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Nerve growth factor stimulation promotes CXCL-12 attraction of monocytes but decreases human immunodeficiency virus replication in attracted population

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The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are key molecules in the central nervous system development, which also exert specific effects on cells of the immune system. With regard to the latter, *in vitro* as well as *in vivo* data suggested that neurotrophins may play a role in human immunodeficiency virus (HIV) infection, especially in perivascular spaces where infiltrated macrophages express NGF. In the present study, we examined the expression of neurotrophins and their receptors in human monocyte-derived macrophages (MDMs) during infection by the R5 prototype HIV1/Ba-L strain. We then assessed to what extent neurotrophins themselves modulate infected macrophage survival and the level of virus production. The data show that neurotrophins and neurotrophin receptors are not modulated during HIV replication. Likewise, exogenous neurotrophins, or alternatively the blocking of neurotrophin receptors, neither modulated MDM sensitivity to HIV infection and replication nor altered their viability. In contrast, NGF clearly increased CD184 expression in macrophages, but this did not sensitize them to the X4 isolate HIV-1/Lai infection. Nevertheless, NGF enhanced monocyte chemotactic response to low CXCL-12 concentration regardless of infection. Surprisingly, CXCL-12–attracted monocytes from NGF-stimulated, HIV-infected cultures produced decreased amounts of virus progeny than their non–NGF-stimulated counterparts. This suggests a preferential effect on uninfected monocytes. Together these findings suggest a role for NGF in the continuous attraction of activated monocytes to the perivascular spaces, contributing to the chronic inflammatory state rather than neuroinvasion by HIV. *Journal of NeuroVirology* (2009) **15,** 71–80.

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Introduction

Macrophages are major targets for human immunodeficiency virus type 1 (HIV-1) infection *in vivo* and play an important role in acquired immunodeficiency syndrome (AIDS) pathogenesis, particularly in solid organs such as the brain (Wiley *et al*, 1986). They constitute a prominent virus reservoir in the body that may account for about 10% of global virus load (Igarashi *et al*, 2001). Beside virus load *per se*, HIV replication in macrophages and HIV-related activation of uninfected macrophages are considered critical in neuronal damage that often occurs in the advanced stages of the disease (Tyor *et al*, 1993). Harrold *et al* suggested that the neurotrophin nerve growth factor (NGF) specifically reduces the production of HIV-1 and enhances CXCR4 expression in macrophages, without affecting CCR5 expression (Harrold *et al*, 2001). In contrast, Garaci *et al* showed that HIV-infected macrophages set up an autocrine NGF loop sustaining macrophage viability and virus replication (Garaci *et al*, 1999), which would be critical for maintaining virus reservoirs (Garaci *et al*, 2003). On the other hand, trafficking of activated and infected monocytes across the blood-brain barrier (BBB), rather than autonomous replication in the isolated central nervous system (CNS) compartment, accounts for both CNS HIV load and the chronic inflammatory activation of brain parenchyma. This is strongly evidenced by anti retroviral therapy efficacy against both cerebrospinal fluid (CSF) viral load and central inflammation markers despite restricted penetration of antiviral drugs (Gras *et al*, 2007; Kaul *et al*, 2001, 2005; Spudich *et al*, 2005, 2006). Chemokines, such as CCL-2 and CXCL-12, regulate leukocyte migration through the BBB (Eugenin *et al*, 2006; Kaul *et al*, 2001; Kelder *et al*, 1998; Persidsky *et al*, 1999; Wu *et al*, 2000) and may thus participate in this chronic activation/invasion. Beside chemokines, neurotrophins exert specific activities in macrophage activation (Barouch *et al*, 2001; Caroleo *et al*, 2001; Susaki *et al*, 1996) and chemotaxis (De Simone *et al*, 2007; Kobayashi and Mizisin, 2001; Samah *et al*, 2008). Among neurotrophins, nerve growth factor (NGF) is overexpressed in perivascular spaces in HIV encephalitis (Boven *et al*, 1999) and sensitizes monocytes to suboptimal CXCL-12 gradient (Samah *et al*, 2008), suggesting it may interplay with chemokines for activated and/or infected monocyte entry into the CNS.

In this work we studied the expression of neurotrophins and their receptors in human monocyte-derived macrophages (MDMs) during infection by the prototype HIV1/Ba-L strain. We also addressed their putative involvement in infected macrophage survival and the level of virus production. We found that these MDM-expressed neurotrophin genes are not modulated during R5 HIV-1/Ba-L strain replication. Likewise, exogenous neurotrophins, or alternatively the blocking of neurotrophin receptors, neither modulated MDM sensitivity to HIV infection and replication nor altered their viability. Nevertheless, NGF specifically increased CD184 (CXCR4) expression in both MDMs and monocytes. Although this did not confer permissiveness to the X4 isolate HIV-1/Lai, it increased sensitivity to CXCL-12–evoked chemotaxis in HIV-infected monocytes. This suggests a participation for NGF in the continuous attraction of inflammatory-activated monocytes to the perivascular spaces. Finally, although NGF increased monocyte attraction, the subsequent amount of virus progeny in transmigrated monocyte cultures was dramatically decreased, suggesting a more potent role on uninfected monocytes.

