High fidelity simian immunodeficiency virus reverse transcriptase mutants have impaired replication in vitro and in vivo

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Abstract

The low fidelity of HIV replication facilitates immune and drug escape. Some reverse transcriptase (RT) inhibitor drug-resistance mutations increase RT fidelity in biochemical assays but their effect during viral replication is unclear. We investigated the effect of RT mutations K65R, Q151N and V148I on SIV replication and fidelity in vitro, along with SIV replication in pigtailed macaques. SIVmac239-K65R and SIVmac239-V148I viruses had reduced replication capacity compared to wild-type SIVmac239. Direct virus competition assays demonstrated a rank order of wild-type > K65R > V148I mutants in terms of viral fitness. In single round in vitro-replication assays, SIVmac239-K65R demonstrated significantly higher fidelity than wild-type, and rapidly reverted to wild-type following infection of macaques. In contrast, SIVmac239-K65R-Q151N was replication incompetent in vitro and in pigtailed macaques. Thus, we showed that RT mutants, and specifically the common K65R drug-resistance mutation, had impaired replication capacity and higher fidelity. These results have implications for the pathogenesis of drug-resistant HIV.

1. Introduction

Antiretroviral therapy (ART) is the mainstay of treatment for people infected with HIV. Suboptimal ART can lead to the emergence of drug-resistant HIV strains, which threatens the efficacy of treatment regimes. The most widely used class of antiretrovirals is the nucleoside/tide reverse transcriptase inhibitors (NRTIs). In vitro studies indicate that some NRTI drug resistance mutants have higher fidelity, making fewer errors during reverse transcription (Menendez-Arias, 2008). Enhanced fidelity of drug-resistant reverse transcriptase (RT) mutations during viral replication may be expected to reduce the ability of the virus to mutate and escape immune or drug pressure (Lloyd et al., 2014). The selection of HIV escape variants from drug and immune pressure is permitted by the rapid accumulation of mutations which provides a wide pool of potentially useful variants (Hu and Hughes, 2012). HIV mutation is affected by numerous factors but a key driver is the error-prone reverse RT (Smyth et al., 2012). The low fidelity of the HIV RT is exploited by NRTIs that mimic natural nucleosides or nucleotides, halting DNA polymerisation upon incorporation (Menendez-Arias, 2008). Tenofovir is one of the most commonly prescribed antiretroviral medications for both treatment and prevention of HIV (Thompson et al., 2012; Grant et al., 2010). The K65R mutation confers resistance to tenofovir, emtricitabine and other widely used NRTIs (Menendez-Arias, 2008; Feng et al., 2006). K65R also decreases the affinity of the RT enzyme for both natural dNTPs and NRTIs resulting in less incorporation of the drug (Garforth et al., 2007). Interestingly, this decreased dNTP affinity makes the mutant enzyme less likely to incorporate incorrect natural nucleotides in biochemical assays (Shah et al., 2000; Mansky et al., 2003). Results of biochemical analyses for RT fidelity have led to the conclusion that HIV containing the K65R mutation has a higher fidelity of replication (Shah et al., 2000; Garforth et al., 2010). However, the effect of K65R on fidelity has not been examined during viral replication,
and biochemical assays often employ purified RT or DNA reporter gene sequences that are not part of the HIV genome (Bebenek and Kunkel, 1995). The recent development of next-generation sequencing technologies has led to important insights into HIV diversity (Yin et al., 2012; Rawson et al., 2015) and has now made it possible to explore replication fidelity during infection.

