

CROI 2013: Basic Science Review

Mario Stevenson, PhD

The 20th Conference on Retroviruses and Opportunistic Infections was held in Atlanta, Georgia, and featured strong coverage in several basic science categories. Presentations on viral reservoir and cure research covered a variety of topics, including approaches to gauge viral reservoir size in patients on suppressive antiretroviral therapy, approaches to reactivate latently infected cells, and the role of residual replication in viral persistence under antiretroviral therapy. Research on viral restriction factors remains a strong feature of the conference, and presentations on the impact of viral restrictions on the establishment of viral reservoirs, as well as strategies that harness the antiviral potential of cellular restrictions, generated a lot of interest. Several studies on the nature of proviral latency left us with the sobering message that elimination of the latent viral pool is going to be an even greater challenge than previously suspected.

Keywords: HIV, pathogenesis, reservoirs, cellular restrictions, eradication

Research on Viral Reservoirs

A population of memory CD4+ T cells harboring latent proviruses is considered the single biggest obstacle to the eradication of HIV-1 from infected individuals. In virologic terms, latency is defined as the phase in which an infected cell is not manufacturing infectious virus but has the capacity to do so under appropriate conditions. The mechanism through which HIV-1 is held in latency is not fully understood. Studies derived predominately from experimental in vitro models of viral latency indicate that latency is dictated at the transcriptional level, and latent proviruses integrated within highly condensed regions of chromatin are relatively inaccessible to cellular transcription factors that promote proviral gene expression. Furthermore, in the quiescent host cell, low levels of host transcription factors create suboptimal conditions for viral gene expression.

Through in vitro studies, latency can be reactivated with agents (eg, valproic acid, vorinostat) that relax chromatin or agents (eg, prostratin, phorbol esters) that activate the nuclear factor (NF)- κ B pathway. Although agents that

activate the NF- κ B pathway have the most profound impact on viral gene expression, they are unlikely to have utility in reactivation of latency, because of the global impact on cellular gene expression. Therefore, researchers have focused their attention on agents that modify chromatin with the rationale that viral reactivation would lead to elimination of the infected cell either due to the cytopathic effects of the virus on the host cell or to elimination of the reactivated cell by immune clearance mechanisms of the host.

Approximately 1 in every 1 million resting CD4+ T cells obtained from HIV-1–infected individuals on suppressive antiretroviral therapy is believed to harbor latent HIV. This number is based primarily on in vitro end-point dilution experiments. However, the number of CD4+T cells harboring viral DNA exceeds this number by 100- to 1000-fold. It has generally been believed that the vast majority of proviruses is defective, and indeed, the majority of proviruses sequenced directly from clinical specimens harbors mutations and inactivating mutations that cripple the provirus or show extensive APOBEC (apolipoprotein B mRNA

editing enzyme, catalytic polypeptide-like)-mediated hypermutations. The fact that the vast majority of proviruses is defective was highlighted in Abstract 371.

Resting CD4+ T cells from patients on suppressive antiretroviral therapy were evaluated for the number of cells harboring provirus and the number that produced RNA following stimulation with CD3/CD28 antibodies or the histone deacetylase (HDAC) inhibitor vorinostat. The study determined that 3% of the proviruses could be induced to produce virions after CD3/CD28 stimulation and less than 1% could be induced following stimulation with vorinostat. Therefore, the vast majority of proviruses is defective rather than latent. These defective proviruses are commonly referred to as graveyard sequences. Although they have no role in the maintenance of infection, they complicate the interpretation of studies that use viral DNA surrogates to gauge the dynamics of the viral reservoirs.

Several presentations provided evidence that a number of silent proviruses are functional yet refractory to reactivation. This suggests that the size of the latent reservoir is considerably larger than estimates based on end-point dilution calculations. In the plenary presentation given by Siliciano (Abstract 16) and further expanded on in Abstract 43, patients' CD4+ T cells were subjected to limiting dilution culture and activated with phytohemagglutinin (PHA). Full-length proviruses were reconstituted from wells that were p24 positive and p24 negative. Approximately 10% of proviruses obtained from p24 negative wells were found to harbor intact open reading frames, and the majority was replication competent in culture. Therefore, functionally intact noninducible

proviruses exceed inducible proviruses by an order of magnitude. It is possible that some integration sites are refractory to even the most potent stimuli that reactivate viral latency. Abstract 375 looked at the nature of integration sites for latent and expressed proviruses. Inactive proviruses were found to be more commonly located in regions of DNA that had a low density of cellular genes and in regions of low transcriptional activity. Latent proviruses were most commonly associated with regions of cellular DNA devoid of recognized histone-effector modules.

