Exposure of resting peripheral blood T cells to HIV-1 particles generates CD25\(^+\) killer cells in a small subset, leading to induction of apoptosis in bystander cells

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Abstract

Apoptosis is a major mechanism whereby HIV-1 depletes uninfected CD4\(^+\) and CD8\(^+\) T cells. We previously showed that resting peripheral blood T cells derived from healthy donors were killed by an apoptotic mechanism after adsorption to gp120-containing, protease-defective HIV-1 (L-2) particles, more effectively than parental wild-type LAI adsorption or rgp120-mediated CD4 cross-linking, followed by mitogenic stimulation. Here, we present evidence that the L-2 particle-based apoptosis was induced both in CD4\(^+\) and CD8\(^+\) cells by generation of effector cells which were mainly derived from a resting memory CD4\(^+\)CD38\(^-\) subset. This subset enhanced the CD25 expression on the surface and secreted IFN-\(\gamma\) in the culture supernatant after L-2 particle exposure. Significant elevation of Fas ligand mRNA was found in the subset by L-2 particle exposure, while expression of Fas antigen on uninfected T cells was induced by exposure to IFN-\(\gamma\). These results indicate that L-2 particles can shift the CD4\(^+\)CD38\(^-\) subpopulation from a resting to an activated state, and this activation leads to killing of bystander CD4\(^+\) and CD8\(^+\) T cells by a Fas-mediated mechanism. In fact, purified CD4\(^+\)CD38\(^-\) cells exposed to L-2 particles were converted into effector cells that were able to kill autologous as well as allogenic target T cells pretreated with IFN-\(\gamma\). Further, we found that the observation of apoptosis due to L-2 particles was a more general phenomenon, that also occurred with Thai primary HIV-1 isolates. These results suggest that such specific types of HIV-1 particles may play a major role in the induction of apoptosis for both bystander CD4\(^+\) and CD8\(^+\) T cells, through inappropriate activation of CD4\(^+\)CD38\(^-\) cells.

Introduction

The depletion of CD4\(^+\) T cells is a major mechanism for pathogenesis in AIDS (1). Recent findings have revealed that this depletion can occur in part by apoptosis which is indirectly attributable to HIV-1 replication (2,3). There are several features ascribed to the process of apoptosis in HIV-1-infected individuals. (i) Apoptosis can be induced in patient's peripheral blood mononuclear cells (PBMC) as an activation-dependent event after mitogenic stimulation (4,5). (ii) HIV-1 can induce apoptosis in HIV-1 genome-negative, uninfected cells, as a bystander effect (6). (iii) Apoptosis in uninfected cells is mediated by Fas–Fas ligand (FasL) interactions and/or an imbalance of Th1/Th2 cytokines (7,8). Finally, apoptosis has been observed not only in CD4\(^+\) T cells but also in CD8\(^+\) T cells (6,9–11).

Recently, HIV-1-induced apoptosis was found to be highly correlated with fusion activity of the virus (12). Therefore, the propensity of HIV-1 for apoptosis can be partly estimated by measuring its syncytia-inducing (SI) activity. In fact, SI viruses...
appear in patients who progress more rapidly to AIDS (13,14). In addition, although continuous virus production is observed even at early infection times as well as during the course of disease (15–17), several studies have revealed that the vast majority of HIV-1 particles in the peripheral blood of infected individuals is non-infectious (18). Therefore, we decided to examine the possible role of SI-type, but defective HIV-1 particles on apoptosis induction in healthy donor PBMC through an activation-dependent mechanism.

Initially, a subclone (named L-2), which produces non-infectious HIV-1 particles, was established by limiting dilution of survivor cells obtained after MT-4 cells had been infected with the HIV-1 LAI strain (19). This subclone was found to carry a provirus with a 1 base insertion in the pol protease, leading to the appearance of a stop codon in the protease gene (20). Therefore, the doughnut-shaped, immature HIV-1 particles in the L-2 cell culture fluid are RT-negative and non-infectious. Surprisingly, these L-2 particles exhibit a higher fusion activity for CD4⁺ T cells, as shown by their syncytia formation in virus-to-cell fusion, than the parental wild-type HIV-1 LAI particles (21). Based on this higher fusion activity, the effect of exposure of PBMC to L-2 particles was examined in detail. After adsorption with the L-2 but not LAI particles, apoptosis was observed for PBMC-derived T cells (PBMC-T) in an activation-dependent manner, using mitogens phytohemagglutinin (PHA) or concanavalin A (Con A), as well as ionomycin (22). In this communication, we provide evidence that L-2 particle-mediated apoptosis occurs for both CD4⁺ and CD8⁺ T cells, and that the trigger for apoptosis is related to acquisition of non-specific killer activity by a subpopulation of CD4⁺ T cells (CD4⁺CD25⁺CD38⁻) after adsorption with the L-2 particles. Interestingly, Thai primary isolates of HIV-1 also exhibited a similar killer activity.

