener et al. 2017).

Effector memory cells have the capacity to move into the tissues and rapidly respond to pathogens *in vivo* (Sallusto et al. 2004), a different role to the central memory T cells which largely stay in lymphoid tissue. Effector memory cells contain a high frequency of genetically identical provirus, perhaps a reflection of their capacity to proliferate in response to a recognised pathogen (von Stockenstrom et al. 2015). We recently demonstrated that on ART, infected effector memory T cells in the peripheral blood have a high frequency of genetically intact provirus (Hiener et al. 2017). Further work into understanding the mechanism of this finding is needed and underway.

Myeloid cells are resistant to the cytopathic effects of HIV (Verani et al. 2005) and have a long lifespan (Zhu et al. 2002), making them excellent candidates for contributing to viral persistence, if at a lower infection frequency (Otero et al. 2003; Josefsson et al. 2013b; von Stockenstrom et al. 2015). However, assays of viral persistence are generally designed for quantifying virus in memory CD4⁺ T cells (Table 11.1), which means that if another cell type is to be examined, the assay must be optimised and modified for the input of these different cell populations.

11.2.3 How Important Are Cells Expressing Activation Markers?

Traditional methods used to quantify HIV persistence on ART generally focus on resting memory CD4⁺ T cells. This is based on the assumption that an activated cell would be productively infected – a source of viraemia – and therefore not considered part of the latent HIV reservoir. As an example, cells that express HLA-DR, a T-cell activation marker, are not commonly included in the classical QVOA (Siliciano and Siliciano 2005). Our understanding of cellular activation is more complex now than when these assays were first developed; however assays have not been modified to take this into account. Memory T cells expressing the HLA-DR activation marker, for example, may be only partially, not fully activated and

therefore may still have the capacity to be latently infected. Indeed, recent studies have found that memory T cells expressing the HLA-DR activation marker in the peripheral blood contain high levels of intact HIV (Cockerham et al. 2014; Lee et al. 2016; Horsburgh et al. 2017). More research is needed to identify markers that differentiate between a latently and productively infected cell. Therefore, researchers should be aware that the strategy used to isolate cells they include in their assays may influence their research outcomes.

11.2.4 Is the Virus Replication-Competent?

Only virus that is replication-competent is infectious and hence will be able to reinstitute HIV infection once ART is ceased. Not all assays that measure viral persistence can determine whether a virus is replication-competent and hence a true representative of the latent HIV reservoir. Some assays, such as QVOA, clearly indicate that a virus is able to replicate and produce progeny. However, the QVOA requires a large number of cells and is labour intensive, making it difficult to use for identifying replication-competent virus within anatomic sites. The classical QVOA provides a numerical read-out and does not include a sequencing step for the genomic analysis of the replication-competent virus, limiting the information available about the virus itself. Variations of the QVOA have been developed however that do allow for some genetic characterisation.

PCR-based assays of cell-associated HIV RNA and DNA are often less labour intensive, however they most commonly only examine a few open reading frames of the virus (Eriksson et al. 2013). PCR-based assays that do not amplify the full-length provirus can overestimate the frequency of latently infected cells as these assays detect both intact and defective virus. As an alternative, the Tat-/Rev-induced limiting dilution assay (TILDA) measures the frequency of cells infected with inducible virus by using limiting dilution and quantification of *tat-/rev*-specific mRNA in cells following activation with a mitogen (Procopio et al. 2015). While this assay gives an estimate of the frequency of infected cells between the QVOA and single-region PCR-based methods (Procopio et al. 2015), it is still subject to the same bias as PCR-based assays, though perhaps to a lesser extent as the *tat/rev* region is commonly deleted in defective proviruses and splicing is a prerequisite for virus replication.

11.3 Available Methods for Measuring HIV Persistence

There are several assays for measuring HIV persistence (Fig. 11.1). Some of these assays measure the free virus in bodily fluids such as plasma and CSF. Other assays delve more deeply into cellular compartments and can determine which cells contain HIV DNA and/or RNA. Other assay types assess the replication-competency of

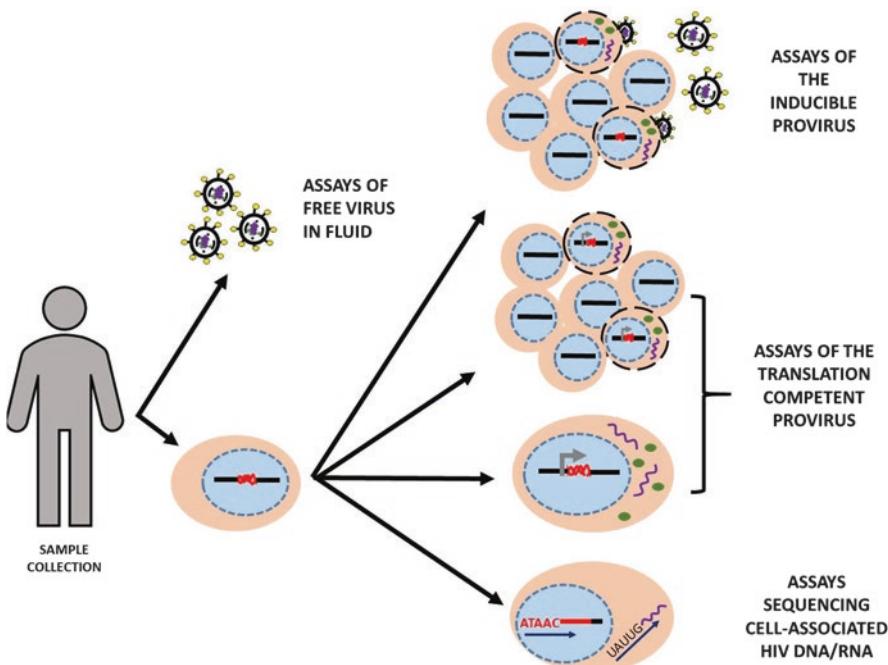


Fig. 11.1 Alternative methods of measuring persistent human immunodeficiency virus (HIV). Integrated HIV cDNA is indicated in red, while HIV RNA is indicated in purple. HIV-specific proteins are indicated by green circles. Grey arrows indicate cellular transcription, while blue ones indicate sequencing. Images are not to scale

the virus. Each of these assays has their own advantages and disadvantages, which must be balanced depending on the aims and expectations of the research question. Moreover, the successful performance of these assays will depend upon access to appropriate facilities and resources.

11.4 Measuring Cell-Free Virus in Body Fluids

Virions in bodily fluids – the CSF and the plasma – are not generally considered part of the latent HIV reservoir. However, they are a good marker of virus production and can also be used to identify latency reversal (Rasmussen et al. 2013; Winckelmann et al. 2017). Sequencing of this cell-free virus as well as virus found integrated in different cells can also give an indication as to the source of viraemia (Ostrowski et al. 1999; Dahl et al. 2014). Hence, while not a measure of the reservoir itself, assays of the free virus are still valuable tools.

When a numerical read-out is needed, clinical assays can detect down to the level of <20 copies/mL. These kits are useful for clinical care, but do not have the sensi-

tivity to measure low-level persistent viraemia (below 20 copies/mL) or incremental increases in viraemia during viral reactivation after treatment with latency-reversing agents (LRA). To detect viral copies at a lower concentration, an assay that can detect down to one copy per well, commonly called the single-copy assay (SCA), is more appropriate (Palmer et al. 2003). This virus can also be extracted and sequenced, and as such the single-genome sequencing assay can provide the genetic composition and diversity of plasma-derived (Palmer et al. 2005; Kearney et al. 2009; Josefsson et al. 2013b; von Stockenstrom et al. 2015) and CSF-derived (Dahl et al. 2014) virions.

11.5 Measurements of Cell-Associated DNA and RNA

Measures of cell-associated DNA allow for the quantification of the number of cells that are infected with either intact or defective virus. Single-region PCR-based methods can be used to quickly and relatively cheaply quantify HIV DNA utilising real-time PCR or digital droplet PCR (ddPCR) read-outs. These methods generally lack the capacity to distinguish between a replication-competent virus and one that is incompetent, however, as they detect a short section of provirus, but the provirus may be defective in a region outside of the assayed region. Therefore, assays which quantify HIV DNA are known to overestimate the frequency of latently infected cells (Eriksson et al., 2013). Alu-PCR can also be used to determine the level of integrated HIV DNA (Butler et al. 2001; Chomont et al. 2009; Liszewski et al. 2009; Vandergeeten et al. 2014). Alu elements are repeated sequences within the human genome: for this PCR, primers are designed to bind to an Alu element as well as the LTR or gag region of an HIV sequence. As this method gives a read-out of the level of HIV integrated near an Alu sequence, it may not detect virus that is integrated far from a target Alu sequence.

Measures of integrated DNA can give an indication of the amount of HIV within cells. As an alternative, cell-associated RNA can also be measured (Elliott et al. 2014; Kiselinova et al. 2014; Hong et al. 2016). This RNA is indicative of viral transcription and hence gives an indication of which cells are transcribing virus. As such, cell-associated RNA can be used as a biomarker of latency reversal, however should not be taken as indicative of the size of the reservoir itself. Cells that are undergoing transcription could express viral proteins and thus be removed from the reservoir due to either viral cytopathic effects or as they are recognised by the immune system. Instead, assays of cell-associated RNA have a place in studies of the efficacy of different LRAs, as an example.

Assays of cell-associated RNA generally either rely on quantitative reverse-transcription PCR or ddPCR for quantification. While ddPCR is advantageous as it does not require an external standard curve, the false-positive rate for this method is much higher, meaning it may not be accurate when assaying participant samples with very low RNA levels during suppressive ART (Kiselinova et al. 2014). The

other main consideration with assays of cell-associated RNA is the site of amplification. Different primers will allow for the detection of either unspliced or multi-spliced RNA, or indeed both.

11.6 Measuring Replication-Competent and/or Inducible Virus in Cells

11.6.1 *The Quantitative Viral Outgrowth Assay (QVOA)*

The QVOA is perhaps the most common assay used to define the frequency of latently infected cells. It is largely considered the gold standard measure of the latent HIV reservoir and has been in use in various iterations since the 1990s. To perform the QVOA, limiting dilutions of CD4⁺ T cells are maximally stimulated with a T-cell-activating mitogen, usually phytohaemagglutinin (PHA), to initiate HIV transcription and replication (Siliciano and Siliciano 2005). To expand the number of virions and thus increase the ability to detect a positive result, these activated cells are cocultured with CD4⁺ T cells from an HIV-uninfected donor or a reporter cell line. The resulting culture supernatant is examined for viral growth using an enzyme-linked immunosorbent assay (ELISA) which detects the viral protein p24.

