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Accessibility
Reduced Viral Replication Capacity of Human Immunodeficiency Virus Type 1 Subtype C Caused by Cytotoxic-T-Lymphocyte Escape Mutations in HLA-B57 Epitopes of Capsid Protein

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Cytotoxic-T-lymphocyte (CTL) escape mutations in human immunodeficiency viruses encode amino acid substitutions in positions that disrupt CTL targeting, thereby increasing virus survival and conferring a relative fitness benefit. However, it is now clear that CTL escape mutations can also confer a fitness cost, and there is increasing evidence to suggest that in some cases, e.g., escape from HLA-B*57/B*5801-restricted responses, the costs to the escape virus may affect the clinical course of infection. To quantify the magnitude of the costs of HLA-B*57/B*5801 escape, a highly sensitive dual-infection assay that uses synonymous nucleotide sequence tags to quantify viral relative replication capacity (RRC) was developed. We then asked whether such CTL escape mutations had an impact equivalent to that seen for a benchmark mutation, the M184V antiretroviral drug resistance mutation of reverse transcriptase (RRCV184 = 0.86). To answer the question, the RRCs were quantified for escape mutations in three immunodominant HLA-B*57/B*5801 epitopes in capsid: A146P in IW9 (RRCI146 = 0.91), A163G in KF11 (RRCG163 = 0.89), and T242N in TW10 (RRTC242 = 0.86). Individually, the impact of the escape mutations on RRC was comparable to that of M184V, while coexpression of the mutations resulted in substantial further reductions, with the maximum impact observed for the triple mutant (RRCp146-G163-N242 = 0.62). By comparison to M184V, the magnitude of the reductions in RRC caused by the escape mutations, particularly when coexposed, suggests that the costs of escape are sufficient to affect in vivo viral dynamics and may thus play a role in the protective effect associated with HLA-B*57/B*5801.

The capacity of human immunodeficiency virus (HIV) to adapt to, or “escape from,” the host cytotoxic-T-lymphocyte (CTL) immune response is well supported by studies of both human infections (31, 45, 46) and experimental infections in the simian immunodeficiency virus (SIV) macaque model (2, 18, 19, 43). CTL escape originates with the introduction, by the error-prone viral reverse transcriptase (RT), of a mutation that disrupts the processing (1, 16), presentation (30, 32, 33), or recognition (11, 48, 51) of a targeted CTL epitope. As a result, the variant viruses expressing the mutation, i.e., CTL escape viruses, increase in frequency over time. However, the ultimate outcome of such CTL escape, both clinically and virologically, is difficult to predict; CTL escape has been associated with loss of virus suppression and disease progression in some cases (7, 10, 20, 26–28, 31, 46) but with continued, or even improved, immune control in others (6, 37). In addition to the potential for clinical impact, vaccine experiments in the SIV macaque model have demonstrated that CTL escape poses a significant threat to the durability and efficacy of CTL-based vaccines (8, 9).

As for any adaptive process, the dynamics of CTL escape, i.e., the direction, rate, and magnitude of change in the population frequency of a CTL escape mutation, are governed predominantly by the impact of the escape mutation on viral relative fitness, a metric that represents the net of all association advantages and disadvantages. The reduced CTL destruction of escape virus-infected cells, i.e., increased escape virus survival, confers an increase in relative fitness as evidenced by the outgrowth of escape viruses. However, a number of CTL escape mutations have been identified in both HIV and SIV that appear to carry a concomitant “fitness cost” (21, 22, 33, 36, 37, 42, 44), likely a result of decreased replication capacity of the escape virus (12, 23, 36). The net impact of these opposing fitness effects, i.e., the relative fitness, determines both the intrapatient and interpatient dynamics of CTL escape, and it is therefore important to begin to develop methods to quantify these effects in a meaningful way.

