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# Enhanced Dendritic Cell-Driven Proliferation and Anti-HIV Activity of CD8<sup>+</sup> T Cells by a New Phenothiazine Derivative, Aminoperazine<sup>1</sup>

Wei Lu,\*<sup>†</sup> Amar Achour,\* Marine Arlie,<sup>†</sup> Li Cao,\* and Jean-Marie Andrieu\*<sup>†</sup>

T cell anergy, apoptosis, and chronic activation of T lymphocytes are prevailing features of HIV infection. The inability to develop an efficient natural antiviral activity in infected patients might be the consequence of a failure of the Ag presentation by dendritic cells (DCs) in chronically activated lymphoid tissues. We have identified a new phenothiazine derivative aminoperazine (APR; 2-amino-10-[3'-(1-methyl-4-piperazinyl)propyl]phenothiazine, C<sub>20</sub>H<sub>26</sub>N<sub>4</sub>S; m.w. 354.51) able to increase (effective dose from 0.1 to 100 nM) the Ag-specific DC-driven proliferation and differentiation of *in vitro* HIV-infected and uninfected normal donor T cells and of T cells from HIV-1-infected patients. The immunomodulatory effect of APR-sensitized DCs were ascribed to soluble factors derived from DCs. APR was also capable of increasing HIV gag-p24-specific proliferation and anti-HIV cytotoxic activity of patients' CD8<sup>+</sup> T cells against autologous B-lymphoblastoid cell lines expressing a HIV *gag* gene, resulting in the suppression of both proviral DNA and supernatant viral RNA in the HIV-1-infected patients' T cell culture. This new phenothiazine derivative (APR) might be used for boosting the immune response of vaccinated individuals and for restoring the immunity of immunocompromised patients. *The Journal of Immunology*, 2001, 167: 2929–2935.

Dendritic cells (DCs)<sup>3</sup> are critical both for presenting Ags to T cells and for regulating T cell proliferative response after Ag presentation. Impaired proliferative responses (anergy) and increased apoptosis of activated T cells within virus-infected lymph nodes are key immunopathological features of HIV infection, leading to the progressive loss of helper CD4<sup>+</sup> T cells and later of CD8<sup>+</sup> T cells, both in the peripheral blood and in lymphoid organs (1–5). The T cell anergy and increased apoptotic cell death of HIV-infected patients is partly correlated with abnormal expression of cytokines (6–8), and therefore with a possible dysfunction of APCs (9, 10), leading to further viral tolerance and viral dissemination.

In the present study, we have tested the possible immunomodulatory effects of aminoperazine (APR), a new phenothiazine derivative characterized by a cationic amphiphilic structure with a bulky hydrophobic core and a nitrogen-linked lateral positive chain (Fig. 1). We demonstrate that APR can increase the ability of DCs to induce the proliferation and differentiation of both HIV-infected and uninfected normal donor T cells in an Ag-specific fashion. We also show that APR can increase the anti-HIV activity

and gag-specific CTL activity of patients CD8<sup>+</sup> T cells expanded with HIV gag-sensitized DCs.

## Materials and Methods

### Purification and culture of T cells

CD3<sup>+</sup> (CD3<sup>+</sup>CD4<sup>+</sup> in some experiments) T cells were purified from PBMC freshly isolated from whole blood of healthy donors or untreated HIV-1-infected patients who had 200–400 CD4 cells/ $\mu$ l and  $\geq 10^4$  plasma HIV RNA equivalent copies/ml with the use of magnetic beads/DETACH-beads (Dyna, Great Neck, NY). Purified cells were resuspended at  $10^6$ /ml in RPMI 1640 containing 20% heat-inactivated human AB serum, nonessential amino acid, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer (referred as human lymphocyte culture medium (HLCM)). Cells were then seeded at  $2 \times 10^6$ /well in 24-well plates (Nunc, Roskilde, Denmark) and were stimulated with 4  $\mu$ g/ml PHA (Sigma, St. Louis, MO) or 0.5  $\mu$ g/ml anti-CD3 mAbs (Becton Dickinson France, Le Pont-de-Claix, France) plus 100 ng/ml anti-CD28 mAbs (BD Pharmingen, Los Angeles, CA) in the absence or the presence of phenothiazine (PT, Sigma), chlorpromazine (CPZ, Sigma), or APR (kindly provided by Institut Necker, Paris, France; APR can be obtained from Institut Necker (156 rue de Vaugirard, 75015 Paris, France) by written request) at indicated drug concentrations. After overnight stimulation with PHA or anti-CD3/CD28 Abs, cells were washed and resuspended at  $10^6$ /ml in HLCM containing the same concentrations of the drug plus 20 IU/ml recombinant human IL-2 (Boehringer Mannheim, Mannheim, Germany) for a 20-day period culture in the presence or the absence of DC with or without a filter as indicated in the result section. Cultures were maintained at 37°C in humidified air at 5% CO<sub>2</sub>. Culture mediums were changed every 2–3 days keeping the cultures at a viable cell density  $\leq 2 \times 10^6$ /well in a constant volume of 2 ml/well. At each time point, viable cell counts were determined by trypan blue exclusion and supernatants were harvested for storage at –20°C.

