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Generation of Recombinant Antibodies against HIV Clade C Envelope

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List of abbreviations:

~:	Approximately
%:	Percent
°C:	Degrees Celcius
α:	Alpha
γ:	Gamma
κ:	Kappa
λ:	Lambda
µl:	Microliter
µm:	Micrometer
µM:	Micromolar

Ab:	Antibody
AIDS:	Acquired immunodeficiency syndrome
APC-Cy7:	Allophycocyanin-cyanine dye 7
APC:	Antigen presenting cell
APOBEC3G:	Apolipotrotein B mRNA-editing enzyme-catalytic polypeptide-like-3G/F-cytosine deaminase
ASCs:	Antibody secreting cells
BIDMC:	Beth Israel Deaconess Medical Center
bp:	Base pair
BSA:	Bovine serum albumin
C1-C5:	Constant regions of gp120
CD:	Cytoplasmic domain of gp41
CD4:	Cluster of differentiation 4
CD4bs:	CD4 binding site
CD4i:	CD4 induced epitopes
cDCs:	Conventional dendritic cells
cDNA:	Complementary DNA
CDR:	Complementarity-defining region
CHR:	C-terminal heptad repeat
cm:	centimeter
CRF:	Circulating recombinant forms
CTL:	Cytotoxic $CD8^+$ T-lymphocytes
CTT:	C-terminal tail of the HIV-1 glycoprotein gp41
DCs:	Dendritic cells
DFCI:	Dana-Farber Cancer Institute
DNA:	Deoxyribonucleic acid
DOC:	Day of challenge
dNTP:	Deoxyribonucleotide triphosphate
DTT:	Dithiothreitol
EBV:	Epstein-Barr virus
E. coli:	*Escherichia coli*
ELISA:	Enzyme-linked immunosorbent assay
FACS:	Fluorescence activated cell sorting
FITC:	Fluorescein isothiocyanate
FP:	Fusion peptide
FACS:	Fluorescence activated cell sorting
FBS:	Fetal bovine serum
FR:	Framework region
GALT:	Gut associated lymphoid tissue
GFP:	Green fluorescent protein
H_2O:	Water
H_2O_2:	Hydrogen peroxide
H chain:	Heavy immunoglobulin chain
HDC:	High dose challenge
HIV:	Human immunodeficiency virus
HRP:	Horseradish peroxidase
IFN:	Interferon
Ig:	Immunoglobulin
IL:	Interleukin

IMGT:	Immunogenetics database
IN:	Integrase
IPTG:	Isopropyl-β-D-thiogalactopyranosid
kB:	kilo base
kD:	kilo Dalton
L chain:	Light immunoglobulin chain
LB-medium:	Luria Bertani medium
LLP:	Lentiviral lytic peptide
LTR:	Long terminal repeat
MA:	Matrix
MAb:	Monoclonal antibody
MBCF:	Molecular biology core facility
MHC:	Major histocompatibility complex
ml:	milliliter
mM:	millimolar
MPER:	Membrane-proximal external region
mRNA:	messenger RNA
nAb:	Neutralizing antibody
NC:	Nucleocapsid
NHR:	N-terminal heptad repeat
NK cell:	Natural killer cell
nm:	nanometers
NSI:	Non-syncytium inducing
NTC:	No template control
OD:	Optical density
OPD:	Ortho-phenyldiamine
PAMP:	Pattern associated molecular pattern
PBMC:	Peripheral mononuclear blood cells
PCR:	Polymerase chain reaction
PE-Cy7:	Phycoerythrin-cyanine dye 7
Pen/Strep:	Penicillin/Streptomycin
pH:	Pondus Hydrogenii
PIC:	Pre-integration complex
Pol:	Polymerase protein
PR:	Protease
PRR:	Pattern recognition receptors
PCR:	Polymerase Chain Reaction
R5 virus:	CCR5-tropic virus
R5/X4 virus:	Dual-tropic virus (uses CCR5 and CXCR4)
RM:	Rhesus macaque
RNA:	Ribonucleic acid
rpm:	Rounds per minute
RT:	Reverse transcriptase
RT-PCR :	Reverse transcription-polymerase chain reaction
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHIV :	Simian-human immunodeficiency viruses
SI :	Syncytium inducing
SIV :	Simian immunodeficiency virus
SIV_{CPZ} :	SIV from chimpanzees
SIVmac:	SIV from rhesus macaques
SIV_{SM} :	SIV from sooty mangabeys
S.O.C.	Super Optimal broth plus glucose
TB :	Terrific broth
TGS Buffer :	Tris-glycine-SDS Buffer
T_H cells/lymphocytes:	T helper cells/lymphocytes
TLR :	Toll-like receptor
TM region:	Transmembrane spanning region of gp41
TRIM5α:	Tripartite motif protein 5α
(FD)U:	(Fast digest) Unit (restriction enzyme Unit)
UV light:	Ultraviolet light
V:	Volts
V1-V5:	Variable loops of gp120
V Ig gene:	Variable immunoglobulin gene
WHO:	World Health Organization
X4 virus:	CXCR4-tropic virus

HIV-1 genes:

env:	encoding envelope	*tat:*	encoding transactivator
gag:	encoding group-specific antigen	*vif:*	encoding viral infectivity factor
nef:	*encoding* negative effector	*vpr:*	encoding viral protein r
pol:	encoding polymerase	*vpu:*	encoding viral protein u
rev:	encoding regulator of virion gene expression		

HIV-1 proteins:

CA:	Capsid (p24)	Rev:	Regulator of virion gene expression
Env:	Envelope protein		
Gag:	Group-specific antigen	RT:	Reverse transcriptase (p66, p51)
gp41:	Glycoprotein 41		
gp120:	Glycoprotein 120	SU:	Surface protein (gp120)
gp160:	Glycoprotein 160	Tat:	Transactivator protein
IN:	Integrase (p32)	TM:	Transmembrane protein (gp41)
MA:	Matrix (p17)		
NC:	Nucleocapsid (p7)	Vif:	Viral infectivity factor
Nef:	Negative effector	Vpr:	Viral protein r
PR:	Protease (p10)	Vpu:	Viral protein u

Amino acid code:

G:	Glycine	C:	Cysteine	N:	Asparagine
P:	Proline	Y:	Tyrosine	E:	Glutamic acid
A:	Alanine	W:	Tryptophan		
V:	Valine	H:	Histidine	D:	Aspartic acid
L:	Leucine	K:	Lysine		
I:	Isoleucine	R:	Arginine	S:	Serine
M:	Methionine	Q:	Glutamine	T:	Threonine
F:	Phenylalanine				

1 Introduction

1.1 The Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) belongs to the family of *Retroviridae* and is classified as a lentivirus (GONDA et al., 1985). Retroviruses are enveloped viruses that enclose two identical positive-sense RNA-strands, which are reverse transcribed into double stranded complementary DNA (cDNA) before integration into the host-DNA. The virus is then replicated as part of the host genome and establishes a persistent infection (reviewed in PETERLIN & TRONO, 2003; TEIXEIRA et al., 2011). HIV is known to be the causative agent of the acquired immunodeficiency syndrome (AIDS) (ROWLAND-JONES & WHITTLE, 2007) and is responsible for a worldwide epidemic. In 2009, the World Health Organization (WHO) estimated approximately 34 million people living with HIV-infection and 2.6 million new infections in the same year (WHO, 2011).

HIV was first isolated in 1983 (BARRÉ-SINOUSSI et al., 1983), two years after the description of acquired immunodeficiency syndrome (AIDS), a disease characterized by steady breakdown of the infected host's immune system. Other isolations of HIV were reported independently (GALLO et al., 1984; LEVY et al., 1984) before it was formally named the human immunodeficiency virus (COFFIN et al., 1986). Shortly thereafter, HIV-2, displaying significant differences in envelope structure relative to previously known HIV variants, was discovered in West Africa (CLAVEL et al., 1986).

HIV-1 and HIV-2 are two genetically different types of HIV sharing 30-60 % homology in structure and sequence (ROWLAND-JONES & WHITTLE, 2007). The diversity in their genetic make-up resulted from different cross-species transmission events. HIV-1 was shown to originate from chimpanzees, whereas HIV-2 displayed similarity to simian immunodeficiency virus (SIV) from sooty mangabeys (SIV_{SM}) (GAO et al., 1999; LEMEY et al., 2003).

HIV-1 is comprised of three groups, namely the M (main), O (outlier) and the N (non-M, non-O) group. The M-group causes most of the global HIV-1 infections and is further divided into 9 subtypes or clades, namely A-D, F-H and J-K (reviewed in LEVY, 2009). In addition to HIV-1 groups M, N and O, another HIV-1 lineage derived from gorillas was recently identified and named the putative (P) group (PLANTIER et al., 2009). Additional variants resulting from recombination events within the clades were identified and named circulating recombinant forms (CRFs) (ROBERTSON et al., 1995).

HIV-2 comprises eight different groups, with groups A and B predominating. Occurrence of HIV-2 is limited to West Africa (ROWLAND-JONES & WHITTLE, 2007), in contrast to the pandemic global spread of HIV-1. HIV-1 clade B is most common in the Western countries, whereas subtype C shows highest transmission rates in India, China and sub-Saharan Africa (reviewed in KARLSSON HEDESTAM et al., 2008; LEVY, 2009).

1.2 HIV-1 genome

Retroviruses are divided into several genera, namely alpha-, beta-, gamma-, delta- and epsilon-retroviruses as well as spuma- and lentiviruses. A characteristic of the genus Lentivirus is a complex genome (Figure 1) encoding for multiple additional proteins when compared to simple retroviruses (WAHEED & FREED, 2010).

The genome of retroviruses is 10 kb in length and is comprised of two identical single stranded RNA molecules. It generally consists of the *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) genes. HIV further requires another

six genes, namely *tat, rev, nef, vif, vpr* and *vpu*, encoding regulatory and accessory proteins that are essential for replication (Figure 1) (PETERLIN & TRONO, 2003). Sixteen different proteins are synthesized from the HIV-1 proviral genome. The Gag protein encoded by *gag* is cleaved into the viral matrix (MA, p17), the capsid (CA, p24), the nucleocapsid (NC, p7) and the late domain p6, whereas Pol is processed into the viral protease (PR, p10), reverse transcriptase (RT, p66, p51) and integrase (IN, p32). The Env precursor protein gp160 is cleaved into the surface (SU, gp120) and transmembrane (TM, gp41) proteins. The two regulatory genes encode the transcriptional transactivator protein (Tat), the main transcriptional regulator of the long terminal repeats (LTRs), as well as the main nuclear-export protein and regulator of virion gene expression (Rev). Additionally, the accessory genes encode the following proteins essential for viral replication and disease progression, the negative effector (Nef), viral infectivity factor (Vif), as well as the viral proteins r (Vpr) and u (Vpu) (reviewed in PETERLIN & TRONO, 2003).

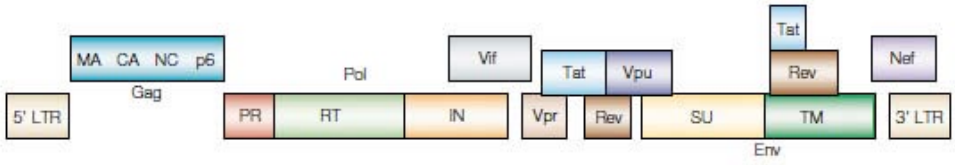


Figure 1: HIV-1 genome
(ROBINSON, 2002)

1.3 HIV-1 particle structure

As a member of the Lentivirus family, HIV-1 exhibits typical virion morphology including a cone-shaped core (Figure 2) (WAHEED & FREED, 2010). The HIV-1 virion is approximately 80-120 nm in diameter. It is covered by the viral envelope derived from the host lipid bilayer membrane and exhibits trimers of non-covalently associated gp120 and gp41. The transmembrane glycoprotein gp41 interacts with the matrix protein, which encircles the cone-shaped core consisting of the capsid. Inside the particle core, two RNA molecules surrounded by the

nucleocapsid protein (not indicated in Figure 2) are packaged together with the enzymes RT and IN. (SIMON et al., 2006)

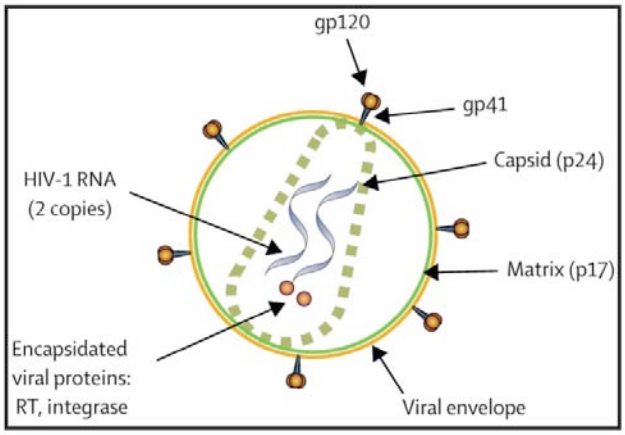


Figure 2: HIV-1 virion structure
(SIMON et al., 2006)

1.4 Replication cycle of HIV-1

The HIV replication cycle can roughly be divided into six major steps ultimately resulting in the formation of progeny virions.

HIV-1 enters the cell by binding (Figure 3; 1) and subsequent fusion (Figure 3; 2) with the cellular membrane. The viral genome is uncoated and the RNA molecules are reverse-transcribed (Figure 3; 3) by the viral enzyme RT. The error-prone RT lacks a proofreading function, resulting in emergence of new escape variants, termed quasispecies. The viral cDNA is then transported into the nucleus via a mechanism enabled by multiple nuclear localization signals within the pre-integration complex (PIC) that covers the viral cDNA and is comprised of RT, IN, MA and Vpr. Upon integration of viral cDNA by the viral enzyme IN (Figure 3; 4), the virus forms LTRs adjacent to the viral genome and is now referred to as a provirus. Proviral DNA is transcribed as part of the host cellular genome and expression is augmented by the viral protein Tat. Viral mRNA species are then exported into the cytoplasm and translated into viral proteins (Figure 3; 5). The viral Rev protein enables transport of non-spliced and singly spliced viral mRNA transcripts out of the nucleus. It is crucial for the transition of the viral life cycle from early-phase, during which only fully-spliced mRNA species can be transported out of the nucleus, to late-phase, which allows transport of single or non-spliced mRNA transcripts. New virions are assembled and released by budding from the cellular

membrane (Figure 3; 6). The replication cycle is completed when the immature virion undergoes maturation by structural rearrangement mediated by the viral enzyme PR (reviewed in PETERLIN & TRONO, 2003; TEIXEIRA et al., 2011).

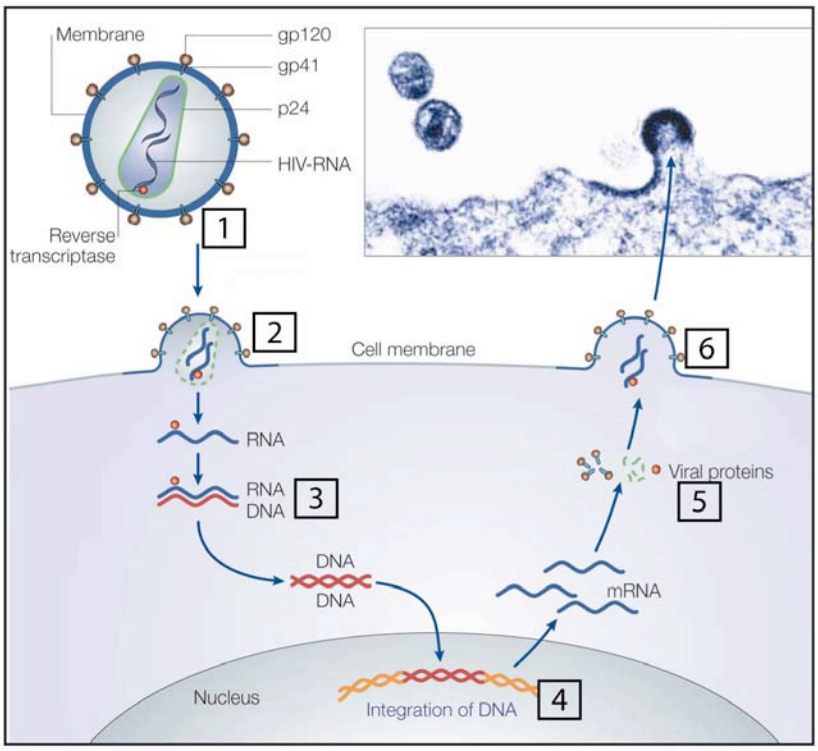


Figure 3: HIV-1 replication cycle
(Modified from SIMON & HO, 2003)

1.5 Molecular entry mechanism of HIV-1

The viral envelope surface displays trimeric spikes consisting of gp120 and gp41 subunits. Upon attachment to the target cell, viral gp120 binds to its primary receptor cluster of CD4 differentiation markers (Figure 4; 2) (DALGLEISH et al., 1984). The engagement of CD4 is followed by a conformational change leading to exposure of the conserved co-receptor binding site. Subsequently, gp120 binds to one of the chemokine co-receptors CCR5 or CXCR4 (Figure 4; 3) (DENG et al., 1996; FENG et al., 1996; MOORE et al., 1997). This induces another reorganization of the viral spike allowing outward rotation of each spike subunit to reveal the viral transmembrane glycoprotein gp41. This step triggers insertion of the hydrophobic gp41 fusion peptide (N-terminal region of gp41) into the host cell membrane (Figure 4; 4), forming a gp41 six-helix bundle (Figure 4; 5), which brings

the viral and cellular membranes in close proximity, ultimately leading to fusion. (PERMANYER et al., 2010)

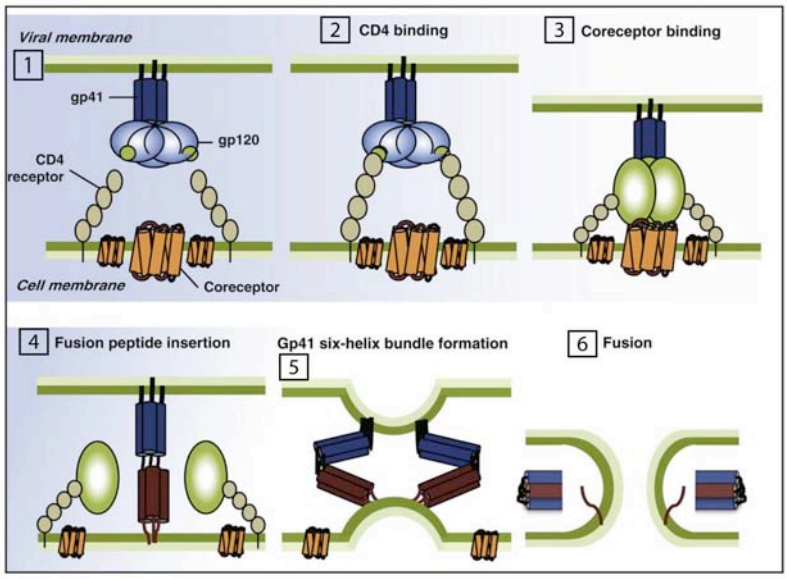


Figure 4: HIV-1 fusion molecular mechanism
(Modified from PERMANYER et al., 2010)

1.6 Structural organization of HIV-1 Env

The surface of HIV-1 is composed of trimers consisting of the non-covalently associated surface glycoprotein gp120 and the transmembrane glycoprotein gp41. Both glycoproteins are generated from their precursor polyprotein gp160 by proteloytic cleavage.

1.6.1 The surface glycoprotein gp120

The envelope glycoprotein gp120 is exposed on the surface of the viral membrane and is crucial for CD4 binding. It is heavily glycosylated, with 50 % of the gp120 surface being covered with carbohydrates (LEONARD et al., 1990; PANTOPHLET & BURTON, 2006). It is comprised of five variable loops exposed on the surface of the virion (V1-V5) and five constant (C1-C5) regions, essential for the interaction with gp41 and fusion (STARCICH et al., 1986; WILLEY et al., 1986).

When they are in a folded conformation, the loops form a two-domain structure and an additional distinct mini-domain termed the “bridging sheet” (Figure 5). The inner gp120 domain faces gp41, whereas the outer domain forms the five variable loops. Both domains together with the bringing sheet form the CD4 binding site (CD4bs). When gp120 is bound to CD4, the interface of these molecules displays two cavities. The first one is large and shallow with a hydrophilic character serving as water buffer between CD4 and gp120. The second cavity, also called the “Phe43 cavity”, is deeper and has a highly conserved hydrophobic character, which is viewed as being very important for CD4 binding. Further conserved structures on gp120 include the chemokine receptor binding sites, which are called CD4-induced (CD4i) epitopes and are revealed upon CD4 engagement (KWONG et al., 1998; WYATT & SODROSKI, 1998).

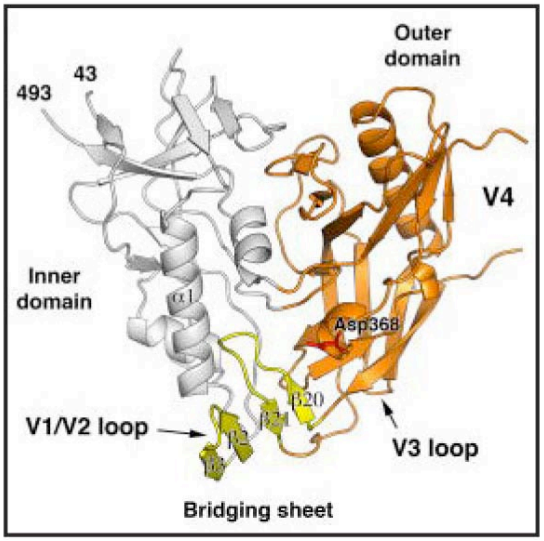


Figure 5: Ribbon structure of gp120
The inner domain is in grey, the outer domain in orange and the bridging sheet in yellow (PEJCHAL & WILSON, 2010)

1.6.2 The transmembrane glycoprotein gp41

The transmembrane glycoprotein gp41 plays a key role in the fusion process upon co-receptor engagement. It is comprised of an ectodomain, a membrane-spanning domain and an endodomain (cytoplasmic domain CD). The ectodomain is further divided into an N-terminal fusion peptide (FP), N-terminal and C-terminal heptad repeat regions (NHR and CHR), a loop region connecting them, a membrane-proximal external region (MPER) and a transmembrane spanning (TM) region (Figure 6) (CHAN et al., 1997; ROUX & TAYLOR, 2007). The MPER is essential for productive fusion and plays an important role in bringing the cellular and viral membranes together (PEJCHAL & WILSON, 2010). The CD is further

comprised of lentivirus lytic peptide, which consists of three amphipathic regions called lentiviral lytic peptide (LLP) 1 to 3 (Figure 20) (MILLER et al., 1991).

