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Nef protein induces differential effects in $CD8^+$ cells from HIV-1-infected patients

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Abstract

Background The Nef protein of HIV-1 is suspected to play a role in the depletion of uninfected $CD4^+$ lymphocytes that leads to AIDS. By contrast its effect on $CD8^+$ cells, whose functions are also deregulated during HIV-1 infection, is presently unclear. Here we describe a number of derangements induced *in vitro* by Nef in $CD8^+$ cells from HIV-1-infected patients.

Design Peripheral lymphocytes from 16 HIV-1^+ subjects and 9 uninfected individuals were cultivated on a Nef-transfected mouse fibroblast layer exposing the carboxyl-terminal region of the viral protein on cell membrane. The cultures were then measured for both apoptosis and proliferation by subdiploid DNA content and Ki67 expression, respectively, whereas the molecular analysis of purified $CD8^+$ cells investigated the Fas-L mRNA levels in Nef-treated CTLs. In addition, we evaluated the Nef-induced variation in the extent of $CD8^+$/HLA-DR^+ subset, which includes non cytotoxic cells secreting T-cell antiviral factor (CAF) and a soluble factor inhibiting the HIV-1 replication.

Results The viral protein induced in peripheral blood lymphocytes (PBL) a moderate tendency to proliferate, as measured by the increment of Ki67 antigen, particularly on the $CD8^+$ subset of HIV-1 infected individuals ($P < 0{\cdot}05$). This profile was particularly evident in cultures from patients with severe $CD4^+$ lymphopenia and paralleled an apparent expansion of the $CD8^+$/$CD57^+$ suppressor cell subset. Molecular analysis of purified $CD8^+$ cells revealed a defective expression of Fas-L mRNA in Nef-cultured CTLs, whereas the viral protein exerted a down modulatory effect on the $CD8^+$/HLA-DR^+ subset ($P < 0{\cdot}05$), thus suggesting a potential inhibition of CAF.

Conclusions These results support a potential role of Nef in the progression of HIV-1 infection as a number of cellular functions are affected in the $CD8^+$ subset. In particular, the defective functions of $CD8^+$ cells induced by the viral protein could contribute, at least partly, to the escape of HIV-1 from the immune control of these cells.

Keywords $CD8^+$ cells, cytolysis, Fas–L, HIV, Nef.
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Introduction

Both the human (HIV-1) and the simian (SIV) immunodeficiency viruses are complex retroviruses whose genomes comprise many regulatory genes, namely *tat*, *rev*, *vif*, *vpu*, *nef*, *vpr* and *vpx* [1,2]. Among them, *vif*, *vpu*, *vpr* and *nef* are not needed for virus replication *in vitro* and are therefore termed 'nonessential' [3].

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The *nef* gene is located at the 3′ of *env* gene and partially overlaps the U3 region of the 3′ LTR. The product of HIV-1 *nef* is a 27–30 kDa protein whose myristoylation at its N-terminus [4] permits its linkage to the cytoplasmic leaflet of the plasma membrane. However, perturbation of the lipid bilayers by the myristoylated protein also leads the carboxyl-terminal portion of Nef to protrude on the cell membrane [5]. Even so, infected lymphocytes usually express Nef in both their cytosol and nuclei and may also solubilize the protein by the cell membrane [6–8].

The biological role of Nef on HIV-1 replication is controversial. In contrast to its *in vitro* inefficacy [9–12], recent studies have shown that the intact *nef* gene is pivotal in maintaining a high virus load in rhesus monkeys infected with the pathogenic SIVmac239 clone, and for

the development of AIDS [13]. In addition, the wild-type *nef* gene from HIV-1 significantly increases the viral load in the SCID-Hu mouse model (a severe combined immunodeficient mouse transplanted with human fetal liver and thymus) [14], whereas its mutated form showing a structural defect in 3′ LTR sequences has been tentatively associated with long-term non-progression of the disease in HIV-1 infected subjects [15]. These observations suggested that *nef* gene and its product could be involved in the lymphocyte depletion that leads to AIDS. Demonstration of the suppressive effect of Nef on T-cell activation as well as the inhibition of $CD4^+$ cell growth [16] and the down-regulation of both TCR-induced IL-2 production [17], and activation of transcriptional factors NF-κB and AP-1 [18] has lent support to this interpretation. In addition, Th1 cytokine secretion is specifically suppressed in T cells incubated with Nef protein, whereas Th2 production is scarcely affected [19].

Nef may directly exert a cytotoxic effect on $CD4^+$ cells by inducing their apoptosis [20,21], just as *env* [22] and *tat* [23] promote this in T cells and contribute to the development of $CD4^+$ lymphopenia. This hypothesis is supported by suppression of the *in vitro* proliferation of $CD4^+$ cells and the fact that membrane expression of the C–terminus domain of Nef in infected lymphocytes cocultured with unprimed T-cell lines activates their suicidal death [20,21,24]. Further investigations have suggested that the Nef-induced down-regulation of CD4 molecules correlates with the intracellular levels of Nef [25], whereas its apoptogenic effect on T and B cells, macrophages and neutrophils during HIV-1 infection is apparently unrelated to the CD95/Fas pathway [26,27]. Conversely, soluble Nef has been shown to induce considerable cellular activation as a result of its superantigen effect on MHC class II molecules [28,29].

This work studied the effect of Nef on peripheral lymphocytes from HIV-1 infected individuals at different stages. $CD4^+$ cells were clearly affected and underwent a variable degree of suppression, whereas $CD8^+$ cells showed an apparent increase in their proliferative rate, especially the $CD8^+/CD57^+$ cells which exhibit a prevalently suppressor phenotype. In addition, Nef inhibited both the expression of Fas-L on cytotoxic $CD8^+$ cells and the proliferation of HLA-DR^+ lymphocytes, namely a population that suppresses HIV-1.