### Generation and culture of DCs

PBMC were isolated from fresh whole heparin-treated blood of healthy donors or HIV-1-infected patients by Ficoll-Hypaque (Eurobio, Les Ulis, France) density gradient centrifugation. PBMC were depleted of CD3<sup>+</sup> and CD19<sup>+</sup> cells with the use of magnetic beads (Dyna) and suspended at  $10^6$ /ml in RPMI 1640 containing 10% heat-inactivated human AB serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 100 mM

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<sup>3</sup> Abbreviations used in this paper: DC, dendritic cell; HLCM, human lymphocyte culture medium; PPD, purified protein derivative; PT, phenothiazine; CPZ, chlorpromazine; APR, aminoperazine; B-LCL, B-lymphoblastoid cell lines; CD45RA, an exon A-dependent epitope of CD45 protein; CD62L, CD62 ligand; IL-1ra, IL-1R antagonist; HAART, highly active antiretroviral therapy; PI, protease inhibitor.

sodium pyruvate, and  $5 \times 10^{-8}$  2-ME supplemented with 250 ng/ml GM-CSF (R&D Systems, Minneapolis, MN) and 100 ng/ml IL-4 (R&D Systems) and cultured for 5 days in 30-ml Teflon bags (VueLife; Afc, Gaithersburg, MD). After this culture period, nonadherent cells were determined to be 95% immature DC based on their morphology and their expressions of CD1a, CD11c, CD40, CD80, CD86, CD4, and HLA-DR assessed by direct immunofluorescence flow cytometry (see below). After three washings with Hanks' buffer, DCs were seeded at  $5 \times 10^5$ /well and cultured with  $2 \times 10^6$  activated autologous T cells in the presence or the absence of a filter or cultured with  $2 \times 10^6$  activated autologous T cell-derived lysate (generated by osmotic shock and sonication of autologous T cells stimulated with anti-CD3/CD28 Abs for 7 days) in the presence or the absence of APR at indicated concentrations.

#### Proliferation assay

PBMC were freshly isolated from 10 untreated patients. Cells were resuspended at a cell concentration of  $5 \times 10^5$ /ml in HLCM and seeded in quadruplicate in 96-well round-bottom microtiter plates (Nunc) containing  $10^5$  PBMC/well. Cells were stimulated with 3000 U/ml purified protein derivative (PPD) of tuberculin (Aventis Pasteur, Lyon, France), or 1  $\mu$ g/ml of HIV env V3 peptide P18 (P18) (Genosys, London, U.K.) in the absence or the presence of APR at indicated concentrations. Microtiter plates were placed in a cell incubator in a humidified atmosphere containing 5% CO<sub>2</sub> for 96 h. Cells were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine (Amersham, Aylesbury, U.K.) for the final 16 h. Cells were harvested on glass fiber filters by an automated multisample harvester; filters were then put in tubes with liquid scintillation fluid and counted on a beta scintillation counter.

For evaluating T cell proliferation in response to viral-Ag-sensitized DCs, purified DCs were seeded in quadruplicate at graded doses (30,000, 10,000, 3,000, and 1,000) in 96-well flat-bottom tissue culture microtiter plates (Nunc) and incubated with 1  $\mu$ g/ml recombinant HIV-1 gag protein p24 (Transgene, Strasbourg, France) for 2 h. Excess Ag was removed by washing;  $10^5$  autologous T cells were then added to each well and cultured for 5 days. T cell proliferation was determined by pulsing the cells with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine (Amersham) for the final 16 h of incubation, and radioactivity was counted in a beta scintillation counter as described above.

#### Apoptosis assay

Apoptotic cells were measured by propidium iodide and FITC-labeled annexin V, a phospholipid-binding protein that preferentially binds to phosphatidylserine exposed at the cell surface in the early phase of apoptosis, using a commercially available kit (Immunotech, Marseille, France). Cells that were negative for propidium iodide and positive for annexin V were identified as apoptotic cells, whereas those positive for both propidium iodide and annexin V were considered pre-necrotic cells.

#### Flow cytometry

CD4<sup>+</sup> and CD8<sup>+</sup> T cell counts as well as their naive (CD45RO<sup>+</sup> or an exon A-dependent epitope of CD45 protein (CD45RA<sup>+</sup>/CD62 ligand (CD62L<sup>-</sup>), memory (CD45RA<sup>+</sup>/CD62L<sup>+</sup>) or Ki-67<sup>+</sup> subsets, and DC (CD1a<sup>+</sup>, CD11c<sup>+</sup>, CD40<sup>+</sup>, CD80<sup>+</sup>, CD86<sup>+</sup>, CD4<sup>+</sup>, and HLA-DR<sup>+</sup>) were assessed by flow cytometry analysis (FACScan, BD Biosciences, San Jose, CA). A panel of mAbs to the following T cell surface markers was used: CD3-PerCP, CD4-FITC, CD8-PE, CD45RO-PE, CD45RA-PE, CD62L-FITC, CD4-PerCP, CD8-PerCP, and Ki-67-PE (Becton Dickinson France); anti-CD1a-FITC, CD11c-PE, CD40-FITC, CD80-PE, CD86-FITC, and HLA-DR-PE (BD Pharmingen).

