Higher expression of human telomerase reverse transcriptase in productively-infected CD4 cells possibly indicates a mechanism for persistence of the virus in HIV infection

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ABSTRACT

Mechanisms involved in survival of productively-infected memory CD4+ cells after initial antigenic stimulation and their subsequent reversion to the resting state are critical for the development of a predominant replication-competent HIV reservoir. These mechanisms may also counter their elimination after HIV reactivation through latency-reversing agents (LRA). Thus, their evaluation is critical when using an appropriate HIV latency model that recapitulates the predominant replication-competent HIV reservoir to develop strategies for HIV eradication. The model for evaluating the possible survival mechanisms after T cell receptor (TCR) stimulation was developed by infecting memory CD4+ cells with an HIV-1C primary isolate and cytokine secretion and gene expression patterns determined. Infected cells showed compromised functionality as evident from 6.8-fold lower secretion of IL-2 than from uninfected control cells. After TCR stimulation, the infected cells showed significantly higher fold increases in CD27 and CCR5 and smaller increases in CD5 mRNA over baseline values. Because CD27 expression may influence telomerase activity through AKT phosphorylation, CD27, human telomerase reverse transcriptase (hTERT) and pAKT expression in productively-infected cells from HIV-infected patients was evaluated by flow cytometry. HIV harbored in memory CD4+ cells was reactivated by HIV-1 envelope peptides, which have been shown to act as effective LRA. P24+CD4+cell showed significantly higher expression of CD27, hTERT and pAKT than P24−CD4+cells. These findings indicate compromised functionality of HIV-infected cells after TCR stimulation, which may interfere with their elimination by the immune system. They also indicate that pAKT and hTERT induction are possible survival mechanisms of productively-infected CD4+cells.

Key words HIV, human telomerase reverse transcriptase, persistence, productively-infected.
a resting state that establishes viral latency has been demonstrated (2). Moreover, it has been shown that the sequences found in rebounding plasma virus are more closely related to those of the actively replicating form of viruses present before initiation of combination therapy than to viruses in a latent pool (4). Hence, it is likely that certain mechanisms help in the persistence of productively-infected cells, which are responsible for the development of HIV reservoirs in these cells.

These mechanisms would also play an important role in reactivation strategies that use LRAs to eliminate viral reservoirs by inducing productive viral replication in CD4+ cells. Many of the LRAs evaluated in clinical trials have not achieved a significant reduction in proviral DNA copies, suggesting the need for more effective latency-reversing interventions and additional strategies for eliminating virus-expressing cells (5). A clinical trial based on romidepsin and therapeutic HIV immunization reported promising results, having demonstrated a significant reduction in the latent HIV reservoir (6). Therapeutic vaccinations have a dual advantage in reactivation strategies because HIV antigens have been shown to act as effective LRAs as well as inducing immune responses directed at eliminating the reactivated HIV reservoir. However, HIV infected CD4+ cells have been shown to secrete IL-10 after activation by HIV antigens (7), which may be one of the mechanisms that contribute to persistence of these cells through immune evasion. To better understand development of HIV latency in these cells and to devise strategies for targeting them after inducing HIV reactivation through HIV antigen, it was important to study persistence mechanisms after TCR stimulation. In order to enable effective targeting, it is also critical to study these mechanisms during preclinical evaluation of LRAs using HIV latency models that simulate in vivo HIV reservoirs.

We here used an in vitro cellular model of HIV latency that we developed by infecting activated CD4 memory cells with primary HIV-1 subtype C isolate, thus recapitulating the predominant replication-competent HIV reservoir and enabling study of persistence mechanisms. Because HIV subtype C accounts for more than 50% of infections globally (8), and as it has been shown that HIV subtypes may influence the establishment and maintenance of latency (9), as well as sensitivity to different LRAs (10, 11), we used a subtype C based model with the aim of achieving the most broadly applicable results possible. We used this model to assess cytokine secretion and gene expression patterns with the aim of understanding the type(s) of immune responses and possible persistence mechanisms induced in them after HIV reactivation through stimulating TCR with CD3/CD28 antibodies. We further evaluated our findings from the latency model by stimulating samples from HIV-infected patients with HIV antigens, which serve as natural TCR stimuli for both memory CD4+ cells and effective LRAs. Overall, the present study aimed to explore the mechanisms of reactions to antigen-specific stimulation, better understanding of which would help in devising strategies for elimination of HIV reservoirs in association with use of HIV antigens as LRAs.