Methods

Viruses

Culture fluids from L-2 cells, a subclone derived by limiting dilution of survivor cells obtained after infection with HIV-1 LAI strain (19), was used as the source of defective particles. The L-2 cells contain pol-defective proviral DNA due to a 1 base insertion of T in the protease gene and therefore L-2 particles released into the conditioned media contain uncleaved p55 Gag, and are negative for RT, as well as integrase (20). Culture fluid of MOLT-4 cells persistently infected with LAI was used as the source of wild-type particles (positive control). Two primary HIV-1 isolates were also used: Hot-017 from an AIDS patient and TNIH-047 from an asymptomatic carrier in Thailand. In contrast to LAI (clade B), these Thai isolates were of clade E as determined by ELISA of plasma antibody using specific synthetic peptides (23). The primary isolates were propagated in healthy donor-derived PBMC and HIV-1 particles were collected from culture fluids by centrifugation at 100,000 g for 30 min, as previously described (24).

Preparation of PBMC-T

PBMC were obtained by centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradients of heparinized venous peripheral blood from seven HIV-1-negative healthy donors. PBMC-T were obtained from PBMC by use of a nylon-wool column (Wako, Osaka, Japan). The following levels of surface markers in the purified PBMC-T fraction were noted by flow cytometry (see below): >95% CD3⁺ and CD4⁺/CD8⁺ ratios of 1.0–1.5.

Conditions for mitogenic stimulation

PBMC-T (2x10⁶/ml) were incubated in RPMI 1640 medium supplemented with 10% FBS (complete medium), with or without a final concentration of 6 ng/ml of L-2 or LAI particles at 37°C for 18 h. As a non-particle control, PBMC-T were incubated with 10 µg/ml of rgp120 protein (Intracel, Cambridge, MA) at 37°C for 1 h, followed by additional incubation with the IgG fraction (1 µg/ml) of an HIV-1-seropositive patient’s serum at 37°C for 17 h in order to permit cross-linking of CD4, as described (25). After incubation, the cells were stimulated with optimal concentrations of PHA (10 µg/ml), Con A (10 µg/ml) or ionomycin (1 µg/ml) in complete medium containing 25 U/ml of rIL-2 for 0, 3 or 4 days, as described (22). As a negative control for mitogenic stimulation, the same lot of PBMC-T was cultured in complete medium without any stimulant or rIL-2 for 3 days.

Subfractionation of PBMC-T

Resting CD4⁺ cells were negatively selected by depletion of CD8⁺, CD11b⁺, CD14⁺ and CD25⁺ cells, as described (26). Briefly, PBMC-T were incubated with a cocktail of mAb recognizing CD8 (OKT8; Ortho Diagnostic Systems, Raritan, NJ), CD11b (Leu-15; Becton Dickinson Immunocytometry Systems, San Jose, CA), CD14 (Leu-M3; Becton Dickinson Immunocytometry Systems) and CD25 (Anti-IL-2R; Becton Dickinson Immunocytometry Systems) at 4°C for 30 min. After washing, the cells were incubated with magnetic bead-conjugated goat anti-mouse IgG antibody (Dynabeads M-450; Dynal, Oslo, Norway) at 4°C for 30 min. Finally, those cells which did not react with these antibodies were harvested by Dynal MPC-E (Dynal). For selection of the CD4⁺CD38⁻ cell subpopulation, PBMC-T were incubated with both the above mAb cocktail and mAb to CD38 (Leu-17; Becton Dickinson Immunocytometry Systems). The relative purities for each fractionated cell population were determined by FACS analysis with specific mAb, as described below.

Measurement of apoptosis

Apoptosis was measured by three methods. (i) Percent cell mortality was measured by the Trypan blue exclusion assay. Briefly, cultured cells were diluted to 1:5 or 1:10 in 0.25% Trypan blue in PBS, then viable and dead cell numbers were counted. The percent cell mortality was calculated as follows: 100×[dead cell numbers/(viable cell numbers + dead cell numbers)]. (ii) Quantitative analysis on apoptotic cells was performed by flow cytometry of cells stained with propidium iodide, as described (27,28). Briefly, cells collected by centrifugation were gently resuspended in a small amount of PBS, then fixed with cold 70% ethanol. After standing on ice for 30 min, cells were centrifuged and the resultant cell pellet was resuspended in RNase A in PBS (500 µg/ml), then incubated at 37°C for 20 min. After centrifugation, the cell pellet was again resuspended in PBS containing 50 µg/ml of propidium
iodide and incubated on ice for 10 min. After centrifugation, the stained cells were resuspended in PBS and then analyzed for the percentage of hypo-diploid DNA content (percent hypo-diploid DNA) using the Becton Dickinson FACScan system (28). (iii) Dead cells were detected by staining with propidium iodide following immunostaining of CD4 or CD8, as described in the assay below.

