TOR VERGATA UNIVERSITY

ROLE OF THE HIV-1 NEF PROTEIN IN THE ACTIVATION OF INFECTED CD4$^+$ T LYMPHOCYTES

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INDEX

ABSTRACT **1**

1 - INTRODUCTION **3**

1.1 The life cycle of HIV-1 3

1.2 HIV-1 pathogenesis 7

1.3 T cell activation during HIV-1 infection 10

1.3.1 T cell activation modulates viral replication 10

1.3.2 Bystander and direct T cell activation 11

1.3.3 The T cell receptor signaling 12

1.4 Role of HIV-1 Nef protein 18

1.4.1 Nef increases HIV-1 replication and infectivity 19

1.4.2 Nef allows immune evasion by HIV-1 20

1.4.3 Down-regulation of CD4 by Nef 21

1.4.4 Effects of Nef on T cell activation 22

2 - MATERIALS AND METHODS **25**

3 - RESULTS **33**

3.1 Nef expression in HIV-1-infected quiescent $CD4^+$ T lymphocytes 33

3.2 Nef increases viral replication and IL-2 production upon activation of HIV-infected T cells 37

3.3 Protein tyrosine phosphorylation upon activation of quiescent HIV-infected T cells 39

3.4 Activation of Akt and NF-κB pathways in HIV-1-infected $CD4^+$ T lymphocytes 42

3.5 CD4/Lck interaction in HIV-1-infected resting T cells 46

3.6 Activation of Lck upon triggering of CD4 in HIV-1-infected cells 48

3.7 Phosphorylation of the TCR ζ-chain upon CD4 stimulation of infected T cells 50

3.8 Nef activity in HIV-1-infected activated $CD4^+$ T lymphocytes…..... 52

3.9 Protein tyrosine phosphorylation upon secondary TCR stimulation of HIV-infected T cells 57

4 - DISCUSSION 60

5 - REFERENCES 67

ACKNOWLEDGMENT 75

PUBLICATIONS 76

ABSTRACT

The replication of HIV-1 is closely linked to the activation state of the cells: in activated memory $CD4^+$ T lymphocytes, HIV-1 readily undergoes multiple rounds of replication, whereas resting T cells are largely refractory to productive infection due to multiple early post-entry replication blocks. Reported evidences suggest that HIV-1 has evolved functions to contrast these blocks. In particular, the product of an early viral gene, Nef, is able to sensitize HIV-1-infected quiescent $CD4^+$ T lymphocytes to activation thus favoring IL-2 production and viral replication in response to TCR stimulation. On the other hand, in productively infected T cells, Nef was shown to impair early activation events during synapse formation with antigen presenting cells that result in reduced release of IL-2. In addition, former studies have shown that Nef associates with several signaling proteins (e.g. Lck, Vav, PI3-K, PAK2), and affects the expression of the CD4 and CD28 co-receptors, but the functional consequences of these interactions in T cell activation is unclear.

This study is focused on the function of the Nef protein in the activation of primary HIV-1-infected $CD4^+$ T lymphocytes. First, the impact of HIV-1 infection, with or without Nef expression, on the steady-state levels and intracellular localization of Lck, CD4 and CD28 was analyzed in both resting and pre-activated $CD4^+$ T lymphocytes. Next, the capacity of $CD4^+$ T lymphocytes infected with wt or Nef-deficient virus to respond to primary and secondary TCR stimulation was investigated. Moreover, CD4 signaling and Lck activation was analyzed after CD4-cross-linking of quiescent T cells infected with wt or Nef-deficient HIV-1. Results show that, despite the steady-state levels of CD4 and Lck are reduced by Nef, the CD4 signaling pathway is preserved in latently infected resting $CD4^+$ T lymphocytes. Upon activation via CD3/CD28 of HIV-1-infected quiescent T cells, early tyrosine-

phosphorylated effector molecules as well as the down-stream NF-κB pathway are induced to the same extent and with the same kinetics in cells infected with wt virus, Nef-deficient virus, and uninfected cells. Conversely, secondary activation via CD3/CD28 of resting HIV-infected T cells, results in a Nef-dependent delay of PLC-γ1 phosphorylation.

Finally, in activated HIV-1-infected T cells the dramatic down-regulation of surface CD4 induced by Nef and late viral proteins (Env, Vpu) as well as the Nef-dependent CD28 down-regulation, may account for the previously described Nef's capacity to impair the formation of the immune synapse. Apparently, that Nef differentially regulates T cell activation depending on the intracellular environment: Nef enhances activation of quiescent cells to favour initial viral replication whereas it inhibits second rounds of TCR stimulation, possibly to avoid apoptosis that would restrict viral replication.

The future identification of target molecules regulated by Nef is an important step for understaing how HIV-1 interferes with T cell function to facilitate viral spread.

1-INTRODUCTION

1.1 The life cycle of HIV-1

The Human Immunodeficiency Virus type 1 (HIV-1) is a member of the lentivirus family of retroviruses and is the etiologic agent of the acquired immunodeficiency syndrome (AIDS). The genome of HIV-1 is 10 Kb in length and encodes 16 distinct proteins. Those proteins that are derived from the *gag* (group specific antigen), *pol* (polimerase), and *env* (envelope) genes are classical structural and enzymatic factors that are required by all retroviruses. In addition, HIV-1 contains open reading frame for six additional regulatory and accessory proteins. The Trans Activator of Transcription (Tat), the Regulator Expression of Virion genes (Rev), the ill-named "Negative Factor" (Nef), the Viral Infectivity Factor (Vif) and the Viral Proteins r (Vpr) and u (Vpu) [1].

HIV-1 enters the body through the exchange of bodily fluids and its major target of infection are cells expressing the CD4 surface antigen, such as T helper (Th) cells, and cells of the monocyte/macrophage lineage including monocytes, macrophages, dendritic cells (DC) and brain microglial cells. Virus-CD4 receptor binding is mediated by a high affinity interaction between the viral outer envelope glycoprotein, gp120, and the CD4 molecule. Subsequentially, the gp120 interacts with one of the additional coreceptors that are strain and target specific and determines the tropism of the virus. The R5 strains of HIV-1 use CC chemokine receptor 5 (CCR5) as their coreceptors and can, therefore, enter macrophages, DCs and T cells, whereas X4 strains use CXCR4 as a coreceptor and can infect T cells only [2] . Early in infection, only R5 viruses are usually detected in infected individuals. At this stage, the virus might need to transit through DCs and

macrophages which in turn could pass the virus to $CD4^+$ T cells possibly during the process of antigen presentation. DCs are likely to have an important role in transporting the virus from its portal of entry to lymphoid organs. These cells can be productively infected by HIV-1 or they can capture the virus trough DC-specific ICAM3-grabbing non integrin receptor (DC-SIGN) and store it in an infectious form before presenting it to T cells that simultaneously become primed for infection. With time, X4 viruses come to predominate which hastens the demise of Th cells, the hallmark of AIDS [3].

Once internalized, HIV-1 is uncoated and its RNA genome is reverse transcribed to a double-stranded DNA that is integrated into the genome of the target cell, yielding the long terminal repeat (LTR)-flaked provirus (Fig. 1). Unlike other retroviruses, HIV-1 does not require disintegration of the nuclear membrane given that nuclear localization signals on integrase (IN), matrix and Vpr proteins ensure that the viral genome passes through the nuclear pores. The linear double stranded cDNA integrates in the genome with a preference for active genes, although other regions, for example heterochromatin-rich centromeric regions, are targeted also [4]. The activity of the integrated viral genome or provirus is greatly influenced by the metabolic and activation state of the host cell and the longevity of the provirus is dictated by the life span of the cell that contains it [5].

In an activated T cell, viral replication is rapid and efficient. The viral long terminal repeat contains binding sites for cellular factors that positively regulate transcription of HIV-1 and that are abundant in activated T cells. However, these host factors are not enough, and the viral Tat protein also markedly augments viral gene expression. The situation is very different in resting lymphocytes. These cells are refractory to viral replication *in vitro* because there are several barriers that preclude

the completion of early steps, such as, inefficient reverse transcription and energy levels too low for effective nuclear import of double-stranded viral genomes [5]. In infected quiescent cells, the provirus might exist in a silent or latent state and it can be rescued upon cell activation. Once they have been activated even partially, T cells become fully permissive for HIV infection. The nature of the induced factors that are responsible for the acquisition of HIV permissiveness is not known yet. Under conditions where integration was undetectable, some genes are already transcribed. All HIV transcripts are derived from a common full-lenght precursor, which also serves as the mRNA for the *gag-pol* genes and the viral genomic RNA. By alternative splicing, this full-lenght precursor generates about 30 different mRNAs, including singly spliced transcripts coding for Env, Vpu, Vpr and Vif, and multiply spliced transcripts coding for Nef-, Tat- and Rev-specific transcripts [6]. Among the multiply spliced transcripts, Nef-encoding mRNA was prominent in quiescent cells, along with lesser levels of Tat mRNA. By comparison, activated cells generated abundant levels of Nef-, Tat- and Rev-specific transcripts. When viral DNA is integrated in the host genome, the provirus behaves like any human gene, with transcription being initiated at the 5' end and terminating at the 3' end. Genomic RNA is exported to the cytoplasm where it is used as a template for translation of Gag full-lenght polypeptide that it is cleaved into the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins. This production is followed by assembly, budding and maturation, in which the new HIV-1 particles are packaged up and sent out to infect new cells [4].

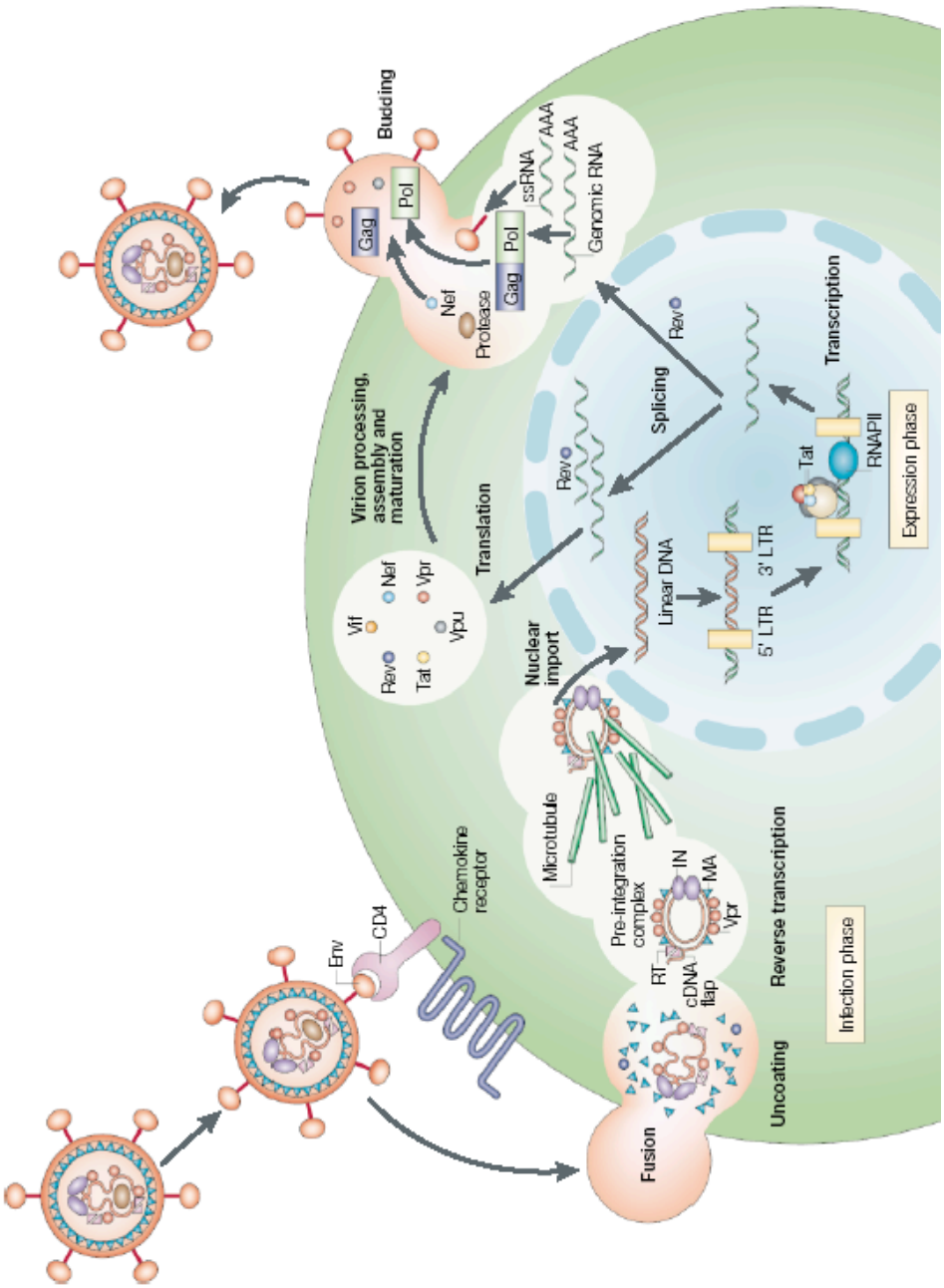

Fig. 1. HIV-1 life cycle (from Nat Rev Immunol, 2003, 3:97)

1.2 HIV-1 pathogenesis

Primary infection of humans with HIV-1 is associated with an acute mononucleosis-like clinical syndrome which appears approximately 3-6 weeks following infection (Fig. 2.). The severity and persistence of the symptoms vary considerably. Significant declines in the levels of $CD4^+$ T lymphocytes in the peripheral blood occur in the first 2-8 weeks following HIV-1 infection. These levels may rebound toward normal as the patients enters the clinically latent stage of disease, although they rarely if ever return to pre-infection levels [6, 7]. The acute syndrome associated with primary HIV-1 infection is accompanied by a burst of viral replication that can be detected in the blood approximately 3 weeks following infection. During this period, infectious virus and viral proteins can be detected in the cell-free plasma, and the number of virions can reach 10^6 to 10^7 per milliliter.

Approximately 3-6 weeks after the infection with HIV-1, specific antiviral immune responses can be detected in association with a decline in the viral burden and a temporary stabilization of the number of $CD4^+$ T cells. Multiple factors probably contribute to the decline in viral titer, including innate and adaptive immune responses, the secretion of suppressing cytokines, and the depletion of $CD4^+$ target cells [7] .

Following the induction of an immune response to HIV-1, there is a relatively long period of latency that is characterized by few, if any, clinical manifestations. The length of the latency is regulated by a complex interplay between the host genetic and immunologic factors and the pathogenic potential of the virus. Throughout the latent period there is usually a continuous, yet variable, decline in the numbers of $CD4^+$ lymphocytes as well as continuous viral replication. Once the $CD4^+$ T cell count falls

below 200 cell/μl, the patient is susceptible to AIDS-defining opportunistic infections and neoplastic diseases. In addition, HIV-1 infection leads to the dysregulation of several leukocyte subpopulations and generalized immune activation with the subsequent development of other infections and malignancies [7]. By progressive destruction of the helper T lymphocyte pool, HIV-1 precludes both the efficient production of antibodies by B cells and the proper function of cytotoxic T lymphocytes (CTLs). In parallel, the virus exploits a great diversity of strategies to alter the cellular arm of the immune response. Indeed, through mechanisms that are not clear as yet, HIV infection leads to B-cell hyperplasia, hypergammaglobulinemia, elevated autoantibody titers, a poor response to neoantigens and mitogens [8]. These abnormalities may contribute to disease progression and development of opportunistic infections.

Several evidence indicate that the HIV-1 induced perturbation of innate immune responses contributes to permissive immunosuppression. For instance, natural killer (NK) cells, display an aberrant phenotype and reduced citotoxic activity [8, 9].

The macrophage lineage play an important role in initial infection with HIV-1 and contribute to pathogenesis. The infected macrophages are resistant to cytopathic effects and persist throughout the course of infection as long-term stable reservoirs for HIV-1 capable of disseminating the virus to tissues [10].

In general, elevated levels of activation are observed in the immune systems of HIV-1-infected individuals and activation marker expression correlates with disease progression [7].

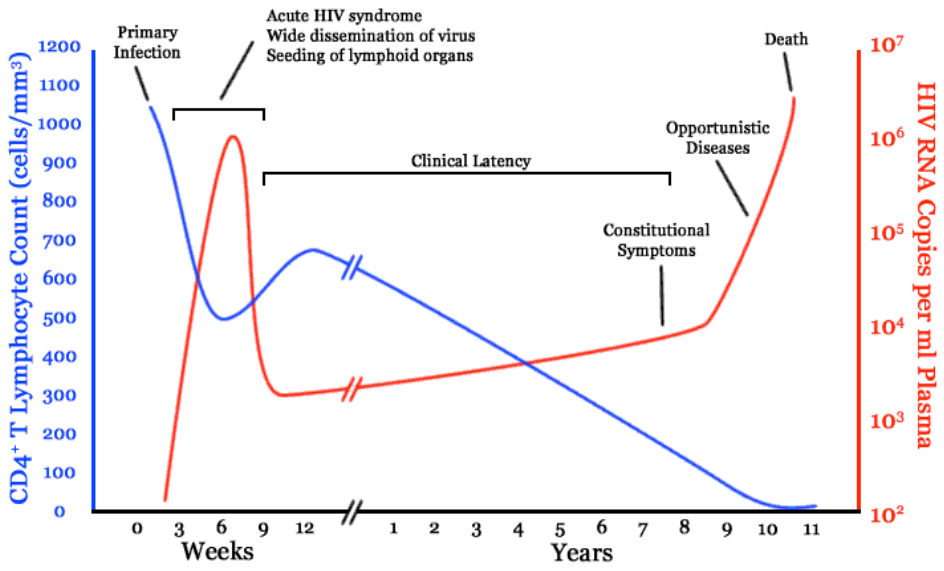

Fig. 2. Viral load and CD4+ T cell count over the course of HIV-1 infection in an untreated patient (from Nat Rev Immunol, 2003, 3:97).

1.3 T cell activation during HIV-1 infection

1.3.1 T cell activation modulates viral replication

As mentioned earlier, the cellular activation state is critical for productive HIV-1 infection. *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 [5, 11]. In particular, some studies indicate that higher HIV-1 LTR activation levels were consistently observed in memory cells if compared to naïve cells [12]. The authors observed that memory CD45RO$^+$ display a more pronounced NFAT (nuclear factor of activated T cells) activation, which may explain higher replication levels [13].

Studies performed with cells derived from HIV-1 patients, showed that memory CD4$^+$ T cells produce fourfold more replication competent virus and contain six fold more provirus than naïve CD4$^+$ T cells [14]. These observations suggest that HIV-1 can enter naïve CD4$^+$ T cells *in vivo* and initiate reverse transcription but it is unable to integrate into genome as efficiently as in memory CD4$^+$ T cells. *In vitro* studies have also demonstrated that naïve CD4$^+$ T cells can be acutely infected with HIV-1 but are less efficient in replicating virus upon physiologic stimulation [15]. The mechanisms responsible for these differences in viral replicative capacity have not been clearly defined and possible explanations include lower levels of deoxynycleoside triphosphate substrates and a deficiency of other cellular factors in naïve cells that are necessary for completion of viral replicative cycle [16].

1.3.2 Bystander and direct T cell activation

HIV infection is known to be associated with changes in the cytokine milieu that can lead to the induction of bystander T cell activation. Bystander activation is defined as activation of a T cell to produce phenotypic or functional changes through a mechanism which is independent of specific T cell receptor stimulation [17]. Numerous studies in mice have shown that cytokines alone can elicit T-cell responses. Cytokines such as IL-2, IL-4, IL-6, IL-7 and IL-15 can render resting T cells susceptible to productive HIV infection, and this might further enhance disease progression.

Previous work has demonstrated that the exogenous viral protein Nef is able to mediate bystander activation of T cells, to increase PBMC proliferation and to enhance HIV-1 replication through IL-15 synthesis induction by the monocyte/macrophage population [18].

Association of the viral envelope gp120 with surface CD4 has been demonstrated to activate pathways in both primary T cells and T cell lines. The gp120 has innate biological activity as a result of a specific interaction with CD4, inducing increases in intracellular levels of inositol triphosphate and of calcium, and in interleukin-2 receptor expression and cell motility [7].

Direct effects of HIV infection on the activation state of human T cells have been described. For instance, it was shown that infection of by HIV-1 renders T cells hypersensitive to T cell receptor and CD28 stimulation [19]. In particular, Nef protein associates with membrane microdomains critically involved in the initiation and propagation of T cell signaling and it primes T cells for activation [20]. Nef promotes the activation of transcription factors such as NFAT and NF-κB in response to CD3/CD28 stimulation that induce enhancement of IL-2 secretion and HIV-1

replication in a stimulus-dependent manner [19, 20]. Another HIV regulatory protein capable to enhance cytokine gene transcription, Tat, is expressed early during infection. Both Nef or Tat enhances IL-2 secretion in Jurkat and primary T cells upon T cell activation [21].

1.3.3 The T cell receptor signaling

The engagement of the T cell receptor (TCR) initiates the earliest events leading to T cell activation. The TCR is a complex consisting of the variable αβ chains noncovalently associated with the nonpolymorphic CD3 proteins. CD3 exist as a series of dimers including γε, δε, and ζζ associated with a single αβ heterodimer. The ζ-chain of the CD3 display an extra-cellular region of 9 amino acids, a trans-membrane domain, and a intra-cytoplasm domain of 113 amminoacids with three ITAM (Immunoreceptor Tyrosine-based Activation Motif). The CD3 and ζ-chain drive the signal transduction induced by antigen recognition and biochemicals events leading to T cell activation.

