Transmembrane TNF-α Facilitates HIV-1 Infection of Podocytes Cultured from Children with HIV-Associated Nephropathy

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ABSTRACT

Studies have shown that podocytes and renal tubular epithelial cells from patients with HIV-associated nephropathy (HIVAN) express HIV-1 transcripts, suggesting that productive infection of renal epithelial cells precipitates development of HIVAN. However, podocytes and renal tubular epithelial cells do not express CD4 receptors, and it is unclear how these cells become productively infected in vivo. Weinvestigated the mechanisms underlying the infection by HIV-1 of podocytes cultured from the urine of children with HIVAN. We observed low-level productive infection on exposure of these cells to primary cell-free HIV-1 supernatants. However, envelope-defective recombinant HIV-1 did not infect the renal epithelial cell lines. Moreover, treatment of podocytes to inhibit endocytic transport or dynamin activity or remove cell surface heparan sulfate proteoglycans reduced infection efficiency. Transfection of CD4−293T cells with a cDNA expression library developed from a podocyte cell line derived from a child with HIVAN led to the identification of TNF-α as a possible mediator of HIV-1 infection. Overexpression of transmembrane TNF-α in cultured CD4−renal tubular epithelial cells, 293T cells, and HeLa cells enabled the infection of these cells; exposure to soluble TNF-α did not. Immunohistochemistry showed TNF-α expression in podocytes of renal sections from children with HIVAN. Furthermore, we found that TNF-α enhanced NF-κB activation and integration of HIV-1 into the podocyte DNA. Finally, inhibition of dynamin activity blocked TNF-α–mediated infection. These data establish a role for transmembrane TNF-α in facilitating the viral entry and integration of HIV-1 into the DNA of renal epithelial cells.


HIV-1–associated nephropathy (HIVAN) is a clinical-pathologic entity seen in HIV+ patients of African ancestry characterized by proteinuria, FSGS, and microcystic tubular dilation leading to renal enlargement and chronic renal failure.1,2 The current pathologic paradigm of HIVAN is that renal epithelial cells (RECs) became productively infected and produce viral transcripts that precipitate these renal lesions in people carrying a genetic predisposition to develop this disease.3−7 However, podocytes and renal tubular epithelial cells (RTECs) do not express significant protein levels of the CD4 HIV-1 receptor or the HIV-1 coreceptors CXCR4 and CCR5,8,9 and despite many years of extensive research, it is unclear how these cells become productively infected in vivo.

Previous studies done in CD4−RTECs cultured from the urine of children with HIVAN showed that these cells develop a low–level productive infection
when exposed to high titer of cell-free viruses derived from these children.\textsuperscript{10} Although RTECs produce significantly lower levels of p24 antigen compared with HIV-1 macrophages, they can transfer viruses and infect cocultured HIV-1–mononuclear cells for at least 20 days in culture.\textsuperscript{10} However, other studies done in cultured primary podocytes or podocyte cell lines derived from HIV-1–persons reported that these cells did not become productively infected.\textsuperscript{9,11} Nonetheless, because viral transcripts have been detected in podocytes in patients with HIVAN,\textsuperscript{3–5} it is necessary to determine whether podocytes cultured from patients with HIVAN can become productively infected or not. Therefore, we carried out this study to determine whether podocytes cultured from the urine of children with HIVAN can sustain a low–level productive infection and identify the mechanisms involved in this process.

RESULTS

Primary Podocytes Cultured from the Urine of Children with HIVAN Became Infected through an Envelope-Dependent Mechanism

As described before,\textsuperscript{12,13} we first cultured podocytes from the urine of two children with HIVAN and expanded podocyte colonies that tested negative for HIV DNA (Figure 1A, Supplemental Figure 1). These cells were characterized as described in a previous study.\textsuperscript{12} They showed the morphologic structure of differentiated podocytes and express the Wilms’ tumor 1 (WT-1) protein and glomerular epithelial protein 1 (GLEPP1) (Figure 1, A–E) as well as synaptopodin, nestin, and nephrin as reported before.\textsuperscript{12} As expected,\textsuperscript{8,15} podocytes showed undetectable protein levels of CD4 and CCR5 and low levels of CXCR4 by RT-PCR and flow cytometry, respectively (Supplemental Figures 1 and 2). However, they were able to sustain a low–level productive infection when exposed to primary cell-free viruses derived from children with HIVAN (1 moi) (Figure 1, B–E). In contrast, control canine tubular epithelial cells (MDCKs) were not infected (Figure 1B). In addition, primary podocytes were infected with the cell-free HIV-NL4–3-GFP or HIV-1/NL4–3-Luc strain (Figure 1, F and G), where the nef gene was replaced by the firefly luciferase gene, and macrotropic (HIV-GFP-YU2) or dual-tropic (HIV-GFP-89.6) viruses\textsuperscript{16,17} (Supplemental Figure 3). In contrast, primary podocytes exposed to the envelope (Env)–defective mutant viruses NL4–3-GFP-Δenv or HIV-1/NL4–3Luc-Δenv were not infected (Figure 1, F and G), suggesting that Env plays a key role in this process.