MATERIALS AND METHODS

Development of latently-infected memory CD4+ cells and HIV reactivation

CD4+ cells were isolated from blood samples of four healthy donors using a commercial kit (Stemcell Technologies, Vancouver, BC, Canada). Memory CD4+ cells were purified from these cells by negative isolation (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the memory CD4+ cells was more than 96% as assessed by staining with CD45RO FITC antibody (BD Biosciences, Franklin Lakes, NJ, USA) using flow cytometry. Memory CD4+ cells were activated using plate-bound anti CD3/CD28 antibodies (2 and 1 μg/mL; BD Biosciences) in the presence of IL-7 (20 U/mL; Roche, Basel, Switzerland) for 48 hrs at 37°C in the presence of 5% CO2. The activated cells were then infected with 100×tissue culture infectivity dose50 of R5 tropic Indian HIV-1 subtype C primary isolate (GenBank accession number: EU908214), which was isolated from a recently infected individual by a co-culture method, after which Env of the virus was sequenced to determine its tropism (12). The infected cells were rested for 3 weeks in the presence of IL-7 (1 ng/mL; R&D Systems, Oakville, ON, Canada), which is required for generation and maintenance of memory cells (12) and Maraviroc (1 μM; NIH AIDS Reagent Program, Germantown, MD, USA) to prevent new rounds of infections with the R5 tropic primary isolate. The cells were further rested without IL-7 for 48 hr thereafter, because IL-7 itself can reactivate HIV replication (13). A control model of uninfected cells from the same donor was also developed for assessing cytokine secretion and gene expression pattern after anti-CD3/CD28 stimulation.

Rested infected memory CD4+ cells were stained to determine reduction in expression of activation markers such as HLA DR and CD25 (BD Biosciences). The cells were fixed by adding 3% formaldehyde and about 30,000 gated CD4+ events acquired within 24 hrs on FACSAria I (BD Biosciences). The data were analyzed using FACSDiva software version 4.0 and FlowJo version 7.1. The rested memory CD4+ cells were stimulated with
anti CD3/CD28 antibodies (5 and 1 μg/mL; BD Biosciences) for 24 hrs.

**Determination of HIV infection**

DNA was extracted from the infected cells using a commercial kit (Qiagen, Hilden, Germany). Alu-gag PCR was performed to determine HIV integration in the host genome, as described elsewhere (14). Productive HIV infection was determined by measuring cell associated HIV gag copies after 3 weeks of resting as well as after HIV reactivation by RT-PCR using TaqMan Universal Master Mix II (Applied Biosystems, Foster, CA, USA) on an ABI 7900HT instrument. Primers and probe used were as follows: forward primer 5’-ACCCATGTATTACAGCATTATCATGAAG-3’, reverse primer 5’-GCTTGATGTCCCCCCTACTGTATT-3’, and TaqMan probe 5’-AGCCACCACACAAGATT-CAAACACCATGT-3’. Cycling conditions were as follows: 50°C for 2 min followed by 95°C for 10 min for polymerase activation, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

**Determination of cytokine secretion pattern of the model after HIV reactivation by anti-CD3/CD28 stimulation**

The supernatants of the stimulated cells were assessed for secretion of various cytokines using a Bioplex Th1/Th2 cytokine kit (Bio-Rad Laboratories, Hercules, CA, USA). Cytokines assessed comprised granulocyte monocyte colony stimulating factor, IFN-γ, IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IL-13 and TNF-α. Assays were performed according to the manufacturer’s instructions. Data were collected and analyzed using a Bio-Plex 200 system and Bio-Plex Manager software (Bio-Rad Laboratories). A five-parameter regression formula was used to calculate the sample concentration from the standard curves.

**Determination of gene expression profile of the model after HIV reactivation by anti-CD3/CD28 stimulation**

The stimulated cells were harvested for RNA extraction using a commercial kit (Qiagen) and cDNA preparation using a RT2 first strand kit (S.A. Biosciences, Hilden, Germany). Gene expression analysis was performed on an ABI 7900HT system (Applied Biosystems) using a T cell and B cell activation kit and RT² Real-Timer SyBR Green qPCR master mix (S.A. Biosciences). For data analysis, the ΔΔCt method was used using hypoxanthine guanine phosphoribosyltransferase, which shows constant expression under different assay conditions, as a housekeeping gene. Fold-changes were calculated as 2^\(\Delta\Delta C_t\) (ΔCt CD3 stimulated cells – ΔCt unstimulated cells) for each gene for both infected and uninfected control models.

