T-tropic human immunodeficiency virus (HIV) type 1 Nef protein enters human monocyte–macrophages and induces resistance to HIV replication: a possible mechanism of HIV T-tropic emergence in AIDS

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Increasing interest has been devoted to the role that monocyte–macrophages play in the pathogenesis of AIDS. The hypothesis of an involvement in AIDS pathogenesis of human/simian immunodeficiency virus (HIV/SIV) Nef also is currently under evaluation by many investigators. The original basis of this hypothesis came from evidence that monkeys infected with a nef-deleted SIV strain failed to develop simian AIDS. Here, we show that treatment of human monocyte-derived macrophages (MDM) with recombinant HIV-1 Nef protein (rNef) induces a strong inhibition of the replication of either macrophage (M- or dual-tropic HIV-1 strains. Through cytofluorimetric analyses, we detected internalization of FITC-conjugated rNef in MDM as early as 6 h after treatment. Confocal microscope observations demonstrated that the intracellular distribution of internalized rNef was identical to that of endogenously produced Nef. Down-regulation of the CD4 HIV receptor detected upon rNef treatment of MDM suggested that the rNef-induced HIV inhibition occurred at the virus entry step. This deduction was strengthened by the observation that CD4-independent infection was totally insensitive to rNef treatment. The specificity of all observed effects was demonstrated by immunodepletion of rNef. Finally, we showed that the resistance to HIV replication induced by rNef treatment in MDM favours the spread of T-tropic over M-tropic HIV strains in doubly infected CD4+ lymphocyte–MDM co-cultures. We propose that extracellular Nef contributes to AIDS pathogenesis by inducing resistance to M-tropic HIV replication in MDM, thereby facilitating the switching from M- to T-tropic HIV prevalence that correlates frequently with AIDS progression.

Introduction

Monocyte–macrophage cells play an essential role in immunological homeostasis as effector cells against microbes and tumours, as antigen-presenting cells (APC), and through the action of several types of cytokines and chemokines released upon activation. AIDS is now considered to be the result of deep immunological malfunctions and not to be due exclusively to the death of CD4+ lymphocytes directly induced as a result of their infection by human immunodeficiency virus (HIV). It is noteworthy that simian immunodeficiency virus (SIV)sm variants unable to replicate in macrophages in vitro showed an impairment in both replication and pathogenicity in vivo (Hirsch et al., 1998). While the possibility that HIV can replicate in human monocyte-derived macrophages (MDM) has been documented since 1986 (Gartner et al., 1986; Koenig et al., 1986), little information on the response of MDM to single HIV proteins is at present available. Recently, the release of macrophage inflammatory protein (MIP)-1α/-1β from MDM transduced by an adenoviral vector expressing Nef has been demonstrated (Swingler et al., 1999). The authors claimed that the releases of chemokines and still unidentified factor(s) induce, respectively, chemotaxis and activation of CD4+
lymphocytes, which consequently become competent for HIV replication (Swingler et al., 1999).

The nef gene is expressed very early and large amounts of Nef protein are synthesized during the HIV replication cycle. Expression of nef is critical for in vitro virus replication in resting peripheral blood lymphocytes (PBLs) (Aiken & Trono, 1995; Chowers et al., 1994; Miller et al., 1994; Spina et al., 1994) as well as for in vivo pathogenesis. A role for Nef therein has been deduced from results obtained in animal models, such as monkeys infected with nef-deleted SIV strains (Kestler et al., 1991) and nef-transgenic mice (Hanna et al., 1998). In addition, molecular studies on HIV isolates from seropositive cohorts indicated the possibility that detection of deleted/mutated nef genes represents a marker for non-progression (Brambilla et al., 1991; Deacon et al., 1995; Kirchhoff et al., 1995), although results inconsistent with this hypothesis have been published also (Huang et al., 1995; Michael et al., 1995). Nef typically induces down-regulation of both CD4 (Aiken et al., 1994; Bandres et al., 1995) and class I major histocompatibility complex (MHC) molecules (Schwartz et al., 1996) through mechanisms that have been described in great detail (Greenberg et al., 1998a; Piquet et al., 1999). Data regarding Nef functions have been obtained mostly by analysing the effects of the endogenously expressed protein. Conversely, relatively few data on the effects of extracellular Nef have been published so far. Indeed, Nef is a cytoplasmic protein that associates with membranes through N-terminal myristoylation (Allan et al., 1985): thus shedding of Nef from infected cells is not an obvious possibility. However, both antibodies (Abs) and cytotoxic T-lymphocytes against Nef have been found in a large proportion of infected individuals (Ameisen et al., 1989; Bahraoui et al., 1990). This suggests that in vivo Nef is processed and presented by APC, as the result of uptake of extracellular Nef possibly released by infected apoptotic cells. In this paper, we report that recombinant Nef protein (rNef) from the T-cell (T)-tropic NL4-3 HIV strain is able to enter human MDM and induce CD4 down-regulation. This correlates with a strong inhibition of HIV replication. We also show that rNef treatment of lymphocyte (T)- and macrophage (M)-tropic HIV doubly infected CD4+ lymphocyte–MDM cocultures led to preferential replication of the T-tropic HIV strain. These observations support the hypothesis that the HIV replication block induced by extracellular Nef in MDM could contribute to the switching from M- to T-tropic HIV strains frequently observed during AIDS progression.

