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The HIV-1 Nef protein has a dual role in T cell receptor signaling in infected CD4$^+$ T lymphocytes

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ABSTRACT

The phenotypic changes that are induced by immune activation in CD4$^+$ T lymphocytes provide an optimal environment for efficient HIV-1 replication in these cells. The pathogenic Nef protein of HIV-1 modulates the T cell receptor (TCR) signaling, but whether this has a positive or negative effect on cellular activation is a matter of debate. Here we have investigated the response to TCR stimulation of primary CD4$^+$ T lymphocytes infected with wt or Nef-deficient HIV-1. Results show that, in freshly isolated quiescent T cells, Nef superinduces NFAT and IL-2 production bypassing early TCR effector molecules. Conversely, the early phosphorylation of PLC-γ1, the induction of NFAT, and the expression of IL-2 are impaired by Nef in sub-optimally activated/resting T cells. Our data indicate that Nef has a dual role in the modulation of TCR signaling aimed at favoring HIV-1 replication and spread in both quiescent and metabolically active CD4$^+$ T lymphocytes.

Introduction

The efficiency of HIV-1 replication in CD4$^+$ T lymphocytes is strongly dependent on the activation state of the cells. While activated T cells are fully permissive for viral replication, quiescent T cells do not support efficient retrotranscription, integration, and expression of the HIV-1 genome due to low levels of nucleotides, ATP, and nuclear transcription activators (Coiras et al., 2009; Stevenson, 2003). Therefore, it is not surprising that HIV-1 has evolved mechanisms to stimulate the signaling pathway of the T cell receptor (TCR). Several experimental evidences indicate that Nef, an HIV-1 protein required for efficient viral replication *in vivo* and AIDS disease progression (Deacon et al., 1995; Kirchhoff et al., 1995), has a key role in the modulation of TCR signaling. Nef is a ~27 kDa myristoylated protein expressed soon after infection that associates with cell membranes and down-modulates an array of cell surface molecules including CD4, MHC class I and class II, CD28, and chemokine receptors, thus favoring at different levels HIV-1 spread (Arien and Verhasselt, 2008; Kirchhoff et al., 2008). In addition, many interactions between Nef and cellular factors belonging to the TCR signaling pathway such as Lck, Vav, and the TCR ζ chain have been described (reviewed by Renkema and Saksela, 2000). Because of the correlation between HIV-1 replication and cellular activation, a positive influence of Nef on T cell signaling could be expected. Experimental evidence supporting such positive effect of Nef has indeed been reported. Nef expression in the Jurkat T cell line or in primary CD4$^+$ T cells increased production of IL-2, a key indicator of T cell activation, upon TCR triggering with antibodies against TCR/CD3 and CD28 co-receptor (Schibeci et al., 2000; Schrager and Marsh, 1999; Wang et al., 2000) or exposure to mitogens that bypass surface receptors (Keppler et al., 2006). Nef also increased the activity of NFAT, another hallmark of T cell activation, in stimulated Jurkat cells transfected with Nef-expressing vectors (Fenard et al., 2005; Manninen et al., 2000) or infected with HIV-1 (Schindler et al., 2006). Importantly, it was reported that Nef sensitizes HIV-infected quiescent CD4$^+$ T lymphocytes to activation, thus favoring IL-2 production and viral replication in response to TCR stimulation (Wu and Marsh, 2001). Finally, Nef expression itself was shown to induce in Jurkat cells a transcriptional program that closely resembles that induced by TCR/CD3 stimulation (Simmons et al., 2001). However, contrasting with these results, several reports have suggested that Nef inhibits T cell activation. This latter phenotype was observed in Nef-expressing T cell clones that were defective for their capacity to respond to stimulation with mitogens or anti-TCR/CD3 antibodies in terms of activation of NF-κB and AP-1 transcription factors or IL-2 production (Bandres and Ratner, 1994; Collette et al., 1996;

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Niederman et al., 1993). Recently, Nef was shown to impair actin remodeling and recruitment at the plasma membrane of Lck and other signaling molecules that normally follow TCR engagement (Haller et al., 2006; Haller et al., 2007; Thoulouze et al., 2006). Thoulouze et al. (2006) also reported that Nef reduced the formation of the immunological synapse (IS) between infected lymphocytes and antigen-presenting cells (APC), thus leading to lower IL-2 production by T cells, although these results were not confirmed in a more recent study (Arhel et al., 2009). It was suggested that these negative effects of Nef may favor HIV-1 replication by preventing full activation and, consequently, activation-induced cell death (AICD) of infected T lymphocytes (Fackler et al., 2007).

The confusion surrounding the effects of Nef on the TCR pathway may reflect differences in the cell systems employed, the types of *nef* alleles used, the duration and the level of Nef protein expression. In addition, the various conditions used to stimulate T cells, including the nature, the relative amount, and the duration of the stimulus, might have diversified effects on Nef function. Finally, the activity of Nef may be influenced by other viral proteins; thus, the impact on T cell activation may vary whether the protein is expressed by itself or in the context of HIV-1 infection.

In the present study, we examined the impact of Nef expression in the capacity of HIV-1-infected $CD4^+$ T lymphocytes to respond to TCR-mediated stimulation. Quiescent and resting $CD4^+$ T lymphocytes, both representing important targets of *in vivo* HIV-1 infection, have been infected with wt or Nef-defective virus, then stimulated with antibodies that cross-link TCR/CD3 and CD28 to mimic antigen stimulation. Results presented herein show that the HIV-1 Nef protein modulates the TCR function either positively or negatively depending on the activation state of the infected T cells.

Results

Role of Nef in the activation of HIV-infected quiescent $CD4^+$ T cells

To explore the capacity of the HIV-1 Nef protein to affect the activation state of quiescent primary $CD4^+$ T lymphocytes, freshly purified $CD4^+$ T cells have been infected with the NL4-3 strain of HIV-1 either wt or Nef-deficient (ΔNef). Both viruses were pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) to allow entry into target cells by endocytosis and bypass the requirement of Nef for optimal viral infectivity (Chazal et al., 2001). After 5 days of culture in the absence of mitogenic stimuli, infected as well as uninfected (n.i.) control cells were harvested to analyze by Western blotting the expression of Nef and of three signaling molecules, Lck, CD4, and CD28, that are known targets of Nef-induced degradation (Greenway et al., 2003). Fig. 1A shows that the Nef protein was readily detected in cells infected with wt virus despite no measurable viral replication occurred (data not shown), in agreement with a previous study showing Nef expression in HIV-infected quiescent T cells lacking viral DNA integration (Wu and Marsh, 2001). If compared to n.i. cells, the steady-state level of CD28 remained unvaried upon infection, while CD4 was reduced by 30–40% ($P<0.05$) in cells infected with wt but not with ΔNef virus. On the other hand, about 20% reduced Lck levels were consistently observed in both wt- and ΔNef-infected cells ($P<0.05$). In addition, the intracellular distribution of CD4, CD28, and Lck in wt-infected cells was analyzed by double-staining with an anti-Nef antibody together with an anti-CD4, anti-CD28 or anti-Lck antibody followed by immunofluorescence microcopy. ΔNef-infected cells could not be identified due to the lack of an HIV-1-specific marker different from Nef to be used for this analysis.

