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Nef-mediated primate lentiviral immune evasion mechanisms

Dissertation

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List of Abbreviations

agm	african green monkey
AIDS	Acquired Immune Deficiency Syndrome
APC	antigen presenting cell
APC (staining)	allophycocyanin
[α-^{32}P] TTP	alpha-32 phosphorus isotope-deoxythymidine triphosphate
bdg	binding
β-gal	β-galactosidase
blu	blue monkey
c	centi (10^{-2})
°C	degree celsius
$CaCl_2$	calcium chloride
CD	cluster designation
Ceat	*Cercocebus atys* (MHC-I)
Ci	curies
CO_2	carbon dioxide
cpz	chimpanzee
CTL	cytotoxic T lymphocyte
deb	de brazza monkey
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

gsn	greater spot-nosed monkey
h	hour
HBS	HEPES buffered saline
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hi-C-SNP	high VL, HLA-C SNP group
HIV	human immunodeficiency virus
hi-WT	high VL, WT group
HLA	human leucocyte antigen
HRP	horse radish peroxidase
Ig	immune globulin
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
l	liter
LB	Luria Bertani
low-C-SNP	low VL, HLA-C SNP group
low-WT	low VL, WT group
LTR	long terminal repeat
m	meter
m	milli (10^{-3})
µ	micro (10^{-6})
M	molarity (mol/l)
mac	macaque
Mamu	*Macaca mulatta* (MHC-I)
MFI	mean fluorescence intensity
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minute

MHC	major histocompatibility complex
mus	mustached monkey
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
ORFs	open reading frames
Patr	*Pan troglodytes* (MHC-I)
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyd
PHA	phytohaemagglutinin
PMSF	phenylmethylsulfonylfluorid
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	rounds per minute
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
sec	second
SIV	simian immunodeficiency virus

smm	sooty mangabey
SNP	single-nucleotide polymorphism
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
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 Discovery, origin and genomic organization of HIV

In 1981, Gottlieb *et al.* described a disease associated with diverse opportunistic infections indicative of a severely defective immune system that was later designated AIDS (Acquired Immunodeficiency Syndrom) (Gottlieb *et al*., 1981). After intensive research the virus causing the immunodeficiency could be isolated from blood samples of AIDS patients in 1983 (Barré-Sinoussi *et al*., 1983). In 1986, this virus was termed Human Immunodeficiency Virus-1 (HIV-1) and assigned to the family of *Retroviridae* in the lentivirus subfamily (Coffin *et al*., 1986). In the same year an HIV-1-related virus was isolated from the blood of an African AIDS patient and named Human Immunodeficiency Virus-2 (HIV-2) (Clavel *et al.*, 1986). The discoverers of HIV, Barré-Sinoussi and Montagnier, have been awarded with the 2008 Nobel prize in Medicine.

Currently, about 33.2 million people are globally infected with HIV and 2.5 million new infections and 2.1 million deaths were reported for last year (www.unaids.org). This makes AIDS one of the most frequent death causes, especially in developing countries. The infection can not be cured, but the development of Highly Active Anti-Retroviral Therapy (HAART), which combines different antiretroviral drugs targeting several steps in the viral replication cycle, has substantially increased the life expectance of HIV-1-infected individuals at least in industrialized countries despite severe side effects (Palella *et al*., 1998). However, complete elimination of HIV is not possible because the virus integrates its genome into that of the host and persists in long living quiescent memory CD4+ T cells (Ho *et al*., 1998). In addition, HIV-1 mutates at high rates and can rapidly become resistant to all available drugs (Hogg *et al*., 2006).

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. Many African nonhuman primate species are naturally infected with related lentiviruses, but do usually not develop disease (Hahn *et al*., 2000; Santiago *et al*., 2002). HIV-1 originated from cross-species transmission of SIVcpz *P.t.t.* (*Pan troglodytes troglodytes*) to humans, giving rise to HIV-1 groups M and N (Keele *et al*., 2006). The origin of HIV-1 O is currently unclear, because its closest SIV relatives have been detected in gorillas. Thus, chimpanzees may have

transmitted HIV-1 group O-like viruses independently to gorillas and humans, or first to gorillas that subsequently transmitted the virus to humans (Van Heuverswyn and Peeters, 2007). SIVcpz itself appears to be a recombinant of lentiviruses now found in red-capped mangabeys (SIVrcm) and greater spot-nosed monkeys (SIVgsn) or a closely related species (Bailes *et al*., 2003). HIV-2 resulted from multiple zoonotic transmissions of SIVsmm from sooty mangabeys (Hirsch *et al*., 1989). Although both HIV-1 and -2 are pathogenic in humans, the simian ancestors of HIV-2 infect their natural hosts (sooty mangabeys) without causing disease (Hahn *et al*., 2000). It has been suggested that SIVcpz does not cause AIDS in chimpanzees but experimental evidence is largely missing and recent data suggest that the life expectance of SIVcpz-infected chimpanzees in the wild is significantly decreased (Hahn, personal communication).

The RNA genomes of HIV and SIV encompass about 9.2 to 9.8 kb and contain the *gag*, *pol* and *env* genes (Fig. 1). The genome is flanked by sequences known as the Long Terminal Repeats (LTR) which act as promoter. *Gag* encodes the capsid (CA), matrix (MA) and nucleocapsid (NC) proteins, *pol* the viral enzymes necessary for replication (reverse transcriptase (RT) and RNase H, protease (PR), integrase (IN)) and *env* encodes the glycoproteins (gp120 and gp41) which are responsible for the infectivity of the virus particle. HIV-1 also possesses several additional genes, i.e. *tat* and *rev* encoding the regulatory proteins and *vif*, *vpr, vpu* and *nef* encoding the accessory proteins (Turner and Summers, 1999; Anderson and Hope, 2004). The HIV-1 accessory proteins modulate infected cells and their local environment to ensure effective viral persistence, replication, dissemination and transmission in *vivo.* However, they are dispensable for viral replication in some cell lines in *vitro* (Malim and Emerman, 2008). The Vif (virion infectivity factor) protein counteracts a cellular restriction factor, APOBEC3G, that inhibits HIV-1 (Sheehy *et al*., 2002). Vpu (viral protein unknown) degrades CD4 and promotes virus release by counteracting the cellular restriction factor tetherin (Geleziunas *et al*., 1994; Neil *et al*., 2006). Vpr (viral protein regulatory) arrests cellular proliferation in the G_2 phase of the cell cycle, promotes cellular differentiation and interacts with cellular proteins involved in DNA repair (Andersen *et al*., 2006; Malim and Emerman, 2008). As outlined below, Nef (negative factor) performs various activities that promote viral immune evasion and replication.

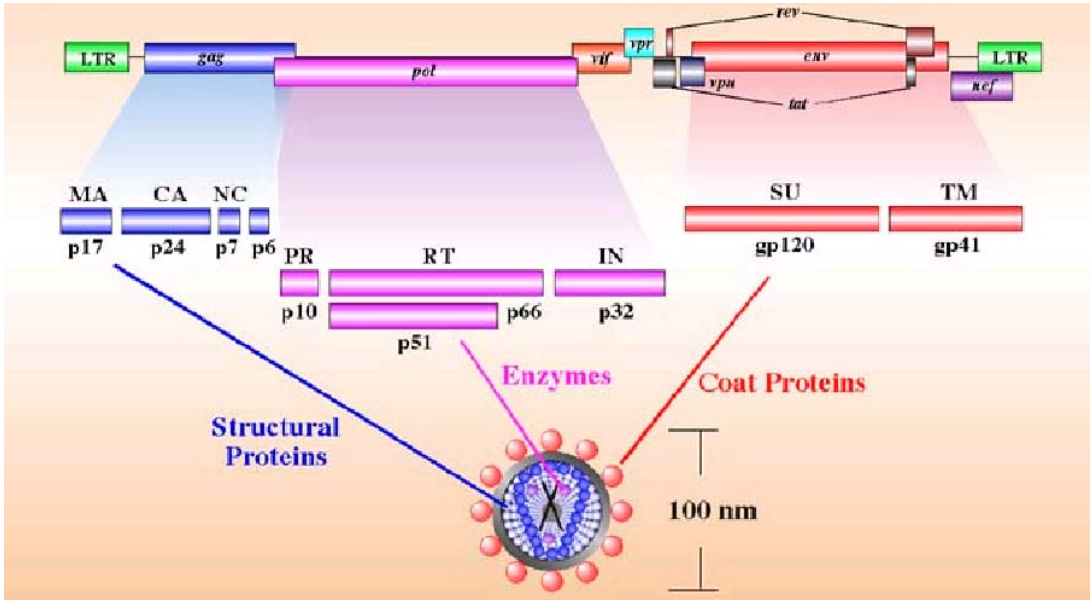


Fig. 1: Genomic organization of HIV-1.
(http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV-1b.jpg)

1.2 Nef: a multifunctional viral persistence factor

The accessory *nef* gene encodes a protein of 27-35 kDa that is abundantly expressed early during the viral life cycle and has a N-terminal myristoylation site which is critical for membrane association and essentially all of its functions (Geyer *et al*., 2001) (Fig. 2). Nef is required for efficient primate lentiviral persistence. In recent non-adapted hosts, such as HIV-1-infected humans or SIV-infected macaques the high viral loads (VLs) are associated with greatly accelerated progression to immunodeficiency (Kestler *et al.*, 1991; Kirchhoff *et al*., 1995). In contrast, SIVs persist efficiently at high levels in their natural hosts like sooty mangabeys, without causing disease (Hirsch *et al*., 1989; Hahn *et al*., 2000). Experimental infections of macaques with SIVmac result in a disease that is remarkably similar to human AIDS and represents a useful model to study HIV pathogenesis and to evaluate antiretrovirals and vaccines (Fultz *et al*., 1989; McClure *et al*., 1990).

One of the most important function of primate lentiviral Nef is the evasion of the host immune response by removing class I MHC molecules (MHC-I) from the surface of infected cells (Bevan and Braciale, 1995; Kirchhoff *et al*., 2004; Münch *et al*., 2005; Yang *et al*., 2006; Schindler *et al*., 2006). In *vivo* studies further supported the relevance of this Nef function as they showed that an amino acid substitution which disrupts the ability of SIV Nef to downmodulate MHC-I consistently reverted in infected macaques and that the Nef activity was fully

restored within 4 weeks after infection. These results demonstrated that there is a strong selective pressure on Nef to maintain MHC-I downregulation, because it provides a selective advantage for viral replication in *vivo* (Münch *et al*., 2001, Swigut *et al*., 2004).

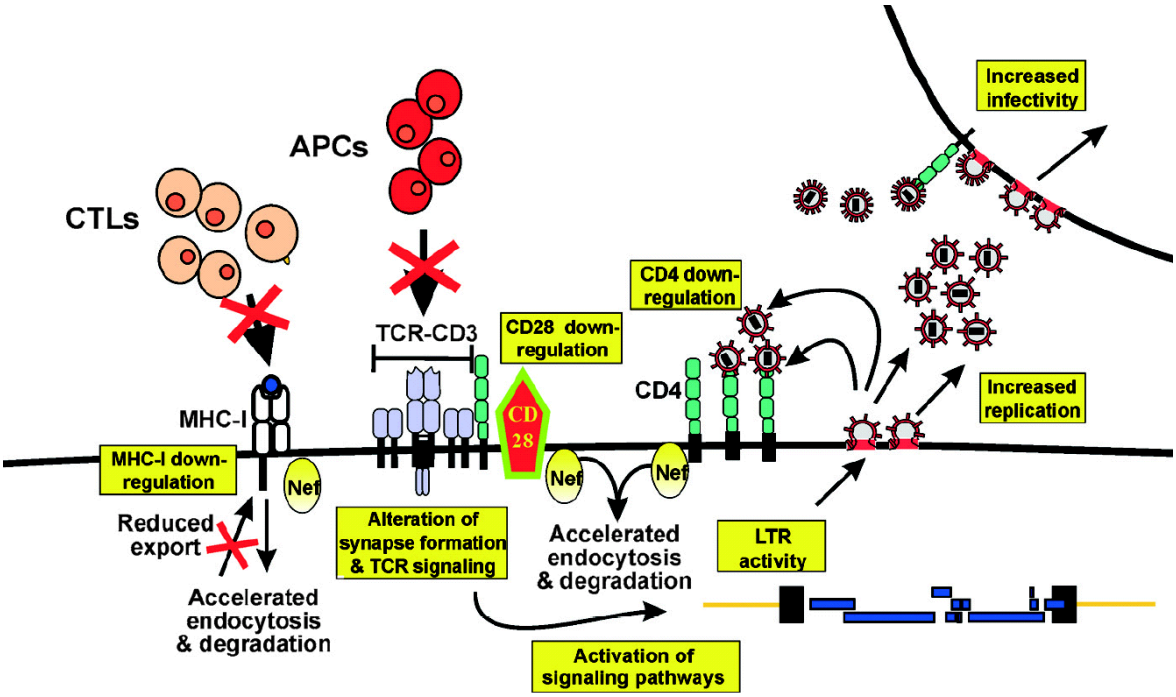


Fig. 2: Manipulation of infected T cells by Nef. Nef reduces MHC-I antigen presentation to avoid CTL lysis; downregulates CD28 and often also CD3 (only HIV-2 and most SIVs) to impair TCR signaling; reduces CD4 surface expression to promote virus release and to prevent superinfection; affects downstream signaling events; and enhances virion infectivity and virus replication. (Kirchhoff *et al*., 2008)

At least two pathways are known through which Nef removes MHC-I from the cell surface: (i) recruitment of AP-1 to the MHC-I cytoplasmic tail and re-routing to lysosomes (Mangasarian *et al*., 1999; Kasper *et al*., 2003; Roeth *et al*., 2004; Kasper *et al*., 2005), and (ii) endocytosis of MHC-I to the trans-Golgi network (TGN) in a PACS-1, AP-1 and clathrin-dependent manner (Le Gall *et al*., 1998; Piguet *et al*., 2000; Blagoveshchenskaya *et al*., 2002; Lubben *et al*., 2007). However, the exact mechanisms are controversial.

Gene products of the highly polymorphic *HLA-A* and *-B* loci, and to a lesser extent of the *HLA-C* locus, present viral peptides for recognition by cytotoxic T lymphocytes (CTLs) (Parham and Ohta, 1996). HLA-C and -E molecules serve as ligands to inhibit the activation of natural killer (NK) cells through interactions with receptors of the KIR and CD94/NKG2 families (Lanier, 2005). HIV-1 Nef downregulates HLA-A and –B, but not -C or –E based on amino acid differences in

the cytoplasmic tails of these molecules where Nef binds to (Le Gall *et al*., 1998; Cohen *et al*., 1999; Williams *et al*., 2002). This selective MHC-I modulation most likely allows HIV-1 to balance escape from CTL lysis with protection from NK cell attack. However, it is unknown if the ability of Nef to specifically remove MHC-I molecules from the surface of infected cells is conserved between other primate lentiviruses and their hosts.

Nef also exerts a variety of other functions to help the virus to persist efficiently in a host. HIV and SIV use the CD4 receptor to infect T cells and macrophages and upon infection remove their primary receptor from the surface, like other viruses (Lama, 2003). Usually, the biological function of CD4 is to assist the T cell receptor (TCR) to activate its T cell following an interaction with an antigen presenting cell (APC) (Miceli and Parnes, 1993). The importance of CD4 removal from the surface of infected cells is already evident from the fact that HIV-1 utilizes three of its gene products, Vpu, Env and Nef, to perform this action (Lama, 2003). Nef removes its primary receptor by enhancing its endocytosis *via* recruitment to AP-2 complexes and directing the receptor to lysosomes for degradation (Aiken *et al*., 1994; Mangasarian *et al*., 1997; Craig *et al*., 1998; Bresnahan *et al*., 1998; Greenberg *et al*., 1998; Piguet *et al*., 1998). In contrast, Vpu and Env interfere with the transport of newly synthesized CD4 to the cell surface (Willey *et al*., 1992a and b; Geleziunas *et al*., 1994). Notably, Nef reduces the surface expression of CD4 molecules with substantially higher efficiency than Vpu and Env (Chen *et al*., 1996; Wildum *et al*., 2006).

Nef also affects MHC-II antigen presentation by upregulating the MHC-II-associated invariant chain (Ii, CD74) (Stumptner-Cuvelette *et al*., 2001; Schindler *et al*., 2003). APCs such as macrophages and dendritic cells, but also activated CD4+ T cells, express MHC-II and are permissive to HIV-1 infection. It is well documented that stable surface expression of Ii prevents antigen presentation (Roche *et al.*, 1992; Stumptner-Cuvelette *et al*., 2002), which might result in impaired helper T cell responses observed in AIDS patients (Betts *et al*., 2001). The ability to upregulate Ii is well conserved in primate lentiviruses and already observed at very low levels of Nef expression, suggesting an important role in HIV-1-infected individuals (Stumptner-Cuvelette *et al*., 2001; Schindler *et al*., 2003).

Furthermore, it has been shown that Nef alleles from HIV-2 and the great majority of SIVs also downregulate TCR-CD3 with high efficiency, whereas those of HIV-1 and its simian counterparts from chimpanzees and some *Cercopithecus* monkeys fail to perform this function (Schindler *et al*., 2006). Efficient T cell activation by APCs requires the interaction of the MHC-II/antigen complex in addition to a costimulatory signal mediated by the binding of CD28 to B7 (Grakoui *et al*., 1999; Huppa *et al*., 2003). HIV-1 Nefs manipulate this process by reducing CD4 surface expression and impairing MHC-II antigen presentation. In comparison to HIV-1 *nef* alleles, most SIV and HIV-2 Nefs are also capable to efficiently downregulate CD3, CD28 and CXCR4, most likely to suppress T cell activation, migration and apoptosis (Kirchhoff *et al*., 2008). The inability of HIV-1 Nefs to interfere with TCR signaling might contribute to the high levels of immune activation and apoptosis associated with progression to AIDS in infected humans (Schrager and Marsh, 1999; Manninen *et al*., 2000; Djordjevic *et al*., 2004; Fortin *et al*., 2004; Krautkramer *et al*., 2004; Fenard *et al*., 2005; Schindler *et al*., 2006).

In addition of being a master manipulator of T cell and APC function, Nef also enhances virion infectivity and viral replication (Chowers *et al*., 1994; Miller *et al*., 1994; Spina *et al*., 1994; Aiken and Trono, 1995; Schwartz *et al*., 1995; Alexander *et al*., 1997; Glushakova *et al*., 1999). The exact mechanisms are still not well understood and the overall effect of Nef on virus spread in *vivo* most likely depend on a large number of cellular properties and interactions. It seems that the combination of all Nef activities contributes to efficient viral replication in *vivo* (Kirchhoff *et al*., 2008).

In summary, primate lentiviral Nef proteins generally perform a large number of activities which help the virus to persist efficiently in the infected host by facilitating evasion of the immune system and by increasing virus spread. High VLs were found to be associated with disease in poorly adapted hosts but not in natural SIV infection. Hence Nef should be considered a “persistence” rather than a “virulence” factor. It is obvious that a large number of viral but also inherent host properties determine the different clinical outcomes of primate lentiviral infections (Sorin and Kalpana, 2006; Fellay *et al*., 2007; Lama and Planelles, 2007; Brass *et al*., 2008).