**Cytotoxicity assay**

Cytotoxic activities of fractionated effector cells obtained after adsorption with HIV-1 particles were measured by cocultivation with autologous or allogenic PBMC-T as target cells at various ratios. Briefly, CD4⁺ CD38⁻ cells, with or without adsorption by HIV-1 particles for 18 h, were co-cultured with autologous or allogenic PBMC-T which had been treated or untreated with human IFN-γ (2000 IU/ml; Hayashibara Biochemical Laboratories, Okayama, Japan) for 18 h at various effector:target (E:T) ratios (1:10 to 1:160) in RPMI 1640 containing rIL-2 with PHA or ionomycin, as described above. After a 4 day co-culture, the percent cell mortality and percent hypo-diploid DNA were measured as described (22). To confirm that the assay system was functioning, target PBMC-T were treated with a Fas antagonist [ZB-4, a mAb (IgG) to Fas antigen; Medical and Biological Laboratories, Nagoya, Japan] at 2 µg/ml according to Estaquier et al. (29), before co-cultivation with effector cells as described (22), then the percent cell mortality and hypo-diploid DNA were similarly measured. Additionally, the effector and target cells were separately co-cultured in 24-well microplates with a cell culture insert (0.4 µm pore size; Falcon; Becton Dickinson, Franklin Lakes, NJ), so that cell-to-cell contact could not occur for the induction of effector killer activity.

**Measurement of cell surface antigens**

Expression of CD3, CD4, CD8, CD25, CD38 and Fas antigens on the cell surface was measured by flow cytometry. Briefly, 1×10⁶ cells were incubated with either mAb to CD3 (OKT3; Ortho Diagnostic Systems), CD4 (OKT4; Ortho Diagnostic Systems/Leu-3a; Becton Dickinson Immunocytometry Systems), CD8 (OKT8), CD25 (anti-IL-2R), CD38 (Leu-17) and Fas (CH-11; Medical and Biological Laboratories, Nagoya, Japan) at 2 µg/ml according to Estaquier et al. (29), before co-cultivation with effector cells as described (22), then the percent cell mortality and hypo-diploid DNA were similarly measured. Additionally, the effector and target cells were separately co-cultured in 24-well microplates with a cell culture insert (0.4 µm pore size; Falcon; Becton Dickinson, Franklin Lakes, NJ), so that cell-to-cell contact could not occur for the induction of effector killer activity.

**ELISA for cytokine levels**

The amount of IFN-γ in the culture supernatant was measured with an ELISA kit (Predica IFN-γ ELISA kit; Genzyme, Cambridge, MA), according to the protocol provided by the manufacturer.

**HIV-1 p24 antigen-capture ELISA**

The amount of virus inoculum was measured using an ELISA for Gag p24 antigen according to the protocol provided by the manufacturer (Intracel).

**Detection of FasL transcripts**

Expression levels of Fasl mRNA in the CD4⁺CD38⁻ cell population before and after adsorption with L-2 particles were semi-quantified by RT-PCR. Briefly, cellular RNA extracted from CD4⁺CD38⁻ cell populations which had been mock adsorbed or adsorbed with L-2 particles for 18 h as described above was reverse transcribed using oligo(dT)₁₆ as a primer. The cDNA was subjected to PCR analysis using oligonucleotide primers specific to FasL mRNA (TTTCTAGTCCTTACGCTACA; nucleotides 387–408, and AGAGGTGATTTAAAAGGTTGCC; 1268–1247) (31) and as a control GAPDH mRNA (GGTTCTGCTAGGATGACATGCG; 325–346, and GGTGGCGAGGGCATTTGCTAG; 521–500) (32). The PCR products of Fasl and GAPDH were separated by agarose gel electrophoresis and blotted onto a Hybond-N Plus membrane (Amerham, Buckinghamshire, UK), followed by hybridization with individual ³²P-labeled internal oligonucleotide probes, as described (15). The hybridized bands were visualized using an image analyzer (Bas-1000; Fuji, Tokyo, Japan). The relative amount of Fasl mRNA was estimated by comparison with the level of GAPDH mRNA.

**Assay for RT activity**

RT activity in the culture supernatant of infected PBMC-T was measured using poly(rA)–oligo–(dT) and [³²P]dTMP as described (33).

