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The *nef* Gene Controls Syncytium Formation in Primary Human Lymphocytes and Macrophages Infected by HIV Type 1

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ABSTRACT

***nef*, the 3′-most open reading frame of HIV, has been reported to enhance HIV replication in various host cell types and to promote *in vivo* replication and pathogenesis. The mechanism underlying the increased *in vivo* viral replication is still unclear. We have examined the effect of a *nef* deletion on the infection of primary human CD4+ T lymphocytes and macrophages, using clones with *nef* and *env* sequences derived, respectively, from T cell- and macrophage-tropic viruses. The deletion of *nef* enhanced the formation of syncytia in CD4+ T lymphocytes infected with macrophage-tropic clones, despite a severalfold reduced viral production. No such enhancement of syncytium formation was observed in CD4+ T lymphocytes infected with a T cell line-tropic clone, but in this clone, the deletion of *nef* imparted a more severe replication defect. A similar increase in syncytium formation was observed in primary human macrophages infected with *nef*-deleted clones compared with wild-type counterparts, except under conditions in which the deletion of *nef* markedly reduced viral replication. We could not demonstrate an enhanced cell surface expression of HIV-1 envelope in lymphocytes infected with *nef*-deficient clones to explain the increased syncytium formation. In enhancing the HIV-1 cytopathic effect, the deletion of *nef* might curtail virus production by infected cells, and thus explain in part the reduced viral load observed *in vivo* in hosts infected with *nef*-deficient viruses.**

INTRODUCTION

THE *nef* GENE is the 3′-most open reading frame (ORF) of HIV, overlapping with the 3′ long terminal repeat (LTR). It is present in all primate lentiviruses, suggesting an important functional role during *in vivo* infection. It encodes a 27-kDa myristoylated protein, which is among the earliest proteins expressed during HIV replication in cell culture.[1] The importance of *nef* has been confirmed by studies with SIV in rhesus macaques, which showed that an intact *nef* ORF was required for the virus to establish a high viral load and to cause disease in animals.[2] Similar evidence that HIV-1 *nef* is required for *in vivo* replication and pathogenicity was obtained using the SCID-hu mouse model.[3,4] In addition, a cluster of patients infected by a common blood product donor, who all remain disease free and with normal CD4 T cell counts 10–14 years after donation, has been found to be infected by HIV-1 quasispecies with various deletions of *nef* sequences extending into the U3 region of the long terminal repeat. This further suggests a role for *nef* in HIV pathogenesis as well.[5]

How *nef* promotes *in vivo* HIV replication and pathogenesis is still unclear. *In vitro*, *nef* promotes HIV replication in primary lymphocytes,[6–9] an effect best revealed when resting peripheral blood lymphocytes are infected prior to stimulation.[8] *nef* has also been found to promote HIV replication in macrophages.[9–11]

The different functions of *nef*, namely, increased infectivity of virions,[9,12] CD4 antigen surface downregulation,[13–15] and interference with T cell signal transduction pathways,[1,15] may all play a role in promoting HIV replication *in vitro* and *in vivo*.

In the present study, we have systematically examined the effect of a *nef* deletion on the infection of primary human CD4+ T lymphocytes and macrophages using clones with *nef* and *env* sequences derived, respectively, from T cell- and macrophage-tropic viruses. We observed that the effect of *nef* on HIV replication varied according to the host cell type and the viral ge-

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netic background. In addition, we observed that the deletion of the *nef* gene enhanced the capacity of HIV to induce syncytium formation under the majority of experimental conditions—all but those in which the deletion of *nef* imparted the most profound replication defect.

MATERIALS AND METHODS

Cells

COS-7 cells (ATCC CRL 1651), obtained from P. Walker (Centre Hospitalier Universitaire Vaudois [CHUV], Lausanne, Switzerland) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Paisley, Scotland) with 10% fetal bovine serum (FBS; PAA, Linz, Austria). Primary $CD4^+$ lymphoblasts and macrophages were prepared from buffy coat fractions from random donors (kindly provided by the Centre de Transfusion Sanguine, Croix Rouge Suisse, Lausanne, Switzerland). Mononuclear cell fractions were prepared by Ficoll-Hypaque separation. Monocyte-derived macrophages (MDMs) were prepared from Ficoll-Hypaque-separated peripheral blood mononuclear cells (PBMCs) by a fibronectin adherence step, plated at 4–5 × 10^5 cells/cm^2, and cultured in RPMI 1640 with 5–10% human AB^+ serum (Sigma, St. Louis, MO) or 10% FBS as previously described.[16] To generate $CD4^+$ T lymphocytes, nonadherent cells from the fibronectin adherence step were subjected to immunomagnetic sorting. The cells were labelled with anti-CD4 monoclonal antibody-conjugated magnetic beads and positively selected by a magnetic cell sorting system (VarioMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer instructions. After the cells were sorted, they were stimulated with phytohemagglutinin (PHA-P, 1 μg/ml; Sigma) at a cell concentration of 10^6/ml. The $CD4^+$ lymphoblasts were maintained in RPMI 1640 with 10% FBS and recombinant interleukin 2 (IL-2, 10 U/ml; provided by G.-P. Corradin, Institute of Biochemistry, University of Lausanne, Switzerland). The purity of the $CD4^+$ cell preparation was controlled by flow cytometry using Dako antibodies (Dako, Untermueli, Switzerland) and was found routinely to be >95% CD4 positive.

Viruses

pNL43 and pNL43Δ*nef* were provided by D. Richman (University of California at San Diego) and have been described.[8,17] To generate a pair of clones derived from pNL43 and pNL43Δ*nef* but capable of infecting macrophages, the *Sal*I(5785)–*Bam*HI(8468) fragment of these clones (i.e., from the *vpr* ORF to the second exon of *rev*, encompassing most of the *env* open reading frame) was replaced by the corresponding fragment from pBaL[18] (provided by B. Cullen, Duke University Medical Center, Durham, NC), thus generating pNL43BE (standing for BaL envelope) and pNL43BEΔ*nef* (Table 1). Of note, in this pair of clones, the *nef* sequence is derived from pNL43, therefore from a T cell line-passaged isolate, HIV-1_{LAI}.

