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Selective Depletion of Low-Density CD8$^+$, CD16$^+$ Lymphocytes During HIV Infection

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ABSTRACT

Two color cytofluorometric analyses of CD3$^-$, CD16$^+$, Leu 19$^+$ natural killer cells (NK) were assessed in HIV seropositive patients, high-risk seronegative homosexuals, and healthy heterosexuals. A selective depletion of lymphocytes bearing NK phenotypes was found among HIV-positive infected patients. When the CD16$^+$ lymphocyte compartment was further dissected, lymphoid cells bearing simultaneously low cell-surface density CD8 and CD16 (Leu 11a or Leu 11c) or Leu 19 epitopes were selectively and significantly decreased. This important depletion of CD8$^+$ NK cells, which in most cases are CD3$^-$, accounts for the decline in low-density CD8$^+$ lymphocytes in HIV positive group, while a significant increase occurs in their CTL pool. Furthermore, in HIV negative high-risk homosexuals, a less profound but significant reduction of this lymphocyte subset was also found. Whether the involvement of the NK compartment, especially NK cells expressing the CD8 marker, may influence the outcome of individuals infected with HIV is still an open question.

INTRODUCTION

THE HIV INDUCED CD4 (T-helper cells) lymphocyte depletion with a corresponding increase of CD8 (T-suppressor and/or cytotoxic pools) lymphoid cells are among the most prominent features of both the acquired immunodeficiency syndrome (AIDS) and HIV infection-related stages.[1–4] Although, the increase in CD8 subpopulation has been interpreted as a defense response,[4] there is still considerable controversy concerning its beneficial role.[5] CD8 lymphocytes are heterogeneous both in phenotype and function, including at least, three different subsets defined by their cytotoxic, suppressor and natural killer (NK) "like" functions.[6,7] Phenotypically, cytotoxic T-lymphocytes (CTL) are defined as CD3$^+$, CD8$^+$, CD11$^-$ (Leu 15), CD16$^-$ (Leu 11a, Leu 11c) cells whereas suppressor cells have been claimed to correspond to CD3$^+$, high cell-surface density (HD) CD8$^+$, CD11$^+$, CD16$^-$ phenotype.[8] The NK cells, initially asso-

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ciated to the HNK-1 (Leu 7) marker, are now defined by the expression of the CD16 and the NKH-1 (Leu 19) markers. The vast majority of this subpopulation is $CD3^-$, while a significant proportion expresses low cell-surface density (LD) CD8 and CD11 markers.[9]

Although it has been established that CTLs are increased during HIV infection, there are scant and conflicting data concerning the NK pool during HIV infection.[4,10,11]

In this investigation, we have assessed the phenotypically defined CTL and NK pool size in HIV-infected patients and compared them to high-risk seronegative homosexuals and healthy heterosexual controls.

METHODS

Subjects

The three groups studied included:

1. 15 healthy heterosexual donors from the blood bank of the Pasteur Institute.
2. 13 apparently healthy homosexuals belonging to a high-risk group who were found to be negative for HIV antigens and HIV antibodies.
3. 32 HIV seropositive patients (HIV^+); this group included 21 seropositive asymptomatic carriers (AC) and 11 with the diagnosis of AIDS (5 cases with opportunistic infections, 5 with Kaposi sarcoma, and 1 case presenting with both).

Detection of HIV antibodies. Serum samples were tested for antibodies to HIV-1 and HIV-2 by an enzyme-linked immunosorbent assay (ELISA) (ELAVIA I and ELAVIA II Ab; Diagnostics Pasteur, Marnes la Coquette, France) and subsequently confirmed by immunofluorescence[12] and/or by Western blot (LAV-blot; Diagnostics Pasteur, France).

Detection of circulating HIV antigens. The presence of circulating HIV-1 p25 antigens was examined by ELAVIA antigen (Diagnostics Pasteur). Briefly, polystyrene plates coated with anti-p25 antibody were incubated with undiluted test serum followed by incubation with anti-p25 antibody coupled to Biotin; the reactivity was revealed by streptavidin coupled to peroxidase enzyme.

Monoclonal antibodies. Monoclonal antibodies (mAbs) coupled to either fluorescein isothyocyanate (FITC) or phycoerythrin (PE) were purchased from Becton-Dickinson (Mountain View, CA) except 4B4 and 2H4 mAbs (Coultronics; Hileah, FL).