**Methods**

**Cell cultures and lymphocyte activation assay.** Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat obtained from 20- to 40-year-old healthy male blood donors. Monocytes were isolated by 1 h adherence of PBMC followed by immunodepletion with anti-CD2, -CD3 and -CD19 monoclonal antibodies (mAbs) (Dako) coupled, in a second incubation step, with anti-mouse IgG Dynabeads (Dynal). The purity of recovered cell populations was assayed by FACS analysis with PE-conjugated anti-CD14 MAb (Becton Dickinson) labelling. Cell preparations staining below 95% positive for CD14 (a cell surface marker specific for monocyte–macrophage cell populations) were discarded. Monocytes were cultured in 48-well plates in RPMI 1640 (Life Technologies) supplemented with 20% heat-inactivated foetal calf serum (FCS).

Monocyteopoietic unilineage cultures were obtained from CD34+ human progenitor cells (HPC) purified by negative selection from PBMC of healthy donors as described (Chelucci et al., 1999). HPC were seeded at 105 cells/ml and induced to monocytic differentiation in 20% FCS medium containing 1 ng/ml interleukin-6, 500 U/ml monocyte-colony stimulation factor and 100 ng/ml FLT3-ligand. Morphology analyses were performed by cyto centrifugation onto glass slides and staining with May–Grunwald–Giemsa (Sigma).

PBMCs were obtained from PBMC adherence supernatants. Purified CD4+ lymphocytes were obtained by immunodepletion of PBMC with anti-CD8, -CD14 and -CD19 mAbs (Dako) followed by treatment with anti-mouse IgG Dynabeads. Lymphocyte expansion was performed by activating cells with 0.5 µg/ml phytohaemagglutinin (Sigma) and cultivating them in the presence of 100 U/ml interleukin-2 (Roche).

HLaCD4 and 293 cell lines were grown in Dulbecco’s modified minimum essential medium supplemented with 10% heat-inactivated FCS. HSB-2 (Ablashi et al., 1987), C8166 and CEMs cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS.

The lymphocyte activation assay was performed by cultivating CD4+ lymphocytes for 2 days in supernatants from rNef-treated MDM. Then, cells were washed, seeded at 3 x 105/ml in 100 µl RPMI–20% FCS in the presence of 2 µCi [methyl-3H]thymidine (2 Ci/mmol; Amersham) and incubated for 16 h at 37 °C. Finally, cells were washed, lysed in 1% SDS buffer and amounts of TCA-insoluble 3H-labelled macromolecules were determined by liquid scintillation counting.

**Virus preparations, infections and detection.** Both ADA and Bal virus preparations were obtained as supernatants of peripheral blood (PB) MDM infected 7 days after purification and plating. Both NL4-3 and 89.6 virus preparations were obtained as supernatants of 293 cells 48 h after transfection of respective molecular clones by the calcium phosphate method (Wigler et al., 1979). The T-tropic NL4-3 strain was titred by scoring the syncytium number on C8166 cells 5 days after infection (Federico et al., 1993). Infectivity titres of supernatants containing M- or dual-tropic HIV strains were evaluated by the limiting dilution method 10 days after infection of day 7 MDM. HIV p24 contents in supernatants were measured by quantitative ELISA (Abbott). Supernatants obtained 48 h after co-transfection of 293 cells with vectors expressing ADA HIV-1 and vesicular stomatitis virus envelope glycoprotein (VSV-G) (molar ratio 1:3) served as source of pseudotyped ADA virions. One ng/106 cells of either parental or pseudotyped ADA HIV-1 was used to infect day 7 MDM. Virus entry was evaluated 48 h thereafter by scoring the number of cells expressing HIV Gag-related products in cytofluorimetric analyses.

**rNef preparation and FITC labelling.** rNef was obtained as a 6 x His tagged fusion protein. The nef gene from HIV strain NL4-3 (Adachi et al., 1986) was amplified by PCR and cloned in-frame with the 6 x His tag into the 5' BamHI/5' SalI sites of pQE 30 vector (Qiagen). Sequences of oligoprimers used in PCR are 5' GTGATGATCCCTTTAGATGATTTTGCAATGGTGG 3' for the 5' end (to obtain rNef mutated in the myristoylation acceptor the underlined codon was changed to GCT, leading to a Gly → Ala substitution), and 5' GTCTGCTGACTCAGCATTTGGAGCTACTC 3' for the 3' end. The correct reading frame of the inserted fragment was checked by sequencing (Sequenase II kit, U8 Biochemical). rNef was purified from bacterial lysates by using Ni-NTA resin (Qiagen) following the manufacturer’s recommendations.
rNef was eluted stepwise with 100, 250 and 500 mM imidazole, and the fractions were collected and analysed by 12% SDS–PAGE. rNef-containing fractions were pooled and dialysed extensively against 1 × PBS to remove the urea completely. Finally, 10 μg aliquots of rNef were analysed by SDS–PAGE and found to be devoid of non-specific bands on Coomassie brilliant blue staining. Recombinant protein preparations were scored as negative for the presence of bacterial endotoxins by using the Limulus amoebocyte lysate assay. Preparation of 0 × His Uvp1 protein (a 21.5 kDa plasmidic DNA–RNA invertase–resolvase) was as previously described (Tosini et al., 1998).

