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The Effects of HIV-1 Nef on CD4 Surface Expression and Viral Infectivity in Lymphoid Cells Are Independent of Rafts*

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The HIV-1 Nef protein is a critical virulence factor that exerts multiple effects during viral replication. Nef modulates surface expression of various cellular proteins including CD4 and MHC-I, enhances viral infectivity, and affects signal transduction pathways. Nef has been shown to partially associate with rafts, where it can prime T cells for activation. The contribution of rafts during Nef-induced CD4 down-regulation and enhancement of viral replication remains poorly understood. We show here that Nef does not modify the palmitoylation state of CD4 or its partition within rafts. Moreover, CD4 mutants lacking palmitoylation or unable to associate with rafts are efficiently down-regulated by Nef. In HIV-infected cells, viral assembly and budding occurs from rafts, and Nef has been suggested to increase this process. However, using T cells acutely infected with wild-type or *nef*-deleted HIV, we did not observe any impact of Nef on raft segregation of viral structural proteins. We have also designed a palmitoylated mutant of Nef (NefG3C), which significantly accumulates in rafts. Interestingly, the efficiency of NefG3C to down-regulate CD4 and MHC-I, and to promote viral replication was not increased when compared with the wild-type protein. Altogether, these results strongly suggest that rafts are not a key element involved in the effects of Nef on trafficking of cellular proteins and on viral replication.

The human immunodeficiency virus type-1 (HIV-1)[1] Nef protein is a critical player of viral pathogenesis (1–3). In cell cultures, many different Nef activities have been reported (4). Nef is a 27 kDa myristoylated protein and is expressed early and abundantly during viral infection. Nef affects the intracellular trafficking of a number of proteins, including CD4, MHC-I and MHC-II, CD28, DC-SIGN, TNF, and HIV-1 Env glycoproteins (5–11). One of the best characterized function of Nef is the down-regulation of CD4, which occurs by a rapid endocytosis of surface molecules (12–14). Reduction of CD4 surface expression may facilitate viral release and infectivity of virions, and may prevent Env-associated cytopathic effects, resulting in an optimal viral replication (6, 15–17). Nef misroutes CD4 and other proteins through an interaction with a number of sorting proteins, including β-COP, AP complexes, and PACS-1 (18–21). Overexpression of Nef also provokes pleiotropic effects on sorting organelles, inducing the accumulation of clathrin-coated pits, endosomes, lysosomes, and multivesicular bodies (22–24).

Nef facilitates viral propagation by different means. Viral particles produced in the presence of Nef are more infectious (25, 26), performing more efficiently the early steps of the replicative cycle (25–28). Nef also exerts direct and indirect positive effects on virus replication in primary human lymphocytes (26, 29–31). Direct effects are particularly visible in lymphocytes activated a few days after infection, in which replication of *nef*-deleted HIV (HIVΔnef) is barely detectable. Nef also indirectly impacts lymphocytes, when expressed in macrophages or in dendritic cells: HIV-infected macrophages or dendritic cells release paracrine factors (chemokines, soluble CD23, and soluble ICAM), which permit the infection of resting T cells (32–34). The capacity of HIV to replicate in non-activated lymphocytes is likely linked to the numerous described effects of Nef on T cell activation. Nef interacts with a number of kinases or other cellular proteins involved in signal transduction pathways (4, 35–37), potentially priming infected cells for activation and protecting them from apoptosis (38–40).

Nef has been suggested to act at the level of rafts to trigger cell activation (41). Rafts are involved in many biological events, including intracellular protein trafficking, signal transduction pathways, entry and release of various virus species (42–45). Regarding HIV, rafts are thought to operate at multiple steps of the viral life cycle. Rafts were initially proposed to act as platforms for virus entry, facilitating interactions between CD4 co-receptors, and incoming virions (44, 46–48). This role has been, however, recently questioned, because CD4 molecules unable to associate with rafts still allow virus entry (49, 50). There is also mounting evidence that rafts are important for HIV-1 assembly and budding. HIV-1 Gag and Env viral structural components are concentrated in rafts or in raft-like complexes (51–57). Viral particles are enriched in cellular raft proteins and lipids, and cholesterol-depleting agents decrease viral infectivity. Interestingly, Nef has been proposed to increase viral infectivity via lipid rafts. In this model, Nef induces accumulation of Gag within rafts, thus facilitating viral release from these membrane domains (58). Nef may also increase synthesis and transport of cholesterol to rafts and progeny virions (59).

Recently, raft targeting by Nef has been reported to be functionally important for CD4 and MHC-I down-regulation as well

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type-1; gp, glycoproteins; WT, wild-type; MHC-I, major histocompatibility class I complex; PBMC, peripheral blood mononuclear cells; IL, interleukin; mAb, monoclonal antibody; PHA, phytohemagglutinin; ELISA, enzyme-linked immunosorbent assay; MOI, multiplicity of infection; GFP, green fluorescent protein.

as infectivity enhancement (60). These conclusions were drawn by comparing the activity of LAT-Nef chimeras accumulating in rafts or excluded from these domains.

Association of cellular and viral proteins with rafts is often regulated by palmitoylation. Palmitoylation is a reversible post-translational fatty acylation of many proteins, such as transmembrane receptors, G-proteins, tyrosine kinases (61–63), as well as viral envelope glycoproteins (64–68). Palmitoylation generally occurs on cysteine residues located close to cellular membranes. The reversible nature of the thioester bound that links palmitate to cysteine allows dynamic changes in protein palmitoylation (63, 69). Palmitoylation regulates protein trafficking and association with plasma membranes and with rafts. It also potentially modulates protein activity, oligomerization, and interaction with other proteins. At least two proteins involved in HIV replication are known to be palmitoylated, the cellular receptor CD4 and the viral Env glycoprotein. Efficient CD4 association with rafts requires palmitoylation, but also interaction with the tyrosine kinase p56Lck (49, 50, 70). CD4 palmitoylation occurs on two membrane proximal residues (49, 71) and also requires a short cluster of positively charged residues within the cytoplasmic tail (50). HIV-1 Env glycoproteins are palmitoylated on two intracellular cysteines (56, 72). Mutating these residues did not affect cell fusion activity or surface expression of HIV-1 Env, but abrogated raft association, virion incorporation, and viral infectivity.

In general, palmitoylation is a reversible and dynamic process. Two major classes of palmitoylthioesterases have been described. One family is lysosomal; the second is cytosolic and removes palmitate moieties from membrane-associated proteins (73). Nef induces CD4 endocytosis and disrupts the association between CD4 and Lck (13, 14, 74). Of note, Nef also efficiently binds to a thioesterase enzyme, although the role of this enzyme in Nef function remains unknown (75, 76).

The central role of rafts in many cellular events parallels the multiple activity of Nef on cellular trafficking, signal transduction pathways, and viral replication. We therefore asked whether rafts intervene in the effects of Nef on CD4 down-regulation, and examined whether the down-regulating activity of Nef on CD4 is associated with a modification of the palmitoylation state of CD4. We also asked whether Nef improves viral infectivity by increasing the association of Gag with detergent-resistant membranes. We report here that Nef-induced CD4 down-regulation is independent of CD4 palmitoylation or raft association. We also show that Nef is associated at rather low levels with rafts. We have designed a palmitoylated Nef mutant, which strongly accumulates within rafts. However, targeting Nef to rafts does not improve CD4 or MHC-I down-regulation and does not facilitate viral replication in non-activated primary lymphocytes. Furthermore, Nef does not modify the raft segregation of Env and Gag proteins. Therefore, rafts are not a key element involved in the effects of Nef on trafficking of cellular proteins and on viral replication.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Infections—A2.01 T cells, Jurkat T cells, and human peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 with Glutamax (Invitrogen) supplemented with 10% fetal calf serum (Sigma) and penicillin-streptomycin (100 IU/ml each, Sigma). A2.01 cells expressing WT and mutant CD4 have been described (49). HeLa cells were maintained in Dulbecco's modified Eagle's medium with Glutamax supplemented with 10% fetal calf serum and penicillin-streptomycin. PBMCs were isolated from healthy donors using Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation.