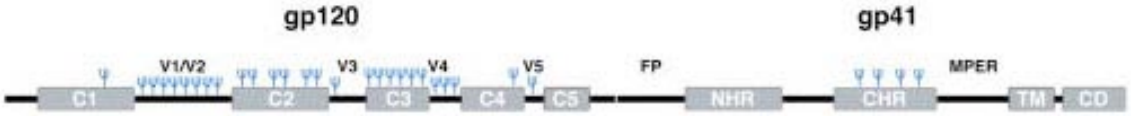


Figure 6: gp120 and gp41 structure
Potential glycosylation sites are indicated by forks; C1-C5: constant regions 1-5; V1-V5: variable regions; FP: fusion peptide; MPER: membrane-proximal external region; NHR and CHR: N- and C-terminal heptad repeat regions TM: transmembrane spanning region; CD: cytoplasmic domain (PEJCHAL & WILSON, 2010)

1.7 HIV-1 cell tropism

The primary receptor of HIV-1 is the CD4 molecule expressed on the surface of cells such as T helper lymphocytes (T_H lymphocytes), macrophages and dendritic cells (DALGLEISH et al., 1984).

The cell tropism of HIV-1 is defined as the ability to infect specific target cells depending on chemokine-receptors expressed on the surface (either CCR5 or CXCR4). During the initial part of infection, HIV-1 mainly uses CCR5 as co-receptor (R5-tropic virus) and productively infects mainly activated T lymphocytes, macrophages and dendritic cells (SIMON et al., 2006). In contrast, as the disease progresses, a switch in co-receptor usage is noted and the more pathogenic X4-tropic viruses emerge, allowing the virus to replace CCR5 with CXCR4 as co-receptor. R5 viruses that further expand their co-receptor usage to both CCR5 and CXCR4 and are referred to as dual-tropic R5/X4 viruses (reviewed in MOORE et al., 2004; LEVY, 2009).

Before the role of the co-receptors CCR5 and CXCR4 was discovered, HIV strains were designated according to their ability to replicate in either macrophages or primary T lymphocytes, but not immortalized T cells. R5 strains were referred to as M-tropic viruses according to their ability to infect primary macrophages. X4 strains using CXCR4 as a co-receptor and infecting established T-lymphocyte cultures were termed T tropic. Because of the ability of X4 viruses to cause cytopathic

changes in cultivated T lymphocytes, they were also termed syncytium-inducing (SI), whereas R5 HIV-1 strains were non-syncytium inducing (NSI) viruses (reviewed in LIN & KURITZKES, 2009).

1.8 HIV-1 pathogenesis

1.8.1 Clinical course of HIV-infection

The clinical course of HIV-1 infection is characterized by a progressive decline in naïve and memory $CD4^+$ T lymphocytes. It can be divided into three phases depending on the symptomatic outcome of the infection, namely the acute phase followed by the asymptomatic phase and finally progression to AIDS.

The acute phase is considered to be the period from time of infection with HIV-1 to emergence of HIV-1 specific antibodies (seroconversion) (COHEN et al., 2011). This time period is associated with clinical signs such as fever, skin rash, oral ulcers ad lymphadenopathy (POPE & HAASE, 2003).

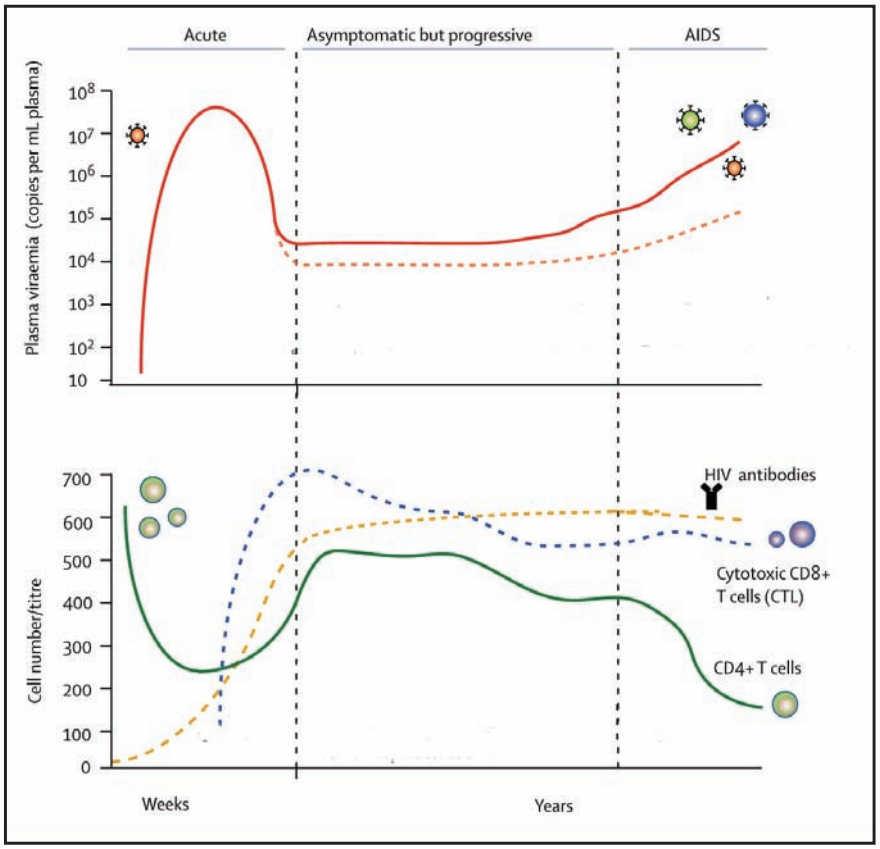


Figure 7: Clinical course of HIV-1 infection
(SIMON et al., 2006)

Following infection, the virus remains latent for a certain time, before it starts replicating explosively to afford peak viremia 21-28 days post-infection (Figure 7 top; red line) (MCMICHAEL et al., 2009). However, the number of $CD4^+$ T lymphocytes gradually decreases as the number of virions in the blood increases (Figure 7; green and red lines). As soon as the immune system develops HIV-1 specific antibodies and cytotoxic $CD8^+$ T lymphocytes (CTLs), the plasma viral load decreases and $CD4^+$ cell counts recover as the asymptomatic phase is entered. Despite the ability of the host to control viremia, the virus continuously maintains its replication, resulting in a gradual decline of naïve and memory $CD4^+$ T lymphocytes (Figure 7 bottom; green line). Ultimately progression to AIDS occurs and is generally accompanied by opportunistic infections, increased incidence of cardiovascular disease and AIDS-associated cancers (LEVY, 2009).

1.8.2 Routes of transmission

HIV is transmitted in various ways, including contaminated blood products, sharing of contaminated needles by intravenous drug abuse and sexual contact involving mucosal surfaces such as those of the oral, vaginal and rectal mucosa. HIV-positive mothers can infect their infants during breast-feeding by oral transmission or in utero, when infants are exposed to maternal fluids within the birth canal (mother-to-child-transmission) (LACKNER et al., 2009). The major transmission route of HIV-1, however, is through the genital or rectal mucosa, mainly when the mucosal site is damaged (COHEN et al., 2011).

1.9 Host immune response

The human defense system against invading pathogens is comprised of two major branches, namely the innate and the acquired immune response, each consisting of a cellular and a humoral compartment. The innate immune system constitutes the first line of defense and uses cells that express pattern recognition

receptors (PPRs) to recognize conserved patterns on microorganisms (= pathogen associated molecular patterns, PAMPs). In contrast, the acquired immune system is defined by the ability to recognize specific antigens and develop immunological memory (AKIRA et al., 2006). Upon infection, the first actions against invading pathogens are unleashed by the innate immune system, providing time for the adaptive immune system to develop a specific response mediated by B and T lymphocytes, when this is required (EGER & UNUTMAZ, 2005).

Infection with HIV-1 leads to major dysregulation of the innate and acquired immune system. Effector cells such as dendritic cells (DCs), natural killer cells (NK cells), $CD4^+$ T-helper lymphocytes and $CD8^+$ T-lymphocyte populations, such as cytotoxic $CD8^+$ T cells (CTLs) are severely impaired (BORROW & BHARDWAJ, 2008; BERNSTEIN et al., 2009; LEVY, 2009; ALTFELD et al., 2011).
In addition to the innate and acquired immune system, the human body produces intrinsic factors that can restrict HIV-1 activity. Restriction factors include the human APOBEC (apolipotrotein B mRNA-editing enzyme-catalytic polypeptide-like-deaminase) proteins, TRIM5α (tripartite motif protein 5α) and Tetherin (SHEEHY et al., 2002; STREMLAU et al., 2004; ULENGA et al., 2008). Moreover, certain genetic factors have been shown to influence HIV-1 disease progression or infection (e.g. homozygous CCR5Δ32 deletion confers resistance to infection with R5-tropic viruses) (LIU et al., 1996; CARRINGTON et al., 1999).

1.10 B lymphocytes

1.10.1 Immunoglobulin structure and diversity

The cell compartment of short- and long-lived plasma cells constitutes less than 1 % of the entire cell population in lymphoid organs (FAIRFAX et al., 2008); yet this compartment is the source of all secreted Abs. The antibody molecule is bivalent and is comprised of two identical heavy chains (H) and two identical light chains (L). The latter can be comprised of either the κ or λ type (Vκ or Vλ).

At protein level, immunoglobulins can be functionally separated into variable and constant domains. The variable domains contain immunoglobulin (Ig) leader regions, followed by hypervariable regions called complementarity-determining regions (CDR 1-3), which are interspersed with four relatively conserved framework regions (FR 1-4). The CDRs from the V_H and V_L chains are responsible for binding of antigens.

The constant domains define the isotype of antibody (IgM, IgA, IgG, IgD and IgE) and also mediate effector functions such as Fc receptor binding (Figure 8; top H and L chain). The isotype of the antibodies can change, whereas the antigen specificity is maintained at the same time. The five main classes of H chain constant domains are further subdivided into subclasses (e.g. IgG 1-4, IgA 1-2).

At nucleotide level, the chains are composed of different gene segments. H chains include constant (C), variable (V), diversity (D) and joining (J) gene segments, whereas the L chains lack D segments (V_H, D_H, J_H and C_H for H chain; V_L, J_L and C_L for L chain) (Figure 8; bottom H and L chain) (LEDER, 1982; TONEGAWA, 1983; DI NOIA & NEUBERGER, 2007; SCHROEDER & CAVACINI, 2010).

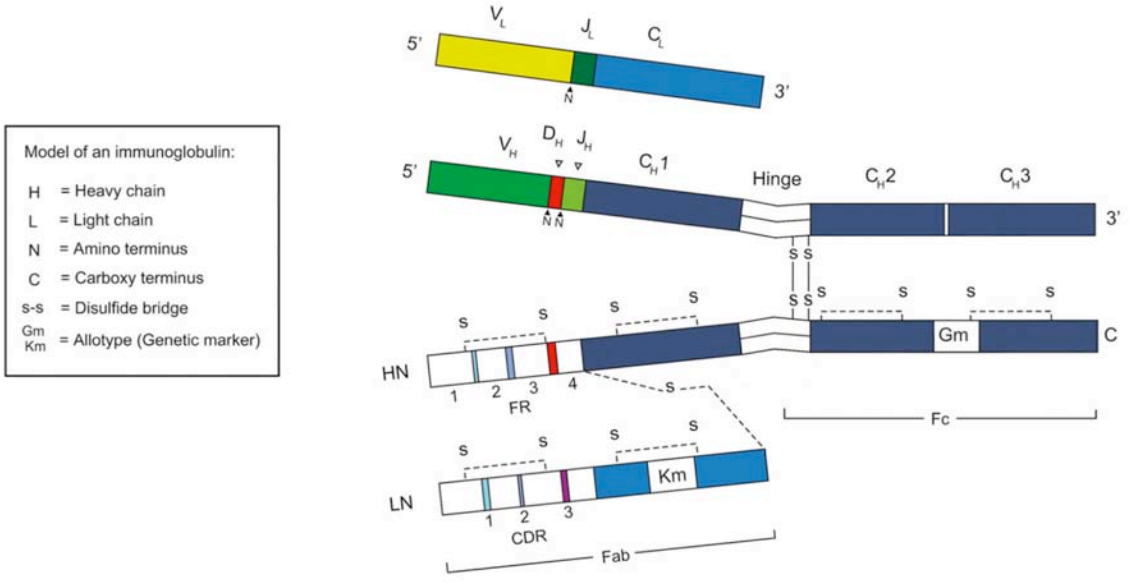


Figure 8: Model of an IgG molecule

The H and L chains on the top display the Ab structure at nucleotide level. The bottom H and L chains represent the protein sequence (SCHROEDER & CAVACINI, 2010)

A human produces more than 10^9 different antibodies during his/her life. This enormous production rate is the result of diversification mechanisms including V-(D)-J recombination of gene segments and somatic hypermutation of B cell receptors leading to high-affinity maturation upon antigen encounter (KIM et al., 1981; MCKEAN et al., 1984; DI NOIA & NEUBERGER, 2007).

1.10.2 Isolation techniques for B cells

Separation of particular cell types from a heterogeneous cell mixture is an important tool enabling the analysis of small numbers of cells, such antigen-specific T or B lymphocytes that display less than 1 % of the total lymphocyte population (OSHIBA et al., 1994; KODITUWAKKU et al., 2003). For the isolation of antigen-specific B cells, three major techniques have been used over the past 30 years, namely capture on an antigen-coated solid matrix, rosetting with antigen-coated red blood cells or magnetic particles and staining with fluorescent antigen followed by isolation by flow cytometric cell sorting. Cell sorting for isolation of fluorescently labeled cells uses fluorescent labeling of specific antigens expressed on the desired cell population. Using multiple fluorescent stains for detection of antigen-specific B cells and subsequent isolation of single cells is considered highly sensitive as well as specific (reviewed in KODITUWAKKU et al., 2003).

The production of human MAbs has used several techniques including immortalization of B cells with Epstein-Barr virus (EBV) (STEINITZ et al., 1977; LANZAVECCHIA et al., 2007), the production of B-cell hybridomas (KOZBOR et al., 1982), phage display libraries (MCCAFFERTY et al., 1990) or generation of MAbs from isolated single B cells (TILLER et al., 2008). (For detailed information on the different techniques, the reader is referred to the stated references as well as to (TILLER, 2011).

1.11 Neutralizing antibodies and implications in vaccine design

Interplay between the cellular and humoral arms of the immune system is crucial for a potent immune response against HIV-1. In fact, induction of both is required for development of an effective vaccine against HIV-1 (ZOLLA-PAZNER, 2004). Neutralizing antibodies (nAbs) inhibiting replication of autologous virus develop within several months. Nevertheless, these nAbs are not able to neutralize escape variants of HIV-1 (DEEKS et al., 2006; GRAY et al., 2007).

Initially, four broadly neutralizing MAbs against HIV-1 (MAbs b12, 2G12, 2F5, 4E10) have been found to exhibit potent neutralization against different HIV-1 clades (BURTON et al., 1994; TRKOLA et al., 1996; ZWICK et al., 2001). The MAb b12 is believed to induce its broad neutralizing capabilities by "mimicking CD4" and competing for the CD4-binding site (CD4bs) (HOXIE, 2010). The second antibody, 2G12, exhibiting an extraordinary structure, neutralizes HIV-1 by targeting high-mannose glycans on an Env-surface region that is absent on host cells (SANDERS et al., 2002; CALARESE et al., 2003). The remaining MAbs, 2F5 and 4E10, target the highly conserved MPER-region within gp41. Albeit displaying the broadest neutralization activity against HIV-1, these MAbs also exhibited cross-reactivity with cardiolipin (CARDOSO et al., 2005). Another human MAb, 447-52D, binds the highly immunogenic V3-domain within gp120 and shows moderate neutralization (GORNY et al., 1992; HOXIE, 2010).

Recently, further cross-clade neutralizing MAbs were isolated, including the VRC MAbs VRC01, VRC02 and VRC03 that displayed broad cross-neutralization of different HIV-1 strains by targeting the CD4bs (WU et al., 2010). Another potent MAb, HGN194, neutralized different HIV-1 subtypes by binding a conserved epitope in the V3 loop of gp120 (CORTI et al., 2010). A class of highly active agonistic CD4bs Abs (HAADs) was isolated from HIV-1 infected individuals and showed high levels of neutralizing activity by binding to a similar surface to that of the CD4bs (SCHEID et al., 2011).

In the field of HIV-1 vaccine development, researchers have faced major obstacles due to the changing nature of the virus, which reflects the highly error-prone nature of viral RT, as well as the existence of HIV-1 subtypes and inter-subtype recombinants. It is highly challenging to elicit broadly neutralizing antibodies, inasmuch as conserved regions on the envelope are hidden, in contrast to heavily glycosylated exposed epitopes, which are highly immunogenic and antigenic (KWONG et al., 1998). Further, HIV-1 integrates into the host genome and exists as latent provirus (ROBINSON, 2002).

AIDS vaccine research has focused on two major approaches; (1) eliciting a nAb response and (2) inducing a cellular immune response. However, while both approaches have been tested in vaccine trials, they have thus far failed to induce protection, emphasizing the challenge of finding an effective protective vaccine against HIV-1 (HOXIE, 2010).

1.12 Non-human primate models and SIV/HIV chimera

Non-human primate models have been widely used in vaccine studies and much information on safety, immunogenicity and efficacy has been gained using these models. Due to numerous similarities between infections with SIV in macaques and HIV-1 infection in humans, the SIV model was initially chosen as the in vivo model for HIV vaccine research. Similarities included points, such as the use of CCR5 as co-receptor, selective depletion of memory T cells in gut-associated lymphoid tissues, viral replication in both activated and resting T lymphocytes, and finally the high virus variability resulting from high mutation rates. However, the asymptomatic disease-free phase varies significantly between HIV-infected individuals and SIV-infected macaques, lasting approximately 8-10 years for humans and 0.5-3 years for macaques. Sequence analysis of SIV revealed a high degree of homology with HIV-2 (ca. 80 %), but only 40-50 % with HIV-1. Due to the low serological cross-reactivity of SIV to HIV-1, direct extrapolation of SIV data to HIV-1 is not possible.

As the relevance of data from SIV-infected monkeys needed to be considered when compared with HIV-infected individuals, chimeric viruses called recombinant simian-human immunodeficiency viruses (SHIVs) were developed. SHIVs were engineered containing a genomic backbone from SIV from rhesus macaques (SIVmac239) and an HIV-1 envelope to allow direct testing of HIV-1 vaccines in macaque animal models (HU, 2005). However, initial SHIV variants did not display biologically relevant results, as they did not exhibit natural characteristics of an HIV-1 challenge, such as CCR5-tropism, mucosal transmission and gradual progression of disease (VLASAK & RUPRECHT, 2006). Consequently, more biologically relevant SHIV strains were developed, mimicking natural HIV-1 transmission in humans. Their features included R5-tropism, mucosal transmissibility, encoded HIV-C envelopes and the ability to cause gradual disease progression (SONG et al., 2006; HUMBERT et al., 2008; SIDDAPPA et al., 2009; SIDDAPPA et al., 2010).

1.13 Aim of the thesis

1.13.1 Background of the study

In 2009, a vaccine study was performed in the Ruprecht lab using several rhesus monkeys (RMs) including the RTr-11 and RVe-10, which were vaccinated according to different strategies. The RM RVe-10 was primed with SHIV-1157ipEL-p *env* DNA, followed by several multimeric protein immunizations with SHIV-1157ipΔV1-V4 Env (envelope protein lacking the variable loops from V1 to V4). After five low-dose and one high-dose challenge with SHIV-1157ipEL-p, this monkey showed peak viremia levels five weeks after the first low-dose challenge (1 x 10^6 RNA copies/ml) (unpublished data). The HIV chimera SHIV-1157ipEL-p (SIDDAPPA et al., 2010) carries the envelope of HIV1157i, a HIV-C strain characterized by R5 tropism. The SHIV backbone was derived from SHIV-1157ipd3N4 (SONG et al., 2006), a SIVmac239 variant with improved virus replication due to additional NF-κB sites within its LTRs.

The RM RTr-11 received three multimeric protein immunizations including HIV1084i gp160 before it was challenged with SHIV-1157ipEL-p. This monkey stayed aviremic (RNA copies constantly below 50 copies/ml), despite multiple low-dose and one high-dose challenge. However, a cryptic response against HIV-1 (i.e. cellular response) together with humoral response by binding antibodies were detected (LAKHASHE et al., 2011).

Mimotopes are defined as sequences that mimic epitopes on the HIV-1 envelope. The mimotope sequences used in this study were previously isolated from RM RTr-11 (Bachler B., unpublished data). They are located at the C-terminal tail (CTT) of the HIV-1 glycoprotein gp41 and are upstream or partially overlapping, respectively, with the previously described 22-residue Kennedy-sequence ^{731}PRGPDRPEGIEEEGGERDRDRS^{752} (Figure 20; Kennedy sequence lies within yellow mark) (CHANH et al., 1986; KENNEDY et al., 1986; HEAP et al., 2005) (numbering system from (RATNER et al., 1985).

The mimotope sequences used in this study will be referred to as Kennedy-proximal mimotopes throughout this thesis.

1.13.2 Generation of mimotope-specific MAbs

The isolation of antigen-specific cells and production of MAbs has been subjected by numerous research groups and only a small number of studies have focused on the generation of macaque antigen-specific MAbs. Previous studies demonstrated that epitopes within the CTT of the HIV-1 glycoprotein gp41 induced nAb-responses in the infected animals. This study focuses on the generation of mimotope-specific MAbs targeting epitopes upstream of and partially overlapping with the previously published Kennedy-sequence and aims to characterize their binding to their corresponding mimotopes.

