The role of Nef protein in the pathogenesis of HIV infection



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Thesis submitted for the Degree of Doctor of Philosophy

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Abstract

Human Immunodeficiency virus (HIV-1) uses various mechanisms to evade immune recognition by cytotoxic T cells. Down-regulation of Human Leucocyte Antigen class I (HLA-I) by the viral protein Nef is one such important mechanism. HLA-A and B are believed to be down-regulated by Nef while HLA-C and E are not. The differences in down-regulation were due to sequence differences between alleles. I evaluated the functional significance of polymorphisms in the cytoplasmic domains of HLA-A and B in Nef mediated HIV-1 pathogenesis. HLA-B cytoplasmic alleles resisted down-regulation mediated by nef compared to HLA-A cytoplasmic alleles and these differences were seen to play a crucial role in maintaining CTL response as shown by live virus ELISPOT assays, viral suppression assays and CD107a secretion. Therefore, we propose that this relative resistance to Nef mediated down-regulation by HLA-B cytoplasmic allele contributes to the high efficacy and better control of HIV infection by HLA-B restricted CTLs.

The partial resistance of HLA-B cytoplasmic domain was maintained in HIV-2 Nef alleles and sub group O HIV-1 viruses. The variability of the HLA-A and B susceptibility appeared to depend on the Nef allele while resistance of HLA-C was global. Substitution of ^{315}DR^{316} to GG and truncation of C terminal amino acids play a role in mediating resistance to Nef. Possibly these amino acid changes lead to different molecular trafficking profiles as observed in wet lab methods, and changes to secondary protein structures as predicted by *in silico* methods resulting in partial resistance.

In addition, the functional significance of CD4 down-regulation by Nef, another important effect was also explored in this thesis. Nef was found to be ineffective in down-regulating CD4 molecules truncated at cytoplasmic domain and the viruses which were produced in cell lines expressing truncated CD4, were found to be defective in terms of replication and infection. Evaluating the potential of these CD4 molecules in gene therapy is highlighted in my *in vitro* studies.

In summary this thesis provides insight into important but unexplored areas of Nef mediated pathogenesis of HIV infection.

Declaration

I declare that the work presented in this DPhil thesis entitled ‘The role of Nef protein in the pathogenesis of HIV infection’ is entirely my own work, except for where the contributions of others have been clearly acknowledged. No part of my thesis has been submitted for any degree or other qualification in this university or elsewhere.

Ushani S. Rajapaksa Hilary term, 2013

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Abbreviations and Acronyms

AIDS	Acquired Immnuno Deficiency Syndrome
AP	Adaptor protein
APC	Allophycocyanin
BSA	Bovine serum albumin
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocytes
DNA	Deoxyribo nucleic acid
E	Effector
EDTA	Ethelendiaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot assay
Env	Envelope
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
GFP	Green fluorescent protein
gp	Glycoprotein
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
IFN	Interferon
IL	Interleukin
IRES	Internal ribosomal entry site
KIR	Killer-cell immunoglobulin like receptor
M	Main
MHC	Major Histocompatibility Complex
MIP	Macrophage inflammatory protein
Nef	Negative factor
NK	Natural killer cells
O	Outlier
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohemagglutinin
Rev	Regulator of expression of virion proteins (HIV)
RT	Reverse transcriptase
SIV	Simian Immunodeficiency Virus
T	Target
Tat	Trans activator
TCR	T cell receptor
TNF	Tumor necrosis factor
Vif	Virion of infectivity
Vpr	Viral protein R
Vpu	Viral protein unique
VSA	Viral suppression assay

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1. Introduction

1.1. Human Immunodeficiency Virus

Since the first isolation of Human Immunodeficiency virus (HIV) in the early 1980s (Barre-Sinoussi, Chermann et al. 1983) the HIV epidemic has expanded through all inhabitable continents and has led to very high morbidity and mortality levels specially in developing world, namely Africa and SE Asia (http://www.who.int/hiv/pub/progress_report2011/regional_facts/en/index.html). First clinical pictures of homosexuals presenting with profound immune suppression associated with opportunistic infections, malignancies, wasting and central nervous system degeneration proved to be a scary picture (http://www.cdc.gov/mmwr/preview/mmwrhtml/lmrk077.htm). However, the advent retroviral therapy has brought much improvement and increased living standards, although it took nearly three decades to curb the increase of morbidity and mortality according to recent WHO data (http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_en.pdf).

HIV is a member of the retroviridae family. It is transmitted through blood/serum products, intravenous drug abuse, sexual intercourse or vertically. The virus mainly infects CD4 expressing cells leading to progressive depletion of immune cells, destroying and impairing their functions.

HIV-1 and HIV-2, both share the same transmission pathways although HIV-2 seems to be less transmissible and results in a less pathogenic clinical picture (Campbell-Yesufu and Gandhi 2011). At present HIV-1 seems to be causing most mortality and morbidity in the world, it was recently reported that HIV-2 incidence is declining (Campbell-Yesufu and Gandhi 2011).

HIV-1 isolates are classified into three different groups, M (main) which encompasses the majority of HIV-1 strains worldwide, group O (Outlier) which is predominant in Cameroon and west central Africa, group N (non M/non O) predominantly in Cameroon and a more recent group P (Plantier, Leoz et al. 2009). Based on genetic sequence analysis of the viral envelope gene, group M of HIV-1 is further subdivided in to at least 11 different clades A-D, F-H, J and K and some 20 circulating recombinant forms (Buonaguro, Tornesello et al. 2007).

The origin of HIV is thought to have occurred through cross species transmission of the simian lentivirus, Simian Immunodeficiency Virus (SIVcpz) from the chimpanzee subspecies *Pan troglodytes troglodytes* to humans through direct exposure to animal blood through hunting, butchering and or consumption of raw meat (Buonaguro, Tornesello et al. 2007). Interspersion of HIV-1 group M, N, and O sequences among different SIVcpz (*Pan troglodytes troglodytes*) lineages in phylogenetic trees indicate that HIV-1 viruses from the three groups originated from no fewer than three separate SIVcpz transmission events. However, while group M viruses appear to have efficiently adapted to the new host species, spreading all around the world and generating multiple genetic subtypes, the other two groups show less efficient adaptations to humans (Buonaguro, Tornesello et al. 2007).

1.1.1. HIV-1, the immune system and pathogenesis

The interaction between the HIV virus and the immune system is complex, especially with the ability of HIV to cause continuous, unrelenting viral replication. It is quite clear now that this virus has a unique mode of viral persistence, unprecedentant ability to replicate even when the host mounts an apparently vigorous, virus specific immune response (Johnson and Desrosiers 2002). Involvement of both adaptive immune system and innate system makes HIV a very robust successful pathogen than most other viruses in pathogenesis.

HIV, while primarily targeting immune system, interact with several important molecules that results in a complicated interaction of a multitude of immune cells. An essential property of HIV-1 and other primate lentiviruses is the use of CD4 and a second cellular receptor (CXCR4 or CCR5) to enter into susceptible cells which makes CD4+ T helper cells, CD4+ cells of the macrophages and some dendritic cells the main target. CCR5 tropic viruses usually form the bulk of founder and transmitting viruses while CXCR4 are rare. However, CD4 and CCR5/CXCR4 are not necessary for HIV infection of all different cells and tissue types affected, sometimes other molecules for example, galactosyl ceramide serve as a viral receptor for brain, bowel, and vaginal cells. Moreover, heterogeneity of co-receptor usage can be observed among isolates from different tissues (e.g. brain and bowel) from the same individual (Killian and Levy 2011).

Once the CD4+ cells are infected, virus is able to make use of cellular mechanisms to produce proviruses. To enable replication, HIV counter intracellular host factors using variety of gene products that restrict virus production which include APOBEC3G/F, TRIM5a, Lv2, tetherin, and more recently SAMHD1 (Takeuchi and Matano 2008; Lim and Emerman 2011).

Founder viruses multiply in CD4+ T cells in gut associated lymph nodes, ultimately resulting in a decline of CD4+ T cells in circulation, but the major depletion of mucosal memory CD4+ T cells (Brenchley, Schacker et al. 2004; Mattapallil, Douek et al. 2005) occur within the first week of infection with subsequent chronic activation resulting in the dysfunction of the immune system (Picker and Watkins 2005). Memory CD4+T cell depletion occurs via direct targeting by the virus and bystander activation (Mattapallil, Douek et al. 2005; Douek, Roederer et al. 2009).

Chronic immune activation is a hallmark of HIV-1 (Douek 2007) and at least partly responsible for pathogenesis as evidenced by the elevated expression of HLA-DR and CD38

on CD8+ cells, leading to telomere shortening, cellular anergy, and immunosenescence (Killian and Levy 2011). It is thought that loss of integrity of the intestinal mucosal barrier that permits bacteria and endotoxins, such as lipopolysaccharide (LPS), to enter the blood stream gives rise to chronic activation (Douek 2007). Compared to HIV-1, the HIV-2 and SIV in their natural hosts show much less systemic immune activation and this was attributed to the down-regulation of the TCR (Schindler, Munch et al. 2006). However, this needs further assessing as it has been demonstrated that TCR down-regulation does not prevent disease progression in HIV-2 (Feldmann, Leligdowicz et al. 2009).

Cell mediated immune responses mediated by cytotoxic CD8+ T cells (CTL) play a significant role in HIV virus control (McMichael and Rowland-Jones 2001). HIV successfully avoids this by accumulating escape mutations in the regions presented by HLA-I restricted epitopes (McMichael, Borrow et al. 2010) and also by down-regulating HLA-I (Collins, Chen et al. 1998). The immune escape occurs earlier on in the infection (Goonetilleke, Liu et al. 2009). As a result CTLs are unable to function efficiently as they are unable to identify cells that are infected with HIV (McMichael and Rowland-Jones 2001).

In addition, it has been shown that HIV has the ability to infect CD8+ T cells (Saha, Zhang et al. 2001). The contribution of Nef in down-regulating CD8β was confirmed recently and this function seems to be preserved through most of HIV-1, HIV-2 and SIV *nef* alleles (Heigele, Schindler et al.). This manipulation of CD8+ T cells suggest direct role in viral immune evasion.

The involvement of B cells, humoral and innate arms of immune system including the role played by NK cells, dendritic cells in HIV pathogenesis is complex (Killian and Levy 2011) and beyond the scope of this dissertation thus is not discussed here.

1.1.2. Disease course of HIV infection

HIV-1 infection progresses through 3 phases. It begins its journey in the human target in the first few weeks after exposure (Acute stage) with prodromal symptoms such as fever, sore throat with pharyngitis and rashes (O'Brien and Markowitz 2012).

Although there is rapid multiplication of virus, within a few weeks the levels in blood decline and it has been shown that this coincides with the appearance of HIV specific CTLs (McMichael, Borrow et al. 2010). During this period the infection and viral production are partially controlled with patients entering the chronic phase (Figure 1.1). Patients are usually asymptomatic and the immune system is competent. However, low viral replication that occurs in lymphoid tissue during this period results in steady destruction and decline of CD4+ T cells. During chronic progression of HIV, the immune responses that occur against other infections may stimulate HIV production and accelerate destruction of lymphoid tissues (Pantaleo, Graziosi et al. 1993; Pantaleo and Fauci 1995). The ability of HIV to replicate partly depends upon the state of activation of the target cells. Different types of cells get activated during a normal antigen specific response to a pathogen. A pool of activated CD4+ T cells provides target cells for HIV replication. The virus life cycle is far more efficient with rapid integration, virion production and death of infected cells in activated cells compared to resting cells (Evans, Khoury et al. 2012). Secondly, activation induces high levels of expression of cytokines such as TNF-α, IL-6, and IL-10 that may either induce virus expression or modulate certain components of the HIV-specific immune response (Pantaleo and Fauci 1995). Indeed, co-infection with cytomegalovirus (CMV), herpes simplex virus (HSV), hepatitis virus, human herpes virus (HHV) 6, and mycoplasma has been observed to increase HIV production and progression (Chowdhury, Munakata et al. 1990; Rosenberg and Fauci 1991; Pantaleo, Graziosi et al. 1993). In fact, immune activation is likely to be a major

contributor of maintenance of viral reservoir (Pantaleo and Fauci 1995; Hatano 2013). Proinflammatry cytokines by activated immune cells including monocytes, macrophages, NK cells, B and T cells, and activation markers i.e. HLA-DR and CD38 are higher in HIV infected patients compared to healthy individuals (Catalfamo, Le Saout et al. 2012). However, whether this is secondary to microbial translocation in the gut, HIV replication or co-infections (Catalfamo, Le Saout et al. 2012; Sandler and Douek 2012), and also the mechanism for the effect of chronic CD4 T-cell activation on CD4 depletion has not yet been established (Sedaghat, German et al. 2008).

The final stage of HIV infection, AIDS, is a collection of symptoms and signs, preceded by increasing viral loads and rapid decline of CD4+ T cell counts. With drop of CD4 counts to below 200 cells/mm3, opportunistic infections and unusual clinical symptoms related to AIDS start appearing such as weight loss, progressive dementia, Kaposi's sarcoma etc.

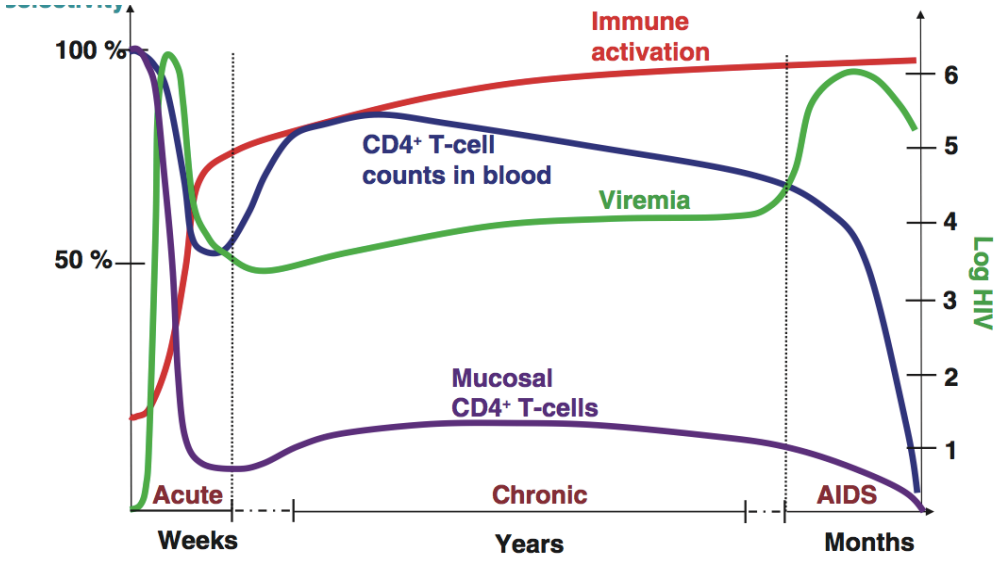


Figure 1.1: **Changes of CD4+ T cells and viraemia with disease progression in HIV-1**. Adapted from (Grossman, Meier-Schellersheim et al. 2006).

1.2. Molecular and biological features of HIV-1

1.2.1. Structure of the virus

HIV-1 is a 100-120µm enveloped virion with a cone shaped cylindrical core. Each virus contains two copies of a RNA genome which are reverse transcribed into DNA in infected cells and integrated into the host genome. Each RNA genome is 9.2 kb long and packaged inside a core of viral proteins and surrounded by a phospholipid bilayer derived from the host cell membrane, that also includes virally encoded membrane proteins gp120 and gp41 (Figure 1.2). The contents are packaged in a cone shaped core composed of p24 capsid protein with a surrounding p17 protein matrix.

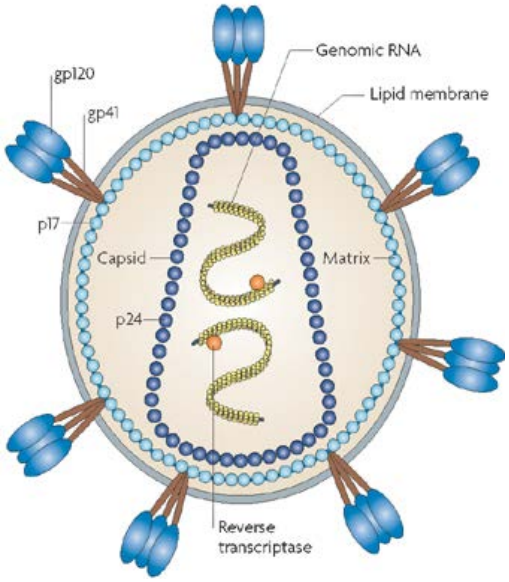


Figure 1.2: Structure of Human Immunodeficiency Virus-1. Adapted from (Karlsson Hedestam, Fouchier et al. 2008).

1.2.2. Genomic details of HIV

HIV is highly heterogenetic. This is due to high level of viral multiplication *in vivo* (10^9 particles per day), error prone DNA synthesis during reverse transcription and high recombination frequencies associated with reverse transcription.

As discussed in section 1.1, HIV-1 viruses are classified into three main groups, group M, O and N, and the new group P (Plantier, Leoz et al. 2009). Group M is subdivided into 11 phylogenetically equidistant clades (A1, A2, B, C, D, F1, F2, G, H, J,and K) with internucleotide distance ranging from 15-20% in the *gag* gene and 20-30% in *env.*

All HIV-1 clades can be found in Africa but clade C viruses are the dominant subtype. Clade A viruses are prevalent in eastern and central African countries while clade B remains prevalent in Europe and North America.

The RNA genome of HIV-1 is flanked by two long terminal repeats and encodes three primary HIV-1 translation products, that are initially synthesized as polyprotein precursors and subsequently processed into 3 structural proteins (Gag, Pol and Env) and six accessory proteins (Vif, Vpr, Nef, Tat, Rev and Vpu) (Figure 1.3).

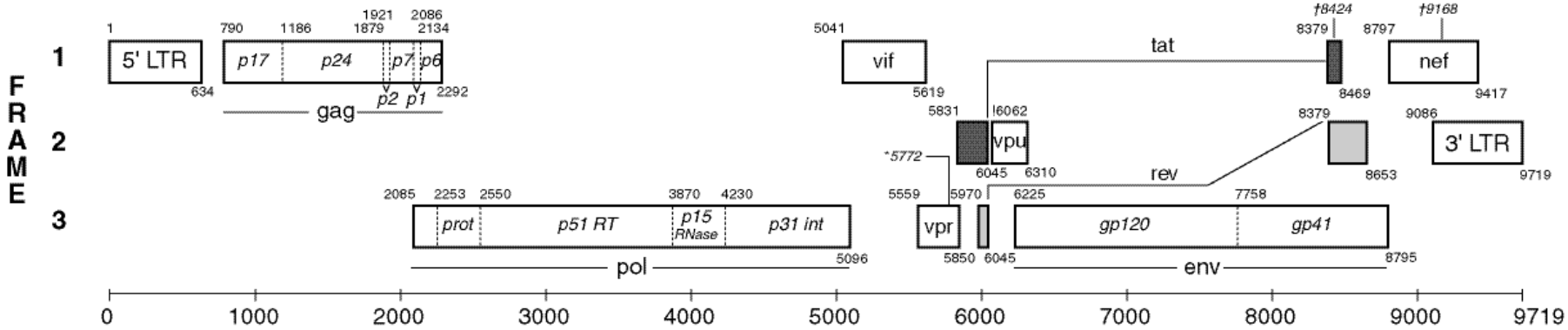


Figure 1.3: Human Immunodeficiency Virus encoded proteins HXB2 strain. Open reading frames are shown as rectangles. The tat and rev spliced exons are shown as shaded rectangles. In HXB2, *5772 marks position of frameshift in the vpr gene caused by an "extra" T relative to most other subtype B viruses; !6062 indicates a defective ACG start codon in vpu; †8424, and †9168 mark premature stop codons in tat and nef. Adapted from http://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html.

Gag

Gag encodes 4 core structural proteins including the matrix (p17), capsid (p24), nucleocapsid and p6. **Matrix** is the N-terminal component of Gag polyprotein and is important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane prior to viral assembly. In addition matrix also facilitates the infection of non-dividing cell types principally macrophages.

Capsid forms the core of the virus particle. Capsid is important for viral infectivity by participating in viral uncoating. **Nucleocapsid** coats the genomic RNA inside the virion core. The primary function of nucleocapsid is to bind specifically to the packaging signal and deliver full length viral RNAs into the assembling virion. The protein **p6** is important for the incorporation of Vpr during viral assembly and also in virus release.

Env

Precursor Env protein is proteolytically cleaved into two functional glycoproteins, surface gp120 and transmembrane gp41. These glycoproteins make up the core and the outer membrane envelope of the virion. They are crucial in HIV-1 binding to host cell receptors and subsequent cell entry. Gp120 binds the target cell surface receptors preceding viral entry and gp41 mediates fusion between the viral and cellular membranes following binding.

Pol

Pol sequence encodes for enzymes which are encapsulated in the virion and important in different steps in the virion life cycle. These include protease, reverse transcriptase and integrase.

Protease is responsible for cleavage of Gag-pol and gag polyproteins into their functional component parts, an essential step in the maturation process of a non-infectious viral particle to a mature infectious virion. Protease has been the prime target in drug development and several protease inhibitors are in wide clinical use.

Reverse transcriptase is responsible for the reverse transcription of the viral RNA genome into double stranded DNA. It catalyses both RNA and DNA dependent DNA polymerisation reactions and contains a RNAse H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the process.

Integrase is involved in integrating the viral genome into host cell chromosome in several reactions that include the removal of two 3' nucleotides from each strand of the linear viral DNA and covalent joining of the processed 3'end to 5'end of the target DNA. Finally, unpaired nucleotides at the viral 5'end are removed and the ends are joined to the target site 3'end generating an integrated provirus flanked by five base pair direct repeats of the target site DNA.

Unlike Tat and Rev that perform an essential regulatory function, Vif, Vpu, Nef and Vpr are accessory proteins that are involved in increasing viral infectivity. Several good reviews have been published on this topic (Malim and Emerman 2008; Kirchhoff 2010) therefore is not discussed in detail here. However, Nef is discussed in section 1.5.

Vif

Virus infectivity factor (Vif) is important in production of highly infectious mature virions (Malim and Emerman 2008). Vif significantly enhances HIV-1 replication in non permissive cells but has no activity in HIV permissive cells. Vif blocks the action of an antiviral protein APOBEC3G (a cytidine deaminase that suppresses reverse transcription and introduces G-

to-A substitutions in the HIV-1 genome, which are detrimental to viral replication) that attacks the viral DNA synthesis in the infected cells.

Vpu

Vpu is found exclusively in HIV-1 (Cohen, Terwilliger et al. 1988). HIV-2 counterpart is known as Vpx. It promotes degradation of newly synthesized CD4 molecules in the endoplasmic reticulum and enhances virion release.

Vpr

Viral protein R is a viral particle incorporated protein, whose function is debatable. Vpr is said to be cytopathic, whether this is mediated through apoptosis or necrosis is not certain. Vpr expression also leads to delay or arrest of cells in the G2 phase of the cell cycle. Vpr also facilitates infection of macrophages.

Tat

Tat is essential for effective viral replication. Tat increases steady state levels of viral RNA several hundred times by directing the formation of a more processive RNA pol II transcription complex in virus infected cells. In the absence of Tat, prematurely and randomly terminated transcripts accumulate.

Rev

Rev regulates expression and usage of viral transcripts by binding to a cis-acting target, Rev Response Element, present in all unspliced and partially spliced viral mRNAs. It exports singly spliced or unspliced viral transcripts from the nucleus to the cytoplasm, where they are either translated or packaged into virions.

1.2.3. Viral life cycle

Fusion, Reverse transcription and Integration-

Infection of a cell with HIV begins following fusion of virion envelope and the membrane of the target cell (Figure 1.4). The binding of envelope glycoprotein gp120 to CD4 and a coreceptor induces a conformational change in gp41. A hydrophobic region of gp41, called the fusion peptide, is exposed and inserts into the target cell membrane promoting virion and cell membrane fusion.

On entering the cell, the virion forms a large preintegration complex containing the viral genome and virion proteins that include reverse transcriptase, integrase, matrix and Vpr. The viral RNA genomes are transcribed into double stranded DNA by reverse transcriptase. The viral DNA and integrase are then transported into the host cell nucleus. With the aid of viral integrase, viral DNA integrates into the host genome and is now known as provirus. This provirus can remain latent with little or no transcription of viral genes.

Replication-

Initiation of HIV gene transcription is linked to activation in T cells. Regulatory genes *rev, tat* and *nef* are expressed during the early stage of viral replication. Tat and its associated cellular proteins promote completion of viral genome transcription. Rev mediates cytosolic delivery of singly and non-spliced message and converts viral gene expression from early stage to late stage. Nef regulates cellular activation and makes the cellular environment favourable to viral gene transcription and expression (Foster and Garcia 2008). The structural proteins Env, Gag and Pol are late gene products.

Assembly and Budding of virion-

The assembly of infectious viral particles begins by packaging full length RNA transcripts of the proviral genome within a nucleoprotein complex that includes the Gag and the Gag-Pol poly-protein precursors. This nucleoprotein complex is then enclosed within a membrane envelope and released from the cell by a process of budding from the plasma membrane. It is interesting to note that the portion of cytoplasmic membrane that is incorporated into the virion does not contain any CD4 molecules. After the virion is separated from the host cell, HIV-1 protease cleaves the Gag and Gag-Pol precursors to form mature Gag and Pol proteins that generate a mature and infectious virion.

1.3. HIV and HLA-class I

1.3.1. HIV, HLA and Immune system

Recent knowledge of genomic studies involving well defined multicentre cohorts of HIV/AIDS patients have led to a clearer understanding of the role of host genetic determinants in viral acquisition, disease progression and transmission. Evidence supporting the importance of specific recognition by T cells is emerging (McMichael, Borrow et al. 2010). Further, the understanding of viral genetic diversity, the much studied aspect of HIV, has somewhat helped in elucidating the mechanism leading to the evolution of viral escape mutants to host immune determinants in particular the MHC associated genes (Kaur and Mehra 2009).

We currently know that at least two alleles, HLA-B*27 and B*57, have been identified as 'protective' against HIV-1 while B*35 and B*53 have been associated with susceptibility (O'Brien, Gao et al. 2001; Kiepiela, Leslie et al. 2004). The way HLA mediated selection drives the evolution of HIV-1 and which circulating variants are more likely to evade immune surveillance of the population are now beginning to become clear. Importantly, the rare HLA alleles in a population bear a selective advantage to the host because these can induce immune responses against pre-adapted viruses (Kawashima, Pfafferott et al. 2009; Rousseau, Lockhart et al. 2009).

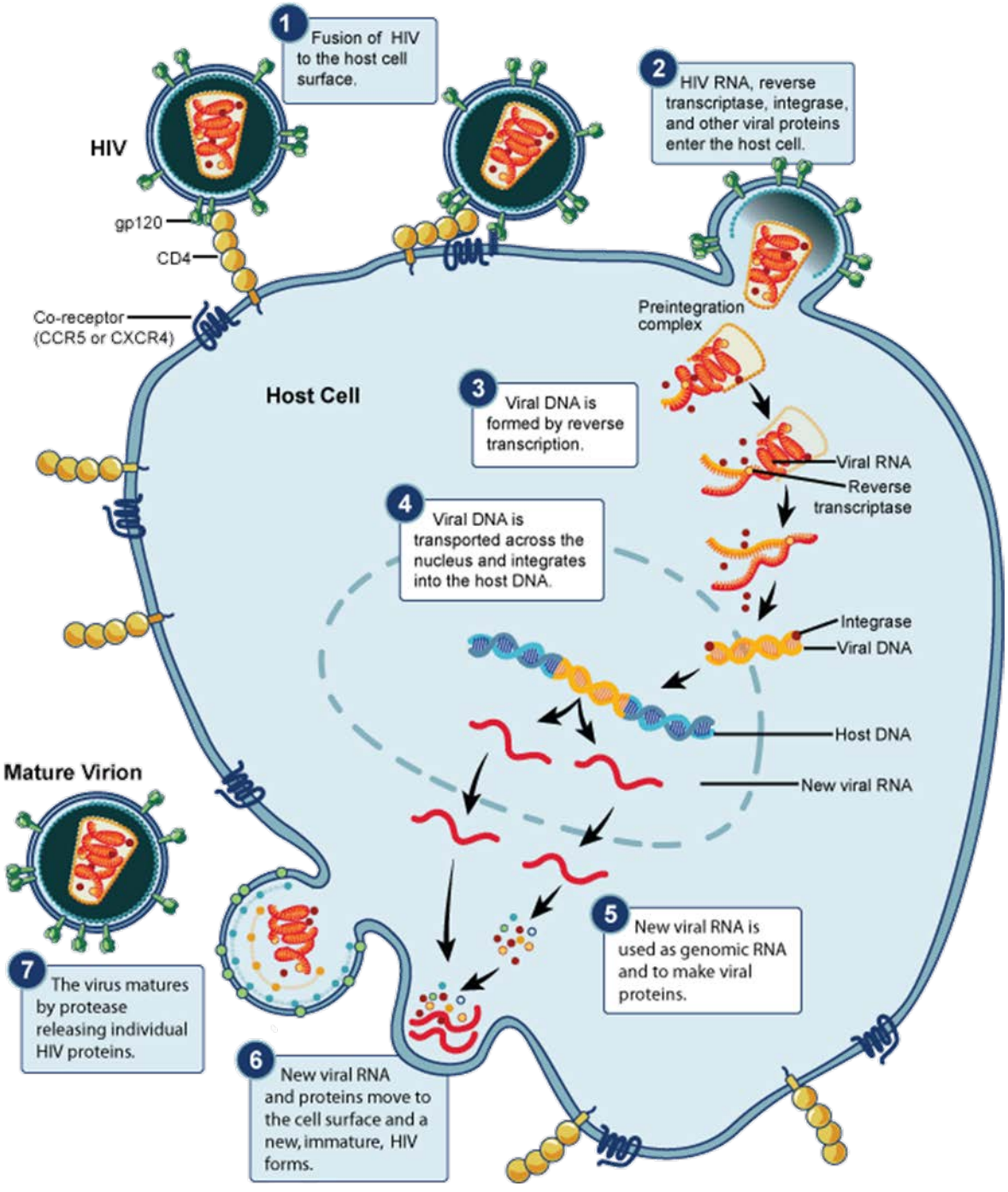


Figure 1.4: Life cycle of HIV-1. Adapted from
http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/hivreplicationcycle.aspx

Immune evasion by HIV occurs via a number of mechanisms including its' ability to rapidly mutate, leading to antigenic escape from T and B cell/ antibody responses, its' ability to down-regulate of Major Histocompatibility Complex-I (MHC) expression from the infected

cell surface and its' destruction of CD4+ T helper cells. MHC-I plays a key and central role in the human body's defence against invading pathogens. HIV is a master of immune evasion and use most if not all of its' proteins to evade immune response. Since several good reviews have been published on immune evasion strategies of HIV (Piguet and Trono 2001; Johnson and Desrosiers 2002; Hansen and Bouvier 2009), this thesis will explore only the aspects that are related to HLA- class I.

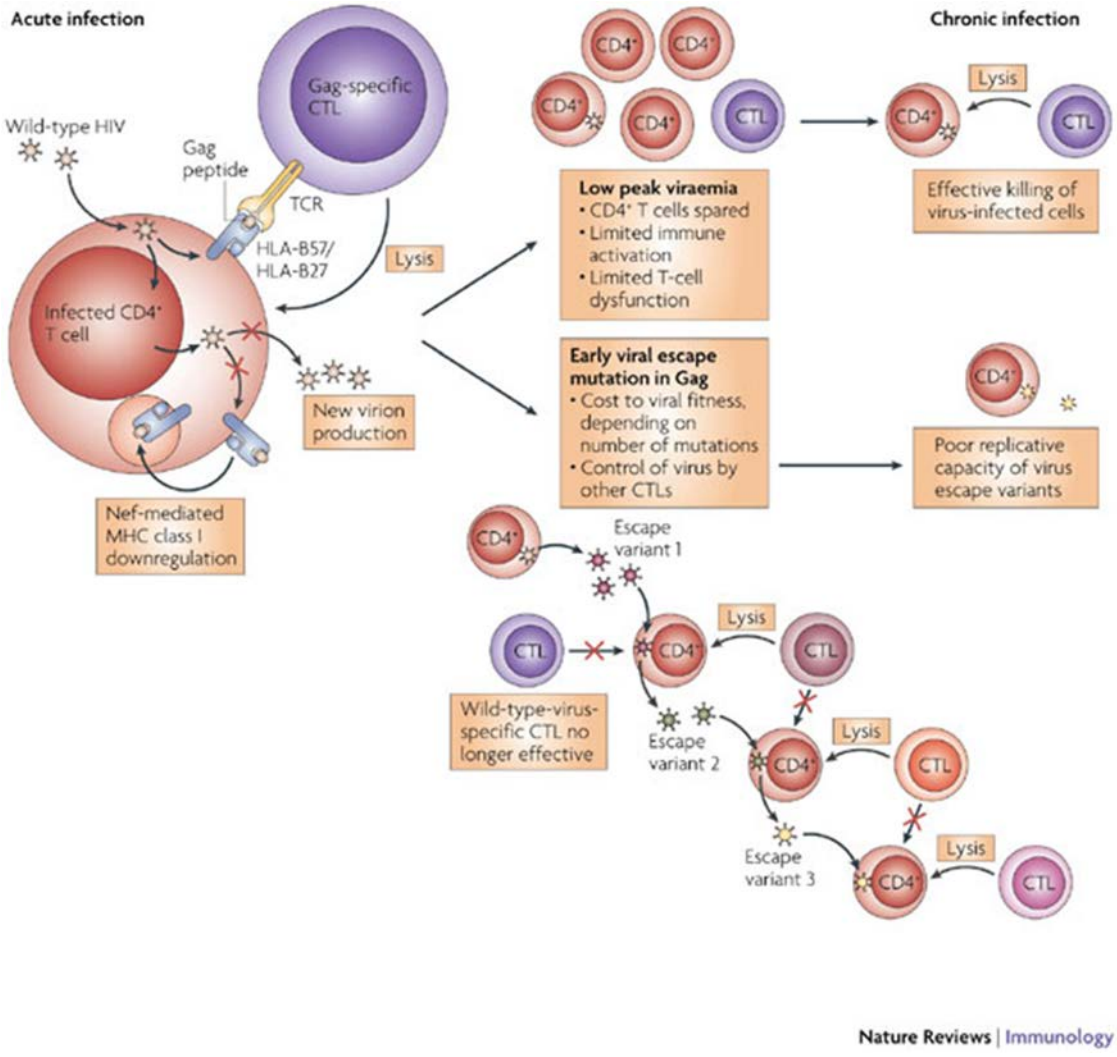


Figure 1.5: Schematic model of immune control of HIV mediated by HLA alleles presenting multilple Gag epitopes. Diagram adapted from Goulder PJR &Watkins DI, Impact of class I diversity on immune control of immunodeficiency viral replication (Goulder and Watkins 2008).

Indeed, it is known that HLA-I is important in mounting an effective immune response against a wide variety of pathogenic microorganisms. This relationship is probably demonstrated best in HIV infection. It has been shown that a critical component of HIV-1 control in the acute phase of infection occurs through CTL (McMichael and Rowland-Jones 2001). It has also been shown that reduction of viral load to a set point level coincides with the initial emergence of HIV specific CTL (Borrow, Lewicki et al. 1994; Koup, Safrit et al. 1994), an increase in viral load when CTL are depleted in the SIV macaque model of HIV (Kuroda, Schmitz et al. 1999; Schmitz, Kuroda et al. 1999), differential disease outcomes associated with polymorphisms in HLA-I restricting CTL responses (Kaslow, Carrington et al. 1996) and evidence linking disease progression to selection of viral escape mutants that can evade CTL response (Phillips, Rowland-Jones et al. 1991; Goulder, Phillips et al. 1997).

Although the sequencing of first detectable virus has shown that a single transmitter founder virus is responsible for ~80% of mucosally transmitted HIV-1 (Keele, Giorgi et al. 2008), the variation of HIV quasispecies is very high. This is a consequence of rapid replication within an individual that is coupled with error prone reverse transcription and frequent switching of template during reverse transcription. It has been reported that total HIV-1 *in vivo* mutation rate is $3x10^{-5}$ per target base pair per cycle of replication (Mansky and Temin 1995). Highly variable quasispecies are fertile substrates for selective pressures favoring the best-adapted, most 'fit' genetic variants to survive (Altman and Feinberg 2004).

With the spread of HIV from one individual to another, genetically diverse viruses confront the one of the most polymorphic gene family in humans, HLA class I and II. These proteins determine the specific peptide sequences (epitopes) to be presented to and to be recognized by host $CD8^+$ and $CD4^+$ T cells respectively. In the confrontation between genetically diverse HIV variants and genetically diverse HLAs, viral variants that harbor mutations in specific

viral epitopes that escape recognition by host immune effector cells namely HIV-specific cytotoxic T lymphocytes are selected (CTLs) (Figure 1.5) (Altman and Feinberg 2004; Goulder and Watkins 2008).

The importance of selective pressure exerted by different CTL in driving viral evolution and relative viral fitness costs associated with different CTL escape mutations influence the variants that emerge in infected individuals, and transmitted to and persist within new hosts (Friedrich, Dodds et al. 2004; Leslie, Pfafferott et al. 2004).

As mentioned previously, an effective way of immune evasion in HIV infection is by down-regulation of HLA-I. It has been shown that reduction in class-I on the surface leads to poor recognition by HIV-1/SIV specific CTL (Collins, Chen et al. 1998; Minang, Trivett et al. 2009). However, the exact role and functional significance of down-regulation of HLA class-I (HLA-I) in HIV infection is incompletely understood. Importantly, the contribution of cytoplasmic tail of HLA-I, the domain that is involved in down-regulation is yet to be elucidated.

HLA-B allele restricted T cell responses have been studied to a greater extent than A or C alleles and are known to be strongly associated with disease control (Kiepiela, Leslie et al. 2004). In fact, HLA-B is shown to be associated with variation in viral set-point, absolute CD4 count and rate of disease progression in a South African cohort of patients (Kiepiela, Leslie et al. 2004). It is yet to be determined whether the close association of HLA-B was due to a stronger and more effective allele specific CTL response or whether it was due to a differential down-regulation of alleles. More importantly the literature does not reveal any attempts of relating these responses to polymorphism seen in the HLA-I cytoplasmic region.

1.3.2. CTL response in acute and chronic HIV infection

As in most other viral infections, CD8+ T cell response is generally dominant among immune responses mediated against HIV although it is unable to clear the infection. Activation of T cells is a striking feature of HIV and is also associated with early and extensive apoptosis leading to release of apoptotic microparticles into circulation and expression of TRAIL (Tumor Necrosis Factor related apoptosis-inducing ligand) and Fas ligands that kill bystander cells (Gasper-Smith, Crossman et al. 2008).

Initial events following HIV transmission are not well defined. It is believed that HIV first breeches the mucosal epithelium and makes initial contact with mucosal Langerhans cells and CD4+ T cells, where they multiply. The first cells to be infected in experimentally infected macaques were found in foci of resident memory T cells that expressed CD4 (McMichael, Borrow et al. 2010). Perhaps early innate immune responses that recruit additional CD4+ T cells to the site enhance this action.

To appreciate the role of CD8+ T cells in HIV infection, and for future implications of CD8 based vaccine it is important to understand how CTLs mediate their function (Masopust 2009). Naïve T cells that are confined to blood, lymph and secondary lymphoid tissues, gets activated in secondary lymphoid tissue when dendritic cells present cognate antigen and costimulatory signals. In the presence of active viral replication, there is massive clonal expansion and changes in gene expression and effector functions. This is followed by dissemination of effector cells into other tissues. Usually the initial immune response results in antigen clearance that is followed by contraction of the effector population. However, a small pool of antigen specific T cells survives and is maintained in lymphoid and non lymphoid tissues indefinitely. Development of memory CD8+ T cells is dependent on IL-15 driven homeostatic division for survival (Becker, Wherry et al. 2002). There are two subtypes

of memory CD8+T cells; central (CTC) and effector memory cells (ETC) respectively. CTC express CD62L and CCR7 while ETC lack these markers. CTC traffic through lymph nodes, spleen and blood, and comprise a long-lived population of memory cells with high proliferative capacity upon reinfection. ETC are usually found in spleen and blood, and putatively re-circulate through the parenchyma of nonlymphoid tissues where they may maintain immediate cytotoxic potential (Masopust 2009).

The early CD8+ T cell responses in HIV are now becoming clear with the ability to recruit and diagnose patients in the acute phase. Initial CTL response is shown to be narrow and directed towards 2-3 epitopes (Goonetilleke, Liu et al. 2009) with high entropy (Bansal, Gough et al. 2005) and mostly to Nef and Env proteins (Goonetilleke, Liu et al. 2009). Most of the amino acid changes in the viruses were selected by CD8+ T cells that recognized epitopes expressed by founder viruses but not the escape mutants (Goonetilleke, Liu et al. 2009). The acute phase CD8+ T cell response occurs in the settings of acute phase proteins and proinflammatory cytokines (Stacey, Norris et al. 2009). As HIV progresses, the breadth of CTL response begins to increase (Altfeld, Kalife et al. 2006; Streeck, Jolin et al. 2009). Interestingly, IFNγ response in primary infection is said to be a poor predictor of viral set point and progression (Gray, Mlotshwa et al. 2009), suggesting that, this assay may not discern CTL function of setting the viral set point. Furthermore, in contrast to our wealth of knowledge on behavior of peripheral blood CD8+ T cells, almost nothing is known about the T cell response at tissue level where the main site of infection lies. It is believed that the responses at the tissue level precede the responses seen at periphery (Altfeld, van Lunzen et al. 2002).

During progression to chronic phase, the number of epitopes that could be targeted by CTL at one time in an individual can increase up to 14 (Addo, Yu et al. 2003) and predicted to be

atleast 30-40% higher in autologous peptides (Altfeld, Addo et al. 2003; Goonetilleke, Liu et al. 2009). The CTL response is broader in progressive disease than controlled disease (Pereyra, Addo et al. 2008). The responses that are detected at the time viral set point, is said to persist till very last stages. However, appearance of negative factors such as PD-1 (Petrovas, Casazza et al. 2006; Trautmann, Janbazian et al. 2006) and CD160 (Peretz, He et al. 2012), up-regulation of transcription factors that inhibit cell proliferation and cytokine secretion, such as BATF-1 suggest that immune function is exhaust and lost (Quigley, Pereyra et al. 2010).

Immunization studies in mice reveal that CD8+ memory cell compartment has enormous expansion capacity without affecting naïve T cell numbers or other pathogen specific CD8+ T cells. Upon stimulation they expand massively and generate IFNγ within few hours. Production of lytic granules requires a bit longer but once activated, effector memory CD8+ T cells can release perforin and granzymes within minutes (Barber, Wherry et al. 2003). Given that CD4+ T cells are important in maintaining CD8 T cell responses, even with a depleted CD4 levels, a strong CD8 response is seen in HIV infection.

1.3.3. Function of CTL in HIV infection

Despite causing immune deficiency, HIV is capable of inducing a strong CTL mediated response, which is important in HIV control. During the acute phase, CTL responses start to rise and reach a peak which coincides with a drop in viral titres, followed by an inverse correlation between viral load and CTL response (Figure. 1.1).

It is said that the acute CTL response is large with as many as 10% of CD8+ T lymphocytes being involved. During chronic phase of infection the expanded CTL stay at a higher

frequency in circulation. This is around 1-2% of cells specific for a single epitope. It is thought that similar numbers exist in lymph nodes (McMichael and Rowland-Jones 2001).

Given the strong evidence that HIV-1-specific $CD8^+$ T cells contribution to the control of HIV-1 through acute and chronic stages of infection, it is important to know what T-cell functions are responsible for this. Most widely known effector function is cytotoxicity or lysis that occur secondary to infection. In addition, production of IFNγ, IL-2, TNF-α, MIP-1α, MIP-1β, and RANTES (CCL5) are also important in viral control (Betts, Nason et al. 2006; Streeck, Brumme et al. 2008). Understanding the relative importance of lytic mechanisms and chemokine, cytokine secretion in the control of HIV is imperative as CD8+ T cells may not display all these effector functions simultaneously and to the same extent (Ferrari, Korber et al. 2011).

During the acute infection, the lytic function probably plays a major role as shown by the rapid decline of infected cells as implied by Goonetilleka *et al* (Goonetilleke, Liu et al. 2009). It has been demonstrated that early T cells lacked cytokines and chemokines (Ferrari, Korber et al. 2011) but express perforin (Hersperger, Pereyra et al. 2010) supporting the above notion.

Once viral set point is established and the disease progresses into chronic phase, other functions may become important (Betts and Harari 2008). It has been demonstrated that controllers have more polyfunctional T cells (Appay, Nixon et al. 2000; Almeida, Price et al. 2007; Betts and Harari 2008) in addition to cytotoxic cells. T cell responses in HIV are reviewed elsewhere (Walker and McMichael 2012), however it is important to understand that all the CTL responses are inseparably connected to HLA-I.

1.3.4. HIV and antibody responses

Acute HIV infection also results in increased number of blood and tissue memory B cells and plasma cells, and a decline in naïve B cells which is associated with class switching of polyclonal B cells (McMichael, Borrow et al. 2010). Replication of HIV in mucosal B cell sites such as Peyer's patches results in lysis of follicular B cells and apoptosis during the first few weeks after infection. This loss results in defects of generation of HIV-1 antibodies and delay in induction of autologous virus neutralizing antibodies which usually arise ~12 weeks or longer after transmission (Wei, Decker et al. 2003). Appearance of heterologous virus neutralising antibodies can take up to years following infection (Richman, Wrin et al. 2003).

In fact, neutralising antibodies occur in most of HIV infected individuals within one year of sero conversion (Moog, Fleury et al. 1997). However, this antibody response is ineffective and lags behind the changes in the virus and rarely targets the contemporaneous virus (Richman, Wrin et al. 2003). For unknown reasons the initial antibody responses are narrow (Wei, Decker et al. 2003) and starts with the first free antibodies against Env glycoprotein gp41 followed by anti gp120 specific antibodies that appear two weeks later in plasma (Tomaras, Yates et al. 2008). This event is followed by appearance of other IgG antibodies to Gag (p24 and p55 appearing around 18 days and p17 later) and integrase p31 (Tomaras and Haynes 2009). Although the initial antibody response to Env is non-neutralising (Tomaras, Yates et al. 2008), the narrow neutralizing antibodies that appear later on can control the virus quasispecies present when they appear but neverthesess results in rapid viral escape (Wei, Decker et al. 2003).

Broadly neutralising antibodies are usually rare in HIV-1 infection. Unlike narrow neutralising antibodies that are generated against variable region of Env protein gp41, broadly neutralising antibodies are generated against conserved regions. Monoclonal antibodies 1b12

recognising CD4 binding site (Kwong, Doyle et al. 2002), 2F5 (Ofek, Tang et al. 2004), Z13 (Nelson, Brunel et al. 2007) and 4E10 (Cardoso, Brunel et al. 2007) recognising membrane proximal region are rarely generated and usually take 20-30 months to develop.

1.3.5. Implications for future vaccine

As described before HIV is still an incurable infection although marked improvement of clinical picture has been brought about by treatment with antiretroviral. Thus far, no single vaccine has been shown to be effective with the most promising candidate that was described, MRKAd5 failing at Phase IIb (STEP trial). Although the hopes for an ideal prophylactic HIV-1 vaccine that will induce protection through concerted actions of T cells and antibodies, where virus neutralizing antibodies slow the incoming virus and provide time for CTL to eliminate any break through virus infected cells is still there, this is providing to be more elusive with HIV vaccine trials not moving beyond Phase II expect for Thai Rv144 which has shown some efficacy in phase III trials (Karasavvas, Billings et al. 2012).

Developing a vaccine that induces broadly neutralizing antibodies is challenging because the Env protein is highly variable. In addition, Env has evolved a number of strategies that make the accessibility of the neutralizing antigenic determinants and induction of corresponding antibody specificities extremely difficult. If a vaccine is to induce an antibody response it has to have a durable protective antibody responses and also prime in order to induce a rapid secondary response. The rarity of broadly neutralizing antibody responses to conserved epitopes in Env emphasise the need to search for B cell subsets that make above.

With the early events of HIV infection becoming clearer, the importance of T cell based vaccine could not be undermined. A vaccine that stimulates CD8+T cell responses to multiple

epitopes, especially to those that are highly conserved would be able to stimulate a broader T cell response. If they recognise common variants of the founder virus epitope sequences, that would limit chances of escape, making the virus more efficient. There is a lot to learn from STEP trial (Hanke 2008). However, with the greater understanding of acute events following HIV transmission and correlates of protection in humans, extrapolating data from animal model to humans, better immunogenic vectors, there is still greater scope for T cell based vaccines.

1.3.6. Structure of HLA- class I

HLA-I is a trimeric molecule expressed on all nucleated cells. Structurally it consists of an alpha heavy chain, a β2 microglobulin chain and a short antigenic peptide (Figure 1.6). It assembles in the endoplasmic reticulum and is transported to the surface.

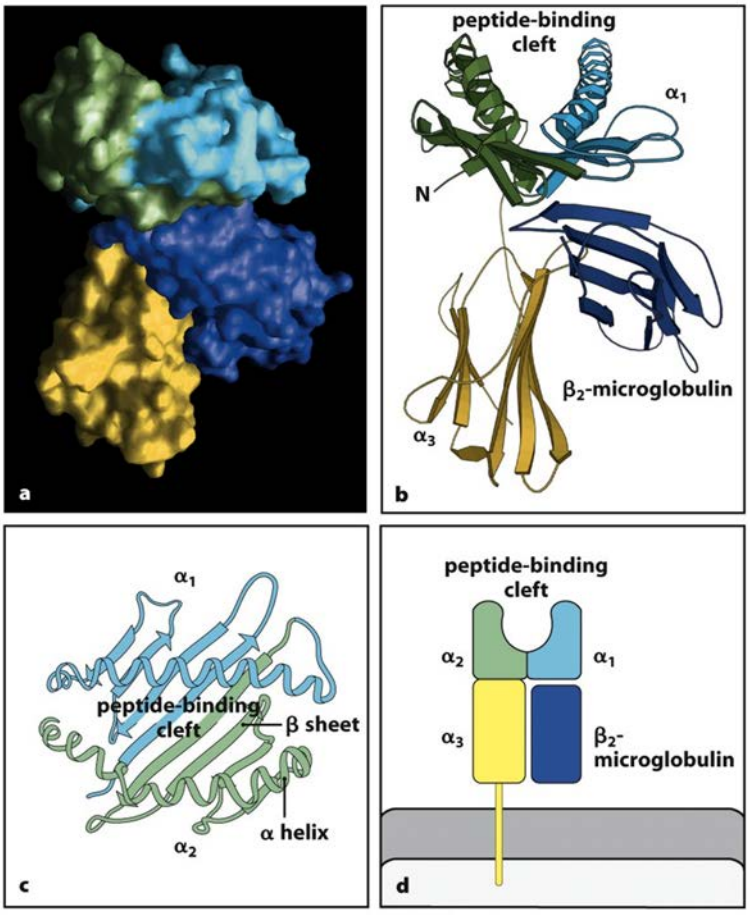


Figure 1.6: The structure of HLA-I molecule determined by X-ray crystallography. a. computer graphic representation of HLA-A2, which has been cleaved from the cell surface by the enzyme papain. The surfaces are colored according to the domains shown in panels b-d. b and c. ribbon diagram of the structure. D. schematic HLA-I molecule as heterodimer of a membrane spanning α chain, non covalently bound to β_2 microglobulin which does not span the membrane. Adapted from Immunobiology 7ed.(©Garland Science 2008)(Murphy).

The HLA-I I heavy chain locus is found on chromosome 6 and is polygenic, encoding the HLA-A, HLA-B, and HLA-C proteins in humans. HLA-A, B and C are considered the classical class I genes while there are also non classical E, F and G. HLA-I genes are highly polymorphic. For example, there are more than 2800 known variants of HLA-B genes, more than 2100 variants of HLA-A genes, and more than 1700 variants of HLA-C genes (http://hla.alleles.org/nomenclature/stats.html). Each HLA-I protein binds to a large number of peptides that share similar primary structural features such as the presence of a particular amino acidic residue at a given position of an octamer or a nonamer sequence.

Polymorphisms of HLA-I variants differ in the structures of their peptide-binding grooves, which in turn impacts the specificity of peptide binding. Because of HLA-I polymorphisms, immune responses can be generated against a diverse array of antigen peptides. Although, homozygosity at any locus is associated with negative outcomes during infections heterogeneity leads to a multitude of reactions that protect you from viral infections.

