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Impact of Fibronectin Fragments on the Transendothelial Migration of HIV-Infected Leukocytes and the Development of Subendothelial Foci of Infectious Leukocytes1

Holly H. Birdsall,2*† Wendy J. Porter, * David M. Green,‡ Jose Rubio,§ JoAnn Trial,§ and Roger D. Rossen3†

Leukocyte infiltrates that can serve as viral reservoirs, and sites for viral replication are found in many organs of HIV-1-infected patients. Patients whose blood leukocytes migrate across confluent endothelial monolayers ex vivo and transmit infectious virus to mononuclear leukocytes (MNLs) lodged beneath this endothelial barrier have a worse prognosis. We evaluated the ability of 110- to 120-kDa fibronectin fragments (FNf), which are found in the blood of >60% of HIV-1-infected patients, to stimulate transendothelial migration and drive productively infected MNLs into a potential perivascular space. FNf induced MNLs to release TNF-α in a dose-dependent fashion; the resulting increase in lymphocyte and monocyte transendothelial migration could be blocked with soluble TNF receptor I. Rather than penetrate deeply into the subendothelial matrix, as is seen with untreated controls, FNf-treated MNLs clustered just below the endothelial monolayer. Treatment with FNf during migration increased subsequent recovery of HIV-infected cells from the subendothelial compartment. FNf treatment also significantly increased the numbers of HLA-DRbright, dendritic-type cells that reverse-migrated from the subendothelial depot to the apical endothelial surface 48 h after migration. Fibronectin fragments can be produced by viral and host proteases in the course of inflammatory conditions. The ability of FNf to stimulate transendothelial migration of HIV-1-infected MNLs may help to explain the dissemination of this infection into cardiac, renal, and CNS tissues. The Journal of Immunology, 2004, 173: 2746–2754.

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3 Abbreviations used in this paper: MNL, mononuclear leukocyte (monocytes and lymphocytes); CS-1, connecting segment; FN, fibronectin; FNf, 110- to 120-kDa FN fragments; TNF sRI, soluble TNF receptor I.
Materials and Methods

**MNls and transendothelial migration under static conditions**

Peripheral blood MNls, prepared by Ficol/Hypaque density centrifugation, were added to the apical surfaces of confluent monolayers of HUVECs (Lonza, Walkersville, MD) or MCF-7 (American Type Culture Collection, Manassas, VA) in triplicate. MNls, isolated weekly (32), were used at the first or second passage after an ~3-fold expansion in serum- and growth factor-containing endothelial cell medium (proprietary recipe; Cell Systems, Kirkland, WA). Endothelial monolayers were grown on a matrix of hydrated collagen (Vitrogen; Celtrix, Palo Alto, CA) containing 20 g/ml native FN (Sigma-Aldrich, St Louis, MO) (19). MNls, suspended in RPMI 1640 (Invitrogen Life Technologies, Grand Island NY) with 10% FCS (HyClone, Logan UT) were allowed to migrate, typically for 2 h, through the endothelial monolayer into the collagen matrix that supported the endothelial cells. To assess how deeply migrating MNls penetrated the collagen gel beneath the HUVECs, we used an inverted microscope to count the number of MNls per high power field in serial planes, spaced at 100-μm intervals through the collagen pad. Three series of planes were counted for each well.

To enumerate the migratory population, nonadherent cells were washed away with Ca2+- and Mg2+-free HBSS, and cells that were adherent, but had not migrated through the endothelium, were then removed with trypsin (19). Migratory MNls were subsequently harvested by digesting the subendothelial matrix with collagenase (Sigma-Aldrich). To enumerate the original and migratory populations, cells were incubated with fluorochrome-labeled lymphocyte-specific (CD45) and monocyte-specific (CD14) mAbs (LeucoGATE; Coulter, Hialeah, FL) and were counted by flow cytometry using FlowQuant beads (Coulter) as an internal reference.

To assess the ability of MNls that had migrated below the endothelial barrier to reverse migrate back from the basal to the apical surface, we allowed MNls to migrate for the customary 2 h. After the nonmigratory cells were washed away with complete medium, we refed the cultures with medium 199 (Invitrogen Life Technologies) containing 20% human type AB serum. Two days later, the reverse-migrating MNls were collected by gently washing the surface of the HUVECs with warm complete medium so as not to disturb the integrity of the endothelium.

