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HIV 2-long terminal repeat circular DNA is stable in primary CD4+T Cells

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ABSTRACT

Treatment resistant latent reservoirs remain a barrier to cure HIV, but the maintenance and properties of these reservoirs are not completely understood. 2-LTR circular HIV DNA has been used to assess ongoing viral replication in HAART treated patients. However, the half-life of this DNA form is still debated with conflicting *in vivo* and *in vitro* data. Prior *in vitro* studies have focused on cell lines or short lived activated cells in cultures of brief duration, while *in vivo* studies have the added complications of cell migration, division, and death. Therefore, we monitored the stability of 2-LTR circles in primary CD4+T cells in a month long culture and compared it to the stability of integrated HIV DNA and T cell receptor excision circles (TRECs), another circular DNA form that is thought to be stable. We found that 2-LTRs, along with TRECs, were stable, suggesting 2-LTRs do not necessarily indicate ongoing replication.

Introduction

Although highly active antiretroviral therapy (HAART) is effective at controlling HIV replication, it is not a cure as a reservoir of treatment resistant cells persists. These latently infected cells, mainly consisting of resting CD4+T cells, can release infectious virus upon stimulation (Chun et al., 1997; Finzi et al., 1999). How this reservoir is maintained in the face of HAART is still an open question. While several hypotheses exist, one possible explanation is that a low level of ongoing replication occurs, in spite of therapy either in sanctuary sites or due to insufficient drug levels, that replenishes the reservoir over time (Coiras et al., 2009; Lafeuillade and Stevenson, 2011; Palmer et al., 2008). Some evidence for this hypothesis is the existence of circular HIV DNA forms including 2 long terminal repeat circles (2-LTRs) in patients on HAART (Sharkey et al., 2000) and the increase in such circles upon Raltegravir intensification (Buzon et al., 2010). This evidence presupposes a short half-life of these circular forms. However, there is still debate over the longevity of 2-LTR circles (Brussel et al., 2003; Butler et al., 2002; Gillim-Ross et al., 2005; Murray et al., 2012; Pierson et al., 2002; Sharkey et al., 2000, 2005; Zhu et al., 2011). While several *in vivo* studies have suggested 2-LTRs are short-lived (Murray et al., 2012; Sharkey et al., 2005; Zhu et al., 2011), it is difficult to determine if decreases in 2-LTRs reflect degradation of the circles themselves or other complicating factors such as cell half-life, division, and migration. *In vitro* studies also show conflicting evidence regarding how long 2-LTRs persist. While some studies using cell lines suggest 2-LTRs are short-lived (Sharkey et al., 2000), other studies using cell lines indicate long 2-LTR half-lives, particularly when cell division and viability are controlled (Butler et al., 2002; Pierson et al., 2002). However, these *in vitro* studies did not use physiologically relevant cells and did not culture the cells for a long period of time (~10 days at most) (Butler et al., 2002; Pierson et al., 2002). Therefore, we chose to examine the stability of 2-LTRs in primary CD4+T cells infected *in vitro* in a month long culture as some *in vivo* studies suggest that 2-LTR circles have a half-life between 8 and 25 days (Murray et al., 2012; Zhu et al., 2011). We also examined the stability of T cell receptor excision circles (TRECs) as a comparison as TRECs are often assumed to be long-lived *in vivo* (Hazenberg et al., 2001; Somech, 2011).

Results and discussion

We first examined the dynamics of total HIV DNA and 2-LTRs in infected primary CD4+T cells in a short time-course, similar to previous *in vitro* studies. We treated the cells with 10 ng/mL IL-7 to maintain the cells in culture and with the integrase inhibitor Raltegravir to increase the levels of 2-LTR circles to more easily detectable levels. We found that total HIV DNA peaked at 2 days post infection while 2-LTR circles peaked 4 days post infection (Fig. 1A), consistent with the literature (Gillim-Ross et al., 2005). Total HIV DNA quickly declined until it reached levels similar to those of 2-LTR circles (~day 4 post infection), suggesting that most of the HIV DNA consisted of 2-LTRs by that time (Fig. 1A). This is consistent with the short half-life of linear unintegrated HIV DNA

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found *in vitro* (Koelsch et al., 2008). Additionally, we saw no decline in 2-LTRs during the 10 day culture, similar to prior *in vitro* studies using cell lines (Butler et al., 2002; Pierson et al., 2002).

