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Tumor Necrosis Factor Alpha Inhibits Entry of Human Immunodeficiency Virus Type 1 into Primary Human Macrophages: a Selective Role for the 75-Kilodalton Receptor

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The proinflammatory cytokine tumor necrosis factor alpha (TNFα) is readily detected after human immunodeficiency virus type 1 (HIV-1) infection of primary macrophages in vitro and is present in plasma and tissues of patients with AIDS. Previous studies have shown that human recombinant TNFα (hrTNFα) enhances HIV replication in both chronically infected promonocytic and T-lymphoid cell lines in vitro. We report here that in contrast to untreated tissue culture-differentiated macrophages (TCDM), in which the proviral long terminal repeat (LTR) could be detected as soon as 8 h postinfection by a PCR assay, TCDM pretreatment for 3 days by hrTNFα markedly delayed its appearance until 72 h after infection with the HIV-1 Ada monocytotropic strain. Moreover the inhibition of formation of the proviral LTR in HIV-1-infected TCDM was directly proportional to the concentration of hrTNFα used. To determine if the inhibition of LTR formation results from blockade of viral entry, we performed a reverse transcription PCR assay to detect intracellular genomic viral RNA as early as 2 h after infection. Pretreatment of primary TCDM by hrTNFα for 3 days and even for only 2 h inhibits 75% of the viral entry into the cells. The inhibition of viral entry by hrTNFα was totally abolished by the use of anti-human TNFα monoclonal antibody. By using TNFα mutants specific for each human TNFα receptor, we showed that the inhibition of HIV-1 entry into TCDM was mediated not through the 55-kDa TNF receptor but through the 75-kDa TNF receptor. Although prolonged (1 to 5 days) TNFα treatment can downregulate CD4 expression in primary human TCDM, surface CD4 levels were not reduced by 2 h of treatment and was therefore not a limiting step for HIV-1 entry. In contrast to the inhibition of viral entry into primary TCDM, pretreatment with hrTNFα did not modify HIV-1 entry into phytohemagglutinin A-activated peripheral blood lymphocytes. TNFα-pretreatment inhibited HIV-1 replication in primary TCDM but not in phytohemagglutinin A-activated peripheral blood lymphocytes as assessed by decreased reverse transcriptase activity in culture supernatants. These results demonstrate that TNFα is able to enhance host cellular resistance to HIV-1 infection and that selective inhibition of HIV-1 entry into primary TCDM by TNFα involves the 75-kDa TNF receptor but not the 55-kDa TNF receptor.

Interactions of $CD4^+$ T lymphocytes and macrophages, which also express the CD4 entry receptor for human immunodeficiency virus (HIV), play a key role in maintaining immunocompetence in HIV-infected individuals. One of the main failure of AIDS is a major dysregulation of cytokine production. Parallel to the Th1/Th2 cytokine switch (5), a chronic activation of the immune system might explain the increased levels of proinflammatory cytokines detected in plasma and tissues of patients with AIDS (12). Among the proinflammatory cytokines detected during the progression of the disease, tumor necrosis factor alpha (TNFα) seems to play a central role. In vitro, TNFα is secreted by primary macrophages infected in culture by HIV type 1 (HIV-1) or treated with envelope glycoprotein gp120 and by HIV-infected monocytes isolated from patients (34). In vivo, membrane-bound TNFα present on the surface of $CD4^+$ T cells might account for the polyclonal activation of B cells (31), and high levels of TNFα detected in plasma and tissues might contribute to the cachexia and fever observed in patients with AIDS (27).

In humans, two TNF receptors (TNF-Rs) with molecular masses of 55 kDa (TNF-R1) and 75 kDa (TNF-R2) have been identified and cloned (29, 57). The two receptors display no homology between their intracellular domains, suggesting that they utilize separate signaling pathways (59). To determine the action mediated by each TNF-R, anti-TNF-R antibodies behaving as receptor agonists or antagonists and TNFα mutants have both been used. TNF-R1 mediates most activities of TNFα such as cytotoxicity, fibroblast proliferation, resistance to chlamydiae, and synthesis of prostaglandin E_2 (10, 11, 52). TNF-R2 has been involved in the proliferation of thymocytes and a murine cytotoxic T-cell line (58), in the TNF-dependent proliferative response of human mononuclear cells (15), in the induction of granulocyte/macrophage colony-stimulating factor secretion (61), and in the inhibition of early hematopoiesis (22).

TNFα enhances HIV-1 replication in chronically infected promonocytic and T-lymphoid cell lines by activation of the nuclear factor NF-κB, which stimulates the long terminal repeat (LTR) of the provirus (9, 41). By contrast, gamma interferon (IFN-γ), which is an important enhancer of TNFα production by macrophages, inhibits HIV-1 growth in primary tissue culture-differentiated macrophages (TCDM) (36). Thus, the role of lymphocyte activation in mediating both host resistance and susceptibility to HIV seems paradoxical. In view of previously reported antiviral actions of TNFα (35, 64), we sought evidence for alternate actions of TNFα in host resis-

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tance to HIV infection. In this report, we describe that human recombinant TNFα (hrTNFα) pretreatment of primary TCDM is accompanied by a significant delay and inhibition of HIV-1 LTR reverse transcript detection which is the consequence of a strong inhibition of viral entry into the TCDM, independent of surface CD4 expression. By using agonistic TNFα mutants specific for either TNF-R1 or TNF-R2, we demonstrate that the inhibition of HIV-1 entry by hrTNFα is mediated through the 75-kDa TNF-R2. These results reveal a previously unknown protective effect of TNFα against HIV-1 infection in primary TCDM and highlight the role of TNF-R2 in anti-HIV defense.

MATERIALS AND METHODS

Isolation and culture of TCDM. Human peripheral blood mononuclear cells were isolated from healthy donors as described previously (8); in short, Ficoll-Hypaque-isolated peripheral blood mononuclear cells were incubated for 1 h in 2% gelatin-coated plates. Adherent TCDM, >94% $CD14^+$ by flow cytometry analysis, were cultivated in RPMI supplemented with 10% (vol/vol) pooled AB human serum for 48 h before transfer either to 50-mm-diameter petri dishes at a density of 4×10^6 cells per dish in a 3-ml final volume or to 48-well plates at a density of 2×10^5 cells per well in a 400-μl total volume. All media and sera were monitored for endotoxin contamination (*Limulus* amebocyte lysate; E-Toxate; Sigma).

Preparation of PHA-activated lymphocytes. Nonadherent cells, >90% of which were peripheral blood lymphocytes (PBL), were harvested after Ficoll-Hypaque isolation and adherence as described above. Three-day-old PBL were cultivated in RPMI with 10% pooled AB human serum supplemented for the first 48 h with phytohemagglutinin A (PHA; 5 μg/ml) before addition of human recombinant interleukin-2 (hrIL-2; 50 U/ml).