**Treatment of MNls**

The media, cytokines, and Abs used in these experiments contained <0.03 endotoxin unit/ml endothaxin as determined by the Limulus amebocyte assay (Associates of Cape Cod, Woods Hole, MA). Purified human FN enriched for the 110- to 120-kDa chymotrypsin fragment, hereafter called FNf, was obtained from Invitrogen Life Technologies (Gaithersburg, MD) and was used to assay migration under flow conditions. This quantity of FNf contains ~105 pg/ml TNF-α and ~100 pg/ml soluble TNF-α, which is 10-fold more TNF-α than that released by MNls as they migrate through HUVECs. To quantify the TNF-α released by migrating MNls, aliquots of the supernatant were collected at the end of the 2-h migration assay and tested with a chemiluminescent TNF-α kit (R&D Systems).

To purify lymphocytes, monocytes were allowed to phagocyte iron beads and were removed with a magnet. Resident monocytes were removed by adherence to plastic for 30 min at 37°C. This protocol leaves <0.1% CD14+ monocytes, as determined by flow cytometry (18, 31). To prepare a T-cell-depleted population, we incubated MNls with anti-CD3-coated iron beads (Dynal Biotech, Brown Deer, WI), at a ratio of 20 beads/T cell, for 30 min on ice. Bead-coated T cells were removed with a magnet, and the cycle was repeated. The resulting population contained <1% CD3-positive T cells by flow cytometry.

**Preparation and assay of HIV-1-infected MNls**

Normal donor MNls were infected with HIV-1 at a multiplicity of infection of 0.01 in the absence of mitogens or added IL-2 (19). This protocol generates ~0.1–0.01% provirus-positive cells, a fraction comparable to what we found in HIV+ patients’ freshly isolated MNls (19). Two strains of HIV-1 were used, the M-tropic UC5 and the T-tropic Phlp (a field isolate propagated in MT2 cells). Cells inoculated in this manner do not produce virus until further stimulated, and there is no detectable p24 in their cytoplasm as measured by flow cytometry. After 3 days, the HIV-infected MNls were washed, resuspended in fresh medium, and used in transmigration studies. Cell viability at that time was >95%, determined with trypan blue. Just before the migration assay, the 3-day-old HIV-infected MNls and an appropriate number of fresh cells from the same donor as a source of fresh resting monocytes. This is necessary because monocytes cultured for 3 days begin to differentiate toward macrophages and are no longer representative of circulating monocytes from peripheral blood. We estimated the frequency of infectious cells in the starting and migratory populations with the limiting dilution assay in which serial dilutions of MNls are added to duplicate wells containing 0.5 × 10⁴ PHA-stimulated allogeneic lymphoblasts in the presence of IL-2 (17). After 7 days we measured the p24 Ag released into the supernatant (ELISA; Coulter Hialeah, FL) to determine which cultures had received MNls with infectious virus. The frequency of infected cells was calculated by reference to published tables (17).

**Migration under shear stress conditions**

MNls were added to medium flowing at 2 dynes/cm² across a HUVEC monolayer in a parallel plate flow chamber (34, 35). HUVEC monolayers used to assay migration under flow conditions were routinely prestimulated overnight with 10 ng/ml TNF-α, because leukocytes will not migrate across unstimulated endothelium under these flow conditions. The endothelial monolayers were washed before the assay began and were videotaped for 10 min after the leukocytes began to flow over the surface. Leukocytes that rolled, adhered, or migrated were identified as previously described (34, 35), except that we used more stringent criteria to define adherent cells (full arrest for 1 min rather than 5 s). We did not observe the formation of strings of cells as has been reported previously (36, 37), but did note that several cells would often migrate through the same spot on the endothelial monolayer. Migrating cells typically assumed a polarized shape, extended a cellular process, tunneled through a narrow constriction, and established adherence to FN in a phase-dense area below the monolayer. After migration, they localized in a focal plane below the endothelial surface and remained phase-dense as they moved below the endothelial cells.

To verify that MNls were migrating below the endothelium, we stained MNls with an FITC-based intracytoplasmic dye (CellTracker Green; Molecular Probes, Eugene, OR) before the migration. After migration, we fixed the endothelial monolayers that provided the base of the flow chamber with paraformaldehyde, then added PE-conjugated anti-CD45 to the apical surface. Cells adherent to the apical side of the HUVECs gave both an FITC and a PE signal. Cells that had migrated through the endothelium gave off an FITC fluorescence color resulting from the cytoplasmic stain beads (CellTracker Brown, Ref. WC1), at a ratio because they were shielded from PE-anti-CD45 Ab by the overlying endothelium, they did not show any PE signal. The subendothelial position of the FITC-positive, PE-negative population was further confirmed by demonstrating that they were located in the focal plane beneath that occupied by the FITC-positive, PE-positive cells that adhered to the apical side of the HUVECs.