CD4 and CD8 are two important co-receptors that participate to the triggering and signal transduction of the TCR. The interaction between CD4 and the non variable regions of MHC II molecules on the surface of the antigen presenting cell (APC) enhances the T cell response to antigenic stimulation. CD4 is a 55 kDa glycoprotein comprised of four Ig-like extracellular domains, a single transmembrane domain, and a short cytoplasmic tail of 38 residues. CD4 is associated noncovalently with a *src*-related tyrosine kinase, p56Lck, through two cysteine residues at positions 420 and 422 of the cytoplasmic domain [22]. The CD4/Lck association is required to

bring Lck in the vicinity of the TCR signaling complex and initiate T cell activation upon antigen recognition [24, 25].

In addition, the outcome of TCR stimulation is regulated by the simultaneous engagement of the costimulatory molecule CD28. Indeed, T cells costimulated through CD28 respond more rapidly to lower levels of TCR occupancy enhancing distinct signaling pathways that lead to gene transcription [26].

The earliest step in intracellular signaling following TCR ligation is the tyrosine phosphorylation of ITAM sequences in the intra-cytoplasm tail of ζ-chain and CD3. The phospho-tyrosine becomes a binding site for SH2 domain of s*rc* kinase (Lck, Fyn**)** and ZAP-70. ZAP-70 binds the transmembrane adapter protein linker for the activation of T cells (LAT) and the cytosolic adapter domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) [27]. These two adapters form the backbone of the complex that organizes effector molecules in the correct spatiotemporal manner to allow for the activation of multiple signaling pathways. LAT contains nine tyrosines that are phosphorylated upon TCR engagement, which bind the C-terminal SH2 domain of PLC-γ1, the p85 subunit of phosphoinositide 3-kinase (PI3K), and the adapters growth factor receptor-bound protein 2 (GRB2) and the adapter downstream of Shc (Gads). PLC-γ1 also directly binds to SLP-76, LAT and Vav1. These interactions collectively are required to stabilize PLC-γ1 in the correct conformation within the complex to allow for its optimal activity. The proximal signaling complex results in the activation of PLC-γ1-dependent pathways including Ca^{2+}- and DAG-induced responses, cytoskeletal rearrangements, and integrin activation pathways. Activated PLC-γ1 then hydrolizes the membrane lipid PI(4,5)P2, producing the

second messengers IP3 and DAG. The production of DAG results in the activation of two major pathways involving Ras and PKC. Ras is a guanine nucleotide-binding protein and is required for the activation of the Raf-1 kinase, which initiates a mitogen-assiciated protein kinase MAPK phosphorilation, which in turn phosphorylate Erk. Erk kinase activity results in the activation of Elk, which contributes to the activation of AP-1, Jun and Fos transcription complex. One critical pathway that PKC regulates involves the NF-κB transcription factor. In resting cells, NF-κB is found in the cytosol associated with inhibitor of NF-κB (IκB) family members that keep NF-κB from moving into the nucleus. Upon T cell activation, IÍκB is phosphorylated by the IκB kinase (IKK) complex, ubiquitinylated, and degraded, allowing NF-IκB to translocate into the nucleus, where it activates genes involved in the function, survival and homeostasis of T cells [28].

TCR recruitment and Vav activation induce Rac/cdc42/MKK and p38 activation. Rac-GTP activates Fos and JNK (c-Jun N-terminal kinase) which phosphorylates c-Jun. TCR-induced increases in intracellular Ca^{2+} levels result in the activation of Ca^{2+} and calmodulin-dependent transcription factors, including the phosphatase calcineurin and the Ca^{2+}-calmodulin-dependent kinase that in turn activate a variety of transcription programs. Activated calcineurin dephosphorylates members of the NFAT family, leading to their translocation to the nucleus. In the nucleus, NFAT isoforms can form cooperative complexes with a variety of other transcription factors, thereby integrating signaling pathways resulting in differential gene expression patterns and functional outcomes. The NFAT/AP-1 interaction integrates Ca^{2+} and Ras signals and results in the expression of genes important for T

cell activation including IL-2. In contrast, NFAT activity in the absence of AP-1 activation induces a pattern of gene expression that results in T cell anergy and a characteristic lack of IL-2 production.

Numerous studies have shown that CD28 promotes T cell proliferation, cytokine production, cell survival and cellular metabolism. Following binding of CD28 to its ligands CD80 or CD86 on APCs, the p85 regulatory subunit of PI3K associates with a cytoplasmic tail of CD28. This regulatory subunit recruits the p110 catalytic subunit of PI3K, which converts PIP2 to phosphatidylinositol (3,4,5) trisphosphate (PIP3) at the membrane [29]. Localized PIP3 generation serves as a docking site for the PDK1 and its target Akt. Akt phosphorylates multiple proteins, and thus affects numerous cellular responses. Activation of Akt enhances the nuclear translocation of NF-kB, which has positive effects on the expression of prosurvival genes. The ability of Akt to promote prosurvival gene expression, coupled with the ability of Akt to inhibit trancription factors that promote cell cycle arrest, results in Akt-driven cell survival and proliferation. Akt also affects optimal trascription of NFAT-regulated genes, such as IL-2 [24].

During HIV-1 infection different viral proteins are involved in alteration of the TCR signaling. In particular the function of Tat is to dysregulate cytokine production resulting in perturbation of the host immune response and enhancement of the retrovirus survival. Previous work demonstrate that Tat impairs the IFNγ-receptor signaling pathway. In addition the Nef protein of HIV-1 has a important role in the activation pathway of T cells. In T cell lines, Nef induces transcription of an array of genes almost identical to that triggered upon exogenous stimulation of T cell receptor

[30]. However, 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 IL-2 production [19], other studies demonstrated that Nef has no effect on T cell activation markers or even reported an inhibitory effect of Nef on T cell activation, such as disruption of immunological synapse and the accumulation and degradation of Lck [19, 20].

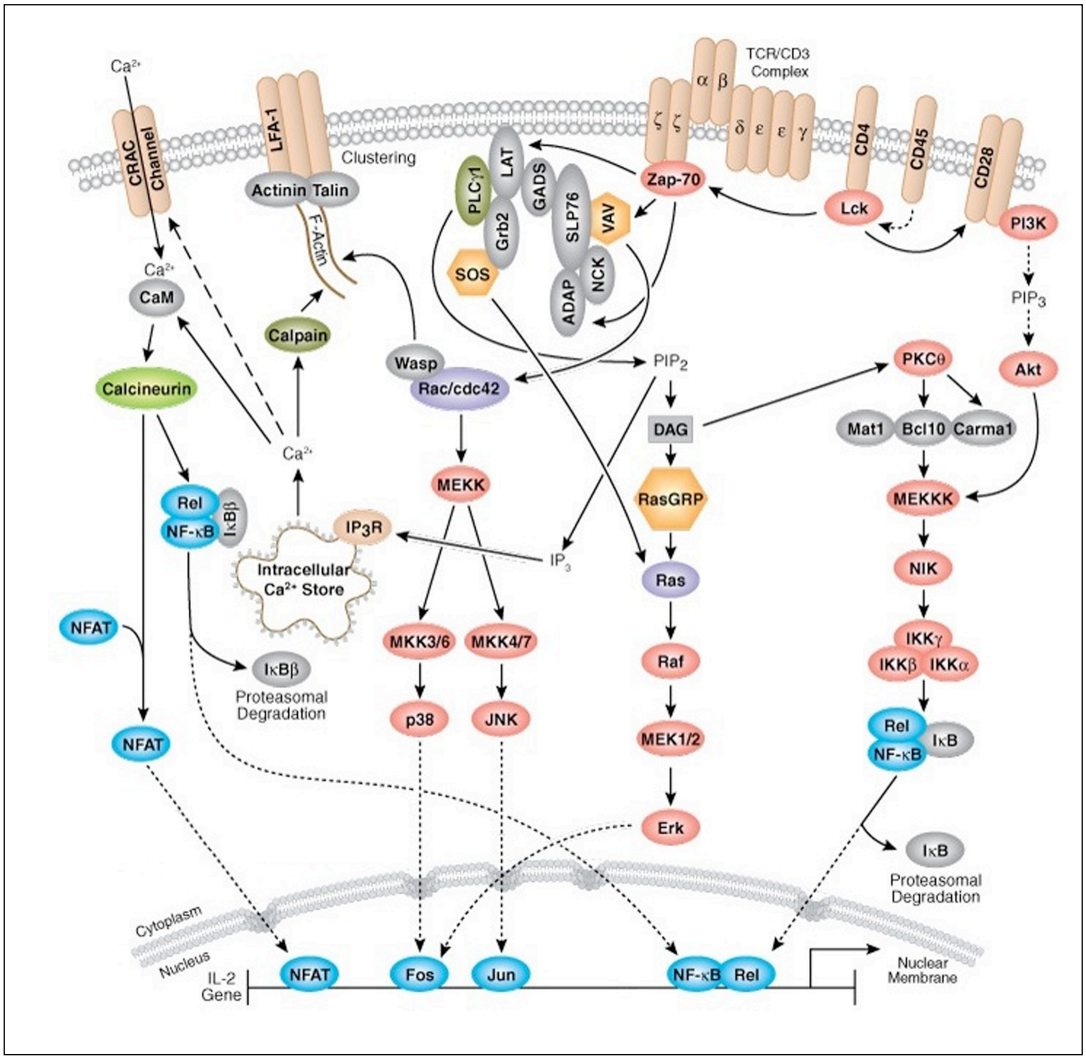

Fig. 3. TCR receptor signaling

T Cell Receptor (TCR) activation promotes a number of signaling cascades that ultimately determine cell fate through regulating cytokine production, cell survival, proliferation, and differentiation. (from Immunol. Rev. 2007)

1.4 Role of the HIV-1 Nef protein

The *nef* gene product is a small protein of about 27 kDa that is syntesized at the earliest stage of viral gene expression. It was originally thought to exert a negative influence on viral replication, hence the acronym Nef for Negative factor. These early results were not confirmed and Nef was instead convincingly shown to stimulate viral growth both in cell culture and *in vivo*. Nef is post-translationally modified by myristoylation of its amino terminus, and this modification targets the protein to the inner surface of the plasma membrane and to the trans-golgi network. Nef is present also in the cytosol, in the nucleus and is incorporated in the virion. To date, no enzymatic activity has been directly attributed to the Nef protein. Nevertheless, several major distinct activities of Nef have been demonstrated *in vitro*: down-regulation of cell surface molecules such as CD4 and MHC-I, alteration of cellular signaling pathways and stimulation of HIV-1 infectivity and replication [31, 32].

The Nef protein contains several domains that are critical for its functic[33]. The N-myristoylation signal of Nef is required for its association with cellular membranes and is critical for virtually all of its biological activities. The conserved proline-rich motif (PxxP) of Nef was accredited as a Src homology domain 3 (SH3)-binding site, with its central prolines, P_{72} and P_{75}, contacting two hydrophobic pockets in the SH3 domain, whereas P_{69} and P_{78} assist the PxxP positioning. Through PxxP, Nef binds SH3-containing signaling molecules such as Hck and Vav. To date, several regions of Nef have been involved in the internalization and trafficking of molecules, events that mediate the degradation of CD4 and MHC-I [34]. Two Nef's motifs located in the C-terminal flexible loop are involved specifically in CD4

internalization: LL_{165} and DD_{175}. The highly conserved endocytic LL_{165} signal is essential for the sorting of Nef into clathrin-coated pits, a process mediated by the interaction with clathrin adaptor complexes AP-1 and AP-2. This motif likely binds adaptor protein complexes, although its recognition domain has not yet been mapped precisely. In the case of the two aspartic acids (DD_{175}) motif, mutation to alanine completely abolishes the ability of Nef to down-regulate CD4. The catalytic subunit H of the vacuolar ATPase (V1H) has been shown to interact with this region, and to promote CD4 internalization by bringing it to the endocytic machinery. The interaction between Nef and PACS-1, which is supposed to mediate retention of MHC I by Nef in the *trans*-Golgi, was mapped to an acidic cluster of four successive glutamic acids in Nef [$EEEE_{(62)}$].

1.4.1 Nef increases HIV-1 replication and infectivity

Lack of Nef protein synthesis compromises the virus infection process by markedly decreasing the ability of HIV-1 to replicate *in vitro* systems [32, 35]. *In vivo* studies confirmed that Nef confers an advantage for virus replication. The positive influence of Nef on viral growth is due, at least in part, to its capacity to enhance the infectivity of virions. Indeed, end-point titrations of isogenic wild-type and *nef*-deleted viruses have determined that virus containing an intact *nef* gene have a greater infectivity per picogram of capsid antigen (p24) than *nef*-deleted virus. The mechanism behind Nef enhancement of infectivity is not clear.

In addition, Nef may increase viral release and infectivity by down-regulating CD4, thus counteracting the negative effect of the receptor on Env incorporation into viral particles and on the output of progeny virions. However, Nef is also able to enhance the viral infectivity of HIV-I particles produced in CD4 negative cells [35,

36, 37]. Several properties of Nef may explain its CD4-independent capacity to improve the function of the viral envelope. Recent evidences suggest that Nef increases the cell-surface levels of Env products and cholesterol which, as a consequence, accumulate into virions increasing their infectivity. In addition, it was suggested recently that Nef's ability to associate with cell membrane lipid rafts may favor viral egress from this sites, a phenomenon that seems to increase HIV-1 infectivity.

1.4.2 Nef allows immune evasion by HIV-1

HIV-1 has evolved several mechanisms to evade the immune defence of the host and establish a chronic infection. The Nef protein reduces the surface expression of MHC-I molecules thus protecting infected cells from CTL recognition and killing . However, MHC-I downregulation can alert NK cells that preferentially lyse target cells with reducted MHC-I expression. Of note, Nef selectively decreases HLA-A and -B, leaving the levels of HLA-C and -E unchanged. This selective MHC-I downregulation has been shown to protect HIV-infected cells from lysis mediated by NK cells expressing inhibitory receptors that are specific for HLA-C and -E [9].

Several evidences suggest that the ability of Nef to disrupt MHC-I-mediate antigen presentation is very important for viral disease pathogenesis *in vivo*. For instance, our group found that Nef proteins derived from rapid progressor patients down-regulate MHC-I more efficiently than proteins derived from slow or non-progressor patients [38].

1.4.3 Downregulation of CD4 by Nef

The best characterized among Nef's activities is down-regulation of cell surface CD4 molecules. Nef acts as a CD4-specific sorting adapter by accelerating receptor endocytosis and misdirecting internalized molecules to lysosomes where they are degraded instead of entering recycling pathways [39]. This process requires a membrane-proximal dileucine motif (LL_{414}) of CD4 that functions as an internalization signal, as well as various residues in Nef that mediate its association with plasma membranes (the myristoylation site G_2), CD4 (WLE_{59}), adaptor protein (AP) complexes (LL_{165}), and the catalytic subunit of the vacuolar ATPase (DD_{175}) . In our laboratory we demonstrated that to accumulate and degrade CD4 Nef uses mechanisms distinct from those used to down-regulate cell-surface CD4 [40].

It is currently unclear how Nef-mediated CD4 down-regulation may increase *in vivo* viral loads and influence pathogenesis. The first effect attributed to this Nef's activity was protection of infected cells against viral superinfection, a phenomenon that can lead to reduced viral replication and premature cell death. On the other hand, it was recently shown that Nef variants defective for CD4 down-regulation were still able to inhibit to some extent HIV-1 superinfection [43]. An important effect accredited to Nef-mediated removal of CD4 consists in avoiding Env sequestration into a CD4-Env complex thus favoring the release and infectivity of HIV-1 particles [44, 45, 46].

Since the disruption of the CD4/Lck complex precedes internalization of CD4 [47], a probable indirect consequence of Nef-induced down-regulation of CD4 is the accumulation of Lck in recycling endosomes. Lck is usually translocated from recycling endosomes to the immunological synapse to sustain signal transduction, but this process is inhibited by Nef, which induce intracellular accumulation of Lck [48].

It was observed that disruption of CD4/Lck complex by Nef correlates tighly with CD4 down-modulation suggesting that both processes are coupled [49, 50], although discordant data have been reported. For instance, it was shown that CD4 mutants that cannot dissociate Lck are efficiently down-regulated by Nef, suggesting that CD4/Lck association is not involved in downregulation by Nef. In addition, by *in vitro* binding of purified proteins, it was observed that Nef associates directly with Lck and inhibits its *in vitro* catalytic activities [53, 54].

1.4.4. Effects of Nef on T cell activation

Despite early reports described a positive effect of Nef on TCR signaling, opposing effects have been demonstrated by a large number of recent papers.

A positive effect of Nef in T cell activation has been attributed to the protein's capacity to lower the T cell activation threshold, that is associated with increased IL-2 production. By examining human T cell line (Jurkat) and primary T lymphocytes after transduction with retroviral vector expressing only the Nef protein of HIV and after CD3/CD28 stimulation, some groups observed that Nef increase of IL-2 production is due to the enhancement of cells secreting IL-2, but not to a higher amount of IL-2 secreted per cell [19, 20]. A positive role of Nef was observed also in quiescent primary $CD4^+$ T cells infected with NL4-3 strain of HIV-1, and 5 days post infection stimulated with anti-CD3/CD28 coated beads. Wu and colleagues showed a stimulus-dependent increase in IL-2 generation and an increase in viral replication when a functional *nef* gene was present in resting cells exposed to HIV [21]. Moreover, some reports reveled that the Nef primes human T lymphocytes for signaling through the CD3/CD28 stimulation promoting the activation of transcription factors such as NFAT and NF-κB [20, 55]. In another study, the effects

of Nef on PI3K-dependent signaling pathways were analyzed. The Jurkat cell line was transduced with Nef-expressing vaccinia virus and the phosphotyrosine-associated fraction of PI3K was precipitated from cells stimulated with PMA and anti-CD28. As shown in this work, the level of PI3K activity was two fold higher in cells expressing Nef [56].

In addition, the TCR signaling events were analyzed in thymocytes transduced with HIV and stimulated through their CD3. This study showed that Nef expressing thymocytes were in a state of activation and hyperresponsiveness with respect to the tyrosine phosphorylation of several substrates of TCR signaling including LAT and MAP kinase [57].

Different results were obtained in a more recent study in which quiescent $CD4^+$ T cells were transfected with expression plasmids encoding a GFP-tagged Nef protein of the SF2 strain of HIV-1. In this experimental model, 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 stimulation [58]. Analogue results were obtained in Jurkat cells transfected with a Nef-encoding plasmid: little or no effect by Nef was observed on T cell activation after CD3/CD28 activation [59].

A negative effect of Nef in T cell activation was observed in other studies showing that the viral protein dysregulates the function of the immunological synapse (SI) [60]. One study proposed that HIV-1 Nef perturbs T cell receptor clustering and internalization and reduces T cell activation following receptor ligation [48]. In this experimental system, primary T lymphocytes and Jurkat T cell line were infected with HIV-1 and then incubated with super-antigen pulsed APC. In addition, Nef reduced the recruitment of Lck and tyrosine-phosphorylated proteins at the IS by both

inducing endosomal retention of Lck and reducing the accumulation of tyrosine-phosphorylation protein at contact site between T cell and APC [48, 61].

Finally, Nef has been proposed to functionally interact with the actin cytoskeleton that drives segregation of surface receptors and receptor-proximal signaling machinaries favouring the stable IS formation. In particular, it was shown that Jurkat T cells expressing a GFP-Nef fusion protein had a markedly impaired formation of actin rings upon contact with anti-CD3 antibody coated surfaces mimicking the IS [62].

2-MATERIALS AND METHODS

Cells and antibodies

293T cells (kindly provided by G. Nolan, Stanford, CA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin. For isolation of primary human $CD4^+$ T lymphocytes. Peripheral blood mononuclear cells (PBMCs) were derived from healthy donors by Ficoll-Hypaque (Amersham Pharmacia Biotech) density gradient sedimentation of buffy coats. $CD4^+$ T cells were purified from PBMC with anti-CD4 mAb-coated magnetic beads (MACS Miltenyi Biotec) according to manufacturer's instructions and cultivated in complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin. The purity of $CD4^+$ T cells was >95% as determined by flow cytometry.

For the analysis of the surface expression of CD4 and CD28 molecules were used, respectively; (PE)-conjugated anti-CD4 (MT310, Dako), and FITC-conjugated anti-CD28 antibodies (BD).

For cells stimulation; we used monoclonal antibody (mAb) anti-CD4 (clone RPA-T4, BD Biosciences), anti-CD3, anti-CD28 (BD Biosciences Pharmingen) cross-linked with goat anti-mouse IgG (Sigma).

For immunofluorescence microscopy, the following antibodies were used; mAb anti-Nef MATG (kindly provided by Schwartz O.), mAb anti-CD4 and anti-CD28 (BD Biosciences Pharmingen), polyclonal rabbit anti-CD4 and anti-Lck (Santa

Cruz Biotechnology), Alexa 488- or Alexa 555-conjugated goat anti-mouse IgG, texas red-conjugated-goat-anti rabbit, IgG.

Western blotting and immunoprecipitation analyses were performed with the following antibodies; polyclonal rabbit serum anti-CD4, mAb anti-p-Tyr, polyclonal rabbit serum anti-Lck and mAb anti-CD3-ζ–chain, polyclonal rabbit serum anti-PLCγ1, polyclonal rabbit serum anti-Vav (all from Santa Cruz Biotechnology), polyclonal sheep anti-Nef (clone 444, kind gift of M. Harris), polyclonal rabbit serum anti-ZAP-70 (Upstate), mAb anti-Akt, monoclonal rabbit IgG anti-IKKα/β, mAb anti-NF-κB, mAb anti-IκB (all from Cell Signaling). As secondary antibodies, HRP-conjugated goat anti-mouse, or anti-rabbit IgG or, alternatively, G-protein-coupled HRP were used.

