Institute of Molecular Virology



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Efficient Nef-mediated down-modulation of TCR-CD3 and CD28 is associated with high CD4+ T cell counts in viremic HIV-2 infection

Dissertation

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List of Abbreviations

AGM	african green monkey
AIDS	acquired Immune Deficiency Syndrome
APC	antigen presenting cell
APC	allophycocyanin
bdg	binding
β-gal	β-galactosidase
blu	blue monkey
c	centi (10^{-2})
°C	degree celsius
$CaCl_2$	calcium chloride
CD	cluster designation
Ci	curies
CO_2	carbon dioxide
cpz	chimpanzee
CTL	cytotoxic T lymphocyte
d.p.i	days post infection
DMEM	Dulbecco´s modified eagle medium
DTT	dithiothreitol
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
Env	envelope
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
Fig.	figure
g	gram
g	(centrifugation) gravity

Gag	group specific antigen
gor	gorilla
h	hour
HAART	highly active antiretroviral therapy
HBS	HEPES buffered saline
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HRP	horse radish peroxidase
Ig	immuneglobulin
Ii	invariant chain (CD74)
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IRES	internal ribosomal entry site
kb	kilobase
KCl	potassium chloride
k	kilo (10^{3})
kDa	kilo-Dalton
Koz	Kozak sequence
l	liter
LB	Luria Bertani
LTNP	long term non progressors
LTR	long terminal repeat
m	meter
m	milli (10^{-3})
µ	micro (10^{-6})
M	molarity (mol/l)
mac	macaque
MFI	mean fluorescence intensity

$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minute
MHC	major histocompatibility complex
n	nano (10^{-9})
N	normality
NaCl	sodium chloride
Na_2HPO_4	disodium hydrophosphate
NK cell	natural killer cell
Nef	negative factor
NFAT	nuclear factor of activated T cells
NIG	nef-IRES-eGFP
NP40	nonidet P 40
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohaemagglutinin
PMSF	phenylmethylsulfonylfluoride
p.i	post infection
Pol	polymerase
P.t.s.	*pan troglodytes schweinfurthii*
P.t.t.	*pan troglodytes troglodytes*
rcm	red-capped mangabey
Rev	Regulator of expression of virion proteins
RIPA buffer	radioimmunoprecipitation buffer
RLU	relative light units
RNA	ribonucleic acid

rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
sec	second
SIV	simian immunodeficiency virus
smm	sooty mangabey
SOE PCR	splice overlap extension PCR
sun	sun-tailed monkey
syk	syke monkey
Tat	transactivator of transcription
Tm	melting temperature
TCR	T cell receptor
TGN	trans-Golgi network
Tris	trishydroxymethylaminomethane
UV	ultraviolet
V1H	catalytic subunit of vacuolar ATPase
Vif	viral infectivity factor
VL	viral load
Vpr	viral protein rapid
Vpu	viral protein out
VSV-G	vesicular stromatitis virus glycoprotein
v/v	volume per volume
w.p.i	week post infection
WT	wild type
w/v	weight per volume
www	world wide web

Amino Acids:

A	Ala	alanine
M	Met	methionine
C	Cys	cysteine
N	Asn	asparagine
D	Asp	aspartic acid
P	Pro	proline
E	Glu	glutamic acid
Q	Gln	glutamine
F	Phe	phenylalanine
R	Arg	arginine
G	Gly	glycine
S	Ser	serine
H	His	histidine
T	Thr	threonine
I	Ile	isoleucine
V	Val	valine
K	Lys	lysine
W	Trp	tryptophan
L	Leu	leucine
Y	Tyr	tyrosine

1 Introduction

1.1 HIV and AIDS

In July 1981 an outbreak had been reported of a rare form of cancer among gay men in New York and California. About the same time, another mysterious disease was reported in seemingly healthy young gay men causing fevers, flu-like symptoms, and a rare pneumonia called *Pneumocystis*. In the beginning these outbreaks were named GRID (gay-related immune deficiency) (7). However, cases started to be seen in heterosexuals, drug addicts, and people who received blood transfusions. This new disease of the immune system was later designated as Acquired Immune Deficiency Syndrome (AIDS). In 1983, the causative agent of AIDS was isolated from patient's blood and named lymphadenopathy-associated virus (7). In 1987 the virus was designated as the Human Immunodeficiency Virus-1 (HIV-1) (16). HIV-1 infects and eliminates CD4+ cells, thereby causing the destruction of the host immune system (57).

Thirty years after the discovery of HIV, about 34 million people are living with the viral infection, more than 25 million people have already died and HIV is still spreading with a rate of almost 2.5 million new infections and about 2 million deaths every year (114). The lack of proper health services, therapy and treatment worsen the situation of the infected people living in third world countries. After spending billions of Euros in rigorous research all over the globe we still can't eradicate the virus from infected individuals, as it integrates into the genome of long lived (half life 8-15 years) memory CD4+ T cells after infection. The combined antiretroviral therapy (HAART) has improved the life expectancy and quality of life drastically (75) but as a result of mutations, HIV may develop resistance to all available drugs (43).

1.2 HIV-1 and HIV-2 (Origin, Type, Genome organization and protein)

There are two distinct types of human immunodeficiency viruses, HIV type 1 and 2. HIV-1 is responsible for the global AIDS pandemic. In comparison to HIV-1, HIV-2 shows a poor capacity for both sexual and vertical transmission in humans and is largely confined to West Africa but has also spread to other parts of Africa, Europe, India, and the United States (45). HIV-1 and HIV-2 were introduced into the human population by zoonotic transmissions during the first half of the 20th century, and are hence very recent human pathogens (37). Many African nonhuman primate species are naturally infected with related lentiviruses, but

usually do not develop disease (83). HIV-1 groups M and N originated by crossing the species barrier from SIVcpz *P.t.t.* (*Pan troglodytes troglodytes*) to humans (48). The origin of HIV-1 group O is not clear, but its closest SIV relatives have been detected in gorillas. Thus, HIV-1 group O virus came into human host crossing the species barriers of chimpanzees and then gorillas or directly from gorillas to human (101). In 2009, a new HIV-1 group was reported to have greater similarity to a SIVgor than to SIVcpz. The virus had been isolated from a Cameroonian woman residing in France who was diagnosed with HIV-1 infection in 2004. The scientists reporting this sequence placed it in a proposed "Group P" pending the identification of further human cases (76).

Notably, SIVcpz itself seems to be a recombinant of primate lentiviruses, its precursors now found in red-capped mangabeys (SIVrcm), greater spot-nosed monkeys (SIVgsn) and mustached monkeys,and was subsequently transmitted to humans (6). HIV-2 originated from multiple zoonotic transmissions of SIVsmm from sooty mangabeys (41). HIV-1 is pathogenic and majority (80%) of HIV-2 infections, are not pathogenic in humans, while simian ancestors of HIV-2, SIVsmm infect their natural hosts (sooty mangabeys) without causing disease (37). Though it was suggested that SIVcpz does not cause AIDS in chimpanzees but the recent findings reported that SIVcpz does cause negative impact on infected chimpanzees. All chimpanzees that harbor SIVcpz in the wild, had higher rate of morbidity and mortality than uninfected hosts (49).

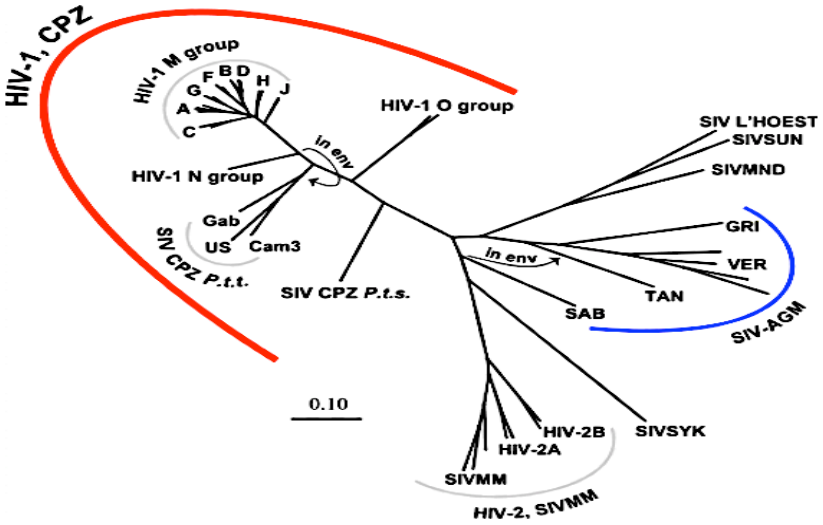

Fig. 1: The Phylogenetic Tree of primate lentiviruses: The tree has been generated by *pol* gene sequences alignment. The sequence clusters clearly indicate that HIV-1 is closely related to SIVcpz, similarly HIV-2 is closely associated to SIVsmm (45).

The RNA genomes of HIV-1 and 2 comprise of about 9.2 to 9.8 kb and contain structural and functional genes, namely *gag*,*pol* and *env* (Fig. 2). The genome is flanked by the Long Terminal Repeat (LTR) sequences, which work as a promoter. *Gag* gene encodes the structural protein capsid (CA), matrix (MA) and nucleocapsid (NC) proteins, *pol* gene encodes functional viral enzymes necessary for replication (reverse transcriptase (RT) and RNase H, protease (PR), integrase (IN)) and *env* gene encodes the structural glycoproteins. HIV-1 and HIV-2 genomes also contain many additional genes e.g. *tat* and *rev* that encode regulatory proteins and *vif*, *vpr, vpu* and *nef* genes encode accessory proteins (103, 4). The HIV-1 accessory proteins help in evasion of various cell mediated and host intrinsic factors. They help in modifying the local environment to ensure effective viral persistence, replication, dissemination and transmission *invivo* (61). However, they are not absolutely essential for viral replication *invitro*.

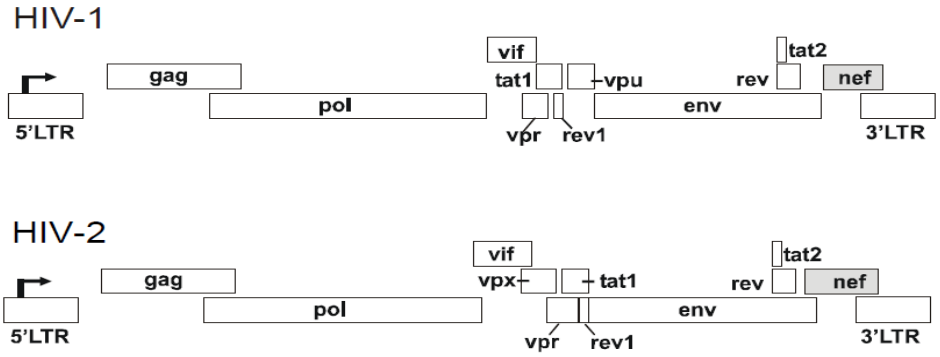

Fig. 2: Genome Organization of HIV-1 and HIV-2: The *nef* gene of HIV-2 is bigger than HIV-1 and it overlaps with *env* gene. In contrasttoHIV-1, HIV-2 does not encode a *vpu* gene. Instead, one extra gene, *vpx* is found in the HIV-2 genome.

The Vif (virion infectivity factor) protein counteracts a cellular restriction factor APOBEC3G that inhibits HIV-1 RNA by deaminating cytidine (C) to uridine (U) (88). Vpu (viral protein u) degrades CD4 and promotes release of budding virions otherwise tethered by the intrinsic restriction factor tetherin (29, 71). The HIV-1 Vpr (viral protein regulatory) arrests cell division cycle in the G_2 phase (3, 61). Nef (negative factor) performs various functions that help the HIV-1 to evade immune system and replicate in infected cells.

1.3 Pathogenesis of HIV-1 and HIV-2 infection

The disease progression of HIV infection may be measured through various outcomes such as plasma viral load, decline in CD4 count and HIV associated opportunistic infections. The hallmark for the onset of immunodeficiency syndrome is the destruction of CD4+ T cells and subsequent loss of immune competence (42). Most of HIV-1 infected individuals develop AIDS in about 6-8 years if left untreated.

In the first phase (acute infection) of HIV, the virus massively depletes the CD4+ memory T cells and in the second (chronic) phase, the immune system slowly lose control. The findings indicate that the HIV-1-encoded proteins prime the infected cells for activation, making them hyper-responsive to TCR-CD3 and co-stimulatory receptors mediated T cell activation signals (26). Although, the hyper-activation of the infected cells favors virus production but high turnover rate of CD4+ T cells leads to activation induced cell death, which led to the hypothesis that CD4+ T cell destruction results in the loss of critical immune effectors and/or regulatory functions, ultimately leading to immune deficiency (31, 65). However, later observations about the relative paucity of infected CD4+ T cells in the chronic phase, the slow loss of CD4+ T cells and the delay between infection and symptomatic immune deficiency, all in the face of continuous, high-level viral replication, were difficult to reconcile with this direct CD4+ T cell destruction hypothesis. To accommodate other observations, several mechanisms for the loss of CD4+ T cells have been proposed, including bystander killing, functional exhaustion and gradual loss of T cell–regenerative capabilities (65), but so far the relative contributions of these mechanisms to the pathogenic sequence have not been definitively established.

However, hyper-activation of the immune system and loss of CD4+ T cells are the typical characteristics of HIV-1 infection, contrary to that, in majority (~80%) of HIV-2 infections, infected people do not progress to AIDS. They have very low/undetectable viral load, the hyper-activation of the immune system doesn't take place and the HIV-2 infected host maintains the CD4 count and become long-term non-progressors (LTNP) (63, 108).

1.4 Multi-functional Nef protein

In all primate immunodeficiency viruses studied so far, a *nef* open reading frame is

present which encodes a protein of 27-35 kDa that is abundantly expressed during the early phase of viral infection. Its mRNA is estimated to represent three quarters of the early viral mRNAs in the cell (36, 58). Studies in Rhesus monkeys have demonstrated that an intact *nef* gene is critical for attaining high virus loads and development of simian AIDS (51, 17). Another study of HIV-1 in the SCID-Hu model also demonstrated the role of Nef to maintain the high viral load (2, 23, 47, 94).In this system, viruses with an intact *nef* ORF replicate faster, achieve higher titers, and deplete thymocytes better than their *nef*-defective counterparts (18). Moreover, long-term survivors of HIV infection i.e. LTNPs are almost the case of either a deletion in the *nef* gene or defective *nef* alleles (21, 55, 62, 79).

Nef performs a wide variety of activities to evade the immune system (as shown in Fig. 3), by down-modulation of CD4, CD28, MHC I, MHC II and CD28 proteins, critical for the formation of an immune synapse (IS), and up-regulation of Ii and enhancement of viral infectivity and replication (68, 70, 83, 85). Lower surface levels of CD28 in infected T cells ensure that these cells can not provide effective help following TCR engagement. Correspondingly, in professional antigen-presenting cells such as macrophages and dendritic cells being the first cells to be infected by HIV, down-modulation of surface MHC I and MHC II ensures poor presentation of HIV peptides to TCRs on helper or cytotoxic T cells. In addition, Nef-mediated reduction in MHC I expression on the surface of HIV-infected cells helps these cells escape from virus-specific CTLs. The overall effect of this strategy is to reduce T cell help for generating effective virus-specific antibodies and cytotoxic responses, as well as reduce the engagement of infected cells and virus-specific CTLs (54).