Methods

Plasmid construction

Full-length *nef* was amplified from pNL4–3 (kindly provided by Dr M. Federico, Laboratory of Virology, Istituto Superiore di Sanità, Rome, Italy) by polymerase chain reaction (PCR) using the primer pair covering the ATG and the 'stop codon' of the native *nef* sequence [4]. The amplification employed 25 cycles at 94 °C for 45″, 58 °C for 30″ and 72 °C for 30″, respectively. The PCR product was then purified and cloned into pTracer eukaryotic expression vector (Invitrogen, Pero, Italy) to create pTracer-*nef*.

Cell lines and transfection procedures

In preparing the feeder layer expressing Nef for human lymphocyte cultures, we adopted a xenogeneic adherent cell line to avoid molecular interactions between syngeneic human cells potentially perturbing the cellular response. Therefore, we used the NIH-3T3 cells, a fibroblast line established from NIH Swiss mouse embryo cultures (ATCC, Rockville, Maryland, USA), which have been successfully employed in transfection studies exploring the response of human NK cells [30]. The 3T3 cells were grown in DMEM (at high glucose rate) medium supplemented with 10% FCS and L-glutamine and subsequently transfected with 5 μg of pTracer-*nef* by Lipofectamine (Gibco-BRL, Life Technologies Italia, San Giuliano Milanese, Italy) according to the manufacturer's instructions. After appropriate selection in G418 at 1 mg mL^{-1}, single clones were isolated and further analysed by RT-PCR to assess the quantitative expression of *nef*. We then selected nine clones showing high levels of *nef* RT-PCR product as evaluated by direct O.D. measurement after extraction from agarose gel (Concert^{TM} Gel Extraction Kit, Gibco BRL, Milan, Italy). In addition, the membrane expression of Nef was estimated by flow cytometry with the anti-Nef monoclonal antibody (MoAb) AE6 (obtained from 'AIDS Research and Reference Reagent Program', Division of AIDS, NIAD, NIH, Bethesda MA, USA). Four clones (3T3–2, −3, −5, and −7) were positive for high membrane expression of Nef as demonstrated by a relative fluorescence intensity higher than one decade. Comparative experiments in preliminary tests in which peripheral blood lymphocytes (PBL) were incubated with each of the Nef-positive transfectants showed a major suppressive effect on $CD8^+$ subsets by the 3T3–7 transfectant. As the protein was expressed on the membrane of more than 95% of cells in this clone, we used the 3T3–7 transformant as adherent layer to culture lymphocytes.

PBL purification and coculturing with Nef-transfected cells

The study included 16 HIV-1 infected patients at different disease stages and 9 healthy donors. Seven patients were arbitrarily considered as severely lymphopenic ($CD4^+$ cells $\leq 300\,\mu L^{-1}$). PBL were purified from heparinized blood using Ficoll Hypaque gradient centrifugation and subsequent removal of adherent cells by incubation for 45 min at 37 °C. The cells were then washed and cultured at $1\times10^6\,mL^{-1}$ in 6-well plates containing the adherent 3T3–7 at 50–60% of confluency in RPMI-1640 plus 10% FCS in the presence of 1 μg mL^{-1} interleukin (IL)-2. After 60 h of coculture, the nonadherent cells were

harvested, washed and used for further analysis. To properly evaluate the effect of Nef, cellular analyses were compared in all instances to parallel cultures from each PBL sample of both patients and controls using non-transfected 3T3 cells as substrate.

Measurement of proliferation and apoptosis in cells stimulated by Nef

Proliferation was assessed by double fluorescence measurement of the expression of Ki67 antigen, in association with subset phenotyping with specific MoAbs to CD4, CD8, and CD16 antigens (Becton-Dickinson, Mountain View, CA, USA). Double fluorescence was also used to measure the size of the $CD8^+/CD57^+$ subset in response to Nef. To evaluate apoptosis, cells were harvested and permeabilized by 70% ethanol for 1 h at +4 °C prior to staining with propidium iodide at $50\,\mu g\,mL^{-1}$ in PBS to detect subdiploid DNA [31,32]. The extent of apoptosis was then expressed as the percentage of cells with subdiploid DNA. These analyses were conducted in a FACScan (Becton-Dickinson) using the CellQuest program.

Preparation of purified $CD8^+$ cell suspension and phenotype analysis

The effect of Nef on T-cell subsets was examined by purifying $CD8^+$ subsets from each PBL sample preincubated with *nef*-transfected cells and the relative control by immunomagnetic isolation using Dynabeads M-450 (Unipath, Milan, Italy). The full procedure recommended by the manufacturer was employed, providing a final enrichment of 92·3% of $CD8^+$ cells. Double fluorescence analysis then evaluated the expression of HLA-DR antigens, which define the activated phenotype of $CD8^+$ cells as response to the incubation with Nef.

Molecular analyses

In view of the proliferative response of $CD8^+$ cells to Nef, we measured the expression of Fas-L mRNA in purified $CD8^+$ populations, because Fas-L is a prevalent marker of cytotoxic cells (CTL) within this subset. Thus, mRNA was isolated from 1×10^6 $CD8^+$ cells in both unstimulated and Nef-stimulated cultures from 11 patients by the guanidium thiocyanate-cesium chloride procedure (Invitrogen, Celbio, Pero, Italy), and transcribed into first-strand cDNA with the Boehringer-Mannheim (Milan, Italy) cDNA kit. The Fas-L wild-type-specific primers were designed in relation to the known gene structure as follows: 5′-GCC CAA GCT TGA AGC AGC CCT-3′ (FW), and 5′-TGC TGT GTG CAT CTG GTC GGT AGA-3′ (RV) related to the exon-2 of the gene. Both primers and cDNA from each $CD8^+$ preparation were added to the PCR mixture (Perkin-Elmer, Cetus, Norwalk, Connecticut, USA) with subsequent amplification in a thermal cycler for 35 cycles (1 min 94 °C, 1 min 65 °C, 1 min 72 °C) and the Fas-L PCR product was visualized on a 1·5 agarose gel with ethidium bromide. Fas-L mRNA was estimated by evaluating the relative bands as 'trace quantity value' (o.d. × mm of each band) by Quantity-One 4·3 software in the Fluor-S gel analyzer (Bio-Rad Labs, Hercules, CA, USA).