Results

Neurotrophin and neurotrophin receptor genes are expressed throughout HIV replication kinetics in MDMs

We have recently described that MDMs express both neurotrophin and neurotrophin receptors genes, but without detectable secreted protein as assessed by enzyme-linked immunosorbent assay (ELISA) (<10 pg/ml; Samah *et al*, 2008). Likewise, neurotrophin concentrations in supernatants remained undetectable in HIV-infected cultures (data not shown). Although cell-associated neurotrophins were readily detectable by immunocytochemistry (Samah *et al*, 2008, and data not shown), this technique does not allow reliable quantitation and we thus used real-time reverse transcriptase–polymerase chain reaction (RT-PCR) in the following experiments.

Gene expression levels of NGF, BDNF, NT3, and NT4 as well as their receptors TrkA, TrkB, TrkC, and $p75^{NTR}$ were quantified twice weekly between days 3 and 21 after *in vitro* infection of MDMs by HIV-1/Ba-L. The kinetic of HIV replication is shown in Figure 1A.

As shown in Figure 1B, although gene expression levels varied over time (NGF in particular), HIV replication had no major effect. With the detection threshold of this technique, which allows ascertaining fourfold modulations (Samah *et al*, 2008), we detected a sevenfold repression of NT3 gene expression at day 3 post infection (p.i.) that did not persist thereafter. Statistical analysis confirmed this result as a trend, with a P value of .06 (two-tailed Student's t test). Likewise at day 21 p.i., NT4 expression increased fourfold whereas that of receptor TrkC decreased sevenfold, but these changes did not reach statistical significance probably due to a high variance ($P > .15$ and $P > .45$, respectively, with two-tailed Student's t test). Finally, only a decrease in TrkB gene expression was detected throughout the full replicative phase of the culture (days 14 to 21), with amplitudes varying between three- and fivefold. Alpha risk was 7% at day 14 p.i. ($P = .07$, two-tailed

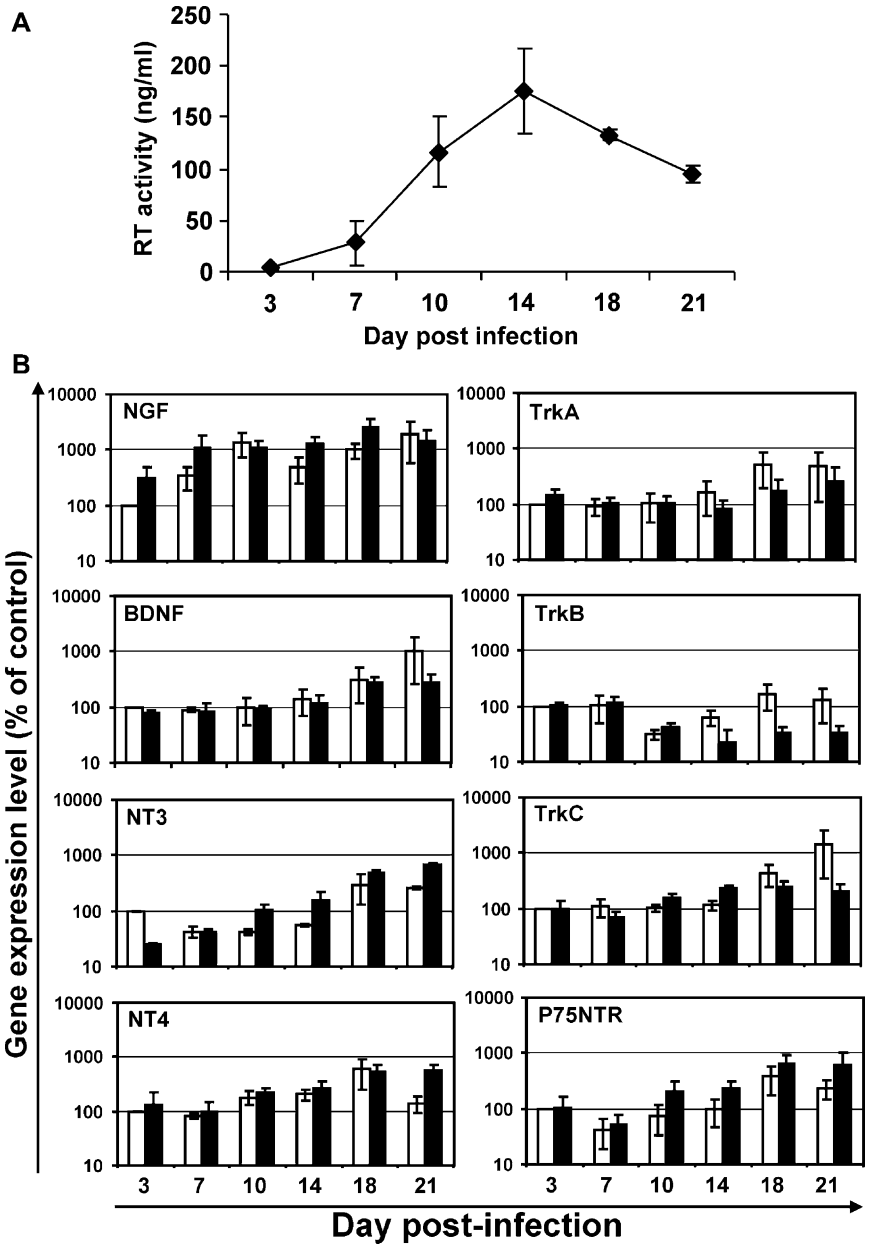

Figure 1 Neurotrophin and neurotrophin receptor expression during HIV replication in MDM. Mature macrophages were infected with HIV-1/Ba-L (m.o.i. = 0.1) and followed up for 21 days thereafter. (**A**) RT activity was measured twice weekly in supernatants. (**B**) NGF, BDNF, NT3, and NT4 and their receptors TrkA, TrkB, TrkC, and $p75^{NTR}$ gene expression levels were measured in uninfected (*open bars*) and infected macrophages (*closed bars*) twice weekly by means of quantitave RT-PCR normalized to GAPDH expression. Results are shown after normalization to day 3 noninfected control expression level. Data are expressed as mean ± SEM of three independent experiments.