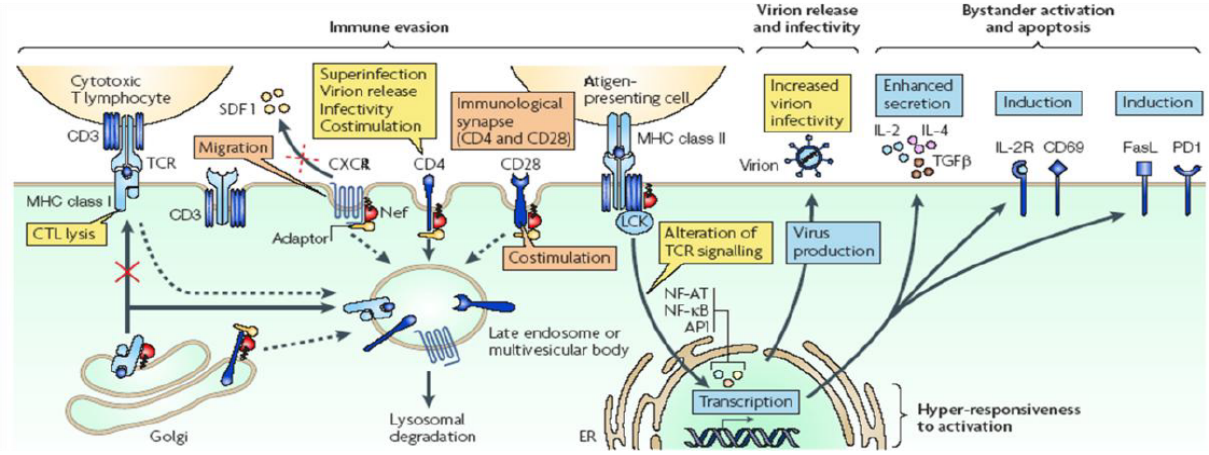

Fig. 3: The Multifunction of Nef to evade the immune system: Nef manipulate the infected cells in many ways to evade the immune system and to promote infection (54).

Many activities of Nef are conserved across different lineages of HIVs and SIVs and in combination they help the virus to persist efficiently in the infected host by facilitating evasion or counteraction of the immune system (56). However HIV-2 and the great majority of SIV Nefs, down-modulate the TCR-CD3 complex from the infected CD4+ T cells. Nef-mediated down-modulation of surface TCR-CD3 impairs the IS formation with dendritic cells or macrophages (5). Subsequently, TCR-CD3 mediated cellular activation does not take place which prevents the cells from hyper activation and activation induced cell death (AICD), thereby contributing in maintaining the CD4+ T cell count in infected hosts (5).

1.5 Scientific aims

HIV-2 infection cause immunodeficiency in humans but an unusually high proportion (~80%) of these infections remain asymptomatic and become long-term non-progressors with undetectable or very low viral loads. The Nef protein of primate lentivirus is a major immune modulator in the host. According to the previously published study (98), the high occurrence of defective *nef* alleles in HIV-2 infected individuals may be responsible for low viral load and the asymptomatic LTNP. So, the first aim of this thesis was, to explore the possible association between Nef functions, viral loads and the disease progressions in HIV-2 infection. We know that Nef protein is the great manipulator of host immune system and modulates various surface receptors including TCR-CD3 (83). Therefore, the second aim of this thesis was to find out the possibility that the differences in Nef's TCR-CD3 modulation function might be responsible for differences in clinical outcome of HIV-2 infections. The lentiviral Nef is a multifunctional protein, performs a striking variety of activities by its four major overlapping domains (30), many of these functions are genetically separable (1, 33, 44, 97). The third aim of my thesis was to find naturally occurring *nef* alleles that are selectively defective in specific functions, with particular focus on TCR-CD3 down-modulation. Furthermore, to map the amino acid residues responsible for specific functions will be interesting tool to study pathogenesis of AIDS.

Therefore, in general, the aim of my thesis was to study the role of Nef function in the clinical outcome of HIV-2 infection.

2 Materials and Methods

2.1 Materials

2.1.1 Bacteria

Escherichia coli XL2-Blue^{TM}: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* F *proABlacI^{q}Z M15 Tn10* (Tet^{r}) Amy Cam^{r} (Stratagene Agilent Technologies, Waldbronn).

2.1.2 Eukaryotic Cells

HEK293T: Human Embryonic Kidney epithelial cells, characterized with high transfectability. These cells have been transformed with adenovirus type 5 and express SV40 (simian virus 40) large T antigen (31).

HeLa: Human Cervical carcinoma epithelial cell line from 31 years old women. These adherent cells are characterized by a particularly large cytoplasmic region and are therefore often used for fluorescence microscopy.

Jurkat: Human T- Cell line.

Jurkat-NFAT: Human T-Cell line stably transfected with luciferase gene under NFAT promoter control (25).

P4-CCR5: HeLa--CD4/LTR lacZ reporter cell line that stably expresses human CD4, CCR5 and CXCR4. It contains the gene for ß-galactosidase under the control of HIV-1-LTR (13).

THP-1: Human monocyte cell line (97).

TZM-bl: It is a HeLa cell line, expressing large amounts of CD4, CCR5 and CXCR4. It contains the luciferase and ß-galactosidase gene under the control of HIV-1 promoter.

2.1.3 Nucleic Acid

2.1.3.1 Oligonucleotide

The listed below oligonucleotides were synthesized by biomers.net GmbH (Ulm).

Table 1: List of primers used for PCR reactions.

No.	Name	5'-Sequence-3'
1	2NefFD	GGGCTATAGGCCTGTTTTCTCTTCCCC
2	SLTR5	ACCTGCTAGTGCTGGAGAGAACCT
3	2NEFFB	TCCGCCAGCTGATTCGCCTCTTGA
4	2LTRRA	AACCTCCCAGGGCTCAATCTGCCAG
5	5RecForward	AGATTGGCAGAATTACACATCAGGACC
6	3RecReverse	TAATTCTGCCAATCTGGGATGATGCCTTCT
7	5-pBRNL43envHpaI	GCT GTTAACTTGCTCAATGCCACAGCC
8	3-pBRNL43MluI	GCACGCGTCAGCTTGTTTCCTTC
9	3-pBRNL43envEnd	CTTATAGCAAAATCCTTTCCAAGCCC
10	5-env-NIG-nef-F2	GGATTTTGCTATAAGATGGGTGCGAGTGGG
11	5-env-NIG-nef-1	GGATTTTGCTATAAGATGGGTGCGAGTGG
12	5-env-NIG-nef-Nveaux1	GGATTTTGCTATAAGATGGGGGCCAGTGG
13	5-env-NIG-nef-Nveaux2	GGATTTTGCTATAAGATGGGGGCGAGTGG
14	5-env-NIG-nef-Nveaux3	GGATTTTGCTATAAGATGGGTGCGAATGG
15	5-env-NIG-nef-Nveaux4	GGATTTTGCTATAAGATGGGTGTGAGTGG
16	3-R2-nef-MluI	GCACGCGTCAACTAAATGGTATCCCTCT
17	3-Nveaux1-nef-MluI	GCACGCGTTAACTAAATGGTATTCCCCT
18	3-Nveaux2-nef-MluI	GCACGCGTTAACTAAATGGTATTCCTCT
19	3-Nveaux3-nef-MluI	GCACGCGTTAATTAGATGGTATTCCTCT
20	3-Nveaux4-nef-MluI	GCACGCGTTAATTAAATGGTATCCCTCT
21	3-Nveaux5-nef-MluI	GCACGCGTCAGTCAAATGGTATTCCCCT
22	3-Nveaux6-nef-MluI	GCACGCGTTAACTATATGGTATTCCCCT
23	3-Nveaux7-nef-MluI	GCACGCGTTAACTATATGGTATCCCTCT
24	3-Nveaux8-nef-MluI	GCACGCGTTAACTGACTGGTATTCCCCT
25	3-Nveaux9-nef-MluI	GCACGCGTTAACTGACTGGTATCCCTCT
26	5-env-nef2-Neth-1	GGATTTTGCTATAAGATG GGATCGGCTGGT
27	5-env-nef2-Neth-2	GGATTTTGCTATAAGATG GGATCAGCTGGT
28	3-MluI-nef2-Neth-1	GCACGCGTTAACTATGTGGTAT
29	3-MluI-nef2-Neth-2	GCACGCGTTAATCTGTGGGTAT
30	3-MluI-nef2-Neth-3	GCACGCGTTAACTATGTGATAT
31	3-MluI-nef2-Neth-4	GCACGCGTCACATGGTATTCCC
32	3-MluI-nef2-Neth-5	GCACGCGTTAATCACATGGTAT
33	3-MluI-nef2-Neth-6	GCACGCGTTACTCAAATGGTAT
34	3-MluI-nef2-Neth-7	GCACGCGTTAGTTTGTAGGTAT
35	3-MluI-nef2-Neth-8	GCACGCGTTATTCACTATATGG

2.1.3.2 Plasmids

PCR®2.1-TOPO®-TA Vector: Vector for direct cloning of PCR products (Invitrogen, Karlsruhe).

pBR-NL43 nef-IRES-eGFP: pBR322 vector containing HIV-1 NL4-3 provirus. The proviral backbone has been engineered in a way that *nef* and *eGFP* are separated via an IRES element which can be expressed from a bicistronic mRNA.

pHIT/G: Expression vector containing envelope protein of vesicular stomatitis virus (VSV-G)

pCG: CMV-based expression plasmid.

2.1.3.3 DNA Marker for Agarose Gel

1 Kb Ladder	Invitrogen, Karlsruhe.

2.1.4 Enzymes

Alkaline phosphatase	Roche, Mannheim.
EDTA-Trypsin	Invitrogen/Gibco, Karlsruhe.
Restriction endonucleases	BioLabs, Frankfurt.
T4 DNA Ligase	Promega, Mannheim.

2.1.5 Reagents

Sigma, München	Agarose-Ultra, Dithiothreitol (DTT), Ethanol, Isopropanol, Methanol, Sodium dodecyl sulphate (SDS), Sodium chloride (NaCl).
Ratiopharm, Ulm	Ampicillin.
BD/Difco, Heidelberg	Bacto Tryptone, Yeast Extract.
Merck, Darmstadt	Bromophenol blue, Glucose, N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES), Hydrochloric acid (HCl), Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4$), Paraformaldehyde (PFA), Phenylmethylsulfonylfluorid (PMSF), Triton X-100, Tween 20.
Applichem, Darmstadt	Calcium chloride ($CaCl_2$), Glycine.
J.T. Baker, Deventer, NL	Disodium hydrophosphate (Na_2HPO_4).
Invitrogen/Gibco, Karlsruhe	Dulbecco´s modified eagle medium (DMEM), Fetal calf serum

	(FCS), Geneticin (G418), Penicillin/Streptomycin sulphate, Roswell Park Memorial Institute medium (RPMI-1640).
Fluka, Neu-Ulm	Ethylenediaminetetraacetate (EDTA).
Biochrom, Berlin	Ficoll separation solution, L-Glutamine.
Roth, Karlsruhe	Glycerol, Potassium chloride (KCl).
Miltenyi Biotec, Gladbach	Interleukin-2 (IL-2).
J.M. Gabler Saliter	Milk powder.
USB Corporation, USA	Nonidet P 40 (NP40), Tris.
Amersham Bios., München	Oligo dT, PolyA.
PAA, Marburg	Phosphate buffered saline (PBS).
Remel, Dartfort, UK	Phytohaemagglutinin (PHA).

2.1.6 Kits

Phire™ Hot Start DNA polymerase Kit	BioLabs, Frankfurt.
dNTPs	Invitrogen, Karlsruhe.
UltraClean 15 DNA Purification Kit	Dianova, Hamburg.
TA Cloning® Kit	Invitrogen, Karlsruhe.
Quick ligation™ Kit	BioLabs, Frankfurt.
Miniprep Kit	Qiagen, Hilden.
Wizard™ Plus Midiprep Kit	Promega, Mannheim.
Gal-screen substrate	Applied Biosystems, USA.
Luciferase assay system	Promega, Mannheim.
NuPAGE® Novex Bis-tris gels	Invitrogen, Karlsruhe.

2.1.7 Media

2.1.7.1 Cell culture media

Adherent and suspension cells were maintained in DMEM or RPMI-1640 respectively, supplemented with 10% (v/v) heat inactivated FCS, 350 µg/ml L-glutamine, 120 µg/ml Streptomycin sulphate and 120 µg/ml Penicillin.

2.1.7.2 Bacterial media

LB-medium: 10 g/l Bacto Tryptone, 5 g/l Yeast Extract 8 g/l NaCl and 1 g/l glucose in distilled water; pH 7.2 was adjusted with NaOH, 100 mg/l ampicillin was added before use.

LB_{AMP} Agar: 15 g/l agarose-ultra in LB-medium, 100 mg/l ampicillin was added before plating.

SOC Medium: 20 g/l Bacto Tryptone, 5 g/l Yeast Extract, 2.5 mM NaCl, 10 mM MgCl2, 10 mM MgSO4 and 20 mM glucose in distilled water.

2.1.8 Solutions and buffers

2.1.8.1 Calcium-phosphate transfection

10x HBS: 8.18% NaCl (w/v), 5.94% HEPES (w/v) and 0.2% Na_2HPO_4 (w/v) in distilled water. For 2x HBS preparation, the 10x stock solution was diluted with distilled water. The pH was adjusted to 7.12 and sterilized by filtration 2 M $CaCl_2$ was prepared and sterilized by filtration.

2.1.8.2 Western Blot

RIPA buffer:	1% Triton X-100 (v/v), 0.15 M NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA and 1 mM PMSF in distilled water.
Sample buffer:	0.5 M Tris, 22% Glycerol, 0.1% Bromophenol blue, 10% SDS in distilled water, adjust pH to 6.8.
Running buffer:	20x NuPAGE Mes SDS buffer (Invitrogen, Karlsruhe) diluted with distilled water.
Transfer buffer:	47.9 mM Tris, 38.6 mM Glycine, 1.3 mM SDS and 20% Methanol (v/v) in distilled water, adjust pH to 8.3.
Wash buffer:	0.2% Tween 20 in PBS.

2.1.8.3 HIV-1 p27 capsid-antigen-ELISA

Lysis solution:	10% Triton X-100 in distilled water.
Wash buffer (10x):	Contains PBS, Tween 20® and 2-chloroacetamide.
Sample diluent:	0.2% Tween 20 in RPMI-1640.

SIV p27 Antigen Standard:	contains detergent-disrupted, heat-inactivated viral antigen, goat serum, Triton X-100® and sodium azide.
SIV p27 Detector Antibody:	contains biotin-labeled polyclonal antibody to SIV, milk, Tween 20®, normal human serum and PBS.
Streptavidin peroxidase:	contains streptavidin conjugated to horseradish peroxidase, PBS, goat serum, and 2-chloroacetamide.
Assay diluent:	contains goat serum, PBS, Triton X-100® and 2-chloroacetamide.
10x Plate Wash Buffer:	contains PBS, Tween 20® and 2-chloroacetamide.
Substrate:	contains tetramethylbenzidine (TMB) and dimethyl sulfoxide.
Substrate Buffer:	contains citrate/acetate buffer, hydrogen peroxide and 2-chloroacetamide.
Stop solution:	1 N HCl.

2.1.8.4 RT assay

RT master mix:	60 mM Tris pH 7.8, 75 mM KCl, 5 mM $MgCl_2$, 0.1% (v/v) NP40, 2.02 mM EDTA pH 8.0, 5 µg/ml PolyA and 0.16 µg/ml Oligo dT in distilled water. Just prior to use 4 mM DTT was added.
Wash buffer:	20x SSC buffer (Eppendorf, Hamburg) diluted in distilled water
[α-32P] TTP:	Perkin Elmer, Rodgau-Jugesheim.

2.1.8.4 Buffers

FACS buffer: 1% FCS in PBS.

50x TAE-buffer: 5Prime, Hamburg.

2.1.9 Antibodies

2.1.9.1 ELISA

rabbit anti-HIV-1 p24	AIDS Repository, Fredrick, USA.
goat anti-rabbit IgG (H+L) HRP	AIDS Repository, Fredrick, USA.

2.1.9.2 FACS

Caltag, Buckingham, UK	anti-CD4-APC.

Becton-Dickinson, Heidelberg	anti-CD3-PE.
Dako, Hamburg	anti-MHC-I-APC.
Ancell, Baypart, USA	anti-CD74-PE.