Despite the limitations of measuring RT fidelity in biochemical assays, such studies have suggested that alterations to the fidelity of RT is detrimental to viral fitness (Lloyd et al., 2014; Dapp et al., 2012). Viral fitness can be defined as the replication capacity for a virus in growth competition experiments in the context of a defined environment (Lauring et al., 2013). Drug-resistant strains have a fitness advantage in the presence of drugs; however, they often lack the same replicative capacity of drug-sensitive strains, as indicated by emergence of compensatory mutations in resistant viruses (Deuzing et al., 2014) or reversion back to wild-type (WT) previously higher fitness in cell free assays. However, the Q151N mutation (glutamine to methionine), which has been shown to have marginally higher fidelity in cell free assays. However, the Q151N mutation (glutamine to asparagine) (Weiss et al., 2000), has a reported fidelity 13-fold higher (Weiss et al., 2004) than WT, which is even higher than K65R (Weiss et al., 2000; Kaushik et al., 2000). K65R and Q151N are both located in highly conserved motifs in SIV. The V148I is not an NRTI escape mutant and was instead discovered in a natural late-stage SIV infection in conjunction with three other RT mutations K173R, S211G and Y302F that may be required for enhanced viral fitness (Diamond et al., 2001, 2003). The fidelity of the V148I alone has been shown to be 8.7-fold higher than WT in reporter gene assays. Additionally, its discovery in SIV makes it an ideal candidate for study in the SIV model (Diamond et al., 2003). Whether the increased fidelity of RT mutations observed in biochemical assays is also observed during virus replication is unclear.

We hypothesised that SIV RT mutations leading to a higher fidelity of replication will have a negative impact on viral fitness. We evaluated the fidelity of important K65R drug resistance RT mutation using next-generation sequencing of a cell infection based model. We further assessed the fitness of the RT mutants K65R, Q151N and V148I mutation in cell-based assays and pig-tailed macaques.

2. Results

2.1. Replication kinetics of SIV RT mutants

Despite the importance of RT fidelity to HIV pathogenesis, most analyses of HIV or SIV RT fidelity have used biochemical assays that may not reflect the complexity of reverse transcription in the context of in vivo systems. To assess the influence of RT fidelity in vitro and in vivo we constructed six SIV variants containing potential high-fidelity variants of RT: K65R, Q151N, V148I or the combined V148I-K173R-S211G-Y302F (IRGF) mutations as well as combined K65R+Q151N and K65R+Q151N+V148I. To assess their replication capacity, we cultured them in a human CD4+ lymphoblast line: CEM.NK.CCR5 (CEM), and determined viral growth kinetics by RT-qPCR. K65R, V148I and IRGF infected CEM cells, with supernatant viral RNA levels increasing over time in culture (Fig. 1). Viruses carrying Q151N (data not shown for SIVmac239-K65R+Q151N and SIVmac239-K65R+Q151N+V148I) could not infect cells, with supernatant viral RNA levels barely above the limit of detection for the assay. K65R, V148I and IRGF all replicated at levels significantly lower than WT at peak infection (day 5, p=0.004, 0.001 and 0.004 respectively).

2.2. Comparative fitness of SIV RT mutants

Since the replication kinetic assays suggested significant differences across the RT mutant SIV strains, to directly compare the relative fitness of the SIV RT mutants, we performed viral competition assays. Both 50:50 and 95:5 ratios of pairs of viruses were used. We focussed on the SIV harbouring RT mutations K65R, V148I and IRGF that were replication competent (Fig. 1). For competition assays starting with 50:50 ratio of virus, the SIVmac239 virus was able to outcompete each of the mutants (Fig. 2A, C, E, G, I and K). The K65R mutant virus outcompeted both V148I-containing mutants. The V148I single mutant virus showed a trend of being slightly more replication competent compared to virus harbouring the IRGF RT mutations (Fig. 2K and L). Similar indications of fitness were obtained with competition experiments initiated with a 95:5 ratio of viruses (Fig. 2B, D, F, H, J and L), however at this ratio, the WT did not overcome K65R during the 7 days culture and K65R could not outcompete the V148I mutants.
Fig. 2. Growth competition assays of mutant and WT virus. Ratios of SIV WT and mutant viruses were co-cultured in CEM.NKcCCR5 cells. Media was harvested over a time-course and viral RNA was extracted, converted to cDNA, amplified and Sanger sequenced. The percentage of mutant to WT virus was quantified from Sanger data using MacVector software. (A) 50/50 ratios. (B) 95/5 ratios. (C) The changing ratio of the viruses was quantified by MacVector software by using the equation $P_n = M_n / (W_n + M_n)$ where $P_n$ represents relative proportion of the mutant at day $n$, $W$ represents WT peak at mutation site and $M$ is the mutant peak height at the mutation site.
Fig. 3. Elucidation of WT and mutant SIV fidelity as determined in cell-based assays. CEM.NKCr.CCR5 cells were either infected separately or co-infected with SIVmac239 and SIVmac239-K65R during a single round of replication. (A) Outline of co-infection fidelity assay with SIVmac239 and SIVmac239-K65R where the K65R region only is amplified for sequencing and analysis. (B) Outline of fidelity assays performed in cells separately infected with SIVmac239 and SIVmac239-K65R where the whole genome was amplified for sequencing and analysis. (C) Overall mutation rates derived for both K65R and WT co-cultures. Co-infection cultures analysed at n=6. Statistical significance determined by paired, nonparametric, Wilcoxon matched-pairs signed rank test. (D) Overall mutation rates derived for both K65R and WT cultured in separate wells. Separate cultures at n=6 with statistical values determined by an unpaired, nonparametric Mann Whitney test. (E) The number of K65R and WT reads used in the analysis of mutation rates in the co-cultures. (F) The amount of viral gag cDNA transcripts present in gDNA preparations from infected CEM.NKCr.CCR5 cells.
completely. Taken together, these data indicate that all mutant viruses were less fit than WT. SIV containing V148I alone or together with the IRGF mutations were also outcompeted by K65R at 50:50 ratios (Fig. 2G and I). The overall “fitness hierarchy” was therefore WT > K65R > V148I ≈ IRGF.

