

LA-UR-19-30331 (Accepted Manuscript)

Accumulated mutations by 6 months of infection collectively render transmitted/founder HIV-1 significantly less fit

Wang, Chu; Liu, Donglai; Zuo, Tao; Hora, Bhavna; Cai, Fangping; Ding, Haitao; Kappes, John; Ochsenbauer, Christina; Kong, Wei; Yu, Xianghui; Bhattacharya, Tanmoy; Perelson, Alan S.; Gao, Feng

Provided by the author(s) and the Los Alamos National Laboratory (2019-12-11).

To be published in: Journal of Infection

DOI to publisher's version: 10.1016/j.jinf.2019.12.001

Permalink to record: <http://permalink.lanl.gov/object/view?what=info:lanl-repo/lareport/LA-UR-19-30331>

Disclaimer:

Los Alamos National Laboratory, an affirmative action/equal opportunity employer, is operated by Triad National Security, LLC for the National Nuclear Security Administration of U.S. Department of Energy under contract 89233218CNA000001. By approving this article, the publisher recognizes that the U.S. Government retains nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or to allow others to do so, for U.S. Government purposes. Los Alamos National Laboratory requests that the publisher identify this article as work performed under the auspices of the U.S. Department of Energy. Los Alamos National Laboratory strongly supports academic freedom and a researcher's right to publish; as an institution, however, the Laboratory does not endorse the viewpoint of a publication or guarantee its technical correctness.

Journal Pre-proof

Accumulated mutations by 6 months of infection collectively render transmitted/founder HIV-1 significantly less fit

Chu Wang , Donglai Liu , Tao Zuo , Bhavna Hora , Fangping Cai , Haitao Ding , John Kappes , Christina Ochsenbauer , Wei Kong , Xianghui Yu , Tanmoy Bhattacharya , Alan S Perelson , Feng Gao

PII: S0163-4453(19)30373-1
DOI: <https://doi.org/10.1016/j.jinf.2019.12.001>
Reference: YJINF 4394



To appear in: *Journal of Infection*

Accepted date: 1 December 2019

Please cite this article as: Chu Wang , Donglai Liu , Tao Zuo , Bhavna Hora , Fangping Cai , Haitao Ding , John Kappes , Christina Ochsenbauer , Wei Kong , Xianghui Yu , Tanmoy Bhattacharya , Alan S Perelson , Feng Gao , Accumulated mutations by 6 months of infection collectively render transmitted/founder HIV-1 significantly less fit, *Journal of Infection* (2019), doi: <https://doi.org/10.1016/j.jinf.2019.12.001>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2019 Published by Elsevier Ltd on behalf of The British Infection Association.

Highlights:

- Mutations in early HIV-1-infection collectively impair fitness of the T/F viruses.
- Fitness loss is associated with reduced viral loads from viremia peak.
- Testing mutations in the cognate T/F genome accurately determinates HIV-1 fitness.

Journal Pre-proof

Accumulated mutations by 6 months of infection collectively render transmitted/founder HIV-1 significantly less fit

Running title: Fitness costs of accumulated mutations in the HIV-1 genome

Chu Wang^{1,2#}, Donglai Liu^{1,2,3#}, Tao Zuo^{1,2}, Bhavna Hora², Fangping Cai², Haitao Ding⁴, John Kappes⁴, Christina Ochsenbauer⁴, Wei Kong¹, Xianghui Yu¹, Tanmoy Bhattacharya⁵, Alan S Perelson⁵ and Feng Gao^{1,2*}

¹National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun 130012, Jilin Province, China

²Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710, United States of America

³Division of the second in vitro diagnostic, National Institute for Food and Drug Control, Beijing 100050, China

⁴Departments of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294, United States of America

⁵Theoretical Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, United States of America

* Corresponding author

E-mail: fgao@duke.edu

Phone number: 1-919-668-6433

Address: 244 Sands Building, 303 Research Drive, DUMC 102359, Durham, North Carolina 27710, United States of America

These two authors contributed equally to this work.

Declarations of interest: none.

Summary

Objective: Viral fitness plays an important role in HIV-1 evolution, transmission and pathogenesis. However, how mutations accumulated during early infection affect viral fitness has not been well studied.

Methods: We generated paired infectious molecular clones (IMCs) for transmitted/founder (T/F) and 6-month (6-mo) viruses post infection from 10 infected individuals to investigate the impact of accumulated mutations on viral fitness by comparing 6-mo viruses to their cognate T/F viruses.

Results: We found that all ten 6-mo viruses were less fit than their cognate T/F viruses. Moreover, the fitness losses of the 6-mo viruses correlated with the decrease in viral loads from the peak of viremia.

Conclusion: These results show that the mutations accumulated during half a year post infection collectively reduce viral fitness and thereby contribute to lowering viral loads.

Keywords: HIV-1, Mutation, Fitness, Transmitted/founder virus, Infection.

Introduction

Viral fitness plays an important role in transmission, pathogenesis, drug resistance, disease progression and vaccine development for HIV-1 (1-9). The viral load (VL) declines dramatically from the peak of viremia during acute HIV-1 infection under the selection pressure by cytolytic T lymphocytes (CTL) and then maintains at a stable set point level as a result of the balance between the host selection pressure and viral replication (10-13). During acute/early infection, immune escape mutations are selected by immune responses, allowing mutant viruses to evade immune selection pressure. These individual mutations can significantly reduce the replicative fitness of HIV-1 (14-19). The reduced viral fitness and lowered viral loads can lead to long-term HIV-1 control and a decreased probability of transmission to new hosts (4-6). However, the fitness lost caused by the immune escape mutations can also be repaired by compensatory mutations that occur within or outside the targeted epitopes (14, 18, 20-23). The restoration of the fitness losses by compensatory mutations may be responsible for the subsequent VL changes and the unsustained disease control (6). A number of mutations (e.g., A146P, A163G, E207D, T242N and I437L in Gag; T68A, K466R in Pol; and N279K, V281A, V281G in Env) selected by CTL and neutralizing antibodies could impair viral replicative fitness (16, 17, 24-27), while some other mutations (e.g., H219Q, I223V, M228I, V247I, G248A and K436R in Gag) could compensate the fitness losses (18, 20, 23, 26, 28). However, how combinations of mutations affect viral fitness has not been elucidated.

Earlier studies have shown that viral fitness may be correlated with set point VLs and thus it may be a good predictor of disease progression and clinical parameter (8, 29-31). However, viral fitness was determined by using quasispecies viral isolates (29, 32, 33), infectious molecular clones containing the gag-protease fragments from acute/early/late viruses (32, 34, 35), or cross sectional studies by comparing viruses from different hosts from different time points (1, 36). Recent studies showed that fitness of transmitted/founder (T/F) viruses play an important role in HIV-1 infection outcomes (8, 37). These studies showed how the viral replication capacity was associated with disease

progression. However, the impact of mutations or gene fragments on viral fitness differences were only analyzed in unrelated viral genome background and compared viruses were not head-on compared in the same culture in these studies. In nearly 80% of HIV-1 infections through mucus areas, a single T/F virus is responsible for productive clinical infection (38, 39). Following establishment of HIV-1 infection, the viruses in the infected hosts gradually accumulate mutations under the host selection pressure. However, it remains unclear how the mutations accumulated in T/F viruses affect viral fitness and how the fitness changes affect VLs *in vivo*.