**Flow cytometry**

Flow cytometric analyses were performed as previously described (38) on an EPICS XL cytometer calibrated daily with Flow-Check fluorospheres (Beckman Coulter, Miami, FL). Photomultiplier tube voltages were adjusted each day to a target range of fluorescent intensities by means of...
Impact of FNf on MNL transendothelial migration

In the presence of FNf, MNLs adhere in greater numbers to endothelial cells, leaving significantly fewer nonadherent cells in the overlying medium. Monocyte adherence, reflected as a depletion of nonadherent cells, was significantly increased within 20 min, and lymphocyte adherence was significantly increased within 40 min after FNf treatment (p < 0.01, by Mann-Whitney U test; Fig. 1). When allowed to remain with the endothelium for 2 h, stimulation with as little as 1 μg/ml FNf significantly increased the numbers of lymphocytes and monocytes that subsequently migrated through the HUVEC monolayer (p < 0.05, by Mann-Whitney U; Fig. 2). Doses of FNf >20 μg/ml caused a small, but statistically significant, additional increase in the numbers of migratory monocytes, whereas lymphocyte migration reached a plateau with 20 μg/ml FNf. Migration was not affected by intact FN (in doses up to 80 μg/ml) or by the CS-1 fragment of FN (in doses up to 250 μg/ml; Fig. 3). The CS-1 fragment binds VLA-4, but does not include the RGD sequence and does not bind VLA-5 (33).

FNf increased the migration of both CD4 and CD8 T cells (Fig. 4; p < 0.05, by Mann-Whitney U test). On the average, two-thirds of the migratory T cells express CD45RO, and one-third express CD45RA, and the migration of both increases with FNf stimulation (not shown).

Exposure to FNf also increased, in a dose-dependent manner, the quantity of TNF-α released by MNLs as they migrate (Fig. 5A). HUVEC monolayers exposed to FNf without addition of MNLs produced <1 ng/ml TNF-α during this 2-h period (data not shown).
Depleted MNLs were stimulated with FNf (50 ng/ml) from normal donors. Results shown are representative of those obtained with cells from three normal donors.

To determine whether TNF-α in FNf-induced migration, MNLs were allowed to migrate across confluent monolayers of HUVECs in the presence of FNf with and without TNF sRI to block TNF-α effects. The number of lymphocytes (■) and monocytes (□) that migrated at the end of 2 h are shown. Error bars show ±1 SD of triplicate determinations. The significant increase in migration of lymphocytes and monocytes caused by FNf was blocked by TNF sRI (0.64 µg/ml; p < 0.05, by Mann-Whitney U test). The results shown are representative of those obtained with MNLs from two normal donors.

To evaluate the effect of TNF-α on lymphocytes vs endothelial cells in terms of migration, we treated them separately or together before or during the migration assay (Fig. 8). When assaying the effect of TNF-α on lymphocyte migration, monocytes were removed because they are a constitutive source of TNF-α (19, 31). Pulse treatment of purified lymphocytes with TNF-α before the migration assay induced a 1.86 ± 0.6-fold increase in migration (mean ± SD of nine donors; p = 0.015, by t test), indicating that TNF-α can directly affect lymphocyte migration (Fig. 8, compare result B to A). Pulse treatment was more effective than continuous treatment of the lymphocytes during the migration (Fig. 8, result B vs C; p = 0.05, three donors, by Mann-Whitney U test). Activation of the endothelium alone with TNF-α also increased migration (Fig. 8, compare result D to A). However, even when the endothelium was maximally stimulated by prior exposure to TNF-α for 18 h, addition expressed diminished quantities of CD11a and CD49e (Fig. 7).

The expression of CD49d did not change significantly on the MNLs exposed to FNf or TNF-α.

