Modulation of specific surface receptors and activation sensitization in primary resting CD4$^+$ T lymphocytes by the Nef protein of HIV-1

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Abstract: The human immunodeficiency virus type 1 (HIV-1) pathogenicity factor Nef increases viral replication in vivo. In immortalized cell lines, Nef affects the cell surface levels of multiple receptors and signal transduction pathways. Resting CD4$^+$ T lymphocytes are important targets for HIV-1 infection in vivo—they actively transcribe and express HIV-1 genes and contribute to the local viral burden and long-lived viral reservoirs in patients undergoing antiretroviral therapy. In vitro, this primary cell type has, however, thus far been highly refractory to experimental manipulation, and the biological activities exerted by HIV-1 Nef in these cells are largely unknown. Using nucleofection for gene delivery, we find that Nef induces a drastic and moderate down-regulation of CD4 and major histocompatibility complex type 1 (MHC-I), respectively, but does not alter surface levels of other receptors, the down-modulation of which has been reported in cell line studies. In contrast, Nef markedly up-regulated cell surface levels of the MHC-II invariant chain CD74. The effect of Nef on these three surface receptors was also detected upon HIV-1 infection of activated primary CD4$^+$ T lymphocytes. Nef expression alone was insufficient to activate resting CD4$^+$ T lymphocytes, but Nef modestly enhanced the responsiveness of cells to exogenous T cell activation. Consistent with such a signal transduction activity, a subpopulation of Nef localized to lipid raft clusters at the plasma membrane. This study establishes the analysis of Nef functions in these primary HIV target cells. Our data support the involvement of modulation of a defined set of cell surface receptors and sensitization to activation rather than an autonomous activation function in the role of Nef in HIV-1 pathogenesis. *J. Leukoc. Biol.* 79: 616–627; 2006.

Key Words: *HIV-1 pathogenesis · immune receptors · T cell activation · flow cytometry · nucleofection*

INTRODUCTION

The Nef protein of human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV) contributes critically to the pathogenic potential of these primate lentiviruses by augmenting virus replication in vivo [1–3]. Numerous activities have been ascribed to Nef, including the modulation of surface levels of an array of immune receptors such as CD3, CD4, major histocompatibility complex type 1 (MHC-I), the invariant chain of immature MHC-II (CD74), mature MHC-II, and the costimulatory receptor CD28 [4–8]. These activities depend on the ability of Nef to serve as a sorting adaptor [9]. In addition, Nef modulates intracellular signaling pathways to lower the threshold of T cell activation [10, 11]. In T cell lines, this Nef activity induces transcription of an array of genes almost identical to that triggered upon exogenous stimulation of the T cell receptor (TCR) [12]. The extent of T cell activation imprinted by expression of Nef is a matter of controversy. Although in some studies, Nef facilitates the cell surface exposure of the early activation marker CD69 as well as interleukin (IL)-2 production, others did not observe effects of Nef on T cell activation markers or even reported an inhibitory effect of Nef on T cell activation [10, 13–16]. As an additional function, Nef enhances production and infectivity of HIV particles [17–20]. How these diverse activities translate into the profound effects of Nef on viral replication in vivo is unknown, but it is generally assumed that the combination of several of these activities is required for the role of Nef as a lentiviral pathogenicity factor. A major limitation of previous studies about Nef-mediated modulation of surface receptors has been the almost exclusive use of constitutively hyperactivated, immortalized cell lines expressing unphysiological surface levels of the receptors in question. Conceivably, this may overemphasize or mask effects of the viral protein, which are relevant in primary target cells of HIV infection.

Macrophages and, most importantly, CD4$^+$ T lymphocytes comprise the major targets for productive HIV-1 infection in vivo. In vitro studies with peripheral blood mononuclear cells (PBMC) have indicated that specific cellular characteristics govern the susceptibility of T cells to productive HIV-1 infection, including activation, maturation, and proliferation. Specifically, memory T cells in culture were found to be preferentially infected by HIV-1 [21–23], and resting and/or naive T

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cells are highly resistant to infection [24–26]. The cellular activation and proliferation state also appear to be critical for productive HIV-1 infection in PBMC in vitro [27, 28].

However, several studies revealed that resting, nonproliferating $CD4^+$ T lymphocytes from infected patients harbor HIV-1 and can exhibit active viral gene expression [29–31]. In a SIV/rhesus macaque study, the productive infection of resting T cells was evident shortly following mucosal transmission, supporting a model in which during the early stages of infection, SIV exploits the greater availability of resting T cells to maintain unbroken chains of transmission [30, 32]. Similarly, infected, resting $CD4^+$ T cells appear to contribute significantly to the HIV latent reservoir and can carry unintegrated as well as integrated HIV proviral DNA. In this context, recent work characterized stably integrated, replication-competent viral genomes in resting $CD4^+$ T cells of viremic patients [33]. These findings are supported by studies in the human ex vivo tonsil histoculture model of HIV infection [34, 35]. In this physiological lymphoid microenvironment, naive, resting $CD4^+$ T cells were also found to be productively infected and contributed to the local viral burden. Finally, HIV gene expression, including Nef and p24, can occur under certain conditions in nonproliferating $CD4^+$ T cells also in vitro [36–38]. Thus, resting $CD4^+$ T lymphocytes are targets for HIV-1 infection in vivo, actively transcribe and express viral genes, and may contribute to viral burden and the long-lived viral reservoir in infected patients.

Notably, Nef provides HIV and SIV with a net replicative advantage of up to 2-logs in vivo [1] as well as in ex vivo tonsil cultures [39, 40]. It is remarkable that similar effects of Nef can be recapitulated in vitro only upon infection of cocultures of resting $CD4^+$ T cells with immature dendritic cells or endothelial cells [41–44]. Given that Nef boosts HIV-1 replication almost exclusively in resting but not activated $CD4^+$ T lymphocytes in these experimental, primary cell model systems, it is conceivable that in vivo Nef also exerts activities to optimize virus replication in this cell population and support their evasion from immune recognition.

Because of the high degree of resistance of resting $CD4^+$ T cells in vitro to infection and conventional transfection procedures, little information is currently available about the biological activity of the pathogenicity factor Nef in these cells. In the current study, the recently introduced Amaxa nucleofection procedure allowed us to overcome this technical barrier and to investigate an array of Nef effects on functionally important surface receptors as well as cellular activation in resting primary $CD4^+$ T lymphocytes. We find that Nef expression modulates a specific set of immune receptors at the cell surface and sensitizes resting lymphocytes for T cell activation.

MATERIALS AND METHODS

Plasmids and viruses

Plasmids encoding for the HIV-1_{SF2} Nef wild-type and the NefG2A, NefEDAA, and NefAxxA mutants as green fluorescent protein (GFP) fusion proteins or bicistronically are based on the plasmid enhanced GFP (pEGFP)-N1 or internal ribosome entry site (pIRES)2-EGFP vector (both from Invitrogen, Carlsbad, CA), respectively [45, 46]. Details about the construction of the proviral clones used for virus production in the Western blot analysis will be described elsewhere (O. T. Fackler and Hans-Georg Kräusslich, unpublished). Briefly, *nef* genes from the HIV-1_{SF2} (wild-type or carrying the indicated mutations) were inserted into the HIV-1_{NL4-3} proviral DNA. A HIV-1_{NL4-3} proviral clone lacking an intact *nef* gene was used as *nef*-negative control (Δ*nef*). pBR HIV-1_{NL4-3} IRES-*gfp* plasmids (Δ*nef*, HIV-1_{NA-7} *nef*, or HIV-2_{BEN} *nef*; ref. [47]), encoding replication-competent viruses with a *nef-IRES-gfp* element, were a kind gift of Dr. Frank Kirchhoff (Universitätsklinikum Ulm, Germany). Virus stocks were generated by transfection of proviral HIV plasmids into 293T cells as described [48]. For production of HIV-1-*IRES-gfp* viruses, proviral DNA was cotransfected with an expression plasmid for the G-protein of the vesicular stomatitis virus (VSV-G). Two days after transfection, culture supernatants were harvested. The HIV-1 p24 antigen concentration of concentrated stocks was determined by a p24 antigen enzyme-linked immunosorbent assay.