The QVOA, however, underestimates the size of the reservoir due to the presence of non-induced proviruses (Ho et al. 2013). Not all provirus may be induced due to silencing from epigenetic changes (Matsuda et al. 2015), transcriptional interference (Lenasi et al. 2008) or the stochastic induction of provirus even with maximal stimulation of the cellular host (Ho et al. 2013). Furthermore, the cells used in the assay are traditionally resting cells, identified by a lack of HLA-DR expression (Siliciano and Siliciano 2005); however it is worth noting that recent work has questioned the appropriateness of only examining resting CD4⁺ T cells (Lee et al. 2016; Horsburgh et al. 2017). The assay is also limited to the use of peripheral blood as large numbers of CD4⁺ T cells are required to conduct this assay. The large amount of participant blood needed to collect enough cells for the assay limits its sensitivity, as well as its use in a clinical setting (Eriksson et al. 2013). The assay is difficult to perform on cell subsets or from cells collected from tissue biopsies.

Furthermore, the use of this assay is limited to certain kinds of research questions. The classical form of the assay does not tell us anything about the virus itself, aside from the fact that it is inducible and can make a p24 protein. Information regarding the compartmentalisation of any viral quasispecies or the development of the viral swarm, for example, is not immediately available. Most importantly QVOA is an indication of replication-competent virus, but how this relates to viral rebound off ART remains unknown.

11.6.2 Variations on the QVOA

There have recently been several modifications to the original QVOA method that allows for more rapid throughput. While the classical QVOA takes up to a fortnight to be completed, the modified assay may be completed in a single week (Laird et al. 2013).

The first modification is with cell purification. Instead of sorting resting CD4⁺ T cells using fluorescence-activated cell sorting (FACS), cells can be purified from peripheral blood mononuclear cells (PBMCs) using two rounds of bead purification. First CD4⁺ T cells are isolated using negative selection from PBMCs, and then resting cells are isolated by depleting any cells expressing CD69, CD25 or HLA-DR (Laird et al. 2013). Purities of 96–97% are reported, which are somewhat lower than what may be achieved by FACS. This may limit the conclusions drawn from this method, if contamination with other cells is too high. However, a similar frequency of infected cells has been reported using both methods.

The next modification is in the expansion phase. Instead of expanding the number of virions present in culture through the addition of HIV-negative donor PBMCs, a single addition of cells from the MOLT-4 cell line is performed (Laird et al. 2013). MOLT-4 cells express high levels of the HIV entry receptor CD4 and co-receptors CCR5 and CXCR4, and as such they are efficiently infected and allow for high-level viral replication. Additionally, as the cells are from a cell line, all sample cells are subjected to the same culture conditions. The use of MOLT-4 cells has been shown to be comparable to the use of uninfected donor PBMCs, and therefore if these cells are available, they may be a good alternative to the use of coculture with HIV-uninfected human PBMCs.

The last modification is an alternative measure of virus production. Instead of using an ELISA for detection of p24 in the culture supernatant, Laird et al. suggest using RT-PCR specific for polyadenylated HIV virus. The advantage of this method is that it is more sensitive; the RT-PCR can be used after 7 days, whereas p24 protein may not be detected using ELISA until after 14 days (Laird et al. 2013). Additionally, the use of RT-PCR may be cheaper in the long term. As such, RT-PCR makes the QVOA a more widely applicable system for determining the levels of replication-competent virus in resting memory T cells.

Other modifications also change the read-out of the QVOA, but this time in order to be able to sequence the virus. One such modification, the qualitative and quantitative viral outgrowth assay (Q²VOA) (Lorenzi et al. 2016), involves performing the viral outgrowth cultures at the limiting dilution as determined by Poisson distribution (i.e., $\leq 30\%$ p24⁺ wells equates to 80% of the wells containing a single replication-competent provirus), such that single-genome sequencing methods can then be used to sequence the virus found in the culture supernatant from each p24⁺ well. This method therefore allows for the characterisation of virus producing p24 protein, and therefore likely replication-competent virus. This method is suited to studies focussed on only translation-competent virus, and not the characterisation of all HIV-infected cells. Q²VOA does still rely on the stimulation of T cells, however,

meaning not every translation-competent virus will be characterised using this assay due to the presence of non-induced proviruses in the latent reservoir (Ho et al. 2013).

Another alternative read-out method, called the quantitative viral outgrowth assay, ultra-deep sequencing assay (QVOA-USDA), has been recently developed (Lee et al. 2017b). Here, the QVOA is performed, but instead of assaying the presence of p24 protein in the culture supernatant, viral RNA is extracted, reverse transcribed into cDNA and the V1-V3 region of *env* sequenced using a deep sequencing method. These sequences are then analysed for the number of distinct lineages detected in each well. It is known that most CD4⁺ T cells are infected by a single HIV provirus (Josefsson et al. 2011; Josefsson et al. 2013a). This means that one unique proviral sequence in a culture should equate to the progeny of one infected cell. Therefore, the number of unique proviral sequences detected in the QVOA can be used, along with the number of cells in each well, to determine the infection frequency and thus the total size of the reservoir (Lee et al. 2017b). As sequences are generated, they may also be analysed to determine if they are intact, as well as for the genetic relationships between sequences. QVOA-USDA therefore allows for more information to be derived from the traditional QVOA analysis.

The QVOA-USDA method is best suited to cells derived from participants treated during chronic infection, as their HIV sequences will be genetically distinct and therefore the diversity of sequences can be used to calculate the infection frequency (Lee et al. 2017b). If treated during acute infection, the virus may not have had enough time to diversify in the region studied (Kearney et al. 2009; Josefsson et al. 2013b; Maldarelli et al. 2013) to be genetically distinct in each cell. This lack of viral genetic diversity will affect the estimation of the overall reservoir size. Additionally, it should be noted that cellular proliferation may increase the number of identical sequences detected in latently infected cells (Chomont et al. 2009; Josefsson et al. 2013b; Maldarelli et al. 2013; Vandergeeten et al. 2013; Cohn et al. 2015; Kearney et al. 2016; Lee et al. 2016). If a latently infected cell proliferates, the proviral genome will be replicated along with the host cell DNA. As such, identical proviruses may belong to more than one original host cell, and the QVOA-USDA calculation may underestimate the size of the reservoir due to this identical provirus. In addition, two different sequences found in culture may be derived from one original host cell, if there was some error in transcription within the cell. The likelihood of this taking place in the region of the genome analysed, however, will be small. Despite the errors that may arise from the QVOA-USDA, the level of replication-competent viruses and the measurement of the latent HIV reservoir by this method is strongly correlated with the traditional QVOA.

Another modification of the QVOA to enhance sensitivity is the murine viral outgrowth assay, or MVOA (Metcalf Pate et al. 2015). In the MVOA, infected T cells or PBMCs are injected into a mouse host, commonly a NOD. Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mouse. The human cells will become activated in

the non-human host, releasing virus into the mouse circulation. The virus released is identified in the murine plasma using HIV-specific RT-PCR. While more of a proof-of-infection assay and not quantitative as such, modifications of this method are possible that would allow for the quantitation of virus in the murine plasma. The MVOA has successfully demonstrated latent infection of cells from both elite controllers and ART-suppressed participants. However, the use of an animal model may not be available in all laboratories, limiting its use. In conducting this assay, the virus is amplified from a xenograft of 25 million PBMCs or 10 million CD4⁺ T cells per animal. Therefore if quantification is required, multiple animals are needed to be injected with a serial dilution of participant cells. The use of MVOA is therefore limited by both the number of cells needed and the space requirements for housing mice, and is not always quantitative.

11.6.3 *The Tat-/Rev-Induced Limiting Dilution Assay*

Similar to the QVOA, in the Tat-/Rev-induced limiting dilution assay (TILDA) resting CD4⁺ T cells are maximally stimulated with PHA and ionomycin in order to induce provirus expression. After stimulation these cells are serially diluted and the resulting provirus is then subjected to a nested PCR performed using primers specific for the *tat/rev* region (Procopio et al. 2015) and the frequency of positive compared to negative wells determined.

TILDA has a number of advantages over both traditional QVOA and PCR-based methods that quantify the amount of integrated DNA in cells. As the cells are induced to produce virus, the assay can first give an indication that the virus has an intact LTR region, which is needed for successful induction. Secondly, the choice of the *tat/rev* region is significant, as this region is often deleted when the viral genome contains a large internal deletion (Ho et al. 2013; Bruner et al. 2016; Hiener et al. 2017). Therefore, TILDA specifically identifies inducible virus that is most likely to be replication-competent. The TILDA assay estimates the number of latently infected cells as 24/10⁶ resting CD4⁺ T cells, a value which is between the estimates using QVOA (underestimation) and PCR-based assays (overestimation).

However, the TILDA assay also suffers from some drawbacks. Firstly, it is now known that maximally stimulating cells does not always induce all replication-competent proviruses, due to the stochastic induction of the provirus in the cell (Ho et al. 2013). Therefore, much like the QVOA, the TILDA will most likely underestimate the amount of inducible proviruses. Furthermore while the *tat/rev* region is usually deleted or mutated in defective proviruses, some defective viruses have an intact *tat/rev* region (Ho et al. 2013; Bruner et al. 2016; Hiener et al. 2017), and thus the measure may not be completely accurate.

11.6.4 Single-Cell Analysis of Inducible Virus

Assays of the inducible virus can also be performed down to the single cell level. An example of one such assay is the recently developed Flow-FISH method (Baxter et al. 2016). Here, CD4⁺ T cells are reactivated with PHA and ionomycin, much like the QVOA, before fluorescent in situ hybridisation (FISH) staining is used to detect *gag-pol* mRNA together with gag protein. The presence of both gag mRNA and protein is detected using flow cytometry and/or confocal microscopy. This assay provides a method of assessing HIV transcription and translation within a single cell. This assay has reported the infection frequency of the viral translation-competent reservoir to be approximately 4.65 HIV^{RNA+/Gag+} translation-competent HIV-infected cells/million, which is similar to the frequencies detected by the QVOA in the same participants, although the numbers from the two assays did not directly correlate (Baxter et al. 2016). This assay may still underestimate the size of the reservoir due to the need to stimulate the cells and the presence of non-induced provirus in the reservoir. The ability to look at the single cell, however, means that more phenotypic information can be gained from the host cell, as multiple stains can be performed in parallel. By staining for cellular markers, cells that are HIV⁺ can be better defined, allowing for a greater understanding of the cells that make up the translation-competent reservoir.