FIGURE 5. Effect of FNf on TNF-α release by MNLs. A, MNLs were added to confluent monolayers of HUVECs in the presence of increasing quantities of FNf. At the end of 2 h, we measured the quantity of TNF-α released into the supernatant above the HUVECs. Error bars indicate ± SD of triplicate determinations. FNf significantly increased the release of TNF-α at all doses tested (p < 0.05, by Mann-Whitney U test). The results shown are representative of those obtained with cells from three normal donors. B, Unfractionated MNLs, monocyte-depleted MNLs, and T cell-depleted MNLs were stimulated with FNf (5 µg/ml) in Teflon jars, and the quantity of TNF-α released in the supernatant was assayed after 24 h. FNf induced release of TNF-α from monocytes, but not T cells. An additional control, consisting of reconstituted MNLs using monocyte-depleted MNLs (e.g., T cells) and T cell-depleted MNLs (e.g., monocytes) is included to show the effects of cell handling. It illustrates that the lower amounts of TNF-α released by purified monocytes compared with unfractionated cells is due to the process of cell handling, because readdition of T cells (in the form of monocyte-depleted MNLs) does not increase the quantity of TNF-α released. In the absence of FNf stimulation, monocytes released <60 pg/ml TNF-α (not shown).

FIGURE 6. Role of TNF-α in FNf-induced migration. MNLs were allowed to migrate across confluent monolayers of HUVECs in the presence of FNf with and without TNF sRI to block TNF-α effects. The number of lymphocytes (■) and monocytes (□) that migrated at the end of 2 h are shown. Error bars show ±1 SD of triplicate determinations. The significant increase in migration of lymphocytes and monocytes caused by FNf was blocked by TNF sRI (0.64 µg/ml; p < 0.05, by Mann-Whitney U test). The results shown are representative of those obtained with MNLs from two normal donors.

FIGURE 7. Effect of FNf or TNF-α on leukocyte adhesion molecules.

MNLs were treated with medium (□), FNf (5 µg/ml; ■), or TNF (10 ng/ml; □) for 1 h at 37°C and then stained for adhesion markers: CD11b, CD11a, CD62L, and CD49e. Cells were treated in a Teflon jar, processed in siliconized tubes, and washed with gelatin-containing buffers throughout to prevent cell loss through adhesion. Data shown are the density of the marker (mcf, mean channel fluorescence) or percentage of CD14+ monocytes or CD45+ CD14+ lymphocytes that was positive for the marker. Delimiters were set using MNLs stained with nonspecific isotype controls. Results are representative of three experiments.
of more TNF-α during the migration assay caused a small, but signifi-
cant, increase in the number of lymphocytes that passed through
the endothelium (Fig. 8, compare result E or F to D).

To evaluate whether TNF-α also influences monocyte migration,
we pulse-treated unfraccionated MNLs. TNF-α treatment in-
creased monocyte migration by 2.08 ± 0.08-fold (p = 0.008) and
migration of the accompanying lymphocytes by 1.95 ± 0.68-fold
(mean ± SD of nine donors; p = 0.008, by paired t test compared
with untreated MNLs; Fig. 9). Dose-response and kinetic analyses
performed to identify the optimum quantity and timing of treat-
ment showed that TNF-α caused a significant increase in MNL
migration when used at doses as low as 1 pg/ml and that exposure
of the leukocytes to TNF-α for <1 min was sufficient to stimulate
migration (data not shown).

FIGURE 9. TNF-α stimulates lymphocyte and monocyte migration.
MNLs or lymphocytes were treated with TNF-α, washed, and allowed
to migrate for 2 h across unstimulated HUVECs. Bars indicate the percentage
of added cells that migrated (mean ± SD of triplicate wells). TNF-α treatment
on the behavior of cells after they had passed through the
endothelium. Untreated MNLs tended to migrate deep into the
collagen pad during the 2-h period, whereas FNf caused MNLs to
accumulate just below the endothelial monolayer (Fig. 11). After
FNf treatment, there were significantly more leukocytes in the first
100 μm of the collagen pad and significantly fewer in the region
300–400 μm below the endothelial monolayer (p < 0.05, by t
test).