Podocyte Cell Lines Derived from Children with HIVAN Became Productively Infected through a Mechanism That Involves HIV Env, Heparan Sulfate Proteoglycans, and Dynamin-Dependent Endocytosis

Here, we used two podocyte cell lines generated from children with HIVAN and control GHOST (3) cells, which express CD4, CXCR4, and CCR5\textsuperscript{18} (Figure 2). The characterization of both podocyte cell lines was described in a previous study.\textsuperscript{12} These cells express the podocyte markers WT-1, synaptopodin, podocalyxin, nestin, and nephrin.\textsuperscript{12} Podocyte cell line 1 carries two APOL1 G1 risk alleles (G1 p.S342G and p.I384M), and podocyte cell line 2 carries two wild-type alleles (p.E150K).\textsuperscript{19} Both podocyte cell lines exposed to cell-free HIV-NL4–3-GFP or HIV-1/NL4–3-Luc were infected in contrast to those exposed to NL4–3-GFP-Δenv or HIV-1/NL4–3Luc-Δenv (Figures 2B and 3B, Supplemental Figure 4). The fusion inhibitor Trimeris, the integrase inhibitor Raltegravir, and the reverse transcription inhibitor Zidovudine (Azidothymidine) all inhibited the infection of GHOST (3) cells and podocytes (Figure 3, A and B, Supplemental Figure 4). In contrast, the HIV-1 protease inhibitor Ritonavir, which inhibits the HIV protease, preventing the cleavage of Gag before budding, did not prevent the infection (Figure 3, A and B). Blocking antibodies against CD4 or the endocytic receptor dendritic cell–specific ICAM-3 grabbing nonintegrin (DC-SIGN)\textsuperscript{9} and the CXCR4 chemical blocker bicyclam did not prevent the infection of podocytes (Figure 3, C and D). However, removing the cell surface heparan sulfate proteoglycans (HSPGs) by Heparitinase III or pretreating cells with the heparin–like drug pentosan polysulfate reduced the infection efficiency (Figure 3, C and D, Supplemental Figure 4). These findings suggest that HSPGs serve as attachment receptors for HIV-1 in podocytes, probably by interacting with the basic residues of gp120 as described in macrophages.\textsuperscript{20,21} To determine whether fusion was detected at the cell membrane, we used the β-galactosidase gene activation assay\textsuperscript{22} but failed to detect gp120 fusion (data not shown).

Alternatively, as described before,\textsuperscript{23} we explored whether HIV-1 could be internalized via endocytosis before fusion. Here, we found that Bafilomycin A1, an inhibitor of the vacuolar proton ATPase that affects the transport from early to late endocytic compartments, decreased the infection of podocytes in a concentration–dependent manner (Figure 3, E and F), although cells treated with NH4Cl (0.4 and 2 mM) were infected (Supplemental Figure 5). In addition, dynasore, a small molecule inhibitor of the dynamin GTPase activity that prevents the scission of clathrin-coated pits from the plasma membrane,\textsuperscript{23} inhibited the infection of both podocyte cell lines (Figure 3, E and F, Supplemental Figure 4). In summary, we concluded that HIV-1 infects podocytes cultured from the urine of children with HIVAN via a CD4-independent mechanism that requires Env, HSPGs, and dynamin-dependent endocytosis.

Identification of TNF-α as a Critical Factor Facilitating the Infection of Cultured Podocytes

To further define the mechanisms facilitating the infection of podocytes cultured from children with HIVAN, we used a cDNA expression library generated by Invitrogen (Carlsbad, CA)\textsuperscript{24} from the podocyte cell line 1.\textsuperscript{12} This cDNA library was transfected into CD4– 293T cells, which were subsequently exposed to HIV-1/NL4–3 puromycin viruses, to allow the
Figure 1. Low-level productive infection of primary podocytes cultured from the urine of children with HIVAN. (A) Primary podocytes cultured from children with HIVAN express WT-1. (B–D) HIV− podocytes cultured from children with HIVAN (1 × 10^5) were exposed for 48 hours to cell-free HIV-1 (1 moi) derived from PBMCs collected from children with HIVAN. Supernatants were treated with DNase I. Subsequently, all cells were washed and trypsinized to remove adherent viruses. Nonpermissive renal canine epithelial cells (MDCKs) and mock-infected podocytes were used as controls. (B) p24 antigen levels were measured by ELISA in the supernatants every 2–3 days. On day 7, all cells were harvested to perform (C) p24 immunohistochemistry studies (red) or (D) IV-DNA PCR using an electrochemiluminescent (ECL) quantitative assay or (B) washed and cocultured with HIV− PBMCs as described in Concise Methods. (E) Primary podocytes cultured from the urine of children with HIVAN undergo differentiation changes, stain positive for GLEPP-1 (blue), and express both GLEPP1 (blue) and p24 antigen (red) 7 days after HIV infection. (F and G) Primary podocytes were exposed for 48 hours to HIV-1/NL4–3-GFP, HIV-1/NL4–3-GFPΔenv, HIV-1/NL4–3-Luc, or HIV-1/NL4–3-LucΔenv (equal moi or p24 amount as described in Concise Methods). (F) Subsequently, all cells were fixed in 4% paraformaldehyde to detect GFP, stained with 1 μg/ml DAPI (blue nuclei), and photographed to detect GFP+ cells (green), nuclei, and cell morphology (phase contrast). (G) Primary podocytes exposed to equal amounts of p24 HIV-1/NL4–3-Luc or HIV-1/NL4–3-LucΔenv were followed as described above and lysed to measure luciferase activity in relative light units (RLUs). Scale bars, 50 μm. *t Test, P<0.05 (n=3).
identification of DNA clones that facilitate the infection of podocytes in puromycin-resistant colonies. We obtained 28 puromycin-resistant colonies and found that one clone was infected with both HIV-1/NL4–3GFP and HIV-1/NL4–3Luc (data not shown). Genomic DNA extracted from this clone was subjected to PCR amplification using the primers flanking the inserts of the cDNA library vector to generate a 1.7-kb DNA fragment. Subsequently, this PCR product was subcloned into the expression vector pGEM-T easy (Promega, Madison, WI) and sequenced, leading to the identification of the full-length TNF-α cDNA clone. To confirm the role of TNF-α, we performed podocyte infection assays in the presence and absence of TNF-α mAbs (T3), siRNA TNF-α expression vectors, dynasore, and podocytes transduced with TNF-α or GFP lentiviruses. We found that TNF-α plays an essential role in this process without affecting the expression of CD4, CXCR4, or CCR5 (Figure 3, G and H, Supplemental Figures 6 and 7).