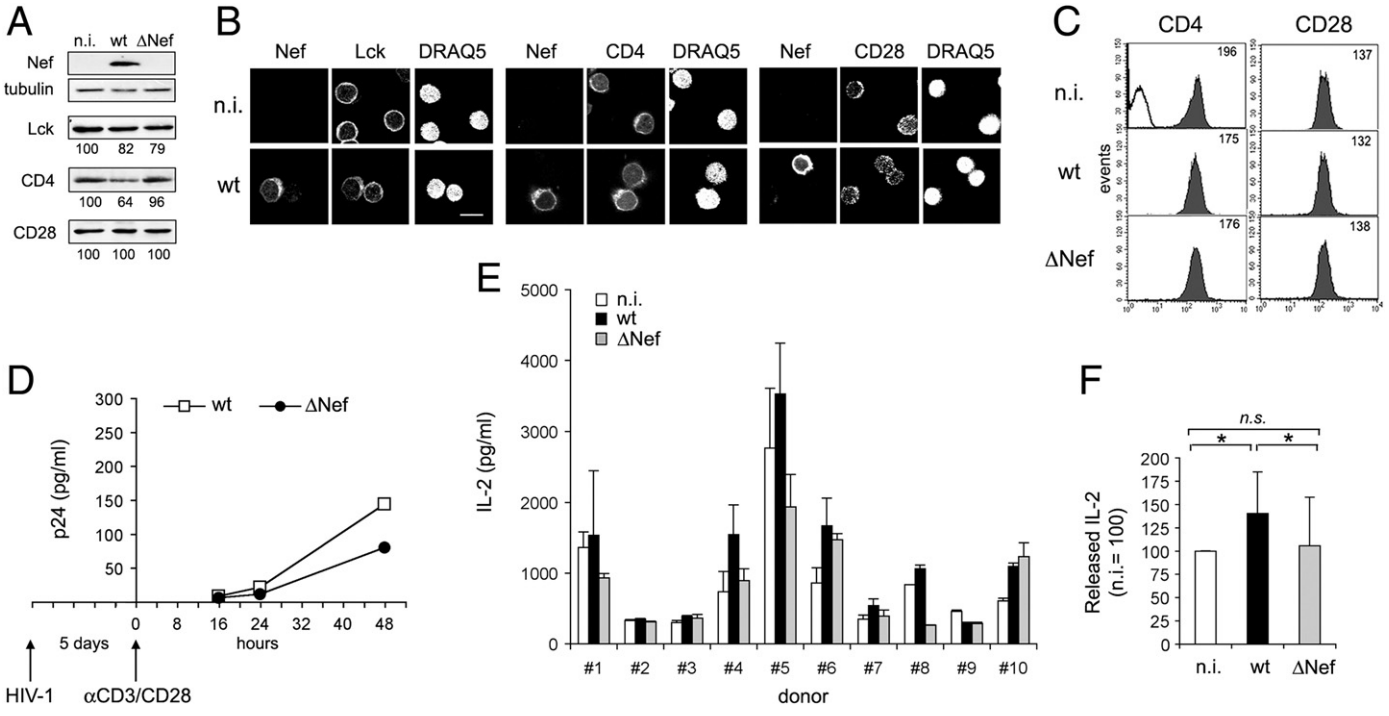


Fig. 1. Analysis of quiescent $CD4^+$ T lymphocytes infected with wt or Nef-deficient HIV-1. Freshly isolated $CD4^+$ T lymphocytes were infected with equivalent p24 doses (250 ng × 10^6 cells) of VSV-G-pseudotyped wt or ΔNef HIV-1 or not infected (n.i.), then cultivated in the absence of mitogenic stimuli for 5 days. Panels A–D show representative results obtained in one out of at least four independent experiments. (A) Whole-cell extracts were analyzed by Western blotting with antibodies against Nef, tubulin, Lck, CD4, and CD28. The level of the latter three proteins was measured by densitometry, normalized by tubulin and expressed by setting at a 100% the value found with n.i. sample. (B) N.i. and wt-infected cells were stained with anti-Nef antibody together with anti-Lck (left panels), anti-CD4 (central panels), or anti-CD28 (right panels) antibody and processed for immunofluorescence microscopy. Nuclei were stained with DRAQ5. Depicted is a representative confocal section through the middle of the cell. Bar: 5 μm. (C) The cell surface expression of CD4 and CD28 was analyzed by flow cytometry. The geometric mean fluorescence intensity (MFI) values of the life cell population are reported. The unshaded histogram shows unlabeled cells. (D and E) At 5 days p.i., n.i., wt- and ΔNef-infected cells have been activated with anti-CD3 and -CD28 antibody as described in Materials and methods. (D) Viral replication was assessed at 16, 24, and 48 h post-activation by measuring p24 in the culture supernatant with specific ELISA. (E) At 16 h post-activation, IL-2 secreted in the supernatant of cells derived from 10 healthy donors was measured by ELISA. (F) The average IL-2 release by cells shown in panel (E) was calculated and expressed by setting n.i. to a 100. *$P=0.02$, *n.s* = not statistically significant.

An average 3.5% of wt-infected cells were intensely stained with the anti-Nef antibody in four independent experiments. As shown in Fig. 1B, if compared to uninfected cells, Nef$^+$ cells maintained the intracellular distribution of CD28 but accumulated both CD4 and Lck in large perinuclear vesicles. Moreover, flow cytometric analysis indicated that the surface level of both CD28 and CD4 did not change significantly upon HIV infection with or without Nef expression (Fig. 1C). Overall, these results indicate that during the 5-day period that follows HIV-1 infection of quiescent T cells, Lck levels are reduced through a Nef-independent mechanism and that the accumulated Nef protein induces the intracellular retention/degradation of CD4 without affecting the cell surface expression of this receptor or CD28.

Then, we tested the role of Nef in the response to stimulation of HIV-infected quiescent T cells, specifically in the production of progeny virions and IL-2. Five days post-infection, n.i., wt- and ΔNef-infected quiescent T cells were activated with anti-CD3 and anti-CD28 cross-linked antibodies then monitored at various time for the release of p24 and IL-2 in the culture medium by specific ELISA. As expected, a positive effect of Nef on HIV-1 replication was consistently observed with a 2-fold higher p24 release at 48 h post-activation by cells infected with wt virus if compared to those infected with ΔNef (Fig. 1D). Upon stimulation, all tested T cell populations derived from healthy donors produced IL-2 with a maximal release after 16 h, although the relative amount of cytokine varied considerably among individuals (Fig. 1E). In 9 out of 10 donors, cells infected with wt virus produced higher amounts of IL-2 if compared to n.i. cells (from 10% to 110% higher levels; Fig. 1E). Conversely, cells infected with Nef-negative virus responded to stimulation producing IL-2 amounts that were lower if compared to wt-infected cells (from 10% to 75% lower levels) in the majority of the donors (8 out of 10). By comparing the average IL-2 release of all tested donors, this was increased by 40% in wt-infected cells if compared to n.i. ($P=0.02$) or ΔNef-infected cells ($P=0.02$), while the latter two cell types did not differ ($P=0.74$) (Fig. 1F). Therefore, we concluded that in this cell system HIV-1 positively influences the production of IL-2 in a Nef-dependent manner.

Early TCR signaling events in quiescent CD4$^+$ T cells are not altered by HIV-1

Next, we asked whether the positive effect of Nef in the activation of HIV-infected quiescent T cells occurred through variations in the phosphorylation of intracellular substrates that follows TCR stimulation. Quiescent CD4$^+$ T cells were infected and incubated for 5 days as described above then stimulated with cross-linked anti-CD3 and anti-CD28 antibodies for variable time before Western blot analysis with an anti-phosphotyrosine (p-Tyr) antibody. Fig. 2A shows that various phosphoproteins were induced 1 min after TCR triggering, then slowly decreased remaining detectable till 30–60 min. Based on their apparent molecular masses, some phosphoproteins corresponded to activated molecules that are distinctive of TCR signaling such as p-PLC-γ1 (140 kDa), p-Vav (95 kDa), p-ZAP-70 (70 kDa), and p-Lck (56 kDa). Thus, the total protein level of these proteins was evaluated by reprobing the membrane with specific antibodies (Fig. 2A) and the efficiency of phosphorylation was calculated by normalizing phospho-bands for their corresponding loading controls. By comparing n.i., wt- and ΔNef-infected cells derived from 5 donors, it was evident that the extent and the kinetics of tyrosine phosphorylation of PLC-γ1, Vav, and ZAP-70 (with a peak after 1 min of stimulation) did not differ significantly (representative results are shown in Fig. 2B). By this analysis, the extent of Lck phosphorylation was extremely variable between different experiments (data not shown) and thus it could not be evaluated. This was possibly due to the high basal level of Lck phosphorylation in this cell system and/or to the presence of other phosphorylated proteins co-migrating with Lck.

During T cell activation, costimulation by CD28 induces the PI3K-dependent activation of the serine/threonine kinase Akt, an event that starts a complex signaling cascade ultimately conducting to proliferation and other cellular activities (Song et al., 2008). Therefore, infected T cells were stimulated via CD3/CD28 then monitored for Akt activation by measuring with Western blotting both the total Akt protein and the form phosphorylated at the regulatory Ser473 site. Apparently, Akt activation/phosphorylation was similarly induced in n.i., wt- and ΔNef-infected T cells (Fig 2C).