11.7 Analysis of Intracellular HIV RNA and DNA Using Sequencing Methods

Single-genome sequencing of RNA and single-proviral sequencing for HIV DNA allow for the genetic analysis of a specific HIV genomic region (Palmer et al. 2005; Kearney et al. 2009; Josefsson et al. 2013b; von Stockenstrom et al. 2015). These methods can be used to investigate the genomic composition of intracellular RNA and DNA from total PBMCs or specific memory T cells sorted from the peripheral blood and anatomic tissue sites. A common factor of these two methods is that they should be conducted at the limiting dilution endpoint as determined by Poisson distribution. In conducting these assays, intracellular HIV RNA is converted to cDNA. This cDNA, or the HIV DNA extracted directly from PBMCs or specific memory CD4⁺ T cells, is serially diluted to a single copy and sequenced. Common target regions for these assays include the p6 region of *gag*, protease and the first 900 nucleotides of reverse transcriptase, or the V1–V3 region of envelope. Individual HIV RNA or DNA genomes are then sequenced allowing for an understanding of not only the size of the reservoir (if the input cell number is known) but also the genetic composition and relationship between intracellular RNA and DNA. These methods can provide important information regarding the infection frequency of specific memory T cells, the dynamics of the reservoir, the amount of latency reversal following administration of a latency-reversing agent and the mixing of virus

between different cellular and anatomical compartments (Josefsson et al. 2013b; von Stockenstrom et al. 2015; Barton et al. 2016; Lee et al. 2016; Wiegand et al. 2017; Winckelmann et al. 2017). Even though these assays provide an in-depth genetic analysis as well as the infection frequency of HIV within specific T-cell subsets, because only a portion of the genome is sequenced, they cannot be used to accurately determine the amount of intact virus within cells.

To determine if a virus is intact and therefore replication-competent using PCR-based methods, full-length HIV genome sequencing should be employed. Methods have been developed that allow for the sequencing of >90% of the genome, which provides a more accurate estimate of the amount of intact HIV within cells and, therefore, the latent HIV reservoir. Two unique ways of performing this assay have been developed thus far.

The first method involves the amplification of four overlapping segments of a single HIV genome which are then subjected to Sanger sequencing (Ho et al. 2013; Bruner et al. 2016). These are then consolidated into one HIV genome and analysed for genetic defects within the entire virus. This work has led to some remarkable breakthroughs, which includes demonstrating the existence of non-induced provirus (Ho et al. 2013). However, this method of full-length HIV sequencing is highly labour intensive due to the need to generate and then analyse four amplicons per unique provirus. This means that it cannot be used to conduct an in-depth analysis of the replication-competent viral reservoir within different T-cell subsets, as an example, as it is not feasible to produce large numbers of HIV sequences using this method.

As an alternative, recently developed sequencing methods are able to amplify and sequence the near full-length HIV genome directly (Hiener et al. 2017; Lee et al. 2017a). One example of this assay is the Full-Length Individual Proviral Sequencing (FLIPS) (Hiener et al. 2017). The FLIPS method amplifies the whole (92%) provirus as one amplicon using the same first-round PCR primers as the previous method (Ho et al. 2013; Bruner et al. 2016) and is run at limiting dilution, meaning a library of full-length, unique HIV genomes can be built (Hiener et al. 2017). This method is dependent on both the availability of an efficient high-fidelity polymerase with the capacity to accurately amplify a 10 kb PCR product, and a next-generation sequencing (NGS) platform that allows for the accurate sequencing of such a comparatively large amplicon. The use of NGS allows for 96 uniquely tagged samples to be sequenced at the same time.

The high-throughput capabilities of NGS and the relatively less labour-intensive PCR protocol mean that FLIPS can be used to conduct in-depth genome-scale analysis of the HIV populations in cells sorted from the peripheral blood and anatomic tissue sites, as it is easier to produce a large number of sequences. However, the method is expensive compared to single-genome/proviral sequencing assays due to the cost of reagents and the use of NGS technology. The identification of a genetically intact HIV genome does indicate a replication-competent provirus; however, full-length HIV genome sequencing may not distinguish between a slowly replicating virus – perhaps caused through a mutation in a non-essential open reading frame – and one that is truly replication-competent. To address this issue, previous

studies of full-length provirus reconstructed the genetically intact proviral sequences in a plasmid and analysed the resulting daughter virions for infectivity and growth kinetics within cells (Ho et al. 2013). Furthermore, full-length HIV genetic analysis may miss key details that make a provirus replication-incompetent, such as integration into a centromere (Lewinski et al. 2005).

11.8 The Future

In terms of new methodology, sequencing of the full-length RNA has not yet been widely developed. However, there are a few potential relevant methods. One example was pioneered by Salazar-Gonzalez et al. (2009) and is much like the fragmental genome cloning of the Ho et al. (2013) full-length DNA sequencing method. Here, viral RNA is extracted from the plasma and cDNA synthesised. To amplify the full-length genome, two “half genomes” are synthesised using two different primer sets specific for the 5' and 3' ends of the genome (Salazar-Gonzalez et al. 2009). PCR is then used to amplify the cDNA, with multiple primer sets used to amplify amplicons encompassing the entire genome (Salazar-Gonzalez et al. 2009). These amplicons are then sequenced and then the full-length HIV RNA can be built. Much like the amplification of four overlapping segments of a single proviral genome (described above), the method is rather labour intensive and may be prone to error due to the multiple sequencing steps and potential for primer mismatch. An equivalent of the FLIPS method for RNA, that is, the amplification of one full-length RNA sequence, is still being optimised.

While the cells which contain latent HIV during effective ART have been identified, exactly which cell subsets are important for the maintenance of the reservoir are only just being elucidated. Additional subsets, such as PD-1⁺ memory CD4⁺ T cells, should also be assayed in order to better understand their contribution to the reservoir. Non-CD4⁺ T cells should be studied to further our understanding of how these cells contribute to the HIV reservoir. These subset-focussed studies are also drawing attention to the manner in which cell subsets are chosen or excluded within traditional assays. As an example, HLA-DR⁺ cells, traditionally not included in the classical QVOA, have been demonstrated to contain a higher infection frequency with intact HIV genomes (Lee et al. 2016; Horsburgh et al. 2017).

Assays of the latent HIV reservoir have largely focussed on the peripheral blood, with some studies also exploring sites of early infection – such as the gut (Chun et al. 2008; Yukl et al. 2010; Imamichi et al. 2011) – as well as sites of immune cell concentration, most commonly the lymph nodes (Rothenberger et al. 2015). Other sites, such as the liver and spleen, which contain cells that can be infected by HIV, may also be important sites of viral persistence. The HIV reservoir in these areas is largely unexplored, owing in part to the difficulty of obtaining participant samples due to the invasive nature of such procedures, as well as the small number of cells able to be assayed in said samples. Studies looking into these sites are also needed in order to gain a clearer picture of the total reservoir in the body, rather than just in the peripheral blood.

Finally, we need to determine how coinfections with other viruses affect the development of the latent HIV reservoir. Coinfection with HIV and herpes simplex virus-2 (HSV-2) is common (Freeman et al. 2006). Given the higher level of immune cell presence and activation due to HSV-2, it is reasonable to assume that coinfection may therefore impact the development of the latent HIV reservoir. In addition, morbidity is increased in HIV-infected individuals coinfected with hepatitis B (HBV), as liver-related mortality has been found to be higher in individuals who are coinfected with the viruses (Thio et al. 2002). In order to develop curative HIV strategies, it will be important to understand the impact of viral coinfections upon the latent HIV reservoir.

11.9 Conclusion

Measuring HIV persistence may be approached in a variety of ways depending on the question that is being asked by the researcher. Measurement of free virus provides a quick way of assaying productive infection, but this virus is not considered a part of the latent HIV reservoir. Methods which utilise PCR to quantify intracellular RNA and DNA overestimate the size of the HIV reservoir in cells and do not demonstrate whether the virus is replication-competent. The QVOA is the gold standard for quantifying the amount of replication-competent virus; however the classical QVOA, along with variations on this method, all underestimate the size of the latent HIV reservoir due to the presence of viruses that are intact but not induced after one round of maximal stimulation. Single-genome/proviral sequencing methods overestimate the size of the reservoir as they do not sequence the entire provirus. Viruses that are intact in the genomic region sequenced may be defective in another genomic regions, for example. Full-length proviral sequencing methods offer an alternative by sequencing an entire integrated provirus; however this method can be labour intensive and expensive. Methods for quantifying the latent HIV reservoir are being continually modified and optimised to answer more complex questions about the cells contributing to the latent HIV reservoir and the virus they contain. These new methods will expand our knowledge about factors driving HIV persistence on ART and requirements for designing curative strategies that specifically target persistent replication-competent HIV.

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Chapter 12

Clinical Interventions in HIV Cure Research



Thomas Aagaard Rasmussen and Ole S. Søgaard

Abstract Research over the past decade has resulted in a much-improved understanding of how and where HIV persists in patients on otherwise suppressive antiretroviral therapy (ART). It has become clear that the establishment of a latent infection in long-lived cells is the key barrier to curing HIV or allowing for sustained ART-free remission. Informed by in vitro and ex vivo studies, several therapeutic approaches aimed at depleting the pool of latently infected cells have been tested in small-scale experimental clinical trials including studies of ART intensification, genome editing, ART during acute/early infection and latency reversal. Many studies have focused on the use of latency-reversing agents (LRAs) to induce immune- or virus-mediated elimination of virus-producing cells. These trials have been instrumental in establishing safety and have shown that it is possible to impact the state HIV latency in patients on suppressive ART. However, administration of LRAs alone has thus far not demonstrated an effect on the frequency of latently infected cells or the time to virus rebound during analytical interruption of ART. More recently, there has been an enhanced focus on immune-based therapies in the onwards search for an HIV cure including therapeutic vaccines, toll-like receptor agonists, broadly neutralising antibodies, immune checkpoint inhibitors, interferon- α and interleukin therapy. In ongoing studies immunotherapy interventions are also tested in combination with latency reversal. In this chapter, the overall results of these clinical interventions ultimately aimed at a cure for HIV are presented and discussed.

Keywords HIV cure · Clinical trials · Histone deacetylase inhibitors · Latency-reversing agents · Immunotherapy

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12.1 Introduction

Combination antiretroviral therapy (ART) to treat HIV infection was widely introduced in the mid-1990s, and this marked a major advance allowing for suppression of viral replication to below the detection limit of clinical assays. ART has significantly improved the immune function of people living with HIV and has transformed a fatal disease into a chronic infection for those with access to ART (Deeks et al. 2013; Volberding and Deeks 2010). Initially, hopes were even raised that the potent viral inhibition by combination ART might lead to the decay and eradication of all HIV-infected cells within few years of therapy (Perelson et al. 1997). However, it was soon demonstrated that HIV establishes a transcriptionally inactive infection in long-lived memory CD4+ T cells and that subsequent activation of these cells may drive viral rebound in the absence of ART (Chun et al. 1995, 1997a, b; Finzi et al. 1997).

