

## Macrophage-dependent Apoptosis of CD4<sup>+</sup> T Lymphocytes from HIV-infected Individuals Is Mediated by FasL and Tumor Necrosis Factor

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### Summary

Apoptosis of bystander uninfected CD4<sup>+</sup> T lymphocytes by neighboring HIV-infected cells is observed in cell culture and in lymphoid tissue of HIV-infected individuals. This study addresses whether antigen-presenting cells such as human macrophages mediate apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals. Uninfected human macrophages, and to a larger degree, HIV-infected macrophages mediate apoptosis of T cells from HIV-infected, but not from uninfected control individuals. This macrophage-dependent killing targets CD4<sup>+</sup>, but not CD8<sup>+</sup> T lymphocytes from HIV-infected individuals, and direct contact between macrophages and lymphocytes is required. Additional analyses indicated that the apoptosis-inducing ligands, FasL and tumor necrosis factor (TNF), mediate this macrophage-induced apoptosis of CD4<sup>+</sup> T cells. These results support a role for macrophage-associated FasL and TNF in the selective depletion of CD4<sup>+</sup> T cells in HIV-infected individuals.

The physiologic regulation of peripheral CD4<sup>+</sup> T cell homeostasis is influenced by the rate of lymphocyte apoptosis. This form of cell death is tightly regulated and is dependent upon the expression of a family of ligands (Fas ligand [FasL], TNF, tumor necrosis factor-related apoptosis-inducing ligand [TRAIL]) and receptors (Fas, TNFR) that mediate apoptosis and upon the induction of susceptibility of these cells to receptor-initiated apoptosis (1–9). Enhanced apoptosis of peripheral uninfected CD4<sup>+</sup> T lymphocytes is postulated to contribute to CD4<sup>+</sup> T cell depletion in HIV-infected individuals (reviewed in references 10–12). Increased spontaneous and activation-induced apoptosis of peripheral CD4<sup>+</sup> T cells from HIV-infected individuals is observed *ex vivo* (13–15) and in lymph nodes of HIV-infected individuals and SIV-infected macaques (16, 17). Importantly, a correlation between CD4<sup>+</sup> T cell apoptosis and CD4<sup>+</sup> T cell depletion has been established in different animal models of AIDS (18–20).

Accumulating data indicate that the increased level of CD4<sup>+</sup> T cell apoptosis in HIV-infected individuals is due to an aberrant upregulation of the physiological mechanisms controlling peripheral CD4<sup>+</sup> T cell apoptosis, namely, the expression level of apoptosis-inducing ligands and receptors and the state of cell susceptibility to such ligand/receptor initiated apoptosis. Fas, FasL, TNF, and TNFR are increased in HIV-infected individuals and inversely correlated with CD4<sup>+</sup> T cell numbers (21–28). In addition, pe-

ripheral CD4<sup>+</sup> T cells from HIV-infected individuals are highly susceptible to FasL/Fas initiated apoptosis (29, 30). The mechanisms whereby the level of apoptosis-inducing receptors/ligands and the state of susceptibility of CD4<sup>+</sup> T cells to apoptosis are increased in the context of HIV infection are unknown, although gp120 cross-linking of CD4 and *tat* and cytokines may be responsible as demonstrated *in vitro* (31–35).

The circumstances whereby a susceptible CD4<sup>+</sup> T cell encounters apoptosis-inducing ligands remains unknown. *In vitro* culture models demonstrate that uninfected CD4<sup>+</sup> T cells undergo apoptosis upon contact by HIV-infected cells (36, 37). Moreover, in lymph nodes from HIV-infected individuals or SIV-infected macaques, the CD4<sup>+</sup> T cells that undergo apoptosis are not HIV-infected, but rather in direct contact with neighboring HIV-infected cells (17). These observations support the requirement of direct cell contact between a susceptible CD4<sup>+</sup> T cell and a cell that potentially expresses apoptosis-inducing ligands. Antigen-presenting cells such as human macrophages are postulated to play a major role in the physiological deletion/apoptosis of activated, and hence susceptible, peripheral T lymphocytes (38) suggesting that apoptosis-inducing ligands expressed by macrophages can mediate apoptosis of susceptible CD4<sup>+</sup> T cells. In fact, differentiated macrophages trigger selective apoptosis of CD4<sup>+</sup> T cells that have been rendered susceptible following CD4<sup>+</sup> cross-linking (39). These ob-

servations, together with the recent identification of FasL expression in human macrophages and its upregulation by HIV infection (40), lead to the hypothesis that antigen-presenting cells such as human macrophages may play a major role both in normal CD4<sup>+</sup> T cell homeostasis and in the enhanced CD4<sup>+</sup> T cell depletion observed in HIV-infected individuals.

In this study, we have evaluated whether human macrophages lead to the selective apoptosis of CD4<sup>+</sup> T cells isolated from HIV-infected individuals. In addition, we have analyzed whether apoptosis-inducing ligands presumably present on human macrophages participate in the apoptosis of CD4<sup>+</sup> T cells. Our results demonstrate that human macrophages induce selective apoptosis of CD4<sup>+</sup> T lymphocytes from HIV-infected individuals through FasL and TNF.

## Materials and Methods

**Patient Population and Isolation of Peripheral Blood Mononuclear Cells.** HIV-infected individuals attending the HIV Clinics at Hennepin County Medical Center (Minneapolis, MN) and Mayo Clinic (Rochester, MN) were selected based on CD4<sup>+</sup> T lymphocyte counts of >100 cells/dl and absence of concomitant opportunistic infections. After obtaining informed consent, 50 ml of whole blood was obtained and PBMC were isolated by density gradient centrifugation (Ficoll Hypaque; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Cells were then cultured in RPMI 1640 supplemented with 10% AB serum (GIBCO BRL, Gaithersburg, MD) and adhered to plastic at 37°C in 5% CO<sub>2</sub> for two 1 h periods. The nonadherent cells (PBL) were pelleted and resuspended in RPMI 1640 10% FBS (Hyclone Laboratories Inc., Logan, UT) at a final concentration of  $1 \times 10^6$  cells/ml.