Transmembrane TNF-α Plays a Crucial Role Facilitating the Infection of Cultured Podocytes

TNF-α is produced as a 212-amino acid-long type 2 transmembrane protein, from which soluble TNF-α (sTNF-α) is
Figure 3. HIV-1 internalization and infection require Env, HSPGs, Bafilomycin A1, Dynamin, and TNF-α. (A and B) GHOST cells and podocytes were exposed for 4 hours to equal amounts of p24 HIV-1/NL4-3-GFP-Denv or HIV/NL4-3-Luc (moi 0.1 or 1 for GHOST cells
released via proteolytic cleavage by a metalloproteinase TNF-α–converting enzyme. To determine the relative role of sTNF-α and transmembrane TNF-α (tmTNF-α), we cloned either the full length TNF-α, tmTNF-α, and a TNF-α inactive mutant (red fluorescent protein [RFP]-TNF-α A160V-Y163L) into the RFP vector pDsRed2-C1 (Clontech Laboratories, Mountain View, CA) using standard methods as described before. These constructs were transfected into CD4−293T cells, which were subsequently exposed to HIV-NL4−3 GFP (1 moi) for 48 hours and fixed (Figure 4, A−C). As shown in Figure 4A, cells transfected with either the fully-length TNF-α open reading frame or tmTNF-α were infected. In contrast, cells transfected with the inactive TNF-α mutant (A160V-Y163L) or exposed to soluble human recombinant TNF-α (sTNF-α) were not infected (Figure 4, A−C), even when sTNF-α induced the activation of NF-κB and the HIV-LTR (Figure 3D). Furthermore, we found that podocytes derived from children with HIVAN express significant mRNA and protein TNF-α levels (Figure 5, A−C) as described in other renal inflammatory diseases.

**DISCUSSION**

The classic mechanism of HIV-1 infection is mediated by the Env glycoproteins gp120 and gp41. These proteins interact with the primary CD4 receptor and the coreceptor CXCR4 or CCR5, which allows the fusion of Env to the plasma membrane, leading to the productive infection of cells. As an alternative but less efficient infection mechanism, HIV-1 can be endocytosed and trapped within endosomal compartments, where it could avoid degradation and escape into the cytoplasm or undergo fusion within endosomes. Here, we uncovered a new role for tmTNF-α facilitating the infection of CD4− podocytes and RTEcs through an Env-dependent mechanism that involves HSPGs, dynamin-dependent endocytosis, and NF-κB activation.

In addition to the CD4 and HIV coreceptor mechanism described above, HIV-1 uses HSPGs as attachment receptors. It is well known that HSPGs interact through electrostatic interactions with several viruses, including the Feline Immunodeficiency Virus (FIV) and HIV-1. Thus, a novel finding of our study is that HSPGs facilitate the infection of podocytes cultured from children with HIVAN, although we did not identify which specific HSPGs are involved in this process. It could be argued, however, that HSPGs only facilitate the infection of laboratory-adapted viruses and therefore, might not play an in vivo role in children with HIVAN. In fact, previous studies showed that heparin only blocks the infection of laboratory-adapted strains of HIV-1 or FIV, the latter through a selective interaction with the FIV surface glycoprotein and CXCR4. However, other studies showed that primary HIV isolates can attach to heparan sulfate syndecans and that HSPGs can modulate the infectivity of primary isolates through an Env strain–dependent but coreceptor–independent mechanism. These studies concluded that HSPGs only facilitate the infection of primary isolates that contain highly positively charged V3−loop Env