We have inferred the whole HIV-1 T/F genomes using the single genome amplification (SGA) method from acute infection samples (25, 38, 40-42) and identified a number of mutations that are associated with T cell and B cell immune selection in those viral genomes (25, 43, 44). Using infectious molecular clones (IMCs) generated for T/F viruses (25, 38, 41, 42), we precisely determined how some of those mutations affected the fitness of the T/F viruses. We found that some of the mutations could cause significant fitness losses while others could compensate the fitness losses (18, 23, 25, 45, 46). However, how the mutations together in the viral genome generated during about half a year after infection affect the viral fitness of their cognate T/F viruses has not been investigated. To understand the overall impact of mutations accumulated by six months of infection on viral fitness, we for the first time assemble a panel of 10 pairs of T/F and 6-mo viruses from the same individuals and determined the impact of these mutations on viral fitness and the association between the fitness losses and VL decreases by comparing replication kinetics of the 6-mo viruses to their cognate T/F viruses in primary CD4⁺ T cells.

Materials and Methods

Human subjects

Plasma samples were obtained from 10 adult subjects (Table 1) enrolled in acute HIV-1 infection cohorts (38). None were treated with antiretroviral drugs until after the time when the samples were collected for analysis. All subjects provided

written informed consent for the collection of samples and subsequent analyses. The study was approved by the Institutional Review Boards of Duke University.

Amplification of viral genome by SGA

Viral RNA (vRNA) was extracted from plasma samples using the PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA). cDNA was synthesized using the SuperScript III reverse transcriptase (Invitrogen) with the primer 07Rev8 (5'-CCTARTGGGATGTGTACTTCTGAACTT-3'; nt 5193–5219 in HXB2) for 5'-half genome, or primer 1.R3.B3R (5'-ACTACTTGAAGCACTCAAGGCAAGCTTTATTG-3'; nt 9611–9642) for the 3'-half or near full-length genomes. The 3'- and 5'-half genomes were amplified by SGA as described previously (42, 45). The two overlap PCR fragments cover the entire viral RNA genome.

Sequence analysis

The PCR amplicons were directly sequenced by the cycle sequencing and dye terminator methods on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). Individual sequences were assembled and edited using Sequencher 4.7 (Gene Codes, Ann Arbor, MI). The sequences were aligned using CLUSTAL W (47), and the manual adjustment for optimal alignment was performed using Seaview v4. The overlapping region between the two half genomes is over 1000 bp long (42, 45). The 5'- and 3'-half genome sequences that shared the identical overlap sequences were put together to correctly generate complete viral genomes that represent the viruses *in vivo*.

Generation of infectious molecular clones

Of the 10 pairs of T/F and 6-mo IMCs, eight T/F IMCs and five 6-mo IMCs were reported before (41, 42, 48, 49). Using the same method, we inferred two additional T/F genome sequences (CH0107 and CH0569) by analyzing the plasma quasispecies in selected patients at sequential time-points during acute and early infection and identified predominant mutations representing >50% of

sequences at polymorphic positions at month 6 for five viruses (CH0040, CH0042, CH0107, CH0162 and CH0569) in this study. The inferred T/F whole genomes were chemically synthesized as three subgenomic fragments (Blue Heron Biotechnology, Bothell, WA) and cloned together through unique restriction enzyme sites to generate T/F IMCs (48). To generate 6-mo IMCs, predominant mutations detected at month 6 were introduced into the corresponding T/F IMCs using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA). All newly generated IMCs were sequence confirmed. The virus stocks were prepared from the supernatants of HEK293T cells transfected with IMCs as previously described (40, 45).

Viral replication kinetics

Blood was obtained from a healthy donor under clinical protocols approved by the Institutional Review Board of Duke University. Peripheral blood mononuclear cells (PBMCs) were isolated by using the Ficoll-Hypaque density gradients. CD4⁺ T cells were negatively selected from PBMCs using a CD4⁺ T cell Isolation Kit II (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Purified CD4⁺ T cells were stimulated with interleukin 2 (IL-2) (32 IU/ml; Advanced Biotechnologies, Columbia, MD) and Dynabead Human T-Activator CD3/CD28 (Thermo Scientific, Waltham, MA) for 3 days. The viral growth kinetic assays were performed with stimulated CD4⁺ T cells as we described before (18). The dynamic viral replication in the culture supernatant was monitored by measuring the p24 concentration using an Alliance HIV-1 p24 ANTIGEN ELISA Kit (PerkinElmer, Waltham, MA). All infections were performed in triplicate.

Parallel allele-specific sequencing (PASS) fitness assay

Stimulated CD4⁺ T cells (5×10^5) were infected with the mixture of two compared viruses at a ratio of 1:1 (2.5 ng of p24 for each virus) both in the single passage and through multiple passages as previously described (17). Specifically, in the multiple passage assay, the supernatant at each passage was harvested at day 5 or day 6 at the peak of the p24 production, and 100 μ l of the supernatant was

passaged onto fresh CD4⁺ T cells (about 5 ng p24 per 5 × 10⁵ cells). The viral replication kinetics was monitored by measuring the p24 concentration in the culture supernatant. All infections were performed in triplicate. To ensure that all the fitness data were comparable, the primary CD4⁺ T cells from the same one individual collected through leukapheresis were used for all fitness comparison experiments.

The PASS fitness assay was performed as we described before (18). The culture supernatants were first treated with DNase I (New England Biolabs, Ipswich, MA) at 37°C for 20 minutes. Then vRNA was extracted and used for cDNA synthesis using a SuperScript III reverse transcriptase (Invitrogen) with the primer lower3 (5'-TTTTTCCTAGGGGCCCTGCAATTT-3'; nt 1998–2021). 20 µl of 6% acrylamide gel mix, containing viral cDNA, 1 µl acrydite-modified forward primer M6F2 (5'-Acry-CTCGACGCAGGACTCGGCTTGCTG-3'; nt 685–708), 0.3% diallyltartramide, 0.1% ammonium persulfate (APS), 0.1% N,N,N',N'-tetramethylethylenediamine (TEMED), 5% Rhinohide and 0.2% bovine serum albumin (BSA), were used to cast the gel on a bind-silane (GE Healthcare Bio-Sciences, Pittsburgh, PA) treated glass slide. The in-gel PCR amplification was then performed in a PTC-200 Thermal Cycler (MJ Research, Hercules, CA) with a mix of 3.3 units of Jumpstart Taq DNA polymerase (Sigma, St. Louis, MO), 1 µM reverse primer M6R2 (5'-TCCTCCCACTCCCTGACATGCTGTCATCATTTTC-3'; nt 1822–1854), 100 µM dNTP mix, 1 x PCR buffer, 0.1% Tween-20, 0.2% BSA, and H₂O (up to 300 µl) under a sealed SecureSeal Hybridization Chamber (Grace Bio-Labs, Bend, OR). After in-gel PCR, the gels were treated with denaturation solution to remove the free DNA strands. Single base extension (SBE) was then performed to distinguish the compared viruses using two different fluorophore labeled bases with sequencing primers that annealed just upstream of the target sites. Different sequencing primers used to detect the bases at different mutated positions were listed in Table S1. Data analysis was performed as previously described (17, 50).

Quantification and statistical analysis

The relative fitness (S_{ij}) in competitive assays and the method of statistical analyses to assess the significance of the measured fitness difference were determined using the mathematical model as described in our previous studies (17, 18, 23, 45).