Monocyte derived macrophages (MDM)<sup>1</sup> were obtained from PBMC from HIV seronegative healthy individuals. Briefly, PBMCs were separated from buffy coats by Ficoll Hypaque density gradient centrifugation, and  $10^8$  cells were resuspended in 50 ml of RPMI 1640 supplemented with 10% AB serum (GIBCO BRL) and placed horizontally in a 75 cm<sup>2</sup> plastic flask (Corning-Costar, Cambridge, MA). 6 d later, nonadherent cells were removed by washing the monolayer three times in RPMI 1640, and adherent cells (MDM) were either HIV- or mock-infected overnight (see below).

**Viral Stocks and HIV Infection.** Supernatants from cultures of PHA-IL2-treated PBMC either infected or not with HIV-SF<sub>162</sub> (obtained from National Institutes of Health AIDS Reference and Reagent Program) were collected at days 6 through 10 after infection. Clarified and filtered supernatants were analyzed for p24 content using a commercial ELISA kit (Cellular Products Inc., Buffalo, NY), and mycoplasma using a commercial RIA kit (Fisher, Pittsburgh, PA). Supernatants were then aliquoted and stored at -120°C.

HIV infection of MDM was performed by incubating the adherent cell monolayer with 2.5 µg of HIV p24 containing supernatant per T75 flask of MDM during 24 h at 37°C 5% CO<sub>2</sub>. Mock infection was performed by incubating MDM with an equal volume of mock-infected culture supernatant. After infection, MDM monolayers were washed extensively and cultured in 10% AB serum (GIBCO BRL). At day 7 after infection, HIV- or

mock-infected MDM were scraped, counted, and reseeded in 24-well plates (Costar) at cell concentrations indicated in each experiment. 5 d later (12 d after infection), cells were used in experiments. Analysis of p24 content in HIV-infected macrophage supernatants was analyzed by a commercial ELISA kit (Cellular Products Inc.). Flow cytometric evaluation of intracytoplasmic p24 was performed randomly in different batches of HIV-infected MDM by fluorescein isothiocyanate conjugated p24 antibody (Virostat, Portland, ME), as previously described (40, 41), demonstrating specific p24 fluorescein in 15–48% of MDM from a variety of donors.

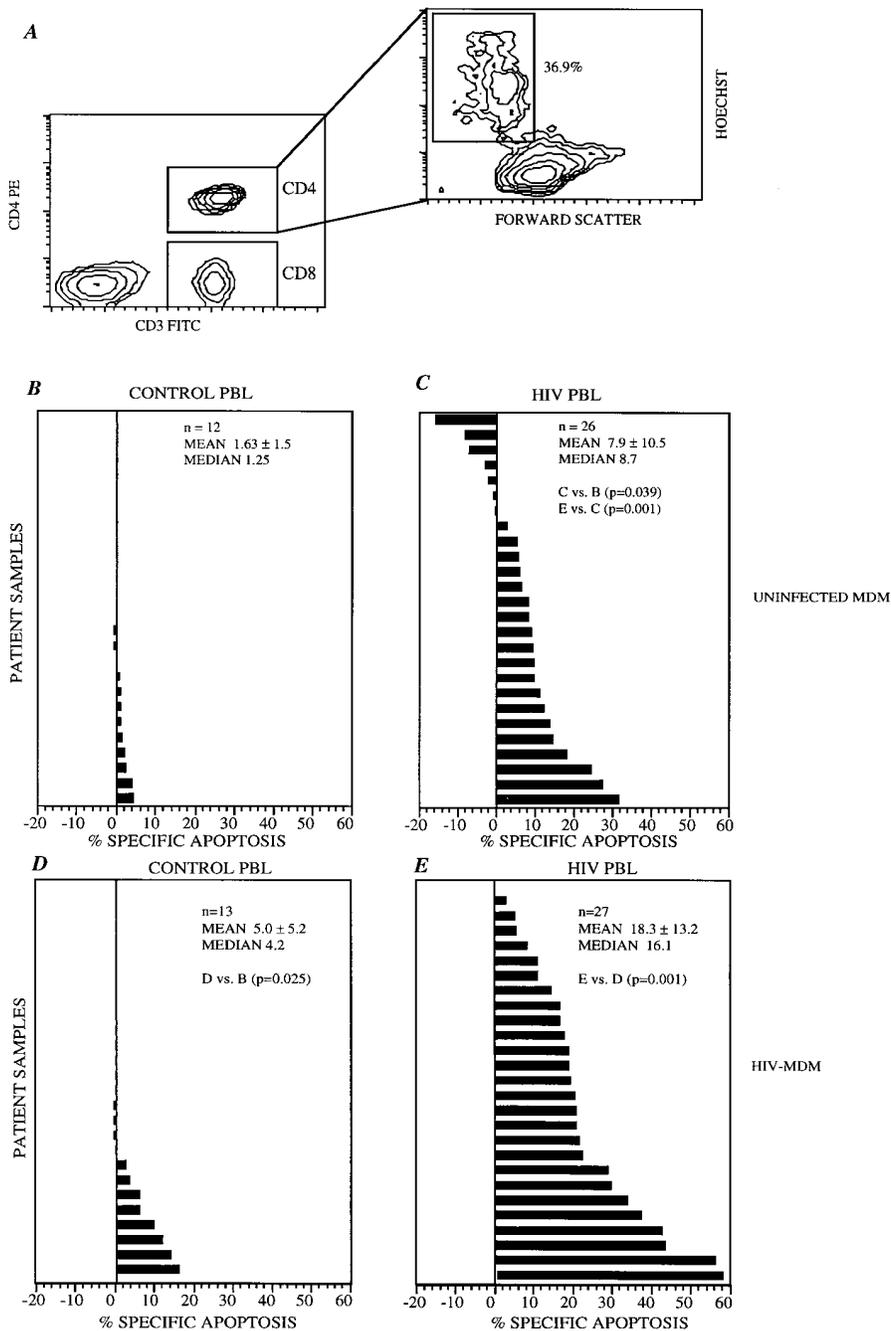
**Antibodies and Fusion Proteins.** Anti-Fas monoclonal antibodies M3 and M33 were used to determine the involvement of Fas/FasL interaction. M3 and M33 bind to Fas, but M3 and not M33 blocks ligand induced killing (2, 5, 40). Fusion proteins containing the Fc portion of mouse IgG1 and the Fas antigen (Fas-Fc), the TNFR (TNFRFc), or CD40 (CD40-Fc) have been previously described (40, 41).