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and podocytes, respectively. Cells were treated with Trimeris (50, 10, and 2 μg/ml), Raltegravir (1, 0.2, and 0.04 μM), Azidothymidine (AZT; 100, 20, and 4 μM), or Ritonavir (10, 2, and 0.4 μg/ml). After 48 hours, all cells were lysed in 1% Triton X-100 buffer to measure the luciferase activity as described in Concise Methods. (C and D) Cells were exposed to the corresponding viruses in the presence or absence of CD4 blocking antibodies (Leu3a; 10, 2, and 0.4 μg/ml), Bicyclam (1, 0.2, and 0.04 μM), or blocking antibodies for DC-SIGN (5, 1, and 0.2 μg/ml), Heparinase III (2, 0.4, and 0.08 U/ml for 2 hours at 37°C before adding HIV-1), or pentosan polysulfate (20, 4, and 0.8 μg/ml). (E and F) Cells were exposed to the corresponding viruses in the presence and absence of Bafilomycin (10, 2, and 0.4 nM) and Dynasore (200, 40, and 8 μM) and lysed to assess their luciferase activity. (G) Podocytes were exposed to HIV-1 viruses in the presence and absence of the anti-TNF-α mAb T3 (10, 2, and 0.4 μg/ml), which was added 1 hour before HIV/NL4-3-Luc. Other cells were transduced with lentiviral-GFP or TNF-α virus as described in Concise Methods. (H) 293T cells were transfected with the pCMV-TNF-α vector and exposed to HIV/NL4-3-Luc (1 moi) in the presence and absence of Dynasore (200, 40, and 8 μM). Subsequently, all cells were lysed to assess their luciferase activity. RLU, relative light unit. *P<0.05 comparing each group with cells infected with HIV/NL4-3-Luc by t test (n=3 per group).
sequences. In general, the switch from CCR5 to CXCR4 tropic viruses during the progression of HIV infection is associated with an increased net positive charge of the V3 loop. In support of this notion, we found that primary isolates cultured from children with HIVAN can infect CD4+ and CXCR4+ HeLa cells. Furthermore, children with HIVAN revealed an upregulated expression of renal HSPGs, which could act as a sink trapping circulating heparin binding growth factors and viral proteins, including TNF-α, FGF-2, and HIV-Tat. Thus, it is tempting to speculate that, during the late stages of HIV infection, renal HSPGs may facilitate the recruitment of highly positive charged X4 viruses and precipitate the onset of childhood HIVAN acting through different mechanisms. More studies are needed, however, to validate this notion.

Figure 4. tmTNF-α facilitates the infection of RECs. (A and B) Nonpermissive kidney embryonic epithelial 293T cells (2×10⁵) were transfected with the plasmid fusion RFP full-length TNF-α (RFP-TNF-α), RFP-TNF-αΔ77–88 (RFP-tmTNF-α), RFP-inactive-TNF-α (A160V-Y163L), or the RFP vector as described in Concise Methods. Twenty-four hours later, these cells were exposed to HIV-1/NL4–3-GFP (1 moi) for 48 hours and then, fixed in 4% paraformaldehyde in PBS to detect GFP + cells. Cell nuclei were stained with 1 μg/ml DAPI, and pictures were taken with an ultraviolet microscope with green (GFP), blue (DAPI), and red (RFP) filters. GFP pictures were merged with RFP pictures to detect cells coexpressing GFP and RFP. RFP pictures were merged with DAPI pictures to show the cell nuclei. Scale bars, 20 μm. (C) 293T cells were transfected with either pCMV-TNF-α or pCMV control vectors, or they were treated with human recombinant TNF-α (hrTNF-α; 10 ng/ml) for 24 hours. Subsequently, the cells were exposed to HIV/NL4–3-GFP (1 moi) for 4 hours, washed extensively as described in Concise Methods, and followed for 7 days in culture. Cell supernatants were collected daily to determine the p24 antigen levels. (D) 293T cells were transfected with either the NF-κB-Luc (p5x NF-κB-Luc) or the LTR-Luc plasmids in addition to the corresponding pCMV-TNF-α or control vectors as described in Concise Methods. A separate group of cells transfected with the pCMV-control vector were treated with hrTNF-α (10 ng/ml), which was added 24 hours after the transfection. All cells were harvested after 48 hours to assess their luciferase activity. (D) Both tmTNF-α and sTNF-α induced the activation of NF-κB and HIV-LTR, but (A–C) only tmTNF-α facilitated the infection of 293T cells. *P<0.05 compared with cells transfected with the pCMV empty vector by t test.
Here, we also show that NF-κB activation seems to be necessary but not sufficient to infect podocytes. This conclusion is made on the basis of the following findings. (1) ATNF-α mAb that blocks NF-κB activation prevented the infection. (2) 293T cells and podocytes were stained with either isotype control or TNF-α antibodies, labeled with PE-conjugated anti-mouse IgG, and processed by flow cytometry as described in Concise Methods. (C) Immunohistochemistry studies in renal sections derived from children with HIVAN show TNF-α protein expression in podocytes. (D) Podocytes, proximal tubular epithelial HK2 cells, RT3 cells, and HeLa cells were transduced with lentivirus carrying full-length TNF-α. Twenty-four hours later, all cells were exposed to either HIV-1/NL4-3-LucΔenv or HIV-1/NL4-3-Luc (equal amounts of p24) and harvested at 48 hours to assess the luciferase activity in relative light units (RLUs). (E) Podocytes (cell line 1) were exposed for 4 hours to HIV/NL4-3-Luc (1 moi) and harvested at 48 hours to assess the luciferase activity in RLUs. *P<0.05 compared with cells exposed to HIV-1/NL4-3-LucΔenv by t test.