The various specificities included: CD3, CD4, CD8, CD11 (Leu 15), CD16 (Leu 11a, Leu 11c) NKH-1 (Leu 19), HNK-1 (Leu 7) and 4B4 (CDw29) and 2H4 (CD45R) as recently defined.[13–15] mAb couples employed in the protocol were: CD4-CD8, CD8-CD11 (Leu 15), CD8-Leu 11a, CD8-Leu 11c, CD8-Leu 19, CD3-CD8, CD8-CDw29, CD8-CD45R, CD8-Leu 7, CD3-Leu 19, CD3-Leu 11c, Leu 11a-Leu 11c, Leu 11a-Leu 19, Leu 7-Leu 11a and Leu 7-Leu 19.

Cytofluorometric analysis

Fifty microliters (50 μl) of peripheral blood were washed twice in phosphate-buffered saline (PBS), incubated with optimal concentrations of a couple of FITC or PE conjugated mAbs for 30 min at 4°C. After two washings with PBS, red blood cells were lysed. The remaining cell pellet was fixed and washed twice (immunolyse and fixation solution were provided by Coultronics, France). Cells were kept at 4°C until cytofluorometric analysis. The analysis was carried out on 10,000 lymphocytes for one or two colors employing an EPICS C-cell sorter (Coultronics) equipped with a single argon 2-W laser beam and logarithmic intensity scales. Lymphocytes were gated using both forward and right angle scatters. The gates also included cells identified as large granular lymphocytes. Experiments performed on five healthy donors comparing phenotyping characteristics and using whole blood versus Ficoll-Hypaque isolated buff coats showed no significant differences. Thus, whole blood was employed throughout the research protocol.

STATISTICAL ANALYSIS

The least significant difference test[16] was employed for statistical analysis of the data.

RESULTS

Major lymphocyte subsets

The first alteration we found when investigating the major phenotypically defined lymphocyte subsets was a significant decrease in the absolute numbers of either CD16$^+$ (Leu 11a, Leu 11c) or NKH-1$^+$ (Leu 19) cells among HIV-infected patients when compared to controls and in the case of Leu 11a or Leu 11c when compared to both controls and high-risk seronegative homosexuals (Table 1). This difference may be linked in part to the decreased lymphocyte count observed in the HIV-positive individuals since only in the case of Leu 11c the observed percentage was significantly lower than in controls. Results obtained with anti-HNK-1 (Leu 7) are noteworthy, since both HIV seropositive and high-risk seronegative individuals showed a nonsignificant increase in the absolute numbers of Leu 7$^+$ cells when compared to controls (Table 1). Double-labeling experiments illustrated that while 90% of CD16$^+$ cells coexpressed Leu 11a and Leu 11c, only 56% of controls, 46% high-risk seronegative subjects and 36% seropositive HIV patients coexpressed Leu 11a-Leu 19. Double labeling with Leu 11c-Leu 7 was present in only 34% of control's lymphoid cells and in 40 and 56% of both high-risk seronegative and HIV-positive individuals (data not shown).

TABLE 1. PHENOTYPICALLY DEFINED LYMPHOCYTE SUBSETS

	Healthy heterosexual controls n = 15 (Group 1)		*HIV negative homosexuals n = 13 (Group 2)*		*HIV positive subjects n = 32 (Group 3)*		*Least significant difference test*		
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*1 vs. 2*	*1 vs. 3*	*2 vs. 3*
Total lymphocytes	2621	799	2.400	543	1.840	871	N.S.	$p < .01$	N.S.
CD4									
%	42	8	39	5	16	10	N.S.	$p < .01$	$p < .01$
AN/mm^3	1092	385	932	238	314	255	N.S.	$p < .01$	$p < .01$
CD8									
%	30	5	31	8	54	12	N.S.	$p < .01$	$p < .01$
AN/mm^3	791	261	755	259	996	503	N.S.	N.S.	N.S.
CD16									
LEU 11a									
%	17	6	21	6	15	9	N.S.	N.S.	$p < .01$
AN/mm^3	421	251	515	181	266	158	N.S.	$p < .05$	$p < .01$
LEU 11c									
%	17	7	19	8	12	6	N.S.	$p < .05$	$p < .01$
AN/mm^3	456	220	463	233	215	146	N.S.	$p < .01$	$p < .01$
NKH-1 (LEU 19)									
%	14	6	12	8	10	4	N.S.	N.S.	N.S.
AN/mm^3	342	112	296	208	187	109	N.S.	$p < .05$	N.S.
HNK-1 (LEU 7)									
%	15	7	24	8	29	11	$p < .05$	$p < .01$	N.S.
AN/mm^3	394	210	583	236	525	273	N.S.	N.S.	N.S.

