

*Invited Review***CROI 2023: Summary of Basic Science Research in HIV****Mario Stevenson, PhD**

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Abstract. *The 2023 Conference on Retroviruses and Opportunistic Infections (CROI) represented the first fully in-person conference since the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic began. CROI continues as the premier conference in which delegates can appraise themselves of almost every facet of HIV/AIDS research as well as emerging and re-emerging pathogens such as SARS-CoV-2 and mpox. The return to an in-person format is particularly important for early-stage investigators, who were faced with challenges of advancing their independent research careers during the SARS-CoV-2 pandemic. The personnel interactions and face-to-face meetings between junior investigators and their peers enable collaboration that is important in the academic development process. A very packed program showcased research advances in basic research, clinical, and epidemiology/public health endeavors around HIV and other pandemic viruses. Session presentation summaries, themed discussion sessions, and scientific workshops condense and assimilate specific areas of research that are particularly useful for delegates who want to see the state of research in areas that may be outside their specific areas of interest. The conference organizers drew on more than 1000 accepted abstracts to assemble a dynamic and engaging program that was appealing to infectious disease researchers worldwide.*

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Virology

In his presentation in the Scott M. Hammer New Investigator workshop, Neil reviewed replication and immune escape strategies of HIV-1 and SARS-CoV-2. Much of the detailed understanding of HIV-1 infection has helped guide investigations into the inner workings of SARS-CoV-2. Both viruses are driven by competing selection pressures, such as those provided by host immune responses and target cell availability, and those pressures drive evolution in HIV-1 envelope glycoprotein and SARS-CoV-2 spike protein. Advances in imaging methods such as single-particle, cryo-electron microscopy (cryo-EM) have allowed visualization of the HIV-1 receptor binding and conformational changes including trimer opening at remarkable levels of resolution (3.7 Å and 3.9 Å).

Work from several groups reveals the HIV envelope glycoprotein binding 3 CD4 proteins in rapid succession that induces conformational changes in the envelope, which exposes coreceptor binding sites. This CD4-induced conformational change in envelope opens new avenues for the development of therapeutics that prevent viral infection. One can see the impact of envelope evolution by following changes in the envelope sequence that occur in a single infected individual over the course of several years. Most of the changes lead to escape from neutralizing antibody and CD8+ T-cell responses. Some changes lead to increased CD4 binding affinity that typically can increase tropism for macrophages as well as changes in coreceptor use.

Similar forces appear at play in the evolution of the spike protein of SARS-CoV-2 and in the emergence of new variants of concern. Most of the changes over time erode the impact of neutralizing antibodies levied against the spike protein. This drives changes in spike protein conformation and alteration of viral biology. These themes were expanded on in presentations by Bieniasz (Abstract 18) and Hodcroft (Abstract 19). Neil reinforced the notion that changes occurring in the SARS-CoV-2 spike protein are not simply due to escape from host humoral immune responses, but change the

basic biology of the spike protein itself. For example, the spike protein of some variants has a more open conformation, but that of the Omicron variant has a more closed conformation, which reduces dependence on the TMPRSS2 protease and is more dependent on endosomal entry than the original Wuhan strain of the virus. This also affects the sensitivity of the virus to antiviral membrane proteins such as interferon-induced transmembrane proteins (IFITMs) that have been shown to inhibit viral infection of diverse viruses such as Ebola virus, influenza A virus, and West Nile virus in different subcellular compartments, and guanylate binding proteins (GBPs) that mediate a broad spectrum of innate immune functions against viruses. Some of these changes are suspected to change the tropism of SARS-CoV-2 for epithelial cells in the upper respiratory tract

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that could potentially alter viral pathogenicity. Numerous lines of evidence suggest that HIV-1 has evolved mechanisms to limit pattern-recognition immune responses as well as to evade type I interferons that are triggered by this pattern-recognition response. This also appears to hold true for SARS-CoV-2 evolution outside of the SARS-CoV-2 spike protein (particularly in ORF6, ORF9, and N) in that some changes increase the level of expression of these viral accessory proteins that antagonize the innate antiviral forces of the cell (see also Abstract 108). These presentations collectively highlight the continuous evolution and adaptation of viruses that although driven primarily by escape from humoral and cell-mediated immune responses, also alter the biology of the virus to achieve greater fitness in the face of a hostile host environment.

