Peripheral blood mononuclear cells of HIV-infected patients contain CD8 T cells that form conjugates with and kill HIV-infected autologous CD4 T cells

Ehud Chorin,[1] Orit Gal-Garber,[2] Yael Yagel,[1] Dan Turner,[1] Boaz Avidor,[1] Gideon Berke[2] and David Hassin[1]

[1]Department of Internal Medicine 'H' and the Kobler AIDS Centre,Tel-Aviv Sourasky Medical Centre, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel, and [2]Department of Immunology, Weizmann Institute of Science, Rehovot, Israel

Summary

Peripheral blood mononuclear cells (PBMC) of untreated, HIV-infected patients contain HIV-specific CD8 T cells as well as their corresponding targets, HIV-infected CD4 T cells. To determine if CD4 T-cell depletion in HIV-infected patients may result from autologous CD8–CD4 T-cell interaction, CD8 and CD4 T cells procured from PBMC of acute and chronic untreated HIV-infected patients were sorted and co-incubated. Formation of CD8-CD4 T-cell conjugates was observed by fluorescence microscopy. Apoptosis of CD4 T cells in conjugation was recorded by digitized images and was further observed and measured by FACS using Annexin staining. Perforin expression in the CD8 T cells was measured using intracellular monoclonal perforin antibody staining. HIV DNA in the conjugated CD4 T cells was detected by *in situ* PCR. We found that $6·1 \pm 0·5\%$ of CD4 T cells from acute HIV-infected patients and $3·0 \pm 0·5\%$ from chronic HIV-infected patients formed CD8–CD4 T-cell conjugates. Annexin binding and cell morphology typical of apoptosis were observed in the conjugated CD4 T cells. The majority of CD8 T cells that had conjugated to CD4 T cells expressed perforin. The conjugated CD4 T cells exhibited nuclear HIV DNA. CD8 T cells and HIV-infected CD4 T cells, both procured from the PBMC of untreated HIV-infected patients, form conjugates. Apoptotic lytic activity has been observed in the conjugated CD4 T cells. We propose that CD4 T-cell annihilation in HIV-infected patients results, at least in part, from the interactions of perforin-rich CD8 T cells with autologous, HIV-infected CD4 T cells.

Keywords: apoptosis; CD4 T cells; CD8 T cells; HIV.

Introduction

The appearance of HIV-1-specific CD8 T cells in acute HIV-1 infection correlates with the decline in both viral load and CD4 T cells.[1–5] The mechanism(s) that brings about progressive decline of CD4 T cells in HIV-infected individuals is controversial.[6–9] A number of processes have been proposed to account for the depletion of CD4 T cells.[10–15] The majority of HIV-infected CD4 T cells are resting memory cells in a latent state of infection, probably expressing only minute amounts of viral proteins, making their actual contribution to CD4 T-cell depletion unknown.[10,16,17]

The aim of this study was to delineate the role that CD8 T cells play in CD4 T-cell depletion during HIV infection. Depletion of CD8 T cells in simian immunodeficiency virus-infected macaques has resulted in a rapid rise in plasma viral load.[2,3,18] Recognition of viral

Abbreviations: AICD, activation-induced cell death; Ab, antibody; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; FCS, fetal calf serum; PS, phosphatidyl serine; PI, propidium iodide; CTL, cytotoxic T lymphocyte; TRAIL, TNF-related apoptosis- inducing ligand; MHC, major histocompatibility complex

peptides by CD8 T cells has been demonstrated to be specific and to eventually result in the killing of target cells by cytotoxic T-cell lymphocytes (CTL).[4,5,14,19,20] CD8 T cells can suppress viral spread by the production of interferon-γ, by the induction of apoptosis via the cell surface receptors Fas–Fas ligand, and by secreted perforin and granzymes. Several studies have also demonstrated that expression of perforin can serve as a marker of ongoing activation and killing capacity of CD8 T cells.[21,22] Apart from their positive effects of controlling viral spread, CTL have been shown to cause tissue damage in viral infections, such as in viral myocarditis[23,24] and viral hepatitis.[25] These observations suggest that the gradual elimination of CD4 T cells observed in HIV-infected patients could be attributed, at least in part, to HIV-specific CD8 T cells. Between 3 and 10% of all CD8 T cells in the peripheral blood mononuclear cells (PBMC) of chronically HIV-1-infected patients are HIV-1-specific,[26–28] which can target HIV-1-infected CD4 T cells in the peripheral blood.[16,26,29] The major HIV reservoir resides in a pool of latently infected resting memory CD4 T cells carrying an integrated form of the viral genome that does not produce virions.[16,17] A suggested approach for the eradication of HIV-1 involves reversing latency in patients on anti-retroviral therapy.[30,31] Cells harbouring induced proviruses could then be lysed by HIV-1-specific CTL,[32] while new rounds of infection are blocked by anti-retroviral therapy.

Here, we report on the interaction of peripheral blood CD8 T cells with autologous, HIV-infected CD4 T cells from HIV-infected patients, with the aim of testing the hypothesis that HIV-specific CD8 T cells can recognize and kill autologous, HIV-infected CD4 T cells.

Materials and methods

Study population

Twenty-two untreated HIV-1-infected male patients from the Kobler AIDS Centre at the Tel-Aviv Medical Centre (Tel-Aviv, Israel) were enrolled in the study along with 22 age-matched healthy male controls. HIV-1 antibodies were detected by ELISA kit (AxSYM HIV-1, 2gO, Abbott Laboratories, Wiesbaden, Germany) and confirmed by Western blot (Inno-LIA HIV1+2 Score, Innogenetics, Gent, Belgium). Acute HIV-1-infected patients were identified by clinical presentation and sero-conversion analysis. The 22 study patients were divided into two groups: acute HIV-1-infected patients ($n = 11$, 3–12 weeks after an acute HIV-1 syndrome) (Table 1) and chronic HIV-1-infected patients ($n = 11$, 6–40 months after an acute HIV-1 syndrome) (Table 2). All patients provided written informed consent to participate according to the guidelines of the institutional Human Subjects Protection Committee. The experiments were conducted on the day blood was drawn.

