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Characterization of HIV-1 Nef and Vpu function with a focus on T cell polarity

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SUMMARY

Expression of accessory proteins during human immunodefiency virus-1 (HIV-1) life cycle is essential to ensure efficient viral replication and spread. Despite of being dispensable for HIV-1 infection of cell lines *in vitro*, use of primary target cells and *in vivo* experiments proved them to be critical key pathogenicity factors. Along with counteraction of restriction factors to facilitate immune response escape, they play an important role as manipulators of host cell fundamental biological processes to favor replication. This work focuses on the study of the accessory proteins. Both these proteins are known to strongly alter intracellular vesicular transport. As a consequence, the composition of the plasma membrane is modified upon their expression, a phenomenon studied in the first part of this thesis. Here, it has been demonstrated the specificity of the Nef- and Vpu-mediated downregulation of tetraspanins, the most drastically affected host receptors, and identification of molecular determinants of both proteins necessary for this activity was pursued. It was also studied the ability of Vpu to retarget the T lymphocyte master regulator Lck, a well described phenomenon induced by Nef. Another yet unstudied field for Vpu examined herein was the induction of extracellular vesicle release. This work revealed that Vpu, like Nef, enhances extracellular vesicle release when expressed by T lymphocytes with comparable or even higher efficiency. The second part of this thesis addressed the effect of these proteins on T cell polarity disruption in the context of T cell migration. Unlike Vpu, Nef has been reported to inhibit F-actin cytoskeleton rearrangements. This requires the interaction of Nef with PAK2 which facilitates a specificity switch of the host kinase resulting in hyperphosphorylation and thus inactivation of the actin severing factor cofilin. As a consequence, the motility of HIV-infected T lymphocytes is hampered. A previous report showed that in murine $CD4^+$ T cells, the central Nef-mediated block *in vivo* and *ex vivo* is at the level of transendothelial and subendothelial migration. Whereas the latter is dependent on Nef-PAK2 on actin dynamics, the effect on transmigration involves other, yet uncharacterized protein interactions and is remarkably paralleled by a pronounced reduction of cell polarization. This thesis shows that Nef also hinders polarization of HIV-1 infected primary human CD4+ T lymphocytes. Vpu was entirely unrequired for this effect. Nef-expression in human T cell lines caused a marked polarization defect conserved among Nef proteins from HIV-1, -2 and simian immunodeficiency virus. The interaction surface of Nef with the Nef-associated kinase complex (NAKC) was found to be the essential molecular determinant for polarization disruption by Nef, both when overexpressed or in HIV-1 infection. Pharmacological inhibition of NAKC components evidenced the involvement of protein kinase C (PKC) in this Nef activity. This work also details setting up of experimental conditions to study the possible implication of this Nef amino acid stretch on transendothelial migration, that is on diapedesis *ex vivo*. Altogether, the results presented here underline the importance of the studied redundant functions between Nef and Vpu in HIV-1 infection and the relevance of these accessory proteins as pathogenicity factors. Also, they highlight the role of Nef as the main HIV-1 negative regulator of T lymphocyte migration.

ZUSSAMENFASSUNG

Die Expression akzessorischer Proteine während des Humanes Immundefizienz-Virus-1 (HIV-1) Replikationszyklus ist essenziell für die effiziente Replikation und Verbreitung des Virus. Obwohl akzessorische Proteine bei der Infektion von Zelllinien *in vitro* nicht notwendig sind, zeigten Experimente mit primären Zielzellen und *in vivo* Versuche, dass diese Proteine kritische Faktoren der Pathogenese darstellen. Neben der Abwehr von Restriktionsfaktoren, um dem Immunsystem auszuweichen, spielen akzessorische Proteine eine wichtige Rolle bei der Manipulation essentieller biologischer Prozesse des Wirts, um die Virusreplikation zu unterstützen. Diese Arbeit behandelte die Rolle der akzessorischen Proteine Nef und Vpu mit genauerer Betrachtung ihrer gegenseitig redundanten Funktionen. Beide Proteine sind dafür bekannt, den intrazelluläre Transport zu verändern. Als Folge, wird die Komposition der Plasmamembran bei ihrer Exprimierung modifiziert, ein Phenomen, das im ersten Teil dieser Arbeit untersucht wurde. In diesem Teil wurde gezeigt, dass das Herunterregulieren von Tetraspanine, die am stärksten herunterregulierten Wirtsrezeptoren während einer Infektion, spezifisch durch Nef und Vpu vermittelt wird. Zusätzlich es wurde versucht, die molekularen bestimmenden Faktoren beider Proteine, die für diesen Effekt verantwortlich sind, zu identifizieren. Weiterhin wurde Vpu auf die Fähigkeit hin untersucht, den T-Zell-Hauptregulator Lck umzuleiten, ein Phänomen dass für Nef bereits ausführlich beschrieben ist. Zusätzlich wurde in dieser Arbeit der Einfluss von Vpu auf die Vesikelfreisetzung untersucht, welches ein bislang unerforschtes Gebiet darstellte. Diese Arbeit zeigte, dass wenn Vpu in T-Zellen exprimiert wird, die Vesikel Freisetzung im verglich zu Nef-Exprimierung genauso oder sogar effizienter verstärkt wird. Im zweiten Teil der Arbeit wurde der Einfluss beider Proteine auf den Zellpolaritätsdefekt und dessen Auswirkung auf die Zellmigration untersucht. Im Gegensatz zu Vpu, es wurde berichtet, dass Nef kann die Umlagerung des Aktin-Zytoskeletts weitgehend beeinträchtigen. Dafür wird die Interaktion zwischen Nef und der Wirtskinase PAK2 benötigt, was eine Veränderung der Spezifität dieser Kinase einleitet und mit einer Hyperphosphorylierung und Inaktivierung des Aktindepolymerisierugsfaktors Cofilin einhergeht. Dies führt zu einer beschränkten Motilität von HIV-infizierten T-Zellen. In $CD4^+$ Mauszellen ist diese Blockade *in vivo* und *ex vivo* auf der Ebene der transendothelialen und subendotelialen Migration bemerkbar. Letztere ist abhängig von der Nef-PAK2 Interaktion und der damit verbundenen Beeinträchtigung des Aktin-Zytoskeletts, jedoch ist der Effekt auf die transendotheliale Migration von anderen Faktoren abhängig und geht bemerkenswerteweise mit einer ausgeprägten Inhibierung der Zellpolarität einher. Weiterhin konnte gezeigt werden, dass der negative Einfluss auf die Zellpolarität auch in HIV-1-infizierten humanen primären CD4+ T Zellen auftritt, dieser jedoch unabhängig von Vpu ist. Die Expression von Nef in T-Zelllinien zeigte einen ausgeprägten Defekt auf Ebene der T-Zellpolarität, der von HIV-1 und HIV-2, bis hin zu Simianes Immundefizienz-Virus konserviert war. Die Interaktionsoberfläche von Nef mit dem Nef-assoziierten Kinase-Komplex (NAKK), wurde als essentielle molekulare Determinante identifiziert, die für die Störung der Zellpolarität verwantwortich war.

Dies konnte in HIV-1-Infektionsversuchen sowie durch die Überexpression von Nef bewiesen werden. Die pharmakologische Inhibierung von NAKK-Komponenten zeigte, dass Protein-Kinase C (PKC) bei diesem durch Nef hervorgerufenen Effekt beteiligt ist. Es wurde in dieser Arbeit zudem eingerichtet ein experimentelles System auf, welches den Einfluss der Aminosäurenabfolge der Interaktionsoberfläche von Nef mit NAKK auf die transendotheliale Migration *ex vivo*, vor allem während der Diapedese, testen lässt. Zusammengefasst zeigen die hier aufgeführten Ergebnisse die Bedeutung der redundanten Wirkweise der Proteine Vpu und Nef während der HIV-1-Infektion und der Relevanz dieser akzessorischen Proteine als pathogene Faktoren. Zusätzlich wird die Hauptrolle von Nef als wichtigster negativer Regulator der T-Lymphozyten-Migration verdeutlicht.

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1 INTRODUCTION

1.1 Retroviruses

1.1.1 General genomic features

Retroviruses are a family of enveloped RNA viruses which share structural, composition and replication properties. They represent one the most broadly spread family of infectious factors in vertebrates, having been identified in all mammals' species as well as some birds, reptiles and fish, and probably the most diverse (Coffin, 1992). The retroviral virions are 80-100 nm in diameter and contain two identical copies of positive single-stranded, linear and non-segmented RNA genome which is 7-12 kb in size depending on complexity. The most exclusive characteristic of the retroviridae family of viruses is its replication strategy that comprises retrotranscription or conversion of the genomic RNA to double-stranded DNA and the subsequent integration of the synthesized DNA in the genome of the infected cell. The first one of these steps is possible thanks to a unique enzyme of this virus family after which they are named: the Reverse transcriptase (RT) or retrotrancriptase (retro, Latin for "backwards") (Baltimore, 1970; Temin and Mizutani, 1970). The integrated DNA or provirus relies upon integration on the cellular machinery for its transcription and translation. All retroviruses contain common coding domains that encode for polyproteins: *gag* (for Group-specific antigen), encoding for the structural virion proteins matrix (MA), capsid (CA) and nucleocapsid (NC), *pol*, containing the expression sequence of the RT and integrase (IN) enzymes, and *env*, the transcription of which results in the expression of the viral envelope protein with its transmembrane (TM) and the surface (SU) components (Vogt, 1997). The latter represents the main differential feature of retroviruses from other retroelements (Kim et al., 2004) and is responsible of the recognition and initial interaction with the host cell. The expression of the viral protease (PR), necessary to cleave the polyproteins into mature proteins during the virion maturation process, follows a different scheme in different viruses.

1.1.2 Classification of retroviruses

Members of the family of retroviruses are categorized in two subfamilies according to their complexity: the orthoretrovirinae, including the genera alpha-, beta-, gamma-, delta- and epsilonviruses, as well as lentiviruses, and the spumavirinae, integrated by the single genus of spumaviruses (Table 1). The human and simian immunodeficiency viruses (HIV and SIV, respectively) are some of the most characteristic representatives of the lentiviruses genus which is characterized by their slow progression to disease (Lentus, Latin for "slow").

The integration of retroviral genomic sequences into the genome of cells of the germ line originated the so called endogenous retroviruses or inheritable retroviral elements (Escalera-Zamudio and Greenwood, 2016; Weiss, 2016). It is considered that 5-8 % of the genes in human genome derive from retroviral elements (Belshaw et al., 2004;

Lander et al., 2001; Seifarth et al., 2005). Retroviral element derived-genes are involved in important physiological processes not only in humans but also in most vertebrate species like placenta formation, brain cognitive functions, immunity against retroelements and some fundamental biological processes such as cell proliferation, apoptosis and cancer (Naville et al., 2016). Upon infection exogenous can recombine with endogenous retroviral elements resulting in the activation of the latter, as observed for murine and feline gammaretroviruses (Rosenberg and Jolicoeur, 1997) or HIV (van der Kuyl, 2012; Vincendeau et al., 2015). The same has been described for human herpesviruses (HHVs) such as human cytomegalovirus (HCMV) (Assinger et al., 2013) or Epstein-Barr virus (EBV) (Brudek et al., 2008; Sutkowski et al., 2001).

Subfamily	Genus	Example
Orthoretrovirinae	Alpharetrovirus	Avian Leukosis Virus (ALV); Rous Sarcoma Virus (RDV); Moloney Murine Leukaemia Virus (MMLV)
	Betaretrovirus	Mouse Mammary Tumor Virus (MMTV); Mason-Pfizer Monkey Virus (MPMV)
	Gammaretrovirus	Murine Leukaemia Virus (MLV); Feline Leukaemia Virus (FLV)
	Deltaretrovirus	Human T-cell Lymphotrophic Virus-1 and 2 (HTLV-1, -2)
	Epsilonretrovirus	Walleye Dermal Sarcoma Virus (WDSV)
	Lentivirus	HIV; SIV; Feline Immunodeficiency Virus (FIV)
Spumavirinae	Spumavirus	Simian Foamy Virus (SFV); Human Foamy Virus (HFV)

Table 1.1: Classification of exogenous retroviruses.

1.2 HIV and Acquired Immunodeficiency Syndrome (AIDS)

In the early 1980s a list of opportunistic infections was described for groups of homosexual and intravenous-drug user patients (CDC, 1981; Gottlieb et al., 1981; Masur et al., 1981). This included *Pneumocystis Carinii* pneumonia, mucosal candidiasis and Kaposi's Sarkoma, a rare type of cancer caused by human herpesvirus-8 (HHV-8), and it was coupled to diminished total lymphocyte counts or lymphopenia and a reversion in the ratio of helper and cytotoxic T cells. The term AIDS was used to refer to the newly characterized clinical picture. Soon after, blood transfusion recipients were defined as a third risk group. With epidemic spread, AIDS became a major public health problem and affected people beyond the initially defined risk groups. Two years were needed until the causative agent of AIDS, a retrovirus later designated HIV, was discovered (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Popovic et al., 1984). The authorship of this discovery remained subjected to a very polemic discussion. The discovery was awarded with the Nobel Prize in 2008 to the French researchers F. Barre-Sinoussi and L. Montagnier, first and last authors of the original article published in 1983.

HIV infection and AIDS represents one of the largest and most lethal pandemics in history having claimed more than 34 million lives so far (WHO, 2015). By the end of 2014 there were 36.9 million people living with HIV around the globe. Among those, 2

million were newly infected in the course of that year. Access to antiretroviral therapy (ART) (See section 1.2.5) is increasing and 15.8 million people were under treatment as of June 2015, up from 13.6 million in June 2014 (UNAIDS, 2016). This resulted in a reduction in the number of people dying from AIDS-related illnesses from 2 million in 2005 to 1.2 million in 2014, a 42% drop since the peak in 2004. Despite of the optimistic data, lack of access to ART is still a problem affecting millions of people especially in developing countries. The most affected area of the globe is Sub-Saharan Africa where 25.8 million people were living with HIV in 2014. This region also accounts for 70% of the total of new infection (WHO, 2015). Probably the most preoccupying piece of information is that worldwide only 54% of people infected by HIV know their status (UNAIDS, 2016; WHO, 2015).

1.2.1 Classification

HIV is the result of a zoonosis. It originated from simian immunodeficiency viruses (SIV) as a result of several cross-species transmissions (Sharp and Hahn, 2011). Most of those events resulted in viral strains that spread in humans to a certain limit. However, one of them occurring from chimpanzee subspecies *Pan troglodytes troglodytes* generated group M of HIV-1 (for "major"), which accounts for most of the infections worldwide (Gao et al., 1999; Keele et al., 2006; Sharp and Hahn, 2011; Van Heuverswyn and Peeters, 2007). Another two transmissions gave rise to the other two described groups of HIV-1: group N (for "no M, no O") , transmitted from the same Chimpanzee subspecies as above (Gao et al., 1999; Keele et al., 2006; Sharp and Hahn, 2011; Van Heuverswyn and Peeters, 2007), and group O (for "outlier", transmitted from Gorilla (Sharp and Hahn, 2011; Takehisa et al., 2009; Van Heuverswyn et al., 2006; Van Heuverswyn and Peeters, 2007). Group M can be further subdivided in nine subtypes or clades (A-K). The genetic variability within clades ranges between 15-20% whereas between clades the range is >30% (Hemelaar et al., 2006; Korber et al., 2001; Luft et al., 2011). The most prevalent clade is C which accounts for 52% of the infections and is predominant in Southern and Eastern Africa and India. 12% prevalence corresponds to clade A which is mostly found in West Africa and Eastern Europe. Clade B with 10% prevalence is mainly distributed in Central and Western Europe, America and Australia (Hemelaar et al., 2006; Killian and Levy, 2011; Luft et al., 2011). Furthermore, over 45 circulating recombinant forms of HIV-1 have also been described to date (Luft et al., 2011). A second type of HIV, HIV-2, resulted from eight independent transmission events from monkey of the species sooty mangabey (*Cercocebus atys*) that generated the described groups A-H (Gao et al., 1992; Hirsch et al., 1989; Lemey et al., 2003).

1.2.2 HIV-1 Genome and structure

The HIV-1 genome spans approximately 10 Kbp and an infectious virion consists of two copies of positive single-stranded RNA comprising 9 open reading frames (ORFs) flanked by long terminal repetitions (LTRs). The ORFs encode 9 different proteins including the 3 characteristic retroviral polyproteins: Gag, Pol and Env (Figure 1.1A). Proteolysis of the latter 3 generates a total of 15 viral proteins, most of which form the

125 nm-sized HIV-1 particles (Figure 1.1B and C) (Briggs et al., 2006). Viral assembly is largely driven by Gag and its isolated expression results in the release of noninfectious virus-like particles (VLPs) (Ganser-Pornillos et al., 2008; Schur et al., 2015). PR-mediated cleavage of the multi-domain polyprotein Gag generates the structural HIV proteins MA, CA, NC and the small p6 protein (Sundquist and Krausslich, 2012). MA interacts with phospholipids to form a layer underneath the viral envelope, an essential phenomenon for the recruitment of Gag to budding sites (Briggs et al., 2009). The mature cone-shaped HIV-1 core is formed by roughly 250 CA hexamers and 12 pentamers distributed at each end of the cone, required for closure of the curved hexagonal lattice (Figure 1.1D) (Briggs and Krausslich, 2011; Ganser et al., 1999; Li et al., 2000). This structure contains NC-coated genome (Briggs and Krausslich, 2011). P6 is required for the correct budding process and release of nascent virions facilitating the recruitment members of the endosomal sorting complexes required for transport (ESCRT) (Friedrich et al., 2016; Gottlinger et al., 1991). As a consequence of a ribosomal frame-shift with a frequency of ~5% the fusion protein Gag-Pol is synthesized (Biswas et al., 2004), PR-mediated processing of which results in the 3 viral enzymes PR, RT and IN. The first of them mediate proteolytic maturation of the Gag and Pol polyproteins. RT and IN are required for conversion of the viral RNA into double-stranded DNA and its integration in the host cell genome, respectively (Craigie and Bushman, 2012; Hu and Hughes, 2012). Finally, the Env polyprotein is cleaved by the host protease furin at the Golgi apparatus to originate glycoprotein (gp) 120 and gp41 (Hallenberger et al., 1992). Both proteins form heterodimers which are located at the viral envelope, being gp120 exposed at its surface and gp41 spaning the lipid bilayer. This heterodimers in turn trimerize to form the so called HIV spikes.

Besides the structural proteins, the HIV-1 genome encodes also 2 regulatory proteins, Tat and Rev (Cullen, 1991), and four accessory proteins, Vif, Vpr, Vpu and Nef (Figure 1.1A) (for detailed information see 1.2.3 and 1.3, respectively). The expression of accessory proteins is characteristic of the complex retroviruses HIV and SIV that distinguishes them from other viruses of the same family. Initially thought to be dispensable for viral replication, as concluded from *in vitro* observations, they have been proven necessary for efficient infection *in vivo*. Critical roles have been described for these proteins not only as counteractors of host innate and adaptive antiviral responses but are also thought to favor viral pathogenesis by targeting bystander cells (see also 1.3) (Malim and Emerman, 2008; Seelamgari et al., 2004).

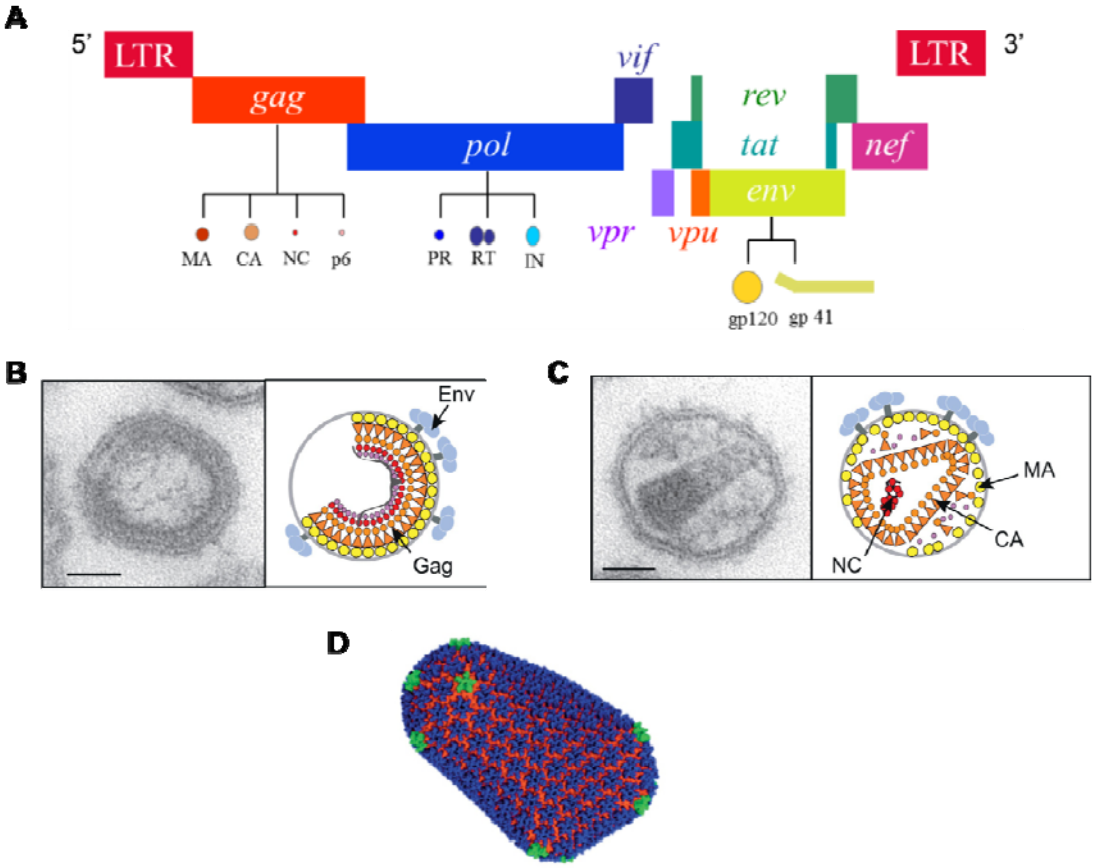

Figure 1.1 HIV-1 Genome and structure. (A) Representation of the HIV-1 genome. The two copies of plus single-stranded RNA comprise the typical retroviral structural genes (*gag*, *pol*, *env*), four accessory genes (*vif*, *vpr*, *vpu*, *nef*) and two regulatory genes (*tat*, *rev*) organized in 9 ORFs and flanked by the LTRs. Processing of the gene products results in a total of 15 different proteins. (B) Electron microgram (left) and schematic cartoon (right) of an immature virion structure. (C) Electron microgram (left) and schematic cartoon (right) of a mature virion structure. Maturation implies virion protein reorganization within the viral particle following proteolysis. Protease activation is therefore essential for maturation. (D) Schematic cartoon of the conical capsid core of a mature virion. (A) (Fackler and Geyer, 2002) (B),(C) (Konvalinka et al., 2015) (D) (Freed, 2015).

1.2.3 Replication cycle of HIV-1

The first phase of viral replication cycle is the entry, which begins with the adhesion of the virion to the host cell and ends with the fusion of the cellular and viral membranes with the release of the virion content into the host cell (Figure 1.2). A first contact between the virion and the host cell can result from unspecific interactions like that of Env with negatively-charged heparan sulphates proteoglycans (Saphire et al., 2001) or be the consequence of more specific interactions like that of Env with α4β7 integrin (Arthos et al., 2008; Cicala et al., 2009) or with pathogen associated molecular patterns (PAMP) receptors such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Geijtenbeek et al., 2000). All these interactions mediated by attachment factors which are not indispensable for HIV-1 pathogenesis increase infection efficiency by bringing Env to the vicinity of the primary viral receptor CD4 and coreceptor (Orloff et al., 1991). The close proximity favors the

interaction of both proteins in which the variable loop V3 of Env plays a critical role and it leads to several conformational changes in Env needed for the subsequent binding to the coreceptor (Chen et al., 2005; Hartley et al., 2005; Kwong et al., 1998). CCR5 and CXCR4 are the two coreceptors of HIV-1 and the viral strains can be classified according to their coreceptor usage as R5 or X4 tropic viruses. There are viral strains which can use both correceptors and are therefore designed as dual or X4R5 tropic (Berger, 1998; Berger et al., 1998). Upon interacting with the coreceptor, the hydrophobic gp41 subunit of Env is exposed and gets inserted in the cell host membrane. After a series of conformational changes, this leads eventually to the formation of a fusion pore through which the viral conical core is delivered into the host cell cytoplasm (Melikyan, 2008). Despite the lack of dependence on low pH (Stein et al., 1987), it is still a matter of discussion whether internalization of the virion-receptor complexes is needed for fusion to occur as opposite to fusion at the cell surface (Daecke et al., 2005; Fackler and Peterlin, 2000; Herold et al., 2014; Miyauchi et al., 2009). It is becoming increasingly clear that both take place and that it depends on the cell type and virus type as well as the disease stage.

Following fusion, the CA conical core structure of HIV-1 is released into the cytoplasm and subsequently disassembled releasing its content, a process known as uncoating (Figure 1.2). The mechanism as well as the precise location or timing of uncoating is not clear yet but it is thought to be a progressive multistep process and to be critical for further post-entry steps, i.e. reverse transcription and nuclear import (Dismuke and Aiken, 2006; Forshey et al., 2002). A partial disassembly of the viral core is enough for RT to start reverse transcribing the viral RNA into DNA (Peng et al., 2014). RT synthesizes dsDNA using the viral RNA genome as a template with an error rate of 1 x 10^5, which account for one error per replication cycle (Abram et al., 2010) The protein complex formed by the disassembling core, the viral RNA genome, RT, the nascent DNA and other viral as well as cellular factors is known as the reverse transcription complex (RTC) (Peng et al., 2014). This complex evolves into the preintegration complex (PIC) as reverse transcription is completed and it is translocated into the nucleus through the nuclear pore. This process is dependent on the viral CA and involves also proteins of the nuclear pore complex such as NUP358 (Schaller et al., 2011).

Once in the nucleus, the DNA is integrated in the cell host genome by the IN, a hallmark of retroviral infection. Integration takes place at the periphery of the nucleus and in the vicinity of a nuclear pore preferentially within actively transcribed chromatin (Marini et al., 2015; Schroder et al., 2002). In addition to IN, members of the NPC and other host factors such as LEDGF/p75 are required for this process (Koh et al., 2013; Marshall et al., 2007; Matreyek et al., 2013; Ocwieja et al., 2011; Shun et al., 2007). Unsuccessful integration results in the formation of defective LTR circles (Farnet and Haseltine, 1991). Two critical aminoacid residues involved in binding to the target DNA have been described for IN and mutations of those have been correlated to disease progression (Demeulemeester et al., 2014).

After integration the provirus can enter latency (see 1.2.5) or undergo gene expression. The latter relies on the cellular transcription machinery and it is regulated by the viral factor Tat, which is expressed early. Activation-dependent host cell transcription factors NF-κB and NFAT are translocated into the nucleus and recruited to the viral promoter at the LTR (Nabel and Baltimore, 1987). The regulatory protein Tat binds to the TAR (transactivation responsive region) RNA element present at the HIV-1 leader sequence. This interaction leads to the recruitment of P-TEFb and other cellular elongation factor to the transcription complex increasing the efficiency of the process (Karn and Stoltzfus, 2012; Laspia et al., 1989). Viral genome transcription follows a complex gene expression pattern. Early completely spliced mRNAs encoding Tat, Rev and Nef are expressed in a short time interval following infection. Larger, incompletely spliced mRNAs which encode for Env and the accessory proteins Vif, Vpr and Vpu and full-length, unspliced encoding for the polyprotein Gag-Pol also acting as the virion genomic RNA are transcribed only late after that (Karn and Stoltzfus, 2012). The unspliced mRNA which will constitute the virion genome and the incompletely spliced mRNA which encodes for the structural proteins is transported out of the nucleus in a Rev-dependent manner, to avoid the otherwise typical degradation of intron-containing transcripts (Sodroski et al., 1986). This requires a threshold level of the Rev protein (Pomerantz et al., 1992). Rev interacts with an elongated stem-loop RNA structure of the *env* gene termed Rev-responsive element (RRE) (Malim et al., 1989).

Once in the cytoplasm, the viral transcripts are translated by the cellular machinery generating the viral proteins (Figure 1.2). The newly synthesized proteins localize then to specialized microdomains at the plasma membrane where assembly of the new virion progeny occurs (Sundquist and Krausslich, 2012). The Gag protein itself is able to mediate all steps of the virion assembly process. Its molecules arrange themselves underneath the plasma membrane inducing its curvature (Gottlinger, 2001). The amino-terminal domain MA is responsible of binding to the plasma membrane and to recruit the Env protein, trimers of which form HIV-1spikes (Tang et al., 2004). The central domain CA mediates protein-protein interactions that stabilize the immature Gag lattice during assembly. Gag-Pol molecules are also recruited to the assembly site. The basic NC domain of Gag interacts with the genomic RNA via its two zinc finger motifs recruiting it to the assembly site (Sundquist and Krausslich, 2012). Finally the carboxy-terminal domain p6 recruits several other viral factors. It also interacts with proteins from the ESCRT pathway namely Tsg101 and ALIX, which the virus hijacks to achieve budding of the viral particles (Bieniasz, 2006; Pornillos et al., 2003). During the budding process the viral PR gets activated, a key event for the subsequent maturation of the immature, non infectious released virions. Cleavage of Gag and Gag-Pol results in a reorganization of the internal protein structure and the formation of the conical core typical of fully mature, infectious virions (Briggs and Krausslich, 2011; Briggs et al., 2009; Schur et al., 2015).

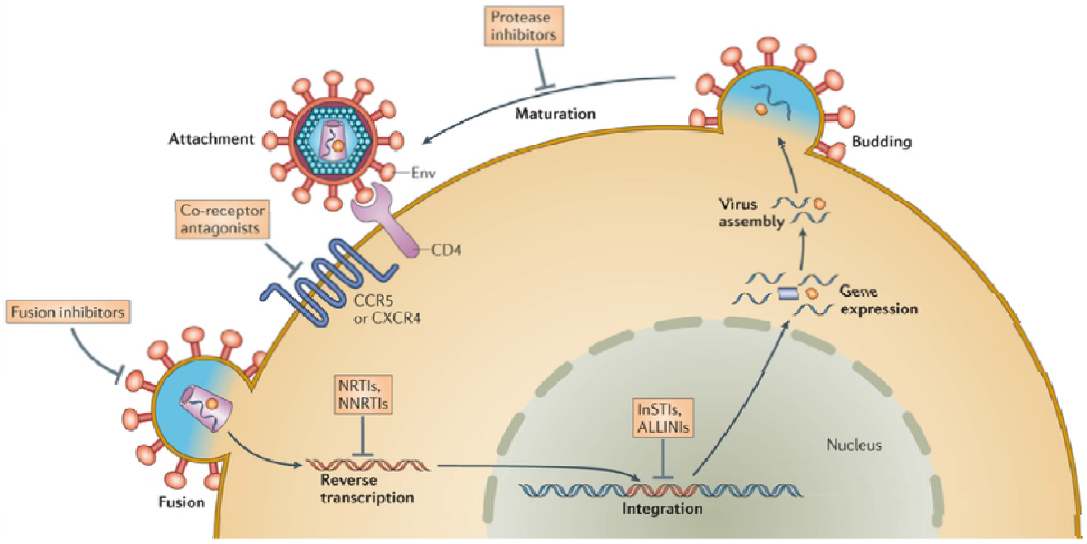

Figure 1.2 HIV replication cycle. The scheme represents the main steps of viral replication and the stages at which antiretroviral drugs block it. (Laskey and Siliciano, 2014).

The dynamics of the early steps of HIV-1 replication are cell type dependent and the differences in the published estimations may be due to variation in receptor and intracellular dNTP pool. Viral entry takes place <1-3 hours after exposure of cells to the virus (Fernandez-Larsson et al., 1992; Platt et al., 2005; Reeves et al., 2002), reverse transcription occurs in the following 6-48 hour (Collin and Gordon, 1994; Donahue et al., 2010; Kim et al., 2012; Murray et al., 2011; O'Brien et al., 1994) and integration takes place 5 hours after completion of reverse transcription (Donahue et al., 2010; Murray et al., 2011). Data derived from time-of-addition experiments show resistance to transcription inhibitors is acquired ~35 hours after infection (Lalonde et al., 2011; Tomaras et al., 2000). The kinetics of the late stages of HIV-1 replication cycle are poorly understood as compared to those of the early stages. This is mostly due to the inherent asynchrony in populations of infected cells. However, published data suggest a temporal gap of ~11 hours between integration and transcription of early genes, a smaller delay between early and late gene expression (~3h) and the need for another 6-12h between late gene expression and new virions release (Holmes et al., 2015). The time required for a complete viral life cycle is estimated to be 48 hours and it interestingly matches to the lifespan of an infected cell *in vivo* (Dixit et al., 2004).

1.2.4 Means of transmission and pathogenesis

HIV is transmitted by sexual, percutaneous and perinatal routes (Cohen et al., 2011; Hladik and McElrath, 2008). The infection is nevertheless predominantly acquired via sexual exposure. This type of transmission accounts for 80% of the infections among adult people (Cohen et al., 2011; Sharp and Hahn, 2011). The squamous stratified epithelium of the vagina, ectocervix, male foreskin and the columnar epithelium of the endocervix and rectum constitute the entry path of HIV. Despite of a lower probability of effective HIV transmission than anal intercourse, vaginal sex accounts globally for

most of the new HIV infections (Figure 1.3) (Hladik and McElrath, 2008). Nevertheless, the mucosa represents a high selective barrier to HIV transmission that results in an infection established by one single virus, the so called founder virus. CD4+ T cells are the main targets of infection by HIV. To a lesser extent the virus also infects cells involved in antigen presentation, including dendritic cells (DCs), monocytes and macrophages (Patterson et al., 1995; Stevenson, 2003; Vremec et al., 2000). All these cells express the virus receptor CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984) and CC-Chemokine receptor-5 (CCR5) or CXC-Chemokine receptor-4 (CXCR4), that act as entry co-receptors for HIV-1 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996; Lee et al., 1999; Patterson et al., 1995; Vremec et al., 2000). Interestingly, the founder virus presents R5 or X4R5 tropism in the great majority of the cases (Keele et al., 2008). There are mainly two differentiated mechanisms for viral spread: cell-free and cell-cell. Direct cell to cell spread takes place via the formation of intercellular contacts known as virological synapses and it has been shown to have a higher efficiency *in vitro* (Chen et al., 2007). Eventually and making use probably of both these mechanisms, the virus disseminates through the blood stream causing a systemic infection.

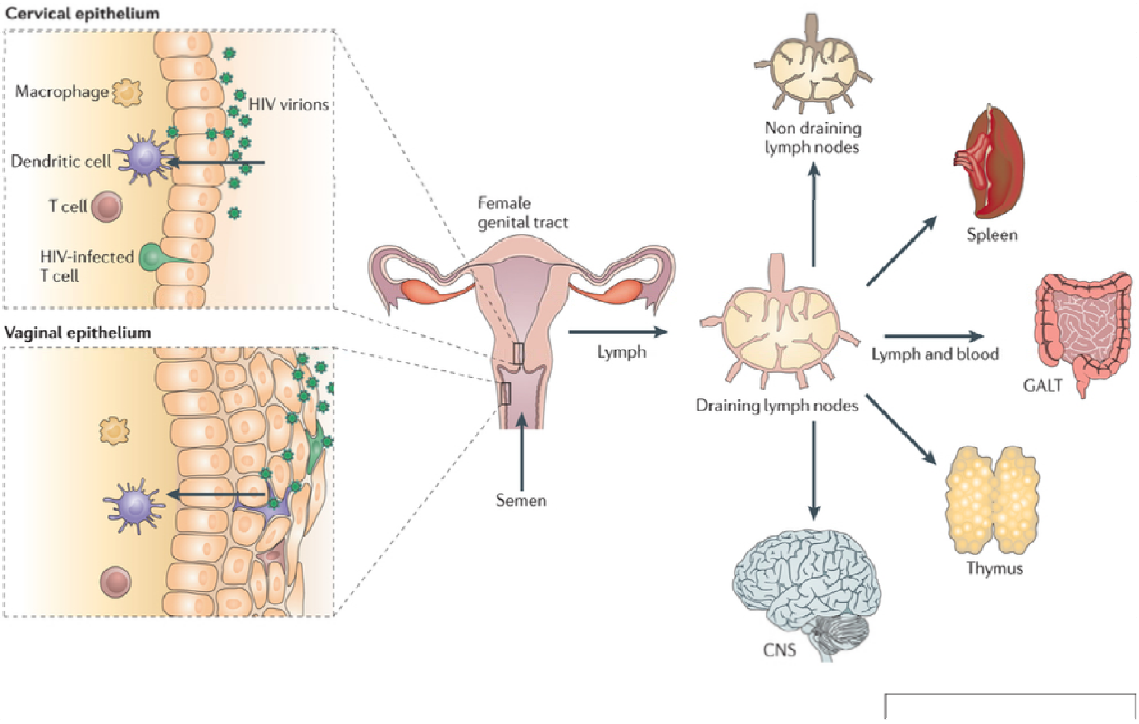

Figure 1.3 HIV-1 early transmission events and spread. In women, vaginal sex transmission requires crossing of the squamous stratified epithelium of the vagina or the ectocervix or of the columnar epithelium of the endocervix. After establishing the first infection in a yet undetermined cell type, the virus reaches the draining lymph node from which it disseminates via the bloodstream to the rest of the organism. CNS, central nervous system; GALT, gut-associated lymphoid tissue. (Fackler et al., 2014).

Many aspects about the first transmission events remain yet unclear however. It is not yet known how the virus crosses the genital or rectal mucosal tract to reach the first target cell. It also remains unclear which target cell is infected by HIV in the first place

and how. Another unanswered question in HIV pathogenesis is how the virus reaches the draining lymph node after local expansion at the mucosal tissue. DCs can also capture HIV virions and release them upon contact with CD4+ T cells resulting in the infection of the latter (Geijtenbeek et al., 2000; Izquierdo-Useros et al., 2014; Izquierdo-Useros et al., 2012; Kwon et al., 2002; McDonald et al., 2003). This phenomenon is termed *trans*-infection and relies on the interaction of the virions with the sialic acid binding molecule Siglec-1 (Izquierdo-Useros et al., 2014; Izquierdo-Useros et al., 2012). Whether it is a DC carrier cell or a migrating infected CD4+ T cell or macrophage that brings the virus to the lymphoid tissue, the outcome of this event is the efficient amplification of the infection due to the high target cell concentration.

The course of HIV infection presents three stages (Figure 1.4). The first of them known as acute phase spans approximately 2 to 4 weeks after a person acquires the virus. Following transmission of the founder virus, HIV replication rapidly increases. Flu-like clinical manifestations are frequent during this phase including fever, headache and rash as a consequence of the innate immune response mounted with prominent induction of proinflammatory cytokines and chemokines. Viral load is high since viruses are spreading throughout the body and massive CD4+ T cell depletion occurs. Transmission of HIV can take place at any stage but the risk peaks at the acute infection phase. Typically between 1 and 3 weeks later the asymptomatic or chronic phase begins. As a response of the antiviral immune response, viral load decreases and the extent in this drop constitutes an important predictor factor for disease progression (Figure 1.4). During this stage HIV replication continues albeit at low levels. In parallel, immune activation drops although not reaching pre-exposure levels. This is maintained during the course of infection, establishing chronic immune activation. Simultaneously, a slow and progressive loss of CD4+ T cells takes place (Figure 1.4). In the absence of treatment, progression to the final phase, AIDS, occurs after 10 years in average. Diagnosis of AIDS is made when a person has CD4+ T lymphocyte counts lower than 200 cell/mm3 and/or development of one or more AIDS defining illnesses. The latter include opportunistic infections and rare malignancies such as candidiasis, cytomegalovirus infections, Kaposi sarcoma, lymphomas, neuro-AIDS (a form of dementia in which HIV-infected microglia is dysfunctional), pneumocystis pneumonia and tuberculosis. Without treatment an AIDS-diagnosed person typically survives around 2 years. In some exceptional cases the progression rate to AIDS varies from the characteristic disease course exposed above. This is the case of rapid progressors, for which the chronic phase lasts about 2 to 3 years, and the long-term-non-progressors (LTNP), which are able to control viremia and maintain CD4+ T cell levels high in the absence of ART (Kumar, 2013).

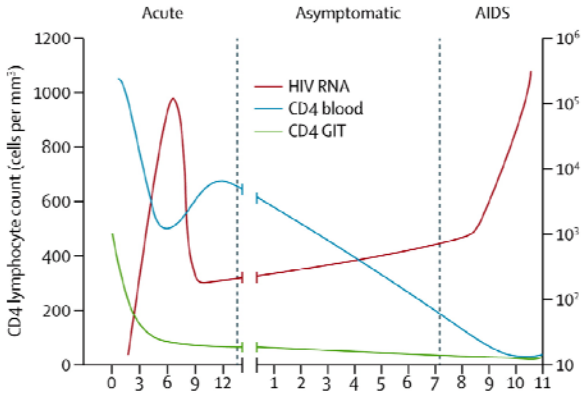

Figure 1.4 Clinical course of HIV-1 infection in the absence of antiretroviral treatment. Following transmission and during the acute phase, the viral load, or viral RNA copies in plasma, increase rapidly and then drop as a result of the patient's immune response to the so called set point. From then on and lasting years, viral load increases in parallel to the loss of CD4+ T cells during the asymptomatic phase, a phenomenon specially marked in the gastrointestinal tract (GIT), until CD4+ T cell counts reach 200 which is the onset of AIDS. (Maartens et al., 2014).

1.2.5 Immune response against HIV-1

The immune system establishes responses against HIV-1 early after transmission which are maintained and develop during the course of the disease. The following provides some information of an investigation field of increasing relevance for HIV researchers and retrovirologists in general.

1.2.5.1 Innate immunity

The innate immunity accounts for the early, unspecific response to HIV-1 when adaptive immunity has not been mounted yet. Within minutes, the immune system is able to sense viral components and from other pathogens through pattern recognition receptors (PRRs), such as Toll like-receptors (TLRs), and PAMP receptors (Altfeld and Gale, 2015). Additionally, DNA sensors like gamma-interferon-inducible protein-16 (IFI16) and cyclic GMP-AMP synthase (cGAS) contribute to HIV recognition at early stages (Gao et al., 2013; Jakobsen et al., 2013a; Sauter and Kirchhoff, 2016). These sensors generally activate signaling cascades leading to induction of interferon and proinflammatory cytokines and chemokines release, which in the HIV-1 infection scenario is termed "cytokine storm" due to the magnitude it reaches (Stacey et al., 2009). As a consequence, an antiviral state is established by the expression of interferon induced genes (ISDs) and innate immune cells are recruited, respectively. The cytokine storm is secreted by natural killer (NK) cells, NKT cells, plasmacytoid dendritic cells (pDCs), monocytes, macrophages and $CD4^+$ T cells.

1.2.5.2 Intrinsic immunity

HIV-1 specific restriction factors were typically categorized as part of the innate immunity. Unlike the prototypical innate immune players which induce an antiviral state through indirect mechanisms, restriction factors rather limit replication and spread in a direct, efficient way. For that reason they are now suggested to form an independent

immune strategy against infection, the intrinsic immunity (Simon et al., 2015). The mediated block occurs throughout the HIV-1 replication cycle (Fackler, 2015). Individual steps restricted include early post-entry, where TRIM5α and Mx10 recognize and destabilize capsid cores (Kane et al., 2013; Stremlau et al., 2004), reverse transcription, interfered by apolipoprotein B mRNA editing enzyme, catalytic peptide-like 3G (APOBEC3G) and SAM domain- and HD domain- containing protein (SAMHD-1) (Baldauf et al., 2012; Bishop et al., 2008; Hrecka et al., 2011; Laguette et al., 2011; Mangeat et al., 2003), virion production, where Schlaffen 11 restricts translation of viral mRNA (Jakobsen et al., 2013b; Li et al., 2012), virion release, blocked by tetherin (see) (Neil et al., 2008; Perez-Caballero et al., 2009) and infectivity of release viral particles, affected by serine incorporator 3/5 (SERINC3/5) (see) (Rosa et al., 2015; Usami et al., 2015). To overcome these host cell barriers, the virus has developed antagonists which establish a dose-dependent response against them. The accessory proteins of HIV-1 often act as counteractors of the restriction imposed by these molecules which typically involves direct interaction. In some cases, no counteraction has been yet found despite of extensive investigation. This is the case of TRIM5α, which is able to bind to incoming viral capsids and hamper the uncoating process and for which no antagonism by an accessory protein has been described to date and only mutations in the Capsid protein induce escape (Stremlau et al., 2004).

1.2.5.3 Adaptive immunity

After the initial unspecific response, a more specific and efficient one based on antigen-recognition is established. This type of immunity presents the advantage of memory acquisition what makes it able to mount faster and stronger responses to the same antigen in the future. Memory development constitutes the foundation for vaccine development. The adaptive immune response comprises two interrelated strategies: the humoral response, or antibody production, and the cellular response. CD4+ T cells are necessary for the correct functioning of both components of the adaptive immunity and therefore the systematic depletion and impaired functionality of these cells developed upon HIV-1 infection results in a compromised performance (Iyasere et al., 2003; Kaufmann et al., 2007).

Cytotoxic T lymphocytes (CTLs) are the major mediators of the adaptive cellular response and HIV-1-specific subsets are involved in the decline of viremia in plasma observed at the acute phase of infection (Koup et al., 1994). The selection pressure imposed by the CTL response leads at this stage to the establishment of a population of viruses known as the quasispecies, leading to escape to founder virus-specific CTLs (Keele et al., 2008; Koup, 1994). A proof of the relevance of CTL response in HIV-1 infection is provided by the viral control observed for LNTP presenting some particular MHC-I alleles (Allen et al., 2005). HIV-1 has developed evasion mechanisms against recognition and lysis consequence of the CTL response which include the accessory proteins Nef and Vpu (Collins et al., 1998; Matusali et al., 2012; Schwartz et al., 1996; Shah et al., 2010).

The humoral response is mediated by antibody producer cells or B cells. Despite of not being natural targets of HIV-1, these cells are dysfunctional in infected individuals and only small subpopulations are HIV-1-specific (Eriksson et al., 1995; Moir et al., 2001). While these subjects show increased antibody production or hypergammaglobulinaemia, the produced antibodies are polyclonal and present low affinity (Shirai et al., 1992). Broadly neutralizing antibodies (bNAbs) targeting several HIV-1 strains and quasispecies can be found in about 20% of the HIV-1 infected patients (Mikell et al., 2011); however they are effective only in 1% of them, the so called elite controllers (Mouquet, 2014).

1.2.6 Therapy and challenges towards the cure of HIV-1 infection

Treatment of HIV infection with antiretroviral therapy (ART) can reduce HIV-associated morbidity, prolong survival and prevent HIV transmission (Antiretroviral_Therapy_Cohort_Collaboration, 2008; Palella et al., 1998; Vittinghoff et al., 1999). Sustained suppression of plasma viremia mediated by ART restores and preserves immune function with a certain recovery in CD4 T cell levels and may reduce also the chronic immune activation observed in HIV infected patients. Furthermore, it delays or prevents the development of drug-resistance mutations (O'Brien et al., 1996; Yamashita et al., 2001). Monitoring CD4 T cell counts and viral load in plasma is critical to identify ART response.

Since the approval in 1987 of zidovudin (AZT), the first anti-HIV drug, more than 25 antiretroviral agents belonging to six different mechanistic classes or families have been approved for the treatment of HIV infection (Table 1.2). The first drugs developed and approved targeted the viral RT. Due to the lack of proofreading activity of this enzyme and the subsequent high mutation rate but also to HIV's high viral turnover and frequency of recombination, selective pressure is established among the viral populations present in a patient to escape the effect of the therapy (Santoro and Perno, 2013). Drug resistance was the main cause of treatment failure until medicaments from two new families of antiretroviral drugs targeting the viral protease were approved in 1995 and 1996. Since then, ART has been administered as a combination of drugs of different families including typically two nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and a third drug from one of the other families: non-nucleoside/nucleotide reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), fusion inhibitors, entry inhibitors or integrase strand transfer inhibitors (INSTI). This combination ART (cART) is also known as high activity antiretroviral therapy (HAART) (Table 1.2). The list of antiretroviral approved drugs has not stopped growing. This constant development of new drugs is essential to maximize safety and tolerability while maintaining or improving clinical efficacy. Thanks to this, typically associated ART side effects in the 1980s and 1990s such as lipodystrophy, strongly linked to the social stigma, are now a problem of the past. The current international (e.g. WHO, 2016; URL: http://www.who.int/hiv/pub/guidelines/earlyrelease-arv/en/) and regional guides (e.g. federally approved HIV/AIDS medical practice guidelines 2016; URL: https://aidsinfo.nih.gov/guidelines) recommend starting treatment following HIV

infection diagnosis regardless of the CD4 counts. This very recent update responds to the results obtained from the international study START (short for "Strategic Timing of Antiretroviral Treatment") (Lundgren et al., 2015) and the study TEMPRANO conducted in Ivory Coast (Danel et al., 2015). Both studies provide the highest levels of evidence that early HIV treatment is associated with reduced frequency of primary outcomes (any serious adverse event -AIDS related or non-AIDS related- or dead). Still today, access to ART remains an important issue affecting ~56% of HIV infected population, especially in low-and middle-income countries.

Drug Family	Mechanism of Action	FDA Approved Drugs
Nucleoside/nucleotide RT inhibitors (NRTI)	Nucleoside/nucleotide analogues lacking a 3'OH group incorporated into the viral DNA. Reverse transcription stops the impossibility of further addition of nucleotides	abacavir, didanosine, emtricitabine, lamivudine, stavudine, zidovudine (AZT), tenofovir
Non-nucleoside RT inhibitors (NNRTI)	Despite structurally diverse, they bind to the same, unique site of RT close to active center inhibiting it	efavirenz, etravirine, nevirapine, rilpivirine
Protease inhibitors	Prevent cleavage of polyproteins	atazanavir, darunavir, fosamprenavir, indinavir, nelfinavir, ritonavir, tipranavir, saquinavir
Fusion inhibitors	Directly bind to gp41 and preventing conformational changes needed for fusion of cellular and viral membranes	enfuvirtide (T-20)
Entry inhibitors	Interaction between gp120 and CCR5 is blocked	maraviroc
Integrase inhibitors	Viral integrase is inhibited (3' processing of viral DNA and/or strand transfer), thereby preventing integration of viral DNA into the host cell genome	raltegravir, dolutegravir, elvitegravir

Table 1.2 Classification and pharmacological mechanism of antiretroviral drugs.