CD4-induced Lck activation is maintained in quiescent T cells infected with wt and Nef-deficient HIV-1

To investigate whether HIV-1 and, more specifically, the Nef protein could modulate Lck activity, infected quiescent CD4$^+$ T cells were stimulated by CD4 cross-linking that should result in the phosphorylation of associated Lck at the Y394 residue and thus in the full activation of the kinase (Holdorf et al., 2002). Total extracts of cells unstimulated or stimulated for variable time (1, 5, and 10 min) were analyzed by Western blotting with anti-p-Tyr antibody followed by reprobing with anti-Lck antibody. As shown in Fig. 3A, CD4 triggering induced after 1 min the phosphorylation of Lck that reached a maximum after 5 min, while the total protein level decreased progressively due to the rapid consumption of activated Lck. In wt- and ΔNef-infected cells, despite the lower steady-state protein level of Lck, the extent and the kinetic of Lck activation/consumption was similar to that observed in uninfected cells. In addition, HIV-infected T cells stimulated via CD4 were tested for the phosphorylation of the TCR ζ subunit, one of the early substrate for Lck's kinase activity (van Oers, 1999). To this end, total cell lysates of n.i., wt- and ΔNef-infected CD4$^+$ T lymphocytes stimulated with cross-linked anti-CD4 antibody for 5 min were immunoprecipitated with anti-p-Tyr antibody and the recovered immunocomplexes were analyzed by Western blotting with anti-ζ chain antibody. Fig. 3B shows that the amounts of ζ chain found in the precipitated phosphoproteins increased upon CD4 stimulation and that this increment was similar in n.i., wt- and ΔNef-infected cell samples. These results were confirmed by the reciprocal analysis of tyrosine-phosphorylated proteins in the anti-ζ-chain immunocomplex (data not shown) and suggest that the function of Lck is maintained in quiescent T cells upon HIV-1 infection with and without Nef expression.

Nef superinduces NFAT during activation of HIV-infected quiescent T cells

Then, we analyzed the impact of HIV-1 infection in the late steps of signal delivery in quiescent T cells stimulated by CD3/CD28 engagement. Specifically, we investigated the NF-κB and NFAT proteins that are key regulators of both T cell activation (Macian, 2005; Schulze-Luehrmann and Ghosh, 2006) and HIV-1 gene expression (Kinoshita et al., 1998; Nabel and Baltimore, 1987). Primary CD4$^+$ T cells were infected and stimulated as described in Fig. 2 then harvested at various times after stimulation to prepare total cell extracts. The expression and activation of proteins belonging to the NF-κB pathway (IKKα/β, IκBα, NF-κB p65) was quantified by performing a Western blotting analysis with antibodies against the total proteins or their phosphorylated forms. As shown in Fig. 4A, after 15 min of stimulation, we observed phosphorylation of regulatory residues in the IKKα catalytic subunit that is specific for activated IKK complex along with phosphorylation of the IKK target protein IκBα. As expected, IκBα phosphorylation coincided with the reduction of total IκBα protein level and the appearance of phosphorylated/activated NF-κB that was maximally induced after about 6 h. These events occurred similarly in cells not infected or infected with HIV-1 either wt or Nef-defective, indicating that the capacity of quiescent CD4$^+$ T cells to activate the NF-κB pathway is not altered by the virus.

Finally, we analyzed the transcription factors NFATc1 and NFATc2 (also called NFAT2 and NFAT1, respectively) that reside in the cytoplasm in a hyperphosphorylated state in quiescent T cells and translocate into the nucleus upon calcineurin-mediated dephosphorylation induced by TCR/CD28 ligation. Cell extracts were analyzed by immunoblotting with antibodies that detected both the inactive

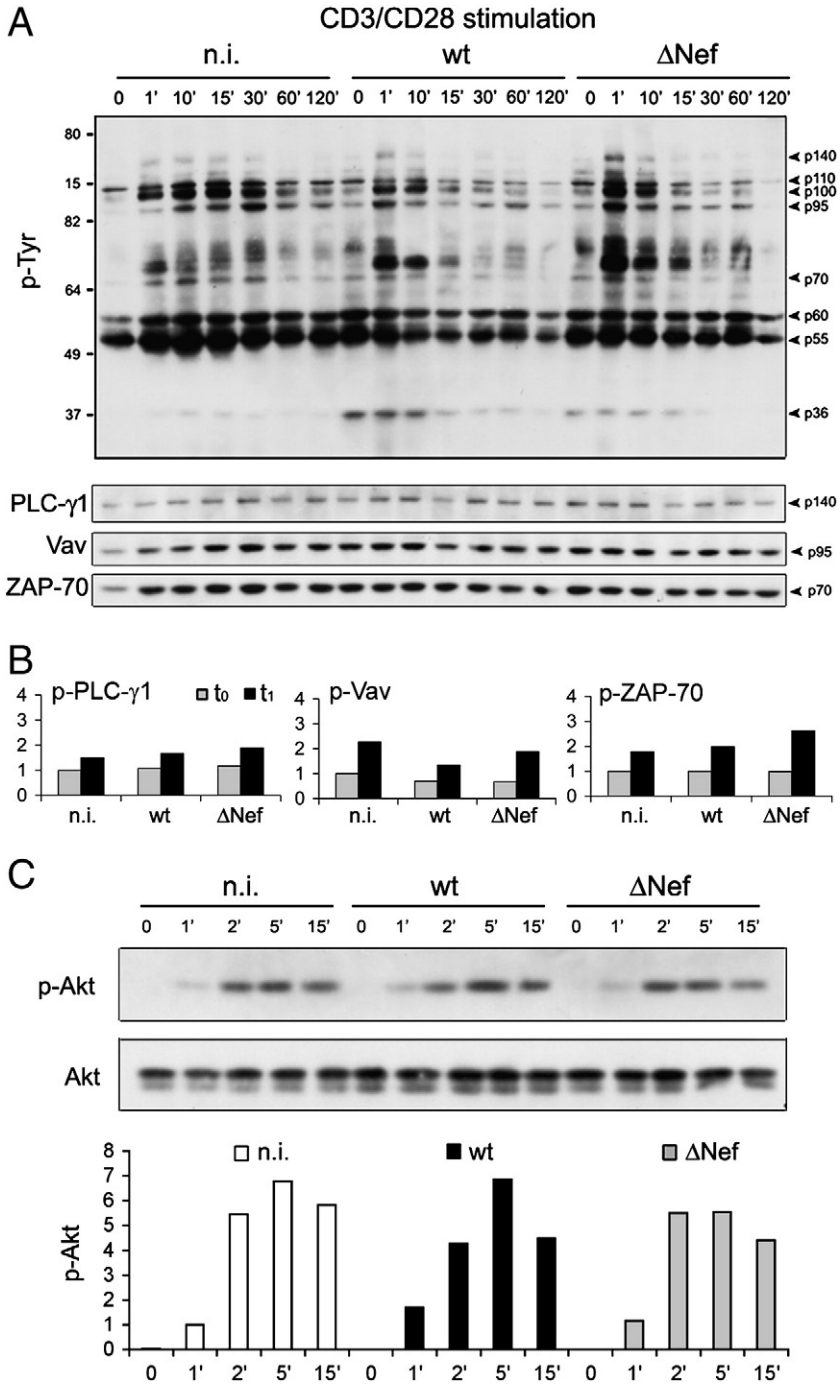


Fig. 2. Phosphorylation of signaling proteins upon primary activation of T cells infected with wt or Nef-deficient HIV-1. Five days p.i., quiescent $CD4^+$ T lymphocytes were stimulated for variable time with cross-linked anti-CD3 and anti-CD28 antibodies. (A) Equal amounts of total cell lysates were analyzed by Western blotting with anti-p-Tyr antibody (top panel). The membrane was stripped and reprobed with specific antibodies anti-PLC-γ1, anti-Vav, and anti-ZAP-70 (lower panels). (B) Phosphorylation of PLC-γ1, Vav, and ZAP-70 measured after 1 min (t_1) of stimulation was calculated by densitometric analysis normalizing phospho-bands for their loading controls and setting to 1 the signal of n.i. cells before stimulation (t_0). (C) Total cell lysates were analyzed by Western blotting with an antibody recognizing the activated Ser473-phosphorylated (p) form of Akt (top). Then, the membrane was reprobed with an antibody against total Akt (center). Phosphorylation of Akt was obtained by normalizing phospho-bands for their loading controls and setting to 1 the value obtained with n.i. cells stimulated for 1 min (bottom). Representative results of 1 out of 3 independent experiments are shown.

phoshorylated NFAT proteins and their active dephosphorylated forms. As shown in Fig. 4B, a large NFATc1 isoform and up to three faster migrating proteins corresponding to active dephosphorylated forms were induced 4 h after stimulation, then almost completely disappeared 18 h post-stimulation. This process was more efficient (3-fold higher signals, $P<0.05$) in cells infected with wt HIV-1 if compared to n.i. or ΔNef-infected cells. As to NFATc2, a major isoform was induced upon stimulation ($P<0.01$) (Fig. 4C). Notably, in cells infected with wt virus, NFATc2 appeared earlier (18 rather than 48 h post-stimulation) than in n.i. and ΔNef-infected cells. These results suggest that the Nef protein is able to superinduce NFAT transcription factors.