2.3. Fidelity of SIVmac239-K65R in vitro

To determine RT fidelity during viral replication, the number of mutations across the SIV genome during viral replication was analysed by deep sequencing of SIV DNA. The short infection assay allows only one round of replication to remove variation in the number mutations that might occur due to slower replication of the mutant. This allows an equal number of transcripts to be analysed for WT and K65R DNA. Similarly, there is an upper limit on the number of transcripts that may be analysed during high throughput sequencing and the cDNA levels produced during the fidelity cultures more than exceeded this. SIV harbouring the K65R mutation and the WT virus were used to infect separate or the same cell cultures (Fig. 3A and B). The most rigorous analysis was co-infection of cells with WT and the K65R mutant viruses since the samples were processed together, thereby eliminating variables associated with separate wells, cellular and viral DNA harvesting, and PCR. In these experiments an 800 bp fragment spanning the K65 codon was PCR amplified and sequenced. Only reads that contained K65 or K65R were analysed, so that any additional mutations could be characterised as resulting from the WT or the K65R virus. For cells infected with either WT or the K65R mutant, approximately 9 kb of the virus was sequenced, where the 5’ long terminal repeat sequence between 0 and 1200 bp was omitted to enable clear genome alignment during analysis.

The co-infection experiment reads containing K65R had a lower mutation rate (higher fidelity) than reads containing K65 (WT) in all 6 replicates (Fig. 3D). Mutation rates were determined by the number of single nucleotide polymorphisms present in the reads. This analysis compares paired samples of K65R containing reads with those containing WT (K65) reads in the same co-infection experiment and the result showed a significantly reduced mutation rate due to K65R ($p=0.031$) (Fig. 3C). The reduced mutation rate after this single round of replication was on average 14% less in the K65R containing reads compared to the WT reads. In the separate cultures infected with the K65R mutant or WT virus, we observed a range of mutation rates across the 6 replicates of each viruses. Although there was on average a 9% lower mutation rate in the K65R cultures compared to WT, analysis of unpaired samples, since the cultures are separate, provided a result that was not significant ($p=0.13$). Overall, the results of these in vitro infection assays are consistent with the reduced fidelity of particular RT mutants observed in previous biochemical assays although the magnitude of the difference observed was much lower (Shah et al., 2000; Mansky et al., 2003; Garforth et al., 2010).

It is possible that the observed lower replication fitness of the RT mutant viruses is due to the mutant RTs functioning at a suboptimal level and poorly reverse transcribing viral RNA into DNA
in infected cells. The lower level of RT function may lead to less mutations independent of fidelity. To address this in these single round replication experiments, we first analysed the number of reads of WT and K65R in the 6 co-cultures (Fig. 3E) and found no significant differences. We then also quantified the cDNA by PCR in the WT and K65R separate cultures and found no significant differences in the generation of cDNA from the 2 viruses across the 6 cultures (Fig. 3F, representative of 2 experiments). We acknowledge that small difference in RT function may not be detected by this single round infection experiments at 6 replicates and concede that small differences in transcribing RNA to cDNA between the RTs could still contribute to the replication and fitness differences observed.

