blood

1997 90: 1365-1372

Tat−Human Immunodeficiency Virus-1 Induces Human Monocyte Chemotaxis by Activation of Vascular Endothelial Growth Factor Receptor-1

Stefania Mitola, Silvano Sozzani, Walter Luini, Luca Primo, Alessandro Borsatti, Herbert Weich and Federico Bussolino

Updated information and services can be found at:

http://bloodjournal.hematologylibrary.org/content/90/4/1365.full.html

Information about reproducing this article in parts or in its entirety may be found online at:

http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:

http://bloodjournal.hematologylibrary.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:

http://bloodjournal.hematologylibrary.org/site/subscriptions/index.xhtml

Tat–Human Immunodeficiency Virus-1 Induces Human Monocyte Chemotaxis by Activation of Vascular Endothelial Growth Factor Receptor-1

By Stefania Mitola, Silvano Sozzani, Walter Luini, Luca Primo, Alessandro Borsatti, Herbert Weich, and Federico Bussolino

Human immunodeficiency virus-1 (HIV-1) Tat protein can be released by infected cells and activates mesenchymal cells. Among these, monocytes respond to Tat by migrating into tissues and releasing inflammatory mediators. In the present study, we have examined the molecular mechanism of monocyte activation by Tat, showing that this viral protein signals inside the cells through the tyrosine kinase receptor for vascular endothelial growth factor encoded by *fms*-like tyrosine kinase gene (VEGFR-1/Flt-1). Subnanomolar concentrations of Tat induced monocyte chemotaxis, which was inhibited by cell preincubation with vascular-endothelial growth factor-A (VEGF-A). This desensitisation was specific for VEGF-A, because it not was observed with FMLP. In addition, the soluble form of VEGFR-1 specifically inhibited polarization and migration induced by Tat and VEGF-A, thus con-

firming the common use of this receptor. Binding studies performed at equilibrium by using radiolabeled Tat showed that monocytes expressed a unique class of binding site, with a kd of approximately 0.2 nmol/L. The binding of radiolabeled Tat to monocyte surface and the cross-linking to a protein of 150 kD was inhibited specifically by an excess of cold Tat or VEGF-A. Western blot analysis with an antibody anti–VEGFR-1/Flt-1 performed on monocyte phosphoproteins immunoprecipitated by an monoclonal antibody anti-phosphotyrosine showed that Tat induced a rapid phosphorylation in tyrosine residue of the 150-kD VEGFR-1/Flt-1. Taken together, these results suggest that biologic activities of HIV-1 Tat in human monocytes may, at least in part, be elicited by activation of VEGFR-1/Flt-1.

© *1997 by The American Society of Hematology.*

MONOCYTES AND macrophages have been implicated in a variety of relevant roles in human human immunodeficiency virus–type 1 (HIV-1) infection. During the latent phase of the disease, these cells represent an important viral reservoir for the transmission of virus to sensitive target cells.[1,2] Productive HIV-1 replication in monocytes and macrophages is triggered by interleukin-6 (IL-6),[3] granulocyte-macrophage colony-stimulating factor (GM-CSF),[4] lipopolysaccharide (LPS),[5] or contact with endothelial cells[6] and often predicts the clinical manifestation of the disease.[2] Furthermore, HIV-1–infected monocytes/macrophages participate in tissue injury by releasing inflammatory cytokines and autacoids.[7-9] The mechanism by which HIV-1 alters monocyte/macrophage gene expression and function is unknown, and a putative effector is the product of the regulatory viral gene *tat*. Tat protein transactivates viral genes, increases the replication rate of virion,[10] and regulates host gene expression, including tumor necrosis factor α and β (TNF), IL-2, IL-6, major histocompatibility complex of class I , IL-2 receptor, p53 tumor-suppressor gene, and superoxide dismutase in lymphoid cells[11-17] and matrix proteins in glioblastoma cells.[18]

Furthermore, Tat can be released by infected cells[19] and acts extracellularly in the microenvironment regulating functions of immunocompetent and mesenchymal cells.[20,21] One of the most striking effects of Tat is the induction of a functional program in endothelial cells related to angiogenesis and inflammation (migration, proliferation, expression of plasminogen activator inhibitor-1 and E selectin, in vivo angiogenesis[22-25]). Subnanomolar concentrations of Tat promote the release of IL-6, transforming growth factor β (TGFβ) and metalloproteinase-9 by human monocytes[11,26] increase their motility[27] and adhesion to endothelial cells.[26]

Given the similarity of Tat basic sequence (aminoacids 42-64) to basic sequence of growth factor (fibroblast growth factor, vascular-endothelial growth factor-A [VEGF-A], hepatocyte growth factor, and heparin binding epidermal

growth factor),[24] we have recently shown that the tyrosine kinase VEGF receptor-2 (VEGFR-2)[28,29] mediates Tat-induced migration of endothelial cells and in vivo angiogenesis.[30] VEGF-A is a dimeric glycoprotein mitogenic for endothelial cells and able to induce angiogenesis and vessel permeability (reviewed in Ferrara et al[31] and Kenneth[32]). Two high-affinity tyrosine kinase receptor for VEGF-A have been identified so far on vascular endothelial cells: VEGFR-1, encoded by the *fms*-like tyrosine kinase (Flt-1) gene,[33] and VEGFR-2, encoded by kinase insert domain-containing receptor (KDR) gene.[28,29] However, the activities of VEGF-A are not restricted to endothelial cells, because it also activates the induction of procoagulant activity by monocytes[34] and their migration by acting through VEGFR-1.[35,36]

In light of these similarities between Tat and VEGF, we have investigated the chemotactic properties of Tat on hu-

man monocytes. This report shows that monocytes express a high-affinity binding site for Tat that induces a rapid (within minutes) cell shape change and migration. Monocytes activation by Tat is associated to phosphorylation of a 150-kD protein that cross-reacted with an anti–VEGFR-1 antibody. Finally, VEGF-A$_{165}$ cross-desensitized with Tat in chemotaxis assay and competed with labeled Tat to monocytes, suggesting the promiscuous use of a common receptor by the two proteins.