For HIV, the molecular steps that immediately follow fusion of the viral membrane with the host cell membrane and precede integration of viral cDNA within host cell DNA remain the least well understood events in the viral replication cycle. This enigmatic phase of replication, collectively referred to as the preintegration

steps of replication, is made of a number of successive events, some that distinguish the basic biology of lentiviruses from animal retroviruses. Once the virus has engaged receptor and coreceptor molecules, the core of the virion, which is made up of a capsid lattice containing genomic viral RNA and viral enzymes (eg, reverse transcriptase, integrase) that catalyze the cDNA synthesis and integration steps, respectively, is released into the cytoplasm of the target cell. Through an as yet poorly understood process, viral nucleic acids are reverse transcribed and transported to the nucleus of the cell, where the integrase enzyme promotes integration of viral cDNA with host cell DNA. This all occurs within a subviral capsid lattice, also referred to as the reverse transcription complex or preintegration complex.

Since the viral integrase needs to remain in association with nascent viral cDNA so that it can catalyze integration of that DNA, the subviral lattice has a mass that would otherwise preclude it from accessing the nuclear compartment of a cell that is not in mitosis. Myeloid cells do not divide, yet are permissive to HIV infection. Therefore, HIV-1 and other lentiviruses have evolved a mechanism that permits access of the preintegration complex to the nucleus. This is a central characteristic that underscores the general ability of lentiviruses to transduce nondividing cells and this property has been exploited for the derivation of lentivirus vectors for transduction of nondividing targets.

As discussed in several presentations (Abstracts 102, 104, 215, 216, and 217), there appear to be characteristics of the core that play several roles in the molecular events involved in cDNA synthesis and nucleic acid transport. The capsid protein that forms the core lattice mediates interactions with cellular factors that help guide the complex through the cytoplasm to promote further interactions that aid in docking the preintegration complex to the nuclear pore. The question remains whether the entire core/preintegration complex structure passes through the core as is, or undergoes some rearrangement that facilitates nuclear transport.

It now appears that the process of reverse transcription is triggered when the core docks at the nuclear pore, as opposed to the conventional view that reverse transcription is initiated prior to or immediately after fusion and proceeds concurrently with core transport through the cell cytoplasm. This likely serves as a viral defense mechanism that helps the virus avoid sensing by the host cell of viral cDNA by cytoplasmic DNA sensors. Mutations within the capsid can interfere with this avoidance mechanism and post-entry steps in viral replication in primary cells.

A number of immune proteins that target the capsid core as it traverses the cytoplasm, such as tripartite motif (TRIM) containing 5 (TRIM5) and TRIM containing 34 (TRIM34), have been identified. Mx2/MxB is an interferon-inducible guanosine triphosphate (GTP)ase that localizes to the nuclear pore complex and that has antiviral activity against a wide variety of viruses including lentiviruses, herpesviruses, and flaviviruses. As discussed in Abstract 104, the N-terminal domain of Mx2, which also harbors the nuclear localization signal mediating nuclear pore localization of Mx2, interacts with HIV-1 capsid and inhibits nuclear import of HIV-1 preintegration complexes. One presentation (Abstract 100) provided mechanistic insight into the antiviral activity of lenacapavir, the first-in-class HIV-1 capsid inhibitor. Although it prevents assembly of the viral capsid lattice during production of virions in the producer cell, it also interrupts preintegration events in viral replication. Lenacapavir binds to the capsid lattice after it enters the cytoplasm and interferes with interactions between the capsid lattice and host cell proteins (such as NUP153, Sec24C, and CPSF6) that are required for postentry functions of the capsid lattice. Intriguingly, capsid lattices stabilized by lenacapavir were able to access the nucleus but underwent abortive infection. In addition to their intrinsic value in management of HIV-1,

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antiviral agents such as lenacapavir can be used as research tools to help shed greater light on the enigmatic, preintegration events in HIV-1 replication.

HIV-1 Persistence and Reservoir Studies

Analysis of the antiviral defenses levied against incoming viral genomes has provided provocative evidence that those defenses may play a key role in the establishment of viral latency. As highlighted in Abstract 37, antiviral defenses assault retroviruses not just as they traverse the cytoplasm, but also within the nucleus. It now appears that some of those antiviral defenses may aid in the establishment of latent HIV-1 infection.

