In depth analysis of the HIV-specific CD8 T cell response in seropositives exposed to a mixed subtype epidemic

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Dedicated to all the bar and sex workers of this planet

Abbreviations

aa	amino acid
Ag	antigen
AIDS	aquired immune deficiency syndrome
CCR5	chemokine receptor, co-receptor for HIV-1
CXCR4	chemokine receptor, co-receptor for HIV-1
CD	cluster of differentiation
CEF	Cytomegalo virus, Eppstein Barr virus, Influenza virus
CMV	Cytomegalo virus
CPTA	4-[(1,4,8,11-tetraazacyclotetradec-1-yl)-methyl]benzoic acid (=anti-coagulant)
CRF	circulating recombinant form
CTL	cytotoxic T lymphocyte
DMSO	Dimethylsulfoxid
dNTP	desoxy-nucleoside triphosphate
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
Env	envelope protein
FACS	"fluorescence activated cell sorting"
FCS	fetal calf serum
FITC	Fluoresceinisothiocyanat
Fmoc	N-alpha-(9-fluorenylmethyloxycarbonyl)-amino acid
FU	follow up
Gag	group specific antigen
GagR1R3	Gag regions aa001 to aa075 and aa248 to aa500
Gag plus number	number of overlapping 15mer peptide within gag
GM-CSF	Granulocyte-Macrophage colony stimulating factor
gp	glycoprotein
H001-H605	sample ID of study subject
HISIS	HIV superinfection study
HIV	human immunodeficiency virus
HLA	human leukocyte antigen

HPLC	high performance liquid chromatography
HVL	high viral load (>50 000 RNA copies/ml)
ICC	intracellular cytokine staining
IFNγ	interferon gamma
IL	interleukin
LTR	long terminal repeat
LVL	low viral load (<50 000 RNA copies / ml)
MHAacd	multi-hybridization assay for subtypes A, C and D
MHC	major histocompatibility complex
ml	milliliter
Nef	negative factor, HIV accessory protein
OLP	overlapping peptide pool
p17	HIV matrix protein of 17 kd (Gag aa001-aa132)
p24	HIV capsid protein of 24 kd
p15	HIV nucleocapsid proteins complexing viral RNA
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	Phycoerythrin
PerCP-Cy5	Peridinin Clorophyll protein covalently bound to Cyanin 5
PHA	Phytohämagglutinin
PCR	polymerase chain reaction
QC	quality control
R10	cell medium that includes 10% FCS
RNA	ribonucleic acid
rpm	rotations per minute
SEB	*Staphylococcus* Enterotoxin B
SFC	spot forming cells
SIV	simian immunodeficiency virus
TCR	T-cell receptor
URF	unique recombinant form
VLP	virus like particle (recombinant HIV Gag PR55, subtype B)

Contents

Introduction

1.1 The Human Immunodeficiency Virus (HIV)

HIV belongs to the family of Retroviridae. Together with other immunodeficiency viruses, such as the simian and feline immunodeficiency viruses, HIV belongs to the genus of lentiviruses. The name is derived from the Latin *lentus*, meaning slow, because of the gradual course of the diseases that these viruses cause.

The closely related human immunodeficiency viruses HIV-1 and HIV-2 are the disease causing agents of the acquired immunodeficiency syndrome (AIDS). Worldwide around 40 million people are now infected with HIV with up to 5 million new infections in the year 2005 (UNAIDS). AIDS is characterized by a profound decrease of CD4 T cells leading to an increased susceptibility a wide variety of opportunistic pathogens. Several bacterial and viral infections, such as Mycobacterium tuberculosis and human Herpes virus 8 (HHV-8), infections with fungi, such as Candida albicans, and infections with the protozoan Pneumocystis carinii can be indicative of AIDS. Frequent diagnoses of such infections in homosexual men in combination with low lymphocyte counts lead to the description of AIDS in 1981 (Gottlieb et al., 1981).The virus was first isolated from the lymph node of an AIDS patient in 1983 (Barre-Sinoussi et al., 1983). The viral sequence was determined in 1985 (Barre-Sinoussi et al., 1983; Muesing et al., 1985; Ratner et al., 1985; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985) and since 1986 the name of the virus was changed from lymphadenopathy associated virus (LAV) to HIV (Coffin et al., 1986).

1.1.1 Genome and replication of HIV

HIV is a retrovirus with a genome of approximately 10kb of size (Ratner et al., 1985; Wain-Hobson et al., 1985). Due to its 5'-Cap-structure and a 3'-polyadenylation, the HIV genome closely resembles an eukaryotic mRNA. Characteristic for retroviruses, the genome contains direct repeats (R) on both ends and two unique sequences, one after the direct repeat at the 5' end (U5), the other immediately preceding the direct repeat at the 3' end (U3) (figure 1.1). The genome consists of the three classical retrovirus open reading frames (ORF), the *gag* (group-specific antigen) ORF, the *pol* (polymerase) ORF and

the *env* (envelope) ORF. In addition to these, the HIV-1 genome contains genes encoding for the small accessory proteins Nef, Vif, Rev, Tat, Vpu, Vpr (Table 1.1). The *gag* ORF encodes for the p17 matrix protein, the p24 capsid protein, the p7 nucleocapsid protein and the p6 link-protein. The *pol* ORF encodes for the viral enzymes reverse transcriptase, integrase and protease and the *env* ORF encodes for the viral glycoprotein gp160, which is cleaved inside the lumen of the Golgi-apparatus into functional gp41 and gp120 by the cellular furin-protease.

Tab. 1.1: Function and localization of small accessory proteins of HIV-1

Gene product	Localisation	Function
Tat ("transactivator of transcription")	• Binds to "RNA-trans-activation response region" at the 5´-end of the proviral DNA	• Regulates trans-activation of the transcription (Klotman et al., 1991)
Rev ("regulator of virion protein expression")	• Binds to „rev responsive element" in unspliced and singly spliced mRNA	• Regulates switch from early to late gene expression • essential for expression of virion proteins • Binding causes translocation of these RNAs into cytoplasm (Hope and Pomerantz, 1995)
Vif ("virus infectivity factor")	• inside the virion, inside the cytoplasm and extracellular in cell culture supernatant of infected cells	• binds to Apobec proteins and excludes them from incorporation into virions (Mangeat et al., 2003)

Tab. 1.1: Function and localization of small accessory proteins of HIV-1

Gene product	Localisation	Function
Nef ("negative regulatory factor")	• is translated from multiply spliced mRNAs • membrane associated through myristoylation	• increases viral replication • affects T cell activation • downregulates CD4 and MHC Class I molecules on surface of infected cells • protects infected cell from superinfection (Michel et al., 2005) • downregulation of MHC Class I helps to evade the HIV-specific CD8 T cell response (Aiken et al., 1994; Collins et al., 1998; Garcia and Miller, 1991) • influences cell cycle machinery (Shaheduzzaman et al., 2002)
Vpr ("viral protein R")	• intracellular, mainly inside the nucleus (Lu et al., 1993), but also inside virion	• participates in the nuclear import of the pre-integration complex (Heinzinger et al., 1994) • influences cell cycle (Goh et al., 1998; He et al., 1995) • regulates NF-κB (Ayyavoo et al., 1997)

Tab. 1.1: Function and localization of small accessory proteins of HIV-1

Gene product	Localisation	Function
Vpu ("viral protein U")	• inside the cytoplasm	• Degradation of CD4 inside ER and transport of HIV-1 Env to the cell surface (Willey et al., 1992) • Assembly and release of virions from the cell (Cohen et al., 1988; Strebel et al., 1988)

Upon viral entry into host cells, the RNA genome is reverse transcribed into double-stranded DNA, which is subsequently integrated into the chromosome. The integrated viral genome, called proviral DNA, is flanked on both ends by long terminal repeats (LTR) made of U3, R and U5 (figure 1.1). The LTR contains cis-active elements that are important for the integration of the viral DNA into the host genome and for the transcription of the retroviral genome (Haseltine, 1991; Klotman et al., 1991; Steffy and Wong-Staal, 1991).

Transcription of the viral genome is initiated upon activation of the host cell and depends on the presence of various cellular transcription factors. Most important are the transcription factors nuclear factor kappa B (NF-κB) and nuclear factor of activated T cells (NF-AT) that are expressed in activated CD4 T cells (Kinoshita et al., 1997; Perkins et al., 1993). The cellular RNA polymerase II complex transcribes the proviral DNA. Early transcripts are multiply spliced and code for the regulatory proteins Tat, Rev and Nef. Tat is a potent transcriptional regulator and functions as an elongation factor that enhances the transcription of the proviral DNA about 100-fold. Rev promotes the export of unspliced or partially spliced RNAs. Later in the replication cycle and with increasing concentration of Rev, unspliced and partially spliced RNA transcripts of the structural proteins and the viral enzymes are translocated efficiently from the nucleus into the cytoplasm.

The *gag* and *pol* ORFs are transcribed into one mRNA. During translation a shift in the ribosomal reading frame occurs with a frequency of around 5% in an uridine-rich region that is located upstream of the *gag* stop codon. This suppresses translational termination at the *gag–pol* border and decoding of the mRNA may proceed past the *gag* stop codon. Therefore about 20-fold more Gag precursor protein than the Gag/Pol fusion protein is being synthesized.

The virion is formed at the plasma membrane by an ordered self-assembly of the viral structural proteins together with the genomic RNA. After closure of the spherical ribonucleoprotein shell, the immature virion is released from the cell and undergoes a functional and morphological maturation. During maturation the Gag precursor protein and the Gag/Pol fusion protein is cleaved into functional proteins by the viral protease.

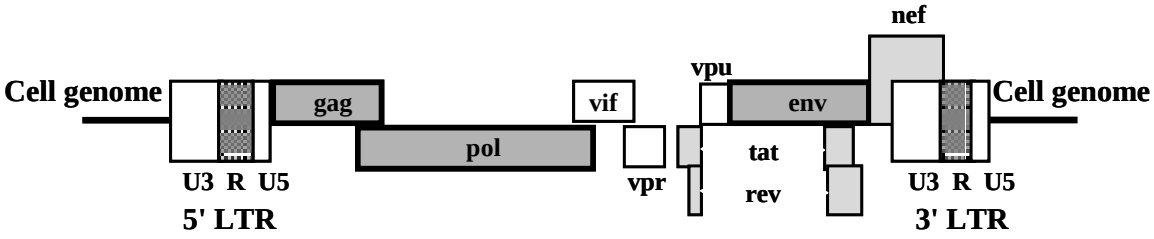


Figure 1.1 Organization of the HIV-1 genome
Besides the *gag*, *pol* and *env* ORFs that are characteristic for all retroviruses, the HIV genome contains additional regulatory genes. (Table 1.1) (according to Feinberg and Greene 1992)

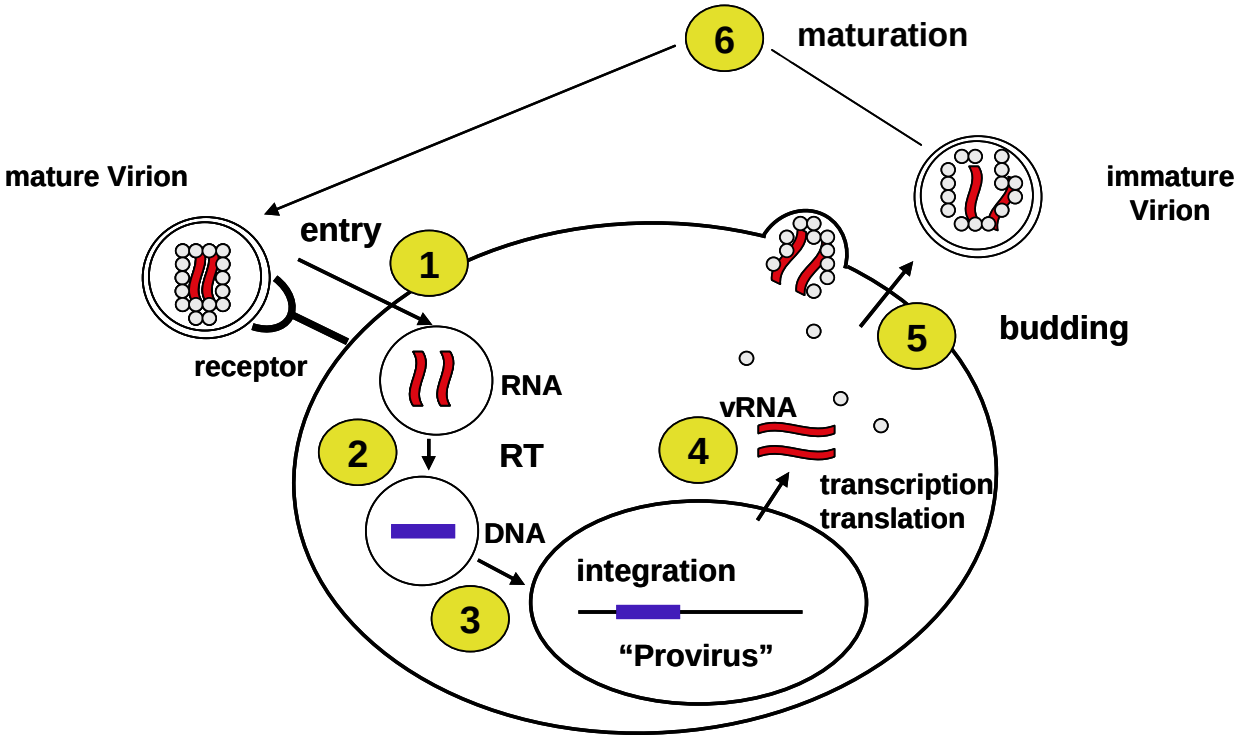


Figure 1.2 Replication cycle of HIV
1. HIV binds to CD4 and a cellular co-receptor on host cells and subsequently enters the cell after the viral membrane fuses with the cellular membrane. **2**. Reverse Transcription (RT) of the 2 viral RNAs into 1 double stranded DNA. **3.** The pre-integration complex is transported into the nucleus and the viral DNA is integrated into the host cell chromosome. **4**. Proviral gene expression and transcription of genomic viral RNAs (vRNA). **5.** Virus self-assembly at the cellular membrane and viral budding. **6.** Maturation of virion and infection of a new cell.

The replication of HIV is coupled with a mutation rate of 2.5 x 10^{-5} per cycle per replication round. Responsible for this mutation rate is mainly the low-fidelity reverse transcriptase (RTase). It lacks the proof reading mechanisms that is common for cellular DNA polymerases and produces "sequence errors" during reverse transcription of the genomic RNA into cDNA.

1.1.2 The HIV virion

The HIV virion is spherical and about 100nm in diameter. It has a lipid membrane, which is derived from the cellular membrane of the infected cell (Figure 1.2). Integrated into the virus envelope is the transmembrane glycoprotein gp41, which is non-covalently associated with the highly glycosylated protein gp120. Besides gp41, cellular membrane proteins are also inserted into the viral membrane. Gp41 and gp120 both are highly variable. The latter interacts with the cellular receptor CD4 and, depending on the HIV strain-specific tropism, either with the cellular co-receptor CCR-5 or CXCR-4. Subsequently gp41 initiates fusion of the viral membrane with the cellular membrane.

All the capsid and nucleocapsid proteins are derived from the Gag precursor protein. The matrix protein p17 is associated with the inner surface of the viral membrane. P17 is important for incorporation of the envelope protein into mature virions (Yu et al., 1992). It also assists with the transport of the pre-integration complex and acts in targeting this complex into the nucleus in quiescent, non-dividing cells (Bukrinsky et al., 1993; von Schwedler et al., 1994). Furthermore, because of its affinity for the cytoplasmic domain of gp41, p17 also plays an important role in positioning viral cores at the budding sites inside the infected cell (Dorfman et al., 1994; Yu et al., 1992).

The capsid of the lentiviruses is conical in shape and consists of the capsid protein p24. Domains mainly at the C-terminus of p24 are essential for the assembly of the spherical ribonucleoprotein shell (Zhang et al., 1996), while the N-terminal region is required for the formation of the mature, cone shaped core. The middle region of p24 contains the “major homology motif”, which is well conserved among different retroviruses and is important for the formation of infectious virions (Mammano et al., 1994). The smaller proteins p7 and p6 are derived from p15. The nucleocapsid protein p7 binds directly to the viral genome whereas p6 links the viral membrane to the viral capsid. Besides the structural proteins the HIV virion contains the viral enzymes reverse transcriptase, integrase and protease and the viral genome, two copies of single-(+) stranded RNA molecules.

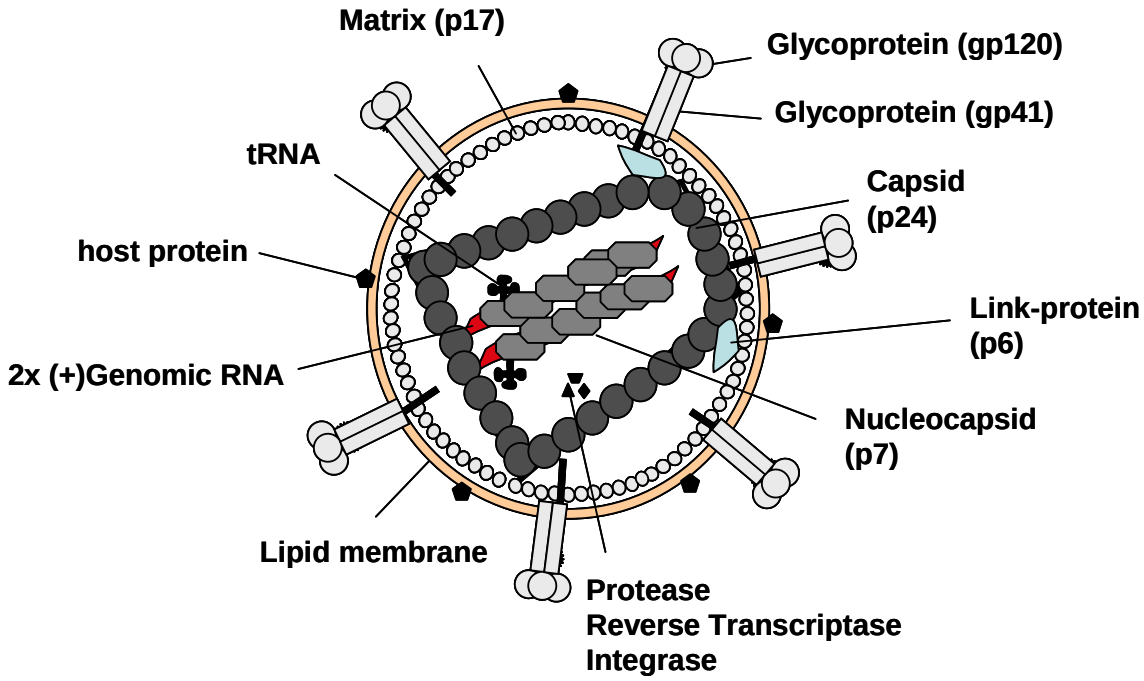


Figure 1.3. Schematic structure of an HIV virion. Inside the virion there is the conical capsid made of the capsid protein p24. The capsid contains the enzymes reverse transcriptase, integrase and protease, cellular tRNAs and the viral genome, two copies of single-(+) stranded RNA molecules, which are complexed by the nucleocapsid protein p7. The capsid is surrounded by a lipid membrane. The viral glycoprotein gp41 and host cell surface proteins are integrated into the viral membrane. Gp120 is non-covalently bound to gp41. The matrix protein p17 and the link-protein p6 are associated with the inner surface of the viral membrane – the latter connects the viral capsid to the membrane.

1.1.3 Phylogeny and evolution of HIV

HIV-1 and HIV-2 are closely related to the simian immunodeficiency viruses (SIV). HIV-2 is related to the SIVsm from the monkey sooty mangaby and HIV-1 to the SIVcpz from the chimpanzee. HIV-2 mainly occurs in Western Africa. HIV-1, which contributes to most of the pandemic, can be subdivided into the three groups, group M (main), group O (outlier) group N (new). For these groups, independent zoonotic transmission events must have occurred, as each of these groups is more closely related to a particular SIVcpz strain than among themselves. Fig.1.4 shows the phylogenetic relationship of SIVcpz to HIV-1 group M. HIV-1 group O and group N appear to be rare and are found

almost exclusively in the Central-West African countries Equatorial Guinea and Cameroon. Group M spread most efficiently into the human population and is the cause of the HIV pandemic. Based on phylogenetic analysis of a large number of HIV-1 sequences collected over the years and the fact that the first identified HIV group M infection case dates back to 1959 (Isolate ZR.59) it is believed that the founder of the HIV-1 group M crossed from chimpanzee to human early during the 20^{th} century (Korber et al., 2000).

HIV-1 is amongst the most genetically variable human pathogen. Geographically defined epidemics can be characterized by the dominance of 9 distinct genetic subtypes of HIV-1 group M (subtypes A, B, C, D, F, G, H, J and K). The phylogenetic analysis of the predominant group M subtypes is shown in figure 1.4. Of the 9 different subtypes, only 4 of the 9 subtypes contribute to 90% of the HIV pandemic (Kijak and McCutchan, 2005). Subtype C is responsible for more than 50% of world wide infections, followed by subtype B, subtype A and subtype D. The global distribution of HIV-1 subtypes is shown in figure 1.5. Parameters contributing to this global pattern of subtype distribution remain speculative. The highest diversity of HIV-1 subtypes and recombinants is seen in the central African countries, which is likely to relate to the long time period since the start of the HIV epidemic in that particular region.

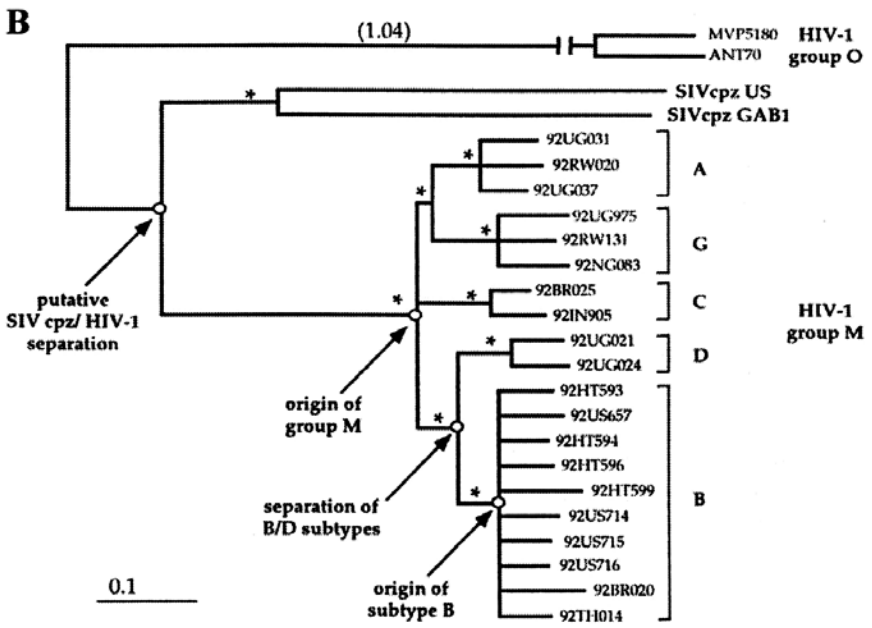


Figure 1.4. Phylogenetic analysis of predominant subtypes of HIV-1 group M and SIV_{CPZ}. The phylogenetic tree shows the genetic distances and relationships between subtypes A, B, C, D and G of HIV-1 group M, SIV_{CPZ} and HIV-1 group O.

1.1.4 Multiple infection and recombination

It is known that an individual can be infected by more than one HIV strain (Altfeld et al., 2002; Hoelscher et al., 2002) that subsequently can give rise to recombinant viruses. The basis for the generation of these recombinant forms of HIV is the infection of the same cell with more than one HIV-1 variant. Jung et al demonstrated that one infected cell can habour multiple genetically distinct proviruses (Jung et al., 2002). As a consequence, two RNA strands that are derived from two different proviruses have the opportunity to assort at the packaging stage. Upon infection of a new host cell, recombination of the two genomic RNA molecules occurs via the mechanism of strand switching of the reverse transcriptase during the process of reverse transcription (An and Telesnitsky, 2002; Jetzt et al., 2000). Within an infected individual carrying multiple HIV subtypes, ongoing inter-strain recombination may contribute to a strong fluctuation of many different molecular HIV-1 forms over time (Gerhardt et al., 2005; McCutchan et al., 2005).

A growing number of HIV-1 recombinant forms has been identified within the last few years. Some epidemics are characterized by so-called circulating recombinant forms (CRFs) or by unique recombinant forms (URFs). A CRF is a recombinant HIV strain that has achieved to spread into a larger population. There are two recombinant forms of global importance. CRF01_AE predominates in Southern Asia and CRF02_AG in some Western and Central African countries (Fig 1.5). At present, there are at least 16 circulating recombinant forms (CRFs) and many unique recombinant forms (URFs) described (http:// www.hiv.lanl.gov/content/hiv-db/CRFs/ CRFs.html). A high proportion of URFs is exclusively seen in the context of an epidemic with mixed HIV subtypes and is indicative for a relatively high frequency of multiply infected individuals. This phenomenon is characteristic for the regional HIV-1 epidemics of Eastern and Central African countries.

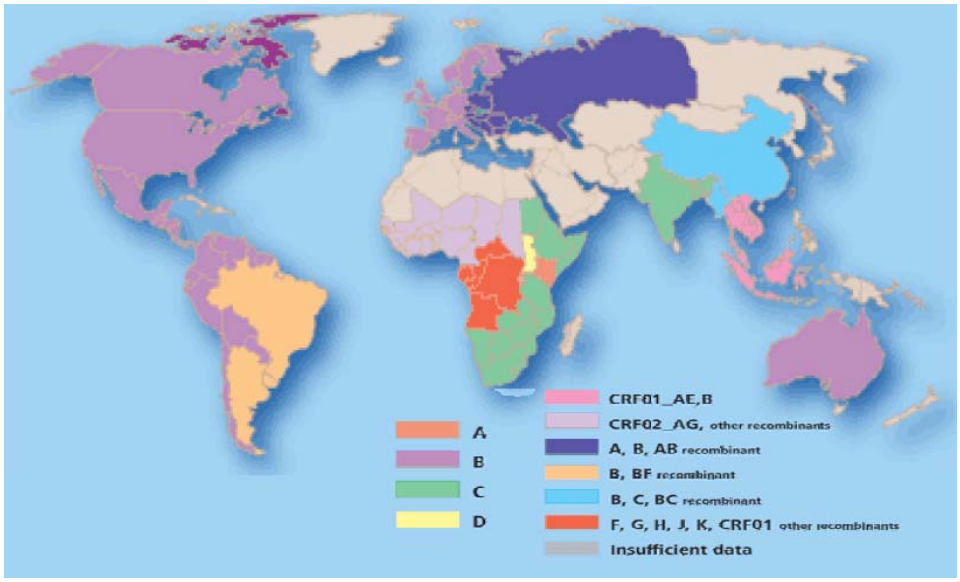


Figure 1.5. Regional epidemic patterns of HIV-1 subtypes and recombinant forms. Shown is the global distribution of subtypes and recombinant forms of HIV-1.

1.2 The human immune system

The meaning of the word "immunity" refers to the protection from an infectious agent. All cells, molecules and organs that contribute to this immunity are summarized in the term "immune system". The immune system can be subdivided into two branches that closely interact with each other: The innate immune system includes phagocytic cells such as neutrophiles and macrophages, natural killer cells (NK cells) that – upon activation - are an early source of IFN gamma (IFNγ) and soluble molecules such as the components of the complement system. The components of the innate immune system have a relatively limited capacity to recognize microbe-specific structural features, but they serve as an important first and early line of defence against many pathogens (Janeway et al., 2003).

The specific immune response is initiated by professional antigen-presenting cells (APC), such as dendritic cells (DCs), macrophages and B cells that can take up antigen. APCs are characterized by high level expression of major histocompatibility complex (MHC) and co-stimulatory molecules on their cell surface, that are essential for the activation of T cells. Especially DCs play a central role in the initiation of the adaptive immune response. Most of the common pathogen entry routes such as the skin and mucosal surfaces are interspersed with DCs. After antigen encounter, DCs can take up antigen in the periphery and migrate to the draining lymph node, where they serve as a potent activator for the T cell response. The adaptive immune response relies on clonal expansion of antigen-specific B cells and T cells. Both of these cell types express a highly specific antigen receptor on their cell surface and can differentiate into "memory cells". Memory cells are long lived and are not depleted after antigen is cleared. Therefore they can contribute to a long lasting immunity to an infection with the same pathogen.

The high specificity of the B and T cell antigen receptor is based on somatic gene rearrangements of highly variable gene segments that code for the antigen binding site of the antigen receptor, namely the B cell receptor (BCR) and the T cell receptor (TCR). The mechanism of somatic gene rearrangement is

unique to lymphocyte development and has evolved with the vertebrates (Janeway et al., 2003).