Cytokine treatment. hrTNFα (Sigma) was added to the TCDM for 2 h to 3 days before infection at concentrations ranging from 0.1 to 250 ng/ml; 100 IU of hrIL-1β (Sigma) per ml, 10 ng of hrIL-10 (DNAX) per ml, and 200 IU of human recombinant IFN-γ (hrIFN-γ; Boehringer) per ml were added to the TCDM for 2 h to 3 days before infection. For detection of reverse transcriptase (RT) activity, TNFα pretreatment consisted of addition of TNFα every day before infection, at the time of infection, and every 2 days afterward. To inhibit the effects of hrTNFα, 2.3 μg of mouse-human chimeric anti-human TNFα monoclonal antibody (MAb) cA2 (kindly provided by M. Feldmann, Kennedy Institute of Rheumatology, London, England) was mixed for 30 min at room temperature with 20 ng of hrTNFα per ml and then added to 4×10^6 TCDM in a 3-ml final volume. MAb cA2 has been previously reported to block TNFα binding to both TNF-R1 and TNF-R2 (54). Agonistic TNFα mutants (kindly provided by W. Lesslauer, Hoffmann-La Roche, Basel, Switzerland) R32W-S86T (arginine at position 32 replaced by tryptophan and serine at position 86 replaced by threonine) and D143N-A145R (aspartic acid at position 143 replaced by asparagine and alanine at position 145 replaced by arginine) called T55 and T75, are specific for human TNF-R1 and TNF-R2, respectively. T55 has more than 5,000-fold-decreased affinity for TNF-R2 compared with wild-type TNFα but only a 2-fold reduction in binding to TNF-R1 (30). T55 elicits a full cytotoxic response in human KYM-1 cells indistinguishable from that of wild-type TNFα (30). T75 has more than 2,500-fold-decreased affinity for TNF-R1 but has 10- to 20-times-lower binding affinity for TNF-R2 compared with wild-type TNFα (30). The biological activity of T75 was similar to that of the agonistic polyclonal antibody RaTNF-R75 (30), which has been shown to stimulate activated human mononuclear cells (15). In our experiments, we used T55 and T75 at 20 and 200 ng/ml, respectively. Cytokines, the anti-human TNFα MAb cA2, and agonistic TNFα mutants were all found to be endotoxin free (*Limulus* amebocyte lysate).

Cell survival. The cell viability after treatment of TCDM for 2 to 72 h with 100 IU of hrIL-1β per ml, 10 ng of hrIL-10 per ml, 200 IU of hrIFN-γ per ml, 20 ng of hrTNFα per ml, 20 ng of T55 mutant per ml, 200 ng of T75 mutant per ml, or both mutants together was assessed by performing a colorimetric reaction based on the capacity of mitochondrial dehydrogenase of living cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide to formazan. The quantity of formazan produced and measured at an optical density of 540 nm is correlated with the number of living cells (42).

Flow cytometry. TCDM were pretreated for 2 to 24 h with 20 ng of TNFα, 20 ng of T55 mutant, or 200 ng T75 mutant per ml or left untreated. TCDM were labeled after fixation with 3% paraformaldehyde in phosphate-buffered saline (PBS), quenched with 10% (vol/vol) goat serum in PBS, and analyzed by flow cytometry. Surface CD4 was detected by using $F(ab')_2$ fragments of mouse anti-human CD4 (MAb Q4120 [immunoglobulin G1] from Q. Sattentau, provided by the Medical Research Council's AIDS Directed Program [ADP318]) followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Fab specific; Sigma). Specific fluorescence was assessed by comparison with an irrelevant isotype-matched control MAb (Serotec, Oxford, England). Relative antigen density was calculated as follows: geometric mean (hrTNFα) − geometric mean (isotype control)/geometric mean (mock) − geometric mean (isotype control).

The characterization of the TCDM population was confirmed by using a mouse anti-human CD14 immunoglobulin G2 MAb (Sigma Immunochemicals).

Preparation of virus. Macrophage-tropic viral stock HIV-1 Ada (H. E. Gendelman, AIDS Reagent Program, National Institute of Allergy and Infectious Diseases) was grown and titrated in TCDM as described previously (8). Endotoxin contamination was avoided, and no exogenous cytokines or growth factors were added to virus stock cultures. At 12 to 15 days following infection, viral supernatants were clarified by centrifugation at 1,200 × *g* for 10 min and passaged through 0.44-μm-pore-size filters. Viral DNA was removed by treatment with 100 μg of DNase I per ml in the presence of 5 mM $MgCl_2$ for 30 min at room temperature. The supernatant was divided into aliquots and stored at −80°C.

Detection of the proviral LTR by PCR. Three-day-old TCDM were cultivated in 48-well plates (2×10^5 cells per well) and treated for 72 h with either hrTNFα (0.1 to 250 ng/ml) or hrIL-1β (100 IU/ml) before infection with HIV-1 Ada (multiplicity of infection [MOI] of 0.12). Before use, the HIV-1 Ada stock was treated for 30 min at room temperature with 100 μg of DNase I per ml supplemented with 5 mM $MgCl_2$. After 1 h of exposure to the virus at 37°C, the cells were washed three times with PBS to remove the unadsorbed inoculum and reincubated in fresh culture medium supplemented with cytokines at 37°C. Then the cells were lysed 0, 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, and 72 h postinfection in a buffer containing 100 mM KCl, 20 mM Tris (pH 8.4), 500 μg of proteinase K per ml, and 0.2% (vol/vol) Nonidet P-40. PCR was performed on cell lysates as described previously (8), using a Programmable Thermal Controller (M.J. Research Inc., Watertown, Mass.). The PCR primer sequences for LTR and human β-globin gene (DNA control) were as follows: HIV-1 LTR primers, CACACA AGGCTACTTCCCTGA (U3 [sense]) and GATCTCTAGTTACCAGAGT CAC (U5 [antisense]) (PCR product, 540 bp); human β-globin, CCTTTGTTC CCTAAGTCCAA (glb [sense]) CCTCACCTTCTTTCATGGAG (glb [antisense]) (PCR product, 238 bp).

Proviral quantitative PCR was performed by comparison of experimental samples with standards containing known amounts of HXB2 DNA (AIDS Reagent Program). The proviral DNA was excised by *Xba*I digestion and titrated in TCDM cell lysate. HXB2 titration was confirmed by comparison with LTR PCR products of known dilutions of ACH-2 cells (6). PCR products were measured by incorporation of ^{32}P via end labeling of the 3′ primer as reported in detail previously (7). Experimental values were extrapolated from a curve fitted to HXB2 DNA titration values, using a hyperbolic function (Sigma Plot; Jandel Scientific, Corte Madera, Calif.).