Student's *t* test) and higher thereafter, suggesting a trend rather than a clear effect. On the whole, HIV may thus not modulate neurotrophin expression in our model of MDMs, nor induce major variations in receptor levels.

NGF specifically increases macrophage expression of CD184

We in turn aimed to investigate neurotrophin ability to modulate HIV infection in macrophages. We first studied the expression level of the HIV receptor and coreceptors CD4, CD184 (CXCR-4), and CD195 (CCR-5). Basal expression levels in untreated macrophages were 30,583 ± 23,316 mean equivalent fluorochrome bound per cell (MEF) for CD4 (mean ± SEM, $n = 5$), 12,237 ± 10,617 MEF for CD184 ($n = 5$), and 4798 ± 325 MEF for CD195 ($n = 5$). As shown in Figure 2, NGF increased CD184 expression level (164% ± 18% of control; $P = .034$ by two-tailed Student's *t* test with unequal variances) and did

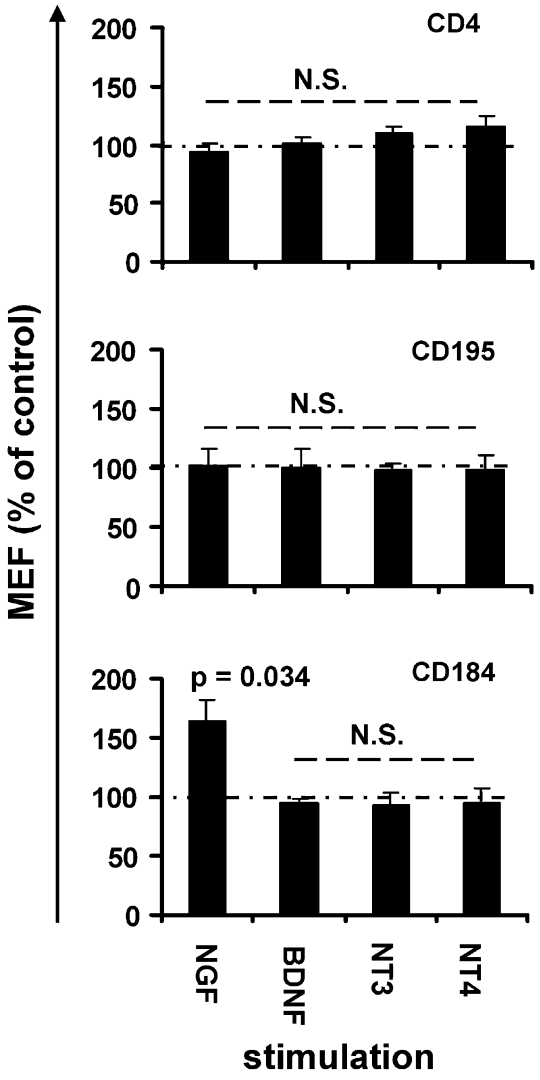

Figure 2 Neurotrophin effect on CD4, CD184, and CD195 expression by MDM. Mature macrophages were treated for 2 days with different neurotrophins. Levels of CD4, CD184, and CD195 membrane expression were then assessed by quantitative flow cytometry. Results are expressed as a percentage of untreated cells mean equivalent fluorochrome bound per cell (MEF) ± SEM for four (CD195) or six (CD4 and CD184) independent donors. NGF: $P = .034$, other conditions non significant (unpaired Student's t test with unequal variances).

not change those of CD4 and CCR5. The other neurotrophins had no effect on the tested receptors. This important modulation of CD184 expression classifies NGF apart from BDNF, NT3, and NT4.

NGF does not sensitize MDMs to HIV-1/Lai productive infection

To assess whether NGF-induced increase in CD184 expression may increase macrophages susceptibility to X4 HIV strain replication, we treated MDMs with NGF from day 1 before infection to day 18 post HIV-1/Lai infection, and measured virus release in supernatants. As shown in Figure 3A, NGF-treated cultures were not susceptible to HIV-1/Lai