Role of Nef in the activation state of HIV-infected resting $CD4^+$ T cells

To further investigate the role of Nef in T cell activation, we analyzed the impact of HIV-1 infection with and without Nef expression in the capacity of pre-activated $CD4^+$ T lymphocytes to

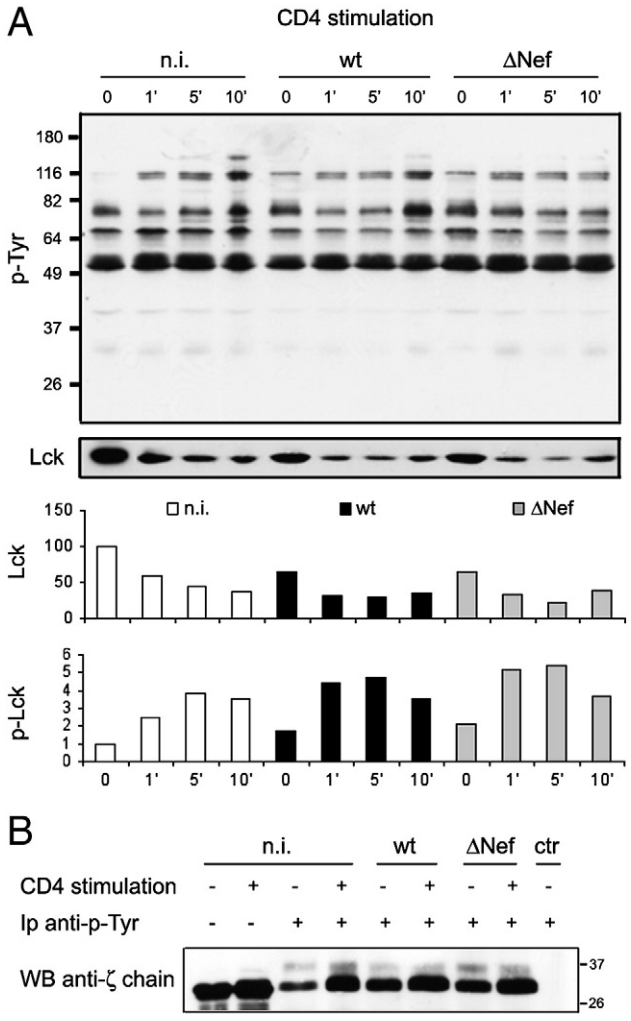


Fig. 3. Lck activation in quiescent HIV-infected CD4$^+$ T cells stimulated through CD4 engagement. (A) Quiescent HIV-infected CD4$^+$ T cells were stimulated 5 days p.i. with cross-linked anti-CD4 antibody for the indicated time, then whole-cell extracts have been prepared and analyzed by Western blotting using antibodies anti-p-Tyr first, then anti-Lck. Total Lck and normalized p-Lck levels were expressed by setting to 100% and 1, respectively, the value measured in n.i. cells before stimulation (t_0). (B) Total extracts of cells stimulated or not by CD4 ligation for 5 min have been immunoprecipitated with anti-p-Tyr antibody then analyzed by Western blotting with anti-ζ-chain antibody. As controls, total lysates of n.i. cells not stimulated or stimulated via CD4 (first and second lanes) and an immunoprecipitation reaction in the absence of cell lysate (ctr lane) were loaded. Results obtained in a representative experiment out of three are shown.

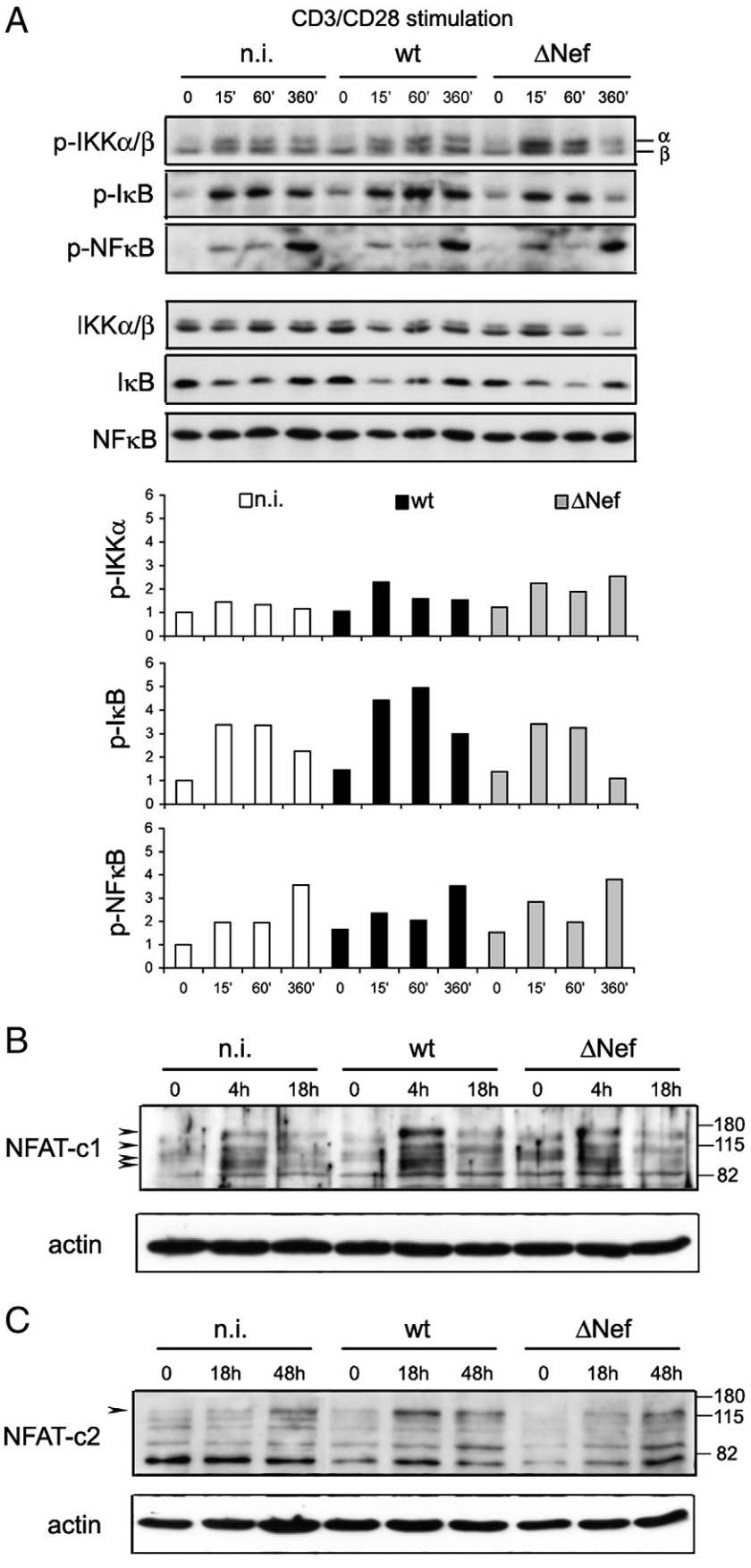
respond to secondary stimulation. First, freshly purified CD4$^+$ T cells have been stimulated through CD3/CD28 ligation and cultivated for 6 days, till when cells stopped proliferating and dumped expression of activation markers such as cell surface CD25 and CD69 (data not shown). Here we will refer to these cells as resting T cells. Next, resting cells have been infected with HIV-1, wt or Nef-defective, or mock infected and further cultivated for 3 days to allow accumulation of the Nef protein (Fig. 5A). If compared to quiescent T cells at 5 days p.i., resting cells infected with wt virus for 3 days displayed from 5- to 7-fold higher amounts of Nef and expressed detectable levels of other viral proteins as determined by Western blotting analysis (Fig. 5B). Concomitantly, a small percentage of p24$^+$ (between 1 and 6%) was detected by flow cytometry in resting but not in quiescent populations of infected cells (Fig. 5C and data not shown). Similarly to quiescent infected CD4$^+$ T cells, HIV-1 infection of resting CD4$^+$ T cells did not affect steady-state CD28 protein levels but slightly reduced total Lck levels (by ~10%; $P=0.05$) in a manner that was independent of Nef expression (Fig. 5A). As to CD4, upon infection with wt and, to a slightly lower extent, ΔNef virus, the total protein amounts were consistently reduced (by 10–20%; $P = 0.03$) and the cell surface expression was strongly suppressed (10- to 40-fold) as determined by Western blotting and FACS analysis, respectively (Fig. 5A and C). The Nef-independent down-regulation of CD4 is consistent with the capacity of the late Vpu and Env viral proteins to induce CD4 degradation (Geleziunas et al., 1994). Conversely, p24$^+$ cells infected with wt but not with ΔNef virus had reduced levels of surface CD28, in agreement with Nef-specific down-regulation of this molecule.