**Determination of Apoptosis in CD4<sup>+</sup> or CD8<sup>+</sup> T Lymphocytes.** Apoptosis was measured using a flow cytometric method previously described (29, 41). PBL were washed in RPMI 10% AB serum (GIBCO BRL), and resuspended in PBS 0.1% azide. Cells were then incubated for 15 min at 4°C with 1 µg/10<sup>6</sup> cells of both FITC-labeled anti-CD3 monoclonal antibody (Becton Dickinson, San Jose, CA) and PE-conjugated anti-CD4 monoclonal antibody (CALTAG Labs., South San Francisco, CA) in the presence of 0.01% sodium azide. Hoechst 33342 (2 µg/ml) (Calbiochem Corp., La Jolla, CA) was added, and cells were incubated for an additional 7 min at 4°C. Cells were then washed and resuspended in ice-cold 0.5% paraformaldehyde in PBS. Flow cytometric evaluation was performed using a FACSTAR+® flow cytometer. 30,000 events were recorded and apoptosis was separately quantitated in the CD4<sup>+</sup> and CD8<sup>+</sup> populations. Apoptotic cells were quantitated by gating on cells with decreased forward angle light scatter and increased Hoechst-specific fluorescence.

**Coincubation of Macrophages and PBLs.** Unless stated,  $5 \times 10^6$  HIV- or mock-infected MDM/well in 24-well plates were washed and incubated for 36 h with  $10^6$  PBL from HIV seropositive or seronegative individuals. In experiments addressing cell-cell contact, 6.5 mm transwell porous cell culture inserts (0.4 µm) (Costar) were placed into wells containing HIV- or mock-infected MDM. PBL were added to the top of the transwell.

In experiments addressing the role of autologous killing of PBLs by HIV-infected MDMs, the methodology was as follows. 200 cc of peripheral blood was obtained and MDMs were cultured, HIV infected, and reseeded as above. In the case of the HIV seronegative individual, 9 d after HIV infection of the MDMs, an additional 50 ml of peripheral blood was obtained, and PBLs were isolated and cultured in media containing PHA (Murex Wellcome, Research Triangle Park, NC) at 10 µg/ml and IL-2 (Hoffmann-LaRoche, Nutley, NJ) at 60 IU/ml to generate lymphoblasts. 14 d after HIV infection of MDM, an additional 50 ml of peripheral blood was drawn from the same individual and PBLs were isolated. PHA and IL-2 blasts or freshly isolated PBLs were then incubated with HIV-infected MDMs in the presence or absence of M3 or M33 Fab antibodies for 24 h. PBL blasts, but not freshly isolated PBLs, from the HIV seronegative individual, were shown to be Fas sensitive as demonstrated by the induction of apoptosis by cross-linked agonist anti-Fas antibody (M3), but not by isotype matched control antibody. In experiments addressing syngeneic apoptosis of CD4<sup>+</sup> T cells in an HIV-infected individual, MDMs were isolated from 300 cc of peripheral blood from this individual and HIV infected in vitro. 14 d after HIV infection of MDM, PBLs were isolated and coin-

<sup>1</sup>Abbreviation used in this paper: MDM, monocyte derived macrophage.



**Figure 1.** Apoptosis of CD4<sup>+</sup> T cells from HIV seropositive individuals by uninfected and HIV-infected macrophages. (A) Analysis of apoptosis can be performed separately on CD4<sup>+</sup> T lymphocytes (CD3 FITC positive, CD4<sup>+</sup> PE positive cells) and on CD8<sup>+</sup> lymphocytes (CD3 FITC positive, CD4<sup>+</sup> PE negative cells). In the plot depicted, 36.9% of cells have decreased forward angle light scatter and increased Hoechst specific fluorescence, indicating that these cells are apoptotic within the CD4 population (right). (B and C) Uninfected macrophages (UNINFECTED MDM) were used as effector cells at 5:1 effector/target ratio against PBL from either HIV seronegative (B) or infected individuals (C). Mean spontaneous apoptosis was  $7.8 \pm 4.9\%$  (B), and  $23.1 \pm 15.1\%$  (C). (D and E) The same experiments repeated with HIV-infected MDM (HIV-MDM) against HIV seronegative (D) or seropositive (E) individuals. Median spontaneous apoptosis was  $8.1 \pm 4.8\%$  (D), and  $23.0 \pm 14.8\%$  (E). In B-E, percent specific apoptosis was calculated by subtracting the amount of apoptosis from PBLs cultured in medium alone from the amount of apoptosis when the same PBLs were coincubated with macrophages as indicated.