Figure 5. TNF-α is expressed in podocytes of children with HIVAN and facilitates the infection of HeLa cells and RECs derived from HIV– persons. (A) RNA was isolated from cultured HeLa cells, 293T cells, and HIVAN podocytes cell lines 1 and 2. RT-PCR studies were done with specific primers for TNF-α and GAPDH as described in Concise Methods. The pCMV-TNF-α plasmid was used as a positive control. (B) HeLa cells, 293T cells, and podocytes were stained with either isotype control or TNF-α antibodies, labeled with PE-conjugated anti-mouse IgG, and processed by flow cytometry as described in Concise Methods. (C) Immunohistochemistry studies in renal sections derived from children with HIVAN show TNF-α protein expression in podocytes. (D) Podocytes, proximal tubular epithelial HK2 cells, RT3 cells, and HeLa cells were transduced with lentivirus carrying full-length TNF-α. Twenty-four hours later, all cells were exposed to either HIV-1/NL4-3-LucΔenv or HIV-1/NL4-3-Luc (equal amounts of p24) and harvested at 48 hours to assess the luciferase activity in relative light units (RLUs). (E) Podocytes (cell line 1) were exposed for 4 hours to HIV/NL4-3-Luc (1 moi). Subsequently, all cells were treated with different concentrations of human recombinant TNF-α (hrTNF-α; 20, 4, 0.8, and 0 ng/ml) and harvested at 48 hours to assess the luciferase activity in RLUs. *P<0.05 compared with cells exposed to HIV-1/NL4-3-LucΔenv by t test.

In support of the latter findings, previous studies showed that TNF-α activates latently infected mononuclear and trophoblastic cell lines and increases the transcytosis of HIV-1 across monolayers of trophoblastic cell lines. Taken together, these findings suggest that tmTNF-α facilitates the infection of podocytes by at least two different mechanisms, enhancing the viral entry process and inducing postintegration viral gene expression via NF-κB activation.

At this time, we do not know the exact mechanisms through which tmTNF-α facilitates the entry of HIV-1 in podocytes. Using a β-galactosidase gene activation assay, we were
Figure 6. tmTNF-α increases the internalization of HIV-1 in podocytes. (A) Podocytes (cell line 1) were transduced with Lac-Z or TNF-α lentiviral vectors and exposed for 2 hours to HIV-iGFP in the presence and absence of Dynasore (20 μM) as described in Concise Methods. Texas Red Transferrin (5 μg/ml) was added 30 minutes before the cells were fixed. Cell nuclei were stained blue with DAPI (1 μg/ml). The pictures were taken under an ultraviolet confocal microscope with green, blue, and red filters to detect GFP, DAPI, and Texas Red Transferrin, respectively. The HIV-iGFP and transferrin pictures were merged to assess their colocalization. (B) Podocytes
unable to detect Env fusion to the cell membrane. This assay, however, could not completely rule out the possibility that low undetectable levels of fusion could occur. We speculate that tmTNF-α may induce the focal expression of adhesion molecules on the cell membrane and in this manner, allow the concentration of viruses on entry spots containing clusters of highly active adhesion and endocytic molecules.53 Indeed, tmTNF-α is associated with lipid rafts, which could play an active role in endocytosis, cell signaling, viral entry, and recruitment of HIV-Tat.12,53,54 However, in a paradoxical manner, previous studies showed that sTNF-α can inhibit the entry of HIV-1 to primary CD4+ macrophages37 as well as the replication of HIV-1 in freshly isolated PBMCs by increasing the expression of HIV-1 suppressive factors (i.e., regulated on activation normal T cell expressed and secreted [RANTES]).55 These findings may not be relevant for podocytes, because they do not express detectable levels of CD4 or CCR5,6 even after TNF-α stimulation. Alternatively, HIV-1 could be internalized via endocytosis before fusion as described before.23 For example, the endocytic receptor DC-SIGN mediates the internalization of HIV-1 in a podocyte cell line derived from an HIV− individual.9 However, these cells were not productively infected,9 and we were unable to block the infection of podocytes cultured from children with HIVAN using DC-SIGN neutralizing antibodies. In addition, podocytes express very low levels of DC-SIGN protein.9 Alternatively, other studies done in primary podocytes or podocyte cell lines cultured from HIV− individuals reported that HIV-1 is internalized via dynasore-mediated endocytosis.11 Dynasore is a small molecule inhibitor of the dynamin GTPase activity that prevents the scission of clathrin-coated pits from the plasma membrane.23 In agreement with this study, our findings suggest that HIV-1 enters podocytes via clathrin-dependent endocytosis. It is worth mentioning that dynasore can inhibit the fusion of HIV-1 in endosomes23 and that dynamins facilitate the escape of HIV-1 from endosomes.23,56 Furthermore, HIV-1 infection via endosomes was reported in macrophages, lymphocytic cells, and HeLa cells.38 Nevertheless, the precise mechanisms through which HIV-1 is released into the cytoplasm and integrated in the nuclei of podocytes are still undefined. It is also unclear whether there is a pH dependency for this route of infection. In this regard, we found that Bafilomycin A, an inhibitor of the vacuolar proton ATPase that impairs the acidification of endosomes, inhibited the infection of podocytes. However, cells exposed to NH4Cl were infected, and Bafilomycin A can block the transport of endocytosed material from early to late endocytic compartments independent of the pH.57 Finally, we found that some viruses traffic to late endosomes/lysosomes, whereas others remain localized to nonacidic compartments, and we have not identified the compartments where the viral fusion occurs. In conclusion, more studies are needed to define how tmTNF-α regulates the processes of viral entry and fusion.