MATERIALS AND METHODS

Tat protein. Recombinant full-length Tat protein (rTat) was obtained by Intracel (London, UK; batch R12.3). rTat (50 ng) gave a unique band of 14 kD (12%) detected by silver staining (Gelcode; Pierce, Europe B.V., Oud Beijerland, The Netherlands). Synthetic Tat (sTat), constituted by 86 aminoacid residues, was from Tecnogen (Piana di Monte Verna, Italy). The purity of this molecule was controlled by reverse-phase high-pressure liquid chromatography and was more than 97%. sTat was eluted from a Spherisorb C18 column (100 × 1 mm; Applied Biosystem, Foster City, CA) for 5 minutes under isocratic gradient (100% solvent A: 0.1% trifluoroacetic acid in water), for 40 minutes under a linear gradient from 0% to 95% solvent B (0.1% trifluoroacetic acid in CH_3CN_2), for 5 minutes under isocratic gradient (B 95%), and finally for 2 minutes under linear gradient to 5% solvent B. rTat and sTat were stored at −80°C in phosphate-buffered saline (PBS) containing 0.1 % human serum albumin (HSA; Farma Biagini, Lucca, Italy) and 1 mmol/L dithiothreitol. To label Tat, rTat (2 μg) was dissolved in 200 μL of sodium phosphate buffer at 20 mmol/L, pH 7.4, without dithiothreitol and transferred in iodogen-coated tubes (50 μg/mL; Pierce), where Tat was iodinated (5 minutes at 4°C) with 0.2 mCi ^{125}I (Amersham, Buchs, UK). Twenty microliters of phosphate buffer at 20 mmol/L, pH 7.2, containing 1% HSA, 0.4 mol/L NaCl, and 0.1% Chaps (Pierce) was added, and the reaction products were separated on Sephadex-G10. The specific activity of the tracer was 1 mCi/127 fmol. [^{125}I] Tat retained its biologic activity as migration of Kaposi's sarcoma cells.[34]

Cloning and expression of the soluble form of VEGFR-1/Flt-1. Human Flt-1 cDNA was cloned from a placental cDNA library. A 2-kb *Hin*dIII fragment of Flt-1 cDNA coding for the first five extracellular Ig-like domains was transfected into *Spodoptera frugiperda* clone 158 (Sf158) cells using a liposome transfection system (Life Technologies GmbH, Eggenstein, Germany) along with purified and *Bsu*-36I–linearized baculovirus DNA (Pharmigen, Hamburg, Germany). After infection, cells were maintained in serum-free medium SF900 II (Life Technologies). Positive baculovirus plaques were identified by RNA expression of Flt-1. The soluble form of VEGFR-1/Flt-1 (sFlt-1) was purified, initially by addition of ammonium sulphate to a final concentrations of 35%, and the resulting precipitate was discarded and the sFlt-1 in the supernatant was precipitated by the addition of ammonium sulphate to a final concentration of 65%. After precipitation, the samples were centrifuged at 2°C. The pellet was dissolved in 10 mmol/L sodium phosphate buffer (pH 6. 2) containing 0.15 mol/L NaCl and applied to heparin-Sepharose CL-6B column (1.5 × 4 cm; Pharmacia Biotech, Uppsala, Sweden). The column was washed with the same buffer containing 0. 3 or 0.6 mol/L NaCl and sFlt-1 was eluted as a single peak with 1.2 mol/L NaCl. The purity of the protein was analyzed using sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) under denaturing and nondenaturing conditions and, after silver staining, there appeared a unique band of 70 kD. Purified protein was identified by NH_2-terminal sequence analysis using Edman degradation on an automatic gas sequencer (Applied Biosystems, Weiterstand, Germany).

Mononuclear cell preparation. Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy blood donors through the courtesy of Centro Trasfusionale, Ospedale Sacco (Milan, Italy). Blood was washed once with saline at 400g to remove plasma and platelets and then centrifuged on Ficoll (Biochrom; Seromed, Berlin, Germany) at 600g for 30 minutes at room temperature. PBMCs were collected at the interface, washed twice with saline, and resuspended in RPMI 1640 with 1% fetal calf serum (FCS; Irvine, Santa Ana, CA). In some experiments, monocytes were further purified (>85% pure) by centrifugation at 450g on a 46% isoosmotic Percoll (Pharmacia Biotech) gradient, as previously described.[37]

Migration assay. Cell migration was evaluated using a chemotaxis microchamber technique,[38] as previously described,[37] using a 5-μm pore size polycarbonate filter (Neuroprobe, Pleasanton, CA). Polycarbonate (migrated cell count at the end point) or nitrocellulose (leading front distance as the end point) were used. sTat, rTat, VEGF-A$_{165}$,[35] and FMLP (Sigma, St Louis, MO) were used as stimuli. Twenty-seven microliters of chemoattractant solution or control medium (RPMI 1640 with 0.1% HSA, 1 U/mL heparin) was added to the lower wells of a chemotaxis chamber (Neuroprobe, Bethesda, MD). Fifty microliters of cell suspension (1.5 × 10^6/mL monocytes) was seeded in the upper chamber. At the end of the incubation (37°C in air with 5% CO_2 for 90 minutes), filters were removed and stained with Diff-Quik (Baxter Spa, Rome, Italy) and five high-power oil-immersion fields were counted. Cross desensitization studies were performed by preincubating cells (2 × 10^6/mL monocytes in PBMCs) with the different agonists at 37°C in constant agitation for 30 minutes. The cells were then washed in RPMI 1% FCS, counted, and tested for their ability to respond to agonist stimulation.[37] Recombinant pertussis toxin (PTox) was from Calbiochem (San Diego, CA). Cells were preincubated with different concentrations of the toxins at 37°C for 90 minutes. At the end of the incubation, cells were washed twice with RPMI and tested for their ability to migrate in response to chemotactic agonists.[37] Ptox treatment did not alter cell viability (>90%) as evaluated by trypan blue dye exclusion.

Polarization assay. Monocyte polarization assay was performed as previously described[39] with minor modifications. Briefly, monocytes (10^6/mL) were prewarmed in polypropylene tubes at 37°C for 5 minutes and then exposed to different concentrations of the agonists for 10 minutes. The reaction was stopped by adding an equal volume (1 mL) of ice-cold phosphate-buffered formaldehyde (10% vol/vol; pH 7. 2). The percentage of cells with bipolar configuration (front-tail) was determined in at least 200 cells for each tube by phase-contrast microscopy at 400× magnification. Each experiment was performed in duplicate.

Binding studies. For binding studies, monocytes (1 × 10^6) were incubated for 2 hours at 22°C in 0.2 mL RPMI 1640 medium containing 20 mmol/L (N-[2-hydroxyethyl]piperazine-N′[2-ethanesulfonicacid]) pH 7.4, 0.1% HSA, 0.2 U heparin, 100 μg/mL soybean trypsin inhibitor, and bacitracin (binding buffer) and 0.7 pmol [I^{125}]-Tat without or with the indicated concentrations of unlabeled polypeptides. Alternatively, monocytes were incubated in the same way with different concentrations of [^{125}I]Tat in the presence of 100-fold excess of cold ligand. At the end of incubation, cell suspension were overlayered on 0.5 mL of dibutylphtalate (Sigma) and centrifuged for 10 minutes at 13,000g, and the pellet was solubilized with 2% SDS in PBS was counted in a γ counter. Specific binding, which was calculated by subtracting from the total the cpm bound after incubation with a 100-fold excess of unlabeled ligand, was approximately 80%. The kd was estimated by Scatchard plot using Ligand program (Elseviere-Biosoft, Cambridge, UK).

