Production of HIV-1 by resting memory T lymphocytes

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Background: The persistence of HIV-1 within resting memory CD4 T cells constitutes a major obstacle in the control of HIV-1 infection.

Objective: To examine the expression of HIV-1 in resting memory CD4 T cells, using an in-vitro model.

Design and methods: Phytohaemagglutinin-activated peripheral blood mononuclear cells were challenged with T cell-tropic and macrophage-tropic HIV-1 clones, and with a replication-incompetent and non-cytotoxic HIV-1-derived vector (HDV) pseudotyped by the vesicular stomatitis virus glycoprotein G. To obtain resting memory CD4 T cells containing HIV-1 provirus, residual $CD25^+$, $CD69^+$ and $HLA-DR^+$ cells were immunodepleted after a 3 week cultivation period.

Results: In spite of the resting phenotype, the majority of provirus-harbouring T cells expressed HIV-1 genomes and produced infectious virus into cell-free supernatant. The expression of HDV dropped by only 30% during the return of activated HDV-challenged cells into the quiescent phase. Although resting memory T cells generated *in vitro* expressed HIV-1 and HDV genome when infected during the course of the preceding T cell activation, they were resistant to HIV-1 and HDV challenge *de novo*. The infected culture of resting memory T cells showed a higher resistance to the cytotoxic effects of HIV-1 in comparison with the same cultures after reactivation by phytohaemagglutinin.

Conclusion: The majority of resting memory T cells infected during the course of a preceding cell activation produces virus persistently, without establishing a true HIV-1 latency. The described system could be used as a model for testing new drugs able to control residual HIV-1 replication in resting memory T cells.

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Introduction

Although highly active antiretroviral therapy (HAA RT) has been successful in reducing HIV–1 plasma viraemia to undetectable levels in a substantial proportion of treated patients, replication–competent HIV persists in resting memory CD4 T cells [1–3]. Virus replication rebounds in these individuals very quickly after therapy interruption and can be induced by cell activation *in vitro* [1–3]. In spite of a subliminal plasma viral load, residual viral replication was detected during the course of HAART in virtually all efficiently treated patients [1,4–7]. It has been unclear whether ongoing residual HIV–1 replication continues predominantly in

resting memory CD4 T cells in peripheral blood or in anatomical or cellular compartments inaccessible to current HIV-1 therapy. More recently, Zhang and colleagues [8] demonstrated the propagation of HIV in resting CD4 T lymphocytes in the tonsils and lymph nodes of infected individuals, and have shown the relative resistance of this cell population to HAART. In agreement with this observation, the majority of circulating virions from infected individuals, especially in the absence of opportunistic infections, appears to have replicated in resting HLA-DR-negative cells [9].

In tissue culture, resting T cells cannot be infected efficiently with HIV-1 unless prestimulated with activation agents such as phytohaemagglutinin and anti-CD3 and CD28 antibody. Without minimal cell stimulation, the infection of resting memory CD4 T cells results in pre-integration latency limited to a few days by a short survival time of free viral DNA that fails to be expressed and is rapidly degraded [10–14]. The treatment of latently infected resting T cells with a variety of activation agents can markedly enhance viral production [1–3,10–19]. These observations point to a close relationship between viral replication and T cell activation. However, several mechanisms, including the paracrine effects of chemotactic and mitogenic cytokines [13,20] or direct transfection of the nuclear factor of activated T cells (NF-AT) [14] can render resting T cells susceptible to HIV infection without changing their resting phenotype. All these factors modify an intracellular environment that can favourably support HIV-1 replication. Interestingly, HIV-1 infection itself, via production of the Nef and Tat proteins, activates both NF-AT and nuclear factor κB, resulting in increased IL-2 secretion and T cell priming [21–23]. To study post-integration events of the viral cycle in resting memory CD4 T cells, we developed an in-vitro culture system in which we followed the return of activated HIV-1-infected T cells into the G_0/G_1 phase and acquisition of the memory CD45RO$^+$ phenotype. We addressed the question of whether HIV-1 provirus persists in this in-vitro culture system in a true latent form [1,24–26] or whether it replicates as in resting memory CD4 T cells in the lymphatic tissues of infected individuals [8]. We also examined the sensitivity of resting memory CD4 T cells to the cytotoxic effects of residual replication of HIV-1.

Materials and methods

Preparation of HIV-1-infected resting memory T cells derived from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) of healthy donors were separated on Ficoll–Hypaque gradients.