Table 1
Percentage of K65R and wt virus in animals K65R-1 and K65R-2.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>7</th>
<th>10</th>
<th>14</th>
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<tr>
<td>Animal K65R-1</td>
<td>K65R</td>
<td>100%</td>
<td>99.8%</td>
<td>0.7%</td>
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<td></td>
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<td>Wt</td>
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<tr>
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<td>K65R</td>
<td>100%</td>
<td>50.6%</td>
<td>12.7%</td>
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<td></td>
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<td>Wt</td>
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2.4. Infection of pigtailed macaques with SIVmac239 RT mutants

The growth assays above suggest a significant impairment of replication of the SIV RT mutants in cell culture-based assays. To determine the infectivity of the mutants in vivo, seven animals were inoculated intravenously with SIVmac239 (WT n = 3, K65R n = 2 and Q151N n = 2 as a non-infectious control). Animals infected with WT or SIVmac239-K65R (n = 5/7) developed a peak of viral plasma viral load within 2 weeks of infection (Fig. 4). One of the 3 SIV-infected animals subsequently controlled SIV replication (indicated with a hexagon). The 2 animals infected with Q151N had no detectable plasma SIV RNA, suggesting a lethal impairment to replication and consistent with the in vitro replication studies. Of the 2 animals infected with the SIV K65R mutant, one subsequently controlled the virus, while the other had a persistent viral load.

To determine if the K65R mutation was maintained or reverted during infection, viral RNA from plasma was sequenced over the viral load. In both animals K65R rapidly reverted to WT during infection, viral RNA from plasma was sequenced over the 14 days post-infection. We also showed that the Q151N mutation ablated growth in vitro and prevented infection in pigtailed macaques. The V148I and combined IRGF mutations were both functional, although they significantly reduced replication capacity compared to both SIVmac239 and the K65R mutant.

SIVmac239 with the Q151N mutation could not establish an infection in vitro and was not able to infect pigtailed macaques in vivo. In addition, we found that the Q151N mutant could not transcribe RNA into cDNA (not shown). The Q151N mutation has not been studied in SIV to date, and the differences between SIV and HIV may have led to the Q151N dysfunction, despite the codon of interest lying in a highly conserved region of RT. Also, our mutagenesis procedure necessitates the introduction of Q151N into both subunits of RT, which could have impaired the virus's function. Overall, the Q151M (rather than the Q151N) mutation is a comparatively more common (Feng et al., 2006; Shirasaka et al., 1995) multi drug-resistant mutation in the clinic. The Q151N mutation (glutamine to asparagine) is much less common and was generated in the context of mutagenesis studies and in our studies results in a non-functional virus in vitro and in vivo.

The K65R mutation on the other hand, has been previously observed in macaque infections following tenofovir therapy (Van Rompay et al., 2007). The appearance of K65R in the continued presence of tenofovir treatment preceded a rebound of viremia; however the plasma viral RNA levels were approximately 10-fold lower in animals infected with the K65R SIV mutant compared to animals infected with WT SIV (Van Rompay et al., 2007). This suggests a likely fitness cost resulting from K65R that is consistent with the fitness cost that we observed. If the virus is maintained artificially through the presence of drugs, it does not replicate as well, even in vivo. In the context of increasing ART usage and pre-exposure prophylaxis, where tenofovir and emtricitabine are commonly prescribed, the K65R mutation may become more prevalent, especially if adherence problems lead to sub-optimal drug exposure (Hamers et al., 2012; Sunpath et al., 2012; Skhosana et al., 2015). While the K65R mutation leads to a fitness cost, infection with SIV strains containing the K65R mutation is possible in rhesus macaques (Tsegaye et al., 2015; Cong et al., 2013). The rapid reversion of the SIV K65R mutation we observed in two infected pigtailed macaques illustrates the in vivo fitness cost of this mutation.