Despite of the efficacy of HAART to control HIV infection, discontinuation of it results in a rebound of the viral load. The reason is the generation of a pool of latently infected cells or reservoir which cannot be targeted by ART due to lack of replication. Similarly, these latently integrated viruses remain invisible to the immune system. The site of integration, interaction with cellular factors and, epigenetics play a pivotal role in the establishment of latency (Lusic and Giacca, 2015; Siliciano et al., 2003). Post-integration latency occurs in resting (central or translational) memory CD4 T cells (Chomont et al., 2009). The reservoir is established early during acute infection, likely within days following initial exposure (Chun et al., 1998a). Therefore, early initiation of HAART might contribute limiting reservoir size. These cells bearing the transcriptionally silent provirus are fully capable of generating infectious virions once they are reactivated by recall antigen or cytokines (Chun et al., 1998b). The frequency of latently infected resting CD4 T cells is 1 in 10^6 cells (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997). However, the decay rate of the reservoir is so slow (half-life of latently infected cells is 43.9 months) that it was estimated it would take 73 years for ART to clear it (Siliciano et al., 2003). It has been later reported the existence of latent, replication-competent proviruses in the reservoir not responding to PHA induction in viral

outgrowth assays. Based on this, eradication of the reservoir would be much more challenging than initially thought (Ho et al., 2013). Evidence for the contribution to the residual plasma viremia by other source than resting CD4 T cells in patients under ART has been provided (Nickle et al., 2003; Ruff et al., 2002). However, no conclusive data about the identity of it could be obtained so far.

Given the great obstacle latency represents towards HIV infection cure, eradicating the viral reservoir is now one of the priorities in this field (Katlama et al., 2013; Richman et al., 2009). The main strategy is to find a way to reactivate cells from the reservoir in patients under HAART. It is assumed that reversion of latency in these cells will lead to dead by the viral cytopathyc effects or due to recognition and killing by the patient's CTLs (Hoxie and June, 2012). Cytokines such as IL-2 and anti-CD3 antibodies have been tested with no success (Chun et al., 2015; Davey et al., 1999). Agonists of PKC for downstream activation of NF-κB (Brooks et al., 2003; Jiang and Dandekar, 2015) or inhibitors of histone deacetylase (HDAC), shown to interact with 5' LTR and induce latency (Jiang et al., 2007; Williams et al., 2006), have arose promising results in in vitro studies (Archin et al., 2009; Korin et al., 2002; Kulkosky et al., 2001; Lehrman et al., 2005; Williams et al., 2004; Ylisastigui et al., 2004). Interestingly, antigen-stimulation of patient cytotoxic lymphocytes (CTL) prior to viral reactivation from the latent reservoir is essential for efficient killing of HIV-1 infected cells *in vitro* (Shan et al., 2012). This suggests a potential need for boosting patient CTL response in order to deplete the HIV reservoir. Targeting latent provirus in cells from the reservoir for excision has also been attempted. Delivery specificity represents the main limitation in this case (Ebina et al., 2013; Hu et al., 2014).

The only case reported of HIV healing in history was that in 2009 of a patient with acute myeloid leukemia, the so called "Berlin patient" (Hutter et al., 2009). This patient received an allogeneic hematopoietic progenitor/stem cells transplant from a donor who was homozygotic for a 32 bp deletion in the *CCR5* allele which confers immunity against HIV infection (Dean et al., 1996; Liu et al., 1996). The CD4 counts of the patient recovered and the viral load remained undetectable following the transplant leading to discontinuation of HAART. No viral load rebound in plasma or in tissues was detected ever since and the size of the reservoir got reduced until becoming undetectable (Allers et al., 2011; Yukl et al., 2013). This case however has remained as unique. Similar attempts did not succeed due to viral tropism shift to X4 strain (Kordelas et al., 2014) or the patients died after the transplant (Passaes and Saez-Cirion, 2014). As opposing to a sterilizing cure as that of the "Berlin patient", a functional cure was reported for 14 patients from the so called VISCONTI cohort (for "VIrologic Sustained CONTrol after Treatment Interruption"). After a limited time of early treatment, they displayed undetectable viremia in plasma while still bearing latent provirus in their reservoir (Saez-Cirion et al., 2013). Other efforts included repression of viral gene expression by engineered zinc-finger transcription factors (Reynolds et al., 2003), genetic manipulation of CD4 T cells to achieve resistance to infection, mostly by modification or knock-down of *CCR5* (Didigu et al., 2014) or by transplantation of

autologous cells redirected to target HIV or manipulated genetically to express antiviral molecules such as bNAbs or nucleic acids (Focosi et al., 2015).

Development of a preventive vaccine is the main goal to achieve eradication given that, despite of the existence of effective therapies, more than half of the HIV-infected population worldwide does not have access to them. The discovery of HIV-1 specific bNAbs in LNTPs provided faith in the possibility of developing a vaccine which could induce its production. Before that all attempts to elicit protecting antibodies against Env, the only accessible protein on the viral surface, did not succeed due to the lack of broadly neutralizing capacity (Wang et al., 2015). Recent promising results derived form *in vivo* work on protection acquired upon passive transmission of bNAbs keep the hope on the potential of this strategy (Hessell et al., 2009; Pietzsch et al., 2012). A second direction towards the development of a vaccine aims at stimulating HIV-specific CTL response. A report showing this type of response in uninfected women highly exposed to HIV serves as a proof of principle supporting this idea (Rowland-Jones et al., 1995). Life-attenuated vaccines like MMR (measles, mumps, rubeola) are not pursued anymore due to safety concerns, despite of the induction of a potent immune cellular responses. As an alternative vaccines based on vectors such as canarypox or adenoassociated virus have and are currently being developed and they are able to elicit both cellular and humoral responses. The most promising results were obtained in a phase III clinical trial that combined two of these vaccines to achieve efficient delivery of *gag*, *protease* and *env* followed by two boost immunizations including *env* from another subtype. As a result, the HIV infection risk was reduced in a 31%. Interestingly, the successful results correlated with antibody-dependent cellular cytotoxicity rather than induction of neutralization.

1.3 Accessory proteins of HIV-1

Besides the prototypical lentiviral structural proteins Gag, Pol and Env and the regulatory proteins Tat and Rev, complex primate lentiviruses express so called accessory proteins with essential functions to ensure efficient viral replication, despite the misleading of their name. The terms accessory genes and proteins derive from initial reports describing that inactivation of these gene products *in vitro* had little or no impact on virus replication in continuous cell lines (Fan and Peden, 1992; Strebel et al., 1988). However, further research focusing on primary cells susceptible to HIV infection or on *in vivo* models dispelled the doubts concerning unneglectable relevance of the accessory proteins during the course of infection (Bour and Strebel, 2000). Primate lentiviral accessory proteins are Vif, Vpr, Nef and Vpu. While the first three are expressed by most HIV-1, HIV-2 and SIV strains, Vpu is only expressed by HIV-1 with the exception of SIVcpz and SIVgsn (Courgnaud et al., 2002; Huet et al., 1990). At each step of the viral cycle certain accessory proteins are expressed and, through the multiple functions exerted by them, these proteins contribute to achieve efficient viral infectivity (Nef and Vif), viral gene expression (Vpr) and optimal virion production (Vpu). Among others, one of the main functions attributed to lentiviral accessory proteins is to fine tune the interactions between the infected cells and the immune system facilitating

escape to innate and adaptive immune responses. Often, that is driven by the counteraction of host cell barriers known as restriction factors which trigger those innate responses (Simon et al., 2015). One of the first restriction factors described was APOBEC3G, a cytidine deaminase which elevates the mutation rate during reverse transcription hampering viral replication (Bishop et al., 2004; Sheehy et al., 2002). HIV-1 Vif promotes degradation of APOBEC3G and prevents its inclusion into nascent virions (Simon et al., 2015). Via counteraction of one or more unknown restriction factors, Vpr antagonizes restriction of Env expression in a macrophage-dependent manner (Collins et al., 2015; Mashiba et al., 2014). In the same cellular system, Vpr arrest cell cycle in G2 phase (Re and Luban, 1997). In addition, it activates the SLX4 endonuclease complex with subsequent clearance of exogenous DNA resulting in the suppression of IFN type I production and hampering antiviral response (Laguette et al., 2014). The following describes in more detail the accessory viral gene products Nef and Vpu as well as some of the cellular processes in which they interfere to enable optimal viral replication.

1.3.1 HIV-1 pathogenesis factor Nef

HIV-1 Nef is a 27-35 kDa myristoylated accessory protein synthesized early in the viral cycle by HIV-1, HIV-2 and SIV. The *nef* coding sequence appears at the 3' end of the viral genome where it overlaps partially with the 3' LTR. Despite of its low concentration and inactivation by the viral PR within the virions (Pandori et al., 1996; Welker et al., 1996), Nef is active at early infection stages and it has been shown to be transcribed and expressed even prior to integration of the provirus (Sloan et al., 2011; Wu and Marsh, 2003). Nef was initially described to have a negative impact on viral replication and transcription and thereby to be involved in establishment of latency, hence its name which stands for "negative factor" (Ahmad and Venkatesan, 1988; Cheng-Mayer et al., 1989; Luciw et al., 1987; Terwilliger et al., 1986). Results from other researchers however refuted these findings soon thereafter (Hammes et al., 1989; Kim et al., 1989). Also, *in vivo* studies showed how macaques infected with SIV harboring insertions or deletions in the *nef* gene abolishing expression of the protein led to a reduction in the viral pathogenicity and slower or no progression to immunodeficiency (Kestler et al., 1991; Whatmore et al., 1995). Furthermore, selective pressure established in the infected monkeys resulted in the reversion of the introduced mutations underlining the importance of the viral product Nef. There are also evidences of the importance of Nef for HIV pathogenesis and progression to AIDS in humans. The study of a cohort of LTNP patients who received blood or blood products transfusion from the same LTNP donor infected by an HIV-1 bearing deletions in the *nef* ORF and absence of other changes in the viral genome is one example of it (Deacon et al., 1995; Kirchhoff et al., 1995). Despite of having all remained asymptomatic during 10 to 14 years after sero-conversion, some of these patients have eventually developed AIDS 20 years after sero-conversion (Gorry et al., 2007), linking expression of the viral factor to high virulence and rapid disease progression while no absolute requirement of an intact *nef* ORF for the development of AIDS can be concluded. Adding up to these observations, studies using a transgenic mice model showed AIDS-like disease

development and CD4 T lymphocyte depletion upon isolated Nef expression by CD4 T cells and cells of the macrophage/dendritic lineages (Hanna et al., 1998). Altogether, the previous studies demonstrate the critical role Nef plays as a pathogenicity factor during infection. While the direct mechanistic fundaments for that remain unclear, multiple Nef functions have been described *in vitro* which might contribute to the pathogenic outcome mediated by the accessory protein, namely interference with host cell vesicular transport, signal transduction and enhancement of virion infectivity (explained more in detail below). In the absence of enzymatic activity, Nef exerts all the above functions via specific protein-protein interactions with a large array of host cell factors. Motifs involved in many of these interactions have been described and they are present throughout the molecule. However, which of those motifs are relevant *in vivo* remains a conundrum. Crystal (Arold et al., 1997; Grzesiek et al., 1997; Lee et al., 1996) and NMR (Grzesiek et al., 1996a; Grzesiek et al., 1997) studies showed that Nef's structure consists of a membrane anchor, a globular core and two highly flexible loops (Figure 1.5). The N-terminal domain of Nef contains an SH4 domain (Geyer et al., 2001). This domain consists of a glycine in position 2 as part of the N-myristoylation consensus sequence MGxxx(S/T) with a tendency to find a lysine or arginine at positions 7 and/or 8 (Resh, 1999). Nef myristoylation and subsequent interaction with cellular membranes is critical for all Nef described activities (Fackler et al., 2006; Giese et al., 2006; Guy et al., 1990; Kaminchik et al., 1994; Yu and Felsted, 1992). Residues from the two flexible loops are involved in transient interactions with proteins of the trafficking machinery relevant for downregulation of cell surface receptors such as CD4 and MHC-I (Atkins et al., 2008; Craig et al., 1998; Dikeakos et al., 2012; Greenberg et al., 1998; Lindwasser et al., 2007; Piguet et al., 2000; Ren et al., 2014; Rhee and Marsh, 1994). Interactions between Nef and signaling proteins containing SH3 domains or with p21-activated kinase (PAK) rely on residues of the central core (Geyer et al., 2001; Lee et al., 1996; Nunn and Marsh, 1996; Renkema et al., 1999; Sawai et al., 1994).

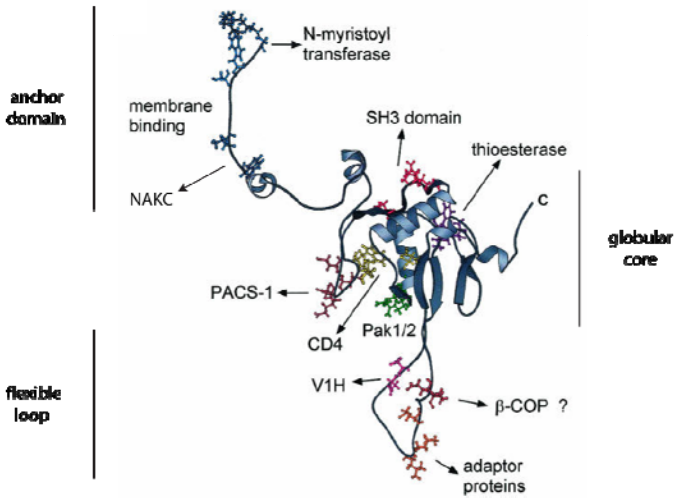

Figure 1.5 Structure of HIV-1 Nef. Structural representation of full-length Nef is derived from a NMR structure assemble. Three main domains can be distinguished (N-t membrane anchoring domain, central core domain and C-t disordered loop) and functional motifs and interaction partners are indicated. Adapted from (Geyer et al., 2001).

1.3.1.1 Enhancement of viral infectivity and replication

Nef has been shown to induce a 3- to 10-fold increase in single-round infectivity assays when viruses containing the intact *nef* gene sequence were compared to viruses with disrupted *nef* genes including a Nef-deficient virus (ΔNef virus). This activity is dependent on Nef's correct association with cellular membranes and its presence in the virus producer but not in the target cells, and due to enhancement of the intrinsic infectivity of the virions rather than an increase in the number of particles released (Aiken and Trono, 1995; Chowers et al., 1994; Miller et al., 1994; Pizzato, 2010; Schwartz et al., 1995b; Spina et al., 1994). Furthermore, it is evolutionarily conserved and maintained through high selective pressure during disease progression (Carl et al., 2001; Munch et al., 2007). The identification of a Jurkat clone (Jurkat E6.1) for which, when used as virus producer cells, the Nef-mediated fold-change in infectivity rose up to 100 shed some light on our understanding of the molecular basis of Nef-induced boost of lentiviral infectivity (Pizzato et al., 2007; Pizzato et al., 2008). In this system, Nef effect on infectivity depended on the GTPase dynamin 2, clathrin and the adaptor complex AP-2, suggesting that Nef exploits the endocytic machinery of the host cell for this activity (Pizzato et al., 2007; Usami et al., 2015). Together with the requirement for functional clathrin-mediated endocytosis, the fact that this activity has been mapped to the PxxP, EEEE66-69 and Δ12-39 motifs, all of them relevant for vesicular trafficking, (Chowers et al., 1994; Fackler et al., 2006; Goldsmith et al., 1995; Pizzato et al., 2007) suggested that the observed effects of Nef on viral infectivity are exerted by counteracting a yet unknown restriction factor. Through two different experimental approaches (proteomics of virions produced in presence or absence of Nef or expression profiling of Nef-sensitive and Nef-insensitive producer cells (Fackler, 2015)), the Pizzato and Göttlinger laboratories identified two members of the SERINC protein family, SERINC3 and 5, as the responsible host cell restriction factors implicated (Rosa et al., 2015; Usami et al., 2015). Both these proteins are incorporated in significant amounts in released HIV-1 particles and the presence of Nef strongly reduced their incorporation. Parallel observations by the same authors regarding the cell surface expression levels and subcellular localization of SERINC3/5 in the presence of Nef indicate that the viral factor retargets them from the plasma membrane to a Rab-7 positive compartment. Also, SERINC5 hinders virion fusion. The current model therefore is that the presence of SERINC3/5 on HIV virions impairs fusion to target cells and Nef counteracts this restriction by redirecting the host factors to an endocytic intracellular compartment reducing thereby their incorporation into budding viral particles (Fackler, 2015)

1.3.1.2 Nef-mediated receptor downregulation: CD4 and MHC-I

The best described *in vitro* Nef function is the modulation of the levels of cell surface receptors. This is thought to have a beneficial impact on infection by avoiding superinfection, mediate evasion of the immune response, or enhance virus release, thus generating the optimal scenario for viral replication (Landi et al., 2011). The main HIV receptor CD4 is found among the surface proteins downmodulated by Nef (Garcia and

Miller, 1991). Nef causes a reduction in CD4 surface levels by mediating its internalization from the plasma membrane via clathrin-mediated endocytosis (Aiken et al., 1994; Chowers et al., 1994; Piguet et al., 1998). CD4 downregulation results in the reduction of superinfection, a phenomenon which may drive to premature cell death due to aggregation of non-integrated proviral DNA, as well as in the optimization of viral spread by preventing redundant infection (Benson et al., 1993; Michel et al., 2005; Wildum et al., 2006). Furthermore, it is thought to promote viral egress by avoiding interaction between newly synthesized gp120 and CD4 within the vesicular system prior to assembly of new virions (Willey et al., 1992b) or at the cell membrane where CD4 can act as a "tether" for the budding virion progeny (Ross et al., 1999). However, this has only been shown for unphysiologically high levels of CD4. The molecular determinants defining this Nef activity are the myristoylation site needed for the interaction with cellular membranes (glycine in position 2) (Aiken et al., 1994; Peng and Robert-Guroff, 2001), the W61-62 motif necessary for the direct interaction with the cytoplasmic tail of CD4 (Grzesiek et al., 1996b) and the dileucin motif in the C-terminal flexible loop (LL168/169) involved in the interaction with the clathrin adaptor AP2 required for CD4 targeting to lysosomal degradation (Craig et al., 1998; Ren et al., 2014; Rhee and Marsh, 1994). The transport pathway that CD4-containing vesicles follow to encounter lysosomes is still unclear but it seems to follow the endosomal sorting complexes required for transport (ESCRT) pathway and to require interaction of Nef with TSG101 and Alix (Amorim et al., 2014; daSilva et al., 2009). Unlike in the case of CD4 where Nef mediates its internalization, Nef modulates the levels of most of the other targeted receptors by altering their anterograde transport. This is the way how Nef reduces the levels of major histocompatibility complex (MHC)-I what is needed to escape adaptive CTL responses (Collins et al., 1998; Schwartz et al., 1996). The absence of MHC-I at the plasma membrane is also sensed by the innate immune system through NK cells. To avoid NK cell-mediated killing, HIV-1 Nef specifically downregulates two of the isoforms of MHC-I, human leukocyte antigen (HLA)-A and -B, but not HLA-C and -E (Cohen et al., 1999). The molecular mechanism of Nef-mediated MHC-I downregulation relies on the interaction of the viral protein with the cytoplasmic tail of the cellular protein and the subsequent formation of a complex with the sorting molecules PACS-1 and -2 and the clathrin adaptor AP-1. All these interactions and complex formation lead to an interference with the anterograde transport of newly synthesized MHC-I molecules and their retargeting from the TGN to lysosomal degradation (Roeth et al., 2004; Williams et al., 2002). A second model suggests that mature MHC-I molecules are internalized from the plasma membrane and targeted to lysosomal degradation through an unclear mechanism (Pawlak and Dikeakos, 2015). The molecular determinants of Nef involved on MHC-I downmodulation are defined and include EEEE66-69 needed for the interaction with the PACS sorting adaptors (Atkins et al., 2008; Piguet et al., 2000), PxxP (80-83) and M20 both involved in the interaction with AP-1 (Greenberg et al., 1998; Hung et al., 2007) (Dikeakos et al., 2012; Jia et al., 2012) as well as the myristoylation site G2 (Peng and Robert-Guroff, 2001). The HIV coreceptor CXCR4 is also downregulated from the cell surface by Nef and the motifs involved on this process are the ones involved on MHC-I

downregulation. Therefore a similar mechanism has been proposed (Venzke et al., 2006). Nef also mediates downmodulation of the other HIV coreceptor, CCR5, as well as other members of the family of the chemokine receptors (Michel et al., 2005; Michel et al., 2006). Downregulation of the correceptors CXCR4 and CCR5 takes place even before proviral integration (Sloan et al., 2010). The reduction in the expression of the HIV correceptors may contribute to prevent superinfection as well as to the impairment on T cell motility mediated by Nef (Venzke et al., 2006). The number of surface receptors and molecules described to be targeted by Nef for downregulation does not stop to grow (Haller et al., 2014). Also, the fact that whole protein families are susceptible to Nef effects and common motifs are responsible for this effect show that the effect of Nef is on general transport platforms rather than on single molecules.

1.3.1.3 Modulation of T cell signaling by Nef

T lymphocytes form transient interactions in which their TCR complexes recognize the antigens bound to MHC-II molecules on the surface of APCs. This leads to the formation of the so called immunological synapse (IS), a complex structure formed by signaling and intercellular adhesion molecules associated to a polymerized F-actin structure, which optimizes TCR signaling and leads to T cell activation (Alarcon et al., 2011). Nef profoundly alters the signaling cascade downstream of the TCR in infected T lymphocytes as well as in cells expressing the viral factor alone. This is mediated via the interaction with several proteins of the TCR signaling cascade (Haller et al., 2006; Iafrate et al., 1997; Schindler et al., 2006; Schrager and Marsh, 1999). This way, Nef fine-tunes the degree of T cell activation favoring viral spread and replication. It has been reported that Nef induces T cell activation prior to proviral DNA integration by lowering the threshold for the TCR signaling cascade leading to efficient viral transcription (Abraham and Fackler, 2012; Baur et al., 1994; Wu and Marsh, 2001). On the other hand, TCR overstimulation can result in cell death (Arnold et al., 2006; Bourgeois and Stockinger, 2006). This phenomenon known as activation induced cell death (AICD) can also be prevented by Nef by interfering with IS formation. To study this in vitro, some of the features of IS formation can be reproduced by seeding T lymphocytes on anti-CD3 or anti-CD3/anti-CD28 antibody-coated surfaces (Fackler et al., 2007). On this artificial system, cells spread and form the characteristic actin ring and proximal TCR signaling can be detected by the formation of distinct signaling complexes or microclusters (MCs). On one hand, Nef inhibits formation of MCs positive for the signaling adaptor Src homology-2 domain-containing leukocyte protein of 76 kDa (SLP-76) to reduce the density of MC (Abraham et al., 2012). This results in a potent inhibition of the early TCR signaling events. Nef's negative effects on the actin cytoskeleton organization are necessary but not sufficient to mediate the observed disruption. On the other hand, Nef retargets members of the TCR signaling cascade such as LAT or Lck to intracellular compartments (Abraham et al., 2012; Haller et al., 2007; Haller et al., 2006; Pan et al., 2012). Unlike in the case of retargeting of LAT, which is consistent with disruption of early TCR signaling events, rerouting of Lck has been associated to the enhancement of distal TCR signaling events. Lck is redirected and accumulated at early and recycling endosomes (RE) as well as at the *trans*-Golgi

network (TGN) in a catalytically active, signaling competent conformation (Haller et al., 2007; Haller et al., 2006; Pan et al., 2013b; Pan et al., 2012; Thoulouze et al., 2006). The enrichment of active Lck at this compartments lead to activation of the RAS GTPase and ultimately to the activation of Erk kinase and production of IL-2 upon exogenous stimulation (Abraham et al., 2012; Haller et al., 2007; Haller et al., 2006; Keppler et al., 2006; Rudolph et al., 2009). Thereby, Nef induces paradoxal effects on TCR signaling potently inhibiting the early steps and enhancing downstream events. Furthermore, Nef from the non-pathogenic viruses HIV-2 and SIV are able to downregulate TCR-CD3. The lack of this activity in Nef from HIV-1 and HIVcpz has been linked high T cell activation levels and display of a virulent outcome of the infection with this viruses with (faster) progression towards AIDS (Munch et al., 2002; Rudolph et al., 2009; Schindler et al., 2006).

1.3.1.4 Inhibition of actin dynamics by Nef

1.3.1.4.1 The actin cytoskeleton

The actin cytoskeleton consists of a highly flexible filament meshwork in constant remodeling that defines the inner structure and the outer shape of the cells. Regulated actin rearrangements ensure fundamental cellular processes such as cell migration, endocytosis and intracellular vesicle trafficking or cell division. Particularly, cytoskeletal reorganization plays a crucial role in multiple aspects of T cell function where it is involved in cell signaling, differentiation, adhesion, membrane reshaping and protrusion formation, molecular and morphological polarization, motility, immune synapse formation and activation, and execution of effector functions (Burkhardt et al., 2008; Dupre et al., 2015; Samstag et al., 2003). Actin polymerization and depolymerization is tightly regulated by actin-binding proteins which act as nucleators as well as upstream promoting factors and regulators. The building block is the actin monomer or globular actin (G-actin). Double-stranded helical actin filaments are assembled when a critical concentration of ATP-bound G-actin is reached. Actin filaments are polar, meaning they have two differentiated ends: the fast-growing barbed ends (plus ends), where actin polymerization takes places, and the pointed ends (minus ends) where actin disassembly occurs (Dupre et al., 2015). Actin nucleators such as formins or the actin related proteins 2/3 (Arp2/3) complex are responsible of initiating polymerization, a thermodynamically disfavored process not taking place otherwise (Devreotes and Horwitz, 2015). While formins are able to promote *de novo* linear actin assembly or the elongation of existing filaments, Arp2/3 complex binds to preexisting filaments and induces formations of branches with an angle of 70º (Figure 1.6) (Insall and Machesky, 2009). Branching is crucial to achieve an elaborated actin filament network. Arp2/3 activity is regulated by proteins of the Wiskott-Aldrich syndrome protein (WASP) family and the WAVE family (Padrick and Rosen, 2010; Pollitt and Insall, 2009). Together with formins, the latter are regulated by small GTPases of the Rho family, including Rho, Rac and Cdc42 (Parsons et al., 2010). These proteins are active when bound to GTP and become inactive when the nucleotide is hydrolyzed to GDP. This inactivation process is catalyzed by GTPase activating proteins (GAPs)

whereas activation by exchange of GDP for free GTP is mediated by guanine nucleotide exchange factors (GEFs) (Bos et al., 2007). Guanosine nucleotide dissociation inhibitors (GDIs) stabilize the inactive form of small GTPases. By being switched on and off, these proteins are able to modulate actin cytoskeleton spatially and temporally according to extracellular triggers. These proteins also regulate kinases such as p21-activated kinase (Pak) or Rho kinase (ROCK) which are necessary for contractility and controlled severance of existing actin filaments (Bokoch, 2003). The latter process is absolutely necessary for rearrangements in the actin cytoskeleton upon a certain stimulus since *de novo* filament synthesis is a slow process which does not account for the required rapid responses. Fragmentation of existing filaments is mediated by the actin severing factor cofilin and provides new barbed ends for further polymerization and nucleation by Arp2/3 complex (Carlier et al., 1997; Ichetovkin et al., 2002; Ichetovkin et al., 2000; Lappalainen and Drubin, 1997). Cofilin induces also disassembly at the pointed end of actin filaments by targeting ADP-bound actin molecules. Profilin induces nucleotide exchange and regenerates ATP-bound actin monomers that can be used for polymerization at the barbed end (Dupre et al., 2015; Wolven et al., 2000). Both cofilin and profilin are then essential to ensure rapid actin dynamics. Phosphorylation of cofilin at a serine residue at position 3 mainly by Lim Kinase (LIMK) downstream of PAK and ROCK leads to its reversible inactivation (Bernstein and Bamburg, 2010). Capping proteins prevent actin monomer association or dissociation at the barbed ends stabilizing the filament (Bear and Gertler, 2009).

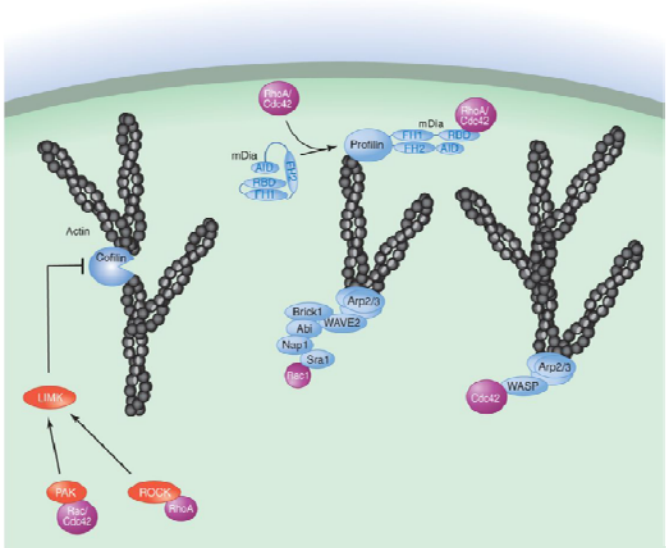

Figure 1.6 Main actors involved in the actin polymerization/depolymerization process. Actin dynamics is a tightly regulated process. Arp2/3 and Cofilin are the central players in polymerization/depolymerization of branched actin filaments. The activity of this two proteins is modulated by a number of effectors regulated in turn by small GTPases of the Rho family, including Rho, Rac and Cdc42 families. (Devreotes and Horwitz, 2015).

1.3.1.4.2 Nef interferes with actin dynamics

HIV manipulates the actin cytoskeleton of the host cell to its own benefit during several steps of the viral cycle including entry into, trafficking within and egress from infected cells (Fackler and Krausslich, 2006). In this context, many viral factors have been shown to interact with components of the actin cytoskeleton. Among them Nef plays a central role as a master inhibitor of actin rearrangements (Fackler and Krausslich, 2006; Stolp and Fackler, 2011). HIV-1 Nef is long known to associate with actin (Fackler et al., 1997; Niederman et al., 1993). Nef was reported to interact with the GEF Vav leading to its activation and ultimately to F-actin depolymerization (Fackler et al., 1999). Further investigations showed that Nef-mediated effects on actin dynamics were dependent on its interaction with the cellular kinase Pak 2 in the context of a labile multiprotein complex together with the GEF Vav and the small GTPases Rac and Cdc42 (Haller et al., 2006; Nunn and Marsh, 1996; Rauch et al., 2008; Renkema et al., 1999; Stolp and Fackler, 2011). Despite of being transient and of very low affinity, the interaction between both proteins reprograms the substrate specificity of the kinase towards the non natural substrate cofilin. Following dissociation of the complex, Pak 2 phosphorylates cofilin what renders the actin severing factor inactive resulting ultimately in a reduction of actin turnover (Stolp et al., 2009). Nef and Pak 2 interact via a hydrophobic patch in Nef consisting of several aminoacids but mutation of one single residue, F195 in SF2 Nef (F191 in NL4-3 and NA7 Nef), has proven to disrupt association of the protein (O'Neill et al., 2006; Rauch et al., 2008). Nef-Pak 2 interaction as well as the effects on actin dynamics are conserved among SIV, HIV-1 and HIV-2 Nef proteins (Rudolph et al., 2009; Stolp et al., 2010). Besides F195/191, additional motifs involved in other functions of Nef are also important for Nef-Pak 2 association. Membrane association via G2 myristoylation and targeting to lipid rafts where Pak 2is recruited via K4K7 are both needed for the interaction (Krautkramer et al., 2004). Integrity of the acidic stretch EEEE66-69 is also required (Baugh et al., 2008; Haller et al., 2007; Stolp et al., 2010; Stolp et al., 2009). The PxxP motif is also involved in the outcome of the interaction between Nef and Pak 2 since it is necessary for the recruitment of Vav, upstream activator of the kinase via Rac and Cdc42 (Fackler et al., 1999; Rauch et al., 2008). The PAK 2 interaction surface of Nef was thought to be exclusively involved in the interaction between both proteins until recently. The exocyst complex (EXOC), an octameric protein complex implicated in vesicular trafficking and actin remodeling, has been shown to interact with Nef via the Pak 2 interacting domain (Mukerji et al., 2012). Mutational, biochemical and functional analysis of its interaction with Nef proved that interaction of Nef and Pak2 is essential to induce the recruitment of EXOC which, after dissociation of Pak 2 from the complex, remain stably associated to the viral factor. Despite of being dispensable for other activities like Nef-Pak 2 downstream functions and interference of intracellular trafficking, Nef-EXOC complex is necessary for TCR-induced actin remodeling inhibition by Nef in the context of IS formation (Imle et al., 2015). This adaptor function of PAK2 for the recruitment of EXOC is independent of the other described PAK2 activities.

1.3.2 Viral protein U of HIV-1 (Vpu)

HIV-1 Vpu is a 16 kDa, integral type I transmembrane (TM) protein encoded by the pathogenic viruses HIV-1 and some related SIV strains, but absent in the less or non pathogenic viruses HIV-2 and other SIVs (Cohen et al., 1988; Courgnaud et al., 2002; Gonzalez, 2015; Huet et al., 1990; Maldarelli et al., 1993; Strebel et al., 1988). As observed for Nef, macaques infected with Vpu deficient hybrid HIV-SIV viruses (SHIV) displayed a 10- to 100-fold reduction in viral titers in plasma as compared to wt SHIV (Hout et al., 2005). Also, recent studies have proven the relevance of Vpu during early HIV-1 spread during acute infection in humanized mice models (Dave et al., 2013; Sato et al., 2012). Vpu is expressed from a bicistronic mRNA that encodes as well for the Env protein. As a result both proteins are produced late in the viral life cycle in a coordinated manner (Schwartz et al., 1990). The translated product has about 81 amino acids (aa) and consists of a short luminal domain and unknown function, a single α-helical TM domain and cytoplasmic tail (Figure 1.7) (Sugden et al., 2016). The TM domain with 23 aa enables the viral protein to form supramolecular complexes by homooligomerization or by interaction with other proteins (Gonzalez, 2015). The cytoplasmic domain is 56 aa long and comprises a flexible hinge region proximal to the plasma membrane followed by two α-helices (H1 and H2) flanking a linker region containing a highly conserved double-serine motif (positions 52 and 56). The subcellular localization of HIV-1 Vpu varies with the different clades. Vpu from clade B localizes preferentially at the TGN while Vpu from clade C is also present at the plasma membrane (Pacyniak et al., 2005).

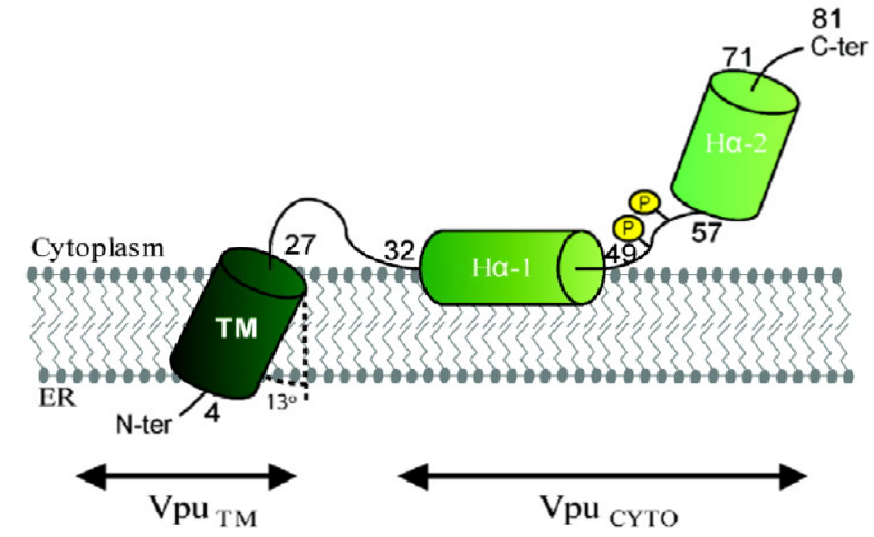

Figure 1.7 Structure of HIV-1 Vpu. The Vpu protein presents a short N-t tail facing the ER lumen, a TM domain and a cytoplasmic C-t domain. The latter consists of a short C-t tail and two helices separated by a flexible hinge containing two constitutively phosphorylated serine residues. The aa positions correspond to the NL4.3 Vpu allele. (Dube et al., 2010a)

1.3.2.1 CD4 downregulation by HIV-1 Vpu

CD4 downregulation by HIV is a relevant phenomenon for virus replication and spread. An evidence for it is that several viral proteins contribute to this activity this activity: Nef, Vpu and Env (Wildum et al., 2006). The strongest downregulation effect is mediated by Nef which is capable of inducing CD4 internalization and degradation (Aiken et al., 1994; Chowers et al., 1994; Piguet et al., 1998). On the other hand, Vpu and Env block transport of newly synthesized CD4 molecules to the plasma membrane (Willey et al., 1992a). Vpu targets molecules of the receptor at the endoplasmic reticulum (ER) to the ER-associated degradation (ERAD) pathway (Binette et al., 2007; Magadan et al., 2010; Schubert et al., 1998; Willey et al., 1992a). Following the interaction of both proteins through their cytoplasmic tails (Bour et al., 1995), the SCF E3 ubiquitin ligase adaptors beta-transducin repeats-containing proteins (β-TrCP) -1 and -2 are recruited to the complex leading to subsequent recruitment of SCF complex. Interaction between Vpu and β-TrCP requires integrity of the canonical sequence DSGXXS of Vpu and phosphorylation of its two serine residues at positions 52 and 56 (Margottin et al., 1998; Schubert et al., 1994). This ultimately results in the serine/threonine-dependent polyubiquitination of the cytoplasmic tail CD4 (Binette et al., 2007; Magadan et al., 2010; Schubert et al., 1998), but not Vpu (Schubert and Strebel, 1994), promoting recruitment of the VCP-UFD1L-NPL4 dislocase complex, a late stage component of the ERAD pathway, and relocalization of CD4 from the ER to the cytoplasm where they are finally delivered to the proteasome for efficient degradation (Binette et al., 2007; Magadan et al., 2010; Schubert et al., 1998).

1.3.2.2 Counteraction of BST-2/tetherin/CD317 by Vpu

Another well described effect of HIV-1 Vpu is the mediation of efficient virion release from infected cells (Klimkait et al., 1990; Strebel et al., 1989; Terwilliger et al., 1989). It was observed that following infection with a Vpu deficient virus the newly produced, budding viral particles remain associated to the surface of infected cells and accumulated at endosomal compartments (Neil et al., 2006). Previous reports showed that this impairment was cell specific (Geraghty et al., 1994; Sakai et al., 1995). Furthermore, it was later described that this restriction could be induced in permissive cells by Interferon (IFN)-I treatment (Neil et al., 2007). These findings suggested the presence of a cell-type specific restriction factor responsible for the observed "tethering" of new virions on the surface of infected cells. The search led to the discovery of tetherin (also known as bone marrow stromal cell antigen 2 (BST2) or CD317) as the IFN-I-inducible molecule mediating virion retention (Neil et al., 2008; Van Damme et al., 2008). Tetherin is a type II integral transmembrane protein which is present at the plasma membrane and also at the TGN and recycling compartments. From the structural perspective, it consists of a short cytoplasmic tail with a conserved dityrosine trafficking motif (YDYCRV), a membrane spaning α-helix and a predominantly α-helical luminal domain with a glycosylphosphatidylinositol (GPI) membrane anchor at the C-terminal end (Arias et al., 2011; Kupzig et al., 2003). This peculiar topology enables tetherin to perform its virion retaining function as part of the

innate antiviral immune response. This occurs through the incorporation of one its membrane anchors into the membrane of the virion, with a preference to be this the GPI anchor (Venkatesh and Bieniasz, 2013), while maintaining the other membrane anchor into the plasma membrane of the producer cell (Lehmann et al., 2011; Perez-Caballero et al., 2009). HIV-1 Vpu was thought to counteract tetherin by interfering with its anterograde transport to the plasma membrane (Dube et al., 2009; Schmidt et al., 2011). For this, both proteins directly interact at the TGN through their respective TM domains (Dube et al., 2010b; McNatt et al., 2013) with the subsequent sequestration of tetherin at this level. This interaction is essential for counteraction and relies on the A10, A14, A18 and W22 residues of Vpu, which form a hydrophobic rim within the membrane spaning region (Skasko et al., 2012; Vigan and Neil, 2010). Subsequently, Vpu mediates targeting of tetherin to proteasomal degradation through the endosomal sorting complex required for transport (ESCRT) pathway (Janvier et al., 2011). This requires recruitment of the β-transducin repeats-containing protein (β-TrCP)-2 subunit of the $SCF^{\beta\text{-}TrCP\text{-}1/2}E3$ ubiquitin ligase and therefore the integrity of the conserved diserine β-TrCP-binding motif (S52,56) (Douglas et al., 2009; Mitchell et al., 2009). $SCF^{\beta\text{-}TrCP\text{-}1/2}E3$ ubiquitin ligase ultimately mediates direct ubiquitination of tetherin and degradation in the lysosomes (Gustin et al., 2012; Tokarev et al., 2011). Vpu-mediated tetherin counteraction has been linked to its downregulation form the host cell surface. However, tetherin antagonism has been observed in the absence of downregulation (Miyagi et al., 2009). Furthermore, β-TrCP recruitment and tetherin degradation can be decoupled from the antagonism of tetherin by Vpu (Dube et al., 2010b; Mitchell et al., 2009; Schmidt et al., 2011; Tervo et al., 2011). The facts that Vpu blocks anterograde transport of newly-synthesized tetherin as well as the transport of recycling tetherin molecules to the plasma membrane (Dube et al., 2011; Schmidt et al., 2011), that Vpu antagonism of tetherin strongly depend on clathrin (Kueck and Neil, 2012; Lau et al., 2011) and that Vpu can interact with adaptor proteins AP-1 and AP-2 (Jia et al., 2014; Kueck et al., 2015), have led to a new mistrafficking model. In this new model Vpu mediates sequestration of tetherin at clathrin-rich areas of endosomes at the TGN by hijacking AP-1-dependent membrane trafficking where the cellular protein is eventually targeted to β-TrCP-dependent degradation. Consistently with this model, it has been also reported that Vpu displaces tetherin from virion assembly sites (McNatt et al., 2013). Recently Pujol et al. have shown that interference with recycling or anterograde transport is not sufficient for counteraction of tetherin-mediated restriction by Vpu and tetherin displacement from assembly sites is strictly dependent on the tetherin- and AP-1-binding motifs and correlates to viral particle release antagonism (Pujol et al., 2016). Inhibition of AP-1 or a reduction in its expression confirmed these results. In sum, Vpu blocks tetherin-mediated viral release restriction by preventing its recruitment to the assembly sites of nascent viral particle in an AP-1-dependent manner.

Besides its virion “trapping” function, tetherin also acts as an innate viral sensor by mediating activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) resulting in antiviral gene expression by the host cell (Galao et al., 2012; Tokarev et al., 2013). Mechanistically, the sequestration of virions on the surface of the

infected cell leads to the formation of tetherin dimer clusters and tyrosine-dependent phosphorylation at the cytoplasmic tails. This is the trigger for intracellular signaling that proceeds by the recruitment of spleen tyrosine kinase (Syk) and ultimately causes activation of NF-κB which is translocated into the nucleus where it induces proinflammatory gene expression (Galao et al., 2014). By counteracting virus retention on the plasma membrane Vpu avoids tetherin-triggered signaling and immune recognition of the infected cells. In addition, it has also been reported that Vpu is able to stabilize IκB, the NF-κB regulator which interferes with its translocation, resulting in the sequestration of the p65 subunit of NF-κB in the cytoplasm (Sauter et al., 2015). HIV however depends on NF-κB activation for viral gene expression and its genome contains two NF-κB binding sites at the 5'LTR (Nabel and Baltimore, 1987). Given the ability of Nef to induce activation of the transcription factor, it has been proposed that Nef acts on NF-κB activity early in the replication cycle promoting its activation and viral gene transcription and Vpu inactivates it at late stages to prevent immune detection of infected cells (Sauter et al., 2015).

1.3.2.3 Other Vpu functions: ion channel and proapoptotic factor

Given the structural similarities between Vpu and the M2 ion channel protein of influenza virus, it was hypothesized that homooligomerization of Vpu could result in the formation of membrane pores (Maldarelli et al., 1993). Further *in vitro* experimentation with artificial lipid bilayers confirmed that Vpu indeed forms selective ion channels for monovalent cations like sodium and potassium (Ewart et al., 1996; Schubert et al., 1996). It was thereby suggested that this activity could be required for its effects on viral release. Despite a discrete number of in vitro and in vivo studies were consistent with this hypothesis (Herrero et al., 2013; Hout et al., 2005; Hsu et al., 2010), more recent studies argue against this based on the fact that mutations which disrupt ion channel activity (e.g. S23A or S23L) do not affect virus release enhancing and mutations that affect virus release (e.g. A14N or A18N) retained ion channel activity (Bolduan et al., 2011; Kuhl et al., 2011; Mehnert et al., 2008; Skasko et al., 2011). Thus, HIV-1 Vpu possesses a viroporin activity for which a clear biological function has yet to be revealed.

As discussed above, HIV-1 Vpu is able to deregulate NF-κB by preventing phosphorylation at serine residues and β-TrCP-dependent subsequent proteasomal degradation of its natural inhibitor IκB (Sauter et al., 2015). The consequences of inhibiting a central transcription factor such as NF-κB can be very important since it has been shown to be involved in the expression of key cellular genes for cell proliferation, cytokine induction and apoptosis (Barkett and Gilmore, 1999; Pahl, 1999). Consequently, Vpu-mediated inhibition of NF-κB in HIV-1 infected cells potentially can play a role in the induction of apoptosis in these cells (Badley et al., 2000; Casella et al., 1999). This idea was strongly supported by a study in Jurkat T cells which displayed two-fold increase in apoptosis levels when infected with a Vpu-deficient virus as compared to wt HIV-1-infected control cells. This effect was dependent on the inhibition of NF-κB-dependent expression of antiapoptotic genes such as the Bcl2

family proteins resulting in an increase in the levels of the apoptosis-inducer caspase-3 (Akari et al., 2001).

1.3.3 Overlap in functions exerted by Nef and Vpu

Despite of the fact that HIV-1 both factors are very distinct in regard to their sequence, structure, organization of their domains and membrane topology, the accessory proteins Nef and Vpu carry out redundant functions. As mentioned above, the downmodulation of CD4 levels from the plasma membrane is the best example. By doing so, they prevent superinfection and premature death of infected cells and optimize viral egress and spread (Benson et al., 1993; Michel et al., 2005; Wildum et al., 2006; Willey et al., 1992b). Both proteins downmodulate other surface receptors and molecules from the plasma membrane of infected cells such as MHC-I, CD1d and poliovirus receptor (PVR) (Chen et al., 2006; Kerkau et al., 1997; Matusali et al., 2012; Moll et al., 2010; Schwartz et al., 1996). A particular case is that of the restriction factor tetherin/CD317/BST-2. As described previously, counteraction of the antiviral effect of this molecule is a well-described activity of Vpu and it is thought to be the result of the displacement form the virion assembly sites (McNatt et al., 2013; Pujol et al., 2016) and, at least in part, of a block in the anterograde transport of newly synthesized as well as recycling tetherin molecules leading eventually to proteasomal degradation (Douglas et al., 2009; Mangeat et al., 2009; Neil et al., 2008). However, recent studies showed that in viruses lacking the *vpu* gene Nef was able to perform such activity (Zhang et al., 2009). In *vpu*-deficient viruses tetherin is counteracted by Nef and this activity was overtaken by Vpu in those viruses that incorporated the *vpu* gene. Exceptions to this are the HIV-1 precursors SIVcpz and SIVgor which, despite of encoding the Vpu protein, express Nef proteins able to counteract tetherin albeit with poor activity (Sauter et al., 2009). In humans, tetherin is insensitive to Nef due to a 5-aminoacid deletion which represents an interspecies transmission barrier (Sauter et al., 2009). Nevertheless, likewise SIVcpz and SIVgor Nef proteins, the HIV-1 group O Nefs are able to counteract tetherin despite of the expression Vpu by these viruses (Kluge et al., 2014).

Another function in which both Nef and Vpu play key roles and is also partially linked to their ability to downregulate proteins from the cell surface is the evasion of innate immune response mediated by natural killer (NK) cells. NK cells as well as other cells of the lymphoid lineage namely monocytes and neutrophils posses so called Fc receptors on their surface (FcγRIIIa; CD16) which recognize and bind the crystallizable (Fc) region of antibodies coating the surface of APCs. Recognition leads to the activation of their cytolitic activity and the killing of the antibody-coated APCs. This process is commonly known as antibody-dependent cell-mediated cytotoxicity (ADCC) (Scully and Alter, 2016). The extent of the ADCC response depends on the antigen being recognized by the antibodies coating the APCs (Rolland et al., 2012). Recently, gp120-CD4 interactions have been linked to anti-HIV mediated ADCC by NK cells (Smalls-Mantey et al., 2013). The interaction facilitates exposure of an epitope in gp120 which is preferentially recognized by non-neutralizing antibodies which mediate ADCC (Bonsignori et al., 2012; Ferrari et al., 2011; Veillette et al., 2014; Wyatt et al., 1995).

By downregulating CD4 from the plasma membrane of infected cells, Nef and Vpu promote escape from ADCC by NK cells (Pham et al., 2014; Veillette et al., 2014). Furthermore, the retention of viral particles on the surface of infected cells mediated by tetherin increases the pool of viral antigens exposed to immune recognition favoring ADCC. Consistently, Vpu-induced decrease in virion tethering results in a significant reduction in Env epitopes exposure and lower NK cell-driven ADCC (Alvarez et al., 2014; Arias et al., 2014; Pham et al., 2014). Altogether, in the context of HIV-1 infection ADCC is prevented by two synergistic strategies: CD4 downregulation by Nef and Vpu, and tetherin counteraction by Vpu. In addition, Nef and Vpu contribute to NK cell-mediated innate response by specifically downregulating NK cell ligands such as NK T and B cell antigen (NTBa), PVR and UL16-binding protein involved in the activation of these cells (Galaski et al., 2015; Matusali et al., 2012; Shah et al., 2010).

All in all, HIV-1 Nef and Vpu perform a number of redundant functions directed to escape host immune responses. These functions are strongly linked to the ability of both accessory proteins to modulate the exposure of a long and largely overlapping list of receptors and surface molecules by the infected cells.