DNA constructs

The following proviral HIV-1 constructs, were used: NL4-3 (wt; NHI Reagent Program), PDS (ΔNef; NHI Reagent Program), HIV-GFP wt and ΔNef (kindly provided by Kirchhoff F.), the mutated DDAA virus was generated in the genetic background of the NL4-3 strain cloned in the pNLblue vector [63]. The DDAA mutation was introduced by standard PCR-based mutagenesis in the NL4-3 *nef* gene, that was amplified by PCR with specific primers introducing *Nco*I and *Not*I sites at the 5' and 3' ends, respectively, and cloned in the *Nco*I/*Not*I sites of pNLblue. All DNA constructs have been confirmed by sequencing of both strands.

HIV-1 production and detection

Stocks of infectious NL4-3 viruses were prepared by transfecting 20 μg of proviral plasmids into 293T cells with the standard calcium-phosphate method. 48 h post-transfection, cell culture supernatants were collected, clarified by low-speed centrifugation, and stored in aliquots at – 80°C.

Viral stocks were titrated by anti-p24 ELISA (Immunogenetics) according to manufacturer's instructions.

HIV-1 infection and stimulation of T cells.

Freshly isolated quiescent $CD4^+$ T cells were infected by incubation with occasional agitation for 4 h at 37°C with 250 ng of p24 x 10^6 cells of VSV-G-pseudotyped wt or Nef-deleted virus. Cells were then washed twice, re-suspended at 5 x 10^6/ml in complete RPMI medium. 5 days post-infection, the cells were either analyzed immediatelly or stimulated. To stimulate the CD4 receptor, infected $CD4^+$ T cells were incubated on ice with 10 μg/ml of anti-CD4 mAb for 30 minutes. Alternativelly, infected $CD4^+$ T cells were incubated with 10 μg/ml of anti-CD3 and anti-CD28 mAb to stimulate the TCR signalling. Then, cells were wash, re-suspended with 20 μg/ml of anti-mouse goat IgG and incubated for variable time at +37°C. Finally, the cells were collected, washed twice with PBS and lysed for western blotting analysis. In order to analyze viral replication and IL-2 production, quiescent T cells have been infected with low viral doses (25 ng p24/ 10^6 cells), stimulated 5 days p.i. via CD3/CD28 described above, then cultivated in the absence of IL-2 for 48 hrs. At various time after stimulation (16, 24, 48 hours), an aliquot of cell culture

supernatant was collected for p24 and IL-2 quantification by specific ELISA (Innotest and Endogen Test respectively) according to manufacturer's instructions.

To infect T cells with the HIV-GFP viruses, the procedure described in ref. 64 was followed. Freshly isolated $CD4^+$ T cells were activated with 1μg/ml of PHA and maintained in culture in complete RPMI medium with IL-2 for three days. Then, the cells were infected as described above and re-suspended at 2 x 10^6/ml in complete RPMI medium. Three days post-infection the cells were stimulated a second time with PHA, maintained in culture with IL-2 for 3 before analysis. To analyze surface CD4 expression, $CD4^+$ T cells were infected with 50 ng p24/10^6 cells, stimulated with PHA and IL-2, then harvested after 3, 5, 7 and, 10 days for FACS analysis.

To analyze secondary activation of infected T cells, freshly isolated $CD4^+$ T cells were first stimulated with anti-CD3 and anti-CD28, mAb as described above, washed and maintained in culture in complete RPMI medium with IL-2 for six days. Then, cells have been infected as described above, re-suspended at 5 x 10^6/ml in complete RPMI medium with IL-2 and, after three days, stimulated again via CD3/CD28 for various time prior analysis.

Flow cytometry

The following procedures were performed at 4°C in phosphate-buffered saline (PBS) containing 0.5% BSA and 0.1% sodium azide. $CD4^+$ T cells were incubated with saturating amounts of PE-conjugated anti-CD4 and FITC-conjugated anti-CD28, for 30 min on ice. After staining, cells were washed three times and re-suspended in 1% para-formaldehyde for 30 minutes at RT, then fluorescence intensities for CD4 and CD28 were analyzed by flow cytometry on a FACS Calibur with CellQuest

software (Becton Dickinson). Parameters were set in order to acquire 10^4 living cells/sample. The amounts of cell surface CD4 molecules were determined as the geometric mean of the specific fluorescence in cells gated (R1 region).

For the simultaneous detection of surface CD4 and intracellular p24, T cells were incubated with anti-CD4 mAb or mouse IgG_1 as isotype control. After 3 washes, cells were incubated with Cy5-conjugated goat anti-mouse IgG. Cells were then washed, fixed and permeabilized with reagents from BD Biosciences and incubated with the PE-conjugated anti-HIV p24 mAb. Finally, cells were washed, resuspended in 1% paraformaldehyde and analyzed by two-colour flow cytometry

Western blotting analysis

$CD4^+$ T lymphocytes uninfected and infected cells were washed two times in PBS 1X and lysed for 20 min on ice in JS buffer (50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1.5 mM $MgCl_2$, 150 mM NaCl, 5 mM EGTA, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, 1μg of pepstatin/ml, 5 ug of aprotinin/ml, 1μg of leupeptin/ml and 1X phosphatase inhibitor cocktail (SIGMA). After centrifugation at 12000 x g for 15 min to 4°C, equal amounts of total cellular extracts (20-30μg) were separated by 10% SDS-PAGE, transferred on nitrocellulose and analyzed by immunoblotting. In order to evaluate the level of molecule phosphorylation, the membranes was blocked with TBS-0.05% Tween, 5% milk for 1h and ibridizated with anti-p-Y antibody diluted 1:1000 and secondary anti-mouse IgG antibody diluted 1:10.000. Next the membrane was stripped with stripping buffer (2% SDS, 62.5% TRIS HCl pH 6,7, 100 mM β-Mercaptoetanol) for 20 min 50°C with gentle agitation to remove the antibodies of first ibridization. After three wash the membrane was re-probed with specific antibodies anti-PLC-γ1 1:200, anti-Vav 1:500,

anti-ZAP-70 1:1000, anti-Lck 1:200, anti-ζ-chain 1:200 in TBS 1X, 0,1% Tween, 5% milk and anti-Akt 1:1000, anti-IKKα/β 1:1000, anti-NF-κB p56 1:1000, anti-IκB 1:1000 in TBS 1X 0,1% Tween 5% BSA and secondary anti-mouse IgG-HRP 1:10000, anti-rabbit IgG-HRP 1:10000. The signals were detected with the ECL normal and ECL Advance system (Amersham Pharmacia Biotech) and proteins' specific signals were quantified by densitometric analysis.

Immunoprecipitation assay

Total cellular extracts of quiescent $CD4^+$ T cells infected with wt and ΔNef virus were subjected to immunoprecipitation with anti-CD4. Equal amounts of total extracts (50-80μg) were incubated with 10 μg/ml of monoclonal mouse anti-CD4 antibody (clone RPA-T4, BD Biosciences) 1h on ice. To immunoprecipitate CD4/Lck complex was added 10% glutathione-sepharose beads according to the manufacturer's instructions (Amersham Pharmacia Biotech) and incubated with gentle rotation for 1 h at 4°C. The beads were then collected by centrifugation at 400 x *g* for 5 min at 4°C followed by supernatant removal, and washed three times at room temperature by addition of 800 μl of JS with inhibitors, inversion three times, and centrifugation. Moreover the supernatant was collected and subjected to the second immunoprecipitation with anti-CD4 antibody. The CD4/Lck complex were eluted by boiling in reducing sample loading buffer at 95°C for 5 min, divided in equal part in two gels, separated by 10% SDS-PAGE and analyzed by immunoblotting with anti-CD4 and anti-Lck specific antibodies.

Analogously, total extracts of quiescent T cells infected with wt and ΔNef virus were subjected to immunoprecipitation by anti-p-Tyr and anti-ζ-chain. Equal amounts of lysetes were incubated with 10 μg/ml anti-p-Tyr or anti-ζ-chain

antibodies 1 h on ice. Then it was added 10% glutathione-sepharose beads according to the manufacturer's instructions (Amersham Pharmacia Biotech) and incubated with gentle rotation for 1 h at 4°C. The beads were then collected by centrifugation at 400 x *g* for 5 min at 4°C followed by supernatant removal, and washed three times at room temperature by addition of 800 μl of JS with inhibitors, inversion three times, and centrifugation. The samples were boiled in reducing sample loading buffer at 95°C for 5 min, divided in equal part in two gels and separated by 10% SDS-PAGE and analyzed by immunoblotting with anti- ζ-chain and anti- p-Tyr respectively.

Immunofluorescence microscopy

Uninfected and infected $CD4^+$ T lymphocytes were counted and equal number of cells 10^5 cells were let to adhere on polilysine-treated glass covers-lips. The covers-lips were rinsed twice with PBS and fixed with 4% para-formaldehyde 30 min RT. The cells were washed twice with PBS and permeabilized/blocked with PBS, 0.05% Saponine, 0.5% BSA for 10 min at RT. Then the cells were incubated for 1 h at RT with monoclonal mouse anti-Nef (1:10000), alone or together with polyclonal rabbit anti-CD4 (1:10), polyclonal rabbit anti-Lck (1:100), the covers-lips were washed twice with PBS and incubated with goat anti-mouse IgG antibody conjugated with Alexa 488 or Alexa 555 (1:100), and Texas Red-conjugated goat anti-rabbit antibody 1:100 1 h at RT. To analyze the CD28 distribution in infected cells we used monoclonal mouse anti-Nef with secondary goat anti-mouse IgG antibody conjugated with Alexa 555, and FITC-conjugated anti-CD28 (1:5) antibodies. After washing twice with PBS the nucleus was permeabilized with PBS/0,15% Triton-X-100 5 minutes RT, then the cover-lips were incubated 10 minutes with DRAQ5 1:5000.

Finally, the covers-lips were washed with 5 μg/ml of DAPI in PBS and mounted on glass slides.

Analogously, we performed the immunfluorescence of HIV-GFP infected cells and uninfected cells. The cells were let to adhere on polilysine treated glass covers-lips, fixed with para-formaldehyde 4%, and permeabilized/blocked with PBS, 0.05% Saponine, 0.5% BSA for 10 min at RT. Then the cells were incubated for 1 h at RT monoclonal mouse anti-CD4 1:400, polyclonal rabbit anti-Lck 1:100 and monoclonal mouse anti-CD28 1:100. Finally, the covers-lips were washed twice with PBS and incubated with Alexa 555-conjugated goat anti-mouse IgG antibody 1:100, goat anti rabbit Texas Red 1:100 secondary antibodies. After washing twice with PBS the nucleus was permeabilized with PBS/0,15% Triton-X-100 5 minutes RT, then the cover-lips were incubated 10 minutes with DRAQ5 1:5000. Finally, the covers-slips were washed with 5 μg/ml of DAPI in PBS and mounted on glass slides.

Images were acquired by Confocal Laser Microscope with immersion objective 60X and Olympus FV1000 software (Pediatric Hospital Bambino Gesù, Rome, Italy).

3-RESULTS

3.1 Nef expression in HIV-1 infected quiescent $CD4^+$ T lymphocytes

The capacity of the HIV-1 Nef protein to affect the activation state of quiescent $CD4^+$ T lymphocytes was investigated. To this aim, a wt and Nef-deficient HIV-1 (ΔNef) viruses were produced by transfecting 293T cells with the NL4-3 molecular clone and its mutated variant containing two premature termination codons in the *nef* coding region [63]. Since ΔNef is less infectious than wt virus due to the absence of Nef expression, the vesicular stomatitis virus glycoprotein (VSV-G) was co-expressed to allow its incorporation into the viral envelope and induce, consequently, viral entry by endocytosis [65]. Primary quiescent $CD4^+$ T lymphocytes derived from healthy donors were infected with equal amounts of VSV-G-pseudotiped wt or ΔNef viruses and cultivated in the absence of mitogen for 5 days during which no measurable viral replication occurred. After this period, the cells were lysed and equal amounts of total cell extracts were analyzed by western blotting with anti-Nef antibody, showing that in cells infected with wt virus Nef expression was readily detected despite the absence of viral replication (Fig. 3A). This observation is in agreement with a previous study showing expression of Nef soon after infection in the absence of viral DNA integration [21]. Next, we investigated the levels of CD4 and Lck that are known targets of Nef-induced degradation. By western blotting, it was evident that CD4 levels were reduced by 30% in cells expressing wt virus if compared to cells infected with ΔNef or not infected, indicating that early during infection Nef was already able to degrade CD4. On the other hand, 20% reduced Lck levels were

observed in both wt- or ΔNef-infected cells, suggesting the existance of a Nef-independent mechanism through which the virus degrades Lck (Fig. 3A).

Next, we analyzed by immunofluorescence the intracellular localization of CD4, Lck and CD28, another molecules down-regulated by Nef. Uninfected and wt-infected T cells were doubly-stained with anti-Nef antibody to identify infected cells and, anti-CD4, anti-CD28 or anti-Lck antibodies. ΔNef-infected cells could not be identified due to the lack of an HIV-1-specific marker different from Nef expressed in infected quiescent cells. Nef^+ cells represented about 3,5% of total cells (average of three experiment), although this measurement probably underestimate the real percentage of infected cells. Indeed, upon stimulation infected cells become 10-20% p24+ after 48h as a consequence of the first round of viral replication. It is likely that, before activation only those that express high levels of Nef could be detected with anti-Nef antibody by immunofluorescence microscopy thus underestimating the efficiency of infected cells. As shown in Fig. 3B, if compared to uninfected cells, in Nef-expressing cells both CD4 and Lck accumulate intracellularly in vesicle-like structures. As opposed to western blotting analysis, an overall reduction of CD4 and Lck proteins could not be appreciated by immunofluorescence microscopy because this latter tecnique is likely inadequate for quantitative analysis. Next, we analyzed CD28 localization in uninfected and wt-infected cells and, as shown in Fig. 3B, this molecules was similarly distributed in the two cell types. Then, the cell surface expression of CD4 and CD28 was analyzed by flow cytometry in uninfected, wt- and ΔNef-infected cells. As shown in Fig. 3C, there was a 10% reduction of the surface CD4 expression in wt- and ΔNef-infected cells if compared to uninfected cells. This weak CD4 reduction was Nef-independent and probably due to receptor down-regulation during the process of viral entry. Therefore, in quiescent HIV-infected

cells Nef is not able to down-regulate surface CD4 expression. This observation also suggests that Nef-induced overall reduction of CD4 resulted from Nef's capacity to retain intracellularly and degrade newly synthesized CD4 molecules. By analysing the cell surface expression of CD28 we observed that there were no differences between uninfected, wt- or ΔNef-infected cells, in agreement with the immunofluorescence analysis shown above.

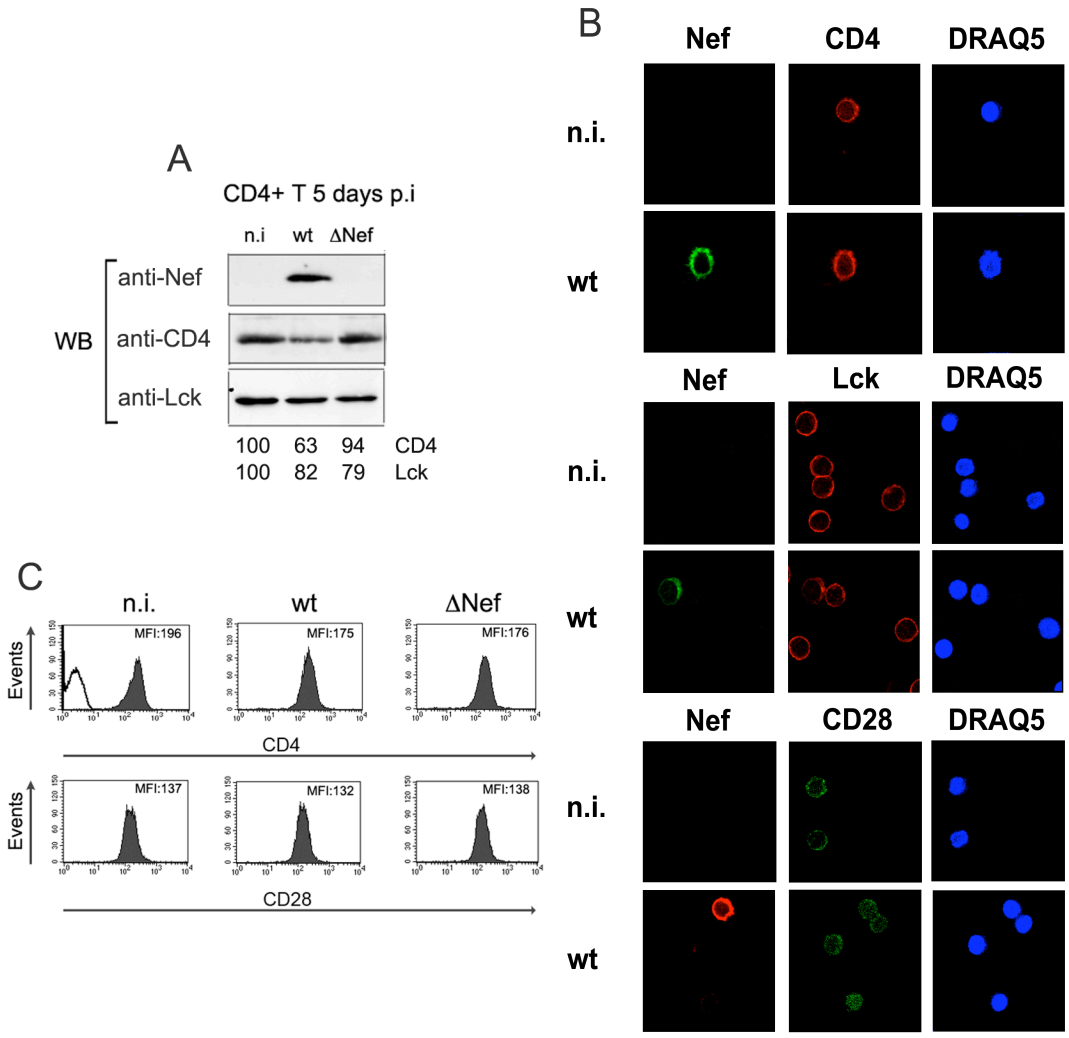

Fig. 3. Analysis of CD4, Lck and CD28 expression in quiescent CD4⁺ T lymphocytes infected with HIV-1

(A-C) Freshly isolated $CD4^+$ T lymphocytes uninfected (n.i) and infected with HIV-1 NL4-3 virus either wt or Nef-deficient (ΔNef) were cultivated for 5 days in the absence of mitogen. (A) The cells were lysed and equal amounts of total cell extracts were analyzed by western blotting with anti-Nef, anti-CD4 and anti-Lck specific antibodies. (B) 5 days post infection (p.i.) cells were counted and equal number of cells were let to adhere on polilysine-treated glass covers-lips. Next, cells were permeabilized and blocked with saponine/BSA to perform the intracellular double-staining with anti-Nef and anti-CD4, anti-Lck, anti-CD28 antibodies. Finally the nucleus was marked with DRAQ5. Images were acquired using confocal mycroscope. (C) 5 days p.i., n.i., wt- and ΔNef-infected cells were analyzed by flow cytometry for the cell surface expression of CD4 and CD28 with specific antibodies.

3.2 Nef increases viral replication and IL-2 production upon activation of HIV-infected T cells

We then investigated the contribution of the HIV-1 Nef protein in both viral replication and T cell activation. Freshly isolated $CD4^+$ T lymphocytes have been infected with VSV-G-pseudotiped wt or ΔNef viruses and five days post infection (p.i.) the cells were stimulated through their T cell receptor with anti-CD3 and anti-CD28 cross-linked antibodies. 16, 24, 48 hrs post stimulation, the cells were monitored for viral release by measuring soluble p24 in the culture supernatant by ELISA. As expected, a positive effect of Nef was consistently observed with a two-fold increase in viral production by cells infected with wt virus if compared to those infected with ΔNef (Fig. 4A).

As described earlier, Nef may favor viral replication by lowering the threshold of T cell activation. Since the increase of IL-2 production represent a hallmark of T cell activation [19, 20, 21], we analyzed whether in our experimental conditions Nef expression altered the release of IL-2 from infected cells. As described above, $CD4^+$ T lymphocytes were infected and, after 5 days, stimulated with anti-CD3 and anti-CD28 cross-linked antibodies. 16, 24, 48 hrs post-stimulation, an aliquot of the culture supernatant was tested for the presence of IL-2 by ELISA. As shown in Fig. 4B, 48 hrs post activation we observed a two-fold increase of IL-2 production from cells infected with wt virus if compared to those infected with ΔNef virus or uninfected. This result is in agreement with other studies [56] and confirms the positive role of the HIV-1 Nef protein in T cell activation.

