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REPORT

Deficiency in DNA damage response, a new characteristic of cells infected with latent HIV-1

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ABSTRACT

Viruses can interact with host cell molecules responsible for the recognition and repair of DNA lesions, resulting in dysfunctional DNA damage response (DDR). Cells with inefficient DDR are more vulnerable to therapeutic approaches that target DDR, thereby raising DNA damage to a threshold that triggers apoptosis. Here, we demonstrate that 2 Jurkat-derived cell lines with incorporated silent HIV-1 provirus show increases in DDR signaling that responds to formation of double strand DNA breaks (DSBs). We found that phosphorylation of histone H2AX on Ser139 (gamma-H2AX), a biomarker of DSBs, and phosphorylation of ATM at Ser1981, Chk2 at Thr68, and p53 at Ser15, part of signaling pathways associated with DSBs, are elevated in these cells. These results indicate a DDR defect even though the virus is latent. DDR-inducing agents, specifically high doses of nucleoside RT inhibitors (NRTIs), caused greater increases in gamma-H2AX levels in latently infected cells. Additionally, latently infected cells are more susceptible to long-term exposure to G-quadruplex stabilizing agents, and this effect is enhanced when the agent is combined with an inhibitor targeting DNA-PK, which is crucial for DSB repair and telomere maintenance. Moreover, exposing these cells to the cancer drug etoposide resulted in formation of DSBs at a higher rate than in un-infected cells. Similar effects of etoposide were also observed in population of primary memory T cells infected with latent HIV-1. Sensitivity to these agents highlights a unique vulnerability of latently infected cells, a new feature that could potentially be used in developing therapies to eliminate HIV-1 reservoirs.

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Introduction

DNA damage response is an important part of cellular networks, responsible for maintaining genome stability. The system monitors DNA integrity, detects DNA lesions, activates cell cycle checkpoints and regulates DNA repair pathways.¹ Among the different types of DNA damage, DSBs are considered to be the most serious, as a single DSB is sufficient to induce cell death.² Improperly repaired DSBs can lead to chromosomal rearrangements resulting in translocations, or deletions, both contributing to cell death or oncogenic transformation.³ DSBs can be caused by direct interaction with a damaging agent, reactive oxygen species (ROS), metabolic processes, impaired DNA damage repair processes, and telomere erosion.^{4–8}

Relationships between viruses and DDR in the host are well described and show that viruses modulate cell metabolism and interact with cellular molecules to activate or deactivate different pathways in DNA damage repair.^{9–12} Virus-induced dysfunction in DNA repair may result from up- or downregulation of key factors involved in DDR. There is also growing evidence that HIV-1 influences DDR during active infection, with most studies focusing on the accessory protein Vpr targeting different factors essential

for host genome integrity and cell cycle arrest.^{13–22} However, nothing is known about DDR in HIV-1 reservoirs, where the virus remains latent.

Here, we used 2 Jurkat-derived cell lines with incorporated HIV-1 provirus to determine whether latently infected cells have specific DDR characteristics that distinguish them from un-infected cells. We asked whether these cells, which do not express the virus, exhibit a greater degree of DSB formation and activated cellular signaling associated with detection of DSBs, when exposed to agents targeting DNA or DDR. Long-term exposure of latent cells to an agent targeting telomeres increased cell death, an effect enhanced by a DNA repair inhibitor. High doses of NRTIs caused greater increases in the DSB biomarker in latent cells, indicating defective repair of induced damage. Moreover, exposing latent cells to the DSB-inducing cancer drug etoposide produced more DSBs than in un-infected cells. Similar effect of etoposide were also observed in population of primary memory T cells infected with latent HIV-1, a latency model, which closely resembles the environment *in vivo*. We discuss how changes in signaling in response to DSB formation, derived from deficient DNA repair and telomere erosion, suggest that HIV-1 latency influences the ability of the host to efficiently respond to DNA damage.

Results

Latently infected cells are more susceptible to agents targeting G-quadruplexes and an inhibitor of DSB repair

Previous reports demonstrated that G-quadruplexes (G4) are formed in HIV-1 sequences and that G4-stabilizing agents, BRACO19 and TMPyP4 have activity against G-rich HIV-1 sequences, inhibiting viral replication.^{23–28} The cellular effects of G4 binding agents have been intensively investigated in anticancer research.^{29,30} Studies showed that G4-stabilizing agents can induce DSBs, and sensitize cells to inhibitors of DNA repair, triggering apoptosis.^{31,32} This characteristic prompted us to investigate whether cells infected with latent HIV-1 could be more susceptible to G4-stabilizing agents and inhibitors of DNA repair, than un-infected cells. We first tested antiviral activity of BRACO19 and TMPyP4 on replication competent infectious HIV-1 virus propagating in PM1

cell culture. Consistent with a previous report, BRACO19 showed strong antiviral activity at as low as 6 μ M concentration, much lower than previously reported,²⁶ inhibiting HIV-1 replication to an undetectable level (p24 ELISA) in a cell culture maintained for 1 w (Fig. 1A, left). TMPyP4 at 25 μ M was also able to inhibit HIV-1 replication and reduced the level of p24 about 100-fold. Viability of PM1 cells was not affected at indicated concentrations of tested G4-stabilizing agents (data not shown). The strong antiviral activity of BRACO19 was also observed in long-term treatment (Fig. 1A, right). Here, the level of virus replication dropped rapidly when infected PM1 cells were exposed to the agent on day 5 post-infection and virus was undetectable by p24 ELISA for up to 3 w post-infection. These data confirm that 2 different G4-stabilizing agents affect HIV-1 replication, suggesting that the agents directly target HIV-1 G-rich sequences capable of forming G-quadruplexes.