Crystallized BSA and rNef were labelled by reacting each protein with dissolved FITC (Pierce) following the manufacturer’s recommendations. Gel filtration chromatography was then performed on the reaction products and recovery of FITC-conjugated protein evaluated by quantitative Western blot analysis as described (d’Aloja et al., 1998).

### rNef immunodepletion

To ensure a total and specific depletion of rNef, complete medium supplemented with 100 ng/ml rNef was incubated for 8 h at 4 °C with a 1:50 dilution of a cocktail containing six different mono- and polyclonal anti-Nef Abs (all obtained from the NIH AIDS Research and Reference Program). As a control, rNef-complemented medium was incubated with irrelevant isotype- and species-matched Abs. Then, immunocomplexes were reacted with detergent-free protein A–Sepharose beads (Sigma) overnight at 4 °C. Afterwards, immunocomplexes bound to protein A–Sepharose were discarded after centrifugation, and supernatants were filtered (0.22 μm pore diameter) and added to MDM cultures. The efficiency of rNef immunodepletion was checked by a quantitative Western blot analysis on the protein A–Sepharose fraction (not shown).

### FACS analyses and chemokine detection

Direct and indirect FACS analyses were performed as previously described (Baiocchi et al., 1997) by using a Becton Dickinson cytofluorimeter. Anti-CD4–PE, PE- or TRITC-conjugated anti-CD14, CD45–FITC, and class II MHC (HLA-DR)–FITC MAbs were obtained from Becton Dickinson. Anti-CXCR4 (clone 12G5) MAb was obtained from the NIH AIDS reagent program, and anti-CCR5 (clone 2D7)–PE MAb was from R&D Systems. All FACS analyses on monocyte–macrophage cells were performed after an Fc-blocking step, carried out by incubating cells for 15 min at room temperature with 10 μg/ml human IgG (Dako).

Percentages of cells expressing intracytoplasmic HIV-1 Gag-related products were evaluated by FACS analyses after treatment with Permeafix (Ortho Diagnostic) for 30 min at room temperature and labelling with a 1:50 dilution of KC57-RD1 PE-conjugated anti-HIV-1 Gag MAb (Coulter). For confocal microscope analyses, > 95% pure monocytes were seeded in complete medium on glass coverslips. After 7 days, medium was replaced and 50 μg/ml BSA–FITC in BSA-free medium or 100 ng/ml of either wild-type or mutated rNef–FITC in complete medium was added. Sixteen hours after treatment, cells were labelled for 1 h at 4 °C with anti CD14–TRITC MAb. Then, cells were fixed in 2% (v/v) formaldehyde buffer, observed, and images were analysed by using a CS4D confocal microscope (Leica).

ELISA kits for the detection of MIP-1α/-1β chemokines in supernatants were obtained from R&D Systems.

### Results

#### rNef treatment specifically induces an HIV replication block in PB monocyte–macrophage cultures

Human PB-derived monocytes were kept in culture for 7 days. Then, rNef from the T-tropic HIV strain NL4-3 was added at 10 or 100 ng/ml and incubated for 6 h. Afterwards, cultures were challenged with M-tropic HIV strains (Bal. or ADA) at m.o.i.s of 0·1 and 0·5. MDM cultures were washed and refed with medium containing appropriate rNef concentrations 24 h after infection and every 3–4 days thereafter. Finally, levels of released HIV particles in supernatants were evaluated in terms of amounts of p24 HIV protein at different days post-infection. As shown in Fig. 1(A), a dose-dependent inhibition of HIV BaL replication was observed in rNef-treated MDM cultures, whatever m.o.i. was used. In addition, a complete block of virus spread was observed after infecting rNef-treated MDM with HIV strain ADA at an m.o.i. of 0·1 (not shown). By increasing the m.o.i. to 0·5, a 1–1·5 log reduction in HIV p24 was detected in supernatants of rNef-treated MDM in comparison to untreated MDM (Fig. 1A). Importantly, cell viability was unaffected by rNef at the concentrations used, as assessed by neutral red viability assay (not shown). Furthermore, macrophage cells protected by rNef treatment appeared morphologically indistinguishable from uninfected cells, while typical cell enlargement, syncytia formation and cell death were detectable at later time-points in control infected cells (not shown). Withdrawal of rNef 7 days after infection did not substantially influence the inhibitory effect on HIV replication (not shown).