Cross-linking. Monocytes (2 × 10^7) were incubated at 22°C in 0.5 mL of binding buffer with 0.5 nmol/L [^{125}I]Tat with or without a 50-fold excess of unlabeled rTat or VEGF-A$_{165}$ for 90 minutes

and then cross-linked with 1 mmol/L disuccimidyl suberate (Pierce) for 30 minutes. Cells were washed twice with PBS and separated by denaturing SDS-PAGE (8%). Radiolabeled proteins were visualized by autoradiography.

Tyrosine phosphorylation and immunoprecipitation experiments. Human monocytes (1×10^7) were washed three times with RPMI 1640 and then twice with PBS containing 1 mmol/L Na orthovanadate. Cells were stimulated for different times with rTat (10 ng/mL) at 4°C. At the end of incubation, cells were centrifuged (1 minute at 130,000g) and then cells were lysed in 1 mL at 4°C in a 20 mmol/L Tris-HCl buffer, pH 7.2, containing 158 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L ethylendiaminetetraacetic acid, 5 mmol/L ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′ tetracetic acid, and protease and phosphatase inhibitors (50 μg/mL pepstatin, 50 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mmol/L phenyl-methyl-sulphonyl fluoride, 500 μg/mL soybean trypsin inhibitor, 100 μmol/L ZnCl$_2$, 1 mmol/L Na orthovanadate, 1 mmol/L NaF, and 2 mmol/L sodium pirophosphate). After centrifugation (20 minutes for 10,000g), supernatants were precleared by incubation for 1 hour with rabbit antimouse IgG-agarose and then incubated for 1 hour with monoclonal antibody 4G10 antiphosphotyrosine (10 μg/mL; UBI, Salt Lake City, UT). Immune complexes were recovered on rabbit antimouse IgG-agarose. Immunoprecipitates were washed four times with lysis buffer, twice with the same buffer without detergents, and once with Tris-buffered saline. Proteins were separated by SDS-PAGE (8%), blotted onto nitrocellulose, and probed with polyclonal antibody anti–Flt-1 (Santa Cruz, Santa Cruz, CA). Immunoreactivity was determined by using the enhanced chemiluminescence reaction (Amersham).

Statistical analysis. Chemotaxis experiments were performed in triplicate. Results are presented as mean ± SD of a representative experiment or as a mean (±SE) of several experiments. Statistical significance was assessed using the Student's t-test.

RESULTS

Induction of human monocyte migration and polarization by Tat. Figure 1A shows that rTat induced a concentration-dependent migration of human monocytes that was statistically significant at the concentration of 1 ng/mL and reached maximal values at 10 ng/mL (5 and 500 pmol/L, respectively). These concentrations are in the range of the active concentrations described for monocytes[26,27] and endothelial cells.[30] At the optimal concentration of 10 ng/mL, the number of monocytes migrating to Tat was 87% ± 13% (n = 3) of that observed in the presence of an optimal (10 nmol/L) concentration of FMLP, a reference chemoattractant. Sta-
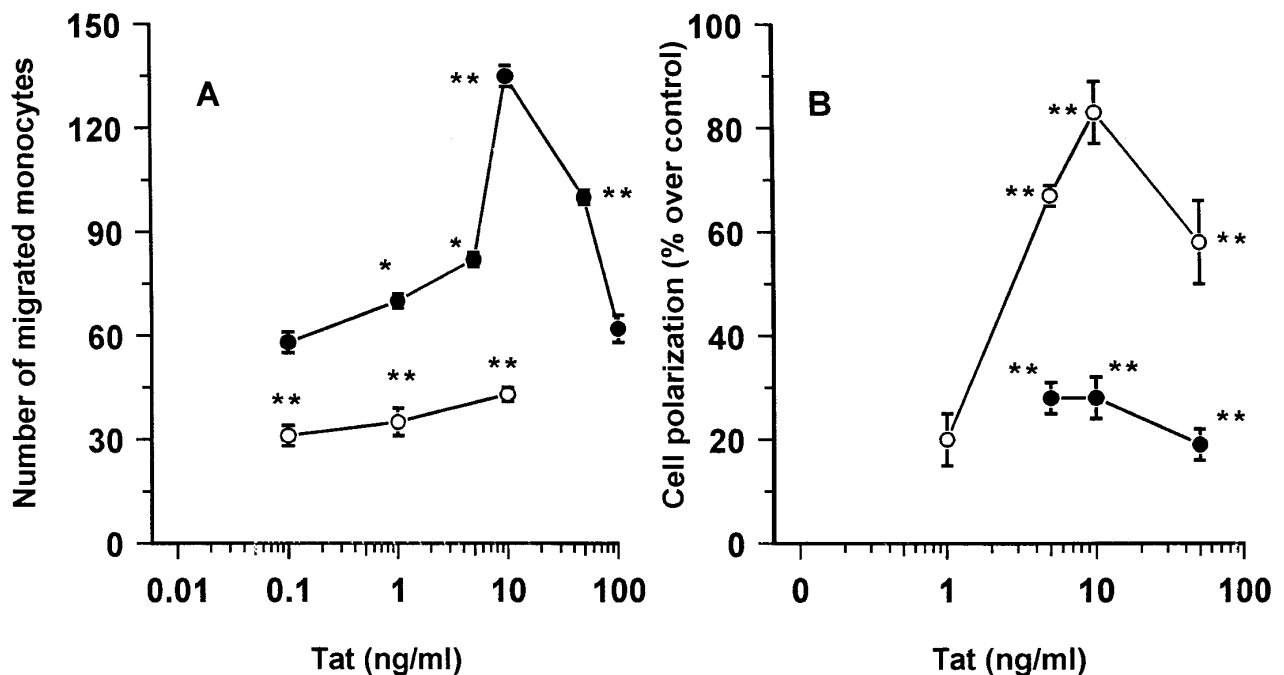