To generate a *nef* deletion mutant in the genetic background of a clone representative of an unpassaged primary isolate, we chose pYU-2,[19] (obtained from B. Hahn and G. Shaw through the NIH AIDS Reagent Repository Program, Bethesda, MD). As we found this plasmid too unstable for convenient manipulations, the *Sac*I(487)-to-*Sac*I(9563) fragment of pYU-2 was used to replace the corresponding fragment in pNL43 (from nucleotide 491 to 9567), thus generating a hybrid plasmid, pNSSY, in which the vector, cellular sequences, and parts of the LTR were derived from pNL43 and all remaining HIV sequences, including *nef*, are from pYU-2 (Table 1). A deletion was introduced in the *nef* ORF of this clone by excising the *Esp*I(8855)–*Bsu*361(9004) fragment. Because this does not give rise to a frame-shifting deletion, the linearized plasmid was subjected to a brief BAL31 nuclease (New England Biolabs, Beverly, MA) digestion before blunting and ligation. Several clones were sequenced across the deletion region, and a clone with a 145-bp deletion with a frame shift leading to a termination

TABLE 1. PROPERTIES OF INFECTIOUS CLONES

Clone[a]	Sal*I(5785)*–Bam*HI (8468) fragment*[b] *derived from:*	Sac*I(491)*–Sac*I (9567) fragment*[c] *derived from:*	nef *ORF*[d]	*Ability to productively infect:*		
				T cells (e.g., CEM)	*Primary lymphocytes*	*Primary macrophages*
pNL43[e]	HIV-1_{LAI}	HIV-1_{NY-5}/HIV-1_{LAI}	Intact	+	+	−
pNL43Δ*nef*[f]	HIV-1_{LAI}	HIV-1_{NY-5}/HIV-1_{LAI}	Deletion nt 71–256	+	+	−
pNL43BE	HIV-1_{BaL}[g]	HIV-1_{LAI}/HIV-1_{BaL}	Intact	−	+	+
pNL43BEΔ*nef*	HIV-1_{BaL}	HIV-1_{LAI}/HIV-1_{BaL}	Deletion nt 71–256	−	+	+
pNSSY	pYU-2[h]	pYU-2	Intact	−	+	+
pNSSYΔ*nef*	pYU-2	pYU-2	Deletion nt 100–245	−	+	+

[a]The sequence of each clone listed here is derived from pNL43, unless mentioned otherwise.

[b]This fragment includes the 3′ end of the *vpr* open reading frame, the first exons of *tat* and *rev*, the *vpu* open reading frame most of the *env* ORF, the second exon of *tat*, and part of the second exon of *rev*.

[c]This fragment extends from LTR to LTR, including most of the HIV genome, in particular *env* and *nef*.

[d]Numbering of nucleotides deleted begins from *nef* ATG. These frameshifting deletions result in a translation stop a termination codons 16 and 20 bp, respectively, after the end of the deletion.

[e]From Adachi *et al.* (1986).[17]

[f]From Spina *et al.* (1994).[8]

[g]From Hwang *et al.* (1991).[18]

[h]From Li *et al.* (1991).[19]

codon 20 bp after the deletion was chosen, potentially encoding a 40-amino acid polypeptide. This clone was named pNSSYΔ*nef* (Fig. 1B). Another pNL43/pYU-2 hybrid infectious clone was obtained by replacing the 308–9383 *Kpn*21 fragment of pNL43 with the 305–9381 corresponding fragment of pYU-2, thus generating pNKKY. A *nef* deletion was generated as in pNSSY. pNKKYΔ*nef* had a 154-bp deletion with a frame shift leading to a termination codon 11 bp after the deletion, and thus encoding potentially a 35-amino acid polypeptide.

Virus stock production

To prepare viral stocks, infectious clones were transfected into COS-7 cells using calcium phosphate coprecipitation.[20] Viral stocks were harvested 48–72 hr after transfection, centrifuged to pellet cell debris, filtered (0.2-μm pore size filter), aliquoted, and stored at −80°C until use. The virion concentration in the stock was assessed by measuring the HIV-1 p24 antigen in the culture supernatant by enzyme-linked immunosorbent assay (ELISA) (HIVAG-1, Abbott Laboratories, Chicago, IL) as indicated by the manufacturer. Infectious titers were determined by performing terminal dilution assays using half-log dilutions of stocks in quadruplicate. Infections were performed as indicated below. The titers were computed according to the method of Reed and Muench as described[21] and expressed as 50% tissue culture infectious dose ($TCID_{50}$).

Infections

Purified PHA-stimulated CD4$^+$ T cells were infected by exposing 10^6 cells to an inoculum standardized to 1000 pg of p24 antigen, except when stated otherwise, for 4 hr in round-bottom polypropylene tubes. The cells were then washed and plated at 10^6 cells/ml, and cultured in RPMI 1640 medium with 10% FBS and recombinant IL-2 (10 U/ml), observed for syncytium formation, counted, and diluted twice a week to 10^6 cell/ml for a 2- to 3-week infection course. To assess viral replication, culture medium samples were harvested for p24 antigen determination by ELISA. Cell aliquots were also saved to determine the genotype of *nef* by polymerase chain reaction (PCR), to determine the presence of Nef protein by Western blotting, and to determine CD4$^+$ and HIV envelope glycoprotein surface expression by flow cytometry. Macrophages were infected by exposing 5×10^5 cells to an inoculum standardized to 1000 pg of p24 antigen, except when stated otherwise, for 4 hr in 1-cm^2 wells. These cells were washed and cultured in RPMI 1640 medium with 10% FBS for 2–3 weeks with weekly changes of medium. The appearance of syncytia was assessed by phase-contrast microscopy twice a week. The syncytia were counted over the entire wells by phase-contrast microscopy, and the syncytium diameter assessed using a reticulated grid ocular. At the same time interval, supernatant samples were harvested to assess viral production. Cells in replicate wells were scraped off for the determination of the genotype of *nef* by PCR.

PCR determination of the nef *genotype*

To verify the genotype of each clone (i.e., the presence or absence of a deletion in *nef*) in infected cells, total nucleic acid extracts were prepared as described[22] from 10^5 to 10^6 cells and amplified using primers that bracket the *nef* ORF (PM-1 [gctgttaacttgctcaaygccacagc], corresponding to pNL43 nucleotides 8645–8670, and PM-2 [gcttattgaggcttaagcagtgg], corresponding to pNL43 nucleotides 9609–9586). PCR products were analyzed on a 1.5% agarose gel.