During retroviral infection, some linear viral DNA molecules undergo end-to-end ligation and recombination to form 1- and 2-long terminal repeat (LTR) circles (viral episomes containing 1 or 2 copies of the LTR sequence). Some viruses, such as Epstein-Barr, can replicate from episomal DNA. However, viral gene expression from unintegrated DNA is extremely inefficient and viruses with mutations in integrase are also replication defective and accumulate repressive epigenetic marks, including trimethylation of lysine 9 on histone H3. Therefore, integration is a necessary step in the replication of HIV.

The processes that limit the expression of unintegrated viral DNA are not well understood. Using a genome-wide clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 knockout screen, the Cullen laboratory identified the host SMC5/6 as orchestrating epigenetic silencing of unintegrated HIV-1 DNA.¹ SMC5/6 was shown to bind to small ubiquitin-like modifier (SUMO)ylate-unintegrated chromatinized HIV-1 DNA. Remarkably, when SMC5/6 expression was blocked, unintegrated DNA directed efficient transcription and integration defective mutants were replication competent. Surprisingly, blocking SMC5/6 expression also prevented establishment of latent HIV-1 infection in CD4⁺ T cells in vitro. The investigators propose that latent infection is a predetermined phenomenon that depends on SUMOylation of unintegrated DNA by the SMC5/6 complex.

This observation has important implications and suggests that HIV-1 latency is not merely a consequence of the activation state of the host cell or the region of chromatin that harbors the provirus, but rather, is an unfortunate side effect of a cellular innate immune response that originally was meant to silence foreign DNA. The current view is that latency is dictated by the activation state of the host cell. In activated cells, there are abundant cellular transcription factors that create an optimal environment for viral transcription, but in quiescent cells, these factors are rate limiting and therefore promote conditions for latency. Furthermore, studies suggest that proviruses integrated within non-genic regions of host cell chromatin are more likely to exist in a latent state and undergo selection over time, perhaps because they are less likely to be transcriptionally active and thus, less likely to be detected by host immune clearance forces. If indeed, latency is driven by epigenetic modification of viral cDNA prior to its integration, this could point to new strategies to limit the establishment of latency or to reverse latency.

The path to a cure for HIV-1 infection will be facilitated by a deeper understanding of the nature of the

viral reservoir and characteristics of viral reservoir cells that might help inform on strategies to eliminate those reservoirs. As highlighted in Siliciano's presentation (Abstract 26) a concerted research effort has revealed some central features of the CD4+ T cell reservoir. The reservoir appears to be established early after infection and establishment of the latent reservoir is perhaps facilitated by initiation of antiretroviral therapy (ART). The reservoir comprises proviruses in memory CD4+ T cells that are expanded through the process of homeostatic proliferation. The intrinsic stability of the latent proviral reservoir in memory CD4+ T cells is a consequence of the longevity of memory CD4+ T cells. So far, host cell signatures that might distinguish a latently infected cell from an uninfected cell have not been identified. These basic characteristics of the memory CD4+ T-cell reservoir enforce the notion that elimination of this reservoir will be a formidable challenge. Researchers are now zeroing in on characteristics of the CD4+ T-cell reservoir at the single-cell level to better understand what might facilitate survival of the reservoir cell as well as host factors that might be differentially expressed in that cell and that could be exploited for therapeutic intervention.

Several excellent presentations highlighted and summarized advances in approaches to viral reservoir analysis that help to give a more detailed picture of the characteristics of individual reservoir cells (Abstracts 2, 3, 4, 135, and 142). A presentation by Roan (Abstract 2) overviewed the application of tools such as cytometry by time of flight (CyTOF) and single-cell RNA sequencing (scRNAseq) to the analysis of reservoir cells at the

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single-cell level. CyTOF uses mass cytometry to simultaneously quantitate various labeled proteins on the surface and the interior of individual cells. scRNAseq involves sequencing of cDNA libraries that were prepared from individual cells. Combined, these methods reveal the transcriptomic and proteomic content of individual

cells in blood and in tissues. A limitation to these approaches is that they must look at many individual cells to find ones that are infected. Roan first discussed how these methods can reveal changes that occur in infected cells using tonsillar CD4+ T cells infected with an indicator virus. She illustrated how, on infection, CD4+ T cells exhibit characteristics not shown by uninfected cells—referred to as virus-induced remodeling. This information was then extrapolated to predict which subsets of T cells are most susceptible to infection. By comparing genes expressed in the infected cells, Roan was able to predict the nature of the original target cell, known as predicted precursor cells. This analysis was also applied to identify the HIV-susceptible cell subsets in the female reproductive tract.