#### Cytokine detection

Cell-free supernatants collected at days 7 and 14 of culture were assayed for TNF- $\alpha$ , IL-1 $\beta$ , IL-1R agonist (IL-1ra), IL-2, IL-4, IL-6, IL-10, IL-12, IL-15, IL-18, and IFN- $\gamma$  using commercial ELISA kits (Quantikine; R&D Systems). The sensitivity of the assay for each cytokine was 4.4 pg/ml for TNF- $\alpha$ , 7 pg/ml for TGF- $\beta$ 1, 1 pg/ml for IL-1 $\beta$ , 22 pg/ml for IL-1ra, 5 pg/ml for IL-2, 10 pg/ml for IL-4, 0.7 pg/ml for IL-6, 3.9 pg/ml for IL-10, 5 pg/ml for IL-12, 1 pg/ml for IL-15, 15 pg/ml for IL-18, and 7 pg/ml for IFN- $\gamma$ , respectively.

#### Viral quantitation assay

Viral production was determined by measuring cell-free HIV RNA by a recently developed multiple primer-induced overlapping amplification assay with a detection threshold of 10 equivalent copies/ml (11). Proviral DNA was determined by a quantitative PCR assay (12).

For evaluation of the antiviral activity of T cells expanded in the presence of viral Ag-sensitized DCs, purified DCs were seeded at  $5 \times 10^5$  in duplicate in 24-well plates (Nunc) and incubated with 1  $\mu$ g/ml recombinant

HIV-1 gag protein p24 (Transgene) for 2 h. Excess Ag was removed by washing, and  $2 \times 10^6$  autologous T cells were added for a 7-day culture in HLCM supplemented with 20 IU/ml recombinant human IL-2 in the presence or the absence of APR. Then,  $2 \times 10^6$  autologous CD4<sup>+</sup> T cells superinfected with 100 50% tissue culture-infective dose of autologous viral isolate as described previously (13) were added to each well and cultured for additional 20 days. Proviral DNA and supernatant viral RNA were measured by the above described viral quantitation assays. Anti-CD4 and anti-CD8 mAbs (clones RPA-T4 and RPA-T8; BD Pharmingen) were used for blocking analysis.

#### Cytotoxicity assay

EBV-transformed autologous B-lymphoblastoid cell lines (B-LCL) were infected with recombinant vaccinia virus containing a gag gene of HIV-1 (14). Vaccinia virus-infected B-LCL were incubated with 100  $\mu$ Ci <sup>51</sup>Cr in a total volume of 200  $\mu$ l for 2 h at 37°C before use as targets. Targets were washed and seeded at  $5 \times 10^3$  cells/well at an E:T of 10:1 in quadruplicate in HLCM and assayed for cytotoxicity in a standard chromium release assay. The percentage of specific lysis was calculated by subtracting the specific <sup>51</sup>Cr release of wild-type vaccinia virus-infected targets (controls) (14). Anti-CD4 and anti-CD8 mAbs were used for blocking analysis.

#### Statistical analysis

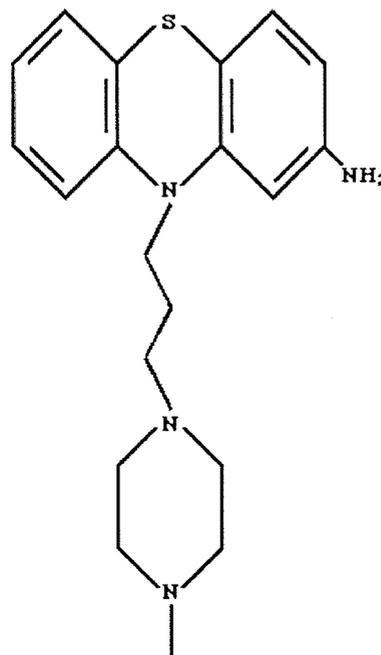
Paired data between different *in vitro* treatments were compared by the *t* test.

## Results

### APR enhances the proliferation of *in vitro* HIV-1-infected and uninfected normal donor T cells and of HIV-1-infected patients' T cells after mitogen or anti-CD3/CD28 stimulation