Latent infection in long-lived CD4+ T cells enables the virus to persist for decades in a quiescent state evading both host immune responses and combination ART. Life-long ART is therefore required to avoid virus rebound and disease progression. The long-term persistence of HIV in latently infected CD4+ T cells is regarded as the primary barrier to curing HIV, but other mechanisms may also contribute to long-term persistence. These include infection of non-CD4+ T cells (Damouche et al. 2015); clonal expansion of cells infected with replication-competent proviruses (Simonetti et al. 2016); low-level residual replication in tissues, possibly driven by insufficient penetration of ART (Lorenzo-Redondo et al. 2016; Rothenberger et al. 2015); or persistence in tissue sites such as lymph node follicles where infected cells may be protected against cytotoxic T cells (CTLs) (Fukazawa et al. 2015). The implications of long-term viral persistence in patients on ART are most dramatically demonstrated by the rapid viral rebound seen upon cessation of ART, even after many years of viral suppression (Davey et al. 1999; Chun et al. 2010). This shows that despite potent viral inhibition by combination ART, replication-competent virus persists in all infected individuals and usually drives viral rebound when therapy is discontinued.

Several lines of research are currently pursued with the long-term aim of developing a cure for HIV or allowing for ART-free remission. Most of these efforts are taking place at the preclinical level, but some strategies have been advanced to the stage of clinical testing. In this chapter we will provide an overview of the various clinical interventions, which in recent years have been investigated with the aim of reducing the frequency of latently infected cells or prolonging the time to viral rebound after interruption of ART. We will discuss these approaches and their perspectives broadly including their potential applicability in the onwards search for an HIV cure.

12.2 HSCT and Chemotherapy for Malignancy

To date there has been only one example of a complete cure for HIV. This occurred in Timothy Ray Brown, who underwent haematopoietic stem cell transplantation (HSCT) for acute myeloid leukaemia (AML) and received HLA-matched stem cells with a homozygous 32-base pair deletion in the gene for CCR5 ($CCR5\Delta 32$) (Hutter et al. 2009). ART was discontinued following HSCT without rebound viremia, and no evidence of HIV replication has been demonstrated since in this individual during over 10 years of follow-up (Yukl et al. 2013; Hutter et al. 2009). It is not known, though, whether the HSCT procedure itself, chemotherapy, myeloablative HSCT conditioning, graft versus host disease (GvHD) or a combination of those factors was primarily responsible for eradicating all infected cells. Notably, subsequent studies of HIV-infected individuals receiving chemotherapy, but not HSCT, for malignancies showed no impact on residual plasma HIV RNA or the frequency of latently infected cells (Cillo et al. 2014).

The case reports of two individuals from Boston subsequently confirmed the ability of HSCT to drastically reduce the frequency of latently infected cells (Henrich et al. 2013). Both HIV-infected individuals on ART underwent reduced-intensity conditioning HSCT for myelodysplastic syndrome and Hodgkin's lymphoma, respectively. In both individuals this led to an extensive depletion of HIV-infected cells; in fact HIV could not be detected in cells or plasma during post-HSCT follow-up while on ART. Still, there was rebound viremia after 12 and 32 weeks when ART was stopped (Henrich et al. 2014). These two cases demonstrated two important points. Firstly, viral persistence capable of causing rebound viremia can occur at a frequency, which is lower than what our current assays can measure in blood and, secondly, even with such profound depletion in the number of infected cells, effective and durable immune surveillance is needed to target any residual virus.

In contrast to Timothy Brown, the two individuals from Boston received CCR5 wild-type donor cells, and this may be an important difference, but there are very few cases on which to base this assessment. In fact, seven other HIV-infected individuals on ART with haematological malignancy have received HSCT with stem cells from homozygous $CCR5\Delta 32$ donors, and most of these recipients have died within 12 months of HSCT (Hutter 2014). Finally, the virus rebound in the Boston patients, despite undetectable levels of HIV in plasma and cells on ART, underscores the importance of conducting analytical treatment interruptions (ATI) to assess the extent of functional depletion of the reservoir following HSCT or other interventions (Kuritzkes 2016).

There are few cases described of HSCT recipients with HIV infection who subsequently discontinued ART (Hutter et al. 2009; Henrich et al. 2014; Avettand-

Fenoel et al. 2007; Kordelas et al. 2014). Thus, as there are many potentially important variables such as conditioning regimen, donor cell CCR5 genotype and GvHD severity, there is still a sound scientific case to be made for conducting ATI post-HSCT although risks and ethical considerations obviously need to be weighed carefully on a case-to-case basis (Sugarman et al. 2016). The scientific considerations around conducting ATI in cure research are discussed in further detail at the end of this chapter.

12.3 Gene Therapy

Genetic engineering is a powerful tool with the capacity to modify gene expression by knockout, activating, editing or silencing of target genes or even site-specific addition of new genes. Genetic engineering is also being employed in HIV cure research using various targeted nuclease systems such as clustered regularly interspaced short palindromic repeats (CRISPR), transcription activator-like effector nucleases (TALEN) and zinc finger nucleases (Gaj et al. 2016). Most gene engineering-based approaches to perturb HIV are still at the very early preclinical stages, but one approach has progressed to clinical trials (Wang and Cannon 2016).

Inspired by the intriguing case report of the ‘Berlin patient’, gene editing aimed at permanently disrupting T cell expression of CCR5, the main co-receptor used for HIV entry, was developed by a biotech company (Sangamo BioSciences) based on the zinc finger nuclease technology. In the initial phase 1 study, zinc finger-mediated CCR5 deletion was achieved *ex vivo*, and genetically modified autologous CD4+ T cells were reinfused in virologically suppressed participants on stable ART (Tebas et al. 2014). The intervention was found to be relatively safe. Interestingly, in one participant, the level of plasma HIV RNA during analytical ART interruption dropped after an initial increase and was below the limit of detection before reinitiation of ART (Tebas et al. 2014). A phase II study using the same approach is now ongoing, and zinc finger nuclease CCR5 editing is also being employed to genetically modify haematopoietic stem cells, which may have a larger potential for proliferation and repopulation of the bone marrow. While the initial results of the Sangamo trial are encouraging, safety concerns related to off-target gene editing, the technical requirements related to the procedure and the significant costs associated with this type of treatment are considerable hurdles to overcome if genetic engineering is to be scaled up to treat millions of people living with HIV infection (Wang and Cannon 2016). In addition, finding a reliable and efficient method of delivering the editing techniques to cells *in vivo* is a major challenge for this new and exciting field of research.

12.4 ART Intensification

The concept of intensifying ART by adding an additional antiretroviral drug to an already suppressive regimen has been investigated in numerous clinical trials. These studies were originally done to investigate whether intensified ART would reduce levels of persistent HIV in plasma or cells but have shown that ART intensification does not further suppress low-level viremia nor does it reduce the frequency of latently infected cells (Dinoso et al. 2009; Gandhi et al. 2010, 2012; McMahon et al. 2010; Gutierrez et al. 2011; Hatano et al. 2011; Llibre et al. 2012; Buzon et al. 2010; Vallejo et al. 2012). However, findings from integrase inhibitor-based intensification studies indicated the presence of residual low-level replication on ART in a subset of individuals and showed how intensification studies can be used to demonstrate this phenomenon. Two randomised clinical trials, in which ART was intensified with the integrase inhibitor raltegravir, reported a transient but significant increase in two-long terminal repeat (2-LTR) circles, considered to be by-products of unsuccessful integration, in participants who received raltegravir compared to controls who received placebo (Buzon et al. 2010; Llibre et al. 2012; Hatano et al. 2013). Integrase inhibitors block the integration of linear HIV DNA, which then becomes circularised by host repair enzymes to form 2-LTR circles. Therefore, an increase in 2-LTR circles suggests that active replication is blocked by inhibition of integration. Moreover, the transient increase in 2-LTR was accompanied by a decrease in immune activation parameters including plasma levels of D-dimer (Hatano et al. 2013) and measures of T cell activation (Buzon et al. 2010). Other studies did not demonstrate an increase in 2-LTR circles following raltegravir intensification, but this may be due to lack of sampling in the early phases of intensification as 2-LTR circles are believed to be relatively short-lived (Gandhi et al. 2012; Vallejo et al. 2012). Collectively, these observations suggest that residual replication may be occurring in some individuals on ART and that this might be more common in individuals receiving protease inhibitors.

Understanding the potential contribution of residual virus replication to HIV persistence on ART is particularly important for curative strategies aimed at reversing HIV latency as a mechanism to eliminating latently infected cells, because such approaches will not be as effective if there is residual virus replication. It is possible, though still controversial, that residual virus replication on ART could be linked to insufficient drug penetration in lymphoid tissues, often referred to as pharmacological sanctuaries (Eisele and Siliciano 2012). This was suggested by a study, which reported tissue concentrations of five of the most commonly used antiretroviral drugs to be much lower in lymphoid tissue than in peripheral blood mononuclear cells (PBMCs), and these lower concentrations correlated with detection of viral RNA in productively infected cells (Fletcher et al. 2014). The investigators subsequently presented a model to explain how persistent HIV replication and viral evolution on ART might be occurring without the emergence of drug resistant variants in blood (Lorenzo-Redondo et al. 2016). Finally, a recently completed randomised controlled clinical trial investigated the effect of ART intensification with the potent

second-generation integrase inhibitor dolutegravir but found no evidence of residual replication in blood.

12.5 Early ART and Posttreatment Control

There is now strong evidence that HIV reservoirs including the latent infection in CD4+ T cells are established in the early phases of primary infection. This was initially demonstrated in a study where initiation of ART within 10 days after infection reduced the frequency of detection of latently infected CD4+ T cells carrying replication-competent virus (Chun et al. 1998). These results have subsequently been confirmed by other studies showing that latent infection is established in the very early phases of acute infection (Ananworanich et al. 2016). The case report of the Mississippi child provided additional insights into the establishment of HIV reservoirs during acute infection and the impact of early ART. In this child, perinatal HIV infection was detected shortly after birth, and the child started on ART 30 hours after birth. ART was then discontinued at age 18 months without rebound viremia (Persaud and Luzuriaga 2014), and no detection of virus in plasma or cells for an extended period until virus rebound finally occurred after 27 months off ART (Luzuriaga et al. 2015). This study thus provided an example that early ART reduces the amount of virus that persists on ART and may set the scene for extended virological control in the absence of ART. Given the major immunological differences between perinatal and adult infection, it is difficult to extrapolate that very early ART during acute adult infection will lead to remission off ART.

