Anti-retroviral therapy in HIV-infected patients: *in vitro* effects of AZT and saquinavir on the response of CD4 and CD8 lymphocytes to interleukin-7

Stéphanie Beq[1], Florence Bugault[1], Jean-Hervé Colle[1], Olivier Lambotte[2], Jean-François Delfraissy[2], Jacques Thèze[1]

[1] Unité d'Immunogénétique Cellulaire, Département de Médecine Moléculaire, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France
[2] Service de Médecine Interne, Assistance Publique–Hôpitaux de Paris, Centre Bicêtre, France

Correspondence : J. Thèze
<jtheze@pasteur.fr>

ABSTRACT. IL-7 is a crucial cytokine regulating lymphopoiesis and peripheral T lymphocyte homeostasis. Plasma IL-7 levels increase during HIV infection and, although antiretroviral therapy (ARV therapy) decreases these levels, they fail to return to normal. Immune reconstitution in most ARV-treated patients is only partial. We tested the possibility that the IL-7R system might be affected by ARV drugs. The effects of the antireverse transcriptase AZT and the anti-protease saquinavir on CD3- and CD3+CD28-induced T lymphocyte stimulation, in the presence (or absence) of IL-7, were studied *in vitro*. Small amounts of the drugs did not interfere with the capacity of IL-7 to stimulate T cell proliferation, but higher concentrations significantly decreased IL-7-induced T cell proliferation both in cells from HIV-infected patients and in cells from healthy donors. IL-7 is known to down-modulate its own receptor on the surface of CD4 and CD8 T lymphocytes *in vitro*. In CD4 lymphocytes from healthy donors or HIV-infected patients, neither AZT, nor saquinavir, nor a combination of the two, interfered with this property. In contrast, AZT + saquinavir worsened the IL-7-induced down-regulation of CD127 expression by CD8 T cells from HIV-infected patients, while no such effect was observed with CD8 T cells from healthy donors. Our data suggest that, under certain conditions, antiretroviral therapy could interfere with the expression and function of the IL-7/IL-7R system, and more particularly it may affect the CD8-lymphocyte compartment of HIV-infected patients.

Keywords: HIV, IL-7 receptor, CD127, AZT, saquinavir, immune restoration

IL-7 is a pleiotropic cytokine essential for the early, human T-cell development [1-3]. In addition, IL-7 plays an essential role in peripheral T cell homeostasis, and is involved in controlling specific immune responses [4, 5]. The interleukin 7 receptor (IL-7R) consists of an α-chain (CD127), which confers specificity, and a γ chain, which is common to several cytokines (IL-2, IL-4, IL-9, IL-15 and IL-21) [6].

Untreated HIV-infected patients show increased plasma IL-7 levels [6, 7], and a negative correlation has been noted between IL-7 levels and the CD4 T cell count, suggesting that IL-7 is part of a feedback loop regulating the size of the CD4 pool [7, 8]. Plasma IL-7 decreases during highly active antiretroviral therapy (HAART), but fails to return to normal. HAART-treated HIV-infected patients show only partial immune reconstitution, and restoration of the CD4 count. Many hypotheses have been discussed to explain the corresponding defects. Here we tested the possibility that IL-7 reactivity could be impaired by antiretroviral (ARV) drugs, an issue that is crucial to the understanding of the role played by IL-7 during HAART-driven immune reconstitution, and to the further documentation of the therapeutic potential of IL-7.

We used an *in vitro* model to address this question. The *in vitro* effects of the antireverse transcriptase AZT, and of the anti-protease saquinavir, were evaluated either alone or in combination, on lymphocytes taken from treatment-naive, viraemic, HIV-infected patients and healthy donors, cultured in the presence, or absence, of IL-7. The proliferation of T cells stimulated by limited amounts of anti-CD3 mAb was investigated first. The IL-7 induced down-modulation of the IL-7Rα chain (CD127) on CD4 and CD8 T lymphocytes was then analyzed. The results suggest that AZT affects T cell proliferation, and that AZT + saquinavir affect the expression of CD127 by CD8 lymphocytes from HIV-infected patients. These results will serve as a basis for further discussion of the putative mode of action of ARV drugs in HAART-treated patients. This investigation into the effects of ARV drugs on the IL-7/IL-7R system should be followed by an extensive analysis of this issue in order to fully characterize the side effects caused by the

various ARV drugs employed in the immune reconstitution of HAART patients.