Primary cells, nucleofection, and T cell activation

Human PBMC from HIV-, hepatitis B virus and C-negative blood donors were purified by Ficoll gradient centrifugation as described [49]. Washed, freshly isolated PBMC were transiently transfected using the nucleofection technology (human T cell Nucleofector kit, 10^7 cells, 5 μg plasmid DNA, Nucleofector Program U-14, Amaxa Biosystems, Germany), according to the manufacturer's instructions. Immediately after nucleofection, cells were transferred into prewarmed culture medium [500 μl RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% L-glutamine (all from Gibco, Grand Island, NY)]. Four hours post-nucleofection, cells were replated in tissue culture plates, which were uncoated or coated with affinity-purified goat anti-mouse immunoglobulin G (H^+L; 10 μg/ml, Jackson ImmunoResearch, West Grove, PA), followed by anti-human CD3 monoclonal antibodies (mAb; 1 μg/ml, clone HIT3a, NA/LE, BD PharMingen, San Diego, CA) and anti-human CD28 mAb (1 μg/ml, clone CD28.2., NA/LE, BD PharMingen). Where indicated, T cell activation was performed by treatment with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (1 μM; both from Sigma Chemical Co., St. Louis, MO) or by treatment with phytohemagglutinin-P (PHA-P; 1 μg/ml, Sigma Chemical Co.) and human recombinant IL-2 (20 nM, kind gift from Chiron Corp., Emeryville, CA).

Virus infection

For the evaluation of Nef expression levels in virus infection, freshly isolated PBMC were stimulated with PHA-P/IL-2 for 48 h, infected with HIV-1 virus stocks, and cultured in the presence of IL-2 for 3 days. Subsequently, an aliquot of the cells was analyzed by flow cytometry for intracellular HIV-1 p24 expression to quantify the number of productively infected cells as recently described [46], and aliquots corresponding to 2×10^4 productively infected cells were analyzed for Nef expression by Western blotting. To monitor effects of Nef (from strain HIV-1_{NA-7}) on cell surface receptor levels of CD74, CD4, and MHC-I in HIV-1-infected $CD4^+$ T cells, activated cells were challenged with concentrated stocks of VSV-G-pseudotyped, replication-competent HIV-1_{NL4-3}-based viruses carrying a *nef*$_{NA7}$ *IRES gfp* element or a Δ*nef IRES gfp* element. Four days post-infection, cells were fixed, stained with the respective mAb, and analyzed by flow cytometry.

Western blotting

Comparable numbers of GFP-positive, plasmid-transfected resting PBMC or p24-positive, HIV-infected, PHA-P/IL-2-activated peripheral blood lymphocytes (PBL) were subjected to denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with a sheep α-Nef-glutathione *S*-transferase antiserum as described [46].

Flow cytometry

Pelleted cells were stained in fluorescence-activated cell sorter (FACS) medium [3% FBS, 0.05% Na-azide in phosphate-buffered saline (PBS)] with the following nonconjugated, phycoerythrin (PE)-, peridinin-chlorophyll (PerCP)-, or allophycocyanin (APC)-conjugated mouse mAb: anti-human CD3 (clone SK7, BD PharMingen), anti-human CD4 (clone RPA-T4, BD PharMingen), anti-human CD8 (clone SK1, BD PharMingen), anti-human CD25 (clone M-A251, BD PharMingen), anti-human CD28 (clone CD28.2, BD PharMingen), anti-human CD69 (clone FN50, BD PharMingen), anti-human CD74

(clone M-B741, Ancell Corp., Bayport, MN), anti-human leukocyte antigen (HLA)-ABC (clone W6/32, DakoCytomation, Carpinteria, CA; clone G46-2.6, BD PharMingen), anti-HLA-A2 (clone BB7.2, BD PharMingen), or anti-human HLA-DR (clone TU36, BD PharMingen). A FACSCalibur with BD CellQuest Pro 4.0.2 software (BD PharMingen) was used for analysis.

Statistical analysis of flow cytometric data

To quantitatively analyze the flow cytometric data, arbitrary gates were introduced into FACS dot blots [see, for example, **Fig. 1**, F–H, N (none), L (low), M (medium), H (high)] to distinguish amongst cells expressing different levels of Nef.GFP proteins. The mean fluorescent intensity (MFI) in the N gate of each sample was set to 100%, and the MFIs in the other gates were calculated as a relative percentage. These relative cell surface receptor levels, following expression of wild-type and mutant Nef proteins, were determined in independent experiments with cells from individual donors (n=3–6). These relative percentages, reflecting the degree of receptor modulation from each of these independent experiments, were used to calculate the arithmetic mean and standard deviation of the down-regulation of the cell surface receptors for all donors (see **Figs. 2** and **3**). The statistical evaluation by Student's *t*-test was based on these values obtained from all independent experiments conducted (***, $P<0.0005$; **, $P<0.005$; *, $P<0.05$).

Intracellular IL-2 staining

PBMC were nucleofected with the Nef.GFP expression constructs and analyzed for intracellular IL-2 by flow cytometry 37 h post-transfection as described [50]. During the entire post-nucleofection period, cells were left unstimulated or cultured on anti-CD3/anti-CD28 mAb-coated plates. Where indicated, previously untreated cells were stimulated with PMA/ionomycin for 4 h prior to analysis. Starting 32 h post-nucleofection, cells were cultivated for 5 h in the presence of the intracellular transport inhibitor Brefeldin A (10 μg/ml, Sigma Chemical Co.). Cells were fixed and permeabilzed using the Cytofix/Cytoperm