Then, resting T cells infected for 3 days have been re-stimulated via CD3/CD28 and monitored for the release of soluble p24 and IL-2 in the culture medium. If compared to infected T cells stimulated for the first time, secondary activated wt- and ΔNef-infected cells produced p24 at earlier time points but the growth disadvantage of Nef-deficient virus was always maintained in 6 tested donors (Fig. 5D shows a representative experiment). Upon secondary stimulation, the production of IL-2 was similar in n.i. and wt-infected cells but increased in cells infected with ΔNef virus (Fig. 5E). This response was evident in 5 donors showing 35–110% higher IL-2 levels with ΔNef, while donor #6 instead showed a modest Nef-mediated increase of IL-2 release. Considering the average IL-2 released by all donors, this was increased by 55% in ΔNef-infected cells if compared to wt-infected ($P=0.02$) and n.i. cells ($P=0.008$) (Fig. 5F). Apparently, in these experimental settings the Nef protein had a negative impact on IL-2 release and was advantageous for viral replication independently of its function in IL-2 regulation. By comparing the levels of IL-2 produced in the quiescent versus resting cell systems (Fig. 5G), uninfected resting cells had a propensity to release higher amounts of cytokine (3-fold induction if compared to quiescent T cells, $P=0.13$), in agreement with their higher metabolic status. This induction was somewhat lower (2-fold, $P=0.16$) or, conversely, significantly higher (5-fold, $P=0.04$) upon infection with wt or ΔNef virus, respectively. These results indicate that IL-2 expression in resting cells is inhibited by Nef rather than stimulated as in quiescent cells.

Nef alters early TCR signaling and NFAT induction in HIV-infected resting T cells

At last, resting T cells, n.i. or infected with wt or ΔNef virus, have been re-stimulated via CD3/CD28 and collected at various time points to investigate the pattern of tyrosine-phosphorylated proteins. T cells responded to secondary stimulation very rapidly, within 30 s, by upmodulating a series of phosphoproteins that, based on their molecular masses, corresponded to the TCR signaling cascade described earlier (Fig. 6A). The efficiency of PLC-γ1 phosphorylation could be easily quantified since p-PLC-γ1 was almost undetectable before T cells re-stimulation. By three independent analyses, following TCR stimulation an average 2.5-fold phosphorylation of PLC-γ1 was reached in n.i. cells as well as in cells infected with wt or ΔNef virus ($P<0.05$). This pick, however, was consistently observed after 1 min in cells infected with wt virus rather than after

Fig. 4. Nef specifically superinduces NFATc1 and NFATc2, not NF-κB, in response to primary activation of HIV-infected T cells. Quiescent HIV-infected CD4$^+$ T cells were stimulated 5 days p.i. via CD3/CD28 for the indicated time, then whole-cell extracts have been prepared and analyzed by Western blotting. (B) Phosphorylation of proteins belonging to the NF-κB pathway (IKKα/β, IκBα, NF-κB) was quantified by using antibodies against the phosphorylated form (top panels), reprobing the membrane with antibodies against the total protein (central panels), and finally normalizing phosphorylated bands for their loading controls (bottom). The basal phosphorylation level measured in n.i. cells before stimulation (t_0) was set to 1. (C and D) Whole-cell extracts were analyzed by immunoblotting with antibodies specific for NFATc1 and NFATc2 antibodies that detect both the inactive phosphorylated and the active dephosphorylated NFAT forms. Arrowheads indicate protein bands up-regulated upon T cell stimulation. The membranes were reprobed with an anti-actin antibody loading control. For each pathway, a representative of at least three experiments is shown.


A
CD3/CD28 stimulation
n.i.
wt
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0 15' 60' 360' 0 15' 60' 360' 0 15' 60' 360'
p-IKKα/β
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p-NFκB
IKKα/β
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wt
ΔNef
p-IKKα
p-IκB
p-NFκB
0 15' 60' 360' 0 15' 60' 360' 0 15' 60' 360'
B
n.i.
wt
ΔNef
0 4h 18h 0 4h 18h 0 4h 18h
NFAT-c1
180
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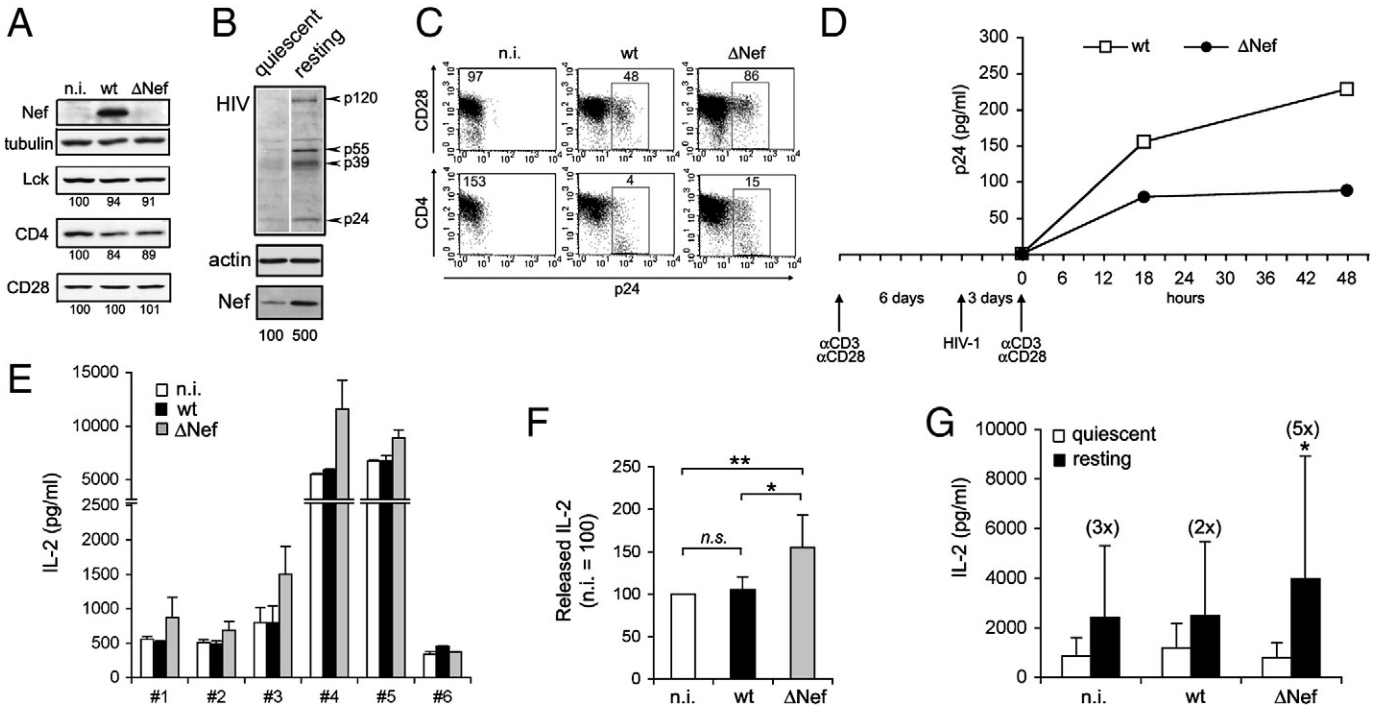