1.4 Tetraspanins (TSPANs)

The members of the TSPAN superfamily of proteins are present at the plasma membrane and intracellular vesicles of virtually all mammalian cells. TSPANs contain 4 transmembrane domains and have small and large extracellular loops (SEL and LEL, respectively) and short N- and C- terminal cytoplasmic tails. The LEL is involved in specific protein-protein interactions and the cytoplasmic regions act as linkers to cytoskeletal and signaling molecules (Tarrant et al., 2003). TSPANs form microstructures at the plasma membrane known as tetraspanin-enriched microdomains (TeMs). These structures act as protein scaffolds similar to but biochemically distinguishable from lipid rafts (Tarrant et al., 2003). Selective sorting of proteins into these microdomains appears to be important for a number of membrane-dependent processes to take place such as cell-cell fusion, cell adhesion, cell motility and sorting of exosomal proteins, among many others (Hemler, 2003; Levy and Shoham, 2005a, b; Perez-Hernandez et al., 2013; Wright et al., 2004; Zoller, 2009). Notably, assembly sites of HIV-1 at the plasma membrane of host infected cells selectively localize at TeMs (Jolly et al., 2007; Nydegger et al., 2006), likely a consequence of Gag-TSPANs interactions (Hogue et al., 2011). Consistently, TeMs also form part of the virological synapses (Nydegger et al., 2006; Welsch et al., 2007). As a result of that, TSPANs are included in viral particles (Orentas and Hildreth, 1993; Ott, 2008). Other human pathogens rely on TSPANs for their replication, i.e. hepatitis C virus (HCV) whose main entry cellular receptor is known to be CD81 (Feneant et al., 2014; Pileri et al., 1998).

1.5 T cell biology: migration and extracellular vesicle release

As central players of the immune system, T cells need to communicate with a vast spectrum of cells to carry out their defensive function. Direct contact with other cells

efficiently help them feel and sense activatory clues and need for immune synergy. Thereby, there is a continuous requirement form active migration and covering of long distances. Another way to send and receive signals is via a more paracrine, or even endocrine, manner which can involve single molecules such as cytokines, hormones or growth factors or complex structures like extracellular vesicles. Now the focus falls on migration and its main molecular and temporal components and on extracellular vesicle release.

1.5.1 T cell migration

Continuous migration is a hallmark of T cell function and immune surveillance (Dupre et al., 2015; Samstag et al., 2003; Serrador et al., 1999). Already at the earliest phase of their life following haematopoiesis, immature T cells or thymocytes need to travel from the bone marrow to the thymus where they mature into naïve T cells. During maturation, TCR-mediated antigen specificity is achieved and cells with intermediate affinity are selected. Once out of the primary lymphoid organs (PLO), naïve T cells are constantly migrating between the bloodstream and the secondary lymphoid organs (SLO, lymph nodes, spleen, tonsils and Payers patches). The so called T cell homing process or migration from the blood to SLO allows for cognate MHC II-antigen complex recognition from the surface of APCs by these cells (Figure 1.8C). The resulting effector T cells migrate then to the inflammation sites where adaptive immune responses are initiated. T cells migrate with high speed reaching 7-10 μm/min, 10 times faster than fibroblasts (0.3-1.3 μm/min) (Gudima et al., 1988). The whole process is tightly regulated and directed by chemokines sensed through chemokine receptors and adhesion molecules on other cells or by the extracellular matrix (ECM) interacting with those present on the surface of T lymphocytes. Migrating T cells have morphological and functional characteristics reminiscent of amoeba. This migration mode requires continuous changes in cell shape and relies on the ability of the cell to polarize which subsequently depends on a highly dynamic cytoskeleton (Figure 1.8) (see 1.5.2). Amoeboid migration is a multistep process comprising four consecutive events: actin-driven protrusion, adhesion to the substrate, myosin-based contraction and disassembly of adhesive contacts (Ridley et al., 2003; Serrador et al., 1999). T cells can adapt the migration mode to the environmental context and stimulus such as chemoattractants, share flow and the cellular/matrix density and composition in the tissue as well as to their own functional status. These adaptations result in two different types of amoeboid migration: a slow migration mode involving strong focal adhesion contacts to the substrate observed in 2D ligand-coated surfaces, a model for T cell migration on endothelial cell monolayers, or a fast migration mode in which contacts with the substrate are more diffuse, used during 3D migration within solid tissues. 2D migration is largely integrin-dependent whereas 3D migration involves multiple cell-cell and/or cell matrix interactions (Dustin, 1997; Dustin et al., 1992; Friedl and Brocker, 2000; Lammermann et al., 2008). T cell can also use a mesenchymal mode of migration associated with matrix degradation in which their cell body acquires an elongated spindle-like shape showing one or more leading pseudopods at the front.

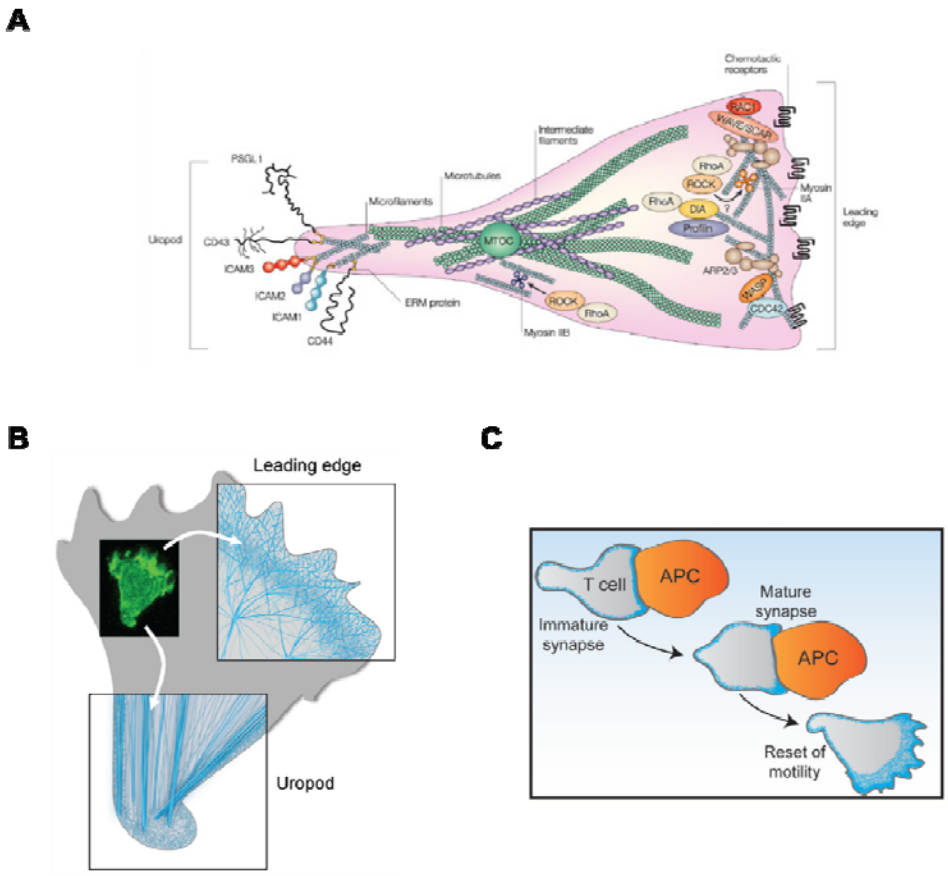

Figure 1.8 Polarization of migrating T cells. (A) Molecular and morphological organization of a polarized T lymphocyte. (B) Cytoskeletal fundamental differences are present in the actin cytoskeleton of a polarized T cell. Actin polymerization takes place mainly at the leading edge resulting in a largely branched actin filament network. Actin filaments at the uropod are linear and form bundles to which myosin associates to promote rear contraction. (C) Lymphocytes scan APCs within the lymph nodes relying on their migration capacity in a "stop and go" fashion. Polarization is a prerequisite for T cell migration and allows for directed migration leading to sequential encounters with APCs. These contacts can last a few minutes in the context of immature IS or be extended in time as in the case of a mature IS resulting from cognate antigen recognition. (A) (Vicente-Manzanares and Sanchez-Madrid, 2004); (B) and (C) (Dupre et al., 2015).

1.5.2 Polarization of migrating T cells

Acquisition of spatial asymmetry or cellular polarization is a fundamental property of most eukaryotic cells and can be permanent, as in the case of an epithelial cell, or transitory, as in migrating lymphocytes or fibroblasts). By acquiring a polarized morphology, the cell establishes and keeps physically separated and functionally differentiated membrane domains (Figure 1.8A). The spatial organization and distinct composition of these domains are essential for a number of basic cellular and physiological processes such as diverse as cell-cell interaction, vesicle secretion, cellular immunity, cell migration, differentiation, development, tissue function and morphogenesis.

In order to migrate, T lymphocytes have to acquire a polarized shape described as a "hand-mirror" which permits conversion of mechanical forces into net cell locomotion

(Figure 1.8) (Stossel, 1993). Polarization is a complex process in which several regulatory pathways integrating spatial and temporal actin cytoskeleton components are involved (Gomez-Mouton et al., 2001; Ridley et al., 2003). Two highly specialized compartments can be distinguished in a polarized T cell: a leading edge at the front and the uropod at the rear (Figure 1.8A) (Friedl and Brocker, 2000; Sanchez-Madrid and del Pozo, 1999; Serrador et al., 1999). The formation of two differentiated compartments leads to an asymmetrical spatial organization optimal for the establishment of net movement associated with the onset of migratory induction. This includes binding to and crawling on the endothelium, migration within the ECM, and response to chemotactic factors.

The leading edge (LE)

It localizes at the front of the cell (Figure 1.8A). Plasma membrane protrusions namely lamellipodia and filopodia emerge here due to high actin polymerization rates defining the direction of migration (Figure 1.8B). This compartment is thereby enriched in F-actin and actin-binding proteins such as talin, vinculin or α-actinin. Chemokine receptors are also concentrated at this end and they direct cell migration by sensing the chemotactic gradient (Gomez-Mouton et al., 2001; Nieto et al., 1997). Molecules involved in the formation of adhesion contacts such as LFA-1 ($\alpha_L\beta_2$ integrin) and the focal adhesion (FAK) localize to this area of the cell and mediate adhesion of the protruded membrane to the substrate (Campanero et al., 1994; Friedl and Brocker, 2000; Nieto et al., 1997).

The proteins N-WASP and WAVE regulate the formation of protrusions at the front of a migrating cell. As reviewed above, they act as effectors of small GTPases and this subsequently regulate Arp2/3, which ultimately mediates branched actin polymerization. However, different small GTPases are involved in generating different types of protrusions. Lamellipodia require Rac activity whereas Filopodia are dependent on Rho and Cdc42. The supramolecular design of these two types of structures enable the cell to perform different functions in relation to migration: by forming lamellipodia the cell elongates increases vastly the length of its plasma membrane, while filopodia, with paralleled actin bundles starting at the plasma membrane, serve as “sensors” to explore the extracellular environment (Figure 1.8A) (Borisy and Svitkina, 2000; Dupre et al., 2015; Samstag et al., 2003). Furthermore, focal adhesions contribute to the stabilization of actin filaments and require activation of integrins which are recruited to the nascent adhesions. Other proteins regulated by Rac like Talin, PKC, Rap1 and PI3K are also involved in actin-driven formation of protrusions (Webb et al., 2002).

The uropod

It is a narrow cytoplasmic projection at the trailing edge of the cell (Figure 1.8A). The motor protein myosin II concentrates here (Campanero et al., 1994; Serrador et al., 1997). Dimers of this protein form bipolar filaments which associate with actin filaments and generate ATP-dependent motion by pulling two actin filaments against each other (Jay et al., 1995; Korn and Hammer, 1988). The intracellular adhesion

molecules (ICAM)-1, -2 and -3, CD43, CD44 and β1 integrins are also polarized to this part of the cell (del Pozo et al., 1995; Friedl and Brocker, 2000; Serrador et al., 1997). The actin-binding proteins of the ezrin radixin moesin (ERM) family link physically the plasma membrane and F-actin (Tsukita and Yonemura, 1999). These proteins activity is dependent on phosphorylation at a threonine residue at the C-terminal by Rho as well as on PIP_2 binding to the N-terminus. When inactivated by dephosphorylation and PIP_2 dissociation, N- and C-termini of proteins of the ERM family interact intramollecularlly preventing binding between the plasma membrane and F-actin. As a result, the actin cytoskeleton disorganizes what is a pre-requisite for adhesive interactions disassembly (Samstag et al., 2003; Tsukita and Yonemura, 1999). In that regard, myosin II has also been suggested to be important by promoting break-down of adhesion contacts with the substrate by direct application of physical stress which results in disruption of the intracellular interaction between integrins and the actin cytoskeleton and/or with their extracellular ligands (Cox and Huttenlocher, 1998).

Thus, polarization enables T cells to acquire the structural organization required to perform amoeboid motility. A fundamental characteristic of this migration mode is the ability to form protrusions at the leading edge of the cell and not at the uropod (Figure 1.8B). To spatially coordinate this, small GTPases are essential. Rac largely controls leading edge protrusion whereas Rho induces lack of them at the uropod. Cdc42 serves as guide to control the overall cellular polarity acquisition process (Bardi et al., 2003; Bustelo, 2002; del Pozo et al., 1999; Lee et al., 2004; Ratner et al., 2003).

1.5.3 Transendothelial migration (TEM)

As discussed above, migration of T cells throughout the body is essential for immunosurveillance and repair of peripheral tissues as well as for efficient immune responses against pathogens and tumor cells. This implies frequent crossing of endothelial barriers limiting blood vessel walls, a complex multistep process known as transendothelial migration (TEM) (Figure 1.9). This is the case for naïve T cells migrating from the bloodstream into the lymph nodes across the high endothelial venules (HEV) or for effector T cells after recognition of cognate antigen when they travel into sites of inflammation. Initially, T cells circulating in the bloodstream establish weak, transient interactions with the endothelial cells of the walls of HEV or postacapillary venules in the vicinity of an inflamed tissue. These weak interactions trigger a process called the leukocyte-adhesion cascade which consists of selectin-mediated tethering and rolling on the endothelium, integrin activation, subsequent firm arrest and crawling and, finally, TEM (Ley et al., 2007; Nourshargh et al., 2010). These sequential but overlapping steps are controlled by the integration of chemotactic and exit cues, mainly chemokines displayed on the luminal and basolateral sides of the endothelium as well as the underlying extravascular tissue and lipid chemoattractants (Alon and Shulman, 2011; Rot and von Andrian, 2004). A crucial step for these events to take place is the activation of the endothelial cells. There are two modes of endothelial activation: a rapid (within minutes) and protein-synthesis-independent leading to expression of preformed adhesion molecules on the plasma membrane (e.g.

P-selectin), and a slow (within hours) and protein-synthesis-dependent mode which involves transcription of trafficking molecules, namely selectins, integrin ligands and chemoattractants. This occurs as a result of inflammatory stimuli such as histamine and PAF in the case of rapid activation and cytokines in the case of slow activation (e.g. interleukin-1β (IL-1β) and tumor necrosis factor (TNF)) (Pober and Sessa, 2007). Circulating T cells in the blood are covered with short microvilli. These structures contain parallel bundles of highly dynamic actin filaments (Majstoravich et al., 2004). The tips of these microvilli is rich in low-affinity adhesion molecules such as L-selectin and α4β7 integrin (Stein et al., 1999), whereas molecules that mediate firm arrest, e.g. LFA-1, are distributed randomly or excluded from microvilli (Berlin et al., 1995; Bruehl et al., 1996). Interaction between lymphocyte glycoproteins (e.g. P-selectin glycoprotein ligand (PSGL-1)) on microvilli with P-selectin and E-selectin molecules on the surface of endothelial cells results in the formation of reversible interactions mediating not only tetherin of lymphocytes on the endothelium (Ley et al., 2007; Zarbock et al., 2011) in the collapse of microvilli (Alon and Feigelson, 2002). PSGL-1 on free lymphocytes in the bloodstream can bind to L-selectin on attached lymphocytes leading to secondary lymphocyte tetherin (Eriksson et al., 2001; Sperandio et al., 2003). Flattening slows down rolling and facilitates topographical availability of chemokine receptors and integrins. The former recognize endothelium-displayed chemokines which mediates the stimulation of GEFs or JAK protein tyrosine kinases and in turn different members of the Rho GTPase family leading to inside-out and the activation of integrins on the surface of the lymphocyte (Alon and Dustin, 2007; Alon and Feigelson, 2009; Carman and Springer, 2003; Ley et al., 2007; Montresor et al., 2013). Activated lymphocyte integrins such as LFA-1, VLA-4 and α4β7 bind to their ligands, e.g. ICAM-1 and vascular adhesion molecule (VCAM)-1, inducing lymphocyte arrest (Figure 1.9) (Nourshargh and Alon, 2014). Upon arrest, lymphocytes protrude and directly translocate their body through the endothelium or crawl in search for exit cues (Phillipson et al., 2009). Crawling requires lymphocyte polarization, relies on the actomyosin machinery and is chemokine-directed and integrin-dependent (Hyun et al., 2012; Shulman et al., 2009). TEM can occur through junctions between adjacent endothelial cells (paracellular TEM) or through the body of these cells (transcellular TEM), being the former the preferred route (Muller, 2011; Nourshargh et al., 2010). Both modes are the result of lymphocyte-driven changes in the endothelium such as the formation of adhesion platforms including ICAM-1, VCAM-1 and different tetraspanins and pentaspanins (e.g. CD9, CD151 or CD47, respectively) (Azcutia et al., 2012). Ligation of these molecules by their ligands on lymphocytes induce signaling in the endothelial cells leading to reduced endothelial barrier properties such as an increase in intracellular Ca2+ (Huang et al., 1993; Pfau et al., 1995), reactive oxygen species (ROS) generation (Deem et al., 2007; Martinelli et al., 2009), activation of Src (Allingham et al., 2007) or activation of p38 mitogen-activated protein kinase (MAPK) (Hu et al., 2000). These events result in the modulation of a number of endothelial targets and the reorganization of the cytoskeleton promoting junction opening and lymphocyte crossing of the endothelial barrier. VE-cadherin internalization and recycling has also been shown to be needed for successful lymphocyte diapedesis

(Weber et al., 2007). In the context of effector T cells migrating into inflamed tissues, TEM is related to enhanced survival and increased effector functions (Nourshargh et al., 2010; Stark et al., 2013). Therefore, TEM not only represents a regulated process for lymphocytes to home to access damaged or infected tissues but also contributes to priming of the extravasated lymphocytes to an efficient immune response performance.

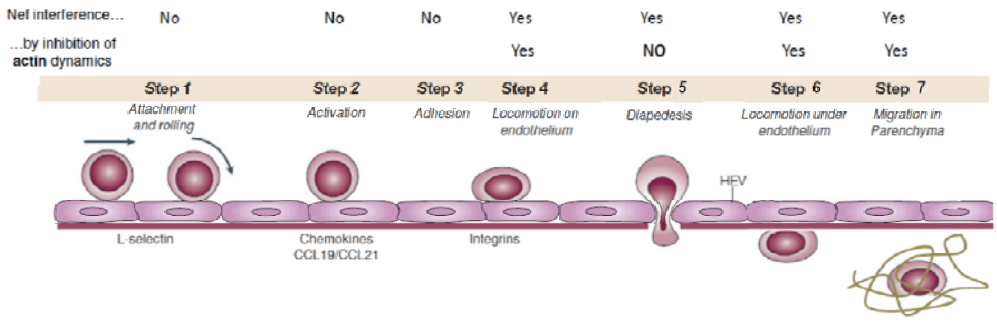

Figure 1.9 Transendothelial migration process. The scheme shows the most important steps of this complex process. Most relevant molecules mediating T cell-endothelial cell interactions are indicated as well as the influence of Nef on the individual steps and the dependency on its ability to disrupt actin dynamics. (von Andrian and Mempel, 2003)

1.5.4 Extracellular vesicle release

Cell communication is essential in multicellular organisms. It can take place by direct cell-cell contact or via transfer of secreted molecules or more complex cell-derived structures such as extracellular vesicles (EV) (Gyorgy et al., 2011). These vesicles are produced by most cell types and are present in the extracellular space. According to their endosomal or plasma membrane origin, they can be classified as exosomes or microvesicles (MVs) (also known as shedding vesicles or ectosomes), respectively (Raposo and Stoorvogel, 2013). MVs are very variable in size ranging between 100 and 1,000 nm in diameter and they are formed by outward budding of the plasma membrane upon activation and crosslinking of cell surface receptors or apoptosis and the correspondent intracellular Ca^{2+} increase (Ratajczak et al., 2006b). The release rate of MVs in steady state is typically low except for tumor cells which release them constantly (Muralidharan-Chari et al., 2010; Raposo and Stoorvogel, 2013). Exosomes, on the other hand, are smaller with sizes comparable to those of viruses ranging between 50 and 120 nm in diameter. These vesicles are formed by inward budding of the membrane of late endosomes resulting in the formation of multivesicular bodies (MVBs). MVBs can then follow two different pathways: either they fuse to lysosomes for degradation, or they fuse to the plasma membrane to release their content to the extracellular space, the so called exosomes (Mittelbrunn and Sanchez-Madrid, 2012; Yanez-Mo et al., 2015). Despite of their important role in intercellular communication, the overall function of exosomes remains unclear. Though, multiple functions of exosomes in immune modulation, tumorigenesis and the propagation of neurodegenerative diseases have been described. Nowadays exosomes raise

indisputable interest as biomarkers and therapeutic targets (Yanez-Mo et al., 2015). Of note, the membrane of both types of EVs displays an array of proteins which are not randomly included. Unknown protein sorting mechanisms play a role in the formation of these vesicles. However, a difference between them is the fact that MVs contain sorted plasma membrane proteins (Cocucci et al., 2007; Moskovich and Fishelson, 2007), while exosomes contain proteins original form the plasma membrane, the endocytic pathway and the cytoplasm (Raposo and Stoorvogel, 2013; Thery et al., 2002; Vlassov et al., 2012). The composition of exosomes reflects their endosomal origin including proteins involved in the formation of MVBs including ESCRT proteins, apoptosis-linked gene 2-interactin protein X (Alix), tumor susceptibility gene 101 (tsg101), Rab GTPases or tetraspanins such as CD9, CD63 and CD81. In the context of T cell biology, exosomes released by these cells have been described to be directed specifically to IS-engaged APCs in a polarized fashion and to contain microRNAs (miRNA) different from the repertoire present in their producer cells (Choudhuri et al., 2014; Gutierrez-Vazquez et al., 2013; Mittelbrunn et al., 2011). Immune cell and T cell-specific proteins have been identified to be enriched in exosomes derived from T cells such as TCR/CD3/ζ, MHC-I and –II, LFA-1, CXCR4 or Lck (Blanchard et al., 2002; Miguet et al., 2006). Additionally, a new type of vesicles derived from a T cell line was described. The so called microvesicle clusters (MCs) differ in the generation mechanism with other EVs which consists in the accumulation of small vesicles underneath the plasma membrane and the subsequent formation of a large ball-like structure containing the small vesicles with the eventual rupture of the plasma membrane limiting this structure and release of the small vesicle to the extracellular space (Muratori et al., 2009).

1.6 Effects of HIV-1 Nef on T cell biology

HIV modulates several aspects of T cell biology to its own benefit. Nef seems to be the master manipulator leading to such modulation. The following provides a review on how Nef affects capital T cell functions such as migration or EV secretion.

1.6.1 Nef interferes with T cell migration

Nef potently interferes with T cell migration (Choe et al., 2002; Hrecka et al., 2005; Janardhan et al., 2004; Lee et al., 2008; Nobile et al., 2010; Park and He, 2009; Stolp et al., 2010; Stolp et al., 2009). As in most other Nef-mediated effects, T cell migration impairment has been linked to Nef-mediated downregulation of membrane receptors and adhesion molecules. Nef reduces surface expression levels of several chemokine receptors including CCR1, CCR2, CCR3, CCR4, CCR5, CXCR1, CXCR2 and CXCR4 (Chandrasekaran et al., 2014; Hrecka et al., 2005; Michel et al., 2005; Michel et al., 2006; Zaitseva et al., 2003). Despite marginal Nef-mediated downregulation of CCR7, a receptor involved in sensing of the chemokines CCL19 and CCL21 expressed by reticular stromal cells in lymphoid organs and required to direct T cell migration within them, it has been reported that Vpu reduces surface levels of this molecule contributing to Nef-induced overall migration defect (Ramirez et al., 2014). The effects of Nef on T cell migration however do not depend on CCR downregulation. Nef inhibits T cell

chemotaxis *in vitro* as reflected from transwell assays in which Nef-expressing cells migrate with significantly reduced frequency through filter pores towards a chemoattractant such as SDF-1α (Choe et al., 2002; Park and He, 2009; Stolp et al., 2010; Stolp et al., 2009). From a molecular point of view, Nef effects on chemotaxis and migration rely on its ability to disrupt actin cytoskeleton by interacting with PAK-2 and inducing substrate specificity change towards cofilin. Dependency on this molecular mechanism has been shown not only *in vitro* but also in complex *in vivo* experimental settings, such as migration of Nef-expressing primordial germ cells in zebra fish (Stolp and Fackler, 2011; Stolp et al., 2009) or homing of Nef-expressing T lymphocytes in mice models (Fujii et al., 2011; Stolp et al., 2012). Nef has been shown to impair T cell homing by strongly reducing the capacity of Nef-expressing T lymphocytes to migrate across HEVs *in vivo* using intravital 2-photon microscopy (Stolp et al., 2012). Influence of Nef on TEM was then assessed *in vitro* using an experimental system that incorporated primary endothelial murine cells as well as controlled shear flow. Transmigration across the endothelium of murine T cells expressing Nef was notably reduced when compared to control cells. In addition, dependency on the PAK-2 binding motif of Nef and, thereby, on impairment of actin cytoskeleton rearrangements by Nef was required for all TEM steps affected except diapedesis (Stolp et al., 2012). Nef-mediated block of TEM thereby relies on other, uncharacterized interaction motif of Nef. Furthermore, Nef as well as Vpu have been also described to induce downmodulation of L-selectin (CD62L), a molecule involved in T lymphocyte tethering and rolling leading to TEM by interacting to its ligands of endothelial cells at HEVs (e.g. GlyCAM-1 or CD34) or other lymphocytes (e.g. PSGL-1) (Haller et al., 2014; Stolp et al., 2012; Vassena et al., 2015). The Nef-mediated interference with T cell migration was observed already at the early stage of polarization. Park and He described a defect polarization in Jurkat T cells on a 2D system consisting of anti-LFA-1-coated surfaces linked to expression of Nef which correlated to impaired chemotaxis and impaired migration through primary endothelial cells towards a chemoattractant (Park and He, 2009). Consistently, Stolp et al. observed a block in polarization of Nef-expressing murine T cells upon adherence to a primary endothelial cell monolayer under shear flow, a system which resembles better a physiologically relevant environment for T lymphocytes (Stolp et al., 2012). In this context, the inability of Nef-expressing cells to polarize correlated to the lack of locomotion on the endothelium and absence of diapedesis events. Of note, the block in polarization was also observed upon plating these cells on fibronectin-coated surfaces and stimulation with Sdf-1α (Stolp et al., 2012). Remarkably, Nef also inhibits the amoeboid motility of macrophages while it enhances the mesenchymal migration of these cells, a migration mode that relies on matrix degradation (Verollet et al., 2015).

1.6.2 Nef induces EV secretion in T cells

Across evolution EV biogenesis and release pathways have been modulated and exploited by various pathogens like viruses providing a potential escape mechanism from the host immune response. Studies with retroviruses showed that viruses are able to hijack the cellular machinery involved in EV generation for the budding of the viral

progeny (Schorey and Bhatnagar, 2008). HIV-1 Nef has been reported to induce alterations within the endosomal compartment and to increase endosome, lysosome and late endosome/MVB formation (Campbell et al., 2008; Sandrin and Cosset, 2006; Schwartz et al., 1995a). Muratori et al. described a Nef-mediated induction of EV release and the self-incorporation of the viral factor in these vesicles with the subsequent delivery to bystander cells (Muratori et al., 2009). These authors postulate the evolutionary advantage of Nef-delivered on exosomes to potentially increase detrimental bystander effects on non-infected cells as well as the induction of viral particle release. Lenassi et al. could also observe Nef incorporation into EVs derived from infected or Nef-expressing immortalized and primary T cells reported and that these Nef-containing EVs promoted apoptotic death of bystander cells (Lenassi et al., 2010). Consistently, Nef-containing exosomes were detected in the cultures of HIV-1 infected cells and the plasma of infected patients (Ali et al., 2010; Campbell et al., 2008; Raymond et al., 2011). Further investigation showed that Nef induces the release of TACE (ADAM-17)/ADAM-10 containing EVs through an intricate mechanism regulated by paxillin which leads to secretion of TNF-α in cells taking up those exosomes. Ultimately, TNF-α secretion is supposed to promote HIV-1 replication in infected cells (Lee et al., 2013). Extensive mutagenesis analysis revealed a conserved motif (VGFPV 66-70) whose integrity is required for Nef-induced EV secretion in immortalized T cell lines transfected for expression of Nef, namely the secretion modification region (SMR) (Ali et al., 2010). This motif forms a binding pocket that facilitates interaction with mortalin, an event suggested to be part of the molecular mechanism underlying Nef-induced EV secretion (Shelton et al., 2012). Unlike what was observed for isolated expression of Nef, mutation in the SMR did not abrogate Nef-induced EV secretion in infected T cell line cultures or humanized mice. However, apoptosis and CD4 T cell depletion were reduced, respectively (Konadu et al., 2015). In addition, humanized mice infected with the virus bearing the SMR Nef mutant displayed decreased cytokine production as compared to those infected with wt Nef encoding virus. Nef-containing exosomes also contain several endogenous retroviral RNA sequences, e.g. transactivation response element miRNA (Narayanan et al., 2013). Finally, the biological relevance of Nef-induced EV release has been highlighted by studies that found not only that these vesicles can render resting CD4 T cells permissive to HIV-1 infection (Arenaccio et al., 2014) but also that they are able to reactivate the latent provirus in a TACE-dependent manner (Arenaccio et al., 2015).

1.7 Aims of the study

The work presented in this thesis is divided in two parts. A minor project focuses on the study of HIV-1 Nef and Vpu redundant functions. The second part corresponds to the main PhD project and investigates the effects of these two proteins on T cell polarity in the context of T cell migration.

1.7.1 Overlapping functions of Nef and Vpu

Proteins of the TSPANs superfamily have been described to be involved in the replication cycle of HIV, conforming platforms for virion assembly and budding

(Nydegger et al., 2006). Recent work of our lab has identified members of the TSPAN family of proteins as specific targets for cell surface downregulation by Nef and Vpu (Haller et al., 2014). The mechanism by which this process takes place remains largely unidentified. Interestingly, Nef and Vpu share a number of redundant functions, many of them related to downregulation of host cell receptors and surface molecules. Of note, no comparative studies have been performed on the interference in host cell trafficking by these two viral factors, e.g. the transport of the host kinase Lck. In addition, it has been described that HIV-1 Nef stimulates the secretion of exosomes in Nef-expressing cells and in infected primary T cells (Lenassi et al., 2010; Muratori et al., 2009). Importantly, nothing was known regarding the effects of Vpu on exosome release at the time this project started. This minor thesis project aimed to provide an answer to the following questions:

- Which are the determinants involved in the mechanism of downregulation of TSPANS by Nef and Vpu?
- Are there other not yet defined shared functions between Nef and Vpu? More specifically:
    - Is Vpu able to block anterograde transport of the host kinase Lck?
    - Is Vpu able to enhance EV release and to induce its own incorporation into EVs?
- Does the effect of Nef on EV release account for the mechanism of TSPAN downregulation from the cell surface? Could this be the case for Vpu as well?

1.7.2 Effects of Nef on T cell polarization and TEM

In the most recent work of our laboratory concerning Nef-mediated T cell migration impairment, Stolp et al. described that *in vivo* and *ex vivo* the predominant inhibition of murine $CD4^+$ T lymphocytes by Nef is at the level of transmigration through endothelial monolayers as well as subsequent subendothelial migration (Stolp et al., 2012). While the inhibition of subendothelial migration is mediated via the effects of Nef on actin dynamics, Nef effects on transmigration involve other, yet uncharacterized protein interactions. Notably, the inability to transmigrate of Nef-expressing cells was paralleled by a marked reduction of cell polarization. This results however have not been yet confirmed for human cells. In this context, we hypothesize that the impairment of T cell polarity mediated by Nef determines the observed defect in TEM, more specifically in diapedesis. This main thesis project aims at testing this hypothesis and, more concretely, at finding an answer to these questions:

- Does the Nef-mediated loss of polarity seen on transduced murine T cells also occur in primary human T cells in the context of infection?
- Is this effect conserved among lentiviral Nef proteins?
- Which are the molecular determinants of Nef involved in this activity, others than those responsible for actin rearrangements disruption?
- Which is the mechanism by which Nef disrupts T cell polarity and the relevance of it in the frame of HIV-1 infection?

- What is the contribution of the disruption in polarity on the ability of T cells to migrate through the endothelium?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Name	Company
Acrylamide Rothipherese Gel 40	Roth
Agarose NEEO	Roth
Ampicillin	Roth
Anti-CD3 containing OKT3 hybridoma supernatant	Kindly provided by Andrea Imle
BEZ235	Synkinase
BSA 100x	NEB
Calcium Chloride	Sigma Aldrich
CCL-21	Peprotech
Cell Tracker Blue CMAC	Thermo Fisher
Cell Tracker Green CMFDA	Thermo Fisher
Cell Tracker Orange CMRA	Thermo Fisher
ColorPlus Prestained Protein Marker (7–175 kDa)	New England Biolabs
Compensation beads	BD
ConcavalinA	Sigma Aldrich
Dabrafenib	SelleckChem
DNA ladder 1kb Plus	Life Technologies
dNTP Set	Fermentas
ECL (supersignal west pico/femto)	Pierce Biotechologies
Gelatin	Merck
GFP-trap beads	chromotek
Glutamin	Gibco
Gö6983	Santa Cruz
HBSS	Sigma Aldrich
High concentration rat Collagen I	BD/Corning
Hoechst 33258	Life Technologies
Imatinib/Gleevec	SelleckChem
IL-2	Biomol
IPA3	Sigma
JetPei	Peqlab
Kanamycin	Roth
Linmount	Linearis
Luciferase Assay Substrate	Promega
Lysisbuffer for cell culture for Luciferase assay 5x	Promega
LY294002 (SF1126)	Synkinase

Matrigel	BD Biosciences/Corning
Magnesium Chloride Hexahydrate	Merck Millipore
MK-2206	Synkinase
Mowiol 4-88 reagent	Merck Millipore
Midori Green Advance	Nippon genetics
Pei	Sigma Aldrich
Penicillin, Streptomycin	Sigma Aldrich
Phalloidin-TRITC	Sigma Aldrich
PIR3.5	Santa Cruz
PKH 26	Sigma Aldrich
PMSF	Sigma Aldrich
Polybrene	Sigma Aldrich
Poly-L-Lysine (0.01% solution)	Sigma Aldrich
Powdered milk	Roth
PP2	SelleckChem
Protease inhibitor cocktail	Sigma Aldrich
Puromycin	Sigma Aldrich
RNAse Inhibitor, Ribolock	Fermentas
RiboLock RNase Inhibitor	Thermo Scientific
Rottlerin	Santa Cruz
SDF-1α, human (CXCL12)	Peprotech
Sorafenib	Synkinase
Sunitinib	Synkinase
SYBR Green	Invitrogen
TEMED	Roth
TMB	Serva
TNF-a	R&D
Total Exosome Isolation (from cell culture) Reagent	Invitrogen
Trametinib (GSK1120212)	Synkinase
Tris	Roth
Triton x-100	Merck Millipore
Trytan Blue	Life Technologies
Vemurafenib	Synkinase
β-Mercaptoethanol	Sigma Aldrich
X-Gal	Thermo Scientific
1 Kb plus DNA ladder	Life technologies

2.1.2 Buffers and solutions

Name	Component	Concentration
Blotting buffer	TrisHCl, pH 8.8	19 mM
	SDS	0.1% (w/v)
	Glycine	192 mM
	Methanol	20% (v/v)
Carbonate solution	Na2CO3	0.2 M (2.2 g)
	destilled H20	100ml
Bicarbonate solution	NaHCO3	0.2 M (1.68 g)
	destilled H20	100 ml
Carbonate/bicarbonate tampon buffer (pH 9.2)	Carbonate solution	4 ml
	Bicarbonate solution	46 ml
	destilled H20	up to 200 ml
DNA loading buffer	TrisHCl, pH 7.5	50 mM
	EDTA	50 mM
	Glyerol	50% (v/v)
	Bromophenol blue	0.25% (w/v)
KEB lysis buffer	EDTA	2 mM
	Glycerol	10% (v/v)
	NP-40	0,5% (v/v)
	NaCl	137 mM
	TrisHCl, pH 8	50 mM
MACS Buffer	PBS	1x
	EDTA	2 mM
	inact. FBS	0.5% (v/v)
3x Non-reducing sample buffer (SDS-PAGE)	TrisHCL, pH 6.8	125 mM
	SDS	2% (w/v)
	Glycerin	10% (w/v)
	Bromophenol blue	2% (w/v)
10x PBS	PBS Dulbecco (Biochrom), pH 7.4	1 pkg/l
1x PBST	Tween 20 in 1x PBS	0.1% (v/v)
3% PFA	paraformaldehyde in 1x PBS	3% (w/v)
Ponceau	Acetic Acid	5% (v/v)
	Ponceau S Red	0.2% (w/v)
RIPA Buffer	Tris-HCl, pH 8	50 mM
	NaCl	150 mM
	SDS	0.1% (w/v)
	Natrium deoxycholate	0.5% (w/v)

	Triton-X 100	1% (v/v)
	Protease inhibitors cocktail	1:1000
Running buffer (SDS-PAGE)	TrisHCl	19 mM
	SDS	0.1% (w/v)
	Glycine	192 mM
6x Sample buffer (SDS-PAGE)	TrisHCl, pH 6.8	390 mM
	SDS	10% (w/v)
	β-Mercaptoethanol	30% (v/v)
	Glycerol	30% (v/v)
	Bromophenol blue	tip of spatula
4x Separating gel stock solution, pH 8.8	Tris	1.5 M
	SDS	0.4% (w/v)
Stacking gel stock solution, pH 6.8	Tris	0.6 M
	SDS	0.4% (w/v)
20% sucrose solution	sucrose in 1x PBS	20% (w/v)
SG-PERT 10x PCR buffer	Tris-HCl, pH 8.0	50 mM
	KCl	200 mM
	(NH4)2SO4	200 mM
SG-PERT 2x lysis buffer	Tris-HCL, pH 7.4	100 mM
	KCl	50 mM
	Glycerol	40%
	Triton-X100	0.25%
SG-PERT 2x reaction buffer 1x PCR buffer	MgCl2	10 mM
	2x BSA	
	dNTP	400 µM
	Forward primer	1:00 PM
	Reverse primer	1 pM
	MS2 RNA	8 ng
	SYBR Green	1:10000
50x TAE buffer, pH 8.3	Tris	2 M
	EDTA	0.1 M
	NaAc	1 M
β-Gal solution	Ferrocyanide	3 mM
	MgCl2	1 mM
	PBS	1x

2.1.3 SDS-PAGE gel

	10% Separating gel	Stacking gel
SDS Seperating Gel stock	1 ml	-
SDS Stacking Gel stock	-	0.3 ml
H2O	1.7 ml	0.8 ml
30% Acrylamide	1.3 ml	200 µl
10% APS	13 µl	8 µl
TEMED	6 µl	3 µl

2.1.4 Antibodies

Antibody	Company/Source	Technique	Dilution
APC mouse anti-human CD317	Biolegend	FACS	1:40
APC mouse anti-human CD4	BD Biosciences	FACS	1:30
APC mouse anti-human HLA-ABC	BD Biosciences	FACS	1:20
APC Mouse anti-human PECAM-1, CD31	eBiosciences	FACS	1:20
FITC Mouse anti-human E-Selectin, CD62E	eBiosciences	FACS	1:20
FITC Mouse anti-human ICAM-1, CD54	Immunotools	FACS	1:20
FITC Mouse anti-human VCAM-1, CD106	eBiosciences	FACS	1:20
Goat anti-Ezrin	Santa Cruz	IF	1:500
Goat anti-mouse IgG, Fc Fragment	Jackson ImmunoResearch (Dianova)	Activating Surface (Coat)	1:1000
Goat anti-Talin	Santa Cruz	IF	1:1000
mouse-anti-CA (183)	kindly provided by Hans-Georg Kräusslich	ELISA	1:1000
Mouse anti-Calnexin	Santa Cruz	WB	1:2000
Mouse anti-HIV-1 p24CA	Kindly provided by Hans-Georg Kräusslich	ELISA	1:1000
Mouse anti-HLA-DR-Brilliant Violet 510 (L243)	Biolegend	FACS	1:20
Mouse anti-Hsc70	Santa Cruz	WB	1:4000
Mouse anti-human CD37	BD Biosciences	FACS	1:5
Mouse anti-human CD53	BD Biosciences	FACS	1:5
Mouse anti-human CD63	BD Biosciences	WB	1:1000
		FACS	1:5
Mouse anti-human CD81	BD Biosciences	WB	1:1000
		FACS	1:5
Mouse anti-human E-Cadherin	Invitrogen	IF	1:24

Mouse anti-human Occludin	Invitrogen	IF	1:24
Mouse anti-human-CD28	BD Biosciences	Activating Surface (Coat)	1:1000
Mouse anti-human-CD3	BD Biosciences	Activating Surface (Coat)	1:1000
Mouse anti-human Lck	Santa Cruz	IF	1:50
Mouse α-TfR (monoclonal)	ZYMED Laboratories	IF	1:1000
Rabbit anti-HIV-1 CA	Kindly provided by Hans-Georg Kräusslich	IF	1:200
Rabbit anti-human Claudin-1	Invitrogen	IF	1:24
Rabbit anti-human ZO-1	Invitrogen	IF	1:12
Rabbit anti-PKCδ	Santa Cruz	IF	1:20
Rabbit anti-Vpu (polyclonal)	FabGennix Inc.	WB	1:1000
Rat anti-CD44	Biolegend	IF	1:800
Rat anti-GFP (monoclonal)	Chromotek	WB	1:1000
Secondary antibodies fluorophore-coupled (Alexa Fluor dyes)	Molecular Probes (Invitrogen)	IF	1:2000
Secondary antibodies horseradish peroxidase-coupled	Jackson ImmunoResearch (Dianova)	WB	1:5000
		FACS	1:200
Sheep anti-HIV-1 p24CA	Kindly provided by Barbara Müller	WB	1:5000
Sheep anti-Nef (polyclonal)	NIBSC Center for AIDS	WB	1:2000

2.1.5 Enzymes

Name	Company
Alkaline Phosphatase, Calf Intestinal (CIP)	New England Biolabs
GoTaq Hot Start DNA Polymerase	Promega
Pfu Polymerase	Promega
Phusion High Fidelity DNA Polymerase	New England Biolabs
Restriction Endonucleases	New England Biolabs
T4 DNA Ligase	New England Biolabs
T4 Polynucleotide Kinase	New England Biolabs

2.1.6 Plasmids

Plasmid	Description	Source
GFP (pEGFP-N1)	CMV driven GFP expression, used for cloning of GFP fusion proteins	Clontech
pAdVAntage	Expression enhancer plasmid	Promega
pDisplay.YFP	yfp was cloned into pDisplay via BglII+Klenow and	Kindly provided

	SalI (pDisplay) or NcoI+Klenow and SalI (yfp insert); YFP is expressed on the cell surface	by Barbara Müller
pNL4.3 IRES.pDisplay.YFP	XhoI site and NcoI site were removed in pDisplay.YFP by Quik-Change PCR, amplified fragment was interted into pNL4.3 IRES.GFP by NcoI and XmaI	Kindly provided by Andrea Imle
pNL4.3 HIV-1 SF2 Nef wt IRES.pDisplay.YFP	Insertion a Mlu I site at 3' of nef from pNL4.3 SF2 Nef, amplified fragment was interted into pNL4.3 IRES.pDisplay.YFP by HpaI and MluI	This work
pNL4.3 HIV-1 ΔNef IRES.pDisplay.YFP	XhoI site and NcoI site were removed in Display.YFP by Quik-Change PCR, amplified fragment was interted into pNL4.3 ΔNef IRES.GFP by NcoI and XmaI	Kindly provided by Andrea Imle
pNL4.3 HIV-1 SF2 Nef Δ12-39.wt IRES.pDisplay.YFP	Sewing PCR Using Hpa-I and Mlu-I on a pNL4.3 HIV-1 SF2 Nef.wt IRES.pDisplay.YFP to insert SF2 Δ12-39 Nef	This work
pNL4.3 HIV-1 SF2 Nef AXXA IRES.pDisplay.YFP	Insertion a Mlu I site at 3' of nef from pNL4.3 SF2 Nef AXXA, amplified fragment was interted into pNL4.3 IRES.pDisplay.YFP by HpaI and MluI	This work
pNL4.3 HIV-1 SF2 Nef F195I IRES.pDisplay.YFP	Insertion a Mlu I site at 3' of nef from pNL4.3 SF2 Nef F195I, amplified fragment was interted into pNL4.3 IRES.pDisplay.YFP by HpaI and MluI	This work
pNL4.3 SF2 Nef	proviral vector expressing SF2 Nef. Standard proviral vector for HIV-1 infections	Fackler lab, (Fackler et al., 2006)
pNL4.3 SF2 ΔNef	proviral vector not expressing Nef due to the presence of two stop codons at the Nef ORF	Kindly provided by Frank Kirchoff
pNL4.3 SF2 Δvpu	proviral vector not expressing Nef due to the presence of two stop codons at the Vpu ORF	Kindly provided by Frank Kirchoff
pNL4.3 SF2 ΔNefΔVpu	proviral vector neither expressing Nef nor Vpu due to the presence of stop codons at the respective ORFs	Kindly provided by Frank Kirchoff
pNL4.3 SF2 Δ12-39 Nef	proviral vector expressing SF2 Nef mutant	Fackler lab, (Fackler et al., 2006)
pNL4.3 SF2 F195A Nef	proviral vector expressing SF2 Nef mutant	Fackler lab, (Fackler et al., 2006)
SF2 Nef.GFP (wt)	Standard Nef allele used, expression of Nef fusion proteins by cloning HIV-1 SF2 nef into pEGFP-N1 via BglII and EcoRI	Fackler lab, (Fackler et al., 1999)
SF2 Nef.GFP Δ12-39	SF2 Nef mutant fused to GFP	Fackler lab, (Michel et al., 2005)
SF2 Nef.GFP AxxA	SF2 Nef mutant fused to GFP	Fackler lab, (Michel et al., 2005)
SF2 Nef.GFP E4A4	SF2 Nef mutant fused to GFP	Fackler lab,

		(Michel et al., 2005)
SF2 Nef.GFP EDAA	SF2 Nef mutant fused to GFP	Fackler lab, (Michel et al., 2005)
SF2 Nef.GFP Δ12-39.F195A	SF2 Nef double mutant fused to GFP	This work
SF2 Nef.GFP F195A	SF2 Nef mutant fused to GFP	Fackler lab, (Michel et al., 2005)
SF2 Nef.GFP G2A	SF2 Nef mutant fused to GFP	Fackler lab, (Michel et al., 2005)
SF2 Nef.GFP LLAA	SF2 Nef mutant fused to GFP	Fackler lab, (Michel et al., 2005)
SF2 Nef.GFP VGFAAA	SF2 Nef mutant fused to GFP	Fackler lab, (Michel et al., 2005)
SF2 Nef.GFP S6A	SF2 Nef mutant fused to GFP	(Wolf et al., 2008)
SF2 Nef.GFP M20A	SF2 Nef mutant fused to GFP	(Chaudhry et al., 2005)
HIV-1 NL4.3.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
HIV-1 NA7.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
HIV-1 8161K9.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
HIV-1 YBF30.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
HIV-2 Ben.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
HIV-2 Cbl23.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV cpz Gab2 Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV cpz Tan3 Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV mac239 Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV gsn Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et

		al., 2006)
SIV mon Val Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV Ben Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV blue Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV blue Nef.YFP RRAA	SIV blue Nef mutant fused to YFP	(Rudolph et al., 2009)
SIV blue Nef.YFP R129A	SIV blue Nef mutant fused to YFP	(Rudolph et al., 2009)
SIV blue Nef.YFP ELAA	SIV blue Nef mutant fused to YFP	(Rudolph et al., 2009)
SIV smm Fwr 1 Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV smm Ffm 1 Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV agm Sab Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV agm Tan 1 Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SIV sykes 51 Nef.YFP	Expression of Nef.YFP fusion protein	(Rudolph et al., 2009; Schindler et al., 2006)
SrcN18.GFP	Expression of N-terminal 18 aa of Src fused to GFP protein	Fackler Lab (Pan et al., 2013)
syn Vpu.GFP (wt)	Standard Vpu allele used, expression of Vpu fusion proteins by cloning syn vpu into pEGFP-N1 via EcoRI and BamHI	(Tervo et al., 2011)
syn Vpu.GFP S52/56A	syn Vpu mutant fused to GFP	Fackler Lab
syn Vpu.GFP A14LW22A	syn Vpu mutant fused to GFP	Fackler Lab
syn Vpu.GFP V25Y29G	syn Vpu mutant fused to GFP	Fackler Lab
syn Vpu.GFP R30K31A	syn Vpu mutant fused to GFP	Fackler Lab
syn Vpu.GFP TM VSV-G	syn Vpu mutant fused to GFP	This work, (Haller et al., 2014)
Lck.GFP	Expression of Lck fusion protein by cloning lck into pEGFP-N1	Fackler Lab (Pan et al., 2012)

2.1.7 Primers

Description	Primer	Sequence 5'->3'

Cloning SF2 Nef and Δ12-39, AXXA and F195I mutants into pNL4.3 IRES.pDisplay.YFP	Fwd	ctaaagaatagtgctgttaacttgctcaatgcc
	Rev	gtactacaaagactgctgaacgcgtcggatc

2.1.8 Kits

Name	Source
μMACS GFP Isolation Kit	Miltenyi Biotec
CD4 isolation kit	Miltenyi Biotec
CD8 isolation kit	Miltenyi Biotec
Detach Kit for Endothelial Cells	PromoCell
NucleoBond PC 500	Macherey-Nagel
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel
NucleoSpin Plasmid	Macherey-Nagel
Pierce BCA protein assay	Thermo Scientific
Pierce microBCA protein assay	Thermo Scientific
QIAfilter Plasmid Midi Kit	Qiagen

2.1.9 Bacterial culture media

Name	Component	Concentration/Quantity	Comments
LB	NaCl	86 mM	add H2O and adjust to pH 7.2, autoclave
	Tryptone	0.5% (w/v)	
	Yeast extract	1% (w/v)	
LB agar plates	agar	1.25%	add when warm into plastic petri dishes
	LB		
TB			
Solution A	glycerine	4 ml	autoclave solutions separately and then mix when <60°C
	H2O	900 ml	
	Trypton	12 g	
	Yeast extract	24 g	
Solution B	H2O	100 ml	
	K2HPO4	12.5 g	
	KH2PO4	2.3 g	

2.1.10 Bacterial strains

E. coli	Genotype	Company

strain		
Stble2	F– mcrA Δ(mcrBC-hsdRMS-mrr) recA1 endA1 lon gyrA96 thi supE44 relA1 λ– Δ(lac-proAB)	Life technologies
DH5-α	F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1	Life technologies

2.1.11 Cell culture media

Cell line/type	Components
Adherent cell lines	DMEM (Gibco)
	FCS 10% (v/v) (Biochrom)
	Penicillin (100 U/ml)
	Streptomycin (100 µg/ml)
Suspension cell lines (except CCR7)	RPMI 1640 (Gibco)
	FBS 10% (v/v)
	Penicillin (100 U/ml)
	Streptomycin (100 µg/ml)
CCR7	DMEM
	FBS 10% (v/v)
	Penicillin (100 U/ml)
	Streptomycin (100 µg/ml)
	2 mM glutamine
	10 mM HEPES (pH 7.4)
	1 mM sodium pyruvate
	1x NEAA (Life Technologies)
	45.76 µM β-Mercaptoethanol
Cell freezing medium	90% (v/v) FBS
	10% (v/v) DMSO
HUVECs	Basal Endothelial Cell Growth Medium (PromoCell)
	EGCM supplement mix 1 or 2 (PromoCell)
Cryo-SFM Medium (for endothelial cells)	PromoCell

2.1.12 Eukaryotic cell lines

Cell line	Description	Reference
Jurkat TAg	Acute T cell leukemia-derived cell line expressing the SV40 large T antigen	(Northrop et al., 1993)
Jurkat CCR7	Acute T cell leukemia-derived cell line stably transfected with CCR7	(Ott et al., 2004)
Jurkat E6.1	Acute T cell leukemia-derived	(Weiss et al., 1984)

	cell line, IL-2 producing	
A3.01	Acute lymphoblastic leukemia-derived	(Folks et al., 1985)
HeLa TZM-bl	Human cervical epithelial cancer cell line, HeLa derivative; contains HIV-1 Tat-regulated luciferase and ß-galactosidase reporter genes	(Wei et al., 2002)
HEK 293T	Human embryonic kidney cells expressing the SV40 large T antigen	(Pear et al., 1993)

2.1.13 Primary cells

Name	Source/Company
Human Peripheral Blood Mononuclear Cells (PBMCs)	Buffy Coats obtained form the Heidelberg University Hospital Blood Bank
Human Lymphocytes	Buffy Coats obtained form the Heidelberg University Hospital Blood Bank
Human Umbilical Vein Endothelial Cells	PromoCell

2.1.14 Cell migration media

Medium	Components
MAM	HEPES
	FCS
	Glutamin
Binding Medium	HBSS cation free
	HEPES
	BSA
	Ca2+
	Mg2+

2.1.15 Consumables

Name	Company
µ-dish 50 mm, low (uncoated)	Ibidi
6-, 12-, 48-, 96-well plates (tissue culture)	BD Biosciences
24-well plate	Orange Scientific
96-well U bottom	Orange Scientific
96-well V bottom	BD
Falcon Conical Tubes 15 and 50 ml	BD Biosciences
Cellview Cell Culture Dishes, Glass Bottom (4 compartments)	Greiner bio-one

Conical Tubes 50 ml	Orange Scientific
Counting chamber	Neubauer-improved Marienfeld-Superior
Cover glasses	Thermo Scientific
Cryotubes 1.5 and 2 ml	Greiner bio-one
Electroporation cuvettes 0.4 cm	Life Technologies
F 96 Maxisorb plate	Nunc
LS column	Miltenyi Biotec
LucentBlue X-ray film	Advansta
Microscope slides	Marienfeld-Superior
PCR tubes 0.2 ml	Sarstedt
PCR 8er SoftStrips 0.2 ml	Biozym Scientific GmbH
Protran BA85 Nitrocellulose Membrane	GE Healthcare Life Science
Reaction tubes 1.5ml	Sarstedt
Syringe Driven Filter unit 0.22, 0.45 µm	Merck Millipore
Tissue culture dishes 15 cm	Orange Scientific
Tissue culture flasks T25, T75, T175	BD Biosciences
Transwell filter 3 µm	Corning
Transwell filter 5 µm	Corning
Tubes for Micro-Rack-System 1.2 ml (FACS)	Steinbrenner Laborsysteme
Whatman blotting paper	GE Healthcare Life Sciences
White plates 96 well	Costar

2.1.16 Equipment

Name	**Company**
AutoMACS Pro Seperator	Miltenyi Biotec
Balance (Explorer)	Ohaus
Biofuge Fresco Table top centrifuge	Heraeus
CFX 96 Real Time PCR detector	BioRad
Climatisation control for Nikon microscope	PerkinElmer
Climatisation control for Spinning disc and Zeiss microscope	EMBL
Electrophoresis system 2-D	Hoefer
Flow cytometer FACS Calibur	BD Biosciences
Flow cytometer FACS Verse	BD Biosciences
Gel iX Imager	INTAS Science Imaging
Gene Pulser Xcell	Bio-Rad
Gliseal	Borer Chemie
Infors HT bacterial shaker	Infors
IX81 S1F-3 microscope	Olympus

J2-Hc Centrifuge with JA-20 or F10-6x500/JA-10 rotor	Beckman Coulter
L8-70M and XL-70 Ultracentrifuge with SW28 and SW32 rotor	Beckman Coulter
Luer-Lok 1, 2, 10, 20 and 50 ml syringes	BD Biosciences
Luminoscan Ascent	Thermo Scientific
Megafuge 1.0R	Heraeus
Migration chamber for flow movies (handmade)	Kindly provided by Ruth Lyck
Multiskan EX ELISA reader	Thermo Scientific
NanoPhotometer	Implen
Nanosight NS300	Malvern
Nikon microscope Ti-E	Nikon
OctoMACS Magnet	Miltenyi Biotec
Open-top polyallomer centrifuge tubes 25 x 89 mm	Seton
pH Meter 761 Calimatic	Knick
Semidry blotter	Cti
Stericup Filterng Devices	Millipore
Syringe Pump	Harvard Apparatus
Thermocycler	Biometra, Eppendorf
Ultra-View ERS-6 spinning disc confocal microscope	PerkinElmer

2.1.17 Software

Name	Source
ApE	Wayne Davis
Cell M	Olympus
Cell Quest Pro	BD Biosciences
CFX Manager	BioRad
Cyflogic	CyFlo Ltd
Endnote	Thomson Reuters
Excel	Microsoft
FACSuite	BD Biosciences
FlowJo V10	FlowJo
Graph Pad Prism 5	GraphPad software
Illustrator CS5	Adobe Systems Inc.
ImageJ	NIH
NIS Elements	Nikon
Photoshop CS5	Adobe Systems Inc.