The production and use of WT and mutant HIV (from NL4.3 or NLAD8 strains) have been described (77, 78). For HIV replication experiments, freshly prepared PBMCs were exposed to the indicated virus stocks (viral input ranging from 20 to 0.02 ng of p24/10^6 cells) for 2 h 30 min, washed twice with phosphate-buffered saline, and cultured in 96-well plates (2×10^5 cells/well, in triplicates). Four days after infection, cells were activated by phytohemagglutinin (PHA, 1 μg/ml, Abbott) and grown in the presence of recombinant interleukin 2 (IL-2, 50 international units/ml, Chiron). Alternatively, HIV replication experiments were performed with PBMC activated (with PHA and IL-2) 3 days prior to infection. Viral replication was monitored by measuring HIV-1 Gag p24 release in supernatants by ELISA (PerkinElmer Life Sciences). Jurkat cells (30×10^6 cells per point) were infected with the indicated virus stocks, (50 ng of p24/10^6 cells), washed with phosphate-buffered saline, and cultured for 1 or 2 days before proceeding with further experiments. Single cycle infections of P4C5 cells were performed as described earlier (77). Viral infectivity was measured 24 h after viral exposure.

Design and Use of Plasmids and Lentiviral Vectors—Nef WT and Nef mock plasmids carry the *nef* gene (from the HIV LAI isolate) under the control of the cytomegalovirus promoter, in a sense and antisense orientation, respectively (19). pHR′Nef lentiviral vector encoding the HIV LAI (also termed R7) *nef* gene (under the control of the elongation factor-1α promoter) was a kind gift from Didier Trono (41). *nef* G3C and G2A mutants were generated using the QuickChange kit (Stratagene). Mutations were introduced in Nef WT plasmid, in pHR′Nef vector, or in pNL4.3 and pNLAD8 proviruses. The accuracy of the mutations was verified by sequencing. The CD4 LL/AA mutant was introduced in the pTRIP CD4 lentiviral vector (49). Lentiviral vector particle production and transduction of A2.01 CD4 T-cells or Jurkat T cells were performed as described (11, 41, 49). The stable integration of the vector into the host DNA allows efficient and long term transgene expression, without selection of cell clones. HeLa cells were cotransfected with Nef, CD4 or HLA-A2 and GFP expression vectors as described (19).

Flow Cytometry and Immunofluorescence Analysis—A2.01 and Jurkat cells were stained with anti-CD4 (SK3-PE, BD Biosciences) and anti-MHC-I (W6.32-FITC, Sigma) mAbs, 6 days after transduction with lentiviral vectors. Anti-Gag staining was performed on permeabilized HIV-infected Jurkat cells using an anti-p24 mAb (KC57-RDE, Beckman Coulter). HeLa cells were stained with anti-CD4 (SK3-PE) or with anti-HLA-A2 (BB7.2) (19) mAbs, 24 h after transfection. Surface levels of CD4 and HLA-A2 were measured in GFP+ cells, which represented the fraction of the cell population (~30–50% of cells) that was transfected. Cells were analyzed by flow cytometry with a FACScalibur apparatus (BD Biosciences). Isotype-matched mAbs were used as negative controls in all experiments. For immunofluorescence studies, Jurkat cells were fixed, permeabilized, and stained with anti-Nef mAb (MATG020) as described (19). Confocal microscopy was performed on a Leica TCS4D instrument. Series of optical sections at ~0.5-μm intervals were recorded and mounted using Adobe Photoshop software.

Detergent-resistant membranes (DRMs) isolation. DRMs were obtained by sucrose flotation after Triton X-100 cell lysis as previously described (49). Briefly, 30×10^6 cells were washed twice in ice-cold TKM buffer (50 mM Tris, pH 7.4, 25 mM KCL, 5 mM $MgCl_2$, 1 mM EGTA) containing phosphatase and protease inhibitors (Roche Applied Science). Cells were then incubated for 1 h on ice in TKM containing 1% Triton X-100 (v/v), in a final volume of 0.375 ml. Cell lysates were loaded onto a 5-ml discontinuous sucrose gradient (5 to 35–40% in w/v) and then centrifuged at equilibrium for 18 h at 4 °C and 200,000 $\times$ *g* (Beckman L70-ultracentrifuge). Fractions were collected from the top of the gradient, and their protein contents were estimated using the Nano-Orange quantification kit (Molecular Probes).

Immunoblotting and Immunoprecipitation—Equal volumes of each fraction of the gradient were analyzed by SDS-PAGE using a 4–12% NuPage Gel (Invitrogen) under reducing (for the analysis of CD4, p56Lck, Gag, Env, and Nef) or non-reducing (for the analysis of CD46) conditions. For immunodetection, the following antibodies were used: CD4 (1F6, Novocastra), p56Lck (sc-13, Santa Cruz Biotechnology), CD46 (J4–48 Immunotech), Gag p24 and p17 (25A+18A mAbs) (79), Env gp120 and gp41 (160A+41A+110H mAbs Hybridolabs, Institut Pasteur, Paris), Nef (MATG020, Ref. 19). Immobilized antigen-antibody complexes were detected with secondary IgG-horseradish peroxidase conjugates (Pierce), revealed using enhanced chemiluminescence (ECL+, Amersham Biosciences), and quantified using an electronically cooled LAS-1000 plus charge-coupled device (CCD) camera system (Image Gauge 3.4 software, Fuji Photo Film Co., Tokyo, Japan). Ganglioside GM1 detection by slot-blot was performed using peroxidase-coupled cholera toxin (Sigma). [^{3}H]palmitate or [^{35}S]Met/Cys metabolic labeling were performed as previously described (49). Briefly, radiolabeled cells were lysed in TKM buffer containing 1% Triton (w/v), and CD4 and Nef were immunoprecipitated using OKT4 and Nef MATG 020 mAbs, respectively. Detection of [^{3}H]palmitate was performed using the enhanced autoradiography kit (EABiotech).

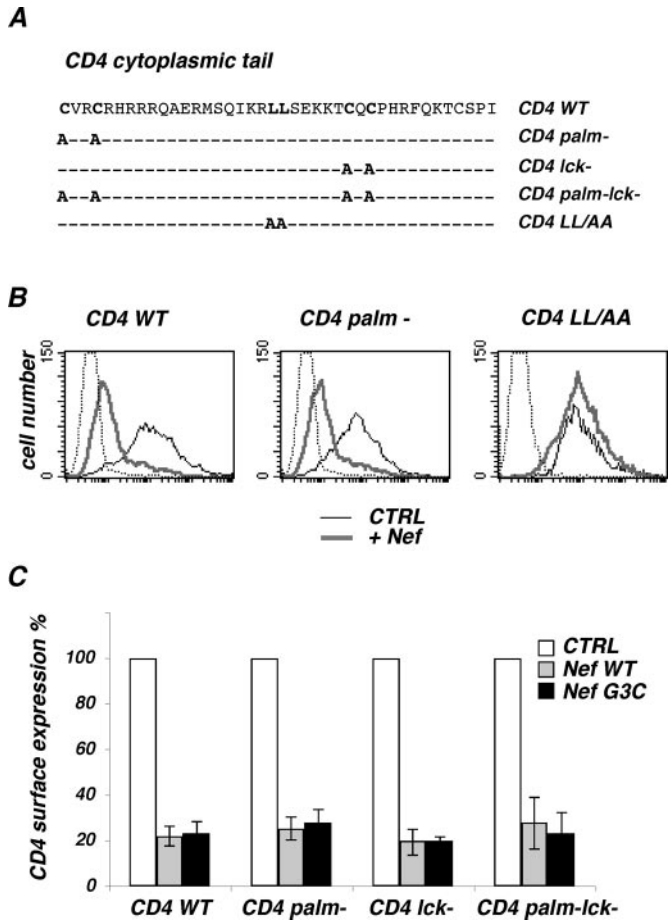

FIG. 1. **Susceptibility of CD4 mutants to Nef-induced modulation.** *A*, amino acid sequence alignment of the cytoplasmic domain of CD4 WT and point mutants. Cysteine residues involved in CD4 palmitoylation and in CD4 association with Lck were mutated, yielding, respectively, CD4 palm$^-$ CD4 lck$^-$ and CD4 palm$^-$ lck$^-$ mutants. The di-leucine motif involved in the interaction of CD4 with the cell sorting machinery is mutated in CD4 LL/AA. *Bold letters* identify amino acid substitutions. *B*, surface levels of WT and mutant CD4 in A2.01 lymphocytes, in the absence or presence of Nef. A2.01 cells expressing the indicated CD4 molecules, transduced with a control (*CTRL curves*) or with a Nef-expressing lentiviral vector. Cells were then stained with an anti-CD4 mAb and analyzed by flow cytometry. *Dotted lines* represent background staining with control isotypic mAb. Data are representative of three experiments. *C*, surface levels of WT and mutant CD4 in HeLa cells in the absence or presence of Nef. HeLa cells were transfected with 1 μg of the indicated CD4 vectors, along with 2 μg of Nef-mock (carrying the *nef* gene in an antisense orientation, CTRL, *white columns*), Nef WT (*gray columns*), or Nef G3C mutant (*black columns*), and 0.075 μg of GFP reporter vector. 24 h later, the surface expression of CD4 was measured in GFP-positive cells by flow cytometry. 100% values corresponded to CD4 surface levels obtained with Nef-mock. Data are mean ± S.D. of three independent experiments.

