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Letters to the Editor

CD4/CXCR4-mediated cell death in AIDS

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Dear Editor,

HIV-1 infection leads to the progressive depletion of $CD4^+$ T cells resulting in immunodeficiency, and apoptosis has been shown to be a major cause of destruction of both infected and uninfected lymphocytes (reviewed by Gougeon[1]). The mechanisms that contribute to HIV-associated lymphocyte apoptosis include direct killing of infected target cells following viral gene expression and cytopathicity, death of bystander cells by proapoptotic viral proteins released by infected cells, killing of HIV-specific effectors following their sequestration into infected lymphoid tissues and contact with infected dendritic cells, and altered expression of cellular apoptosis regulatory molecules on lymphocytes and antigen-presenting cells (APC) as a consequence of HIV-driven immune activation.[1]

T cells from HIV-1-infected individuals show enhanced spontaneous and activation-induced apoptosis.[1] They also exhibit an increased sensitivity to CD95-mediated apoptosis, which is paralleled by enhanced expression of CD95 and CD95 ligand (CD95L).[2–4] Aberrant immune activation, due to persistent replication of HIV, can lead to dysregulation of death receptor pathways and there is considerable evidence that HIV-1 and HIV-1-encoded proteins are involved in this process. For example, crosslinking of CD4 by HIV-1 envelope gp120 induces apoptosis of HIV-infected cells[5] and it can trigger apoptosis of bystander $CD4^+$ T cells.[6] The discovery of the coreceptor CXCR4 prompted the investigation of its role in HIV-1-induced apoptosis. Infection of lymphocytes and lymphoid tissues with CXCR4-dependent HIV-1 leads to depletion of $CD4^+$ T cells,[7] and apoptosis induced by gp120 in syncytia can be blocked by the CXCR4 antagonist AMD3100 and the agonist SDF1.[8] AMD3100 was also shown to inhibit CD4 and CD8 T cell apoptosis in peripheral blood mononuclear cells from HIV-infected subjects. In order to investigate the role of CD4/CXCR4-mediated apoptosis as a potential indirect mechanism of CD4 T-cell depletion in AIDS, we analyzed the apoptotic response of T cells from HIV-infected subjects following coligation of both CD4 and CXCR4 receptors.

To mimick gp120 binding and to activate both receptors individually, we used mAbs against CD4 (HP2/6) and CXCR4 (12G5). These mAbs interfere with binding of gp120, as determined by surface staining, or inhibit HIV-1 infection, respectively.[9] Although ligation of CD4 and CXCR4 was shown to mediate a rapid cell death in T-cell lines and transfected B-cell lines, apoptosis induction in peripheral lymphocytes from HIV-1-infected individuals showed a later onset (at 6–8 h) with a maximal effect observed after 18 h of stimulation (data not shown). Peripheral blood lymphocytes (PBLs) from a representative cohort of 157 HIV-1-infected individuals were found more sensitive to CD4/CXCR4-induced apoptosis in comparison to lymphocytes from 78 healthy individuals ($P<0.001$). Each receptor could individually mediate apoptosis, and engagement of both receptors showed a partially additive effect, although variability was seen among patients (not shown). The variability of the results from one donor to the other reflects the entire complexity of the mechanisms that control life and death in T lymphocytes (recently reviewed by Arnold *et al.*[10]) The HIV-1-infected individuals were divided into two groups: patients that had not received any treatment (antiretrovirals (ARV)-naive patients, $n=52$) and ARV-treated patients ($n=105$). The latter received various combinations of ARV, including inhibitors of HIV reverse transcriptase and inhibitors of HIV protease. Specific CD4/CXCR4-triggered cell death was significantly higher in lymphocytes from both groups of HIV-1^+ patients compared to control individuals ($P<0.001$), and treated patients showed a decreased susceptibility to CD4/CXCR4-induced apoptosis compared to ARV naive patients, although the difference was not statistically significant (Figure 1a). No correlation was observed between CD4/CXCR4-triggered cell death and CD4 counts in both groups. Given the multiparametric origin of CD4 T-cell depletion, thought to be the consequence of altered homeostatic mechanisms, including HIV-driven exacerbated cell death, hardly compensated by the production of recent thymic emigrants and peripheral homeostatic proliferation, the correlation between apoptosis levels and CD4 T-cell counts is not always found.

