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G. 知的所有権の取得状況

1. 特許取得

1) 発 明 人；石坂幸人、長谷川正勝、野原 聡
発明の名称；「新規核移行ペプチド」
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2) 発 明 人；田中勇悦
発明の名称；「ヒト免疫不全ウイルス感染阻害物質」、抗CXCR4抗体A120のHIV抑制活性について国内、国外へ琉球大学の知財部門を通して出願中

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Ⅲ. 研究成果の刊行物・別刷

Effect of lentivirus encoding HIV-1 Nef-U3 shRNA on the function of HIV-specific memory CD4$^+$ T cells in patients with chronic HIV-1 infection

Takuya Yamamoto[a,b,c], Assia Samri[c], Anne-Geneviève Marcelin[d], Yu-ya Mitsuki[a], Calvez Vincent[d], Brigitte Autran[c] and Yasuko Tsunetsugu-Yokota[a]

Objective: To determine whether HIV-1-specific CD4$^+$ T cells with proliferative capacity are eliminated or functionally defective because of HIV-1 reactivation.

Design: The loss of proliferative capacity by HIV-1-specific CD4$^+$ T cells compromises the host's ability to maintain protective immunity against HIV-1 and is a hallmark of disease progression. We used a recombinant lentivirus encoding an HIV-specific short hairpin (sh)RNA (Lenti shNef366) with known HIV-inhibitory activity to analyze the functional state of HIV-1-specific CD4$^+$ T cells.

Methods: T lymphocytes from untreated chronically HIV-infected patients with documented high viral loads (above 10 000 HIV-RNA) were transduced with Lenti shNef366, and the proliferation, differentiation, and cytokine production of HIV-specific CD4$^+$ T cells were analyzed.

Results: Lenti shNef366 restored the proliferation of HIV p24-specific CD4$^+$ T cells in eight of 12 patients tested, affecting primarily CD27$^+$ or CD28$^+$ CD4$^+$ T cells that were at an intermediate stage of differentiation. Although cytokine production by CD4$^+$ T cells remained poor after transduction with Lenti shNef366, improved proliferative capacity was associated with significantly higher levels of expression of CD107a.

Conclusion: In chronic stages of HIV-1 infection with high levels of HIV replication, proliferation-competent HIV-specific CD4$^+$ T cells in an intermediate stage of differentiation are present but are exquisitely and strongly impaired. Blocking HIV reactivation may restore a key functional property of memory T cells.

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Keywords: chronic HIV-1 infection, HIV-specific memory CD4$^+$ T cells, lentiviral shRNA transduction, proliferative responses

Introduction

The establishment of persistent HIV infection eventually leads to early quantitative and functional defects in HIV-specific CD4$^+$ T cells [1,2]. HIV-specific CD4$^+$ T cells rapidly lose their proliferative capacity or the ability to produce IL-2 or both under conditions of high viral load (HiVL). Two key functions of memory T cells and disease progression are closely associated with defects in proliferation of IL-2 or IFN-γ/IL-2 production by

[a]Department of Immunology, National Institute of Infectious Diseases, Shinjuku-ku, [b]Division of Cellular and Molecular Biology, Department of Cancer Biology, The Institute of Medical Science, The University of Tokyo, Shirokane-dai, Minato-ku, Tokyo, Japan, [c]Laboratoire d'Immunologie Cellulaire et Tissulaire, INSERM U543, and [d]Virology Laboratory, UPRES EA2387, Hopital Pitie-Salpetriere, Universite Pierre et Marie Curie, Paris, France.

Correspondence to Dr Yasuko Tsunetsugu-Yokota, MD, PhD, Department of Immunology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan.

Tel: +81 3 5285 1111 x2133; fax: +81 3 5285 1156; e-mail: yyokota@nih.go.jp

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$CD4^+$ T cells [2–8]. Substantial numbers of IFN-γ^+ HIV-specific $CD4^+$ T cells persist in most individuals with active HIV-1 infection [9–12], but the levels of this subset of T cells do not correlate with disease progression [10,12] except in long-term nonprogressors (LTNPs) [5]. Thus, it appears that functional status, and not the absolute number, of HIV-specific $CD4^+$ T cells is important for controlling viral replication. A full understanding of the mechanisms that underlie the loss of HIV-specific memory $CD4^+$ T cells and methods of restoring these cells is important, as antigen-specific $CD4^+$ memory T cells are required for the maintenance of effective memory $CD8^+$ T cells, a key element of the protection against HIV [13–15].