In order to demonstrate that the observed antiviral effect was specifically induced by rNef, MDM were cultivated in rNef immunodepleted medium after infection with HIV. The efficiency of immunodepletion was evaluated by Western blot analysis of the protein A–Sepharose-precipitated fraction (not shown). As shown in Fig. 1(B), panel I, immunodepletion prevented the inhibitory effect of rNef. This result indicates that the observed phenomena did not depend on the possible presence of contaminating bacterial products in rNef preparations. In order to exclude a possible contribution of the 6 × His tag experiments were repeated with MDM treated with a 6 × His-tagged irrelevant recombinant protein (Uvp1, a plasmid DNA invertase–resolvase) (Tosini et al., 1998). As shown in Fig. 1(B), panel II, amounts of Uvp1 equivalent to the 6 × His-tagged rNef used to observe the rNef-specific HIV inhibition were totally ineffective. Hence, we could exclude the possibility that the 6 × His tag has per se a negative influence on M-tropic HIV replication in MDM.

#### rNef is internalized by MDM

We asked how MDM interact with rNef, i.e. through binding of a specific receptor and/or cell internalization. MDM were incubated for 1 h at 4 °C with 100 ng/ml of FITC-labelled rNef and analysed by FACS. Fig. 2 shows that there was no rNef binding on MDM cell membrane, indicating the absence of a highly represented specific receptor recognizable by rNef. However, cells scored positive in FACS analysis as early as 6 h after the pulse at 37 °C. Levels of rNef internalization peaked 16 h after treatment (Fig. 2) and
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Fig. 1. (A) rNef treatment specifically induces HIV resistance in MDM. Amounts of HIV p24 in supernatants from MDM untreated (●) or treated with 10 (▲) or 100 (◆) ng/ml of rNef at different times after infection with HIV strain Bal at an m.o.i. of 0.1 (panel I) or 0.5 (panel II) or with HIV strain ADA at an m.o.i. of 0.5 (panel III) are shown. Data from one experiment representative of eight (I), five (II) and two (III) independent experiments are reported. (B) Amounts of HIV p24 in supernatants from MDM at different times after infection with HIV strain Bal (m.o.i. 0.1) and cultivation (panel I) in complete medium (●), in rNef-containing medium pre-treated with specific anti-Nef Abs (▲) or with isotype- and species-matched irrelevant Abs (◆), or (panel II), in complete medium (●), in the presence of 100 ng/ml of rUvp1 (▲) or in the presence of 100 ng/ml of rNef (◆) are shown. Data from one representative experiment of three (I) and two (II) are reported.

progressively declined thereafter (not shown). We reproduced these results on both 7-day-old (Fig. 2A) and 14-day-old (Fig. 2B) MDM cultures. In general, we observed a more efficient rNef uptake in the less-aged MDM cultures. Very similar results were obtained by using day 14 HPC-derived monocytoid cultures (not shown). Conversely, neither binding nor internalization of rNef were observed in CD4+ lymphocytes, either quiescent or activated by supernatants from rNef-treated MDM (not shown).

Studies performed by transiently transfecting Nef–green fluorescence protein (GFP) expression vectors have demonstrated that N-terminal myristoylation allows Nef to localize in a punctate pattern, predominantly in perinuclear regions and at the cell margin (Greenberg et al., 1998 b). In order to determine the intracellular localization pattern of internalized rNef, confocal microscopy analyses were carried out. MDM were treated for 16 h with BSA–FITC (Fig. 3A) or rNef–FITC (Fig. 3B), and then labelled for 1 h at 4 °C with a TRITC-conjugated MAb recognizing an extracellular epitope of the CD14 molecule. We observed that rNef was indeed internalized and localized in an intracytoplasmic pattern highly reminiscent of that described for cells endogenously expressing Nef (Fig. 3B, C). Of note, signals from both an anti-CD14 TRITC MAb and rNef–FITC were coincident in discrete regions (Fig. 3B). Considering that rNef–FITC apparently failed to bind the outer side of the MDM cell membrane (see above), it is likely that the localization of internalized rNef at the inner side of the cell membrane was the basis of the coincident signals. The evidence that rNef localized both in an intracytoplasmic punctate pattern and at the cell margin suggests that at least part of the internalized rNef undergoes myristoylation. In order to prove such an hypothesis, confocal microscope observations were carried out after treatment of MDM with an FITC-conjugated rNef mutated in the acceptor signal for myristoylation (∆myr-rNef). As shown in Fig. 3(E, F), ∆myr-rNef retains the ability to enter MDM but the typical punctate pattern observed in wild-type rNef–FITC-treated MDM was no longer detectable. More importantly, the absence of yellow spots at the cell membrane (as reproducibly observed by analysing more than 500 ∆myr-rNef-positive cells), indicates...
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that the rNef mutant localized at the cell margin far less efficiently than wild-type rNef. These data are in agreement with those already published on endogenously expressed Nef (Greenberg et al., 1998b), and suggest that myristoylation is important for the localization of rNef at the cell margin.