DONORS AND METHODS

Patients

Patients selected for this study were naive of any antiretroviral treatment, and presented with a detectable viral load at inclusion. Patient characteristics are given in *table 1*. Viral load at inclusion was very heterogeneous and ranged from 9,560 to 750,000 mRNA copies/mL. CD4 counts ranged from 220 to 803 CD4/mm^3.
Healthy donors were from the *Établissement Français du Sang*, rue Lecourbe, 75015 Paris.

Chemicals

RhIL-7 was supplied by Cytheris (Vanves) France. The antireverse transcriptase (RT) inhibitor AZT (Zidovudine) was obtained from Sigma (Paris, France). The antiprotease saquinavir was from Roche (Paris, France). AZT and saquinavir solutions were prepared in accordance with the manufacturers' instructions.

Proliferation assay

Freshly isolated PBMC from HIV-infected patients were resuspended and seeded at 2.10^5 PBMCs per well in 96-well, flat-bottomed microtitre plates coated with 0.1 µg/mL anti-CD3 monoclonal antibody (clone UCHT 1 from DAKO) and with goat anti-mouse IgG (H + L) (Beckman Coulter). When indicated, stimulation was also induced by soluble anti-CD3 or anti-CD3 + anti-CD28 mAb. PBMC were cultured in RPMI 1640 medium supplemented with 0.5% AB human serum in the presence or absence of rhIL-7 (10 ng/mL). A mixture of AZT and saquinavir (1 µM/10 nM and 10 µM/100 nM of AZT and saquinavir, respectively) was prepared and added at the start of the culture. The ARV drugs (AZT 0.1, 1, 10, 100 µM) or saquinavir (1, 10, 100 nM/1 µM) were also tested alone. The microtitre plates were placed in a cell incubator, in a humidified atmosphere containing 5% CO_2 for 96h. Proliferation was assessed by incorporation of [^{3}H] thymidine (0.125 µCi/well) for the last 17 hours of the culture.

Flow cytometry analysis of CD127 expression

Freshly isolated PBMC from HIV-infected patients were seeded in 24-well plates containing 2.10^6 PBMC per well in medium alone (RPMI 1640, serum AB 0.5%) or in the presence of rhIL-7 (10 ng/mL). When indicated, AZT or saquinavir were added as described above. Flow cytometry was performed after six days of culture. Immunostaining was performed with conjugated mAbs. CD4-APC (clone MT310) and CD8-APC (clone DK25) were from DAKO (Paris, France). The anti-CD127 PE-conjugated mAb (R34-34) was from Coulter-Immunotech (Marseille, France). The stained preparations were analyzed on a cytofluorometer (FASCalibur) running Cellquest software (Becton Dickinson).

Statistical analyses

Statistical analyses were performed using the Statview version 5.0 with paired or unpaired tests selected depending on the parameter. Significant variations were defined as $p < 0.05$.

RESULTS

IL-7-induced T cell proliferation in PBMC from HIV-infected patients, and healthy donors: effects of the antiretroviral drugs AZT and saquinavir

We first studied the impact of the antiretroviral drugs on T cell proliferation *in vitro*. AZT and saquinavir were tested in combination at two concentrations: 1µM AZT+10 nM saquinavir, and 10 µM AZT+100 nM saquinavir. The results obtained with lymphocytes from five HIV-infected patients are shown in *figure 1A*. IL-7 alone did not induce T cell proliferation. However, in the presence of small amounts of coated anti-CD3 (0.1 µg/mL), IL-7 increased T cell proliferation 5-fold ($p < 0.01$). Although the low drug concentration (1 µM AZT + 10 nM saquinavir) failed

Table 1
Characteristics of viremic HIV-infected patients at inclusion

Patient	Age	Gender	Months after HIV detection	Stage of infection	VL	CD4/mm^3	CD8/mm^3
D12	28	M	36	A	12800	447	531
D13	27	M	14	A	57700	566	1613
D14	56	M	39	A	750000	494	1656
D15	32	F	1	B	84600	367	751
D16	33	F	9	A	19400	320	1454
D17	33	M	78	A	26300	524	1007
D18	41	M	21	A	177000	444	906
D19	24	F	1	A	9560	386	1958
D21	45	M	8	A	25000	803	1032
D22	21	F	2	A	53100	364	2113
D23	30	F	3	A	17300	220	464
D24	37	M	120	A	145000	601	1709
D25	35	F	180	A	44800	579	1421

VL: viral load in mRNA copies/mL. All patients were treatment naive at inclusion.