1.3 A HLA-C SNP is associated with improved control of HIV-1

The clinical course of HIV-1 infection is highly variable and it is largely unknown why the plasma VL during the chronic phase of infection varies by up to five orders of magnitude (O`Brien and Nelson, 2004; Fellay *et al*., 2007). It is well documented that the decline in VLs after the acute phase of infection correlates with the appearance of HIV-specific CTLs (Koup *et al.*, 1994; Borrow *et al*., 1994). Therefore, the CTL response most likely plays a key role in suppressing viral replication since HIV-1-infected individuals with high VLs progress to AIDS substantially more rapidly than those who are capable to achieve better control of HIV-1 (Mellors *et al*., 1996; Mellors *et al*., 1997; de Wolf *et al*., 1997). Furthermore, it has been shown that specific HLA alleles are associated with particularly rapid or attenuated courses of HIV-1 infection (Kaslow *et al*., 1996; Carrington *et al*., 1999; Migueles *et al*., 2000).

In addition to the high variability of HIV-1, the ability of the Nef protein to selectively downregulate MHC-I molecules may explain why CTL responses are rarely capable to achieve long-lasting effective control over virus replication (Collins and Baltimore, 1999). Recent evidence suggests that in some HIV-1-infected individuals the elaborate balance to avoid both NK cell and CTL lysis by selective Nef-mediated downmodulation of HLA-A and –B may be disturbed. A whole-genome study identified two single-nucleotide polymorphisms (SNPs) located within the *MHC-I* gene region as major determinants of low setpoint VLs (Fellay *et al*., 2007). One of them is located with the *HLA-B*5701* gene, which has previously been associated with low VLs and protection against progression to AIDS (Migueles *et al*., 2000; Altfeld *et al*., 2003). The second most significant SNP, rs9264942, was detected upstream of the *HLA-C* gene (HLA-C SNP) and homozygous copies of this allele were associated with an about 10-fold reduction in VLs. It is known that HLA-C molecules can in addition to present self-peptides to NK cells also present viral peptides to CTLs (Goulder *et al*., 1997; Adnan *et al*., 2006). Since HIV-1 Nef is not able to remove HLA-C from the cell surface it may be particularly effective in restricting HIV-1 replication. Thus, the HLA-C SNP might help infected individuals to control HIV-1 by affecting the efficiency or the quality of HLA-C-mediated antigen presentation (Fellay *et al*., 2007).

Although the average VLs in HLA-C SNP individuals are lower than in "wildtype" individuals they also vary (Fellay *et al.*, 2007). The efficient persistence of HIV-1 in a subset of people with the polymorphism suggests that they are either unable to mount an effective HLA-C-mediated immune response or that the virus "learns" to counteract it. Usually, the need to avoid NK cell lysis is associated with a strong selective pressure against effective HLA-C downmodulation by Nef. However, this could be different in HIV-1-infected HLA-C SNP individuals, who might be able to mount effective HLA-C-mediated CTL responses leading to effective elimination of virally infected cells and therefore low VLs.

1.4 Scientific aims

The ability to downregulate human MHC-I from the surface of infected cells is highly conserved between HIV and SIV Nef alleles and allows these viruses to evade the host immune response. HIV-1 Nef is capable of selectively downmodulating HLA-A and -B, but not -C or -E molecules to evade both CTL and NK cell surveillance. It remained elusive, however, whether this ability is shared by other primate lentiviruses and whether it is also conserved in the SIV/macaque model. Thus, the first goal of this thesis was to clarify if selective downmodulation of specific MHC-I molecules is a common property of HIV and SIV Nefs.

Some HIV-1-infected individuals carry a SNP, rs9264942, upstream of the *HLA-C* gene, which is associated with reduced VLs. HIV-1 is unable to downmodulate HLA-C from the cell surface. Thus, HLA-C-mediated antigen presentation may be particularly effective in inducing CTL lysis of HIV-1-infected cells. The HLA-C SNP may help infected individuals to control HIV-1 because it affects the efficiency or the quality of HLA-C-mediated antigen presentation. Thus, the second aim of this thesis was to determine whether HIV-1 Nef variants capable of downmodulating HLA-C emerge in some high VL HIV-1-infected individuals carrying the polymorphism or whether other changes in Nef functions facilitate effective viral persistence in this genetic background. The overall aim of this thesis was to obtain better insights into the mechanisms allowing HIV and SIV to evade the host immune response, to understand how a better control of HIV-1 replication can be achieved.

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´ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r}) Amy Cam^{r}] (Bullock *et al*., 1987) (Stratagene, Agilent Technologies, Waldbronn)

2.1.2 Eukaryotic cells

293T: human renal epithelial cell line which was transformed with adenovirus type 5 and expresses SV40 (simian virus 40) large T-antigen (Graham *et al*., 1977)

P4-CCR5: HeLa-CD4/LTR-lacZ reporter cell line, expresses CD4, CXCR4, and CCR5 receptors, contains *β-galactosidase* gene under the control of the HIV-1 promoter (Carneau *et al*., 1994).

Jurkat: human T cell line

Jurkat-NFAT: human T cell line stably transfected with a plasmid expressing the *luciferase* gene under the control of the NFAT promoter (Fortin *et al.*, 2004)

THP-1: human acute monocytic leukemia cell line (Tsuchiya *et al*., 1980)

CD8-MHC-I fusion Jurkats: Jurkat T cell lines expressing the extracellular and transmembrane domains of the CD8α chain (residues 1-208) fused to different MHC-I cytoplasmic tail sequences were established by retroviral transduction (Specht *et al*., 2008; DeGottardi *et al*., 2008). The cells were kindly provided by David T. Evans.

721.221: 721.221 B cell line (Shimizu and DeMars, 1989) stably expresses MHC-I chimeric molecules containing the extracellular and transmembrane protein of Cw4 and the cytoplasmic tails of either HLA-A2, -B7 or -Cw4 (Cohen *et al*., 1999).

2.1.3 Nucleic acids

2.1.3.1 Oligonucleotides

Oligonucleotides were ordered from Biomers (Ulm):

1) 5-pBRNL4-3envHpaI GCT*GTTAAC*TTGCTCAATGCCACAGCC

2) 3-pBRNL4-3envEnd CTTATAGCAAAATCCTTTCCAAGCCC

3) 5-env-NIG-HLACnef	GGATTTTGCTATAAGATGGGGGGCAAATGG	
4) 3-nefHLAC1-MluI	GC*ACGCGT*CAGCAGTTCTTG	
5) 3-nefHLAC2-MluI	GC*ACGCGT*CAGCAGTCTTTG	
6) 3-nefHLAC3-MluI	GC*ACGCGT*CAGCAGTCTCAG	
7) 3-nefHLAC4-MluI	GC*ACGCGT*CAGCAGTTCTCG	
8) 3-nefHLAC5-MluI	GC*ACGCGT*CAGCAGTTCTGG	
9) 3-nefHLAC6-MluI	GC*ACGCGT*CAGCAGTCCTTG	
10) 3-nefHLAC7-MluI	GC*ACGCGT*CAGCAGTCTTTG	
11) 3-nefHLAC8-MluI	GC*ACGCGT*CAGCAGTCTTTATAG	
12) 3-nefHLAC9-MluI	GC*ACGCGT*CAGCAGTTAGTTC	
13) 3-nefHLAC10-MluI	GC*ACGCGT*CAGTCTTGGTAG	
14) 3-nefHLAC11-MluI	GC*ACGCGT*TAGTCCTTGTAATAC	
15) 5pBRNL4-3nef HpaI	GTGGAACTTCTGGGACGCAGG	for sequencing

2.1.3.2 Plasmids

All plasmids contain the gene coding for ampicillin resistance and can be selected after transformation by selective media.

PCR 2.1-TOPO-TA vector: cloning vector for PCR fragments (Invitrogen, Karlsruhe)

pBR-NL4-3-nef-IRES-eGFP: modified pBR-322 vector expressing the HIV-1 NL4-3 provirus with *nef* and *eGFP* from a bicistronic mRNA with the help of an IRES element (Schindler *et al.,* 2003; Schindler *et al.*, 2005)

pBR-NL4-3-nef-vpu- -env*-IRES-eGFP: modified pBR-NL4-3-nef-IRES-eGFP vector not expressing vpu (start codon mutated) and env (3 stop codons introduced)

pHIT60: vector which expresses the envelope protein of the vesicular stromatitis virus (VSV-G) (Schindler *et al*., 2003)

2.1.3.3 HIV and SIV isolates

Patient HIV-1 *nef* alleles were from the SHCS CHAVI cohort, kindly provided by Amalio Telenti (table 1). Further used proviral constructs have been described previously (Schindler *et al*., 2003; 2006; Takehisa *et al*., 2007).

Table 1: Overview of patient Nefs from the SHCS CHAVI cohort with patient VLs and CD4 counts.

nef alleles		VL	CD4 counts
		(log genomic viral RNA copies/ml plasma)	(CD4+ T cells/ml blood)
hi-C-SNP	3477	4.836	500
	5122	4.045	
	5542	4.496	276
	5565	4.836	
	5661	5.095	421
	3551	4.685	171
	3620	4.774	514
	5549	5.602	75
	5796	4.716	551
	5958	4.550	387
	5969	4.555	285
	6017	4.826	43
	6056	4.935	917
	6116	4.606	410
	6129	4.660	271
	6296	4.528	416
	6301	4.583	1067
	6349	5.172	374
	6401	4.954	693
low-C-SNP	3478	2.201	601
	5569	3.231	478
	5615	2.798	318
	5319	2.906	334
	5527	2.362	1051
	5541	2.402	400
hi-WT	5466	5.345	
	5505	5.299	
	5535	5.694	285
	5602	5.293	500
	5613	5.357	
	5619	5.404	316
	5631	5.857	400
	5633	5.466	238
	5859	5.618	407
	6069	5.716	965
	6193	5.394	718
	6230	5.476	322
	6231	5.321	500
	3085	4.957	87
	3968	4.109	1034
	3975	4.072	729
	5478	4.081	263
	5578	5.215	421
	5585	4.395	380
low-WT	5492	1.806	303
	5691	2.000	881
	5696	2.319	763
	5896	2.463	638
	6012	2.289	752
	6050	2.540	643

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, Sodiumdodecylsulphate (SDS), Sodium chloride (NaCl)
Ratiopharm, Ulm	Ampicillin
BD/Difco, Heidelberg	Bacto-trypton, 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/Streptomycinsulphate, Roswell Park Memorial Institute medium (RPMI-1640)
Fluka, Neu-Ulm	Ethylene Diamine Tetraacetate (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, Obergünzburg	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 for western blot	Invitrogen, Karlsruhe
Annexin V-PE apoptosis kit	Becton-Dickinson, Heidelberg
Human CD4+ T cell RosetteSep Kit	StemCell Technology, Köln

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 Streptomycinsulphate and 120 µg/ml Penicillin

2.1.7.2 Bacterial media

LB-medium: 10 g/l Bacto-trypton, 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-trypton, 5 g/l yeast extract, 2.5 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ 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 p24 capsid-antigen-ELISA

Lysis solution: 10% Triton X-100 in distilled water

Wash buffer: 20x wash concentrate (KPL, Maryland, USA) was diluted in distilled water

Sample diluent: 0.2% Tween 20 in RPMI-1640

Primary antibody diluent: 10% FCS and 2% normal mouse serum (Sigma, München) in RPMI-1640

Secondary antibody diluent: 2% normal mouse serum, 5% normal goat serum (Invitrogen/Gibco, Karlsruhe) and 0.01% Tween 20 in RPMI-1640

Substrate: TMB Peroxidase Substrate System (KPL, Maryland, USA)

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 were added.

Wash buffer: 20x SSC buffer (Eppendorf, Hamburg) diluted in distilled water

[α-^{32}P] TTP: Perkin Elmer, Rodgau-Jugesheim

2.1.8.5 Others

FACS buffer: 1% FCS in PBS

50x TAE-buffer: 5Prime, Hamburg

2.1.9 Antibodies

2.1.9.1 Western Blot

Pooled sera from plasma samples of 22 HIV-1-infected patients were used for the detection of HIV-1 protein expression.

rabbit polyclonal anti-GFP	Abcam, Cambridge, USA
alkaline-phosphatase goat anti-human IgG	Bethyl laboratories, Montgomery, USA
alkaline-phosphatase goat anti-rabbit IgG	KPL, Maryland, USA

2.1.9.2 ELISA

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

2.1.9.3 FACS

Caltag, Buckingham, UK	anti-CD4-APC, anti-CD25 (IL2R)-APC, anti-annexin V-APC
Becton-Dickinson, Heidelberg	anti-CD28-PE, anti-CD69-PE, anti-CD8-PE
Dako, Hamburg	anti-MHC-I-PE
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 digest
- dephosphorylation of DNA with alkaline phosphatase
- ligation of DNA fragments using T4-DNA-ligase

2.2.1.2 Plasmid DNA preparation

Plasmid DNA for transfection was prepared using the Wizard^{TM} Plus Midiprep Kit and plasmid DNA for cloning and sequencing was prepared using the Miniprep Kit,

according to the manufacturer´s protocol. The DNA concentration and quality was determined using a spectrophotometer (Eppendorf, Hamburg).

2.2.1.3 Isolation of DNA from agarose gel

Electrophoretically separated DNA fragments were visualized on a UV screen (366 nm) (Syngene, USA), and the bands were isolated using a scalpel. Subsequently the DNA was purified using the UltraClean 15 DNA purification Kit, according to the manufacturer´s instructions.

2.2.1.4 Polymerase chain reaction (PCR)

All PCR reactions were performed with the Phire^{TM} 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^{TM} Kit according to the manufacturer´s instructions.

2.2.1.6 DNA Sequencing

2 µg of the DNA sample were heat dried in a tube at 58°C. Sequencing was performed by MWG-Biotec/Operon (Ebersberg).

2.2.2 Bacterial methods

2.2.2.1 Bacterial culture

The used plasmids contained a gene coding for ampicillin resistance. Therefore, transformed bacteria were grown in LB medium or on LB agar plates containing ampicillin. The bacteria were grown in LB medium for 12-16 h at 37°C on a shaker and the inoculated LB agar plates were incubated at 37°C.

2.2.2.2 Bacterial transformation

Ligated DNA was incubated with 15 µl of *Escherichia coli XL2 Blue*^{TM} 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 transformed cells were incubated at 37°C on a shaker for 30 min and plated on LB agar plates containing ampicillin. For pool cloning, half of the transformed cells were inoculated directly in LB medium and one half was plated on the agar plates. 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 cells were maintained in 25 cm^2 or 75cm^2 cell culture flasks (Sarstedt, Nümbrecht) with supplemented DMEM or RPMI-1640, respectively, in an 37°C incubator with 5% CO_2. The cells were splitted 1:10 or 1:20 regularly twice a week.

2.2.3.2 Isolation of primary blood cells

Buffy coat (lymphocyte concentrate from 500 ml whole blood), obtained from the blood bank (Ulm), was diluted 1:3 with PBS containing 2% FCS. Ficoll separating solution was overlayed with the diluted blood and centrifuged at 1600 g for 20 min without brakes. The white interphase layer formed by peripheral blood mononuclear cells (PBMCs) was transferred in a fresh tube and washed twice with PBS containing 2% FCS.

For the isolation of CD4+ T cells the diluted blood was incubated with the Human CD4+ T cell RosetteSep Kit according to the manufacturer´s instructions. After 20 min incubation time the cells were processed like PBMCs.

After separation and washing 2x10^6 cells/ml were cultured in supplemented RPMI-1640. For viral infection cells were pre-stimulated with 1 µg/ml PHA and 10 ng/ml IL-2 for 3 days.

2.2.4 Protein and enzyme methods

2.2.4.1 HIV-1 p24 antigen ELISA

Viruses were quantified based on their p24 core antigen content using the HIV-1 p24 antigen ELISA system (AIDS Repository, Fredrick, USA) according to the manufacturer´s instructions. Briefly, virus stocks were lysed with Triton X-100 solution resulting in the release the p24 capsid protein. The lysed and diluted antigens were transferred in p24 antibody coated microwell plates (AIDS

Repository, Fredrick, USA) where the p24 antigen binds to the monoclonal mouse antibody. After incubation the unbound capsid proteins were removed by washing. For the quantification of the p24 protein the “sandwich”-ELISA method was used. First a polyclonal rabbit anti-HIV-1 p24 antibody was added, which binds to the p24 protein. Then the samples were incubated with a goat anti-rabbit antibody conjugated with horse radish peroxidase (HRP) followed by the addition of TMB peroxidase substrate which results in the development of a blue color. The reaction was stopped with 1 N HCl. The colour intensity, which is proportional to the amount of p24 capsid antigen in ng/ml, was measured at 450 nm and 650 nm with the Thermomax microplate reader (Molecular devices, UK).

2.2.4.2 Reverse transcriptase (RT) assay

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

2.2.4.3 Western blot

Cell lysates from transiently transfected 293T, expressing the proviral HIV-1 constructs with the different *nef* alleles and *eGFP* were prepared with RIPA buffer. Viral proteins were separated by SDS-Poly acrylamide gel electorphoresis using NuPAGE® Novex Bis-tris gels (Invitrogen, Karlsruhe), as recommended by the manufacturer. The proteins in the gel were transferred onto a nitrocellulose membrane (Millipore, Schwalbach) using a Trans-Blot SD Semi-Dry electrophoretic Transfer Cell (BioRad, München). The transferred membranes

were blocked for 1 h with 10% milk solution and then incubated overnight with the respective primary antibodies. Unbound antibodies were removed with washing buffer. 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 added to develop the color.

2.2.5 Cloning of patient *nef* alleles in proviral vectors

PCR products of patient *nef* alleles were kindly provided by Amalio Telenti. Splice overlap extension (SOE) PCR was used to clone the already PCR amplified *nef* alleles from the patients into proviral vectors. For this standard PCR reactions were used with specific 5´primers for *nef* including an overlap to NL4-3 *env* (primer 3, see 4.3.1) and specific 3´primers for the end of the *nef* genes including a MluI restriction site (primer 4 to 14, see 2.1.3.1). In a second PCR reaction the NL4-3 *env* region was amplified (primer 1 and 2, see 2.1.3.1). Both PCR products were separated on an agarose gel and purified using the UltraClean 15 DNA purification Kit. The purified PCR fragments were combined in another PCR reaction using the primers 1 and 4 to14. These PCR products were again purified and cloned as a pool into the PCR 2.1-TOPO-TA vector (TA Cloning® Kit) according to the manufacturer´s protocol. Single clones were sent for sequencing. By using the introduced restriction sites, the *nef* alleles were cloned as pools into the pBR-HIV-1-NL4-3-IRES-eGFP and pBR-HIV-1-NL4-3-vpu- -env*-IRES-eGFP vectors for further analyses. Some single clones were also cloned into the pBR-HIV-1-NL4-3-IRES-eGFP vector.

2.2.6 Viral methods

2.2.6.1 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). At a confluence of 50-75% the cells were used for transfection. For this 5 µg DNA was mixed with 13 µl 2 M $CaCl_2$ and the total volume was made up to 100 µl with water. This solution was added dropwise to 100 µl of 2xHBS. The transfection cocktail was vortexed for 5 sec and added dropwise to the cells. The transfected

cells were incubated for 8-16 h before the medium was replaced by fresh supplemented DMEM. 48 h post transfection, virus stocks were prepared by collecting the supernatant and centrifuging it at 1300 rpm for 3 min. Virus stocks were stored at 4°C for up to two weeks.
For the preparation of VSV-G pseudo-typed viruses 5µg DNA of the proviral constructs and 1µg of the pHIT60 plasmid were used.