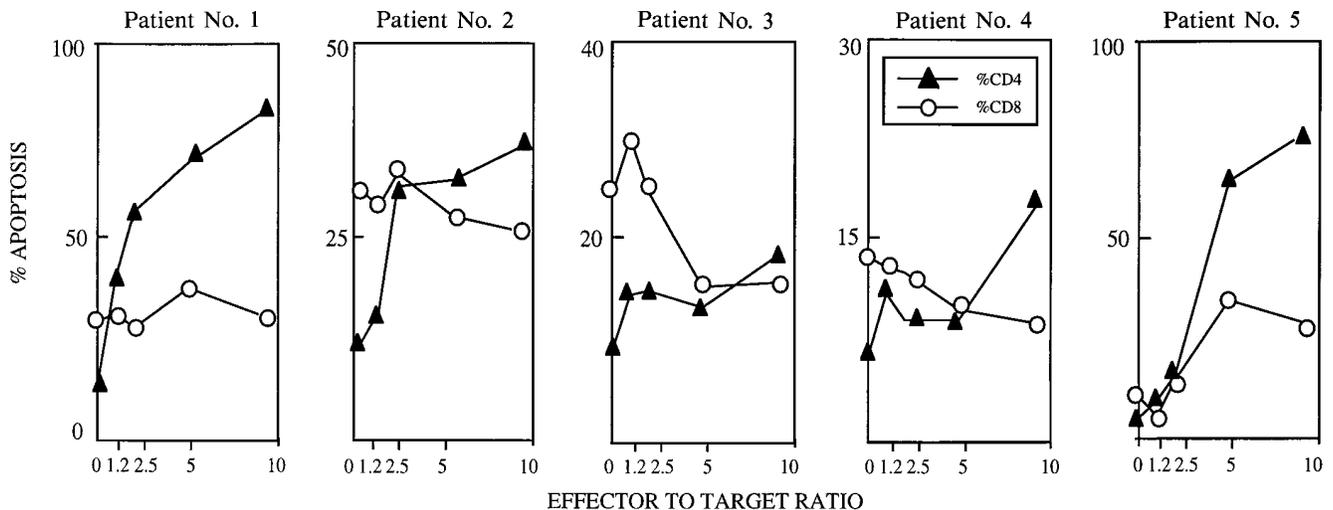
cubated with HIV-MDM in the presence or absence of M3 or M33 Fab antibodies for 24 h.

**Statistical Analysis.** Comparison of the amount of apoptosis that was observed with different HIV seropositive or seronegative PBL donors after coincubation with either uninfected or HIV-infected macrophages were made using a one way analysis of variance.

## Results

**Primary Human MDM Trigger Apoptosis of CD4<sup>+</sup> T Cells from HIV-infected Individuals.** To determine whether MDM trigger apoptosis of CD4<sup>+</sup> T cells, 14-d-old MDM from

HIV seronegative healthy donors were incubated for 36 h with freshly isolated PBL from HIV-infected individuals or HIV seronegative healthy donors. Thereafter, nonadherent cells were harvested and analyzed using flow cytometry to detect apoptosis within CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes. A representative example of this analysis is depicted in Fig. 1 A. A minimal degree of CD4<sup>+</sup> T cell apoptosis (median of 1.25%) is observed in PBL from HIV seronegative healthy control donors (Fig. 1 B). However, when MDM are incubated with PBL from HIV-infected individuals, a larger degree of CD4<sup>+</sup> T lymphocyte apoptosis is observed (median of 8.7%,  $P = 0.039$ ) (Fig. 1 C), suggesting enhanced sus-



**Figure 2.** HIV-infected macrophages induce selective apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals. 10<sup>6</sup> PBL from HIV-infected and HIV seronegative healthy individuals were coincubated with varying amounts (0, 1, 2, 5, and 10 × 10<sup>6</sup>/well) of HIV-infected macrophages for 36 h, and stained with anti-CD3 FITC, anti-CD4<sup>+</sup> PE, and Hoechst 33342. The observed amounts of apoptosis for CD4 lymphocytes (CD3<sup>+</sup>, CD4<sup>+</sup>) and CD8 lymphocytes (CD3<sup>+</sup>, CD4<sup>-</sup>) are shown. Spontaneous apoptosis is defined as the degree of apoptosis of PBL incubated in the absence of MDM.

ceptibility of CD4<sup>+</sup> T cells from HIV-infected individuals to MDM-mediated apoptosis.

Previous studies in lymph nodes from HIV-infected individuals indicate that those cells that are undergoing apoptosis are not HIV-infected, but are in direct contact with HIV-infected ones (17). Therefore, we tested whether HIV infection of MDM further enhances the MDM-mediated CD4<sup>+</sup> T cell apoptosis in PBL from HIV-infected individuals. Using the same experimental design as described above, PBL from HIV seronegative healthy controls and from HIV-infected individuals were incubated with 14-d-old HIV-infected MDM. A significant increase in CD4<sup>+</sup> T cell apoptosis is observed in PBL from HIV-infected individuals incubated with HIV-MDM (median 16.1%) as compared to the degree of CD4<sup>+</sup> T cell apoptosis in PBL from HIV seronegative individuals (median 4.2%, compare Fig. 1, E and D,  $P = 0.001$ ). In addition, HIV-infected MDM, as compared to uninfected ones, further increase the apoptosis of CD4<sup>+</sup> T cells from HIV seronegative individuals (compare Fig. 1 D with B,  $P = 0.025$ ) and HIV-infected individuals (compare Fig. 1, E with C,  $P = 0.001$ ).

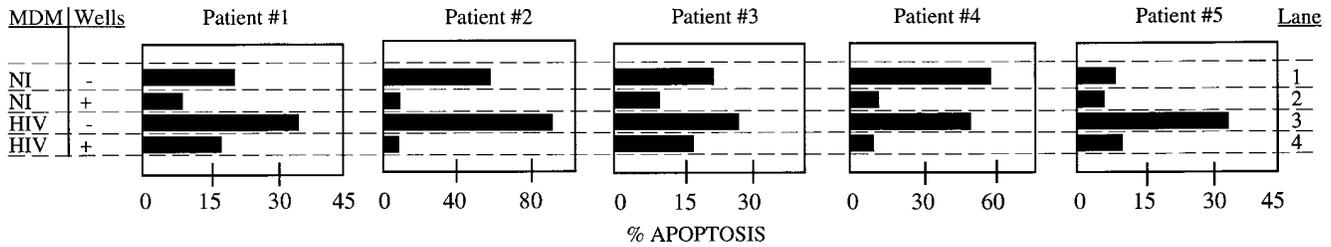
Altogether, these results indicate that uninfected, and to a larger degree, HIV-infected MDM selectively induce apoptosis of CD4<sup>+</sup> T cells, and that CD4<sup>+</sup> T cells from HIV seropositive individuals are inherently more susceptible to MDM and HIV-MDM-mediated apoptosis.

