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Upregulation of Fas Ligand Expression by Human Immunodeficiency Virus in Human Macrophages Mediates Apoptosis of Uninfected T Lymphocytes

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Apoptosis has been proposed to mediate CD4$^+$ T-cell depletion in human immunodeficiency virus (HIV)-infected individuals. Interaction of Fas ligand (FasL) with Fas (CD95) results in lymphocyte apoptosis, and increased susceptibility to Fas-mediated apoptosis has been demonstrated in lymphocytes from HIV-infected individuals. Cells undergoing apoptosis in lymph nodes from HIV-infected individuals do not harbor virus, and therefore a bystander effect has been postulated to mediate apoptosis of uninfected cells. These data raise the possibility that antigen-presenting cells are a source of FasL and that HIV infection of cells such as macrophages may induce or increase FasL expression. In this report, we demonstrate that HIV infection of monocytic cells not only increases the surface expression of Fas but also results in the de novo expression of FasL. Interference with the FasL-Fas interaction by anti-Fas blocking antibodies abrogates HIV-induced apoptosis of monocytic cells. Human monocyte-derived macrophages from healthy donors contain detectable FasL mRNA, which is further upregulated following HIV infection with monocytotropic strains. HIV-infected human macrophages result in the apoptotic death of Jurkat T cells and peripheral blood T lymphocytes. Interruption of the FasL-Fas interaction abrogates the HIV-infected macrophage-dependent death of T lymphocytes. These results provide evidence that human macrophages can provide a source of FasL, especially following HIV infection, and can thus participate in lymphocyte depletion in HIV-infected individuals.

The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of CD4$^+$ T lymphocytes. Recent reports estimate the daily loss of CD4$^+$ T cells to be between 3.5×10^7 and 2×10^9 cells (20, 54). Proposed causes of CD4$^+$ T-cell depletion in the setting of human immunodeficiency virus (HIV) infection include both direct and indirect mechanisms. Direct HIV-mediated cytopathic effects (43) and syncytium formation (53) occur in vitro but have not been observed in vivo. Studies of lymphoid tissue from infected individuals, while demonstrating clusters of infected cells, did not note syncytium formation (11, 42, 43). Studies derived from HIV-hSCID mouse models demonstrate that the degree of CD4$^+$ depletion following HIV infection does not correlate with the in vitro cytopathicity of the viral strain (37). In addition, recent data derived from lymph node studies indicate that it is not the virus-replicating cells but the uninfected neighboring cells that are dying (13, 14, 36). These data, together with the fact that very few peripheral blood mononuclear cells are actively infected with HIV (58) in spite of the rapid and aggressive destruction of circulating CD4 T lymphocytes (20, 54), clearly argue for an indirect mechanism(s).

Although a variety of indirect mechanisms have been identified by using different in vitro models (3, 13, 18, 45, 61), HIV-induced apoptosis has emerged as a potential candidate. Apoptosis or programmed cell death is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (3, 27). HIV-induced apoptotic cell death has been demonstrated not only in vitro but also, more importantly, in infected individuals (3, 13, 38). Peripheral blood mononuclear cells from HIV-infected individuals undergo apoptosis ex vivo (19, 28, 35, 41), and cell death by apoptosis has been observed in both HIV-infected lymph nodes (12, 13, 38) and gut tissue (26). Furthermore, apoptosis and CD4$^+$ T-lymphocyte depletion is tightly correlated in different animal models of AIDS (5, 17), and, importantly, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (17). The signal(s) and the receptors responsible for inducing apoptosis in these models of HIV infection remain undefined. Candidate signals include HIV envelope proteins (4), HIV Tat (31), and putative superantigens (7), while potential receptors to mediate apoptosis include the T-cell receptor (4, 39) and CD4 (4, 25). One common feature provided by the in vivo and in vitro studies of HIV-induced apoptosis is that cellular activation is required for its induction (3, 4, 8, 13, 19). Advances in the understanding of the biology of apoptosis indicate that a family of receptors may mediate apoptosis of many cell types (47). Among them is Fas, which, upon its ligation either by cross-linking antibody (21, 52) or by the Fas ligand (FasL) (49), results in the apoptotic cell death of the Fas-bearing target cell. Recent data indicate that FasL-Fas interaction, in conjunction with T-cell receptor activation, results in apoptosis in antigen-primed and transformed T cells (1, 2, 6, 10, 22, 44). Thus, the requirement for T-cell activation appears to be a common feature of apoptosis mediated by FasL-Fas interactions and of HIV-induced apoptosis in vitro. Moreover, circulating T lymphocytes from HIV-infected individuals have a higher degree of activation

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than do those from uninfected individuals (16), have increased expression of Fas in their surface (9, 23), and are more susceptible to Fas-mediated killing (23). Altogether, the data suggest that uninfected but primed or activated T lymphocytes from HIV-infected individuals may undergo apoptosis after encountering FasL.

FasL has so far been described to be expressed only in lymphoid cells (50) and not in cells of the monocyte lineage. However, data derived from HIV animal models imply that accessory cells such as macrophages can trigger T-cell depletion (36). The inability of HIV to infect chimpanzee macrophages has been suggested as an explanation for the lack of apoptosis and development of AIDS in HIV-infected chimpanzees (17, 46). In addition, in the HIV-hSCID mouse, it is the monocytotropic and not the lymphotropic strains of HIV that lead to T-lymphocyte depletion (36, 37). Therefore, apoptosis of lymphocytes in lymph nodes from infected individuals could be mediated by HIV-infected accessory cells that confer a death signal to neighboring primed uninfected T lymphocytes.

