

Tracking the culprit: HIV-1 evolution and immune selection revealed by single-genome amplification

Zabrina L. Brumme and Bruce D. Walker

Early control of HIV-1 infection is determined by a balance between the host immune response and the ability of the virus to escape this response. Studies using single-genome amplification now reveal new details about the kinetics and specificity of the CD8⁺ T cell response and the evolution of the virus during early HIV infection.

The extraordinary genetic diversity of HIV-1 remains one of the most fundamental challenges to AIDS vaccine design. Globally, the diversity among HIV-1 subtypes may exceed 35% in the viral envelope sequence (1). Within an infected individual, quasispecies diversity arises as a result of host and other selective pressures and can surpass the extent of diversity in influenza viruses during an outbreak (1). When HIV is transmitted from person to person, however, a dramatic evolutionary bottleneck occurs, with ~80% of heterosexual infections apparently initiated by a single variant (2). After transmission, mutational escape and reversion rapidly shape HIV evolution (3, 4). These effects are so dramatic, in fact, that they are detectable at the population level (5–10). The existing global HIV-1 diversity, therefore, has arisen as a result of the >50 million cumulative infections that have occurred since the genesis of the epidemic (11), via continual cycles of infection bottlenecks followed by intrahost viral evolution.

Determining the exact kinetics and dynamics of the duel between host and virus in the early stages after infection has been a challenge, in part because of the difficulty of identifying very early cases of HIV transmission and the uncertainty in pinpointing the specific viral

sequence responsible for establishing infection. CD8⁺ T cells have long been thought to be instrumental in the initial decline in plasma viremia (12, 13), but a precise definition of the earliest adaptive antiviral responses remains elusive in part because most immunological studies have used reference or consensus rather than autologous virus reagents. Although methods to identify transmitted HIV *env* sequences have recently been developed (14), to date these have not been used to define the entire sequence of the transmitted founder virus or to comprehensively define the earliest immune responses to the infecting strain.

In this issue, papers by Salazar-Gonzalez et al. (on p. 1273) (15) and Goonetilleke et al. (on p. 1253) (16) help address these fundamental knowledge gaps through the comprehensive virologic and immunological assessment of individuals with acute HIV infection. In a sense, they have performed the ideal experiment. By identifying persons before seroconversion, pinpointing the transmitted virus, and assessing immune responses to that particular variant as it evolves, they provide a novel view of host and viral dynamics during the earliest stages of infection.

Revelations of early infection

The studies by Salazar-Gonzalez et al. (15) and Goonetilleke et al. (16) both used an optimized version of the single-genome amplification (SGA) technique originally described by Palmer

et al. (17, 18) to determine the full-length transmitted virus sequence and to characterize early intrahost viral evolution. This technique has recently become the gold standard for the characterization of the transmitted founder virus. SGA involves extraction of HIV RNA from plasma, followed by its full-length *in vitro* reverse transcription into cDNA. The cDNA is then endpoint-diluted such that <20% of reactions yield an amplicon by nested polymerase chain reaction (PCR), which is then sequenced directly. Although SGA is considerably more costly and labor-intensive than traditional molecular cloning approaches,

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it eliminates many of the confounding effects that previously complicated the identification of the transmitted virus, including *in vitro* PCR recombination, *Taq*-induced nucleotide misincorporation, PCR template sampling bias, and cloning errors.

Using this SGA technique, Salazar-Gonzalez et al. (15) extend their earlier studies of HIV-1 envelope (14) to reconstruct the full-length founder virus

Z.L. Brumme and B.D. Walker are at the Ragon Institute of MGH, MIT and Harvard, Charlestown MA 02129; Z.L. Brumme is at the Faculty of Health Sciences, Simon Fraser University, Burnaby BC V5A 1S6, Canada.

CORRESPONDENCE

B.D.W.: bwalker@partners.org

sequence in 12 acutely infected individuals identified before full antibody seroconversion. The use of SGA allowed the unambiguous identification of every nucleotide in >95% of the sequenced genomes, and full reconstruction of all founder viruses, including one case of dual-variant transmission (15). The founder sequences encoded complete open reading frames for all genes. All of the resulting virions were replication competent and CCR5 tropic. However, despite the CCR5 tropism, the founder viruses were unable to replicate in autologous monocyte-derived macrophages, suggesting that HIV-1 replication in macrophages does not contribute substantially to virus production in the early stage of infection.

In the companion paper, Goonetilleke et al. (16) define longitudinal proteome-wide T cell responses in three individuals using synthetic overlapping 18-mer peptides matched to the transmitted virus and genetic variants arising within each individual, an approach that has traditionally been precluded by cost and sample availability. Comprehensive analysis across all expressed viral proteins revealed that immune escape occurs as early as 25–32 d after estimated infection. This is a few days earlier than previously reported in humans (3), but is consistent with studies in nonhuman primate models of SIV infection (19). These new data demonstrate that some of the earliest HIV-specific CTL responses are directed against previously uncharacterized epitopes (16). This finding may otherwise have been overlooked if individuals had not been recruited so early, and/or if consensus or optimal peptides had been used to screen for CD8⁺ T cell responses.

The study by Goonetilleke et al. also provides a novel perspective on the contribution of CTLs to acute-phase viremia decline. Previously, this contribution has been inferred based on temporal correlations (12, 13), CD8⁺ T cell depletion studies in nonhuman primates (20, 21), and epidemiological data (22). Using mathematical modeling, Goonetilleke et al. (16) suggest that CTLs play a causal role in the resolution of acute phase viremia. This observation, which will require confirmation in larger studies of

individuals with diverse outcomes, provides further support that CTLs are important in establishing the viral set point.