The closely related HLA class I alleles HLA-B*57 and HLA-B*5801 are notable for restricting immunodominant CTL responses that effectively suppress HIV viremia and for being associated with improved long-term clinical outcomes of HIV infection (3, 29, 38, 41). Three of the epitopes that are most strongly targeted by the HLA-B*57/B*5801-restricted CTL response are located in the HIV capsid protein (CA; p24) (3), and CTL escape mutations have been identified for each of them: A146P for IW9 (16), A163G for KF11 (14), and T242N for TW10 (33). The clinical impact of HIV escape from HLA-B*57/B*5801 responses remains unclear; although there are reports of breakthrough viral replication following CTL escape in HLA-B*57/B*5801 epitopes, there are also reports of continued virus suppression despite the presence of CTL escape viruses (6, 7). The latter observation suggests the possibility that escape from HLA-B*57/B*5801 responses may come at a fitness cost that is sufficiently high so as to affect the capacity of
the escape virus to replicate to high viral loads. Indeed, the recent descriptions of reduced growth kinetics associated with the T242N mutation in the TW10 epitope (12, 36) and of improved clinical outcomes (lower viral load and higher CD4 count) associated with transmission of viruses expressing the A146P and/or T242N mutations to individuals who do not express HLA-B*57/B*5801 (13) support such a hypothesis.

Here, we describe our efforts to further investigate the impact of HLA-B*57/B*5801 escape mutations on in vivo viral dynamics by quantifying the magnitude of the reductions in the viral relative replication capacities (RRC) of the A146P, A163G, and T242N escape mutations, both individually and, perhaps more physiologically relevant, in combination. Specifically, we address the question of whether the impact of these mutations is sufficient to potentially reduce viral replication in vivo. To do so, we developed a new synonymous-sequence tag dual infection assay that allows direct comparison of the growth of wild-type and variant viruses for the sensitive quantification of RRC and assessed whether the fitness cost for CTL escape mutations is comparable to that seen for a standard drug resistance mutation in the same assay. We provide a benchmark of in vivo relevance by quantifying the RRC of the RT M184V antiretroviral drug resistance mutation, which causes a well-described and clinically important decrease in HIV replication capacity. We then applied the assay to the quantification of the RRC associated with the capsid A146P, A163G, and T242N HLA-B*57/B*5801 escape mutations. In the case of CA T242N, we also assayed the RRC of the alternative CA T242S substitution and of the putative T242N compensatory mutation H219Q (12, 33). The results demonstrate that viral expression of these CTL escape mutations is associated with a fitness cost by virtue of reduced replication capacity. That the cost is cumulative with additional mutations, and that by comparison to RT M184V, the magnitude of the reduction is sufficient to have an impact on in vivo viral loads.

