UNIVERSITÀ DEGLI STUDI DI VERONA

DIPARTIMENTO DI
SCIENZE DELLA VITA E DELLA RIPRODUZIONE

DOTTORATO DI RICERCA IN
BIOSCIENZE

CICLO XXIII

TESI DI DOTTORATO

# Humanization of murine neutralizing monoclonal antibodies against HIV-1 through a new approach based on lentiviral vectors

S.S.D. BIO 11

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# ABBREVIATIONS

| | |
|---|---|
| **ADCC** | Antibody-Dependent Cellular Cytotoxicity |
| **AIDS** | Acquired Immunodeficiency Syndrome |
| **BSA** | Bovine Serum Albumin |
| **CXCR4** | C-X-C chemokine receptor type 4 |
| **CCR5** | C-C chemokine receptor type 5 |
| **CD4** | Cluster of Differentiation 4 |
| **cDNA** | Complementary DNA |
| **CH** | Constant region of Heavy chain |
| **CHO** | Chinese Hamster Ovary |
| **CK** | Constant region of K light chain |
| **CL** | Constant region of Light chain |
| **CMV** | Cytomegalovirus |
| **cPPT** | Central Polypurine Tract |
| **CTS** | Central Termination Signal |
| **DEAE** | Dextran Diethylaminoethyl-Dextran |
| **DIS** | Dimerization Signal |
| **DNA** | Deoxyribonucleic Acid |
| **dNTP** | Deoxyribonucleotide |
| **Fab** | Fragment Antigen Binding |
| **Fc** | Fragment Crystallizable |
| **FBS** | Fetal Bovine Serum |
| **gp120** | Glycoprotein 120 (kDa) |
| **gp41** | Glycoprotein 41 (kDa) |
| **HAART** | Highly Active Antiretroviral Therapy |
| **HIV** | Human Immunodeficiency Virus |
| **HLA** | Human Leukocyte Antigen |
| **HR** | Heptad Repeat region |
| **HTLV-III** | Human T-lymphotropic virus |
| **IAVI** | International AIDS Vaccine Initiative |
| **Ig** | Immunoglobulin |

| | |
|---|---|
| **IgA** | Immunoglobulin A |
| **IgD** | Immunoglobulin D |
| **IgE** | Immunoglobulin E |
| **IgK** | Immunoglobulin K (light chain) |
| **IgKv** | Variable region of IgK |
| **IgG** | Immunoglobulin G |
| **IgGv** | Variable region of IgG |
| **IgL** | Immunoglobulin L (light chain) |
| **IgM** | Immunoglobulin M |
| **IN** | Integrase |
| **IRES** | Internal Ribosome Entry Site |
| **LAV** | Lymphadenopathy Associated Virus |
| **LTR** | Long Terminal Repeats |
| **mIg** | Membrane Ig |
| **NK** | Natural Killer |
| **OD** | Optical Density |
| **ORF** | Open Reading Frame |
| **PBMC** | Peripheral Blood Mononuclear Cell |
| **PBS** | Primer Binding Site / Phosphate Buffer Saline |
| **PCP** | Pneumocystis Pneumonia |
| **PCR** | Polymerase Chain Reaction |
| **PFA** | Paraformaldehyde |
| **PIC** | Preintegration Complex |
| **polyA** | Polyadenilation Signal |
| **PPT** | Polypurine Tract |
| **PR** | Protease |
| **Rev** | Regulator of Expression of Viral proteins |
| **RNA** | Ribonucleic Acid |
| **RRE** | Rev Responsive Element |
| **RT** | Reverse Transcriptase |
| **SIN** | Self-Inactivating |
| **SIV** | Simian Immunodeficiency Virus |
| **ssRNA** | Single-stranded RNA |
| **SU** | Surface Subunit |

| | |
|---|---|
| **SV40** | Simian Virus 40 |
| **TAR** | Transactivation Response Element |
| **Tat** | Transactivator |
| **TCID50** | 50% Tissue Culture Infective Dose |
| **TM** | Transmenbrane Subunit |
| **UNAIDS** | United Nations programme on HIV/AIDS |
| **VH** | Variable region of heavy chain |
| **VL** | Variable region of light chain |
| **VSV-G** | Vescicular Stomatitis Virus Glycoprotein |
| **WPRE** | Woodchuck Post-Regulatory Element |

# ABSTRACT

More than 25 years after the discovery of HIV, AIDS still represents a pandemic emergency. Antiretroviral therapies are becoming more and more effective, but no real cure has yet been found to eradicate it. In order to develop a preventive vaccine, the isolation of new immunogens, capable to stimulate humoral immune response towards highly conserved viral epitopes, is pivotal.

In a past work performed by our lab, the fusion between HIV-1 envelope and cell membrane was exploited to produce fusion intermediates exposing viral conserved epitopes. Such fusion complexes were used to immunize mice to elicit antibody production, and to produce hybridomas population expressing antibodies against HIV-1.

During the course of the present work of thesis, hybridoma populations were screened for antibody production and neutralization activity. Cells were gradually subcloned down to single cell population in order to identify broad-spectrum monoclonal antibodies. In order to stabilize antibody expression, and to obtain antibodies supernatants close to human antibody, IgG mRNA sequences of the selected monoclonal population were cloned to be humanized.

Viral vectors expressing humanized light and heavy IgG chains were produced by matching human constant regions with murine variable regions. Such viral vectors were used to co-infect CHO cells in order to obtain a cell line stably expressing humanized antibodies. Transgenes integration was confirmed by amplification of genomic DNA and humanized antibody production was quantified by ELISA assay.

Obtained results show that viral vectors can integrate transgenes inside target cells and, more precisely, that our method works to produce humanized antibodies. Moreover, preliminary functional tests show that the produced antibody is working.

Nevertheless, such protocol needs to be devised, as antibody expression remained at low concentration values to perform functionality assays. Optimization of this method and tests of antibody functionality will allow to the release of a rapid and easy protocol to produce cell line stably expressing the antibody of interest.

Antibody characterization and analysis could be favored by a stable production source.

# RIASSUNTO

A più di 25 anni dalla scoperta del virus HIV, l'AIDS rappresenta ancora un'emergenza pandemica. Le terapie antiretrovirali stanno diventando sempre più efficaci, ma non è stata ancora trovata una vera cura. Al fine di sviluppare un vaccino preventivo, è fondamentale l'isolamento di nuovi immunogeni, in grado di stimolare la risposta immunitaria umorale verso epitopi virali altamente conservati.

Nel nostro laboratorio, sono stati prodotti intermedi di fusione che esponessero epitopi virali conservati. Tali complessi di fusione sono stati utilizzati per immunizzare dei topi in modo da sollecitare la produzione di anticorpi, e per produrre ibridomi che esprimessero anticorpi contro HIV-1.

Nel corso del presente lavoro di tesi, popolazioni di ibridomi sono state sottoposte a screening per testare la produzione di anticorpi e l'attività neutralizzante. Le cellule sono state subclonate in passaggi successivi fino a popolazioni derivate da singola cellula al fine di individuare anticorpi monoclonali ad ampio spettro. Al fine di stabilizzare l'espressione degli anticorpi, e di ottenere anticorpi simili ad anticorpi umani, le sequenze di mRNA di IgG della popolazione monoclonale selezionata sono state clonate per essere umanizzate.

Vettori virali esprimenti catene umanizzate leggere e pesanti di IgG sono stati prodotti clonando le regioni costanti umane in frame con le regioni variabili murine. Tali vettori virali sono stati usati per co-infettare cellule CHO in modo da ottenere una linea cellulare che esprimesse stabilmente anticorpi umanizzati. L'integrazione dei transgeni è stata confermata dall'amplificazione del DNA genomico e la produzione di anticorpi umanizzati è stata quantificata mediante test ELISA.

I risultati ottenuti mostrano che i vettori virali sono in grado di integrare i transgeni all'interno delle cellule bersaglio e, più precisamente, che il nostro metodo funziona per la produzione di anticorpi umanizzati. Inoltre, i test preliminari mostrano che l'anticorpo prodotto denota attività interessante.

Tuttavia, tale protocollo deve essere messo a punto e ottimizzato, in quanto l'espressione di anticorpi è su valori di concentrazione troppo bassi per la

produzione in larga scala. L'ottimizzazione di questo metodo e dei test di funzionalità degli anticorpi permetterà la pubblicazione di un protocollo rapido e semplice per produrre linee cellulari che esprimano stabilmente l'anticorpo di interesse. La caratterizzazione e l'analisi degli anticorpi sarà quindi favorita da una espressione stabile.

# 1. INTRODUCTION

## 1.1 HIV-1 discovery, origin and evolution

In 1981, Gottlieb and colleagues of Los Angeles (California, USA) reported five cases of patients affected by pneumonia caused by *Pneumocystis carinii* and associated to heavy immune system deficiencies (Gottlieb *et al*., 1981). Such pathologic condition was considered, to date, extremely rare and usually associated to patients already affected by other immunodeficiencies. At the same time, more reports rose concerning American homosexual males showing pneumocystis associated with Kaposi's sarcoma, a vascular cancer typically found in patients over 60. The disease was characterized by high mortality, mainly due to the onset of several other opportunistic infections (Lerner and Tapper, 1984), as the one caused by *Candida albicans*. In the next years a rise of reported cases was noticed even in other geographic zones and ethnic groups. Disease transmission was proved to be associated with sexual intercourses, exchange of infectious blood contaminated syringes and from mother to child during pregnancy: such infectious agent was thus transmittable by sex, blood and maternally (Freedman *et al*., 1989).

Epidemiologic data collected by the Center of Disease Control in Atlanta (Georgia, USA), confirmed that this syndrome was an infection previously unknown, caused by a viral agent and characterized by strong immune deficiency. It was named AIDS, that is, Acquired Immunodeficiency Syndrome (Marx, 1982). In 1983, at the Pasteur Institute in Paris, and in 1984 at National Cancer Institute in Bethesda (Maryland, USA), AIDS causative agent was isolated from peripheral blood of patients. French group directed by Luc Montagnier named it Lymphadenopathy Associated Virus (LAV) (Barre-Sinoussi *et al*., 1983), whereas the American team directed by Robert Gallo named it HTLV-III (Popovic *et al*., 1984). In 1985, when the viral genome was published, the concurrence of retroviral sequences of LAV and HTLV-III was discovered. Afterwards, in 1986, the International Committee on Taxonomy of viruses named such viral agent as Human Immunodeficiency Virus type 1 (HIV-1).

HIV is a genetically related member of the *Lentivirus* genus of the *Retroviridae* family. Lentiviruses are so called due to the typical chronic course of the disease, with a long period of clinical latency, persistent viral replication and involvement of the central nervous system.

The virus is classified, on genome sequence basis, in two types: HIV-1 and HIV-2 They present a sequence homology higher than 85% and share a large number of genes and several studies claim they derived by zoonosis, a transition of a virus of primates to humans (Gao *et al*., 1999). The site of origin was located most likely in sub-Saharan Africa, from which the disease spread in USA across the Caribbean and later in Europe and in the rest of the world. Indeed, documentation is present since the 50s-60s of independent events of transition to humans from chimpanzees (*Pan troglodytes troglodytes*) infected by SIVcpz or, in case of HIV-2, from collared mangabey (*Cercocebus torquatus atys*) infected by SIVsm. The modality of transition was correlated with hunting and consumption of monkey meat, common in some African indigenous populations. After transition to humans, HIV-1 accumulated several genetic mutations, mainly due to the activity of reverse transcriptase enzyme (lacking a proofreading activity) and to genetic recombination events.

Depending on differences on *env* gene, four HIV-1 groups were identified: M, N, O and P. Group M (Major) is the most common one and it is responsible of pandemic. Moreover, it is subdivided into eleven subtypes (clades), from A to K (Requejo, 2006), based on the genomic divergence from a common ancestor and on the geographical position. Group O (Outlier) is present in Central and West Africa and it is responsible for less than 5% of worldwide HIV-1 infections. Group N (New, non-M/non-O) was isolated in few individuals in West Africa. Group P was discovered only in 2009 (Plantier *et al*., 2009). HIV-2 is confined in the geographic areas containing HIV-1 groups N and O and presents a much more limited transmission risk.

The various groups and subtypes derive by at least seven independent events of zoonosis, whereas the whole group M derives by a single transition event (Gao *et al*., 1999). The geographic distribution (figure 1.1) of clades belonging to group M seems to resemble the modality of transmission of HIV-1: clade B is mainly located in Europe and North America, where infections due to homosexual

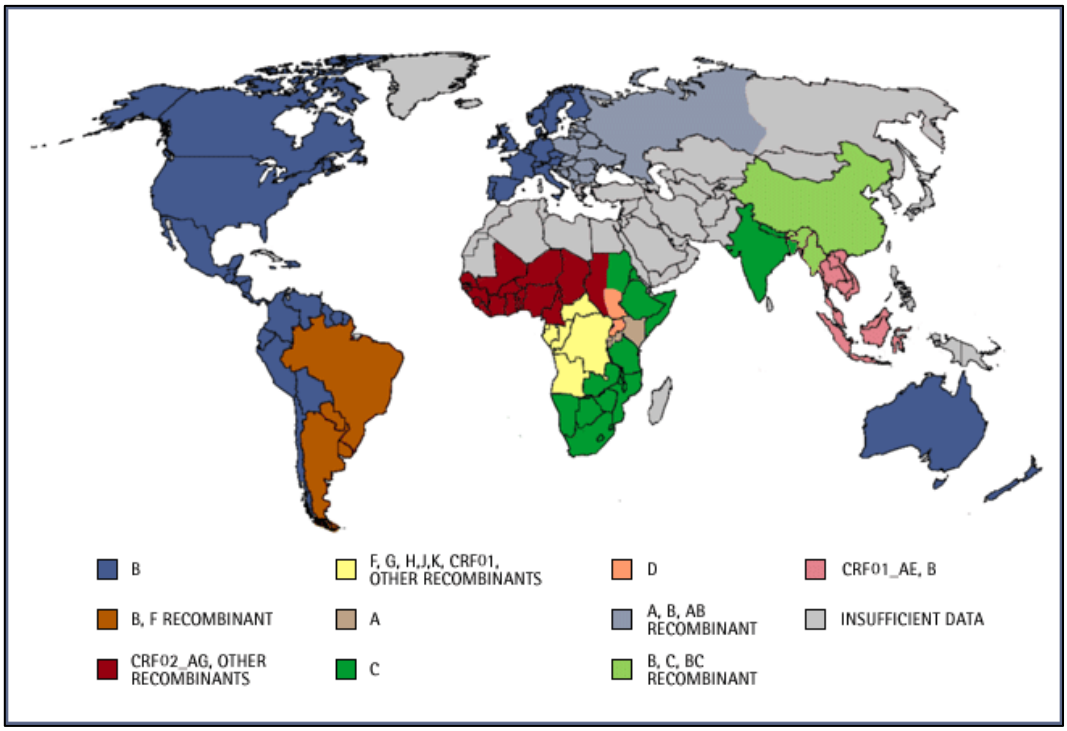


*Figure 1.1 Schematical representation of HIV-1 subtypes and clades distribution. Clade C and A are predominant in Africa, India and South-East Asia whereas Clade B is mainly found in America and Europe. (Obtained from http://www.pbs.org/wgbh/pages/frontline/aids/atlas/clade.html).*

relationships and blood contacts (infected needles and transfusions) seems to prevail. Clades C, A and E are predominant in Africa, India and South-East Asia where transmission occurs mainly mucosally and by heterosexual contacts. According to data provided by International AIDS Vaccine Initiative (IAVI), clade C covers 48% of global infections, whereas clade A and B cover respectively 25% and 16%.

## 1.2 Clinical course of HIV infection

HIV-1 has the ability to infect CD4+ lymphocytes and a variety of other cells in the body, including monocytes and thymocytes (Nilsson *et al*., 2007, Ho Tso Fang *et al*., 2008). The virus enters target cells via cell surface molecules, including CD4 and chemokine co-receptors (CXCR4, CCR5) (Berger *et al*., 1999). During the first few weeks of infection, the patient is often affected by a flu-like illness and a rash, an illness termed acute HIV-1 infection syndrome (Levy, 2006). This initial phase of HIV-1 infection is followed by a gradual deterioration of the immune function (figure 1.2).

CD4+ cells, also called "T-helper cells," play a central role in the immune response, signaling other cells such as the cytotoxic T cell and the B cells to perform their functions (Fahey *et al*., 1990). Normally, a healthy person has a CD4+ count of 800 to 1200 CD4+ T cells per cubic millimeter ($mm^3$) of blood. As CD4+ cells are destroyed by HIV and as these cells decrease in number, leaks develop in the immune repertoire (Nilsson *et al*., 2007). Once the CD4+ count falls to 500 $mm^3$, half of the immune reserve has been destroyed and minor infections including cold sores (herpes simplex), condyloma (warts) and fungal infections, thrush and vaginal candidiasis, may occur. These infections are troublesome but not life threatening. However, as the CD4+ count falls to 200 cells/$mm^3$, the patient becomes particularly vulnerable to the serious opportunistic infections and cancers characteristic of AIDS, the end stage of HIV-1 disease. AIDS is defined as a CD4+ count of 200 cells/$mm^3$, or the presence of a serious infection, such as PCP (pneumocystis pneumonia), toxoplasmosis, cytomegalovirus infections of the eye or intestine, as well as debilitating weight

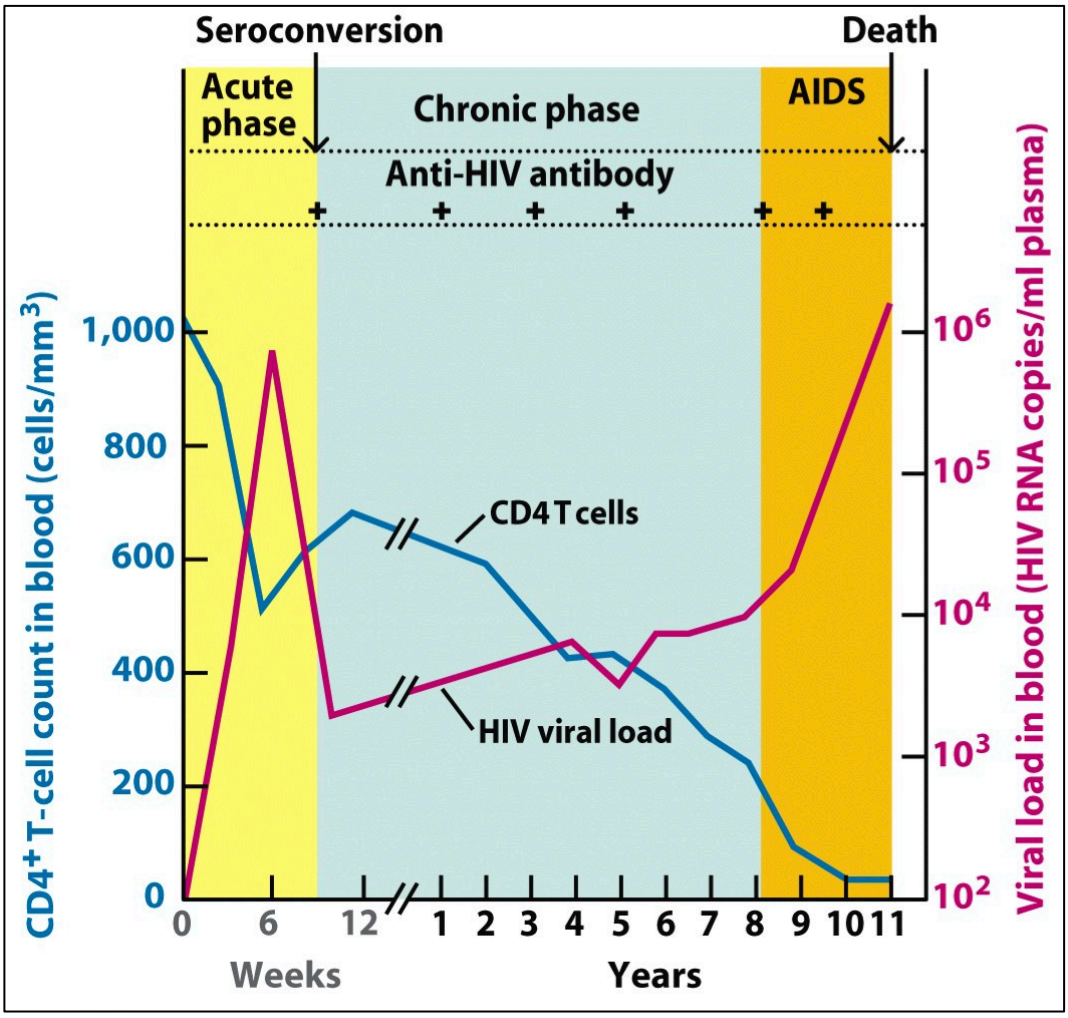


*Figure 1.2 Representative graph relating CD4+T-cells with (blue line) with viral load (red line) during acute and chronic phase, as well as in full-blown, AIDS when no antiretroviral therapy is being used. (Adapted from Immunology, Kuby, Sixth Edition, 2007)*

loss, diarrhea, HIV dementia and cancers, Kaposi's sarcoma and lymphomas (Center for Disease Control and Prevention, 1992).

## 1.3 HIV-1 structure and genome

HIV-1 virion (figure 1.3) has a diameter of 100 nm and contains a genome constituted by positive polarity single-stranded RNA (ssRNA). The genome is present in double copy and enclosed inside an icosahedral protein structure named capsid.

The viral external structure is a phospholypidic bilayer called envelope, derived by the cell membrane, and generated during the process of viral budding from host cell. Therefore the envelope is constituted both by cell proteins that are incorporated during budding (i.e. HLA – Human Leukocyte Antigen – class I and II proteins or adhesion proteins) and viral proteins as the gp160. Such complex is a heterodimer composed of trimers of the transmembrane gp41 and the external surface spanning gp120 glycoproteins, which are strongly glycosylated. Both proteins are required for the early phase of infection process, permitting the attachment and the fusion of the envelope with the cell membrane. The binding between gp120 and gp41 is not covalent and therefore gp120 may be shed spontaneously within the local environment and detected in the serum, as well as within the lymphatic tissue of HIV- infected patients.

Anchored to the inside of the envelope, it is present an electron-dense protein structure called matrix, mainly constituted by protein p17. The matrix surrounds a protein structure called capsid, which is mainly constituted by the viral protein p24 and contains the viral genome combined with a nucleoprotein and the enzymes Reverse Transcriptase, Integrase and Protease.

The HIV-1 genome (figure 1.4) encodes the three structural genes *gag*, *pol*, and *env*. In addition to these genes, HIV-1 genome presents a complex combination of other regulatory/accessory genes.

The protein-encoding regions are flanked by 5' and 3' LTRs (Long Terminal Repeats), which consist of 3' unique element (U3), repeat element (R) and 5' unique element (U5), and harbor some of the *cis*-acting elements of the genome. The c*is* elements contain signals important for provirus integration into the host genome (ATT repeats, which are located at the 5' and 3' ends of provirus DNA),

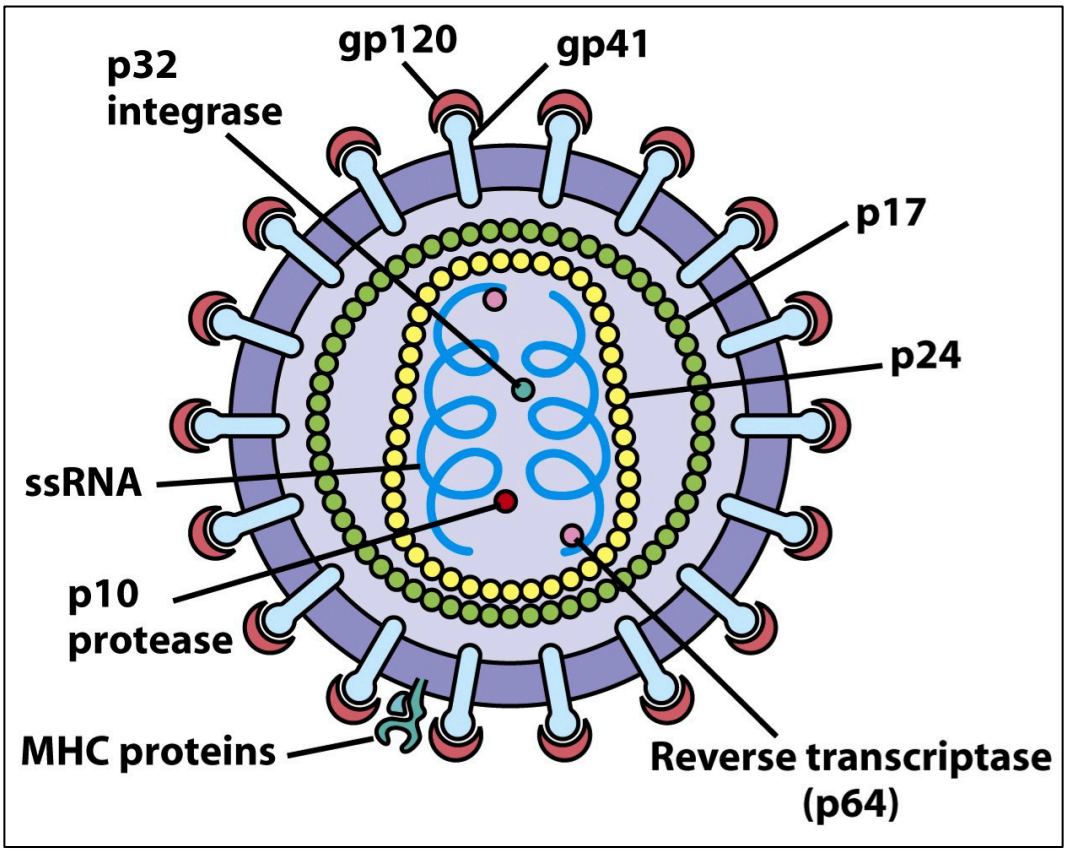


*Figure 1.3 Schematic representation of HIV-1 virion. Viral proteins are shown, located on the phospholypidic envelope (gp120, gp41), on the matrix (p17), on the capsid (p24) and at core level (protease, integrase, reverse transcriptase) where the genome is located. Moreover, cell protein are shown (MHC) as the envelope is derived from cell membrane during budding. (Adapted from Immunology, Kuby, Sixth Edition, 2007)*

enhancer/promoter sequences, transactivation response element (TAR), and polyadenylation signal (polyA). Besides the two LTRs there are other cis-acting sequences including the primer binding site (PBS); viral RNA packaging/ dimerization signals (ψ and DIS); central polypurine tract (cPPT) and the central termination sequence (CTS), leading to the formation, during reverse transcription, of a three stranded DNA structure called the central DNA Flap. In addition, there is the Rev Responsive Element (RRE) and the purine-rich region (polypurine tract; PPT), which provides a second RNA primer for the initiation of plus strand DNA synthesis by virus-specific reverse transcriptase (Srinivasakumar, 2001; Spirin *et al*., 2008). Between the LTRs, the provirus includes nine open reading frames (ORFs). The *gag-pol*, *gag* and *env* ORFs are encoding structural proteins and enzymes that are typical of all retroviruses. Additional ORFs encode for essential regulatory proteins (*tat* and *rev* genes) and accessory proteins (*vif*, *vpu*, *vpr*, and *nef* genes). There are nineteen protein products encoded by the provirus.