Detection of viral RNA by RT-PCR. Three-day-old TCDM were cultivated in six-well plates (4×10^6 cells per well) and treated for 2 to 72 h with 20 ng of hrTNFα, 100 IU of hrIL-1β, 10 ng of hrIL-10, or 200 IU of hrIFN-γ per ml before infection with HIV-1 Ada (MOI of 1.0). Before use, HIV-1 Ada stock was treated for 30 min at room temperature with 100 μg of DNase I per ml supplemented with 5 mM $MgCl_2$. After 1 h of exposure to virus at 37°C, the cells were washed three times with PBS to remove the unadsorbed inoculum and reincubated in fresh culture medium at 37°C. Cells were then treated with 0.25% (wt/vol) trypsin in PBS for 10 min at room temperature, washed once in PBS, and subsequently treated with RPMI supplemented with 10% fetal calf serum for 10 min at room temperature. After extensive washes with PBS to eliminate extracellular virions, total cellular RNA was extracted by using 1 ml of RNAzol (Cinna/Biotex, Houston, Tex.) with 20 μg of *Escherichia coli* rRNA as carrier. To test the validity of the entry assay, we compared two different temperatures, 37 and 4°C. For each sample, a 10-μl RNA aliquot was stored at −20°C while the remaining 10 μl was reverse transcribed in a final volume of 50 μl containing 800 U of Moloney murine leukemia virus RT (Gibco-BRL), 2.5 mM deoxynucleoside triphosphate (dNTP), 5 μg of $oligo(dT)_{12-18}$, and 31 U of RNase inhibitor (all from Pharmacia) for 1 h at 37°C. PCR amplification was performed in a 50-μl volume in the presence of 10 ng of each primer per ml, 0.25 mM each dNTP, 1 U of *Taq* polymerase, 50 mM KCl, and 10 mM Tris-HCl (pH 8.4), using a Programmable Thermal Controller (M.J. Research) operating the following temperature steps: 60 s at 94°C and then 30 cycles (30-s denaturation at 94°C; 30-s annealing at 60°C; 60-s extension time at 72°C). The $MgCl_2$ concentrations used for *gag* and human β-actin cDNA amplifications were 3.0 and 2.5 mM, respectively. The following primer pairs were used: *gag* (5′ primer, GGTACATCAG GCCATATCACC; 3′ primer, TGACATGCTGTCATCATTTCTTC [PCR product, 627 bp]) and human β-actin (5′ primer, GATGCAGAAGGAGATCA CTG; 3′ primer, AGTCATAGTCCGCCTAGAAG [PCR product, 205 bp]). PCR products were separated by 2% agarose electrophoresis and visualized by ethidium bromide staining. A DNA ladder (type VI; Boehringer) was used for molecular weight determination.

After blotting onto a nylon membrane (Hybond-N^+; Amersham), using 0.4 M NaOH for the transfer, the membrane was prehybridized for 3 h at 45°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate)–1% (wt/vol) blocking agent–0.1% (wt/vol) *N*-lauroylsarcosine–0.02% (wt/vol) sodium dodecyl sulfate (SDS). Primer gag^{1669} (5′-ACCGGTCTACATAGTCTC-3′) (55) (100 pM) was labeled with digoxigenin as instructed by the manufacturer (Boehringer) and used as a probe. Hybridization was performed overnight at 45°C by using the digoxigenin-labeled probe gag^{1669}. The membrane was washed twice with 2× SSC–0.1% (wt/vol) SDS at room temperature for 5 min and then twice with 2×

SSC–0.1% (wt/vol) SDS at 45°C for 15 min. Digoxigenin-labeled probes were detected by using Lumigen PPD (Boehringer). The quantification of the labeled amplified *gag* fragments was achieved by using a photodensitometer. To compare the amounts of genomic viral RNA in different samples, we assayed equal amounts of cell lysates standardized by sequential dilution of β-actin cDNA.

RT activity assay. Culture supernatants (65 μl) were incubated for 10 min at 4°C in 10 μl of lysis buffer containing 0.5% Triton X-100, 0.75 M KCl, and 50 mM dithiothreitol. Then 25 μl of reaction mixture containing 0.2 M Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 4 mM EGTA, 3 μCi of [^{3}H]thymidine triphosphate (48 Ci/mmol; Amersham), and 5 μg of poly(rA)-oligo(dT) (Pharmacia) was added, and reverse transcription was performed at 37°C for 1 h. The reaction was stopped with 20 μl of 120 mM tetrasodium diphosphate decahydrate in 60% trichloroacetic acid. Precipitates were spotted onto DE81 paper, and radioactivity was measured with a Beckman LS 1800 liquid scintillation spectrometer. To determine intracellular RT activity, the cells were washed twice with PBS and then lysed in hypotonic buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 1.5 mM $MgCl_2$) supplemented with 1% (vol/vol) Nonidet P-40 as described previously (62). Assays were carried out in quadriplate.

Microscopy. Three-day-old TCDM were pretreated with 20 ng of hrTNFα per ml, 20 ng of hrTNFα per ml mixed with 2.3 μg of anti-huTNFα MAb per ml, or 2.3 μg of anti-huTNFα MAb per ml alone or were left untreated. Cultures were observed daily by contrast microscopy for appearance of syncytia.

Statistical analysis. Figures show the means of independent experiments and standard deviations.

RESULTS

Inhibition of HIV-1 LTR formation in TNFα-pretreated TCDM. To determine the effects of TNFα on early stages of the HIV-1 replicative cycle in TCDM, we analyzed proviral LTR synthesis by PCR assay (Fig. 1). We measured the time course of proviral LTR synthesis in untreated TCDM or in cells that had been pretreated for 3 days with 20 ng of hrTNFα per ml (Fig. 1A). β-Globin was used as an internal control. The absence of proviral LTR in untreated TCDM 0 and 2 h after infection indicated that the DNase I-treated viral stock itself contained no residual HIV-1 DNA. The HIV LTR signal appeared clearly at 8 h postinfection in untreated TCDM and increased subsequently with time, indicating cumulative entry and reverse transcription. In contrast, TCDM pretreatment with 20 ng of hrTNFα per ml for 3 days reduced and delayed the LTR signal until 72 h postinfection, although a dim but inconstant LTR signal could be detected at 24 h postinfection in some experiments. The amount of HIV LTR signal detected 24 h after infection in TCDM pretreated for 3 days with hrTNFα was dose dependent (Fig. 1B). To measure the inhibition of proviral LTR synthesis, a quantitative PCR assay was performed 24 and 48 h after infection of TCDM that had been either untreated or pretreated for 3 days with 10 ng of hrTNFα per ml (Fig. 1C). In untreated infected TCDM, the LTR copy number doubled between 24 and 48 h postinfection. In contrast, pretreatment with 10 ng of hrTNFα per ml for 3 days reduced HIV LTR to a constant 10 to 12 LTR copies per 10^3 cells, which represented 44 and 20% of the LTR levels detected in untreated TCDM at 24 and 48 h postinfection, respectively.

Inhibition of HIV-1 entry into TNFα-pretreated TCDM is mediated before reverse transcription and is independent of surface CD4 expression. To determine if the inhibition of proviral LTR formation after hrTNFα pretreatment results from a blockade of HIV-1 entry prior to reverse transcription, we assayed intracellular viral genomic RNA at 2 h after infection of TCDM at a high MOI (i.e., 1.0). A problem inherent in such an entry assay is to differentiate between virus that has entered the target cells and virus that has simply bound to the cell surface. As reported previously (37), we showed that a brief treatment with trypsin followed by extensive washing of the cells is an effective technique for removal of noninternalized virus from target cells. Thus, to detect intracellular viral RNA, we trypsinized the TCDM at 37°C 1 h after infection and washed them thoroughly to eliminate the cell surface-bound virions. Then we assayed *gag* sequence by reverse transcription of HIV-1 genomic RNA and subsequent cDNA amplification by PCR.