We hypothesize that abnormal regulation of FasL-Fas interactions may play a pivotal role in HIV pathogenesis. To explore this concept, we first examined whether HIV infection of host cells could lead to FasL expression. Using monocytic cell lines that result in death following HIV infection, we demonstrate that infection of U937 cells with HIV results in the de novo expression of FasL and that FasL mediates HIV-induced apoptosis. Next, we tested whether HIV-mediated expression of FasL could be detected in relevant accessory cells such as human macrophages. We demonstrate that FasL is detectable in uninfected macrophages and that its expression is upregulated following HIV infection to result in selective killing of uninfected CD4 T-lymphocytic cells. These results indicate that FasL expression is present in nonlymphoid cells such as monocyte-derived macrophages and can be upregulated by HIV infection, events that could be critical in mediating T-lymphocyte depletion in HIV-infected individuals.

MATERIALS AND METHODS

Cells, antibodies, fusion proteins, and reagents. The U937 promonocytic and the Jurkat T-lymphoblastoid cell lines were obtained from the American Type Culture Collection, passaged in RPMI 1640 (BioWhittaker, Walkersfield, Md.) supplemented with glutamine, antibiotics, and 5 to 10% fetal bovine serum (Clontech, Palo Alto, Calif.), and verified routinely to be *Mycoplasma* negative by a radioimmunoassay (Fischer, Pittsburgh, Pa.). Monocyte-derived macrophages (MDM) were prepared from peripheral blood mononuclear cells obtained from buffy coats from healthy donors at our blood bank. After Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) density gradient centrifugation, 10^8 peripheral blood mononuclear cells were placed horizontally in a T75 tissue culture flask containing 30 ml of RPMI 1640 supplemented with antibiotic, glutamine, and 10% AB serum (Gibco BRL, Gaithersburg, Md.) and incubated at 37°C under 5% CO_2. Six days later, nonadherent cells were removed by washing the adherent population three times with RPMI 1640. At this point, MDM were over 90% $CD14^+$ as detected with My4, a fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibody (Coulter, Hialeah, Fla.). The immunoglobulin G (IgG) anti-Fas antibodies M38 and M33 have been described previously (1). The FasFc fusion protein consists of the extracellular domain of Fas linked to a mutated Fc portion of human IgG1. The TNFRFc fusion protein consists of the extracellular domain of the p75 tumor necrosis factor (TNF) receptor fused to the Fc portion of human IgG1. 7C11 IgM anti-Fas antibody ascites was a generous gift from Michael J. Robertson, Dana-Farber Cancer Institute, Boston, Mass.

HIV infection. HIV strains IIIB and SF_{162} were obtained from the National Institutes of Health AIDS Reference and Reagents Program. IIIB was amplified in U937 cells, and SF_{162} was amplified in phytohemagglutinin (PHA) (Murex Wellcome, Research Triangle Park, N.C.) and interleukin-2 (IL-2) (Hoffman La Roche, Nutley, N.J.)-stimulated PBMN cells. Both strains repeatedly tested negative for *Mycoplasma* strains by RIA (Fischer). For HIV infection of U937 cells, 10^6 freshly thawed and exponentially growing cells were infected for 15 h at 37°C under 5% CO_2 with 1 ml per 10^6 cells of 10^5 cpm of reverse transcriptase activity-containing supernatant. Cells were then washed three times with RPMI 1640, adjusted to a concentration of 0.25×10^6/ml, and passaged every 3 days starting at the same concentration. Mock infection of U937 cells was performed under the same conditions, except that the supernatant was generated from uninfected U937 cells. Infection of MDM was performed with SF_{162}. A 2.5-ng sample of p24-containing supernatant was used to infect an MDM-containing T75 flask for 15 h at 37°C and 5% CO_2, after which the cells were washed three times with RPMI 1640 and passaged with RPMI 1640–10% FBS (Clontech) once a week. For mock infection of MDM, the same volume of supernatant from PHA- and IL-2-treated but uninfected blasts was used. Culture supernatants were assayed for HIV infection by measurement of reverse transcriptase activity (34) and by p24 antigen testing (Cellular Products, Inc., Buffalo, N.Y.). HIV-infected MDM were assayed by flow cytometry, and over 60% of cells were found to express intracytoplasmic p24 (34).

For experiments requiring the use of soluble CD4, the HIV-infected supernatant was incubated in the presence of soluble CD4 (50 μg/ml) obtained from the National Institutes of Health AIDS Reference and Reagents Program for 1 h at 4°C prior to infection (40). For these experiments, HIV supernatant and mock-infected supernatant were also preincubated at 4°C for 1 h prior to infection.