Aliquots of 2×10^8 PBMC depleted of monocytes by adherence to a plastic culture flask were activated with phytohaemagglutinin-P (Difco, Franklin Lakes, NJ, USA) at 2 μg/ml in RPMI 1640 supplemented with 200 U/ml recombinant IL-2 (Chiron, Seresne, France), 15% fetal calf serum and antibiotics for a period of 3 days. After cell-clump disintegration 3 days later, 2×10^7 peripheral blood lymphocytes (PBL) per millilitre were treated for one hour with the anti-CD8 antibody at saturating concentration at 4°C. The cell suspension was incubated with magnetic beads coated with goat anti-mouse antibody (Miltenyi Biotech, Bergisch Gladbach, Germany) and the positively labelled cells were removed as recommended by the manufacturer. CD4 T cells were then infected with HIV-1 NL4-3 [27] or HIV-1 AD8 [28] at a multiplicity of infection of 0.01 or 0.1 tissue culture infectious doses (TCID)/cell at 10 days after phytohaemagglutinin activation. Alternatively, CD4 T cells were transduced by HIV-1-derived vector (HDV) (kindly provided by Dr D.R. Littman, Skirball Institute of Biomolecular Medicine, NY, USA), prepared and used as described by Unutmaz et al. [13]. HDV pseudotyped by vesicular stomatitis virus (VSV) glycoprotein G typically had a titre of 2×10^6 TCID HeLa/ml. PBL were cultured at a concentration of 10^6 cells/ml in the presence of IL-2, and fed and analysed every 3 or 4 days until the expression of activation markers CD25 and HLA-DR had diminished to below 15 and 30% of the maximum level, respectively. Approximately 3 weeks after phytohaemagglutinin activation, the residually activated T cells were removed from the cell culture by incubation with monoclonal antibodies (mAb) against CD25, CD69 and HLA-DR followed by magnetic bead separation. Typically, $5–10 \times 10^6$ cells were recovered after immunodepletion. In some experiments, the resting T cells generated *in vitro* were re-activated by co-culture with autologous PBMC lethally irradiated at an X-ray dose of 3000 rad in the presence of 2 μg/ml of phytohaemagglutinin.

Titration of HIV-1-infected cells

The endpoint titre of HIV-1 NL4-3-infected T cells was determined by syncytia formation after the co-culture of twofold dilutions of target cells with C8166 indicator T cells. The identical endpoint titre values were determined by a readout of p24^{gag} in the culture supernatant. Tenfold cell dilutions in duplicate were assayed for the presence of non-spliced HIV-1 RNA and of HIV-1 DNA in semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR) and PCR, respectively, by the detection of a 322 nt amplification product of *gag* as described previously [29]. Tenfold dilutions of H9 cells chronically infected with HIV-1 NL4-3 and of the plasmid pNL4-3 standard DNA, amplified in parallel, served as an external standard. Productive infection was assessed by measuring p24^{gag} in the culture medium using an HIV-

1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Beckman Coulter SA, Paris, France).

Antibodies

The mAb anti-CD8 (clone B9-11), anti-CD4 (clone 13B8-2), anti-CD25 (clone B1.49.9), anti-CD69 (clone TP1-55-3), anti-CD45-RA (clone ALB 11), and anti-CD45-RO (clone UCHL1) were from Beckman Coulter SA (Paris, France). The mAb anti-CD8 (clone MEM-31), anti-CD25 (clone MEM-181), anti-HLA-DR (clone MEM-12) were prepared and characterized in the Prague laboratory. The mAb anti-bromodeoxyuridine was purchased from Pharmingen Inc. (San Diego, CA, USA).

Immunofluorescence analysis

For analysis of cell surface marker expression, 2×10^5 cells were washed in phosphate buffered saline containing 0.5% fetal calf serum and 0.02% sodium azide and incubated for 45 min at 4°C in the presence of the respective antibodies at a twofold saturating concentration. After washing, cells were incubated for 30 min with a goat anti-mouse Ig antibody coupled to phycoerythrin or to fluorescein isothiocyanate. For the simultaneous labelling of HLA-DR and p24^{gag}, the cell surface antigens were stained by HLA-DR antibodies L243 coupled to peridinin chlorophyll protein or allophycocyanin purchased from Pharmingen Inc. (San Diego, CA, USA). For the immunodetection of intracellular expression of HIV-1 p24^{gag}, the cells were fixed and semipermeabilized with Cytofix-Cytoperm (Pharmingen), and stained with anti-p24 antibody KC57 RD1 coupled to phycoerythrin (Coulter, Miami, FL, USA). Cells were then washed, fixed in 1% formaldehyde and analysed after gating on live lymphocytes using a FACScan and CELLQuest software (Becton-Dickinson, Le Pont de Claix, France).

Cell cycle analysis

For evaluating the presence of cycling cells in the cultures, the method of simultaneous labelling of RNA and DNA with pyronin Y and 7-amino-actinomycine D (7AAD) was used [14]. For bromodeoxyuridine analysis, resting cells were cultivated for 3 days in the presence of 10 μM bromodeoxyuridine, fixed in 70% ethanol, treated with 2 N HCl for 30 min to denature DNA and labelled with anti-bromodeoxyuridine antibody.

