Antibody Against Integrin Lymphocyte Function-Associated Antigen 1 Inhibits HIV Type 1 Infection in Primary Cells Through Caspase-8-Mediated Apoptosis

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Abstract

HIV-1 infection induces formation of a virological synapse wherein CD4, chemokine receptors, and cell-adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1) form localized domains on the cell surface. Studies show that LFA-1 on the surface of HIV-1 particles retains its adhesion function and enhances virus attachment to susceptible cells by binding its counterreceptor intercellular adhesion molecule 1 (ICAM-1). This virus–cell interaction augments virus infectivity by facilitating binding and entry events. In this study, we demonstrate that inhibition of the LFA-1/ICAM-1 interaction by a monoclonal antibody leads to decreased virus production and spread in association with increased apoptosis of HIV-infected primary T cells. The data indicate that the LFA-1/ICAM-1 interaction may limit apoptosis in HIV-1-infected T cells. This phenomenon appears similar to anoikis wherein epithelial cells are protected from apoptosis conferred by ligand-bound integrins. These results have implications for further understanding HIV pathogenesis and replication in peripheral compartments and lymphoid organs.

Introduction

ONE OF THE MOST CRITICAL STEPS in the life cycle of human immunodeficiency virus type-1 (HIV-1) occurs when viral proteins assemble at the plasma membrane of a newly infected cell and bud to form new viral particles. Acquisition of host cellular constituents by HIV-1 during the budding process is a key property of HIV-1 biogenesis. In addition to virally encoded proteins, HIV-1 can incorporate a vast array of cellular proteins, including CD43, CD55, CD59, and HLA-DR.^{1–5} Included among the cellular membrane proteins incorporated into virus particles are adhesion molecules such as CD44.⁴ Using the CD44-hyaluronate system, we demonstrated for the first time that the adhesion molecules acquired by budding HIV-1 particles retain their function.⁶

Another key adhesion molecule incorporated into nascent HIV-1 particles is lymphocyte function-associated antigen 1 (LFA-1), a member of the leukocyte integrin subfamily of adhesion molecules. LFA-1 is found on cells of leukocyte lineage including neutrophils, monocytes, and lymphocytes.⁷ Upon binding its counterreceptors, intercellular adhesion molecules (ICAMs), LFA-1 participates in the formation of

immunological synapses, T cell activation, and leukocyte trafficking to sites of infection and inflammation.^{8–11} LFA-1 was first implicated in HIV-1 infection with the observation that treatment of susceptible cells with an anti-LFA-1 monoclonal antibody (Mab) blocked HIV-1-induced syncytia.¹² Through interaction with their cognate receptors, the presence of functional adhesion molecules, such as LFA-1, on the HIV-1 membrane serves to enhance virion binding to target cells, which has important implications for virus attachment, infectivity, and tropism.^{2,6,13}

While early studies established that the LFA-1/ICAM-1 interaction was not required for HIV-1 infection, it has been shown that antibodies against LFA-1 can dramatically increase neutralization of primary HIV-1 strains by AIDS anti-serum and gp120 Mab.^{13–16} These results indicate that LFA-1 significantly contributes to the overall binding avidity of HIV-1 to susceptible cells, and as such can work to facilitate virus infection. Moreover, HIV-1 has been shown to also incorporate the LFA-1 ligand ICAM-1 during the budding process. Virally expressed ICAM-1 dramatically increased the infectivity of HIV-1 when exposed to cells expressing functional or activated LFA-1 molecules.¹⁷ Others have shown that

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coexpression of ICAM-1 with the HIV-1 envelope glycoprotein on both infected cells and virus particles can dramatically increase virus-induced syncytium formation and infectivity, respectively.^{17–19} Taken together, these findings illustrate the significant contribution made by adhesion molecules present on the surface of HIV-1 particles to virus attachment.

Incorporation of cellular proteins into the HIV-1 membrane appears to be a selective process. The presence of ICAM-1 and MHC class II adhesion molecules in the viral envelope has been shown to increase HIV-1 infectivity through binding to LFA-1 and CD4, their respective counterreceptors, on target cells.^{17,20} Notably, other cell surface proteins, such as CD45, CXCR4, and CD4, are not incorporated into the virion.^{4,21,22} Selective incorporation of cellular proteins into the viral membrane is largely due to HIV-1 particles budding from cholesterol/glycolipid-enriched membrane lipid rafts.²³ It is unknown whether cell adhesion molecules act solely by enhancing binding events to T cells. Given the many signaling pathways linked to adhesion molecules it is possible that adhesion molecules contribute to HIV infection and pathogenesis in other ways as well. Recent studies show that gp120 binds directly to the integrin $\alpha_4\beta_7$ on CD4/CCR5 T cells by way of a tripeptide in the V1/V2 loop of gp120.²⁴ This interaction leads to activation of LFA-1, thereby facilitating formation of virological synapses and intercellular spread of HIV-1. This appears to be an important mechanism of early virus spread in the gut and possibly the basis for the biological filter selecting for R5 virus transmission.^{25–28}

Another possible way in which LFA-1/ICAM-1 interactions could impact HIV-1 infection is suggested by the role of integrins in apoptosis. In epithelial and endothelial cells, there has been extensive study of anoikis, apoptosis resulting from integrin detachment from ligands.^{29,30} A similar requirement for anchorage dependence has also been described in T lymphocytes.^{29,31,32} More recently, integrin-mediated death has been described wherein cells, though attached, undergo apoptosis due to the presence of unligated integrins. The apoptosis-related cysteine aspartase caspase-8 has been implicated in these processes.^{33,34} Moreover, there is considerable evidence that caspase-dependent apoptosis is induced by HIV-1 infection. It has been shown that caspases 3, 6, 8, and 9 are activated by various HIV-1 proteins.^{35–45} Thus, caspases may represent a link between integrins and HIV-1 infection.