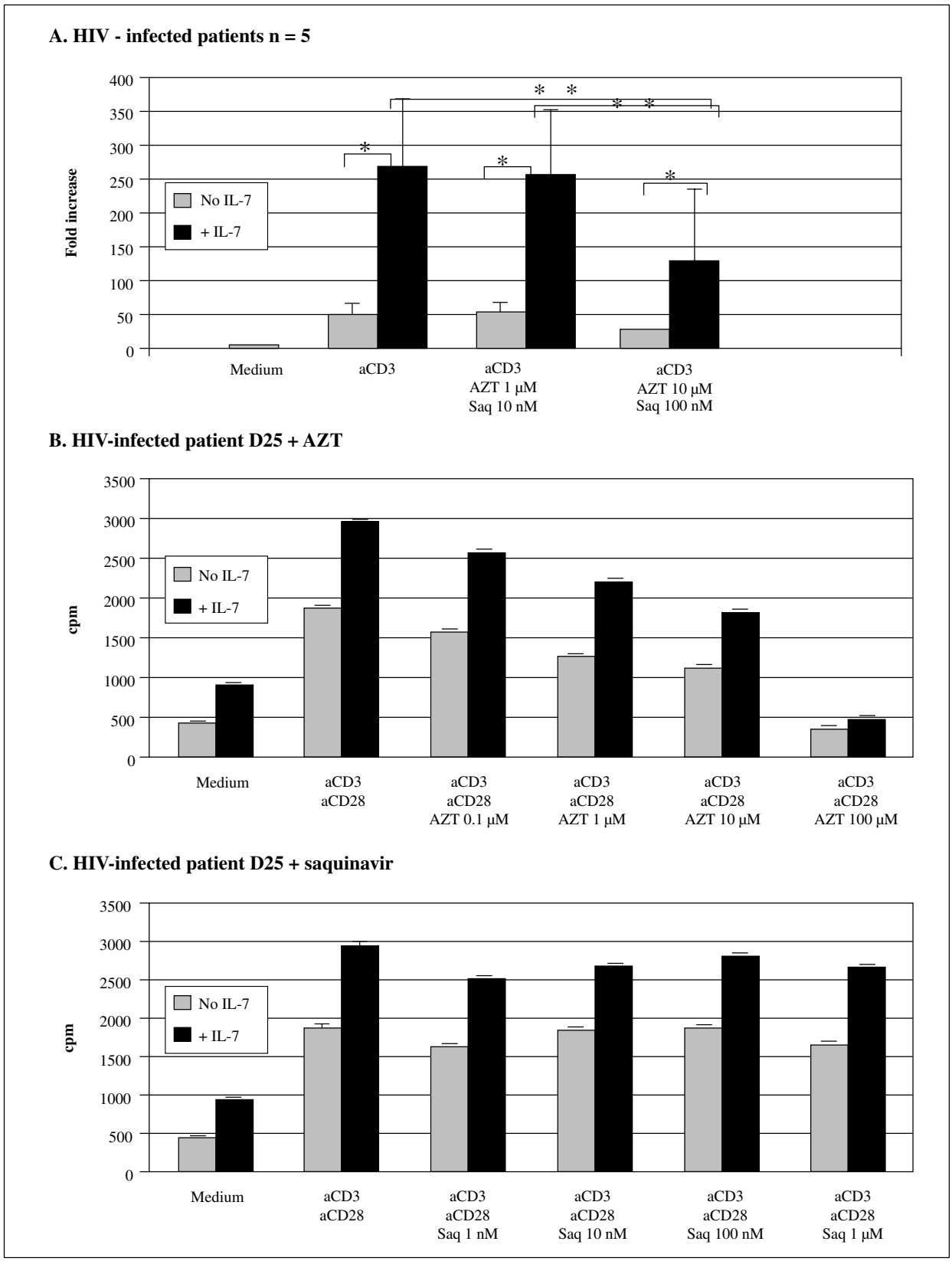

Figure 1

Proliferation of PBMCs in the presence of IL-7 and antiretroviral drugs.