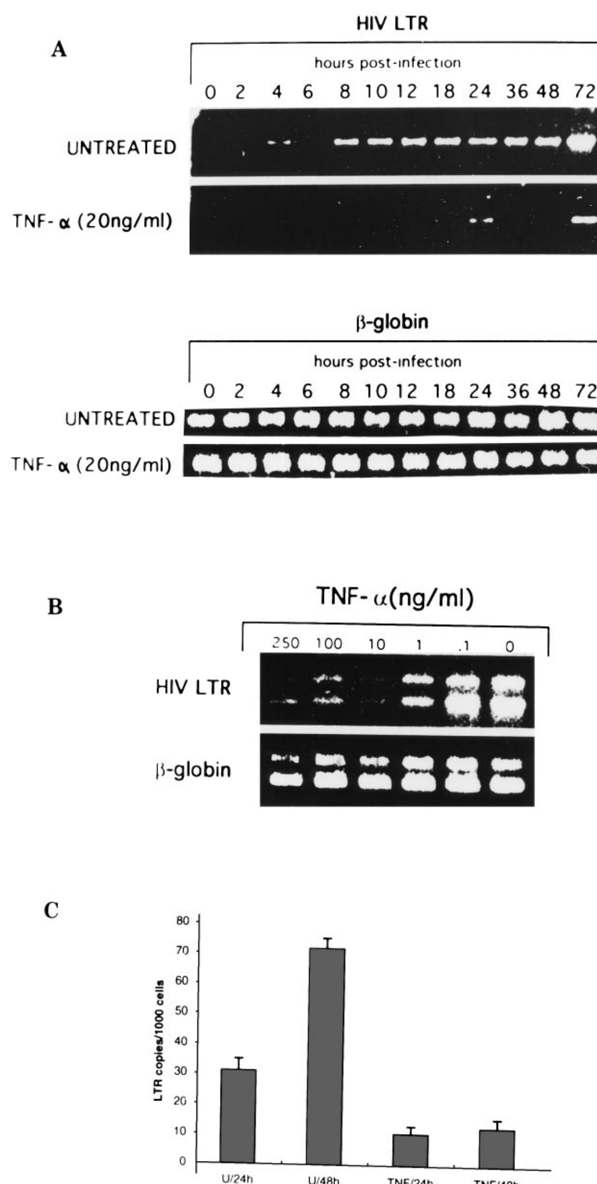

FIG. 1. Inhibition of LTR proviral synthesis in TCDM treated with hrTNFα. (A) Time course up to 72 h postinfection by HIV-1 Ada (MOI of 0.12) of TCDM pretreated with 20 ng of hrTNFα per ml for 72 h before infection or left untreated. Two percent agarose gels with PCR products for HIV LTR (540 bp) and human β-globin (238 bp) are shown. (B) Dose response of inhibition of LTR proviral synthesis by hrTNFα. TCDM were pretreated for 72 h with increasing concentrations of hrTNFα before infection by HIV-1 Ada (MOI of 0.12). Proviral LTR was analyzed at 24 h after infection. Two double-loaded 2% agarose gels with PCR products for HIV LTR (540 bp) and human β-globin controls (238 bp) are shown. (C) Quantitation of inhibition of LTR proviral synthesis by hrTNFα. TCDM were left untreated or were pretreated for 72 h with 10 ng of hrTNFα per ml before infection by HIV-1 Ada (MOI of 0.12). Proviral LTR products were measured by quantitative PCR at 24 and 48 h postinfection as described in Materials and Methods. The results are representative of four independent experiments.

To validate our entry assay, we analyzed viral RNA in TCDM infected and trypsinized at either 37 or 4°C. In contrast to infection at 37°C, virus can bind at 4°C, but neither fusion

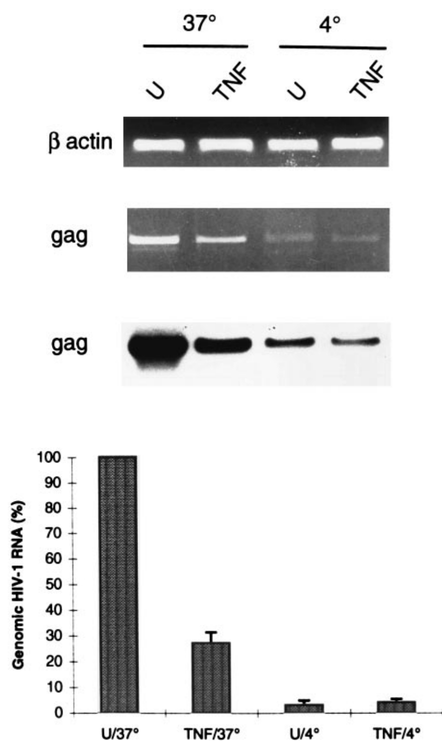

FIG. 2. Validation of an entry assay and inhibition of HIV-1 entry into TCDM by hrTNFα. TCDM were pretreated for 72 h with 20 ng of hrTNFα per ml or left untreated and, at either 37 or 4°C, infected with HIV-1 Ada (MOI of 1.0), trypsinized, and washed thoroughly. Viral RNA was extracted, reverse transcribed, and then amplified by PCR; PCR products for HIV *gag* (627 bp) and human β-actin (205 bp) were visualized on 2% agarose gels. The Southern blot shows that the amplified 627-bp *gag* fragment is specifically detected by an internal gag[1669] probe. The histogram summarizes the results of three independent experiments performed at 37 and 4°C for the detection of genomic HIV-1 RNA *gag* in TCDM pretreated for 72 h with 20 ng of hrTNFα per ml or left untreated.

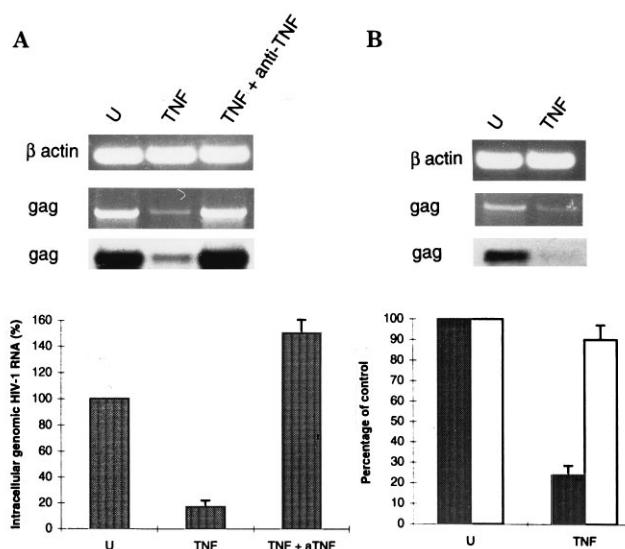

FIG. 3. Inhibition of HIV-1 entry into TCDM by hrTNFα is totally abolished by an anti-human TNFα MAb and does not depend on surface CD4 expression. (A) TCDM were pretreated for 72 h with 20 ng of hrTNFα per ml or 20 ng of hrTNFα per ml mixed with 2.3 μg of anti-human TNFα MAb per ml or left untreated before infection with HIV-1 Ada strain (MOI of 1.0). Anti-human TNFα MAb blocks totally the inhibition of HIV-1 entry into TCDM observed with hrTNFα. The histogram summarizes the results of three independent entry assay experiments. (B) TCDM were pretreated for 2 h with 20 ng of hrTNFα per ml or left untreated before infection with HIV-1 Ada strain (MOI of 1.0). In parallel with the measurement of HIV-1 entry, the TCDM surface CD4 expression was determined by flow cytometry as described in Materials and Methods. The histogram summarizes the results of three independent entry assays (■) and surface CD4 experiments (□); pretreatment for only 2 h with hrTNFα inhibited HIV-1 entry into TCDM but did not modify significantly TCDM surface CD4 expression. Entry assays were performed as described in Materials and Methods. Viral RNA was extracted, reverse transcribed, and then amplified by PCR; PCR products for HIV *gag* (627 bp) and β-human actin (205 bp) were visualized on 2% agarose gels. The Southern blot demonstrates that the amplified 627-bp *gag* fragment is specifically detected by an internal gag[1669] probe.