Prior studies from the same laboratory indicated that genital tract T cells are more susceptible to HIV infection than their counterparts in blood. Those cells were identified as memory CD4+ T cells. Naive CD4+ T cells were spared from HIV-1 infection. Within the memory T-cell population, T-effector memory and central T-resident memory cells were preferentially infected, and T-central memory cells were preferentially spared from infection. Roan was able to extend this by examining the protein signature of productively infected cells to identify the proteins that might be remodeled by infection. This analysis revealed that HIV infection downregulates T-cell receptor (TCR) signaling apparatus and promotes expression of factors such as surviving that promote T-cell survival and homing. This paints a more detailed picture of an infected cell and indicates that HIV-1 infection of the cell dampens the ability of the cell to respond to adaptive immune responses mediated through TCR signaling while simultaneously remodeling promoting their ability to survive and disseminate infection by inducing their migration to draining lymph nodes and retention in lymph node follicles.

Roan's laboratory has more recently extended the analysis to assess the glycan content of infected cells. This approach involves labeling of glycans with tagged lectins, an approach termed CyTOF-Lec. Through this modification, Roan was able to ask whether HIV-1 preferentially infects cells with specific glycan profiles and whether those profiles were remodeled after infection. HIV-1 was found to preferentially infect cells expressing high levels of fructose and sialic acid and further upregulated those glycans upon infection. Interestingly, the expression levels of sialic acid appeared to discriminate among a population of similar cells for susceptibility to infection. Sialic acid also plays a role in promoting evasion from natural killer (NK)-mediated recognition. Therefore,

HIV appears to select for cell subsets that might be able to provide refuge from NK-mediated killing.

These *in vitro* studies are now being extended to examine characteristics of reservoir cells from ART-suppressed individuals with HIV. The challenge to this analysis is that there is no marker that would aid in selecting the reservoir cells for analysis. Prior studies have circumvented this by *ex vivo* reactivation of reservoir cell, which in itself, would change the phenotype of the reservoir cell. Roan addressed this through the same approach used to predict the original transcriptomic and proteomic content of a cell infected *in vitro*. First, an atlas of all memory T-cell types is assembled. The information from a reactivated, infected cell is then matched against the atlas to reveal the identity of the original nonreactivated cell—the predicted phenotype. This analysis revealed the identity of several markers on memory T cells that could enable enrichment of reservoir cells from infected individuals followed by high-dimensional single-cell analysis that would not have been possible with unenriched cells. This allowed defining some of the characteristics of nonreactivated reservoir cells. One of those characteristics was the presence of viral RNA transcripts that have previously been used to define a component of the reservoir as the “expressed” viral reservoir. Prior studies have indicated that levels of cell-associated viral RNA in CD4+ T cells from individuals on ART can predict the time to viral rebound if ART is interrupted, and that the origin of rebound viremia includes cells with expanded proviruses that harbored viral transcripts prior to ART interruption.^{2,3}

Abstract 106 discussed studies that attempted to assess the fraction of infected cells harboring viral RNA and whether there is a relationship between level of plasma viremia and the percentage of cells that harbor viral RNA. The frequencies of cells harboring viral RNA (unspliced, genomic RNA) were compared between untreated individuals with high levels of viremia, untreated individuals with low levels of viremia, and individuals on ART. Approximately 20% of infected cells from viremic and aviremic individuals were found to express viral RNA at any point in time. Noncontrollers were found to have a 20-fold higher frequency of viral RNA-containing cells than viremic controllers. There was also a direct correlation between number of viral RNA-containing cells and levels of plasma viremia. Surprisingly, however, the fraction of infected cells containing viral RNA was not associated with the level of plasma viremia. However, levels of viral RNA in single cells were correlated with plasma viremia and cells from viremic noncontrollers had higher levels of viral RNA per cell than those from viremic controllers.