In addition to the challenges involved in reactivation of latent proviruses, it is as yet unclear whether the level to which proviruses can be reactivated is sufficient to ultimately result in the clearance of the reactivated cell. Elimination would require viral reactivation to the extent that the cell succumbs to viral cytopathic effect or that expression of viral proteins and presentation of antigen on the infected cell surface leads to killing of infected cells by cytotoxic effector cells such as cytotoxic T lymphocytes (CTLs). Unfortunately, agents being used in “shock and kill” trials to eliminate the latent reservoir, such as vorinostat, exert a rather modest effect on viral gene expression *in vitro* and *in vivo*, raising the concern that this modest induction may not translate into sufficient priming of the host cell for CTL recognition.

Abstract 383 evaluated the ability of HIV-specific CTL clones established from an elite controller to recognize latently infected CD4+ T cells after stimulation with vorinostat *in vitro*. Treatment of infected cells with vorinostat alone did not lead to reductions in p24, whereas addition of cytokines such as interleukin (IL)-2 and IL-15 resulted in recognition of infected target cells by HIV-specific CTLs. Therefore, strategies employing cytokine augmentation of CTL function may be more effective in clearing latently infected cells following their reactivation.

The 19th Conference on Retroviruses and Opportunistic Infections (CROI 2012) featured presentations documenting the first use of vorinostat in patients aimed at reducing the size of

the latent reservoir.¹ Progress of a similarly designed study (Abstract 50LB) was presented at CROI 2013. The aims of the study were to determine the safety and tolerability of numerous doses of vorinostat in HIV-1-infected individuals on suppressive antiretroviral therapy. Patients received vorinostat once daily for 14 days. Numerous doses of vorinostat were well tolerated. Grade 1 or 2 adverse events such as nausea, diarrhea, and fatigue were observed in 90% of patients. Unspliced viral RNA and viral DNA levels were determined in CD4+ T cells from blood and rectal tissue. There was an approximately 3-fold increase in cell-associated RNA during the vorinostat dosing that remained elevated when vorinostat was discontinued. There were no substantial changes in viral DNA levels. Thus, although vorinostat appears to be well tolerated, no discernible impact on the viral reservoir was observed, at least based on measures of total viral DNA. However, the study cannot rule out effects of vorinostat on a subpopulation of latently infected cells. The investigators’ analysis focused on total viral DNA, which would measure both functional and defective proviruses. However, the majority of proviruses determined by total DNA measures is defective and insensitive to agents that reactivate viral latency. As such, an impact on a subpopulation of latently infected cells would go unnoticed.

It is likely that efficient purging of latently infected cells will require treatment with agents that would potentially activate viral gene expression. Efforts to identify small molecules that can reactivate viral latency were highlighted by Hazuda during her delivery of the 18th Bernard Fields lecture. Importantly, these screening efforts are being validated with primary cell models of HIV-1 latency. As such, agents that emerge from these screens will be more likely to induce viral reactivation in latently infected CD4+ T cells *in vivo*.

Although antiretroviral therapy can sustain suppression of plasma viremia to below detectable levels, treatment interruption leads to rapid viral recrudescence. The persistent nature of HIV-1

infection is believed predominantly to be a consequence of the intrinsic stability of latently infected CD4+ T cells. However, several lines of experimental evidence support the view that antiretroviral therapy is not fully suppressive, and as a result, there is a low degree of residual replication in some patients on suppressive antiretroviral therapy. It is also possible that this residual replication can contribute to maintenance of viral reservoirs through replenishment. Treatment intensification studies have attempted to broach the issue of residual infection under antiretroviral therapy. If there were residual replication in patients on suppressive antiretroviral therapy, increasing the level of suppression would be predicted to perturb some viral parameter. Unfortunately, the majority of treatment intensification studies has employed different agents, different study designs (eg, treatment intensification interval), and different surrogates with which to gauge the impact of intensification on the viral reservoir. As a result, no clear picture has emerged from treatment intensification studies as to whether there is residual replication or whether intensification further impacts the viral reservoir.