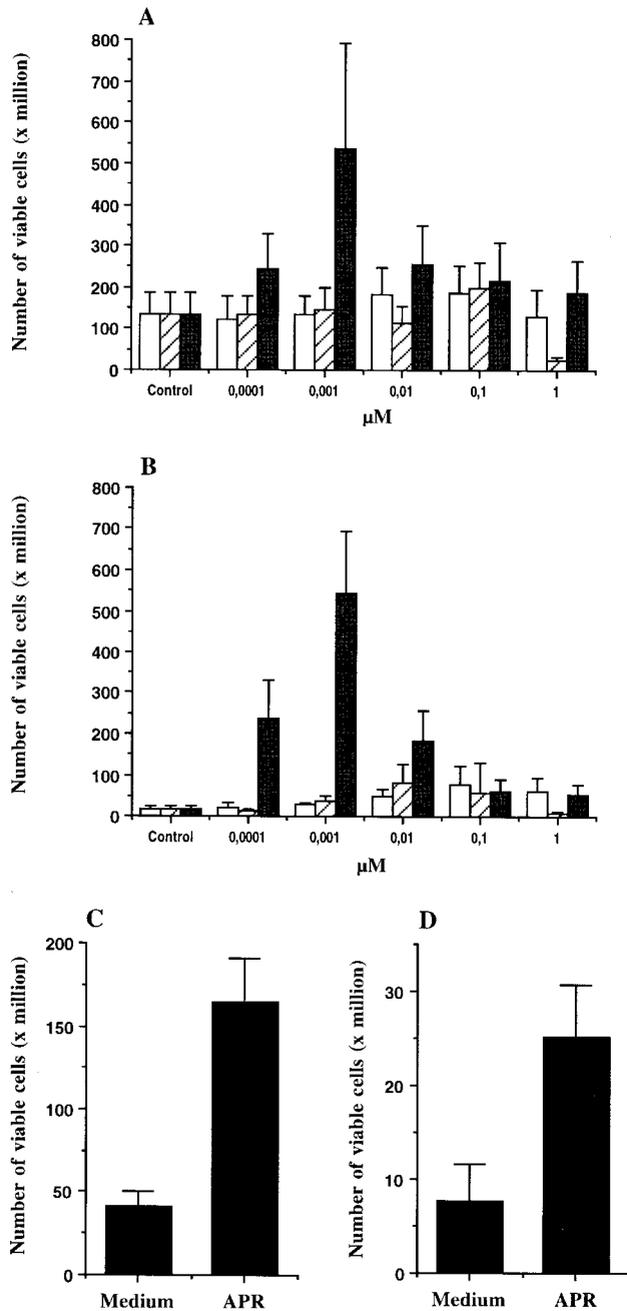
The survival of PHA-stimulated normal T cell cultures in the presence of DCs, increased by 1- to 5-fold (at day 14) with 0.1–10 nM APR (peak response at 1 nM) (Fig. 2A). In addition, the reduced survival of *in vitro* HIV-infected T cells was almost completely corrected to the level of uninfected cells with 1 nM APR (Fig. 2B). This corresponds to a 10- to 25-fold increase in survival. PT or CPZ (another PT derivative) showed only a minor, if any effect, on T cell survival (<1-fold and A and B).

Significantly, APR (1 nM) could also increase the survival of T cells from 6 HIV-infected patients (mean  $\geq$ 3-fold, ranging from 1- to 5-fold; patient 1 had 800 CD4 cells/ $\mu$ l, patient 2 had 664 CD4



**FIGURE 1.** Chemical structure of the new phenothiazine derivative APR (2-amino-10-[3'-(1-methyl-4-piperazinyl)propyl]phenothiazine, C<sub>20</sub>H<sub>26</sub>N<sub>4</sub>S; m.w. 354.51).

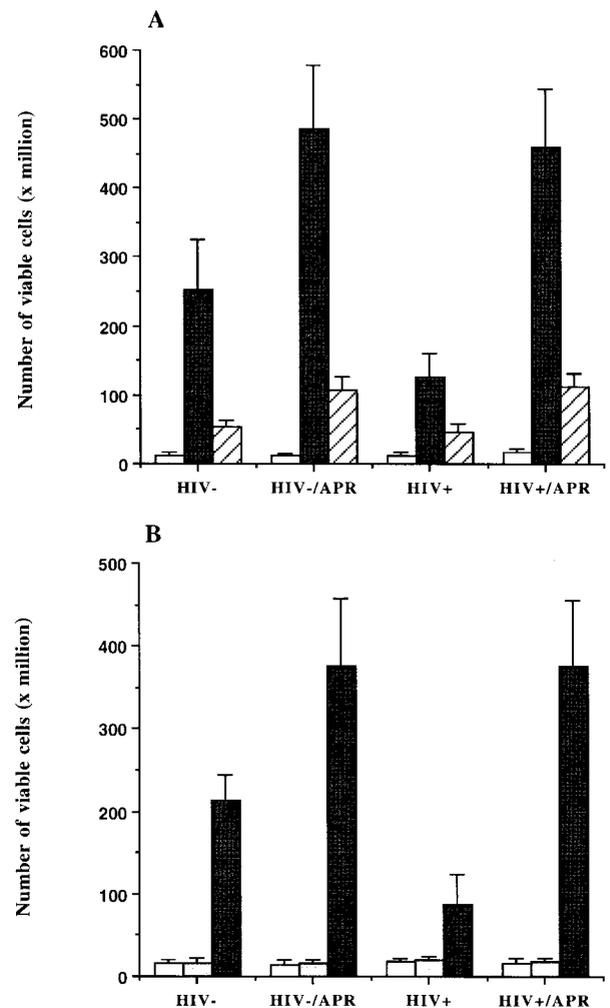
cells/ $\mu\text{l}$ , patient 3 had 504 CD4 cells/ $\mu\text{l}$ , patient 4 had 484 CD4 cells/ $\mu\text{l}$ , patient 5 had 267 CD4 cells/ $\mu\text{l}$ , and patient 6 had 214 CD4 cells/ $\mu\text{l}$ ) cultured with DCs and stimulated with either PHA (Fig. 2C) or anti-CD3/anti-CD28 Abs (Fig. 2D). The magnitude of the increase in T cell survival by APR was inversely correlated with the patient's CD4 count ( $p < 0.01$ ). Analysis by flow cytometry demonstrated that the naive (CD45RO<sup>+</sup> or CD45RA<sup>+</sup>/CD62L<sup>-</sup>) and memory (CD45RA<sup>+</sup>/CD62L<sup>+</sup>) subsets of both CD4 and CD8 T lymphocytes were equally expanded by APR (data not shown).