In this study, we investigated the role of LFA-1 in HIV-1 infection and spread in primary T cells. We observed that an anti-LFA-1 Mab, H52, which blocks adhesion, strongly inhibited HIV-1 release and infection in these cells. The antibody could be added as late as 24 h after HIV-1 infection and retain its ability to inhibit virus production and infection without affecting virus protein expression in the infected cells. The ability of H52 to mediate postentry inhibition of HIV-1 replication prompted us to investigate apoptosis of infected cells as a possible mechanism for antibody inhibition of virus replication. To that end, we examined the role of caspase-8-dependent apoptosis in limiting HIV-1 infection when H52 is present. We observed an increase in active caspase-8 expression that correlated with decreased viral protein expression upon H52 treatment of infected cells. Furthermore, we found that blocking caspase-8 activity reversed the observed H52-mediated inhibition of HIV-1 production. These results suggest that LFA-1-mediated signaling in HIV-1-infected cells

may be necessary to prevent apoptosis and concomitantly promote intercellular virus spread.

Materials and Methods

Cells and monoclonal antibodies

Peripheral blood mononuclear cells (PBMCs) from normal donors were isolated from leukopaks (All Cells, Emeryville, CA) by Ficoll-Hypaque (Amersham) density centrifugation. Aliquots were cryopreserved and stored in a liquid N₂ freezer. Cells were cultured at 37°C, 5% CO₂ in complete RPMI (cRPMI): 1:1 volume of RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) and AIM-V medium supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 µg/ml streptomycin, 100 U/ml penicillin, L-glutamine, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES). For each experiment, PBMC were thawed and stimulated with phytohemagglutinin (PHA) (Roche, Indianapolis, IN) (5 µg/ml) for 3 days at 37°C in cRPMI, 5% CO₂. PHA blasts were then grown in cRPMI supplemented with 50 U/ml recombinant human interleukin-2 (IL-2) (Roche Applied Science). PM1 and 293T cell lines were maintained, respectively, in cRPMI and complete DMEM (cDMEM): Dulbecco modified Eagle medium (DMEM) supplemented with 10% FCS, L-glutamine, and HEPES. A culture of PM1 cells chronically infected with HIV-1_{IIIIB} was established in the laboratory and maintained in cRPMI.

The Mabs against LFA-1 β -subunit (CD18) used in this study were produced in our laboratory and are well characterized as previously reported: H52 blocks LFA-1 function and PLM-2 does not.^{46–49}

Virus preparation

HIV-1_{NL4.3} viral stocks were prepared by transfecting 293T cells with pNL4.3 DNA using the Ca₂PO₄ method.⁵⁰ pNL4.3 is a full-length infectious molecular clone of HIV-1.⁵¹ This vector was provided by Dr. Malcolm Martin through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Briefly, 293T cells were plated 24 h before transfection at 50% confluency in cDMEM in 150-cm² flasks. All solutions were brought to room temperature before use. Immediately before transfection, 15 µg of pNL4.3 DNA and 0.5 µg of transfection control pMAX EGFP (AMAXA) were added to 50 µl of 2.5 M CaCl₂ and 440 µl of distilled water. Five hundred microliters of 2X BES-buffered saline (BBS) solution [50 mM BES, 280 mM NaCl, 1.5 mM Na₂HPO₄ (pH 6.95)] was slowly added drop by drop with gently mixing to the DNA mixture and incubated at room temperature for 15 min. The DNA-BBS mixture was finally added drop by drop to the plated 293T cells before incubation at 37°C, 5% CO₂. Virion-containing supernatants were collected 3–5 days after transfection, centrifuged at 4000 rpm to remove cell debris, and filtered through a 0.45-µm filter. The virus was concentrated by ultracentrifugation at 100,000 × g through a cushion of 20% sucrose in phosphate-buffered saline (PBS). The pelleted virus was resuspended in cRPMI, then aliquots were made and stored in a liquid N₂ freezer. The virus was quantified by anti-p24^{Gag} immunocapture ELISA.

Infection assays

PHA-activated PBMCs were washed with RPMI and resuspended in cRPMI supplemented with IL-2 at 50 U/ml for

48 h. Cells were infected with HIV-1_{NL4.3} (60 ng p24 per 5×10^5 cells) by spinoculation⁵² (1200 $\times$ g, 2 h). This was followed by a 16–24 h incubation at 37°C, 5% CO₂ before washing to remove input virus. Cells were left untreated or treated with H52 (20 μ g/ml) or PLM-2 (20 μ g/ml) and incubated at 37°C, 5% CO₂ in the presence of IL-2. At time points indicated, culture supernatants were removed, virus was lysed with 1% Triton X-100, and Gag p24 measured by a standard immunocapture ELISA.

Homotypic adhesion

PHA-activated PBMCs (1×10^6 cells) were infected with HIV-1 and treated with Mabs as previously described.⁴⁶ Twenty-four hours after antibody addition the cells were examined for homotypic adhesion using a light microscope connected to a digital camera. Representative pictures of each well were taken at $\times 40$ magnification.

Adhesion assay

Ninety-six-well microtiter plates (Costar, Cambridge, MA) were coated overnight with goat antihuman IgG, Fc γ specific (Jackson ImmunoResearch) (10 μ g/ml in 10 mM Tris, pH 9.5). The plates were blocked with adhesion buffer (phenol ref-free RPMI/0.5% BSA) for 1 h at room temperature and 50 μ l of recombinant soluble ICAM-Ig (R&D Systems) at 2 μ g/ml was then added to the plates and incubated for 2 h at room temperature. The plates were then washed three times with adhesion buffer.