CD nomenclature was adjusted from the Third Human Leukocyte Differentiation Antigens Workshop (Oxford, Sept. 1986). CD16 was simultaneously assessed by Leu 11a and Leu 11c mAbs. The least significant difference test[13] was employed throughout the protocol. AN = absolute number.

CD16$^+$ subpopulations

In controls, an important part of the CD16$^+$ subpopulation was found to coexpress CD8 antigen.[9] In Table 2, we have depicted the analysis of CD16$^+$ cells according to the presence or absence of the CD8 marker. The data indicate that there is a decrease in the absolute numbers of the CD16$^+$, CD8$^-$ cells in the HIV-positive group; however, the compromise was more pronounced within the CD8$^+$, CD16$^+$ subset since both percentage and absolute numbers were significantly decreased. Despite higher absolute numbers of CD8$^+$ lymphoid cells in HIV-positive patients, values under 40/mm^3 of CD16$^+$, CD8$^+$ cells were observed in 1 of 15 healthy controls, 1 of 13 high-risk seronegative homosexuals as compared to 12 of 21 AC and 9 of 11 AIDS patients (the difference between AC and AIDS failed to achieve statistical significance). Similar results were observed when Leu 19$^+$, CD8$^+$ or CD8$^-$ lymphoid cells were investigated. These results are in contrast with a significant increase in CD8$^+$, Leu 7$^+$ cells found among the HIV-positive group (data not shown). In Table 2, we also show that 1% or less of lymphoid cells shared CD3 and CD16 or Leu 19, compared to 17% and 14% of lymphocytes expressing these markers.

Lymphocyte subsets among CD8

These results prompted us to investigate the lymphocyte subsets among CD8 (Table 3). Cells were analyzed in relation to the presence of low (LD) or high (HD) cell-surface density CD8 and also according to the coexpression of CD11, CD16, CD3 and Leu 7 markers. A significant decrease in LD CD8 lymphocytes was found in HIV-positive patients. In fact, these LD CD8 subpopulations can be displayed into two main subsets, phenotypically defined as CD3$^+$, LD CD8$^+$, CD 16$^-$ and CD3$^-$, LD CD8$^+$, CD16$^+$ (Fig. 1). The histograms show the CD8$^+$ lymphocytes of a control (A,C) and of an AIDS patient (B,D) in

TABLE 2. CD16$^+$ LYMPHOCYTE SUBSETS

	Healthy heterosexual controls n = *15* *(Group 1)*		*HIV negative homosexuals* n = *13* *(Group 2)*		*HIV positive subjects* n = *32* *(Group 3)*		*Least significant difference test*		
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*1 vs. 2*	*1 vs. 3*	*2 vs. 3*
CD8$^-$, Leu 11a$^+$									
%	12	4.9	17	6	13	8	N.S.	N.S.	N.S.
AN/mm^3	312	160	397	143	248	181	N.S.	N.S.	$p < .01$
CD8$^+$, Leu 11a$^+$									
%	5	2.8	4.6	3.3	2.2	1.7	N.S.	$p < .01$	$p < .05$
AN/mm^3	143	98	113	96	42	36	N.S.	$p < .01$	$p < .05$
CD8$^-$, Leu 11c$^+$									
%	11	4.9	14	8	8	4	$p < .05$	N.S.	$p < .01$
AN/mm^3	274	140	349	194	152	105	N.S.	$p < .05$	$p < .01$
CD8$^+$, Leu 11c$^+$									
%	7	3	5	2.8	2.7	1.7	N.S.	$p < .01$	$p < .05$
AN/mm^3	179	106	129	87	62	72	N.S.	$p < .01$	$p < .05$
CD3$^+$, Leu 11c$^+$									
%	0.4	0.4	0.4	0.3	1	1	N.S.	N.S.	N.S.
AN/mm^3	12	10	12	10	14	13	N.S.	N.S.	N.S.

Assessment of CD16 lymphoid cell subsets included those lymphocytes bearing only both Leu 11a and Leu 11c epitopes or else coexpressing either CD8 or CD3 antigens.