Plasma viral load and CD4 and CD8 T-lymphocyte counts

Plasma HIV-1 RNA levels were determined by the COBAS AmpliPrep/COBAS TaqMan HIV-1 test (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Levels of RNA in plasma were considered undetectable when they were lower than 40 copies/ml. CD4 and CD8 T-cell counts were assessed using Tritest CD3 FITC/CD4 PE/CD45 PerCP and Tritest CD3/FITC/CD8 PE/CD45 PerCP reagents (Becton Dick-

Table 1. Conjugations and cytolytic activity of CD4–CD8 T cells in acute HIV infection

Case	Time since acute HIV syndrome (weeks)	Laboratory tests			Conjugate formation (%)		Cytolytic activity (%)	
		CD4	CD8	Viral load	HIV	Control	HIV	Control
1	3	961	1395	42 100	7·9	1·4	25	13
2	3	975	1100	22 800	8·1	1·6	31	8
3	3	580	1160	31 474	6·1	1·0	30	14
4	4	1035	4278	780 610	4·5	0·6	18	5
5	8	780	860	2407	4·4	0·6	19	8
6	8	1260	2296	454	6·5	1·0	13	5
7	12	735	1242	19 300	3·6	1·0	14	5
8	12	550	1350	125 974	3·3	0·6	20	8
9	4	368	1610	1 000 000	5·6	0·6	12	6
10	3	469	2263	177 517	8·2	1·3	16	5
11	5	420	540	49 416	8·2	1·3	8	5
Average		739	1645		6·1	0·9	19	8
SE		69	362		0·58	0·06	2·4	1·09

Table 2. Conjugation and cytolytic activity of CD4–CD8 T cells in chronic HIV infection

Case	Time since acute HIV syndrome (weeks)	Laboratory tests			Conjugate formation (%)		Cytolytic activity	
		CD4	CD8	Viral load	HIV	Control	HIV	Control
1	73	340	1020	96 318	3·8	1·1	23	23
2	363	480	1416	5218	0·6	0·5	11	8
3	>52	960	1376	6300	1·2	0·6	2	2
4	64	400	800	8685	3·5	1·0	13	12
5	60	672	798	71 000	2	0·8	14	12
6	>52	720	2160	2498	5·5	1·0	12	13
7	130	323	850	189	2·8	1·0	9	13
8	26	456	1146	85 117	2·2	0·6	5	8
9	104	520	580	694	5·6	0·6	10	8
10	31	870	1537	30 923	3·6	0·6	17	6
11	130	574	1160	30 690	4	0·9	3	5
Average		574	1077		3·1	0·9	11	8
SE		63	130		0·48	0·06	2·05	1·09

inson, Franklin Lakes, NJ) according to the manufacturer's instructions. Analysis was carried out on a FACSCaliber machine, using CELLQUEST 3.3 software (Becton Dickinson).

Sorting of CD4 and CD8 T cells from the PBMC of HIV-infected patients

Thirty millilitres of whole blood was collected into vacutainer tubes containing EDTA. The PBMC were separated over a Ficoll-Hypaque density gradient. CD8 and CD4 T cells were sorted: positive CD4 T cells were separated using Dynabeads FlowComp Human CD4 (Invitrogen 113.61D; Invitrogen, Carlsbad, CA), followed by positive selection for CD8 T cells using Dynabeads FlowComp Human CD8 (Invitrogen 113·62D) according to the manufacturer's protocol, resulting in isolated cells that were bead-free and antibody-free, phenotypically unaltered and suitable for flow cytometry and functional studies. The purity of the isolated cells was determined by FACS analysis to be 89–92%.

Conjugate formation of CD8–CD4 T cells

Isolated CD4 T cells were labelled with the fluorescent dye calcein, 1–2 μM (Molecular Probes #C-1430; Molecular Probes, Eugene, OR) in PBS for 1 hr at 37°.[33] Conjugates were formed by mixing 10^6 CD8 T cells with an equal number of calcein-labelled CD4 T cells suspended in 1 ml PBS–fetal calf serum. The suspension was allowed to stand for 10 min after which the cells were co-centrifuged at 185 *g* for 10 min at room temperature, re-suspended and placed on ice. The number of conjugates formed was recorded by a blinded reviewer using a haemocytometer under fluorescence microscopy.[34] At least 1000 cells were scored for each patient. The per cent conjugation is the number of conjugated CD4 T cells divided by the total number of CD4 T cells × 100. Counting CTL target cell conjugates under a microscope has been shown to be both accurate and specific.[21,34,35]

Quantification of CD4 T cells in apoptosis following CD8–CD4 T-cell interaction

Sorted CD4 and CD8 T cells were allowed to form conjugates that were incubated for 1·5 hr at 37°. The cells were then allowed to adhere to poly-L-lysine-coated glass slides, stained with 5 μl annexin V-FITC (Calbiochem, San Diego, CA) and anti-CD8 allophycocyanin-conjugated antibody (BioLegend, San Diego, CA) for 15 min at room temperature, and recorded by fluorescence microscopy. Binding of annexin V-FITC was used to measure CD4 T-cell apoptosis. In viable cells, phosphatidyl serine is located on the cytoplasmic surface of the cell membrane. During apoptosis, phosphatidyl serine is exposed on the outer cell surface, which enables binding of annexin V-FITC. Binding of propidium iodide (PI) to nucleic acid has been used to detect advanced apoptotic cells. The annexin V-FITC apoptosis detection kit (Calbiochem PF032) was employed following conjugate formation between sorted CD8 and CD4 T cells compared with stand-alone CD8 and CD4 T cells as control, according to the manufacturer's instructions. Briefly, equal numbers of sorted CD8 and CD4 T cells were mixed and allowed to form conjugates, followed by incubation at 37° for 2 hr. The apoptotic activity of CD8 effectors against conjugated CD4 cells was measured using FACS: annexin-positive and PI-positive cells represented cells in different stages of apoptosis. The cytolytic activity was the percentage of total cells in apoptosis. The difference between the average of isolated live CD4 and CD8 T cells compared with CD8–CD4 T cells in conjugation reflected CD8 T cell-induced killing.

Recording CD4 T-cell apoptosis following conjugation with CD8 T cells

Isolated CD4 T cells were labelled with 1–2 μM fluorescent dye calcein. Conjugates were formed by mixing half a million CD8 T cells with an equal number of calcein-labelled CD4 T cells suspended in 1 ml RPMI–10% FCS. Then, 2×10^5 cells suspended in 300 μl medium were plated in eight-well flat-bottomed plates, culture area 0·8 cm²/well (Lab-Tek™, Swedesboro, NJ) and placed on an inverted microscope. i (Cell R, Olympus, Tokyo, Japan). Ten different regions of interest for a single HIV patient were recorded simultaneously with 10 different regions of interest for one healthy control in each experiment. The temperature was maintained at 37° in a 5% CO_2 environment throughout the experiments. The region of interest was photographed under fluorescence microscopy before and after the recording to recognize the calcein-labelled CD4 T cells.