Flow cytometry. For analysis of surface Fas expression, 10^6 mock-infected or HIV-infected U937 cells were washed and resuspended in 100 μl of phosphate-buffered saline (PBS)–0.1% azide and incubated with 1 μg of either IgM anti-Fas antibody (Upstate Biotechnology, Inc., Lake Placid, N.Y.) or IgM isotype control antibody MOPC 104E (Organon Teknika) for 15 min at 4°C. The cells were then washed and resuspended in 100 μl of PBS–0.1% azide and incubated with goat anti-mouse IgM FITC-conjugated antibody (Becton Dickinson, San Jose, Calif.) for 15 min at 4°C. The cells were then washed and fixed in 0.2% paraformaldehyde. Subsequently, they were analyzed on a FACSTAR+ flow cytometer. For p24 staining, cells were preincubated with 10 μg of mouse myeloma protein (Sigma, St. Louis, Mo.) for 15 min at 4°C, washed and permeabilized with ice-cold 100% methanol for 15 min, washed, and stained either with IgG isotype control FITC antibody (Becton Dickinson) or with anti-p24 FITC antibody (Virostat, Portland, Maine) for 15 min. The cells were then washed in PBS and fixed in 2% paraformaldehyde and analyzed as above.

Detection of apoptosis in U937 cells. Apoptosis was measured by multiparameter flow cytometry. Cells were incubated in 1 ml of 20-μg/ml propidium iodide (Sigma) for 30 min at 4°C, washed and permeabilized with 25% ethanol–PBS solution, and washed and counterstained with 7 μl of 1-mg/ml Hoechst 33342 (Calbiochem, La Jolla, Calif.) (30). Propidium iodide was used to exclude dead cells, and Hoechst 33342 was used to evaluate the DNA content. Following fluorescence-activated cell sorter (FACS) analysis, data were analyzed by the MODFIT software program on F-DIP-N3-MOD settings. Only the propidium iodide-negative cells (representing the viable population) were analyzed for apoptosis. Cells with hypodiploid DNA content were considered to be those cells undergoing apoptosis. The detection of apoptosis in this way was confirmed by using an apoptosis enzyme-linked immunosorbent assay detection kit (Boehringer-Mannheim, Indianapolis, Ind.). Cell viability was measured by trypan blue staining.

Chromium release assays. Jurkat target cells were labeled with ^{51}Cr (Amersham, Arlington Heights, Ill.) as described previously (57) and pretreated either with phorbol myristate acetate (Sigma) (10 μg/ml) and ionomycin (Calbiochem) (500 ng/ml) or with 10 μg of OKT3 antibody (American Type Culture Collection) per ml before being added to wells as indicated. Spontaneous release was measured by incubating 50 μl of ^{51}Cr-labeled Jurkat T cells with 150 μl of culture medium alone, and maximum release was measured by incubating 50 μl of ^{51}Cr-labeled Jurkat T cells with 150 μl of 10% Triton X-100. Cocultivation of ^{51}Cr-labeled Jurkat T cells with MDM was done at a 1:1 effector/target ratio in a total volume of 200 μl in the presence or absence of 10 μg of fusion protein or $F(ab')_2$ antibodies per ml as indicated. For supernatant transfer experiments, 50 μl of target cells was added to 150 μl of supernatant from either mock- or HIV-infected macrophages. After 15 h of cocultivation, the culture supernatant was harvested and ^{51}Cr activity was analyzed. Percent specific lysis was calculated as follows: [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100 = specific release. The spontaneous ^{51}Cr release in these experiments was ≤25%. Experiments were performed in triplicate wells, and the standard deviation from the mean was less than 10%.

Flow-cytometric determination of PBL apoptosis. At 14 days after mock or HIV infection, MDM were washed and coincubated with 10^6 stimulated peripheral blood lymphocytes (PBLs). PBL stimulation was achieved by incubating monocyte-depleted PBLs for 10 days with 10 μg of PHA per ml and 60 IU of IL-2 per ml. After 30 h of PBL-MDM coincubation, nonadherent cells (containing <5% MDM) were collected and stained with anti-CD3 FITC antibody (Becton Dickinson); this was followed by a 7-min incubation with 1 μg of Hoechst 33342 per ml at 4°C. After being washed, cells were fixed in 0.5% paraformaldehyde and analyzed on a Becton Dickinson FACSTAR Plus flow cytometer. CD3 bright cells were analyzed for Hoechst 33342 content, with the percentage of Hoechst 33342 bright cells representing the apoptotic fraction (23). Specific apoptosis was determined by subtracting spontaneous apoptosis from experimental values.

Northern (RNA) blot hybridization. Total cellular RNA was harvested with RNAzol (Cinna/Biotecx Laboratories, Inc., Houston, Tex.). Where indicated, poly(A) RNA was isolated by using an oligo(dT) extraction kit (Qiagen, Chatsworth, Calif.). Samples were electrophoretically separated on a 1% SeaKem agarose gel (FMC Bioproducts, Rockland, Maine). The gel was then capillary transferred overnight onto a Hybond N^+ (Amersham) membrane with 20× SSC