nor virus entry should occur. Therefore, after HIV-1 infection, trypsinization, and intensive washes at 4°C, we expected to detect no viral RNA in cell lysates. At 4°C, residual viral genomic RNA after trypsinization and extensive washes represented less than 5% of that of nontrypsinized TCDM (data not shown), demonstrating the efficiency of trypsinization even at low temperatures as reported previously (37). Taking into account that the binding affinities between HIV-1 and cell surface are similar at 4 and 37°C (21) and that trypsinization is efficient at both 4 and 37°C (data not shown), our results show that the detection of genomic viral RNA in untreated trypsinized TCDM was 20 times higher at 37°C than at 4°C (Fig. 2), suggesting that at least 95% of viral RNA detected at 37°C by our RT-PCR assay was intracellular. At 4°C, the amount of genomic viral RNA detected in untreated or hrTNFα-pretreated TCDM was less than 5% of that of untreated TCDM at 37°C (Fig. 2).

At 37°C, pretreatment with 20 ng of hrTNFα per ml for 3 days decreased the intracellular amount of viral genomic RNA to about one-fourth of that of untreated TCDM (Fig. 2), suggesting that hrTNFα inhibits HIV-1 entry into TCDM prior to reverse transcription. The amount of *gag* detected in TNFα-pretreated TCDM ranged from 10 to 25% of that of untreated cells, depending on variability between donors (data not shown). The absence of amplified *gag* fragment in HIV-1-infected TCDM RNA extracts not submitted to reverse transcription but amplified by PCR confirmed that our DNase I-treated viral stock did not contain any residual viral DNA (data not shown). Figure 3A confirmed the inhibition of HIV-1 entry into TCDM by hrTNFα pretreatment for 3 days. Moreover, the mixing of hrTNFα with an anti-TNFα MAb totally restored virus entry, even increasing it to 140%, suggesting endogenous production of TNFα in TCDM cultures.

We reported previously that pretreatment with 10 ng of hrTNFα per ml for 24 h downregulates surface CD4 expression in TCDM (18). To determine if the modulation of surface CD4 expression plays a role in inhibition of HIV-1 entry by TNFα, we pretreated TCDM with hrTNFα for 2 h. Figure 3B shows that pretreatment with 20 ng of hrTNFα per ml for 2 h, while inhibiting about 75% of viral entry, did not modulate surface CD4 expression, suggesting that surface CD4 expression level is not the limiting step for HIV-1 entry into hrTNFα-pretreated TCDM. The downregulation of surface CD4 expression in primary TCDM observed after 20 ng/ml TNFα treatment for 24 h was mediated through the 55-kDa TNF-R1 and not through the 75-kDa TNF-R2, as shown by flow cytometry (data not shown).

In contrast to primary TCDM, pretreatment of PHA-activated PBL with 20 ng of hrTNFα per ml for 2 h and even for 3 days (data not shown) did not modify viral entry into these cells (Fig. 4), demonstrating that the inhibition mediated by hrTNFα is macrophage specific.

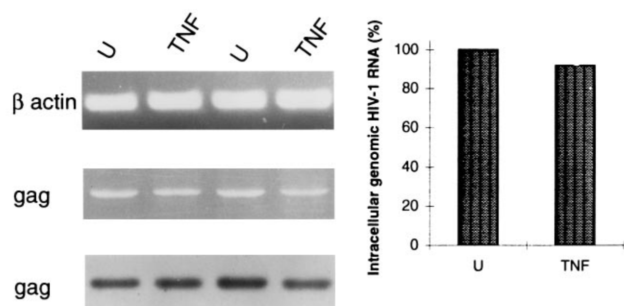

FIG. 4. Human rTNFα does not inhibit HIV-1 entry into PHA-activated PBL. PHA-activated PBL were pretreated for 2 h with 20 ng of hrTNFα per ml or left untreated before infection with HIV-1 Ada strain (MOI of 1.0). An entry assay was performed as described in Materials and Methods. Viral RNA was extracted, reverse transcribed, and then amplified by PCR; PCR products for HIV *gag* (627 bp) and β-human actin (205 bp) were visualized in duplicate on 2% agarose gels. The Southern blot demonstrates that the amplified 627-bp gag fragment is specifically detected by an internal gag[1669] probe. The histogram summarizes the results of the two independent entry assay experiments.

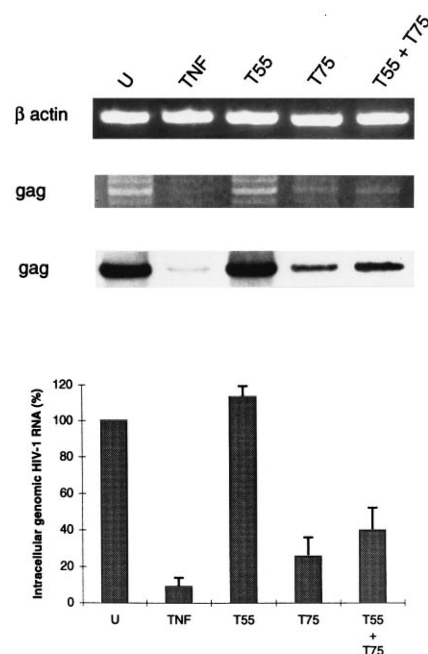

FIG. 5. Inhibition of HIV-1 entry into TCDM by hrTNFα is mediated through TNF-R2 but not through TNF-R1. TCDM were pretreated for 72 h with 20 ng of hrTNFα per ml, 20 ng of T55 mutant per ml, 200 ng of T75 mutant per ml, or both T55 and T75 mutants mixed together, or left untreated, before infection with HIV-1 Ada strain (MOI of 1.0). In contrast to T55 mutant, hrTNFα, T75 mutant, and to a lesser extent the mixture of T55 and T75 mutants inhibit HIV-1 entry into TCDM. An entry assay was performed as described in Materials and Methods. Viral RNA was extracted, reverse transcribed, and then amplified by PCR; PCR products for HIV *gag* (627 bp) and β-human actin (205 bp) were visualized on 2% agarose gels. The Southern blot demonstrates that the amplified 627-bp *gag* fragment is specifically detected by an internal gag[1669] probe. The histogram summarizes the results of three independent entry assay experiments.

Inhibition of HIV-1 entry into TNFα-pretreated TCDM is mediated through the 75-kDa TNF-R2 and not the 55-kDa TNF-R1. TNFα binds to the TCDM surface through two distinct TNF receptors of 55 kDa (TNF-R1) and 75 kDa (TNF-R2). To discriminate between the roles of TNF-R1 and TNF-R2 in regard to inhibition of HIV-1 entry by TNFα, we pretreated TCDM with 20 ng of hrTNFα per ml, 20 ng of TNF-R1-selective mutant (T55) per ml, 200 ng of TNF-R2-selective mutant (T75) per ml, and both mutants together (Fig. 5). T55 mutant did not modify HIV-1 entry into TCDM. In contrast, T75 mutant inhibited viral entry into TCDM, but not as efficiently as hrTNFα. Pretreatment of TCDM by T55 and T75 mutants mixed together showed a two-thirds inhibition of HIV-1 entry compared with untreated TCDM. Taken together, these data suggest that inhibition of HIV-1 entry into TCDM by hrTNFα can be mediated through TNF-R2 but not through TNF-R1 alone.