HIV receptors: MDM down-regulate CD4 upon rNef treatment

In order to investigate the mechanism of HIV inhibition induced by rNef, we first analysed the effects of internalized rNef in terms of HIV receptor and co-receptor expression. It has been reported that Nef expressed either by infecting virus or by transfected vector is able to induce CD4 down-regulation (Aiken et al., 1994; Piguet et al., 1999). MDM cultures were treated with rNef at the most effective concentration for blocking HIV replication, and CD4 expression was evaluated 6 and 16 h thereafter. Treatment with rNef induced CD4 down-regulation in both 7-day-old (Fig. 4 A) and 14-day-old (Fig. 4 B) MDM. Significantly, similar results were obtained by treating HPC-derived monocytopenic cultures at day 14 (Fig. 4 C), 18 and 22 (not shown) of differentiation/maturation. In general, CD4 down-modulation appeared stronger in less-aged cell cultures where, consistently, more efficient rNef internalization was observed (see above). The specificity of the effect on CD4 expression was checked by a 16 h treatment of 7-day-old MDM with rNef-immunodepleted medium (Fig. 4 D).

We also tested a possible influence of rNef treatment on expression of the major HIV co-receptors (i.e. CXCR4 and CCR5) (Alkhatib et al., 1996; Choe et al., 1996; Cocchi et al., 1995; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). FACS analyses showed no variations in the expression of either co-receptor. This was observed consistently in both rNef-treated MDM and HPC-derived monocytopenic cultures, irrespective of the age of the culture (not shown). Expression of other membrane markers (i.e. CD14, CD45 and HLA-DR) was also unaffected by rNef treatment (data not shown), indicating that rNef does not induce non-specific receptor down-modulation in MDM.

rNef treatment of MDM induces CD4-dependent inhibition of HIV entry

We observed that rNef treatment of MDM induces a CD4 down-regulation correlating with a strong impairment of HIV replication. Thus, a block at the virus entry step could be envisaged. In order to determine more specifically the point of action of the rNef-induced HIV inhibition, we utilized a CD4-
Fig. 3. For legend see facing page.
Fig. 4. Treatment with rNef specifically induces CD4 down-regulation in monocyte-macrophages. (A, B) Levels of CD4 expression (panels III) as measured by FACS analyses of 7-day-old (A) and 14-day-old (B) MDM, untreated (lines a) or treated for 6 h (lines b) and 16 h (lines c) with 100 ng/ml of rNef are reported. (C) Levels of CD4 expression (panel III) as measured by FACS analyses of HPC-derived monocytopoietic cultures on day 14 of maturation/differentiation, untreated (line a) or treated for 16 h with 100 ng/ml of rNef (line b) are shown. In all analyses, mean fluorescence intensities of cells treated with isotype-matched IgG–PE did not exceed 1.5. Panels (I) show cell populations selected for analyses. Panels (II) report levels of CD14 expression in the analysed cell populations (M1: range of CD14 positivity). (D) Levels of CD4 expression as measured by FACS analyses of 7-day-old MDM untreated (lane b) or treated for 16 h with 100 ng/ml of rNef-complemented medium, before (lane c) or after addition of anti-Nef Abs (lane d) or isotype- and species-matched irrelevant Abs (lane e) and removal of immunocomplexes with protein A–Sepharose. Lane a, histogram of fluorescence intensity from MDM labelled with PE-conjugated isotype-matched IgG.

Fig. 3. rNef internalized by MDM localizes in an intracytoplasmic punctate pattern and at the cell margin in a myristoylation-dependent manner. Confocal microscope analyses of 7-day-old MDM 16 h after treatment with 50 µg/ml of BSA–FITC in BSA-free medium (A), with 100 ng/ml of rNef–FITC (B) or with Δmyr-rNef–FITC (E) in complete medium and 1 h labelling at 4 °C with an anti-CD14 TRITC MAb are shown. Images (B) and (E) were also separately analysed by means of FITC (C, F) or TRITC (D, G) fluorescence. Arrows indicate the most evident coincident signals from rNef–FITC and the anti-CD14 TRITC MAb.
Fig. 5. HIV pseudotyping is sufficient to overcome the block imposed by rNef treatment. Seven-day-old MDM were treated or not for 16 h with 100 ng/ml of rNef and then infected with either (VSV-G) ADA HIV or the parental HIV strain (1 ng per 10^5 cells, corresponding approximately to an m.o.i. of 0.2). After 2 h adsorption, cells were washed extensively and cultured for 48 h. Afterwards, the extent of virus entry was evaluated by means of FACS analyses for the presence of intracellular HIV Gag-related products. Quality control of pseudotyped virus preparations was performed by infecting a CD4^-human lymphoblastoid cell line (HSB-2). Percentages of HIV Gag-positive cells reported are from one experiment representative of two independent experiments.