Cell staining with surface and intracellular monoclonal antibodies

For cell surface staining, the cells were incubated with anti-CD8 allophycocyanin-conjugated antibody (BioLegend) in 50 μl PBS, 2% fetal calf serum for 20 min on ice. For intracellular perforin staining, cells were first stained with a cell surface antibody as described above, washed, then permeabilized using FACS-Perm2 (BD Biosciences, San Jose, CA) and incubated with an anti-perforin-FITC antibody (BD Pharmingen, San Diego, CA) for 30 min at room temperature. The cells were then washed and seeded on poly-L-lysine-coated slides and recorded by fluorescence microscopy.

Cytometric analysis

CELLQUEST software on a FACSCalibur (Becton Dickinson) was used for the cytometric analysis. A total of 100 000 events were acquired for each analysis. Lymphocytes were identified by gating on forward and side scatter of the CD8 T cells, and the percentage of perforin-expressing cells was determined (Table 3).

Identification of HIV-infected CD4 T cells by *in situ* PCR

We used *in situ* PCR of HIV DNA to test if CD4 T cells in the conjugates are infected by HIV. This method was previously described by Bagasra for PBMC of HIV-infected patients.[26,36]

Primers

The PCR primers were designed to recognize integrated HIV DNA in the CD4 T cells of clinical samples. The pri-

Table 3. Percent perforin-expressing CD8 T cells

Case	Acute stage (%)	Chronic stage (%)	Control (%)
1	75	48	16
2	35	22	14
3	49	33	16
4	34	26	6
5	35	18	12
6	20	45	13
7		24	14
8		24	18
9		10	16
10			7
11			17
Average	41·3	27·7	12·8
SE	4·4	3·6	2·8

mer pair from the HIV long terminal repeats was: ltr-f-GCCTCAATAAAGCTTGCCTTGA, ltr-r: GGCGCCACTG CTAGAGATTTT.

Sequencing of the PCR product

DNA from CD4 T cells of HIV-infected patients was amplified and sequenced. We selected a sequence of 56 nucleotides from the amplified DNA shared by several patients and synthesized and tagged it with a fluorescin dye to be used as a probe. The selected sequence 5′-CAC AAC AGA CGG GCA CAC ACC TAC TTT AAG CAC TCA AGG CAA GCT TTA TTG AGG CA-3′.

In situ PCR

Following conjugation, CD4 and CD8 T cells were fixed on slides in 4% paraformaldehyde in 1× PBS, amplified and hybridized with the fluorescein-tagged probe using frame-seal incubation chambers.

Statistical analysis

All data were analysed by using one-way analysis of variance. Statistical differences among means were considered significant at $P < 0·05$. A post hoc test (Tukey–Kramer) was performed when the interaction between treatments was significant. The JMP version 5.1 was used for all analyses. Values are presented as mean ± standard error of the mean.

Results

CD8 and CD4 T cells from the PBMC of HIV-infected patients form conjugates

The ability of CD8 T cells procured from HIV-1-infected patients to form conjugates with autologous

CD4 T cells was determined (Fig. 1; Tables 1 and 2). Conjugate formation between CD8 T cells and their target cells was previously studied and found to be specific.[21,34,35] The present experiments included cells from healthy controls, acute HIV-1-infected patients and chronic HIV-1 infected patients. PBMC were sorted for CD4 and CD8 T cells, the CD4 T cells were labelled with the green fluorescent tag calcein. Equal numbers of CD8 and CD4 T cells were interacted, recorded and counted (Fig. 1). A mean of 6·1 ± 0·5% of the CD4 T cells from acute HIV-1-infected patients and 3·0 ± 0·5% from chronic HIV-infected patients formed CD8–CD4 T-cell conjugates, compared with 0·9 ± 0·06% from HIV-negative controls ($P < 0.0001$) (Tables 1 and 2; Fig. 3).

Apoptosis of conjugated CD4 T cells

We continuously recorded the apoptotic process in conjugated CD4 T cells in tissue culture under a microscope (Fig. 2a–c, see Supporting information, Videos. S1–S3). Digitized images were acquired every 2 min for up to 3 hr using a live cell imaging interface. The CD4 T cells were identified by the green fluorescence of calcein. We recorded cells derived from six HIV-infected patients as well as six healthy controls. Three representative films and snapshots thereof are shown in Fig. 2(a–c). Figure 2(a) illustrates conjugation of CD4–CD8 T cells, eventually leading to apoptosis of CD4 T cells from the PBMC of an acute HIV-infected patient. Figure 2(b,c) illustrate similar events in two chronic HIV-infected patients. CD4 target cells can be seen to have undergone apoptosis even after the CD8 T cell had detached from it (Fig. 2c; see Supporting information, Video S3) as previously demonstrated.[21] No comparable events of conjugation of CD4–CD8 T cells resulting in CD4 T-cell apoptosis were observed in healthy controls.

To verify that the observed apoptosis was indeed of CD4 T cells that had conjugated to CD8 T cells, the cells were labelled with an anti-CD8 antibody (blue) and the apoptosis marker Annexin V-FITC (green). Annexin binding and morphology typical of apoptosis (i.e. cell shrinkage, plasma membrane blebs and formation of apoptotic bodies) were observed under the microscope. Apoptosis was detected in CD4 T cells that had conjugated to CD8 T cells during the acute (Fig. 2d; 1–3) as well as the chronic stages of HIV infection (Fig. 2d; 4–6).

Conjugation of CD8–CD4 T cells and cytolytic activity in acute and chronic HIV-1 infection

We applied FACS analysis to quantify the apoptosis of conjugated CD4 T cells from HIV-1-infected patients during both the acute and chronic phases of HIV-1 infection using the Annexin V-FITC apoptosis detection kit and compared their findings with those of healthy controls (Fig. 3a). We observed differences between both the numbers of intact CD4 and CD8 T cells (Annexin-negative, PI-negative) and the number of cells in early apoptosis (Annexin-positive, PI-negative) with the number of cells in late apoptosis (Annexin-positive, PI-positive). The cytolytic activity is the percentage of cells in apoptosis out of the total number of cells (two different experiments in Fig. 3a). It emerged that 90% and 88% of the cells of the control were intact compared with 69% and 66% of the acute HIV-infected patients and 83% and 80% of the chronic HIV-infected patients. It appeared that the main difference between the acute and chronic HIV-infected patients stemmed from the increased late apoptosis within the acute HIV-infected patients (Fig. 3a).