Powerpoint	Microsoft
Quantity One	BioRad
SnapGene Viewer	GSL Biotech
UCSF Chimera 1.10.1	UCSF
Velocity	Perkin Elmer
Word	Microsoft

2.2 Molecular biology methods

2.2.1 Transformation and culture of bacteria

50 µl of StblII or DH5a bacteria were thawn on ice. Bacteria were added onto 1 µg of plasmidic DNA or a ligation reaction. The mix was incubated for 5 min on ice and then transferred to the heat block to undergo heat shock for 90 seconds at 42ºC. Bacteria were immediately taken onto ice and incubated for 10 minutes. 1 ml of LB medium was added and bacterial culture was shaken at 37ºC for one hour. Cultures were then centrifuged, supernatant was discarded and the pellet was resuspended in 50-100 µl LB medium to be spread on LB-agar plates containing the appropriate antibiotic. After overnight growth at 37ºC, a single colony was picked and resuspended in 5 ml (Miniprep) or 200 ml (Maxiprep) of LB medium to be grown overnight.

2.2.2 Isolation of plasmidic DNA from bacteria

2.2.2.1 Miniprep

NocleoSpin® Plasmid kit (Macherey-Nagel) was used according to manufacturer's instructions. 3 ml of bacterial culture were centrifuged (10.000 rpm, 5', 4ºC). The supernatant was discarded and bacteria were resuspended in 300 µl of buffer S1 containing RNase A. After addition of 300 µl of lysis buffer S2, the tubes were inverted 6 times and incubated at room temperature for 5 minutes. 300 µl of buffer S3 were then added to neutralize the reaction. Following a centrifugation step to get rid of the precipitates, the supernatant was transferred and made run through a mini spin column, washed with buffer Wash and eluted with 30 µl Elution buffer in subsequent centrifugation steps at 13.000 rpm during 5 minutes at 4ºC.

2.2.2.2 Maxiprep

Nucleobond PC 500 kit was used following the manufacturer's instructions. 200 ml of bacterial culture were spun (5.000 rpm, 15', 4ºC) and resuspended in 12 ml of ice cold buffer S1. 12 ml of lysis buffer S2 were added and the lysate was incubated for 5 minutes at room temperature. The reaction was then neutralized with 12 ml of ice cold buffer S3 and incubated for 10 minutes on ice. After a centrifugation step to get rid of the cell debris, the clarified lysate was applied to and run through a previously equilibrated Nucleobond 500 PC column and washed twice with washing buffer. Next, bacterial DNA was eluted with 15 ml of elution buffer and precipitated with 15 ml of

isopropanol. After centrifugation the pelleted DNA was washed one last time with 70% ethanol and air-dried. DNA was finally dissolved in 100 µl H_2O for at least 2 hours at 4ºC.

2.2.2.3 DNA concentration and purity

DNA concentration and purity were measured using a Nanophotometer (Implen) and 1 µl of the DNA solution. The DNA concentration was quantified by measuring optical density (OD) at 260 nm. To determine the purity of the isolated DNA protein concentration was quantified by measuring OD at 280 nm. Purity was considered acceptable when the ratio OD_{260}/OD_{280} resulted in a value between 1.8 and 2.

2.2.3 Polymerase chain reaction (PCR)

For each standard PCR reaction the following components were handled on ice and added into 0,2 ml tubes:

Component	Volume
H2O	31 µl
Phusion HF Buffer 5x	10 µl
dNTP (10 mM stock)	1 µl
Forward primer (10 µM predilution)	2.5 µl
Reverse primer (10 µM predilution)	2.5 µl
Template DNA (10 ng/µl)	1 µl
DMSO	1.5 µl
Phusion Polymerase	0.5 µl

The thermocycling program used was the following:

Step	Temperature	Duration	
1) Initial denaturation	98°C	30 sec	
2) Denaturation	98°C	10 sec	
3) Annealing	55°C	30 sec	30 x
4) Elongation	72°C	1 min per kbp	
5) Final extension	72°C	7 min	
6) store		4°C	

5 µl of the obtained PCR products were loaded and run on a 1% agarose gel in order to monitor successful amplification.

2.2.4 Electrophoretic separation of DNA

Separation DNA purification was achieved through electrophoresis in agarose gel. 3 µl of Midori Green were added to 50 ml of 1% agarose in TAE Buffer 1x (w/v). DNA samples were mixed with DNA Sample Buffer prior to being loaded onto the gel. Size was evaluated via comparison to 1 kb DNA Ladder which was, when possible, loaded in a central lane of the gel. Gels were run at 80 V for 30-40 minutes. DNA was then visualized under UV light. If needed, DNA fragments were excised by using a fresh scalpel and the DNA for their subsequent purification.

2.2.5 PCR product and DNA purification

Purification of PCR products as well as of agarose gel-embedded DNA was performed using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. 50 µl of H_20 were used for elution of DNA.

2.2.6 DNA restriction

Both for analytical and preparative restriction, DNA was digested with the appropriate restriction enzymes in the following proportions for a 25 µl reaction:

Component	Volume
Restriction enzyme A	1 µl
Restriction enzyme B	1 µl
DNA	1 µl
CutSmart Buffer	2.5 µl
Nuclease-free water	To 25 µl

For analytical DNA restriction the reaction was incubated at 37ºC for 30 minutes. Incubation time was increased to a minimum of 60 minutes for preparative purposes and more restriction enzyme was added if necessary.

2.2.7 Ligation

In order to perform ligation, the digested and purified vector and insert were mixed in a way that a vector-to-insert molar ratio between 1:2 and 1:3 was achieved. 2 µl of 10x T4 ligase buffer and 1 µl of T4 DNA ligase were added to the DNA mix and incubated for 2h at room temperature before it was used for transformation of bacteria (see).

2.3 Cell biology Methods

2.3.1 Cell line culture

Cells were cultivated in the appropriate culture media at 37°C and 5% CO_2, humidified atmosphere. Passage of adherent cells was carried out 2 to 3 times a week depending on the growth speed. For that purpose, cells were washed with PBS and trypsin/EDTA was added for detachment adjusting the volume so it would just cover the bottom of the

flask with a thin layer (1 ml for T75; 2 ml for T175). Detachment was monitored by eye. A 9x volume of complemented medium was added into the flask to inactivate trypsin and detachment was facilitated by pipetting medium on the still undetached cells. Splitting was carried out at 1:10 dilution (dependent on growth rate and experiment plans). Suspension cell lines were cultured at 0.3-1 x 10^6 cell/ml in their respective vulture media. Splitting was typically performed at 1:4 dilution every 2-3 days, depending on growth rate and experiment plans. A3.01 cells were observed to show higher polarization rates after transfection when denser, so for polarity assay they were allowed to grow up to 2 x 10^6 cell/ml.

2.3.2 Freezing and thawing of cells

Cell line stocks storage was achieved by cryoconservation in liquid nitrogen. To that end, 1x10^7 cells were centrifuged at 1200 rpm for 5 minutes and the pellet was resuspended in 1 ml freezing media and transferred into a cryotube. Cells were frozen at -80°C either in freezing containers for 24 hours before they were taken to a liquid nitrogen-containing tank for long term storage. Thawing of cells was carried by submerging the bottom of the tube in the water-bath at 37°C. Thawed cells were rapidly transferred to 15 ml falcon tubes containing 10 ml of pre-warmed medium and spun down. Cell pellets were resuspended in the appropriate media (see) and cultured initially in low volumes (3-5 ml) which were progressively increased once cells had recovered from freezing-thawing stress. HUVECs were frozen the same gradual way in Cryo-SFM medium (PromoCell) in a concentration of 10 x 10^6 cell/ml. When thawed, they were directly added onto supplemented ECGM.

2.3.3 Cell transfection

2.3.3.1 Electroporation

Transient transfection of plasmidic DNA was achieved by electroporation using a Gene Pulser Xcell (Bio-Rad). To that end, the appropriate number of cells was resuspended in non-supplemented medium, since the presence of FCS is known to diminish transfection efficiency. For A3.01 cells, 18 μg of plasmidic DNA were used and 1 x 10^7 cells were pulsed at 350 V and 950 μF. For CCR7 cells, 60 μg of plasmidic DNA were used and 1 x 10^7 cells were pulsed at 250 V and 950 μF. Immediately after pulsing, cells were transferred into 6-well plates containing 3 or 5 ml pre-warmed supplemented medium (for polarity assay or all other assays, respectively). Cells were then culture at 37ºC and 5% CO_2 until they were used for the subsequent assay.

2.3.3.2 Cationic polymer transfection using Pei

HEK293T cells were seeded 24 hours earlier in 15-cm culture dishes (5x10^6 cells/dish). DNA and Pei were mixed in a 1:3 in 7.5 ml Optimem/culture dish. The transfection mix incubated for 60 min at room temperature and added then dropwise to the cell culture. This type of transfection was used for HIV-1 or lentiviral particle production (see 2.3.8 and 2.3.9).

2.3.4 Treatment with pharmacological inhibitors

The impact of pharmacological inhibition of NAKC members in T cell polarity was evaluated on A3.01 transfected cells. To that end, 2 hours pot-transfection cells were harvested, resuspended in 4 ml of supplemented RPMI, split into two and transferred into p24-well plates. The appropriate volume of each inhibitor was then pipetted onto the culture and, after gentle shacking of the plate, cells were cultured for another 6 hours until being subjected to polarity assay (see).

2.3.5 Isolation of human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Ficoll gradient from buffy coats of healthy individuals provided by the Blood Bank of Heidelberg University Hospital. Buffy coats were diluted 1:1 with sterile 1x PBS and 34 ml of the mix were used to overlay 15 ml of Ficoll. The two-phase obtained solution was centrifuged at room temperature at 2000 rpm without break for 30 min. PBMCs accumulated at the interphase were collected and washed with sterile 1x PBS and centrifuged at 1600 rpm for 7 min. Typically the obtained pellets were red, so lysis of erythrocytes was performed by resuspending the pellet in 5 ml ACK lysis buffer for 5 min, mixing the cell suspension every 1 minute. Next, an excess of 1x PBS was added and cell suspension centrifuged at 1400 rpm for 7 min. After one last wash and centrifugation step (1200 rpm, 7 min), monocyte depletion was performed by incubating 2 x 10^8 cells in 20 ml of supplemented RPMI on a T175 flask in horizontal position for 2 hours. The supernatant was collected and cells were resuspended in fresh RPMI++ containing 10 ng/ml of IL2 at a concentration of 3 x 10^3 cell/ml. Cells were then activated for their subsequent infection (see 2.3.6 and 2.3.7).

2.3.6 Standard lymphocyte activation

For standard activation, 10 ng/ml of IL-2 and 2 μg/ml of PHA-p were added to the supplemented RPMI medium of isolated PBMSs and they were incubated for 72 hours.

2.3.7 Activating surface: anti-CD3/anti-CD28 activation

In order to activate EV-producer A3.01 human T lymphocytes, activating surfaces were used; i.e. anti-CD3 and anti-CD28 antibody-coated surfaces. Goat anti-mouse IgG, Fc Fragment (Jackson Immunoresearch) antibody was diluted in carbonate/bicarbonate buffer solution at a concentration of 0.5 mg/ml and applied on to the surface to be coated. After 2h incubation at room temperature, the antibody solution was sucked away and the surface was washed 3 times with 1x PBS containing 0.5% FCS. Then a carbonate/bicarbonate tampon buffer based solution containing anti-CD3 and anti-CD28 antibody (both at 0.5 mg/ml) was applied to the surface. Following three final washings, cells were added onto the surface (flask or culture plate) and incubated overnight at 37ºC and 5% CO_2.

2.3.8 3 x 3 lymphocyte activation

This protocol was developed by Dr. Andrea Imle in order to optimize infection with pNL43 IRES.pDisplay.YFP sortable HIV-1 virus. Tissue culture flasks were coated

with anti-CD3 antibodies contained in OKT3 hybridoma supernatant overnight at room temperature with mild shaking 24 hours prior PBMC isolation. PBMC from three donors were isolated as detailed in 2.3.4 and, following $CD8^+$ T cell-depletion by magnetic isolation according to the manufacturer's protocol with an autoMACS pro separator (Miltenyi Biotec), cells from the three donor were pooled and subjected to activation in parallel with 0.5 µg/ml PHA (low PHA), 5 µg/ml PHA (high PHA) or activating (anti-CD3 coated) surfaces for 72 h at $3x10^6$ cells/ml. Cells activated by the three different methods were pooled and infected (see).

2.3.9 HIV-1 production

HEK293T cells 6 x 10^6/15-cm culture dish cells were seeded at one day before transfection. For each 15-cm plate, 25 µg of proviral DNA were added in 1.5 ml of Optimem containing 75 µl Pei (1 mg/ml) and mixed by inverting the tube once or twice. The mix was incubated for 60 min at room temperature and added gently dropwise to each culture dish. Supernatants were harvested two and three days after transfection, filtered using 0.45 µm pore size filters. Typically virus was subjected to concentration by ultracentrifugation of the cleared supernatant through a 20% sucrose cushion (24.000 rpm, 1.5-2 h). The pelleted virus was resuspended in supplemented RPMI medium, aliquoted and stored at -80°C. Virus titers were evaluated by p24 ELISA or SG-PERT (see 2.3.17 and 2.) and infectivity was determined by Blue cell assay (see 2.3.19). Half of the cells on a 15 cm culture dish were lysed in 2x sample buffer and protein expression was analyzed by Western Blot.

2.3.10 p24 ELISA

Mouse-anti-CA 183 antibody was used to coat Maxisorb 96-well plates overnight at room temperature in a humidified atmosphere. Following three washing steps with PBS-T, plates were blocked with 200 µl 10% FCS/PBS for 2 h at 37°C. Viral supernatants were inactivated by mixing them with 10% Triton at a 1:10 ratio. 100 µl of these samples and of p24 standard were pipetted into each well of the plate in duplicates. Seven 1:1 serial dilutions of the latter were prepared in PBS-T resulting in concentrations ranging between 50 ng and 0.78 ng p24. A negative PBS-T control was also included. Virus samples were diluted 1:20, 1:200, 1:2000 and 1:20000. The plates were incubated overnight in a wet chamber at room temperature. The next day and after 3 washing steps with PBS-T, plates were incubated with polyclonal rabbit-anti-CA serum for 1 h at 37°C. Plates were washed again prior addition of a peroxidase-conjugated goat-anti-rabbit antibody for 1 h at 37°C. After one last washing step, 100 µl of peroxidase substrate were added to each well. Development of blue color was facilitated by gentle shaking and the reaction was stopped 2-5 min later by the addition of 50 µl of 0.5M H_2SO_4 to each well inducing a color change to yellow. p24 concentration was finally correlated to photometric measurements performed using a Multiskan EX photometer by interpolation in the linear range of the standard curve.

2.3.11 SG-PERT

Quantification of HIV-1 particles in cell culture supernatant was performed by the SYBR Green-I product-enhanced reverse transcriptase (SG-PERT) assay (Pizzato et al.,

2009; Vermeire et al., 2012). To that end, serial 1:10 dilutions of standard virus supernatant with known reverse transcriptase activity and plane dilution buffer as negative control were used. 5µl of virus supernatant samples or standard were lysed with 5µl of 2x SG-PERT lysis buffer containing RNase inhibitor (RiboLock). After 10 minutes incubation at room temperature, 90 µl of 1x SG PERT PCR buffer were added. 10 µl of lysed virus samples and standards were transferred to PCR tubes and 10 µl of 2x SG-PERT reaction buffer containing GoTaq Hotstart Polymerase (1 µl polymerase for 100 µl 2x reaction buffer) were added. Accumulation SYBR Green signal was detected by RT-PCR using the following thermocycling program:

Step	Temperature	Duration	
RT reaction	42°C	20 min	
Taq Activation	95°C	2 min	
Denaturation	95°C	5 sec	30 x
Annealing	60°C	5 sec	
Extension	72°C	15 sec	
Acquisition	80°C	7 sec	

Concentration of RT activity per µl was assessed according to the standard curve using CFX Manager software.

2.3.12 Infectivity assay: blue cell assay

As a way to characterize the infectivity of HIV-1 virus after its production a HeLa based reporter system was used, the so called blue cell assay. The target cells for this assay, TZMbl cells, stably express two reporter genes under the HIV promoter, namely luciferase and β-galactosidase. One day prior to being infected, 5 x 10^3 TZMbl cells were seeded in each well of a 96-well plate and they were cultured at 37ºC and 5% CO_2. The following day, serial dilutions of the concentrated virus were added on the cells starting with a 1:10^2 dilution and diluting it further to 1:10^6 and the plate was placed in the incubator for another 48 hours. After this time, supernatant was sucked away, cells were washed with PBS and they were fixed in ice cold 1:1 (v/v) acetone/methanol mixture. Following incubation at -20ºC, 100 µl of the reaction substrate, β-Gal supplemented with 200 µg/ml X-Gal, were added onto each well and the plate was incubated for 3 hours at 37ºC. After this incubation time the number of infected cells was evaluated by eye due to the blue coloration derived from the β-galactosidase activity and the processing of the substrate and subsequent oxidation of the subproduct. 2 x 10^4 blue cell units of virus were used to infect 2 x 10^5 target cells (see 2.3.9).

2.3.13 HIV-1 infection

Infection of primary lymphocytes was carried out by standard spin infection. The format chosen for infection was a U-bottom, 96-well plate containing 2 x 10^5 PBMCs (CD8+ T cell-depleted or not) and an amount of virus to yield at least 2 x 10^4 blue cell units (MOI=1 or more, being never higher than 3) with a total volume of 100 µl/well. To achieve the mentioned conditions, concentrated virus and activated cells were

resuspended in the required amount of supplemented RPMI medium containing 4 µg/ml of Polybrene. The plates were then centrifuged at 2000 rpm for 90 minutes at 37ºC and incubated 4-6 h at 37ºC and 5% CO_2 prior to medium change to avoid toxicity by polybrene. Cells were cultured in supplemented RPMI containing 10 ng/ml of IL2. 72 h later, cells were either subjected to polarity assay or purified by magnetic sorting (see).

2.3.14 Sorting of pNL4.3 IRES pDisplay.YFP infected cells

Following 3x3 stimulation (see) and infection with pNL4.3 IRES.pDisplay.YFP, $CD8^+$ depleted lymphocytes were sorted and enriched. Magnetic sorting is only successful if more than 2% of lymphocytes are infected, as determined by the number of YFP positive cells detected by FACS. $1x10^7$ total cells were resuspended in 97 µl of ice cold MACS buffer and 3 µl anti-GFP beads (Miltenyi Biotec) were added (for 2% infected cells) or a higher volume depending on the percentage of infected cells. After mixing and 15 minutes incubation at 4ºC, a washing step with MACS buffer was performed to get rid of the non-bound beads and cell were pelleted down. Cells were the resuspended in 500 µl of MACS buffer and applied to pre-equilibrated LS columns placed on an OctoMACS magnet. 4 washing steps with 500 µl MACS buffer were carried out and elution of YFP positive cells took place as columns were removed from the magnet in 1 ml MACS buffer. Eluted cells were subjected to a second round of magnetic selection by being applied to a second pre-equilibrated LS column. After the second elution step, isolation efficiency was determined by flow cytometry and also samples collected during the complete process. As a result of this process YFP positive, infected cells were enriched to 90-96%.

2.3.15 Production of lentiviral vectors

As a way to deliver exogenous DNA into A3.01 cells for expression of GFP, Nef.GFP or Vpu.GFP, lentiviral vectors were used. To that end, 6 x 10^6 HEK293T cells were seeded per 15 cm tissue culture dish. One day later, cells were transfected using 711 µl of Pei and 112.5 µg of the pWPI expression vector including the *gfp*, *nef* and *gfp* or *vpu* and *gfp* genes, 73 µg of pPax2 vector containing all structural lentiviral genes for packaging and the protease gen, 40 µg of VSV-G vector for pseudotyping of the lentiviral particles favoring fusion to most cell types and 11.5 µg of pAdvantage for enhancement of expression. The described amounts of DNA and Pei were added to 7.5 ml optimem medium and the transfection mix was added gently to the cell culture dropwise after 60 minutes incubation at room temperature. Cell supernatant was collected 72 hours later, clarified by filtration through 45 µm pore-size filters and subjected to concentration by 20% sucrose cushion ultracentrifugation (24000 rpm, 1.5-2 h). Pellets were then resuspended in supplemented RPMI, aliquoted (typically 100 µl) and stored at -80ºC.

2.3.16 Transduction of A3.01 cells

One aliquot of the produced lentiviral vectors was used for titration. Different volumes of lentiviral particles (typically 60, 30 and 10 µl) were added onto 2 x 10^6 A3.01 cells contained in a total volume of 400 µl/well of a 24-well plate. Cells were centrifuged at

2000 rpm for 90 minutes and incubated for 48 hours at 37ºC and 5% CO_2. After this time, transduction efficiency was analyzed by measuring the expression of by FACS using untransduced cells as negative control for establishing the gating. Once determined the optimal volume for transduction, A3.01 cells were transduced following the same protocol used for the prior titration.

2.3.17 Lck retargeting assay

One day after transfection, 5 x 10^5 A3.01 cells contained in 50 µl of supplemented RPMI were seeded onto coverslips previously coated with 0.01% Poly-L-Lysin. After incubation 10 minutes incubation at 37ºC, cells were fixed using 3% PFA in PBS (v/v) and incubated for another 15-30 minutes at room temperature, permeabilized with 0.1% Triton-X100 for 2 minutes and blocked in 1% BSA in PBS (v/v) for 30 minutes at room temperature. After washing with 1x PBS, mouse anti-human Lck antibody (1:50, in 1% BSA in PBS, Santa Cruz) was added and incubated for 2 h at room temperature. Coverslips were washed 3 times with 1x PBS and incubation with the secondary goat anti-mouse antibody coupled to Alexa-568 (1:2000, in 1x PBS, Invitrogen) was carried out for 1 h at room temperature protected from light. After 3 washing steps with 1x PBS, coverslips were mounted on microscopy slides using Linmount, let dry for 2 h at room temperature protected from light and stored at 4ºC until analyzed by microscopy. When cotransfected for ectopic expression of Lck.RFP, cells were directly mounted after fixation.

2.3.18 Polarity assay

6 hours post-transfection/post-inhibitor treatment or 72 hours post-infection, 3-5 x 10^5 A3.01 cells or monocyte depleted, standard activated PBMCs respectively, contained in 100 µl of supplemented RPMI were seeded onto Fibronectin-coated coverslips (30 µg/µl, 1 hour at room temperature) and placed in the incubator for 2 hours. After this time, they were taken out of the incubator very gently to avoid shaking and they were fixed using 3% PFA in PBS (v/v) and incubated for another 15-30 minutes at room temperature. Cells were then permeabilized with 0.1% Triton-X100 for 2 minutes and blocked in 1% BSA in PBS (v/v) for 30 minutes at room temperature. Following washing with 1x PBS, mouse anti- CD44 antibody (1:800, in 1% BSA in PBS, Biolegend) was added and incubated for 2 h at room temperature. Coverslips were washed 3 times with 1x PBS and incubation with the secondary goat anti-mouse antibody coupled to Alexa-568 (1:2000, in 1x PBS, Invitrogen) was carried out for 1 h at room temperature protected from light. After washing with 1x PBS, coverslips were mounted using Mowiol, let dry for 2 h at room temperature protected from light and stored at 4ºC until analyzed by microscopy.

2.3.19 Immunofluorescence

Cell phenotype frequencies were quantified using an epifluorescence microscope (Olympus IX81 S1F-3). At least 100 transfected cells or 30 infected cells were evaluated for their phenotype as compared to untransfected or uninfected neighboring. Lck retargeting

confocal images were acquired using a spinning disc confocal microscope (Perkin Elmer Ultra-View ERS-6). All other images were acquired using the epifluorescence microscope.

2.3.20 Flow Cytometry

This technique was used to assess infection efficiency by determination of the percentage of p24-CA positive cells (72 hours post-infection), transfection efficiency by determination of the percentage of GFP or RFP positive cells (typically 48 hours post-transfection), the effect of the expression of Vpu and Nef and their mutants on the expression of cell surface proteins, namely CD4, MHC-I, CD317 and Tetraspanins (48 hours post-transfection), and to quantify the proportion of migrated cells in chemotaxis assay. To this end, cells were inactivated with 10% Triton at a 1:10 ratio for 90 minutes (only for infected cells), washed with PBS and transferred to a suitable format (1.5 ml reaction tubes, 1.2 ml FACS tubes or V-bottom 96-well plate). Following a centrifugation step at 1200 rpm for 5 minutes, cells were resuspended in 100 µl PBS or a 1:20 dilution of the appropriate antibody and incubated on ice in the dark for 60 minutes. After washing with PBS supplemented with 0.5% FCS, cells were taken up in 200 µl PBS 0.5% FBS for flow cytometry analysis using the BD FACS Calibur or the BD FACS Verse. 10,000 events were typically acquired and analyzed using the Cyflogic or FlowJo software.

2.3.21 Chemotaxis assay

24 hours post-transfection, Jurkat CCR7 cells were starved in DMEM medium containing 0.5% FCS for 2-4 hours at 37ºC and 5% CO_2. Meanwhile, 450 µl starvation medium containing 10 ng/ml SDF-1α or not were added to the wells of a 24-well plate. Next, transwells (5 µm pore size, Corning) pre-equilibrated with the same starvation medium were applied onto the wells and simultaneously 100 µl starvation medium containing 1 x 10^6 cells were pipetted into the transwell. Following 2 hours incubation at 37ºC and 5% CO_2, cells were harvested and total GFP-expressing cells cell numbers of the input and of the chemoattracted cells were quantified by flow cytometry. Nef Inhibitory effects were evaluated as the ratio of the percentage of migrated GFP-expressing cells and transfection efficiency relative to GFP controls, which were arbitrarily set to 100%.

2.3.22 Extracellular vesicle isolation

EV-producer cells were cultured in supplemented RPMI medium containing 10% exosome/EV-deprived FCS. The latter was obtained by ultracentrifugation of the normally used, inactivated FCS at 28000 rpm for 16 hours. Two different methods were used to isolate EVs from cell culture supernatant: the standard differential ultracentrifugation method and a commercial isolation reagent.

The standard differential ultracentrifugation method was performed as previously established (Thery et al., 2006). Cell supernatants were harvested, sequentially clarified by centrifugation for 20 minutes at 4ºC at 2000 x g first (JA20 rotor, 5000 rpm) and at then at 10000 x g (JA20 rotor, 11300 rpm) and filtered using 0.45 µm pore size filter

(Merck Millipore). Cleared supernatant was then transferred to ultracentrifugation tubes and run at 100000 x g at 4ºC for 70 minutes to ensure 60 minutes at maximum speed. Extremely carefully to not disturb the pellet and leaving about 100 µl, supernatant was transferred into a fresh tube and run again at the same speed and temperature and for the same time. Even more carefully than before, supernatant was removed and pellet was resuspended in 30-50 µl of KEB lysis buffer.

Alternatively, the Total Exosome Isolation (form cell culture media) reagent (Invitrogen) was used. In this case, cleared supernatants were mixed with the reagent in a 2:1 (v/v) ratio. The mixture was incubated at 4ºC overnight and centrifuged at 10000 x g for 1 hour at 4ºC. Supernatant was extremely carefully removed and pellet was resuspended in 30-50 µl of KEB lysis buffer.

Protein quantification was measured directly after EV isolation using the BCA or the microBCA protein assay kits according to manufacturer's instructions and samples were analyzed by SDS-PAGE and Western Blotting.

2.3.23 Transendothelial migration assay

At day one, 50-mm µ-dishes (Ibidi) were coated with Matrigel (BD/Corning) at a concentration of 0.225 mg/ml. To that end a silicone ring with the size of a 96-well plate well was placed into the dish to delimitate the cell culture area and Matrigel (handled on ice) was added and incubated for 1 hour at room temperature. Freshly acquired, dense HUVECs were detached using the Detach Kit for Endothelial Cells (PromoCell) according to manufacturer's instructions, stained with trypan blue and counted. 2-4 x 10^4 cells contained in 100 µl of ECGM were added into the silicone rings. Four hours later, the medium was replaced for fresh medium containing 200 U/ml of TNF-α and incubated overnight (between16 and 18 hours). The following day, HUVECs were activated by addition of Sdf-1α to a final concentration of 1 µM. $CD8^+$ depleted T lymphocytes stained or not with Cell Tracker dyes (Thermo Fisher) were diluted in MAM (preferentially) or binding medium migration media at a concentration of 2 x 106 cell/ml. Flow movie chamber was assembled and connected to a syringe plugged onto a Syringe Pump (Harvard Apparatus) through the output tube. The input tube was submerged into the lymphocyte solution. Flow movies were acquired using an inverse light microscope (10 or 20 x objective, Nikon Ti-E) and a withdrawing program with shear flow values of 0.1 dyn/cm2 (0.028 ml/min) for the accumulation phase and 5 dyn/cm^2 (1.390 ml/min).

2.3.24 Software and statistical analysis

Data analysis and statistical calculations were performed using Microsoft Excel and GraphPad Prism. Image editing was carried out using Adobe Photoshop and Illustrator CS4. Video editing was performed with ImageJ. Lck accumulation was assed using a free-hand tool to define the boundary and the pixel intensity measuring tool of ImageJ. Analysis of DNA sequences and cloning strategies were performed and defined using Ape and Snapgene software and NCBI Basic Local Alignment Set Tool (BLAST) and

Expasy Translate free online tools. Statistical significance was calculated by Mann-Whitney-U-test analysis (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; n.s., not significant).

2.4 Biochemistry methods

2.4.1 Cell and extracellular vesicle lysis

A3.01 (or other cell lines) EV-producing cells and EV preparations were lysed in ice cold KEB lysis buffer. Typically the cell pellet obtained in the first step of EV isolation (see) was lysed in 100 µl of KEB buffer and the EV pellet obtained at the end of the isolation process was resuspended in 10-20 µl of the same buffer. Cell lysates and EV preparations were handled on ice and stored at -80ºC following protein quantification and to SDS-PAGE analysis. 293T virus-producer cells were lysed in 500 µl 6x sample buffer for half a 15-cm culture dish.

2.4.2 Protein quantification

In order to measure cell lysate and EV sample protein content and to be able to normalize loading for SDS-PAGE, the Pierce BCA protein assay and the Pierce microBCA Pierce assay were used according to manufacturer's instructions.

2.4.3 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used for qualitatitive and quantitative protein detection. To this end, the electrophoresis system 2-D (Hoefer) was used. Preparation of 10% separating and stacking gels was conducted as detailed in the materials section (see). Samples in 6x sample buffer were boiled at 95ºC for 10 minutes. EV-producer cell lysates were mixed with 3x non-reducing sample buffer as well as EV preparations, adjusting the volume of sample buffer for the latter so the desired amount could fit in the gel wells when loading. Volumes corresponding to 10^6 cells of cell lysate-3x sample buffer mix and EV-sample volumes containing equal amounts of protein also mixed with 3x sample buffer were boiled as described above. Gels were mounted on the running chamber containing SDS running buffer. After 5 minutes wait for cooling, samples were loaded onto the gel and run at 30 mA/gel for about 1 hour. ColorPlus prestained protein marker was used as a protein size standard.

2.4.4 Western blot

Following SDS-PAGE, the resolved protein content of the samples was transferred onto nitrocellulose membranes using a semi-dry blotting apparatus (Cti). To this end, Whatman paper was dipped in blotting buffer and used to make a sandwich containing the SDS polyacrylamide gel and the membrane. Blotting was achieved after 60 minutes at 100 mA/gel. Membranes were then blocked for 1 hour in 5% milk in PBS-T, washed 3 times with PBS-T, cut (if necessary) and incubated overnight with the primary antibodies at 4ºV. After 3-6 10-minute-long washes with PBS-T, the membranes were incubated with hoseradish peroxidase-coupled secondary antibodies for 60 minutes. Following 4 10-minute-long washes, the enhance chemoluminiscence (ECL) substrate

was added homogenously on the membrane surface and wiped off after one minute. Film exposure times were variable depending on the sample.

3 RESULTS: OVERLAPPING FUNCTIONS BETWEEN NEF AND VPU

As it has been already reviewed in the introduction, redundant functions between the HIV-1 pathogenicity factors Nef and Vpu have been previously reported. All of these are related to the ability of these two proteins to reduce surface expression levels of a short list of receptors, such as CD4, MHC-I, CD1d, or PVR (Chen et al., 2006; Galaski et al., 2015; Garcia and Miller, 1991; Kerkau et al., 1997; Matusali et al., 2012; Moll et al., 2010; Schwartz et al., 1996; Willey et al., 1992a). We have further studied Nef and Vpu overlapping functions which resulted in an expanded list of host cell membrane receptors targeted by both viral proteins, as well as describing the shared abilities to internalize the host kinase Lck to intracellular compartments and to modify extracellular vesicle (EV) secretion (Haller et al., 2014).

3.1 Both Nef and Vpu are able to downmodulate a broad spectrum of cell surface host receptors

One of the most described effects of the HIV-1 accessory proteins Nef and Vpu is the modulation of surface levels of host cell receptors. To analyze the breadth of this effect and to identify the target specificity of the viral factors, we studied by flow cytometry the susceptibility of a panel of receptors after expression of the viral proteins. A3.01 human T lymphocytes were transiently transfected for the expression of HIV-1 Nef.GFP, HIV-1 Vpu.GFP or SIV_{Mac239} Nef.YFP. The expression of these proteins was only slightly lower to that obtained after infection in this cell system (Fig. 3.0B). The reduction in surface expression of the analyzed cell receptors was calculated as the ratio between the mean fluorescence intensity (MFI) values obtained for GFP/YFP positive cells (transfected) and for negative cells (untransfected) from the same sample (Fig. 3.1A, R3 gate *vs* R2 gate) (Haller et al., 2014). This methodology proved to be suitable for determining the effect of isolated expression of the accessory proteins on the levels of individual cell surface receptors (Fig. 3.0A, primary data shows efficient downregulation or no effect on CD4 and CD49a, respectively, by the expression of HIV-1 Nef, HIV-1 Vpu and SIV_{Mac239} Nef). A panel of 105 receptors was analyzed this way (Fig. 3.0D and E). The results show that a number of receptors were unaffected by the expression of the viral factors, proving the specificity of the surface level modulation mediated by them. Receptor upregulation was a rare event (that took place only for CD69 in the presence of Nef). Nef was observed to downmodulate a considerable number of these receptors: 36 out of 105 showed reduction in their plasma membrane level to an extent of <65%, including previously but also newly identified Nef targets with an interesting relation to HIV Virology, such as CD37 (Fig. 3.1A), Lamp-1 (CD107a), Lamp-2 (CD107b), ICAM-1 (CD102), or gamma interferon receptor (CD119). Interestingly, there was a significant overlap between receptors that were downregulated by HIV-1 Nef and HIV-1 Vpu. 32 receptors were identified to have diminished levels in the presence of the latter accessory protein to an extent of at least 80%, being all of them targeted as well by Nef. No significant receptor upregulation was observed in this case. Overall, the effect mediated by Vpu was less pronounced

than that of Nef, except for CD164, CD71 and CD317 (data not shown). Taken together, this results show there is a vast but specific overlap in the set of cell surface receptors targeted by both viral proteins.

Upon analysis of the characteristics of the newly identified targets of Nef and Vpu, we detected amongst the studied host cell receptors several belonging to the tetraspanin (TSPAN) superfamily of proteins. HIV-1 Nef and Vpu lowered the levels of all TSPAN proteins included in the study (CD37 [also referred to as TSPAN-26], CD53 [TSPAN-25], CD63 [TSPAN-30], CD81 [TSPAN-28], CD151 [TSPAN-24], and CD231 [TSPAN-7]). Furthermore, the effect of Nef on CD37 and CD81 was the strongest seen in the complete study. Also in this case, Vpu affected the surface expression levels of all these proteins to a lesser extent than Nef (Fig. 3.0C).

3.2 Downregulation of TSPANs from cell surface by HIV-1 Nef and Vpu is the result of a specific activity

Given the observed specificity of Nef and Vpu for members of the TSPAN family of transmembrane proteins, we next wondered if the observed downregulation could be the result of an artifact caused by unspecific effects on membrane trafficking due to membrane association and insertion, respectively. To study this, we utilized transfected A3.01 human T cells (over)expressing Nef.GFP or Vpu.GFP and compared them to cells (over)expressing N18-src.GFP, the N-terminal domain of the src protein fused to GFP, and pDisplay.YFP. The reason why the latter two proteins were chosen as controls is that they are targeted to the cell membranous system (preferably to the plasma membrane) by the same mechanism as the viral proteins. N18-src.GFP contains a SH4 domain susceptible to myristoylation that favors anchoring at the inner leaflet of membrane (Geist et al., 2014; Haller et al., 2014; Pan et al., 2013b), as in the case of Nef. pDisplay.YFP consists of the transmembrane domain of CD4 spanning the membrane similarly to the Vpu protein (Haller et al., 2014). Forty eight hours after transfection, cells were stained with antibodies targeting CD4 and several TSPANs and analyzed by Flow Cytometry. The relative remaining surface expression of the receptors was calculated as the ratio between the MFI of intermediate-high expressing cells and that of GFP-negative from the same sample (Fig. 3.1A, gates R3 vs R2). Expression of Nef.GFP and Vpu.GFP resulted in diminished levels of the five studied TSPAN members, CD27 (data not shown), CD37, CD53, CD63, and CD81. The reduction was in all cases less pronounced than that seen for the control receptor CD4 (Chaudhuri et al., 2007; daSilva et al., 2009; Garcia and Miller, 1991; Ren et al., 2014) (First set of Fig. 3.1B). The unrelated control proteins N18src.GFP and pDisplay.YFP did not cause significant downregulation of any of the transmembrane proteins. Thus, HIV-1 Nef and Vpu target proteins from the TSPANs superfamily for downregulation in a specific manner.

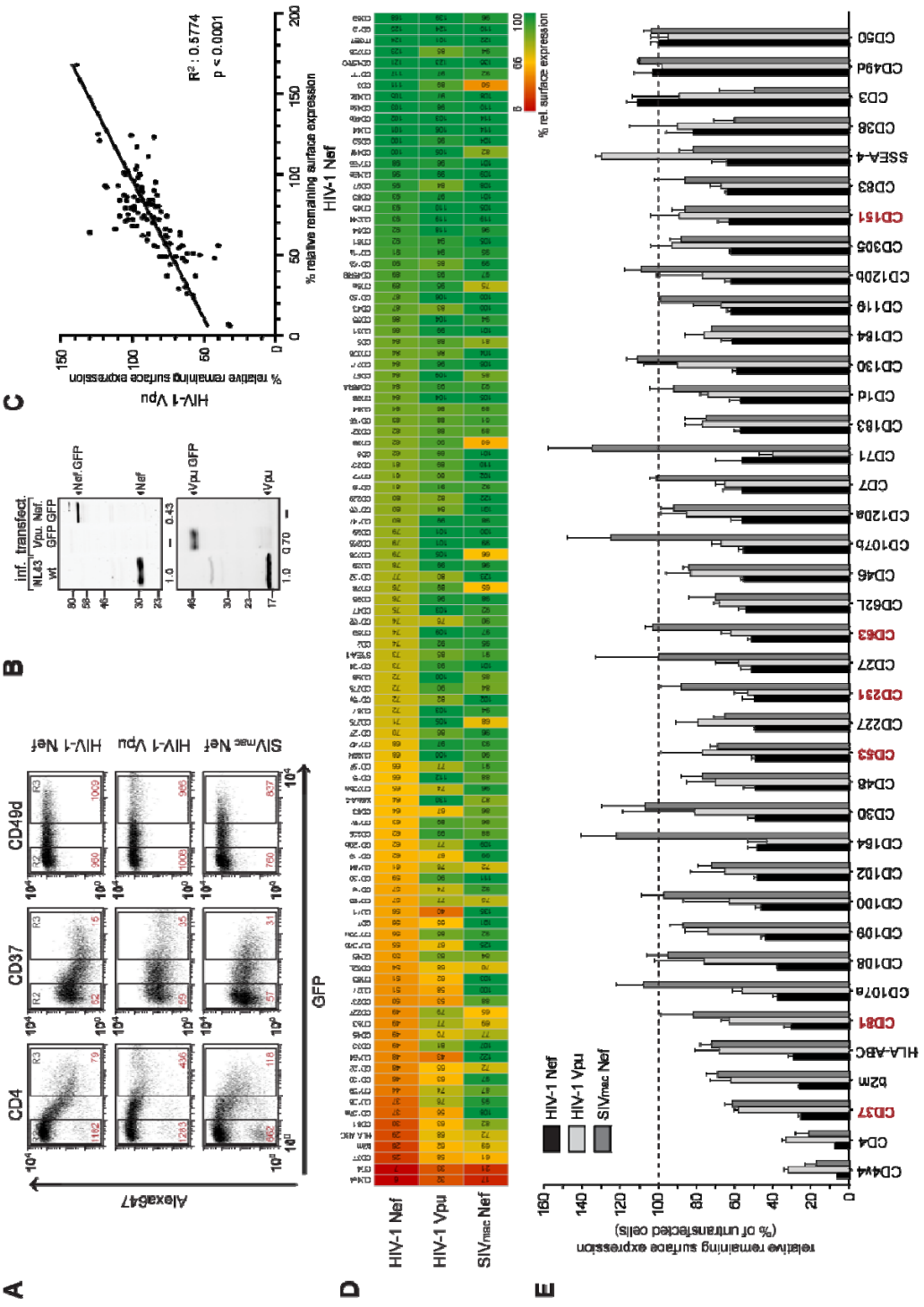

Fig. 3.0 Lentiviral Nef and Vpu share the ability to downregulate a multiplicity of markers from the cell surface. A3.01 cells were transiently transfected with expression plasmids for GFP fusion proteins for HIV-1 Nef, HIV-1 Vpu, and SIVmac239 Nef (YFP fusion protein) and stained 48 h later with the individual antibodies in the Lyoplate human cell surface marker screening panel (BD). Surface expression of the respective markers was investigated by flow cytometry. (A) Representative flow cytometry dot plots of gated living cells. The MFI (YGeoMean) of untransfected (gate R2) and medium- to high-GFP-expressing (gate R3) cells is indicated in red for the respective surface receptor. (B) Western blot analysis of A3.01 CD4 T cells infected with wt HIV-1 or transiently transfected with expression plasmids for GFP fusion proteins for HIV-1 Nef or HIV-1 Vpu. A total of 1 _ 105 infected or transfected cells were lysed and subjected to Western blotting for expression analysis of Nef (top) and Vpu (bottom). Intensities of the Nef and Vpu bands were quantified by using ImageStudioLite software (Li-Cor). Numbers below the panels indicate Nef and Vpu expression levels relative to that observed for cells infected with wt HIV-1, which was arbitrarily set to 1.0. (C) Correlation analysis of the cell surface receptor downregulation activity of HIV-1 Vpu and HIV-1 Nef. (D) Heat map diagram of the relative remaining surface expression (percent) of the individual markers screened for. Red indicates strong downregulation, yellow indicates medium downregulation, and green indicates no downregulation from the cell surface. The factors are ordered according to the identified surface levels upon HIV-1 Nef expression. The numbers represent mean values of the MFI ratio of transfected to untransfected cells (R3/R2) from three independent experiments. b2m, ß-2 microglobulin; ITGB7, integrin-ß7 (see also Table S1 in the supplemental material). (E) Diagram of all receptors showing 65% relative cell surface expression upon expression of HIV-1 Nef or Vpu or SIVmac Nef and two unaffected surface receptors (CD49d and CD50). Values are the arithmetic means and standard deviations from three independent experiments. Highlighted in red are members of the tetraspanin (TSPAN) family. Work performed by Dr. Claudia Haller. SDS-PAGE performed by Birthe Müller.