Inhibition of HIV-1 entry into TCDM is mediated by both TNFα and IFN-γ but not by IL-1β and IL-10. The proinflammatory cytokine IL-1β shares biological functions with TNFα, and both IFN-γ and IL-10 have been reported previously to exhibit an anti-HIV activity. We therefore tested hrIL-1β, hrIFN-γ, and hrIL-10 in regard to HIV-1 entry into TCDM, using the intracellular genomic RNA assay described above (Fig. 6). Pretreatment with 200 IU of hrIFN-γ per ml for 2 h inhibited viral entry into TCDM, but to a lesser extent than 20 ng of hrTNFα per ml, with approximately 35% residual viral entry compared with the untreated control. In contrast, pretreatment with 100 IU of hrIL-1β or 10 ng of hrIL-10 per ml did not inhibit viral entry into TCDM. In agreement with the absence of inhibition of viral entry into hrIL-1β-pretreated TCDM, we showed that the formation of proviral LTR was not modified after 100 IU/ml hrIL-1β pretreatment for 3 days (39). Inhibition of HIV-1 entry into TCDM is therefore specifically related to the proinflammatory cytokine TNFα and to a lesser extent to IFN-γ.

Pretreatment with TNFα inhibits HIV-1 replication in TCDM but not in PHA-activated PBL. To determine if the inhibition of HIV-1 entry into TNFα-pretreated TCDM could account for a sustained inhibition of HIV-1 replication, we monitored the RT activity in the supernatant of infected TCDM. Three-day-old TCDM were pretreated with 20 ng of hrTNFα per ml, 20 ng of hrTNFα per ml mixed with 2.3 μg of anti-human TNFα MAb cA2 per ml, or 2.3 μg of anti-human TNFα MAb cA2 per ml or were left untreated before infection with HIV-1 Ada (MOI of 0.10). While syncytia appeared at day 12 after infection in untreated TCDM (Fig. 7A), TNFα-treated TCDM did not show any syncytia (Fig. 7B). When TNFα and the anti-TNFα MAb were mixed and added to TCDM before and throughout infection, syncytia appeared at day 12 postinfection as in untreated TCDM (Fig. 7C). Addition of the anti-human TNFα MAb alone to HIV-1-infected TCDM led to more syncytium formation than in untreated infected TCDM (Fig. 7D). Taken together, these data suggest that TNFα pretreatment inhibits syncytium formation in HIV-1 Ada-infected TCDM. Absence of syncytium formation in TNFα-pretreated TCDM correlated with sixfold-lower RT activity in culture supernatants of TNFα-pretreated TCDM infected with HIV-1 Ada (MOI of 0.10) than in untreated infected control cells (Fig. 8). By contrast, addition of the anti-human TNFα MAb to TNFα-pretreated infected TCDM restored RT activity levels almost totally, similar to those of untreated infected TCDM (Fig. 8). Pretreatment of HIV-1-infected TCDM with the anti-human TNFα MAb alone showed higher RT activity levels than untreated infected TCDM (Fig. 8), in agreement with enhanced syncytium formation, suggesting endogenous secretion of TNFα by HIV-1-infected TCDM. TNFα pretreatment of human TCDM decreased both extracellular and intracellular RT activity (data not shown). In contrast with TCDM, TNFα pretreatment did not inhibit HIV-1 growth in PHA-activated PBL (Fig. 8). These results show that TNFα pretreatment could specifically inhibit replication of a HIV-1 monocy-

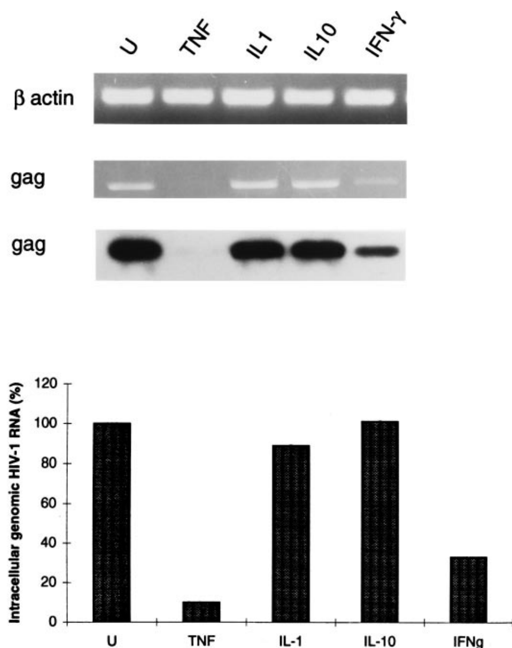

FIG. 6. Selective effects of different cytokines on HIV-1 entry into TCDM. TCDM were pretreated for 2 h with 20 ng of hrTNFα, 100 IU of hrIL-1β, 10 ng of hrIL-10, or 200 IU of hrIFN-γ per ml, or left untreated, before infection with HIV-1 Ada strain (MOI of 1.0). In contrast to hrIL-1β and hrIL-10, hrTNFα and to a lesser extent hrIFN-γ inhibited HIV-1 entry into TCDM. An entry assay was performed as described in Materials and Methods. Viral RNA was extracted, reverse transcribed, and then amplified by PCR; PCR products for HIV *gag* (627 bp) and β-human actin (205 bp) were visualized on 2% agarose gels. The Southern blot demonstrates that the amplified 627-bp *gag* fragment is specifically detected by an internal gag[1669] probe. The histogram summarizes the results of two independent entry assay experiments.

totropic strain in primary TCDM but not in PHA-activated PBL.

DISCUSSION

Our results demonstrate that inhibition of HIV-1 entry into primary TCDM by TNFα involves the 75-kDa TNF-R2 but not the 55-kDa TNF-R1. Inhibition of HIV-1 entry into TCDM is independent of surface CD4 expression levels and occurs specifically in primary TCDM but not in PHA-activated PBL. Among the other cytokines tested, only IFN-γ inhibits HIV-1 entry into primary TCDM, suggesting that T lymphocyte-macrophage activation which results in both TNFα and IFN-γ production is an important aspect of acquired immunity against HIV-1 infection.