Results

Preparation of HIV-1-infected resting T cells

To prepare resting CD4 T cells with integrated HIV-1 provirus, we infected the phytohaemagglutinin-activated PBMC, depleted of monocytes and CD8 T cells, with HIV-1 and subsequent to a prolonged cultivation period, immuno-depleted residually activated cells (Fig. 1a). The cell division of PBMC infected 10 days after phytohaemagglutinin activation was gradually slowing down for approximately 20 days after phytohaemagglutinin activation (Fig. 1b). The activation status of cells after HIV-1 infection was monitored by the expression of the early and late activation markers CD25 and HLA-DR, respectively (Fig. 1c). Immunofluorescence of CD25, maximal at 5 days after phytohaemagglutinin activation, progressively diminished, whereas that of HLA-DR gradually increased for 15 days and then slowly decreased. The residually activated cells were removed from the cell culture by incubation with mAb against CD25, CD69 and HLA-DR, followed by magnetic bead separation 20 days after phytohaemagglutinin activation. The negatively selected T cell population expressed only 1.2% of maximal CD25 and HLA-DR levels (Fig. 1c). It was free of CD8 cells, more than 98% cells were CD3 (not shown) and revealed strong membrane expression of CD4 (Fig. 1d) and of the memory marker CD45RO (Fig. 1e). The cell cycle status of resting memory cells was examined by 7AAD for DNA and by pyronin Y for RNA [14]. In contrast to the PBMC population analysed 6 days after phytohaemagglutinin activation (Fig. 1f), less than 1% of negatively selected HIV-1-infected cells were out of the G_0/G_1 phase of the cell cycle at 20 days after phytohaemagglutinin activation (Fig. 1g). Bromodeoxyuridine incorporation confirmed that less than 1.5% of cells replicated their DNA (not shown). On the basis of both criteria, the expression of activation markers and the cell cycle analysis, a nearly pure population of resting memory T cells was derived from phytohaemagglutinin-activated PBMC infected with HIV-1.

Production of HIV-1 by resting memory T cells generated *in vitro*

Because HIV-1 replication is intimately related to the activation of T cells, we investigated whether T cells infected during the activation phase maintain HIV-1 production after their return into the resting state. The proportion of infected cells determined by co-culture and the quantity of p24^{gag} in cell-free supernatant were assayed before and after the depletion of residually activated cells performed at 20 days after phytohaemagglutinin activation (Fig. 2a). In repeated independent experiments the proportion of infected cells ranged from 4 to 40%, and the virus production from resting T cells ranged from 2 to 12 ng p24^{gag}/ml of cell-free supernatants. This residual replication represented on average 5% of HIV-1 production from fully activated PBMC secreting $100-500$ ng p24^{gag}/ml (not shown). A cell culture containing 4% of HIV-1-infected cells and producing 6 ng p24^{gag}/ml of the cell-free supernatant was used to demonstrate the residual replication of HIV-1 and its reactivation by phytohaemagglutinin (Fig. 2a,b). A sevenfold decrease in HIV-1 production at day 7 after immunodepletion of residually activated
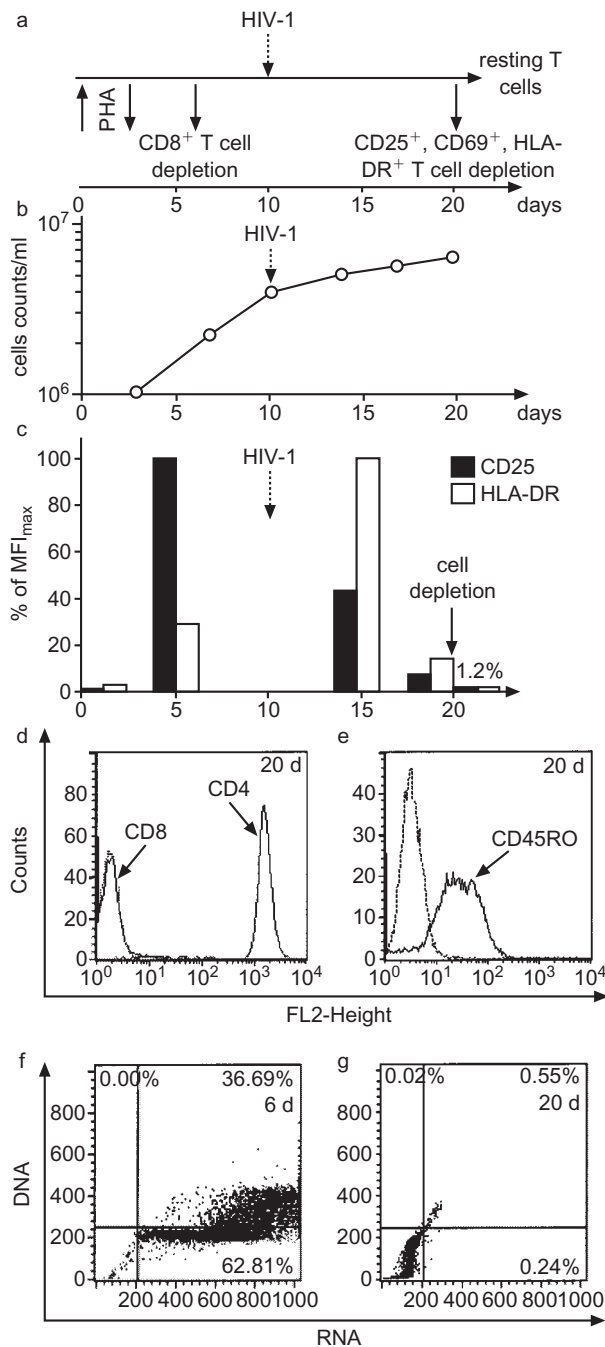
Fig. 1. Characteristics of HIV-1-infected resting memory CD4 T cells generated *in vitro*. (a) Time schedule of cell culture and HIV-1 challenge experiments. T cell culture was infected with HIV-1 NL4-3 at 10 days after phytohaemagglutinin activation of peripheral blood mononuclear cells (PBMC). (b) Cell division of infected cells (○). Cell cultures were fed and adjusted to a concentration of 10^6 cells/ml each 3 or 4 days. The curve shows the superposition of cell counts. (c) Kinetics of cell-surface expression of CD25 and HLA-DR in the same HIV-1-infected culture as shown in (b) was determined by FACS analysis and expressed as the percentage of the mean to maximal fluorescence intensity of tested cells. MFI, mean fluorescence intensity. Immunofluorescence of CD4 and CD8 (d) and CD45RO (e) after depletion of activated cells. Dotted lines represent negative controls. The DNA and RNA content was determined by 7-amino-actinomycine D, for DNA, and pyronin Y, for RNA, 6 days (f) and 20 days (g) after phytohaemagglutinin activation of PBMC in the same cell culture as shown in (b) and (c), infected with HIV-1 at 10 days after phytohaemagglutinin activation. Numbers displayed in each quadrant of (d) and (e) are percentages of positive cells.