Western blotting

Immunodetection of HIV and Nef proteins was performed as described, using a polyclonal sheep antiserum raised to recombinant pNL43 Nef,[23] except that proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Polyscreen; New England Nuclear [NEN] Research Products, Boston, MA) and 3% dried nonfat milk in phosphate-buffered saline (PBS) was used as a blocking agent. To assess the pres-

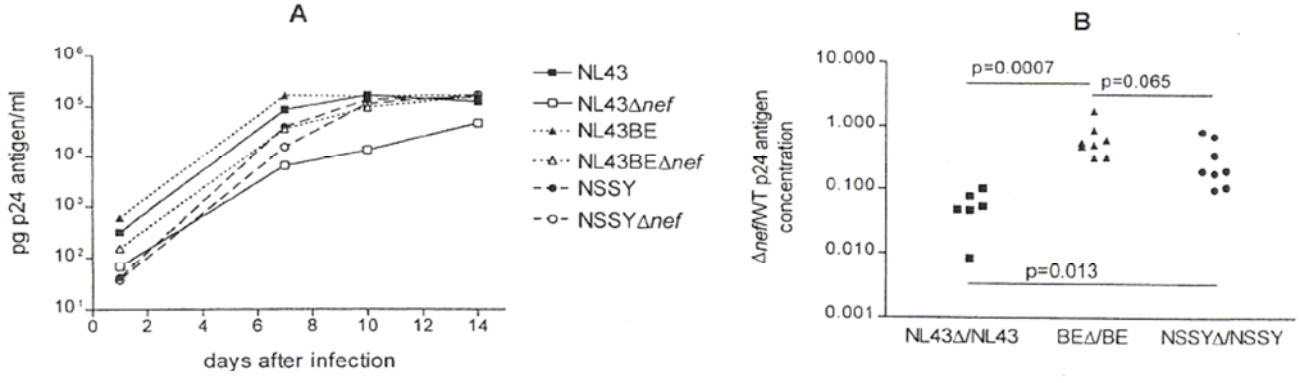

FIG. 1. (A) Effect of *nef* deletion on the replication of various clones in PHA-prestimulated CD4$^+$ T lymphocytes, as assessed by p24 antigen production in the supernatant. Cells (10^6) were infected with an inoculum containing 1000 pg of p24 antigen of each transfection stock, washed, and the cultures split twice a week to 10^6 cells/ml in medium containing rIL-2 (10 U/ml) at which times the p24 antigen content of the culture supernatants was determined. *nef* deletion imparted a larger replication defect to pNL43 than to the other clones. (**B**) The ratio of the p24 antigen production in culture supernatants harvested at the time of late exponential increase [e.g., day 10 in the experiment depicted in (**A**)] by *nef*-deleted clones compared with their wild-type counterparts was computed in six experiments in which all three pairs of clones were tested side by side. Statistical comparisons were performed using the two-tailed Mann–Whitney test. *p* Values between clones are indicated.

ence of other HIV proteins in cell extracts, replicate membranes were treated in the same manner except that the first antibody was an HIV-infected patient serum, diluted 1:200, and the second antibody was a rabbit anti-human IgG–horseradish peroxidase (HRP) conjugate (P0214; Dako) diluted 1:1000. Immunoreactive proteins were revealed using the Renaissance system (NEN).

Viral envelope and CD4$^+$ cell surface expression

Viral envelope and CD4$^+$ cell surface expression was assessed by flow cytometry. For staining experiments, 10^6 lymphocytes were incubated with 5% normal rabbit serum in staining buffer (PBS with 2% FBS and 0.1% sodium azide) for 30 min on ice. Several monoclonal antibodies were screened for the surface envelope staining of cells infected with the various clones. For staining of cells infected with pNL43 (envelope derived from HIV-1_{LAI}) or pNL43BE (envelope derived from HIV-1_{BAL}), monoclonal antibody IAM2G12 (MRC AIDS Reagent Project 3064[24]) gave the highest specific signal while for pNSSY (envelope derived from pYU-2), monoclonal antibody MN215 (MRC AIDS Reagent Project 3064[25]) appeared to stain more brightly. The cells were incubated for 1 hr on ice with primary antibody (10 μg/ml) in staining buffer. After two washes, the cells were incubated with the secondary antibody [rabbit anti-human IgG F(ab')$_2$ linked to fluorescein isothiocyanate (FITC)] (F 0315; Dako) diluted 1:20 for 30 min. In some experiments, the CD4 surface antigen was detected using the R-phycoerythrin (RPE)-labeled CD4 v4 antibody (clone L120; Becton Dickinson, San Jose, CA) diluted 1:20. This antibody recognizes amino acids 306–370 of the antigen, independent of the binding of HIV gp120. After two washes, the cells were fixed in 1% paraformaldehyde in PBS and analyzed using a FACS Vantage (Becton Dickinson) flow cytometer.

Statistical analysis

Statistical comparisons were performed with the tests indicated in Results, using Graphpad Prism software (San Diego, CA) or Stata software (Stata Corporation, College Station, TX).

RESULTS

Construction of infectious clones

To assess the effect of *nef* on the course of productive infection of primary human cells by HIV, we used infectious clones in which deletions in the *nef* ORF had been introduced (Table 1). pNL43 and pNL43Δ*nef*[8,17] have been described. They could be used to infect primary lymphocytes only, as these clones are T cell line tropic and cannot infect primary macrophages. In contrast, pNL43BE and pNL43BEΔ*nef* could infect both primary lymphocytes and macrophages, as they contain a *Sal*I–*Bam*HI fragment from pBaL, including a macrophage-tropic *env* gene. They retained, however, *nef* sequences from pNL43, i.e., a clone from a laboratory-adapted T cell line-tropic HIV strain. A second pair of macrophage-tropic clones, pNSSY and pNSSYΔ*nef*, were constructed using sequences from the macrophage-tropic clone pYU-2, which has been directly cloned from the brain tissue of an AIDS patient.[19] In these clones, almost all HIV sequences, including *env* and *nef*, originate from this macrophage-tropic clone.

Effect of nef *on HIV infection in primary CD4$^+$ lymphocytes*

Replication. The major goal of the present article is to report the effect of *nef* on HIV-mediated syncytium formation in primary cells. However, the infection experiments described here represented several rounds of infection (the relatively low titers of the transfection stocks used here did not allow the observation of endpoints in the frame of a single infection cycle). Hence, any effect of *nef* on HIV replication had a confounding effect on syncytium formation that must be taken into account when presenting syncytium formation data.