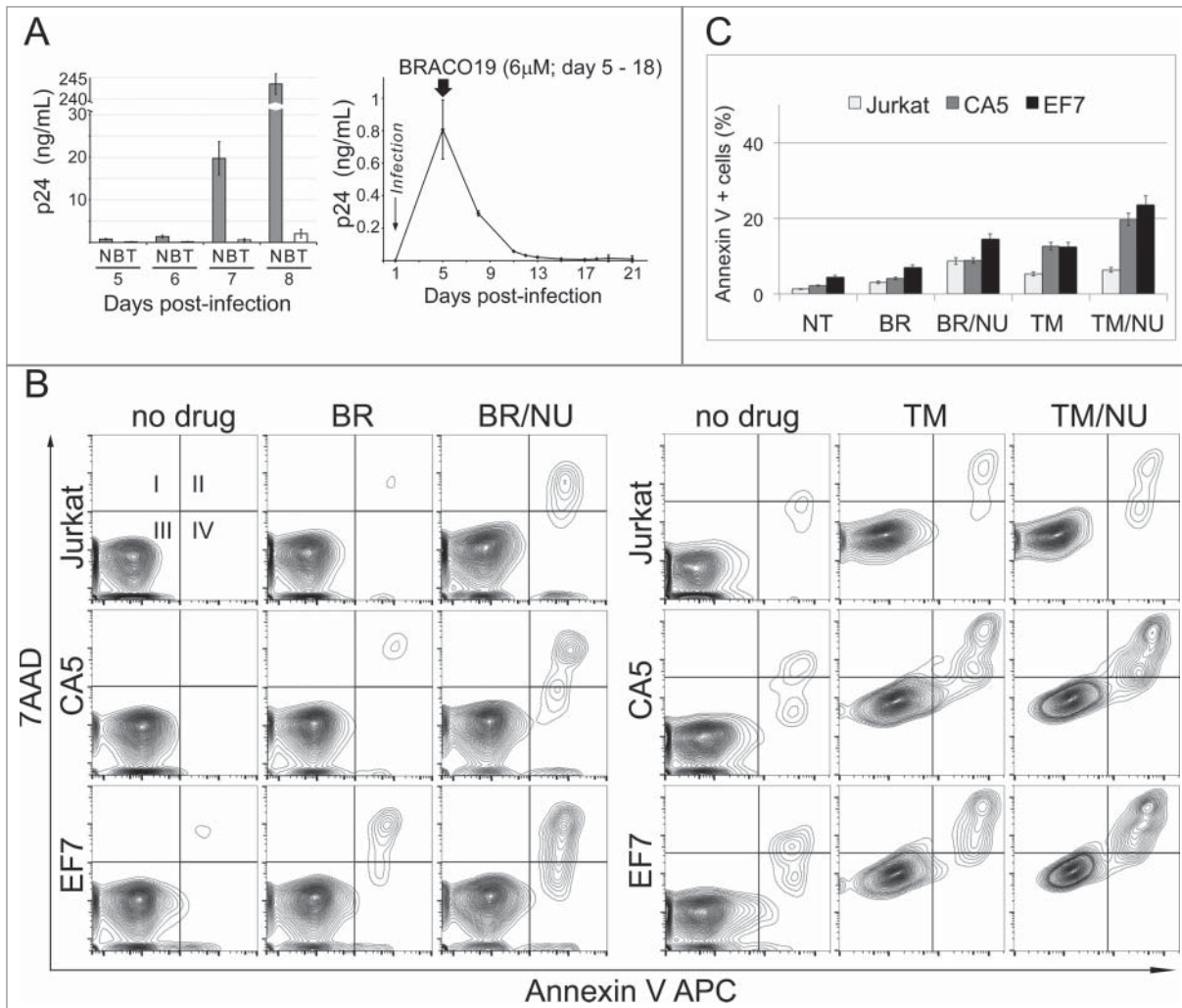


Figure 1. Latently infected cells are susceptible to G4 binding agents and an inhibitor of DSB repair. (A) Antiviral activity of TMPyP4 and BRACO19. Left panel, PM1 cells were infected with replication-competent HIV-1 NL4-3 and exposed to 6 μ M BRACO19 (B) or 25 μ M TMPyP4 (T). Samples were collected on days 5, 6, 7 and 8 post-infection and HIV-1 *gag* p24 was assayed in cell-free culture supernatants by ELISA. N – not treated infected PM1 cells. Right panel, BRACO19 displays strong antiviral activity. The level of virus replication dropped rapidly when infected PM1 cells were exposed to the agent on day 5 post-infection and virus was undetectable by p24 ELISA for up to 3 w post-infection. (B) Representative contour plots of flow cytometric analyses showing that Jurkat-derived HIV-1 latently infected cells CA5 and EF7 show increased susceptibility to G4-binding agents and a DNA repair inhibitor. The cultures were maintained in the presence of 6 μ M BRACO19 (BR), 15 μ M TMPyP4 (TM), and in combination with 1 μ M NU7441 (NU), an inhibitor of DNA-PK involved in DSB repair and telomere maintenance. Apoptosis was analyzed at day 6 (left panel), and day 8 (right panel). Live (Square III), early apoptotic (IV), and late apoptotic/dead cells (II) were discriminated based on binding of Annexin V APC and the uptake of 7AAD. (C) The graph shows changes in a population of cells, which stained positively with Annexin V APC (mean of triplicate experiments). NT – not treated cells.

To test the effects of G4-stabilizing agents and a DNA repair inhibitor on HIV-1 latently infected cells, we used 2 Jurkat-derived T cell lines, CA5 and EF7 with established HIV-1 latency.^{33,34} Both cell lines have an integrated single copy of a full-length HIV-1 genome, which is not expressed, but can be activated upon induction with TNF α producing infectious replication-competent virions. We first tested susceptibility of CA5 cells to G4-stabilizing agents at different concentrations and in combination with a DNA repair inhibitor by analysis of cell viability using a Vi-CELL Cell Viability Analyzer. Cells were seeded in the presence of BRACO19 (3 μ M and 6 μ M) or TMPyP4 (5 μ M and 15 μ M), and also in the presence or absence of the inhibitor 2-N-morpholino-8-dibenzothiophenyl-chromen-4-one (NU7441, 1.5 μ M), targeting DNA-dependent protein kinase (DNA-PK).³¹ DNA-PK is required for the non-homologous end-joining (NHEJ) pathway of DNA repair, which rejoins double-strand breaks. The number of live cells was determined 48h later. No changes in cell viability were observed at all tested concentrations of G4 binding agents alone or in combination with the DNA-PK inhibitor (data not shown).