A) Results from five patients are presented as x-fold increase over proliferation in medium alone. IL-7-increased T cell proliferation induced by anti-CD3 mAb was always significant (* $p < 0.01$). The effects of AZT + saquinavir at two different concentrations are shown (for the highest concentration ** $p < 0.05$).

B) Effect of AZT on T cell proliferation in the presence, or the absence of IL-7. T cells were stimulated *in vitro* by soluble anti-CD3 + anti-CD28 mAbs as indicated in Donors and Materials. Results from one representative HIV-infected patient are shown.

C) Effects of saquinavir on T cell proliferation in the presence or absence of IL-7. PBMC were stimulated as above. The results are from one representative HIV-infected patient.

to affect IL-7-induced T cell proliferation ($p < 0.01$), the high drug concentration (10 µM AZT + 100 nM saquinavir) significantly decreased IL-7-induced proliferation ($p < 0.05$) *(figure 1A)*. Similar experiments were performed with PBMC from five healthy individuals. Here again, IL-7 alone did not induce T cell proliferation *(table 2)*. IL-7-induced proliferation was observed only in the presence of limited amounts of anti-CD3 *(table 2)*. At low doses, AZT + saquinavir were found to reduce the proliferative responses. At higher doses of the drug combination, the data indicated that the ARV drugs had a negative effect on CD3 + IL-7-induced T cell responses, as with PBMC from HIV-infected patients.

The effects of the drugs were further investigated by testing AZT and saquinavir separately in PBMC from HIV-infected patients. AZT alone inhibited anti-CD3 + anti-CD28-induced T cell proliferation in a dose-dependent manner. The results obtained in a representative HIV-infected patient (D25) are shown *(figure 1B)*. At low concentrations (0.1, 1, and 10 µM) AZT failed to have a significant impact, but at 100 µM it markedly inhibited ^{3}H TdR incorporation induced by anti-CD3 + anti-CD28 in the presence or absence of IL-7. In contrast, saquinavir failed to affect anti-CD3 + anti-CD28-induced T cell proliferation in the presence or absence of IL-7. These results obtained with a representative, HIV-infected patient (D25) are shown in *figure 1C*.

CD127 cell surface expression by CD4 and CD8 T lymphocytes from HIV-infected patients: effects of IL-7 in the presence of AZT and saquinavir

IL-7 is known to down-modulate its own receptor on the surface of CD4 T cells. Freshly isolated PBMC from HIV-infected patients were cultured with, or without IL-7 (10 ng/mL), and AZT, or saquinavir, or both, added at the start of the culture period as indicated in *figure 2*. CD127 expression of the T cells was measured by flow cytometry after six days. The results shown are those obtained with lymphocytes from one representative individual (D15) *(figure 2A)*. It can be seen that IL-7 (10 ng/mL) caused a significant reduction in CD127 expression. Adding AZT (10 µM) + saquinavir (100 µM) did not modify the amplitude of this down-modulation. An analysis of the results obtained with five HIV-infected patients is shown *figure 2B*. When IL-7 was added to the culture, neither AZT (10 µM) nor saquinavir (100 nM), nor a combination of the two, affected CD127 cell surface down-modulation in CD4 T cells ($p > 0.2$; *figure 2B*).

We then studied the effects of IL-7 on CD127 expression by CD8 lymphocytes from HIV-infected patients. We showed that, in the absence of IL-7, the constitutive expression of CD127 was unaffected by the presence of AZT or saquinavir alone. However, a combination of the two antiretroviral drugs reduced basal, steady state CD127 expression ($p < 0.03$, *figure 3*). Culturing these CD8 T lymphocytes from HIV-infected patients in the presence of IL-7 down-modulated their CD127 surface expression ($p < 0.01$). Such IL-7-induced down-modulation of CD127 was unaffected by AZT or saquinavir alone ($p > 0.4$). It is interesting to note that when the two drugs were combined, IL-7-induced CD127 down-regulation was more pronounced *(figure 3)*. The down-regulation of the basal constitutive expression of CD127 caused by AZT + saquinavir in CD8 T cells from HIV-infected patients, and the worsening of the IL-7-induced CD127 down-modulation on CD8 lymphocytes from these patients were both statistically significant ($p < 0.03$*)*.

In comparison, we analyzed the effects of AZT and saquinavir on CD127 expression and modulation on the surface of both CD4, or CD8 T cells from healthy individuals. We observed that AZT + saquinavir affected neither CD127 basal expression by CD4 nor CD8 T cells from healthy individuals, nor the IL-7-induced down-regulation of CD127 expression on the cells from these donors *(table 3)*.