TABLE 3. LYMPHOCYTE SUBSETS AMONG CD8

		Healthy heterosexual controls n = 15 (Group 1)		*HIV negative homosexuals n = 13 (Group 2)*		*HIV positive subjects n = 32 (Group 3)*		*Least significant difference test*		
		Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*1 vs. 2*	*1 vs. 3*	*2 vs. 3*
$CD8^+$										
*Low density:	%	37	12	32	8	18	7	N.S.	$p < .01$	$p < .01$
	AN/mm³	297	136	245	123	169	94	N.S.	$p < .01$	$p < .01$
*High density:	%	63	12	68	8	82	7	$p < .01$	$p < .01$	N.S.
	AN/mm³	492	168	508	167	826	445	N.S.	$p < .01$	$p < .01$
$CD8^+$, $CD11^+$										
*Low density:	%	14	8	11	6	5.4	4	$p < .05$	$p < .01$	$p < .05$
	AN/mm³	114	89	88	60	44	26	N.S.	$p < .01$	$p < .05$
*High density:	%	4	2	8	8	3.6	3.8	$p < .05$	N.S.	$p < .05$
	AN/mm³	34	25	65	75	30	23	$p < .05$	N.S.	$p < .01$
$CD8^+$, $CD16^+$										
*Low density:	%	19	11	13	6	4.8	4	$p < .05$	$p < .01$	$p < .01$
	AN/mm³	153	99	107	75	45	40	$p < .05$	$p < .01$	$p < .05$
*High density:	%	2.5	2.3	1.7	1	1.6	1.8	N.S.	N.S.	N.S.
	AN/mm³	19	18	13	10	17	23	N.S.	N.S.	N.S.
$CD8^+$, $CD3^+$										
*Low density:	%	12	6	14	6	11	7	N.S.	$p < .05$	$p < .05$
	AN/mm³	91	45	88	30	93	37	N.S.	N.S.	N.S.
*High density:	%	62	17	61	20	81	9	N.S.	$p < .01$	$p < .01$
	AN/mm³	486	180	504	204	850	430	N.S.	$p < .01$	$p < .05$
$CD8^+$, Leu 7^+										
*Low density:	%	12	7	10	6	8	5	N.S.	N.S.	N.S.
	AN/mm³	95	76	77	60	81	78	N.S.	N.S.	N.S.
*High density:	%	16	14	19	12	26	11	N.S.	$p < .01$	N.S.
	AN/mm³	121	102	217	268	262	172	N.S.	N.S.	N.S.
$CD8^+$, $CD11^-$										
	%	75	14	78	11	89	7	N.S.	$p < .01$	$p < .01$
	AN/mm³	590	203	574	164	907	471	N.S.	$p < .05$	$p < .01$

Low-density (LD) or high-density (HD) surface membrane expression of CD8 antigen was exhaustively investigated within CD8 as well as in those lymphoid cells coexpressing CD16 (Leu 11c), CD11 (Leu 15), CD3 or HNK-1 (Leu 7) epitopes. Assessment of both T-suppressor cells ($CD3^+$, $CD8^+$, $CD11^+$) and CTL's ($CD3^+$, $CD8^+$, $CD11^-$) was performed according to Clement *et al.* (8) and Nicholson *et al.* (4).

double-labeling experiments either with Leu 11a or CD3 mAbs. Depletion of the LD $CD8^+$-Leu $11a^+$ subset in the AIDS patient is clearly demonstrated (4%) when compared to the control (24%), whereas LD $CD8^+$ lymphocytes coexpressing $CD3^+$ did not show significant modifications. The depletion of $CD3^-$ LD $CD8^+$ lymphocytes parallels the decrease of LD CD8, Leu $11a^+$ in the HIV-positive group. Interestingly, the latter subset appears to be severely affected in HIV-positive patients regardless of the mAbs employed to identify the cells (Leu 11a, Leu 11c or Leu 19). Moreover LD $CD8^+$, Leu 7^+ lymphocytes remained unchanged. Concomitantly, among the same HIV-positive patients, HD $CD8^+$ cells were signifi-

