Inhibition of HIV-1 Integration in *Ex Vivo*-Infected CD4 T Cells from Elite Controllers[▽]

Maria J. Buzon,^{1,2} Katherine Seiss,² Robert Weiss,³ Abraham L. Brass,² Eric S. Rosenberg,¹
Florescia Pereyra,^{2,4} Xu G. Yu,² and Mathias Lichterfeld^{1*}

Infectious Disease Division, Massachusetts General Hospital, Boston, Massachusetts¹; Ragon Institute, Boston, Massachusetts²;
Division of Nephrology, University of California, Davis, California³; and Infectious Disease Division, Brigham and
Women's Hospital, Boston, Massachusetts⁴

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Elite controllers spontaneously maintain undetectable levels of HIV-1 replication for reasons that remain unclear. Here, we show that in elite controllers, direct *ex vivo* infection of purified CD4 T cells without prior *in vitro* activation results in disproportionately low levels of integrated HIV-1 DNA relative to the quantity of reverse transcripts, while the levels of two-long terminal repeat (2-LTR) circles were excessively elevated relative to those of integrated HIV-1 DNA. This indicates that chromosomal HIV-1 integration is inhibited in *ex vivo*-infected CD4 T cells from elite controllers. This defect in HIV-1 integration was unrelated to p21, a host protein that can restrict early HIV-1 replication steps, and was not visible following infection of *in vitro*-activated CD4 T cells from elite controllers. These data contribute to increasing evidence that intrinsic inhibition of specific HIV-1 replication steps plays an important role in the ability of elite controllers to maintain undetectable viral loads.

Despite encouraging findings in the recent RV144 HIV-1 vaccine study (13), correlates of immune protection against HIV-1 remain poorly understood and continue to represent a high-priority area of research (7). Since individuals who spontaneously clear HIV-1 infection do not exist, efforts to identify effective mechanisms of immune defense have focused on elite controllers, a group of persons who maintain undetectable levels of viral replication in the absence of antiretroviral therapy, although residual low-level viremia remains detectable in most of these patients by ultrasensitive detection techniques (12). Current views suggest that effective suppression of HIV-1 replication in these patients is likely to involve a synergistic interplay between multiple innate and adaptive immune defense mechanisms and may be facilitated by specific polymorphisms in the human HLA class I gene locus (8, 11). Strong, highly functional HIV-1-specific CD8 T cell responses have been described for the majority of elite controllers (2), and these cells are able to effectively restrict HIV-1 replication, at least in *in vitro* experiments (15). In addition to HIV-1-specific T cell responses, recent studies from two separate laboratories indicated that cell-intrinsic inhibition of HIV-1 replication steps can also importantly contribute to HIV-1 immune defense in elite controllers (6, 14). In these investigations, it was shown that *in vitro* infection of CD4 T cells from elite controllers consistently resulted in lower levels of HIV-1 replication than *in vitro* infection of CD4 T cells from progressors and healthy volunteers. This reduced susceptibility to HIV-1 involved inhibition of early viral replication steps and was associated with a selective upregulation of p21, a host protein from

the cyclin-dependent kinase inhibitor family that can modulate HIV-1 replication in macrophages (1), hematopoietic stem cells (16), and CD4 T cells (6). Intrinsic inhibition of HIV-1 replication steps in elite controllers was also suggested by a recent study in which HIV-1 DNA was quantified in direct *ex vivo* assessments of purified CD4 T cells (9). These investigations demonstrated that in comparison to HIV-1 patients with highly-active antiretroviral therapy (HAART)-mediated suppression of viral replication, elite controllers had significantly lower levels of chromosomally integrated HIV-1 DNA but elevated levels of HIV-1 2-long terminal repeat (2-LTR) circles, an episomal HIV-1 DNA form that results from aborted integration of HIV-1 DNA into host chromosomes. This pattern closely resembles alterations in HIV-1 replication dynamics observed after exposure to pharmaceutical HIV-1 integrase inhibitors and suggests that at least under specific circumstances, HIV-1 integration is restricted in elite controllers. However, following *ex vivo* infection of CD4 T cells from elite controllers, using spinoculation protocols, no evidence for cell-intrinsic inhibition of HIV-1 integration in CD4 T cells from elite controllers was found (9). Yet, intrinsic restriction of HIV-1 replication may not be visible after infection of CD4 T cells by spinoculation (14).