Cells were pinocytotically labeled with horseradish peroxidase (HRP) at 1 mg/ml in RPMI for 30 min at 37°C and then washed with RPMI.⁵³ Then 100 μ l of labeled cells (3.5×10^5 cells) in RPMI was preincubated with 100 μ l of indicated concentrations of H52 or PLM-2 Mabs for 1 h at 37°C and then added to the coated wells. The plates were centrifuged briefly (2 min) at 300 $\times$ g, incubated at 37°C, 5% CO₂ for 30 min, and washed three times with PBS to remove unbound cells. TMB substrate (0.2 M Na acetate/citrate, pH 4.0, 24 μ g/ml tetramethylbenzidine, H₂O₂) containing 1% Triton X-100 was then added to the wells to lyse the bound cells and detect HRP. After 20 min at room temperature, 0.5 M H₂SO₄ was added to stop the reaction. The absorbance was measured at 450 nm in a Cambridge 750 microplate reader.

Flow cytometry

PHA-activated PBMCs (1×10^6 cells) were washed and stained with H52 (10 μ g/ml) or PLM-2 (10 μ g/ml) for 1 h on ice. Cells were washed and stained with a 1:50 dilution of Alexa Fluor 488 goat antimouse for 1 h on ice. Cells were then washed and analyzed for Mab binding. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, San Jose, CA) using Cell Quest (Becton Dickinson) data acquisition and analysis software.

PHA-activated PBMCs (1×10^6 cells) were infected with HIV-1 and treated with Mabs as described above. Twenty-four hours after antibody treatment cells were processed for flow cytometry analysis. For intracellular p24 staining, cells were fixed in 2% paraformaldehyde, permeabilized with 0.02% saponin, and stained with a 1:200 dilution of KC57-RD1 α -p24 antibody. For Annexin V staining, unfixed cells were washed once in PBS and once in Annexin V binding buffer

(BD Pharmingen). Cells were then stained with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) for 10 min at room temperature, Annexin V [(BD Pharmingen) according to the manufacturer's protocol], and an antibody against CD3 [CD3-Alexa Fluor 700 (BD Pharmingen)], for 20 min on ice. Cells were vortexed, fixed and permeabilized using the Cytofix/Cytoperm kit (Pharmingen). Cells were washed with and resuspended in 100 μ l Perm/Wash buffer and incubated with a 1:200 dilution of the KC57-FITC α -p24 Mab (Beckman Coulter) for 20 min on ice. Data were collected on a FACS Aria (BD Biosciences) instrument and analyzed with FACSDiva (BD Biosciences) and Flowjo (Tree Star) software.

PHA-activated PBMCs were treated with either 2 mM camptothecin (1:250 dilution) or anti-CD95 (50 μ g/ml) and protein G (10 μ g/ml). To these samples were added either medium alone, H52 Mab (20 μ g/ml), or PLM-2 (20 μ g/ml) followed by culture for 24 h. Addition of protein G has been reported to significantly enhance the efficiency of the anti-Fas Mad-induced apoptosis, presumably by cross-linking Fas receptors.⁵⁴ Cells were harvested and analyzed for apoptosis using an Annexin V-PE apoptosis detection kit (BD Pharmingen) following the manufacturer's protocol. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, San Jose, CA) using Cell Quest (Becton Dickinson) data acquisition and analysis software.

Western blotting

PHA-activated PBMCs (1×10^6 cells) were infected or not with HIV-1 and treated with Mabs where indicated as described above. Twenty-four hours after antibody treatment cells were solubilized in RIPA lysate buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, protease inhibitor cocktail (Roche)]. Protein concentrations were determined using a Pierce BCA Protein Assay kit. Five micrograms of protein from each sample was loaded onto a 4–12% NuPAGE Bis-Tris polyacrylamide gel (Invitrogen). Proteins were transferred onto a nitrocellulose membrane by standard techniques. The beta subunit of LFA-1 was detected by immunoblot analysis using H52 (10 μ g/ml) and PLM-2 (10 μ g/ml) followed by antimouse IgG secondary antibody. Other specific proteins were detected by immunoblot analysis with a 1:1000 dilution of rabbit polyclonal antibody against cleaved caspase-8 (Cell Signaling), 1:1000 dilution of rabbit anti- β -tubulin antibody (Sigma), and 1:500 dilution of rabbit HIV-1 human serum. Primary antibodies were detected with a 1:10,000 dilution of horseradish peroxidase-conjugated specific goat antirabbit antibody (Jackson ImmunoResearch) and enhanced-chemiluminescence reagents (Thermo Fisher Scientific, Rockford, IL). The intensities bands were measured using Image J software.

Detection of caspase activity

Five hundred chronically infected PM1/IIIB cells were incubated with 1×10^6 PHA-stimulated PBMCs for 24 h. At 24 h, H52 (20 μ g/ml) was added. Caspase activity was measured according to kit instructions (Clontech, Palo Alto, CA) using caspase substrates, DEVD-AFC and IETD-AFC, which fluoresce upon cleavage. Briefly, at day 3 postinfection, cells were pelleted and lysates were incubated with the appropriate substrates for 1 h at 37°C in a 96-well microplate. Fluorescence

was detected in a Cytoflour 2300 microplate reader (Millipore, Billerica, MA).

Caspase inhibition assay

PHA-activated PBMCs were washed and incubated for 24 h with HIV-1_{NL4.3} (50 ng p24) in 1 ml of RPMI. Cells were then washed with RPMI and resuspended in cRPMI supplemented with IL-2 at 50 U/ml. Caspase inhibitors z-VAD-fmk and z-IETD-fmk (50 μ M) in DMSO or DMSO alone (as vehicle control) were incubated with the infected PHA blasts for 1 h at 37°C, 5% CO₂ prior to the addition of H52 (20 μ g/ml). Culture supernatants were removed, virus was lysed with 1% Triton X-100, and Gag p24 was measured by a standard immunocapture ELISA. To access the effect of caspase inhibitors on apoptosis, z-VAD-fmk and z-IETD-fmk (50 μ M) in DMSO and DMSO (as vehicle control) were incubated with infected PHA blasts for 24 h at 37°C, 5% CO₂ in the presence or absence of H52. The cells were then analyzed for Annexin V binding by flow cytometry as stated previously.