2.2 Methods

2.2.1 DNA methods

2.2.1.1 General methods

The following methods were performed according to Maniatis *et al.*, 1989:

- Plasmid DNA isolation after alkaline lysis of bacteria
- Ethanol and isopropanol precipitation of DNA
- Determination of DNA concentration
- Agarose gel electrophoresis
- Restriction digestion
- Dephosphorylation of DNA with alkaline phosphatase
- Ligation of DNA fragments using T4 DNA ligase

2.2.1.2 Plasmid DNA preparation

Plasmid DNA for transfecting various cells was prepared using the Wizard™ Plus Midiprep Kit and plasmid DNA for cloning and sequencing was prepared using the Miniprep Kit, according to the manufacturer's instructions. The DNA concentration and quality was established using a spectrophotometer (Eppendorf, Hamburg).

2.2.1.3 Isolation of DNA from Agarose gel

Electrophoretically separated DNA fragments were analyzed on a UV screen (366 nm) (Syngene, USA), and the bands of interest were isolated from agarose gel using a scalpel. Subsequently the DNA was purified using the UltraClean 15 DNA purification Kit, according to the manufacturer's protocol.

2.2.1.4 Polymerase chain reaction (PCR)

All PCR reactions were performed with the Phire™ Hot Start DNA polymerase Kit in a PTC-100 Programmable Thermal Controller (MJ Research Inc). The following PCR conditions were used: (1) Initial denaturation: 94°C, 4 min (2) Denaturation: 94°C, 1 min (3)

Annealing: Tm-5°C (based on primer Tm), 1 min (4) Extension: 72°C (1 min/kb) 30-33 cycles (6) Final extension: 72°C, 7 min.

2.2.1.5 DNA ligation

Vector and insert DNA were mixed in the ratio of 1:3 and ligated using the Quick ligation™ Kit according to the manufacturer's instructions.

2.2.1.6 DNA Sequencing

2 µg of the plasmid DNA sample was prepared in a tube. Sequencing of DNA samples were performed by MWG-Biotec/Operon (Ebersberg).

2.2.2 Bacterial methods

2.2.2.1 Bacterial culture

The plasmids contained ampicillin resistance gene as a selectable marker. Therefore, transformed bacteria were grown in LB medium or on LB agar plates containing ampicillin. The bacterial culture was incubated for 12-16 h at 37°C on a shaker.

2.2.2.2 Bacterial transformation

Ligated DNA was transformed in 15 µl of *Escherichia coli XL2-Blue*™ cells on ice for 20 min. After the cells were heat-shocked for 30 sec at 42°C, they were incubated on ice for 2 min, followed by the addition of 200 µl SOC medium. The transformants were incubated at 37°C on a shaker for 30 min and plated on LB agar plates containing ampicillin. The colonies were counted to confirm the cloning efficiency.

2.2.3 Cell culture

2.2.3.1 Adherent and suspension cell culture

The adherent and suspension cell lines were cultured in 25 cm^2 or 75cm^2 cell culture flasks (Sarstedt, Nümbrecht) with supplemented DMEM or RPMI-1640 respectively, in a 37°C incubator with 5% CO_2. The cells were regularly passaged by splitting 1:10 or 1:20 twice a week.

2.2.3.2 Isolation of primary blood cells

Buffy coat (concentrated lymphocyte from 500 ml of blood), obtained from the blood bank (Ulm), was diluted to 1:3 with PBS containing 2% FCS. Ficoll separating solution was overlaid with the diluted blood and centrifuged at 2000 rpm for 20 min without brake and accelerator. The white interphase layer of peripheral blood mononuclear cells (PBMCs) was collected in a fresh tube and washed twice with PBS containing 2% FCS. The PBMCs mere cultured at concentration of $2x10^6$ cells/ml in supplemented RPMI-1640. For viral infection, cells were pre-stimulated with 1 µg/ml PHA and 10ng/ml IL-2 for 3 days.

2.2.4 Protein and enzyme methods

2.2.4.1 Reverse transcriptase (RT) assay

Virus production was quantified by measuring the reverse transcription activity of the virus (Sears *et al.,* 1999). The culture supernatants were taken for the assay and diluted to 1:5 with PBS, 5 µl of the diluted samples were transferred into a U-shaped 96-wells plate (Greiner Bio-one, Frickenhausen) and incubated with 25 µl of RT master mix containing 25 µCi of the [α-32P] TTP isotope (per 96-well plate). After 2-3 h incubation at 37°C, 5 µl of the reaction mixture was spotted onto Whatman paper (VWR International, Darmstadt). The membrane was air dried and washed on a rocker in 2x SSC buffer, three times each for 10 min, subsequently once for 1 min with 95% ethanol. The paper was air dried and exposed overnight to a Fuji imaging plate Type BAS-III (Fuji Photo Film Co., LTD). Next day intensity of spots were measured with a Fujifilm FLA-3000 (Fuji Photo Film Co., LTD). The reverse transcription activity was estimated by measuring the spot intensity, using the Aida Image Analyzer V.3.11 (Isotopenmeßgeräte GmbH).

2.2.4.2 Western blot

Quantification of virion release from transiently transfected 293T cells expressing the proviral HIV-1 constructs with the mutant *nef* alleles was performed with RIPA buffer. Viral proteins were separated by SDS-Polyacrylamide gel electrophoresis using NuPAGE® Novex Bis-tris gels (Invitrogen, Karlsruhe), as instructed by the manufacturer. The protein in the gel were transferred onto a nitrocellulose membrane (Millipore, Schwalbach) using a Trans-Blot SD Semi-Dry electrophoretic Transfer Cell (BioRad, München). Then the membrane was

blocked for 1 h with 10% milk solution and then incubated overnight with the respective primary antibodies. Secondary antibodies conjugated with phosphatase were added to the membrane and incubated for 1 h at room temperature. After washing the membrane, BCIP/NBT phosphatase substrate (KPL, Maryland, USA) was used to develop the color.

2.2.5 Viral methods

2.2.5.1 Virus stock preparation by transfection of 293T cells

Virus stocks were generated by transient transfection of 293T cells using the calcium-phosphate precipitation method. One day before transfection, $0.2x10^6$ 293T cells were seeded in 6-well plates (Greiner Bio-one, Frickenhausen). When the cells were 50-75% confluence, the transfection was performed. 5 µg of DNA was mixed with 13 µl of 2 M $CaCl_2$ and the volume was made to 100 µl with water. The DNA mixture was added drop-wise to another tube containing 100 µl of 2x HBS solution. The transfection mixture was vortexed for 5 sec and added drop-wise to the 6-wells plate containing cells. The transfected cells were incubated for 8-16 h and the media was changed with by fresh supplemented DMEM. Forty eight hours post transfection, supernatant was collected by centrifugation at 1300 rpm for 3 min. The virus stocks were stored at 4°C for up to two weeks. For the preparation of VSV-G pseudo-typed viruses, 5 µg DNA of the proviral constructs and 1µg of the pHIT60 plasmid were used for transfection.

2.2.5.2 Infection in PBMCs

For infection experiments, 1 million suspension cells (PBMC) in 3 ml supplemented RPMI-1640 were taken and incubated with 50 ng p24 of VSV-G pseudo-typed virus stocks and bicistronically expressing Nef and eGFP at 37°C for 4-6 h. PBMCs where further incubated in the presence of 10 ng/ml IL-2. Forty eight hours post infection, 1 ml aliquot from the culture was collected and stained (as described in Section 2.2.5.4) for CD4 surface expression and analyzed flow cytometrically. To this culture, 1 ml fresh supplemented RPMI-1640 media containing PHA was added to final concentration of 1 µg/ml and incubated further. The next day of stimulation an aliquot of the culture was collected and stained for CD3, CD69 and CD28 surface expression. Two days post stimulation, the activation marker CD25 and apoptotic marker AnnexinV were measured. After 3 days of stimulation, infected

cells were further analyzed for AnnexinV flow cytometrically.

2.2.5.3 Jurkat-NFAT assay

50000 Jurkat-NFAT cells were infected with 1.5 ng p24 virus stocks in a V-shaped 96-wells plate (Greiner Bio-one, Frickenhausen). Two days post infection the cells were stimulated with 1 µg/ml PHA and 16 h post stimulation, the luciferase activity of the cell lysate was determined using the Luciferase assay system. For this, cells were centrifuged for 3 min at 1300 rpm, the medium was removed and cells were lysed in 40 µl of lysis buffer. The lysed cells were transferred into a 96F-Nunclon-delta white micro-well plate (NuncTM, Langenselbold) and 50 µl luciferase buffer was added. For the measurement, an Orion Microplate Luminometer (Berthold Detection systems, Pforzheim) was used and the luciferase activity was given as relative light units/second (RLU/sec) using the computer program Simplicity 4.02 (Berthold Detection Systems).

2.2.5.4 Flow cytometry analysis: Modulation of surface markers

Infected cells were washed with 500 µl FACS buffer and stained with 100 µl FACS buffer containing 1-10 µl PE- or APC-conjugated antibodies (as per the manufacturer's instructions). The cells were incubated for 30 min at 4°C and then washed with 1 ml FACS buffer to remove unbound antibodies The cells were fixed with 200 µl buffer containing 2% PFA and incubated for 30 min at 4°C. For flow cytometry analyses, a FACS Calibur from Becton-Dickinson was used.

2.2.5.5 T cell activation and apoptosis of PBMCs

Pre-stimulated PBMCs or CD4+ T cells were infected with VSV-G pseudo-typed virus stocks as described under Section 2.2.6.1. Two days post infection, a second stimulus (1 µg/ml PHA) was added. Aliquots of the cultures were taken and the expression of CD69 (1 day post stimulation), CD25 (2 and 3 days post stimulation) and the amount of apoptotic cells (2 and 3 days post stimulation) were measured by FACS analysis. For the determination of the apoptosis rates, the binding of AnnexinV to apoptotic cells was measured. For this, an aliquot of the cell culture was washed with 500 µl PBS and then incubated with AnnexinV binding buffer containing 5 µl AnnexinV-APC. After 15 min of incubation in the dark at room temperature, 200 µl AnnexinV binding buffer containing 3% PFA was added for fixation followed by FACS analysis of the samples.

2.2.5.6 Infectivity assay (β-galactosidase assay)

TZM-bl/P4R5 (modified HeLa cells); these adherent cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. One day before infection, these cell lines were seeded (10,000 cells/well) in Flat-bottom 96-wells plate (Greiner Bio-one, Frickenhausen). Virus stocks were normalized for p24 content and infections were performed with various concentrations. Two days post infection, the supernatants were removed and 40 µl of 1:1 diluted Gal-screen® substrate in PBS was added to each well. After 30 min incubation at room temperature, the cell lysates were transferred into a Flat-bottom 96-wells plate Nunclon-delta white micro-well plate (Nunc™, Langenselbold) and the light emission was measured with an Orion Microplate Luminometer (Berthold Detection systems, Pforzheim). The enzyme activity was calculated as relative light units/second (RLU/sec) using the computer program Simplicity 4.02 (Berthold Detection Systems).

2.2.5.7 Viral replication

To analyze the effect of patient's *nef* alleles on viral replication, stimulated PBMCs were infected with different concentrations of p24 normalized virus stocks, bicistronically expressing Nef and eGFP. Three days post infection, an aliquot of the culture was taken and the amount of infected cells was estimated by measuring the amount of infected eGFP+ cells, flow cytometrically. The supernatants were stored at -20°C and RT assay was performed to determine the virus production. The culture volume was maintained by adding fresh supplemented RPMI-1640 media containing IL-2. Further samples were collected at regular intervals of 2 days.

2.2.6 Computer programs and data analyses

For the analysis of nucleotide and peptide sequences the following programs were used:

- Sequence reverse complementor (http://bioinformatics.org/sms/rev_comp.html)
- Alignment program MultiAlin V5.4.1 (http://prodes.toulouse.inra.fr/multalin/)
- DNA/amino acid program Expasy-tool (http://www.expasy.org/tools/dna.html)
- Gene construction Kit V2.0 program from Bob Gross and Anders Putte

- Sequence analysis program Chroma 1.62
- Phylogenetic tree construction by the Bayesian method using the general reversible (GTR) model of evolution

For FACS analyses the computer program CellQuest-Pro from Becton Dickinson was used.

Statistical analyses were performed with the PRISM program version 4.0 (Abacus Concepts, Berkeley, CA, USA) and Microsoft Excel. Correlation analysis were performed with Pearson correlation method, P-values were calculated using the two-tailed unpaired Student's T-test.

3 Results

3.1 Patient characteristics and sample details

To understand the functional role of Nef in the dichotomy of disease, I analyzed *nef* alleles derived from biological virus clones isolated from a total of 37 HIV-2-infected subjects. Twenty six of them (15 viremic, 11 non-viremic) were from the Guinea-Bissau cohort (109) and 11 individuals (6 viremic, 5 non-viremic) were from the Rotterdam cohort (10, 11, 105, 106). Some characteristics of these HIV-2-infected individuals are summarized in Table 2.

The viral loads (VLs) of these HIV-2 infected individuals have been analyzed in relation to CD4+ T cell counts and are shown in Figure 4. The VLs in the viremic group were 3.87±0.65 (Log10 copies/ml) ranging from 3.13 to 5.52 (Figure 4A). In the non-viremic group, the VLs were usually below the detection threshold of about 100 viral RNA copies/ml. On average, the CD4+ T cell counts in the non-viremic individuals (760.5±69.32 N=11) were 1.6-fold higher than those in the viremic individuals (452±77, N=19) (Figure 4B). The CD4+ T cell counts demonstrated a significant but imperfect inverse association with the viral RNA loads (Figure 4C). All of the non-viremic HIV-2-infected individuals, bar one (N2B9), had CD4 counts >500/µl (Table 2).

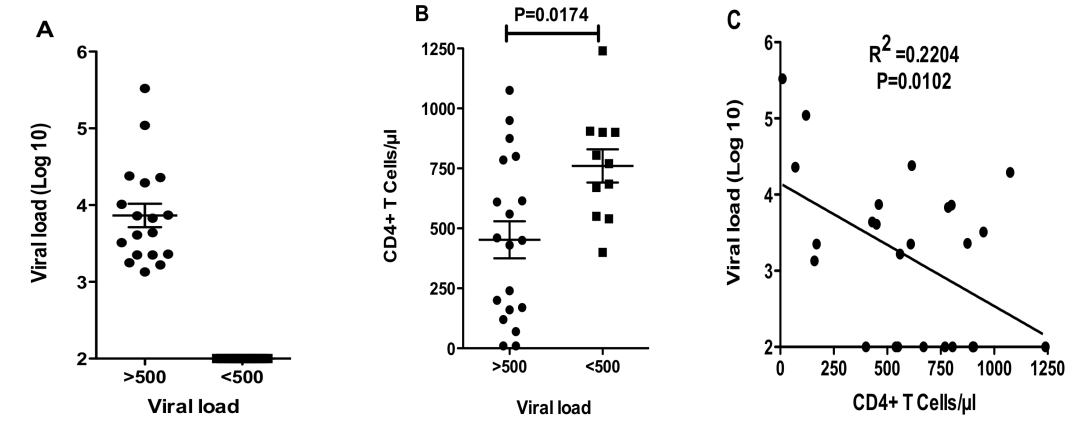

Figure 4: Viral loads and CD4+ T cell counts of study subjects. (A) Viral loads and **(B)** CD4+ T cell counts in HIV-2-infected individuals at the time of sampling for *nef* analysis. Horizontal bars represent average values in each group. (**C**) Pearson correlation between the Viral Loads and the CD4+ T cell counts.