Several mechanisms have been proposed to explain the T-cell defects in HIV infection, including direct virus-induced elimination of infected $CD4^+$ T cells by apoptosis, anergy or exhaustion, any of which would affect both infected and uninfected cells. Initial loss of proliferative potential or the ability to produce IL-2 might reflect active HIV replication, as memory $CD4^+$ T cells represent the major reservoir for HIV-1 [16], and HIV-specific memory $CD4^+$ T cells are the predominant targets for HIV-1 [1]. However, defects in $CD4^+$ T-cell proliferation and cytokine production are corrected only if antiretroviral therapy (ART) is introduced early, at the time of primary infection [2], not late in the disease [17], which indicates that these cells might be deleted early [8]. HIV-specific $CD4^+$ T-cell defects might reflect anergy or exhaustion, rather than deletion, as the defects can be reversed *in vitro* by blocking cell-surface molecules such as PD-1 or CTLA-4, which induce negative signals [18–20]. Given that latent HIV-1 infection targets predominantly HIV-specific memory $CD4^+$ T cells, it is important to develop novel strategies that induce robust HIV-specific $CD4^+$ T-cell responses while protecting these cells from new infection with reactivated HIV-1 [21].

The therapeutic potential of RNAi in combating HIV-1 infection was first demonstrated *in vitro* using synthetic siRNAs that targeted HIV-1 [22–24], though in these early experiments, the effect was transient. siRNAs are generated from single-stranded RNA precursors that form a characteristic short hairpin structure, so-called shRNAs, which can be synthesized from DNA templates under control of the RNA polymerase III promoter. We and others have developed a lentivirus vector-based RNAi system for generating shRNAs that target HIV-1 [21,25,26]. We demonstrated that a lentivirus-encoded shRNA that targeted the U3-Nef region of HIV-1 (Lenti shNef366) inhibits HIV-1 replication in primary macrophages and reduces HIV-1 infectivity [21].

In the current study, we used our lentiviral-based RNAi system to examine the role of HIV-1 reactivation in suppressing the proliferative capacity of HIV-specific memory $CD4^+$ T cells in various differentiation and polyfunctional states in individuals with chronic HIV-1-infection. Our findings suggest that intracellular inhibition of HIV-1 through the expression of siRNAs can restore an important function of HIV-1-specific memory $CD4^+$ T cells.

Materials and methods

Study patients

We selected 12 HIV-infected patients with HiVL from the IMMUNOCO cohort of standard progressors [27]. The patients exhibited a median plasma viral load of 76 850 HIV-1 RNA copies/ml (range approximately 10 800–320 000) and median CD4 cell counts of 488 cells/μl (range 166–770). The clinical characteristics of the patients are presented in Table 1. We received informed consent from all patients, and the protocol was approved by the Pitié-Salpétrière ethical committee.

Peripheral blood mononuclear cells (PBMCs) from normal individuals were prepared from buffy coats provided by the Blood Bank of Japan Red Cross (Tokyo, Japan). The protocol for the utilization of PBMCs from

Table 1. Clinical characteristics.

Patients	Date	ARV	CD4 (cells/ ml)	Viral load (copies/ ml)	HLA
A277 10	14-06-93	No	479	19 400	A1 A19-2(31), B8 B22(55)
A231 20	22-06-94	No	599	10 800	A28-A19.2(31), B14-B12(44)
A211 10	20-01-93	No	484	17 800	A2 A32, B21(49) B7
A221 10	19-04-93	No	770	11 200	A10(26) A32, B22(55) B12(44)
A226 00	24-03-92	Yes	407	22 000	A9(23) A34, B53-52
A219 00	03-03-92	No	506	220 000	A2 A9(24), B15(62) B16(30)
A219 10	15-01-93	No	502	64 000	A2 A9(24), B15(62) B16(30)
A239 00	29-06-92	No	558	140 000	A1-A, B15(57)
A239 10	14-06-93	No	483	320 000	A1-A, B15(57)
A202 10	–93	No	395	28 000	A3 A9(24), B16(38)
A205 10	28-01-93	No	502	13 000	A32, B17 B12(44)
A241 10	06-09-93	No	166	56 000	A3 A32, B35B16(38)

ARVs, antiretrovirals; HLA, human leukocyte antigen; ND, not detectable.

normal individuals was approved by the ethical committee of the National Institute of Infectious Diseases, Japan.