Monocytes that encounter proinflammatory stimuli after they
migrate through vascular barriers in vitro may differentiate into
dendritic cells and reverse-migrate through the endothelial mono-
layer to the apical surface (17, 40). This reverse migration is
thought to model the movement of dendritic cells from tissue into
lymphatics en route to lymph nodes (40). To evaluate whether FNf

FIGURE 10. TNF-α stimulates lymphocyte migration under flow con-
ditions. Purified lymphocytes were treated with 10 ng/ml TNF-α (■) or
medium alone (□) for 10 min, washed, and allowed to interact with
HUVECs under shear stress conditions. Bars indicate the numbers of cells
that rolled, adhered, or migrated within 10 min (mean ± SD). TNF-α
induced a significant increase in the number of cells that migrated, but did
not change the number that only rolled or adhered. Results are represen-
tative of those obtained with two donors’ MNLs pretreated with TNF-α
before the migration and seven donors’ MNLs exposed to TNF-α through-
out the migration.
treatment enhances reverse migration, we washed away the non-migratory cells and refed the cultures. Two days later we collected the cells that emerged from the apical side of the HUVEC monolayer. We found that FNf increased both the number of HLA-DR$^{\text{dim}}$ monocytoid cells that reverse-migrated and the fraction that expressed high levels of HLA-DR, a characteristic of dendritic cells (Fig. 12). The number of lymphocytic cells (CD3-positive, CD19-positive, or CD56-positive cells) that reverse-migrated was not significantly increased by FNf treatment.

**Impact of FNf on transendothelial migration of HIV-infected MNLs in vitro**

HIV-1-infected MNLs display increased quantities of CD11a, CD18, CD29, and CD49d and become more adherent to endothelial cells, more likely to engage in homotypic adhesive interactions, and more likely to migrate across endothelial barriers (19, 20). Activated normal donor lymphocytes are also more responsive to the migration-enhancing effects of TNF-α (31). Consequently, we postulated that stimulation with FNf might increase migration of HIV-infected cells to a greater degree than uninfected cells. To test this hypothesis, we infected normal donor MNLs with the T-tropic (Phlp) or the M-tropic (UC5) strain of HIV-1 for 2 days without exogenous mitogens or cytokines. Under these conditions, ~0.01–0.1% of the leukocytes became infected. These HIV-infected MNLs were then allowed to migrate across endothelial monolayers in the presence or the absence of FNf. We measured the numbers of HIV-infected MNLs recovered from the collagen pad by means of a limiting dilution assay in which MNLs were incubated with highly susceptible PHA-stimulated lymphoblasts in the presence of IL-2. The limiting dilution assay is designed to detect any cell capable of transmitting HIV, including leukocytes with latent virus that can be reactivated with stimulation. With both T-tropic and M-tropic HIV-1 strains, FNf treatment increased the total number of migratory MNLs and the number of HIV-infected MNLs recovered from the collagen pad 2–24 h later (Fig. 13). Fig. 13 shows the effects of FNf on these infected MNLs, expressed as the fold increase in the number of migratory cells after FNf compared with the increase in the number of infected MNLs treated with medium alone. FNf treatment induced a 2- to 5-fold increase in the number of HIV-infected cells that migrated (p = 0.02 for cells collected after 24 h; p = 0.008 for 2 and 24 h harvests analyzed together; by Wilcoxon test). At the same time, FNf induced only a <2-fold increase in the total number of cells that migrated. If infected and uninfected cells responded in the same way to FNf, then the fold increase in migration caused by FNf should be the same for HIV-infected and uninfected cells. However, as shown in Fig. 13, the increase in recovery of HIV-infected cells among the migratory cells consistently exceeded the increase in the total number of MNLs recovered from the subendothelial compartment. This was equally true for cultures that were

**FIGURE 11.** Effect of FNf on depth of migration below endothelium. MNLs were allowed to migrate across confluent monolayers of HUVECs and into the collagen pad in the presence (dotted line) or the absence (solid line) of FNf. At the end of 2 h, the number of migratory cells that accumulated at consecutive 100-μm intervals below the endothelium were counted. Results are presented as the fraction of the total migratory population at each level. Error bars indicate ±SD of nine replicate determinations. *, Significantly different values (p < 0.05, by t test).

**FIGURE 12.** Effect of FNf on differentiation of reverse-migrating dendritic cells. MNLs were allowed to migrate across confluent HUVEC monolayers in the presence or the absence of FNf. Nonmigratory cells were washed away after 2 h, cultures were refed, and 2 days later, MNLs reverse-migrating to the apical side of the HUVECs were collected. Results show the numbers of lymphocytic (CD3$^+$, CD19$^+$, or CD56$^+$), monocytoid (CD3$^+$CD19$^+$CD56$^-$HLA-DR$^{\text{dim}}$), and dendritic-type cells (CD3$^+$CD19$^+$CD56$^-$HLA-DR$^{\text{bright}}$). Error bars indicate the results of triplicate determinations, and results are representative of three donors. The number of reverse-migrating dendritic cells was significantly increased in the presence of FNf (p = 0.05, by Mann-Whitney U test).