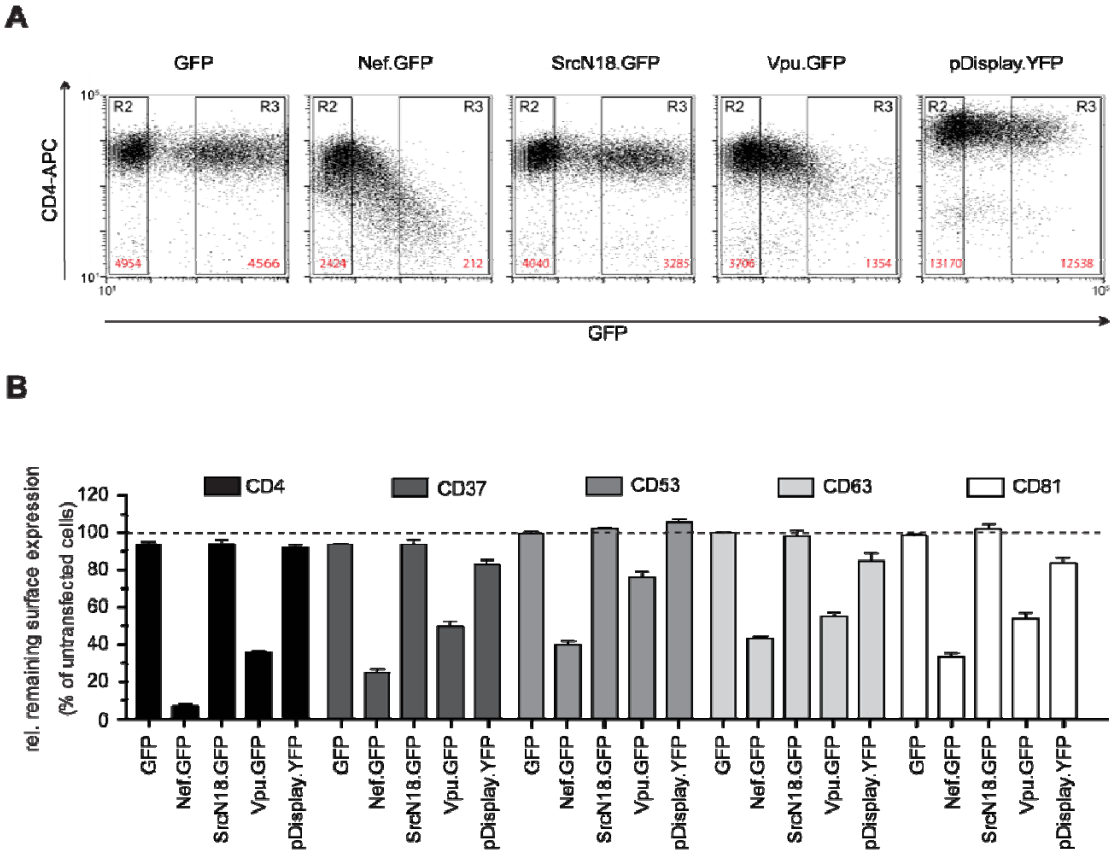

Fig. 3.1 HIV-1 Nef and Vpu target cell surface TSPANs for downregulation in a specific manner. A3.01 human T lymphocytes were transfected with expression plasmids for Nef.GFP, Vpu.GFP, the N-terminus domain of Src fused to GFP (SrcN18.GFP), pDisplay.YFP or GFP as a control. 48 hours later, cells were stained with antibodies targeting different TSPANs or CD4 to study surface expression levels by flow cytometry. (A) Representative flow cytometry dot plots of the CD4 levels of gated living cells. Mean Fluorescence Intensity (MFI, YGeoMean) of untransfected (gate R2) and medium- to high-GFP-expressing (gate R3) cells is shown in red for each of the different conditions. (B) Depicted protein levels were calculated and plotted using the MFI of GFP positive cells relative to that of untransfected cells from the same sample. Displayed are the arithmetic means and standard deviation of three independent experiments. For reference, dotted line corresponds to no downregulation (100% remaining surface expression).

3.3 Different motifs of Nef and Vpu are important for TSPAN proteins downregulation from the cell surface

As the next step in this study, we wanted to define the molecular determinants by which Nef and Vpu mediate downregulation of TSPANs. To this end, A3.01 human T cells were transfected with expression plasmids encoding GFP fusions of Nef, Vpu, or well-characterized mutants of the viral proteins. CD4 and tetherin, two well described downregulation targets of the accessory proteins (Bolduan et al., 2013; Chaudhuri et al., 2007; daSilva et al., 2009; Garcia and Miller, 1991; Goffinet et al., 2009; Neil et al., 2008; Schmidt et al., 2011; Tervo et al., 2011), were analyzed as controls. Immunostaining of these cells was performed 48 hours later and plasma membrane levels of stained receptors were analyzed by flow cytometry as the ratio between the

MFI values of intermediate-high GFP-expressing and untransfected cells from the same sample (as shown in Fig.3.1A, R3 vs R2).

Motif	Relevance/loss of interaction	Mutant
MGxxxS(1–6)	Membrane association	G2A
S(6)	Phosphorylation by nPKC	S6A
M(20)	MHC-I downregulation	M20A
N-t amphipatic helix	NAKC formation	Δ12-39
EEEE(66–69)	MHC-I downregulation	E4A4
VGF(71–73)	MHC-I downregulation	VGFAAA (VGF)
PxxP(80–83)	MHC-I downregulation	AxxA
ExxxLL(164–169)	AP-2 binding , CD4 downregulation	LLAA
ED(178,179)	CD4 downregulation	EDAA
F(195)	Pak2 association	F195A

Table 3.1 HIV-1 SF2 Nef mutants used in this study.

Transient expression of Nef.GFP causes a pronounced reduction of CD4 levels on the cell surface of A3.01 T cells (from 90.1% of GFP control cells to 6.2% of Nef.GFP-expressing cells). Expectedly, mutations in the acidic stretch (EEEE66-69; E4A4), needed for interaction with PACS adaptor involved in receptor downregulation (Piguet et al., 2000), the VGF motif (VGFAAA), involved in SH3-domain interactions between Nef and host cell proteins (Meuwissen et al., 2012), the PxxP motif (AxxA), central structure of the SH3 domain interaction surface in Nef (Collette et al., 1996; Saksela et al., 1995), and the hydrophobic patch (F195A), essential for the interaction with PAK2, resulted in marginal or no effects on the extent of Nef-mediated CD4 downregulation (see table 1.3). The Nef mutant unable to become myristoylated (G2A), deficient for most described functions of the accessory protein, and the 12-39 deletion mutant of Nef (Δ12-39), which lacks an amphipathic alpha-helix needed for the interaction with a multi-molecular complex consisting of at least two cellular adaptor proteins and four kinases, NAKC (Baur et al., 1997), were largely deficient for this activity. As expected, modifications targeting the C-terminal flexible loop, LL168/169 and ED178/179 residues (LLAA and EDAA), interacting with the endocytic machinery, abrogated this Nef activity (Chaudhuri et al., 2007; Fackler et al., 2006; Keppler et al., 2006). Surprisingly, the downregulation Nef mutant-profile differed for the individual TSPANs and none of the analyzed molecular determinants emerged as essential for any of the studied host proteins. Despite the unspecific G2A mutant, the acidic stretch and the VGF and proline-rich motifs also accounted for a discrete partial loss of function in 3 out of 4 TSPANs studied (all but CD37). In the case of CD37, along with the G2 mutation only mutation in the dileucine motif led to partial inhibition of downregulation as compared to wt Vpu. Also, no similarities to CD4-downregulation profile (or that of MHC-I or CXCR4, differing to the CD4 one and described elsewhere (Mangasarian et al., 1999; Meuwissen et al., 2012; Venzke et al., 2006)) were observed.

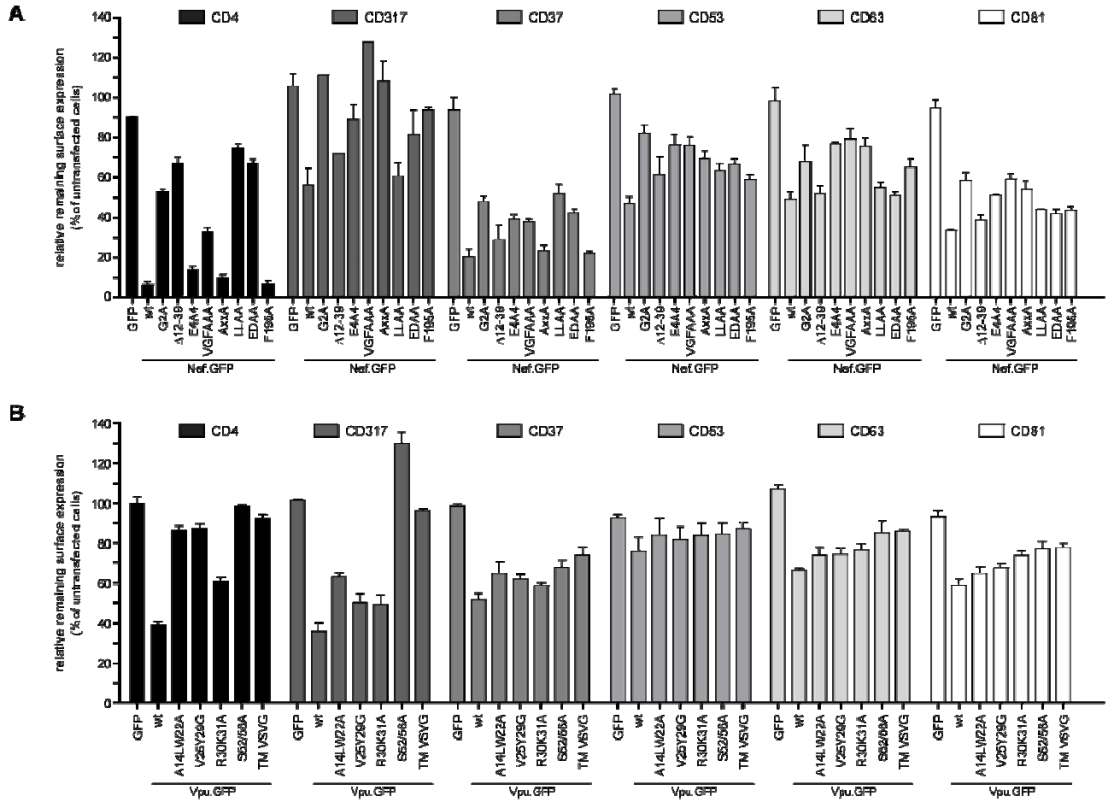

Fig. 3.2 Alteration of individual TSPANs cell surface levels by Nef and Vpu depends on distinct molecular determinants. A3.01 T lymphocytes were transfected with expression plasmids for various Nef.GFP (A) and Vpu.GFP (B) proteins or GFP control. After 48 hours, cells were harvested and immunostained for flow cytometry analysis of surface receptor levels. The graphs show relative protein levels as the MFI ratio of gated GFP positive cells and untransfected cells within the same sample (R3 *vs* R2, as in figure 1). The arithmetic mean and standard deviation of the means from three experiments (two in the cases of Nef.G2A, Δ12-39, and VGFAAA) in triplicates are plotted.

Vpu expression also caused an important reduction in CD4 surface expression levels, although less pronounced as that induced by Nef (from 99.7% of GFP control cells to 39.2% of Vpu.GFP-expressing cells). Conversely, Vpu caused a more potent downregulation on exposed levels of tetherin (from 101.4% to 35.6%). Modification of the diserine motif necessary for the interaction with beta-transducin repeat-continuing protein (β-TrCP) (S52/56A) caused a loss of Vpu-mediated downregulation of both CD4 and tetherin (see table 1.4). The same result was observed when the transmembrane (TM) domain was replaced by that of the vesicular stomatitis virus G protein (TM VSVG) (Magadan et al., 2010). While all other mutations tested did not affect Vpu-mediated downregulation of tetherin, changes were observed for CD4. Mutations targeting a Vpu motif essential for lipid raft association (V25Y29G) (Fritz et al., 2012) or that needed for interaction through the transmembrane domain (A14LW22A) (Vigan and Neil, 2010) resulted in disruption of CD4 downmodulation. Mutations in a motif necessary for the natural localization of Vpu at the trans-Golgi network (TGN) (R30A,K31A) (Dube et al., 2009) did not affect downregulation. Regarding effects of Vpu mutants on TSPAN surface levels, mutations at the diserine motif or the TM domain of Vpu showed a partial loss of the effect. All the other mutants affected only slightly, if at all, the surface levels of TSPANs as compared to

those in presence of wt Vpu. Of note, in the case of Vpu-mediated downregulation, the tested mutants generated a comparable profile for each of the proteins.

Motif	Relevance/loss of interaction	Mutants
DS(52)GNES(56)	β-TrCP-dependent degradation	S52/56A
AxxxAxxxW(14–22)	Tetherin downregulation	A14LW22A
R(30)K(31)	TGN localization	R30K31A
V(25)Y(29)	Lipid raft association	V25GY29G
TMD	CD4 downregulation	TM VSVG (TMD replaced by VSVG TMD)

Table 3.2 HIV-1 NL4.3 Vpu mutants used in this study.

Overall, these results show that the determinants of Nef and Vpu relevant for downregulation of proteins of the TSPAN superfamily are different to those previously described for other target proteins of these viral factors. Furthermore, distinct mechanisms might explain Nef-mediated downregulation of individual TSPANs.

3.4 Nef and Vpu target Lck for relocalization to an intracellular compartment

A consequence of the Nef-mediated modification of intracellular trafficking is the relocalization of the Src kinase and peripheral membrane protein Lck from the plasma membrane to recycling endosomes and *trans*-Golgi network (RE/TGN) to fine tune TCR signaling and favor viral replication (Pan et al., 2013b; Pan et al., 2012). Since Nef and Vpu share specificity for downregulation of a number of receptors as described above, we wanted to study whether Vpu can as well exert another well characterized Nef activity such as rerouting of the TCR signaling master switch Lck. A3.01 cells were therefore transfected with expression vectors for GFP versions of both accessory proteins or the empty GFP-expressing plasmid as control. Under these three conditions, cells were analyzed by immunofluorescence for the localization of endogenous or overexpressed Lck. The latter was achieved by cotransfecting the cells with a plasmid expressing the RFP-labeled kinase (Fig 3.3B). The frequency of Nef-expressing cells displaying retargeted endogenous Lck to intracellular compartments was close to 100%. Only a marginal fraction of Vpu-expressing cells showed this cytoplasmic accumulation when compared to GFP control cells, which showed no detectable cytoplasmic Lck signal (Fig 3.3C; 91.7 +/- 3.8% of Nef.GFP-expressing cells showing accumulation, 18.7 +/- 5.6% of Vpu.GFP-expressing cells and 0% of GFP-expressing cells). The magnitude of the accumulation in single cells was determined as the ratio between accumulated and total Lck signals (i.e. pixel quantification of cytoplasmic *vs* total Lck signal). Accordingly, the extent of the relative accumulation per individual cell studied was in average significantly higher also in the case of Nef-expressing cells than in the case of Vpu-expressing cells. The obtained value was significantly higher for cells expressing either of the viral proteins when compared to GFP control cells (Fig 3.3E; 6.4 +/- 4.8% of the total per cell Lck signal at intracellular compartments in GFP-expressing cells, 57.2 +/- 14.3% in Nef.GFP-expressing cells and 27.4 +/- 11.2% in Vpu.GFP-expressing cells).

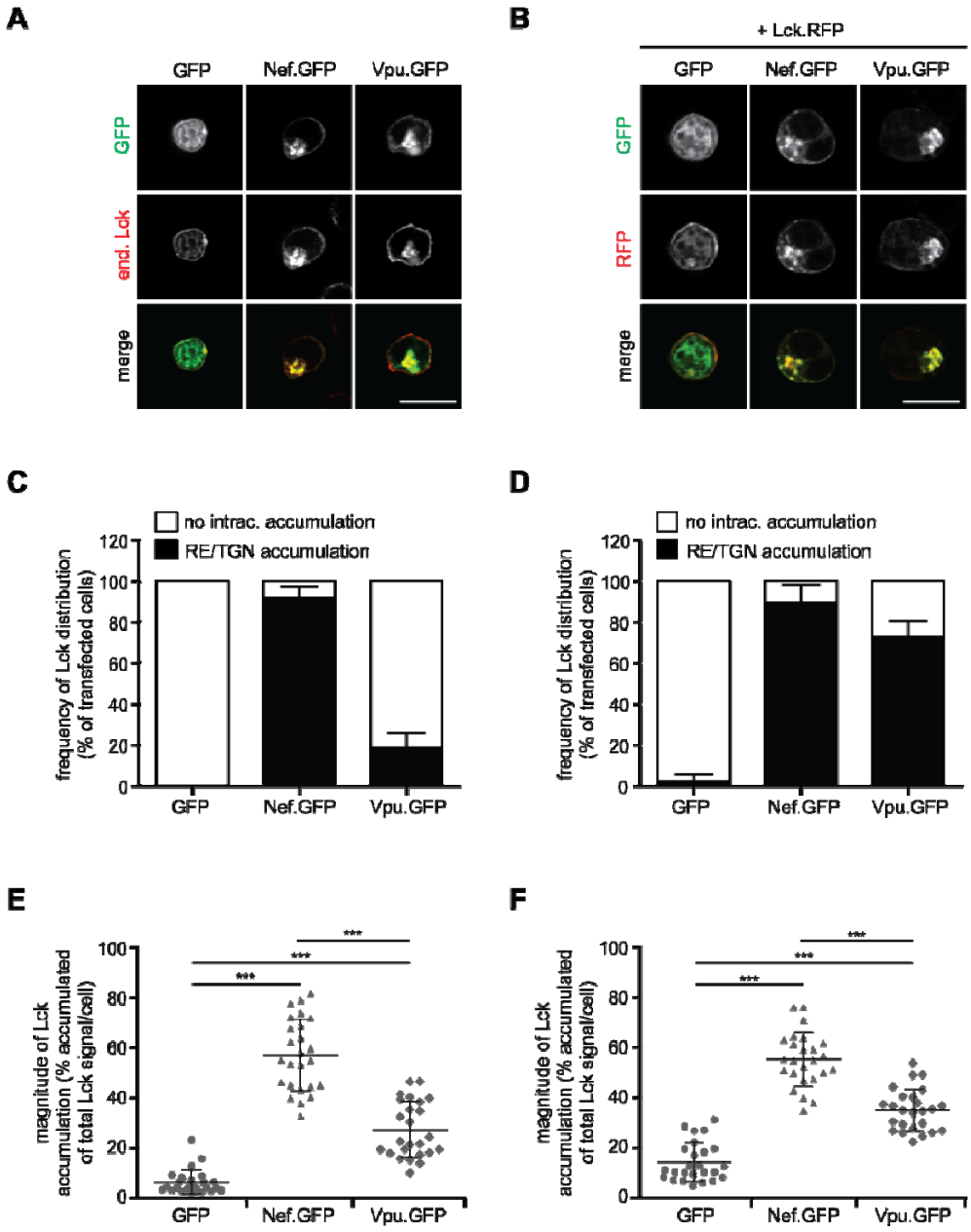

Fig.3.3 Nef and Vpu affect the subcellular localization of Lck. A3.01 T lymphocytes were transfected with expression vectors for GFP, Nef.GFP or Vpu.GFP alone (A, C and E) (endogenous Lck) or co-transfected with an expression vector for Lck.RFP (B, D and F). 24 hours later, cells were fixed on poly-L-lysine-coated cover glasses and analyzed by confocal microscopy. To study endogenous Lck, an intermediate step was required in which cells were immunostained. Representative micrographs in (A and B) show the relocalization of Lck mediated by both accessory proteins compared to GFP control cells. Scale bars = 10 µm. (C and D) Frequency cells showing Lck accumulation (recycling endosomes/*trans*-Golgi network). GFP-high-expressing cells were evaluated for their Lck localization and classified into two groups: showing Lck intracellular accumulation or with Lck at its usual plasma membrane localization. Depicted are mean values and standard deviation of data from three independent experiments in which 100 cells were considered per condition. (E and F) Single-cell analysis of the relative Lck accumulation. Signal intensity was evaluated as the pixel quantification of the cytoplasmic compartment or of the whole cells. Shown is the intracellular Lck signal as a percentage of the total Lck signal for each of the cells analyzed. Each symbol corresponds to the value obtained for an individual cell. Bars indicate mean values of 25 cells studied per condition. Statistical analysis was performed by the Mann-Whitney U test (***, $P < 0.0001$).

When Lck.RFP was overexpressed, no changes were observed in the frequency of Lck cytoplasmic accumulation and its extent per single cell when compared to endogenous Lck in Nef-expressing cells (Fig. 3.3C *vs* D and E *vs* F) (Fig. 3.3D and F; 89.7 +/- 6.4% frequency of cells showing accumulation and 55.4 +/- 10.9% relative accumulation per single cell, respectively). In Vpu-expressing cells co-transfected with Lck.RFP, however, the frequency of accumulation increased to 72.7 +/- 5.8% (Fig. 3.3D). Single cell analysis, however, did not reveal important differences to what was observed for single-cell endogenous Lck relative accumulation in the same cells (Fig. 3.3E *vs* F) (Fig. 3.3F; 35.2 +/- 8.3% relative accumulation per single cell in Vpu.GFP-expressing cells). Altogether, these results show that, as it was already described for Nef, Vpu can affect the localization of peripheral membrane proteins like Lck. Nevertheless, as observed for the downregulation of TSPANs and most of the studied membrane receptors, the extent of Vpu-mediated effect on Lck retargeting to RE/TGN is less potent as compared to that mediated by Nef. Also, Vpu seems to block anterograde transport of Lck molecules directly after their synthesis but, unlike Nef, it has no effect on those that already reached their steady-state distribution.

3.5 Extracellular vesicles (EV) can be purified from human T cells culture supernatant

EVs are shed by most cell types (Thery et al., 2002; Yanez-Mo et al., 2015). Thought to be cellular waste, they remained out of the scope of research until they were discovered to contain enriched proteins and microRNAs (Ratajczak et al., 2006a; Valadi et al., 2007). Today, EVs have been shown to play an important role in many physiological processes in complex organisms and to be key pathological and disease factors (Schorey et al., 2015; Yanez-Mo et al., 2015). Among the proteins which are actively sorted into EVs, proteins of the TSPAN superfamily are some of the best characterized, serving therefore as EV detection markers (Thery et al., 2002). HIV-1 Nef has been described to boost EV release in cells transiently expressing the protein but also in the context of infection. It also has been shown to induce its own inclusion in EVs from these cells to potentially alter metabolism and intracellular signaling in bystander cells favoring pathogenesis and disease progression (Lenassi et al., 2010; Muratori et al., 2009). We thereby hypothesized the release of TSPAN proteins on EV as a potential mechanism of downregulation by Nef and, presumably, also by Vpu. Furthermore, we aimed at understanding this process in a physiologically relevant system in the context of HIV-1 disease, namely HIV-1 target cells. In order to set up a system to study the effects of both HIV-1 accessory proteins on the production and composition of EV, we analyzed the capacity of different human T cell lines to release EV under different conditions. Initially we checked E6 Jurkat cells (Fig. 3.4A). After an overnight starvation, they were cultured in control or stimulation RPMI++ media, containing 20 µg/ml of phytoemagglutin (PHA). The FCS added to this medium was previously depleted form EVs by ultracentrifugation (UC). EV isolation was then performed by the differential UC method (Thery et al., 2006). To analyze the results, we used as output the Western

Blot (WB) signal of TSPANs as exosomal markers (Lotvall et al., 2014). TSPAN WB needed to be performed in non-reducing conditions as described elsewhere (Thery et al., 2006). Cells responded to PHA stimulus by increasing EV release in comparison to control cells, as observed by the increase in the WB signal of both TSPANs used as detection markers CD63 and CD81. Intrigued by the decrease in (and even absence of) signal seen for later time points, known that EV should have high stability and therefore long half-life (Kalra et al., 2013), we added two other human T cell lines to the next pre-experiment: A3.01 and JTag cells. Cells were culture for 16 hours in EV-depleted FCS-containing medium under control or stimulating conditions (PHA). Supernatants were cleared, ultracentrifuged and EV preparations were analyzed by Western Blot. As noted before, EVs derived from Jurkat E6 cells were unstable over time and TSPAN marker detection was not stably achieved (Fig. 3.4B). A3.01 cells- and JTag cells-derived EV detection, however, worked efficiently and cells responded to stimulus by a boost release of EVs inferred from stronger WB signals, as expected. Unlike what was observed for E6 cells, the signal for both TSPANs obtained from cell lysates (CLs) with equal protein amounts to that of corresponding EV preparations (equivalent to 10^6 cells) was lower. This effect was strongly marked for CD63, in particular in A3.01 cells. Given the strong signal obtained from EV preparations for both markers, CD63 and CD81, in the case of A3.01 cells, we utilized these as EV source thereon.

In order to find a more potent, T cell-specific stimulus which could be used as positive control in future experiment if needed, we analyzed the effect of culturing cells on activating surfaces or, in other words, anti-CD3 and anti-CD28 antibody coated surfaces. This way we should trigger a more specific signaling by direct cross-linking of TCR. Following overnight starvation, A3.01 T cells were stimulated with PHA or TCR-specific antibodies in EV-depleted FCS-containing medium. Unstimulated cells were used as control. After 5 days in culture, SNs were cleared and EVs were isolated by UC. The WB signal obtained by TCR-specific stimulation with antibodies was stronger as that obtained by stimulating with PHA for both CD63 and CD81 from the EV preparations (Fig. 3.4C). Of note, both stimulation methods were efficient in enhancing shedding of EV when compared to unstimulated control cells whose EV prep showed very low signal for CD81 and CD63 at the displayed exposure time. Surprisingly and unlike what was observed in the previous experiment (Fig. 3.4B), the CD63 signal from CLs was much more intense in all cases as compared to the corresponding EV preps. Moreover, the CD63 signal from CLs increased in response to the antibody stimulus. In contrast, the CD81 signal from the CL samples was undetectable except in the control cells-derived sample. Altogether, the TCR-specific antibody treatment resulted in an increased EV release as compared to stimulation with PHA (Fig. 3.4C).

Finally we compared two EV isolation methods, ultracentrifugation *vs* a commercial lipophilic reagent. A3.01 cells were starved overnight, cultured under control or stimulating conditions and harvested after 16 hours. The FCS in the culture media had been previously depleted of EVs. Supernatants were then cleared and either subjected to successive centrifugation and UC steps or incubated overnight with the Exosome

Isolation Reagent (Invitrogen, Carlsbad CA) and pelleted down by centrifugation. EVs obtained with the reagent generated considerably more TSPAN signal as those obtained by ultracentrifugation (Fig. 3.4D). Of note, the CD63 WB signal from CL of the reagent samples and from the CL obtained by UC was so intense that higher exposure times were not possible to achieve. As a consequence CD81 bands are faint (reagent samples) or not visible (UC samples). However, given the very low specificity of the isolation mechanism and the high risk of impurity in the final preparation, we decided to use the standard sequential UC as the EV isolation method thereon.

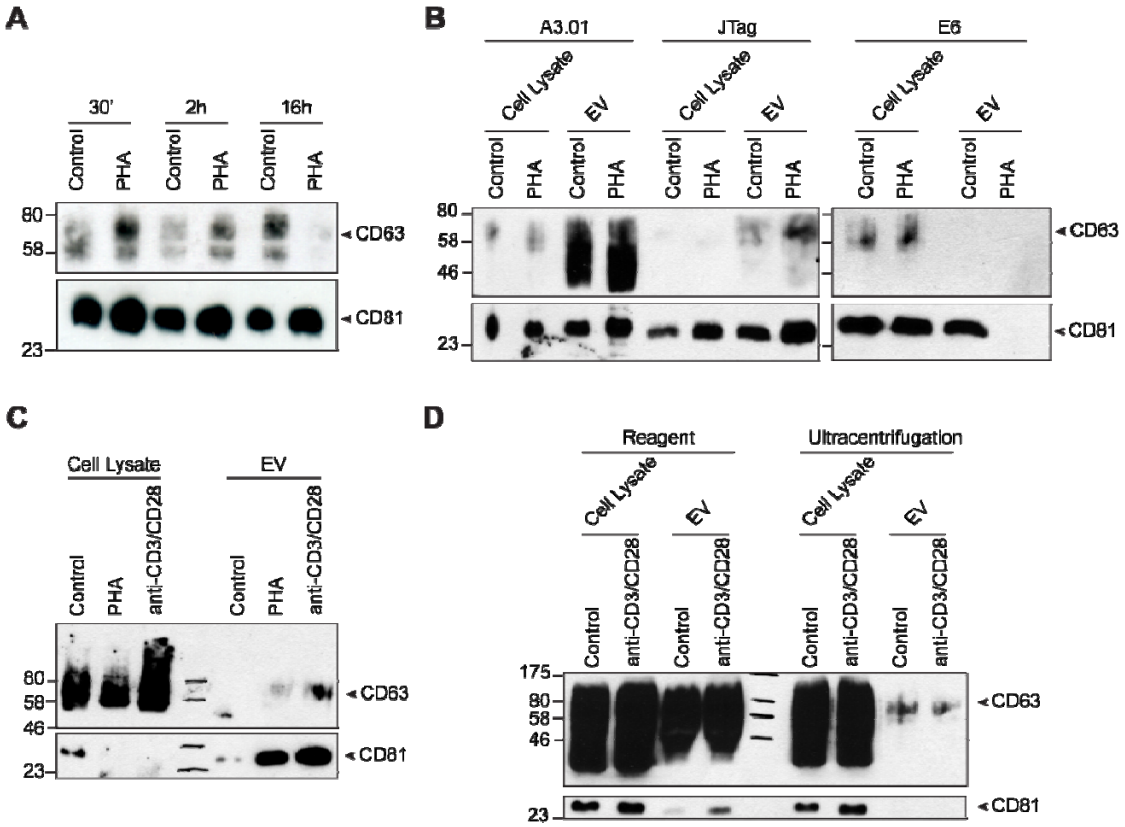

Fig. 3.4 Setting up extracellular vesicle (EV) isolation and detection in T lymphocytes. (A) 5 x 10^7 Jurkat E6 cells were cultured in exosome deprived medium (EDM) in the absence or presence of 20 µg/ml PHA during 30 minutes, 2 hours or 16 hours. PHA addition was coordinated timewise to collect the respective supernatants simultaneously. EV from the cleared supernatants were isolated by differential ultracentrifugation and analyzed by non-reducing SDS-PAGE. Volumes of EV preparation corresponding to 1 x 10^6 cells were loaded. (B) Three different T cell lines were tested for their capacity to respond to a general stimulus that elevates extracellular vesicle release. 5 x 10^7 cells were cultured as in A after a 24-hour starvation step in 0.5% FCS containing medium. After 16 hours, EVs were isolated by differential ultracentrifugation. Volumes corresponding to 1 x 10^6 cells from the cell lysates were loaded as well as equal amounts of protein of the EV samples. (C) 10 x 10^6 serum-starved cells were stimulated either by addition PHA to a concentration of 20 µg/ml or by cultivation on anti-CD3/anti-CD28 pre-coated bottles. After 5 days, EV were isolated and analyzed as in B. (D) Experiment performed as in C but comparing EV isolation by ultracentrifugation *vs* Exosome Isolation Reagent (Invitrogen).

3.6 HIV-1 Nef stimulates extracellular vesicle release and its own export to the extracellular space by this mechanism

As mentioned above, previous reports showed that, when expressed by cells, HIV-1 Nef boosts EV release and induces its own inclusion in EVs with subsequent effects on bystander cells (Lenassi et al., 2010; Muratori et al., 2009). In order to reconfirm the published information at our laboratory, we used pre-starved A3.01 human T lymphocytes transiently expressing Nef.GFP fusion protein or GFP as a control. Cell numbers were normalized so that an equal number of GFP-expressing cells were contained in the culture for each condition. Cells were cultured in EV-depleted FCS-containing medium. 3 days post-transfection, cell cultures were harvested, cleared and ultracentrifuged. After lysis, the protein concentration of EVs and CLs was measured. Interestingly, the amount of total protein was close to 2-fold higher in the Nef-expressing cell-derived EV preparation than in GFP-expressing cell-derived EV preparation (Fig. 3.5C). However, protein levels in cell lysates from both conditions did not differ (Fig. 3.5B). This increase in protein might be due to a boosted EV secretion mediated by Nef. Consistent with these results, WB analysis of both preparations under non-reducing conditions showed an increase in total EV release as interpreted form the higher CD63 and CD81 signal obtained for the Nef-derived sample as compared to the GFP-derived sample (Fig. 3.5A). In this case, TSPAN WB signals form EV samples were more intense than those from the corresponding CLs. Nef.GFP could be detected in EVs shed by cells transiently expressing this protein when proving with an anti-GFP antibody (Fig. 3A, upper panel). Notably, the Nef-specific antibody was not able to detect this (Fig. 3A, second panel from the top). That might reflect the low concentration of Nef on EVs despite of its strong presence in the CL and the higher sensitivity of the anti-GFP antibody. The heat-shock cognate 70 kDa protein (Hsp70), another described exosomal marker (Escola et al., 1998; Raposo et al., 1996; Thery et al., 1999), did not show significant enrichment in EVs as compared to CL nor for Nef-samples neither for GFP-samples. Expectedly, calnexin, and ER marker used as a negative control (Lotvall et al., 2014), was present in CL but not in EVs. Thus, with these results we have been able to reproduce and confirm the previously published information which defines HIV-1 Nef as able to potentiate the secretion of EV and to use these shed EV vesicles to deliver itself to the extracellular compartment.

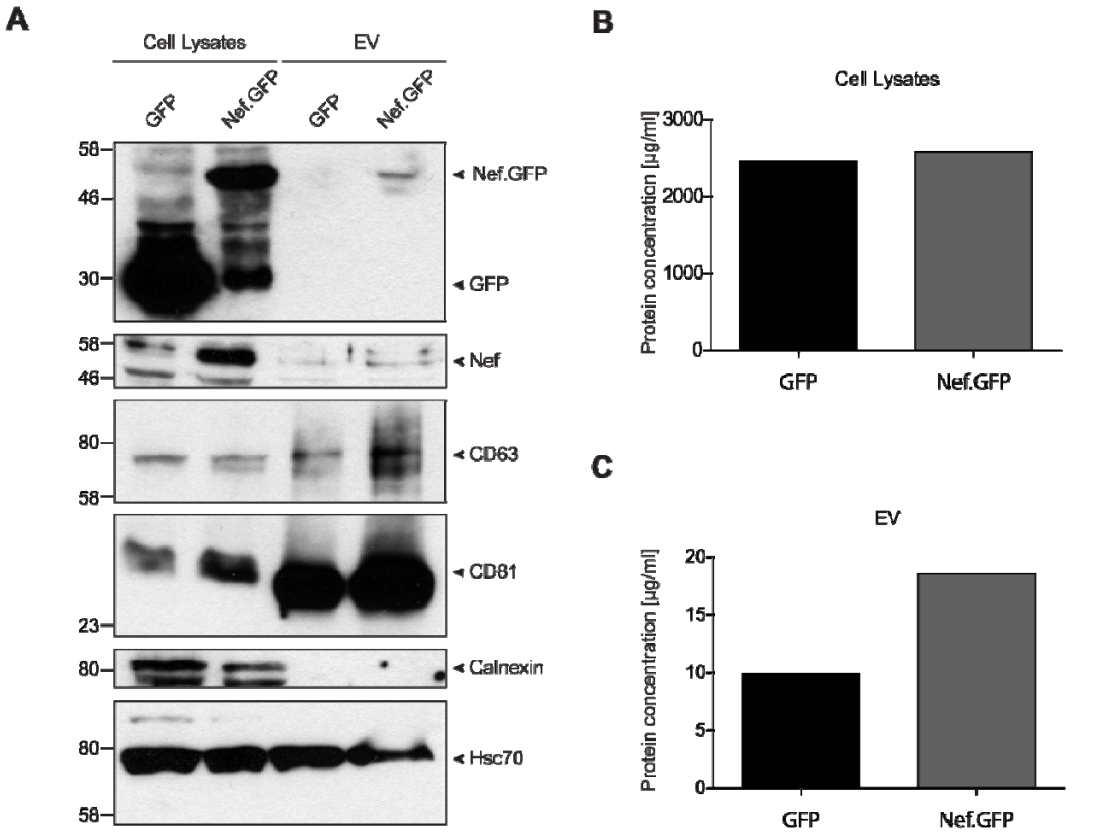

Fig. 3.5 HIV-1 Nef promotes EV production and is incorporated in EVs. (A) 6 x 10^8 cells per condition were starved during 24 hours in 0.5% FCS-containing medium and transfected with expression vectors for GFP or Nef.GFP. 3 days later, cell culture medium was harvested and EVs were isolated by ultracentrifugation. Samples were analyzed by Western Blotting for different exosomal markers and control markers. Detection of CD63 and CD81 was achieved under non reducing conditions (absence of 2-mercaptoethanol). Proteins levels were measured by the BCA assay in the case of cell lysates (B) and micro-BCA assay for EV (C).

3.7 HIV-1 Nef and Vpu boost secretion and stimulate their own export into EV

HIV-1 Nef interferes with migration of T cells both *in vitro* and *in vivo* (Choe et al., 2002; Hrecka et al., 2005; Janardhan et al., 2004; Lee et al., 2008; Park and He, 2009). Nef exerts this effect by altering the actin dynamics of the cell. The molecular mechanism governing this relies on the interaction between Nef and the cellular kinase PAK2 through the viral factor's hydrophobic patch (Stolp et al., 2010; Stolp et al., 2012; Stolp et al., 2009). The interaction of both proteins is very transient (Imle et al., 2015) but, upon dissociation of the complex, leads to a substrate specificity switch toward phosphorylation of the actin severing factor Cofilin. Hyper-phosphorylation of Cofilin results in its inactivation and, thereby, in a reduction in actin turnover affecting chemokine-induced actin cytoskeleton rearrangements that cause an arrest of migration. In a more recent study, our laboratory described the Nef-mediated interference with T cell homing *in vivo* and transendothelial migration (TEM) *in vitro* (Stolp et al., 2012). Both effects were dependent on the PAK2 interacting motif of Nef and, thereby, on the disruption of actin dynamics. However, a more detailed analysis into the multi-step

TEM process uncovered a lack of dependency on the integrity of that motif for diapedesis, the central step of this intricate process. Furthermore, Nef was also observed to affect the morphological polarization of the cells upon attachment to the endothelium, a prerequisite for efficient migration performance. Given the need for an extreme polarization of cells for transmigration through the endothelium, we hypothesized that a deep understanding of the mechanism of Nef-mediated polarization disruption would shed some light onto how the viral protein alters the TEM process. In this work we aimed at unraveling the molecular determinants of Nef involved both in polarization and TEM interference in the context of infection of primary human T lymphocytes.

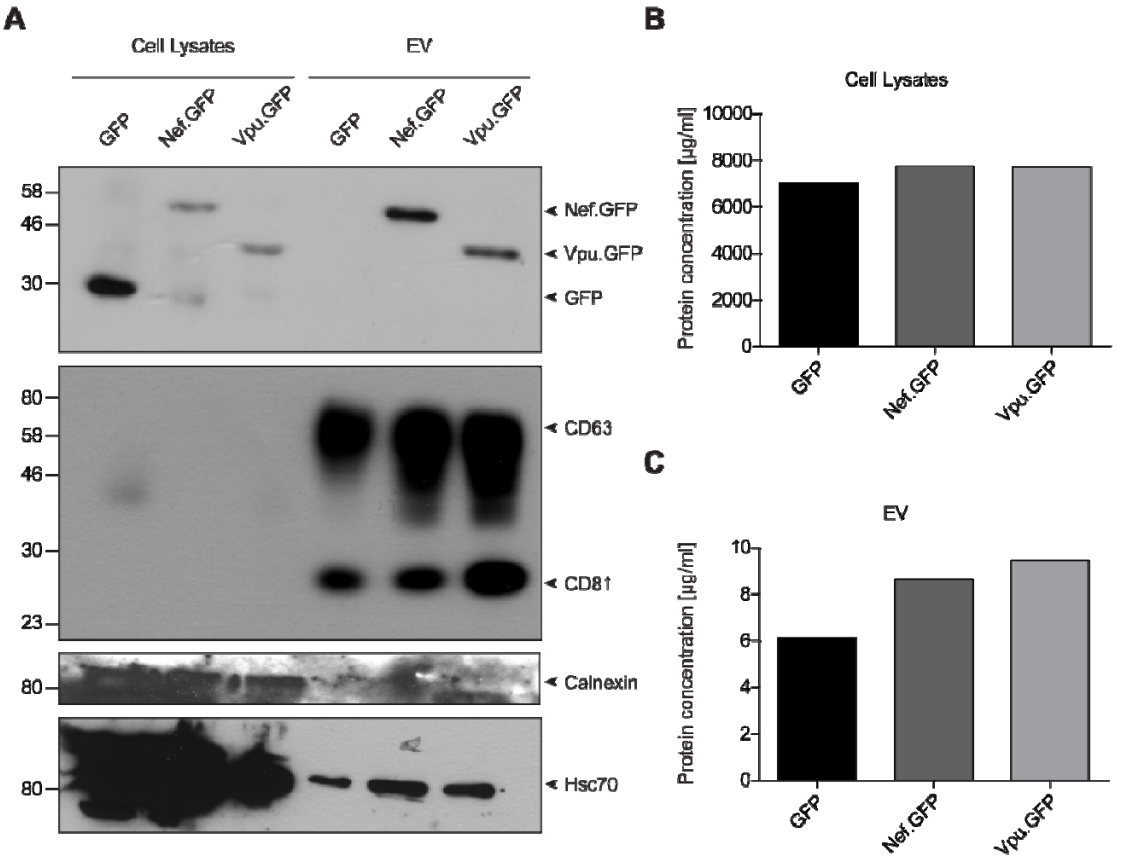

Fig. 3.6 HIV-1 Vpu is able to boost EV release and is incorporated in EVs. (A) 3 x 10^8 cells per condition were transduced using lentiviral expression vectors for GFP, Nef.GFP and Vpu.GFP. After 36 hours, transduction efficiency was evaluated by flow cytometry and equal amounts of GFP-positive cells were starved for 24 hours in 0.5% FCS-containing medium. 72 hours later, EV were isolated by ultracentrifugation and analyzed by Western Blotting for different exosomal and control markers. (B and C) Protein levels measured by the BCA assay in the case of cell lysates (B) or micro-BCA assay for EV (C).

4 RESULTS: HIV-1 NEF INTERFERES WITH T CELL POLARIZATION AND TEM

HIV-1 Nef interferes with migration of T cells both *in vitro* and *in vivo* (Choe et al., 2002; Hrecka et al., 2005; Janardhan et al., 2004; Lee et al., 2008; Nobile et al., 2010; Park and He, 2009). Nef exerts this effect by altering actin dynamics in the cell. The molecular mechanism governing this was described by our laboratory and it relies on the interaction between Nef and the cellular kinase PAK2 through the viral factor's hydrophobic patch (Stolp et al., 2010; Stolp et al., 2009). The interaction of both proteins is very transient (Imle et al., 2015) but, upon dissociation of the complex, it results in activation of the kinase and leads to a substrate specificity switch toward phosphorylation of the actin severing factor Cofilin. Hyper-phosphorylation of Cofilin results in its inactivation and, thereby, in a reduction in actin turnover affecting not only migration but also chemokine-induced actin cytoskeleton rearrangements following TCR cross linking. In a more recent study, our laboratory described the Nef-mediated interference with T cell homing *in vivo* and transendothelial migration (TEM) *in vitro* (Stolp et al., 2012). Both effects were dependent on the hydrophobic interacting surface of Nef required for its association with PAK2 and, thereby, on the disruption of actin dynamics. However, a more detailed analysis into the multi-step TEM process uncovered a lack of dependency on the integrity of that motif for diapedesis, the central step of this intricate process. Furthermore, Nef was also observed to affect the morphological polarization of the cells upon attachment to the endothelium, a prerequisite for efficient migration performance. Given the need for an extreme polarization of cells in order to perform transmigration through the endothelium, we hypothesized that a deep understanding of the mechanism of Nef-mediated polarization disruption would shed some light onto how the viral protein alters the TEM process. In this work we aimed at unraveling other molecular determinants of Nef involved both in polarization and TEM interference in the context of infection of primary human T lymphocytes.

4.1 Uninfected primary human T cells polarize in a simple 2D in vitro system

In order to determine the conditions under which we could study polarization of primary human T lymphocytes *in vitro* we isolated these cells from Buffy coats with blood of healthy donors. After 72-hours activation with interleukin-2 (IL-2) and phytoemagglutin (PHA), the cells were incubated on Fibronectin (FN)-coated surfaces for 15 minutes and fixed. A chemokine, SDF1-α, was used to stimulate polarization (data not shown), however integrin signaling triggered by crosslinking of FN proved to be sufficient for this. Cells were fixed and stained for the visualization of polarity markers. Well described markers were chosen to this purpose (Fig. 4.1A) (Freeley et al., 2012; Samstag et al., 2003; Stolp et al., 2012; Vicente-Manzanares and Sanchez-Madrid, 2004). Staining of the leading edge markers Talin and PKC-ζ did not provide conclusive information in terms of protein localization since they appeared at the uropod or at the leading edge depending on the individual cell studied. Moreover, immunofluorescence signal for these two markers was in general rather low. Conversely, staining of the

uropod markers Ezrin and CD44 was more robust resulting in a strong signal for the cells mainly at the plasma membrane which was polarized to the rear of the cell if it was morphologically polarized or elongated. The frequency of morphologically polarized cells under these conditions ranged between 30 and 45% depending on the donor. Parallel analysis of uropod marker polarization resulted in frequencies around 70% (Fig. 4.1B). Acquisition of expertise with this experimental set up as well as optimization of the immunofluorescence staining led to an increase in detection of cell polarization based on morphology as well as markers, achieving lymphocyte polarization frequencies ranging from 50 to 70%. In addition, CD44 was chosen as the best polarity marker given the stronger immunofluorescence signal and the lower background. Analysis of cell polarization over time did not reveal strong differences between the various time-points analyzed; however for two of the studied donors polarity frequencies increased until 2 hours and dropped after that, so we decided to fix cell after 2 hours incubation on FN-coated cover glasses in subsequent experiments (Fig 4.1C).

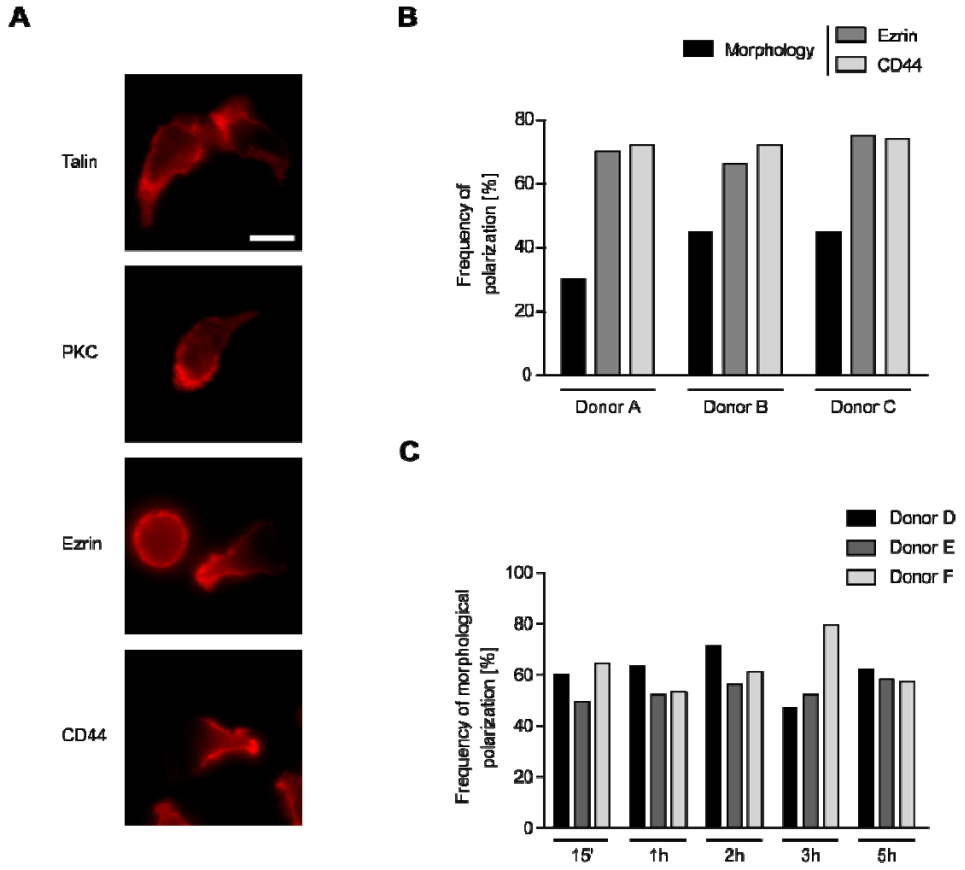

Fig. 4.1 Establishing timing and markers of polarization of Human Peripheral Blood Mononuclear Cells (PBMCs). Human PBMCs were seeded onto Fibronectin (FN)-coated cover glasses and let attach at 37ºC for various time periods, fixed and stained for known polarity markers. Samples were then analyzed by epifluorescence microscopy. (A) Representative epi-fluorescence micrographs showing polarization of the leading edge markers Talin and PKC-ζ and of the uropod markers Ezrin and CD44 2 hours post-seeding. Scale bar = 5 µm. (B) Quantification of polarized cells from three healthy donors. Cells were incubated on FN-coated surface for 15 minutes. A total of 100 cells were analyzed for morphological polarization (black bars) and, among morphologically polarized cells, the frequency of polarization of Ezrin (dark grey bars) and CD44 (light grey bars) markers was evaluated. (C) Morphological polarization of PBMCs from three healthy donors. Different timepoints after seeding were studied.