Statistical analyses

Mean values of cellular subsets and phenotype expression between groups were compared by Student's *t*-test and, in several instances, by the Wilcoxon test as a nonparametric method.

Results

Nef induces a variable proliferation on PBL

Since Nef has been consistently reported to suppress T cells [16,20,21], we first assessed the extent of apoptosis in PBL cultures from both HIV-1-infected patients and healthy controls after 60 h of incubation with the *nef*-3T3–7 transfectant. An apparent inhibition of apoptosis was noted in both groups of cultures. Nef induced a variable though significant decrease in the relative extent of the mean subdiploid DNA peaks (M1 ± SD): 14·2 ± 5% and 10·7 ± 3% in Nef-treated cultures, as compared to 32·4 ± 4% and 19·6 ± 3% in untreated cultures from patients with severe ($CD4^+ \leq 300\,\mu L^{-1}$) and moderate ($> 300\,\mu L^{-1}$) lymphopenia ($P < 0{\cdot}02$ in both instances). The control cultures from uninfected donors showed a similar, though lower decline of apoptosis: mean M1 8·7 ± 3% in Nef-stimulated vs. 14·6 ± 3 in untreated cultures. However, this variation was not different in a statistical mode ($P > 0{\cdot}2$). These data are in agreement, and were in apparent concordance with reference values from our previous work [32], though the prolonged xeno-antigen stimulation by 3T3 cells in increasing the PBL apoptosis independently of Nef cannot be excluded. Representative profiles of this down-modulated apoptosis in a patient with severe $CD4^+$ lymphopenia (Pt. # 1) and a control subject are shown in Fig. 1. In both instances, the subdiploid DNA cell population was quantitatively reduced in cells harvested after incubation with the 3T3–7 clone (right panels). Morphological features of cell activation and proliferation, namely the expansion of both size and forward scatters, were also evident in both the euploid and the hyperdiploid cell populations gated in M2 and M3, respectively. These individual variations of fluorescence intensity were not statistically evaluated, though they were clearly detected in cells cultured in the presence of Nef and occurred in most preparations.

Further experiments were addressed to identify the cellular subset(s) with a prominent tendency to proliferate. Double fluorescence flow cytometry was used to measure Ki67 expression in $CD16^+$, $CD4^+$ and $CD8^+$ subsets