Experimental infection of non-human primates with simian immunodeficiency virus (SIV) offers several advantages over studying acute HIV infection in humans, because this model allows for a better control of the timing between infection and ART initiation. These animal models have therefore been used to investigate the impact of early ART and whether this might lead to virological remission after withdrawal of ART. In a study by Whitney et al., rhesus macaques were infected intrarectally and then put on ART 3, 7, 10 and 14 days postinfection (Whitney et al. 2014). In animals who initiated ART on day 3, viral RNA and DNA were not detected in peripheral blood, and there was no detectable anti-HIV immune response. However, when ART was discontinued at week 24, there was rebound viremia in all monkeys although this occurred later in animals that started ART at day 3 compared to those that started ART at later time points (Whitney et al. 2014). This study thus showed that the SIV reservoir is seeded very early after infection. Again, there are important immunological and virological differences between human and monkey infection, including the Vpx-mediated degradation of SAMDH1, which facilitates more efficient infection of myeloid cells during acute SIV infection (Lagquette et al. 2011).

A range of studies have examined the effect of early ART on the size of the HIV reservoir during human infection and consistently found that early ART is associated with a lower frequency of cells carrying replication-competent virus (Archin

et al. 2012b; Strain et al. 2005; Buzon et al. 2014) and HIV DNA (Jain et al. 2013; Gianella et al. 2011). Investigations arising from the RV254/SEARCH010 study, one of the best-characterised cohorts with regard to timing of ART initiation during acute HIV infection, showed that there is a gradual increase in the size of the reservoir during the first few weeks of infection (Ananworanich et al. 2012). Also, long-term follow-up showed that starting ART during acute infection dramatically limits reservoir size compared to untreated infection and that this difference increases over time (Ananworanich et al. 2016). Collectively, these studies have provided consistent evidence that starting ART early after infection is associated with a smaller reservoir size and preserved anti-HIV immune responses. However, difficulties in identifying infected individuals during acute infection have limited the size of the studies, and prospective controlled clinical trials of early ART are challenging to conduct.

In addition to limiting the frequency of latent infection in CD4+ T cells, early ART may also enhance chances for virological control after stopping ART. This phenomenon, termed posttreatment control (PTC), was initially described in the French VISCONTI cohort (Saez-Cirion et al. 2013). In contrast to elite controllers, posttreatment controllers (PTCs) usually present with high plasma viremia and low CD4+ T cell counts during acute infection. Additionally, they do not display strong anti-HIV CD8+ T cell responses and rarely carry protective HLA alleles (Rouzioux et al. 2015; Saez-Cirion et al. 2007, 2013; Goulder and Walker 2012). As such they may constitute a relevant model for achieving ART-free remission and identify the immunological characteristics associated with virological control.

Few studies have been able to confirm the frequency with which PTC occurs, but an analysis of the SPARTAC study provided further insight. The SPARTAC study was originally conducted to investigate the impact of short-course ART during primary HIV infection (Investigators et al. 2013). In a recent analysis, it was shown that virological control off ART (defined as plasma viral load <400 copies/mL for >16 weeks) occurred spontaneously in 7.9% of untreated individuals but in 15.1% of those whom initially received short-course ART before stopping treatment (Martin et al. 2017). Furthermore, virological control occurred at an even higher frequency of 18.6% among those who received the longest duration of ART (48 weeks). These data thus support a role of ART in inducing PTC but also underline that spontaneous control in the absence of ART is not uncommon, and this may confound estimates of PTC. These data thus emphasise the need for a control group when the primary endpoint of a study is PTC.

12.6 Latency-Reversing Agents: Shock and Kill

12.6.1 Shock and Kill

A small fraction of resting memory CD4+ T cells carries quiescent but replication-competent provirus (Chun et al. 1997b; Finzi et al. 1997). This reversibly non-productive state of infection is termed HIV latency (Eisele and Siliciano 2012), and

due to the long-lived nature of memory CD4+ T cells, it is setting the scene for long-term viral persistence in infected individuals on ART. In the absence of viral protein expression during latent infection, these cells do not express viral proteins and, therefore, their infected status remains invisible to the immune system and unresponsive to ART (Fig. 12.1). Developing therapies with the capacity to deplete this latent viral reservoir, primarily residing within long-lived CD4+ T cells, has become a high priority in HIV research. One approach towards this aim is usually referred to as ‘shock and kill’ (Deeks 2012). Shock and kill is characterised by the use of pharmacological agents to reverse HIV latency and stimulate latently infected cells to express viral proteins as this would theoretically expose such cells to immune-mediated killing or virus-mediated cell lysis. A large number of so-called latency-reversing agents (LRAs), i.e. compounds which induce HIV production in latently infected cells, have been investigated primarily at the preclinical level (Shang et al. 2015; Darcis et al. 2015), but a few candidates have also

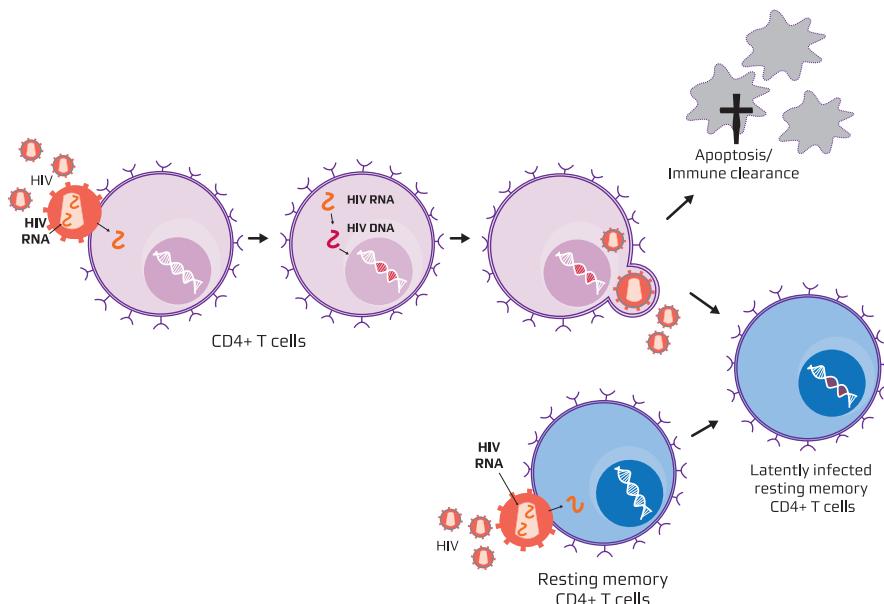


Fig. 12.1 Establishment of HIV latency. After viral entry into the CD4+ T cell, the viral genomic RNA is reverse transcribed to HIV DNA and integrated into the host cell genome. Viral proteins and genomic viral RNA are then produced using the host cell transcriptional and translational machinery, which leads to the assembly and release of new virions that may go on to infect new cells. Most productively infected CD4+ T cells die in this process due to viral- or immune-mediated cell lysis. Occasionally, a newly infected cell may revert to a resting memory state where transcription of the virus is silenced, thus giving rise to long-lived latently infected resting memory CD4+ T cells. Resting memory CD4+ T cells may also be infected directly in their resting state in the presence of stimuli, such as certain chemokines. The transcriptional silencing during latent infection in long-lived CD4+ T cells facilitates evasion from immune-mediated clearance, and latently infected long-lived CD4+ T cells are the primary mechanism for HIV persistence on ART

undergone testing in small-scale clinical trials. Below we will summarise and discuss the results of the few clinical trials of LRAs conducted thus far (Table 12.1). Because histone deacetylase inhibitors (HDACi) have played a key role in testing the shock and kill strategy, we will initially consider the latency mechanisms impacted by these drugs.

12.6.2 Histone Deacetylation: A Mechanism of HIV Latency

In resting memory CD4+ T cells, several restrictions to HIV expression are contributing to maintaining HIV latency. These restrictions include site and orientation of HIV integration, accessibility of key transcription factors, inhibition of transcriptional elongation, RNA interference, DNA methylation and histone deacetylation, reviewed in Ruelas and Greene (2013) and Siliciano and Greene (2011). The effects of histone acetylation/deacetylation on chromatin condensation and viral gene expression are of particular interest for cure-related interventions because several licensed drugs are capable of altering this equilibrium (Falkenberg and Johnstone 2014). The counteracting mechanisms of histone deacetylases (HDACs) and histone acetyl transferases (HAT) control the degree of histone acetylation/deacetylation, which in turn influences chromatin condensation and transcriptional activity (Minucci and Pelicci 2006). During the transcriptionally repressive state of HIV latency, HDACs are recruited to the HIV 5' long terminal repeat (LTR) where they induce chromatin condensation and transcriptional repression by promoting histone deacetylation (Van Lint et al. 1996; Dahabieh et al. 2015). The importance of histone deacetylation for maintaining HIV latency has been clearly demonstrated by the consistent reversal of HIV latency and expression of HIV upon stimulation with inhibitors of HDACs in models of HIV latency (Shan et al. 2014; Archin et al. 2009a, b; Wightman et al. 2012; Matalon et al. 2010; Rasmussen et al. 2013; Laird et al. 2015; Bullen et al. 2014). Through HDAC inhibition, HDACi promote histone acetylation, which induces chromatin relaxation and allows binding of key transcription factors to the HIV LTR, thereby promoting HIV transcription (Van Lint et al. 1996; Verdin et al. 1993) (Fig. 12.2).

12.6.3 Histone Deacetylase Inhibitors

In the initial clinical proof-of-concept study testing the shock and kill hypothesis, the anticonvulsant valproic acid (VPA), which exerts HDAC inhibition though requiring very high concentrations for in vitro efficacy (Ylisastigui et al. 2004; Matalon et al. 2010), was added to intensified ART for 12 weeks. A decline was reported in the frequency of resting CD4+ T cells carrying replication-competent HIV in the initial study (Lehrman et al. 2005), but additional studies failed to demonstrate any effect of VPA on the latent reservoir (Archin et al. 2008, 2010; Routy

Table 12.1 Clinical trials of latency-reversing agents

Clinical trial intervention	Trial design and size	Effects on HIV latency			Effects on HIV reservoir and viral rebound			
		HIV transcription (CA-US HIV RNA)	Plasma HIV RNA	HIV DNA	qVOA	TILDA	ATI	Refs
Vorinostat 400 mg	Single-arm study, single dose of vorinostat ($n = 8$)	4.8-fold increase ^a	No change	No change	ND	ND		Archin et al. (2012)
Vorinostat 400 mg	Single arm study; daily vorinostat for 14 consecutive days ($n = 20$)	2.7-fold increase ^b	No change	No change	ND	No change	ND	Elliott et al. (2014)
Vorinostat 400 mg	Single-arm study; vorinostat 3 days/week for 8 weeks ($n = 5$)	No overall change ^a	No change	No change	ND	ND		Archin et al. (2014)
Panobinostat 20 mg	Single-arm study; panobinostat 3 days/week every other week for 8 weeks ($n = 15$)	2.9-fold increase ^b	Significant increase in detection rate (non-quantitative)	No change	ND	ND	Median time to viral rebound (>1000/mL) 17 days	Rasmussen et al. (2014)
Romidepsin 5 mg/m ²	Single arm study; romidepsin once weekly for 3 weeks ($n = 6$)	3.8-fold increase ^b	Quantifiable increase in five of six participants (range 21-119/mL)	No change	No change	ND		Søgaard et al. (2015)
Vacc-4x followed by romidepsin 5 mg/m ²	Single-arm study; Vacc-4x immunizations followed by romidepsin once weekly for 3 weeks ($n = 20$)	3.1-fold increase ^b	Quantifiable increase in 7 of 16 participants; overall not statistically significant	40 % decrease	38% decrease	ND	Median time to Viral rebound (>50/ml) 14 days	Let et al. (2016)