As one of the major criticisms of previous 2-LTR stability studies *in vitro* was the short duration of the experiments (Sharkey et al., 2005), we wanted to examine the stability of 2-LTRs in primary cells for a longer period of time. In addition, we also wanted to compare the stability of 2-LTRs with integrated HIV DNA and T cell receptor excision circles (TRECs), another type of circular DNA, both of which are thought to persist for the life of the cell. TRECs are generated during TCR recombination and have been used to assess thymic output and the age and division history of T cells (den Braber et al., 2012; Hazenberg et al., 2001; Jamieson et al., 1999; Somech, 2011). While TRECs are assumed to be long-lived based on *in vivo* data (Douek et al., 1998; Hazenberg et al., 2001; Livak and Schatz, 1996; Somech, 2011; van Gent et al., 2011), *in vitro* data is limited and the precise half-life of TRECs is currently unknown (Hazenberg et al., 2001; Somech, 2011; van Zelm et al., 2007). Therefore, we decided to measure TRECs in addition to 2-LTRs to compare the stability of different circular DNA forms and to add to both the TREC and HIV literature. As TRECs are enriched in naïve CD4+T cells (Douek et al., 1998), we decided to examine both TRECs and 2-LTR circles in naïve cells cultured for a month *in vitro*.

We therefore infected naïve CD4+T cells with X4 tropic HIV again in the presence of IL-7 and Raltegravir for the reasons stated above. As integrase inhibitors do not completely inhibit integration in our *in vitro* system (>98% inhibition based on an infected, untreated control, data not shown), we were able to quantify 2-LTR circles, integrated HIV DNA and TRECs. To control for any cell division, we labeled the cells with CFSE and quantified cell division during the culture. We also monitored cell viability to control for cell death.

We found that both 2-LTR circles and integrated HIV DNA were stable in naïve cells during the 30 day culture (Fig. 1B). We next examined levels of TRECs over time *in vitro* and found that TRECs were stable in naïve cells (Fig. 1C). To determine the apparent half-lives of the HIV intermediates and TRECs, we used linear regression to generate a best fit line through the plot of $\log_{10}$ quantities per cell over time as previously described (Brussel et al., 2003). We found that none of the slopes of the best fit lines were statistically different than zero, indicating no significant decline in 2-LTRs, integrated HIV DNA or TRECs (Fig. 1D).

As cell death or proliferation could affect our results, we examined cell viability and division. We found that the naïve cells did not divide during the month long culture, while bulk CD4+T cells did (Fig. 1E), likely due to central memory homeostatic proliferation. This is consistent with prior data indicating that naïve cells only divide in the presence of IL-7 in ~50% of donors (Azevedo et al., 2009). Therefore, cell division did not affect our results. Additionally, we found no significant change in the viability of the cells treated with IL-7 over time, whereas infected cells without additional cytokines died within 7 days (data not shown). Thus, neither cell death nor proliferation altered our findings that 2-LTRs and TRECs are stable *in vitro*.