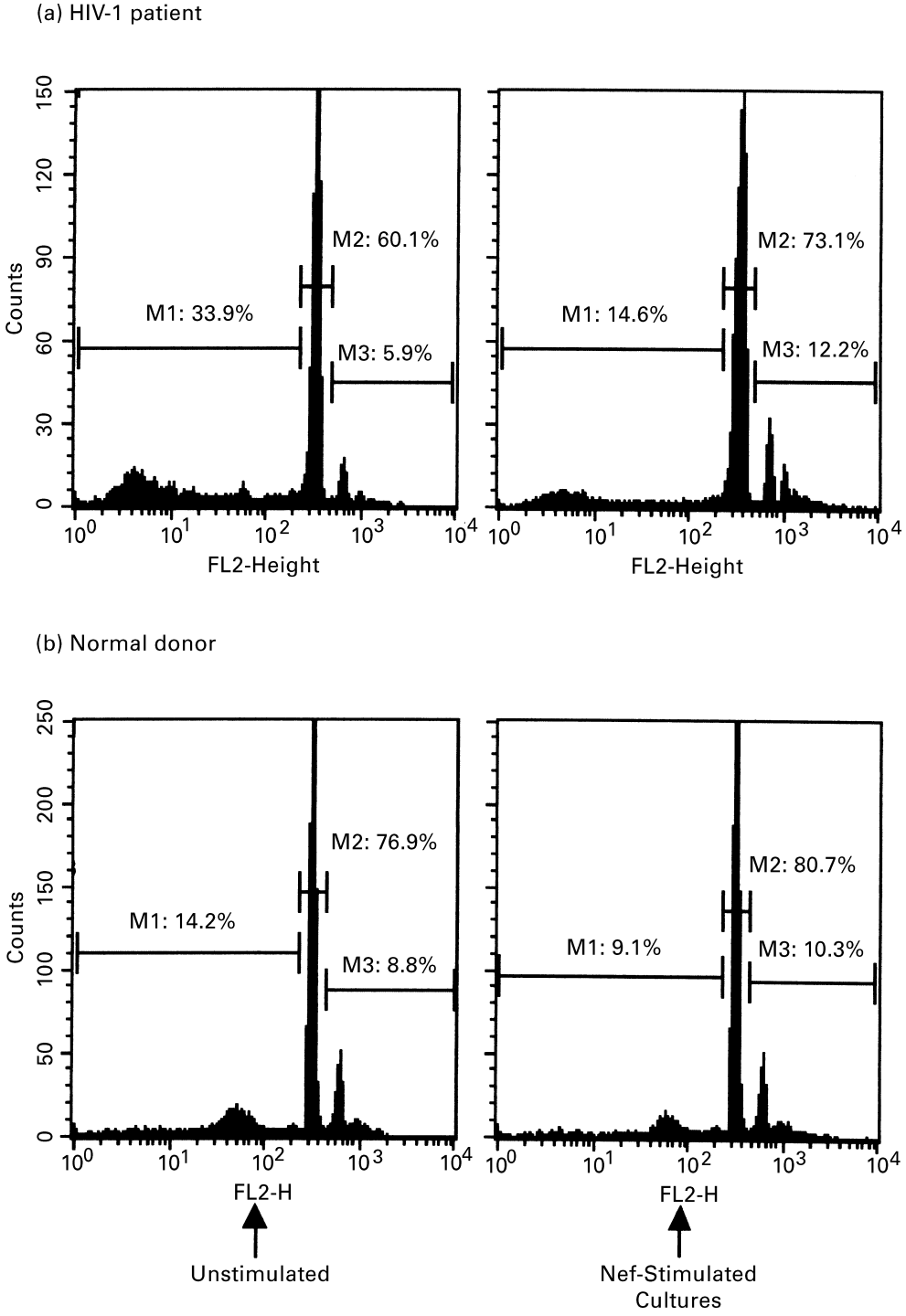


Figure 1 Variation of ploidy in PBL from a severely lymphopenic HIV-1 patient and a normal donor after 60 h of incubation in the presence of myristoylated Nef protein of HIV-1 exposed by the membrane of transfected 3T3–7 fibroblasts (right panel). This treatment usually reduced M1 peak corresponding to the extent of the subdiploid DNA content in apoptotic cells as compared to the control culture with untransfected fibroblasts (left panel). By contrast, M2 (euploid cells) and M3 (hyperdiploid cells) populations were proportionally increased. The pattern is representative of most cultures from patients where inhibition of apoptosis reflected the relative tendency to proliferate.

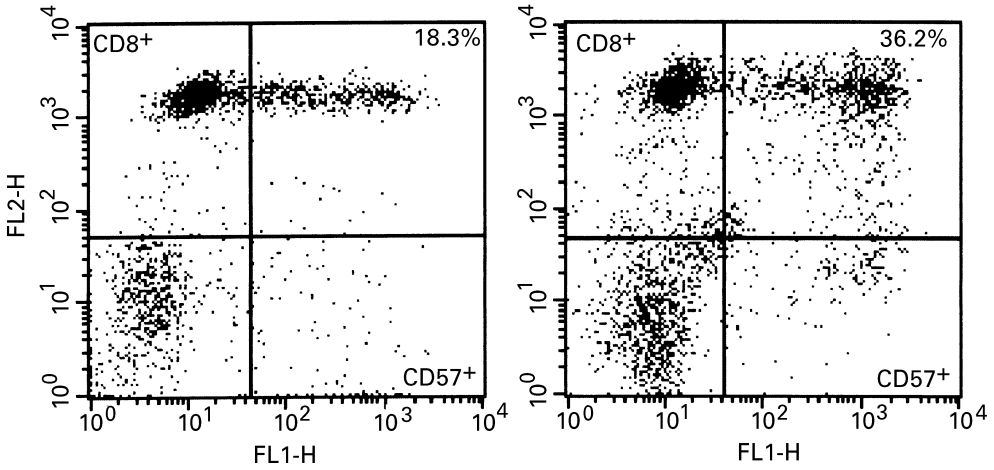
Table 1 Peripheral levels of $CD4^+$ and $CD8^+$ cells and variation of Ki67 antigen expression on specific T-cell subsets after 60-cultures of PBL in the presence of Nef expressed by the membrane of 3T3-7 transfected fibroblasts as layer (Nef-treated) compared to control cultures using untransfected cells (control). Significant variations included an increase of the Ki67 expression in $CD8^+$ subset from both groups of patients with severe as well as moderate $CD4^+$ lymphopenia ($P < 0.05$ in both instances). In addition, a slight though significant decrease of $CD4^+$ subset was also observed in cultures from control donors ($P < 0.02$)

	Peripheral levels (cells μL^{-1})		Per cent expression of Ki67 antigen on T-cell subsets					
			CD16		CD4		CD8	
	CD4	CD8	control	Nef-treated	control	Nef-treated	control	Nef-treated
HIV-1$^+$ patients								
(a) Severely lymphopenic ($\leq$300 $CD4^4$ cells mL^{-1})								
Patient:								
1	167	483	8·2	7·8	6·2	3·5	15·8	29·8
2	206	471	6·4	5·8	12·3	10·4	21·1	26·4
3	188	290	6·5	6	9·8	9	18·7	21·6
4	47	368	3·1	2·4	7·1	5	19	32·3
5	109	409	3·5	2·6	8·3	4·1	19·6	34·4
6	28	421	2·1	1·8	5·4	4·8	21·5	29·6
7	95	287	8·3	9	11·4	9·6	20·4	24·1
		M ± SD	5·4 ± 2	5 ± 2	8·6 ± 2	6·6 ± 2	19·4 ± 1	28·3 ± 4
(b) Moderately lymphopenic (>300 $CD4^+$ cells mL^{-1})								
Patient:								
8	539	901	9·5	10·2	12·3	10·6	31·8	43·6
9	421	1,080	13·4	11·2	22·6	21·4	30·4	36·2
10	406	780	10·4	8	18·4	20·2	26·5	31·5
11	605	980	14·5	15·2	20·1	16·3	19·4	24·3
12	427	1,010	11·6	10	17·4	16·5	30	31·4
13	705	1,606	9·6	8·5	21·3	20·4	28·4	41·5
14	578	865	11·1	10	15·2	14·1	26·4	36·2
15	335	820	7·3	6·1	11	9·2	30·5	38·5
16	390	680	8·7	9·5	9·6	8	21·3	28
		M ± SD	10·6 ± 2	9·8 ± 2	16·4 ± 4	15·1 ± 4	27·1 ± 2	34·5 ± 6
Normal donors (ND):								
1	1,780	792	11	8·6	26·4	20·3	15·6	16·3
2	2,010	900	15·4	15·1	31·3	28·4	21·4	23·6
3	2,300	980	7·8	6·5	28	21·5	18·8	20·4
4	1,950	970	10	8·7	34·1	30·6	21·2	22·4
5	2,600	1,270	13·4	8·4	36·6	19·4	29·4	31·2
6	1,870	1,085	9·5	8·2	42·1	26	13·6	15·6
7	2,300	980	7·8	6·5	28	21·5	18·8	20·4
8	2,900	1,450	12·4	11·6	40·2	36·4	28·4	29·9
9	1,870	906	11·8	12·1	30·4	26·3	21·8	23
		M ± SD	11 ± 2	9·5 ± 2	33 ± 5	25·6 ± 5	21 ± 5	22·5 ± 5