Fig 1. Effect of rTat on human monocyte chemotaxis and polarization. **(A)** Human monocytes (1.5 × 10⁶/mL in PBMCs) were tested for their ability to migrate across a 5-μm pore-size polycarbonate filter in response to different rTat concentrations (●). Results of one experiment (performed in triplicate and representative of at least 3 independent experiments) are shown. In the same experiment, migration to an optimal concentration (10 nmol/L) of FMLP was 149 ± 9 monocytes. **P < .001; *P < .01 versus control (no rTat) by paired Dunnett's test. The effect of Ptox on rTat-induced migration was evaluated by preincubating cells with 1 μg/mL PTox for 90 minutes (○). **P < .001 versus respective Ptox-untreated cells. Results of one experiment (performed in triplicate and representative of at least 3 independent experiments, mean ± SD) are shown. **(B)** Percoll-purified human monocytes (10⁶/mL) were incubated with different concentrations of sTat for 10 minutes (○). The reaction was stopped and the percentage of cells with bipolar configuration (front-tail) was determined. Data are expressed as the percentage of increase over control (0%). In the absence of the agonist (control), 23.6% ± 4% (n = 3) monocytes were in the polarized configuration. Results are the mean ± SD of three independent experiments performed in duplicates. In the same experimental conditions, the effect of 10 nmol/L FMLP was 133% ± 19% (n = 3) increase over control. **P < .001; *P < .01 versus control (no rTat) by paired Dunnett's test. The effect of sFlt-1 on rTat-induced polarization was evaluated by preincubating cells with 210 ng/mL for 10 minutes (●). **P < .001 versus sFlt-1–untreated cells. Results of one experiment (performed in triplicate and representative of at least 3 independent experiments, mean ± SD) are shown.
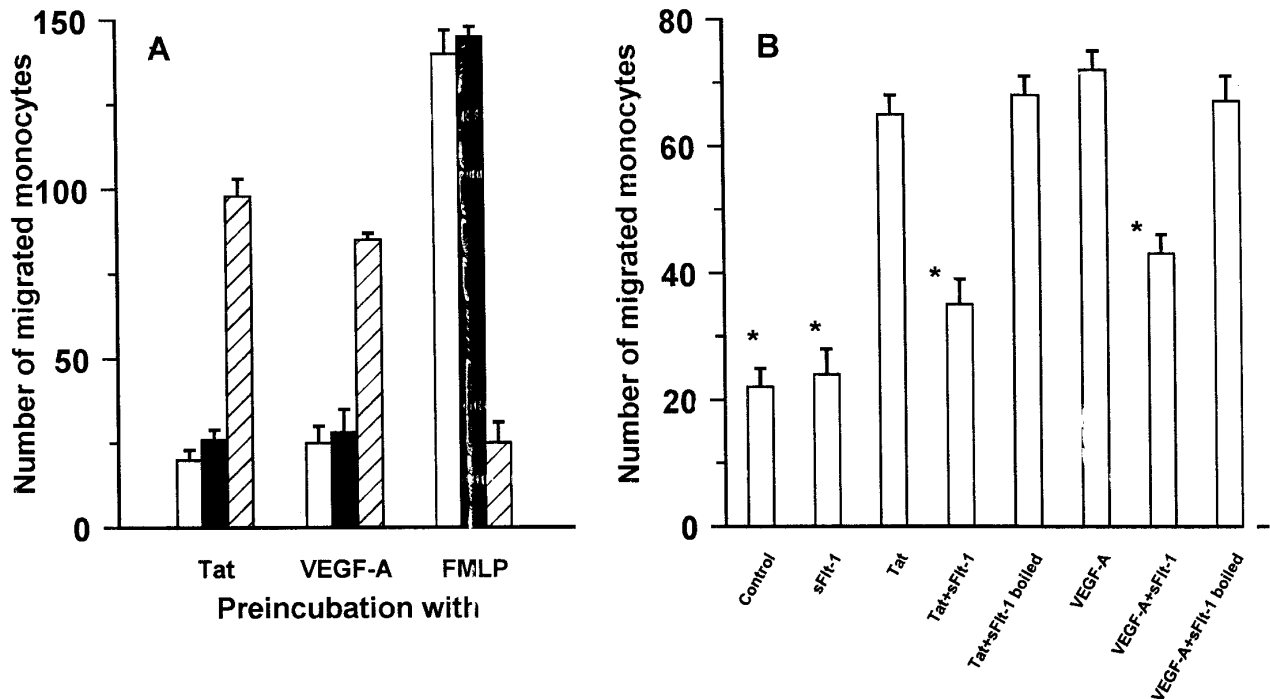
Fig 2. rTat and VEGF-A$_{165}$ activate human monocytes through the same receptor. (A) Homologous and heterologous desensitization of monocyte migration by rTat (□), VEGF-A$_{165}$ (■), and FMLP (▨). Monocytes were preincubated with optimal chemotactic concentrations of the agonists (10 ng/mL rTat, 10 ng/mL VEGF-A$_{165}$, and 10 nmol/L FMLP) at 37°C for 30 minutes. The cells were then washed and assayed for their migration toward homologous of heterologous stimuli used at the same concentrations. Results are expressed as percent of activity with respect to relative control group (cell incubated with medium and tested against the 4 single agonists). The mean numbers (±SE) of three separate experiments performed in triplicate are reported. (B) Monocyte migration by optimal chemotactic concentrations of the agonists (10 ng/mL rTat and 10 ng/mL VEGF-A$_{165}$) was evaluated in the absence or in the presence of 210 ng/mL sFlt-1. Results (mean ± SD) of one experiment (performed in triplicate and representative of at least 3 independent experiments) are shown. In the same experiment, migration to an optimal concentration (10 nmol/L) of FMLP was 114 ± 7 monocytes. *P = .05 or lower versus rTat or VEGF-A$_{165}$.

tistical analysis of checkerboard experiments performed with nitrocellulose filters showed that monocyte migration in response to rTat was mainly directional migration (chemotaxis), with a minor but consistent chemokinetic component (data not shown). Boiled rTat was inactive (data not shown). Identical results were obtained with sTat (data not shown). Monocytes exposed to rTat or sTat acquired a polarized morphology that was maximal at 10 minutes (data not shown) and was concentration-dependent, with a peak observed at 10 ng/mL (Fig 1B). These data suggest a direct effect of rTat on monocyte cytoskeleton reorganization, a prerequisite for cell movement.[40] Tat-induced monocyte migration was sensitive to the action of PTox (Fig 1A and data not shown). At the highest concentration tested (1 μg/mL PTox), monocyte migration to 10 ng/mL rTat was inhibited 79% ± 9% (n = 3; P < .01; Fig 1A). In the same assay conditions, chemotaxis to FMLP was inhibited 81% ± 7% (n = 3), as previously reported.[37] These data are consistent with the sensitivity to PTox of most chemotactic agonists,[41,42] including those using tyrosine kinase receptors (ie, VEGF-A$_{165}$[35]).