1.2.1 The humoral immune response

The humoral immune response to infection involves the production of pathogen-specific antibodies by activated B cells, the binding of antibodies to the pathogen and direct neutralization or elimination of the pathogen by phagocytic cells or the complement system. B cells express an antigen specific membrane associated BCR that is secreted upon antigen binding and activation of the B cell. The secreted form of the BCR is referred to as an antibody. Depending on its specificity, the BCR can bind proteins, carbohydrates, DNA or various other molecules. In contrast to pathogen-specific CD8 T cells, B cells do not need antigen presentation in the context of the MHC, and therefore are less restricted in their capacity to recognize antigens. However, they do require "help" by pathogen specific CD4 T cells to produce antibodies of high affinity and efficiency. An effective humoral immune response is the main determinant of protective immunity after vaccination and most of the antiviral vaccines that are currently available are based on the induction of a strong and efficient antibody response (Janeway et al., 2003).

1.2.2 Antigen presentation by MHC class I molecules

After a virus has used the cells's biosynthetic machinery to express its viral proteins, these proteins are partly degraded by the proteasome inside the cytoplasm. The proteasome cleavage products are peptides of 8-11 amino acids length that are actively transported by the transporter proteins – TAP1 and TAP2 - into the lumen of the endoplasmatic reticulum, where they are loaded onto MHC class I molecules (Niedermann et al., 1999). The MHC-peptide complex is then transported to the cell surface and presented to CD8 T cells. All nucleated cells are capable to express MHC class I molecules and its expression is upregulated by various cytokines. Specific CD8 T cells that recognize antigenic peptides in the context of MHC class I molecules are activated and differentiate into armed effector cells.

MHC class II molecules are almost exclusively expressed on the surface of professional antigen presenting cells (APCs) and present antigenic peptides to

CD4 T cells. Antigens are engulfed by APCs and degraded inside the endosomal compartment. Antigenic peptides are then loaded onto the MHC class II molecules and translocated to the cell surface (Janeway et al., 2003).

The genes encoding for the MHC molecules are highly polygenic. There are more than 500 MHC class I and app. 500 MHC class II variant genes described. These variant genes are referred to as alleles and the human MHC molecules are referred to as Human leukocyte antigens (HLA). Polygeny differs between the 3 HLA class I genes A, B and C. The HLA-B genes are the most variable with more than 380 different alleles described by the year 2001. HLA-C alleles are the least polygenic with 93 alleles described by 2001 (Janeway et al., 2003).

The protein products of individual HLA alleles can differ from one another by up to 20 amino acids, making each variant protein quite distinct. The polymorphism is largely confined to amino acids lining the peptide binding groove and affects the peptide binding properties of a HLA molecule. Particular amino acid residues anchor the peptide into pockets within the peptide binding groove. Stable binding of a peptide to a particular HLA molecule depends on these anchor residues and the amino acids lining the peptide binding groove. Anchor residues do not need to be identical for all peptides that bind to the same HLA molecule, but are always related – hydrophobic, polar, charged or aromatic. The set of anchor residues binding to a given HLA molecule is called a sequence motif (Janeway et al., 2003). For example HLA-B0702 predominantly presents peptides that bear the hydrophobic amino acids proline or valine in position 2 and a leucine at the C-terminus. HLA-B1510 presents peptides with a histidin in position 2 and a leucine residue at C-terminus (http://www.hiv.lanl.gov/content/immunology motif_scan/ motif_ scan). Individual differences in the expressed HLA alleles therefore can have a profound effect on how individuals can deal with a particular pathogen. As a consequence, the specificity of T cell recognition within an individual is largely dependent on the set of HLA alleles expressed.

On a population level the MHC polymorphism has one important advantage, because not all individuals in a population will be equally susceptible to a given pathogen and its spread can therefore be limited. At an evolutionary time scale exposure to certain pathogens can select for particular HLA alleles within a given population. For example the HLA-B53 allele has been shown to be associated with recovery a potentially lethal form of Malaria (Hill et al., 1992). This allele is very common in West African population, where Malaria is endemic and rare elsewhere. Curiously, HLA-alleles also influence the body scent of an individual and can affect reproductive behaviour (Singh, 2001; Yamazaki et al., 1979). Potential partners with different HLA-alleles to oneself are preferred as mating partners. These findings indicate the importance of the HLA gene locus for human evolution.

1.2.3 The cellular immune response

In contrast to B cells, T cells only recognize antigen after presentation through MHC molecules. T cells express the antigen-specific T cell receptor complex that consists of the T cell receptor (TCR), CD3 and either co-receptor CD4 or CD8. CD4 and CD8 bind to conserved regions of the MHC Class II and Class I molecules respectively. The activation of naïve T cells requires not only recognition of MHC presented peptides by the TCR, but also the presence of the co-stimulatory molecules CD80 and CD86 that are expressed on the surface of professional antigen presenting cells (Janeway et al., 2003).

CD4 T cells, also called T helper cells (T_H cells), are central to the function of the immune system as they are specialized to provide “help” to other cells. CD4 T cell help is essential for the generation of an effective antibody response, for the activation of macrophages and the maintenance of an effective CTL response. The role of CD4 T cells has intensively been studied in a CD4 knock-out mouse model. Upon experimental infection with the LCMV virus, CD4 knock-out mice have an impaired antibody response and a decreased frequency of antigen-specific memory CD8 T cells (Rahemtulla et al., 1991; von Herrath et al., 1996). In HIV infected humans, the important role of CD4 T cells is also exemplified. Once most of the CD4 T cells are

eliminated during the HIV infection, individuals are highly susceptible to a whole range of opportunistic infections.

CD4 T cells can be subdivided according to their cytokine secretion pattern. T_H1 cells predominantly secrete IFNγ, Tumor Necrosis Factor alpha (TNFα), interleukin 2 (IL-2) and granulocyte macrophage colony stimulating factor (GM-CSF), whereas T_H2 cells predominantly secrete IL-4, IL-5, IL-6 and IL-10. As a consequence both subsets differ in their respective function. T_H1 cells are mainly specialized to activate macrophages infected with intracellular pathogens such as Mycobacteria or Leishmania species (Paulnock et al., 1992). T_H2 cells predominantly help B cells in the induction of an efficient antibody response and can be important in the downregulation of inflammatory responses by secretion of IL-10. Differentiation into either T_H1 or T_H2 cells is largely dependent on the cytokine microenviroment during the inductive phase of T cell priming (Janeway et al., 2003).

The function of CD8 T cells is to kill cells infected with intracellular pathogens. They are important to eliminate pathogens that are not susceptible to elimination by antibodies due to their intracellular location. Upon encounter with an infected cell the lytic granules move towards the cell-cell contact area and release the effector molecules perforin and granzymes. Perforin inserts and forms pores into the target cell membrane, whereas granzymes enter through these pores and induce apoptosis of the target cell. For this reason CD8 T cells are often referred to as cytotoxic T lymphocytes (CTL). Antigen specific CD8 T cells can also secrete various cytokines such as IFNγ and TNFα and various other cytokines. IFNγ directly inhibits replication of some viruses and increases expression of MHC class I molecules and other molecules involved in peptide loading of the MHC class I molecules (Pamer and Cresswell, 1998).

1.2.4 The HIV-specific immune response

Detection of HIV-specific antibodies after seroconversion is the method of choice to diagnose an HIV infection. Most infected individuals mount a strong humoral immune response against a variety of HIV-1 proteins in the course of their infection. Antibodies targeting epitopes within the highly variable viral envelope protein gp120 may have the capacity to efficiently neutralize the

virus. However, one typical characteristic of HIV-1 is that it rapidly escapes these antibody responses by the continuous generation of mutant viruses (Fenyo et al., 1996). Simultaneously, this high antigenic variability of the viral envelope glycoproteins also contributes to the poor cross-neutralizing capacity for different HIV-1 strains. Preferential destruction of HIV-specific CD4 T-helper cells by HIV (Douek et al., 2002) may further decrease efficiency of the HIV-specific humoral immune response.

Several observations underline the importance of the humoral immune response in HIV infection. Antibodies that activate the classical pathway of the complement system may in part contribute to the control of plasma viremia after acute infection (Aasa-Chapman et al., 2005). Passive transfer of neutralizing antibodies against a highly pathogenic SHIV strain can protect Rhesus macaques from infection (Aasa-Chapman et al., 2005; Mascola et al., 1999; Mascola et al., 2000). Furthermore immunization with Env encoding DNA and recombinant Env protein resulted in sterile immunity of monkeys against SHIV-HXBc2 challenge (Letvin et al., 1997). A recent study in humans has demonstrated a delay of HIV-1 rebound after cessation of antiretroviral therapy through passive transfer of human neutralizing antibodies (Trkola et al., 2005).

Unfortunately the induction of broadly cross-reactive antibodies with the capacity to neutralize a diversity of primary virus isolates has not been achieved until today. Some potentially neutralization epitopes are poorly immunogenic (Liang et al., 1999; Zwick et al., 2001), others are masked by neighbouring N-linked glycans (Reitter et al., 1998). One highly conserved antigenic region, a conserved co-receptor binding site, is exposed only for a very limited time period during infection of a new host cell. Antibodies directed against this conserved co-receptor binding site have been found to neutralize a wide range of HIV-1 primary isolates and even HIV-2 primary isolates (Shaw G., Talk at the AIDS Vaccine Conference Lausanne 2004). In this regard attempts to achieve sterilizing immunity by vaccination still hold some promises.

It is generally accepted that HLA Class I restricted CD8 T cell responses largely contribute to the control of HIV-1 replication in infected individuals. The appearance of HIV-specific CD8 T cells is temporally associated with the decline of plasma viremia shortly after acute infection (Borrow et al., 1994; Koup et al., 1994). During the chronic phase of infection, an inverse correlation between the frequency of CD8 T cells specific for (two) HLA-A2 restricted epitopes and the viral load was found (Ogg et al., 1998). Perhaps, the most conclusive data about the protective role of CD8 T cells comes from the SIV-rhesus macaque model, in which depletion of CD8 T cells leads to a transient rise in SIV plasma viremia and loss of CD4 T cells until the CD8 T cell subset recovers (Jin et al., 1999; Schmitz et al., 1999). However, the parameters determining efficient control of viral replication had still to be defined. With improvements of the technology to rapidly and accurately characterize CD8+ T cell responses in HIV-1 seropositive individuals, many new T cell epitopes have been detected, permitting identification of immunodominant regions across the whole HIV proteome. This resulted in the establishment of Gag and Nef as the most frequently targeted HIV-1 proteins contributing to more than half of the total HIV-specific T cell response in most individuals (Addo et al., 2003; Frahm et al., 2004). That the Gag-specific CD8 T cell response may play a special role in slowing down progression to AIDS was first shown in 1995 (Riviere et al., 1995) and numerous more recent studies supported this finding (Edwards et al., 2002; Masemola et al., 2004; Novitsky et al., 2003; Ramduth et al., 2005). In contrast, other studies have not found a correlation between the Gag-specific T cell response and the viral load (Addo et al., 2003; Betts et al., 2001). Surprisingly, Nef- or Env-specific CD8 T cell responses have never been associated with efficient viral control or even found to be positively correlating with a high viral load, indicating that CD8 T cells of different specificity may differ in their respective antiviral efficiency (Betts et al., 2001).

The CD8 T cell specificities found in one individual are dependent on the HLA Class I background. The identification of HLA class I alleles that contribute to a slow HIV disease progression has been vigorously pursued over the years. Particular HLA class I alleles, such as B57 (Altfeld et al., 2003), B5801 and B4201 (Kiepiela et al., 2004) are associated with slower disease progression,

while other HLA class I alleles, such as B5802, B4501 and B1510 (Kiepiela et al., 2004) appear to correlate with a higher viral load. Direct evidence of how "protective" HLA alleles may exert this effect is still lacking, although it has been shown that within a given population frequent HLA alleles can influence the predominant amino acid sequences found in targeted HIV epitopes (Kiepiela et al., 2004; McMichael and Klenerman, 2002).

HIV amino acid sequence variability plays an important role in both HIV-specific humoral and cell-mediated immune responses. The viral Env and Nef proteins are both very variable with up to 30% sequence differences, whereas Gag and Pol are more conserved between different HIV-1 isolates (<10% sequence differences). To complicate matters for the HIV-specific immune response, even within an HIV-1 infected individual, the HIV mutant spectrum, or "quasispecies" can reach a divergence of 10% (Meyerhans et al., 1989; Plikat et al., 1997). Sequence variability within CD8 T cell epitopes varies and HIV-1 can escape pressure from the CD8 T cell response by mutating targeted epitope sequences. Nonetheless, some antigenic protein regions are well conserved because of their particular importance for viral fitness. Amino acid point mutations within these epitopes may lead to a severely decreased replicative capacity of the virus (Goulder and Watkins, 2004). To identify such CD8 T cell epitopes or protein regions with a high density of such epitopes may contribute to HIV vaccine design. The whole extent to which HIV genetic diversity impacts T cell epitope recognition is not completely established. It was only very recently that peptides representing multiple subtypes have been used to screen for multiple subtype reactivity (Barugahare et al., 2005; Coplan et al., 2005). It now appears that sequence variability does not always directly predict the degree of cross-subtype CD8 T cell recognition, although the level of cross-subtype responses is substantial for HIV-1 Nef and strong, but somewhat lower, for the Gag protein (Coplan et al., 2005).

1.3 Pathogenesis of HIV

HIV and the related SIV cause a depletion of CD4 T cells in infected individuals. CD4 T cells are very important regulators of the immune system. They "help" B cells to produce high affinity antibodies, they activate APCs and

they play a role in the maintenance of memory CD8 T cells (von Herrath et al., 1996). The respective depletion of CD4 T cells during an HIV infection is the cause for a whole range of virus-induced immune defects called AIDS.

Infection with HIV generally occurs after transfer of body fluids such as blood, semen or vaginal secretion from an infected person. Unprotected sex is the most common mode of transmission, followed by perinatal transmission from an HIV-infected mother to the child. Transmission through breast feeding also accounts for a high number of HIV infections in young children - especially in highly affected populations of Sub-Saharan Africa. Besides these, contact with contaminated blood or blood products can cause infection with HIV.

During the initial infection, the virus is either introduced directly into the blood system or through lesions of the mucous membranes. Another mechanism how the virus crosses the mucosal barrier is transcytosis (Bomsel H., 1997). The epithelial cells of the mucosal surfaces lining the reproduction tract are interspersed with a dense network of dendritic cells. The virus attaches to DCs via a protein called DC-SIGN (Geijtenbeek et al., 2000). Binding to DC-SIGN stabilizes the otherwise fragile virus and upon activation the DC migrates to the draining lymph node, where this “hitch-hiking” virus encounters a large population of CD4 T cells (Geijtenbeek and van Kooyk, 2003). After initial replication, the virus disseminates quickly into the blood stream and into other primary lymphatic organs. The lymphatic system is the primary place of HIV replication and serves as an important viral reservoir.

HIV primarily infects and destroys the memory CD4 T cells (Chun et al., 1997; Spina et al., 1997). Most of the bodies’ memory CD4 T cells are found in the gut associated lymphatic tissues (GALTs) – predominantly in the lamina propria. The gut is a primary entry site for food and water borne pathogens and hence the GALTs are among the largest and most dynamic of all primary lymphatic organs. To prevent pathogens from entering the body, the lamina propria harbours a high level of the activated memory CD4 T cells. These cells co-express CD4 and the HIV co-receptor CCR5 (Veazey et al., 2000). This activated CCR5 positive memory subset of CD4 T cells is easily infected by

HIV and contributes to the high level of early virion production. GALTs therefore are viewed as the most important site of active viral replication and T cell depletion during acute infection.

The acute HIV disease is often characterized by flu-like symptoms, which usually disappear after viral replication is brought under control by the HIV-specific immune response. Plasma viral load drops to the "viral set point" and simultaneously CD4 T cell levels rise although not quite to the levels seen before HIV infection (Fig.1.6). The plasma viral load at the viral set point correlates well with the time from infection to the onset of AIDS symptoms (Mellors et al., 1996).

The dynamic of the CD4 T cell depletion during the acute infection has been studied extensively in the SIV-Rhesus macaque model and resembles the dynamic of HIV infection in humans. However, it should be considered that SIV is usually introduced intravenously with a high number of virions. The extent of memory CD4 T cell destruction is immense during the acute phase of infection. After inoculation SIV eliminates around half of the host's memory CD4 T cells within 2 weeks (Mattapallil et al., 2005). In contrast naïve CD4 T cells are virtually unsusceptible to SIV infection (Mattapallil et al., 2005). Interestingly, the majority of infected cells during the acute infection were resting CD4 T cells and infected cells were not restricted to the gut associated lymphoid tissue (Mattapallil et al., 2005). Although these infected resting memory CD4 T cells produce less virions than activated dividing cells, they serve as a major viral reservoir. Therefore the acute phase of infection is likely to play a key role in SIV and HIV pathogenesis. The mechanisms contributing to this rapid destruction of the memory CD4 T cell subset are not well understood, but both viral induced cytolysis and CTL mediated mechanisms could explain this dramatic CD4 T cell loss (Johnson and Kaur, 2005; Mattapallil et al., 2005).

A gradual decline of CD4 T cells marks the chronic phase of HIV infection. As a consequence of the continuously falling CD4 T cell counts, a variety of opportunistic infections and cellular malignancies can occur in the infected

individual (Fig. 1.6). The asymptomatic chronic phase of infection can vary tremendously between infected individuals, but usually lasts for 5-15 years and is marked by a high turn-over rate of virions (Pantaleo et al., 1993). 10^9 to 10^{10} new virions are generated and eliminated each day and it is thought that 99% of the new virions are generated by newly infected CD4 T cells (Ho et al., 1995; Perelson et al., 1996; Wei et al., 1995). However, most destroyed CD4 T cells are replenished by newly generated cells. During chronic infection, the plasma viremia is commonly at comparatively low levels (Piatak, Jr. et al., 1993) and the frequency of infected CD4 T cells is fewer than 1 in 1000 (Haase, 1999).

The final AIDS phase of HIV infection is characterized by profound changes of primary lymphoid organ architecture. The network of follicular dendritic cells is destroyed and the germinal centers are exterminated. The lymphoid organ function collapses and with it the control of HIV replication and other opportunistic infections. Primary HIV strains isolated during the AIDS phase often display an altered cellular tropism. During acute and early infection HIV mostly binds to the target cell through the cellular co-receptor CCR5, while HIV isolates from later stages of infection often bind to the co-receptor CXCR-4 (Connor et al., 1997; Scarlatti et al., 1997). In contrast to the CCR5-tropic strains, primary isolates of CXCR4-tropic strains replicate rapidly in cell culture, contribute to high virion titers and mediate the formation of a high number of syncytial forming cells. The appearance of CXCR-4 tropism is associated with a bad prognosis (Scarlatti et al., 1997). It is not completely understood, what mechanisms contribute to this changing tropism and virulence, but it has been shown in a Rhesus macaque-$SHIV_{DH12R}$ model that infection of Rhesus macaques with this CXCR-4 tropic SHIV strain results in a massive loss of naïve CD4 T cells (Nishimura et al., 2005). In human HIV infection, sequence differences in the V3 region of gp120 correlate with this shift in cellular tropism (Liu et al., 1990; O'Brien et al., 1990).

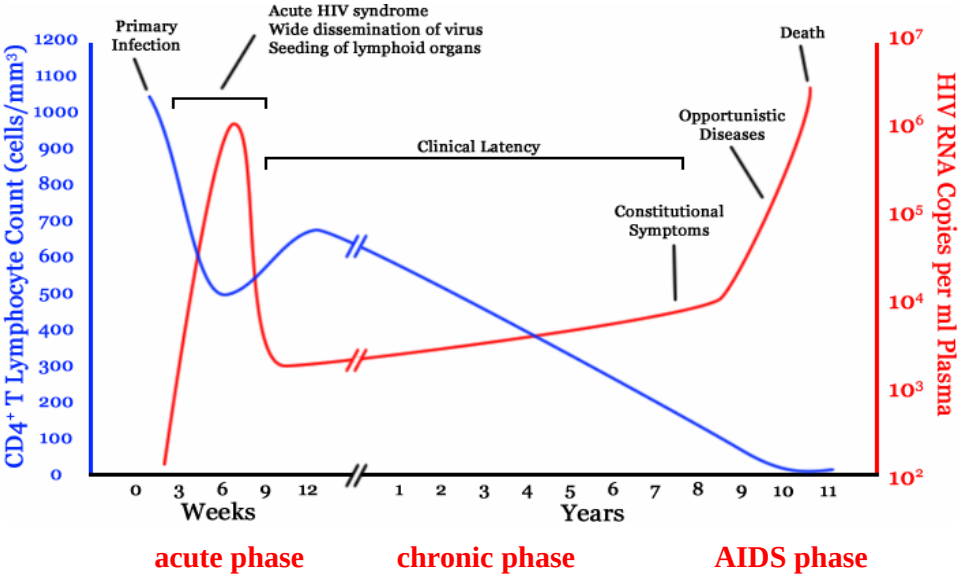


Figure 1.6. HIV and CD4 T cell levels over the course of an untreated HIV-1 infection. The acute phase of an HIV infection are often characterized by an acute influenza-like viral illness with high titers of virus in the blood. An adaptive immune response follows, which controls viral replication, but does not eradicate the virus. At the same time, CD4 T cell levels are largely restored. The chronic phase is asymptomatic in most HIV infected individuals and usually lasts for several years with a continuous decline of CD4 T cells. Opportunistic diseases and other symptoms become more frequent during the AIDS phase as the CD4 T cell count falls, starting at around 200-400 cells/µl.

1.4 The HIV superinfection study

The HIV Superinfection Study (HISIS) is an open cohort study conducted in Mbeya region in south-western Tanzania. The HISIS cohort consists of 600 high risk female bar-workers aged 18-35 years, who were recruited from 14 high-transmission areas within Mbeya region. The HIV prevalence at the beginning of the HISIS study in 2001 was app. 70%. The HIV incidence was high during the first year of the study but dropped subsequently (Hoffmann et al., 2004). The study was designed to investigate various aspects of HIV infection: I) the molecular characterization of the regional HIV-1 epidemic, II) the identification of subjects with multiple infection of different HIV subtypes, III) the evolution of viral quasispecies in individuals infected with multiple HIV-1 strains, IV) the analysis of the HIV-specific cellular immune response in the context of a multi-subtype epidemic and V) the identification of co-factors and parameters that increase the risk of HIV infection such as other sexually transmitted diseases and sexual behaviour. To enable a detailed analysis of the

infecting HIV strains, the cohort was followed up for a total of 4.3 years with 17 follow ups in 3-month intervals.

1.5 Objectives

Increasing evidence supports an important role for the HLA class I restricted CD8 T cell response in the control of HIV viral replication in vivo. Consequently, most of the current vaccine strategies are based at least in part on the induction of a strong HIV-specific CD8 T cell response. However, it is not completely understood why some HIV-infected individuals appear to control viral replication over extended time periods, while others progress rapidly despite a strong HIV-specific CD8 T cell response.

The anti-viral efficiency of the HIV-specific CD8 T cell response may be influenced by two important parameters, I) the HLA Class I background of the infected individual and II) the genetic variability of the virus. Hence advances in HIV vaccine design will require not only a better understanding of the immune responses that control this retrovirus but also a better understanding of these responses in the context of individual host genetics and an understanding of the implications of HIV diversity.

In this work three central questions concerning the HIV-specific CD8 T cell response were addressed:

1.) How do favourable HLA class I alleles contribute to the HIV-specific CD8 T cell response? What determines the anti-viral efficiency of the CD8 T cell response in general?

2.) What is the degree of HIV cross-subtype recognition within antigenic regions that are frequently recognized by HIV-specific CD8 T cells?

3.) Does the increased diversity of viral “quasispecies” within multiply HIV-1 infected individuals affect the HIV-specific CD8 T cell response?

To answer these questions, the HIV-specific CD8 T cell response was studied in the context of a mixed HIV subtype epidemic. The HIV-specific responses against Gag, Nef and Env were mapped to individual peptides that were representative of the locally occurring HIV subtypes A, C and D. Simultaneously, the genetic diversity of infecting HIV-1 strains was characterized and the HLA class I background of the study subjects was determined for each of the study subjects.

A number of fundamental insights into the HIV-specific T cell response could be derived from the analysed data.

2. Materials and methods

2.1 Chemical reagents

Brefeldin A	Sigma, Deisenhofen
DMSO	Sigma, Deisenhofen
EDTA	Becton Dickinson, Heidelberg
FACS Lysing Solution	Becton Dickinson, Heidelberg
FBS	Gibco, Invitrogen, Karlsruhe
Ficoll-Hypaque	Sigma, Deisenhofen
Gentomycin (50mg/ml)	Sigma, Deisenhofen
HEPES	Gibco, Invitrogen, Karlsruhe
Iso-Propyl alcohol	BMLS-Dar Es Salaam
L-glutamine	Gibco, Invitrogen, Karlsruhe
Nova-Red Substrate	Vector, Burlingame, California
PBS	Sigma, Deisenhofen
Penicillin/Streptomycin	Gibco, Invitrogen, Karlsruhe
PHA	Sigma, Deisenhofen
RPMI 1640	Gibco, Invitrogen, Karlsruhe
SEB	Sigma, Deisenhofen
Streptavidin-horseradish peroxidase	Pharmingen, San Diego
Trypane blue	Sigma, Deisenhofen
Tween-20	Sigma, Deisenhofen

2.2 Characteristics of study subjects

600 female bar workers were enrolled in the Mbeyan region, Tanzania between September and November 2000 for a prospective HIV Superinfection Study (HISIS) within the Mbeya Medical Research Programme (MMRP). After giving informed consent, each participant provided blood samples at enrolment and every three months for a four years period. During the study, all participants received health care that included treatment of all acute infectious diseases, of sexually transmitted diseases, and since 2003, cotrimoxazole prophylaxis for opportunistic infections for women with CD4+ T-cell counts

below 200. Since September 2004, all women with low CD4 counts have access to antiretroviral treatment within the Southern Highland Care and Treatment Programme. The HIV status of all study subjects was determined using two diagnostic HIV enzyme-linked immunoassays (ELISA), Enzygnost® Anti HIV1/2 Plus, Dade Behring, Liederbach, Germany and Determine™ HIV 1/2, Abbott, Wiesbaden, Germany). Discordant results were resolved using a Western Blot assay (Genelabs Diagnostics, Geneva, Switzerland). The genetic subtypes of HIV-1 were determined with a fluorescent genotyping assay, MHAacd (see 2.7). In April 2003, fifty-six of the HIV-1 positive participants were enrolled into the HISIS-CTL study, and HIV specific T-cell responses were measured for 4 consecutive follow ups (FU). Study subjects who did not belong to the HISIS cohort, were chosen from two cohorts: One cohort that evaluated the HIV prevalence and circulating subtypes (CODE study) in the Mbeya region and the other belonged to a pre-study for evaluation of surrogate markers for tuberculosis activity. After giving informed consent each subject was screened for HIV infection and their immune response to HIV was assessed.

2.2.1 Determination of CD4 T cell counts by immunophenotyping

The determination of the CD4 T cell count from whole blood was based on fluorescent activated cell sorting (FACS). FACS allows the characterization of single cells depends on hydrodynamic focussing. During this process the cells are accelerated and pass the laser light in the flow cell. Simultaneously the size and the granularity of the cells are determined by measuring the forward and the sideward scattered light, respectively and allows differentiation of lymphocytes from monocytes and granulocytes.

Immunophenotyping is the identification of cell surface antigens characteristic of subsets of leucocytes utilizing fluorescence-labeled monoclonal antibodies. Fluorescence-labeled antibodies bind to the leucocyte subset-specific surface antigens. The fluorescence of the chromophore is being activated by a laser and the emitted light is detected with an optical system. During this work a FACSCalibur (Becton Dickinson, Heidelberg) was used enabling measurement of 4 different chromophores simultaneously.

Procedure

The determination of CD4 cell counts was performed using the MultiTEST IMK Kit with TruCOUNT Tubes (Becton Dickinson, Heidelberg). Each TruCOUNT tube includes a defined number of small particles that are detected via fluorescence activation. This allows direct quantification of the cell concentration in the blood sample. The MultiSET software provides a lab-report with multi-colored dot plots and a physician's report, which shows the patient data and the normal reference ranges. The results are reported as the percentage of positive cells per lymphocyte population or as an "absolute count" which shows up the number of positive cells per microliter of blood. Briefly, 50µl of whole blood, collected into CPTA tubes, was added to the TruCount tubes. 10 µl of MultiTEST antibody mix (CD3-FITC-labeled; CD8-PE-labeled; CD45-PerCP-labeled; CD4-APC-labeled) was added, the tubes gently vortexed and incubated for 15 minutes in the dark at room temperature. To lyse the red blood cells, 450 µl of FACS lysing solution (Becton Dickinson, Heidelberg) was added, the sample gently vortexed and incubated for 15min in the dark. The acquisition and analysis was performed using the FACSCalibur together with the MultiSET software.