Table 2: Samples of HIV-2-infected individuals examined

Subject	Sex	Age[a]	CD4[b]	PVL[c]	Group[d]	R5/X4[e]	Group	References
N2A4	Female	83	n.k.	bql	non-viremic	n.k.	A	25, 108
N2A7	Female	72	n.k.	bql	non-viremic	n.k.	A	25, 108
N2A8	Female	67	n.k.	bql	non-viremic	n.k.	A	25, 108
N2A9[f]	Female	71	685	bql	non-viremic	n.k.	A	25, 108
N2B7	Female	38	n.k.	bql	non-viremic	n.k.	A	25, 108
N2B9	Female	33	400	bql	non-viremic	n.k.	A	25, 108
N2C6	Female	41	805	bql	non-viremic	n.k.	A	25, 108
N2D7	Male	60	n.k.	bql	non-viremic	n.k.	A	25, 108
N2D8	Female	49	905	bql	non-viremic	n.k.	A	25, 108
N2E10	Female	60	1240	bql	non-viremic	n.k.	A	25, 108
N2F8	Female	79	540	bql	non-viremic	n.k.	A	25, 108
RH2-3[f]	n.k.	n.k.	770	bql	non-viremic	R5	A	10, 11, 104, 105
RH2-8	n.k.	n.k.	900	bql	non-viremic	R5	B	10, 11, 104, 105
RH2-13[f]	n.k.	n.k.	900	bql	non-viremic	R5	A	10, 11, 104, 105
RH2-14[f]	n.k.	n.k.	550	bql	non-viremic	R5	A	10, 11, 104, 105
RH2-22[f]	n.k.	n.k.	670	bql	non-viremic	R5	B	10, 11, 104, 105
P2A12	Female	83	615	24002	viremic	n.k.	A	25, 108
P2B5	Female	59	610	2247	viremic	n.k.	A	25, 108
P2C11	Female	63	460	7456	viremic	n.k.	A	25, 108
P2C12[f]	Female	74	160	1350	viremic	n.k.	A	25, 108
P2C5	Female	59	170	2259	viremic	n.k.	A	25, 108
P2C9	Female	75	875	2298	viremic	n.k.	A	25, 108
P2E5	Female	70	n.k.	1781	viremic	n.k.	A	25, 108
P2E6	Male	57	800	7260	viremic	n.k.	A	25, 108
P2E9[f]	Male	36	450	4111	viremic	n.k.	A	25, 108
P2F10	Female	33	1075	19576	viremic	n.k.	A	25, 108
P2F12	Female	38	785	6792	viremic	n.k.	A	25, 108
P2F4	Male	43	560	1655	viremic	n.k.	A	25, 108
P2F5	Male	52	430	4366	viremic	n.k.	A	25, 108
P2F7[f]	Female	74	n.k.	10129	viremic	n.k.	A	25, 108
P2G8	Female	59	950	3212	viremic	n.k.	A	25, 108
RH2-1[f]	n.k.	n.k.	240	>500	viremic	R5	A	10, 11, 104, 105
RH2-5[f]	n.k.	n.k.	120	110000	viremic	R5	A	10, 11, 104, 105
RH2-7[f]	n.k.	n.k.	10	>500	viremic	R5	A	10, 11, 104, 105
RH2-24	n.k.	n.k.	70	23000	viremic	R5/X4	A	10, 11, 104, 105
RH2-26[f]	n.k.	n.k.	10	330000	viremic	R5/X4	B	10, 11, 104, 105
PH2-1[g]	n.k.	n.k.	200	>500	viremic	n.k.	A	10, 11, 104, 105

[a]Age at the time of sampling. [b]Absolute CD4 T cell counts per µl blood. [c]Copies of plasma viral RNA/milliliter. [d]Grouped based on Viral Loads. [e]R5/X4 co-receptor usage was reported (104, 105). [f]Two and [g] three viral clones were derived from infected individuals respectively. Abbreviations: n.k., not known; bql, below quantifiable limit.

In comparison, the CD4 counts in viremic HIV-2 infected individuals varied

considerably from 10/µl to 1075/µl and 11 out of 19 individuals had CD4 counts <500/µl (Figure 4B). Notably, the relative old age of most HIV-2 infected subjects at the time of sample collection (mean age 58.95±16.1 in the viremic and 59.45±17.0 in the non-viremic cluster) probably contributed to lower levels of CD4+ T cells. In accordance to published data (25, 89), these results suggest that a considerable number of HIV-2-infected subjects do not progress immunodeficiency disease since they efficiently control the virus.

3.2 Proviral DNA constructs expressing *nef* alleles derived from HIV-2 infected individuals

To address the genetic composition of the 50 HIV-2 *nef* alleles derived from 37 infected individuals (16 non-viremic and 21 viremic), they were sequenced and analyzed phylogenetically with reference to HIV-2 group A, B, AB and SIVmac 239 *nef* alleles. Patient specific clusters based on these Nef amino acid sequences verified that the *nef* alleles were from different HIV-2-infected individuals (Figure 5). With the exception of three patients (RH2-8; RH2-22 and RH2-26) who were infected with group B viruses, all individuals were infected with HIV-2 group A strains (Figure 5; Table 2). When I aligned the deduced amino acid sequences of these HIV-2 Nefs, I found that though there were huge variations in the sequences of these Nefs, but several domains and putative protein interaction sites relevant for HIV-1 Nef functions (30) were maintained and conserved in HIV-2 Nefs e.g., the N-terminal myristoylation signal, the acidic region, a diarginine motif, a diacidic putative V1H binding site and C-proximal adaptor-protein interaction site (Figure 6). Interestingly, the central P(xxP)3 motif which interacts with cellular kinases, exhibited wide inter-sample variation. These data indicate that distinct differences in Nef sequences rather than gross tertiary structural changes are responsible for variation in specific functions, which may play role in disease outcome.

Notably, all *nef* genes analyzed contained intact reading frames that predicted full-length Nef protein sequences, which is in contrast to a previous study (98).

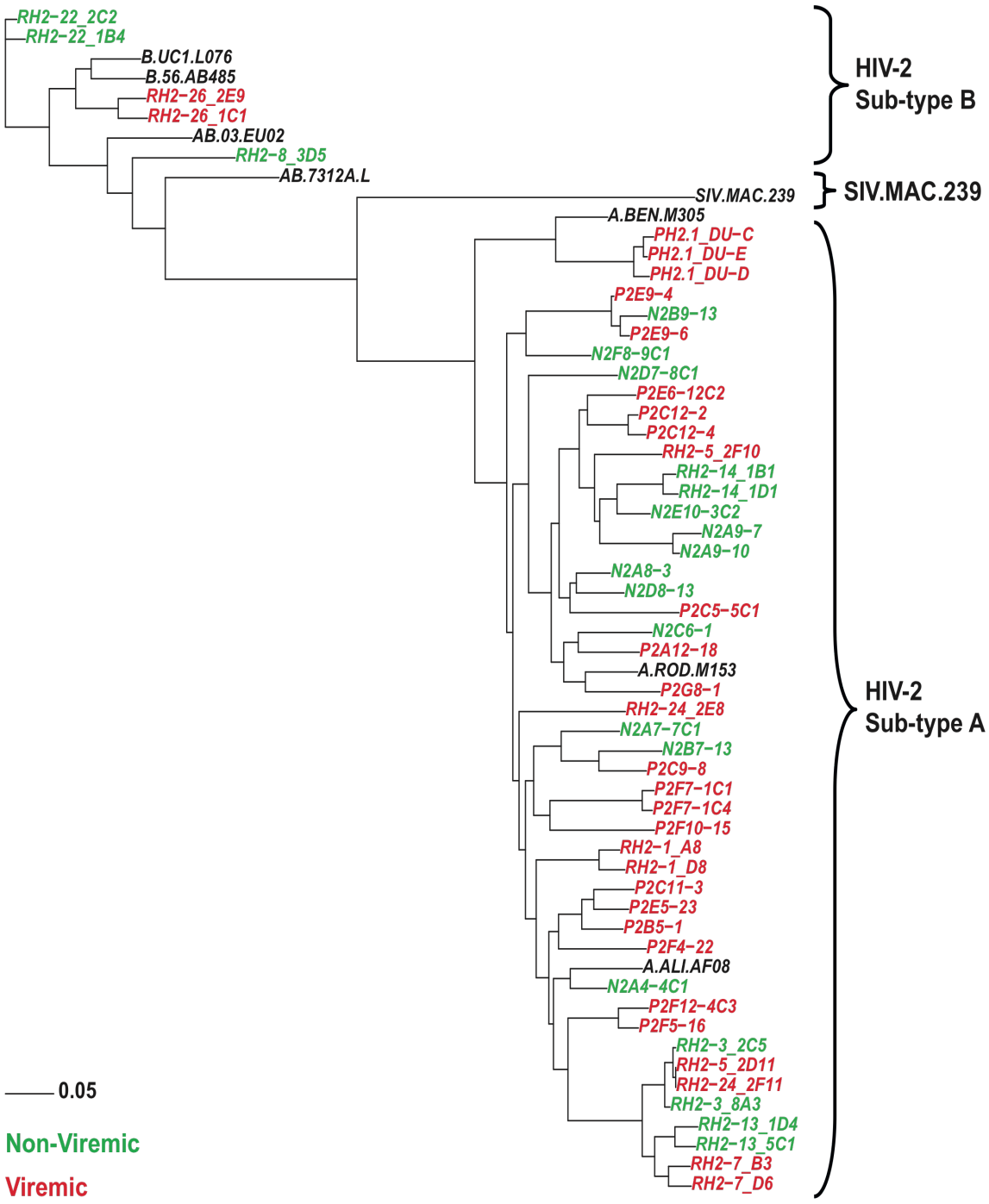

Figure 5: Evolutionary relationships among HIV-2 Nef sequences. The phylogenetic tree of deduced amino acid sequences constructed using the minimum evolution criterion as implemented in PHYML v2.4.4 (34). Bootstrap node support was calculated from 10,000 replicates. Black is representing reference; green is representing *nef* alleles derived from non-viremic and red is representing *nef* alleles from viremic HIV-2 infected individuals.

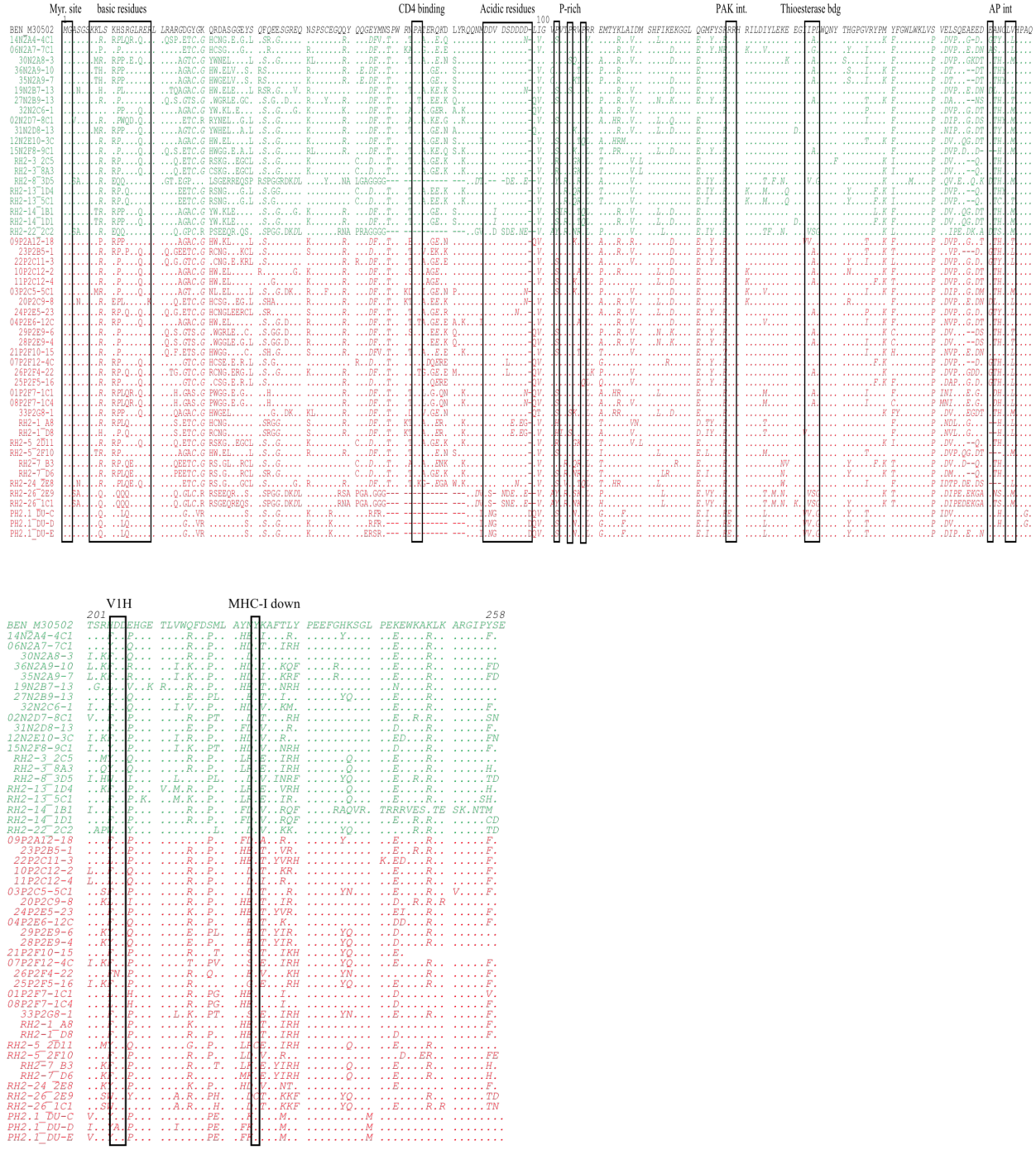

Figure 6: Alignment of HIV-2 Nef Sequences. The HIV-2 BEN sequence is shown for reference. Some conserved sequence elements in Nef, including the N-terminal myristoylation signal, PA residues known to be critical for CD4 down-modulation by 239wt Nef, the acidic and proline rich regions, a diarginine motif, a C-proximal adaptor protein (AP) interaction site, a diacidic putative V1H binding site and a Y residue involved in MHC-1 down-regulation (29, 92) are indicated schematically. Dots indicate identity with the BEN Nef sequence and dashes indicate gaps introduced to optimize the alignment.

To study the role of Nef functions in HIV-2 infection, I cloned all 50 *nef* alleles derived from 37 HIV-2-infected individuals; 16 non-viremic (<500 RNA copies/ml) and 21 viremic (>500/ml) into an HIV-1 NL4-3-based IRES-eGFP proviral vector co-expressing Nef and eGFP from bicistronic RNAs (Figure 7A) (82, 83). The rational for using HIV-1 backbone was that the *env* and *nef* do not overlap in the genome so keeping the Env intact we could analyzes the role of Nef in our assays, while in case of HIV-2 backbone, *env* and *nef* overlap which would make analyzing Nef more difficult (Fig 2). Besides that, HIV-1 is better characterized in *in vitro* assays than HIV-2.

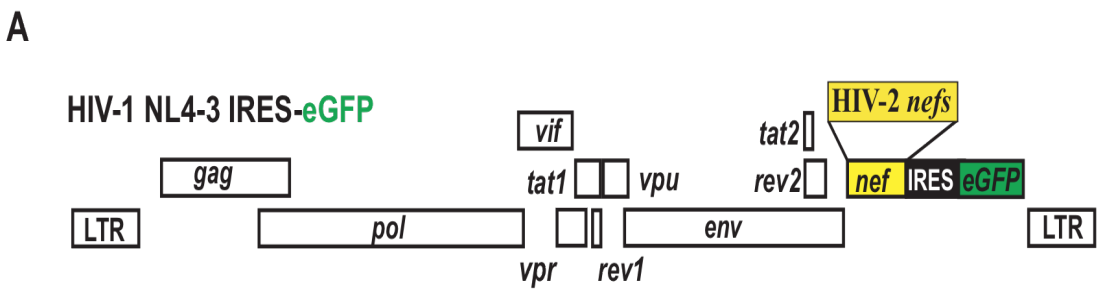

Figure 7: (A) Schematic representation of proviral HIV-1 IRES-eGFP construct encoding HIV-2 *nef* allele.