2 Materials and Methods

2.1 Materials and animals

Animals:

The plasma samples used from this study were derived from Indian-origin RM (*Macaca mulatta*). The monkeys were kept at the Yerkes National Primate Research Center (YNPRC) (Atlanta, GA), fully accredited by the Association for assessment and Accreditation of Laboratory Animal Care International (AAALAC). All procedures were approved by the Institutional Animal and Care and Use Committees of Dana-Farber Cancer Institute and Emory University.

Mimotopes:

The Kennedy-proximal mimotopes are comprised of 12 amino acids (Figure 9). For practical reasons, two conserved sequences of 10 amino acids (in green), derived from phage protein p3, were added to the N- and C- terminal of each mimotope sequence (in bold). Mimotopes were provided by Barbara Bachler, MSc (University of Veterinary Medicine Vienna, DFCI).

- BB-m-12-C5: PLVVPFYSHS**GHFQPYTARSPS**GGGSAETVES
- BB-m-12-E7: PLVVPFYSHS**TAFQLYTQRPTN**GGGSAETVES
- BB-o-12-E7: PLVVPFYSHS**FMFQPYTTRVIS**GGGSAETVES
- BB-o-12-G2: PLVVPFYSHS**GDFQTWIDRSVR**GGGSAETVES

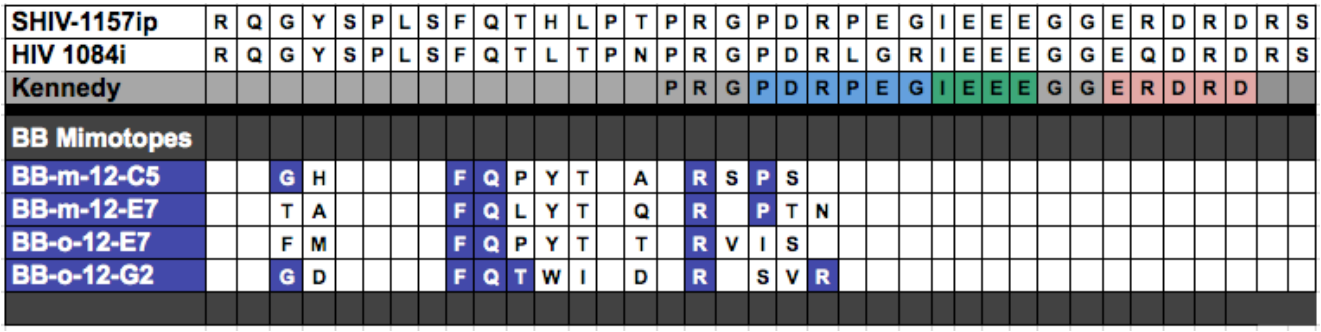


Figure 9: Alignment of Kennedy-proximal epitopes
SHIV-1157ip, HIV 1084i and the Kennedy sequence along with the mimotope sequences isolated from RTr-11 are aligned. Amino acids in the blue indicate identity between the aligned sequences. The color code within the Kennedy sequence represents the three separate peptides (HEAP et al., 2005) (Alignment generously provided to the author by Bachler B.)

List of plasmids:

- **pET-GFP.II**: The original vector pET-22b(+) vector was provided by Novagen (MerckKGA, Darmstadt, Germany) and contains an ampicillin resistance gene as well as a cloning region displaying a T7 promoter. The complete GFP sequence (~800bp) was previously cloned into the pET-22b(+) vector in this laboratory by Sholukh A.

- **pFUSE-vectors:** The vectors were provided by InvivoGen (San Diego, CA). They are composed of human IgG1 constant heavy regions, as well as human constant κ and λ regions. They were used for the expression of RM Ig heavy- and light-chains and carried zeocin or blasticidin resistance genes, respectively.
  pFUSE-CHIg-hG1: used for expression or RM γ heavy-chain
  pFUSE2-CLIg-hk: used for expression of RM κ light-chain
  pFUSE2-CLIg-hl2: used for expression of RM λ light-chain

- **pNCS-mWasabi:** The vector was provided by Allele Biotechnology and Pharmaceuticals (San Diego, CA). It was used for the expression of GFP-protein and served as source of the GFP-gene.

List of primers (All primers were obtained from Invitrogen (Camarillo, CA))

Step	Primer name	5'-3' sequence
		Mimotope PCR
	Fw-M2-22	CGTGAATTCGGCCCTTTAGTGGTACTTTTCTATTCTCACTCT
	Rev-M2-22	GATAAGCTTCTAACTTTCAACAGGTTTCGGCCGAACCTCC
		Semi-nested PCR primers
Forward primer γ	VH-1	SAGGWSCAGCTGGTRCAATCCGG
(Fw-mix γ)	VH-2	CAGGTGACCTTGAAGGAGTCTGG
	VH-3/5/7	SAGGTGCAGYTGGTGSAGTCTGG
	VH-4/6	CAGGTGCARCTGCAGGAGTCRGG
	VH-5	GAGGTGCAGCTGGTGCAGTCTGG
	VH-6	CAGGTACAGCTGCAGCAGTCAGG
	VH-7	CAGGTGCAGCTGGTGCAATCTGG
Forward primer κ	Vκ-1	GACATYCAGATGWCCCAGTCTCC
(Fw-mix κ)	Vκ-2	GATAYTGTGATGACCCAGACTCC
	Vκ-3	SAAATWGTRWTGACKCAGTCTCC
	Vκ-4	GACATYGTGMTGACCCAGTCTCC
	Vκ-5	GAAACGACACTCACGCAGTCTCC
	Vκ-6	GAWRTTGTGMTGACWCAGTCTCC
	Vκ-7	GACATTGTGCTGACCCAGTCTCC
Forward primer λ	Vλ-1	CAGTCTGTRCTGACVCAGCCDCC
(Fw-mix λ)	Vλ--2	CAGKCTGCCCYGAYTCAGYCTCC
	Vλ-3A	TCCTCTGGGCTGACTCAG
	Vλ-3B	TCCTMTGAGCTGACACAGCCDCC
	Vλ-4	CAGCYTGTGCTGACTCARTCGCC
	Vλ-5	MAGSCTRTGCTGACTCAGCCRRC
	Vλ-6	AATTTTATGCTGACTCAGCCC
	Vλ-8/7	CAGACTGTGGTGACYCAGGAGYC
	Vλ-9	CAGCYTGTGCTGACTCARCCACC
	Vλ-10	CAGGCAGGGCTGACTCAGCCACC
Reverse primer γ	γ-PCR1-Rev	GGACAGCCKGGAAGGTGTGC
	γ-PCR2-Rev	GCCTGAGTTCCACGACACGGTCAC
Reverse primer κ	κ-PCR1-Rev	GAGGCAGTTCCAGATTTCAA
	κ-PCR-2-Rev	GGTGCAGCCACAGCTCGTTTGAT
Reverse primer λ	λ-PCR1-Rev	CCGCGTACTTGTTGTTGCTCTGT
	λ-PCR2-Rev	CAGAGGAGGGCGGGAASAGA

N = A+G+C+T; V = A+C+G; D = A+T+G; B = T+C+G; H = A+T+C; W = A+T; S = C+G; K = T+G; M = A+C; Y = C+T; R = A+G;

		pFUSE cloning PCR
Forward primer γ	IGHV3-71	5'GCCGAATTCGCCACCATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCT ATTTTAAAAGGTGTCCAGTGTGAGGTGCAGCTGGTGCAGTCTGGGG3'
	IGHV4-4	5'GCCGAATTCGCCACCATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCT ATTTTAAAAGGTGTCCAGTGTCTGGTGCAGCTGGTGGAGTCTGGCC3'
	IGHV4-39	5'GCCGAATTCGCCACCATGGAGTTTGGGCTGAGCTGGGTTTTCCTTGTTGCT ATTTTAAAAGGTGTCCAGTGTCAGGTGCAGCTGGTGCAATCGGGC3'
Forward primer κ	IGKV1-12	5'GACACCGGTGCCACCATGGACATGAGGGTCCCCGCTCAGCTCCTGGGGC TCCTGCTGCTCTGGCTCCCAGGTGCCAGATGTGATATTGTGATGACCCAGT3'
Forward primer λ	IGLV3-21	5'GACACCGGTGCCACCATGGCCTGGGCTCTGCTGCTCCTCACTCTCCTCAC TCAGGACACAGGGTCCTGGGCCTCCTATGTGCTGACTCAGCCACCC3'
	IGLV2-14	5'GACACCGGTGCCACCATGGCCTGGGCTCTGCTGCTCCTCACTCTCCTCAC TCAGGACACAGGGTCCTGGGCCCAGGCTGCCCTGACTCAGTCTCCG3'
Reverse primer γ	Pan-Rev-γ	CTTGGTGCTAGCTGAGGAGACGGTGAC
Reverse primer κ	Pan-Rev-κ	TCGCGTACGTCGTTTGATATCCAAGTGGGTCCC
Reverse primer λ	Pan-Rev-λ	CTGACCTAGGACGGT(G/C)AGCCGGGTC
		Sequencing primers
pET-GFP plasmids	T7 promoter	TAATACGACTCACTATAGGG
	T7 terminator	GCTAGTTATTGCTCAGCGG
pFUSE plasmids	Pan-pFUSE-seq-Fw	CTGTTCTGCGCCGTTACAGATCCAAG

List of restriction enzymes:

All fast digest (FD) restriction enzymes were obtained from Fermentas (Glen Burnie, MD).

- FD® *Age*I: 5'-A^CCGGT-3'
- FD® *Avr*II 5'-C^CTAGG-3'
- FD® *BsiW*I 5'-C^GTACG-3'
- FD® *EcoR*I: 5'-G^AAT TC-3'
- FD® *Hind*III: 5'-A^AGCTT-3'
- FD® *Nco*I 5'-C^CATGG-3'
- FD® *Sal*I: 5'-G^TCGAC-3'
- FD® *Nde*I: 5'-CA^TATG-3
- FD® *Nhe*I: 5'-G^CTAGC-3'
- FD® *Xho*I: 5'-C^TCGAG-3'

List of mAbs:

APC-Cy7 mouse anti-human CD3	BD Pharmingen (San Diego, CA)
APC mouse anti-human CD27	
FITC mouse anti-human CD4	
PE-Cy7 mouse anti-Human IgG	
CD19-PE mAb	Beckman Coulter (Brea, CA)
Rabbit anti-monkey-horseradish peroxidase (HRP) secondary antibody	Sigma-Aldrich (St. Louis, MO)
Goat anti human Ab-HRP	Jackson Laboratories (Bar Harbor, ME)
Anti-HIV-1 mAb 2G12	Polymun Scientific (Vienna, Austria)

List of products:

293F cells	Invitrogen (Camarillo, CA)
Amicon®Ultra Centrifugal Filter Devices	Millipore (Billerica, MA)
Ampicillin	Sigma-Aldrich (St. Louis, MO)
BD Falcon™ conical tubes	BD Biosciences (Bedford, MA)
BD Falcon™ Multiwell™ 6 well plate	BD Biosciences (Bedford, MA)
BD Luer-Lok™ Syringe 3 ml	BD Biosciences (Bedford, MA)
BD Falcon™ Petri Dish	BD Biosciences (Bedford, MA)
β-Mercaptoethanol	Sigma-Aldrich (St. Louis, MO)
Carbonate- Bicarbonate Buffer Capsule	Sigma-Aldrich (St. Louis, MO)
Corning® Syringe Filters, 0.2 µm	Corning Incorporated (Corning, NY)
DNA Loading Dye	Fermentas (Glen-Burnie, MD
dNTP mix 10 mM, 50 mM	Fermentas (Glen-Burnie, MD)
DTT 0.1 M:	Invitrogen (Camarillo, CA)
ELISA 96-well Micro-plates	Greiner Bio-One (Monroe, NC)
Eppendorf Safe-Lock Tubes	Eppendorf (Westbury, NY)
Ethidium Bromide	Invitrogen (Camarillo, CA)
Fetal Bovine Serum (FBS)	Sigma-Aldrich (St. Louis, MO)

GeneJET™ Plasmid Miniprep Kit	Fermentas (Glen-Burnie, MD)
GeneRuler™ 100bp Plus DNA Ladder:	Fermentas (Glen-Burnie, MD)
GIBCO®FreeStyle™ 293 Expression Medium	Invitrogen (Camarillo, CA)
GIBCO®Trypan Blue Stain 0.45:	Invitrogen (Camarillo, CA)
Glycerol	Sigma-Aldrich (St. Louis, MO)
Gp41 protein HxB2 (ARP 680)	CFAR (Hertfordshire, UK)
Halt™ Protease Inhibitor Cocktail (100x)	Thermo-Fisher Scientific (Waltham, MA)
Hard-Shell® Thin-Wall 96-Well Skirted PCR Plates	Bio-Rad Laboratories (Hercules, CA)
HotStarTaq DNA Polymerase	QIAGEN (Valencia, CA)
HyClone® HyPure endotoxin free water	Thermo-Fisher Scientific (Waltham, MA)
Hydrogen Peroxide (H_2O_2) Solution 30 % (w/w)	Sigma-Aldrich (St. Louis, MO)
Igepal CA-630	Sigma-Aldrich (St. Louis, MO)
Imidazole ($C_3H_4N_2$)	Sigma-Aldrich (St. Louis, MO)
Imperial Protein Stain	Thermo-Fisher Scientific (Waltham, MA)
IPTG	Fermentas (Glen-Burnie, MD)
Laemmli Sample Buffer	Bio-Rad Laboratories (Hercules, CA)
LB-medium	Sigma-Aldrich (St. Louis, MO)
LB-Agar	MP Biomedicals (Solon, OH)
L-Glutamine 200 mM	Invitrogen (Camarillo, CA)
Magnesium sulfate ($MgCl_2$) 25 mM	QIAGEN (Valencia, CA)
Millex®-GV Syringe Drive Filter Unit	Millipore (Billerica, MA)
Mini-PROTEAN® TGX™ precast gel	Bio-Rad Laboratories (Hercules, CA)
Mini-PROTEAN® Tetra Cell tank	Bio-Rad Laboratories (Hercules, CA)
NucleoSpin® Extract II kit	Macherey-Nagel (Bethlehem, PA)
NucleoSpin® Plasmid kit	Macherey-Nagel (Bethlehem, PA)
N-Z-Caze Plus 3 %	Sigma-Aldrich (St. Louis, MO)
OneShot ® Top10 Chemically competent *E. coli*	Invitrogen (Camarillo, CA)
Ortho-phenyldiamine (OPD) tablets	AMRESCO (Solon, OH)
Page Ruler™ Plus Prestained Protein Ladder	Thermo-Fisher Scientific (Waltham, MA)
Phosphate-buffered saline (PBS)	Invitrogen (Camarillo, CA)
Penicillin (10,000U)-Streptomycin (10,000 µg)	Invitrogen (Camarillo, CA)
Pfu DNA Polymerase Reaction Buffer	Agilent Technologies (Santa Clara, CA)
PfuTurbo® Hotstart DNA Polymerase	Agilent Technologies (Santa Clara, CA)
Platinum® *Taq* DNA Polymerase High Fidelity	Invitrogen (Camarillo, CA)
Platinum® *Taq* High Fidelity PCR Buffer	Invitrogen (Camarillo, CA)
QIAGEN Plasmid Midi kit:	QIAGEN (Valencia, CA)
Random Hexamers 50 µM	Life Technologies (Carlsbad, CA)
Rnase™ OUT (Recombinant ribonuclease inhibitor)	Invitrogen (Camarillo, CA)
RPMI Media 1640	Invitrogen (Camarillo, CA)
Sodium Chloride (NaCl)	Thermo-Fisher Scientific (Waltham, MA)
Sodium Phosphate (NaH_2PO_4):	Thermo-Fisher Scientific (Waltham, MA)
SoluLyse™	Genlantis (San Diego, CA)
Steriflip® 0.45 Filter devices	Millipore (Billerica, MA)
Sulfuric acid (H_2SO_4)	Thermo-Fisher Scientific (Waltham, MA)
SYBR® Safe DNA gel stain	Invitrogen (Camarillo, CA)
TAE-Buffer	Thermo-Fisher Scientific (Waltham, MA)
TB-blasticidin agar	Invitrogen (Camarillo, CA)
TB-blasticidin medium	Invitrogen (Camarillo, CA)
TB-zeocin agar	Invitrogen (Camarillo, CA)
TB-zeocin medium	Invitrogen (Camarillo, CA)
T4 DNA Ligase	Fermentas (Glen-Burnie, MD)
*Trans*IT-PRO® Transfection kit	Mirus BIO (Madison, WI)
TGS 10x electrophoresis buffer	Bio-Rad Laboratories (Hercules, CA)
TurboCells™ Chemically Competent *E. coli*:	Genlantis (San Diego, CA)

TurboCells™ BL21 Chemically Competent *E. coli*	Genlantis (San Diego, CA)
Tween 20	Sigma-Aldrich (St. Louis, MO)
UltraPure™ Agarose	Invitrogen (Camarillo, CA)
Vivapure Maxiprep columns	Sartorius Stedim (Edgewood, NY)

List of buffers and media:

- ### Purification of GFP-mimotope proteins

Diluting Buffer	50 mM NaH_2PO_4
	0.6 M NaCl
	30 mM imidazole
	pH 8.0

Washing Buffer B	50 mM NaH_2PO_4
	0.3 M NaCl
	25 mM imidazole
	pH 8.0

Washing/Binding Buffer A	50 mM NaH_2PO_4
	0.3 M NaCl
	15 mM imidazole
	pH 8.0

Elution Buffer	50 mM NaH_2PO_4
	0.3 M NaCl
	200 mM imidazole
	pH 8.0

- ### ELISA solutions:

Coating Buffer	Carbonate- Bicarbonate Buffer Capsule
	1 capsule for 100 ml dH_20; pH 9.6

Blocking Buffer- PBSCT 3 %	PBS pH 7.2
	3 % N-Z-Caze Plus
	0.5 % Tween 20

OPD solution	Ortho-phenylenediamine (OPD) tablets
	Hydrogen Peroxide (H_2O_2) Solution 30 % (w/w)

- ### Tissue culture medium R15:

R15 medium:	RPMI
	15 % FBS
	1 % Pen/Strep
	2 mM L-glutamine

2.2 Methods

2.2.1 Cloning of mimotopes

The mimotopes were cloned into the pET-GFP.II expression vector at the N-terminus of the GFP-sequence to enable future expression of recombinant GFP-mimotope proteins. Prior to expression, the integrity of both the mimotope and the GFP-sequence was verified by sequence analysis.

2.2.1.1 Amplification of the pET-GFP.II vector

The pET-GFP.II expression vector was obtained from Dr. Anton Sholukh (DFCI), who had previously cloned the GFP-sequence into the commercially obtained pET-22b(+) vector. A plasmid stock of the pET-GFP.II expression vector was prepared by transformation and subsequent plasmid purification.

2.2.1.1.1 Transformation of pET-GFP.II vector

Transformation was performed using TurboCells™ Chemically Competent *E. coli* according to the manufacturer's protocol. In short, 10 µl of plasmid DNA was mixed with 50 µl of TurboCells™ Chemically Competent *E. coli*. The mix was incubated on ice for 5 min, followed by a heat shock at 37°C for 60 sec and subsequent incubation on ice for 2 min. The transformation reaction was diluted with 200 µl TurboCells Transformation Buffer, from which 100 µl was spread onto previously prepared agar plates containing 100 µg/ml Ampicillin. The agar plates were incubated at 37°C for 14-16 h overnight. Single colonies were selected and used for subsequent plasmid purification.

2.2.1.1.2 Plasmid purification

DNA was isolated from successfully transformed competent bacteria. Single clones from each transformation were incubated in 5 ml Luria Bertani (LB)-medium supplemented with 75 µg/ml ampicillin with shaking at 220 rpm at 37°C for 14-16 h overnight. Plasmid purification was performed using the GeneJET™ Plasmid Miniprep Kit according to the manufacturer's protocol. Purified DNA was eluted in 50 µl EB-Buffer.

2.2.1.1.3 Assessment of plasmid DNA concentration

The concentration of the purified plasmid DNA was assessed using the NanoDrop1000 Spectrophotometer. Two µl of each plasmid-DNA sample was pipetted onto the instrument's fiber optic cable and the concentration was measured using the "Nucleic Acid" application module for double-stranded DNA ('DNA-50'-setting). The concentration of DNA was assessed by measuring the absorption of UV light by DNA at 260 nanometers (nm) wavelength.

2.2.1.1.4 Verification of pET-GFP.II vector

The purified pET-GFP.II vector was digested and subsequently loaded onto a 1.5 % UltraPure™ Agarose gel to verify its identity. Digestion was performed using the restriction enzyme for FD® *Nde*I and FD® *EcoR*I to cut out the GFP-sequence. Briefly, 250 ng of the pET-GFP.II vector was digested for 20 min at 37°C using 1 unit (U) of FD® *Nde*I and FD® *EcoR*I in FastDigest® Buffer, respectively, and adjusted with ultrapure H_2O (Millipore) to a total volume of 20 µl.

The digested pET-GFP.II vector was loaded onto a 1.5 % UltraPure™ Agarose gel containing 500 ng/ml ethidium bromide. Samples were mixed with 6 x Loading Dye before they were loaded and the gel was run in TAE-Buffer at 100 volts (V) for 40 min. The separated vector-backbone and GFP-fragment were identified using ultraviolet (UV) light by comparing their fragment sizes with the GeneRuler™ 100 bp Plus DNA Ladder. The gels were photographed using the Molecular Imager® Gel Doc™ XR and analyzed using the Quantity One 1-D Analysis Software Version 4.6.3.

2.2.1.2 Amplification of mimotope DNA

Mimotope DNA was amplified and restriction sites for subsequent cloning of the mimotopes into the pET-GFP.II expression vector were introduced by PCR.

All PCR reactions were performed using 1 µl mimotope DNA, 5 µl 10 x *Pfu* DNA Polymerase Reaction Buffer, 0.5 µl 10 mM dNTPs, 1 µl each of the forward primer Fw-M2-22 and reverse primer Rev-M2-22 (working concentration: 5 pmol/µl), 1 µl *PfuTurbo*® Hotstart DNA and ultrapure H_2O up to a final volume of 50 µl. Negative controls contained ultrapure H_2O instead of DNA.