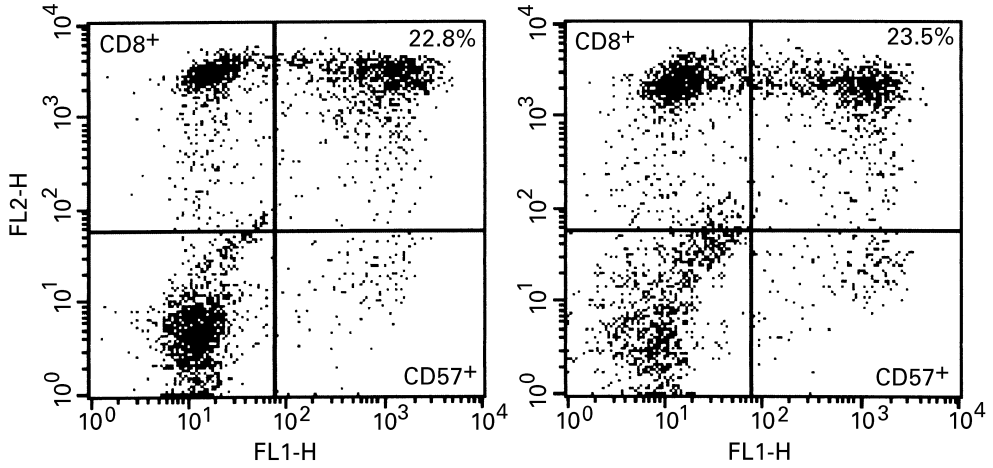
following a 60-h-culture with Nef. Table 1 shows the distribution of relative values of peripheral $CD4^+$ and $CD8^+$ cell counts in association with the effect of Nef in groups of cultures from patients and controls. Nef generally had little effect on the proliferation of $CD16^+$ and $CD4^+$ cells, because their mean values within each group showed a decrease, thus suggesting an apparent suppression. On comparing single values within each subset, however, it was found that patients with severe lymphopenia were variably affected in relative $CD4^+$ cell proliferation. Similarly, a proliferative decrease was prevalent in the uninfected controls and the mean values of suppression were significant ($P < 0·02$), thus confirming the susceptibility of uninfected cells to Nef-induced apoptosis [17,21]. In contrast, a clear-cut trend to proliferation was detected in the $CD8^+$ subset. The increase of positive cells was higher in patients than in the controls and the difference between mean values was significant ($P < 0·05$ in both instances) in each group. We also recorded a moderate

Figure 2 Cytofluorimetric evaluation of changes in the size of $CD8^+/CD57^+$ subset in HIV-1^+ patients with and without severe lymphopenia (peripheral $CD4^+$ cells $< 300\ \mu L^{-1}$) and in a normal subject. The fluorescence profiles are related to single subjects and show representative patterns for each group of cultures. Incubation of PBL in the presence of Nef (right panels) induced a major subset growth in cultures from lymphopenic patients, as in section (a), and a poor increase in those from nonlymphopenic patients (b) and healthy controls (c). Left panels refer to control cultures prepared with untransfected fibroblasts.

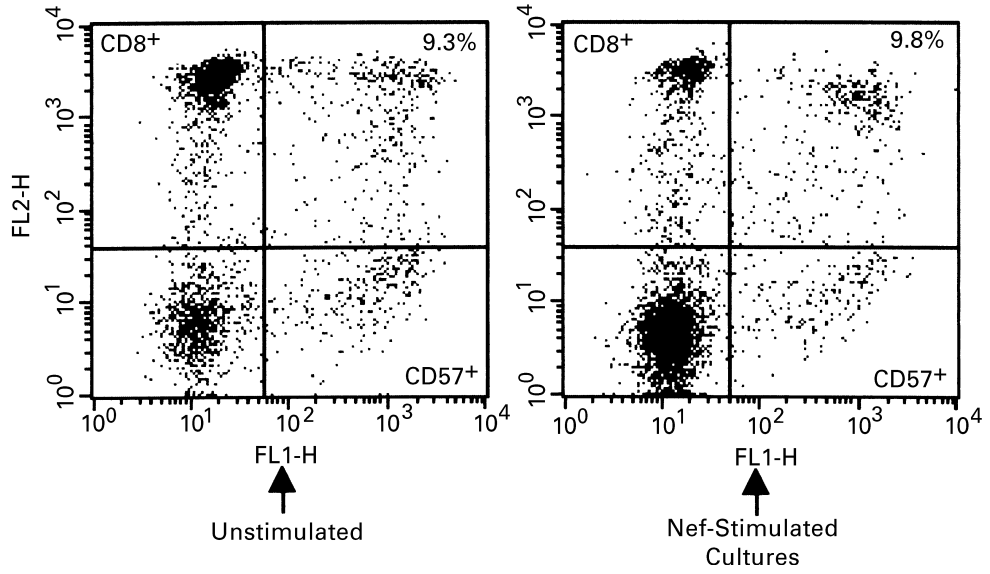
(a) Severely lymphopenic patient (patient no. 4)



(b) Moderately lymphopenic patient (patient no. 15)



(c) Normal donor (no. 1)



though not significant tendency to proliferation ($P > 0{\cdot}1$) in cultures from healthy controls.