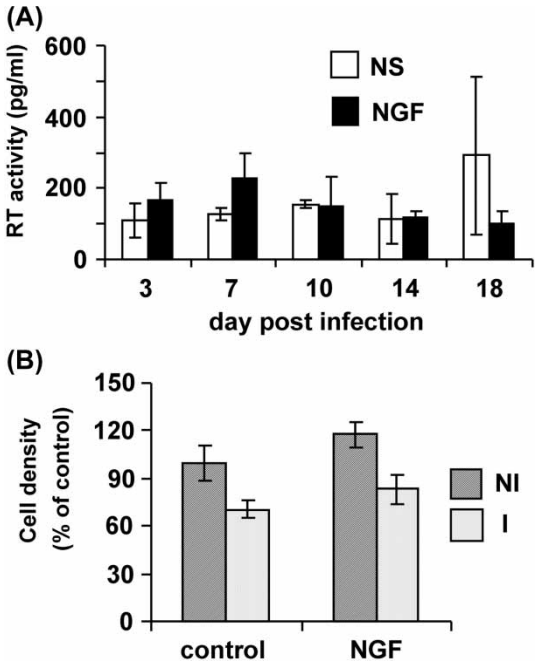

Figure 3 NGF effect on HIV-1/Lai replication and macrophage survival. Mature macrophages were infected with HIV-1/Lai (m.o.i. = 0.1) and followed up for 18 days thereafter. (**A**) Macrophages were treated by NGF from day 1 before infection to day 18 post infection. Culture medium was renewed every 3 to 4 days and RT activity measured. (**B**) Cell viability was assessed on day 18 p.i. by tetrazolium salt (MTT) assay in infected cultures (I) and noninfected controls (NI). Data are expressed as a percentage of control mean ± SD; experiment was performed in quadruplicate.

replication. As NGF might increase cell survival, or alternatively provide apoptosis signals, we measured cell viability in both cultures and found no modulation by NGF treatment (Figure 3B).

HIV-1/Ba-L replication in MDMs is independent of neurotrophin signaling

To assess whether neurotrophins may regulate HIV-1/Ba-L replication in macrophages, we treated infected MDMs with neurotrophins from day 1 before infection to day 18 post HIV-1/Ba-L infection and measured RT activity in supernatants every 3 to 4 days. As shown in Figure 4A, the quantity of virus produced throughout the kinetic was not changed by continuous neurotrophin treatment. We verified in the same cultures that cell viability was not modified by long-term neurotrophin treatment (Figure 4B).

Although we did not detect any secreted neurotrophin by ELISA in MDM supernatants, and keeping in mind that neurotrophins were readily detected by immunocytochemistry, we aimed to verify whether or not an autocrine loop existed that might entail rapid consumption of the secreted ligand. We thus treated HIV-replicating macrophages with neutralizing anti-neurotrophin antibodies (or the corresponding irrelevant controls) at day 10 post infection when high replication occurs, and assessed virus production kinetic until day 14 p.i.

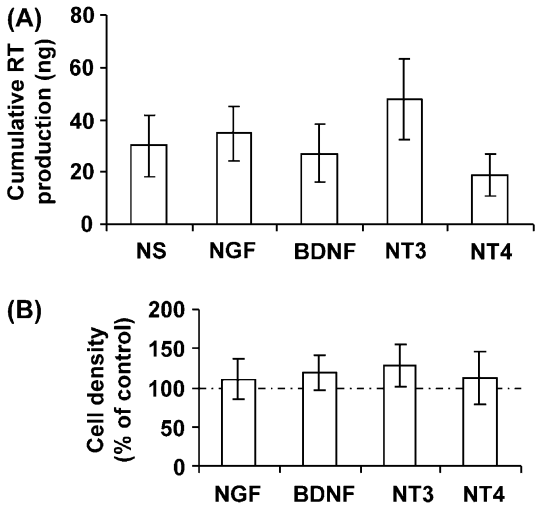

Figure 4 Neurotrophin effect on HIV-1/BaL replication and macrophage survival. Mature macrophages were infected with HIV-1/Ba-L (m.o.i. = 0.1) and followed up for 18 days thereafter. (**A**) Macrophages were treated by neurotrophin from day 1 before infection to day 18 post infection. Culture medium was renewed every 3 to 4 days and RT activity measured. Results are shown as mean ± SD of cumulated RT production throughout the kinetic. (**B**) Cell viability was assessed on day 18 p.i. by tetrazolium salt (MTT) assay. Data are expressed as a percentage of control mean ± SD. Experiment was performed in quadruplicate for two independent donors.

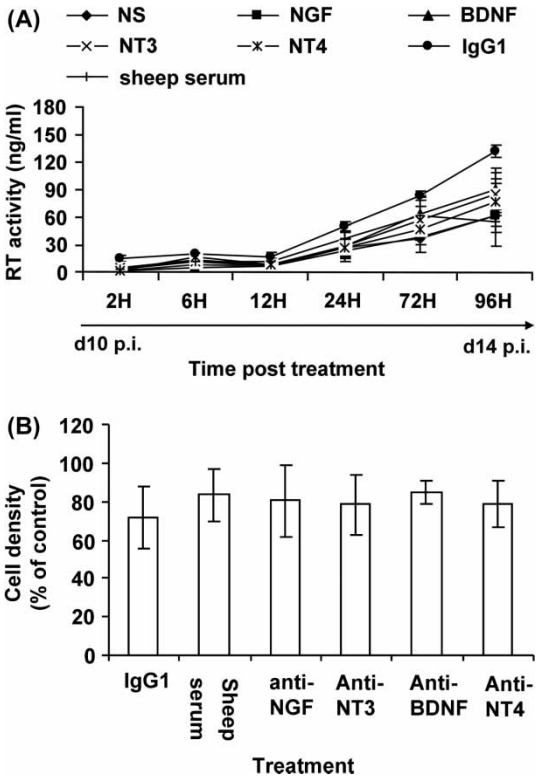

Figure 5 Effect of neurotrophin neutralization on HIV replication and MDM survival. Mature macrophages were infected with HIV-1/Ba-L (m.o.i. = 0.1) and followed up for 14 days thereafter. At day 10, cultures were washed and neutralising antibodies to neurotrophins added. (**A**) RT activity was measured 2, 6, 12, 24, 72, and 96 h after neurotrophin neutralization. (**B**) Cell viability was assessed by tetrazolium salt (MTT) assay, at day 14 post infection, 4 days after neurtrophin neutralization. Results are normalized to control untreated cells. Data are mean ± SD of triplicate determinations. Two independent donors were tested with similar results.