The mimotope PCR reactions were run on the GeneAmp® PCR System 9700 cycler using the following cycle program:

1. Initial denaturation at 95°C for 2 min
2. 30 cycles:
   Denaturation at 95°C for 30 sec
   Annealing at 58°C for 30 sec
   Elongation at 72°C for 30 sec
3. Final elongation at 72°C for 10 min

Amplification of the mimotope DNA was verified by agarose gel electrophoresis (2 % agarose gel) using 10 µl of the PCR-products as described in section 2.2.1.1.4.
Successfully amplified mimotope DNA was purified to prevent interference by non-specific DNA during the subsequent cloning procedure. Purification was performed using the NucleoSpin® Extract II kit according to the manufacturer's protocol for PCR clean-up. DNA was eluted in 30 µl NE-Elution Buffer. Concentrations of purified PCR products were assessed as previously described in section 2.2.1.1.3.

2.2.1.3 Restriction enzyme digest of mimotope and pET-GFP.II vector DNA

Restriction digests of the mimotopes and pET-GFP.II vector were performed to enable subsequent ligation into the vector-backbone. Ten µl of the purified mimotope DNA and 500 ng of pET-GFP.II, respectively, were digested separately using 1 U of FD® *Hind*III and FD® *EcoR*I in FastDigest® Buffer in a total volume of 20 µl (volume was adjusted with ultrapure H_2O). The reaction mixture containing FD® *Hind*III was incubated in a 37°C waterbath for 40 min, before FD® *EcoR*I was added and incubation was continued for another 20 min. The reaction was stopped by thermo-inactivation at 80°C for 10 min.

2.2.1.4 Ligation of mimotope fragment into pET-GFP.II vector

To enable transformation of the pET-GFP.II-mimotope plasmids into competent cells, previously digested mimotope and pET-GFP.II vector DNA was mixed together for ligation. The pET-GFP.II vector and the mimotope fragments were mixed at a molar ratio of 1:3 using approximately 3 ng of mimotope DNA per 57 ng of vector DNA. DNA mixtures were mixed with 1 U of T4 DNA Ligase and T4 DNA Ligase Buffer and were adjusted with ultrapure H_2O to a total volume of 20 µl. To prevent transformation of re-ligated pET-GFP.II

vector DNA, digested vector was linearized using FD® *Sal*I at the previously mentioned conditions for restriction digest.

2.2.2 Screening for integer pET-GFP.II-mimotope plasmids

GFP-mimotope encoding plasmids were sequenced to exclude the existence of mutations within both the mimotope and GFP-sequence, thus enabling productive expression of the GFP-mimotope proteins.

The previously ligated pET-GFP.II-mimotope plasmids were used for transformation of TurboCells™ Chemically Competent *E. coli*. Transformation was performed as described previously in section 2.2.1.1.1. Ten µl of plasmid DNA was used for transformation and single colonies were selected for subsequent plasmid purification. DNA was isolated from successfully transformed competent bacterial cells as previously described in section 2.2.1.1.2. Plasmid DNA was eluted in 50 µl EB-Buffer and concentration was assessed (see section 2.2.1.1.3).

The GFP-II-mimotope plasmids were sequenced in order to verify the integrity of the mimotope- and GFP-sequence. Sequencing was performed at the Dana-Farber Cancer Institute Molecular Biology Core Facilities (DFCI- MBCF). The reactions were prepared by mixing approximately 50 ng/µl DNA with 3.2 pmol of either the T7 forward or reverse primer (T7 promoter or T7 terminator primer, respectively) in a total volume of 12 µl before being brought to the sequencing facility. Sequencing analysis was performed using the DNAStar® Lasergene Version 8.1.5 and FinchTV Version 1.4.0. The original mimotope and GFP sequences required for analysis were obtained from Barbara Bachler, MSc. (University of Veterinary Medicine Vienna, DFCI) and Dr. Anton Sholukh (DFCI), respectively.

2.2.3 Expression of GFP-mimotope proteins

Plasmids containing integer GFP and mimotope DNA sequences were subsequently used for expression of recombinant proteins using expression competent TurboCells™ BL21 (DE3) Chemically Competent *E. coli*.

2.2.3.1 Screening for protein expressing clones

Transformation of verified plasmids was performed as previously described for TurboCells™ in section 2.2.1.1.1. Several colonies were selected from each plate and used for small-scale expression to screen for protein-expressing clones. Each colony was

transferred into 2 ml of LB-medium supplemented with 100 µg/ml of ampicillin and incubated for 14-16 h at 37°C shaking at 220 rpm. The following day, 50 µl of the culture was inoculated into each of two tubes containing 1 ml of LB-medium without ampicillin. After incubation for 2 h at 37°C with shaking at 220 rpm, protein expression was induced in one of the tubes by adding IPTG (final concentration 1 mM). The second tube was not induced and was kept as a negative control. The cultures were incubated for another 3 h under the same conditions and then centrifuged at 3000 rpm for 10 min. The pellet was resuspended in 100 µl ultrapure H_2O.

The cell suspension was subsequently used for analysis by SDS-PAGE to detect expression of the desired proteins. For this purpose, 10 µl of the bacterial cell suspension was mixed with 20 µl Laemmli Sample Buffer supplemented with β-mercaptoethanol. The mixture was boiled for 2 min to allow denaturation of the proteins and loaded onto a Mini-PROTEAN® TGX™ precast gel. The gel was run in Tris/Glycine/SDS (TGS) premixed electrophoresis buffer in a Mini-PROTEAN® Tetra Cell tank at 100 V for approximately 10 min and another 25 min at 200 V. The Page Ruler™ Plus Prestained Protein Ladder was loaded in addition to the samples to allow identification of protein band sizes. Gels were stained with Imperial Protein Stain according to the manufacturer's protocol.

2.2.3.2 Expression of GFP-mimotope proteins

Protein-expressing bacterial clones were transferred into 5 ml of LB-medium supplemented with 100 µg/ml ampicillin and incubated 14-16 h at 37°C with shaking at 220 rpm. Additionally, the mWasaby plasmid was used for expression of the GFP-protein. The following day, a 5 ml volume of each bacterial culture was added to 50 ml of fresh LB-medium without ampicillin and incubated under the same conditions as before. Incubation was done for 2-3 h before optical density (OD) was measured using the Beckman DU®640 Spectrophotometer at a wavelength of 600 nm. When an OD of 1 was reached, IPTG (final concentration 1 mM) was added and incubation was resumed for another 5 h. The cultures were then centrifuged at 10,000 rpm for 20 min using the Beckman Avanti™ J-25 Centrifuge and the pellet was used for isolation of proteins. The identity of the proteins was confirmed by loading 5-10 µg per well and performing SDS-PAGE as described in section 2.2.3.1.

2.2.3.3 Purification of GFP-mimotope proteins

Isolation of the GFP-mimotope fusion proteins from *E. coli* was performed using the Vivapure Maxiprep columns. Before use, the columns were washed with HyPure endotoxin-free water and equilibrated with Washing Buffer A, both at 500 x *g* for 2 min. (All centrifugation steps were performed with the Sorvall Legend X13 centrifuge.)
The bacterial pellet was resuspended 10 ml of SoluLyse™ supplemented with Halt™ Protease Inhibitor Cocktail to induce lysis. The lysate was centrifuged at 10,000 rpm for 20 min and the supernatant was collected. The pellet was resuspended and centrifuged one more time as described above. The combined supernatants were filtered through 0.45 mm Steriflip® filters before adding an equal volume of Diluting Buffer. The samples were then applied onto the equilibrated Vivapure columns and centrifuged at 100 *x g* for 20 min. To maximize protein binding to the column, the flow-through fraction was passed through the same column again. The columns were washed at 500 x *g* with 10 ml of Washing Buffer A and Washing Buffer B, respectively, before the GFP-mimotope fusion proteins were eluted with 10 ml of Elution Buffer at 500 x *g*. The elution step was repeated if GFP was not eluted completely. Eluted protein was subsequently transferred to PBS by three dilution-concentration steps using an Amicon®Ultra Centrifugal Filter (10K). The concentrated proteins were sterile-filtered through a 0.22 μm Millex®-GV Syringe filter, and the resulting filtrate (ca. 400-500 µl) was either transferred into endotoxin-free Eppendorf Safe-Lock Tubes and stored at 4°C, or mixed with 50 % glycerol for long-term storage at -20°C.

2.2.3.4 Assessment of protein concentration and confirmation of identity

The concentration of proteins was assessed using the Beckman DU®640 Spectrophotometer. A 20 µl protein aliquot was diluted to 1 ml with PBS and the OD was measured at the wavelength of 493 nm. The following formula was used for calculation of protein concentration:

$$\textbf{C (mg/ml)} = \frac{\text{OD (493)}}{\text{70,000 M-1 cm-1 x 1 cm}} \text{ x 32,000 x dilution}$$

Formula description:

Concentration in mg/ml; OD (493): optical density (OD) measured at 493 nm; 70,000 M^{-1} cm^{1}: molar extinction coefficient; 32,000: molecular weight of GFP

SDS-PAGE gel electrophoresis was performed to confirm the identity of the GFP-fusion proteins. 10 µg of protein in a total volume of 10 µl (adjusted with ultrapure H_2O) was mixed 1:1 with Laemmli Sample Buffer previously supplemented 1:20 with β-mercaptoethanol. Protein gel electrophoresis was performed as previously described in section 2.2.3.2.

2.2.4 Screening of monkey plasma samples

Expressed GFP-mimotope fusion proteins were analyzed by enzyme-linked immunosorbent assay (ELISA) to screen for monkey plasmas containing mimotope-specific neutralizing antibodies.

ELISA 96-well microplates were coated with 200 ng protein diluted in 100 µl Coating Buffer and incubated overnight at 4°C. Next day, the plates were washed 3 x with 300 µl of distilled H_20 per well. All washing steps were performed using the automatic ELISA-microplate washer. The 96-well plate was subsequently blocked with 200 µl of 3% PBSCT blocking buffer per well for 2 h at room temperature before being washed as previously described. 100 µl of plasma, previously diluted 1:600 with 3 % PBSCT, was added per well and incubated over night at 4°C. Next day, the plate was washed as described above and 100 µl of rabbit anti-monkey-horseradish peroxidase (HRP) secondary antibody, previously diluted 1:2000 with 3 % PBSCT, was added per well. Washing was repeated 10 times under the same conditions, before adding 100 µl OPD substrate solution per well to initiate the chemical reaction. The reaction was stopped using 100 µl 1 N H_2SO_4. The ELISA plate was read using the automated reader at 492 and 620 nm for detection of the Ab-complex and background absorption, respectively.

2.2.5 B cell isolation and amplification of variable Ig domains

Monkey plasmas bearing mimotope-specific neutralizing antibodies were chosen for subsequent isolation of B cells. Single B cells were sorted into 96-well plates and B cell RNA was reverse-transcribed. Subsequently, semi-nested PCR using the same forward primer mixture and two different reverse primers was performed for amplification of the desired immunoglobulin genes. The verified immunoglobulin genes were cloned into appropriate pFUSE-vectors for expression and subsequent co-transfection of 293F cells.

2.2.5.1 Isolation of mimotope-specific B cells

To isolate mimotope-specific memory B cells out of a heterogeneous cell mixture, FACS analysis was performed. Blood from selected monkeys was shipped from the Yerkes National Primate Research Center (YNPRC) and used for isolation of peripheral blood mononuclear cells (PBMCs). In short, blood samples were centrifuged to separate PBMCs from plasma before they were pelleted and resuspended in R15 medium for counting. Subsequently, a final concentration of 20 x 10^6 cells per ml was resuspended in PBS supplemented with 0.2 % of ultrapure bovine serum albumin (BSA).

For specific identification of memory B cells, MAbs APC-Cy7 mouse anti-human CD3, anti-CD19-PE, APC mouse anti-human CD27 and PE-Cy7 mouse anti-Human IgG were used for labeling. CD4 was labeled with MAb FITC mouse anti-human CD4 as a compensation control for green auto-fluorescing cells. Control samples were prepared for each labeled MAb separately in addition to one control containing all anti-CD MAbs and GFP and one tube containing only cells without MAbs. Five µl of each labeled mAb was used for 100 µl of cell suspension containing 2 x 10^6 cells, except in the case of anti-CD19-PE, of which a larger volume (40 µl) was used. The experimental samples were prepared in a total volume of 1 ml containing 20 x 10^6 cells per tube. 0.5 mg/ml of the previously mixed GFP-mimotope proteins was added to the cells of each sample along with 100 µl of each anti-CD mAb, except in the case of mAb anti-CD19-PE, of which 600 µl was used. No FITC mouse anti-human CD4 was added to the experimental samples.

The labeled PBMCs were subsequently brought to the Beth Israel Deaconess Medical Center (BIDMC) Sorting Facility for FACS analysis. Positively selected B cells with a $CD3^-$, $CD19^+/CD27^+/IgG^+$ and GFP^+ phenotype were sorted into separate wells of Hard-Shell® 96-Well PCR Plates. Memory B cells were loaded into 16 µl of a previously prepared reverse transcription buffer. The mixture was prepared in a total volume of 16 µl containing 4 µl of 5 x Superscript III buffer, 1.25 µl 0.1 M DTT, 0.625 µl 10 % Igepal CA-630, 1 µl RNase OUT and 9.125 µl of ultrapure H_2O. The 96-well plate containing sorted B cells was stored at -80°C until use. Dr. Anton Sholukh (DFCI) performed the labeling of infectious PBMCs with mAbs conjugated with fluorescence dyes.

2.2.5.2 Reverse transcription and amplification of Ig cDNA (RT-PCR)

Reverse transcription is commonly used to convert RNA into cDNA that can be used as template for subsequent amplification. B cells were sorted into wells containing a

reverse transcription buffer reaction for B cell lysis prior to RNA-extraction. Reverse transcription of the Ig-RNA was performed directly in the 96-well plates used for sorting. Four µl of a reverse transcription mixture containing 1 µl of 50 mM dNTPs, 1.62 µl of 50 µM random hexamers, 0.25 µl SuperScript III RT (200 U/µl) and 1.13 µl ultrapure H_2O were added to the isolated B lymphocytes. The reactions were reverse-transcribed on the C1000 Thermal Cycler using the following cycle program: 42°C – 10 min, 25°C – 10 min, 50°C – 60 min, 94°C – 5 min. After completing reverse transcription, the reactions were stored at -20°C until further use or directly used for amplification.

Subsequent semi-nested PCR was performed to amplify Ig-cDNA for γ heavy and κ or λ light chains. All three Ig-chains were amplified on separate Hard-Shell® 96-Well PCR Plates using a mixture of different forward primers for γ (Fw-mix γ), k (Fw-mix k) and λ (Fw-mix λ). Different reverse primers for PCR1 and PCR2 were used: γ- PCR1- & 2-Rev for variable heavy (VH) fragments, κ-PCR1- & 2-Rev and λ-PCR1- & 2-Rev for variable light (VL)-κ and λ fragments, respectively. (For primer sequences refer to list in Materials section).

Both PCR reactions were performed at the same conditions using 1 µl 50 mM dNTPs, 5 µl 10 x HotStarTaq Buffer, 1 µl 25 mM $MgCl_2$, 1 µl of both forward and reverse primer (working concentration: 5 pmol/µl), 0.4 µl Qiagen HotStarTaq and ultrapure H_2O in a final volume of 50 µl. Five µl of RT-reaction and 3 µl of the first PCR was used as the template for first and second PCR, respectively. The reactions were run on the GeneAmp® PCR System 9700 cycler using the following cycle program:

1. Initial denaturation at 95°C for 15 min
2. 50 cycles:
   - Denaturation at 95°C for 30 sec
   - Annealing at 54°C (γ) or 50°C (κ, λ) for PCR1 and 57°C (γ) or 52°C (κ, λ) for PCR2, respectively, for 30sec
   - Elongation at 72°C for 50 sec
3. Final elongation at 72°C for 10 min

After the second PCR, 30 µl of PCR-product was loaded onto a 1.8 % UltraPure™ Agarose gel containing 500 ng/ml ethidium bromide to verify amplification of the corresponding γ heavy chain and κ or λ light chain. Agarose gel electrophoresis (1.8 % agarose gel) was performed as described in section 2.2.1.1.4. Amplified DNA from corresponding Ig-pairs

(either γ and κ chains or γ and λ pairs) was purified directly or extracted from gels that had been previously run to cut out the individual bands. Both procedures were performed using the Macherey-Nagel gel extract kit for "DNA extraction from agarose gels" and for "PCR clean-up" according to the manufacturer's protocol. DNA was eluted in 30 µl NE-elution buffer and concentration was assessed (described in section 2.2.1.1.3).

2.2.6 Sequencing of Ig fragments

After semi-nested amplification of individual γ, κ and λ Ig chains from single sorted B cells, purified DNA was sequenced to allow selection of specific forward primers for subsequent pFUSE cloning PCR. For sequencing, the reverse primers used for PCR2 for each Ig chain were used (γ, κ and λ PCR2-Rev primers). Sequencing of PCR-fragments was performed at the BIDMC Sorting Facility. Twelve µl of undiluted DNA along with a primer dilution of 0.4 pmol/µl in a total volume of 12 µl were prepared in separate tubes.
Sequences were analyzed using the Immunogenetics (IMGT) database (www.imgt.org) as well as DNAStar® Lasergene Version 8.1.5 FinchTV Version 1.4.0, respectively for sequence alignments.

2.2.7 Cloning of Ig chains

Heavy and light chain fragments of corresponding Ig pairs were cloned into pFUSE expression vectors to purify plasmid DNA for subsequent co-transfection of 293F cells and expression of MAbs.

2.2.7.1 pFUSE cloning PCR

After sequencing, individual forward primers bearing specific cloning sites for γ, κ or λ chains, a Kozak sequence and a specific leader peptide sequence were selected. Different forward primers were used, depending on the gene family of each Ig fragment (For the different V gene families, refer to Table 2). The reverse primers Pan-Rev γ, κ and λ were used for amplification and introduction of cloning sites for each heavy and light chain.
All PCR-reactions were performed using 2 µl of the first PCR product, 1 µl of 10 mM dNTPs, 5 µl of 10x Platinum® *Taq* High Fidelity PCR Buffer, 2 µl of 50 mM $MgSO_4$, 0.2 µl Platinum® High Fidelity *Taq* DNA Polymerase and 37.81 µl ultrapure H_20 to make up a total volume of 50 µl. For each reaction, 1 µl of Pan-Rev-γ, Pan-Rev-κ or pan-Rev-λ

reverse primer (5 pmol/µl) was added according to the appropriate Ig chain as well as 1 µl of each previously selected forward primer (5 pmol/µl) (The primer sequences are listed in the Materials and Method section). The NTC contained ultrapure H_2O instead of DNA.
The PCR-reactions were run on the GeneAmp® PCR System 9700 cycler using the following cycle program:

1. Initial denaturation at 95°C for 2 min
2. 50 cycles:
   Denaturation at 95°C for 30 sec
   Annealing at 40-50°C for 30 sec
   Elongation at 72°C for 50 sec
3. Final elongation at 72°C for 10 min

Amplification of the heavy and light chains was verified by gel electrophoresis using a 1.8 % UltraPure™ Agarose gel containing 1 x SYBR® Safe DNA gel stain. 30 µl of the PCR-products was loaded onto the agarose gel as described in section 2.2.1.1.4. Desired bands were cut out of the gel and the DNA was extracted using the Macherey-Nagel gel extract kit for "DNA extraction from agarose gels". Extraction was performed according to the manufacturer's protocol. DNA was eluted in 30 µl NE-elution buffer and the concentration of extracted DNA was measured as described in section 2.2.1.1.3.

2.2.7.2 Digestion of pFUSE PCR products

Amplified heavy and light chain fragments as well as the corresponding pFUSE vectors were digested to enable subsequent ligation of the fragment with the corresponding vector backbone. The γ heavy chain and the corresponding vector pFUSE-CHIg-hG1 were digested with FD® *Nhe*I and FD® *EcoR*I. The κ light chain and corresponding vector pFUSE2-CLIg-hk were digested with FD® *Age*I and FD® *BsiW*I. The λ light chains and the corresponding vector pFUSE2-CLIg-hl2 were digested with FD® *Age*I and FD® *Avr*II. 200 ng of Ig fragment DNA was digested in a reaction containing 2 µl of 10x FD® Buffer, 1 U of each FD® restriction enzyme and ultrapure H_20 in a final volume of 20 µl. Digestion of the pFUSE vectors was performed using 2 U of the corresponding enzymes, except for FD® *Nhe*I, of which 3 U were added to the digestion mixture.
The digestion reaction was performed at 37°C for 20 min, followed by heat inactivation at 80°C for 5 min. Subsequently, the reaction was cleaned up using the Macherey-Nagel DNA extract kit for "PCR clean-up" according to the manufacturer's protocol. DNA was

eluted with 30 µl NE elution buffer and the DNA concentration of the digestion reaction was measured (see section 2.2.1.1.3).

2.2.7.3 Ligation of Ig fragments into pFUSE vectors

Previously digested heavy and light chain fragments as well as pFUSE vector DNA were subsequently used for ligation of the Ig fragments into the vector backbones. The ligation reaction was mixed using a molar ratio of vector to insert of 1:3, meaning that 3.6 ng vector DNA per 1 ng of Ig fragment DNA was used. The reaction was adjusted with T4 DNA Ligase Buffer, 1 U of T4 DNA Ligase and ultrapure H_20 up to a final volume of 20 µl. After overnight incubation, the T4 DNA Ligase was heat-inactivated at 80°C for 5 min before adding either FD® *Xho*I or FD® *Nco*I to the heavy and light chain containing reactions, respectively. An additional 20 min incubation time at 37°C was used to ensure linearization of re-ligated vector DNA and prevent transformation of pFUSE vectors lacking Ig DNA. As in the other experiments, the enzymes were thermo-inactivated at 80°C for 5 min upon completion of the reaction.

2.2.7.4 Transformation of integer pFUSE plasmids

For transformation of ligation mixtures OneShot® Top10 Chemically competent *E. coli* were used. The manufacturer's transformation protocol was followed with a few differences. Briefly, 10 µl of plasmid DNA was added to 50 µl of competent cells and the mixture was kept on ice for 30 sec followed by heat-shock at 42°C for 30 sec and ice-cooling for another 2 min. 250 µl of Super Optimal broth plus glycose (S.O.C.) medium was added to each vial before allowing the cells to divide for 1 h at 37°C with shaking at 225 rpm. Transformation reactions were quickly spun down for 10 sec before removing 200 µl of the medium. The cells were resuspended in the residual 100 µl of S.O.C. medium and the whole volume was plated onto previously prepared Terrific Broth (TB) agar plates.