**Flow cytometry analysis of reactivated HIV infected CD4+ cells from HIV-infected patients**

To determine whether the findings of the gene expression analysis from the in vitro model would also occur under ex vivo conditions, blood samples from ten HIV-infected ART-naive patients with CD4 counts ranging from 235–670 cells/mL of blood (median, 413 cells/mL) were collected. A pool of HIV-1 subtype C 15-mer consensus Env peptides (Cat No.: 9499, NIH AIDS Reference Reagent Program) was used to stimulate PBMCs because HIV-1 Env reportedly to causes maximum HIV reactivation of cells from HIV-infected patients (15). After stimulation for 5 hrs, P24-expressing cells were characterized for expression of CD27 (a marker required for long-term maintenance of memory T cells) and hTERT. The stimulated PBMCs were treated with a Cytofix/Cytoperm solution (BD Biosciences) and stained with antibodies against the following markers: CD3 PE-TR, CD4 APC-Cy7, CD27 PE (BD Biosciences) and P24 (KC57)-FITC (Beckman Coulter, Fullerton, CA, USA). To determine hTERT expression, unstimulated and Env stimulated cells were treated with Permeabilization III buffer (BD Biosciences, Franklin Lakes, NJ) after surface staining with CD3 APC and CD8 PECy5. The permeabilized cells were incubated with 1:100 diluted purified rabbit anti human TERT antibody (NeoBioLab, London, UK) for 30 minutes at room temperature. The cells were then washed and treated with anti-rabbit antibody conjugated with PE for 30 minutes at room temperature along with P24 (KC57)-FITC (Beckman Coulter). pAKT expression was determined in HIV-infected and -uninfected CD4 T cells at baseline and after stimulation for 30 mins with HIV-1 Env. The cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) and stained with antibodies against the following markers: CD3 PE-TR, CD4 APC-Cy7, P24 (KC57)-FITC and anti AKT (pS473) Alexa Fluor 647 (BD Biosciences, Beckman Coulter and Invitrogen, Carlsbad, CA, USA). The cells were fixed by adding 3% formalde-hyde and acquired within 24 hrs to get 30,000 gated CD4 events on FACSAria I (BD Biosciences). The data were analyzed using FACSDiva software version 4.0 and FlowJo version 7.1.

**Ethical considerations**

This study was approved by the institutional Ethics Committee and conducted in accordance with nationally and internationally accepted ethical guidelines.
**RESULTS**

**Development of HIV latency in an in vitro model and subsequent HIV reactivation following anti-CD3/CD28 stimulation**

Activated memory CD4+ T cell isolated from blood samples of healthy individuals were infected with Indian HIV-1 C primary isolate and rested for 3 weeks in the presence of IL-7 and maraviroc to induce HIV latency. Development of post-integration HIV latency as determined by Alu-gag PCR showed a range of 22,000–35,000 integrated proviral DNA copies per million CD4+ cells of the model. Cell-associated HIV gag copies were undetectable at the end of the resting period, indicating transcriptional silencing and induction of HIV latency. Flow cytometry analysis of the cells showed presence of less than 4% activated cells as determined by HLA-DR (range, 1.8–2.5%) and CD25 (range, 0.8–3.5%) expression, confirming induction of resting state in these cells (Fig. 1a and b). Cell-associated gag copies became more numerous, indicating reactivation of HIV replication upon reactivation with anti CD3/CD28 antibodies (Fig. 1c).

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**Fig. 1. Development of in vitro model of HIV latency** (a) and (b) show representative displays of the cells of the model expressing activation markers, namely (a) HLA DR and (b) CD25 at the end of a resting period of 3 weeks in the presence of IL-7 and maraviroc and without IL-7 in the culture for the last 48 hrs. (c) Detection of HIV reactivation in an HIV-1 latency model by RT-PCR assay. HIV reactivation was assessed by measuring HIV gag RNA copies by RT-PCR at baseline and after stimulation with anti-CD3/CD28 antibodies as indicated on the X axis. Number of copies after stimulation was absolutely quantified by plotting a standards curve using the standards of known values. The bars and whiskers indicate mean and SEM for the HIV gag copies as detected in the three different models. Lower limit of detection (20 copies) of the assay is indicated by a horizontal line in the figure.
**Defective functionality of latently-HIV infected cells after stimulation with anti-CD3/CD28 antibody**