MATERIALS AND METHODS
Molecular clones. The pMJ infectious molecular HIV type 1 subtype C (HIV-1C) clone (39) served as the backbone for all viruses used in this study. Several subclones were constructed to provide templates for site-directed PCR mutagenesis. For subcloning convenience, pMJ4 was modified by the deletion of the Nef4140 and Nef4436 primers. This resulted in the selection of the synonymous nucleotide substitutions A8940T, C8943G, C8946G, and T8949C, located in the middle of the forward TGG) primers, the BamH I/XhoI fragment was cloned back into the T242S primer on the wild-type template but would also result in approximately a small number of synonymous nucleotide substitutions in one of the sites that would cause maximal, and approximately equal, destabilization of mismatch binding with both the wild-type and synonymous primer on the wild-type template but would also result in approximately equal annealing temperatures for each of the primers with its matched template. This resulted in the selection of the synonymous nucleotide substitutions A8940T, C8943G, C8946G, and T8949C, located in the middle of the forward primer binding site, and creation of the matching forward primer, ABI nef_for_syn (CAACACTGCGGCGAACAAT). Next, the synonymous substitutions were introduced into the pCLB9 MJ4 nef subclone by QuickChange II (Stratagene, La Jolla, CA) site-directed PCR mutagenesis for the manufacturer’s protocol with primers as designed by the online Stratagene QuikChange primer Design software. The mutagenesis was confirmed by sequencing with Nef4140 (GAGGGCTATCTGCAATATAC) and Nef4436 (GCTGCCCTTATAGCTCATTGGT CTT) Sybr green quantitative PCR primers were selected using the ABI Primer Design software. Next, the primer binding sites were examined to identify a small number of synonymous nucleotide substitutions in one of the sites that would cause maximal, and approximately equal, destabilization of mismatch binding with both the wild-type and synonymous primer on the wild-type template but would also result in approximately equal annealing temperatures for each of the primers with its matched template. This resulted in the selection of the synonymous nucleotide substitutions A8940T, C8943G, C8946G, and T8949C, located in the middle of the forward primer binding site, and creation of the matching forward primer, ABI nef_for_syn (CAACACTGCGGCGAACAAT). Next, the synonymous substitutions were introduced into the pCLB9 MJ4 nef subclone by QuickChange II (Stratagene, La Jolla, CA) site-directed PCR mutagenesis for the manufacturer’s protocol with primers as designed by the online Stratagene QuikChange primer Design software. The mutagenesis was confirmed by sequencing with Nef4140 (GAGGGCTATCTGCAATATAC) and Nef4436 (GCTGCCCTTATAGCTCATTGGT CTT) primers, the BamH I/XhoI fragment was cloned back into pMJ4/Δapa/Δbam to produce the full-length infectious molecular clones using Polyfect transfection reagent (Qiagen, Valencia, CA) according to a modified manufacturer protocol. Briefly, at 1 day prior to transfection, 2.8 × 10⁹ HEK 293 cells were seeded in a T75 flask. For the transfection, 15 μg of highly purified plasmid DNA, at a minimum concentration of 1 μg/ml, was diluted to a 150-μl final volume in Dulbecco modified Eagle medium without supplements, 115 μl of Polyfect reagent was added, and the solution was mixed by gentle pipetting and incubated for 10 min at room temperature. During the incubation, the medium removed from the 293 cells to be transfected, they were washed once in cold PBS, and then 7 ml of fresh medium was added. After the 10-min incubation, the transfection mixture was transferred to the flask, swirled gently to mix, and incubated for 3 h at 37°C with 5% CO₂. After 3 hours, the medium was removed and discarded, the cells were washed once with PBS, and 7 ml of fresh medium was added before returning the cells to the incubator. Transfection supernatant was harvested after 72 h, filtered through a 0.2-μm filter, and stored in aliquots at −80°C.

The initial screen of virus stocks was conducted by p24 enzyme-linked immunosorbent assay (Perlman-Erimer, Waltham, MA) per the manufacturer’s protocol; stocks that contained less than 10 ng of p24 per ml were discarded. Titers of stocks with a sufficient p24 titer were subsequently determined on CDS-depleted PBMC by 50% tissue culture infective dose per a standard protocol (NIH-NIAID-DAIDS).

ncf synonymous-sequence tag. First, the ABI nef_for wt (GCAACACAGC GCAATAAT) and ABI nef_reverse (CGTCGCCCTTATAGCTCATTGGT CTT) Sybr green quantitative PCR primers were selected using the ABI Prism Primer Design software. Next, the primer binding sites were examined to identify a small number of synonymous nucleotide substitutions in one of the sites that would cause maximal, and approximately equal, destabilization of mismatch binding with both the wild-type and synonymous primer on the wild-type template but would also result in approximately equal annealing temperatures for each of the primers with its matched template. This resulted in the selection of the synonymous nucleotide substitutions A8940T, C8943G, C8946G, and T8949C, located in the middle of the forward primer binding site, and creation of the matching forward primer, ABI nef_for_syn (CAACACTGCGGCGAACAAT). Next, the synonymous substitutions were introduced into the pCLB9 MJ4 nef subclone by QuickChange II (Stratagene, La Jolla, CA) site-directed PCR mutagenesis for the manufacturer’s protocol with primers as designed by the online Stratagene QuikChange primer Design software. The mutagenesis was confirmed by sequencing with Nef4140 (GAGGGCTATCTGCAATATAC) and Nef4436 (GCTGCCCTTATAGCTCATTGGT CTT) primers, the BamH I/XhoI fragment was cloned back into pMJ4/Δapa/Δbam to produce the full-length infectious pMJ4-nef⁵⁷⁷, and the synonymous substitutions were reconfirmed by sequencing the full-length clone with the NeF440 and NeF446 primers.