Figure 3(b) and Tables 1 and 2 compare CD4 T cells, CD8 T cells, viral load, conjugate formation and cytolytic activity observed in 11 acute (Table 1) and 11 chronic
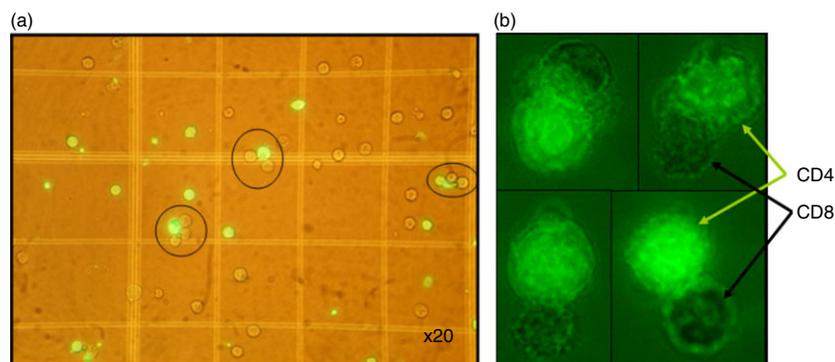
Figure 1. Conjugate formation: CD4 and CD8 T cells were sorted from peripheral blood mononuclear cells (PBMC) of HIV-1-infected patients. Calcein-labelled CD4 T cells (green florescence) and non-labelled autologous CD8 T cells (one million each) were allowed to form conjugates by co-centrifugation followed by re-suspension. (a) Conjugates were counted in a haemocytometer chamber under fluorescence microscopy, – magnification × 20. (b) CD4 (green) and CD8 cells in conjugates, magnification × 100.
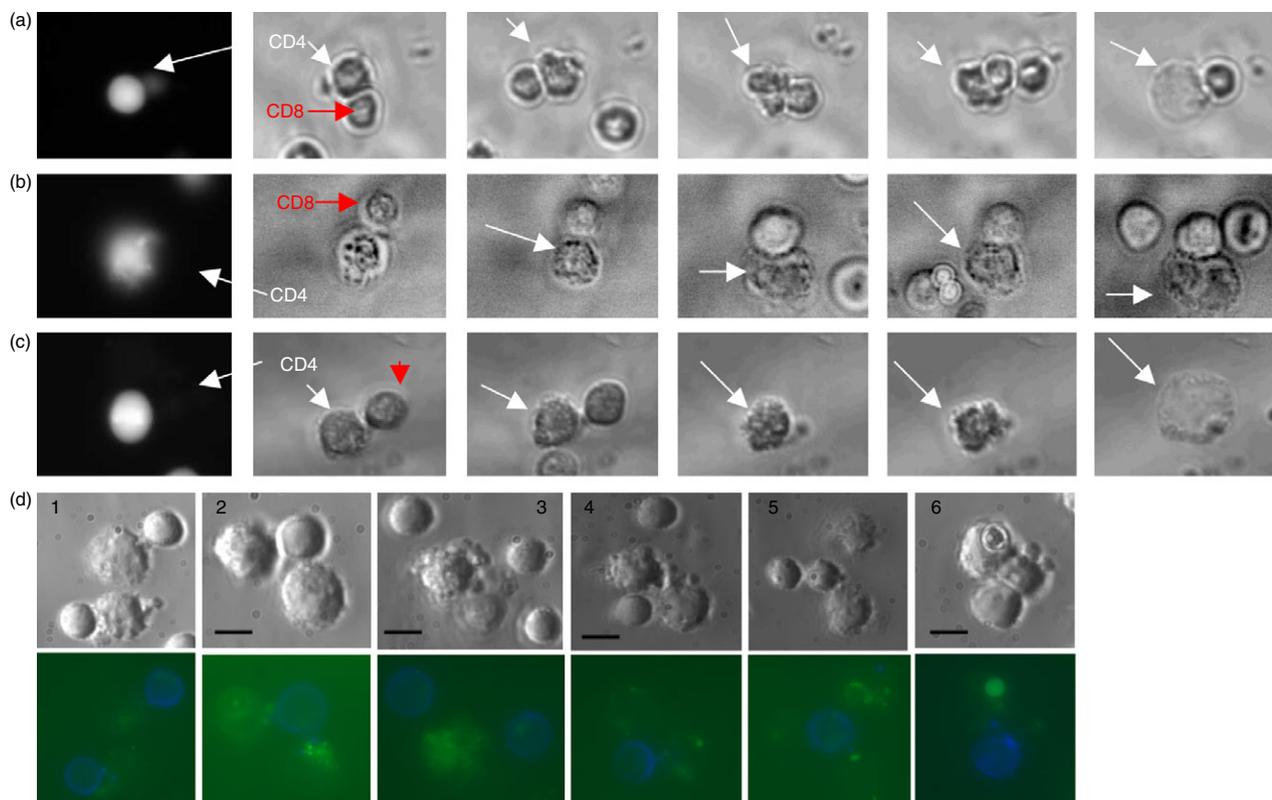
Figure 2. CD8-mediated killing of autologous conjugated CD4 T cells during acute and chronic HIV infection. (a–c, video S1–S3) We continuously recorded the apoptotic process of the conjugated calcein-labelled CD4 T cells in tissue culture under a microscope. Three representative films and snapshots from the films are shown. (a) CD4 and CD8 T cells conjugate, eventually leading to CD4 T-cell apoptosis from the peripheral blood mononuclear cells (PBMC) of an acute HIV-infected patient. (b and c) Events similar to (a) in two chronic HIV-infected patients. (c, video S3) The CD4 target cells were able to undergo apoptosis even after the CD8 T cell had detached from it. (d 1–6) CD8 T cell (blue) and the apoptosis marker annexin V (green) staining of conjugates. Only CD4 T cells that had conjugated to CD8 T cells demonstrate apoptosis (annexin staining, cell shrinkage, plasma membrane blebs and formation of apoptotic bodies) during both the acute (d 1–3) and chronic (d 4–6) stages of HIV infection. Each scale bar is 10 μm.

(Table 2) HIV-1 infected patients with cells of healthy controls. Conjugate formation in the acutely infected patients was $6·1 \pm 0·5\%$, significantly higher compared with the $3·1 \pm 0·5\%$ of the chronically infected patients, and the $0·9 \pm 0·06\%$ for the healthy controls ($P < 0·0001$ for each) (Fig. 3b). Cytotoxic activity in the acutely infected patients was $18·6 \pm 2·4\%$, significantly higher compared with $10·9 \pm 2·0\%$ in the chronically infected patients and $8·8 \pm 1·0\%$ in healthy controls ($P = 0·0008$) (Fig. 3b).