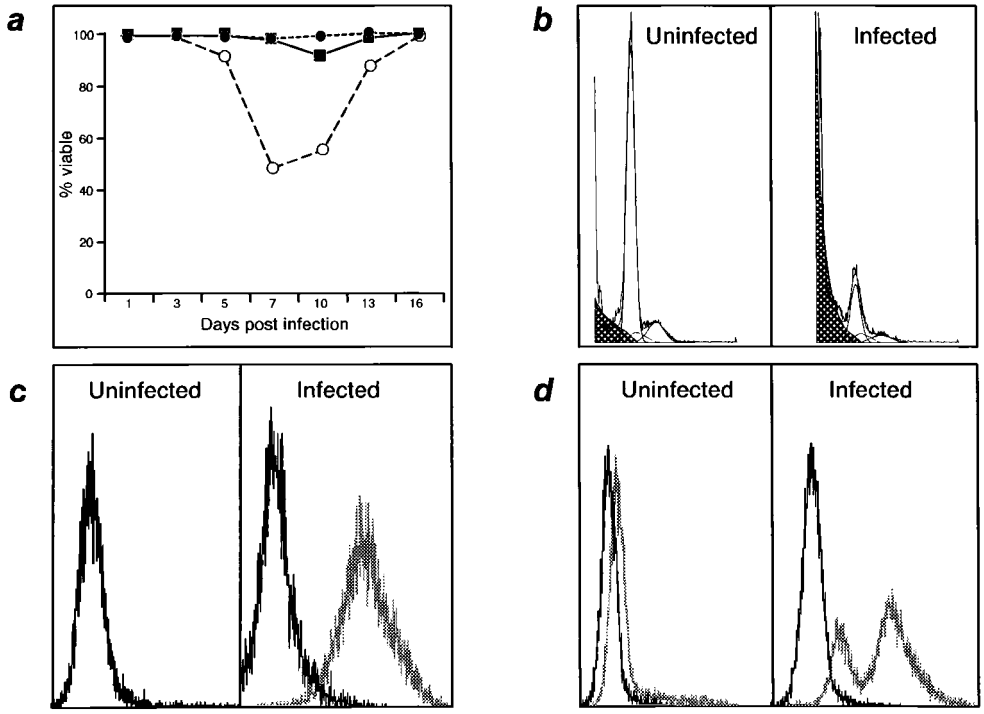

FIG. 1. HIV induces U937 cell death by apoptosis and increases Fas expression. (*a*) U937 cells were either mock infected (●), HIV infected (○), or infected with HIV preincubated with sCD4 (■). Cell survival was analyzed every 3 days by trypan blue staining. (*b*) A total of 10^6 mock-infected (Uninfected) and HIV-infected (Infected) U937 cells were harvested on day 6 postinfection and analyzed for apoptosis by FACS. The hypodiploid population is shaded. (*c*) A total of 10^6 mock-infected (Uninfected) or HIV-infected (Infected) U937 cells were harvested on day 6 postinfection and stained with anti-p24 FITC (grey) or isotype (black) control antibodies. (*d*) A total of 10^6 mock-infected (Uninfected) or HIV-infected (Infected) U937 cells were incubated with anti-Fas (grey) or isotype control (black) antibodies and counterstained with goat anti-mouse IgM FITC antibody.

(1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as a buffer. The membrane was prehybridized with 5× Denhardt's solution–50% formamide–5× SSC–200 μg of sonicated salmon sperm DNA (Sigma) per ml–1% sodium dodecyl sulfate (SDS) solution for 4 h at 65°C. A riboprobe was transcribed by using a T7 polymerase riboprobe system (Promega, Madison, Wis.) with the human FasL cDNA as the template. The cDNA vector was linearized with *Sal*I (Gibco BRL, Gaithersburg, Md.), extracted with phenol-chloroform, and transcribed. After confirmation of the size of the expected riboprobe, the percentage of [^{32}P]UTP incorporation was calculated and 50×10^6 cpm of riboprobe was added to the prehybridization solution. Hybridization was allowed to proceed for 12 to 15 h. The membrane was subsequently washed with increasingly stringent SSC-SDS solutions. Following autoradiography, membranes were stripped with boiling 1% SDS in diethylpyrocarbonate-treated water and membranes were then exposed to film for at least 12 h to ensure no residual radioactive signal. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were [^{32}P](d)CTP labeled with a random-primer DNA-labeling kit (Boehringer Mannheim). Membranes were prehybridized in 0.5 M phosphate buffer (NaH_2PO_4, Na_2HPO_4 [pH 7.0])–7% SDS in diethylpyrocarbonate-treated water for 1 h at 65°C. Then 20×10^6 cpm of labeled probe was added to the prehybridization buffer, and the mixture was incubated for 12 to 15 h at 65°C. Membranes were washed and exposed as described above.

RESULTS

HIV infection of U937 cells results in death by apoptosis. To develop a model in which to study the mechanisms involved in HIV-mediated cell death, we used the cell line U937, which is known to die following HIV infection (34). Cultures of U937 cells were either infected with HIV (IIIB) or mock infected. Mock-infected cells remained viable during the entire period of observation (16 days), whereas over 50% of the HIV-infected U937 cells died between days 5 and 7 postinfection (Fig. 1A). In parallel, HIV stock was preincubated with soluble CD4 prior to U937 infection to neutralize infectious virus. Neutralized HIV did not result in the death of U937 cells (Fig. 1A), indicating that infectious HIV was responsible for the observed cell death. Similar results were obtained with the HIV strain LAV_{BRU} (data not shown). To determine whether HIV-mediated death of the U937 cell line was due to apoptosis, U937 cells were analyzed for DNA content by FACS analysis. A significant increase in hypodiploid DNA is observed in the HIV-infected population on day 6 postinfection (24 h immediately preceding death), indicating that HIV-mediated death was due to apoptosis (Fig. 1B). This finding was confirmed in additional experiments by detection of cytosolic histone-DNA complexes by a commercially available enzyme immunoassay (Boehringer Mannheim) (data not shown). As shown in Fig. 1C, intracytoplasmic p24 was detected in over 90% of cells within the HIV-infected U937 cultures at day 6 postinfection, indicating that the cells undergoing apoptosis are HIV infected.