RESULTS

Non-palmitoyated CD4 Is Efficiently Down-regulated by Nef—Preferential lipid raft localization of Nef (41, 80) and CD4 (49, 50, 81) suggested that Nef-induced CD4 down-regulation may be linked to distribution of both molecules within these membrane microdomains. To test this hypothesis, we first studied the sensitivity to Nef of CD4 mutants, which were targeted to non-raft domains. Both CD4 palmitoylation and its association with p56Lck contribute to the presence of CD4 in rafts (49, 50, 70, 81). Palmitoylation of CD4 occurs on two cysteine residues (Cys-419 and Cys-422, Fig. 1*A*) located in the membrane-proximal region of the CD4 cytoplasmic tail (49, 71). We previously described the design of A2.01 T cells stably expressing either CD4 WT or CD4 palm$^-$ (49). In A2.01 CD4 palm$^-$ cells, CD4 surface levels were similar to those observed in CD4 WT cells (Fig. 1*B*). However, cell fractionation experiments indicated that CD4 palm$^-$ is much less associated with raft fractions (49). With the WT molecule, about 50% of CD4 is found within rafts, whereas this proportion drops below 25% with CD4 palm$^-$ (49). Of note, the cytoplasmic tail of CD4 also carries a di-leucine motif (Fig. 1*A*), which is required for interaction of the molecule with endocytic sorting pathways, as well as for Nef-induced down-regulation (13). As a control, this di-leucine-negative CD4 mutant was stably expressed in the same cell line, yielding A2.01 CD4 LL/AA cells (Fig. 1*A*).

We determined whether these various CD4 molecules were down-regulated by Nef. To this aim, A2.01 cells were transduced with a HIV-1_{LAI} Nef-expressing lentiviral vector. Nef expression, as monitored by Western blot and immunofluorescence, was similar in all cell lines, and was observed in more than 90% of cells (not shown). Flow cytometry analysis indicated that Nef efficiently down-regulated CD4 WT and CD4 palm$^-$ (Fig. 1*B*). Similar results were obtained upon transient transfection of HeLa cells with CD4 and Nef expression plasmids, indicating that this phenomenon is not restricted to A2.01 T cells (Fig. 1*C*). As expected, in A2.01 CD4 LL/AA cells, CD4 surface levels were unaffected by Nef (Fig. 1*B*), whereas MHC-I molecules were significantly down-regulated (not shown).

Binding between CD4 and p56Lck requires two C-terminal cysteine residues (Cys-445 and Cys-447) (82). Mutating these additional cysteines yielded the CD4 palm$^-$ lck$^-$ mutant (Fig. 1*A*), which is associated with rafts at low levels (about 5%, Ref. 49). Surface expression of CD4 palm$^-$ lck$^-$ was particularly low in A2.01 T cells (not shown); likely because p56Lck prevents constitutive endocytosis of CD4 (83). To study the effect of Nef on this mutant, we thus performed transient transfection experiments in HeLa cells (Fig. 1*C*). CD4 palm$^-$ lck$^-$ was down-regulated by Nef as efficiently as CD4 WT. Altogether, these results show that CD4 palmitoylation, as well as CD4 association with rafts, are not required for efficient Nef-induced down-regulation.

Nef Does Not Affect CD4 Association with Rafts and Palmitoylation—We documented further the role of rafts in Nef-induced CD4 down-regulation. We examined whether Nef affects CD4 partition within these microdomains. To this aim, CD4 WT A2.01 T cells expressing (or not) Nef were lysed in 1% Triton X-100 and separated into 10 fractions, using sucrose gradient sedimentation. Low density fractions (fractions 3–5) corresponded to lipid rafts and were enriched in gangliosides like GM1, as detected by the binding of the cholera toxin subunit B (Fig. 2*A*). Higher density fractions (8–10) corresponded to non-raft proteins, soluble in Triton X-100. As expected, CD46, a measles virus receptor known to associate with Triton X-100 soluble membranes (84), was only found in non-raft fractions (Fig. 2*A*).

Immunoblotting with anti-CD4 mAbs and analysis by densitometry indicated that CD4 segregated in both raft and non raft fractions, (40 and 60%, respectively). Of note, equal volumes of each fraction were loaded for the analysis. This allowed a precise assessment of the relative quantities of the studied molecules, regardless of the total protein content. Only a minor amount of the total protein content was recovered in low density fractions (about 5%, not shown), confirming that CD4 is particularly enriched in these fractions. In Nef-expressing cells, the overall amounts of CD4 were decreased. This was not surprising, since Nef significantly reduces the half-life of CD4 by inducing its degradation in a lysosomal compartment (13, 14). Interestingly, the partition of CD4 between raft and non-raft fractions was similar in the absence or presence of Nef (about 40% of CD4 found in low density fractions, Fig. 2*A*).

The hypothesis that Nef induces the localization of CD4 to

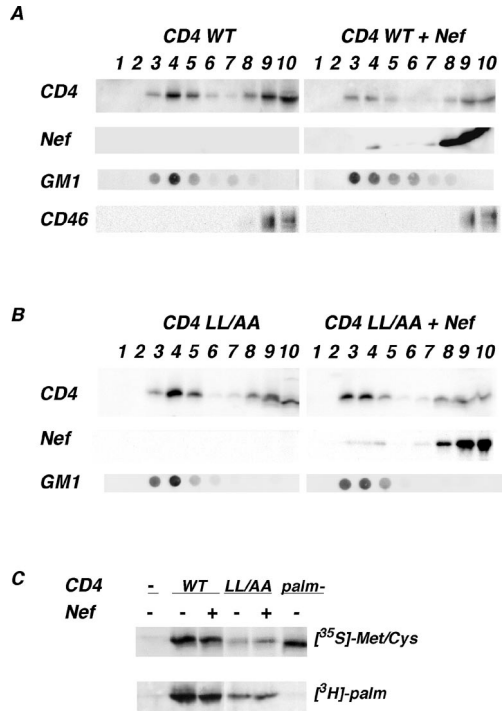

FIG. 2. **Nef does not affect CD4 distribution into rafts and palmitoylation.** *A* and *B*, raft distribution of CD4 WT (*A*) or CD4 LL/AA (*B*) and Nef in A2.01 lymphocytes. A2.01 CD4 WT cells (*A*) or A2.01 CD4 LL/AA cells (*B*) expressing or not expressing Nef were lysed in 1% Triton X-100. Lysates were loaded onto a step sucrose gradient. Equal volume fractions were analyzed by Western blotting for the presence of CD4, Nef, CD46, and GM1. Data are representative of three independent experiments. *C*, CD4 palmitoylation was monitored in A2.01 cells expressing the indicated CD4 molecules in the presence or absence of Nef. Cells were metabolically labeled with [^{3}H]palmitate for 4 h. Cell lysates were then analyzed by immunoprecipitation with an anti-CD4 mAb. Cells were also labeled with [^{35}S]Met/Cys for 3 h to visualize overall amounts of synthesized proteins. The slightly lower levels of labeling of CD4 LL/AA are likely caused by experimental variations in the number of cells used for each point and are independent of Nef expression. Data are representative of two independent experiments.

raft domains of a lesser detergent resistance was also envisaged. This possibility was based on the structural heterogeneity of membrane lipids that form a mosaic of domains in membranes. At low concentrations of Triton X-100 (0.3%), partition of CD4 between rafts and non-rafts was similar with or without Nef (not shown). This confirms that Nef does not significantly affect the partition of CD4 between rafts and non-rafts.