Perforin expression in sorted and conjugated CD8 T cells from HIV-1-infected patients

Perforin expression was determined in sorted CD8 T cells procured from PBMC of HIV-1-infected patients and from the healthy controls. CD8 T cells were labelled with CD8 and perforin antibodies, and 10^5 cells were analysed by FACS for perforin expression (Table 3). There were no significant differences in perforin expression between the acute ($41 \pm 4·4\%$) and chronic ($27 \pm 3·6\%$) groups of

infected patients: both were significantly different from the healthy controls ($12 \pm 2·8\%$; $P < 0·0001$ for each) (Fig. 4a,b). Immunofluorescent staining was applied to determine perforin expression in individual conjugated CD8 T cells. Perforin expression (green) in conjugated CD8 T cells (blue) was found in 60% of CD8 T cells found in conjugation with CD4 T cells in both the acute (Fig. 4c) and chronic HIV-1-infected patients (Fig. 4d).

Conjugated CD4 T cells are HIV-infected

In situ PCR was used to identify integrated HIV DNA in conjugated CD4 T cells in four HIV-infected patients. Previous studies employing *in situ* PCR to identify chronic HIV-infected CD4 T cells within the PBMC of HIV-infected patients found an average of 6–8% infected cells.[26,29] Figure 5(a, b) illustrates conjugation of CD4–CD8 T cells from the PBMC of a 42-year-old patient, 2 years after infection, with a viral load of 25 471 copies/ml and CD4 count of 666/mm^3. Figure 5(a) demonstrates a mixture of CD8 and CD4 T cells in which 10% of the
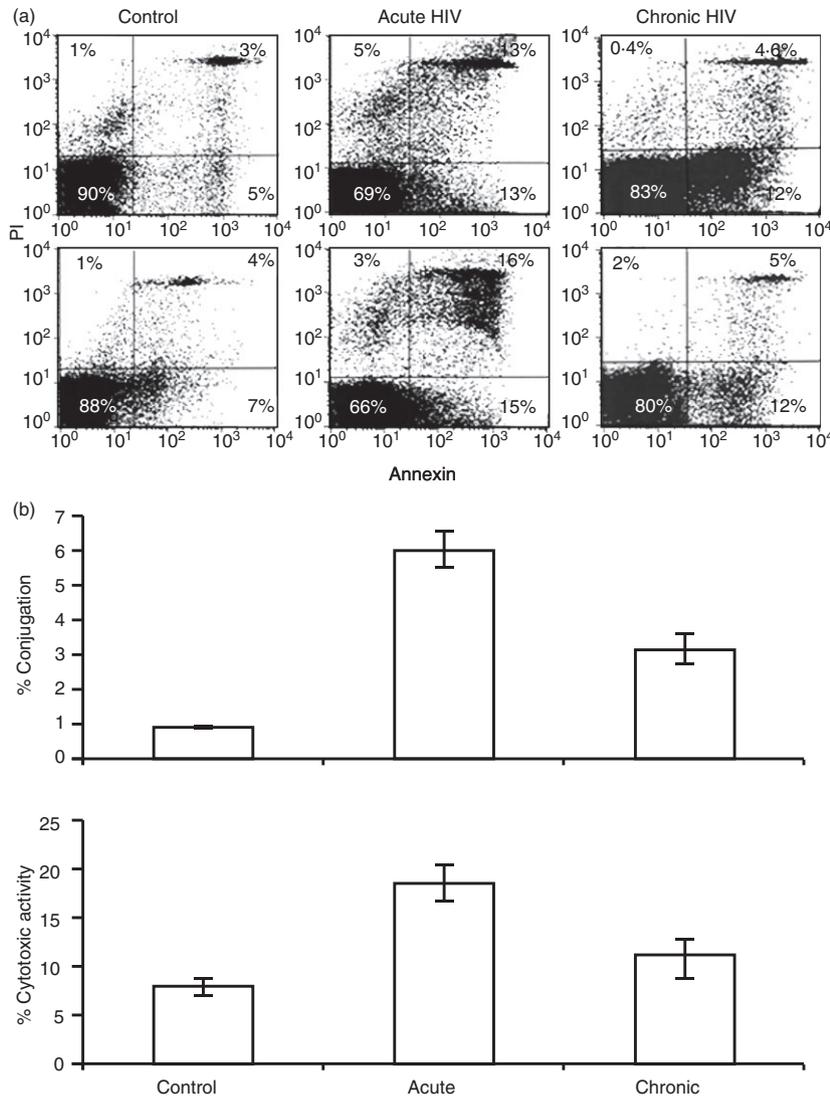
Figure 3. Conjugate formation and lytic activity. Killing activity was measured with an annexin V-(FITC) apoptosis detection kit and analysed by FACS where annexin and PI-positive cells represent cells in different stages of apoptosis. (a) Two different experiments are presented: 90% and 88% of the cells in the non-infected control group are intact compared with 69% and 66% in the acute HIV-infected patients and 83% and 80% in the chronic HIV-infected patients. The main difference between the acute and chronic HIV-infected patients stems from the increased late apoptosis occurring in the former. (b) Statistical analysis of the CD8-CD4 T-cell conjugate formation and cytotoxic activity between 11 acute and 11 chronic HIV-infected patients and healthy controls. Average ± SE; bars with different letters differ at $P < 0.05$ (Tukey–Kramer test).

CD4 T cells have integrated HIV DNA in their nucleus. All conjugates demonstrated an HIV DNA within them (Fig. 5b). Figure 5(c) illustrates conjugation of CD4–CD8 T cells from the PBMC of another chronic HIV-infected patient.