Results

Inhibition of LFA-1-mediated cell adhesion inhibits HIV-1 production in primary cells

To determine the stage of the HIV-1 replication cycle at which anti-LFA-1 Mab H52 inhibits infection, purified virus, produced by transfecting 293T cells with proviral HIV-1 DNA (NL4.3), was added to PHA-stimulated PBMCs for 24 h. The infected cells were washed and then cultured for an additional 6 days. Either anti-LFA-1 Mab H52 or an isotype-matched nonfunction blocking control Mab PLM-2, both of which bind to the β -subunit of LFA-1,⁴⁶ was added to the culture at various times. As shown in Fig. 1A, H52 blocked HIV-1 production when added at time 0 and maintained in the culture for 7 days but only modestly limited virus production measured at day 7 when present only during the initial 24-h

period. Notably, addition of H52 24 h after infection inhibited virus production to levels similar to that observed when the antibody was present during the entire culture period. These data indicated that inhibition of HIV-1 production by H52 is a postentry effect.

To examine the consequence of adding the LFA-1 Mab at later time points, we performed a time course assay. The inhibitory effects of H52 on HIV-1 production decreased when the antibody was added at later time points (Fig. 1B), a finding consistent with antibody-induced death of newly infected cells. Furthermore, the inability of the control anti-LFA1 Mab PLM-2 to affect HIV-1 production demonstrates that inhibition of HIV-1 production required inhibition of LFA-1 adhesion. Inhibition of LFA-1 functional activity by H52 but not by PLM-2 was confirmed using a phorbol ester-induced LFA-1 adhesion assay (data not shown). Since only a small percentage of primary cells is infected at 24 h, antibody-induced death of those infected cells will have a dramatic effect when cumulative virus production is measured at day 7 as spread of the virus through the culture will be prevented. Antibody-induced death of infected cells should occur regardless of the time at which the antibody is added; conversely, antibody addition at later times after infection will have only a marginal effect since the virus will have had the opportunity to spread throughout the culture.

The assays above were performed with an X4-tropic virus that utilizes CXCR4 as coreceptor. CXCR4 through SDF-1-mediated signaling is linked to activation of integrins, including LFA-1, to high avidity states.⁵⁵ CC chemokines can also affect the activation state of integrins.⁵⁶ Therefore we examined the effect of H52 on HIV infection of primary T cells by a panel of primary HIV isolates with characterized tropisms for CCR5 and CXCR4. As seen in Table 1, only viruses that utilize CXCR4 as coreceptor were sensitive to inhibition by H52. These results along with the data above indicated that LFA-1 blockade of infection of primary T cells was specific to CXCR-tropic viruses.

FIG. 1. H52 inhibition of virus production in primary cells. **(A)** Peripheral blood mononuclear cells (PBMCs) (1×10^6) were stimulated with PHA-L, infected with HIV-1 (50 ng p24) for 24 h, and then cultured for 6 days for a total culture period of 7 days. In some wells, the cells were washed to remove input virus at 24 h and H52 (20 μ g/ml) was present during the remainder of the 7 day culture (2nd bar). In other wells, the cells were incubated with monoclonal antibody (Mab) H52 (20 μ g/ml) for 1 h prior to adding virus and then for 24 h before washing away input virus and antibody followed by culture for 6 days (3rd bar). Finally, in some wells, the Mab H52 (20 μ g/ml) was added 1 h prior to the virus and was added back after the 24 h wash and was present for the remainder of the 7-day culture (4th bar). After 7 days, culture supernatants were removed and HIV-1 was measured by p24 ELISA. **(B)** Twenty-four hours after infecting PBMCs with HIV as above, cells were washed and cultured in cRPMI. Mabs H52 (20 μ g/ml) or PLM-2 (20 μ g/ml) were added at the time points indicated. Culture supernatants were removed from all samples at day 7 and HIV-1 was measured by p24 ELISA. The data represent the means $\pm$ standard errors (SEM) from three independent experiments **(A)** or triplicate samples of a single experiment but representative of three separate experiments **(B)**. **p* value of <0.05 versus the control by Student's two-tailed *t* test.

TABLE 1. BIOLOGICAL CHARACTERIZATION OF ANTI-LFA-1-SENSITIVE HIV-1

<i>Virus</i>	<i>LFA-1-sensitive</i>	<i>Replication in PBMCs</i>	<i>CPE in PBMCs (day 7)</i>	<i>CPE in MT2 cells (day 7)</i>	<i>CR usage: CXCR4/CCR5</i>
97-003	No	Low	No	No	CCR5
97-004	No	Moderate	No	No	CCR5
97-025	No	Low	No	No	CCR5
97-063	No	Low	No	No	CCR5
97-099	No	Low	No	No	CCR5
97-174	No	Low	No	No	CCR5
97-177	No	Low	No	No	CCR5
97-409	Yes	High	Yes	Yes	CCR5/CXCR4
97-534	Yes	High	Yes	Yes	CCR5/CXCR4
97-598	No	Moderate	No	No	CCR5

A panel of clinical HIV isolates was obtained from the NIH AIDS Reagent program and was characterized for use of chemokine receptors on GHOST cells. The viruses were used to infect phytohemagglutinin (PHA)-activated PBMCs, or MT2 cells that express high levels of CXCR4 and show high levels of CPE when infected by X4-tropic viruses. The effect of H52 on infection of primary T cells by the viruses was determined exactly as described in Materials and Methods. Viruses were scored as "sensitive" if the antibody inhibited infection by more than 80%. Replication was rated by infecting 1 million PBMCs with 10 ng (p24) of the virus and after 24 h, input virus was washed away followed by culture for 6 days. Gag p24 was measured by ELISA (low: <250 pg/ml; moderate: 250 to 1000 pg/ml; high: >1000 pg/ml). CPE was scored as "Yes" if balloon cells at least 10 cell diameters in size were noted in any of the cultures.

LFA-1, lymphocyte function-associated antigen 1; PBMCs, peripheral blood mononuclear cells; CPE, cytopathic effect.