Overall, our results do support previous studies, which suggest that changes in viral replication fidelity lead to a fitness cost (Dapp et al., 2012; Furio et al., 2007), however a link between the two will need to be formally demonstrated in future studies. Previous studies report the reversion of high-fidelity NRTI-resistant HIV in patients, suggesting reduced fitness (Brodard et al., 2005; Zaccarrelli et al., 2004). Similarly, the existence of compensatory mutations that partially restore fitness provide yet more evidence for the interrelationship between fidelity and fitness. K65R and Q151M in particular have a variety of compensatory mutations that have been characterised in both HIV-1 and HIV-2 (Feng et al., 2006; Svarovskaia et al., 2008; Cong et al., 2007; Trevino et al., 2011). This decrease in fitness is likely due to a lower proccessivity of RT and a lowered affinity for dNTPs (Garforth et al., 2007). This is supported by the finding that an increased dNTP concentration during K65R RT (−)ssDNA synthesis shows similar efficacy to WT RT (Xu et al., 2009). It is important to note that cell lines, such as
the CEM.NKr.CCR5 we studied, have a higher level of dNTPs compared to primary CD4\(^+\) cells (Heinemann et al., 1990; Amie et al., 2013) and it will be important to study primary CD4 T cells in future work. High levels of dNTPs can potentially abrogate the lowered affinity of mutant RTs, providing a relative advantage to SIV RT mutants in cell line cultures (Xu et al., 2009). In vivo, higher fidelity will also potentially reduce evolutionary potential, as it would lead to fewer variants being produced during replication. While it would have been interesting to investigate the fidelity of SIV\textsubscript{mac239}-K65R in vivo by deep sequencing technologies as was performed in vitro, obtaining such data in this study was prohibited by (a) the rapid reversion of K65R (Fig. 4) and (b) our inability to obtain a sufficient amount of viral products from early time points with low viral load (Fig. 4). The different growth rates of the viruses we observed in vitro (Fig. 1) would further complicate the analysis of such data, as fewer mutations may have been a result of fewer rounds of replication, rather than a lower fidelity. Studying SIV\textsubscript{mac239} K65R fidelity in macaques in the presence of tenofovir therapy would partially address this in future studies, although if differences in growth rates were observed this would complicate analyses of mutation rates (Fig. 5).

Although many studies have explored the fidelity of these NRTI-resistant mutations in vitro, to our knowledge this is the first use of next-generation sequencing to analyse the mutation rate of the drug-resistant K65R mutation in a cell-based system, rather than with purified RT kinetics-based assays or a reporter gene. The data obtained with this deep sequencing illustrate the decrease in mutation rate caused by the K65R mutation in SIV RT, after a single replication cycle, more thoroughly than conventional sequencing. The use of a cell-based system and direct analysis of mutations in the viral genome is likely to be more reflective of infection than reporter gene assays. Interestingly, the reported fidelity of HIV–K65R varies widely depending on the assay used. Reporter gene assays give the highest reported fidelity of approximately 8-fold greater than WT (Shah et al., 2000), while a cell-based reporter gene assay suggests a 1.51-fold higher fidelity for K65R compared to WT. Our finding that the K65R mutation’s modest fidelity increase is more in line with the cell-based reporter system, rather than the cell-free system. This may indicate that fidelity differences are less dramatic in systems that more accurately reflect natural infection (Warrillow et al., 2009). In this regard there are studies that have proposed that overall fidelity of WT RT is higher in vivo than in vitro data would suggest (Abram et al., 2010; Mansky and Temin, 1995).

We are confident that the results reflect true differences in mutation rate from the mutant RTs of the viruses above background mutation rates since the in vitro infection-based fidelity assay outlined here contained two layers of control. First, amplicon products were made from the original WT and K65R plasmids used in the transfection to produce virus particles. Amplicons were produced from separate and combined plasmids for the individual and co-cultured fidelity samples, respectively. Second, cellular DNA from uninfected CEM.NKr.CCR5s were prepared by the same method as experimental amplicons with equivalent plasmid amounts spiked into the lysate during the final stages of extraction. Amplicons were produced from these mock gDNA samples, with separate and combined plasmids as above. This level of analysis of background mutation rates allowed us to compare the mutation rates between the WT and K65R RTs.