The *gag* gene encodes the structural proteins of the core (p7, p6), the capsid (p24) and the matrix (p17). The *pol* gene encodes enzymes crucial for viral replication, as the reverse transcriptase that converts viral RNA into DNA and has ribonuclease H (RNase H) activity (RT; heterodimer p66/p51 and RNase H), the integrase (IN; p31) that incorporates the viral DNA provirus into host chromosomal DNA and the protease (PR; p11) that cleaves large Gag and Pol protein precursors into their components. *gag* and *pol* genes are transcribed as a whole mRNA, then translated into 160 KDa protein (p160, Gag-Pol precursor), which is later cleaved by viral protease. Such cleavage determines the formation of Pol proteins and of Gag-precursor (p55), later cleaved into its elements.

The *env* gene encodes the glycoproteins in the viral envelope: the surface subunit gp120 (SU) and the transmembrane subunit gp41 (TM). They are both trimers essential for recognition of cell surface receptors and for entry into target cells. Env is translated into an 88 KDa protein that, in Golgi apparatus, undergoes to N-glycosylation with 25-30 complex residues, raising its molecular weight to 160 KDa (gp160). Env glycosylation is necessary to gain viral infectivity (Capon and Ward, 1991). Gp160 is then cleaved by cell protease furine to form gp120 and gp41 (Melikyan, 2008).

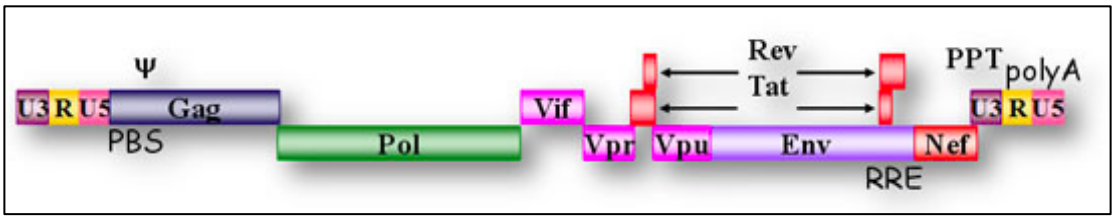


*Figure 1.4 Schematic representation HIV-1 genome. Structural genes (gag, pol, env) are represented on the genome and on the virion. Furthermore accessory genes (Rev, Tat, Nef, Vif, Vpr and Vpu) are shown on the genome, as well as special sequences like LTR (U3-R-U5), PBS (Primer Binding Site), packaging signal ψ, RRE (Rev Responsive Element), PPT (Polypurine tract) and plolyadenilation signal (polyA). (Obtained from http://tcf.epfl.ch/page-20833.html).*

The *tat* gene encodes for Tat (transactivator, p14), a 14 KDa protein that is expressed very early after infection and promotes the expression of HIV-1 genes (Perkins *et al*., 1989). Tat begins its activity by binding the TAR sequence and stabilizes the transcriptional elongation process and to increase viral genes transcription about 1000-fold. Such increase is achieved by avoiding early termination of transcription, thus permitting longer transcripts production. This is obtained through Tat association with cellular T-cyclin (Kao *et al*., 1987; Seelamgari *et al.,* 2004; Barboric & Peterlin, 2005; Bolinger & Boris-Lawrie, 2009; Cheng *et al.,* 2009).

The Rev protein (regulator of expression of viral proteins, p19), coded by the *rev* gene, ensures the export from nucleus to cytoplasm of the correctly processed messenger and genomic RNA, thus enabling the transition from early to late phase of HIV-1 genes expression. Rev can bind with high affinity and specificity to a highly structured cis-acting RNA element present within the coding region of the HIV-1 mRNA called Rev Responsive Element (RRE) (Levin *et al.,* 2009; Malim and Cullen, 1991).

The *nef* gene encodes a 27 KDa (p27) multifunctional protein with GTPasic ability. Its name (Nef) derives from studies indicating its transcription inhibiting activity (Negative Factor) (Stumptner-Cuvelette *et al.,* 2001). Moreover, such protein can bring to internalization and consequent down regulation of molecules present on cell surface as the CD4 receptor (Rhee and Marsh, 1994) and class I MHC molecules (except HLA-C and HLA-E) (Cohen *et al*., 1999), thus protecting infected cells from recognition by CD8 cytotoxic lymphocytes (Swigut *et al*., 2004) and from lysis acted by Natural Killer cells (NK). Furthermore, Nef is secreted from infected cells to act on B-cells by inhibiting immunoglobulin class switch process (Qiao *et al*., 2006). This way Nef can reduce the production of IgG, IgA and IgE, which are necessary counteract against the virus inside the organism, especially in mucous membrane and bloodstream (Seelamgari *et al.,* 2004; Anderson & Hope, 2005; Foster & Garcia, 2008).

Vpr is 15 KDa protein involved in in the arrest of the cell cycle to promote the LTR function. This protein also enables the reverse transcribed DNA to gain access to the nucleus in non-dividing cells such as macrophages (Seelamgari *et al.,* 2004; Barraud *et al.,* 2008; Oku- mura *et al.,* 2008).

Vpu is a protein necessary for the correct release of virus particle, whereas the *vif* gene codes for a small protein (Vif) that enhances the infectiveness of progeny virus particles (Seelamgari *et al.,* 2004).

## 1.4 HIV-1 replication cycle

The entry pathway of HIV-1 can be divided into three major events: virus binding to the cell, activation and fusion. The viral envelope trimeric complex, composed of the heterodimer proteins gp120 and gp41, is essential for virus recognition and entry into target cells. Indeed, the gp41 subunit contains a fusogenic hydrophobic peptide at its amino terminus, which is essential for fusion of the viral and cellular membranes. HIV gp120 binds a 58 kDa monomeric glycoprotein, known as CD4, which is expressed on the cell surface of about 60% of circulating T-lymphocytes, on T-cell precursors within the bone marrow and thymus, on monocytes/macrophages, eosinophils, dendritic cells and microglial cells of the central nervous system. The CD4 molecule normally functions as a co-receptor for the major histocompatibility complex class II molecule during T-cell recognition of a foreign antigen. Upon gp120 binding to the CD4 protein, the virus envelope complex undergoes a structural change, exposing a specific domain in the gp120 able to bind chemokine receptors on the cell membrane (figure 1.5). These molecules are recognized by chemotactic cytokines, as chemokines, small proteins that mediate the homing and recruitment of immune cells in the course of inflammation. These receptors are classified on the basis of the position of disulfide-like cysteine residues, as well as their angiogenic effects. Most common co-receptors used by HIV are CXCR4 and CCR5, but other potential co-receptors have been described (Alkhatib and Berger, 2007). The α-chemokine SDF-1 (stromal cell-derived factor 1) is the natural ligand of CXC4, whereas CCR5 is a receptor for the β-chemokine family (RANTES, macrophage inflammatory proteins MIP-1-α and MIP-1-β). CXCR4 is expressed on many cell types, including T-lymphocytes, whereas CCR5 is present on monocytes/macrophages, dendritic cells and activated T-lymphocytes.

The differential expression of chemokine receptors on cell targets has been shown to be a major determinant of the HIV-1 tropism (figure 1.6). In fact, there are HIV-1 strains preferentially binding the β-chemokine receptor CCR5 present

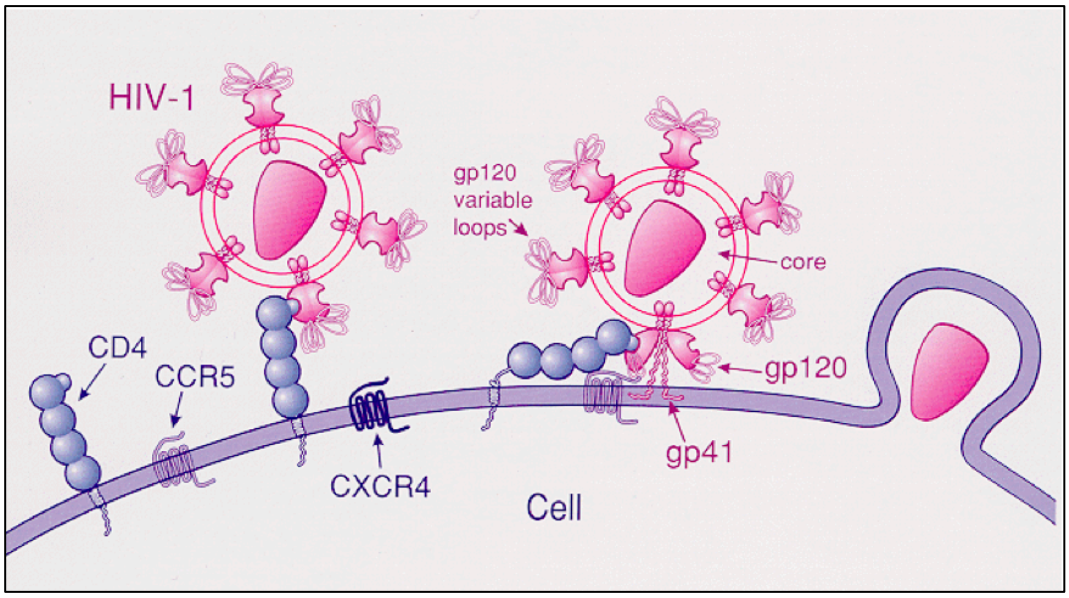


*Figure 1.5 Phases of HIV-1 fusion with cell membrane acted by gp120 and gp41. HIV-1 receptor (CD4) and co-receptors (CCR5 and CXCR4) are also shown.*

mainly in macrophages and CD4+ T-cells (Coakley *et al*., 2005). These strains are also known as macrophage-tropic (M-tropic) or R5 viruses. Conversely, other isolates use preferentially CXCR4 for entry and replicate in primary CD4+ T-cells, which also express CXCR4. These strains are known as T-lymphocyte-tropic (T-tropic) or X4 viruses. Finally, there are HIV-1 isolates that are able to bind to both CCR5 and CXCR4 receptors. These strains are termed dual tropic or X4R5 viruses.

In infected patients it is possible to find a heterogeneous viral population with different tropism. It is known how some individuals are partially resistant to HIV-1 infection thanks to a mutation called CCR5-Δ32, which is named by a deletion in the gene coding CCR5 that prevents the receptor exposure on the cell membrane. Such mutation is mainly present in Northern Europe people (Katzenstein *et al*., 1997), who result protected even when bearing functional CXCR4 molecules (Margolis and Shattock, 2006). In semen both X4 and R5 tropic viruses are present and they can be transferred during sexual contacts. Nevertheless studies reported that transmission is selective for R5 tropic viruses. Indeed, sperm cells bear CCR3 and CCR5 on their membrane but not CXCR4 (Muciaccia *et al*., 2005) and vaginal cervix epithelial cells rather stop X4 strains through mucin (Berlier *et al*., 2005; Margolis and Shattock., 2006). On the other hand it is possible to observe the development of X4 tropic and CD4-indipendent variants during late phases of a syndrome caused by an *in vivo* R5 infection (Zehrouni *et al*., 2004). These variants are strongly virulent and can cause a rapid loss of T lymphocytes and an immune system breakdown.

The double binding of gp120 to both the CD4 and one chemokine receptor allows a more stable two-pronged attachment of the virus, which, in turn, allows the N-terminal hydrophobic portion of gp41 (called fusion peptide) to penetrate the target cell membrane. The heptad repeat regions (HR1 and HR2) of gp41 interact, causing the collapse of the extracellular portion of gp41 into a hairpin. This loop structure brings the virus and cell membranes close together, allowing fusion of the membranes and subsequent entry of the viral capsid.

Following membrane fusion, the virus uncoats into the cytoplasm of the target cell freeing the viral RNA (uncoating). The conversion of viral RNA into proviral DNA takes place because of the action of the Reverse Transcriptase and the Integrase. Through its Ribonuclease H active site, the reverse transcriptase begins

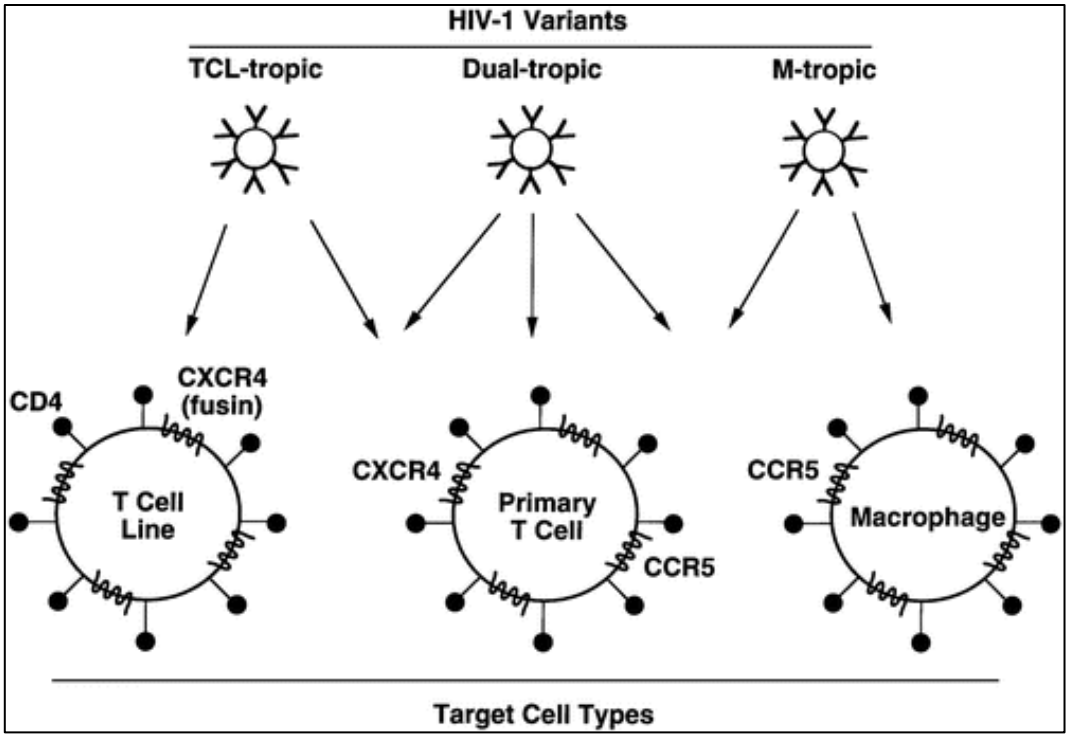


*Figure 1.6 Receptor and co-receptors of HIV-1 and relative tropisms. CD4 acts as the main receptor whereas CCR5 and CXCR4 are co-receptors. CCR5 is mainly express on macrophages/monocytes and dendritic cells. CXCR4 is expressed on many cells as T-lymphocytes (Berger et al., 1999).*

the reverse transcription of viral RNA in the cytoplasm that occurs as a minus-strand polymerization, starting at the primer-binding site, until viral RNA is transcribed into a RNA/DNA hybrid double helix. Then, the ribonuclease H site breaks down the RNA strand and the polymerase active site of the reverse transcriptase completes a complementary DNA strand to form a double helix DNA molecule, which is integrated within the cell genome by the viral enzyme Integrase. Acting on LTR sequences, this protein cleaves nucleotides of each 3' ends of the double helix DNA, transfers the modified provirus DNA into the cell nucleus and facilitates its integration into the host genome. The integration of proviral DNA and the expression of the provirus require that target cell is in an activated state. Indeed highly transcribed regions, as genes, are preferential hotspot for integration (Schröder *et al*., 2002). Monocytes/macrophages, microglial cells, and latently infected quiescent CD4+ T-cells contain integrated provirus and are important long-living cellular reservoirs of HIV-1 (Chun *et al*., 1997). Upon cell activation, transcription of proviral DNA into a messenger RNA occurs. Transcription process initially results in the early synthesis of regulatory HIV-1 proteins such as Tat and Rev.

Viral messenger RNA coding for long fragments migrates into the cytoplasm, where structural proteins of new virions are synthesized. The proteins coded by *pol* and *gag* genes form the nucleus of the maturing HIV particle; the gene products coded by the *env* gene form the glycoprotein spikes of the viral envelope. The cleavage of the precursor molecules by the HIV-1 Protease is necessary for the generation of infectious viral particles. The formation of new viral particles is a stepwise process: two viral RNA strands associate together with replication enzymes, while core proteins assemble over them forming the virus capsid. This immature particle migrates towards the cell surface. The large precursor molecules are then cleaved by the HIV-1 protease, resulting in new infectious viral particles, which bud through the host cell membrane, thus acquiring a new envelope. During the budding process, the virus lipid membranes may incorporate various host cell proteins and become enriched with phospholipids and cholesterol. Differently from T-lymphocytes, where budding occurs at the cell surface and virions are released into the extracellular space, the budding process

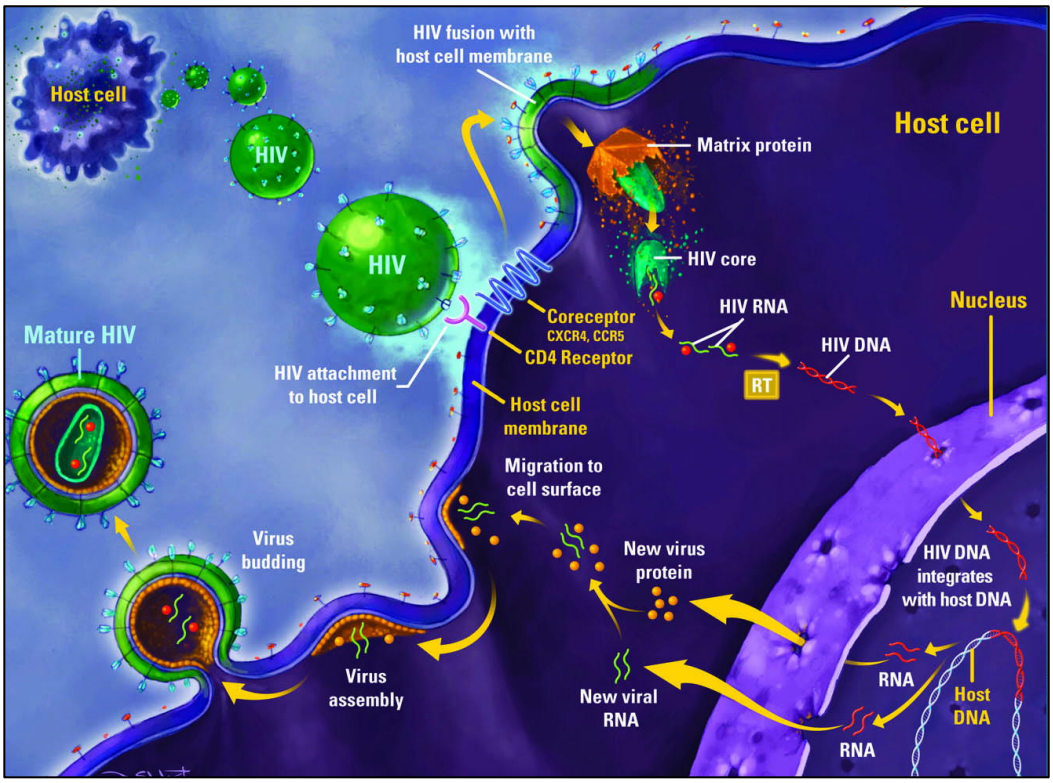


*Figura 1.7 Simplified design of HIV-1 replication cycle (HIV Web Study – www.HIVwebstudy.org)*

in monocytes and macrophages results in the accumulation of virions within intracellular vacuoles, which are then released.
HIV-1 replication cycle is schematically shown in figure 1.7

## 1.5 HIV-1 vaccine: state of the art

UNAIDS (United Nations programme on HIV/AIDS) estimates that 34 million people now live with HIV-1 infection, and 2 million become newly diagnosed each year (figure 1.8). Currently available therapy is called HAART (Highly Active Antiretroviral Therapy) and, by means of antiretroviral drugs, it can suppress viral replication, increasing life expectancy among those infected, but cannot cure infection. Several modalities can reduce HIV-1 infection rates in persons at risk of exposure, including screening of blood donors, condom usage, male circumcision, pre-exposure to chemoprophylaxis and finally, use of vaginal gels, like Tenofovir, containing microbicides (Bailey *et al*., 2007; Grant *et al*., 2010; Karim *et al*., 2010). Although these interventions may reduce susceptibility to the virus, in order to limit HIV-1 diffusion a highly efficacious preventive vaccine would be desirable. Since 1987, more than 30 candidate HIV-1 vaccines were tested on non-human primate models and advanced to human clinical trials (Ross *et al*., 2010). These candidates were based on exploitation of envelope proteins (VAX trial) (Pitisuttithum *et al*., 2006; Flynn *et al*., 2005), of first generation human adenoviral vectors expressing *gag*, *pol* and *nef* genes (STEP trial) (Gray *et al*., 2010), and of vectors, derived from canaripox, coding for clade B and E envelope proteins (RV144 trial) (Rerks-Ngarm *et al*., 2009).
In particular, STEP trial, did not prove to be effective in limiting HIV-1 infections and reducing post-infection viremia. O'Brien *et al*. (2009) demonstrated that, in subjects enlisted in the trial, presence of anti Ad5 antibodies, directed against capsid proteins, and against proteins expressed by the genome of used adenoviral vector, implies a decreased immunogenicity of the vector and a potential risk of increased HIV-1 infection (Buchbinder *et al*., 2008). RV144 trial resulted, on the other hand, partially effective (31%) in reducing infective capacity of HIV-1. Even administration of Tat protein to volunteers (Ensoli *et al*., 2010), seems to induce an increase of CD4+ and CD8+ T cells, improving HAART effectiveness,

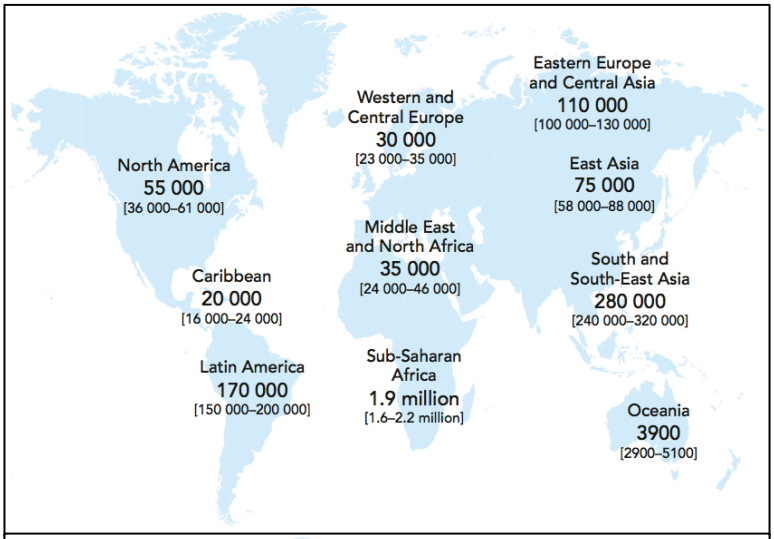


*Figure 1.8 A Estimated number of adults and children newly infected with HIV, 2008 (Obtained from UNAIDS).*

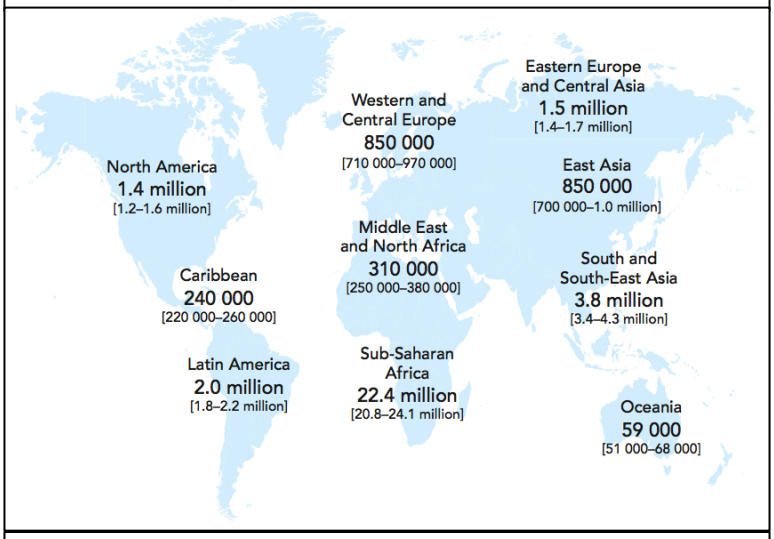


*Figure 1.8 B Adults and children estimated to be living with HIV, 2008 (Obtained from UNAIDS).*

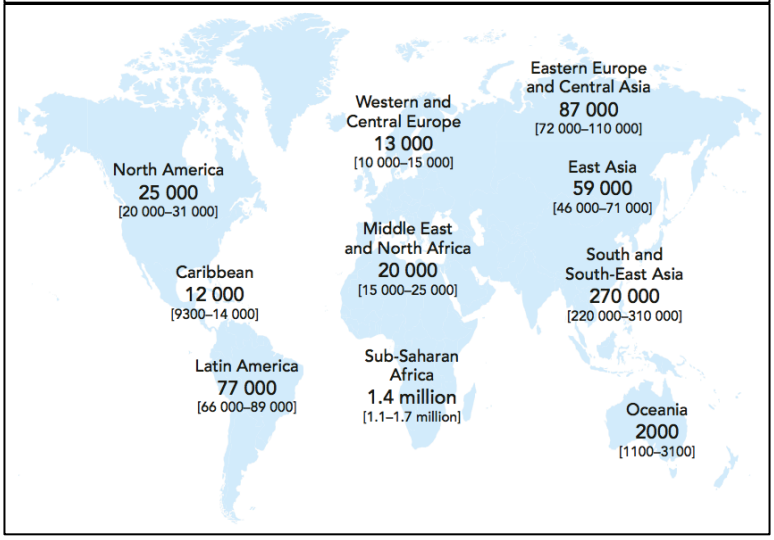


*Figure 1.8 C Estimated adult and child deaths due to AIDS, 2008 (Obtained from UNAIDS).*

by containment and reduction of viral progression. Nevertheless, to date a protective vaccine that could induce both a strong mucosal immunity, block the virus on its site of infection, and neutralize antibodies against HIV is still missing, and there are many difficulties in generating it. Variability is the main problem, allowing the virus to overcome host immunity and the effects of therapeutic (drugs) and prophylactic (vaccines) interventions (Menéndez-Arias, 2002). HIV variability is a consequence of at least three peculiar features: 1) the "error-prone" mechanism of action of the virus enzyme reverse transcriptase, that introduces, on average, one substitution per genome per replication round (Sarafianos *et al*., 2009); 2) the very rapid viral replication, that generates a high number of virions per day (estimated around $10^{10}$) in the infected individual (Ho, 1997) and 3) the occurrence of recombination processes between two or more different HIV-1 viruses within the same infected individual. Another major obstacle in HIV-1 vaccine development is that conserved Env epitopes, capable of stimulating broad spectrum neutralizing antibodies, are poorly immunogenic because they are masked by carbohydrates, and exposed only transiently during infection phases.