Discussion

AIDS is characterized by a failure of the immune system to overcome HIV-1 infection, resulting in persistent viral infection with an insidious disease progression. This process occurs despite a strong HIV-1-specific CD8 T-cell response.[1–3] The precise role of CD8 T cells during the clinical course of HIV-1 infection has not yet been defined. CD8 T cells are an essential component of an effective host immune response to viral infections.[5,37] There are HIV-specific CD8 T cells as well as latently infected CD4 T cells in the PBMC of untreated HIV-1-infected patients. The biology of latent HIV-infected CD4

T cells is still an enigma. The latent reservoir in resting CD4 T cells is the major barrier to HIV-1 eradication. Studies that used sensitive *in situ* PCR to identify chronic HIV-infected CD4 T cells within the PBMC found that between 0·1–13·5% of those CD4 T cells are positive for HIV DNA (average of 6 and 8%).[26,29] Recently, Siliciano and Siliciano published a study that provides a molecular basis for understanding measures of the latent reservoir.[17] Through an analysis of proviruses that did not give rise to infectious virus following a single round of T-cell activation (non-induced proviruses), they provided a definitive explanation for the large discrepancy between results of PCR and culture assays of latent reservoir size. The identification of intact, non-induced proviruses indicates that the size of the latent reservoir may be much greater than previously thought. They reconstructed full-length, intact non-induced proviruses from multiple patients, and all showed growth kinetics comparable to induced proviruses from the same patient and a reference isolate.
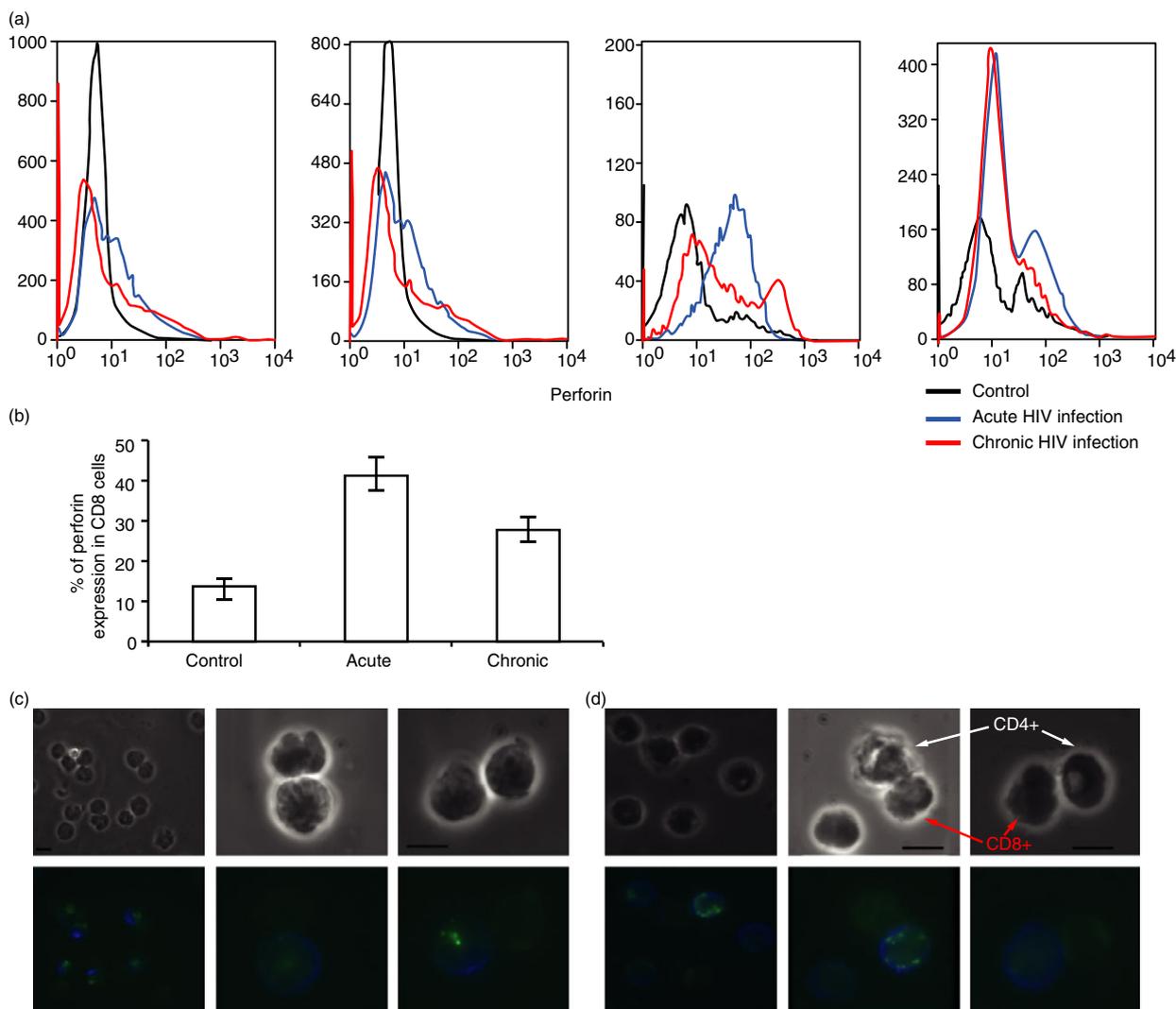
Figure 4. Perforin expression in CD8 T cells from peripheral blood mononuclear cells (PBMC) of acute and chronic HIV-infected patients and healthy controls. (a) FACS analysis of perforin expression in CD8 T cells in acute HIV infection (blue), chronic HIV infection (red), and non-infected controls (black) (four different experiments are presented). (b) Statistical analysis of perforin expression in sorted CD8 T cells of acute and chronic HIV-infected patients and healthy controls (average ± SE), bars with different letters differ at $P < 0.05$ (Tukey–Kramer test). (c) Immunofluorescence of CD8 (blue) and perforin (green) in conjugated CD8–CD4 T cells of acute and (d) chronic HIV-infected patients. Each scale bar is 10 μm.

These intact non-induced proviruses are not detected in standard culture assays.

The frequency of cells harbouring HIV pro-viral DNA in lymph nodes and gut biopsies is comparable to the frequency of chronic HIV-infected memory CD4 T cells in PBMC.[16] Studies of CD8 T cells and chronically HIV-infected CD4 T cells in the PBMC are important to understand the events taking place in the lymphoid tissue of HIV-infected patients.