It is worth mentioning that tmTNF-α, the precursor of sTNF-α, can act as both a ligand and a receptor.25 Thus, in vivo, tmTNF-α could induce cell to cell contact–dependent signaling and raise the concentration of sTNF-α in the proximity of TNF receptors.25 These events could promote the expression of cell adhesion and signaling molecules involved in viral entry and replication. Furthermore, tmTNF-α can activate the noncanonical NF-κB pathway acting through TNF receptor 2, which has higher affinity for tmTNF-α than sTNF-α.25,31 Through all of these mechanisms, tmTNF-α may facilitate the formation of synapses between REcs and HIV+ mononuclear cells, leading to the accumulation of viruses in this space. Thus, the high viral titers used in this study may become physiologically relevant in the context of these virologic synapses given that Trojan horse–like interactions are the most efficient mechanism to infect REcs cultured from children with HIVAN.10 We also confirmed that, in children with HIVAN, podocytes express TNF-α mRNA and protein as described in other renal inflammatory diseases.28–33 Moreover, the expression of TNF-α in podocytes and RTEcs is upregulated in HIV-Tg26 mice with HIVAN33 as well as in many other inflammatory human renal diseases.28–32 and tmTNF-α may prime REcs to become infected with HIV-1. Finally, we have shown that podocytes that express either the APOL1 wild-type or the G1 risk alleles that predispose to HIVAN58 can be infected. Therefore, the APOL1 G1 risk alleles seem to be dispensable for this process. Nonetheless, given that TNF-α can induce the expression of APOL1 in REcs,59 we speculate that tmTNF-α may affect the outcome of childhood HIVAN acting through both mechanisms.

In summary, we have uncovered a new role for tmTNF-α in facilitating the infection of podocytes cultured from children with HIVAN. We propose that REcs cultured from children transduced with Lac-Z or TNF-α lentiviral vectors were cultured in the presence and absence of human recombinant TNF-α (hTNF-α; 10 ng/ml) for 24 hours. Subsequently, all cells were exposed for 2 hours to HIV-iGFP as described above. LysoTracker Red (25 nM) was added 30 minutes before the cells were fixed. The picture were taken under an ultraviolet confocal microscope with green, blue, and red filters for GFP, DAPI, and LysoTracker, respectively. The HIV-iGFP pictures were merged with the LysoTracker pictures to assess their colocalization. (C and D) The number of HIV-iGFP was counted in red filters for GFP, DAPI, and LysoTracker, respectively. The particles were expressed as percentage changes relative to controls. (E and F) Results were confirmed doing RT-PCR viral entry assays. Here, podocytes were transduced with GFP or TNF-α lentiviral vectors overnight and exposed to HIV/NL4-3-GFP or mock-infected supernatants for 2 hours. Subsequently, the cells were trypsinized and harvested as described in Concise Methods. RNA was extracted, treated with DNase I as described in Supplemental Material, and used as a template for the RT-PCR quantitative studies with specific primers for HIV-1 Pol. As a positive control, we used the NL43-GFP plasmid (0.1 ng) as template. *P<0.05 comparing each group with its respective control by t test (n=5 per group).
with HIVAN are primed by tmTNF-α to become latently or productively infected, albeit at low levels, when exposed to a high viral load. More studies are needed to validate this hypothesis and elucidate how tmTNF-α facilitates the viral entry and integration processes in these cells. These findings may have wider clinical implications for the pathogenesis of childhood HIVAN and other AIDS–related illnesses, including the vertical transmission of HIV-1.51

**CONCISE METHODS**

**Materials**

The plasmids for the HIV-1 infectious clones pYU2; p89.6; pNL4–3. Luc.E.~R~; pNL4–3; pNL4–3.ΔE-EGFP; pBlue3'LTR-luc-A (pLTR-Luc); GHOST (3) CD4+/CXCR4+; CCR5+ cells; the fusion inhibitor Trimeris/roche T6-20; the reverse transcription inhibitor Zidovudine (Azidothymidine); the integrase inhibitor Raltegravir; the protease inhibitor Ritonavir; and the CCR5 inhibitor bicyclam JM-2987 were all obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, National Institutes of Allergy and Infectious Disease, National Institutes of Health and are described in Supplemental Material. Heparitinase III and NH4Cl were obtained from the National Institute of Allergy and Infectious Disease, National Institutes of Health and are described in Supplemental Material. Heparitinase III and NH4Cl were obtained from the National Institutes of Health and are described in Supplemental Material.

**Plasmid Constructs**

Several plasmids were constructed as described in Supplemental Material. Briefly, the pNL4–3–GFP was constructed as described before.60 The pNL4–3–Luc was constructed by excising the DNA fragment containing luciferase gene coding sequence from pNL4–3–Luc.E.~R~ vector and inserting it into the BamHI and XhoI sites of pNL4–3–Luc. The pNL4–3–LucEnv was constructed by excising the DNA fragment containing luciferase gene coding sequence from pNL4–3–Luc.E.~R~ vector and inserting it into the BamHI and XhoI sites of pNL4–3–LucEnv (pNL4–3–GFPΔEnv). pNL4–3–Puro was constructed by PCR amplification of puromycin N-acetyl transferase gene from pPUR vector (Clontech Laboratories) using primers described in Supplemental Material. The full-length TNF-α cDNA clone was obtained by screening the podocyte cDNA library as described below. The amplified cDNA was digested and inserted into the XhoI and BamHI sites of pDsRed-C1 (pRFP) vector (Clontech Laboratories). This plasmid was designated as pRFP-TNF-α. pRFP-inactive-TNF-α (pRFP-TNF-α A84V-Y87L)27 and pRFP–tm-TNF-α (pRFP-TNF-α Δ1–12) were constructed by using overlap extension PCR techniques26 as described in detail in Supplemental Material. All plasmids were confirmed by nucleotide sequence analysis.