## 1.6 Fusion complexes

In order to obtain broad-spectrum highly neutralizing antibodies against HIV-1, which are essential for vaccine production, the study of new immunogens is extremely important. In particular, a strong humoral response against viral conserved regions would be advisable.

As already explained, HIV-1 infects host cells by membrane fusion, mediated by the envelope glycoproteins gp120/gp41, the CD4 receptor and the co-receptors CCR5 and CXCR4. Fusion is triggered during co-receptor binding, when the hydrophobic ''fusion peptide'' region of gp41 is exposed and inserted into the membrane of the target cell. The triple-coiled trimeric structure of gp41 exists in both the pre-hairpin and the 6-helix bundle configuration (Doms and Moore, 2000). Both conformations are fusion intermediates. The transition from the pre-hairpin to the 6-helix bundle configuration may determine the apposition of the viral envelope and the cellular membrane (Melikyan *et al*., 2000) and the mixing of the lipid bilayers (Kliger *et al*., 2000), resulting in the fusion of the 2 membranes, viral entry into the target cell and initiation of infection. During the fusion process, conserved epitopes are exposed that may be necessary for

induction of virus neutralizing antibodies. It is known that, while CD4-induced conformational changes in the gp120/gp41 occur over a wide temperature range (Sullivan *et al*., 1998), the insertion of the fusion peptide requires a temperature higher than 25°C (Doranz *et al*., 1999). The transition between the pre-hairpin and the 6 helix bundle is blocked at 21°C (Melikyan *et al*., 2000) and the extension of the fusion pore does not occur if the temperature is below 31°C (Golding *et al*., 2002).

Our group exploited these features to produce fusion complexes that were used to immunize mice and produce neutralizing antibodies (Zipeto *et al*., 2006). CHO cells expressing CD4-CCR5-CXCR4 were co-cultivated with CHO cells expressing HIV-1 *env* gene. Cells co-cultivation at sub-optimal temperatures allowed the formation of fusion intermediates that expose conserved epitopes. Fusion complexes were ‘‘synchronized’’, using different temperatures and different types of fixation, at intermediate conformations on which conserved epitopes might be exposed. They were then used to determine which conditions resulted in the best induction of HIV-1 neutralizing antibodies. Mice were immunized with fusion complexes and later they were sacrificed, and their serum was harvested. Antibodies were purified from serum and analyzed for reactivity against viral epitopes and for eventual cytotoxic effect against cells. Fusion complexes resulted to be immunogenic in mice and reactivity was proved not to be due to antibodies directed against cell receptors.

Additional mice were then immunized with the fixative/temperature combination giving the highest neutralizing activity, and using the myeloma cell fusion technique, several hybridomas were produced.

## 1.7 Immunoglobulins

Immunoglobulins are a group of globular glycoproteins found in the serum and body fluids and play a vital role in the immune response. Since they are capable of specifically recognizing and binding to antigens, they are also known as antibodies. Immunoglobulins are also found on the surface of B cells where they are inserted through the cell membrane (mIg). When B cells are activated by antigens, they proliferate and may differentiate into plasma cells, which produce and secrete large amounts of antibody. This antibody binds the same antigen as the mIg on the B cell from which the plasma cell was derived (i.e. they have the

same binding specificity).

In humans and rodents there are five major classes of immunoglobulin (IgG, IgA, IgM, IgE and IgD), which differ from each other in size, charge, amino acid sequences and carbohydrate content. Within the classes, there are distinct differences (heterogeneity) and subclasses can be distinguished, (e.g. IgG1, IgG2, IgG3 and IgG4 in human and IgG1, IgG2a, IgG2b and IgG3 in mice). The number of subclasses varies depending on the host species.

The basic structure of all immunoglobulins consists of two pairs of chains (heavy and light, 50-60 kDa and 23 kDa respectively) linked together by, disulphide bonds (figure 1.9). The heavy chains dictate the class of immunoglobulin, which are μ chains in IgM, γ chains in IgG, α chains in IgA, ε chains in IgE and δ chains in IgD. By contrast, the light chain types are the same for all immunoglobulin classes. Each immunoglobulin molecule contains light chains of one of two types, either lambda (λ) or kappa (κ). These chains are antigenically distinct and only one type of light chain is present in any single antibody molecule. They are usually free of carbohydrate components. The ratio of κ/λ chains in human immunoglobulins is about 60:40, whereas in mouse, rat and rabbit immunoglobulins it is about 95:5. The four-chain structure is seen in IgG, IgD and IgE. By contrast, IgA occurs in both monomeric and polymeric forms (comprising more than one basic four-chain unit structure) whilst IgM occurs as a pentamer with five basic units. Each of the chains comprises a number of globular regions (called domains) formed by intra-chain disulphide bonds. At the amino terminal of both the heavy and light chains is a single variable region or domain (VL or VH) consisting of 110 amino acids; such region is essential for the recognition and the binding to antigens. The variable regions of one light chain and one heavy chain form one of the antigen-binding sites of the antibody. The number of heavy chain constant regions (CH) in an antibody molecule varies; being three in IgG, A and D and four in IgM and E. Light chains have only one constant region (CL). These regions are called constant because there is very little difference in the secondary and tertiary structure of the proteins in these areas.

There are several genes that code for immunoglobulins and unusually each antibody chain molecule is the product of more than one of these. The variable region is coded for by a V gene and the constant region by a Cγ, Cμ, Cα, Cε or Cδ

gene.

Individual CH genes are organized such that different exons code for the structural domains of the protein. Thus, the C genes contain four exons to code for the four constant domains of the μ chain. Each of the Cγ genes has an additional small exon between the first and second CH domain exons that encodes the hinge region. The Cδ gene is organized somewhat differently from the other H chain genes in that it contains an extended hinge and lacks a CH2 domain.

Each antibody class may exist in either a secreted or membrane-bound form. With the exception of IgD, the carboxy-terminal sequences of the secreted forms are contiguous with the terminal C region domain. By contrast, the unique sequence of the membrane-bound forms is encoded by an exon (or exons) downstream of the terminal CH exon. This sequence comprises a series of 26 hydrophobic amino acids, which spans the plasma membrane and a hydrophilic, cytoplasmic tail varying in size from 3 to 28 amino acids. For IgD, the exons for both the secreted and membrane-bound forms of the molecule are separated. The production of membrane-bound or secreted immunoglobulin is probably regulated at the level of RNA processing.

V region genes code for the variable regions of the antibody. Specific V region genes associate with a constant region product of either a heavy chain or a light chain, i.e. VH with CH and VL with CL gene products. However, any of the products of the CH genes may associate with any of those of the VH genes.

Kappa chains have V region subgroups differing in the number and position of amino acid substitutions and deletions. These molecules share a degree of structural similarity, which distinguishes them from lambda V or VH region products. Similar subgroups also exist for lambda and heavy chain V regions.

All V gene products have regions that are relatively conserved, and it is these that define the type of the V gene product (VH or VL) and the subgroup to which it belongs. In addition, they have extremely variable zones or ‘‘hot spots’’. These are the hypervariable regions, which are involved in the formation of the antigen-binding site. Light chains have three hypervariable regions whilst heavy chains have four.

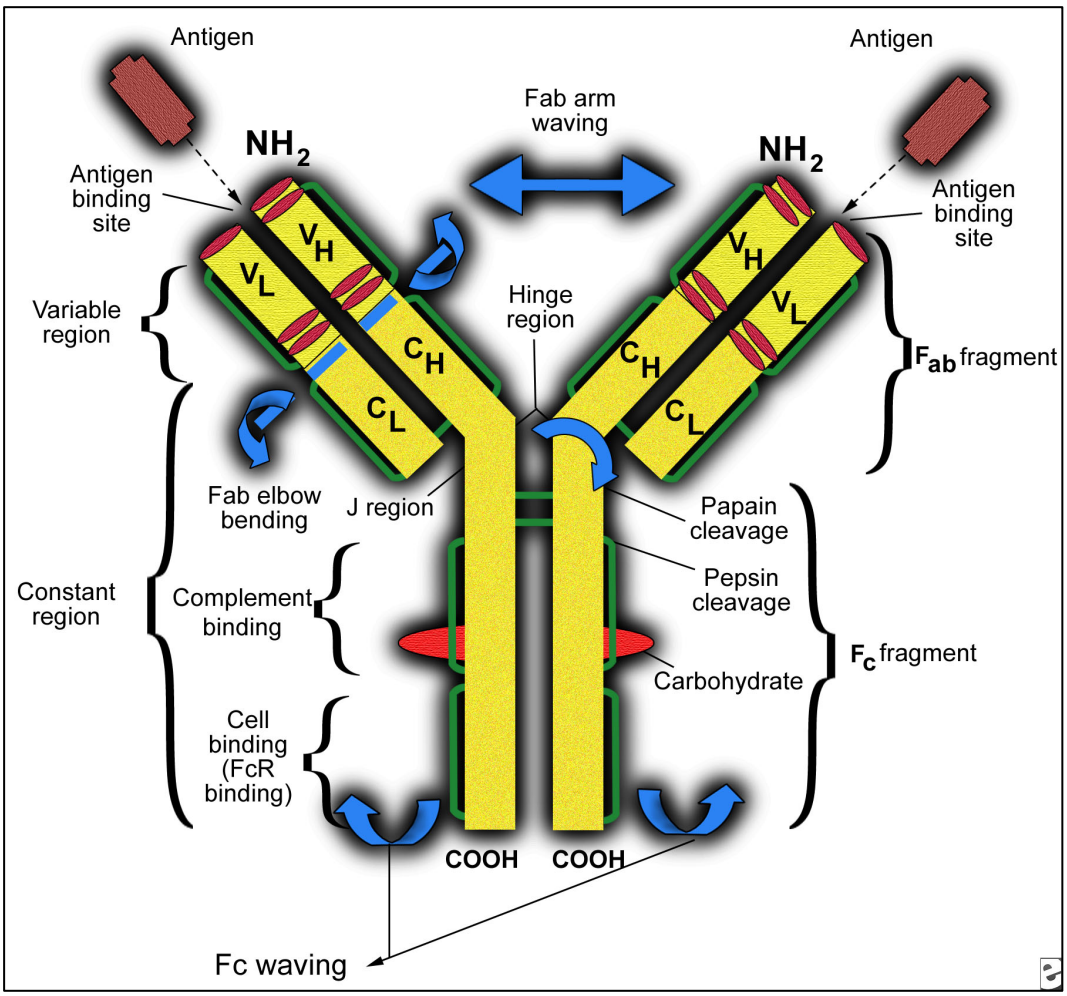


*Figure 1.9 Schematic representation of IgG structure (Obtained from http://emedicine.medscape.com/article/136897-overview).*

All immunoglobulins are covalently bonded to carbohydrates in the form of simple or complex side-chains. This carbohydrate may assist in secretion of the antibody by plasma cells and may affect the biological functions of the molecule, which are associated with the constant regions of the heavy chains. Relying on enzymatic digestions acted either by papain or pepsin, in immunoglobulins it is possible to distinguish two functional domains: the Fab fragment (Fragment antigen binding), where the antigen specificity of the molecule resides, consists of the two VH–CH1 domains and the two VL–CL domains; the Fc fragment (Fragment crystallizable) consists of the CH2 and CH3 domains (and CH4 in IgM and IgE) of both heavy chains (figure 1.9). The hinge region binds these two fragments. As its name suggests, this area allows slight relative motion between the two fragments. This mobility is important in antigen binding. The hinge region contains a high number of proline and cysteine residues; the latter form inter-chain disulphide bonds, which maintain the integrity of the molecule. These bonds also prevent folding in this area making it especially vulnerable to enzymatic cleavage. The number of these disulphide bonds varies between classes and subclasses of antibody (Bengten *et al*., 2000).

### 1.7.1 Immunoglobulin G

IgG (figure 1.9) comprises 70–75% of the circulating immunoglobulins. IgG has a molecular weight of 146 kDa, it is the major immunoglobulin produced during a secondary immune response and is the only antibody with antitoxin activity. As already said, both humans and mice produce four subclasses of IgG. These molecules vary in molecular weight, the number and position of inter-chain disulphide bonds and in some functional properties. Many cells (including monocytes/macrophages, B cells, NK cells and some T cells) bear molecules on their surface, which bind IgG through the Fc region. These are known as Fc receptors. Fc receptors on macrophages, some T cells and killer cells allow these cells to bind antibodies attached to specific antigens on cells (target cells) and can lyse these target cells through a mechanism known as antibody-dependent cellular cytotoxicity (ADCC). IgGs are also found in mucous membrane secretions and therefore have a role in immunity to infection at mucosal surfaces (Bengten *et al*., 2000).

## 1.8 Lentiviral Vectors

Viral vectors have gained their popularity in basic research and gene therapy applications because of their high rates of gene transfer, that are far superior to those achieved with non-viral methods. Numerous types of virus-derived gene transfer systems are available to date. These genetic vehicles are based either on DNA viruses or on RNA viruses. The corresponding vectors either integrate into the host genome or express their genetic information episomally. Retroviral vectors, due to their ability to integrate into the host DNA, are widely used in both cell biology and biomedicine. Retrovirus-based systems (Buchschacher, 2001), unlike episomal viruses (herpes simplex virus — HSV, adenovirus), offer stable and long-term transgene expression. A serious drawback of using retroviral vectors in clinical trials is their natural disposition to integrate near promoters and regulatory regions (Wu *et al.,* 2003; De Palma *et al.,* 2005; Tsukahara *et al.,* 2006; Deichmann *et al.,* 2007; Daniel & Smith, 2008) and to induce insertional tumors due to the presence of potent transcriptional enhancers in viral long terminal repeats (LTRs). Other limitations to the application of retroviral vectors are their ability to insert only relatively small expression cassettes and their low production titer. Moreover they can infect only dividing cells. This is because viral preintegration complex (PIC), a large nucleoprotein complex responsible for viral cDNA integration, cannot enter the cell nucleus and requires disassembly of the nuclear envelope during mitosis (Roe *et al.,* 1993; Lewis & Emerman, 1994). Some of these drawbacks can be avoided by using lentiviral vectors. Lentiviruses have evolved different strategies to interact with the host cell chromatin and do not integrate preferentially into close proximity of transcription start sites but rather favor introns in chromosomal regions rich in expressed genes (Schroder *et al.,* 2002; Mitchell *et al.,* 2004; De Palma *et al.,* 2005; Laufs *et al.,* 2006; Yang *et al.,* 2008a; Felice *et al.,* 2009; Wang *et al.,* 2009). Other advantages of lentivirus-based vectors, important for research and gene therapy applications, are their ability to infect both dividing and non-dividing cells, relatively large cargo capacity (7–8 kb without affecting vector titer) and target specificity achieved by pseudotyping. These features make lentiviral vectors promising agents in research and medicine. Consistently with this information, a HIV-1 based lentiviral vector is an optimal solution to integrate the transgenes of interest. Nevertheless, when using HIV-1-based vector it is imperative to develop a vector-production system

that minimizes the risk of reconstituting a replication-competent lentivirus (RCL). First-generation lentiviral vectors were produced by transient co-transfection of the packaging plasmid, which also contained the transgene, and of the envelope plasmid; therefore a single recombination event could determine the formation of a RCL. For this reason the viral genome was split into multiple fragments to minimize the potential formation of replication-competent viruses; hence, second- and third-generation lentiviral vectors were developed. Second-generation lentiviral vectors need transient co-transfection of plasmids separately expressing the transfer vector genome, the viral structural components (Gag, Pol and Rev), and a heterologous envelope protein. Each of these components have undergone several refinements in recent years, with due emphasis being put on developing vector systems capable of producing high titer supernatants, but also having improved biosafety profiles. Indeed, in third-generation vectors, the structural components are divided in two constructs expressing Gag-Pol (the former) and Rev (the latter) and are both co-transfected with the envelope plasmid and the transfer vector.

In both second- and third-generation vectors, the transfer (integrating) vector contains two main regions that express the viral RNA genome following transfection during vector production: *1*) a multiple cloning site for the insertion of various expression cassettes and *2*) flanking long-terminal repeats (LTRs) that have several distinct functions. First, the 5'-LTR can act like an RNA pol II promoter. Second, the 3'-LTR acts to terminate transcription and promote polyadenylation. Third, the LTR has recognition sequences necessary for integration into the genome. Moreover the vector presents important *cis*-acting DNA elements, as the central polypurine tract sequence (cPPT) and, eventually the woodchuck post-regulatory element (WPRE), which enhance the transduction efficiency and transcript stability, respectively (Follenzi *et al*., 2000; Park and Kay, 2000; Zufferey *et al*., 2004; Zennou *et al*., 2000). The cPPT is a small DNA fragment found in the *pol* gene of HIV that is usually cloned 5' to the internal promoter region, whereas the WPRE is cloned 3' to the inserted transgene so that it is in close proximity to the poly(A) signal in the 3'-LTR. An important feature in the third-generation lentiviral transfer plasmid is a 400-bp deletion in the U3 region of the 3'-LTR, which debilitates the 5'-LTR RNA pol II promoter activity following integration (Park and Kay, 2000; Zufferey *et al*., 1998). Since this

deletion renders the LTR largely transcriptionally inactive (Zufferey *et al*., 1998), it also provides important features to SIN vectors: the vector has a decreased chance of insertional mutagenesis due to transcription from the 3′ LTR of the integrated provirus; the lentiviral vector becomes Tat-indipendent. This is a particular advantage as Tat expression is toxic for cells.

# 2. AIM OF THE WORK

This work is focused on the stabilization of the production of broad-spectrum neutralizing antibodies against HIV-1. Mice were immunized with fusion complexes obtained by co-cultivation of cells expressing HIV-1 envelope proteins (gp120-gp41) with cells expressing receptors used by the virus to enter host cells (CD4-CXCR4-CCR5). Cell membranes fusion was blocked using non-permissive temperatures and various fixatives. Mice sera were analyzed for IgG concentration. Fusion complexes demonstrated to be strong immunogenic agents and were used for immunization of additional mice. Mice spleen cells were used to produce hybridomas in combination with myeloma cell line X63.Ag8.653 (mouse myeloma).

This work can be divided into two parts:

1) Isolation and of new neutralizing antibodies directed against HIV-1
2) Production of viral vectors expressing humanized light and heavy chains of IgG, and use of such vectors to obtain a cell line stably expressing humanized IgG.

Starting from hybridoma supernatants the aim of the work was to identify broad-spectrum neutralizing antibodies against HIV-1 and to stabilize their expression. An additional objective was to humanize such antibodies in order to have a better relation with control antibodies (Tri-Mab) used for neutralization assays, and to use them for possible therapeutic purposes. In order to do this, human IgG constant regions (heavy and light) were cloned into two different viral vectors, in which later murine variable regions, obtained from mentioned mice hybridomas, were added. Such vectors were used to transduce CHO cells, which were then cloned to single cell populations in order to obtain a cell line stably expressing humanized IgG. The functionality of the produced antibody was then tested.

# 3. MATERIALS AND METHODS

## 3.1 Eukaryotic Cell lines

The TZM-bl cell line consists of engineered HeLa cells expressing the HIV-1 receptors (CD4, CXCR4, CCR5) and two different reporter genes coding for *Photinus pyralys* luciferase enzyme (*luc* gene) and for beta-galactosidase (*LacZ* gene), both placed under control of HIV-1 LTR. These cells are maintained under selective conditions (G418, final concentration 50 μg/ml) in complete Dulbecco's Modified Eagle Medium (DMEM - 50% Fetal Bovine Serum, 1% glutamine, 1% penicillin/streptomycin). Variants of such cell line (TZM-bl FcγRI and TZM-bl FcγRIIb) were also used.

CHO is a cell line derived from the ovary of an adult Chinese hamster (*Cricetulus griseus*). CHO cells are used in studies of genetics, toxicity screening, nutrition and gene expression, particularly to express recombinant proteins. Today, CHO cells are the most commonly used mammalian hosts for industrial production of recombinant proteins.

293T/17 cells derive from HEK-293 (Human Embryonic Kidney) and constitutively express the simian virus 40 (SV40) large T antigen. They are used to produce retroviruses, pseudoviruses and viral vectors.

3T3 cells come from a cell line established in 1962 at the Department of Pathology in the New York University School of Medicine. The 3T3 cell line has become the standard fibroblast cell line but it was originally obtained from Swiss mouse embryo tissue.

## 3.2 Pseudovirus production

### 3.2.1 Transfection of 293T/17 cells

3 x $10^6$ 293T/17 cells were seeded in a 100 mm dish containing 10 ml of complete DMEM (FBS 10%, Penicillin-streptomycin 5%, Glutamine 5%) and incubated overnight to show a monolayer confluence of about 50-80% on transfection day. In a sterile tube, 1,5 μg of Env plasmid DNA and 3 μg of backbone plasmid DNA (pSGΔenv) were added to the appropriate volume of pure DMEM (serum-free), so that the total volume of the mixture were 33,5 μl. Into a second sterile tube, 201,3

µl of pure DMEM were mixed with 16 µl of FuGENE 6 reagent (Roche). The entire content of the plasmid DNA mixture from the first tube was transferred to the second tube containing the FuGENE solution, mixed by pipetting and incubated 30 minutes at room temperature to allow the complex formation. The entire amount of transfection complexes was added to 293T/17 cells, seeded the day before. The cells were incubated for about 5 hours at 37°C in a 5% $CO_2$ incubator to allow the entrance of the plasmids into the cells; later the medium containing DNA-FuGENE complexes was decanted and replaced with 5 ml of fresh complete DMEM. The transfected cells were incubated at 37°C, 5% $CO_2$ for 48 hours. The culture supernatant containing pseudovirus was harvested and the FBS concentration in the virus-containing culture medium was adjust to 20%. It was then filtered through a 0.45-µm filter, distributed into aliquots of appropriate volume and stored at -80°C.

### 3.2.2 Pseudovirus titration (TCID50 Assay)

A volume of 100 µl of grow medium per well was placed in all wells of a 96-well flat-bottom culture plate. An amount of 25 µl of pseudovirus was placed into the first 4 wells of the first column and mixed; 5-fold serial dilutions were performed by transfer of 25 µl from each well to the next one, for a total of eleven dilutions. From the 11th dilution, 25 µl were discarded. Wells in column 12 served as cell controls (no virus added). A volume of 100 µl of TZM-bl cells (10.000 cells/100 µl DMEM containing 25 µg DEAE dextran/ml) was added to all wells, to obtain a DEAE-Dextran final concentration of 10 µg/ml. The cells and pseudovirus were incubated at 37°C, 5% $CO_2$ for 48 hours.

A volume of 100 µl of culture medium was removed from each well, leaving approximately 100 µl. Steadylite Plus Reagent (100 µl) (Perkin Elmer) was dispensed to each well and incubated at room temperature for 2 minutes to allow complete cell lysis. The solution was mixed with the cells and 150 µl were transferred to a corresponding 96-well black plate to read the plate in a luminometer (Victor3 – Perkin Elmer – 482 nm). The TCID was calculated using the Montefiori's TCID50 macro.

(*http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm*)

### 3.3 Neutralization assay

A volume of 150 µl of growth medium was placed in all wells of column 1 of a 96-well flat-bottom culture plate. This column was used as cell control. Aliquots of 100 µl of growth medium were placed in all wells of column 2 (column 2 will be the virus control) and 40 µl were added in all wells of columns 3-12. A volume of 11 µl of test samples was added to each well in column 3 and 4 of row H. To test other samples, 11 µl of each sample were added to each well in columns 5 and 6, 7 and 8, 9 and 10, 11 and 12 of row H.

The samples were mixed in row H and 50 µl were transferred to row G. The transfer was repeated serially, diluting samples through the columns to row A (these are serial 3-fold dilutions). After final transfer and mixing were completed, 50 µl were discarded from the wells in columns 3-12, row A.

A vial containing the pseudovirus preparation was thawed in a room temperature water bath and the pseudovirus was diluted in grow medium to achieve a concentration of 2000 TCID50/ml. A volume 50 µl of cell-free virus (100 TCID50) was dispensed to all wells in columns 2-12, rows A through H. The plate was incubated at 37°C, 5% CO2 for 1 hour.

A suspension of TZM-bl cells, at a density of 1 x $10^5$ cells/ml in growth medium, containing DEAE dextran (37.5 µg/ml) was prepared. A volume of 100 µl of cell suspension (10000 cells per well) was dispensed to each well in columns 1-12, rows A through H, to obtain a DEAE dextran final concentration of 15 µg/ml. The plate was incubated at 37°C, 5% $CO_2$ for 48 hours.

Part of culture medium (150 µl) was removed from each well, leaving approximately 100 µl. A volume 100 µl of Steadylite Plus Reagent (Perkin Elmer) was dispensed to each well and incubated at room temperature for 2 minutes to allow complete cell lysis. After mixing, 150 µl of solution were transferred to a corresponding 96-well black plate and read in a luminometer (Victor3 – Perkin Elmer – 482 nm).

The neutralization percentage was determined according to following formula:

$$\left[1 - \frac{RLU_{(cells+serum\ sample+viruus)} - RLU_{(cells\ \text{only})}}{RLU_{(cell+virus)} - RLU_{(cells\ \text{only})}}\right] x100$$

## 3.4 Concentration of antibody supernatants

When necessary, supernatants of antibody producing clones were concentrated using centrifugal filter units (Amicon Ultra 50 kDa 15 ml – Millipore). Supernatants were centrifuged 30 minutes at 4000 g.

## 3.5 Production of cells expressing humanized IgG

### 3.5.1 Lentiviral Vectors Production

5 x $10^6$ 293T/17 cells were seeded in a 10 cm dish, approximately 24-30 hours before transfection and incubated at 37°C, 5% $CO_2$.

The plasmid DNA mix was prepared by adding 3 μg ENV plasmid, 5 μg Packaging plasmid (pMDLg/p RRE or CMV R8.74), 2,5 μg of pRSV-REV and 10 -15 μg of either pTY2-CMV-humanized_IgG-IRES-PURO-WPRE or pTY2-CMV-humanized_IgK-IRES-NEO together. The plasmid solution was made up to a final volume of 450μl with 0,1 XTE/$dH_2O$ (2:1). Finally 50 μl of 2,5M $CaCl_2$ was added and the solution was incubated 5 minutes at RT.

The precipitate was formed by dropwise addition of 500 μl of a 2x HBS solution to the 500 μl DNA-TE-$CaCl_2$ mixture while vortexing at full speed. The precipitate was added to the 293T/17 cells immediately following the addition of the 2X HBS. The CaP-precipitated plasmid DNA was allowed to stay on the cells for 14-16 hours, after which the medium was replaced with fresh medium for virus collection to begin.

The cell supernatant was collected 24 hours after change of medium, filtered through a 0,45- μm filter and distributed into aliquots of appropriate volume and stored at -80°C.

### 3.5.2 Cells co-transduction

5 x $10^6$ CHO cells were seeded in a 10 cm dish, approximately 24-30 hours before transduction and incubated at 37°C, 5% $CO_2$. A volume of 200 μl of both freshly prepared viral vectors (expressing humanized light and heavy chains) was added to cell medium. After 48 hours from co-infection cells were placed under selective conditions by adding neomycin (100 μg/ml) and puromycin (5 μg/ml). After one week of selective conditions the protocol proceeded to single cell cloning.