4.2 HIV-1 Nef interferes with polarization of infected primary human T cells

HIV-1 Nef's effects on T lymphocyte polarization are largely unstudied. Only two reports described that Nef can affect T cell polarity but did not study the mechanism or relevance of this phenomenon (Park and He, 2009; Stolp et al., 2012). Park and He (Park and He, 2009) described a Nef-mediated polarity loss on Jurkat T cell lines inducible for the expression of Nef.GFP or GFP-tagged Nef mutants using crosslink of LFA-1 as polarization stimulus. They, however, did not study the phenotype in the context of infection. Dr. Bettina Stolp-Rastätter from our laboratory (Stolp et al., 2012) observed a defect in polarization of murine T cells transduced for isolated expression of SF2 Nef.GFP and the PAK2-interaction deficient mutant, SF2 F195A Nef , both when seeded on FN-coated cover glasses in the presence of SDF1-α and after attachment to the endothelium *in vitro*. In the present work, we addressed the question from a more physiologically relevant point of view by using PBMCs from healthy donors which were infected *in vitro* with a laboratory strain of HIV-1. Activation is required to render primary human lymphocytes susceptible to productive infection by HIV-1 (Stevenson et al., 1990). After the standard activation with IL-2 and PHA for 72 hours, cells were infected with NL4.3 HIV-1 viruses expressing or lacking expression of the Nef protein. 72 hours post-infection, cells were harvested, seeded on FN-coated cover slips and incubated for 2 hours at 37ºC before being fixed with 3% PFA/PBS. Under these conditions, non-infected cells were established to polarize at least to a frequency of 50%. To visualize cells from these samples we stained them with antibodies recognizing CD44 and p24/CA, as markers of polarization and infection, respectively. In order to exclude dead cells from the analysis, they were stained with the 7-amino-4-chloromethylcoumarin (CMAC) cell tracker dye (Invitrogen, Carlsbad CA) and discriminated from viable cells by the characteristic loss of the dye (Fig. 4.2A, intense blue epifluorescent signal *vs* dim blue epifluorescent signal, respectively; only for cells from donors 5 to 7). Quantification of polarized cells in these samples illustrates the negative effect caused by the wt HIV-1 infection in relation to the values for uninfected cells from the same samples (Fig. 4.2B, black bar and first white bar of each data set, respectively). Frequency of polarization of infected PBMCs was largely decreased (from 47.3% polarization of uninfected to 3.3% polarization of wt HIV-1 infected for donor 1; from 53.6% to 6.7% for donor 2; from 63.2% to 26.7% for donor 3; from 45.3% to 16.7% for donor 4; from 70.0% to 8.3% for donor 5; from 54.0% to 6.7% for donor 6; from 59.0% to 15.0% for donor 7). This reduction of polarization frequency was less pronounced or not observable, depending on the donor, when cells were infected with the ΔNef virus (grey bars as compared to black bars in the same figure; 13.33% polarization of ΔNef HIV-1 infected for donor 1; 33.3% for donor 2; 50.0% for donor 3; 53.3% for donor 4; 28.3% for donor 5; 18.3% for donor 6; 36.7% for donor 7). The differences become more obvious when these results are shown as relative to values of non-infected cells from the same sample (Fig. 4.2C; from 7.1% relative polarization of wt HIV-1 to 27.5% relative polarization of ΔNef HIV-1 infected for donor 1; from 12.4% to 52.7% for donor 2; from 42.2% to 88.8% for donor 3; from 36.8% to 103.9% for donor 4; from 11.9% to 41.1% for donor 5; from 12.4% to 36.7% for donor 6; from

25.4% to 52.4% for donor 7). It is appreciable in all cases that polarity values increase in the absence of Nef, even reaching levels of control uninfected cells for two donors. For these two donors Nef was sufficient to inhibit T cell polarization while other factors may contribute to this effect in the other donors. From these results we conclude that HIV-1 infection disrupts polarization of human primary T lymphocytes in vitro and that Nef plays a critical role in this polarity disruption.

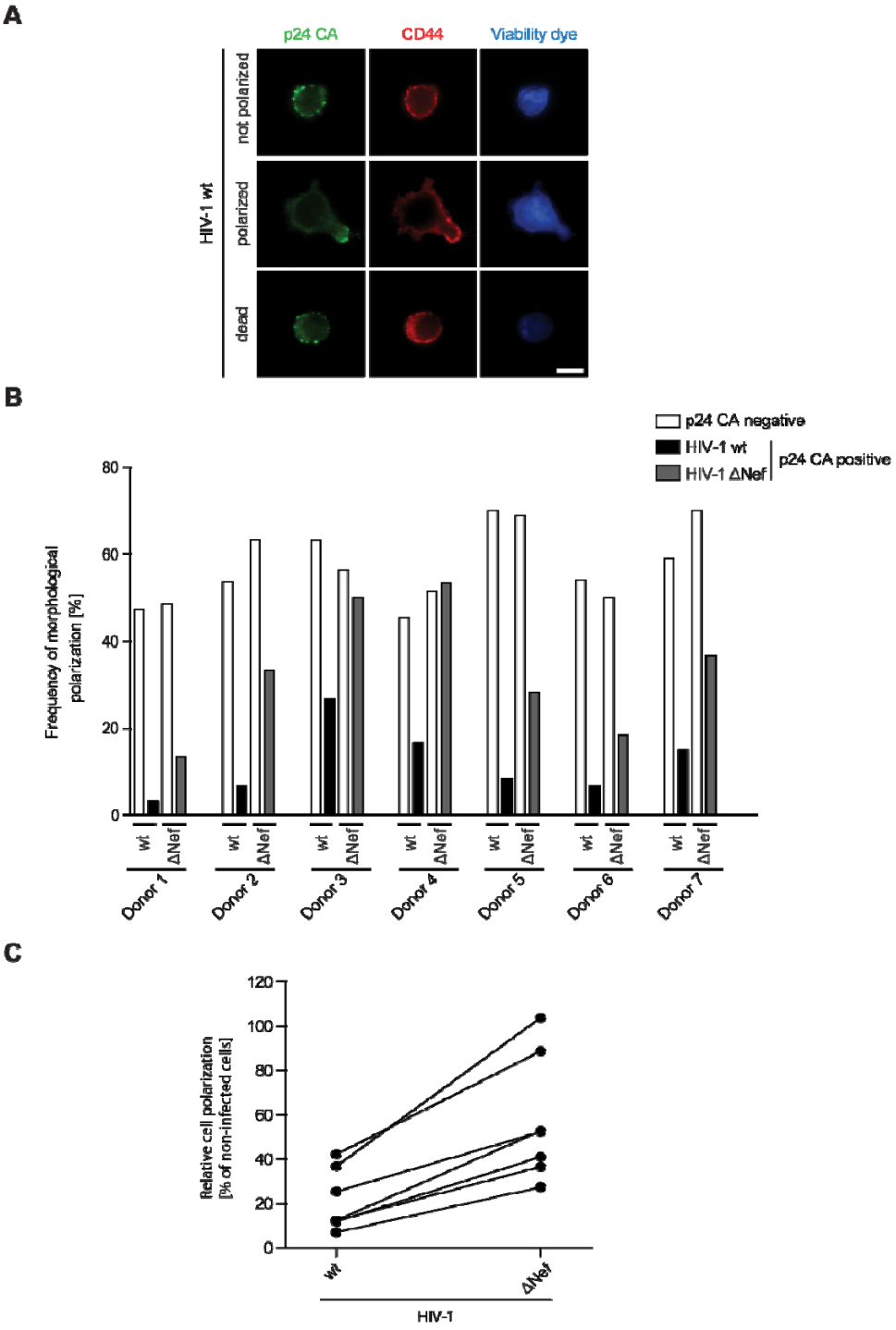

Fig. 4.2 HIV-1 Nef impairs polarity of primary infected human T cells. Human PBMCs were activated for 72 hours with 2 µg/ml PHA and 10 µg/ml IL-2 and spin-infected with wt and ΔNef versions of NL4.3 SF2 Nef HIV-1 in the presence of 4 µg/ml polybrene. After 72 hours, cells were seeded on FN-coated cover glasses, incubated for 2h at

37ºC and fixed. Cells were stained for CD44 and HIV-1 p24-Capsid, as polarity and infection markers, respectively. For donors 5 to 7, CellTracker™ Blue CMAC viability dye was used to discriminate dead cells. (A) Representative epi-fluorescence micrographs of the studied morphotypes: polarized (upper row) or not polarized (middle row). Dead cells (lower row) were not considered. Scale bar = 5 µm. (B) Histogram of the polarization values observed for HIV-1 wt-infected (black) and ΔNef-infected (grey) cells. Polarization values of non-infected cells (white) from the same cover glass are shown as controls. At least 30 cells were counted per condition. (C) Relative frequency of polarization was calculated as the ratio between polarity frequency values of infected cells (p24 CA positive, black and grey bars in B) and those of non-infected control cells from the same cover glass (p24 CA negative, white bars in B).

4.3 HIV-1 Vpu is dispensable for disruption of T cell polarity

As addressed by Haller et al. (Haller et al., 2014) and extended in the first part of this doctoral thesis, HIV-1 Nef and Vpu share several activities such as downregulation of a multitude of host cell surface receptors, rerouting the cellular kinase Lck form the plasma membrane to cytoplasmic compartments and induction of EV secretion. Since polarization frequencies did not reach levels of uninfected control cells for several of the donors studied when their cells were infected with the *nef* deleted HIV-1 virus, we wondered whether Vpu could act synergistically with Nef. To answer this question we included the Vpu deficient as well as the double deficient (lacking expression of both Nef and Vpu) counterparts of the NL4.3 virus used in the previous experiment. After cell fixation and immunostaining for CD44 and p24-Capsid (Figure 4.3A), cell polarity was quantified. As we had observed before, HIV-1 ΔNef induced a weaker reduction in polarization frequency than HIV-1 wt (Fig. 4.3B and C; from 23.3% polarization of wt HIV-1 infected to 46.7% polarization of ΔNef HIV-1 infected for donor 8; from 33.3% to 43.3% for donor 9; from 23.3% to 50.0% for donor 10; from 59.7% polarization relative to non-infected of wt HIV-1 to 97.3% polarization relative to non-infected of ΔNef HIV-1 infected for donor 8; from 66.6% to 92.1% for donor 9; from 44.0% to 100.0% for donor 10). In the case of the three donors studied here, polarization frequencies completely equaled those of uninfected control cells. No significant differences were observed, however, between the polarization levels of cells infected with the ΔVpu virus and the wt virus (16.7% polarization of ΔVpu HIV-1 infected for donor 8; 23.3% for donor 9; 16.7% for donor 10; 40.7% polarization relative to non-infected of ΔNef HIV-1 infected cells for donor 8; 47.6% for donor 9; 39.8% for donor 10). The effect of the double deficiency in Nef and Vpu expression did not adjust to a clear trend and the donor variability in polarity frequency was high. Thus, the Vpu protein of HIV-1 does not affect polarity of human T lymphocytes in the context of infection. Taken together with the results presented in 4.3, for 5 out of ten donors, the absence of Nef completely prevented disruption of polarization in HIV-1 infected cells. For the other 5 donors, the observed partial effect accounted for more than a two-fold increase in polarization as compared to wt HIV-1 infected cells. Together with result from 4.3, the results shown here support the idea that HIV-1 Nef is the major player in disruption of polarity of infected human T cells. Therefore, we focused on studying Nef as the key factor in T cell polarity impairment from here on.

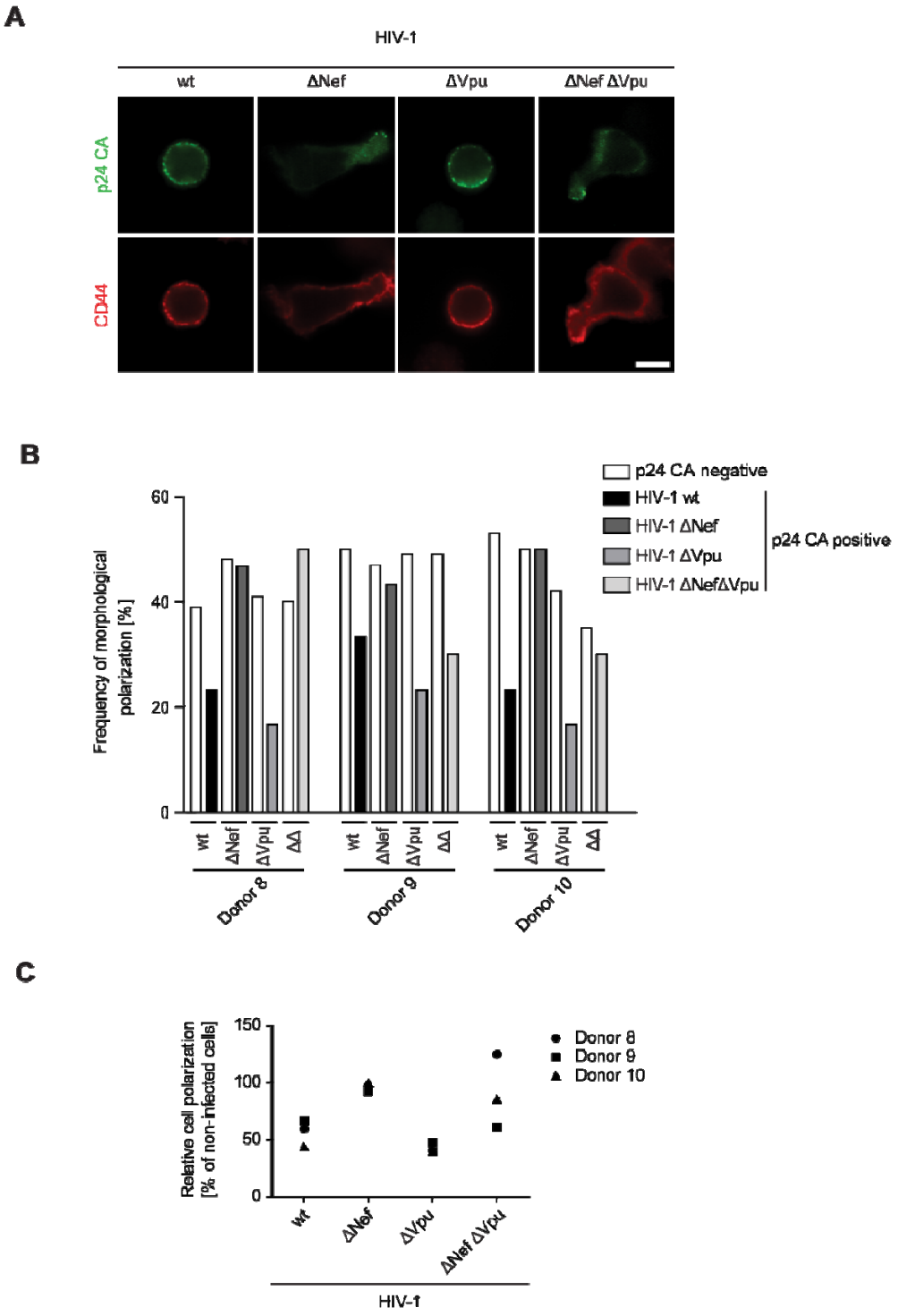

Fig. 4.3 HIV-1 Vpu does not affect polarity of primary human T cells. Pre-activated human PBMCs (2 µg/ml PHA and 10 µg/ml IL-2 for 72 hours) were spin-infected with HIV-1 wt NL4.3 SF2 Nef and its ΔNef, ΔVpu and ΔNefΔVpu derivatives in the presence of 4 µg/ml polybrene. Another 72 hours later, cells were seeded on FN-coated cover glasses, incubated for 2h at 37ºC, and fixed. Staining for HIV-1 p24-Capsid and CD44 was performed. Dead cells were identified by loss of the live-cell dye CellTracker™ Blue CMAC in all samples. (A) Representative epi-fluorescence micrographs. Scale bar = 5 µm. (B) Polarity quantification. Coloured bars indicate polarization levels of infected cells in comparison with the polarization levels of uninfected cells from the same cover glasses (white bars). 100 cells were counted per condition. (C) Relative frequency of polarization of infected cells from donors 10 to 12 (p24 CA positive, coloured bars in B) as compared to non-infected control cells (p24 CA negative, white bars in B) from the same cover glass.

4.4 Establishment of an experimental system to study Nef-mediated effects on T cell polarity

To further understand Nef's effects on polarity we wanted to search for molecular determinants involved in the phenotype as well as to evaluate the prevalence of this phenotype in lentiviral evolution. For that we needed a cellular system in which we could express various Nef mutants and alleles. To that end, we tested nucleofection as delivery method of an expression vector for GFP into PBMCs using the AMAXA kit (Lonza, Basel, Switzerland) and evaluated polarization of these cells. This resulted in frequencies of polarization as low as 2% for GFP-expressing cells (Fig. 4.4A and B). Values were only slightly higher for GFP-negative cells from the same coverslip or mock control cells; however they did not exceed 21%. Under these circumstances, the dynamic range needed to see the consequences of Nef expression would not be achieved. Moreover, polarization frequency values of control (untransfected) cells were importantly reduced respect to the usual values observed for control (uninfected) PBMCs (Figures 4.1C, 4.2B and 4.3B *vs*). Therefore we can conclude that the process of nucleofection itself negatively affected the capacity of cells to polarize. Thereby nucleofection was ruled out as a gene delivery method for subsequent experiments. As a next attempt, A3.01 cells were tested for polarity. These cells showed a frequency of polarization of around 50% for all the timepoints studied (Figure 4.4C). When transfected for the expression of GFP or Nef.GFP, polarity values dropped marginally for GFP-expressing control cells but expression of Nef caused a 2-fold reduction in cell polarization. In such situation, the dynamic range of Nef's effect on polarity was broad enough to make possible the molecular studies we wanted to pursue (Figure 4.4 D and E). Thus, A3.01 cells turned out to be a suitable system for subsequent molecular studies to further enlarge our understanding of Nef-mediated effect on T cell-polarity.

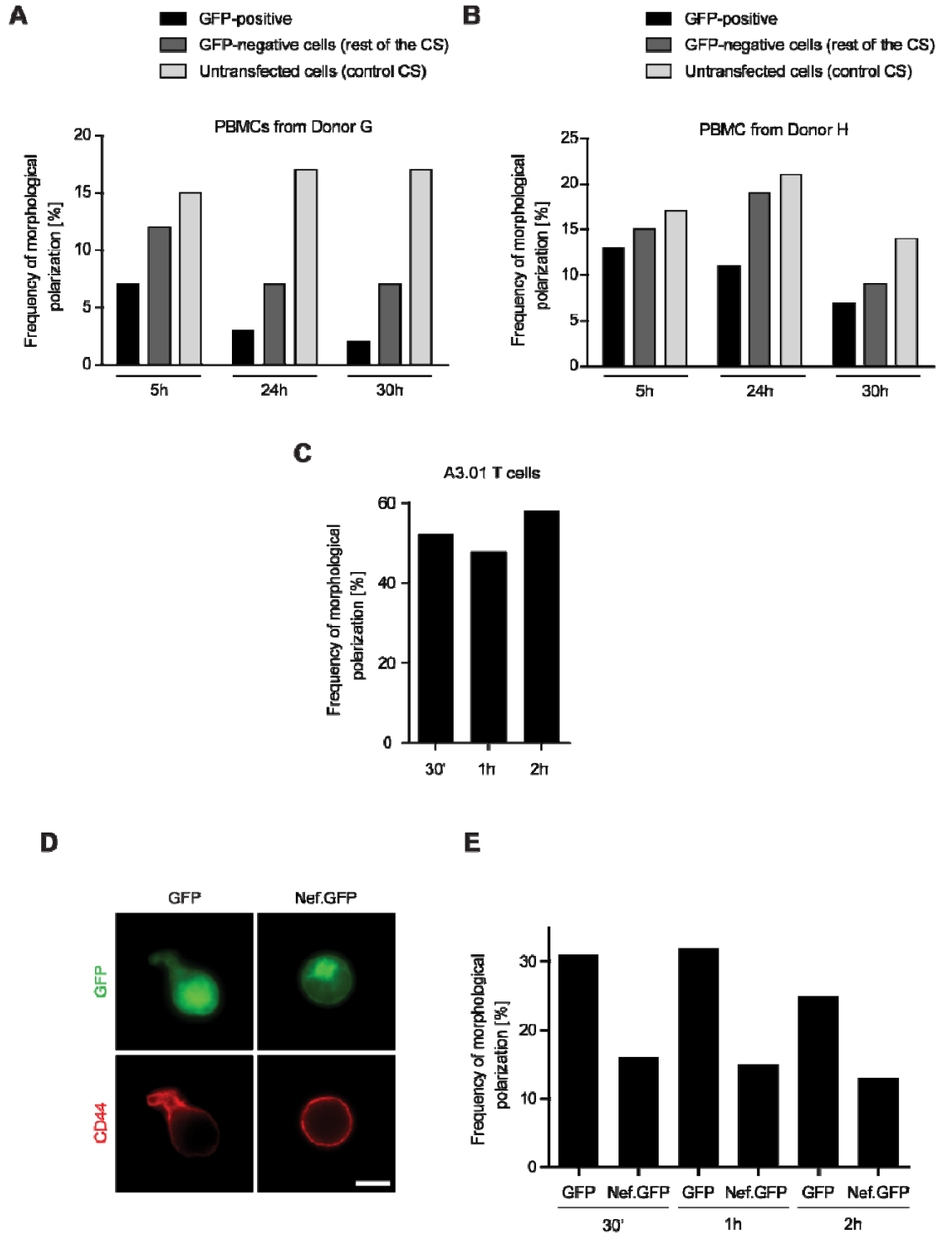

Fig. 4.4 A3.01 T lymphocytes are a suitable system for *in vitro* polarity studies. (A and B) Pre-activated (2 µg/ml PHA and IL-2 10 µg/ml for 72 hours) human PBMCs isolated from two healthy donors were nucleofected with an expression vector for GFP. Polarity assays were performed 28 hours later and cells were fixed at various timepoints. Histogram bars depict frequency of polarization of GFP-positive cells (black bar) compared to those of GFP-negative cells from the same coverslip (dark grey bar) or mock control cells (light grey) from a different coverslip. (C) Polarization values of human A3.01 T lymphocytes subjected to polarity assay. Cells were fixed at the indicated timepoints. n = 100 cells. (D) Representative epi-fluorescent micrographs of A3.01 cells transfected with expression plasmids for GFP or SF2 Nef.GFP. 6 hours post-transfection, cells were subjected to polarity assay and fixed at different timepoints. Shown are cells fixed 1 hour post-transfection. Scale bar = 5 µm. (E) Quantification of cells shown in (D). n = 100 cells.

4.5 T cell polarity disruption is an evolutionarily conserved effect of lentiviral Nef proteins

Primate lentiviruses, including human pathogenic viruses HIV-1 and HIV-2 as well as SIVs, have in common, among other features, the accessory *nef* gen. Functions of the viral gene products important for HIV-1 biology are conserved and conserved throughout evolution. This is the case for most of the described functions of Nef. However, some functional differences have been also reported as the ability of HIV-2 Nef to downregulate CD3 from the plasma membrane which cannot be performed by HIV-1. Lack of this activity correlates to high T cell activation levels observed in infected subjects and with virus pathogenicity displaying (faster) progression towards AIDS (Munch et al., 2002; Rudolph et al., 2009; Schindler et al., 2006). According to the capacity to downmodulate CD3, Nef alleles can be categorized into two groups: Group 1, containing Nef alleles of HIV-1 and SIVcpz, unable to downmodulate CD3, and Group 2, including Nef alleles of less- or non-pathogenic viruses HIV-2 and SIV that downregulate CD3. Differences between Nef alleles can help determining at which point in lentiviral evolution a specific activity was developed. All previously shown results were obtained with the HIV-1 SF2 Nef allele, the standard allele used for *in vitro* studies in our laboratory. To study the evolutionary relevance of Nef-mediated polarity disruption we transfected A3.01 human T lymphocytes with expression plasmids for GFP, HIV-1 SF2 Nef.GFP and a panel of YFP-tagged Nef alleles from HIV-1 (NL4.3, NA7, 8161K9, YBF30), HIV-2 (Ben, Cbl23), and SIV (Cpz Gab2, Cpz Tan3, gsn, mon Val, mac239, blue, smm Fwr 1, smm Ffm 1, agm Sab 1, agm Tan 1, sykes 51). Six hours after transfection, cells were incubated at 37ºC on FN-coated coverslips for 1 hour, fixed and stained for the visualization of the polarity marker CD44 (Figure 4.5A). All alleles tested reduced the frequency of polarization when expressed by A3.01 cells ranging from 9.7 +/- 3.8% for SIV Nef mac239 to 40.7 +/- 9.3% for HIV-1 YBF30 as compared to GFP-expressing control cells, with 46.8 +/- 7.1%. In addition, all of them showed no statistical differences when compared to the effect caused by HIV-1 SF2 Nef except two Nef alleles, HIV-1 YBF30 Nef and SIV cpz Tan 3 Nef (Figure 4.5B; p values of 0.0315 and 0.0400, respectively; p value for SIV Nef Gab 2 of 0.0625). These two Nef alleles were totally or partially deficient for many other studied Nef functions, including Lck relocalization, TCR-induced actin dynamics or induction of tyrosine phosphorylation (Rudolph et al., 2009). A comparative analysis of the polarity values of Nef alleles categorized into group 1 or group 2 evidenced a statistically significant difference between them, suggesting Nef's effects on polarity may be linked to the CD3 downregulation capacity of these alleles (Figure 4.5C; p value < 0.0001). To address whether CD3 downmodulation is indeed coupled to the Nef-mediated disruption of polarity, we made use of a panel of SIV Blue Nef mutants with specific defects in downregulating surface receptors (Rudolph et al., 2009). Modification of the ExxxL motif of SIV Blue Nef generates a mutant which specifically downregulates CD3 but not other receptors (E181A/L185A; ELLA). R129/130A (RRAA) and R129A mutations result in CD3 downmodulation deficient mutants. Surprisingly, ELLA mutant turned out to be deficient in T cell polarity impairment while RRAA and R129A performed as

efficiently as SIV Blue Nef wt (Figure 4.5D; 19.0 +/- 2.3% for SIV wt Blue; 24.1 +/- 0.8% for RRAA; 14.2 +/- 6.3% for R129A; 42.7 +/- 10.4%). However, comparison of cell polarization frequencies for HIV-1 SF2 Nef, unable to downregulate CD3, with those of SIV ELLA Blue Nef, selectively targeting CD3 for downregulation, allows us to rule out a CD3 downregulation dependency for the observed Nef-mediated disruption of T cell polarity.

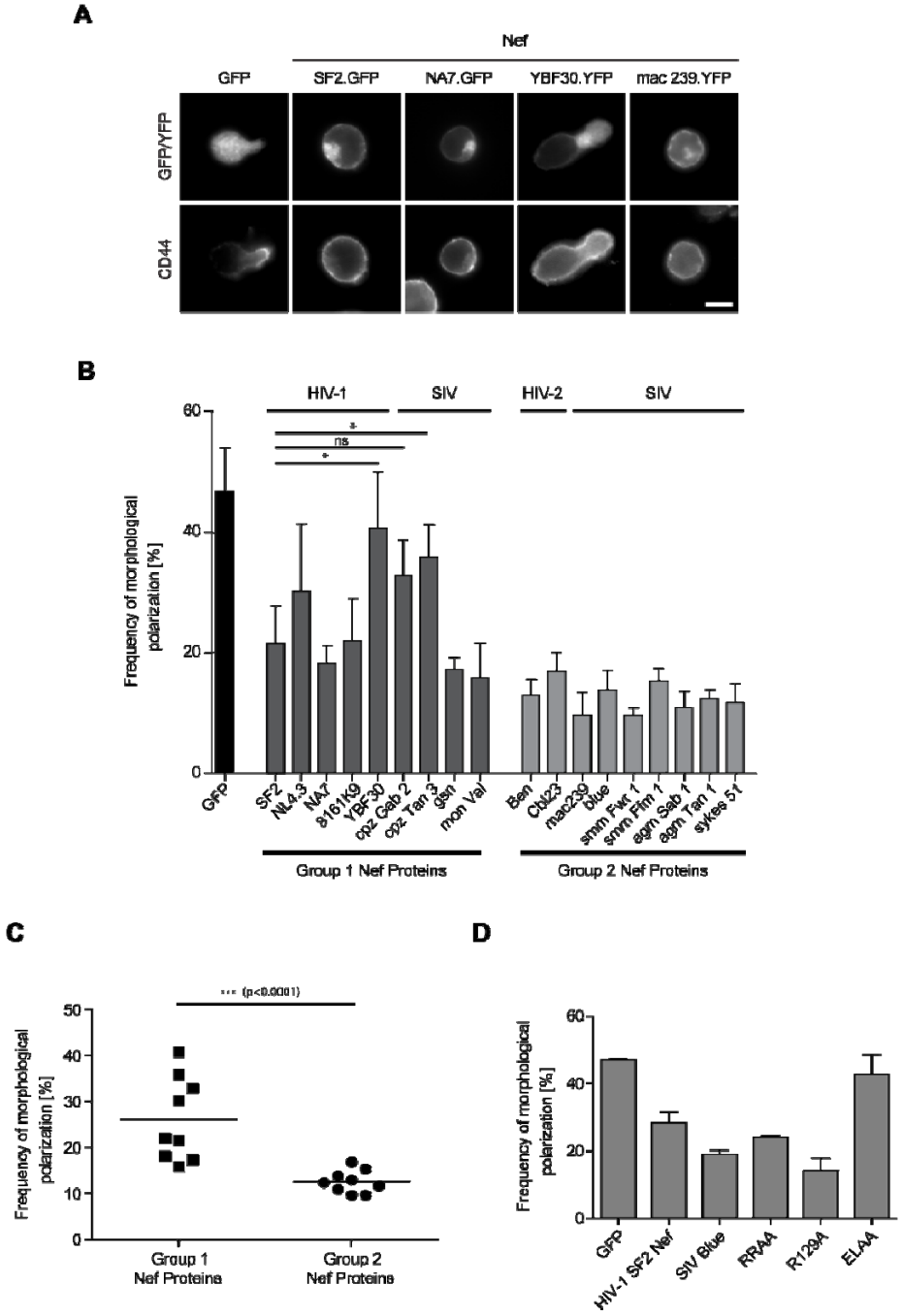

Fig. 4.5 Polarity disruption is an evolutionarily conserved activity of Nef proteins. A3.01 T lymphocytes were transiently transfected with expression plasmids for GFP, SF2 Nef.GFP or YFP versions of various SIV, HIV-1 and HIV-2 Nef alleles. 6 hours post-transfection, cells were seeded on FN-coated cover glasses and incubated for 1h at 37ºC, fixed and stained for CD44. At least 100 cells were counted for each condition. (A) Representative epi-fluorescence micrographs of transfected cells. Scale bar = 5 µm. (B) Histogram bars depict polarization levels of

transfected cells. Nef alleles were categorized into tow group: group 1, or Nef alleles able to downregulate the CD3 surface receptor, and group 2, or Nef alleles unable to downregulate CD3. Shown are arithmetic mean values and standard errors from three independent experiments. Statistical significance as assessed by the Man-Whitney U test is indicated (*, $P < 0.05$). (C) Batch comparison of the two groups of Nef alleles. Bars indicate mean values for each group. Statistical analysis was performed by the Man-Whitney U test (***, $P < 0.05$). (D) A3.01 cells were transfected with expression vectors for GFP, SF2 Nef.GFP or YFP versions of SIV Blue Nef and three of its mutants. Polarity assay was performed as described above and polarization quantification was carried out. Arithmetic means and standard errors are plotted.

4.6 The NAKC interacting surface of Nef is important for T cell polarity impairment

As described above (see section 4), the molecular determinant/s of Nef that mediate the impairment of transendothelial migration and polarization are unknown. To identify these determinants, we transfected A3.01 human T cells with expression plasmids for GFP, Nef.GFP or mutants thereof. After 6 hours, cells were incubated at 37ºC on FN-coated coverslips for 1 hour, fixed and stained for the polarity marker CD44 (Figure 4.6A). Frequency of polarization was quantified for transfected cells. As observed previously, the isolated expression of Nef resulted in an approximate 2-fold decrease in polarization frequency as compared to GFP-expressing control cells (Figure 4.6B; 24.7 +/- 2.4% for SF2 wt Nef and 48.4 +/- 3.7% for GFP). As expected, the PAK2-interacting deficient mutant, F195A Nef.GFP (Stolp et al., 2010; Stolp et al., 2009), did not cause impairment and displayed polarization levels comparable to those of control cells, 48.2 +/- 4.0%. Expression of G2A Nef mutant, unable to anchor into membranes due to lack of the myristoyl-acceptor glycine 2 and, therefore, largely deficient in all Nef functions (Fackler et al., 2006; Geyer et al., 1999), also resulted in a frequency of polarization similar to that of GFP-expressing control cells, 44.2 +/- 12.4%. On the other hand, mutations of serine at position 6, the phosphorylation site by novel PKC (nPKC) required for efficient viral transcription and infectivity (Wolf et al., 2008), and of methionine at position 20, needed for downmodulation of HLA-I (Tomiyama et al., 2002), did not show relevance for T cell polarity impairment (polarization frequencies of 28.2 +/- 7.2% and 23.2 +/- 1.4%, respectively). The same was observed for sequence modifications targeting the motifs LL168/169 and ED178/179 at the C-terminal flexible loop, essential for CD4 downregulation through the interaction with the endocytic machinery (adaptor complex and V-ATPase, respectively) (Bresnahan et al., 1998; Craig et al., 1998; Lu et al., 1998), (polarization frequencies of 23.4 +/- 6.7% and 28.9 +/- 6.4%, respectively). Mutations at the acidic stretch (EEEE66-69), needed for interaction with PACS adaptor involved in receptor downregulation (Piguet et al., 2000), the VGF motif, the integrity of which is needed for SH3-domain interactions between Nef and host proteins (Meuwissen et al., 2012), or the Proline rich motif (PxxP), part of the SH3 domain interaction surface in Nef (Collette et al., 1996; Saksela et al., 1995), resulted in intermediate reduction in polarity as compared to the expression of SF2 wt Nef and GFP (35.7 +/- 2.2%, 31.9 +/- 4.8%, 4.6 +/- 4.4%, respectively). Interestingly, expression of a Nef version lacking an amphipathic alpha-helix necessary for the interaction with a multi-molecular complex consisting of at least two adaptor proteins and four kinases, NAKC (Baur et al., 1997), did not affect cell

polarization levels as compared to GFP-expressing control cells, displaying a frequency of polarization of 50.8 +/- 8.2%. Thus, the interacting surface of Nef implicated in the formation of the NAKC is required for inhibition of polarity by Nef. This suggests the implication of one or more of the factors and/or kinases involved in the complex in the molecular mechanism governing this effect.

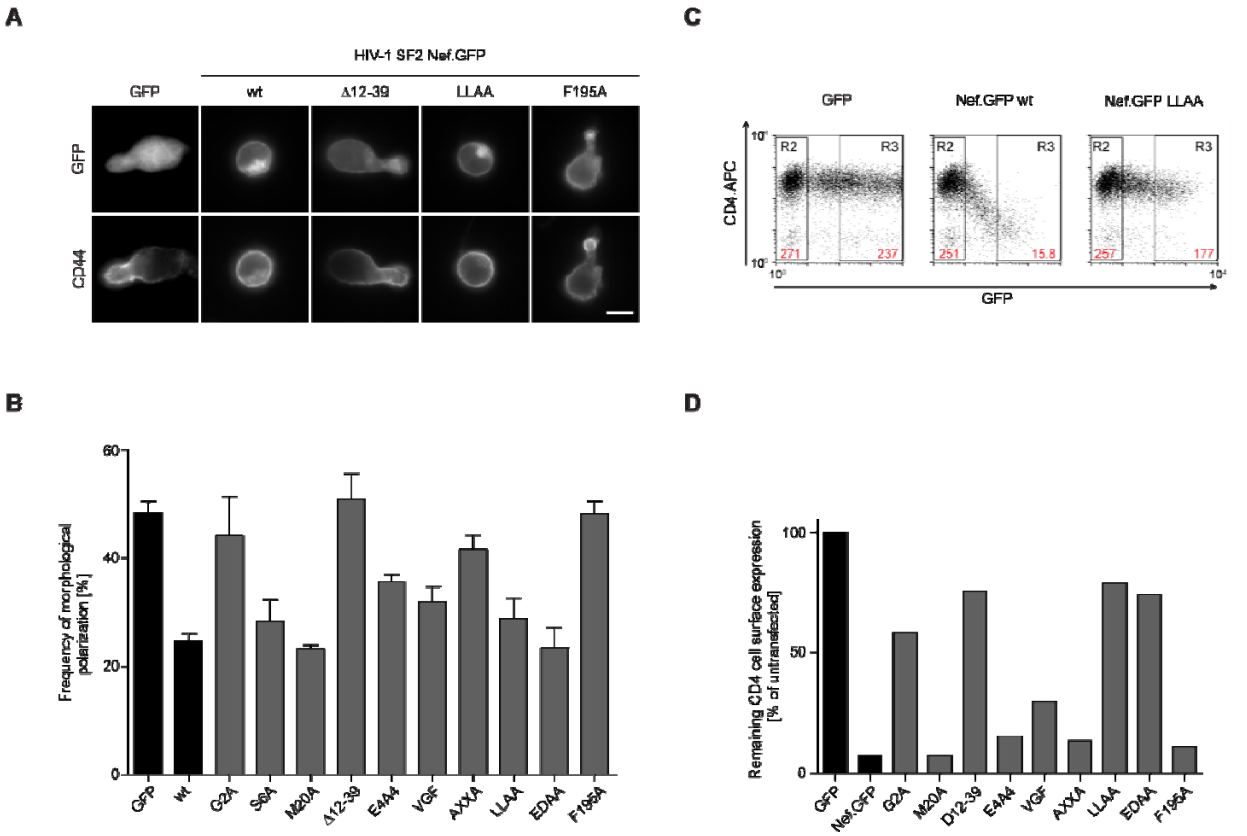

Fig. 4.6 Mapping the phenotype: correct NAKC formation necessary for T cell polarity. A3.01 cells were transfected with expression plasmids for GFP, SF2 Nef.GFP or various mutants. 6 hours after transfection, cells were incubated at 37ºC for 1 hour on FN-pre-coated cover glasses, fixed and immunostained for CD44. (A) Representative epi-fluorescence micrographs of transfected cells. Scale bar = 5µm. (B) Histogram showing polarization levels of cells transfected with the indicated mutants. Bars correspond to the arithmetic mean and standard error of three independent experiments. At least 100 cells were counted for each condition. (C) Cells from the same source as those used in (A) and (B) were stained in parallel with APC-coupled antibodies targeting CD4 to evaluate by flow cytometry the downregulation capacity of each of the mutants studied. Representative dot plots of gated living cells transfected with GFP, wt Nef.GFP or LLAA Nef.GFP are shown. MFI values are shown in red for the gated populations. (D) Histogram depicts the remaining surface CD4 levels obtained by dividing the MFI of transfected cells by that of untransfected cells (R3 gate *vs* R2 gate, respectively). All values were normalized to that of GFP transfected cells, arbitrarily set to 100.

To make sure of the functionality of the constructs used in the previous mapping experiment, we evaluated CD4 surface expression levels of cells from the same source. Cells were harvested 8 hours post-transfection and stained them with an APC-coupled antibody recognizing CD4 for subsequent analysis by flow cytometry. Membrane exposed CD4 levels were calculated as the ratio between the MFI values of intermediate/high-GFP-expressing cells and those of untransfected cells (GFP-negative)

form the same sample (Figure 4.6C, gate R3 *vs* gate R2). As expected, modifications in Methionine at position 20, the acidic stretch (EEEE66-69), the VGF motif, the PxxP motif and the hydrophobic patch (F195) had little to no effect in the expression of CD4 (Collette et al., 1996; Fackler et al., 2006; Meuwissen et al., 2012; Piguet et al., 2000; Saksela, 2011; Saksela et al., 1995). The non-myristoylatable mutant of Nef displayed a two-fold reduction in CD4 expression at host cell plasma membrane (Fackler et al., 2006; Geyer et al., 2001; Giese et al., 2006). As expected, structural changes in the C-terminal flexible loop, and more specifically at LL168/169 and ED178/179 residues, abrogated this Nef activity (Chaudhuri et al., 2007; Fackler et al., 2006; Keppler et al., 2006). Finally and also expectedly, deletion of the 12-39 region of Nef caused also no significant difference in remaining CD4 expression when comparing to that of GFP-expressing control cells, or, in other words, this mutant was deficient for CD4 downregulation (Figure 4.6D) (Fackler et al., 2006).

Taken together, the in vitro mapping allowed to define a protein determinant previously unrelated to migration phenotypes, namely the NAKC interaction surface (12-39), as essential for Nef-mediated T cell polarity disruption together with the PAK2-interacting surface.

4.7 NAKC interaction surface of Nef is required for disruption of T cell polarity in the context of HIV-1 infection

We next wanted to validate the results from the mapping experiment in a more physiologically relevant set up. We utilized the experimental design used in the beginning of this work (see 4.1 and 4.2) in which we infected pre-activated primary PBMCs from healthy donors and subjected them to our polarity assay 72 hours later. In this case, together with NL4.3 HIV-1 wt SF2 Nef and its Nef-deficient version we included viruses bearing F195I and Δ12-39 Nef mutants. CD44 and p24-Capsid were used as polarity and infection markers, respectively (Figure 4.7A). Evaluation of polarization frequencies once more showed a strong reduction in polarity of HIV-1 wt infected cells as compared to non infected cells from the same sample (Figure 4.7B; from 66.4% polarization of uninfected to 14.6% polarization of wt HIV-1 infected for donor 11; from 56.1% to 19.7% for donor 12; from 69.9% to 32.2% for donor 13). This difference became less pronounced or even not observable for cells infected with the ΔNef virus (from 71.7% polarization of uninfected to 44.4% polarization of ΔNef HIV-1 infected for donor 11; from 45.1% to 62.0% for donor 12; from 68.1% to 73.5% for donor 13). Of note, polarization levels of cells infected with both Nef-mutant viruses also resulted in polarization frequencies similar to those of uninfected cells and ΔNef-infected cells for all three donors studied (polarization frequency of 52.1, 49.2 and 67.6%, for cells from donors 11 to 13 infected with Δ12-39 Nef HIV-1, respectively; 52.2, 50.8 and 67.1% for cells from donors 11 to 13 infected with F195I HIV-1 virus, respectively). Thus, the amphipathic alpha-helix at the N-terminal region of Nef responsible for the formation of a protein/kinase complex is also required for the interference with polarization processes in T cells.

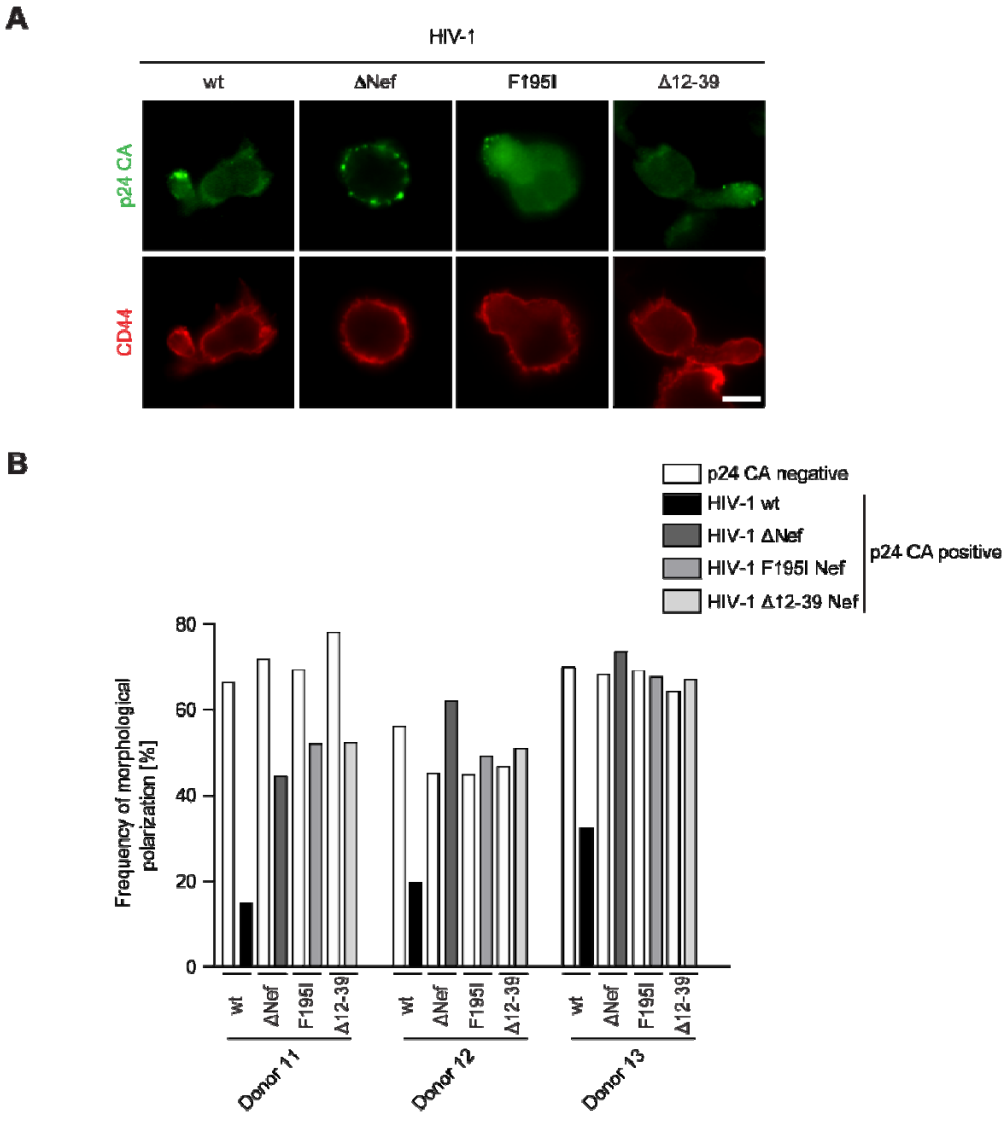

Fig. 4.7 The NAKC-interaction surface of Nef is also relevant in the context of infection. Pre-activated (PHA 2 µg/ml and 10 µg/ml IL-2 for 72 hours) human PBMCs from healthy donors were spininfected in the presence of 4 µg/ml polybrene. Cells were then incubated on FN-coated coverslips at 37ºC for 2 hours, fixed and stained for CD44 and HIV-1 p24-Capsid. (A) Representative images of infected cells acquired with an epifluorescence microscope. Scale bar = 5 µm. (B) Histogram of the polarization levels of cells infected with HIV-1 wt (black) and or its ΔNef, F195I Nef, Δ12-39 Nef derivatives (three tones of grey: dark to light, respectively) compared to uninfected cells from the same coverslip (white). At least 100 cells were counted for each condition.

4.8 Pharmacological inhibition of NAKC members unravels implication of PKC as part of the Nef-mediated polarity disruption mechanism

In order to search for host cell factors which could potentially be implicated in the molecular mechanism of the impairment of T cell polarity by Nef, we targeted members of NAKC by pharmacological inhibition. First of all the potential cytotoxicity of this drugs was studied by evaluating viability rates of A3.01 cells pot-treatment at different timepoints by exclusion of 7AAD dye. After the corresponding adjustment of concentration, none of the inhibitors caused cytotoxic effects on target cells (data not

shown). A3.01 cells were transfected with expression plasmids for GFP or Nef.GFP followed by addition of inhibitors or DMSO to the culture 1 hour later and cells were cultured for 6 hours in the presence of the inhibitors (see Table). After harvesting, cells were seeded on FN-coated coverslips, incubated for 1 hour at 37ºC, fixed and stained for CD44 (Figure 4.8A). Addition of DMSO did not change frequencies of polarization of GFP- and Nef-GFP transfected cells as compared to the same DMSO-untreated cells. Treatment of GFP-transfected cells with the inhibitors caused no significant change in the frequency o polarization as compared to control and DMSO-treated cells. The only exception was observed for the addition of rottlerin, a PKC inhibitor targeting preferentially PKC-δ and less efficiently all the other PKC isoforms as well as other cell proteins including MAPK. This inhibitor induced a decrease in the frequency of polarization to the levels seen for untreated and DMSO-treated Nef.GFP-transfected cells. On the other hand, treatment of Nef-expressing cells with the inhibitors resulted in no significant increase in the frequency of polarization excluding Gö6983, a pan protein kinase C (PKC) inhibitor which targets all PKC isoforms except PKC μ (Figure 4.8B; 35.8 +/- 6.8% as compared to 20.0 +/- 6.2% of Nef.GFP-expressing DMSO-treated cells) (He et al., 2014; Lin et al., 2014; Prgomet et al., 2015; Young et al., 2005). The drug caused a recovery in the frequency of polarization to that observed for control and DMSO-treated GFP-transfected cells. Of note, no difference was observed for GFP-expressing cells cultured in the presence of Gö6983 as compared to untreated and DMSO-treated GFP-expressing control cells (Figure 4.8C; 4.4 +/- 2.9% and 43.2 +/- 4.4%, respectively), providing evidence that the observed recovery in polarity levels is Nef-specific. Importantly, treatment with Rottlerin resulted in an even stronger reduction in the polarization frequency levels of Nef.GFP-transfected cells as compared to the Nef.GFP control and DMSO-treated cells. Since this drug showed a low target selectivity affecting indistinctly GFP- and Nef-GFP expressing cells, we concluded that Rottlerin treatment results in an unspecific reduction in the frequency of polarization which does not relate to Nef expression. Thus, from the Nef-specific effect on of polarization levels observed after treatment with a PKC inhibitor, we presume PKC plays a key role as a host cellular Nef partner in mediating disruption of T lymphocyte polarity.