**MDM Trigger Apoptosis of CD4<sup>+</sup>, but not CD8<sup>+</sup>, T Lymphocytes.** Because CD4<sup>+</sup> T lymphocytes are preferentially depleted over CD8<sup>+</sup> T cells in HIV-infected individuals, we sought to determine whether MDM induced apoptosis of T lymphocytes is restricted to CD4<sup>+</sup> T cells. For this, PBL from five different HIV seropositive individuals (HIV-PBL) were incubated with HIV-infected MDM (HIV-MDM) at different effector (HIV-MDM) to target (HIV-PBL) ratios. After 36 h of coincubation, PBLs were analyzed using flow

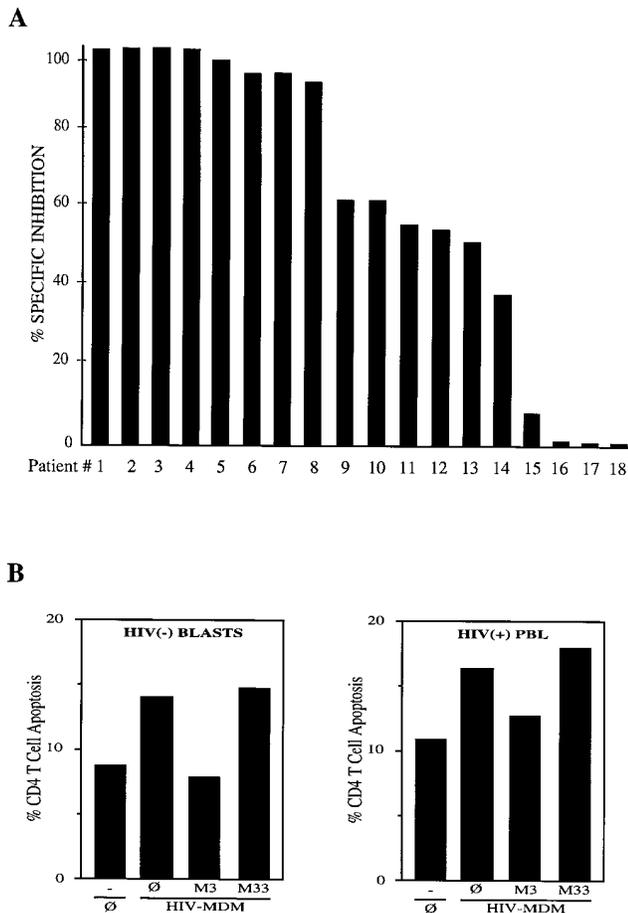
cytometry to determine the level of apoptosis with CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Spontaneous apoptosis was higher in CD8<sup>+</sup> than CD4<sup>+</sup> T cells (Fig. 2). Increasing the effector to target ratio resulted in a progressive increase in the level of CD4<sup>+</sup> T cell apoptosis in all patients, an effect that was not observed in CD8<sup>+</sup> T lymphocytes from the same HIV-infected individual (Fig. 2). These results indicate that MDM can trigger selective apoptosis of susceptible CD4<sup>+</sup> T lymphocytes.

**Cell Contact Is Required for HIV-MDM-mediated Apoptosis of CD4<sup>+</sup> T cells from HIV-infected Individuals.** Human macrophages express apoptosis-inducing ligands such as FasL and TNF (40, 42). Because these two apoptosis-inducing ligands may participate in CD4<sup>+</sup> T cell apoptosis and are known to be expressed in both cell-associated and soluble form (21), we investigated whether direct cell contact between MDM and PBL is required for CD4<sup>+</sup> T cell apoptosis. HIV- or mock-infected MDM were coincubated with PBL from another series of five HIV-infected individuals, in the presence or absence of transwells. Apoptosis of CD4<sup>+</sup> T cells was then analyzed by flow cytometry. CD4<sup>+</sup> T cell apoptosis mediated by mock-infected MDM (NI) was decreased in the presence of transwells in all patients tested (Fig. 3, compare lanes 1 and 2). Similarly, the higher level of CD4<sup>+</sup> T cell apoptosis mediated by HIV-infected MDM (HIV) was also decreased in the presence of transwells (Fig. 3, compare lanes 3 and 4). These results support the requirement for direct contact between MDM and PBL to result in the selective apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals.

**Fas/FasL Interactions Participate in the Apoptosis of CD4<sup>+</sup> T cells from HIV Seropositive Individuals Triggered by HIV-MDM.** We have previously described that FasL is partly responsible for the HIV-MDM-mediated apoptosis of Fas-susceptible targets such as Jurkat T cells and PHA-IL2-



**Figure 3.** Contact between HIV-MDM and PBL is required to induce apoptosis of T lymphocytes. Macrophages that were either HIV- or mock-infected were coincubated with PBL from HIV seropositive individuals with either the two cell populations in direct contact (-), or with contact interrupted (+) by a semipermeable transwell. The mean spontaneous apoptosis in these experiments was  $11.0 \pm 3.4\%$ .