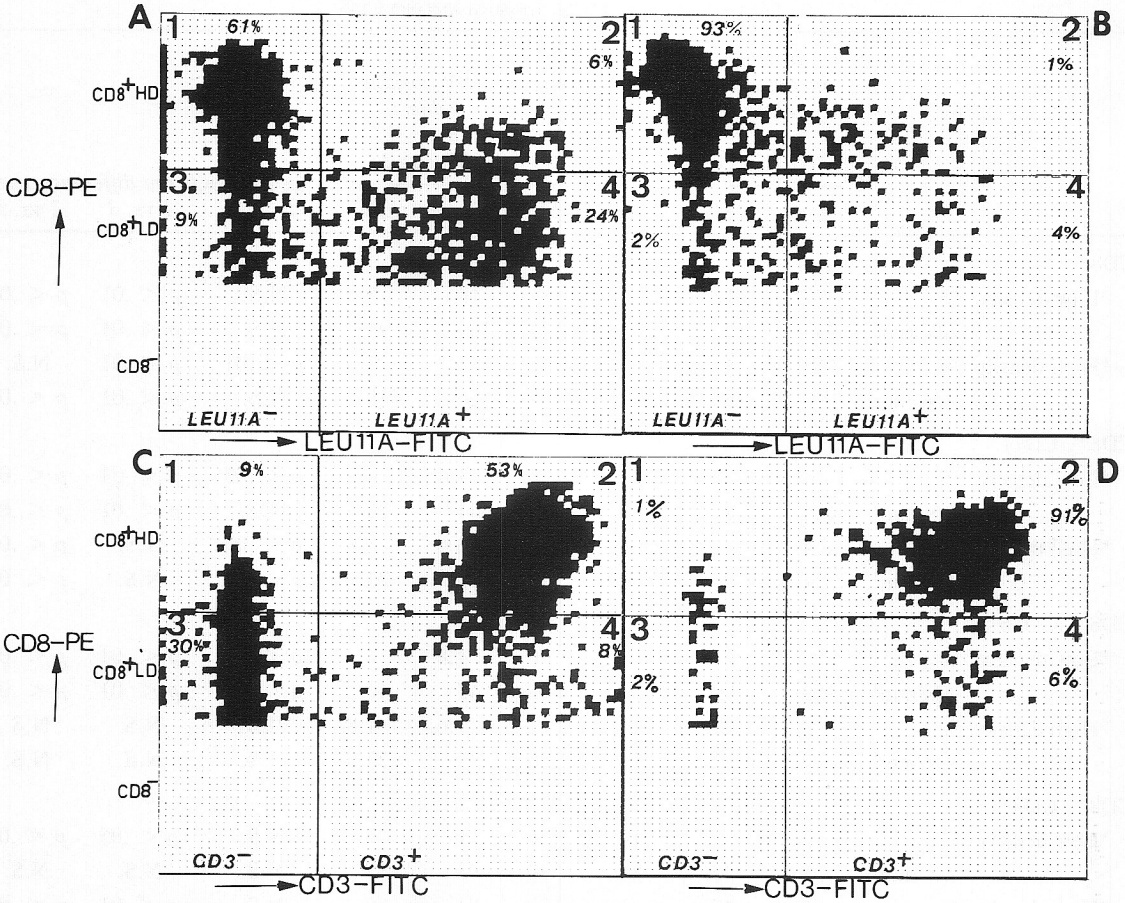

FIG. 1. Comparative study of CD8 positive cells (CD8 negative cells were excluded) displayed according to low (LD) or high (HD) cell-surface density, and to coexpression of either Leu 11a or CD3, in a control subject (A,C) and an AIDS patient (B,D).

cantly increased within the CD8 compartment. However, cells coexpressing HD CD8 and either CD11 or CD16 were comparable to controls (Table 3).

Two additional findings were of relevance when analyzing the subsets among the $CD8^+$ pool. A significant increase in CTLs ($CD8^+$, $CD11^-$) was present in HIV-positive patients while their suppressor cells ($CD8^+$, $CD11^+$) remained unaltered. Secondly, among the high-risk seronegative group it was apparent that their LD CD8 lymphocytes were also diminished, reaching significance in the case of LD $CD8^+$, $CD16^+$ lymphocytes. In contrast, their suppressor cells were significantly elevated while the CTL's pool remained comparable to those in the control group.