As shown in Fig. 1A, all three wild-type clones replicated to a high level in PHA-stimulated CD4$^+$ T cells. The deletion of *nef* resulted in impaired replication compared with the wild-type clone. Interestingly, however, the replication defect imparted by the same *nef* deletion was more obvious in the background of pNL43 than in pNL43BE, as shown in Fig. 1A. Figure 1B shows the ratio of the p24 antigen production in culture supernatants harvested at the time of late exponential increase (e.g., day 10 in the experiment depicted in Fig. 1A) by *nef*-deleted clones compared with their wild-type counterparts in six experiments in which all three pairs of clones were tested side by side. These data demonstrate the significantly greater replication defect imparted by the *nef* deletion in pNL43 than in pNL43BE (two-tailed paired Mann–Whitney test, p values indicated on the graph). These two clones share the same *nef* region but differ by a *Sal*I–*Bam*HI fragment that encompasses parts of or the whole ORF of *vpr*, *tat*, *rev*, *vup*, and *env*. This suggests that the effect of *nef* on HIV replication depends on genetic determinants located in this *Sal*–*Bam*HI fragment of the HIV genome.

In each experiment, the genotype of the various clones was verified by PCR for the *nef* ORF, with the length of the PCR product indicating the absence or presence of the deletion as expected (not shown). The deletions introduced in the *nef* ORF of pNL43 and pNSSY are frame shifting and should lead to the production of truncated peptides of 29 and 40 amino acids, respectively. To verify the absence of Nef protein in cells infected with Δ*nef* clones, CD4$^+$ lymphocytes infected with the various clones were harvested at a late time point, when all clones were producing similar amounts of virus (e.g., day 14 in the experiment depicted in Fig. 1A), as indeed indicated by the similar amounts of HIV proteins detected by Western blotting using a patient serum in cells infected by wild-type clones and the corresponding *nef* deletion mutant. In contrast, Western blotting detected immunoreactive Nef in cells infected by wild-type clones but not in cells infected by *nef* deletion mutants (not shown).

Syncytium formation. Early in the present experiments, we were struck by the observation that CD4$^+$ T lymphoblast cultures infected with clones pNL43BEΔ*nef* and pNSSY Δ*nef* displayed increased numbers of syncytia and, in some experiments, larger syncytia than their wild-type counterparts (see, e.g., Fig. 3A and B). This observation was repeated in seven independent experiments. This was observed despite the slightly lower replication rate of these clones in CD4$^+$ lymphocytes. Such an

enhanced syncytium formation was not observed in $CD4^+$ T cells infected with the *nef*-deleted mutant of pNL43, but it should be stressed that this clone had a more striking replication defect than did the other *nef* deletion mutants (see Fig. 1B), which may prevent it from expressing enhanced syncytium-inducing power.

To quantify syncytium formation, we counted syncytia per 10 microscope fields at a power of ×200. In six experiments, we found at the time of late exponential p24 antigen increase (e.g., day 10 in the experiment depicted in Fig. 1A) a mean (±SEM) of 16 ± 13 syncytia per ten ×200 fields in cells infected by pNL43 versus 4.5 ± 3.6 in cells infected by pNL43Δ*nef* (not significant by the two-tailed Mann–Whitney test). In contrast, cells infected by pNSSYΔ*nef* displayed 63.7 ± 25.7 syncytia per ten ×200 fields versus 19.2 ± 8.5 in cells infected with pNSSY ($p = 0.026$, Mann–Whitney). Since differences in syncytium formation were observed among cells infected by the different clones, we wondered whether this would result in differences in the number of viable cells in the cultures. Actually, counting live cells by trypan blue exclusion (which was performed biweekly in every experiment with lymphocytes) did not reveal any consistent difference in cell viability among cultures infected with the different clones. Reports have suggested that Nef may have a cytostatic effect, particularly in T cells.[26] We therefore also performed tritiated thymidine-incorporation experiments to check whether differences in cytopathic power could be compensated for by a differential effect of infection on cell proliferation. This proliferation assay did not reveal any significant difference in thymidine incorporation in cells infected by the different clones in two experiments (not shown).

Effect of nef *on HIV infection in primary macrophages*

Replication. When tested on *in vitro*-differentiated monocyte-derived macrophages, the effect of *nef* on HIV replication varied with the genetic background of the clone in which the *nef* deletion was introduced, but also with the particular macrophage preparation used for the infection. Indeed, of nine infections performed with macrophages from random donors, the deletion of *nef* in the context of pNL43BE had little effect on HIV replication, with less than twofold differences in p24 antigen production in the culture supernatant (see, e.g., Fig. 2A). Note that the effect of the *nef* deletion could not be tested in macrophages in the context of pNL43, as this clone does not enter macrophages. In contrast to pNL43BE, the deletion of *nef* affected positively or negatively the replication of pNSSY, depending on the macrophage batch. For instance, in the experiment depicted in Fig. 2A, an enhanced virus production by pNSSYΔ*nef* was observed, compared with pNSSY. We then computed the ratio of the p24 antigen production by *nef*-deleted clones compared with wild types at the time of late exponential increase in virus production (e.g., day 7 in the experiment depicted in Fig. 2A) in nine separate experiments. Figure 2C shows how this ratio varied significantly from 0.065 to 13.02 in the context of pNSSY, compared with a ratio close to 1 for pNL43BE (variance ratio test, $p = 0.0081$). In the experiment depicted in Fig. 2A, infections were also performed with 10- and 100-fold reduced multiplicities of infection (MOIs, i.e., respectively, 100 and 10 pg of p24 antigen/5×10^5 macrophages). A similar effect of the *nef* deletion was observed at the different MOI (not shown). To exclude the fact that differences of p24 antigen levels in the culture supernatant might reflect not so much differences in virus production, but differences in virus budding and release, infected macrophages were lysed at the end of the experiment shown in Fig. 2A in the p24 antigen assay lysis buffer, and assayed for p24 antigen content. Intracellular p24 antigen contents paralleled supernatant concentrations (Fig. 2B). In all these experiments, the presence or absence of a *nef* deletion was verified by PCR.