1.3.7. Genes responsible for HLA-I down-regulation in HIV and their role

HIV in a similar manner to most of the other viruses evades cell-mediated immune response by reducing surface expression of HLA-I. In HIV genome, *nef*, *tat* and *vpu* have been described to be responsible for HLA-I down-regulation (Kerkau, Bacik et al. 1997).

Nef is the most well characterised HLA-I manipulator that is described elsewhere in this chapter (section 1.5). Tat, the potent transcription regulator, was initially found to repress HLA-I (Howcroft, Strebel et al. 1993) which was later called into question (Matsui, Warburton et al. 1996). However, both groups observed a transient down-regulation of MHC-I and later attempts revealed that repression and transcription were separable and mediated by two functional domain of Tat (Brown, Howcroft et al. 1998; Verhoef, Bauer et al. 1998).

Vpu induces a rapid loss of newly synthesized endogenous MHC-I alpha chains thereby affecting cell surface expression of MHC-I molecules (Kerkau, Bacik et al. 1997). This combined with the known functions of Vpu, led to the suggestion that Vpu might act on MHC-I similarly as on CD4 (Kerkau, Bacik et al. 1997). However, further research has to be done to elucidate the precise mechanisms.

1.4. HIV and CD4

Another important aspect of Nef in HIV infection is its ability to down-regulate CD4 molecule from the surface of cells which renders the immune system vulnerable while increasing susceptibility to other opportunistic infections and neoplasms. CD4 is a glycoprotein expressed on the surface of mature T helper cells, regulatory T cells, immature thymocytes, dendritic cells, monocytes and macrophages. CD4 is found on approximately 65% of the T cells in blood and is the main receptor used by HIV to gain access into the host T cell.

1.4.1. Structure of CD4

CD4 is 55 kd molecule belonging to the immunoglobulin superfamily. It is composed of a highly charged cytoplasmic domain (amino acids 396-422), a single hydrophobic transmembrane domain (amino acids 372-395) and four distinct extracellular domains D1 to D4 (amino acids 1- 371). The first two domains are packed together to form a rigid rod that is joined by a flexible hinge to a similar rod formed by the third and fourth domains (Figure 1.7).

CD4 binds class II molecules through a region that is located mainly on the lateral face of the first domain. The role of CD4 is believed to be to stabilize the interaction between T cell receptors and class II major histocompatibility complex molecules on the antigen presenting cells.

The intracellular tail of CD4 interacts strongly with a cytoplasmic tyrosine kinase called Lck and brings Lck close to the intracellular signalling components associated with the T cell receptor.

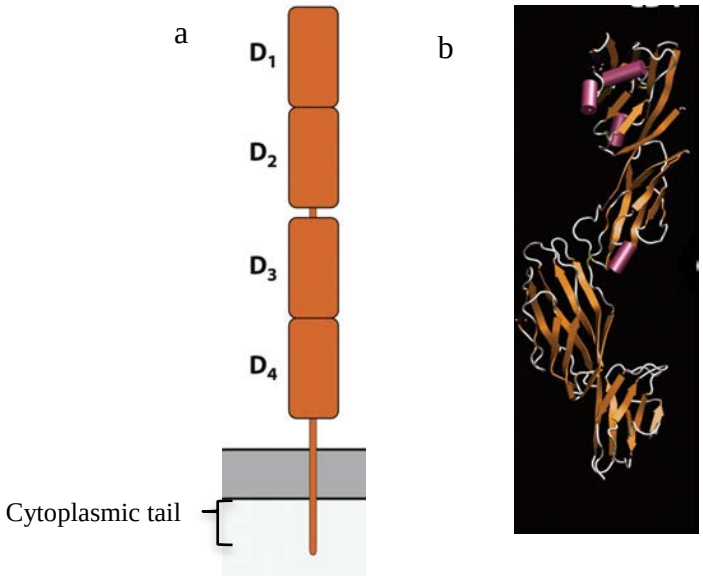


Figure 1.7: The structure of CD4 molecule. a. Schematic and b. Crystal structure of CD4 Adapted from Immunobiology 7ed. (Garland Science 2008) (Murphy)

1.4.2. Complex relationship between HIV and CD4

HIV infection is initiated by binding of gp120, a protein in the viral envelop, to its primary receptor CD4 and subsequent binding to chemokine coreceptor CCR5 or CXCR4. This leads to a series of conformational changes in gp41, facilitating fusion of the outer membrane of the virus to that of the host cell. Following infection there is a progressive reduction in the number of T cells possessing CD4 receptors. Despite understanding the mechanism of HIV induced CD4 down- modulation (mediated by Nef, Vpu and Env), the functional significance of this is not completely understood (Lama, Mangasarian et al. 1999). Preventing premature

death by avoiding multiple super infection is one of the well-described functions (Benson, Sanfridson et al. 1993).

It is also thought that in analogy with influenza virus neuraminidase, CD4 down-regulation prevents trapping and aggregation of outgoing virions on the surface of producer cells (Lama, Mangasarian et al. 1999). Infection with HIV culminates in release of progeny virions by budding. In CD4+ T cells it is believed that HIV buds from the plasma membrane and the budding virion accumulates a coating of cell membrane that does not contain any CD4. If CD4 molecules are present on the cell membrane they could possibly bind to the gp120 of the budding virus and interfere with the budding process.

Further, if CD4 down-modulation does not occur in the host cell, the chance of incorporating CD4 on to the virion during budding is high. Incorporated CD4 could possibly bind to gp120 on the virus and it is reasonable to hypothesise that these viruses are going to be defective, as they no longer have functional gp120. However, this model is not yet supported by evidence.

The mechanism of CD4 down-regulation is described in detail in chapter 6 of this thesis.

1.5. Nef

The importance of Nef was discovered when a few individuals who were infected with viruses which had gross deletions in Nef gene remained healthy with normal CD4 counts and failed to progress to AIDS after more than a decade of infection (Deacon, Tsykin et al. 1995; Kirchhoff, Greenough et al. 1995; Salvi, Garbuglia et al. 1998). The crucial role played by Nef was further confirmed in animal models (Kestler, Ringler et al. 1991; Hanna, Kay et al. 1998).

The primate lentivirus *nef* gene encodes a 27 kD membrane associated phosphoprotein (Figure 1.8). Originally the protein was labelled as negative factor due to early reports of it in inhibitory effects on HIV. But it is now clear that Nef is dispensable for virus spread (Geyer, Fackler et al. 2001) but important for viral replication and disease progression. Nef is synthesized from the first mRNA detected post infection. It has also been noted that within the first few hours of infection unintegrated Nef can still lead to pathogenic activities (Gillim-Ross, Cara et al. 2005; Sloan, Donahue et al. 2010). Furthermore, it has been observed that Nef is important in many of the virulent activities of HIV including disruption of cell signalling and increasing virulence. These activities are however are mediated through different motifs found on the Nef (Figure 1.8).

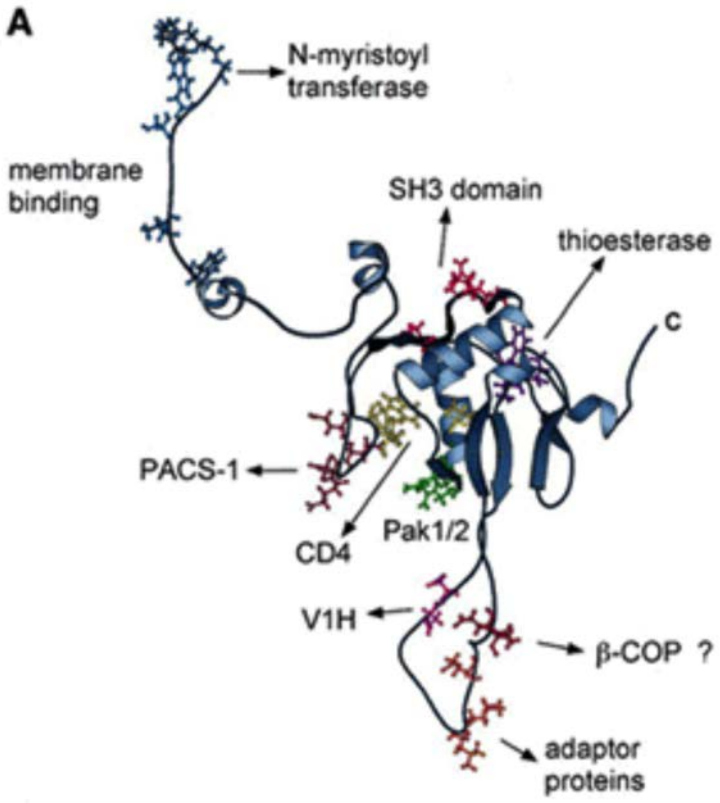


Figure 1.8: Functional motifs in HIV-1 Nef and their locations in full length structure as derived from NMR structure assembly. Binding sites for the known interactive proteins are indicated. Adapted from Geyer et al 2001(Geyer, Fackler et al. 2001).

1.5.1. Mechanism of Nef mediated class I down-regulation

Nef mediated class I down-regulation is probably one of the most important mechanisms through which HIV infected cells evade MHC restricted CTL recognition. Knowledge on how Nef pertubes trafficking of MHC-I and other immune receptors is important because it may provide new insights into mechanisms regulating HIV persistence and antigen presentation, and it may also offer new strategies for vaccine development.

As mentioned previously, down-regulation of HLA-I is important in protecting cells against killing by CTL. Researchers have demonstrated that Nef mediated reduction of MHC-I from cell surface of HIV infected T cells is of sufficient magnitude to protect against killing by

CTL (Collins, Chen et al. 1998). Down-regulating HLA-A and B would help escape from cytotoxic T cells but removal of cell surface MHC-I proteins may expose the infected cells to attack by NK cells, but presence of HLA-C and HLA-E sends inhibitory signals to NK cells. It has also been demonstrated that Nef alleles isolated from patients early in the infection are more effective in HLA down-regulation compared to the ones obtained later in the course of disease (Carl, Greenough et al. 2001). This explains the important role played by Nef protein in evading immune system in the early phase of infection.

Literature depicting mechanisms have shown that at least three distinct subdomains of Nef are required for down-regulation of Class-I, which are N terminal alpha helix with a conserved Methionine at position 20, SH3 binding polyproline motif ^{72}PXXP^{75} and an acidic domain ^{62}EEEE^{65} (Greenberg, Iafrate et al. 1998; Mangasarian, Piguet et al. 1999; Akari, Arold et al. 2000). There is data to support defective retrograde receptor trafficking – also known as recycling and also defective anterograde trafficking in which the newly synthesized MHC-I complex is misrouted in an AP1 dependent manner from trans-golgi network to endolysosomes for degradation (Yi, Rosales et al.). Nef directly binds to MHC-I (Williams, Roeth et al. 2002) which leads to exposure of cryptic tyrosine motif (YSQA) (Le Gall, Erdtmann et al. 1998) that facilitates direct binding to mu unit of AP-1 (Singh, Lau et al. 2009).

The cytoplasmic domain of HLA-I has a highly conserved 9 serine residues expanding through its' conserved region of 33 amino acids. Yet, interestingly Nef activity on MHC-I is not consistent throughout all the MHC alleles. Nef down-regulates HLA-A and B but not C and E. Selective down-regulation of HLA-I proteins is ascribed to the differences in the amino acid sequence in cytoplasmic tail region of HLA molecule. This was mapped to a tyrosine based sequence (YSQAASS) in this domain (Cohen, Gandhi et al. 1999) which is the same

region required for binding Nef (Roeth and Collins 2006). It was first shown that mutation of tyrosine residue at position 321 abrogates Nef dependent down-regulation (Le Gall, Erdtmann et al. 1998; Cohen, Gandhi et al. 1999). Subsequently, Cohen demonstrated that presence of ^{321}tyrosine, ^{325}alanine and ^{328}aspartate were critical for Nef dependent down-regulation of HLA-I proteins.

1.5.2. Mechanism of Nef mediated CD4 down-regulation

The mechanism of Nef mediated CD4 down-regulation is discussed in detail in chapter 6.

1.6. The aim of the project

After nearly three decades, we are yet to understand the complete picture of HIV pathogenesis. There is no doubt that Nef plays a significant role in HIV immune evasion and disease progression. As previously described it has been demonstrated that a diverse HIV-1, HIV-2 and SIV *nef* genes down-modulate human HLA from the surface of infected T cells (Kirchhoff, Schindler et al. 2004; Munch, Schindler et al. 2005) which is of sufficient magnitude to evade CTL mediated cytotoxic killing. Indeed, Nef mutations have been correlated with protection from progression to AIDS in HIV infected individuals (Kirchhoff, Greenough et al. 1995; Salvi, Garbuglia et al. 1998) and SIV infected rhesus macaques (Swigut, Alexander et al. 2004). However, whether the reported slow progression to AIDS is due to the poor replicative ability of Nef defective virus or whether it was due to a better CTL responses in the context of abolished MHC-I down-regulation or both is not yet known. Several researchers have shown that the down-regulation of MHC-I expression on HIV-1 infected cells by HIV-1 Nef, is associated with significant loss of virus suppressive capacity of HIV-1 specific CTL (Schwartz, Marechal et al. 1996; Collins, Chen et al. 1998; Tomiyama, Akari et al. 2002; Tomiyama, Fujiwara et al. 2005; Adnan, Balamurugan et al. 2006). Furthermore, Minang *et a*l. demonstrated that a defective CTL mediated viral suppression of SIV infected CD4+ cells by SIV specific CTL clones, which could not be explained by the level of expression of IFNγ or CD107a, could be explained by a differential level of down-regulation of MHC-I (Minang, Trivett et al. 2009). They further confirmed that a subtle difference in surface expression MHC is sufficient to overcome SIV Nef induced resistance to CTL mediated suppression.

Deciphering the role played by HIV Nef protein in down-regulation of Human Leucocyte Antigen Class I in HIV infection and disease progression forms the basis of my thesis. We

have noted that even though HLA-I A, B and C alleles are highly polymorphic, the cytoplasmic tail is somewhat conserved. Therefore if differences were found to exist in cytoplasmic domain amino acid sequences, they may affect the binding of HLA-I to Nef protein. If these differences in the membrane proximal complex of HLA-I lead to differential expression of HLA-I molecules on surface when associated with Nef, it may affect the antigen presentation to the immune system. If so these differences in MHC-I down-regulation may play an important role in disease progression in humans.

1.6.1. Specific aims of my thesis are

1. To investigate whether there is any polymorphism in the cytoplasmic tail of HLA-I alleles.
2. If there is any polymorphism, could this determine the level of down-regulation mediated by HIV Nef? If so we can identify a motif?
3. To investigate whether there is a difference in Nef mediated down-regulation of HLA-A and B alleles, and what are the functional consequences for T cell responses.
4. To investigate whether there are any differences in down-regulation by Nef associated with HLA-C alleles and the effect on CTL recognition
5. To investigate whether there is any difference in down-regulation between *nef* alleles of HIV-1, HIV-2 and SIV which could have consequences for HIV pathogenesis
6. To test the hypothesis that HIV virus infected cells subsequently produce defective viruses if the CD4 is not down-regulated

No studies have been carried out so far to assess the role played by different cytoplasmic isoforms of HLA-I in Nef mediated down-regulation which may have an important effect in

CTL mediated immune response. However, DeGottardi and his group recently demonstrated that several Nef alleles including SIV239 and HIV-2 differentially down-regulate MHC class I of Rheses Macaques and Sooty Mangabys, where the difference in class I genes were in cytoplasmic domains (McCutcheon JA 1995; DeGottardi, Specht et al. 2008).

In summary, after identifying a total of 16 polymorphisms in the cytoplasmic region of HLA-A, B and C alleles, I created 16 cell lines stably expressing HLA-A2 chimeric molecules (fusion of extracellular and transmembrane domains of HLA–A0201 to different HLA-I cytoplasmic isoforms) in each Jurkat cell line to assess the role of HIV Nef in HLA-I down-regulation. In this thesis I have assessed the Nef mediated down-regulation of HLA-A2 chimera of these cell lines. We observed that HLA-B was partially resistant to Nef/HIV-1 mediated down-regulation. This was preserved in most of the HIV-2 Nef alleles that we used. Interestingly, this resistance was enough to mediate control of infection with laboratory-adapted clade B virus. We also observed that HIV-1 viruses generated in the presence CD4 that was not down-regulated, were defective in replication and infection. Taken together, this thesis gives novel insight and answers several important questions relating to HIV pathogenesis.

2. Materials and Methods

2.1. Molecular Biology

2.1.1. Polymerase Chain Reaction (PCR)

Desired genes were amplified using the proof reading DNA polymerase Pfu (Stratagene). The following protocol in a Hybaid thermal cycler was used. The exact annealing temperature was established for each primer pair used in a given amplification reaction. Extension was carried out for 1 minute for every 1000bp of product.

Typical Pfu programme: 95°C 5minutes, 60°C 1minute, 72°C 2minute – 1 cycle

95°C 1minutes, 60°C 30seconds, 72°C 2minute - 30 cycles

A typical PCR reaction mix contains

10X reaction buffer	5µl
5mM dNTP mix	5µl
Forward primer (4pmol/µl)	5µl
Reverse primer (4pmol/µl)	5µl
Pfu	2.5U
Template DNA	10-100ng
H_20	to make up 50µl final volume

2.1.2. HLA typing and generating chimeric HLA-A2-fusion molecules

The GenBank search returned 7 HLA-A, 2 HLA-B and 7 HLA-C cytoplasmic isoforms. Primer pairs as specified below were used to alter HLA-A2 cytoplasmic tail sequences as detailed by PCR mutagenesis (Table 2.1). An HLA-A0201 wild type construct inserted into a pcDNA3.1 vector (Clontech) acted as template. The forward primer introduced a *Bam*HI restriction site. For A2-A2, A2-A26, A2-A11 and A2-A43, the reverse primer introduced both

desired point mutation and *Not*I restriction site to the gene terminus. For A2-A24, A2-A80, A2-A32, A2-B35, and A2-B57 the desired mutation was introduced in one round of PCR, the product purified by gel extraction, and then extended with primer A2R2Not/B57NotR/B35NotR to introduce the second restriction site (Table 2.2). Similar steps followed for A2-C chimera as indicated in Table 2.2.

Name of the primer	Sequence
A2F2Bam	ACG GGA TCC ACC ACC ATG GCC GTC ATG GCG CCC CGA
A2R2Not	ATA GTT TAG CGG CCG CTC ACA CTT TAC AAG CTG TGA GAG ACA CAT CAG A
A2M1RNot	ATA GTT TAG CGG CCG CTC ACA CTT TAC AAG CTG TGA GAG ACA TAT CAG A
A2M2RNot	ATA GTT TAG CGG CCG CTC ACA CTT TAC AAG CTG TGA GAG ACA CAT AGC CCT GAG CTC TGT CAC T
A2M3RNot	ATA GTT TAG CGG CCG CTC ACA CTT TAC AAG CTG TGA GAG ACA CAT CAG AGC CCT GGG CAC TGT CAC TGC TTG CAG CCT GAG TGT AAC T
A2M4RNot	ATA GTT TAG CGG CCG CTC ACA CTT TAC AAG CTG TGA GAG ACA TAT CAG AGC CCT GGG CAC TGT CAC TGC TTG CAG CCT GAA AGT AGC T
A2M5R	TGT GAG AGA CAC ATC AGA GCC CTG GGC ACT GTC ACT GCT TGC AGC CTG AGA GTA GCT CCC TCC TTT TCT ATC TGA GCT GTT CCT CCT
A2M6R	TGT GAG AGA CAC ATC AGA GCC CTG GGC ACT GTC ACT GCT TGC AGC CTG
A2R	T CCA CAT CAC AGC AGC GAC CAC
B57F	T GTG GTC GCT GCT GTG ATG TGG AGG AGG AAG AGC TCA **GGT GGA** AAA GG
B57NotR	ATA GTT TAG CGG CCG CTC A AGC TGT GAG AGA CAC ATC AGA GCC CTG GGC ACT GTC GCT **GCA** CGC AGC
B35NotR	ATA GTT TAG CGG CCG CTC A AGC TGT GAG AGA CAC ATC AGA GCC CTG GGC ACT GTC GCT **GGA** CGC AGC
CwR1	GCT GGA CGC AGC CTG AGA GCA GCT CCC TCC TTT TCC ACC TGA GCT CTT CCT CCT|CCA CAT CAC AGC AGC GAC CAC
CwR2Not	TCG ATG CGG CCG CTC AGG CTT TAC AAG CGA TGA GAG ACT CAT CAG AGC CCT GGG CAC TGT TGC TGG ACG CAG CCT GAG AGC
CwR1	AGC TCC CTC CTT TTC CAC CTG
CwF1	CAG GTG GAA AAG GAG GGA GCT ACT CTC AGG CTG CGT CCA GC
CwR2	TGG ACG CAG CCT GAG AGC AG
CwF2	CTG CTC TCA GGC TGC GTC CAC CAA CAG TGC CCA GGG CTC TG
CwR3	TCG ATG CGG CCG CTC AGG CTT TAC AAG TGA TGA G
CwR4	ACG CAG CCT GAG AGC AGC TC
CwF4	GAG CTG CTC TCA GGC TGC GTG CAG CAA CAG TGC CCA GGG C
CwR6	ACG CAA CCT GAG AGC AGC TC
UR7	TCG ATG CGG CCG CTC A GGC TTT ACA AGC GAT GAG AGA CTC ATC AGA GCC CTG GGC ACT GTT G
UR13F	GGAGGAGGAAGAGCTCAGATGGAAAAGGAGGGAGC
UR13R	GCTCCCTCCTTTTCCATCTGAGCTCTTCCTCCTCC
UR 14F	GGAGGAGGAAGAGCTCAGGTAGAAAAGGAGGGAGC
UR14R	GCTCCCTCCTTTTCTACCTGAGCTCTTCCTCCTCC
UR15F	GGAGGAGGAAGAGCTCAGATAGAAAGGAGGGAGC
UR15R	GCTCCCTCCTTTTCTATCTGAGCTCTTCCTCCTCC
UR16	ATA GTT TAG CGG CCG CTC ACA CTT TAC AAG CTG TGA GAG ACA CAT CAG A

Table 2.1: List of primers used to make the HLA class-I alleles. (Restriction sites are underlined and the point mutations highlighted)

Allele name		Primers used
A2-A2:	RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV	A2F2Bam/A2R2Not
A2-A26:	.....M.....	A2F2Bam/A2M1RNot
A2-A11:	.....T.....	A2F2Bam/A2M3RNot
A2-A43:	.....F.....M.....	A2F2Bam/A2M4RNot
A2-A24:	..N.....	A2F2Bam/A2M5→ A2F2Bam/A2R2Not
A2-A80:	.K...V.....	A2F2Bam/A2M6→ A2F2Bam/A2R2Not
A2-A32:	....L.....M.....	A2F2Bam/A2M7→A2F2Bam/A2R2Not
A2-B35:	.....GG.....	B57F/B57NotR → A2F2Bam/ B57NotR
A2-B57:	.....GG.....C.....	B57F/B35NotR → A2F2Bam/B35NotR
A2-C1205:	.....GG....C.....N.....E..I...A	CwR1/Cw2Not→A2F2Bam/A2R
A2-C0822:	.....GG.....N.....E..I...A	A2F2Bam/CwR1→CwF2/UR7→A2F2Bam/UR7
A2-C0430:	.....GG....C.....TN.....E..I...A	A2F2Bam/CwR2→CwF2/UR7→A2F2Bam/UR7
A2-C0750:	.....GG....C.....N.....E..IT..A	A2F2Bam/CwR3
A2-C0711:	.....GG....C....C.N.....E..I...A	A2F2Bam/CwR4→CwF4/UR7→A2F2Bam/UR7
A2-C0704:	.....GG....C....C.N.....E..IT..A	A2F2Bam/CwR4→CwF4/CwR3→A2F2Bam/CwR3
A2-C0719:	.....GG....C..V.C.N.....E..IT..A	A2F2Bam/CwR6→CwF4/CwR3→A2F2Bam/CwR3
G_{315}D:	.....DG.....C.....	UR13F/UR13R
G_{316}D:	.....GR.....C.....	UR14F/UR14R
G_{315}DR:	.....DR.....C.....	UR15F/UR15R
A2-B57$_{340}$CKV:	.....GG.....C.....CKV	A2F2Bam/UR16

Table 2.2: HLA-I cytoplasmic tail isoforms and primers used to generate each corresponding HLA-A2 construct.

2.1.3. Making chimeric CD4 molecules

Three 293T cell lines stably expressing wild type CD4 (CD4/WT), CD4 truncated at cytoplasmic domain (CD4/420T), and CD4 molecule truncated at amino acid position 430 (CD4/430T) were prepared as below.

Primer pairs specified below were used to alter CD4 cytoplasmic tail sequences as detailed by PCR mutagenesis (Table 2.3). A CD4 wild type construct inserted into a pcDNA3.1 vector (Clontech) acted as template. The forward primer introduced a *Bam*HI restriction site and the reverse primer a *Not*I restriction site. The desired truncation was introduced in one round of PCR and the product was purified by gel extraction (QIAGEN).

Name of the primer	Sequence
CD4BAMH1 F'	ACGGGATCCACCACCATGAACCGGGGAGTCCCTTTTAGG
CD4/420TR'	TCGATGCGGCCGCTCAAATGGGGCTACATGTCTTCTG
CD4/WTR'	TCGATGCGGCCGCTCAGACACAGAAGAAGATGCCTAGCC
CD4/430TR'	TCGATGCGGCCGCTCACTCTGCTTGGCGCCTTCGGTG

Table 2.3: Primers used to generate each corresponding HLA-A2 construct.

(Restriction sites are underlined)

2.1.4. Gene mutagenesis

2.1.4.1. Site directed mutagenesis using QuikChange II XL Site-directed mutagenesis kit (Agilent Technologies.)

The QuikChange II XL kit was used to make point mutations, replace amino acids, and delete or insert single or multiple adjacent amino acids. The basic protocol required a supercoiled double-stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers, each complementary to opposite strands of the vector and both

containing the desired mutation (see Figure 1). The oligonucleotide primers were extended during temperature cycling by *PfuUltra* HF DNA polymerase, without primer displacement. Extension of the oligonucleotide primers generated a mutated plasmid containing staggered nicks. Following temperature cycling, the product was treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5´-Gm ATC-3´) is specific for methylated and hemi-methylated DNA, and was used to digest the parental DNA template and to select for mutation containing synthesized DNA. DNA isolated from almost all *E. coli* strains is Dam methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA incorporating the desired mutations was then transformed into XL10-Gold ultra competent cells.

2.1.4.2. Site directed mutagenesis using in-house method

The procedure is essentially the same as in previous section and used to make mutations in large vectors (>14kb). However, primer designing involved two complementary strands of oligonucleotide with non-overlapping regions at the end to facilitate extension. The primers were extended using PrimeSTAR® HS DNA Polymerase (Takara Bio Inc). The PCR product was then digested with *Dpn*I, at 37^0C for 2 hours. The nicked vector was transformed in to One shot® Stbl3™ chemically competent *E. coli* cells.

2.1.5. Gene cloning

2.1.5.1. Lentiviral vector preparation

Vector DNA (2μg) was digested with appropriate restriction enzymes in accordance to instruction of the supplier (New England Biolabs). The vector was dephosphorylated by adding Shrimp Alkaline Phosphatase directly to the digested vector at a concentration of 0.1U/ml and incubated for 1 hour at 37^{O}C. The vector was purified by agarose gel electrophoresis.

2.1.5.2. Preparation of construct

Constructs were initially cloned into Topo vector using following recipe.

PCR product	4μl
Salt solution	1μl
Topo vector	1μl
Total volume	6μl

The above were mixed gently and left at room temperature for 10 minutes, and were then left on ice for chemical transformation. Two microliters of the above mix were added to One Shot® chemically competent E coli and mixed gently. This was incubated on ice for further 30 minutes. Cells were then heat shocked for 30 seconds in a water bath at 42^{0}C without shaking. These cells were subsequently transferred to ice for 2 minutes before adding 250μl of SOC medium (Invitrogen). The tubes were capped tightly and were shaken horizontally (220rpm) at 37^{0}C for 1 hour.

Fifty microliters of each transformation reaction were spread on prewarmed Kanamycin selective agar plate. After overnight incubation colonies were selected and expanded in 5ml of terrific broth containing Kanamycin and DNA extracted through mini prep. DNA (21.5µl from extracted 50µl volume) was digested with *Bam*HI (0.5µl) and *Not*I (0.5µl) with appropriate buffer (Buffer 3, 2.5µl) in a final volume of 25µl for one hour and then they were run on gel. Products that contained appropriate band size were sent for sequencing. The constructs that had correct sequence were ligated to lentiviral vector.

2.1.5.3. Ligation to lentiviral vector

Ligation is usually performed at room temperature for 2 hours or at 16^{O}C overnight. Typically a 20µl reaction volume was used with 20ng of vector DNA and 100ng of insert DNA with 2 units of T4 ligase (New England Biolabs) in commercial buffer.

2.1.5.4. Transformation

2.1.5.5. Bacterial strains used

DH5∝ (Invitrogen) were used for the propagation of DNA. For larger constructs such as proviral plasmids, either HB101 or Stbl3 (Invitrogen) were used for transformation.

2.1.5.6. Transformation reaction

Competent cells were thawed on ice. Ten nanograms of plasmid DNA or 10 µl of ligation product was added to 100 µl of competent cells and mixed gently by tapping. The cells were then incubated on ice for 30 minutes, heat shocked at 42^{0}C for 45 seconds followed by

incubation on ice for further 2 minutes before adding 500 μl of S.O.C. medium. These were returned to a shaking incubator for 1 hour at 37^0C. The cells were then spread on plates containing appropriate antibiotic (Kanamycin for selection during initial Topo cloning and Ampicillin in the sub cloning round) and incubated overnight at 37^0C.

2.1.5.7. DNA isolation

QIAprep spin miniprep kit (QIAGEN) was used for small-scale plasmid DNA preparation (as manufacturers' protocols). Approximately 3 mls culture were used which gave a yield of approximately 15 μg plasmid DNA in a volume of 50μl TE buffer.

When large amount of DNA preparation is required an equilibrium centrifugation in Caesium chloride-ethidium bromide gradient ultracentrifugation method was used as described below. Briefly, cells were grown in 1l of Terrific broth at 37^0C overnight. Cells were harvested by centrifugation at 4000 rpm for 15 minutes. Pellet was resuspended in 100 ml of P1 solution and mixed well. This was followed by addition of 100 ml of P2 buffer to lyse cells. This suspension was neutralised by adding 100 ml P3 buffer after 5 minutes incubation at room temperature. The precipitates were removed by centrifugation at 4000rpm for 30 minutes at 4^0C. The supernatant was decanted into a fresh bottle through clean filter. The nucleic acid was precipitated by adding equal volume of isopropanol at room temperature for at least one hour. DNA was pelleted by centrifuging at 4000rpm for 30minutes and air dried before dissolving with 10 ml TE buffer.

The solution weight was adjusted to 12g. Twelve grams of CsCl and 1ml Ethidium bromide (10mg/ml) was added to the solution. Insoluble protein was removed by centrifugation for 30 minutes at 4000rpm in Heraeus Megafuge 2.0R centrifuge. The supernatant was then

transferred to Beckman Quick seal tubes and heat sealed. The DNA was banded by centrifugation at 48000 rpm in Beckman 70.1Ti rotor in a Beckman L7-65 ultracentrifuge for 48 hours. Bands are drawn off through the side of the tube using syringe fitted with 19 gauge needle, with a second needle inserted at the top of the tube to act like a vent. Ethidium bromide was removed by extraction with water saturated butanol. DNA was precipitated by adding two volumes of 100% ethanol and spinning at 4000rpm for 20minutes. Pellet was washed twice with 70% isopropanol. The pellet was first air dried and then dissolved in TE buffer.

2.1.5.8. DNA sequencing

All DNA constructs generated in the thesis were sequenced using the MRC Human Immunology Unit service. Primers used for sequencing are listed in table 2.4.

Construct/Vector	Primer name	Primer sequence
Constructs in Topo vector	M13F	5´-GTAAAACGACGGCCAG-3´
	M13R	5´-CAGGAAACAGCTATGAC-3´
Constructs in lentiviral vector	FSFFV	5'-TGCTTCTCGCTTCTGTTCG-3'
	RWPRE	5'-CCACATAGCGTAAAAGGAGC-3'
pNL4-3	NL432 Nef SEQ F'	CTCTTGATTGTAACGAGGATTGTGG
	NL432 Nef SEQ R'	GGAACTCCCAACTCAGGTGATCC
	NL432-M20A-F'	CTGTAAGGGAAAGAGCGAGACGAGCTGAGC
	NL432-M20A-R'	TCTTTCCCTTACAGCAGGCCATTCCAATCAC

Table 2.4: Primers used for sequencing and generating M20A mutants in this thesis.

2.2. Tissue culture

2.2.1. Cell culture

Jurkat cells expressing CD4+ (JJK), MT2 and C8166 cells were grown in RPMI (Sigma) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50U/ml penicillin and 50mg/ml streptomycin. 293T cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% FCS, 2mM L-glutamine, 50U/ml penicillin and 50mg/ml streptomycin.

JJK, MT2, C8166 and 293T cells were split every 3-4 days.

2.2.2. Preparation of human peripheral blood mononuclear cells (PBMC)

Blood samples were usually obtained from healthy volunteers or otherwise buffy coats used. Venous blood was drawn in to collection tubes containing heparin and separated by Ficoll Hypaque (Sigma) density centrifugation. Briefly blood was layered onto Ficoll hypaque and spun for 20 minutes at 2000 rpm on a tabletop centrifuge (Heraeus labofuge) without brakes at room temperature. PBMC layer is separated out and washed twice with preheated R10 at 2000 rpm for 10 minutes. If cells are being used for T cell clone expansion, PBMC are irradiated using sealed Ceasium source, 4000 rads.

2.2.3. T cell clones

CD8+ T cell clones were expanded by addition of mixed (three donors) irradiated PBMC and PHA (5μg/ml) using approximately $5x10^6$ PBMC per $1x10^6$ T cell clones. The clones were

grown in RPMI supplemented with 10% AB negative human serum, 2mM L-glutamine, 50U/ml penicillin, 50mg/ml streptomycin and 100-200 U/ml recombinant IL-2. 2-3 days later 100-200 U/ml IL-2 was added. Media was regularly changed and cells split when confluent. T cells were used on day 13 for viral suppression assays. For IFNγ ELISPOT they were used 10-14 days and for other assays up to 16 days post stimulation. T cell clones were restimulated 12-21 days later as described before. Cells could be frozen after 7 days of the cycle and then be used in T cell assays 2 days after thawing and IL-2 addition, but this was very rarely done. Fresh clones were used most of the time.

2.2.4. Electroporation of Jurkat cells

Electroporation makes use of the fact that the cell membrane acts as an electrical capacitor unable (except through ion channels) to conduct electric current. When the cell membrane is subjected to a high voltage electric field it results in temporary breakdown and formation of pores that are large enough to allow macromoleules to enter. During the time when pores are open, nucleic acid can enter the cells and sometimes the nucleus.

Jurkat cell were grown in R10 at density of $0.5x10^6$/ml and the media was changed to fresh media the day before transfection. On the day of transfection $4\text{-}6x10^6$ JJK (per experiment) were spun down and washed with RPMI1640. All residual media was carefully removed. Cells were resuspended in 0.4ml RPMI1640. Cells were gently mixed with 30 μg DNA and transferred to a 4 mm electroporation cuvette (BioRad). Then the cuvette was placed in the holder of the electroporation apparatus (BTX Electro cell manipulator 600) and shocked once at 260 V, 1070 μF, 72 Ω. Soon after electroporation cells were transferred to prewarmed 2ml

of R10 in a 6 well plate. The cells were returned to incubator at 37^0C, 5% CO_2. Cells are analysed using appropriate assays after 24 hours.

2.2.5. Transfection of human T cells using Nucleofector kit

Transient transfection of freshly isolated PBMC or cell lines was performed using the T cell nucleofector kit (Amaxa). Briefly, $1x10^6$ cells were pelleted and resuspended in 100µl of room temperature Nucleofector solution (this depends on the type of cell line that is used). Cell suspension was mixed with 5µg of DNA and transferred to a cuvette supplied in the kit. The cuvette was placed on the holder of the electroporator (Amaxa) and relevant programme (U14 for PBMC, V5 for JJK cells) was used. Cells were rested for 10 minutes and then diluted into 2ml of R10 and incubated at 37^0C, 5% CO_2 until relevant assays were performed.

2.2.6. Transfection of monolayer cells using Lipofectamine Transfection reagent

Lipofectamine based transfection uses the ability of a polycationic synthetic lipid, mixed with phosphatidyl-ethanolamine or DOPE, a fusogenic lipid with an amine-group with a strong positive charge to form a link with phosphate group of DNA neutralizing the negative charge of phosphate group. This lipid-DNA mixture results in the formation of structures that can fuse and pass across the plasma membrane to deliver DNA into the cell.

The day before transfection, 2-6 x 10^4 cells are plated in 500 µl of growth medium (with the usual amount of serum) in 24 well plate without antibiotics so that cells will be 50-80% confluent at the time of transfection.

For each transfection sample, 0.2-0.4 µg DNA is diluted in 25 µl of Opti-MEM® I Reduced Serum Medium without serum and mixed gently. Lipofectamine™ 0.5-5 µl were diluted in 25 µl of Opti-MEM® I Medium (or other medium) without serum and mixed gently. Diluted DNA was mixed with diluted Lipofectamine™ (total volume = 50 µl) and incubated for 15-45 minutes at room temperature.

For each transfection, 0.15 ml of Opti-MEM® I Medium was added to the tube containing the complexes (total volume = 200 µl) and mixed gently. Growth medium was removed from 293T cells and replaced with 0.2 ml of growth medium without serum. 0.2 ml of diluted complexes from previous steps was added to each well and mixed gently by rocking the plate. Incubate cells at 37°C in a CO_2 incubator for 5 hours.

Then 0.4 ml of growth medium containing 2X the normal concentration of serum was added without removing the transfection mixture. If toxicity was observed after transfection, medium was replaced with fresh, complete medium (with normal amount of serum).

2.2.7. Transfection of monolayer cells using Calcium phosphate co precipitation method

This method was used in most 293T cell transfection experiments. Calcium phosphate transfection method is based on the principle that, by slowly mixing a HEPES buffered saline solution with a solution containing calcium chloride and DNA, a precipitate containing calcium phosphate and DNA will form which will adhere to the cell surface and will be taken up by the cells.

Cells were plated in D10 media the day before the transfection expecting to reach a confluency of 50-80% on the day of transfection. For each well of a 6 well plate (99.4 cm^2 surface area) 5μg of DNA was mixed with 100 μl of 0.25 M $CaCl_2$ solution in a sterile eppendorf tube. 100 μl of ice cold 2xHEPES buffer was added to DNA-$CaCl_2$ solution in a drop wise manner while vortexing the solution. The precipitation was allowed to form at room temperature for 20 minutes. The solution was then added to wells in a drop wise manner and mixed with the medium gently, without agitating cells. The plates were then returned for further incubation for 8-16 hours at 37^0C, 5% CO_2. The medium was replaced with fresh D10 after this incubation. Either the cells were harvested 24-48 hours later for analysis or if this was used to make pseudotyped viruses, the supernatant was harvested 48, and 72 hours later.

2.3. Antibodies, plasmids and viruses

2.3.1. Antibodies. The following antibodies were used in this thesis.

Description	Host	Conjugate	Clone	Supplier	Cat. No.	Others
Anti HLA-ABC	Mouse	AlexaFluo®	W6/32	AbD serotec	MCA81A647	IgG2a monoclonal
Anti HLA-ABC	Mouse	Purified	W6/32	Alistair		
Anti HLA-A2	Mouse	Phycoerythrin	BB7.2	AbD Serotec	MCA2090PE	IgG2b monoclonal
Anti HLA-A2	Mouse	AlexaFluor®	BB7.2	AbD Serotec	MCA2090A647	IgG2b monoclonal
Anti HLA-A2	Mouse	Purified	BB7.2	Alistair		
Anti HLA-A3	Mouse	Biotin	4i153	Abcam	Ab31572	IgM monoclonal
Anti HLA-B7	Mouse	Purified		AbD Serotec	MCA986	IgG1
Anti CD4	Mouse	APC	MT310	Dako		
Anti CD107a	Mouse	PECy7	H4A3	BD	555802	IgG1κ
Anti CD107a	Mouse	Phycoerythrin	H4A3	BD	555801	IgG1κ
Anti CD107a	Mouse	PerCP	1D4B	ebiosciences	46-1071-82	
Anti IFN gamma	Mouse	FITC	25723.11	BD	340449	IgG2b
Anti IFN gamma	Mouse	APC	25723.11	BD	341117	IgG2b
Anti CD4	Mouse	APC	SK3	BD	435771	IgG1κ
Anti CD4	Mouse	Phycoerythrin	SK3	BD	345769	IgG1κ
Anti CD4	Mouse	FITC	SK3	BD	345768	IgG1κ
Anti CD8	Mouse	PerCP	SK1	BD	345664	IgG1κ
Anti CD8	Mouse	APC-H7	SK1	BD	641400	IgG1κ
Anti CD8	Mouse	FITC	SK1	BD	345772	IgG1κ
Anti CD3	Mouse	PECy5	HIT3a	BD	555341	IgG2a
AntiCD3	Mouse	PerCP	SK7	BD	345766	IgG1κ
Coulter Clone KC57-RD1	Mouse	Phycoerythrin	FH190-1-1	Beckman Coulter	6604667	IgG1
Anti mouse	Donkey	APC		eBiosciences		IgG
Anti rabbit	Goat	HRP	Polyclonal	Invitrogen	656120	IgG (H+L)
Anti-Human	Goat	HRP		Invitrogen	AHI0404	
Anti HIV-1 p24	BALB/C	Purified	05-009	NIBSC	ARP3243.3	Monoclonal
Anti tubulin	Mouse	Purified		Abcam	Ab56676	IgG2b
Antigp120	Goat	Purified	Polyclonal	Abcam	Ab21179	IgG
Anti mouse	Sheep	HRP		GE Healthcare		IgG
Anti rabbit	Goat	HRP				IgG
Anti goat	Donkey	HRP	Polyclonal	Abcam	Ab6885	IgG (H+L)

Table 2.5:Antibodies used in this thesis.

2.3.2. Vectors used in the thesis

Plasmids encoding wild type or mutant Nef-GFP fusion proteins created as described before (Drakesmith, Chen et al. 2005) and pIRES2-EGFP (Clontech), pCDNA3.1 Hygro (Invitrogen), and pHR-SIN-BX-IRES-Em were gifts from Drs. X. Xu and Demin Li.

Truncated form of pBR322 (New England BioLabs) with Nef inserts that were used in chapter 4 were obtained through the AIDS Reagent Program Division of AIDS, NIADS, NIH.

2.3.3. Viruses used

Proviral HIV-1 construct NL4-3 and constructs containing different *nef* alleles were obtained from AIDS reagent programme. The M20A NL4-3 construct was generated using specific PCR that introduced site directed mutagenesis. The sequences were confirmed by sequencing both strands of DNA insert.

Laboratory adapted strains of clade B virus, IIIB and MN viruses (kind gift of Dr. Tao Dong) were also used in some of the assays.

2.4. Virology

2.4.1. Generation of VSV pseudotyped viruses

A replication incompetent recombinant lentivirus was generated using the three plasmid system by cotransfection of 293T cells with calcium phosphate. A DNA mix composed of 1.5μg lentiviral vector with inserted HLA-A2 chimeric sequence, 1μg envelop plasmid; pMD.G, expressing the vesicular stomatitis virus G-envelope protein, and 1μg packaging plasmid pCMVΛR8.91, containing the HIV-1 gag/pol, tat, and rev genes required for efficient lentivirus production were used.

Supernatant was collected at 24hr, 48hr, and 72hr post-transfection, and stored at -80°C until they were concentrated. The viruses were concentrated by ultracentrifugation at 30,000 rpm for 1.5 hours under sterile conditions. The pellet was resuspended in 200μl RPMI 1640, and aliquoted for storage at -80°C or used directly to infect target cells.

2.4.2. Generation of stable HLA-A2 fusion construct expressing Jurkat cell lines

JJK cells in the exponential growth phase were infected with the packaged virus. $1x10^6$ JJK cells were pelleted and supernatant drawn off. One aliquot of lentivirus was used to infect cells. The virus aliquot was added to cells and returned to incubation for 2 hours at room temperature. Cells were mixed every ½ hour before adding 1ml of R10 and returning to 24 well plates for culturing. After 5 days cells were checked for HLA-A2 expression and positive cells were selected by flowcytometric sorting.

2.4.3. Generation of HIV viruses

HIV-1 IIIB virus was used for initial viral assays. The virus stocks were generated by infecting $1x10^7$ C8166 cells. In summary, the cells were spun down in 15ml falcon tubes and mixed with one vial of concentrated HIV-1 IIIB virus stock and incubated at 37^0C in 5% CO_2 for 1.5 hours. At the end of the incubation period, cells were washed twice with RPMI and resuspended in R10 and returned to the incubator. The supernatant was harvested on D4, D7 and D10, and the virus concentration was measured using p24 capture ELISA (Immunodiagnostics).

2.5. Functional assays

2.5.1. Viral suppression Assay

Viral suppression assays were carried out by incubating freshly infected cell lines with CD8+ CTL clones at ratios of 1:1, 1:2, 1:4 and 1:8 on D12/13 of stimulation. Infected cells incubated without CTL were used as positive control. At predetermined time points either cells were harvested for flow-cytometric based analysis and/or supernatant was harvested for analysis of p24 antigen using p24 Capture ELISA kit (Immundiagnostics).

HIV-1 IIIB virus, C8166, HIV Gag P17 specific (G10), B8 Nef specific (B3), B51 Gag specific clone SM342 B2 Nan, A24 Nef specific (B80), A24 Gag specific CTL clone and A3 Nef specific (FLK3) CTL clones were kind gifts of Dr. Tao Dong.

2.5.2. Live virus IFNγ ELISPOT assay

The production of IFNγ in CTL that were co-cultured with target cells were measured on D6 of infection (the highest infection rate of Jurkat cells was achieved on D6). The cells were incubated at E:T = 400:20000 after correcting for infectivity rate. All ELISPOT assays were performed using the human IFNγ ELISPOT kit (Mabtech) according to the manufacturer's instructions. Briefly, the ELISPOT plates are coated with 5μg of anti IFNγ monoclonal antibodies for at least 2 hours at room temperature or overnight at 4^{o}C. Antibodies were then washed 6 times with PBS and blocked with R10 at 37^{0}C for 2 hours to prevent non specific binding. The cells were incubated at above concentration in R10 overnight (12-14 hours) before developing according to manufacturer's instructions. The plates were read using automated ELISPOT reader and AID ELISPOT 3.1.1 HR software (Autoimmune Diagnostika)

ELISPOT assays utilising virus-infected cells were carried out in the Category III laboratory. Uninfected cells pulsed with relevant peptide (peptide concentration 2 μg/ml), PHA and/or PMA + Ionomycin were used as positive controls. HLA mismatched clones incubated with infected target cells, target cells alone, media alone were used as internal negative controls. Wells containing spot numbers greater than the mean + 2 SD of three negative control wells (HLA mismatched clones incubated with target cells) were regarded as positives. Values of T cell responses were all background subtracted and presented as SFU/10^6 CD8+ T cells.

2.5.3. Flow based HLA-I down-regulation assay

Transduced cells were stained for surface molecules using a panel of antibodies after 24 hours. The panel included monoclonal anti bodies against HLA-A2, MHC-I, CD4 and HLA-A3 (Figure 2.5). Cells were washed in PBS, pelleted by centrifugation at 1500rpm for 5 minutes and the supernatant removed completely before incubating with the pre-titrated amounts of antibodies. The cell were incubated on ice for 20 minutes and washed with 2ml of FACS wash (PBS+2%FCS+0.01% sodium azide).

If a secondary antibody is used, the staining steps were repeated. Intracellular staining was carried out by first permiabilising cells with cytofix/cytoperm (BD Biosciences) for 20 minutes at 4^0C. Cells were then washed with 1xcytoperm buffer (BD Biosciences) twice before incubating with intracellular antibodies. Washed cells were fixed with fixing reagent before performing analysis using Dako CyAn flowcytometer with summit software.

2.5.4. FACS based HLA-I/CD4 internalisation assay

The internalisation assay was performed essentially as described previously (Le Gall, Buseyne et al. 2000). In brief, Jurkat/293T cells were stained with anti-A2/CD4 specific antibody in phosphate buffered saline, 0.5% BSA and 2 mM EDTA for 20 minutes on ice. Cells were washed twice with FACS wash containing 0.5% BSA to remove all unbound antibodies. BSA instead of FCS was used to prevent beta microglobulin replacement from FCS. Supernatant was discarded and cells were resuspended in RPMI media supplemented with 0.5% BSA. An aliquot was taken out at zero time point to determine the steady state surface expression and samples returned to the incubator at 37^0C. Aliquots were collected at different time points aliquots to determine the level of surface expression of A2/CD4. Previously titrated secondary antibody was added and incubated on ice for 20 minutes. Cells were washed with ice-cold FACS wash and analysed using the FACS as soon as possible.

$$\text{Percentage internalised} = \frac{\text{MFI of A2/CD4 at } T^0 \text{ time point- MFI of A2/CD4 at Tm time point}}{\text{MFI of A2/CD4 at } T^0 \text{ time point}} \text{X100\%}$$

2.5.5. FACS based HLA-I /CD4 recycling assay

A measurement of the rate of recycling of internalised molecules was performed as described before (Le Gall, Buseyne et al. 2000). Briefly cells were harvested and treated with 150µg/ml cycloheximide, EDTA (2 mM) for 2-3 hours in RPMI1640 supplemented with 10% FCS. The cells were then harvested and an aliquot removed and placed on ice. The remainder of the sample was stripped of stainable HLA-A2 or CD4 by washing in an acidic buffer 50mM glycine, 100mM NaCl (pH 3.0 for Jurkat cell lines expressing A2 chimera and pH 2.2 for 293T cells expressing CD4 chimera, pH were optimised in a pilot study for the two cell lines) twice at room temperature for 1 min. The stripped cells were washed in PBS and incubated at

37^0C, 5%CO_2 medium without serum for the required period of time in the presence of 150µg/ml cycloheximide. Samples were incubated in media without FCS to avoid substitution of bovine ß2 microglobulin present in FCS for the human ß2 microglobulin removed with the striping protocol. Cells were harvested and placed on ice and stained for HLA-A2 or CD4 using wash buffers that included BSA instead of FCS.

Steady state surface levels was defined as 100% and the fluorescence intensity at T^0 time point after the acidic wash was defined as 0%.

The amount of molecules recycled was calculated as below

$$\text{Percentage recycled} = \frac{(\text{MFI of A2/CD4 at } T^m \text{ time point} - \text{MFI of A2 at } T^0 \text{ time point})}{\text{MFI of A2 at } T^0 \text{ time point}} \times 100\%$$

2.6. Protein Biochemistry

2.6.1. SDS PAGE electrophoresis and Western blotting technique

Cells were harvested and lysed with 1% NP40 containing protease and phosphatase inhibitors for 30 minutes on ice. After removing cellular debris by centrifuging at 13000rpm for 20 minutes at 4^0C the lysate was mixed with loading buffer. The remaining sample stored at -20^0C.

Before loading onto Gel (NuPAGE® 2-12% Bis-Tris Gel Cat. No. NP0323BOX) lysate was boiled for 5 minutes with reducing agent (DTT 100 mM) and loading buffer (Thermo Scientific Cat No. 39001) to denature the protein. After setting up the gel tank, samples were loaded with prestained Benchmark protein ladder. The gel was run at 70 V for 30 minutes until the sample formed a sharp band and then it was increased to 140 V until the dye reached the bottom of the gel. Proteins were then transferred from NuPAGE gel to PVDF membrane using a semi-dry transfer cell (Bio-Rad) after methanol activation of membrane. Nonspecific binding was blocked by incubating membrane with blocking buffer (PBS containing 2-10% milk and 0.01% Tween-20 for 1 hour at room temperature). Subsequently, blocked membrane was incubated with primary antibody diluted in blocking buffer for 1 hour at room temperature or overnight at 4^0C. After 3 washes with washing buffer (PBS-Tween) the membrane was incubated with Horse Radish Peroxidase conjugated secondary antibody at room temperature for 1 hour. The membrane was washed with PBS-Tween for 10 minutes (3-4 times) and developed using chemiluminescent detection kit from BioRad.

2.7. Data analysis

Data analysis was performed with Flowjo Version 9.0.1, Graph Pad Prism V5.0a and SPSS Statistics v17.0. The *p* values were calculated with the Students *t*-test using SPSS software (IBM) with a two-tailed distribution.