As shown in Figure 5A, neurotrophin neutralization did not affect HIV replication, ruling out an autocrine control of HIV replication through neurotrophin signaling. Again, we verified that cell viability was not different in neurotrophin-neutralized versus control cultures, at day 14 p.i., after 4 days of neutralization (Figure 5B).

NGF increases monocyte transmigration in response to suboptimal CXCL-12 concentrations

The NGF-evoked increase in CD184 expression levels led us to assess whether this was accompanied by an increase in CXCL-12–induced chemotaxis. To avoid issues that may arise from cell detachment, we choose to perform chemotaxis assay on peripheral blood mononuclear cells (PBMCs) and analyze monocyte migration. We previously verified that NGF increased CD184 expression on monocytes as well (data not shown). Activated $CD16^{++}/CD14^{-}$ monocytes preferentially crossed the filter chamber, with their percentage varying from 19% ± 1% of total monocytes in input cells to 54% ± 7% in migrated cells. Nevertheless, this selective migration of CD16-expressing monocytes was neither modulated by NGF nor by CXCL-12, and we thus choose to analyze total numbers of migrated monocytes thereafter.

Analysis of variance (ANOVA) showed that infection, NGF treatment, and CXCL-12 presence affected monocyte migration ($P = .0006$, $P < .0001$, and $P < .0001$, respectively). *Post hoc* Fisher possibly least significant difference (PLSD) test confirmed these results. For three different CXCL-12 concentrations, both Fisher and Bonferroni-Dunn tests showed an effect of CXCL-12 on monocyte migration, ruling out a possible artefact from multiple group testing ($P < .0001$ for control versus each concentration), but no dose effect could be evidenced.

As shown in Figure 6, monocytes from untreated uninfected PBMC cultures migrated in response to CXCL-12 gradient, with a maximum 48% ± 2% migration at 600 ng/ml CXCL12. According to increased CD184 expression, NGF pretreatment increased monocyte responsiveness to CXCL-12, with a maximum migration of 53% ± 3% reached at 300 ng/ml CXCL-12.

Monocytes from HIV-1–infected PBMC cultures exhibited an increased spontaneous migration (28% ± 1% versus 21% ± 2%). In HIV-1–infected cultures, NGF increased monocyte sensitivity to 100 ng/ml CXCL-12 (59% ± 5% versus 46% ± 4%). This effect was lower at 300 ng/ml CXCL-12 and no longer detectable at 600 ng/ml CXCL-12, suggesting a saturation effect. Of note, NGF effect on migration may not be due to modulations of monocyte survival

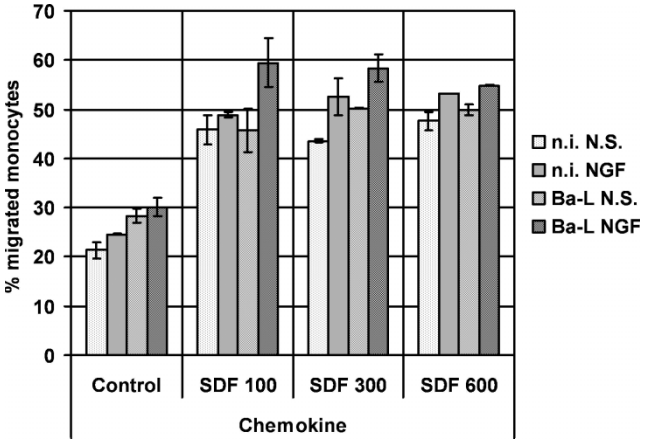

Figure 6 NGF sensitizes monocyte to CXCL-12 gradient attraction. PBMCs were treated or not with NGF for 24 h and infected or not with HIV-1/BaL (m.o.i = 0.1) for another 24 h in NGF containing medium. PBMCs were then assessed for chemotaxis. Spontaneous (control) and CXCL-12-induced chemotaxis was assessed, whereas input cells were placed in a plate well without filter. Migrated cells were recovered from the bottom compartment of the chemotaxis chamber, stained for CD14 and CD16 and counted and analyzed by flow cytometry. The proportion of $CD14^+/CD16^-$, $CD14^+/CD16^+$, and $CD14^-/CD16^{++}$ cells in migrated monocyte was not modulated by NGF (data not shown) and we thus considered the numbers of migrated monocytes from all three subpopulations in the figure. The mean ± SD of % migrated monocytes in response to different CXCL-12 doses (duplicate wells) are shown. ANOVA analysis confirmed that the effects of infection ($P = .0006$), NGF pretreatment ($P < .0001$), and CXCL-12 ($P < .0001$, no dose effect) were significant. Results are from one representative experiment of three. n.i.: not infected. N.S.: not stimulated by NGF.