Next, we wanted to know whether long-term exposure to G4-stabilizing agents and the DNA repair inhibitor would affect the viability of latent cells. The cultures were maintained in the presence of these drugs for 1–2 w and were monitored for viability and apoptosis by flow cytometry at days 6 and 8. The long-term exposure to 6 μ M BRACO19 resulted in a sharp decline in viability at a similar rate for all cells after 13–16 d (data not shown). The combination of BRACO19 with NU7441 (1 μ M) affected EF7 more (about 14.5% apoptotic/dead cells) than CA5 and Jurkat (about 8%), which showed increased susceptibility by day 6 (Fig. 1B, left and 1C). However, both latent cell lines showed increased sensitivity to TMPyP4 (15 μ M). The susceptibility of the cells to TMPyP4 was analyzed at day 8 (Fig. 1B). The apoptosis assay indicated that latently infected cells were entering apoptosis and death at a faster rate (12% of Annexin V positive cells), than un-infected cells (5%) (Fig. 1C). Importantly, this effect was further accelerated by the presence of 1 μ M NU7441. Here, about 6% of un-infected Jurkat cells were apoptotic/dead, compared with 19–23% of the latently infected cells.

In conclusion, cells infected with latent HIV-1 are more susceptible to agents targeting G-quadruplex structures when used alone and this effect is exacerbated by the combination with the DNA repair inhibitor. Results also indicate that the 2 G4-stabilizing agents have different antiviral activities in cells, and that tested latent cell lines show different levels of susceptibility to compounds targeting DNA and DDR. These differences may result from different affinities of these 2 drugs for G-quadruplexes formed by RNA and DNA sequences. Finally, increased susceptibility to agents targeting DNA and DDR suggests that the DNA damage response in infected cells is affected by HIV-1 latency.

DDR in latently infected cells responds less efficiently to NRTI-induced DNA damage

To determine whether efficiency of DDR is affected by HIV-1 latency, we analyzed cellular response to a high

concentration of nucleoside analogues (NAs). NAs are used in treatment of cancer and viral infections. Antiviral NAs are designed to inhibit replication of the viral genome, but at high concentrations they induce DDR in cells.^{35–37} This response is related to mobilization of the cellular DNA repair system to remove NAs, which become incorporated into host genomic DNA. NAs also affect telomerase activity and induce production of ROS, leading to further DNA damage. Cells with compromised DDR should display an increased level of DNA damage resulting from inefficient DNA repair of lesions produced by NAs. We analyzed DDR by exposing cells to high doses of the anti-HIV class of NAs called nucleoside reverse transcriptase inhibitors (NRTIs), choosing AZT (azidothymidine, zivudine) and d4T (stavudine), which are known to incorporate into genomic DNA, affect telomerase and to induce oxidative stress.^{36,38–46}

The effects of high doses of NRTIs on DNA damage response in latent cells were analyzed by assessing the level of gamma-H2AX (γ H2AX), which is a biomarker of DSBs. The γ H2AX protein is a histone H2A variant, H2AX, phosphorylated on serine 139 in response to DSBs. Jurkat and Jurkat-derived latently infected cell lines were initially treated with AZT at 5, 25, 125 μ M for 1–2h, and the levels of γ H2AX were measured by flow cytometry (data not shown). Greater increases in the level of γ H2AX were observed for both CA5 and EF7 cells at the highest tested concentration of AZT, evident by a right shift of fluorescence intensity of the fluorophore BV421 conjugated with anti- γ H2AX antibody. After 24h of treatment, up to 53–60% of latent cells showed the highest fluorescence intensity, whereas only 26% of Jurkat cells responded (Fig. 2A; left). Similar increases were observed when these same cells were exposed to high doses (100 μ M) of d4T (Fig. 2A; right). Surprisingly, we observed that both Jurkat-derived latently infected HIV-1 cell lines show increases in γ H2AX levels before NRTI exposure, when compared with un-infected cells. Here, roughly 18–22% of CA5 and 9–11% of EF7 populations showed highest fluorescence intensity of γ H2AX-BV421, compared with only about 2–5% of the un-infected Jurkat cell population.

To confirm that the NRTI-induced effects are related to DDR, the levels of γ H2AX were analyzed in each phase of the cell cycle by checking γ H2AX distributions *versus* DNA content.⁴⁷ Results show that in response to the AZT and d4T treatments, the levels of the DSB biomarker were elevated in every phase (G1, S, G2), which is consistent with an alteration of the DDR. In addition, accumulation of the γ H2AX signal in S-phase indicates that high doses of NRTIs caused cell cycle arrest in the context of DNA damage. This is consistent with previous reports indicating that AZT causes replication inhibition and accumulation of cells in S-phase.³⁸

In conclusion, greater increases in the level of γ H2AX in response to high doses of NAs indicate that latent cells do not process NA-induced damage in DNA at the same rate as un-infected cells. This suggests that DDR is likely less efficient in latently infected cells. In fact, untreated latently infected cells initially appear to have small increases in the level of γ H2AX, indicating that defective DDR is a fundamental property of latently infected cells.