**Generation of the Podocyte cDNA Library and Screening**

The library from the podocyte cell line 1 was generated by Invitrogen’s construction cDNA library services using standard methods.24 This cell line, derived from a child with HIVAN, was described and characterized in detail in a previous study.12 The full-length cDNA library was transfected into 293T cells, which were subsequently exposed to 1 moi HIV-1/NL4–3 Purino viruses. After 48 hours, puromycin was added to a final concentration of 1 μg/ml. All visible colonies detected after approximately 10–14 days in culture were expanded. Total cellular DNA was then extracted from approximately 10^6 cells and purified using the Qiagen DNeasy Tissue Kit (Qiagen, Germantown, MD). The transfected plasmids were recovered from the cell colonies by PCR with T7 and SP6 primers complementary to the cDNA library vector pCMVSPORT6.1. The PCR products were digested with restriction enzymes MspI and KpnI and ligated back into the pCMVSPORT6.1 vector.

**Human Samples and Cell Culture**

These experiments were approved by the Institutional Review Board of the Children’s National Medical Center (protocol no. 00000002) and carried out in accordance with the principles of the Declaration of Helsinki. Renal sections from children with HIV renal diseases (n=4) were obtained from the Children’s National Medical Center. Human PBMCs were derived from HIV– donors or children with HIVAN as described in detail in a previous study.10 All primary podocytes and podocyte cell lines were characterized as described in detail before.12 Briefly, these cells express the podocyte markers WT-1, GLEPP114 (Figure 1), synaptopodin, nestin, podocalyxin, and nephrin12 and do not express endothelial or RTEc markers as we described before.10,12,61 The RTEc line (RT3) and primary podocytes were cultured from the urine of HIV+ or HIV– children with renal diseases and characterized as described before.10,12 Podocytes cell lines 1 and 2 were generated from children with HIVAN, immortalized, and characterized as described in detail before.12 The methods and primers used to characterize the expression of CD4, CXCR4, and CCR5 by RT-PCR are described in Supplemental Material. These podocyte cell lines were genotyped for the APOL1 risk variants as described before.7,19,58 Podocytes were cultured in DMEM supplemented with 10% FBS and penicillin (100 U/ml)-streptomycin (100 μg/ml). HeLa cells, 293T cells, and the renal proximal tubular epithelial cell line HK2 were obtained from the American Tissue Culture Collection (Manassas, VA) and cultured using the media recommended by the American Tissue Culture Collection.

**Virus Production and Infection Assays**

Primary podocytes were exposed to cell-free HIV-1 supernatants (1 moi) collected from PBMCs derived from children with HIVAN and titrated as described before.10 Before use, HIV-1 supernatants were treated with 50 U/ml RNAase–free DNase I (Qiagen) for 1 hour at 37°C to eliminate contaminant DNA. Subsequently, cells were exposed to HIV-1 for 48 hours, treated with trypsin at 0.05% at 37°C for 5 minutes, washed three times with PBS to eliminate adherent viruses, and cultured in fresh culture media for at least 7 days. HIV-1–specific amplification products were detected using an electrochemiluminescent assay as previously described.10 Expression of p24Ag antigen was detected by immunocytochemistry and ELISA as described before.10 HIV-1 was rescued from podocytes infected for 7 days by adding HIV– PBMCs activated with phytohemagglutinin-A and IL-2 as described before.10 In other experiments, recombinant HIV-1 viruses were prepared in 293T cells
transfected with pNL4–3-Luc, pNL4–3-LucΔenv, pNL4–3-GFP, pNL4–3-GFPΔEnv, and pNL4–3-Puro vectors. Cells were cotransfected with proviral DNA p89.6 or pYU2 with pNL4–3-LucΔenv or pNL4–3-GFPΔEnv vector at a 1:1 ratio to generate viral particles containing both wild-type and luciferase/GFP reporter virions with Env proteins. Transfections were performed using the Lipofectamine 2000 (Life Technologies, Carlsbad, CA) following the manufacturer’s protocol. Viruses present in the supernatant of 293T transfected cells were collected by centrifugation, filtered through a 0.24-μm-pore size filter, and quantified by the p24 ELISA Kit (ZeptoMetrix, Buffalo, NY). Viral titration was done in GHOST cells (2×10^6 per well) exposed to different viral dilutions for 48 hours as described in detail in Supplemental Material.