Preparation of recombinant lentivirus

The preparation of Lenti cont and Lenti shNef366 was previously described [21]. In brief, 293T cells were transfected either with a replication-incompetent lentiviral vector expressing enhanced green fluorescent protein (EGFP) alone or shNef366 + EGFP together with an expression plasmid for VSV-G and Rev and the packaging plasmid pCAG-HIVgag/pol using the calcium phosphate precipitation method. Lentivirus infection was monitored by EGFP fluorescence. The recombinant HIV-1 encoding the fluorescent protein DsRed (HIV-1_{NL-D}) was produced by 293T transfection as described previously [28].

Lentiviral infection of CD4+ T cells and peripheral blood mononuclear cells

CEM cells were infected with HIV-1_{NL-D}, washed and then superinfected with lentivirus at multiplicity of infection (MOI) of 1–2. After extensive washing, the cells were cultured and analyzed by FACScalibur (BD Biosciences, San Jose, California, USA) at 3 or 7 days after infection. Latently HIV-1-infected ACH-2 cells were infected with lentivirus, cultured for 1 week, and then stimulated with 0.05 or 0.5 ng of TNF-α for the reactivation of HIV-1 [29]. The culture supernatant was collected 2 days after cultivation. Viral production in the cell-culture supernatants was determined using a Gag p24 antigen ELISA kit (RETRO TEC; ZeptoMetrix, Buffalo, New York, USA).

Frozen PBMCs with a cell viability of greater than 85% were separated into enriched T cell (>95% $CD3^+$) and non-T-cell fractions using a human T-cell enrichment kit (StemCell Technologies, Vancouver, Canada). The enriched T-cell fraction was infected with lentivirus by spinoculation [30] for 2 h, and then the cells were washed extensively and recombined with the non-T-cell fraction.

T-cell proliferation assay

T-cell proliferation was assessed by BrdU incorporation. Frozen PBMCs were transduced with lentivirus as described above. One day postinfection (dpi), cells were stimulated with either 2 μg/ml of recombinant HIV Gag p24 (Protein Science, Meriden, Connecticut, USA), 10 μg/ml of human cytomegalovirus (CMV) extract (Behring, Liederbach, Germany), 10 μg/ml of purified protein derivative (PPD) (Statens Institut, Copenhagen, Denmark) or 1 μg/ml of staphylococcal enterotoxin B (SEB). Cells were cultured for 3 days, incubated for 1 h with BrdU (100 ng/ml), and then intracellular staining was carried out using allophycocyanin (APC)-conjugated anti-BrdU antibody in combination with surface staining as previously described [31]. Approximately 100 000 events were acquired by FACScalibur, and data were analyzed using Flowjo software (Tree Star, Inc., San Carlos, California, USA). As the small number of $BrdU^+$ $CD4^+$ T cells within this population made a direct analysis of their phenotype impractical, we analyzed the phenotype of all $CD4^+$ T cells obtained from each donor and estimated the frequency of $BrdU^+$ $CD4^+$ T cells at a particular differentiation stage.

Analysis of cytokine production by HIV-specific $CD4^+$ T cells

PBMCs were transduced with lentivirus and then, 1 dpi, cells were either not stimulated or stimulated with recombinant HIV Gag p24 and incubated for an additional 48 h. Cells were incubated with Gag p24 and 0.5 μl of anti-CD107a antibody (BD Biosciences) for 1 h, followed by incubation with monensin (BD Biosciences) and brefeldin A (Sigma-Aldrich, St Louis, Missouri, USA) for an additional 5 h. The following antibodies were used (labeling moiety in parentheses): anti-CD3 (APC-Cy7), anti-CD4 (PerCP), anti-CD8 (biotin), anti-MIP-1β (Phycoerythrin), CD107a (pacific blue), anti-IL-2 (APC), and IFN-γ (Phycoerythrin-Cy7) (BD Biosciences) and anti-CD69 (Phycoerythrin-Texas Red; ECD) (Beckman Coulter, San Jose, California, USA). An aqua fluorescent reactive dye (ViViD; Invitrogen, Carlsbad, California, USA) was used to exclude dead cells from the analysis. Cells were analyzed using a LSRII system (BD Biosciences), and data files were processed using FlowJo and FACSDiva software (BD Biosciences). Analysis of the polyfunctional phenotype of $CD4^+$ cells was carried out using PESTLE (version 1.5. 4) and SPICE (version 4.1. 6) software, which were generously provided by Dr Mario Roederer (National Institutes of Health, Bethesda, Maryland, USA).