Results

T/F and 6-mo viruses from the same individuals are replication-competent

Individual escape mutations selected by T cell immune responses can cause fitness losses and lead to the lowered VLs during acute infection (10, 51-54). However, it is not known whether 6-mo viruses carrying multiple mutations are less fit than their cognate T/F viruses since such paired viruses were not available for comparison before. To investigate the impact of accumulated mutations during early infection on viral fitness, we studied 10 individuals who were infected with single T/F viruses and were followed from acute infection (Fiebig stages I-IV) for two years of infection (38). Relatively few mutations at month 6 after infection were predominant in the HIV-1 genome. However, the escape mutations selected by hosts can be clearly identified. We previously generated five IMCs for the 6-mo viruses that carried all predominant mutations and compared their relative resistance to type 1 interferon inhibition to their cognate T/F viruses (41, 48). All predominant mutations identified in the 6-mo viruses in each individual were introduced into their cognate T/F genome to generate the additional five pairs of T/F and 6-mo IMCs. Together with the IMCs for T/F and 6-mo viruses from our previous studies (41, 42, 48, 49), we generated 10 pairs of T/F and 6-mo IMCs from 10 HIV-1-infected individuals (Table 1). Comparison of the 6-mo and their cognate T/F viral genome sequences showed an average of 16 (7-27) predominant point mutations (i.e. present in $\geq 50\%$ of the viral population) in their genomes (Figure 1 and Table S2).

We first determined the replication capacity of each paired T/F and 6-mo viruses from the same individual by culturing each virus independently in purified primary CD4⁺ T cells from an HIV-1 negative donor. While most 6-mo viruses replicated as well as their cognate T/F viruses, differences in replication kinetics

between some paired T/F and 6-mo viruses were also observed (Figure 2). The replication kinetics of the T/F and 6-mo viruses from CH0042, CH0058, CH0077 and CH0569 laid on top of each other (Figure 2A-2D), while those from CH0236 and CH0850 were parallel to each other (Figure 2E and 2F). In contrast, the 6-mo viruses from CH0040, CH0107, CH0162 and CH0470 showed relatively slower replication kinetics than their corresponding T/F viruses (Figure 2G-2J). This suggests that such replication differences *in vitro* might translate into fitness losses significant *in vivo*. These results indicate that overall both T/F and 6-mo viruses from each individual replicated well *in vitro* but some 6-mo viruses appeared replicatively impaired compared to their cognate T/F viruses.

6-mo viruses are less fit than their cognate T/F viruses.

We next determined the relative fitness of 6-mo viruses in a competitive fitness assay using the PASS fitness method as we reported before (17, 50). The competitive fitness assay in which both compared viruses are grown together in the same culture is more sensitive and accurate than the parallel fitness assay in which compared viruses are grown independently (17, 55, 56). Equal amounts (2.5 ng of p24) of each of the T/F and 6-mo viruses from the same individual were mixed together to infect primary CD4⁺ T cells. The mixed viruses initially replicated exponentially as determined by measuring p24 concentrations in the culture supernatants (Figure S1). The viruses harvested from days 1, 3 and 5 or 6 post infection were used to determine the relative fitness of the 6-mo viruses by assessing their relative abundance in the culture using the PASS fitness method. Among 10 pairs of T/F and 6-mo viruses, eight 6-mo viruses were outgrown by their cognate T/F viruses at various paces during the culture (Figure 3A). Three 6-mo viruses (CH0040, CH0107 and CH0470) were almost completely outcompeted by their T/F viruses by day 3 or day 5 (Figure 3A). These 6-mo viruses only accounted for less than 6.5% of the viral population at the end of comparison. Their fitness compared to their cognate T/F viruses was lower by between 24% ± 2% and 50% ± 8%. All three 6-mo viruses also showed relatively slower replication kinetics than their cognate T/F viruses when they were cultured

individually (Figure 2). Five other 6-mo viruses (CH0058, CH0162, CH0236, CH0569 and CH0850) were outgrown by their T/F viruses at a slower pace (Figure 3A). These 6-mo viruses were less fit than their cognate T/F viruses by between $9\% \pm 27\%$ and $57\% \pm 35\%$. The fitness losses were statistically significant for all these eight 6-mo viruses, except the CH0569 6-mo virus for which the fitness loss could not be statistically evaluated because the data did not fit our mathematical model of viral competition (17, 18) although the 6-mo virus was as quickly outcompeted by the T/F virus in all three replicates by day 3 as other 6-mo viruses (Figure 3A).

Statistical differences in fitness were not observed between the T/F and 6-mo viruses for CH0042 and CH0077, although the proportion of the CH0077 6-mo did gradually decrease while its T/F virus continuously increase during the culture (Figure 3A). We previously showed that fitness costs for some mutant viruses could only be detected after the compared viruses were passaged together multiple times (17, 23). Therefore, to investigate whether the CH0042 and CH0077 6-mo viruses were less fit than their cognate T/F viruses, we determined their relative fitness by passaging the mixture of cell-free viruses for each T/F and 6-mo pair to fresh CD4⁺ T cells three times. Both of the CH0042 and CH0077 6-mo viruses were gradually outcompeted during passaging (Figure 3B), as we had previously observed for CH0077 mutants which did not show fitness differences by the single passage fitness assay but had significantly fitness differences when passaged multiple times (17, 23). The results showed that the CH0042 and CH0077 6-mo viruses were $23\% \pm 4\%$ and $143\% \pm 40\%$ less fit than their T/F viruses in the multiple passage fitness assay. These results demonstrate that all ten 6-mo viruses were less fit than their cognate T/F viruses although the fitness losses of the majority of the 6-mo viruses can be detected in the single passage assay while two of them require the multiple passage assay to detect the fitness differences.

Viral fitness losses are associated with low viral loads

Studies have shown the correlation between *in vitro* HIV replication capacity and

the level of plasma VLs in chronically infected individuals (1, 2, 29, 32, 36). However, impacts of viral fitness on viral loads have been studied only with quasispecies viral isolates which were propagated by coculturing PBMCs from both HIV-positive and -negative individuals (1, 2, 29, 32, 36). With well-defined viral fitness and VL data for viruses around the peak of viremia and six months after infection from the same infected individuals, we sought to investigate whether the relative fitness levels of 6-mo viruses with accumulated mutations in the viral genome were associated with the VL levels. The VLs at month 6 decreased about 2 logs (range from 0.52 to 4.63 logs) from the peak of viremia in these 10 participants (Table 2 and Figure S2). To understand how viral fitness costs associated with VL decreases, we determined the levels of VL decrease from the peak of viremia by calculating the quotient between the viremia peak and month 6 VLs (Table 2). When the levels of fitness losses were plotted against the quotients of peak levels over VLs at month 6, the high levels of fitness losses showed a moderate but significant positive correlation with low VLs at month 6 ($R^2 = 0.48$; F-test $p = 0.027$; Figure 4).

Discussion

To understand how accumulated mutations together in the HIV-1 genome affect viral fitness and VLs during early infection, we generated a panel of 10 unique T/F and 6-mo virus pairs in which both were from the same infected individuals. We found that the 6-mo viruses were significantly less fit than their cognate T/F viruses and the fitness losses were associated with low VLs.