Immunoblotting with an anti-Nef mAb indicated that the viral protein is also found in these low density fractions, albeit at a low ratio (about 2% of total amounts of Nef, Fig. 2*A*). This confirmed previous reports showing that Nef partially associates with rafts (41, 60, 80).

We also examined the effects of Nef on CD4 segregation within rafts in a situation in which CD4 is no longer down-regulated by the viral protein. To this aim, we performed experiments in cells expressing CD4 LL/AA, a mutant that is unable to interact with the clathrin-dependent cell sorting machinery and hence is not modulated by Nef (Fig. 2*B*). No significant differences in raft localization of CD4 LL/AA were observed with or without Nef (Fig. 2*B*). This confirmed that Nef does not modify CD4 segregation within detergent-resistant membrane microdomains, even in the absence of CD4 down-regulation.

We next asked whether Nef has an impact on the palmitoylation state of CD4. To this aim, CD4 WT and CD4 LL/AA A2.01. T cells, expressing or not Nef, were metabolically labeled with [^{3}H]palmitate. Cells were also labeled with [^{35}S]Met/Cys to visualize overall amount of synthesized proteins. After immunoprecipitation with anti-CD4 antibodies, the incorporation of [^{3}H]palmitate or [^{35}S]Met/Cys was analyzed by autoradiography (Fig. 2*C*). Both CD4 WT and CD4 LL/AA incorporated palmitate, regardless of the presence or the absence of Nef. As expected, the CD4 palm$^-$ mutant was normally synthesized but failed to incorporate palmitate (Fig. 2*C*). Thus, Nef efficiently down-regulates CD4 without significantly affecting its palmitoyation state or its raft localization.

Design of a Nef Mutant (NefG3C) That Significantly Accumulates in Rafts—Nef has been previously reported to associate with rafts (41, 80). In A2.01 cells, we observed the presence of the viral protein in detergent-resistant membrane microdomains. However, the proportion of Nef within rafts was rather low (about 2%) (Fig. 2*A*). Similar results were obtained in Nef-expressing Jurkat cells (Fig. 3*B*), indicating that these low levels are not caused by special features of A2.01 cells. We asked whether Nef association with rafts is involved in its biological activities. We thought to design a Nef mutant that preferentially accumulates within rafts. We noticed that a number of tyrosine kinases, including Yes, Fyn, Lck, and Hck are enriched in raft microdomains (61, 85, 86). This accumulation is mediated by a double acylation at the N-terminal end of the kinases. The glycine at position 2 is myristoylated, whereas the cysteine at position 3 is palmitoylated (Fig. 3*A*). Nef is also myristoylated at position 2, which allows association of the protein with cellular membranes but lacks the cysteine residue at position 3. We thus designed the Nef G3C mutant to induce palmitoylation of the additional cysteine (Fig. 3*A*). Wild-type (Nef WT), Nef G3C, as well as a non-myristoylated mutant (Nef G2A) were then stably expressed in Jurkat cells by lentiviral-mediated gene transfer. Cell fractionation experiments indicated a 10-fold increase in levels of NefG3C in low density fractions, when compared with the wild-type protein (Fig. 3*B*). As expected, the non-myristoylated Nef G2A mutant was not detected in rafts (Fig. 3*B*). Furthermore, metabolic labeling experiments indicated that Nef G3C was palmitoylated, whereas neither Nef WT nor Nef G2A incorporated [^{3}H]palmitate (Fig. 3*C*).

We also compared the intracellular localization of wild-type and mutant Nef proteins by immunofluorescence staining and confocal microscopy analysis. Nef WT staining appeared mostly as intracellular dots, located at the plasma membrane and in the perinuclear region (Fig. 3*D*). Intracellular localization of Nef G3C was similar, except that the staining was slightly brighter in some cells (Fig. 3*D*). As previously reported in numerous studies (see for example Refs. 87 and 88), the non-myristoylated Nef mutant showed a diffuse intracytoplasmic staining, consistent with its lack of association with cellular membranes (Fig. 3*D*). Altogether, these results indicate that Nef is not spontaneously palmitoylated and only minimally associated with rafts. Adding a cysteine residue at the N terminus allows palmitoylation of the protein and induces the accumulation of Nef in raft domains.

Impact of Nef Association with Rafts on CD4 and MHC-I Cell Surface Expression—We then evaluated the impact of an increased localization of Nef within rafts by comparing the behavior of Nef WT and Nef G3C in various biologic assays. We first examined the ability of wild-type and mutant Nef proteins to down-regulate surface expression of CD4 and MHC-I molecules. To this aim, transduced Jurkat cells expressing either Nef WT, Nef G3C, or Nef G2A were stained with anti-CD4 and anti-MHC-I antibodies and analyzed by flow cytometry (Fig. 4*A*). Nef WT and Nef G3C efficiently down-modulated both molecules, whereas the non-myristoylated mutant was ineffective. The relative efficiencies of Nef WT and Nef G3C in down-

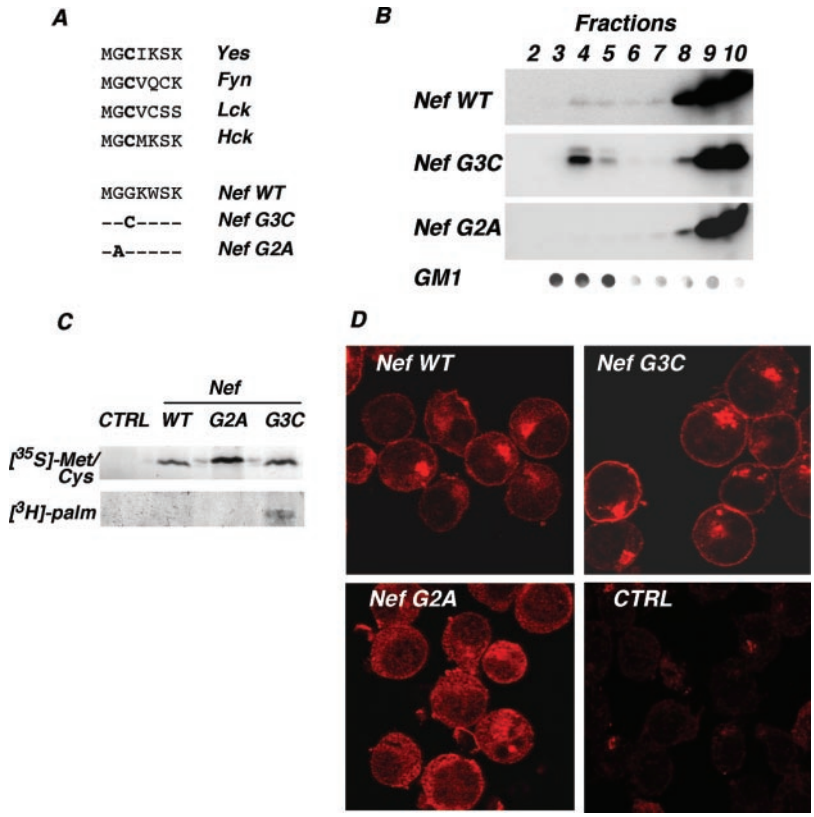

FIG. 3. **Design of a palmitoylated Nef mutant (Nef G3C), which accumulates in rafts.** *A*, amino acid sequence alignment of the N-terminal end of the indicated tyrosine kinases, and of Nef WT and point mutants. Tyrosine kinases are myristoylated (on glycine residue at position 2) and palmitoylated (on cysteine residue at position 3). Nef G3C mutant carries a potential palmitoylation site. Nef G2A is defective for myristoylation. *B*, raft distribution of wild-type and mutant Nef proteins. Jurkat cells were transduced with lentiviral vectors expressing the indicated Nef molecules. Cells were then lysed in 1% Triton X-100, and lysates were loaded onto a step sucrose gradient. Equal volume fractions were analyzed by Western blotting for the presence of Nef and GM1. Data are representative of three independent experiments. *C*, palmitoylation of wild-type and mutant Nef proteins. Jurkat cells expressing the indicated Nef molecules were metabolically labeled with [^{3}H]palmitate for 4 h. Cell lysates were then analyzed by immunoprecipitation with an anti-Nef mAb. Cells were also labeled with [^{35}S]Met/Cys for 3 h to visualize overall amounts of synthesized proteins. Non-transduced cells were analyzed as a negative control (*CTRL*). Data are representative of two independent experiments. *D*, subcellular localization of WT and mutant Nef proteins. Jurkat cells expressing the indicated Nef proteins were stained with anti-Nef mAb. Localization of Nef was examined by confocal microscopy. Non-transduced cells were analyzed as a negative control (*CTRL*). Representative fields are shown. Data are representative of three experiments.