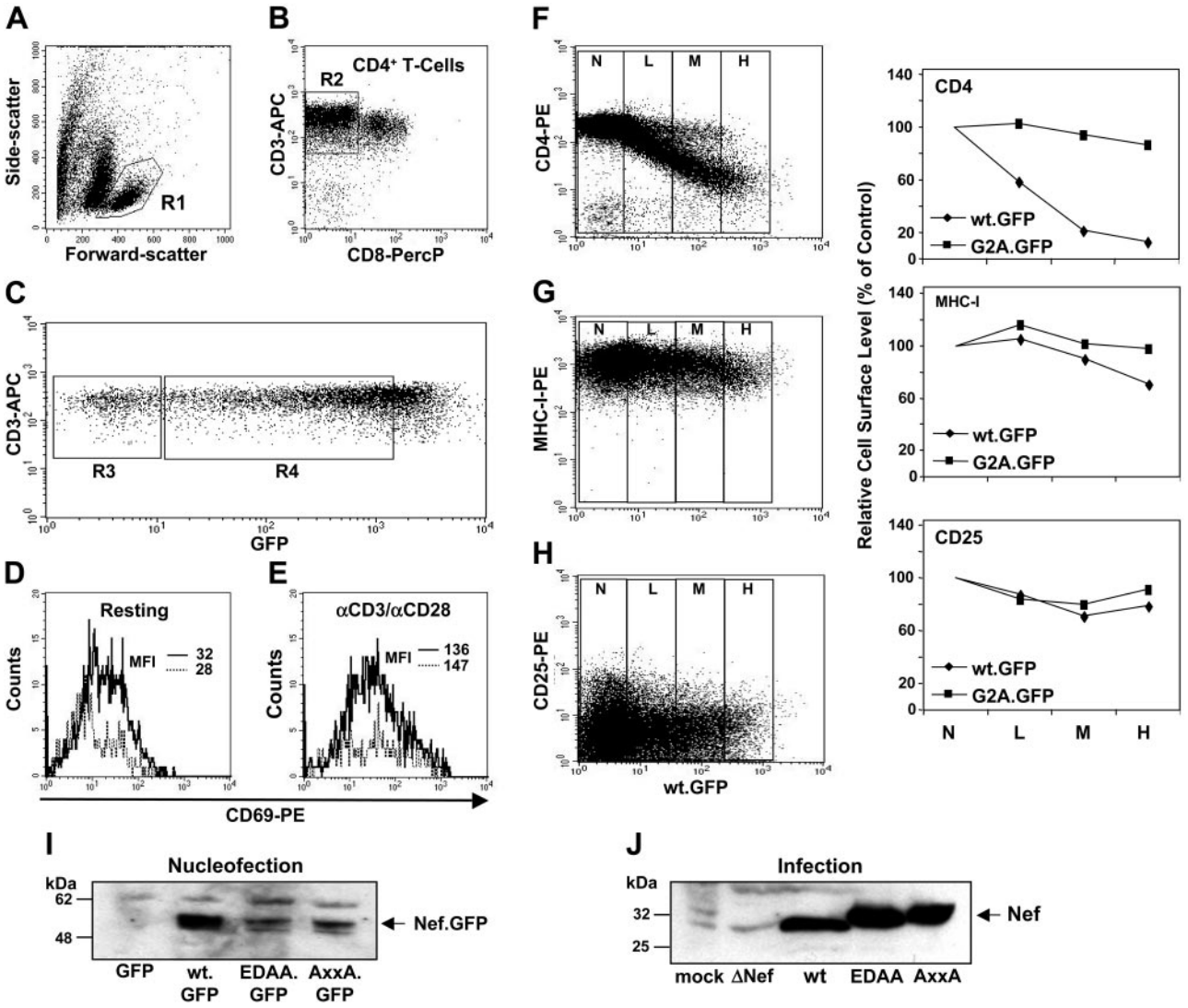

Fig. 1. Analysis of nucleofected resting $CD4^+$ T cells, which were transfected with an expression plasmid for GFP using Amaxa nucleofection, and live (A, gate R1) $CD3^+/CD8^-$ (B, gate R2) $CD4^+$ T lymphocytes were analyzed 36 h post-transfection. CD69 surface levels of GFP-negative (C, gate R3; D and E, dotted lines) or GFP-positive (C, gate R4; D and E, solid lines) $CD4^+$ T cells 36 h post-nucleofection. Note that high GFP-expressing cells were excluded from the analysis. Cells were cultured without stimulation (D, Resting) or activated by triggering the TCR and CD28 (E, αCD3/αCD28). (F–H) FACS analysis of surface-expressed CD4 (F), MHC-I (G), and CD25 (H) on resting $CD4^+$ T lymphocytes 36 h post-nucleofection. Original dot plots are shown on the left. Gates indicate no (N), low (L), medium (M), and high (H) GFP expression levels. On the right, cell surface receptor levels in the N gate were set to 100%, and the MFIs of the other gates were plotted relative to that. (I, J) Western blot analysis of Nef expression levels in resting PBMC expressing wild-type.GFP (wt.GFP) following nucleofection (I) or in PHA-P/IL-2-activated PBMC infected with the indicated HIV-1 recombinants (J). The percentage of GFP- or HIV-1 p24-positive cells was determined by flow cytometry, and cell lysates corresponding to 2×10^4-positive cells were analyzed by anti-Nef Western blotting in parallel on the same SDS-PAGE.

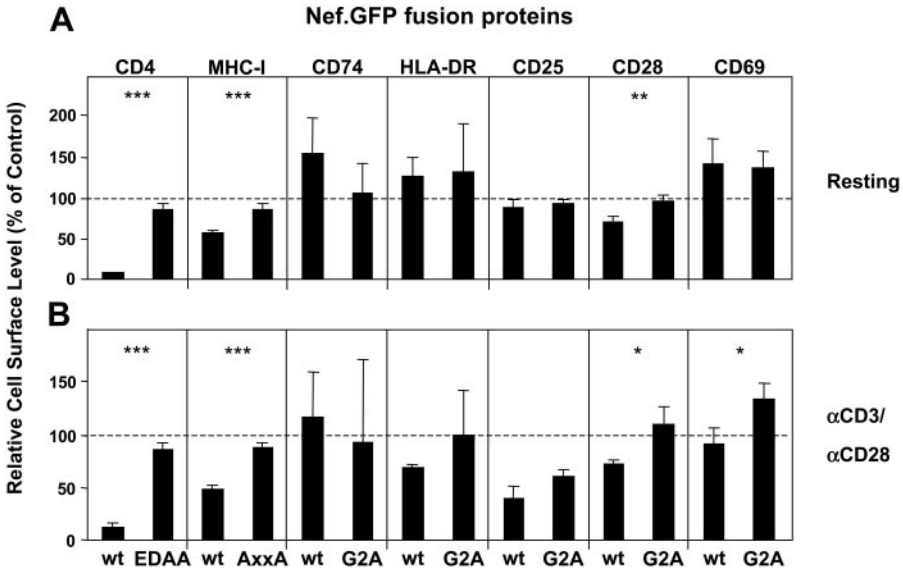

Fig. 2. Modulation of cell surface levels of receptors on $CD4^+$ T cells by GFP fusion proteins of Nef. Cells were transfected by nucleofection with the indicated expression constructs and analyzed by flow cytometry 36 h post-transfection without stimulation (A) or following activation on anti-CD3/anti-CD28 mAb-coated plates (B). Presented are receptor cell surface levels of live $CD4^+$ T cells with high GFP expression relative to GFP-negative cells in the same sample, which were set to 100%. Values represent the mean plus standard deviation from experiments with PBMC from three to six donors (see Materials and Methods for details). The statistical significance of the differences in cell surface levels in the presence of wt.GFP relative to the indicated Nef mutants was analyzed by Student's *t*-test (***, $P<0.0005$; **, $P<0.005$; *, $P<0.05$).

kit (BD PharMingen) and subsequently stained with a PE-conjugated anti-human IL-2 mAb (clone MQ1-17H12, BD PharMingen).

Lipid raft clustering and confocal microscopy

The analysis of lipid raft incorporation of GFP or Nef.GFP fusion proteins was essentially carried out as described for Jurkat cells [45]. Prior to nucleofection, $CD4^+$ T lymphocytes were isolated by depletion of $CD8^+$ T cells from freshly prepared PBMC using a PE-conjugated anti-CD8 mAb followed by anti-PE-mAb-coated magnetic cell sorter microbeads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. Subsequently, nucleofection was performed as described for bulk PBMC. Eighteen hours post-transfection, cells were incubated with Alexa 594-conjugated cholera toxin (CTx; Molecular Probes, Junction City, OR) for 30 min on ice, subsequently cross-linked by incubation with anti-CTx antibody (Sigma Chemical Co.) for 30 min at 4°C and 10 min at 37°C, seeded on poly-L-lysine-coated coverslips, and fixed with 3% paraformaldehyde (PFA)/PBS (CTx clustering). A fraction of the cells from the same transfection was seeded on coverslips without prior cross-linking and stained with CTx following PFA fixation (CTx staining). Images were taken with an LSM 510 confocal laser scanning microscope (Zeiss, Thornwood, NY), attached to an Axiovert inverted microscope with a 100× oil immersion objective, and processed using Adobe Photoshop (San Jose, CA).