The activity of HIV-specific CD8 T cells against HIV-1 infected autologous CD4 T cells as it occurs *in vivo* has never been successfully tested. The published studies that attempted to assess the function of CD8 T cells had involved the use of target cells that were either infected *in vitro* by HIV or coated with HIV proteins, and so did not authentically represent the *in vivo* setting.[27] Assays employing epitope-specific CTL lines involve pre-incubation with interleukin-2 that up-regulates perforin expression, creating an artificially vigorous cytotoxic activity.[21]

To overcome those limitations, we studied CD8-CD4 T-cell interaction with cells procured from the blood of untreated HIV-infected patients. Those cells were neither manipulated nor stimulated *in vitro*. We demonstrated that CD8 T cells procured from the blood of untreated HIV-1-infected patients express perforin and form conjugates with autologous CD4 T cells during both the acute and chronic phases of the infection. Using annexin PI, we
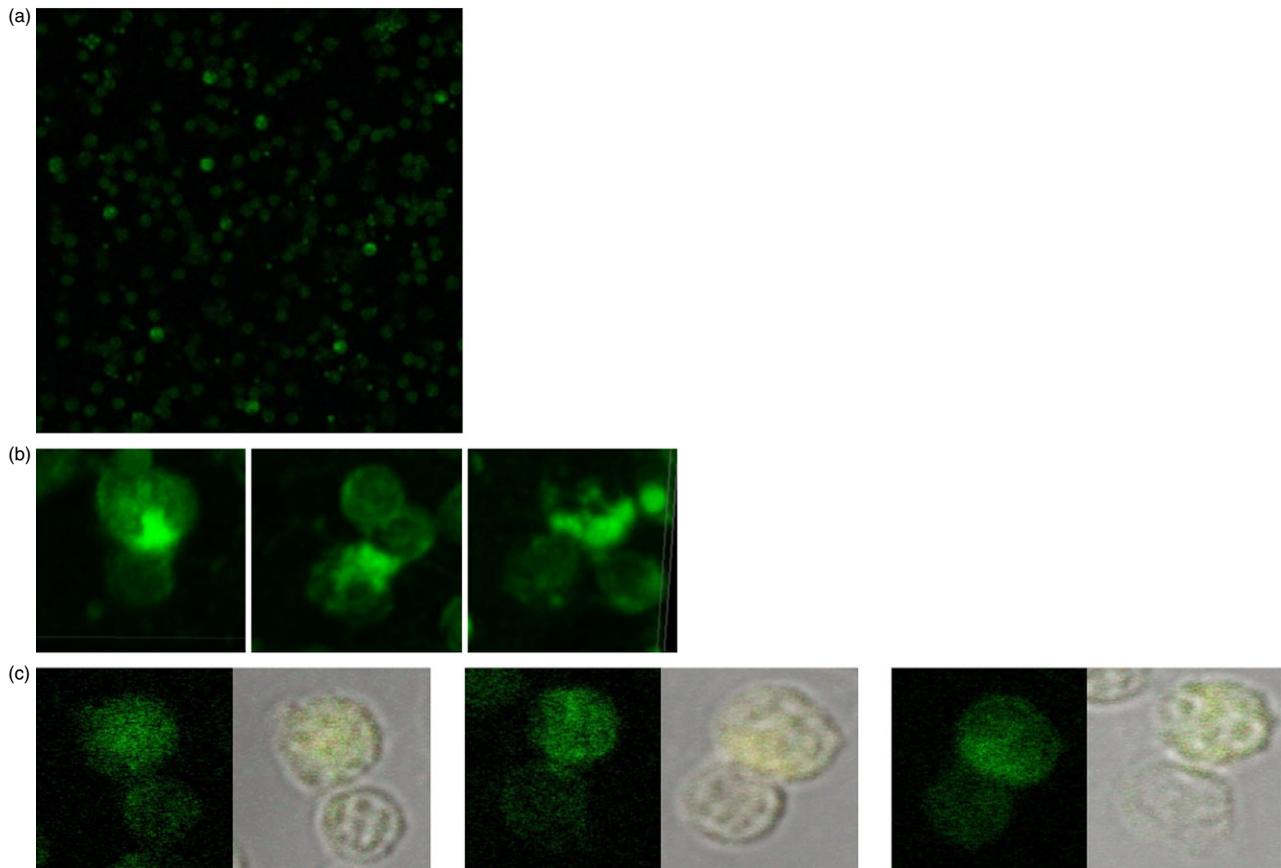
Figure 5. The CD4 T cells in the conjugates are HIV- infected. We demonstrated that the CD4 T cells in the conjugates are HIV-infected with *in situ* PCR for identifying the integrated HIV DNA in the cells (green). The conjugation of CD4–CD8 T cells are from the peripheral blood mononuclear cells (PBMC) of a 42-year-old patient, 2 years after infection, with a viral load of 25 471 copies/ml and CD4 count of $666/mm^3$. (a) A mixture of CD8 and CD4 T cells in which 10% of the CD4 T cells have integrated HIV DNA in their nucleus. (b) Conjugated CD4 T cells with integrated HIV DNA in their nucleus (c) Conjugated CD4 T cells with integrated HIV DNA in their nucleus from the PBMC of another patient. All conjugates demonstrated an HIV-infected cell within them

measured significant apoptotic lytic activity in conjugated CD4 T cells during the acute period of the infection but less so during the chronic period (Tables 1 and 2). We were able to follow and document apoptosis of CD4 T cells while conjugated with CD8 T cells in both the acutely and chronically infected patients (Fig. 2a–c). Annexin staining enabled us to observe the apoptosis of the CD4 T cells conjugated to the CD8 T cells (Fig. 2d). The results of *in situ* PCR of HIV DNA confirmed that CD4 T cells in the conjugates are indeed infected by HIV (Fig. 5).

The decline in CD4 T cells in the peripheral blood of HIV-infected patients during the chronic stage of infection is slow, a feature that may be indicative of controlled elimination of CD4 T cells. We demonstrate that HIV-specific CD8 T cells can recognize HIV-infected CD4 T cells (Fig. 1; Tables 1 and 2). These results are in accord with the number of latent HIV-infected CD4 T cells found by others in the PBMC of HIV-infected patients by sensitive *in situ* PCR.[26,29] The frequency of latently infected cells detected in the viral outgrowth assay is 300-fold lower than the frequency of resting $CD4^+$ T cells that harbour proviruses detectable by PCR.[38] Of the integrated DNA, 12% has a complete genome of the virus.[17,38]

We demonstrate that perforin expression in conjugated CD8 T cells was considerably higher during the acute and chronic phases of HIV infection compared with the CD8 T cells of uninfected controls (Table 3; Fig. 4). The high conjugate formation and cytotoxic activity we have observed during acute HIV infection correlated well with the known concomitant decline in the number of CD4 T cells during this stage of infection. We demonstrate that the killing of CD4 T cells by CD8 T cells continues during the chronic period of HIV infection as well, albeit at a much slower pace. In addition, the majority of CD8 T cells conjugated to CD4 T cells during both the acute and chronic phases of HIV infection expressed high levels of perforin (Fig. 4).

Based on these results, we propose that the annihilation of CD4 T cells in HIV-1-infected patient results, at least

in part, from an ongoing autologous interaction of CD8 T cells with HIV-infected CD4 T cells initiated by conjugate formation and culminating in gradual elimination of the conjugated CD4 T cells. We assume that a dynamic balance is established between HIV-infected CD4 T cells and HIV-specific CTL. It is likely that the virus manipulates the immune system to maintain a low-grade infection, so achieving prolonged survival combined with efficient virus spread.