2.2.6.2 Infection of primary cells and cell lines

For infection of suspension cells (primary or cell lines) 1 million cells were incubated with 50 ng p24 of VSV-G pseudo-typed virus stocks at 37°C for 4-6 h. Then 3 ml supplemented RPMI-1640 was added. PBMCs or CD4+ T cells where further incubated in the presence of 10 ng/ml IL-2. 3 days post infection cells were used for FACS analyses.

2.2.6.3 Jurkat-NFAT assay

50000 Jurkat-NFAT cells were infected with 1.5 ng p24 virus stock in a 96-V-well plate (Greiner Bio-one, Frickenhausen). 2 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 lysis buffer. The lysed cells were transferred into a 96F-Nunclon-delta white micro-well plate (Nunc^{TM}, 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).
An aliquot of the cells was also used for FACS analyses and stained (see 2.2.6.4.1) for CD69 and CD28 expression, one day post stimulation. Two days post stimulation the CD25 surface expression was determined.

2.2.6.4 FACS analysis

2.2.6.4.1 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 (see manufacturer).

The cells were incubated for 30 min at 4°C and then washed with 1 ml FACS buffer to remove unbound antibody. The cells were fixed with 200 µl FACS buffer containing 2% PFA and incubated for 30 min at 4°C. For FACS analyses a FACSCalibur from Becton-Dickinson was used.

2.2.6.4.2 T cell activation and apoptosis of primary blood cells

Prestimulated PBMCs or CD4+ T cells were infected with VSV-G pseudo-typed virus stocks as described under 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) was 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 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.

2.2.6.5 Infectivity assay (β-galactosidase assay)

One day before infection, P4-CCR5 cells were seeded (5000 cells/well) in F-96-well plates (Greiner Bio-one, Frickenhausen). Virus stocks were normalized based on their p24 content and infections were performed with various concentrations. Three days post infection, the supernatants were removed and 40 µl of 1:1 diluted Gal-sceen® substrate in PBS was added to each well. After 30 min incubation at room temperature, the cell lysates were transferred into a F-96-Nunclon-delta white micro-well plate (Nunc™, Langenselbold) and the light emission was monitored with an Orion Microplate Luminometer (Berthold Detection systems, Pforzheim). The enzyme activity was measured as relative light units/second (RLU/sec) using the computer program Simplicity 4.02 (Berthold detection systems).

2.2.6.6 Viral replication

To analyze the influence of the patient *nef* alleles on viral replication, prestimulated 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 determined by FACS analysis measuring the amount of infected GFP+ cells. The supernatants of the taken culture were stored at -20°C and RT assay was performed to determine the virus production. The taken culture volume was replaced by adding fresh supplemented RPMI-1640 containing IL-2. Further samples were collected at regular intervals of 2 to 3 days.

2.2.7 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.

For the evaluation of the RT assay the computer program Aida Image Analyzer V.3.11 (Isotopenmeßgeräte GmbH) was used.

Statistical analyses were performed with the PRISM program version 4.0 (Abacus Concepts, Berkeley, CA, USA) and Microsoft Excel. P-values were calculated using the two-tailed unpaired Student´s-T-test.

3. Results

3.1 Specific modulation of MHC-I molecules

Nef helps primate lentiviruses to persist efficiently in their respective hosts, by evading the immune system, e.g. by impairing MHC antigen presentation. It is shown that HIV-1 selectively removes HLA-A and -B, but not -C or -E molecules from the surface of infected T cells to evade lysis by both CTL and NK cells (Cohen *et al*., 1999; Williams *et al*., 2002). It remained elusive, however, whether this ability is conserved between HIV-1 and also occurs in experimentally SIV-infected rhesus macaques and in the original chimpanzee and sooty mangabey hosts of HIV-1 and HIV-2, respectively.

3.1.1 MHC-I cytoplasmic tail selection

To analyze if the Nef-mediated selective MHC-I modulation in different hosts is preserved, MHC-I molecules representative for human (HLA), chimpanzee (Patr: *Pan troglodytes*), rhesus macaque (Mamu: *Macaca mulatta*) and sooty mangabey (Ceat: *Cercocebus atys*) were selected. Rhesus macaques are used as animal model and chimpanzees and sooty mangabeys are the hosts of SIVcpz and SIVsmm, which are the precursors of HIV-1 and HIV-2, respectively (Fultz *et al*., 1989; Hirsch *et al*., 1989; McClure *et al*., 1990; Hahn *et al*., 2000). The selected cytoplasmic tail sequences represent the gene products of multiple MHC-I alleles or known *MHC-I* loci from the different species. The amino acid tail sequences and the positions of the Y321, A325 and D328 residues in HLA-A and –B, known to be critical for Nef interaction (Cohen *et al*., 1999; Williams *et al*., 2002), are indicated in Fig 3.

As expected from the close relationship between humans and chimpanzees, the comparison of their MHC-I cytoplasmic tail sequences revealed that the HLA and Patr *MHC-I* loci of *-A*, *-B* and *-C* are identical. However, the Patr-E tail is shorter than the HLA-E with a G319R substitution (Fig. 3) (Mayer *et al*., 1988; Lawlor *et al*., 1990; Adams and Parham, 2001). Further, sequence comparisons showed that the rhesus macaque Mamu-A*01 sequence, which is representative for the majority of Mamu-A molecules, is identical to the HLA-A tail consensus. Sequence alignments of Mamu-B molecules revealed at least 20 different cytoplasmic tail variants, while the most common sequence, represented by Mamu-B*01, is also identical to the HLA-B consensus (Fig. 3). The

cytoplasmic domains of Mamu-E molecules are variable and different from their human counterparts (Fig. 3) (Boyson *et al*., 1995). Recent sequence analysis of sooty mangabey MHC-I molecules identified nine Ceat-A and -B alleles with cytoplasmic tails identical to those of rhesus macaques. An allele of the *Ceat-E* locus, Ceat-E*01, was also identified with a predicted cytoplasmic domain sequence not found in either human or rhesus macaque MHC-I molecules (Fig. 3) (Kaur, unpublished data). Altogether, the most important MHC-I alleles of humans, their counterparts chimpanzees and sooty mangabeys as well as from the rhesus macaque model were analyzed in this thesis.

```
            310        320        330        340
             .          .          .          .
HLA-A       RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV  Mamu-A*01, -A*02, -A*11, -A*05, -A*13, -A*14,
                                                Ceat-A*02, -A*04
HLA-B       .....GG.....................       Mamu-B-*01, B*02, -B*04, -B*06, -B*17,
                                                -B*28, -B*36, -B*39, -B*40, -B*45, -B*48,
                                                -B*49, -B*55, -B*69, -I*04, -I*07, Ceat-B*02,
                                                -B*04, -B*07
HLA-C       .....GG....C.....N......E..I...A
HLA-E       .K...GG......K.EW.......ESHSL
Patr-E      .K.......R...K.EW.....

Mamu-B*30   .....GA.....................
Mamu-B*03   .....GG.........N...........  Mamu-B*08, -B*11, -B*21, -B*37, -B*66,
                                           Ceat-B*03, -B*05, -B*08
Mamu-B*44   .....GG.........V...........
Mamu-B*20   .....GV.....................M.
Mamu-B*29   .....GG.........V.N.........  Mamu-B*53, -B*70, Ceat-B*12
Mamu-B*47   .....GG.........CN..........
Mamu-B*67   .....GG.....................M.  Mamu-B*68
Mamu-B*46   .....GG.........V...........K.
Mamu-B*50   .....GG.....F....N..........
Mamu-B*24   .....GG..R..................
Mamu-B*26   .....GGR....F...........E.....  Mamu-B*57
Mamu-B*61   .....GG.....F..V.N..........
Mamu-B*05   ..*..GG.........W...........
Mamu-B*07   .....GG.....F....N.......E....
Mamu-B*27   .....GG.........V..........
Mamu-B*43   .....GE.....F....K.N.....E..M.
Mamu-B*64   .....GGR-.....V.N..........
Mamu-B*19   .....GG.....F....K..P....E..M.  Mamu-B*22, -B*41, -B*58, -B*65
Mamu-I*01   .....GG...........N..........  Mamu-I*02, -I*03, -I*08, -I*09, -I*10, -I*11
Mamu-E*01   .....G.........SC...T....E......A  Mamu-E*03, -E*07, -E*09
Mamu-E*02   W....G...R.....SC.........E....Y.A
Mamu-E*0201 .....G.........LC...T...EE....Y.T
Mamu-E*05   .....G...W.....VG...S....D....Y.P
Ceat-E*01   .....G.........VC..G.....E....Y.LA
```

Fig. 3: Cytoplasmic tails of human, rhesus macaque and sooty mangabey MHC-I sequences. Amino acid sequences corresponding to the cytoplasmic domains of human (HLA), rhesus macaque (Mamu) and sooty mangabey (Ceat) MHC-I molecules are aligned to the cytoplasmic tail sequence of HLA-A. MHC-I sequences indicated in bold were selected for the analysis of selective downregulation by Nef. Additional rhesus macaque and sooty mangabey MHC-I molecules with identical cytoplasmic domains are listed to the right of each index sequence. The positions of residues previously shown to contribute to selective HLA downregulation by HIV-1 Nef are shaded in gray (Cohen *et al*., 1999). Amino acid identity is indicated with a dot, translational stop sites are indicated with asterisks and residues are numbered according to Boyson *et al.*, 1996.

3.1.2 Selection of *nef* alleles representing most primate lentiviral lineages

To determine whether specific MHC-I modulation is a general property of primate lentiviruses the following Nefs were selected based on their species origin

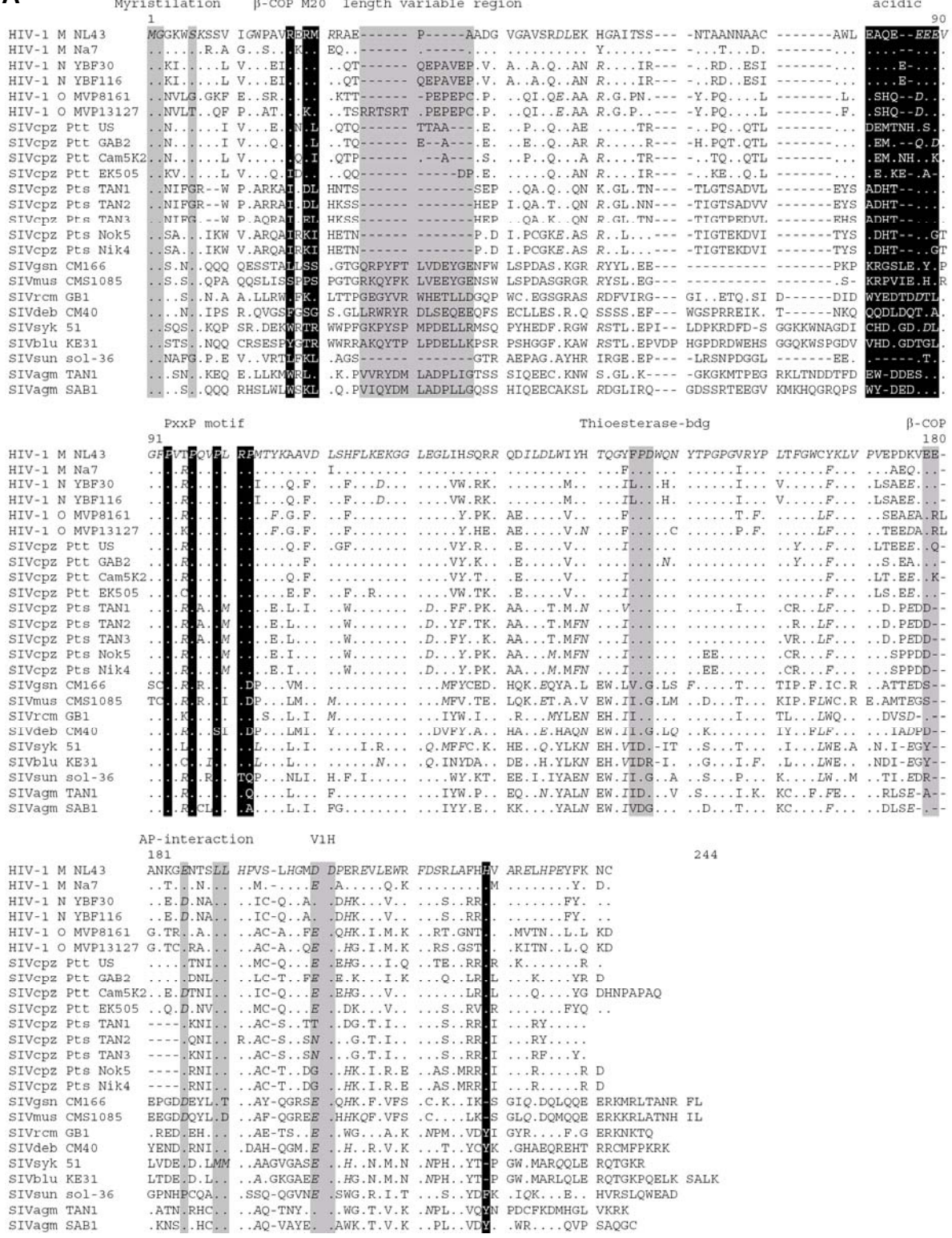
and phylogenetic relationship (Schindler *et al*., 2006). The first set encompasses the HIV-1 M, N and O groups, which arose from independent transmissions of SIVcpz *P.t.t.* and/or SIVgor to humans (Hahn *et al*., 2000; Keele *et al*., 2006; Van Heuverswyn and Peeters, 2007) as well as SIVcpz from both *P.t.t.* and from *P.t.s.* (*Pan troglodytes schweinfurthii*) that has not been found in humans (Hahn *et al*., 2000). Notably, the great majority of SIVcpz *nef* alleles used in this study does not contain adaptive changes to human cells, because they were PCR-amplified directly from uncultured chimpanzee material, such as the spleen (US), PBMC (GAB2 and Ch-Ni), plasma (Ch-No), and feces (TAN1, TAN2 and TAN3) (Kirchhoff *et al*., 2004; Takehisa *et al*., 2007). Furthermore, a variety of SIVs from different primate lentiviral lineages was tested in this thesis, including the precursors of SIVcpz, which are SIVrcm from red-capped mangabeys (*Cercocebus torquatus*), as well as SIVgsn and SIVmus from greater spot-nosed and mustached monkeys (*Cercopithecus nictitans* and *mona*, respectively), which most likely recombined in chimpanzees (Bailes *et al*., 2003). The second set encompasses *nef* alleles from SIVsmm, a virus adapted to its natural host; SIVmac, obtained after experimental infection of rhesus macaques; HIV-2, which resulted from multiple zoonotic transmissions of SIVsmm from sooty mangabeys to humans (Hahn *et al*., 2000); and HIV-1 as control. Altogether, Nef alleles from both human viruses, their precursors, the virus used within the SIV/macaque model as well as viruses from most other primate lentivirus lineages were included in the analysis.

3.1.3 Motifs involved in MHC-I modulation by HIV-1 Nefs are poorly conserved in other primate lentiviruses

To examine whether residues proposed to be critical for MHC-I downmodulation by HIV-1 are conserved within other primate lentiviruses, an alignment of the amino acid sequences of the Nef alleles selected for this analysis was made (Fig. 4). The alignment reveals substantial variation in the M20 residue, the β-COP RxR motif within the N-terminal α-helix, the acidic region and the proline-rich motif, which have all been implicated in MHC-I downmodulation and degradation by HIV-1 Nef (Greenberg *et al*., 1998; Mangasarian *et al*., 1999; Blagoveshchenskaya *et al*., 2002; Schaefer *et al*., 2008). M20 (numbering refers to the alignment shown in Fig. 4) in the N-terminal α-helix is conserved in HIV-1 Nefs

but not in those of HIV-2 or SIV (Fig. 4). The RxR motif, published to be a binding site for β-COP and needed for maximal MHC-I degradation (Schaefer *et al*., 2008), is also not conserved in some SIVs as well as in SIVcpz *P.t.s.* and one HIV-2 strain (Fig. 4). The acidic region (EEEE65), proposed to represent a PACS-1 binding site (Piguet *et al*., 2000), is conserved in HIV-1 group M and N Nefs but

A



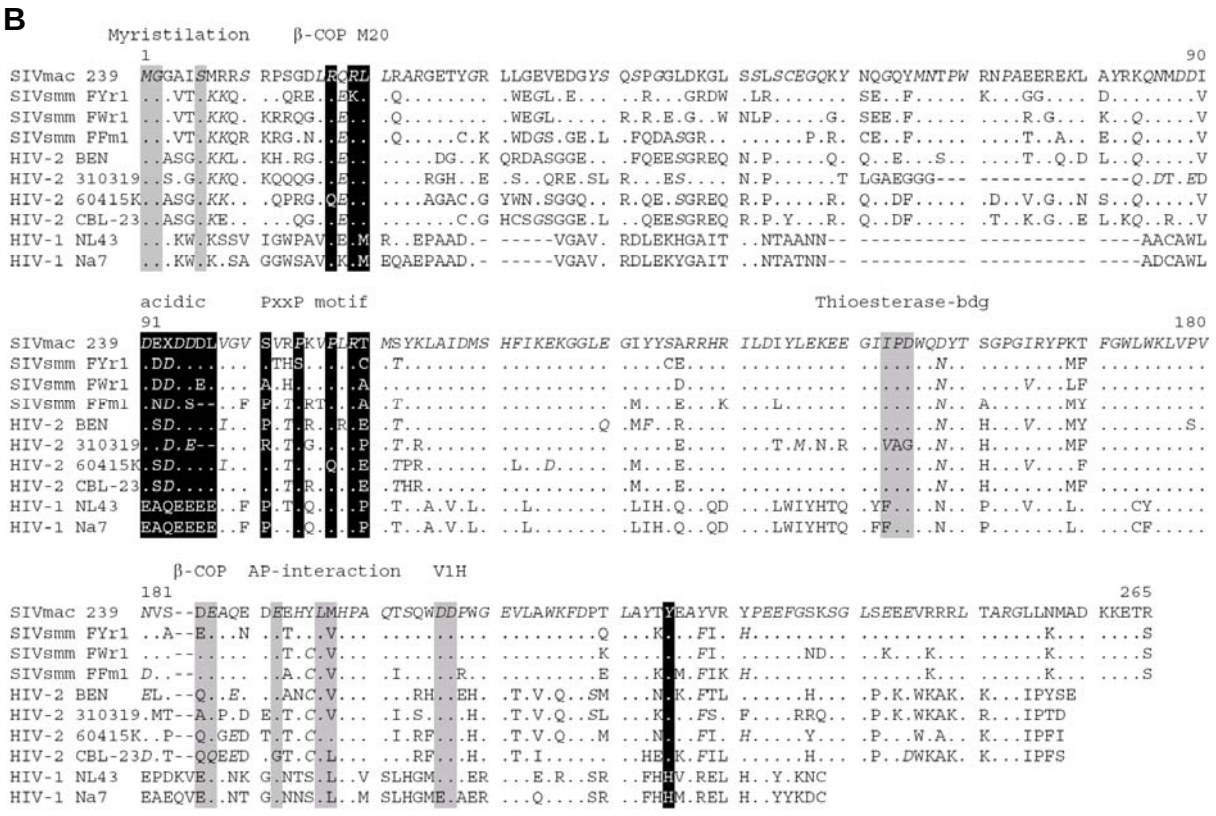


Fig. 4: Amino acid alignment of HIV and SIV Nef proteins.
(A) In the first set Nef alleles of HIV-1, SIVcpz and other SIVs are compared to the NL4-3 sequence and (B) in the second set Nef alleles of HIV-2, SIVsmm and HIV-1 are compared to the SIVmac sequence. The M20 residue, the RxR βCOP motif, the acidic region, the proline-rich domain and the tyrosine at the C-terminal part, which have all been implicated in HIV and SIV MHC-I downmodulation and degradation are shaded in black and some conserved sequence elements in Nef are shaded in gray. Dots indicate identity with the reference sequences, dashes indicate gaps introduced to optimize the alignment and asterisks stop codons.

variable in all remaining Nef sequences (Fig. 4). It is controversial, whether PACS-1 is required for Nef-mediated rerouting of MHC-I to the TGN (Piguet *et al*., 2000; Blagoveshchenskaya *et al*., 2002; Lubben *et al*., 2007). The proline-rich motif $(PxxP)_3$ implicated in binding of cellular kinases and MHC-I modulation (Greenberg *et al*., 1998; Yamada *et al*., 2003), is preserved in HIV-1 and SIVcpz Nefs, however, the last proline is changed mostly to aspartic acid or glutamine in many SIV Nefs (Fig. 4). Similarly, a thioesterase binding site (FPD123), known to be critical for multiple HIV-1 Nef functions including MHC-I downmodulation (Roeth and Collins, 2006), is preserved in SIVcpz but altered in the remaining HIV-2 and SIV Nef sequences. A tyrosine in the C-terminal region of SIVmac Nef also plays a role in MHC-I downregulation (Swigut *et al*., 2000) and is present in most SIV Nefs (Fig. 4). Thus, the domains previously implicated in

MHC-I removal from the cell surface and binding by HIV-1 Nef show substantial variation between the HIV-1, HIV-2 and SIV Nef sequences investigated.