Syncytium formation. The increased syncytium formation in macrophages infected with *nef*-deleted clones compared with their wild-type counterparts was also a striking observation (see, e.g., Fig. 3C and D). It was observed in all nine experiments mentioned in Fig. 2C in the background of pNL43BE, and in seven of nine experiments in the background of pNSSY. In those two experiments in which the deletion of *nef* hampered most the replication of pNSSYΔ*nef*, no increased syncytium formation was observed in cells infected with this clone compared with pNSSY. Figure 4 shows the effect of the *nef* deletion on the cytopathic effect of the two macrophage-tropic clones. This experiment was chosen as the replication rate of *nef* deletion mutant clones was similar to that of the wild type (Fig. 4A), so that replication was not a confounding factor for syncytium formation. In both genotypic backgrounds, the deletion of *nef* led to a substantial increase in syncytium formation in terms of number (Fig. 4B) and size (Fig. 4C). A similar increase in syncytium formation was documented quantitatively in five separate experiments in which this type of measure was performed. As mentioned in Materials and Methods, another clone derived from pYU-2 was constructed (called pNKKY), whose coding sequences come from pYU-2 except for the last four Nef amino acids. Infections with this clone and its *nef*-deletion mutant revealed an effect of Nef on HIV replication entirely similar to those observed with pNSSY in primary lymphocytes and macrophages (not shown).

Effect of Nef on HIV envelope surface expression in primary $CD4^+$ T lymphocytes

The determinants of HIV-induced membrane fusion are the envelope glycoproteins. We therefore wondered whether cells infected with *nef*-deletion mutants would express more HIV envelope at the cell surface than would cells infected with their wild-type counterparts, particularly at times when increased syncytium formation was observed. Figure 5 demonstrates the staining of infected $CD4^+$ T cells for CD4 antigen and HIV envelope, using anti-V3 loop monoclonals, 9 days after infection. At that time, all clones except pNL43Δ*nef* were producing a similar amount of virus (see Fig. 1) but $CD4^+$ T cell cultures infected with *nef*-deleted macrophage-tropic clones were displaying increased syncytium formation. Each clone productively infected a large proportion of the cells, as demonstrated by cell surface expression of HIV envelope. However, cells infected with *nef*-deleted clones did not stain more brightly for HIV envelope than did nef^+ counterparts. In some instances, we observed a reduced fraction of envelope-negative/CD4-positive cells in cultures infected with nef^+ clones compared with their nef^- counterparts. This would be consistent either with a population of yet uninfected cells (consistent with the delayed

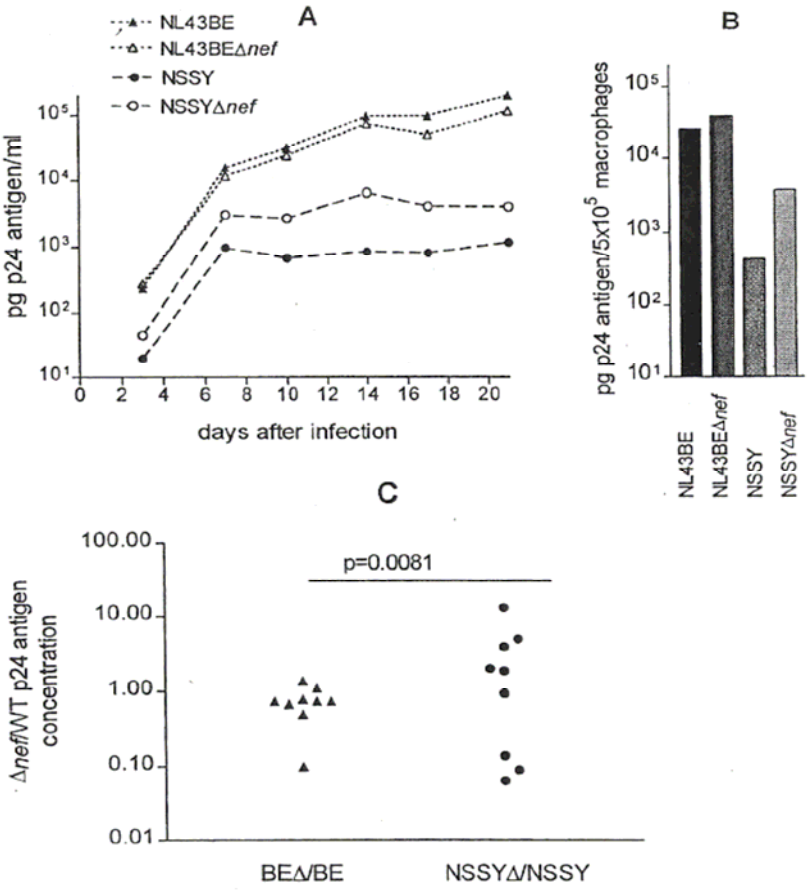

FIG. 2. Effect of *nef* deletion on the replication of pNL43BE and pNSSY in macrophages (pNL43 does not replicate in these cells). Cells (5×10^5) were infected with an inoculum containing 1000 pg of p24 antigen of each transfection stock, washed, and the virus replication assessed by p24 antigen production in the supernatant over the next 3 weeks (**A**) and in the cells on day 21 [(**B**) the macrophage monolayer was lysed in p24 antigen assay lysis buffer, 5×10^5 cells/ml]. To show the differential effect of *nef* on HIV replication in various cell batches, the ratio of the p24 antigen production in culture supernatants harvested at the time of late exponential increase [e.g., day 7 in the experiment depicted in (**A**)] by *nef*-deleted clones compared with their wild-type counterparts was computed in nine experiments in which the two pairs of clones were tested side by side (**C**). In all nine experiments performed, *nef* deletion had little influence on the replication of pNL43BE, as in the experiment depicted in (**A**). In contrast, varying effects were observed using pNSSY (and pNKKY). The statistical comparison was performed by the variance ratio test, and the *p* value is indicated.

replication rate of nef^- viruses) or with the effect of *nef* down-regulating surface CD4 expression early in infection, when envelope is not yet expressed. Thus, we could not find evidence of increased expression of envelope in the absence of Nef to explain the increased syncytium formation in $CD4^+$ T cells. Similar observations were made at two time points in two separate experiments.

DISCUSSION

In the present study, we describe an increased level of syncytium formation in primary human lymphocytes and macrophages infected by *nef*-deleted clones compared with wild-type counterparts. We assessed the effect of *nef* not only in the context of a T cell-tropic clone (pNL43) and a chimeric clone with a macrophage-tropic envelope capable of infecting macrophages (pNL43BE), but also in the context of a clone in which *env* and *nef* itself come from a macrophage-adapated virus (pNSSY).