Statistical analysis

The Graphpad Prism v.5.0 software package (GraphPad Software, Inc., San Diego, California, USA) was used for statistical analysis. Intergroup comparisons were performed using the Mann–Whitney *U* test (univariate nonparametric group analysis). A two-tailed *P* value of less than 0.05 was considered significant.

Results

Lenti shNef 366 can block HIV-1 replication/reactivation in $CD4^+$ T cells

Upon stimulation of PBMCs with Gag p24, T cells from individuals that are chronically infected with HIV-1 lose their proliferative capacity *in vitro*, and this defect correlates well with HiVL [2]. To determine whether the loss of proliferation was caused by HIV-1 reactivation followed by virus-mediated cell killing or the depletion or anergy of Gag-specific $CD4^+$ T cells after chronic antigen stimulation *in vivo*, we used an HIV-specific RNAi system to inhibit HIV-1 reactivation. Previously, we reported that a lentivirus-encoded shRNA directed against the

U3-overlapping region of *nef* (Lenti shNef366) inhibits HIV-1 replication in primary macrophages [21]. The putative mechanism of inhibition by RNAi involved early provirus synthesis or late mRNA transcription and decreased infectivity of secondary HIV-1 particles produced by Lenti shNef366-transduced cells [21].

The ability of Lenti shNef366 to inhibit HIV-1 replication in T cells was examined by superinfecting CEM cells that were either mock infected or infected with HIV-1_{NL-D}, with control lentivirus (Lenti cont) or Lenti shNef366. The expression of EGFP (lentivirus) and DsRed (HIV-1) was monitored by FACS. At 3 dpi (upper panel), the frequency of $DsRed^+$ $EGFP^+$ cells was lower in cells transduced with Lenti shNef366 (1.35%) than in cells transduced with Lenti cont (5.22%), though the level of HIV-1 infection (total $DsRed^+$ cells) was similar in Lenti cont and Lenti shNef366-transuced cells [11.74 (6.52 + 5.22) and 10.47 (9.12 + 1.35) %, respectively] (Fig. 1a, upper panels). The frequency of double-positive ($DsRed^+$ $EGFP^+$) cells remained low in cells transduced with Lenti shNef366 (1.17%), whereas the frequency of double-positive cells transduced with Lenti cont was higher (9.79%) at 7 dpi (lower panels). Moreover, the number of Lenti Nef366-transduced cells infected with HIV (total $DsRed^+$) was three-fold lower than the number of control cells infected with HIV [25.09 (15.3 + 9.79) vs. 7.39 (6.22 + 1.17%)], which supports the notion that HIV-1 replication is blocked not only in cells that express Lenti Nef366 but also in nontransduced cells, probably because of the reduced infectivity of secondary HIV-1 virions produced by Lenti Nef366-transduced cells.

To determine whether Lenti shNef366 intervened in the reactivation of latently HIV-infected ACH-2 cells, lentivirus-transduced ACH-2 cells were stimulated with TNF-α and the level of Gag p24 in the culture supernatant was measured. As shown in Fig. 1b, Lenti shNef366 suppressed HIV-1 production, despite the fact that only a quarter of the total cell population expressed EGFP (data not shown). Hence, we postulated that Lenti shNef366-mediated inhibition of HIV-1 replication/reactivation and reduced infectivity by progeny virus might improve the proliferative capacity of Gag-specific $CD4^+$ T cells.