cells correlated with an eightfold decrease in cell viability during the same period. The production of p24^{gag} per viable cell thus remained constant. Similar to the constant virus production into the cell-free super-natant, the proportion of HIV-1-infected T cells determined by co-culture and normalized to viable cell count was also stable (4%, Fig. 2a). The proportion of HIV-1-infected T cells remained stable when the culture of resting memory T cells was kept in the presence of 5 µM zidovudine during 4 days after the depletion of residually activated T cells (not shown). The stability of the infected population in the presence

of ziduvudine suggests that new infectious cycles did not occur in this cell system and that additional rounds of infection were not necessary to sustain the residual replication of HIV-1.

Although the α chain of the IL-2 receptor CD25 was virtually absent on the surface of HIV-1-infected resting memory T cells (Fig. 1c), cell culture survival was reduced three to four times by the removal of IL-2 at the moment of immunoselection of residually acti-vated cells (Fig. 2a). Therefore, resting memory T cells were continuously cultured in the presence of IL-2. In
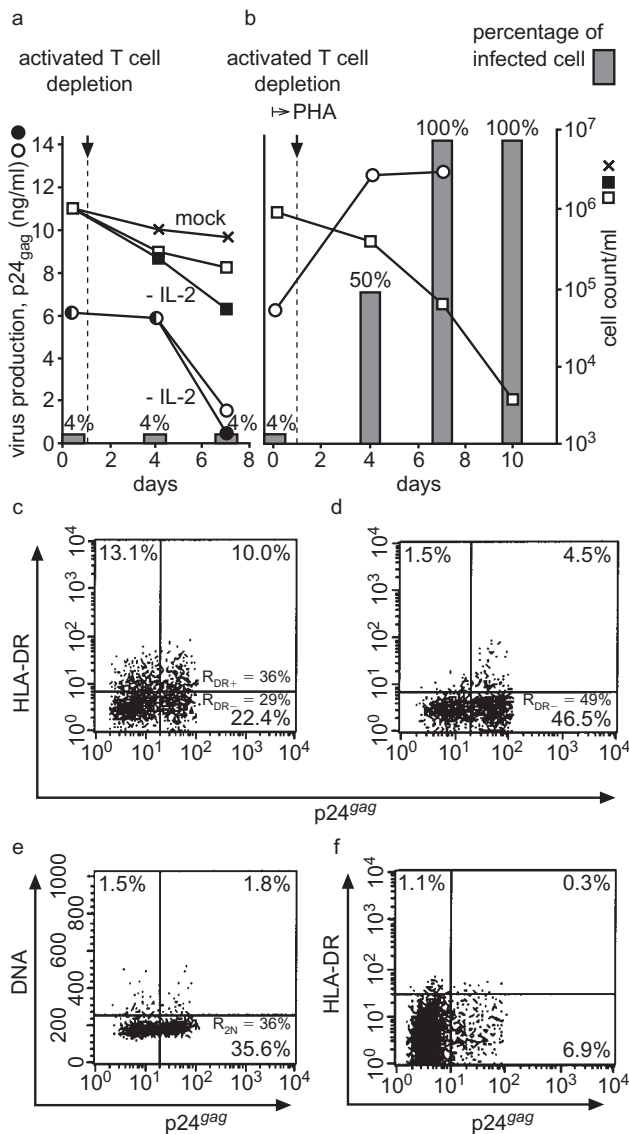
Fig. 2. HIV-1 production and expression of the HIV-1 protein p24^{gag} by resting memory T cells generated *in vitro*. CD4 T cells were infected with HIV-1 NL4-3 [a, b, multiplicity of infection (m.o.i.) 0.01 tissue culture infectious doses (TCID)/cell and c, d, e, m.o.i. 0.1 TCID/cell] or HIV-1 AD8 (f, m.o.i. 0.1 TCID/cell) at 10 days after phytohaemagglutinin (PHA) activation of peripheral blood mononuclear cells (PBMC) (see experimental protocol in Fig. 1a). (a) Depletion of residually activated cells by magnetic beads 20 days after phytohaemagglutinin activation coincides with day 0 of time scales. IL-2 was continuously present in the cell culture medium up to this moment. Time-points on the left of the vertical dotted line represent samples taken just before immunodepletion. Cell cultures were fed and adjusted to a concentration of 10^6 cells/ml each 4 days. After immunodepletion, PBMC were split and cultured in the presence (open symbols, □, ○) or in the absence (closed symbols, ■, ●) of IL-2. ×, □, ■, cell viability determined by trypan blue exclusion (□, mock-infected culture). The curves show the superposition of viable cell counts. ○, ● , HIV-1 production in cell-free supernatant as determined by p24^{gag} at the end of a 4 day cultivation period. The shadowed columns represent the percentage of infected cells determined by the co-cultivation of infected cells with C8166 indicator cells and normalized to viable cell counts. (b) HIV-1 production by resting memory T cells generated *in vitro* after their reactivation by phytohaemagglutinin. The immunofluorescence of the p24^{gag} and HLA-DR (c, d, f) or p24^{gag} and 7-amino-actinomycine D (e)-double-labelled cells was determined before (c) and 4 days after (d, e, f) depletion of CD25, CD69 and HLA-DR-positive cells performed at 20 days after PBMC activation. The numbers displayed at the corner of each quadrant are the percentage of positive cells. R_{DR+}, R_{DR-} and R_{2N} are percentages of p24^{gag+} cells in the subpopulations of HLA-DR-positive, HLA-DR-negative and diploid (2N) cells, respectively.