Disulfiram 500 mg	Single arm study; daily disulfiram for 14 consecutive days ($n = 16$)	ND	Significant increase post-dosing (SCA)	ND	No change	ND	ND	Spivak et al. (2014)
Disulfiram 500-2000 mg	Dose escalation study; 3 dose arms of 500, 1000 and 2000 mg disulfiram daily for 3 days ($n = 30$)	1.7 to 2.1-fold increase across dose-arms ^b	Significant increase post-dosing in the 2000 mg dose-arm (SCA)	No change	ND	ND	ND	Elliott et al. (2015)
Bryostatin 10-20 µg/m ²	Single-dose placebo-controlled study comparing bryostatin-1 at 10 and 20 µg/m ² ($n=12$)	No change ^b	No change	ND	ND	ND	ND	Gutierrez et al. (2016)
Interleukin-7	Randomised-controlled trial comparing ART intensification with or without IL-7 ($n = 29$)	No change ^b	Significant increase in detection rate (non-quantitative)	Significant increase	ND	ND	ND	Kallama et al. (2016)

^a Analysed in resting CD4+T cells

^b Analysed in total CD4+ T cells

ATI analytical treatment interruption, CA-US RNA cell-associated unspliced HIV-RNA, IL-7 interleukin-7, ND not done, qVOA quantitative viral outgrowth assay, TLLDA *tat/rev* induced limiting dilution assay, SCA single copy assay

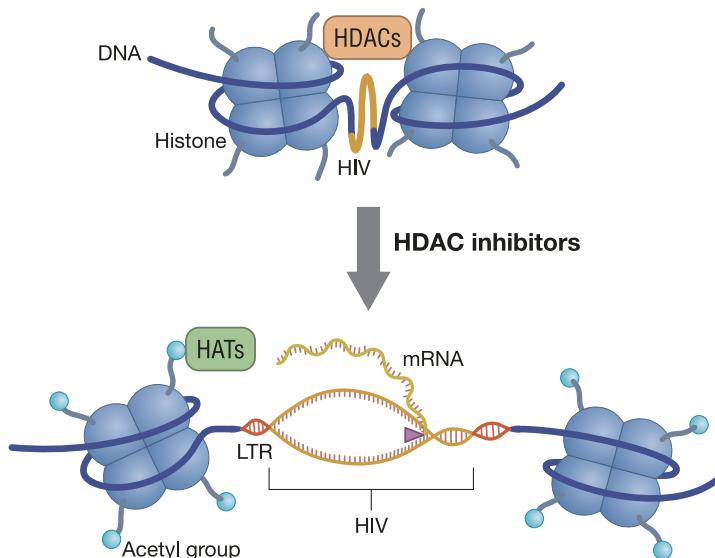


Fig. 12.2 Disruption of HIV latency by HDAC inhibitors. The activity of HDACs suppresses HIV expression by promoting deacetylation of lysine residues on histone tails and keeping the chromatin in a compacted state. Inhibition of HDACs by HDACi promotes histone acetylation by HATs, which leads to chromatin relaxation and initiation of transcription. *HDACs* histone deacetylases, *HDACi* histone deacetylase inhibitors, *HATs* histone acetyl transferases, *LTR* long terminal repeat. Original publication in *Human Vaccines and Immunotherapeutics* (2013 Apr;9(4):790–9) (169), reprinted here with permission from publisher

et al. 2012). Recognising that latency reversal with VPA may be too weak, HDACi with much higher potency then became the focus of investigation. Vorinostat, approved by the American Food and Drug Administration (FDA) in 2006 for the treatment of cutaneous T cell lymphoma (Mann et al. 2007), was the first potent HDACi to be tested in HIV-infected individuals on suppressive ART. In this study, a single dose of 400 mg vorinostat led to an almost fivefold increase in HIV transcription as measured by cell-associated unspliced (CA-US) HIV RNA (Archin et al. 2012a) in resting CD4+ T cells, thus demonstrating for the first time that the state of HIV latency could be disrupted in patients on ART. In a subsequent multiple-dose study, daily vorinostat (400 mg) for 14 days also significantly increased HIV transcription as measured by CA-US HIV RNA in unfractionated CD4+ T cells (Elliott et al. 2014). In contrast, in another multiple-dose study where vorinostat was given 3 days a week for 8 weeks, levels of HIV transcription (again measured as CA-US HIV RNA) were overall not increased above baseline when measured after 11 and 22 doses (Archin et al. 2014).

The highly potent HDACi panobinostat, which was approved by the FDA in 2015 for the treatment of multiple myeloma (Laubach et al. 2015), has also been

tested in a clinical trial for its ability to reverse HIV latency in HIV-infected patients on ART (Rasmussen et al. 2014). Panobinostat was added to suppressive ART three times per week every other week for 8 weeks in 15 HIV-infected patients. This resulted in a significant increase in HIV transcription measured as levels of CA-US HIV RNA in total CD4+ T cells and panobinostat also increased the detection rate of plasma HIV RNA using a semi-quantitative assay (Rasmussen et al. 2014). However, the most convincing effect of reversing latency in the clinical setting was seen in a pilot study of the HDACi romidepsin, which inhibits HDACs with even higher potency (Wei et al. 2014). In this study, three infusions of romidepsin (5 mg/m²) not only significantly increased HIV transcription but also led to increases in plasma HIV RNA, which in five of six individuals on suppressive ART, were readily quantifiable using a standard clinical assay (Cobas Taqman, limit of quantification 20 copies/mL) (Sogaard et al. 2015).

Collectively, these studies of HDACi provided evidence that it is possible with a clinical intervention to disrupt the state of HIV latency and for some interventions to the extent that this increases plasma HIV RNA. The latter is important because this is associated with viral protein expression, which is a requirement for immune recognition. However, despite reversing HIV latency, none of the clinical trials to date using HDACi alone added to ART have been able to demonstrate a decrease in the frequency of latently infected cells (Rasmussen and Lewin 2016; Rasmussen et al. 2016), whether this was measured as cell-associated HIV-1 DNA, infectious units per million (IUPM) using a quantitative viral outgrowth assay (QVOA) (Laird et al. 2013) or inducible multiply spliced HIV-1 RNA using the *tat/rev*-induced limiting dilution assay (TILDA) (Procopio et al. 2015). Finally, rebound viremia occurred within an expected time frame in all individuals who consented to analytical interruption of ART following panobinostat treatment (Rasmussen et al. 2014).

12.6.4 Disulfiram

The latency-reversing potential of disulfiram and its metabolites was initially discovered in a drug library screen (Yang et al. 2009). Subsequent investigations have revealed that disulfiram activates HIV transcription through depletion of the phosphatase and tensin homolog (PTEN), which results in activation of the Akt signalling pathway (Doyon et al. 2012). Given that disulfiram have been used safely for many years to treat alcoholism, these observations immediately formed the basis for testing the effect of disulfiram on HIV latency in a clinical trial. In this study, standard dose disulfiram (500 mg) was given daily for 14 days to HIV-infected individuals on ART (Spivak et al. 2014). Although there was no overall effect on plasma HIV RNA using an assay with single-copy sensitivity (single-copy assay; SCA), a transient increase in plasma HIV RNA was noted in a post hoc analysis in subjects

with immediate post-dose sampling available and in subjects with higher concentrations of disulfiram in plasma (Spivak et al. 2014). To further explore these observations, a subsequent dose-escalation study was undertaken wherein 30 HIV-infected individuals on ART received 3 days of disulfiram either at dose 500 mg (licensed dose), 1000 mg or 2000 mg. In all three dose cohorts, disulfiram treatment led to an increase in the levels of CA-US HIV RNA, though the magnitude of the increase was slightly less than what was seen in the HDACi studies (Elliott et al. 2015). Furthermore, disulfiram at 2000 mg/day resulted in a significant increase in plasma HIV RNA. Interestingly, increases in CA-US HIV RNA and plasma HIV RNA were seen both on-disulfiram and post-dosing. The mechanism behind this prolonged effect on HIV production is not well understood, but similar post-intervention changes were observed in trials of vorinostat and panobinostat at 70 and 28 days of follow-up, respectively (Rasmussen et al. 2014; Elliott et al. 2014). However, subsequent extended follow-up for 2 years after vorinostat with sampling performed every 6 months showed that levels of HIV transcription and plasma HIV RNA were not increased at any of these time points relative to pre-vorinostat levels (Mota et al. 2017). Also, whole-genome microarray analyses before and after panobinostat showed that gene expression profiles had normalised 24 weeks post-panobinostat. Finally, similar to the studies of HDACi, latency reversal by disulfiram was not associated with a decrease in the frequency of latently infected cells. None of the studies using disulfiram has included analytical interruption of ART.

12.6.5 Protein Kinase C Agonists (*Bryostatin-1*)

Activation of protein kinase C (PKC) has shown great promise as a mechanism to reverse HIV latency in vitro and ex vivo, and in these investigations PKC agonists appear among the most potent LRAs, especially when used in combination with HDACi (Laird et al. 2015; Bullen et al. 2014). In terms of their effect on HIV latency, bryostatin-1 and prostratin are the most well-characterised compounds in this group. Prostratin potently activated latent HIV in vitro (Kulkosky et al. 2001) but given its toxicity may not proceed to clinical studies in HIV. Bryostatin-1 is a naturally occurring PKC agonist isolated from the marine bryozoan *Bugula neritina* (Ramsdell et al. 1986) and is in clinical development for several malignancies including multiple myeloma and renal cell carcinoma (Plimack et al. 2014). Due to concern of its potential toxicity exerted through T cell activation, clinical investigations have progressed slowly, and bryostatin-1 was only just recently tested in a small-scale clinical trial. In this study, 12 HIV-infected individuals were randomised to placebo or a single dose of bryostatin-1 at 10 µg/m² or 20 µg/m² (Gutierrez et al. 2016). No significant toxicities were seen at these doses, but bryostatin-1 did not demonstrate any effect on either PKC activity or HIV transcription, likely due to the low plasma concentrations observed with the dosages used (Gutierrez et al. 2016).