3.1.4 Selective HLA-A and -B modulation is conserved between HIV-1 and its simian counterpart SIVcpz

First, the selective removal of HLA-molecules from the surface of infected cells by different HIV-1 groups and their counterpart SIVcpz, was determined. In agreement with previous studies (Kirchhoff *et al*., 2004; Schindler *et al*., 2006) my data showed that the great majority of HIV-1 and SIVcpz Nefs efficiently downregulate endogenous human MHC-I (examples shown in Fig. 5, upper panel). Using the CD8-HLA chimeras it could be demonstrated that Nefs from HIV-1 group M, N and O specifically downmodulate HLA-A and -B, but not -C or -E (Fig. 5). The levels of HLA-A and -B removal are similar and correlate with each other and that of endogenous MHC-I (Fig. 5C). Unexpectedly, the HIV-1 O 8161 Nef moderately upregulates HLA-C surface expression (Fig 5A, lane 5 and 5B). Altogether, these results demonstrate that selective downmodulation of HLA-A and -B is a conserved property of all three groups of HIV-1.

A similar pattern of HLA-molecule removal from the surface of infected cells was found with Nefs derived from SIVcpz *P.t.t.,* the simian counterpart of HIV-1, as well as with Nefs from SIVcpz *P.t.s.*, which has not been detected in humans (Hahn *et al*., 2000) (Fig. 5B). This result is not unexpected, because molecules of the *HLA*- and *Patr-A*, *-B* and *-C* loci share identical cytoplasmic domain sequences and the HLA-E and Patr-E tail sequences are also similar (Fig. 3). The obtained results were confirmed using 721.221 cells expressing endogenous HLA-molecules.

Altogether, the ability to selectively downmodulate HLA-A and -B, but not -C or -E to avoid CTL and NK cell lysis, is well conserved between Nefs from all HIV-1 groups as well as from SIVcpz of both chimpanzee subspecies. Due to identical *A*-, *B*- and *C*-loci in chimpanzees the specific modulation of MHC-I molecules is most likely also preserved in this natural host.

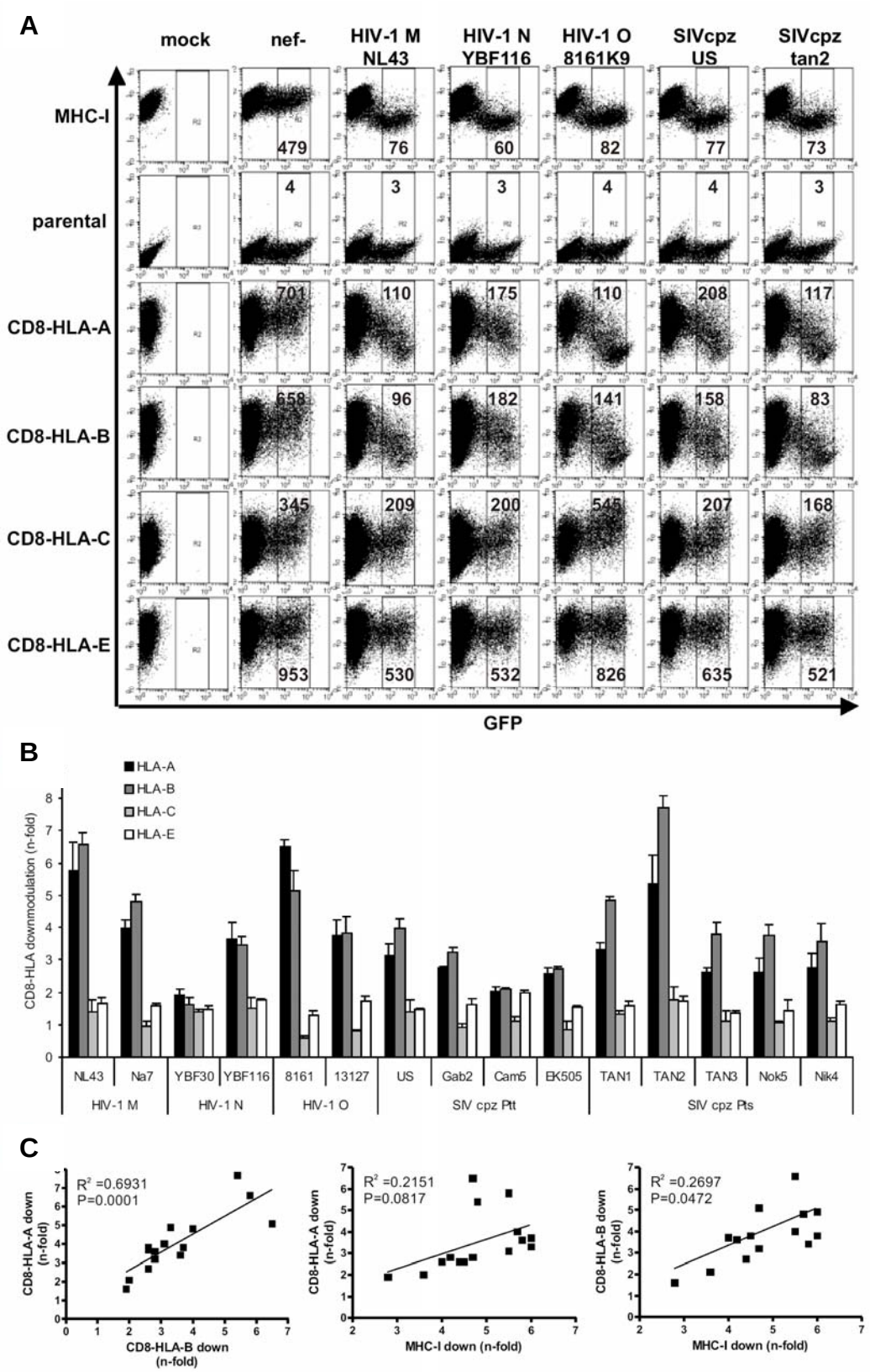


Fig. 5: HIV-1 and SIVcpz Nef alleles selectively downmodulate CD8 fusions to HLA-A and –B tails. Flow cytometric analysis of Jurkat T cell lines stably expressing CD8 fusions containing the cytoplasmic tails of the indicated HLA molecules. Cells were infected with HIV-1 recombinants expressing eGFP alone or together with the *nef* alleles indicated. (A) Representative examples of primary FACS data. The range of eGFP expression and the MFIs used to calculate receptor downmodulations are indicated. (B) Quantitative assessment of Nef-mediated downregulation of the indicated CD8-HLA chimeras. (C) Correlation of the efficiencies of Nef-induced HLA-A and -B downmodulation between one another and with endogenous human MHC-I.

3.1.5 Specific Nef-mediated MHC-I modulation is conserved between recent and natural hosts of primate lentiviruses

The specific Nef-mediated modulation of MHC-I molecules in humans, representing a recent host which progresses to AIDS, and sooty mangabeys, representing a natural host which shows no progression to AIDS, was determined. For the analysis HIV-2 and SIVsmm *nef* alleles were used as HIV-2 causes a pathogenic infection in humans and SIVsmm is non-pathogenic in sooty mangabeys. Earlier publications have already shown that the Nefs from both viruses efficiently downregulate endogenous human MHC-I (Münch *et al*., 2005; Schindler *et al*., 2006). Results of the present thesis demonstrated that Nefs from HIV-2 and the majority of SIVsmm strains also specifically downmodulate HLA-A and -B, but not -C or -E (Fig. 6B). The tested Nefs also selectively remove Ceat-A and -B molecules while leaving Ceat-E on the surface of infected cells (Fig. 6A). These results were expected, because the cytoplasmic tails of MHC-I-A and -B molecules of humans and sooty mangabeys are identical or highly similar (Fig. 3). However, sooty mangabeys have no *C*-loci and their Ceat-E*01 tail sequence is different from HLA-E, but still contains a substitution within a residue critical for Nef interaction (Cohen *et al*., 1999). Thus, the ability to specifically modulate MHC-I molecules to evade the immune system is well conserved between HIV-2 and its simian counterpart SIVsmm within their hosts representing recently and naturally infected hosts.

3.1.6 Selective HLA modulation is conserved between different lineages of primate lentiviruses

Next, it was determined if specific human MHC-I modulation is also conserved in other primate lentiviral lineages, including those from the precursors of SIVcpz, i.e. SIVrcm and SIVgsn/mus/mon (Bailes *et al*., 2003). My results demonstrated that *nef* alleles from SIVgsn, SIVmus, SIVsyk (from sykes monkeys), SIVblu (from blue monkeys) and SIVagm sab1 (from african green sabaeus monkeys) selectively remove HLA-A and –B from the surface of infected cells (Fig. 7). In comparison, the SIVrcm, SIVdeb (from de brazza monkeys), SIVsun (from sun-tailed monkeys) and SIVagm tan1 (from african green tantalus monkeys) Nefs display little if any effect on HLA molecule surface expression

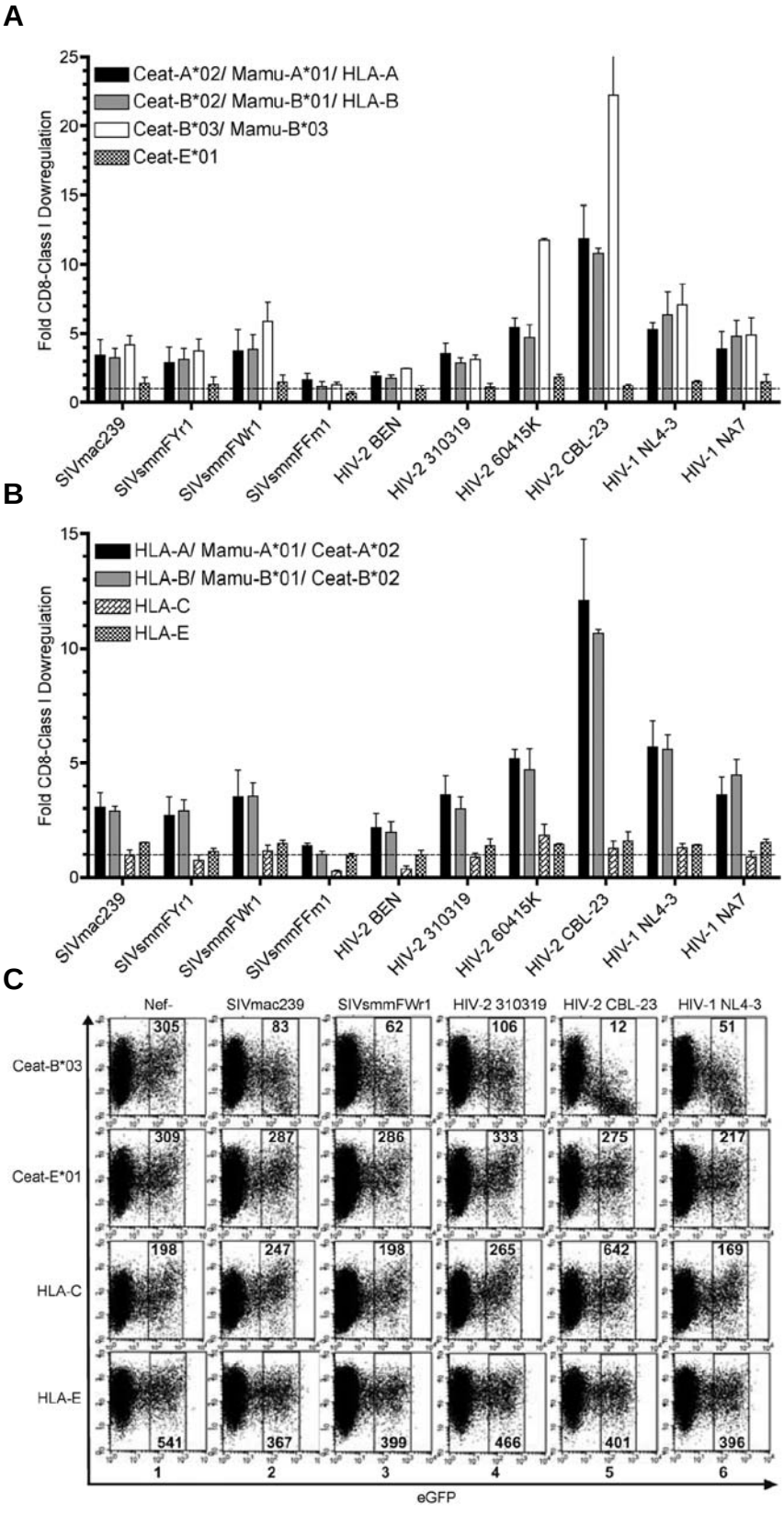


Fig. 6: Selective downmodulation of CD8 fusions to sooty mangabey and human MHC-I molecules by Nefs of SIVmac/smm and HIV. Flow cytometric analysis of Jurkat T cell line stably expressing CD8 fusions containing the cytoplasmic tails of the indicated sooty mangabey (Ceat) and human (HLA) molecules. Cells were infected with HIV-1 recombinants expressing eGFP alone or together with the *nef* alleles indicated. Quantitative assessment of Nef-mediated downregulation of the indicated (A) CD8-Ceat and (B) CD8-HLA chimeras. (C) Representative examples of primary FACS data for the modulation of CD8-Ceat and –HLA fusions. The range of eGFP expression and the MFIs used to calculate receptor downregulation are indicated.

(Fig. 7A), however, they clearly reduce endogenous human MHC-I surface expression (Fig. 7).

In summary, the ability to selectively downregulate specific human MHC-I molecules from the surface of infected cells is conserved between all groups of primate lentiviruses including HIV and its simian counterparts.

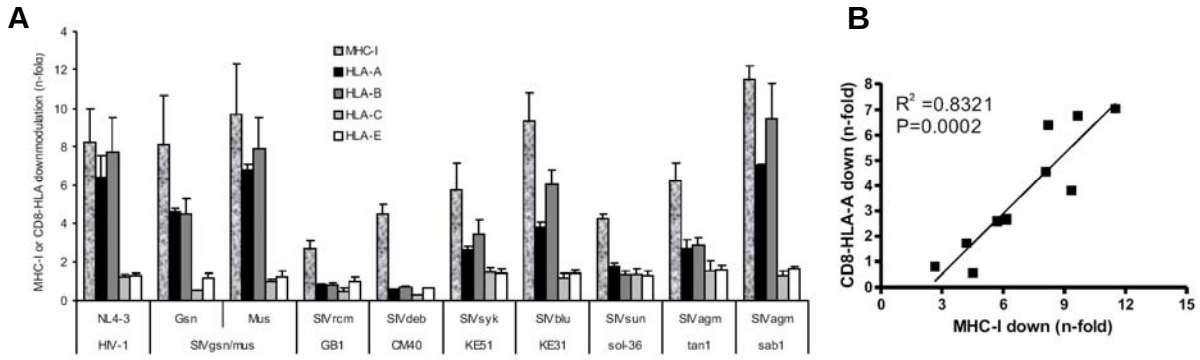


Fig. 7: Selective downmodulation of HLA-A and -B is conserved between highly divergent primate lentiviral Nefs. (A) Quantitative assessment of Nef-mediated downmodulation of MHC-I and the indicated CD8-HLA chimeras. (B) Correlation between the efficiency of Nef-induced CD8-HLA-A and endogenous human MHC-I downregulation.

3.1.7 Specific MHC-I modulation in the rhesus macaque model

SIV infection of rhesus macaques is an important animal model for the development of AIDS vaccines and for the analysis of primate lentiviral pathogenesis (Fultz *et al*., 1989; McClure *et al*., 1990). Therefore, it is important to know whether the immune evasion mechanisms, such as selective MHC-I modulation to evade NK cell and CTL lysis is preserved in this common model for AIDS in humans. Thus, the abilities of HIV-1, HIV-2 and SIVmac in selective removal of Mamu- and HLA-MHC-I molecules were compared in this thesis. SIVmac Nef only specifically downregulates CD8 fusions to the Mamu-A, -B*01 and -B*03 tails, while leaving the others on the surface of infected cells (Fig. 8). Additionally, HIV-1 and HIV-2 Nefs reduce the surface expression of Mamu-B*07 or other Mamu-B and -E molecules, respectively (Fig. 8). Furthermore, like the previously tested HIV-1 and HIV-2 Nefs, SIVmac also selectively downmodulates HLA-A and -B (Fig. 6B). Altogether, the ability of HIV and SIVmac Nef to specifically downregulate MHC-I molecules from the rhesus macaque model is generally conserved.