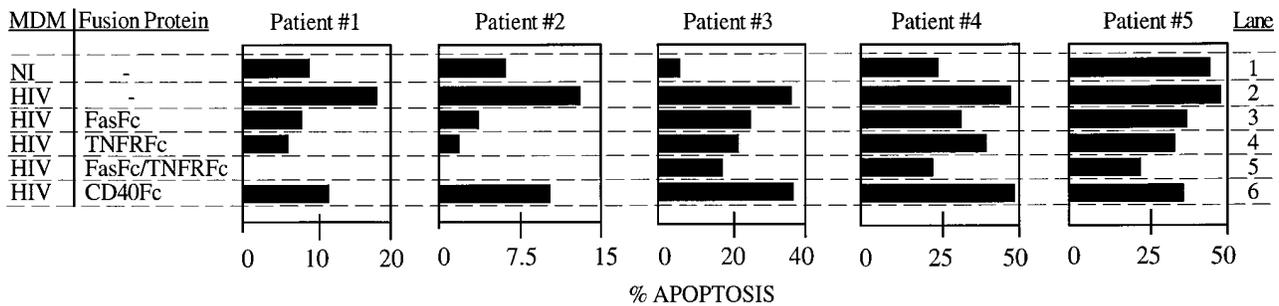


**Figure 4.** (A) MDM induced apoptosis of CD4<sup>+</sup> T lymphocytes is partially mediated by FasL. PBL from HIV-infected individuals were incubated alone or with HIV-infected macrophages at an effector/target ratio of 5:1, either in the absence or presence of blocking (Fab' M3) or nonblocking (Fab' M33) anti-Fas antibodies (10  $\mu\text{g}/\text{well}$ ). In all cases, Fab' M33 did not significantly alter the level of apoptosis seen in the PBL coincubated with macrophages without antibody. In order to determine the contribution of FasL to MDM killing of PBLs, percent specific inhibition was calculated as follows: ([apoptosis of PBL coincubated with MDM - apoptosis of PBL coincubated with Fab'3 and MDM] / [apoptosis of PBL coincubated with MDM - apoptosis of PBL incubated alone]). The mean spontaneous apoptosis was  $20.7 \pm 13.7\%$ . (B) Syngeneic apoptosis of susceptible CD4<sup>+</sup> T cells by HIV-infected MDM. (Left) Lymphocyte blasts from an HIV seronegative individual (HIV(-) BLASTS) were incubated with  $3 \times 10^6$  syngeneic HIV-infected MDM (HIV-MDM) either alone ( $\emptyset$ ) or in the presence of blocking (Fab' M3) or nonblocking (Fab' M33) antibodies (10  $\mu\text{g}/\text{well}$ ). Spontaneous apoptosis was calculated by

treating T cell blasts (40). Based on the above results, we questioned whether HIV-MDM triggered apoptosis of CD4<sup>+</sup> T cells from HIV seropositive individuals is mediated through FasL/Fas interactions. PBL from a series of 18 HIV-infected individuals were incubated with HIV-infected MDM (HIV-MDM). The role of FasL was analyzed by adding blocking (M3 Fab'') or nonblocking (M33 Fab'') anti-Fas antibodies to wells in which MDM-HIV and PBL were coincubated. In a subgroup of patients (patients No. 1-8), Fas blocking antibodies (M3) completely reversed the CD4<sup>+</sup> T cell apoptosis triggered by MDM-HIV (Fig. 4 A). However, in additional patients, M3 incompletely reversed CD4<sup>+</sup> T cell apoptosis (patients No. 9-14) or had no effect (patients No. 15-18). In those patient samples in which M3 blocked HIV-MDM triggered apoptosis, the nonblocking M33 had no effect. From these results, it is inferred that apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals triggered by HIV-infected MDM is partially mediated by FasL.

The above experiments were performed under allogeneic conditions in which MDM, obtained from healthy HIV seronegative individuals and infected in vitro with HIV, were coincubated with freshly isolated allogeneic PBLs from a series of HIV-infected individuals. While FasL/Fas interactions are presumed to be unaffected by allo-antigens, we performed additional experiments to address this issue. First, we determined whether lymphocyte blasts that were generated by incubating PBL from an HIV seronegative individual with PHA and IL-2, would become apoptotic upon incubation with syngeneic HIV-MDM. It has been previously demonstrated that PHA-IL-2-treated lymphocytes become susceptible to FasL-dependent apoptosis (40). In addition, we tested whether FasL/Fas interactions participated in the MDM-HIV-triggered apoptosis of syngeneic lymphocyte blasts. As shown in Fig. 4 B (left), HIV-MDMs increased the spontaneous level of apoptosis of syngeneic CD4<sup>+</sup> T cell blasts, an effect that was reversed by

incubating PBL blasts without macrophages ( $\emptyset$ ). Incubation with cross-linked agonistic M3 anti-Fas antibody lead to 27% CD4<sup>+</sup> T cell apoptosis. Freshly isolated PBLs had a spontaneous level of CD4<sup>+</sup> T cell apoptosis of 2% which was not modified (3%) upon coincubation with HIV-infected MDM. (Right) Freshly isolated PBLs from an HIV-infected individual (HIV(+)) PBL were incubated with syngeneic HIV-infected MDMs ( $3 \times 10^6$ ) in the absence or presence of Fab M3 or M33 (10  $\mu\text{g}/\text{ml}$ ) antibodies. Cross-linked M3 antibodies induced 15% of CD4<sup>+</sup> T cell apoptosis.