DISCUSSION

In addition to its central role in lympopoiesis, IL-7 plays a critical role in controlling T cell homeostasis. It protects T cells from death, and accelerates T cell reconstitution by enhancing T cell proliferation [5, 9-11]. IL-7 also plays a role in the specific immune response and, more particularly, it potentiates CD8 cytotoxicity [12-14]. Some publications have also shown that IL-7 potentiates the specific responses to viral antigens, including HIV antigens [13, 15]. All these properties highlight the potential involvement of IL-7 in HIV infection. We have previously shown that plasma IL-7 levels before HAART can be used to predict the magnitude of CD4 T cell regeneration during treatment [8]. Despite increased levels of IL-7, immune reconstitution remains limited after HAART, and the reasons for this impairment are not well understood. Here we studied the possibility that ARV drugs may interfere with IL-7R expression and function, and to do this we measured

Table 2
IL-7-induced T cell proliferation of PBMCs from healthy donors[a]

Healthy donors	H12	H13	H14	H15	H16	Mean
Media	156	56	123	49	78	**92**
IL-7	209	163	239	374	344	**266**
αCD3 0.1 µg/mL	964	938	3199	12752	9775	**5526**
IL-7 + αCD3 0.1 µg/mL	6663	7054	11528	20939	19286	**13094**
IL-7 + αCD3 0.1 µg/mL + AZT1 µM + saq 10 nM	4446	4921	6242	13068	10258	**7787**[b]
IL-7 + αCD3 0.1 µg/mL + AZT 10 µM + saq 100 nM	2588	3376	2849	8114	7768	**4939**[b]

[a] T cell proliferation was measured by ^{3}H Td R incorporation and expressed in cpm.
[b] Responses in the presence of ARV are significantly different from those in the absence of ARV ($p < 0.002$).