DISCUSSION

To our knowledge this is the first report of a profound and significant depletion of $CD3^-$, $CD16^+$, Leu 19^+ lymphoid cells, associated with the natural history of HIV infection. The diminished NK pool seems to parallel the progressive diminution of the CD4 lymphocyte compartment which represents the hallmark of the immunoclinical spectrum of HIV infection. Previous studies have failed to recognize such a decrease, probably because of the systematic use of HNK-1 (Leu 7) monoclonal antibody as the appropriate reagent to quantify the NK pool. Within this context, we also report herein that only with the simultaneous use of either Leu 11a or Leu 11c and Leu 19 mAbs can the NK compartment be fully evaluated. In fact,

reagents recognizing the CD16 epitope (Leu 11 series) may behave more as "pan NK marker" than reagents recognizing the NKH-1 antigen (Leu 19). An analysis of the NK pool showed that two-thirds of the compartment contained $CD3^-$, $CD8^-$, $CD16^+$ lymphoid cells while 37% coexpressed CD8. The depletion observed, not only in patients with full-blown AIDS but also in ACs, seems to involve both subsets. We suggest that the diminished $CD3^-$, $CD8^-$, $CD16^+$, Leu 19^+ subset may be related in part to the lymphopenia intimately associated to HIV, since the difference found between HIV-positive patients and controls or high-risk seronegative subjects was in absolute numbers and not in percentages. Nevertheless, the depletion observed within $CD3^-$, $CD8^+$, $CD16^+$, Leu 19^+ cells appears to be more severe as both percentages and absolute numbers were affected. Remarkably, these results were observed despite increased numbers of $CD8^+$ cells in HIV-positive patients. These data led us to further dissect NK cells bearing CD8. As the vast majority of them are LD $CD8^+$, $CD16^+$, Leu 19^+ lymphocytes, the observed depletion is located mainly in this subset. Furthermore, the depletion of LD $CD8^+$, $CD11^+$ is also probably related to the diminished NK compartment, since NK cells very frequently express the latter marker.[9] In addition, LD $CD8^+$, $CD16^+$, Leu 19^+ NK cells were more severely depleted among AIDS patients when compared to the AC group, although these differences were not statistically significant. Even though multimarker analysis was not performed, we have indirect evidence arising from two marker analyses, suggesting that this NK LD $CD8^+$ subset appears to coexpress simultaneously both 4B4 and 2H4 antigens (data not shown). It was of interest to find that high-risk seronegative subjects who tested consistently negative for both HIV antigens and antibodies also showed a significant decrease in LD $CD8^+$, $CD16^+$, Leu 19^+ lymphoid cells. This latter finding appears unrelated to other concomitant viral infection frequently diagnosed in high-risk homosexuals, since at least in seropositive cytomegalic viral infection (CMV) carriers, as Gratama *et al.*[17] recently showed, the $CD3^-$, $CD16^+$ lymphocyte levels were nonsignificantly elevated when compared with seronegative healthy carriers. In addition, the CMV seropositive group exhibited a normal lytic activity against K562 cells and normal levels of LD$CD8^+$ lymphocytes. Whether the depletion of $CD3^-$, LD $CD8^+$, $CD16^+$ cells may represent an early event during HIV infection is currently under investigation in our laboratory.

Taken together, these data indicate that HIV-infected patients may not only have selective depletions of regulatory lymphoid cells (CD4, CD16/Leu 19 subsets) but also have a significant effector cells imbalance demonstrable by an increase in the CTL subset and a depletion of the NK pool. The available information on the functional status of CTLs in HIV infection is conflicting. While Ruscetti *et al.*[18] have concluded that IL-2 activated T-cells were unable to lyse HTLV-I or HIV infected cells, Walker *et al.*[19] and Plata *et al.*[20] have found intact CTL responses to appropriate HIV-infected targets. Although CD8 antigen has been shown to be important in CTL's function through its interaction with MHC class I antigens,[21] its role in unrestricted NK cells remains unknown.

In contrast, numerous studies have suggested that AIDS patients have a defective NK activity against appropriate targets.[22–24] Bonavida *et al.*[25] reported the inability of NK cells from AIDS patients to be triggered and release soluble factors, while Ruscetti *et al.*[18] showed that IL-2 activated Leu 11^+, Leu 19^+ large granular lymphocytes from healthy donors had substantial cytotoxic activity against both HTLV-I and HIV-infected targets. Furthermore, they also showed that this cell population was also susceptible to be infected by HIV. Whether the lack of IL-2 synthesis due to selective depletion of both CD4 and CD16 lymphocytes or the consequences of viral-cell interaction (i.e., viral products, direct cytopathic effect) or both are related to the underlying mechanism of NK cells depletion remains to be clarified. Whether the progressive depletion of the NK cell compartment may severely compromise the outcome of individuals infected with HIV is still an open question.

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