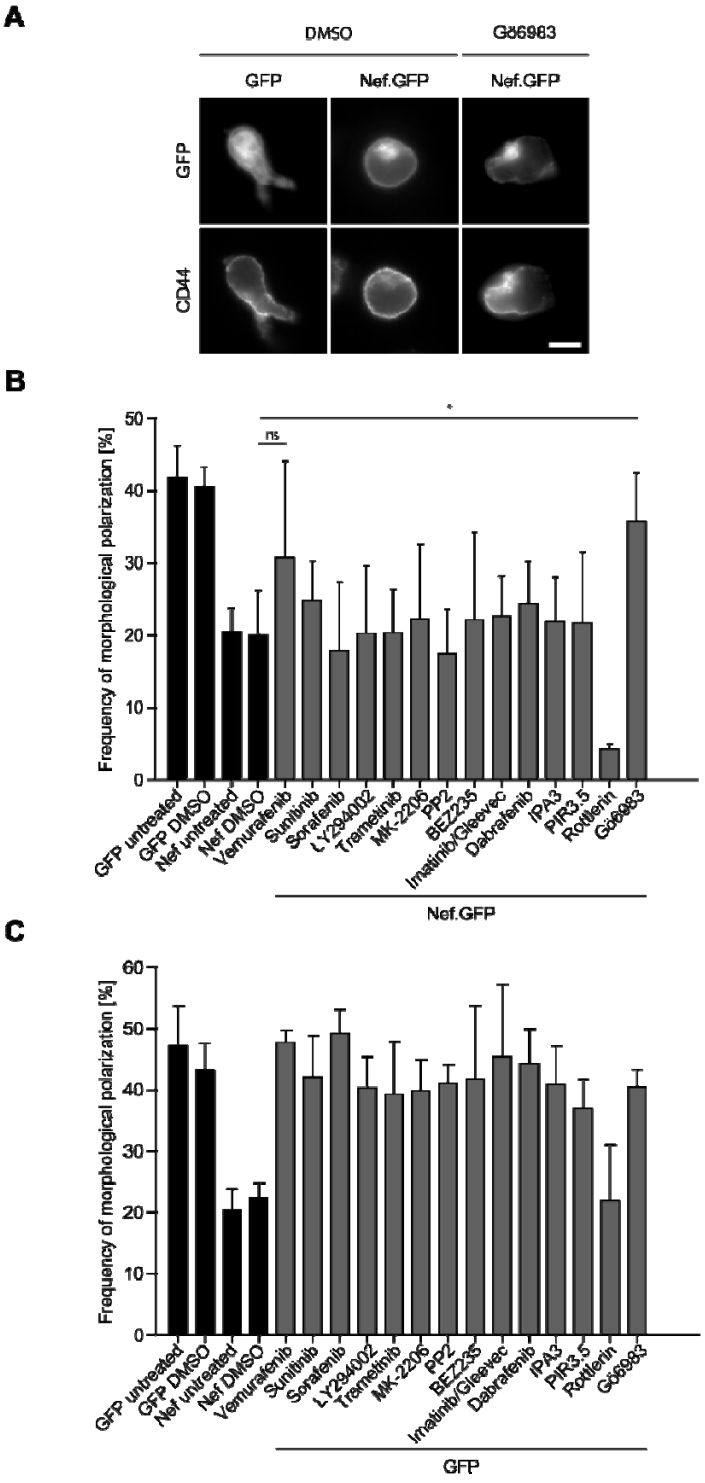

Fig. 4.8 Pharmacological inhibition of NAKC members discloses involvement of PKC in the Nef-mediated impairment of T cell polarity. A3.01 cells were transfected with GFP or Nef.GFP. One hour later, the inhibitors were added to the culture (see working concentrations in Table) and cells were incubated for another six hours. DMSO was used as the internal control. Cells were then incubated for one hour on FN-coated coverslips, fixed and stained for CD44. (A) Representative epi-fluorescence micrographs of transfected control and Gö6983-treated cells. Scale bar = 5µm. (B) Polarization frequency values of inhibitor-treated Nef.GFP transfected cells (grey) compared to GFP and Nef.GFP untreated and DMSO-treated controls (black). At least 100 cells were counted for each condition. Depicted are the arithmetic means and standard deviation from three independent experiments. (C) Frequency of polarization of inhibitor-treated GFP-transfected cells compared to the same controls as in (B). At least 100 cells counted per condition. Arithmetic means and standard deviation from three independent experiments are shown. Statistical significance as assessed by the Man-Whitney U test is indicated (*, $P < 0.05$).

4.9 The NAKC interaction surface of Nef is not involved in chemotaxis inhibition toward SDF-1α *in vitro*

A well described activity impaired by Nef is T lymphocyte chemotaxis toward the cytokine SDF-1α through a porous filter system (Choe et al., 2002; Lee et al., 2008; Park and He, 2009; Stolp et al., 2010; Stolp et al., 2009). With a pore size sufficiently small, this artificial system can resemble in a not very physiological manner the central steps of TEM diapedesis. In order to see if the defect in T cell polarization described here accounts for the previously observed disruption in diapedesis (Stolp et al., 2012), we wanted to test the 12-39 deleted Nef mutant in this assay and compare it to Nef wt and the rest of the relevant mutants. To that end, we transfected CCR7 T lymphocytes for the expression of GFP, Nef.GFP and various Nef mutants as GFP fusions. 24 hours after transfection, cells were starved in 0.5% FCS-containing medium in order to boost response to the chemokine. Cells were seeded onto transwell inserts of 5 μm pore size and placed on 50 μg/ml SDF-1α-containing media. Migration of GFP-positive cells was evaluated by flow cytometry after culturing the cells at 37ºC and 5% CO_2 for 2 hours (Figure 4.9A). The proportion of migrated cells was calculated as the ratio between the percentages of GFP-positive cells from the cells migrated through the filter (total migrated cells at the lower chamber) and the cells added initially on the filter (input). The obtained value is then normalized to those obtained for GFP-transfected control cells arbitrarily set to 100%. Expression of Nef abrogated almost completely migration as compared to GFP-expressing control cells (Figure 4.9B; 4.4 +/- 2.3% migrated cells expressing Nef.GFP). Mutation in the flexible loop (ED178/179), responsible for the interaction with the endocytosis machinery, did not induce any changes to the proportion of wt Nef-expressing cells (4.9 +/- 1.8% migrated cells). As described previously, by mutating the Phenylalanine residue from the hydrophobic patch (F195) responsible of the interaction with PAK2 the Nef-mediated inhibition of migration was significantly reduced in comparison to the wild type protein (Stolp et al., 2009). However, this effect did not reach GFP-expressing control levels (43.5 +/- 5.0% F195A Nef-expressing migrated cells). This underlines the importance of the actin cytoskeleton dynamics for T cell migration toward chemotactic stimulus. Deletion of the amphipathic helix forming the interaction surface with NAKC caused only a marginal reduction in the inhibition of migration caused by wt Nef (12.9 +/- 5.0% migrated cells). We wondered then whether simultaneous disorganization of the hydrophobic patch and deletion of NAKC-interaction surface could completely inhibit Nef function. The effect of the double mutation on chemotaxis in vitro did not provide further loss of Nef-mediated inhibition of migration as compared to F195A mutation (46.4 +/- 6.8% migrated Δ12-39.F195A Nef.GFP-expressing cells), indicating that the effect mediated by this double mutant was exclusively due to disorganization of the hydrophobic patch.

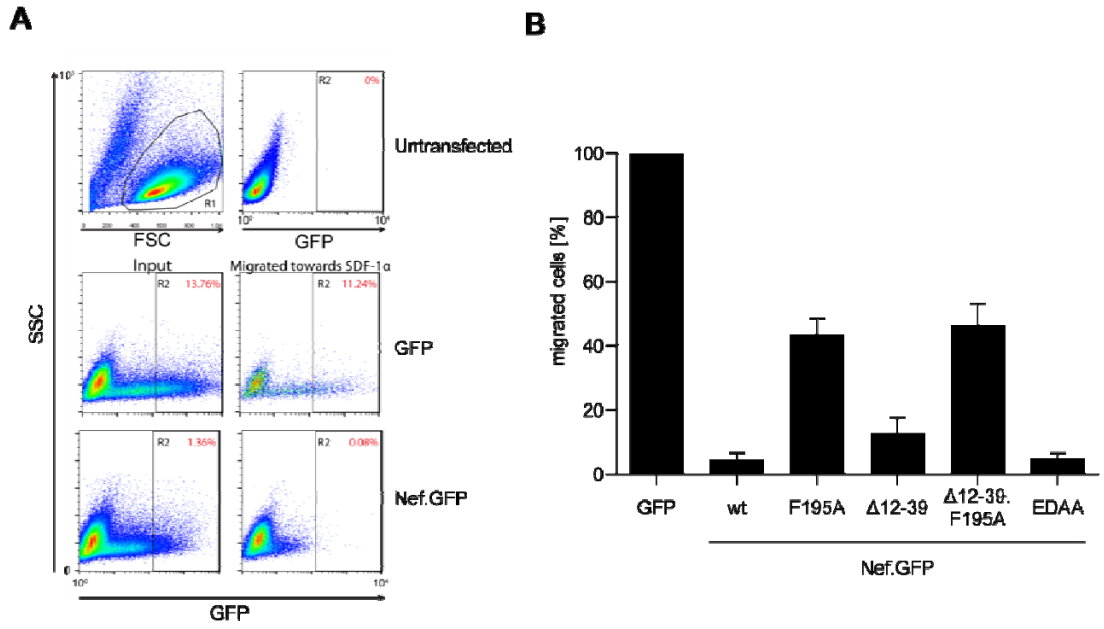

Fig. 4.9 NAKC-interacting surface of Nef does not play a role in an *in vitro* chemotaxis assay. CCR7 human lymphocytes were transfected with expression plasmids encoding GFP, Nef.GFP or four Nef mutants. After 24 hours, cells were starved in 0.5% FCS-containing medium for 2-4 hours and their ability to migrate through a transwell (5 µm pore size) toward 50 ng/ml SDF-1α was studied over 2h. (A) Representative dot blots of the INPUT and the migrated fractions. (B) Histogram depicts the proportion of migrated cells calculated as the ratio between the percentages of GFP-positive-gated cells (Gate R2) of total migrated cells and INPUT (Shown in red). Arithmetic means with standard deviations from three independent experiments are shown. Values were normalized to those of GFP transfected cells, arbitrarily set to 100.

4.10 Monitoring polarity of Nef-expressing T cells over time shows fundamental differences with control cells and cells expressing Nef mutants

All the results shown so far had been obtained in a steady state, from infected or transfected human lymphocytes after attachment to FN-coated surfaces and fixation. That experimental set up provided information on how many cells could polarize at a certain timepoint, however it did not provide insight about the dynamics of this cellular process. Had GFP or Nef-GFP round-shaped cells been round over the whole incubation time until fixation (1 or 2 hours)? Or were they rather subjected to cycles of polarization and depolarization? In other words, is polarity a "yes or no" phenotype or is it subjected to temporal regulation? To address this, A3.01 human T cells were transfected with expression plasmids for GFP, Nef.GFP, and GFP fusions of the Nef mutants Δ12-39, F195A and the double mutant Δ12-39.F195A. 6 hours after transfection, cells were stained with PKH26 membrane dye and seeded on FN-coated MatTek microscopy plates. Time lapse microscopy videos were then acquired over 1 hour (Figure 4.10).

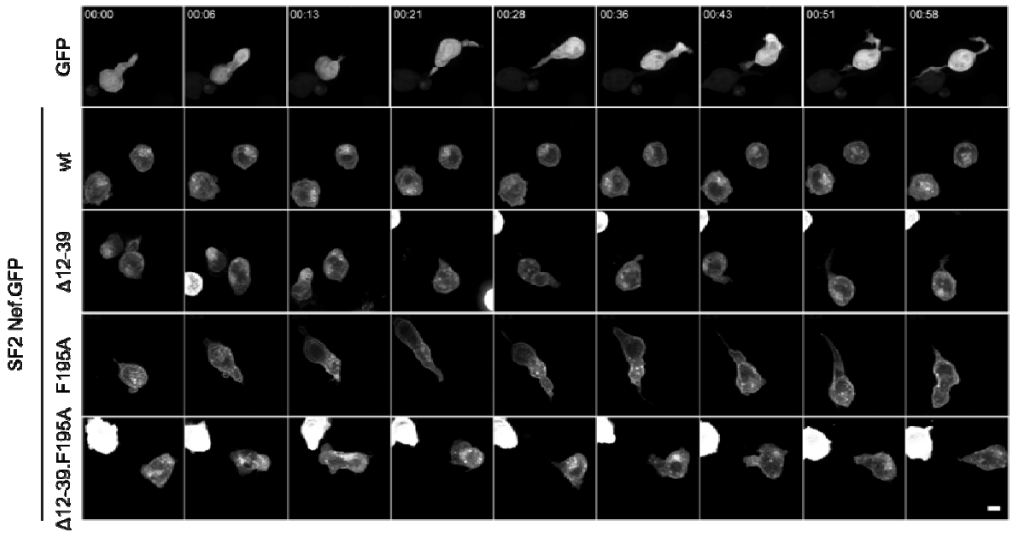

Fig. 4.10 Nef interferes with T cell polarity in a sustained manner over time and its mutants revert this effect. Still images from time lapse microscopy videos of A3.01 cell polarization in the presence of Nef. Cells were transfected with expression plasmids encoding SF2 Nef.GFP, Δ12-39 Nef.GFP, F195A Nef.GFP or the double mutant Δ12-39.F195A Nef.GFP and 6 hours later they were stained with the PKH26 membrane dye (Sigma-Aldrich, St. Louis, MO) and seeded on FN-pre-coated MatTek (four-chambered petri dishes for live cell imaging). Acquisition of one frame every 1.5 minutes results in time lapse videos of approximately 1 hour length. Confocal spinning disk microscope was handled by Nikolaos Tsopulidis

Analysis of the videos revealed that polarization of GFP-expressing or untransfected control cells is a highly dynamic process in which cell shape cycles between elongated and round, with a tendency to rather long elongation periods as compared to those in which the cell remains round (Figure 4.11). Of note, when analyzed over 60 minutes, all of the studied control cells were able to polarize at least once during the observation period. On the other hand, Nef.GFP-expressing cells displayed an almost complete lack of polarization over the complete 1 hour. If occurring at all, polarization periods in this case were rare and with a clear tendency to be very short (for one single frame in most cases). For 9 out of 15 cells polarization events were inexistent during the acquisition time. Unlike wt SF2 Nef-expressing cells, expression of the three analyzed mutants resulted in much higher frequency of polarization over time. These cells therefore showed a behavior more similar to that of GFP-expressing cells. However, some fundamental differences were observed in the effect these mutants exerted on T cell polarization. Firstly, polarization of all the studied cells was not observed for none of the three mutants. There were cells which did not polarize over the complete 1 hour, and the frequency of these cells was the highest for Δ12-39 Nef (4 cells out of 15), followed by F195A Nef (2 cells out of 15) and the double mutant Δ12-39.F195A Nef in the last place (1 cell out of 15). Furthermore, Δ12-39 Nef-transfected cells showed in general longer periods of no polarization as compared to the other two mutants or control cells. Conversely, F195A Nef-expressing cells had a higher tendency to remain polarized during the complete 1 hour of acquisition or to display shorter periods of no polarization

as compared to the other two mutants or control cells. The extent of the elongation of polarized cells differed also between the mutants and control cells. Cells expressing F195A Nef had a tendency to be very elongated. Δ12-39 Nef-expressing cells on the other hand generally polarized less keeping a round cell body with a rather short but well defined uropod. Δ12-39.F195A Nef-expressing cells and GFP-expressing and untransfected control cells kept also an easily recognizable round cell body but with a longer uropod in general. All in all, the double mutant resembled better the phenotype shown by GFP-expressing or untransfected control cells.

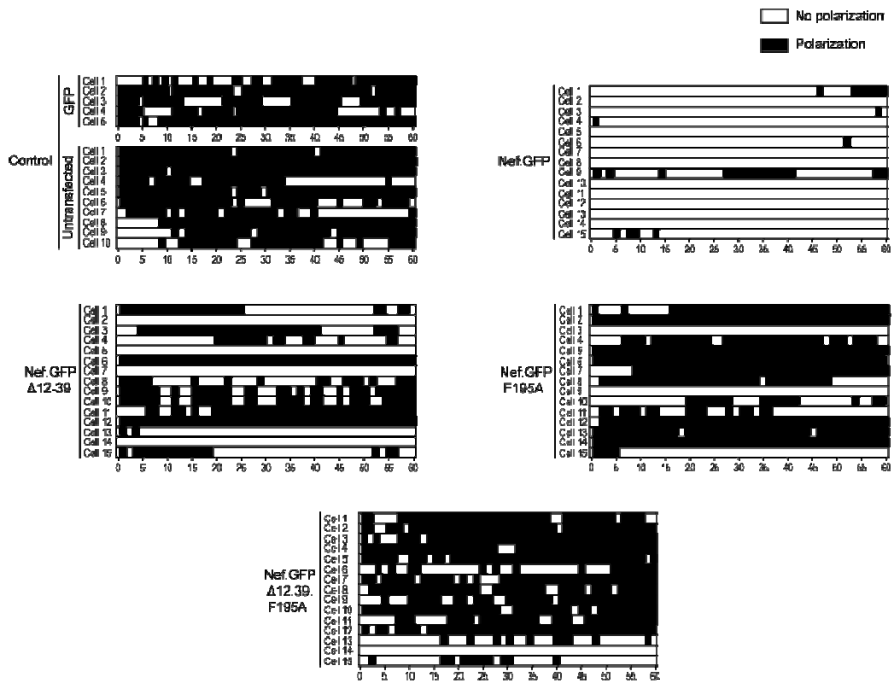

Fig. 4.11 Nef interferes with T cell polarity in a sustained manner over time and its mutants revert this effect. Analysis of the polarity assay over time shown in Figure 10. Polarization (black) and no polarization (white) periods for individual cells are depicted over the acquisition time (in minutes). Fifteen cells where analyzed per condition.

Analysis over time of the area covered by cells from the same videos was also performed as an evaluation of the polarization extent (Figure 4.12). As expected, Nef.GFP-expressing cells displayed an almost 2-fold reduction in the area covered over time as compared to GFP-expressing control cells (213.73 +/- 106.14 μm^2/h *vs* 397.5 +/- 75.9 μm^2/h, respectively). Only a subtle change could be observed in the area covered over time for the Δ12-39 Nef mutant as compared to wt Nef (284.8 +/- 138.2 μm^2/h). F195A and Δ12-39.F195A were defective for this Nef effect and did not cause a reduction in respect to GFP-expressing control cells (394.4 +/- 152.9 μm^2/h and 462.4 +/- 181.2 μm^2/h, respectively)

Taken together, the results shown so far describe a Nef-induced polarity defect in human PBMCs in the context of infection and identify the α-helical motif 12-39, required for the assembly of the NAKC, as a new determinant for this effect together

with F195, involved in the interaction with PAK2 and responsible for actin dynamics disruption. Further characterization of the requirement of these motifs, and thereby of T cell polarization, for TEM via an artificial chemotaxis *in vitro* assay led to no conclusive results. Therefore, the following presents the setting up of a more physiologically meaningful TEM experimental system intended to help us understanding the contribution of polarity impairment to the negative effects of Nef on T cell homing.

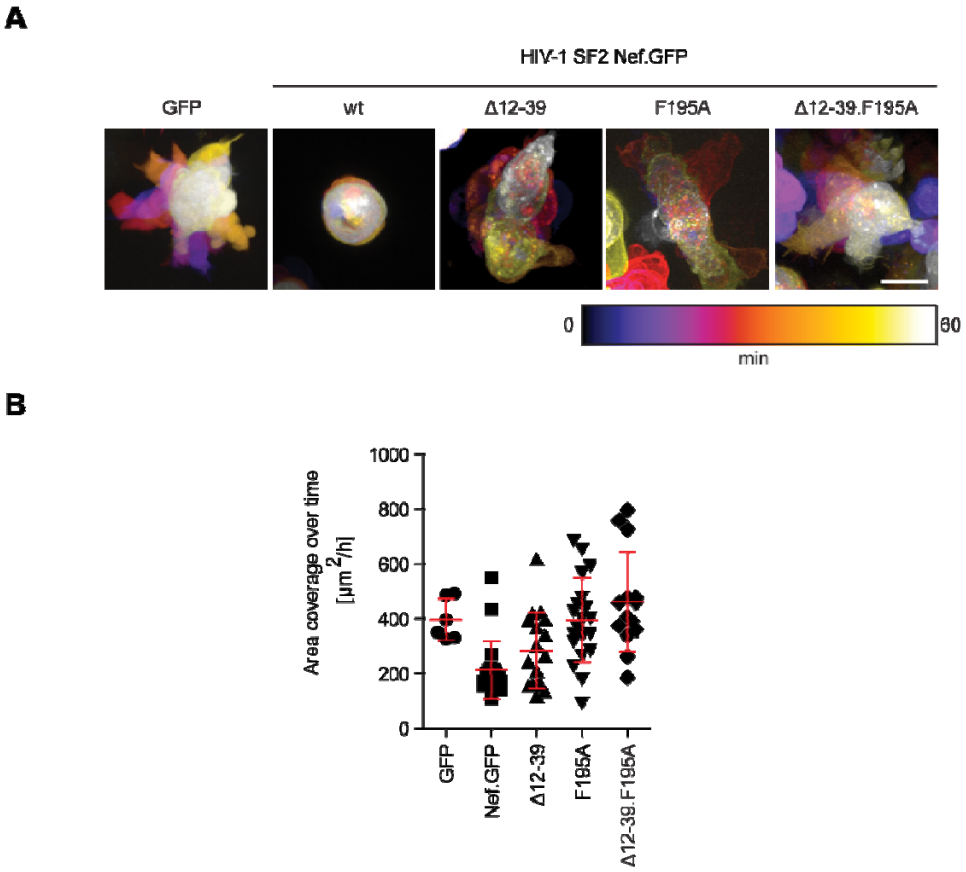

Fig. 4.12 Nef interferes with T cell polarity in a sustained manner over time and its mutants revert this effect. (A) Time projection of videos analyzed in Figure 10 shown as temporal colour code. Scale bar = 5 µm. (B) Depicted is the single-cell area coverage over one hour. Each symbol corresponds to the value obtained for an individual cell. Bars represent the arithmetic mean and standard deviation for at least 6 analyzed cells. Analysis was performed by Nikolaos Tsopulidis using FIJI.

4.11 Establishment of an *in vitro* system to study transendothelial migration of primary infected human T lymphocytes

T lymphocytes constantly migrate between blood and secondary lymphoid organs. This is a hallmark of lymphocyte function that leads to their activation, antigen-specificity development and efficient immune surveillance. It also requires constant crossing of the vascular endothelium by the complex, multistep process of TEM (Ley et al., 2007; Nourshargh and Alon, 2014). Successful transmigration is typically followed by

subendothelial locomotion and penetration into the underlying tissue. The study of TEM using primary human lymphocytes and endothelial cells in vitro is something that has been already performed and established by others (Boscacci et al., 2010; Muller and Luscinskas, 2008; Stolp et al., 2012). Primary human umbilical vein endothelial cells (HUVECs) are typically used to that end. These cells are able to form continuous monolayers which impose some difficulties for the lymphocytes to migrate through them. This makes of it an active process which requires strong stretching or polarization of the transmigrating cells to push their cell bodies through, and more specially their voluminous nucleus. Our laboratory has previously described that Nef interferes with TEM and subendothelial tissue migration both *in vivo* and *ex vivo* imposing a defect on T cell homing. While the inhibition of subendothelial migration is mediated via the effects of Nef-PAK2 on actin dynamics, Nef effects on transmigration involve other, yet to be determined protein interactions. Notably, the inability to transmigrate of Nef-expressing cells was paralleled by a marked reduction of cell polarization. Therefore, we hypothesized that the observed new polarity defining Nef determinant 12-39 may be required for hindering TEM.

In order to study the effects of deletion of the 12-39 deletion of Nef in TEM we designed an experiment in which infected and sorted human PBMCs will then be tested for transmigration through a primary HUVEC monolayer under shear flow. This highly complex experimental set up needed to be first established in our lab. To achieve attachment of lymphocytes to the HUVECs monolayer, the endothelial cells have to be activated e.g. with tumor necrosis factor-α (TNF-α) which stimulates surface expression of key adhesion molecules involved in TEM such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet/endothelial adhesion molecule-1 (PECAM-1) (Pober and Cotran, 1990; Pober and Sessa, 2007). TNF-α concentration is critical for the correct activation and if it is too high this can result in cell aggregation or death (Figure 4.13A). Evaluation of the formation of continuous monolayers requires monitoring over time. We did this by seeding cells simultaneously on cell culture plates suitable for microscopy and transwell inserts, both coated with matrigel. The first ones allowed us visualizing the monolayer and the second ones facilitated a system in which we could measure trans-endothelial electrical resistance (TEER), as a method to evaluate the integrity of the monolayer. We seeded cells in two different concentrations, 3.125×10^4 cell/cm^2 (low cell concentration) or 1.25×10^5 cell/cm2 (high cell concentration) treated with TNF-α 4.3 ng/ml (200 U/ml) or untreated. We then monitored over a period of 30h the formation and evolution of the HUVEC monolayers both visually and by TEER measurements. Despite of visually looking confluent, the monolayers were not completely sealed as inferred from the TEER measurements (Figure 4.13B and C, images of cells at low concentration not shown). No differences were detected at any studied timepoint between cells seeded at any concentration or treated with TNF-α when compared to empty matrigel-coated transwell inserts.

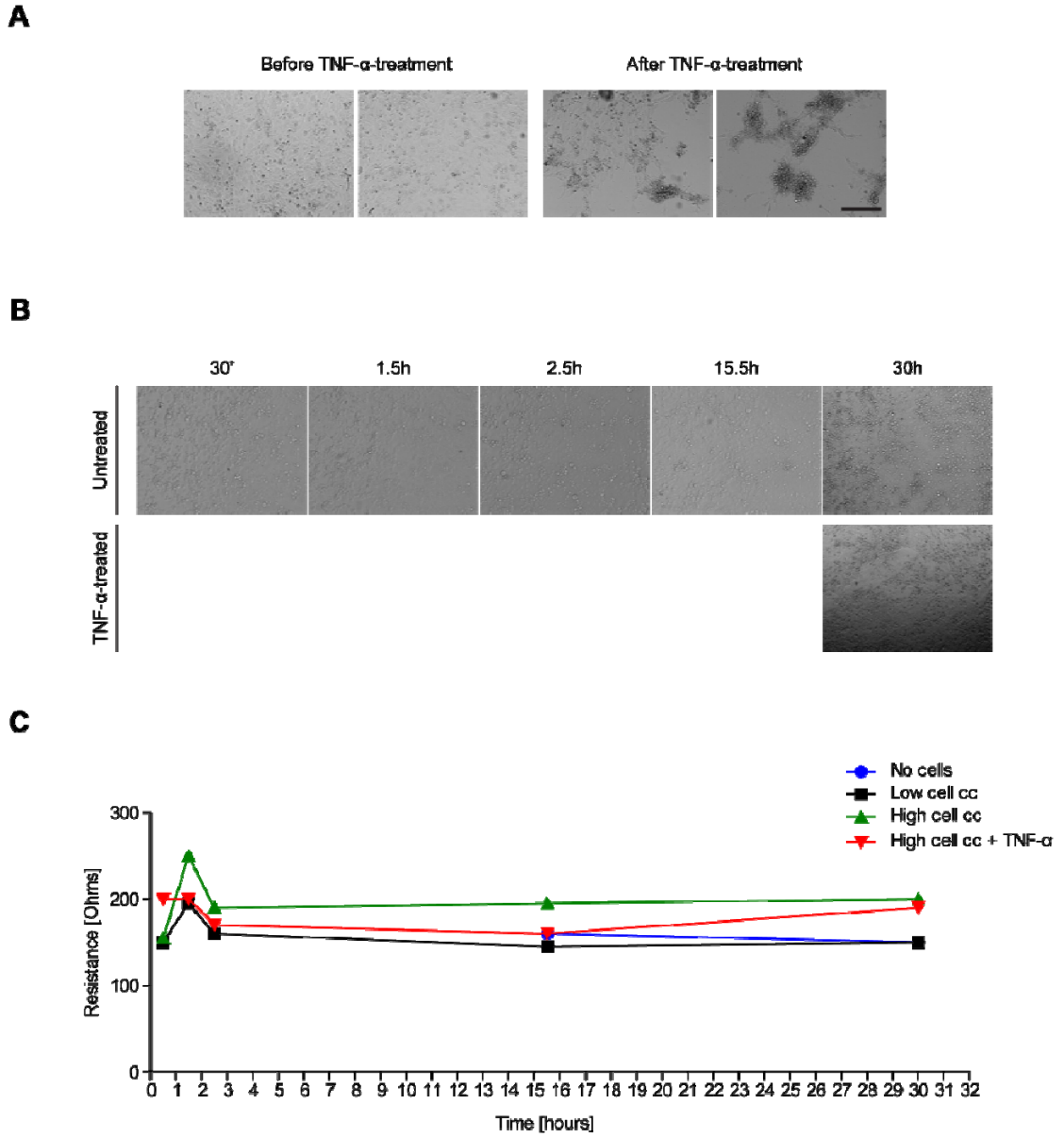

Fig. 4.13 A system to study transendothelial migration *in vitro*: Human Umbilical Venule Endothelial Cells (HUVECs). (A) White field micrographs showing the effects of a too high concentration of TNF-α (25 ng/µl). Scale bar = 500 µm. (B) HUVECs were seeded at various concentrations on Matrigel-coated cell culture dishes with cover glass-like bottom, which allow microscopy visualization. White field micrographs of HUVECs seeded at high concentration (1.25 x 10^5 cell/cm^2) over 30 hours. (C) Cells from the same source as in B were seeded on Matrigel-coated transwells at concentrations of 3.125 x 10^4 cell/cm^2 (Low cell concentration) or 1.25 x 10^5 cell/cm^2 (High cell concentration) and treated when indicated with TNF-α 4.3 ng/ml (200 U/ml) for 16 hours. Measurement of the Trans Endothelial Electrical Resistance (TEER) was performed as an evaluation of the integrity of the monolayer. TEER values for empty transwells were used for comparison.

Endothelial cells construct confluent monolayers by formation of tight junctions (TJ) between them (Beese et al., 2010). This provides structural anchoring between the cells keeping them at close distance. Therefore, we studied TJ formation by seeding HUVECs on matrigel-coated cell culture plates suitable for microscopy at a concentration of 1.25 x 10^5 cell/cm^2 and treating them with 4.3 ng/ml TNF-α for 16

hours. Cells were fixed 30 hours after seeding with PFA, Methanol of Acetone/Methanol and stained for the TJ markers Claudin-1, E-Cadherin (data not shown), ZO-1 and Occludin (Figure 4.14). In line with the TEER measurements, no tight junctions were observed for any of the fixation conditions studied. To test why we did not observe TJ, we used hepatocarcinoma cell line Huh7 as a positive control. Staining of these cells resulted in the characteristic net pattern evidencing antibodies did efficiently and specifically stain their target proteins (Figure 4.14).

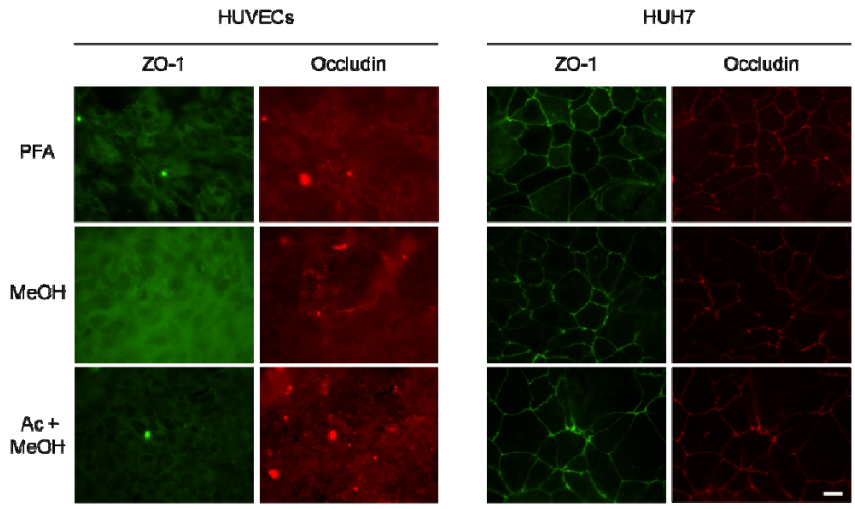

Fig. 4.14 A system to study transendothelial migration *in vitro*: Human Umbilical Venule Endothelial Cells (HUVECs). HUVECs seeded at a concentration of 1.25 x 10^5 cell/cm2 on Matrigel-pre-coated culture dishes were fixed using 3% PFA/PBS, Methanol or an Acetone/Methanol mix (1:1) after 16 hours of treatment with 4.3 ng/ml (200 U/ml) TNF- α (30 hours post-seeding) and stained for the Tight Junction markers ZO-1 and Claudin. Huh7 cells were used as a positive control. Epi-fluorescence micrographs of fixed stained cells are shown. Scale bar = 10 µm.

We next speculated that the matrix coating of the cell culture surface on which the HUVECs grow could be the limiting factor to achieve a confluent monolayer. Time and cell seeding concentration were also key factors to be assessed. We therefore tested other coatings namely FN, gelatin and collagen and compared them to matrigel. We seeded cells on coated-cell culture plates suitable for microscopy at three different densities, 9.375 x 10^4, 1.094 x 10^5 and 1.25 x 10^5 cell/cm^2, and monitored TEER over a longer time period of 65 hours. At this late time-point bright field images were also acquired to visually evaluate the integrity of the endothelial monolayers. HUVECs grew on all four matrices (Figure 4.15A). However, cell-free areas were clearly visible on gelatin- and collagen- coated plates at all concentrations. Cells on collagen grew not only as a 2D culture but also formed 3D structures which mirrored those observed in angiogenesis reports (Klose et al., 2015; Martin et al., 2008). On the other hand, endothelial monolayers grown on FN and matrigel seemed to be rather confluent when analyzed by eye. A more careful analysis of HUVECs seeded on matrigel-coated plate evidenced the appearance of cell-free areas or holes (Figure 4.15B), which could

explain the previous lack of success when measuring TEER (Figure 4.13C). In line with the cell-free spaces observed in the bright field images of HUVECs cultured on matrigel-, gelatin- and collagen-coated cell culture plates, the TEER values obtained for these samples did not show a significant change as compared to those of control empty transwell inserts coated with the same matrices (Figure 4.15C). In spite of the promising appearance of the endothelial monolayers grown on FN, the TEER values for these samples did not significantly differ from those of control empty transwells either.

We now believe that in order to form a continuous monolayer where HUVECs interact closely among them and form tight junctions, they need to be seeded at a lower concentration (not seeded to density) and proliferate. This way when they get to touch each other junctions will be developed resulting in a confluent monolayer. Increasing the content in growth factors and specially Vascular Endothelial Growth Factor (VEGF) allows better and faster proliferation of HUVECs. These difference in growth rate compared to the regular medium cannot be observed for early passage (Figure 4.15D), but it is very prominent for passages there on (passage 3 and beyond).

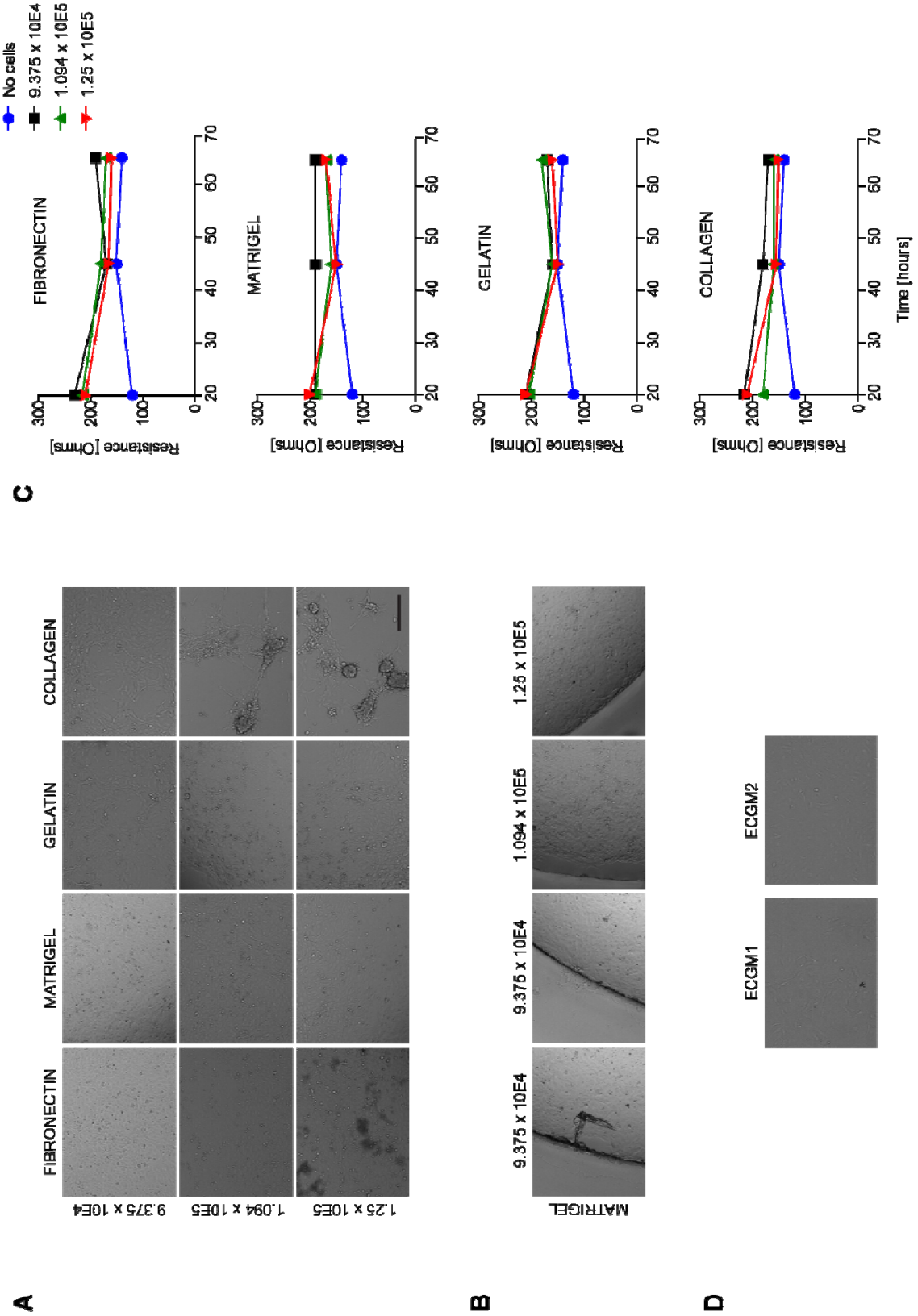

Fig. 4.15 A system to study transendothelial migration *in vitro*: Human Umbilical Venule Endothelial Cells (HUVECs). (A) Three different concentrations of HUVECs were tested this time: 9.375 x 10^4, 1.094 x 10^5 and 1.25 x 10^5 cell/cm^2. Cells were seeded on culture dishes or transwells coated with different matrices, from biological (Fibronectin and Collagen) or artificial (Matrigel and Gelatin) sources. White field images were taken 65 hours after seeding. (B) Holes in the HUVECs monolayer evidence technical issues of the coating with matrigel. (C) TER measurements of cells shown in (A) over 65 hours. For comparison, empty transwells were used. (D) Images show proliferation of HUVECs in two different endothelial growth media at passage 2 (after one split in the laboratory).

4.12 Human lymphocytes attach, crawl and perform TEM under shear flow

Despite of not having achieved a continuous monolayer, we wanted to test whether our designed experimental set up can potentially work once endothelial cells handling is optimized. With this aim, we isolated PBMCs from Buffy coats of healthy donors followed by a CD4+ cell selection and activation with PHA and IL-2 for 72 hours. In parallel we seeded HUVECs on matrigel-coated culture plates and acquired an endothelial monolayer relatively high in confluence in certain areas as assessed by eye. Activation of the endothelial cells with was performed by adding TNF-α at a concentration of 4.3 ng/ml (200 U/ml) for 16 hours. Coating the monolayer with CCL-21 shortly before the TEM assay is necessary for T cells to adhere and roll on it (Bao et al., 2010; Shamri et al., 2005; von Andrian and Mempel, 2003). CD4+ lymphocytes were then perfused on the HUVECs utilizing a handmade TEM flow chamber and a syringe pump that allows adjustment of the flow. At a constant shear flow of 1.5 dyn/cm^2, a high number of PBMCs successfully attached to the endothelial cells (Figure 4.16A). Furthermore, they displayed a highly motile behavior with a clear preference to migrate on or specially underneath the endothelial cells. Lymphocytes crawling on cell-free, matrigel-coated plastic were very rare. By the end of the video all cells appear to be underneath the endothelial cells.

A closer look at the video in an area of highly confluent endothelium revealed a cell performing TEM (Figure 4.16B). The observed diapedesis event is easily detectable by the characteristic progressive darkening of the cell body as it migrates through the endothelial cell layer and the observable uropod tip which is the last part of the cell in becoming dark.

Thus, from this preliminar experiment we conclude that the *in vitro* TEM assay we plan to perform with primary infected human T lymphocytes could potentially work since uninfected cells are able to adhere to and crawl on HUVECs, transmigrate and migrate subendothelially.

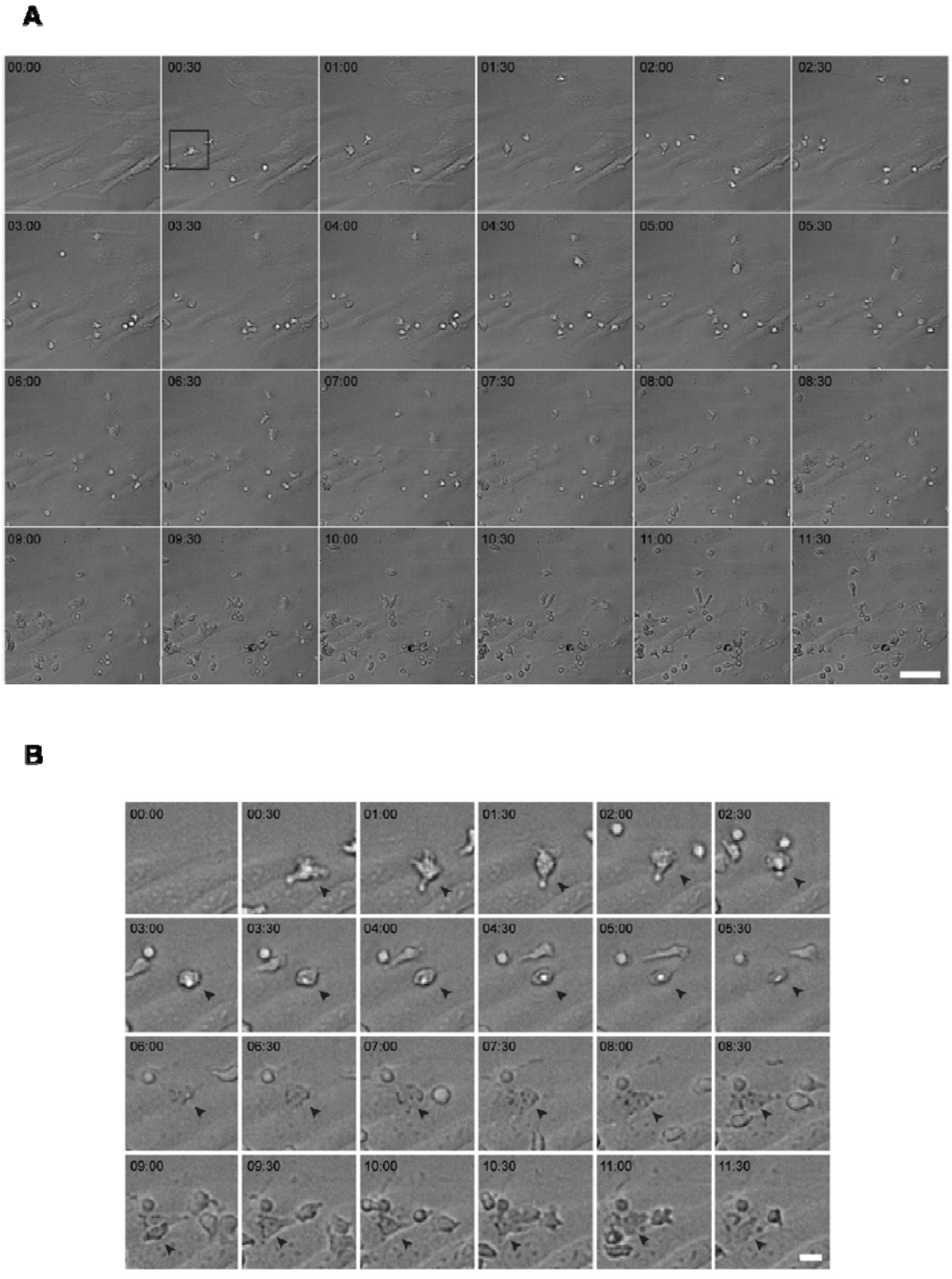

Fig. 4.16 Human primary T lymphocytes adhere on and transmigrate through a HUVECs monolayer. Activated primary human CD4+ lymphocytes (PHA 2 µg/ml and 10 µg/ml IL-2 for 72 hours) were applied to TNF-α-stimulated, CCL-21-coated primary HUVECs under shear flow (1.5dyn/cm^2). (A) Still images of time lapse microscopy video of a representative TEM field of view. The black rectangle on the second frame corresponds to the enlarged area shown in (B). Scale bar = 50 µm. (B) Zoom from images in (A) showing a cell presumably undergoing diapedesis. Black arrows point to the specific cell before and after extravasation. Scale bar = 10 µm.

4.13 Sorting of HIV-1 infected cells will allow studying effects of Nef on TEM in the context of infection

HIV-1 infection efficiency of primary T lymphocytes in vitro is very low even with pre-activation of the target lymphocytes. Working with a homogeneously infected population is essential in our case since the presence of non infected, and thereby no Nef-expressing cells for example would lead to an elevated number of transmigration events and therefore to an underestimation of the Nef effect. In order to achieve a homogeneously infected population, Dr. Andrea Imle developed in our laboratory a proviral plasmid which allows sorting of infected cells. This provirus originates from the NL4.3 HIV-1 backbone and it contains sorting cassette expressing YFP fused to the transmembrane domain of Platelet Derived Growth Factor Receptor (PDGFR) (Figure 4.17A). Since infection efficiencies are even lower as with the parental NL4.3 HIV-1 virus strain, an optimized activation method was also developed by Dr. Andrea Imle to achieve higher infection rates (up to 3-6%). After infection with such virus, incubation with anti-YFP antibody-coupled magnetic beads allows for positive selection of infected cells and achievement of a highly pure infected population (over 90% HIV-1 infected cells) (Figure 4.17C). The original provirus generated by Dr. Andrea Imle contained NL4.3 *nef*. In order to be able to compare our previous results obtained by studying Nef-mediated effects on polarity, we needed to substitute this NL4.3 *nef* with the SF2 *nef* used previously. We also included Δ12-39 *nef*, F195A *nef* and AXXA *nef* genes to be able to evaluate whether Nef motifs important for T lymphocyte polarity disruption play a role as well in TEM as we hypothesize. Cloning of all *nef* versions was successful as judged from the SDS-PAGE and Western Blotting of virus producer 293T cell lysates (Figure 4.17B). Nef was detected at the right size (~27kDa) in all producer cell lysates except for those transfected with a ΔNef provirus, whose *nef* gene expression is blocked by an artificially included early stop codon. (second and third lanes). As expected the 12-39 deletion of Nef resulted in a slightly higher electrophoretic mobility (~22kDa). Unprocessed Gag p55, intermediate-processed Gag p41 and the capsid protein p24 CA were also detected for all producer cell lysates although in variable levels, which mirror differences in gel loading as concluded when comparing to the WB signal of a house keeping gene, e.g. transferring receptor (TfR). Further successful confirmation was obtained by sequencing the cloned proviral plasmids.

Infected cell-sorting worked appropriately for cells infected with wt, ΔNef and Δ12-39 Nef viruses (shown in Figure 17.C for wt infected cells). The yield of infected cells obtained ranged between 3 and 7%. This was sufficient (>2%) for cell-sorting.

Taken together, this NL4.3-backbone based, sortable virus provides a perfect tool to study TEM allowing for purification of a heterogeneous population obtained after *in vitro* infection.

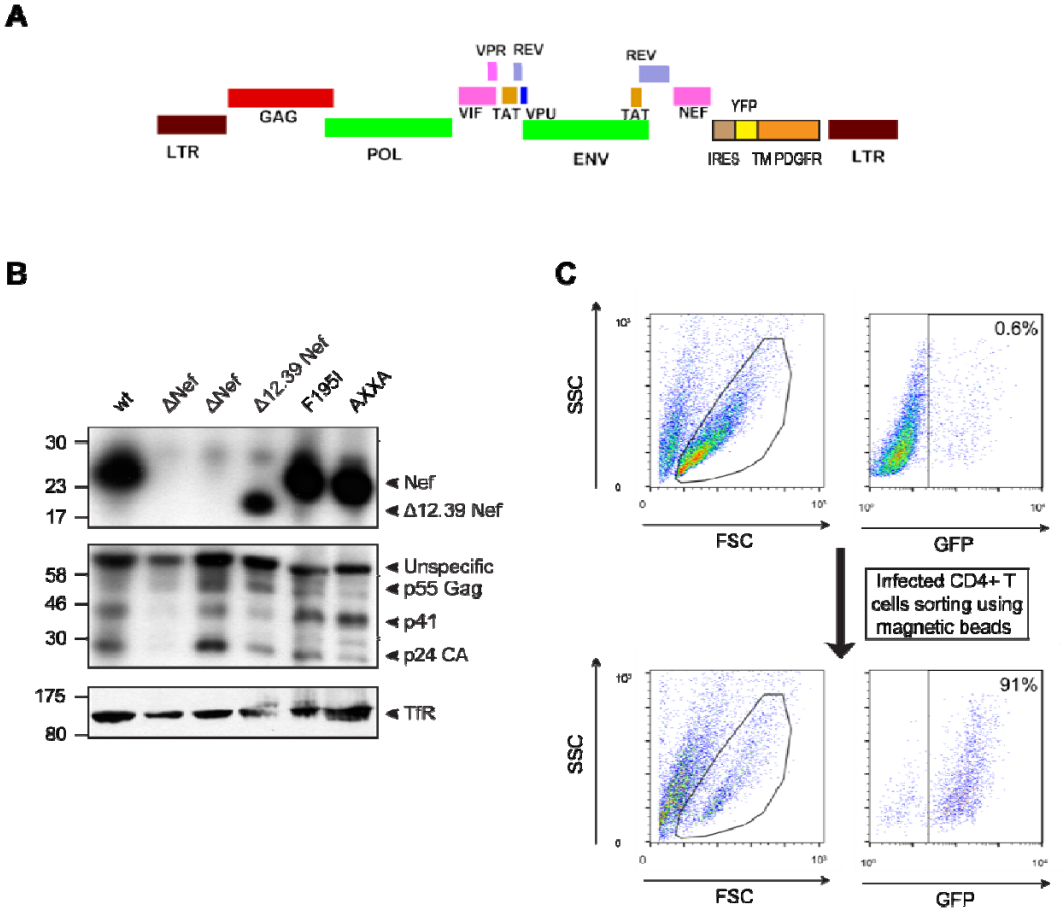

Fig. 4.17 Sortable viruses expressing Nef mutants relevant for polarity disruption as tools to study TEM of primary human lymphocytes. (A) Schematic diagram of the modified, NL4.3-backbone-based HIV-1 provirus construct designed and generated by Dr. Andrea Imle in our laboratory: HIV pNL4.3 IRES.pDisplay.YFP. It incorporates a sorting cassette consisting on YFP followed by the transmembrane domain of PDGFR thus allowing purification of infected cells form a virus exposed population using anti-YFP-antibody-coupled magnetic beads. (B) SDS-PAGE of the lysates of 293T virus-producer cells. The *nef* gene was substituted for those encoding Δ12-39, F195I or AXXA Nef mutants and compared to the parental wt virus as well as a Nef deficient variant. (C) Representative dot blots of the wt virus-infected population before and after sorting. Left plots, percentages of infected cells are stated in black on the upper right corner.