TurboCells™ Chemically Competent *E. coli* were used for transformation of purified plasmid DNA. The procedure was performed according to the manufacturer's protocol (see section 2.2.1.1.1). One µl of plasmid DNA was used for transformation and 100 µl of S.O.C. medium containing transformed cells was plated onto TB agar plates.

For the transformation of the heavy chain plasmid pFUSE-CHIg-hG1 containing the cloned γ chain fragment, TB agar plates containing the antibiotic zeocin were used, whereas for transformation of the pFUSE2-CLIg-hk and pFUSE2-CLIg-hl2 plasmids containing κ or λ

fragments, respectively, blasticidin-containing agar plates were used. The plates were incubated for 14-16 h at 37°C before selecting single colonies for plasmid purification.

2.2.7.5 Purification and sequencing of pFUSE plasmid DNA

Colonies from transformed OneShot® competent cells were grown in 1.5 ml of TB medium containing either zeocin (zeocin-TB) or blasticidin (blasticidin-TB), depending on the Ig chain, at 37°C with shaking at 250 rpm for 7 h. Plasmid DNA was isolated using the NucleoSpin® Plasmid kit according to the manufacturer's protocol and eluted in 30 µl AE-elution buffer.

Colonies from transformed competent TurboCells™ were grown in 30 ml of TB medium (either zeocin- or blasticidin- TB) for 14-16 h, before DNA was purified using the standard protocol for DNA isolation of the QIAGEN Plasmid Midi kit and was eluted in 150 µl EB elution buffer. DNA concentration of all plasmids was assessed as described in section 2.2.1.1.3.

Purified plasmid DNA from both transformation reactions was sequenced at the DFCI-MBCF sequencing facility. For this purpose, 12 µl of undiluted DNA were mixed with 0.65 µl of 5 µM Pan-pFUSE-seq-Fw forward primer before being sent for sequencing.

2.2.8 Expression of MAbs and characterization

2.2.8.1 Co-transfection of 293F cells with Ig plasmid DNA

Purified plasmid DNA from heavy chains and the corresponding κ or λ light chains was co-transfected into 293F cells, a suspension cell line derived from human embryonic kidney cells. Co-transfection was performed in 6-well plates containing 1 x 10^6 293F cells in a total volume of 2 ml per well. The *Trans*IT-PRO® Transfection Kit was used for transfection. Plasmid DNA was prepared by mixing heavy and light chain plasmid DNA at a molar ratio of 1:1. The plasmid DNA mixture was added to 200 µl of serum-free GIBCO®FreeStyle™ 293 Expression Medium. Subsequently, 2 µl of *Trans*IT-PRO and 1 µl of PRO Boost Reagent were added per transfection reaction before incubating the reaction for 30 min at room temperature to allow DNA complex formation. For the negative control, the transfection reagents were mixed with Free Style™ 293 Expression Medium without DNA. Each transfection mixture was then added to single wells of previously seeded 293F cells and incubated in the Reach-in CO_2. Incubator for 72 h at 37°C and 8 % CO_2 shaking in the Forma Benchtop Orbital Shaker at 130 rpm. The cell supernatant containing MAbs

was harvested by centrifuging the cell suspension in 15 ml BD Falcon™ conical tubes at 3000 rpm for 10 min at 4°C using the GS-6R Centrifuge. Twenty µl of penicillin/streptomycin (stock concentration: penicillin: 10,000 U, streptomycin: 10,000 µg) and 2 µl of the 100 x Halt™ Protease Inhibitor Cocktail were added to the cell free supernatant before filtration through a 0.2 µm Corning® Syringe Filter and a BD Luer-Lok™ 3 ml Syringe. The MAbs were stored at + 4°C until use.

2.2.8.2 Testing of expression of MAbs

The expression of MAbs was tested by SDS-PAGE. Twenty µl of the harvested transfection supernatant along with 200 ng of the mAb 2G12 were mixed separately with 20 µl Laemmli Sample Buffer and boiled for 2 min and before loading 30 µl of the mixture onto a 7.5 % Mini- PROTEAN® TGX™ precast gel. The mAb 2G12 was loaded to compare its size to the expressed MAbs. Ten µl of Page Ruler™ Plus Prestained Protein Ladder were loaded as a reference.

The gel was run in the Mini-PROTEAN® Tetra Cell tank in TGS buffer at 100 V for 20 min and 200 V for another 20 min before treating it with Imperial Protein Stain for 5-12 h. Destaining of the gel was performed with distilled H_2O for 5-7 h.

2.2.8.3 Testing of binding capabilities of MAbs

The expressed MAbs were tested for their binding capabilities to the mimotopes used for isolation of B cells. The procedure for ELISA is described in section 2.2.4.

The 96-well microplate was coated with 200 ng per well with the GFP protein, the GFP-mimotope fusion proteins and the gp41 (HxB2) protein before incubated overnight at 4°C. Next day, the MAbs and plasma samples were diluted 1:2 and 1:600, respectively, with PBSCT 3 % to a total volume of 100 µl before overnight incubation. Two different secondary Abs were used for detection, namely the rabbit-anti-monkey-HRP for detection of the MAbs and the goat-anti-human-HRP for detection of nAbs in the plasma samples. The chemical reaction was initiated by OPD as previously described and read at 492 and 620 nm (see section 2.2.4)

3 Results

3.1 Background of the study

In the course of a vaccine study, the rhesus macaques RVe-10 and RTr-11 were challenged with SHIV-1157ipEL-p. One of these monkeys, RTr-11, was subject of another study, during which mimotopes were isolated out of the plasma of this monkey (Bachler B., unpublished data). The observed mimotopes included those mimicking regions within the viral gp41, expressed upstream of the previously published Kennedy-mimotope on the viral gp41 glycoprotein (CHANH et al., 1986; KENNEDY et al., 1986). These Kennedy-proximal mimotopes are hypothesized to be important for neutralization.

In this study, we sought to isolate antigen-specific B cells from the plasma of RM RTr-11 and RVe-10 in order to express recombinant mimotope-specific antibodies targeting these mimotopes.

3.2 Cloning of mimotopes into pET-GFP.II vector

3.2.1 Amplification of pET-GFP.II vector and mimotope DNA

Mimotope and pET-GFP.II vector DNA was amplified to increase the initial yield of DNA for use in subsequent cloning procedures. For this purpose, we purified vector DNA from transformed cells and verified its identity by restriction enzyme digest to cut out the previously cloned GFP-sequence.

Figure 10 A displays the characteristic digestion pattern with the upper band representing the empty vector backbone and the lower band with ~800 base pairs (bp) displaying the GFP-fragment.
Mimotope DNA was amplified by PCR, during which at the same time restriction enzyme sites for subsequent cloning were introduced. Successfully amplified bands with ~100 bp are displayed in Figure 10 B.

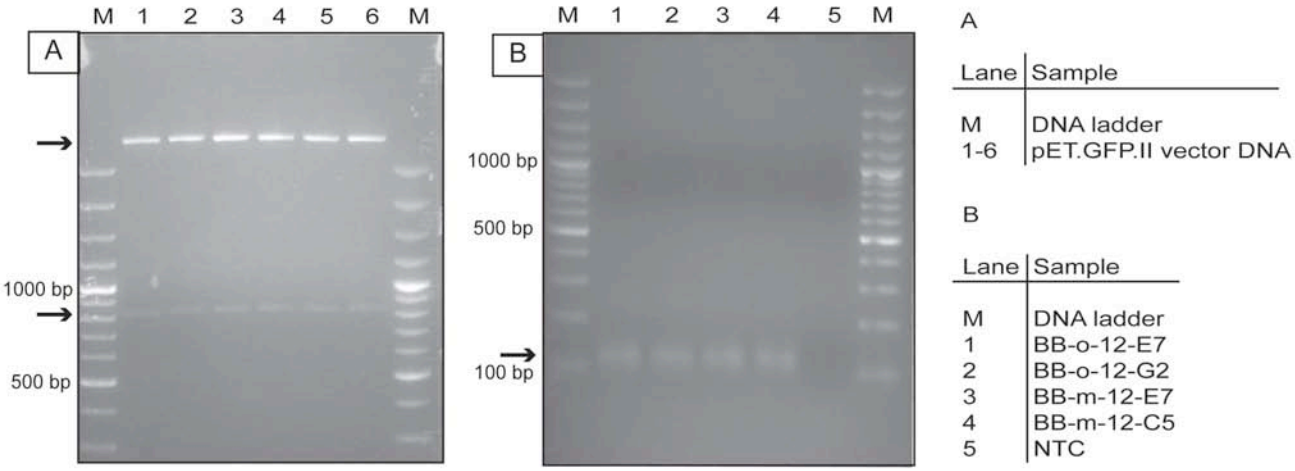


Figure 10: Amplification of pET-GFP.II-vector and mimotope DNA
A) Amplification of pET-GFP.II vector DNA. The digested vector-backbone and GFP-protein are indicated with arrows. B) The amplified mimotope DNA is indicated with an arrow. NTC= no template control

Amplified vector and mimotope DNA were then transformed into competent cells and 1-4 colonies from each transformation were collected.

3.2.2 Screening for integer pET-GFP.II-mimotope plasmids

Next, we sought to screen for integer pET-GFP.II-mimotope plasmids to express GFP-mimotope fusion proteins. Plasmid DNA was isolated from the previously collected colonies and was subsequently sequenced.

We analyzed the sequences by aligning the original mimotope and GFP sequences with those obtained after the cloning procedure. We verified the integrity of the GFP and mimotope sequences for each of the mimotopes and denoted the number of the clones at the end of each mimotope name as follows:

- BB-o-12-E7.2, BB-o-12-G2.2, BB-m-12-E7.1, BB-m-12-C5.1

3.3 Expression of GFP-mimotope proteins

The previously verified plasmids were used to express recombinant GFP-mimotope fusion proteins. Expression of the desired fusion proteins was performed using competent cells that express the T7 RNA polymerase, required to initiate transcription of the desired genes. Upon addition of isopropyl-β-D-thiogalactopyranosid (IPTG), the T7 RNA polymerase is induced, allowing the expression of the desired proteins.

3.3.1 Screening for GFP-mimotope protein-expressing clones

To find a clone that expresses the GFP-mimotope fusion proteins upon induction, multiple clones had to be screened. For this purpose, we transformed competent cells with plasmid DNA and tested approximately four colonies per transformation for their protein expression capabilities. Each tested clone was cultured in two separate tubes; one containing IPTG and the other used as negative control lacking IPTG.

BB-o-12-E7.2, BB-o-12-G2.2, BB-m-12-E7.1 and BB-m-12-C5.1 exhibited clones with increased GFP-mimotope protein expression upon induction. Figure 11 displays the clones of each plasmid, exhibiting a significant difference in protein expression upon IPTG induction (+, ~35 kDa) compared to the non-induced controls (-). The clones that were chosen for high yield protein expression are encircled.

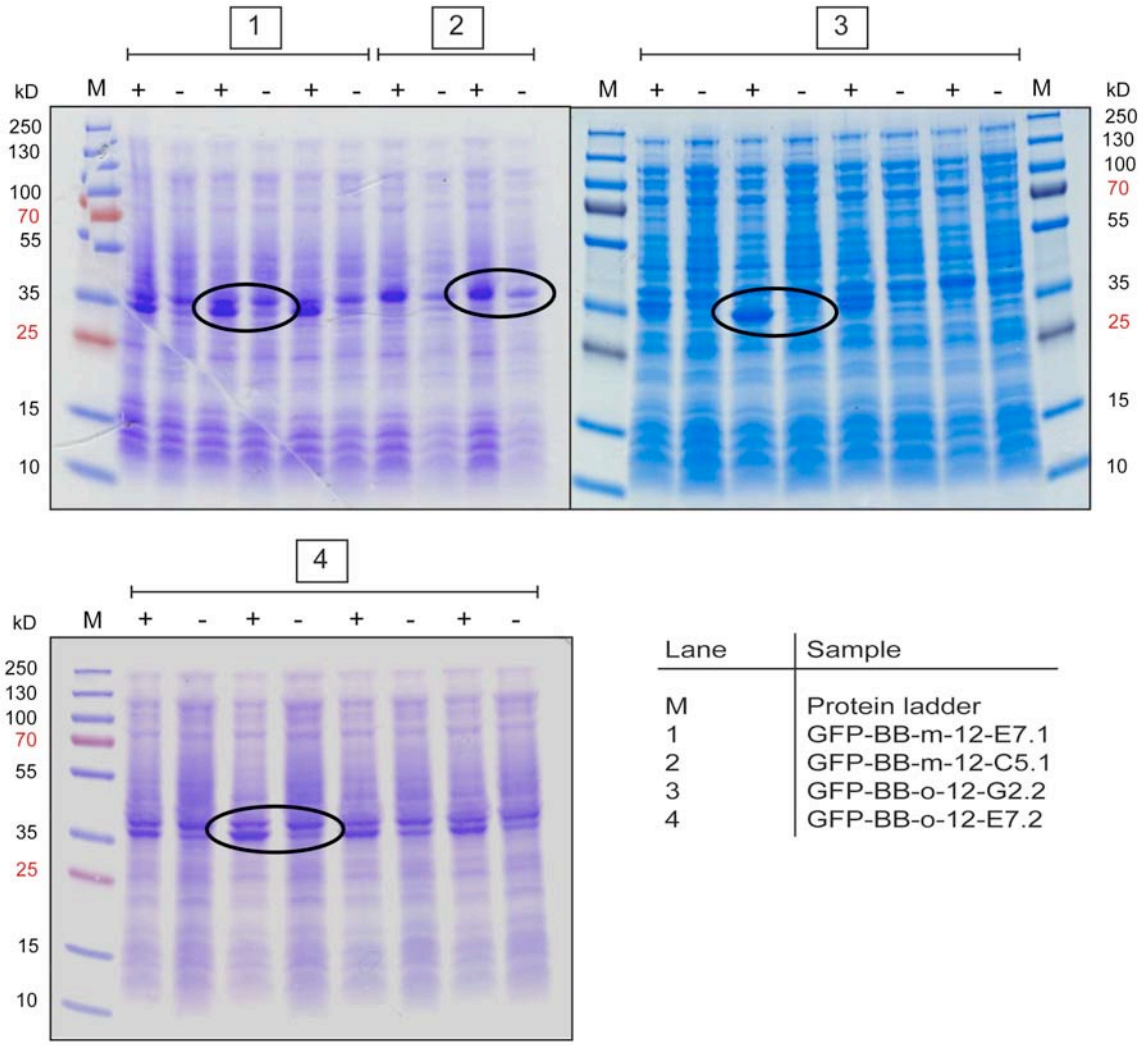


Figure 11: Screening for GFP-mimotope protein expressing clones

The induced expression of GFP-mimotope proteins is shown. Several clones per transformation were tested for each pET-GFP.II-mimotope plasmid. For each clone, two reactions were prepared, including an induced (+) sample and a non-induced negative control (-). Clones with a significant induction of protein expression are encircled.

3.3.2 Expression and purification of GFP-mimotope proteins

After we identified protein-expressing clones, high yields of GFP-mimotope proteins were expressed in order to screen for RMs bearing binding Abs against the mimotope motifs. Previously selected clones (encircled in Figure 11) were cultured in large amounts and induced with IPTG, before they were purified.

Figure 12 displays the expression of the desired GFP-mimotope proteins GFP-BB-m-12-E7.1, GFP-BB-m-12-C5.1, GFP-BB-o-12-E7.2 and GFP-BB-o-12-G2.2 as well as the recombinant protein GFP.

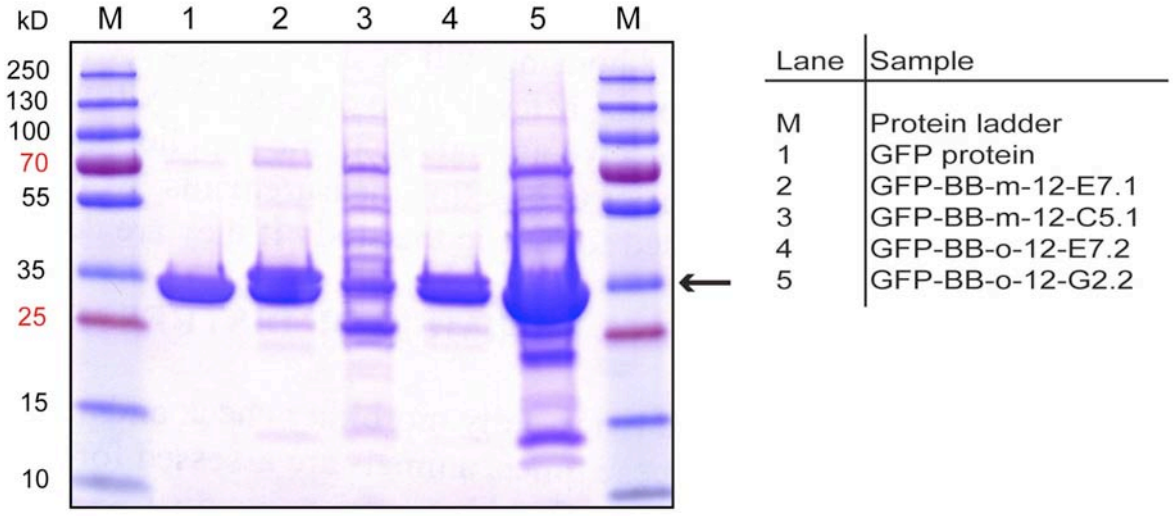


Figure 12: Expressed GFP-mimotope proteins
Expressed GFP-mimotope proteins (~34 kD) are indicated with an arrow.

3.4 Screening of RM plasma samples for binding Abs

The next step was to find RMs bearing Abs that specifically target the mimotope motifs presented by the GFP fusion proteins. Thus, we tested plasma samples from multiple RMs by ELISA. This method is based on specific binding of antibodies to their corresponding antigens and subsequent visualization by a chemical reaction between enzyme-conjugated antibody and substrate.

3.4.1 Testing of GFP-mimotope protein reactivity

Initially, we asked whether the recombinant GFP-mimotope proteins were recognized by Abs present in plasma samples from the RM RTr-11, from which the mimotope-motifs had been isolated. Several plasma samples from this monkey were tested for the presence of mimotope-specific Abs including plasma drawn before the first vaccination (pre-immune serum; June 10, 2008) as well as before the first low-dose of challenge (DOC; May 27, 2009) and the high-dose challenge (HDC; July 15, 2009) with SHIV-1157ipEL-p. We coated ELISA plates with the expressed GFP-mimotope fusion proteins and added the plasma samples before the reaction was visualized.

The mimotope motifs presented on the recombinant GFP-mimotope proteins GFP-BB-o-12-E7.2 and GFP-BB-m-12-E7.1 displayed significant reactivity with the DOC plasma sample indicating the presence of high titers of specific Abs. However, the level of mimotope-specific Abs seemed to decrease in the course of the vaccine study, as the reactivity with the GFP-mimotope proteins was lower at the day of HDC compared to that on the DOC. The GFP-mimotope proteins GFP-BB-o-12-G2.2 and GFP-BB-m-12-C5.1 did not exhibit any reactivity with the tested plasma samples and therefore were excluded from further work (Figure 13). The pre-immune plasma control was negative correlating with the assumption that it should not contain any binding Abs against the mimotope motifs. GFP was used as second negative control to exclude plasma samples showing reactivity against it.

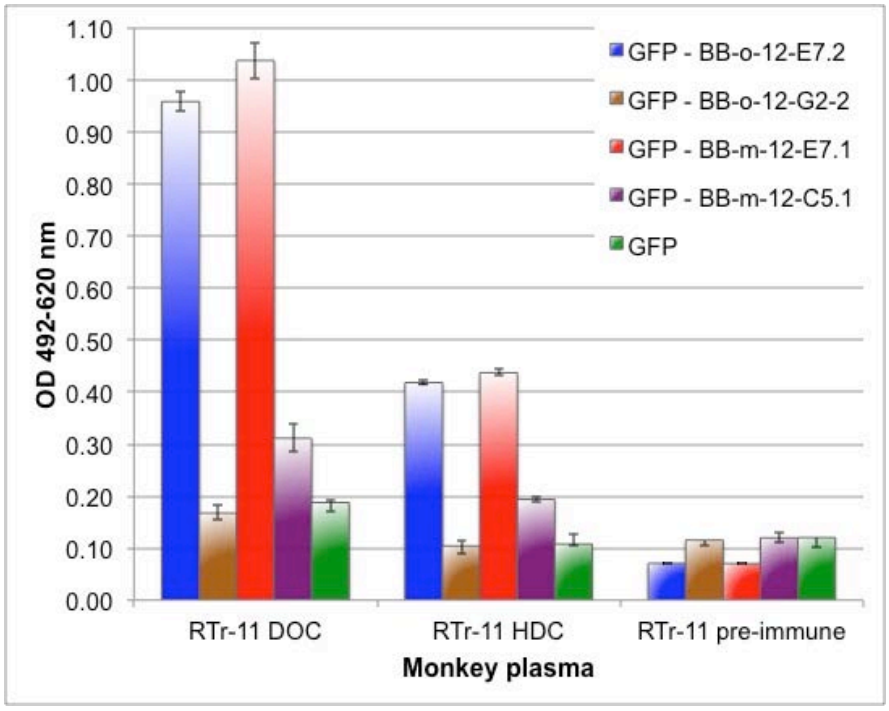


Figure 13: Reactivity of RTr-11 plasma against Kennedy-proximal mimotopes
Different plasma samples from RTr-11 (DOC, HDC, pre-immune) were tested for the presence of specific Abs against the mimotope motifs from BB-o-12-E7.2, BB-m-12-E7.1, BB-o-12-G2.2 and GFP-BB-m-12-C5.1. The pre-immune plasma and GFP served as negative controls for binding. The ELISA reaction was read at 492-620 nm.

3.4.2 Screening of RMs with mimotope-specific Abs

After we confirmed the reactivity of the fusion proteins GFP-BB-o-12-E7.2 and GFP-BB-m-12-E7.1 with plasma from RTr-11 drawn at the DOC (Figure 13), we screened for other monkeys containing mimotope-specific Abs. An ELISA was performed as described before by coating plates with the GFP-mimotope fusion proteins and adding plasma samples of each monkey.