**Figure 5.** FasL and TNF participate in MDM-induced apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals.  $5 \times 10^6$  uninfected (NI) or HIV-infected (HIV) macrophages were incubated with  $10^6$  PBL from five different HIV-infected individuals in the presence or absence of 20  $\mu$ g FasFc, TNFRFc, or CD40Fc or 10  $\mu$ g of each of TNFRFc and FasFc for 48 h. Analysis of CD4<sup>+</sup> T cell apoptosis was done as described in Fig. 1 A. Observed amounts of apoptosis for CD4 T cells are shown. Mean spontaneous apoptosis was  $23.5 \pm 9.8\%$ .

anti-Fas blocking (M3) but not nonblocking (M33) antibodies. The susceptibility of lymphocyte blasts to FasL was confirmed by their coincubation with cross-linked anti-Fas antibodies (see legend Fig. 4 B). This suggests that the HIV-MDM-mediated, FasL-dependent, apoptosis of susceptible CD4<sup>+</sup> T cells can also be observed in a syngeneic model. These observations were confirmed using MDM and PBL obtained from an HIV seropositive individual. MDM were HIV infected in vitro and coincubated 14 d later with freshly isolated PBL. As shown in Fig. 4 B (right), HIV-infected MDMs enhanced the level of spontaneous apoptosis of freshly isolated CD4<sup>+</sup> T lymphocytes, an effect that was reduced by anti-Fas blocking (M3), but not by nonblocking (M33) Fab antibodies. These results suggest that the enhanced FasL-dependent apoptosis of CD4<sup>+</sup> T cells of this HIV-infected individual is also observed using syngeneic HIV-infected MDM.

**TNF Participates in the MDM-triggered Apoptosis of CD4<sup>+</sup> T cells from HIV Seropositive Individuals.** TNF is present as a soluble or cell membrane bound form (6), is produced by macrophages (42), and, as for FasL, can cause apoptosis of activated T cells (8, 9). To determine whether TNF participates in the apoptosis of CD4<sup>+</sup> T lymphocytes mediated by MDM, PBLs from five HIV-infected individuals were coincubated with uninfected and HIV-infected MDM for 48 h in the presence or absence of the human TNFRFc fusion protein. This incubation period was selected to allow for possible differences in the timing of induction of T lymphocyte apoptosis by FasL and TNF (9). In parallel, we tested possible additive or synergistic effects between TNF and FasL using FasFc fusion proteins. As shown in Fig. 5, FasFc inhibited the HIV-MDM induced apoptosis of CD4<sup>+</sup> T lymphocytes from the five HIV-infected individuals (compare lanes 2 and 3). However, TNFRFc also reduced CD4<sup>+</sup> T cell apoptosis from the same individuals to a similar degree to that observed with FasFc (compare lanes 2–4). Interestingly, combining of TNFRFc and FasFc resulted in an additive inhibition of HIV-MDM mediated apoptosis of CD4<sup>+</sup> T cells (lane 5). CD40Fc, included to control for the specificity of fusion protein dependent inhibition, reduced the MDM-mediated apoptosis of CD4<sup>+</sup> T cells in three patients, albeit to a lower degree than FasFc or TNFRFc

(lane 6). Altogether, these results indicate that FasL and TNF participate in the selective apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals mediated by human macrophages.

## Discussion

This report provides evidence of a cellular interaction that selectively mediates CD4<sup>+</sup> T cell apoptosis of HIV-infected, but not uninfected, individuals. This finding is noteworthy in several regards. First, macrophage-induced apoptosis is selective for CD4<sup>+</sup> T lymphocytes. Second, HIV infection of macrophages further enhances CD4<sup>+</sup> T cell apoptosis from HIV-infected individuals. Third, CD4<sup>+</sup> T cells from HIV-infected individuals are inherently more susceptible to macrophage-derived apoptosis-triggering ligands, including FasL and TNF.

A large number of studies addressing apoptosis of PBL from HIV-infected individuals have demonstrated that ex vivo activation of PBL from HIV-infected individuals results in enhanced CD4<sup>+</sup>, but also CD8<sup>+</sup> T cell apoptosis (14, 15, 18, 32, 43), hence raising doubts about the relevance of this observation to the in vivo CD4<sup>+</sup> T cell depletion observed in HIV-infected individuals. Our results indicate that macrophage-PBL interactions do not further enhance the spontaneous apoptosis of CD8<sup>+</sup> T cells, but selectively increases CD4<sup>+</sup> T cell apoptosis. This in vitro finding may be of relevance to the pathogenesis of HIV where selective CD4<sup>+</sup> T cell depletion is observed, and which correlates with animal models of AIDS where ex vivo apoptosis of PBL is primarily enhanced within CD4<sup>+</sup> T cells (18). It is possible that differences in susceptibility to macrophage-bearing apoptosis-inducing ligands (FasL and TNF) between CD4<sup>+</sup> and CD8<sup>+</sup> T cells account for such selectivity. Alternatively, the frequency or affinity of the interaction between macrophages and CD4<sup>+</sup> T cells is higher than between macrophages and CD8<sup>+</sup>.

If interactions between macrophages and susceptible CD4<sup>+</sup> T cells participate in CD4<sup>+</sup> T cell depletion in HIV-infected individuals, uninfected MDM may suffice to mediate apoptosis of susceptible CD4<sup>+</sup> T cells, and thus not depend solely on the apoptosis-inducing capabilities of HIV-

infected macrophages. Alveolar macrophages from HIV-infected individuals are highly activated (potentially expressing apoptosis-inducing ligands) (44–48). While the role of these macrophages in AIDS pathogenesis is unknown, their presence correlates with decreased CD4<sup>+</sup> T cells in alveolar fluid (49), thus suggesting that organs with high lymphocyte toxicity such as the lung may be involved in CD4<sup>+</sup> T cell depletion via macrophage–lymphocyte interactions.