RESULTS

Efficient transfection of primary, resting $CD4^+$ T lymphocytes by nucleofection

Nucleofection is an electroporation-based method using a karyophilic solution, which allows for rapid nuclear delivery of plasmid DNA without requiring nuclear membrane breakdown. We first sought to explore the suitability of the nucleofection procedure for the analysis of Nef functions on surface receptors and the activation state in this cell type. Nonstimulated PBMC from healthy donors, freshly isolated by Ficoll-gradient centrifugation, were nucleofected with the pEGFP-N1 expression vector. Thirty-six hours post-transfection, a prominent population of cells was viable, as judged by flow cytometric forward-/side-scatter analysis (Fig. 1A, gate R1) or 7-amino-actinomycin D (7-AAD) staining (data not shown). $CD4^+$ T lymphocytes were identified indirectly as $CD3^+/CD8^-$ (Fig. 1B, gate R2) to also allow subsequent assessment of the effect of Nef on CD4 cell surface levels. Routinely, 30–65% of $CD4^+$ T cells expressed the GFP transgene. To clarify whether cells were activated by the nucleofection procedure per se, we quantified the surface expression of activation markers on GFP-positive cells (Fig. 1C, gate R4) relative to GFP-negative cells (Fig. 1C, gate R3). No significant differences were detected for CD69 (Fig. 1D) or the late activation markers CD25 and HLA-DR (data not shown). As an activation control and staining reference, transfected resting cells from the same donor were activated by cultivation on tissue-culture plates coated with anti-CD3/anti-CD28 antibodies. GFP-positive and -negative cells exhibited a marked and comparable induction of CD69 (approximately fourfold, Fig. 1E). Thus, nucleofection allowed for efficient transfection of resting, primary $CD4^+$ T lymphocytes without inducing an elevated activation state and without impairment of the responsiveness of cells to T cell activation.

Comparable Nef expression levels in nucleofected and infected resting $CD4^+$ T cells

Next, we compared expression levels of Nef in resting PBMC following nucleofection of expression plasmids encoding different GFP fusion proteins of Nef from HIV-1_{SF2} to expression levels in PHA-P/IL-2-activated PBMC, which were productively infected with HIV-1_{NL4-3} carrying or lacking various nef_{SF2} genes [46]. Based on flow cytometric analyses, 2×10^4 cells, which were positive for GFP (nucleofection) or HIV-1 p24 (infection), respectively, were analyzed by Western blotting with an anti-Nef antibody. Nucleofection (Fig. 1I) resulted in transient Nef expression levels, which were comparable or lower with those achieved by productive HIV infection (Fig. 1J), demonstrating that this transfection procedure does not result in unphysiologically high Nef expression levels. Expression of the mutant Nef.GFP proteins was slightly reduced relative to wild-type.GFP, most likely as a result of the loading normalization based solely on absolute numbers of GFP-positive cells and not on relative expression levels of the GFP-positive cell population. It is important that no significant proteolytic cleavage resulting in the accumulation of GFP was

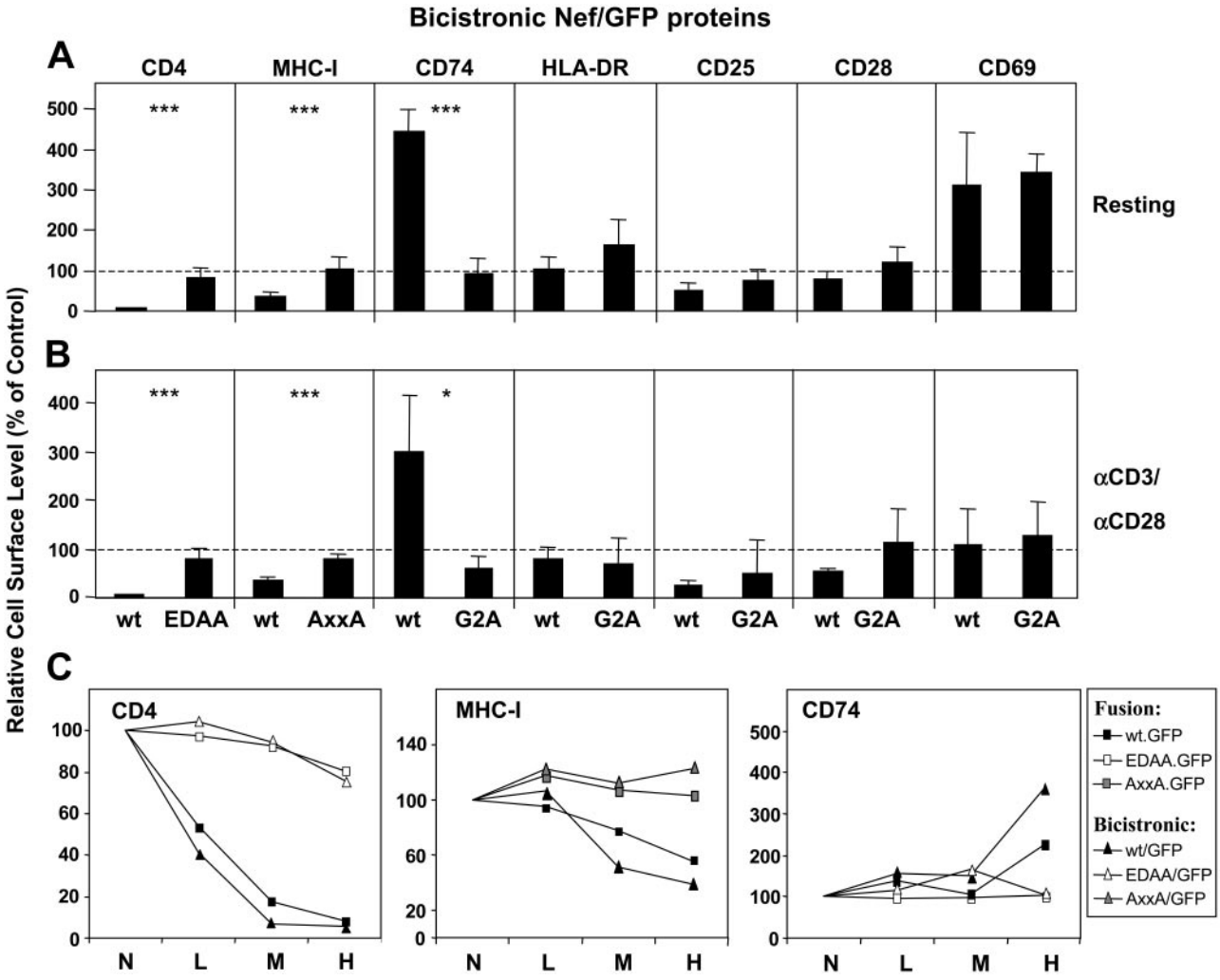

Fig. 3. Modulation of cell surface levels of receptors on CD4$^+$ T cells expressing Nef from the bicistronic pIRES2-eGFP vector. Analyses were performed in analogy with those described in the legend to Figure 2. (C) Effects of the indicated GFP fusion proteins (wt.GFP, EDAA.GFP. AxxA.GFP) or bicistronically expressed Nef proteins (wt/GFP, EDAA/GFP, AxxA/GFP) are shown side-by-side for all GFP intensities.

detected for any of the Nef mutants, thus allowing for a direct correlation between GFP positivity and Nef.GFP expression levels in the subsequent FACS-based functional analyses.