Interaction of Tat with Flt-1 on human monocytes. On the basis of previous observations in endothelial cells,[30] the ability of rTat to cross-desensitize with VEGF-A$_{165}$ in terms of chemotaxis was investigated. As shown in Fig 2A, pre-

treatment of monocytes with either 10 ng/mL rTat or 10 ng/mL VEGF-A$_{165}$ resulted in more than 75% (P < .01 by Dunnett' test) of inhibition of a subsequent response to the two agonists. On the contrary, there was no (<20% inhibition) heterologous deactivation between rTat or VEGF-A$_{165}$ and FMLP (Fig 2A).

Of the two VEGF-A$_{165}$ receptors, human monocytes express on their surface only VEGFR-1/Flt-1.[35,36] The soluble, truncated product of VEGFR-1/Flt-1 (sFlt-1) is a competitor of VEGF-A[37] and has been used to verify the role of this receptor on the biologic response of Tat on monocytes. sFlt-1 inhibited both monocyte polarization (Fig 1B) and monocyte chemotaxis (Fig 2B) to rTat. The effect was concentration dependent, with an IC$_{50}$ of 12 ng/mL for chemotaxis (not shown). At the highest concentration of sFlt-1 tested (210 ng/mL), inhibition of chemotaxis to rTat was 71% ± 10% (n = 4; P < .01). In the same experimental conditions, also monocyte migration to VEGF-A$_{165}$ was significantly inhibited (56% ± 9%; n = 3; P < .01), whereas chemotactic response to FMLP was not affected (120% of control; n = 2; not shown). sFlt-1 did not change monocyte basal migration and boiled sFlt-1 was inactive (Fig 2B). These data strongly suggest the Tat and VEGF-A$_{165}$ share common receptors on human monocytes.

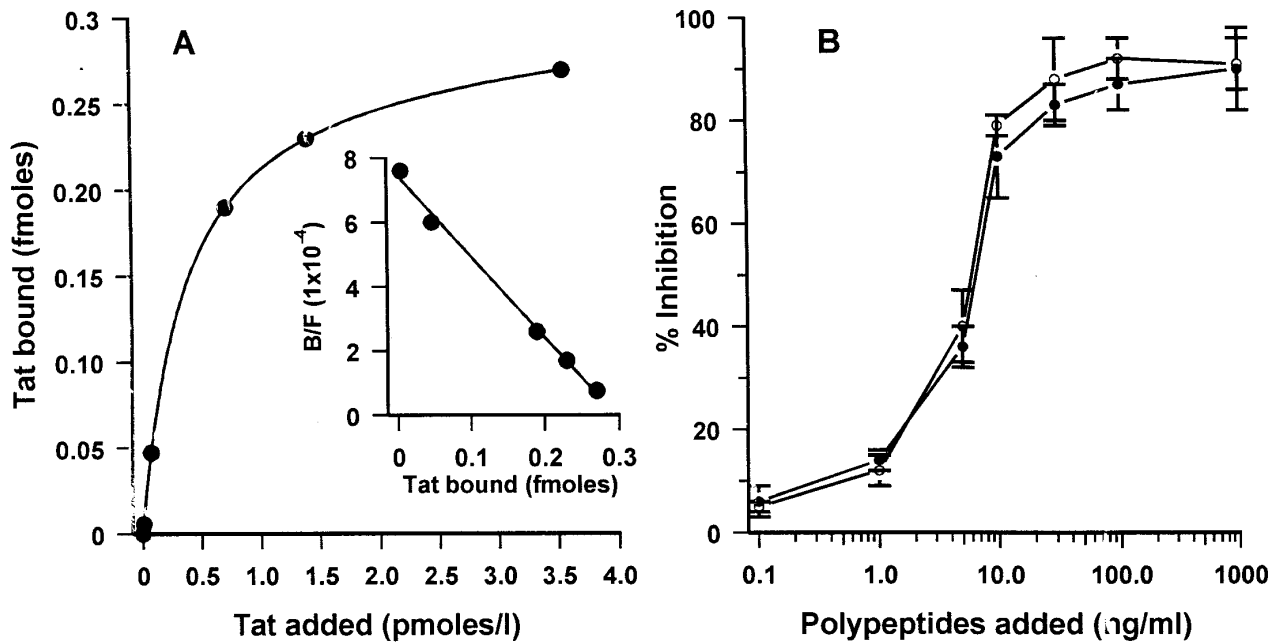To test the presence of high-affinity binding sites for Tat

Fig 3. Specific binding of [^{125}I]Tat to monocytes and curve displacement with cold rTat and VEGF-A$_{165}$. (A) Specific [^{125}I]Tat binding curve to monocyte at equilibrium and Schatchard analysis. For specific binding studies of Tat, human monocytes (1 × 10^6/ 0.2 mL of binding buffer) were incubated with increasing concentrations of [^{125}I]Tat in the presence of a 100-fold excess of unlabeled ligand at 22°C for 120 minutes. At the end of incubation, cell suspension were overlayered on 0.5 mL of dibutylphtalate and centrifuged for 10 minutes at 13,000g, and the pellet was solubilized with 2% SDS in PBS was counted in a γ counter. (B) Ligand displacement curve at equilibrium of [^{125}I]Tat binding to monocytes by rTat and VEGF-A$_{165}$. Human monocytes (1 × 10^6) were treated in the same way as in (A) with 0.7 pmol of [^{125}I] without or with the indicated concentrations of unlabeled rTat (○) or VEGF-A$_{165}$ (●). Each experiment was repeated at least three times.

on human monocytes, cells were incubated with increasing concentrations of [^{125}I]Tat in the presence of 100-fold amounts of cold molecule. The binding evaluated at equilibrium indicated the presence of a high-affinity binding site and the calculated kd was 280 ± 78 pmol/L (n = 3) with 320 ± 100 receptors per cell (n = 3; Fig 3A). As reported in chemotaxis experiments (Fig 2A), VEGF-A$_{165}$ competed with [^{125}I]Tat for the binding to monocytes (Fig 3B), and a concentration of 100 ± 12 pmol/L (n = 4) of unlabeled VEGF-A$_{165}$ blocked half of the binding of the labeled ligand. Figure 3B shows that unlabeled rTat also inhibits the binding of [^{125}I]Tat to monocytes, with a IC$_{50}$ of 204 ± 67 pmol/L.

The affinity binding site for Tat was further characterized by cross-linking experiments (Fig 4A). Two [^{125}I]Tat-containing major complexes with molecular weights of 92, 163, and 180 kD were detected when the ligand was cross-linked to the intact cells and displaced by 50-fold excess of unlabeled rTat or VEGF-A$_{165}$, indicating the interaction specificity and the cross-competition between the two polypeptides for the same protein (Fig 4A), confirming the biologic observation that VEGF-A$_{165}$ has a competitive effect on Tat-induced monocyte movement (Fig 2A). A third complex of 90 to 100 kD was present, but was not displaced by an excess of cold ligands.