To overcome this possible limitation, we performed a detailed investigation of HIV-1 reverse transcription and integration in directly *ex vivo*-isolated CD4 T cells that were infected without spinoculation or prior to *in vitro* activation. For this purpose, CD4 T cells from elite controllers (HIV-1 viral load, <50/74 copies/ml; CD4 T cell count, 618/ μ l [363 to 1,001/ μ l]) recruited from the International HIV Controllers Study (www.hivcontrollers.org) and reference cohorts of HIV-1-negative volunteers and untreated HIV-1 progressors (viral load, 98,000 copies/ml [7,560 to 449,000 copies/ml]; CD4 T cell count, 488/ μ l [199 to 1,000/ μ l]) were *ex vivo* purified by negative immunomagnetic selection (purity, >90%). Afterwards, cells

* Corresponding author. Mailing address: Infectious Disease Division, Massachusetts General Hospital, Boston, MA 02114. Phone: (617) 724-4076. Fax: (617) 726-5611. E-mail: mlichterfeld@partners.org.

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FIG. 1. Inhibition of HIV-1 integration in purified, *ex vivo*-infected CD4 T cells from elite controllers. CD4 T cells from elite controllers, HIV-1-negative persons, or HIV-1 progressors were *ex vivo* infected with HIV-1 without prior *in vitro* activation. (A) Summary of the quantity of LRTs, 2-LTR circles, and integrated HIV-1 DNA in the three study cohorts. RT, reverse transcripts. (B) Ratios of the indicated HIV-1 DNA forms from the three study cohorts. Autologous HIV-1 DNA levels in CD4 T cells without exogenous HIV-1 infection were subtracted from corresponding HIV-1 DNA quantities after *ex vivo* infection. Significance was tested using Mann-Whitney U tests. Data are presented as box-and-whisker plots, indicating the median, interquartile ranges, and minimum and maximum values.

were infected with a yellow fluorescence protein (YFP)-encoding vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 virus (3) (50% tissue culture infective dose [TCID₅₀] of 5,000) that infects cells independently of coreceptor-mediated entry processes and causes only a single round of infection, thus allowing for detailed assessments of individual early HIV-1 replication steps (4). After two washes, cells were plated at a concentration of 5×10^5 cells/ml in 24-well round-bottom plates in RPMI medium supplemented with 10% fetal calf serum (FCS) but without the addition of exogenous interleukin-2 (IL-2). Forty-eight hours after infection, cell lysates were collected and subjected to quantification of HIV-1 late reverse transcripts (LRT) and 2-LTR circles; chromosomally integrated HIV-1 was detected in cell lysates collected 96 h after infection using PCR protocols described in our previous work (5, 6).

Following infection of *ex vivo*-isolated CD4 T cells with YFP-encoding VSV pseudotyped HIV-1, the proportions of YFP-positive CD4 T cells were 0.58% (0.17 to 1.4%) in elite controllers, 1.4% (0.77 to 2.14%) in progressors, and 1.79% (1.07 to 2.58%) in HIV-1-negative persons. As summarized in Fig. 1, infection of CD4 T cells resulted in significantly lower levels of LRT and integrated HIV-1 DNA in CD4 T cells from