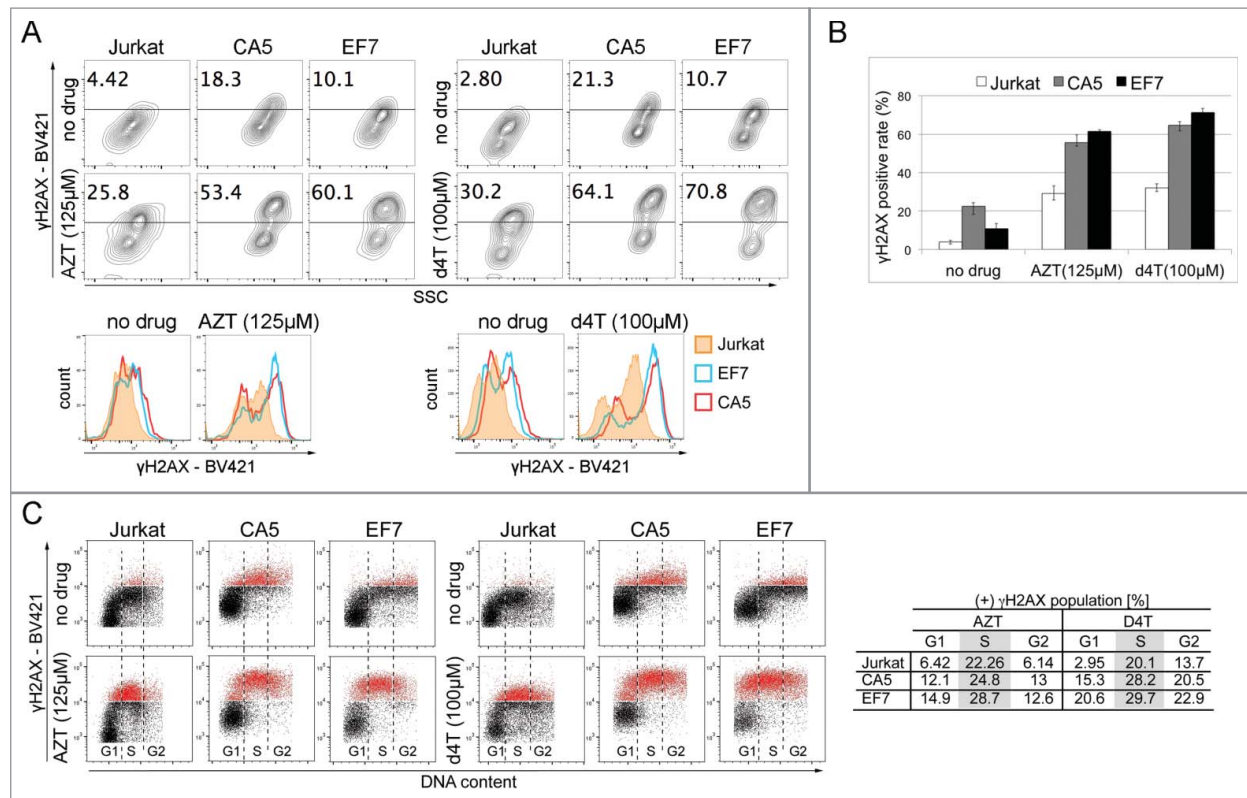


Figure 2. DNA damage response in latently infected cells to high doses of NRTIs. (A) Representative counter plots of 3 experiments showing that the level of γ H2AX, a DSB biomarker, is increased in untreated CA5 and EF7 cells, and 24h after exposure to high concentrations of AZT (125 μ M; left) and d4T (100 μ M; right). The cells were stained with anti-H2AX (pS139) mAb conjugated to BV421 to detect γ H2AX and analyzed by flow cytometry. The gate represents the plot area with the highest fluorescence intensity determined by BV421 labeling. The numbers indicate the percentage of the γ H2AX positive population with the highest fluorescence intensity. Below, histograms show differences in fluorescence intensities between Jurkat and the 2 latent HIV-1 cell lines before and after treatment with high doses of NRTIs. (B) Population of γ H2AX positive events detected for Jurkat and latent cells, and in response to high doses of AZT and d4T. (C) NRTIs-induced increases in γ H2AX level are related to DNA damage. Distribution of γ H2AX in cell cycle phases was analyzed by staining intensity for γ H2AX vs. DNA content (7-AAD). Plots show that high doses of AZT and d4T caused a shift toward high fluorescence intensity of BV421 (in red) detecting γ H2AX in each cell cycle phase (G1, S and G2), which is consistent with cellular response to DNA damage. Significant accumulation of signal observed for S-phase suggests that the cell cycle arrest derives from replication inhibition.

Latently infected cells are more susceptible to an agent that promotes formation of DSBs

An increased level of the DSB marker in response to high doses of NAs suggests that mechanisms of DNA damage repair and/or telomere maintenance are antagonized by HIV-1 latency. DNA repair deficiency is true and well described for different types of cancer cells, and, therefore, is a major consideration in anti-tumor drug development efforts. To determine whether HIV-1 latency affects cellular sensitivity to a cancer drug that induces DSBs in cells, we exposed latently infected cells to etoposide, an inhibitor of DNA topoisomerase II. The cells were treated with 50 μ M etoposide for 1 to 2h, then harvested and analyzed for the level of etoposide-induced γ H2AX (Fig. 3). Flow cytometry analyses of the γ H2AX levels show that after about 1h of treatment in both latently infected cell lines most of detected events (77–87%) were recorded with the high fluorescence intensity of fluorophore BV421 corresponding to positive detection of γ H2AX. However, for Jurkat cells, about 45% of all events were still not detected in the range of high fluorescence intensity. After 2h of exposure, most of the events were recorded in the range of high fluorescence intensity for all cells. However, the distribution of fluorescence intensities observed on histograms showed that right shifts toward high fluorescence intensities of fluorophore BV421 were greater for latent

cells than for un-infected cells suggesting that the number of DNA lesions per cell had been greater in the latent cells.

These results indicate that exposing latently infected cells to etoposide caused formation of DSBs at higher rate than in un-infected cells. In conclusion, increased levels of the DSB biomarker observed in untreated cells and after etoposide treatment suggest that cells with established HIV-1 latency have deficiencies in DNA repair mechanisms, and are more susceptible to targeting of DDR.

Signaling of DNA damage is activated in cells infected with latent HIV-1

The elevated levels of γ H2AX in cell lines infected with latent HIV-1 suggest that these cells are experiencing more DNA damage than the uninfected parental ones, which is triggering a cellular response to conduct repairs. To determine whether signaling of DNA damage is chronically activated in latently infected cells, we analyzed the proteins Ataxia telangiectasia mutated (ATM), serine/threonine kinase Chk2, and tumor suppressor p53, all involved in specific signaling in response to DSBs. ATM is a serine/threonine kinase that regulates cell cycle checkpoints and DNA repair.⁴⁸ It is a primary DSB sensor recruited to DNA lesions to phosphorylate the histone H2AX on Ser139. In response to DSBs, ATM is activated by