Co-infection of cells with the WT and mutant viruses allowed mutations to be linked directly to reads containing either K65R and WT, while minimising confounding factors such as PCR-induced errors or differences been genomic DNA preparations. A limitation of the co-infection studies we used is the relatively short reads can be analysed with the Illumina sequencing technology employed. More advanced technologies allowing longer reads from individual viruses to be studied would allow a greater in depth analysis of mutation rates. There is the potential for recombination and APOBEC-induced hypermutation to be further analysed with longer reads. Indeed, RT mutations and changes in fidelity may well impact both recombination and hypermutation as additional sources of HIV and SIV sequence variation (Sadler et al., 2010; Delviks-Frankenberry et al., 2011; Monajemi et al., 2012).

In conclusion, our results show that the important K65R RT mutation, in the setting of SIV, has reduced replication capacity, reduced fidelity, and reverts rapidly in macaques during acute infection. Understanding the relationship between NRTI resistance, fidelity and viral fitness could lead to a better grasp of HIV evolution and assist in efforts to minimise drug resistance and control the spread of HIV.

4. Methods

4.1. Animals

Seven juvenile male pigtailed macaques (Macaca nemestrina) were exposed to WT or mutant SIV\textsubscript{mac239} by either an intramuscular injection of 300 mg of the pKP55-SIV\textsubscript{mac239} expression plasmid as previously described (Kent et al., 2001b), and/or intravenous delivery of virus particles or virus particles with infected CEM.NKr.CCR5 cells produced through the cell culture techniques described below. Serial blood samples were taken from the animals over 24–30 wks. Macaques were sedated with ketamine during all procedures and the Commonwealth Scientific and Industrial Research Organisation Animal Health Animal Ethics Committees approved all studies.

4.2. Cell line and virus sources

The pKP55-SIV\textsubscript{mac239} plasmid was a generous gift from Professor Damian Purcell, originally obtained from Dr Keith Peden (Kent et al., 2001a). CEM.NKr.CCR5 cells were sourced from National Institute of Health AIDS reagent repository.

4.3. Generation of mutations in SIV\textsubscript{mac239} reverse transcriptase

Mutations were introduced to the RT sequence of the pKP55-SIV\textsubscript{mac239} (Genebank: M33262.1) expression plasmid using a “seamless cloning” approach, where 772 bp of DNA containing the mutated regions (from nucleotides 3281 to 4053) was inserted into a construct containing SIV sequence with this region removed. DNA cassettes containing mutations (comprising SIV\textsubscript{mac239} residue 3281–4053) were synthesised containing the mutations by DNA 2.0 (Menlo Park, CA, USA). The regions from nucleotides 2161–3281, 3281–4053 and 4053–5382 were arbitrarily termed A, MID and B respectively. Restriction sites (BspQ1 and HindIII) were added 3’ of B and 5’ of A using PCR. A and B were cloned separately into pAcquire (Alchemiy Biosciences, Melbourne, VIC). Fragment A was isolated as an insert sub-cloned into linearised pAcquire-r-B, to form an A–B construct, which served as a vector into which the synthetic DNA was subcloned in, via “seamless cloning”. This resulted in the removal of the HindIII and BspQI sites and established an A-MID-B cassette where the MID fragment was girt by A and B. This cassette was isolated by restriction digest and subcloned into pKP55-SIV\textsubscript{mac239}. All constructs were fully sequenced to ensure no extraneous mutations were added during the cloning process. All restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA).
4.4. Virus production in HEK 293T cells and CEM.NKrtCCR5 infection

Five or 15 μg of plasmid was used to transfect confluent HEK293T cells in T25 or T75 cell culture flasks, respectively, using Fugene (Promega, Wisconsin, USA) according to the manufacturer’s instructions. Cells were washed with PBS 24 h post-transfection to remove plasmid. 48 h Post-transfection, supernatant was harvested and treated with Benzonase (250 U/ml) (Sigma Aldrich, Saint Louis, MO, USA), and DNase I (Worthington, Lakewood, NJ, USA) for 45 min at 37 °C to remove remaining plasmid. Virus was then used to infect CEM.NKrtCCR5 cells. Cells were infected with equal amounts of virus (1.4 × 10^4 copies of gag RNA), as determined through a viral load qPCR reaction on DNase-treated (Ambion, Carlsbad, CA, USA) transfection supernatant as previously described (Mason et al., 2009). CEM.NKrtCCR5 cells (2 × 10^5) were spinoculated with virus in 200 μl of RPMI/10%FCS/Penicillin-Streptomycin-Glutamine for 2 h at room temperature at 1200 g. Virus was washed away from cells 24 h post-infection and culture supernatants were collected after a day – 1 wash on days 1, 3, 5, and 7 post-infection.