Supernatants of anti CD3/CD28 antibody stimulated latently-infected cells and uninfected control cells showed increased titers of IL-2, the predominant cytokine secreted by central memory cells. Latently-infected cells showed a 6.8-fold lower secretion of IL-2 (mean +/− SEM = 68.49 +/- 18.87) after anti-CD3/CD28 stimulation than the same number of uninfected cells [mean +/− standard error of mean (SEM) = 470.2 +/- 68.47] (Fig. 2a). Titers of the other cytokines did not increase over baseline values after stimulation.

**Gene expression analysis showed a significant increase in CD27 and decreased CD5 mRNA in infected CD4+ cells**

Expression of different genes associated with T cell activation upon stimulation with anti CD3/CD28 antibodies was further assessed in three independent experiments. Upregulation of CD5 (P = 0.05), CD7 and CD45 mRNA was found to be weaker in cells of the *in vitro* latency model upon anti CD3/CD28 stimulation than in those of the uninfected control model in all three experiments. Contrarily, mRNA of CD27 (P = 0.05), CCR5 (P = 0.05) and CD28 were highly upregulated in the infected cell model after anti CD3/CD28 stimulation (Fig. 2b).

**hTERT induction in productively-infected cells from HIV-infected patients**

After reactivation of HIV, PBMCs from HIV-infected individuals were also characterized phenotypically by flow cytometry using HIV Env, which has previously been shown by us to induce HIV replication. Representative displays and the gating strategy used for the flow cytometry assays are shown in Fig 3. Productively-infected CD4+ cells were identified by intracellular P24 expression. Because CD27 and CD28 expression has been shown to be associated with preserved telomerase activity, which occurs mainly through AKT phosphorylation, hTERT and...
Phosphorylated AKT (pAKT) expression were compared between P24-expressing and P24-negative CD4+ cells, along with CD27 expression. The frequency of CD27-expressing P24+CD4+ cells was found to be significantly higher than that of CD27-expressing P24−CD4+ cells in both unstimulated (P = 0.010) and Env-stimulated (P = 0.024) PBMCs (Fig. 4a). There was a significant reduction in frequency of P24−CD4+ expressing CD27 after Env stimulation as compared to their baseline values (P = 0.024). Similarly, P24+CD4+ cells also showed greater expression of pAKT in unstimulated (P = 0.04) and Env stimulated (P = 0.07) PBMCs than in P24−CD4+ cells (Fig. 4b). The MFI and frequency of hTERT expressing P24+CD4+ cells was higher than those of P24−CD4+ cells in unstimulated (P = 0.028 and P = 0.114, respectively) and Env stimulated (P = 0.028 in both cases) PBMCs (Fig. 5a and b). Because P24+CD4+ cells after Env stimulation originated from P24−CD4+ cells under the unstimulated condition, the strength of expression of hTERT was compared between these populations. Both frequency and MFI of hTERT expression after Env stimulation were significantly higher in P24+CD4+ cells (P = 0.028 in both cases) than in P24−CD4+ cells under the unstimulated condition, indicating upregulation of hTERT expression in P24-expressing cells after Env stimulation.

**DISCUSSION**

Understanding survival mechanisms in productively-infected CD4 cells are critical for inferring the possible mechanisms that lead to establishment of HIV reservoirs in them and for devising strategies for...
targeting these mechanisms, along with HIV reactivation strategies. We used memory CD4+ cells infected with an HIV-1 subtype C primary isolate to develop our model for representing the predominant replication-competent HIV reservoir in patients from developing countries infected with subtype C virus. The presence of integrated HIV proviral DNA copies and undetectable viral transcription at the end of the resting period confirmed development of post-integration HIV latency in our model. The frequency of the infected cells found in our model is similar to that reported earlier after *in vitro* HIV infection (16). Fewer than 4% of cells expressed activation markers like HLA DR and CD25, indicating induction of a resting state in most of the cells, as also reported by another HIV latency model (17). After activation with anti-CD3/CD28 antibodies, they also secreted only IL-2, which is known to be the predominant cytokine secreted by central memory CD4+ cells (18). Treatment with anti-CD3/CD28 antibodies induced HIV reactivation in the cells, as evidenced by an increase in cell-associated HIV *gag* RNA copies.