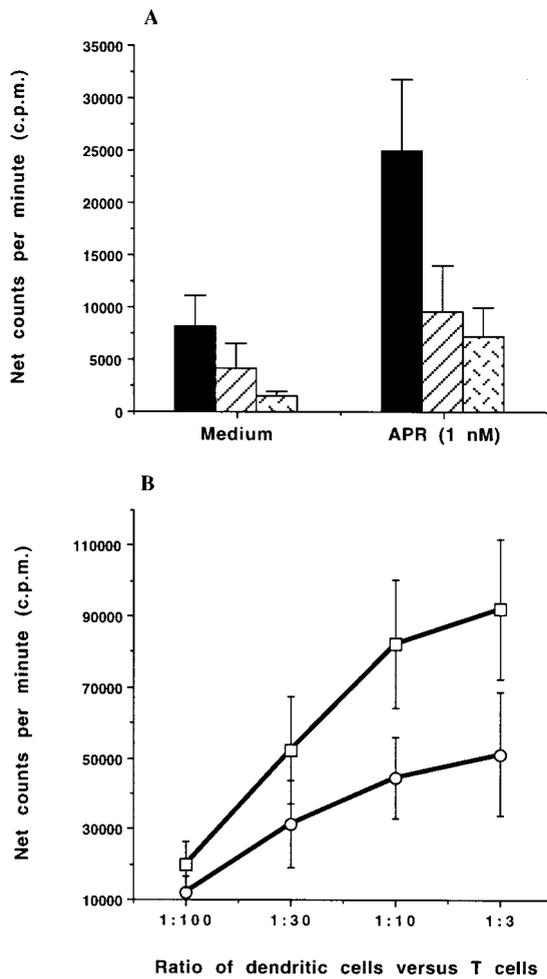
**FIGURE 2.** Survival (day 14) of normal (A) and exogenously (in vitro) HIV-infected (B) T cells stimulated with PHA and cultured with DC in the presence of 0.1–1000 nM PT (□), CPZ (▨), or APR (■). Effects of APR (1 nM) on the survival (day 11) of six untreated HIV-infected patients T cells stimulated with PHA (C) or anti-CD3/CD28 Abs (D) in the presence (■) of DCs. Data were expressed as the mean  $\pm$  SD of triplicate experiments in A and B and as the mean  $\pm$  SE of the measurements obtained with five untreated patients samples in C and D.

*The immunomodulatory effects of APR are mediated via soluble factor(s) released from DCs in the presence of activated T cells*

The target of APR for the observed increased T cell survival might be the T cells or DCs. To answer this question, we purified T cells from normal and HIV-infected patients. These cells were cultured either alone or separated from DCs by a filter. The increased survival was partial and was observed when T cells were cultured in the presence of DCs separated by a filter but not in their absence (Fig. 3A). To test whether the partial nature of the observed increase in T cell survival promoted by DCs separated by a filter reflected a decreased concentration of activated T cell-derived soluble factors, we used an activated T cell lysate (generated by osmotic shock and sonication of autologous T cells stimulated with anti-CD3/CD28 Abs for 7 days) which contains a high concentration of T cell-derived soluble factors. Supernatants derived from DCs cultured with activated autologous T cell lysate in the presence of APR (1 nM), but not from DCs cultured alone, increased the survival of normal (2-fold) and HIV-infected T cells (5-fold)



**FIGURE 3.** Effects of APR (1 nM) on the survival (day 14) of normal (HIV<sup>-</sup>,  $n = 5$ ) or HIV-infected patients (HIV<sup>+</sup>,  $n = 10$ ) T cells stimulated with PHA. T cells were measured alone (□), cultured with autologous DCs in the absence (■) or the presence (▨) of a filter (A) or with supernatants derived from autologous DC culture (▨) or from DCs cultured with activated autologous T cell lysate (■) (B). Data were expressed as the mean  $\pm$  SE of the measurements obtained with 5 normal donors or 10 patient samples.