The rhesus macaque also represents a recently infected host, like humans (see 3.1.5). The precursor of SIVmac is, like for HIV-2, SIVsmm from sooty

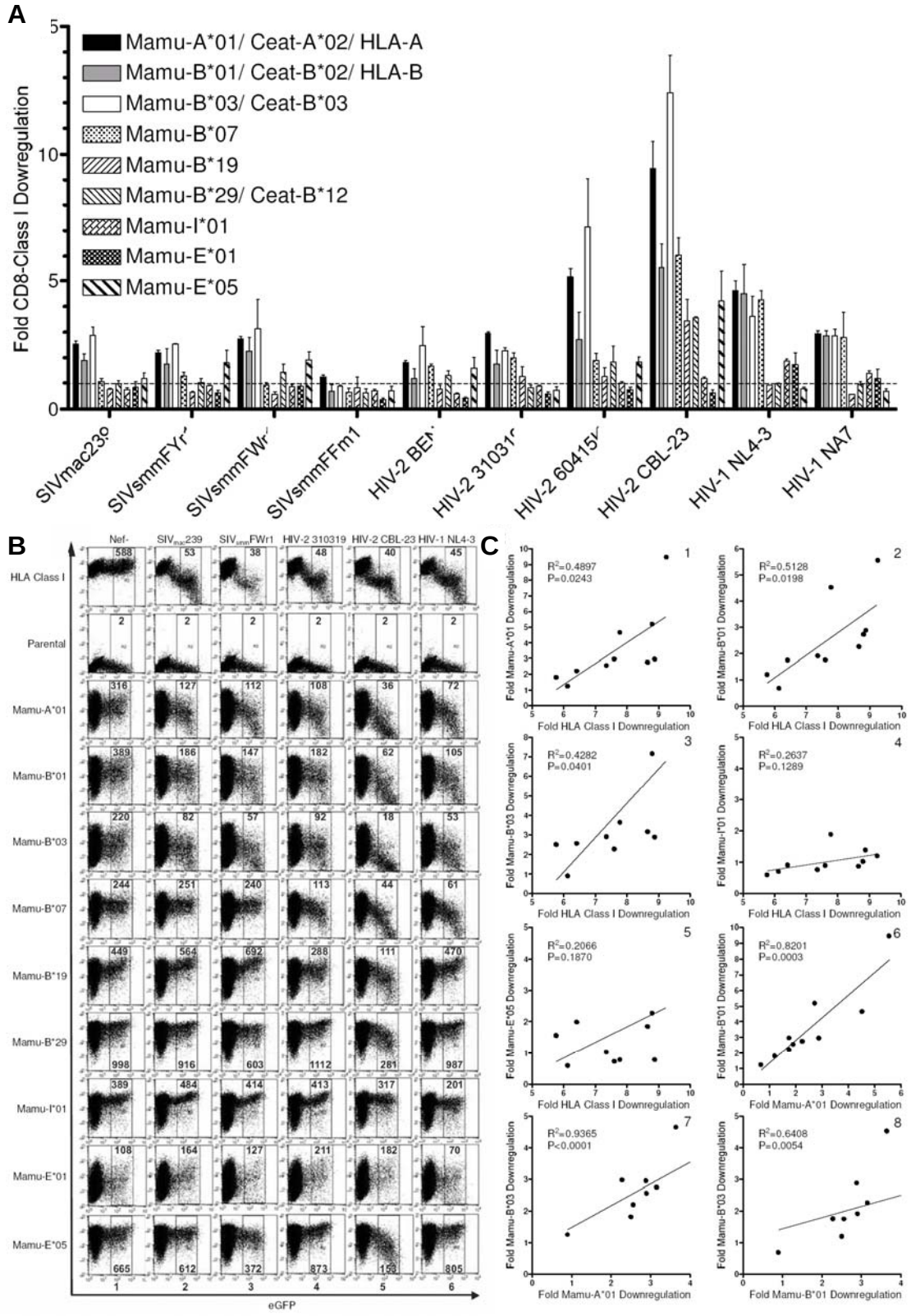


Fig. 8: Selective downmodulation of CD8 fusions to rhesus macaque MHC-I molecules by Nefs of SIVmac/smm and HIV. Flow cytometric analysis of Jurkat T cell lines stably expressing CD8 fusions containing the cytoplasmic tails of the indicated rhesus macaque (Mamu) molecules. Cells were infected with HIV-1 recombinants expressing eGFP alone or together with the *nef* alleles indicated. (A) Quantitative assessment of Nef-mediated downregulation of the indicated CD8-Mamu chimeras. (B) Representative examples of primary FACS data. The range of eGFP expression and the MFIs used to calculate receptor downmodulations are indicated. (C) Correlations between Nef-mediated downmodulation of specific CD8-Mamu fusions with each other and with endogenous human MHC-I.

mangabeys (Hirsch *et al*., 1989; Hahn *et al*., 2000). The present results show that specific removal of Mamu- and Ceat-MHC-I molecules is generally conserved between SIVmac and its counterpart SIVsmm within their recently and naturally infected hosts, respectively.

In summary the data suggest that the ability to selectively modulate MHC-I molecules is generally shared by different primate lentiviral Nefs to simultaneously evade CTL and NK cell lysis.

3.2 Effects of a SNP near the *HLA-C* gene on HIV-1 Nef function

Some HIV-1-infected individuals carry a SNP, rs9264942, which is located upstream of the *HLA-C* gene (HLA-C SNP) and associated with reduced VLs (Fellay *et al.*, 2007). As outlined above, HIV-1 is unable to downmodulate HLA-C from the cell surface. Thus, HLA-C-mediated antigen presentation may be particularly effective in mediating CTL lysis of HIV-1-infected cells. Therefore, the HLA-C SNP may help infected individuals to control HIV-1 because it affects the efficiency or the quality of HLA-C-mediated antigen presentation. One aim of this thesis was to examine, whether HIV-1 Nef variants capable of downmodulating HLA-C emerge in some HLA-C SNP HIV-1-infected individuals or whether other changes in Nef facilitate effective viral persistence in patients with this polymorphism.

3.2.1 Generation and molecular characterization of proviral HIV-1 constructs expressing patient *nef* alleles

To assess if Nef plays a role in the development of high or low setpoint VLs in HLA-C SNP individuals, *nef* alleles from 50 HIV-1-infected individuals with, “C-SNP”, (n=25) or without, “WT” (wildtype), (n=25) the polymorphism were analyzed. The Nefs were further subdivided to distinguish those derived from people with high (“C-SNP” $10^{4.8\pm0.3}$, n=19 and “WT” $10^{5.2\pm0.6}$, n=19) and low (“C-SNP” $10^{2.7\pm0.4}$, n=6 and “WT” $10^{2.3\pm0.3}$, n=6) setpoint VLs (table 1). On average, the VLs differed by more than two orders of magnitude between the “high” and “low” groups but were relatively homogeneous within the hi-C-SNP, low-C-SNP, hi-WT and low-WT groups (Fig. 9A). In comparison, the CD4+ T cell counts showed substantial intra-group variations (Fig. 9B) and correlated only weakly with the setpoint VLs (Fig. 9C).

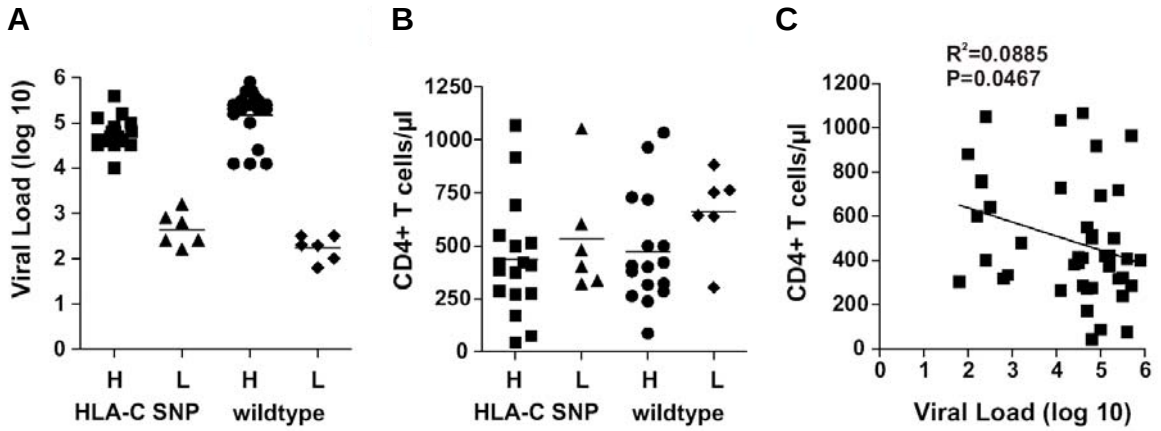


Fig. 9: Characteristics of the HIV-1-infected individuals analyzed. (A) Setpoint VLs and (B) CD4+ T cell counts in the HIV-1-infected individuals at the time of PBMC or plasma sampling for *nef* analysis. Patient samples were grouped based on the presence (HLA-C SNP) or absence (wildtype) of the rs9264942 SNP and the VLs (H, high; L, low). (C) Correlation between the setpoint VLs and CD4+ T cell counts.

For functional analyses, *nef* alleles from all patients were cloned in bulk into the replication-competent HIV-1-NL4-3-IRES-eGFP proviral construct coexpressing Nef and eGFP from bi-cistronic RNAs (Schindler *et al.*, 2003, Schindler *et al*., 2005). To examine the accuracy of these constructs three to 12 individual proviral clones derived from each patient (a total of 224 clones) were sequenced. Sequence analysis revealed that 195 of the 224 proviral NL4-3 constructs (87.1%) contain intact full-length Nef open reading frames (ORFs). The remaining *nef* alleles contain premature stop-codons and frameshift mutations at various positions.

Phylogenetic analysis showed that the great majority of *nef* alleles form patient-specific clades (Fig. 10), but there were three exceptions. Nef amino acid sequences from patient 5466 form two distinct clusters and show a high degree of diversity at their N-terminus. In contrast, Nefs derived from patients 5859 and 5661 are very closely related with only two consistent differences, i.e. T45S/S46N and I194V. To exclude the possibility of contamination additional independent *nef* alleles from the patients were cloned and analyzed and the results confirmed that patient 5466 contains two groups of *nef* alleles varying specifically in their 5` region, whereas all Nef sequences derived from individuals 5859 and 5661 are closely related. More importantly, the sequence analysis showed that most *nef* ORFs are intact and specific for the respective patient samples.

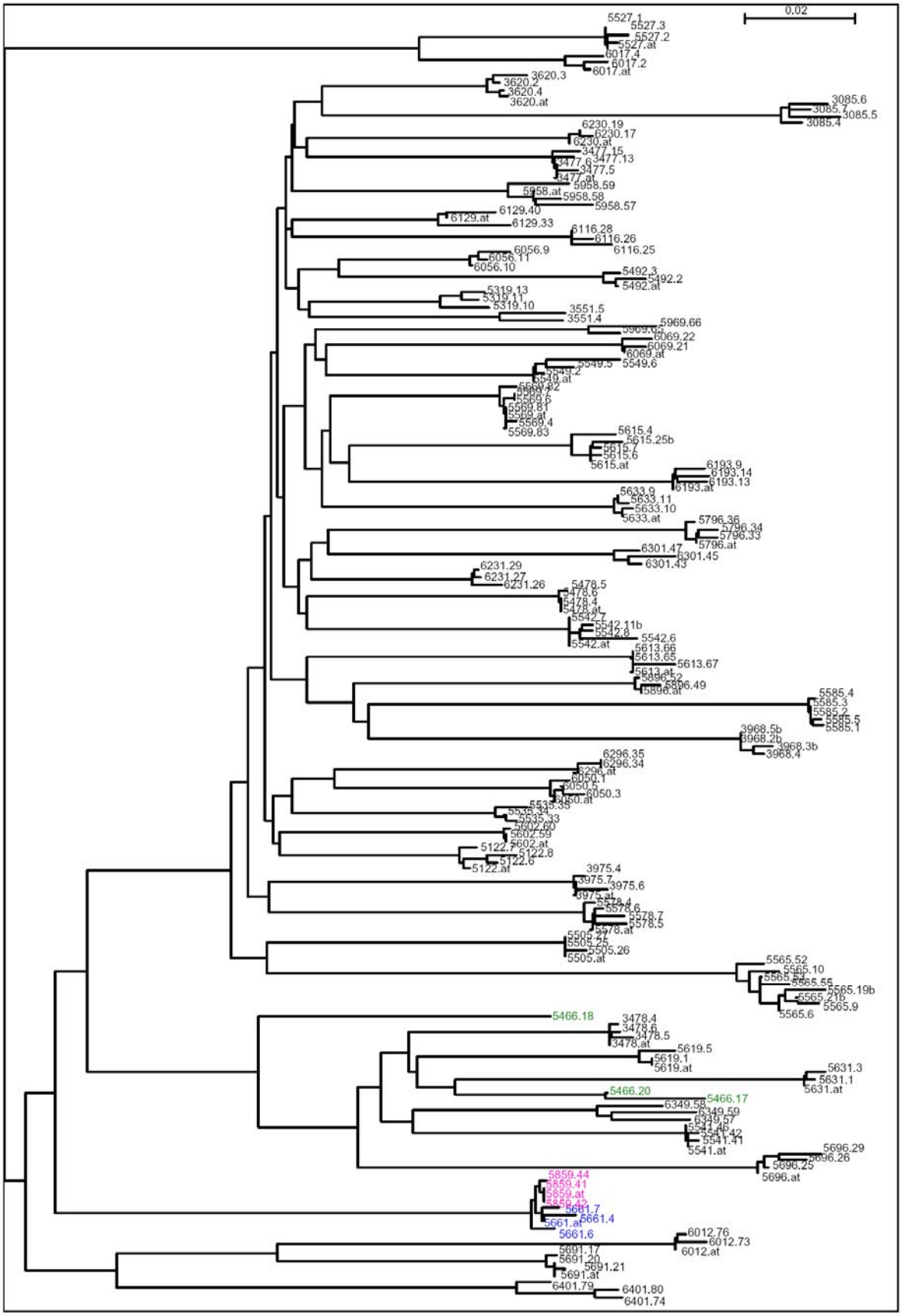


Fig. 10: Evolutionary relationship among HIV-1 Nef sequences derived from individuals with or without the HLA-C SNP. The phylogenetic tree was constructed by the Bayesian method using the general reversible (GTR) model of evolution. The scale bar indicates 0.02 substitutions per site. *nef* alleles from patient 5466, 5859 and 5661 are highlighted in green, pink and blue, respectively.

Evaluation of all consensus Nef amino acid sequences revealed that motifs of known functional importance, such as the N-terminal myristoylation signal, the acidic region, the $(PxxP)_3$ motif, the ExxxLL endocytosis motif in the C-loop and a V1H interaction site (Geyer *et al.*, 2001), are usually highly conserved (Fig. 11). However, two of three clones derived from patient 3968 have a P97L substitution in the proline-rich region and *nef* alleles from five individuals contain small in frame deletions within the di-acidic motif in the C-terminal loop that interacts with ß-COP (Benichou *et al.*, 1994). The sequence analysis did not reveal any obvious group-specific sequence differences.

To ensure the expression of the *nef* alleles western blot analyses of 293T cells transfected with the proviral constructs were made and confirmed that all of them expressed the p24 core antigen and eGFP. Nef could also be detected in most but not all samples, most likely because the pooled patient sera used did not detect all heterologous primary Nef variants. However, the subsequent analysis showed that all proviral constructs express functional Nef proteins.

3.2.2 High VLs in patients with the rs9264942 SNP are not associated with Nef-mediated downmodulation of HLA-C

To determine whether *nef* alleles capable of downmodulating HLA-C may emerge in some HIV-1-infected individuals, particularly in those with the HLA-C SNP, Jurkat T cells expressing the CD8-human MHC-I chimeras were transduced with HIV-1-NL4-3-IRES-eGFP proviral constructs expressing the Nef pools isolated from the patients. Flow cytometric analyses showed that Nefs derived from the great majority of patients clearly removed HLA-A and -B fusions as well as endogenous MHC-I from the surface of infected cells (Fig. 12A and B). In contrast, none of the *nef* alleles analyzed reduced the surface expression of HLA-E chimeras, whereas some Nefs were capable of downmodulating HLA-C, albeit usually with substantially lower efficiency than HLA-A and -B (Fig. 12A). This was confirmed by experiments using the 721.221 cell line expressing the unaltered HLA-Cw4 molecule (Cohen *et al.*, 1999). However, Nefs from a single individual of the low-WT group downregulated HLA-A, -B and -C with comparable efficiency suggesting that in rare cases Nef may evolve towards better interaction with the HLA-C cytoplasmic tail (Fig. 12A). On average, *nef* alleles derived from patients with high VLs are slightly more active in downmodulating MHC-I compared to

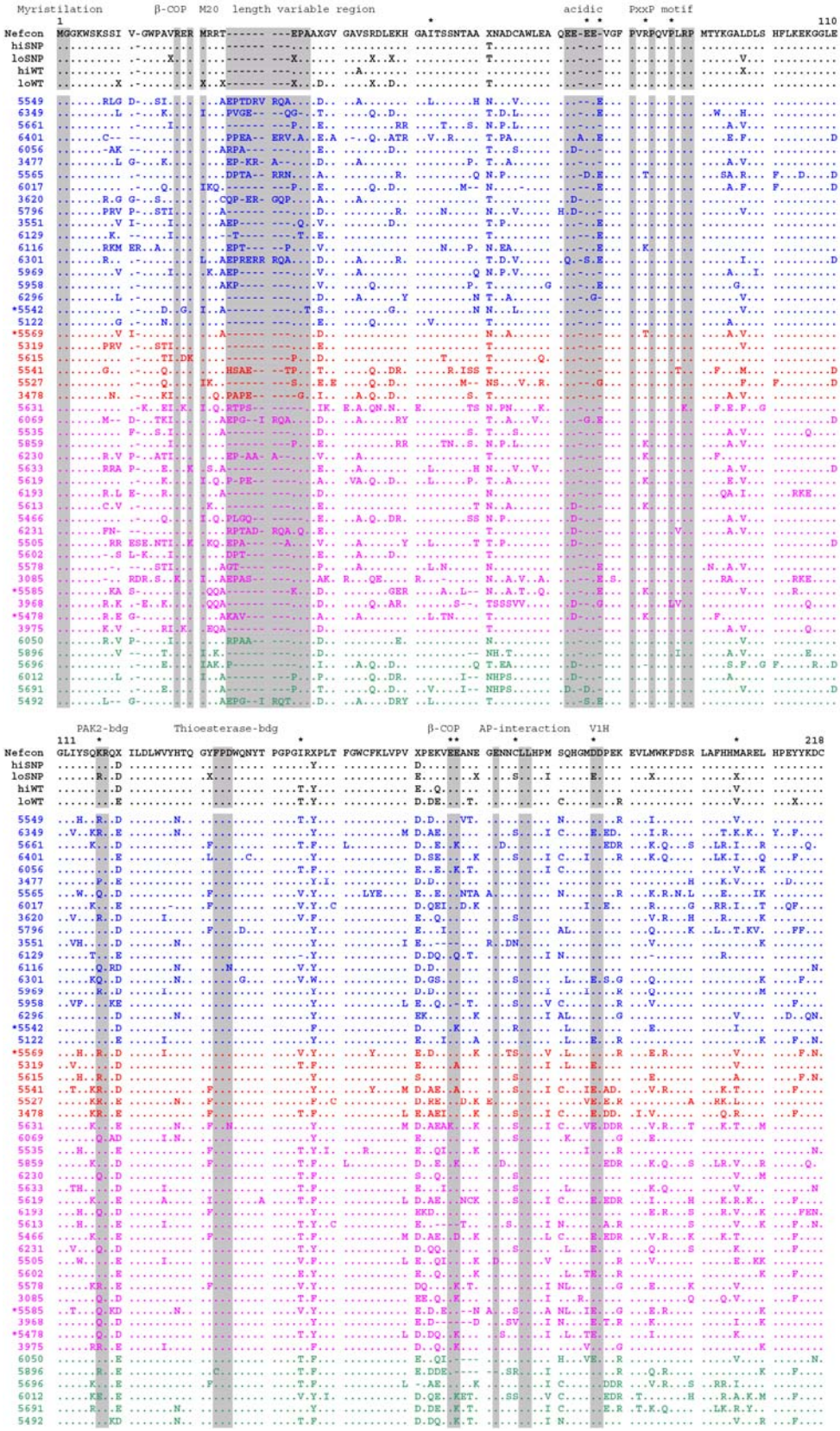


Fig. 11: Amino acid alignment of consensus Nefs isolated from patients. The consensus Nef sequences from HIV-1-infeced individuals grouped based on their genotype and VLs are shown in the upper panels. Nefs derived from plasma RNA are indicated by an asterisk. The order of sequences corresponds to the VLs in the respective patients. Some conserved sequence elements in Nef are shaded in gray. Asterisks above the alignment indicate positions where amino acid variations were observed; dots indicate identity with the consensus sequence, dashes indicate gaps introduced to optimize the alignment.