2.2.2 Determination of plasma viral load

Plasma viral load was determined with a COBAS Amplicor TM analyzer (Roche Diagnostics, Penzberg, Germany) that allows complete automatization of the amplification and detection steps of the Polymerase Chain Reaction testing process. The principle of this assay is briefly described in the following. As a first step, HIV-1 RNA is isolated from plasma by lysis of virus particles with a chaotropic agent followed by precipitation of the RNA with alcohol. The HIV-1 RNA then serves as a template for the amplification of a sequence of 142 bases located in a highly conserved region of the HIV-1 *gag* gene. The nucleotide sequence of the biotinylated primers has been optimized to yield equivalent amplification of Group M subtypes of HIV-1. This region is reversely transcribed into DNA and subsequently amplified by the enzyme *Thermus thermophilus* DNA polymerase. As a quantitative standard, a known amount of control RNA (Quantitative Standard target RNA,

included in the Amplicor 1.5 reagent kit, Roche Diagnostics) is treated in the same way. The amplicon is then chemically denatured to form single stranded DNA. The denatured amplicon preparations are added to designated wells of a microwell plate coated with HIV-1 specific oligonucleotide probes and a series of 5-fold dilutions of the amplicons is made. The biotin-labeled HIV-1 amplicons hybridize to the plate-bound HIV-1 specific probes and are captured onto the plate. This specific hybridization of the amplicons increases the overall specificity of the reaction. Following the hybridization reaction, the microwell plate is washed to remove unbound material and avidin-conjugated horseradish peroxidase (Av-HRP) is added to the plate. The avidin binds to the biotinlabeled amplicons captured by the plate-bound oligonucleotide probes. The microwell plate is washed again to remove unbound Av-HRP and the substrate mixture containing hydrogen peroxide and tetramethylbenzidine (TMB) is added to each well. The reaction is stopped by the addition of dilute sulfuric acid and the absorbance of each well is read at 450 nm. To calculate the original concentration of the HIV-1 RNA, the Total HIV-1 optical density (OD) is divided through the total control RNA OD and multiplied by 40 x the number of control RNA copies that was used. The total HIV-I OD of the specimen is calculated by multiplying the OD of the HIV well with the lowest OD in the range of 0.2 to 2.0 OD units by the dilution factor associated with that well. Similarly, the Total control OD for the specimen is calculated by multiplying the OD of the control well with the lowest OD in the range of 0.3 to 2.0 OD units by the dilution factor associated with that well.

Procedure

The standard specimen processing procedure that was used throughout the study allows the determination of the viral load from 400 to 750 000 RNA copies/ml. Whole blood was collected into standard blood collection tubes containing the anticoagulant ethylenediaminetetraacetate (EDTA, Sarstedt). Plasma centrifugation was performed at 1000x g for 20 minutes. 700µl of plasma / sample was then stored in screw-cap tubes at -80°C until viral load detection according to the instruction of the manufacturer. Viral Load determination was performed in part by trained laboratory personal according to the standard operating procedure accessible at http://aactg.s-

3.com/pub/download/vir/AMPLICOR-HIV-1-MONITOR-TEST-Standard-Method-Version-1.5.pdf.

2.2.3 HLA typing

HLA-A, -B, and -C class I alleles were typed by DNA sequencing on the automated sequencer ABI 3700 (Applied Biosystems, Applied Biosystems, Foster City, USA) essentially as described by Turner et al. (Turner et al., 1998). DNA was extracted from 200 000 PBMC using the High Pure viral nucleic acid kit (Roche Diagnostics, Mannheim). Exons 2 and 3 (encoding the peptide binding groove) were amplified in a first round PCR as previously described (Turner et al., 1998). The initial exon 2-3 amplicon then served as a template for the nested and/or hemi-nested amplification of exon 2. Exon 3 was similarly amplified in a nested fashion. Both the first round and nested PCR primers are located in the introns flanking the amplified exon(s) (Turner et al., 1998). The nested PCR primers are alternately tailed with an M13 universal DNA sequencing primer sequence. Bidirectional M13 cycle sequencing of exon 2 and of exon 3 was executed with BigDye V3.1. HLA DNA sequence typing analysis was completed with Assign SBT software (Conexio Genomics). DNA sequence ambiguities were resolved using the PEL-FREEZ SSP Uni tray kit (Invitrogen, USA). The HLA-typing of the specimen was facilitated by Steve Cate and Dr. William Hildebrand (University of Oklahoma Health Science Center).

2.3 Separation, counting and cryo-preservation of peripheral blood mononuclear cells

2.3.1 Buffers and solutions

Freezing medium

RPMI 1640	
Heat-inactivated FBS	20 % (v/v)
DMSO	10% (v/v)
Penicillin	100 U/ml

Streptomycin	100 µg/ml
Gentomycin	0.25% (v/v)

Fetal Bovine Serum was heat-inactivated for 30 minutes at 56°C.

R10 medium

RPMI 1640	
Heat-inactivated FBS	10 % (v/v)
L-Glutamine	2mM
Hepes buffer	10mM
Penicillin	100U/ml
Streptomycin	100µg/ml

Fetal Bovine Serum was heat-inactivated for 30 minutes at 56°C.

Trypane Blue solution (prepared with double destilled water)

Trypane blue	0.36 % (w/v)
NaCl	0.9 % (w/v)

Trypane Blue solution was sterile filtrated with a syringe micro-filter

2.3.2 Blood collection and plasma centrifugation

From each subject 50 ml of blood was collected into CPTA tubes and delivered to the processing lab within 4 hours. The plasma was then separated from the cellular blood components by centrifugation at 400x g for 15 minutes at room temperature, dispensed into Plasma-Cryotubes and stored at -20°C for further serological assays or at -180°C for determination of the plasma viral load. The plasma was then replaced by adding the same amount of sterile PBS.

2.3.3 Isolation of peripheral blood mononuclear cells (PBMC)

Before adding the diluted cellular blood components, 15ml of Ficoll-Hypaque (Sigma, Deisenhofen) was added to the Leucosep tubes (Greiner, Frickenhausen, Germany) and centrifuged for 1 min. at 400x g. Leucosep tubes allow the separation of PBMC from diluted blood by Ficoll-Hypaque density gradient centrifugation. The diluted cellular blood components were then transferred into two 50ml Leucosep tubes. Centrifugation causes the PBMC to form a distinct layer at the PBS-Histopaque interface. Cells of higher density –

namely the granulocytes and erythrocytes – will be trapped in the lower part. Blood components of low density - the blood platelets - will remain in the supernatant.

Procedure

After adding the diluted blood into the Leucosep tubes, these were centrifuged at 1000 x g for 10 minutes at room temperature without using the centrifuge brake. After centrifugation the supernatant above the PBMC layer was carefully removed and the PBMC were transferred into a 50ml polypropylene tube (Becton Dickinson, Heidelberg). After transfer, the tubes were filled up to 50ml with sterile PBS and centrifuged at 250g for 10 minutes at room temperature (the centrifuge break was adjusted to level 5). Subsequently, the supernatant was carefully discarded. This washing and centrifugation step was repeated once to get rid of any residual Ficoll-Hypaque and to dilute out the blood platelets. The pelleted PBMC were then re-suspended in a total of 10ml of pre-warmed (37°C) R10 medium, pooled into a single tube and kept at 37°C and 4.5% CO_2 until PBMC were counted (see 2.3.4) and the amount of PBMC needed for the IFNγ-ELISPOT assay was removed. PBMC that were not directly used were cryo-preserved (see 2.3.5)

2.3.4 Cell counting

Accurate and consistent cell counting is a essential for the enumeration of antigen-specific PBMC with the IFNγ-ELISPOT assay (see 2.5). Cells were counted after trypane blue staining in a Neubauer cell counting chamber. Trypane blue is exclusively staining dead cells. The Neubauer cell counting chamber has 4 quadrants, with each quadrant having a grid of 16 squares. 10µl of PBMC sample was added into 90µl trypane blue, mixed well and added below the coverslide. For each sample all 4 quadrants were counted. The cell concentration is calculated from the mean number of cells/quadrant multiplied by 10 000 and the dilution factor.

2.3.5 Cryo-preservation of cells

PBMC for cryo-preservation were centrifuged at 250 x g for 10 minutes and the supernatant was discarded. Then the cell pellet was resolved gently by tapping. After any cell clumps were resolved, the tubes were transferred into a box with crushed ice. To adjust the cell concentration to 10 x 10^6 PBMC/ml the appropriate amount of 4°C cold freezing media was added. Without delay cryotubes were then transferred into special freezing containers ("Mr. Frosties", Nalgene) and frozen down to -80°C inside the freezer. The freezing containers contain iso-propanol and approximately cool down with a rate of 1°C/minute, which is considered optimal for the cell quality. After a maximum of 2 weeks, cryo-preserved cells were transferred into liquid nitrogen for long term storage.

2.3.6 Thawing of frozen PBMC

Vials of frozen PBMC were thawed inside a 37°C waterbath and cells immediately transferred into 10ml of prewarmed R10 medium containing 2U/ml Benzonase (Novagen, Merck). Benzonase is a DNAse that is added to digest DNA released from dead cells that can contribute to cell clumping. The cell suspension was then centrifuged at 250 x g, the supernatant carefully discarded and the cell pellet resolved in 10ml of pre-warmed R10 medium. After another centrifugation step, cells were resolved in R10 medium and cultivated overnight at 37°C in 4.5% CO_2 to exclude dying cells from the assay.

2.4 Synthetic peptides and peptide matrices

PBMC were screened for HIV-specific CD8 T cell responses by stimulation with overlapping peptide pools (OLP) representing Gag and Nef from isolates of subtypes A, C and D. OLPs consisted of 15mer or 18mer peptides overlapping by 11 amino acids covering the entire Gag, Nef or Env proteins Sequences are based on HIV-1 isolates 90CF402 (subtype A Gag, AAB38823), Du422 (subtype C Gag, CAD62240) 98UG57143 (subtype D Gag, AF484514), 92UG037 (subtype A Nef, AAC97549), DU151 (subtype C Nef, AAL05314) and 94UG114 (subtype D Nef, AAC97574), Du179 (subtype C Env, CAD62243) and 98UG57143 (subtype D Env, AF484514).

Tab. 2.1: Peptides for restimulation

Peptide set	Isolate	Length	N° of peptides (matrix)	Supplied by
A-Gag	90CF402	15mer	123 (11 x 11)	Anaspec. Inc, San Diego
C-Gag	Du422	15mer	123 (11 x 11)	NMI, Tuebingen
D-Gag	98UG57143	15mer	123 (11 x 11)	NMI, Tuebingen
A-Nef	92UG037	15mer	51 (7 x 7)	NMI, Tuebingen
C-Nef	Du422	15mer	49 (5 x 10)	NMI, Tuebingen
D-Nef	94UG114	15mer	51 (7 x 7)	NMI, Tuebingen
C-Env	Du179	18mer	113 (11 x 11)	NMI, Tuebingen
D-Env	98UG57143	15mer	179 (13 x 14)	NMI, Tuebingen
CEF	CMV, EBV, Influenca A	8-12mer	23 (no matrix)	Henry M. Jackson Foundation

Procedure

Peptides were synthesized using Fmoc chemistry and standard solid-phase techniques with free amino termini. All peptides were >80% pure as determined by HPLC, mass spectroscopy, amino acid analysis and N-terminal sequencing. The A Gag peptides were synthesized at the Henry M. Jackson Foundation and by Anaspec Inc (San Jose, USA). The rest of the peptides were synthesized by the Natural and Medical Sciences Institute (University of Tuebingen, Germany). Lyophilized peptides were resolved at a stock concentration of 10mg/ml in cell culture grade DMSO (Sigma, Deisenhofen).

To minimize the number of PBMCs used, initial screening for T-cell responses was performed using the OLPs in a matrix format. Subtype A, C, and D Gag

peptide sets were pooled in an 11 x 11 format, the A- and D-Nef peptide sets were pooled in a 7 x 7 format and the C-Nef peptide set was pooled in 10 x 5 formats. To assure consistent quality of the assay, peptide matrices were aliquoted at a concentration of 50µg/ml/peptide into 96-well micro racks (Neolab, Heidelberg). Micro racks were sealed using plate sealers (Millipore, Schwalbach) and stored at -80°C until use. For each working day, one 96-well aliquot was thawn and peptides were diluted with R10 medium to a concentration of 4µg/ml/peptide**.**

2.5 The IFNγ-ELISPOT assay

The enzyme linked immuno spot (ELISPOT) assay for detecting individual cytokine-secreting cells is based on the use of highly specific antibodies against cytokines. The technique resembles a cytokine-specific Sandwich ELISA with the exeption that the bottom of each well is made of a polyvinylidene difluoride membrane. After the detection step, each IFNγ-secreting cell results in one spot on the membrane, where the primary antibody had immobilized the secreted IFNγ. Its 96-well format, high sensitivity and consistency allows screening for antigen specific PBMC in a "high-throughput" manner, which is crucial to map and enumerate HIV-specific cellular immune responses to individual peptides or peptide pools. In the HISIS-CTL study, the IFNγ-ELISPOT assay was combined with overlapping pooled peptide technology and has allowed efficient quantification and mapping of HIV-1-specific CD8+ T-cell responses by measuring interferon-gamma (IFNγ) release (Addo et al., 2003; Betts et al., 2001). Whether specific CD8 or CD4 T cells are responsible for IFNγ secretion has been resolved in a second experiment using flow cytometry with the intracellular cytokine staining protocol (see 2.6).

Procedure

Buffers and solutions

PBS without Mg^{2+} und Ca^{2+}

NaCl	8.0 g/l
KCl	0.2 g/l
Na_2HPO_4 x 12 H_2O	2.9 g/l
KH_2PO_4	0.2 g/l

Wash buffer (made with PBS)

FBS	1.0 % (v/v)
Tween 20	0.001% (v/v)

R10 medium

RPMI 1640

Heat-inactivated FBS	10 % (v/v)
L-Glutamine	2mM
Hepes buffer	10mM
Penicillin	100U/ml
Streptomycin	100µg/ml

Fetal Bovine Serum was heat-inactivated for 30 minutes at 56°C.

NoveRed substrate solution (included in the NoveRed substrate kit, Vector Laboratories)

15ml of double distilled Water

reagent 1	3 drops
reagent 2	2 drops
reagent 3	2 drops
Hydrogen Peroxidase	2 drops

The composition and the principle of the NovaRed substrate are company secretes. However, the principle of the colour reaction is likely to be based on the generation of free OH· radicals by the horseradish peroxidase and a color reaction that is dependent on the presence of free OH· radicals. The substrate

solution is light sensitive and was prepared 5 minutes before adding into the microplate wells inside 50ml Polypropylene tubes that were wrapped in tin foil.

IFNγ-ELISPOT assays were performed as previously described with minor modifications (Mashishi and Gray, 2002). Polyvinylidene difluoride plates (Millipore Multiscreen-IP 96well, MAIPN0B10, Schwalbach) were coated with 50µl of anti-IFNγ monoclonal antibody 1-D1k (2µg/ml) (Mabtech, Stockholm, Sweden) overnight at 4°C and then blocked for a minimum of 2 hours with R10 medium. Peptides were added directly to the wells at a final concentration of 2µg/ml along with 1 x 10^5 of fresh PBMC in 50µl of R10 medium and were incubated at 37°C in 4.5% CO_2. After 16 to 18 hours, plates were extensively washed 3 times with PBS and 3 times with wash buffer, followed by incubation with a biotinylated anti-IFNγ monoclonal antibody (2 µg/ml) (clone 7-B6-1; Mabtech) at room temperature for 3 hours. After six more washes with wash buffer, 2µg/ml of streptavidin-horseradish peroxidase (Pharmingen, San Jose) was added to the wells, and the plates were incubated for another hour at room temperature. Spots were visualized by adding 100µl of NovaRed substrate (Vector, Burlingame) and 10-15minutes of incubation in the dark. The reaction was stopped by flushing the wells 2 times with water.

Duplicate wells containing only PBMC and R10 medium were used as negative controls. Wells containing PBMC and 4µg/ml phytohemagglutinin (PHA) served as positive controls. Spots per well were counted with an Immunospot (Cellular Technology Ltd., Cleveland, Ohio) automated plate counter. Peptide responses were confirmed by using individual peptides in the ELISPOT assay with freshly isolated PBMC during FU14.

2.5.1 Monitoring and evaluation of the assay consistency

Differences in cell counting, pipetting or inconsistent sensitivity of the ELISPOT assay can hamper the quantitative analysis of T cell responses. Therefore assay variation and sensitivity was monitored throughout all follow ups using 4 batches of Quality control PBMC (QC-PBMC) that were donated by the same voluntary HIV-negative blood donor on 4 different time points.

The QC-PBMC responded to the CEF peptide pool containing 23 peptides from CMV, EBV, Influenza with a mean of 1400 spot forming cells (SFC)/10^6 PBMC and a standard deviation of 247 SFC/10^6 PBMC. QC-PBMC were always recovered from frozen one day in advance of the IFNγ-ELISPOT assay (see 2.3.6). Two wells containing unstimulated PBMC and one containing CEF-stimulated PBMC were included on each Gag-peptide test plate. The maximum fluctuation of the assay - highest SFC: lowest SFC (fig 2.1) - was 1.82 for FU12, 1.88 for FU13, 1.92 for FU14 and 1.7 for FU15.

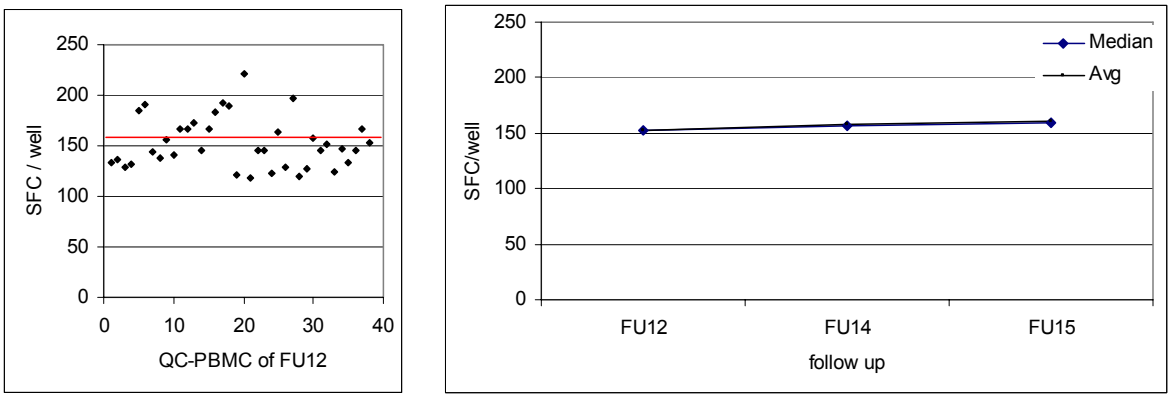


Figure 2.1. IFNγ-ELISPOT quality control throughout the HISIS-CTL study. Shown is the SFC/well of QC-PBMC after restimulation with the CEF peptide pool throughout follow up (=FU) 12 (left panel). The median is indicated in red. The median and average SFC/well for FUs 12, 14 and 15 are shown in the right panel.

2.6 Intracellular cytokine staining (ICS) from whole blood

The type cells and cytokine production was measured by flow cytometry with different fluorochrome-labled antibodies (see 2.2.1). Positive peptide-specific responses or responses to virus like particles (VLP-Pr55, generously provided by Prof. Ralf Wagner, University of Regensburg, Germany) were tested. To determine, whether the antigen-specific T cell responses were mediated by CD4 or CD8 T cells, the intracellular IFNγ expression was measured directly from whole blood.

Procedure

250 µl of heparinized whole blood was added into polypropylene tubes and stimulated with either individual peptides (2µg/ml final), Staphylococcal enterotoxin B (SEB, 10µg/ml final) (Sigma, Deisenhofen) as a positive control, or left without stimulant as a negative control. All tubes contained 25µg/ml of co-stimulatory anti-CD28 and anti-CD49d monoclonal antibodies (Pharmingen, San Diego, CA), 25µg/ml of protein transport inhibitor brefeldin A (Sigma) and were incubated for 6 hours at 37°C in the presence of 4.5% CO_2. Samples were then processed by lysing, fixation, permeabilization and staining according to the BD Bioscience standard whole blood assay analysis protocol (included in the package insert of the Becton Dickinson Fastimmune Kit for intracellular cytokine detection kit). Cells were stained with anti-IFNγ-FITC (Pharmingen, clone 4S.B3), anti-CD4-PE (Pharmingen, clone RPA-T4), anti-CD8+-PerCp-Cy5.5 (BD Biosciences, clone SK1) and anti-CD3-APC (Pharmingen, clone UCHT1). Data acquisition was performed using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg), and data sets were analysed with FlowJo software (version 4) (TreeStar, Cupertino, CA). Responses of at least 3 times the negative control and at least 0.06% of CD8+ T cells were scored as positive.

Tab 2.2: Antibodies used for ICS

Antigen	Clone	Conjugate	pupose	Company
IFNγ	4S.B3	FITC	staining	Pharmingen
CD4	RPA-T4	PE	staining	Pharmingen
CD8	SK1	PerCp-Cy5.5	staining	BD Bioscience
CD3	UCHT1	APC	staining	Pharmingen
CD28	555725	none	co-stimulation	Pharmingen
CD49d	555501	none	co-stimulation	Pharmingen

2.7 Molecular characterization of the Virus by the Multi-Hybridization Assay

The Multi-Hybridization Assay (MHA) is a real-time PCR based technique that was first introduced and validated against complete genome sequences for the ability to accurately distinguish subtypes and recombinant forms within amplified genomic regions (Hoelscher 2002). It was developed primarily for rapid discrimination of subtypes A, C and D and recombinant forms of HIV-1, but is also capable of detecting dual infections. The principle of the MHA is to amplify five short regions distributed along the HIV-1 genome in a separate first round PCR that does not discriminate between subtypes. Each amplicon is then distributed to three second-round PCRs, each with a different subtype-specific probe labeled at the 5' end with 6-carboxyfluorescein and a quencher at the 3'end (provided by Applied Biosystems, Darmstadt). Nested PCR primers and probes for the five genome regions, are termed gag , pol, vpu, env 1 and env2.

Procedure

The template DNA was extracted from 200 000 PBMC using the High Pure viral nucleic acid kit (Roche Diagnostics, Mannheim). The first round PCR contained 250µM of each dNTP, 1.5mM $MgCl_2$, 50mM KCl, 15mM Tris-HCl pH8.0, 1µM primers, with 3 Units AmpliTaq GOLD (Applied Biosystems, Darmstadt). The amplicons were 412-633 base pairs (bp) in length. The thermocycle routine was: 1 cycle of 95°C 10 minutes, then 35 cycles of 95°C 15 seconds, 52°C 45 seconds, 72°C 90 seconds in an ABI9600 Thermocycler (Applied Biosystems, Darmstadt). The second round PCR reactions contained 2µl of first round product, 250µM of each dNTP, 3.5mM $MgCl_2$, 480nM primers, 220nM probe, 1.5 Units AmpliTaq GOLD in 1x Taqman Universal Master Mix (Applied Biosystems, Darmstadt). PCR amplification was in a 96-well spectrofluorometric ABI7700 sequence detection system (Applied Biosystems, Darmstadt) at 95°C for 15 second, 55°C 1 minute, and 60°C 1 minute for 30-35 cycles. Fluorescence intensity was monitored during every cycle, and samples were considered positive, when the fluorescence intensity increased exponentially over at least 5 consecutive cycles. The MHA was in part performed by Martina Gerhardt at the Institute for Tropical Medicine and

Infectious Diseases in Munich and at the US Military HIV Research Program in Rockville.

2.8 Prediction of CD8 T cell epitopes by HLA-specific binding motifs

HLA binding motifs were predicted with the online HLA motif scanner tool at http://www.hiv.lanl.gov/content/immunology/motif_scan/motif_scan. This tool allows to find HLA anchor residue motifs within protein sequences for specified HLA alleles. Binding motifs are predicted predominantly on the basis of two HLA motif libraries (Marsh et al., 2000; Rammensee et al., 1999). HLA B4201 and B8101 binding motifs have been added to the database by Yusim et al. (Yusim et al., 2004). Epitopes can vary in length from 8 to 11 amino acids. It should be noted that binding motif prediction does not consider proteasome cleavage preferences within a given amino acid sequence.

Table 2.3. Binding motifs of selected HLA B alleles considered in this work. Anchor residues displayed in round brackets are preferred but not dominant. Shown is the binding motif of the 8mer epitope. For prediction of 9-11mer epitopes, amino acids are inserted in the position -1 before the C-terminal position. This position is indicated as x.

HLA allele	8 mer Motif	scource
B0702	x-[P]-x-x-x-x-x-[L(F)]	(Marsh et al., 2000)
B0702	x-[P(V)]-x-x-x-x-x-[L]	(Rammensee et al., 1999)
B1503	x-[QK]-x-x-x-x-x-[YF]	(Rammensee et al., 1999)
B1510	x-[H]-x-x-x-x-x-[L(F)]	(Rammensee et al., 1999)
B4201	x-[P]-x-x-x-x-x-[L]	(Yusim K et al., 2004)
B57	x-[ATS]-x-x-x-x-x-[FW]	(Marsh et al., 2000; Rammensee et al., 1999)

Table 2.3 (continuation)

HLA allele	8 mer Motif	scource
B5801	x-[ATS]-x-x-x-x-[WF]	(Marsh et al., 2000)
B5801	x-[AST(G)]-x-x-x-x-[FW(Y)]	(Rammensee et al., 1999)
B5802	x-[ST]-x-x-x-[R]-x-[F]	(Marsh et al., 2000; Rammensee et al., 1999)
B8101	x-[P]-x-x-x-x-[L]	(Yusim K et al., 2004)

2.9 Statistical analysis

Statistical analysis of the data was performed in part with the "Prism V3.00 Software" (Graphpad). The statistical tests used are indicated in the results chapter. In general, non-parametric tests were used to describe and analyze the data, since cellular immune response data generally does not follow a normal distribution. In chapter 3.2, responses of the population to an individual epitope (single peptide) were calculated as median values of all subjects responding to a particular peptide in the original screening assay. To determine if there was a statistical difference in response to peptide variants among the three peptide sets, the responses were analyzed using the Friedman Test - a non-parametric, repeated measures ranking test for comparing three or more treatment groups. When the null hypothesis was rejected ($p<0.05$), a post hoc test (Dunn's multiple comparison test) was used to test for response differences between the pairs of groups.

3. Results

3.1 Recognition of multiple epitopes within two regions of Gag is associated with a low viral load

A recent report by Kiepiela et al 2004 demonstrated a dominant influence of HLA class I B alleles on the HIV disease progression in chronically HIV-infected individuals. Particular B alleles, namely B5801, B57, B4201 and B8101 were significantly associated with a lower than median viral load, whereas others, such as B5802 and B4501 were associated with a higher than median viral load. It was further demonstrated that HLA B alleles contribute most to the HIV-specific CD8 T cell response (67% of CD8 T cell responses restricted by B alleles) if compared with HLA A or HLA C alleles ((Kiepiela et al., 2004), indicating that there may be a mechanistic link between the HIV-specific CD8 T cell response restricted by a particular HLA B allele and the corresponding viral load.

Therefore in order to study the mechanism underlying the beneficial effect of protective HLA class I alleles, the HLA alleles of 53 study subjects from the HISIS-CTL cohort (described in chapter 2.2) were typed and the plasma viral load and CD4 counts determined. At the same time the HIV-specific CD8 T cell response of the study subjects was examined for responses to Gag, Nef and Env with an IFNγ-ELISPOT assay and intracellular cytokine staining for IFNγ.

3.1.1 HLA class I alleles B5801, B8101 and B0702 are associated with a low viral load during chronic HIV-1 infection

As stated above, particular HLA class I B alleles are associated with a low viral load during chronic infection. Therefore the HLA allele expression and the plasma viral load were determined for study subjects within the HISIS-CTL cohort as described in the materials and methods chapter. The most common HLA B alleles were B4201 (n=12) followed by B5301 (n=10), B5802 (n=9), B8101 (n=9), B0702 (n=8) and B1510 (n=8). The “protective” B alleles B5801 and B5703 were less common (n=4) (Fig. 1A).

Median plasma viral load for the HISIS-CTL cohort was 198 500 RNA copies/ml, a value much higher than seen in most other studies (Kiepiela et al.,

2004 ; Masemola et al., 2004; Mellors et al., 1996; Novitsky et al., 2003),. B5801 expressing subjects had the lowest median viral load (17 250 RNA copies/ml), followed by subjects expressing B8101 (19 360 RNA copies/ml), B0702 (45 350 RNA copies/ml), B5703 (67 250 RNA copies/ml) and B4403 (85 800 RNA copies/ml). In contrast B5802, B4501, B1510 and B4201 had a median plasma viral load of close to or above 200 000 RNA copies/ml. These results indicate that similar associations between particular HLA-B alleles and plasma viral load are seen in the HISIS-CTL cohort and the cohort of the Kiepiela study, except for allele B4201 (Kiepiela et al., 2004).