To discriminate between the early stages of the virus life cycle targeted by hrTNFα in HIV-infected primary TCDM, we performed two different assays. The presence of viral genomic RNA in the cells was measured by an RT-PCR assay performed after trypsinization of the cells, and the subsequent proviral LTR synthesis was analyzed by a PCR assay as reported previously (8). Trypsinization which allows the shedding of cell surface-bound virions and is followed by extensive washes has been reported previously as an efficient method to measure intracellular virions; in agreement with our data, the residual cell surface-bound virions after trypsinization have been reported to represent fewer than 5% of input virions (37). The detection of viral genomic RNA is of interest because, in contrast to the detection of proviral DNA by PCR, it provides an earlier detection of viral entry into the cells and focuses on adsorption and fusion steps but does not depend on reverse transcription. hrTNFα pretreatment inhibits both appearance of viral genomic RNA and proviral LTR synthesis in HIV-1-infected TCDM. This finding demonstrates that hrTNFα affects an early step of the virus life cycle after (and including) adsorption and prior to reverse transcription, although we cannot exclude an additional direct effect of TNFα on reverse transcription. The inhibition of viral entry into TCDM was specifically mediated through TNFα because the addition of an anti-TNFα MAb totally restored viral entry even to higher levels than in untreated infected cells, suggesting production of endogenous TNFα in culture. Interestingly, the inhibition of HIV-1 entry occurred with immediate maximal antiviral effect after TNFα pretreatment for only 2 h. The 75% inhibition in viral entry of TNFα-treated TCDM could correspond either to a 75% decrease in the number of infected cells or to a 75% decrease in the amount of viral RNA detected per cell, although we were not able to discriminate between these possibilities. The inhibition of viral entry into primary TCDM was not due to a toxic effect of TNFα on the primary TCDM, as assessed by identical cell survival in untreated TCDM and TCDM treated with hrTNFα, T55 mutant, T75 mutant, and both mutants together (data not shown). As lipopolysaccharide has been reported to inhibit HIV-1 growth in primary human TCDM (25), we tested TNFα, T55 mutant, and T75 mutant stocks for the presence of endotoxin and found all to be endotoxin free (data not shown).

The entry step inhibited by TNFα could be virus-cell binding, fusion, or uncoating. The sensitivity to HIV-1 infection has been reported to correlate closely with surface CD4 levels in monocytoid cell line subclones expressing different amount of surface CD4 (2), but the opposite has also been demonstrated in human T-cell lines (24, 56). We have previously shown that both surface and total CD4 are downregulated in TCDM at the level of transcription after hrTNFα pretreatment for 1 to 5 days (18, 23). After hrTNFα pretreatment for 2 h, a 75% decrease in virus entry was observed in parallel with stable levels of surface CD4 expression, and the surface CD4 downregulation in TNFα-treated human primary TCDM was mediated through the 55-kDa TNF-R1 but not through the 75-kDa TNF-R2. All of these data taken together suggest that inhibition of HIV-1 entry into primary TCDM by TNFα is mediated through the 75-kDa TNF-R2, but not through the 55-kDa TNF-R1, and is independent of modulation of surface CD4 expression. Apart from the absolute number of CD4 molecules on the cell surface, the binding affinity of gp120 for CD4 may also be important; this affinity has been shown to vary, with viruses exhibiting different cell tropism and cytopathicity (21). We cannot exclude that TNFα inhibits fusion between the virion envelope and the cell or that viral RNA is degraded intracellularly in activated TCDM. Recently an HIV-1 entry cofactor, fusin, that allows CD4-expressing nonhuman cell types to support HIV-1 envelope-mediated cell fusion and HIV-1 infection has been described (13). Fusin acts preferentially for T-cell line-tropic isolates in comparison with its activity with macrophage-tropic HIV-1 isolates. An HIV entry cofactor such as fusin, not isolated so far, might be implicated in inhibition of HIV-1 entry into TCDM by TNFα.

During AIDS pathogenesis, HIV-1 infected macrophages produce proinflammatory cytokines (20, 28, 44). TNFα has been reported to stimulate HIV-1 replication in chronically infected promonocytic and lymphoid cell lines through activation of NF-κB and subsequent transactivation of the proviral LTR (9, 41, 46), whereas the role of TNFα in regard to HIV-1 replication in primary TCDM is more contradictory, either enhancing or having no effect (25, 33). We have confirmed that

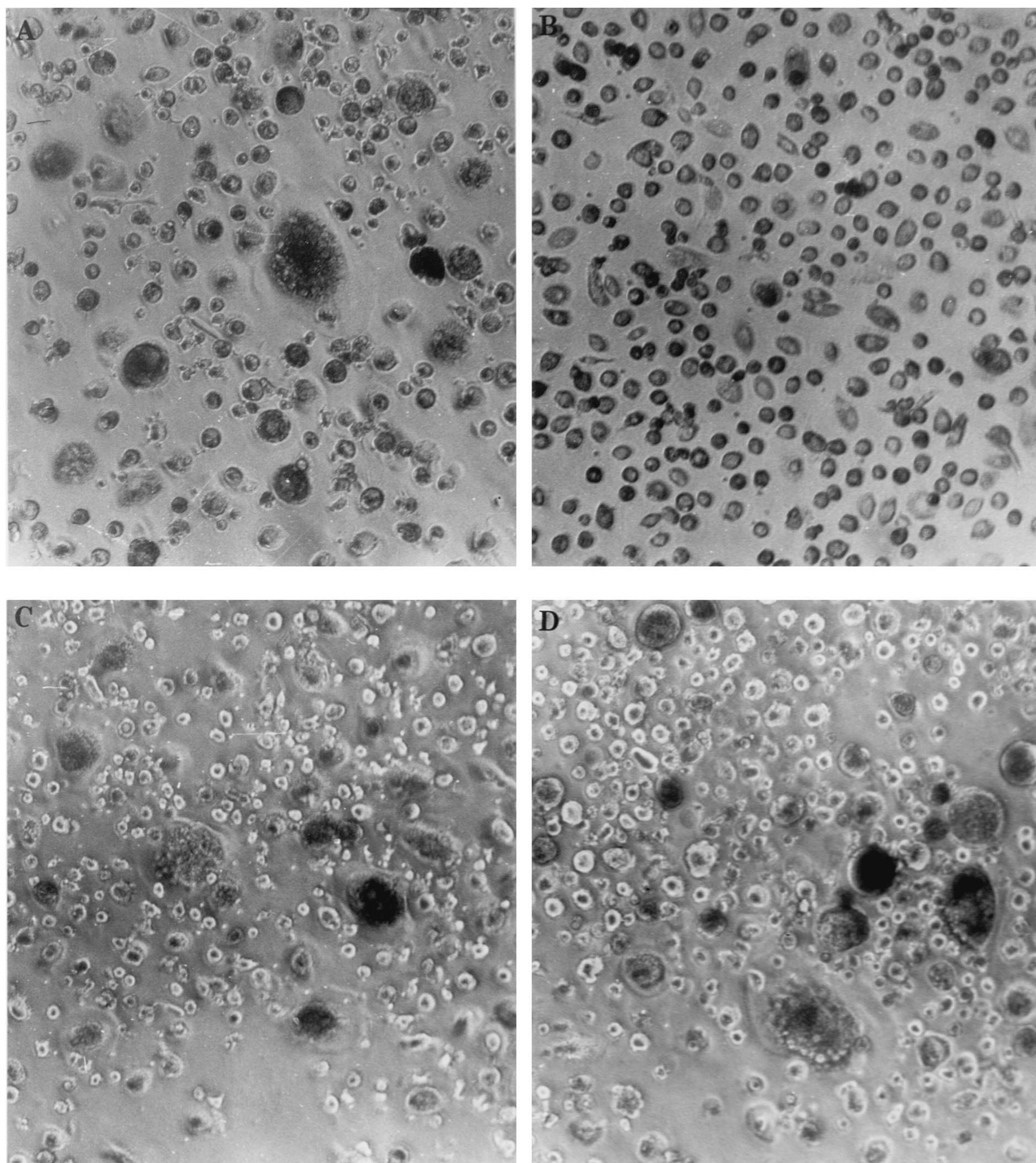

FIG. 7. Inhibition of syncytium formation in TNFα-pretreated TCDM infected with HIV-1 Ada. Three-day-old TCDM were left untreated (A) or pretreated for 72 h as described in Materials and Methods with 20 ng of hrTNFα per ml (B), 20 ng of hrTNFα per ml mixed with 2.3 μg of anti-human TNFα MAb per ml (C), or 2.3 μg of anti-human TNFα MAb per ml alone (D) before infection with HIV-1 Ada (MOI of 0.10). Syncytium formation was observed at day 12 postinfection in untreated TCDM (A) and in TCDM treated with a mixture of TNFα and anti-human TNFα MAb (C) or treated with anti-human TNFα MAb alone (D) but not in TNFα-pretreated TCDM (B). Phase-contrast microscopy; magnification, ×60. The results are representative of five independent experiments.