With the aim of identifying possible mechanisms of persistence, we compared cytokine secretion and gene expression patterns after anti-CD3/CD28 stimulation between infected and uninfected control models that we had developed in a similar way from the same donor. The infected cells showed compromised functionality, as evidenced by the smaller amounts of IL-2 secreted by
them after anti-CD3/CD28 stimulation. Local production of small amounts of IL-2 has been shown to adversely affect development of IFN-γ producing effector CD8+cells (19), which may in turn affect immune elimination of the reservoir harbored in memory CD4+ cells. Anti-CD3/CD28-stimulated infected cells also reportedly show lesser fold increases in mRNA of CD7 and CD45, both of which are also reportedly downregulated in HIV infection (20, 21). The functional significance of downregulation of these markers in HIV reservoirs needs to be investigated further. Lower fold increases in expression of CD5 in infected cells than in uninfected cells would also indicate a survival advantage for these cells by increasing their responsiveness to IL-15, as has been shown in cytomegalovirus infection (22). IL-15 has also been shown to act as a LRA (23); hence, the significance of lesser CD5 gene up-regulation in this regard needs to be understood.

Contrarily, the infected cells showed stronger up-regulation of CCR5, CD27 and CD28 than did the uninfected cells. HIV has been shown to upregulate CCR5 expression on CD4+ lymphocytes (24). Although CCR5 signaling has been shown to play an anti-apoptotic role in monocytes (25), it needs to be evaluated in the case of CD4+ lymphocytes. Because CD27 was significantly upregulated in our in vitro model of HIV latency after anti-CD3/CD28 stimulation, we determined CD27 expression on productively-infected cells in HIV-infected patients after stimulating them with HIV-1 Env to rule out a bystander effect in the model (not all cells of the model were infected). Productively-infected CD4+ cells also showed higher CD27, confirming the in vitro assay findings. Stronger CD27 expression has been implicated in long-term maintenance of memory T cells (26). CD27+ cells have also been shown to have lower functionality than CD27 negative cells in terms of both effector cytokine secretion and antigen recall response (27). The role of stronger CD27 expression in lowering IL-2 secretory response by infected cells needs to be investigated. Interestingly, there was a significant downregulation of CD27 expression on P24-CD4+ cells after Env stimulation, indicating the maturation of these cells that is known to occur through repeated antigenic stimulation (28).

Given that the loss of CD27 and CD28 expression has been shown to be associated with lower AKT phosphorylation, which ultimately decreases telomerase activity, pAKT and hTERT expression on productively-infected cells in HIV-infected individuals has been studied (29). pAKT and hTERT expression are reportedly significantly stronger in productively-infected than P24-negative CD4 cells. The PI3K/Akt pathway is known to be a key pathway for cell survival and its blockade has been shown to limit HIV-1 recovery from latently infected T cells (30). hTERT is the catalytic subunit of telomerase complex and its expression is positively correlated with telomerase activity in both normal and malignant human cells (31). Telomerase is essential for maintaining the replicative capacity of memory T cells by preventing shortening of telomere length of the cells and thereby their senescence (32). hTERT expression has also been shown to protect cells from apoptosis apart from protecting them from senescence (33). HIV has been shown to induce telomerase activity in monocyte-derived macrophages, conferring resistance against oxidative stress on them (34). One group of researchers has also shown that hTERT activity is down-regulated in CD4+ cells in HIV infection (35). However, the effect of bystander cells in such downregulation needs to be investigated. One latency model has identified a role of P53 downregulation in establishment and maintenance of latency in central memory cells (36). Researchers have shown that AKT enhances P53 degradation (37) and that a P53-dependent pathway affects hTERT expression (38) suggesting interlinking of these mechanisms.

Thus, the subtype C based model presented in this study could prove to be valuable tool for broader use in evaluating LRAs for both their effects on HIV reactivation and on the persistence of HIV-infected cells after reactivation, especially in developing countries. The present study identified novel mechanisms of persistence such as compromised functionality and CD27 as well as hTERT expression on HIV-infected memory CD4+ cells. It would be advisable to devise strategies for countering these mechanisms when HIV reactivation is attempted through TCR stimulation.

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DISCLOSURE

The authors have no known conflicts of interest.

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