ASPcR. Allele-specific quantitative PCR (ASPCR) was performed on the Applied Biosystems Prism 7500 instrument using the Quantitect SYBR green RT-PCR kit (Qiagen, Valencia, CA) per the manufacturer’s protocol in a 25-μl reaction volume. Briefly, each reaction mixture consisted of 12.5 μl 2X Quantit- TECT SYBR green RT-PCR master mix, 1.25 μl each of 6 μM stocks of allele-specific forward and common reverse primers, 0.25 μl Quantitect RT mix, 7.75 μl of distilled water, and 2.0 μl of sample. The cycling conditions consisted of a 30-min RT step at 50°C, a 15-min initial activation and denaturation step at 95°C, 40 cycles of 1 s at 95°C and 1 min at 60°C, and a melting curve consisting of 1 min at 60°C followed by ramping to 95°C with continuous sampling.
The quantification standards consisted of near-full-length linear plasmid DNA generated by XmaII digest of pMJ4-nefWT and pMJ4-nefSYN following agarose gel purification and spectrophotometric copy number quantification. Independent standard curves were generated for both the nefWT and the nefSYN allele-specific reactions by quantification of a series of seven 10-fold dilutions of the linearized plasmid standards (5 copies/μL × 5 × 10^6 copies/μL) quantified in duplicate in each of the three independent assays. The cumulative data for each reaction, comprising six data points at each standard dilution, were used to generate a standard curve by regression. The standard curve was calibrated to each subsequent run by including in each run the 5 × 10^6 standard in duplicate and using the crossing-threshold data to adjust the y intercept of the standard curve.

HIV-1C capsid mutants. All capsid escape mutations were considered in the context of the HIV-1C consensus capsid. Recreation of the HIV-1C consensus capsid in MJ4 required the introduction of 10 nucleotide substitutions (C1225G, G1272C, A1446C, G1532C, C1545T, A1554C, T1656C, T2619G, and G2620C [HXB2 numbering]) by successive rounds of QuikChange II (Stratagene, La Jolla, CA) site-directed mutagenesis of the pCLBl2 Gag subclone per the manufacturer’s protocol using primer sets as designed by the online Stratagene QuikChange primer design software. Successful mutagenesis was confirmed by sequencing each round by following with Sanger sequencing with Gag551 and Gag984 primers prior to cloning back mutants into the full-length clones with Gag551, Gag984, Nef9410, and Nef4436 primers.

Next, the substitutions A146P (G1225C), A163G (C1277G), H219Q (C1446A), H219R (C1446G), and T242S (C1514G) were introduced into the HIV-1C consensus capsid by QuikChange II (Stratagene, La Jolla, CA) site-directed PCR mutagenesis of the HIV-1C consensus capsid subclone. Double and triple mutants were produced by successive rounds of mutagenesis. All mutagenesis was confirmed by sequencing with Gag551 and Gag984 primers prior to cloning back into the full-length clones with Gag551, Gag984, Nef9410, and Nef4436 primers.

RESULTS

Quantification of the impact of mutations on the RRC of HIV comprised three steps: dual infection of target cells with “wild-type” and “mutant” viruses, differential quantification of the “wild-type” and “mutant” viruses by nef synonymous-tag ASPCR, and calculation of the RRC of the mutant. The dual infection assay provides a powerful technique for quantifying the impact on HIV replication capacity of a mutation of interest, e.g., drug resistance or CTL escape, because it allows direct comparison of viral growth without the nonspecific variation that can affect parallel but independent infections. However, the assay requires the differential quantification of nearly isogenic viruses from a mixed sample. To accomplish this without disrupting any native virus function, we constructed two recombinant HIV-1C backbones, named MJ4-nefWT and MJ4-nefSYN, that differed by only four synonymous nucleotide substitutions in nef: A2925T, C2926G, C2926A, and T9267C (Fig. 1A). These synonymous sequence tags served as artificial “alleles” to allow differential quantification of the two viruses by ASPCR using tag-specific forward primers and a common reverse primer in parallel but independent reactions (Fig. 1B and C). The nefWT-specific and the nefSYN-specific ASPCRs exhibited linear quantification across a 7-log-unit range of plasmid reference standard concentrations from 5 copies/μL to 5 × 10^6 copies/μL (Fig. 1D and E, respectively) with nearly identical high reaction efficiencies as indicated by the slope of the nefWT and nefSYN standard curves (slope = −3.23 and −3.26, respectively). The tag specificity of each reaction was demonstrated by quantification of mixed template samples containing known quantities of the MJ4-nefWT and MJ4-nefSYN plasmid reference standards; the nef synonymous-tag ASPCR accurately quantified the absolute and relative quantities of the two templates across a broad range of sample mixes (Table 1).