Strongly selected mutations generally predominate in the viral genome (>80% of the viral population) by 6 months of infection, although a few of mutations are present at 50% of the viral population (41, 48). However, our analysis of viral sequences shows that all strongly selected mutations are present in the 6-mo viruses. Thus, the 6-mo viruses that were generated by introducing all the strongly selected mutations are real and represent the predominant viruses in the sample (41, 48). All ten 6-mo viruses were less fit than their cognate T/F viruses in the single or multiple passage competitive fitness assays. The fitness costs are most

often caused by T cell escape mutations during acute/early infection but such fitness losses can also be compensated by additional or preexisting mutations (18, 20-23). The 6-mo viruses carried an average of 16 (7-27) point mutations, and these accumulated mutations together in each viral genome had caused a fitness loss. Since the majority of mutations in the 6-mo viruses are predominant or present in all detected viral genomes in most cases, these mutations were included in the 6-mo viral genomes (48). Thus, phenotypes of the 6-mo viruses could well represent the majority of the viruses *in vivo* by the time (41, 48).

T cell responses have been thoroughly analyzed using autologous overlapping peptides based on the whole proteome sequences of the T/F genomes for six (CH0040, CH0042, CH0058, CH0077, CH0162 and CH0470) of the 10 subjects (12, 44). T cell responses were detected in all six subjects and escape mutations were found in most of the identified T cell epitopes. The fitness costs caused by these T cell escape mutations might contribute to the reduced viral loads. Half of these mutations (9 of 18) were found in highly conserved epitopes (Table S2). It is expected that such mutations in the function-restrained regions will cause fitness losses. A few of these T cell escape mutations were confirmed to impair viral fitness in our studies (17, 23). No mutations were also found in about one-third (17 of 46) of the identified T cell epitopes (2.8 epitopes without mutations per each of the six subjects; Table S2). This suggests that these epitopes are still sensitive to the T cell responses so that the T cell responses can exert selection pressure on viral replication and thus lower VLs. Our earlier study also showed that a strongly selected mutation that is independent of T cell responses and neutralizing antibodies can also result in a significant fitness loss (46). Taken together, the reduced viral loads from peak viremia is a combination of effects of fitness-reducing mutations, compensatory mutations and T cell immune responses.

Immune escape mutations often revert back to the consensus sequence of the general HIV-1 population sequences. It has been thought that such reversion mutation may render the viruses more fit. By comparing to subtype B or C consensus sequences, 30 reversion mutations were identified in ten 6-mo viral

genomes (an average of three per virus) after six months of infection (Table S2). We previously studied two reversion mutations (V247I and I64T) in CH0077 (23). The V247I mutation in Gag made the mutant peptide partially less recognized by the T cell response than the wild type TW10 peptide, suggesting that it is a partially resistant mutation. It had no detectable fitness effects on its cognate CH0077 T/F virus but could compensate the fitness loss caused by the T242N mutation in the TW10 epitope in Gag. The I64T reversion mutation in Tat is a synonymous mutation in a T cell epitope in Rev (12). Since it did not change amino acid in the epitope, it could be a simple reversion mutation. However, it did not have any impact on fitness of its cognate CH0077 T/F virus. These results suggest whether a reversion mutation is an escape, reversion or compensatory mutation needs to be experimentally validated.

The VLs by six months of infection were nearly 2 logs lower than those around the peak of viremia, the time when the T/F virus sequences were inferred. Our data show a moderate, but statistically significant, linear correlation between the log VL decrease and viral fitness cost (Pearson's correlation $r = 0.69$, 95% Confidence Interval [0.11, 0.92], Adjusted $R^2 = 0.41$, F-test $p = 0.027$ with residuals normally distributed with Shapiro Wilk $p = 0.73$). This indicates that the significant reduction in fitness of 6-mo viruses may play an important role in lowering VLs during early infection as previously suggested (14-16, 27, 57). The association between fitness costs and VL decreases in this study was clearly demonstrated by analyzing 10 pairs of the T/F and cognate 6-mo viruses in which each pair was from the same individual, rather than by introducing mutations to unrelated viral genomes or by quasispecies viral isolates. Therefore, the direct comparison of viral fitness between the T/F viruses and the corresponding 6-mo viruses that carry the mutations accumulated in the same hosts can help to more precisely determine how the fitness affects VLs.

Funding

This work was supported by National Institutes of Health (NIH) grants [R01AI087520, R01AI028433, R01OD011095]; NIH grants to the Center for

HIV/AIDS Vaccine Immunology [AI067854]; the Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery [AI100645]; the National Natural Science Foundation of China [31670162]; the Key Projects in the National Science & Technology Pillar Program in the Thirteenth Five-year Plan Period [2018ZX10731101-001-010]; Program for JLU Science and Technology Innovative Research Team [2017TD-05]; National Postdoctoral Program for Innovative Talents [BX20180124]; and China Postdoctoral Science Foundation [2018M641786]. Portions of this work were done under the auspices of the U.S. Department of Energy under contract 89233218CNA000001.

Acknowledgments

We thank Barton Haynes for his tremendous support, Beatrice Hahn and George Shaw for making viral sequences and infectious molecular clones available for this study, and Anna Berg for technical support.

Declarations of interest

The authors have no competing interests to declare.

References

1. Quinones-Mateu ME, Ball SC, Marozsan AJ, Torre VS, Albright JL, Vanham G, et al. A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. *J Virol.* 2000 Oct;74(19):9222-33. PubMed PMID: 10982369. Pubmed Central PMCID: PMC102121.
2. Troyer RM, Collins KR, Abraha A, Fraundorf E, Moore DM, Krizan RW, et al. Changes in human immunodeficiency virus type 1 fitness and genetic diversity during disease progression. *J Virol.* 2005 Jul;79(14):9006-18. PubMed PMID: 15994794. Pubmed Central PMCID: PMC1168764.
3. Cong ME, Heneine W, Garcia-Lerma JG. The fitness cost of mutations associated with human immunodeficiency virus type 1 drug resistance is modulated by mutational interactions. *J Virol.* 2007 Mar;81(6):3037-41. PubMed PMID: 17192300. Pubmed Central PMCID: PMC1865994.
4. Chopera DR, Woodman Z, Mlisana K, Mlotshwa M, Martin DP, Seoighe C, et al. Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage. *PLoS Pathog.* 2008 Mar;4(3):e1000033. PubMed PMID: 18369479. Pubmed Central PMCID: PMC2265427.
5. Goepfert PA, Lumm W, Farmer P, Matthews P, Prendergast A, Carlson JM, et al. Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *J Exp Med.* 2008 May 12;205(5):1009-17. PubMed PMID: 18426987. Pubmed Central PMCID: PMC2373834.
6. Crawford H, Lumm W, Leslie A, Schaefer M, Boeras D, Prado JG, et al. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. *J Exp Med.* 2009 Apr 13;206(4):909-21. PubMed PMID: 19307327. Pubmed Central PMCID: PMC2715113.
7. Shakirzyanova M, Ren W, Zhuang K, Tasca S, Cheng-Mayer C. Fitness disadvantage of transitional intermediates contributes to dynamic change in the infecting-virus population during coreceptor switch in R5 simian/human