Efficiency of lentiviral transduction of primary $CD4^+$ T cells

Because the transduction efficiency of lentivirus in primary T cells is crucial, we assessed the infectivity of Lenti shNef366 and Lenti cont using GFP fluorescence. The T-cell-enriched fraction of PBMCs was infected with lentivirus by spinoculation. On the basis of infectivity of lentivirus in 293T cells, we defined 100 ng of p24 per 1×10^5 cells as 1 MOI and adjusted the MOI to achieve the highest level of GFP expression possible in primary T cells without toxicity. After lentivirus infection, T cells were stimulated with anti-CD3 and anti-CD28 antibodies, either immediately or 1 dpi, and the level of GFP expression in $CD4^+$ T cells was analyzed by FACS 2 dpi. As seen in Fig. 2a, 64.8% (donor 1) and 60.4% (donor 2) of $CD4^+$ T cells were infected with Lenti cont at an MOI of 0.5, whereas 68.8% (donor 1) and 43.8% (donor 2) of $CD4^+$ T cells were infected with Lenti shNef366 at an MOI of 1.0. There was no difference in transduction efficiency between $CD4^+$ T cells activated immediately and those activated 1 dpi. Therefore, in all subsequent experiments, we used an MOI of 0.5 and an MOI of 1.0–2.0 for Lenti cont and Lenti shNef366, respectively, to achieve comparable transduction efficiencies.

Effect of lentivirus transduction on $CD4^+$ T-cell phenotype and proliferation *in vitro*

We first examined whether lentivirus transduction affected the differentiation phenotype of resting $CD4^+$ T cells or the proliferative capacity of SEB-stimulated $CD4^+$ T cells from healthy donors. To ensure that only T cells were transduced by lentivirus, we separated the T cells from total PBMCs, infected them with lentivirus and then recombined the infected T cells with the non-T-cell fraction.

We used FACS to analyze the two major differentiation phenotypes of $CD4^+$ T cells ($CD45RA^+CCR7^+$, naive; and $CD45RA^-CCR7^+$, central memory) 2 dpi, when the maximum level of GFP expression was achieved. The results of two donors are shown in Fig. 2b. In mock infected PBMCs, naive and central memory cells constituted 43.4 and 28.2% (donor 1) and 84.9 and 8.1% (donor 2), respectively, of total $CD4^+$ T cells (left panels). In lentivirus-infected PBMCs, GFP^+ cells constituted 32.5 and 72.9% of total $CD4^+$ T cells for donor 1 and 2, respectively (middle panels). The percentage of naive and central memory cells was similar in the GFP^+ $CD4^+$ T-cell population of each donor (right panels), which indicated that $CD4^+$ T-cell differentiation is not modulated by lentivirus infection.

To examine the proliferation state of $CD4^+$ T cells, lentivirus-transduced PBMCs were stimulated with SEB for 3 days (beginning 1 dpi), and then BrdU uptake was analyzed. As shown in Fig. 2c, there were similar levels of BrdU uptake in SEB-treated Mock-transduced, Lenti cont-transduced and Lenti shNef366-transduced primary $CD4^+$ T cells (10.5, 12.5 and 10.7%, respectively). These results indicated that lentiviral transduction has little effect on the proliferation of antigen-stimulated $CD4^+$ T cells.

Lenti shNef366 restores HIV-specific $CD4^+$ T-cell proliferation in peripheral blood mononuclear cells from HIV-1 infected individuals

Using our system of lentivirus-mediated inhibition of HIV, we wanted to evaluate the impact of HIV-1