To further confirm that Tat uses VEGFR-1/Flt-1, we studied the tyrosine phosphorylation of the receptor after stimulation. rTat or VEGF-A$_{165}$ induced the phosphorylation in tyrosine residues of a 150-kD protein recognized by anti–VEGFR-1/Flt-1 antibody (Fig 4B). Heat-inactivated

rTat was inactive, showing the specificity of the phenomenon (data not shown).

DISCUSSION

Tat protein of HIV-1 activates biologic functions of mesenchymal cells, including monocytes and endothelial cells.[11,13,20,21-27] We have previously shown that Tat binds VEGFR-2/KDR expressed on endothelial cells and activates its tyrosine kinase activity.[30] Furthermore, antiserum neutralizing VEGFR-2/KDR or an excess of soluble form of KDR inhibited in vitro and in vivo activation of endothelial cells triggered by Tat, suggesting that this viral protein mimics VEGF-A.[30]

Besides migration and proliferation of endothelial cells,[31,32] VEGF induces directed migration of monocytes and expression of procoagulant activity.[34] This effect is mediated by a specific interaction with a single class of binding site with an apparent kd of 300 to 500 pmol/L.[44] In contrast to endothelial cells, which express both VEGFR-1/Flt-1 and VEGFR-2/KDR,[28,29,33] human monocytes express only VEGFR-1/Flt-1, which accounts for the observed biologic activities.[35,36]

Indeed, the observations that VEGF-A activates the migration of monocytes by a specific receptor-dependent mechanism[31,35,36] and that rTat activates VEGFR-2/KDR in endothelial cells raise the possibility that a VEGFR is also essential for the migration and the invasiveness properties of human monocytes triggered by this HIV-1 protein.[26,27]
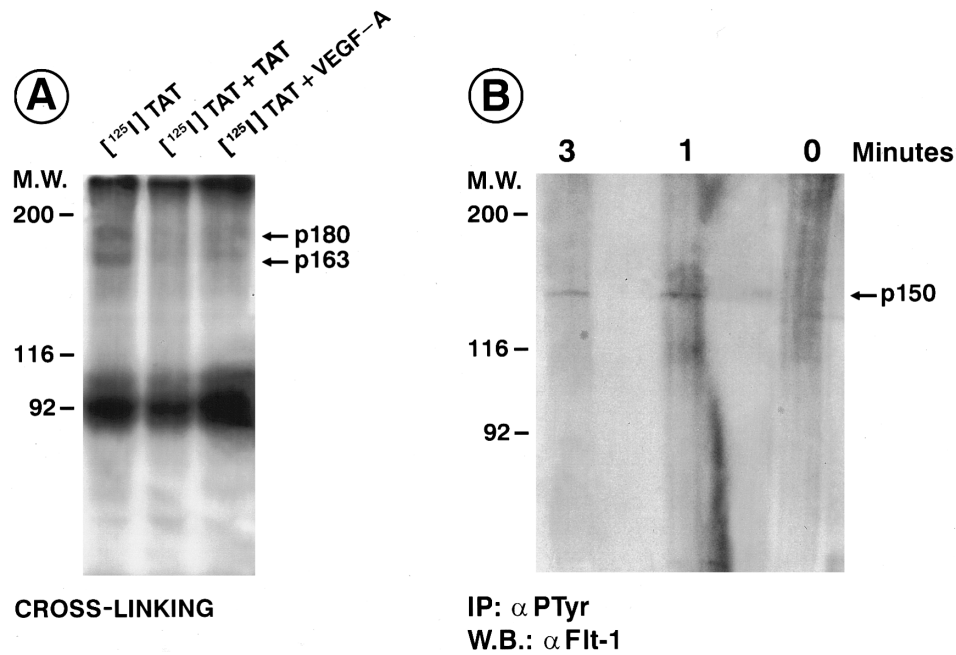
Human monocytes stimulated by subnanomolar concen-

Fig 4. Cross-linking of [^{125}I]Tat to monocytes and effect of rTat on tyrosine autophosphorylation of the VEGFR-1/Flt-1. (A) Monocytes (2 × 10^7) were incubated at 22°C in 0.5 mL of binding buffer with 0.5 nmol/L [^{125}I]Tat with or without 50-fold excess of unlabeled rTat or VEGF-A$_{165}$ for 90 minutes and then cross-linked with 1 mmol/L disuccimidyl suberate for 30 minutes. Solubilized proteins were separated by denaturing SDS-PAGE (8%) and visualized by autoradiography. (B) Human monocytes (1 × 10^7) were stimulated with rTat (10 ng/mL) in RPMI 1640 medium containing 1 mmol/L Na orthovanadate at 4°C. Cell lysates were immunoprecipitated with antiphosphotyrosine monoclonal antibody. After SDS-PAGE (8%) separation, proteins were immunoblotted with an anti–VEGFR-1/Flt-1 antibody and visualized by enhanced chemiluminescence technique. Each experiment was repeated at least two times.

trations of rTat start to migrate. This biologic effect shows a cross-desensitization with VEGF-A$_{165}$, but not with FMLP. Furthermore, monocyte migration elicited both by rTat and VEGF-A$_{165}$ is blocked by Ptox, and as a whole these data suggest that the two molecules use an overlapping pathway to induce monocyte motility. sFlt-1, which lacks in transmembrane and kinase domains and inhibits biologic activities of VEGF-A on endothelium,[43] has been used to support this hypothesis. sFlt-1 inhibited the polarization and the migration induced by rTat and VEGF-A$_{165}$, but was ineffective on the activity of FMLP. This suggests that sFlt-1 competes with membrane spanning VEGFR-1/Flt-1, as shown in endothelial cells,[43] and thus can function as specific antagonist. However, it is possible to speculate that sFlt-1 makes a heterodimer with the membrane receptor that cannot signal inside monocytes upon rTat or VEGF-A$_{165}$ binding.