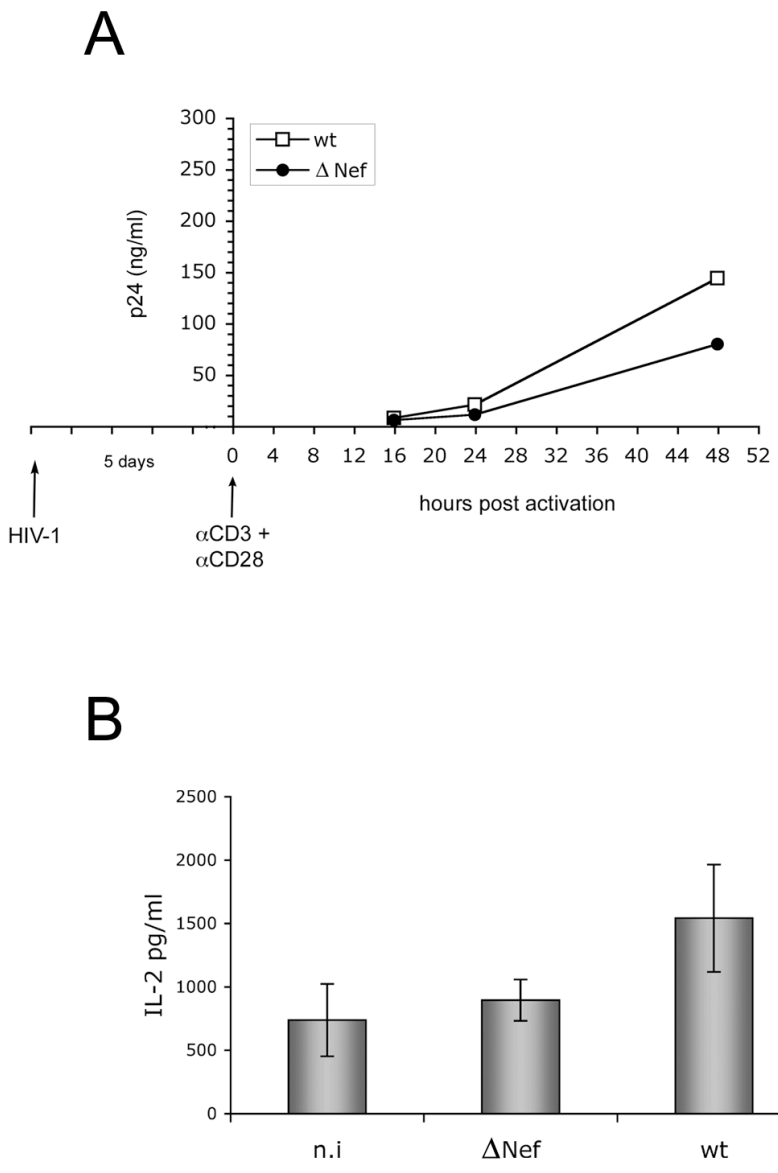

Fig. 4. Nef enhances viral replication and IL-2 production in response to CD3/CD28 stimulation in quiescent CD4$^+$ T lymphocytes infected with HIV-1
(A, B) Freshly isolated CD4$^+$ T lymphocytes uninfected (n.i) and infected with HIV-1 NL4-3 virus either wt or Nef-deficient (ΔNef) were cultivated for five days in the absence of mitogen. Next, the cells were activated with anti-CD3 and anti-CD28 cross-linked antibodies. (A) 16, 24, 48 hrs post stimulation, the supernatant was collected for p24 quantification by ELISA one out of three independent experiments is shown. (B) 48 hrs post activation an aliquot of the culture supernatant was tested for the presence of IL-2 by ELISA. Results shown represent the average value of three independent experiments.

3.3 Protein tyrosine phosphorylation upon activation of quiescent HIV-1-infected T cells

As shown in previous work, Nef profoundly manipulates HIV target cells by altering a variety of signal transduction pathways and protein sorting processes [58]. With respect to $CD4^+$ T lymphocytes, Nef is known to affect the TCR signal transduction. Successful TCR signal initiation induces a characteristic cascade of tyrosine phosphorylation events, Ca^{2+} release and the activation of transcription factors [19]. Thus we investigated the role of Nef in the TCR signaling of primary HIV-1 infected $CD4^+$ T lymphocytes. Freshly purified $CD4^+$ T cells were infected with equal amounts of VSV-G-pseudotyped wt or ΔNef virus or not infected and cultivated in the absence of mitogen for 5 days. The cells were then stimulated at 37°C for variable time (0, 1, 10, 15, 30, 60, 120 minutes) with anti-CD3 and anti-CD28 cross-linked antibodies. Finally, cells were lysed and equal amounts of total cell extracts were analyzed by western blotting. By probing the membrane with anti-p-Tyr antibody, the typical pattern of protein phosphorylation of activated T cells was observed: p140, p95, p70, p56, p36 corresponding to p-PLC-γ1, p-Vav, p-ZAP-70, p-Lck and p-ζ-chain of TCR complex respectively (Fig. 5A). Next, the membrane was stripped with reducing buffer and re-probed with specific antibodies anti-PLCg-γ1, antia-Vav, antia-ZAP-70 (Fig. 5B). The relative phosphorylation was calculated by measuring bounds by densitometry, normalizing phospho-bands for their loading controls, and setting to 1 the signal of uninfected cells at time 0. By comparing uninfected, wt- and ΔNef-infected cells, it was evident that upon TCR stimulation the extent and the

kinetic (with a peak after 1' of stimulation) of tyrosine phosphorylation of PLC-γ1, Vav, and ZAP-70 did not differ significantly (Fig. 5C). By this analysis, the extent of Lck phosphorylation was extremely variable between different experiments and thus it could not be evaluated. This was possibly due to the high basal level of Lck phosphorylation and/or to the presence of other phosphorylated protein co-migrating with Lck.

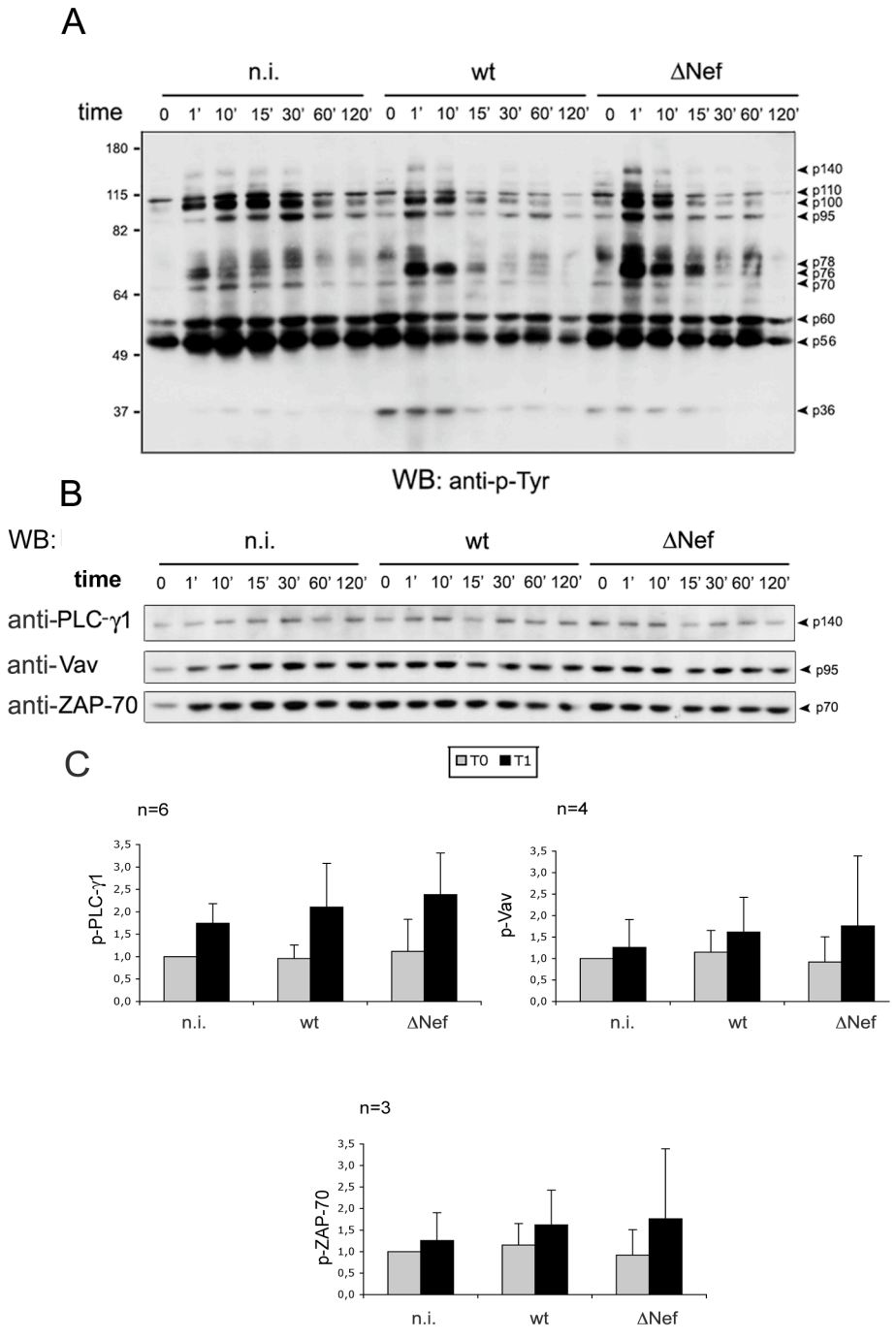

Fig. 5. Activation of HIV-infected T cells via CD3/CD28

Five days post infection, quiescent $CD4^+$ T lymphocytes were stimulated for variable time with cross-linked anti-CD3 and anti-CD28 antibodies (A). Equal amounts of cell lysates were analyzed by western blotting with anti-p-Tyr antibody. (B) The membrane shown in panel (A) was stripped and re-probed with specific antibodies anti-PLC-γ1, anti-Vav, anti-ZAP-70. (C) The average phosphorylation of PLC-γ1, Vav, ZAP-70 measured after 1 minute of stimulation in 6, 4 and 3 independent experiments is shown.

3.4 Activation of Akt and NF-κB pathways in HIV-1-infected CD4+ T lymphocytes

Since Akt has an important role in the T cell activation pathway [29], we investigated its phosphorylation/activation in the context of wt- and ΔNef-infected $CD4^+$ T lymphocytes. Primary $CD4^+$ T lymphocytes uninfected or infected for 5 days were stimulated through CD3/CD28 as described above and, at various times after stimulation, the cells were lysed. Equal amounts of total cell extracts were analyzed by western blotting with anti-Akt antibody and, after stripping, the membrane was re-probed with anti-p-Akt specific antibodies (Fig. 6A). The rate of Akt phosphorylation was obtained by densitometric analysis and by normalizing Akt phospho-bands for their loading control. The ratio between p-Akt/Akt in uninfected cells before the stimulation (T0) was set to 1. We observed that in each cell sample (n.i., wt, ΔNef), the Akt protein became phosphorylated to a similar extent upon stimulation and that the peak of phosphorylation was reached after five minutes (Fig. 6B). Thus, Akt phosphorylation did not differ significantly in uninfected or HIV-1-infected cells, with or without Nef expression.

To examine whether Nef could affect the NF-κB pathway, we analyzed the level of phosphorylation of IKK, IκB and NF-κB in primary $CD4^+$ T lymphocytes uninfected or infected with wt and ΔNef virus for 5 days. The cells were either treated or not with anti-CD3 and anti-CD28 antibodies for variable time (15, 60, 360 minutes) then collected, lysed and equal amounts of cell extracts were analyzed by western blotting with anti-IKK antibodies specific for α and β isoforms, anti-IκB and anti-NF-κB (p50) antibodies. Next the membrane was stripped and re-probe con anti-IKK, anti-

IκB and anti-NF-κB specific antibodies (Fig. 7A). The relative phosphorylation was calculated by densitometric analysis normalyzing phospho-bands obtained by anti-p-Tyr antibody for their loading control with specific antibody and setting to 1 the signal of uninfected cells at time 0. As shown in the Fig. 7B, the phosphorylation of IKK, IκB and NF-κB was induced and reached a peak after 15, 60 minutes and 6 hrs respectively. By comparing uninfected with wt- and ΔNef-infected cells, we observed that there were no differences in the phosphorylation level of IKK, IκB and NF-κB.

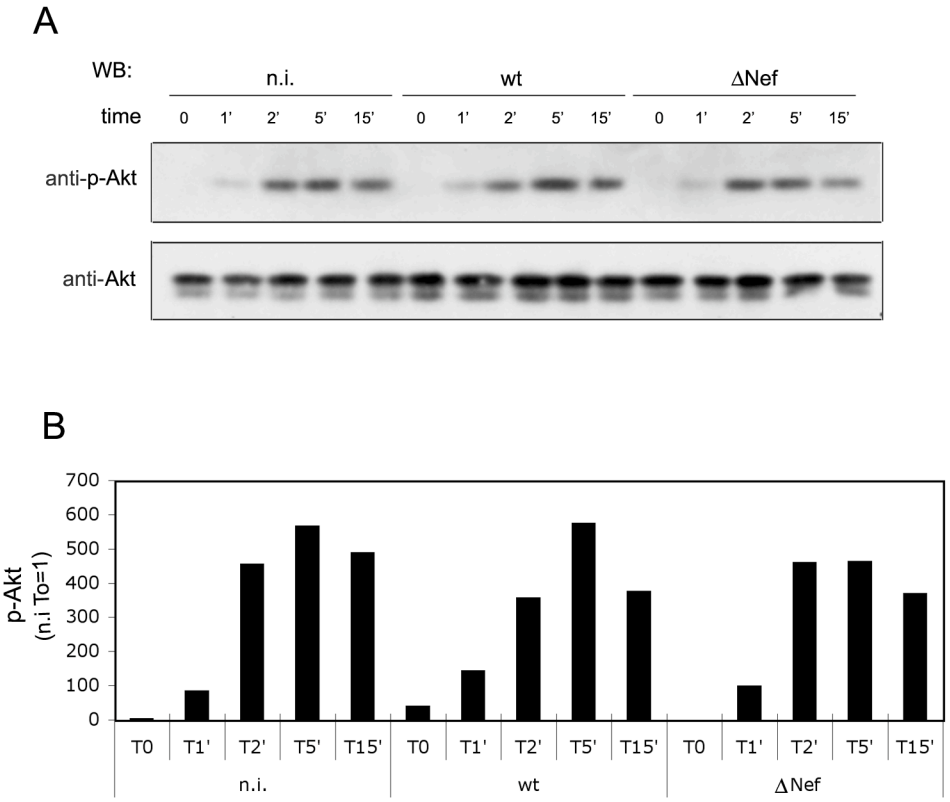

Fig. 6. Effect of Nef on Akt activation in HIV-1-infected cells.

(A) Quiescent $CD4^+$ T cells were infected and five days post infection were stimulated for variable time (0, 1, 2, 5, 15 minutes) with cross-linked anti-CD3 and anti-CD28 antibodies. Then equal amounts of cell extracts were analyzed by western blotting. The membrane was immunoblotted with anti-p-Akt antibody and, after stripping, re-probed with anti-Akt specific antibody. (B) Relative Akt phosphorylation was obtained by densitometric analysis normalizing phospho-bands for their loading controls and setting to 1 the signal of n.i. cells at T0. Results shown are representative of three independent experiments.

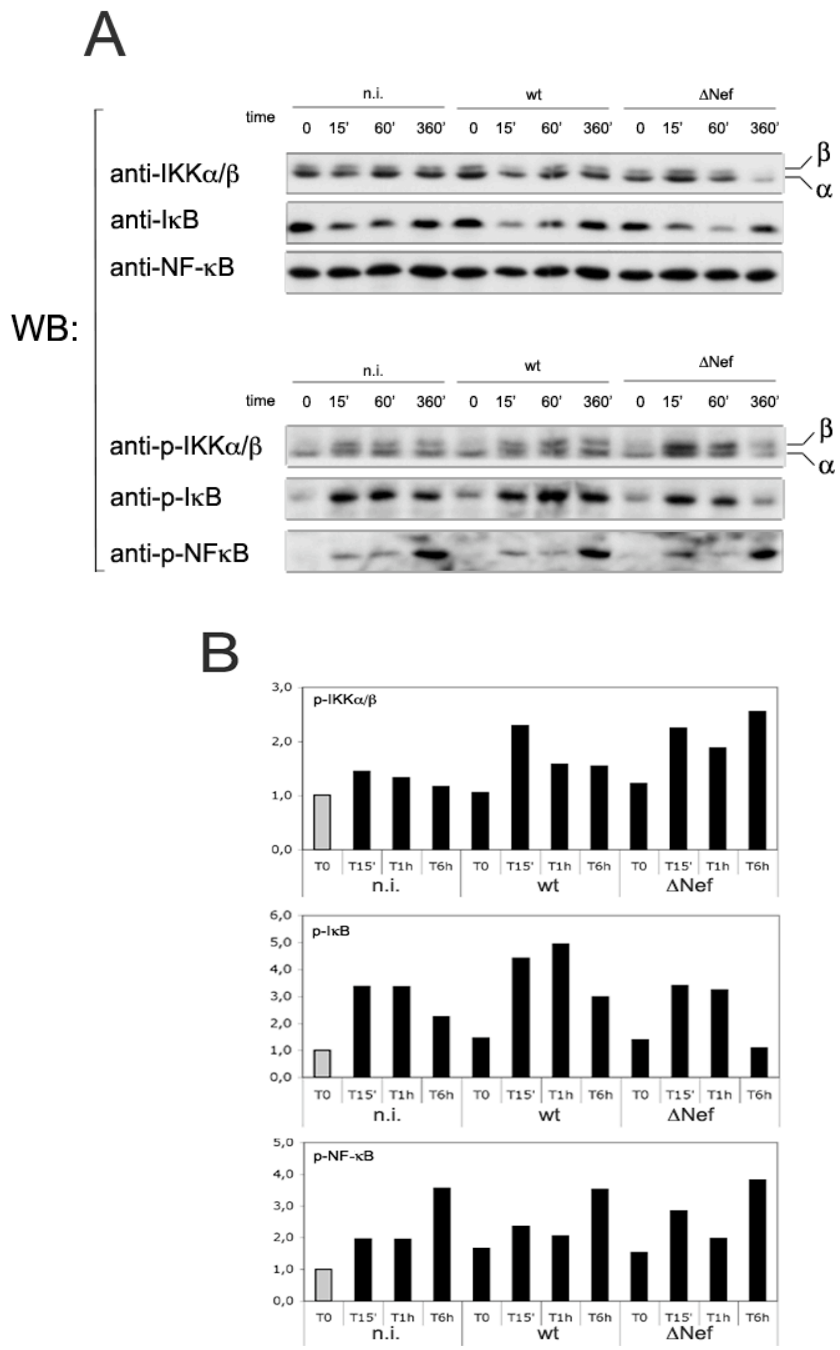

Fig. 7. Activation of the NF-κB pathway in HIV-1-infected cells.

(A) $CD4^+$ T cells were uninfected, infected with wt or ΔNef virus. Five days post infection the cells were stimulated for 0, 15, 60, 360 min with cross-linked anti-CD3 and anti-CD28 antibodies. Equal amounts of cell lysates were analyzed by western blotting with anti-p-IKKα/β, anti-p-IκB and anti-p-NFk-κB antibodies. After stripping the membrane was re-probed with anti-IKK, anti-IκB and anti-NFk-κBa/b, antibodies. (B) The phosphorylation level of the proteins in one representative experiment out of three is shown. The signal of n.i. cells at time 0 was set to 1.

3.5 CD4/Lck interaction in HIV-1-infected resting T cells

Next, we investigated the association of Lck with CD4 in HIV-1-infected T cells, with and without Nef expression, by a co-immunoprecipitation analysis. Primary quiescent $CD4^+$ T lymphocytes uninfected, infected with wt or ΔNef HIV-1, were harvested five days post infection to prepare total cell extracts. As shown by western blotting (Fig. 3A), HIV-1 expression results in decreased levels of CD4 and Lck in a Nef-independent manner, while the intracellular re-distribution of both CD4 and Lck occur only if the viral Nef protein is expressed. Equal amounts of total cell extracts were subjected to two rounds of immunoprecipitation with anti-CD4 antibody. Western blotting analysis of cell lysates before and after anti-CD4 immunoprecipitation confirmed that the overall amount of cell-associated CD4 was bound to antibodies (Fig. 8A). In addition, Lck levels were almost completely removed from the cell lysate upon anti-CD4 immunoprecipitation indicating that the vast majority of Lck is associated to CD4 within primary T cells. The immunoprecipitated proteins were divided in two aliquots that were analyzed by western blotting with anti-CD4 and anti-Lck antibody (Fig. 8B). The levels of both CD4 and Lck in the immunoprecipitated samples were measured by densitometry and the relative amount of Lck was divided by the corresponding CD4 amount. This results shows that CD4 and Lck associates efficiently in HIV-1-infected T cells, despite the levels of both proteins are reduced and, due to Nef's activity, relocalized within intracellular compartments.

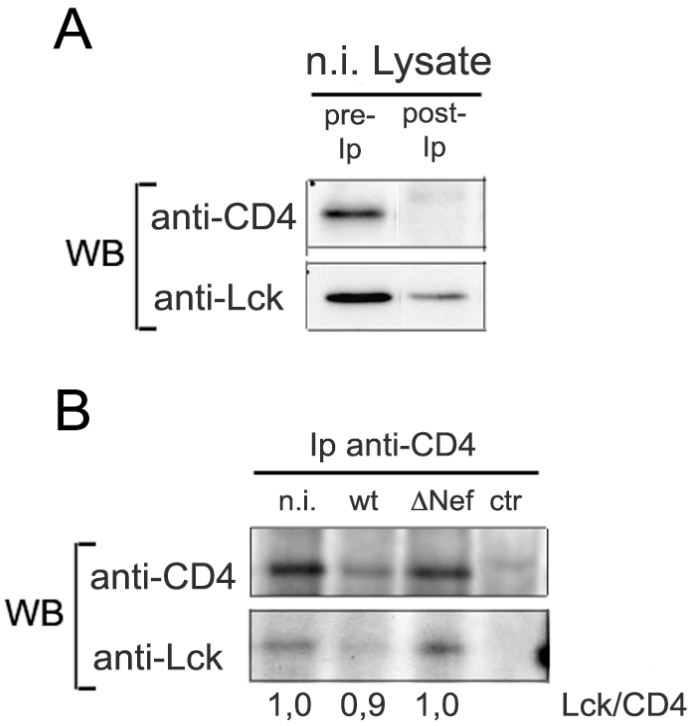

Fig. 8. CD4/Lck association in HIV-1 infected quiescent CD4$^+$ T lymphocytes

5 days post infection, total cell extracts of uninfected, wt- and ΔNef-infected cells were quantified and subjected to immunoprecipitation with anti-CD4 antibody. (A) The lysates before and after two rounds of anti-CD4 immunoprecipitation were analyzed by western blotting with anti-CD4 and anti-Lck specific antibodies. (B) Immunocomplexes were analyzed as described in panel (A) and the relative amount of Lck was divided by the amount of immunoprecipitated CD4 in each sample. The Lck/CD4 ratio was calculated by setting to 1 that found in the n.i. sample. As control, lane ctr corresponds to an immunoprecipitation reaction performed without cellular lysate.