**Results**

**Apoptosis induction in both CD4⁺ and CD8⁺ cell populations after adsorption with L-2 particles**

We previously showed that PBMC-T derived from healthy donor PBMC were killed by an apoptotic mechanism after adsorption of L-2 particles and mitogenic stimulation (22). Specifically, apoptosis was observed in 40–50% of the PBMC-T at 3 days after PHA, Con A or ionomycin stimulation, following adsorption. We initially thought that L-2 particle-mediated apoptosis occurred only in CD4⁺ cells found among the PBMC-T, because PBMC-T contained an almost equal amount of CD4⁺ and CD8⁺ cells, and L-2 particles containing gp120 should adsorb to only CD4⁺ cells. However, after further analysis, we found that apoptosis occurred in both CD4⁺ and CD8⁺ cells. PBMC-T were initially mock adsorbed or adsorbed with L-2 particles for 18 h. After stimulation with PHA, Con A or
cells were more sensitive than CD8
distributed both into CD4
particle-adsorbed PBMC-T (relative to mock treatment) was
propidium iodide-stained dead cells found among the L-2
with propidium iodide. The results show that an increase in
of the cell surface with CD4 or CD8 markers and treatment
were determined by flow cytometry, subsequent to staining
ionomycin for 3 days, the dead cells in the PBMC-T population
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subpopulations of PBMC-T after exposure to L-2 particles, followed by
mitogenic stimulation. (A) Healthy donor-derived PBMC-T which
had been adsorbed with L-2 particles followed by PHA stimulation
for 3 days were stained with anti-CD4 or anti-CD8 mAb, then with
propidium iodide, as described in Methods. Numbers in each flow
cytometric profile show the quadrant status of the corresponding dot-
blot histogram. (B) The surface markers on viable cells which survived
apoptosis by exposure to L-2 particles were also stained with anti-
CD4 or anti-CD8 mAb. The number in each histogram shows the
percentage of CD4+ or CD8+ cells among the surviving PBMC-T.
Dashed lines represent the profile of staining with the same amount
of control mouse IgG.

Fig. 1. Occurrence of apoptosis in both CD4+ and CD8+ cell
subpopulations of PBMC-T after exposure to L-2 particles, followed by
mitogenic stimulation. (A) Healthy donor-derived PBMC-T which
in L-2 particle-adsorbed PBMC-T (relative to mock treatment) was
distributed both into CD4+ and CD8+ cell fractions (Fig. 1A).
In addition, viable cells which survived adsorption with L-2
particles and mitogenic stimulation were also detected in
both CD4+ and CD8+ cell fractions (Fig. 1B). The ratio of
survivor CD4+ to CD8+ cells in the L-2 particle-adsorbed
PBMC-T after mitogenic stimulation for 3 days was significantly
higher (65.7–34.6%) than that in mock-adsorbed PBMC-T
(40.0–58.0%). The same results were observed in three
independent PBMC-T preparations. Thus, CD8+ cells seem
to be more sensitive to L-2 particle-mediated apoptosis than
CD4+ cells. However, one would have expected that CD4+ cells were more sensitive than CD8+ cells to binding to gp120
derived from L-2 particles. Therefore, our result, although
indirect, was the first suggestion that there could be a
requirement for a subpopulation of CD4+ cells with effector
function in apoptosis induction of both CD4+ and CD8+ cells.

Shift of the CD4+ CD38+ subpopulation from a resting to an
activated state after L-2 particle exposure
In contrast to apoptosis induced with L-2 particles, wild-type
LAI particles or rgp120-mediated CD4 cross-linking did not
induce notable apoptosis in PBMC-T under the same condi-
tions (22). This result also correlates with the observed
aggregation of host PBMC-T, seen only with L-2 particle
exposure (data not shown). It led us to then examine whether
a cellular activation event occurred with PBMC-T, after expo-
sure to L-2 particles.
PBMC-T which had been adsorbed with L-2 particles for
18 h were thus analyzed by flow cytometry for the expression
of CD25, an activation marker. As shown in Fig. 2(A), the
expression level of CD25 on the surface of PBMC-T was
significantly elevated in L-2 particle-exposed PBMC-T. The
percentages of CD25+ cells formed in the PBMC-T cell
population after adsorption was estimated to be 37.1 ± 2.7%
for seven different samples. Values of 19.4 ± 6.3, 17.2 ± 7.1
and 15.9 ± 2.7% were formed respectively after adsorption
with LAI particles, treatment with rgp120 or mock treatment.
After mitogenic stimulation, much higher levels (>85%) of
CD25 expression on the cell surface were observed in all
PBMC-T, irrespective of their adsorption with L-2 or LAI
particles or treatment with rgp120 (data not shown).

The distribution of three other surface markers on the
cell population showing an activation phenotype was next
determined by two-color flow cytometry of L-2 particle-
exposed PBMC-T. After adsorption with L-2 particles for 18
h, PBMC-T were incubated either with mAb to CD4, CD8 or
HIV-1 Env gp120. After subsequent incubation with a FITC-
conjugated secondary antibody, the cells were stained with PE-
conjugated anti-CD25 mAb, as described in Methods. The
flow cytometry pattern revealed that only those gp120+ cells in
the CD4+ but not CD8+ cell population among L-2 particle-
adsorbed PBMC-T (Fig. 2B) enhanced the expression of
CD25. CD25 expression for the CD4+ cell population was
higher than mock-adsorbed PBMC-T, regardless of whether
OKT4 or Leu-3a was used as the CD4 surface marker (31.6
and 22.7%, as compared to 13.4%). CD25 expression was
not significantly present on the CD8+ cell population (5–7%).