In addition, NetPhos (Blom, Gammeltoft et al. 1999), Scansite (Obenauer, Cantley et al. 2003), NetSurfP (Petersen, Petersen et al. 2009) and I-TASSER (Roy, Kucukural et al. 2010) software were used to analyse phosphoprediction and tertiary structures of chimeric molecules.

2.8. Reagents and Solutions

2.8.1. Buffers:

2.8.1.1. 1% NP40 Lysis buffer

50mM Tris HCl pH 7.4
150 mM NaCl
1mM EDTA
1% NP40

1 miniRoche P1tablet per 10ml of buffer
1:200 NaF
1:200 Sodium orthovanadate

2.8.1.2. Loading buffer

0.3M Tris HCl
5% SDS
50% glycerol
0.004% Bromophenol blue

2.8.2. Bacterial growth medium

2.8.2.1. LB medium (Luria Bertani Medium)

10g Trypton, 5g yeast extract, 10g NaCl dissolved in 1l of deionized H_2O, pH adjusted to 7.4 and sterilised by autoclaving for 20 minutes at 15 psi (1.05kg/cm^2) on liquid cycle

2.8.2.2. Terrific broth

12g Tryptone, 24g yeast extract, 8ml glycerol were dissolved in 900ml litre deionized H_2O and autoclaved. Allow the medium to cool down to 60^{0}C or less and add 100ml sterile

solution of 0.17M KH_2PO_4, 0.72M K_2HPO_4. Sometimes TB powder is purchased from manufacturer (Invitrogen/Sigma)

2.8.3. Large scale plasmid isolation buffer

- **Buffer P1** - 50 mM Tris pH7.4, 10 mM EDTA
- **Buffer P2** - 200 mM NaOH, 1% SDS
- **Buffer P3** - 3M potassium acetate pH 5.5 (Dissolved in 1kg potassium acetate in 1.6l of H_2O followed by mixing with 2.4l Acetic acid)

2.8.4. Other buffers used

- **TE Buffer** – 10 mM Tris pH 8.0, 1 mM EDTA
- **PBS Tween 0.05%** - 0.5 ml Tween 20 (Sigma), 1l PBS, mixed on magnetic stirrer
- **2x HEPES buffered saline (HeBS)** - 16.4 g NaCl (0.28 M final), 11.9 g HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; 0.05 M final), 0.21 g Na_2HPO_4 (1.5 mM final), 800 ml H_2O. The pH was titrate 7.05 with 5N NaOH. H_2O added to 1 liter. Filter sterilize through a 0.45μm nitrocellulose filter. This is tested for transfection efficiency and stored at -20 □in 50ml aliquots
- **2.5M $CaCl_2$ buffer** - 183.7 g $CaCl_2.2H_2O$ (Sigma; tissue culture grade), H_2O to 500 ml. Filter sterilize through a 0.45-μm nitrocellulose filter (Nalgene). Stored at -20 □in 10-ml aliquots

3. Cytoplasmic domain of HLA class I B alleles resist down-regulation mediated by HIV Nef than HLA-A

3.1. Introduction

The role of HLA class I (HLA-I) in HIV infection has been investigated to a great extent over the last two decades. Inspite of this, the results so far had been confusing and have failed to provide convincing answers to simple questions like why certain alleles are protective and certain alleles are not. Out of the classical HLA class I alleles, HLA-B has been studied extensively due to its ability to associate with natural killer cells and KIR. There is multifaceted evidence supporting the fact that HLA-B is more protective against HIV than HLA-A (Kiepiela, Leslie et al. 2004; Goulder and Watkins 2008). This is somewhat puzzling given that both HLA-A and HLA-B alleles are expressed to the same extent on cells. This protection is not entirely explained by a confounding effect of a few highly protective HLA-B types such as B57, B58, B27 (Stephens 2005). Some argue that the increased protection is due to a better HLA-B restricted cytotoxic T cells while there is also evidence to support that this is due to polyfunctionality of HLA-B associated T cell responses (Almeida, Price et al. 2007; Harari, Cellerai et al. 2007) although these could be secondary to better control of virus by the mentioned HLA types. It also has been argued that HLA-B drives evolution of HIV-1 infection (Kiepiela, Leslie et al. 2004).

However, it is often forgotten that HIV leads to the down-regulation of most of the classical HLA-I molecules. The less emphasis paid on this aspect is probably due to the fact that degree of HLA-I down-regulation is variable and depends on the type of cells examined. Also to add to the confusion is the involvement of multiple genes of HIV (Nef, Tat and Vpu) and their involvement at different levels of life cycle to reduce surface expression of HLA-I (described in Chapter 1).

After nearly three decades, we are yet to understand the complete picture of HIV pathogenesis. There is no doubt that Nef plays a significant role in HIV immune evasion and

disease progression. As previously described, it has been demonstrated that a diverse HIV-1, HIV-2 and SIV Nef alleles down-modulate human HLA from the surface of infected T cells (Kirchhoff, Schindler et al. 2004; Munch, Schindler et al. 2005) and that, at least with HIV-1, it occurs to sufficient extent to evade CD8+ T cell (CTL) mediated cytotoxic killing (Collins, Chen et al. 1998). Indeed, Nef mutations have been correlated with protection from progression to AIDS in HIV infected individuals (Kirchhoff, Greenough et al. 1995; Salvi, Garbuglia et al. 1998) and SIV infected rhesus macaques (Swigut, Alexander et al. 2004). However, it has not been demonstrated that the reported slow progression to AIDS is due to the poor replicative ability of Nef defective virus or whether it was due to a better CTL response in the context of abolished MHC-I down-regulation or both. Several researches have shown that direct down-regulatory effect of HIV-1 Nef on MHC-I expression of HIV-1 infected cells is associated with significant loss in the virus suppressive capacity of HIV-1 specific CTL (Schwartz, Marechal et al. 1996; Collins, Chen et al. 1998; Tomiyama, Akari et al. 2002; Tomiyama, Fujiwara et al. 2005; Adnan, Balamurugan et al. 2006). It has also been described that subtle difference in surface expression MHC-I is sufficient to overcome SIV Nef induced resistance to CTL mediated suppression and this viral suppression was independent of IFNγ and CD107a expression (Minang, Trivett et al. 2009).

Deciphering the role played by HIV Nef in down-regulation of HLA-I in HIV infection and disease progression forms the basis of this chapter. Nef's mechanism on HLA-I down-regulation has been extensively investigated. DeGottardi and his group recently demonstrated that several Nef alleles including SIV239 and HIV-2 differentially down-regulate MHC-I of Rheses Macaques and Sooty Mangabys, the difference of MHC-I being the differences in cytoplasmic domains (McCutcheon JA 1995; DeGottardi, Specht et al. 2008). However, the apparent involvement of the polymorphism of HLA-I cytoplasmic domain has not yet been studied. Limited information available on this aspect to study the role played by different

cytoplasmic isoforms of HLA-I in Nef mediated down-regulation which may have an important effect in CTL mediated immune response forms the reason for my study.

In this study I have used cytoplasmic domains that are present in HLA sequence database (**http://www.ebi.ac.uk/imgt/hla/**) and have generated cell lines that stably express a chimeric molecule that has one of these cytoplasmic domains fused to an extracellular and transmembrane domains of HLA-A*0201. We have noted that even though HLA-I A, B and C alleles are highly polymorphic, the cytoplasmic tail is well conserved. Therefore if differences were found to exist in cytoplasmic domain amino acid sequences, they may affect the binding of HLA-I to Nef protein. If these differences in the membrane proximal complex of HLA-I lead to differential expression of HLA-I molecules on surface when associated with Nef, it may affect the antigen presentation to the immune system. If so these differences in MHC-I down-regulation may play an important role in disease progression in humans. I have demonstrated that HLA-B cytoplasmic domain is less susceptible to down-regulation mediated by Nef of HIV-1 and HIV IIIB infection compared to HLA-A. We also demonstrate that this leads to better control of HIV infection by HLA-B alleles.

Specific aims we are trying to address in this chapter are,

1. To evaluate polymorphism of the cytoplasmic tail of HLA-I alleles
2. To assess the role of polymorphism in determining the level of down-regulation of HLA-I in HIV Nef
3. To investigate whether there is a difference in Nef mediated down-regulation of HLA-A and B alleles, if so does it have an antiviral efficacy of HLA-A and B restricted T cell responses
4. To investigate whether there is any differences in down-regulation associated with HLA-C alleles and whether it has any effect on CTL recognition

In summary, after identifying a total of 16 types of polymorphisms in the cytoplamic region of HLA-A, B and C alleles, I created 16 cell lines stably expressing HLA-A2 chimeric molecules (fusion of extracellular and transmembrane domains of HLA–A*0201 to different HLA-I cytoplasmic isoforms) to assess the role of HIV Nef in HLA-I down-regulation. I have assessed HIV-1_{SF2} Nef mediated down-regulation of HLA-A2 chimera of these cell lines. I also confirmed that we can see a similar pattern of down-regulation with a laboratory strain of HIV, IIIB virus. From here, I went on to demonstrate that there is a statistically significant difference in down-regulation of HLA-B compared to HLA-A cytoplasmic alleles which makes HLA-B alleles more efficient in control of viral infection in terms of IFNγ secretion and CD107a secretion. In this chapter I have also shown that native HLA-B alleles are down-regulated to a much lesser degree than HLA-A and this leads to better control of HIV infection by HLA-B restricted CTL than HLA-A.

3.2. Results

3.2.1. Polymorphism of HLA-I cytoplasmic domain

It has been shown that the cytoplasmic domain sequence is important in determining Nef mediated down-regulation of HLA-I, where HLA-A and B are believed to be susceptible while HLA-C cytoplasmic allele is resistant (Cohen, Gandhi et al. 1999). A previous study looking at simian MHC-I down-regulation by Nef of SIV, HIV-1 and HIV-2, demonstrated that MHC-I down-regulation depends on polymorphism of the Mamu, Ceat MHC cytoplasmic domains but gave little information on human HLA-I (DeGottardi, Specht et al. 2008). Looking at literature we observed that consensus sequences for HLA-A, B and C alleles have been used for assessment of HLA-I cytoplasmic domain (Wonderlich, Williams et al. 2008). To find out whether there is any polymorphism of cytoplasmic domain in classical HLA class-I A, B and C, we first searched the GenBank (**http://www.ebi.ac.uk/imgt/hla/**). Whilst HLA-A, B and C locus has hundreds of alleles, the cytoplasmic tail domain is highly conserved with GenBank analysis revealing only small variations within cytoplasmic domain (Table 3.1 and Table 3.2).

Gene products of multiple alleles shared many of the cytoplasmic sequences of HLA-A, HLA-B and HLA-C. There were at least 7 HLA-A cytoplasmic alleles, 2 HLA-B alleles and 7 HLA-C alleles. Six of seven HLA-C, but none of the HLA-A and –B cytoplasmic regions have the Y321C and D328N variations, which are critical in Nef protein association (Cohen, Gandhi et al. 1999). Sequence variations in the tyrosine-based motif were also detected in two HLA-A isoforms; HLA-A*11 with S322T and HLA-A*43 with S322F, and HLA-B*57 (S326C). Interesting, it was noted that most of the protective and disease susceptible HLA-B alleles that have been described so far cluster separately (Table 3.2).

To carry out the rest of the experiments I generated 16 cell lines expressing HLA-A2 extracellular and transmembrane domain fused to different cytoplasmic isoforms in table 3.1 using a lentiviral system to transfect the commonly used CD4+ T cell line Jurkat (JJK). The cell lines were thus named as A2-cytoplasmic allele it is fused to. A series of checkpoints confirmed success at different stages of cell line production i.e. site directed mutagenesis, cloning into lentiviral vector, production of lentivirus, transfection of cell lines, sorting Jurkat cell lines to obtain HLA-A2 wild type and mutant constructs expressing cells (data not shown).

Classical HLA-I	**Allele name**	**Amino acid sequence**	**No. of HLA types known to share**
HLA-A	A2-A2	^{310}RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV^{342}	51
	A2-A26	^{310}.....M.....^{342}	34
	A2-A11	^{310}.....T.....^{342}	37
	A2-A43	^{310}.....F.....M.....^{342}	1
	A2-A24	^{310}..N.....^{342}	18
	A2-A80	^{310}.K...V.....^{342}	1
	A2-A32	^{310}....L.....M.....^{342}	1
HLA-B	A2-B35	^{310}.....GG.....^{339}	67
	A2-B57	^{310}.....GG.....C.....^{339}	48
HLA-C	A2-C1205	^{310}.....GG....C.....N.....E..I...A^{342}	73
	A2-C0822	^{310}.....GG.....N.....E..I...A^{342}	1
	A2-C0430	^{310}.....GG....C.....TN.....E..I...A^{342}	1
	A2-C0750	^{310}.....GG....C.....N.....E..IT..A^{342}	1
	A2-C0711	^{310}.....GG....C....C.N.....E..I...A^{342}	1
	A2-C0704	^{310}.....GG....C....C.N.....E..IT..A^{342}	25
	A2-C0719	^{310}.....GG....C..V.C.N.....E..IT..A^{342}	3

Table 3.1: Aligned cytoplasmic domain sequences of cell lines generated representing HLA-A, B and C alleles in the database. Dots represent residues that match with HLA-A*0201 sequence. Highlighted amino acids are believed to be crucial residues that are important in Nef mediated HLA-I down-regulation (Cohen, Gandhi et al. 1999). The numbering corresponds to the residue number in HLA-Cw4.

Allele name	**Known class-I alleles**
HLA-A A2-A2	A*6901, A*6871, A*6836, A*6822, A*6817, A*6808, A*6803, A*6802, A*6801, A*1105, A*0289, A*0281,A*0279, A*0277, A*0268, A*0264, A*0260, A*0257, A*0251, A*0248, A*0233, A*0230, A*02279, A*02269, A*02266, A*02265, A*02264, A*02259, A*02253, A*02251, A*0224, A*02249, A*02230,A*0222, A*0221, A*0218, A*0217, A*0216, A*0213, A*02124, A*0212, A*0211, A*0209, A*0210, A*0207, A*0206, A*0205, A*0204, A*0203, A*0202, A*0201
HLA-A A2-A26	A*2501, A*2502, A*2601, A*2602, A*2603, A*2604, A*2605, A*2607, A*2608, A*2615, A*2650, A*2901, A*2902, A*2903, A*3101, A*3104, A*3132, A*3146, A*3201, A*3202, A*3205, A*3217, A*3301, A*3303, A*3307, A*3349, A*3401, A*3402, A*4301, A*6601, A*6602, A*7401, A*7402, A*0265
HLA-A A2-A11	A*3601, A*3025, A*3008, A*3004, A*3002, A*3001, A*1177, A*1175, A*1174, A*1162, A*1161, A*1160A*1156, A*1150Q, A*1125, A*1112, A*11110, A*1107, A*1104, A*1103, A*1102, A*1101, A*0378, A*0377, A*0339, A*0326, A*03134, A*0304, A*0302, A*0301, A*0120, A*0125, A*0114, A*0109, A*0103, A*0102, A*0101
HLA-A A2-A43	Aw*43
HLA-A A2-A24	A*2461, A*2422, A*2420, A*24152, A*24143, A*24142, A*24129, A*24128, A*2410, A*2408, A*2407 A*2404, A*2403, A*2402, A*2326, A*2309, A*2306, A*2301
HLA-A A2-A80	A*8001
HLA-A A2-A32	A*32
HLA-B A2-B35	B*1402, B*49, B*15, B*15/41, B*1501, B*15011, B*1501101, B*1502, B*1504, B*1508, B*1510new, B*1511, B*1512, B*1513, B*1514, B*1515, B*1516, B*1517, HLA-B*15170101, B*1518, HLA-B*1525, B*1519, B*1520, B*1521, B*1522, B*1523, B*1542, B*1554, B*1801, B*3502, B*3515, B*3511, B*3512, B*3541, B*3565Q, B*38new, B*390101, B*39011, B*3906v, B*3913v, B*510111, B*510113, B*510114, 510115, B*510116, B*51022, B*5104, B*5108, B*5301, B*53v, HLA-B*5401, B*5501, B*14, B*15, B*18, B*3801, B*35, B*38, B*51, B*51-cd3.3, B*73, B7301, Bw46, Bw58, Bw65, Bw72, Bw78
HLA-B A2-B57	B*07, B*27, B*4002, B*57, B*8101, B*0703, B*0705, B*07021, B*07021, B*0706, B*0707, B*0750 B*07ne, B*07xx 362, B*1301 B*1302, B*130203, B*27052 B*2706, B*2711, B*2732, B*3701, B*3702, B*4007 B*4201, B*4202, B*4208, B*44, B*44021, B*4403, B*4407, B*4805, B*5701 B*5706, B*5711, B*8101, B*0701, B*0703, B*0704, B*0802, B*13, B*44.2, B*44BO, B*4803, Bw41, Bw42, Bw62, B*44new
HLA-C A2-C1205	C*0346, C*1802, C*1801, C*1604, C*1602, C*1601, C*1517, C*1516, C*1513, C*1505, C*1504, C*1503, C*1502, C*1436, C*1423, C*1403, C*1222, C*1219, C*1213, C*1208, C*1205, C*1204, C*1203, C*1202, C*0841, C*0840, C*0831, C*0827, C*0825, C*0824, C*0821, C*0820, C*0804, C*0803, C*0802, C*0801, C*0624, C*0602, C*0509, C*0508, C*0503, C*0501, C*0471, C*0470, C*0442, C*0406, C*0403, C*0401, C*0361, C*0340, C*0313, C*03100, C*0306, C*0305, C*0304, C*0303, C*0302, C*0223, C*0221, C*0220 C*0216, C*0211, C*0210, C*0202, C*0140, C*0130, C*0121, C*0114, C*0108, C*0106, C*0104, C*0103, C*0102
HLA-C A2-C0822	C*0822
HLA-C A2-C0430	C*0430
HLA-C A2-C0750	C*0750
HLA-C A2-C0711	C*0711
HLA-C A2-C0704	C*0767, C*0766, C*0757, C*0756, C*0749, C*0742, C*0736, C*0730, C*0729,C*0726, C*07172, C*07170, C*07149, C*0704, C*0702, C*0701, C*07235, C*0704, C*0702, C*0701, C*0752, C*0793, C*0759,C*0746, C*07208
HLA-C A2-C0719	C*0719, C*0718, C*0706

Table 3.2: Complete list of alleles shared by cytoplasmic domain sequences.

3.2.2. Expression of HLA-C is lower than HLA-A or HLA-B alleles

It has been described that surface expression of HLA-C is lower than HLA-A or HLA-B alleles (Zemmour and Parham 1992; Schaefer, Williams et al. 2008). This has been attributed to unstable mRNA of the heavy chain of HLA-C (McCutcheon JA 1995), unstable HLA-C heavy chain and inefficient association of β microglobulin as opposed to other classical HLA-I (Neefjes and Ploegh 1988; Shimizu and DeMars 1989; Setini, Beretta et al. 1996).

In my attempts to make HLA-A2-C chimera expressing cells (as described in section 2.1.2), by transducing JJK cells with VSV pseudotyped virus containing different HLA-I cytoplasmic alleles, I experienced very low expression of HLA-A2-C chimera on infected cell lines. The expression of HLA-A2-C was at least 10 times less than A and B chimera except A2-C1205. Repeated transduction experiments with increasing amounts of viral titres also failed to make cell lines that expressed similar amounts of HLA-A2-C chimera on the surface as the HLA-A or B lines except for A2-C1205 (Figure 3.1).

It has been described that isoleucine at 337 position is responsible for accelerated internalisation of HLA-C (Schaefer, Williams et al. 2008), resulting in lower expression of surface HLA-C. This could be the reason for inability to get HLA-A2-C to express to the same extent as A2-B or A2-A, although higher expression of A2-C1205 is not explained.

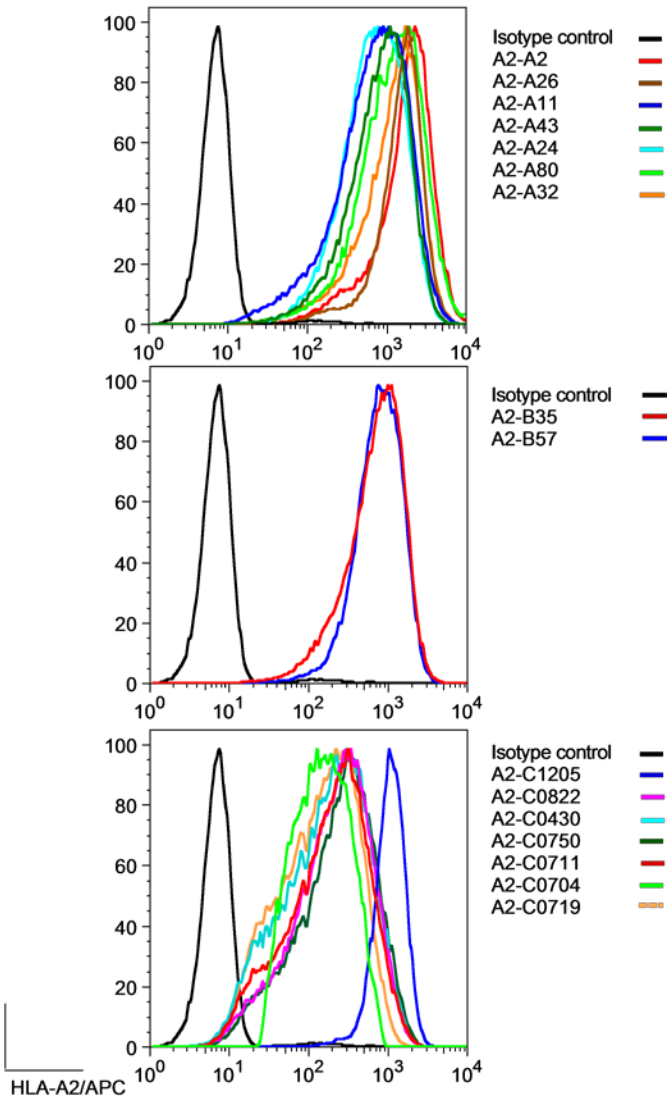


Figure 3.1: Expression of HLA-A2 chimera in cell lines. Representative histograms demonstrating expression of HLA-A2-A, -B, and -C chimera on the surface of Jurkat cell lines.

3.2.3. HLA-I down-regulation mediated by HIV-1_{SF2}Nef depends on the cytoplasmic domain of HLA-I

HIV-1 Nef induced down-regulation of HLA-I is mediated through its interaction with the cytoplasmic domain of HLA-I and clathrin adaptor protein complex-1. To assess the role of polymorphic cytoplasmic domain of HLA-I in Nef mediated down-regulation, I transiently transfected my cell lines with HIV-1_{SF2}Nef (Figure 3.2 a, b and c). As a negative control a mutant HIV-1_{SF2}Nef (HIV-1_{G2A}Nef/GFP) that does not down-regulate HLA-I was used (Figure 3.2 a, b and c). Additionally, antibodies against CD4, HLA-A3 and W6/32 were used as controls (data not shown here).

The down-regulation of HLA-A2 was compared between cells gated on GFP (described in Methods) (Fig. 3.2d). The fold down-regulation of HLA-A chimera ranged from 3 to 7 with A2-A24 demonstrating the highest down-regulation. The least mean fold down-regulation was observed in A2-A43 and A2-A26 chimera. Chimeric cells lines expressing HLA-B alleles demonstrated a relatively low down-regulation compared to HLA-A, followed by HLA-C, which showed the least down-regulation. The difference in cumulative fold down-regulation of HLA-A compared to HLA-B was found to be highly statistically significant ($p < 0.01$). There was no significant difference in cumulative fold down-regulation between HLA-B and HLA-C.

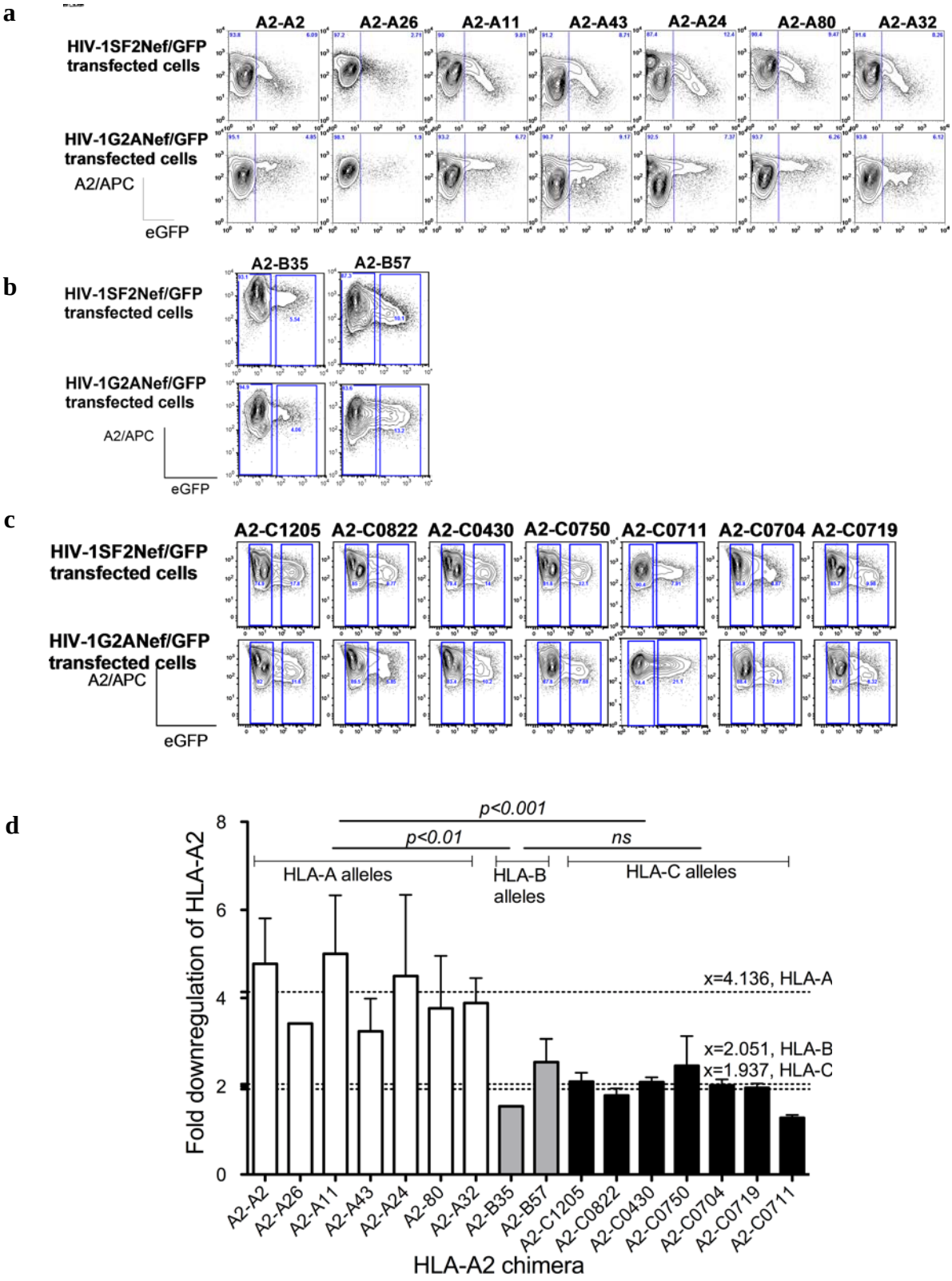


Figure 3.2: **Differential down-regulation of HLA-A, B and C alleles with Nef.** (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2-A chimera, (**b**) HLA-A2-B chimera, (**c**) HLA-A2-C chimera measured 24 hours after transiently transfecting with plasmids expressing HIV-1_{SF2}Nef/GFP or control plasmid HIV-1_{G2A}Nef/GFP. (**d**) The fold down-regulation of A2 chimera in the presence of HIV-1_{SF2}Nef/GFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SEM, x=mean fold down-regulation of HLA-A2.

3.2.4. The pattern of down-regulation of HLA-I cytoplasmic alleles in HIV-1 IIIB infection is consistent with HIV-1 $_{SF2}$Nef mediated down-regulation

To confirm that polymorphism in the cytoplasmic domain of HLA-I lead to differential down-regulation during presence of HIV infection, we used a laboratory strain of HIV-1, III B virus. JJK cells expressing A2 chimeric molecules were infected with HIV-IIIB and the degree of down-regulation of HLA-A2 chimera of infected cells were compared with uninfected cells (Figure 3.3a, b and c)**.**

Indeed, we observed a pattern of down-regulation consistent to what was seen with HIV-1$_{SF2}$Nef (Figure 3.3d). The highest mean fold down-regulation was noted with A2-A24 and lowest with A2-A43. The chimeras containing HLA-B cytoplasmic domains had very low fold down-regulation with a mean of 1.56. Confirming previous results, a significantly lower level of fold down-regulation was noted with HLA-B domains compared to HLA-A ($p<0.05$, student t test; Figure 3.3d). HLA-A2 with HLA-C cytoplasmic domains were not down-regulated.

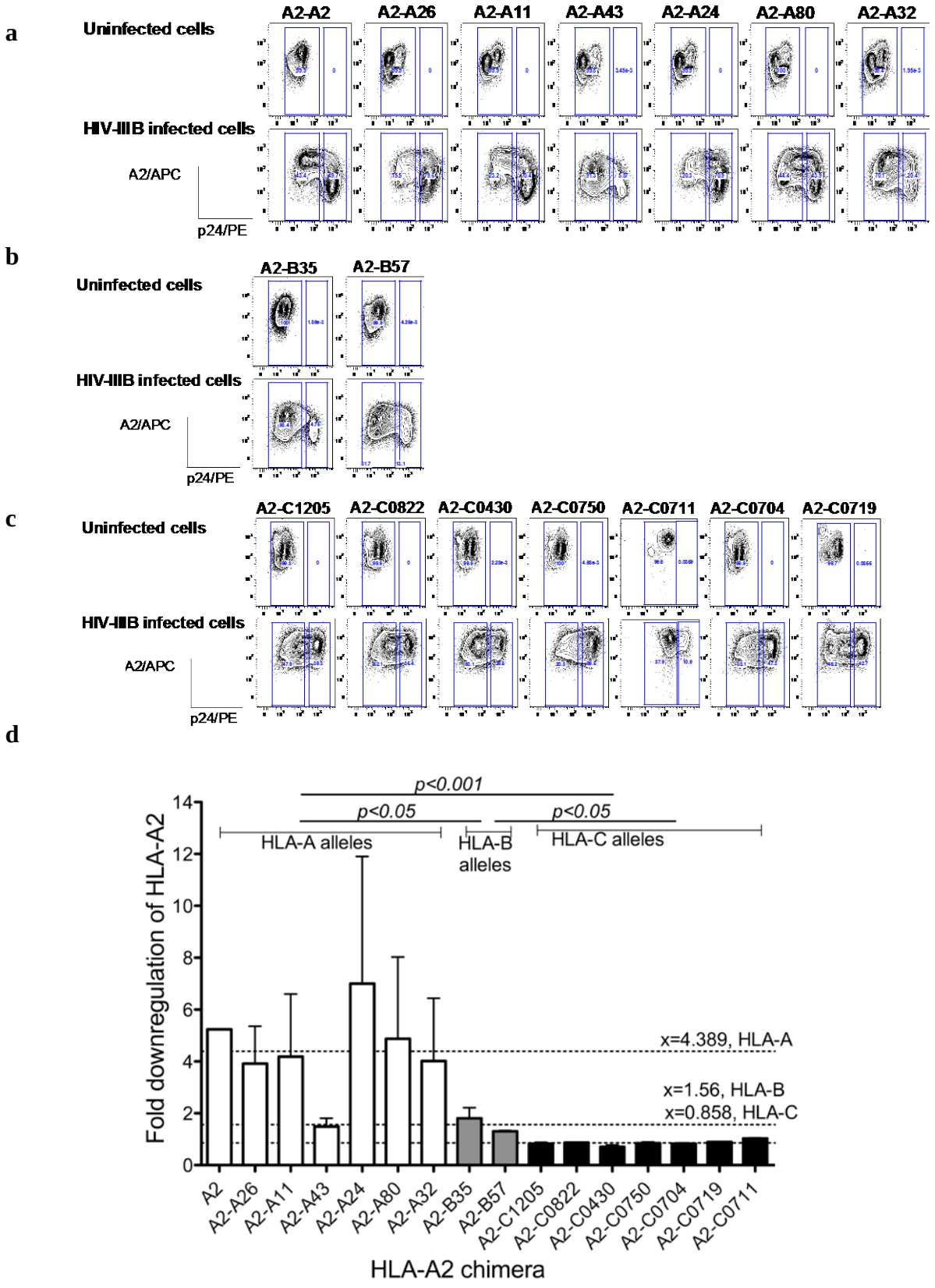


Figure 3.3: Differential down-regulation of HLA-A, B and C alleles in HIV-IIIB infection (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2-A chimera (**b**) HLA-A2-B chimera (**c**) HLA-A2-C chimera measured 6 days post infection with HIV-IIIB virus. (**d**) The fold down-regulation of A2 chimera in infected cells were calculated by comparing MFI of A2 in p24 positive cells (described in detail in methods section). Each bar represents the mean of three independent experiments+ SEM, x=mean fold down-regulation of HLA-A2.

3.2.5. Assessing the relationship between A2 restricted Gag p17 specific CTL function and down-regulation of A2 chimera mediated by HIV-1 IIIB

Literature provides evidence supporting down-regulation of HLA-I from the surface leads to protection of infected cells from HLA-I restricted CD8+ T cells. This is a mechanism through which many viruses including HIV evade CTL response (Collins, Chen et al. 1998). Since my previous findings demonstrated a relationship between HLA-I down-regulation and cytoplasmic domain sequence, we then wanted to assess the contribution of differential HLA-I down-regulation on antiviral activity mediated by CTL.

As a first step, an assay was designed to assess the ability of my chimeric cell lines to cross present to a A2 Gag p17 (SL9) specific CTL clone (G10 clone). The generated chimeric cell lines were pulsed with 2mM SLYNTVATL and then cocultured with G10 clone to measure response by IFNγ ELISPOT. We noted that all of generated cell lines could present SL9 peptide (Figure 3.4a). We also observed that the IFNγ response to HLA-A2-C (except C1205) chimera were low and clustered separately from A and B (Figure 3.4a). Autologous B cells pulsed with Gag p17 peptide SLYNTVATL 2mM, PHA stimulation and HLA mismatched CTL clone were used as controls.

Secondly, to assess whether HIV-1 infected chimeric cell lines can present HIV antigens to G10 clone, to workout the optimum Effector(E):Target(T) ratio and to determine whether the number of SFU in an IFNγ ELISPOT assay relates to the level of down-regulation of A2-A chimera, randomly selected several infected cell lines were incubated with G10 clones at different E:T ratios (Figure 3.4b). We observed that IIIB infected HLA-A2 chimeric cells could present peptides to G10 clone and the level of response (as measured by spot forming units / 10^6 cells) in ELISPOT assay appeared to correlate with the level of A2 molecules on the surface. The best E:T ratio was determined as 1:50. This ratio yielded the highest measure.

More inaccurate numbers were produced by the automated ELISPOT reader when the E:T ratio was increased, and response declined with lower target cell numbers.

Due to the difficulty in optimising HIV infectivity rates in all sixteen cell lines at the same time, a selected group of generated chimeric cell lines were used for preliminary experiments. In an attempt to overcome this difficulty in subsequent experiments, we assessed the feasibility of using cells that expressed similar amounts of GFP/Nef protein as target cells. Cells expressing similar amounts of GFP/Nef were obtained following transient transfection with HIV-1_{SF2}Nef and FACS sorting. Target cells were then pulsed with equal amount of SLYNTVATL peptide and cocultured with A2 restricted Gag specific CTL clones in IFNγ ELISPOT assay. However, these attempts were unsuccessful and could be due to following reasons. Firstly, following electroporation cells were not fit enough to undergo a FACS sorting the next day and the cells that were sorted only transiently expressed GFP thus lost GFP expression very quickly. Secondly, in keeping with previous research findings, I also noticed that pulsing cells with peptides overcomes HIV mediated down-regulation (data not shown)

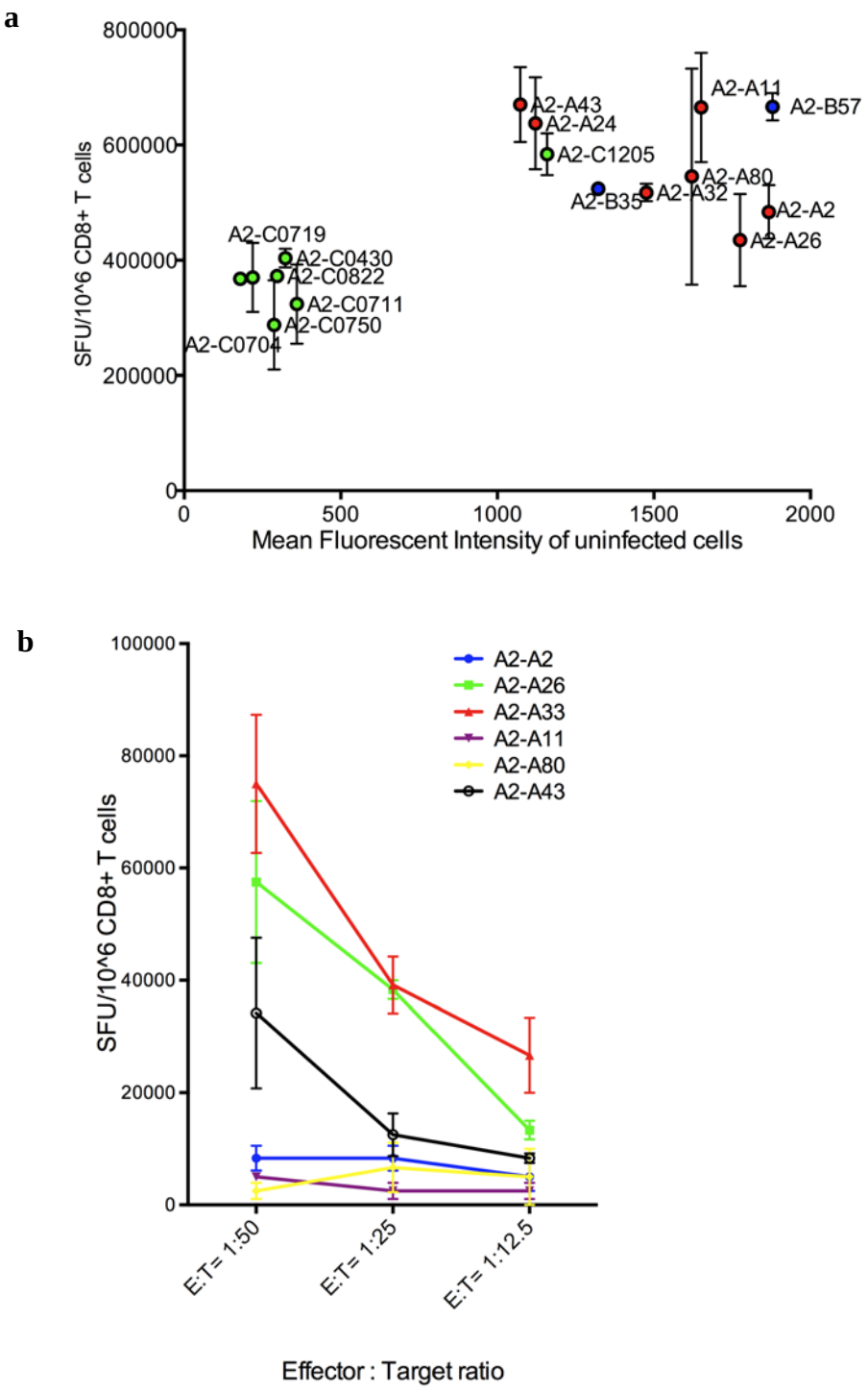


Figure 3.4: Experimental system used to investigate the relationship of CTL response and HLA-I down-regulation. (**a**) The degree of cross presentation depends on the amount of HLA-I molecules on the surface shown by cross presentation of chimeric cell lines pulsed with SL9 peptide (**b**) Cross presentation of HIV-IIIB infected cells to Gag p17 specific CTL also depend on the degree of down-regulation of A2-A chimera. The figure is the summary of mean + SEM of three experiments. A2-A33 cell line was not used for any other experiments as subsequent GenBank search revealed that the cytoplasmic tail sequence was updated.

3.2.6. Effect of HLA-A2 chimera down-regulation on functional outcome of CTL- IFNγ response is determined by the degree of down-regulation

CTL response to HIV is recognised as an important determinant of progression of HIV disease (McMichael, Borrow et al. 2010). Among the studied qualitative immune correlates of CTLs in HIV infection, polyfunctionality (Betts, Nason et al. 2006) and HIV suppressive ability (Saez-Cirion, Lacabaratz et al. 2007) are seen to be associated with spontaneous control of HIV and slower disease progression (discussed in section 1.2.2). Polyfunctional CTL are those that exhibit multiple effector functions - degranulation and production of antiviral factors- IFNγ, tumour necrosis factor (TNF)-α, Interleukin 2 and macrophage inflammatory protein (MIP)-1β simultaneously upon antigen encounter.

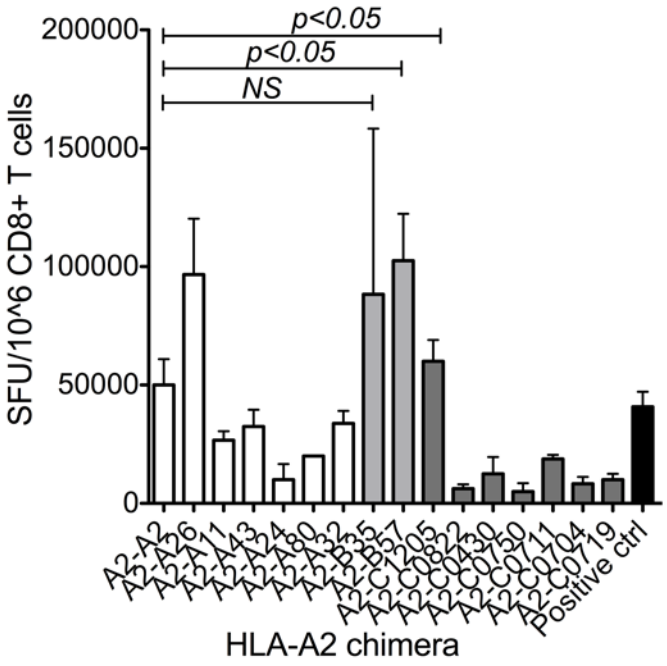
It has been shown that the Nef mediated down-regulation of HLA-I can result in escape from CTL responses. This has been attributed to low levels of HLA-I on the surface. It has also been shown that functional outcomes upon antigen engagement depend on the strength of the stimulus (Viola and Lanzavecchia 1996; Almeida, Price et al. 2007), directly affected by the level of antigen sensitivity (Almeida, Sauce et al. 2009) and also by antigen concentrations (Valitutti, Muller et al. 1996; Price, Sewell et al. 1998; Betts, Price et al. 2004). Our hypothesis is that the strength of the stimulus could correlate with the amount of antigen presenting epitopes on the surface of virus-infected cells, thus producing more polyfunctional and HIV suppressive responses when CD8 + T cells are cocultured with alleles that are not down-regulated.

As an initial assessment, we carried out a live virus IFNγ ELISPOT assay to see whether there was a differential response between the cell lines when they are infected with HIV-IIIB (equal levels of infectivity determined by flow based p24 staining) just before plating with A2 restricted Gag p17 specific CTL).

We consistently saw greater response (more spot forming units) with HLA-B chimera than HLA-A (Figure 3.5a). Interestingly less SFU were noted with HLA-C except for A2-C1205. This could be due to low expression of HLA-C alleles on the surface of cells (Figure 3.1).

On further assessment of the relationship between degree of down-regulation of HLA-A2 chimera and HIV-1 IIIB infection, we noted that there is a correlation between the amount of HLA-A2 on the surface of infected cells and IFNγ response. We used the mean fluorescent intensity of anti HLA-A2 conjugated antibody on infected cells and fold down-regulation of A2 chimera vs the number of SFU that were obtained when cells were cocultured with A2 restricted Gag p17 specific CTL (Figure 3.5b and 3.6). An inverse linear relationship between fold down-regulation of HLA-A and B alleles and SFU were noted. HLA-C was not included in the calculation of linear relationship as -C alleles resisted down-regulation and also had a very low number of SFU as a direct result of lower expression on the surface.

a



b

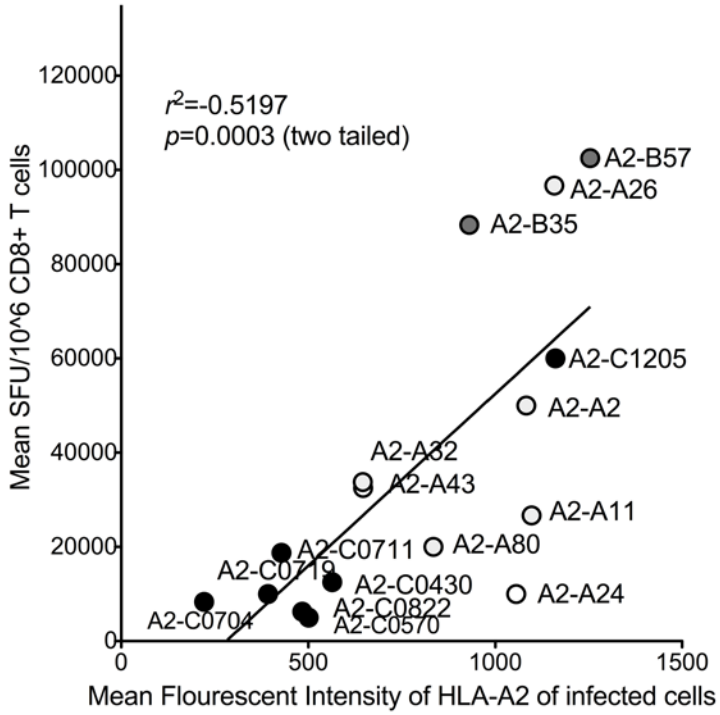


Figure 3.5: Control of HIV infection by A2 restricted Gag p17 specific CTL. (**a**) Results of live virus ELISPOT assay carried out with E:T = 400:20000. Bar represents mean of three independent experiments+SD. (b) Correlation MFI of A2 of infected cells and mean spot forming units.

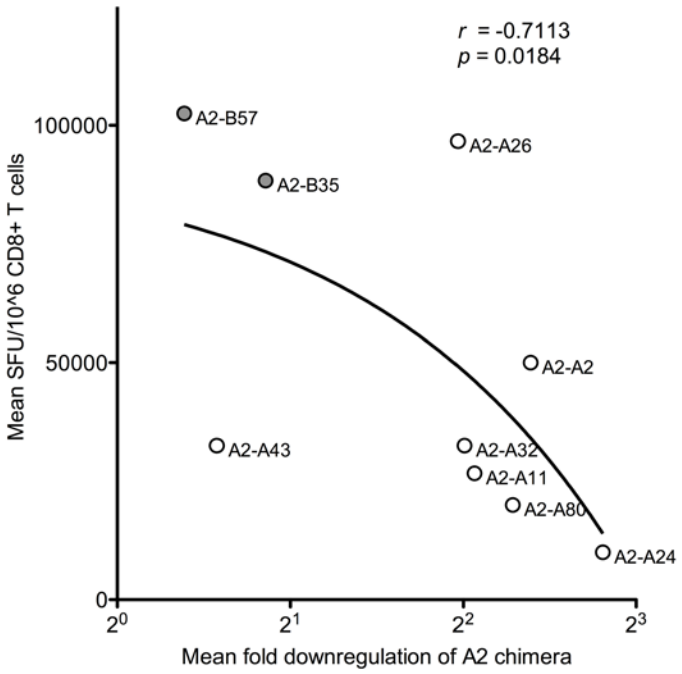
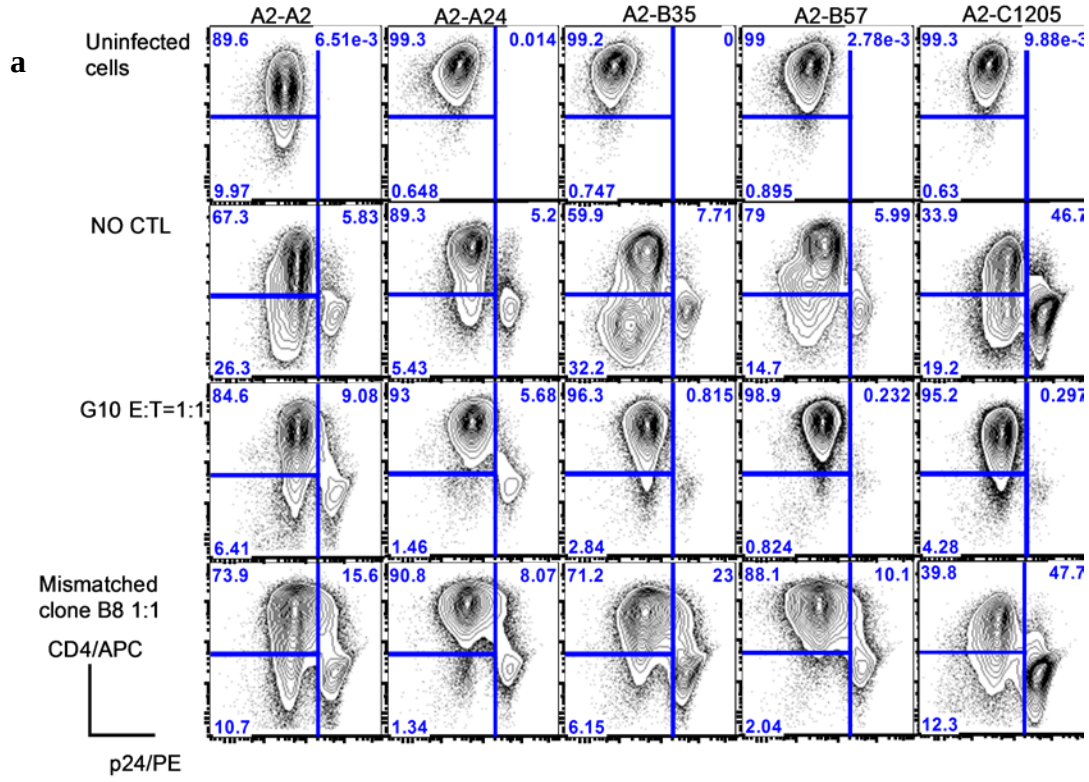


Figure 3.6: Correlation between level of down-regulation of A2 chimera (HLA-A and HLA-B) of HIV-IIIB infected cells and mean spot-forming units. Data is a summary of three independent experiments. p, spearman correlation (one tailed).

3.2.7. The moderate resistance to down-regulation of HLA–B in HIV leads to a better CTL control

Next I assessed the effect of differential down-regulation of HLA-A chimera on viral suppression in an *in vitro* viral suppression assay (Yang, Kalams et al. 1997; Saez-Cirion, Shin et al. 2010). Infected cells were cocultured with HLA-I restricted CTL clones at different E:T ratios and at different time points either p24 levels in the supernatant or p24 positivity was assessed using flow cytometry. We saw that HLA-B alleles are better at controlling infection (Figure 3.7). This demonstrates that the magnitude of HLA-A2 down-regulation by HIV is related to control by CD8+ T cells and viral replication in co-cultured samples (measured by p24 capture ELISA assays, Immunodiagnostics and flow based VSA).