The equivalence of the nefWT and nefSYN tags with respect to virus replication was demonstrated by quantification of the RRC associated with the nefSYN tag. For example, a representative dual infection with an inoculum comprising 43% MJ4-nefWT and 57% MJ4-nefSYN resulted in a 3-log-unit increase of each virus with approximately equal growth kinetics (Fig. 2A) and a cumulative log-linear growth phase from day 2 to day 6 (Fig. 2B), thereby defining the measurement period as day 2 to day 6. The relative frequencies of the two viruses varied slightly during log-linear growth (Fig. 2C), and thus the per-day change in log-transformed ratio of MJ4-nefSYN to MJ4-nefWT ranged from −0.16 to 0.28, resulting in an RRC of 1.00 for MJ4-nefWT in this experiment. The RRC associated with expression of the nefSYN sequence tag was quantified in a total of eight such dual-infection experiments using inoculation with different initial virus ratios ranging from 70% MJ4-nefWT/30% MJ4-nefSYN to 20% MJ4-nefWT/80% MJ4-nefSYN, resulting in a mean RRC of 1.00, which was not significantly different from wild type. Following validation of quantification by ASPCR and dem-
onstration of the equivalence of the two nef synonymous-se-quence tags, we established a clinically relevant benchmark for the assay by quantifying the RRC associated with the RT antiretroviral drug resistance mutation M184V. Multiple dual-infection assays were conducted at a range of M184/V184 inoculum ratios and with RT V184 expressed in both the MJ4-nefWT and MJ4-nefSYN virus backbones. In contrast to the aforementioned synonymous-tag dual-infection experiments,

FIG. 1. nef synonymous-sequence tag ASPCR. (A) Tagged recombinant HIV-1C backbones were constructed by introduction of four synonymous nucleotide substitutions into the nef gene of the MJ infectious clone. (B to D) Allele-specific quantitative PCR using a common reverse primer paired with a forward primer specific for the nefWT (B) or nefSYN (C) tag allowed efficient and sensitive quantification over a wide range of nefWT (D)- and nefSYN (E)-tagged template concentrations.
the RT M184/RT V184 dual infections showed a clear impact of the V184 mutation on viral replication as demonstrated by growth kinetics (Fig. 3A) and changes in relative virus frequency plot over the log-linear growth phase (Fig. 3B) from a representative experiment (initial inoculum of 40% MJ4-nef WT and 60% MJ4-nef SYN). Quantification of RRC values in 11 individual dual infections resulted in a mean RRCV184 of 0.86, a statistically significant decrease from the defined wild-type RRC of 1 (Fig. 4A).

Similar to the quantification of RRC V184, the impact on RRC of HLA-B57 capsid CTL escape mutations, individually and in combination, was quantified by conducting multiple dual-infection assays across a range of initial mutant/wild-type ratios with the mutation expressed in the MJ4-nef WT and MJ4-nef SYN virus backbones in an equal number of experiments (all mutations and mutation combinations were analyzed in 10 dual-infection assays except T242N, which was analyzed in 11). The A163G and T242N mutations caused a statistically significant decrease in RRC (mean RRC = 0.89 and 0.86, respectively) while the A146P mutation had a decrease in RRC mean of 0.91. Similar to the quantification of RRC V184, the quantification of RRC of the T242N mutation was not statistically significant.