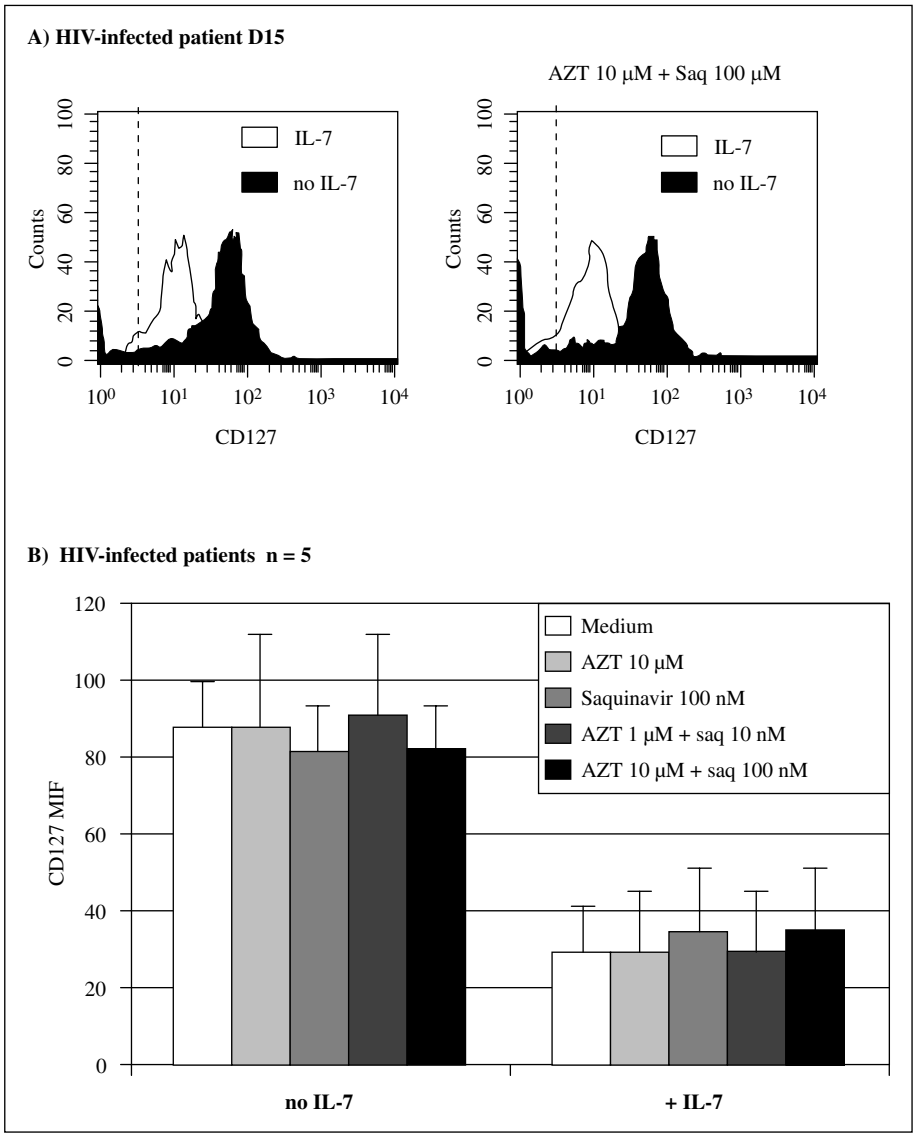

Figure 2

CD127 expression of CD4 T lymphocytes from HIV-infected patients.
PBMC were cultured in complete medium alone or with IL-7 (10 ng/mL). When indicated AZT and/or saquinavir were added at the indicated doses. CD127 expression of the CD4 lymphocytes was analyzed by flow-cytometry after six days of culture.
A) Fluorescence histogram showing intensity of CD127 expression on CD4 T cells from one representative HIV-infected patient. Black curves show CD127 fluorescence observed on CD4 lymphocytes cultured in complete medium alone. White curves show CD127 fluorescence observed on CD4 lymphocytes cultured in complete medium plus IL-7. The dotted line indicates the location of MFI with isotype control mAb.
B) Data from 5 HIV-infected patients expressed as mean CD127 MIF on CD4 cells. IL-7-induced down modulation of CD127 was statically significant ($p < 0.04$). Differences in levels of CD127 expression between CD4 T cells from cultures in the presence or absence of ARV drugs were not significant ($p > 0.2$).

the effects of AZT and saquinavir on CD127 expression and IL-7R function.

With regards to T cell proliferation, we observed that IL-7 was able to increase the cellular proliferation of PBMC from HIV-infected patients and normal donors when these cells were co-stimulated by limited amounts of anti-CD3 mAb. In the presence of the ARV drugs, we observed a significant reduction in IL-7 co-stimulatory capacity, both with PBMC from healthy individuals and from HIV-infected patients. Such effects may, in part, be attributed to the direct effect of AZT on T cell proliferation *(figure 1B)*. We cannot rule out that AZT has toxic effects, although our study of CD4 and CD8 T cells by FACS argued against this possibility *(figures 2 and 3)*. Saquinavir alone did not show any effect on IL-7-dependent T cell proliferation.

We then tested the effects of AZT and saquinavir on constitutive CD127 expression and IL-7-induced CD127 down-modulation. When examining CD4 lymphocytes taken either from healthy individuals or from HIV-infected patients, we observed that the drugs had no impact on CD127 expression, either alone, or in combination. In contrast, CD127 basal expression on the surface of CD8 T cells from HIV-infected patients was decreased when these cells were cultured in the presence of high-dose AZT and saquinavir. In addition, the two drugs further decreased CD127 expression levels when the cells from HIV-infected patients were cultured in the presence of IL-7. These effects were not seen when AZT + saquinavir were tested in CD8 lymphocytes from healthy individuals.