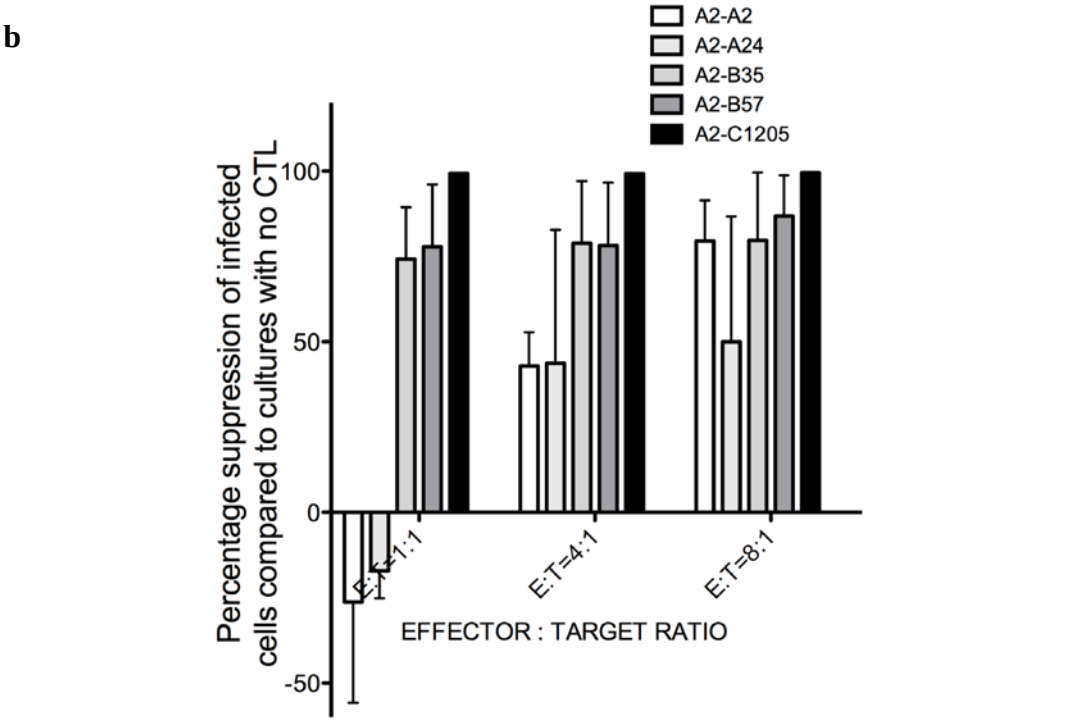


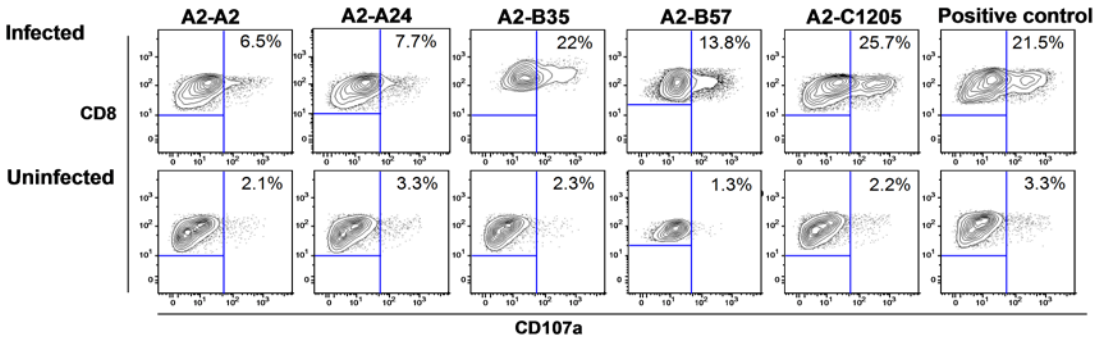
Figure 3.7: Control of HIV infection by HIV-1 Gag p17 specific CTL. (**a**) Flow based viral suppression on day 4 post infection. Percentage of p24 positive cells at different E:T ratio is compared against infected cells incubated without CTL. Only E:T=1:1 is shown. (**b**) Percentage suppression of p24 antigen in the supernatant of the cocultured cells relative to the cells incubated without CTL. A bar represents mean of threee experiments +SD.

3.2.8. Induction of CD107a in HIV-1 A2 Gag specific CTL clone by HIV infected transfected jurkat cells is associated with the ability to suppress virus

CD107a, also known as lysosomal associated membrane protein 1 (LAMP-1), is a degranulation marker which is representative of the lytic capacity of infected cells by CTL. Having demonstrated differential down-regulation mediated by cytoplasmic domain results in differential IFNγ secretion and viral inhibitory capacity, we wanted to investigate whether differential down-regulation affects CTL lytic capacity demonstrated by CD107a expression.

We saw that CD107a secretion was highest in the cell lines that have chimeric alleles that do not get down-regulated by HIV (Figure 3.8). Thus we can hypothesise that the density of HLA-A2 on the surface of target cell plays a role in the capacity for CD8+T cells to degranulate.

a



b

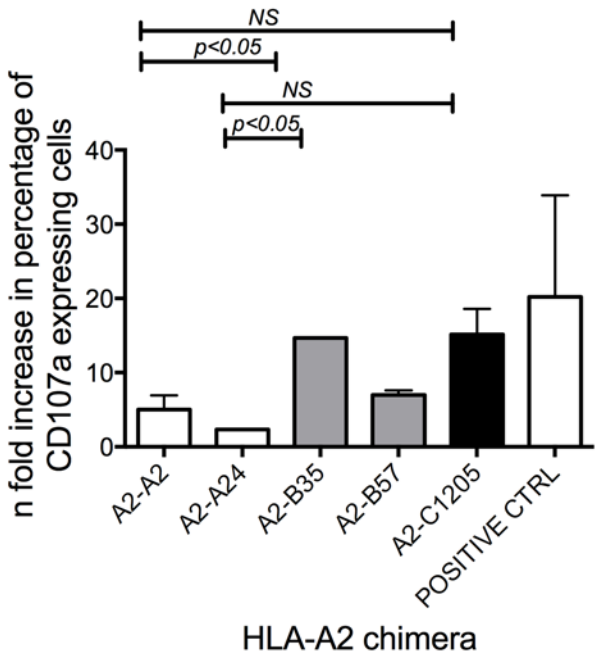


Figure 3.8: CD107a secretion by CD8+ T cells depend on the level of HLA-I on the surface of target cells. (**a**) Differential CD107a expression in A2 restricted HIV Gag p17 specific CD8+ T cell clone (G10) was measured in a flow based assay after coculturing with Jurkat cells stably expressing A2 chimera a 5 h assay in the presence of a protein transporter inhibitor (Monensin). HIV-1 IIIB infected Jurkat cells were used on day 6 of infection. HLA-A2 restricted HIV Gag p17 specific CD8+ T cell clones were added to infected cells in 1:1 ratio. (**b**) Fold increase of CD107a positive cells were calculated by comparing against cells which were cocultured with uninfected Jurkat cells. Bars represent means plus standard deviations for three independent experiments.

3.2.9. The level of down-regulation of HLA-I of antigen presenting cells determines CD8+T cell polyfunctionality

Polyfunctional cells are those that exhibit multiple effector functions (degranulation and production of antiviral factors) simultaneously upon antigen encounter. This is usually assessed after stimulation with cognate peptides by multiparametric flowcytometry (eg. mobilization of CD107a, IFNγ, tumour necrosis factor α, interleukin-2 and macrophage inflammatory protein 1-β). We wanted to replicate a situation where our selected cell lines representing HLA-A, HLA-B and HLA-C are infected and cocultured with G10 CTL clone. Since it has been shown that antigen sensitivity is a major determinant of CD8+ T cell polyfunctionality our hypotheses was when there is more antigen on the surface of presenting cells they enhance polyfunctionality. However, because Nef down-regulates class I of HLA-A alleles, fewer viral antigens are seen on the surface of HIV infected cells. Since HLA-B is not down-regulated more antigens are present on the surface of cells. Thus we believe HLA-B restricted T cells tend to be more polyfunctional.

Indeed, we were able to demonstrate that G10 CTLs exposed to A2-B35 and A2-B57 have both higher IFNγ and CD107a expression (Figure 3.9). In addition A2-C1205, which had a high HLA-C expression (this allele completely resisted Nef mediated down-regulation), also elicited a high IFNγ and CD107a expression. Although the expression of IFNγ and CD107a were shown in different assay systems these experiments were carried out on the same CTL clone using the same target cells. This supports the notion that presence of more antigens on the surface with HLA-I molecules causes CD8+T cells to show a polyfunctional profile.

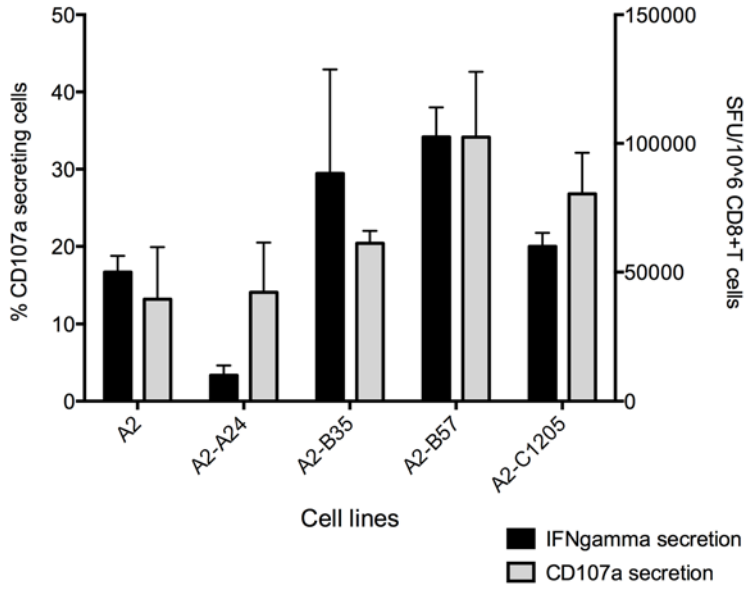


Figure 3.9: Assessment of profiles that constitutes multifunctionality in selected cell lines. Data are summary of three independent experiments described in sections 3.2.6 and 3.2.8. Error bar=SEM.

3.2.10. Resistance of native HLA-B to down-regulation by Nef is consistent in cell lines.

Our previous experiments confirmed that down-regulation of chimera containing HLA-A cytoplasmic domains were significantly higher than chimera containing HLA-B cytoplasmic domains in HIV infection. This difference can be considered to be a true function of the cytoplasmic domain as the extracellular and transmembrane domain were same in all of the above chimeric molecules where lentiviral vector and the initial Jurkat cell line were essentially the same.

Although it has been briefly noted that HLA-B alleles were less efficiently down-regulated than HLA-A (Cohen, Gandhi et al. 1999), this effect has not been confirmed. To see whether the native HLA-B alleles resist down-regulation compared to HLA-A, we employed two frequently used primate cell lines, Jurkat and 293T cells. These were either infected with

HIV-IIIB or transfected with HIV-1_{SF2}NEF-GFP and down-regulation of native HLA-A and HLA–B alleles were assessed using specific antibodies for these alleles. A limitation in this study was unavailability of HLA-I specific antibodies, thus we were only able to assess the expression of A2, A3 and B7. Confirming previous findings (Cohen, Gandhi et al. 1999) we noted a higher degree of down-regulation of HLA-A alleles compared to HLA-B in both Nef and HIV infection in tested cell lines (Figure 3.10).

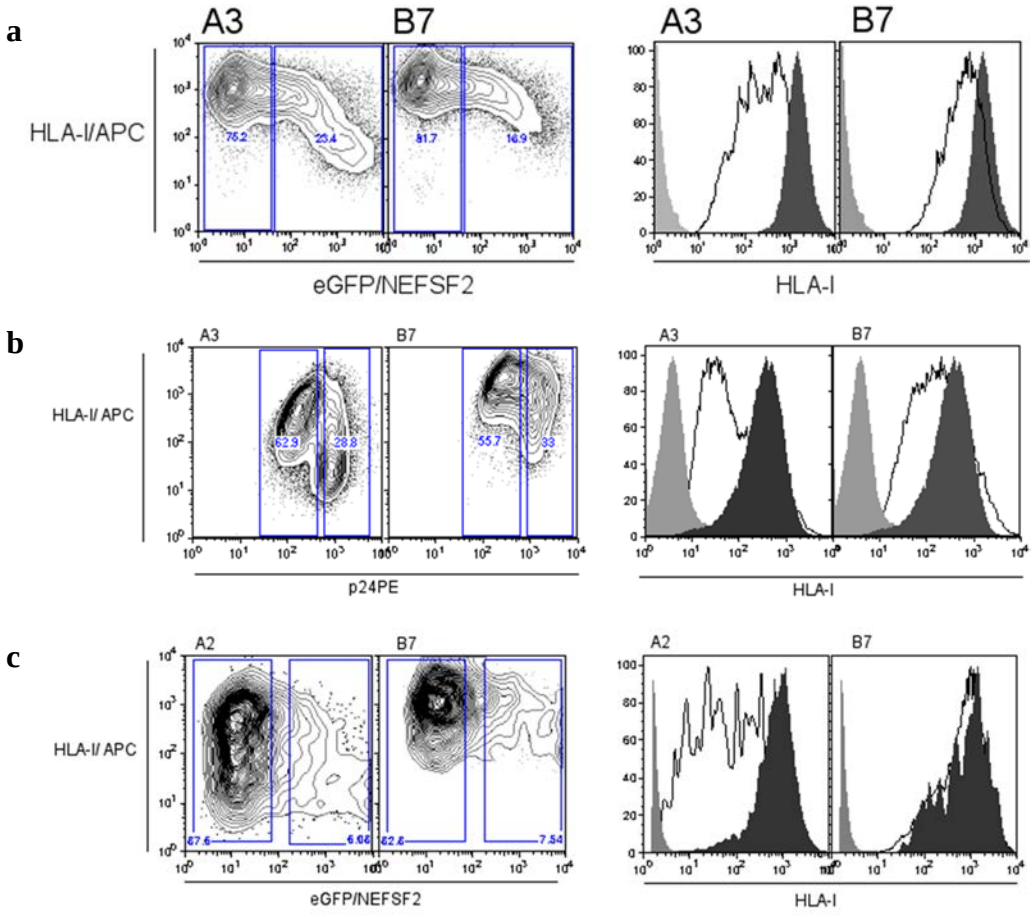


Figure 3.10: **Comparison of down-regulation of native HLA-A and HLA-B alleles among selected cell lines.** (**a**) Representative histogram showing down-regulation of HLA-A3 and HLA-B7 in Jurkat cell line expressing HIV-1_{SF2}NEF-GFP and (**b**) representative histogram showing down-regulation of HLA-A3 and HLA-B7 in HIV IIIB infected Jurkat cells. (**c**) Representative histogram showing down-regulation of HLA-A2 and HLA-B7 in HIV-1_{SF2}NEF-GFP expressing 293T cells. N=3. The magnitude of HLA-A/B down-regulation was measured 6 days post infection or 24 hours post electroporation.

3.2.11. Impact of differential down-regulation of native HLA-A and HLA-B on CTL mediated response.

To investigate the hypothesis that resistance of HLA-B alleles to Nef mediated down-regulation plays a significant role in providing better control over HIV by HLA-B restricted CTL, we sought to provide indirect evidence, since it is difficult to find HLA-A and B specific CTL clones that behaved in perfect concordance. We used MT2 cells (which express HLA-A24 and HLA-B51) as targets, HLA-A24 restricted Nef specific and B51 restricted Gag specific clone as HLA-I matched clones and a B8 restricted Nef specific clone as a mismatched clone. We infected target MT2 cells with either pNL4-3 virus or the mutant M20A pNL4-3 virus which lacks the ability to down-regulate HLA-I. Our hypothesis is that CTLs are able to mount a good effector response (IFNγ secretion and/or degranulation measured by CD107a) when the cells are infected with a virus which abrogates down-regulation of HLA-I. Also if a particular HLA-I allele is resistant to pNL4-3 mediated down-regulation, the response it elicits when cocultured with HLA-I restricted CTLs should be more or less similar to cells infected with a M20A pNL4-3. To prove our hypotheses we carried out a live virus ELISPOT assay to assess the level of IFNγ response following overnight incubation (Figure 3.11 and 3.12) followed by comparison of percentage of IFNγ (Figure 3.13a and b) and CD107a secreting cells (Figure 3.14 a and b).

We consistently observed that the CTL activity of B51 specific cells when incubated with pNL4-3 or M20A pNL4-3 virus infected cells was similar. At 40% infectivity, the MT2 cells that were cocultured with B51 Gag specific CTL clone demonstrated an ELISPOT response of 64% (infected with pNL4-3) and 67.2% (infected with M20A pNL4-3) respectively compared to the cells that were pulsed with the relevant peptide. The difference between these means was not statistically significant. However, with A24 Nef specific CTL clone, the

ELISPOT responses that were obtained for M20A pNL4-3 and pNL4-3 virus infected cells were 44.9% and 30.8% respectively and the difference of mean was statistically significant. Similar differences were seen with lower infectivity rate (25%) (Figure 3.11) and also when cells were cocultured with A24 Gag specific CTL clone (Figure 3.12). This difference in the magnitude of A24 specific CTL responses to target cells infected with these viruses, support the hypothesis that the degree of down-regulation of HLA-I plays a very important role. Our findings were also confirmed in a flow-based assessment of IFNγ (Figure 3.13) and CD107a (Figure 3.14).

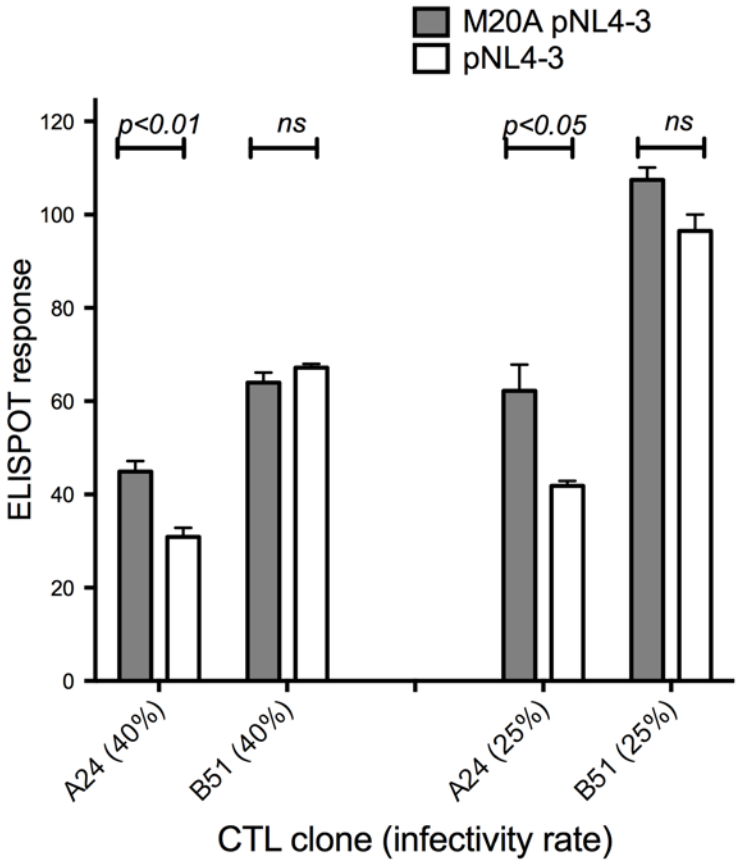


Figure 3.11: **HLA-B restricted CTL have better IFNγ response that is secondary to resistance to down-regulation mediated by Nef**. Live virus IFN-γ ELISPOT assay was performed with pNL4-3 or M20A pNL4-3 virus-infected MT2 cells at different infectivity ratios. These were cocultured either with HLA-A24 Nef and HLA-B51 Gag-restricted CTL clones at E:T= 400:20,000 overnight. SFU were normalized by comparing SFU obtained for each clone with mean SFU obtained for MT2 cells pulsed with relevant peptide. All data are representative of three independent experiments. Error bars, SEM, P as calculated by Student's t test.

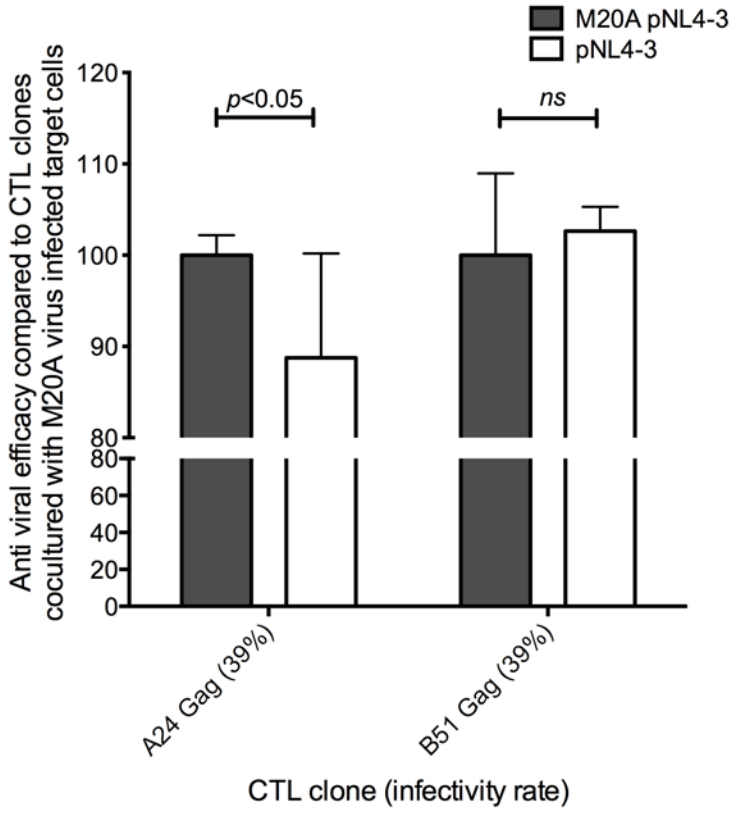


Figure 3.12: **HLA-B restricted Gag CTL elicit better IFNγ response that is secondary to resistance to down-regulation mediated by Nef**. Live virus IFN-γ ELISPOT assay was performed with pNL4-3 or M20A pNL4-3 virus-infected MT2 cells at ~40% infectivity. These were cocultured with Gag restricted CTL clones, HLA-A24 and HLA-B51 at E:T= 400:20,000 overnight. IFNγ response, termed as anti viral efficacy, was calculated by comparing IFNγ response to the response that was generated when CTLs were cocultured with M20A pNL4-3 virus infected cells. All data are representative of three independent experiments. Error bars, SEM, P as calculated by Student's t test.

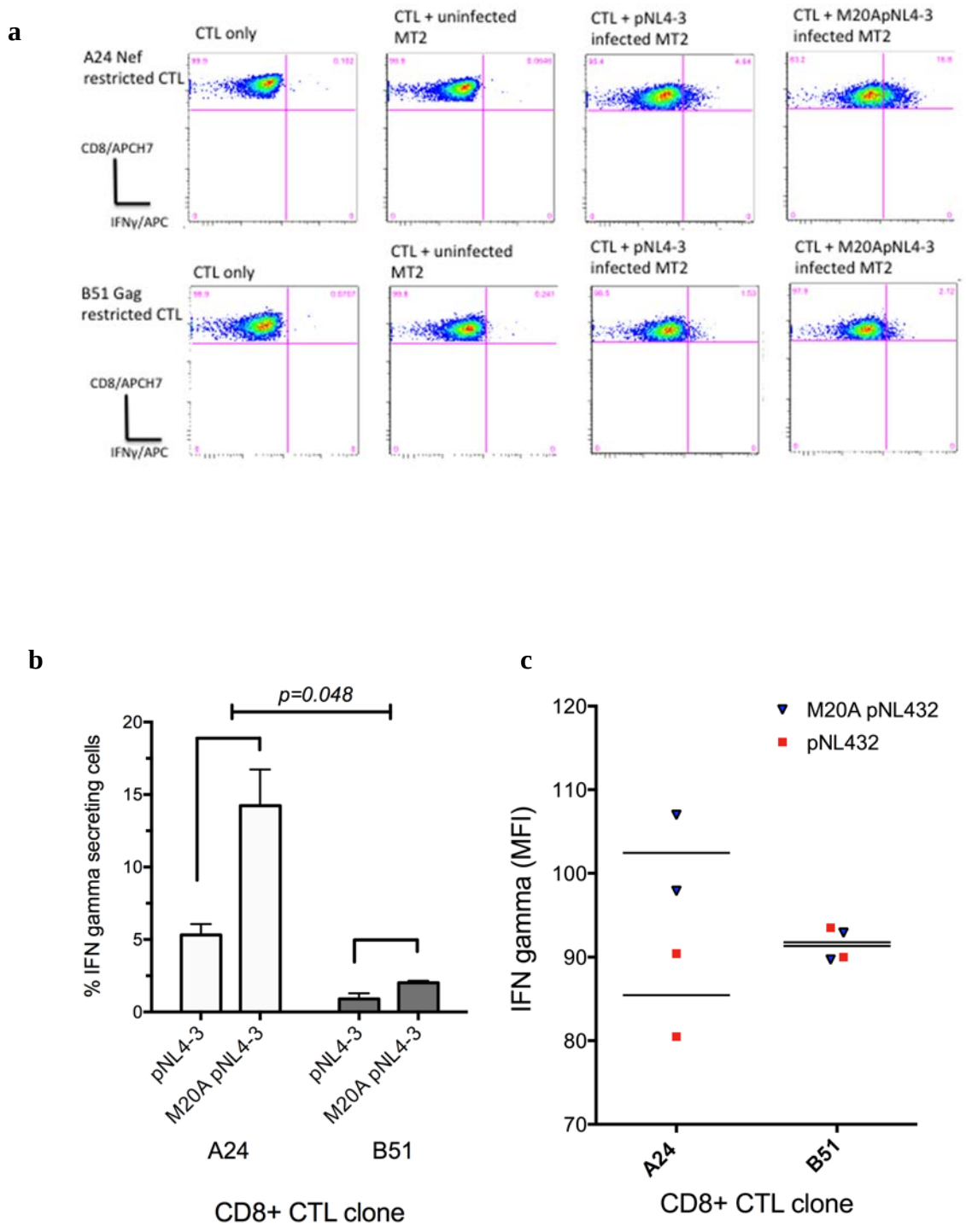


Figure 3.13: IFNγ secretion by CD8+ T cells depends on the level of HLA-I on the surface of target cells. (**a**) Flowcytometric IFNγ assay. Differential IFNγ expression in A24 Nef restricted CD8+ T cell clone and B51 Gag specific CD8 clones cocultured with MT2 cells in a 5 h assay in the presence of a protein transporter inhibitor (Monensin). MT2 cells infected with pNL4-3 or M20A pNL4-3 virus were used on day 4 of infection. CTL clones were added to infected cells in 1:1 ratio. Target cell infectivity rate is 20% thus effective E:T ratio is 5:1. (**b**) Percentage of IFNγ secreting cells were calculated by comparing against cells that were cocultured with uninfected MT2 cells. Bars represent means plus standard error of mean for two independent experiments (**c**) Mean fluorescent intensity of IFNγ secretion in CTL after subtracting the back ground value.

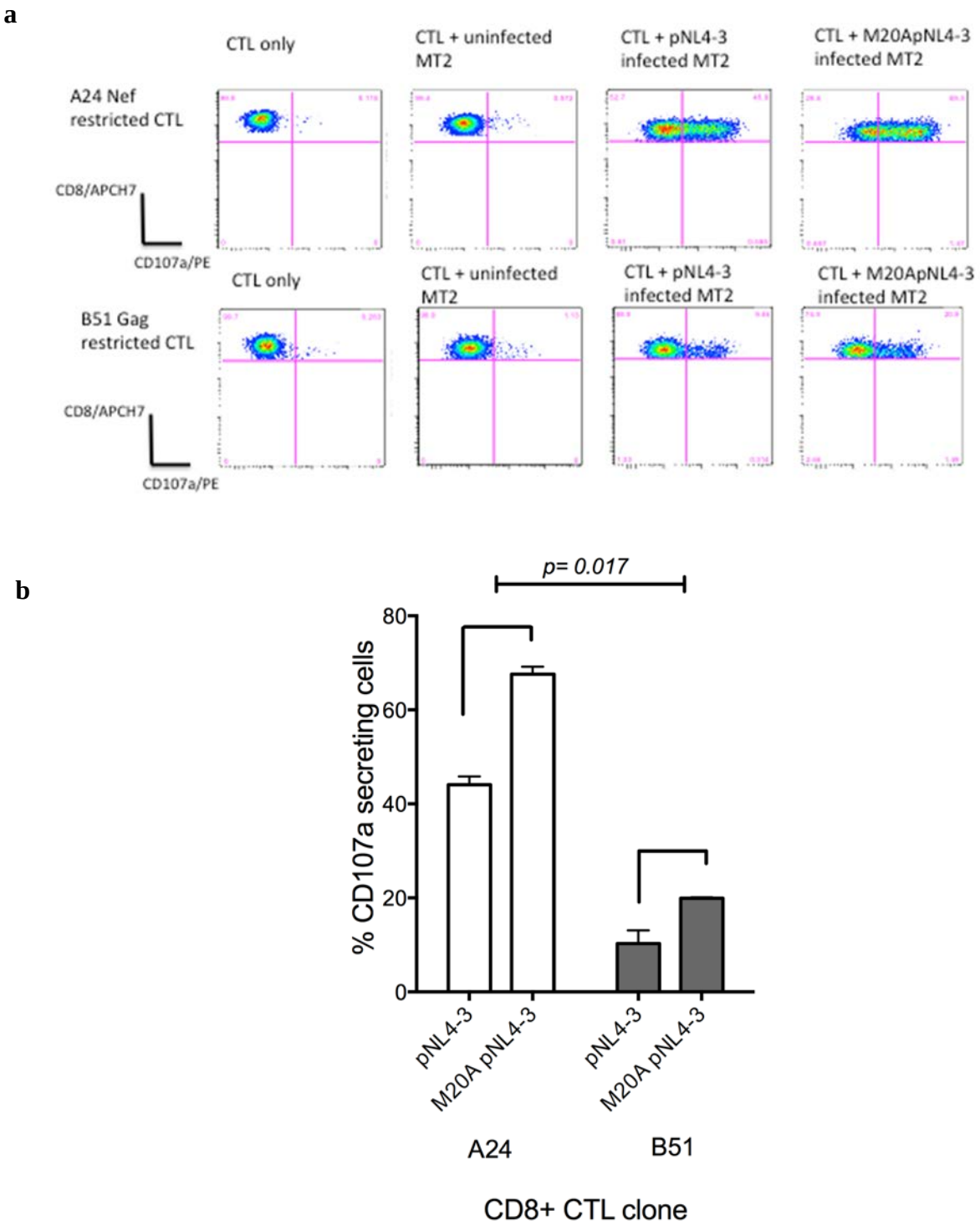


Figure 3.14: CD107a secretion by CD8+ T cells depend on the level of HLA-I on the surface of target cells. (**a**) Flowcytometric degranulation assay. Differential CD107a expression in A24 restricted HIV Nef specific CTL clone and B51 Gag specific CTL clones cocultured with MT2 cells in a 5 hour assay in the presence of a protein transporter inhibitor (Monensin) as concurrent staining of IFNγ was carried out. pNL4-3 or M20A pNL4-3 virus infected MT2 cells were used on day 4 of infection. CD8+ T cell clones were added to infected cells in 1:1 ratio. Target cell infectivity rate is 20% thus effective E:T ratio is 5:1. (**b**) Percentage of CD107a secreting cells in were calculated by comparing against cells which were cocultured with uninfected MT2 cells. Bars represent means plus standard deviations for two independent experiments. The mean differences between % CD107a secretion by A24 and B51 CTL that were cocultured with wild type and mutant viruses, were statistically significant.

3.2.12. Granzyme and perforin secretion as a function of CD8+ T cells

CTL activation follows T-cell receptor ligation with specific peptide presented by MHC class I plus costimulation and cytokine signals which leads to the secretion of cytolytic molecules, perforin and granzyme. We analysed whether differential down-regulation of HLA-I resulted in differences in the expression of these effector molecules. We observed little difference in the frequencies of perforin or granzyme secretion (data not shown). However, the comparison of mean fluorescent intensity of granzyme between CTLs cocultured with target cells infected with pNL4-3 and M20A pNL4-3 virus showed that there was a bigger difference in HLA-A alleles compared to HLA- B (Figure 3.15 a and b).

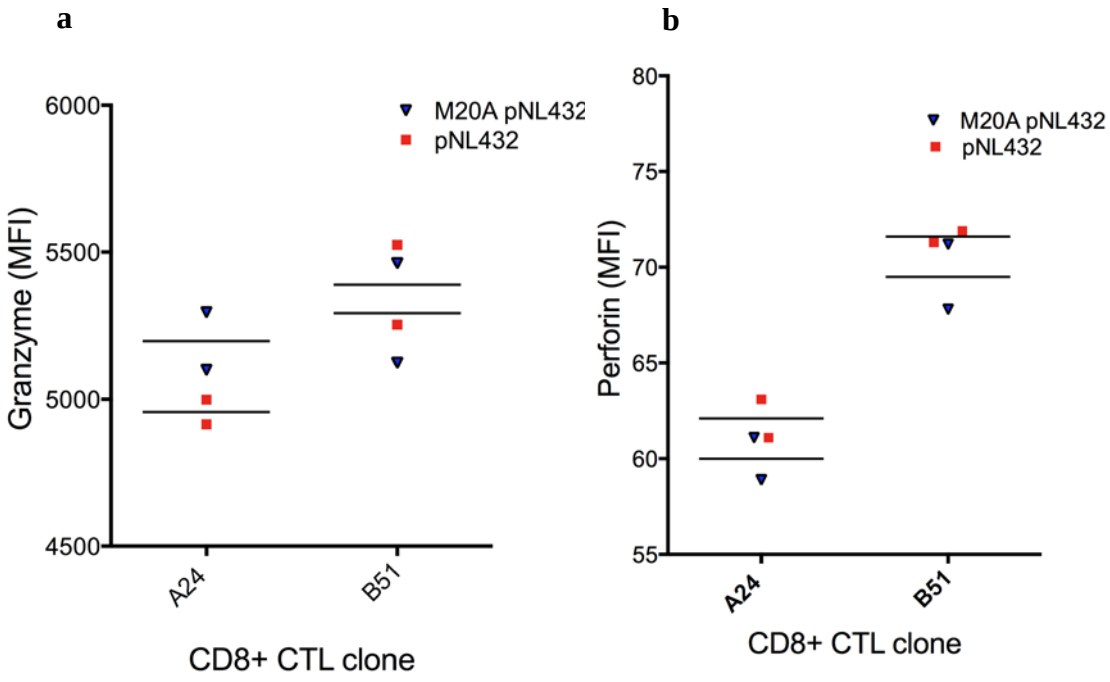


Figure 3.15: Differential (**a**) Granzyme and (**b**) Perforin expression in A24 restricted HIV Nef specific CD8+ T cell clone and B51 Gag specific CD8 clones cocultured with MT2 cells in a 5 h assay in the presence of a protein transporter inhibitor (monensin). pNL4-3 or M20A pNL4-3 virus infected MT2 cells were used on day 4 of infection. CTL were added to infected cells at 1:1 ratio. Target cell infectivity rate is 20% thus effective E:T ratio is 5:1.

3.3. Discussion

This is the first study detailing the role played by HLA-I cytoplasmic domain in Nef mediated differential down-regulation of HLA-I alleles (Rajapaksa, Li et al. 2012). Previous work has demonstrated that HLA-A and B are down-regulated in HIV infection but not HLA-C nor E (Cohen, Gandhi et al. 1999). Simian MHC-I down-regulation by Nef is said to be preserved in SIV, HIV-1 and HIV-2 (Munch, Schindler et al. 2005; Specht, DeGottardi et al. 2008; Schmokel, Li et al. 2009) and depends on polymorphism of the MHC-I cytoplasmic domain (DeGottardi, Specht et al. 2008) but there is very little information on human HLA-I. This is understandable as it is widely held that there is little or no variation within HLA-I cytoplasmic domain. However, available HLA-I sequencing data (**http://www.ebi.ac.uk/imgt/hla/**) demonstrated that the cytoplasmic domain is indeed polymorphic and is shared by different HLA-I alleles. It is therefore only reasonable to hypothesis that different HLA-I cytoplasmic alleles may vary in their susceptibility to Nef mediated down-regulation. This thesis compares the degree of down-regulation between cytoplasmic alleles with special emphasis on differences between HLA-A and HLA-B allele response to Nef.

To investigate the role of HLA-I cytoplasmic domain in Nef mediated HLA-I down-regulation, Jurkat cell lines expressing chimeric molecules that contain HLA-A0201 extracellular and transmembrane domains fused to the different cytoplasmic tails of HLA-I found in the database, were generated. These individual cell lines were initially transfected with Nef and later infected with clade B virus, HIV-IIIB. It had been briefly noted that HLA-B down-regulation is comparatively less than HLA-A (Cohen, Gandhi et al. 1999; Specht, DeGottardi et al. 2008). Confirming this we also observed that HLA-B down-regulation is subtler with a statistically significant difference between cumulative fold down-regulation of chimera containing HLA-A and HLA-B cytoplasmic tails.

It is also widely accepted that HLA-B alleles have a superior function compared to HLA-A alleles (Kiepiela, Leslie et al. 2004; Goulder and Watkins 2008). As mentioned earlier all of the strongly protective HLA alleles are B (Carrington and O'Brien 2003; Pereyra, Jia et al. 2010). However, this does not entirely account for HLA-B restricted T cell responses and slower disease progression. Although it has been suggested that protective HLA-B57 and B27 select a more broadly responding T cell repertoire in the thymus (Kosmrlj, Read et al. 2010), this alone does not explain superiority of HLA-B alleles. Perhaps a more attractive explanation for better B alleles is functionally superior HLA-B restricted T cell response.

Evidence suggests that CD8+ T cells play a central role in control of HIV infection, similar to other disease (McMichael and Rowland-Jones 2001; McMichael, Borrow et al. 2010). Qualitative attributes of CD8+ T cells have come under major scrutiny and it has been identified that polyfunctionality (Betts, Nason et al. 2006) and HIV suppressive activity (Saez-Cirion, Lacabaratz et al. 2007) are associated with spontaneous control of HIV infection and slower disease progression. Interestingly it has been shown that HLA-B restricted HIV-1 specific T cells are more polyfunctional, have higher avidity, and show greater clonal turnover compared with HLA-A restricted T cells (Almeida, Price et al. 2007; Harari, Cellerai et al. 2007).

Efficient CTL mediated control of HIV-1 (Collins, Chen et al. 1998) and SIV (Minang, Trivett et al. 2009) has been shown with *nef* alleles that have reduced HLA-I down-regulatory activity. A limitation in many of these studies is the use of viruses with wild type *nef* alleles that mediate complete down-regulation of HLA-I, or *nef* deleted/mutant viruses that mediate no down-regulation (Collins, Chen et al. 1998; Tomiyama, Akari et al. 2002; Adnan, Balamurugan et al. 2006). The effect of intermediate levels of down-regulation of MHC-I of SIV Nef revealed that subtle differences in MHC-I down-regulation had marked impact on

CTL mediated viral suppression (Minang, Trivett et al. 2009). However, there is limited direct evidence of differential Nef mediated down-regulation of HLA-I and its effects on CTL mediated suppression of HIV.

Therefore, it is important to understand the role of polymorphic HLA-I cytoplasmic domain in control of HIV infection by CTL, which has not been explored so far. Here we demonstrate that cytoplasmic domain differences play a major role in Nef mediated differential down regulation of HLA-I. We also show that HLA-B molecules avoid reduced CTL cell recognition of HIV infected cells by resisting Nef mediated down-regulation. Although the resistance to Nef mediated down-regulation of B allele is incomplete, it appears to be sufficient enough to maintain an effective CTL response. Moreover, resistance of down-regulation maintained control of viral replication by CTL and lead to elimination of infected cells in *in vitro* viral suppression assays.

We were also able to demonstrate greater levels of degranulation (CD107a secretion) in cell lines expressing HLA-B chimera when cocultured with A2 gag restricted CTLs. There was higher degranulation in HLA-A2-C1205 cell line too. The similarities between these alleles were that they resisted Nef mediated down-regulation. Indeed, it is reasonable to speculate that higher expression of HLA-I molecules on the surface of infected cells lead to a polyfunctional profile. We observed greater IFNγ response in ELISPOT assays, more degranulation and IFNγ secretion in cell lines expressing HLA-B alleles in repeated experiments where infected chimeric cell lines were cocultured with the A2 Gag p17 specific CTL clone. IL-2, TNFα and MIP-1β secretion have not been assessed here, but our findings support the concept of 'CD8+ T cell populations are governed by the level of antigen stimulation' (Valitutti, Muller et al. 1996; Viola and Lanzavecchia 1996; Betts, Price et al. 2004; Almeida, Sauce et al. 2009).

Recent evidence suggests that there is no difference between polyfunctionality of HLA-B and HLA-C restricted CTL responses (Mkhwanazi, Thobakgale et al. 2010). This was also observed in our experimental set up except that overall low surface expression of HLA-C was not accounted for. Similarities observed between trafficking of HLA-B and C alleles also support the common origin of these alleles (Discussed in Chapter 5).

Together these findings provide an important insight into understanding how CTL could exert better control over HIV-1 infection when they are restricted by HLA-B compared with HLA-A. Our results are consistent with current understanding of better recognition of HIV-1 infected cells by HLA-B restricted T cells compared with HLA-A restricted T cells, although high viral setpoints associated with HLA-B*3502 and B*5802 are not explained. Differential down-regulation may also explain why a greater diversity of peptides can be presented by HLA-B compared with HLA-A alleles (Goulder and Watkins 2008). But this does not explain why protective HLA-I alleles, especially HLA-B mostly targets Gag epitopes (Borghans, Molgaard et al. 2007).

HLA-C molecules are known to resist Nef mediated down-regulation (Cohen, Gandhi et al. 1999). It is also known that the expression of natural HLA-C molecules on the cell surface is lower than HLA-A and B (Zemmour and Parham 1992). Increased turnover of mRNA of the heavy chain of HLA-C (McCutcheon JA 1995), unstable HLA-C heavy chain, inefficient association of beta microglobulin as compared with other classical HLA-I (Neefjes and Ploegh 1988; Shimizu and DeMars 1989; Setini, Beretta et al. 1996), and low availability of specific peptides have been attributed as causes for this (Falk, Rotzschke et al. 1993). Consistent with previous published data (Schaefer, Williams et al. 2008) chimeric HLA-C expression was repeatedly low (with the exception C1205) in our transduction assays. Yet the expression was sufficient enough to assess the down-regulatory activities mediated by Nef.

However, importantly this observation in a A2-C chimeras system clearly demonstrates the role of HLA-C cytoplasmic domains in its low surface expression. In general T cell recognition of HLA-A2-C chimera was weak. We also saw a strong linear correlation between level of HLA expression after exposure to HIV-1 and CTL recognition.

It is important to understand the behaviour of endogenous HLA-A and HLA-B alleles in the presence of HIV-1 Nef, if we are to generalise our findings. The number of cell lines that could be used for this experiment was limited by the availability of specific HLA-I antibodies. However, we were able to demonstrate that HLA-A alleles are more susceptible to Nef mediated down-regulation than HLA-B alleles. Moreover, a greater IFNγ response in ELISPOT assays and greater suppression of HIV cells infected with pNL4-3 by HLA-B specific CTL clones compared to HLA-A specific CTL clones, indicates that lower down-regulation mediated by Nef leads to better control by CTL. Avidity of CTL clones that are used (A24 Nef and B51 Gag restricted) plays a big role in IFNγ response and viral control, but it does not influence this study results as the variable that is assessed, is the ELISPOT response in different viruses (i.e. wild type pNL4-3 and M20A pNL4-3) that were used for the study.

Also no difference were seen in the percentage of CD107a expression by B51 Gag restricted CTL in coculture with MT2 cells infected with pNL4-3 and M20A pNL4-3. This indicates that the presence of a functional Nef does not affect the ability of CTL recognising B51 to degranulate as opposed to HLA-A24 CTL which showed a statistically significant difference ($p<0.05$) in degranulation when infected with wild type and mutant viruses. We also found a difference in the expression levels of granzyme between A24 restricted CTL in culture with WT versus mutant virus infected cells; but not with B51 clones. In contrast to granzyme, secretion of perforin was very low and no difference was observed. Perhaps previously

described inability of HIV-specific CD8+ T cells to produce perforin (Appay, Nixon et al. 2000; Harari, Enders et al. 2009), or secretion of preformed perforin to medium during CTL culture (Appay, Nixon et al. 2000) may have resulted in this.

Another important observation in this study was the variability of the susceptibility of different HLA-A cytoplasmic alleles. Particularly interesting was the low susceptibility of A-26 and A-43 to Nef mediated down-regulation and higher susceptibility of A-24. A*26 is considered to be a protective allele (O'Brien, Gao et al. 2001) although there are reports to the contrary (Rohowsky-Kochan, Skurnick et al. 1998). A*2501, A*3201 and A*7401 which are also considered to be protective alleles in clade B and C infections share the A26 cytoplasmic allele (Kaslow, Carrington et al. 1996; Fellay, Ge et al. 2009; Koehler, Walsh et al. 2010; Pereyra, Jia et al. 2010; Goulder and Walker 2012). Both A*2501 and A*3201 carry Bw4 motifs that have been described to be associated with protection in HIV-1. A*7401 differs from A*3201 by not carrying the residues that constitute the Bw4 motif, is nonetheless consistently protective against HIV disease progression. In some populations HLA-A*7401 is in linkage disequilibrium with B*5703, another protective B allele, but the protective activity of A*7401 is said to be independent of B alleles (Matthews, Adland et al. 2011). Although, I have not investigated it, I hypothesize that the resistance of the cytoplasmic tails of these alleles to down-regulation mediated by Nef could be contributing in an additive way to CTL mediated control of HIV.

A2-A24 chimera was dramatically down-regulated by HIV-1_{SF2}Nef and HIV-IIIB. Although HLA-A*24 carries a Bw4, motif it is known to be a HIV susceptible allele (de Sorrentino, Marinic et al. 2000). A*2301, that shares a cytoplasmic tail with A*24, is also known as an allele favourable to HIV progression (Chen, Winchester et al. 1997; Mackelprang, Carrington et al. 2010). MacDonald et al (MacDonald, Fowke et al. 2000) speculate that the reasons for

the A*2301 association with HIV progression range from presenting T cell antagonist peptides leading to a narrow T cell repertoire, to presenting fewer conserved epitopes, and being more tolerant to escape mutations (MacDonald, Fowke et al. 2000). In the current study we demonstrated that CTLs are unable to suppress HIV infection in chimeric A2-A24 cell line and also had low degranulation and IFNγ response. This suggests that *in vivo*, the same mechanisms could be responsible for escape from CTL recognition following down-regulation of A*24/A*2301, eventually contributing to disease progression.

As previously explained we believe that the intracellular domain contributes to the HIV control by modulating the expression of HIV epitopes. We have successfully demonstrated that HLA-B alleles resist down-regulation mediated by Nef of HIV-1 and that HLA-B is more similar to HLA-C than HLA-A alleles. This is indeed a novel concept, as it was always believed that there was not much of a difference between HLA-A and HLA- B alleles. There is evidence to show that HLA B and C have common origins. In fact it is believed that HLA-C originated by duplication when divergence of old world monkeys to apes occurred (Boyson, Shufflebotham et al. 1996; Fukami-Kobayashi, Shiina et al. 2005). More interestingly it has been shown that with regards to polyfunctionality that there was no difference between HLA-B and C restricted T cells (Mkhwanazi, Thobakgale et al. 2010).

It is also interesting to think why HLA-A alleles are extensively down-regulated but not HLA-B. We can easily hypothesise that if all HLA alleles are down-regulated from the surface of infected cells, these cells will easily escape CTL recognition. This will lead to extensive cell infection and subsequent cell death that is not a good survival method for virus. Thus for the viruses to survive and propagate they would need a continuous supply of target cells. In addition, differential down-regulation could lead to selection of virus variants, which

is advantageous to virus. If the virus acquires mutations that do not compromise fitness, they would be able to evolve more efficiently and spread better.

On the other hand this differential down-regulation could be an adaptation of the immune system. We know that HLA-I alleles specifically act as ligands for NK cells. NK cells are component of the innate immune system and missing self HLA or missing ligand recognition on target cells activates them. NK cells have the ability to act as cytotoxic cells and also to produce copious amounts of inflammatory cytokines IFNγ and TNFα. Unregulated activation of immune system during infection could lead to extensive tissue damage. Perhaps to avoid this, the immune system may have adapted to differentially down-regulate class I.

However, our study is important, as this is the first study detailing importance of differences in HLA-A and B cytoplasmic domains and could explain how HLA-B alleles better control HIV compared to HLA-A. Indeed our study is unique because we have used controls for all our variables; i.e to demonstrate the differences in effector functions due to cytoplasmic domains, we generated chimera containing HLA-A2 on the surface fused to different A, B and C cytoplasmic alleles and used the same A2 restricted Gag specific CTL clone, to demonstrated the effects of both HLA-A and HLA-B, we used same target CD4 cell lines that express both thus overcoming the limitation of adjusting infectivity rates etc., and to demonstrate that changes in antiviral efficacy is caused by Nef mediated Class I down-regulation mechanisms rather than other viral effects, a single mutation in Nef (M20A) was made. Even though these are *ex vivo* experiments, they explain the data of previous researches detailing better HIV control by HLA-B alleles. However, it would be interesting to test these results in a HIV cohort to better explain how HLA-B alleles as a whole are good in controlling HIV than HLA-A. Our findings have important implications to the field of immunology. Firstly, this study provides evidence to support why targeting HLA-B responses

might be beneficial in designing T cell based vaccines. We speculate that our findings also provide an explanation as to how HLA-B drives evolution of HIV-1 (Kiepiela, Leslie et al. 2004), by resisting down-regulation and exposing epitopes that will lead to better CTL recognition that drives the virus to mutate.

4. Contribution of Nef alleles of HIV-1, HIV-2 and Simian Immunodeficiency virus on selective modulation of HLA class I

4.1. Introduction

As discussed in chapter 3, the down-regulation of MHC-I by Nef is an important immune evasion strategy of HIV-1 (Collins, Chen et al. 1998). It has been shown that Nef mediated down-regulation of HLA-I decreases the susceptibility of infected cells to recognition and destruction by virus specific CTL. The down-regulation of MHC-I by Nef is important for viral survival, as it is preserved through Simian and Human Immunodeficiency virus type 2 (Munch, Schindler et al. 2005; Schmokel, Li et al. 2009). However, it's functional significance is somewhat unclear as HIV-2 causes a less virulent outcome in infected human hosts and SIV causes asymptomatic infection in its natural host. But importantly, rapid restoration through nucleotide reversion and/or compensatory changes in rhesus macaques that were infected with SIV Nef mutants that disrupt MHC-I down-regulation indicates a strong selective pressure to maintain this function *in vivo* (Munch, Stolte et al. 2001; Swigut, Alexander et al. 2004).

It is said that HIV-1 originated following transmission of SIV_{cpz} from chimpanzee (*Pan troglodytes troglodytes*) (Gao, Bailes et al. 1999). SIV_{cpz} crossed the species barrier on three independent occasions resulting in HIV-1 groups M, N and O (Hahn, Shaw et al. 2000; Schmokel, Li et al. 2009). On the other hand there is strong evidence to support (phylogenetically, geographically and epidemiologically) that human HIV-2 and macaque SIV_{mac} originated from cross species transmission of SIV_{smm} from Sooty mangabeys and that human transmission occured more than 8 times (Hahn, Shaw et al. 2000). Sooty mangabeys are the natural hosts of SIV_{smm} infection although they resist CD4 depletion and progression to AIDS. Understanding the adaptations of SIV_{smm} for immune evasion is important and will give insight into the evolutionary origins of HLA-I down-regulation for this group of primate lentiviruses.

The Nef-MHC-I association always occurs through the cytoplasmic domain of MHC-I that is relatively conserved. This conservation indicates the importance of the cytoplasmic domain in the function of HLA-I *in vivo*. Orthologous HLA-A and HLA-B are found in Rhesus macaques, but not HLA-C (Boyson, Shufflebotham et al. 1996). Orthologous HLA-C have been seen only in gorillas, chimpanzees and orang-utans (Boyson, Shufflebotham et al. 1996). Absence of HLA-C in old world monkeys implies that it is a recent duplication of HLA-B locus that occurred after divergence of apes from old world monkeys. Interestingly, HLA-C was found to be resistant to Nef mediated down-regulation, the resistance mediated by cytoplasmic domain (Le Gall, Erdtmann et al. 1998; Cohen, Gandhi et al. 1999). Retention of HLA-C even at low levels may mean that it can still inhibit natural killer (NK) cells that have HLA-C specific ligand KIR2DL (Cohen, Gandhi et al. 1999).

Three essential domains of Nef; an N terminal alpha helix with a conserved methionine at position 20, a SH3 binding polyproline domain and an acidic domain, are required for HLA-I down-regulation (Greenberg, Iafrate et al. 1998; Akari, Arold et al. 2000; Blagoveshchenskaya, Thomas et al. 2002). However, in contrast to HIV-1, SIV Nef does not require a PxxP motif for MHC-I down-regulation, but instead uses a unique amino acid sequence at the C terminal end of SIV Nef molecule that is not present in HIV-1 Nef (Swigut, Iafrate et al. 2000). This amino acid sequence is preserved in HIV-2 Nef but its necessity for MHC-I down-regulation has not yet been assessed. Although, MHC-I down-regulation is preserved in HIV-2 (Munch, Schindler et al. 2005; Schindler, Munch et al. 2006), the details of its significance is limited.