spite of the IL-2-dependence on cell culture viability, the residual replication of HIV-1 continued in the absence of IL-2 (Fig. 2a). HIV-1 production normalized to a viable cell count was the same in the treated IL-2 and in the mock-treated culture. The cell viability of the resting memory T cell culture infected with HIV-1 decreased more rapidly than that of mock-infected culture, suggesting that HIV-1 elicits cytotoxic effects in the quiescent T cell population.

The population of resting T cells containing 4% of HIV-1-positive cells determined by co-culture with indicator cells (Fig. 2a) and 6% of HIV-1-positive cells determined by PCR (not shown) was reactivated by lethally irradiated autologous PBMC in the presence of phytohaemagglutinin (Fig. 2b). The proportion of HIV-1-positive T cells and HIV-1 production into the cell-free supernatant rapidly increased up to the levels

observed in fully activated PBMC. This shows that HIV-1 present in resting memory T cells generated *in vitro* can spread rapidly from less than 10% of infected cells to the rest of the culture after T cell reactivation. Concomitantly, cell viability rapidly dropped. The half-life of the phytohaemagglutinin-reactivated T cell culture during the exponential phase of cell-death in comparison to that of the HIV-1-infected resting memory T cell culture was approximately 0.9 and 3 days, respectively (Fig. 2a,b). HIV-1 cytotoxicity was thus more important in the phytohaemagglutinin-reactivated population of resting memory T cells than in the original resting T cell culture.

Residual production of HIV-1 in single resting T cells

The production of HIV-1 in infected resting T cells was also examined by simultaneous labelling of HLA-

DR and protein p24^{gag} before (Fig. 2c) and 4 days after the depletion of residually activated cells (Fig. 2d). To illustrate the upper limit of HIV-1 infectivity in resting memory T cells generated *in vitro*, a cell culture in which 33% of cells were HIV-1 positive in the co-culture assay was analysed in this part of the experiment, instead of the culture containing 4% of HIV-1-positive cells assayed in the part shown in Fig. 2a,b. As expected, a high proportion of purified resting memory T cells expressed intracellular p24^{gag} after the depletion of residually activated cells (R_{DR-} 49%, Fig. 2d). In repeated experiments, proportions of HIV-1-positive cells determined by FACS analysis were similar to those determined by co-culture; thus, 4% of HIV-1-positive cells determined by co-culture (Fig. 2a) corresponded to 5.6% of p24^{gag+} cells determined by FACS analysis (not shown). To confirm the resting status of productively infected T cells generated *in vitro*, DNA was stained by 7AAD simultaneously with the labelling of p24^{gag} (Fig. 2e). A comparable percentage of HIV-1 surface antigen–positive cells was detected on the surface of HLA-DR–negative cells (46.5%, Fig. 2d) and non-dividing (2N) cells (36%, Fig. 2e). Only a small fraction of p24^{gag+} cells (1.8%) was found in the cycling cell population.

Comparable percentages of p24^{gag+} cells and similar mean fluorescence intensity (MFI) of p24^{gag} were detected in the population of HLA-DR–positive (R_{DR+} 43%) and HLA-DR–negative (R_{DR-} 29%) cells just before the immunodepletion of residually activated cells (Fig. 2c). In addition, the HIV-1 p24^{gag} production in the cell-free supernatant and the percentage of HIV-1-positive cells determined by co-culture were quite similar just before and 4 days after the immunodepletion of residually activated T cells (Fig. 2a). This suggests that HIV-1 is expressed and produced at a similar level in HLA-DR–negative and weakly positive T cells. This also suggests that HLA-DR weakly positive T cells present within the immunodepleted resting T cell population (Fig. 2d) are not the principal producers of HIV-1, detected in the cell-free supernatant by p24^{gag} assay (Fig. 2a).

To avoid possible bias by targeting subpopulations of PBL expressing predominantly the CXCR4 instead of the CCR5 co-receptor, we repeated the above experiments with the R5-dependent HIV-1 AD8. Similar to HIV-1 NL4-3, the expression of p24^{gag} of HIV-1 AD8 in resting T cells (Fig. 2f) and the secretion of p24^{gag} into the cell-free supernatant (not shown) indicated that resting T cells infected via CCR5 co-receptor produce HIV-1.