To evaluate the impact of HIV replication on the susceptibility of patients' lymphocytes to apoptosis, patients were stratified according to their level of plasmatic viral load. Greater sensitivity to CD4/CXCR4-mediated apoptosis was significantly correlated with high viral load levels (>5000 copies/ml) in therapy naive patients. In contrast, no correlation with the viral load could be found in ARV-treated patients, neither in the total group of patients nor in the individual treatment subgroups (Figure 1a). These observations suggest that coligation of CD4 and CXCR4 by CXCR4-tropic strains may contribute to the destruction of bystander uninfected CD4 T cells during chronic HIV-1 infection, provided these cells are primed for CXCR4-induced apoptosis. *In vivo* circulating immune complexes and replication-incompetent viruses that contain gp120 could induce cell death in a similar manner.[11] Moreover, during HIV-1 infection, there is an increase of circulating anti-CD4 antibodies and more importantly of soluble gp120, anti-gp120 antibodies, and a large number of defective virions, all of which can lead to a

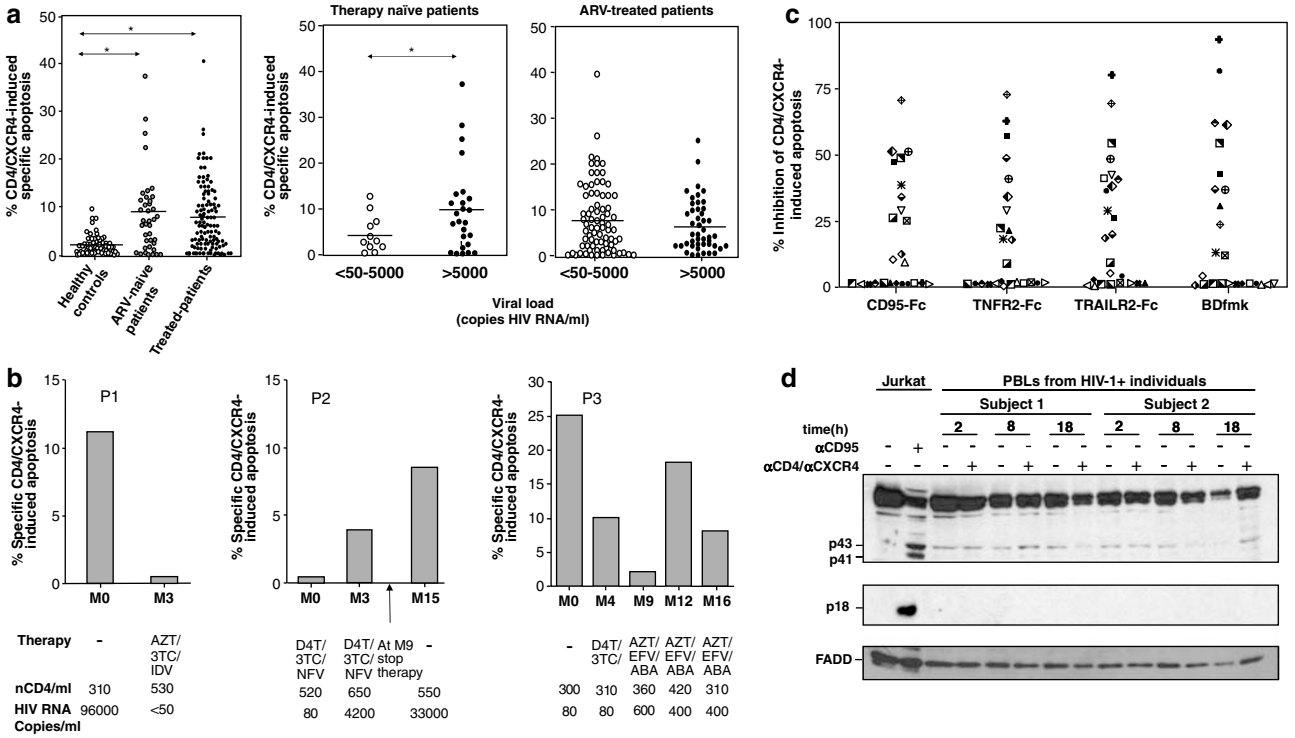

Figure 1 CD4/CXCR4-induced apoptosis in HIV-1 infection. (**a**) PBMC from HIV-1-infected patients or age-matched healthy donors were isolated within 4 h after venipuncture and depleted from monocytes/macrophages by adherence for 2 h at 37°C. PBLs from HIV-1-infected patients, either naive of therapy ($n=52$) or who received ARV treatment ($n=102$) were compared to that of control subjects ($n=78$) for apoptosis levels following overnight incubation in the presence of 10 μg/ml mAbs against CD4 and CXCR4, crosslinked with 50 μg/ml of sheep anti-mouse Ig. Specific apoptosis, measured by FACS following FSC/SSC criteria, was calculated according to the formula 100 $\times$ (% experimental cell death in the presence of mAbs)/[100–% spontaneous cell death in the absence of mAbs). Specific CD4/CXCR4-induced apoptosis is significantly higher in both groups of HIV-infected patients *versus* control subjects (*$P<0.001$, Mann–Whitney test). In therapy naive patients, higher levels of specific CD4/CXCR4-mediated apoptosis were observed in patients with high HIV RNA viral load (>5000 copies/ml) as compared to patients with low viral load (<5000 copies/ml) ($P<0.05$), whereas in the ARV-treated patients, no significant difference could be seen, neither in the total group of treated patients nor in the individual treatment subgroups (NRTI *versus* NRTI/NNRTI *versus* NRTI/PI) (not shown). (**b**) Longitudinal follow-up for CD4/CXCR4 apoptotic response in three representative patients. The parallel evolution of sensitivity to CD4/CXCR4-induced apoptosis, antiretroviral treatment, plasmatic HIV RNA viral load and CD4 T-cell numbers are presented from the first time point of the study (M0) to the last one, varying from 3 to 16 months. (**c**) The role of caspases and the CD95-, TNF- and