Nef down-regulates CD4 and to a lesser extent, MHC-I from the surface of resting CD4$^+$ T cells

As a first functional characterization, we assessed the effect of Nef.GFP (wt.GFP) on surface levels of CD4, MHC-I, and CD25 in resting CD4$^+$ T cells (Fig. 1, F and G). The NefG2A.GFP mutant (G2A.GFP), which is to a large degree functionally impaired as a result of its lack of myristoylation and membrane association, served as a negative control. Arbitrary gates were introduced [Fig. 1, F–H, N (none), L (low), M (medium), H (high)] to distinguish amongst cells expressing different levels of the Nef proteins. The MFI in the N gate of each sample was set to 100%, and the MFIs in the other gates were plotted relative to that. For CD4, a strong and dose-dependent down-modulation was induced by wt.GFP, and ~10% of surface-exposed CD4 remained on high expressing cells (Figs. 1F and 2A). Modulation of MHC-I was much less pronounced (Figs. 1G and 2A): Significant and specific down-modulation (ranging between 39% and 45%) was only detected in cells expressing high levels of the wt.GFP protein. This is in agreement with results from immortalized cell lines, in which significantly higher Nef expression is required for the down-modulation of MHC-I than for down-modulation of CD4 [51]. These moderate effects on cell surface MHC-I levels were observed when overall MHC-I levels (mAb W6/32, Figs. 1G and 2A) or the specifically Nef-sensitive HLA-A2 (mAb BB7.2) were analyzed (data not shown). In contrast, the cell surface levels of CD25 remained unaffected (Fig. 1H). Thus, in resting CD4$^+$ T cells, expression of HIV-1 Nef reduces the cell surface exposure of CD4 and MHC-I molecules, two well-established, receptor-modulating activities of the viral protein previously reported in cell lines.

Nef's receptor-modulating capacity is selective in resting and activated CD4$^+$ T cells

Subsequently, we performed a comprehensive analysis of specific effects of Nef on surface levels of receptors, which have

been reported to be targeted by the pathogenicity factor. Receptor levels on high GFP-expressing $CD4^+$ T cells (H) relative to non-GFP-expressing $CD4^+$ T cells (N) were analyzed independently in PBMC from three to six donors, and the statistical significance of the results was verified by Student's *t*-test. For CD4 and MHC-I, mutations in Nef, disrupting its interaction with the endocytic machinery (EDAA) [52] or with SH3 domain-containing proteins (AxxA) [53], respectively, were used as reference control (see ref. [46] for details about constructs). For CD74, HLA-DR, CD25, CD28, and CD69, the NefG2A mutant was used as a specific negative control.

It is notable that little donor variability was observed. Among the seven receptors analyzed, only surface exposure of CD4 and MHC-I was affected by wt.GFP fusion proteins with the highest statistical significance (Fig. 2A, $P<0.0005$). Down-regulation of CD28 by the wt.GFP fusion protein was detectable, albeit with lower significance ($P<0.005$). For the activation markers CD25 and HLA-DR as well as for CD74, no specific changes in surface exposure were noted. The early activation marker CD69 was slightly up-regulated in cells expressing wt.GFP but also in cells expressing the myristoylation-deficient G2A.GFP protein. Similarly, we failed to detect Nef-induced alterations of cell surface CD3 (data not shown), which agrees with the finding that this receptor is targeted by Nef from SIV, but not HIV-1 [54].

To address whether exogenous T cell activation of resting $CD4^+$ T cells could affect the receptor-modulating activity of Nef, cells were stimulated on anti-CD3/anti-CD28 mAb-coated plates subsequent to nucleofection (Fig. 2B). The pattern observed in activated $CD4^+$ T cells was remarkably similar to that seen in resting cells—CD4 and MHC-I were the main receptors down-modulated by Nef ($P<0.0005$). The slight reduction of CD28 and CD69 surface levels induced by wt.GFP relative to the G2A.GFP control upon activation of $CD4^+$ T cells occurred with lower statistical significance ($P=0.021$ and 0.027, respectively). Again, as seen before for unstimulated cells, no wt.GFP-specific changes were found for the three activation markers or CD74 on activated $CD4^+$ T cells. Of note, the late activation marker CD25 was reduced approximately twofold by wt.GFP as well as by the nonmyristoylated mutant. We conclude that cell surface receptor modulation of Nef.GFP fusion proteins is restricted to a specific set of molecules in primary $CD4^+$ T lymphocytes.

Bicistronically expressed HIV-1 Nef markedly up-regulates CD74

For functional in vitro analyses, some laboratories routinely use carboxy-terminal GFP fusion proteins of Nef [45, 51, 55], and other groups use Nef, expressed independently from GFP using bicistronic expression vectors [47, 56]. However, the relative activity of Nef under these experimental approaches has not been addressed in primary HIV target cells. At the protein level, the ratio of Nef:GFP was 1:1 for the fusion constructs, and the bicistronic pIRES2-EGFP vectors yielded an ~5:1 ratio [46]. For most surface receptors, results obtained for bicistronically expressed Nef (wt/GFP) matched those seen with the wt.GFP fusion protein in resting as well as in activated $CD4^+$ T cells (Figs. 2 and 3, A and B). For CD4 and MHC-I down-modulation, the slightly higher potency of wt/GFP compared with wt.GFP within identical GFP gates (Fig. 3C, L, M, H) likely reflects the approximate fivefold higher relative Nef expression levels. It is notable that no specific modulation of CD28, CD69, and CD25 was observed for wt/GFP relative to the G2A/GFP control, and CD69 was up-regulated in resting cells and CD25 down-regulated in activated cells expressing both Nef proteins. It is surprising that although wt.GFP had no effect on CD74 (Fig. 2), wt/GFP enhanced CD74 levels approximately fourfold (Fig. 3A, $P<0.0005$). As wt/GFP-expressing cells with a fivefold lower GFP intensity than the highest wt.GFP-expressing cells analyzed still displayed notable up-regulation of CD74, the increased Nef expression from the bicistronic constructs is unlikely to fully account for this striking difference. Thus, these results suggest a specific role of the Nef carboxyterminus in this activity, which is masked in the context of the Nef.GFP fusion protein. In summary, with the notable exception of CD74, bicistronically expressed Nef proteins affect cell surface receptor densities with comparable specificity and efficiency as Nef.GFP fusion proteins in primary $CD4^+$ T cells.