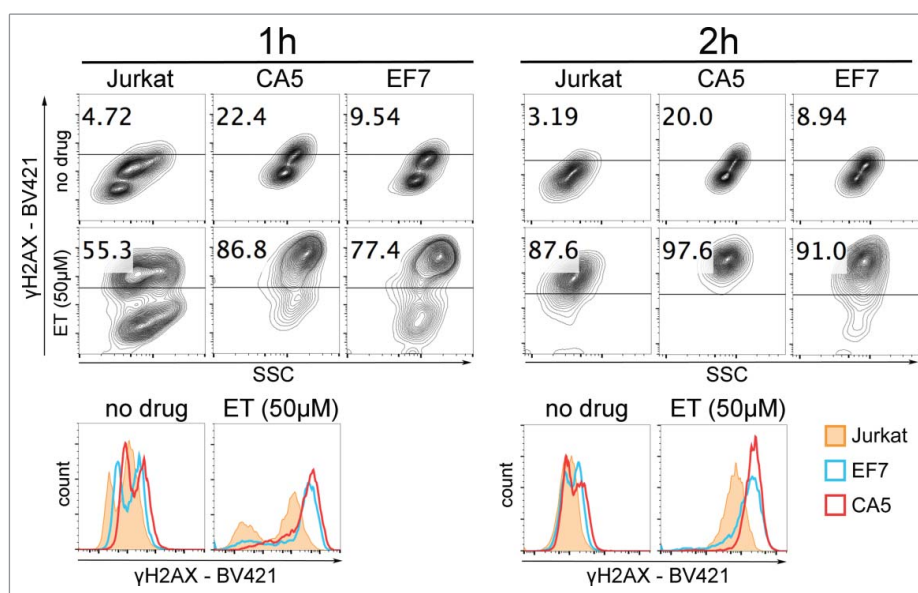


Figure 3. Effect of etoposide on cells infected with latent HIV-1. Jurkat cells and cells latently infected with HIV-1, CA5 and EF7 were exposed to 50 μ M etoposide (ET) for about 1h (left) and 2h (right). Collected cells were washed and prepared for analysis of the γ H2AX levels by flow cytometry. The gate represents the plot area with the highest fluorescence intensity determined by BV421 labeling. The numbers indicate the percentage of the γ H2AX positive population with the highest fluorescence intensity. Below, histograms show differences between Jurkat and the 2 latent HIV-1 cell lines in distribution of fluorescence intensities of fluorophore BV421 used for detection of γ H2AX.

autophosphorylation on Ser1981.⁴⁹ The protein further recruits other factors including the effector protein kinase Chk2 and the tumor suppressor p53. ATM phosphorylates Chk2 at Threonine 68, and this Thr68-phosphorylated form of Chk2 localizes at sites of DNA breaks.^{50,51} p53 becomes activated in response to different stressors, but in the case of DNA damage, phosphorylation of Serine 15 on p53 is required to permit local acetylation of histones and relaxation of chromatin.⁵²⁻⁵⁵

To determine whether this signaling pathway associated with DNA damage is activated in latently infected cells, we analyzed the levels of phosphorylated forms of ATM at Ser1981, Chk2 at Thr68 and p53 at Ser15 (Fig. 4). These levels were assessed by flow cytometry after staining with monoclonal antibodies that specifically detect only phosphorylated forms of these factors. Our data show that phosphorylated forms of the analyzed factors are elevated in cells infected with latent HIV-1. This is evident from a right shift of fluorescence intensity of detected fluorophore-conjugate antibodies in the 2

latently infected cell types, compared with intensities detected in un-infected cells.

In summary, increases in the level of the DSB biomarker γ H2AX, as well as phosphorylated forms of ATM, Chk2 and p53, indicate that signaling linked to DNA damage is induced in cells infected with latent HIV-1.

Primary memory T cells infected with latent HIV-1 are more susceptible to etoposide

We also used a HIV-1 latency model, which closely resembles the environment *in vivo* to determine whether latent HIV-1 infection influences DNA damage response. Here, we used a method developed by Bosque and Planelles^{56,57} to generate latently HIV-1 infected cells derived from human primary CD4⁺ T lymphocytes (Fig. 5A). The efficiency of established latency in this model varies from about 15% to 50%, depending on the blood donor.⁵⁶ Peripheral blood mononuclear cells

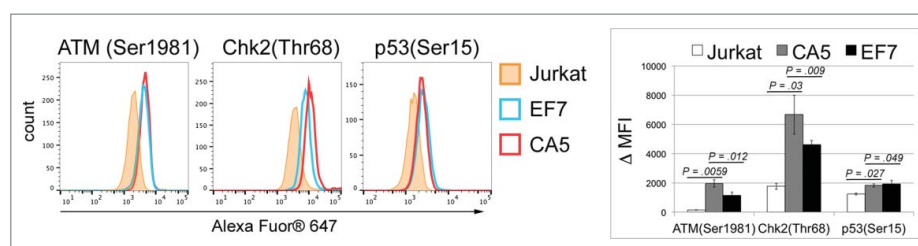


Figure 4. Activation of the DNA damage response pathway in latently infected cells. Representative histograms illustrating increases in levels of phosphorylated forms of ATM(Ser1981), Chk2(Thr68) and p53(Ser15) in 2 latently infected cells, CA5 and EF7, as compared with un-infected parental Jurkat cells. To detect phosphor-p53(Ser15), samples were stained with mAb conjugated with Alexa Fluor[®] 647. To detect phosphor-ATM(Ser1981) and phosphor-Chk2(Thr68), samples were stained with specific mAb, followed by staining with secondary antibody conjugated with Alexa Fluor[®] 647. A right shift of fluorescence intensities detected for the 2 latently infected cells corresponds to increases in the level of analyzed factors involved in detection of DSBs. On right, levels of phosphorylated forms of ATM, Chk2 and p53 in cells were compared using median of fluorescence intensities (MFI). The graph shows relative MFIs calculated from 3 experiments using MFI values of unstained samples as controls in the p53(Ser15) analysis; and samples stained only with secondary antibodies conjugated with Alexa Fluor[®] 647 in analysis of ATM(Ser1981) and Chk2(Thr68). Significance (*p*-value) was determined by 2-tailed paired-samples *t* test analysis.