In Fig. 2(B), we also note that after L-2 particle adsorption,
~60% of PBMC-T showed a shift to the gp120+ fraction (see
the quadrant status of the dot-dot blot histogram using anti-HIV-
1 Env in Fig. 2B). About two-thirds of these gp120+ cells were
found to be positive for CD25 expression. A similar level
(55%) of CD4+ cells expressed CD25 when OKT4 was used
as anti-CD4. In contrast, slightly less (48%) of the CD4+ cells
expressed CD25 when Leu-3a was used as anti-CD4. This
result is consistent with a down-regulation of CD4 from the
cell surface by masking with adsorbed L-2 particle-derived
Env gp120, since Leu-3a recognizes the gp120-binding site
on CD4 molecules, which is different from the site recognized
by OKT4 (34). Thus, in summary most of the CD4+ but not
CD8+ cells were sensitive to adsorption by L-2 particles
and ~60% of the CD4+/gp120+ cell population shifted to
expression of CD25 after exposure to L-2 particles.

Next, the CD4+ resting T cell-enriched fraction was nega-
tively selected by depletion of CD8+, CD11b+, CD14+ and
CD25+ cells from the PBMC-T population, as described in
Fig. 2. Appearance of the cell activation marker CD25 antigen on PBMC-T after exposure to L-2 particles. (A) Flow cytometric analysis was performed on PBMC-T 18 h after mock adsorption or adsorption with L-2 or LAI particles, or treatment with rgp120 protein and anti-HIV-1 antibody, as described in Methods. PBMC-T were then stained with anti-CD25 mAb, followed by second antibody staining with FITC-conjugated anti-mouse IgG. Dashed lines represent the profiles of staining with control mouse IgG. (B) Cell surface antigens of the same PBMC-T used in (A) were analyzed by two-color FACS. Cell surface antigens were stained with anti-CD4 (1, OKT4; 2, Leu-3a), anti-CD8 or anti-Env gp120 mAb, followed by FITC-conjugated second antibody. Thereafter, the cells were further stained with PE-conjugated anti-CD25 mAb. Positive and negative cell percentages in each of the flow cytometric profiles are shown by the quadrant status of the dot-blot histogram.

Fig. 3. Cell surface phenotype of T cells activated by adsorption with L-2 particles. (A) CD4<sup>+</sup> cells enriched from PBMC-T as described in Methods were analyzed by flow cytometry with anti-CD4 mAb. As a control, the cell fraction was stained with normal mouse IgG (dashed line). (B) The same CD4<sup>+</sup> cells were mock treated. Cells were stained with anti-CD25 mAb, as in Fig. 2(A). (C) The same CD4<sup>+</sup> cells adsorbed with L-2 particles for 18 h were first stained with either anti-CD38, -45RO or -45RA mAb, followed by FITC-conjugated second antibody. Thereafter, the cells were further stained with PE-conjugated anti-CD25 mAb, essentially as described in Fig. 2(B). Numbers (%) in each flow cytometric profile show the quadrant status of the dot-blot histogram.

Fig. 4. Cell mortality of PBMC-T induced by exposure to L-2 particles followed by mitogenic stimulation before or after the depletion of CD25<sup>+</sup>-activated cells. The percent cell mortality was calculated for cells that have been adsorbed with L-2 particles, followed by PHA, Con A, ionomycin or no (None) stimulation for 3 days. A comparison was drawn between L-2 particle-exposed cells without (shaded bars) or with (open bars) depletion of CD25<sup>+</sup> cells before mitogenic stimulation. The percent cell mortality of mock-treated PBMC-T was used as control (solid bars). Results are calculated according to the formula provided in Methods.

Methods. This fraction was positive for CD4 expression by >95% (Fig. 3A), but since it was depleted had <3% of CD25 (data not shown). In contrast to this low value for CD25 expression (resting cells), FACS analyses by two-color staining with PE-conjugated anti-CD25 mAb revealed that a significant enhancement of CD25 expression was observed after 18 h L-2 particle adsorption, compared with mock adsorption (Fig. 3B). The percentage of CD25<sup>+</sup> cells estimated over five different PBMC-T samples derived from four healthy donors showed an enhancement to the level of 43.2 ± 5.3% cells after L-2 particle adsorption, while the control showed only 7.9 ± 2.2% cells after mock adsorption. The subset which had an enhanced CD25 expression after exposure to L-2 particles was further characterized by two-color FACS analyses (Fig. 3C). This subset of cells mainly resided in the CD38<sup>+</sup> population (40% enhanced over 7% spontaneous expression; Fig. 3C). This was in contrast to the slight level
of CD25 expression in the CD38+ population (8% enhanced over 2% spontaneous expression). In addition, expression levels of CD25 on the CD45RA+ subpopulation were similar regardless of whether they were adsorbed with L-2 particles or mock adsorbed (12 and 9% respectively). By contrast, about half of the CD45RO+ memory T cell population after exposure to L-2 particles showed an enhanced expression of CD25 (28% enhanced over 5% with spontaneous expression). Thus, one can now further define the subset which exhibits enhanced CD25 expression after adsorption with L-2 particles to one which is predominantly a resting memory CD4+ T cell population (CD4+CD25+CD38+CD45RO+CD45RA+). Almost 100% of this population was also positive for both CD11a and CD28 expression (data not shown).