**FIGURE 13.** Effect of FNf on migration of HIV-infected MNLs. MNLs from six normal donors were infected with HIV (UC5 for no. 1–5 and Phlp for no. 6) for 48 h, then allowed to migrate across confluent monolayers of HUVECs in the presence or the absence of FNf (5 μg/ml for donors 1–3 and 20 μg/ml for donors 4–6). The migratory population was harvested at 2 h (inset for donors 1–3) and 24 h (donors 1–6) to quantify both the total numbers of MNLs (■) and the numbers carrying infectious HIV (■■). Data are presented as the fold difference in results with FNf-treated cells compared with infected cells treated with medium alone. Actual cell numbers are presented in Table I. FNf induced a significant increase in the migration of HIV-infected MNLs (p = 0.008 for all time points; p = 0.02 for 24 h harvests alone; by Wilcoxon test).
harvested within 2 h of migration (see inset, Fig. 13). This demonstrates that FNF preferentially promotes the migration of HIV-1-infected cells. The original cell populations in these experiments contained varying numbers of infected cells, ranging from 82–1122/million MNLs (Table I). However, the level of infection did not appear to influence the total number of MNLs, the number of HIV-infected cells that migrated, or the response of these cells to stimulation with FNF.

Discussion

These studies were undertaken to investigate why some, but by no means all, HIV-1-infected men whose blood contains productively infected leukocytes have circulating MNLs that can transport infectious virus across endothelial barriers in vitro and disseminate HIV-1 among a large number of uninfected lymphocytes and monocytes drawn to the same sites, presumably by agents that caused the infected cells to migrate in the first place (17). The clinical significance of these observations was initially obscure until, with continued observation of this cohort of patients, it became apparent that complications associated with progression of HIV-1 infection occurred more often in those subjects whose MNLs exhibited this behavior (17). Recognition that FN fragments are present, at least intermittently, in the plasma of many HIV-1-infected patients (38) and that these FNf are among the triggers that cause monocytes to release cytoplasmic stores of TNF-α (26–28) caused us to consider the possibility that FNF may be one of the agents that induces MNLs, particularly HIV-1-infected MNLs, to migrate across endothelial barriers.

The experiments presented in this report suggest that an important effect of FNF is to stimulate MNLs to release TNF-α. The central role of TNF-α in driving MNL migration is confirmed by demonstrating that the increase in MNL migration induced by FNF is blocked by TNF sRI. A focal increase in TNF-α has many effects. It up-regulates endothelial expression of selectins, ICAMs, and VCAM-1, facilitating adhesive interactions that promote leukocyte transendothelial migration (31). The experiments presented in this report suggest that the TNF-α may also directly stimulate MNL migration, even when the endothelium is not activated and even under shear stress conditions. One mechanism may be the modulation of adhesion molecules involved in transendothelial migration and translocation across protein matrices. TNF-α and FNF induce monocytes to shed CD49e and CD62L to increase the expression of CD11b. They also induce lymphocytes to decrease their expression of CD11a and CD49e, but have little effect on CD49d. One can visualize several ways in which changes in adhesion molecule expression might affect leukocyte motility. Migration involves a dynamic process of attachment and detachment. It is possible that a diminution in cell surface adhesins may prevent a cell from being tethered and allow it to migrate more easily. The phenotypic data shown in Fig. 7 are a single snapshot after 60 min of stimulation. These markers continue to change with time after exposure to FNF. For example, at 24 h, monocyte CD62L and CD11b return to normal, but CD49d and CD49e become elevated on FNF-treated cells. Lymphocyte CD11a and CD49e return to normal, but CD11b decreases to levels below those seen on untreated MNLs (not shown).