- immunodeficiency virus-infected macaques. *J Virol.* 2010 Dec;84(24):12862-71. PubMed PMID: 20943985. Pubmed Central PMCID: PMC3004298.
8. Claiborne DT, Prince JL, Scully E, Macharia G, Micci L, Lawson B, et al. Replicative fitness of transmitted HIV-1 drives acute immune activation, proviral load in memory CD4+ T cells, and disease progression. *Proc Natl Acad Sci U S A.* 2015 Mar 24;112(12):E1480-9. PubMed PMID: 25730868. Pubmed Central PMCID: PMC4378387.
 9. Theys K, Libin P, Pineda-Pena AC, Nowe A, Vandamme AM, Abecasis AB. The impact of HIV-1 within-host evolution on transmission dynamics. *Curr Opin Virol.* 2018 Feb;28:92-101. PubMed PMID: 29275182.
 10. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol.* 1994 Jul;68(7):4650-5. PubMed PMID: 8207839. Pubmed Central PMCID: PMC236393.
 11. Borrow P, Lewicki H, Wei X, Horwitz MS, Peffer N, Meyers H, et al. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat Med.* 1997 Feb;3(2):205-11. PubMed PMID: 9018240.
 12. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Gnanapavan V, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med.* 2009 Jun 8;206(6):1253-72. PubMed PMID: 19487423. Pubmed Central PMCID: PMC2715063.
 13. Cohen MS, Shaw GM, McMichael AJ, Haynes BF. Acute HIV-1 Infection. *N Engl J Med.* 2011 May 19;364(20):1943-54. PubMed PMID: 21591946. Pubmed Central PMCID: PMC3771113.
 14. Martinez-Picado J, Prado JG, Fry EE, Pfafferoth K, Leslie A, Chetty S, et al. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol.* 2006 Apr;80(7):3617-23.

- PubMed PMID: 16537629. Pubmed Central PMCID: PMC1440414.
15. Miura T, Brockman MA, Schneidewind A, Lobritz M, Pereyra F, Rathod A, et al. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. *J Virol.* 2009 Mar;83(6):2743-55. PubMed PMID: 19116253. Pubmed Central PMCID: PMC2648254.
 16. Troyer RM, McNevin J, Liu Y, Zhang SC, Krizan RW, Abraha A, et al. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. *PLoS Pathog.* 2009 Apr;5(4):e1000365. PubMed PMID: 19343217. Pubmed Central PMCID: PMC2659432.
 17. Song H, Pavlicek JW, Cai F, Bhattacharya T, Li H, Iyer SS, et al. Impact of immune escape mutations on HIV-1 fitness in the context of the cognate transmitted/founder genome. *Retrovirology.* 2012;9:89. PubMed PMID: 23110705. Pubmed Central PMCID: PMC3496648.
 18. Liu D, Zuo T, Hora B, Song H, Kong W, Yu X, et al. Preexisting compensatory amino acids compromise fitness costs of a HIV-1 T cell escape mutation. *Retrovirology.* 2014;11:101. PubMed PMID: 25407514. Pubmed Central PMCID: PMC4264250.
 19. Arcia D, Acevedo-Saenz L, Rugeles MT, Velilla PA. Role of CD8(+) T Cells in the Selection of HIV-1 Immune Escape Mutations. *Viral Immunol.* 2017 Jan/Feb;30(1):3-12. PubMed PMID: 27805477.
 20. Brockman MA, Schneidewind A, Lahaie M, Schmidt A, Miura T, Desouza I, et al. Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. *J Virol.* 2007 Nov;81(22):12608-18. PubMed PMID: 17728232. Pubmed Central PMCID: PMC2169025.
 21. Crawford H, Prado JG, Leslie A, Hue S, Honeyborne I, Reddy S, et al. Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. *J Virol.*

- 2007 Aug;81(15):8346-51. PubMed PMID: 17507468. Pubmed Central PMCID: PMC1951305.
22. Schneidewind A, Brockman MA, Yang R, Adam RI, Li B, Le Gall S, et al. Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *J Virol.* 2007 Nov;81(22):12382-93. PubMed PMID: 17804494. Pubmed Central PMCID: PMC2169010.
 23. Song H, Hora B, Bhattacharya T, Goonetilleke N, Liu MK, Wiehe K, et al. Reversion and T cell escape mutations compensate the fitness loss of a CD8+ T cell escape mutant in their cognate transmitted/founder virus. *PLoS One.* 2014;9(7):e102734. PubMed PMID: 25028937. Pubmed Central PMCID: PMC4100905.
 24. Boutwell CL, Rowley CF, Essex M. 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. *J Virol.* 2009 Mar;83(6):2460-8. PubMed PMID: 19109381. Pubmed Central PMCID: PMC2648284.
 25. Gao F, Bonsignori M, Liao HX, Kumar A, Xia SM, Lu X, et al. Cooperation of B cell lineages in induction of HIV-1-broadly neutralizing antibodies. *Cell.* 2014 Jul 31;158(3):481-91. PubMed PMID: 25065977. Pubmed Central PMCID: PMC4150607.
 26. Shahid A, Olvera A, Anmole G, Kuang XT, Cotton LA, Plana M, et al. Consequences of HLA-B*13-Associated Escape Mutations on HIV-1 Replication and Nef Function. *J Virol.* 2015 Nov;89(22):11557-71. PubMed PMID: 26355081. Pubmed Central PMCID: PMC4645636.
 27. Murakoshi H, Koyanagi M, Chikata T, Rahman MA, Kuse N, Sakai K, et al. Accumulation of Pol Mutations Selected by HLA-B*52:01-C*12:02 Protective Haplotype-Restricted Cytotoxic T Lymphocytes Causes Low Plasma Viral Load Due to Low Viral Fitness of Mutant Viruses. *J Virol.* 2017 Feb 15;91(4). PubMed PMID: 27903797. Pubmed Central PMCID: PMC5286884.

28. Navis M, Schellens I, van Baarle D, Borghans J, van Swieten P, Miedema F, et al. Viral replication capacity as a correlate of HLA B57/B5801-associated nonprogressive HIV-1 infection. *J Immunol.* 2007 Sep 1;179(5):3133-43. PubMed PMID: 17709528.
29. Trkola A, Kuster H, Leemann C, Ruprecht C, Joos B, Telenti A, et al. Human immunodeficiency virus type 1 fitness is a determining factor in viral rebound and set point in chronic infection. *J Virol.* 2003 Dec;77(24):13146-55. PubMed PMID: 14645571. Pubmed Central PMCID: PMC296087.
30. Kouyos RD, von Wyl V, Hinkley T, Petropoulos CJ, Haddad M, Whitcomb JM, et al. Assessing predicted HIV-1 replicative capacity in a clinical setting. *PLoS Pathog.* 2011 Nov;7(11):e1002321. PubMed PMID: 22072960. Pubmed Central PMCID: PMC3207887.
31. Fraser C, Lythgoe K, Leventhal GE, Shirreff G, Hollingsworth TD, Alizon S, et al. Virulence and pathogenesis of HIV-1 infection: an evolutionary perspective. *Science.* 2014 Mar 21;343(6177):1243727. PubMed PMID: 24653038.
32. Campbell TB, Schneider K, Wrin T, Petropoulos CJ, Connick E. Relationship between in vitro human immunodeficiency virus type 1 replication rate and virus load in plasma. *J Virol.* 2003 Nov;77(22):12105-12. PubMed PMID: 14581547. Pubmed Central PMCID: PMC253754.
33. Arnott A, Jardine D, Wilson K, Gorry PR, Merlin K, Grey P, et al. High viral fitness during acute HIV-1 infection. *PLoS One.* 2010;5(9). PubMed PMID: 20844589. Pubmed Central PMCID: PMC2936565.
34. Wright JK, Novitsky V, Brockman MA, Brumme ZL, Brumme CJ, Carlson JM, et al. Influence of Gag-protease-mediated replication capacity on disease progression in individuals recently infected with HIV-1 subtype C. *J Virol.* 2011 Apr;85(8):3996-4006. PubMed PMID: 21289112. Pubmed Central PMCID: PMC3126116.
35. Prince JL, Claiborne DT, Carlson JM, Schaefer M, Yu T, Lahki S, et al. Role of transmitted Gag CTL polymorphisms in defining replicative capacity and early HIV-1 pathogenesis. *PLoS Pathog.* 2012;8(11):e1003041. PubMed