TRAIL-systems in CD4/CXCR4-mediated apoptosis was tested in PBLs from 25 HIV$^+$ individuals that were preincubated for 40 min in the presence of CD95-Fc (50 μg/ml), TNF-R2-Fc (30 μg/ml), TRAIL-R2-Fc (30 μg/ml) or BD-fmk (50 μg/ml) and the αCD4/αCXCR4 mAbs, being transferred to sheep anti-mouse Ig coated plates. Each symbol represents an individual patient. (**d**) Caspase 8 activation during CD4/CXCR4-mediated apoptosis was analyzed by Western blot in cellular lysates from 10^6 PBLs from HIV-1$^+$ individuals treated as indicated in the figure. As a positive control, Jurkat cells were either left untreated or treated for 5 h with 1 μg/ml αCD95 mAbs. The positions of the active fragments p43, p41 and p18 of caspase 8 are indicated

chronic and significant degree of CD4-mediated activation and cell death in uninfected CD4$^+$ cells[12,13]

Highly active ARV therapy (HAART), combining HIV reverse transcriptase and protease inhibitors, leads to a decrease in plasmatic and lymphoid tissue viral load, that may reach undetectable levels, and a concomitant increase in CD4 T-cell numbers. Several mechanisms are involved in the quantitative restoration of CD4$^+$ T cells, including homeostatic peripheral proliferation, central (thymic) production of new T cells and increased lymphocyte survival (reviewed by Gougeon[1]). To analyze the impact of HAART on CD4/CXCR4-mediated apoptosis and to understand the links between apoptosis and virological or immunological parameters, we performed a follow-up of 26 HIV-1-infected individuals, including patients who stopped their therapy for clinical or virological reasons. Three representative cases are shown in Figure 1b. Patient 1 presented with a high viral load (96 000 HIV RNA cp/ml), low CD4 counts and significant levels of specific CD4/CXCR4-induced apoptosis. Initiation of HAART suppressed the viral load to undetectable levels by M3, restored a high number of CD4 T cells (ΔTCD4 >200 in 3 months of HAART) while the specific CD4/CXCR4-triggered apoptosis was suppressed. Patient 2 was under HAART when he presented at the first time point (M0), with a very low viral load (80 copies/ml) associated to significant number of CD4 T cells (520 cells/μl) and no specific CD4/CXCR4-induced apoptosis. By M3, a slight increase in apoptosis paralleled a rise in viral load (80–4200 copies/ml), and further interruption of therapy at M9 led to an increase in both viral load and apoptosis by M15, whereas the CD4 count decreased. These two cases confirm the correlation between the CD4/CXCR4 apoptotic response and HIV replication, as well as the impact of therapy on the concomitant suppression of viremia and apoptosis. Finally, in therapy naive Patient 3, the low blood CD4 count (300/μl) was associated with high levels of CD4/CXCR4-induced apoptosis. Initiation of bitherapy by M4 was followed by a slight increase in CD4 T cells paralleled with a decrease in apoptosis by M9, and introduction of HAART