**FIGURE 4.** Effect of APR (1 nM) on the proliferative responses of 10 HIV-infected patients T cells to TCR/CD28 cross-linking (■), PPD (▨), or V3-P18 (▩) in the presence of autologous DCs (A) or HIV gag p24-sensitized autologous DCs (○, DC alone; □, DC plus APR) (B). Data were expressed as the mean  $\pm$  SD of the measurements: obtained with 10 patient samples.

(Fig. 3B). Similar results were observed with supernatants derived from DCs cultured with activated autologous or heterologous T cell lysates in the presence of APR (1 nM) (data not shown).

*APR increase the proliferation of HIV-1-infected patients T cells after recall Ag stimulation*

We next tested the effects of APR on the proliferation of T cells from HIV-infected patients in response to recall Ags, which is known to be depressed. In response to PPD and HIV env V3-specific Ag P18, the proliferation of T cells from HIV-infected patients cultured in the presence of DCs, increased in the presence of APR (1 nM) by 2- to 3-fold as shown by the [ $^3$ H]thymidine uptake T cell index. This increase was similar to that observed in the control group stimulated with anti-CD3/CD28 Abs (Fig. 4A). The [ $^3$ H]thymidine uptake of patients' T cells in response to HIV gag p24-sensitized DCs was also increased by APR (1 nM; Fig. 4B).

*APR decreases the production of most detectable cytokines in HIV-infected patients T cells after anti-CD3/CD28 stimulation*

The interaction between activated T cells and DCs initiates a series of events leading to the increased or decreased secretion of cytokines and other unidentified soluble factors, which shape the fate (survival or depletion) of T cell populations (15). We therefore measured a panel of cytokines in the supernatants of T cells from 10 untreated HIV patients cultured with autologous DCs. In the presence of APR (1 nM), the concentrations of IL-1 $\beta$ , IL-1ra, IL-6, IL-10, IL-12, and IFN- $\gamma$  started to decline at day 7 (2-fold) and dropped significantly ( $\geq 10$ -fold) at day 14 of culture (Table I). TNF- $\alpha$  and IL-4 were undetectable in the supernatants collected at days 7 and 14 of each culture. In contrast, TGF- $\beta$ 1 and IL-2 were not affected by APR. The expression of the cell cycle marker Ki-67 and the fraction of apoptotic cells decreased after 14 days of culture in the presence of APR (Table I). We also directly tested the role of IL-2 on the effect of APR. When recombinant human IL-2 was withdrawn from the standard lymphocyte culture medium, we observe a 50% reduction in T cell survival. Nevertheless, the increased T cell survival promoted by APR was similar (5-fold at 14 days of culture) irrespective of the presence of exogenous IL-2 (data not shown).

**Table I.** Effects of APR (1 nM) on the apoptosis and membrane Ki-67 expression of HIV-infected patients' (n = 10) T cells stimulated with anti-CD3/CD28 Abs and cultured with DCs and on their cytokines production in the supernatant of culture<sup>a</sup>

	Day 7 of Culture		Day 14 of Culture	
	Medium	APR	Medium	APR
% apoptotic T cells	12.3 $\pm$ 4.1	12.6 $\pm$ 5.7	20.6 $\pm$ 8.4	10.1 $\pm$ 7.3*
% Ki-67 <sup>+</sup> T cells	47.8 $\pm$ 14.5	48.1 $\pm$ 14.9	21.7 $\pm$ 5.1	8.4 $\pm$ 3.1*
TNF- $\alpha$ (pg/ml)	UND <sup>b</sup>	UND	UND	UND
TGF- $\beta$ 1 (pg/ml)	0.41 $\pm$ 0.27	0.42 $\pm$ 0.28	UND	UND
IL-1 $\beta$ (pg/ml)	185 $\pm$ 155	64 $\pm$ 48*	77.6 $\pm$ 65.2	UND
IL-1Ra (pg/ml)	4136 $\pm$ 2395	2077 $\pm$ 1421*	1736 $\pm$ 934	27 $\pm$ 16*
IL-4 (pg/ml)	UND	UND	UND	UND
IL-6 (pg/ml)	4306 $\pm$ 1032	2458 $\pm$ 726*	1669 $\pm$ 489	64 $\pm$ 45*
IL-10 (pg/ml)	166 $\pm$ 124	84 $\pm$ 81*	73 $\pm$ 68	UND
IL-12 (pg/ml)	389 $\pm$ 357	164 $\pm$ 137*	149 $\pm$ 106	UND
IL-15 (pg/ml)	6.2 $\pm$ 1.7	6.1 $\pm$ 1.8	UND	UND
IL-18 (pg/ml)	54.6 $\pm$ 17.8	55.3 $\pm$ 18.9	UND	UND
IFN- $\gamma$ (ng/ml)	10.4 $\pm$ 8.6	5.5 $\pm$ 4.1*	5.7 $\pm$ 3.2	1.3 $\pm$ 1.1*

<sup>a</sup> Data were expressed as the mean  $\pm$  SD of the measurements.