elite controllers than in cells from HIV-1-negative persons or progressors, as described in our earlier findings (6). In contrast, no significant differences were found between 2-LTR quantities from elite controllers and those from the two reference cohorts. These data resulted in significantly reduced ratios of LRT to 2-LTR and integrated DNA to 2-LTR in elite controllers in comparison to those for HIV-1-negative persons or progressors, indicating that relative to LRT and integrated HIV-1 DNA quantities, 2-LTR circles were disproportionately elevated in elite controllers. Moreover, ratios of integrated DNA to LRT were also significantly lower in elite controllers than in HIV-1-negative persons or progressors, consistent with a disproportionate decrease of integrated HIV-1 DNA relative to LRT levels in elite controllers. Overall, this altered pattern of early HIV-1 replication products strongly suggests a defect at the level of HIV-1 integration in *ex vivo*-infected CD4 T cells from elite controllers.

Prior studies have shown that p21, a host protein from the cyclin-dependent kinase inhibitor family that is highly upregulated in CD4 T cells from elite controllers, can inhibit HIV-1 integration in hematopoietic stem cells (16) and may also be involved in restriction of early HIV-1 replication steps in macrophages (1) and CD4 T cells (6). To analyze whether p21 is

FIG. 2. Inhibition of HIV-1 integration in *ex vivo*-infected CD4 T cells from elite controllers is unrelated to p21. CD4 T cells from elite controllers or HIV-1-negative persons were *ex vivo*-infected with HIV-1 without prior *in vitro* activation. Experiments were performed in the presence of a small molecule inhibitor of p21 (striped bars) or the carrier DMSO as a control (solid bars). (A) Quantitation of levels of LRT, 2-LTR, and integrated HIV-1 DNA in the two study cohorts. (B) Corresponding ratios of indicated HIV-1 DNA forms. Significance was tested by Mann-Whitney U tests or paired Wilcoxon tests, as appropriate. Data are presented as box-and-whisker plots, indicating the median, interquartile ranges, and minimum and maximum values.

involved in the observed inhibition of HIV-1 integration in *ex vivo*-infected CD4 T cells from elite controllers, we performed HIV-1 infection experiments with *ex vivo*-isolated CD4 T cells in the presence of a small molecule inhibitor of p21 (#15; concentration of 2 μ M) that selectively eliminates p21 through proteasomal degradation (10); control cells were treated with the carrier dimethyl sulfoxide (DMSO) only. In line with prior work (6), the addition of the p21 inhibitor had no effect on LTR, 2-LTR circles, and integrated HIV-1 DNA in CD4 T cells from HIV-1-negative persons, likely as a result of low-baseline p21 expression in these individuals (Fig. 2). However, following inhibition of p21 in CD4 T cells from elite controllers, LRT and 2-LTR circles significantly increased and reached levels similar to those in HIV-1-negative persons; this suggests that p21 inhibition can overcome intrinsic restriction at the level of HIV-1 reverse transcription in elite controllers. In contrast, integrated HIV-1 DNA only weakly increased after p21 inhibition in CD4 T cells from elite controllers and remained significantly lower than in control cells from HIV-1-negative persons. Moreover, inhibition of p21 resulted in lower ratios of integrated DNA to 2-LTR and integrated DNA to LRT than in control cells, indicating that the disproportionate decrease of integrated HIV-1 DNA relative to LRT and 2-LTR circles described above cannot be corrected by the silencing of p21 (Fig. 2). Overall, this suggests that the intrinsic

inhibition of HIV-1 integration in *ex vivo*-infected CD4 T cells from elite controllers is unrelated to p21.