H52 inhibits LFA-1-mediated cell-to-cell adhesion in primary cells

Cell-to-cell transmission, made possible through the virological synapse (VS), is the most efficient mechanism by which HIV-1 infects T cells.⁵⁷ VS formation relies heavily on LFA-1-mediated adhesive interactions with ICAMs; thus LFA-1-mediated adhesion plays a critical role in cell-to-cell transmission.⁵⁸ To examine the effect of H52 on LFA-1-mediated adhesion in primary T cells, PHA-activated T cells were treated with increasing concentrations of H52 and PLM-2 and then added to soluble ICAM-1-coated plates. H52 inhibited cell adhesion to ICAM-1 in a dose-dependent fashion with inhibition observed at concentrations of 0.8 μ g/ml and higher (Fig. 2A). We next tested the antibody for inhibition of LFA-1-mediated cell-to-cell adhesion (agglutination) of PHA blasts. As seen in Fig. 2B, H52 strongly inhibited cell-to-cell adhesion of primary cells while the control antibody PLM-2 did not block cell adhesion, regardless of HIV-1 infection. To validate that both H52 and PLM-2 bind to LFA-1 molecules on PHA-activated T cells, PHA-activated T cells were either lysed for western blot analysis or fixed and stained for flow cytometry assays. As shown in Fig. 2C, H52 and PLM-2 both recognize the 93-kDa beta subunit of LFA-1 on PHA-activated T cells (left panel) and bind to more than 90% of the cells (right panels). These data confirm previously published data showing that H52 and PLM-2 bind to human CD18.⁴⁶ Altogether, these results confirm that H52 prevents LFA-1-mediated cell-to-cell adhesion in primary T cells in the presence and absence of HIV-1 infection.

Inhibition of LFA-1-mediated adhesion reduces the number of HIV-1-infected primary cells

We have demonstrated that H52 is able to block virus production when added at an early time point in a 7-day culture of HIV-1-infected primary T cells. We used flow cytometry to assess the frequency of infected cells when H52 or the control antibody was added to cultures of primary T cells 24 h after adding HIV-1. Cells were collected 24 h later, fixed,

permeabilized, and stained with an antibody against HIV Gag. As seen in Fig. 3A, there was a 50% reduction in the number of Gag-positive cells in cultures treated with H52 as compared to untreated cells. By contrast, there was no change in the percent of Gag-positive cells in cultures treated with the control antibody. Moreover, the mean channel fluorescence intensity of the positive cells was similar in all three cases, indicating that while the number of cells infected by HIV-1 was reduced by H52, the level of viral protein expression was not affected (Fig. 3B). This indicates that the effect of H52 is not due to reduced viral protein expression. Collectively, the results indicated that treatment of infected primary cells with the function blocking anti-LFA-1 antibody greatly reduced the number of HIV-positive cells.

Inhibition of LFA-1-mediated adhesion induces caspase-8 activity in HIV-1-infected primary T cells

Since H52 is capable of blocking HIV-1 production as late as 24 h after exposing cells to the virus, it appears that it must be blocking infection at a stage after viral entry. Caspase-dependent apoptosis has been associated with both HIV-1 infection and lack of integrin engagement. We hypothesized that blockade of LFA-1 may induce caspase activity. To test this idea, primary T cells were infected with HIV-1 for 24 h and the LFA-1 antibodies were added as described above. Twenty-four hours later the cells were lysed and subjected to immunoblot analysis. The expression of active caspase-8 was markedly increased in HIV-1-infected cells treated with H52, seen by an increase in band intensity (Fig. 4A). Active caspase-8 expression in the infected cells treated with the control antibody remained unchanged. This increase in active caspase-8 expression was not seen in cells infected with the virus but not exposed to H52 or in uninfected cells exposed to H52. The increase in active caspase-8 levels induced by H52 was associated with a significant decrease in cell-associated HIV-1 p24 as seen by decreased band intensity (Fig. 4A). Using a microplate-based fluorescence caspase assay, we also examined the effect of the antibodies on caspase-8 activity. Consistent

FIG. 2. H52 strongly reduces phytohemagglutinin (PHA)-mediated cell-to-cell adhesion of PBMCs. **(A)** Horseradish peroxidase (HRP)-labeled PHA-activated PBMCs were either left untreated or treated with varying concentrations of H52 or PLM2 Mabs for 1 h at 37°C and then added to a 96-well plate coated with soluble intercellular adhesion molecule-1 (ICAM-1). After incubation for 30 min at 37°C, TMB substrate containing 1% Triton X-100 was added to lyse the cells and detect pinocytosed HRP. The reaction was stopped by the addition of 0.5 M H₂SO₄ and the absorbance was measured at 450 nm. **(B)** PHA-activated PBMCs were infected with HIV-1 (60 ng p24) or exposed to medium alone (mock) for 24 h. Twenty-four hours after infection, mock-infected and infected cells were incubated with H52 (20 μ g/ml) or PLM2 (20 μ g/ml). Twenty-four hours after Mab treatment, cell agglutination was observed under an inverted microscope at a magnification of $\times 40$. **(C)** PHA-activated PBMCs were either lysed for immunoblotting or fixed and stained for flow cytometry. The specificity of H52 (10 μ g/ml) and PLM2 (10 μ g/ml) for the beta-subunit of lymphocyte function-associated antigen 1 (LFA-1) (CD18) was confirmed by immunoblotting (left panel) confirming previous data. The binding of both antibodies to PHA blasts was confirmed by flow cytometry (right panel). The data represent the means $\pm$ SEM from triplicate samples of a single experiment and are representative of three separate experiments **(A)**. **p* value of <0.05 versus the control by Student's two-tailed *t* test.

with the immunoblot results, we observed an increase in caspase-8 functional activity when H52 was added to HIV-1-infected primary T cells (Fig. 4B). The antibody-induced increase in caspase-8 activity peaked at day 3 after infection (data not shown). We also observed a slight increase in caspase activity with HIV-1 infection alone; this is not surprising given the evidence for HIV-1 induction of caspase-dependent apoptosis.^{37,45} Taken together, these findings suggest that inhibition of LFA-1 function with an Mab results in induction of caspase-8 activity in HIV-1-infected primary T cells.