### Table 1. Differential quantification of mixed plasmid template samples by nef synonymous ASPCR

<table>
<thead>
<tr>
<th>Sample composition (%)</th>
<th>nef synonymous-tag ASPCR copies/μl % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMJ4-nef WT</td>
<td>99 1 449,719 4,781</td>
</tr>
<tr>
<td>pMJ4-nef SYN</td>
<td>95 5 446,684 24,475</td>
</tr>
<tr>
<td>pMJ4-nef WT</td>
<td>80 20 367,034 89,644</td>
</tr>
<tr>
<td>pMJ4-nef SYN</td>
<td>70 30 318,377 139,766</td>
</tr>
<tr>
<td>pMJ4-nef WT</td>
<td>60 40 307,776 198,032</td>
</tr>
<tr>
<td>pMJ4-nef SYN</td>
<td>50 50 239,559 233,320</td>
</tr>
<tr>
<td>pMJ4-nef WT</td>
<td>40 60 177,828 258,501</td>
</tr>
<tr>
<td>pMJ4-nef SYN</td>
<td>30 70 137,479 304,565</td>
</tr>
<tr>
<td>pMJ4-nef WT</td>
<td>20 80 92,195 344,423</td>
</tr>
<tr>
<td>pMJ4-nef SYN</td>
<td>10 90 25,119 440,471</td>
</tr>
<tr>
<td>pMJ4-nef WT</td>
<td>1 99 4,063 425,677</td>
</tr>
</tbody>
</table>

Note: nef synonymous tagged templates were quantified in parallel nef WT- and nef SYN-specific quantitative PCRs. Mixed samples consisted of known quantities of linearized pMJ4-nef WT and pMJ4-nef SYN plasmid standards. ASPCR-derived template frequencies in the sample calculated from copy number values.
and the T242S alternative escape mutation did not cause a statistically significant change in RRC (mean RRC\textsubscript{S242} = 0.95) (Fig. 4B). Coexpression of escape mutations caused further reductions in RRC: RRC\textsubscript{P146-G163} = 0.46, RRC\textsubscript{P146-N242} = 0.73, RRC\textsubscript{G163-N242} = 0.78, and RRC\textsubscript{P146-G163-N242} = 0.62. All reductions caused by expression of multiple escape mutations were statistically significant (Fig. 4C).

In addition to the three primary HLA-B57 escape mutations in capsid, we also assayed the impact on replication capacity of the H219Q capsid mutation, an apparent compensatory mutation for T242N. Expression of the H219Q mutation individually resulted in a small mean increase in replication capacity relative to wild type (RRC\textsubscript{Q219} = 1.08) which achieved borderline statistical significance, coexpression of H219Q with T242N restored wild-type replication (RRC\textsubscript{Q219-N242} = 0.95), and coexpression with the alternative T242S mutation had a minimal effect (RRC\textsubscript{Q219-S242} = 0.92) (Fig. 4D).

**DISCUSSION**

The adaptation of HIV to the host CTL immune response, manifested as the appearance and increase in population frequency of variant viruses expressing CTL escape mutations, represents a serious challenge to the control of virus replications.
tion by CTL immune responses, whether induced by infection or vaccination. However, the evidence that CTL escape mutations, like those conferring antiretroviral drug resistance, can impart a viral relative fitness cost (21, 22, 33, 36, 37, 42, 44) raises the possibility that with sufficient understanding of the mechanisms and dynamics of the process, it may be possible to anticipate, mitigate, and perhaps even manipulate CTL escape (4). Indeed, recent reports suggest that CTL escape-associated fitness costs may contribute to the protective effect associated with host expression of particular HLA class I alleles such as HLA-B57 (6, 37). In order to develop sufficient understanding of CTL escape so as to some day co-opt this viral advantage for patient benefit, it is important not only to move from qualitative to quantitative analyses of the replicative cost of CTL escape mutations but also to begin to consider the impact on viral fitness of the coexpression of multiple escape mutations rather than just individual mutations in isolation. The results of this study demonstrate that HLA-B57-associated CTL escape mutations in the HIV-1C capsid cause significant reductions in viral replication capacity when expressed both individually and, as shown for the first time, in combination. We also demonstrate that the magnitude of such CTL escape mutations is equivalent to that seen for the well-known RT M184V antiretroviral drug resistance mutation and thus is sufficiently large so as to have a potential impact on virus replication in vivo.