HIV-1-derived vector genome is persistently expressed in activated and resting T cells

To compare the expression of the HIV-1 genome in activated and resting T lymphocytes without confound-

ing the effects of repeated infectious cycles and the death of infected T cells, we challenged phytohaemagglutinin-activated PBMC with HDV [13], and followed its expression up to the return of T cells into the quiescent phase (Fig. 3). HDV is a replication-incompetent and non–cytopathic vector deleted for the *vif, vpr, vpu, env* and *nef* genes, and pseudotyped with VSV glycoprotein G. The expression of HDV can be monitored by the fluorescence of the green fluorescent protein (GFP), inserted in the open reading frame of the *nef* gene. HDV can challenge primary T lymphocytes but is unable to form infectious progeny. There-
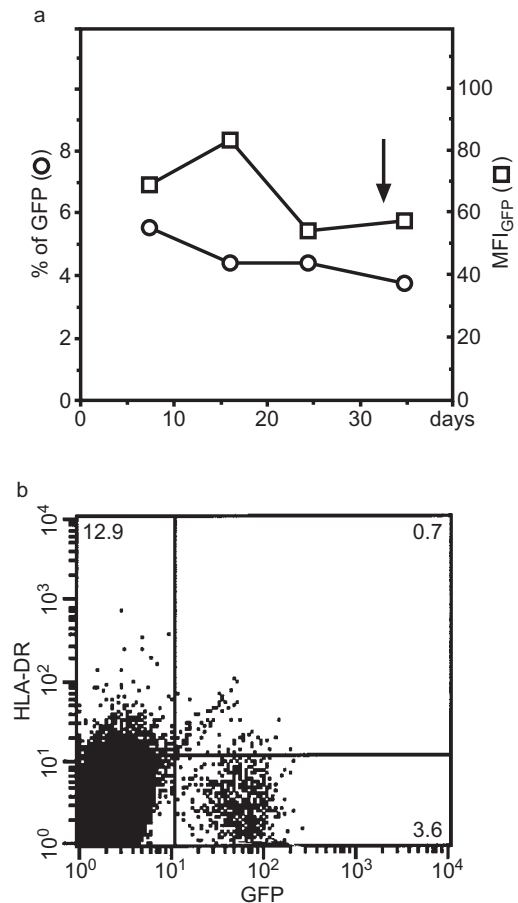

Fig. 3. Expression of HIV-1-derived vector genome in activated and resting memory T cells generated *in vitro*. (a) CD4 T cells were challenged with HIV-1-derived vector (HDV) at 3 days after phytohaemagglutinin activation of peripheral blood mononuclear cells (PBMC) at a multiplicity of infection of 1 tissue culture infectious dose/cell and the expression of its genome was followed by the production of green fluorescent protein (○) and by mean fluorescence intensity (MFI)$_{GFP}$ (□). Tha arrow indicates the time of depletion of CD25, CD69 and HLA-DR-positive cells. (b) The immunofluorescence of the HLA-DR and GFP was determined after the depletion of CD25, CD69 and HLA-DR-positive cells 36 days after PBMC activation. Numbers displayed at the corner of each quadrant are percentages of positive cells.

fore, GFP detected in resting T cells is expressed from HDV proviruses formed just after the challenge of activated T cells.

We found that the percentage of GFP-positive cells and the MFI_{GFP} decreased by only 30% over the period of 38 days after phytohaemagglutinin activation, during the return of activated T cells to the quiescent state (Fig. 3a). The MFI of CD25 diminished by 95% during the same period (not shown). After the depletion of residually activated cells, 33 days after phytohaemagglutinin activation, 3.6% of resting T cells expressed GFP (Fig. 3b). This result shows that the expression of the HIV-1 genome established in activated T cells is kept stable in the majority of T cells during their return to the resting phenotype. We speculated that a 20 times higher production of replication-competent and cytopathic HIV-1 NL4-3 in the cell-free supernatant of activated T cells, in comparison with resting T cells, is related to multiple virus cycles in dividing activated T cells.

Resting memory T cells generated *in vitro* are refractory to HIV infection *de novo*

To test whether mock-infected resting memory T cells generated *in vitro* are resistant to productive infection with HIV-1 *de novo* to the same extent as resting T cells from non-stimulated PBMC [10–14,30], we infected both cell cultures with HIV-1 NL4-3 and determined the titres of infected T cells at one day later by co-culture (Fig. 4). Resting CD4 T cells generated *in vitro* (Fig. 4B, column c) were approximately 150 times more resistant to HIV-1 infection than the control, consisting of fully activated lymphocytes (Fig. 4B, column b). They were only twofold less resistant to HIV-1 infection than resting memory T cells isolated directly from PBMC (Fig. 4B, column a). The resistance of mock-infected resting memory T cells generated *in vitro* to HIV infection *de novo* correlates with the stability of the infected resting T cell population in the presence of zidovudine, indicating the absence of new infectious cycles in this culture. The absence of new infectious cycles in the cell culture containing the constant proportion of HIV-1-producing resting T cells normalized to a viable cell count (4%, Fig. 2a) indicates that the survival of both HIV-1-infected and non-infected resting T cells is quite similar.