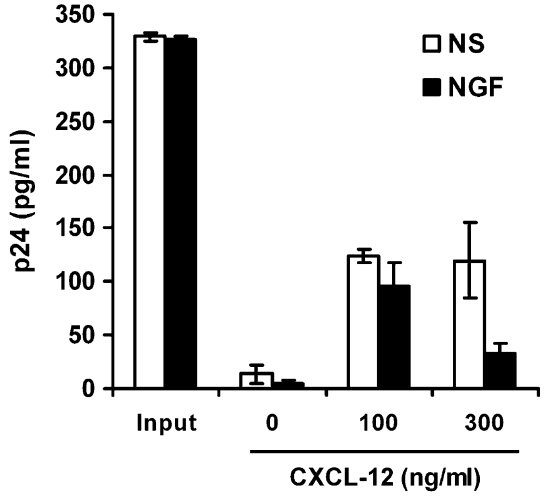

Figure 7 NGF decreases HIV-1 progeny in transmigrated infected monocyte cultures. Freshly elutriated monocytes were treated or not with NGF for 24 h, infected with HIV-1/BaL (m.o.i. = 0.1), washed, and then cultured for another 24 h in NGF-containing medium. Monocytes were then washed and assessed for chemotaxis for 3 h using 0, 100, and 300 ng/ml CXCL-12 (0, 100, 300; horizontal axis). Migrated cells were then cultured until day 14 post infection and p24 production was measured. Input cells were directly seeded into the wells without a chemotaxis step, in order to measure the HIV production potential of the monocytes. ANOVA analysis confirmed that the effects of NGF pretreatment ($P = .0279$) and CXCL-12 ($P = .0034$, no dose effect) were significant. Data are from one representative experiment of two tested donors.

or activation, as neither monocyte percentage, nor the relative abundance of $CD14^+/CD16^-$, $CD14^+/CD16^{dim}$, and $CD14^-/CD16^{bright}$ monocytes subpopulations were affected in treated PBMCs (data not shown).

NGF dramatically decreases HIV progeny in transmigrated monocyte cultures

The NGF-evoked increase in monocyte transmigration led us to assess if this could also help HIV passage. We thus performed the same experiment as above but with elutriated monocytes, and quantitated virus progeny in migrated cell cultures.

As shown in Figure 7, unstimulated monocytes that had migrated in response to CXCL-12 produced HIV p24, with a maximum of 124 ± 6 pg/ml HIV-1 p24 at 100 ng/ml CXCL-12. This effect of CXCL-12 on HIV production grossly parallels its effect on monocyte transmigration. In contrast, NGF pretreatment decreased p24 production in migrated monocyte cultures, with a maximum of 73% inhibition at 300 ng/ml (Figure 7). This effect contrasts with the positive action of NGF pretreatment on cell migration, which was even higher in infected cultures versus uninfected ones (Figure 6). Moreover, this effect of NGF may not be due to a repression on HIV replication in infected monocytes, as NGF treatment did not modulate HIV production in input cultures (Figure 7, input).

ANOVA analysis showed that the effect of CXCL-12 (positive effect on virus progeny) as well as that of NGF (decreased virus progeny) were significant ($P = .0034$ and $P = .0279$, respectively). Again, the Fisher and Bonferroni-Dunn *post hoc* tests confirmed the result.

These results show that although NGF promotes the transmigration of monocytes in HIV-infected cultures, it specifically promotes noninfected monocyte attraction and even reduces the migration of HIV-infected cells.

Discussion

In this study, we first assessed neurotrophin and neurotrophin receptor gene expression in HIV-infected MDMs. Consistent with previous works showing that basal NGF production by MDMs is very low (Achim and Wiley, 1996; Garaci *et al*, 1999; Samah *et al*, 2008; Soontornniyomkij *et al*, 1998), we did not detect any neurotrophin in MDM supernatants by ELISA (limit of detection: 10 pg/ml), although cell-associated neurotrophin tested positive by immunocytochemistry (Samah *et al*, 2008).

In our model, HIV replication did not significantly modify neurotrophin and neurotrophin receptor gene expression. These data are consistent with previous findings (Harrold *et al*, 2001) but also differ from the ones of Garaci *et al*. who described a HIV-induced autocrine loop involving NGF, TrkA, and $p75^{NTR}$ driving both HIV-1–infected macrophage survival and virus production (Garaci *et al*, 1999). We further assessed the possibility that neurotrophins may affect HIV replication and HIV–infected cell viability in our system, by exogenous neurotrophin treatment and alternatively by experiments using blocking antibodies. Again, both the level of HIV production and cell viability were independent on neurotrophin signaling. The discrepancy between our data and the ones of Garaci *et al* may not be due to HIV strain, as we both used the Ba-L strain of HIV-1. We have also performed experiments using adherence for monocyte sorting, and the results were not different from elutriation (data not shown). The NGF autocrine loop that Garaci *et al* (1999) described thus deserves further investigations.