Fig. 5. Analysis of resting CD4$^+$ T cells infected with wt or Nef-deficient HIV-1. Freshly isolated CD4$^+$ T lymphocytes were stimulated via CD3/CD28, cultivated for 6 days till when they stopped growing and reacquired a resting phenotype, infected with VSV-G-pseudotyped wt and ΔNef HIV-1 or not infected, then cultivated for 3 more days. (A) At 3 days p.i., whole-cell extracts were prepared and analyzed by Western blotting with antibodies against Nef, tubulin, Lck, CD4, and CD28 as described in Fig. 1. (B) Lysates of resting T cells 3 days p.i. and quiescent T cells 5 days p.i., both infected with wt virus, were analyzed Western blotting with an anti-HIV human serum, anti- Nef, and anti-actin antibody. Based on their apparent molecular masses, some viral proteins reacting with the anti-HIV-1 serum are indicated. The levels of Nef were normalized by actin and expressed by setting quiescent cell sample at a 100%. (C) Expression of cell surface CD4 or CD28 and intracellular p24 was analyzed by two-color flow cytometry at 3 days p.i. The MFI value specific for CD4 and CD28 of n.i. cells and of gated p24$^+$ infected cells is indicated. (D) Resting cells were re-stimulated 3 days p.i. by CD3/CD28 ligation and viral replication was assessed 18 and 48 h later by measuring p24 in the culture supernatant with specific ELISA. Results shown in panels A–D are representative of six independent experiments. (E and F) At 16 h post-activation, IL-2 secreted in the supernatant of cells derived from 6 healthy donors was measured by ELISA. The values obtained for each donor (E) and the average release of IL-2 (F) are shown. (G) A comparative analysis of IL-2 release between resting cells in panel E ($n=6$) and quiescent cells shown in Fig. 1E ($n=10$) was performed. *$P<0.05$, **$P<0.001$.

30 s as in n.i. and ΔNef-infected cells (Fig. 6A shows a representative experiment).

To test whether the delayed kinetics of PLC-γ1 phosphorylation in wt-infected cells also resulted in altered downstream signaling events, the activation of NFAT was analyzed as described earlier. As to NFATc1, its active isoforms were already abundantly expressed before TCR re-stimulation and no differences were observed between n.i., wt- or ΔNef-infected cells at the time points analyzed (Fig. 6B). On the other hand, a large active NFATc2 isoform transiently appearing 18 h post-activation accumulated to about 3-fold higher extents in ΔNef-infected cells compared to wt-infected and n.i. cells ($P<0.01$). Overall, these results support a negative role of Nef in the response to secondary activation by HIV-infected resting T cells.

Discussion

Previous studies, despite controversial, have highlighted the importance of the HIV pathogenicity factor Nef in the modulation of T cell activation. Here we have investigated the role of Nef in the TCR-mediated activation of primary CD4$^+$ T lymphocytes undergoing HIV-1 infection. We employed for this study either freshly isolated quiescent CD4$^+$ T cells or *in vitro* pre-activated CD4$^+$ T cells that returned to an arrested state after few days in culture. These two cell populations consist in CD4$^+$ T lymphocytes with a naïve or memory phenotype and in resting/sub-optimally activated CD4$^+$ T lymphocytes, respectively; thus, they recapitulate the features of the *in vivo* T cell targets of HIV-1 infection (Coiras et al., 2009). We found that, upon TCR-mediated stimulation of HIV-infected quiescent CD4$^+$ T cells, the activation of NFATc1 and NFATc2 transcription factors was enhanced in a Nef-dependent manner. This result is in line with the capacity of Nef to enhance NFAT-dependent transcription of reporter genes shown in Jurkat cells (Fenard et al., 2005; Manninen et al., 2000; Schindler et al., 2006). We found that NFAT super-activation correlated with higher release of IL-2 and enhanced viral replication induced by Nef, confirming and extending previously reported data (Wu and Marsh, 2001). Indeed, numerous experimental findings showed that, in activated T cells, induction of both the IL-2 promoter and the HIV-1 long terminal repeat (LTR) depend critically on the activity of NFAT (Chow et al., 1999; Cron et al., 2000; Fortin et al., 2001; Kinoshita et al., 1998). Of note, increased production of IL-2 can boost HIV-1 replication through autocrine mechanisms, for instance, by further stimulating NFAT (Barlic et al., 2004) and, in addition, can promote viral spread in bystander CD4$^+$ T cells through their recruitment and activation. In HIV-infected quiescent T cells, the phosphorylation/activation of proximal TCR/CD3 and CD28 effectors (i.e., PLC-γ1, Vav, ZAP-70, Akt) occurred normally as in uninfected cells, suggesting that to induce NFAT activation Nef uses mechanisms that are downstream in the pathway. This observation is in line with a study performed in Jurkat cells showing that Nef activates the calcium/calcineurin pathway independently of the TCR and synergizes with the Ras pathway in potently inducing NFAT-dependent transcription (Manninen et al., 2000). However, the detailed mechanism that connects Nef to calcium metabolism and NFAT activation remains to be elucidated. A previous report showed that the serine/threonine kinase Pak1 is implicated in NFAT activation in T cells (Yablonski et al., 1998). Since Nef associates with activated Pak2 (Renkema and Saksela, 2000), another member of the Pak family of closely related kinases, it would be interesting to investigate the role of the Nef/Pak2 complex in the regulation of NFAT activity. Surprisingly, upon activation of primary quiescent CD4$^+$ T cells, we