In a parallel experiment, we determined the resistance of resting T cells to HDV challenge (Fig. 4C). HDV pseudotyped with the VSV glycoprotein G can enter T cells irrespectively of CD4, CCR5 and CXCR4 receptors. The titres of HDV transduced cells were determined by FACS analysis of the GFP signal. HDV expressed its genome in 0.3% of resting T cells (Fig. 4C, column a,c) and in 2.7% of activated T cells (Fig. 4C, column b).
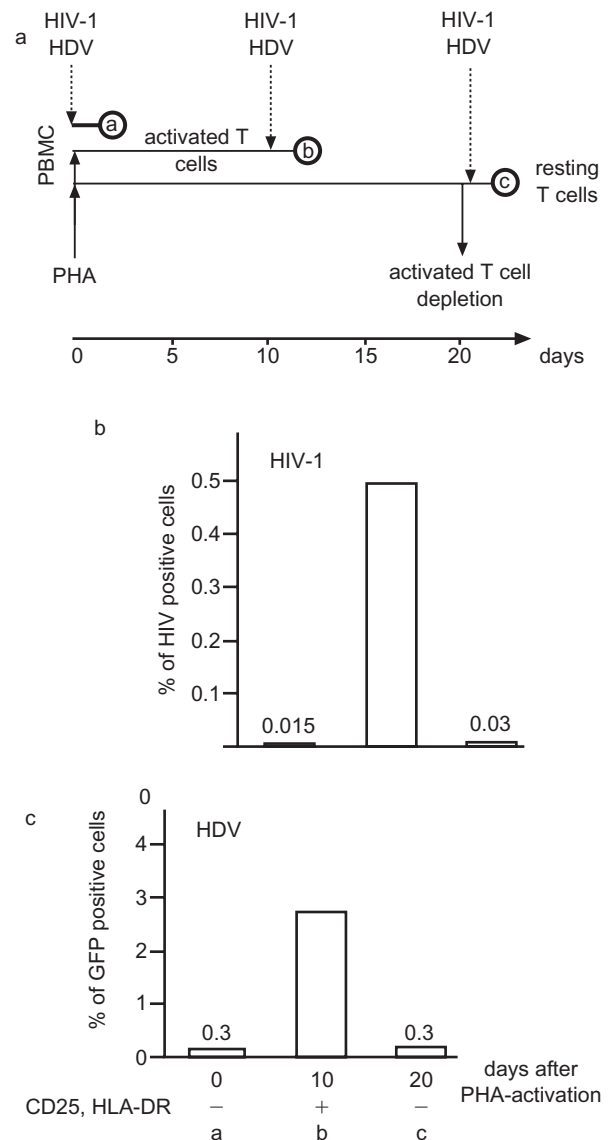

Fig. 4. Resistance of resting memory T cells generated *in vitro* to HIV and HIV-1-derived vector challenge. (A) Time schedule of cell culture and HIV-1 and HIV-1-derived vector (HDV) challenge experiments. (a) Non-stimulated peripheral blood mononuclear cells (PBMC) of healthy donor, depleted of CD8, CD25, CD69, HLA-DR and CD45RA just after purification on Ficoll gradient; (b) phytohaemagglutinin (PHA)-activated PBMC; (c) phytohaemagglutinin-activated PBMC after return to quiescent state and the immunodepletion of residually activated cells. Dotted arrows indicate the time of challenge. Open circles with letters a, b, and c indicate the time of analysis. (B) T cells infected with HIV-1 NL4-3 at a multiplicity of infection (m.o.i.) of 0.1 tissue culture infectious doses (TCID)/cell. The percentage of HIV-1 infected cells was determined at 1 day post-infection by co-cultivation of infected cells with C8166 indicator cells. (C) T cells transduced with HDV at m.o.i. 1 TCID/cell. The percentage of HDV transduced cells was determined by FACS at 3 days after challenge. The numbers at the top of the columns are percentages of the HIV-1 genome-expressing resting T cells. GFP, green fluorescent protein.

Latent infection of resting memory T cells

In order to estimate the proportion of latently infected cells in the culture of resting memory T cells generated *in vitro*, we determined the difference between provirus-harbouring and provirus-expressing cells. The titres of T cells harbouring HIV-1 provirus and those producing non-spliced genomic HIV-1 RNA and infectious virus were measured by PCR and RT−PCR of *gag* and by co-culture with C8166 indicator cells, respectively (Table 1). A cell culture in which 33% of T cells were HIV-1 positive in co-culture assay was analysed in this experiment. The ratios of HIV-1 RNA positive and infectious virus producing cells to HIV-1 DNA positive cell titres indicate that approximately 60% of provirus-harbouring resting memory T cells expressed the HIV-1 genome. This result suggests that viral latency defined by the absence of non-spliced HIV-1 RNA and of the production of virus particles was established in approximately 40% of infected resting T cells.

Discussion

Our results demonstrate that resting memory T cells generated *in vitro* produce residual amounts of virus when infected during the course of a preceding T cell activation. Although the production of replication-competent and cytopathic HIV-1 is strongly reduced in resting T cells in comparison with activated T cells, the expression of replication-incompetent and non-cytopathic HDV genome is only slightly diminished. Virus production in a high proportion of resting memory T cells generated *in vitro* is reminiscent of the expression of the HIV-1 genome in the HLA-DR and Ki67 T cells described by Zhang *et al.* [8] in the lymphoid

tissues of infected individuals. Only a moderate reduction in HIV-1 expression in resting T cells in comparison with activated T cells was observed in the lymphoid tissue of HAART-treated individuals [8]. We speculated that the many infectious cycles accomplished by the replication-competent virus in dividing activated T cells accompanied by the death of infected cells are the major factors responsible for the differences in the production of HIV-1 in activated and resting T cells.