5 DISCUSSION

5.1 Overlapping functions between HIV-1 Nef and Vpu

The work shown in the section 3 of this dissertation is partly incorporated or related to a recent study of our laboratory which aimed at improving understanding the breadth of the Nef- and Vpu-mediated downmodulation of cell surface molecules and membrane receptors (Haller et al., 2014). In this study a parallel analysis of 105 host cell membrane proteins present in human A3.01 T cells was performed. One third of them were newly identified as targets of HIV-1 Nef-mediated downregulation (36/105). Interestingly, a number of cell membrane markers were shown to be affected by HIV-1 Vpu expression (32/105), all of which were targeted as well by HIV-1 Nef. Overall, the effects mediated by HIV-1 Vpu (or SIVmac239 Nef) on the surface levels of the receptors were less marked than those seen for HIV-1 Nef. This likely mirrors the capacity of Nef to not only block the anterograde transport of newly synthesized receptor molecules but also to induce their internalization (Casartelli et al., 2006; Giolo et al., 2007; Laguette et al., 2010; Laguette et al., 2009; Matusali et al., 2012; Roeth and Collins, 2006; Wonderlich et al., 2011). Vpu however can only block anterograde transport without inducing internalization (Bolduan et al., 2013; Dube et al., 2011; Kerkau et al., 1997; Lindwasser et al., 2007; Pujol et al., 2016; Schmidt et al., 2011; Willey et al., 1992a). Therefore, the ultimate effect induced by Vpu will depend strongly on the degree of turnover, i.e. endocytic internalization and anterograde transport of the specific cargo molecule, and when these processes are slow, as for relatively stable membrane protein pools, Vpu will fail to achieve the downregulation extent facilitated by Nef.

Of note, the surface levels of all members of the TSPAN superfamily of transmembrane proteins studied were affected by both HIV-1 Nef and Vpu, two of which appeared among the most strongly downregulated molecules in the screen. The Thali laboratory had previously observed that CD81 -a representative member of the TSPAN family also included in our study-, despite of being present at the assembly sites of HIV-1 (Nydegger et al., 2006), is largely downregulated from the infected cell surface (Krementsov et al., 2009). As a confirmation of our findings, in a parallel, independent study they could describe Vpu as the responsible factor for TSPANs downregulation in the context of infection (Lambele et al., 2015). A third study by Matheson et al. also obtained results consistent to the ones we have shown (Matheson et al., 2015). They identified downregulation of TSPANs among many other molecules from the plasma membrane of CEM-T4 T cells infected with a VSV-g pseudotyped HIV-1 reporter virus. To that end, they used a systematic, not-candidate-based screening approach combining plasma membrane profiling (PMP; PM enrichment through selective aminooxy-byotinilation) with tandem mass tag (TMT) and stable isotope labeling by aminoacids in cell culture (SILAC)-based quantitative proteomics. With this technical set up, these authors have been able to quantitate a much larger panel of proteins (2320, among which 804 were already reported to locate at the plasma membrane) and thereby have identified a large number of downregulation targets upon HIV-1 infection.

Interestingly, their preoteomics approach correlated well with quantification by flow cytometry and together with TSPANs CD37/53/63/81/82 they identified other known downregulation targets such as CD28, CCR7 or NTB-A. Taken together, our results and the results obtained by others identify a new family of receptors as major target for downmodulation by HIV-1 accessory proteins.

In this work, a first attempt towards understanding the mechanism by which the two lentiviral accessory proteins induce TSPANs surface level reduction has been carried out by studying the contribution of several well-described motifs of each of these proteins in downregulation. Despite of no clearly comparable downregulation profiles applying to all TSPANs studied, clear differences in the mechanisms by which each of the viral factors induce downregulation of these molecules can be glimpsed from the overall data by Haller et al. (Haller et al., 2014). Here, we also observed that the overlap in specificity between HIV-1 Nef and Vpu applies to other proteins different than membrane molecules and receptors. The host cell peripheral protein Lck was observed to be targeted by both lentiviral proteins to intracellular accumulation. The data again suggest that two different mechanisms converge in the same effect here. One last activity described for Nef which remained unexplored for Vpu is the enhancement of EV secretion. We describe here that this represents one more shared function between the two accessory proteins and that, unlike what we could see for surface receptor downregulation and Lck rerouting to intracellular compartments, Vpu shows more pronounced effects than Nef.

5.1.1 Specific Nef- and Vpu-mediated TSPAN downregulation and determinants involved

The fact that numerous surface receptors were unaffected by the expression of HIV-1 Nef or Vpu (or SIV Nef) suggests that of those which were efficiently downregulated were selectively targeted by the viral proteins. We however wanted to strengthen experimentally this point. To that aim, we compared the effect mediated by the expression of Nef and Vpu on TSPANs surface levels to that of completely unrelated proteins with similar membrane anchoring mechanisms, namely N18src.GFP and pDisplay.YFP (Geist et al., 2014; Pan et al., 2013b). As expected, none of the unrelated proteins induced reduction in the levels of the TSPANs studied whereas the HIV-1 accessory proteins caused a marked effect. This strategy provides thereby evidence that the effect induced by HIV-1 Nef and Vpu does not result from an artifact such as an unspecific disturbance of membrane transport associated to membrane association and insertion of the viral factors, respectively.

In this study, we attempted for the first time to define molecular determinants in Nef and Vpu required for TSPAN downmodulation. We addressed this by analyzing the effect of mutations targeting motifs implicated in well-described functions or protein-protein interactions of these proteins. Of note, our mutant screen did not identify a motif leading to loss of TSPAN downregulation function upon induction of sequence changes. The downregulation profile obtained for the mutants tested was however similar for the

TSPANs studied. In the case of Nef a partial effect was observed for all three mutants targeting motifs within the SH3-interacting domain (EEEE66-69 and the VGF and PxxP motifs), involved in a number of protein-protein interactions required for interference with intracellular trafficking and cell membrane receptor downregulation, e.g. trafficking of Lck or surface downregulation of MHC-I (Collette et al., 1996; Meuwissen et al., 2012; Piguet et al., 2000; Saksela et al., 1995). The only exception was CD37 for which a partial effect could be observed when the dileucine motif at the C-terminal flexible loop implicated in CD4 downregulation via endocytic components, was mutated (LL168/169) (Craig et al., 1998; Chaudhuri et al., 2007). Also, a slightly less pronounced effect on CD37 downregulation was observed for mutations at the flexible loop and the SH3-binding domain of Nef (ED178/179, and EEEE66-69 and VGF respectively). For Vpu-induced downregulation a partial effect was observed for mutations at the transmembrane motif and the diserine motif, required for the interaction with β-TrCP and tetherin, respectively. Neither for Nef nor for Vpu the downregulation profiles obtained in this mutant screen resembled that of receptors with already described mechanisms, like CD4 or tetherin (used as controls in this study) or MHC-I.

HIV-1 Vpu has also been shown to induce the intracellular accumulation of and colocalize with TSPANs at a TGN marker-positive compartment when overexpressed (Haller et al., 2014) and also in the context of infection (Lambele et al., 2015). Importantly, robust coimmunoprecipitation with TSPANs has been shown by us (Haller et al., 2014) and direct interaction has been observed independently by Lambelé et al. between CD81 and Vpu using fluorescence resonance energy transfer (FRET) (Lambele et al., 2015). These authors have also shown that pharmacological inhibition of lysosomal and proteasomal degradation blocks the Vpu-induced decrease in total CD81 content suggesting Vpu mediates surface downregulation of this TSPAN. Furthermore, we have observed that Vpu coimmunoprecipitated with TSPANs even after disruption of TEMs by harsher buffer conditions (unpublished data) (Haller et al., 2014). This argues against a model in which Vpu coassembles with TSPANs at specific membrane microdomains, but rather reflects a direct protein-protein interaction as part of the Vpu-mediated TSPAN downregulation from the host cell surface, intracellular accumulation and degradation. Given the central role of Vpu for TSPAN cell surface downregulation upon HIV-1 infection, dissecting the mechanism of this interaction would likely bring some clarification as to how specific downregulation of TSPAN take place in the sole presence of Vpu and in the context of infection. Weak (Haller et al., 2014) or no interactions (Lambele et al., 2015) were described for HIV-1 Nef with TSPANs. Colocalization between Nef and TSPAN molecules at perinuclear compartments was also less marked as that seen for Vpu (Haller et al., 2014). These observations suggest that Nef may alter TSPAN intracellular transport indirectly as it has been described for the induced accumulation of Lck at RE/TGN for which association and colocalization with Nef are not required (Pan et al., 2013b; Pan et al., 2012).

Taken together, the results shown here and those reported by Haller et al. suggest that HIV-1 Nef and Vpu may affect trafficking of TSPANs, amongst a wide range of cellular cargo, by different mechanisms. Moreover, the downregulation of this large spectrum of target membrane proteins likely mirrors the alteration induced by Nef and Vpu of general transport routes of which the downregulated molecules are cargo and argues against the "direct connector model", previously described for specific molecules like CD4 or MHC-I (Mangasarian et al., 1997).

5.1.2 HIV-1 Nef and Vpu overlapping functions extend to peripheral membrane proteins such as Lck

HIV-1 is known to utilize it accessory protein Nef to finely modify basal T cell activation levels and the responsiveness to TCR engagement (Abraham and Fackler, 2012; Baur et al., 1994; Fackler and Baur, 2002; Simmons et al., 2001). The block in some TCR proximal signaling transduction events like the reorganization of the actin cytoskeleton, TCR signaling-related protein microcluster formation or tyrosine phosphorylation significantly contribute to reduce AICD and prolong the life of infected cells (Abraham et al., 2012; Haller et al., 2007; Haller et al., 2006; Schindler et al., 2006; Thoulouze et al., 2006). Perturbances in distal signaling events such as the Nef-dependent intensification of the Ras-Erk signaling contribute to promote replication (Pan et al., 2012; Schrager et al., 2002). Our laboratory showed recently that Nef-induced manipulation of proximal and distal TCR signaling events rely on one the relocalization of the Src kinase master switch Lck to RE/TGN compartments (Pan et al., 2012). This Nef effect is highly specific since the closely related Src family kinase Fyn cellular distribution remains unaltered. Whereas the Nef-induced retargeting of a central player in early TCR signal transmission such as Lck results in block of TCR signaling at initial steps, the intracellular pool of Lck is highly active and contributes to signaling specifically to the Ras-Erk cascade to promote viral replication (Pan et al., 2013a; Pan et al., 2012).

In this work we have described for the first time that HIV-1 Vpu can also induce relocalization of Lck towards intracellular compartments. However, the magnitude of the intracellular accumulation as well as the frequency of cells showing accumulation was significantly lower than that seen in Nef-expressing cells. The Vpu-mediated effect became more pronounced when the analysis was restricted to newly synthesized Lck. Under these conditions Vpu (or Nef) and Lck were synthesized simultaneously and the amount of Vpu-expressing cells showing intracellular Lck accumulation was comparable to that in Nef-expressing cells. Nevertheless, the magnitude of the Vpu-effect remained lower. As seen in the case of the downregulation effect caused by the two lentiviral proteins, the more pronounced effect observed for Nef than for Vpu might reflect the ability of Nef to retarget the whole cellular pool of Lck by altering transport to as well as from the plasma membrane. On the contrary, Vpu is only able to block anterograde transport of newly synthesized Lck molecules to the plasma membrane, being unable to target Lck molecules which have reached already their steady-state distribution. Our results show that HIV-1 Vpu can potentially retarget the peripheral

membrane protein Lck. However, the fact that the observed Vpu-mediated effect is exclusively significant when Lck is overexpressed is probably mirroring the lack of biological relevance of this process.

5.1.3 Vpu, like Nef, enhances EV release and exploits this strategy to induce its own export

Enhancement of EV release is another activity which has been described for Nef but remained unstudied for Vpu. HIV-1 Nef expressed alone or in the context of viral infection enhances EV release (Campbell et al., 2008; Lenassi et al., 2010; Muratori et al., 2009). Furthermore, it induces its self-incorporation into the EVs. This leads to the induction of intracellular signaling and transport changes in bystander cells upon uptake of the shed EVs generating a favorable environment for viral replication and spread. Of note, Nef-positive EVs have been reported not only to be present in cell culture but also in plasma of HIV-infected patients (Raymond et al., 2011) It has been described that the released EVs triggered cell death in recipient cells, thus providing a potential mechanism for the bystander CD4+ T loss which represents a hallmark of HIV infection (Lenassi et al., 2010). Consistent with these studies, we have been able to detect Nef in EVs derived from A3.01 human T cells upon isolated expression of the accessory protein *in vitro*. Furthermore, we could observe a marked Nef-induced enhancement of EVs release in these cells as concluded from the higher CD63 and CD81 WB signal obtained as well as a strong increase in total protein content in the EV preparation when compared to GFP-expressing cells-derived control EVs. Of note, the CD63 signal and the total proteins content of CLs from both Nef-expressing and control cells showed comparable levels. The CD81 signal from lysates of Nef-expressing was stronger as that seen for GFP-expressing control cells, however the difference was not as marked as that seen between the CD81 signal of EVs derived from both cell cultures. Viewing these results, we can conclude we have confirmed the induction of EV release by Nef previously reported by others.

Importantly, here and for the first time we have observed that enhancement of EV release can also be observed upon isolated expression of HIV-1 Vpu. Moreover, we have seen that the effect mediated by Vpu, in contrast to what was observed for cell surface receptor downregulation or rerouting of Lck, was stronger than the effect mediated by Nef. Of note, this novel observation has set up the basis to identify the presence of Vpu in EVs in the plasma of HIV-infected patients in a subsequent study by our collaborators, thus highlighting the physiological relevance of Vpu incorporation into EVs (Lee et al., 2016). Interestingly, these authors observe abnormally high amounts of EVs in the plasma of infected patients and detect both accessory proteins in them. Also interesting is that the biochemical analysis of those EVs shows absence of CD81, unlike what has been seen in this work. This is a surprising finding knowing that CD81 is a prototypical exosomal marker present in exosomes from most cell types and it might reflect the proteome of the main producer cells.

Based on the Western Blot signal obtained and despite of being expressed at a comparable degree in producer cells, EVs contained lower levels of Vpu than of Nef. Interestingly, the amount of CD81 and CD63 present in EVs from Vpu-expressing cells was considerably higher than that in Nef-expressing cells suggesting a more potent boost in EV release induced in the former case. This is consistent with slightly higher total protein content in EVs derived from Vpu- than for Nef-expressing cells and no visible difference in the same parameter for CL from these cells. Importantly, both proteins enhance significantly exosome release as compared to

It is important to point out that the results shown in Section 3.7 (and also in Section 3.6) of this work have been obtained only once and would need further repetition to make the data and the conclusions from this part more robust. Nevertheless, we believe that the validation of previously published observations regarding Nef-induced secretion enhancement and its own inclusion onto released EVs (Campbell et al., 2008; Lenassi et al., 2010; Muratori et al., 2009) represents a strong argument contributing to the credibility of the obtained Vpu results in regard to EV release.

Amongst the aims of this part of this doctoral work there was testing the hypothetical mechanism of TSPAN downregulation by enrichment and export onto EVs. Understanding which motifs of Nef and Vpu are important for the described effect on EV release enhancement was also of our interest. Technical issues however kept us from going that far into mechanistic research. The main reason for that was the low amounts of EVs obtained from T cell line cultures. This forced us to scale up the experimental settings and to use very high amounts of plasmidic DNA for transfection (section 3.6) or of lentiviral vectors for transduction (section 3.7). In this context, testing Nef and Vpu mutants would have required extremely high volumes of cell culture (litters) and it would have been unfeasible concerning time and resources. A recent publication by Choudhuri et al. showed that T cell vesicle secretion occurs in a very polarized manner directed to the immunological synapse formed upon recognition of cognate antigen-MHC-I complexes (Choudhuri et al., 2014). This is consistent with the increase in EV release we observed upon stimulation of A3.01 T cells with activating surfaces coated with TCR triggering antibodies. Furthermore, it offers an explanation for the low secretion levels observed for T cells in culture.

5.1.4 Evolutionary perspective and functional advantage of redundancy in biological activity between HIV-1 Nef and Vpu

The appearance of the *vpu* gene in some SIV precursors and its inter-species transmission to HIV resulted in a duplication of the manipulative potential on the host cell physiology mediated by the accessory proteins Nef and Vpu. As we describe here, Nef and Vpu alter intracellular transport, endocytosis and exocytosis leading to modification of cell surface molecule exposure of a broad range of molecules like TSPANs, retargeting of peripheral membrane proteins like Lck and enhanced vesicle secretion. The fact that Nef and Vpu are expressed early and late in the replication cycle, respectively, ensures to achieve efficiently vesicular trafficking modification at

all time points of its life cycle. It also allows for adaptation to different cellular environments given the mechanistic divergence of Nef and Vpu for the activities studied in this work. Furthermore, it provides a safeguarding mechanism and preventing the loss of those activities by disrupting mutation acquisition or counteraction.

In contrast to the redundancy of function observed in our work, HIV-1 Nef and Vpu still posses well differentiated, distinct activities that are not shared. Examples of this are the enhancement in viral infectivity and actin rearrangements disruption by Nef, and counteraction of tetherin and boost of viral release by Vpu. Altogether, the complexity of activities, shared or specific, carried out by the accessory proteins Nef and Vpu of HIV-1 might have been the consequence of a detailed adaptation of the virus to achieve efficient infection in humans.

From the functional point of view, it is tempting to speculate about the benefits generated by the studied processes affected by both HIV-1 Nef and Vpu, which in some cases are already known:

- TSPANs have been described to disrupt membrane fusion by a yet to be identified mechanism (Symeonides et al., 2014; Takeda et al., 2003). Additionally, these molecules are present in HIV virion envelopes and it has been described that assembly occurs preferentially at TEMs (REFERENCES). In this scenario, TSPANS inhibit cell-to-cell fusion by binding of Env expressed at the membrane of infected cells and CD4 of neighboring cells (Gordon-Alonso et al., 2006; Weng et al., 2009) probably at the hemifusion step (Symeonides et al., 2014). It is easy to hypothesize that the described increase in virus cell-to-cell transmission results, at least partially, from the Nef- and Vpu-mediated downregulation of TSPANs from the plasma membrane (Casartelli et al., 2010; Haller et al., 2011; Malbec et al., 2013).
- As mentioned before, Nef-mediated Lck rerouting to RE/TGN results in the inhibition of proximal TCR signaling steps avoiding AICD while downstream signaling is favored by the highly active intracellular Lck pool increasing viral replication. The redundancy of this activity by the Vpu protein likely permits efficient Lck targeting also at late steps of the viral cycle and provides a backup preventing from loss of this relevant function.
- The incorporation of HIV-1 Nef and Vpu onto EVs provides a potentially strong strategy to alter the physiology of bystander cells (Muratori et al., 2009). This could translate into higher infection rates by rendering neighboring cells more permissive to infection or even induce and explain bystander cell death (Lenassi et al., 2010), a hallmark of AIDS.

5.2 Effects of HIV-1 Nef on T cell polarity and TEM

The negative impact of Nef on T cell motility has been extensively described by us and others (Choe et al., 2002; Hrecka et al., 2005; Janardhan et al., 2004; Lee et al., 2008; Nobile et al., 2010; Park and He, 2009; Stolp et al., 2010; Stolp et al., 2012; Stolp et al., 2009). Expression of the viral factor compromises the ability of these cells to undergo actin cytoskeleton rearrangements essential for efficient directional migration and results in chemotaxis block (Nobile et al., 2010; Stolp et al., 2010; Stolp et al., 2009). Our laboratory described the molecular mechanism by which Nef interferes with T cell actin dynamics and thereby with the motility of these cells to be the consequence of the interaction between the accessory protein and the host cell kinase PAK2 (Stolp et al., 2010; Stolp et al., 2009). The interaction results in a shift in the specificity of the kinase towards a non-natural target, the actin severing factor cofilin. In this scenario, the phosphorylation of cofilin leads to its inactivation and thereby to a reduced actin turnover in the cell (Stolp et al., 2009). A subsequent study also by our laboratory reported that isolated expression of Nef in murine CD4+ T lymphocytes inhibits homing to lymph nodes *in vivo* using a mouse model (Stolp et al., 2012). Detailed investigation showed that the main block takes place at the level of migration through the endothelium as well as subendothelial migration. The latter appeared to be dependent on the effects of Nef on cell actin dynamics. The former however relies on other, yet unknown Nef molecular determinant(s). Notably, the impairment in TEM was paralleled by a block in polarization of T cells expressing Nef both on the endothelium and on collagen-coated surfaces under chemotactic stimulus. Only Park et al. have also addressed the question of how does Nef affect T cell polarization (Park and He, 2009). These authors used a Nef-inducible Jurkat T cell line and anti-LFA-1 antibody-coated surfaces to stimulate polarization of these cells. Importantly, both mentioned studies described for the first time a Nef effect on T cell polarization in the context of migration. However, many open questions remained such as if the Nef-induced polarity block can be observed in the context of infection of human primary cells and what is the relevance of it. The mechanism governing this Nef activity was completely unexplored as well.

Here, we used a fibronectin stimulus-based 2D system to describe for the first time that HIV-1 SF2 Nef can hinder polarization of human PBMCs in the context of infection and of A3.01 T cells expressing the viral factor alone. Taken together, the results shown in this doctoral dissertation demonstrate that Nef is necessary and sufficient to cause this effect. Furthermore, together with the PAK2-interacting motif of Nef we identified the α-helical 12-39 stretch, required for the assembly of a kinase complex necessary for viral replication and enhanced EV secretion, as important for this activity (Figure 5.1). Further experimental work suggests that the relevant host protein among those forming the complex implicated on the Nef-induced polarity block is PKC. Future experiments should aim at identifying the isoform(s) involved and improve our understanding of the underlying mechanism. Besides, we have also set the basis here for a complex experiment to study the effects of Nef on transmigration of T cells through the endothelium *ex vivo* in the context of infection. This experiment intends to test our

hypothesis that the TEM defect mediated by Nef is ultimately due to the polarization defect.

5.2.1 Specific role of Nef on T cell polarization block in the context of infection

As mentioned above previous studies targeting effects of Nef on T cell polarity where not performed with physiological HIV-1 target cells and used isolated expression of the lentiviral protein (Park and He, 2009; Stolp et al., 2012). Here we have studied primary human target cells of HIV-1 in the context of infection. According to our observations, the polarity loss occurs also as a result of infection. HIV-1 wt infection caused at least a two-fold decrease in the frequency of polarization in cells from all donors except for one case. In this scenario, preventing expression of Nef led to higher polarization frequencies in cell form all 13 donors studied. Thus Nef appears as the major player being necessary and often sufficient to mediate polarity disruption. The extent of the effect caused by Nef on T cell polarity was however subject to marked donor variability. Of note, cells from 7 out 13 donors analyzed showed polarization frequencies which equaled those of uninfected cells when infected with a virus lacking expression of Nef. Importantly, the impact of wt HIV-1 on polarization levels was not as strong as that seen for cells from the remaining 6 donors which did not recover polarization levels of uninfected cells when Nef was absent. While for the latter 6 donors the frequencies of polarization upon HIV-1 wt infection ranged between 3.3 and 15.0%, the values for the former 7 donors only ranged between 14.6 and 33.3%. Thus the stronger the effect of wt HIV-1 infection on the frequency of polarization of PBMCs is the lower are the probabilities to see a recovery to levels obtained for uninfected cells by subtracting Nef from the system.

5.2.2 A system to study T cell polarization in vitro

In order to study further study the effects of Nef on T cell polarity we needed to establish in our laboratory an experimental set up for that. Despite of unsuccessful efforts to nucleofect human PBMCs to study the observed polarity defect induced by Nef, we could finally find a much simpler and more versatile system. We thereby tested different human T cell lines on surfaces coated with different extracellular matrix molecules and under different conditions (data not shown). A3.01 human T cells proved to polarize on fibronectin-coated surfaces without the need for chemokine stimulation. Of note, these cells were able to polarize to similar levels of uninfected PBMCs even after transfection with a GFP expression vector. The fact that these cells can be easily transfected or transduced for delivery of expression plasmids makes of our fibronectin-based 2D system a perfect set up for the study of T cell polarity under different conditions, e.g. after manipulation of our cells for expression of a variety of ectogenic proteins or following different pharmacological treatments.

Notably, unlike in previous studies (Park and He, 2009; Stolp et al., 2012), chemokines were not utilized since fibronectin-integrin crosslinking was enough to induce polarization both of PBMCs and A3.01 cells *in vitro*. In this regard and consistent with this experimental set up, downregulation of LFA-1 has been reported as one of the

mechanisms for Nef-mediated interference with T cell adhesion to the endothelium and with polarization, thereby inhibiting TEM (Park and He, 2009).

5.2.3 Conservation of Nef-mediated T cell polarization defect in evolution

This study reveals that the observed impact on T cell polarization by Nef is highly conserved among lentiviral proteins from SIV, HIV-1 and HIV-2. 18 Nef alleles, as well as specific SIV Nef mutants lacking CD3-downregulation activity, were studied for their ability to inhibit polarity in A3.01 human T cells. Notably, the HIV-1 YBF30 and the SIVcpz Tan 3 alleles failed or caused an intermediate decrease in polarization frequency levels, respectively. However, these alleles were previously reported to induce intermediate effect or to fail, respectively, in several Nef activities, namely Lck accumulation, inhibition of immunological synapse formation and concomitant tyrosine phosphorylation events (Rudolph et al., 2009). Additionally, SIVcpz Tan 3 Nef was also shown to be unable to inhibit membrane ruffles upon chemokine stimulation, chemotaxis and cofilin hyperphosphorylation induction (Stolp et al., 2010). This may indicate a global lack of most general Nef functions and our finding is consistent to it. Despite of the total independency of CD3 stimulus in our experimental set up, our data suggested initially dependency on CD3-downregulation as the overall effect of Nef proteins form HIV-1 and SIV unable to downregulate CD3 seemed less active in blocking T cell polarity than those of HIV-2 and other SIV strains able to do so. In order to study this implication we tested a panel of SIVblue Nef mutants with altered membrane receptor downregulation capacities as compared to the corresponding wt protein. Altogether, using these SIV Nef mutants helped us drawing the conclusion that Nef-mediated inhibition of T cell polarity is independent of CD3-downregulation activity. However, to our surprise, the trend observed for these mutants reflected exactly the opposite to what we observed with the whole set of alleles studied. Unlike the initial results with all the alleles, this other experiment showed that the two mutants unable to downregulate CD3 (R129/130A and R129A) were able to block polarity of A3.01 cells while the mutant downregulating selectively CD3 was not (E181A/L185A). Since CD4 downregulation is dispensable for the Nef effect on T cell polarity (see below), the only possible explanation for the lack of function of the ELAA mutant should be linked to CD28 downregulation, which seems unlikely, or to other undetermined consequences of the mutation at the ExxxL motif for this precise Nef allele.

5.2.4 12-39 stretch of Nef is important for T cell polarization impairment

In search for molecular determinants implicated in T cell polarity block mediated by Nef we tested a panel of HIV-1 SF2 Nef mutants in our 2D fibronectin-based experimental system. As expected, the generally defective G2A mutant unable to anchor into membranes was unable to block polarity of A3.01 cells (Fackler et al., 2006; Geyer et al., 1999). Consistent to that observed previously, the F195A mutant specifically unable to interact with PAK2 and thereby to interfere with actin dynamics was also incapable of interfere with T cell polarity (Stolp et al., 2012). Interestingly, the PxxP motif had an intermediate, yet pronounced effect in our mutant screen, the integrity of which was described to be important for the inhibition of T cell motility (Park and He,

2009). Importantly, our mutant screen defined a third region of Nef needed for polarity disruption, the 12-39 α-helical stretch which acts as a platform for the assembly of a multimolecular complex consisting of several host cell kinases and adaptor proteins. The formation of this complex has been reported to be necessary for viral replication (Witte et al., 2008), extracellular vesicular secretion induction (Muratori et al., 2009) and downregulation of CD4 form the plasma membrane (Fackler et al., 2006). Further confirmation of the implication of the 12-39 Nef stretch on T cell polarity disruption was achieved by infection experiments on human PBMCs were a HIV-1 virus bearing the deletion mutant showed polarization levels comparable to uninfected cells unlike wt HIV-1 infected cells which were strongly impaired for polarization. Here we describe for the first time the implication of this mutant in a migration-related phenotype. Since mutations at the flexible C-terminal loop involved in interaction with the endocytic machinery and thereby required for CD4 downregulation (Bresnahan et al., 1998; Craig et al., 1998; Lu et al., 1998) had no effect on T cell polarization and behaved like Nef wt we conclude CD4 downregulation is dispensable for polarity disruption and the 12-39 stretch must be involved by a different mechanism.

5.2.5 Possible mechanism of T cell polarity block via 12-39 motif, role of PKC

In the absence of enzymatic activity, the HIV-1 Nef protein exerts its functions by interacting with other proteins and molecules in the host cell. The mechanism by which Nef interferes with T cell polarization via the 12-39 stretch remains elusive. Our data suggest however that Nef might carry out this activity via PKC signaling since pharmacological inhibition of the other NAKC components in A3.01 cells did not induce changes in the frequency of polarization when compared to Nef-expressing cells (Figure 5.1).

Given that, besides the generally defective mutant of Nef G2A, the only two mutants of Nef relevant for T cell polarity disruption are F195A and Δ12-39, and knowing that the former hinders cellular actin rearrangements with high specificity, it is tempting to think that the latter acts also affecting the cytoskeletal organization. Interesting results were obtained from a large comparison of lentiviral Nef proteins for the conservation of PAK2-based hyperphosphorylation of cofilin as a strategy to hamper cell actin dynamics (Stolp et al., 2010). This study described a strong interaction between SIVcpz Tan 3 Nef and PAK2 but yet this Nef allele was unable to interfere with actin rearrangements. Thus, PAK2 association via the F195 (or equivalent residue) is necessary but not sufficient for the observed disruption in actin dynamics. Therefore, there might be other relevant motifs implicated in this Nef activity. On the other hand, a previous study by Haller and colleagues from our laboratory included an assay evaluating the inhibition of the F-actin ring formation at the immunological synapse, a structural scaffold to establish morphological and molecular polarity as well as directed secretion in this scenario. In that study it was reported that the Δ12-39 Nef mutant was partially dysfunctional as compared to Nef wt suggesting the implication of this Nef stretch in actin rearrangements inhibition (Haller et al., 2006). Additionally, a marked dependency on F-actin integrity has been reported for diapedesis as well as

subendothelial migration (Cinamon et al., 2001). In line with this, F-actin regulators control both T-lymphocyte polarization and crawling on the endothelium (Faroudi et al., 2010; Gerard et al., 2009; Heasman et al., 2010; Nombela-Arrieta et al., 2007; Shulman et al., 2006). Interestingly, the role of PKC, a component of the NAKC, in the regulation of actin dynamics and its impact on cell polarization and migration has been extensively studied in T lymphocytes. In this context, it has been reported that PKCβ_I was required for human T cell polarization and migration *in vitro* upon LFA-1-dependent stimulation. It also colocalized during migration on anti-LFA-1 or ICAM-1 (the cognate ligand of LFA-1) with the MTOC and microtubules at the uropod what suggests a role for this kinase on microtubule cytoskeleton rearrangements (Volkov et al., 1998; Volkov et al., 2001). Colocalization with MTOC and microtubules at the uropod under similar experimental conditions has also been described for PKCδ and pharmacological inhibition of this kinase hampered migration in a 3D matrix model (Fanning et al., 2005; Volkov et al., 1998). This kinase was reported to phosphorylate cytoskeletal adaptor paxillin contributing to LFA-1 activation and leading to adhesion (Romanova et al., 2010). Furthermore, phosphorylation of PKCδ has been shown to occur upon chemokine stimulation (Cronshaw et al., 2006). PKCε interacts with and phosphorylates Rab5a at the centrosomal region upon chemokine or integrin stimulation and regulates vesicular trafficking to the leading edge in migrating T cells. These events have been shown to be necessary for Rac1 activation, actin reorganization and T cell migration (Ong et al., 2014). The most studied PKC isoform in T cells is PKCθ due to the fact that its expression is more restricted to cell from the immune lineages and because it polarizes to the contact area between lymphocytes and APC or immune synapse (Zhang et al., 2013). Moreover, this kinase is central in antigen-dependent T cell activation and proliferation and induces chemokine secretion via activation of NF-κB, AP-1 and NFAT. Whereas PKCθ localizes at the uropod of murine cells following chemokine stimulation and it regulates distribution of MTOC and ERM proteins (Cannon et al., 2013), this protein appears to localize at the leading edge of chemokine-stimulated human lymphocytes (Freeley et al., 2012). Pharmacological inhibition *in vitro* and *ex vivo* experiments with PKCθ-deficient cells from knock-down mice show a marked reduction in migration towards chemokines when compared to the untreated or control counterparts, respectively (Cannon et al., 2013; Shahabi et al., 2008). *In vivo* studies however suggest that the observed effect is not as relevant as it could be expected from *in vitro* experiments (Cannon et al., 2013). Also, it is still unclear whether the impact of inhibition or lack of expression of PKCθ on migration are in fact the consequence of deficient T cell activation and adhesion due to impeded signaling events downstream of TCR (Letschka et al., 2008). Ultimately, atypical PKCs are known to regulate permanent polarity of epithelial cells (Chen and Zhang, 2013). Similarly, chemical inhibition or the use of dominant negative mutants of atypical PKCs part of polarity complexes in T cells following chemokine and integrin signaling showed impairment in polarization, migration and crawling on the endothelium under shear flow (Chen et al., 2008; Gerard et al., 2007; Gerard et al., 2009; Real et al., 2007). Furthermore, the F-actin bundling protein L-plastin has been reported to colocalize at the leading edge with and to become phosphorylated by PKC leading to defects in the

activation of Rac1 and Akt phosphorylation upon chemokine stimulation. PKCζ is part of the Par3 complex, involved in the establishment of polarity in well-studied cell systems like the epithelium or neurons. The inhibition of this atypical PKC in T lymphocytes prevents CCL21-induced LFA-1 polarization (Giagulli et al., 2004). Furthermore, the polarization of this PKC isoform has been described during asymmetric division in T cells, a process strictly dependent on cell polarity (Chang et al., 2007). Ultimately, all this events led to inhibition of T cell polarization and migration on ICAM-1-coated surfaces in an LFA-1 activation- or adhesion-independent manner. Altogether, PKCs play key roles in T cell polarity and motility and this is mainly related to their function as cytoskeletal regulators and key signaling master molecules. Therefore, it seems reasonable that Nef could affect T cell polarity or even migration via the manipulation of PKC proteins.

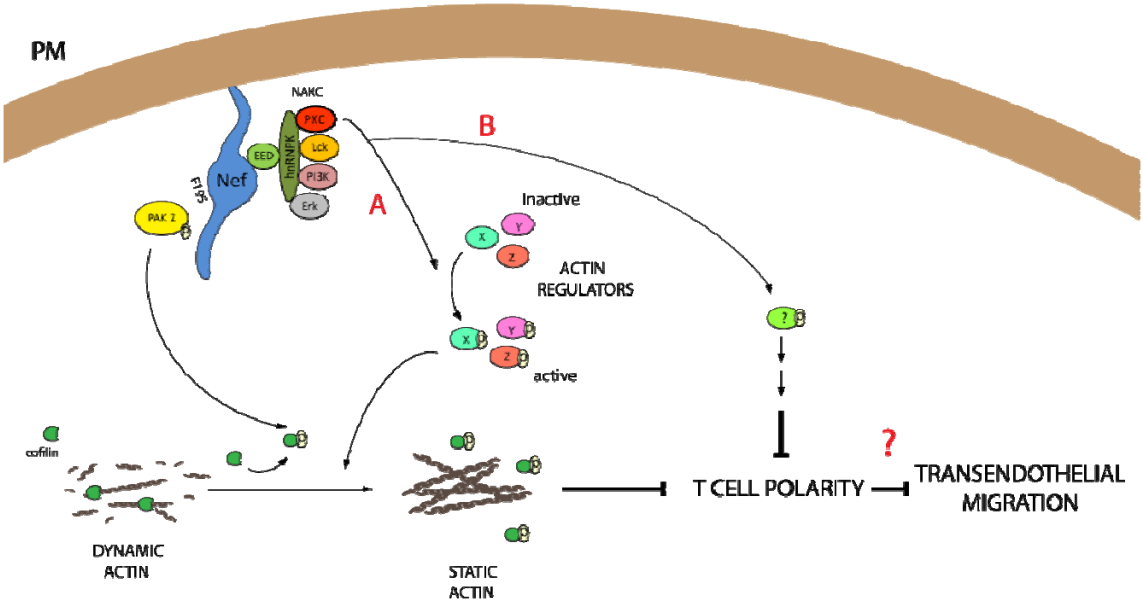

Fig 5.1 Final model: Nef interferes with T cell polarity via two different mechanisms involving either PAK-2 or PKC being the latter potentially the missing pathway resulting in transendothelial migration. Previously reported Nef's interaction with PAK-2 leads to hyperphosphorylation and thus inactivation of cofilin interfering thereby with the actin cytoskeleton dynamics. Alternatively, Nef possesses another determinant, the 12-39 α-helical stretch, through which the viral protein interacts with adaptor proteins of the NAKC. This complex includes PKC which presumably acts as an effector of other factors involved (A) or not (B) in actin regulation. As a consequence, T cell polarity is disrupted. Whether the exposed hypothesis, that is the a further impairment of transendothelial migration downstream in this pathway, is true will subsequently be examined using the experimental system set up in this work.

5.2.6 What do we learn from polarity monitoring by live microscopy?

Live microscopy has worked efficiently in the present study to observe the dynamics of the process and how Nef modifies the behavior of A3.01 human T cells in this regard. One question we initially asked was "how does polarity take place", "is it a transient mechanism that takes places frequently or in cycles or is it a rather a permanent process

after which the cell remains long term elongated". Our data shows that control A3.01 cells polarize and depolarize constantly. They cycle between a rounded shape and an elongated one and at least for this cell type there is no clear predominance of one over the other state. This observation is consistent with the polarization values we have quantified generally for control cells of around 50% (40-60%). Nef-expressing cells however do not polarize that frequently. They remain round and, if at all, they polarize only very short intervals and with very low frequency. Altogether, the fact that we can see some Nef-expressing cells which polarize means that Nef does not completely block polarization capacity but it makes it much less frequent. If a Nef-expressing cell is able to polarize or not is probably dependent on the basal activity of that specific cell. Unlike wt Nef, the studied Nef mutants are unable to cause this effect. The way cells expressing these mutants polarize however is different suggesting different intensity in the effect mediated by each of them. Interestingly, expression of a Nef combining the mutations Δ12-39 and F195A results a phenotype that more closely resembles that of GFP-control cells. Altogether, we have learnt that polarity is a cyclic mechanism with high frequency of elongation-rounding events and Nef is able to reduce the frequency of this process significantly spacing the elongation events.

5.2.7 Possible implication of T cell polarity impairment in TEM and migration

Previous work from our laboratory described a lack of dependency on the PAK-2 interacting domain for diapedesis, the central step of TEM, and therefore we decided to search for another molecular determinant responsible of that (Stolp et al., 2012). Given the parallel block on T cell polarity observed while studying TEM, we decided to characterize that effect aiming at learning more about both processes. We now know that both the 12-39 amino acid stretch and the F195 residue are important for Nef-mediated polarity disruption. Whether the 12-39 deletion plays a role in inhibition of diapedesis or not is something we can only learn by performing the TEM study which has been set up and established in the present work. However, unpublished *in vivo* work form Dr. Sheetal Kaw from our laboratory suggest an important role of the 12-39 motif in T cell homing. The expression of the 12-39-deleted Nef by murine $CD4^+$ T cells is enough to restore the block in T cell homing, as it was noticed before for F195A Nef mutant (Stolp et al., 2012). The effect observed is stronger at early timepoints (4 hours post-transfer into the mice) than that seen for the latter mutant. The impact of the expression of both mutants becomes equally important at a later timepoint (24 hours post-transfer into the mice). These results suggest that these two mutations may act through two different mechanisms to affect T cell homing yet leading to a convergent effect. Also, 12-39 is essential to mediate block of T cell homing at early timepoints as compared to F195 which enhances the implication of two different molecular mechanisms with different kinetics but an equal final consequence (Figure 5.1).

5.2.8 T cell migration inhibition upon HIV-1 infection: the big picture

HIV-1 infection of T cells results in a reduction in the migration of these cells. Previous studies' results together with the ones presented here have pinpointed Nef as the main negative regulator of lymphocyte motility in this context. They also suggest that Nef

disturbs T cell migration at multiple steps. Despite of the increasing amount of information on how Nef exerts this activity, the reason why inhibition of migration of T cells is beneficial for viral infection and spread remains a conundrum. Some authors think that by rendering infected cells static, the chances to meet other cells of the immune system and thereby being recognized and cleared are minimized (Park and He, 2009). It has been also suggested based on the inability of Nef-expressing cells to migrate towards the chemoattractant sphingosin-1-monophosphate S1P, involved in lymph node egress, that HIV-1 infected cells' density in the lymph nodes can thereby increment resulting in a maximization of the cell-to-cell spread (Stolp et al., 2012). Given the capital role of migration for T cell function and immune surveillance, the deficiencies in polarity, migration and TEM observed for Nef-expressing cells may correlate with the incapacity of HIV-1 infected individuals to mount an efficient humoral response (Moir and Fauci, 2009). In contrast to this and other studies, *in vivo* data with humanized mice by Murooka et al. showed that most HIV-1 productively infected cells migrate actively likely promoting cell-to-cell spread through the formation of virological synapses and resulting in high viremia (Murooka et al., 2012). These discrepancies may reflect

6 OUTLOOK

In this work, some of the functions exerted by the accessory proteins of HIV-1, Nef and Vpu, have been investigated. Our data shows a redundancy of functions among the two viral proteins that includes downregulation of TSPANs form the expressing-cell plasma membrane, Lck retargeting to intracellular compartments or enhancement of EV vesicle release. Despite of providing interesting clues about the molecular determinants involved in the first of those functions, TSPANs downregulation, our data could not provide definitive information on this regard. None of our mutants led to a clear cut effect in this assay and, in the case of Nef, the profiles obtained for the different molecules studied were even different to each other suggesting that different determinants could be involved in downregulation of individual TSPANs. In order to address this question in the future, screening of a larger Nef and Vpu mutant panel should be pursued. Even combination of mutations could help shedding some light on this. Concerning EV release, the main limitation faced for performing mechanistic studies was the small amounts of vesicles obtained from T cell cultures. Increasing culture size provided only small help improving the detection of EV markers. Alternatively, other cells different than our T cell lines were tested. Primary monocyte-derived macrophages did not release substantial amounts of EV upon transduction for expression of Nef.GFP or Vpu.GFP. No better results were obtained with HUH7 cells and the same delivery method. Other cell types/lines may be tested in the future in order to establish a suitable experimental set up that allows mechanistic studies.

The data shown in the second part of this thesis defines Nef as the main negative regulator of T cell polarization upon HIV-1 infection. A new Nef determinant implicated in the formation of the NAKC has been identified as necessary for T cell polarity disruption, besides the already defined motif specifically interfering with F-actin dynamics. We hypothesize that the polarity defect correlates to the Nef-mediate block on TEM since the central step of this intricate process, diapedesis, implies a strong polarization of the T cells to squeeze it cellular body and nucleus through the endothelium. Here, the experimental conditions for the TEM experiment have been established. Conducting this experiment will prove whether the hypothesis formulated above is correct and will provide valuable information about the mechanism by which Nef specifically interferes with TEM, i.e. diapedesis. In this line, it has been previously postulated that the Nef-mediated block in diapedesis may rely on the downregulation of key TEM molecules such as CCR7 and CD62L by the viral protein (Stolp et al., 2012). Since a new determinant involved in polarity disruption has been identified and with a potential relevance for diapedesis inhibition, it would be interesting to evaluate the downregulation capacity of these molecules. Investigating the importance of the 12-39 stretch of Nef in the inhibition of F-actin sheet like protrusions would be of high interest as well since this has been shown to be required not only to achieve successful TEM but also for efficient migration in highly constrained environments, e.g. high density collagen. It would be of significance to address the LFA-1 downregulation behavior for the Δ12-39 mutant due to the implication of this molecule not only on the acquisition of

polarity through fibronectin crosslinking but also in adhesion to the endothelium. A more long-term goal would be to explore the role of well-known polarity establishing factors like Par3 or Scribble described to be essential for other cell systems, i.e. neurons and epithelial cells, in the context Nef expression and HIV-1 infection of T cells.

One last point would somewhat combine learnt lessons from the two parts of this dissertation. As mentioned above, by interfering with T cell migration HIV-1 may favor immune recognition escape of infected cells. However, the still functional, non infected surveillant $CD4^+$ T cells are able to migrate and encounter them. By releasing extracellular vesicles loaded with viral signal in form of Nef and Vpu, it could be possible that infected cells affected the migration of those uninfected CD4+ T cells that escape apoptosis induction that has been described for Nef-expressing cell-derived EVs. This could be tested experimentally by exposing uninfected T cells to Nef- and Vpu-loaded EVs previous to migration assay.

Interference with migration is increasingly recognized as essential for HIV-1 pathogenesis. A profound understanding of the mechanism that governs it will help developing new therapeutic targets and eventually shorten the distance towards a cure.

ABBREVIATIONS

Abbreviation	Decscription
2D	two dimensions
3D	three dimensions
ADCC	antibody-dependent cellular cytotoxicity
ADP	adenosinediphosphate
AICD	activation-induced cell death
AIDS	acquired immunodeficiency virus
ALIX	ALG-2-interacting protein X
ALV	avian leukosis virus
AMP	adenosinemonophosphate
AP	adaptor protein complex
APC	allophycocyanin
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ART	antiretroviral therapy
Arp 2/3	actin related proteins 2/3
ATP	adenosinetriphosphate
bNAbs	broadly neutralizing antibodies
BSA	bovine serum albumin
BST	bone marrow stromal antigen
CA	capsid
cART	combination antirretroviral therapy
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
CDC	Center for Disease Control
Cdc42	Cell division control protein 42
CD4 T cell	T cell positive for CD4
CD8 T cell	T cell positive for CD8
cGAS	cyclic GMP-AMP synthase
cm2	square centimeter
CMAC	7-amino-4-chloromethylcoumarin
C-t, C-terminal	Carboxy-terminal end
DAG	diacylglycerole
CTL	cytotoxic CD8 positive T lymphocyte
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DN	dominant negative
DNA	desoxiribinucleic acid

dNTP	deoxynucleoside triphosphate
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGFR	endothelial growth factor receptor
ELISA	enzyme linked immunosorbent assay
Env	envelope protein
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport
EV	extracellular vesicle
EXOC	exocyst
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FIV	feline immunodeficiency virus
FLV	feline leukaemia virus
FN	fibronectin
FRET	Förster resonance energy transfer
G	guanine
Gag	group-specific antigen
GAP	GTPase activating proteins
GDP	guanosinediphosphate
GFP	green fluorescent protein
GEF	guanine nucleotide exchange factor
GMP	guanosinemonophosphate
gp	glycoprotein
GPCR	G-Protein coupled receptor
GTP	guanosinetriphosphate
h	hour
HAART	high activity antiretroviral therapy
HCMV	human cytomegalovirus
HDAC	histone deacetylase
HFV	human foamy virus
HHV	human herpes virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTLV	human T-cell lymphotrophic virus
HUVEC	human umbilical vein endothelial cell
ICAM	imtercellular adhesion molecule

IL	interleukin
IFN	interferon
IN	integrase
IRES	internal ribosomal entry site
IS	Immunological synapse
ISG	interferon stimulated genes
kb	kilobases
kbp	kilobasepairs
kDa	kiloDalton
Lamp	Lysosome-associated membrane glycoprotein
LAT	linker for the activation of T cells
LB	lysogeny broth
Lck	lymphocyte-specific protein tyrosine kinase
LEDGF	lens epithelium-derived growth factor
LFA	lymphocyte function-associated antigen
LTNP	long-term-non-progressor
LTR	long terminal repeat
MA	matrix
MAPK	mitogen-activated protein kinase
MC	microclusters
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
ml	mililiter
mM	millimolar
MLV	murine leukaemia virus
MMLV	moloney murine leukaemia virus
MMTV	mouse mammary tumor virus
MMP	matrix metalloprotein
MPMV	Mason-Pfizer monkey virus
mRNA	messenger RNA
NAKC	Nef-associated kinase complex
NC	nucleocapsid
Nef	negative factor
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NGFR	nerve growth factor receptor
NK	natural killer cell
nm	nanometer
NNRTI	non-nucleoside reverse transcriptase inhibitor

nPKC	novel protein kinase C
NRTI	nucleoside reverse transcriptase inhibitor
N-t, N-terminal	Amino-terminal end
OD	optical density
ORF	open reading frame
PACS	phosphofurin acidic cluster sorting protein
PAGE	polyacrylamide gel electrophoresis
PAK	p21 activated kinase
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFR	platelet derived growth factor receptor
PECAM	platelet/endothelial adhesion molecule
PE	phycoerythrin
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PIC	pre-integration complex
PIP2	phosphatidylinositol-bisphosphate
PIP3	phosphatidylinositol-trisphosphate
PHA	phytoemagglutin
PKC	protein kinase C
PM	plasma membrane
Pol	polymerase
PR	protease
PRR	pattern-recognition receptor
P-TEFb	positive transcription elongation factor
PVR	poliovirus receptor
RDV	rous sarcoma virus
RE	recycling endosomes
Rev	regulator of expression of virion proteins
RFP	red fluorescent protein
RNA	ribonucleic acid
ROCK	Rho kinase
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
RRE	Rev responsive element
RT	reverse transcriptase
RTC	reverse transcription complex

SAMHD1	SAM domain and HD domain-containing protein 1
SD	standard deviation
SDF-1α	stromal cell-derived factor 1α
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERINC	serine incorporator
SFV	simian foamy virus
SG-PERT	sybr green one step PCR-enhanced reverse transcriptase assays
SH	src homology (domain)
SHIV	simian-human immunodeficiency virus
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
S1P	sphingosine-1-phosphate
ß-TrCP	beta-transducin repeat-continuing protein
SU	surface component of envelope
t	time
TAR	transactivation responsive region
Tat	trans-activator of transcription
TCR	T cell receptor
TEER	trans-endothelial electrical resistance
TEM	transendothelial migration
TGN	trans-Golgi network
TJ	tight junctions
TLR	toll-like-receptor
TM	transmembrane component of envelope
TM	transmembrane domain (of a protein)
TNF-α	tumor necrosis factor-α
TRIM5α	Tripartite motif-containing protein alpha
TRITC	Tetramethylrhodamineisothiocyanate
TSPAN	tetraspanin
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
Vif	viral infectivity factor
Vpu	Viral protein U
VSVG	vesicular stomatitis virus glycoprotein protein
WASP	Wiskott-Aldrich syndrome protein
wt	wild type
WDSV	Walleye dermal sarcoma virus

WHO	World Health Organization
YFP	yellow fluorescent protein
ZAP-70	Zeta-chain-associated protein kinase 70
µg	microgram
µl	microliter

PUBLICATIONS

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