Nef modulates cell surface levels of CD4, MHC-I, and CD74 also in activated HIV-1-infected $CD4^+$ T cells

We next sought to confirm the cardinal effects of HIV-1 Nef on the cell surface exposure of CD4, MHC-I, and CD74 molecules in the context of a HIV-1 infection. As resting $CD4^+$ T lymphocytes are refractory to productive HIV-1 infection, and our above analyses had demonstrated that Nef acts on these three receptors independently of the cellular activation state (Figs. 2 and 3), this analysis was performed in PHA-P/IL-2-activated $CD4^+$ T cells, which were infected with VSV-G-pseudotyped HIV-1 *IRES gfp* viruses, lacking a *nef* gene (**Fig. 4A**) or carrying a $nef_{\text{HIV-1 NA-7}}$ gene (Fig. 4B). In HIV-infected T cells, cell surface levels of CD74 were up-regulated drastically in a Nef-dependent manner, ranging from seven- to 11-fold for different donors (Fig. 4C). Simultaneous monitoring of CD4 and MHC-I surface levels on infected cells (open and shaded bars, respectively) revealed a marked down-modulation of both receptors by Nef to levels ranging between 25% and 40% of those present on cells infected with the Δ*nef* virus (solid bars; Fig. 4D). It is notable that the seemingly low activity of Nef for CD4 down-modulation in this experimental setting probably underlies the additional CD4 down-modulating activity of two other HIV-1 proteins, namely Vpu and Env [57]. In contrast, Nef is the only viral gene product that affects cell surface levels of MHC-I in HIV-1-infected T cells (data not shown). Thus, the three major receptor modulation activities of Nef observed in nucleofected resting $CD4^+$ T lymphocytes can also be detected in activated primary $CD4^+$ T cells infected with HIV-1.

Nef expression is insufficient to trigger IL-2 production in resting $CD4^+$ T lymphocytes but enhances their responsiveness to exogenous activation

Given the ability of Nef to interfere with signal transduction and cellular activation, we next investigated whether Nef ex-

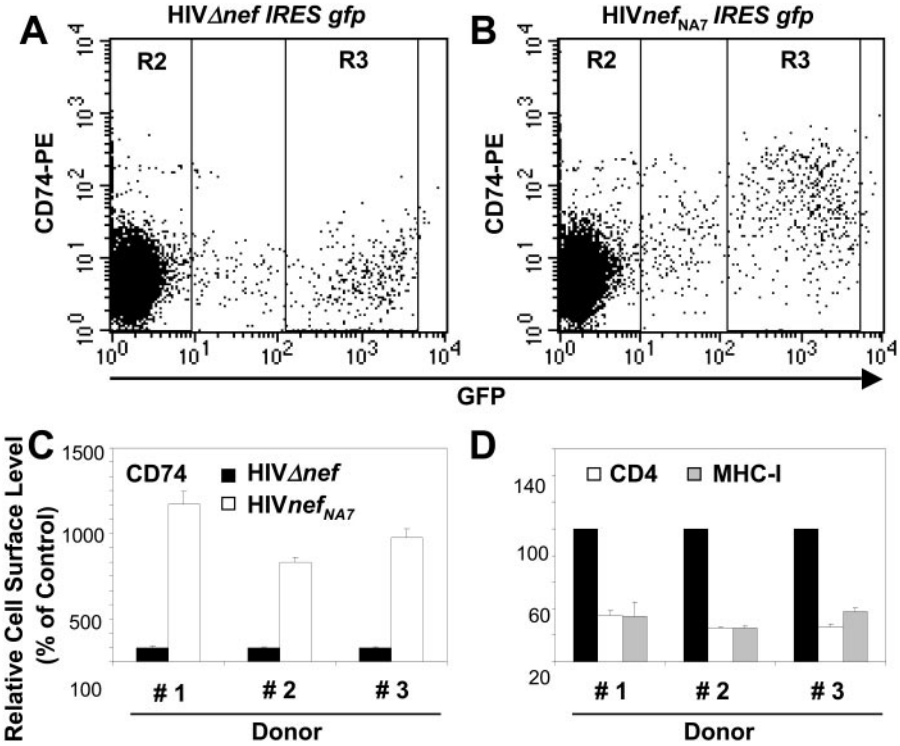

Fig. 4. HIV-1 Nef up-regulates CD74 and down-regulates CD4 and MHC-I in infected primary $CD4^+$ T lymphocytes. PHA-P/IL-2-activated primary human PBL were infected with replication-competent VSV-G-pseudotyped HIV-1 *nef*$_{NA7}$ *IRES gfp* or HIV-1$_{NL4-3}$ Δ*nef IRES gfp* viruses. Four days post-infection, cells were fixed, stained with the respective mAb, and analyzed by flow cytometry. FACS dot plots of cell surface levels of CD74 (*y*-axis) relative to GFP expression (*x*-axis) are shown (A, B). The MFI of cell surface CD74 (C) and CD4 and MHC-I (D) was quantified on infected cells (gate R3) relative to the MFI of GFP-negative, noninfected cells (gate R2) in principle, as described in the legend to Figure 1. Values obtained for cells infected with HIV Δ*nef* viruses (solid bars) were arbitrarily set to 100%. Shown are arithmetic means of triplicates + SD from three different donors.

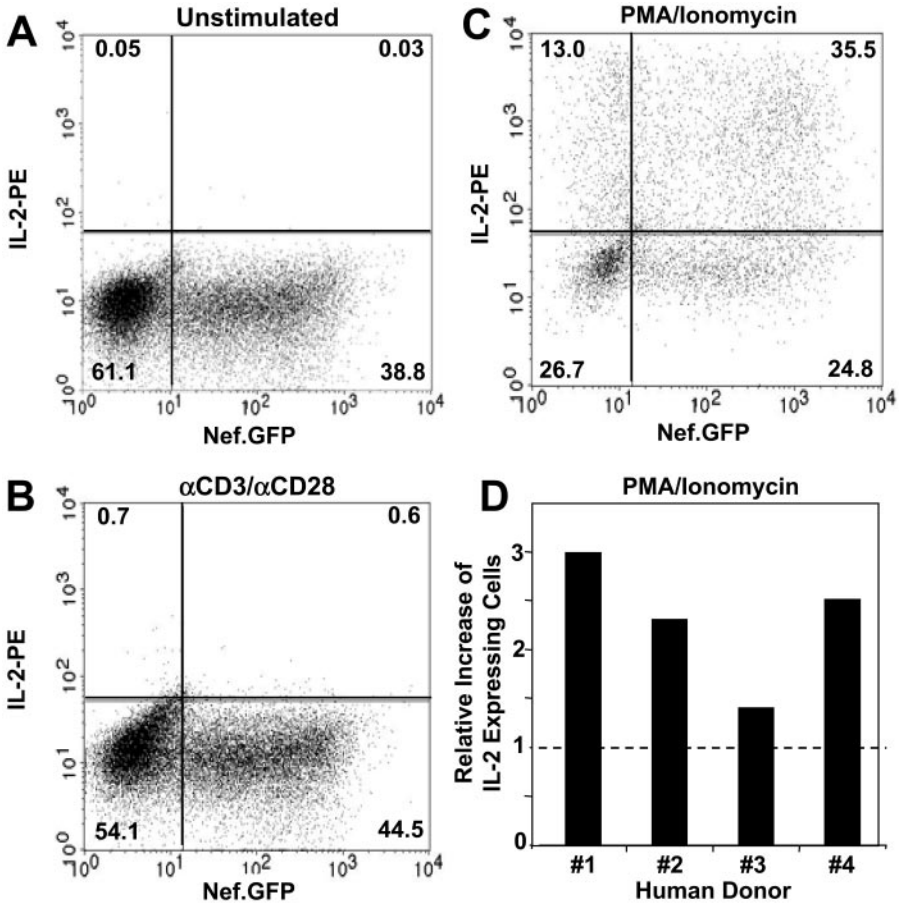

Fig. 5. Effect of Nef.GFP on IL-2 production in $CD4^+$ T lymphocytes. PBMC were nucleofected with the Nef.GFP expression constructs and analyzed for intracellular IL-2 by flow cytometry 37 h post-transfection without stimulation (A), following cultivation on anti-CD3/anti-CD28 mAb-coated plates (B) or following PMA/ionomycin stimulation (C). Numbers indicate the percentage of cells in the respective quadrants. (D) Quantitative representation of the effects of Nef.GFP on IL-2 production upon PMA/ionomycin treatment. The ratios of IL-2-positive cells were calculated for transfected cells (Nef.GFP-positive, upper right vs. lower right quadrant) and nontransfected cells (Nef.GFP-negative, upper left vs. lower left quadrant). The ratio of these values was used to express the relative increase of IL-2-expressing cells upon Nef expression. Shown are results for PBMC from four different donors.