Caspase inhibitors reverse anti-LFA-1 antibody-mediated inhibition of HIV-1 infection

If caspases or caspase-dependent apoptosis are involved in the H52-mediated inhibition of HIV-1 infection, caspase inhibitors should reverse H52 inhibition of infection. We evaluated the effect of z-VAD-fmk, a pan-caspase inhibitor, and z-IETD-fmk, a caspase-8 inhibitor, on inhibition of HIV-1 infection by H52 and on Annexin V expression. To evaluate the effect of the inhibitors on inhibition of infection, the experiment was performed as described previously. Primary T cells were infected with HIV-1 for 24 h. The inhibitors were added followed by incubation for 1 h. The Mabs were then added and the cultures were incubated for an additional 48 h. As shown in Fig. 5, both inhibitors abrogated H52-mediated inhibition of HIV-1 infection (Fig. 5A and B). At 3 days postinfection (2 days post-Mab treatment), when infection had not

spread thoroughly through the culture, the level of infection in the presence of H52 and the inhibitors was more than 90% of the level of infection seen with no H52, indicating very little inhibition of infection with H52 in the presence of the inhibitors (Fig. 5B). However, caspase inhibitors also decreased overall virus production in the absence of H52 treatment. This latter observation may reflect the finding that Casp8p43, a cleavage product of caspase-8, is a potent activator of HIV LTR.⁵⁹ This suggests that caspase-8 may have opposing effects in the present study: promoting HIV-1 transcription and initiation of apoptosis. To confirm that abrogation of H52 inhibition of infection was associated with blockade of apoptosis, we analyzed infected cells cultured with H52 alone or H52 plus the caspase-8 inhibitor by flow cytometry for Annexin V binding. As seen in Fig. 5C, in the presence of the caspase-8 inhibitor Annexin V binding in the presence of H52 was similar to that of control cells (no antibody). This result showed that the caspase inhibitor blocked H52-induced apoptosis in HIV-1-infected primary cells.

Inhibition of LFA-1-mediated cell adhesion increases apoptosis in HIV-1-infected primary cells

We have shown that anti-LFA-1 antibody blocks infection or spread of HIV-1 in primary T cells. This could result from death of either the infected cells or the surrounding uninfected cells, either of which would limit the spread of virus to other cells. To determine which of these two populations was in fact

FIG. 3. H52 reduces HIV-1 infection in primary cells. PHA-activated PBMCs were infected with HIV-1 (60 ng p24) or exposed to medium alone for 24 h and treated with H52 (20 μ g/ml) or PLM2 (20 μ g/ml) for 24 h postinfection. **(A)** At various time points cells were fixed, permeabilized, and stained with KC57-RD1 (α -p24 antibody), analyzed by flow cytometry. Data shown are percent positive cells. **(B)** Forty-eight hours after adding the antibodies, infected cells were fixed and permeabilized before labeling with KC57-RD1 (gray curve) or an isotype-matched control antibody (dashed curve). Flow cytometry analysis was performed and the intracellular expression of p24 was determined. Percent positive cells is shown in the top panels and mean fluorescence intensity (MFI) of the positive population is shown in the lower panels. The quantitation represents the means $\pm$ SEM of the results of three independent experiments **(A)**. * p value of <0.05 versus the control by Student's two-tailed t test.

undergoing apoptosis in our model system, we employed an Annexin V binding assay. Primary cells were infected with HIV-1 and the Mabs were added 24 h later. The cells were cultured for an additional 24 h and then stained with Annexin V-PE and examined by flow cytometry. Anti-CD3 and anti-HIV-1 Gag Mabs were also used to allow gating on T cells and HIV-1-positive cells, respectively. Gating on the CD3-positive cell population to ensure only T cells were being captured, we analyzed Annexin V staining profiles in both intracellular Gag-positive and Gag-negative populations in HIV-1-infected cultures. In examining the noninfected cells (Gag-negative) there is little or no difference in Annexin V expression between the untreated, PLM-2-treated, and H52-treated cells (Fig. 6). However, H52 treatment substantially increased Annexin V binding to infected (Gag-positive) cells (Fig. 6). The results demonstrate that H52 selectively increased Annexin V binding in the HIV-1-infected cell population indicating that the inhibitory effect of H52 on HIV-1 replication in primary T cells results from enhanced apoptosis of infected cells. This

result is consistent with a model in which anti-LFA-1 blocks spread of HIV-1 by eliminating the infected cells through programmed cell death.

H52 Mab does not affect apoptosis induced in primary cells by camptothecin or anti-CD95

To evaluate the specificity of the LFA-1/ICAM-1 interaction on HIV-induced apoptosis, we sought to investigate whether LFA-1/ICAM-1 interactions could limit apoptosis in primary T cells treated with known apoptosis-inducing agents. Primary T cells were incubated with either camptothecin or anti-CD95 and protein G to induce apoptosis in the presence of medium alone, H52, or PLM-2 (both at 20 μ g/ml). Twenty-four hours after treatment, cells were analyzed for Annexin V binding via flow cytometry as described above. As shown in Fig. 7, blocking LFA-1/ICAM-1 interactions in primary T cells with H52 Mab had little or no effect on apoptosis induced by well-characterized proapoptotic agents (white bar

FIG. 4. H52 induces caspase-8 activity in HIV-1-infected PHA-stimulated PBMCs. PHA-activated PBMCs were infected with HIV-1 (60 ng p24) or exposed to medium alone for 24 h. **(A)** Cells were washed, treated with H52 (20 μ g/ml) or PLM2 (20 μ g/ml) for 24 h, and cell lysates were analyzed for active caspase-8 by western blot analysis. The intensities of active caspase-8 and HIV-1 p24 bands were measured using Image J software and normalized to the β -tubulin band of its corresponding treatment (indicated below the lanes). **(B)** Twenty-four hours postinfection, H52 (20 μ g/ml) was added to the cell cultures. At day 3 postinfection, cells were lysed and caspase-8 activity was measured using a conjugated caspase-8 substrate (IETD-AFC) that fluoresces upon cleavage (Clontech). Caspase-8 activity from control cells (no HIV-1, no H52) was subtracted. Four replicate infections were performed for each condition. Error bars show the standard deviation **(B)**. *p* values (Student's *t* test) are shown for comparisons between sample groups.

vs. dotted bar). These results suggest that the role of the LFA-1/ICAM-1 interaction in limiting apoptosis in HIV-1-infected primary T cells may be specific to this biological setting but not relevant in others.