A major proliferative effect is detectable in CD8$^+$/CD57$^+$ cells

Additional phenotyping tests identified the CD8$^+$ subsets sensitive to Nef. The effect of Nef on the CD8$^+$/CD57$^+$ subset, which displays a suppressor phenotype, was investigated by measuring the extent of the double positive CD8$^+$/CD57$^+$ subset on harvested PBLs from all cultures. An apparent increase in the percentage of these cells was observed in most Nef-stimulated patient cultures from patients showing the proliferative trend by Ki67 expression. Major elevations of CD8$^+$/CD57$^+$ cells (100% of increase) occurred prevalently in Nef-stimulated cultures from the severely CD4$^+$ lymphopenic patients ($P < 0{\cdot}01$). In fact, the mean value of $16{\cdot}8 \pm 4\%$ of CD8$^+$/CD57$^+$ cells in cultures from this group rose to $35{\cdot}1 \pm 6\%$ after incubation with Nef. Cell cultures from nonlymphopenic patients also showed a slight increment of mean levels ($24{\cdot}6 \pm 3\%$ vs. $20{\cdot}3 \pm 7\%$ in unstimulated cultures), whereas no evident variations were observed in the control cultures from the uninfected donors ($10{\cdot}9 \pm 2\%$ vs. $9{\cdot}8 \pm 3\%$ of CD8$^+$/CD57$^+$ cells in unstimulated cultures). Figure 2 compares representative profiles from a severely lymphopenic (patient no. 4), a nonlymphopenic patient (patient no. 15) and a control donor (normal donor no. 1): the CD8$^+$/CD57$^+$ increase was doubled in the first patient, whereas it was small in the second. The pattern relative to the control shows that Nef has little effect on CD8$^+$/CD57$^+$ population in cultures from healthy donors. These data suggested that Nef stimulation results in greater expansion of suppressor CD8$^+$ cells when CD4$^+$ cells are severely depleted as in the advanced stages of HIV-1 infection.

Nef down-regulates Fas-L expression in cytotoxic CD8$^+$ cells from HIV-1-infected patients

We next looked to assess whether or not Nef was able to affect the CD8$^+$ cells displaying the cytolytic phenotype (CTL). Fas-L expression was then explored in these cells by measuring the relative mRNA levels of purified CD8$^+$ cells from most patient cultures, five patients with severe and six with moderate lymphopenia. In addition, CD8$^+$ cells were harvested from the cultures of five healthy controls. We observed an apparent diminution of the Fas-L bands in Nef-exposed CD8$^+$ cells from all culture samples. Figure 3 illustrates this pattern in a representative subject from each group, namely from patients no. 4 and 15, and normal donor no. 6. The quantitative evaluation by the Fluor-S analyzer confirmed a general decrease of Fas-L expression after treatment with Nef (lanes b). We found a difference in the Fas-L mRNA trace quantity values, which were apparently higher in patients with severe CD4$^+$ lymphopenia (0·112 of mean reduction value as compared to 0·087 o.d.×mm of the other group of patients). However, these variations were not statistically evaluated in comparing groups of samples because of the high intragroup variability.

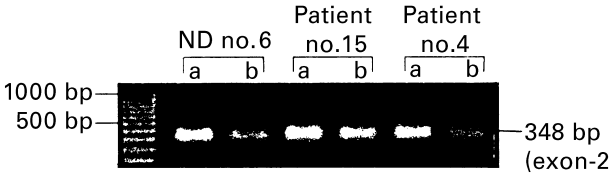


Figure 3 Comparative PCR amplification of Fas-L mRNA in CD8$^+$ cells purified from cultures stimulated by Nef (lane b) with respect to unstimulated cultures (lane a). The treatment down-regulated Fas-L mRNA in all instances, particularly in patients with severe CD4$^+$ lymphopenia (patient no. 4 is representative of this group).

Suppressor anti-HIV-1 CD8$^+$ lymphocytes are inhibited by Nef

Lastly, the role of Nef on noncytotoxic HIV-1-specific suppressor CD8$^+$ lymphocytes in cultures from HIV-1-infected subjects was examined. This anti-HIV-1 response appears to be associated primarily with activated CD8$^+$ cells expressing both HLA-DR and CD28 antigens and acts through a soluble inhibitory factor termed CAF (CD8$^+$ T-cell antiviral factor) [33]. Therefore, our analysis investigated the effect of Nef on this specific subset by measuring in double fluorescence the expression of HLA-DR molecules in purified CD8$^+$ cells from four patients with severe and 5 with moderate lymphopenia. Nef down-regulated HLA-DR in most cultures. Patients with the lowest CD4$^+$ levels showed a significant suppression of their CD8$^+$/HLA-DR$^+$ population in response to Nef: the mean number declined from $32{\cdot}9 \pm 8$ to $15{\cdot}1 \pm 7$ ($P < 0{\cdot}02$). A similar effect was also observed in single patients with moderate CD4$^+$ lymphopenia. However, although the decrement of the mean value of HLA-DR$^+$ cells ranged from $37{\cdot}6 \pm 4\%$ in untreated cultures to $32{\cdot}1 \pm 6\%$ in the presence of Nef, it was not statistically significant ($P > 0{\cdot}2$).

Figure 4 reports the cytofluorimetric pattern of two patients from each group and gives an example of the suppressive effect of Nef on these cells, which was higher than 50% in the CD8$^+$ population of both patients with severe CD4$^+$ lymphopenia (section a). Similarly, a lower suppression of proliferation was demonstrable in both patients with moderate lymphopenia (section b), whose average value was about 5%, and reflected the results

Figure 4 Representative patterns of cytofluorimetric assays related to quantitative variations of activated (HLA-DR$^+$) CD8$^+$ cells in cultures from patients with severe (a) and moderate (b) lymphopenia in response to 60 h of incubation with Nef. This treatment induced a substantial decrease in this subset, especially in patients with advanced disease.