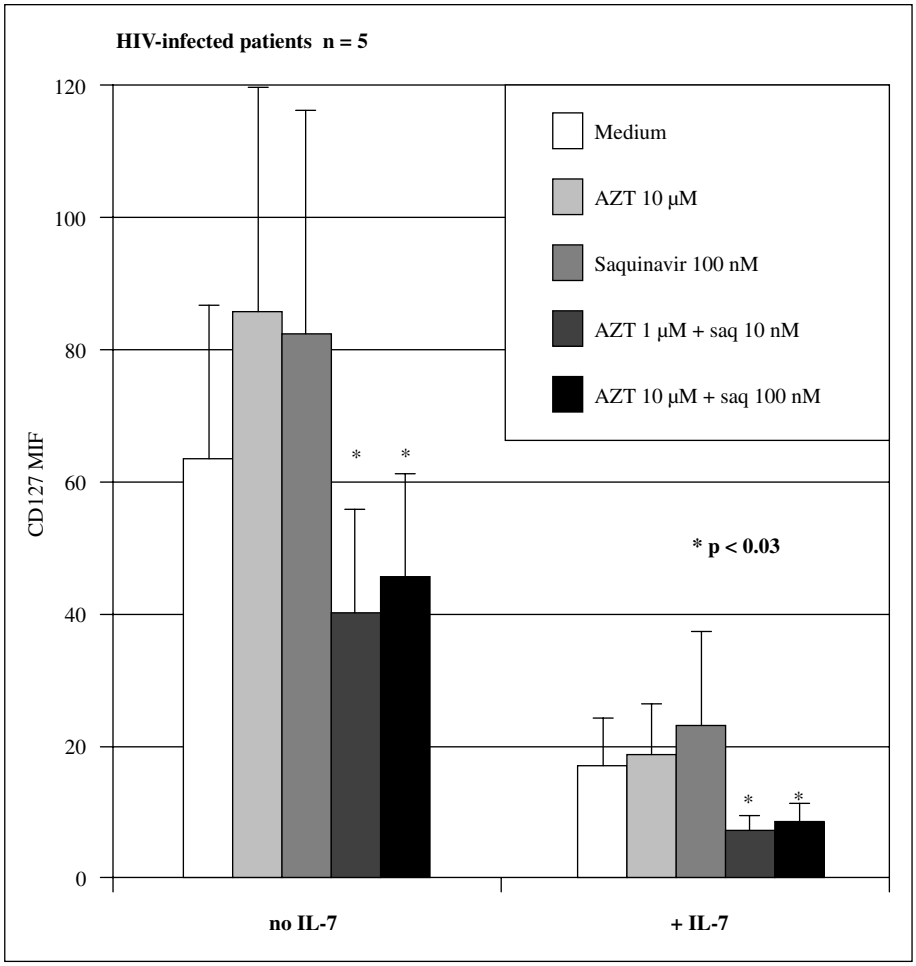

Figure 3

CD127 expression of CD8 T lymphocytes from HIV-infected patients.
PBMCs from five HIV-infected patients were cultured and the CD127 expression on CD8 T cells analyzed as indicated in *figure 2*. Data from five HIV-infected patients were expressed as mean MFI on CD8 T cells. CD127 IL-7-induced down-modulation of CD127 was statistically significant ($p < 0.04$). When combined, AZT (10 µM) and saquinavir (100 nM) significantly decreased the CD127 expression levels of CD8 lymphocytes cultured either in the presence or absence of IL-7 ($p < 0.03$).

Table 3
CD127 expression by CD4 and CD8 lymphocytes from healthy donors in the presence of IL-7 and antiretroviral drugs[a]

A. CD127 expression (MFI) by CD4+ cells from healthy donors						
Healthy donors	**H2**	**H3**	**H4**	**H5**	**H6**	**Mean**
Media	70	117	79	64	75	**81 ± 21**
AZT 1 µM + saq 10 nM	69	114	76	67	74	**80 ± 19**
AZT 10 µM + saq 100 nM	68	97	82	58	62	**73 ± 16**
Media + IL-7	15	36	8	9	8	**15 ± 12**[b]
AZT 1 µM + saq 10 nM + IL-7	23	52	9	8	8	**20 ± 19**[b]
AZT 10 µM+ saq 100 nM + IL-7	14	44	8	9	8	**17 ± 16**[b]
B. CD127 expression (MFI) by CD8+ cells from healthy donors						
Healthy donors	**H2**	**H3**	**H4**	**H5**	**H6**	**Mean**
Media	69	106	71	49	47	**68 ± 24**
AZT 1 µM+saq 10 nM	73	97	77	49	43	**68 ± 22**
AZT 10 µM+saq 100 nM	67	97	86	48	47	**69 ± 22**
Media + IL-7	23	44	9	7	7	**18 ± 16**[b]
AZT 1 µM+saq 10 nM+IL-7	33	54	8	7	7	**22 ± 21**[b]
AZT 10 µM+saq 100 nM + IL-7	19	47	9	7	9	**18 ± 17**[b]

[a] CD127 expression was measured at the surface of CD4 and CD8 T lymphocytes after six days of culture in the absence (media) or the presence of IL-7. The Mean Fluorescence Intensity (MFI) of the positive cells is reported.
[b] Down modulation of CD127 by CD4 and CD8 lymphocytes was always significant when compared to the MFI of the same cells in the absence of IL-7 ($p < 0.002$).