Based on radioligand binding experiments, monocytes expressed a single class of Tat binding sites with a kd of approximately 0.2 nmol/L, similar to that reported for VEGF-A on monocytes.[43] The displacement of [^{125}I]Tat by an excess of VEGF-A$_{165}$ again confirms the hypothesis that Tat uses a VEGFR. [^{125}I]Tat was specifically cross-linked into two major complexes with molecular weights of 163 and 180 kD. These complexes were displaced by an excess of Tat or VEGF-A$_{165}$. Because the molecular mass of VEGFR-1/Flt-1 in monocytes is 150 kD (Fig 4), we can speculate that these complexes correspond to VEGFR-1/Flt-1 containing, respectively, one and two molecules of labeled

Tat. A third complex of 90 to 100 kD was observed, but not displaced by an excess of cold rTat, suggesting an unspecified interaction due to the cross-linking procedure. However, we cannot exclude that the last complex is the result of a specific binding with a membrane protein that binds Tat with low affinity and high capacity (ie, an integrin[45]). The lack of displacement of [^{125}I]Tat by the addition of cold rTat could be explained by the fact that experimental conditions have been standardized to study the high-affinity binding sites

The tyrosine kinase domain of VEGFR-1/Flt-1 is implicated in the ligand-dependent signal transduction in intact cells and the same receptor can be weakly phosphorylated on tyrosine residues upon VEGF-A challenge.[33,46,47] Therefore, the rapid tyrosine phosphorylation of VEGFR-1/Flt-1 in rTat-stimulated monocytes suggests that the complex Tat/receptor transduces motogenic signals.

Experimental evidences have indicated that some extracellular activities of Tat can be mediated through specific interactions between the RGD-containing region and integrin receptors.[25,27,45,48] Indeed, we cannot exclude a cooperation between VEGFR-1/Flt-1 and integrins in monocyte response to Tat.

The data presented in this report suggest that HIV-1-Tat could play an active role in monocyte extravasation and subsequent tissue damage in AIDS patients. Furthermore, the activation of monocyte VEGFR-1/Flt-1 by Tat present in serum or tissues[20,25] could participate to the generalized

impairment of immune system in infected subjects. A previous report has shown that monocytes from patients with AIDS have a selective defect of chemotaxis.[49] Our preliminary data demonstrate that Tat desensitizes monocytes to unrelated chemotactic stimuli such as monocyte chemotactic peptide-1 (MCP-1; monocytes preincubated for 2 hours in control medium [% over the basal migration; n = 3]: Tat [10 ng/mL], 75 ± 12; VEGF-A$_{165}$ [10 ng/mL], 72 ± 9; MCP-1 [50 ng/mL], 102 ± 15; and monocytes preincubated for 2 hours with 10 ng/mL rTat [% of the basal migration; n = 4]: Tat [10 ng/mL],10 ± 3; VEGF-A$_{165}$ [10 ng/mL], 12 ± 2; MCP-1 [50 ng/mL], 15 ± 3]) and suggest that continued exposure to this viral protein could induce a loss in monocyte functions. We can also presume that Tat-VEGFR-1/Flt-1 interaction is instrumental in the defective function of professional antigen-presenting cells, including monocytes, as showed for VEGF-A.[50]

ACKNOWLEDGMENT

REFERENCES

1. Griffin GE, Leung K, Folks MT, Kunkel S, Nabel GJ: Activation of HIV gene expression during monocyte differentiation by induction of NF-kB. Nature 339:70, 1989

2. Pantaleo G, Graziosi C, Demarest Butini L, Montroni M, Fox CH, Orenstein JM, Kotler DP, Fauci AS: HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362:355, 1993

3. Poli G, Bressler P, Kinter A, Duh E, Timmer WC, Rabson A, Justement JS, Stanley S, Fauci AS: Interleukin 6 induces HIV expression in infected monocytic cells alone and in synergy with TNF(by trascriptional and post-trascriptional mechanisms. J Exp Med 172:151, 1990

4. Koyanagi Y, O'Brien WA, Zhao JQ, Golde DW, Gasson JC, Chen ISY: Cytokines alter production of HIV-1 from primary mononuclear phagocytes. Science 241:1673, 1988

5. Pomerantz RJ, Feinberg MB, Trono D, Baltimore D: Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human HIV-1 expression. J Exp Med 172:253, 1990

6. Gilles PN, Lathey JL, Spector SA: Replication of macrophage-tropic and T-cell–tropic strains of human immunodeficiency virus type 1 is augmented by macrophage-endothelial cell contact. J Virol 69:2133, 1995

7. Dhawan S, Weeks BS, Soderland C, Schnaper HW, Toro LA, Asthana SP, Hewlett IK, Stetler-Stevenson WG, Yamada SS, Yamada KM, Meltzer MS: HIV-1 infection alters monocyte interactions with human microvascular endothelial cells. J Immunol 154:422, 1995

8. Genis P, Marti J, Berton EW, Boyle T, Gelbard HA, Dzenko K, Keane RW, Resnick L, Mizrachi Y, Volsky DJ, Epstein LG, Gendelman HE: Cytokines and arachidonic metabolites produced during human immunodeficiency virus (HIV)-infected macrophage-astroglia interactions: Implications for the neuropathogenesis of HIV disease. J Exp Med 176:1703, 1992

9. Le Naour R, Raoul H, Mabondzo H, Ripoll L, Bartholeyns J, Romet-Lemon JL, Dormont J: Functional consequences of monocyte/macrophage infection by HIV1. Res Immunol 143:49, 1992

10. Vaishnaw YN, Wong-Staal F: The biochemistry of AIDS. Annu Rev Biochem 60:577, 1991

11. Gibellini D, Zauli G, Re MC, Milani D, Furlini G, Caramelli E, Capitani S, La Placa M: Recombinant human immunodeficiency virus type-1 (HIV-1) Tat protein sequentially up-regulates IL-6 and TGF-β1 mRNA expression and protein synthesis in peripheral blood monocytes. Br J Haematol 88:261, 1994

12. Scala G, Ruocco MR, Ambrosino C, Mallardo M, Giordano V, Baldassarre F, Dragonetti E, Quinto I, Venuta S: The expression of the interleukin 6 gene is induced by the human Immunodeficiency virus 1 Tat protein. J Exp Med 179:961, 1994

13. Westendorp MO, Shatrov VA, Schulze-Osthoff K, Frank R, Kraft M, Los M, Krammer PH, Droge W, Lehmann V: HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. EMBO J 14:546, 1995

14. Siekevitz M, Feinberg MB, Holbrook N, Wong-Staal F, Greene WC: Activation of interleukin 2 and interleukin 2 receptor (Tac) promoter expression by the trans-activator (tat) gene product of human T-cell leukemia virus, type 1. Proc Natl Acad Sci USA 84:5389, 1987

15. Buonaguro L, Buonaguro FM, Giraldo G, Ensoli B: The human immunodeficiency virus type 1 Tat protein transactivates tumor necrosis factor beta gene expression through a TAR-like structure. J Virol 68:2677, 1994

16. Li CJ, Wang C, Friedman DJ, Pardee AB: Reciprocal modulations between p53 and Tat of human immunodeficiency virus type 1. Proc Natl Acad Sci USA 92:5461, 1995