Chapters 1 and 3 of this thesis discuss how little is known about HLA-A, B and C allele cytoplasmic tail differences. Polymorphic cytoplasmic tail and its role in differential down-regulation of HLA-I mediated by HIV-1 clade B Nef was assessed in Chapter 3. We saw that

down-regulation of consensus HLA-I molecules could not be extrapolated to all HLA-I alleles as noticeable differences were observed between them (Rajapaksa, Li et al. 2012) and even subtle differences had marked impact on CTL mediated viral suppression capacity (Minang, Trivett et al. 2009; Rajapaksa, Li et al. 2012).

Previously (DeGottardi, Specht et al. 2008) it has been reported that the susceptibility of Mamu-I to HIV-2 *nef* alleles depends on the cytoplasmic domain sequence of the Mamu-I allele. Their experimental model contained different HIV-2 *nef* alleles and a variety of Mamu and sooty mangabey MHC (Ceat) cytoplasmic domains, consensus HLA-A, -B and -C alleles. Yet it is not known whether the HLA-I cytoplasmic alleles differ in their susceptibility to Nef from other HIV-1 subgroups, HIV-2 or SIV.

In this chapter, a panel of stable Jurkat T cell lines expressing extracellular and transmembrane domain sequences of HLA–A2 fused to cytoplasmic domain sequences of different HLA A, B and C alleles (generated and discussed in chapter 3) were used to investigate selective down-regulation by Nef of HIV-1, HIV-2 and SIV that infect primate species. The *nef* alleles of these lentiviruses except for MVP5180/HUT78 (subgroup O) have been cloned into replication competent HIV-1 (NL4-3) based proviral vector designed to coexpress Nef and eGFP from a bicistronic RNA (Schindler, Munch et al. 2006). Nef and eGFP are expressed at correlating levels and is mediated by HIV-1 LTR promoter (Schindler, Munch et al. 2005). MVP5180 virus was isolated from a Cameroon patient (obtained from NIHAIDS reagent programme) and grown in C8166 cell line. Stable Jurkat T cell lines expressing A2-chimera were infected with these HIV-1 NL4-3 based viruses and MVP 5180.

In addition, the amino acid sequences of human HLA-I have been compared to rhesus macaque MHC class molecules (Mamu) and sooty mangabeys MHC class molecules (Ceat). Taken together this will help us understand, at least partly, why certain alleles are more

susceptible and / or resistant to Nef, how the relationship between Nef and MHC-I evolved from macaques to humans and importantly whether there is a difference between HIV-2 and HIV-1 Nef mediated HLA-I down-regulation.

4.2. Results

4.2.1. Mamu and Ceat MHC-I to HLA-I

To assess the role of HLA-I cytoplasmic domain in Nef mediated selective HLA-I modulation in other HIV-1 subgroups and HIV-2, we used Jurkat T cell lines that express chimeric A2 molecules fused to different cytoplasmic domains that were generated in chapter 3. Previous research by Kirchoff group (DeGottardi, Specht et al. 2008) demonstrated that MHC-I A and B allele down-modulation was preserved in other primate lentiviruses. They used a selective group of cytoplasmic tail sequences representing gene products of multiples MHC-I from different species which included rhesus macaque (Mamu; *Macaca mulatta*); animal model of HIV-1 and sooty mangabey (Ceat; *Cercocebus atys*) and chimpanzee, hosts of SIV_{smm} and SIV_{cpz}.

Sequence comparison of MHC I-A cytoplasmic alleles revealed more variation within cytoplasmic domain of HLA-A in contrast to Mamu-A, which had very little polymorphism (Figure 4.1). Mamu-A*01 sequence which is representative of majority of Mamu-A shared the cytoplasmic domain sequence of HLA-A*0201 and consensus HLA-A tail sequence.

However, sequence alignments of Mamu-B revealed at least 20 different cytoplasmic tail variants compared to HLA-B alleles. The most common sequence represented by Mamu-B*01 is identical to HLA-B consensus sequence which is as same as HLA-B*35. In addition, there are some Ceat-A and -B alleles with cytoplasmic tails identical to those of rhesus macaques and HLA-A*02 and HLA-B*35 (DeGottardi, Specht et al. 2008). Most of the Mamu-B alleles were found to be sharing S322F polymorphism in the cytoplasmic tail with A43. Rhesus macaques lacked a counterpart to HLA-C.

Sequence comparison of Mamu-B and HLA-B demonstrate that both have truncated cytoplasmic domains. Most of the Mamu-B alleles share ^{315}GG with HLA-B except Mamu-B*43, B*30 and B*20 (Figure 4.1)

Allele name	Amino acid sequence	MHC-I types known to share
A2-A2	^{310}RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV^{342}	HLA-A*0201, A*6801, (Table 3.1) Mamu A*01, A*02, A*11, A*05, A*13, A*14 Ceat A*02, A*04
A2-A26	^{310}.....M.....^{342}	See Table 3.1
A2-A11	^{310}.....T.....^{342}	See Table 3.1
A2-A43	^{310}.....F.....M.....^{342}	See Table 3.1
A2-A24	^{310}..N.....^{342}	See Table 3.1
A2-A80	^{310}.K...V.....^{342}	See Table 3.1
A2-A32	^{310}....L.....M.....^{342}	See Table 3.1
A2-B35	^{310}.....GG.....^{339}	HLA-B*35, B*51, (See Table 3.1) 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
A2-B57	^{310}.....GG.....C.....^{339}	See Table 3.1
Mamu B*05	^{310}..*..GG.....W.....^{339}	Mamu B*05
Mamu B*07	^{310}.....GG.....F....N.....E....^{339}	Mamu B*07
Mamu B*27	^{310}.....GG.....V.....^{336}	Mamu B*27
Mamu B*43	^{310}.....GE.....F....K.N.....E..M.^{339}	Mamu B*43
Mamu B*64	^{310}.....GGR.....V.N.....^{339}	Mamu B*64
Mamu B*19	^{310}.....GG.....F....K..P....E..M.^{339}	Mamu-B*19, B*22, B*41, B*58, B*65,
Mamu B*30	^{310}.....GA.....C.....^{339}	Mamu B*30
Mamu B*03	^{310}.....GG.....N.....^{339}	Mamu B*03, B*08, B*11, B*21, B*37, B*66 Ceat B*03, B*05, B*08
Mamu B*44	^{310}.....GG.....V.....^{339}	Mamu B*44
Mamu B*20	^{310}.....GV.....M.^{339}	Mamu B*20
Mamu B*29	^{310}.....GG.....V.N.....^{339}	Mamu B*29, B*53, B*70, Ceat B*12
Mamu B*47	^{310}.....GG.....CN.....^{339}	Mamu B*47
Mamu B*67	^{310}.....GG.....M.^{339}	Mamu B*68
Mamu B*46	^{310}.....GG.....V.....K.^{339}	Mamu B*46
Mamu B*50	^{310}.....GG.....F....N.....^{339}	Mamu B*50
Mamu B*24	^{310}.....GG..R.....^{339}	Mamu B*24
Mamu B*26	^{310}.....GGR....F.....E.....^{339}	Mamu B*26, B*57
Mamu B*61	^{310}.....GG.....F..V.N.....^{339}	Mamu B*61
A2-C1205	^{310}.....GG....C.....N.....E..I...A^{342}	See Table 3.1
A2-C0822	^{310}.....GG.....N.....E..I...A^{342}	C*0822
A2-C0430	^{310}.....GG....C.....TN.....E..I...A^{342}	C*0430
A2-C0750	^{310}.....GG....C.....N.....E..IT..A^{342}	C*0750
A2-C0711	^{310}.....GG....C....C.N.....E..I...A^{342}	C*0711
A2-C0704	^{310}.....GG....C....C.N.....E..IT..A^{342}	See Table 3.1
A2-C0719	^{310}.....GG....C..V.C.N.....E..IT..A^{342}	C*0719, C*0718, C*0706

Figure 4.1: Aligned cytoplasmic domain sequences of cell lines generated representing HLA-A, B and C alleles in the database and macaque MHC-I alleles that were used by DeGottardi *et al* (DeGottardi, Specht et al. 2008). Dots represent residues that match with HLA-A*0201 sequence. Highlighted amino acids are believed to be crucial residues that are important in Nef mediated HLA-I down-regulation (Cohen, Gandhi et al. 1999). The numbering corresponds to the residue number in HLA-Cw4. Adapted from DeGottardi, 2008 (DeGottardi, Specht et al. 2008).

4.2.2. Selection of Nef alleles representing HIV-1, HIV-2 and some primate lentiviral lineage

In the chapter 3, we demonstrated the involvement of the cytoplasmic domain in the differential down-regulation of HLA-I using the popular laboratory strains, HIV-1_{SF2}Nef and HIV-1 IIIB virus. To determine whether cytoplasmic domain dependent differential down-regulation of HLA-I is conserved in other HIV-1 and HIV-2, a panel of molecular clones that express lentiviral *nef* (DeGottardi, Specht et al. 2008) were obtained from NIH/AIDS reagent programme. The selection was based on species origin, phylogenetic relationship and the ease of comparison of their behaviour against primate MHC-I described elsewhere (Schindler, Munch et al. 2006; DeGottardi, Specht et al. 2008) (Figure 4.2. and 4.3.). In addition, popular SIV_{mac}239 and SIV_{smm}FFm1 Nef were also included in the panel. SIV_{smm} contains a Nef allele from a virus adapted to its natural host. SIV_{mac} was obtained after experimental infection of rhesus macaques.

To examine whether the motifs that are critical in HLA-I down-regulation are conserved between *nef* genes, the amino acid sequences of the Nef that were used were aligned (Figure 4.2). Sequence analysis reveals noticeable variation between *nef* alleles. These are seen in ^{20}M, β-COP, acidic (^{62}EEEE^{65}) and proline rich regions (Greenberg, Iafrate et al. 1998; Mangasarian, Piguet et al. 1999; Akari, Arold et al. 2000; Schaefer, Wonderlich et al. 2008). Methionine at position 20 is conserved in HIV-1 Nef but not in any of the HIV-2 nor SIV (Figure 4.2). RxR motif, that is needed for β-COP binding and MHC-I down-regulation (Schaefer, Wonderlich et al. 2008) was not conserved in HIV-2 60415k Nef. The acidic region ^{62}EEEE^{65} that binds PACS-1 (Piguet, Wan et al. 2000) is conserved in HIV-1 groups M and N but variable in remaining Nefs (Figure 4.2). The proline rich motif $(PxxP)_3$ is implicated in binding of cellular kinases and MHC-I modulation (Greenberg, Iafrate et al.

1998) and is preserved in HIV-1 Nef. However, the first and the last prolines are changed to glutamic acid residues in HIV-2.

Additionally, a thioesterase binding site described to be important in MHC-I down-modulation and some other Nef functions (Roeth and Collins 2006), is only preserved in HIV-1 subgroups and is absent in all of HIV-2 and SIV *nef* alleles. A tyrosine in the C terminal region of SIV_{mac} Nef that has been described to play an important role in MHC-I down-regulation (Swigut, Iafrate et al. 2000) is preserved in SIV_{mac}, SIV_{smm} and HIV-2 *nef* alleles. These regions are implicated in HLA-I down-regulation and degradation. Altogether, this demonstrates a vast variation between HIV-1, HIV-2 and SIV *nef* sequences. HIV-2 *nef* alleles closely resembled SIV and clustered together (Figure 4.3)

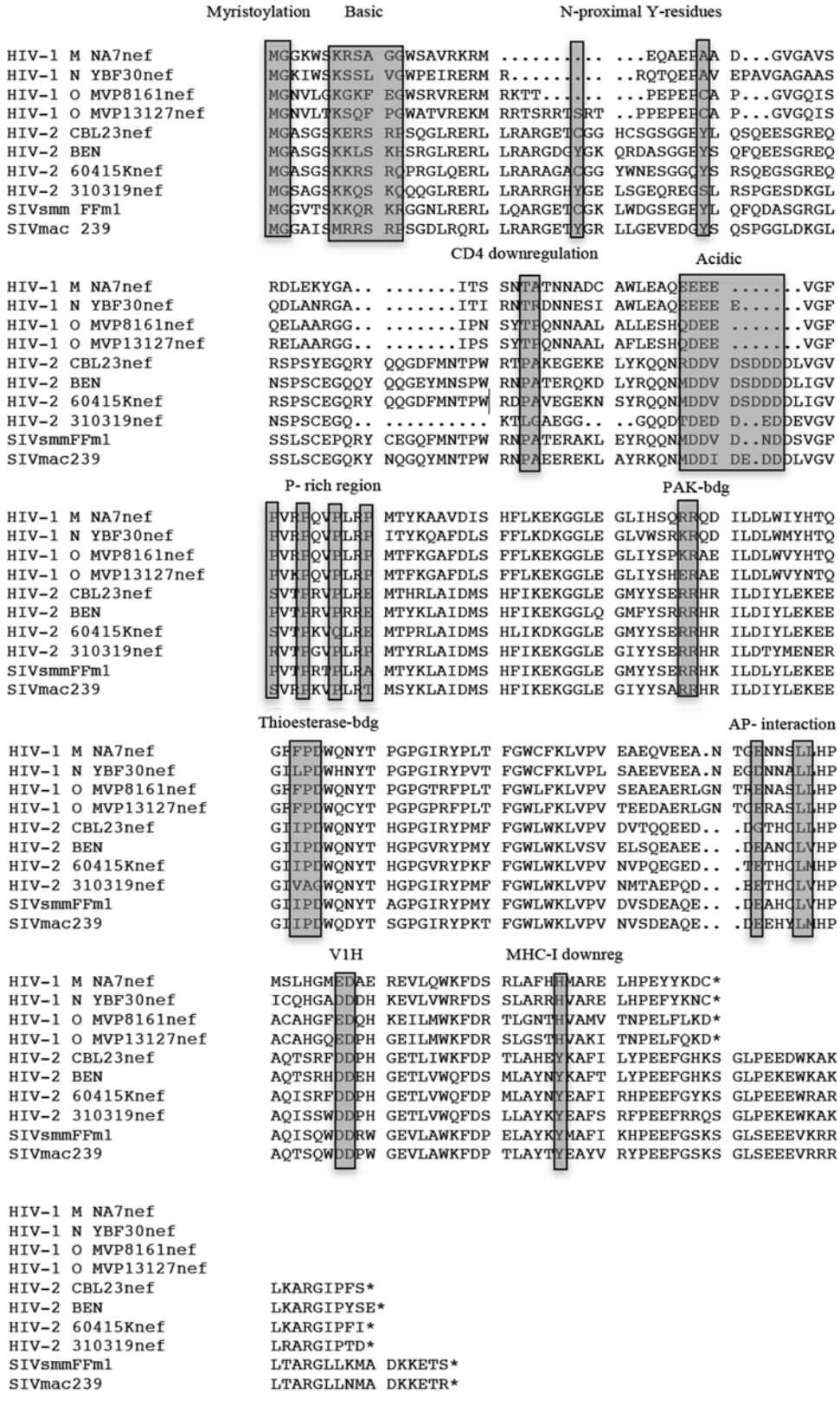


Figure 4.2: Sequence alignments of Nef alleles that are used in the study. The *nef* alleles of HIV-1, HIV-2 and SIVs are compared to the NA7 sequence. The ^{20}M residue, the RxR β-COP motif, the acidic region, the proline-rich domain and the tyrosine at the C-terminal part, which have all been implicated in HIV and SIV MHC-I down-modulation and degradation and some conserved sequence elements in Nef (the N-terminal myristolyation signal, N-proximal tyrosines, PA residues known to be critical for CD4 down-modulation by Mac239wt Nef, a diarginine motif, a C-proximal adaptor protein interaction site and a diacidic putative V1H binding site) are shaded in gray. Dots indicate gaps introduced to optimize the alignment and asterisks the stop codons. Adapted from Munch et al (Munch, Schindler et al. 2005).

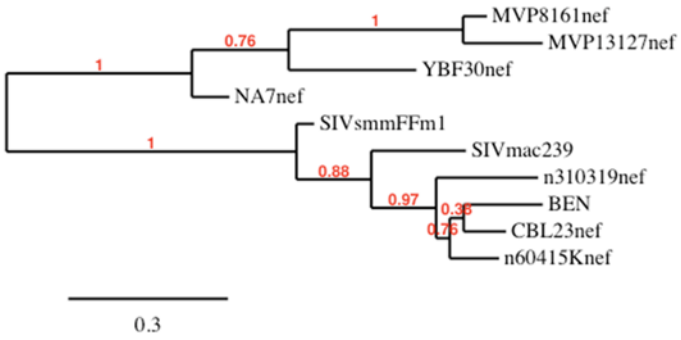


Figure 4.3: Evolutionary relationships between HIV-1, HIV-2 and SIV Nef sequences used in this study.

4.2.3. HLA-I down-regulation mediated by *nef* alleles from HIV-1

First, we wanted to assess the effects of HIV-1 subgroup-O Nef on different HLA-I cytoplasmic alleles (Figure 4.4, 4.5 and 4.6). Although, there was a wide variability between efficiencies of down-regulation, in agreement with previous data (Munch, Schindler et al. 2005; Schindler, Munch et al. 2006), HLA-A and B down-regulation was conserved in *nef* alleles HIV-1 subgroup M and O, as was the resistance of all HLA-C alleles. Several attempts to generate group N virus YBF30 and viruses of clade A, C & D failed, and due to time constrains these will be carried out at a later date.

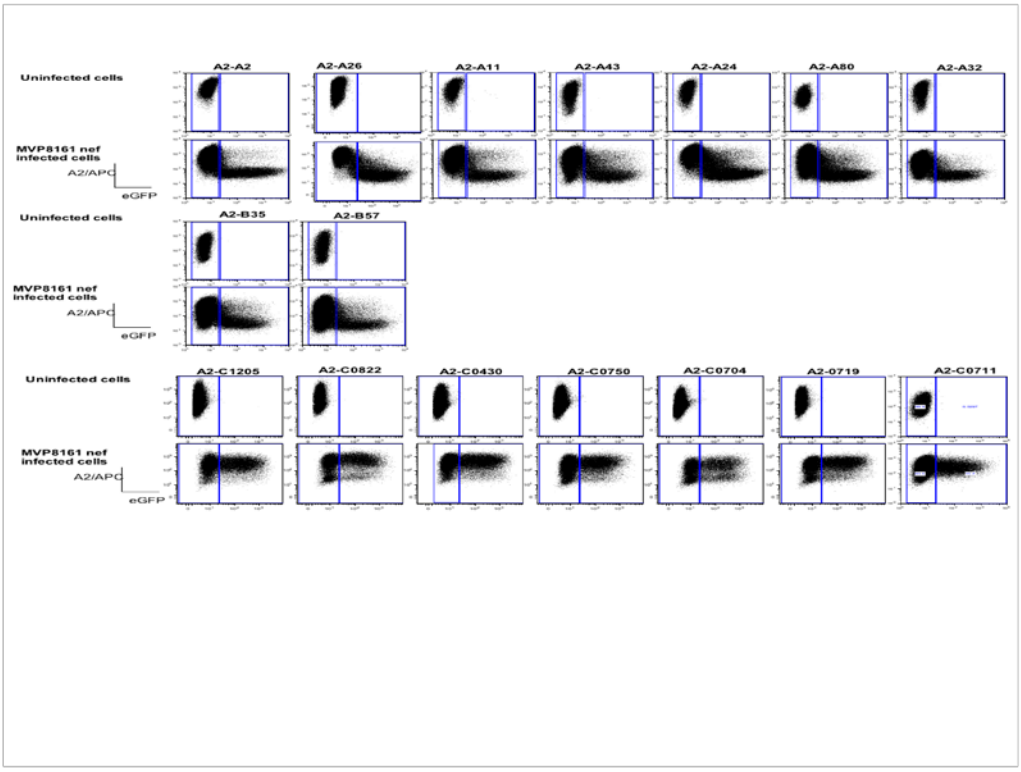
The efficiency of Nef mediated down-regulation differed according to the cytoplasmic tail. A2-A43 had the lowest down-regulation among the HLA-A cytoplasmic tails in all subgroup O and M *nef*. An unusually high degree of fold down-regulation of A2-A26 was noted with

MVP8161 *nef* (~13 fold) (Figure 4.4) compared to other *nef* alleles that were used. Similar to HIV-IIIB, MVP8161 demonstrated a slight up-regulation of HLA-C surface expression (Figure 3.3 and Figure 4.4).

It was observed that HLA-B alleles were less susceptible to MVP5180/HUT78 (Figure 4.5) and MVP8161 (Figure 4.4) mediated down-regulation with a mean fold down-regulation of 1.0259 and 3.2813 respectively. The cumulative differences between A and B alleles, A and C alleles and also B and C alleles reached statistically significant levels with both these *nef* alleles ($p<0.05$). Similar results were obtained with NA7 *nef* allele (Figure 4.6).

Altogether our results demonstrate that selective down-regulation of HLA-A and B, and resistance of HLA-C is conserved in the subgroup O and M of HIV-1. However, the sensitivity of HLA-B to *nef* alleles were comparatively less than HLA-A.

a



b

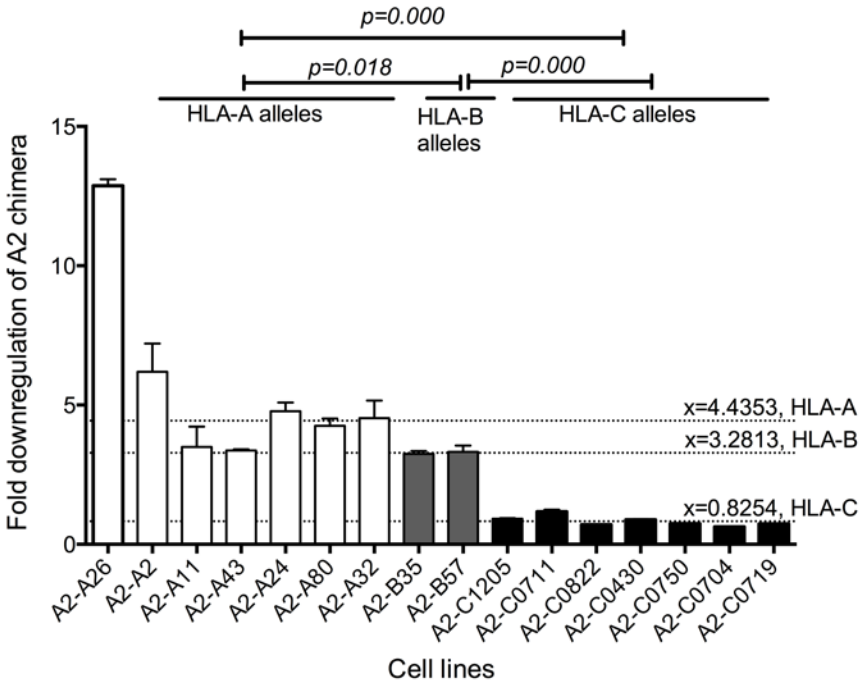


Figure 4.4: **Differential down-regulation of chimeric HLA-A, B and C with subtype O virus Nef MVP8161.** (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection (**b**) The fold down-regulation of A2 chimera in the presence of MVP8161/GFP was calculated as described in the methods. Each bar represents the mean of two independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

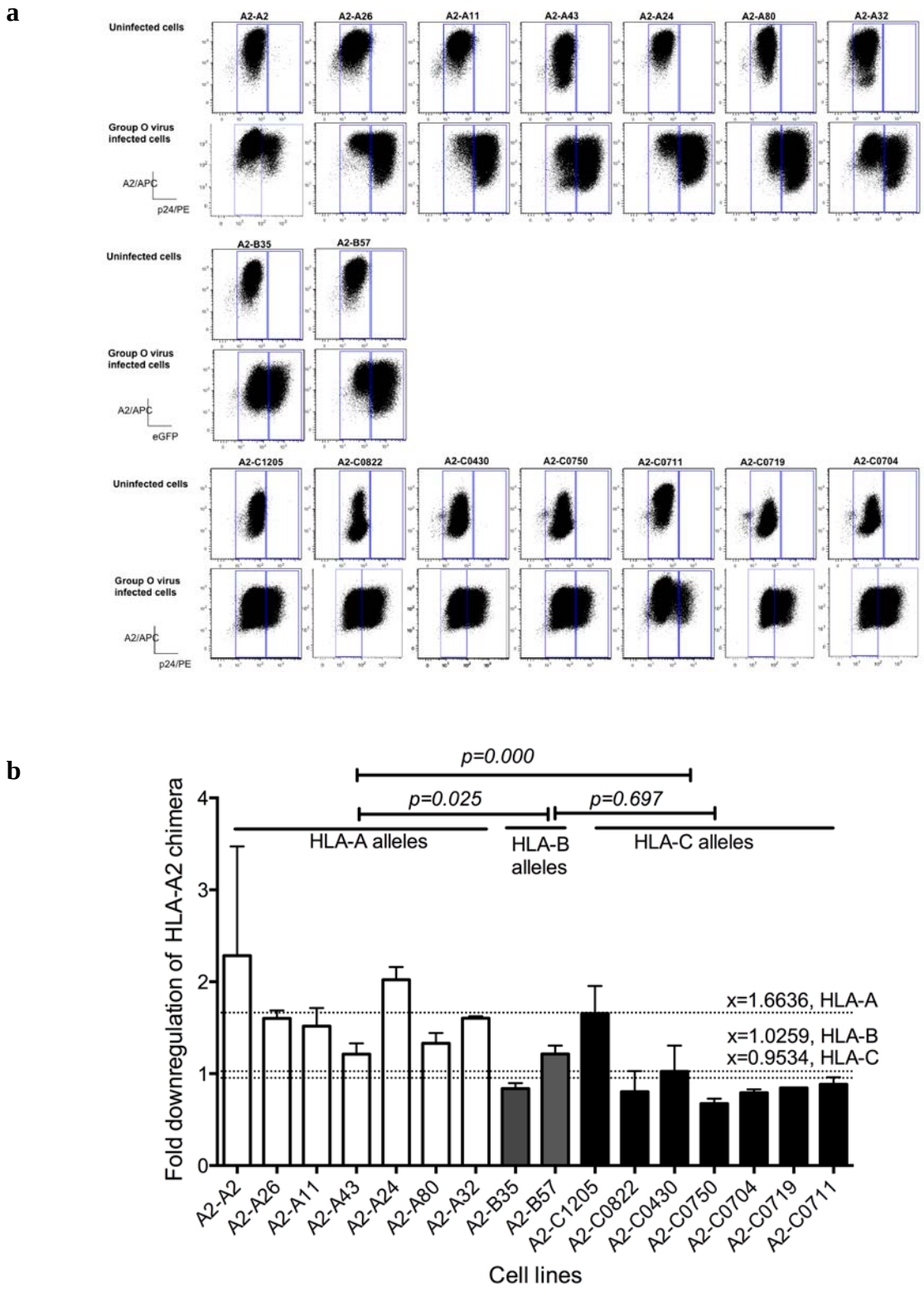
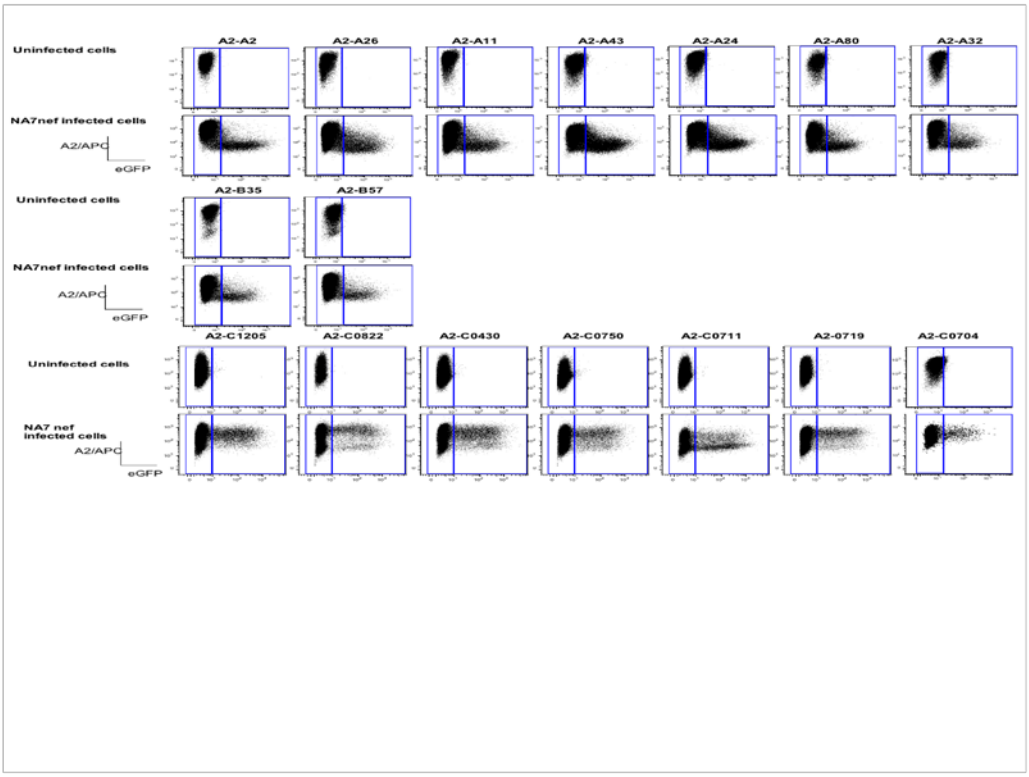


Figure 4.5: Differential down-regulation of chimeric HLA-A, B and C with MVP5180/HUT78 subtype O virus. (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of MVP5180 as calculated as in methods. Each bar represents the mean of three independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

a



b

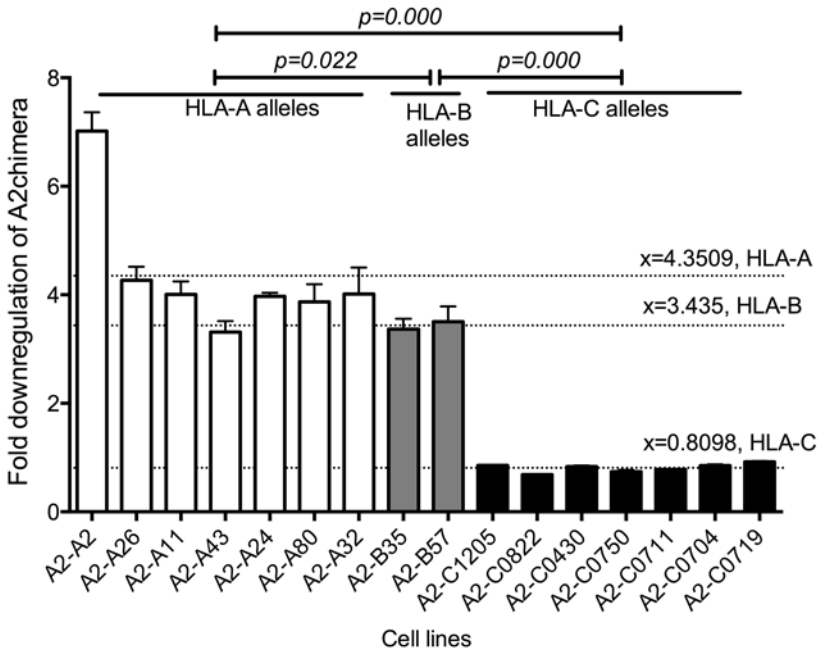


Figure 4.6: Differential down-regulation of chimeric HLA-A, B and C with subtype M NA7 virus. (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of NA7/GFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

4.2.4. Specific HLA-I down-regulation mediated by HIV-2 and SIV$_{smm}$ *nef* alleles

There is limited availability in the literature on the association of HLA-I down-regulation and HIV-2 infection. HLA-I down-regulation is preserved in HIV-2 viruses (Munch, Schindler et al. 2005; Schindler, Munch et al. 2006). These studies used consensus HLA-I cytoplasmic sequences to measure the effect of Nef. Contribution of MHC-I down-regulation in the immune response in HIV-2 infection is poorly studied. This requires more attention as the clinical cause of HIV-2 is completely different to that of HIV-1 in spite of sequence similarities between the viruses. Here, we assessed the susceptibility of different HLA-I cytoplasmic alleles to HIV-2 Nef. Four HIV-2 *nef* alleles that originated from patients were used for this assay: 60415k, CBL23, BEN and 310319 (Kirchhoff, Jentsch et al. 1990; Schulz, Whitby et al. 1990; Gao, Yue et al. 1994; Owen, Ellenberger et al. 1998).

Interestingly, and similar to HIV-1 (chapter 3), the cytoplasmic domain sequence was found to be playing a major role in Nef mediated HLA-I down-regulation in HIV-2 (Figure 4.7, 4.8, 4.9, 4.10). All HLA-A alleles were down-regulated but with variable efficiencies. In contrast to other *nef* alleles we saw a very high degree of down-regulation of HLA-A and B alleles with CBL23 (Figure 4.8). Among the HIV-2 *nef* alleles that were used in this study, the lowest fold down-regulations for HLA-A and B alleles were observed with 310319 Nef.

Similar to group O *nef* alleles A2-A43 had a lower down regulation, ranging from a mean fold down-regulation of ~1.8 in 310319 Nef to ~5 in CBL23 Nef, an interesting observation that needs closer attention since both HIV-2 and HLA-A*43 are found in the African subcontinent.

More interestingly, we noted a partial resistance to down-regulation of HLA-B in HIV-2 60415k Nef, 310310 and BEN Nef with cumulative difference between HLA-A and -B alleles

reaching statistically significant levels (Figure 4.7, 4.9, 4.10). Indeed, it has been noted that several Mamu-B alleles demonstrated some degree of resistance to SIV_{smm}FWr1 and HIV-2 310319 (DeGottardi, Specht et al. 2008). We noted that cumulative down-regulation of HLA-B by SIV_{smm} was not much different to mean fold down-regulations of HLA-A alleles (Figure 4.11.). Although obvious disparities exist between HLA-B and Mamu-B alleles, it appears that resistance of HLA-B to HIV-2 Nef mediated down-regulation, is greater and more global in humans as oppose to macaques.

Altogether, these data indicate that HLA-A down-regulation is conserved in HIV-2 and SIV_{smm} Nef but efficiencies depend on cytoplasmic domains. HLA-B down-regulation is partial in most HIV-2 *nef* alleles probably indicating an adaptation of HLA-B to Nef mediated down-modulation. Of interest is the observation that the difference between mean fold down-regulation of HLA-A and HLA-B in HIV-2 nef was not as great as in what was observed with HIV-IIIB and SF2 (Figure 3.2, 3.4) (1-1.5 fold difference in HIV-2 versus 2-3 fold in HIV-1). As expected all HLA-C cytoplasmic alleles resisted Nef mediated down-regulation.

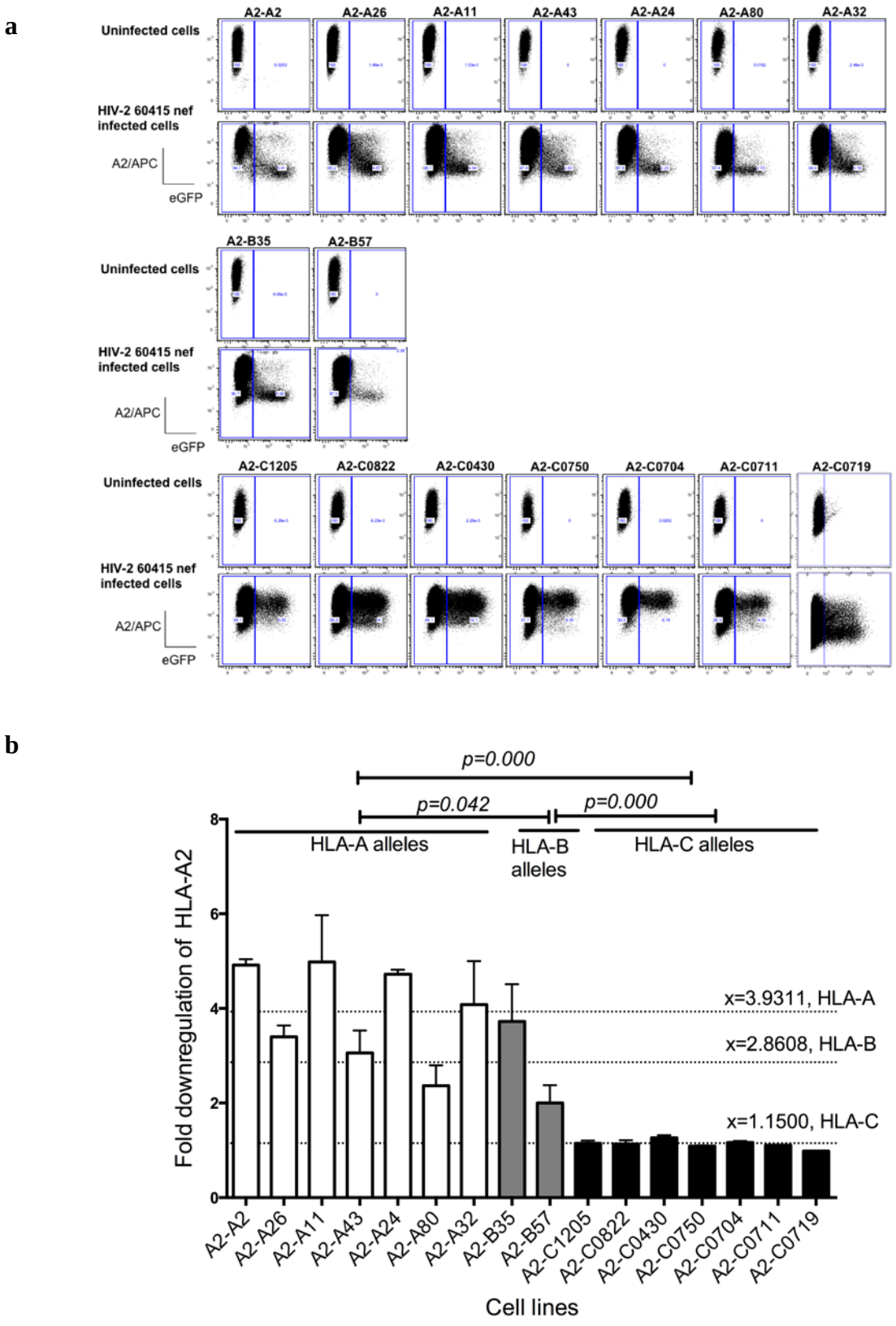


Figure 4.7: **Differential down-regulation of chimeric HLA-A, B and C with HIV-2 60415k.** (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of HIV-2 60415k Nef/eGFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

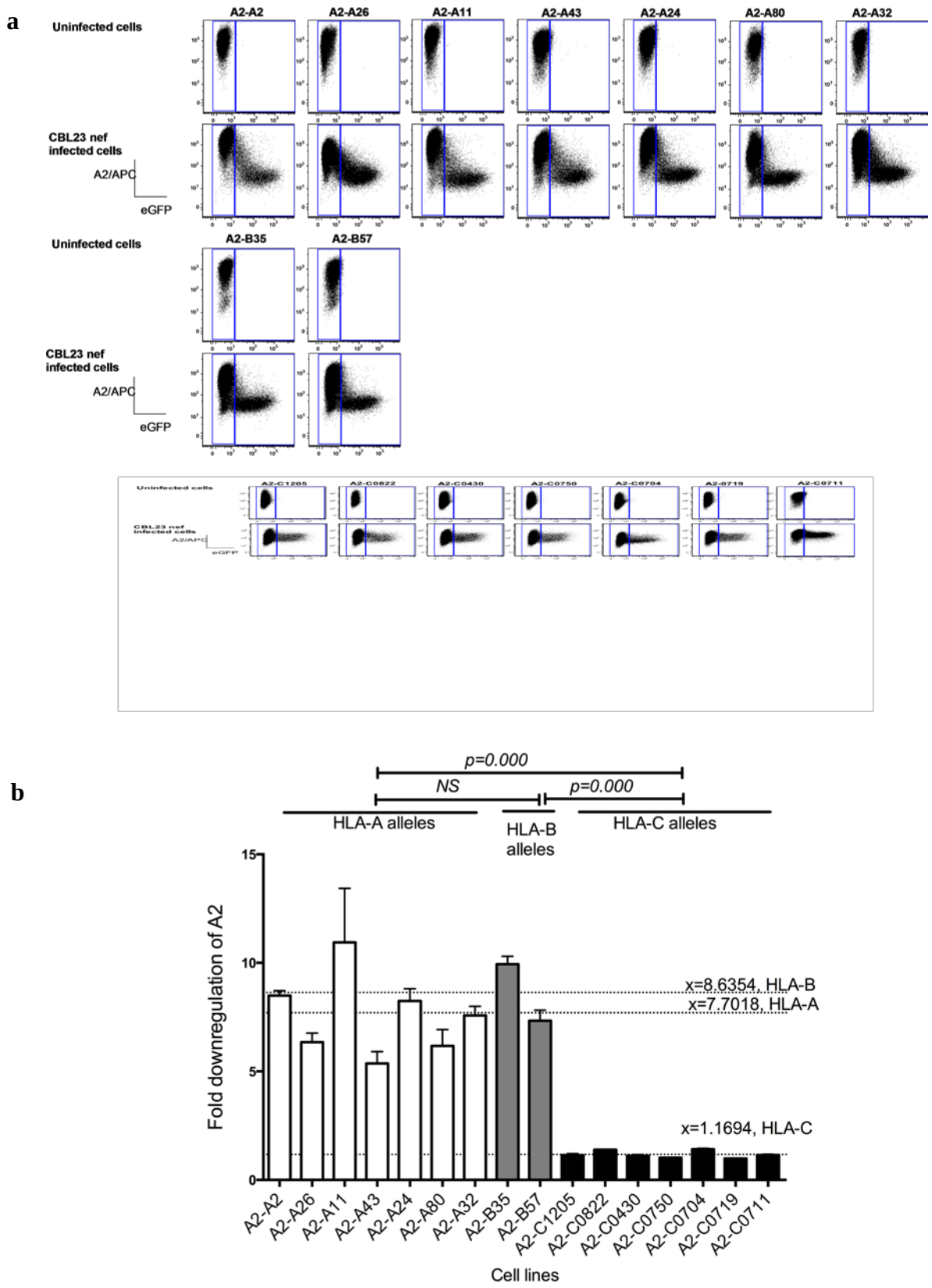
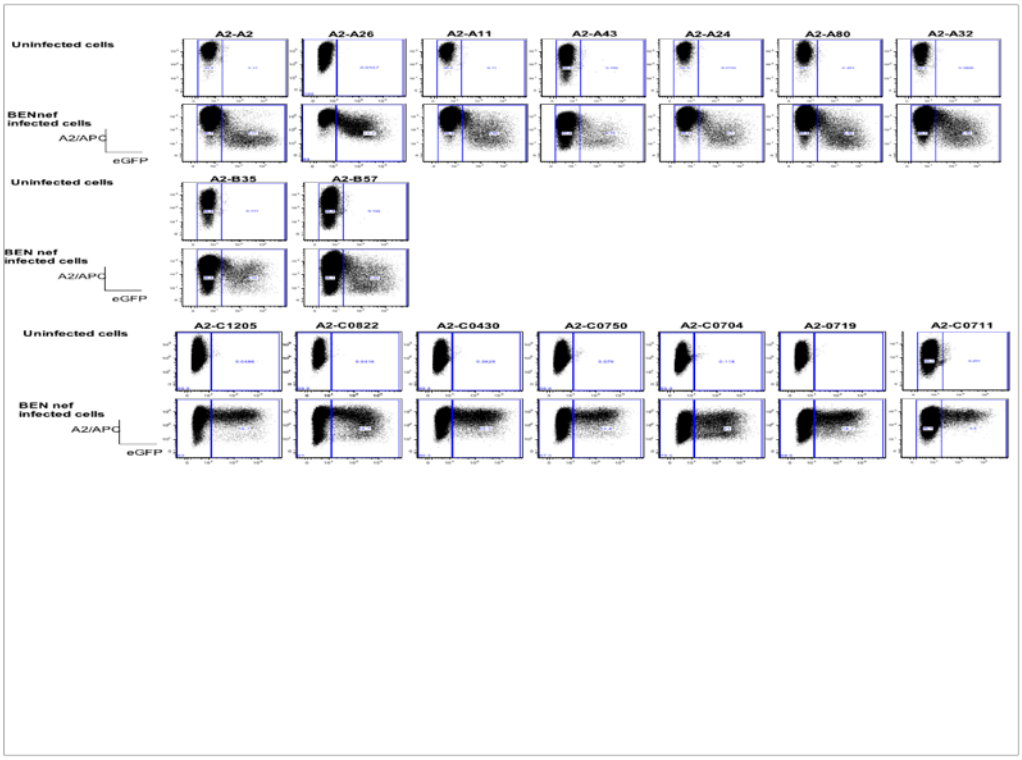


Figure 4.8: **Differential down-regulation of chimeric HLA-A, B and C with HIV-2 CBL23 Nef.** (**a**)Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of HIV-2 CBL23 Nef/eGFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

a



b

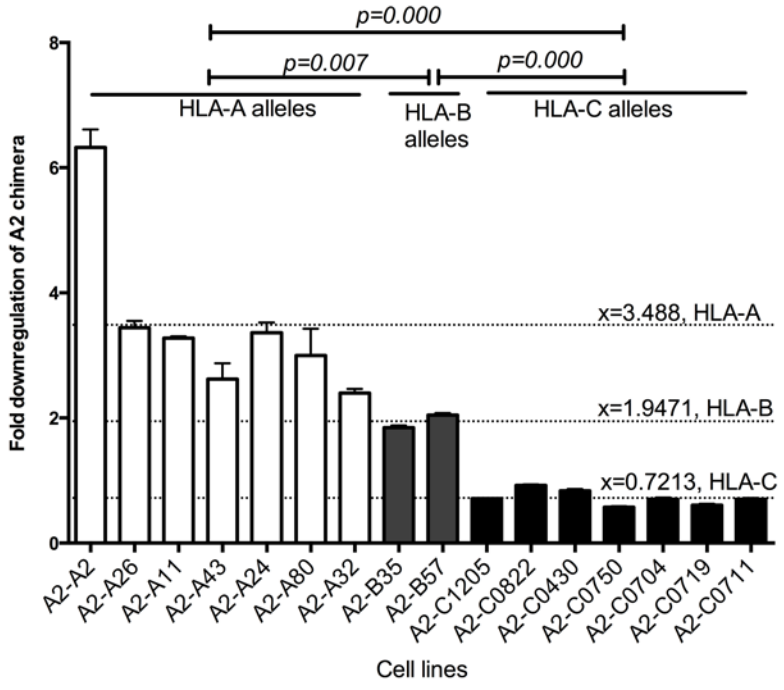


Figure 4.9: Differential down-regulation of chimeric HLA-A, B and C with HIV-2 BEN Nef. (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of HIV-2 BEN Nef/eGFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

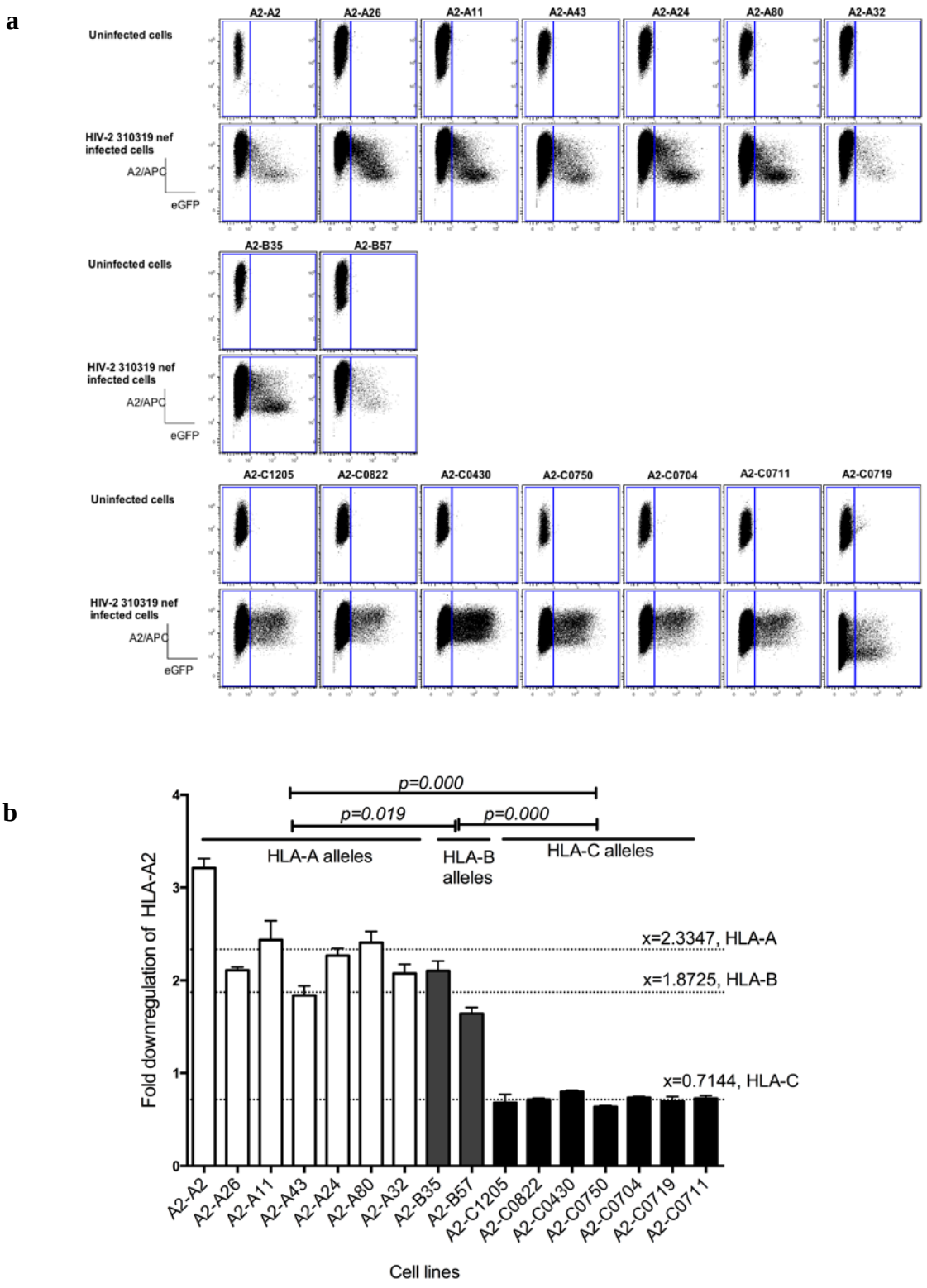


Figure 4.10: Differential down-regulation of chimeric HLA-A, B and C with HIV-2 310319 Nef. (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of HIV-2 310319/eGFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

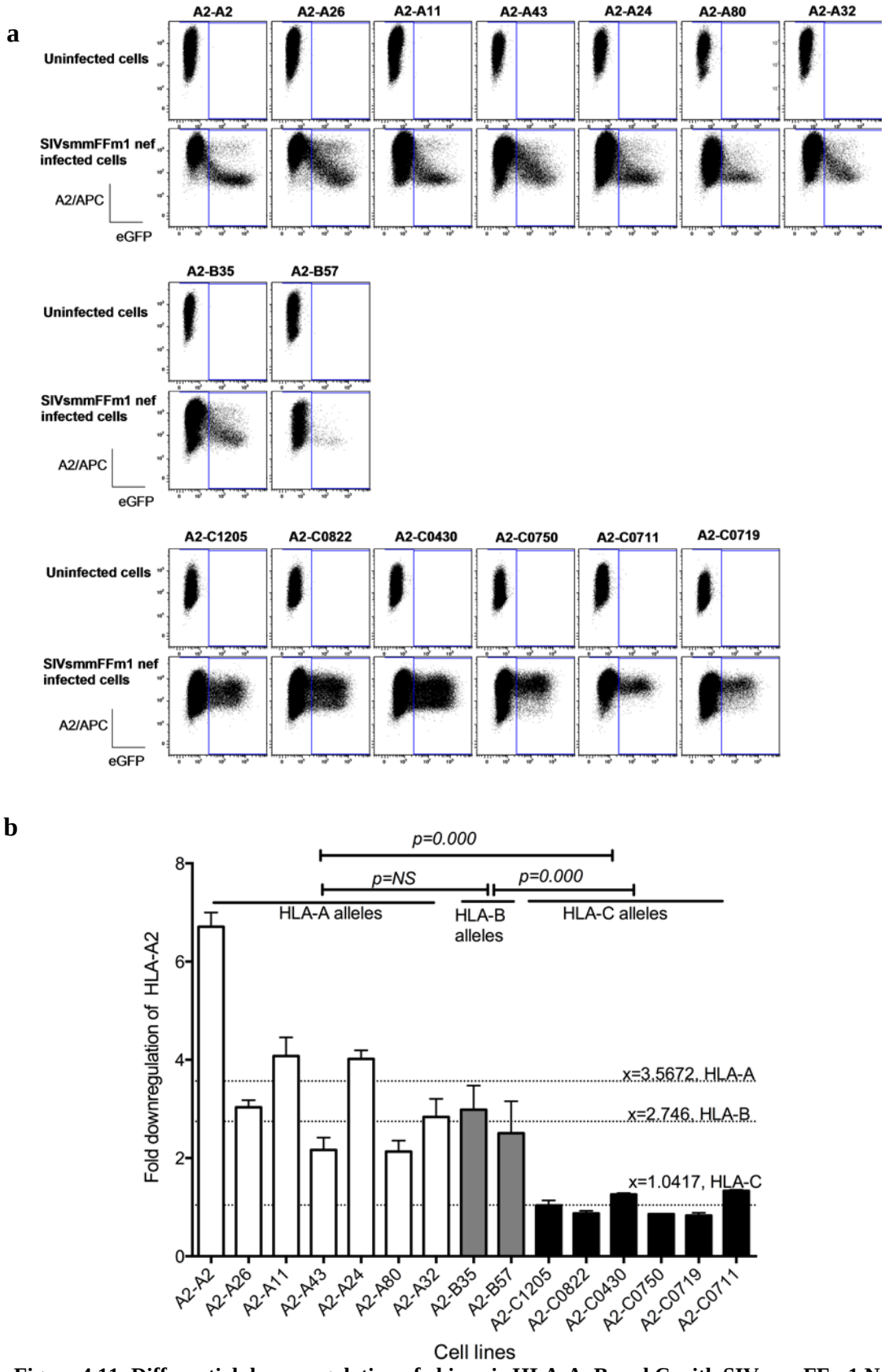


Figure 4.11: **Differential down-regulation of chimeric HLA-A, B and C with SIVsmmFFm1 Nef.** (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of SIVsmmFFm1/eGFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SD, x=mean fold down-regulation of HLA-A2.