- PMID: 23209412. Pubmed Central PMCID: PMC3510241.
36. Blaak H, Brouwer M, Ran LJ, de Wolf F, Schuitemaker H. In vitro replication kinetics of human immunodeficiency virus type 1 (HIV-1) variants in relation to virus load in long-term survivors of HIV-1 infection. *J Infect Dis.* 1998 Mar;177(3):600-10. PubMed PMID: 9498438.
 37. Yue L, Pfafferott KJ, Baalwa J, Conrod K, Dong CC, Chui C, et al. Transmitted virus fitness and host T cell responses collectively define divergent infection outcomes in two HIV-1 recipients. *PLoS Pathog.* 2015 Jan;11(1):e1004565. PubMed PMID: 25569444. Pubmed Central PMCID: PMC4287535.
 38. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proc Natl Acad Sci U S A.* 2008 May 27;105(21):7552-7. PubMed PMID: 18490657. Pubmed Central PMCID: PMC2387184.
 39. Abrahams MR, Anderson JA, Giorgi EE, Seoighe C, Mlisana K, Ping LH, et al. Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. *J Virol.* 2009 Apr;83(8):3556-67. PubMed PMID: 19193811. Pubmed Central PMCID: PMC2663249.
 40. Jiang C, Parrish NF, Wilen CB, Li H, Chen Y, Pavlicek JW, et al. Primary infection by a human immunodeficiency virus with atypical coreceptor tropism. *J Virol.* 2011 Oct;85(20):10669-81. PubMed PMID: 21835785. Pubmed Central PMCID: PMC3187499.
 41. Fenton-May AE, Dibben O, Emmerich T, Ding H, Pfafferott K, Aasa-Chapman MM, et al. Relative resistance of HIV-1 founder viruses to control by interferon-alpha. *Retrovirology.* 2013;10:146. PubMed PMID: 24299076. Pubmed Central PMCID: PMC3907080.
 42. Parrish NF, Gao F, Li H, Giorgi EE, Barbian HJ, Parrish EH, et al. Phenotypic properties of transmitted founder HIV-1. *Proc Natl Acad Sci U S A.* 2013 Apr 23;110(17):6626-33. PubMed PMID: 23542380. Pubmed Central PMCID:

PMC3637789.

43. Bar KJ, Tsao CY, Iyer SS, Decker JM, Yang Y, Bonsignori M, et al. Early low-titer neutralizing antibodies impede HIV-1 replication and select for virus escape. *PLoS Pathog.* 2012;8(5):e1002721. PubMed PMID: 22693447. Pubmed Central PMCID: PMC3364956.
44. Liu MK, Hawkins N, Ritchie AJ, Ganusov VV, Whale V, Brackenridge S, et al. Vertical T cell immunodominance and epitope entropy determine HIV-1 escape. *J Clin Invest.* 2013 Jan;123(1):380-93. PubMed PMID: 23221345. Pubmed Central PMCID: PMC3533301.
45. Song H, Hora B, Giorgi EE, Kumar A, Cai F, Bhattacharya T, et al. Transmission of Multiple HIV-1 Subtype C Transmitted/founder Viruses into the Same Recipients Was not Determined by Modest Phenotypic Differences. *Sci Rep.* 2016 Dec 2;6:38130. PubMed PMID: 27909304. Pubmed Central PMCID: PMC5133561.
46. Liu D, Wang C, Hora B, Zuo T, Goonetilleke N, Liu MKP, et al. A strongly selected mutation in the HIV-1 genome is independent of T cell responses and neutralizing antibodies. *Retrovirology.* 2017 Oct 10;14(1):46. PubMed PMID: 29017536. Pubmed Central PMCID: PMC5634943.
47. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994 Nov 11;22(22):4673-80. PubMed PMID: 7984417. Pubmed Central PMCID: PMC308517.
48. Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, et al. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med.* 2009 Jun 8;206(6):1273-89. PubMed PMID: 19487424. Pubmed Central PMCID: PMC2715054.
49. Parrish NF, Wilen CB, Banks LB, Iyer SS, Pfaff JM, Salazar-Gonzalez JF, et al. Transmitted/founder and chronic subtype C HIV-1 use CD4 and CCR5 receptors with equal efficiency and are not inhibited by blocking the integrin

- alpha4beta7. PLoS Pathog. 2012;8(5):e1002686. PubMed PMID: 22693444. Pubmed Central PMCID: PMC3364951.
50. Cai F, Chen H, Hicks CB, Bartlett JA, Zhu J, Gao F. Detection of minor drug-resistant populations by parallel allele-specific sequencing. *Nat Methods*. 2007 Feb;4(2):123-5. PubMed PMID: 17206150.
 51. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science*. 1999 Feb 5;283(5403):857-60. PubMed PMID: 9933172.
 52. Matthews PC, Prendergast A, Leslie A, Crawford H, Payne R, Rousseau C, et al. Central role of reverting mutations in HLA associations with human immunodeficiency virus set point. *J Virol*. 2008 Sep;82(17):8548-59. PubMed PMID: 18596105. Pubmed Central PMCID: PMC2519667.
 53. Ganusov VV, Goonetilleke N, Liu MK, Ferrari G, Shaw GM, McMichael AJ, et al. Fitness costs and diversity of the cytotoxic T lymphocyte (CTL) response determine the rate of CTL escape during acute and chronic phases of HIV infection. *J Virol*. 2011 Oct;85(20):10518-28. PubMed PMID: 21835793. Pubmed Central PMCID: PMC3187476.
 54. Klooverpris HN, Stryhn A, Harndahl M, van der Stok M, Payne RP, Matthews PC, et al. HLA-B*57 Micropolymorphism shapes HLA allele-specific epitope immunogenicity, selection pressure, and HIV immune control. *J Virol*. 2012 Jan;86(2):919-29. PubMed PMID: 22090105. Pubmed Central PMCID: PMC3255844.
 55. Maree AF, Keulen W, Boucher CA, De Boer RJ. Estimating relative fitness in viral competition experiments. *J Virol*. 2000 Dec;74(23):11067-72. PubMed PMID: 11070001. Pubmed Central PMCID: PMC113186.
 56. Wu H, Huang Y, Dykes C, Liu D, Ma J, Perelson AS, et al. Modeling and estimation of replication fitness of human immunodeficiency virus type 1 in vitro experiments by using a growth competition assay. *J Virol*. 2006 Mar;80(5):2380-9. PubMed PMID: 16474144. Pubmed Central PMCID: PMC1395363.

57. Weber J, Gibson RM, Sacka L, Strunin D, Hodek J, Weberova J, et al. Impaired human immunodeficiency virus type 1 replicative fitness in atypical viremic non-progressor individuals. *AIDS Res Ther.* 2017;14:15. PubMed PMID: 28331526. Pubmed Central PMCID: PMC5359922.