HIV-induced apoptosis of U937 cells is mediated by FasL-Fas interactions. Fas is a cell surface receptor that has been shown to trigger apoptosis following its cross-linking by certain antibodies or by interaction with its ligand (21, 49, 52). To test whether Fas is present in U937 cells, flow-cytometric analysis with an anti-Fas antibody was performed and demonstrated that uninfected U937 cells express a moderate amount of this receptor on their surface. The amount of cell surface Fas was further upregulated in the HIV-infected population (Fig. 1D). Although FasL has so far been shown to be expressed by lymphoid cells (50), we hypothesize that this ligand was present in the promonocytic U937 cell line and that it is further upregulated following HIV infection. As shown in Fig. 2A, Northern blot analysis revealed detectable levels of FasL mRNA in HIV- but not mock-infected U937 cells on day 5 postinfection, which were further increased (threefold) on day

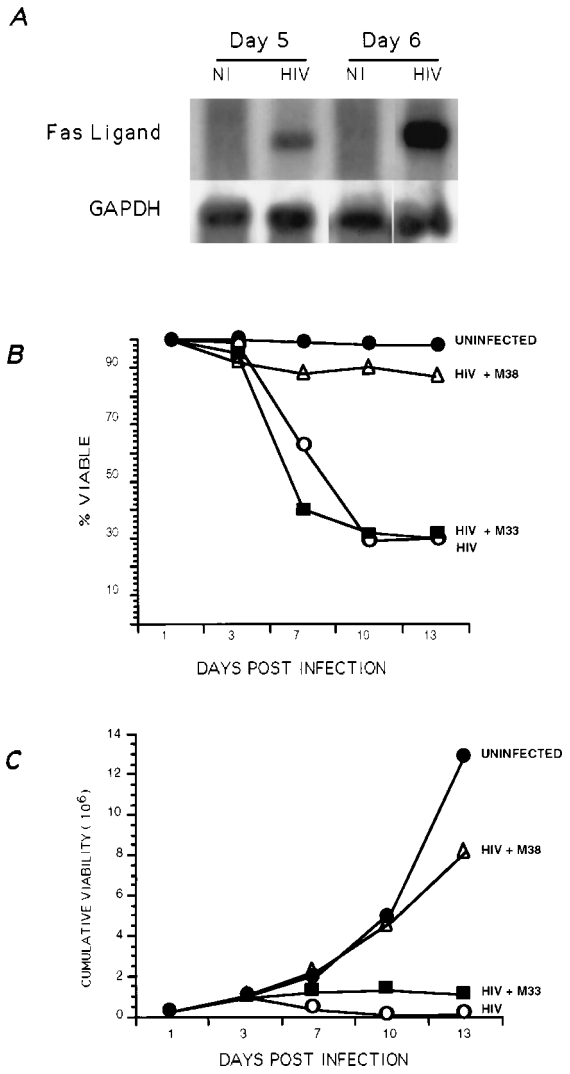

FIG. 2. FasL mRNA is induced by HIV infection, and FasL-Fas interaction mediates the apoptotic death induced by HIV infection in U937 cells. (A) Total RNA was harvested from mock (NI) and HIV-infected (HIV), and poly(A) was isolated by oligo(dT). Northern hybridization for Fas ligand and for GAPDH was performed. (B) U937 cells were mock infected (●) or HIV infected and cultured in the absence (○) or presence of 10 μg of M33 antibody per ml (■) or 10 μg of M38 antibody per ml (△). (C) The same experimental approach as in panel B, except that in this experiment, the cumulative number of viable cells is plotted against time postinfection. These experiments were performed in triplicate wells with a standard deviation of <10% and are representative of three additional experiments.

6 after HIV infection (Fig. 2A), preceding the reduction of cell viability. These data indicate that the promonocytic U937 cell line does not constitutively express FasL mRNA but that HIV infection results in its induction. Therefore, the HIV-induced apoptosis of U937 cells appears to be due to the de novo expression of FasL and not solely to the increased level of Fas on the infected cell.

To determine whether FasL-Fas interactions participate in HIV-mediated apoptosis, we used blocking and nonblocking Fas-specific antibodies. While both M38 and M33 are anti-Fas antibodies which bind to but do not stimulate Fas, M38 (but not M33) blocks the induction of apoptosis induced by FasL and anti-Fas IgM antibodies (1). Mock-infected or HIV-infected U937 cells were cultured with the various Fas-specific antibodies, and cell viability (Fig. 2B) and cumulative cell number (Fig. 2C) were determined. The death rate of HIV-infected U937 cells cultured with nonblocking M33 antibody was similar to that of untreated HIV-infected U937 cells, while HIV-infected cells cultured with M38 blocking antibody survived. M33 or M38 had no detectable deleterious or stimulatory effects on mock-infected cells (data not shown).

HIV-infected MDM have increased FasL expression and mediate apoptosis of uninfected T-lymphoblastoid cells. As noted above, accumulating evidence points to accessory cells such as macrophages as key cells in mediating apoptosis of uninfected T lymphocytes in different in vivo models of AIDS. In addition, human macrophages are a major reservoir of persistent HIV replication and are refractory to HIV-induced cytopathogenicity (15). For these reasons, we examined whether infection of human MDM with monocytotropic strains of HIV results in increased FasL expression and, if so, whether this mediates apoptosis of uninfected T cells.