4.2.5. Comparison of HLA-I modulation to Mamu-I modulation in rhesus macaque model

The animal model for the development of HIV/AIDS vaccine is SIV infection of rhesus macaques (McClure, Anderson et al. 1990). Previous studies have shown that SIV_{mac} Nef down-regulate Mamu-A, -B*01 and -B*03 but leaves B*07, B*19 and B*29 on the cell surface. However, other HIV-1 and HIV-2 *nef*s reduced surface expression of Mamu B*07 and other B alleles to differing degrees respectively. In the same study, HIV-2 310319 and HIV-1 pNL4-3 *nef* alleles failed to down-regulate Mamu-B*19 and B*29 (DeGottardi, Specht et al. 2008). On careful inspection it could be seen that a lot of the Mamu-B alleles were partially resistant to SIV_{mac}/HIV-2 310319 Nef mediated down-regulation compared to Mamu-A alleles.

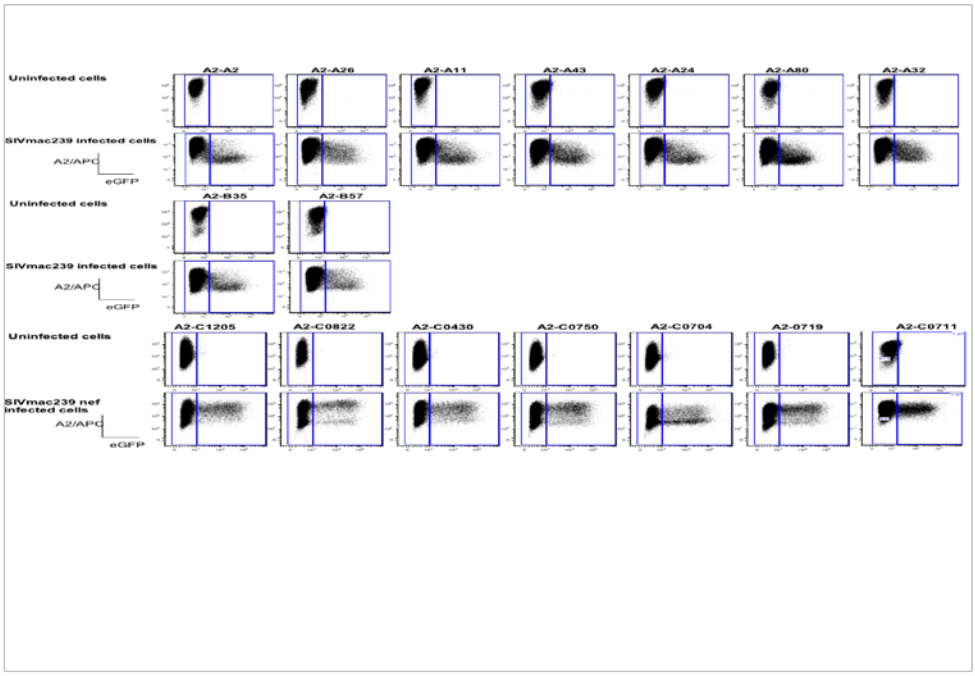
Here, we assessed the ability of SIV_{mac} to down-regulate HLA-I cytoplasmic alleles (Figure 4.12). Similar to HIV-1 and HIV-2, SIV_{mac} was able to down-regulate HLA-A and B alleles while HLA-A2-C chimera demonstrated clear resistance. The pattern of susceptibility of HLA-I alleles was similar to HIV-IIIB. A2-A43 demonstrated the least susceptibility to down-regulation with A2-A26 closely following it. Interestingly, A26, A43 and most Mamu-B alleles that have been found to be resistant to Nef mediated down-regulation demonstrate polymorphism at positions ^{322}serine and ^{335} valine (discussed later).

We noted that cumulative HLA-B allele down-regulation by SIV_{mac} compared to HLA-A, failed to reach statistically significant level (Figure 4.12, student t test, $p>0.05$). The fold down-regulation for the 7 HLA-A chimera ranged from ~2-5 fold, whereas the HLA-B chimera were both approximately 2 fold-down-regulation. Collectively there was no difference statistically between A and B. The differing susceptibility of Mamu-B to Nef seems to be lost in humans concurrent to loss of polymorphism of HLA-B tails. Although

seemingly this suggests that humans have lost a layer of immune response, perhaps it may not so, as there are polymorphisms in the HLA-A cytoplasmic tail compensating for this.

The animal model of HIV-1, SIV infected rhesus macaques, like HIV-1 in humans represents a recently infected host. The precursor of SIV_{mac} is, like for HIV-2, SIV_{smm} from sootymangabeys (Hirsch *et al.*, 1989; Hahn *et al.*, 2000). However, the present results demonstrating the reduced susceptibility of the HLA-B to HIV-1 Nef versus higher and more varied Nef susceptibility of the Mamu-B alleles to the SIV_{mac} Nef (DeGottardi, Specht et al. 2008), raises the question as to what extent the results obtained in the macaque model can be applied to the human and its impact on T cell vaccine development.

a



b

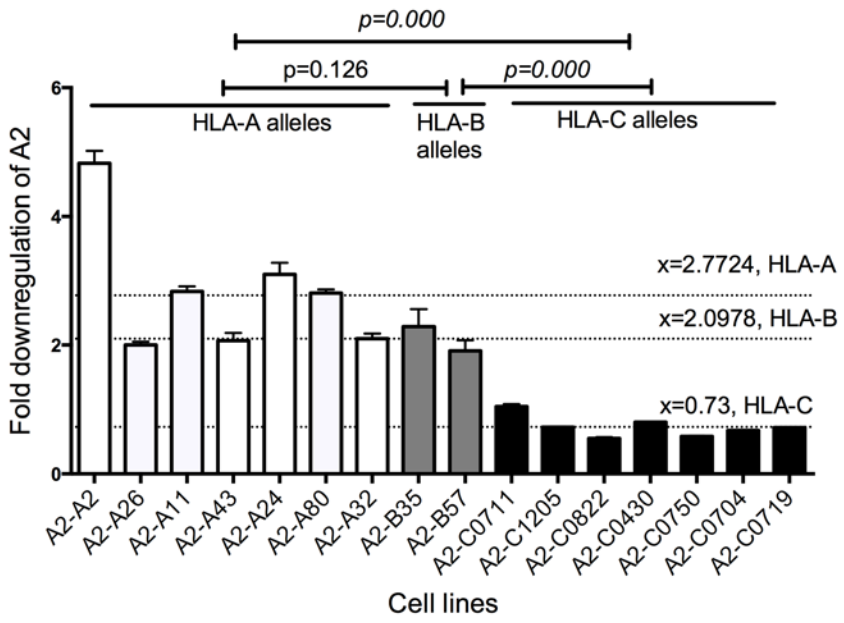


Figure 4.12: **Differential down-regulation of chimeric HLA-A, B and C with SIVmac239 Nef.** (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A2 chimera measured 4 days after infection. (**b**) The fold down-regulation of A2 chimera in the presence of SIVmac/eGFP as calculated as in methods. Each bar represents the mean of three independent experiments+ SEM, x=mean fold down-regulation of HLA-A2.

4.2.6. Selective modulation of native HLA-A and B between HIV-1 counterparts

We next assessed the selective down-modulation of endogenous HLA-A and HLA-B alleles by HIV-1 subgroup Nef counterparts. Although it has been shown that HLA-A and HLA-B down-modulation is a conserved property of all three groups, the major limitation was the antibody that these researchers used. W6/32 is an anti class I antibody and binds to HLA-A, HLA-B and also HLA-C molecules on the surface. In a context where HLA-A and HLA-B are highly expressed but down-regulated and HLA-C is expressed at lower levels but is not removed from the surface, W6/32 binding show the cumulative results of all these effects. In our experimental context we used allele specific anti bodies overcoming this problem. We used Jurkat cells that inherently express HLA-A3 and HLA-B7 and specific antibodies that bind to them.

We noted that both HLA-A3 and HLA-B7 were down-regulated by HIV-1 viruses of group M and O but to a variable level (Figure 4.13). HLA-A3 down-regulation was more marked compared to HLA-B7 in which the down-regulation was incomplete.

a **b**

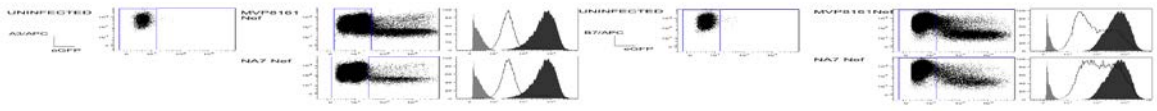


Figure 4.13: **Differential down-regulation of native HLA-A and B with Nef alleles representing HIV-1 subgroups.** (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A3 (**b**) HLA-B7 in subgroup O- MVP8161 and sub group M- NA7 virus. n=3. Solid grey- negative control, solid black- uninfected cells, solid white- Nef expressing cells.

4.2.7. Selective native HLA-A and B modulation by HIV-2 and simian counterparts

The down-regulation of the native HLA-I alleles of Jurkat cells with Nef of HIV-2 and simian counterparts was next assessed. HIV-2 causes a pathogenic infection which progress slowly to AIDS in humans while SIV_{smm} is non pathogenic to sooty mangabeys, who are natural hosts and shows no progression to AIDS.

All HIV-2 *nef* alleles that were used in this study were able to down-regulate native HLA-A3 and B7 (Figure 4.14). In general, the sensitivity of HLA-B7 to Nef mediated down-regulation was less than A, the exception being CBL23 Nef which down-regulated A3 and B7 to similar extents.

Interestingly, HLA-A3 down-regulation was more extensive and reached the maximum level at the lowest level of Nef. This was in contrast to B7, which was more gradual with down-regulation correlating with the level of Nef GFP expression. This behaviour was only observed in VSV pseudotyped 'molecular viruses' but not with 'natural lentiviruses' generated in T cell lines (e.g. HIV-IIIB virus discussed in chapter 3 and MVP5180 virus used in the previous section). Whether this behaviour was due to the experimental set up that we have used is not known.

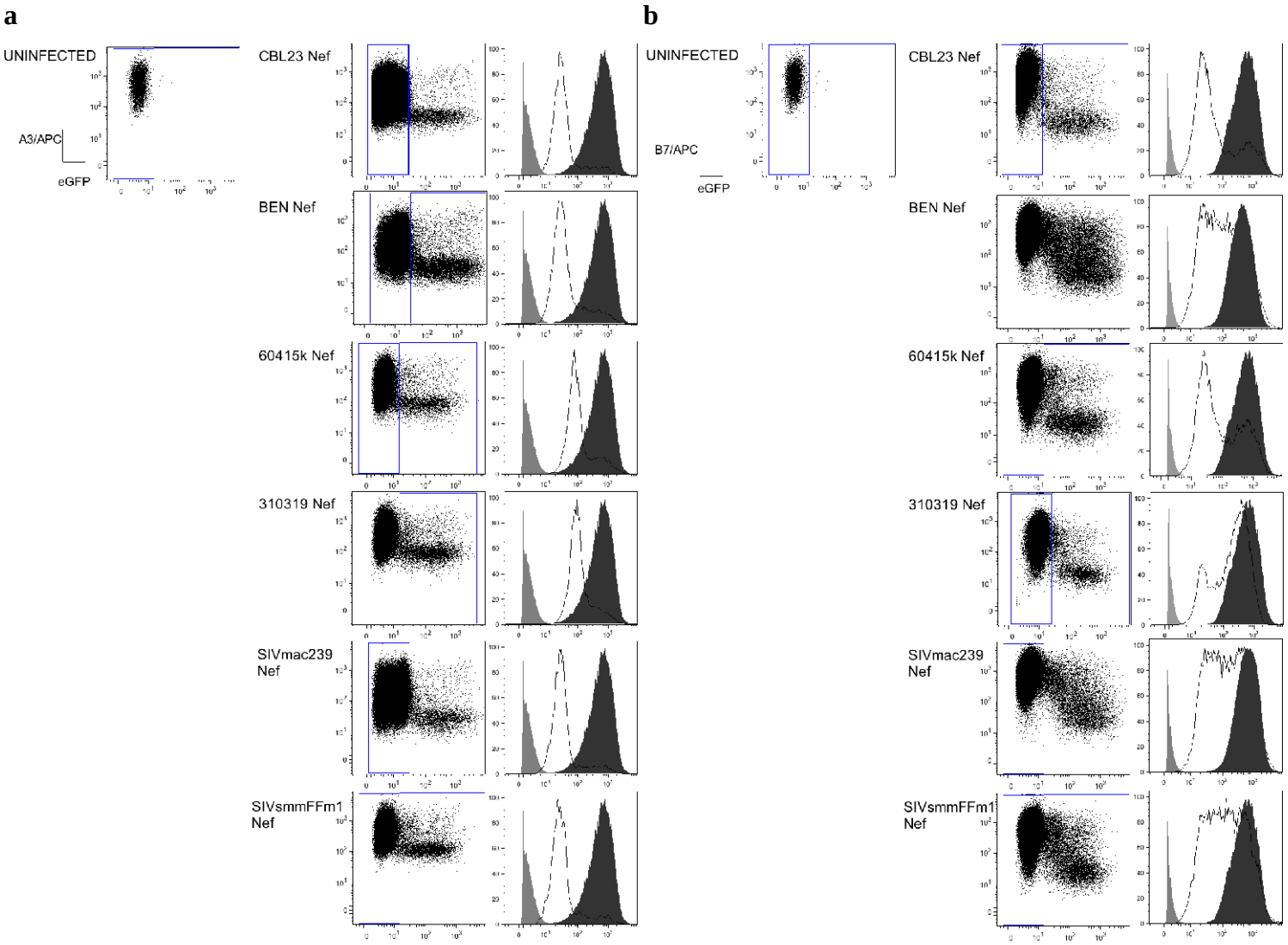


Figure 4.14: Differential down-regulation of HLA-A, B with HIV-2, SIV_{mac} and SIV_{smm} Nef (**a**) Representative flow cytometry demonstrating magnitude of down-regulation of HLA-A3, (**b**) HLA-B7, measured 4 days after infection with molecular clones containing Nef/eGFP. n=3 independent experiments. Solid grey- negative control, solid black- uninfected cells, solid white- Nef expressing cells.

4.2.8. Correlation of native allele and HLA-I cytoplasmic domain down regulation

We then assessed the relationship between the fold down-regulation of HLA-A, -B and –C by Nef in the chimeric cell lines and with native alleles. Endogenous HLA-A3 and B7 down-regulation correlated with their HLA-A2 chimera counterparts down-regulation by linear regression analysis for A2, B35 and B57 and several other HLA-A cytoplasmic alleles (data not shown) but not A2-C1205 (Figure 4.15.) Additional correlations were observed for pairwise comparisons between A2-B35, A2-B57, A2-A43 (Figure 4.16.). These observations indicate that the down regulation of heterologous HLA-A2 chimeric proteins accurately predict the down regulation of full length HLA-I. This also demonstrates that the magnitude of down-regulation is a function of the relative potency of each Nef variant.

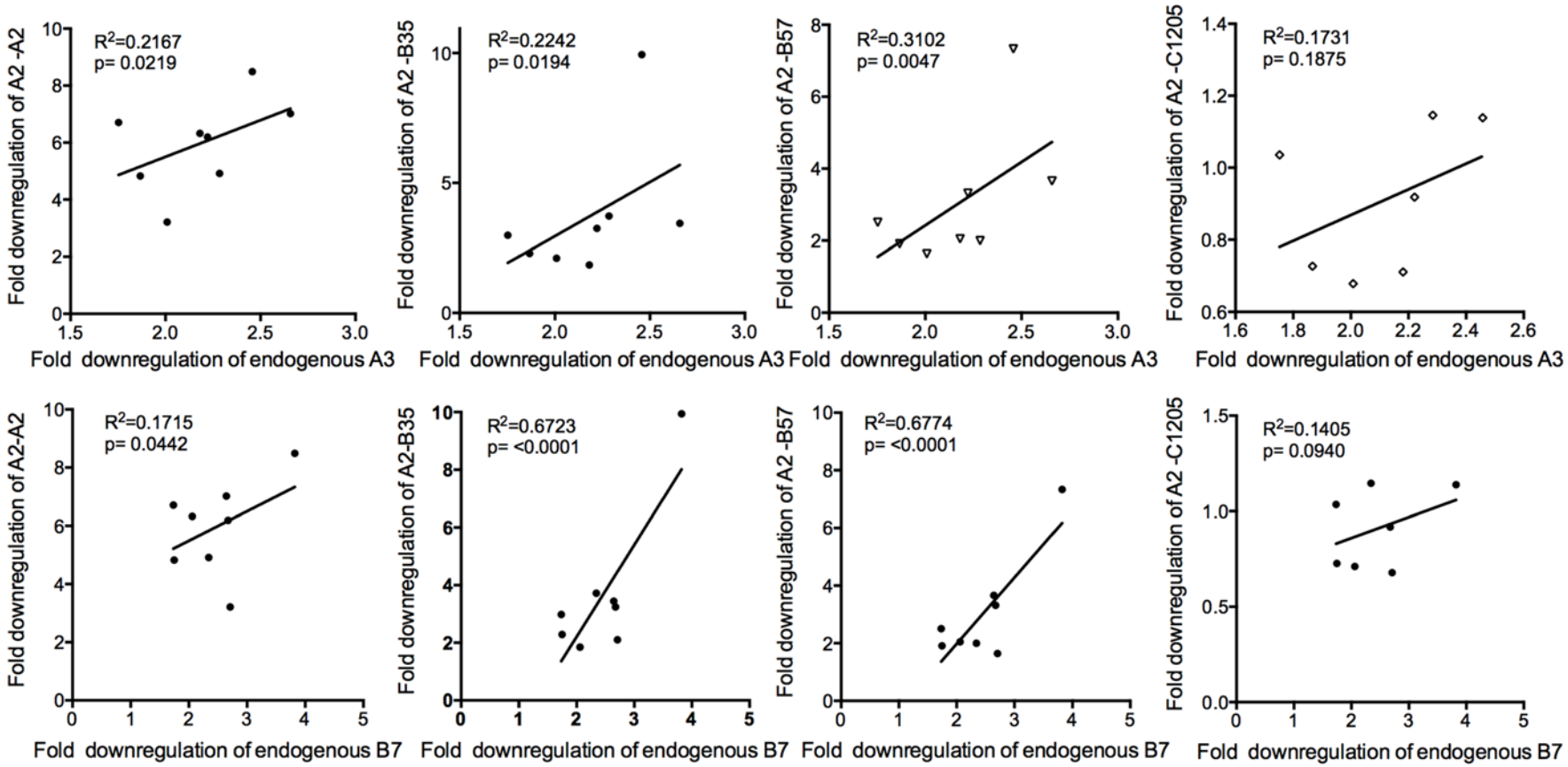


Figure 4.15: **Down-regulation of selected HLA-A2 chimera was compared to down-regulation of endogenous HLA-class I molecules and to other HLA-A2 chimera by linear regression analysis.** Comparison of endogenous A3 and B7 versus A2-A2, A2-B35, A2-B57 and A2-C1205 are shown in panels 1-8. Each data point is the mean fold down-regulation of three independent experiments.

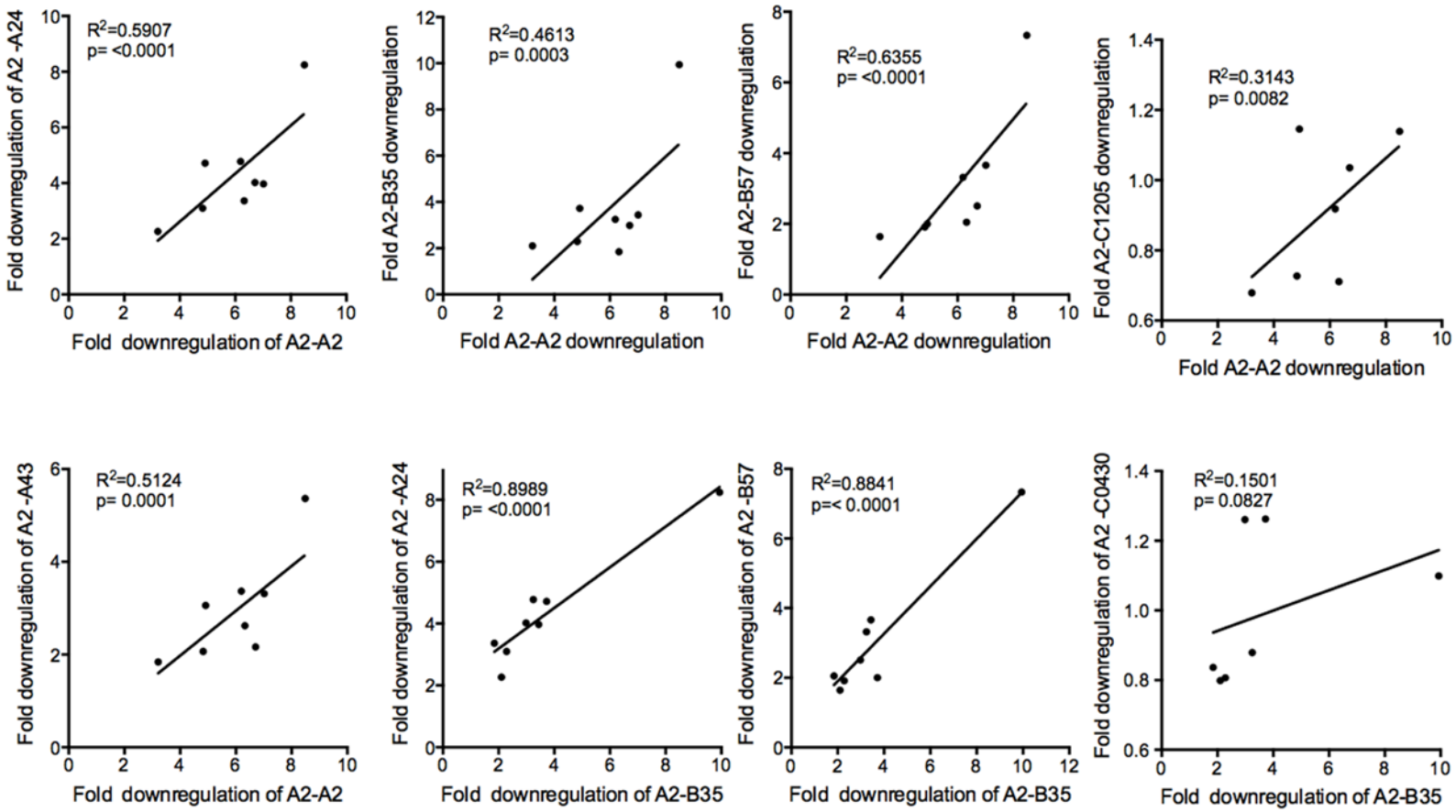


Figure 4.16: Down-regulation of selected HLA-A2 chimera was compared to down-regulation of endogenous HLA-class I molecules and to other HLA-A2 chimera by linear regression analysis. Comparison of endogenous A3 and B7 versus A2-A2, A2-B35, A2-B57 and A2-C1205 are shown in panels 1-8. Each data point is the mean fold down-regulation of three independent experiments.

4.3. Discussion

MHC-I down-regulation is an important immune evasion mechanism in primate lentiviruses that is mediated through its cytoplasmic domain. In humans, HLA-A and B alleles are down-regulated while HLA-C and –E are not. Mutation of tyrosine at 321 position in HLA-C (Cohen, Gandhi et al. 1999) and cytoplasmic tail truncation in HLA-E (Williams, Roeth et al. 2002) render these molecules resistant to Nef mediated down-modulation. This differential down-regulation, mainly mediated by accessory protein Nef, is important in the immune response to HIV. Down-regulation of HLA-A and B helps infected cells to evade recognition by CD8+ T cells (Collins, Chen et al. 1998). Perhaps to prevent NK cell mediated responses, that are activated when cells do not present MHC-I on their surface, HLA-C molecules have adapted to resist Nef mediated down-regulation (Cohen, Gandhi et al. 1999). The previous chapter examined the effect of the polymorphic cytoplasmic domain of HLA-I on down-regulation using two popular clade B virus *nef* alleles, SF2 and HIV-IIIB. Although, it is said that HLA-I down-regulation is preserved in all lentiviral infections (DeGottardi, Specht et al. 2008; Specht, DeGottardi et al. 2008), there is little information as to what extent this contributes towards immune evasion, where HIV-2 causes a more protracted disease course than HIV-1 and SIV causes asymptomatic disease in it's natural host. This chapter explores HLA-I allele down-regulation by HIV-1 and HIV-2 *nef* alleles. A simultaneous attempt has also been made to compare HLA-I down-regulation to Mamu-I in rhesus macaques.

The relationship between primates and lentiviruses is quite complex. SIVs have been infecting their primary hosts for a long period of time, possibly hundreds of thousands of years. Evolutionary origins and transmission patterns of primate lentiviruses demonstrate very close relationships between viruses from non-human primate species to one another, compared to viruses from another species. These 'host specific clusters' also appear to have

evolved in a 'host dependent' manner (Hahn, Shaw et al. 2000). This coevolution probably led to the less virulent nature of SIVs in their natural hosts. However, cross species transmission of these viruses (to unnatural hosts) has always resulted in pathology. As mentioned earlier, SIV_{smm} transmission from sooty mangabeys to rhesus macaques resulted in the animal model of AIDS (McClure, Anderson et al. 1990).

All primate lentiviruses (SIV, HIV-2 and HIV-1) have their own Nef protein counterparts and MHC-I down-regulation is preserved in this group of lentiviruses (Schindler, Munch et al. 2006; DeGottardi, Specht et al. 2008; Specht, DeGottardi et al. 2008). However, comparatively much less is known about the function of Nef in HIV-1 subgroups N, O and HIV-2. Most of the available data are limited to HIV-1 subgroup M, especially clade B viruses. Sequence comparison of the *nef* alleles of the HIV-I subgroups M, N and O showed that motifs known to play a role in HLA-I down-regulation are highly conserved in HIV-1 but not so much in HIV-2 and SIV. Not surprisingly, HIV-2 *nef* sequences were noted to be more similar to SIV.

It is interesting to see how MHC-I evolved through the species. Old world monkeys only have MHC-A and MHC-B alleles. MHC-C has arisen following recent duplication of MHC-B allele. HLA-A, B and C demonstrate a high degree of polymorphism in human population. These polymorphisms, determined by exon 2 and 3 are highest in HLA-B alleles. Although HLA-I allelic variability is very wide, for the purpose of this thesis we only consider the cytoplasmic domain sequences.

Humans have at least 7 HLA-A cytoplasmic alleles (Rajapaksa, Li et al. 2012) but rhesus macaques have no variation in the cytoplasmic domain of Mamu–A alleles (DeGottardi, Specht et al. 2008). However, rhesus macaques have at least 19 Mamu-B cytoplasmic alleles whereas humans only have two. It makes one wonder why such a big allelic variation occurs

between the two species. Allelic polymorphism has been shown to be absent in Mamu-B compared to HLA-B, in pedigree macaques (Otting, Heijmans et al. 2005). Rhesus macaques are able to diversify gene numbers and gene combinations to ensure that different individual mounts distinct responses for the same pathogen. Frequent crossing over processes in Mamu-A and Mamu B regions and differential expression helps in maximising and maintaining the diversity of the population (Otting, Heijmans et al. 2005). Cytoplasmic allele differences seen in Mamu-B alleles seem to be adding an additional layer of polymorphism to tackle pathogens just as in humans.

In the next chapter, we demonstrate that truncation of C terminal amino acids and replacement of two glycines at 315 and 316 enable HLA-B alleles to be more resistant to Nef. Sequence analysis showed that similar to HLA-B, Mamu-B is three amino acids shorter than HLA-A. Almost all Mamu-B alleles shared the two glycine residues at 315 and 316. Therefore, we expected HLA-B alleles to behave in a similar way to Mamu-B alleles as described before (DeGottardi, Specht et al. 2008). However, we noted that HLA-B was comparatively more resistant than Mamu-B in our experiments. This difference in Nef susceptibility could be due to polymorphic nature of Mamu-B cytoplasmic alleles that seems to have disappeared in HLA-B.

The results of this chapter indicate that susceptibility of HLA-A, B and C alleles in Jurkat cell lines to Nef were highly variable. HLA-A and B chimera were down-regulated by *nef* alleles with different efficiencies which seemed to depend on the *nef* allele sequence. Among the subgroup O alleles, MVP5180 had the lowest mean down-regulation of around 1.5 fold where as in MVP8161 it was about 7 fold. Similar variation was seen among HIV-2 *nef* alleles with CBL23 having as high as 8 fold average and 310319 having an average of 2.3 fold down-regulation. Altogether, this indicates that the efficiency of MHC-I down-regulation is Nef

dependant, an observation that has been noted previously (DeGottardi, Specht et al. 2008). As expected, HLA-C alleles resisted all Nef mediated down-regulation.

Complementing our previous findings (Rajapaksa, Li et al. 2012) HLA-A allele down-regulation by other HIV-1 groups and HIV-2 were found to be dependent on the cytoplasmic allele sequence. In general HLA-A*0201, A*2401 were more susceptible to Nef mediated down-regulation with A2-A43 being the least Nef susceptible HLA-A allele. HLA-A*43 is a rare allele found in south Africans (Martell, Du Toit et al. 1989) and A*43 cytoplasmic allele was shown to be less susceptible to HIV-1_{SF2}Nef and HIV-IIIB mediated down-regulation (Rajapaksa, Li et al. 2012). Sequence comparison of A2-A43 and A2-A2 shows the substitution of ^{322}serine with pheynylalanine in the Nef binding region. Secondary structure prediction programmes also demonstrate that ^{321}Tyrosine is buried from the surface of the protein thus potentially providing less access to kinases (discussed in chapter 5). Whether the resistance of this allele is mediated through these residues is not known and worth investigating.

Similar to A*43, Mamu-B*07 and B*19 share S322F. Indeed, it had been noted that in spite of majority of Mamu-B alleles being susceptible to Nef mediated down-regulation, Mamu-B*07, B*19 and B*29 were not down-regulated by SIV_{mac}239, SIV_{smm} FWr1, HIV-2 310319 and HIV NL4-3 (DeGottardi, Specht et al. 2008). Mamu-B*50, B*26, B*61, B*43 also share the same amino acid, but they have not been tested for their susceptibility to Nef. Although it was shown that the cytoplasmic truncation and ^{315}GG substitution in the HLA-B tail render it more resistant to Nef (discussed in the chapter 5) the above variant could be adding another layer of resistance.

Additionally, A2-A26 was also noted to be partially resistant to *nef* alleles from most HIV-1 and HIV-2 groups except for MVP8161. As discussed in chapter 3, this cytoplasmic tail is

shared by some of the protective alleles e.g. HLA-A*2501, A*7401. A2-A26 and A2-A43 both have a methionine instead of a valine at position 335. Whether this amino acid plays a role in Nef mediated HLA-I down-regulation has not yet been described. However, there was an exceptionally high level of down-regulation of A2-A26 allele with MVP8161, which again confirms that not only is variation HLA-I important, but also variation in Nef proteins for down-regulation.

Similar to HIV-1_{SF2}Nef and HIV-IIIB (Rajapaksa, Li et al. 2012), the cumulative down-regulation of HLA-B, compared to HLA-A was statistically significant ($p<0.05$) in HIV-1 group M and O, and in HIV-2 *nef* alleles, BEN, 310319 and 60415k. Careful observation of the elegant study of DeGottardi et al (DeGottardi, Specht et al. 2008) reveals that susceptibility of Mamu-B*01 to $SIV_{mac}239$ and HIV-2 310319 Nef mediated down-regulation was also partial. This was in agreement with our findings as Mamu-B*01 is the rhesus macaque counterpart of HLA-B. Preserving HLA-B on the surface appears to be an important phenomenon, which could be observed in Nef of lentiviruses that are infecting humans. Altogether, it is reasonable to speculate that lentiviruses that were transmitted to humans may have selected Nef alleles that allowed partial expression of HLA-B on the surface for survival advantages.

It has been demonstrated that subtle differences in HLA-I down-regulation mediated by Nef & cytoplasmic tail association have marked impact on CTL mediated antiviral efficacy (Rajapaksa, Li et al. 2012). This has been observed in SIV infection of rhesus macaques as well (Minang, Trivett et al. 2009). It would be interesting to assess the differential effect of HIV-2 Nef on CTL response as HIV-2 causes a more protracted disease in spite of sequence similarities. Since the literature on CD8 T cell responses and immune activation in HIV-2 is

limited, the implications of the differential down-regulation of HLA-I that I observed are not clear. However, this study provides first insight into HLA-I susceptibility to HIV-2 Nef.

Furthermore, we observed that HLA-B susceptibility to SIV_{mac} mediated down-regulation was lower than HLA-A, but this did not reach a statistically significant level ($p>0.05$). The variability of Mamu-B allele down-regulation was noted with SIV_{mac} Nef and the polymorphism of its' cytoplasmic tail provided an additional layer of immune control. Although it seems like humans have lost this advantage in HLA-B, it may have been compensated for by the polymorphism of HLA-A cytoplasmic tails. The most interesting observation is much higher resistance of HLA-B to HIV-2 Nef compared to Mamu-B alleles. This seems to have occurred concurrent to the loss of HLA-B cytoplasmic tail polymorphism. Perhaps this indicates a human adaptation to resist lentiviral infection, but needs more in-depth study. However, this also raises the question of reliability of SIVmac model for HIV.

The results obtained with the transfected HLA-A2 chimeric molecules were attempted to reproduce with native HLA-A and HLA-B alleles in naïve Jurkat cells. As explained in chapter 3, this type of experiment is difficult to extend because of the limited availability of HIV-I susceptible cell lines with HLA matched antibodies. However, it could be seen that results that were shown with HIV-1 were reproducible with most of the *nef* alleles.

An interesting observation with HLA-A3 was the more dramatic Nef mediated down-regulation with high levels of down-regulation achieved even at low expression of Nef. In contrast to this, HLA-B7 demonstrated a more gradual decline of surface expression, which correlated with the amount of intracellular Nef . These results were only observed when molecular clones were used. Whether this was due to the different experimental set up is unknown. Results obtained with HIV-1 subgroup O virus MVP5180/HUT and HIV-IIIB (described in chapter 3) certainly supports this hypothesis. MVP5180 and HIV-IIIB were all

intact viruses generated in HIV permissive cell line C8166. But, HIV-2, SIV and MVP8161 viruses that were used in this study were VSV pseudotyped (to achieve high infectivity rates) viruses, generated using pNL4-3 based proviral vector with the *nef* alleles of interest cloned into original *nef* position. Questions have been raised before about the physiological relevance of results achieved in *in vitro* studies with high Nef expression (Johnson and Desrosiers 2002; Swigut, Alexander et al. 2004). It might be worthwhile assessing whether the high Nef expression abrogates differential down-regulation and thereby leading to misinterpretation of Nef associated results, when similar kind of experimental setup is used.

One limitation of this study was not studying other circulating clade viruses and subgroup N, which would have given a complete picture. Subgroup N virus that was acquired from NIH AIDS reagent program could only be grown in PBMC and did not grow well in my hands thus I was unable to study its ability to down-regulate different HLA-I alleles. Molecular clones of clade A, C and D have now been received thus study of their effects are in the pipeline.

Altogether our results show that cytoplasmic tail dependent differential down-regulation of HLA-I alleles is preserved in all tested HIV *nef* alleles. Comparatively, HLA-B was found to be more resistant to Nef mediated down-regulation in both HIV-1 and HIV-2. But the difference between cumulative mean fold down-regulation of HLA-A and HLA-B appears to be less marked with HIV-2 *nef* compared to HIV-1. HLA-C resistance to Nef was preserved in all HIV types that were tested. Importantly, this study provides the first insight into HIV-2 Nef and HLA-I cytoplasmic tail association.

5. Understanding the mechanism of differential down-regulation of HLA class I cytoplasmic alleles by Nef

5.1. Introduction

The chapter 3 of this thesis demonstrated that Nef mediated down-regulation of HLA-I alleles was variable and the cytoplasmic tail played a significant role in this process. It was seen that HLA-B allele down-regulation was partial and played a significant role in maintaining CTL mediated control of HIV infection. Chapter 4 confirmed the previous findings by demonstrating that above characteristic appears to be well preserved in Nef of HIV-2 and the HIV-1 subgroup O. In this chapter, I attempt to identify trafficking signals responsible for partial down-regulation with special focus on determining HLA-B allele resistance.

HLA-I polypeptide chains are synthesised in the cytosol and assembled in the endoplasmic reticulum with the aid of a cascade of proteins (Murphy). They are transported to the cell surface with endogenous 8-10 amino acid long peptides in their peptide-binding groove that stabilizes the HLA-I molecule. The cytoplasmic domain of HLA-I contains 9 highly conserved serine phosphorylation sites (Guild and Strominger 1984). The spontaneous internalization of HLA-I requires presence of full-length cytoplasmic tail (Capps, Van Kampen et al. 1989; Vega and Strominger 1989). It was then hypothesised that MHC I internalization is regulated by phosphorylation (Capps, Van Kampen et al. 1989), a theory that was later questioned (Eichholtz, Vossebeld et al. 1992). However, the lysosomotropic drug Primaquine inhibits HLA-I phosphorylation and recycling to the cell surface (Capps and Zuniga 2000), suggesting that phosphorylation either regulates or reflects MHC I trafficking to and from the cell surface.

The mechanisms of HLA-I endocytosis and recycling were briefly described in chapter 1. It is understood that sorting signals located in the cytoplasmic tail of transmembrane proteins are recognized by adaptor protein (AP) complexes. The AP complexes are involved in formation of transport intermediates such as clathrin-coated pits and clathrin coated vesicles.

Involvement of AP-1, that targets transgolgi network and plasma membrane proteins to endosomes, is described in Nef's mechanism of HLA down-regulation (Wonderlich, Williams et al. 2008; Singh, Lau et al. 2009).

Sorting motifs in cytoplasmic tails can either be leucine based or tyrosine based (LΦ and YXXΦ, where X is any amino acid residue and Φ is an amino acid with a bulky hydrophobic chain i.e. L or I). The μ chain of the AP complex binds to the tyrosine based motifs (Ohno, Stewart et al. 1995; Owen and Evans 1998) and the ligand of leucine based signals are either the μ or β chains of AP-1 and AP-2 (Bremnes, Lauvrak et al. 1998).

Tyrosine 321 is conserved between HLA-A and HLA-B alleles but not in HLA-C and HLA-E, and has shown to be responsible for Nef susceptibility (Cohen, Gandhi et al. 1999). Both HLA-A and B contain a tyrosine based YSQA motif. Although this does not confer to the classical YXXΦ motif, tyrosine at this site can be considered as part of a degenerated YXXΦ motif (YSQA) and Nef discloses this motif to AP-1 that is otherwise cryptic (Le Gall, Buseyne et al. 2000). HLA-C has a leucine based motif LΦ which has been described to be responsible for its rapid internalization and low surface expression (Schaefer, Williams et al. 2008).

This chapter looks at the differences in the sequences of the consensus cytoplasmic domains of HLA-A and HLA-B molecules and tries to determine the trafficking signals that are responsible for the differential down-regulation observed. I propose a mechanism for the differential down-regulation by Nef using *in silico* and laboratory evidence.

5.2. Results

5.2.1. Internalization profile of chimera cell lines containing HLA-A and HLA-B allele cytoplasmic domain

The down-regulation of HLA-I alleles with Nef was thought to be due to the effects of both down-regulation and diversion of class I molecules to lysosomal degradation pathway, and also due to blocking the release of newly synthesised molecules from Golgi (Foster and Garcia 2008). We wanted to assess whether there was a difference in internalisation of chimeric HLA-A, HLA-B and HLA-C alleles. Consensus HLA-A allele that corresponds to A2, HLA-B which corresponds to B57, and HLA-C1205 were selected for these experiments.

We observed that at steady state the internalisation of HLA-B and HLA-C alleles were much more rapid than HLA-A allele at early time points (Figure 5.1 and 5.2) before converging after 30 minutes. Five minutes after starting the experiment, nearly 4 times more of A2-B57 compared to A2-A2 was internalised. However, in the presence HIV-1_{SF2}Nef, the down-regulation of HLA-A2 increases, but this was less pronounced with HLA-B and HLA-C alleles (Figure 5.3).

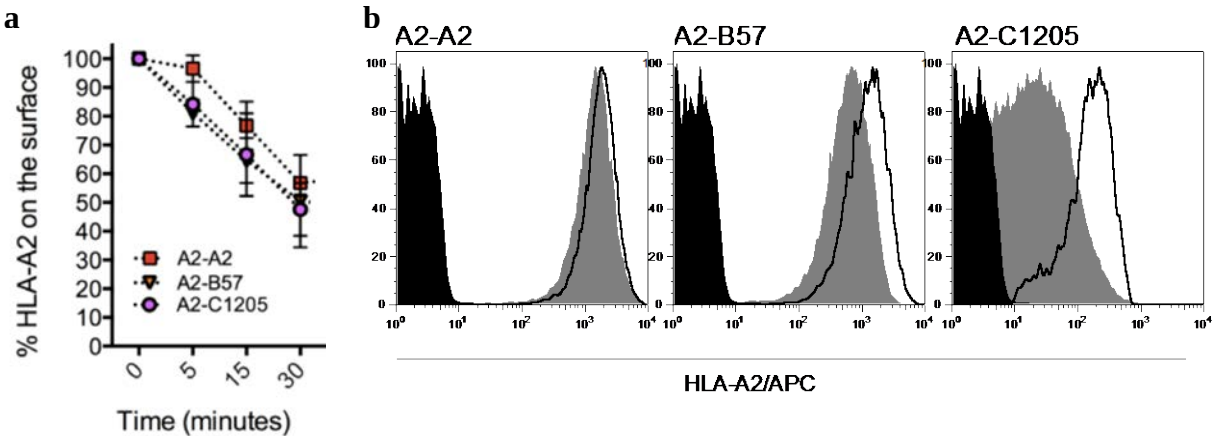


Figure 5.1: Internalisation of A2-B57 and A2-C1205 is higher than A2-A2 chimera at steady state. (a) Internalization profiles of HLA-A chimera. Each data point is summary of three independent experiments, Error bar=SEM. (b) Representative histogram showing the surface expression of HLA-A2 15 minutes post staining in untransected /uninfected chimeric cell lines. Black line-A2 expression at the commencement of experiment, solid ash - A2 expression 15 minutes later, solid black- unstained cells.

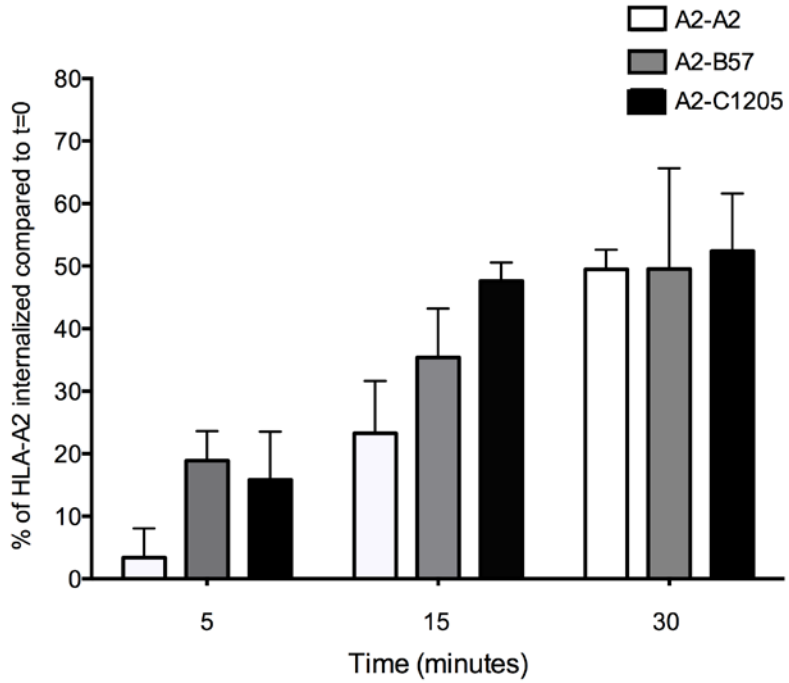


Figure 5.2: Comparison of internalisation profiles of selected A2 chimeric molecules at steady state. Each bar represents the mean of three independent experiments, Error bar=SEM.

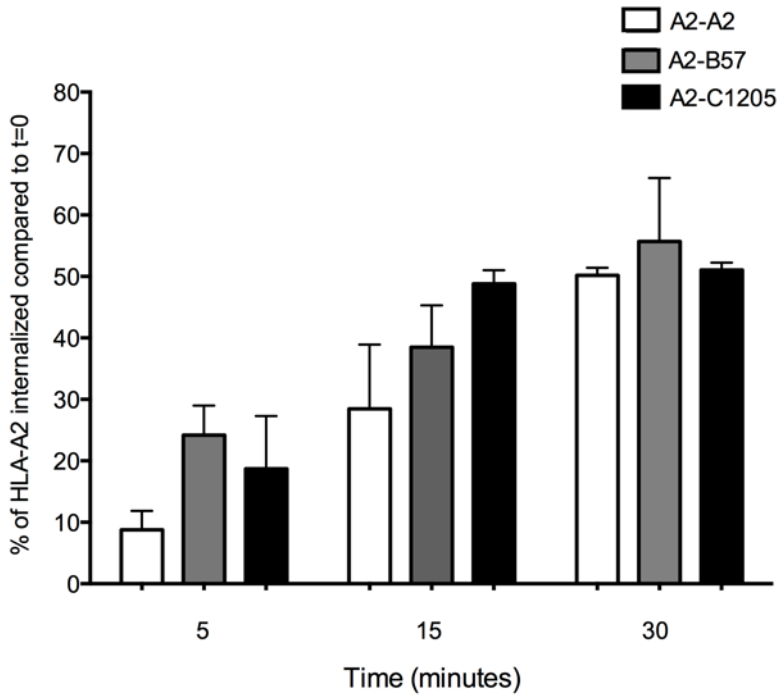


Figure 5.3: Comparison of internalisation profiles of selected A2 chimeric molecules in the presence of HIV-1_{SF2}Nef GFP. Each bar represents the mean of three independent experiments, Error bar=SEM.

5.2.2. Chimera containing HLA-B cytoplasmic tails are recycled differently to HLA-A

A potential secondary means of HLA-I down-regulation is endocytosis in the absence of recycling (Capps and Zuniga 2000). Nef redirects internalized HLA-I to lysosomal compartment for degradation and these have been found in late endosomal, and lysosomal compartments (Yi, Rosales et al.). Herein we have assessed the HLA-I recycling and the contribution of the cytoplasmic domain. Briefly, cells were incubated in cyclohexidine for 2-3 hour to inhibit neo-protein synthesis. Surface molecules were then stripped by washing with glycine buffer. This procedure prevents *de-novo* protein syntheses, and the surface molecules that are recovered and detected by flowcytometry will be considered as recycled molecules.

Surprisingly, limited recycling of HLA-A2 chimera compared to HLA-C1205 chimera was observed at steady state (Figure 5.4.a). Chimera containing the HLA-C1205 cytoplasmic allele was recycled to near pre-strip levels after 90 minutes (Figure 5.4.a). Recycling of A2 cytoplasmic tail was impaired in the presence of Nef (Figure 5.4.b). However, more HLA-C1205 chimera compared to A2 were recycled by 90 minutes. Interestingly, HLA-B57 allele chimera took a middle position where it recycled HLA-I molecules to near pre-strip level at both steady state and in Nef expressing cells.

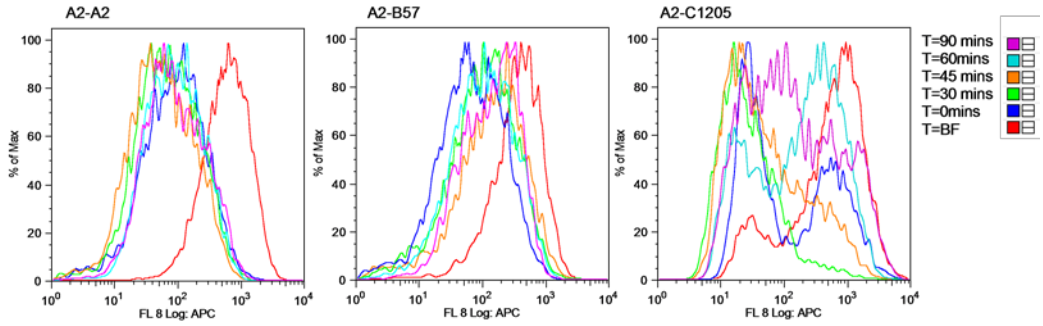


Figure 5.4.a: Recycling of HLA-A2 chimera at steady state. Representative histogram of two independent experiments. BF=Before stripping.

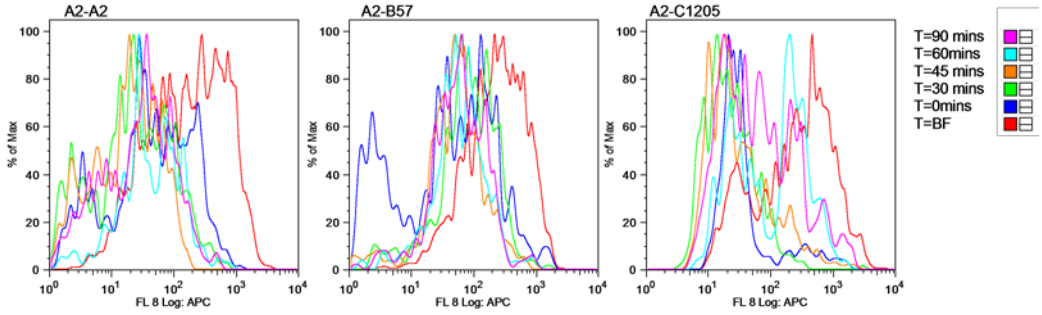


Figure 5.4.b: Recycling of HLA-A2 chimera in HIV-1 Nef_{SF2}GFP expressing cells. Representative histogram of two independent experiments. BF=Surface expression before stripping.

5.2.3. Identification of the trafficking signal in the HLA-B cytoplasmic domain that resists down-regulation by HIV-1_{SF2}Nef and HIV IIIB

The comparison of cytoplasmic domains of consensus HLA-A and B alleles revealed two major differences (substitution of two glycine residues at 315, 316 to aspartic acid and arginine plus a truncation of tail by three amino acids).