Hatano and colleagues (Abstract 42) examined the impact of raltegravir intensification in patients on suppressive antiretroviral therapy. The study was similar in design to a study previously published by Buzon and colleagues.² Both studies examined the impact of raltegravir intensification on the frequency of 2-long terminal repeat (2-LTR) circles. During infection of the cell, a linear viral cDNA is generated by reverse transcription. The linear genome then integrates within chromatin to form the provirus. In the presence of raltegravir, integration and formation of the provirus is blocked. As a result, the linear precursor to the integrated provirus is converted to circular forms of viral cDNA harboring either 1 or 2 copies of the LTR. Therefore, if raltegravir is applied in cells that are supporting *de novo* infection, there is a specific increase in the abundance of 2-LTR forms. These forms can then be quantitated using

polymerase chain reaction (PCR) and primers that span the unique circle junction created when the LTRs ligate end to end.

Abstract 42 presented results obtained with 31 subjects on suppressive antiretroviral therapy who were aviremic for at least 1 year. 2-LTR circles were analyzed at weeks 0, 1, 2, and 8 by droplet digital PCR (ddPCR). In contrast to real-time PCR, ddPCR does not require comparison with copy number standards. This facilitates quantitation of target molecules in samples obtained at different intervals and also facilitates comparison of data obtained from different laboratories. The impact of raltegravir intensification on 2-LTR circle dynamics was remarkably similar between the 2 studies. There was a rapid and transient increase in 2-LTR circle frequency by 2 weeks postintensification and a subsequent decline to baseline. The Buzon study² observed an increase in 2-LTR circles in 30% of raltegravir-intensified patients, whereas the Hatano study (Abstract 42) reported an increase in 2-LTR circles in over half of the subjects undergoing raltegravir intensification.

Two conditions must be satisfied in order for raltegravir to induce increases in frequency of 2-LTR circles. First, there must be infectious virions in order to initiate an infection event. Second, there must be active reverse transcription in order to generate the linear cDNA that is the substrate for 2-LTR circles. Therefore, an increase in 2-LTR circle formation following raltegravir intensification can only occur if there is *de novo* infection. This demonstrates that even in the presence of antiretroviral regimens comprising inhibitors of protease and reverse transcription, *de novo* infection persisted in a substantial fraction of individuals on suppressive antiretroviral therapy. It is tempting to speculate that residual infection is occurring at tissue locations that are inefficiently accessed by certain classes of antiretroviral drugs and that raltegravir more readily accesses those sites to impact residual infection.

Although the raltegravir intensification studies provide direct evidence for

de novo infection in patients on suppressive antiretroviral therapy, they fall short of being able to prove that there is ongoing replication in these patients. In the virologic definition of ongoing replication, productively infected cells release virions that go on to initiate productive infection of neighboring cells. Ongoing replication would be expected to result in sequence evolution. However, there is little evidence for viral sequence evolution in patients on suppressive antiretroviral therapy. It is important to point out that because of sampling limitations, low levels of sequence evolution could go unnoticed. With the information at hand, the best way to reconcile these results is to invoke a model in which *de novo* infection under suppressive antiretroviral therapy is a result of limited rounds of infection that is perhaps driven by a chronic virus source. In this scenario, the chronically infected cell releases virions that infect neighboring cells, but those cells do not infect other cells.

Abstract 173LB presented intriguing evidence for a chronic viral source in patients on suppressive antiretroviral therapy. In the study, gut-associated lymphoid tissue (GALT) was obtained from patients on suppressive antiretroviral therapy who interrupted their therapy. GALT was analyzed by *in situ* hybridization to identify the source of viral recrudescence when treatment is interrupted. In 3 subjects, foci of viral RNA-positive cells emerged simultaneously at numerous anatomic sites and shortly after treatment was interrupted. This simultaneous and focal appearance of viral RNA-expressing cells following treatment interruption was unexpected, as current models indicate that HIV-1 recrudescence originates from a clonal viral source that fuels exponential viral growth. Although it is possible that the emergence of viral RNA-expressing cells was the result of latent cell reactivation, it is difficult to envision simultaneous reactivation of latently infected cells at numerous anatomic sites. Alternatively, viral recrudescence may originate from cells that are productively infected yet are held in check by suppressive antiretroviral therapy.