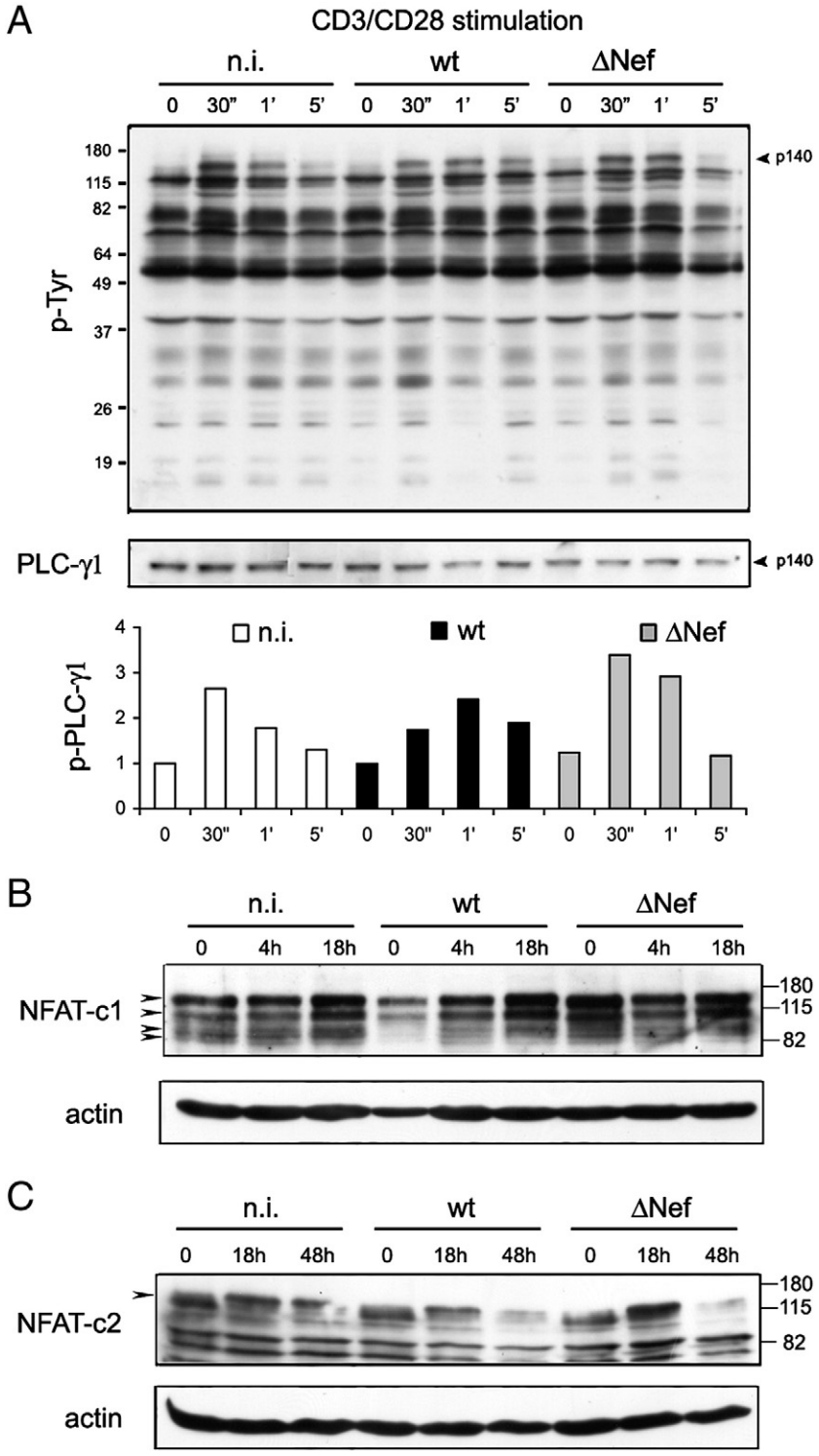

Fig. 6. PLC-γ1 phosphorylation and NFAT activation upon secondary stimulation of T cells infected with wt or Nef-deficient HIV-1. Resting $CD4^+$ T lymphocytes infected with wt or ΔNef viruses as described in Fig. 5 have been re-stimulated 3 days p.i. via CD3/CD28 for the indicated times. (A) Equal amounts of total cell lysates were analyzed by Western blotting with anti-p-Tyr antibody (top), followed by reprobing with anti-PLC-γ1 antibody (center). The phosphorylation of PLC-γ1 was calculated by normalizing phospho-bands for their loading controls and setting to 1 the signal of n.i. cells at t_0 (bottom). (B and C) The cells were analyzed for the activation of NFATc1 and NFATc2 as described in Fig. 4C and D. One representative out of three independent experiments is shown.

found that HIV-1 did not alter the NF-κB pathway. Nevertheless, Nef-induced activation of NFAT may also result in higher NF-κB activity due to the capacity of NFAT to bind the κB regulatory elements in the LTR and to synergize with NF-κB and the viral Tat protein in the enhancement of HIV-1 transcription (Kinoshita et al., 1997).

The analysis of the response to secondary CD3/CD28 activation of resting HIV-infected $CD4^+$ T cells gave different results. In this cell system we showed for the first time that Nef expression resulted in delayed activation of the early signaling molecule PLC-γ1 and impaired super-induction of NFATc2, in agreement with the capacity

of Nef to reduce the recruitment of the TCR-proximal signaling machinery previously reported in two studies (Haller et al., 2006; Thoulouze et al., 2006). In those previous reports, either Jurkat cells or pre-activated primary $CD4^+$ T lymphocytes have been infected with wt or ΔNef HIV-1, then stimulated by engagement with APC or immobilized anti-CD3/CD28 antibodies. In the paper by Thoulouze et al. (2006) the production of IL-2 by Jurkat cells was higher in ΔNef-infected cells if compared to cells infected with wt virus or not infected, suggesting that the inhibitory function of Nef in this cell system suppressed the positive effect on IL-2 expression of other viral proteins (*e.g.* Tat). Similarly, we found that the average IL-2 release by resting T cells was higher if cells were infected with ΔNef virus. Nevertheless, with cells derived from 1 out of 6 donors we observed the opposite phenomenon of a slightly positive effect of Nef on IL-2 production, in agreement with another study performed with resting infected lymphocytes co-cultured with APC (Arhel et al., 2009). Discrepancies may arise from subtle differences in the metabolic state of the cells possibly due to minor variations in the experimental procedures employed or even to donor-dependent features that have not been identified, as yet. We envision that Nef can exert its negative function on IL-2 expression only when the extent of cellular activation has gone beyond a specific threshold. Importantly, we found that the capacity of Nef to enhance viral replication in resting T cells was independent of the protein's function in IL-2 production. Likely, the relative amount of IL-2 produced upon secondary stimulation of resting T cells largely exceeds that required for efficient HIV-1 gene expression and may not represent the best parameter to evaluate the function of Nef in this cell system.

Overall, results obtained in this study indicate that Nef acts as a positive or negative modulator of the TCR pathway depending on the quiescent or active state, respectively, of the infected T cell. Possibly, the levels and/or the availability of Nef and other viral or cellular factors that are influenced by the cell metabolism determine the impact of Nef in the response of infected T cells to activation. Taking advantage of improved assays for HIV-1 integration and reverse transcription (Agosto et al., 2007; Swiggard et al., 2005; Vatakis et al., 2007), it would be interesting to compare the levels of integrated viral DNA and accumulated viral transcripts in quiescent and resting T cells prior as well as after TCR stimulation. While these aspects warrant further investigation, results obtained herein suggest that the inhibitory effect of Nef may result, at least in part, from the protein's capacity to down-regulate surface CD4 and CD28 co-receptors, a phenomenon that we observed in resting but not in quiescent $CD4^+$ T cells infected with HIV-1. It is expected that reduced cell surface expression of CD4 will not allow efficient recruitment of Lck at the activated TCR (Holdorf et al., 2002). Analogously, cells with low surface CD28 levels should be impaired in the response to stimulation with anti-C28 antibody, form aberrant IS with APC, and respond to both stimuli with reduced activation of PLC-γ1 (Michel et al., 2001) and Lck (Holdorf et al., 2002). These negative effects will not occur in HIV-infected quiescent $CD4^+$ T cells in which CD4 and CD28 are not down-regulated by Nef, as shown here for the first time. It is possible that the CD4 and CD28 down-regulation activities of Nef require higher levels of protein expression (shown in Fig. 5B) and/or some cellular events linked to T cell activation. At least for CD4, the latter hypothesis is more likely, since the previously described capacity of Nef to induce intracellular retention and degradation of neo-synthesized CD4 molecules (Rose et al., 2005) was readily detected in quiescent $CD4^+$ T cells 5 days post-infection with HIV-1. In these cells, the intracellular accumulation and reduced total protein expression were also observed for Lck. Surprisingly, the decrease in total Lck protein levels was found also in quiescent cells infected with ΔNef virus as well as in wt- and ΔNef-infected resting T cells. These results indicate that, during HIV-1 infection of both quiescent and resting T cells, Lck levels are reduced by some viral factor(s) different from Nef that warrants to be characterized in future studies. Moreover, we found that, despite reduced Lck expression, wt- and ΔNef-infected quiescent T cells sustained normal Lck activation as measured by CD4 triggering with specific antibody. Therefore, the role of Nef in T cell activation cannot be ascribed to the protein's capacity to modulate the function of Lck. Since most activities of Nef that can have an impact on the TCR signaling pathway (CD4 and CD28 down-regulation activities; Lck re-localization; association with activated Pak2 kinase) are genetically separable, mutated Nef proteins with single functional defects might provide a means to investigate the role of each activity in the responsiveness of HIV-infected cells to stimulation.

The seemingly contradictory effects of Nef on TCR signaling may reflect a dual strategy evolved by HIV-1 to endorse its propagation in T cells. In quiescent $CD4^+$ T cells that are permissive for HIV-1 gene expression but resistant to productive replication, by increasing the cellular response to TCR activation, HIV-1 can favor rapid and efficient synthesis of viral particles. This phenomenon may have important consequences for *in vivo* viral burden since quiescent $CD4^+$ T lymphocytes carrying HIV-1 proviral DNA contribute significantly to the long-lived viral reservoirs in infected patients (Ostrowski et al., 1999; Zhang et al., 1999). On the other hand, by attenuating the ability of sub-optimally activated T cells to respond to external TCR triggering, HIV-1 may protect productively infected cells from AICD, hence favoring viral replication. In support of this model, several studies with *ex vivo* or *in vitro* HIV-infected T cells indicate that exposure to cytokines can trigger viral replication even in the absence of TCR engagement and full T cell activation (reviewed by Coiras et al., 2009). Interestingly, other lymphotropic viruses such as the human T-lymphotropic virus type 1 (HTLV-1) and herpesvirus saimiri (HSV) use this dual strategy to modulate TCR signaling to successfully replicate and spread in the host T cells (Jerome, 2008). In particular, a striking similarity exists between Nef and the HTLV-1 $p12^I$ protein that is able to increase intracellular calcium and NFAT activation bypassing proximal TCR effectors (Ding et al., 2002) as well as to inhibit phosphorylation of PLC-γ1, Vav, and LAT after TCR ligation (Fukumoto et al., 2007). Recent work provided evidence that the positive and negative effects of $p12^I$ are mediated, respectively, by the full-length protein residing in the endoplasmic reticulum and by a smaller proteolytically cleaved isoform located at the Golgi compartment and plasma membrane (Fukumoto et al., 2009). Further studies will be required to establish whether the previously described secondary modifications (proteolytic cleavage, phosphorylation, and myristoylation) and/or the localization at specific sub-cellular compartments determine the phenotype of Nef in the regulation of TCR signaling. The molecular dissection of Nef's activity in the function of the TCR will enhance our understanding of how this viral protein increases HIV-1 replication and may help the development of novel strategies for the treatment of AIDS.