Therefore, although viremic noncontrollers and controllers have similar frequencies of cells harboring viral RNA, viremic controllers have fewer infected cells, and as a consequence, fewer total cells expressing viral RNA. The investigators concluded that the natural control of HIV replication is not due to inhibition of proviral expression but to factors (such as cytotoxic T lymphocytes [CTLs], NK cells, and host factors) that limit viral replication from cells harboring expressed proviruses. Collectively, these analyses are providing deeper insight into reservoir characteristics. These approaches will hopefully address fundamental questions regarding the viral reservoir. For example, is the “expressed” viral reservoir distinct from the latent viral reservoir? What

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percentage of reservoir cells is transcriptionally active? If most reservoir cells are transcriptionally active, will they also express viral proteins, and if so, can this be exploited for viral reservoir clearance strategies?

Betts (Abstract 3) also overviewed single-cell approaches for viral reservoir characterization at the DNA level. The approaches use the HIV provirus as the tag for the reservoir because, by definition, any reservoir cell must harbor a copy of the viral genome. In addition, using the provirus as a tag circumvents the need to reactivate the infection and as such, the original identity of the reservoir cell can be maintained.

One of the most recent approaches to reservoir analysis at the DNA level is FINDseq, developed by the Boritz research group, which uses a viral DNA probe to sort low numbers of reservoir cells in batches of approximately 100 cells that then underwent transcriptomic analysis. That approach revealed that viral DNA-containing cells exhibited transcriptomic signatures consistent with antideath and antiproliferative characteristics to the infected cell. This analysis was not optimal since it was not at single-cell resolution and was limited to the transcriptomic content of the cell.

Some of these issues were addressed in Abstract 1435, which described an approach called PHEPseq that combines viral DNA profiling with cell surface protein characterization. The approach targets viral DNA with

primer-probe sets that identify intact versus defective (with deletions) provirus and allows identity of the cell to be determined from analysis of cell surface proteins. As such, the memory phenotype of the infected cell harboring intact, defective, and clonally expanded proviruses could be determined. Finally, Betts highlighted the utility of ASAPseq that detects viral DNA in genes in open chromatin and simultaneously uses oligo-tagged antibody to give information on the nature of the infected cell. The analysis showed that viral DNA was present in almost every CD4+ T-cell subset from individuals with HIV-1 infection on ART. As with data gleaned from other single-cell approaches, provirus-containing cells exhibited a trend toward expression of cell survival proteins. Taken together, these different approaches point toward a phenotype of a reservoir cell that has a propensity for survival and activation, a feature that favors reservoir longevity and renewal of viral replication when conditions for that replication are favorable.

Ho (Abstract 4) assessed approaches being adopted in her laboratory to answer basic questions on reservoir characteristics such as features of the reservoir cell that enable viral persistence in the face of viral cytopathicity and immune clearance. Ho uses an approach (ECCITE-seq) that allows analysis of transcriptomic profiles of infected cells together with their memory phenotype and their T-cell clone size. This method was applied to individuals at acute viremia and after 1 year of ART. Over this interval, the most clonally expanded CD4+ T cells exhibited a cytotoxic CD4+ cell phenotype expressing granzyme A and B and perforin. In addition, T-cell clones with the same antigen and immune program also contained expanded proviruses indicating that antigen responsiveness drives clonal proviral expansion. HIV-positive granzyme B-positive clones upregulated SERPINB9, which inhibits granzyme B. This supports a model in which the infected cell protects itself from self-inflicted granzyme B killing, again enforcing the notion that HIV infection remodels the infected cell for survival and self-protection. Although these characteristics appear representative of cells within the “expressed” viral reservoir, it is unclear whether those same characteristics are exhibited by reservoir cells that are not expressing HIV. Regardless, these studies provide a baseline for future studies to zero in on cell surface markers that could aid in the specific identification (and removal) of reservoir cells. 

All cited abstracts appear in the virtual CROI 2023 Abstract eBook, available online at www.CROIconference.org

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Financial relationships with ineligible companies within the past 24 months: Dr Stevenson reported no relevant financial affiliations. (Updated May 11, 2023)

Reviewer 1 reported consultant or received advisor fees from Antiva, Assembly Biosciences, Generate Biomedicines, and IGM Biosciences, and fees for participation in review activities, eg, data monitoring boards, statistical analysis, or endpoint adjudication committees with Gilead Sciences, Inc. (Updated June 30, 2023) Reviewers 2 and 3 reported no relevant financial relationships with ineligible companies. (Updated April 23, 2023)

All relevant financial relationships with ineligible companies have been mitigated.

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