regulating CD4 and MHC-I were then compared in a dose-response transient transfection assay (19). HeLa cells, which are HLA A2-negative, were cotransfected with GFP, CD4, or HLA A2, and different amounts of Nef vectors. In this assay, the levels of Nef expression were proportional to the amount of transfected Nef vector (19). Transfection efficiencies were in the range of 30–50%, as detected by GFP expression 24-h post-transfection (not shown). As expected, CD4 was efficiently down-regulated by Nef WT (Fig. 4*B*). A maximal 90% decrease was observed with 1 μg of transfected plasmid, whereas 50% down-regulation occurred with 0.05 μg of DNA. HLA A2 surface expression was also reduced by Nef, and reached a 70% reduction with 2 μg of DNA. Interestingly, similar dose-response curves were observed with Nef G3C, both for CD4 and HLA A2 (Fig. 4*B*). Of note, Nef G3C efficiently down-regulated CD4 palm$^-$ and CD4 palm$^-$lck$^-$ mutants (Fig. 1*C*). Altogether, these results indicate that accumulation of Nef in rafts does not improve its ability to down-regulate the cell surface expression of CD4 and MHC-I molecules.

Impact of Nef Association with Rafts on HIV-1 Replication—Nef exerts direct positive effects on virus replication in primary human lymphocytes (26, 29–31), probably by facilitating early (entry or post-entry) steps of the replicative cycle (25–28). In some studies, it has been reported that *nef*-deleted HIV (HIVΔnef) replicated poorly only in primary CD4$^+$ cells stimulated after infection, when virus inoculum was low (30, 31). In others, the replicative defect was also manifest in fully activated PBMCs and was independent of the multiplicity of infection (MOI) (26, 29). We have therefore examined the effect of Nef G3C on viral replication in primary lymphocytes. To this aim, we introduced the G3C mutation in the *nef* gene of the X4 strain HIV-1 NL4.3. Wild-type (HIV WT), *nef*-deleted (HIVΔnef), and Nef G3C-encoding (HIV G3C) viruses were produced by transient transfection of 293 T cells. Similar levels of p24 production were obtained in cell supernatants (not shown), strongly suggesting that Nef G3C does not affect the efficiency of viral release from producer cells. Viruses were then used to infect primary lymphocytes. The influence of Nef on viral replication is more marked when lymphocytes are infected before activation (30, 31). PBMCs were thus exposed to viral preparations (at various inoculum, ranging from 20 to 0.02 ng of p24/10^6 cells), and cultures were activated (with PHA and IL-2) 4 days later. Viral replication was then assessed by measuring p24 production in cell supernatants (Fig. 5*A*). At a high MOI (20 ng), we did not observe any significant differences between strains. However, when the MOI was reduced (2 and 0.2 ng of p24), replication of HIVΔnef was barely detectable. Interestingly, HIV WT and HIV G3C replicated with equivalent efficiencies, regardless of the MOI. Exposing cells to 0.02 ng of p24 was below the threshold allowing viral replication (Fig. 5*A*). Of note, we did not observe any differences between HIV WT and HIV G3C with cells from two other donors (not shown).

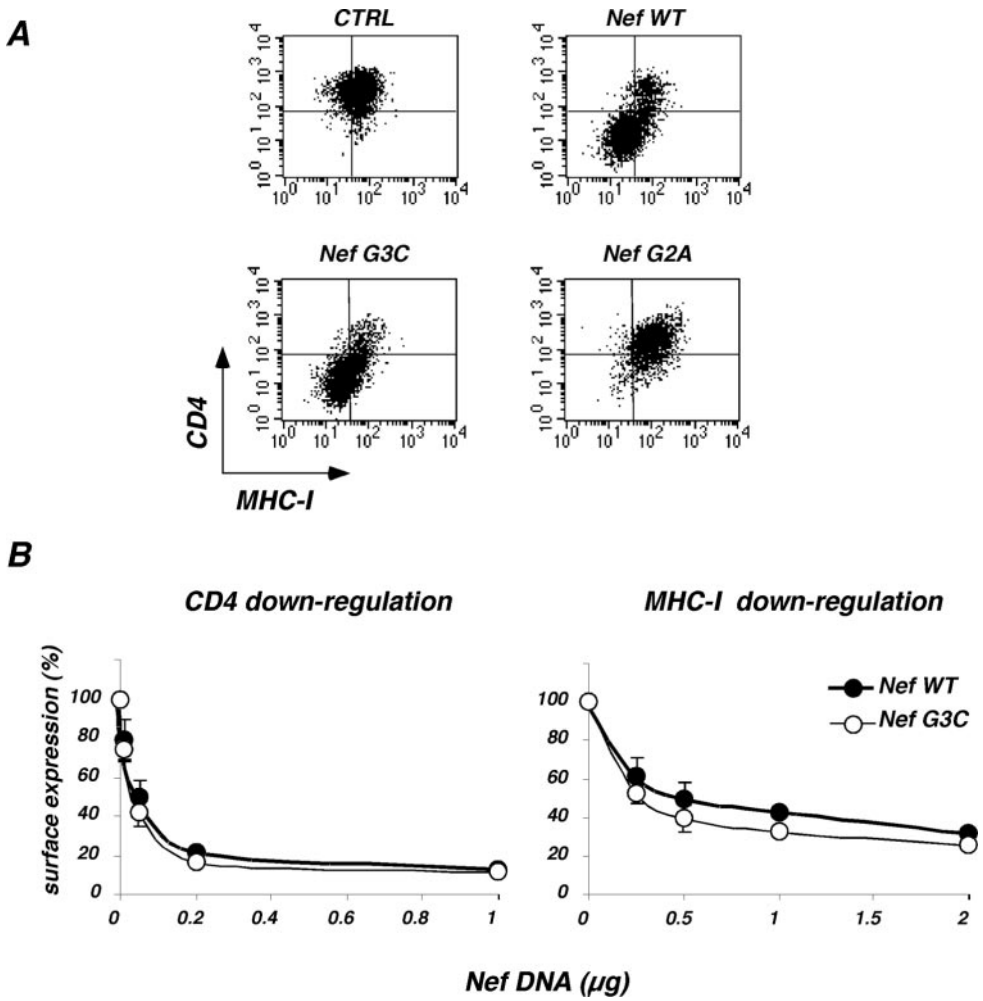

FIG. 4. **Modulation of CD4 and MHC-I by palmitoylated Nef (Nef G3C).** *A*, two-color flow cytometry analysis of Jurkat cells. Surface expression of CD4 and MHC-I was analyzed in Jurkat cells expressing the indicated Nef proteins. Non-transduced cells were analyzed as a negative control (*CTRL*). *B*, dose-dependent CD4 and MHC-I modulation in response to Nef WT and Nef G3C. HeLa cells were transfected with 1 μg of CD4 plasmid (*left panel*) or HLA-A2 plasmid (*right panel*), along with the indicated amounts of Nef WT and Nef G3C plasmids, and 0.075 μg of GFP reporter vector. 24 h later, the surface expression of CD4 (*upper panel*) or HLA-A2 (*lower panel*) was measured in GFP-positive cells by flow cytometry. 100% values corresponded to surface levels obtained in the absence of Nef. Data are mean ± S.D. of three independent experiments.

We also examined viral replication in PBMCs activated by PHA and cultivated with IL-2 prior to infection (Fig. 5*B*). Cells were infected with a low inoculum (0.2 ng of p24/10^6 cells). NL efficiently replicated in this experimental system, and levels of p24 production reached 250 ng/ml at day 6 post-infection. HIVΔnef demonstrated a slight decreased viral replication, with a peak of 100 ng/ml p24 at day 10 post-infection. Again, replication of HIV G3C was similar to that of wild-type virus. We then introduced the *nef G3C* gene in another viral isolate, the R5 strain NLAD8. No significant difference in viral replication was detected between strains encoding Nef WT or Nef G3C (Fig. 5*B*). Similar results were observed at other MOI (not shown).