Discussion

In the present study, we demonstrate that inhibition of LFA-1/ICAM-1-mediated adhesion with an Mab can limit HIV-1 infection and spread in cultures of primary T cells. In addition, we report that this loss of adhesion leads to an increase in caspase-8 activity and apoptosis only in cells infected

with HIV-1. Together our results suggest that LFA-1-mediated cell-cell adhesion may be required to prevent programmed cell death in HIV-1-infected primary T cells. This appears to be a novel mechanism by which an LFA-1 antagonist inhibits HIV-1 infection: inducing the T cell equivalent of anoikis as normally observed in epithelial cells. Since HIV-1 replication is known to occur primarily in lymphoid organs where cell-cell interactions are facilitated,⁶⁰ our observations are highly relevant to HIV-1 pathogenesis. We found that the effect of the anti-LFA-1 was specific to HIV-1 strains that utilize CXCR4 as coreceptor. Thus signaling through CXCR4 but not other chemokine receptors appears to be responsible

FIG. 5. Caspase inhibitors reverse inhibition of HIV infection and induction of apoptosis by H52. HIV-1 (50 ng p24) was incubated with 1×10^6 PHA-stimulated PBMCs for 24 h at 37°C, 5% CO₂. The cells were then washed and incubated with DMSO (control) or the caspase inhibitors, 50 μ M [z-VAD-fmk (pan-caspase) or z-IETD-fmk (caspase-8)] for 1 h at 37°C, 5% CO₂. H52 (20 μ g/ml) was added and after 3 days of culture, supernatants were removed and HIV-1 p24 was measured by ELISA. Black bars show infection with no treatment, and gray bars show infection in the presence of H52 **(A)**. **(B)** Infection in the presence of H52 and either caspase inhibitors or vehicle as a percent of control (no H52). **(C)** To confirm that abrogation of H52 inhibition of infection was associated with inhibition of apoptosis, we performed flow cytometry analysis for Annexin V binding on HIV-infected cells exposed to H52 in the presence of DMSO or IETD for 24 h. Data shown are the percent of cells showing positive Annexin V binding. The data from **(A)** and **(C)** represent the means $\pm$ SEM from triplicate samples of a single experiment and are representative of three separate experiments.

FIG. 6. H52 induces apoptosis of HIV-1-infected cells. PHA-activated PBMCs were infected with HIV-1 (60 ng p24) or exposed to medium alone for 24 h and left either untreated or treated with H52 (20 μ g/ml) or PLM2 (20 μ g/ml) for 24 h. Cells were stained with CD3-Alexa Fluor 700, Annexin V-PE, and KC57-FITC. The cells were subjected to flow cytometry analysis and gated on CD3⁺ cells for analysis of the total T cell population. Red, untreated cultures; blue, H52-treated cultures; orange, PLM2-treated cultures.

for protecting primary T cells from unliganded LFA-1-induced death. This is interesting in that CXCR4 is known to mediate rapid activation of LFA-1 to a high avidity state.⁵⁵ Furthermore, CXCR4 has been shown to prevent detachment-induced death—anoikis—in tumor cells.⁶¹ Thus signaling through CXCR4 but not other chemokine receptors appears to be responsible for protecting primary T cells from unliganded LFA-1-induced death.

Cell-to-cell transmission is the most efficient means of HIV-1 infection.⁶² The virus has evolved mechanisms to allow it to take advantage of normal immunological processes including formation of virological synapses, analogous to immunological synapses formed between T cells and antigen-presenting cells. These cell-cell junctions, stabilized by integrins, facilitate receptor/coreceptor interactions with gp120 while supporting signaling pathways resulting in cell activation. The counterreceptor interaction between the integrin LFA-1 and ICAM-1 is one that is critical for immune recognition and activation. Since this interaction is also critical for the formation and stabilization of cell-to-cell adhesion processes it is not surprising that HIV-1 exploits integrins for its benefit.⁶³ Virological synapses formed between HIV-1-infected T cells and uninfected target cells involves recruitment of HIV-1 receptors, coreceptors, adhesion molecules, and cytoskeletal proteins, and, like immunological synapses, relies on LFA-1-mediated adhesion to ICAM-1.^{58,64} This in turn facilitates infection by directed budding and fusion of newly generated virions to interacting target cells. Interestingly, since adhesion molecules are recruited to virological synapses where virus budding also occurs, this may explain the efficient incorporation of adhesion molecules by HIV-1. Furthermore, since these adhesion molecules are the ones mediating cell-cell adhesion they would also likely facilitate virus-cell interactions thereby increasing the likelihood of infection. Our studies demonstrate that loss of LFA-1-mediated adhesion to ICAM-1 not only reduces cell-to-cell adhesion but also inhibits

viral production and infection. This inhibition of infection occurred in PBMCs stimulated with PHA-L, a lectin that cross-links several cell surface molecules thereby increasing LFA-1's expression and avidity for ICAM-1.⁶⁵ These results are in line with data from recent studies showing that small molecule inhibitors of LFA-1/ICAM-1 interactions prevent cell-to-cell adhesion and HIV infection.^{66,67}