4.5. SIV quantification

SIV RNA (viral load) was quantified as previously described, with some modifications (Mason et al., 2009). Fresh, EDTA-treated whole blood was centrifuged at 950 × g for 12 min at room temperature. Plasma was recovered and stored at −80 °C. Alternately, cell culture media was used during in vitro assays. In all cases, RNA was isolated using a QiAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. SIV viral load was determined by qPCR by a Superscripts III RT-PCR reaction (Invitrogen, Carlsbad, CA, USA) using Taqman Universal PCR Mastermix (Applied Biosystems Inc., Foster City, CA, USA). A 142 bp product in SIV gag was amplified using the primers 5′-ATTAGTAGGTGGACAGAACACG-3′ (forward) and 5′-CACCAGATGGACACACAGATAT-3′ (reverse) so that sequences from 1269 to 10405 and WT virus, were incubated as above. For these co-infections, a single product was amplified over the K65 region of SIV RT (primers: 5′-CTCTAGACCATGTTGGACGCT-3′ forward and 5′-CAGCTCCTCTTGGCTGGCAACT-3′ reverse) so that sequences from either the WT or K65R input virus could be analysed.

4.6. Enzyme linked immune-sorbent assay

ELISA plates were coated with whole AT-2 inactivated SIVmac239, kindly supplied by Dr Jeff Lifson (Mason et al., 2009), at 100 ng/well overnight. Plates were blocked with 5% skim milk powder in PBS/0.1% Tween for 1 h. After washing, dilutions of heparinised macaque plasma were added and incubated for 4 h. After washing away plasma, HRP-conjugated rabbit anti-human IgG (P0214 Dako, North Sydney, NSW) was incubated for 1 h. ELISAs were visualised using TMB (Thermo Fischer Scientific, Rockford, IL, USA). The TMB reactions was stopped with the addition of 100 μl of 1 M sulphuric acid and quantified on a CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany).

4.7. Viral competition assays

To assess relative viral replicative capacity, 50:50 and 95:5 ratios of two virus combinations were prepared and validated by Sanger sequencing. Prepared virus ratios were co-cultured in competition assays as previously described (Telwatte et al., 2015) with some modifications. Briefly, 2 × 10^5 CEM.NKrtCCR5 cells were infected with 100 μl of the combined viruses by 1 h spinoculation at 1200 × g at room temperature. Virus was washed out at 12 h post-infection and cell culture media samples were collected at 1, 2, 3 and 7 days post-infection. Viral RNA was extracted, converted to cDNA, and amplified by PCR with the SIV RT forward primer 5′-GATGGTCAGTTGGAGGAAGCTC-3′ and reverse primer 5′-CAGGCTCTCTTGTGGCAACT-3′. PCR products were purified and Sanger sequenced. All virus ratios were quantified by MacVector software (version 13.0.4, MacVector, Inc., Cary, NCA, USA) as previously described (Carr et al., 2009) by using the equation \( P_n = M \frac{W_n - M_n}{W_n} \) where \( P_n \) represents relative proportion of the mutant at day \( n \), \( W \) represents WT peak at mutation site and \( M \) is the mutant peak height at the mutation site. Where relevant in homopolymeric runs, pure virus controls were sequenced and used to remove background signal. Independent assays were performed at \( n \geq 3 \) for each condition.