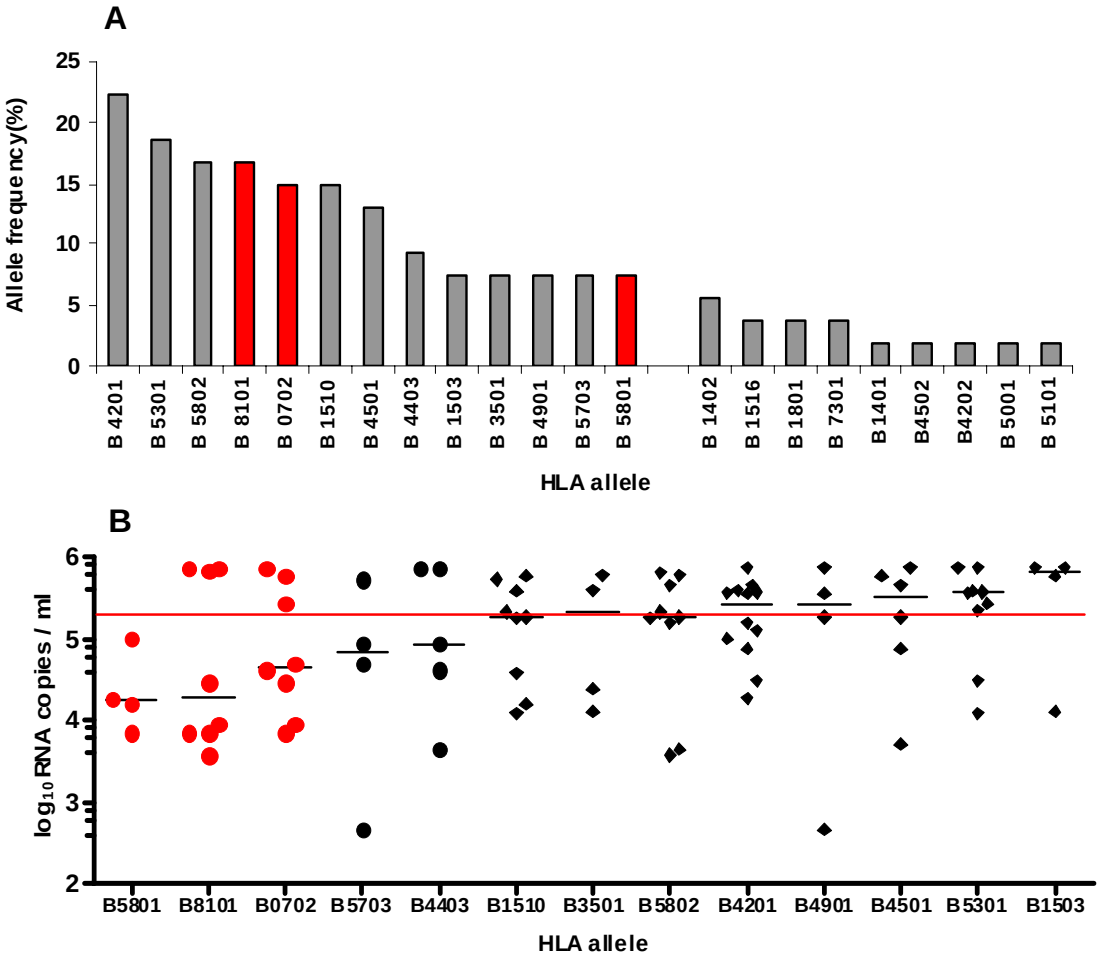


Fig. 3.1. HLA allele frequencies and plasma viral load among HISIS-CTL subjects. (**A**) Frequencies of HLA class I alleles in the HISIS-study cohort. Alleles that were associated with low median plasma viral load of below 50 000 are indicated in red (n=53). (**B**) HLA class I expression and plasma viral load in chronically infected study subjects (n=55). The red line indicates the median viral load of the HISIS-CTL cohort (198 000 RNA copies / ml).

3.1.2 Protective HLA B alleles are associated with a broad Gag-specific CD8 T cell response

Next it was examined whether subjects expressing HLA alleles that were associated with a median plasma viral load of below 50.000 RNA copies/ml (B5801, B8101 and B0702 - for simplicity referred to as "protective" HLA alleles) differed in the number of Gag, Nef or Env epitopes recognized. Therefore the peptide recognition by PBMCs was tested with the IFNγ-ELISPOT assay using overlapping peptide matrices of Gag, Nef and Env from HIV subtypes A, C and D. Responses to individual peptides were confirmed in a second step by single peptide retesting. To determine the phenotype of the responding cells, a total of 82 individual peptide responses from 20 study subjects were subjected to cytokine flow cytometric analysis for IFNγ production and confirmed the same peptide responses as detected with the ELISPOT assay. Eighty-nine percent of responses were from CD8+ T-cells (data not shown).

For each tested HIV protein, the frequency of responding CD8 T cells and the number of CD8 T cell epitopes recognized was analyzed and compared for study subjects expressing "protective" HLA alleles and those that were associated with a viral load of above 50 000 RNA copies/ml (all other B alleles) (Fig.3.2). There was a significant difference between subjects expressing "protective" HLA B alleles and non-expressing subjects in the number of epitopes recognized (single peptide or the first of a pair of overlapping peptides) within Gag with a median number of 4 and 2 recognized epitopes respectively (p-value = 0.0035, Mann-Whitney Test). The frequency of total Gag-specific CD8 T cells was not significantly different between both groups, although the median Spot-forming cells (SFC)/10^6 PBMC was doubled for subjects expressing a "protective" HLA allele with 2840 SFC/10^6 PBMC. For Nef and Env specific CD8 T cell responses, there were no differences between both groups in the number of epitopes recognized (data not shown for Env) or in the frequency of specific CD8 T cells (data not shown). Within Nef, the number of epitopes recognized was a median of 2 and the median frequency was 1600 SFC/10^6 PBMC for either group. However, three of four HLA B5801 expressing subjects had a very broad Nef response with 11 or 12 Nef epitopes recognized. The HLA alleles B57 and B44, both of which were

associated with a median viral load below 100 000 RNA copies/ml, were not associated with a broad Gag or Nef epitope recognition (data not shown).

Taken together these results suggest that the HIV-specific CD8 T cell response of subjects expressing "protective" HLA class I alleles B5801, B8101 and B0702 (n=18) is characterized by a broad number of epitopes recognized within Gag.

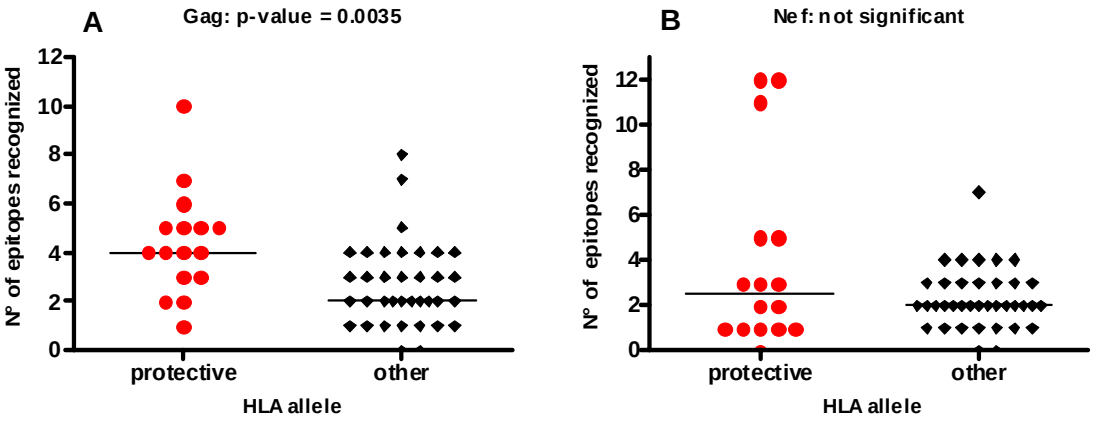


Figure 3.2. Expression of "protective" HLA Class I alleles correlates with a broad Gag-specific CD8 T cells response. Shown is the number of recognized epitopes for (**A**) Gag and (**B**) Nef from study subjects who express "protective" HLA B alleles (n=16, red dots) and from study subjects expressing other HLA B alleles (n=37, black dots). "Protective" HLA alleles were defined as those associated with a median viral load below 50 000 RNA copies/ml in the HISIS CTL-cohort (alleles B5801, B8101 and B0702). Recognition of two consecutive peptides was counted as 1 epitope response. Statistical analysis was performed using the Mann Whitney test.

3.1.3 Linear relationship between the number of Gag epitopes recognized and the plasma viral load

As stated above a significant difference was found for the HIV-specific CD8 T cell response for subjects expressing "protective" HLA alleles B5801, B8101 and B0702 was the number of recognized Gag epitopes. Neither was the frequency of Gag-specific CD8 T cells significantly elevated, nor appeared the Nef or the Env specific response to be any different between subjects expressing "protective" HLA B alleles and subjects who do not express a "protective" HLA allele. Could a broad number of recognized Gag epitopes be

beneficial to the HIV-infected subject and serve as a predictive marker for disease progression in general? To clarify this question, we compared the breadth of epitope recognition and the frequency of protein-specific CD8 T cells with the plasma viral load and the CD4 cell count. As shown in figure 3.3 there was a negative linear relationship between the number of recognized epitopes within Gag and the plasma viral load [p-value = 0.0016 (Spearman-Rank Test), R-linear = -0.36] and a positive linear relationship between the number of recognized epitopes within Gag and the CD4 cell count [(p-value = 0.013 (Spearman-Rank Test), R-linear = 0.33]. The frequency of Gag-specific CD8 T cells was not correlating with a low plasma viral load. Neither did the Nef- or Env-specific CD8 T cell response correlate with a low plasma viral load nor with the CD4 cell counts (data not shown). However, up to 5 recognized epitopes within Nef, there was a positive linear relationship between the number of recognized epitopes and the plasma viral load and a negative linear relationship with the CD4 cell counts. This relationship is strong up to 5 Nef epitopes recognized (93% of subjects fall into this group) and is reversed with recognition of more than 6 Nef epitopes (4 subjects only, 3 of the 4 express B5801).

These results indicate that the breadth of the Gag-specific CD8 T cell response appears to be of particular importance for the control of viral replication during chronic HIV-1 infection. In contrast Nef and Env-specific responses did not contribute to efficient control of viral replication, suggesting that CD8 T cells of different specificities may vary in their respective antiviral efficiency.

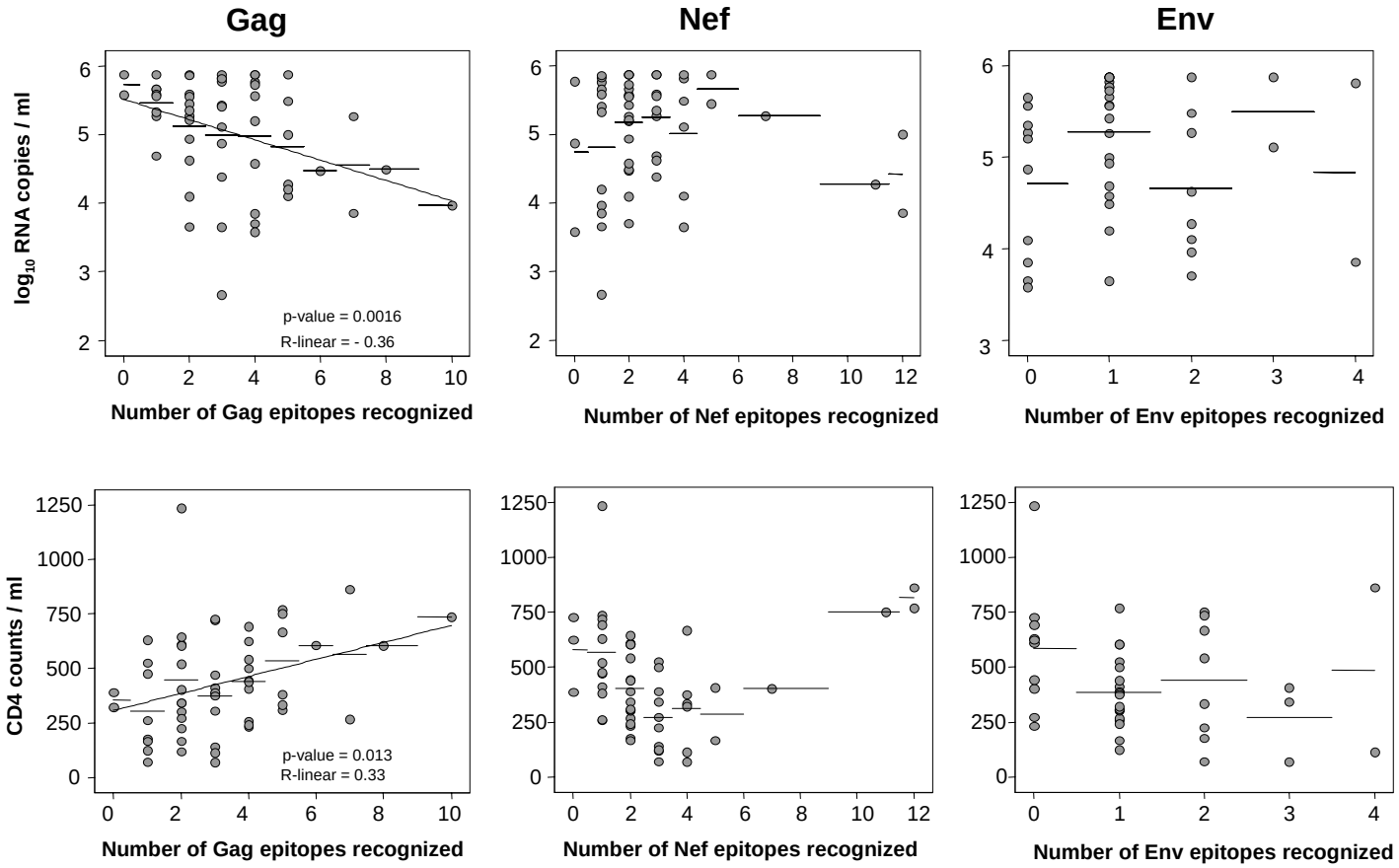


Figure 3.3. Linear relationship between Viral load and CD4 counts with the number of recognized epitopes within Gag, but not within Nef or Env. Shown are **(A)** the linear regression analysis of the viral load and (**B**) the CD4 count versus the number of epitopes recognized per subject for Gag (left panels), Nef (middle panels) and Env (right panels). Recognition of two consecutive peptides was counted as 1 epitope response. Statistical analysis was performed using the Spearman-Rank Test.

3.1.4 Eighty-nine percent of subjects with efficient control of viral replication recognize multiple epitopes within 2 regions of Gag

The finding that neither Nef- nor Env-specific CD8 T cell responses were associated with a lower viral load raised the question, whether there are differences in the antiviral efficiency between different Gag-specific peptide responses. To approach this question, Gag peptides recognized by subjects with a viral load below 50 000 RNA copies/ml (n=18; low viral load (LVL)-group) were compared with those recognized by subjects with a viral load above 50 000 RNA copies/ml (n=36; high viral load (HVL)-group) (Fig.4). There were striking differences in the CD8 T cell specificities between these two groups. The average LVL-subject not only recognized more peptides within the whole Gag protein (factor 1.41), but also differed in the preferential Gag regions targeted compared to the HVL-group. LVL-subjects recognized peptides within the N-terminal region of Gag (GagR1, aa001-aa075, peptides 1-16) almost twice as often (factor 1.80) compared to the HVL-group. Peptides within the C-terminal Gag region (GagR3, aa248-aa500, peptides 62-120) were recognized twice as often (factor 2.02), whereas peptides within the middle region of Gag (GagR2, aa076-aa247, peptides 17-61) were recognized equally among both groups (factor 0.93). These findings suggest that Gag regions 1 and 3 are rich in epitopes whose recognition is important for efficient viral control.

For the whole Gag protein, recognition of 5 or more epitopes is required to significantly reduce the median viral load (p-value=0.047, Mann-Whitney Test, data not shown) and significantly increase the median CD4 cell count (p-value=0.024). However, only a limited number of 11 subjects recognized that many epitopes within the Gag protein, 7 of which had a viral load below 50 000 RNA copies/ml. By excluding the central region of Gag R2, recognition of 2 or more epitopes within GagR1R3 correlated strongly with a low viral load (p-value=0.015) and a higher CD4 cell count (p-value= 0.016) (fig.3.5). 16 of 29 subjects, who recognized 2 or more epitopes within GagR1R3 had a viral load below 50 000 RNA copies/ml, whereas only 3 of 26 subjects who recognized only 0 or 1 epitope within GagR1R3 were falling into this category

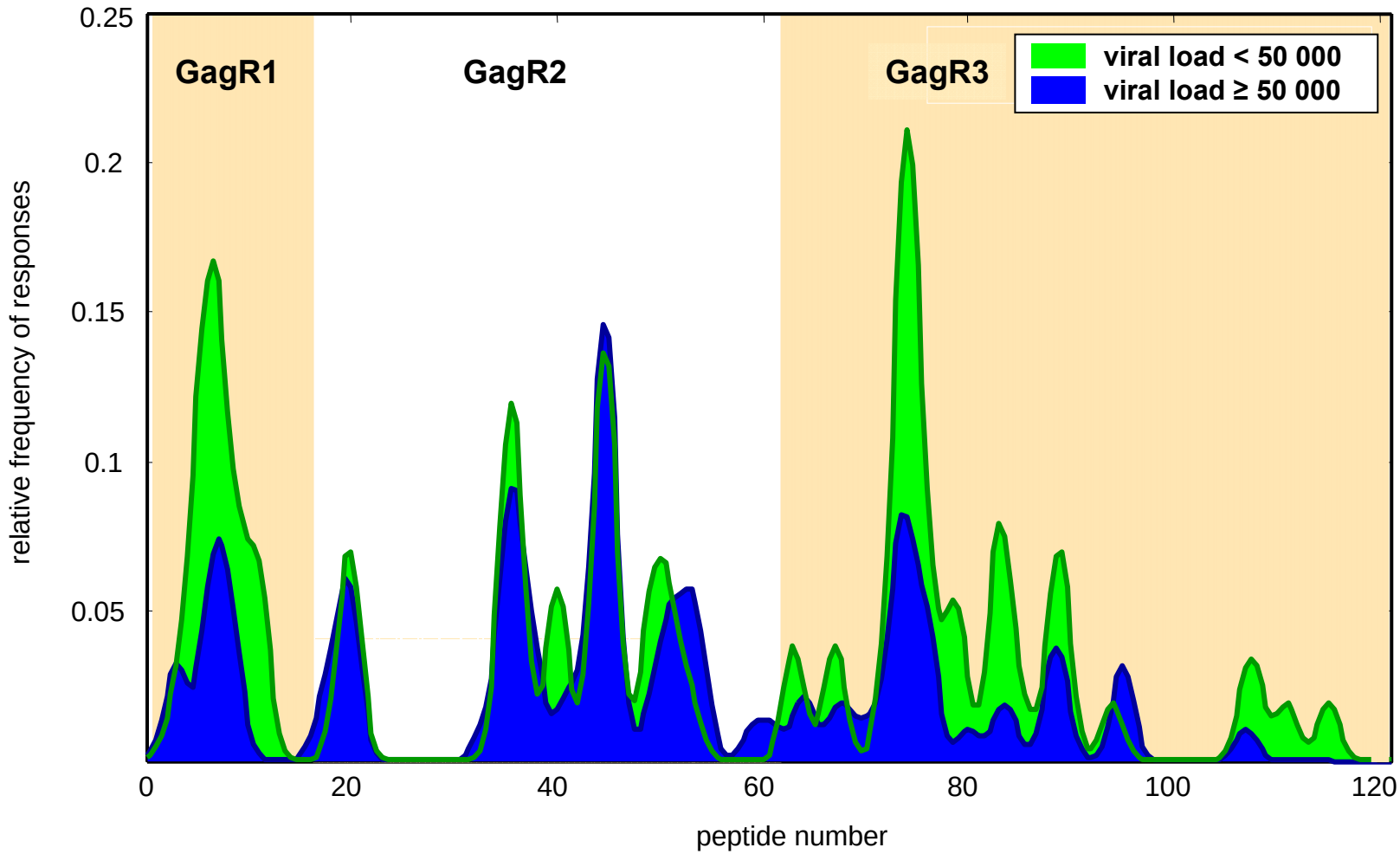


Figure 3.4. Comparison of Gag-specific CD8 T cell specificities of subjects with a plasma viral load below (n=19) or above (n=36) 50 000 RNA copies/ml. Shown is the frequency of responses after smooth local average with Gaussian kernel weights and a standard deviation (bandwidth) of 1 peptide.

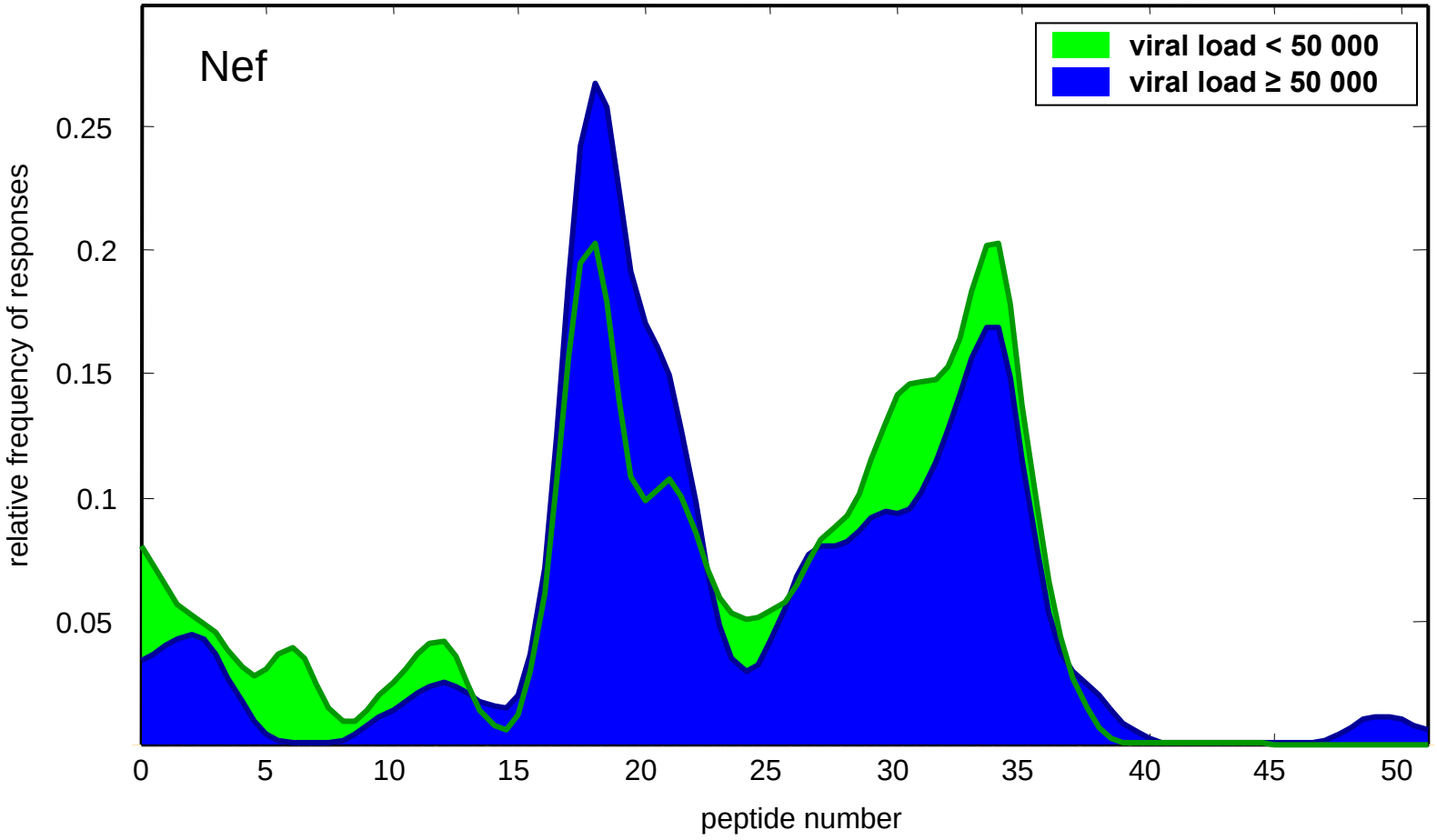


Figure 3.4. Comparison of Nef-specific CD8 T cell specificities of subjects with a plasma viral load below (n=19) or above (n=36) 50 000 RNA copies/ml. Shown is the frequency of responses after smooth local average with Gaussian kernel weights and a standard deviation (bandwidth) of 1 peptide.

– two of these were expressing B5703 (indicated as red boxes in fig.3.5). Hence, 16 of 19 (86%) subjects with a viremia below 50 000 RNA copies/ml targeted at least 2 epitopes within GagR1R3. The strong correlation of protective HLA-B-alleles B5801, B8101 and B0702 expression with a broad epitope recognition within GagR1R3 (p-value=0.0022, data not shown), suggests that the low viremia may indeed be the result of the antiviral efficiency of a HIV-specific CD8 T cell response that is targeting multiple epitopes within GagR1R3.

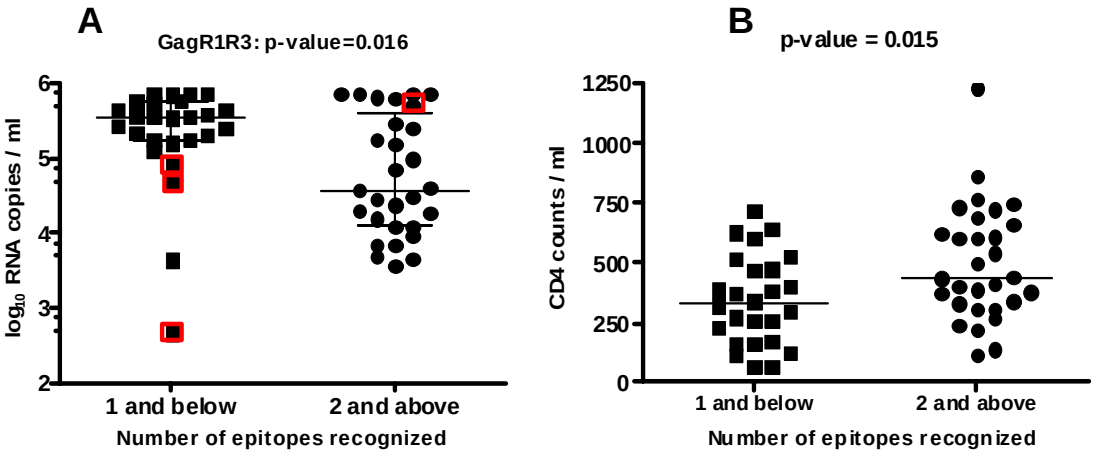


Figure 3.5. CD8 T cell recognition of multiple epitopes within Gag regions aa001-aa075 and aa248-aa500 (Gag R1R3) is associated with efficient viral control. Shown is the viral load (**A**) and the CD4 cell count (**B**) of subjects with a broad (n=29) or narrow (n=26) CD8 T cell response against GagR1R3. Cut-off value was 2 epitopes recognized. The respective median and the interquartile range are indicated. HLA B5703 expressing subjects are indicated as red squares in the left panel. Recognition of 2 consecutive peptides was counted as 1 epitope response. Statistical analysis was performed using the Mann Whitney test (two tailed).

3.1.5 Recognition of multiple epitopes within Gag R1R3 is not associated with the Gag-specific CD4 T cell response

The antiviral efficiency of a CD8 T cell response could theoretically be influenced by the frequency in HIV-specific CD4 T cells. In order to analyse this, the whole blood of 38 study subjects was tested for IFNγ and IL-2 expressing CD4 T cells after stimulation with subtype B virus like particles VLP-Pr55 during FU16. As shown in figure 3.6 there was no association of the

VLP-Pr55-specific CD4 T cell response with the GagR1R3 specific CD8 T cell response. In general only low frequencies of Gag-specific CD4 T cells were found that were predominantly secreting IFNγ and very little IL-2.