In $CD4^+$ lymphocytes, the deletion of *nef* diminished in a modest manner the replication of the macrophage-tropic clones pNL43Be and pNSSY, while a more severe replication defect was observed using pNL43. This clone differs from pNL43BE by a *Sal*I(5785)–*Bam*HI(8468) fragment that includes the 3′ end of the *vpr* open reading frame, *tat* and *rev* first exons, the *vpu* open reading frame, most of the *env* ORF, the second exon of *tat*, and part of the second exon of *rev*. Our data therefore confirm previous reports showing that the deletion of *nef* impairs HIV replication to a modest extent in stimulated primary $CD4^+$ lymphocytes,[7–9,11,27] but suggest that the expression of the phenotype of *nef* on HIV replication in lymphocytes depends partly on some genetic determinant located in this *Sal*I–*Bam*HI fragment, perhaps in the envelope gene as suggested for macrophages by Malykh *et al.*[28] It has been shown that the replication defect of nef^- viruses is most evident when $CD4^+$

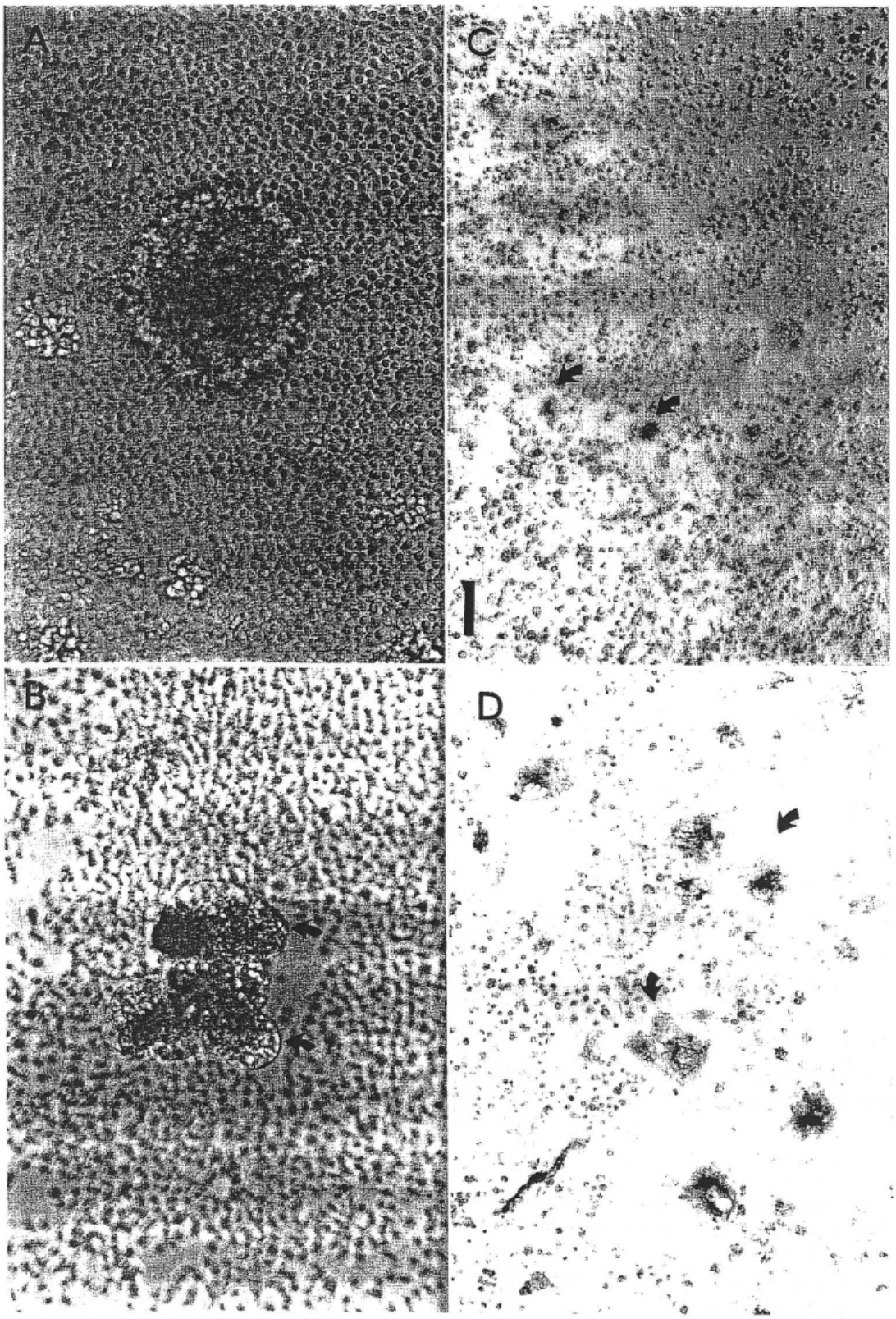

FIG. 3. Phase-contrast microscopy of infected cells. Purified $CD4^+$ T lymphocytes infected with pNL43BE (**A**) and pNL43BEΔ*'''nef* (**B**), 10 days after infection. Lymphocytes infected with pNSSYΔ*nef* showed a similar increased syncytium formation compared with pNSSY in this experiment. Macrophages infected with pNSSY (**C**) and pNSSYΔ*nef* (**D**) 14 days after infection. Macrophages infected with pNL43BEΔ*nef* showed a similar increased syncytium formation compared with pNL43BE. Bar: 200 μm (**A** and **B**) or 40 μm (C and D).