It has been reported recently that MDM transduced with a Nef expression vector are induced to release MIP-1α and MIP-1β chemokines (Swingler et al., 1999). Consistently, we detected an increase in release of these chemokines in supernatants of rNef-treated MDM (Table 1). In order to test whether masking of CCR5 HIV co-receptors through binding with chemokines secreted by MDM upon rNef treatment could play a role in the observed HIV inhibition, we repeated HIV challenge experiments by using the 89.6 HIV strain. This is a dual-tropic HIV strain that uses CCR5 and/or CXCR4 as co-receptors to enter MDM (Yi et al., 1998). To avoid possible interference by activated lymphocytes that may contaminate MDM isolated from PB, the infection experiments were performed on day 14 HPC-derived monocytopoietic cultures. These are unilineage cell cultures more than 99% pure, containing less than 1% of neutrophilic granulocytes (an irrelevant cell type in terms of HIV infection) (Chelucci et al., 1999) as the only detectable contaminants. As shown in Table 2, rNef treatment induced a strong dose-dependent inhibition of HIV 89.6 replication also. No measurable levels of stromal derived factor-1 (the natural ligand for CXCR4) (Oberlin et al.,

### Table 1. Amounts of MIP-1α and MIP-1β released in supernatants of monocyte–macrophage cells treated with 100 ng/ml of rNef

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<thead>
<tr>
<th>Cell type</th>
<th>MIP-1α</th>
<th>MIP-1β</th>
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<tr>
<td></td>
<td>Ctrl</td>
<td>rNef</td>
</tr>
<tr>
<td>PB MDM</td>
<td>0.45</td>
<td>7.47</td>
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<tr>
<td>HPC-derived monocytes</td>
<td>0.23</td>
<td>10.8</td>
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Chemokine contents of clarified supernatants of 10^6 MDM (day 7) or HPC-derived monocytes (day 14) treated with rNef for 16 h or left untreated were determined. For PB MDM, chemokine contents were also measured after treatment with rNef-complemented medium and addition of either anti-Nef or isotype- and species-matched irrelevant antibodies (Abs), and removal of immunocomplexes with protein A-Sepharose. Data are expressed in ng/ml and are from one representative of two (for MDM) and three (for HPC-derived monocytopoietic cultures) independent experiments. ND, Not done.

### Table 2. Amounts of p24 HIV in rNef-treated HPC derived monocytopoietic cultures infected with the dual-tropic HIV strain 89.6

Day 14 HPC-derived monocytopoietic cultures treated or not with rNef were infected at an m.o.i. of 0.1 with HIV strain 89.6. Supernatants were collected 10 days post-infection and HIV p24 amounts measured. Values were calculated as pg/ml normalized for 10^6 cells. Results are representative of two independent experiments.

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<tr>
<th></th>
<th>Ctrl</th>
<th>10 ng/ml rNef</th>
<th>100 ng/ml rNef</th>
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<tr>
<td>HIV p24</td>
<td>912</td>
<td>280</td>
<td>&lt; 25</td>
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It has been reported recently that MDM transduced with a Nef expression vector are induced to release MIP-1α and MIP-1β chemokines (Swingler et al., 1999). Consistently, we detected an increase in release of these chemokines in supernatants of rNef-treated MDM (Table 1). In order to test whether masking of CCR5 HIV co-receptors through binding with chemokines secreted by MDM upon rNef treatment could play a role in the observed HIV inhibition, we repeated HIV challenge experiments by using the 89.6 HIV strain. This is a dual-tropic HIV strain that uses CCR5 and/or CXCR4 as co-receptors to enter MDM (Yi et al., 1998). To avoid possible interference by activated lymphocytes that may contaminate MDM isolated from PB, the infection experiments were performed on day 14 HPC-derived monocytopoietic cultures. These are unilineage cell cultures more than 99% pure, containing less than 1% of neutrophilic granulocytes (an irrelevant cell type in terms of HIV infection) (Chelucci et al., 1999) as the only detectable contaminants. As shown in Table 2, rNef treatment induced a strong dose-dependent inhibition of HIV 89.6 replication also. No measurable levels of stromal derived factor-1 (the natural ligand for CXCR4) (Oberlin et al.,
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Fig. 6. rNef treatment of CD4+ lymphocyte–MDM co-cultures induces preferential replication of T- over M-tropic HIV strains. (A) Flow diagram of the setup of co-culture experiments. (B) Purified CD4+ lymphocytes were cultivated for 2 days in medium conditioned with rNef-treated MDM, and thereafter infected with HIV BaL strain (calculated m.o.i. 0.02). After an additional 3 days, CD4+ lymphocytes were either (panel I) cultivated for an additional 7 days, and M-tropic HIV infectious units in supernatants measured, or (panel II) superinfected with NL4-3 at an m.o.i. of 0.001 and, after 1 h of virus adsorption, washed extensively. Then, autologous co-cultures were set up in 0.3 ml RPMI–20% FCS by seeding 10⁶ doubly infected lymphocytes with 5 x 10⁴ MDM untreated (white bars) or pre-treated for 6 h (black bars) with 100 ng/ml of rNef. Co-culture supernatants were collected 7 days after NL4-3 challenge, clarified and infectious units (IU) of NL4-3 or BaL measured. Results were obtained for cells from four different healthy donors (I–IV). Panel (III), infectious units of NL4-3 or ADA as measured in supernatants of CD4+ lymphocyte–MDM autologous co-cultures set up as described in (B), panel (II), except that HIV ADA was used as the M-tropic infecting strain. Data were obtained for cells from three additional healthy donors (V–VII).
1996) were detected in supernatants of rNef-treated cultures (not shown). This result runs against a major role of chemokine-mediated CCR5 blocking in the rNef-induced HIV resistance observed in MDM.