CD38 is also an activation marker. However, the expression level of this antigen was similar on the CD4+ T cells before (24.5%) and after (25.1%) adsorption of L-2 particles (Fig. 3C), although the CD38 expression was strongly enhanced after mitogenic stimulations, as in CD25 (data not shown).

Failure to induce apoptosis in L-2 particle-adsorbed PBMC-T by depletion of CD25+ cells before mitogenic stimulation
Since the subpopulation of CD4+CD38 cells was activated to an enhanced level of CD25 expression after adsorption of L-2 particles, we reasoned that it was these cells that might play a significant role in L-2 particle-induced apoptosis of PBMC-T. To confirm this possibility, the effect of depletion of this subset on apoptosis induction was examined. As shown in Fig. 4, the depletion of CD25+ T cells from PBMC-T after exposure to L-2 particles lead to a significant reduction of apoptosis, similar to the level observed in mock adsorbed cells after mitogenic stimulation for 3 days. This result clearly indicates that enhanced CD25 expression on this small subset of CD4+CD38+ cells could be the trigger for apoptosis induction we have observed in PBMC-T after L-2 particle adsorption.

Fas system working on L-2 particle-induced apoptosis
Our previous report showed up-regulation of both Fas and FasL in PBMC-T after exposure to L-2 particles (22), indicating Fas system involvement in L-2 particle-mediated apoptosis. Several papers have described the importance of IFN-γ for up-regulation of Fas antigen itself and Fas-mediated apoptosis (35). Consistent with this, we found that the CD4+CD38+ subset prepared from PBMC-T secreted a significant amount of IFN-γ after adsorption with L-2 particles for 18 h, while the culture fluid from mock-adsorbed CD4+CD38+ cells had an undetectable level of IFN-γ (Fig. 5A). In addition, RT-PCR revealed ~5-fold elevation of FasL mRNA in the L-2 particle-exposed CD4+CD38+ cells relative to that found in the same subset which had been mock treated (Fig. 5B). Furthermore, the percentage of Fas antigen-positive cells in PBMC-T was increased by incubation with exogenous IFN-γ for 18 h, as expected (Fig. 5C). The percentage of Fas antigen-positive cells estimated over three different PBMC-T samples derived from three healthy donors was 34.0 ± 1.4% without treatment and 70.0 ± 5.3% with IFN-γ treatment. However, this up-regulation of Fas antigen expression was mainly due to low-density Fas-positive cells, but not high-density Fas-positive cells. Thus, enhanced FasL transcription in putative effector cells, as well as expression of Fas antigen on target cells induced by exposure to IFN-γ, appear to correlate with L-2 particle-mediated apoptosis of PBMC-T.

Generation of non-specific killer cells in the PBMC-T after adsorption with L-2 particles
As shown earlier, target cells in PBMC-T for apoptosis were derived from both CD4+ and CD8+ subsets (Fig. 1). This result, taken together with the observed activation of CD4+CD38-, but not CD4+CD38+ or CD8+ cell populations (Figs 2 and 3), failure of apoptosis induction by depletion of CD25+ cells (Fig. 4) and involvement of the Fas–FasL system (Fig. 5), supports a working hypothesis that L-2 particle-mediated stimulation of a CD4+CD38- cell subset in healthy donor-derived PBMC-T provides a non-specific killer activity, which could be the trigger essential for apoptosis induction of CD4+ and CD8+ cells in PBMC-T. Therefore, we decided to measure the cytotoxic activity of cell populations which had been mock or L-2 particle adsorbed and used as effector cells against autologous and allogenic resting PBMC-T as target cells.

Initially, we tried labeling of target cells with 51Cr or [3H]thymidine; however, the cytotoxic assay failed, presumably due to...
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The resting state of the primary PBMC-T. Thus, we decided to assay for cell killing activity by the CD4⁺CD38⁻ subset after co-culturing of autologous or allogenic PBMC-T which had been previously cultured with human IFN-γ for 18 h as target cells. The percent cell mortality and percent hypo-diploid DNA were measured in the co-cultures at various E:T ratios (1:20, 1:40, 1:80 or 1:160) in the presence of stimulants such as PHA or ionomycin (Fig. 6). Both assays revealed that effector cells induced significant levels (>40%) of apoptosis in autologous, as well as allogenic, target cells at 1:20 and 1:40 E:T ratios. There was no apparent enhanced cell mortality (<20%) in co-cultures with mock-treated effector cells (Fig. 6) or IFN-γ-untreated target cells (data not shown).

Using an E:T ratio of 1:40 as experimentally effective, both the percent cell mortality and percent hypo-diploid DNA showed a significant increase in L-2 particle-mediated apoptosis after treatment of autologous as well as allogenic target cells with IFN-γ (Fig. 7). Treatment of target cells with a Fas antagonist reduced cell mortality down to about the control levels seen with mock treatment. Further, similar cytotoxicity results were found using both PHA (Fig. 7A) and ionomycin stimulation (Fig. 7B), post L-2 or L-2/IFN-γ treatment.