3.3 HIV-2 Nefs modulate cellular receptors

To determine the abilities of HIV-2 Nefs from individuals with different VLs for interfering with the surface expression of CD3, CD4, CD28 and MHC-I, human peripheral blood mononuclear cells (PBMCs) were transduced with HIV-1 expressing different HIV-2 Nefs and analyzed by flow cytometry. All proviral clones containing *nef* alleles (n=50) derived from the HIV-2 infected individual in the Guinea-Bissau cohort and the Rotterdam cohort, as described earlier, were co-transfected with vesicular stomatitis virus *envelope* (VSV-G) to HEK-293T cells, and pseudo typed HIV-1 virions with HIV-2 *nef* were produced. HIV-1 virions pseudo-typed with the VSV-G envelope protein were used to transduce the cells with comparable efficiency independent of their Nef coding regions.

My results revealed that the *nef* alleles from viremic and non-viremic individuals all code for a functionally active Nef proteins, which modulated the various surface receptors analyzed, albeit with variable efficiency (Figure 7B-E). The effect of Nef on CD4 down-

modulation (hVL: 2.5±0.5; lVL: 2.6±0.6; values give average of n-fold down-modulation ±SD) was comparatively low because the Vpu and Env expression, present in HIV-1 proviral constructs, also contributed to reduce CD4 cell surface expression (59). Most of the HIV-2 *nef* alleles from both the groups down-modulated TCR-CD3 efficiently (hVL: 6.1±2.5; lVL: 5.8±1.9) from the cell surface (Figure 7C), while the effects on CD28 (hVL: 3.6±1.4; lVL: 3.8±2.0) and MHC-I (hVL: 3.0±0.8; lVL: 3.1±0.8) were modest. HIV-1 and 2 Nefs have many common features of modulation of various surface receptors but their amino acid sequences have got huge variations. Most of the HIV-2 Nefs derived from non-viremic and viremic HIV-2 infected individuals were fully active in down-modulating CD4, CD3, CD28 and MHC-I although they have genetic variations (deletions or point mutations) in domains known to be important for HIV-1 Nef function. In comparison, two HIV-2 Nefs (RH2-5_2D11 and RH2-26_2E9) that contain Y to C mutations at position 234, in an otherwise highly conserved C-terminal Tyr residue in SIVmac239 Nef that is crucial for MHC-I down-modulation (70, 96), were inactive in modulating MHC-I (Figure 6). Most importantly, *nef* alleles derived from viremic and non-viremic HIV-2-infected subjects down-modulated CD4, CD3, CD28 and MHC-I surface receptors with similar efficiency and did not differ significantly.

The primate lentivirus Nef may also modulate MHC class II antigen presentation by reducing the mature MHCII and up-regulating the cell surface expression of the immature MHCII (invariant chain-Ii) (85, 93). To establish the role of HIV-2 Nefs in MHC class II antigen presentation, I used the human monocytic leukemia THP-1 cell line, which still maintains several characteristics of monocyte-derived macrophages and expresses high levels of MHCII (102). I found that HIV-2 Nefs up-modulated invariant chain-Ii surface expression with efficiencies ranging from 1.2-to 10.0-fold (Figure 7F). I did not find remarkable differences in the up modulation of cell surface invariant chain-Ii by Nefs derived form viremic and non-viremic HIV-2 infected individuals, notably, *nef* alleles from viremic HIV-2 infected individuals were slightly more active (4.7±0.4) than those from non-viremic individuals (3.7±0.4) but this difference was essentially due to two unusually active *nef* alleles in the high VL group and yet the variation was not significant (p=0.08).

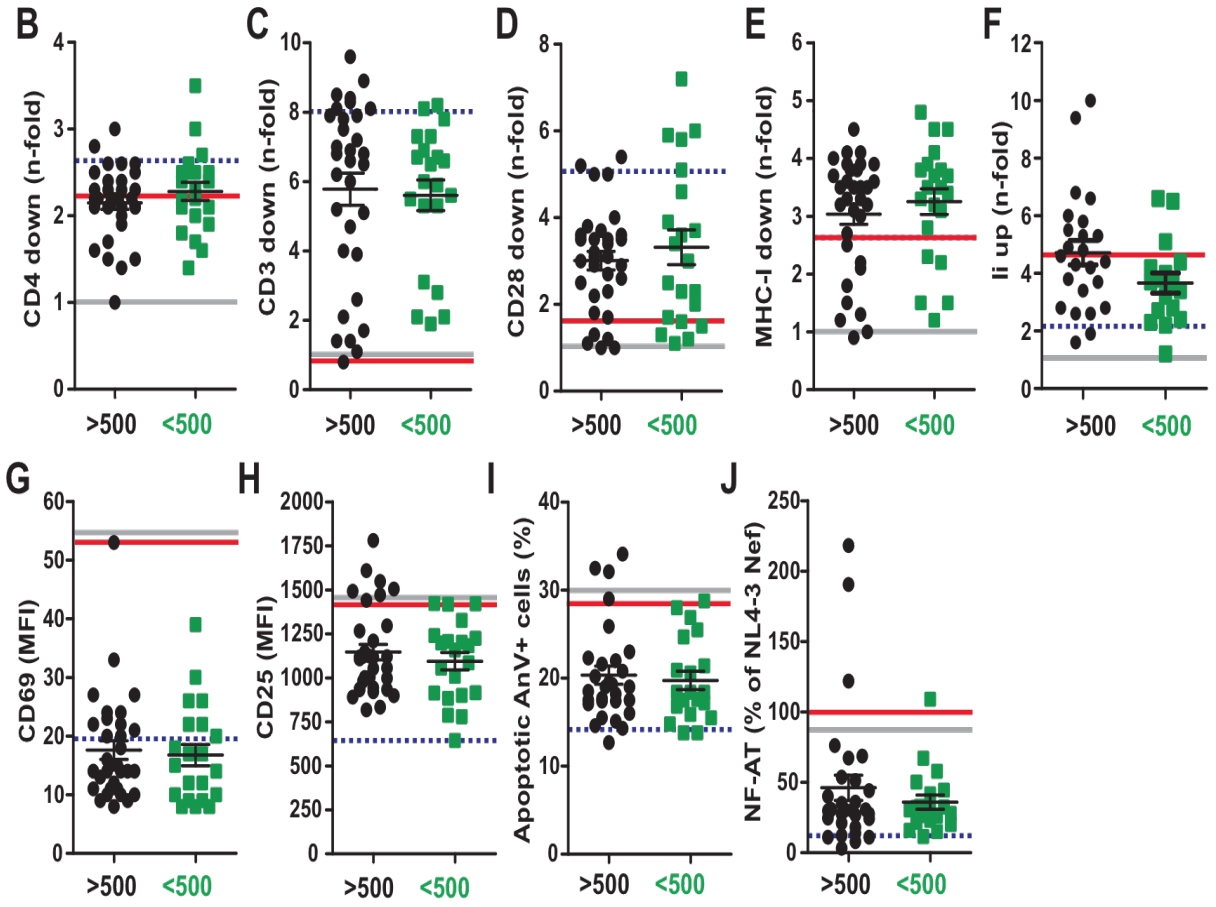

Figure 7: Functional characterization of HIV-2 *nef* alleles. (B to E) Quantification of ability of Nef in down-modulation of CD4 (B), CD3 (C), CD28 (D) and MHC-I (E) on human PBMCs infected with HIV-1 *nef/eGFP* constructs. HIV-2 *nef* genes were grouped on Viral Loads. Each symbol represents the n-fold down-modulation of the mentioned receptor molecule. (F) The potency of HIV-2 *nef* alleles in up modulating Ii (CD74) surface expression on THP-1 cells. (G to I) Surface expression of CD69 (G), CD25 (H), and levels of apoptotic cells (I) in PBMCs culture transduced with HIV-1 *nef/eGFP* constructs. (J) Suppression of induction of NFAT in CD4 T-cell cultures transduced with viral constructs expressing HIV-2 *nef* alleles. The results obtained for the HIV-1 *nef/eGFP* control constructs are indicated by lines: red, NL4-3 nef; broken blue, HIV-2 BEN nef and grey, disrupted *nef* gene. Data points in panels B to J represent average of triplicate experiments and horizontal bars represent average activities in that group.

3.4 Most HIV-2 Nefs suppress T-cell activation and apoptosis

It has already been published that HIV-2 Nefs prevent the responsiveness of virally infected T cells to activation by down-modulation of TCR-CD3 (69, 83). To investigate if the diverse viral loads in HIV-2 infected subjects are linked with differential activation status of *in vitro* HIV transduced PBMCs with *nef* alleles derived from the respective individuals, I examined the surface expression levels of early (CD69) and late (IL-2 receptor-α chain, IL-2Rα or CD25) activation markers in T cell activation. I discovered that all 50 HIV-2 *nef* alleles (except one) reduced the induction of CD69 expression (Figure 7G). Likewise, most of the HIV-2 Nefs also inhibited induction of late activation (IL-2R expression) and activation-induced cell death (Figure 7H and I). The nuclear factor of activated T cells (NFAT) regulates transcription of IL-2 gene expression, a hallmark of T cell activation. It has been established that NFAT activation is suppressed by HIV-2 Nef that down modulate TCR-CD3 (83). To assess whether *nef* alleles from the non-viremic and viremic HIV-2 infected individuals affect NFAT induction differentially in infected cells, I transduced Jurkat T cells stably transfected with the luciferase gene under the control of an NFAT-dependent promoter (26). I found that the majority of *nef* alleles inhibited NFAT activation (Figure 7J) with the exception of a few *nef* alleles (n=4).

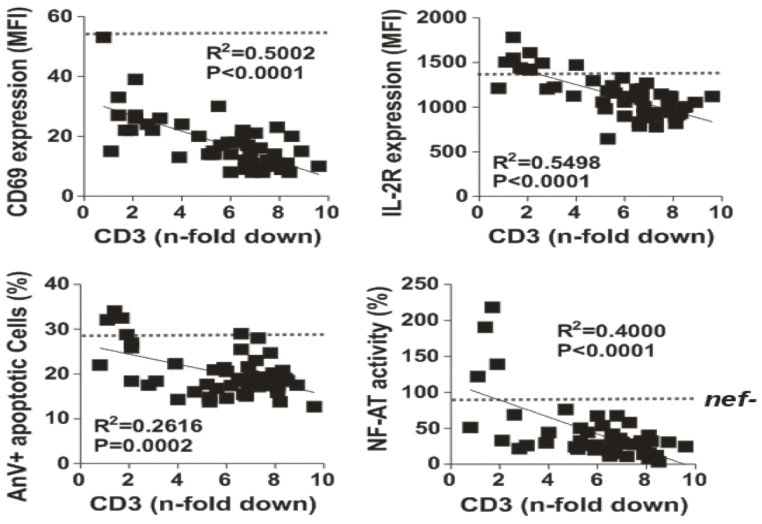

Figure 8: Down-modulation of TCR-CD3 by HIV-2 Nefs correlates with suppression of T cell activation and programmed death. Pearson correlation between the surface expression of CD69 and IL-2R (CD25) on HIV-1 transduced human PBMCs, the percentage of apoptotic cells, or the levels of NFAT-driven luciferase gene activity and the efficiency of TCR-CD3 down-modulation by the corresponding HIV-2 *nef* alleles. Values in panels represent average of triplicate experiments.

In general, Nefs derived from HIV-2 infected viremic and non-viremic individuals had similar effects in their ability to inhibit T-cell activation and apoptosis. However, this is in accordance with the published studies (5, 69, 83), suppression of early and late activation and NFAT expression and apoptosis correlated significantly with one another as well as with the ability of TCR-CD3 down-modulation (Figure 8). Therefore, we observed that down-modulation of TCR-CD3 and CD28 (key ligand and co-stimulatory factor of T cell activation) by HIV-2 Nefs suppress the responsiveness of virally infected T cells to stimulation and induction of programmed cell death irrespective of viral loads in HIV-2 infected subjects.

3.5 HIV-2 *Nef*-alleles derived from viremic individuals are efficient in promoting viral spread

Nef was named "negative" factor but later proved to be positive for the virus because of its role in facilitating viral spread by promoting viral immune evasion and also by enhancing virion infectivity and by promoting viral replication in primary cells and thus critical for high viral loads (15, 66, 68, 92). To investigate whether efficient replication of HIV-2 *in vivo* is associated with an increased capability of Nef to increase virion infectivity, I infected a HeLa-CD4/LTR-lacZ indicator cell line, P4-CCR5 with HEK-293T cell-derived virus stocks normalized for their p24 antigen, and established the β-galactosidase activities two days post infection. Most HIV-2 *nef* alleles enhanced virion infectivity (with two exceptions) but with varying efficiencies. Notably, HIV-2 Nefs are less efficient than HIV-1 Nefs in enhancing the HIV infectivity (Figure 9A). We did not find significant difference in promoting the virion infectivity by Nef derived from viremic and non-viremic HIV-2-infected individuals (Figure 9A).

Subsequently, I analyzed whether *nef* alleles from non-viremic and viremic HIV-2-infected individuals differ in their ability to enhance viral spread in infected PBMC cultures. The HIV-1 NL4-3 *nef/eGFP* (83) constructs I used to generate the virus in my experiments, are replication competent and that allowed us to measure the virally infected PBMCs by counting eGFP+ cells using flow cytometrically. I discovered that the viruses generated with proviral constructs containing intact *nef* alleles spread with considerably higher efficiency than their disrupted *nef* counterparts (Figure 9B). Interestingly, *nef* alleles derived from viremic HIV-2-infected individuals were more efficient in promoting viral spread in PBMCs

than those derived from non-viremic HIV-2-infected subjects (Figure 9B) which has been shown significant in Figure 9C. I determined the mean collective number of HIV-1-infected eGFP+ cells for the whole set of 50 proviral constructs. Notably, we discovered that viral spread was significantly promoted more by the Nefs derived from the viremic HIV-2 infected individuals compared to their non-viremic counterparts (186.1±11.7 versus 143.1±13.3; P = 0.02) (Figure 9C). I used TZM-bl indicator cells for mirroring the effect of Nefs in enhancing virion infectivity. I found HIV-2 Nefs were less effective than HIV-1 Nefs enhancing virion infectivity contrary to enhancing the viral spread measured by counting the number of virally infected GFP+ PBMCs (Figure 9). The T cells expressing HIV-1 Nefs show higher levels of apoptosis than those expressing HIV-2 Nefs (69, 83) and that may be the possible explanation for this finding.