Materials and methods

Cells and viruses

293T cells and primary human $CD4^+$ T lymphocytes purified as described previously (Cerboni et al., 2007) were maintained in Dulbecco's modified Eagle's medium and RPMI 1640 medium, respectively, both supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin–streptomycin (all from GIBCO-BRL). Stocks of infectious VSV-G-pseudotyped NL4-3 virus, either wild type (NIH Reagent Program) or unable to express Nef due to two stop mutations (ΔNef) (Chowers et al., 1994), were prepared by transfecting with the standard calcium-phosphate method 20 μg of proviral plasmid and 3.5 μg of pCMV-VSV-G into 293T cells and collecting cell culture supernatant after 48 h. Viral stocks were titrated by anti-p24 enzyme-linked immunosorbent assay (ELISA) according

to manufacturer's instructions (Immunogenetics). For each experiment, at least three different stocks of viruses were used.

T cell infection and activation

Primary $CD4^+$ T cells, either freshly isolated (quiescent) or activated 6 days earlier as described below (resting), were infected by incubation for 4 h at 37 °C with 250 ng of $p24 \times 10^6$ cells of VSV-G-pseudotyped wt or ΔNef virus. Then, cells were washed twice and cultivated at 5×10^6/ml in complete RPMI medium. The medium was supplemented with 100 IU of human recombinant interleukin-2 (IL-2)/ml in the case of resting T cells. T cell activation was performed either immediately after cell purification or 5 days post-infection (p.i.) by incubating $CD4^+$ T cells on ice for 30 min with phosphate-buffered saline (PBS) alone or supplemented with anti-CD3 (HIT3a) and anti-CD28 (CD28.2) or with anti-CD4 (RP4-T4) NA/LE monoclonal antibodies (mAb) from BD Biosciences each at 10 μg/ml. Next, cells were washed twice with cold PBS, resuspended with 20 μg/ml of anti-mouse goat IgG (GAM; Sigma) and incubated for variable time at 37 °C or not (t_0). Finally, cells were centrifuged, washed twice with PBS and either replaced in culture or lysed for Western blot analysis. To analyze HIV-1 replication and IL-2 production, infected $CD4^+$ T cells were activated via CD3/CD28 as described, resuspended at 5×10^6/ml in complete medium then an aliquot of cell culture supernatant was collected after 16–18, 24, and 48 h for quantification by ELISA of p24 and IL-2 (Endogen) and replaced with fresh medium.

Western blot and immunoprecipitation analysis

$CD4^+$ T lymphocytes were washed twice with PBS and lysed in JS buffer (50 mM Tris–HCl [pH 8.0], 1% NP-40 [vol/vol], 1.5 mM $MgCl_2$, 150 mM NaCl, 5 mM EGTA, 10% glycerol [vol/vol], 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml of pepstatin, 5 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 1× phosphatase inhibitor cocktail 2 (all Sigma's reagents)) for 20 min on ice. After centrifugation, equal amounts of clarified whole-cell lysate (20–30 μg) were resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane (Schleicher & Schuell). Where indicated, total cell lysates were immunoprecipitated with a standard procedure using mAb PY99 anti-p-Tyr (0.2 μg for 80 μg of total cell lysate) from Santa Cruz Biotechnology and Gamma Bind Sepharose (Amersham Pharmacia Biotech) prior SDS-PAGE. Immunoblot analysis was performed by standard methods using 0.05–0.1% Tween-20 (vol/vol) and either non fat dry milk (5%) or bovine serum albumin (5%) as blocking reagent. The following antibodies were used: anti-HIV-1 human serum (kind gift of Dr. M. Federico, ISS, Rome, Italy; used at 1:500 dilution), polyclonal rabbit serum anti-CD4 (H370; 1:200), anti-Lck (3A5; 1:200), anti-PLCγ1 (1:200), and mAb anti-p-Tyr (PY99; 1:1000) and anti-CD3-ζ (6B10.2; 1:200) from Santa Cruz Biotechnology; polyclonal rabbit serum anti-Vav (1:500) and anti-ZAP-70 (1:1000) from Upstate Biotechnology; mAb anti-β-actin (AC15; 1:10000) and anti-α-tubulin (DH1A; 1:10000) from Sigma; anti-Nef polyclonal sheep serum (ARP444; 1:400); anti-CD28 mAb (CD28.2; 1:500) from BD Biosciences; rabbit antibodies anti-Akt (C67E7; 1:1000), anti-p-Akt (D9E; 1:2000), anti-NF-κB p65 (C22B4; 1:1000), anti-p-NF-κB p65 (93H1; 1:1000), anti-p-IκBα (14D4; 1:1000), anti-IKKα (1:1000), anti-p-IKKα/β (16A6; 1:1000), anti-NFATc1 (H-110; 1:200), anti-NFATc2 (M-300; 1:200), and mouse mAb anti-IκBα (L35A5; 1:1000) from Cell Signaling. As secondary antibodies, Protein G (Bio-Rad), anti-mouse, anti-rabbit or anti-goat IgG (Amersham Pharmacia Biotech) conjugated to horseradish peroxidase (HRP) were used. When indicated, the membrane was stripped with 2% SDS (w/w), 62.5 mM Tris–HCl (pH 6.7), 100 mM β-mercaptoethanol for 20 min at 50 °C, then washed three times and reprobed. The proteins were detected with the ECL and ECL advance systems (Amersham Pharmacia Biotech) and proteins' specific signals were quantified by densitometry.

Confocal microscopy

$CD4^+$ T lymphocytes were let adhere on polilysine-treated glass coverslips and fixed with 4% paraformaldehyde in PBS for 30 min at RT. Immunofluorescence staining was performed at RT in the presence of 0.05% saponin and 0.5% bovine serum albumin (Sigma) as permeabilizing and blocking agent, respectively. Cells were incubated for 1 h with anti-Nef mAb MATG020 (1:10000) together with rabbit antibody anti-CD4 (H370, Santa Cruz Biotechnology; 1:10) or anti-Lck (73A5, Upstate Biotechnology; 1:100). As controls, antibodies were replaced with purified mouse IgG or rabbit serum. After washing, cells were reacted for 1 h with Alexa488-conjugated GAM (Molecular Probes; 1:100) and Texas Red-conjugated goat anti-rabbit antibody (Jackson Immunoresearch; 1:100). To simultaneously analyze Nef and CD28, cells were first incubated for 1 h with FITC-conjugated anti-CD28 mAb (BD Biosciences; 1:5), then reacted with normal mouse serum (20 min at RT), with anti-Nef mAb, and, finally, with Alexa555-conjugated GAM (Molecular Probes; 1:100). Nuclei were permeabilized with 0.15% Triton X-100 for 5 min and stained with DRAQ5 (Molecular Probes; 1:5000) for 10 min. Finally, coverslips were mounted on glass slides and confocal imaging was performed on Olympus Fluoview FV1000 confocal IX81 inverted microscope equipped with FV10-ASW version 1.6 software.

Flow cytometry

For simultaneous detection of surface CD4 or CD28 and intracellular p24, HIV-infected or uninfected T cells were incubated with phycoerythrin (PE)-conjugated anti-CD4 or anti-CD28 mAb (BD Biosciences). Next, cells were fixed and permeabilized with reagents from BD Biosciences and incubated with the fluorescein (FITC)-coupled anti-p24 mAb (KC57-RD1; Beckman Coulter). Finally, cells were fixed with 1% paraformaldehyde and analyzed by two-color flow cytometry on a FACSCalibur with CellQuest software (BD Biosciences).

Statistical analyses

All experiments were performed at least three times and a representative experiment is presented. The amounts of IL-2 are expressed as the mean ± SD. Statistical calculation of the mean differences was performed with the Student's *t*-test or, for comparisons shown in Fig. 5G, with the non-parametric Mann–Whitney/Wilcoxon two-sample test. A value of $P<0.05$ was considered statistically significant.

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