## 3.6 ELISA Assays

IgG concentration of hybridomas supernatants was detected using Mouse-IgG Elisa Kit (Roche). IgG concentration of supernatants of humanized IgG producing CHO cells was detected with Human IgG FastELISA Kit (RD Biotech). Both assays were performed following manufacturer's instructions.

### 3.6.1 ELISA on whole cells

An amount of $5x10^4$ 3T3.T4.CCR5 cells was seeded in complete DMEM in a 96 well flat-bottom culture plate approximately 24 hours before the assay and incubated at 37°C, 5% $CO_2$. Cells were washed three times in PBS without calcium and magnesium and fixed with 0,2% PFA, O/N at 4°C. After PBS wash, cells were treated 10 minutes at room temperature with glycine 100 mM pH 7 (100 µl/well) and washed again. Cells were blocked in PBS milk 4% for 2 hours at 37°C and washed twice with PBS-tween 20 0,1%. Antibodies from mouse sera were diluted in PBS-BSA 2% and 50 µl of samples were added in duplicate in each wells and incubated 90 minutes at room temperature. After three washes with PBS tween 20 0,1%, a 1:3000 dilution in 1% BSA of a goat-anti-mouse horse radish peroxidase conjugated antibody (Dako, Denmark) was incubated for 60 min at room temperature, plates were washed with T-PBS and developed using the Sigma Fast OPD Peroxidase Substrate (Sigma). The developed color was read at $OD_{490}$.

## 3.7 Nucleic acids extractions from eukaryotic cells

### 3.7.1 Genomic DNA extraction

An amount of 1 x $10^6$ CHO cells was seeded in a 60 mm dish, approximately 24-30 hours before extraction and incubated at 37°C, 5% $CO_2$. A suspension of trypsinized cells was prepared and 750 µl of Lysis Buffer and 30 µl Proteinase K were added. The cells were incubated overnight at 55°C with gentle shaking. A spin of 8 minutes at 4°C was performed and the supernatant was collected into a clean tube. A volume of 250 µl of saturated NaCl (~6M) was added and the solution was mixed by inversion to precipitate the proteins. The sample was centrifuged at max speed in a microcentrifuge (~12600 g) for 10 minutes at 4°C to spin down the proteins. The supernatant was collected into a clean tube, avoiding transfer of the white precipitate. A volume of 500 µl of isopropanol was added

and the solution was mixed by inversion until a white thready material was visible. A max speed centrifuge (~12600 g) of 10 minutes at 4°C was carried out and the pellet was washed with 70% EtOH (100-800 µl). The genomic DNA was air-dried for 45-60 minutes and dissolved in 100µl $H_2O$ containing RNase. $OD_{260}$ or $OD_{260/280}$ was measured to determinate the genomic DNA concentration.

### 3.7.2 RNA extraction and RT-PCR

Templates for human constant and murine variable IgG regions were obtained from human PBMC and from clones isolated from hybridomas. Total RNA was extracted from cells using miRNeasy mini kit with RNase-free DNase set (Qiagen) following manufacturer's instructions. RNA concentration was measured using a spectrophotometer (Nanodrop). GoScript RT kit (Promega) was used to reverse transcribe total RNA to cDNA with random primers. Manufacturer's instructions were followed using samples of 4 µg RNA.

## 3.8 Bacterial protocols

### 3.8.1 Bacterial transformation

Calcium chloride competent Top10 cells (E. coli) were thaw on ice for 5 minutes. 50 µg of circular DNA was added to cells and gently stir with tip. The cells were incubated on ice for 30 minutes to thaw competent cells. The heat shock was obtained putting the tube with DNA into water bath at 42°C for 20 seconds. The tube was put back on ice for 2 minutes to reduce damage to the cells. One ml of LB medium (with no antibiotic added) was added to the cells and incubated for 1 hour at 37°C. Cells were harvested at 5000 rpm for 5 minutes and about 100-150 µl of the resulting culture was spread on warmed LB plates (with the appropriate antibiotic added – usually Ampicillin or Kanamycin). The transformed cells were grown overnight at 37°C. For TA-cloning protocol (Invitrogen), provided MachT1 cells were used.

### 3.8.2 Plasmid DNA extraction from bacterial cells

Midipreps were performed using Qiagen Plasmid Midi Kit.

Bacterial culture was harvested after 12–16 h of growth by centrifuging at 6000 g for 15 min at 4°C. The pellet was completely resuspended in 4 ml Buffer P1. A volume of 4 ml Buffer P2 was added, mixed by inverting the sealed tube 4–6 times, and incubated at room temperature (15–25°C) for 5 min. During the

incubation, the cap was screwed onto the outlet nozzle of the QIAfilter Cartridge, and placed in a convenient tube. A volume of 4 ml pre-chilled Buffer P3 was added to the lysate, and mixed immediately and thoroughly by inverting 4–6 times. The lysate was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 min. The QIAGEN-tip was equilibrated by applying 4 ml Buffer QBT, and the column was allowed to empty by gravity flow. The cap was removed from the QIAfilter Cartridge outlet nozzle. The plunger was inserted into the QIAfilter Cartridge, and the cell lysate was filtered into the equilibrated QIAGEN-tip. The lysate was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed with 2 x 10 ml Buffer QC. DNA was eluted with 5 ml Buffer QF. DNA was precipitated by adding 3.5 ml room-temperature isopropanol, mixed, and centrifuged at 15000 g for 30 min at 4°C. The supernatant was decanted. The DNA pellet was washed with 2 ml of room temperature 70% ethanol and centrifuged at 15000 g for 10 min. The supernatant was decanted. The pellet was air-dried for 5–10 min and DNA was redissolved in a suitable volume of $H_2O$.

## 3.9 PCR amplification

Reagents for PCR were purchased from 5 Prime. Primers were synthetized by Invitrogen.

### 3.9.1 Primers for human IgG constant regions

Reverse primers for amplification of human constant regions (heavy and light) were designed by modification of primers described by Dr. Gary McLean (McLean et al., 2000): in particular, restriction sites were changed from *Xho*I to *Sal*I (light chain) and from *Hind*III to *Hpa*I to clone the amplified sequences in our vectors. Forward primers were designed from scratch (Table 3.1).

### 3.9.2 Primers for murine IgG variable regions

Forward primers for murine variable regions were based on Mouse-Ig Primer set (Novagen) changing restriction site to *AgeI* (light chain) and *NheI* (heavy chain), whereas reverse primers were made to match the frame of human constant regions.

**Table 3.1** Primer for amplification of human IgG constant regions

| **light chain - IgK** | | |
|---|---|---|
| **Primer** | **Sequence** | **Tm** |
| **HuIgKcF** | ATATAT**ACCGGT**TTCATCTTCCCGCCA | 59.4°C |
| **HuIgKcR** | ATATATAT**GTCGAC**TCAACACTCTCCCCTGT | 59.6°C |
| **heavy chain - IgG** | | |
| **Primer** | **Sequence** | **Tm** |
| **HuIgGcF** | ATATATA**GCTAGC**TCGGTCTTCCCCCTGG | 61.8°C |
| **HuIgGcR** | ATCCACAT**GTTAAC**TCATTCACCCGGAGACA | 62.3°C |

**Table 3.2** Primer for amplifications of light chain variable regions of murine IgG

| Group | Primer | Sequence | Tm |
|---|---|---|---|
| **group A** | **MuIgKvFA** | GG**ACCGGT**ATGRAGWCACAKWCYCAGGTCTTT | 62-66.3°C |
| **group B** | **MuIgKvFB** | GG**ACCGGT**ATGGAGACAGACACACTCCTGCTAT | 65.6ºC |
| **group C** | **MuIgKvFC** | ACTA**ACCGGT**ATGGAGWCAGACACACTSCTGYTATGGGT | 66.3-67.7°C |
| **group D** | **MuIgKvFD1** | ACTA**ACCGGT**ATGAGGRCCCCTGCTCAGWTTYTTGGIWTCTT | 67.2-69.5°C |
| | **MuIgKvFD2** | ACTA**ACCGGT**ATGGGCWTCAAGATGRAGTCACAKWYYCWGG | 64.5-68.5°C |
| **group E** | **MuIgKvFE1** | ACTA**ACCGGT**ATGAGTGTGCYCACTCAGGTCCTGGSGTT | 68.8-70.3°C |
| | **MuIgKvFE2** | ACTA**ACCGGT**ATGTGGGGAYCGKTTTYAMMCTTTTCAATTG | 62.4-67.3°C |
| | **MuIgKvFE3** | ACTA**ACCGGT**ATGGAAGCCCCAGCTCAGCTTCTCTTCC | 68.4°C |
| **group F** | **MuIgKvFF1** | ACTA**ACCGGT**ATGAGIMMKTCIMTTCAITTCYTGGG | 63-69.1°C |
| | **MuIgKvFF2** | ACTA**ACCGGT**ATGAKGTHCYCIGCTCAGYTYCTIRG | 63.7-71.3°C |
| | **MuIgKvFF3** | ACTA**ACCGGT**ATGGTRTCCWCASCTCAGTTCCTTG | 63.8-65.8°C |
| | **MuIgKvFF4** | ACTA**ACCGGT**ATGTATATATGTTTGTTGTCTATTTCT | 57.5°C |
| **group G** | **MuIgKvFG1** | ACTA**ACCGGT**ATGAAGTTGCCTGTTAGGCTGTTGGTGCT | 67.4°C |
| | **MuIgKvFG2** | ACTA**ACCGGT**ATGGATTTWCARGTGCAGATTWTCAGCTT | 62.6-64.3°C |
| | **MuIgKvFG3** | ACTA**ACCGGT**ATGGTYCTYATVTCCTTGCTGTTCTGG | 62.7-66.4°C |
| | **MuIgKvFG4** | ACTA**ACCGGT**ATGGTYCTYATVTTRCTGCTGCTATGG | 61.3-66.7°C |
| **reverse** | **MuIgKvR** | ATAT**ACCGGTT**ACAGTTGGTGCAGCATCAGCCCG | 66.5°C |

**Table 3.3** Primer for amplification of heavy chain variable regions of murine IgG

| Group | Primer | Sequence | Tm |
|---|---|---|---|
| **group A** | **MuIgHFA** | GG**GCTAGC**ATGRASTTSKGGYTMARCTKGRTTT | 60.4-70°C |
| **group B** | **MuIgHFB** | GG**GCTAGC**ATGRAA TGSASCTGGGTYWTYCTCTT | 63.7-68°C |
| **group C** | **MuIgHFC1** | ACTA**GCTAGC**ATGGACTCCAGGCTCAATTTAGTTTTCCT | 64.6°C |
| | **MuIgHFC2** | ACTA**GCTAGC**ATGGCTGTCYTRGBGCTGYTCYTCTG | 64.2-70.5°C |
| | **MuIgHFC3** | ACTA**GCTAGC**ATGGVTTGGSTGTGGAMCTTGCYATTCCT | 65.9-69.8°C |
| **group D** | **MuIgHFD1** | ACTA**GCTAGC**ATGAAATGCAGCTGGRTYATSTTCTT | 61.8-64.7°C |
| | **MuIgHFD2** | ACTA**GCTAGC**ATGGRCAGRCTTACWTYYTCATTCCT | 61.4-66.4°C |
| | **MuIgHFD3** | ACTA**GCTAGC**ATGATGGTGTTAAGTCTTCTGTACCT | 62.2°C |
| **group E** | **MuIgHFE1** | ACTA**GCTAGC**ATGGGATGGAGCTRTATCATSYTCTT | 62-65°C |
| | **MuIgHFE2** | ACTA**GCTAGC**ATGAAGWTGTGGBTRAACTGGRT | 60.5-64.9°C |
| | **MuIgHFE3** | ACTA**GCTAGC**ATGGRATGGASCKKIRTCTTTMTCT | 61.3-67.7°C |
| **group F** | **MuIgHFF1** | ACTA**GCTAGC**ATGAACTTYGGGYTSAGMTTGRTTT | 61-66°C |
| | **MuIgHFF2** | ACTA**GCTAGC**ATGTACTTGGGACTGAGCTGTGTAT | 63.6°C |
| | **MuIgHFF3** | ACTA**GCTAGC**ATGAGAGTGCTGATTCTTTTGTG | 61.1°C |
| | **MuIgHFF4** | ACTA**GCTAG**CATGGATTTTGGGCTGATTTTTTTTATTG | 60.7°C |
| **reverse** | **HuIgG1R** | ATATA**GCTAGC**TGGGGGTGTCGTTTTGGCG | 64.7ºC |
| | **HuIgG2aR** | ATATA**GCTAGC**TGGGGCTGTTGTTTTGGCT | 63.1ºC |
| | **HuIgG2bR** | ATATA**GCTAGC**GTGGGGTGTTGTTTTGGCT | 63.1ºC |

A total of 15 degenerated forward primers for mouse light chain were used. They were divided into 7 groups (A-G) as indicated by the manufacturer. One reverse primer was designed (details in Table 3.2).
Fifteen degenerated forward primers for mouse heavy chain were used. They were divided into 6 groups (A-F) as indicated by the manufacturer. Three reverse primers (IgG1, IgG2a, IgG2b) were designed (details in Table 3.3).

### 3.9.3 PCR reactions

All PCR were performed considering a final volume of 50 μl; dNTPs final concentration was 200 μM; a total amount of 10 pmoles for each primer was used. Concerning primer pools, the final amount was calculated as the sum of all primers of the pool.
PCR to amplify constant regions consisted in an initial denaturation at 94°C for 4 minutes, 40 cycles divided in 30 seconds denaturation (94°C), 30 seconds annealing (58°C) and 1 or 2 minutes elongation (72°C) for light or heavy chain respectively, plus 10 minutes of final elongation.
All PCR reactions for variable regions were performed with an initial denaturation of 94°C for 4 minutes, 40 cycles consisting of 30 seconds denaturation (94°C), 30 seconds annealing (58°C) and 1 minute elongation (72°C).
PCR to reveal humanized IgGs were performed using forward primer of mouse variable regions and reverse primers of human constant regions. For both heavy and light chains, 35 cycles were used, consisting in 30 seconds denaturation (94°C), 30 seconds annealing (58°C) and 2 minutes elongation (72°C).

## 3.10 Vectors

Vectors pTY2-CMV-GFP-IRES-NEO (Neo) (10040 bp) and pTY2-CMV-IRES-PURO-WPRE (Puro) (10021 bp) were kindly provided by Dr. Andrea Cara (ISS – Rome, Italy), and were used to clone IgG light and heavy chain respectively. They both contain a CMV promoter, after which it is possible to insert a transgene (Neo bears the sequence for GFP), followed by an IRES region. Downstream this sequence the vectors present a gene for antibiotic resistance (Neomycin on Neo and Puromycin on Puro). Vector Puro presents an additional sequence (WPRE) that enhances transgenes expression. Defective LTR (ΔU3) sequences are present

in both vectors at the extremities of the coding sequences (upstream CMV and downstream Neomycin or WPRE sequence).

### 3.11 Cloning IgG regions

Reagents were purchased from Invitrogen (TOPO TA Cloning Kit), Promega (T4 Ligase) New England Biolabs (endonucleases) and Qiagen (Plasmid MIDI kit). Fresh PCR products (human constant regions and mouse variable regions of IgK and IgG) were directly cloned in pCR2.1 vector (Invitrogen) and expanded transforming *E. coli* cells Mach1$^{TM}$-T1$^{R}$ (Invitrogen). Screening digestions with *Eco*RI were performed to find positive clones. Human constant light chain regions fragments were then digested with *Age*I-*Sal*I and ligated into pTY2-CMV-GFP-IRES-NEO; Human constant heavy chain regions were cloned into pTY2-CMV-IRES-PURO-WPRE using *Nhe*I-*Hpa*I digestions. Subsequently they were expanded by transformation of *E. coli* cells TOP10. When vectors pTY2-CMV-human_constant_IgK-IRES-NEO and pTY2-CMV-human_constant_IgG-IRES-PURO-WPRE were ready, the protocol was repeated with murine variable regions: light chain regions were digested with *Age*I whereas for heavy chain regions *NheI* was used. All endonucleases reactions were performed following manufacturer's instructions. Following endonucleases reactions, all products were extracted from agarose gel using Qiagen Gel Extraction Kit. Fragments of acceptor vectors were dephosphorylated using Shrimp Alkaline Phosphatase (SAP – Promega) and ligated using T4 DNA Ligase (Promega). All protocols where performed following manufacturer's instructions. For ligation reactions an insert:vector ratio of 9:1 was used.

### 3.12 Sequencing

PCR and cloning products were sequenced by BMR Genomics.

# 4. RESULTS

## 4.1 Antibody analyses by neutralization assays

### 4.1.1 Hybridomas screening for neutralization activity

Hybridomas were obtained from spleen cells of mice immunized with fusion complexes of CHO-CD4-CCR5 and CHO-gp120/gp41. A total of 64 selected hybridomas supernatants were tested for neutralization activity against several HIV-1 isolates belonging to panel B (figure 4.1). The used isolates have different sensitivity to neutralization: AC10.0-29 (AC10), moderately sensitive, 91US005.11 (91US), 6535-3 (6535), QHO692-42 (QHO), sensitive. Neutralization assays were performed on the TZM-bl cell line using 200 TCID50 of each pseudovirus. The antibody Tri-Mab, constituted by three different anti-Env monoclonal antibodies derived from LTNP patients (2G12, 4E10, 2F5), was used as positive control at 1 μg/ml. Neutralization activity was expressed as a lowering of light emitted by luciferase, expressed by TZM-bl and transactivated by the pseudovirus once infection has taken place. Mean activity of all polyclonal populations showed values settling around 30% for isolates 6535 and QHO, whereas values obtained against 91US and AC10 have a mean of about 20% and 0,5% respectively. Among all supernatants analyzed, the one produced by polyclonal population SM24 showed values ranging from 50% on QHO to 80% on 6535. These results indicated SM24 as a possible candidate for subcloning.

### 4.1.2 Comparison between SM24 and positive control

Further analyses on SM24 were done comparing supernatant with Tri-Mab positive control in a neutralization assay against isolates QHO, 6535 and AC10. This assay was performed on TZM-bl cell line using the same amount of SM24 and Tri-Mab with scale dilution going from 2 μg/ml to 0,12 μg/ml of antibody against 200 TCID50 of each pseudovirus. For all tested isolates both SM24 and

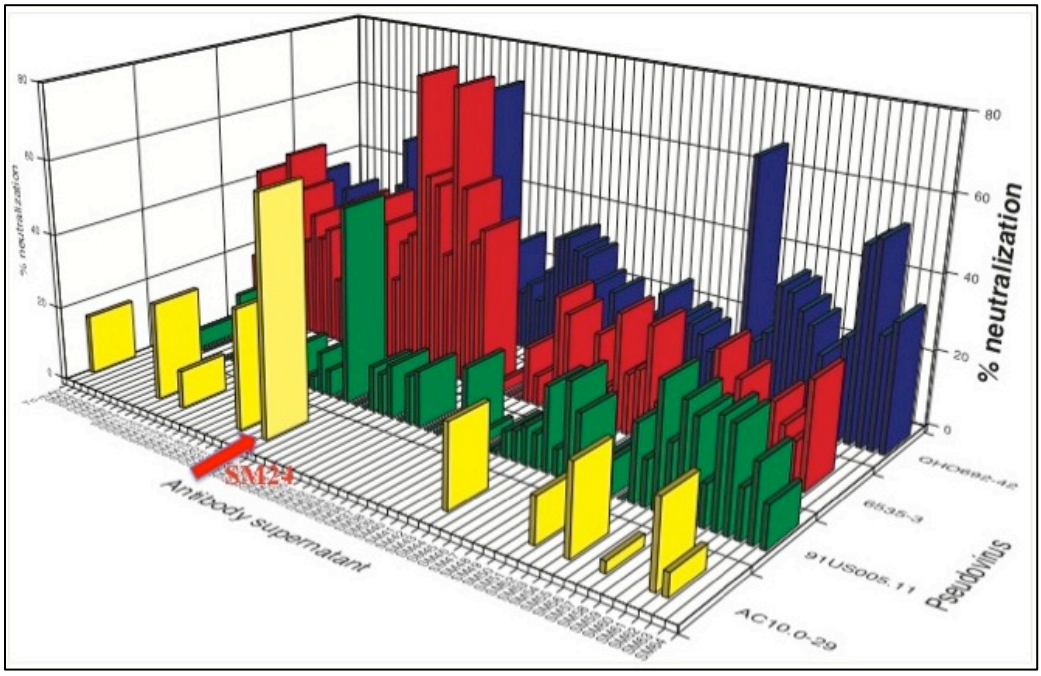


*Figure 4.1 Screening of 64 supernatants, obtained from mouse hybridomas, for neutralizing activity against four HIV-1 isolates. Neutralizing activity varies among different samples but it is possible to see how supernatant SM24 shows high values for all viral isolates.*

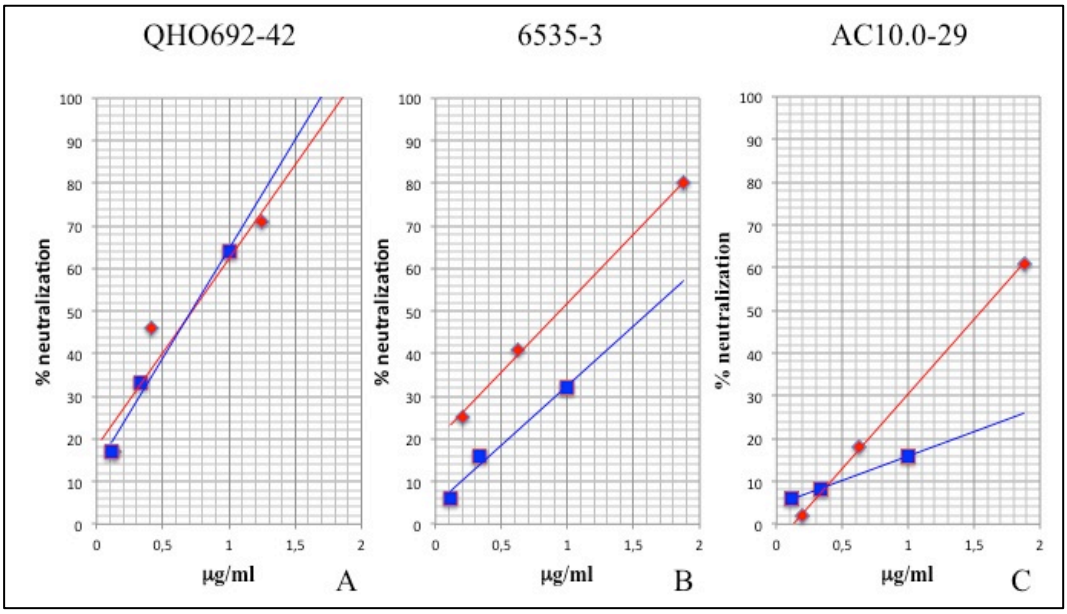


*Figure 4.2 The panel shows three different neutralization assays comparing neutralizing activities of sample SM24 (red) and Tri-Mab control (blue), against three different HIV-1 pseudoviruses. A) Against QHO692-42, SM24 and control show very similar trendlines. B) Neutralizing activity of SM24 against 6535-3 shows a linear trendline, proportional to Tri-Mab, but always about 20% higher. C) Values against AC10.0 show result in a much sharper trendline for SM24.*

Tri-Mab showed a decreasing trend of neutralization activity, proportional to the decrease of antibody concentrations. SM24 supernatant showed a neutralization activity closely comparable with positive control when directed against QHO isolate, going from about 70% at 1,3 μg/ml to 25% at about 0,3 μg/ml (figure 4.2 A). In particular, analyses against pseudovirus 6535 showed how the neutralization trendline of both SM24 and Tri-Mab lowered linearly from 1,8 μg/ml to about 0,2 μg/ml, giving values going from 80% to 25% for SM24 and from 55% to 5% for Tri-Mab. Therefore, for every tested value, SM24 showed a higher neutralization curve than the reference Tri-Mab (figure 4.2 B). Using the same range of antibody concentrations against AC10 isolate, the given decreasing trend is milder for Tri-Mab varying from 25% to 5%, and sharper for SM24, lowering from 60% to 1% (figure 4.2 C). Thus, even in this case, SM24 neutralizing activity resulted to be higher than Tri-Mab activity in both cases.

### 4.1.3 Specificity of SM24 IgGs

Since the amount of neutralization activity of the supernatants could depend from antibodies directed against the viral receptors used to produce fusion complexes, a test was performed to ensure that IgGs in SM24 supernatant were mostly directed to HIV-1 epitopes. Antibody supernatant was pre-adsorbed on CHO cells expressing CD4 and CCR5 receptors, and then used in a neutralization test against isolate 6535 used at 200 TCID50. Pre-adsorbed supernatant was analyzed and compared to non pre-adsorbed sample using equivalent scale dilutions going from 1:5 to 1:135 and data were reported as percentage of luminescence lowering (figure 4.3). With dilutions up to 1:10, neutralization trendline of pre-adsorbed sample shows no differences with non-pre-adsorbed SM24 reaching 80% for both. Differences in neutralization activity could be observed with increasing dilutions, going from 55% to 50% (normal and pre-adsorbed respectively) when samples were diluted 1:50, and from 40% to 25% for 1:100 dilution. However, despite the drop of the curve, neutralization values of pre-adsorbed samples remain high even at low concentrations.

Moreover, to test the specificity of SM24 supernatant we performed a neutralization assay against a pseudovirus with the envelope VSV-G

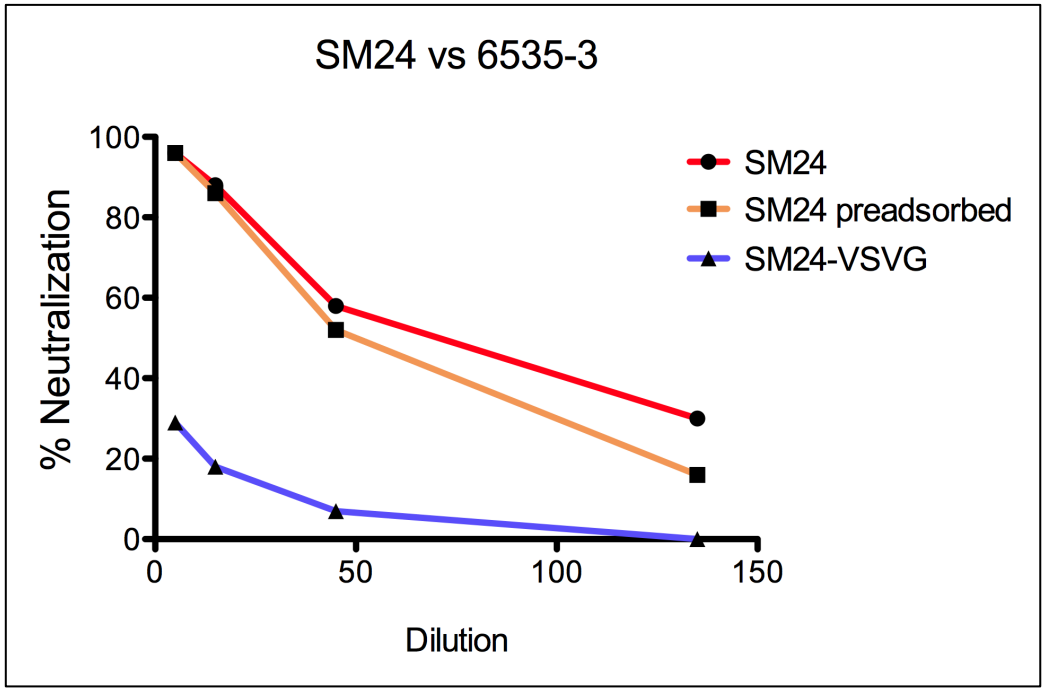


*Figure 4.3 Trend of neutralizing activity of SM24 supernatant dilutions against viral isolate 6535-3 before pre-adsorption on cells expressing cell receptors for CD4 (circle) and after adsorption (square). The blue line (triangle) represents the activity against a pseudovirus expressing VSV-G.*

(Vescicular Stomatitis Virus Glycoprotein), which has no similarities with HIV-1 glycoprotein gp120/gp41. Supernatant diluted up to 1:10 neutralizes VSV-G at about 25% but the trendline shows a very quick decrease as the sample is diluted. The difference between the neutralization curves of SM24 directed against isolate 6535 and VSV, indicated that antibodies produced by SM24 population were specifically directed against HIV-1 and neutralize isolate 6535 at about 60% when undiluted.