We next compared the infectivity of HIV, HIVΔnef, and HIV G3C in a single cycle assay, using P4C5 cells as targets. P4C5 cells carry an integrated HIV-LTR *LacZ* cassette, which is activated by Tat upon HIV-1 infection. β-Galactosidase expression levels correlate with infection efficiency in a single cycle viral replication assay (77). As expected, *nef*-deleted virus was at least 5-fold less infectious than its wild-type counterpart (Fig. 5*C*). No significant difference in infectivity was observed between HIV and HIV G3C virions, produced either from transfected 293T cells or from infected PBMCs (Fig. 5*C*). Similar results were observed with NL4.3 and NLAD8 viral strains (Fig. 5*C*).

Altogether, these data confirmed the importance of Nef for optimal viral replication. However, inducing the accumulation of Nef in rafts does not improve virion infectivity or viral propagation in primary lymphocytes, whatever the activation state of the cells, the multiplicity of infection, and the tropism of the virus.

Nef Does Not Affect Raft Segregation of HIV-1 Gag and Env Proteins—A number of studies have provided evidence for the selective assembly and budding of HIV-1 virions from lipid rafts (51–53). The viral envelope glycoprotein is palmitoylated, potentially promoting its localization within rafts (56, 72). The Gag precursor p55 is also preferentially found in rafts, or in raft-like detergent-resistant complexes, where oligomerization and maturation may be facilitated (51–55). Nef has been proposed to improve viral budding from rafts, resulting in an increased viral infectivity (58). We took advantage of the existence of viruses expressing Nef WT and Nef G3C to examine the influence of Nef on the raft segregation of viral structural proteins. To this aim, Jurkat cells were infected with HIV, HIVΔnef, and HIV G3C (NL4.3 strain, 50 ng of p24/10^6 cells). At day 2 post-infection, no significant cytopathic effect was observed, whereas between 16 and 35% of the cells were productively infected, as assessed by intracellular Gag p24 staining and flow cytometry analysis (Fig. 6*A*). Cells were lysed and fractionated with the aim to study raft repartition of viral proteins. Fractions 3–5 and 8–10 of the sucrose gradient were

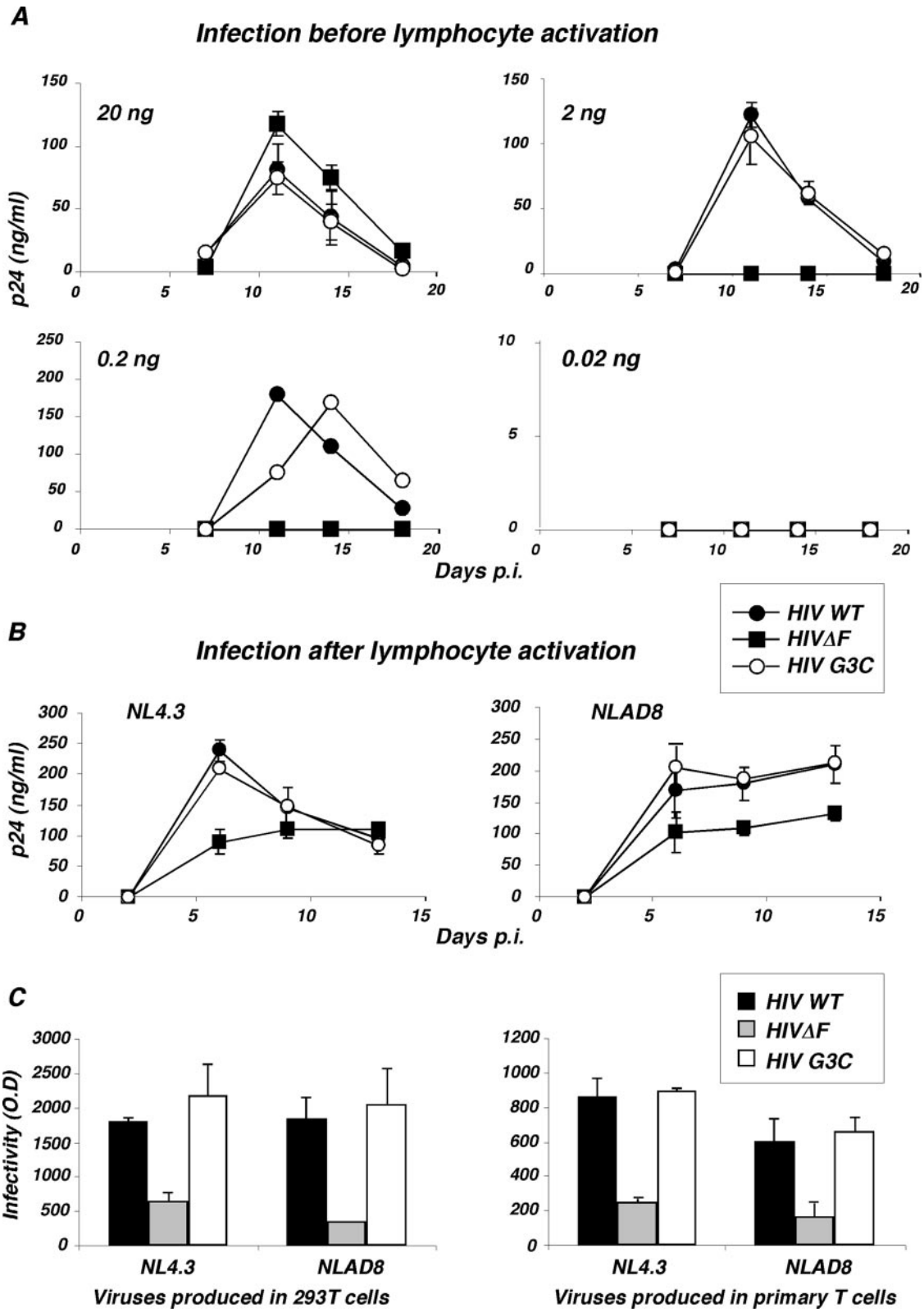

FIG. 5. **Contribution of Nef and palmitoylated Nef to viral replication.** *A*, HIV replication in PBMCs activated 4 days after infection. Non-activated PBMCs were infected on day 0 with isogenic HIV, HIV G3C, and HIVΔnef clones (NL4.3 strain) at four different inoculum (20, 2, 0.2, and 0.02 ng of p24/10^6 cells). Cells were then activated 4 days later and cultivated in the presence of IL-2. Viral replication was followed by measuring p24 release in cell supernatants at the indicated days post-infection. Data are mean ± S.D. of triplicates and are representative of experiments performed with cells from three donors. *B*, HIV replication in activated PBMCs. Viral replication was similarly examined in PBMCs activated 3 days before viral exposure. Viral inoculum was 0.2 ng of p24/10^6 cells. Two different viral isolates were used, the X4 strain NL4.3 (*left panel*) and the R5 strain NLAD8 (*right panel*). Data are mean ± S.D. of triplicates and are representative of experiments performed with cells from three donors. *C*, virion infectivity in a single cycle assay. P4C5 reporter cells (HeLa CD4+ CCR5+ cells carrying an integrated HIV LTR *LacZ* cassette) were exposed to 5 ng of the indicated viruses, produced either by transfection of 293 T cells (*left panel*) or during viral replication in PBMCs (*right panel*). Infection was assessed by measuring β-galactosidase activity in cell extracts 24 h later. Data are mean ± S.D. of triplicates and are representative of three independent experiments. Similar results were obtained at lower MOI (not shown).