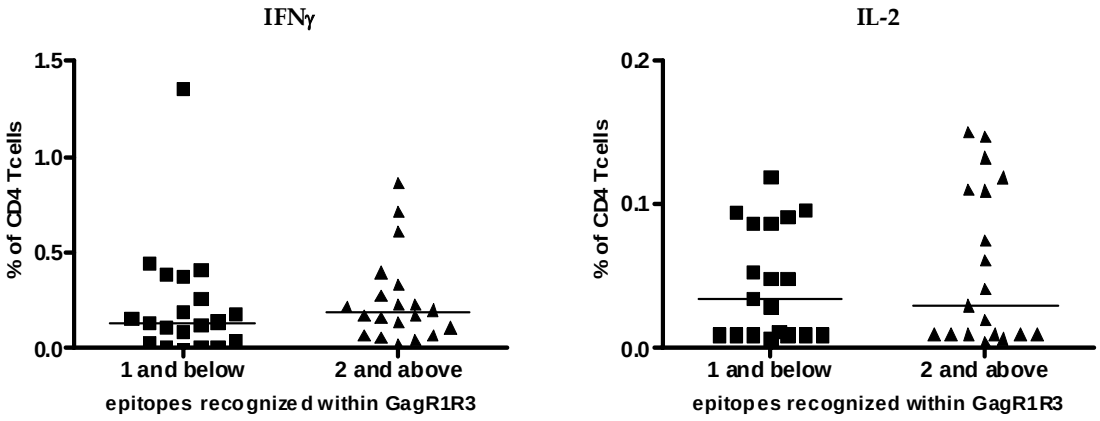


Figure 3.6 Frequency of Gag-specific CD4 T cells in subjects who do recognize (n=20) or do not recognize (n=18) multiple epitopes within GagR1R3. Shown is the frequency of IFNγ (left panel) or IL-2 (right panel) expressing Gag-specific CD4 T cells detected after restimulation with VLP-Pr55. The background was subtracted. The median is indicated.

3.1.6 Recognition of multiple epitopes within Gag R1R3 is associated with the maintenance of a low viral load

HIV infection is characterized by a typical course of the plasma viremia. During the acute phase, plasma viremia rises to peak levels within the first 3 months and, with the initiation of the HIV-specific immune response, is subsequently dropping to the "viral load set point" (introduction fig.1.6). The viral load set point also allows estimating the time to AIDS (Mellors et al., 1996). After primary infection, usually stays on a steady-state level with relatively little variation until it starts to rise again during the final AIDS phase of HIV infection. This typical course of viremia indicates that the outcome of an HIV-infection is largely determined during the acute phase of HIV-infection. Therefore it was interesting to see whether the recognition of multiple epitopes within GagR1R3 would be associated with the establishment of the viral set point viremia and subsequent steady-state viral load levels.

To address that question, the course of the plasma viremia was studied in 19 subjects that seroconverted during the HISIS study. In the group recognizing 2 or more epitopes (n=12) the course of plasma viremia is typical for HIV infection (fig. 3.7 top panel). During the acute phase viral load rises to a median peak level of 286 000 RNA copies/ml and drops down subsequently to a steady state level of 10-20 000 RNA copies/ml for the next 24 months. Only 2 of 12 subjects have a viral load of above 50 000 RNA copies/ml at 24 months past seroconversion. In stark contrast the course of plasma viremia of subjects recognizing less than two epitopes within GagR1R3 (n=7; HLA B57 expressing subjects excluded) is strikingly different. Plasma viral load rises to peak levels with a median of 403 000 RNA copies/ml during acute infection and subsequently stays at a steady-state viremia of 150 000 to 350 000 RNA copies/ml over the next 24 months. At 24 months after seroconversion none of this group has a viral load below 50 000 RNA copies/ml and only one just below 100 000 RNA copies/ml. Hence viral replication is never brought under efficient control in any of these subjects. These results indicate that after the acute phase peak viremia, the recognition of multiple epitopes within the GagR1R3 may strongly contribute to a low viral set point and the maintenance of a low steady-state viremia.

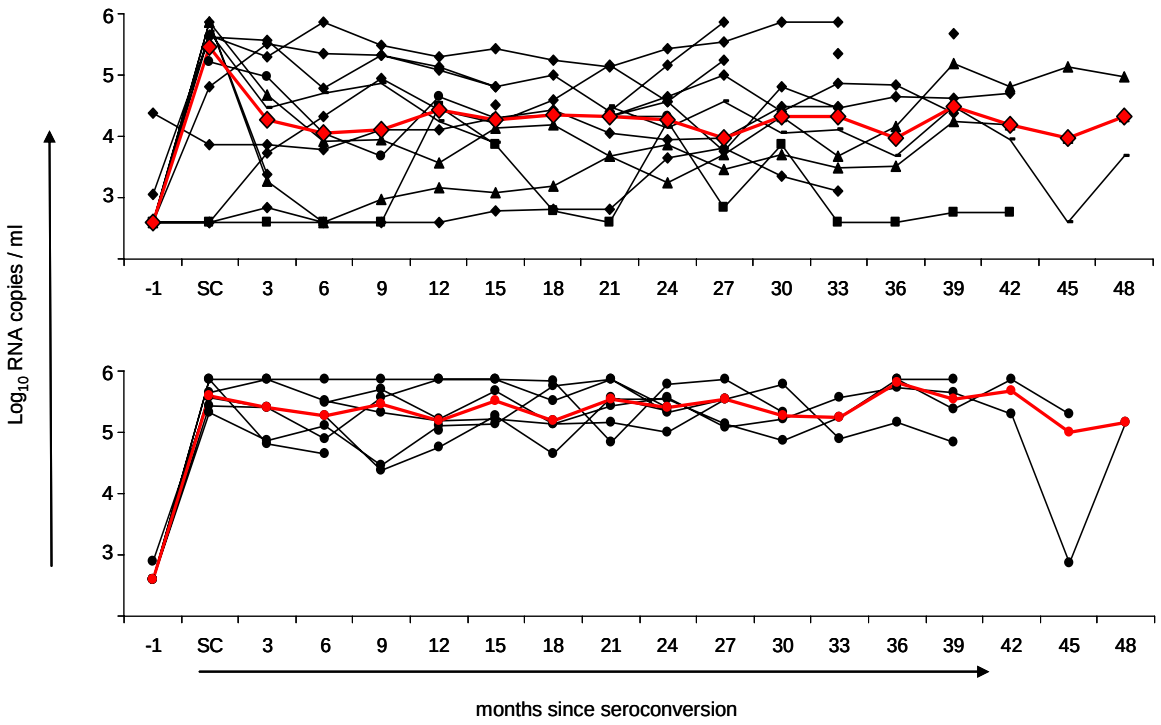


Figure 3.7. Dynamics of the plasma viral load after seroconversion (SC) Shown is the viral load since seroconversion of study subjects who (**A**) recognized two or more epitopes (n=7) or (**B**) 0 or 1 epitope (n=12) within the Gag regions aa001-aa132 and aa297-aa500. The median viral load is indicated in red. Gag peptide responses were determined during FU14 with the IFNγ-ELISPOT assay. Three HLA-B5703 expressing subjects were excluded from analysis. Recognition of 2 consecutive peptides was counted as 1 epitope response.

3.2 A minimal set of 15 peptides can cover the CD8 T cell response against HIV-1 Gag and Nef of subtypes A, C and D in a population affected by a mixed epidemic

The genetic variability of HIV-1 is a major obstacle for both vaccine design and immunological screening methods (Brander 2003; Walker 2001, Gaschen 2003). Geographically defined epidemics can be characterized by the dominance of distinct genetic subtypes of HIV-1. At least 9 subtypes and 16 circulating recombinant forms (CRFs) are currently described (http://www.hiv.lanl.gov/content/hiv-db/CRFs/CRFs.html). To complicate matters, intra-subtype variability can account for amino acid sequence differences of up to 25%, depending on the protein considered. Even within a single HIV-1 infected individual, the HIV mutant spectrum or so-called quasispecies that developes over the years of infection can reach a divergence of 10% (Meyerhans et al., 1989, Plikat et al., 1997). Yet, how this genetic diversity translates into an immunologically relevant diversity is not completely established.

In this chapter the influence of subtype diversity on the CD8 T cell recognition was examined. Subtypes of the infecting HIV-1 strains were analysed, T cell responses were mapped to individual peptides within Gag and Nef. Furthermore the relative cross-recognition of subtype A-, C- and D-specific peptide variants was examined for the most frequently recognized antigenic peptides. The results were used for the design of a peptide pool of the most frequently recognized HIV-1 peptides within Gag and Nef, which was tested for recognition with seropositive subjects outside the HISIS cohort.

3.2.1 Subtype C and AC/ACD recombinant viruses make up the majority of HIV-1 infections in the Mbeya region of Tanzania

To determine the subtype of the infecting strain of HIV-1 in each seropositive a Multi Hybridization Assay (MHAacd) (Hoelscher et al., 2002) was performed on up to 14 follow-ups per participant. This assay probes for the genetic subtype in multiple regions of the HIV-1 genome, one of which is the *gag* gene relevant to this study. As shown in figure 3.7, HIV-1 infections in the HISIS-CTL cohort include subtypes A, C and D, with predominance of subtype C and subtype C-containing unique recombinant forms (URFs). 19 subjects were infected with HIV-1 subtype C, 3 with subtype A, and 29 subjects were infected with recombinant forms of HIV-1, mostly AC and ACD. The *gag* region subtype distribution closely matched that observed over the entire HIV-1 genome for the 56 analysed subjects.

Additionally, 27 *gag* gene sequences from discarded HIV positive blood donations from the blood bank of the Mbeya Referral Hospital confirmed that the subtype A, C and D Gag peptide sets used in this study were consistent with and generally representative of HIV-1 strains circulating in Mbeya region (fig 3.8).

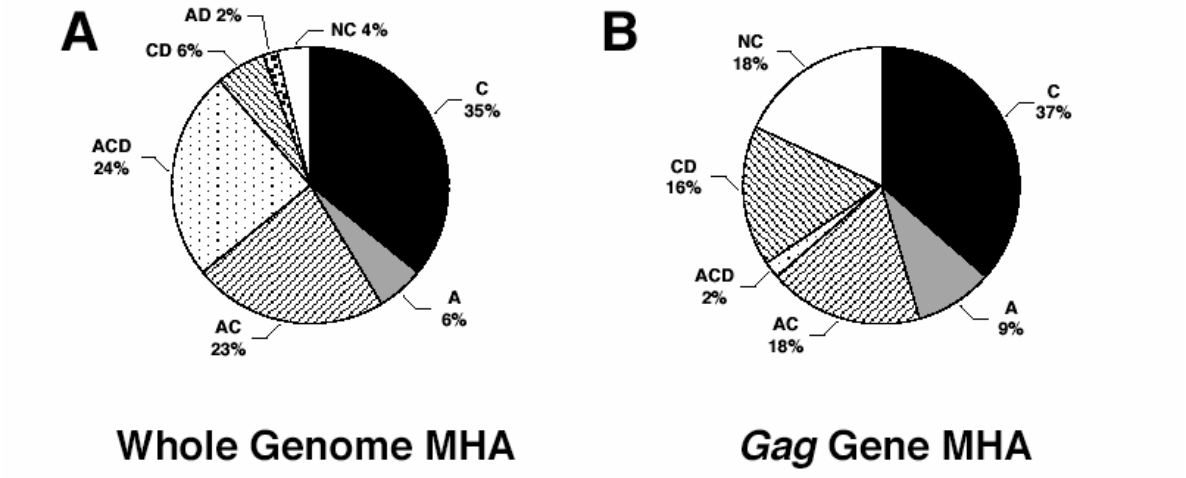


Figure 3.7. HIV-1 subtype distribution within the HISIS-CTL cohort. Shown are the results of the Multi Hybridization Assay analysis for the whole genome (**A**) and the *gag* gene (**B**). The frequencies of the detected pure and recombinant HIV-1 strains are given in percent. (NC- not conclusive)

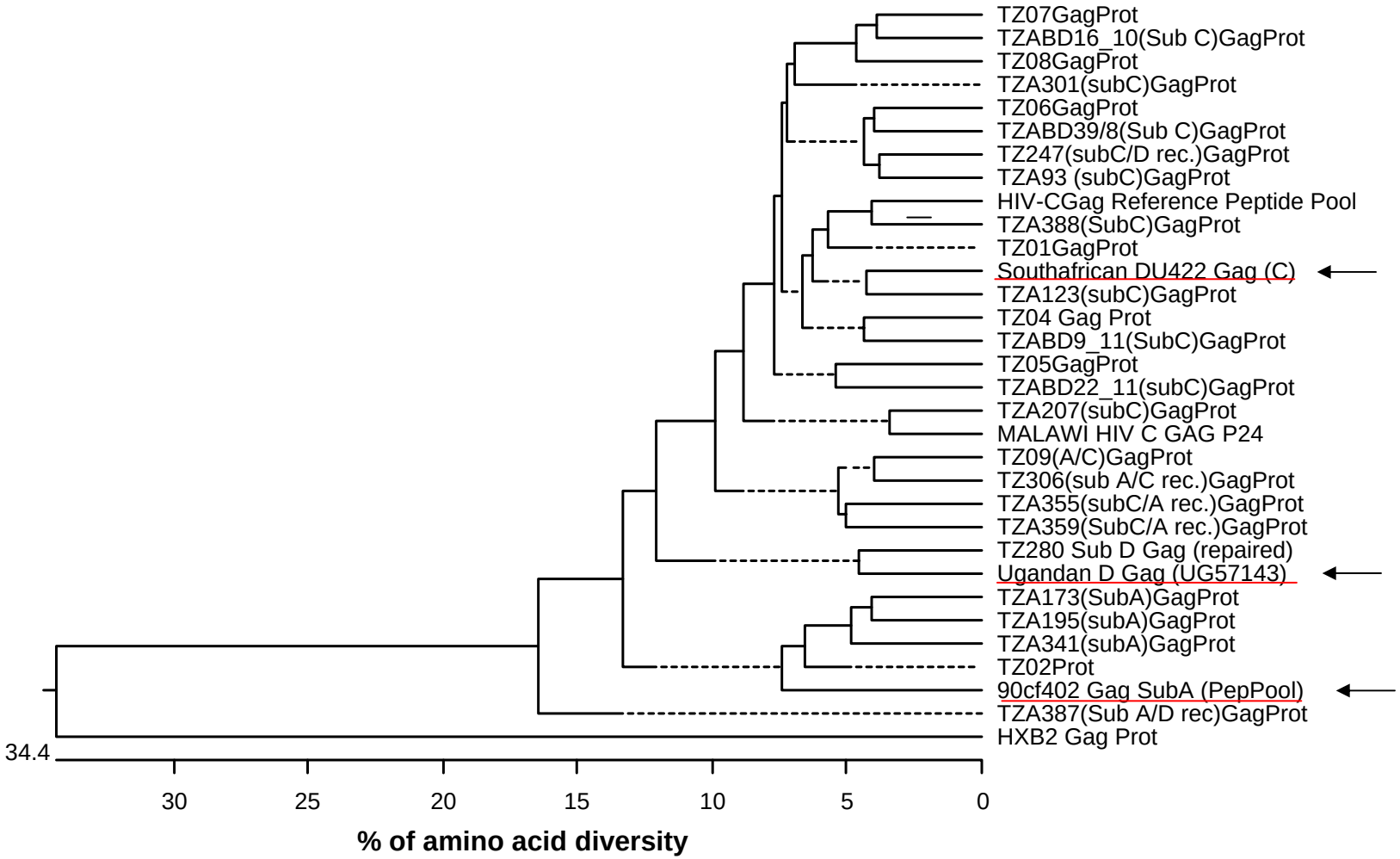


Figure 3.8. Phylogenetic relationship between subtype A, C and D Gag peptide sets and HIV Gag protein sequences from the Mbeya region in Tanzania.

3.2.2 HIV-1 Gag and Nef are frequent targets of a broad CD8 T cell response in HIV-1 infected Tanzanians

A peptide matrix-based IFNγ-ELISPOT assay was used to screen for HIV-1 Gag- and Nef-specific T-cell responses in the 56 HISIS-CTL cohort subjects. To maximize detection in this mixed-subtype epidemic, three peptide sets, representing subtypes A, C and D Gag were selected. Confirmation of positive peptide responses was carried out 3 months later during the next follow up using only the peptide inducing the highest response from the A, C or D peptide sets. Ninety-six percent (n=54) of the HISIS-CTL cohort responded to at least one Gag peptide, while 93% responded to at least one nef peptide, and all study subjects responded to PHA. A total of 82 individual HIV-specific responses were subjected to cytokine flow cytometric analysis for IFNγ production to determine the phenotype of the responding cells and confirmed the same responses as detected with the ELISPOT assay. Eighty-nine percent of responses were from CD8 T cells (data not shown).

To compare the responses among the three peptide sets, the frequency of responses against each linear pool of 11 consecutive Gag peptides, derived from the screening assay, was determined for the cohort. Subtype C peptides were recognized more frequently compared to subtype A or D peptides across the entire Gag protein (fig. 3.9 A). This is expected for this epidemic as subtype C and C-containing recombinants predominate. Further analysis of all individual Gag responses underscores the superiority of the subtype C peptides for detecting CD8+ T-cell responses in this cohort (fig 3.9 B). Of the 180 Gag-specific responses detected, 149 (83%) were detected with the C-peptides, 103 (57%) with the D-peptides and 91 (51%) with the A-peptides. The Venn diagram of relative cross-recognition of individual linear peptide pools (fig. 3.9 B) also shows that 98 (54%) of the responses detected by the C-peptides were also detected by the subtype A- and D-peptides, but 51 (28%) were detected only with the subtype C-peptides. Subtype A- and D peptide sets detected 31 responses not detected by the C-peptides. Of note, 56 (31%) of the responses were detected with all three peptide sets and may represent broadly crossreactive CD8+ T-cell epitopes.

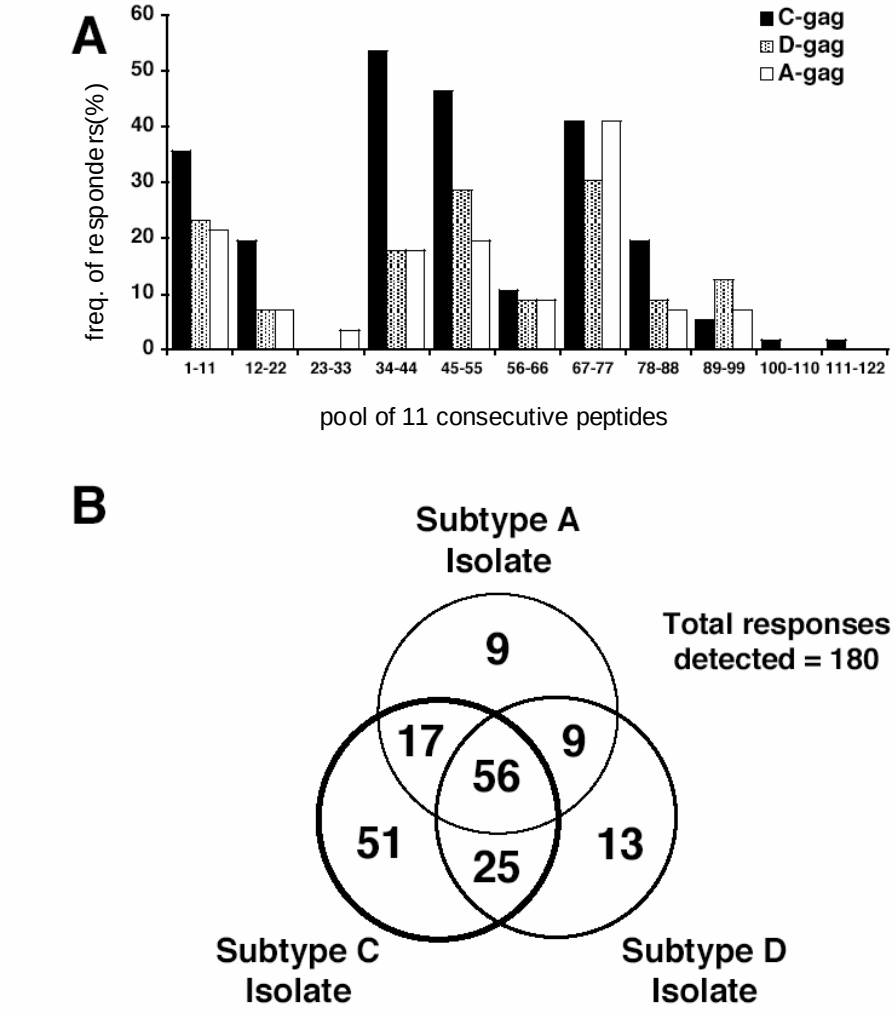


Figure 3.9. Comparison of recognition among the HIV-1 Gag peptide sets within the HISIS-CTL cohort. Fifty-six HIV-1 positive study subjects were screened with a peptide matrix-based IFNγ-ELISPOT assay for recognition of subtype A, C, and D Gag-specific peptide sets. **(A)** Peptide pools containing 11 successive overlapping peptides are represented on the x axis and the corresponding frequency of responding study subjects (n=56) with a response to the subtype A, C or D peptide pool is represented on the y axis. (**B)** Subtype-specific distribution of the total number of detected T-cell responses against all Gag peptide pools.

A composite frequency distribution of the response to any individual peptide from either Gag or Nef was constructed (fig. 3.10, panels A and B). The p24 protein of Gag was targeted with the highest frequency of subjects (91%), followed by p17 (56%) and the p15 proteins (15%). The breadth of Gag recognition for each individual was a median of 3 epitopes (a single peptide or pair of adjacent overlapping peptides), with a range of 0-10 epitopes (fig. 3.10, panel C). The magnitude of single peptide responses ranged from 70-5850 SFC/10^6 PBMC. As reported by others (Addo et al., 2003; Frahm et al., 2004; Masemola et al., 2004; Novitsky et al., 2002), regions with hot spots of immune recognition within the Gag protein were detected: aa10-aa47 (recognized by 45% of subjects); aa143-aa197 (64%) and aa291-aa321 (46%). The Nef protein was targeted by 93% of study subjects (n=52). As shown in figure 3.10 (panel B) the majority of responses against Nef were detected in the central region of the protein, which is in agreement with previous findings (Addo et al., 2003; Frahm et al., 2004; Masemola et al., 2004; Novitsky et al., 2002). Hot spots of immune recognition were detected at the highly conserved regions aa78-aa108 (targeted by 78% of subjects) and aa125-aa159 (71%). The breadth of Nef-specific responses was a median of 2 epitopes per subject with a range of 0-12 (fig. 3.10, panel D). The magnitude of single peptide responses ranged from 70-3440 SFC/10^6 PBMC. Thus, both gag and nef gene products are frequent and consistent targets of the CD8+ T-cell responses in the HISIS-CTL cohort.

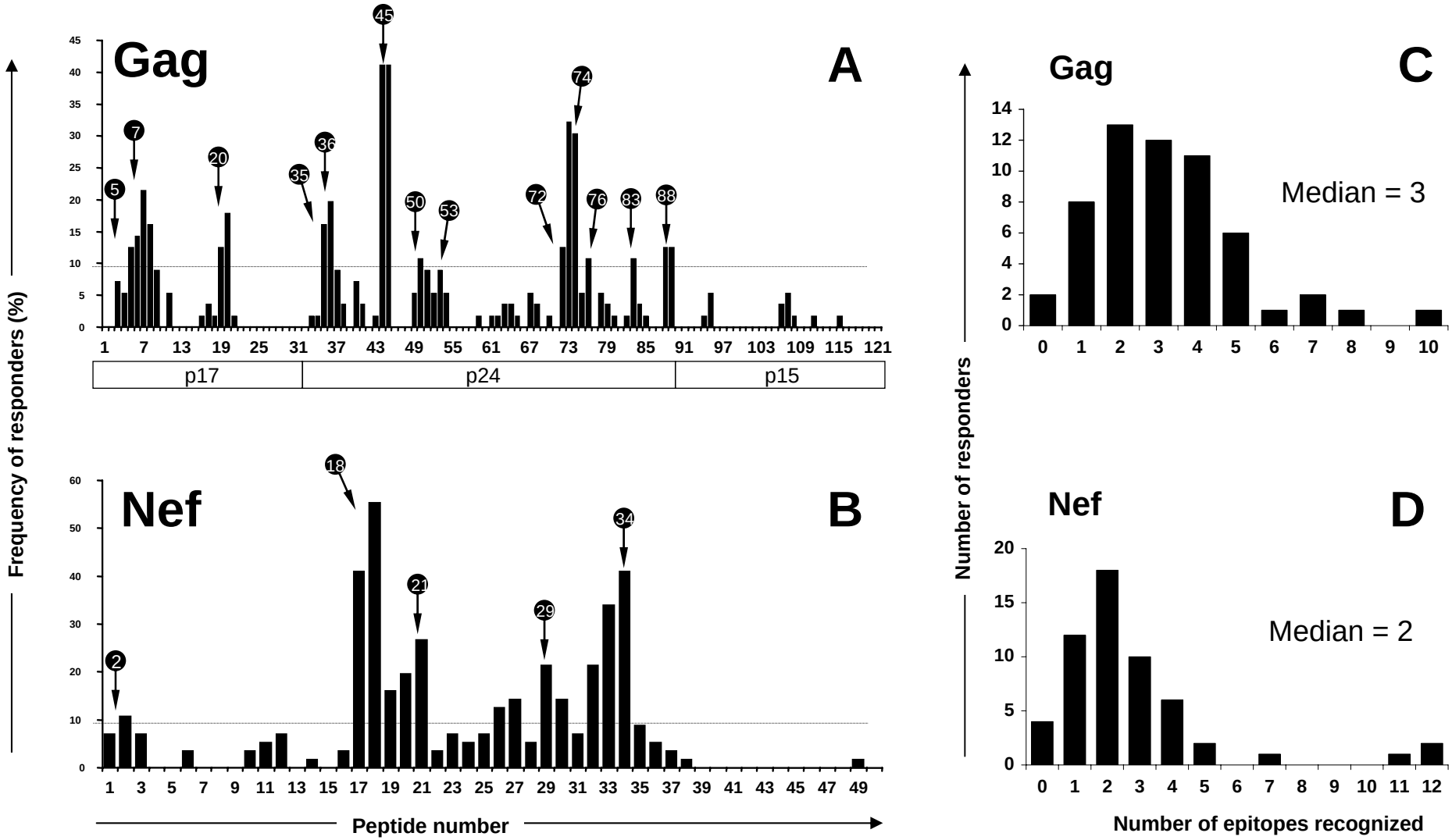


Figure 3.10. Frequency distribution and breadth of the Gag- and Nef-specific T-cell responses detected in the HISIS-CTL cohort. Individual peptide responses were confirmed for the 56 HIV-1 positive study subjects in an IFNγ-ELISPOT assay and are shown for Gag **(A)** and Nef **(B)**. The frequency of responders is given in percent. Peptides that were further analysed for cross-recognition are highlighted with arrows. Peptide numbers are represented on the x axis. The breadth of the Gag **(C)** and Nef **(D)** response per study subject is also shown. Responses to 2 consecutive peptides were counted as 1 epitope response.

Table 1. HLA-associations of the most commonly recognized peptides from the HISIS-CTL cohort.