### 4.1.4 Selection of oligoclonal populations

SM24 hybridoma cell population was subcloned into subgroups of 20 cells/well. A total of 18 supernatants of oligoclonal populations were then screened to identify samples with the highest neutralization activity. A neutralization assay was performed for 18 supernatants in comparison to SM24 and to Tri-Mab (5 µg/ml). A wider set of HIV-1 isolates was used: it included additional *env*-expressing-pseudoviruses such as SF162, TROcl-11 (TRO) and pREJ04541 (pREJ) besides AC10, 6535 and QHO. Except some samples as aC12, eC6 and gC1, all oligoclonal supernatants resulted to have a good level of neutralization (figure 4.4 A). Generally, the neutralization activity against SF162 isolate was higher than SM24 but still lower than the Tri-Mab, whereas other pseudoviruses were neutralized better by the supernatants than by the Tri-Mab. For example, neutralization values against isolate 6535 where generally very high, with a range going from 50% to 90%; QHO isolate was neutralized from 30% to 70%; neutralization values against AC10 vary from 30% to 90%; values against TRO and pREJ present a wider range going respectively from 20% to 90% and from 5% to 80%.

For each sample we calculated mean neutralization values against all used isolates and the range of the spectrum covered by the supernatants, to choose which oligoclonal populations were to be subcloned to single cell (figure 4.4 B). As showed by the neutralization assay, samples gB11 and cG11 gave values higher than other samples (above 75%) and low differences among isolates (10-12%). Together with 9 more supernatants, they were analyzed by whole-cells-ELISA assay, performed in order to measure supernatants reactivity against

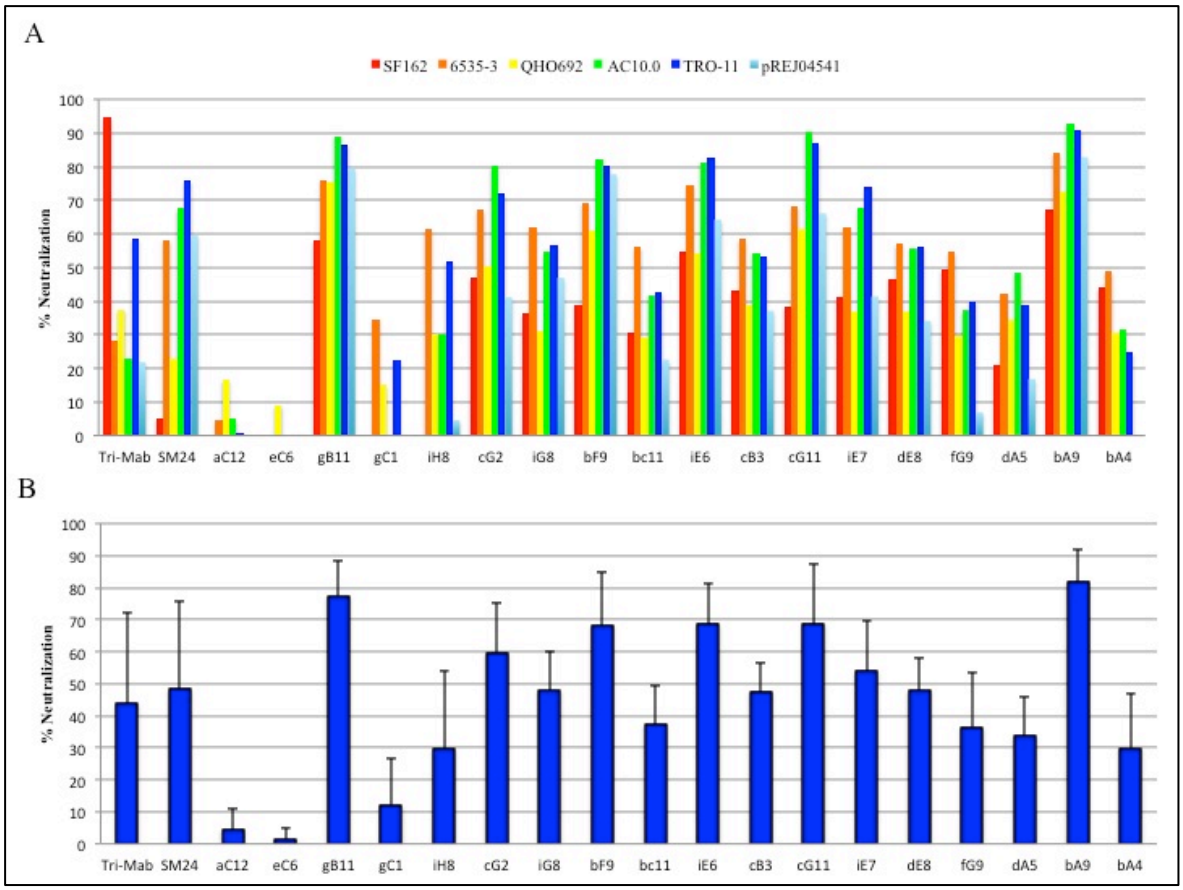


*Figure 2.4 A) Neutralization analyses performed on 18 oligoclonal supernatants against 6 different viral isolates and compared with SM24 and Tri-Mab. B) Mean neutralization activity of oligoclonal supernatants.*

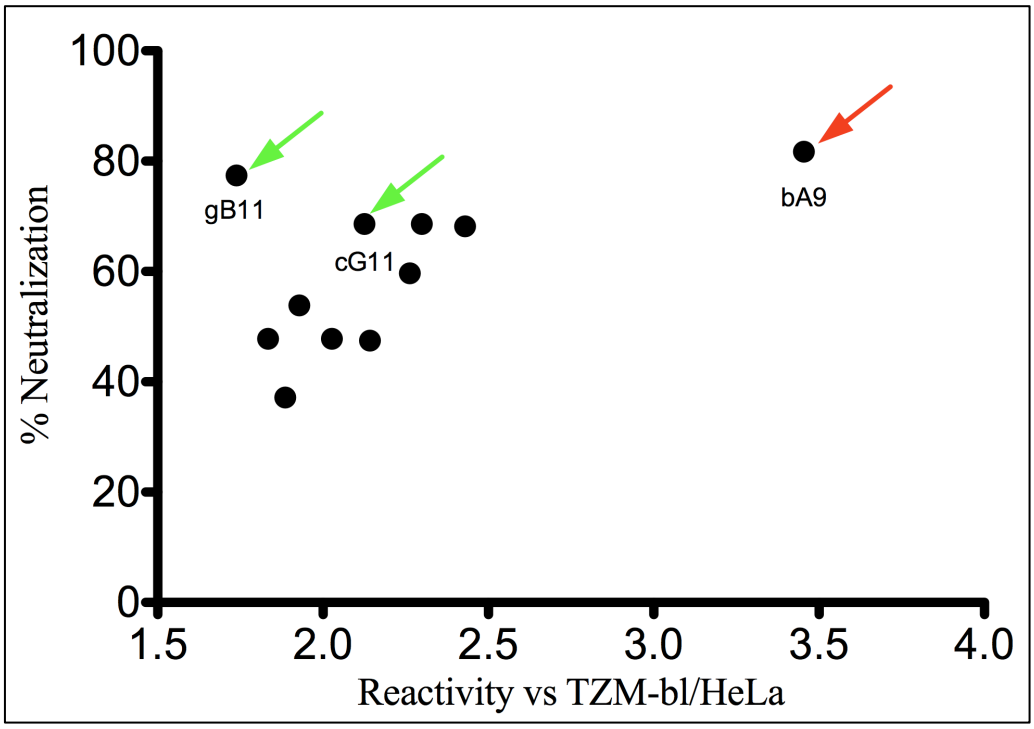


*Figure 4.5 Comparison between mean neutralization activity of oligoclonal supernatants and their reactivity against target cells for neutralization assay. Samples gB11 and cG11 show the best ratio neutralization/reactivity therefore are chosen to proceed with the study.*

TZM-bl cells, and to exclude those containing cell specific IgGs (figure 4.5). Comparing reactivity against cells obtained by ELISA assay and mean neutralizations obtained before, it was possible to observe 5 clones neutralizing at 40-50% with TZM-bl reactivity with values between 1,8 and 2,2; 4 clones, neutralize at about 60-70% and showed reactivity between 2,2 and 2,5. Clone bA9 gave a high neutralization percentage (about 80%) but a very high reactivity against cells (about 3,25). Finally, clones gB11 and cG11 showed a mean neutralization value of about 80% and 70% respectively, and a low reactivity against cells (1,7 and 2,1). Therefore, populations gB11 and cG11 resulted to be the best available, with the highest neutralization activity, and the lowest reactivity against cells.

### 4.1.5 Selection of monoclonal antibodies

gB11 and cG11 oligo-populations were subcloned to single cell populations; supernatants of about one hundred clones from each progenitor were then tested for neutralization activity against isolate 6535 (sensitive) as previously described. On 72 clones derived from cG11 (figure 4.6 A), 63 showed neutralization activity. 30% of such clones present an activity within 10%; 50% of them neutralized within 20%; 25% reached neutralization between 20% and 30%. The 5 remaining clones surpassed 30% of neutralization activity; among them, clone 8F9 reached 39%. 50 gB11 clones were found and 31 showed neutralization activity (figure 4.6 B). Among them, 21 clones reached 10% of max neutralization, 11 clones gave a neutralization range between 10% and 20%, 2 clones surpassed 20% and one clone (10F12) showed a neutralization percentage of 35%.

From here on our attention was focused on gB11 clones. Neutralization activity of supernatants was compared with reactivity against target cells, as done for oligoclonal supernatants. As shown in figure 4.7, clone 10F12 presented both the higher neutralization and the lower reactivity against TZM-bl cells. Clone 10F12 was thus chosen to proceed with our studies. Firstly, it was tested for neutralizing activity against more viral isolates in comparison with Tri-Mab, as done for oligoclonal supernatants. Such assay was performed using 5 μg/ml antibody. Excluding activity against TRO-11 values against all other viral

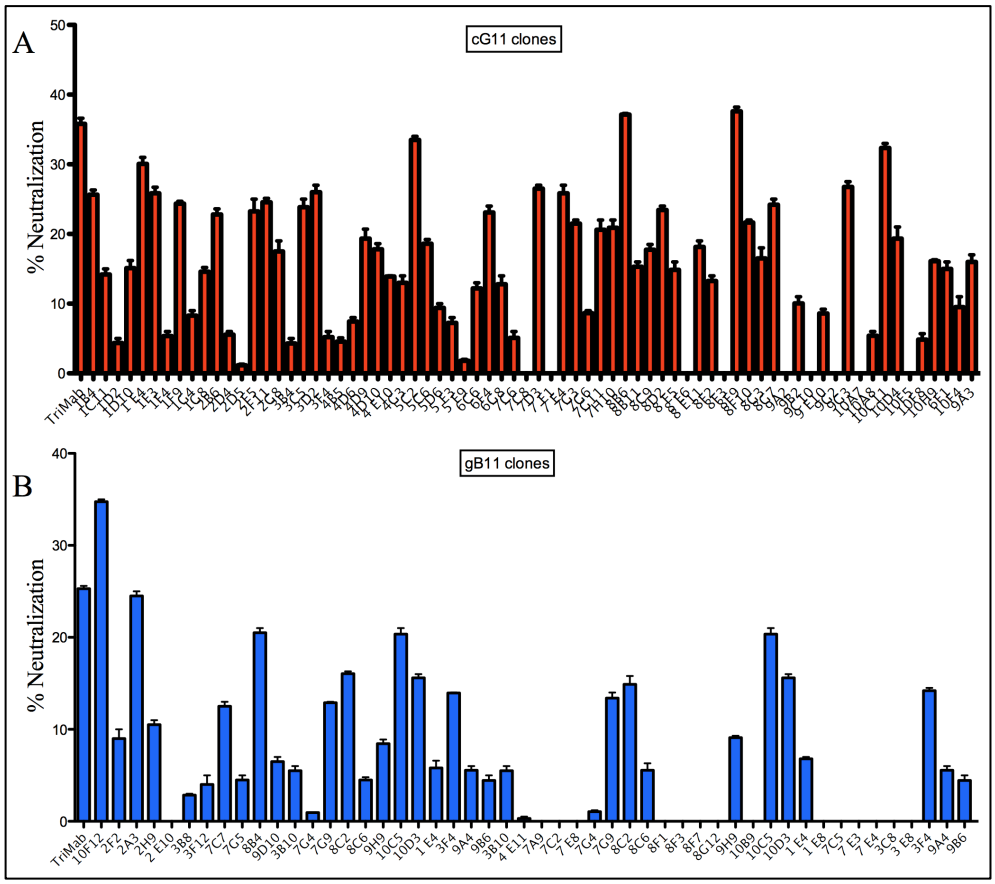


*Figure 4.6 Initial screening of supernatants of monoclonal population obtained from cG11 (A) and gB11 (B). Neutralization assays were performed against viral isolate 6535-3. Higher neutralization values are found for clones 8F9 (A) and 10F12 (B).*

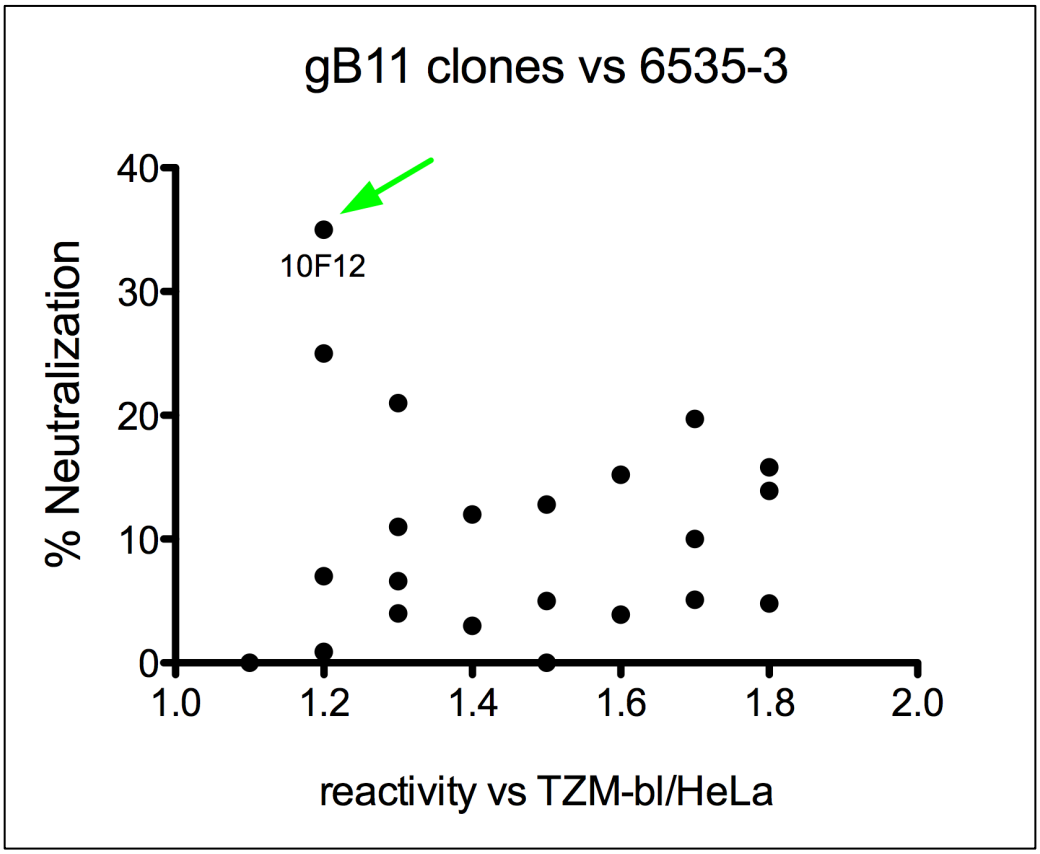


*Figure 4.7 Comparison between neutralization activity of monoclonal supernatants from gB11(against isolate 6535-3) and their reactivity against target cells for neutralization assay. Sample 10F12 the best ratio neutralization/reactivity therefore is chosen to proceed with the study.*

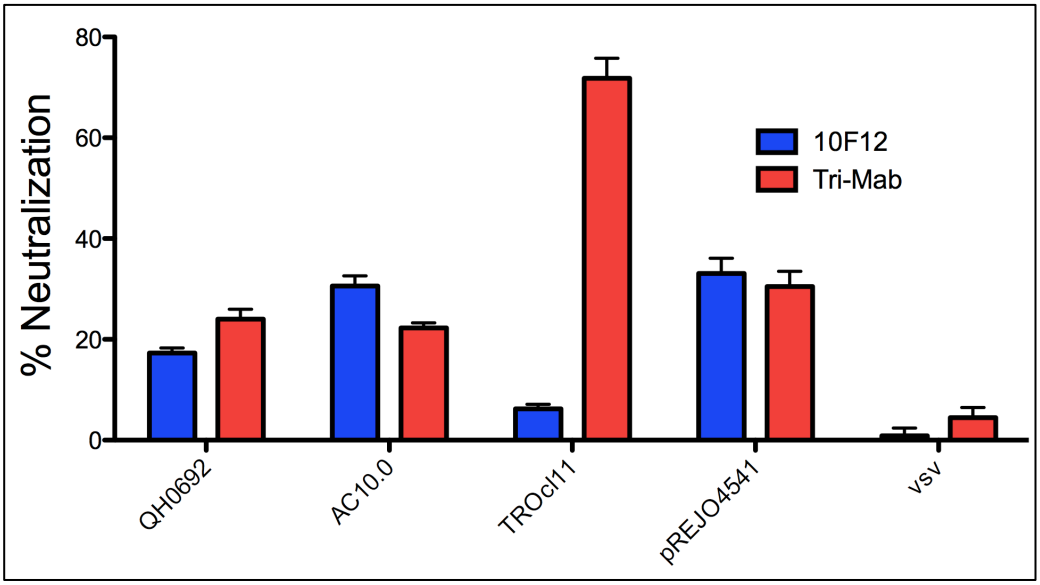


*Figure 4.8 Analyses of neutralization activity of clone 10F12 against several HIV-1 isolates and against VSV-G, compared with neutralizing activity of control Tri-Mab. For all isolates, except TROcl11, values of 10F12 and Tri-Mab are comparable. Activity against VSV-G is closed to 0, excluding unspecific neutralization.*

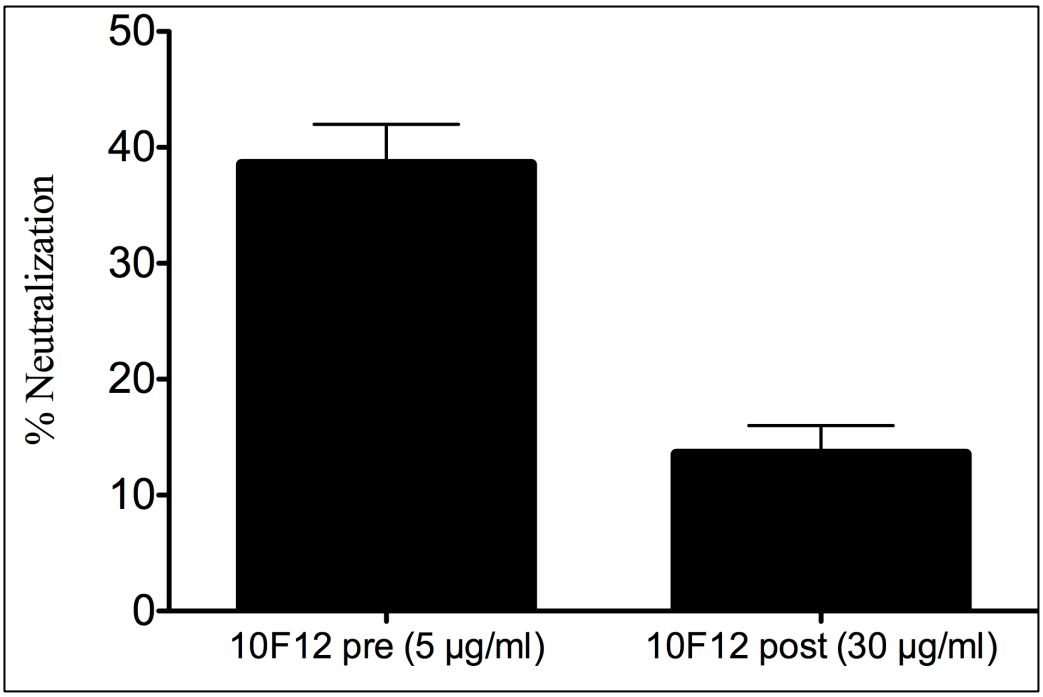


*Figure 4.9 Comparison of neutralization activity of supernatant 10F12 before and after clone expansion.*

isolates were closely comparable. Neutralization values against QHO and AC10 were found around 20%-25%, whereas values against pREJ04541 were about 40%. Moreover, neutralization activity against VSV-G pseudovirus was taken into account, in order to exclude unspecific activity (figure 4.8). In order to produce high titers of antibody, 10F12 cells were cultured into bioreactors, used to concentrate IgGs secreted in culture medium. Neutralization assays were repeated at each subsequent culture steps in bioreactors; tests showed a decrease of neutralization activity as cultures proceeded. Figure 4.9 shows the comparison of neutralization activity for 10F12 antibody against pseudovirus 6535-3 before and after expansion. It is possible to see that, whereas first analyses showed a neutralization activity of 35% using 5 μg/ml, after expansion a concentration of 30 μg/ml is needed in order to neutralize the pseudovirus at about 11%.

## 4.2 Construction of vectors expressing humanized IgGs

In order to stabilize antibody expression our aim was to produce a robust cell line, stably expressing the antibody produced by clone 10F12. Furthermore, we decided to humanize our murine antibody.

### 4.2.1 Amplification of human constant regions starting from PBMCs

Starting from human PBMCs, total RNA was extracted and retrotranscripted to cDNA, which was then used as template for PCR amplification of IgG human constant regions of both light and heavy chains. PCR products were analyzed by agarose gel electrophoresis (figure 4.10). Human constant light chain (Human CK) product is noise-free and shows, as expected, a band of about 300 bp, whereas human constant heavy chain (Human CH) analysis showed a smear and an unspecific band of about 1200 bp. Nevertheless the specific band of about 980 bp gives a much stronger signal.

### 4.2.2 Amplification of murine variable regions from clone 10F12

10F12 supernatant was used to isotype the IgGs produced by 10F12 cells, and resulted to express murine light chain K and heavy chain G2b. Clone 10F12 was then used to extract total RNA, then retrotranscripted by random primers and used as template for amplification of murine light and heavy chains variable regions. Based on isotyping results, PCRs were performed using a subtype

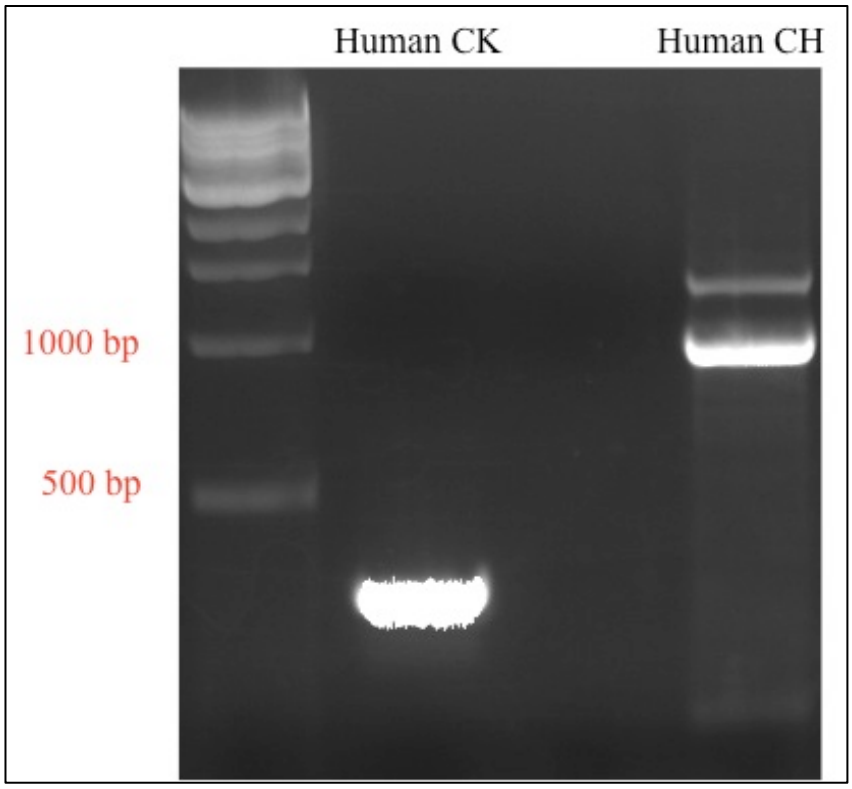


*Figure 4.10 PCR amplification of human IgG constant regions: on the left we find light constant region (Human CK) around 300 bp; on the right is the heavy constant region (Human CH) of about 980 bp.*

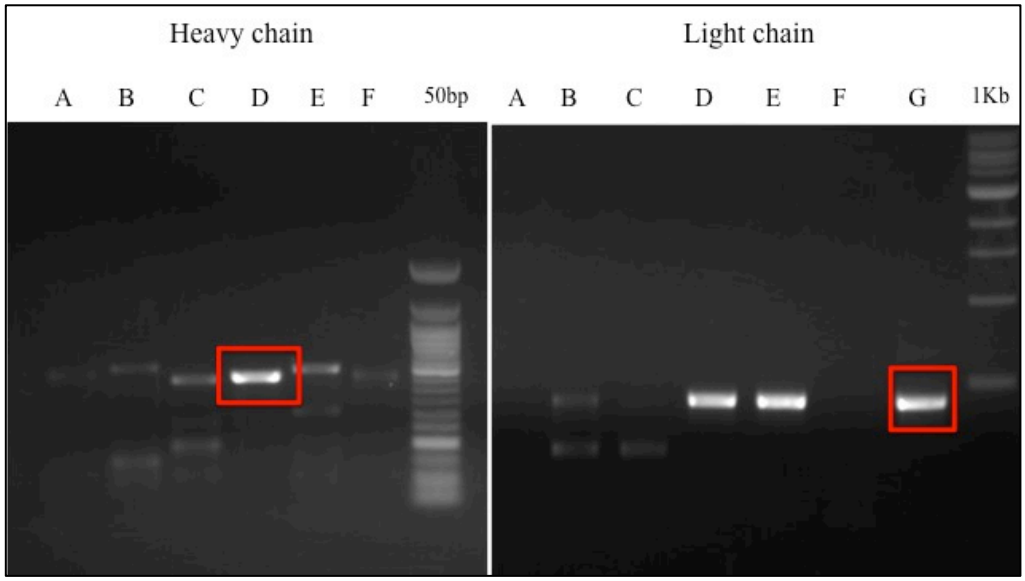


*Figure 4.11 PCR amplification of murine IgG variable regions, performed using pools of degenerated primers. On the left heavy chain variable regions are visible, whereas on the right there are light chain variable regions. Red squares indicate the chosen fragment to proceed with our studies.*

specific reverse primer, IgK for light chain and IgG2b for heavy chain. As forward primer, we used pools of degenerated primers, divided in seven groups for light chain (A-F) and six groups for heavy chain (A-G) each of them including from one to four primers. PCR products were analyzed for correct size by agarose gel electrophoresis. Expected size for both variable regions was about 420 bp. On the left of figure 4.11, results for murine heavy chain variable region are shown; each lane indicates the group of forward primers used for amplification. Products obtained with primers group A and F give a weak signal; results obtained with groups B, C and E show strong background and weak specific band, whereas group D resulted in a strong and clean signal of correct size.