A set of 26 RMs was altogether tested for the presence of specific Abs (data not shown). We found four monkeys, RRi-11, RDc-11, REc-11 and RVe-10, showing reactivity to the recombinant fusion proteins in addition to RTr-11. As shown earlier, RTr-11 exhibited high binding of the fusion proteins GFP-BB-o-12-E7.2 and GFP-m-12-E7.1 at the DOC (Figure 13 and Figure 14);

however, there was no detectable presence of mimotope-specific Abs in the most recently isolated plasma (February 2, 2011). The RM RDc-11 displayed increased binding six weeks post-DOC (June 1, 2009) compared to the DOC (June 1, 2009). Monkeys REc-11 and RVe-10 exhibited high binding titers at their days of challenge (June 1, 2009 and June 2, 2009, respectively) for both GFP-mimotope proteins; however, binding decreased six weeks after the DOCs (July 13, 2009 and July 14, 2009, respectively) and was not detectable in the most recently drawn plasma samples (February 22, 2011 and May 19, 2010, respectively) (Figure 14). The naïve serum sample is comprised of pooled naïve sera from different monkeys and served as negative control for the ELISA. Additionally, pre-immune plasma samples were used as negative controls for each RM, if available.

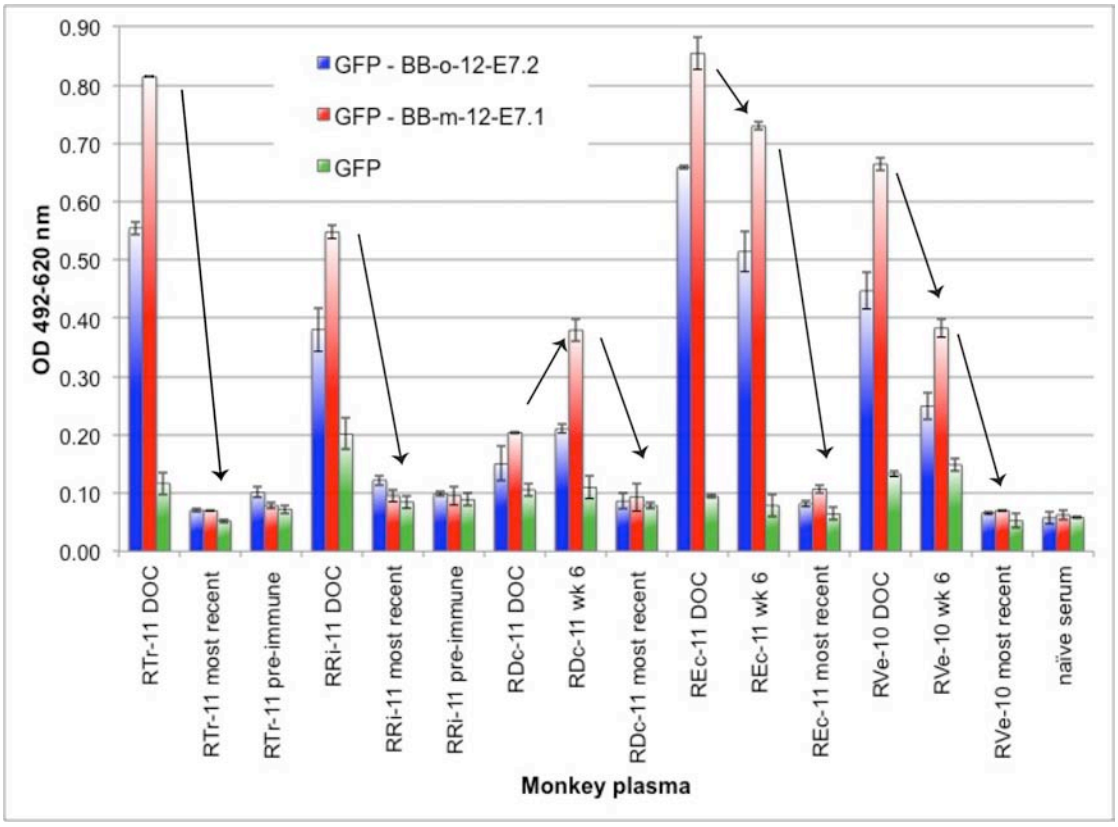


Figure 14: Screening for RM plasma samples containing mimotope-specific Abs
The RMs RTr-11, RRi-11, RDc-11, REc-11 and RVe-10 were tested for the presence of specific Abs against the mimotope motifs BB-o-12-E7.2 and BB-m-12-E7.1. Naïve serum and pre-immune samples served as negative controls. The ELISA reaction was read at 492-620 nm.

The RMs RTr-11, RRi-11 as well as REc-11 and RVe-10 were of particular interest for subsequent isolation of B cells, as their plasma contained mimotope-specific Abs. However, as we were limited in time, we selected two of these monkeys. One of them was RM RTr-11, as it was the monkey, from which the Kennedy-proximal mimotopes were isolated. The second monkey, RVe-10, was chosen randomly as there was no significant difference in the binding of the GFP-mimotope proteins compared that of RRi-11 and REc-11.

3.5 B cell isolation and amplification of Ig domains

3.5.1 Isolation of mimotope-specific B cells

Antigen-specific B cells were isolated using fluorescence activated cell sorting based upon their specific light scattering and fluorescence. Briefly, a heterogeneous cell mixture is labeled with multiple MAbs tagged with different fluorescent dyes. This enables identification of a particular cell type based on the binding of labeled MAbs to specific cell surface antigens and subsequent detection of their fluorescence activity.

Blood samples from the RMs RTr-11 and RVe-10 were drawn in April 2011 and PBMCs were isolated. Next, the surface antigens CD3, CD19, CD27, IgG and CD4 were labeled using MAbs conjugated with different fluorescent dyes, before recombinant GFP-mimotope proteins were added to the cell mixture. Using this labeling strategy, we specifically selected memory B cells that recognized the GFP-mimotope proteins displaying a $CD3^-$, $CD19^+/CD27^+/IgG^+$ and GFP^+ phenotype to sort them into single wells.

The following analysis performed for RTr-11 represents the sorting strategy for both monkeys. (The sorting analysis for RM RVe-10 is listed in the Appendix. Figure 21) Gating of specific memory B cells was performed by first selecting lymphocytes out of the heterogeneous PBMC population (Figure 15 A, P1). Subsequently, single cells were separated from cell doublets (Figure 15 B & C, P2 and P3), followed by

selection of $CD3^-$ B lymphocytes out of a heterogeneous mixture of $CD3^+$ T and $CD3^-$ B lymphocytes (Figure 15 D, P4). The next gating step specifically selected $CD27^+/CD19^+$ positive B cells (Figure 15 E, P5), followed by the selection of GFP^+/IgG^+ memory B cells (Figure 15 F, P6). To exclude auto-fluorescent cells from sorting, CD4-presenting PBMCs were labeled as fluorescence compensation controls. Additionally, a control sample lacking GFP-mimotope proteins, but including recombinant GFP was set up to discriminate auto-fluorescence (Figure 15 H).

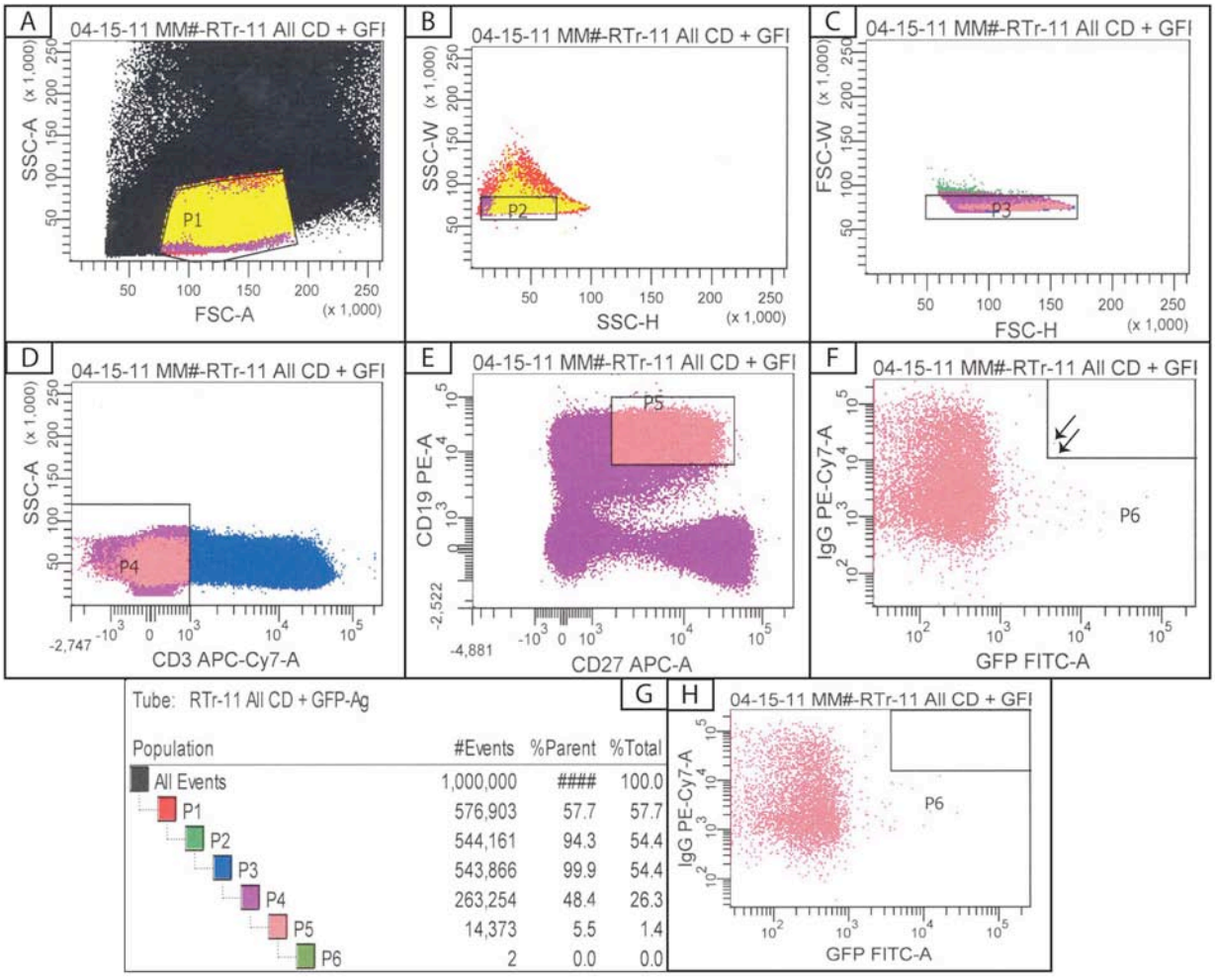


Figure 15: Sorting strategy for RM RTr-11

B lymphocytes were selected out of a heterogeneous PBMC cell mixture (A, P1) as well as out of cell doublets (B, P2 and C, P3). Memory B cells showing a $CD3^-$ (D, P4), $CD27^+/CD19^+$ (E, P5) and IgG^+/GFP^+ (F, P6) phenotype were selected. FITC labeling of CD4 (H, P6) was used to discriminate between GFP and auto-fluorescence.

Figure 15 displays the sorting results for $1x10^6$ PBMCs exhibiting two mimotope-specific memory B cells in P6 (Figure 15 F with arrows and G). The numbers of PBMCs analyzed for RTr-11 and RVe-10 were 63 x 10^6 and 67 x 10^6, respectively. Finally, 29 and 25 mimotope-specific memory B cells were isolated for RTr-11 and RVe-10, respectively.

3.5.2 RT-PCR of Ig variable domains

Next, we amplified variable domains of immunoglobulin genes from single sorted memory B cells to sequence and subsequently clone them into expression vectors. Single B cells isolated in wells of a 96-well plate were used for reverse transcription of Ig heavy and light chain mRNA into cDNA gene transcripts. The synthesized cDNA of Ig heavy chains and both Ig light chains (κ or λ) was then amplified by semi-nested PCR in three separate plates. Because the V genes of the γ, κ and λ Ig chains consist of different gene families, we used forward primers mixtures for the respective of V_H, Vκ and Vλ framework region 1 (FR1) regions. The reverse primers for first and second PCR of each particular Ig chain annealed at C_H, Cκ and Cλ regions.

Figure 16 exemplifies the amplification of Ig chain fragments for RVe-10 as selection strategy for both monkeys (The results for RTr-11 are shown in the Appendix; Figure 22). Each gel displays amplification of either the γ, κ or λ chain fragment. A matching Ig pair displays an amplified γ heavy chain and either the κ or λ light chain from the same B cell. Figure 16 shows the successful amplification of four corresponding Ig pairs for RVe-10. Two pairs comprised of γ heavy and κ light chains are circled in red, whereas the two pairs consisting of γ heavy and λ light chain fragments are indicated with yellow circles. A summary of the successfully amplified matching Ig pairs and the designated sorting numbers for RTr-11 and RVe-10 are shown in Table 1.

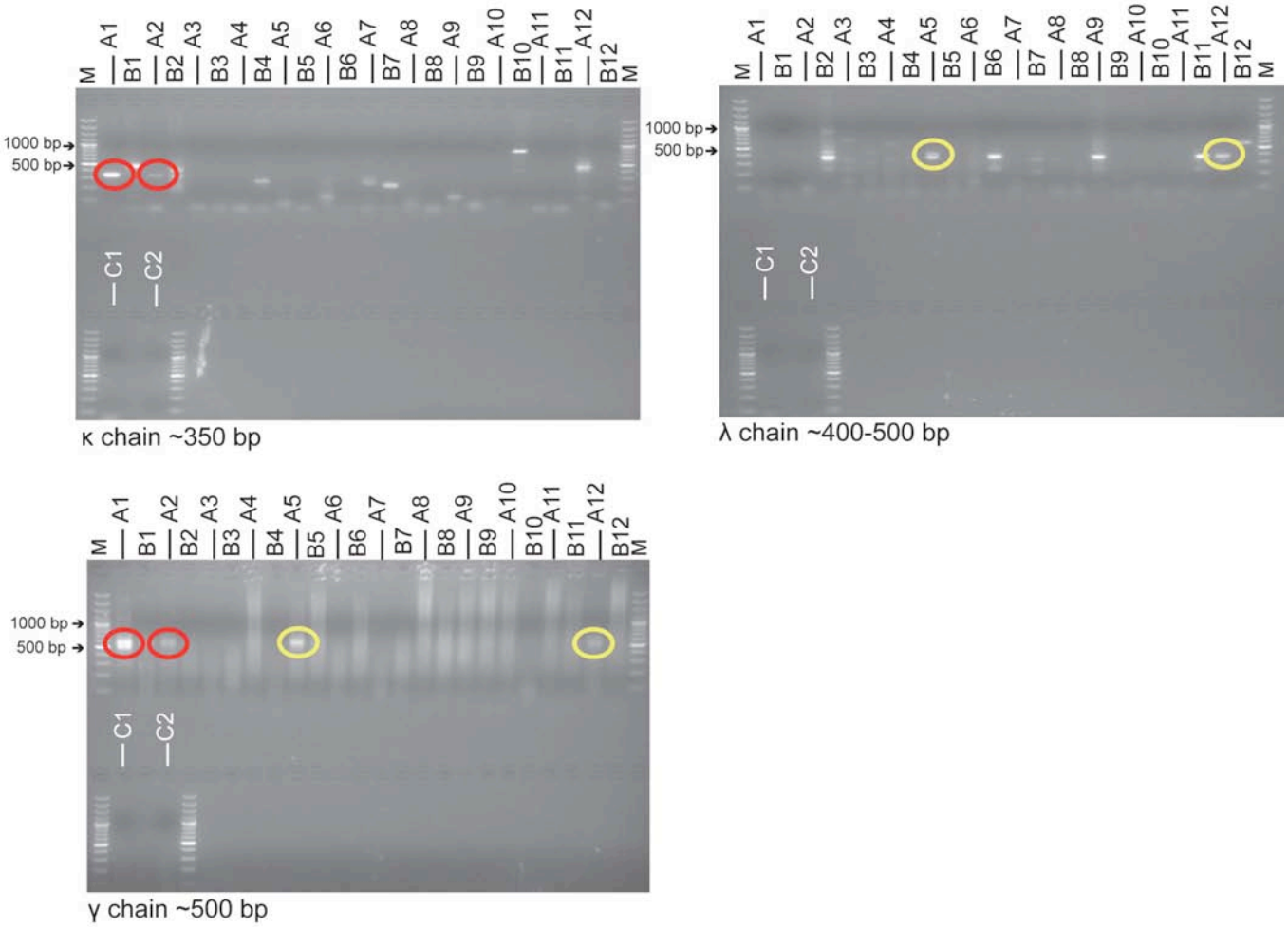


Figure 16: Ig fragments from RM RVe-10 amplified after RT-PCR

The amplification of Ig chain fragments from each single sorted B cell from RM RVe-10 is shown. Corresponding γ heavy and κ light chains are circled in red. The yellow circles indicate Ig pairs exhibiting γ heavy and λ light chains. The letter and number combinations (A1-A12, B1-B12) refer to the well positions on the 96-well plates where the particular chain was amplified.

Table 1. Ig pairs for RMs RTr-11 and RVe-10 after RT-PCR

Ig fragments are designated according to their sorting number and location on the 96-well plate.

Matching Ig-pairs	heavy chain	light chains
RVe-10 (Sorting 35)	35A1-γ	35A1-κ
	35A2-γ	35A2-κ
	35A5-γ	35A5-λ
	35A12-γ	35A12-λ

Matching Ig-pairs	heavy chain	light chains
RTr-11 (Sorting 36)	36A5-γ	36A5-λ
	36A9-γ	36A9-κ
	36A12-γ	36A12-κ
	36B5-γ	36B5-κ
	36B12-γ	36B12-κ

The matching Ig pairs listed in Table 1 were used for subsequent sequencing analysis to select integer Ig pairs for expression of monoclonal antibodies (MAbs).

3.6 Sequencing analysis of Ig fragments

Ig fragments from matching pairs were sequenced to identify their immunoglobulin families and enable selection of forward primers for pFUSE cloning PCR. Sequencing of individual Ig chains was performed using the same reverse primers as in the second PCR reaction. We analyzed the RM Ig sequences using the database IMGT and compared them to human Ig sequences. Comparison to human Ig sequences was done, because of the high availability of human Ig sequences and the high degree of Ig genome similarity to RMs (KUWATA et al., 2011). Table 2 summarizes the V and J gene families, the degree of identity to the human germline sequence and productivity of each Ig fragment. The designation of gene family is exemplified by the V gene IGHV3-66*02 of 35A1- γ:

- IGH: Ig heavy chain; V3: V gene family; 66: subfamily; 02: allele location.

The analysis of most of the γ heavy chains fragments resulted in productive and rearranged fragments, whereas most of the light chains displayed unproductive rearrangement (Table 2). Three Ig pairs, 36A12-γ/36A12-κ, 36B12-γ/36B12-κ and 36B5-γ/36B5-κ, were excluded from further work, as the light chains could not be identified by IMGT analysis. However, the residual Ig pairs, listed in Table 2, were selected for subsequent cloning. We used all Ig fragments for subsequent cloning, regardless of the productivity and rearrangement resulting from IMGT analysis, as errors during sequencing of the PCR fragments could have lead to the productivity state.

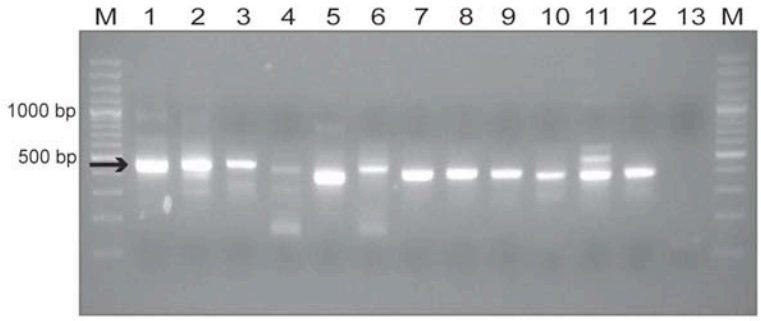
Table 2. Sequence analysis of Ig heavy and light chain fragments

Ab name	V gene	Identity	J gene	Identity	Productivity & arrangement
RVe-10:					
35A1-γ	IGHV3-66*02	93 %	IGHJ3*01	86 %	productive & rearranged
35A1-κ	IGKV1-17*02	77 %	-	-	no rearrangement
35A2-γ	IGHV3-43*02	83 %	IGHJ4*02	65 %	productive & rearranged
35A2-κ	IGKV1-27*01	76 %	IGKJ2*04	40 %	rearranged (no junction)
35A5-γ	IGHV4-4*07	74 %	IGHJ3*01	78 %	productive & rearranged
35A5-λ	IGLV3-9*01	85 %	IGLJ3*02	68 %	unproductive &rearranged
35A12-γ	IGHV4-4*02	73 %	IGHJ5*02	84 %	productive & rearranged
35A12-λ	IGLV3-21*01	89 %	IGLJ1*01	62 %	productive & rearranged
RTr-11:					
36A5-γ	IGHV3-48*03	48 %	-	-	no rearrangement
36A5-λ	IGLV2-14*01	89 %	IGLJ1*01	61 %	unproductive & rearranged
36A9-γ	IGHV3-h*01	91 %	IGHJ3*01	90 %	productive & rearranged
36A9-κ	IGKV1-6*01	87 %	IGKJ4*01	61 %	unproductive & rearranged
Excluded					
36A12-γ	**36B12-γ**	**36B5-γ**			
36A12-κ	**36B12-κ**	**36B5-κ**			

3.7 Cloning of Ig chains into pFUSE vectors

We used the obtained sequences and the information of the gene families to find annealing forward primers for pFUSE cloning PCR. Each forward primer was comprised of a restriction site, a Kozak and heavy or light chain Ig leader peptide sequence at its 5' end and was specific for the individual Ig V gene. The reverse primers, also containing a restriction site for subsequent cloning, annealed at the conserved intersection between the J and C gene of each γ, κ or λ Ig fragment.

All γ, κ and λ chains were successfully amplified (Figure 17), exhibiting bands with a size of 400-500 bp. The negative control (no template control NTC) did not display any band.