17. Howcroft TK, Strebel K, Martin MA, Singer DS: Repression of MHC class I gene promoter activity by two-exon Tat of HIV. Science 260:1320, 1993

18. Taylor JP, Cupp C, Diaz A, Chowdhuhury M, Khalili K, Jimenez SA, Amini SA: Activation of expression gene coding for extracellular matrix proteins in Tat-producing glioblastoma cells. Proc Natl Acad Sci USA 89:9617, 1992

19. Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gentelman R, Morgan RA, Wingfield P, Gallo RC: Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J Virol 67:277, 1993

20. Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH: Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp 120. Nature 375:497, 1995

21. Lotz M, Clark-Lewis I, Ganu I: HIV-transactivatior Tat induces proliferation and TGF beta expression in human articular chondrocyes. J Cell Biol 245:365, 1994

22. Barillari G, Buonaguro L, Fiorelli V, Hoffman J, Michaels F, Gallo RC, Ensoli B: Effects of cytokines from activated immune cells on vascular cell growth and HIV-1 gene expression: Implications for AIDS-Kaposi's sarcoma pathogenesis. J Immunol 149:3727, 1992

23. Hofman FM, Dohadwala MM, Wright AD, Hinton DR, Walker SM: Exogenous tat protein activates central nervous system-derived endothelial cells. J Neuroimmunol 54:19, 1994

24. Albini A, Benelli R, Presta M, Rusnati M, Ziche M, Rubartelli A, Paglialunga G, Bussolino F, Noonan D: HIV-1 tat protein is a heparin-binding angiogenic growth factor. Oncogene 12:289, 1996

25. Ensoli B, Gendelman R, Markham P, Fiorelli V, Colombini S, Raffeld M, Cafaro A, Chang H, Brady JN, Gallo RC: In vitro and in vivo evidence that basic fibroblast growth factor and human immunodeficiency virus type-1 Tat protein synergize in inducing Kaposi's sarcoma development. Nature 371:674, 1994

26. Lafrenie RM, Wahl LM, Epstein JS, Hewlett IK, Yamada KM, Dhawan S: HIV-1-Tat modulates the function of monocytes and alters their interactions with microvessel endothelial cells. J Immunol 156:1638, 1996

27. Lafrenie RM, Wahl LM, Epstein JS, Hewelett IK, Yamada KM, Dhawan S: HIV-1-Tat protein promotes chemotaxis and invasive behaviour by monocytes. J Immunol 157:974, 1996

28. Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB: Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene 6:1677, 1991

29. Millauer B, Wizgmann-Voos S, Schnurch H, Martinez R, Moller NPH, Risau W, Ulrich A: High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72:835, 1993

30. Albini A, Soldi R, Giunciuglio D, Giraudo E, Benelli R, Primo L, Noonan D, Salio M, Camussi G, Rockl W, Bussolino F: The angiogenesis induced by HIV-1 Tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. Nat Med 2:1371, 1996

31. Ferrara N, Houck K, Jakerman L, Leug DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocrinol Rev 13:18, 1992

32. Kenneth AT: Vascular endothelial growth factor, a potent and selective angiogenic agent. J Biol Chem 271:603, 1996

33. De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The *fms*-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989, 1992

34. Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC, Pan YCE, Olander JV, Connolly DT, Stern D: Vascular permeability factor: A tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J Exp Med 172:1535, 1990

35. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marmé D: Migration of human monocytes in response to vascular endothelial growth factor (VEGF-A$_{165}$) is mediated via the flt-1/VEGF-A$_{165}$ receptor. Blood 87:3336, 1996

36. Clauss M, Weich H, Breier G, Knies U, Wolfgang R, Waltenberg J, Risau W: The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implication for a functional role of placenta growth factor in monocyte activation and chemotaxis. J Biol Chem 271:17629, 1996

37. Sozzani S, Luini W, Molino M, J'lek P, Bottazzi B, Cerletti C, Matsushima K, Mantovani A: The signal transduction pathway involved in the migration induced by a monocyte chemotactic cytokine. J Immunol 147:2215, 1991

38. Falk W, Goodwin RH Jr, Leonard EJ: A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. J Immunol Methods 33:239, 1980

39. Locati M, Zhou D, Luini W, Evangelista V, Mantovani A, Sozzani S: Rapid induction of arachidonic acid release by monocyte chemotactic protein-1 and related chemokines—Role of Ca2+ influx, synergism with platelet-activating factor and significance for chemotaxis. J Biol Chem 269:4746, 1994

40. Wilkinson PC: Cell locomotion and chemotaxis: Basic concepts and methodological approaches. Methods 10:74, 1996

41. McPhail LC, Harvath L: Signal transduction in neutrophil oxidative metabolism and chemotaxis, in Wheeler JG, Abramson J (ed): The Natural Immune System: The Neutrophil. Oxford, UK, Oxford, 1993, p 63

42. Murphy PM: The molecular biology of leukocyte chemoattractant receptors. Annu Rev Immunol 12:593, 1994

43. Kendall RL, Thomas KA: Inhibition of vascular endothelial growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 90:10705, 1993

44. Shen H, Clauss M, Ryan J, Schmidt AM, Tijburg P, Borden L, Connoly D, Stern D, Kao J: Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. Blood 81:2767, 1993

45. Brake DA, Debouck C, Biesecher G: Identification of an Arg-Gly-Asp (RGD) cell adhesion site in human immunodeficiency virus type 1 transactivation protein, tat. J Cell Biol 111:1275, 1990

46. Waltemberger J, Claesson-Welsh L, Siegbahn A, Shibuyha M, Heldin CH: Differential signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem 269:26988, 1994

47. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shybuya: A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene 10:135, 1995

48. Zauli G , Gibellini D, Celeghini C, Mischiati C, Bassini A, La Placa M, Capitani S: Pleiotropic effects of immobilized versus soluble recombinant HIV-1 Tat protein on CD3-mediated activation, induction of apoptosis, and HIV-1 long terminal repeat transactivation in purified CD4$^+$ T lymphocytes. J Immunol 157:2216, 1996

49. Poli G, Bottazzi B, Acero R, Bersani L, Rossi V, Introna M, Lazzarin A, Galli M, Mantovani A: Monocyte function in intravenous drug abusers with lymphadenopathy syndrome and in patients with acquired immunodeficiency syndrome: Selective impairment of chemotaxis. Clin Exp Immunol 62:136, 1985

50. Gabrilovich DI, Chen HL, Giris KR, Cunningham HT, Meny GM, Nadaf S, Kavanaugh D, Carbone DP: Production of vascular endothelial growth by human tumors inhibits the functional maturation of dendritic cells. Nat Med 2:1096, 1996