Although additional studies are required to determine whether residual infection in antiretroviral therapy-suppressed patients is a result of ongoing replication or limited rounds of infection, the Buzon² and Hatano (Abstract 42) studies provide some indication that *de novo* infection under antiretroviral therapy may be clinically significant. The SMART (Strategies for Management of Antiretroviral Therapy) study³ demonstrated that D-dimer levels were associated with increased risk of cardiovascular disease and mortality in HIV-1-infected individuals and that increased D-dimer was independently associated with venous thromboembolic events. Data presented by the Hatano group (Abstract 42) indicated that raltegravir intensification resulted in a substantial decrease in D-dimer levels. Although the Buzon study² did not evaluate the impact of raltegravir intensification on markers of cardiovascular comorbidities, raltegravir intensification led to a reduction in the frequency of activated CD8+ T cells by 24 weeks postintensification. A number of studies indicate that high levels of CD8+ T cell activation in HIV-1 infection are associated with poorer prognosis and that higher CD8+ T cell activation is associated with lower treatment-mediated CD4+ T cell gains.⁴ Going forward, it will be important to determine whether raltegravir intensification over the long term has an impact on the size of the persistent viral reservoirs. This will determine whether residual infection sustains the reservoirs that persist in the face of antiretroviral therapy.

HIV-1 Molecular Virology and Immunopathogenesis

Primate lentiviruses encode a set of accessory proteins whose functions are to oppose the antiviral activities of cellular restrictions. The viral infectivity factor (Vif) protein counteracts the APOBEC 3 cytidine deaminases; the viral protein unique (Vpu) protein of HIV-1 and negative regulatory factor (Nef) proteins of HIV-2/simian immunodeficiency virus (SIV) counteract tetherin; and the viral protein X (Vpx)

proteins of HIV-2/SIV counteract the antiviral activities of SAMHD1 (sterile alpha motif [SAM] domain and HD domain-containing protein 1), which is the most recently identified cellular restriction. SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase that regulates the level of the deoxynucleotides in the cell. By reducing cellular deoxynucleotides, SAMHD1 creates conditions that are inefficient for reverse transcription of viral cDNA. Primate lentiviruses have therefore evolved vpx to antagonize the activity of SAMHD1. The Vpx protein promotes proteosomal degradation of SAMHD1, thereby raising cellular deoxynucleotide levels, which in turn allows efficient reverse transcription of viral cDNA following infection of the cell. Since the discovery of SAMHD1, it has generally been believed that this protein is a myeloid cell-specific restriction that regulates infection of macrophage and dendritic cells.

Abstract 109 presented evidence that SAMHD1 can restrict HIV-1 infection of resting CD4+ T cells. To counteract SAMHD1, the Vpx protein must be packaged with virus particles. When Vpx was packaged within HIV-1, or if intracellular nucleotide levels were elevated by addition of exogenous deoxynucleotides, HIV-1 efficiently infected resting CD4+ T cells. Furthermore, individuals with Aicardi-Goutières syndrome contain an inactivating mutation in SAMHD1; resting cells from these individuals were permissive to HIV-1 infection. Although neutralization of SAMHD1 improved conditions for resting cell infection, it did not remove a block to virion production, indicating the existence of additional barriers to productive infection in resting T cells. Several predictions extend from this line of investigation. HIV-2 and SIV encode Vpx and are thus able to neutralize SAMHD1. Therefore, one would predict that the bottleneck to resting cell infection imposed upon HIV-1 is absent in HIV-2 and SIV. And, therefore, one would expect a much larger resting cell and myeloid cell reservoir in HIV-2-infected humans and SIV-infected

monkeys. The overarching question as to why HIV-1 has not evolved the ability to neutralize SAMHD1 remains unaddressed.

Tetherin (also known as BST2/CD317) is a host cell restriction that manifests its antiviral activity by inhibiting the release of virus particles from the surface of the infected cell. The Vpu and Nef proteins of primate lentiviruses neutralize the antiviral activities of tetherin, to promote the release of virions from the cell surface. There is some evidence that the antiviral effect of tetherin is minimal during cell-to-cell transfer of viral particles at the virologic synapse. Abstract 110 presented evidence that tetherin exhibits antiviral activities that extend beyond the physical retention of viral particles at the cell surface. Evidence was presented that human tetherin has the capacity to recruit and signal through a complex that includes TRAF 6 (tumor necrosis factor [TNF] receptor-associated factor 6) and TAK1 (transforming growth factor- β [TGF- β]-activated kinase 1) and that induces NF- κ B activation. This activation appears to occur in concert with virion retention. HIV-1 Vpu mutants that were unable to counteract tetherin induced an inflammatory cytokine response in primary CD4+ T cells. This has implications for antiviral strategies aimed at counteracting Vpu, as inhibition of Vpu would lead to tethering of viral particles to the cell surface and activation of an inflammatory cytokine cascade that could be deleterious to the host.