MDM were either mock or HIV (SF_{162}) infected, and FasL mRNA levels were analyzed on day 14 postinfection. As shown in Fig. 3A and in contrast to U937 cells, low but detectable levels of FasL mRNA were present in the mock-infected MDM. The basal expression of FasL mRNA in mock-infected MDM was observed in additional experiments with different donors and irrespective of detectable or undetectable levels of lipopolysaccharide present in the culture medium (data not shown). However, infection of MDM with a monocytotropic strain of HIV resulted in a fourfold increase in the level of FasL mRNA over that of FasL mRNA in uninfected MDM when normalized to GAPDH mRNA as detected by scanning densitometry. To determine if the heightened expression of FasL mRNA in HIV-infected MDM is capable of inducing apoptotic cell death of a heterologous Fas-sensitive target, a cytotoxicity assay was performed with HIV-infected MDM as effectors and ^{51}Cr-labeled Jurkat T cells (uninfected) as targets. At 14 days after mock or HIV infection, MDM were harvested and incubated with ^{51}Cr-labeled Jurkat T cells in the presence or absence of $F(ab')_2$ M38 or M33 antibodies. HIV-infected MDM resulted in a specific, albeit moderate, lysis of the target cells (Fig. 3B). The role of FasL-Fas interactions in the HIV-infected MDM-mediated killing was confirmed by the blocking effect of M38 but not M33 antibodies.

We next tested whether direct cell contact is required between the FasL-expressing HIV-infected MDM and Jurkat T cells to result in the death of the latter. In addition, and because of the potential release from HIV MDM of soluble forms of FasL (51) and cell-free virions that could influence the killing of Jurkat T cells, we examined whether cell-free supernatant and paraformaldehyde-fixed MDM mediated the killing of Jurkat T cells. To generate supernatants from HIV-infected MDM and paraformaldehyde-fixed HIV-infected MDM, we first confirmed that such HIV-infected MDM selectively mediated the killing of uninfected Jurkat T cells by Fas-FasL interactions (Fig. 4A). Incubation of ^{51}Cr-labeled Jurkat T cells with cell-free supernatant from HIV-infected MDM generated from the experiment shown in Fig. 4A did not result in any detectable killing of these target cells (Fig. 4B). Further, paraformaldehyde fixation of HIV-infected MDM used in the experiment in Fig. 4A did not abrogate their ability to mediate the lysis of Jurkat target cells (Fig. 4C). Overall, this set of experiments indicates that direct contact between an HIV-infected macrophage and its target cell is required to mediate apoptosis through FasL-Fas interaction.

HIV-infected MDM induce apoptosis of PBL. To determine whether FasL-expressing MDM kill nontransformed T cells, activated PBLs were coincubated with mock- or HIV-infected

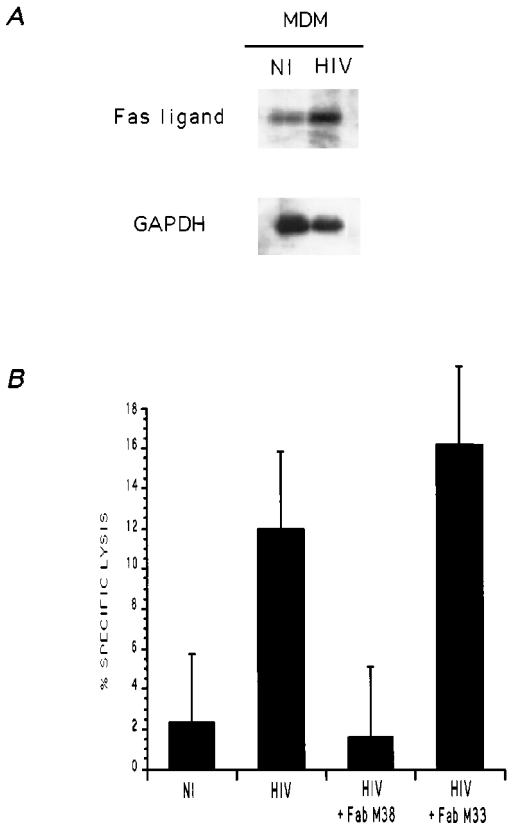

FIG. 3. HIV-infected macrophages have increased levels of FasL mRNA, and HIV-infected MDM mediate killing of Jurkat T cells through FasL-Fas interaction. (A) MDM were HIV infected (HIV) or mock infected (NI). Northern blot analysis of MDM total RNA (14 days postinfection or mock infection) was performed with a FasL riboprobe followed by hybridization with GAPDH to normalize for total amounts of cellular RNA. (B) MDM, 14 days old, mock infected (NI) or HIV infected (HIV) were incubated with ^{51}Cr-labeled Jurkat T cells. F(ab′)$_2$ M38 and M33 Fas-specific antibodies (10 μg/ml) were added or not added to the cytotoxicity assay.