Peptide #	Subtype	Sequence	HLA-Association	Comments	FRP-pool	Analysed for Crossreactivity
Gag5	D	EKIRLRPGGKKKYRL	B4201/B8101 (4/5)**	Described*	n	y
Gag7	C	GKKHYMLKHIVWASR	A2301 (8/11)	Not described	y	y
Gag20	C	SLYNTVATLYCVHEK	A02/A6802 (7/8)	Described*	y	y
Gag35	C	GQMVHQAISPRTLNA	B5802 (4/5)	Not described	n	y
Gag36	D	HQAISPRTLNAWVKV	B57/B58 (8/8)	Described*	y	y
Gag45	C	TPQDLNTMLNTVGGH	B7/B42/B8101(20/22)	Described*	y	y
Gag50	C	MLKDTINEEAAEWDR	B5301 (3/6)	Described*	y	y
			or A6802 (4/6)	Not described		
Gag53	C	WDRLHPVHAGPIAPG	A6802 (4/5)	Not described	y	y
Gag72/73	C	GPKEPFRDYVDRFFK	B8101 (6/7)	Not described	y (Gag73)	y
Gag74/73	C	YVDRFFKTLRAEQAT	B1503/B1510 (9/12)	Described*	y (Gag73)	y
Gag76	C	LRAEQATQEVKNWMT	B5301/B5801 (5/6)	Described*	y	y
Gag83	C	TILRALGPGATLEEM	B1503/B1510 (4/8)	Not described	y	y
		TILRALGPGATLEEM	and B8101(4/8)	Not described	y	
Gag88	D	ACQGVGGPSHKARVL	B7/B42/B8101 (9/9)	Described*	y	y
Nef2	A	WSKSSIVGWPAVRER	{B7/B42/B8101 (4 of 6)}	Not described	y	y
Nef18	C	PVRPQVPLRPMTYKA	B7/B42/B8101 (18/24)	Described*	y	sequence identical
Nef21	C	YKAAFDLSFFLKEKG	{B1503/10/16 (7/12)}	Not described	y	y
Nef29	C	WVYHTQGYFPDWQNY	B5703 (4/9)	Described*	y	y
Nef33/34	C	VRYPLTFGWCFKLVP	A2301(10/21)	Described*	y (Nef33)	sequence identical
			or A6802(8/21)	Not described		
			B5301 (8 of 21)	Described*		

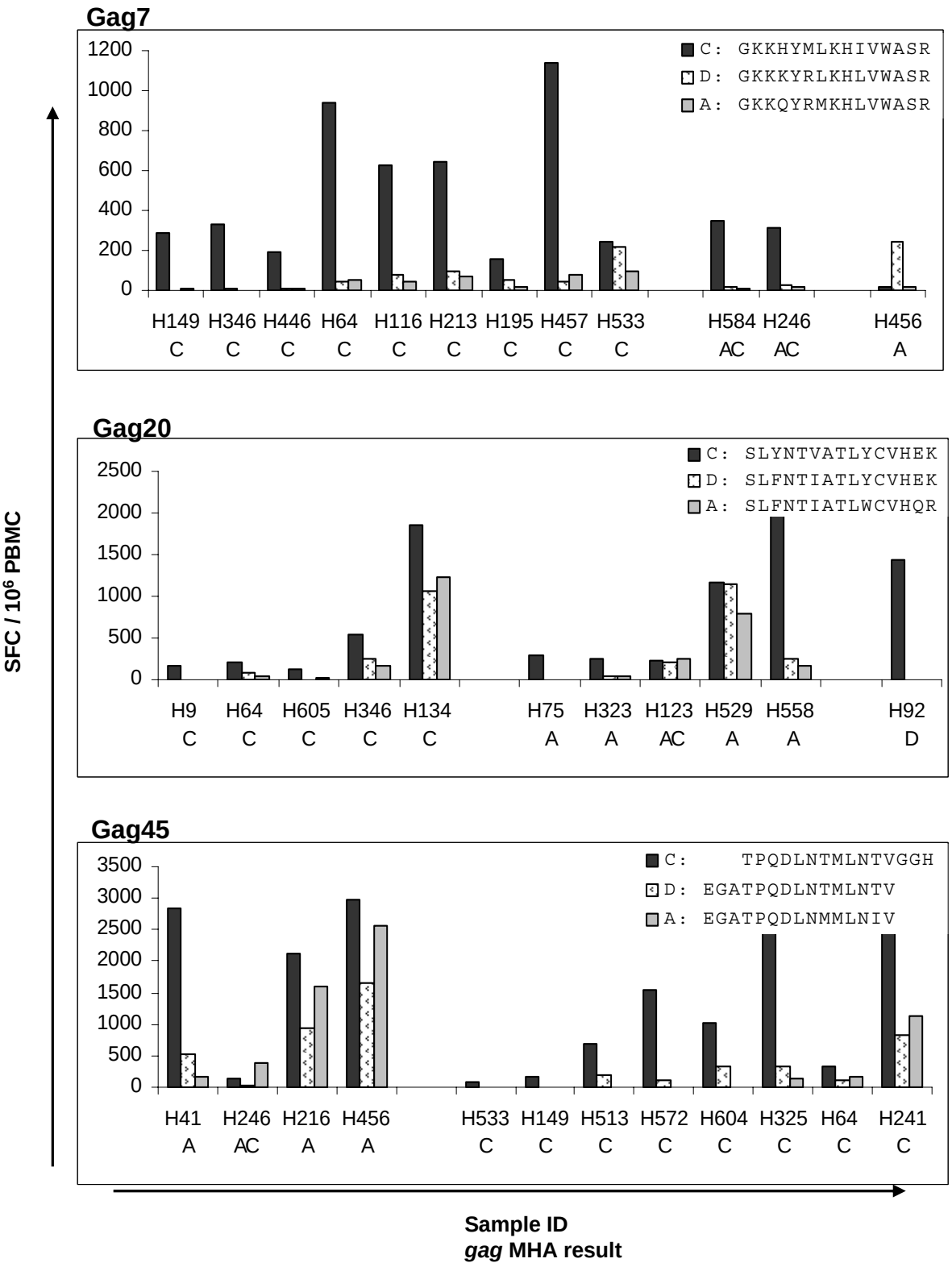
* described in HIV Immunology and HIV/SIV Vaccine Databases 2005: http://www.hiv.lanl.gov/immunology;
** X of Y responding subjects express indicated HLA class I allele
FRP-pool = included in pool of frequently recognized peptides

3.2.3 Frequently recognized peptides are associated with common HLA alleles in the cohort

HLA-typing of the A, B and C alleles from the 56 subjects in the cohort was performed as described in Methods and Materials. Allele frequencies for each detected allele are shown in figure 3.1. The 13 most frequently recognized Gag peptides and the 5 most frequently recognized Nef peptides (denoted by arrows in figure 3.10) were analyzed in greater detail. In addition, responses to each epitope (single peptide or a pair of 2 consecutive peptides) correlated with possession of a single common HLA-allele or HLA super-family present in the cohort (table 3.1). Many of the responses could be attributed to minimal epitopes and HLA associations. Seven of the peptides were displaying 14 associations with HLA alleles and are described in this work for the first time. 12 peptides contained previously described epitopes with known HLA-allele restrictions. Strikingly, all of the subjects in the cohort recognized one or more of these 18 peptides.

3.2.4 Discordance in the recognition of subtype derived peptide sets is a result of both sequence variation and frame-of-epitope positioning within a 15mer peptide

To determine the extent of cross-recognition in commonly recognized antigenic regions, the most frequently recognized peptides within Gag and Nef were analyzed for disparity of recognition among the different peptide sets used for screening. The median response of confirmed responders to the A, C and D variants of 16 peptides was analyzed. Figure 3.11 shows recognition of subtype A, C and D peptide variants for study subjects responding to peptides Gag5, Gag7, Gag20, Gag45, Gag73/74 and Gag83 and is exemplary for the analysis of all 16 antigenic regions shown in Figure 3.12. Additionally in figure 3.11 the *gag* MHA result for each study subject is shown below the sample ID. Two peptides (Nef18 and Nef34) with identical epitope sequences for all subtype variants were excluded from figure 5 as there was no difference in the magnitude of recognition among the three versions of these peptides. A statistical difference in the median response to the three peptide variants was


Gag7
C: GKKHYMLKHIVWASR
D: GKKKYRLKHLVWASR
A: GKKQYRMKHLVWASR
1200
1000
800
600
400
200
0
H149 C
H346 C
H446 C
H64 C
H116 C
H213 C
H195 C
H457 C
H533 C
H584 AC
H246 AC
H456 A
Gag20
C: SLYNTVATLYCVHEK
D: SLFNTIATLYCVHEK
A: SLFNTIATLWCVHQR
2500
2000
1500
1000
500
0
H9 C
H64 C
H605 C
H346 C
H134 C
H75 A
H323 A
H123 AC
H529 A
H558 A
H92 D
Gag45
C: TPQDLNTMLNTVGGH
D: EGATPQDLNTMLNTV
A: EGATPQDLNMMLNIV
3500
3000
2500
2000
1500
1000
500
0
H41 A
H246 AC
H216 A
H456 A
H533 C
H149 C
H513 C
H572 C
H604 C
H325 C
H64 C
H241 C
SFC / 10^6 PBMC
Sample ID
gag MHA result

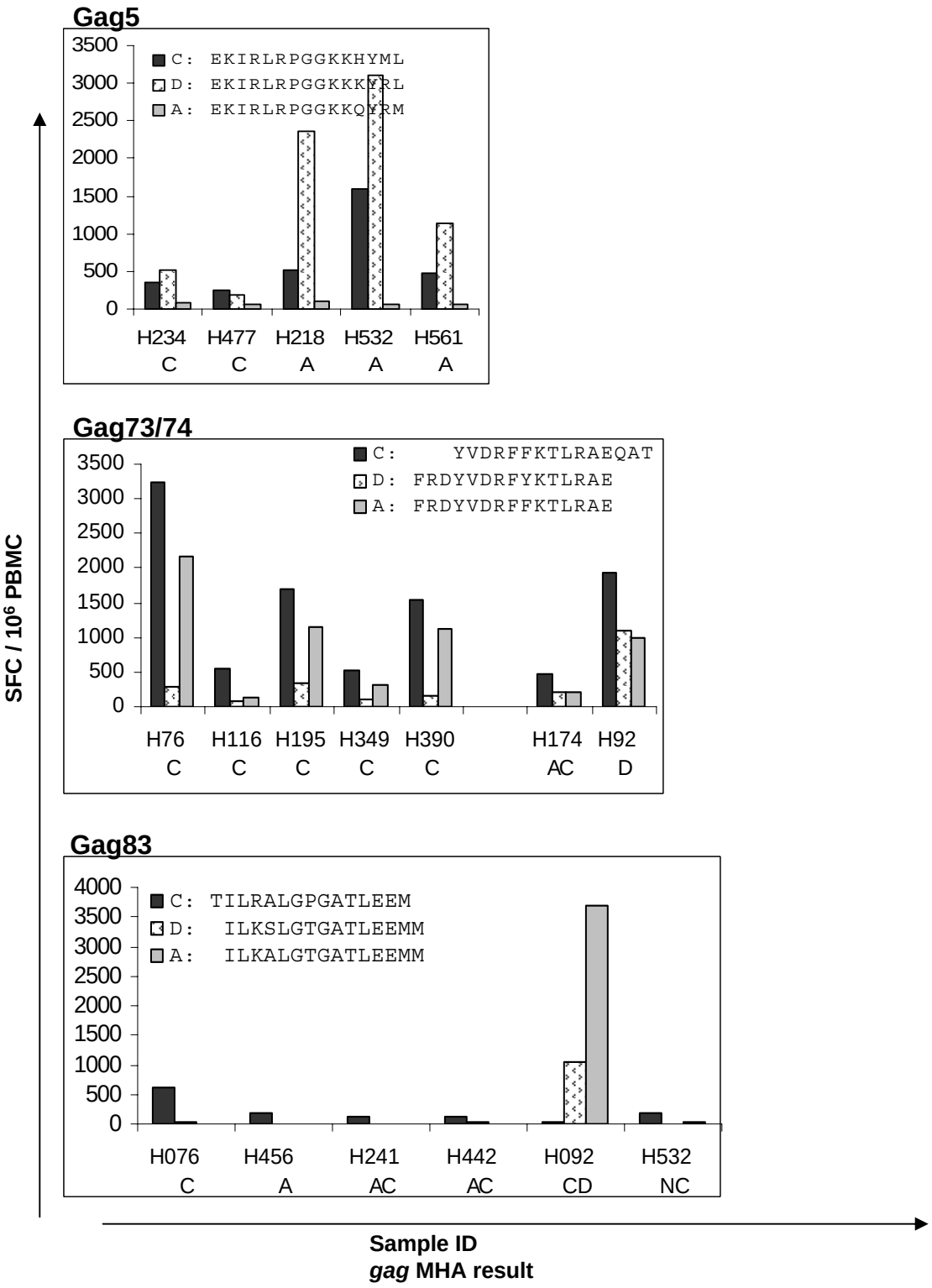


Figure 3.11. Recognition of subtype A, C and D derived peptide variants for antigenic regions Gag7, Gag20, Gag45, Gag5, Gag73/74 and Gag83. Shown is the SFC/10^6 PBMC of confirmed responding subjects after peptide stimulation *ex vivo*. The sample ID and the *gag* MHA results of each subject is given on the x axis.

observed in 11 of the 16 responses (Friedman test, $p<0.05$). The pair, or pairs, of peptides that elicited different responses were then identified using Dunn's multiple comparison test. In those cases that didn't reach statistical significance, a trend towards a difference in the median response was evident (fig. 3.12 E, L and N).

During this study the observed magnitude of T cell responses measured against multiple overlapping peptides sets was affected not only by sequence differences of the peptides, but was also due to insertion/deletion polymorphisms affecting the relative positioning of the epitopes. Disparate recognition of 10 of 16 antigenic regions could be directly attributed to sequence variation within the compared peptides (reduction in median magnitude of responders), while frame-of-epitope shifting (the position of a minimal 9-mer or 10-mer epitope within a 15-mer peptide) contributed to diminished recognition of 4/16 epitopic regions (fig.3.12 F, G, J and K). As demonstrated in figure 3.11 differences in the magnitude of response to the three peptides could, in the p17 region, be directly attributed to sequence variation between the peptide sets. In 9 cases it was impossible to determine whether discordant peptide recognition was the result of point substitution or a result of the epitope position within the 15-mer peptide. In spite of sequence identity of the minimal B42/B81-restricted epitope TL9 (TPQDLNTML; fig. 3.11C and fig. 3.12 F), the median response of 11 subjects to the subtype C peptide (1030 SFC/10^6 PBMC) was decreased over 5 fold when using the subtype D peptide (180 SFC/10^6 PBMC). Here, only the position of the minimal epitope within each 15-mer differs between the two peptides and affects the detected magnitude of the T-cell response. The T→M substitution in the subtype A peptide (containing the minimal epitope in the same position as the subtype D peptide) resulted in a further two-fold reduction in median recognition (90 SFC/10^6 PBMC) and was recognized poorly by Gag45 responders (fig. 3.11). Of note, the preceding peptide in the subtype C pool (Gag44) showed only a slightly decreased frequency of recognition compared with peptide Gag45 even though the minimal epitope is in a different frame in these two peptides. Similar observations were made for the antigenic regions Gag74 and Gag72 (fig. 3.12 J and K). Nonetheless, for Gag p17 the relative

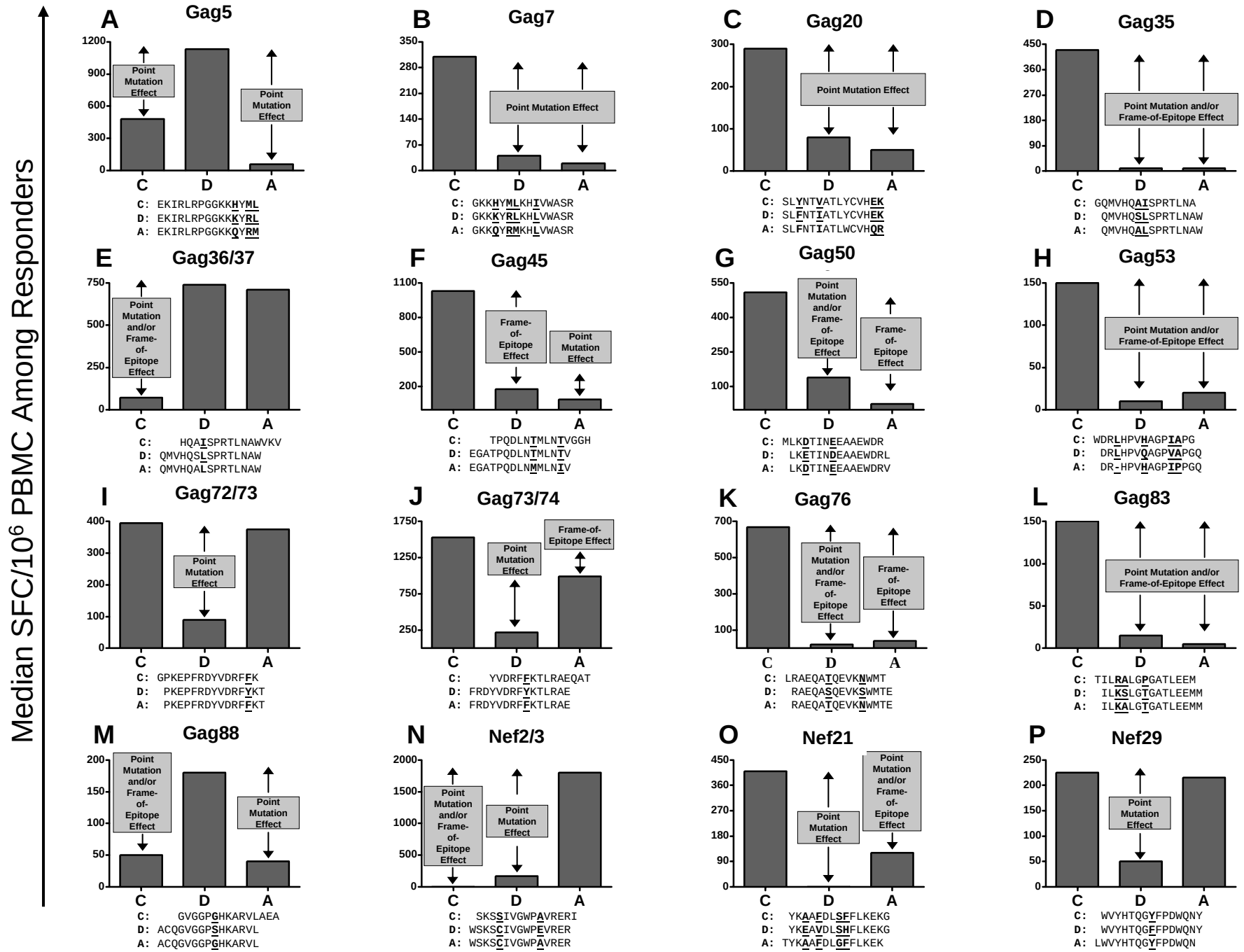

Median SFC/10^6 PBMC Among Responders
A
Gag5
Point Mutation Effect
Point Mutation Effect
C: EKIRLRPGGKKHYML
D: EKIRLRPGGKKKYRL
A: EKIRLRPGGKKQYRM
B
Gag7
Point Mutation Effect
C: GKKHYMLKHIVWASR
D: GKKKYRLKHLVWASR
A: GKKQYRMKHLVWASR
C
Gag20
Point Mutation Effect
C: SLYNTVATLYCVHEK
D: SLFNTIATLYCVHEK
A: SLFNTIATLWCVHQR
D
Gag35
Point Mutation and/or Frame-of-Epitope Effect
C: GQMVHQAISPRTLNA
D: QMVHQSLSPRTLNAW
A: QMVHQALSPRTLNAW
E
Gag36/37
Point Mutation and/or Frame-of-Epitope Effect
C: HQAISPRTLNAWVKV
D: QMVHQSLSPRTLNAW
A: QMVHQALSPRTLNAW
F
Gag45
Frame-of-Epitope Effect
Point Mutation Effect
C: TPQDLNTMLNTVGGH
D: EGATPQDLNTMLNTV
A: EGATPQDLNMMLNIV
G
Gag50
Point Mutation and/or Frame-of-Epitope Effect
Frame-of-Epitope Effect
C: MLKDTINEEAAEWDR
D: LKETINDEAAEWDRL
A: LKDTINEEAAEWDRV
H
Gag53
Point Mutation and/or Frame-of-Epitope Effect
C: WDRLHPVHAGPIAPG
D: DRLHPVQAGPVAPGQ
A: DR-HPVHAGPIPPGQ
I
Gag72/73
Point Mutation Effect
C: GPKEPFRDYVDRFFK
D: PKEPFRDYVDRFYKT
A: PKEPFRDYVDRFFKT
J
Gag73/74
Point Mutation Effect
Frame-of-Epitope Effect
C: YVDRFFKTLRAEQAT
D: FRDYVDRFYKTLRAE
A: FRDYVDRFFKTLRAE
K
Gag76
Point Mutation and/or Frame-of-Epitope Effect
Frame-of-Epitope Effect
C: LRAEQATQEVKNWMT
D: RAEQASQEVKSWMTE
A: RAEQATQEVKNWMTE
L
Gag83
Point Mutation and/or Frame-of-Epitope Effect
C: TILRALGPGATLEEM
D: ILKSLGTGATLEEMM
A: ILKALGTGATLEEMM
M
Gag88
Point Mutation and/or Frame-of-Epitope Effect
Point Mutation Effect
C: GVGGPGHKARVLAEA
D: ACQGVGGPSHKARVL
A: ACQGVGGPGHKARVL
N
Nef2/3
Point Mutation and/or Frame-of-Epitope Effect
Point Mutation Effect
C: SKSSIVGWPAVRERI
D: WSKSCIVGWPEVRER
A: WSKSCIVGWPAVRER
O
Nef21
Point Mutation Effect
Point Mutation and/or Frame-of-Epitope Effect
C: YKAAFDLSFFLKEKG
D: YKEAVDLSHFLKEKG
A: TYKAAFDLGFFLKEK
P
Nef29
Point Mutation Effect
C: WVYHTQGYFPDWQNY
D: WVYHTQGFFPDWQNY
A: LWVYHTQGYFPDWQN

Figure 3.12. Comparison of the median T-cell responses to subtype-derived peptide variants of the 16 most frequently recognized antigenic regions in gag (n=13) and nef (n=3). Shown is the median SFC/10^6 PBMC of study subjects after peptide stimulation *ex vivo*. Peptide numbers and sequences are annotated. The response to 2 dominant regions in Nef (Nef18 and Nef33/34) is not shown, because the assumed epitope sequences are identical among the 3 subtypes.

effect of point mutations on T-cell recognition could be resolved for all three peptides sets as well as for subtype A and D peptide sets in Gag p24.

Does the subtype of the infecting HIV-1 strain influence the peptide-specific CD8 T cell recognition? Peptide Gag7 was predominantly recognized by study subjects that were infected with a subtype C Gag expressing HIV strain. 3 of 4 subjects who were exposed to a subtype A Gag expressing strain recognized the subtype A variant of Gag45 better than the subtype D variant, wheareas only 2 of 8 subjects infected with a C Gag expressing strain recognized the A Gag variant better than the subtype D variant (fig. 3.11). Together with the observation that the C Gag peptide set was superior for detecting responses in the HIV-1 subtype C dominated epidemic in Mbeya region, these results suggest that the T cell recognition of variant epitopes is in part dependent on the subtype of the infecting HIV strain. However, for some of the analysed Gag-specific peptide responses, the subtype of the infecting strain did not appear to play a major role on which peptide variant would be recognized with the highest frequency of responding PBMC.

3.2.5 A minimal set of 15 peptides is recognized by 94% of HIV seropositives outside the HISIS cohort

While no single peptide set was capable of detecting all of these responses optimally, a composite peptide pool consisting of peptides representing subtypes A, C and D across the immuno-dominant regions would be the best configuration to detect HIV-1-specific CD8 T-cell responses in HIV-infected subjects in Tanzania. To test this hypothesis, a pool of frequently recognized peptides including 10 of the most frequently recognized Gag peptides and 5 of the most frequently targeted nef peptides was designed. Responses to these peptides were associated with HLA class I alleles common in the Mbeya

region. The 15 peptides included in the pool are denoted in Table 1 (frequently recognized peptides; FRP-pool). Within the HISIS-CTL cohort, 96% of subjects expressed at least one, and 77% expressed two or more alleles known to restrict responses to the FRP-pool. The FRP-pool was then tested on 50 HIV seropositive and 25 HIV seronegative individuals from two other studies (as described in Materials and Methods), outside the HISIS-CTL cohort. The ELISPOT assay was performed using 2.5 x 10^5 PBMC/well and the cut-off value was 20 SFC/10^6 PBMC. Strikingly the FRP-Pool was recognized by 94% of HIV seropositives, while it was not recognized by any of the HIV seronegative study subjects (fig. 3.13).

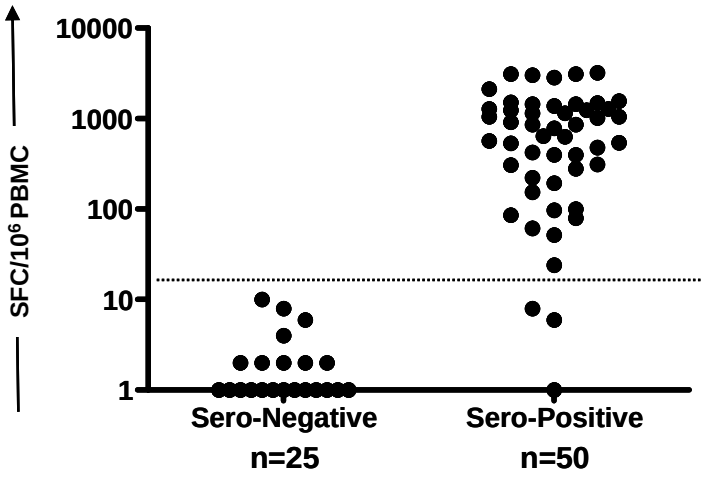


Figure 3.13. A pool of 15 frequently recognized HIV-1 Gag- and Nef-derived peptides (FRP-pool) was tested for recognition by PBMC from 50 HIV-1 seropositive individuals arbitrarily chosen from an HIV cohort in the same epidemic region as the HISIS-CTL cohort. Study subjects from the HISIS-CTL cohort were excluded. To demonstrate assay specificity, 25 HIV seronegative individuals were included. Shown are the SFC/10^6 PBMC for each individual.

3.3 Infection with multiple HIV-1 strains contributes to a broadening Gag-specific CD8 T cell response

In chapter 3.1, evidence was presented that one of the main determinants for the immunological outcome of an HIV-1 infection appears to be the HLA background of the infected individual. Additional parameters are the duration of the infection (Addo et al., 2003; Goulder et al., 2001) and potentially the viral diversity within an HIV-infected individual on the HIV-specific CD8 T cell response. Infection with multiple HIV strains has been shown to increase diversity of viral quasi-species by ongoing inter-strain recombination. This resulted in a strong fluctuation of many different molecular forms of HIV-1 within multiply infected individuals (Gerhardt et al., 2005; McCutchan et al., 2005). Hence the diversity of the viral quasi-species is increased and, with the ever changing molecular recombinant forms, much more dynamic in individuals infected with multiple HIV-strains. This in turn is likely to affect the HIV-specific immune response, as it will at least transiently be exposed to different amino acid variants of the same epitope. Jung et al. have suggested that the number of distinct antigenic epitopes presented by a single cell may also be increased, thereby broadening immune recognition (Jung et al., 2002).

Only little is known on how the immune system will react to the exposure to multiple variants of the same virus. Infection to multiple HIV subtypes might induce a broadly cross-reactive immune response, but also could give rise to the least immunogenic strain by recombination of the two strains. In one of the first reported cases of an HIV-superinfection, Altfeldt et al. have observed i) a decreasing frequency and ii) a changing pattern of CD8 T cell epitope specificities upon superinfection with a second subtype B virus (Altfeld et al., 2002; Jost et al., 2002). After inter-subtype superinfections the CD8 T cell response appears to adapt to the incoming strain (Ramos et al., 2002; Yang et al., 2005). However, there have only been few case studies published on this subject so far.

The high proportion of multiply infected study subjects in the HISIS-CTL cohort allowed to address the question of how the increased viral diversity in

multiply infected individuals affects the HIV-specific CD8 T cell response on a population level. This issue was addressed in the 3rd part of this PhD thesis.

3.3.1 Dual infected study subjects add up to 50% of the HISIS-CTL cohort

The MHAacd allows determination of the infecting subtype and to distinguish infections with a single HIV strain from infections with multiple HIV strains. The subtype distribution of this cohort is described in chapter 3.2. As exemplified in figure 3.14, indications for an infection with multiple HIV subtypes are i) the binding of probes from 2 or 3 different subtypes to a single genomic region in the MHAacd during one or more FUs or ii) a changing pattern of subtype-specific probes that bind to the same genomic region. For example, study subject H123, who seroconverted at the beginning of the study (FU1), was first infected an **AD** recombinant strain and then superinfected during FU2 with a different ACD recombinant virus. Study subject H001 is a pure subtype C infection, whereas study subject H561 was infected with an AC recombinant strain during FU8. In total, the MHAacd results indicate that 50% (n=28) of the HISIS-CTL study subjects were infected with more than one HIV strain.

H001: single infected

	Gag	Pol	Vpu	Env1	Env2
FU0		C	C	C	C
FU1	C	C	C	C	
FU2			C	C	C
FU3	C	C	C	C	C
FU4	C	C	C	C	C
FU5	C	C	C	C	C
FU6	C	C	C	C	C
FU7	C	C	C		C
FU12	C	C	C	C	C
FU13	C	C	C	C	C

H561: inf. with AC-recombinant

	Gag	Pol	Vpu	Env1	Env2
FU4					
FU5					
FU6					
FU7					
FU8					
FU9	A	C	A		C
FU10	A	C	A		C
FU11	A	C	A		C
FU12	A	C			C
FU13	A	C	A		C

H123: Dual infected

	Gag	Pol	Vpu	Env1	Env2
FU0					
FU1	A	**A**			D
FU2			C		
FU3	A	**C**	C	A	**ACD**
FU4	A	C	C	A	**AD**
FU5	A	C	C	A	**ACD**
FU6	A	C	C	A	**ACD**
FU7	A	C	C	A	**AD**
FU12	A	C	C	A	D
FU13	A	C		A	D

Figure 3.14 MHAacd patterns of single and multiply infected subjects: Shown are the results of the analysis for subtypes A, C and D for the 5 genomic regions Gag, Pol, Vpu, Env1 and Env2 at multiple follow ups (3 months intervals). Indications for multiple infection in **bold**)

3.3.2 Infection with multiple HIV strains is associated with a broad Gag-specific CD8 T cell response

Infection with multiple HIV strains increases the diversity of the viral quasi-species and exposes the human immune system to multiple amino acid variants of the same HIV protein. Thus it may be hypothesized that infection with multiple HIV strains may broaden the HIV-specific T cell response. To test this hypothesis, the number of recognized epitopes within Gag and Nef was compared in multiply and single infected subjects (fig. 3.15 and 3.16). The breadth of the Gag-specific CD8 T cell response was a median of 3 epitopes recognized for both multiply and single infected subjects with a range of 0-10 epitopes recognized in multiply infected subjects and 0-6 epitopes recognized in single infected subjects. The mean breadth was 3.6 and 2.8 epitopes recognized for multiply and single infected subjects respectively. Of note is that 28.6% (8 of 28) of multiply infected subjects recognized 5 or more epitopes within Gag, whereas only 10.7% (3 of 28) of single infected subjects recognized that many epitopes. Similar differences were observed in the recognition of subtype A, C and D Gag-specific peptide pools as shown in figure 3.15. 62 study subjects of the HISIS-CTL cohort were tested with subtype-specific peptide pools. Each of the 11 peptide pools contains 11 consecutive overlapping peptides, which subdivide gag into 11 antigenic regions: pools number 1A to 3A cover for Gag p17, 4A to 8A cover p24 and pools 9A to 11A cover p15. Responses to Gag44/45 and Gag88/89 were detected within 2 consecutive pools, but were counted as one pool response only, because in both cases the respective response is directed against a single epitope: Gag44/45 was counted as a pool 5 response and Gag88/89 as a pool 8 response. A median of 3 peptide pool responses were detected with subtype C Gag peptides within both groups. For the subtype A and D Gag peptides, the median number of pools recognized was 2 and 1.5 for multiply infected subjects respectively, but only 1 for subtype A and D Gag peptides for single infected subjects. With the subtype C peptides, 5 or more peptide pools were recognized in 18.8% (n=6/32) of multiply infected and only 6.3% (2 of 32) of single infected subjects, corresponding numbers for the subtype D peptides were 13.3% (4 of 32) in multiply infected versus 6.3% (2 of 32) in single infected subjects and for the subtype A peptides 13.3% (4 of 30) of multiply

infected and 0% of the single infected. Hence there was a clear tendency that the increased viral diversity of quasi species contributes to a broadening of the Gag-specific CD8 T cell response in subjects infected with multiple HIV-1 strains.