The techniques used in these studies allowed the authors to show that the well-characterized “immunodominant” CD8⁺ T cell epitopes in HIV-1 (23) may not be the earliest targets of the acute phase CTL response in all individuals. Instead, some of the earliest targets include novel epitopes not currently featured in HIV immunology and sequence databases (<http://www.hiv.lanl.gov>) and thus would not have been evaluated in previous studies, which identified B*57-TW10 among the earliest known escaping epitopes in studies of large cohorts (24, 25). Goonetilleke et al. argue that the decline in peak viremia is driven in part by the recognition (and subsequent escape) of these novel epitopes. The classical immunodominant responses, on the other hand, seem to arise later and

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may instead be instrumental in maintaining viral set point (16).

The combination of virus sequence data and early CD8⁺ T cell responses also provides important insights into immune escape. Recently, HLA-restricted CTL escape mutations have been mapped at the population level (7–9, 26, 27), demonstrating that escape pathways are reproducible and broadly predictable based on host HLA allele expression. These population-level studies also indicate that HIV-1 is limited, or constrained, in its ability to mutate in response to immune pressures (28), raising the possibility that these constraints could be exploited for vaccine design. In addition to these known escape pathways, however, each individual is also likely to develop atypical (or possibly unique) mutations in the context of their autologous viral sequence and CD8⁺ T cell repertoire.

These “personalized” mutations may not be frequent enough to reach statistical significance in population-based studies, but they may impact the individual’s disease course as profoundly as the selection of known escape variants. Indeed, Goonetilleke et al. (16) show that some of the earliest responses (and escape events) arise against novel epitopes whose sequences differ to some extent from consensus virus sequences. This finding underscores the importance of expanding our knowledge of immune escape through detailed individual-level studies. Ultimately, the integration of macro- and microviewpoints will bring us closer to designing a vaccine that will address the combined challenges of intraindividual and global HIV sequence diversity.

Caveats and practical considerations

Despite the importance of these studies, some caveats merit mention. First, although SGA currently represents the gold standard for identifying transmitted founder viruses, the technique is still limited by in vitro errors introduced by reverse transcriptase (although not *Taq*) enzymes (18). Its chief drawback, however, is a practical one: SGA is prohibitively cost- and labor-intensive, and thus remains inaccessible to many researchers. It is therefore important to stress that, depending on the type of study being undertaken, SGA may not be required. For studies seeking a single (consensus) sequence per patient, for example, conventional approaches remain appropriate.

Similarly, although the use of proteome-wide autologous 18-mer peptides allowed the identification of novel, ultra-early CD8⁺ T cell responses in these studies, the cost of custom full-proteome peptide synthesis renders this technique impractical as a routine approach. Although consensus/reference peptides may underestimate responses by up to 30% (29), their use has been invaluable to our understanding of HIV-specific immune responses, and they will remain an important instrument in our research toolkit until alternative approaches become more accessible. Furthermore, even with the use of autologous peptides, some responses may still go undetected

because of the positioning of the epitope within the longer 18-mer peptide (30).

It is also imperative that future studies of HIV-specific cellular immune responses incorporate functional studies of CTL. Although IFN- γ ELISpot assays are useful in the characterization of response specificity, they do not take into account the complex intracellular processing and presentation of epitopes on infected cells. ELISpot assays also re-

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veal little about the actual antiviral activities of CTL, which may differ according to antigen exposure, epitope specificity, and other factors (31–34).

Several scientific questions also remain. Notably, the authors' observation that reversions (i.e., escape mutations selected in the donor that revert to the consensus sequence in a recipient who lacks the restricting HLA allele) are far less frequent than escape events contrasts with previous reports (35, 36). This deserves further study, but, as it stands, the observations by Goonetilleke et al. are consistent with population-level HIV adaptation in response to selection pressures imposed by HLA class I alleles (6–8, 10)—a finding with profound implications for the future of the epidemic (10).

The incidence and clinical significance of extremely early escape events also remain somewhat unclear. Among the three subjects selected for detailed immunological analysis by Goonetilleke et al., all went on to achieve viral set points near or below the 25th percentile of observed values at the population level (37), and two of these individuals expressed the protective HLA-B*5701 allele (22). Further work is needed to determine whether such early escape occurs commonly in acute infection, or whether these potent, early CTL responses (and/or the potential viral rep-

licative costs associated with these mutations [38–40]) represent key drivers of HIV immune containment in individuals who successfully control viremia. If such potent, ultra-early responses are critical to establishment of the viral set point, this would have profound implications for vaccine design and argues strongly for expanded study of early responses and their associated replicative costs to the virus.

Finally, as the authors acknowledge, the documentation of early positive selection events that were not explained by escape from CD8⁺ T cells, neutralizing antibodies, or reversion does not rule out the possibility that innate immune responses (41) may play a critical role in early HIV control.

The studies by Salazar-Gonzalez et al. (15) and Goonetilleke et al. (16) take us a step closer to understanding the earliest events after acute HIV infection, and clearly demonstrate that broader collaboration on specific cohorts, including the incorporation of immunological and virologic data, can fuel biomedical advances that would otherwise not ensue. Such collaboration will clearly be needed to solve the puzzle of how HIV has, and continues to be, shaped by immune selection. The big challenge that remains is how best to exploit this new information to bring us closer to the generation of an effective vaccine that can overcome the enormous challenge of HIV evolution and diversity.

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