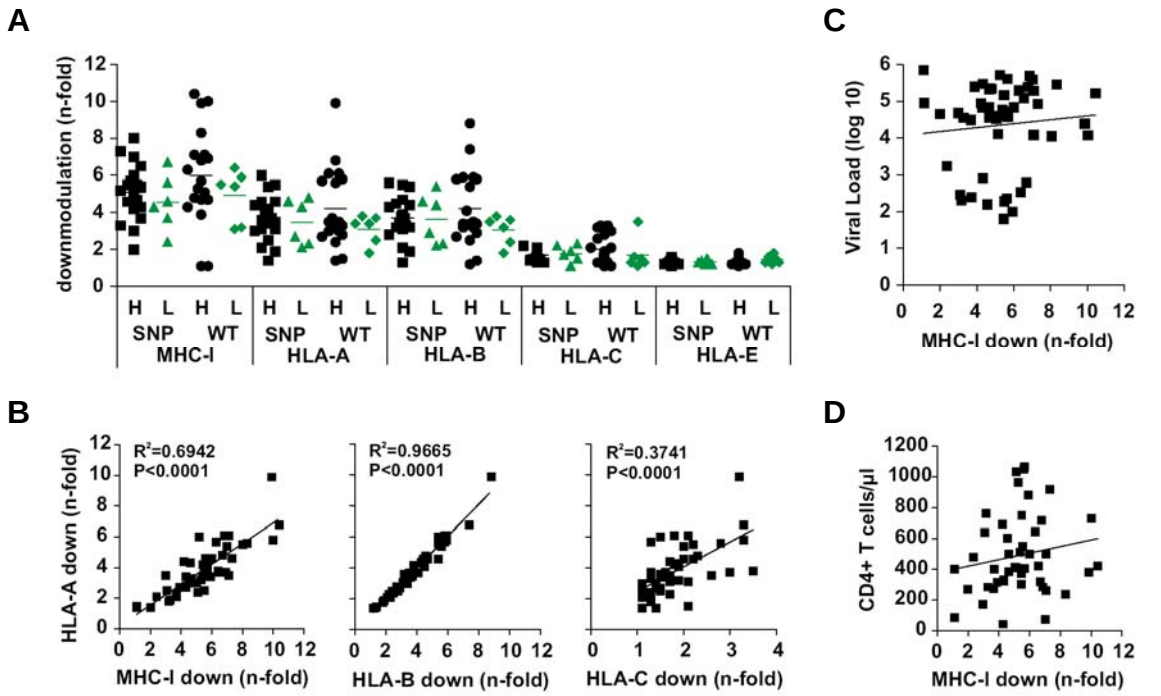


Fig. 12: Modulation of HLA-molecules by Nef alleles derived from patients with and without the HLA-C SNP. (A) Quantitative assessment of Nef-mediated downmodulation of endogenous human MHC-I and CD8-HLA fusions in Jurkats. HIV-1 *nef* genes were grouped by the genotypes and VLs of the patients from which they were amplified; the low VL Nefs are colored green. (B) Correlation analyses between downmodulation of HLA-A and MHC-I, HLA-B and HLA-C, (C) between the efficiency of Nef-mediated MHC-I downregulation and the patient VLs and (D) between MHC-I downmodulation and CD4+ T cell counts of the patients.

those derived from individuals with low VLs (Fig. 12A), but the differences are not statistically significant. There is also no correlation between Nef-mediated downregulation of MHC-I or the CD8-HLA fusions with the VLs or CD4+ T cell counts of the respective patients (Fig. 12C and D). Thus, high setpoint VLs in HIV-1-infected individuals are not associated with significantly increased Nef-mediated removal of MHC-I from the surface of infected cells. Further, effective viral persistence in individuals with the HLA-C SNP is not due to the acquisition of changes allowing Nef to downmodulate HLA-C.

3.2.3 High setpoint VLs are associated with effective modulation of CD4, CD28 and Ii by Nef in HLA-C SNP individuals

Nef is also capable of manipulating the function and interaction of APCs and T cells by modulating the surface expression of several other receptors involved in antigen presentation (Kirchhoff *et al*., 2008). To analyze whether the different VLs or the HLA-C SNP in some individuals are associated with changes in the receptor modulation abilities of Nef, the downregulation of CD4, one of the

best characterized and most relevant Nef activities, was determined. For this, proviral constructs defective in *vpu* and *env* were used since both gene products also reduce CD4 cell surface expression (Lama, 2003), thereby masking the effect of Nef. Unexpectedly, *nef* alleles from the hi-C-SNP group are significantly more active in downmodulating CD4 than Nefs within the low-C-SNP group (3.8±0.2 vs 2.9±0.2; p=0.027) (Fig. 13A). In contrast, Nefs from WT individuals with low VLs are all highly active in CD4 downregulation (4.8±0.3; p=0.0008) (Fig. 13A).

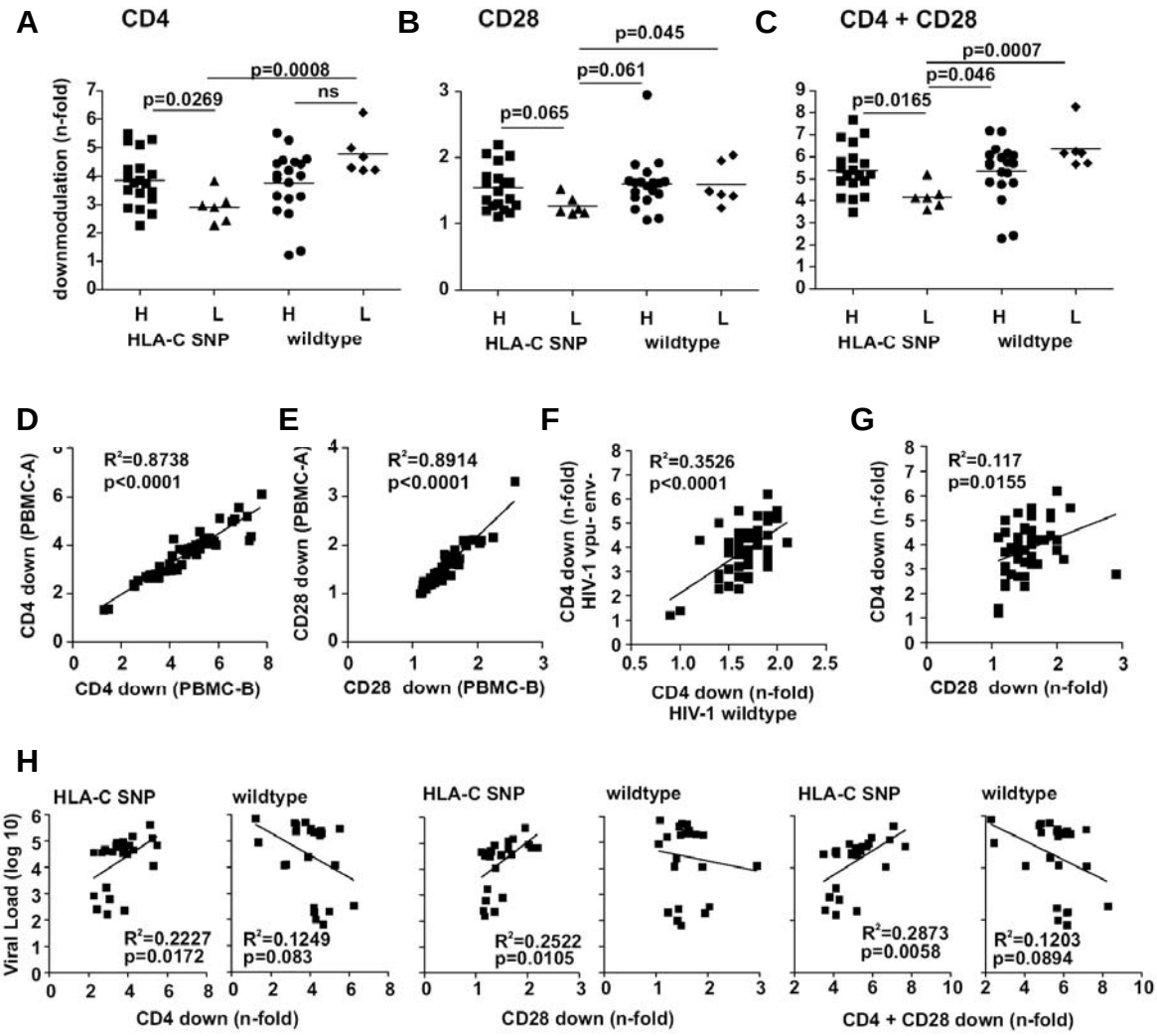


Fig. 13: Nef alleles derived from individuals with the HLA-C SNP and low VLs are poorly active in downmodulating CD4 and CD28. (A and B) Quantitative evaluation of Nef-mediated downregulation of CD4 and CD28 on PBMCs infected with HIV-1 Nef/eGFP (vpu- env* for CD4 modulation) constructs. Nefs were grouped by the genotypes and VLs of the patients from which they were amplified. (C) Quantitative evaluation of the synergistic effect of CD4 and CD28 downmodulation by the patients Nef alleles. (D) Correlation between CD4 or (E) CD28 downregulation in different PBMC donors. (F) Correlation between CD4 downmodulation in the NL4-3 vpu- env* or WT virus background and (G) between CD4 and CD28 downregulation in PBMCs. (H) Correlation analyses of CD4, CD28 or the sum of CD4 and CD28 downmodulations with patient VLs.

The evaluation of CD28 downmodulation revealed that the effects of patient Nefs were usually weaker than on CD4 (Fig. 13B). Notable, within the C-SNP group *nef* alleles from low VL individuals were usually less active in CD28 downregulation than those derived from high VL patients (Fig. 13B). Further analyses showed that the Nef effects on CD4 and CD28 cell surface expression were highly reproducible in PBMCs from different donors (Fig. 13D and E). As expected, a significant correlation was observed between the efficiencies of Nef-mediated CD4 downmodulation in the context of the *vpu-env* defective and the wt viruses, although the detectable effects were considerably weaker in the latter context (Fig. 13F). Furthermore, a weak correlation between the efficiency of CD4 and CD28 downregulation was found (Fig. 13G), which is in agreement with the previous finding that both functions are mediated by overlapping but distinct domains (Swigut *et al.*, 2001). More importantly, Nef-mediated effects on CD4 and CD28 correlate with the VLs from patients within the C-SNP group but not within the WT group (Fig. 13H). Furthermore, a strong correlation was found between the VLs and the sum of CD4 and CD28 downregulation within the C-SNP group (Fig. 13C), probably because both receptors are co-stimulatory ligands of TCR signaling and the effects of Nef on CD4 and CD28 may synergize to manipulate the functionality of T cells. Altogester, these data suggest that viruses infecting HLA-C SNP individuals have to improve their indirect immune evasion mechanisms for counteracting the strong immune response within these patients.

It has been published that Nef also downmodulates CXCR4 to disrupt ordered trafficking of infected leukocytes between local microenvironments (Hrecka *et al.*, 2005). Therefore, the ability to do so was determined for patient *nef* alleles in both Jurkats and PBMCs (Fig. 14A), because this chemokine receptor is internalized in primary T cells upon stimulation, which aggravates testing of this Nef function in PHA stimulated PBMCs. In agreement with previous studies (Hrecka *et al.*, 2005) all tested HIV-1 *nef* alleles had only weak effects on CXCR4 surface expression (Fig. 14B). Similarly to the results obtained on downregulation of CD4 and CD28, effective Nef-mediated removal of CXCR4 was usually associated with low VLs in the WT group of individuals, whereas the opposite trend was observed in individuals with the polymorphism (Fig. 14B and C). Although the overall effects and modulation differences were weak, these results

indicate that suppression of T cell migration may be more beneficial for effective viral persistence in the presence of the HLA-C SNP.

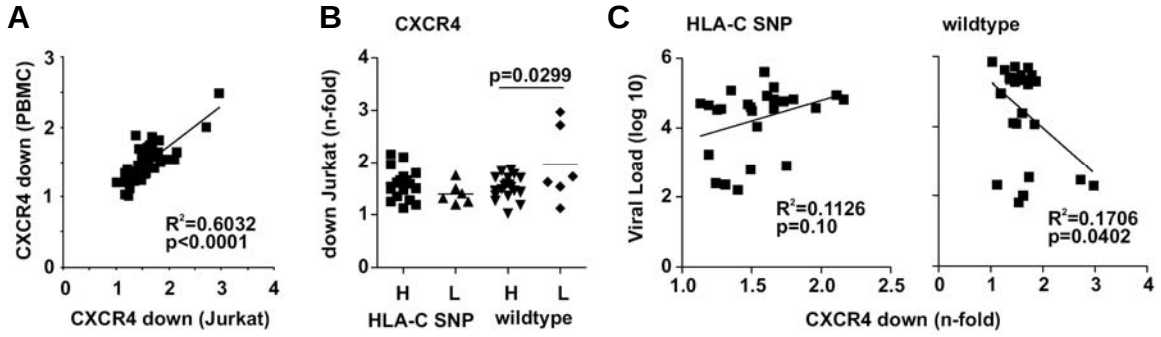


Fig. 14: Modulation of CXCR4 by Nef alleles derived from patients with and without the HLA-C SNP. (A) Correlation between CXCR4 downregulation by patient Nef alleles on Jurkats and PBMCs. (B) Quantitative evaluation of Nef-mediated downmodulation of CXCR4 on Jurkats infected with HIV-1 Nef/eGFP constructs. Nefs were grouped by the genotypes and VLs of the patients from which they were amplified. (C) Correlation of CXCR4 downregulation with patient VLs.

Nef impairs antigen presentation not only by manipulating T cells, but also by altering receptor surface expression on infected APCs, i.e. by upregulating the invariant chain (CD74, Ii) associated with the immature MHC-II-complex (Stumptner-Cuvelette *et al.*, 2001). Flow cytometric analysis revealed that the great majority of patient Nefs enhanced Ii surface expression on infected monocytic THP-1 cells (examples shown in Fig. 15A). *Nef* alleles from individuals with the HLA-C SNP and low VLs were significantly less active in upmodulating Ii than those obtained from individuals with high VLs (4.8±0.4 vs 3.1±0.6; p=0.048) (Fig. 15B). It was striking that only 4 of 19 hi-C-SNP Nefs (21.1%) but 4 of 6 low-C-SNP Nefs (66.7%) upregulated Ii less than 3-fold (Fig 15C). Further it could be observed that VLs in the C-SNP but not in the WT group significantly correlated with the efficiency of Nef-mediated upmodulation of Ii (Fig. 15D). These results support the assumption that ineffective suppression of MHC-II antigen presentation by Nef may be beneficial for individuals with the HLA-C SNP to control HIV-1 replication.

3.2.4 Correlation between Nef-dependent T cell activation and VLs in HLA-C SNP patients

It is well known that HIV-1 Nef proteins interact with various cellular factors involved in signal transduction (Renkema and Saksela, 2000; Greenway *et al.,* 2003) which might result in an altered responsiveness of virally

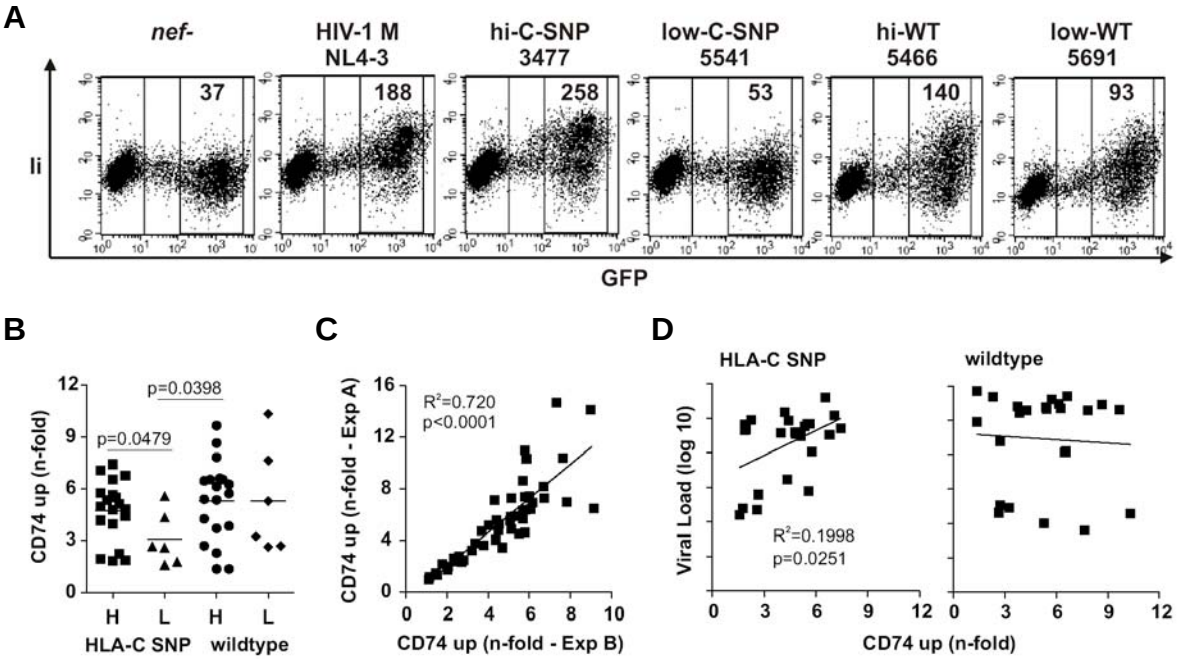


Fig. 15: Nef alleles derived from individuals with the HLA-C SNP and low VLs are poorly active in upmodulating Ii (CD74). (A) Representative examples of primary FACS data. The range of eGFP expression and the MFIs used to calculate receptor upregulation are indicated. (B) Quantitative evaluation of Nef-mediated upmodulation of Ii on THP-1 cells infected with HIV-1 Nef/eGFP constructs. Nefs were grouped by the genotypes and VLs of the patients from which they were amplified. (C) Correlation analysis between Ii upregulation results obtained in independent experiments. (D) Correlation of Ii upmodulation with patient VLs.

infected T cells to stimulation (Skowronski *et al.,* 1993; Schrager and Marsh, 1999; Wang *et al.,* 2000; Fortin *et al.,* 2004; Fenard *et al.,* 2005). One consequence is the hyper-activation of the nuclear factor of activated T cells (NFAT) (Fortin *et al.*, 2004), which regulates *IL-2* gene expression, a hallmark of T cell activation. To assess whether the patient *nef* alleles from the four groups differentially affect T cell activation, Jurkats stably tranfected with the *luciferase* reporter gene under the control of a NFAT-dependent promoter were transduced with the proviral HIV-1 constructs and their responsiveness to activation by PHA was measured. The results were surprising, as they showed a highly significant difference in the levels of NFAT-dependent luciferase activity between *nef* alleles from the hi-C-SNP and hi-WT groups (124.1±12.1, n=19 vs 181.2±10.6, n=19; p=0.0011) (Fig. 16A). Further, an inverse correlation between NFAT activation and VL within the C-SNP group was observed (R^2= 0.2715; p=0.0076) (Fig. 16B). The effects on NFAT activation varied substantially between the primary HIV-1 Nefs. The majority of the Nef alleles induced T cell activation as reported previously (Fortin *et al*., 2004), whereas a total of eight Nefs from the hi-C-SNP (n=6) and low-WT (n=2) groups suppressed NFAT activation (Fig. 16A).