Our result showing an increase in CD184 expression but no modification in CD4 and CD195 in NGF-stimulated MDMs can classify NGF apart from the other neurotrophins. This result is supported at the gene expression level by Harold *et al*. (2001) who showed enhanced CD184 and decreased CD195 RNA expression in NGF-treated macrophages by semi-quantitative RT-PCR. Our result demonstrating that NGF sensitizes monocytes to suboptimal CXCL-12 doses and attracts them (Samah *et al*, 2008; and the present data) is consistent with Kobayashi and Mizisin (2001) who showed an increased monocyte transmigration in response to NGF and NT3. NGF may accordingly participate in the chronic inflammatory activation of the brain parenchyma. In contrast, we show here that NGF treatment decreased HIV-1 production in migrated monocyte cultures, suggesting a beneficial role of NGF against HIV passage into the brain. As it did not modify virus production in input cells, i.e., the very same cells that were used for transmigration assay, but directly seeded in culture, the decrease in virus progeny would rather provide evidence for a selective negative effect of NGF on HIV-infected monocytes, as opposed to its positive effect on noninfected monocytes. CXCL-12 is broadly expressed in the CNS, where the hippocampus is its primary site of expression (Tham *et al*, 2001). In this context, the increased expression of NGF in perivascular spaces during neuroAIDS (Boven *et al*, 1999) is striking as it may contribute to an increase in CXCL-12 binding sites, as well as the attraction of activated monocytes into these spaces. This is a hallmark of HIV-induced chronic inflammation of the brain (Persidsky *et al*, 1999). Boven *et al*. (1999) also showed that perivascular macrophages and multinucleated giant cells are themselves the cellular sources of NGF in HIV encephalitis (HIVE). It is possible that the increase in perivascular expression of NGF arises from the afflux of macrophages at these locations but also from the increased NGF production per cell. The positive staining of cell-associated NGF that we previously described in our model (Samah *et al*, 2008) is nevertheless consistent with the macrophagic origin of NGF in HIVE.

On the other hand, De Simone *et al*. recently reported a chemotactic activity of NGF toward differentiated microglial cells, which was regulated by transforming growth factor (TGF)-β (De Simone *et al*, 2007). Given that TGF-β is also up-regulated in astrocytes in perivascular spaces during HIVE (Boven *et al*, 1999), further studies would determine how TGF-β and NGF may interplay with CXCL-12 for monocyte attraction.

In conclusion, we found that HIV replication in MDMs and infected MDM survival may not depend on neurotrophin signaling. On the other hand, NGF increases monocyte migration in response to CXCL-12, but exerts a clearcut repression of HIV replication in migrating monocyte populations. Accordingly, NGF may take an active part in the continuous influx of activated monocytes through the blood-brain barrier but also largely limit HIV penetration into the brain.

Materials and methods

Human monocyte isolation and differentiation

Monocytes were isolated from buffy coats from healthy seronegative donors by density gradient centrifugation and countercurrent elutriation as previously described (Figdor *et al*, 1983). Monocyte preparations were more than 95% pure, as assessed by flow cytometry (data not shown). Monocytes were seeded into 75-cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM)-glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal calf serum (Bio West, Nuaillé, France) and 1% antibiotic mixture (penicillin-streptomycin-neomycin, 100 ×; Invitrogen). Macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 and 1 ng/ml, respectively) purchased from Abcys (Paris, France) were included in the medium from days 0 to 6. These relative concentrations of cytokines maintained a neutral environment with respect to activation marker expression (HLA-DR, CD163, CD206), which remained similar to that of MDM cultured in medium alone (data not shown). In these conditions, cell survival during differentiation was good. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Blood monocytes adhered to plastic after 1 h, spontaneously detached after 48 h, and retained a monocyte-like

appearance for 5 days. After 3 days, monocytes were washed with phosphate-buffered saline (PBS), and dispensed into 48-well plates (3×10^5 cells/well) in M-CSF– and GM-CSF–supplemented medium. On days 5 to 6, cells were washed and fresh medium was added without growth factors. On days 7 to 8, mature cells were stimulated with neurotrophins and/or infected.

Viruses, infection, and quantification of HIV-1 replication

We used the R5 strain HIV-1/Ba-L (Gartner *et al*, 1986) and the X4 strain HIV-1/Lai (Barre-Sinoussi *et al*, 1983). These viruses were amplified in vitro using only human PHA-activated peripheral blood mononuclear cells (PBMCs). Clarified cell-free PBMC culture supernatants were ultracentrifuged at $360{,}000 \times g$ for 10 min at 4°C just prior to use to eliminate soluble factors such as cytokines and avoid nonspecific modulations. MDMs were infected at a multiplicity of infection (m.o.i.) of 0.1 at days 7 to 8 of culture. At day 1 post infection (p.i.), cells were thoroughly washed to remove residual virus. Supernatant was collected twice weekly until day 18 p.i. and stored at −20°C to measure reverse transcriptase (RT) activity using a commercial kit (RetroSys; Innovagen, Lund, Sweden). RT activity values were corrected according to the results of an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay to normalize data with respect to cell number.

Recombinant cytokines and biologically active substances

Recombinant human NGF, BDNF, NT3, NT4 were purchased from R&D Systems (Minneapolis, MN) and used at 4 ng/ml for NGF, 20 ng/ml for brain-derived neurotrophic factor (BDNF), 60 ng/ml for NT3, and 30 ng/ml for NT4 as recommended by the manufacturer.

Antibodies

Neutralizing mouse anti-NGF and anti-NT3 monoclonal antibodies were purchased from R&D systems. Neutralizing sheep anti-BDNF and anti-NT4 polyclonal antibodies were purchased from Abcys (Paris, France).

According to manufacturers' instructions, we used anti-NGF and anti-NT3 at 1 μg/ml, anti-BDNF at 1:10 dilution, and anti-NT4 at 1:200 dilution. Irrelevant matched antibodies were used at the same concentrations as controls.