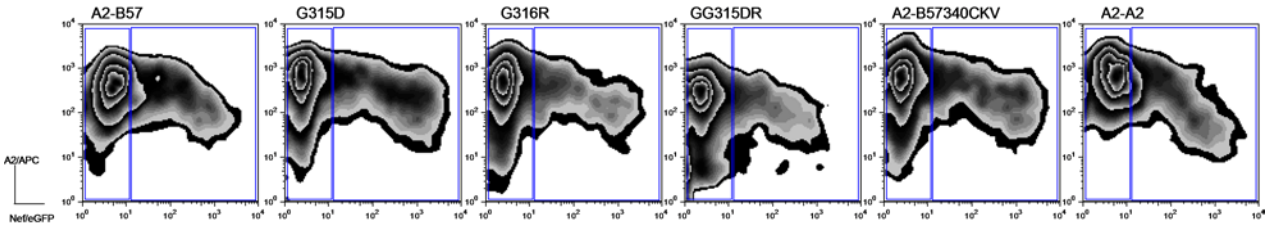
To investigate which amino acid/s is/are responsible for resistance to down-regulation mediated by HIV-1, we carried out site directed mutagenesis to the cytoplasmic domain of HLA-B so that with each mutation introduced to HLA-B, it gradually became a HLA-A (Figure 5.5).

Name	Amino acid sequence
A2-B57	^{310}RRKSSGGKGGSYSQAACSDSAQGSDVSLTA^{339}
G_{315}D	^{310}.....D.....^{339}
G_{316}R	^{310}......R.....^{339}
GG_{315}DR	^{310}.....DR.....^{339}
A2-B57$_{340}$CKV	^{310}.....CKV^{342}
A2-A2	^{310}.....DR.....S.....^{342}

Figure 5.5: Aligned transmembrane and cytoplasmic domain sequences of cell lines generated by employing site directed mutagenesis to HLA-B cytoplasmic isoform, A2-B57.

Figure 5.6 shows that G→D at position 315 makes the chimeric molecule more susceptible to HIV-1_{SF2}Nef mediated down-regulation as seen in G^{315}D and GG^{315}DR, supporting the hypothesis of the presence of ^{315}Glycine in the HLA-B renders it moderately resistant to HIV-1_{SF2}Nef mediated down-regulation (Figure 5.6). However, in HIV-IIIB infected cells the effect of this mutation seems to be less prominent and instead, having a slightly shorter cytoplasmic domain seems to render HLA- B resistant to HIV mediated down-regulation (Figure 5.7).

a



b

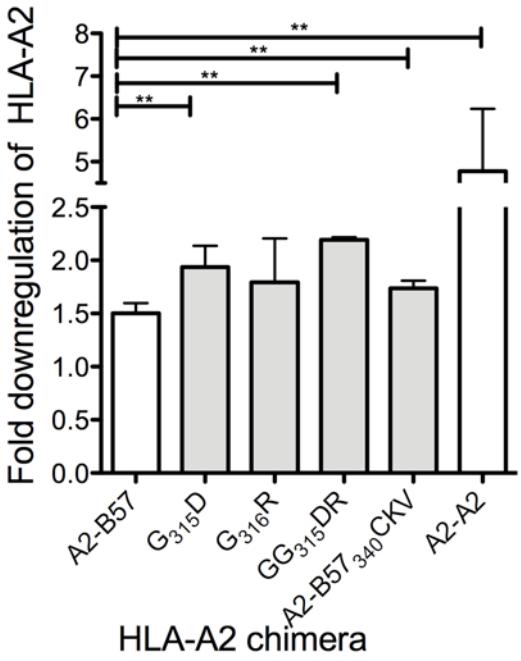


Figure 5.6: Differential down-regulation of mutant B alleles in the presence of HIV-1$_{SF2}$Nef (a) Representative flowcytometry plots (b) Magnitude of downregulation of mutated HLA-B alleles in the context of HIV-1$_{SF2}$Nef. Magnitude of HLA-A2 downregulation was measured 24 hours after electroporating cell lines either with plasmids expressing HIV-1$_{SF2}$Nef-GFP or HIV-1$_{G2A}$Nef/GFP (data not shown). The fold downregulation was calculated as in methods. Each bar represents the mean of four independent experiments+ SD, ** $p<0.05$, ***$p<0.0001$.

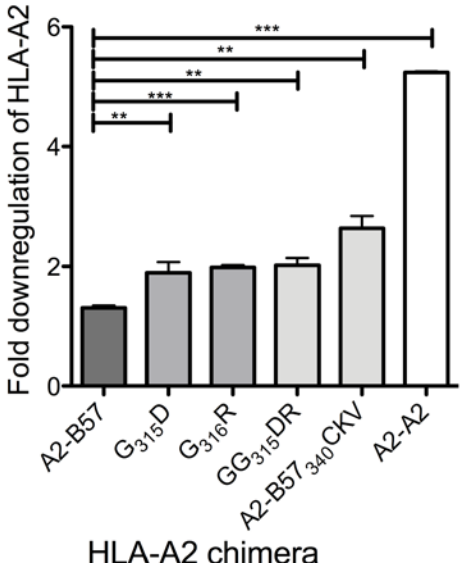


Figure 5.7: Differential down-regulation of mutant B alleles in the presence of HIV-IIIB virus infection. The fold down-regulation was calculated as in methods. Magnitude of HLA-A2 down-regulation was measured 6 days post infection. Each bar represents the mean of four independent experiments+ SD, ** $p<0.05$, ***$p<0.0001$.

5.2.4. Identification of possible phosphorylation sites

Post-translational modification could play a major role in trafficking and molecular conformity. Phosphorylation is one such modification that plays an important role in trafficking of transmembrane molecules. Phosphorylation of serine and tyrosine residues is imperative for HLA-I trafficking in the cells.

Here, using bioinformatics programmes, possible phosphorylation sites that could be mediating partial resistance to Nef, were predicted in the cytoplasmic domain of HLA-B. Six putative sites (Figure 5.8) were chosen as potential phosphorylation sites (Ser-5, Tyr-1) for both B57 and A2. Stringency of phosphorylation site predictors was increased as they have tendency to over-predict. Only those motifs that showed a NetPhos score of 0.5 or greater were selected to investigate further (Blom, Gammeltoft et al. 1999). These motifs were also analyzed using Scansite (Obenauer, Cantley et al. 2003).

It was observed that both HLA-B and HLA-C cytoplasmic domains lacked phosphorylation at ^{313}S and ^{314}S when analysed at medium stringency by Scansite (Obenauer, Cantley et al. 2003), although HLA-A, B and C shared these amino acids.

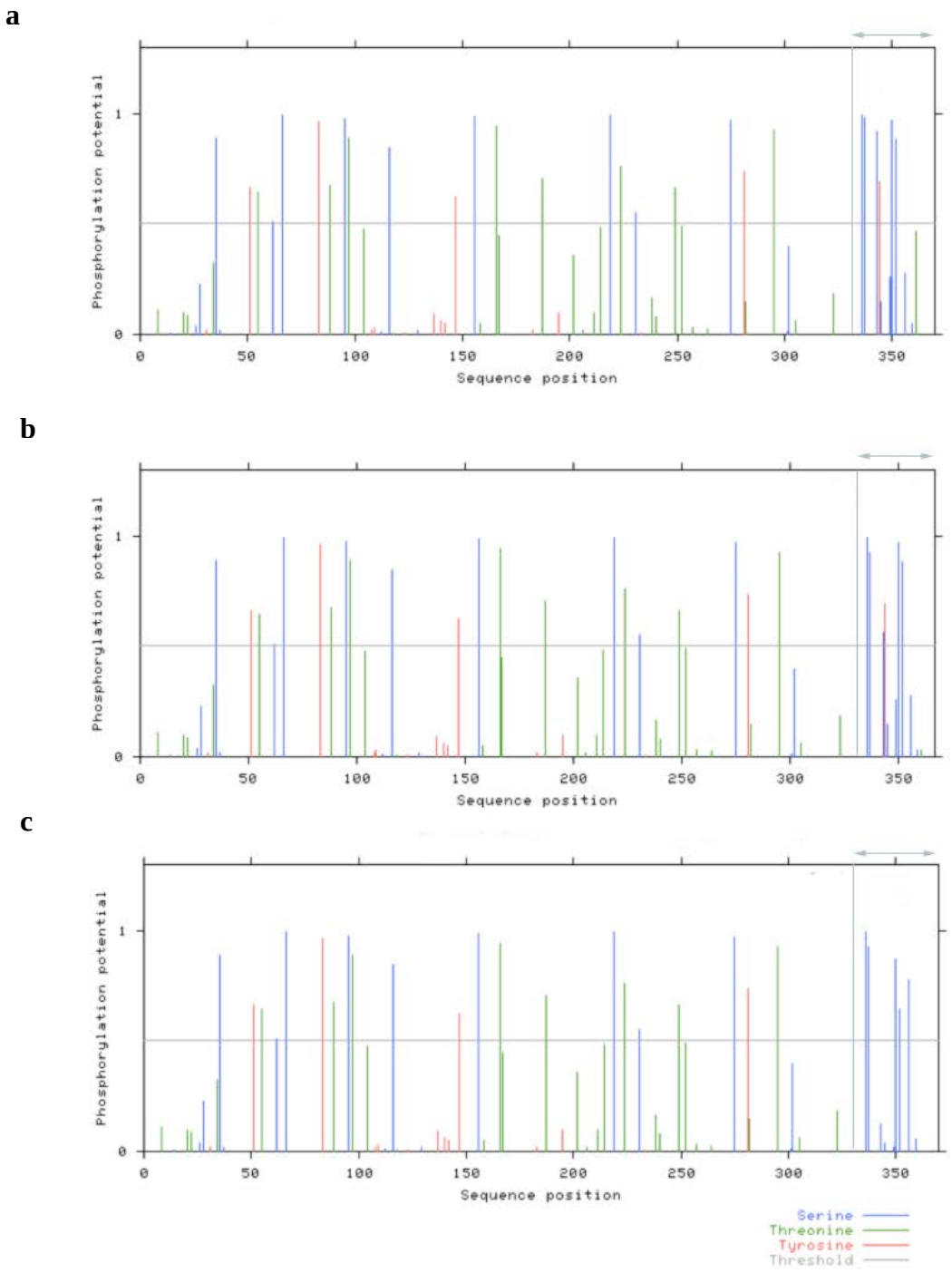


Figure 5.8: Phosphorylation sites predicted by NetPhos 2.0 programme for the chimeric A2 molecules; (a) A2-A2, (b) A2-B57 and (c) A2-C1205. The cytoplasmic domain is marked with a ←→

5.2.5. Surface accessibility of phospho amino acids

It is also imperative that the molecules that are to be phosphorylated to have surface accessibility (Kreegipuu, Blom et al. 1998). Both [313]serine and [314]S were implicated in the previous section as residues that might mediate resistance to Nef mediated down-regulation of –B57 cytoplasmic tail, were exposed on the surface of the molecule. A closer assessment was carried out to see whether other residues that are phosphorylated are buried from the surface using NetsurfP (Petersen, Petersen et al. 2009). Surprisingly, [321]tyrosine in the HLA-B cytoplasmic tail was predicted to be buried by NetSurfP compared to HLA–A2 which was exposed (Figure 5.9). HLA-C lacks phosphorylation at this site because it's cytoplasmic tail lacks [321]tyrosine. Although the predicted phosphorylation score for [322]serine was low, it was buried in HLA-C cytoplasmic tail compared to A and B.

Protein	Site	Amino acid	Region of phosphorylation site	NetPhos score[1]	Scansite score (percentile)- medium stringency[2]	NetSurfP[3]
B57 tail	313	S	AVMWRRK**S**SGGKGGS	0.993	0.4559(0.792%)	E
	314	S	RRKS**S**GGKG	0.927	Not predicted	E
	320	S	GKGG**S**YSQA	0.563	Not predicted	E
	327	S	QAAS**S**DSAQ	0.969	Not predicted	E
	329	S	ASSD**S**AQGS	0.883	Not predicted	E
	321	Y	KGGS**Y**SQAA	0.693	Not predicted	B
A2 tail	313	S	AVMWRRK**S**SDRKGGS	0.995	0.7195(0.969%)	E
	314	S	VMWRRKS**S**DRKGGSY	0.982	0.7113(0.881%)	E
	320	S	RKGG**S**YSQA	0.918	Not predicted	E
	327	S	QAAS**S**DSAQ	0.969	Not predicted	E
	329	S	ASSD**S**AQGS	0.883	Not predicted	E
	321	Y	KGGS**Y**SQAA	0.693	Not predicted	E
C1205tail	313	S	AVMWRRK**S**SGGKGGS	0.993	0.4559(0.792%)	E
	314	S	RRKS**S**GGKG	0.927	Not predicted	E
	320	S	GKGG**S**CSQA	0.121	Not predicted	E
	327	S	QAAS**S**DSAQ	0.869	Not predicted	E
	329	S	ASSN**S**AQGS	0.645	Not predicted	E
	333	S	SAQG**S**DESL	0.778	Not predicted	E

Figure 5.9: Summary of most likely predicted phosphorylation sites.
[1] (Blom, Gammeltoft et al. 1999)
[2] (Obenauer, Cantley et al. 2003)
[3] (Vega and Strominger 1989; Petersen, Petersen et al. 2009)

Site	Amino acid	Region of phosphorylation site	HLA-A2 Predicted kinases (Scansite score) at low stringency[1,2]	HLA-B35 Predicted kinases (Scansite score) at low stringency[1,2]	HLA-C1205 Predicted kinases (Scansite score) at low stringency[1,2]
313	S	AVMWRRKSSDRKGGS	Clk2 Kinase (0.7195) Calmodulin dependent kinase 2 (0.5290) AMP kinase (0.6581)	AMP kinase (0.6644)	AMP kinase (0.6644)
314	S	VMWRRKSSDRKGGSY	Clk2 Kinase (0.7113) PKC delta(0.5207) ProteinKinaseA (0.5285)	PKC α/β/γ (0.5471) ProteinKinaseA (0.5764) AkT Kinase (0.7329) AMP Kinase (0.7027)	PKC α/β/γ (0.5471) ProteinKinaseA (0.5764) AMP kinase (0.7107)
320	S	SSDRKGGSYSQAASS	PKC delta(0.5229) ProteinKinaseA (0.5432) Calmodulin dependent Kinase 2 (0.6130) PDK1binding(0.9812)	ProteinKinaseA (0.5858) PDK1binding(0.9671)	Not predicted
322	S	GGKGGSYSQAASSDS	DNAPK(0.6360) PDK1binding(0.9534)	DNAPK(0.5763) PDK1binding (0.9387)	Not predicted
333	S	SSNSAQGSDESLIAC	Not predicted	Not predicted	DNAPK(0.5257)

Figure 5.10: Interacting enzymes predicted by Scansite for representative HLA-I cytoplasmic tails. The score and sequence of the site are indicated. For domains with an entry in the Weizman Institute's GeneCard database (http://bioinformatics.weizmann.ac.il/cards), the name is listed.
[1] (Obenauer, Cantley et al. 2003)
[2] Only scores above 0.5 is shown.Highlighted are the sites predicted when scanned under medium stringency

A number of interacting enzymes were also predicted for phosphorylation sites in Scansite (Figure 5.10). Clk2 kinase was implicated in the phosphorylation of ^{313}serine and ^{314}serine in the A2 cytoplasmic tail, and needs further investigation.

5.2.5.1. Could a conformational change in 3D structure be responsible for differential susceptibility to Nef

We predicted a 3D structure using the I-TASSER server (Roy, Kucukural et al. 2010) for A2-A2 chimeric molecule and introduced point mutations that were present on HLA-B57 and C1205 tail to compare whether a conformational change could be assigned that could have consequences for the differential susceptibility to Nef (Figure 5.11). Indeed, the 3D predictions demonstrated a conformational change in the B57 with more compactly packed side chains of the amino acids in the cytoplasmic tail compared to A2. These changes probably allow less access of ^{321}tyrosine to kinases.

a



Figure 5.11: I-TASSER structural modelling for HLA-A2-A2 and A2-B57 chimeric molecules. (**a**) Overlapped structures of A2 and B57. (**b**) Chimera containing HLA-A2 cytoplasmic domain. Side chains of amino acids 313-316 are shown in yellow and side chains of rest of the amino acids constituting cytoplasmic tail are shown in Green. (**c**) Chimera containing HLA-B57 cytoplasmic domain. Side chains of amino acids 313-316 and ^{321}Tyrosine are shown in blue and side chains of rest of the amino acids constituting cytoplasmic tail are shown in Purple.

b



Figure 5.11: I-TASSER structural modelling for HLA-A2-A2 and A2-B57 chimeric molecules. (**a**) Overlapped structures of A2 and B57. (**b**) Chimera containing HLA-A2 cytoplasmic domain. Side chains of amino acids 313-316 are shown in yellow and side chains of rest of the amino acids constituting cytoplasmic tail are shown in Green. (**c**) Chimera containing HLA-B57 cytoplasmic domain. Side chains of amino acids 313-316 and ^{321}Tyrosine are shown in blue and side chains of rest of the amino acids constituting cytoplasmic tail are shown in Purple.

c

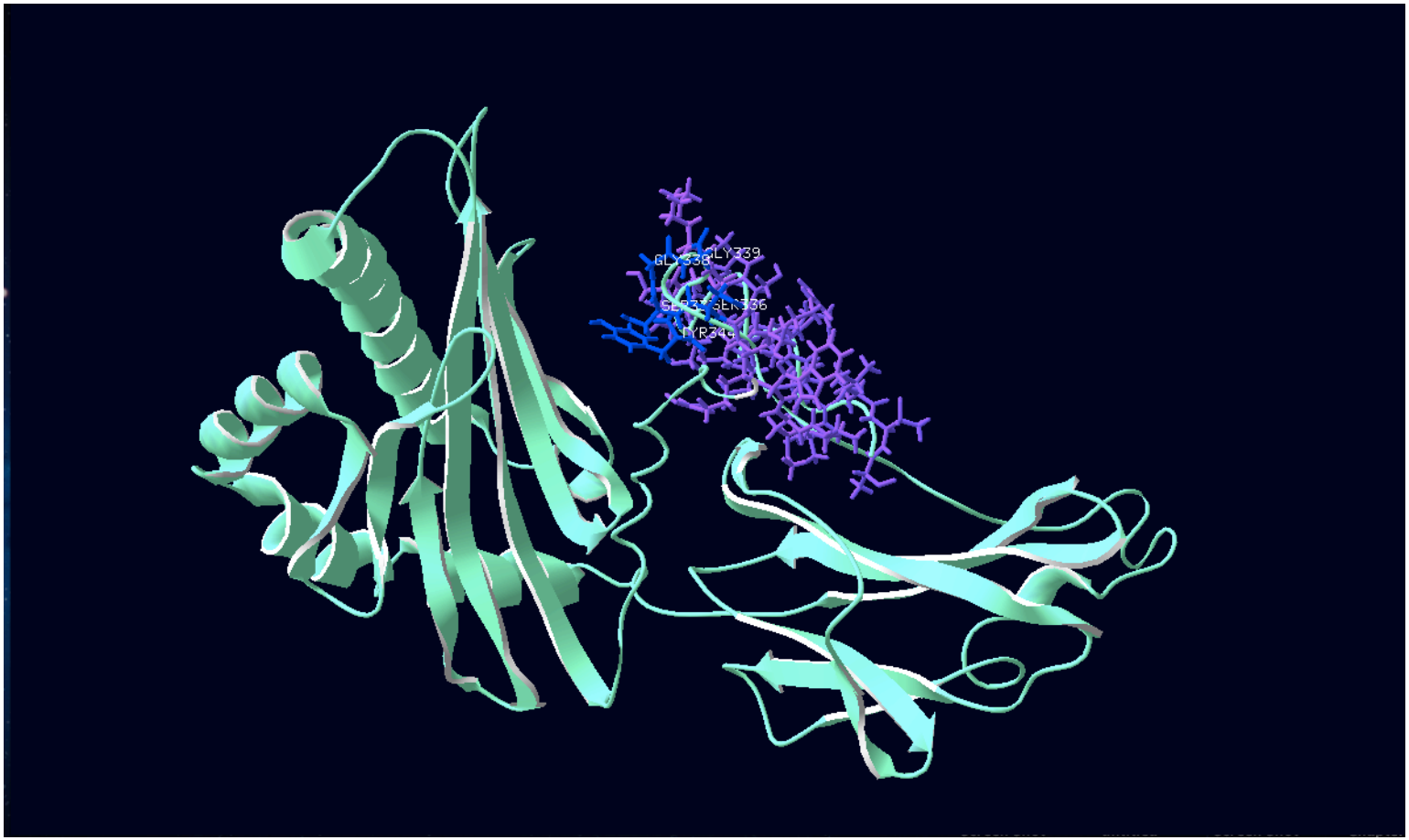


Figure 5.11: I-TASSER structural modelling for HLA-A2-A2 and A2-B57 chimeric molecules. (**a**) Overlapped structures of A2 and B57. (**b**) Chimera containing HLA-A2 cytoplasmic domain. Side chains of amino acids 313-316 are shown in yellow and side chains of rest of the amino acids constituting cytoplasmic tail are shown in Green. (**c**) Chimera containing HLA-B57 cytoplasmic domain. Side chains of amino acids 313-316 and ^{321}Tyrosine are shown in blue and side chains of rest of the amino acids constituting cytoplasmic tail are shown in Purple.

5.2.6. Comparison of HLA-A*02 and A*43

In addition to HLA-B, it was also observed that sensitivity of A43 cytoplasmic allele to Nef was much less with HIV-1 and HIV-2 (Chapter 3 and 4). A*4301 is an interesting allele that is found in the African population (Martell, Du Toit et al. 1989)and is shown to be having an intermediate protective effect in HIV-1 infection (Kiepiela, Leslie et al. 2004). Indeed, the ability of HIV specific CTL to recognise infected cells inversely correlates with sensitivity to Nef.

Sequence comparison of A2 and A43 revealed (Figure 3.1) two differences namely, S322F and V335M substitutions. Phosphorylation prediction programmes predict that $^{322}S \rightarrow ^{322}F$ substitution in A43 renders ^{320}S significantly less likely to be a site for phosphorylation. An additional threonine phosphorylation site was predicted, while the phosphorylation score of ^{321}Y was lowered (Figure 5.12 and 5.13). In addition, Scansite does not predict phosphorylation of ^{321}Y of A43, probably because it appears to be buried from the surface, therefore hidden from the kinases.

These results highly suggest that phosphorylation of ^{321}Y is crucial for susceptibility to Nef, and that the changes in amino acid sequences in the adjoining region that possibly alter the secondary structure, results in changes to the phosphorylation status of the allele leading to an altered Nef susceptibility.

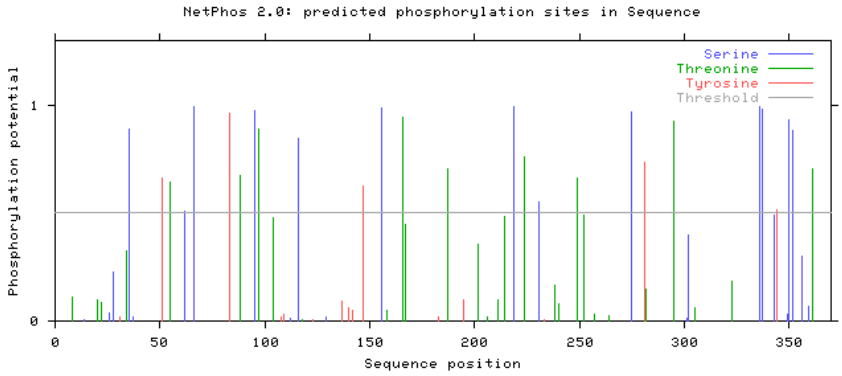


Figure 5.12: Phosphorylation prediction sites with NetPhos 2.0(Blom, Gammeltoft et al. 1999)

Protein	Site	Amino acid	Region of phosphorylation site	NetPhos score[1]	Scansite score (percentile)- medium stringency[2]	NetSurfP[3]
A43 tail	313	S	AVMWRRK**S**SDRKGGS	0.995	0.7195(0.969%)	E
	314	S	VMWRRKS**S**DRKGGSY	0.982	0.7113(0.881%)	E
	320	S	RKGG**S**YSQA	0.491		E
	327	S	QAAS**S**DSAQ	0.932		E
	329	S	ASSD**S**AQGS	0.883		E
	321	Y	KGGS**Y**SQAA	0.516		B
	338	T	DMSL**T**ACKV	0.708		E

Figure 5.13: **Summary of most likely predicted phosphorylation sites**. Highlighted are the differences compared to A2 cytoplasmic sequence.

[1] (Blom, Gammeltoft et al. 1999)
[2] (Obenauer, Cantley et al. 2003)
[3] (Petersen, Petersen et al. 2009)

5.3. Discussion

In this chapter we propose a model for resistance of HLA-B alleles to Nef using *in silico* and wet lab methods. In our attempts to decipher the mechanisms of differential susceptibility of HLA-I alleles to Nef, we first compared the cytoplasmic domains of HLA-A and –B as we have shown a clear difference in Nef mediated down-regulation, which reached statistically significant levels (Rajapaksa, Li et al. 2012). Indeed the cytoplasmic domain plays the main role in expression of HLA-I on the surface of the cells (Davis, Reyburn et al. 1997) best evidenced by high expression of HLA-A and B in contrast to low expression of HLA-C that is solely dependent on cytoplasmic tail (discussed in Chapter 3).

It was evident that differences in cytoplasmic domain resulted in differential susceptibility to HIV Nef. Nef is said to be responsible for MHC-I endocytosis and rerouting of internalised MHC-I towards a degradation pathway. This led us to assess the internalisation profiles of representative HLA-A and B cytoplasmic alleles. Indeed, the internalisation profile of A2 was very different to B35 and B57 (B35 acted in a similar manner to B57, data not shown). At steady state both B alleles were rapidly internalised and acted in a similar pattern to the HLA-C1205 cytoplasmic allele. The differences between internalisation of A and B have been observed before, except that previous research looked at the whole HLA-B allele not just cytoplasmic tail (Gruda, Achdout et al. 2007). Our results confirm that cytoplasmic tail on its' own could mediate the differential internalisation. Similarity between HLA-C and HLA-B somewhat supports the speculation of common origins/ recent duplication (Boyson, Shufflebotham et al. 1996). Interestingly, in the presence of Nef, there was a higher degree of A2 down-regulation compared to B57 and implicated a slight decrease in sensitivity of B57 to Nef mediated internalisation. This finding was similar to HLA-C results.

Internalisation plays a big role in Nef mediated HLA-I down-regulation. The determinants of internalisation are contained in the cytoplasmic tail of MHC-I (Le Gall, Erdtmann et al. 1998). Cohen et al observed three residues that are important for HLA-I down-regulation; ^{325}alanine, ^{328}aspartate and ^{321}tyrosine would lie on the same face if they were displayed as an alpha helix, implicating a potential surface for Nef interaction (Cohen, Gandhi et al. 1999). Although YXXΦ forms a cryptic sorting motif, Nef facilitates HLA-I binding to AP-1 (Le Gall, Erdtmann et al. 1998).

On closer observation it could be seen that the HLA-I cytoplasmic tail is largely conserved and rich in sites for phosphorylation, with 9 serine residues, one threonine and one tyrosine. Indeed, HLA antigens are phosphorylated at these residues with different kinases (Guild, Erikson et al. 1983; Guild and Strominger 1984). Phosphorylation undoubtedly controls HLA-I trafficking as shown by altered recycling in the presence of Primaquine, an antimalarial that inhibits phosphorylation (Capps and Zuniga 2000). This prompted us to assess the contribution of phosphorylation sites in the cytoplasmic tail towards trafficking.

Post-translational modification prediction programmes predicted extra phosphorylation sites for A2 cytoplasmic domain at positions ^{313}S and ^{314} S compared to B35/B57/C1205 cytoplasmic domains. Although these serine residues are common to A, B and C alleles, additional phosphorylation sites mediated either by Protein kinase C delta or Clk2 Kinase are predicted only for HLA-A2. Indeed, in as far back as 1984, Guild, et al demonstrated that HLA-B cytoplasmic tail lacked phosphorylation sites compared to HLA-A (Guild and Strominger 1984). They demonstrated that B7 allele was comparatively less phosphorylated than A2 by cAMP dependent protein kinase, but at that time did not identify the functional significance.

Comparison of HLA-A and HLA-B allele cytoplasmic domain sequences revealed two major differences: a truncated cytoplasmic domain and substitution of ^{315}Aspartate and ^{316}Arginine with two glycine residues. Our attempts to gradually mutate -B57 cytoplasmic domain to become similar to –A2 clearly demonstrated that both these motifs play a role in increased down-regulation of A2. The substitution of ^{315}DR→GG might be responsible for the hypophosphorylation of HLA-B*07 compared to HLA-A*02 that Guild, et al speculated (Guild and Strominger 1984).

Interestingly, *in silico* experiments revealed that in addition to absence of phosphorylation of ^{314}serine, the important ^{321}tyrosine is not exposed on the surface of the protein. It has been described that kinase specific sites needed to be accessible from the surface (Kreegipuu, Blom et al. 1998). Mutation of tyrosine is known to abrogate susceptibility to Nef (Cohen, Gandhi et al. 1999). Thus we propose that a hidden tyrosine prevents potential phosphorylation and interaction with AP-1 that is essential for rerouting HLA-I.

Furthermore, it is said that inhibition of tyrosine phosphorylation results in an enhanced shedding and a slight down-regulation of cell surface folded MHC-I by unknown means (Santos, Antoniou et al. 2006). This means that if an allele is defective in tyrosine phosphorylation, Nef activity may be masked by constitutively depressed levels of cell surface MHC.

Taken together, herein we propose a model (Figure 5.14) that would explain the partial resistance of HLA-B cytoplasmic tail to Nef mediated down-regulation. We propose that substitution of GG for DR at positions 315 and 316 leads to a conformational change in the secondary/tertiary structure of HLA-B cytoplasmic domain. Subsequently, potential phosphorylation at ^{313}S and ^{314}S are either reduced or prevented. The conformational change also results in 'burrowing' ^{321}tyrosine from the surface of the protein. Exposed ^{321}Y is

probably needed for constitutive phosphorylation and for the degenerative 'YxxΦ' motif to function as an internalisation signal, and possibly a buried tyrosine residue can no longer act as a part of the sorting signal for AP-1. Furthermore, ^{321}Y, ^{325}A and ^{328}D are needed to lie on the same face of alpha helical structure for Nef, HLA-I binding. Conformational changes probably prevent one or all of these, ultimately resulting in perturbed HLA-B trafficking. We propose that this lack of interaction between AP-1 and cytoplasmic tail might be responsible for HLA-B resistance to Nef mediated down-regulation.

HLA-C is known for it's resistance to Nef and studies have pinpointed ^{321}Y to be a major determinant. In *silico* experiments reveal that protein phosphorylation sites of HLA-B and C are very similar. Thus, we speculate that HLA-B acts in a very similar manner to HLA-C, however, the partial resistance to Nef in HLA-B compared to complete resistance in C is probably due to presence of the hidden Y whose function is not fully abolished.

Indeed, our results confirm that substitution of GG for DR and truncation in combination leads to resistance of down-regulation of HLA-B alleles, although only part of this mechanism is explored. Further investigation looking into details including mutations to mimic phosphorylation and some other missing mutants are being carried out. Additionally, the proposed lack of interaction between AP-1 and the HLA-I cytoplasmic tail that might be responsible for HLA-B resistance to Nef mediated down-regulation, could be confirmed by dephosphorylating these sites and analyzing its effects on CLk2 kinase binding and inhibition.

There is limited literature on MHC-I recycling (Capps and Zuniga 2000; Basha, Lizee et al. 2008) but clear involvement of phosphorylation has been documented. Primaquine, inhibits both MHC-I phosphorylation and its recycling to the cell surface, suggesting that phosphorylation is related to MHC-I recycling, but whether it's a causative or an incidental association has not been established (Capps and Zuniga 2000). It has been shown that MHC-I

chimera containing mutation of tyrosine failed to colocalise with early/late endosomal or lysosomal compartments (Basha, Lizee et al. 2008). This is compatible with our observations in recycling experiments. The resistance of HLA-C1205 tail to traffic through lysosomal degradation pathway might be resulting in its resistance to down-regulation in the presence of Nef.

It was also interesting to note that amino acid substitution S322F in A43, resulted in adjacent ^{321}tyrosine being not exposed on the surface of protein secondary structure and ^{320}serine less susceptible to phosphorylate. Conformational changes that resulted following amino acid substitution clearly affected secondary structure formation and similar to HLA-B cytoplasmic alleles resulted in partial resistance to Nef. The amino acid substitutions also seem to have exposed ^{338}threonine to phosphorylation. This implies that point mutations in the tyrosine region could affect secondary structure i.e. substitution of bulky charged side chain for a small polar side chain, and have consequences for the susceptibility to Nef. Although, Nef is a substrate for protein kinase C, the contribution of this on differential down-regulation is not explained.

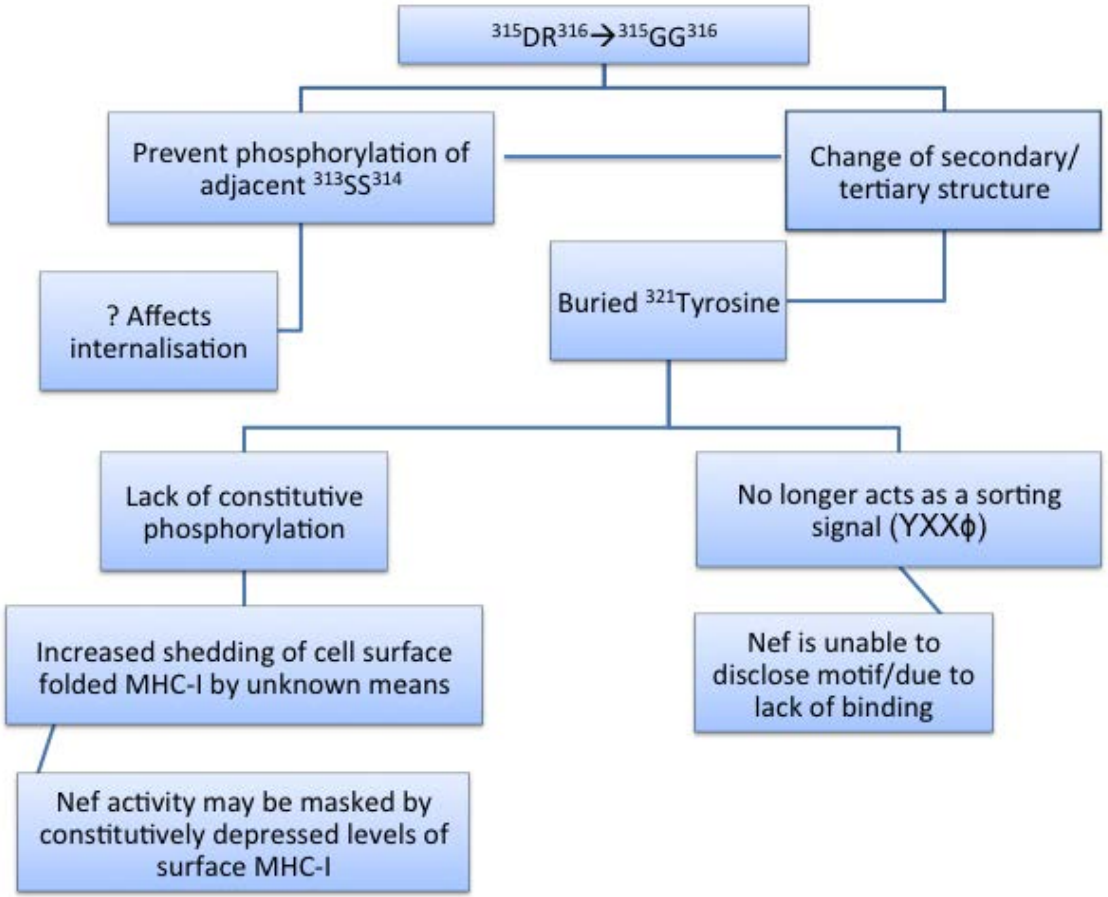


Figure 5.14: **Schematic of proposed model for mechanism mediating partial resistance of HLA-B cytoplasmic allele to Nef.**

Validation of our results and proposed mechanism may be provided by comparative Nef binding assays, feasible either by comparative immunoprecipitation or surface plasmon resonance. This would determine whether binding of Nef to MHC-I cytoplasmic tails and recruitment of AP-1 is in fact perturbed, and whether this is responsible for resistance to down-regulation that was seen with HLA-B cytoplasmic allele and also A43.

The implications of this study relate both to the clinical significance of Nef-HLA-I interaction as detailed in chapter 1 and to mechanisms of Nef perturbed and constitutive HLA-I trafficking. As HIV adapts to the HLA of population, the finding that HLA-B cytoplasmic tails are less susceptible to Nef was not expected. Underpinning molecular reasons for this phenomenon is highly relevant to understand the relationship between HLA-I trafficking, especially HLA-B and Nef. Especially the differences that were seen between –A and B, and between A2 and A43 warrant further investigation.

5.3.1. CD4 and Nef

5.4. Introduction

HIV pathogenesis is brought about by mainly targeting the CD4+ T cells in the immune system. CD4 glycoprotein serves as the main entry molecule of target cells and is down-regulated during the course of infection. The down-regulation of CD4 is believed to be important for increasing viral pathogenicity by increasing infectivity and immune deficiency (Foster and Garcia 2008). Although there are multitudes of research looking at the mechanism of CD4 down-regulation and its functional significance in HIV infection, the exact role of this mechanism is not completely understood, neither has been used for effective control of virus replication, control etc.

As discussed in chapter 1, the first step of HIV and SIV infection involves an interaction between gp120 and CD4, which facilitates binding of other co-receptors that catalyse the fusion reaction between the host cell membrane and the viral envelop and subsequent internalisation.

During the course of HIV-1 infection, the number of CD4+ cells in the host decline. Details of CD4 trafficking at cellular level in acute infection have come from macaque - SIV model. It has been noted that between 30-60% of gut associated CD4+ T cells become infected during acute infection and most of them disappear within few days. With the loss of most of memory CD4+ T cells, the body is left with a severely debilitated repertoire of CD4+ T helper cells.

An inverse relationship between HIV transmembrane glycoprotein precursor gp160 production and surface expression of CD4 suggesting intracellular complexes between gp160 and CD4 was first noted in 1992 (Crise, Buonocore et al. 1990; Marshall, Diamond et al. 1992). It was also shown that HIV infection resulted in both reduced CD4 mRNA levels and amounts of CD4 protein that could be precipitated (Hoxie, Alpers et al. 1986). Later, three

proteins of HIV genome, Env, Vpu and Nef were postulated to be responsible for this down-regulation at different cellular levels indicating that this down-regulatory activity is crucial for viral survival in host cells (Piguet, Schwartz et al. 1999). The newly synthesized CD4 molecules are retained in the endoplasmic reticulum (ER) by envelope protein of HIV-1 and SIV (Malim and Emerman 2008). Vpu leads to rapid degradation of the molecules that are retained in ER (Malim and Emerman 2008). Nef, accessory protein leads to down-regulation of CD4 from the surface of cells.

5.4.1. CD4 internalisation

Despite understanding the mechanism of HIV induced CD4 down-modulation (mediated by Nef, Vpu and Env), as mentioned before, the functional significance of this phenomenon is not completely understood (Lama, Mangasarian et al. 1999). High expression of surface CD4 has been shown to effectively block cell to cell transmission of virions in culture although it did not protect cells from exogenously added viruses (Marshall, Diamond et al. 1992). Lower levels of surface CD4 expression prevented multiple super infection (Benson, Sanfridson et al. 1993). It is thought that premature death of infected cells is prevented by CD4 down-regulation.

Infection with HIV culminates in release of progeny virions by budding. The budding virion accumulates a coating of cell membrane that does not contain any CD4. Presence of CD4 on cell membrane could interrupt the virion budding by possibly binding to gp120. It is thought that in analogy with influenza virus neuraminidase, CD4 down-regulation prevents trapping and aggregation of outgoing virions on the surface of producer cells (Lama, Mangasarian et

al. 1999). However, it was recently shown that Nef mediated CD4 down-regulation is dispensable for functionality and optimal output of viral particles (Neri, Giolo et al. 2011).

It is also thought that CD4 down-regulation could also regulate T cell activation pathways to favour viral gene expression for instance by increasing intracellular Lck Tyrosine kinase as seen in other systems.

5.4.2. Mechanism of Nef mediated CD4 down-regulation

Nef, one of the early-encoded proteins of HIV, is an important mediator of CD4 down-regulation. The CD4 down-regulation occurs in a sequential manner with Nef acting as a receptor specific sorting adaptor. The Nef effect is exerted at a posttranslational level and the integration of HIV genome into hosts' is not essential for this function (Gillim-Ross, Cara et al. 2005).

The Nef of HIV-I associates with a region in the cytoplasmic domain of CD4 molecule that includes the membrane proximal 20 amino acids plus the dileucine motif (Aiken, Konner et al. 1994; Grzesiek, Stahl et al. 1996). Direct binding of Nef to this small region of CD4 was first demonstrated through Nuclear Magnetic Resonance imaging showing that a pocket formed by non-contiguous amino acids WLE^{59}, GLL^{97}, ^{106}R and ^{110}L bound to a peptide corresponding to amino acids of CD4 tail (QIKRLLSEKKT) although with low affinity (Grzesiek, Stahl et al. 1996). The significance of this binding however, is questionable as the association of CD4 and $p56^{lck}$ is strong and $p56^{lck}$ stabilizes CD4 on the surface of cells and prevents rapid internalisation (Pelchen-Matthews, da Silva et al. 1998).

In general, CD4 internalisation can occur in a serine dependent (as in phorbol ester) or in an independent manner (as in gangliosides) (Geleziunas, Bour et al. 1994). Nef mediated CD4 down-regulation occurs in a different manner to phorbol ester and does not require phosphorylation of the cytoplasmic domain (Garcia and Miller 1991). Nef stimulates CD4 internalization by dissociating CD4 from $p56^{lck}$ (Src like tyrosine kinase) that binds CD4 cytoplasmic tail that normally inhibits CD4 endocytosis. Site directed mutagenesis studies have revealed two closely associated cysteine residues in CD4 cytoplasmic tail at 420 and 422 (Position 445 and 447 in GenBank Accession sequence AAB51309.1) positions and two cysteine in $p56^{lck}$ Nh2 terminus (positions 20 and 23) that are essential for CD4 –$p56^{lck}$ binding. Although Nef induces accumulation of Lck (Thoulouze, Sol-Foulon et al. 2006) in endosomes this does not appear to be related to CD4 down-regulation as it has been demonstrated that L164A/L165A mutant Nef failed to down-regulate CD4 in spite of rearranging cellular localization of Lck (Craig, Pandori et al. 1998; Thoulouze, Sol-Foulon et al. 2006).

Interaction between Nef and clathrin associated AP-2 is well described. AP-2 (α/σ2 subunits) binds to the dileucine motif in Nef (Foster and Garcia 2008). This dileucine motif exhibits a canonical ^{160}EXXXLL^{165} sequence and also requires two acidic residues within the loop, ^{174}(E/D)D^{175} for efficient Nef binding. In the absence of diacidic residues there is only weak binding of the dileucine motif, which is peculiar to Nef (Lindwasser, Smith et al. 2008). AP-2 binding facilitates formation of clathrin coated pits that are internalised.

Cystein residues that are necessary for $p56^{lck}$ are found in the same regions as the membrane proximal dileucine motif of CD4. Although they functions as internalisation signals, it has been demonstrated that Cystein residues are dispensable for Nef mediated CD4 down-regulation but not the dileucine motif (Aiken, Konner et al. 1994).

In the endosome Nef connects CD4 with β subunit of COP-I coatomers, which then targets CD4 for lysosomal degradation. GTPase Arf1 has also been shown to be involved in CD4 lysosomal targeting through formation of a complex (Faure, Stalder et al. 2004).

Recently it was noted that C terminal $M_{168}L_{170}$ are also important in AP-2 mediated binding of Nef (Jin, Cai et al. 2012). This was in addition to regions that were described before e.g. WL^{58}, $MGxxxS^{6}$, LL^{165} and DD^{175} (Geyer, Fackler et al. 2001). In addition, a strong Nef induced reduction of the half-life with an anterograde transport of nascent CD4 molecule was demonstrated in transfected epithelial cell lines (Rose, Janvier et al. 2005).

Interestingly, it has also been shown that soluble CD4 (sCD4) can neutralise most HIV-1 strains (Daar, Li et al. 1990), although at lower concentrations it can lead to modest increase of infectivity in some strains (Sullivan, Sun et al. 1998).

Here, we postulate that if the CD4 molecule is truncated to an extent where it could still be expressed on the surface but not bind Nef, that these cells could be infected with HIV yet no down-regulation of CD4 occurs. CD4 is expected to form complexes with gp120 and interfere with the budding of the virus, and subsequent reduction in viral release. This led to a further hypothesis that in the absence of CD4 down-regulation perhaps the viruses that are produced might incorporate CD4 on the surface of infected cells and could be defective as envelope interference is expected. ‘Structurally and functionally different virus’ might be defective in terms of infection and replication. However, this model is yet to be supported by evidence and might explain the functional significance of Nef mediated CD4 down-regulation in HIV.

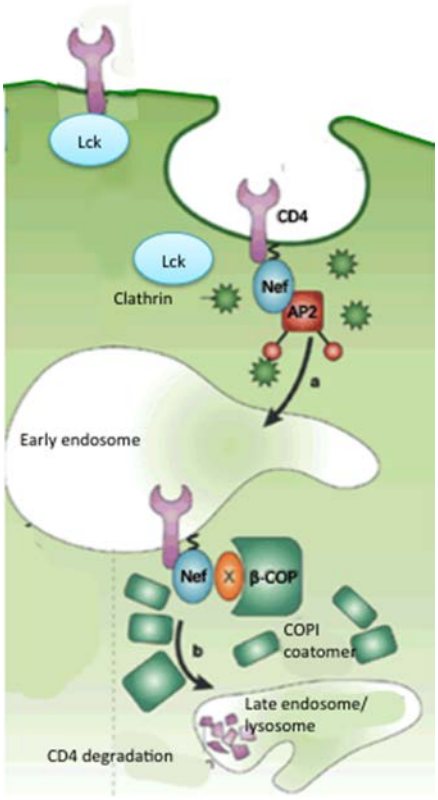


Figure 6.1: The steps of Nef-induced CD4 down-modulation. At the plasma membrane, Nef connects the cytoplasmic tail of CD4 with clathrin-coated pits after displacing Lck through an interaction with adaptor protein 2 and the vacuolar ATPase (v-ATPase), triggering rapid endocytosis of the CD4 receptor (**a**). In the early endosome, Nef then interacts with the COPI coatomer, which targets CD4 for lysosomal degradation (**b**). Adapted from (Peterlin and Trono 2003)

Despite the advances that have occurred in the field of antiretroviral therapy over the last decade, HIV-1 infection still cause epidemic numbers in some sub Saharan and southeast Asian countries. Anti-retro viral therapy has proven to be very effective, still costly, involving multi drug regimens that are sometimes not tolerated by a significant number of patients. Although treatment is effective in keeping viral numbers low, it does not eradicate the virus and results in a rapid rebounce when therapy is discontinued. Alternatively, by engineering the body's immune cells to resist infection, an effective control of HIV-1 replication could be achieved, which is still being explored.

As early as in 1990, Baltimore suggested a form of gene therapy named 'intracellular immunization' in which genes encoding a proteins interfering growth of HIV might be introduced to a patient's T cells. This would interfere with subsequent infection of susceptible T cells (Baltimore 1988). Indeed this avenue has been explored by atleast half a dozen leading laboratories around the world but still there is no consensus treatment method and the search for ultimate is continuing. Majority of focus has been on either blocking the main receptor CD4 (Buonocore and Rose 1990) or coreceptors CCR5 (Holt, Wang et al.) or CXCR4. A recent major breakthrough in HIV therapy was the apparent cure of HIV infection in a patient who was diagnosed with acute myeloid leukaemia who received a stem cell graft from a CCR5 Δ32/Δ32 donor (Allers, Hutter et al. 2011; Hutter and Ganepola 2011).

5.4.3. Learning outcomes for this chapter

The purpose of this study is to answer two main questions related to HIV control. For a long time it has been debated whether the CD4 down-regulation prevents trapping and aggregation of outgoing virions on the surface of producer cells (Lama, Mangasarian et al. 1999; Neri, Giolo et al. 2011). In CD4+ T cells it is believed that HIV buds from the plasma membrane and the budding virion accumulates a coating of cell membrane that does not contain any CD4. If CD4 is present on the cell membrane it could possibly bind to gp120 of budding virion and interfere with the budding process. Presence of CD4 on the virus envelope is also expected to interfere with the functions of gp120 i.e. binding to CD4 on target cells. One can hypothesise that defective viruses be produced when no down-modulation of CD4 occurs in the host cell. However, this model is yet to be supported with evidence.

Contrasting finding have been put forward that supports the notion that CD4 down-regulation may not be so important in optimal output and functionality of the viruses in primary T cells.

Here, we reassess whether the presence of CD4 molecule on the surface of infected cells interfere with release of HIV and also whether the generated HIV viruses are defective in replication and infection due to sequestration of CD4 into the virion, as these questions still remain partly unanswered. Most of the research studies that have tried to answer this question used either Nef deleted or Nef mutated viruses that resisted CD4 down-regulation. The limitation of extrapolating results of such studies is that the Nef sequence that is acquired by a patient is a factor that cannot be controlled; where as mutations of CD4 can be easily controlled. If our hypothesis proves to be right it will have important implications in future management of HIV with potential to use mutant CD4 as a gene therapy.

Specific objectives:

1. To generate retroviral vectors with mutant CD4 that can be used as potential gene therapy target to prevent progression of HIV.
2. To characterise mutant CD4 functions in terms of expression of CD4 on the surface, internalisation, recycling and efficiency of viral production and release by chimeric cell lines.
3. To assess the quality of the HIV virus produced by CD4 mutant cells i.e. infectivity rate, replication, and CD & envelope incorporation into virus.

5.5. Results

5.5.1. Generation of CD4 constructs with truncated cytoplasmic domains

Nef protein needs important residues in membrane proximal dileucine motif of CD4 that function as internalisation signals (Aiken, Konner et al. 1994) in addition to the residues in Nef (WL^{58}, $MGxxxS^{6}$, LL^{165} and DD^{175}) (Geyer, Fackler et al. 2001) to mediate CD4 down-regulation, an important pathological feature of HIV/AIDS. To test our hypothesis, three CD4 constructs were generated as follows; one expressing wild type CD4 (CD4/WT), one with CD4 truncated at cytoplasmic domain (CD4/420T), and the third construct expressing CD4 molecule truncated at amino acid position 430 (CD4/430T) which has been truncated in such a way that there is no Nef binding (Figure 6.2). Premature truncations were introduced by using reverse primers designed with stop codons as given in methods section 2.1.3. The constructs were initially cloned into topo vector, and then subcloned into lentiviral vector pHR-SIN-BX-IRES-Em.

a

CD4 mutant	Sequence of the part of transmembrane and cytoplasmic domain of CD4 molecule
CD4/WT	^{416}IFFCVRCRHRRRQAERMSQIKRLLSEKKTCQCPHRFQKTCSPI*
CD4/430T	^{416}IFFCVRCRHRRRQAE*
CD4/420T	^{416}IFFCV*

b

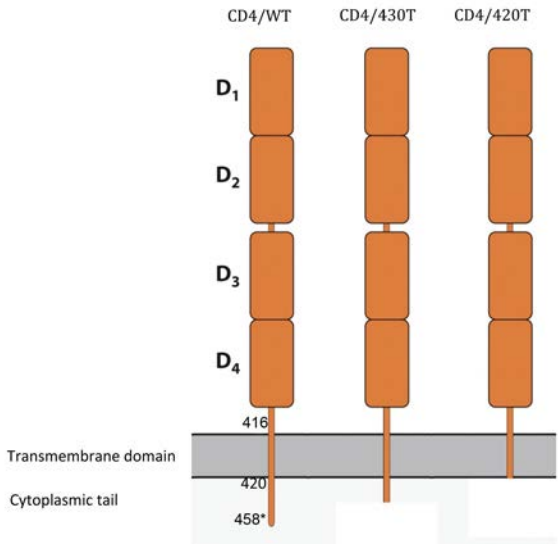


Figure 6.2: Chimeric CD4 molecules expressed on 293T cell lines used in this study. a) Sequences used b) Schematic CD4 molecules. CD4/WT cell line expresses the wild type CD4 molecule. CD4/420T express a CD4 molecule truncated at 420a.a. i.e. this molecule has no cytoplasmic tail; CD4/430T is truncated at 430 a. a. position thus preventing binding of Nef

5.5.2. Stable expression and production of CD4 on the surface of cells

To examine our hypotheses we generated stable cell lines expressing above constructs. Briefly, the constructs that were cloned in to retroviral vector, were transfected into 293T cells to generate pseudotyped viruses. Fresh 293T cells were then transduced with concentrated pseudotyped lentiviruses. Expression of CD4 was verified by cell surface FACS staining for CD4 (Figure 6.3). Before carrying out our experiments we wanted to assess whether the surface expression of CD4 on sorted transfected cell lines were stable. The surface expression of CD4 remained stable through 15 passages (data not shown).