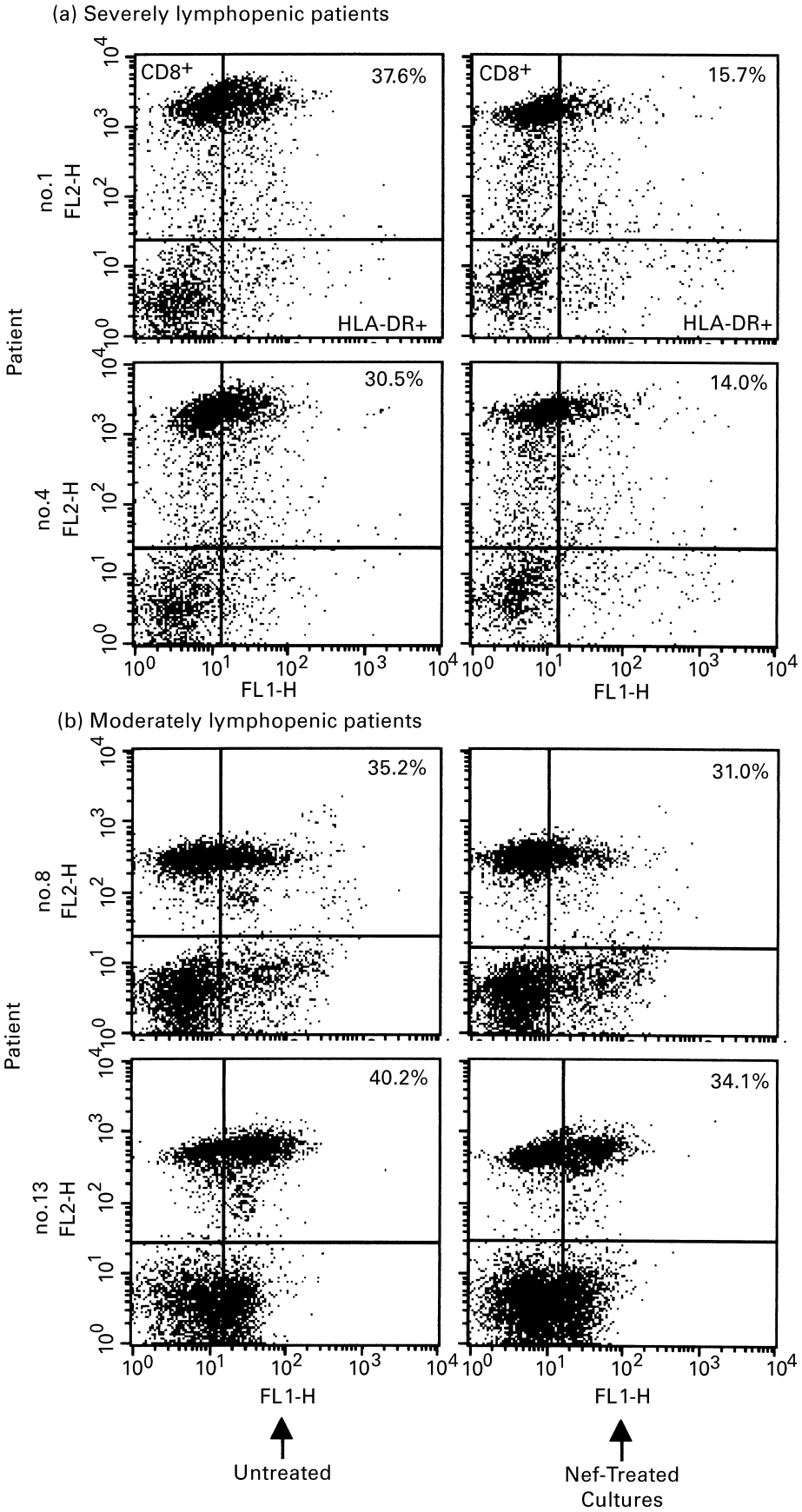

(a) Severely lymphopenic patients
CD8+
37.6%
HLA-DR+
CD8+
15.7%
HLA-DR+
30.5%
14.0%
Patient
no.1
no.4
FL2-H
FL1-H
(b) Moderately lymphopenic patients
35.2%
31.0%
40.2%
34.1%
no.8
no.13
Untreated
Nef-Treated Cultures

Table 2 Variation of relative fluorescence intensity of HLA-DR antigen expression in $CD8^+$ cells from HIV-1^+ patients after their culture in the presence of Nef

HIV-1^+	Patient	Index of HLA-DR expression on $CD8^+$ cells*	
		Untreated culture	Nef-treated cultures
Severely lymphopenic	1	9.81	6.27
	3	12.33	5.11
	4	11.85	6.98
	6	8.42	4.13
Moderately lymphopenic	9	10.42	9.5
	10	8.52	8.31
	12	11.45	10.18
	14	9.47	6.53
	15	7.51	6.18

*The HLA-DR expression index was defined as the ratio of mean fluorescence channels (specific MoAb/isotype-matched control MoAb).

of other patients with similar $CD4^+$ cell levels. Lastly, Table 2 includes the variations of mean values in fluorescence intensity of DR expression following treatment with Nef. In most instances we observed a variable suppression of DR antigens in $CD8^+$ cells incubated with Nef. However, such variations of relative fluorescence intensity in DR expression were not calculated in statistical terms because of the small number of samples. These results suggested that activated anti-HIV-1 suppressor $CD8^+$ cells could be directly inhibited by Nef in culture.

Discussion

Our study on the effect of Nef on $CD8^+$ cells during HIV-1 infection has shown that, in addition to its cytolytic effect on other T cells, it stimulates the growth of $CD8^+/CD57^+$ subset *in vitro*, and down-modulates both the expression of Fas-L, a death factor of CTLs, and the proliferation of $CD8^+$ cells activated to HIV-1; namely the $CD8^+/HLA\text{-}DR^+$ subset. These immune dysfunctions of $CD8^+$ cells are significant in patients with severe lymphopenia and probably contribute to the progression of HIV-1 infection to AIDS.

Recent work has provided new insights into the function of Nef during HIV-1 disease. Originally seen as a negative regulator for virus replication [9] it is now being shown to play a positive role in replicating HIV-1 in primary T cells and T-cell lines [34–36]. It also down-regulates cell surface levels of CD4 molecules [37,38] and induces cytolysis of $CD4^+$ cells in either its soluble [21] or its myristoylated form [20,24]. Although the myristoylated Nef peptide is anchored on the internal sheet of the plasma membrane by the N-terminal glycine, the myristoylation procedure induces a disordering effect on the lipid bilayers, resulting in nonlamellar phases of the whole membrane layer and extrusion of the other terminal portion of the viral protein [39] [40]. Exhibition of the carboxy-terminal domain of Nef on the HIV-1-infected T-cell surface is critical in provoking cell death by cytotoxicity in uninfected $CD4^+$ lymphocytes [5,24], whereas interaction of Nef with specific cellular kinases has suggested that it interferes with signalling pathways that promote T-cell activation [41].