pression in resting CD4$^+$ T lymphocytes affected IL-2 production, a hallmark of T cell activation (**Fig. 5**). Nucleofected cells were cultivated for 5 h in the presence of Brefeldin A, starting 32 h post-transfection and then analyzed for intracellular IL-2 expression by flow cytometry. As shown in Figure 5A, Nef.GFP expression alone was insufficient to induce IL-2 production. Activation of the cells on anti-CD3/anti-CD28 mAb-coated plates during the relatively short course of the experiment resulted in a minor increase in intracellular IL-2, which was also not influenced by Nef.GFP (Fig. 5B). Later analysis was precluded by the decreasing viability of nucleofected cells in the absence of stimulation (data not shown). In contrast, IL-2 production was potently induced by treatment of cells with PMA and ionomycin for 5 h (Fig. 5C). It is interesting that IL-2 production was more pronounced in the Nef.GFP-expressing population relative to the GFP-negative population. The ratio of IL-2-positive cells among nonexpressing cells compared with Nef.GFP-positive cells revealed a 1.5 to threefold increase of IL-2-producing cells upon Nef.GFP expression (Fig. 5, C and D). IL-2 production in control cells expressing GFP alone was indistinguishable from nontransfected cells, excluding nonspecific effects of the nucleofection procedure or GFP expression. In summary, in line with the inability of Nef to up-regulate activation markers at the cell surface (Figs. 2 and 3), Nef.GFP did not trigger the production of IL-2 in resting CD4$^+$ T lymphocytes but modestly enhanced IL-2 production in the context of a potent T cell stimulus.

A subpopulation of cellular Nef.GFP localizes to plasma membrane lipid raft clusters in resting CD4$^+$ T lymphocytes

As signal transduction properties of Nef in T cell lines depend on the incorporation of a Nef subpopulation into lipid rafts, which serve as platforms for TCR signaling [11, 45, 58], we sought to investigate the subcellular localization of Nef in resting CD4$^+$ T cells. For this analysis, CD8-depleted resting T cells, which had been nucleofected with plasmids encoding GFP or Nef.GFP proteins, were incubated with fluorescently labeled CTx, which was subsequently clustered with anti-CTx antibodies [45]. **Figure 6A** depicts representative cells with CTx-positive lipid raft clusters at the plasma membrane. GFP and G2A.GFP were distributed diffusely throughout the nucleus and the cytoplasm without pronounced accumulation at cellular membranes or CTx-positive clusters. Occasionally, small accumulations of GFP and G2A.GFP, which did not overlap with the CTx staining, were detected at the plasma membrane. In contrast, Nef.GFP prominently accumulated at the plasma membrane, and distinct wt.GFP patches were observed, which displayed significant colocalization with CTx-positive clusters, revealed by the yellow color in merged images (Fig. 6A, right panel, see arrows). In z-sections, more distant to the coverslip, wt.GFP was also detected in yet-to-be defined cytoplasmic structures (data not shown and Fig. 6B). To monitor the effect of the clustering procedure on the localization of Nef, cells were also analyzed following fixation and CTx staining without prior clustering (Fig. 6B). This procedure

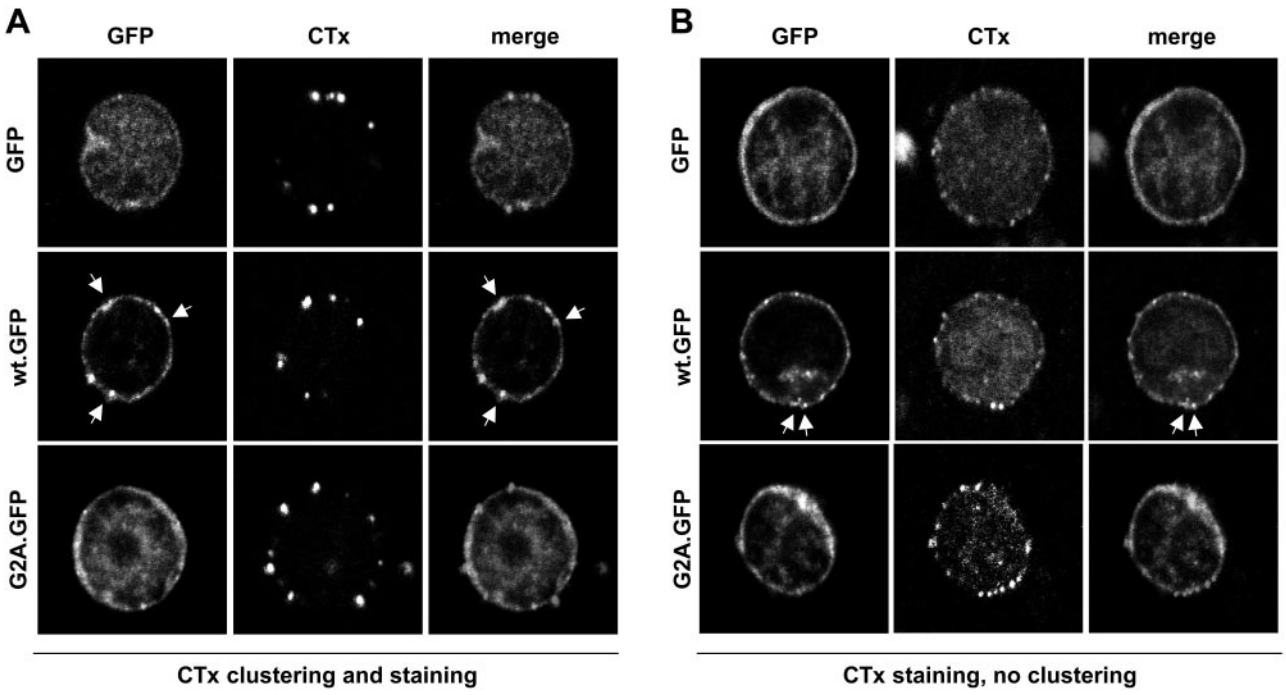

Fig. 6. Subcellular localization of GFP and Nef.GFP proteins in resting CD4$^+$ T lymphocytes. Nucleofected resting CD4$^+$ T lymphocytes expressing the indicated GFP or Nef.GFP fusion proteins were subjected to raft clustering by incubation with Alexa 594-conjugated CTx and subsequent cross-linking with the anti-CTx antibody (A) or CTx staining after fixation without cross-linking (B). GFP and Alexa 594 fluorescence, respectively, was analyzed by confocal microscopy. The merge panel depicts the overlay of both fluorescence channels. Presented are single representative sections of nucleofected CD4$^+$ T lymphocytes from the same donor. Similar results were obtained with cells from two additional donors. The arrows indicate the colocalization of wt.GFP with CTx patches at the plasma membrane.

resulted in a significantly weaker CTx staining and revealed diffuse intracellular signals as well as relatively small patches at the plasma membrane. It is important that the overall distribution of the GFP and wt.GFP proteins analyzed was unchanged relative to cells that underwent CTx clustering, and a fraction of wt.GFP colocalized with CTx plasma membrane patches. Together, these results demonstrate that in resting primary $CD4^+$ T cells, Nef resides partially in plasma membrane lipid rafts from where signaling activities of the viral protein are believed to occur.