4.8. In vitro fidelity cultures

In vitro fidelity during viral replication was assessed in a single round infection of cells with SIV viral particles containing either WT RT or the K65R mutation. The virus undergoes reverse transcription, cellular DNA is harvesting and SIV DNA subjected to deep sequencing. Briefly, 1.5 × 10^6 CEM.NKrtCCR5 cells were infected with equal amounts of SIVmac239-K65R or WT (1 × 10^11 copies of gag RNA), as determined by qPCR. Virus was incubated for 18 h before being washed away from cells. Supernatant was removed and cells were resuspended in 200 μl PBS. Genomic and viral DNA was then harvested from cells using a Qiagen DNA mini kit according to the manufacturer’s instructions. Separate transfections were performed for each well. Six virus products, spanning the SIV genome from 1209 to 10405 bp, were PCR amplified from the harvested DNA using Phusion™ high-fidelity taq polymerase (New England Biolabs, Ipswich, MA, USA) according to the manufacturer’s instructions, conditions available upon request. Additionally, cells co-infected with equal amounts of the K65R mutant and WT virus, were incubated as above. For these co-infections, a single product was amplified over the K65 region of SIV RT (primers: 5′-CTCTAGACCATGTTGGACGCT-3′ forward and 5′-CAGGCTCTCTTGGCTGGCAACT-3′ reverse) so that sequences from either the WT or K65R input virus could be analysed.

4.9. Deep Sequencing

Samples for deep sequencing were prepared using the Nextera XT DNA sample Preparation kit (Illumina, San Diego, CA) according to the manufacturer’s instructions as previously described (Gooneratne et al., 2014), with some modifications. Amplicon concentrations were determined using a Qubit dsDNA HS Assay Kit according to the manufacturer’s instructions. Samples were pooled by genome to ensure even copy numbers of each amplicon. Pooled samples were fragmented into pieces ranging from 177–2733 bp (average 621 bp) and simultaneously tagged using an engineered transposome. The single co-infection PCR product was fragmented to an average of 442 bp. The PCR step was reduced to 9 cycles and 3 ng was used as the initial input to the protocol to minimise resampling.

4.10. Bioinformatics and statistical analyses

The sequence data was processed using Geneious (Version 7.1.9). The forward and reverse paired-end reads were converted to the same relative orientation using the Illumina NexteraXT Forward/Reverse short read kit and the pooled reads were trimmed using an error probability limit of 0.005. The trimmed reads were then mapped to the reference genome for either the WT SIVmac239 or the K65R variant (GenBank: M33262.1). All single-nucleotide polymorphisms (SNPs) were identified, regardless of the coverage.
or variant frequency. Despite the Geneious trimming process, a small subset of reads per sample (average of ~1% of reads across all samples) exhibited an unexpected high number of SNPs at the read ends. Therefore an additional quality control was applied to remove reads with more than 4 SNPs at the ends of the reads. For the sequences obtained from the separated K65R and WT cultures, the mutation rates were determined as the ratio of mismatch SNPs to all nucleotides across all reads per sample. The six K65R and six WT mutation rates were compared using a Mann–Whitney test. For the sequences obtained from the co-cultured K65R and WT, the reads were identified as K65R or WT based on the nucleotide at genome reference position 3301 (A for WT and G for K65R). If a read did not span position 3301, or had a nucleotide other than A or G at this position, it was deemed uninformative and excluded from further analysis. The K65R and WT reads were then realigned against the K65R and WT reference genomes, respectively, to identify all SNPs. Six paired K65R and WT mutation rates were calculated from the identified mismatches and compared using a Wilcoxon test. T tests were performed on both the amount of virus gag transcripts harvested from separate CEM.NKrCCR5 cell gDNA preparations and the number of K65R and WT reads obtained from co-cultures. The statistical analysis was performed using Graphpad Prism 6.

Statistics for growth assays were performed using SPSS software version 18, (IBM, Armonk, NY, USA). The significance of the differences between the replication of WT and mutant SIV was analysed using a Kruskal–Wallis test at day 5, which is usually peak infection. This was followed by 4 Mann–Whitney U tests, comparing each mutant to WT singly, and a Bonferroni correction, leading to a new alpha level of 0.0125.

Acknowledgements

We thank Dr. Robert Center, Prof Damian Purcell and Dr. Keith Peden for SIV reagents and Sushama Telwatte for expert advice. This work was supported by Australian NHMRC Fellowships (1013221 to WRW and 1041832 to SJK), NHMRC Program grant 510448 and an Early Career Researcher grant from the University of Melbourne.

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