Real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR)

Gene expression levels were measured by real time quantitative RT-PCR as already described (Porcheray *et al*, 2006), using previously described primers (Samah *et al*, 2008). Briefly, RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was treated with 5 U RNase-free DNase (Invitrogen) for 15 min at room temperature, and DNase was then inactivated by heating for 5 min at 95°C. RNA was reverse-transcribed in optimal conditions, as previously defined. PCR reactions were performed in the ABsolute qPCR SYBR Green Fluorescein Mix (ABgene, Epsom, UK), in an Icycler apparatus (Bio-Rad). Primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research) (Rozen and Skaletsky, 2000), and the PCR conditions were determined to obtain a linear amplification of a single amplicon at the expected size. A melting curve analysis of the PCR products was performed after each amplification to further verify the absence of nonspecific signals. GAPDH gene was used as a housekeeping gene (for GAPDH primer sequence, see Porcheray *et al,* 2006). Each measurement was performed in duplicate, and the accepted C_T variation between replicates was less than one cycle.

Flow-cytometric analysis of cell surface molecule expression

MDMs were washed with PBS and detached from the plastic by thorough scraping with a rubber policeman. Nonspecific sites were saturated by incubation at 4°C for 30 min with 10% heat-inactivated normal human AB serum (Sigma, Saint Louis, MO) in PBS. Cells (5×10^5/test) were then incubated for 30 min at 4°C with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) against human CD4, CD195 (CCR5), CD184 (CXCR-4), or irrelevant isotype-matched controls (BD Biosciences, Mountain View, CA) in PBS. The cells were then washed twice with cold PBS, fixed in 200 μl CellFix (BD Biosciences), and their fluorescence was assessed with an LSR flow cytometer (BD Biosciences). Viable cells were gated using forward and side light-scatter patterns. The mean equivalent fluorochrome bound per cell (MEF) was determined with the Fluorosphere kit (Dako, Glostrup, Denmark). This made it possible to eliminate variation due to cytometer settings and day-to-day performance variability, as well as differences in stimulation dependent autofluorescence levels.

ELISA

We quantified NGF, BDNF, NT3, NT4 proteins in cell culture supernatants using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Chemicon [Chandlers Ford, UK], Tebu-Bio [Le Perray en Yvelines, France], and R&D systems, respectively). To quantify p24 HIV-1 protein levels in cell culture supernatants, we used ELISA kit purchased from Zeptomatrix (New York, NY). All measurements were performed according to the manufacturer's instructions and in duplicate.

Viability assay
Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma). Briefly, cells were washed twice in PBS and incubated in the presence of 100 µl of MTT solution (5 mg/ml) for 2 h at 37°C. We then added 0.2 ml of extraction buffer (8% HCl in isopropanol). The solution was transferred to a 96-well plate, and optical density was measured at 540 to 630 nm. The optical density (OD) of blank wells (lacking cells, but prepared in the same way) were subtracted from the values obtained for the test wells. We therefore used the MTT assay in subsequent experiments to normalize data to a fixed number of cells. MTT assays were performed for every treatment on two additional replicates of each culture condition.

In vitro *monocyte chemotaxis assay*
Chemotaxis assay was carried out in duplicates as described (Badr *et al*, 2005, 2008) using peripheral blood mononuclear cells (PBMCs) and monocytes.

Freshly isolated PBMCs were first treated for 24 h with medium or NGF before infection with HIV-1/BaL (0.1 m.o.i.), and then cultured for an additional 24 h in NGF-containing medium. Stimulated/infected cells were then tested for chemotaxis. A total 6×10^5 cells in 150 µl of prewarmed culture medium were transmigrated through 5-µm pore size bare filter Transwell inserts (Costar, Cambridge, MA) for 3 h at 37°C in response to medium or 100, 300, or 600 ng/ml CXCL-12 (R&D systems).

After PBMCs were collected in the lower chamber, input and migrated cells were stained with CD14-FITC and CD16-PE (BD Bioscience) monoclonal antibodies (mAbs) prior to data collection. The cells were counted and analyzed for fluorescence with a LSR flow cytometer (BD Bioscience) for 52 s. After exclusion of cell debris and selection of the monocyte subpopulation by forward and side scatter gating, fluorescence analysis was performed, which allowed for discrimination and counting of $CD14^+/CD16^-$, $CD14^+/CD16^{dim}$, and $CD14^-/CD16^{bright}$ monocytes. Results are expressed as the percentage of migrated monocytes relative to the number of monocytes added at the start of the assay (input cells).

Freshly elutriated monocytes were subjected to exactly the same transmigration protocol, and then cells migrated in the lower chamber were maintained in culture plate until day 14 post infection. Culture medium was renewed every 3 to 4 days and p24 was measured.

Statistical analysis
Two-group comparisons were performed using two-tailed Student's *t* test. When the analysed data were percent of control, the two-tailed Student's *t* test for unequal variance was used. Student's *t* tests were performed using Excel 2003 software (Microsoft, Redmond, WA).

Transmigration data were analyzed using analysis of variance (ANOVA). Fisher possibly least significant difference (PLSD) *post hoc* test was performed for each effect. For CXCL-12 effect, with multiple groups due to different concentrations used, a Bonferroni-Dunn *post hoc* test was also applied to add a multiple-group analysis correction. ANOVA and *post hoc* tests were performed using StatView 5.0 software (Abacus, Berkeley, CA).

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