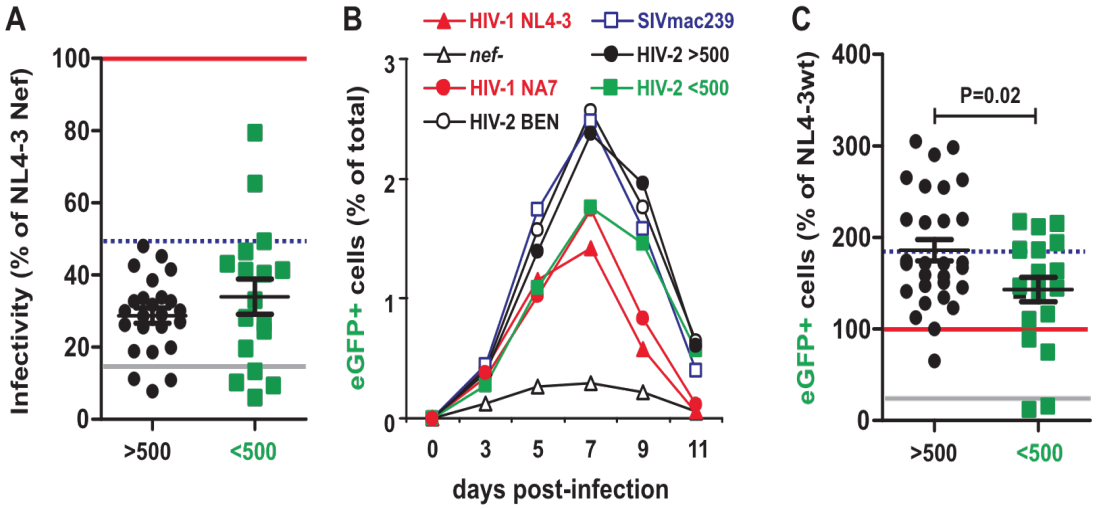

Figure 9: HIV-2 Nef-mediated enhancement of viral infectivity and spread in PBMCs. (A) Viral infectivity enhancement by HIV-2 *nef* alleles. TZM-bl reporter cells were infected with HIV-1 *nef/eGFP* constructs with virus stocks having 1 ng p24 antigen. Mean values of triplicate experiments for each HIV-2 *nef* allele are shown and compared to the infectivity of the virus expressing the NL4-3 Nef (100%). (B) Percentages of virally infected GFP+ cell levels detected in PBMCs derived from three different donors, infected with HIV-1 *nef/eGFP* constructs from each group. The average values for the entire group of HIV-2 *nef* alleles (viremic, n = 23; non-viremic, n = 22) are shown (C) The average cumulative numbers of HIV-1-infected GFP+ cells were estimated for each of the 50 proviral constructs. Average of triplicate experiments values are shown relative to the proviral HIV-1 NL4-3 Nef (*nef+)* construct (100%).

3.6 HIV-2 Nefs do not counteract human tetherin to allow virion release

Tetherin (CD317/BST-2), an interferon-induced membrane protein, restricts the release of newly formed virions from infected cell surfaces (72). While HIV-1 encodes Vpu to counteract tetherin, SIVsmm, the progenitor of both HIV-2 and SIVmac, do not encode a Vpu protein (54). The Nef protein of SIVsmm antagonizes Sooty Mangabeys tetherin was shown in earlier studies (110). To analyze the ability of HIV-2 Nef to counteract human tetherin, HEK-293T cells were co-transfected with human tetherin and *nef* alleles derived from viremic and non-viremic HIV-2 infected individuals. Culture supernatants were harvested 24 hours later and TZM-bl indicator cells were infected with 50µl of supernatants. Infectious virion release was determined by quantifying β-galactosidase activity 48 hrs post infection. In my experiments, I did not find human anti-tetherin activity in HIV-2 Nefs (Figure 10) while these Nefs significantly counteracted Sooty Mangabeys and Rhesus Macaques tetherin. This is in accordance with the report suggested that the HIV-2 Env was responsible for antagonizing human tetherin (39, 100). Anti-tetherin activity of Nefs for Sooty Mangabeys and Rhesus Macaque tetherins indicating HIV-2 still carry some characteristics of its original precursor.

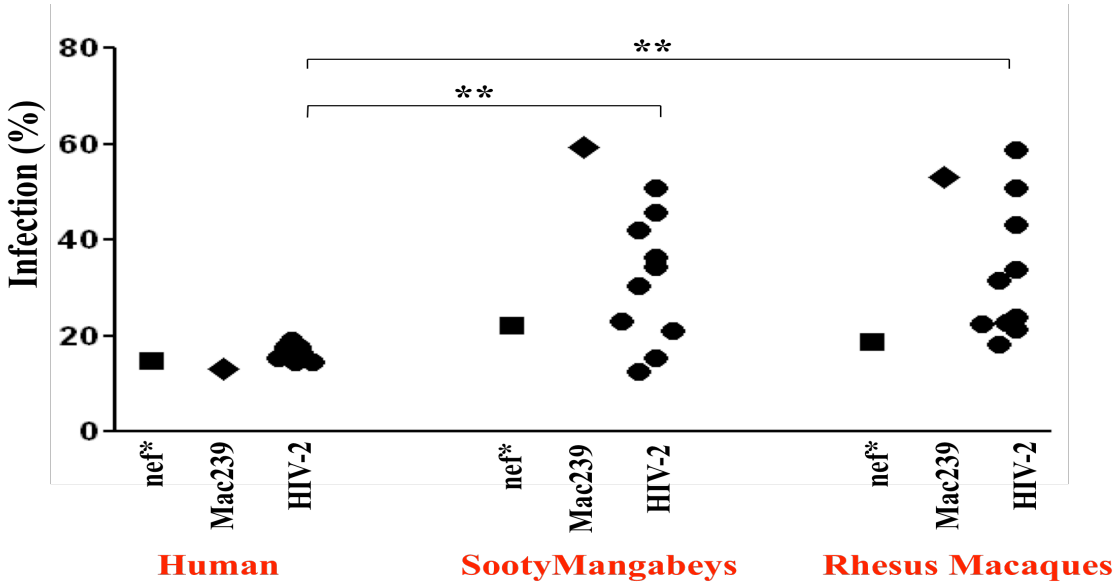

Figure 10: HIV-2 Nef did not counteract human tetherin. Virion release from HEK-293T cells following co-transfection of various tetherins (indicated red) with plasmids expressing HIV-2 Nefs from viremic and non-viremic groups. The *nef** and Mac239 *nef* expression vectors were used as controls. Releases of infectious virions were mirrored in TZM-bl indicator cells. To normalize, the percentage of counteraction was set to 100% in the absence of tetherin.

3.7 Efficient Nef-mediated down-modulation of TCR-CD3 and CD28 correlates with high CD4+ T cell counts in viremic HIV-2-infected individuals

Next, I wanted to investigate any of the examined Nef functions are linked with CD4+ T cell counts in HIV-2-infected individuals. Interestingly, my analyzes revealed that the ability of Nef to down modulate TCR-CD3 correlated significantly with higher numbers of CD4+ T cell counts in viremic HIV-2-infected individuals (R^2=0.2244; P=0.0109) but not in non-viremic HIV-2-infected individuals (Figure 11A).

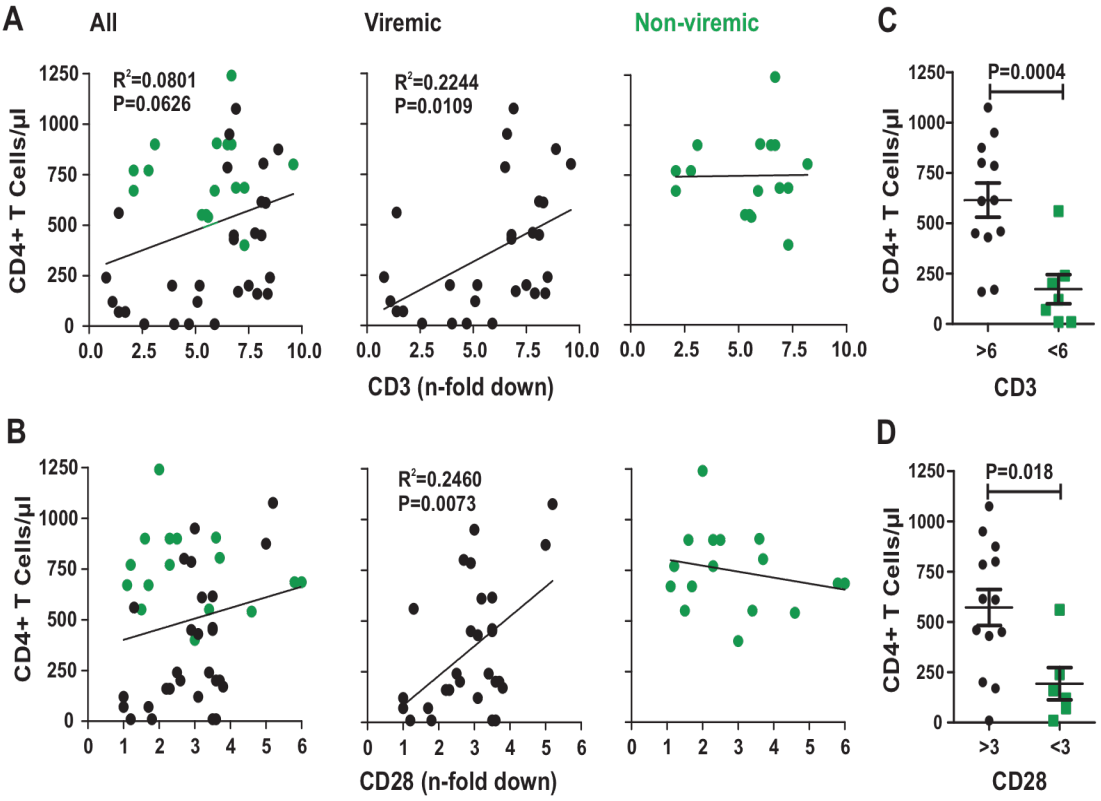

Figure 11: Correlation between Nef-mediated TCR-CD3 and CD28 down-modulation in virally infected PBMCs and the CD4+ T cell counts in HIV-2-infected individuals. Pearson correlation with CD4+ T-cell counts in HIV-2-infected individuals and n-fold down-modulation of (A) CD3 and (B) CD28. Assays were performed in human PBMCs and statistically analyzed using the PRISM 4:0 package. The data derived from viremic and non-viremic individuals were represented with black and green, respectively. (C, D) Evaluation of the CD4+ T-cell counts in viremic individuals infected with HIV-2 strains expressing *nef* alleles that were showing high or low activity *in vitro* down-modulation of (C) TCR-CD3 and (D) CD28. Average values ±SD and P values are indicated.

A similar correlation was also observed in Nef mediated CD28 down-modulation with CD4+ T cell counts in viremic individuals (R^2=0.2460; P=0.0073), as in TCR-CD3, however the correlation was not significant in non-viremic HIV-2 infected individuals. I did not find any correlation between CD4+ T cell counts and any other Nef functions investigated (Figure 11B).

To further explore the possible significance of TCR-CD3 and CD28 surface modulation by Nef in the immunological outcome of HIV-2 infection, I analyzed the CD4+ T cell counts in individuals infected with HIV-2 strains and the activity of respective Nefs in these two functions. This analysis suggested a relevance in the ability of Nef to down module TCR-CD3 and CD28 with CD4+ T cell counts in infected individuals; the Nefs derived from viremic individuals that efficiently down-modulate TCR-CD3 (>6-fold) had 3.6-fold higher CD4+ T cell counts than those infected with viruses that showed only modest (<6-fold) activity in this Nef function (615±85 N=12 vs 173±73 N=7; p=0.0025) (Figure 11C). Likewise, the CD4+ T cell counts were significantly higher in individuals infected with HIV-2 strains that effectively (>3-fold) down-regulated CD28 (542±88, N=14; 193±80; N=6) (Figure 11D). I did not observe any such correlations in non-viremic individuals or for any other Nef functions.

3.8 A single I132T substitution selectively disrupts HIV-2 Nef-mediated down-modulation of TCR-CD3

Correlation analyses showed that Nef-mediated TCR-CD3 down-modulation efficiency correlated significantly with its ability to down-modulate CD4, CD28 and MHC-I but not with the up-modulation of Ii or enhancement of viral infectivity or viral replication (Figure 12A). Furthermore, we examined if the variations in sequences of these HIV-2 *nef* alleles might generate selectively defective phenotypes, as shown in Figure 6. Notably, I found some *nef* alleles were selectively impaired in modulating CD3, CD4, CD28, MHC-I or Ii (examples shown in Figure 12B), which is in agreement with the results of previous studies reporting that Nef functions are genetically separable but mediated by overlapping domains (1, 32, 43, 93).

One pair of *nef* alleles derived from viremic HIV-2 infected patient RH2-1, who showed low levels of CD4+ T cell counts (240/µl) at the time of sampling (Table 2), were exceptionally interesting because one of them was selectively defective in their capability to

down-modulate TCR-CD3 (Figure 13A) and consequently to modulate the responsiveness of T cells to stimulation (Figure 13B, C).

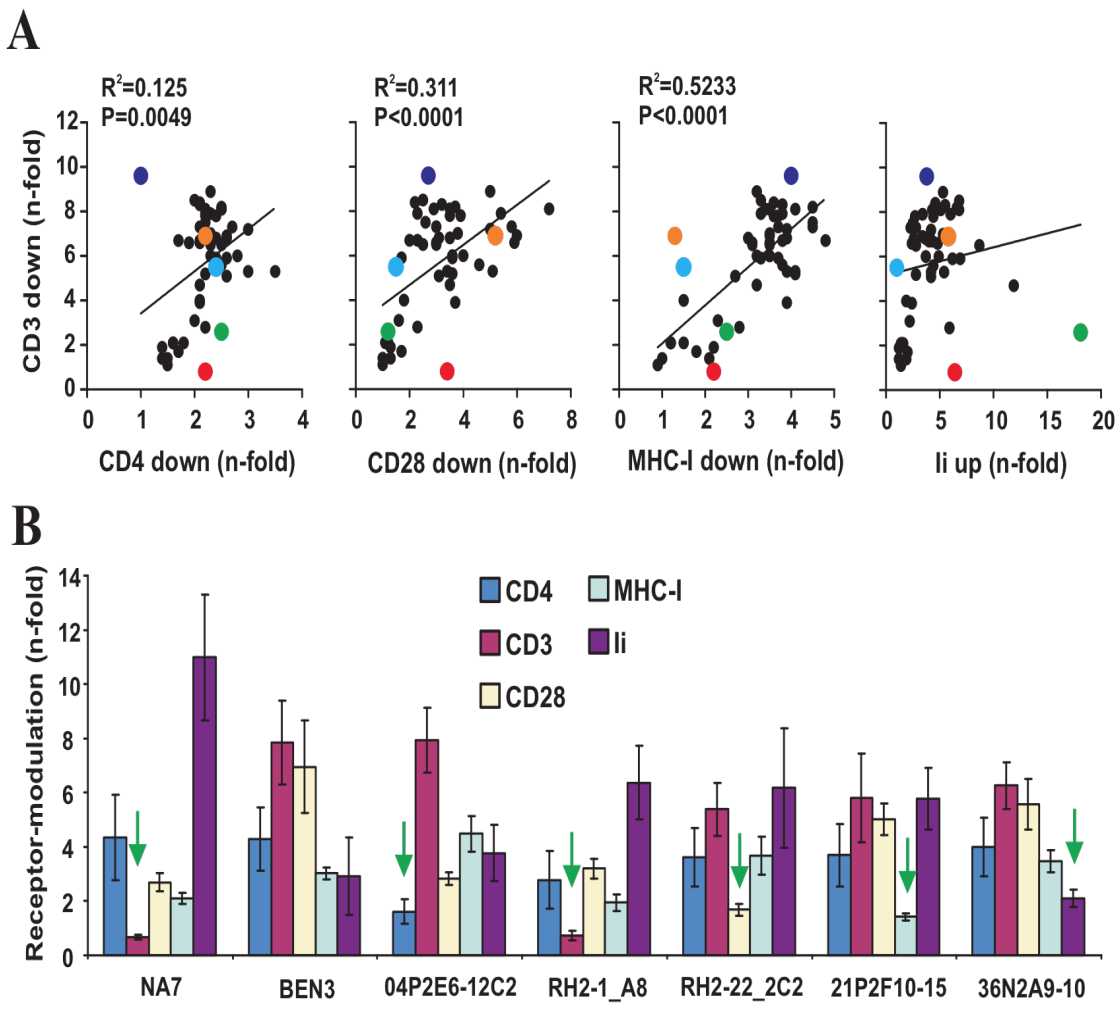

Figure 12: Identification of HIV-2 Nefs impaired in specific functions. (A) Pearson correlation analyses of n-fold down-modulation of TCR-CD3 by HIV-2 Nefs and surface modulation of CD4, CD28, MHC-I and Ii. The *nef* alleles selectively impaired in specific functions are represented with enlarge colored symbols: CD3 (red); CD4 (blue); CD28 (green); MHC-I (orange); Ii (light blue). (B) Representation of HIV-2 Nef selectively impaired in specific activities. The NA7 Nef representing HIV-1 is shown as a scale for comparison on PBMCs or THP-1 cells (Ii). Panel A and B represented average values of four independent experiments. Average values ±SD and P values are indicated.