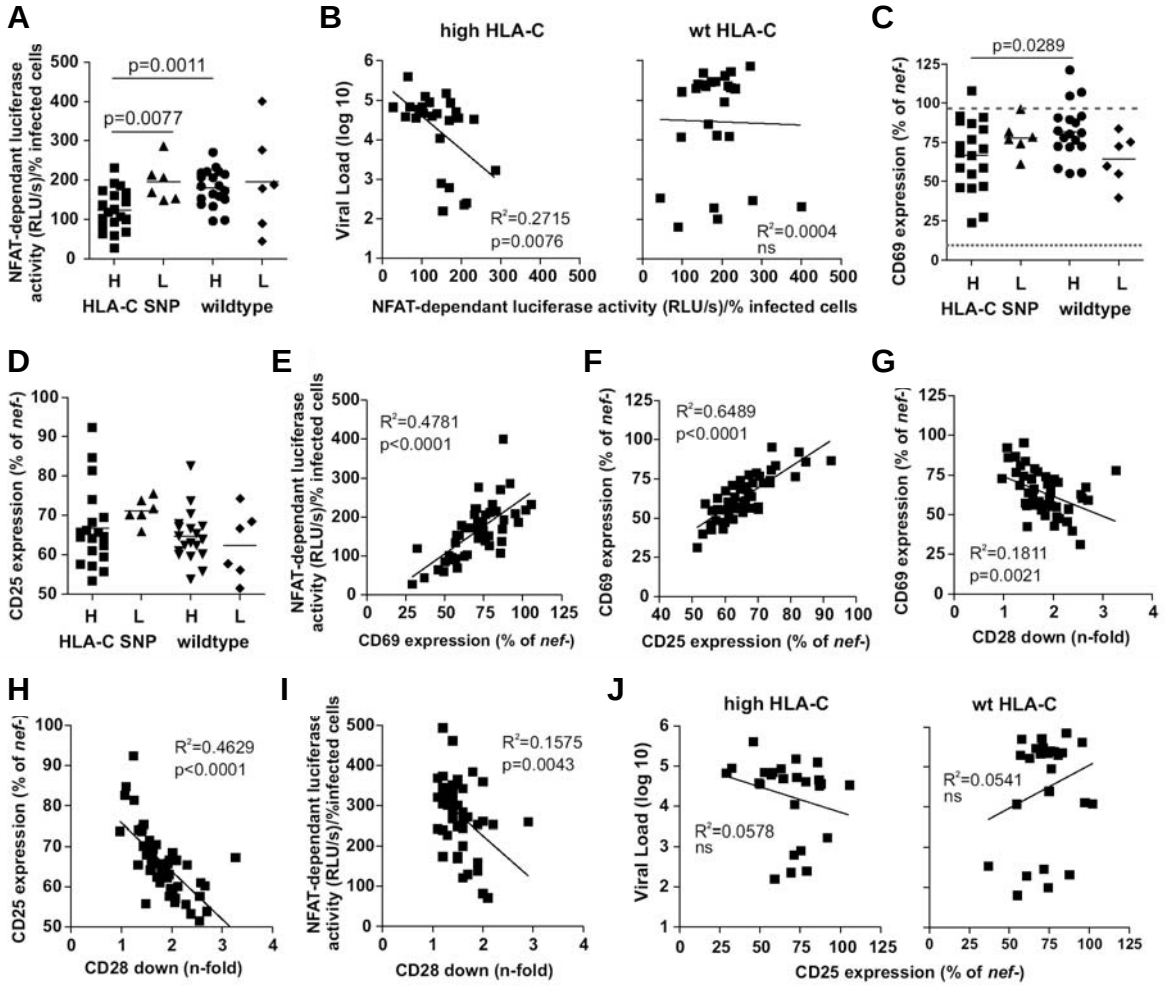


Fig. 16: Nef alleles derived from individuals with the HLA-C SNP and high VLs show reduced activity in inducing NFAT expression. (A) Quantitative evaluation of Nef-mediated induction of NFAT expression, measured in luciferase reporter activity in Jurkats infected with HIV-1 Nef/eGFP constructs, followed by PHA stimulation. Nefs were grouped by the genotypes and VLs of the patients from which they were amplified. (B) Correlation analysis between NFAT activity and the patient VLs. (C and D) Quantitative evaluation of Nef-mediated induction of CD69 and CD25 expression on PHA stimulated Jurkat-NFAT cells. (E) Correlations between Nef-induced CD69 surface expression and NFAT activity and (F) between CD25 and CD69 surface expression. (G - I) Correlations of Nef-mediated CD28 downmodulation with CD69, CD25 or NFAT expression. (J) Correlation analysis between CD25 surface expression and patient VLs.

In addition to NFAT activation the expression levels of CD69 and CD25, early and late T cell activation markers, respectively, were also measured, to further assess the activation status of the infected Jurkat-NFAT cells. On average, infected Jurkats expressing the *nef* alleles of the hi-C-SNP group expressed lower levels of CD69 and CD25 than those of the low-C-SNP group (Fig. 16C and D). However, these differences were less pronounced in comparison to the results obtained by the NFAT assay, possibly because the latter is more sensitive and has a higher dynamic range. Overall, however, the levels of CD69 and CD25 expression correlated with one another and with the NFAT-dependent luciferase activities (Fig. 16E and F) and high levels of activation were usually associated

with low VLs in individuals with the HLA-C SNP, whereas the opposite trend was observed in WT individuals (Fig. 16J). Notably, the values of NFAT activation, as well as CD69 and CD25 expression levels correlated inversely with the efficiency of CD28 (Fig. 16G-I) but not CD4 or MHC-I downmodulation by patient Nefs. Thus, although the effects of Nef on CD28 cell surface expression were relatively weak (Fig. 13B), they obviously affect the responsiveness of HIV-1-infected T cells to stimulation. This indicates that a reduced responsiveness of virally infected T cells to activation might be more beneficial for effective viral persistence in individuals with the polymorphism than in those with the WT genotype.

Next, the consequences of different activation levels for the fate of HIV-1-infected human PBMCs and CD4+ T cells were examined. Flow cytometric analyses showed that the levels of CD69 expression did not differ significantly between *nef* alleles derived from any of the four patient groups (Fig. 17A). In comparison, the expression of Nefs derived from the low-C-SNP group was associated with increased CD25 surface expression, indicating enhanced late T cell activation (Fig. 17B). All tested *nef* alleles had similar effects on CD69 and CD25 expression in infected PBMCs and CD4+ T cells obtained from different donors (R^2 generally >0.4, $p<0.0001$; n=4). Further analyses revealed that Nef proteins from WT individuals were generally associated with higher percentages of apoptotic cells than those derived from individuals with the HLA-C SNP (38.8± 1.1% vs 34.09±0.5%; p=0.0004) (Figure 17C). Nefs from low VL patients induced higher levels of apoptosis than those from high VL patients, although this difference was only significant within the C-SNP groups (Figure 17C). Moreover, levels of CD25 expression correlated inversely with the efficiency of Nef-mediated downmodulation of CD28 (R^2=0.383; $p<0.0001$) and directly with the NFAT induction in Jurkats (R^2=0.1894; p=0.0016) and with the percentage of apoptotic PBMCs (R^2=0.4118; $p<0.0001$). Altogether, low VLs in patients with the polymorphism are usually associated with reduced downmodulation of CD28 (R^2=0.2522; p=0.0105) (Fig. 13H), increased expression of CD25 (R^2=0.1617; p=0.0463) and enhanced levels of apoptosis (R^2=0.1564; p=0.0503) in HIV-1-infected PBMCs (Fig. 17D and E). These associations further support that enhanced Nef-mediated suppression of T cell activation and apoptosis is particularly relevant for effective viral persistence in individuals with the HLA-C SNP.

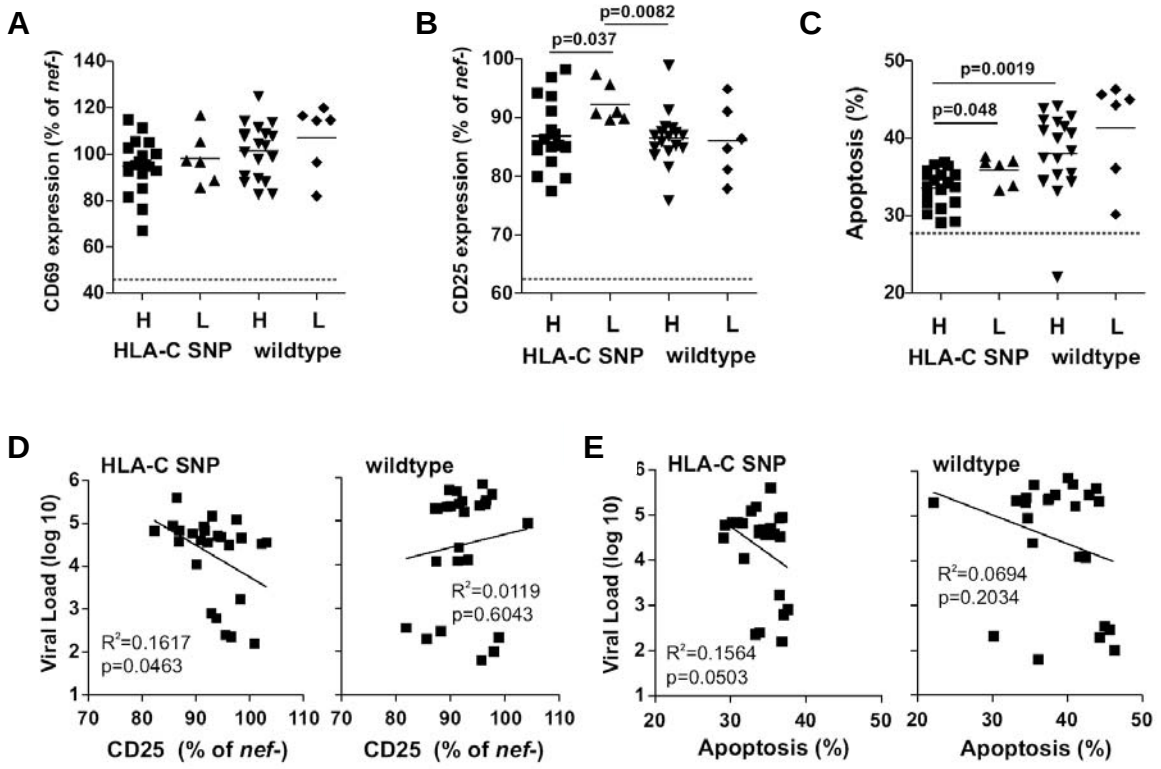


Fig. 17: Nef alleles derived from individuals with the HLA-C SNP and high VLs show reduced activity in inducing T cell activation in PBMCs. (A - C) Quantitative evaluation of Nef-mediated induction of CD69 and CD25 expression and apoptosis in PBMCs infected with HIV-1 Nef/eGFP constructs, followed by PHA stimulation. Nefs were grouped by the genotypes and VLs of the patients from which they were amplified. (D and E) Correlation analyses of Nef-induced CD25 expression or apoptosis rates with patient VLs.

3.2.5 Nef alleles from individuals with the HLA-C SNP are particularly active in promoting virion infectivity but not viral spread

HIV-1 Nef also enhances the infectivity of progeny virions (Chowers *et al.*, 1994). To examine whether the presence of the HLA-C SNP or the high or low VLs are associated with differences in this Nef function, a HeLa-CD4/LTR-lacZ indicator cell line, P4-CCR5, which expresses CD4 together with the CXCR4 and CCR5 coreceptors was infected (Carneau *et al.*, 1994) and the β-galactosidase (β-gal) activities were measured two days later. All bulk HIV-1 *nef* alleles enhanced HIV-1 infectivity, albeit with differential efficiencies (Fig. 18A). On average, Nefs from the hi-C-SNP group were significantly more active in enhancing virion infectivity than those derived from the remaining three groups (8.0±0.7 vs 4.9±0.6; p=0.0205) (Fig. 18A). Statistical analyses showed that high VLs correlated with effective enhancement of virion infectivity only in patients with the HLA-C SNP (R^2=0.2961; p=0.0073) (Fig. 18B). The relative potencies of *nef* alleles in increasing HIV-1 infection were similar at a 10-fold lower infectious dose,

although the magnitude of Nef-mediated enhancement was substantially increased (Fig. 18C).

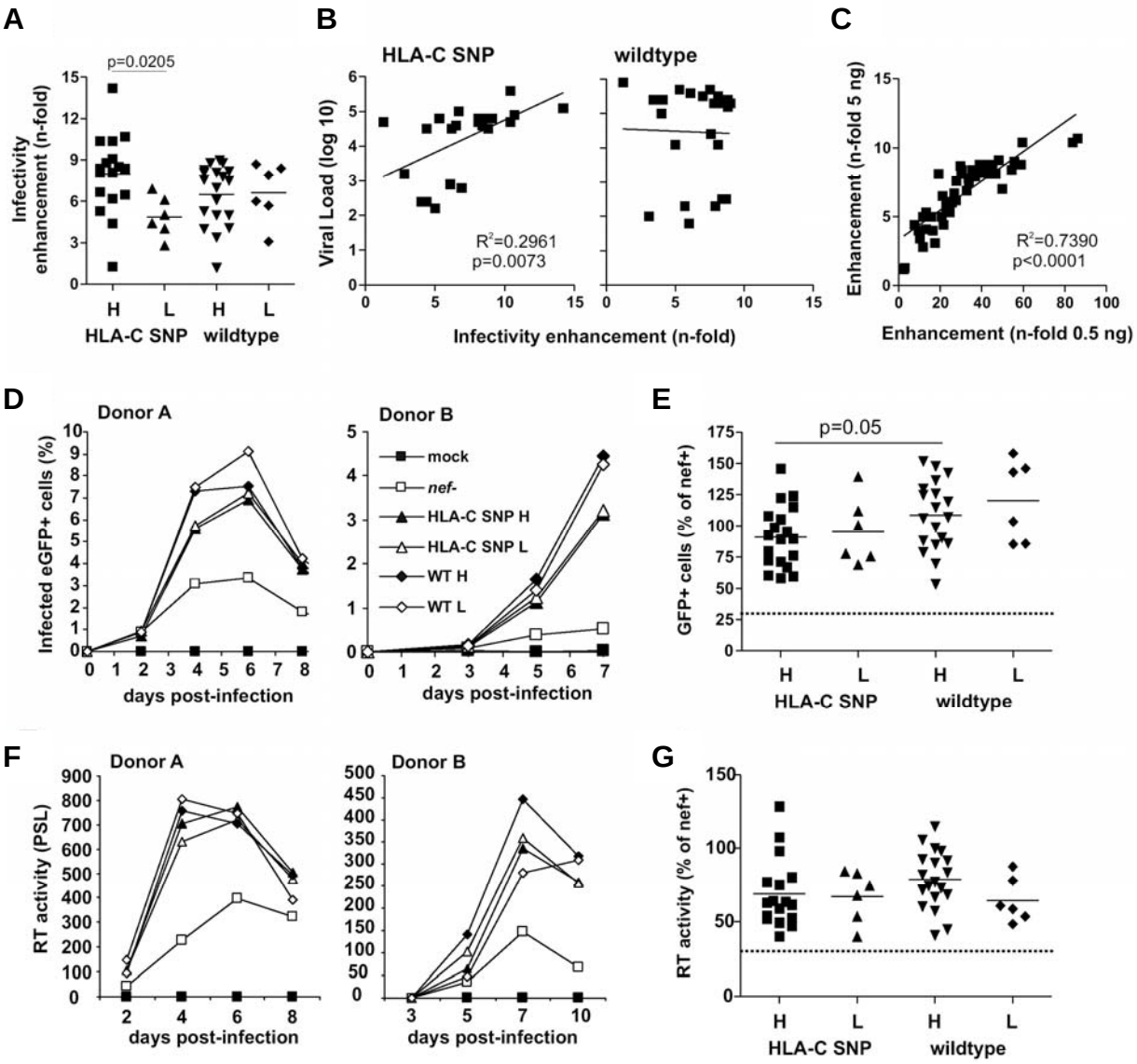


Fig. 18: Nef alleles derived from individuals with the HLA-C SNP and high VLs are particularly active in enhancing virion infectivity but enhance viral spread in PBMCs with reduced efficiencies. (A) Quantitative evaluation of Nef-enhanced infectivity, measured in β-gal reporter activity in P4-CCR5 indicator cells infected with HIV-1 Nef/eGFP constructs. Nefs were grouped by the genotypes and VLs of the patients from which they were amplified. (B) Correlation analyses of Nef-enhanced infectivity with patient VLs and (C) with different infectious doses. (D) Average levels of viral replication in PBMCs from two different donors, infected with HIV-1 Nef/eGFP constructs. (E) Quantitative assessment of cumulative infected PBMC values over 10 days for each of the patient Nef alleles. (F) Average levels of virus production, measured in reverse transcriptase activity in the supernatant from infected PBMCs from two different donors. (G) Quantitative evaluation of cumulative virus production over 10 days for each of the patient Nef pools.

Finally, it was determined, if the four *nef* allele groups differ in their ability to promote viral spread in infected PBMCs (Miller *et al.*, 1994; Spina *et al*., 1994). The HIV-1-NL4-3-IRES-eGFP constructs, expressing the different patient *nef* pools are replication competent and have the advantage that both the amount of

virally infected GFP+ cells as well as the levels of virus production can be readily determined. On average, HIV-1 strains expressing primary *nef* alleles were spreading with about 3- to 5-fold higher efficiency in the PBMCs than the *nef*- control virus (Figure 18D). Altogether, analyses using PBMCs from different donors showed that Nefs derived from WT individuals were slightly more replication competent than those from patients with the C-SNP (Fig. 18D and E), although, they were less effective in promoting virion infectivity (Fig. 18A). This differences remained significant if only *nef* alleles from individuals with high VLs were included in the analysis (108.4±6.4 vs 91.12±5.7; p=0.05) (Fig. 18E). Statistical evaluations showed that the efficiency of patient *nef* alleles in promoting viral spread correlated with that of CD4 (R^2=0.1339; p=0.009) but not with the enhancement of virion infectivity. Furthermore, no significant differences in Nef-mediated enhancement of virus production, measuring reverse transcriptase activity in the culture supernatants, could be detected between the four groups of *nef* alleles (Fig. 18F and G). There was no association observed between the ability of the Nefs to promote viral spread or production in pre-stimulated PBMCs and the setpoint VLs of the patients (Fig. 18E and G).

4. Discussion

HIV-1 Nef helps the virus to evade the host immune response by downmodulating HLA-A and -B, but not -C and -E to avoid killing by CTLs and NK cells (Cohen *et al*., 1999). One aim of this thesis was to determine if the ability to selectively downregulate specific MHC-I molecules is conserved between primate lentiviruses in different hosts. The results show that the selective removal of MHC-I from the surface of infected cells is a generally conserved property of primate lentiviruses in recently, naturally and experimentally infected hosts.