(PBMC) isolated from healthy blood donors were used for negative selection of primary naïve CD4⁺ T cells. The cells were primed toward differentiation into nonpolarized (NP) cells, which resemble the central memory T cells (T_{CM}) phenotype characterized by simultaneous expression of CD27 and CCR7 (Fig 5B).^{58,59} Infection with HIV-1 was performed when cells were still in an activated state (Day 0, Fig 5A), and latency was established by progression of activated cells to a quiescent, memory-like state. For infections, we used VSV-G pseudotyped single cycle HIV-1 virus with defective env (DHIV), and HIV-GFP, a NL4-3 virus with EGFP incorporated into env. Progression toward a quiescent state was monitored by flow cytometry analysis of the p24 or GFP level in cells, which reflects the fraction of cells still in an active state (Fig 5C and Supplement Fig. 1A). By day 10, cultured cells exposed to HIV-1 were in a quiescent state with only about 1% of cells expressing viral genes.

To determine whether HIV-1 latency affects DNA damage response in primary memory CD4⁺ T, we generated latently infected and un-infected cells from 7 blood donors and treated them with etoposide to assess cellular sensitivity to a DNA targeting drug. At day 12 or 14 post-infection, cells were exposed to 25 μ M etoposide for 1 h, then washed and processed for analysis of the level of γ H2AX. All infected cells showed greater increases in the γ H2AX level in response to etoposide when compared with un-infected cells from the same donor (Fig. 5D, E and Supplementary Fig. 1C). Among 7 analyzed blood donors, latently infected cells generated from 2 donors showed greater increases of 40–60% in the levels of γ H2AX, whereas for un-infected cells the increases were in a range of 16–18% in response to etoposide treatment (Fig. 5D, Donor 1 and 2).

These results support the conclusion that HIV-1 latency negatively influences DNA damage response in infected cells.

Discussion

Our studies focused on determining whether cells with incorporated silent HIV-1 provirus have specific characteristics related to the DNA damage response that distinguish them from un-infected cells. We initially focused on G4 binding agents that stabilize G-quadruplexes, since they were demonstrated to have activity toward G-rich RNA and DNA sequences of HIV-1.^{25–27} In anticancer research, G4 binding agents were shown to trigger formation of DSBs and to induce synthetic lethality when combined with DNA repair inhibitors.^{31,32} We hypothesized that similar effects might be induced in HIV-1 latent reservoirs by targeting HIV-1 provirus incorporated into the host chromosome. We found that 2 different G-quadruplex-stabilizing agents, BRACO19 and TMPyP4 have strong antiviral activity against replication-competent HIV-1, suggesting that G4-stabilizing agents directly target G-rich sequences in HIV-1. To determine whether the presence of the provirus in latent cells can render the cells more susceptible to G4-stabilizing agents and DNA repair inhibitors, we used 2 Jurkat cell lines latently infected with HIV-1. We found that both cell lines show an increased rate of apoptosis/death compared with un-infected cells when cultures are maintained for 1 w in the presence of TMPyP4. The effect further increased when the agent was combined with a DNA repair inhibitor targeting DNA-PK crucial for DSB repair and telomere maintenance.^{60,61} Intriguingly, BRACO19 had a smaller effect compared with TMPyP4 against latent cells despite its ability to completely block spreading infection in PM1 cell cultures. These different effects indicate that BRACO19 and TMPyP4 may have different affinities to G-quadruplexes formed in RNA and DNA. Increased susceptibility of latent cells to TMPyP4 suggests that this G4 agent targets proviral sequences and

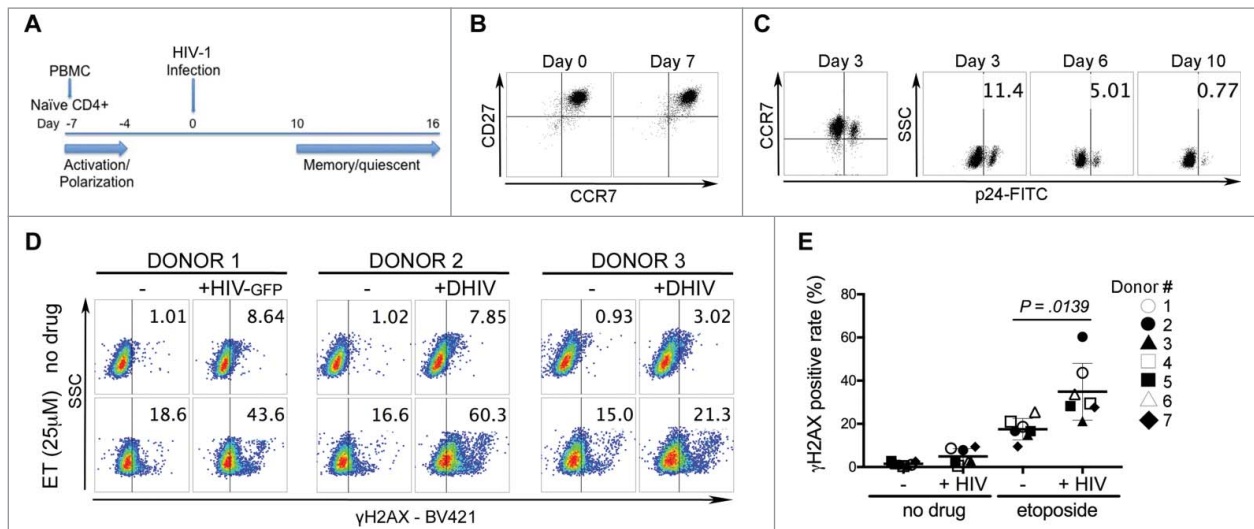


Figure 5. Effect of etoposide on the population of primary memory T cells infected with latent HIV-1. (A) Procedure used for the generation of cultured T_{CM} cells latently infected with HIV-1. Naïve CD4 T cells selected from PBMC were activated with α CD3/ α CD28 beads in the presence of α IL-4, α IL-12 and TGF- β 1 for 3 d (Day -7 to -4), and then cultured in the presence of IL-2 to differentiate into nonpolarized (NP) cells. On day 0, cells were infected with VSV-G-pseudotyped HIV-1 that expresses GFP reporter (HIV-GFP), or without it (DHIV). The cultures were maintained for another 2 w in the presence of IL-2. (B) Cultured cells show simultaneous expression of CD27 and CCR7 on day 0 and 7, which also characterizes T_{CM} cells. (C) Cells infected with DHIV (shown for donor 2) have characteristics of central memory T cells. The cells return to a quiescent state by day 10 post-infection. (D) Cultured T_{CM} cells were prepared from 7 donors and at day 12 or 14 post-infection, HIV infected and un-infected cells were assessed for the level of γ H2AX after 1 h treatment with 25 μ M etoposide (shown for 3 donors). The numbers in the gates indicate the percentage of cells recorded with the high fluorescence intensity corresponding to positive detection of γ H2AX. Values corresponding to 7 donors are shown in panel (E), where each symbol represents a different donor and horizontal lines indicate media values. Significance was determined by 2-tailed paired-samples *t* test analysis (*p*-value).