For murine light chain variable region (figure 4.11 on the right), primers groups A and F gave no amplification and groups B and C showed weak bands and unspecific signals; finally, groups D, E and G gave strong bands of correct sizes, but product G resulted in a cleaner signal. Products obtained with primers from group D (variable region of heavy chain) and from group G (variable region of light chain) were selected.

### 4.2.3 Generation of vectors expressing human constant regions

PCR products of light and heavy chains constant regions, obtained from human PBMCs, were cloned into plasmid pCR2.1 and used to transform *E. coli* cells. Colonies were then screened by *Eco*RI digestion and analyzed on agarose gel to confirm fragments insertion. Expected bands were about 350 bp for light chains and 950 bp for heavy chains. As shown in figure 4.12 A, most of light chain constant regions were successfully cloned into plasmid pCR2.1; exceptions are samples 4 and 7, showing no insertion, and 9,10 and 11, whose fragments resulted higher than expected. Concerning constant heavy chain, figure 4.12 B shows how about 50% of colonies contain the inserts of interest; exceptions are samples 3, 5 and 9, showing non insert, and samples 6 and 11 showing bands with sizes differing from expectations. One colony for constant-IgK (sample 3) and one for constant-IgH (sample 2) were then chosen to be amplified by midiprep and ligated into pTY2-CMV-GFP-IRES-NEO and pTY2-CMV-IRES-PURO-WPRE respectively, which express Neomycin (former) and Puromycin

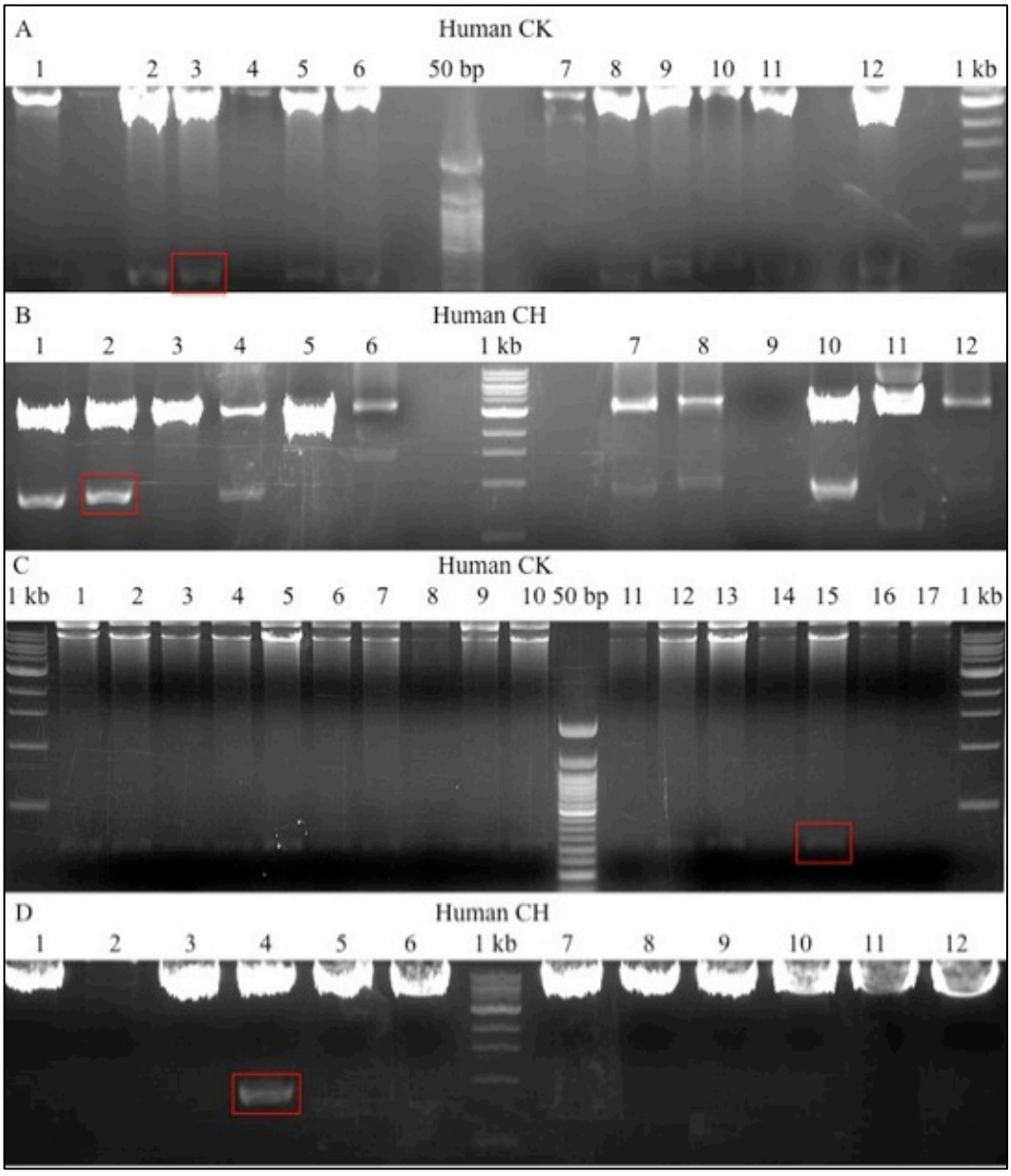


*Figure 4.12 Sequential steps for production of vector containing human IgG contant regions. A) EcoRI digestions of minipreps after TA cloning protocol for constant light chain regions. B) EcoRI digestions of minipreps after TA cloning protocol for constant heavy chain regions. C) Screening for successful insertion of constant light chain regions inside pTY2-CMV-GFP-IRES-NEO by AgeI-SalI digestion. D) Screening for successful insertion of constant heavy chain regions inside pTY2-CMV-IRES-PURO-WPRE by NheI-HpaI digestion.*

(latter) resistance. Once again, ligation products were used to transform *E. coli* cells and colonies were then screened by enzymatic digestion, *Age*I-*Sal*I for K-chain and *Nhe*I-*Hpa*I for H-chain. Digestion products were then analyzed on agarose gel. As shown on figure 4.12 C, light chain colonies were almost all positive, containing Human CK inserts. Concerning constant regions of heavy chains (figure 4.12 D) one colony containing the insert (sample 4) was found. By amplification of positive colonies we obtained vectors expressing human constant regions of light and heavy chains, which will be used to build vectors expressing humanized chains.

### 4.2.4 Generation of vectors expressing humanized heavy and light chains

PCR products obtained from hybridomas cell clone 10F12 were inserted into plasmid pCR2.1 and used for *E. coli* cells transformation. Colonies were screened by *Eco*RI digestion and molecular weight controlled on agarose gel. Figure 4.13 A shows results for variable light chain (IgKv) on the left; almost all colonies show presence of the insert with bands of about 420 bp. On the right side are the colonies for variable heavy chain, which confirm occurred insertion with bands of about 420 bp. One sample for each chain was then chosen to be ligated into vectors bearing human constant light and heavy chains. Ligation products were used to transform *E. coli* cells, and colonies were then analyzed on agarose gel. Cloning of human constant regions employed *Age*I-*Sal*I and *Nhe*I-*Hpa*I digestions for light and heavy chains respectively; insertion of murine variable chain regions makes use of *Age*I and *Nhe*I digestions, attaching variable regions upward to constant regions. Because of this, screening by digestion was not possible, since *Age*I or *Nhe*I reactions would not tell us the correct orientation of inserts; therefore positive clone were identified by PCR, using reverse primers of constant regions and forward primers of variable regions. Thus we can verify not only the insertion of variable regions, but also their correct orientation. In figure 4.13 B we can see PCR products for humanized IgK: samples 2, 3, 6, 7, 11 and 12 show clean bands of correct sizes (about 750 bp). Concerning PCR products for humanized IgG (figure 4.13 C) colonies 3, 6, 7, 11 and 12 show cleaner signals, with bands of the correct size (about 1400 bp) and an upper unspecific band. IgK sample 12 and IgG sample 7

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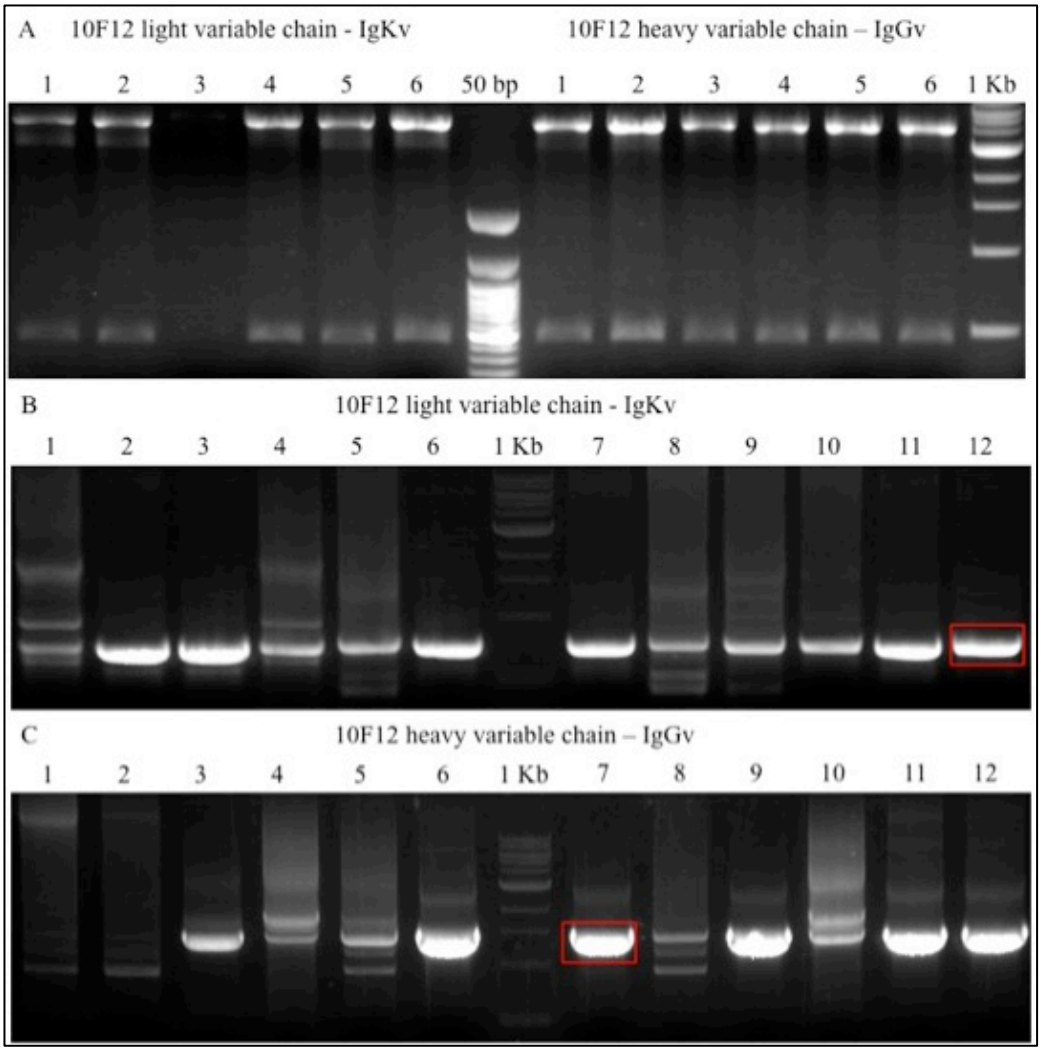


*Figure 4.13 Sequential step for production of vectors expressing humanized IgG and IgK. A) EcoRI digestions of minipreps after TA cloning protocol for murine variable light chains (left) and heavy chains (right). B) PCR Screening for successful insertion of variable light chain regions inside pTY2-CMV-GFP-IRES-NEO; insertion is correct when PCR amplifies a full length sequence for humanized IgK (750 bp). C) PCR screening for successful insertion of variable chain regions inside pTY2-CMV-IRES-PURO-WPRE ; insertion is correct when PCR amplifies a full length sequence for humanized IgG (about 1400 bp).*

were selected to proceed with our studies. Figure 4.14 summarizes the protocol used to produce vectors expressing humanized light and heavy chains.

### 4.2.5 Vectors control

Original vectors, as well as newly generated ones have a high probability to form recombinants because of the presence of LTR regions. Thus, they were digested with different restriction enzymes, comparing new vectors with original vectors. pTY2-NEO vectors were digested with *Age*I, *Bam*HI, and *Pst*I: *Age*I digestion should linearize original vector and separate murine variable light chain (420 bp) from new generated vector; *Bam*HI should give same results on both vectors since humanized IgK replaced GFP and non of them shows a *Bam*HI site; *Pst*I digestion should also result as very similar bands for both vectors, with a 20 bp difference in upper bands. On pTY2-PURO vectors we used *Nhe*I, *Bam*HI and a mix of *Age*I and *Sal*I. NheI should linearize original vector and separate murine variable region (about 420 bp) on the new one; *BamHI* digestion is expected to result in three bands on original vector and for bands on the new one. In this case the two upper bands will be identical, whereas the lower band of original vector (927 bp) will be replaced by two heavier bands due to the presence of a restriction site on IgG. Digestion performed using *AgeI-SalI* will result as two bands for pTY2-CMV-IRES-PURO-WPRE vector and three bands for pTY2-CMV-humanized_IgG-IRES-PURO-WPRE vector, due to the presence of an additional *AgeI* site on the transgene. As showed on figure 4.15 all our expectations were validated.

## 4.3 Production and analysis of humanized IgGs

### 4.3.1 Lentiviral vectors construction

The newly constructed vectors expressing humanized IgK and IgG were used to produces two third generation lentiviral vectors, one for each chain, expressing VSV-G envelope proteins and bearing humanized IgK or IgG respectively. This was done performing two separate quadruple co-transfections in 293T cell line of either pTY2-CMV-Humanized_IgK-IRES-NEO or pTY2-CMV-humanized_IgG-IRES-PURO-WPRE with ENV plasmid (VSV-G), pMDLg/ pRRE and pRSV-REV. After 48 hours from transfection cells supernatants, containing lentiviral vectors, were harvested and filtrated (figure 4.16).

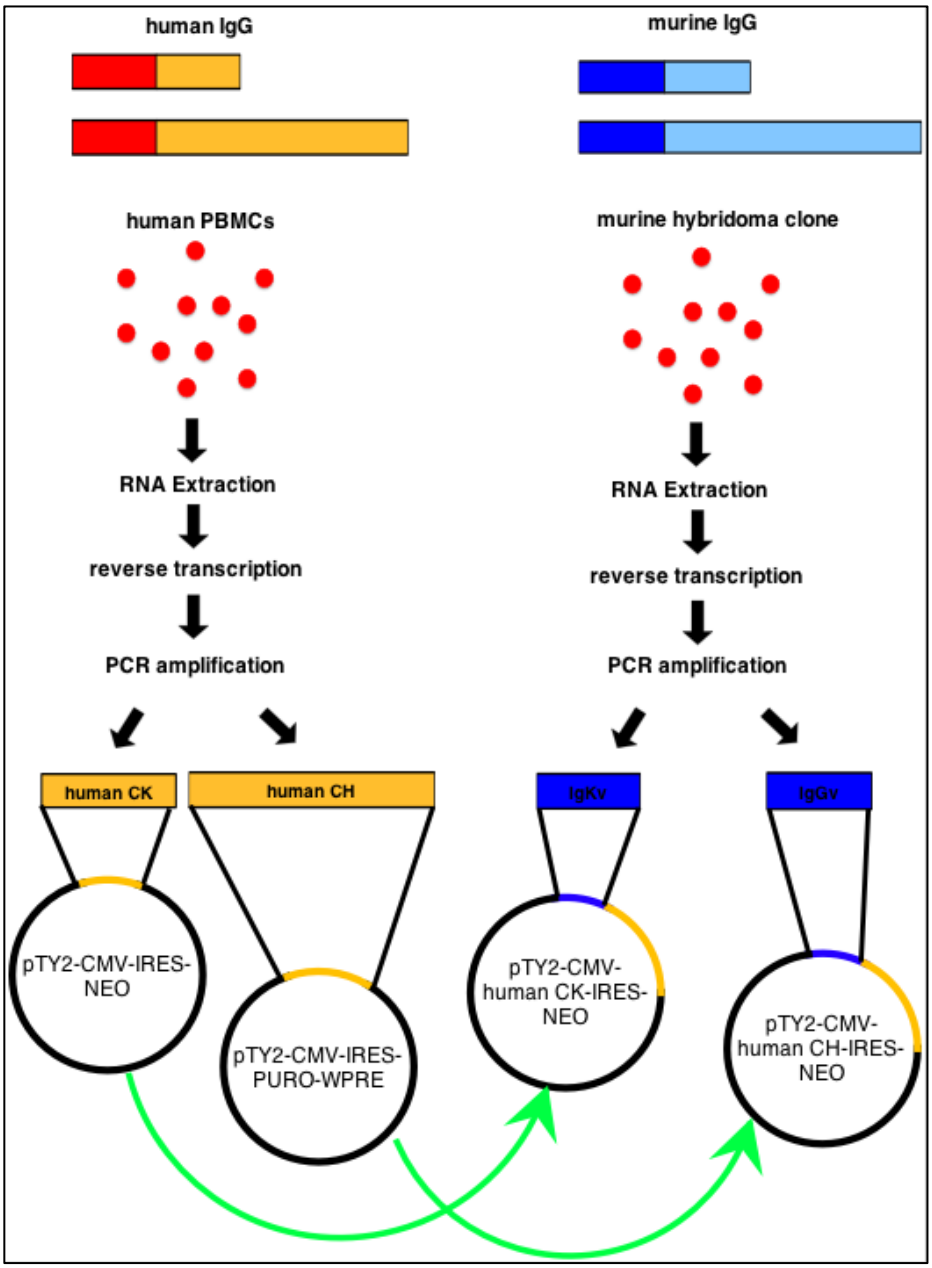


*Figure 4.14 Schematic representation of the steps involved to produce vectors bearing constant regions of human IgG and final vectors expressing humanized IgG.*

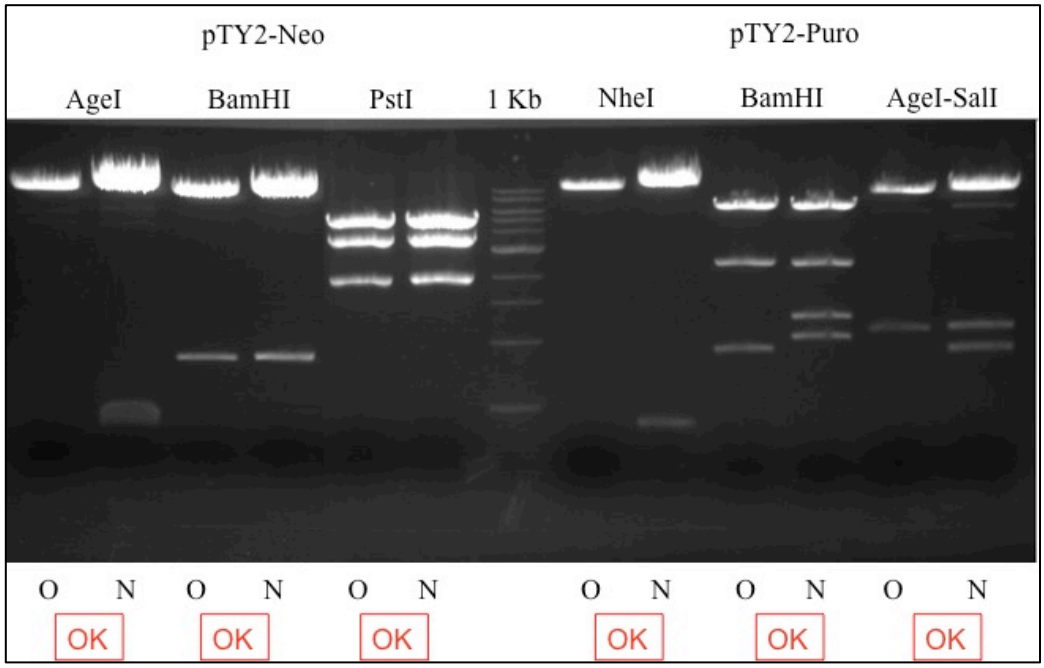


*Figure 4.15 Control digestion for final vectors pTY2-CMV-Humanized_IgK-IRES-NEO (left) and pTY2-CMV-humanized_IgG-IRES-PURO-WPRE (right). "O" indicates the original vectors, whereas "N" stands for new vectors. All digestions confirmed the correct insertion of humanized chains.*

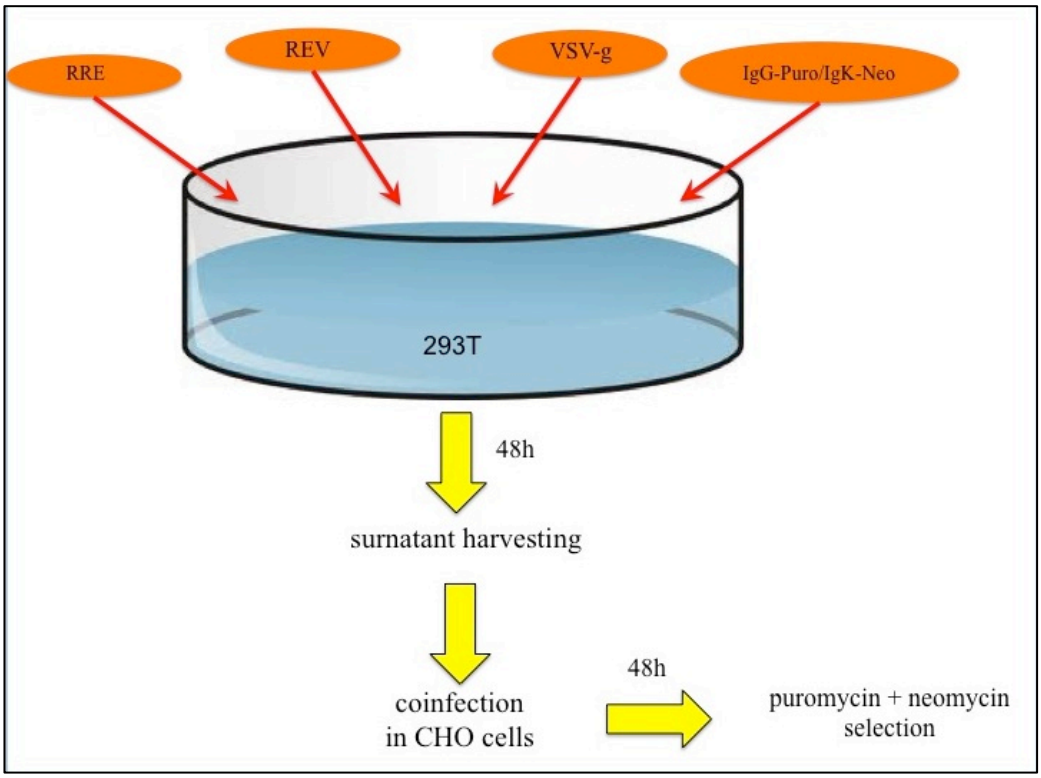


*Figure 4.16 Schematic representation of production of viral vectors expressing humanized IgG light and heavy chains, and their use to produce a cell line stably expressing humanized IgG.*

### 4.3.2 Productions of the CHO cell line expressing humanized IgGs

Freshly prepared lentiviral vectors were used to co-infect the CHO cell line. Lentiviral vectors have the ability to integrate their genome inside host cell genome. CHO cells infected by both vectors will develop resistance to Puromycin and Neomycin. After 48 hours from co-infection cells were placed under selection conditions with Puromycin (5 μg/ml) and Neomycin (100 μg/ml). As a negative control, this was performed on non-infected cells. After 24 hours non-infected cells were dead whereas co-infected cells showed a healthy population (figure 4.16 – 4.17).

### 4.3.3 Analysis of transgenes integration

To verify the presence of humanized IgGs in the genome of CHO cells, genomic DNA of resistant and non-resistant cells was extracted and used to perform PCR of both chains, using murine variable chains primers as forward, and human constant chains primers as reverse. DNA extracted from non-infected CHO cells was used as negative control whereas plasmids expressing humanized chains were used as positive controls. Figure 4.16 shows on the left side, amplifications results for humanized murine heavy chain (MuHu-IgG): genomic DNA of co-infected cells was amplified to the correct size (about 1400 bp), as well as the positive control, represented by pTY2-CMV-humanized_IgG-IRES-PURO-WPRE; genomic DNA of normal CHO cells gave no amplification as expected. On the right side PCR products for humanized murine light chain (MuHu-IgK) can be analyzed: as for MuHu-IgG, genomic DNA of co-infected cells gave a single clean signal of the correct size (about 750 bp), obtained as well with positive control, which showed unspecific bands due to its high concentration; genomic DNA of CHO cells, and blank sample, gave no signal.

### 4.3.4 Analysis of humanized IgG production

After one week of selective conditions, co-infected CHO cells were cloned to single cell populations. Growing clones were expanded from 96-well plate to 6-well plate. In order to verify the expression of humanized IgG, and select the clone with higher antibody production, an ELISA assay directed against human Fc was performed on the supernatants of CHO clones.