Stimulation with FNF increased the recovery of HIV-infected cells among the migratory population. Infection with HIV activates leukocytes. HIV-infected cells express increased quantities of integrins (20, 41–43), adhere in greater numbers to endothelial monolayers (20, 41–43), and have a competitive advantage over uninfected cells when migrating across monolayers of resting endothelial (19). It is not surprising then that HIV-infected cells would also migrate preferentially over endothelium that has been activated by TNF-α released in response to FNF. A similar effect was seen using endothelium stimulated with LPS to up-regulate vascular adhesion molecules (data not shown). In one experiment, 12-fold more HIV-infected MNLs traversed the LPS-activated endothelium compared with resting endothelium, whereas the total number cells that migrated was only 13% higher for LPS-stimulated HUVECs (325 HIV-infected cells and 1.58 million total MNLs migrated across unstimulated HUVECs compared with 4491 HIV-infected cells and 1.79 million total MNLs migrating across LPS-stimulated HUVECs).

In addition to enhancing the transendothelial migration of HIV-infected cells, FNF may have stabilized cell surface HIV, thereby enhancing transmission of virus to the PHA-stimulated lymphoblasts. Recent reports suggest that diverse forms of matrix FN can stabilize virions of HIV-1 on the surface of infected cells and help facilitate the transmission of infectious virus (24, 25). We have also found that addition of FNF to cultures of in vitro infected MNLs stimulated with IL-2 increases by 2- to 4-fold the quantity of p24 generated over the next 3 days (data not shown).

Leukocyte integrins are involved in interactions with FN. Leukocytes can interact with immobilized FN using both VLA-4 (CD29/CD49d) and VLA-5 (CD29/CD49e). The 110-kDa FN fragment used in these studies includes the RGD sequence and binds to CD49e (27). The other FN fragment we used, CS-1, does not contain the RGD sequence and does not bind to VLA-5. The CS-1 fragment is closer to the C terminus of the FN molecule and binds to VLA-4. Neither the CS-1 fragment nor intact FN induced TNF-α release or affected the migration of MNLs.

After exposure to FNF, monocytes shed CD49e and are less able to migrate through collagenous matrices rich in native FN (23). FNF-induced loss of CD49e may also explain the failure of treated MNLs to penetrate deeply into FN-collagen matrices below the endothelial barrier in the present report. Colocalization of large numbers of infected and uninfected cells in a compact site is likely to promote interactions that facilitate the transfer of HIV-1. Accumulation of these foci of infected and uninfected MNLs just under the endothelial surface may also enhance reverse migration.

### Table I. Effect of FNF on migration total MNLs and HIV-infected MNLs

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<td>6,058,400</td>
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</table>

* These same data are shown in Fig. 13, but presented there as the fold increase in FNF-treated cultures compared to sham-treated cultures.

To calculate the numbers of HIV-infected cells that migrated under each condition, we multiplied the total number of migratory cells by the frequency of HIV-infected MNLs in that population, as measured with the limiting dilution assay. For the starting population, data are reported as the number of HIV-infected cells per million MNLs.

In each experiment, 11 HUVEC wells, representing 11 million starting MNLs, were treated with FNF, and an equal number of wells were treated with medium. Migratory cells from the 11 replicate wells were pooled for the limiting dilution assay. The data shown here are the numbers of sham-treated cells that migrated; Fig. 13 shows the fold increase induced by FNF treatment.

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FIBRONECTIN FRAGMENTS AND MIGRATION OF HIV-INFECTED CELLS

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of the subset of infected MNLs that develop characteristics of dendritic cells. To the extent that this process is replicated in vivo, these experiments suggest that circulating FNf in HIV-1-infected patients could help disseminate HIV-1 in extravascular soft tissue depots within an infected host. Whether the development of these perivascular foci help to protect the infected cells within from the actions of antiretroviral drugs remains to be determined.

Cell surface display of HLA-DR, a phenotypic characteristic of monocytes that have begun to differentiate into dendritic cells (44), is prominently seen on FNf-stimulated monocytes, especially those that reverse-migrated. Release of TNF-α, as a result of FNf stimulation, may induce monocytes to differentiate toward dendritic cells (40, 45). If these developing dendritic cells were to take up HIV-1 from the subendothelial depots, they would be highly effective at infecting T cells that entered the same subendothelial microenvironment (46).

Proteolytic enzymes released by replicating virus and those produced by host inflammatory responses to HIV-1 or to secondary microbial infections may help to break down plasma or tissue FN (41, 49–49). Prospective studies of the prevalence of circulating FN fragments in HIV-1-infected subjects together with additional studies of their effects on the functioning of cells of the immune response system may deepen our understanding of the ability of these byproducts of the inflammatory response to influence the development of extravascular foci of infection that appear to participate in the cardiac, renal, and CNS complications of HIV-1 infections.

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References