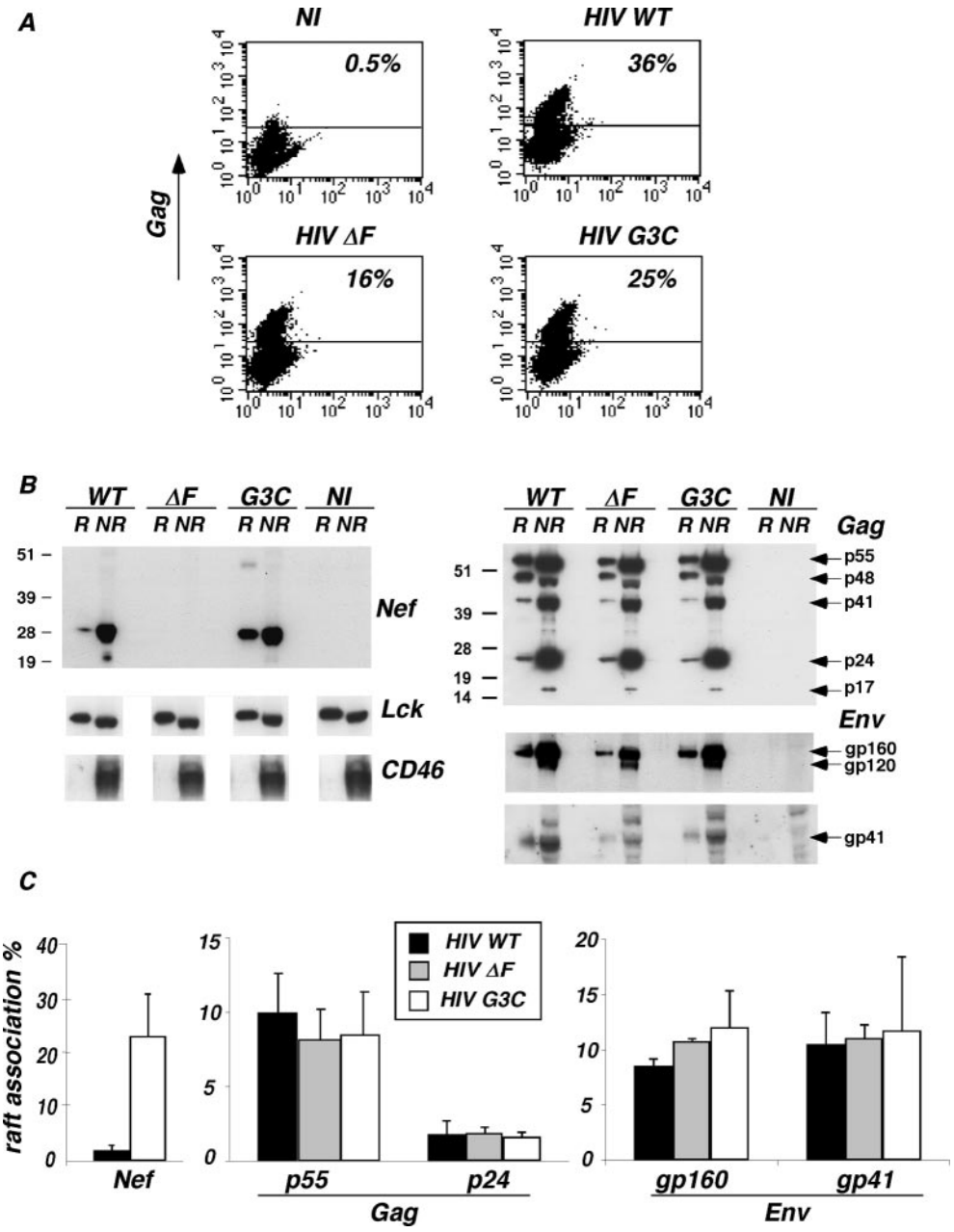

FIG. 6. **Nef does not affect Gag and Env association with rafts during viral replication.** *A*, productive HIV infection of Jurkat cells. Jurkat cells were infected with a high viral inoculum (50 ng of p24/10^6 cells) with the indicated viral strains. At day 2 post-infection, the fraction of productively infected cells (in %) was assessed by p24 intracellular staining and flow cytometry analysis. The *x*- and *y*-axes represent staining with a FITC-labeled control isotypic mAb and a phycoerythrin-labeled anti-p24 mAb, respectively. Background staining was calculated on noninfected cells (*NI*). *B*, raft distribution of Gag, Env, and Nef in Jurkat cells described in *A*. Cells were lysed in 1% Triton X-100. Lysates were loaded onto a step sucrose gradient. Rafts (*R*, fractions 3–5) and non-raft (*NR*, fractions 8–10) fractions were pooled and analyzed by Western blotting for the presence of Nef, Gag, and Env. To verify that the fractionation procedure was correct, blots were probed with mAbs against p56 Lck, which is enriched in rafts, and against CD46, which is only found in non-rafts. Data are representative of three independent experiments. *C*, quantitative analysis of Gag, Env, and Nef proteins in raft and non-raft fractions. Samples treated as in *B* were quantified by densitometry scanning of Western blots, to measure amounts of Nef, Gag (p55 and p24), and Env (gp160 and gp41). The % of each protein found within rafts is depicted. Data are mean ± S.D. of three independent infection experiments.

pooled, allowing the discrimination of raft and non-raft material, respectively. As expected, the tyrosine kinase p56Lck was particularly enriched in raft fractions, whereas CD46 was only found in non-raft fractions (Fig. 6*B*).

We first examined the association of Nef with detergent-resistant membranes. Only a small fraction of Nef WT was found within rafts (Fig. 6*B*). A quantitative analysis of three independent experiments indicated that 2.1% of the viral protein was associated with Triton X-100-resistant domains (Fig. 6*C*), whereas 12-fold more Nef G3C accumulated in these structures (Fig. 6, *B* and *C*). Interestingly, with Nef G3C, the Western blot analysis demonstrated the presence of a band with a higher apparent mass (about 50 kDa), only in the raft fractions (Fig. 6*B*). This species may correspond to Nef dimers, or to a complex between Nef and an uncharacterized protein. The intensity of this slow migrating band was increased when the gel was run under non-denaturing conditions (not shown), suggesting that disulfide bridges are involved in formation of this band. As expected, no anti-Nef reactive signal was detected in non-infected (NI) cells, nor in cells infected with the *nef*-deleted virus (Fig. 6*B*). Altogether, these results indicate that low amounts of Nef can be found within rafts not only when ex-

pressed without other viral proteins, but also in the context of productive HIV infection. The palmitoylated Nef mutant massively accumulates within rafts during viral infection.

Immunoblotting with anti-Gag antibodies revealed interesting features. With wild-type virus, Gag proteins were found in raft and non-raft fractions (Fig. 6*B*). Both Gag precursor (p55), Gag-processing intermediates (p48 and p41), and final products (p24 CA and p17 MA) were detected in soluble fractions. A similar profile of cleavage was observed in low density, detergent-resistant fractions. However, the relative amounts of precursors and processed proteins varied between raft and non-raft fractions. A densitometry analysis of the membrane indicated that the ratio of processed over unprocessed Gag (p24/p55 ratio) was 28% in low density fractions, whereas much more p24 was found in non-raft fractions. Once cleaved, p24 as well as other intermediates will lack the myristoylated anchor and might then be released from cell membranes.

The same profile of Gag repartition and processing was observed with HIV WT, HIVΔnef, and HIV G3C (Fig. 6*B*), suggesting that Nef does not increase Gag cleavage nor association with raft-like structures. The amounts of Gag proteins in each fraction were then precisely quantified by two techniques. Gag p55 and p24 levels were assessed by densitometry scanning, and Gag p24 were also measured by ELISA, using an assay that does not recognize the p55 precursor. With wild-type virus, 10% of Gag p55 was found in low density fractions (Fig. 6*C*). The proportion of processed p24 was lower (1.9% when measured by density scanning (Fig. 6*C*) and 2.5% by ELISA, not shown). Interestingly, similar quantitative results were obtained with HIV WT, HIVΔnef, and HIV G3C viruses, both for Gag p55 and p24 proteins (Fig. 6*C*). Therefore, neither Nef nor Nef G3C affect the partitioning of Gag proteins into detergent-resistant structures.

Of note, similar results were observed when a lower concentration of Triton X-100 (0.3% instead of 1%) was used to lyse cells (not shown). This indicates that Nef does not induce the localization of Gag products to membrane domains of lower detergent resistance.

We then studied the repartition of Env proteins using anti-gp120 and anti-gp41 mAbs (Fig. 6*B*). With wild-type virus, the gp160 precursor as well as gp120 and gp41 mature proteins were found in non-raft fractions. In the three experiments reported in Fig. 6*C*, 8.5 and 10.5% of gp160 and gp41 were found within rafts, respectively. This ratio was similar for HIVΔnef and HIV G3C viruses (Fig. 6*C*).

Altogether, these results indicate that viral structural proteins Gag and Env are significantly enriched in detergent-resistant complexes in HIV-infected Jurkat cells. However, Nef does not seem to participate in this enrichment, because it occurs with a similar efficiency with HIVΔnef and with a virus carrying a palmitoylated mutant of Nef.