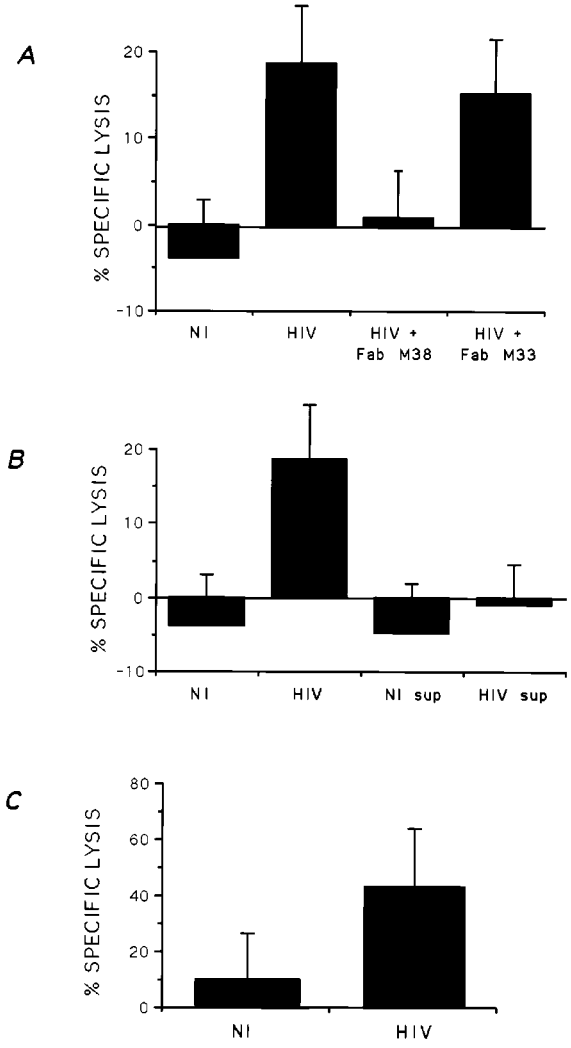

FIG. 4. Cellular interaction of HIV-infected MDM with Jurkat T cells is required for apoptosis. (A) MDM, 14 days postinfection, mock infected (NI) or HIV infected (HIV), were incubated with ^{51}Cr-labeled unstimulated Jurkat T cells in the presence or absence of F(ab′)$_2$ M38 and M33 (10 μg/ml). (B) Same experimental design as for panel A, except that supernatants (sup) from mock-infected (NI) or HIV-infected (HIV) MDM were assayed for their ability to induce Jurkat cell death. (C) MDM, 14 days after mock infection (NI) or HIV infection (HIV), were harvested, washed in PBS, fixed for 5 min in 2% paraformaldehyde, and washed three times prior to incubation in the cytotoxicity assay. Samples were incubated in duplicate.

MDM. Previous data indicate that activation of Fas-bearing lymphocytes is required to render these cells sensitive to Fas-mediated lysis (2, 6, 10, 22, 23, 44). PHA- and IL-2-activated, monocyte-depleted PBLs were coincubated with mock- or HIV-infected MDM, and the amount of apoptosis in T cells was determined by multiparameter flow cytometry. As indicated in Fig. 5, HIV-infected MDM incubated with activated peripheral blood T cells induce T-cell apoptosis, which is partially reversed with the anti-Fas blocking antibody (M38) but not with M33. Interestingly, mock-infected MDM are also able to mediate some degree of apoptosis of activated PBLs via FasL-Fas interaction, albeit to a lower degree than that mediated by HIV-infected MDM. Freshly isolated PBLs from healthy donors were refractory to mock- and HIV-infected MDM-mediated apoptosis (data not shown). In summary, the above results indicate that MDM express functional FasL and that it can be upregulated by HIV infection. Direct cell contact between MDM and a Fas-susceptible target, including peripheral T lymphocytes, will result in apoptotic death of the latter.

DISCUSSION

It has been difficult to reconcile the relatively low frequency of HIV-infected CD4$^+$ T cells in HIV-infected patients (58) with the large number of CD4$^+$ T cells reported to die on a daily basis (20, 54). Thus, it seems likely that direct viral effects do not account for the high level of cell death. An indirect mechanism whereby HIV can mediate the death of uninfected T cells must therefore be responsible. Our studies demonstrate that HIV infection is a novel and efficient stimulus of FasL, which can lead to apoptosis of Fas-bearing cells. Additionally, we provide a model whereby accessory cells such as macrophages not only serve as an HIV reservoir but also can play a crucial role in CD4$^+$ T-lymphocyte depletion.

Of all the indirect mechanisms postulated so far to play a role in CD4 depletion in HIV-infected individuals (3, 13, 18, 36, 45), only apoptosis has been clearly identified to be occurring in patients and in animal models of HIV-induced AIDS (12, 13, 36, 37). Data from these studies confirm that it is not the productively HIV-infected cell that is dying but the uninfected bystander cells, further pointing to an HIV-triggered

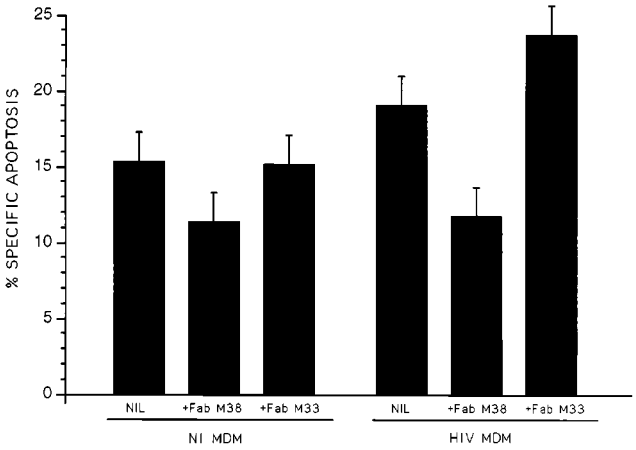

FIG. 5. Mock- and HIV-infected MDM mediate apoptosis of PBLs. MDM, 14 days postinfection, mock infected (NI) or HIV infected (HIV), were coincubated for 30 h with PHA- and IL-2-stimulated PBLs in the presence of M33 or M38 (10 μg/ml). Multiparameter flow cytometry gating on $CD3^+$ target cells was used to quantitate for specific apoptosis. Samples were incubated in duplicate, and this experiment is representative of a total of three additional experiments.