In other organs such as lymphoid tissue, uninfected antigen-presenting cells but also HIV-infected macrophages, can participate in further enhancing apoptosis of susceptible CD4<sup>+</sup> T cells. Studies from lymph nodes indicate a bystander effect in the apoptosis of uninfected cells by HIV-infected neighboring cells (17); thus, future studies should characterize the type of HIV-infected cell that interacts with susceptible bystander CD4<sup>+</sup> T cells. The key role of HIV-infected macrophages in causing CD4<sup>+</sup> T cell depletion is inferred from hu-SCID HIV mouse models. Mosier et al. demonstrated that monocytotropic viruses resulted in enhanced and accelerated apoptosis of the repopulated CD4<sup>+</sup> T cell population, suggesting that macrophages might play an important role in CD4<sup>+</sup> T cell depletion upon HIV infection (50). The enhanced FasL-dependent apoptosis of freshly isolated CD4<sup>+</sup> T lymphocytes from HIV-infected individuals triggered by HIV-infected MDM is most likely a result of enhanced FasL expression or synthesis. However, the possibility that CD4<sup>+</sup> T cells become infected during the 24–36-h incubation period with HIV-MDM cannot be completely excluded. The fact that uninfected MDM also induce FasL-dependent apoptosis of CD4<sup>+</sup> T lymphocytes from HIV-infected individuals, albeit to a lower degree than HIV-infected MDMs, points to macrophage-related mechanisms. Moreover, HIV infection of MDM increases the RNA steady state levels of FasL, and this correlates with increased apoptosis of susceptible target cells through FasL-dependent mechanisms (40). Lastly, fixing of HIV-infected MDMs does not abrogate the selective apoptosis of CD4<sup>+</sup> susceptible T lymphocytes (40). The percentage of HIV-infected MDM, as detected by intracytoplasmic p24 (which may overestimate the number of HIV-infected cells by taking into account that MDM can phagocytize p24), was variable in each experiment, reflecting donor variability and macrophage susceptibility to HIV infection. This variability of HIV infection may have impacted the range of apoptosis observed. Additional studies are in progress to determine whether HIV-infected macrophages and/or uninfected macrophages present in the HIV-infected culture is/are responsible for the enhanced apoptosis of susceptible CD4<sup>+</sup> T lymphocytes by FasL or TNF. Additional studies should also focus on determining which of the different subpopulations present within MDM cultures express functional apoptotic-inducing ligands.

Macrophages are thought to play a role in the physiological CD4<sup>+</sup> T cell depletion in healthy individuals (38). Our results support this observation, and point to macrophages as having a major role in FasL-dependent apoptosis of susceptible CD4<sup>+</sup> T cells from HIV-infected patients (29, 30).

The state of activation, and thus of susceptibility, of the CD4<sup>+</sup> T cell, and not the state of differentiation or HIV infection of the macrophage, may be the critical factor regulating CD4<sup>+</sup> T cell apoptosis. Abundant data supports that CD4<sup>+</sup> T cells from HIV-infected individuals, even at early stages of the disease, are highly activated (51, 52). Although the mechanism(s) leading to aberrant T cell activation in HIV-infected individuals remain unknown, they may be related to the degree of viral load. Decreases in viral load correlates with decreased CD4<sup>+</sup> T cell depletion (53, 54). If HIV-dependent mechanisms result in CD4<sup>+</sup> T cell activation and potential susceptibility to apoptosis, reductions of viral load should decrease the state of T cell activation and hence susceptibility to apoptosis.

Characterization of the molecules responsible for inducing apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals is important to advance our knowledge of the cause of CD4<sup>+</sup> T cell depletion. The participation of FasL in mediating the apoptosis triggered by MDM was not unexpected based on the recent identification of FasL in MDM (40) and the observed enhanced susceptibility to FasL in PBL from HIV-infected individuals (29). However, it was surprising to observe that TNF could also play a role in inducing apoptosis of CD4<sup>+</sup> T lymphocytes from HIV-infected individuals. Recent studies indicate that Fas ligand triggers apoptosis of T lymphocytes earlier than TNF (9). Although we did not address this time sequence in our experimental design, MDMs and CD4<sup>+</sup> T lymphocytes were coincubated for 48 h so that the potential effect of both apoptotic inducing ligands could be detected. The role of TNF as an apoptotic inducing ligand in macrophages may be relevant to HIV pathogenesis. Increased TNF and TNFR levels are observed in HIV-infected individuals and correlate with CD4<sup>+</sup> T cell depletion (26, 27), and our results suggest that this receptor/ligand family may play a key role in directly causing CD4<sup>+</sup> T cell apoptosis. Such prediction is supported by the recent observation that TNF, aside from FasL, is partially responsible for activation-induced T lymphocyte apoptosis in animal models (9, 29), and that antigen-induced apoptosis of CD4<sup>+</sup> T cells from HIV-infected individuals may be mediated by TNF (55). Our results also indicate that while Fas ligand and TNF appear to be the major mediators of apoptosis of CD4<sup>+</sup> T lymphocytes by MDMs, in a small number of patients, additional MDM-mediated apoptosis independent from TNF and/or Fas ligand was observed. This suggests that other ligands known to induce apoptosis may play a role. TRAIL (Apo-2L) is a new member of the TNF family with apoptotic inducing capability (7, 55). Likewise, lymphotoxin has also been recently suggested to play a role in the apoptosis of CD4<sup>+</sup> T lymphocytes from HIV-infected individuals (56). Based on this, additional experiments should determine whether any of these two ligands participate in MDM-induced apoptosis of CD4<sup>+</sup> T lymphocytes from HIV-infected individuals.

Although our study was aimed at identifying basic mechanisms that participate in the enhanced apoptosis of CD4<sup>+</sup> T cells, their relevance to HIV pathogenesis needs to be validated in clinical studies. Correlation of viral load and

CD4<sup>+</sup> T cell number with susceptibility of CD4<sup>+</sup> T cells to FasL and TNF needs to be established. Level and localization of the expression of these ligands in lymphoid tissue and organs from HIV-infected individuals also needs to be addressed. Characterization of the mechanisms that lead to

enhanced susceptibility to lymphocyte apoptosis and the abnormal regulation of apoptosis-inducing ligands in HIV-infected patients would allow future therapeutical interventions to reduce or control virus-dependent enhanced CD4<sup>+</sup> T cell depletion.

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