The fact that the anti-LFA-1 antibody could be added to T cell cultures 24 h after adding HIV-1 indicated that the antibody was possibly mediating its effect through a postentry mechanism. Considering that apoptosis is an important

FIG. 7. H52 Mab does not affect apoptosis induced by camptothecin or anti-CD95. PHA-activated PBMCs were treated with either 2 mM camptothecin (1:250 dilution) or CD95 (50 μ g/ml) and protein G (10 μ g/ml). To these samples were added medium alone (white bar), H52 Mab (20 μ g/ml; dotted bar), or PLM2 Mab (20 μ g/ml; hashed bar) for 24 h. Cells were stained with Annexin V-PE and analyzed by flow cytometry. The data represent the means $\pm$ SEM from triplicate samples of a single experiment and are representative of three separate experiments.

cellular mechanism linked to both HIV-1 and integrin biology, we investigated apoptosis as a mechanism of HIV-1 inhibition by H52. Our data indicate that HIV-1-infected T cells are induced to undergo programmed cell death if interactions between the integrin LFA-1 and its counterreceptor are blocked. As noted above, this phenomenon appears similar to anchorage dependence in epithelial and endothelial cells.^{29,30,68} In fact, these adherent cells normally require integrin-mediated adhesion in order to survive, and the downstream signals resulting from integrin activation cooperate with those from soluble factors to prevent apoptosis.^{69–73} Lack of integrin-mediated adhesion or inappropriate adhesion results in apoptosis that in this scenario has been termed anoikis, Greek for “homelessness.”⁷⁴ Our data are consistent with observations made in other cell types in which integrin adhesion can counteract signals for apoptosis and provide “survival signaling.”^{34,75} We propose that cell adhesion mediated by LFA-1 in HIV-1-infected T cells results in antiapoptotic signals and cell survival.

The ability of HIV-1 to induce apoptosis in infected and uninfected cells is believed to account for much of the depletion of CD4⁺ T lymphocytes and the immune dysregulation that leads to acquired immune deficiency syndrome (AIDS).^{76–78} While our studies did not identify specific HIV-1 proteins that lead to activation of caspase-8 to initiate apoptosis when the LFA-1/ICAM-1 interaction is blocked, induction of caspase activity has been attributed to HIV-1 Env, protease, and the accessory proteins tat, nef, vpr, and vpu.^{35–38,44,45,79–81} Our data showed that X4 tropic and dual tropic viruses induced LFA-1-dependent resistance to apoptosis but not R5 tropic viruses. This result suggests that proteins specifically associated with X4/dual tropic viruses may be responsible for the phenomenon. An obvious candidate is the envelope glycoprotein, which determines viral chemokine receptor specificity. Further studies with viral protein expression constructs or mutant virus clones are needed to clearly define which viral protein is responsible for LFA-1-dependent survival of T cells.

Although our results showed that integrin engagement is important in HIV-1 infection, they are not necessarily at odds with previous studies showing that primary LFA-1-deficient cells from individuals with leukocyte adhesion deficiency (LAD) efficiently propagate HIV-1 infection.^{14,16} This may be explained in part by the expression on lymphocytes of other integrins, such as VLA-4, which mediates intercellular adhesion functions similar to LFA-1. Also, it appears that the presence of an unligated or antagonized integrin may be a positive signal for apoptosis, while the absence of that integrin is not necessarily detrimental. Studies of $\alpha_v\beta_3$ adhesion in endothelial cells have shown that integrin antagonists cause endothelial cell death and block development,^{82–84} while knockout mice that lack this integrin can develop normally.⁸⁵ This has been explained by conclusions drawn from the studies of integrin-mediated death (IMD), in which an unligated integrin directly induces apoptosis.³⁴ IMD was observed for β_1 and β_3 integrins.³⁴ β_2 is evolutionarily related to β_1 and β_3 , and it contains an amino acid sequence with homology to the region in β_1 and β_3 linked to IMD.

Altogether, the data presented in this study provide evidence that treatment with agents that block LFA-1/ICAM-1 interactions may help reduce the spread of HIV-1, not only by inhibiting cell-to-cell transmission, but by leading to the death

of infected cells. As in our model system, early administration of an LFA-1 antagonist to HIV-1-infected persons could have a profound affect by drastically reducing the burden of infected cells. Furthermore, these agents might also limit the migration of infected cells to lymph nodes in the early stages of infection. Both of these actions would be predicted to suppress viral loads and limit spread of the virus in the infected host. Recent reports indicate that integrin $\alpha_4\beta_7$ on CD4/CCR5 T cells serves as a gp120 receptor. Binding of gp120 to this integrin leads to activation of LFA-1 and efficient formation of the virological synapse, critical for HIV-1 infection in these T cells.²⁴ These T cells are major targets of HIV in the gut. Our results indicate that the LFA-1 antagonist may also offer protection from the devastating depletion of gut T cells normally associated with HIV infection.

The ability of HIV-1 to propagate itself depends on a careful interplay between viral and host molecules. The virus has been shown to modulate expression of dozens of host proteins. It is well established that HIV-1 infects activated T cells and that HIV-1 infection up-regulates the expression of ICAM-1 and LFA-1 in lymphoid tissues and peripheral blood lymphocytes.^{86,87} It is also well established that replication of HIV-1 in lymphoid organs is sustained and highly robust.^{88,89} This may in part be related to the fact that in lymphoid tissue T cells make extensive and efficient contact with surrounding cells, including dendritic cells, through integrin-ligand interactions. Thus, HIV-1-infected T cells would be protected from apoptosis through integrin-mediated adhesion. Moreover, it is thought that integrins are involved in viral transmission in the lymph nodes early in the infection process. We have shown that H52 inhibits HIV-1 infection by a novel mechanism involving caspase activity and apoptosis. Agents that target integrins may have the potential to prevent or attenuate HIV-1 infection.

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Author Disclosure Statement

No competing financial interests exist.

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