12.6.6 Latency-Reversing Interventions Thus Far: Shock But No Kill

As outlined in the above sections, several clinical trials have shown that increasing HIV transcription and viral protein production in HIV-infected individuals on suppressive ART is possible through the use of LRAs, especially HDACi. Still, in none of these studies did this result in a decrease in the frequency of latently infected cells. Understanding why reversing HIV latency in clinical trials was not associated with an elimination of latently infected cells is important for the onwards development of more effective interventions, including the development of LRA combination approaches or combining latency reversal with immune enhancement therapy. There are several possible explanations. First, cytotoxic T lymphocytes (CTLs) play an important role in controlling HIV replication during acute infection, but chronic infection is characterised by an impaired cytolytic capacity of CD8+ T cells, which is not restored by ART (Hersperger et al. 2010). Thus, HIV-specific CTL responses may be inadequate to efficiently target virus-expressing cells. It is also possible that viral epitopes induced by LRA treatment are insensitive to CTL killing. This situation may arise because during untreated HIV infection, there is a high rate of virus sequence evolution driven by the high mutation rate of HIV reverse transcription (Cuevas et al. 2015). The initial adaptive immune response by CTLs results in a selection pressure, which means that mutations that confer CTL resistance are quickly acquired and archived in long-lived CD4+ T cells (Borrow et al. 1997). In fact, it was recently shown that in patients initiating ART more than 3 months after HIV infection, CTL escape variants completely dominated the latent viral reservoir (Deng et al. 2015). Second, it is possible that the extent of latency reversal achieved in clinical trials of LRAs thus far only mobilises a minor proportion of the latent reservoir. More potent interventions, possibly achieved through combining LRAs, may mobilise a higher proportion of latently infected cells and also more efficiently induce virus-mediated cytolysis. Finally, clinical studies of LRAs thus far have primarily focused their analyses on peripheral blood, whereas the majority of HIV-infected cells reside in lymphoid tissues, primarily gut-associated lymphoid tissue and lymph nodes. A better understanding of persistence mechanisms in tissues and how LRAs might impact those mechanisms could pave the way for more effective strategies in future studies (Fig. 12.3).

12.6.7 Combining Latency-Reversing Agents with Immunotherapy

The cumulated experience with the use of LRAs in small-scale clinical trials in HIV, including the demonstrated effect on HIV latency but also the lack of an effect on the frequency of latently infected CD4+ T cells, has inspired combination studies where an LRA is combined with immunotherapy. In the first of these studies, the

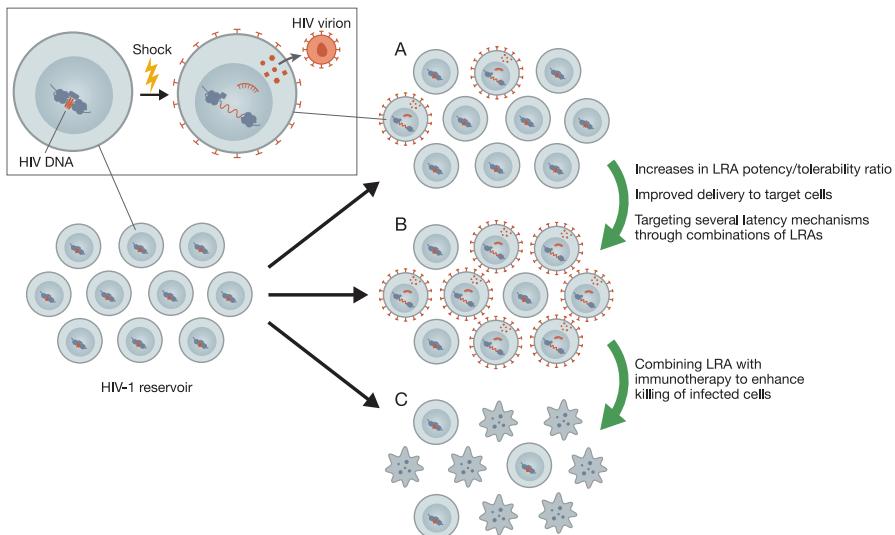


Fig. 12.3 Optimisation of shock and kill therapies. The figure illustrates the total pool of cells latently infected with replication-competent HIV-1 (left side) and how LRAs may activate production of HIV transcripts and viral proteins (enlargement). Panels A, B and C are schematic representation of shock and kill approaches with various degrees of impact on the latent HIV-1 reservoir and indication of factors that may lead to improvement in shock and kill approaches. (A) Illustrates the extent of latency reversal achieved with current LRA studies. (B) Illustrates how optimisation of LRA interventions may enhance the magnitude of latency reversal. (C) Illustrates how combinations of immunotherapy and LRAs may augment killing of latently infected cells. *LRA* latency-reversing agent. Original publication in *Trends in Microbiology* (2016 Feb;24(2):90–7) (90), reprinted here with permission from publisher

therapeutic peptide-based HIV vaccine Vacc-4x (Bionor Pharma), with rhGM-CSF as local adjuvant, was administered intradermally six times in a prime-boost sequence and then followed by three infusions of romidepsin at a 5 mg/m^2 dose (Leth et al. 2016). This combined intervention was associated with a moderate decrease in the frequency of latently infected CD4+ T cells as measured by the level of HIV DNA and, for those with assessable levels, quantitative viral outgrowth, but did not delay the time to viral rebound during ATI (Leth et al. 2016). In an ongoing study, HIV-infected individuals who started ART <6 months after infection received HIV vaccination containing a conserved RNA sequence (HIV_{cons}) using first chimp adenovirus vector and then modified vaccinia virus (MVA) vector in a prime-boost sequence. Study participants were subsequently given three infusions of romidepsin at a 5 mg/m^2 dose before ART was interrupted to assess the effect on virological control. Remarkably, although final results are still awaited, preliminary data showed that virological control off ART was achieved for five of 13 individuals (Mothe et al. 2017). Finally, inspired by post hoc observations of correlates of effect in a previous study of panobinostat (Olesen et al. 2015), panobinostat is combined with pegylated interferon- α 2a in an ongoing study (NCT02471430).

12.7 Immune Checkpoint Inhibitors

T cell stimulatory and inhibitory pathways play a key role for maintaining the delicate balance between protective immunity against foreign pathogens and preventing autoimmunity. Co-inhibitory T cell signalling occurs through ligand binding to immune checkpoint receptors expressed on the surface of CD4+ and CD8+ T cells (Schildberg et al. 2016). In malignant diseases, enhanced inhibitory signalling through immune checkpoints on CD4+ and CD8+ T cells dampens T cell responses against malignantly transformed cells and facilitates their escape from immune destruction. These pathways are therefore exploited therapeutically in the cancer field, in particular through the recent development and clinical use of monoclonal antibodies to block immune checkpoints as a way to enhance antitumour immune responses (Sharma and Allison 2015). The programmed death 1 (PD1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) pathways have proven the most clinically relevant targets so far. Monoclonal antibodies against CTLA4 (ipilimumab) and PD1 (nivolumab and pembrolizumab), including combination blockade, now have regulatory approval and have provided major improvements in the treatment of melanoma and non-small cell lung cancer (NSCLC) (Weber et al. 2015; Robert et al. 2015a, b; Postow et al. 2015) and are also investigated in other malignancies (Boutros et al. 2016). In addition, several other antibodies that block CTLA4, PD1, programmed death-ligand 1 (PD-L1) or other immune checkpoints such as T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), lymphocyte activation gene-3 (LAG-3) or T cell immunoglobulin and mucin domain-containing 3 (TIM-3) are concurrently investigated against various malignancies (Park et al. 2016).

The expression of immune checkpoints and the associated negative T cell signalling is also of interest in HIV cure research. Chronic HIV infection is characterised by increased negative signalling through immune checkpoints, which leads to T cell exhaustion and is associated with disease progression (Trautmann et al. 2006; Day et al. 2006; Chew et al. 2016). Therefore, blocking immune checkpoints as a strategy to enhance HIV-specific T cell responses against virus-expressing cells has attracted considerable interest (Porichis and Kaufmann 2012). This is supported by previous studies, which demonstrated that blocking CTLA4 or PD1 enhances HIV- and SIV-specific T cell function (Porichis et al. 2011; Velu et al. 2009; Amancha et al. 2013). In addition, expression of immune checkpoints may identify CD4+ T cells harbouring HIV as exemplified by a study that revealed higher levels of HIV DNA in PD1-expressing memory T cells in blood compared to their PD1-negative counterparts (Chomont et al. 2009). CTLA4-expressing cells from viremic patients were also shown to contain more HIV DNA than CTLA4-negative cells (El-Far et al. 2015). Another study demonstrated that inducible p24 expression was higher in peripheral T follicular helper cells (Tfh) than peripheral non-Tfh after 48 weeks on ART and that the highest level of inducible virus was found in PD1-expressing peripheral Tfh (Pallikkuth et al. 2015). Finally, these observations were recently corroborated by a study, which showed that lymph node CD4+ T cells that express

PD1, the majority of which are Tfh, are the major source of infectious virus in patients on suppressive ART for up to 14 years (Banga et al. 2016). Collectively, these studies demonstrate that blocking negative T cell signalling through immune checkpoints could have a positive impact on HIV-specific T cell responses but also that immune checkpoint expression, particularly PD1, plays a key role in HIV persistence. The latter may explain why activating T cell function through immune checkpoint blockade could also activate expression of latent HIV; this was a key observation in a case report of an HIV-infected patient who received anti-CTLA4 (ipilimumab) for melanoma (Wightman et al. 2015). In this report, there was an increase in HIV transcription of up to 19.6-fold relative to pre-ipilimumab and a cyclical decrease in residual plasma HIV RNA (Wightman et al. 2015).

Despite this body of evidence from in vitro studies, animal studies and case reports, very little interventional research has been performed to investigate the effect of immune checkpoint inhibitors on HIV-specific immunity and HIV persistence in infected individuals on ART. This is primarily due to a concern of immune-related adverse events (irAEs), which is a well-known risk with the clinical use of immune checkpoint inhibitors and has been described consistently in cancer studies (Eigentler et al. 2016). The frequency of \geq grade 3 irAEs is around 2% in studies with nivolumab or pembrolizumab but higher with ipilimumab and considerably higher when ipilimumab and nivolumab were combined (Eigentler et al. 2016).

Only one clinical trial thus far has investigated immune checkpoint blockade in HIV-infected individuals without malignancy. This was a dose-escalation study of the compound BMS-936559, a monoclonal antibody to PD-L1. Data from the first dose cohort showed an increase in HIV-specific CD8+ T cell responses in two of six individuals treated with a single infusion of low-dose (0.3 mg/kg) anti-PD-L1 but no effect on plasma or cell-associated HIV. The study was terminated prematurely because of retinal toxicity in a concurrent monkey study and, additionally, one individual in the human trial developed hypophysitis 36 weeks after receiving low-dose anti-PD-L1.