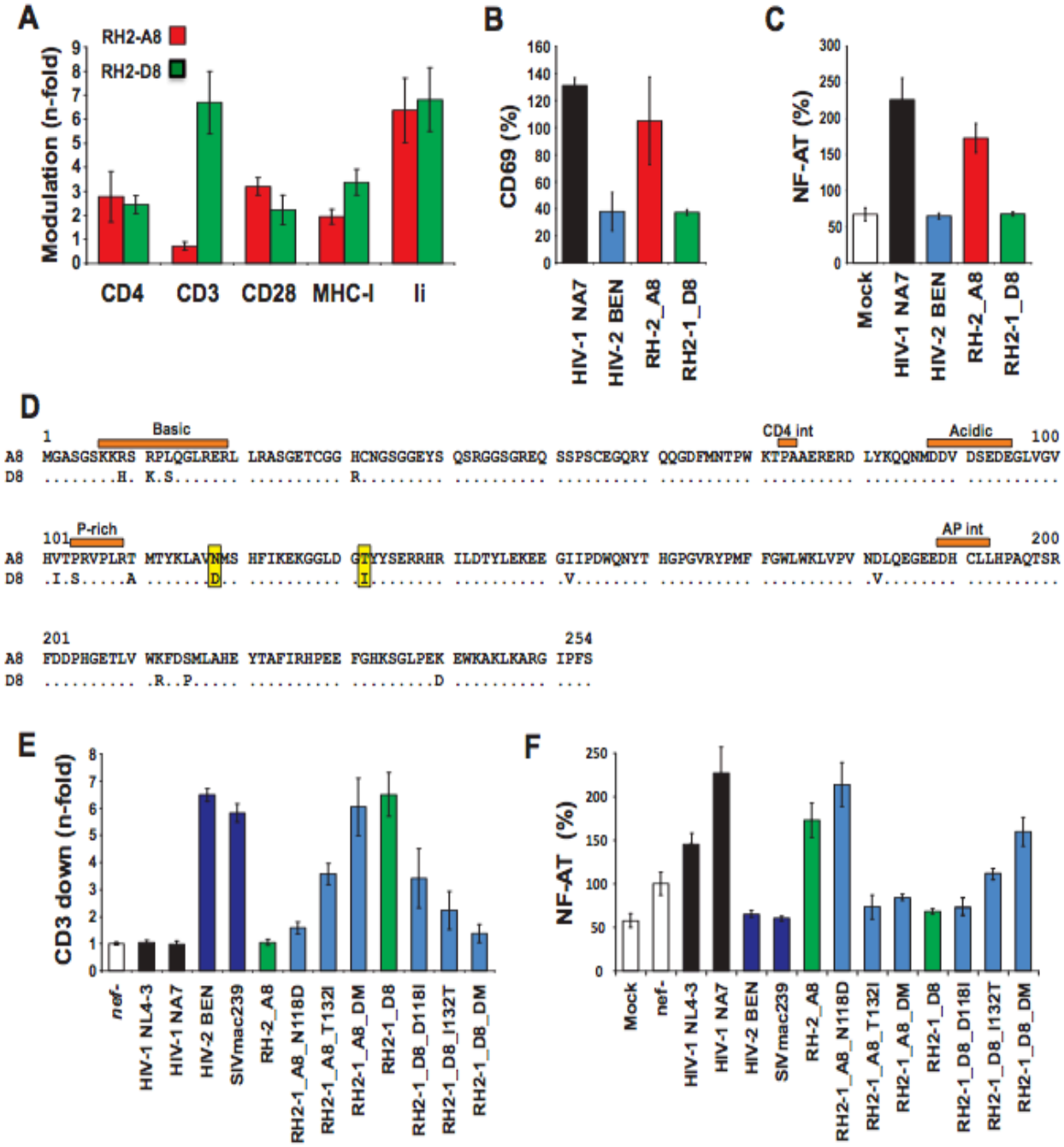

Figure 13: An I132T mutation in the core domain of HIV-2 Nef selectively disrupts down-modulation of TCR-CD3. (A-C) Analysis of functional ability of the RH2-1_A8 and RH2-1_D8 Nef proteins in modulating (A) various receptors, (B) early T cell activation and (C) NFAT activity. (D) Alignment of the RH2-1_A8 and RH2-1_D8 deduced amino acid sequences. (E) The RH2-1_A8 Nef is selectively impaired in TCR-CD3 down-modulation function which enhances the responsiveness of virally infected T cells to stimulation. (F) Analysis of Nef-mediated NFAT-activation in Jurkat cells following transduction with the indicated HIV-1 *nef/eGFP* constructs and subsequent stimulation with PHA. Panel A to C and E to F are showing average (±SD) of four independent experiments.

Assessment of the nucleotide and its deduced amino acid sequences of the RH2-1_A8 and RH2-1_D8 Nefs revealed a total of 32 variations in nucleotide and 14 variations at amino acid sequences, together with five in the conserved core domain, already reported to be important for Nef-mediated down-modulation of TCR-CD3 (81, 95)(Figure 13D). Site-directed mutational analyses showed that from Asparagine (N) to Aspartic acid (D) at position 118 (N118D) and Threonine (T) to Isoleucine (I) at position 132 (T132I) changes were enough to confer CD3 down-modulation activity to the inactive RH2-1_A8 Nef and the reverse changes disrupted the activity in the RH2-1_D8 Nef (Figure 13B).

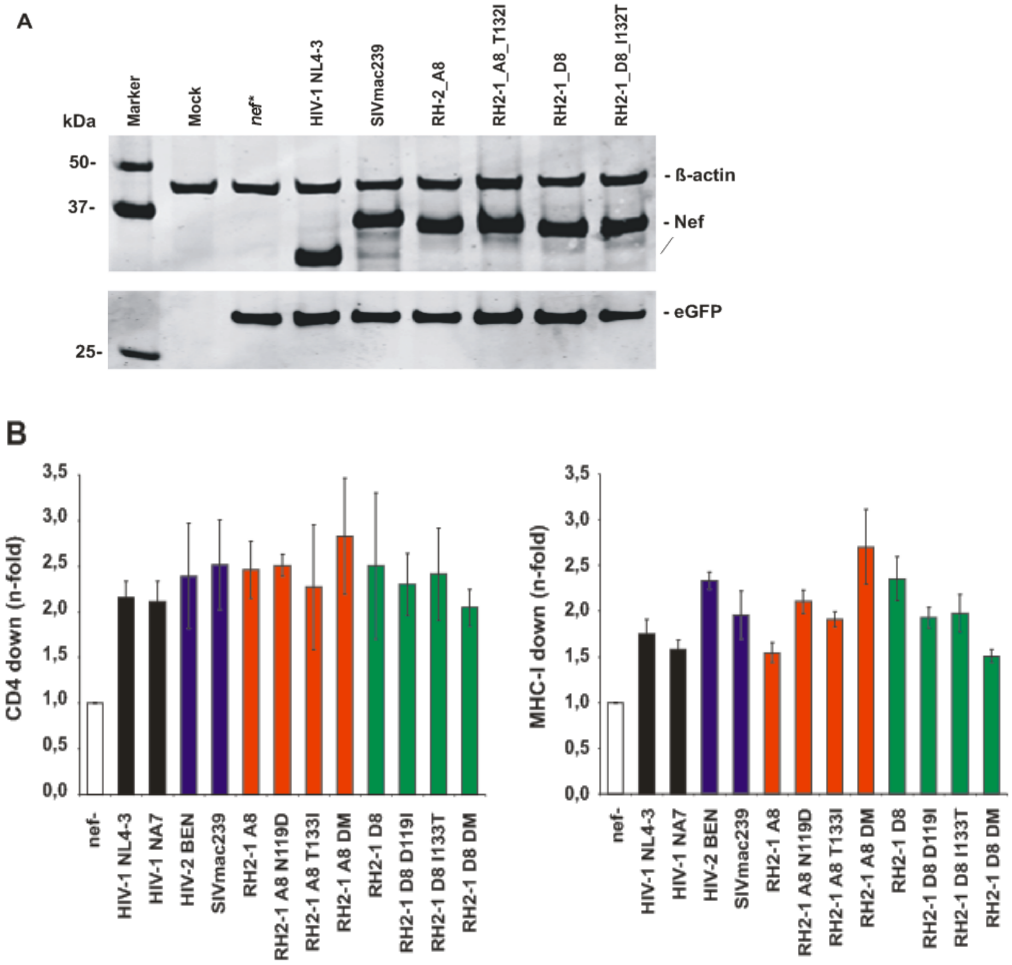

Figure 14: The T132I changes does not impair Nef expression. (A)Western blot analysis of HEK-293T cells transfected with pCGCG vectors containing HA-tagged *nef* alleles followed by an IRES element and GFP. (B) The N118D and T132I substitution mutations in RH2-1 A8 Nef and the reverse mutations in the RH2-1 D8 Nef do not affect down-modulation of CD4 and MHC-1 in PBMCs. Average values ±SD from four experiments are indicated.

Importantly, these two mutations also affected the influence of Nef on the responsiveness of virally infected cells to stimulation (Figure 13F) but had little if any effect on its cellular expression (Figure 14A) or other Nef activities, like modulation of CD4, CD28 and MHC-I (Figure 14B and data not shown).

These two, N118D and T132I substitution mutations affected the ability of Nef to down-modulate TCR-CD3 (Figure 13E) but because of the bigger impact of T132I, I investigated it in detail. Recently, Kim and colleagues have reported that the core domain of SIVmac239 Nef interacts with the cytoplasmic domain of the CD3 zeta chain (53). The crystal structure of Nef revealed that the Isoleucine residue at 132 position is located just at the bottom of a cavity that accommodates the cytoplasmic tail of CD3 zeta (53), suggesting that it may play a direct role in this interaction (52). To further address the role of Isoleucine, I investigated the effect of the I132T substitution on intra-cellular localization of a fusion between the cytoplasmic tail of TCR-CD3 and CD8 (60). In agreement with the FACS analysis, the I132T substitution in HIV-2 Nef was unable to down-modulate TCR-CD3, the control HIV-1 NL4-3 Nef did not co-localize with the CD8-CD3 fusion and did not significantly affect its sub-cellular localization (Figure 15, upper left). In contrast, the SIVmac239 Nef considerably co-localized with CD8-CD3-CT and re-localized it to the peri-nuclear space of the cells and to punctuate structures. Remarkably, single I132T mutation in RH2-1_D8 was enough to disrupt the co-localization with CD8-CD3 fusion and rearrangement in intracellular compartments, while the reciprocal in the RH2-1_A8 Nef had the reverse effect (Figure 15 and 16). These findings suggest a critical role of the I132 residue in HIV-2 Nef for interaction and internalization of TCR-CD3.

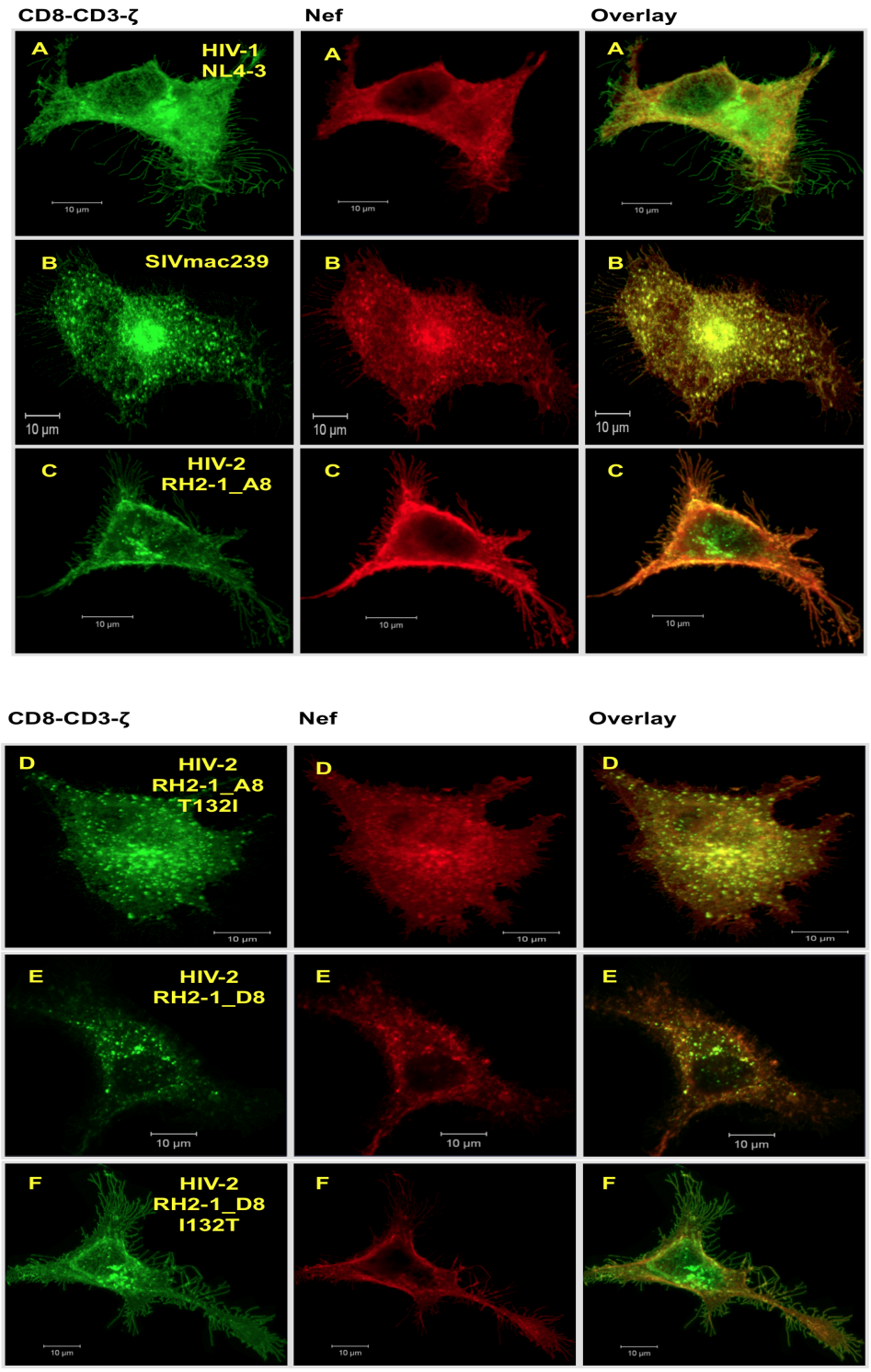

Figure 15: Effect of the T132I mutation in the HIV-2 Nef on sub-cellular localization of CD8-fusions with the cytoplasmic domain of the CD3 zeta chain.

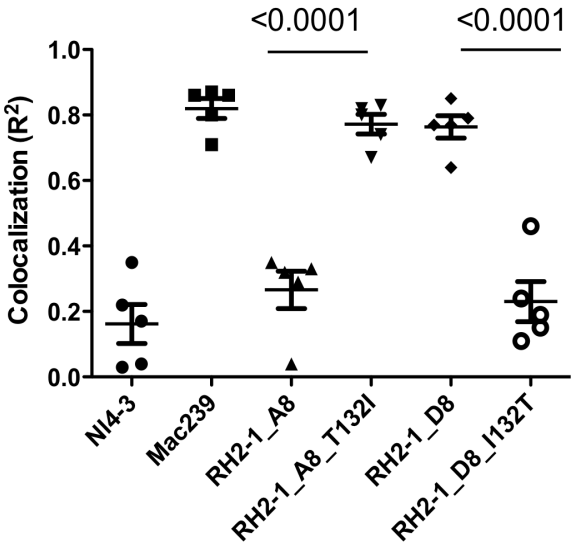

Figure 16: The T132I change affects the colocalization of HIV-2 Nef with CD8-fusions with the cytoplasmic domain of the CD3 zeta chain. The correlation coefficient (R^2) values, were generated from 5 different samples in each Nef variant using the ZEN-2010 software from the Carl Ziess, Germany.