Interestingly the Nef-specific CD8 T cell response appeared to be unaffected by the exposure to multiple HIV strains. Both groups had a very similar breadth of Nef specific CD8 T cell responses that could be explained by the highly focused Nef-specific CD8 T cell response observed in many study subjects.

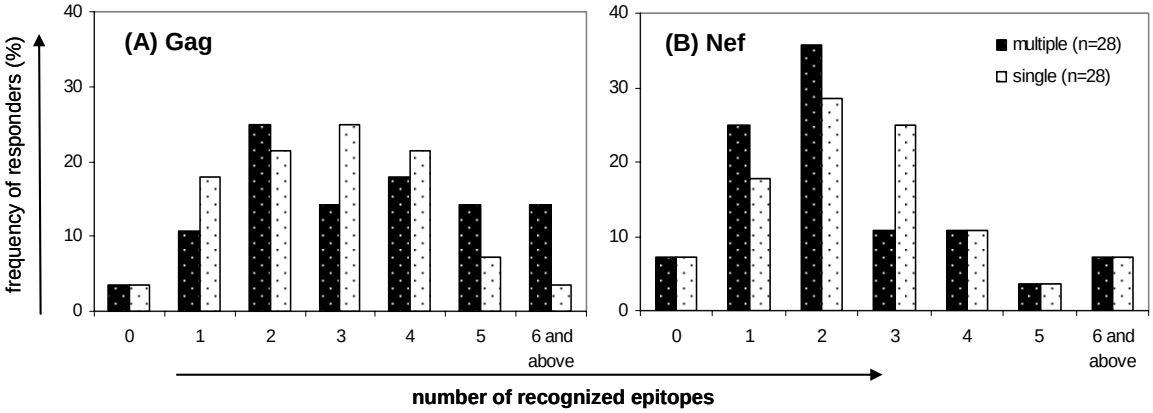


Figure 3.15 Breadth of the HIV-1 Gag (A) and the HIV-1 Nef (B) specific T-cell response in single infected (white bars, n=28) and multiply infected (black bars, n=28) study subjects. Shown is the frequency of responders and the number of epitopes recognized by the different study subjects is given. Responses to 2 consecutive peptides were counted as 1 epitope response**.**

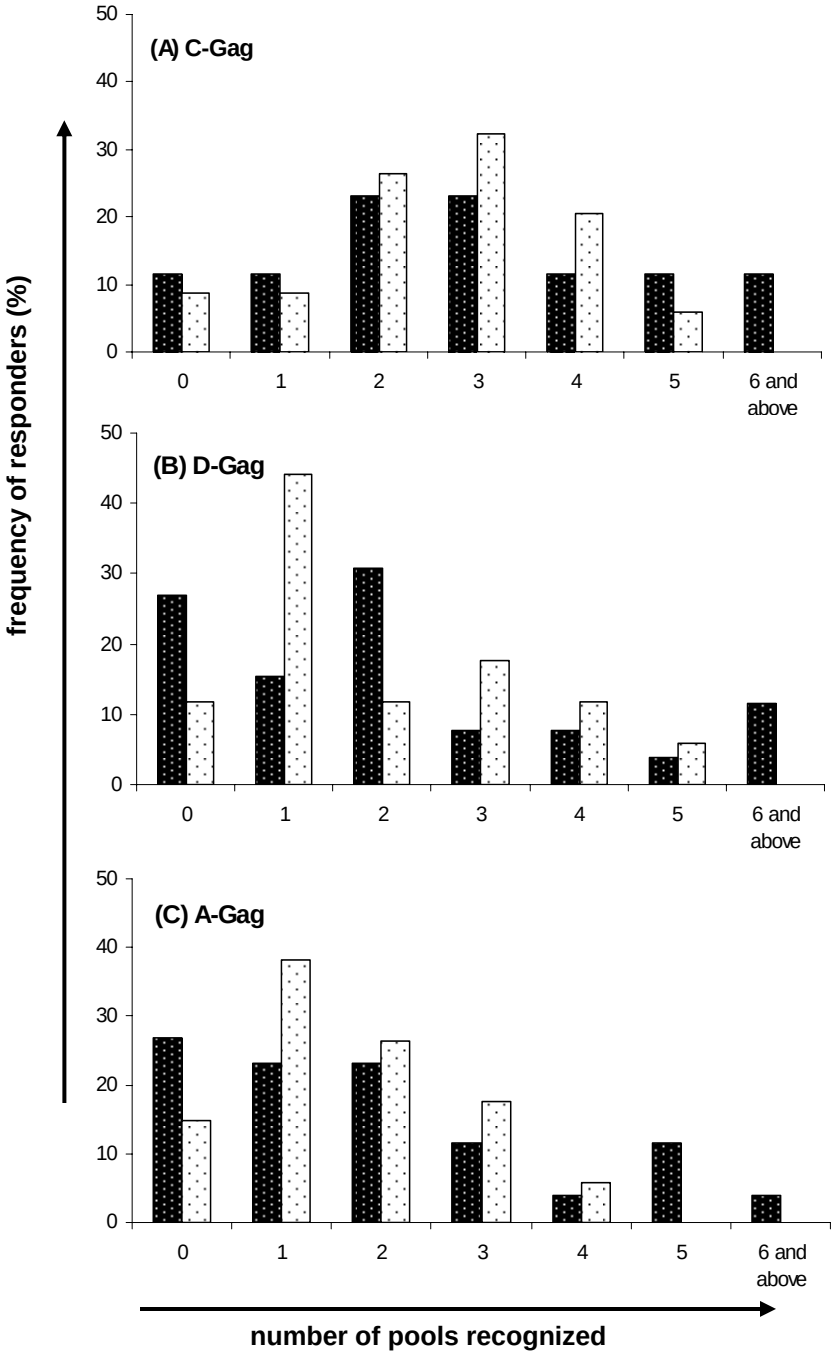


Figure 3.16. Breadth of subtype-specific Gag peptide pool recognition in single and multiply HIV-1 infected individuals. The number of subtype A, C or D Gag derived peptide pools recognized by dual infected (n=30, black bars) and single infected (n=31, white bars) and the corresponding frequency of responders is given. The peptide pools contained eleven successive overlapping peptides. Responses against Gag peptides 44/45 and 88/89, which are detected in two pools, were counted as a single pool response.

3.3.3 Infection with multiple HIV-strains may contribute to better cross-recognition of antigenic regions within p17, p15 and the C-terminal part of p24

Due to the genetic diversity of the HIVpandemic, determination of parameters that contribute to T cell cross-recognition of different HIV subtypes has import implications for HIV vaccine design. Multi-subtype vaccines have been designed and are currently going into phase I/II clinical trials in the near future. Studying the CD8 T cell response in single and multiply infected subjects allowed addressing the question, whether infection with multiple different subtypes may contribute to better cross-recognition of antigenic regions within Gag. For this reason, cross-recognition of the 11 subtype A, C and D derived peptide pools was analyzed. As shown in figure 3.17A, the percentage of pool responses detected with all three peptide sets was 34.5% (40 of 116 analyzed responses) for multiply infected subjects and 23.1% (25 of 108 analyzed responses) for single infected subjects. To determine whether this difference in cross-recognition is seen throughout the whole Gag protein or whether this difference in cross-recognition is restricted to particular regions within Gag, it was next analysed which peptide pools were recognized within all three peptide sets. As shown in figure 3.17B it is mainly p17, the C-terminal region of p24 and p15 that contribute to this difference. 6 of 9 peptide pools are cross-recognized better by multiple infected study subjects, while for 3 peptide pools the differences were marginal. This results suggests that cross-recognition of antigenic regions is improved in subjects infected with multiple HIV strains.

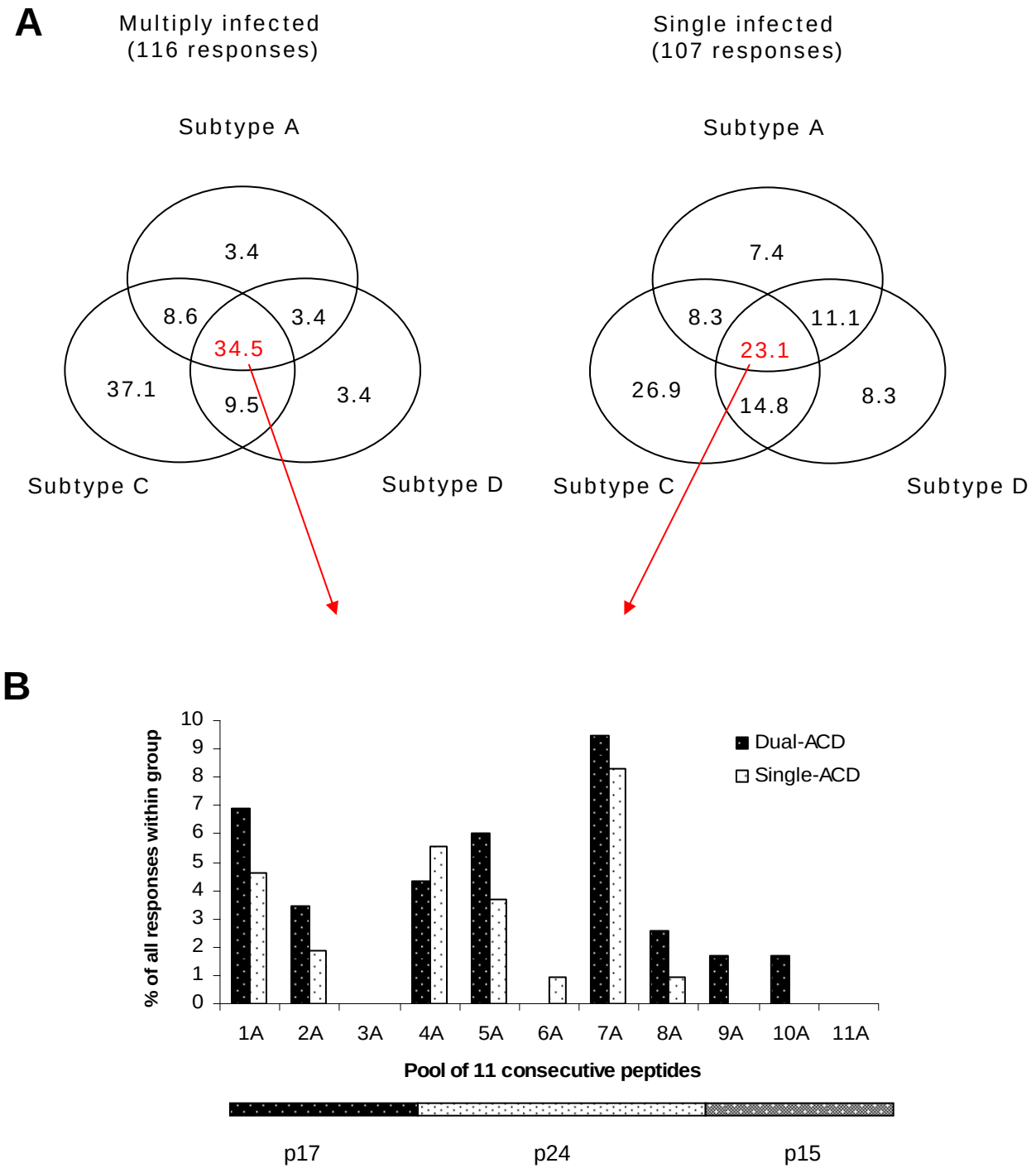


Figure 3.17. Cross-recognition of Subtype A-, C- and D-Gag peptide pools in dual and single infected study subjects. (A) Shown is the percentage of total analysed pool responses for each group (dual, n=116 / single, n=107). **(B)** Shown are the individual peptide pool responses that were detected with all three peptide sets. The y axis shows the percentage of total analysed pool responses for dual infected (black bars) and single infected (white bars).

3.3.4 Frequently recognized Gag peptides are targeted by both single and dual infected subjects

Finding immune correlates of protection from infection is an important task for HIV vaccine design. In the context of the HISIS-study one important goal was the identification of CD8 T cell epitopes that may protect from superinfection. For this reason the most frequently recognized antigenic regions were examined for recognition by single and multiply infected study subjects. As shown in table 3.2, each of the frequently recognized antigenic regions within Gag was targeted by both, single and multiply infected study subjects.

Three peptide responses were somehow remarkable: Responders to Gag5 had a low median viral load of 23.000 RNA copies/ml. Apparently 5 of the 7 responders were recent seroconverters (seroconversion < 3.5 years), suggesting that this response may be lost in the course of chronic HIV infection. 6 of the 8 Gag36 responders with an HLA B5703 or B5802 restricted response against the epitope ISPRTLNAW were single infected. Median viral load of Gag83 responders was only 15.800 RNA copies/ml, but still 6 of 8 subjects were multiply infected. This observation does not argue in favour of a "protective" role of this particular peptide response.

Table 3.2. Recognition of individual Gag peptides by multiply and single infected subjects and median viral load

Peptide#	Sequence	number of multiply infected responders	number of single infected responders	median Viral Load (RNA copies/ml)	N° of subjects seroconverting < 3.5 years
Gag5	EKIRLRPGGKKKYRL	4	3	23300	5 of 7
Gag7	GKKHYMLKHIVWASR	6	5	266000	1 of 11
Gag20	SLYNTVATLYCVHEK	8	5	197600	4 of 13
Gag35	GQMVHQAISPRTLNA	6	3	158000	5 of 9
Gag36	HQAISPRTLNAWVKV	2	6	92600	3 of 8
Gag45	TPQDLNTMLNTVGGH	13	10	158000	8 of 23
Gag50	MLKDTINEEAAEWDR	3	3	221000	1 of 6
Gag72	GPKEPFRDYVDRFFK	3	3	708000	3 of 6
Gag74	YVDRFFKTLRAEQAT	6	6	110250	5 of 12
Gag76	LRAEQATQEVKNWMT	3	3	182700	1 of 6
Gag83	TILRALGPGATLEEM	6	2	15800	3 of 8
Gag88	ACQGVGGPSHKARVL	6	3	74300	3 of 9

4. Discussion

4.1 Identification of a CD8 T cell mediated mechanism that contributes to the maintenance of a low viral load steady-state level

One of the main objectives of the HISIS-CTL study was to clarify determinants of the anti-viral efficiency of the HIV-specific CD8 T cell response and to examine the influence of the expression of HLA class I alleles on the HIV-specific CD8 T cell response.

The results of the HISIS-CTL study are best understood in the context of the study of Kiepiela et al. Frequency of HLA alleles and the associated respective median viral load closely matched that observed in the Kiepiela study (Kiepiela et al., 2004). Nonetheless, the median viral load among the HISIS-CTL study subjects was more than 5 fold higher than the median viral load observed in the Kiepiela study. Study subjects from both studies did not receive antiretroviral treatmenr (ARV). Other comparable studies conducted in the US, in Zambia or in South Africa also reported much lower median viral loads than that observed in the HISIS-CTL cohort, with median viral loads of ~18 000, 37 000 and 11 500 RNA copies/ml respectively (Mellors et al., 1996; Novitsky et al., 2003; Masemola et al., 2004). The protocol used to determine the plasma viral load was identical for the Kiepiela and the HISIS-CTL study and was approved by the U.S. Food and Drug Administration (FDA). Thus technical reasons for the observed difference can be excluded.

Grobler et al. have observed a significantly higher viral load set point in 4 dually infected subjects compared with 15 single infected subjects (Grobler et al., 2004), indicating that the high proportion of multiply infected subjects within the HISIS-CTL study could be a reason for the high viral load observed. However, in the HISIS-CTL study there was no difference in the viral loads of multiply and single infected subjects.

The most likely reason for the high median viral load of the HISIS-CTL study could be longer infection times. It is known that late in the course of HIV infection plasma viral levels rise (see introduction fig.1.6). 33 subjects were infected for more than 3.5 years while 22 study subjects seroconverted during

the last 3.5 years. These seroconverters had a median viral load of 40 600 RNA copies/ml at 24 months past seroconversion, which is in the range observed in most other studies. Therefore the high median viral load observed in within the HISIS-CTL cohort is likely to reflect a high proportion of subjects who have been infected for extended time periods.

HLA class I restricted CD8 T cell is thought to play a key role for containing viral replication. They are likely to contribute to the decline of plasma viremia during acute infection and to the establishment and to the maintenance of the steady-state virus level during chronic infection (Borrow et al., 1994; Koup et al., 1994; Masemola et al., 2004; Novitsky et al., 2003; Ogg et al., 1998). However, it is not completely understood, why the disease in some individuals progresses rapidly even in the presence of a strong CD8 T cell response, but not in others. Various studies have not found any correlation between the CD8 T cell response and viral load (Addo et al., 2003; Cao et al., 2003; Frahm et al., 2004) or even found a positive linear relationship of the frequency of Nef- or Env-specific CD8 T cells and the plasma viral load (Betts et al., 2001). Because of these difficulties to clearly define parameters important for an efficient HIV-1-specific CD8 T cell response in HIV-infected humans, the most conclusive data about the importance of the HIV-specific CD8 T cells is derived from the SIV-Rhesus Macaque model. In experimental SIV infection, depletion of CD8 T cells leads to a transient rise in SIV plasma viremia and loss of CD4 T cells until the CD8 subset recovers (Jin et al., 1999; Schmitz et al., 1999).

A different way of looking at parameters affecting the HIV-specific CD8 T cell response is to study the restricting elements for CD8 T cell recognition of HIV - the HLA class I alleles. Kiepiela and collegues found that i) viral load, absolute CD4 cell counts and the rate of disease progression was strongly associated with particular HLA B alleles and ii) that HIV-1 specific CD8 T cell responses were mainly restricted by HLA B alleles (Kiepiela et al., 2004). HLA-B alleles associated with a beneficial effect during HIV-1 infections were B4201, B57, B5801 and B8101 whereas B5802, B18, B4501 were associated with a high viral load. With the exception of HLA B 4201, the HLA allele/viral

load associations observed in the HISIS-CTL study closely match those reported in the Kiepiela study. However, in contrast to the Kiepiela study, HLA B4201 expression was not associated with a low viral load.

The data presented in chapter 3.1 may provide a link between the expression of the HLA class I alleles B0702, B5801 and B8101, and a particular "protective" pattern of CD8 T cell epitope recognition observed in subjects expressing these HLA alleles. So far nobody has elucidated the mechanism underlying the beneficial effect of particular HLA alleles during HIV infection (Walker and Korber, 2001). The targeting of multiple epitopes within the Gag regions aa001-aa075 (GagR1) and aa248-aa500 (GagR3) appears to play a major role for efficient control of HIV viral replication over extended time periods. Another interesting observation was that co-expression of HLA alleles B8101 with B0702 or B5801 appeared to strengthen the relationship of these alleles with a very broad epitope recognition pattern within whole Gag and GagR1R3 and a low viral load. Contrary, co-expression of non-protective HLA-alleles, such as B5802, B4501, B1510 or B5301 was not associated with broad recognition of epitopes within GagR1R3, but instead with a 30x higher median viral load. This result together with the strong inverse linear relationship observed between the number of epitopes recognized within GagR1R3 and the viral load suggests that the antiviral effect of the CD8 T cell response is in part predictable by the number of epitopes recognized within this region.

How can we exclude the possibility that the recognition of multiple epitopes within GagR1R3 is a mere reflection of an early stage of HIV infection? As shown in figure 3.6, the recognition of multiple GagR1R3 epitopes during the chronic phase of HIV-1 infection was strongly associated with a low median viral load set point during the early phase and the maintenance of a low steady-state level of plasma virus during the chronic phase of HIV-1 infection. With the exception of two HLA B57 expressing subjects, none of the subjects that did not target multiple epitopes within GagR1R3 significantly reduced primary plasma viremia. The high viral load set-point and steady state viremia persists in these subjects in the absence of an efficient CD8 T cell response. These results indicate that the differences in the viral load set point and steady-state

level seen among HIV-infected individuals is strongly dependent on the quality of the HIV-specific CD8 T cell response. The viral load steady state-level has been shown to predict the rate of disease progression (Mellors et al., 1996). Therefore the breadth of GagR1R3-specific CD8 T cell response may in part predict the rate of disease progression.

Differences in the CD8 T cell recognition pattern between subjects with or without efficient viral control were greatest within the C-terminal region of Gag p24 and p15 (GagR3) followed by N-terminal p17 (GagR1). It is possible that Gag regions R1 and R2 contain protein domains, whose function may easily be disrupted by point mutations. For example p17 is important for recruitment of p24 to the budding site and therefore has to interact with p24 and with cytoplasmic tail of gp41. The C-Terminal region of p24 together with p2 are essential for proper assembly of the RNP shell (Zhang et al., 1996) and are crucial for HIV morphogenesis (Gottlinger et al., 1989; Krausslich et al., 1995; Pettit et al., 1994); p10 for effective encapsidation of the viral genome (Bess, Jr. et al., 1992; Clavel and Orenstein, 1990; Gorelick et al., 1990) and effective virus assembly (Jowett et al., 1992) and p6 is essential for the last steps of viral budding (Gottlinger et al., 1991). These results indicate that targeting of multiple epitopes located in Gag regions important for several steps of viral morphogenesis may be associated with high antiviral efficiency.

A changing pattern or loss of epitope recognition in the course of HIV-1 is common and can deteriorate the antiviral efficiency of the CD8 T cell response (Goulder et al., 1997). Escape from CD8 T cell recognition during the acute phase has been shown to be a hallmark of SIV infection. (O'Connor et al., 2002). However, targeted HIV-epitopes vary in their susceptibility to point mutation (O'Connor et al., 2002) and therefore CD8 T cells of different specificities may have very different long term affects. Therefore loss of GagR1R3 epitope recognition or exhaustion of GagR1R3 epitope-specific CD8 T cells is likely to contribute to disease progression. However, viral escape from the GagR1R3 specific CD8 T cell response may be deleterious to the viral fitness, because mutations within these regions might prevent virus assembly. This interpretation is supported by the finding that HLA-associated HIV

sequence polymorphisms are much more often observed in the genes encoding for Nef, Tat and Vif than for the proteins encoded in the *gag* gene (Kiepiela et al., 2004). In the SIV/Rhesus macaque model, escape from a Tat-specific CD8 T cell response is much more rapid than escape from a Gag response (O'Connor et al., 2002), a finding supported by Jones and colleagues in HIV-1 infection. 71% of the HIV-1 Tat epitope responses selected escape variants, whereas only 26% of Gag-specific responses selected such mutants in a similar time frame (Jones et al., 2004). Additional data supporting this view can also be derived from an *in vitro* system to determine HIV-1 mutational escape from cytotoxic T cells. It was demonstrated that escape from Nef-specific CD8 T cell responses was more rapid and more consistent than escape from Gag-specific responses (Yang et al., 2003a). Therefore functional constraints may hamper the rapid escape of HIV-1 from CD8 T cells targeting epitopes within GagR1R3.

A different explanation could be that exhaustion of HIV-specific CD8 T cells may in part be dependent on epitope specificity and avidity of the T cell receptor. CD8 T cells recognizing epitopes within GagR1R3 would be less prone to exhaustion and hence display a preserved functionality. A declining cytotoxicity of HIV-specific responses was observed in two studies despite persistent recognition of autologous epitopes of the infecting virus (Draenert et al., 2004b; Koibuchi et al., 2005).

A broad recognition of epitopes within the Gag regions R1 and R3 appears to be of particular importance for an efficient control of viral replication. In contrast to the antiviral efficiency of the Gag-specific CD8 T cell response, the breadth of Nef or Env epitope recognition did not appear to be of major importance for viral control. Interestingly, an opposite trend for the Nef-specific T cell response compared to the Gag-specific response was observed in this work. A similar relationship of the Nef-specific T cell responses with a higher viral load has also been observed in other studies (Betts et al., 2001; Masemola et al., 2004; Novitsky et al., 2003). These differences in antiviral efficiency between the Gag- and Nef-specific responses indicate that T cell specificity plays a role in determing functionality of a CD8 T cell. *In vitro*

antiviral activity of CTL clones varies in dependency of epitope specificity (Yang et al., 2003b). A recent report suggested that Nef-mediated HLA Class I down regulation may in part dependent on the epitope presented and that Nef can modifiy the antiviral efficiency of CD8 T cells of different specificities (Tomiyama 2005, JI).

That the Gag-specific CD8 T cell response may play a special role in slowing down progression to AIDS was first shown in 1995 (Riviere et al., 1995) and numerous more recent studies supported this finding (Edwards et al., 2002; Masemola et al., 2004; Novitsky et al., 2003; Ramduth et al., 2005). However, most of these studies found that a high frequency of Gag-specific CD8 T cells is associated with efficient control of viral replication (Novitsky et al., 2003; Ogg et al., 1998), a finding not supported by the HISIS-CTL study. Two studies, the Masemola and the Ramduth study, found a significantly lower viral load in individuals who preferentially targeted the Gag protein (indicated by a frequency of Gag-specific CD8 T cells compared to other HIV proteins) (Masemola et al., 2004; Ramduth et al., 2005). One possible explanation for these different observations could be that the frequency of HIV-specific CD8 T cells is to some degree reflective of the number of epitopes targeted (Addo et al., 2003). That both parameters, the breadth and the frequency of the Gag-specific T cell response, correlate with a low viral load has been found by Edwards and colleagues and provides a link between our findings and those of the other studies concerning the anti-viral effect of the Gag-specific T cell response (Edwards et al., 2002; Masemola et al., 2004; Novitsky et al., 2003; Ramduth et al., 2005). The data presented in this PhD thesis suggests that the breadth and specificity of the HIV-specific CD8 T cell response is a major determinant of the antiviral efficiency, but not the frequency of all Gag-specific CD8 T cells. In our HISIS-CTL study an inverse linear relationship between the GagR1R3-specific CD8 T cell frequency and the viral load was found.

What other factors could contribute to differences in antiviral efficiency? The breadth of CD8 T cell recognition can play a role in the containment of HIV during chronic HIV infection (Chouquet et al., 2002). This effect may be explained by a study that suggested that escape from T cell recognition is less

likely in the presence of a broad HIV-specific T cell response (Jones et al., 2004). Interestingly, Gag is the dominant target of the HIV-specific CD4 T cell response (74% of HIV-specific CD4 T cells target the Gag protein, (Ramduth et al., 2005), which theoretically could contribute to an efficient Gag-specific CD8 T cell response due to good support by Gag-specific CD4 T cells. After restimulation with virus like particles (VLP Pr55, subtype B), we did not find any association of the Gag -specific CD4 T cell response with the GagR1R3-specific CD8 T cell response and in general found relatively low frequencies of Gag-specific CD4 T cells that were predominantly secreting IFNγ. The frequencies of IL-2 secreting CD4 T cells were very low. However, it is possible that subtype B VLPs are not the optimal stimulants to detect CD4 responses in subjects infected with non-B subtypes.