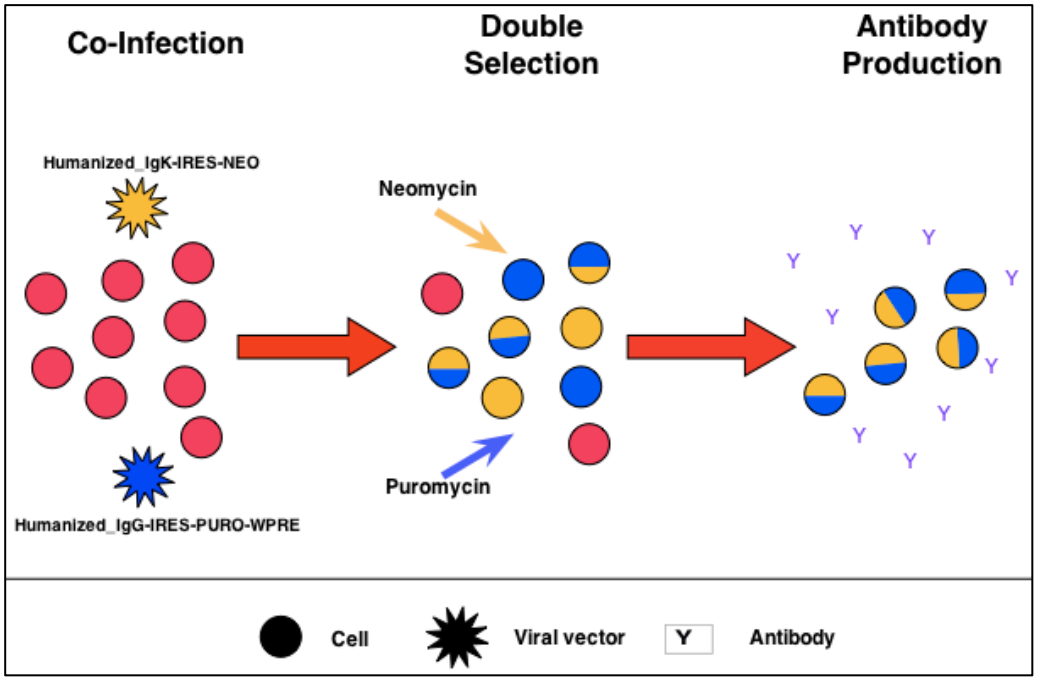


*Figure 4.17 Schematic representation of co-infection of CHO cells using viral vectors expressing humanized light and heavy chains; with double selection (neomycin and puromycin) only cells with integrated copies of both transgenes survive. Such cells produce humanize antibodies.*

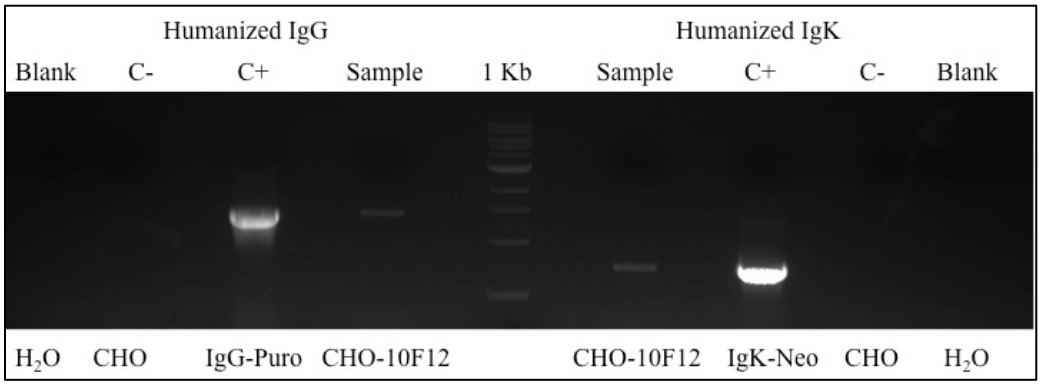


*Figure 4.18 PCR analyses for integration of humanized heavy (left), and light (right) chain inside CHO genome. Genomic DNA of co-infected CHO cells was used. As positive controls pTY2-CMV-humanized_IgG-IRES-PURO-WPRE and pTY2-CMV-Humanized_IgK-IRES-NEO were used. Normal CHO cells genomic DNA was used as negative control.*

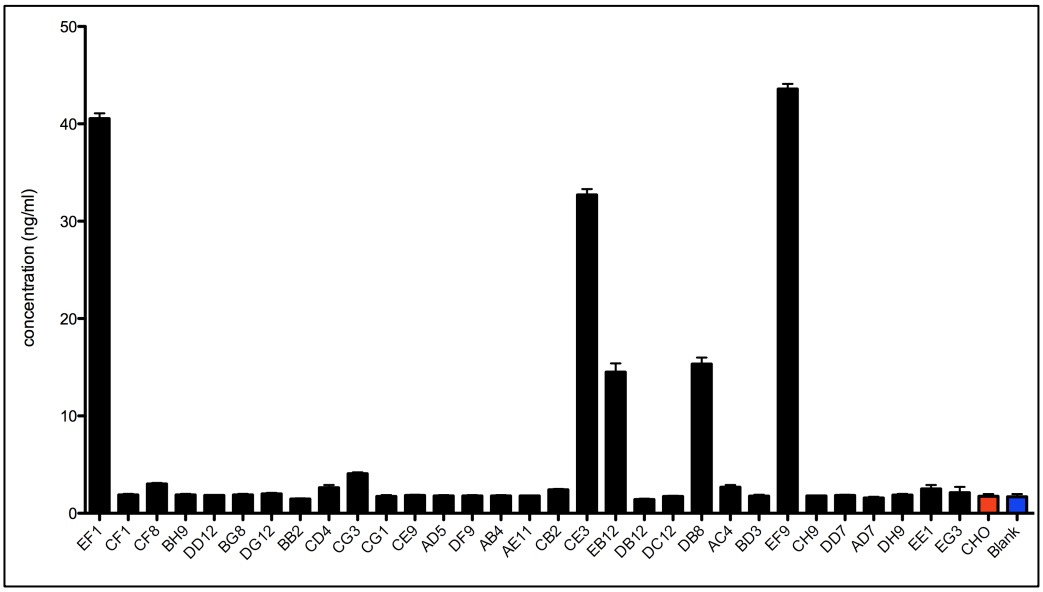


*Figure 4.19 ELISA screening for humanized IgG production on clones derived from co-infected CHO cells. Clones EF1, CE3, EB12, DB8, and EF9 show to produce humanized IgG. Normal CHO supernatant (red) is the negative control, whereas complete DMEM (blue) is used as blank.*

The supernatant of non-infected CHO cells was used as negative control, and complete DMEM was used as blank. Most of the tested supernatants (twenty-six on thirty-one), gave values comparable to negative control and blank samples. The remaining five clones gave higher values, included inside standard curve scale. These are: EB12 (13,6 ng/ml), DB8 (14,7 ng/ml), CE3 (32,1 ng/ml), EF1 (41,1 ng/ml) and EF9 (43,1 ng/ml).

### 4.3.5 Neutralization test on humanized antibody 10F12

A preliminary neutralization assay was performed on concentrated humanized 10F12 (h10F12) antibody supernatant of CHO clone EF9. As controls, murine 10F12 (m10F12) antibody was used, as well as human monoclonal antibodies 2F5 and 4E10. The sample h10F12 was used at a concentration lower than 1 µg/ml, whereas controls were used at 30 µg/ml (m10F12) and at 10 µg/ml (2F5 and 4E10). Viral isolates QHO, AC10 and VSV-G (negative control) were used at 200 TCID. As target cell, TZM-bl, TZM-bl-FcγRI and TZM-bl-FcγRIIb were used in order to verify different pathways of neutralization specific for human antibodies. In figure 4.20a, neutralization values against isolate AC10 are shown: on TZM-bl cells, neutralization activity of the h10F12 is about 70%, compared to 90% of m10F12, 75% of 2F5 and 65% of 4E10. Values with TZM-bl-FcγRI and TZM-bl-FcγRIIb are similar for h10F12, going from 70% to 80%, whereas the activity of the murine antibody is about 50%; 2F5 and 4E10 kept high neutralization values going from 80% to 95%. Neutralization activity against isolate QHO of the humanized antibody remained around 80% for all types of cells (figure 4.20b), resulting slightly higher than 2F5 and 4E10 on TZM-bl and on TZM-bl-FcγRIIb cells, whereas on TZM-bl-FcγRI human monoclonal antibodies values are slightly higher. Neutralization assay against VSV-G (figure 4.20c) showed an unspecific activity of humanized antibody supernatant on TZM-bl and TZM-bl-FcγRI cells (50% and 20% respectively), whereas on TZM-bl-FcγRIIb cells, values were negative. Murine 10F12 showed unspecific values against TZM-bl (15%) and negative values on the derivative cells. Values of 2F5 and 4E10 against VSV-G were close to 0% or negative, on all types of cells.

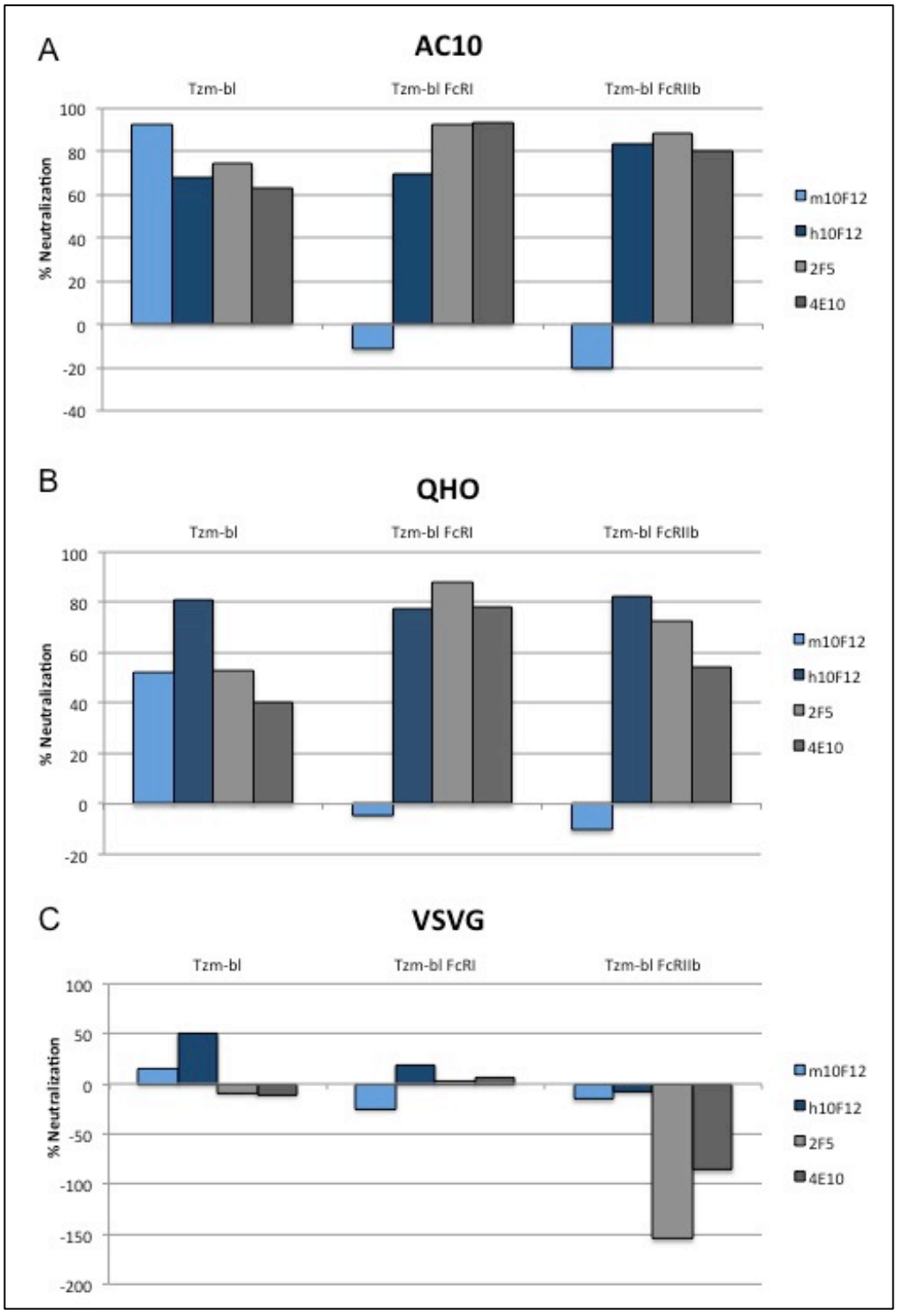


*Figura 4.20 % of neutralization performed using the humanized antibody supernatant (h10F12), murine 10F12 (m10F12) and human monoclonal antibodies 2F5 and 4E10 against viral isolate AC10 (A), QHO (B) and the negative control VSV-G. Obtained values indicate the functionality of humanized antibody.*

# 5. DISCUSSION

Many broad-spectrum neutralizing antibodies currently available were identified in HIV-seropositive patients (e.g. 2F5, 4F10, 2G12, b12, 17b, HJ16, HGN194, HK20, PG16) (Qakkelaar *et al*., 2007; Corti et al., 2010; Pejchal et al., 2010). Therefore, the immunogenic structures that activate such antibodies *in vivo* are unknown. Moreover, some of them (2F5, 4E10) show certain reactivity against self antigens (Haynes *et al*., 2005). Thus, the exploitation of known antigenic structures is highly necessary for the development of new immunogenic agents and the isolation of new broad-spectrum antibodies.

The immunization with fusion complexes to induce a broad-spectrum neutralizing response is based on the possibility to obtain fusion intermediates between HIV-1 proteins gp120/gp41 and cell receptors, using sub-optimal temperatures. In these conditions conserved structures and epitopes, usually hidden inside the protein structure of the viral envelope, are exposed and stabilized. Using fusion intermediates as immunogenic agents induced a specific immune response in mice, which was able to neutralize the infection of competent cells by different viral isolates *in vitro* (Zipeto *et al*., 2006).

Despite such results, indicating the possibility to use fusion complexes to elicit a neutralizing antibody response against HIV-1, the employment of immunogenic agents constituted by fixed cells cannot be applied in clinical studies. Moreover, the purification of fusion complexes (described in Zipeto *et al*., 2006 and Xiao *et al*., 2003) cannot be exploited for large-scale production of a vaccine. The isolation and study of monoclonal neutralizing antibodies obtained with fusion complexes, could allow the exploitation of such antibodies in protection studies on animal models (*in vivo*).

For isolation of neutralizing monoclonal antibodies, mice immunized with fusion complexes fixed with PFA were employed for hybridoma populations production. To select hybridomas secreting antibodies of interest, a functional screening was used, based on usage of neutralizing activity against single infection cycle HIV-1 pseudoviruses (Li *et al*., 2005). Such protocol is highly sensitive, standardized and reproducible (Louder *et al*., 2005). About 8% of hybridomas populations, tested with four different pseudoviruses, were able to show a neutralizing activity above

40%. Few of them showed high neutralization values against less sensitive clades; among them, sample SM24 showed neutralization values between 50% and 80%, generally higher than the positive control (Tri-Mab). Such results indicate production of neutralizing antibodies from immunized mice as well as a good immunogenic function of fusion complexes.

Since the use of fusion complexes can bring to development of antibodies directed against either cell receptors (CD4, CCR5, CXCR4) or other cellular proteins, SM24 supernatant was pre-adsorbed on CHO cells expressing CD4-CXCR4-CCR5, which were used to produce fusion complexes. In addition, its specificity was tested against a pseudovirus with VSV-G envelope.

Sample SM24 proved to contain antibodies specific for HIV-1 envelope epitopes as the whole neutralizing activity was not affected by removal of reactivity against CD4, CCR5 and CXCR4 molecules. Such results prove the specificity of SM24 antibody supernatant against HIV-1. Moreover they indicate possible exposure, in fusion complexes, of important, highly immunogenic, HIV-1 conserved epitopes.

Supernatants of polyclonal populations contain antibodies directed against different epitopes. On one hand, this could result in a synergic activity of antibodies and a consequent higher neutralization rate; on the other hand, the population with higher growth rate could become predominant, resulting in an excess of one particular antibody, which is not necessarily the one with higher neutralization. Indeed, side-expansion of polyclonal population SM24 brought to gradual reduction of neutralizing activity (data not shown). For this reason, and in order to isolate cellular clones responsible for the observed activity, total population of the chosen sample was subdivided into smaller cellular sub-groups (oligoclones). Neutralization assays performed on oligoclone supernatants using different pseudoviruses, some of which highly resistant to neutralization, showed a neutralizing activity of at least 30% with all used pseudoviruses for almost all sub-populations confirming the values obtained for SM24.

In order to exclude samples whose activity was not HIV-1 specific, samples with higher neutralization were tested for their reactivity against target cells. Two samples showing the higher neutralization/reactivity ratio were chosen for single-cell sub-cloning. Supernatants of resulting clones were then tested again for neutralization activity against an easily neutralized pseudovirus, showing a certain degree of neutralization activity. General neutralization of oligoclonal

supernatants resulted higher when compared to monoclonal supernatants; the reason might be due to a synergic effect of combined antibodies.

As gB11 supernatant showed a lower reactivity against target cells, and therefore it is HIV-1 specific, we chose to proceed our studies by analyzing antibody supernatants obtained by single cell cloning of gB11 oligoclone. After the first screening against viral isolate 6535-3 samples were analyzed for their reactivity against target cells, in order to exclude unspecific neutralization activity. Sample 10F12, which showed the higher neutralization values, resulted to be also the sample with lower reactivity against target cells. It was thus tested against different HIV-1 isolates, in order to verify its broad-spectrum activity. As control a VSV-G pseudovirus was also used, to further exclude unspecific reactivity. Results show that the reactivity of sample 10F12 was highly similar to that of Tri-Mab for all tested viral isolates except one (TRO-11). The results obtained in the analyses of 10F12 antibody supernatant indicated a possible broad-spectrum activity of such antibody, which is also characterized by HIV-1 specificity and low toxicity over cells; therefore clone 10F12 was designed as a good candidate for further analyses. 10F12 cells were thus placed into bioreactors, to obtain medium with high antibody concentration, which was harvested and analyzed once a week.

Unfortunately, after some cultivation cycles, the neutralization ability of supernatant 10F12 dramatically lowered. Reasons for such loss of neutralizing activity are not confirmed, but antibody producing clones derived from hybridomas need to be kept in constant sub-cloning conditions, in order to maintain a high antibody activity. Indeed, it is well known that additional problems can arise when the growth of “stable” high-yield hybridomas is scaled up for mass production and either synthesis or activity drop off for no apparent reason (Albright & Janick, 1987).

As a consequence, stable production of a functional antibody is absolutely necessary. Indeed, in order to fully understand the features of an antibody, several analyses for characterization are needed. Therefore, our aim was the cloning of sequences of the 10F12 cells that encode murine IgG protein, in order to include them in a stable expression system.

A further objective was the humanization of IgG antibody produced by the clone 10F12. Tri-Mab, the positive control used in our neutralization, assay is a mixture

of three human monoclonal antibodies; therefore the usage of humanized antibodies for our test would permit a more reliable comparison of neutralizing activity. Moreover, it is known how human antibodies, compared to murine, show a more flexible structure thanks to the hinge region, which separates Fc from Fab (Dorrington and Klein, 1981). As a result, human antibodies can adapt their shape more easily when compared to murine antibodies. Thus, there is the possibility that humanization of a murine antibody could result in a more functional protein. Finally, a humanized antibody could be administered to patients for therapeutic trials.

In order to humanize our antibodies we designed groups of primers to amplify the constant regions of light and heavy chains of human IgG, and variable regions of light and heavy chains of murine IgG. This way it would have been possible to match variable and constant regions inside expression vectors, thus producing full-length sequences for humanized light and heavy chains.

For primer design, our study was inspired by the work of McLean (McLean *et al*., 2000), who produced immunoglobulin expression vectors to be used in the production of recombinant Ig molecules in transfected mammalian cells. In particular, reverse primers for human constant light and heavy chains were designed on the primers described as κ-antisense and γ-antisense, changing the cleavage sites inside them. Forward primers for the same regions were newly designed in order to match the cleavage site placed on murine variable regions and to obtain a functional open reading frame. Primers for murine variable regions were based on commercial primers produced by Novagen. Punctual amplification of DNA fragment of the correct size demonstrated the proper functioning of the designed primers.

The main innovation in this work is the development of a method to produce a cell line that could stably express a recombinant antibody. The usage of lentiviral vectors to transduced eukaryotic cells is an optimal solution to achieve a stable expression, due to their ability to integrate the transgene inside the genome of target cells. When the transgene is integrated inside an active region, with no interruption of genes vital for host cells, it will provide a stable cell line expressing the recombinant protein of interest, which in our case is a humanized antibody against HIV-1. To isolate cells with successful transgene integration single cell cloning is essential, in order to select producing clones.

Nearly 70% of all recombinant proteins produced today are made in Chinese Hamster Ovary (CHO) cells (Jayapal et al., 2007). Their adaptive ability and ease of maintenance have been exploited in many fields of basic bioresearch. The choice of host cells for protein expression has a profound impact on product characteristics and maximum attainable yields. CHO cells possess many of the characteristics needed for a good host cell, as the ability to be adapted and grow in suspension instead of adherence, allowing volumetric scalability and use of stirred-tank bioreactors. Moreover they are amenable to genetic modifications allowing easy introduction of foreign DNA and expression of large amount of the desired protein.

Results demonstrated successful insertion of both the transgenes (light and heavy chain), each transduced by a single lentiviral vector. Evidence can be seen in the growth of co-infected CHO cells after establishment of selective conditions. As mentioned before, downstream both transgenes there is an IRES region, after which genes for antibiotic resistance were placed (neomycin after light chain and puromycin after heavy chain). Therefore, in cells growing under selective conditions the selected transgenes were necessary integrated inside an active region. As a matter of fact, PCR amplification of humanized light and heavy chains, obtained from the genomic DNA of co-infected CHO cells, clearly confirmed the integration of our transgenes inside the cell genome. Altogether, obtained results confirmed the ability of viral vectors to integrate inside the host cell.

After single-cell cloning, supernatants were analyzed for production of humanized antibodies, and 5 producing clones were identified. Thus ELISA assays confirmed transgenes integration and expression.

In order to analyze the activity of the “humanized” antibody, a functional assay based on calculation of neutralization activity was performed. Therefore, the clone with the highest antibody production (EF9) was expanded, and supernatant was then concentrated to increase antibody concentration. Afterwards the concentrated supernatant was analyzed by neutralization assay, performed on two more variants of TZM-bl (TZM-bl-FcγRI and TZM-bl-FcγRIIb), which are specific to analyze human antibodies that neutralize by Antibody-Dependant Cellular Cytotoxicity (ADCC). The results of the neutralization assay indicated high functionality of the humanized antibody. Indeed neutralization values of humanized 10F12 (h10F12)

obtained against viral isolate QHO and AC10 were predominant over the values obtained with original 10F12 (m10F12). Indeed, even though murine 10F12 showed higher values against AC10 on TZM-bl cells, it is used at 30 μg/ml, whereas the humanized antibody concentration was lower than 1μg/ml. Moreover, m10F12 values against QHO are about 50%, whereas values for h10F12 are over 80%. The murine 10F12 did not show neutralizing activity using TZM-bl-FcγRI and TZM-bl-FcγRIIb cells, as they are used to specifically test human antibodies. The observation that h10F12 worked on FcR-expressing cells, is an additional evidence of the production of humanized antibodies. Furthermore, comparing h10F12 with positive controls 2F5 and 4E10, results are far more encouraging as neutralization values are analogous. Concentrating the humanized antibody eventually brought to the concentration of other proteins in cell supernatant, which could interfere with the assay. These preliminary results are encouraging, but they need to be confirmed after large-scale production and protein G purification of the antibody. Nevertheless for TZM-bl-FcγRI and TZM-bl-FcγRIIb, which bind to Fc region, the test is much more specific. Indeed the low neutralization values observed against VSV-G on these cells show high specificity against HIV-1. Moreover, values obtained with TZM-bl-FcγRIIb suggest that the produced antibody is specific for ADCC mediated neutralization, similarly to 2F5 reference antibody on TZM-bl-FcγRI, as recently described (Tudor and Bomsel, 2011). Therefore, even though clones gave low antibody titers (a maximum of 50 ng/ml for non-concentrated EF9 supernatant) results obtained with humanized 10F12 suggests the functionality of the humanized antibody.

There could be multiple reasons for low antibody production. Though CHO cells are not a lymphoblastoid cell line, they are used to successfully produce high yields of recombinant antibody. Nevertheless a soluble sialidase that can degrade recombinant glycoproteins expressed in Chinese hamster ovary (CHO) cells was isolated (Warner *et al*., 1993) so it would be necessary to check the activity of such protein in our co-infected cells. On the other hand, the heavy chain:light chain polypeptide ratio inside the host cell is an extremely limiting factor. In particular, in stably transduced cells, light chain expression needs to be slightly higher than heavy chain as overabundant heavy chain is not folded and is trafficked to the proteasome for degradation (Schlatter *et al*., 2005). Though vectors titration was possible by p24 analysis, the result would not have been a

real infection titration, as count of p24 does not reveal the number of infective particles; therefore the amounts of infective particles of viral vectors were not measured for co-infection. In this case, there are good chances of disequilibrium of insertion of one chain over the other. For this reason, protocol for co-infection needs to be devised. Finally, the primers designed for human heavy chain constant regions can amplify any of human IgG isotype (IgG1, IgG2, IgG3, IgG4), and amplification of constant regions was performed on cDNA derived from human PBMCs, without the chance to select the isotype before cloning. On the other hand, reverse primers for murine variable chains are specific for the murine isotype (IgG1, IgG2a, IgG2b) and hybridomas clone 10F12 produced murine isotype IgG2b. Eventually non-IgG2 human constant regions were matched with variable regions of murine IgG2b. Though no information confirming possible issues deriving from such condition was found, it could, after all, upset the normal production of the antibody protein.

When all this possibilities are taken into account, an antibody production of 50 ng/ml, against a mean production of 1 μg/ml for hybridomas (20-fold higher) is not so embarrassing. The results obtained are a good proof of concept for the method discussed in this work.

As future perspective, confirming the functionality of antibodies produced with this method is our first objective. Our intention is to use the viral vectors described in this work to humanize a well-characterized murine antibody and use the humanized antibody in western blots and ELISA assays thus further verifying its functionality.

In order to minimize variables, after isotyping of murine antibody, human cells producing the corresponding human isotype will be used to extract RNA and amplify the corresponding constant heavy chains. Furthermore a method to measure the viral vectors titer will be studied, and humanized antibodies will be purified with protein G.

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# Acknowledgements

This piece of work had a very problematic creation. Few people know how many times I was tempted to give up. Research life is hard, and only with a lot of patience and passion someone can hope to achieve something. So first of all I want to thank all the people that were close to me during the last three years.

I would like to thank Dr. Donato Zipeto, who welcomed me in his team and followed my work from the beginning, with all that this implies; I thank him for his belief in me no matter what, for his advices, and for sharing computer information with me keeping up my "nerd attitude", event though only on Mac computers.