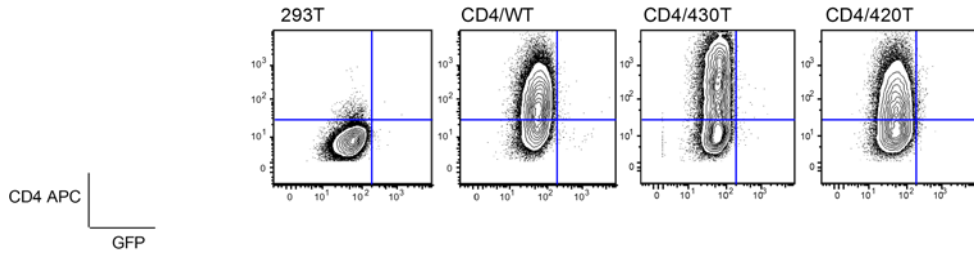


Figure 6.3: Expression of CD4 on 293T cell lines that were generated. Representative flowcytometric graphs are shown.

It was important to assess the amount of CD4 produced by generated stable cell lines to make sure that the cell lines were comparable and that truncation of CD4 cytoplasmic tail does not affect translation of the protein. Western blot analysis of total cell lysates of generated cell lines demonstrated that the amounts of CD4 proteins in them were very similar (Figure 6.4).

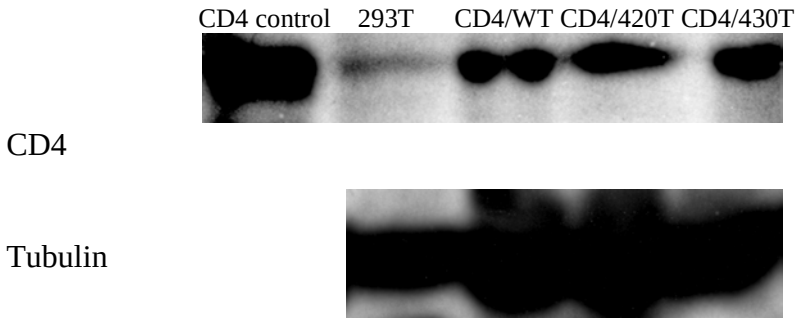


Figure 6.4: Comparison of CD4 proteins in 293T cell lines that were generated. Total cell lyates were analysed by Western blotting using antibodies against CD4 and tubulin.

5.5.3. Truncated CD4 molecules resist down-regulation with both HIV-1$_{SF2}$Nef and NL4-3 virus

Previous research has demonstrated that truncation of CD4 cytoplasmic tail resulted in abolished Nef CD4 binding which eventually lead to resistance to Nef mediated effects (Aiken, Konner et al. 1994). To assess the behaviour of truncated CD4 molecules in the presence of Nef, I transiently transfected the generated cell lines with HIV-1$_{SF2}$Nef (Figure 6.5a). CD4/WT showed a 2.5 fold down-regulation in the presence of HIV-1$_{SF2}$Nef while CD4/430T and CD4/420T resisted Nef mediated down-regulation which were statistically significant ($p<0.05$)(Figure 6.5b).

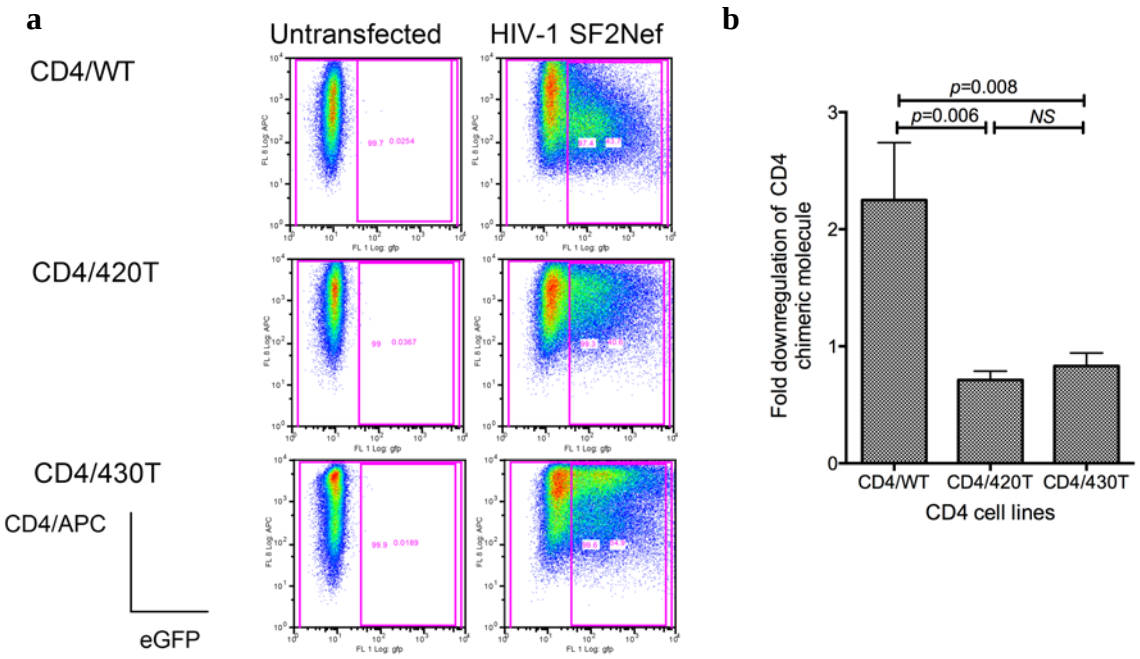


Figure 6.5: CD4 down-regulation mediated by HIV-1$_{SF2}$Nef depends on the cytoplasmic domain sequence. (**a**) Representative flowcytometric plots of CD4 behaviour in the presence of HIV-1$_{SF2}$Nef GFP. (**b**) Fold down-regulation of CD4 from the surface of stable cell lines transfected with HIV-1$_{SF2}$Nef /GFP compared to untransfected cells. Results are the summary of three independent experiments. A bar represents mean+SD.

To assess whether, the results are consistent with HIV virus infection CD4 expressing cell lines were transfected with pNL4-3 virus. As expected, there was no down-regulation of truncated CD4 molecules, CD4/420T and CD4/430T as oppose to CD4/WT (Figure 6.6).

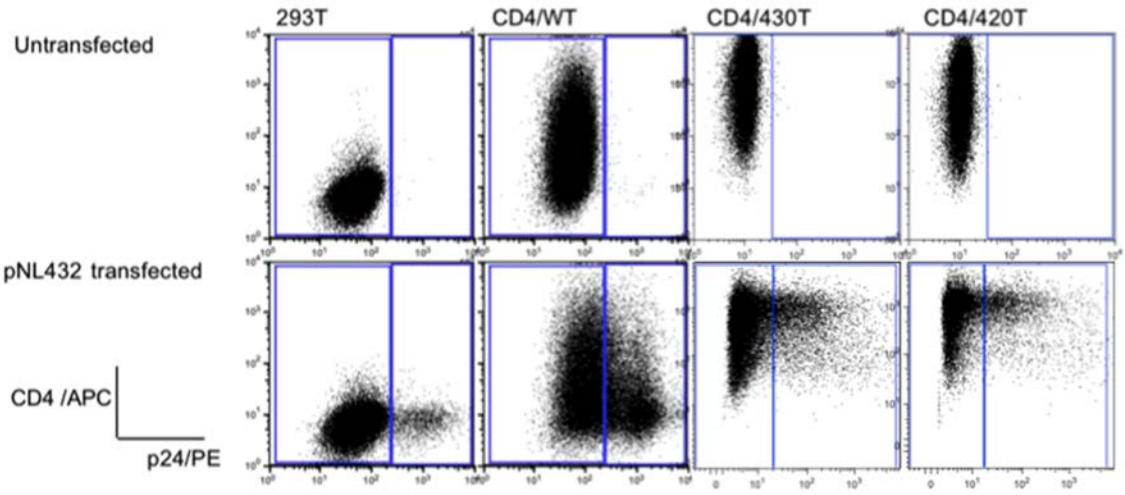


Figure 6.6: CD4 down-regulation in chimeric cell mediated by HIV-1 virus depends on the cytoplasmic tail sequence. Representative flowcytometric plots of CD4 down-regulation with pNL4-3.

5.5.4. Truncated CD4 molecules resisted internalisation compared to wild type CD4

The cytoplasmic domain plays a major role in regulating cell surface molecules. Lack or a modification of the CD4 cytoplasmic domain can have a huge impact on a cell ranging from intracellular signalling to differentiation of T helper subsets (Brown, Moskowitz et al. 1997). We know that CD4 down-regulation occurs due to accelerated endocytosis (Oldridge and Marsh 1998). Recently it was shown that in 293T cells that co express CD4 and HIV-1, the capacity of Nef to enhance the virion incorporation of Env products and the release of viral particles was mediated by retention-degradation of neo-synthesized CD4 rather than by accelerated receptor endocytosis. Different results were observed in primary $CD4^+$ T

lymphocytes in which Nef-mediated CD4 down-regulation occurred primarily by accelerated internalization (Neri, Giolo et al. 2011).

Next, we wanted to characterize the mutant CD4 molecules on the cell lines that we have generated. To examine whether there were any differences in internalization of CD4 molecules in our cell lines, we carried out a flow based internalization assay in the absence and presence of Nef. At steady state both CD4/430T and CD4/420T resisted internalization compared to CD4/WT which had ~85% of initial CD4 present on the surface at the end of an incubation period of 90 minutes (Figure 6.7).

As expected, Nef reduced surface expression of CD4/WT to about 70%. CD4/430T and CD4/420T were less susceptible to Nef. Although internalization of truncated CD4 were similar in untransfected cells, Nef slightly increased internalization of CD4/430T (Figure 6.7a and b).

Taken together, our data demonstrate that molecules with truncated cytoplasmic tails are less susceptible to internalization at steady state. However, it was observed that CD4/430T was slightly more susceptible than CD4/420T to Nef mediated down-regulation.

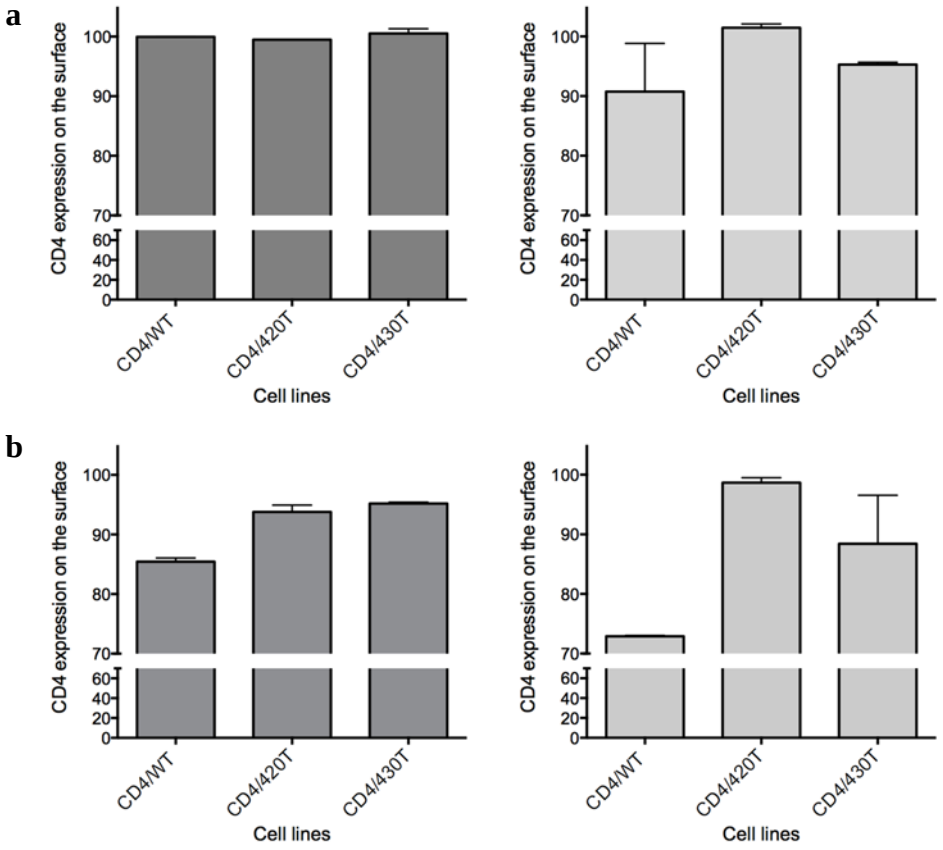


Figure 6.7: Differential internalisation of chimeric CD4 molecules in generated cell lines. (a) The percentage of CD4 expressed at the surface of cell after 45 minutes as a fraction of CD4 at t=0 (b) percentage of CD4 expressed at the surface of cell after 90 minutes as a fraction of CD4 at t=0 is demonstrated. Dark grey – untransfected CD4 chimeric cell line, light grey- Nef expressing CD4 chimeric cell lines. Data is the summary of two independent experiments. Error bar= SD

5.5.5. Recycling profile of chimeric CD4 molecules

Nef inhibits CD4 recycling to the cell membrane. Internalised CD4 molecules are misdirected to lysosomes for degradation (Oldridge and Marsh 1998). More recently a strong Nef mediated inhibition of CD4 anterograde path way was demonstrated in epithelial cells (Rose, Janvier et al. 2005). To further characterise the behaviour of the generated truncated CD4 compared to wild type, we sought to look at recycling of CD4 to the surface (Figure 6.8). The recycling assay was carried out as described (Chapter 2).

We noted that all the CD4 molecules were rapidly recycled and restored to pre stripping levels within 30 minutes of the start of the assay. There was no difference in recycling between CD4 chimera expressing cells at steady state. However, in the presence of Nef, WT/CD4 levels could not be restored to pre stripping level which was in contrast to truncated CD4 that were rapidly restored.

This finding was in agreement with previous reports where reduced recycling of CD4 has been noted with Nef expression (Rose, Janvier et al. 2005). No reduction in recycling of truncated CD4 in the presence of Nef implies that Nef is unable to retain truncated CD4 inside cells.

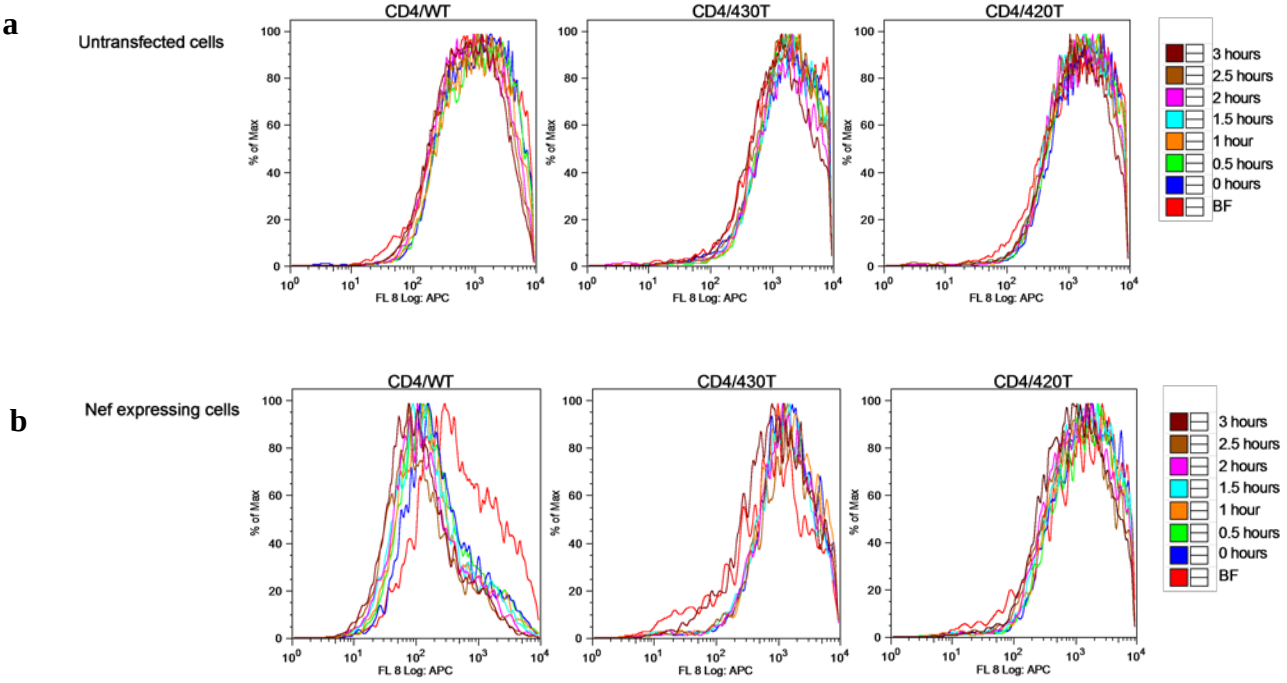


Figure 6.8: **Differential recycling of chimeric CD4 molecules in the generated cell lines at steady state (a) and Nef expressing cells (b).** Differential CD4 recycling as shown by histogram at different time points. Data is representative of two independent experiments that were carried out. BF= CD4 expression before surface molecules were stripped.

5.5.6. The efficiency of HIV release is dependent on the presence of CD4 cytoplasmic domain

It has been shown that CD4 down-regulation prevents superinfection by HIV-1 and also assists the release of infective virions. Ross et al (Ross, Oran et al. 1999) showed that removal of surface CD4 by Nef allowed release of progeny virions that would otherwise be trapped by CD4 at the cell membrane.

Thus one can hypothesise that if CD4 is not down-regulated this will affect the virion release by CD4 cell lines. But Neri et al (Neri, Giolo et al. 2011) demonstrated that virion retention in HIV transfected cells does not occur through surface CD4 down-regulation.

In the light of these contrasting findings we wanted to re-examine the role of CD4 on virion release. First we examined whether the truncated CD4 accumulated at a higher level than wild type CD4 with same amount of expression vector using western blotting (Figure 6.9). Indeed, we noted CD4 accumulation with CD4/430T and CD4/420 T cell lysates but not CD4/WT.

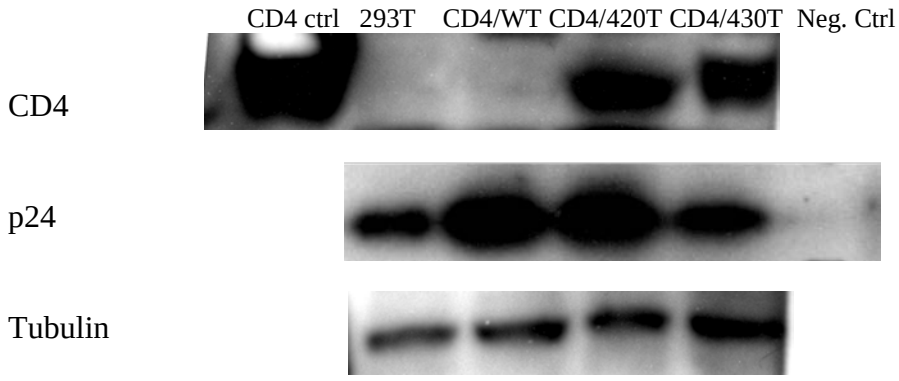


Figure 6.9: **Truncated CD4 accumulates in HIV-1 infection.** Total cell lysates were analysed by Western blotting using antibodies against CD4, p24 and tubulin.

To assess the effect of CD4 on viral release we transfected CD4/WT, CD4/430T and CD4/420T cell lines with pNL4-3 molecular clone. 48 hours after transfection p24 was measured in cellular lysates (Figure 6.10) and cell culture media by western blot and Elisa (Figure 6.11) and the efficiency of viral release (Figure 6.12) was calculated.

In contrast to Neri et al (Neri, Giolo et al.) there was no difference in intracellular sequestration of p24 in the cell lines that lacked or expressed CD4. However, the release of HIVpNL4-3 virus was not similar in the cell lines. Naïve 293T cells had the highest viral release supporting the notion that the absence of CD4 results in higher release of viruses. Surprisingly, there was no difference in viral inhibition between CD4/WT and CD4/430T. However, CD4/420T cell line demonstrated the strongest inhibition of viral release (Figure 6.11 and 6.12).

In summary, these findings indicate that HIV-1 virion release is dependent on a motif that lies between amino acid 416 and 430 in the CD4 cytoplasmic domain. However, viral release appears to be independent of endocytosis pattern.

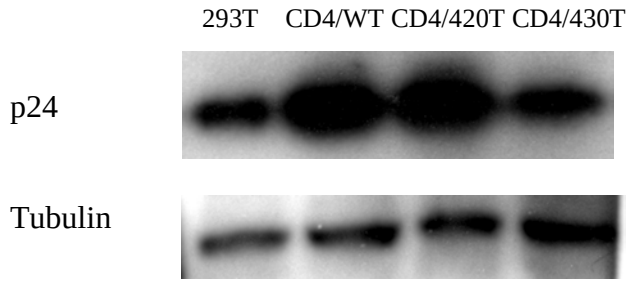


Figure 6.10: Infectivity of 293T cell line transfected with pNL4-3 determined by western blotting of cellular lysate.

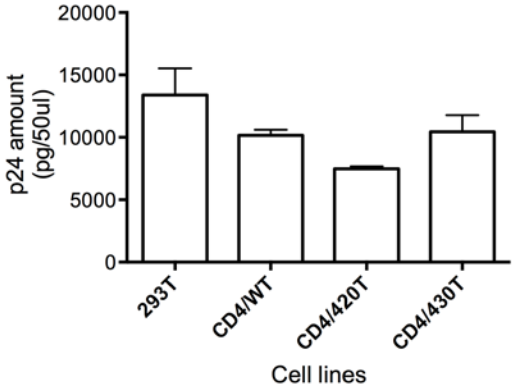


Figure 6.11: 293T cells release HIV-1 virions in a CD4 cytoplasmic tail dependent manner. Viral release by pNL432 transfected cell lines was measured using p24 capture ELISA of culture supernatant. Results are shown as mean +/-SD of three independent experiments. Culture supernatant was collected 48 hours after transfection

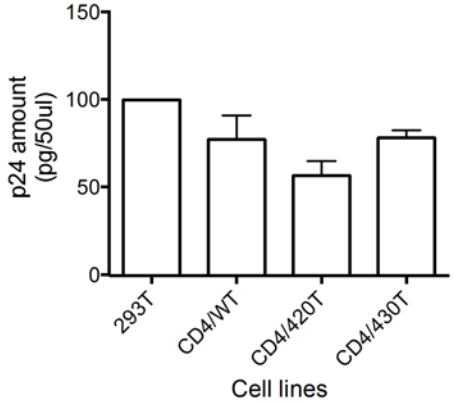


Figure 6.12: The efficiency of HIV-1 virion release is CD4 cytoplasmic tail dependent. The efficiency of viral release in generated cell lines transfected with pNL432 was measured by comparing to efficiency of 293T cells that had no CD4. The release of virus in 293T cells that have no CD4 was set at a 100%. Results are shown as mean +/-SD of three independent experiments. Culture supernatant was collected 48 hours after transfection.

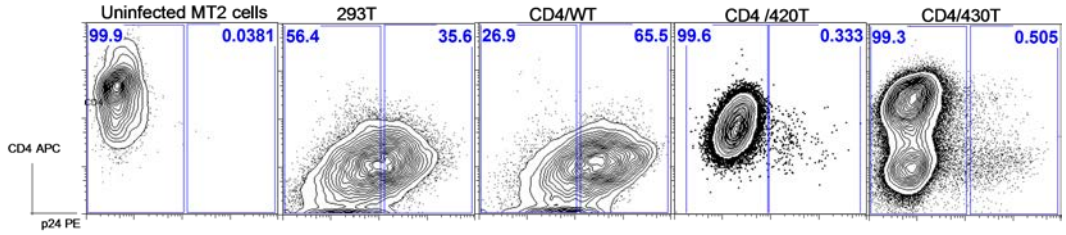
5.5.7. The ability of the HIV-1 virus produced by CD4 mutant cell lines to infect HIV susceptible cell lines MT2 and C8166

Next, to assess the degree of infectivity of viruses generated in mutant CD4 cell lines, HIV permissive cell lines, MT2 cells and C8166 cells were used. The advantage of using these cells is the ability to detect infectivity easily under a microscope. Two million cells were infected with 1000pg of pNL4-3 viruses that were generated either in naïve 293T cells or CD4 chimera expressing 293T cells.

We noted syncytia formation as early as day 3 in MT2 cells infected with viruses produced in 293T cells and CD4/WT expressing cells but not in MT2 cells infected with virus generated in CD4/420T and CD4/430T expressing cell lines. The infectivity rates assessed using flowcytometry were highest with viruses generated in 293T and CD4/WT expressing cells but much lower in cells infected with virus generated in CD4/420T and CD4/430T cells (Figure 6.13 and 6.14). These differences were shown to be statistically significant ($p=0.000$).

Taken together, these data demonstrate that the viruses generated in CD4 cytoplasmic tail truncated cell lines were unable to establish infection in HIV permissive CD4+ T cell lines MT2 and C8166 cells.

a



b

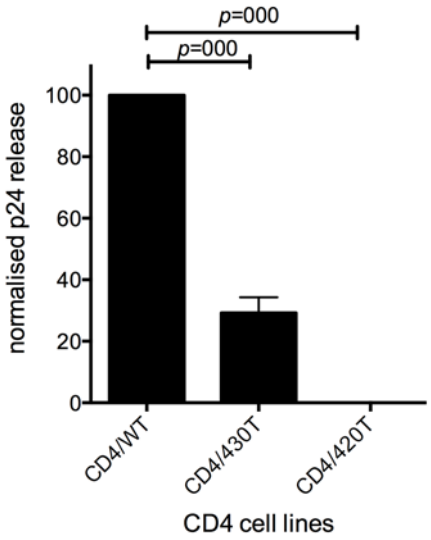


Figure 6.13: Infectivity of pNL4-3 viruses in MT2 cells. a) Representative flow graphs demonstrating infectivity rate of MT2 cells on day 6 after infecting with viruses generated in mutant cell lines b) Viral release by MT2 cells measured using p24 capture ELISA 6 days later. Each bar represents mean of three independent experiments, Error bar =SD. p24 release is normalised to CD4/WT virus infected cells.

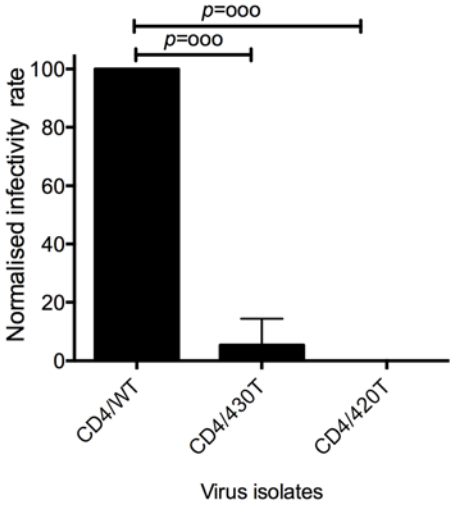


Figure 6.14: Infectivity of pNL4-3 viruses made in CD4 mutant cell lines in C8166 cells. Infectivity was determined by measuring p24 levels in the supernatant using the p24 Capture ELISA kit on day 6 post infection. Each bar represents mean of three independent experiments, Error bar= SD. Infectivity rate is normalised to CD4/WT virus infected cells.

5.5.8. The ability of the HIV virus produced in CD4 mutant cell lines to replicate in a HIV susceptible cell lines MT2 cells

One hypothesis of ours was that the viruses produced in cell lines expressing truncated CD4 would not be able to infect target cells effectively and once infected they would not be able to replicate. To assess the second part of the hypothesis, we examined the replication of pNL4-3 viruses that were generated in chimeric CD4 expressing cell lines in a HIV permissive cell line MT2 (Figure 6.15). Supernatant was taken out of triplicate wells for p24 assays. Only MT2 cells infected with viruses generated in 293T cell lines expressing wild type CD4 had detectable p24 levels on day 4. However, p24 levels in the supernatant of the cells infected with viruses generated in 293T cells also picked up with time. Viruses generated in cell lines expressing truncated CD4 molecules did not replicate as efficiently as the ones that were generated in 293T and WT/CD4 cell lines. This data supports our hypothesis that truncated CD4s results in generation of viruses that are defective in replication.

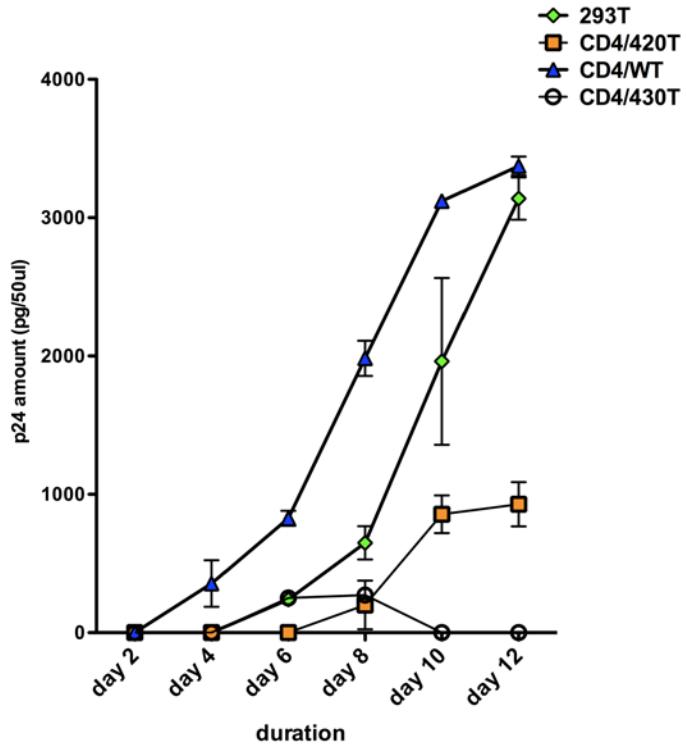


Figure 6.15: Replication of pNL4-3 virus in a HIV permissive cell line MT2. Founder viruses were generated by transfecting stable cell lines expressing different CD4 molecules with pNL43-2 plasmid. 293T cells that do not express CD4 were used as control. Each bar represents mean of three experiments, Error bar= SD.

5.6. Discussion

CD4 is the main entry molecule of HIV virus. Soon after discovery of HIV-1, it was noted that progression of HIV results in a low number of CD4+ T cells and also a reduction of cell associated surface expression of CD4. CD4 down-regulation is mainly mediated by Nef and it has been noted that this activity is relatively higher in *nef* alleles isolated after immunodeficiency than those isolated during early asymptomatic stages of infection in HIV-1 and SIV (Carl, Greenough et al. 2001; Patel, Yu Kimata et al. 2002). However, it is unclear how Nef associated CD4 down-regulation influence pathogenicity.

CD4 down-regulation must be important for viral survival, perhaps not surprisingly three viral proteins out of nine, adapting to the function. Several pathogenic roles have been proposed for this mechanism, ranging from modulation of T cell activation to prevention of super infection and facilitating release of virions (Benson, Sanfridson et al. 1993; Lama 2003).

Recently, it was described that infectivity of the viruses produced in 293T cells was independent of CD4 down-regulation but correlated with Env incorporation that occurred with Nef expression (Neri, Giolo et al. 2011). In contrast to this, early reports observed reduced infectivity of HIV viruses produced by 293T cells expressing CD4 (Lama, Mangasarian et al. 1999).

Most of these *in vitro* studies utilised Nef mutants that were defective in CD4 down-regulation, limiting our understanding as to whether the effects that were described were in fact due to motifs found in CD4 or Nef. The knowledge that was generated so far had limited use in therapeutics, as *nef* alleles that are acquired during infection could not be controlled. The current study was designed to reassess the role of CD4 down-regulation on infectivity and replication of HIV, especially to overcome the limitation of using Nef mutants and to

assess the possibility of using mutant CD4 as a therapeutic agent to circumvent HIV spread in T cells.

This study employed CD4 molecules truncated at C terminal end to assess the effect on HIV virion production. In summary, our data indicated that the viruses that were produced in the presence of truncated CD4 that were resistant to Nef mediated down-regulation, were also defective in infecting and replicating in HIV permissive CD4+ cell lines.

It was important to characterise the CD4 mutants to interpret our results and for use of constructs in future studies aiming at therapeutic interventions. At steady state the recycling of mutant CD4 molecules were very similar to wild type CD4. However, confirming previous findings, it was clearly seen that recycling of wild type CD4 was impaired compared to CD4 with truncated cytoplasmic domains in the presence of Nef.

C terminal truncation clearly led to slightly defective internalisation of CD4/420T and CD4/430T, compared to CD4/WT at steady state. Similar results have been observed before (Pelchen-Matthews, Armes et al. 1989). Perhaps not surprisingly, Nef enhanced internalisation of CD4/WT leading to around 30% difference of CD4 on the surface of cells compared to truncated molecules, 90 minutes after initial assessment.

Both CD4/430T and CD4/420T were resistant to Nef mediated internalisation yet the efficiency of viral release of CD4/430T was as high as CD4/WT. In contrast to this, CD4/420T demonstrated a defective viral release. However, the pNL4-3 viruses produced by both these cell lines were defective in infection and replication. Furthermore, the two cell lines retained/accumulated CD4 following HIV infection. These findings confirm that the residues mediating susceptibility of CD4 cytoplasmic tail to Nef is found at C terminal end beyond amino acid 430 (just short of dileucine motif). Impaired virion release by CD4/420T

but not CD4/430T indicates that the motif responsible for optimal viral output is contained between amino acids 420 and 430 of the CD4 cytoplasmic domain. These findings also imply that virion release and functionality (determined by infectivity and replication) are completely different entities mediated by two domains of CD4.

To the contrary, Neri *et al* in their paper concluded that the functionality and virion release were two independent functions of Nef (Neri, Giolo et al. 2011). Our results imply that the viral release is either dependent on a motif that is located in the cytoplasmic domain of CD4; between amino acids 420 and 430 or the presence (and/or absence) of the cytoplasmic domain. The use of a full length CD4 mutant that was resistant to Nef down-regulation by the above group and especially with this mutation being located after amino acid 430 in the cytoplasm could be the reason behind their interpretation.

As discussed before, there is still debate as to whether the down-regulation of CD4 from the surface of infected cells facilitates effective release of HIV virus. It could be hypothesised that presence of CD4 on the surface of infected cells probably lead to either incorporation of CD4 into the viral envelope and/or reduced incorporation of envelop proteins into virion. Incorporation of CD4 into viral membrane could interfere with envelop protein function by binding to gp120 leading to a reduction in 'free gp120' that could bind to CD4 on naïve target cells. This may subsequently lead to viruses that are ineffective/defective in infection and replication.

Indeed, there are reports of Nef mutants defective in CD4 down-regulation losing the capacity to enhance Env incorporation into virions and to stimulate viral replication in primary T cells (Glushakova, Munch et al. 2001; Lundquist, Zhou et al. 2004; Fackler, Moris et al. 2006). However, reports supporting the notion of Nef mutants defective in CD4 down-modulation maintaining the capacity to enhance viral activity are also present (Goldsmith, Warmerdam et

al. 1995; Glushakova, Munch et al. 2001; Lundquist, Tobiume et al. 2002). Previous studies have reported that CD4 down-regulation results in decreased progeny virus production from infected CD4+ 293T producer cells (Ross, Oran et al. 1999).

Although we did not observe any accumulation of viral products (determined by p24) in the chimeric cell lines expressing truncated CD4 that would suggest viral sequestration, our findings confirm that absence of cytoplasmic domain of CD4 leads to a definite reduction in virion release. Importantly, data presented here show that the viruses that are generated in CD4/420T and CD4/430T are defective in replication and infection, demonstrating that indeed Nef mediated CD4 down-regulation is important for optimal functionality of virus particles in these cell lines.

One of the limitations of this study was not including a HIV virus that does not express Nef. It would have been better to have a Nef deleted virus as an additional control. On the other hand current study proves that expression of CD4 on the target cell surface results in defective viruses. Determination of constitutes of the virions (incorporation of Env and CD4) released by CD4 chimeric cell lines would have given a more complete picture. Another limitation was not extending these results in to primary cells. Time permitting these are aspects that we are planning to pursue further.

CD4 down-regulation is a conserved property of HIV-1, HIV-2 and SIV (Schindler, Munch et al. 2006), which makes one believe that it might be of importance for lentiviral replication. Perhaps CD4 counteraction is needed after infection which otherwise would lead to production of defective viruses. It has been said that reduction of surface CD4 at least partly contribute to prevention of viral super infection, which otherwise can result in cytopathy.

Recent evidence suggests that CD4 down-regulation of Nef poorly correlated with capacity to stimulate viral infectivity and replication (Neri, Giolo et al. ; Neri, Giolo et al. 2011). This was more in line with others who reported of *nef* mutants that were impaired in CD4 down-regulation, yet preserved infectivity (Goldsmith, Warmerdam et al. 1995). To the contrary, there are reports of loss of Env incorporation into virions and stimulation of viral replication in primary T cells by Nef mutants that are resistant/defective of CD4 downregulation (Glushakova, Munch et al. 2001; Arganaraz, Schindler et al. 2003; Fackler, Moris et al. 2006).

We found that deficient CD4 down-regulation inversely correlated with protein capacity to stimulate viral infectivity and replication in HIV permissive cell lines. Although these results are preliminary they have important implications in developing therapeutics for HIV/AIDS. Importantly, to our knowledge this is the first study that used truncated CD4 molecules to assess this function instead of mutant *nef* alleles. Assessing these mutants in an animal model will provide important insight on HIV pathogenesis. It is not known whether the lack of CD4/ truncated CD4 cytoplasmic tail affect differentiation and activation of cells. It is said that protein tyrosine kinase $p56^{lck}$ is an essential participant in thymocyte maturation and T cell activation. But overexpression of transgenic CD4 that cannot associate with $p56^{lck}$ can nevertheless rescue helper cell lineage (Killeen and Littman 1993). Perhaps having truncated CD4 in addition to wild type CD4 might just be sufficient in making defective viruses that are unable to further infect or replicate, which would benefit from further studies.

6. Discussion

General Discussion

With well into the fourth decade after discovery, HIV still proves to be a step ahead of human, causing high morbidity and mortality. The brutal attack had been difficult to counter as HIV rapidly adapts to the new host and successfully evade the immune system. HLA-I and CD4 down-regulation mediated by Nef are important pathogenic features that contribute towards increased virulence and successful establishment of progressive disease. Of the classical HLA-I alleles, HLA-B alleles are best described to provide protection against HIV. However, the mechanism by which HLA-B provides protection is not fully explained. In this thesis, I have investigated how HLA-B alleles provide protection in HIV by partially resisting Nef mediated down-regulation to the contrary to reported findings (Rajapaksa, Li et al. 2012). I also investigated the importance of cytoplasmic tail of CD4 in CD4 down-regulation, subsequent virion release and optimal functionality of HIV.

The crucial role played by Nef in HIV pathogenesis first came in to light when a cohort of patients who were long term non progressors were found to be infected with a Nef deleted virus (Learmont, Geczy et al. 1999; Birch, Learmont et al. 2001). Subsequent research illustrated that Nef is a major manipulator of surface molecules that increased virulence and in doing so makes HIV a master of immune evasion (Foster and Garcia 2008). Nef mediated HLA-I down-regulation is a key mechanism through which HIV infected cells evade HLA-I restricted CD8+ cytotoxic T cells recognition (Collins, Chen et al. 1998). CD8+ T cells play a crucial role in controlling viral infections including HIV. Thus escaping recognition either by mutating the epitope or down-regulating the epitope is important for viral survival.

Interestingly, it was demonstrated that down-regulation of classical HLA-I alleles is different, with HLA-A and B being susceptible to down-regulation, in contrast to HLA-C. The differential down-regulation was attributed to sequence differences found in the cytoplasmic

domain (Cohen, Gandhi et al. 1999), and provided first glimpse of the importance of HLA-I cytoplasmic tail in HIV infection. Although the mechanism of HLA-I down-regulation mediated through co-involvement of cytoplasmic domain and Nef was further studied, less emphasis was placed on studying the polymorphisms of the tail and its direct role in pathogenesis.

In this study we have used chimeric molecules that expressed HLA-A*02 extracellular and transmembrane domain fused to cytoplasmic tail sequences that were reported in GenBank to investigate our hypothesis that protection to HIV mediated by HLA-B allele was in fact mediated through an altered susceptibility to Nef. We found that the HLA-B cytoplasmic tail was less polymorphic than HLA-A and/or –C and it partially resisted Nef mediated down-regulation. We also demonstrated that this decrease in HLA-I down-regulation was sufficient enough to control HIV-1 replication in culture and it also resulted in increased recognition by CD8+ T cells, degranulation and secretion of cytokines than observed in the presence of HLA-A. The fact that we were able to validate the results obtained with chimeric molecules in native HLA-I alleles confirmed that indeed these results are concrete.

The data that I have presented here provide important insight into understanding the complex interplay between HIV and HLA. It has been known for many years that HLA-B alleles are more protective than HLA-A subtypes (Kiepiela, Leslie et al. 2004; Goulder and Watkins 2008). Not surprisingly, unbalanced and extensive studies on HLA-B due to its close association with natural killer cells and KIR (Carrington, Martin et al. 2008), and also confounding effect of a few highly protective HLA-B types such as B*57, B*58 and B*27 (Stephens 2005) would have shaped this thinking. There is also evidence supporting that high avidity, polyfunctionality and rapid clonal turnover mediated by HLA-B restricted CTL leads to better protection (Almeida, Price et al. 2007; Harari, Cellerai et al. 2007), with

polyfunctionality thought to be determined by the level of antigen sensitivity (Valitutti, Muller et al. 1996; Betts, Price et al. 2004; Almeida, Sauce et al. 2009). Supporting this, our results demonstrated that higher expression of HLA-I on the surface led to high levels of IFNγ and CD107a secretion. Although IL-2, MIP-1β and TNFα responses are not included (experiments currently being carried out), the differences that were observed with IFNγ and CD107a between HLA-A and B alleles imply that high expression of HLA-I molecules leads to a polyfunctional profile that is observed with HLA-B restricted CTL by previous researchers (Almeida, Price et al. 2007).

Partial resistance of HLA-B alleles to Nef mediated down-regulation results in more antigens being present on the surface of infected cells. This could be advantageous for the virus especially if it facilitates selecting escape mutations that are favourable. However, if the mutations result in a fitness cost, it would ultimately challenge the viral survival in the infected host. Although, the direct contribution of an impaired HLA-I down-regulation has on the viral evolution i.e. selection pressure and escape, has not yet been demonstrated, perhaps the partial resistance of HLA-B alleles to Nef could be the reason behind the observed HLA-B driven HIV-evolution that is described elsewhere (Kiepiela, Leslie et al. 2004; Leslie, Pfafferott et al. 2004). Future research needs to be designed to address this hypothesis which will shed important insight into understanding the complex HIV-HLA-I relationship and designing therapeutics especially T cell mediated vaccines i.e. targeting HLA-B rather than HLA-A restricted responses. Viral evolution assays have been described previously (Yang, Sarkis et al. 2003) and this technique is established in our lab. As a follow-up study, it would be worthwhile adapting the viral evolution assays to determine whether chimera containing HLA-B cytoplasmic tails lead to generation of more escape mutations than HLA-A. The initial steps to optimise effector to target ratio are currently being carried out. The CTL clone (A2 Gag p17 specific) that I used had a very high avidity and effectively killed HIV infected

cells in the culture. Therefore, at effector to target ratios that were tested (1:10 and 1:30), I could not obtain sufficient viral yields to infect fresh cells for a third round. Readjusting effector to target ratios to very low levels or using a CTL clone with a lower avidity that will allow the virus to replicate and mutate will aid in testing the above hypothesis.

The results that I have presented in this thesis are *in vitro* based. It would be interesting if we could relate our findings to the clinical picture. Assessing the association between HLA-I cytoplasmic tail biology with disease progression, CD4 and vireamia levels in an established cohort, or in a new cohort of patients and controls (similar to other cohort studies where HLA-I epitope associations are analysed) may provide insight into importance of the HLA-I cytoplasmic tail domain in immune response to HIV. A second limitation related to the lack of sequence information for the majority of the cytoplasmic tails of HLA-I alleles in the database (**http://www.ebi.ac.uk/imgt/hla/**); this problem could be overcome by carrying out a study as described above.

Nef mediated MHC class I down-regulation occur in HIV-1, HIV-2 and SIV infections (DeGottardi, Specht et al. 2008; Specht, DeGottardi et al. 2008; Schmokel, Li et al. 2009). Chapter 4 demonstrated that partial resistance of HLA-B to Nef was conserved in subgroup O and HIV-2 *nef* alleles. Perhaps the variability in susceptibility seen in Mamu-B alleles to SIV and HIV Nef were due to high polymorphism of Mamu-B alleles (DeGottardi, Specht et al. 2008). Rhesus macaques have only one Mamu–A allele unlike humans, and it has been reported that allelic polymorphisms are also absent in Mamu-B (Otting, Heijmans et al. 2005). In contrast to this, polymorphism that maps to exon 2 and 3 are more prevalent in humans especially in HLA-B, perhaps not requiring an additional layer of variability to be achieved through HLA-I cytoplasmic domain. However, our results showed that HLA-B alleles were more resistant to HIV-1 and HIV-2 *nef* alleles (chapter 4), compared to Mamu-B described

elsewhere (DeGottardi, Specht et al. 2008). This seems to have occurred concurrent to the loss of HLA-B cytoplasmic tail polymorphism. Perhaps this reflects dynamics between human T cells, NK cells and older viruses such as herpes viruses like CMV, and just so happen to help in the setting of HIV, a relatively new infection. Furthermore in the process of adaptation to humans, HIV may have selected Nef alleles that were unable to down-regulate HLA-B for survival advantages, but would benefit from more in-depth study. However, this also raises the question of reliability of SIV_{mac} model for HIV. Resistance of HLA-C to Nef was preserved in HIV-2 and SIV.

The clinical course of HIV-2 is very different to HIV-1. Asymptomatic disease is protracted, with lower plasma HIV viral loads and lower mortality rates compared to HIV-1 (Marlink, Kanki et al. 1994). Although Nef has been shown to be a main virulent factor for HIV-1, the role mediated by Nef in determining pathogenicity of HIV-2 is not well understood. Whereas HLA-I and CD4 are similarly down-regulated by HIV-1 and HIV-2 Nef (Munch, Schindler et al. 2005), CD3 and CD28 down-regulation is more efficient in HIV-2 infections and is presumed responsible for reduced immune activation observed (Munch, Schindler et al. 2005). An inverse correlation between the level of CTL activity and proviral loads implicate that CTLs play a key role in controlling HIV-2 replication (Ariyoshi, Cham et al. 1995). However, CTL recognition and the response towards HIV-2 infected target cells is not studied to the same extent as HIV-1, perhaps due to difficulties in isolating HIV-2 restricted T cells, which might be secondary to low viral loads *in vivo*. Although there are reports of early differentiated and highly avid CTLs in HIV-2 (Leligdowicz, Onyango et al. 2010), the contribution of HLA-I down-regulation in CTL recognition has not yet been studied. It would be interesting to find out whether HIV-2 Nef mediated down-regulation of HLA-I leads to evasion of CTL response, similar to HIV-1 (Collins, Chen et al. 1998) and SIV (Minang, Trivett et al. 2009). Our system could be adapted to assess effects of HIV-2 Nef (in a HIV-1

virus backbone) on CTL (HIV-1 restricted) recognition. This would overcome the limited availability of HIV-2 restricted CTLs clones to carry out *in vitro* studies.

In addition, it would also be worthwhile amplifying HIV-1 and HIV-2 *nef* alleles from patients at different clinical stages and exploring their response to different HLA-I cytoplasmic alleles, as almost all of the *nef* alleles that were used in my studies were lab adapted strains.

It was interesting to note that replacement of ^{315}DR with two glycine residues and truncation of cytoplasmic domain could bring about a big difference in the way Nef recognises/binds to HLA-B. Indeed, the above amino acid substitutions resulted in a different intracellular trafficking pattern of HLA-B compared to HLA-A and confirmed that it behaved similar to HLA-C. However, the most striking finding was that an important amino acid required for Nef susceptibility, ^{321}tyrosine, (Cohen, Gandhi et al. 1999), was predicted to be buried and less accessible to kinases in *in silico* experiments of HLA-B cytoplasmic tail. Conformational changes that are predicted to be brought about by substitution of aspartic acid and arginine by two glycine residues (in 3D imaging), perhaps results in a different phosphorylation profile. This finding led us to propose a model for the mechanism of resisting Nef mediated down-regulation by HLA-B in chapter 5.

Although appreciating that our model is hypothetical, phosphorylation studies, comparative Nef binding assays, made feasible either by immune-precipitation or surface plasmon resonance, are required to validate our theory. Comparison of level of phosphorylation of chimeric molecules can be carried out using anti-phospho tyrosine and anti-phospho serine antibodies. Immunoprecipitation of chimeric HLA-A2 molecules and probing with anti-Nef and anti AP-1 anitbodies would provide evidence as to whether our hypothesis that ‘when tyrosine is buried it can no longer act as sorting motif that binds to adaptor proteins and Nef is

true. Co-localisation studies with very high resolution imaging are now required to show how chimeras containing HLA-B and HLA-A cytoplasmic tails are trafficked through the cells. This set of experiments will hopefully decipher the mechanism behind reduced susceptibility of HLA-B cytoplasmic tail to Nef.

Another limitation in interpreting trafficking signals clearly in our studies was the lack of few chimeras that would correctly pinpoint the motifs involved in resistance to Nef mediated down-regulation i.e.^{315}aspartic acid with added CKV at 5' end and ^{316}arginine with CKV at 5' end. These mutants are being generated at the moment.

Our study is distinctive, as we have used a cellular system where only the cytoplasmic tail of the chimeric molecule is different. This enables us to understand the effects mediated through the cytoplasmic tail and could easily be adapted to study the relationship of other diseases and HLA-I, with emphasis on cytoplasmic tail. This system could be used to evaluate the trafficking of alleles mediated through cytoplasmic tail regions or the evolution of microbes that might result due to subsequent differential down-regulation.

Nef-mediated CD4 down-regulation, also mediated via the cytoplasmic domain, is another important and pathological feature of HIV (Lama, Mangasarian et al. 1999; Piguet, Schwartz et al. 1999). Although depletion of CD4 was shown to protect cells from super-infection (Benson, Sanfridson et al. 1993), whether it results in more efficient viral release (Lama, Mangasarian et al. 1999; Neri, Giolo et al. 2011) or optimal functionality is debated (Neri, Giolo et al. 2011). The impact of CD4 depletion on developing new therapeutics is shadowed by altered interpretation of results due to usage of Nef mutants, as the *nef* allele that is acquired during HIV infection is beyond patients' control compared to a molecule in the cellular system that could be controlled.

In this thesis, I generated CD4 mutant cell lines and demonstrated that in the absence of CD4 down-regulation, the viruses that were produced were defective in infection and replication. Although we could not determine whether the virus defects were secondary to accumulation of CD4 in budding virions due to time constraints, future studies to probe the viruses that were generated in CD4 mutant cell lines with anti-CD4 antibodies either in a Western blot or an ELISA assay is in progress. However, viral release seemed to be mediated through a different domain and indicated that the absence of cytoplasmic domain (rather than the presence of partial tail) led to impaired viral release. It would have been interesting to have identified the definitive sequence mediating this effect and also to have extended this to study T cells. Since the constructs are in a lentiviral vector this should be straightforward and will provide us with insight as to whether we could replicate our findings in the presence of a full length CD4, in addition to truncated CD4. If so this perhaps may well be a foundation step in a more elaborate study leading to gene therapy in HIV where CD4 with truncated cytoplasmic tail is used to prevent further replication and infection of HIV.

Altogether, this thesis addresses several important questions that have been prominent in the HIV field for several years i.e. why HLA-B is more protective, whether CD4 down-regulation affects virion release and/or results in defective viruses. While providing some answers and explanations for these concepts, this thesis also opens up new avenues that will benefit from future studies. Perhaps this will also influence how future therapeutics are designed, including T cell mediated vaccines or gene therapy based strategies.

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