3.6 Activation of Lck upon triggering of CD4 in HIV-1-infected cells

We investigated whether the HIV-1 Nef protein is involved in the regulation of Lck activation upon triggering of the surface CD4 receptor. Uninfected, wt- and ΔNef-infected $CD4^+$ T lymphocytes were stimulated five days post infection with cross-linked anti-CD4 antibody for variable time (0, 1, 5, 10 min). Next the cells were collected and lysed. The total cell extracts were quantified and equal amounts were analyzed by western blotting with anti-p-Tyr antibody followed by re-probing with anti-Lck specific antibody (Fig. 9A). Levels of total and Tyr-phosphorylated Lck (p-Lck) were calculated by densitometric analysis and expressed by setting to 100 and 1, respectively, the signal of n.i. cells at time 0. As shown in Fig. 9B, upon CD4 stimulation the phosphorylation of Lck rapidly increased while the protein levels decreased, possibly due to the rapid consumption of activated Lck. Despite the basal levels of Lck were lower in wt- and ΔNef-infected cells, after 1' of CD4 stimulation about 50% of the Lck protein disappeared in both infected and uninfected cells. In addition, the phosphorylation of Lck was maximally induced 5 minutes after the CD4 stimulation. Importantly, the kinetic as well as the extent of Lck phosphorylation was similar in uninfected and HIV-1 infected cells. Thus, despite Lck is reduced and re-localized, the function of this kinase in maintained, in HIV-1-infected cells consistently with the full capacity of these cells to respond to TCR activation.

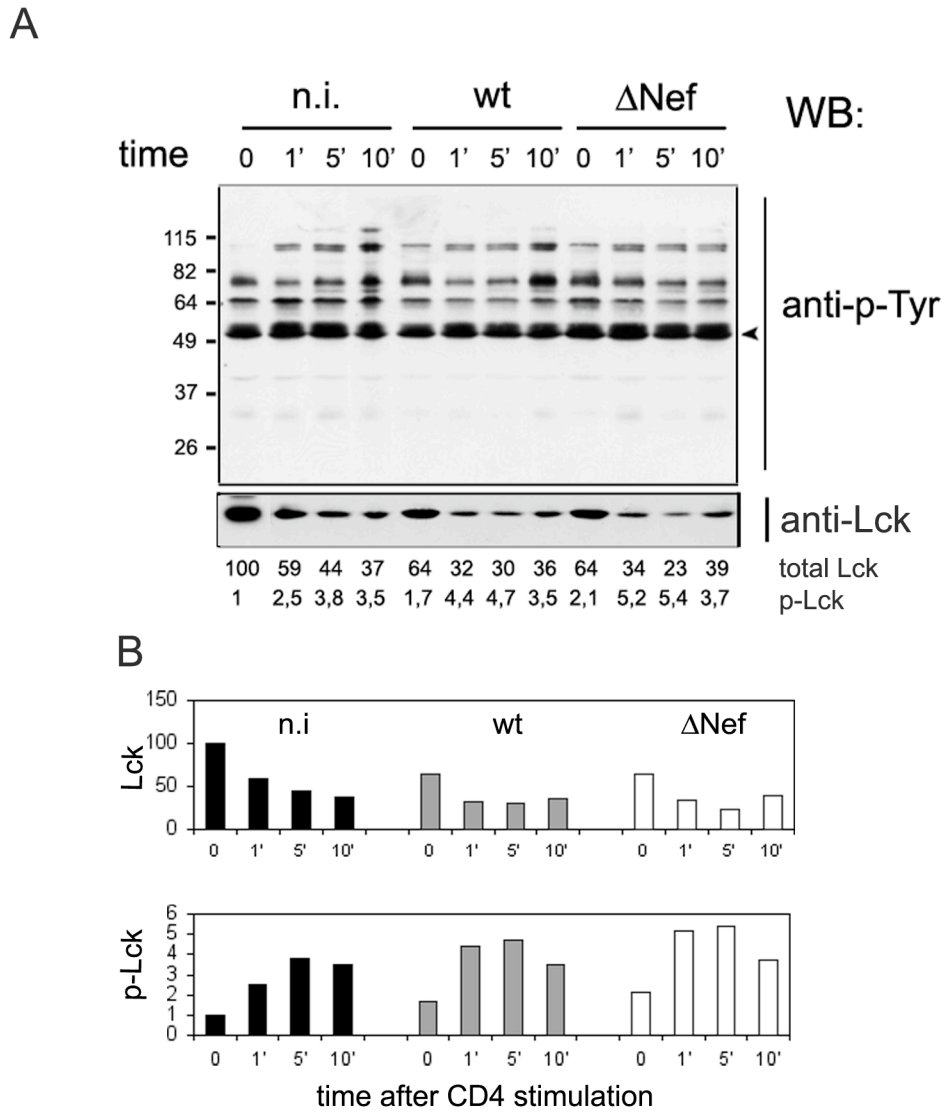

Fig. 9. Lck phosphorylation upon CD4 stimulation of HIV-1-infected CD4⁺ T cells.

(A) CD4⁺ T lymphocytes uninfected, infected with wt or ΔNef virus, were stimulated with cross-linked anti-CD4 antibody for variable time (0, 1, 5, 10 min). To investigate the level of Lck phosphorylation, equal amounts of cell extracts were analyzed by western blotting with anti-p-Tyr (Top). After stripping the membrane was re-probed with anti-Lck (Bottom). (B) Lck and p-Lck in n.i., wt- and ΔNef-infected cells was calculated by densitometric analysis and expressed setting to 100 and 1 the value obtained for n.i. cells at time T0, respectively. Results of one representative experiment out of three are shown.

3.7 Phosphorylation of the TCR ζ-chain upon CD4 stimulation of infected T cells

Then we analyzed the capacity of Lck to phosphorylate its first target, the ζ-chain of the TCR, in cells infected with HIV-1. Five days post infection, uninfected, wt- and ΔNef-infected $CD4^+$ T lymphocytes were stimulated with cross-linked anti-CD4 antibody for 5 min at 37°C and then lysed. Equal amounts of the total cell extracts were immunoprecipited with anti-p-Tyr antibody and the recovered immunocomplex were analysed by western blotting with anti-ζ-chain specific antibody. Fig. 10A shows that, as expected, the amounts of ζ-chain found in the precipitated phospho-proteins increased upon 5' of CD4 stimulation that corresponds to maximal Lck activation. However, this increment was similar in the n.i., wt and ΔNef sample. Moreover, the reciprocal analysis of Tyr-phosphorylated proteins in the anti-ζ-chain-immunoprecipitated protein complex was performed (Fig. 10B). Upon stimulation, in the anti-ζ immuno-complexes a major species of 30 kD phospho-band accumulated and some of slower migrating ζ-chain-related phosphoproteins appeared. The extent of the Tyr-phosphorylated 30 kD band was measured and normalized for its loading control. Results show that tyrosine-phosphorylation of the ζ-chain, both basal and induced by the CD4/Lck signaling pathway, is maintained in primary T cells infected by HIV-1 with and without Nef expression.

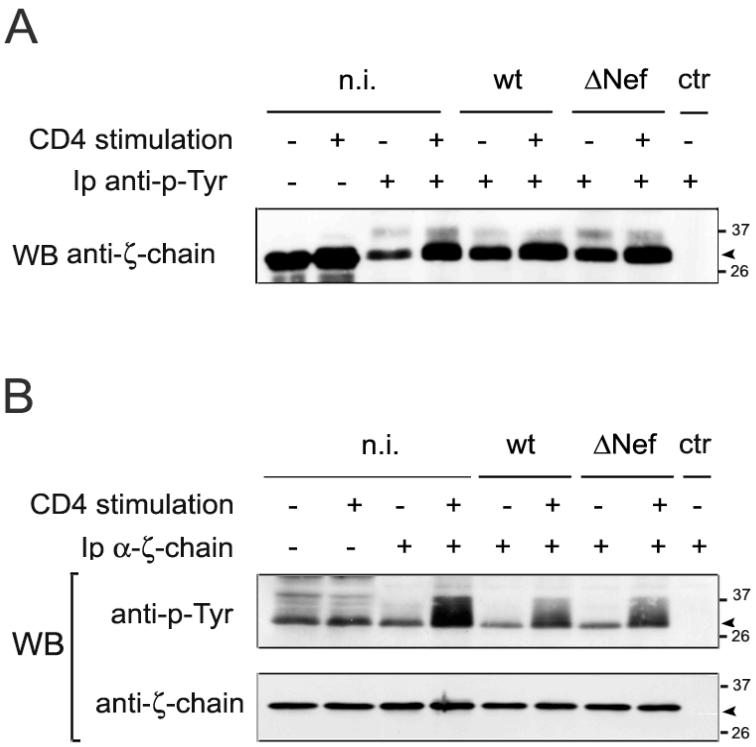

Fig. 10. Phosphorylation of the TCR ζ-chain in HIV-1-infected T cells.

Five days post infection of resting $CD4^+$ T lymphocytes, n.i., wt- and ΔNef-infected cells were stimulated or not with cross-linked anti-CD4 antibody for 5 min at 37°C. (A) The total cell extracts were immunoprecipitated with anti-p-Tyr antibody and analyzed by western blotting with anti-ζ-chain antibody. (B) Immunoprecipitation was performed with anti-ζ-chain antibody followed by western blotting with anti-p-Tyr antibody. After antibody stripping, the membrane was re-probed with anti-ζ-chain antibody. The ctr lanes correspond to control immunoprecipitation reactions performed in the absence of cell lysate. The arrow indicate the major 30 kD ζ-chain band.

3.8 Nef activity in HIV-1-infected activated $CD4^+$ T lymphocytes

Next, we investigated the function of Nef in the context of HIV-1-infected activated $CD4^+$ T lymphocytes. First, we measured surface CD4 down-regulation that is the best characterized activity of Nef. To this aim, wt and ΔNef viruses, together with the DDAA mutated virus that expresses a previously described Nef mutant, $NEFDD_{178}AA$, defective for CD4 down-regulation, were used to infect freshly-isolated primary $CD4^+$ T lymphocytes. After viral exposure, T cells were activated and analyzed by flow cytometry at various time points for the intracellular accumulation of p24 and the cell-surface expression of CD4. Infected p24+ cells became detectable after 5 days (1-8 %) then increased numerically till reaching a maximum after about 10 days (20-40%). At 5 days post-infection (p.i.), the surface CD4 density of cells infected with wt virus was reduced to 20% (considering 100 % the expression in uninfected cells, n.i.) while cells infected with ΔNef or DDAA virus maintained 50-80% of CD4 expression (Fig. 11) in agreement with previous studies showing that Nef is the major determinant of CD4 down-modulation during the early phases of viral infection [43]. However, at 10 days p.i., a 5-fold reduction of surface CD4 was also achieved in cells infected with ΔNef or DDAA virus, likely due to the activity of the viral Vpu and Env proteins accumulating in late stages of infection. These results show that Nef is not required to achieve maximal inhibition of surface CD4 expression in late stages of HIV-1 infection in primary T lymphocytes, thus suggesting that Nef's activity on CD4 may be irrelevant when the virus is

productively assembled and released by these cells. Then we analyzed by immunofluorescence microscopy the intracellular localization of CD4, as well as two additional Nef's targets, CD28 and Lck, in productively infected $CD4^+$ T cells. For this purpose, we stimulated $CD4^+$ T lymphocytes with PHA and IL-2 and after three days, cells were infected with VSV-G-pseudotyped wt-GFP or ΔNef-GFP viruses. These viruses derive from the NL4-3 wt and ΔNef viruses, respectively, in which the *nef* genes is followed by an internal ribosome entry site, (IRES) and the sequence coding for the green fluorescent protein (GFP) [64]. Three days post infection, the cells were treated a second time with PHA and IL-2 to boost viral replication and monitored for GFP expression by flow cytometry. Next, cells were analyzed by 2-colours immunofluorescence to investigate GFP expression and the intracellular localization of CD4, Lck and CD28. As shown in Fig. 12, in this experimental conditions both wt-GFP and ΔNef-GFP virus were able to down-regulate and degrade efficiently CD4 if compared to uninfected cells, consistently with the results obtained by flow cytometric analysis. By analyzing the cellular distribution of Lck, we observed that in uninfected and ΔNef-GFP-infected T cells Lck was localized below the cellular membrane while in the presence of wt-GFP virus it accumulated in intracellular vesicles, similarly to what observed in infected quiescent T cells.

As shown in Fig. 12, if compared to uninfected cells, the CD28 expression level was reduced in GFP+ cells infected with wt-GFP but not with ΔNef-GFP virus. This result confirm that CD28 down-regulation in HIV-1-infected cells is due to the activity of Nef. In sum, in productively infected T cells, HIV-1 is able to down-

regulate efficiently surface CD4 and CD28 receptors, that are instead expressed at almost normal levels in infected quiescent T lymphocytes.

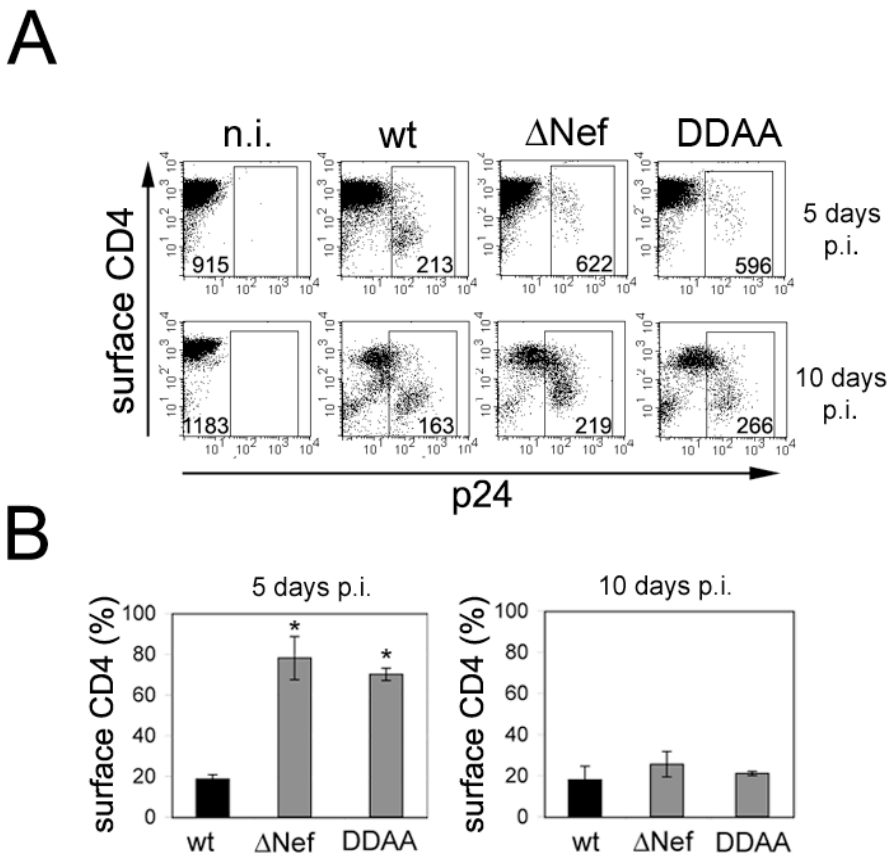

Fig. 11. CD4 surface levels in primary CD4$^+$ T cells infected with HIV-1 expressing wt or mutated Nef and than activated. (A) Freshly isolated CD4$^+$ T lymphocytes were not infected (n.i.) or infected with HIV-1 (50 ng p24/10^6 cells) either wild-type (wt), Nef-deficient (ΔNef), or expressing a mutated Nef protein defective for CD4 down-regulation (DDAA), and activated after infection. At various times p.i., expression of cell-surface CD4 was analyzed together with intracellular p24 expression by two-colour flow cytometry. The mean fluorescence intensity (MFI) values specific for CD4 of n.i. cells and of p24+ infected cells (gated in region R1) at 5 and 10 days p.i. are shown. (B) The mean ± SD intensity of fluorescence relative to the surface expression of CD4 in n.i. and infected cells was determined as shown in panel (A) in three independent experiments with CD4$^+$ T cells derived from three healthy donors. Data were calculated by setting to a 100% the CD4 cell-surface expression of n.i. cells. Significant differences between wt and other viruses, as calculated by paired *t*-test, are indicated: *, $P < 0.05$.

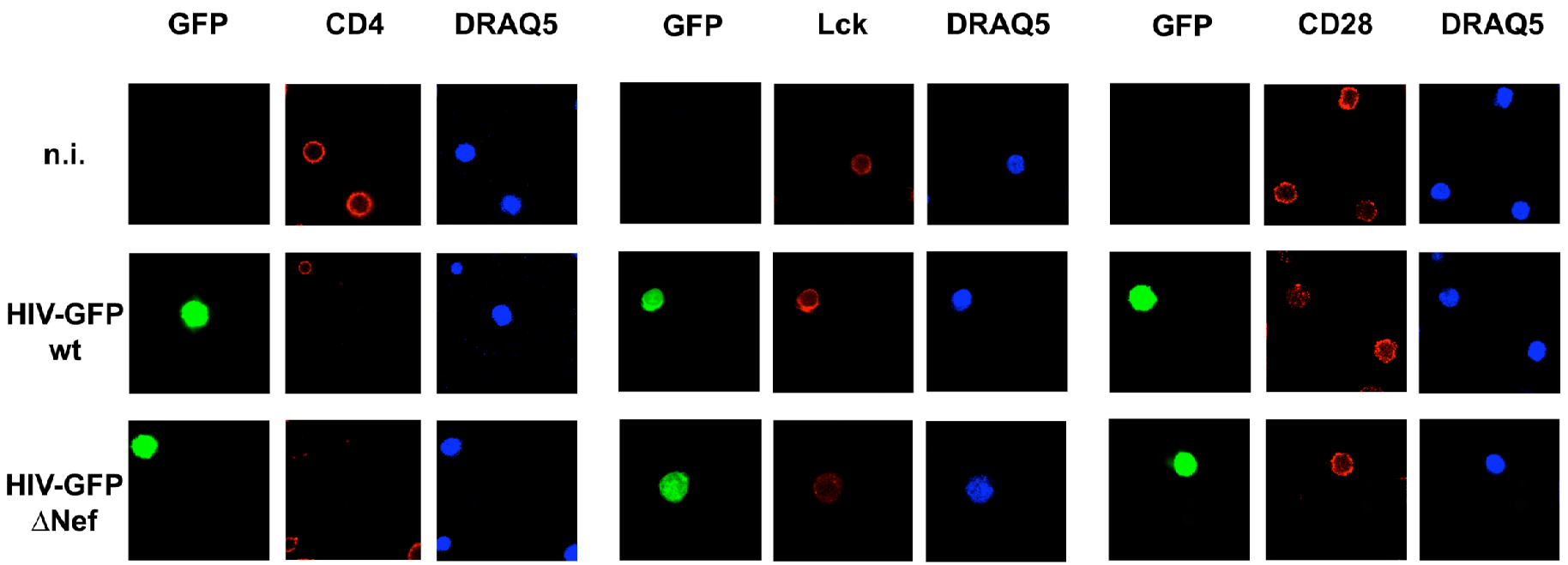

Fig. 12. Analysis of CD4, Lck and CD28 expression in productively $CD4^+$ T lymphocytes infected with HIV-1
Freshly isolated $CD4^+$ T lymphocytes were stimulated with PHA, IL-2 and after three days infected with VSV-G-pseudotyped wt-GFP or ΔNef-GFP viruses. Three days post infection, the cells were treated a second time with PHA and IL-2, next the cells were counted and equal number of cells were let to adhere on polilysine treated glass cover-lip. Next, cells were permeabilized and blocked with saponine/BSA to perform the intracellular staining with anti-CD4, anti-Lck and anti-CD28 antibodies. Finally the nucleus was marked with DRAQ5. Images were acquired using Confocal Laser Microscope.