Our results indicate that 2-LTRs, like TRECs, are stable in primary CD4+T cells *in vitro*. While some studies have suggested that 2-LTRs are long-lived *in vivo* (Brussel et al., 2003), recent reports suggest that 2-LTR circles have relatively short half-lives *in vivo*, between 8 and 28 days (Murray et al., 2012; Zhu et al., 2011). However, these studies admit that the calculations could be due to cellular half-lives or migration of cells containing 2-LTR circles. Our data suggests that the short *in vivo* half-lives of 2-LTRs are likely due to these confounding *in vivo* factors rather than the stability of the circles themselves. Additionally, the reported short half-life of circular HIV DNA *in vivo* suggests our month-long culture is sufficient to determine whether the stability of the cells or the 2-LTRs were responsible for 2-LTR declines in patients. While other *in vitro* studies showed 2-LTR circles were stable in cell lines (Butler et al., 2002; Pierson et al., 2002), their short time courses and use of non-primary cells limited their ability to address both the stability of 2-LTRs *in vivo* and the ability to measure ongoing replication using this circular HIV intermediate. Our studies show that 2-LTRs are stable in primary over a 30 day culture and that 2-LTR degradation cannot be responsible for their apparent short half-life *in vivo*. These results have important implications for the HIV field.

Previous studies suggested that 2-LTRs could be used as a marker for ongoing replication due to their short half-lives (Sharkey et al., 2000, 2005). Our results suggest that the presence of 2-LTRs is not sufficient to identify new rounds of replication. Together, our data showing the stability of 2-LTRs in primary cells along with *in vivo* studies indicating 2-LTRs but not integrated HIV DNA disappear over time suggests that the proliferation or the migration of cells with 2-LTRs occurs *in vivo*, confounding interpretations of ongoing replication. However, this is not to say that 2-LTRs will not increase during new rounds of replication, merely that 2-LTRs alone may not be sufficient to identify these periods. Overall, our data suggest that 2-LTRs themselves are stable in CD4+T cells but dynamics or migration of infected cells results in the shorter apparent half-life of 2-LTRs *in vivo*. Thus, the mere presence of 2-LTRs is not sufficient to determine periods of ongoing replication.

Materials and methods

Cells

Primary CD4+T cells were purified from leukapheresis-enriched PBMC using Rosette Sep (Stemcell Technologies) and were obtained through the University of Pennsylvania's Center for AIDS Research Human Immunology Core. After infection, cells were cultured in RPMI containing 20% heat inactivated FCS and 10 ng/mL IL-7 (R&D Biosystems).

Cell sorting

CD4+T cells were purified as above and were stained with CD45RO PE-Texas Red (Beckman Coulter) and CCR7 PECy7 (BD Bioscience). Naïve CD4+T cells (CD45RO–, CCR7+) were sorted using a FACSAria II Cell Sorter (BD Bioscience) and were >98% pure.

CFSE staining

An aliquot of infected cells was labeled using CellTrace CFSE Cell Proliferation Kit (Invitrogen) as per the manufacturer's instructions immediately after infection.

Virus and infection

NL4-3 viral stocks were prepared by 293 T transfections by the University of Pennsylvania's Center for AIDS Research molecular virology core. Cells were sorted as described above and spinoculated the same day at a concentration of 1×10^7 cells/mL at 1200×g at 25 °C for 2 h with 280 ng of p24 per $1e^6$ cells. After spinoculation, cells were washed twice in CO_2 independent media (Invitrogen) and treated with 50 μg/mL Dnase 1 (Roche) and 10 mM $MgCl_2$ to remove plasmid DNA. Cells were then cultured in the presence of 1 μM Raltegravir (Merck) and 1.25 μM saquinavir (Roche).