The restoration of wild-type-level replication capacity by coexpression of the H219Q mutation with the T242N escape mutation supports its previously described role as a compensatory mutation for T242N (12, 33). However, the slight increase in replication capacity associated with individual expression of the H219Q mutation is consistent with prior descriptions of the effect of this mutation on HIV replication (24, 25) and suggests that H219Q may increase replication more generally rather than as a specific compensation for the T242N escape mutation. Indeed, H219Q has also been described to compensate for decreased replication associated with protease inhibitor resistance mutations (40).

The dual-infection assay, defined here as the inoculation of parallel but independent monocultures, the nonspecific variation that is inevitably introduced during manipulation of the assay is controlled because it affects both viruses equally. In contrast to, for example, an M184V-selective antiretroviral mutation that is commonly used as a benchmark of replication capacity (34), the dual-infection assay provides two advantages over the insertion of heterologous sequence tags (34) or fluorescence genes (17, 50) into the HIV genome. First, the use of synonymous tags avoids the disruption of any viral proteins and allows consideration of the impact of mutations in the context of a native virus. Second, although we placed the synonymous tags in nef to be consistent with previous methods, the flexibility to place synonymous tags in close proximity to any mutation of interest can reduce, or in theory eliminate, the potential for recombination between the mutation and the detector tag. Although the use of low-MOI inocula and short-duration infections also results in negligible recombination, the placement of the synonymous tag in close proximity to the mutation of interest would provide for greater experimental flexibility, including assays using high-MOI infection. It is important to note, however, that although it is undesirable, recombination is not a fatal flaw; the rearrangement of mutation-tag linkage does not cause erroneous detection of mutation-specific differences where there are none, i.e., false positives, but rather it decreases the power to detect mutation-specific differences that truly exist, i.e., increases false negatives.

The interpretation of results of in vitro assays of relative fitness parameters suffers from the need to extrapolate the in vitro-derived values to the viral infection in vivo. Although extrapolation is an inherent weakness of any in vitro assay, the quantification of the RRC associated with the antiretroviral drug resistance mutation RT M184V provides a clinically relevant point of reference, or benchmark, against which the values for other mutations can be compared. The RT M184V mutation is well suited for this task because not only has it been demonstrated by in vitro analysis to cause a significant reduction in viral replication capacity (34), but it is also known to contribute to decreased viral loads in vivo. In fact, the impact is so great that an M184V-selective antiretroviral is often continued following the emergence of resistance in order to maintain the presence of the M184V variant in the patient (15, 40, 49). The value of this mutation as a benchmark of replication capacity is further increased by the apparent constancy of its impact; assayed here in the context of a HIV-1C genetic background, the impact on viral replication capacity (RRC = 0.86) is quite comparable to that observed in previous studies in which it was expressed in a HIV-1B genetic background. For example, in the most directly comparable study, Ly and Kuritzkes describe a 7% reduction in replication capacity, approximating an RRC of 0.93, caused by expression of RT M184V in a HIV-1B background (34).

The capacity of HIV to escape from CTL-mediated immune pressure is an important aspect of the pathogenesis of HIV infection and is also likely to challenge any vaccine design...
based on eliciting CTL immune responses. The ability to sensiti-
vatively quantify the impact of CTL escape mutations on HIV
replication capacity in a relevant and reproducible manner is
an important step in acquiring the data necessary for teasing
apart the contributions of multiple, and at times opposing,
components of net relative fitness, a driving force of CTL
escape dynamics. In this study, we focused our efforts on quan-
tifying the specific impact of several important HLA-B*57/
B*5801 escape mutations, individually and in combination,
by engineering them into a recombinant virus with a defined
genetic background. This approach provides for a very “clean”
determination of the intrinsic impact of specific mutations of
interest, and as additional CTL-associated mutations in HIV
are identified by careful analysis of clinical viral sequences, it
will continue to be useful for fine mapping of the relative
impacts of such mutations on viral replicative capacity. In
addition, however, future studies expanding the approach to
include larger segments of clinically derived sequence, e.g., gag in
toto, will provide important comparators for evaluating the
recombinant-derived RRCs. The continued investigation of
these mechanisms and dynamics of CTL not only increases our
understanding of the factors underlying the clinical progres-
sion of HIV infection but also contributes important informa-
tion toward the eventual development of a durable vaccine
construct.

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