3.9 Protein tyrosine phosphorylation upon secondary TCR stimulation of HIV-1-infected T cells.

In order to study the role of Nef in early signaling events following secondary TCR stimulation of HIV-1 infected T cells, $CD4^+$ T lymphocytes were first activated with anti-CD3 and anti-CD28 cross-linked antibodies and maintained in culture with IL-2 for 6 days. At that point, cells have stopped growing and returned to a resting state. Then, the cells were infected with VSV-G-pseudotyped wt or ΔNef viruses or uninfected. Three days post infection the cells were stimulated a second time throught CD3/CD28 for variable time (0, 30'', 1', 5'), then harvested to prepare total cell extracts. Equal amounts of total cell extracts derived at T0 were analyzed for the expression of Nef, CD4, and Lck by western blotting. Fig. 13A shows that Nef accumulated in wt-infected cells and that the steady-state levels of both CD4 and Lck were reduced in both wt- and ΔNef-expressing cells (CD4 was reduced by 10-20%, Lck was reduced by 20%). Therefore, the impact of HIV-1 expression on CD4 and Lck levels in resting $CD4^+$ T lymphocytes that has been previously activated is similar to that observed in freshly isolated quiescent T cells. In the presence of ΔNefvirus, steady-state CD4 levels are maintained in quiescent T cells but reduced in resting cells that have been previously activated, possibly due to some expression of Env and Vpu in the latter conditions. Then, cell lysates derived from unstimulated as well as stimulated cells, were analyzed by western blotting with anti-p-Tyr antibody. Upon secondary activation, some phosphorylated proteins were up-modulated: p-140, p-95, p-70 and p-36 that likely correspond to p-PLC-γ1, p-Vav, p-ZAP-70 and p-ζ-chain of TCR, respectively (Fig. 13B). Next the membrane was stripped and reprobed

with anti-PLC-γ1 specific antibody (Fig. 13C). After densitometric analysis, we normalized p-PLC-γ1 for its loading control and values were expressed by setting to 1 the signal of uninfected cells at time 0. As shown in Fig. 13D, by comparing uninfected, wt and ΔNef-infected cells we observed that in the presence of wt virus the peak of phosphorylation of PLC-γ1 was delayed. Indeed, uninfected and ΔNef-infected cells reached the peak of PLC-γ1 phosphorylation 30 seconds after stimulation, while wt-infected cells reached the peak 1 minute after the stimulation.

In this experimental condition following secondary TCR stimulation of HIV-1 infected cells it was evident that Nef has a negative effect on T cell signaling slowing down the activation of an important signaling molecule, PLC-γ1.

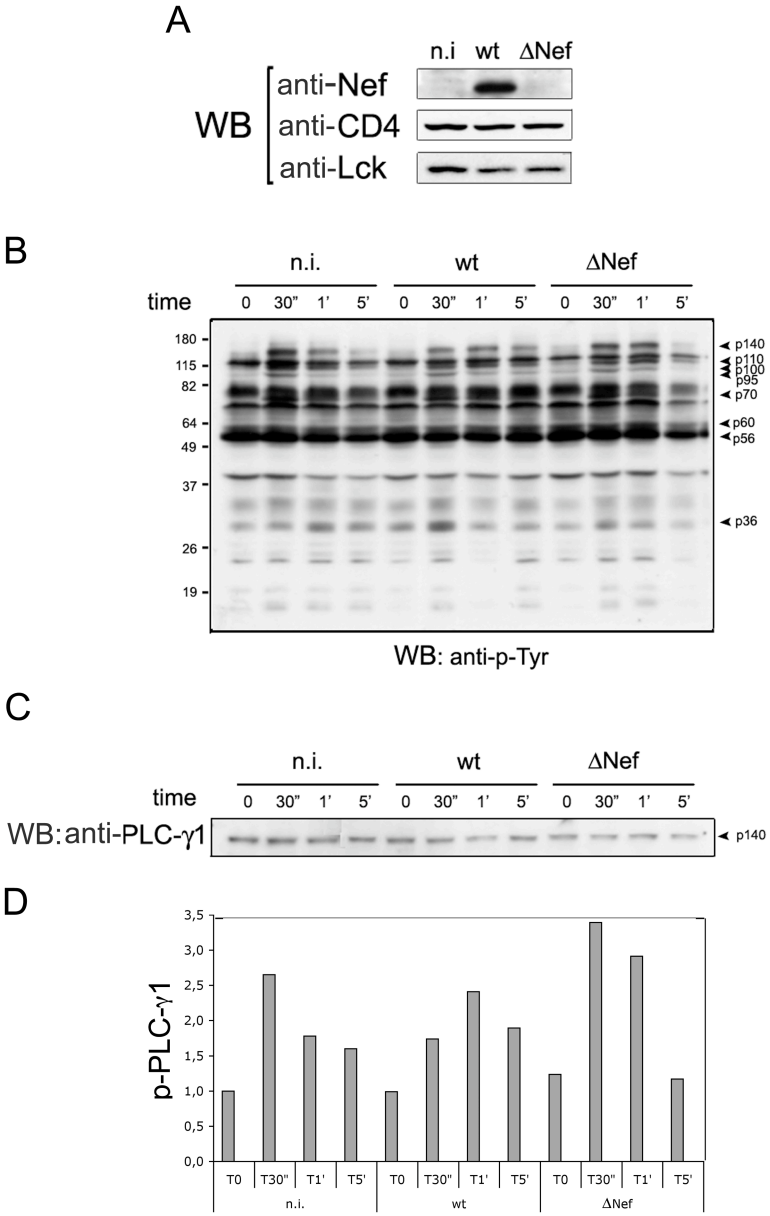

Fig. 13. Secondary TCR stimulation of HIV-1 infected cells.

Pre-activated $CD4^+$ T lymphocytes that have returned to a resting state were infected with VSV-G-pseudotyped wt and ΔNef virus. Three days post infection, the cells were re-stimulated with anti-CD3/CD28 cross-linked antibodies for 0, 30",1', 5' minutes and then lysed. Equal amounts of total cell lysates were analyzed by western blotting with anti-Nef, anti-CD4 or anti-Lck antibody (A) or anti-p-Tyr antibody (B). (C) The membrane shown in (B) was stripped and re-probed with anti-PLC-γ1 specific antibody. (D) The relative phosphorylation of PLC-γ1 is shown by setting to 1 the signal of n.i. cells at time 0. This experiment is one rapresentative out of three independent experiments.

4-DISCUSSION

The capacity of HIV-1 to infect $CD4^+$ T lymphocytes is closely linked to the activation state of the cells: in activated $CD4^+$ T cells HIV-1 readily undergoes multiple rounds of replication, whereas resting T cells are refractory to productive infection. However, some studies indicate that HIV-1 infection does not require full T cell activation and that it might actively modulate the activation profile of target lymphocytes. Various viral proteins are known to be involved in the alteration of signaling pathways, transcriptional activation and intracellular protein trafficking in the infected cells. A key regulator of T cell activity is the Nef viral protein. Nef has an essential role in the pathogenesis of AIDS that can be explained by its numerous activities including the modulation of cell surface expression of an array of receptors (including CD4, MHC class I and II, and chemokine receptors) and the disturbance of multiple signaling pathways. Nevertheless, the function of Nef in T cell activation is at present a controversial issue. On one hand, several studies indicate that Nef has a positive effect on signaling pathways related to the TCR. In particular, it was shown that Nef sensitizes the cells to activation thus favoring IL-2 production and viral replication in response to TCR stimulation. On the other hand, some studies showed that Nef has no effect on IL-2 expression induced by TCR triggering or even impairs early activation events during synapse formation with antigen presenting cells that result in reduced release of IL-2. In addition, discordant evidences have been reported on the role of Nef in the function of the Lck kinase that is a key regulator of T cell activity. It was shown that Nef either has no effect on Lck, activates Lck, or impairs Lck function by reducing its overall expression levels, promoting its intracellular retention, and inhibiting its recruitment at the immunological synapse. Some controversy could be explained by the use of different experimental systems that can

influence the activity of Nef (e. g. primary cells versus cell lines, transient expression of Nef versus infection with HIV-1). In addition, Nef might have diversified or even opposite effects on T cell activation depending on the intracellular environment. It is also important to take into consideration the capacity of Nef to interfere with the expression of the T cell accessory molecules CD4 and CD28. By down-regulating CD4, Nef may induce the de-localization/degradation of receptor-associated Lck or, alternatively, may increase Lck activation. Besides, reduction of surface CD28 levels by Nef should impair TCR signaling events that follow CD3/CD28 triggering including Lck activation. Moreover, Nef-induced down-regulation of surface CD4 and CD8 should result in an inefficient synapse formation between HIV-infected T cells and APCs. All these issues have been considered in the present study that is focused on the role of the HIV-1 Nef protein in the activation of primary $CD4^+$ T lymphocytes.

First, the impact of HIV-1 infection, with or without Nef expression, on the steady-state levels and intracellular localization of Lck, CD4, and CD28 was investigated in both resting and pre-activated $CD4^+$ T cells. In addition, the capacity of $CD4^+$ T lymphocytes infected with wt or Nef-deficient virus to respond to primary and secondary TCR stimulation was investigated. Finally, the CD4 signaling pathway and Lck activation that follow the triggering of surface CD4 receptor was analyzed in resting T cells infected with wt or Nef-deficient virus.

Freshly isolated $CD4^+$ T lymphocytes derived from healthy donors have been infected with HIV-1 of the NL4-3 strain, either wt or defective for Nef expression (ΔNef) or uninfected (n.i.) and analyzed after 5 days by means of western blotting, immunofluorescence microscopy, and flow cytometry with antibodies specific for Nef, Lck, CD4, and CD28. Results show that, despite the absence of viral replication,

both wt- and ΔNef-infected cells had slightly reduced expression of surface CD4 (about 10% less) if compared to n.i. cells, possibly due to receptor down-regulation during viral entry. However, only in Nef-expressing wt-infected cells the steady-state expression level of CD4 was reduced by 30% and the receptor accumulated intracellularly in vesicle-like structures. Therefore, in resting T cells Nef cannot induce internalization of surface CD4 possibly due to some unknown cellular factor(s) regulated by T cell activation. However, the capacity of Nef to retain and degrade newly synthesized CD4 molecules is maintained in resting T cells. Analogously, in this cell setting, Nef promoted the re-localization of Lck in vesicle-like intracellular compartments, as described previously in T cell lines and in activated primary $CD4^+$ T lymphocytes. Of note, a 20% reduction in the overall Lck expression was observed in both wt- and ΔNef-infected cells, indicating that a viral factor different from Nef induces Lck degradation. These results also suggest that, in HIV-infected cells, the degradation and re-distribution of Lck occurs through mechanisms that are independent of surface CD4 down-regulation by Nef. As to CD28, its steady-state protein level and intracellular localization were not affected by HIV-1 infection of resting T cells with or without expression of Nef. Thus, like CD4, surface CD28 is resistant to Nef-mediated down-regulation if cells are in a resting state.

Since it was suggested that Nef disrupts the association of Lck with CD4, we tested by co-immunoprecipitation assay the relative amount of Lck bound to CD4 in resting T cells wt- or ΔNef-infected if compared to n.i. cells. Results show that HIV-1, with or without expression of Nef, does not alter the formation of the CD4-Lck complex. Then, to determine whether Lck could be normally activated, resting n.i., wt- or ΔNef-infected T cells have been treated for variable time with cross-linked

anti-CD4 antibody. As expected, upon CD4 triggering the tyrosine-phosphorylation of Lck rapidly increased while the protein levels decreased, possibly due to the consumption of activated Lck. Importantly, in HIV-1 expressing cells, the kinetic and the extent of tyrosine-phosphorylation of Lck and its earliest substrate, the TCR ζ-chain, were similar to that observed in uninfected cells. Thus, in HIV-infected resting T cells, the function of the Lck kinase in maintained despite its overall reduction and re-localization. Next, by primary TCR activation via CD3/CD28 triggering of resting n.i, wt- or ΔNef-infected T cells, we observed an hyper-responsiveness of cells infected with wt virus as shown by 2-fold increased IL-2 production compared with n.i. or ΔNef-infected cells. In addition, following TCR engagement, higher levels of viral replication were achieved with wt virus if compared to ΔNef. This is consistent with a previous report showing that Nef sensitizes resting T cells leading to increased cellular activation and viral replication. Overall, these results suggest that, in resting $CD4^+$ T lymphocytes, Nef modulates intracellular signaling pathways to lower the threshold of T cell activation.

Then, we investigated early and late signaling events upon activation via CD3/CD28 of n.i, wt- or ΔNef-infected T cells. We found that the typical pattern of tyrosine-phosphorylated effector molecules that are induced early after TCR stimulation was not modified by HIV-1 infection, with or without Nef expression. In particular, significant differences were not found measuring the extent and the kinetics of tyrosine-phosphorylation of PLC-γ1, Vav, and ZAP-70. Analogously, we did not observe differences in the Akt and NF-κB pathways. Therefore, further analysis is needed to identify the mechanism(s) and the cellular target(s) that mediates Nef's capacity to sensitize primary T cells to activation.

The analysis described above were also performed on HIV-1-infected primary $CD4^+$ T lymphocytes that have been previously activated and thus are fully permissive to viral replication. By flow cytometry we observed that T cells infected with wt, ΔNef and DDAA (a mutated virus expressing a Nef mutant defective for CD4 down-regulation) became p24+ 5 days post infection (p.i.) and increased numerically till reaching a maximum after 10 days. At 5 p.i., the surface CD4 density of cells infected with wt virus was reduced to 20% while ΔNef-, DDAA-infected cells maintained 50-80% of CD4 expression. However, at 10 days p.i., a 5-fold reduction of surface CD4 was also achieved in cells infected with ΔNef or DDAA virus, likely due to the activity of the viral Vpu and Env proteins expressed in late stages of infection. Therefore, in primary T lymphocytes CD4 down-regulation by Nef is important in early phases of viral infection whereas it is not required to achieve maximal inhibition of surface CD4 expression in late stages of the HIV-1 life cycle. These results indicate that Nef's activity on CD4 may be irrelevant when the virus is productively assembled and released by these cells, as opposed to what suggested in previous reports. By immunofluorescence microscopy, we observed that in $CD4^+$ T lymphocytes productively infected with either wt or ΔNef virus, the CD4 completely disappeared within cells. In addition, by means of the same analysis we found that in wt- but not ΔNef-infected cells Lck accumulated in intracellular vesicles and CD28 expression was strongly reduced, analogously to what observed in infected quiescent T cells. These results confirm that the Lck re-localization and CD28 down-regulation occurring in HIV-1-infected T cells are two specific function of the viral Nef protein.

Finally, we analyzed the role of Nef in early signaling events following secondary TCR stimulation of HIV-1-infected T cells. $CD4^+$ T cells were pre-activated with anti-CD3/CD28 cross-linked antibodies and after 6 days infected with

wt and ΔNef virus. By that time, cells had stopped growing and returned to a resting state according to the disappearance of surface activation markers (CD69 and CD25, data not shown). Three days p.i., the cells were stimulated a second time by CD3/CD28 triggering and immediately harvested and analyzed by western blotting. As to CD4 and Lck expression, we observed that the steady-state levels of CD4 and Lck were reduced (10-20% and 20% respectively) in both wt- and ΔNef-expressing cells. Therefore, the impact of HIV-1 expression on CD4 and Lck levels in resting $CD4^+$ T lymphocytes that has been previously activated was similar to that observed in quiescent T cells, with the exception of a higher reduction of CD4 in ΔNef-infected cells. Moreover, the pattern of early protein tyrosine-phosphorylation was investigated. By comparing uninfected, wt- and ΔNef-infected cells we observed that in the presence of wt virus the peak of phosphorylation of PLC-γ1 was delayed. Therefore, following secondary TCR activation we observed a negative effect of Nef on the T cell activation pathway. This effect may result from Nef's capacity to dislocate Lck intracellularly, since recruitment and activation of Lck is central in determining the outcome of TCR engagement. Nevertheless, this explanation is not satisfactory given that Lck re-localization is induced by Nef also in quiescent T cells that are instead sensitized to activation by the viral protein and respond with normal PLC-γ1 phosphorylation. These results confirm and extend previous data showing a Nef-dependent impairment of cells productively infected with HIV-1 to properly form the immunological synapse with APCs. In former studies, TCR stimulation as induced by contact with surfaces coated with anti-CD3 antibody or with APCs was investigated in pre-activated HIV-infected T cells expressing high p24 levels. We envision that p24+ T cells undergoing productive HIV-1 replication could poorly respond to synapse formation due to the drastic reduction of surface CD4 and CD28,

important accessory molecules that cooperate to the reorganization of the contact zone at the synapse and its downstream signaling. This phenomenon should strictly depend on the expression of the Nef protein since it is absolutely required for CD28 down-regulation. Here, we analyzed secondary TCR stimulation in pre-activated $CD4^+$ T lymphocytes that have reached a resting state before HIV-1 infection and that should better represent latently infected memory T cells circulating *in vivo*. The observed Nef-dependent delayed activation of PLC-γ1 may account for a reduced signaling capacity of HIV-infected $CD4^+$ T lymphocytes upon encounter with APCs that may have important consequences for the pathogenic outcome of the infection.

In sum, our data support the existence of a dual role for the pathogenic Nef protein of HIV-1. Through mechanisms not yet identified, Nef behaves as an inducer of intracellular signaling pathways in quiescent $CD4^+$ T lymphocytes to favor initial viral replication. On the other hand, Nef is an inhibitor of TCR-mediated secondary activation, possibly to avoid apoptosis that would restrict viral production.

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Short Communication

The Pro78 residue regulates the capacity of the human immunodeficiency virus type 1 Nef protein to inhibit recycling of major histocompatibility complex class I molecules in an SH3-independent manner

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The Nef protein is a crucial pathogenicity factor of human immunodeficiency virus type 1 (HIV-1) that contains a proline-rich motif consisting of four conserved prolines: Pro69 (P69), P72, P75 and P78. P72 and P75 were shown to bind Src homology domains 3 (SH3) and have been implicated in many biological functions of Nef, including downmodulation of cell-surface major histocompatibility complex class I (MHC-I). P78 is involved together with P69 in positioning of the Nef–SH3 complex and it has been shown to be essential for Nef activity of MHC-I downmodulation. It is shown here that alteration of P78 affects recycling of MHC-I molecules to the cell surface, but does not interfere with SH3 binding. In addition, it is demonstrated that P72 and P75, and thus the SH3-binding capacity, are fully dispensable for Nef activity on MHC-I.

The Nef protein of human immunodeficiency virus type 1 (HIV-1) is a critical factor for high virus replication and disease progression in humans and animal models (Deacon *et al.*, 1995; Hanna *et al.*, 1998; Kirchhoff *et al.*, 1995). Nef has several activities: downmodulation of cell-surface molecules such as CD4 and major histocompatibility complex class I (MHC-I), alteration of cellular signalling pathways and stimulation of HIV-1 infectivity and replication (reviewed by Fackler & Baur, 2002; Federico, 2004; Peterlin & Trono, 2003). Conserved amino acid residues and motifs in Nef have been characterized for their role in specific activities and interactions (Arold & Baur, 2001; Geyer *et al.*, 2001), but it is still unclear which one(s) of these ultimately mediate(s) the pathogenic potential of Nef. A conserved proline-rich motif (PxxP) of Nef was accredited as a Src homology domain 3 (SH3)-binding site, with its central prolines, Pro72 (P72) and P75, contacting two hydrophobic pockets in the SH3 domain, whereas P69 and P78 assist the PxxP positioning (Arold *et al.*, 1997; Lee *et al.*, 1996). Through PxxP, Nef binds SH3-containing signalling molecules such as Hck (Saksela *et al.*, 1995) and Vav (Fackler *et al.*, 1999). Although the PxxP motif has been involved in most Nef functions (Greenberg *et al.*, 1998; Hanna *et al.*, 2001; Iafrate *et al.*, 1997; Mangasarian *et al.*, 1999; Saksela *et al.*, 1995), some studies have shown that it is dispensable for Nef activity on MHC-I (Riggs *et al.*, 1999) or CD4 (Saksela *et al.*, 1995) and HIV-1 replication and pathogenesis (Kawano *et al.*, 1997). In addition, it is unclear whether the role of the PxxP motif in the various biological functions of Nef is invariably mediated by its binding to SH3-containing proteins. As for MHC-I downmodulation, P78 rather than P69/P72/P75 was shown to be crucial (Yamada *et al.*, 2003). Besides, conflicting reports exist on the putative role of PI3-K (Blagoveshchenskaya *et al.*, 2002; Kasper & Collins, 2003) and Hck (Chang *et al.*, 2001; Greenberg *et al.*, 1998; Mangasarian *et al.*, 1999) as relevant SH3 ligands for Nef activity on MHC-I.