induces DSBs, sensitizing latent cells. Alternatively or additionally, a slow decline in cellular viability observed during long-term exposure to TMPyP4 may occur because its inhibitory effects are associated with targeting telomeres, not proviruses. The deleterious effects on telomeres would be observed after a delay, since their targeting causes telomeres to become shorter, and eventually induces apoptosis or growth arrest. This reasoning suggests that potential defects in telomere maintenance in latently infected cells sensitized them to TMPyP4 targeting of telomeres. Such a result would indicate that HIV-1 latency influences the cellular environment well beyond the site of integration.

Activation of DDR by exposing cells to high doses of NAs is evident from the level of phosphorylated Ser139 on histone H2AX, known as γ H2AX. This effect in cells is related to DNA lesions induced directly by NAs incorporated into genomic DNA, or indirectly by targeting telomerase or ROS-induced oxidative damage.³⁵⁻⁴⁶ The phosphorylation occurs soon after DNA damage and accumulates around the break site to attract proteins involved in repair and chromatin remodeling. We found that the level of γ H2AX is much greater in both cell lines infected with latent HIV-1, than in un-infected cells, when they were exposed to high concentrations of the NRTI-type of NAs that we tested. This indicates that latently infected cells have lower ability to deal with deleterious effects caused by high concentrations of NRTIs. Moreover, exposing HIV-1 latent cells to etoposide resulted in formation of DSBs at a faster rate than in un-infected cells. We also observed similar susceptibility of latently infected cells to etoposide in generated latently infected memory T cells derived from human primary CD4⁺ T lymphocytes, indicating that normal cells with established HIV-1 latency have deficiencies in DNA repair mechanisms. In fact, our data show that the impact of HIV-1 latency on DNA damage or DDR is already evident in untreated cells, where not only γ H2AX is elevated, but also phosphorylated forms of ATM at Ser1981, Chk2 at Thr68 and p53 at Ser15 are present, indicating DNA damage. This phosphorylation signaling is linked to formation of DSBs, but other types of DNA damage would also induce phosphorylation of these factors, for example oxidative DNA damage, formation of covalent DNA adducts, replication stress and SSBs.⁶²⁻⁶⁶ This indicates that in latently infected cells, the DDR signaling pathway is activated in response to DNA damage suggesting that DNA repairs are not processed efficiently, or there is a factor raising the background level of DNA damage.

The relationship between viruses and DDR is well described.⁹ Viruses interact with cellular mechanisms responsible for the recognition and repair of DNA lesions, and activate or deactivate individual DNA damage response pathways. They can induce genetic instability by interfering with factors responsible for recognition and repair of DNA damage. DNA damage is also caused by virus-induced oxidative stress.^{10,11} Deregulation of DNA repair by viruses facilitates their replication and may contribute to development of cancer. Particularly, studies on the retrovirus human T cell leukemia virus (HTLV) show the magnitude of virus ability to deregulate DDR in its host.⁹ The virus was found to affect each type of DNA damage repair mechanism. In addition, viruses cause telomere dysfunction, which may result in shortening of telomeres causing

chromosome instability, or they may enhance telomere maintenance and bypass cellular senescence.¹² In the case of HIV-1, the virus-encoded protein Vpr was found to influence DNA damage response through several mechanisms. For example, Vpr targets uracil DNA glycosylase (Ung2) for degradation.^{15,16} Ung2 initiates base excision repair (BER) to remove uracil from DNA. Interestingly, Vpr was found to induce formation of γ H2AX, BRCA1 and 53BP1 nuclear foci, but without detection of DSBs. Together with Vpr-induced ATR activation and chromatin binding of replication protein A (RPA), this signaling is linked to activation of G2 cell cycle arrest due to replication stress.^{13,14,21,22,67} It was also demonstrated that Vpr induces the premature activation of SLX4com, which coordinates the repair of replication-born DSBs.¹⁷⁻¹⁹ This Vpr-induced activation of SLX4com was proposed to lead to abnormal cleavage of DNA replication intermediates.

HIV-1 Tat protein was also found to down-regulate expression of DNA-PKs, which are important in repair of DSBs and in maintaining telomeres.^{20,68} Finally, HIV-1 infection negatively regulates the telomerase activity in CD4⁺ T cells, CD8⁺ T cells and Jurkat cells, but increases telomerase activity in infected macrophages.⁶⁹⁻⁷²

In the case of latently infected cells, the modulation of DDR by HIV-1 may involve viral proteins expressed at a very low level (e.g. reservoirs established in macrophages).⁷³ Potentially, non-coding RNAs produced from pro-virus sequences may modulate DDR. This concept is new and as yet unexplored, but studies showed that HIV-1 TAR microRNA, expressed during latent infection, down-regulates ERCC1, which is involved in nucleotide excision repair (NER), and is also important for DSB repair.^{74,75}

Finally, oxidative stress induced by viral infections may contribute to DNA damage. Affected redox regulation is a common feature of retrovirus and RNA virus infections, and has been shown to be associated with HIV-1 infection.¹¹ During active HIV-1 infection, cells generate a large amount of RNS and ROS, and this is accompanied by the deregulation of antioxidant enzymes increasing the level of DNA damage.⁷⁶⁻⁷⁸ Consistent with this notion, HIV-1 proteins gp120, Tat and Vpr are known to induce ROS.⁷⁹⁻⁸¹