To detect HIV–1–specific DNA by PCR, GHOST cells and podocytes (5×10^5) were cultured overnight and exposed to 1 moi HIV-1/NL4–3-GFP supernatants (treated with DNase I as described above) for 2 hours. Subsequently, the cells were washed as described above to eliminate adherent viruses, cultured in fresh medium, and harvested at 30 minutes or 48 or 72 hours after infection. DNA was extracted from these cells using the Qiagen DNeasy Blood and Tissue Kit; 100 ng DNA was subjected to 30 cycles of PCR in a total volume of 50 μl containing 0.2 μM oligonucleotide primers, 200 μM each deoxynucleotide, 50 mM KCl, 10 mM Tris, pH 8.3, 8 mM MgCl2, and 1.25 U Taq DNA polymerase (Promega). Each cycle comprised a 30-second denaturation step (94°C), a 30-second annealing step (55°C for each pair of primers), and a 30-second extension (72°C). After agarose gel electrophoresis, amplified fragments of the correct size were photographed with the Bio-Rad Gel Imaging System (Bio-Rad, Hercules, CA). The following HIV–specific pol primers were used: sense primer (5’-TTCTTCAGAG-CAGACCAG-3’) and antisense primer (5’-ACTTTTGGGCCATCATT-3’). The viral entry assays were done as described in detail before. Briefly, cells were transduced with Lentiv-TNF-α or LentigFp overnight and exposed to cell-free HIV/NL4–3-GFP (1 moi) for 2 hours. Quantitative PCR was performed using Bio-Rad iQ SYBR Green Mix and the following HIV–specific pol primers: sense primer (5’-TTCTTCAGAG-CAGACCAG-3’) and antisense primer (5’-ACTTTTGGGCCATCATT-3’) or LTR primers: sense primer (5’-GGCTCATAAAGCTGCGGCTTG-3’) or antisense primer (5’-TGACTAAAAGGTCTGAGGGATCT-3’). As positive controls, we used 0.1 ng pNL43GFP plasmid as a template.

Role of TNF-α
To assess the activity of the TNF-α plasmids, 293T cells (1×10^5 per well) were transfected first with 0.02 μg pLTR-Luc or 0.5 μg pcMV-TNF-α, pcMV-tm-TNF-α, pcMV-inactive-TNF-α, or pcMV empty vector, pRL-TK (0.02 μg) was added for normalization of transfection efficiency. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Monitoring of light emission was performed for both firefly and Renilla luciferase activity. Raw data were normalized by calculating the ratio of firefly to Renilla luciferase light emission values. Lentiviruses for TNF-α or GFP were generated with the ViraPower HiPerform Lentiviral FastTiter Gateway Expression Kit (Life Technologies) according to the manufacturer’s protocol. Briefly, the pLenti-TNF-α or control vector pLenti-GFP was cotransfected with ViralPower Packaging Mix into 293FT cells, and virus was harvested after transfection for 48 hours. TNF-α immunohistochemistry studies in renal sections were done as previously described using a mouse monoclonal TNF-α antibody (52B83; Novus Biologicals, LLC, Littleton, CO). In other experiments, 5×10^4 per well GHOST (3) cells or podocytes were transfected using Lipofectamine 3000 (Life Technologies) with 10 nM human TNF-α 27mer siRNA duplexes or control siRNA, both purchased from OriGene Technologies (Rockville, MD). The next day, the cells were exposed to 10 ng/ml p24 of HIV-1/NL4–3-Luc for 48 hours and then, harvested to assess their luciferase activity using the Promega Luciferase Reporter Assay System. Alternatively, RNA was extracted from the cells using Trizol and treated with DNase I. The cDNA was synthesized by using the Superscript III First-Strand Synthesis System (Life Technologies), and PCR was done using specific primers for TNF-α and GAPDH as described before. To detect the NF-κB promoter activity, cells were transfected with 0.5 μg p5x NF-κB-Luc per well and processed to assess the luciferase activity as described above.

Confocal Imaging, Flow Cytometry, and Immunohistochemistry Studies
Cells were cultured in six-well plates at 2×10^5 per well and transfected with pRFP-TNF-α, pRFP-tm-TNF-α, pRFP-inactive-TNF-α, or pRFP for 24 hours. Subsequently, the cells were exposed to 1–3 moi HIV-1/NL4–3-GFP (approximately 20–60 ng p24Equiv/ml) for 48 hours, washed, and fixed with 4% paraformaldehyde diluted in PBS for 20 minutes. Nuclei were stained with 1 μg/ml DAPI. Cells were observed under ultraviolet microscopy using a Nikon Model TE300 Microscope (Nikon, Tokyo, Japan) with filters for PE, GFP, and DAPI. Fluorescence images were captured with the IPLCA1.4MPX Color USB2.0 Microscope Digital Camera System (ImagingPlanet, Goleta, CA) and processed with Adobe Photoshop software (Adobe Systems, Inc., San Jose, CA). To identify HIV receptors/coreceptors, all cells were fixed with 4% paraformaldehyde in PBS for 10 minutes and incubated with specific antibodies against human CD4, CXCR4, CCR5, TNF-α, or the corresponding mouse isotype control antibodies for 30 minutes. Subsequently, the cells were mixed with the PE–conjugated anti–mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes and then, analyzed by flow cytometry on a two–laser, four-color FACS-Canto (BD Immunocytochemistry Systems, San Jose, CA).

Statistical Analyses
Statistical analysis was performed using GraphPad Software Inc. (San Diego, CA) Graph Prism 5.0. For parametrically distributed data, we used the t test or ANOVA when more than two groups were compared. When the data were not normally distributed, we used the nonparametric Mann–Whitney U test or the Kruskal–Wallis test when more than two groups were compared. P values <0.05 were considered statistically significant.

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DISCLOSURES
None.

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