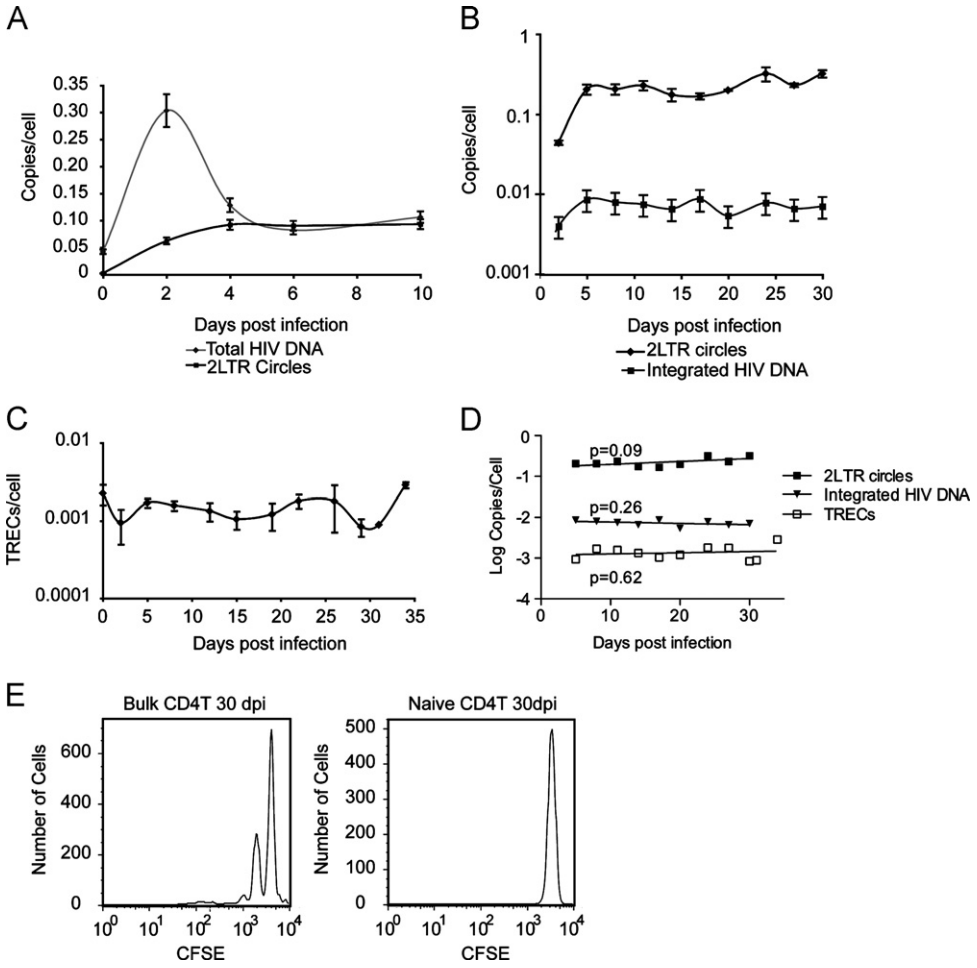

Fig. 1. 2-LTR circles, TRECs and integrated HIV DNA are stable over time in primary CD4+T cells. In (A) CD4+T cells were infected with HIV in the presence of the integrase inhibitor Raltegravir. Cells were cultured in 10 nm/mL IL-7. Total HIV DNA and 2-LTR circles were measured between 0 and 10 days post infection. A representative of three experiments is shown. In (B) naïve CD4+T cells were infected with HIV and cultured in the presence of Raltegravir, saquinavir, and IL-7. 2-LTR circles and integrated HIV DNA were measured over a period of 30 days. 2-LTRs were not above baseline at $t=0$. A representative of three experiments is shown. In (C) TRECs were measured in the same cells shown in B. Bars represent the standard deviations of the measurements (A–C). A representative of two experiments is shown. In (D) the $\log_{10}$ of the values in B and C were graphed over time and a linear regression was performed to calculate half-lives of the different HIV DNA species. In (E) infected bulk CD4+T cells or naïve CD4+T cells were labeled with CFSE and cell division measured over time. Graphs shown are for 30 days post infection.

PCR measurements

2-LTRs, total HIV DNA (using RU5 primers), and integrated HIV DNA were quantified as previously described (Graf et al., 2011; Pace et al., 2012). Integrated HIV DNA was first measured at 2 days post infection to conserve sample. TRECs were measured as previously described (Douek et al., 1998).

Statistical analysis

Linear regression analysis was performed using Graphpad Prism 5.

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