Dendritic cells internalize HIV-1 particles through a nonfusogenic mechanism and transmit virions to bystander CD4+ T cells by a process known as *trans*-infection. Early studies identified a C-type lectin known as DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) as the capture receptor for viral particles on dendritic cells. Abstract 134 presented evidence that HIV-1 capture and transmission to T cells is mediated by a sialic acid-binding lectin called SIGLEC-1 (sialic acid-binding immunoglobulin-like lectin 1; also known as CD169). SIGLEC-1 was

upregulated in lipopolysaccharide-matured dendritic cells that are highly capable of mediating *trans*-infection. SIGLEC-1 captured HIV-1 particles through interaction with sialyllactose-containing gangliosides. *Trans*-infection could be blocked with antibodies to SIGLEC-1, and silencing of SIGLEC-1 by RNA interference blocked viral capture and *trans*-infection. Therefore, SIGLEC-1 appears to be a key myeloid cell receptor that mediates HIV-1 *trans*-infection by dendritic cells.⁵ The identification of SIGLEC-1 as the primary receptor for HIV-1 *trans*-infection by dendritic cells was reinforced by data presented in Abstract 107. These studies have substantial implications for the design of agents that prevent dendritic cell-mediated transmission at mucosal surfaces and in lymphoid tissue.

Pathogenic HIV and SIV infection are associated with increased permeability of the gastrointestinal (GI) tract. Increased GI tract permeability permits translocation of microbial products that drive inflammation of lymphoid tissue and exacerbate conditions for viral replication. Morbidity and mortality in HIV-1-infected individuals on suppressive antiretroviral therapy is associated with elevated levels of microbial translocation and immune activation. Although antiretroviral therapy improves integrity of the GI tract, it does not completely restore it. Investigators have begun to turn their attention to the composition of the enteric microbiome. Research has revealed a synergistic relationship between the gut microbiota and mucosal responses to viral pathogens. Abstract 53 examined microbial translocation and GI tract immunology in SIV-infected macaques undergoing antiretroviral therapy with and without probiotics and prebiotics. Symbiotic treatment in conjunction with antiretroviral therapy enhanced CD4+ T cell reconstitution and reduced fibrosis of lymphoid follicles. Abstract 54 examined the enteric virome in pathogenic SIV infection. Thirty-two previously undescribed enteric viruses were identified in macaques undergoing pathogenic SIV infection that were not observed in nonpathogenic SIV infection. Therefore, undiagnosed

enteric viral infections may contribute to mucosal damage and impaired immune response to viral antigens during pathogenic infection. A highly tractable model with which to examine the interplay of the gut microbiota and GI immune function was described in Abstract 52. The study utilized an intestinal loop model that allows examination of early mucosal responses to probiotic and pathogenic bacteria in the GI tract. This model will direct the design of strategies aimed at establishing a healthy microbiome and that restore the integrity and immune function of the GI tract. 

Financial Affiliations: Dr Stevenson has served as a consultant for Merck.

A list of all cited abstracts appears on pages 90-95 and is available online at www.iasusa.com.

Additional References

1. Archin NM, Liberty AL, Kashuba AD, et al. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature*. 2012;487(7408):482-485.
2. Buzon MJ, Massanella M, Llibre JM, et al. HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. *Nat Med*. 2010;16(4):460-465.
3. Kuller LH, Tracy R, Belloso W, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med*. 2008;5(10):e203.
4. Hunt PW, Martin JN, Sinclair E, et al. T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. *J Infect Dis*. 2003;187(10):1534-1543.
5. Izquierdo-Useros N, Lorizate M, Puentes MC, et al. Siglec-1 is a novel dendritic cell receptor that mediates HIV-1 trans-infection through recognition of viral membrane gangliosides. *PLoS Biol*. 2012;10(12):e1001448.

Top Antivir Med. 2013;21(2): 42-46
©2013, IAS–USA

Correction

There was an error in the figures accompanying the article “Update of the Drug Resistance Mutations in HIV-1: March 2013” published in Volume 21, Issue 1, of *Topics in Antiviral Medicine*. On page 9, one of the elvitegravir-associated resistance mutations at position 92 was listed incorrectly. The correct designation is E92Q/G not E92Q/C.

The figures and downloadable slides posted on our website (www.iasusa.org) show the correct designation, but the error remains in the printed journal copies and in the folded pocket cards inserted with the issue. Corrected pocket cards are available on request through our website, where updates are posted as they become available. We regret any inconvenience this error has caused.