indirect mechanism (13, 14, 36). Cytotoxic cytokines produced by infected cells and/or cytotoxicity to the uninfected bystander cells through direct contact with an infected cell are possible candidate mechanisms. TNF and FasL are two molecules that could fit either of the two hypothetical mechanisms. They belong to a ligand/receptor family which has been implicated in mediating cytotoxicity-apoptosis of cells bearing receptors to the respective ligands (47). The level of circulating TNF is increased in HIV-infected individuals (60), and membrane-bound TNF correlates with disease progression and has been found on the surface of T lymphocytes within HIV-infected lymph nodes (33). The recently cloned FasL (50), which mediates cytotoxicity of Fas-bearing cells (50), can be found in soluble form (51) and has been implicated in the depletion of PBLs as part of the physiological homeostasis of the immune system (48). In the experimental models of HIV-mediated apoptosis used in this study, it is FasL rather than TNF which regulates apoptosis, although we cannot completely exclude a role for TNF in mediating apoptosis in vivo. FasL is expressed in T-lymphocytic cell lines and T-cell hybridomas following T-cell activation (1, 6, 10, 22, 49, 50). Our results indicate that HIV infection can also induce FasL expression. Further, the data demonstrate that FasL is expressed in nonlymphoid cells, thus expanding the potential role of FasL in immunoregulation.

The mechanism(s) by which HIV increases the expression of FasL remains to be studied. To date, the HIV envelope gp160 protein and Tat can lead to apoptosis (29, 31, 32) and both proteins can synergize with FasL expression induced by TCR activation (56). Other HIV proteins will, upon contact with a target lymphocyte, predispose the targeted cell to undergo apoptosis only if the cell is activated. Extracellular gp120/160, through cross-linking of the surface CD4 receptor, will render the lymphocyte susceptible to apoptosis triggered by activation, i.e., TCR cross-linking (4). HIV Tat will result in apoptosis of the host cell upon growth factor withdrawal (31). The underlying mechanism of these observations remains to be elucidated. Although T-cell activation has been shown to be sufficient to trigger apoptosis of lymphoid cell lines, resting PBLs do not undergo apoptosis ex vivo (24, 55, 59). The priming effect mediated by CD4 cross-linking could activate a signal transduction pathway that is required before the activation-induced death can occur. Alternatively, it could induce the de novo expression of a putative receptor necessary for the T-cell activation-triggered ligand to exert its cytotoxic function. Because recent data have established that T-cell activation results in de novo FasL expression (1, 2, 6, 10, 22), it is theoretically possible that cross-linking of the CD4 receptor by gp160 results in the expression of the Fas receptor, which, upon its coupling to the FasL induced by T-cell activation, results in apoptosis. This scenario could also be applied to the fact that T lymphocytes from HIV-infected individuals but not uninfected controls undergo activation-induced apoptosis ex vivo (19, 28, 35, 41), contain elevated levels of Fas on their surface (9, 23), and are intrinsically more susceptible to Fas-dependent killing (23).

Although relevant information about the physiological regulation of FasL is lacking, as in the context of AIDS pathogenesis, FasL could become expressed on the surface of lymphocytes by physiological processes such as antigen presentation of naive T lymphocytes. Moreover, the higher percentage of T lymphocytes described to be activated in HIV-infected individuals (16) could correlate with increased surface FasL expression in these cells. On the basis of recent data indicating increased susceptibility of lymphocytes from HIV-infected individual to Fas-mediated killing (23), it can be extrapolated that encounter of such "primed" lymphocytes with cells that express FasL would result in their elimination by apoptosis. Our data indicate that FasL RNA transcripts can be detected in uninfected human-derived macrophages, although whether these cells express on their surface FasL capable of mediating apoptosis of Fas-bearing cells is unknown. Our results with the Fas-sensitive Jurkat T-cell line indicate that macrophages do not mediate detectable apoptosis of this target cell unless the macrophage is HIV infected. However, this could be due to the sensitivity of the assay and thus cannot exclude a functional role of FasL in uninfected antigen-presenting cells. In addition, we demonstrate that uninfected MDM are capable of killing activated PBLs and that the magnitude of killing is enhanced by HIV-infected MDM. These data imply that in HIV-infected individuals, the rate-limiting step would not be solely the "constitutive" expression of FasL in antigen-presenting cells and in other lymphocyte populations but the "priming" of uninfected lymphocytes for effective FasL-Fas interactions. In addition, HIV-induced upregulation of FasL in human macrophages would amplify this "physiological," situation resulting in a more efficient depletion of Fas-bearing primed uninfected lymphocytes. Thus, HIV-infected macrophages and/or related antigen-presenting cells such as dendritic cells could serve as a continuous source not only of HIV but also of FasL in infected individuals. Future studies should address the expression of the FasL in antigen-presenting cells, especially in HIV-infected individuals, at the level of lymphoid tissue. In addition, HIV-hSCID mouse or simian immunodeficiency (SIV)-macaque models will be valuable tools to test potential therapeutic interventions in AIDS by interfering with FasL-Fas interactions.

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