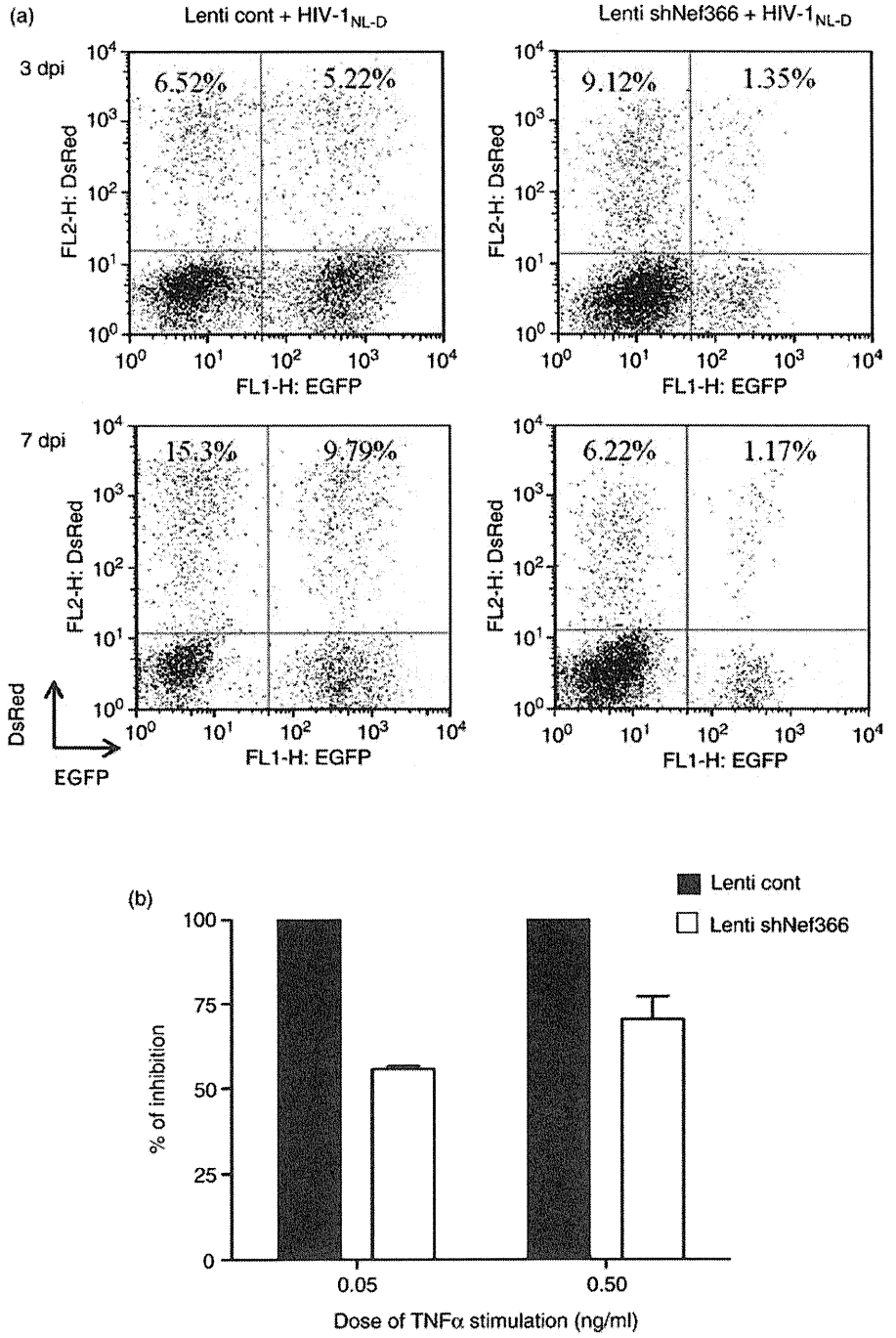

Fig. 1. Effect of Lenti shNef366 on T cells. (a) CEM cells infected with HIV-1_{NL-D} were transduced with Lenti cont (left panels) or Lenti shNef366 (right panels). Cells were collected and analyzed by FACS at 3 (upper panels) and 7 (lower panels) dpi. The data are representative of three independent experiments. (b) ACH-2 cells were transduced with Lenti cont or Lenti shNef366. At 7 dpi, the cells were stimulated with 0.05 or 0.5 ng/ml of TNF-α. Cell-culture supernatants were collected 2 days after stimulation, and the levels of Gag p24 antigen were measured by ELISA. The level of p24 Gag in stimulated ACH-2 culture supernatants was set as 100%, and percentage inhibition of HIV-1 production was calculated. The data represent the means and standard deviation (SD) of three independent experiments.

reactivation on $CD4^+$ T-cell proliferation. Previously, we investigated HIV-1-specific $CD4^+$ T-cell responses in treatment-naive, chronically HIV-infected individuals [5], including the proliferative response (^{3}H-thymidine uptake) to recombinant Gag p24. Although proliferative capacity was greatly enhanced by the depletion of $CD8^+$ cells, it was not affected by the addition of zidovudine (ZDV, 5 μmol/l) in four patients tested (unpublished