Journal Pre-proof

Table 1. Demographic and clinical data from study subjects

Subject	Gender	Clade	Country	Fiebig stage	IMC type	Reference
CH0040	M	B	USA	I/II	T/F, 6-mo	(48) and this study
CH0042	M	C	South Africa	IV	T/F, 6-mo	(42) and this study
CH0058	M	B	USA	I/II	T/F, 6-mo	(48) and (41)
CH0077	M	B	USA	I/II	T/F, 6-mo	(48) and (41)
CH0107	F	C	South Africa	I/II	T/F, 6-mo	This study
CH0162	M	C	South Africa	III	T/F, 6-mo	(42) and this study
CH0236	M	C	South Africa	I	T/F, 6-mo	(41)
CH0470	M	B	USA	IV	T/F, 6-mo	(42) and (41)
CH0569	M	C	South Africa	I/II	T/F, 6-mo	This study
CH0850	M	C	Malawi	I/II	T/F, 6-mo	(41)

Table 2. Correlation between relative fitness of 6-viruses and peak viremia/6-mo VL ratios

Subject	Viral load (vRNA copies/ml)		Quotient (Peak/month 6)	Relative fitness
	Peak viremia	Month 6		
CH0040	2,197,248	29,453	75	-0.24 ± 0.02
CH0042	340,000	102,000	3	0.02 ± 0.02
CH0058	394,649	205	1925	-0.17 ± 0.01
CH0077	179,031	1,680	107	-0.03 ± 0.02
CH0107	>10,000,000	232	>43103	-0.50 ± 0.08
CH0162	>10,000,000	102,793	>97	-0.20 ± 0.02
CH0236	>750,000	209,000	>4	-0.09 ± 0.27
CH0470	264,882	28,456	9	-0.31 ± 0.02
CH0569	1,000,000	324	3086	-0.57 ± 0.35
CH0850	1,200,417	253,675	5	-0.20 ± 0.06

Figure captions

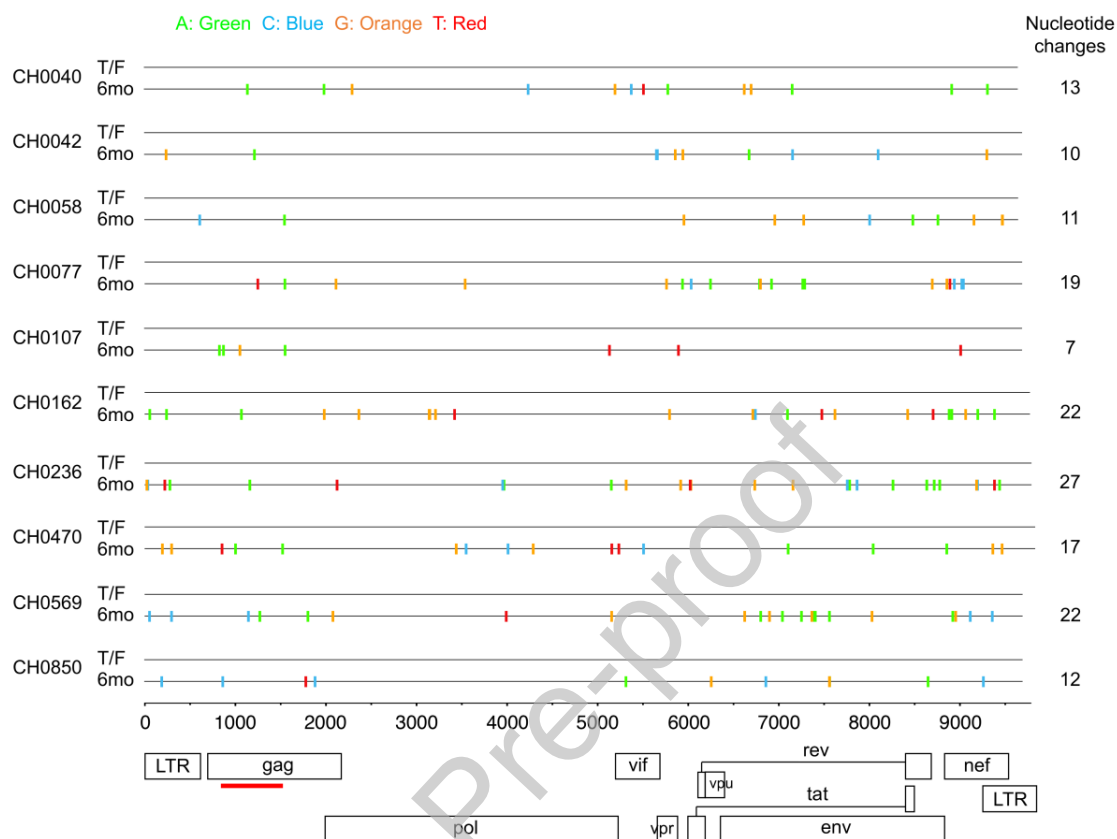


Figure 1. Diagrammatic representation of the nucleotide differences between the T/F and 6-mo IMC genome sequences from the same individuals. The T/F and 6-mo IMC sequences from each subject are represented by horizontal black lines. The horizontal axis indicates nucleotide positions in the comparison beginning at the start of the U3 region of the 5' LTR and extending to the end of the U5 region of the 3' LTR, based on HXB2 reference sequence numbering (<http://www.hiv.lanl.gov/content/sequence/HIV/REVIEWS/HXB2.html>). Nucleotide differences between the T/F and 6-mo IMC sequences are indicated by ticks on the 6-mo sequence, with the color of the tick indicating the base present in the 6-mo genome sequences (A in green, C in blue, G in orange and T in red). The total number of nucleotide differences between each T/F and 6-mo IMC pair is indicated. The red line indicates the region targeted by the sequencing primers in the PASS fitness assay.