DISCUSSION

In vivo, resting $CD4^+$ T lymphocytes have been identified unambiguously as a reservoir for HIV infection [29–31, 33]. The analysis of effects of the HIV pathogenicity factor Nef on viral replication in several in vitro and ex vivo systems suggested that the manipulation of this target cell population might be an important activity of the viral protein [41–44]. In light of the proposed roles of Nef as a facilitator of T cell activation, viral replication, as well as of immune evasion, we sought to identify cardinal functions of the viral pathogenicity factor, which are exerted in this important primary cell type. Because of their high degree of in vitro resistance to infection or transfection, resting $CD4^+$ T cells have thus far been refractory to the detailed analysis of Nef activities. In this study, the recently introduced Amaxa nucleofection methodology was used to achieve transfection efficiencies of up to 65%. The transfection procedure per se had neither appreciable effects on the activation state of cells nor on their sensitivity to exogenous TCR stimulation. This experimental set-up therefore allowed a first functional analysis of HIV-1 Nef in resting $CD4^+$ T cells.

Down-regulation of the CD4 receptor by Nef was the most striking effect (more than 90% reduction of surface exposure), apparent already at low Nef expression levels and markedly more efficient than seen in most immortalized cell lines [6, 46, 52, 59]. The down-regulation of CD4 has been genetically correlated with the effects of Nef on lentiviral replication and pathogenesis [40, 60, 61]. Proposed consequences of this activity include the interference with HIV superinfection, prevention of Env-CD4-mediated, proapoptotic signaling, and increased infectivity and release of virus particles [46, 62–67]. The efficacy of CD4 down-regulation by Nef in these primary T cells supports its putative in vivo relevance.

In comparison, we found down-modulation of cell surface MHC-I to be moderate (up to 45% reduction of surface exposure), even at high Nef expression levels. This was true not only for the *nef* allele from HIV-1_{SF2} but also for alleles from HIV-1_{NL4-3}, HIV-1_{NA-7}, HIV-2_{NEP}, and $SIV_{mac\ 239}$ (data not shown). These moderate levels of Nef-mediated MHC-I down-modulation in primary $CD4^+$ T lymphocytes are in good agreement with a recent report using activated primary cells (ref. [68]; data not shown) or HIV-1 infection of T cell lines (data not shown) but are much less pronounced than the up to 300-fold reduction reported in primary T cells by others [69, 70]. Although the reason for this discrepancy remains unclear, another study reported that a twofold reduction in surface exposure of a specific HLA-B2 molecule was sufficient to markedly reduce the killing of HIV-infected cells by select, HIV-1-specific cytotoxic T cells [68]. Thus, the moderate Nef-mediated reduction of MHC-I molecules we find on resting $CD4^+$ T cells could well be sufficient to facilitate immune escape of infected cells and viral persistence.

We report here for the first time that on resting $CD4^+$ T cells, Nef triggered an approximate fourfold up-regulation of CD74. It is notable that this effect was even more pronounced (seven- to 11-fold) in infected, activated primary human $CD4^+$ T lymphocytes. MHC-II molecules efficiently present antigenic peptides to $CD4^+$ T cells to initiate and establish primary and secondary immune responses, respectively. For antigen presentation, MHC-II α and β chains associate with the invariant chain chaperone CD74 during biosynthetic transport and are ultimately sorted to the endocytic pathway, leading to the degradation of CD74. This maturation step allows MHC-II complexes to bind antigenic peptides, which are subsequently presented at the cell surface. An increase of surface levels of the invariant chain CD74 of immature MHC-II molecules by Nef is therefore believed to inhibit MHC-II-restricted peptide presentation to specific T cells and may thus affect the induction of antiviral immune responses [7, 71]. According to our results, this phenotype of HIV-1 Nef is also clearly exerted in primary T cells. Future analyses will be required to address the physiological relevance of this Nef activity in primary, professional antigen-presenting cells. In contrast, the Nef-mediated down-modulation of the costimulatory receptor CD28, which was reported previously in T cell lines [5], does not appear to be a robust phenotype in primary $CD4^+$ T cells. Notably, the recently observed down-regulation of the CCR5 and CXCR4 coreceptors occured also in HIV-infected primary $CD4^+$ T cells and was Nef-dependent [46].

It is remarkable that the expression of Nef alone did not specifically affect surface levels of the activation markers CD25 and HLA-DR. Of note, CD69 was up-regulated and CD25 down-regulated by Nef wild-type as well as its G2A mutant, especially when expressed from the bicistronic vector. This may reflect nonspecific effects of the pIRES-EGFP vector or yet-undefined, myristoylation-independent activities of Nef. Of note, surface exposure of early and late activation markers was affected differentially, suggesting that the effects of wild-type and G2A Nef reflect alterations of the specific intracellular transport routes used by the individual receptors rather than a direct impact on the cellular activation state. Thus, in clear contrast to studies performed in immortalized T cell lines [10, 13–16], Nef expression alone neither induced appreciable activation of primary resting T lymphocytes nor blocked their sensitivity to exogenous T cell activation. Rather, expression of Nef.GFP modestly enhanced PMA/ionomycin-induced IL-2 production. These results are consistent with the model that expression of Nef alone is unable to trigger appreciable T cell activation but instead, lowers the threshold for the activation by costimulation [10, 11, 72–74]. In line with such a signal transduction activity, a subpopulation of Nef was present in plasma membrane lipid rafts of resting $CD4^+$ T lymphocytes. However, the observed Nef-mediated sensitization of these cells to exogenous T cell activation was moderate. Although several factors have been described, which restrict the permis-

sivity of resting T cells to HIV replication [75–78], the full array of molecular events during $CD4^+$ T lymphocyte activation, which determine permissivity to HIV-1 replication as well as specific, indicative cell surface markers, have remained elusive. Clearly, the induction of a replication-permissive environment by Nef does not require full activation in coculture of resting $CD4^+$ T lymphocytes with immature dendritic cells [41] or endothelial cells [79]. Similarly, treatment of resting $CD4^+$ T lymphocytes with select cytokines allows efficient HIV replication without the appearance of classical T cell activation markers [36]. The molecular definition of this state of T cell activation, which renders resting cells permissive for HIV replication, and the mechanism used by Nef to facilitate this process represent important goals for future studies. The experimental approach established in this study opens the possibility to perform such analyses in pathobiologically relevant HIV target cells.

Together, the results presented demonstrate that HIV-1 Nef has the capacity to perturb host cell machineries selectively, which govern intracellular signaling and transport pathways in resting $CD4^+$ T lymphocytes. Nef exerts its receptor-modulating activities on a specific set of molecules but not all receptors previously implicated in cell lines. Furthermore, our results support a model in which Nef lowers the threshold for activation of infected resting $CD4^+$ T cells, but that expression of the accessory protein alone is insufficient to trigger T cell activation. These results provide insight into the breadth, magnitude, and potential significance of individual Nef actions in an important primary target cell population and thus enhance our understanding of how Nef boosts HIV replication in vivo.

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