Discussion

In my PhD thesis, I performed a detailed functional analysis of the role of the accessory Nef protein in the virological and immunological outcome of HIV-2 infection. I analyzed 50 *nef* alleles derived from viremic and non-viremic HIV-2 infected individuals. All these alleles were expressing functional Nef protein, without showing significant difference in modulating several surface receptors, NFAT-activation or viral infectivity. I discovered that the efficiency of Nef mediated down-modulation of TCR-CD3 from infected CD4+ T cells correlates with high CD4 counts in viremic HIV-2 infected individuals. I did not find a similar correlation in non-viremic subjects, pointing towards the fact that viral presence modulates host immune functions. Notably, Nef mediated down-modulation efficiency of CD28 from infected CD4+ T cells also correlates with high CD4 counts in viremic HIV-2 infected individuals and not in non-viremic infected individuals. My data most likely explain the apparent disagreement between two previous studies showing that the down-regulation of the T cell receptor by HIV-2 Nef does not protect against disease progression (24), although it correlated with high stable CD4+ T cell counts in natural SIV infection i.e., inability to down-modulate TCR-CD3 associated to loss of the CD4+ T cell (79). In addition, I found that some of the *nef* alleles included in my study were defective in specific function. Moreover, Isoleucine to Threonine substitution mutation was enough to create a Nef protein that was selectively defective in TCR-CD3 down-modulation function.

The majority of HIV-2 infected individuals do not develop disease and become long-term non-progressors with undetectable viral loads (25, 89). Contrary to this, SIVsmm-infection in sooty mangabeys leads to high levels of viral replication without development of disease (77). It is known that intact and functional *nef* genes are critical for efficient viral replication *in vivo,* while defective *nef* genes are associated with three to five orders of magnitude reduced viral loads in SIVmac-infected macaques (51). Defective *nef* gene has been also observed in several long-term survivors of HIV-1 infection with exceedingly low viral loads (21, 55). Thus, it was unexpected that *nef* alleles from viremic and non-viremic HIV-2-infected individuals analyzed in my PhD thesis did not differ significantly in modulating the surface expression of CD4, TCR-CD3, CD28, MHC-I and il (CD74), T-cell activation, programmed cell death and virion infectivity. In fact, all the HIV-2 *nef* alleles

included in this study were intact and functionally active in most assays performed, implying that these activities are beneficial for virus replication *in vivo* and help the HIV-2 to persist efficiently in the infected hosts. These results are contrary to previous study suggesting that defective *nef* alleles may be responsible for non-progressive HIV-2 infection (98). Truncation in Nef protein was either due to introduction of premature stop codon in *nef* ORF as a result of G to A hypermutation or gross deletion in the ORF. The deletion is caused by frame shift mutations that expressed totally different protein or sometimes introduced premature stop codons. Notably, most of these truncated *nef* alleles were derived from asymptomatic HIV-2 infected individuals having very low/undetectable viral load (98). The very low viremia makes PCR amplification prone to artifacts that may possibly explain mutation in Nef ORF that led the truncation in Nef protein. Truncation in Nef ORF was often given the reason for low viral load and lower transmissibility of the virus (21, 51, 55). The length of *nef* allele is being described by the PCR amplification using viremia (viral plasma or PBMCs) as template, but low viral load makes the amplification more prone to artifacts resulting in mutations. Therefore, one cannot deny the possibility of truncation as effect rather than cause. In fact, my data suggest that many HIV-2 infected individuals can effectively control the virus despite expression of fully functional *nef* alleles.

The *nef* alleles included in my PhD thesis were derived from 15 viremic and 11 non-viremic HIV-2 infected individuals. All the non-viremic individuals had stable and high CD4 counts (except N2B9) and very low/undetectable viral loads, or in other words, they were non-progressors. It is logical to assume that viruses may only affect the clinical and immunological outcome of HIV-2 infection when active viral replication takes place and viruses are present at detectable levels. Consequently, it is unpredictable that the only significant association between Nef function, observed in this study, and detectable levels of viremia in HIV-2-infected individuals was a modestly increased activity in promoting viral spread in PBMC cultures.

Among the Nef functions examined, I found that TCR-CD3 and CD28 down-modulation correlated significantly with high CD4+ T cell counts in viremic individuals, but such correlations were not observed in non-viremic HIV-2 infected individuals. It is quite convincing that HIV-2 Nef suppresses T cell activation by reduction in key ligand CD3 and co-stimulatory molecule CD28. In contrast to HIV-2 Nefs, HIV-1 Nefs generally lack the

CD3 down-modulation ability and have considerably lesser effect on CD28 cell surface down-modulation (69, 83). HIV-1, its simian precursor, SIVcpz (SIV that infects chimpanzee) and closely related *Cercopithecus* viruses (112) posses another accessory gene *vpu*. The Vpu protein antagonizes a host restriction factor called as tetherin (also named as BST2 or CD317) which tethers budding virions at cell surface (72, 73, 104). HIV-2 and its precursor, SIVsmm do not contain *vpu* gene, the Nef protein in these viruses down-modulates surface CD3, and therefore prevents activation of the CD4+ T cells. On the other hand, those viruses that are equipped with *vpu*, Nefs have lost the CD3 down-modulation function, consequently infected CD4+ T cells may get activated and undergo apoptosis. The acquisition of *vpu* in viral lineage that gave rise to HIV-1 during the course of evolution led the emergence of lentiviruses that can cause damagingly high levels of chronic immune activation. The Vpu protein counteracts the host tetherin induced in response to inflammation. The Vpu may help primate lentiviruses to cope with shortcoming of losing of Nef in CD3 down-modulation function (54).

Although, Nef-mediated TCR-CD3 down-modulation function of primate lentiviruses suppress the responsiveness of infected T cells to activation but this is not always enough to prevent the disease progression, especially in HIV-2 infected hosts, so the ability of Nef to down modulate TCR-CD3 as well as to some extent CD28, which is involved in co-stimulation, may usually diminish damaging immune activation and slow down the loss of CD4+ T cells and so the progression of immunodeficiency. My results support the hypothesis that Nef-mediated down-modulation of TCR-CD3 suppresses the activation of infected CD4+ T cells in viremic HIV-2 infected individuals. However, removal of TCR-CD3 and CD28 from the surface of infected CD4+ T cells may prolong the disease progression and decelerate the loss of CD4+ T cells, but suppression in activation of these infected cells do not protect the host from development of immunodeficiency. Many *nef* alleles, inefficient in down-modulating TCR-CD3, derived from non-viremic HIV-2 infected individuals, suggest that loss of viral replication is not directly associated with CD3 down-modulation function. Rather, significant correlation between efficient CD3 down-modulation and T cell activation as well as apoptosis indicates that CD3 down-modulation may delay the onset of AIDS in HIV-2 infection, but that does not preclude disease progression that differentiate the HIV-2 infection from SIVsmm infection to their natural hosts.

The HIV-2 Nef reduces expression of TCR-CD3 and CD28 on infected CD4+ T cells,

in addition to that the Nef down-modulate MHC-I surface expression which also contribute in suppression of activation induced CD4+ T cell killing. A significant correlation has been observed between efficient MHC-I down-modulation and stable high CD4+ T cell counts in SIVsmm (84). The protective role of Nef mediated MHC-I down-modulation has also been reported in HIV-1 infection where *nef* alleles from long-term non-progressors were efficient in down-modulating MHC-I but are otherwise defective (13, 99). Although I did not find a significant trend between Nef-mediated MHC-I down-modulation and high CD4+ T cell counts, but in my study, Nef mediated TCR-CD3 down-modulation correlated with MHC-I down-modulation function suggesting that HIV-2 Nef also suppresses MHC-I expression, as it does with TCR-CD3 expression in infected CD4+ T cells. Similarly, I have found a significant trend between Nef-mediated TCR-CD3 down-modulation and expression levels of early and late activation markers (CD69 and IL-2R) of the cells. I also observed a significant trend between Nef mediated TCR-CD3 down-modulation and apoptotic cells as well as NF-AT activation in infected CD4+ T cells. Furthermore, the results of my study showed that HIV-2 Nefs better control the expression levels of negative immune regulators, programmed death (PD)-1 (also known as CD279) and CTLA-4 on infected cells than HIV-1. The role of the PD-1 receptor in the pathogenesis of AIDS has been reported (19). The high levels of PD-1 receptor expression helps HIV-1 specific CD8+ T cell exhaustion, because blocking the interaction of PD-1 receptor to its ligand enhances the cellular ability to proliferate and to produce cytokines in HIV-1 infected individuals (19, 101). The HIV-2 Nef protein plays a central role by manipulating the surface expression of CD3, CD4, MHC-I, CD74 and CD28 critical for the formation of an immune synapse (5). Lower surface levels of CD3 and CD28 in infected T cells ensure the inefficient delivery of co-stimulatory signals and interfere in effective TCR engagement even if possible.

The accessory Nef proteins of primate lentiviruses can be divided in two categories: those that are capable of down-modulating TCR-CD3 and that do not exert this activity (83). Previous studies indicated that the amino acid residues responsible for the TCR-CD3 down-modulation ability of Nef protein, are located in the conserved globular core domain (81, 95). But the exact residues have not been mapped and mutations had pleiotropic effects, pointing that they have broad-spectrum disruptive effects may be involved in damaging the highly structured core domain. Few residues in the SIVmac239 Nef involved in modulations of

TCR-CD3 cell surface expression have already been described (81, 95). Likewise in the conserved core domain of Nef protein, the I132T mutation has been identified in my study. Single point mutation in HIV-2 Nef which is selectively defective in TCR-CD3 down-modulation function has not been reported. The discovery of I132T Nef variants in HIV-2 infected subject was highly specific and exclusively affect CD3 down-modulation function of the Nef. Consequently, our data support that residue I132 in Nef is directly involved in the interaction with the cytoplasmic domain of the CD3 zeta chain (Figure 17). It is noticeable that as the consequence of only I to T mutation at 132 position in RH2-1, HIV-2 gives rise to a Nef variant which selectively lost the CD3 down-modulation function and hence lacks the capability to effectively suppress the responsiveness of virally infected T cells to stimulation (5, 83). Among the HIV-1 stains, the strain M is pandemic and responsible for the majority of AIDS cases all over the world (20).

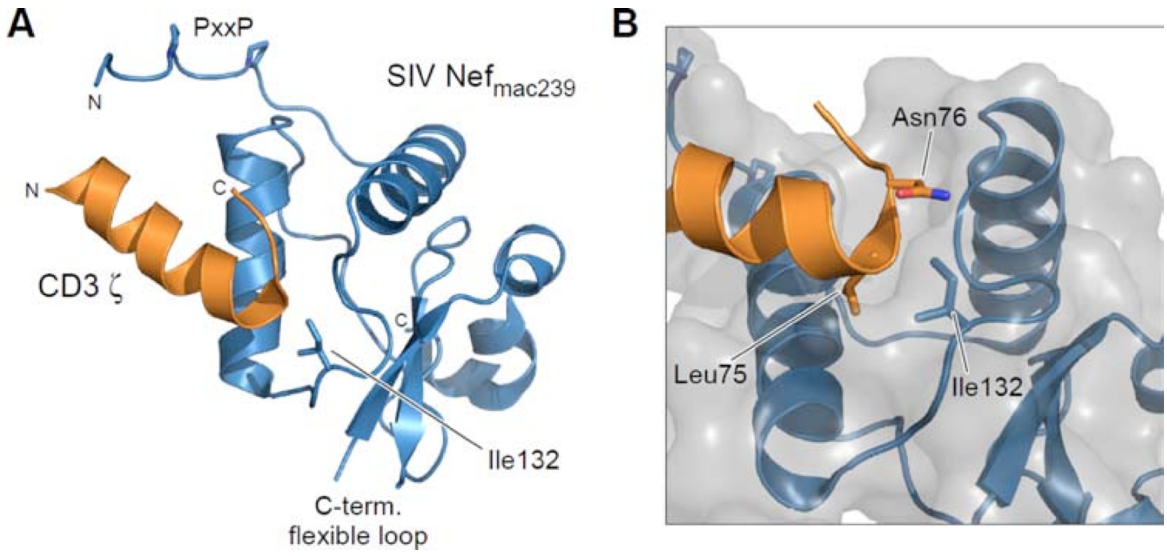

Figure 17: Structural model of Nef core domain interaction with CD3 zeta: (A) Ribbon model of SIVmac239 Nef (blue) core domain interacting with the first ITAM motif of CD3 zeta (orange) (52, 53). (B) L75 and N76 of CD3 zeta interacts with predicted HIV-2 Nef core crevice formed by I132 of Nef. The I132T mutation in HIV-2 Nef allele RH-2_A8 and reverse in RH-2_D8 is enough to determine the interaction between Nef and zeta (52, 113)

Although the single I132T mutation in Nef is enough to generate “HIV-1 like” phenotype of HIV-2 but still this point mutation is very rare in natural infection in human, in fact, I explored 122 HIV-2 Nef sequences present in the Los Alamos National Laboratory (LANL) (111) database and there we found none of the Nef sequences present has the Isoleucine to Threonine mutation at corresponding position as 132 in RH2-1 Nef. T cell hyper activation also leads to interferon-induced tetherin expression and that will hinder the release of budding virions. Therefore, it may not be advantageous to lose the CD3 down-modulation function possibly because HIV-2 is not well equipped with *vpu* gene, product of which can help to counteract the host defense induced by increased immune activation (54) or just nature has made them like that. However, findings of my PhD research raise the possibility that HIV-2 could potentially evolve into a more virulent form by just single change in *nef* gene that is capable of losing “protective” Nef function.

Summary

Whether Nef-mediated down-modulation of TCR-CD3 protects lentivirus-infected hosts against damaging high levels of immune activation, is under debate. In my thesis, I tried to address this issue, taking HIV-2 Nef as a model system, since its ability to down-modulate the TCR-CD3 greatly varied; some of them were highly efficient while others were impaired and have HIV-1 Nef like phenotype for the function. This wide spectrum of the down-modulation function among the HIV-2 Nefs allowed us to analyze the significance of TCR-CD3 down-modulation and it's role in activation of CD4+ T cells. The outline of my thesis suggests that Nef-mediated down-modulation of TCR-CD3 and CD28 play role in protection of viremic HIV-2-infected individuals against loss of CD4+ T cells. These two basic functions play role in activation of the cells and seemingly delay the activation induced exhaustion and apoptotic death of virally infected CD4+ T cells. Nevertheless, there is room left to explain the role of HIV-2 Nef on T cell activation which may be linked with the overall systemic levels of immune activation, exhaustion and control of the virus replication may be responsible for not progressing the disease in majority of the infections. However, in accordance, the significant correlation of the level of Nef-mediated down-modulation of TCR-CD3 and the levels of immune activation in HIV-2-infected individuals with comparably low viral loads have been reported (20). Another remarkable discovery of my study is the identification of naturally occurring HIV-2 *nef* alleles that are selectively impaired in specific functions like surface modulation of CD4, CD28, CD74, MHC-I and more importantly down-modulation of TCR-CD3. These selectively defective Nefs open up ways to study the role of CD4 down-modulation, T cell activation or antigen presentation in viral immune evasion, and effect of these specific functions in pathogenicity in animal models. These selectively defective Nefs in specific functions can play significant role to study the detailed mechanisms involved in AIDS progression *in vivo* and may be the possible remedy.

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