Lane	Sample	Lane	Sample
M	DNA ladder	7	35A1-κ
1	35A1-γ	8	35A2-κ
2	35A2-γ	9	36A9-κ
3	35A5-γ	10	35A5-λ
4	35A12-γ	11	35A12-λ
5	36A5-γ	12	36A5-λ
6	36A9-γ	13	NTC

Figure 17: pFUSE cloning PCR of Ig fragments
Ig variable fragments of each γ, κ and λ Ig chain were amplified. Bands with 400-500 bp are indicated with the arrow. NTC: no template control

We cloned the amplified pFUSE PCR fragments into their corresponding pFUSE expression vectors. The plasmids were sequenced to rule out the possibility of mutations that might have occurred during the cloning procedure. The sequences of the Ig V regions were compared to the original sequences that were obtained after semi-nested PCR. Further, the integrity of the leader sequence and the constant plasmid regions was verified. We were able to verify the integrity of four Ig pairs, 35A1 (γ and κ), 35A12 (γ and λ), 36A5 (γ and λ) and 36A9 (γ and κ) compared to their original sequences after RT-PCR (data not shown).

Because of the analysis in Table 2 showing unproductive rearrangements of the each γ, κ and λ Ig V fragments, we analyzed the newly obtained sequences again using the IMGT database. We were able to confirm the gene families from the last analysis and also verified the productive rearrangement of the Ig fragments after cloning, except for 36A5-γ, for which the not rearranged state was confirmed (see Table 2). Table 3 summarizes the verified Ig pairs that were considered eligible for the expression of MAbs.

Table 3. Ig pairs for expression of MAbs

RVe-10 (Sorting 35)			
35A1-MAb		**35A12-MAb**	
35A1-γ	35A1-κ	35A12-γ	35A12-λ

RTr-11 (Sorting 36)			
36A5-MAb		**36A9-MAb**	
36A5-γ	36A5-λ	36A9-γ	36A9-γ

3.8 Expression and characterization of MAbs

3.8.1 Expression and detection of MAbs

In the next step, we used the verified Ig pairs listed in Table 3 to express mimotope-specific MAbs and characterize their binding capabilities. For this purpose, we co-transfected 293F cells using plasmid DNA from the γ heavy chain and the corresponding κ or λ light chain. The heavy and light chain DNA were mixed in a molar ratio of 1:1 and successful expression was achieved with three out of four MAbs, namely 35A1, 35A12 (both isolated from RM RVe-10) and 36A9 (isolated from RTr-11). No expression was detected in the case of 36A5 (Figure 18).

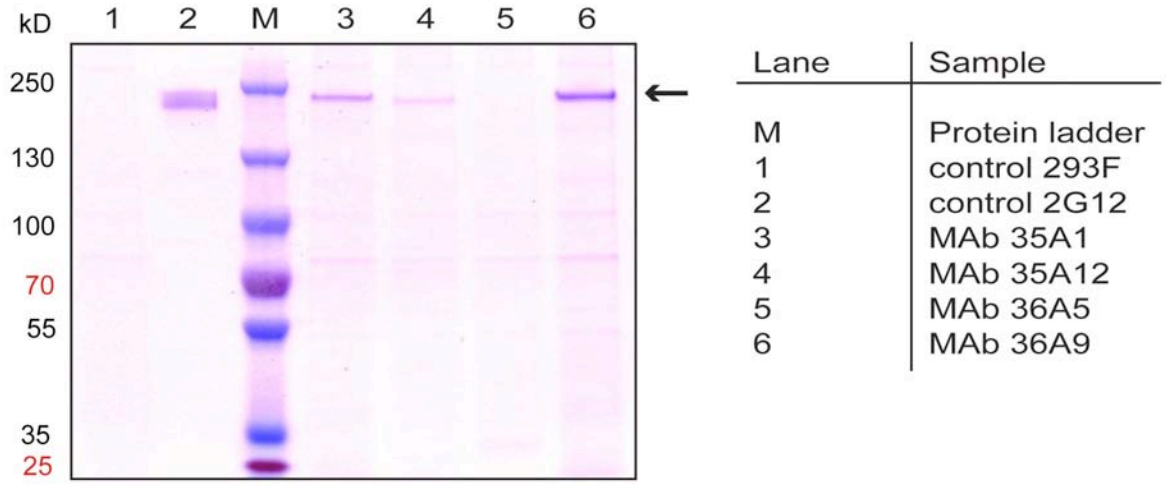


Figure 18: Expressed MAbs

Expression of MAbs using 293F cells is illustrated. The expressed MAbs with ~240 kD are indicated with an arrow. Supernatant from 293F cells transfected without DNA was used as a negative control (Lane 1). MAb 2G12 was used as a positive control (Lane 2).

3.8.2 Characterization of binding capabilities of MAbs

Finally, we sought to characterize the expressed MAbs by testing their binding to the mimotopes used for the isolation of B cells. For this purpose, we coated ELISA plates with the GFP-mimotope proteins GFP-BB-o-12-E7.2 and GFP-BB-m-12-E7.1 as well as the GFP and HIV-1 gp41 proteins before adding MAbs 35A1, 35A12 and 36A9.

MAbs 36A9 did not bind to the presented mimotope motifs. However 35A12, displayed binding not only to the GFP-mimotope proteins but also to the GFP protein (Figure 19). 35A1 showed no significant binding, but also reacted with GFP. The DOC plasma sample from RVe-10 was used as a positive control for the presence of specific Abs binding to the mimotope motifs. Naïve serum and supernatant from the 293F-transfection were used as negative controls.

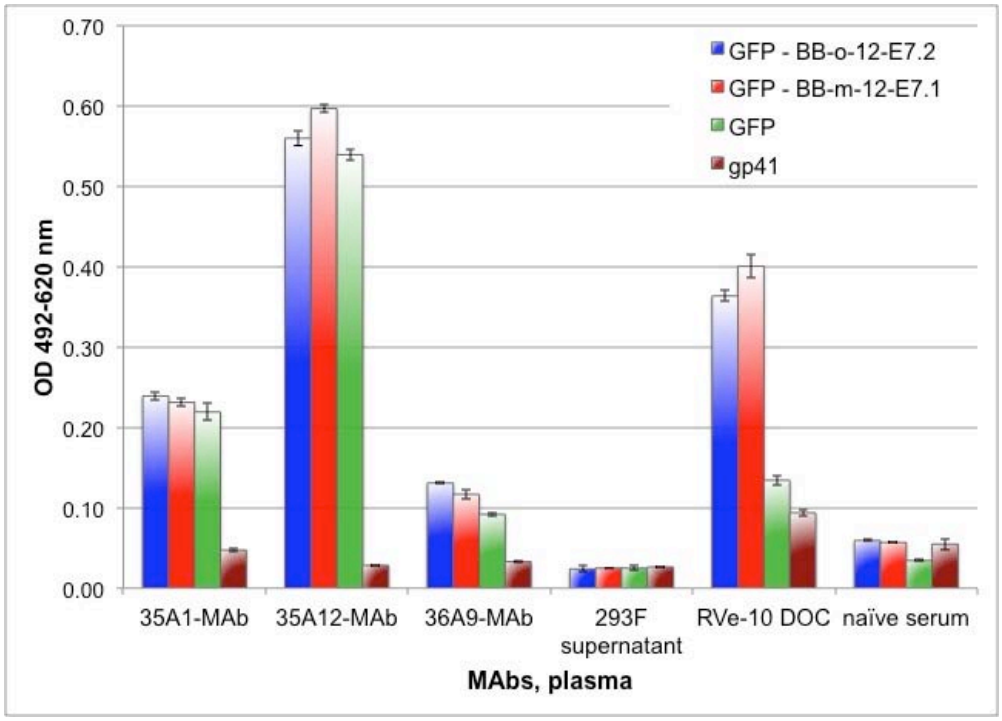


Figure 19: Binding of generated MAbs

The binding capabilities of the expressed MAbs were characterized. The DOC plasma sample from RVe-10 was used as a positive control. Naïve serum and 293F supernatant from the transfection without DNA were used as negative controls.

4 Discussion

In this study, a highly efficient method was used to generate recombinant MAbs derived from RMs. Using this method, we were able to isolate single antigen-specific B cells from a heterogeneous PBMC mixture from two RMs and succeeded in amplifying variable regions of Ig genes in order to clone them into expression vectors. Finally, we succeeded in expressing three different MAbs, with one of these found to be specific for GFP.

4.1 Expression of GFP-mimotope proteins

This study is based on selection of mimotope-specific Abs using the Kennedy-proximal mimotopes within the HIV-1 gp41 glycoprotein, illustrated in Figure 9. Epitopes within the transmembrane glycoprotein were already shown to induce nAbs (CHANH et al., 1986; KENNEDY et al., 1986). Further, the Kennedy-proximal mimotopes BB-o-12-E7, BB-o-12-G2, BB-m-12-E7 and BB-m-12-C5 were interesting because they were isolated from RM RTr-11, one of the monkeys that stayed aviremic despite several SHIV challenges during a vaccine study, performed in the Ruprecht lab in 2009 (LAKHASHE et al., 2011).

We successfully cloned all mimotope sequences into a vector containing GFP, which would be required for future selection strategies of mimotope-specific B cells during fluorescence activated cell sorting. During our screening for clones expressing the desired GFP fusion proteins (Figure 11), we encountered non-specific induction of proteins with the same size of ~34 kD, despite the absence of IPTG. The vector pET-GFP.II used for cloning of the mimotopes carries the T7 *lac* promoter, which is induced by IPTG to express previously cloned genes. However,

undesired protein expression was obvious in the absence of IPTG (Figure 11), resulting from unspecific inducing factors in the LB medium used for the growth of expressing cells. However, Hu and colleagues reported that a basal level of expression in the absence of any inducers occurs and depends on different factors of the experimental setup, such as the integration site of the gene transcript or the structure of the integrated DNA (HU & DAVIDSON, 1990). Therefore, only those clones with significant expression, compared to the non-induced control, were chosen for high-yield expression of recombinant GFP-mimotope proteins.

4.2 Mimotope-specific Abs in RM RTr-11

When we tested the recombinant fusion proteins with plasma from RTr-11 to detect mimotope-specific Ab responses, only the mimotope motifs from BB-o-12-E7 and BB-m-12-7 were recognized, in contrast to BB-o-12-G2 and BB-m-12-C5 (Figure 13). Lack of recognition by Abs in the plasma might be due to conformational changes resulting from the presence of the co-expressed GFP protein as well as the p3 phage protein, which was added at the N- and C-terminal to the mimotope sequence.

The Kennedy-proximal mimotopes represent specific epitopes on gp41. However, they do not reflect the exact sequence. The two mimotopes that were not recognized by Abs in the plasma of RTr-11 may have had slightly different amino acid sequences relative to the original epitope. This could have resulted in expression of conformationally altered proteins not recognized by any binding Abs in the plasma of RTr-11.

4.3 B cell sorting and amplification of Ig variable domains

The isolation of single B cells by flow cytometry is a highly efficient method that allows generation of antibodies of defined B cell origin (WARDEMANN et al., 2003; TILLER et al., 2008). Based on the expression of surface markers, different B cell populations can be isolated and analyzed using this method. With our strategy,

we aimed to specifically isolate memory B cells with a $CD3^-/CD27^+/CD19^+$ and IgG^+ phenotype (Figure 15).

After acute viral infection, memory B cells that express receptors specific for a particular antigen develop, and in doing so, contribute to formation of an immunological memory. They can survive for long periods in immunological niches, such as the bone marrow (HYLAND et al., 1994; SLIFKA et al., 1995), and are capable of differentiation into Ab secreting cells (ASCs) and secretion of specific antibodies upon re-encountering the original antigen (MCHEYZER-WILLIAMS & MCHEYZER-WILLIAMS, 2005). However, continuous maintenance of ASCs and constant production of antigen-specific Abs has also been proposed to occur in an antigen-independent way as a homeostatic mechanism to preserve the serological memory (LANZAVECCHIA et al., 2006).

With our selection strategy, we aimed to select those memory B cells containing genetic information of recombined and matured antibodies elicited during the vaccine study, performed in the Ruprecht lab in 2009 (LAKHASHE et al., 2011). We successfully isolated single B cells for the RMs RTr-11 and RVe-10 (Figure 15) and used this method to generate RM-derived MAbs.

Commonly used methods of production of human MAbs include immortalization of B cells with Epstein-Barr virus (STEINITZ et al., 1977; LANZAVECCHIA et al., 2007), screening of antibody phage display libraries and generation of recombinant MAbs from single B cells (WARDEMANN et al., 2003; TILLER et al., 2008). However, the former two methods are less efficient than the third in producing specific high-affinity Abs (LANZAVECCHIA et al., 2006; MOHAPATRA & JUAN, 2008). In contrast, isolation of single B cells using flow cytometry in combination with RT-PCR allows high-throughput screening of single B cells for MAbs (LIAO et al., 2009); further, by using rearranged mRNA transcripts as templates, this approach does not introduce any bias when the diversity of Ab repertoires is analyzed, inasmuch as low abundant Ig variations can be detected in addition to highly expressed Abs (WANG & STOLLAR, 2000). Using B cell sorting by flow cytometry and single-cell RT-PCR for amplification of Ig V genes, Tiller and

colleagues established a protocol with which to analyze the Ig gene repertoires of the human and murine genome (TILLER et al., 2008; TILLER et al., 2009). Further, Wrammert and colleagues succeeded in isolating high-affinity human MAbs against the influenza virus and generated human MAbs from antigen specific antibody-secreting cells (ASCs) after influenza vaccination (WRAMMERT et al., 2008). We used this method in the current study and derived antigen-specific MAbs from RM using single memory B cells as templates for amplification of variable Ig regions.

4.4 Cloning of Ig fragments

Following RT and semi-nested PCR of variable Ig genes from a single B cell, we successfully amplified variable Ig fragments for both monkeys (Table 2). However, before we could clone them into mammalian expression vectors, it was necessary to introduce specific sequences including, Kozak and Ig leader sequence, in order to enable future expression of MAbs. The Kozak sequence "GCC(A/G)CCATGG" was named after its discoverer, who noted the essential role of this sequence for initiation of protein translation in eukaryotic cells (CHANH et al., 1986; KENNEDY et al., 1986). Integration of an Ig leader sequence into the variable Ig fragment was necessary to enable secretion of Ig by the cell. The importance of the leader sequence was discovered early by researchers including Sasada R. and Koren R., who found it to be essential for outward transport of proteins from cells (KOREN et al., 1983; SASADA et al., 1988). The N-terminal Ig leader peptide mediates translocation of the protein to the lumen of the endoplasmic reticulum, where it is removed by proteolysis prior to the completion of protein synthesis (SASADA et al., 1988).

The RM V fragments containing the aforementioned sequences were successfully cloned into mammalian expression vectors to express chimeric MAbs comprising regions of RM Ig variable and human constant parts. However, we were unable to clone two of six pairs of the Ig variable fragments (see Table 2 and Table 3), namely 35A2 (γ and κ) and 35A5 (γ and λ). Upon analysis of the Ig heavy and light chain sequences, the γ heavy chain of 35A2 was found to contain a restriction

enzyme site for *EcoR*I, which we used for cloning of the γ heavy chains. The γ heavy chain fragment was probably cut at its endogenous and exogenously introduced restriction site, preventing ligation with the vector. However, we did not find any endogenous restriction sites within the sequence of 35A5 λ. Although there was a clear band after pFUSE cloning PCR (Figure 17, lane 10) and insertion of the cloning restriction sites was verified by sequence analysis, this Ig chain could not be cloned despite several attempts to do so.

To avoid ligation-dependent cloning, current research has focused on finding alternatives to directly accomplish transformation without the need to first digest and ligate the vector and insert. Kurosawa and colleagues have published a technique denoted as target-selective homologous recombination, which is based on homology between flanking sequences in both the vector and the insert. They constructed a suitable vector and designed primers to achieve homologous flanking sequences on both constructs. Additionally, they introduced flanking sequences from the amplified variable gene into the vector to increase cloning specificity. Both constructs were then mixed to allow homologous recombination and were then introduced into competent cells (KUROSAWA et al., 2011). However, it must be noted that, in order to accomplish homologous recombination, the sequences of the unknown Ig variable region has to be identified first, before the vector containing flanking sequences of both the primers used for amplification and the desired gene itself could be generated. Keeping in mind the considerable number of V, (D) and J gene segments for heavy and light chains (50 V, 25 D and 6 J gene segments for human heavy chains) (TILLER et al., 2008), the work required to construct the correct sequences into the vector seems to be very laborious.

Nevertheless, we were able to successfully clone four Ig pairs, 35A1 (γ and κ), 35A12 (γ and λ), 36A5 (γ and λ) and 36A9 (γ and κ), for expression of MAbs (Table 3). Although sequence analysis of their PCR fragments after semi-nested PCR suggested unproductive rearrangement for some of the Ig light chains (Table 2), we could verify a productive and rearranged state of all the cloned Ig chains. However, the results obtained after the first sequencing were probably due to sequencing errors and the default analysis settings of the IMGT database.

For sequencing of these Ig fragments, we used the reverse primers from the second of the semi-nested PCRs. These reverse primers annealed at the constant parts of the individual Ig fragments and enabled sequencing up to the V gene (see Figure 8 for reference). However, as sequencing efficiency is known to decrease, the V genes were not completely sequenced. The IMGT database compares query sequences starting from the V gene. If the V gene region is not properly sequenced, inaccurate sequences may lead to a frameshift resulting in unproductive or non-rearranged sequences.

4.5 Expression and characterization of MAbs

We were able to generate three mimotope-specific Abs (MAbs 35A1, 35A12 and 36A9; Table 3) using amplified variable Ig domains from single B cells. The fourth MAb (36A5) could not be expressed, probably because of non-rearranged V, D and J gene segments of the γ heavy chain (Table 2).

Generation of human MAbs has been the object of numerous studies (TILLER et al., 2008; LIAO et al., 2009; WU et al., 2010). In contrast, the amount of available information on the development of macaque MAbs is more easily manageable. Reports on the isolation of MAbs from RM include the recent publication by Kuwata and colleagues, who were able to isolate MAbs from a SIV-infected RM that neutralized several SIV strains in vitro (KUWATA et al., 2011). However, while this study may be a useful contribution to neutralization studies in the SIV model, direct extrapolation of the findings to human AIDS-vaccine research is more debatable. In contrast, our study focused on isolating MAbs from RMs infected with a SHIV isolate (LAKHASHE et al., 2011) that expresses the envelope glycoprotein of an R5-tropic HIV-1 clade C strain (SONG et al., 2006). As HIV-1 C causes over 56 % of all HIV-1 infections worldwide (UNAIDS, 2011) and the majority of these infections occurs transmucosally and is caused by R5 strains (DERDEYN et al., 2004), this primate model is clearly more useful for the development of a vaccine against HIV-1.

Other studies include that by Zhang and colleagues, who were able to produce specific MAbs against the parasite *Enterocytozoon bieneusi* isolated from RMs. However, for the production of parasite-specific MAbs they used BALB/c knockout mice that were previously infected with isolated parasite spores (ZHANG et al., 2005). Fofona and colleagues detected Env-specific B lymphocytes from SIV-infected macaques and quantified them using synthesized SIVmac239 gp120 and gp140 proteins (FOFANA et al., 2011).

The three MAbs 35A1, 35A12 and 36A9 were characterized for binding capabilities to their respective antigens. We observed no binding or moderate binding for 36A9 and 35A1, respectively, and GFP-specific binding for 35A12 (Figure 19). The GFP-specificity of 35A12 was probably due to non-specific binding of the B cell to GFP instead of the mimotope during preparation of the PBMCs for FACS-sorting.

For detection of mimotope-specific binding by MAbs, we used the GFP-Kennedy fusion proteins, which resulted in no binding of the MAbs 35A1 and 36A9. Although the expressed MAbs should exhibit binding to the directly exposed mimotope-motifs within the GFP-mimotope fusion proteins, it is questionable whether this assay is suitable for detection of specific binding to Kennedy-proximal mimotopes. The Kennedy-proximal mimotopes as well as the Kennedy epitope are located within the C-terminal tail (CTT) of gp41. This region has traditionally been referred to as the “intracytoplasmic domain”, reflecting its assumed location within the HIV-1 virion. However, this traditional model (Figure 20 A) has been objected to by other research groups, who questioned the static intracellular presence of the CTT and suggested extracellular exposure on the surface of the virion. An alternative model for the CTT-location has emerged, suggesting the existence of multiple membrane-spanning domains (MSD), in contrast to a single domain as claimed by the traditional model. Importantly, the CTT domain was now proposed to be located on the extracellular surface (Figure 20 B) (HOLLIER & DIMMOCK, 2005; STECKBECK et al., 2010).

Steckbeck and colleagues re-investigated these conflicting positions, intending to find evidence to differentiate between the two models, but what they found, in fact, was evidence consistent with both. They demonstrated exposure of the Kennedy epitope on Env-expressing cells due to binding to the epitope by specific MAbs, but no exposure on intact virions, as suggested by failure of specific MAbs to bind and neutralize the virus (STECKBECK et al., 2010).