To further investigate the role of the PxxP motif of Nef, we performed various analyses based on an *in vivo*-selected mutation. We showed previously that two Nef proteins, NP5-7 and NP5-8, derived from a non-progressor patient (Casartelli *et al.*, 2003a), were impaired in MHC-I downmodulation and partially defective in CD4 downregulation (Casartelli *et al.*, 2003b). As both proteins contained a leucine at position 78, we restored P78 in NP5-8 by mutagenesis and tested the resulting NP5-8P78 protein for both CD4 and MHC-I downregulation activities by means of a

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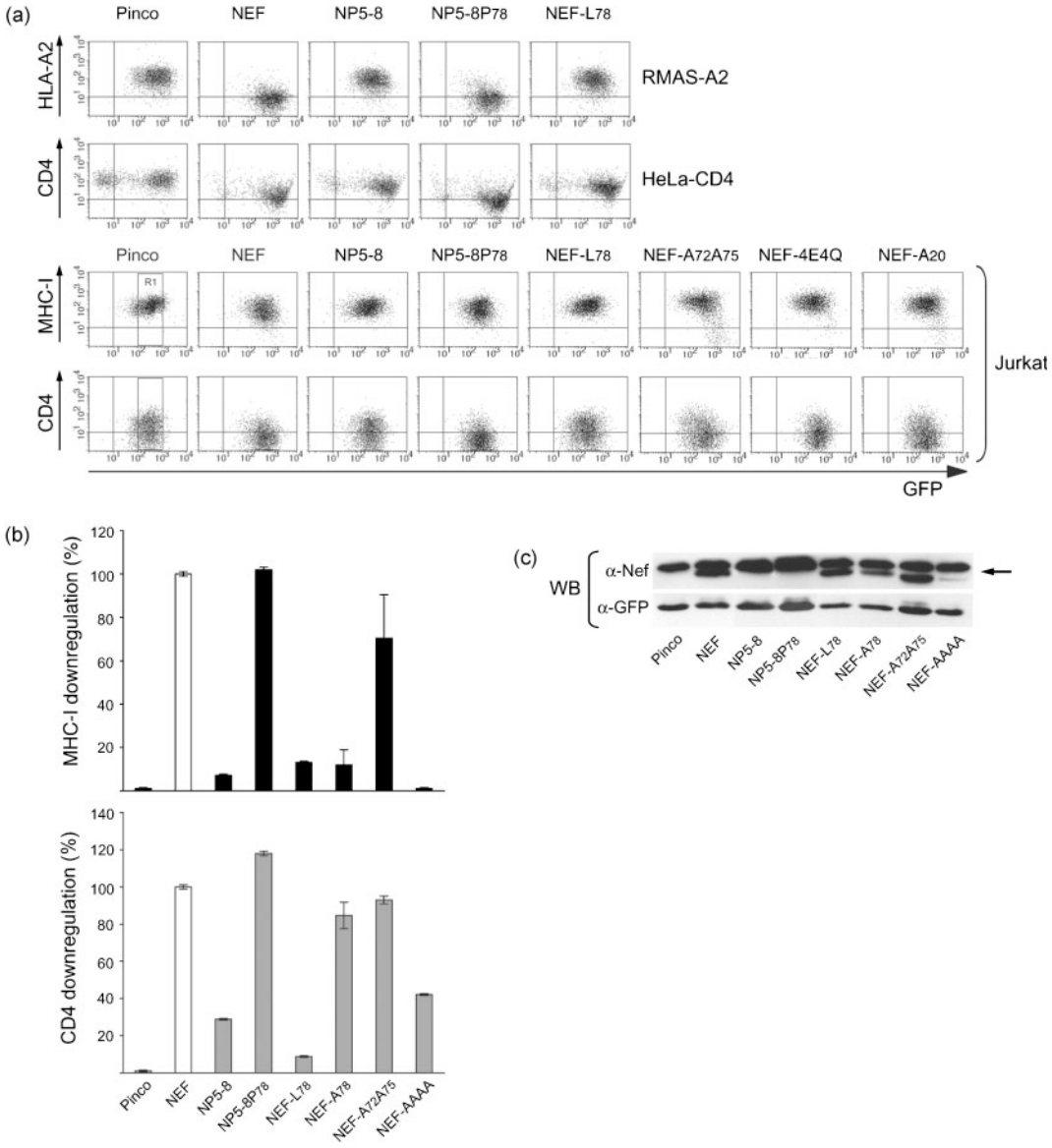

Fig. 1. Role of P78 in Nef activity of MHC-I and CD4 downmodulation. (a) Downregulation of cell-surface MHC-I and CD4 by wild-type and mutated Nef proteins. Cells were infected as described previously (Casartelli *et al.*, 2003b) with empty retrovirus (Pinco) or with recombinant Pinco retroviruses expressing NL4-3-derived Nef (NEF), NP5-8 or the indicated mutants. Specific mutations have been introduced into NEF and NP5-8 by standard site-directed mutagenesis based on recombinant PCR. After 48 h, cells were analysed by two-colour flow cytometry for expression of GFP encoded by the Pinco vector and of cell-surface MHC-I (Jurkat cells, top panels), human leukocyte antigen A2 molecules (HLA-A2; RMAS-A2 cells) or CD4 (HeLa-CD4 cells; Jurkat, bottom panels). HLA-A2 and CD4 stainings were performed as described previously (Casartelli *et al.*, 2003b). For MHC-I staining, a phycoerythrin (PE)-conjugated anti-HLA-A/B/C antibody (Becton Dickinson) was used. (b) Jurkat cells were infected and analysed for expression of MHC-I (top) and CD4 (bottom) as described in (a). The geometric mean fluorescence intensities (MFI) specific for MHC-I or CD4 were evaluated in cells expressing medium levels of GFP fluorescence [gated in the R1 region in (a)]. Values are expressed as a percentage of Nef activity (empty bars) of MHC-I (filled bars) and CD4 (shaded bars) downregulation. Reported values are the means ± SD of three independent experiments. (c) Immunoblotting analysis of NEF, NP5-8 and their mutants. Lysates of Phoenix cells transfected with the indicated clones were immunoblotted with an anti-Nef antibody (upper panel; ARP3026; MRC AIDS Reagent Project) as described previously (Casartelli *et al.*, 2003b). The arrow indicates the Nef protein bands. A cross-reacting, slower-migrating band present in the lysates is also visible. Due to their slower migration (Casartelli *et al.*, 2003b), NP5-8 and NP5-8P78 co-migrate with the cross-reacting band. The blot was then stripped and reprobed with an anti-GFP antibody (Clontech) to evaluate transfection efficiency (lower panel). Similar results were obtained with lysates of cells shown in (a) (not shown).

retrovirus-based transduction system in RMAS-A2 (MHC-I), HeLa-CD4 (CD4) and Jurkat (CD4 and MHC-I) cells, followed by two-colour flow cytometry (Casartelli et al., 2003b). Nef proteins derived from the NL4-3 viral strain, either wild type (NEF), with P78 mutated into a leucine (NEF-L78) or mutated at residues M20 and EEEE65 [shown previously to be required for MHC-I downmodulation (reviewed by Arold & Baur, 2001; Geyer et al., 2001)] (NEF-A20 and NEF-4E4Q, respectively; shown only for Jurkat; Fig. 1a) were also tested. As shown in Fig. 1(a), the L78P substitution restored NP5-8 activity on both CD4 and MHC-I fully. The detrimental effect of L78 was unrelated to the allelic background, as NEF-L78 was also impaired (Fig. 1a, b), and was not caused by altered steady-state protein expression (Fig. 1c) or aberrant subcellular distribution (data not shown). However, in line with a previous report (Yamada et al., 2003), a Nef mutant in which P78 was substituted with alanine (NEF-A78) was defective for MHC-I downregulation, but was active on CD4, similarly to NEF-A20 and NEF-4E4Q. It is likely that, although P78 has no direct role in CD4 downmodulation, selected amino acid substitutions induce conformational changes resulting in a Nef protein defective for this activity.

As P78 may participate in the formation of a Nef–SH3 complex (Arold et al., 1997; Lee et al., 1996), functional defects of L78 mutants might be associated with an altered SH3-binding capacity. We therefore investigated the contribution of the SH3-binding property to Nef activities by analysing two mutants in which either the central two or all four prolines of PxxP were replaced by alanines (NEF-A72A75 and NEF-AAAA, respectively). Fig. 1(a, b) shows that NEF-A72A75 was fully active on CD4 and partially impaired in MHC-I downmodulation. Of note, the steady-state expression level of the mutated protein was lower than that of the wild type (Figs 1c, 2a), in line with previous studies showing NEF-A72A75 protein instability (Craig et al., 1999; Iafrate et al., 1997). As MHC-I downmodulation requires significantly higher intracellular Nef concentrations than does CD4 downmodulation (Liu et al., 2001), low protein amounts should affect Nef activity on MHC-I preferentially. Consistently, NEF-AAAA, which was barely detectable by immunoblotting analysis (Fig. 1c), lost its activity on CD4 and MHC-I partly and completely, respectively. Thus, NEF-AAAA was disregarded for further analysis. To test whether the NEF-A72A75 defect in MHC-I downregulation was due to reduced protein expression, we titrated the retroviral particles expressing NEF or NEF-A72A75 by one to four spin-infection cycles (Fig. 2a, b). We found that cells infected with the same amount of virus displayed lower MHC-I downmodulation, as well as lower Nef protein amounts, when NEF-A72A75 was expressed in place of NEF, as expected for an unstable protein. However, in cells expressing similar levels of NEF and NEF-A72A75 following two and three spin infections, respectively, the extent of MHC-I downmodulation was equivalent (Fig. 2b). Thus, NEF and NEF-A72A75 display identical MHC-I downmodulation activities, for which the SH3-binding facet

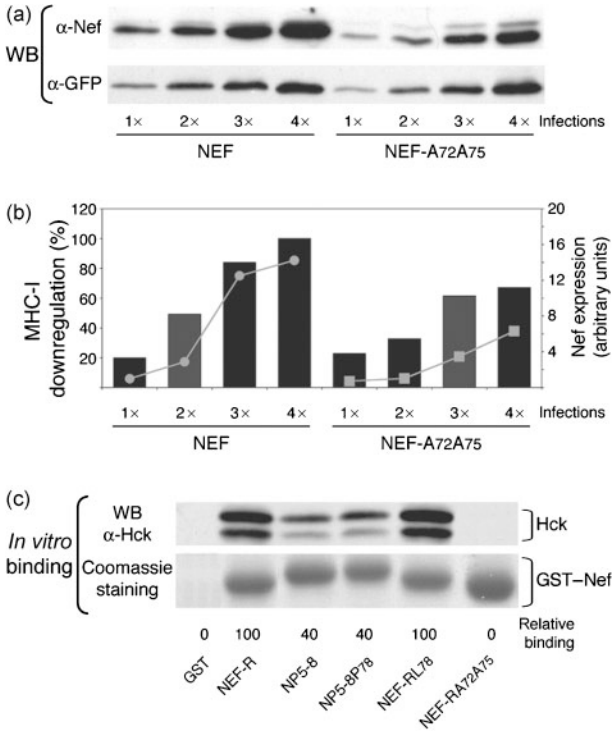

Fig. 2. The MHC-I downmodulation activity of Nef is independent of its SH3-binding capacity. (a, b) Jurkat cells were infected with one to four spin-infection cycles with retroviruses expressing NEF or NEF-A72A75. (a) The steady-state expression of Nef proteins was evaluated by immunoblotting cell lysates with the anti-Nef antibody mAb 158 (Fackler et al., 1997) (upper panel). Next, the blot was stripped and probed with anti-GFP antibody (lower panel). (b) An aliquot of cells was employed to assess MHC-I downregulation (columns) as described in Fig. 1(b). The downregulation efficiencies were calculated by considering as 100% the activity of NEF in cells infected by four infection cycles. Grey columns indicate similar MHC-I downmodulation activities. Grey lines indicate Nef protein levels as quantified by densitometry of blots shown in (a). Arbitrary densitometric units for Nef-specific bands were calculated by considering as 1 unit the value corresponding to the NEF-specific band in 1×-infected cells. (c) In vitro binding of Hck to NEF-R, NP5-8 and their derivatives. The upper panel shows proteins bound to GST-fusion proteins revealed by immunoblotting with an anti-Hck antibody (sc-72; Santa Cruz Biotechnology). Coomassie staining (lower panel) was used to evaluate sample loading. The value corresponding to Hck was normalized for that of GST-fusion protein in the same sample, both measured by densitometry and expressed as a percentage of the Hck protein bound to GST–NEF-R. Representative data from one of three independent experiments are shown.

of the PxxP motif is fully dispensable, at least in this experimental system. In $CD4^+$ T cells infected with HIV-1 carrying the NEF-A72A75 mutant, MHC-I downregulation was reduced (78% of the activity of wild-type virus), concomitant with a lower Nef protein expression (36% of the wild-type NEF; unpublished data), suggesting that, also in

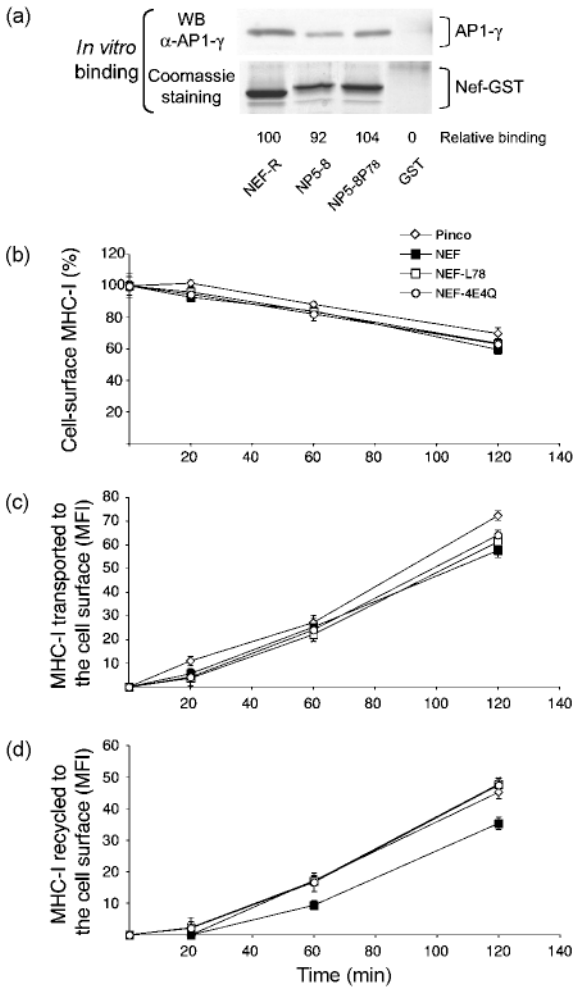

Fig. 3. Role of P78 in Nef capacity to bind AP-1 (a) and regulate MHC-I trafficking (b–d). (a) *In vitro* binding of AP-1 to GST–Nef fusion proteins. GST alone, GST–NEF, GST–NP5-8 and GST–NP5-8P78 were produced and tested for their capacity to bind AP-1 from Jurkat cell lysates as described previously (Casartelli *et al.*, 2003b). Relative AP-1-binding activity was calculated as the amount of AP-1 γ subunit detected by immunoblotting (top panel) normalized for the amount of GST-fusion protein detected by Coomassie staining (bottom panel) and expressed as a percentage of the value measured for GST–NEF. (b) Rates of MHC-I internalization. Jurkat cells were infected with retroviruses expressing NEF, NEF-L78, NEF-4E4Q or with the Pinco empty virus. At 48 h, MHC-I endocytosis was analysed at the indicated time points as described by Kasper & Collins (2003), but with W6/32 anti-MHC-I mAb. The relative amount of cell-surface MHC-I was expressed by considering as 100 % the initial MHC-I expression of each sample. (c) Transport of newly synthesized MHC-I to the cell surface. Jurkat cells transduced as in (b) were treated or not with cycloheximide and stripped of MHC-I molecules by low-pH treatment as described by Kasper & Collins (2003). Then, cells were incubated at 37 °C and 5 % CO_2 for the indicated time and stained with PE-conjugated anti-HLA-A/B/C mAb. The amount of MHC-I transported to the cell surface was determined by subtracting from each sample the corresponding MHC-I staining remaining after stripping, then subtracting the MFI of the cycloheximide-treated cells from the MFI of the untreated cells. (d) Recycling of MHC-I to the cell surface. Transduced Jurkat cells were treated as described in (c). The amount of recycled MHC-I was determined by subtracting the corresponding MFI remaining after stripping from the MFI of the cycloheximide-treated cells at each time point. Reported values are the means ± SD of duplicates from one representative experiment out of three.

primary HIV-1-infected lymphocytes, the SH3-binding surface of Nef is important for protein stability, but not for its activity on MHC-I.

We then analysed the SH3-binding capacity of NP5-8 and NP5-8P78 by testing their ability to form a complex with Hck. NEF, NEF-A72A75 and NEF-L78 were mutated by T71R substitution to optimize Nef–SH3 interaction (Saksela *et al.*, 1995), generating NEF-R, NEF-RA72A75 and NEF-RL78. The T71R substitution did not alter CD4 or MHC-I downregulation activities (data not shown). All variants were expressed as glutathione *S*-transferase (GST)-fusion proteins and tested for their capacity to bind Hck from U937 cellular lysates [as described by Lee *et al.* (1995)]. NP5-8 and NEF-R did not differ from the corresponding mutated variants, NP5-8P78 and NEF-RL78, respectively, in their relative Hck-binding capacity (Fig. 2c). The binding specificity was confirmed by the absence of Hck associated with GST–NEF-A72A75 or GST alone. Measurement of the capacity to associate with p21-activated kinase (Krautkrämer *et al.*, 2004), which depends strictly on the SH3-binding capacity of Nef (Manninen *et al.*, 1998), confirmed that P78 has no role in Nef–SH3 interactions (data not shown).

As the clathrin adaptor-protein complex AP-1 is required for Nef-mediated MHC-I downmodulation (Le Gall *et al.*, 1998; Roeth *et al.*, 2004), we tested the ability of NP5-8 to form a complex with AP-1 by an *in vitro* binding assay (Fig. 3a). No difference in association with AP-1 was detected for NEF, NP5-8 or NP5-8P78. Thus, the functional defect induced by L78 cannot be attributed to inefficient *in vitro* AP-1 binding, although we cannot exclude the possibility that L78 might interfere with the *in vivo* interaction between Nef and AP-1 that is relevant for MHC-I downmodulation.

To gain insights into the mechanism by which P78 regulates Nef activity on MHC-I, we examined MHC-I trafficking in Jurkat cells infected with retroviruses expressing NEF, NEF-L78 and the NEF-4E4Q mutant by a method described by Kasper & Collins (2003). In agreement with previous reports (Kasper & Collins, 2003; Larsen *et al.*, 2004), we observed that NEF stimulated MHC-I internalization from the cell surface slightly (Fig. 3b) and reduced both transport of newly synthesized class I molecules (Fig. 3c) and recycling of internalized MHC-I to the cell surface (Fig. 3d). Interestingly, NEF-L78 displayed the same phenotype as NEF-4E4Q, being unable to reduce MHC-I recycling.

Here, we provide evidence that P78 is required for Nef activity of MHC-I downmodulation without contributing to Nef interactions with SH3-containing proteins. Besides, we demonstrate that P78 mediates the capacity of Nef to inhibit recycling of MHC-I to the cell surface. Further studies are needed to investigate the role of P78 in Nef interactions with cellular cofactors regulating MHC-I retention. Of note, our results also demonstrate that the SH3-binding capacity of Nef is fully dispensable for downmodulating MHC-I. Previous studies performed with NEF-A72A75 (Blagoveshchenskaya *et al.*, 2002; Greenberg *et al.*, 1998; Mangasarian *et al.*, 1999) or with dominant-negative Hck (Chang *et al.*, 2001) suggested a role for SH3 interactions in Nef activity on MHC-I. Our results suggest that the reduced capacity of NEF-A72A75 to downmodulate MHC-I should be ascribed to a low protein amount rather than defective SH3 binding. It is likely that the association of dominant-negative Hck with the SH3-binding site of Nef inhibits Nef activity on MHC-I indirectly as a consequence of steric hindrance and/or allosteric effect. The uncoupling of Nef activity on MHC-I from SH3-binding capacity will have implications for our understanding of the cellular pathways exploited by the viral protein and for attempts to interfere therapeutically with its pathogenic functions.

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Short Communication

Internalization and intracellular retention of CD4 are two separate functions of the human immunodeficiency virus type 1 Nef protein

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The pathogenic Nef protein of the human immunodeficiency virus type 1 (HIV-1) downregulates CD4 by inducing its endocytosis and by inhibiting the transport of the receptor to the cell membrane. By means of *in vivo*-selected mutations, we show that L37, P78 and E177 residues of Nef are required for its effect on CD4 internalization and recycling but dispensable for Nef-induced retention and degradation of intracellular CD4. Of note, the function of Nef on the anterograde transport of newly synthesized CD4 molecules is irrelevant in cells with a slow constitutive CD4 turnover such as T cell lines. Moreover, we show that a mutated CD4 that is unresponsive to Nef-mediated endocytosis, CD4LL$_{144}$AA, is retained intracellularly and degraded by Nef like wild-type CD4. Thus, Nef's abilities to enhance endocytosis and induce intracellular retention of CD4 are mediated by separate protein surfaces and occur through distinct mechanisms.

The multifunctional Nef protein of the human immunodeficiency virus type 1 (HIV-1) is a critical determinant for viral replication and pathogenesis (Deacon *et al.*, 1995; Kestler *et al.*, 1991; Kirchhoff *et al.*, 1995). CD4 downregulation is Nef's best studied activity, for which a pathogenetic role has been proposed (Lama, 2003). Nef accelerates CD4 endocytosis by acting as an adaptor that specifically connects the receptor to clathrin adaptor protein (AP) complexes. In addition, Nef inhibits CD4 recycling to the cell membrane, presumably by misdirecting internalized molecules to lysosomes for degradation (Oldridge & Marsh, 1998). More recently, a strong Nef-mediated inhibition of the CD4 anterograde pathway was also demonstrated in epithelial cells (Rose *et al.*, 2005). Whether the activities of Nef in the CD4 endocytic and anterograde pathways are mediated by common protein surfaces and molecular interactions remains to be established. In this study, the link between the Nef-mediated mechanisms of CD4 downregulation have been investigated by means of *in vivo*-selected mutations of Nef.