DISCUSSION

We have studied here the involvement of rafts at various steps of Nef activity. We have examined the association of Nef with rafts, the potential impact of this association on Nef-induced CD4 and MHC-I down-regulation, the effects of Nef on CD4 palmitoylation and raft localization, the role of raft-associated Nef during viral replication, and the effects of Nef on raft association of Gag and Env. Our results suggest that Nef impacts trafficking of cellular proteins and viral infectivity independently of these membrane microdomains.

Nef has been previously reported to be associated with rafts (41, 58, 80). Our results extend and modify these initial observations. We first performed a series of experiments using Jurkat or A20.1 CD4+ T cells, expressing Nef through lentiviral-mediated gene transfer. Our quantitative analysis indicated that only a minor fraction of Nef (2%) was found associated with detergent insoluble cellular fractions. This percentage is within the range of the overall amount of cellular proteins resistant to detergent treatment. We found equivalent results in cells acutely infected by HIV. Thus, these low levels of raft-associated Nef are similarly observed in the absence of other viral proteins, or during ongoing viral replication. Efficient raft association of intracellular proteins, which do not carry any transmembrane domain, often requires protein modification by multiple acylations. For example, Yes, Lck, Fyn, and Hck tyrosine kinases are enriched in rafts through a process requiring both myristoylation and palmitoylation (61, 85, 86). Nef is a myristoylated protein. Removing the myristoylation site abrogates Nef association with membranes and hence with rafts (41). Nef lacks a N-terminal cysteine potentially allowing palmitoylation. We thus generated a Nef mutant (Nef G3C) carrying this residue. Interestingly, Nef G3C dramatically associated with rafts. About 25% of Nef G3C was found in detergent-resistant membranes. Metabolic labeling experiments indicated that Nef G3C, and not the wild-type protein, is palmitoylated. We conclude from these results that Nef is not particularly enriched in detergent-resistant membranes. Inducing Nef palmitoylation significantly increases raft association of the viral protein. We used this palmitoylated mutant to document further the involvement of rafts in the activity of Nef.

Palmitoylation regulates the trafficking and localization of a number of cellular proteins (61, 62). For example, stable expression at the plasma membrane of Lck, CCR5 or the transferrin receptor requires palmitoylation (85, 89–93). Regarding CD4, both palmitoylation and association with Lck mediate preferential raft localization (49, 50, 70, 81). We show here that CD4 mutants which are no longer palmitoylated, and/or which do not associate with Lck, are still down-modulated by Nef. Moreover, Nef does not significantly affect the partition of CD4 within rafts. Metabolic labeling experiments demonstrated that palmitoylation of CD4 is not affected by Nef. This was true for wild-type CD4 molecules, but also for CD4 LL/AA, a mutant, which is not endocytosed by Nef. Finally, Nef G3C behaves as wild-type Nef in down-regulating wild-type or palmitoyl-free CD4 molecules. MHC-I molecules are also down-regulated by Nef, albeit through distinct mechanisms and trafficking pathways than CD4 (7, 20). MHC-I molecules are not particularly enriched in rafts (not shown). A comparative dose-response analysis indicated that Nef WT was as efficient as Nef G3C for down-regulating MHC-I. Therefore, as for CD4, enrichment of Nef within raft does not improve its activity on MHC-I. We conclude that Nef-induced CD4 and MHC-I down-regulation does not directly involve raft microdomains.

Of note, opposite conclusions were drawn in a recent report (60). Using a LAT-Nef chimera, Alexander *et al.* (60) induced an exclusive localization of Nef within rafts (90% of LAT-Nef was found in rafts). This high raft association was apparently mediated by the presence of a transmembrane domain within LAT, as well as by two palmitoylation sites. A mutant form of the chimera, lacking the cysteine residues allowing palmitoylation (LAT-NefAA), was associated with membranes but excluded from rafts. LAT-Nef was more efficient than LAT-NefAA at down-modulating CD4 and MHC-I, and at enhancing viral infectivity. The authors concluded that rafts are functionally important for Nef activity. However, LAT-Nef was much less active than wild-type Nef. The reason for the poor activity of this chimera is not clear yet, but may be related to the addition of the LAT transmembrane domain and/or to its exclusive accumulation within rafts. Discrepancies between our interpretations and those by Alexander *et al.* may be because of

the use of different Nef proteins carrying or not carrying a transmembrane domain.

We also assessed the contribution of rafts during Nef-mediated enhancement of viral replication. To this aim, we generated an infectious HIV-1 molecular clone expressing NefG3C, and compared replication of HIVΔnef, HIV WT, and HIV G3C. As expected (30, 31), HIVΔnef replicated very poorly in primary lymphocytes activated a few days after viral exposure. In this experimental system, which allows a precise analyze of the involvement of Nef during viral growth, wild-type Nef and NefG3C promoted viral replication with a similar efficiency, regardless of the MOI. Additionally, in single cycle replication assays, HIVΔnef virions were about 5-fold less infectious than their wild-type counterparts, and we did not observe any improvement of viral fitness with HIV G3C.

Whether "classical" rafts are platforms for HIV-1 assembly and budding remains controversial. Evidence for raft localization of Gag was initially based on the observation that a significant fraction of total intracellular Gag protein is resistant to extraction by cold Triton X-100 (51–53). Moreover, a chimeric Gag protein with enhanced raft localization displayed an increase ability to bud from the cell (53). Viral envelope glycoproteins are palmitoylated and also enriched in raft domains (56, 94). These observations led to a model in which rafts act as a meeting point allowing efficient concentration of viral proteins and virion release. However, it has been suggested that Gag proteins assemble in "barge-like" raft structures, of higher density than "classical" rafts, and which fail to incorporate traditional lipid raft components (53–55). Rafts are likely a heterogeneous population, differing in their lipid and protein composition, function, and sensitivity to detergents. Whatever the type of rafts, it has been reported that Gag concentration in detergent-resistant membranes was dependent on Nef (58). We have analyzed here the association of Gag and Env with Triton X-100-resistant structures, during acute HIV infection of Jurkat cells. In agreement with previous reports, we found a strong enrichment of viral proteins in rafts. About 10% of Gag p55 and Gp160 were found in these domains. However, we observed similar raft association of Gag and Env for HIVΔnef, HIV WT, and HIV G3C viruses. The discrepancy with the report by Zheng *et al.* (58) may reflect the use of different experimental systems. These authors isolated lipid rafts from 293T cells transfected with wild-type or Δnef proviruses and did not analyze Env proteins. We used T lymphocytes acutely infected with replicating viruses, which might represent a more relevant model, and we precisely quantified both Gag and Env proteins in raft and non-raft fractions. We conclude from these experiments that Nef does not increase infectivity and replication of HIV by modifying the presence of viral proteins in lipid rafts, at least during acute infection of Jurkat cells.

The membrane of HIV-1 virions contains high levels of cholesterol and sphingomyelin, an enrichment that was explained by the budding of virus through specific membrane microdomains (51, 52, 57). Nef has been proposed to increase the synthesis and transport of cholesterol to the site of viral budding (59). It will be of interest to examine the cholesterol content of HIV G3C virions, to document further the role of lipids in Nef function.

In lymphocytes, rafts represent efficient structures for the assembly of the signaling machinery (42, 95). Rafts are reorganized after TCR engagement, allowing gathering of a number of signaling proteins (CD3zeta, Lck, ZAP70, LAT . . .). Only a small fraction of Nef is found within rafts. It has been proposed that this small fraction represents the functional pool of Nef, priming T cells for activation (41). A recent report demonstrated that Nef activates p21-activated kinase (Pak) via recruitment into lipid rafts (88). In this study, a palmitoylated Nef-GFP fusion protein induced significantly higher levels of Pak activity than the non-palmitoylated protein. It will be now worth examining whether the palmitoylated Nef mutant is a superinductor of T cell activation after TCR stimulation.

An analysis of naturally occurring viral sequences published in the literature or present in the Los Alamos data base, revealed a total absence of Nef proteins carrying a cysteine residue at position 3 (not shown). Given the high mutation rate of HIV during natural infection, this would argue for a negative selection pressure against Nef palmitoylation, and hence against high levels of Nef association with rafts. Whatever the role of Nef during lymphocyte activation, our results strongly suggest that rafts do not significantly contribute to the effects of Nef on CD4 and MHC-I trafficking and on viral replication.

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