In conclusion, while immune checkpoint blockade certainly represents a uniquely interesting immunotherapy intervention in HIV cure research that warrants further investigation, this research must be advanced carefully given the well-known risk of irAEs. The current approach to this dilemma is to initially characterise immunological and virological effects among HIV-infected individuals who receive immune checkpoint blockade as part of cancer treatment (e.g. studies NCT02408861, NCT02595866).

12.8 Toll-Like Receptor Agonists

Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system. TLRs recognise and bind molecular structures that are broadly shared by pathogens, so-called pathogen-associated molecular patterns (PAMP) (Kanzler et al. 2007). This binding then induces activation of innate immune

signalling but also helps shape adaptive immune responses (Iwasaki and Medzhitov 2004). Molecular structures of malignantly transformed cells are also recognised by TLRs, and their binding induces tumour-directed innate immune activation (Liu and Zeng 2012). Therefore, agonists of TLRs are investigated for their potential effect in the treatment of various malignancies (Krieg 2007), but these immunological effects are also relevant for HIV cure research. In HIV, most studies have focused on agonists to TLR7 and TLR9, which are both expressed intracellularly on endolysosomes – TLR7 in plasmacytoid dendritic cells (pDCs) and conventional DCs and TLR9 in pDCs and B cells (Kawai and Akira 2010). Several in vitro studies indicated that certain TLR agonists (TLR2, TLR7 and TLR9) could activate HIV from latency (Scheller et al. 2004, 2006) and thus potential function as latency-reversing agents in vivo. Another important function of certain TLR agonists is their ability to activate antigen-presenting cells (APC), increase B cell maturation, augment cross-priming of T cells and induce natural killer (NK) cell activation (Offersen et al. 2016; Buitendijk et al. 2014). These immune stimulatory effects directly augment killing of infected cells but may also be used to potentiate vaccine responses (Krieg 2006).

While several studies have investigated TLR agonist-adjuvanted vaccination in HIV patients, the experimental use of adjunctive TLR agonist treatment added to long-term ART has only recently entered clinical testing. MGN1703, a novel TLR9 agonist, induced antiviral innate immune responses and boosted NK cell-mediated viral inhibition in vitro (Offersen et al. 2016). The effect of MGN1703 on antiviral immunity and the HIV reservoir was investigated in a phase 1b/2a trial in which 15 virologically suppressed HIV-infected individuals on ART received 60 mg MGN1703 s.c. twice weekly for 4 weeks (Vibholm 2017). Shortly after administration, MGN1703 induced pronounced activation of pDCs and increased levels of plasma interferon- α 2. Subsequently, proportions of activated cytotoxic NK cells and CD8 $^{+}$ T cells expanded significantly. In 6 of 15 participants, plasma HIV-1 RNA blips from 30 c/mL up to >1500 copies/mL were observed during MGN1703 treatment. This subset of participants demonstrated increased capacity of CD8 $^{+}$ T cells to mediate HIV-specific viral inhibition and also displayed a significant reduction in levels of integrated HIV DNA levels in intestinal tissue, suggesting that TLR9 stimulation with MGN1703 activated latent HIV and enhanced antiviral immunity, both key outcomes in HIV eradication therapy.

The oral TLR7 agonists, GS-9620 and GS-986, have been administered to ART-suppressed simian immunodeficiency virus (SIV)-infected rhesus macaques and induced consistent increases in plasma viral RNA and T cell activation (Whitney et al. 2016). Moreover, two of nine TLR7-treated monkeys had no inducible virus in blood and lymph node tissue, and these two animals also displayed virological control when ART was withdrawn (Whitney et al. 2016).

Based on these encouraging preclinical and clinical findings, multiple trials investigating the effects of TLR7 and TLR9 agonists alone or in combination with other interventions such as therapeutic HIV vaccines and broadly neutralising antibodies (bNAbs) against HIV *env* are now underway.

12.9 Interleukin Therapy (IL2, IL7, IL15)

The strategy of eliminating latently infected cells through activating HIV expression was initially tested using interleukin-2 (IL-2). Early studies actually suggested that IL-2 therapy might impact on the frequency of resting cells harbouring replication-competent virus (Chun et al. 1999b), but rebound viremia occurred rapidly when ART was interrupted (Chun et al. 1999a). Moreover, additional studies could not establish an effect of IL-2 on the pool of latently infected CD4+ T cells or HIV production (Dybul et al. 2002; Stellbrink et al. 2002), and when IL-2 was used in combination with anti-CD3 antibody OKT3, this led to detrimental T cell activation and irreversible CD4+ T cell depletion (van Praag et al. 2001). Subsequently, the homeostatic cytokine IL-7 was investigated with a similar aim of reversing HIV latency and facilitating the elimination of latently infected cells. Several studies showed that IL-7 induced virus outgrowth ex vivo in resting CD4+ T cells of HIV-infected patients on ART (Lehrman et al. 2004; Wang et al. 2005) and two small clinical trials reported that IL-7 administration to HIV-infected individuals on ART caused transient increases in plasma HIV RNA in around half of study participants (Levy et al. 2009; Sereti et al. 2009). However, a study that aimed to identify the source of the detected plasma HIV RNA analysed HIV RNA and HIV DNA sequences present before, during and after transient viremic episodes, and these results indicated that the release of virus originated from a pre-existing pool of HIV RNA rather than activation of silent proviruses (Imamichi et al. 2011).

A recent clinical study among 32 HIV-infected subjects found, as expected, that treatment with recombinant human IL-7 increased CD4+ T cells of predominantly naïve and central memory phenotype (Levy et al. 2012). Transient low-level viremia was seen in a minority of study subjects indicating at least some effect on HIV latency, but levels of total HIV DNA increased per mL blood though not per million CD4+ T cells. A similar conclusion was reached in the ERAMUNE-01 trial, which was designed to investigate whether IL-7 treatment added to intensified ART would impact on HIV persistence in HIV-infected individuals on ART. In this study, 29 HIV-infected individuals received ART intensification with raltegravir plus maraviroc added to their baseline ART regimen and were then randomised to intensification alone or an additional 3 weekly injections with IL-7 (Katlama et al. 2016). As seen previously, IL-7 treatment led to increases in CD4+ T cells, predominantly of a central memory phenotype, and also caused an increase in the level of HIV DNA measured in PBMCs. During follow-up, levels of HIV DNA remained elevated per mL blood but not per million PBMCs (Katlama et al. 2016). Collectively, these studies showed that although IL-7 treatment has some latency-reversing effect, which theoretically could have facilitated the elimination of virus-producing cells, the homeostatic effects of this cytokine induced proliferation of memory T cells including latently infected cells and actually expanded the pool of latently infected cells.

More recently, IL-15 and IL-15 superagonists have also been explored in cure-related research. In an in vitro model, both IL-15 and two IL-15 superagonists (and also IL-2) primed latently infected cells for CD8+ T cell recognition. Additionally,

therapeutically relevant concentrations of the IL-15 superagonist ALT-803 primed latently infected CD4+ T cells for CD8+ T cell recognition ex vivo (Jones et al. 2016). This compound is currently tested in a proof of principle clinical trial for its effect on HIV persistence in HIV-infected individuals on ART (estimated enrolment 10, NCT02191098).

12.10 Interferon-Alpha

IFN- α is essential for immunological control of HIV infection (e.g. by induction of antiretroviral restriction factors) (Abdel-Mohsen et al. 2014). Clinical studies have demonstrated that long-term treatment with exogenous IFN- α can lead to viral suppression and a decline in integrated HIV DNA (Azzoni et al. 2013), total HIV DNA (Sun et al. 2014) and CA-US HIV RNA (Moron-Lopez et al. 2016). In one trial, dosing of pegylated IFN-2 α in the absence of ART led to viremic control in 9 of 20 individuals as long as IFN-2 α was continued. The considerable biochemical and neurological side effects from pegylated IFN-2 α treatment make this strategy less appealing than standard combination ART, but the results are nevertheless intriguing because they suggest that IFN-2 α treatment can contribute to virological control in chronically infected patients.

12.11 Broadly Neutralising Antibodies

Unlike ART, bNAbs can engage the host immune system by virtue of their Fc effector domains and thereby accelerate clearance of cell-free virus (Lu et al. 2016), induce antibody-dependent cytotoxicity (ADCC) (Bruel et al. 2016) and/or cellular phagocytosis (ADCP) to kill infected envelope-expressing CD4+ T cells and also produce immune complexes that activate dendritic cells to become antigen-presenting cells generating potent CD8+ T cell responses (Nishimura et al. 2017). Some classes of bNAbs can prevent cell-cell HIV transmission, while it is unclear if some ARVs are able to block cell-cell virus transmission (Chun et al. 2014). Based on these known mechanisms, it has been proposed that bNAbs could play a role in achieving remission off ART by eliminating *env* expressing cells and boosting host immunity. So far, only one study investigated the effect of bNAbs on the size of the HIV reservoir in viremic participants and found no impact of the bNAb VRC01 on total and integrated HIV DNA in peripheral CD4+ T cells (Lynch et al. 2015). In individuals on long-term ART, the absence or very low expression of HIV *env* on infected cells may significantly impair the ability of bNAbs to clear these cells (Bruel et al. 2016). Thus, new trials are underway that will examine the effect of combining bNAbs with latency-reversing agents in an attempt to reduce the size of the latent reservoir (NCT02850016 and NCT03041012).

12.12 The Use of Analytical Treatment Interruptions in Cure Research

The evaluation of strategies to achieve long-term remission of HIV infection will require demonstration of efficacy through ATI studies (Li et al. 2015). However, there are concerns about stopping combination ART, including patient safety, selection of HIV drug resistance and increased risk for HIV transmission. Many of these concerns originate from the results of the SMART study in which participants were randomised to continuous or intermittent ART (El-Sadr et al. 2006). In the SMART study, participants on intermittent ART experienced significantly more serious non-AIDS adverse events than in the continuous ART arm. Subsequently, studies have found increased risk of a variety of non-AIDS defining conditions in individuals who are viremic for an extended period of time (e.g. >16 weeks) compared to fully suppressed individuals. However, short and closely monitored ATIs (e.g. <16 weeks) have generally been found to be safe, most likely because the time with virus-induced inflammation is relatively short (Rasmussen et al. 2014; Davey et al. 1999; Leth et al. 2016; Investigators et al. 2013).

Currently there are no biomarkers which can reliably predict time to viral rebound or posttreatment control, when ART is stopped (Li et al. 2015). The identification of reliable surrogate markers for time to viral rebound would greatly accelerate the HIV cure efforts, but the process of identifying and validating such biomarkers will require participants who are willing to volunteer to have their ART stopped for this effort. Until such biomarkers are identified and validated, well-controlled and tightly monitored ATIs are required to fully evaluate interventions with the ultimate aim of curing HIV infection or inducing sustained control of HIV replication in the absence of ART.

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