Altogether, the data show that the ARV drugs have significant effects on the IL-7-driven proliferation of PBMC from healthy donors and from HIV-infected individuals. The mechanisms involved are therefore not strictly HIV-dependent. Nevertheless, they may have a substantial negative impact during the partial immune reconstitution that takes place following HAART. With regard to their effects on IL-7R expression, the results indicate that these ARV drugs have no effects on the IL-7R expression of CD4 lymphocytes. In contrast, the drugs selectively impair the expression of CD127 by CD8 lymphocytes from HIV-infected patients. Finally, the combined effects of inhibition of T cell proliferation and the decrease in CD127 expression by CD8 T cells, may significantly affect restoration of the cytotoxic T cell response in HAART-treated patients

Further studies on the effects of ARV drugs on the IL-7/IL-7R system are needed to unravel the mechanisms by which, despite efficient control of HIV replication, ARV drugs may hinder immune reconstitution of the immune system during HAART.

Acknowledgements. *Marie-Thérèse Rannou is greatly acknowledged for her constant and precious help during the present study. This work was supported by Grants from Agence Nationale de Recherche sur le Sida and Cytheris SA.*

REFERENCES

1. Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, *et al.* Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 1994; 180: 1955.
2. von Freeden-Jeffry U, Solvason N, Howard M, Murray R. The earliest T lineage-committed cells depend on IL-7 for Bcl-2 expression and normal cell cycle progression. *Immunity* 1997; 7: 147.
3. Puel A, Ziegler SF, Buckley RH, Leonard WJ. Defective IL-7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Genet* 1998; 20: 394.
4. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* 2000; 1: 426.
5. Fry TJ, Connick E, Falloon J, Liewehr DJ, Spritzler J, Steinberg SM, *et al.* A potential role for interleukin-7 in T-cell homeostasis. *Blood* 2001; 97: 2983.
6. Beq S, Rannou MT, Fontanet A, Delfraissy JF, Theze J, Colle JH. HIV infection: pre-highly active antiretroviral therapy IL-7 plasma levels correlate with long-term CD4 cell count increase after treatment. *AIDS* 2004; 18: 563.
7. Napolitano LA, Grant RM, Deeks SG, Schmidt D, De Rosa SC, Herzenberg LA, *et al.* Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. *Nat Med* 2001; 7: 73.
8. Beq S, Delfraissy JF, Theze J. Interleukin-7 (IL-7): immune function, involvement in the pathogenesis of HIV infection and therapeutic potential. *Eur Cytokine Netw* 2004; 15: 279.
9. Fry TJ, Christensen BL, Komschlies KL, Gress RE, Mackall CL. Interleukin-7 restores immunity in athymic T-cell-depleted hosts. *Blood* 2001; 97: 1525.
10. Fry TJ, Mackall CL. Interleukin-7: master regulator of peripheral T-cell homeostasis? *Trends Immunol* 2001; 22: 564; [comment].
11. Fry TJ, Mackall CL. What limits immune reconstitution in HIV infection? Divergent tools converge on thymic function. *AIDS* 2001; 15: 1881; [comment].
12. Carini C, Essex M. Interleukin-2-independent interleukin-7 activity enhances cytotoxic immune response of HIV-1-infected individuals. *AIDS Res Hum Retroviruses* 1994; 10: 121.
13. Carini C, McLane MF, Mayer KH, Essex M. Dysregulation of interleukin-7 receptor may generate loss of cytotoxic T cell response in human immunodeficiency virus type 1 infection. *Eur J Immunol* 1994; 24: 2927.
14. Lalvani A, Dong T, Ogg G, Patham A, Newell H, Hill AV, *et al.* Optimization of a peptide-based protocol employing IL-7 for in vitro restimulation of human cytotoxic T lymphocyte precursors. *Jl of Immunoll Methods* 1997; 210: 65.
15. Bui TT, Dykers SL, Hu CR, Faltynek CR, Ho RJ. Effect of MTP-PE liposomes and interleukin-7 on induction of antibody and cell-mediated immune responses to a recombinant HIV-envelope protein. *J Acquired Immune Deficiency Syndromes* 1994; 7: 799.