In order to examine the possible contribution of soluble factor(s) released from effector cells for this non-specific cell killing, effector and target cells were separately cultured in 24-well microplates with a cell culture insert of 0.4 μm pore size. No killing of IFN-γ-treated target cells by L-2 particle-exposed effector cells was observed (data not shown). This result suggests that the cell-to-cell contact between effector and target cells is critical for apoptosis induction by L-2 particles.

Fig. 6. Non-specific killer activity of the L-2 particle-exposed CD4⁺CD38⁻ subset against autologous and allogenic PBMC-T. The effector CD4⁺CD38⁻ cell fraction was prepared as described in Methods, adsorbed with L-2 particles for 1 h at 37°C, followed by incubation for 17 h at 37°C. As targets, the autologous (C) and allogenic (□) PBMC-T were cultured at 37°C for 18 h in complete medium containing 2000 IU/ml of human IFN-γ. Effector and target cells were then co-cultured at E:T ratios of 1:20, 1:40, 1:80 or 1:160 in complete medium containing 10 U/ml of rIL-2 in the presence of PHA (10 μg/ml) (A) or ionomycin (1 μg/ml) (B) at 37°C for 4 days in a CO₂ incubator. For controls, mock-treated effector and autologous (○) or allogenic (□) target cells were similarly co-cultured. The percent cell mortality of co-cultured total PBMC-T was calculated according to the formula provided in Methods.

Fig. 7. Cell killing activity of L-2 particle-exposed CD4⁺CD38⁻ cells is enhanced by IFN-γ treatment, but suppressed by Fas antagonist treatment of target cells. The effector CD4⁺CD38⁻ cells were mock adsorbed or adsorbed with L-2 particles as in Fig. 6. The PBMC cultured in the absence (None) or presence of IFN-γ as in Fig. 6 were used as targets. Effector and autologous target cells were co-cultured at an E:T ratio of 1:40, as described in Fig. 6, in the presence (open bars) or absence (solid bars) of Fas antagonist (2 μg/ml) at 37°C for 4 days. Similarly, effector and allogenic target cells (shaded bars) were co-cultured at an E:T ratio of 1:40. The percent cell mortality and the percent hypo-diploid DNA of co-cultured total PBMC-T were calculated as in Methods. Error bars represent standard deviations from the results obtained from more than seven different co-cultures. (A) and (B) refer to stimulation of cells with PHA or ionomycin respectively, as previously described.
Similar induction of apoptosis in healthy donor-derived PBMC-T targets by Thai primary HIV-1 isolates

In order to evaluate how broadly specific the system was for defective particle-mediated apoptosis of bystander uninfected T cells, we next examined apoptosis induction by Thai primary HIV-1 isolates. The rate of apoptosis induction by these primary isolates was again estimated by percent cell mortality (Fig. 8A) and percent hypo-diploid DNA (Fig. 8B) using co-culture assays between primary isolate-exposed PBMC-T and IFN-γ-pretreated PBMC-T target cells. Two HIV-1 isolates examined induced significant apoptosis, at a level similar to that found with L-2 particle-exposed effector cells. Similar results were found regardless of whether PHA or ionomycin was used to stimulate the treated cells (Fig. 8). Apoptosis by Hot-017 or TNIH-047 appeared to occur prior to viral replication, because no viral RT activity was found in culture supernatants of the co-cultures 4 days after adsorption (data not shown). Thus, it appears that two wild-type Thai primary isolates can carry out an apoptosis-inducing activity for bystander T cells that had only previously been seen for defective L-2 particles.

Discussion

In this study, we have presented evidence that non-specific killer activity critical for the induction of apoptosis in bystander CD4+ and CD8+ T cells during HIV-1 infection may be induced upon activation of cell surface CD25 antigen in a resting T cell subset with the phenotype of CD4+CD38+. The striking killer activity in this CD4+CD38+CD25− subset appears to be elicited by specific binding of certain types of HIV-1 particles. Our initial starting point to uncover this activity involved the use of defective but highly SI-type L-2 particles and a laboratory strain of LAI as a control; however, it has now been extended to primary HIV-1 isolates as well.

Our findings can be summarized as follows. (i) A non-specific killer activity can be induced in a CD4+CD38− cell subset of a normal PBMC-T cell population, after adsorption with L-2 particles; this was evidenced by a significant induction of apoptosis (>40%) of both autologous and allogeneic PBMC-T target cells by co-culture with this subset of activated CD4+CD38− effector cells at an E:T ratio of 1:40 (Fig. 6). Further, prior depletion of this CD4+CD38−CD25− subset from L-2 particle-adsorbed PBMC-T abrogated the induction of apoptosis (Fig. 4). (ii) IFN-γ strongly enhances the level of L-2 particle-mediated apoptosis (Fig. 7), correlating with the enhanced expression of Fas antigen on target cells (Fig. 5B). In fact, IFN-γ was found to be released from CD4+CD38− cells after exposure to L-2 particles (Fig. 5A). (iii) L-2 particle-induced apoptosis in PBMC-T is mediated by the Fas system, because a Fas antagonist can block apoptosis (22; Fig. 7). (iv) Cell-to-cell contact between effector and target cells appears critical for the induction of L-2 particle-mediated apoptosis (data not shown). (v) Finally, a similar induction of apoptosis to that found with defective L-2 particles after adsorption to healthy donor-derived PBMC-T and mitogenic stimulation is also seen after exposure of the CD4+CD38− subset to Thai primary isolates of HIV-1 (Fig. 8). Furthermore, induction of apoptosis appears independent of viral SI or non-SI phenotype for the Thai strains. On the other hand, a laboratory strain, LAI, which has maintained in a T cell line tissue culture system for >10 years, did not show such apoptosis induction in this system using PBMC (22).