TNFα can stimulate HIV-1 replication in primary TCDM when added only at the time of infection, as measured by RT activity and appearance of syncytia in culture (data not shown). By contrast, iterative daily TNFα pretreatment before infection followed by TNFα addition at the time of infection and every 2 days after infection inhibits HIV-1 replication in primary TCDM. In this case, HIV-1 growth can be restored by an anti-human TNFα mouse-human chimeric MAb. Iterative TNFα pretreatment of primary PHA-activated PBL did not inhibit HIV-1 replication, suggesting that inhibition of HIV-1 replication by TNFα pretreatment is macrophage specific. The inhibition of HIV-1 replication was not due to a toxic effect of TNFα on the primary TCDM, as assessed by identical cell survival in untreated and TNFα-treated TCDM. As lipopolysaccharide has been reported to inhibit HIV-1 replication in primary human TCDM (25), we tested our TNFα stock for the

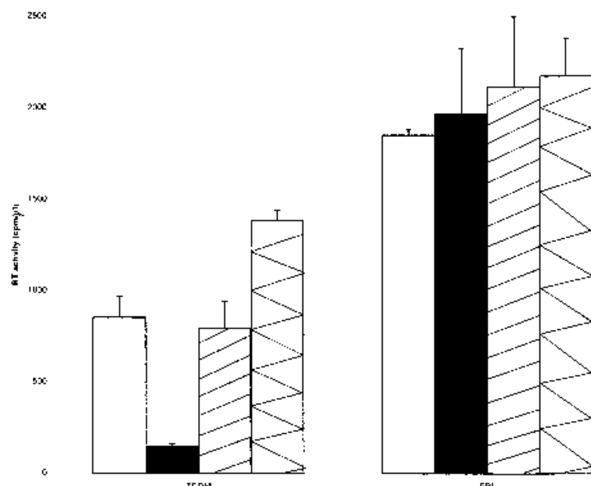

FIG. 8. Decreased RT activity in culture supernatants of TCDM, but not of PHA-activated PBL, pretreated with TNFα and then infected with HIV-1 Ada. RT activity was measured at day 14 postinfection in culture supernatants of 3-day-old TCDM or PHA-activated PBL left untreated (▩) or pretreated for 72 h as described in Materials and Methods with 20 ng of TNFα per ml (■), 20 ng of TNFα per ml mixed with 2.3 μg of anti-human TNFα per ml (□), or 2.3 μg of anti-human TNFα MAb per ml alone (▤) and then infected with HIV-1 Ada (MOI of 0.10). The results are representative of five independent experiments.

presence of endotoxin, which was absent in all cases (data not shown). Our stock of TNFα was biologically active, as demonstrated by stimulation of HIV replication in promonocytic U1 cell line, in agreement with previous reports (45). In agreement with previous reports (4), we have shown that HIV-1 replication in a U1 promonocytic cell line which contains two copies of the integrated HIV genome is stimulated by TNFα through TNF-R1 and not through TNF-R2 (19). TNF-R1 has been involved in NF-κB activation (26, 32), although TNF-R2 could also play a role (51). Taking into account that the signaling pathways mediated by the two TNF-Rs are distinct (59), it would be of interest to discriminate more accurately the effect of each in regard to HIV-1 replication in primary macrophages.

The beneficial function of TNFα in cellular immunity to parasitic pathogens such as *Leishmania* species (60) is well established, and TNFα binding to TNF-R1 is involved in defense against intracellular bacteria such as *Listeria monocytogenes*, as shown by the fact that mice lacking this receptor die from disseminated infection (17, 49). Enhanced host resistance has also been reported for TNFα against vesicular stomatitis virus (63), cytomegalovirus (43), herpes simplex virus (48), and vaccinia virus (53). Our results show that inhibition of HIV-1 entry into primary TCDM by TNFα involves TNF-R2 but not TNF-R1. In vitro, the antiviral activity of fibroblasts has been shown to be induced by TNF-R1 (65); by contrast, in vivo, mice lacking TNF-R1 mount an apparently normal immune response when challenged with vaccinia virus or lymphocytic choriomeningitis virus (50). Therefore, the role of each TNF-R in antiviral defense is still uncertain and might depend on cell type, viral challenge, and step of the virus life cycle studied. Soluble TNF-R2 has been detected in the circulation of patients with AIDS at significantly higher levels than in control subjects and has been proposed as a marker of disease progression. The adsorption of heat-inactivated HIV-1 to isolated human monocytes triggers the release of both TNFα and soluble TNF-R2 but not that of TNF-R1 (47). In patients with AIDS, plasma TNFα might bind preferentially to the soluble TNF-R2 rather than to the cell surface TNF-R2, therefore preventing the TNFα-mediated protective effect against HIV-1 infection in TCDM.

Apart from proinflammatory cytokines, IFNs (14, 16) and IL-10 (1) are produced by HIV-1-infected macrophages. Both IFNs and IL-10 inhibit HIV-1 growth in TCDM (36, 38). IL-10 has been reported to delay the appearance of proviral LTR in HIV-1-infected TCDM (38), although our results show that the amount of viral genomic RNA measured in the cells 2 h postinfection is not modified in IL-10-pretreated TCDM compared with untreated control cells. The decrease of intracellular pool of nucleotide precursors reported previously as a rate-limiting parameter for reverse transcription in primary TCDM (40) could contribute to a slower initiation of reverse transcription by IL-10, which is known to downregulate a range of activities in macrophages. The IFNs have been reported to block the virus life cycle at multiple levels, i.e., entry, transcription, and virion assembly, and budding (36). In agreement with the reported inhibition of the appearance of proviral DNA in IFN-γ-pretreated TCDM infected by HIV-1 (36), our results confirm that IFN-γ inhibits HIV-1 entry into TCDM. Both IFN-γ and TNFα have been previously reported to stimulate HIV-1 replication in U1 cells, although the increased virus expression observed in IFN-γ-treated cells was not mediated through the induction of endogenous TNFα secretion (3). Since IFN-γ is a potent inducer of macrophage activation, we cannot exclude the possibility that the inhibitory effect of IFN-γ on HIV-1 entry into primary TCDM is mediated through the production of endogenous TNFα. IFN-γ and TNFα are often involved synergistically in anti-infectious immunity (63), and both cytokines inhibit HIV-1 entry into primary TCDM, suggesting that T cell-macrophage activation could be a key mechanism of antiviral defense in AIDS. This study provides the first evidence that TNFα, through the 75-kDa TNF-R2, can play a host-protective role in HIV-1 infection by inhibiting viral entry into primary macrophages. Therefore, the role of TNFα in HIV infection is complex, and further studies are needed to determine whether inhibition of entry and enhancement of HIV infection by TNFα might be uncoupled in primary macrophages.

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