The comparison of the survival of mock-infected and HIV-1-infected resting memory T cell culture suggests that HIV-1 elicits cytotoxic effects in the quiescent T cell population (Fig. 2a). The constant proportion of HIV-1-infected T lymphocytes in the population of resting memory T cells generated *in vitro* (Fig. 2a) and the resistance of uninfected resting T cells to HIV-1 (Fig. 4B) indicate that infected and uninfected cells are being lost in infected culture in similar proportions. This could be a consequence of the finite life-span of cells stimulated and maintained under the same conditions in infected cell culture. The infected culture of resting memory T cells showed a higher resistance to the cytotoxic effects of HIV-1 in comparison with the same cultures after reactivation by phytohaemagglutinin (Fig. 2a,b). The relative resistance of resting memory T cells to HIV-1 cytotoxicity could be one reason why the HIV-1 genomic RNA-positive and Ki67 resting T cells survive in the lymphoid tissue of HAART-treated individuals for a longer time than infected activated T cells [8]. Other factors, such as the differences in antigen presentation and sensitivity to HIV-1-specific cytotoxic T lymphocytes or differences in sensitivity to antiretroviral drugs could also be attributed to a higher resistance of infected resting T cells in comparison with activated T cells to HAART.

Although resting memory T cells infected during the course of a preceding T cell activation express the HIV-1 and HDV genome, they are resistant to HIV-1 and HDV challenge *de novo*, like resting T cells in non-stimulated PBMC. A higher percentage of HDV than HIV-1-expressed genome suggests that restriction at the virus−cell fusion step can be important for the resistance of resting T cells to HIV-1 infection. Despite this difference, the majority of resting T cells remained resistant to HDV infection. Therefore, post-entry restriction plays a fundamental role in the resistance of resting T cells to HIV-1 infection.

The expression of HDV in resting memory T cells is of further interest with respect to a possible role of accessory and regulatory genes in the expression of the HIV-1 genome. Among them, only the *tat* and *rev* genes are intact in HDV. HIV-1 proteins Tat and Nef have recently been implicated in the modulation of the expression of cellular signalling molecules resulting in

Table 1. Percentage of HIV-1 NL4-3 infected resting memory T cells generated *in vitro* expressing viral genome.

	HIV-1 DNA[a]	HIV-1 RNA[b]	Infectious cells[c]
Positive T cells (%)	50	31	33
Provirus harbouring T cells (%)[d]	100	62	66

CD4 T cells were infected with HIV-1 NL4-3 at a multiplicity of infection of 0.1 tissue culture infectious doses/cell at 10 days after phytohaemagglutinin activation (see Fig. 1a). The percentage of HIV-1 provirus and its expression was determined after the depletion of residually activated cells 20 days after phytohaemagglutinin activation from limiting dilution.
[a]Determined from limiting cell dilution by polymerase chain reaction (PCR) of *gag*.
[b]Determined from limiting cell dilution by reverse transcriptase (RT)−PCR of *gag*.
[c]Determined from limiting cell dilution by co-culture with C8166 indicator cells.
[d]Percentage of HIV-1 DNA (PCR positive) cells was arbitrarily adjusted to 100%.

the activation of NF-AT and nuclear factor κB [21–23]. The expression of the HDV genome in resting T cells indicates that the deleted genes, including the *nef* gene, are dispensable for HIV-1 production in these cells and suggests a possible role of Tat in the regulation of the residual replication of HIV-1.

Virus latency was established in approximately 40% of infected resting memory T cells. We assume that a part of the CD25, CD69 and HLA-DR–negative virus-producing cell population could be in the process of transition to a resting state without achieving its final step. In such cells, the expression of CD25, CD69 and HLA-DR may decrease and proliferation stop before the expression of the HIV-1 genome is shut off. Although some cells may stay in this intermediate state, others may eventually revert to resting memory T cells with integrated provirus in a true latent form. A slow but constant decrease of the expression of HDV genome during the return of activated T cells to the resting state (Fig. 3a) might reflect the entry of the HDV genomes into the latent phase.

Conclusion

New approaches are necessary to inhibit the residual replication of HIV-1 and to eliminate persistent HIV-1 reservoirs. The weak proportion of infected resting memory lymphocytes in HAART-treated patients, estimated to be lower than 1000 cells/ml of blood and the apparent lack of specific markers on their surface, are the principal obstacles to the study of HIV latency and persistence. Our system represents a simple, and well-defined model containing a high proportion of HIV-1–infected resting T cells. Within the limitations of an artificial in-vitro model, this system documents unappreciated aspects of HIV-1 regulation. The high efficiency of HIV-1 infection in resting T cells generated *in vitro* makes it possible to use this system for the study of the mechanism of resistance to HIV-1 inhibitors, as well as for testing new drugs able to inhibit persistent virus replication. Latently infected T cells present in this culture system represent another attractive target for testing drugs that can activate HIV-1 latency and to purge the reservoir of HIV-1 infection.

Acknowledgements

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