<sup>b</sup> UND, Undetectable (i.e., below the detection threshold of commercial ELISA kits).

\*,  $p < 0.01$  as compared with culture medium alone.

Table II. Effects of APR (1 nM) on the cellular proviral DNA and the supernatant virion RNA of 10 untreated HIV-infected patients' T cells stimulated with anti-CD3/CD28 Abs and cultured with DCs<sup>a</sup>

	Day 7 of Culture		Day 14 of Culture	
	Medium	APR	Medium	APR
Log proviral HIV DNA (cp./10 <sup>6</sup> cells) <sup>b</sup>	4.5 ± 1.2	4.5 ± 1.1	4.7 ± 1.3	4.8 ± 1.4
Log HIV RNA in the supernatant (eq cp./ml)	4.6 ± 0.8	4.8 ± 0.9	5.7 ± 1.4	5.9 ± 1.8

<sup>a</sup> Data were expressed as the mean ± SD of the measurements.

<sup>b</sup> cp., Copies; eq, equivalent.

APR increases the anti-HIV activity of patients' CD8<sup>+</sup> T cells expanded by HIV-gag-sensitized autologous DCs

We have recently shown that the HIV-antiviral activity of CD8<sup>+</sup> T cells is progressively lost during the course of the HIV infection (13). We therefore examined whether this activity could be restored by APR, by measuring cell-associated proviral DNA and supernatant viral RNA. APR had no effect on the HIV proviral DNA load (copies/10<sup>6</sup> cells) and supernatant RNA concentration of T cells from infected patients that had been expanded by anti-CD3/anti-CD28 stimulation in the presence of DCs (Table II). However, the activation of T cells resulting from TCR/CD3 cross-linking may not enrich for the anti-HIV-specific CD8<sup>+</sup> T cell subsets. We therefore stimulated T cells by coculture with autologous DCs sensitized with HIV gag protein p24. Under these experimental conditions, the proviral DNA of CD4<sup>+</sup> T cells was decreased by 1 log<sub>10</sub> ( $p < 0.01$ ) in the absence of APR and by 2 log<sub>10</sub> ( $p < 0.001$ ) in its presence (Table III). The HIV RNA in the supernatant of the same cultures decreased by 2 log<sub>10</sub> ( $p < 0.001$ ) in the absence of APR and by 3 log<sub>10</sub> ( $p < 0.001$ ) in its presence. Addition of anti-CD8 but not anti-CD4 Abs to the T cell culture abolished these antiviral activities (data not shown).

APR enhances the gag-specific CTL activity of T cells from HIV-infected patients

To explore the mechanism underlying the increased antiviral activity promoted by APR, we evaluated the HIV gag-specific CTL activity in five untreated HIV-infected patients. T cells cultured alone or expanded with p24-sensitized DCs in the presence of APR were incubated with autologous B-LCL expressing an HIV gag gene (14). Weak gag-specific CTL activity was observed in these five untreated patients with a relatively low CD4 count (mean ± SD 259 ± 42 cells/μl). This observation is in keeping with our previous report that a low gag-specific CTL activity is associated with advanced stage of the disease (13). In the absence of APR, killing of B-LCL was increased (85%) by

p24-sensitized-DC-stimulated autologous T cells (E:T ratio 10:1;  $p = 0.02$ ). In the presence of APR, the gag-specific CTL activity was further increased (136%) ( $p < 0.01$ ). By contrast, T cells expanded in the presence of APR alone had no influence on gag-specific CTL activity. The CTL-mediated B-LCL killing of p24-sensitized-DC-stimulated T cells was decreased by the addition of anti-CD8 Abs, whereas it was unaffected by the addition of anti-CD4 Abs (Fig. 5).

## Discussion

The observation that APR could increase normal and HIV-infected T cell survival/proliferation after mitogen or Ag stimulation suggests its potential application as an adjuvant in HIV vaccination. Because the increased T cell survival/proliferation is also observed with the supernatant of DCs cultured with activated T cell lysates (generated by osmotic shock and sonication of autologous T cells stimulated with anti-CD3/CD28 Abs for 7 days) that contain high concentration of intracellular soluble proteins such as cytokines and/or chemokines, soluble factor(s) secreted by DCs are probably involved in the observed effect of APR. The fact that T cell survival is partially increased by APR when the T cells and DCs are separated by a filter indicates that a direct contact with activated T cells is required for the optimum secretion of soluble factors by DCs. Although the precise action of APR on DCs remains to be elucidated, its hydrophobic core might facilitate its attachment to the cell membrane, and its lateral positive chain could allow the interaction with polar phospholipid moieties or with other negatively charged molecules.