In conclusion, our results indicate that cells infected with latent HIV-1 show affected DNA damage response and have increased susceptibility to different agents inducing damage in DNA and to agents targeting the DDR. To our knowledge this is the first report indicating that cells with silent HIV-1 infections have some impairment preventing them from making efficient repairs. This defect likely results from ability of the latent HIV-1 virus to modulate the cellular environment and interact with mechanisms directly or indirectly linked to DNA damage repair, a relationship already described for some DNA viruses and retroviruses. Our results suggest that NRTIs should eliminate HIV-1 reservoirs in patients on HAART, since they induce DNA damage. This is not the case, probably because NRTIs used in therapy are found in blood at much lower concentrations (at least 25–50 times), than concentrations we used in cell cultures.⁸²

Our results reveal new features of latently infected cells, which could be used in targeting to enhance strategies currently designed to eliminate HIV-1 reservoirs. They also suggest that HIV-1 reservoirs could potentially be eliminated without virus

reactivation. There are many further questions regarding these new features of latently infected cells. First, future experiments should investigate what DNA damage repair mechanisms are affected. It would also be important to determine whether virus proteins or non-coding RNAs are associated with these defects during latency. Finally, it would be important to test whether pre-existing conditions related to less efficient DDR in cells support establishment of HIV-1 latency.

Material and methods

Reagents

BRACO19 (Sigma, Cat No. SML0560) and TMPyP4 (EMD Millipore, Cat No. 613560) were resuspended in water, aliquoted and stored in -20°C . AZT (Matrix Scientific, Cat No. 069595), d4T (Sigma-Aldrich, Cat No. Y0000408), NU7441 (Apexbio Technology LLC, Cat No. A831525) and Etoposide (EMD Millipore, Cat No. 341205) were resuspended in DMSO and stored in -20°C .

Cell culture and plasmids

Jurkat-derived latently infected T cells CA5 and EF7 have been described earlier.^{33,34,83} Infectious molecular clone pNL4-3 and PM1 neoplastic CD4⁺ T cell line expressing both the CCR5 and CXCR4 co-receptors were obtained from Carrie Dykes. HIV-1-GFP (pNL4-3-deltaE-EGFP) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). DHIV was obtained from Vicente Planelles.⁵⁶ PM1, Jurkat, CA5 and EF7 cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated fetal bovine serum obtained from HyClone. 293T cells were maintained in Dulbecco's modified Eagle medium supplemented as for RPMI 1640.

Cultured T_{CM} cells and their latent infection

Naïve CD4⁺ T cells were isolated from peripheral blood mononuclear cells (PBMC) from healthy donors and cultured T_{CM} cells were generated and infected as described previously.^{56,57}

Generation of virus stocks

293 T cells were transiently transfected with 40 μg of pNL4-3 plasmid DNA by lipofection (SuperFect®, Qiagen, Cat No. 301305); supernatants were harvested after 72 h, and stored at -80°C . HIV-1 virus capsid protein (p24) quantitation was performed on virus stocks using an ELISA (PerkinElmer, Cat No. NEK05000). Single cycle VSV-G pseudotyped viruses were generated as described elsewhere.^{56,84}

Infection and virus replication in the presence of G4-binding agents

Volumes of virus stock corresponding to a total of 25ng of p24 were used to infect 1×10^6 PM1 cells in 1ml medium at 37°C

for 1h. After washing with PBS, the infected cells were resuspended in 5ml of medium without or with G4-binding agent BRACO19 or TMPyP4 at final concentration 6 μM and 25 μM , respectively. The cultures were incubated at 37°C in 5% CO_2 . At 3, 4, 5, 6, 7 and 8 d post-infection, 2.5ml of culture (cells and supernatant) was removed for testing and the same volume replaced with fresh medium with or without drugs. The culture supernatant was harvested after centrifugation for p24 measurement. In long-term exposure to 6 μM BRACO19, the agent was added to infected culture at day 5 post-infection. A half volume of culture (cells and supernatant) was removed each day to maintain cells density between 0.5 and 1×10^6 of cells, and the same volume replaced with fresh medium with drug. The culture was maintained for 3 w.

Flow cytometric analysis

Cell apoptosis was examined by flow cytometry analysis of APC Annexin V and 7-aminoactinomycin (7-AAD) staining (BD Biosciences). Sample fluorescence of 10,000 cells was analyzed by using a BDTMLSRII flow cytometer (BD Biosciences). Cell populations were analyzed using FlowJo software (Version 10.2). The intracellular levels of γH2AX , and phosphorylated forms of ATM(Ser1981), Chk2(Thr68) and p53(Ser15) were detected in cells fixed with BD CytofixTM Fixation Buffer (Cat No. 554655) and permeabilized with BD PhosflowTM Perm Buffer III (Cat No. 558050), then washed and blocked with BD PharmingenTM Stain Buffer (Cat No. 554656). To detect γH2AX and phosphor-p53(Ser15) samples were stained with specific monoclonal antibodies conjugated to BV421 (BD Horizon, Cat No. 564720) and Alexa Fluor®647 (Cell Signaling Technology, Cat No. 8695), respectively. For detection of phosphor-ATM(Ser1981) and phosphor-Chk2(Thr68), samples were stained with specific rabbit mAb (Cell Signaling Technology, Cat No. 13050 and 2197), followed by staining with anti-rabbit secondary antibody, IgG (H^+L), F(ab')₂ fragment conjugated with Alexa Fluor®647 (Cell Signaling Technology, Cat No. 4414). Intracellular p24 Gag expression was detected with monoclonal antibody conjugated with FITC (KC57-FITC, Coulter Clone, Cat No. 6604665) and analyzed as described previously.⁵⁶ The phenotype of cultured primary T cells was analyzed by staining with mAs CD27-PE (BD PharMingen, Cat No. 557330) and CCR7- Alexa Fluor®647 (BD PharMingen, Cat No. 560816). Sample fluorescence of 10,000 cells was analyzed by using a BDTMLSRII flow cytometer (BD Biosciences) or Accuri C6 (BD Biosciences). Statistical analysis was performed with GraphPad Prism (GraphPad Software, La Jolla, CA). The 2-tailed paired-samples *t* test analysis was used to calculate the *p*-value ($\alpha = .05$).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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