We subsequently investigated whether an inhibition of HIV-1 integration is also detectable in CD4 T cells from elite controllers that were infected after *in vitro* activation. Our previous work has shown that following *in vitro* activation, CD4 T cells from elite controllers were significantly less susceptible to HIV-1 infection (6); this appeared to be related to blockages at the level of HIV-1 reverse transcription and mRNA transcription, while a possible inhibition of viral integration in these cells remained unclear. To investigate this further, we activated CD4 T cells from elite controllers and HIV-1-negative persons using CD3/CD8-bispecific antibodies (0.5 μ g/ml) and IL-2 (50 IU/ml) as described before. After 5 days, CD4 T cell populations without contaminating CD8 T cells (<0.1%) were infected with the VSV-G pseudotyped HIV-1 virus (TCID₅₀ of 1,000) and plated at a concentration of 5×10^5 cells/ml in RPMI medium supplemented with 10% FCS and IL-2. Cell lysates collected after 18 h were used for quantification of LRTs and 2-LTR circles, while samples obtained after 48 h were used for assessments of integrated HIV-1 DNA. Overall, we observed that LRT and integrated DNA levels were significantly lower in elite controllers than in HIV-1-negative persons, as demonstrated previously (6). The levels of 2-LTR circles showed a similar pattern and were also re-

FIG. 3. Uncompromised HIV-1 integration in CD4 T cells from elite controllers infected after *in vitro* activation. CD4 T cells from elite controllers or HIV-1-negative persons were activated with CD3/CD8-bispecific antibodies and IL-2 for 5 days before being infected with HIV-1. (A) Summary of the quantities of late reverse transcripts, 2-LTR circles, and integrated HIV-1 DNA in the two study cohorts. (B) Ratios of the indicated HIV-1 forms in the two study cohorts. Significance was tested using Mann-Whitney U tests. Data are presented as box-and-whisker plots, indicating the median, interquartile ranges, and minimum and maximum values.

duced in elite controllers compared to those in HIV-1-negative persons (Fig. 3). Ratios of LRT to 2-LTR, integrated HIV-1 DNA to 2-LTR, and LRT to integrated HIV-1 DNA were not significantly different between the two study cohorts, suggesting that reduced levels of integrated HIV-1 DNA in *ex vivo*-activated CD4 T cells from elite controllers represent a consequence of reduced HIV-1 reverse transcripts and not an independent restriction at the level of HIV-1 integration.

In this study, we analyzed early HIV-1 replication steps in purified CD4 T cells that were infected directly *ex vivo* without prior activation or spinoculation. We showed that exogenous HIV-1 infection of directly *ex vivo*-isolated CD4 T cells from elite controllers leads to disproportionate reductions in the levels of integrated HIV-1 DNA relative to those of LRT and 2-LTR circles; moreover, the levels of 2-LTR circles were disproportionately increased relative to those of LRT and integrated HIV-1 DNA. This specific pattern is consistent with a block at the level of chromosomal HIV-1 integration and corresponds well to the recent description of increases in 2-LTR quantities relative to that of chromosomally integrated HIV-1 DNA in *ex vivo*-isolated CD4 T cells from elite controllers (9). In combination, these studies strongly suggest that at least under specific circumstances, the efficacy of HIV-1 integration can be markedly reduced in CD4 T cells from elite controllers and warrant further studies to identify molecular mechanisms that contribute to such a block. Notably, chromosomal inte-

gration of HIV-1 DNA depends on a number of different host proteins, and alterations in the expression or function of such proteins may lead to conditions that only insufficiently support HIV-1 integration in elite controllers. Moreover, it is possible that specific molecular inhibitors that block host proteins required for effective HIV-1 integration and in this way exert an indirect effect on chromosomal HIV-1 integration are available in CD4 T cells from elite controllers. Importantly, activation levels of CD4 T cells from our elite controller cohort were slightly elevated in comparison to those of HIV-1-negative persons, as determined by surface expression levels of HLA-DR in direct *ex vivo* assessments (data not shown); this indicates that defective HIV-1 integration in CD4 T cells from these patients cannot be attributed simply to reduced activation of CD4 T cells in elite controllers. Overall, the studies presented here contribute to increasing evidence that intrinsic restriction of HIV-1 replication plays an important role in the ability of elite controllers to maintain undetectable viral loads and may stimulate future mechanistic studies to identify cell-intrinsic inhibitors of chromosomal HIV-1 integration in CD4 T cells from elite controllers.

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