The analysis of HIV-1 and SIVcpz *P.t.t.* Nefs revealed that the precursors of HIV-1 M and N are able to specifically remove MHC-I molecules in its new human host without adaptation. It was also found that this property is conserved in Nefs from the other subgroup of chimpanzees, SIVcpz *P.t.s*., which were not detected in humans (Hahn *et al*., 2000). Sequence analysis of MHC-I tails from human and chimpanzee revealed that the *MHC-I-A*, *-B* and *–C* loci of both species are identical. The cytoplasmic tail of Patr-E differs from HLA-E, however, both molecules contain a mutation in the A325 residue, preventing Nef binding and removal from the cell surface (Cohen *et al*., 1999; Williams *et al*., 2002).

The comparison of Nef sequences showed that only some motifs known to be involved in the interaction of MHC-I with Nef are conserved between HIV-1 and SIVcpz (Fig. 4A). However, on average SIVcpz Nefs were as potent as HIV-1 Nefs in selectively downmodulating HLA-A and –B. It remains to be determined whether the divergent motifs still perform their proposed functions, whether changes elsewhere in Nef can compensate for mutations in these regions, and/or whether the same function can be mediated by alternative regions in these Nef alleles. This assumption is further confirmed by HIV-1 Nef sequence comparisons of group M, N and O, which showed that motifs known to play a role in MHC-I regulation are highly conserved, but the magnitudes of modulation differed substantially.

Further, the results of this thesis demonstrate that additionally to HIV-1 and SIVcpz, which constitute one of the clades of the primate lentiviral lineages, also most viruses from others clades, like HIV-2/SIVmac/SIVsmm (SIVsmm clade), SIVagm (SIVagm clade), SIVsun (SIVlhoest clade), SIVsyk (SIVsyk clade) and SIVgsn/SIVmus (SIVgsn clade), (Courgnaud *et al*., 2003; Apetrei *et al*., 2004) are able to selectively remove MHC-I molecules from the surface of infected cells. Additionally, the analysis included viruses, which are not grouped in any of the

clades (Georges-Courbot *et al.*, 1998). Previous studies have shown that the precursors of SIVcpz, i.e. SIVrcm and SIVgsn/mus, most likely recombined in chimpanzees (Bailes *et al*., 2003) but that the SIVcpz Nef was entirely derived from SIVrcm (Schindler *et al*., 2006). I found that the tested SIVrcm *nef* allele does not efficiently downmodulate MHC-I (Fig. 7A), although it was previously shown to remove CD4 and TCR-CD3 from the cell surface (Schindler *et al*., 2006). Thus, it will be interesting to further analyze whether the inability of SIVrcm Nef to downregulate human MHC-I is merely a coincidence for this particular isolate or whether adaptive changes are required to interact more efficiently with MHC-I molecules from humans and chimpanzees.

Altogether, Nef-mediated selective modulation of HLA-molecules to evade the immune system is a conserved property of HIV-1 M, N and O, which arose from independent transmissions of SIVcpz *P.t.t.* and possibly SIVgor to humans. SIVcpz from both chimpanzee subspecies are already capable of exerting this action in their natural hosts, indicating that it was not necessary for SIVcpz *P.t.t*. to ”learn” this feature in humans. Furthermore, analyses of *nef* alleles from highly divergent primate lentiviruses revealed that the ability to selectively modulate MHC-I molecules is generally conserved.

A further aim of this study was to determine if Nef exerts the same immune evasion mechanism of selective MHC-I downregulation in recently, experimentally and naturally infected hosts. There were at least eight independent transmissions of SIVsmm to humans resulting in HIV-2. It is well documented that sooty mangabeys, the natural host of SIVsmm, do not develop AIDS despite high VLs (Hirsch *et al*., 1989). My data show that Nefs from HIV-2 and its simian counterpart, SIVsmm reduce the surface expression of HLA- and Ceat-A, -B, but not HLA-C, -E and Ceat-E*01 (Fig. 6). This was expected since sequence analysis from sooty mangabey MHC-I alleles (Kaur, unpublished data) revealed that most Ceat cytoplasmic domains are identical to human cytoplasmic tails (Fig. 3). Although the cytoplasmic domain of Ceat-E*01 differs from HLA-E (Fig. 3), Ceat-E is not downregulated by HIV-2 and SIVsmm, probably because both molecules contain a mutation in a residue critical for Nef binding (Cohen *et al*., 1999; Williams *et al*., 2002).

SIVsmm is not only the origin of HIV-2. It was also experimentally transmitted to rhesus macaques resulting in SIVmac, which causes AIDS in its

new host (Hirsch *et al*., 1989; Hahn *et al*., 2000). This makes the SIV/macaque model an important animal model for HIV research (Fultz *et al*., 1989; McClure *et al*., 1990). Therefore, it is relevant to determine if selective MHC-I modulation is preserved in these monkeys. A lack of conservation in this major immune evasion mechanism would limit the possibility to transfer data obtained with this model to humans. Data of my thesis showed that the selective downregulation of Mamu- and Ceat-MHC-I molecules is also a general property of SIVmac and SIVsmm. Thus, this immune evasion mechanism probably helped SIVsmm to cross the species barrier from its natural sooty mangabey host to rhesus macaques resulting in the development of a pathogenic virus in its new experimental host. Further results demonstrated that Nefs from HIV-1 and HIV-2 as well as SIVmac, are capable of selectively downregulating HLA- and Mamu-MHC-I molecules, although minor differences were found regarding the Mamu-B and –E modulation by HIV (Fig. 6 and 8). Previously it was published that rhesus macaques have at least four *Mamu-A* loci and an undefined and variable number of *Mamu-B* loci on any given rhesus MHC-I haplotype. Many molecules of the *Mamu-B* loci have unique amino acid differences in their cytoplasmic domains (Otting *et al*., 2005) (Fig. 3), where Nef binds to exert its action (Cohen *et al*., 1999; Williams *et al*., 2002). Results of this thesis showed that Mamu-A, which cytoplasmic tail sequence is identical to HLA-A, and some Mamu-B molecules, with tail sequences identical or similar to HLA-B are downmodulated by HIV as well as SIVmac Nefs. Earlier it was published that all of the rhesus macaque MHC-I molecules known to bind CTL epitopes of SIV have cytoplasmic tails identical to one of these three molecules (Miller *et al*., 1991; Watanabe *et al*., 1994; Voss and Letvin, 1996; Evans *et al*., 2000).

Little is known about the ligands for KIR receptors in rhesus macaques. The monkeys do not express orthologues of *HLA-C* (Boyson *et al*., 1996). Hence, the *C*-locus appears to represent a recent duplication of the *B*-locus that occurred after the divergence of apes from Old World monkeys (Boyson *et al*., 1996; Adams *et al*., 2001; Fukami-Kobayashi *et al*., 2005). The sequence conservation between *E*-locus alleles of different primate species (Boyson *et al*., 1995; Knapp *et al*., 1998) indicates that rhesus macaque MHC-I-E molecules serve as ligands for CD94/NKG2 receptors on NK cells (Braud *et al*., 1997; Braud *et al*., 1998; Lee *et al*., 1998a; Lee *et al*., 1998b). Results of this thesis

suggest that the inability of SIVmac Nef to downregulate some Mamu-B, -I and -E molecules may reflect a role for these molecules in the inhibition of NK cell responses. Indeed, the cytoplasmic domains of Mamu-B*29, -I and both -E molecules have substitutions in one of the residues implicated in Nef binding (Cohen *et al*., 1999; Williams *et al*., 2002), which are also found in HLA-C and -E. HIV Nefs also reduce the surface expression of Mamu-B and -E*05 molecules, which are not affected by SIVmac. This might not be beneficial for the human viruses if these Mamu-MHC-I molecules are involved in NK cell inhibition. However, HIV-1 and -2 are not adapted to the rhesus macaque host and probably make ”mistakes” in MHC-I modulation. Differences in the interaction between HIV or SIVmac Nef with the specific MHC-I cytoplasmic tails could also contribute to the variations observed in rhesus macaque MHC-I modulation. It was already published that HIV-1 uses in comparison to the HIV-2/SIVmac lineage different surfaces of Nef to downregulate MHC-I molecules (Swigut *et al*., 2000) (Fig. 4). Altogether, the principle of Nef-mediated selective MHC-I modulation in natural, recent and experimental hosts is generally conserved.

So far, results of this thesis suggest that primate lentiviruses are usually capable of maintaining an elaborative balance to counteract both antiviral immune responses, CTL and NK cell lysis. However, new published data indicate that the balance is disturbed in some HIV-1-infected patients. Specifically it has been shown that a SNP upstream of the *HLA-C* gene is associated with low VLs in these patients (Fellay *et al*., 2007). Although the average VLs in HLA-C SNP individuals are lower than in “wildtype” individuals they are also highly variable. The efficient persistence of HIV-1 in a subset of people with the polymorphism suggests that they are either unable to mount an effective HLA-C-mediated immune response or that the virus “learns” to counteract it, e.g. by the emergence of Nef proteins capable of removing HLA-C from the surface of infected cells. To determine if virus variants capable of reducing HLA-C surface expression may emerge in *vivo*, 50 *nef* allele pools isolated from HIV-1-infected patients with or without the HLA-C SNP and with low or high VLs were analyzed. It was found that all Nefs have only weak effects on HLA-C surface expression and that high VLs in the HLA-C SNP group are not associated with an acquired ability to downmodulate HLA-C. Further the results demonstrate that the most active Nefs are derived from the WT group, but how Nef acquired this ability and whether these weak effects

are sufficient to manipulate NK cell or CTL lysis remains unknown. An explanation for the inability of Nefs from the hi-C-SNP group to downmodulate HLA-C might be, that Nef may be unable to acquire the capability to efficiently remove HLA-C from the surface of infected cells to escape CTL attack or that the disadvantage of increased lysis by NK cells also impedes the emergence of such variants. Both of these possibilities would explain why HIV-1 may develop indirect mechanisms to circumvent improved HLA-C-mediated immune control.

My results also revealed that the patient Nefs usually selectively remove HLA-A and -B but not -C and -E from the surface of virally infected T cells. Yet, only a weak and non-significant association between the efficiency of MHC-I modulation by Nef and high setpoint VLs was observed. This seems surprising, because previous studies in both HIV-1-infected individuals and in the SIV/macaque model, clearly support a relevant role of Nef-mediated MHC-I downregulation in viral immune evasion and effective persistence (Collins and Baltimore, 1999; Desrosiers, 1999, Carl *et al*., 2001; Münch *et al*., 2001; Swigut *et al*., 2004). However, the selective pressure for MHC-I modulation is dependent on the host CTL response. Nef is targeted by CTLs (Culmann *et al.*, 1989; Choppin *et al*., 1992) and escape mutations may reduce its ability to remove MHC-I from the surface and increase the susceptibility of virus-infected cells to CTL lysis (Ali *et al*., 2003; Ueno *et al*., 2008; Brumme *et al*., 2008). Sequence analyses supported these possibilies, as the Nefs tested in the present study show variations in known CTL epitopes. Thus, different setpoint VLs and ineffective Nef-mediated downmodulation of MHC-I may result from both effective CTL responses, where escape mutations in Nef occur, or lacking CTL responses, where MHC-I downregulation seems not to mediate a selective advantage.

In this thesis, several other variations in Nef functions that correlate with setpoint VLs in individuals with the HLA-C SNP were identified. Specifically, high VLs in HLA-C SNP patients correlate with increased Nef-mediated regulation of CD4, CD28, CXCR4 and the Ii. Previously it has been shown that these four surface receptors are genetically separable in their modulation and hence most likely are independently selected *in vivo* (Geyer *et al*., 2001). Notably, they all target the interaction of APCs with CD4+ helper T cells, which requires the interaction of the peptide-MHC-I complex presented by professional APCs with the

TCR-CD3 complex and CD4 on the T lymphocytes (Bromley *et al*., 2001; Dustin, 2008). Nef-mediated upmodulation of Ii most likely prevents effective MHC-II antigen presentation by APCs (Stumptner-Cuvelette *et al*., 2001) and downregulation of CD4 may impair APC-T cell interaction and communication, because CD4 acts as a coreceptor of the TCR complex and binds a section of the MHC molecule different from the TCR-CD3 complex (Gay *et al*., 1987; de Vries *et al*., 1989; Gao *et al*., 2002). The lack of the CD28 co-stimulatory signal also prevents lasting T cell activation and may render the HIV-1-infected T cells anergic and hence non-responsive to further stimulation (Vingerhoets *et al*., 1995; Berridge, 1997). Finally, CXCR4 downmodulation disrupts ordered trafficking of infected T cells between local microenvironment (Hrecka *et al*., 2005) and may reduce their conjugate formation with APCs. Obviously, the concerted effect of Nef on the surface expression of all four receptors is a better indicator of the overall disruptive effect on the functional interaction between APCs and T cells than the individual activities. This assumption is supported by the fact that the correlation in HLA-C SNP patients with high VLs becomes substantially more significant when the cumulative (R^2=0.3312, p=0.0026) or average (R^2=0.3310, p=0.0038) activities in CD4, CD28, CXCR4 and Ii modulation were used for the calculation. Altogether, the ability of Nef to manipulate the interaction and communication between APCs and T cells, which is essential for the initiation and maintenance of an effective specific antiviral immune response, is particularly important for effective viral persistence in individuals with the HLA-C SNP, as this might compensate for the disability of HIV in HLA-C modulation within these patients.

My results showed that other Nef features, like reduced levels of NFAT activation, CD25 expression and apoptosis as well as an increased potency to enhance virion infectivity, are also specifically associated with high VLs in individuals with the HLA-C SNP. The first three phenotypes could just be side effects of the increased suppression of co-stimulation through CD28 in cell activation, as these Nef functions inversely correlate with CD28 downmodulation. The data also suggest that HIV-1 *nef* variants, which cause stronger levels of activation-induced apoptosis may lead to accelerated depletion of CD4+ T cells in *vivo* (Groux *et al*., 1992). Further, lower levels of T cell activation should be associated with decreased viral gene expression (Cullen, 1991). This explains why Nef alleles from hi-C-SNP individuals are less active in promoting viral spread,

although they are more effective in enhancing virion infectivity. Additionally, in the presence of an effective HLA-C-mediated cellular immune response, lower levels of viral gene expression should be beneficial for the virus because they reduce viral antigen presentation to CTLs (Tomiyama *et al*., 2002). Results also showed that enhanced modulation of CD4, CD28, CXCR4 and Ii is not associated with high VLs in WT HIV-1-infected individuals. To the contrary, on average *nef* alleles from these patients with low VLs are usually more active in downregulating CXCR4 and CD4 than those derived from individuals with high VL (Fig. 13 and 14). The latter is unexpected since it has been previously shown that Nef-mediated removal of CD4 from the surface of infected cells is enhanced in late stage AIDS patients and correlates with the efficiency of viral replication (Arganaraz *et al*., 2003). My observation that the association of some Nef features with high VLs is dependent on the host genotype, suggests that the HLA-C SNP alters the selective pressure exerted on these specific Nef functions. It is assumed that Nef helps the virus to balance T cell activation to levels sufficient for permissive HIV-1 replication but low enough to prevent activation-induced cell death before the viral replication cycle is completed. It will be of interest to examine whether the rates of disease progression differ in infected individuals with and without this polymorphism irrespectively of the VLs.

Altogether the results of this thesis demonstrate that Nef-mediated selective downmodulation of specific MHC-I molecules to evade both CTL and NK cell lysis, is well conserved between primate lentiviruses in different hosts. Recently it was published that genetic host factors, like the HLA-C SNP are associated with a better immune control, leading to low VLs in these patients (Fellay *et al*., 2007). However, in some HLA-C SNP individuals high VLs are observed. The data of this thesis suggest that in these patients HIV-1 may evolve to circumvent improved HLA-C-mediated immune control by indirect mechanisms. Importantly, HIV-1 does not acquire the capability of efficiently downmodulating HLA-C even in a host environment where this may be advantageous for effective viral persistence. Therefore, strategies to induce HLA-C-mediated immune responses may allow to achieve a better control of viral replication and to reduce the transmission of HIV-1 between humans.

5. Summary

One of the best established and conserved Nef activities between primate lentiviruses is the downmodulation of human major histocompatibility complex class I (MHC-I) molecules. Previous studies have shown that HIV-1 Nef is capable of selectively downmodulating human leucocyte antigen (HLA) -A and -B, but not -C and -E molecules to evade both, cytotoxic T lymphocyte (CTL) and natural killer (NK) cell surveillance. This selectivity is based on amino acid differences in cytoplasmic domains of MHC-I molecules. It remained elusive, however, whether the ability of specific MHC-I modulation is conserved between HIV and its simian precursors, as well as in different lentiviral lineages and in recently and naturally infected hosts. Further, the SIV/rhesus macaque model is used for in *vivo* studies of HIV, but it is unknown whether the immune evasion mechanism of selective MHC-I downmodulation is also conserved in this experimentally infected host.

The present thesis shows that the selective MHC-I modulation is a conserved property of most primate lentiviruses in their different hosts. The precursors of HIV-1 and HIV-2, i.e. SIVcpz and SIVsmm, exert this function in their new human host as well as in their natural chimpanzee or sooty mangabey hosts. Thus, this Nef-mediated immune evasion mechanism is a viral property within recently and naturally infected hosts. Furthermore, Nefs from highly divergent primate lentiviruses, not reported to cross the species barrier, also selectively modulate HLA molecules. The results also demonstrated that selective MHC-I downregulation is generally conserved in the SIV/rhesus macaque model.

Previously it has been shown that genetic host factors, like a single-nucleotide polymorphism (SNP), rs9264942, which is located upstream of the *HLA-C* gene (HLA-C SNP) are associated with a better immune control, leading to low viral loads (VLs) in HIV-1-infected patients. HIV-1 is unable to remove HLA-C from the surface of infected cells to prevent HLA-C-mediated antigen presentation to CTLs. However, adaptive evolution of HIV-1 to its host, allows the virus to ”learn” how to efficiently persist in some HLA-C SNP individuals, causing high VLs. Thus, one goal of this study was to determine which adaptive changes in Nef occur in these patients. The present data show that HIV-1 does not acquire the capability of efficiently downmodulating HLA-C even in a host environment where this may be advantageous for effective viral persistence. Interestingly, HIV-1 may evolve to circumvent improved HLA-C-mediated immune control by indirect

mechanisms. Thus, the association of some Nef features with high VLs is dependent on the host genotype, suggesting that the HLA-C SNP alters the selective pressure exerted on these specific Nef functions, as Nef is unable to evolve the ability of HLA-C downmodulation.

Altogether, primate lentiviral Nefs efficiently manipulate the immune system in different hosts, by the important and highly conserved mechanism of specific MHC-I downregulation to avoid CTL and NK cell lysis. Genetic host factors, like a HLA-C SNP can disrupt the balance between both immune evasion mechanisms, leading an improved immune control of the virus. However, high VLs are also observed in HLA-C SNP individuals and are associated with the emergence of Nef variants, which manipulate the antigen presenting process by indirect mechanisms, rather than by downregulating HLA-C. Thus, the selective pressure in *vivo* to avoid the removal of HLA-C from the surface of infected cells, even in hosts where this might be beneficial, supports the potency of antiviral therapies aiming to induce HLA-C-mediated immune responses to achieve a better control of viral replication.

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