rNef treatment of MDM favours the replication of T- over M-tropic HIV strains in co-cultivated CD4+ lymphocytes

The induction by extracellular Nef treatment of resistance of MDM to HIV replication could represent a factor that severely limits the spread of HIV strains targeting these cells in vivo (i.e. M- and dual-tropic HIV). Attempting to define possible pathogenic implications of our experimental observations, we questioned whether such resistance could in some way favour the replication of T-tropic versus M-tropic HIV strains when MDM are co-cultivated with autologous CD4+ lymphocytes infected with both types of HIV strain. Thus, co-culture experiments were set up as summarized in Fig. 6(A). We treated purified, quiescent CD4+ lymphocytes from four different donors with supernatants from rNef-treated MDM. Consistent with data from Swingler et al. (1999), we noticed activation of quiescent CD4+ lymphocytes, as revealed by [3H]thymidine incorporation assay (not shown). After 48 h, CD4+ lymphocytes were infected with the M-tropic HIV strain Bal at an m.o.i. of 0.02. We observed that this treatment allowed the BaL strain to replicate in CD4+ lymphocytes (Fig. 6B, panel I). After an additional 3 days, the infected CD4+ lymphocytes were superinfected with the T-tropic NL4-3 strain by using a dose (i.e. m.o.i. 0.001) 20-fold lower than that used for M-tropic challenge. Then, co-cultures with autologous, rNef-treated MDM were set up and maintained for an additional 7 days. The relative titres of either M- or T-tropic infectious viral particles were measured in supernatants collected at days 4 and 7 from the co-culture. While results at day 4 probably reflected an intermediate condition (not shown), data obtained at day 7 were more clearly interpretable (Fig. 6B, panel II). Titres of M-tropic HIV in day 7 supernatants from the four co-cultivations in the presence of rNef were significantly and consistently reduced with respect to those in untreated co-cultures, whereas relative titres of T-tropic HIV were considerably higher (Fig. 6B, panel II). Absolute increases in titres of NL4-3 in supernatants of rNef-treated with respect to untreated co-cultures were 27-, 25-, 3- and 18.6-fold for the four different donors. Consistently, titres of M-tropic infectious viral particles showed decreases of 2.2-, 3.7-, 7.8- and 60-fold in the supernatants from the four different rNef-treated compared to untreated co-cultures (Fig. 6B, panel II). All these results were fully reproduced by using ADA as the M-tropic HIV strain for cells isolated from three additional healthy donors (Fig. 6B, panel III).

As we failed to detect direct interactions between rNef and CD4+ lymphocytes, these results indicate that favoured replication of T- over M-tropic HIV strains was a result of effects caused by rNef on MDM.

Preferred replication of T-tropic HIV correlates with the presence of MDM

We established that replication of T-tropic over M-tropic HIV strains is favoured in CD4+ lymphocyte–MDM co-cultures in the presence of rNef. This phenomenon may be the consequence of a block of M-tropic HIV replication in rNef-treated MDM or, alternatively, of the effect(s) of soluble factors (e.g. chemokines; see Table 1) released by rNef-stimulated MDM. Of note, enhancement of T-tropic HIV replication induced in lymphocytes by chemokine treatment has been described previously (Dolei et al., 1998; Kinter et al., 1998). In order to define better the mechanism of T-tropic preference in rNef-treated co-cultures, CD4+ lymphocytes were doubly infected as described above and cultivated in the absence of MDM but in the presence of medium conditioned by autologous MDM previously treated or not with rNef. Seven days after NL4-3 challenge, supernatants were collected and titres of M- and T-tropic viruses were determined. Neither reproducible variations in M-tropic HIV nor increases in T-tropic HIV amounts were detected (data not shown). This result supports the idea that preferential replication of T-over M-tropic HIV in rNef-treated CD4+ lymphocyte–MDM co-cultures is the consequence of a block in HIV replication induced in MDM.

Discussion

The observation that rNef is efficiently internalized by human MDM led us to evaluate its effects on these cells. Here, we demonstrate that replication of either M- or dual-tropic HIV strains in MDM is severely impaired after treatment with rNef.

It has been shown that rNef treatment of CD4+ replicating T-cells induces inhibition of cell growth and, upon cross-linking through anti-Nef Abs, apoptosis (Fujii et al., 1996; Okada et al., 1997). We did not detect anticellular effects with a large range of rNef doses (from 0.05 to 5 µg/ml) on both lymphocyte and monocyte cell populations. As our findings were obtained mostly in non-replicating cell populations, it may be inferred that anticellular effects of Nef are addressed mainly to events involved in cell duplication.