Targeting of particular Gag epitopes is not the only way to efficiently control viral replication. No evidence indicated HLA B57 mediated recognition of multiple epitopes within Gag or GagR1R3. Nonetheless B57 was associated with a relatively low median viral load (fig 3.1). Hence the antiviral determinant of HLA B57 restricted CD8 T cell responses is likely to differ from the mechanism described above. This hypothesis is supported by Migueles and colleagues, who found comparable Gag-specific CD8 T cell responses in HLA B57 expressing Long-Term Non-Progressors (LNTPs) and progressors (Migueles et al., 2003). The fact that 89% of the study subjects with a viral load below 50 000 RNA copies/ml recognized 2 epitopes emphasizes the importance of mechanism for maintaining a low viral load and a high CD4 cell count**.** However, in contrast to HLA B57 expression or a homozygous deletion in the CCR5 coreceptor gene that only affect a small minority of individuals, the recognition of multiple GagR1R3 epitopes contributes to efficient viral control in most individuals with maintained low viral loads and therefore the latter observation is of general importance on a population level.

The individual HLA class I background largely determines, which peptides can be presented to CD8 T cells. The finding that certain HLA alleles like B57 or B5801 are associated with long-term non-progression, whereas other alleles,

such as B5802, are associated with more rapid disease progression indicates that protective HLA alleles enable for a particularly effective CD8 T cell response (Carrington et al., 1999; Kaslow et al., 1996; Kiepiela et al., 2004; Migueles et al., 2000). However, a direct relationship between the expression of "protective" HLA alleles and an efficient CD8 T cell response has not been demonstrated by any of these previous studies. The capacity for the presentation of GagR1R3 epitopes appears to be strongly affected by the HLA molecules expressed by one individual. Therefore the HLA background appears to be one of the main determinants enabling a broad epitope recognition within Gag. One central question arising at this point is what causes this difference in the epitope presentation pattern of the "protective" HLA alleles B0702, B5801 and B8101. The sequence binding motif for "protective" HLA alleles may enable the presentation of a much broader variety of epitopes as suggested by Nelson GW et al (Nelson et al., 1997). Experimentally we have found that 7 of the 10 most frequently recognized epitopes within the Gag protein were presented by these alleles. Contrary it was only 3 recognized epitopes for the "non-protective" alleles, such as B1510, B4501 and B5802. In line with Nelson et al, I have found only a limited number of predicted epitopes within Gag in general, but especially within GagR1R3 for B5802 and B1510.

Another possible determinant for the antiviral efficiency of a CD8 T cell response could be a high variability among recognized eoitopes, allowing the virus to easily escape recognition. 47 Gag protein sequences from all predicted epitopes for both "protective" and "non-protective" were aligned and the degree of amino acid variation was determined for each position (data not shown). There was no evidence to support this hypothesis.

One interesting observation is that often 2-3 predicted epitopes restricted by HLA-B5801 are clustering in very close proximity to each other – often sharing one of the anchor residues (exemplified in fig. 4.1). Practically this could be an advantage: If recognition of an epitope would be lost due to mutational escape of one anchor residue, the second epitope could replace the primary response within the same region. A similar phenomenon has also been

described for 2 HLA B57 presented epitopes, one of which was entirely contained within the other (Goulder et al., 2000). Further indication that a relatively similar hypothetical mechanism may play a role can be derived from the fact that 3 subjects co-expressing HLA B0702 and B8101 have a low viral load (<29 500 RNA copies/ml). Some epitopes within Gag are restricted by both B0702 and B8101 and the predicted anchor residues of these two alleles are identical capacity (Marsh et al., 2000; Yusim K et al., 2004). One might hypothesize that targeting of the same epitope by two different HLA-alleles also minimizes the potential for HIV to escape this CD8 T cell response. Therefore targeting of the same epitopic region by CD8 T cells of slightly different specificity might contribute to the anti-viral efficiency of a HIV-specific CD8 T cell response.

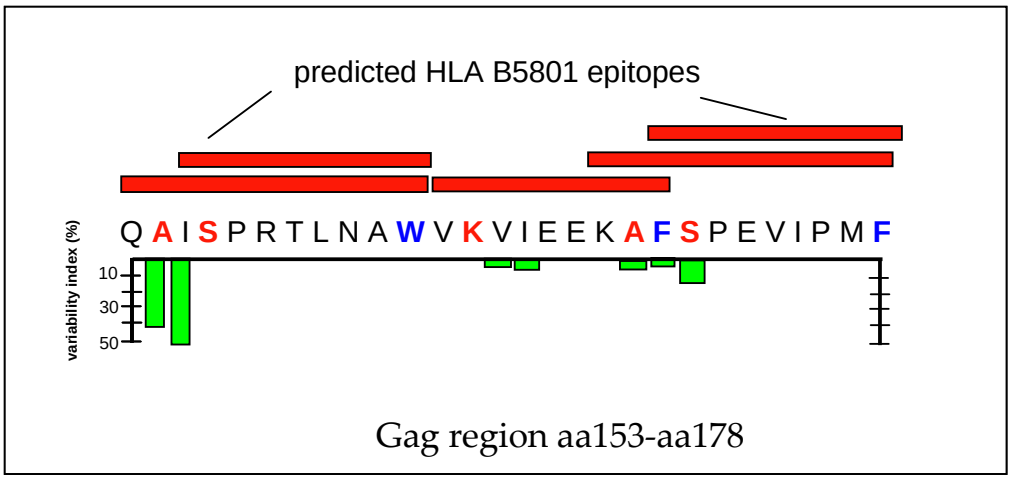


Figure 4.1 Clustering of predicted epitopes for HLA-B5801. Shown is the Gag region aa153 – aa178 and predicted HLA B5801 epitopes (red bars) Epitope prediction was based on the motif x-[AST(G)]-x-x-x-x-x-[FW(Y)] (SYFPEITHI Database). Potential anchor residues in 2nd position are colored red and blue for the C-terminal anchor residues. The variability index is calculated by dividing the number of amino acid substitutions by the number of aligned sequences (n=47) and multiplying the results by 100.

The Kiepiela study is emphasizing the effect of "protective" HLA alleles on human (and viral) evolution. In this context, our data strongly suggests that the advantage to have "protective" HLA alleles, with the exception of HLA B57, is

dependent on their capability to enable the CD8 T cell targeting of multiple regions within GagR1R3 in particular. Of further interest is that a high plasma viral load is associated with increased mother to child transmission in HIV infected pregnant women (Dickover et al., 1996; Mayaux et al., 1997; Pancino et al., 1998). Those who control HIV replication due to CD8 T cells are less likely to transmit the virus perinatally. In fact inheriting HLA genes enables human kind to pass on a "protective pattern" of T-cell recognition in the face of a viral epidemic. This possibility is not given for B cells and the humoral immune response. Contrary Kiepiela and collegues have reported an accumulation of "non-protective" HLA-alleles in HIV positive children born by HIV infected mothers (Kiepiela et al., 2004). Inheritability of the HLA genes in combination with the somatic rearrangement of the T cell receptor genes therefore may actually be crucial in the face of a viral epidemic with the potential to wipe out large parts of the human population. Additionally the differences found between the CD8 T cell response restricted by "protective" HLA alleles and non-protective HLA-alleles stresses the importance of the polymorphism found within the HLA genes. Furthermore the findings of the HISIS-CTL study strongly support the idea that the evolution of the HLA class I gene locus is driven by the relationship of the CD8 T cell response and endemic pathogens.

In the absence of antiretroviral therapy it is possible that HLA allele mediated CD8 T cell pressure is attenuating the virus as well (Brander and Walker, 2003) and may lead to "coexistence" of HIV and the human population in the future, similar to SIV in their respective natural hosts. Furthermore one might speculate that other persistent viruses that now coexist "peacefully" with the human host, such as CMV and to a lesser degree Hepatitis B and C, may have been much more destructive in the human host initially and drove the evolution of the human HLA gene locus. This may give individuals expressing "protective HLA alleles" a selective advantage in the face of a viral epidemic and might therefore also be a major force of human evolution in general. `

However, with the advent of anti-viral drugs, this role of the HLA genes on human evolution may be replaced by a rather cultural mode of evolution,

where other (non-molecular) factors play a more dominant role i.e. education, sexual behaviour, financial status of the individuum or else. In this aspect the human kind clearly differs from other animals.

4.2 Cross-recognition of HIV epitopes

Because of the extensive genetic variation of HIV, the identification of conserved, cross-reactive epitopes is a key element in HIV vaccine development. The study described here was designed to characterize CD8 T cell responses in an East African cohort of HIV-1 seropositive individuals in a mixed-subtype environment, and to better define optimal immunogenic peptide sets. In concordance with other studies, certain regions of Gag and Nef were recognized by a high frequency of study subjects. Many of the immunodominant regions detected during the HISIS-CTL study match frequently targeted antigenic regions from studies conducted with individuals from different ethnic groups and with peptides based on HIV-1 subtypes B and C (Addo et al., 2003; Frahm et al., 2004; Masemola et al., 2004; Novitsky et al., 2002). This suggests that the pattern of immunodominant regions within gag and nef is comparable across many HLA backgrounds and among diverse HIV-1 subtypes in different regions of the world. It will simplify HIV-1 vaccine design if many of the same epitopes are targeted in multiple HLA backgrounds and across many HIV-1 subtypes, because these regions can be specifically selected for vaccine antigens. Additionally, several new HLA/epitope associations were detected (Table 3.1) that will facilitate subsequent research on vaccine design and evaluation in the region of Mbeya.

Interestingly, the subtype of the infecting strain and the recognition of subtype specific peptide were not necessarily associated. Only for Gag7 we did find that predominantly subtype C Gag exposed subjects were mounting a response. For Gag45 the subtype A peptide variant was usually recognized better in subtype A exposed subjects than in subjects exposed to HIV-1 C or D. There was no strong association between the infecting subtype and the CD8 Tcell recognition of subtype-specific peptides.

Complete cross-recognition of subtype A, C and D peptides, i.e. the same number of responders with a similar magnitude response was seen only for completely conserved epitopes. Amino acid substitutions within epitopes were capable of dramatically decreasing or completely abrogating the frequency and magnitude of detected responses. Interestingly, for most of the studied antigenic regions the majority of responders always recognized the three different peptide variants in a similar fashion, while a small minority of responders differed in their pattern of subtype A, C and D variant recognition. This pattern was independent of the restricting HLA-allele. These findings and previously published reports (Charini et al., 2001; Haas et al., 1996), suggest that minor sequence variation within epitopes, some of which may be subtype-associated or subtype specific, can influence the ultimate breadth of the CD8 T cell response that is generated. This concept, in the context of different HLA backgrounds and HIV-1 subtypes, would suggest that the optimal HIV-1 vaccine may not be one based on a single subtype, but either one based on multiple subtypes or one that could contain epitopes from multiple subtypes that generate the most broadly cross-reactive response.

Despite the remarkable degree of flexibility and adaptability of the HIV-specific T-cell response (Boissonnas et al., 2002; Buseyne et al., 2001; Haas et al., 1996), the initial encounter with antigen may play a critical role in the subsequent response to antigenic variants (Klenerman and Zinkernagel, 1998), including those developed against the viral quasispecies within an individual and upon re-exposure to a different HIV-1 strain or subtype. HIV dual and superinfections are being described with increasing frequency (Altfeld et al., 2002; Gerhardt et al., 2005; McCutchan et al., 2005), and it is possible that the specifics of the response to the initial infection may influence susceptibility to re-infection. Unfortunately even a broad and specific T cell response does not guarantee protection from HIV superinfection (Altfeld et al., 2002).

A further important result of the described study for immune assessment laboratories is the limited extent of complete cross-recognition between peptide sets based on subtypes A, C, and D. As Figure 2B highlights, only 56 of 180 pool responses were detected regardless of which peptide set was used, and no

two peptide sets substituted for the combination of the three. The observed superiority of the subtype C Gag peptide set for detecting T-cell responses is likely to reflect the predominance of HIV-1 subtype C and C-containing recombinants in the population studied. In addition, the location of the epitope within some C Gag 15mer peptides may be favourable for stable binding of the peptide to the HLA molecule. Despite identical amino acid sequences of the epitopes, the C Gag peptides Gag45, Gag50, and Gag76 were recognized with a much higher magnitude than the corresponding A or D Gag peptides. Interestingly, subtype C Nef peptides had no obvious advantage for detecting Nef-specific responses if compared with subtype A and D Nef peptide sets. In fact all of the responses against epitopes outside the central region of Nef were only detected with either subtype A and/or D Nef peptide sets.

Some previous studies have highlighted the "frame-of-epitope" effect, in which the position of a minimal 9- or 10-mer epitope within a 15-mer peptide can have a profound effect on the magnitude of the T cell response (Beattie et al., 2004; Draenert et al., 2004a). In the HISIS-CTL study the C Gag peptide set was offset relative to both the A Gag and D Gag peptide sets due to a 3 amino acid deletion of the C Gag sequence at the beginning of p24.

A composite pool of 15 peptides covering the most frequently recognized epitopes was designed and tested. This optimal pool mostly contained subtype C derived peptides, but required the inclusion of peptides from subtypes A and D as well. The success of this pool in detecting CD8 T cell responses within a regional epidemic (94% of subjects tested positive, with no false positives), signifies that this approach may be a practical solution to the problem screening for vaccine generated CD8 T cell responses from samples of limiting cell quantities, serving as the best single reagent for this population. A further advantage of this pool of frequently recognized peptides is that it includes many antigenic regions that are recognized within different ethnicities and HIV-epidemics (Addo et al., 2003; Frahm et al., 2004; Masemola et al., 2004; Novitsky et al., 2002). The quest for a broadly protective HIV-1 vaccine has been frustrated by obstacles at many levels. Problems inherent in host and viral variability, selection and evaluation of appropriate vaccine trial cohorts, and

laboratory assays for the measurement of immune responses are being addressed and, to varying degrees, overcome. The strengths of systematic evaluations of T cell responses, supported by host and viral genotypes and conducted with more optimal laboratory reagents, are apparent from this and other studies.

4.3 Increased viral diversity is associated with a broadening of Gag-specific CD8 T cell responses

Infection with more than one HIV-1 strain has recently become a focus in HIV research. Infection with multiple strains is followed by an increased diversity of viral "quasispecies" and causes a significant fluctuation in molecular recombinant forms of HIV-1 (Gerhardt et al., 2005; McCutchan et al., 2005). Furthermore infection with multiple viral strains is associated with a higher viral load set point and rapid disease progression (Gottlieb et al., 2004; Grobler et al., 2004). However, the effect that dual infection might have on the HIV-specific CD8 T cell response has been studied in 5 superinfected individuals only (Altfeld et al., 2002; Jost et al., 2002; Ramos et al., 2002; Yang et al., 2005). The HISIS study was designed to systematically evaluate intersubtype dual and superinfections in a large high-risk cohort. During the HISIS-CTL study the Gag and Nef specific CD8 T cell response of 56 HIV-infected subjects was analyzed, half of them infected with more than one HIV strain. This allowed addressing the question, whether the increased diversity of viral "quasi species" within multiply HIV-1 infected individuals affects the HIV-specific CD8 T cell response.

One interesting question is whether many of the multiply infected subjects were in fact superinfected at some timepoint. Superinfection has been shown to be relatively rare if compared to coinfection with multiple HIV-strains (Gottlieb et al., 2004; Grobler et al., 2004). Gonzales et al did not detect any case of superinfection in 718 individuals (Gonzales et al., 2003). In the HISIS-CTL study no distinction between superinfection and co-infection was done, except for subject H123 (McCutchan et al., 2005). Based on the findings of Gottlieb, Grobler and Gonzales, it can be assumed that the majority of the

multiply infected subjects in the HISIS-CTL study were coinfected with different HIV-1 strains.

In the HISIS-CTL study, infection with multiple strains appeared to broaden the Gag-specific CD8 T cell response in many, but not all subjects. A broadening CD8 T cell response could be explained by the increased viral diversity observed in cases of multiple HIV-1 infection (Gerhardt et al., 2005; McCutchan et al., 2005). This finding further emphasizes the flexibility of the CD8 T cell response, which seems to adapt upon exposure to multiple HIV-1 strains. It has been observed before that sequence mutations in targeted epitopes during the course of HIV-infection are followed by adaptation of the CD8 T cell response (Feeney et al., 2005; Haas et al., 1996). So far the HIV-specific CD8 T cell response has only been studied in superinfected individuals. In these cases a changing pattern of CD8 T cell epitope recognition has been reported, with some newly emerging and some disappearing responses (Altfeld et al., 2002; Jost et al., 2002; Ramos et al., 2002; Yang et al., 2005).

One interesting observation in the HISIS-CTL study is that the distribution of subjects with a certain breadth of epitope recognition within Gag differs between the two groups. While this distribution within single infected subjects is almost a Gaussian distribution, it appears to be skewed towards a broadening T cell response in multiply infected subjects. 8 of 11 subjects with a very broad Gag-specific CD8 T cell response were infected with multiple strains and most of these control viral replication over extended time periods. At the same time many of these subjects expressed “protective” HLA alleles B5801, B8101 or B0702, indicating that the capacity for a broadening CD8 T cell response in multiply infected individuals may in part be dependent on the HLA class I background. Nonetheless it would be interesting to see, whether infection with multiple strains could in some cases be beneficial. This point of view would be against the “doctrine” that infection with multiple strains is rather associated with a high viral load set point and rapid disease progression (Gottlieb et al., 2004; Grobler et al., 2004). In the only confirmed case of superinfection in the

HISIS-CTL study, the viral load decreased after superinfection (subject H123, described in (McCutchan et al., 2005).

In multiply infected subjects cross-recognition of subtype A, C and D derived peptide pools appears to be enhanced, particularly in Gag regions less conserved, namely the p17 matrix protein, p15 and the C-terminal part of p24. This finding is noteworthy as CD8 T cell targeting of these regions are important to maintain control over HIV replication during the chronic phase of infection and possibly contribute to a reduced viral load set point (chapter 3.1, 4.1).

For the development of HIV-1 vaccines that are based on the induction of CD8 T cell responses, the human diversity of the HLA Class I alleles may prevent achieving equivalent and "protective" CD8 T cell responses on a population level, a problem not met by "traditional" vaccines that usually rely on the induction of neutralizing antibodies. The finding that infection with multiple HIV-1 strains broadens cross-recognition of different subtypes emphasizes the possible benefit of multi-subtype vaccines. Inclusion of multi-subtype variants in vaccines may maximize breadth of the HIV specific T cell response independent of the HLA-background of the vaccinees, an effect that would be highly desirable.

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5. Summary

The importance of HLA class I restricted CD8 T cell response in the control of HIV infection is generally accepted, yet few studies have shown a correlation of the CD8 T cell response with markers of HIV disease progression. Disease progression is dependent on several factors such as the virus load at set-point, rarely occurring mutations in the HIV co-receptor genes or the HLA class I alleles expressed by an HIV infected individual. It has been shown previously that particular HLA class I alleles are associated with a low plasma viral load. However, a relationship between these "protective" HLA class I alleles and an efficient HIV-specific CD8 T cell response has not yet been demonstrated. In order to study the mechanism underlying the beneficial effect of "protective" HLA class I alleles, 53 HIV-1 seropositive individuals from a high-risk cohort in southwest Tanzania were HLA-typed and the plasma viral load and CD4 counts were determined. The recognition of CD8 T cell epitopes within Gag, Nef and Env was analysed using a gamma interferon ELISPOT assay and freshly isolated peripheral blood mononuclear cells (PBMC).

Gag and Nef were frequent targets of the HIV-specific CD8 T cell response with a median recognition of 3 Gag, 2 Nef and 1 Env epitopes per individual. The HLA class I alleles B5801, B8101 and B0702 were associated with a low median plasma viral load. At the same time expression of these correlated with a broader recognition of Gag epitopes ($P = 0.0035$), if compared with all other common HLA class I alleles. Further analysis of the Gag-specific T cell response revealed an inverse linear relationship between the number of Gag epitopes recognized and the plasma viral load ($R = -0.36$, $P = 0.0016$). Particularly, recognition of multiple epitopes within two regions of Gag (aa001-aa075 and aa248-aa500) was strongly associated with the maintenance of a low viral load, indicating that this pattern of HIV-specific CD8 T cell recognition is important for the control of viral replication during the chronic phase of HIV infection.

In the second part of the present work, recognition of Gag and Nef variant epitopes was analysed using HIV-1 subtype A, C and D derived peptides. 83% of the Gag-

specific responses were detected with subtype C derived peptides, whereas only 51% and 57% of responses were detected with subtype A or D peptides, respectively. The superiority of the subtype C Gag peptides for detecting CD8 T cell responses may in part reflect the predominance of subtype C and C-containing recombinant HIV-1 strains within the studied cohort. Nonetheless, screening for CD8 T cell responses with any one subtype-specific peptide set detected fewer responses and underestimated the breadth and the magnitude of responses within individuals, compared to the combined peptide sets from the three subtypes. Cross-subtype recognition for the 16 most frequently recognized peptides was identical only when the peptide sequences were invariant; in 9 of these 16 peptides, diminished recognition was the result of subtype-related sequence variation, and a frame-of-epitope effect diminished or abrogated recognition in 4 peptides. A minimal set of 15 frequently recognized Gag and Nef peptides was then designed and tested with cells from 50 HIV seropositive individuals and elicited responses in 47 of them, at a mean frequency of 715 SFC/10^6 peripheral blood mononuclear cells. Therefore incomplete cross-recognition between subtypes A, C, and D can be partially overcome using a defined peptide set representing frequently recognized epitopes, and potentially, by adjusting epitope frame within peptides. These strategies can help to define optimal vaccine epitopes.

In the third part of the present work, the Gag-specific CD8 T cell response of individuals infected with a single HIV strain was compared with the response observed in individuals infected with multiple HIV-1 strains. Breadth of Gag epitope recognition and cross-recognition of subtype-specific peptide pools was enhanced in multiply infected study subjects, whereas CD8 T cell recognition of Nef or Env appeared to be unaffected. Therefore the increased viral diversity in individuals infected with multiple HIV-1 strains affects the Gag-specific T cell response. As a consequence inclusion of multiple Gag variants in a HIV vaccine is likely to enhance vaccine-induced CD8 T cell responses and cross-recognition of HIV epitopes.

5. Zusammenfassung

Es ist allgemein akzeptiert, daß die HLA Klasse I restringierte CD8 T Zellantwort bei der Kontrolle der Virusreplikation während einer HIV Infektion eine entscheidene Rolle spielt. Allerdings konnten nur wenige Studien eine Korrelation zwischen der HIV-spezifischen CD8 T Zellantwort und dem Krankheitsverlauf nachweisen. Der Krankheitsverlauf hängt von verschiedenen Faktoren ab, darunter die Viruslast nach der Primaervirämie, selten vorkommenden Mutationen in den HIV Korezeptorgenen, oder den HLA Klasse I Allelen. So geht beispielsweise die Expression bestimmter HLA Klasse I Allele mit einer niedrigen Plasma Viruslast einher. Eine Verbindung zwischen solchen “protektiven” HLA Klasse I Allelen und einer effizienten HIV-spezifischen CD8 T Zellantwort konnte jedoch bisher noch nicht nachgewiesen werden. Zur Beantwortung des zugrundeliegenden Mechanismus, der mit „protektiven“ HLA Allelen verbunden ist, wurden 53 HIV infizierte Frauen einer Hochrisikokohorte im Südwesten Tansanias bezüglich des HLA Typs, der Viruslast im Plasma und der CD4 T Zellzahl charakterisiert. Gleichzeitig wurde die Gag-, Nef- und Env-spezifische CD8 T Zell Antwort unter Verwendung eines Interferon gamma ELISPOT Assays bestimmt .

Am häufigsten fanden sich in den Studienteilnehmerinnen CD8 T Zellantworten gegen Epitope in Gag und Nef, weniger häufig gegen Epitope in Env (Median: 3 Gag, 2 Nef und 1 Env erkannte Epitope/Individuum). Die HLA Klasse I Allele B5801, B8101 und B0702 waren mit einer niedrigen Viruslast assoziiert. Gleichzeitig korrelierte die Expression von einem oder zwei dieser Allele mit einer breiteren Erkennung von Gag Epitopen (P =0,0035, Median:4 erkannte Epitope/Individuum) verglichen mit anderen HLA Klasse I Allelen (Median: 2 erkannte Epitopen/Individuum). Die weitere Analyse der Gag-spezifischen CD8 T Zell Antwort offenbarte ein inverses lineares Verhätnis zwischen der Anzahl der erkannten Gag Epitope und der Viruslast im Plasma (R =-0,36 ; P = 0,0016). Des weiteren war die Erkennung von multiplen Epitopen in 2 Regionen des Gag Proteins (as001-as075 und as 248-500, GagR1R3) stark mit einer dauerhaften Kontrolle der Virusreplikation nach Serokonversion assoziiert. Zusammenfassend lassen diese Ergebnisse darauf schließen, daß die Erkennung multipler Epitope im

Gag Protein, ganz besonders in GagR1R3, wichtig für die Kontrolle der Virusreplikation während der chronischen Phase der HIV Infektion ist.

Im zweiten Teil der vorliegenden Arbeit wurde die Erkennung von HIV-1 Subtyp A-, C- und D-spezifischen Gag und Nef Epitopvarianten durch CD8 T Zellen untersucht. 83% der Gag-spezifischen Epitopantworten wurden mit den Subtyp C Peptiden detektiert, während lediglich 51% und 57% der Gag-spezifischen Epitopantworten mit den Subtyp A beziehungsweise Subtyp D Peptiden detektiert wurden. Die erhöhte Frequenz Subtyp C-spezifischer Antworten könnte zum Teil mit dem vorherrschenden Vorkommen Subtyp C und Subtyp C-beinhaltenender rekombinanter HIV-I Stämme in Südwest Tanzania zusammenhängen. Dennoch würde die Anzahl der Gag-spezifischen Epitopantworten durch die ausschließliche Verwendung von Subtyp C-spezifischen Peptiden unterschätzt. Die Erkennung von Subtyp-spezifischen Peptidevarianten durch CD8 T Zellen war lediglich für Peptide gleicher Aminosäuresequenz identisch. Für 9 der 16 am häufigsten erkannten Peptide konnte eine abgeschwächte Erkennung von Varianten des selben Peptids mit Aminosäuresequenzunterschieden erklärt werden. Für 4 Peptide konnte eine abgeschwächte Erkennung von Peptidvarianten mit einer unterschiedlichen Positionierung des Epitops innerhalb der Peptidvarianten erklärt werden. Aufgrund dieser Ergebnisse wurde ein minimales Set aus 15 der am häufigsten erkannten Peptiden zusammengestellt Dieses bestand vor allem aus Subtyp C-spezifischen, aber auch aus Subtyp A- und D-spezifischen Peptiden und wurde anschließend mit peripheren mononuklären Blutzellen („peripheral blood mononuclear cells“, PMBC) von 50 HIV infizierten Personen auf Erkennung getestet. Dieser Peptidpool wurde mit einer durchschnittlichen Frequenz von 715 SFC/10^6 PBMC von 47 dieser Personen erkannt. Zusammenfassend könnte diese Strategie dazu beitragen, optimale CD8 T Zell Epitopevarianten zu identifizieren, die in HIV Impfungen beinhaltet sein sollten.

Im dritten Teil der vorliegenden Arbeit wurde die Gag-spezifische CD8 T Zell Antwort von Studienteilnehmerinnen verglichen, die mit einem oder mehreren verschiedenen HIV-1 Stämmen infiziert waren. Eine Mehrfachinfektion führt

dazu, daß mehr Gagepitope erkannten werden. Darüber hinaus war auch die gleichzeitige Erkennung von subtypspezifischen Peptiden in diesen Studienteilnehmerinnen besser ausgeprägt. Die Nef- und Env-spezifische CD8 T Zellantwort schien durch eine Mehrfachinfektion nicht beeinflusst.

Für einen potentiellen HIV Impfstoff lässt sich aus der vorliegenden Arbeit schließen, daß es entscheidend sein kann, eine effiziente CD8 T Zellantwort zu induzieren. Vor allem sollten hierbei Gag-spezifische Epitopevarianten verschiedener Subtypen berücksichtigt werden.

Aus der vorliegenden Arbeit hervorgegangene Publikationen

Christof Geldmacher, Jeffrey R. Currier, Martina Gerhardt, Antelmo Haule, Leonard Maboko, Deborah Birx, Clive Gray, Andreas Meyerhans, Josephine Cox and Michael Hoelscher

In a mixed subtype epidemic, the HIV-1 gag-specific T cell response is biased towards the infecting subtype

(AIDS, in press)

Christof Geldmacher, Jeffrey R. Currier, Eva Herrmann, Antelmo Haule, Ellen Kuta, Francine McCutchan, Steffen Geis, Oliver Hoffmann, Leonard Maboko, Carolyn Williamson, Deborah Birx, Andreas Meyerhans, Josephine Cox and Michael Hoelscher

A broad Gag-specific CD8 T cell response contributes to efficient viral control during chronic HIV infection

(Manuskript eingereich bei Jounal of Virologyt)

Christof Geldmacher, Martina Gerhardt, Jeff Currier, Andreas Meyerhans, Leonard Maboko, Oliver Hoffmann, Deborah Birx, Francine McCutchan, Clive Gray, Josephine Cox and Michael Hoelscher

Analysis and Comparison of T cell responses in single and dual HIV infected individuals in Southwest Tanzania.

(Manuskript in Vorbereitung)