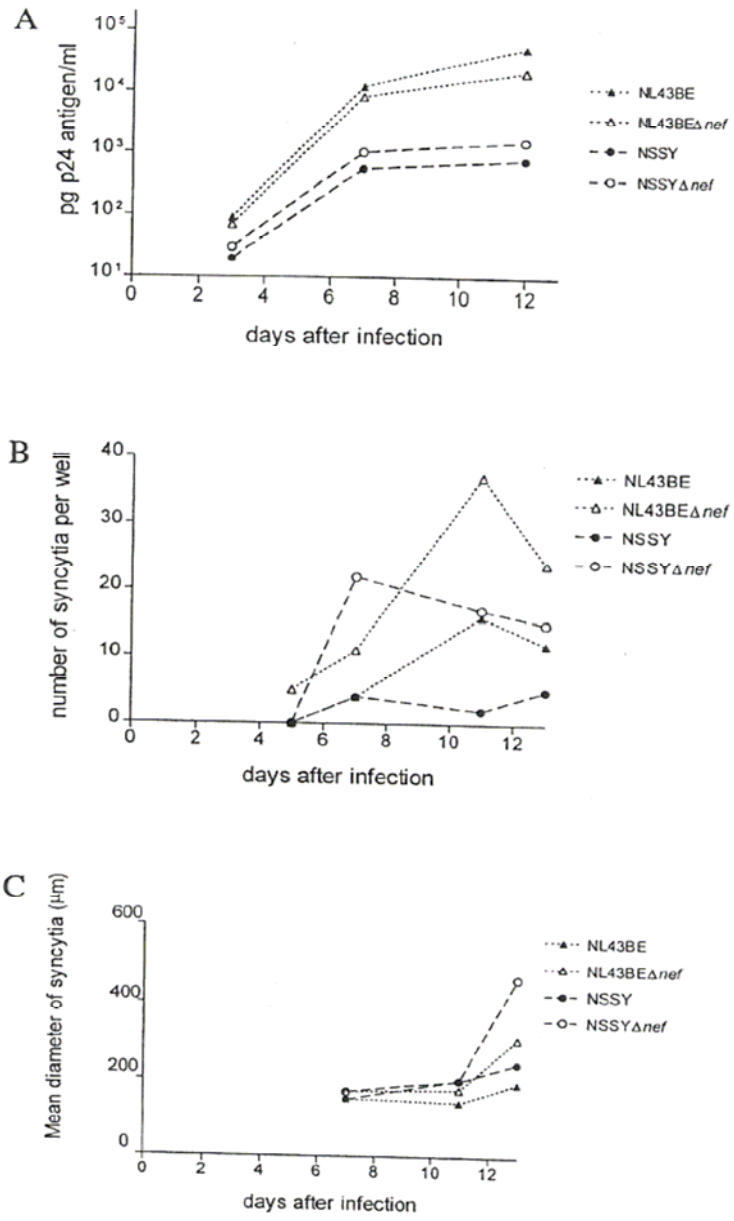

FIG. 4. Demonstration of increased syncytium formation in macrophages infected with *nef*-deletion mutants, in an experiment in which the confounding effect of *nef* on replication was absent as demonstrated by the p24 antigen production in the culture supernatants (**A**). Effect of *nef* deletion on the number (**B**) and size (**C**) of syncytia.

T cells are infected before stimulation.[8] In the present study, we were interested in the effect of *nef* on HIV cytopathic effect. Thus we elected conditions that allowed us to observe such a cytopathic effect with *nef*$^-$ viruses, i.e., we infected PHA-prestimulated cells in the presence of IL-2.

The effect of *nef* on HIV replication in human macrophages has been reported by several investigators. Using clones in which *nef* was derived from T cell-adapted viruses,[9,10] or from a macrophage-tropic primary isolate,[11] they observed a modest delay of replication with *nef*-defective clones. Hattori *et al.*, using an HIV-2 clone, found that the deletion of *nef* had little effect on viral replication in macrophages.[29] As with lymphocytes, we observed a differential effect of *nef* on HIV replication in macrophages, depending on the clone in which the *nef* deletion was introduced. Using pNL43BE, the *nef* of which originates from a T cell-adapted virus, no discernible effect of *nef* on replication was observed. In contrast, using pNSSY, the *nef* of which originates directly from the brain of an HIV-infected patient, *nef* displayed a varying effect on HIV replication depending on the macrophage batch. In some experiments in which an enhanced replication of the *nef*-defective mutant was observed, the performance of a terminal dilution titration documented increased infectivity of the *nef*-defective virus stock when standardized to the p24 antigen content. It is unclear at this point whether the variable effect of *nef* on the replication of pNSSY in macrophages was

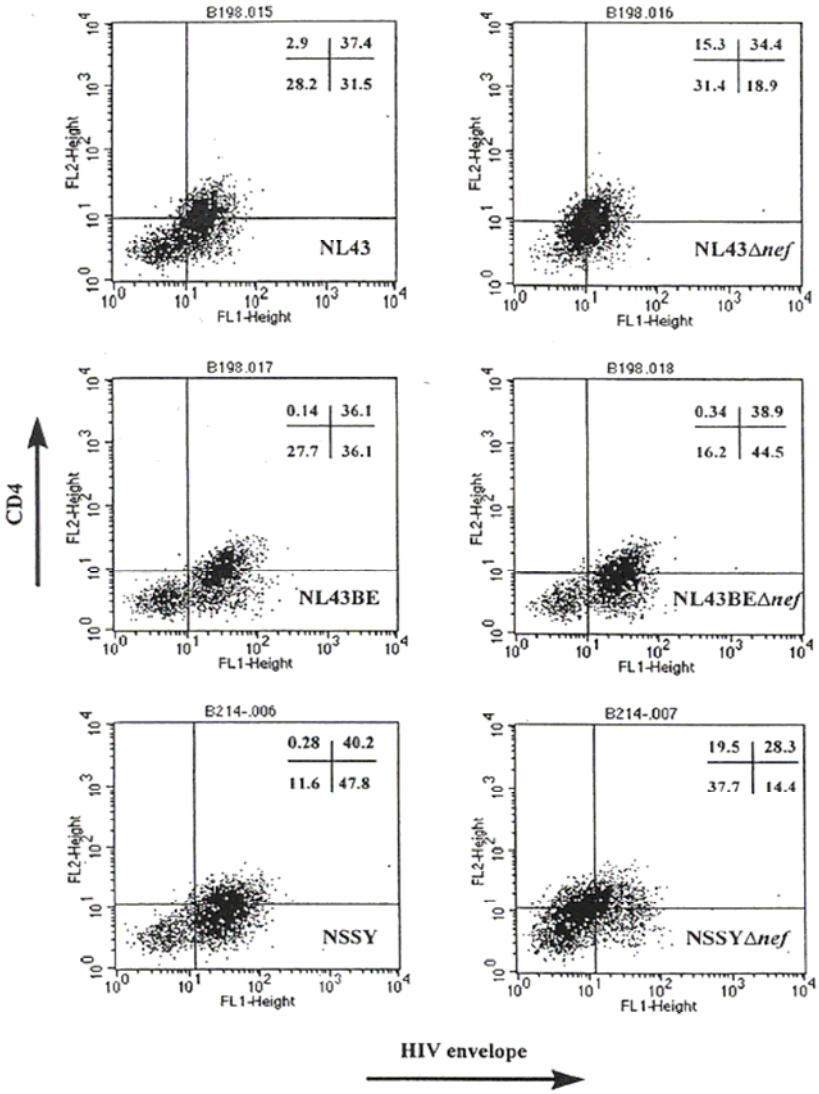

FIG. 5. Determination of CD4 antigen and HIV envelope surface expression in infected CD4$^+$ lymphocytes. Cells were stained with appropriate monoclonal antibodies to detect surface envelope on cells infected with the various clones. pNL43 and pNL43BE, MAb IAM2G12; pNSSY, MAb MN215. Env–antibody binding was detected using an FITC-labeled secondary antibody. The CD4 surface antigen was detected using the RPE-labeled CD4 v4 antibody. This antibody recognizes amino acids 306–370 of the CD4 antigen, independent of the binding of HIV gp120. The cells were fixed and analyzed by gating on intact cells and setting thresholds on unstained cells. Each graph depicts the staining of cells harvested on day 9 in the experiment depicted in Fig. 1A, with green fluorescence (FL1) measuring envelope expression on the *x* axis and red (FL2) measuring CD4 expression on the *y* axis. The percentage of cells in each quadrant is shown on the graphs.

cell donor dependent, as macrophages were prepared from random blood donors.