It has been reported that a Nef peptide (aa 123–160) binds class II DR antigen on Raji cell membrane (Torres & Johnson, 1994). However, in our experiments, no binding with membrane of either monocyte–macrophages or lymphocytes was observed. This appears consistent with data reported by Okada et al. (1997) who demonstrated Nef binding in lectin-stimulated lymphocytes only. It is conceivable that, in our conditions, cells did not expose levels of class II DR and/or possible accessory molecules sufficient to allow Nef binding. However, we cannot formally exclude possible low-level expression of a potential cell-surface Nef receptor that could not be revealed by FACS analysis on rNef–FITC-treated cells.
The evidence that rNef apparently does not bind MDM at 4 ºC suggests that rNef crosses the MDM cell membrane by exploiting the constitutive endocytic MDM machinery. Quantitative differences in rNef uptake between 7- and 14-day-old MDM cultures could be related to decreased pinocytic/phagocytic activity in older cell cultures.

We observed that CD4 exposure is down-regulated by rNef treatment. This result reproduced a typical effect widely observed in CD4+ cells endogenously expressing Nef (Aiken et al., 1994; Anderson et al., 1994; Bandres et al., 1995; Garcia & Miller, 1991; Greenway et al., 1994; Rhee & Marsh, 1994). Importantly, immunodepletion of rNef prevents the negative effect on MDM CD4 expression and, at the same time, induces virus rescue in infected MDM. However, we cannot formally exclude the possibility that as yet unidentified soluble factor(s) induced by rNef treatment may cooperate with rNef action in down-regulating MDM CD4. Infection with pseudotyped HIV allowed us to identify virus entry as the critical step involved in rNef-induced virus restriction.

rNef was obtained by bacterial recombinant technology, and thus it was not N-terminally myristoylated. However, the typical intracytoplasmic punctate pattern and the location of rNef–FITC at the cell margin, clearly detectable in confocal analyses of MDM, are reminiscent of observations for endogenously expressed GFP–Nef (Greenberg et al., 1998b), and could be suggestive of intracellular myristoylation of rNef. The hypothesis that rNef reaches the cell membrane after myristoylation was supported by the fact that internalized rNef–FITC mutated in the myristoylation signal failed to locate to the cell margin.

By use of doubly infected CD4+ lymphocyte–MDM co-cultures, we tried to reproduce events occurring in lymph nodes of seropositive patients when CD4+ lymphocytes prevalently replicating M-tropic HIV quasispecies interact with both infected and uninfected infiltrating monocyte–macrophages. We reasoned that the HIV replication block induced in MDM by extracellular Nef could limit strongly spread of M-tropic virus, normally sustained by the ability to target both lymphocytes and MDM. Conversely, no inhibitory effects should be observed in the replication of HIV T-tropic strains which are unable to enter MDM. Our data appear in line with such an hypothesis. Increased amounts of a T-tropic HIV strain in rNef-treated co-cultures could be the consequence of enhanced availability of target cells in conditions where M-tropic replication is severely impaired in MDM. Of note, we evaluated the replication efficiency of T-tropic strains in activated CD4+ lymphocytes as being approximately 20-fold greater than that of M-tropic strains (unpublished observations).

The detection of anti-Nef Abs in sera from AIDS patients (Ameisen et al., 1989; Bahraoui et al., 1990) was considered to be an indication that Nef is present in vivo in an extracellular form. This hypothesis was confirmed by Fujii et al. (1996) who detected free Nef antigen by ELISA in a majority of HIV patients. Even if here we describe effects mediated by doses of rNef (10–100 ng/ml) somewhat exceeding those detected in patients’ sera (1–10 ng/ml) (Fujii et al., 1996), significantly higher Nef concentrations would conceivably be found in those microenvironments (e.g. lymph nodes) where macrophages and infected lymphocytes tightly interact. However, whether there is a specific secretion pathway for unmyristoylated Nef or the presence of extracellular Nef in vivo is the consequence of lysis of dead infected cells remains a matter of question. Also, effects on MDM of already myristoylated Nef possibly released by HIV-infected cells should be investigated.

It is conceivable that factors released in vivo by MDM as a result of an inflammatory stimulus (either dependent or not on HIV infection) attract and activate both infected and uninfected CD4+ lymphocytes that can be induced to productive HIV replication and cell death. Nef released by dead cells may be internalized by uninfected macrophages that, as a consequence, are induced to produce additional amounts of HIV stimulating factors (included chemokines) becoming, at the same time, resistant to HIV. This mechanism may be at least in part the basis of the switch from M- to T-tropic HIV strains (Cheng-Mayer et al., 1988; Fauci, 1996; Schellekens et al., 1992; Schuitemaker et al., 1992; Tersmette et al., 1989) frequently observed in seropositive patients progressing to AIDS.

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