Our present study emphasizes that, in contrast with the down-regulation of $CD16^+$ and $CD4^+$ cells, the myristoylated form of Nef from *nef*-3T3–7 transfectant induces a number of functional defects within the $CD8^+$ subset that may promote the escape of both HIV-1 and infected cells from immune control. However, as it has been reported that soluble Nef may affect proliferation of $CD4^+$ cells [21], further cellular suppression by soluble Nef molecules possibly released in our cultures by either transfected fibroblasts or infected cells cannot be ruled out. The first derangement induced by Nef concerns the increase of $CD8^+/CD57^+$ cells. This subset is phenotypically distinct because of its prevalent suppression of B-cell differentiation, proliferation and Ig secretion [42,43]. Although no specific antiviral cytotoxicity has been attributed to this population [44], its oligoclonal expansion occurs in several clinical conditions including cytomegalovirus infection [45], Crohn's disease [46], common variable immunodeficiency [47], and HIV-1 infection [48]. In this disease as well as in the immune deficiency of bone marrow transplantation recipients [49], suppression was related to a 20-kDa, heat-stable suppressor factor [50] that inhibits lectin-driven proliferation and cytolysis [51]. In our study, we observed that a broad expression of Ki67 antigen was presented by $CD8^+$ cells cultured in the presence of Nef and paralleled the expansion of the $CD57^+$ cell subset, in particular in patients with severe $CD4^+$ lymphopenia. By contrast, mostly stable or weakly increased values of $CD57^+$ cells were detected in cultures from moderately lymphopenic patients and the controls. We interpreted the differential proliferative response to Nef as a result of the divergent levels of $CD4^+$ cells *in vivo*. As patients with AIDS or severe lymphopenia have a minimal expression of the myristoylated protein on infected $CD4^+$ cells as a result of critical lymphocyte depletion, exposure of their PBL to the high virus antigen load as provided by *nef*-3T3–7 cells could have resulted in a major stimulation and growth of the $CD57^+$ population.

Our study also points to the down-regulation of Fas-L expression in cytotoxic $CD8^+$ cells treated with Nef. Fas-L is the functional coreceptor of Fas and induces apoptosis in Fas^+ target cells by oligomerization of the receptor. This mechanism is used by most effector cells and Fas-L is considered a major phenotypic marker of CTLs. The apparent down-modulation of mRNA in treated cells in our study was in line with the relative increase of the $CD57^+$ subset, especially in patients with severe lymphopenia. As Nef-induced cytolysis of uninfected lymphocytes is independent of Fas/Fas-L system [26,27], this finding supports the view that the viral protein does not act through the Fas/Fas-L pathway. On the other hand, similarly defective Fas-L mRNA expression has been described in advanced HIV-1 infection [52–54], suggesting that the antigenic charge of soluble Nef may partly contribute to Fas-L down-regulation, though defective CTL function in this disease could be the result of the increased suppression by $CD57^+$ cells [45,48]. Our study, in fact, documented $CD57^+$ expansion in advanced infection following exposure to Nef, though its suppression of Fas-L via other mechanisms cannot be excluded.

Another interesting finding was the detrimental effect of Nef on HIV-1 suppressor $CD8^+$ lymphocytes resulting in a defective expression of HLA-DR molecules. These data are in apparent contrast with the increase of $Ki67^+$ cells within the same subset. However, previous studies have pointed out a possible discrepancy between both cell cycle and activation antigen expression in $CD4^+$ and $CD8^+$ T cells in patients receiving the HAART (highly active antiretroviral treatment) therapy, especially during the chronic HIV-1 infection [55] [56]. In particular, though in the presence of a proliferative trend involving the total T-cell population, activation of $CD8^+$ T cells measured by both CD38 and HLA-DR expression was significantly affected in this subset [57]. Our results showing a similar inhibition of HLA-DR molecules in response to Nef in patients with advanced disease are in line with these observations. On the other hand, $CD8^+$ cells expressing both HLA-DR and CD28 markers are thought to suppress HIV-1 replication by CAF in $CD4^+$ lymphocytes and macrophages [33]. We observed an evident decline of this population in cultures treated with Nef. Suppression of HIV-1 replication by CAF in naturally or acutely infected $CD4^+$ cells has been described as dose-dependent and correlated with clinical state and $CD4^+$ cell levels in peripheral blood [58]. Although we did not measure the extent of CAF secretion in cultures, the depletion of $HLA\text{-}DR^+$ cells after incubation with Nef suggests a parallel reduction in the release of the suppressive factor, particularly in patients with advanced disease.

Our data on the effect of Nef on $CD8^+$ cells support recent studies describing its multifactorial role in the depletion of immune cells during HIV-1 infection. In addition to its cytolysis of uninfected $CD4^+$ lymphocytes, Nef may down-regulate the MHC class I on infected cells to promote their escape from cytolysis of functional CTLs [59]. In this context, its down-modulation of their expression of Fas-L and its inhibition of suppression by non cytotoxic anti-HIV-1 $CD8^+$ cells are ways of escaping immune surveillance. Additional work is required to investigate the molecular defects of such Nef-related deregulations of $CD8^+$ cells, though they may be the multiple outcome of a single deviation primarily induced by either soluble or myristoylated Nef. For instance, alteration in the secretion of $CD8^+$ cell cytokines could be a primary event leading to differentiated dysfunctions within the subset. Preliminary data from our laboratory support this hypothesis, in that $CD8^+/CD57^+$ cells treated with Nef showed high mRNA expression of several suppressive cytokines, including IFN-γ and IL-4, which could significantly influence both Fas-L expression in CTLs and CAF secretion in activated $CD8^+$ cells.

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