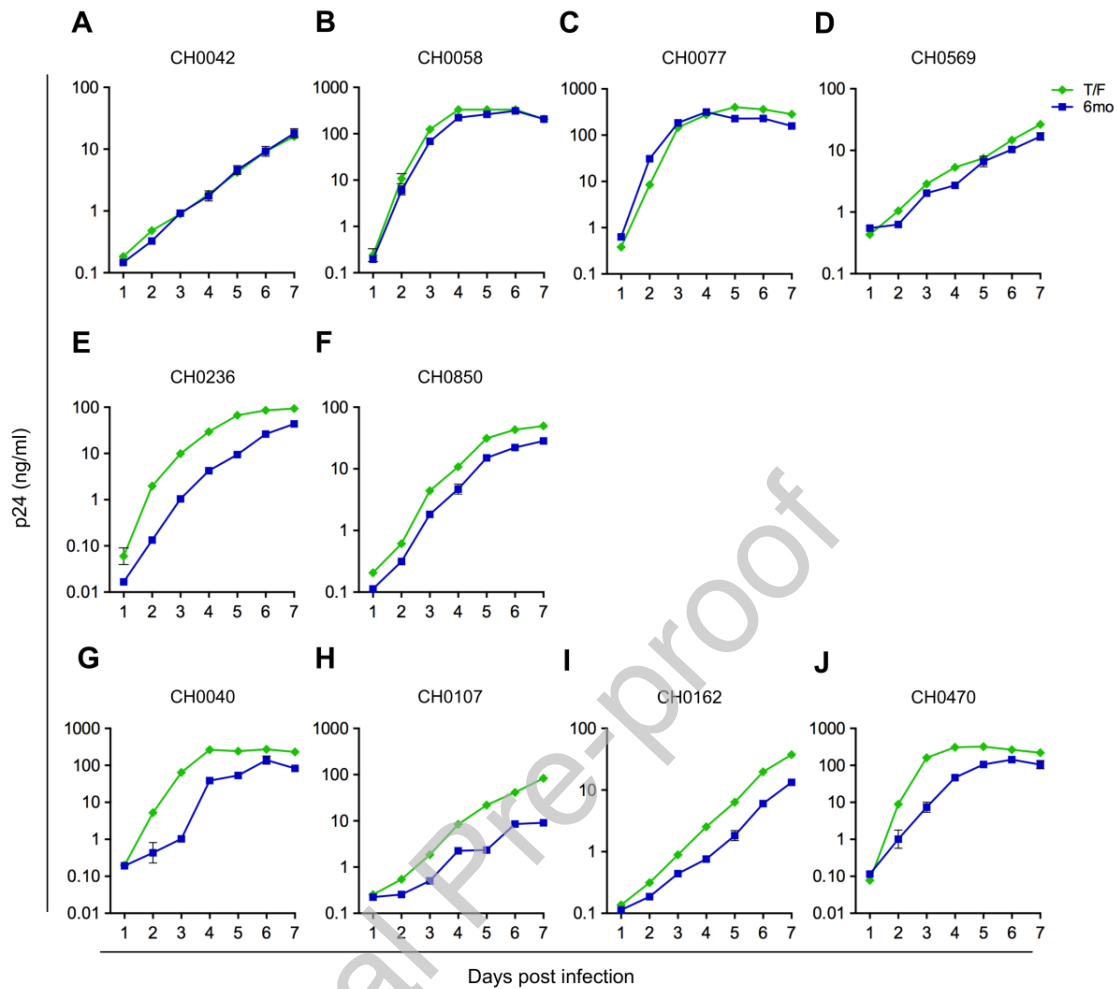


Figure 2. Viral growth dynamics of the 6-mo and T/F viruses from the same individuals. (A-J) The replication kinetics of ten 6-mo viruses and their cognate T/F viruses were determined by measuring p24 concentrations in the cell culture supernatants. Each virus was cultured independently in triplicate. Mean value \pm standard deviation (SD) is shown.

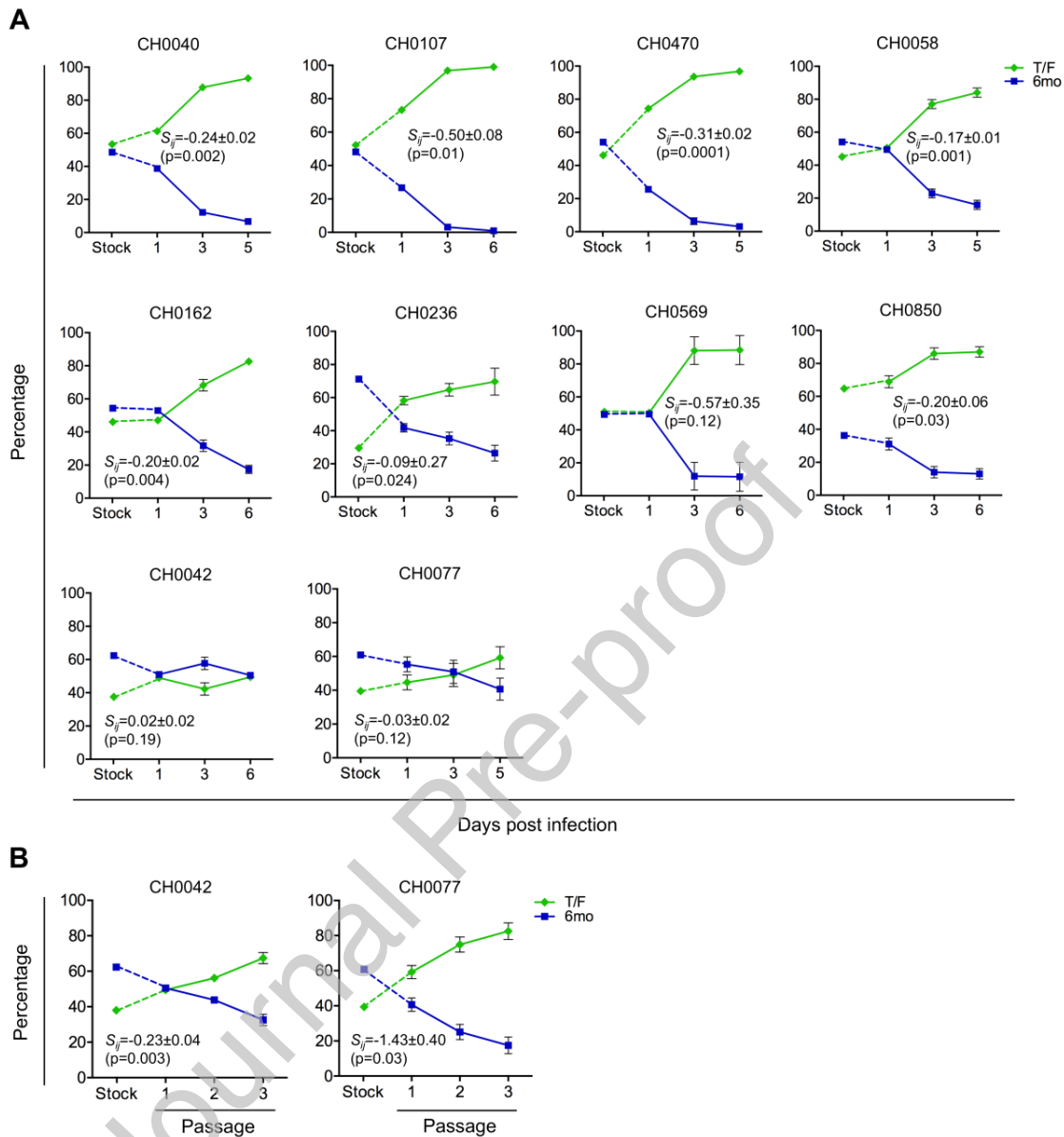


Figure 3. Determination of relative fitness of 6-mo viruses. (A) Equal amounts (2.5 ng p24) of the 6-mo viruses and their respective cognate T/F viruses were mixed to infect freshly purified CD4⁺ T cells, and the proportion of each virus in the culture supernatants at days 1, 3 and 5 or 6 after infection was determined by the PASS fitness assay. (B) Equal amounts (2.5 ng p24) of the 6-mo virus and its cognate T/F virus was mixed to infect freshly purified CD4⁺ T cells. Cell-free viruses were harvested 5 or 6 days after infection, and supernatants were used to infect fresh CD4⁺ T cells for 3 passages. The proportion of each virus in the

culture at the last day of each passage was determined by the PASS fitness assay. All experiments were carried out in triplicate. Mean value \pm SD is shown.

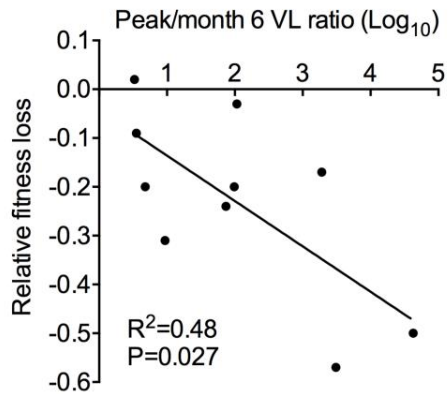


Figure 4. Association between fitness losses and viral load decreases. The quotients were determined between the viral loads between the peak of viremia and month 6, and then plotted against the relative fitness for each T/F and 6-mo virus pairs. The best-fit straight line has an intercept of -0.04 ± 0.08 and a slope of -0.09 ± 0.03 .