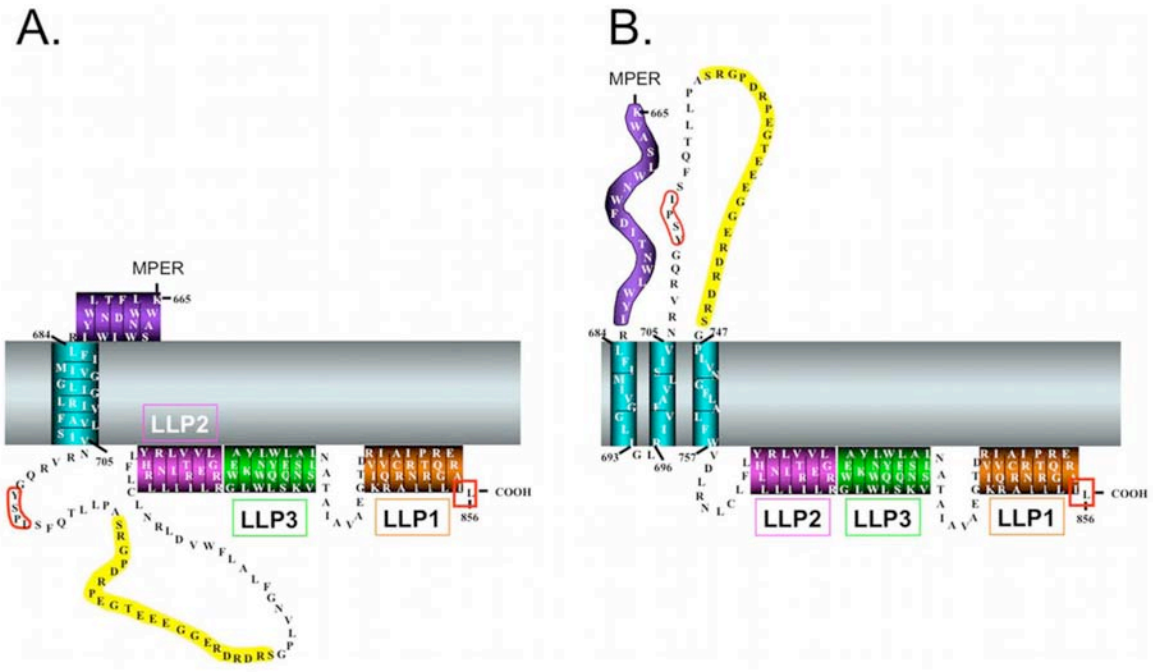


Figure 20 Conflicting gp41 CTT models
A) Traditional model of gp41 CTT with one membrane-spanning domain and completely intracytoplasmic CTT sequence. B) Alternative model with three membrane-spanning domains and extracellular Kennedy sequence (yellow).(STECKBECK et al., 2010)

The dynamic model advocated by Steckbeck and colleagues supported previous studies, which had proposed a dynamic CTT topology. Before Steckbeck, Heap and colleagues had already reported temperature-dependent neutralization of HIV-1 using MAb SAR1, indicating a transient exposure of the C-terminal tail of gp41 (HEAP et al., 2005). SAR1 was specific for the amino acid sequence “GERDRDR”, which is located within the Kennedy sequence (Figure 20 yellow sequence) and was reported to neutralize more effectively following attachment of virus to the cell, in contrast to the failure to bind in standard neutralization assays

(READING et al., 2003). Consistently with Steckbeck's discovery, the virus seemed to be sensitive to neutralization by SAR1 upon exposure of the Kennedy epitope after binding to the target cell (STECKBECK et al., 2010). Furthermore, Kennedy and coworkers suggested, along with their discovery of the epitope, an extracellular exposure that did not fit with the cytoplasmic CTT model (KENNEDY et al., 1986; HEAP et al., 2005). More recently, Lu and colleagues demonstrated inhibition of cell fusion between viral and cellular membranes by Abs specific for LLP1 and LLP2, which are assumed to be in the intracellular space. However, binding by Abs may have been possible due to their transient exposure (LU et al., 2008).
Based on these indications and Steckbeck's discovery of an exposed CTT region in Env-expressing cells, it might be reasonable to investigate the binding of MAbs targeting the Kennedy-proximal mimotopes (Figure 9) to Env-expressing cells.

4.6 Implications for the future – the potential of MAbs

The strategy used in this study to isolate single antigen-specific B cells for subsequent amplification of variable domains of macaque Ig chains, constitutes a highly efficient and unbiased method, with the potential for high-throughput analysis. However, some points of the procedures can still be improved.
Flow cytometry allows analysis of a high number of cells, as exemplified by the number of cells analyzed for RTr-11 and RVe-10 during this study (63 x 10^6 and 67 x 10^6, respectively). Based on the fluorescence of previously used dyes, specific cell types can be identified. During this study, recombinant GFP was used as fluorescent marker to detect B cells that specifically recognized GFP-mimotope proteins. Memory B cells with a $CD3^-$, $CD19^+/CD27^+/IgG^+$ and GFP^+ phenotype were identified (Figure 15 F; P6). Similarities in excitation and emission spectra of recombinant GFP (excitation and emission of 493 nm and 509 nm, respectively (ALLELEBIOTECH, 2011) and auto-fluorescent cells (NIEBYLSKI & PETTY, 1993) make their distinction difficult and may result in false positive results leading to sorting of cells other than the ones of interest. The sorting efficiency of our experiments, defined as the percentage of successfully amplified Ig pairs out of the

total number of cells sorted, was 14 % and 20 % for RTr-11 and RVe-10, respectively. However, sorting efficiency can be increased and false positive results minimized by replacing GFP with another fluorescent protein with an excitation spectrum different than that of mononuclear cells. This could increase the number of sorted B cells, resulting in a higher number of amplified Ig pairs.

After fluorescence activated cell sorting, single B cells were used to amplify variable Ig domains by RT-PCR. Synthesized cDNA was amplified using a reverse primer annealing to constant regions of the γ heavy and κ or λ light chains and mixtures of specific forward primers, as required by number of different gene families for heavy and light chains (SCHROEDER & CAVACINI, 2010). In this step, forward primers annealing to the more conserved Ig leader sequences could be used instead of a mixture of forward primers. However, in order to do so, macaque Ig leader sequences have to first be described as not all of which are yet known.

During the cloning of Ig variable domains into pFUSE-vectors, specifically designed primers were used that included insertions, such as the Kozak- and the Ig leader peptide-sequence. However, due to their considerable length of approximately 90 to 100 bases, these primers are less stable than shorter primers during handling; moreover non-specific annealing during PCR can occur. Thus, shorter primers may afford better amplification of desired DNA.
Finally, a new assay for characterization of the Kennedy-proximal mimotopes should be established as discussed earlier.

Use of Abs as therapeutic agents was recognized early with Robert Koch's group pioneering work in the 1890s, when they used sheep antiserum against diphtheria toxin to immunize a patient already afflicted with the disease (MARASCO & SUI, 2007). Pooled human sera have found broad use to treat infections including tetanus or hepatitis B by passive immunization. However, their use can have negative side effects, such as autoimmune reactions or potential anaphylactic immune responses, thus favoring MAbs for treatment. Effective MAbs have many potential applications, including use against potential bioterrorism agents, such as anthrax. Further, they can be used to provide immediate treatment following

exposure to certain life-threatening toxins, such as the botulinum neurotoxin. Immunocompromised individuals, who cannot be treated with existing vaccines, could perhaps be treated with specific MAbs, inasmuch as their immune systems are not exposed to immune response-inducing agents (SMITH et al., 2009).

Effective use of MAbs against SHIV infection was demonstrated recently by Cherie and colleagues, who passively immunized newborn macaques with IgG, before challenging them with $SHIV_{SF612P3}$. They observed an increase in virus control of vaccinated monkeys compared to non-immunized macaques (NG et al., 2010). Other passive transfer studies in macaques provided further evidence of the potent capacity of MAbs to protect against challenge with SHIVs. In order to evaluate protection of infant macaques from HIV-1 transmission occurring intra- or postpartum (during or after delivery of infants), Baba and colleagues treated pregnant macaques with human IgG1 MAbs before intravenous challenge with SHIV-vpu^+ to observe protection against infection. Furthermore, newborn macaque infants were similarly protected after treatment with the same MAbs followed by SHIV challenge (BABA et al., 2000). Additionally, Mascola and colleagues demonstrated protection of vaginally SHIV-challenged macaques, using the more natural occurring mucosal mode of transmission. By testing different combinations of the MAbs 2F5, 2G12 and HIV-Ig (purified polyclonal IgG derived from HIV-1 positive donors), they observed different levels of protection (MASCOLA et al., 2000). However, despite MAb-mediated protection by neutralization, it should be kept in mind that other mechanisms, including Fc-mediated effector mechanisms such as ADCC, are involved in fighting virus infection (HESSELL et al., 2007).

The importance of finding a broadly nAb against HIV-1 is highlighted by the hypervariable and fast evolving nature of the virus in response to Abs. Steady production of neutralizing Abs by the immune system leads to continuous selective pressure and drives evolution of new HIV-1 variants (RICHMAN et al., 2003). Changes within the highly immunogenic glycan shield of the virus confer protection against neutralization by autologous neutralizing Abs (WEI et al., 2003).

Selective pressure leading to loss of sensitivity to MAbs was also demonstrated during passive immunization studies, including the one performed by Trkola's and Manrique's laboratory, who used mixtures of three neutralizing MAbs (2G12, 2F5 and 4E10) (TRKOLA et al., 2005; MANRIQUE et al., 2007). Thus, finding a broadly neutralizing MAb targeting an epitope conserved within different HIV clades would confer protection despite the presence of viral escape mutants, thereby bringing us a big step closer to finding an effective AIDS vaccine. Apart from its potential for commercial benefits, this work would be useful in the elucidation of critical steps in viral entry and replication.

5 Zusammenfassung

Das Humane Immundefizienz Virus (HIV) ist der kausative Erreger des Immundefizienz-syndroms AIDS und ist für eine weltweite Epidemie mit ungefähr 34 Millionen infizierten Menschen im Jahr 2009 verantwortlich. Die hohe Prävalenz unterstreicht die Notwendigkeit eines sicheren und effektiven Impfstoffes. Die Entwicklung eines breit neutralisierenden monoklonalen Antikörpers würde nicht nur konservierte Epitope in der Virushülle offenbaren, sondern uns auch einen großen Schritt voranbringen, einen effektiven AIDS-Impfstoff zu finden.

Im Verlauf einer Vakzinstudie wurden zwei Rhesusaffen (*Macaca mulatta)* nach mehrmaliger Immunisierung mit SHIV-1157ipEL-p inokuliert. Interessanterweise blieb einer dieser Affen, trotz mehreren SHIV-Inokulationen, avirämisch. Plasma dieses Affen wurde in einer weiteren Studie verwendet, um Mimotope (Sequenzen, die Epitope auf der Virushülle imitieren) zu isolieren, die in der C-terminalen Domäne vom viralen gp41 lokalisiert sind.

In dieser Studie wurden diese Mimotope verwendet, um Antigen-spezifische B-Zellen von Rhesusaffen zu isolieren. Variable Immunglobulin-Regionen von Rhesusaffen wurden von singulär isolierten B-Zellen amplifiziert, um Mimotop-spezifische monoklonale Antikörper zu exprimieren und ihre Bindungsaffinität zu charakterisieren.

Es gelang uns, B-Zellen von beiden Rhesusaffen zu isolieren und variable Immunglobulin-Gene zu amplifizieren. Wir generierten erfolgreich drei Antigen-spezifisch monoklonale Antikörper, wobei einer von ihnen GFP-spezifisch war.

Stichwörter: Monoklonale Antikörper; Einzelzell-PCR; Rhesusaffe; HIV-1

6 Summary

The human immunodeficiency virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS) and is responsible for a worldwide epidemic with approximately 34 million infected individuals in 2009. The high prevalence of HIV-1 underlines the urgent need of a safe and effective vaccine. Generation of a broadly neutralizing monoclonal antibody (MAb) would not only reveal conserved epitopes within the viral envelope but also importantly, bring us a big step closer to finding an effective AIDS-vaccine.

In the course of a vaccine study, two rhesus macaques *(Macaca mulatta)* were challenged with SHIV-1157ipEL-p after several immunizations. Interestingly, one of these monkeys remained aviremic, despite several challenges. Plasma from this monkey was used in another study to isolate mimotopes (sequences mimicking epitopes within the viral Env), located within the C-terminal tail of the viral gp41.

In this study, these mimotopes were used to isolate antigen-specific B cells from rhesus monkeys. Single B cells were used to amplify variable regions of rhesus monkey immunoglobulin genes to express and characterize binding capabilities of mimotope-specific MAbs.

We were able to isolate B cells from both rhesus macaques and amplified variable immunoglobulin genes. We successfully generated three antigen-specific MAbs, with one of these found to be specific to GFP.

Keywords: Monoclonal antibody; Single cell PCR; Rhesus macaque; SHIV; HIV-1

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8 Appendix

8.1 Sorting strategy for RM RVe-10

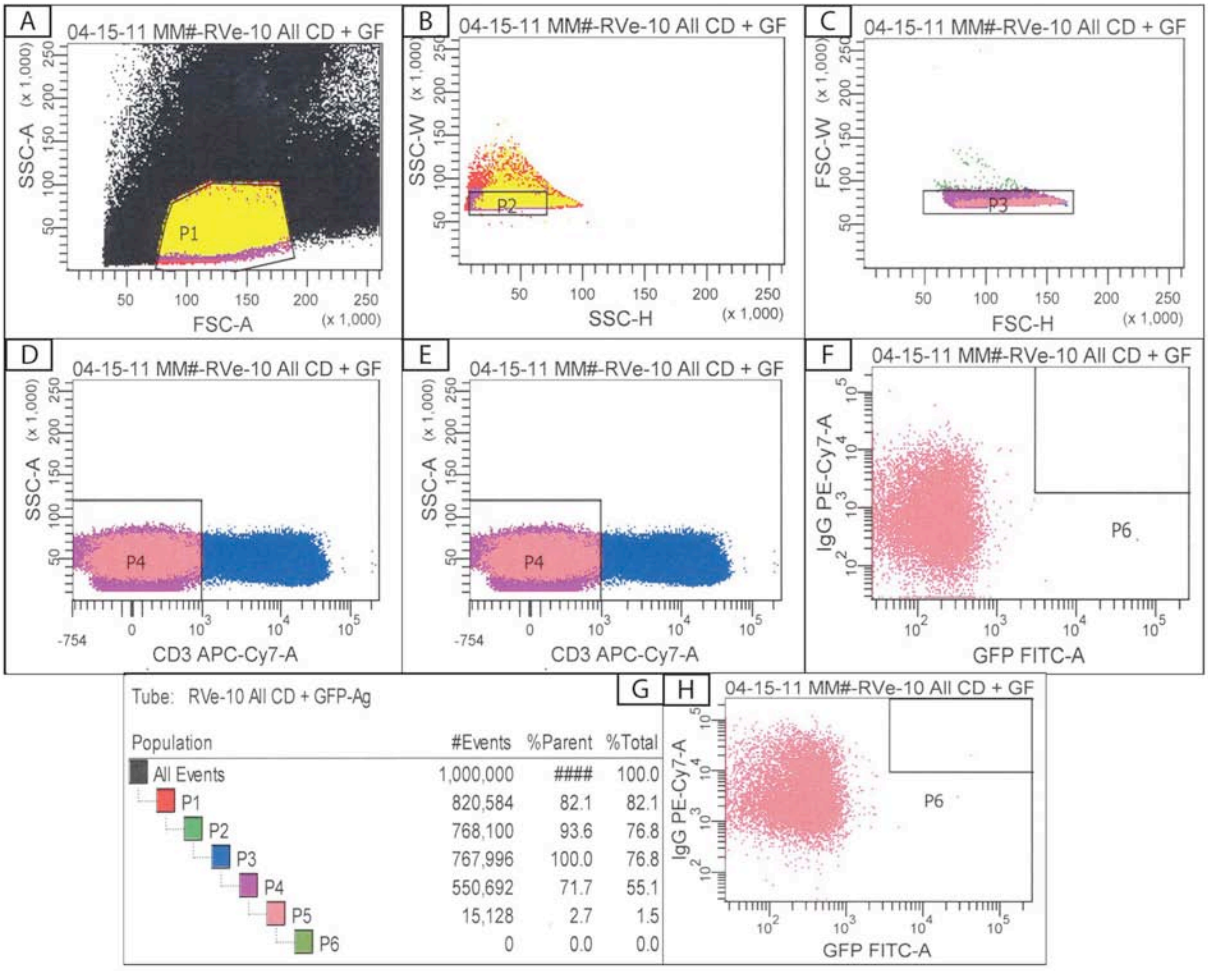


Figure 21: Sorting strategy for RM RVe-10

The description for the gating strategy can be found in Figure 15. No mimotope-specific memory B cells were detected out of $1x10^6$ PBMCs. A total of 67×10^6 PBMCs was analyzed for RVe-10.

8.2 RT-PCR results for RM RTr-11

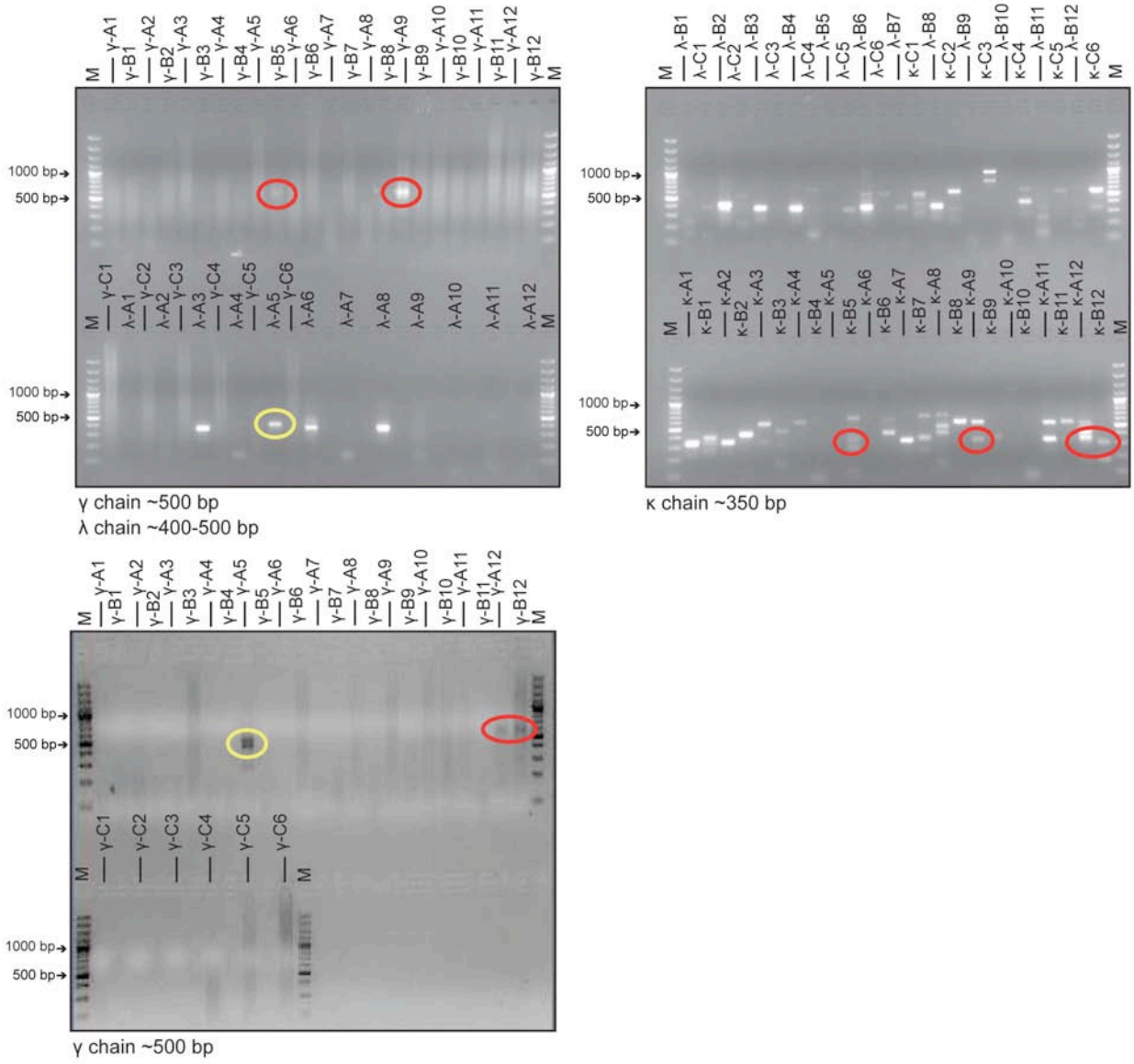


Figure 22: Ig fragments from RM RTr-11 amplified after RT-PCR

The amplification of Ig chain fragments from each single sorted B cell from RM RTr-11 is shown. The γ heavy chain was performed twice. Corresponding γ heavy and κ light chains are circled in red. The yellow circles indicate Ig pairs exhibiting γ heavy and λ light chains. The letter and number combinations (A1-A12, B1-B12, C1-C12) refer to the well positions on the 96-well plates where the particular chain was amplified.

8.3 List of devices

Beckman Avanti™ J-25 Centrifuge	Beckman Coulter (Brea, CA)
Beckman DU®640 Spectrophotometer	Beckman Coulter (Brea, CA)
ELISA automatic micro-plate washer	BioTek Instruments (Winooski, VT)
ELISA reader (MikroWin 2000 software)	Berthold Technologies U.S.A. (Oak Ridge, TN)
Milli-Q Advantage A10 Ultrapure Water Purification System	Millipore (Billerica, MA)
Molecular Imager® Gel Doc™ XR (Quantity One 1-D Analysis Software Version 4.6.3)	Bio-Rad Laboratories (Hercules, CA)
NanoDrop1000 Spectrophotometer (Software: ND-1000 V.3.7.1	Thermo-Fisher Scientific (Waltham, MA)
Sorvall Legend X13 Centrifuge	Thermo-Fisher Scientific (Waltham, MA)
PCR cyclers:	
PCR cycler C1000 Thermal Cycler	Bio-Rad Laboratories (Hercules, CA)
GeneAmp® PCR System 9700	Applied Biosystems, Life Technologies (Carlsbad, CA)
Eppendorf Master cycler gradient	Eppendorf (Westbury, NY)
Tissue culture:	
GS-6R Centrifuge	Beckman Coulter (Brea, CA)
Reach-in CO_2 Incubator	Thermo-Fisher Scientific (Waltham, MA)
Forma Benchtop Orbital Shaker	Thermo-Fisher Scientific (Waltham, MA)

8.4 External facilities

- Beth Israel Deaconess Medical Center (BIDMC) Cell Sorting Facility: Boston, MA
- Dana-Farber Cancer Institute Molecular Biology Core Facilities (DFCI-MBCF): Boston, MA
- Yerkes National Primate Research Center (YNPRC): Atlanta, GA

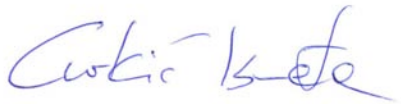
9 Declaration

Herewith I declare that the thesis

“Generation of Recombinant Antibodies against HIV Clade C Envelope“

has been conducted entirely on my own without any impermissible help. All used references and sources of information are quoted and listed.

This bachelor thesis has not been previously submitted to any university.



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