Acknowledgements

Disclosures

The authors have no financial or commercial conflicts of interest.

References

1 Douek DC, Brenchley JM, Betts MR et al. HIV preferentially infects HIV-specific CD4+ T cells. Nature 2002; 417:95–8.

2 Jin X, Bauer DE, Tuttleton SE et al. Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 1999; 189:991–8.

3 Schmitz JE, Kuroda MJ, Santra S et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 1999; 283:857–60.

4 Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 1994; 68:6103–10.

5 Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, Farthing C, Ho DD. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994; 68:4650–5.

6 Alimonti JB, Ball TB, Fowke KR. Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. J Gen Virol 2003; 84:1649–61.

7 Davenport MP, Petravic J. CD8+ T cell control of HIV – a known unknown. PLoS Pathog 2010; 6:e1000728.

8 Gougeon ML, Piacentini M. New insights on the role of apoptosis and autophagy in HIV pathogenesis. Apoptosis 2009; 14:501–8.

9 Thomas C. Roadblocks in HIV research: five questions. Nat Med 2009; 15:855–9.

10 Cummins NW, Badley AD. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. Cell Death Dis 2010; 1:e99.

11 Doitsh G, Cavrois M, Lassen KG, Zepeda O, Yang Z, Santiago ML, Hebbeler AM, Greene WC. Abortive HIV infection mediates CD4 T cell depletion and inflammation in human lymphoid tissue. Cell 2010; 143:789–801.

12 Ferri KF, Jacotot E, Blanco J et al. Apoptosis control in syncytia induced by the HIV type 1-envelope glycoprotein complex: role of mitochondria and caspases. J Exp Med 2000; 192:1081–92.

13 Dyrhol-Riise AM, Stent G, Rosok BI, Voltersvik P, Olofsson J, Asjo B. The Fas/FasL system and T cell apoptosis in HIV-1-infected lymphoid tissue during highly active antiretroviral therapy. Clin Immunol 2001; 101:169–79.

14 Silvestris F, Cafforio P, Frassanito MA, Tucci M, Romito A, Nagata S, Dammacco F. Overexpression of Fas antigen on T cells in advanced HIV-1 infection: differential ligation constantly induces apoptosis. AIDS 1996; 10:131–41.

15 Brenchley JM, Price DA, Schacker TW et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 2006; 12:1365–71.

16 Chomont N, El-Far M, Ancuta P et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med 2009; 15:893–900.

17 Ho YC, Shan L, Hosmane NN et al. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. Cell 2013; 155:540–51.

18 Klatt NR, Shudo E, Ortiz AM et al. CD8+ lymphocytes control viral replication in SIV-mac239-infected rhesus macaques without decreasing the lifespan of productively infected cells. PLoS Pathog 2010; 6:e1000747.

19 Mortara L, Letourneur F, Gras-Masse H, Venet A, Guillet JG, Bourgault-Villada I. Selection of virus variants and emergence of virus escape mutants after immunization with an epitope vaccine. J Virol 1998; 72:1403–10.

20 Nixon DF, Townsend AR, Elvin JG, Rizza CR, Gallwey J, McMichael AJ. HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. Nature 1988; 336:484–7.

21 Hassin D, Garber OG, Meiraz A, Schiffenbauer YS, Berke G. Cytotoxic T lymphocyte perforin and Fas ligand working in concert even when Fas ligand lytic action is still not detectable. Immunology 2011; 133:190–6.

22 Meiraz A, Garber OG, Harari S, Hassin D, Berke G. Switch from perforin-expressing to perforin-deficient CD8+ T cells accounts for two distinct types of effector cytotoxic T lymphocytes in vivo. Immunology 2009; 128:69–82.

23 Hasin Y, Eilam Y, Hassin D, Fixler R. The effect of cytotoxic lymphocytes on contraction, action potential and calcium handling in cultured myocardial cells. Adv Exp Med Biol 1995; 382:229–38.

24 Hassin D, Fixler R, Shimoni Y, Rubinstein E, Raz S, Gotsman MS, Hasin Y. Physiological changes induced in cardiac myocytes by cytotoxic T lymphocytes. Am J Physiol 1987; 252(1 Pt 1):C10–6.

25 Cerny A, Chisari FV. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. Hepatology 1999; 30:595–601.

26 Bagasra O, Hauptman SP, Lischner HW, Sachs M, Pomerantz RJ. Detection of human immunodeficiency virus type 1 provirus in mononuclear cells by in situ polymerase chain reaction. N Engl J Med 1992; 326:1385–91.

27 Migueles SA, Laborico AC, Shupert WL et al. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. Nat Immunol 2002; 3:1061–8.

28 Migueles SA, Osborne CM, Royce C et al. Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. Immunity 2008; 29:1009–21.

29 Patterson BK, Till M, Otto P, Goolsby C, Furtado MR, McBride LJ, Wolinsky SM. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science 1993; 260:976–9.

30 Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ. The challenge of finding a cure for HIV infection. Science 2009; 323:1304–7.

31 Deeks SG. HIV: shock and kill. Nature 2012; 487:439–40.

32 Shan L, Deng K, Shroff NS et al. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. Immunity 2012; 36:491–501.

33 Schiffenbauer YS, Kalma Y, Trubniykov E, Gal-Garber O, Weisz L, Halamish A, Sister M, Berke G. A cell chip for sequential imaging of individual non-adherent live cells reveals transients and oscillations. Lab Chip 2009; 9:2965–72.

34 Berke G, Gabison D, Feldman M. The frequency of effector cells in populations containing cytotoxic T lymphocytes. Eur J Immunol 1975; 5:813–8.

35 Berke G. Killer Lymphocytes, 1st edn. Berlin: Springer, 2007.

36 Bagasra O. Protocols for the in situ PCR-amplification and detection of mRNA and DNA sequences. Nat Protoc 2007; 2:2782–95.

37 Zinkernagel RM, Doherty PC. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. Adv Immunol 1979; 27:51–177.

38 Eriksson S, Graf EH, Dahl V et al. Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. PLoS Pathog 2013; 9:e1003174.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Video S1. Illustrates conjugation of CD4–CD8 T cells, eventually leading to apoptosis of CD4 T cells from the PBMC of an acute HIV-infected patient.

Video S2 and S3. Illustrate similar events in two chronic HIV-infected patients. In video S3 the CD4 T cell can be seen to have undergone apoptosis even after the CD8 T cell had detached from it.