The observation that improvement by APR of the survival/proliferation of T cells from HIV-infected patients correlates with a significant decreased production of all detectable cytokines (Table I) despite ongoing active viral replication (Table II) is consistent with the reported T cell recovery in HIV-infected patients experiencing a virological failure under highly active antiretroviral therapy (HAART) which include at least one HIV protease inhibitor

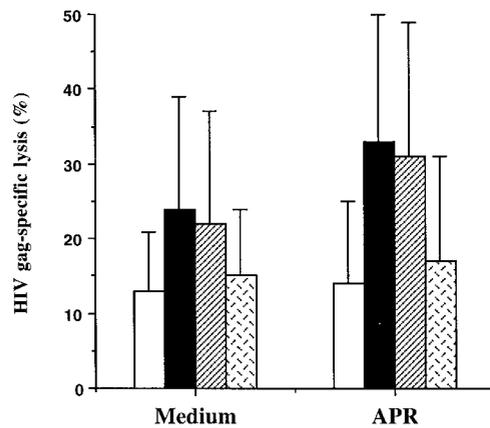
Table III. Effects of APR (1 nM) on the proviral DNA and the supernatant virion RNA of 10 super-infected patients' CD4<sup>+</sup> T cells cultured with T cells expanded by HIV-gag-sensitized DCs<sup>a</sup>

	Day 7 of Culture		Day 14 of Culture	
	Medium	APR	Medium	APR
CD4 <sup>+</sup> T cell culture				
Log proviral HIV DNA (cp./10 <sup>6</sup> cells) <sup>b</sup>	4.7 ± 1.3	4.7 ± 1.4	4.8 ± 1.4	4.8 ± 1.7
Log HIV RNA in the supernatant (eq cp./ml)	5.0 ± 1.7	5.1 ± 1.5	5.5 ± 1.6	5.3 ± 1.7
CD4 <sup>+</sup> /gag-sensitized DC-expanded T cell coculture				
Log proviral HIV DNA (cp./10 <sup>6</sup> cells)	3.8 ± 1.1	2.0 ± 0.5*	3.7 ± 1.7	2.2 ± 0.4*
Log HIV RNA in the supernatant (eq cp./ml)	3.1 ± 0.7	1.5 ± 0.2*	3.8 ± 1.3	1.8 ± 0.3*

<sup>a</sup> Data were expressed as the mean ± SD of the measurements.

<sup>b</sup> cp., Copies; eq, equivalent.

\*,  $p < 0.01$  as compared with culture medium alone.



**FIGURE 5.** HIV-1 gag-specific CTL activity against autologous B-LCL infected with recombinant vaccinia virus containing an HIV-1 *gag* gene. Results represent the mean  $\pm$  SD of percent specific lysis (E:T ratio 10:1) of target cells by patient T cells alone ( $\square$ ) and by patient T cells expanded with p24-pulsed DCs ( $\blacksquare$ ) or p24-pulsed DCs plus anti-CD4 Ab ( $\hbar$ ) or p24-pulsed DCs plus anti-CD8 Ab ( $\boxtimes$ ) in the absence or the presence of APR. Data were expressed as the mean  $\pm$  SD of the measurements obtained with five untreated patient samples.

(PI) (16–20). This is not surprising because recent *in vitro* studies from our group (21) and from others (22) have shown that PIs increase the proliferative index and decrease the apoptosis of PBMC from infected patients. In addition, T cell recovery in HAART-treated patients also correlates with a simultaneous down-regulation of all detectable markers of activation (including cytokines) and T cell apoptosis (23–25). In this perspective, the rapid decline of most cytokines in APR-treated cultures might be interpreted as a consequence of the re-entry of a majority of T cells into the  $G_0$  cell cycle phase, as suggested by the decreased expression of the cell cycle marker Ki-67 and of the percent of apoptotic cells after 14 days of culture in the presence of APR (Table I). The same mechanism may explain the decreased cytokine expression observed in HAART-treated patients.

Although HAART promotes a dramatic decrease (3–4  $\log_{10}$ ) of plasma HIV and a significant recovery of the T cell compartment in a majority of patients (26, 27), the pool of CD4<sup>+</sup> T cells harboring replication-competent provirus is only slightly reduced (<1  $\log_{10}$ ) even after years of therapy (28–30). In these patients, the persistent lack of anti-HIV immunity despite the rapid normalization of T cell reactivity (31, 32) might indicate that an adequate Ag presentation might be failing to trigger an efficient HIV-specific immune response. This is suggested both by the lack of improvement of anti-HIV activity despite the increased DC-driven T cell proliferation by APR (Table II) and by the gag-specific CTL activity enhanced, when HIV-1 gag-sensitized DCs were added to patients' T cells in the presence of APR (Table III and Fig. 5). It is generally accepted that a MHC class II-restricted CD4-mediated enhancement of CD8<sup>+</sup> CTL might be involved in gag-specific presentation by DCs. However, soluble Ags can be also processed and presented by DCs through an MHC class I-restricted pathway *in vitro* (33, 34) and *in vivo* (35).

Taken together, our findings indicate that DC-driven T cell proliferation and Ag recognition can be increased *in vitro* by APR, leading to the efficient expansion of viral Ag-specific CD8<sup>+</sup> T lymphocytes of HIV-infected patients. These findings might have practical implications for immune-based therapies of HIV infection and of diverse immunodeficiencies.

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