As noted above, expression of the T cell activation marker, CD25, is enhanced on the surface of CD4+CD38− cells after adsorption with L-2 particles and after mitogenic stimulation. This does not occur after adsorption with LAI particles or treatment with rgp120 protein, indicating that the cell killing activity involves a specific activation of such cells by L-2 particles. CD4+CD38−CD25− cells exhibit enhanced gp120 on their surface, presumably from L-2 particles, which have a 4-fold increased level of gp120 compared to LAI particles (22). This is supported by findings that these cells are also CD45RO+ and CD45RA−, suggesting that they are a subset of memory CD4+ T cells. Therefore, activation appears to involve interaction(s) between gp120 on virion particles and CD4 on a specific set of T cells. In addition, recent findings have shown that co-receptor molecules such as CXCR4 and CCR5 chemokine receptors play a role in virus entry for both lymphotrophic and monocytotropic HIV-1 respectively (36–39). These new co-receptors might also be involved in the activation and subsequent apoptotic events.

Other reports that support our hypothesis of L-2 particle-induced activation for this subset of CD4+ T cells are (i) a strong cell-activating capacity of HIV-1 particles has been observed during the course of virion adsorption (40) and
(ii) permanent activation of immune responses or immune cells is observed in HIV-1-infected individuals (41,42). As a consequence of this polyclonal activation of the immune system, there appears to be an enhanced progression to AIDS for such HIV-1-infected patients. Additionally, CD4+ T cells once activated could act as a modulator of immune responses by causing apoptosis induction in activated immune cells through the Fas–FasL system (43). Thus, our results, taken together with the reports on infected patients, suggest that HIV-1 particles could prime inappropriate activation of a subset of PBMC-T in order to generate effector CD4+ cells, which in turn can kill bystander CD4+ and CD8+ immune target cells.

Several reports have focused on the possible function of HIV-1-related proteins such as soluble gp120 and/or Tat to prime signals for apoptosis induction in bystander cells (25,44–46). Although these reports have revealed the ability of these proteins to induce apoptosis by using the Fas–FasL system in CD4+ T cells, they mostly examined the effect with T cell lines and large amounts of recombinant proteins. In their hands, soluble gp120 was shown to prime apoptosis only in T cell lines or activated PBMC, but not in freshly prepared resting PBMC (47,48). This is consistent with our previous paper which showed that gp120 protein plus HIV-1-infected patient sera did not induce a significant level of apoptosis in primary PBMC-T even under conditions inducing effective cross-linking of CD4. In contrast, the same PBMC-T underwent apoptosis after adsorption with only a small amount of L-2 particles (~2.0 ng particles/10^6 PBMC-T) (22).

Finally, we want to note that not only defective L-2 particles, but infectious Thai HIV-1 particles can also induce apoptosis: Hot-017 and TNII-047, which were respectively isolated from an AIDS patient and an asymptomatic carrier, showed strong induction of killer activity for the CD4+CD38- cell fractions as did L-2 particles. There is an enhanced amount of gp120 molecules on L-2 particles compared with LAI particles (22). The L-2 cells were shown to carry a provirus with mutations at env gp41, vpr and nef in addition to pol protease (20). Thus, there might be similarity on an unknown function of the gp120 molecule on the surface of the particles between L-2 and some population in the primary isolate HIV-1 and LAI particles produced from different cells on their apoptosis-inducing activities. However, there were no apparent effects, i.e. high apoptosis-inducing activity in primary isolate particles from MOLT-4 and no apoptosis-inducing activity in LAI from PBMC (data not shown).

In conclusion, our results suggest a mechanism for the apoptosis seen in bystander CD4+ and CD8+ cells of HIV-1-infected individuals. We propose that the activation of a CD4+CD38- subset after exposure to certain HIV-1 wild-type or defective L-2 particles leads to apoptosis of bystander cells. It thus may be important in follow-up studies with HIV-1-infected patients at different stages of disease to monitor the relative number of activated effector subset (CD4+CD25+CD38-) cells in order to determine their likely progression to AIDS.

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Abbreviations

Con A concanavalin A
FasL Fas ligand
PBMC peripheral blood mononuclear cell
PBMC-T T cell enriched from PBMC
PE phycoerythrin
PHA phytohemagglutinin
SI syncytia-inducing

References

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