(AZT, EFV, ABA) (M12) stabilized the viral load to 400 HIV RNA copies/ml. However, no CD4 T-cell restoration occurred (310 at M16 *versus* 360 at the initiation of HAART). Of note, the lack of immunological response to HAART was paralleled with the persistence of CD4/CXCR4-triggered apoptotic response. When the total subset of 26 HIV-1-infected individuals was analyzed, subdivided into two groups (group A: untreated individuals or individuals without a change in therapy between the different measurements, $n=12$; group B: individuals with newly initiated therapy or a change in drug combinations whose treatment was considered successful according to clinical, virological and immunological criteria; $n=14$), we found that successful treatment significantly reduced CD4/CXCR4-induced apoptosis ($P=0.02$, Mann–Whitney test).

To investigate the role of caspases and the CD95, TNF and TRAIL receptor/ligand systems, we measured CD4/CXCR4-triggered apoptosis in peripheral blood lymphocytes from 25 patients, in the presence or absence of CD95-Fc, TNF-R2-Fc or TRAIL-R2-Fc fusion proteins that inhibit the respective receptor/ligand interactions, or the broad spectrum caspase inhibitor BD-fmk. In 12 out of 25 patients tested, percentages of apoptosis inhibition ranged from 0 to 20% (inhibition range in healthy donors) and were therefore considered insignificant given the low levels of specific cell death (Figure 1c). This suggests that in these patients (named R for resistant), CD4/CXCR4-mediated apoptosis is independent of the known death systems, as suggested by our previous work.[9] In the remaining 13 patients, significant levels of apoptosis inhibition were observed in the presence of one or more inhibitors (Figure 1c). The blocking pattern showed great variability within this group (named S for sensitive). CD4/CXCR4-mediated apoptosis could be partially inhibited by all four inhibitors tested in five patients, by three inhibitors in three patients, two inhibitors in three patients and one inhibitor in two patients. A statistically significant decrease in the mean levels of CD4/CXCR4-mediated apoptosis was observed in the presence of TRAIL-R2-Fc ($P=0.02$) and BD-fmk ($P=0.03$), thus suggesting a role for the TRAIL system and caspases in CD4/CXCR4-mediated cell death in the S group. As a corollary, a recent study has shown that treatment of primary T cells with gp120 results in the upregulation of TRAIL death receptor expression and acquired sensitivity to TRAIL-mediated cell death, which requires the chemokine coreceptor CXCR4.[14] Comparison of R *versus* S patients did not show any correlation between susceptibility of CD4/CXCR4-triggered cell death to one or more inhibitors and CD4 counts, viral load or antiretroviral treatment. To verify the apparent caspase-independent phenomenon of CD4/CXCR4-triggered apoptotic response, caspase 8 processing and poly(ADP-ribose) polymerase (PARP) cleavage were tested by immunobloting in PBL lysates from five representative R patients. Data from two of them are shown in Figure 1d. No caspase 8 processing into its p43, p41 and p18 fragments (Figure 1d) and no PARP cleavage (data not shown) was observed following treatment of PBL from R patients with αCD4/αCXCR4 mAbs, while both events occurred in αCD95-treated Jurkat cells used as positive control. Furthermore, in the same subjects, no formation of subdiploid nuclei was detected, even 96 h after induction of cell death (data not shown), thus suggesting lack of polynucleosomal DNA fragmentation. CD4/αCXCR4-triggered cell death in R patients might be autophagy, as suggested by a recent study demonstrating that HIV-infected cells that express gp120 induce autophagy in uninfected CD4 T cells via CXCR4.[15]

Taken together, the results of this study highlight the importance of CD4- and CXCR4-mediated apoptosis in $CD4^+$ T-cell depletion in AIDS, and show that viral replication acts as the driving force behind sensitization for HIV receptor/coreceptor death signals. Moreover, we show that multiple mechanisms of CD4/CXCR4-induced apoptosis are operative in HIV-1 infection. The results of this study could have important implications for the development of therapeutic strategies. The use of anti-chemokine receptor mAbs has been suggested as an approach to prevent HIV-1 infection, but our findings suggest that they could have detrimental effects. Therefore, natural chemokine receptor ligand derivatives or small peptide inhibitors might be considered for therapy.

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