We previously described Nef proteins defective in CD4 downregulation that were derived from HIV-1-infected patients (Casartelli *et al.*, 2003b). Although amino acids required for Nef's activity on CD4 were maintained in the defective proteins, some substitutions at highly conserved residues have occurred (Casartelli *et al.*, 2003a). The RP2-7 protein presented glutamine in place of leucine at position 41 (corresponding to position 37 in Nef of the NL4-3 strain), and RP4-11 displayed lysine instead of glutamic acid at position 178 (position 177 in NL4-3 Nef). Hence, we restored the conserved amino acids by mutagenesis, creating RP2-7L$_{41}$ and RP4-11E$_{178}$. In addition, we introduced *in vivo*-selected mutations in NL4-3 Nef (NEF), generating NEFQ$_{37}$ and NEFK$_{177}$. The wild-type and mutated proteins were tested for their CD4 downregulation activity in HeLa-CD4 cells by a previously described retrovirus-based transduction system followed by two-colour flow cytometry (Casartelli *et al.*, 2003b). The Q41L and E178K back-mutations fully restored the capacity of patient-derived Nefs to downregulate CD4 (Fig. 1a). In addition, NEFQ$_{37}$ and NEFK$_{177}$ lost activity, indicating that *in vivo*-selected substitutions impaired Nef function aside from the allelic background. The detrimental effect of the substitutions could not be attributed to protein instability, since they had no effect on the MHC-I downregulation activity (Casartelli *et al.*, 2003b) and did not alter the protein's steady-state expression, as shown by immunoblotting analysis (Fig. 1b). Therefore, two novel residues of Nef important for its activity of CD4 downregulation have been identified: L37, which resides in a conserved alpha helix (αH2) of the N-terminal flexible region (Geyer *et al.*, 1999), and E177, which belongs to one of the two clusters of acidic residues that delimit the

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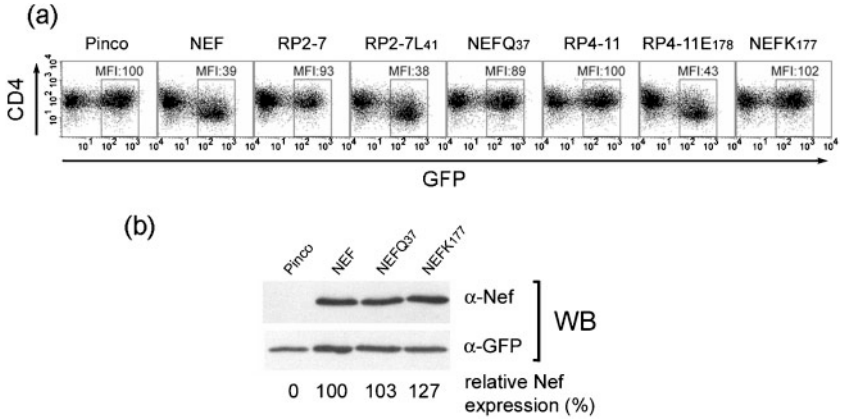

Fig. 1. Identification of novel Nef residues required for CD4 down-modulation. (a) HeLa-CD4 cells were infected with retrovirus either empty (Pinco) or expressing the indicated proteins and analysed 48 h after infection by flow cytometry for expression of GFP encoded by the vector and cell-surface CD4 as described previously (Casartelli *et al.*, 2003b). The mean fluorescence intensity (MFI) values specific for CD4 in GFP+ cells are indicated. (b) Lysates (20 μg) of infected HeLa-CD4 cells expressing NEF or the indicated mutants were immunoblotted with anti-Nef and anti-GFP antibody as described (Casartelli *et al.*, 2003b). Nef expression was normalized for that of GFP and expressed as percentage of wild-type NEF. The figure is representative of three independent experiments.

C-terminal loop and likely maintain the loop flexible through their reciprocal repulsion (Geyer & Peterlin, 2001). In accordance, by simultaneous substitution of 2–3 residues with alanines, both the αH2 helix and the acid cluster have been involved in Nef-mediated CD4 down-regulation in previous studies (Greenberg *et al.*, 1997; Iafrate *et al.*, 1997). The introduction of the polar Q37 residue should predictably alter the structure of the αH2 helix. Since the N-terminal region of Nef is important for high-affinity CD4 binding (Preusser *et al.*, 2001), the disruption of the αH2 helix may potentially perturb the Nef–CD4 interaction. The positively charged K residue in position 177 should alter the C-terminal loop, thus reducing the exposure of resident motifs (LL165 and ED175) critical for Nef functions in protein trafficking (Geyer *et al.*, 2001). In pull-down assays with cellular lysates, $NEFK_{177}$ associated with AP complexes or vacuolar ATPase as efficiently as wild-type NEF (data not shown). However, these assays may not reflect the capacity of Nef to form functional complexes required for effective CD4 down-regulation in intact cells.

To address the nature of the defects introduced by the *in vivo*-selected mutations, trafficking of CD4 was evaluated in Jurkat cells 48 h after transduction with retroviruses expressing NEF, $NEFQ_{37}$ or $NEFK_{177}$. We also tested $NEFL_{78}$, a protein with a patient-derived P78L substitution abrogating both CD4 and MHC-I downregulation (Casartelli *et al.*, 2006), and two mutants, $NEFG_2A$ and $NEFLL_{165}AA$, that have lost the capacity to associate with the cell membrane and AP complexes, respectively, and thus cannot downregulate CD4 (Geyer *et al.*, 2001). In Jurkat cells, surface CD4 was reduced approximately twofold by NEF, whereas it was not affected by the mutants (Fig. 2a). As measured by a FACS-based endocytosis assay (Kasper & Collins, 2003), the expression of NEF increased the endocytic rate of residual surface CD4 (twofold higher initial rates), resulting in 80 % internalization in 60 min, rather than 40 % as observed in cells expressing $NEFQ_{37}$, $NEFL_{78}$, $NEFK_{177}$ or $NEFG_2A$ or in control cells (Fig. 2b). To evaluate the CD4 recycling rate, cells were cultivated for 3 h in the presence of a protein synthesis inhibitor, cycloheximide, then treated with trypsin (0.25 % in PBS 0.5 M EDTA for 20 min at 37 °C) until surface CD4 was completely removed (data not shown). The treated cells were washed, incubated with cycloheximide at 37 °C and the appearance of recycled CD4 on the cell surface was measured over time. The rate of CD4 recycling was slow (15 % in 180 min), and NEF expression reduced this rate almost twofold (8 % in 180 min) (Fig. 2c). On the contrary, expression of $NEFQ_{37}$, $NEFL_{78}$, $NEFK_{177}$ or $NEFLL_{165}AA$ resulted in a very modest reduction of the CD4 recycling rate (~13 % in 180 min). This assay was also performed without cycloheximide to determine the transport of newly synthesized CD4 over time (the amount of surface CD4 reappearing in cycloheximide-treated cells was subtracted from that reappearing in untreated cells). The constitutive transport of CD4 to the cell membrane was very slow (~6 % in 180 min), in cycloheximide-treated with the slow catabolism and long half-life (>24 h) of this molecule in T lymphocytes (Moller *et al.*, 1990) and, importantly, did not vary significantly upon expression of wild-type or mutated Nef (data not shown). Next, the intracellular distribution and steady-state expression of CD4 in T cells expressing wild-type or mutated Nef proteins were analysed by immunofluorescence microscopy and immunoblotting analysis, respectively. To this aim, CEM cells were used rather than Jurkat cells because of their higher CD4 expression levels. As expected, cell-surface CD4 disappeared in cells expressing NEF while it was maintained upon expression of $NEFQ_{37}$, $NEFL_{78}$, $NEFK_{177}$ or $NEFLL_{165}AA$ (Fig. 2d). The intracellular CD4-specific punctate staining was not altered by either wild-

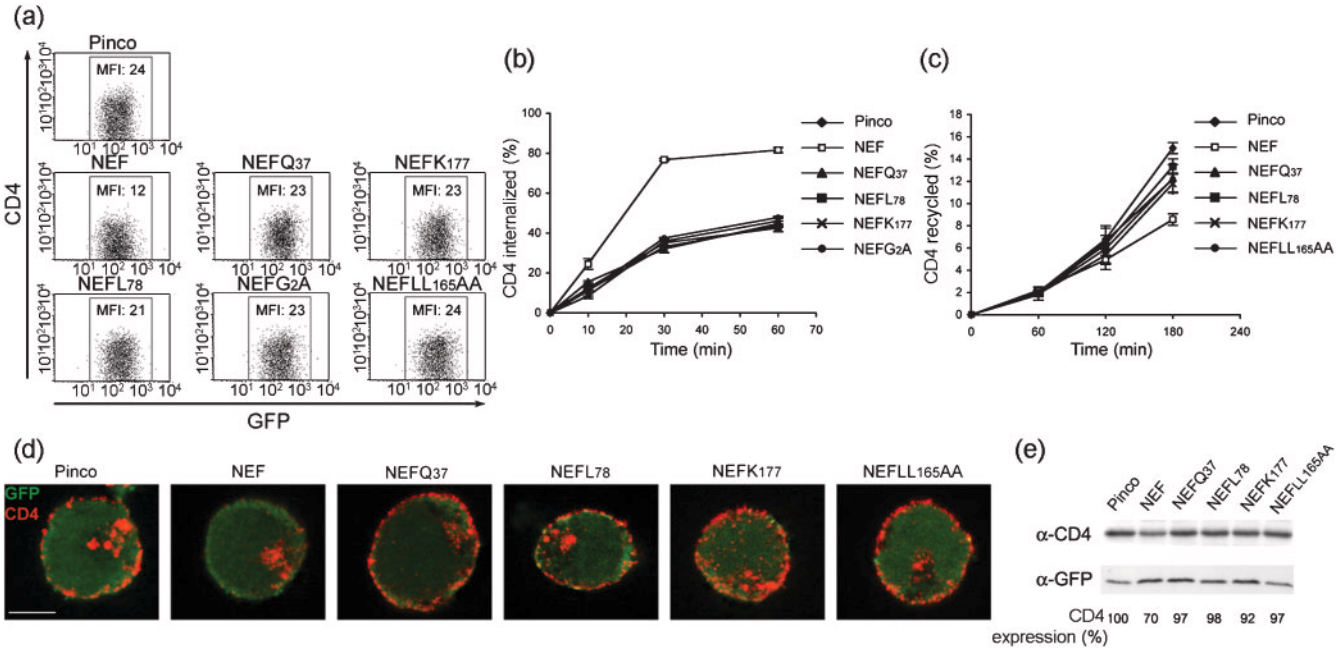

Fig. 2. Nef mutants have impaired capacities to accelerate endocytosis and delay recycling of CD4. (a) Jurkat cells were infected with retrovirus either empty (Pinco) or expressing the indicated proteins and analysed as described in Fig. 1(a). The rates of CD4 internalization (b) and recycling (c) were analysed in Jurkat cells 48 h after infection with retroviruses expressing the indicated Nef proteins or with empty virus. CD4 endocytosis was analysed as described (Casartelli *et al.*, 2006), but with an anti-CD4 antibody (RPA-T4; BD Biosciences). The recycling assay was performed as described (Casartelli *et al.*, 2006) with the exception that surface molecules were removed by trypsin and a PE-conjugated anti-CD4 antibody (BD Biosciences) was employed. Values are expressed as percentage of surface CD4 initially associated to each cell sample. The means ± SD of triplicates from one representative experiment out of three are reported. (d) CEM cells infected as shown in (a) were stained with anti-CD4 mAb and Cy3–goat anti-mouse IgG and processed for microscopy as described by Fackler *et al.* (2000). Images were acquired using a Zeiss Axiovert 200 microscope and ApoTome Imaging system. Merged GFP/CD4 images of central planes are shown. Bar, 5 μm. (e) Total cell lysates (50 μg) of infected CEM expressing the indicated proteins were analysed by immunoblotting with anti-CD4 (H370; Santa Cruz Biotechnology) and anti-GFP antibodies. The level of CD4 was expressed by considering 100 % that observed in Pinco-infected cells.

type or mutated Nef proteins. Therefore, Nef has no effect on the distribution of CD4 within T cells. As shown by immunoblotting analysis (Fig. 2e), wild-type but not mutated Nef proteins induced an approximately 30 % decrease in total cell-associated CD4, in accordance with lysosomal degradation of CD4 molecules internalized by Nef. Thus, Nef-mediated CD4 downregulation in T cells can be attributed mainly to accelerated internalization and reduced recycling of CD4. Results also show that both activities are defective in Nef variants containing *in vivo*-selected mutations. In different cell systems such as HeLa or 293T cells transduced with CD4 expression vectors, the transport to the cell membrane of both newly synthesized and recycling CD4 molecules was shown to be strongly reduced by Nef, thus resulting in the receptor's accumulation in perinuclear vesicles and reduced steady-state expression levels (Rose *et al.*, 2005). In HeLa cells, the effects of Nef on CD4 endocytosis and recycling are modest (Rose *et al.*, 2005), likely because they are masked by the rapid constitutive turnover of CD4 in these cells (Pelchen-Matthews *et al.*, 1991). We then analysed the distribution of CD4 in transfected HeLa-CD4 cells expressing the mutated Nef proteins as GFP fusions, their wild-type counterparts or GFP alone. The construction of GFP-fusion expression vectors and transfection were performed as previously described (Fackler *et al.*, 2000). To optimize staining of surface CD4, immunofluorescence microscopy was performed without prior permeabilization of cells. The cell-surface CD4 disappeared almost completely in cells expressing NEF–GFP or the RP2-7L_{41}–GFP and RP4-11E_{178}–GFP back-mutants while, in cells expressing NEFL_{78}–GFP, RP2-7–GFP, RP4-11–GFP or NEFLL_{165}AA–GFP, it was maintained to the same extent as in cells expressing GFP alone or not transduced (Fig. 3a). Of note, the intracellular localization of defective Nef proteins did not differ from that of wild-type Nef (mainly in perinuclear vesicles, at the plasma membrane and, to a small extent, diffused in the cytoplasm), suggesting that functional defects were not due to protein delocalization. An exception consisted in some nuclear localization and in a higher

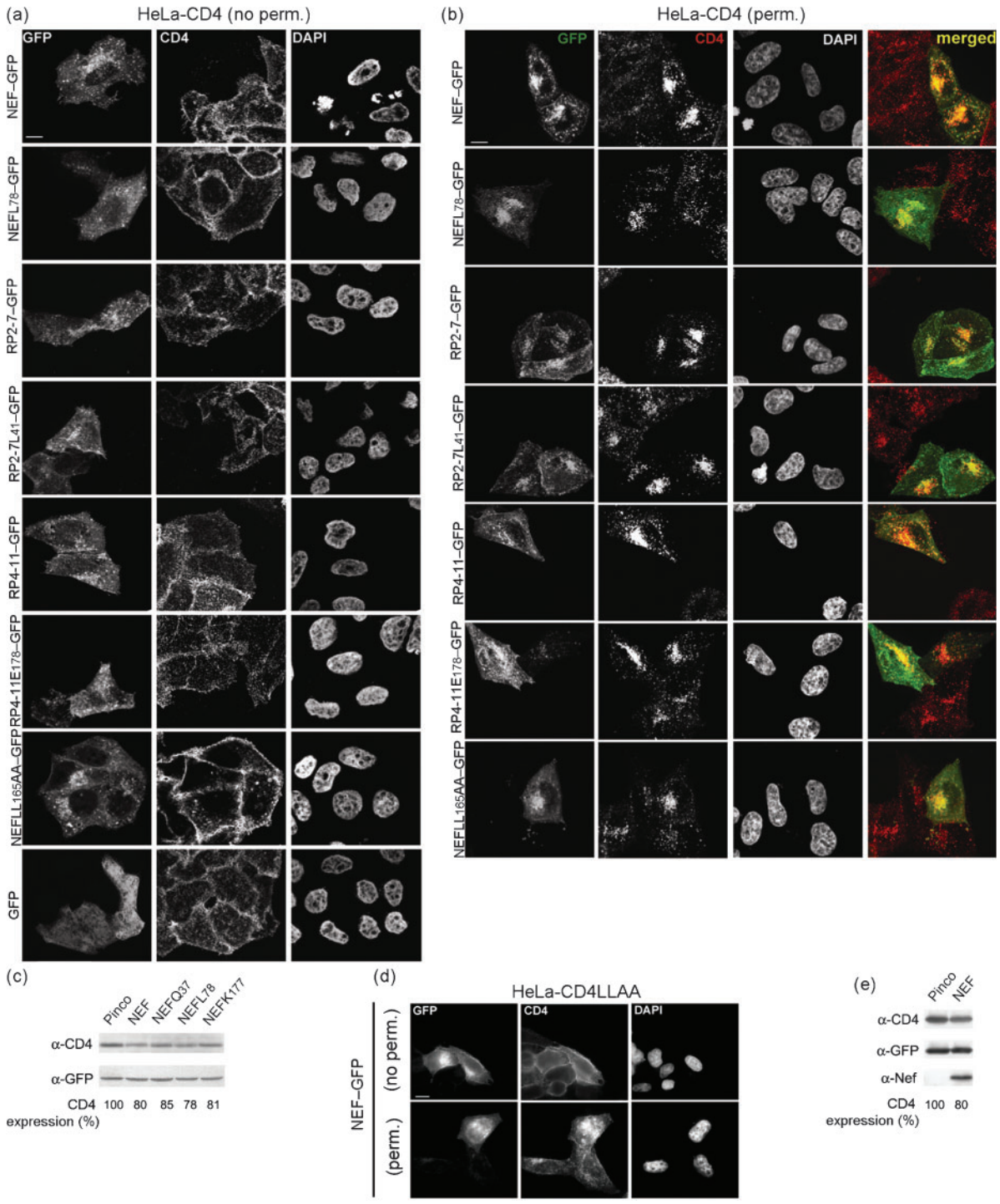

Fig. 3. Nef mutants conserve the ability to retain and degrade CD4 that is independent from CD4 endocytosis. (a, b) Hela-CD4 cells expressing GFP alone or the indicated GFP-tagged Nef proteins, either without (a, no perm.) or with permeabilization (b, perm.), were stained and analysed by microscopy as described in Fig. 2(d). Nuclei were stained with DAPI. Either basal planes (a) or 3D-reconstructions of all planes (b) are shown. Individual channels corresponding to GFP, CD4 and DAPI or merged GFP/CD4 images (only in b) are shown. Bar, 10 μm. (c) Lysates of HeLa-CD4 cells infected as in Fig. 1(a) and expressing the indicated proteins were analysed by immunoblotting as described in Fig. 2(e). (d) HeLa-CD4LLAA cells transfected with NEF–GFP expression plasmid were stained as in (a, b). Images were acquired by a SPOT-2 CCD digital camera (Diagnostic Instruments). (e) HeLa-CD4LLAA cells were infected with empty or NEF-expressing retrovirus as described in Fig. 1(a), lysed and analysed by immunoblotting with anti-CD4, anti-GFP and anti-Nef antibodies. The level of CD4 was expressed by considering 100 % that observed in Pinco-infected cells. Data are representative of three independent experiments.

diffuse cytoplasmic distribution of $NEFL_{78}$–GFP and $NEFLL_{165}AA$–GFP. The intracellular CD4 localization was analysed in permeabilized cells (Fig. 3b). Compared to control cells, the CD4-specific staining was less dispersed throughout the cytoplasm and accumulated in a perinuclear area in cells expressing NEF–GFP. The NEF- and CD4-specific fluorescence largely overlapped, indicating that the majority of CD4 co-localized with Nef in a vesicular compartment. The nature of NEF–GFP+ vesicles, either CD4+ or not, is heterogeneous since few expressed markers of early endosomes (EEA1) or Golgi compartment (giantin), most corresponded to recycling compartments (as determined by transferrin uptake over time), and none was positive with staining specific for multivesicular bodies (CD63) or lysosomes (LysoTracker; Invitrogen) (data not shown). Surprisingly, the same accumulation of CD4 in GFP+ perinuclear vesicles was observed in cells expressing $NEFL_{78}$–GFP, RP2-7–GFP, RP4-11–GFP or $NEFLL_{165}AA$–GFP, indicating that mutants defective for CD4 downregulation conserved the capacity of wild-type Nef to retain CD4 within these cells. Next, we analysed by immunoblotting the steady-state expression of CD4 in HeLa-CD4 cells expressing NEF, $NEFQ_{37}$, $NEFL_{78}$ or $NEFK_{177}$. Both wild-type and mutated Nef induced an approximately 20 % decrease in total cellular CD4 (Fig. 3c), suggesting that retained CD4 molecules are in part redirected to a degradation pathway. Thus, L37, P78 and E177 residues of Nef are required for its effect on CD4 endocytosis and recycling, but dispensable for the protein's capacity to induce the retention and degradation of intracellular CD4. These results suggest that to induce CD4 retention Nef uses mechanisms distinct from those used to internalize surface CD4. This hypothesis was confirmed by analysing the role of Nef on the receptor's distribution and steady-state expression in HeLa-CD4LLAA cells that stably express a CD4 mutant in which leucines 143 and 144, required for Nef-mediated endocytosis, have been substituted with alanines (Aiken *et al.*, 1994; Bentham *et al.*, 2003). Although its surface levels were not altered, $CD4LL_{144}AA$ accumulated in a perinuclear region upon NEF–GFP expression (Fig. 3d). In addition, the amounts of cell-associated $CD4LL_{144}AA$ were reduced by 20 % when Nef was expressed (Fig. 3e). Therefore, Nef-induced CD4 endocytosis does not contribute to retention and degradation of intracellular CD4 that results from an independent activity of Nef. It is conceivable that, during its anterograde pathway, nascent CD4 encounters and binds Nef in some vesicular compartment in which, consequently to the formation of a CD4–Nef complex, it is retained and eventually redirected to a degradation pathway. In agreement with this model, the $LL_{144}AA$ mutation of CD4, that does not interfere with the formation of a CD4–Nef complex (Bentham *et al.*, 2003), had no impact in the Nef-mediated block of CD4 transport.

Taken together, these results show that Nef uses distinct surfaces and mechanisms to enhance endocytosis and inhibit transport of CD4. Apparently, the effects of Nef on CD4 endocytosis and transport are readily detected in cells with a slow (e. g. T cells) and fast (e. g. HeLa-CD4) constitutive CD4 turnover, respectively. Since the function of Nef on CD4 transport was not detected in T cell lines, its relevance as well as its impact on HIV-1 spread should be further investigated, preferably in primary T lymphocytes that have physiological CD4 expression. The molecular dissection of Nef-mediated CD4 downregulation, an HIV-1 function implicated in pathogenesis (Lama, 2003), may help the development of new strategies for the treatment of AIDS.

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