Thus, our data using stimulated lymphocytes are consistent with the increasing amount of data showing that *nef* acts as a positive factor for HIV replication *in vivo* and *in vitro*.[1,30] In contrast, we present evidence that in macrophages, deletion of *nef* has a variable effect on HIV replication, including enhanced replication, an as yet unreported phenomenon in these cells. The mechanism for this increased infectivity is unknown but might reflect yet another mechanism of action of Nef. In our experiments, we cannot ascertain whether the different effect of the *nef* deletion on the replication of pNL43BE and pNSSY is due to a difference of Nef itself, i.e., pNL43BE Nef being nonfunctional in macrophages (an allele effect, which has already been described using lymphocytes[31–35]), or to a difference in the genetic background of these clones, i.e., the pNL43BE background being nonpermissive for the expression of a functional Nef phenotype. Experiments are underway to address this question. In any case, the observation that only the *nef* allele from a macrophage-adapted clone displays a phenotype in macrophages suggests a phenomenon of adaptation of *nef* to macrophages.

The most important observation in this article was that cultures of primary cells, whether stimulated CD4$^+$ lymphocytes

or macrophages, infected with *nef*-defective viruses generally displayed greater numbers and in some experiments larger syncytia than with wild-type viruses.

As already discussed, the *nef* deletion consistently reduced HIV replication in $CD4^+$ T lymphocytes. Thus, any increased syncytium formation in these cells could only truly reflect an increased capacity of Δ*nef* clones to induce syncytium formation. Strikingly increased syncytium formation was observed in lymphocytes infected with Δ*nef* clones compared with pNL43BE or pNSSY (Fig. 3A and B). No such effect was observed with the Δ*nef* clone derived from pNL43. Note, however, that this clone produced a more severe replication defect in lymphocytes than did the deletion mutants of macrophage-tropic clones, perhaps preventing the observation of an increased cytopathic effect. An alternative explanation would be that the effect of the *nef* deletion on syncytium formation would depend on using a coreceptor other than CXCR4, the coreceptor used by pNL43.[36,37] The variable effect of *nef* on HIV replication in macrophages had obviously a confounding effect on syncytium formation in these cells. However, the general observation in every experiment of increased syncytium formation in cells infected with pNL43BEΔ*nef*, which had a similar replication rate compared to its wild-type counterpart, as well as with pNSSYΔ*nef* in all experiments but those two in which the greatest replication defect of pNSSYΔ*nef* (including the experiment displayed in Fig. 4), demonstrates that *nef* affects syncytium formation in macrophages independent of its effect on replication.

The observation that *nef* may affect syncytium formation is not totally unprecedented. Indeed, Talbott *et al.*[38] have mentioned unpublished data suggesting that *nef* inhibits the formation of syncytia by the HIV-2 KR clone in H9 cells. Also, Schwartz *et al.*[39] have studied the effect of *nef* on Env surface expression and syncytium formation in human $CD4^+$ T cells (CEM). Using stable transfection, they showed that *nef* reduced the CD4 cell surface expression, and, when expressed from recombinant vaccinia vectors, the surface expression of HIV envelope glycoproteins in a CD4 binding-dependent manner, with a concomitant decrease in syncytium formation. This effect of *nef* on envelope surface expression was easily observed by flow cytometry. They argued that by reducing the HIV cytopathic effect, this effect might contribute to the increased viral production. While these experiments were performed with cell lines, our data represent the first report of such an effect of *nef* in primary cells. The observations by Schwartz *et al.*[39] provide a potential mechanism for our observation in primary cells. It should be noted, however, that *in vitro*-differentiated macrophages are cells that express little, if any, surface CD4 antigen (our unpublished observation, and Ref. 40), suggesting that a CD4-independent mechanism may also exist for *nef* to control syncytium formation in macrophages.

We attempted to test whether cells infected with *nef*-deleted mutants would express more HIV envelope and CD4 antigen when compared with wild type-infected cells, and could not demonstrate such an effect. It is not excluded, however, that cells infected with a *nef*-deleted clone expressed transiently a higher surface level of CD4 and *Env* that might be responsible for enhanced syncytium formation, as it has been demonstrated that the effect of *nef* on CD4 antigen expression is discernible only early during the infectious cycle, before *env* and *vpu* expression mediate CD4 down regulation.[41]

Of note, Briand *et al.* have demonstrated that the expression of the $p56^{lck}$ tyrosyl kinase in T cell lines, probably also by stabilizing the expression of CD4 at the cell surface, enhanced HIV-1-induced syncytium formation.[42] Alternatively, Nef itself might act on the cell membrane, as it has been described to be expressed at the surface of infected cells and mediate interactions with $CD4^+$ cells[43] as well as to mediate cytotoxicity for these cells.[44]

It may appear at first sight paradoxical to report that the deletion of *nef* induces an enhanced cytopathic effect in HIV major host cell types while such a deletion *in vivo* has been observed in various systems to attenuate the virulence of SIV and HIV in terms of viral load and pathogenesis.[2–5] However, as suggested by Schwartz *et al.*,[39] an increased cytopathic effect may curtail virus production with a reduced *in vivo* viral load. This net effect of increased cytopathicity is also predicted by mathematical modeling of HIV infection.[45] Net, by downmodulating MHC class I antigens, has been shown to protect infected primary cells against killing by cytotoxic T lymphocytes *in vitro*.[46] It appears therefore that Nef may protect infected cells not only from CTL-mediated death, but also from direct viral cytopathic effect. In conclusion, we demonstrate in the present study that a functional Nef has different effects on replication in primary cells depending on the host cell type and the HIV clone, but that it represses syncytium formation in primary $CD4^+$ lymphocytes and in macrophages by macrophage-tropic clones, a mechanism explaining perhaps in part the reduced viral load of *nef*-deficient viruses observed *in vivo*.

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