I want to thank doctors Paola Rossolillo and Andrea Matucci; they were there when I started and guided my first steps in this lab. We shared funny moments and they endured my systematic disorganization with a smile on their face…usually….thank you guys!

I would like to thank also Prof. Bertazzoni and his team (doctors Marco Turci, Paola Righi, Cecilia Bender, Carlo Bidoia, Gianfranco di Gennaro) with whom I shared everything during my first year. Looking back, we were a bit packed in the lab at the time. They helped me to get accustomed with the place and to think of my work. Thank you guys!

My thanks also go to Prof. Morandi and Prof. Mottes without whom I probably would not have started this PhD course. Thank you very much!

Thanks to all the guys in the department (I cannot list them all, you know who you are) for all the coffee breaks, the help (taken and given), confidences, and for all the evenings spent together. I had the possibility to know new people and make some friends in here.

Then I want to thank my partners in this journey, doctors Dalila Astone, Alessandra Ruggiero, Almudena Blanco Miranda and Serena Ziglio, for the help at work, the nights together, all the laughs, the recipes, the cultural exchanges, the computer lesson (I gave), the life lessons (I learned) and….yes, even for tango lessons…I still think it is not for me though!

Dalila: she believed in me even when I didn't, she helped me a lot with my work encouraging me, and inspiring my reasoning, sometimes gently, sometimes harshly, but always for my own good. She was also a very good friend to me, we

shared lots of funny and sad moments, always helping each other and confiding in each other. I can hardly think of anyone whom I count on so much in these last years, neither I can think of anyone so devoted to her job, that at the same has a so strong will to live. I think the balance is not perfect but we will work on it ok? So for all these reasons, Dalila, I want to say thank you, thank you very much!

Alessandra: she made a mess in the lab with her joy and her desire-to-learn. She was a very good friend to me, as well as a good student. Always positive and unleashed, she was also very sweet. Thanks for everything Ale!

Almudena: she brought some Spanish feeling to us, along with her sympathy. She had a very hard time and showed us she could stand it. I think I could learn something from her. Now she is going to go back to Spain, so I want to say thank you Almudena, it was nice to know you!

Serena: she arrived in the last year of my PhD, and from her I could learn that being quiet has nothing to do with being helpless. She showed me to have some spirit and the will to learn. I would like to have her same attitude sometimes. We shared the lab and we got along pretty well. I want to thank you Serena and I wish your time in Tenerife would be ok!

Last but not least, I want to thank all the people in my life that were close to me during these years even though they were not in with me in the lab: my family and my friends. Thanks to all!

# Abstract and posters presented during PhD cycle XXIII

1. Rossolillo Paola, Matucci Andrea, **Racchiolli Pierpaolo**, Lara Mainetti, Stefania Dispinseri, Scarlatti Gabriella, Zipeto Donato. *Broad spectrum neutralizing antibodies against HIV-1 elicited by immunizing with fusion complex.* Europrise Meeting, November 2008, Malta.

2. Rossolillo Paola, Matucci Andrea, **Racchiolli Pierpaolo**, Scarlatti Gabriella, Zipeto Donato. *Identification of anti-HIV-1 broad spectrum neutralizing antibodies raised with fusion complex as immunogen.*
11th Annual IHV International Meeting, September 2008, Baltimore, USA.

3. Matucci Andrea, Rossolillo Paola, Turci Marco, **Racchiolli Pierpaolo**, Siccardi Antonio, Beretta Alberto, Zipeto Donato. *Presence and role of HLA-C in HIV-1 infection.*
11th Annual IHV International Meeting, September 2008, Baltimore, USA

4. Marco Turci, **Pierpaolo Racchiolli**, Dalila Astone, Alessandra Ruggiero, Antonio Siccardi, Alberto Beretta, Donato Zipeto.
*HLA-C presence increases human immunodeficiency virus type 1 (HIV-1) infectivity by interacting with the envelope glycoprotein gp120/41.*
9th National Congress of the Italian Society of Virology, September 2009, Orvieto, Italy

5. Dalila Astone, Paola Rossolillo, Andrea Matucci, **Pierpaolo Racchiolli**, Alessandra Ruggiero, Donato Zipeto.
*Broad spectrum neutralizing antibodies against HIV-1elicited by immunizing with fusion complexes and CD4-indipendent gp120/41s*
9th National Congress of the Italian Society of Virology, September 2009, Orvieto, Italy

6. Marco Turci, **Pierpaolo Racchiolli**, Dalila Astone, Donato Zipeto.
HLA-C presence increases human immunodeficiency type 1 (HIV-1) infectivity by interacting with the envelope glycoprotein gp120/41.
European Society for Virology, 4th European Congress for Virology, September 2010, Cernobbio, Italy.

7. Dalila Astone, Paola Rossolillo, Andrea Matucci, **Pierpaolo Racchiolli**, Andrea Cara, Donato Zipeto.
*Complexes and CD4-independent Env for the Induction of Broad Spectrum Neutralizing Antibodies Against HIV-1.*
Institute of Human Virology 12th Annual International Meeting, October 2010, Tropea.

8. Almudena Blanco, Serena Ziglio, **Pierpaolo Racchiolli**, Dalila Astone, Marco Turci, Donato Zipeto.
*HLA-C increases HIV-1infectivity by interacting with the envelope glycoprotein gp120*
Europrise, Rational Design of HIV Vaccines and Microbicides, November 2010 Lisbon, Portugal.

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# 137 Presence and role of HLA-C in HIV-1 infection

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**Background:** A whole genome association study reported a SNP at -35Kb from the HLA-C gene strongly associated to HIV-1 viral set point and HLAC expression level (Fellay J. et al., 2007). HLA-C is not down-modulated by HIV-1 Nef and can be specifically incorporated in viral membrane enhancing infectivity and resistance to neutralizing antibodies (Cosma A. et al., 1999). We investigated the role of HLA-C in modulating HIV-1 infectivity using cell fusion and pseudovirus infection models and the interaction between HLA-C and Env at membrane level and in purified fusion complexes.
**Methods:** Human cell lines expressing different HIV-1 gp120/gp41 were specifically silenced for HLA-C expression. Cells or pseudoviral particles were used for fusion and single-cycle infection analysis with TZM-bl cells. Fusion efficiency and viral infectivity were compared. Fusion complexes from fusing cells were purified and the molecular proximity of HLA-C and Env was analyzed by using BRET2.
**Results:** The absence of HLA-C significantly decreased the fusion efficiency of cells expressing different R5 and X4 tropic gp120/gp41s. Similarly, pseudovirus infectivity was significantly reduced if they were produced on HLA-C silenced 293T cells. The X4 tropic NDK Env was insensitive to HLA-C at higher infectious doses. VSV-G pseudovirus used as control was not sensitive to HLA-C presence. HLA-C could be detected associated to gp120 in cells taken before fusion, albeit a stronger association was evident during fusion process with target cells.
**Conclusions:** The co-expression of HLA-C with X4/R5 HIV-1 Env increases the fusogenicity of cell lines and the infectivity of pseudotyped viruses. This data point to a specific interaction between HLA-C and gp120 on the cell surface membrane. HLA-C and gp120 increase their association during fusion complex formation. This interaction can stabilize Env trimers, increasing the kinetic of conformational changes or the exposure of the receptor and/or coreceptor binding site favoring membrane fusion. Inhibiting the HLA-C/Env interaction might be of great importance in reducing virus infectivity and become the rationale for the development of new inhibitors of HIV-1 entry.

# 143 Broad Spectrum Neutralizing Antibodies Against HIV-1 Elicited by Immunizing with Fusion Complexes

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**Background:** The development of neutralizing antibodies against HIV-1 is of pivotal importance for the development of an AIDS vaccine. We immunized mice with fusion complexes and elicited antibodies with neutralizing activity against heterologous HIV-1 isolates (Zipeto et al., Microb Infect 2006).

**Methods:** We prepared murine hybridomas from mice whose sera showed the highest neutralizing activity. Their supernatants were tested for reactivity against cells expressing CD4 and CCR5, gp120/41, and the gp120/CD4 complex formed by capture ELISA. Hybridoma antibodies with no reactivity against HIV-1 receptors were selected. IgGs were purified and tested for their neutralizing activity (1-5 µg/ml) using both the TZM-bl neutralization assay (Li M. et al, J Virol 2005) and pNL4-3.Luc.R-E- based pseudoviruses on U87R5 cells with the standard group B Env panel. Specificity was tested against the VSV-G envelope protein. The neutralizing activity of selected antibodies was confirmed using the PBMC-based neutralization assay (Polonis VR et al, Virol 2008).

**Results:** We screened 150 different hybridoma groups; 8% showed a neutralizing activity higher than 40% and 1 between 50 and 80% against the different pseudoviruses tested, similar or higher than the Tri-mAb positive control. Cells from this hybridoma group were cloned; 7% showed a neutralizing activity higher than 40% and 2% higher than 50%. Among the latter as many as 44% showed a neutralizing activity higher than 75% against the different pseudoviruses tested.

**Conclusions:** Hybridomas produced from mice immunized with fusion complexes secrete antibodies neutralizing a panel of HIV-1 clade B Envs. We are screening monoclonal antibodies for their broad spectrum neutralizing activity against HIV-1. The isolation of such monoclonal antibodies will be of great interest for the development of an AIDS vaccine.

Società Italiana di Virologia

# 9th NATIONAL CONGRESS OF THE ITALIAN SOCIETY OF VIROLOGY

Orvieto (TR)
Palazzo del Capitano del Popolo

September 7 - 9, 2009

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## HLA-C presence increases human immunodeficiency type 1 (HIV-1) infectivity by interacting with the envelope glycoprotein gp120/41.

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INTRODUCTION: HLA-C is among the major genetic determinants for host control of HIV-1 and is incorporated into HIV-1 envelope, associates with HIV-1 gp120/41 and increases virus infectivity. Aim of this study is to obtain a detailed analysis of the interaction between HLA-C and HIV-1 gp120/41 to understand its biological consequences. METHODS: We prepared fluorescent HLA-C and gp120/41, to study their interaction during biosynthesis, intracellular transport and membrane localization using BiFC and FLIM. To identify gp120 domains involved in the association with HLA-C, different deletion mutants, as well as an HLA-C insensitive gp120, will be tested. RESULTS: Gp120/41 and HLA-C, tagged with the two YFP fragments Yc and Yn, were produced in mammalian cells and their expression verified. BiFC experiments revealed a direct proximity of gp120/41 and HLA-C, with the putative interaction localizing at the RE level. Colocalization analysis between complemented gp120/41 and HLA-C and the Golgi vescicules will be performed with using Organellight Golgi-RFP. In addition, the Venus fluorescent tag will be tested. DISCUSSION: The finding that HLA-C is involved in increasing HIV-1 infectivity through a direct association with gp120 suggests that gp120 trimers conformation on HIV-1 envelope is influenced by HLA-C. This interaction involves the expression of neutralization epitopes. This study will allow to define the molecular interaction between HLA-C and gp120, leading to the indentification of specific HLA-C binding regions in gp120. This will allow to influence viral infectivity by interfering with this specific interaction.

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## HPV16 affects the expression of ErbB-family receptors during carcinogenesis.

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The ErbB tyrosine kinase receptors are involved in a complex network of signal transduction pathways, playing a key role in regulating many cellular functions. Co-expression of these receptors favours omo-hetero-dimerization among them, enhancing tyrosine-kinase activity promoting the phosphorylation of several tyrosine residues which leads to a complex signalling cascade. The third oncogene of HPV, the E5 protein, interacts with the trasductional pathway of different growth factor receptors including the EGFR. Moreover the HPV-16 E5 can abrogate ErbB4-induced c-Jun protein expression resulting in increasing cell proliferation. Beside the E5, all the early proteins of high risk HPV may regulate the growth factor signalling pathways and therefore, the relationship among all these viral genes and the ErbB-family receptors has been analysed. In this study the W12 cell line in which all the phases of carcinogenesis take place during passages, including integration and E2 regulation loss, was utlised. RT-PCR, western-blot and immunoprecipitation were employed to detect viral and cellular expression at early (W12E) and late passages (W12G). The EGFR expresssion was very high in W12G, and its expression correlated with disease progression. The highest difference was seen in the expression of ErbB3 and ErbB 4 receptors with a reduction from the early to the late passages of W12. These data suggest that the expression of ErbB family is finely tuned with a clear inhibition of the ErbB 4 expression during transformation. Since the expression of this receptor is limited to a less aggressive behaviour, its expression could be useful as favourable prognostic marker.

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## Effect of the expression of influenza A virus NS1 protein in human and avian cell lines.

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The NS1 protein of influenza A virus is a 24 kDa multifunctional protein that plays a key role during infection by counteracting cellular antiviral activities and now is recognizes as pathogenicity factor of the virus. Recently it has been demonstrated that NS1 protein exists in different forms distinguished in the carboxy terminal region's length. These variants can be generated by the introduction of a premature stop codon in the ns1 gene that in turns produces truncated protein's variants. We have focused our work on three forms of NS1 protein, identified in a screening of 40 avian influenza isolates (subtype H7N1 and H7N3) circulating in Italy between 1999 and 2003: a full-length form, of 230 aa, and two truncated forms, of 224 aa and 220 aa. Interestingly, all and only the viruses classified as high pathogenic express the intermediate form (224aa) of NS1 protein. We want to identify cellular specific partner of interaction of the three forms of NS1 protein and, in case, to determine the involvement of specific kinase that may explain the different outcome of viral infection. For this we optimize the method of expression of the three forms of the viral protein in human and avian cell lines and we want to identify specific cellular partners by co-immunoprecipitation and mass spectrometry. Moreover we intend to perform kinase activity assay to analyze the capacity of NS1 protein to interfere with specific cellular pathway and then to promote viral replication and pathogenicity.

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## New immunogens for the induction of broad spectrum neutralizing antibodies against human immunodeficiency virus type 1 (HIV-1).

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INTRODUCTION: Broad spectrum neutralizing antibodies against HIV-1 are considered a central point for the development of an AIDS vaccine. Conserved epitopes which are transiently exposed during the fusion process between viral envelope and target cell, might also be present on CD4-independent gp120/41. Fusion complex intermediates and CD4-independent gp120/41s represent new immunogens that could induce broad spectrum neutralizing antibodies. METHODS: We immunized mice with fusion complexes, showing the induction of broad spectrum neutralizing antibodies. We prepared murine hybridomas and screened for neutralizing activity of secreted antibodies. We immunized mice and rabbits with CD4-independent HIV-1 envelope glycoproteins to evaluate their immunogenicity. RESULTS: Fusion complexes were immunogenic and induced antibodies with neutralizing activity against heterologous HIV-1 isolates. 8% of hybridoma antibodies showed a neutralizing activity higher than 40%, and 2% had an activity higher than 70%. This was specific for HIV-1 and was not due to the presence of receptor-coreceptor antibodies. Hybridoma supernatants containing antibodies with the highest and broadest neutralizing activity are being tested for further characterization. Sera from mice and rabbits immunized with CD4-independent gp120/41s were able to neutralize the infectivity of heterologous HIV-1 isolates in the presence of soluble CD4. DISCUSSION: The induction of broad spectrum neutralizing antibodies against HIV-1, using fusion complexes and CD4-independent gp120/41s, represents an important step toward the development of a vaccine against HIV-1/AIDS.

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## Construction and characterization of recombinant fowlpox viruses expressing human papilloma virus E6 and E7 oncoproteins.

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Human papilloma virus (HPV)-16 is the most prevalent high-risk mucosal genotype and the expression of the E6 and E7 proteins, which can bind the p53 and p105Rb host cell-cycle regulatory proteins, is related to its tumorigenicity. Virus-like-particle (VLP)-based immunogens developed recently have proven to be successful as prophylactic HPV vaccines. However, given the high number of individuals already infected with HPV and the absence of expression of the L1 structural protein in HPV-infected or HPV-transformed cells, an efficient therapeutic vaccine targeting the non-structural E6 and E7 oncoproteins is required.

In this study, two new fowlpox virus (FPV) recombinants encoding the HPV-16 E6 and E7 proteins were engineered and evaluated for their correct expression *in vitro*, with the final aim of developing a therapeutic vaccine against HPV-related cervical tumors.

Although vaccinia viruses expressing the HPV-16 and HPV-18 E6 and E7 oncoproteins have already been studied, due to their natural host-range restriction to avian species and their ability to elicit a complete immune response, FPV recombinants may represent efficient and safer vectors also for immunocompromised hosts.

The results indicate that FPV recombinants can express correctly the E6 and E7 oncoproteins, and they should represent appropriate vectors for their expression in human cells. After genetically modifying the E6 and E7 oncogenes to avoid their binding to p53 and p105Rb cellular substrates, and after evaluating their innocuity and immunogenicity, these avipox-based putative vaccines can be examined for specific elimination of HPV-positive tumor cells.

EUROPEAN SOCIETY for VIROLOGY

# Abstracts

Cernobbio, Italy
April 7 – 11, 2010

## KEYNOTE ABSTRACTS

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## DIFFERENCES IN THE FRAMESHIFT-REGULATING P1-SITE IN TREATMENT-NAIVE AND PI-RESISTANT HIV ISOLATES

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**Background:** In HIV, viral enzymes are cleaved out of gag-pol precursor proteins, which result from a programmed ribosomal frameshift-event in p1. Two Gag cleavage-sites (CS) (p7/p1, p1/p6-gag) and two Pol CS (p7/TFP, TFP/p6-pol) are encoded by the frameshift-regulating site and might contribute to differences in the frameshift-efficiency and thereby the amounts of viral enzymes.
**Methods:** We analysed the p1 nucleotide sequence of 968 isolates from patients infected with HIV subtype-B, of whom 644 were treatment-naive and 324 treatment-experienced failing PI therapies. The frameshift-efficiency was determined with a dual-luciferase assay.
**Results:** 21 of 52 nucleotide positions (nt2085-2136) showed no variability compared to HxB2. Mutations at five positions significantly accumulated in PI-resistant HIV and were mainly associated with Gag CS- but also non-CS-mutations. Only mutation ntA2098G resulted in CS-mutations in Gag (I437V) and Pol (05G/05S) reading frames. Viruses with 437V/05G or 437V/05S showed different resistence profiles in the protease. The analysis of sequences (n=66) harbouring different changes in the nucleotide sequence revealed no significant difference in frameshift-efficiency. L449F lead to a significant increase in frameshift-efficiency (HxB2: 6.7%, L449F: 9.6%) due to a second slippery site and I437V in combination with additional polymorphism (I437V+polymorphisms: 10.6%), but both effects could be reversed by natural polymorphisms. Only 25 of 81 isolates harbouring L449F or I437V showed a moderate increased frameshift-efficiency.
**Conclusions:** An increase in frameshift-efficiency is not a general mechanism of drug-resistance. In PI-resistant HIV treatment-associated mutations occur at both C-terminal Gag CS and at Pol CS TFP/p6-pol.

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## DIVERSITY OF PLASMA AND PROVIRAL GAG SEQUENCES DURING HIV INFECTION

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**Background**: The rate of progression of HIV infection varies amongst patients, with some remaining healthy for >10years. Evidence suggests differences in the host, such as HLA-type and co-receptor polymorphisms play a role in progression to AIDS, but less is known about the viral contribution to immune collapse (IC) and in particular diversity within the viral swarm.
**Methods**: A small cohort of HIV patients (n=7) were investigated. Gag isolates were cloned and sequenced both from proviral and plasma samples, including where available, longitudinal samples. Changes in the viral swarm were determined and the diversity within each sample calculated and an association with CD4+ count and VL assessed.
**Results**: During control of HIV infection, there was minimal diversity (average 1.7%) of Gag amino acid sequences in both plasma and provirus from each patient. Surprisingly, it was evident that proviral sequences were distinct from those in the plasma, suggesting compartmentalisation of virus. From samples studied at IC (CD4+ T cells <500), the average diversity was 0.78%, lower than that seen during latency, and this was associated with a higher VL (average 328,097 *vs*. 49,246 copies/ml in latency). For one patient (005) diversity during IC was slightly greater (2.38%) and may be explained by a recombination event between two viral isolates.
**Conclusion:** Results suggest that once IC occurs, diversity decreases albeit slightly, possibly as viral fitness is at its peak. Future monitoring of the cohort as others progress towards IC, and the inclusion of more patients, will further our understanding of this phenomenon.

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## HLA-C PRESENCE INCREASES HUMAN IMMUNODEFICIENCY TYPE 1 (HIV-1) INFECTIVITY BY INTERACTING WITH THE ENVELOPE GLYCOPROTEIN GP120/41

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**Background.** HLA-C is one of the major genetic determinants for host control of HIV-1 infection. HLA-C is incorporated into HIV-1 envelope, associates with HIV-1 gp120 and increases virus infectivity. A detailed analysis of the interaction between HLA-C and HIV-1 gp120 is important to understand its role and its biological consequences.
**Methods.** We prepared fluorescent-tagged versions of HLA-C and gp120 and used them to study their localization during biosynthesis, intracellular transport and membrane localization with techniques based on Bi-Molecular Fluorescence complementation (BiFC). To identify gp120 domains involved in the association with HLA-C, we will test different deletion mutants, as well as an HLA-C insensitive gp120 we recently characterized.
**Results.** HIV-1 gp120 and HLA-C were tagged at the C-terminal with YFP or Venus C-term or N-term fragment (YC, VC, YN, VN). The fusion proteins were transiently expressed in mammalian cells and analyzed by western blot to check their correct expression. BiFC experiments revealed a direct proximity of gp120 and HLA-C in living cells using different combination of tagged proteins. Colocalization analysis between the complemented tagged proteins and the Golgi apparatus or Endoplasmic Reticulum (ER) was performed using Immuno-Fluorescence and Organellight Golgi-RFP. The interaction between the two proteins was located at ER and Golgi level.
**Conclusions.** The finding that HLA-C molecules are involved in increasing HIV-1 infectivity through a direct association with gp120 suggests that the conformation of gp120 trimers on the envelope of HIV-1 particles might be influenced by HLA-C. This interaction involves the expression of neutralization epitopes.
This study will allow to define more precisely the molecular interaction of HLA-C and gp120 leading to the possible identification of specific HLA-C binding regions in gp120. This will open possibilities of interfering with viral infectivity by suppressing the association with HLA-C.

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## SEMEN ENHANCES HIV INFECTION

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**Background**. Although semen (SE) is the major vector for HIV transmission, its impact on viral infectivity is poorly defined with conflicting effects reported. We have explored the influence of SE on HIV infection in a systematic fashion and now describe methodologies that permit the evaluation of SE by minimizing its intrinsic cytotoxic properties.
**Methods:** To minimize the toxic effects of SE, we first incubated virions rather than cells with SE followed by adding small volumes of these HIV/SE mixtures to comparatively large volumes of cell cultures.
**Results:** Using these conditions we consistently found that SE effectively enhances the infectiousness of HIV independently of its genotype, the virus producer or the target cell type. We showed that the enhancing activity of SE involves the formation of high molecular weight aggregates and can be observed under acidic pH conditions or in the presence of vaginal fluid. We found that the ability of SE to promote HIV infection is conserved between different donors and that the magnitude of enhancement correlated with the level of reactivity with antisera raised against SEVI, an HIV enhancing amyloid previously isolated from SE. Our data also revealed that SE and SEVI substantially reduce the antiretroviral efficacy of microbicides.
**Conclusions**: Our findings suggest that SE, the major vehicle propelling the expansion of the HIV pandemic, potently enhances the infectivity of HIV and that SEVI amyloid fibrils in semen contribute to this effect. This HIV boosting effect of SE should be considered in the future efforts to develop effective microbicides.

# 12TH Annual International Meeting of the INSTITUTE OF HUMAN VIROLOGY

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## Fusion Complexes and CD4-Independent Env for the Induction of Broad Spectrum Neutralizing Antibodies Against HIV-1

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**Introduction:** Broad spectrum neutralizing antibodies against HIV-1 are essential for the development of a humoral anti-AIDS vaccine. We used fusion complexes and CD4-independent gp120 as new immunogens to induce neutralizing antibodies blocking the infectivity of different primary isolates of HIV-1.

**Methods:** Spleen cells from mice immunized with fusion complexes were used to prepare murine hybridomas. Secreted antibodies were screened for their neutralizing activity using the pseudovirus standard neutralization assay. In parallel, the immunogenicity of CD4-independent Env, on which conserved epitopes might be exposed, has been tested.

**Results:** Among antibodies secreted by hybridoma clones, 8% showed a neutralizing activity higher than 40% (1 μg/ml), and the best ones showed neutralization levels as high as 80% against the pseudovirus B panel, reaching neutralization levels similar or higher than the Tri-mAb control. 10 hybridoma clones showing 80% or higher neutralization levels were selected and re-cloned by limiting dilutions. Panel C evaluation is ongoing. Preliminary results using a 1:1000 sera dilution from mice immunized with CD4-independent Env showed a neutralizing activity of 40-60% and, as expected, a 2-3 folds neutralization increase in the presence of sCD4.

**Conclusions:** Monoclonal antibodies obtained by immunizing with fusion complexes showed a broad spectrum neutralizing activity against all panel B pseudoviruses, as well as against a group of selected laboratory isolates. Sera from mice immunized with CD4-independent Env showed neutralizing activity against heterologous Envs that increases in the presence of sCD4, suggesting the elicitation of antibodies against the conserved coreceptor binding site. In conclusion, fusion complexes and CD4-independent Env represent potential new immunogens that can induce neutralizing antibodies with activity against a wide panel of HIV-1 isolates.

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## HLA-C Associates with Env and Increases HIV-1 Infectivity

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**Introduction:** Host cell proteins are specifically incorporated into HIV-1 envelope during budding. Virionic HLA-C reduces HIV-1 susceptibility to neutralizing antibodies (Cosma 1999). A polymorphism in the 5' region of the HLA-C gene has been associated to individual variations in set point viral loads (Fellay 2007), suggesting a role of HLA-C expression levels in modulating HIV-1 infectivity. We have reported (Matucci 2008; Baroni 2010) that HLA-C in the HIV-1 envelope associates to Env increasing viral infectivity of both R5 and X4 tropic viruses. The purpose of this study is to elucidate this interaction and exploit it for generating new immunogens capable of conferring protective immunity.

**Methods:** Using recombinant HLA-C and Env molecules fused to fluorescent tags, we are studying their association using the bimolecular fluorescence complementation (BiFC) technique. BiFC allows the analysis of the interaction between associated proteins in living cells and to study their co-localization into cellular compartments. Env-deletion mutants and Env swapped domain recombinants are being tested to identify protein domains involved in their association. HLA-C coded by different alleles will be analyzed for their association with Env to study the influence of HLA-C polymorphisms in increasing HIV-1 infectivity.

**Results:** Preliminary results on Env-HLA-C association and sub-cellular compartmentalization, using a specific marker for ER, reveal a direct proximity between these proteins, suggesting an early association at the ER level. Similarly, analysis of co-localization between Golgi apparatus and Env-HLA-C reveal the presence of the complementation signal between the two proteins in Golgi vesicles.

**Conclusions:** BiFC assays allows an efficient visualization of HLA-C and HIV-1 Env association in living cells. Preliminary results obtained suggest an association between the two proteins at the ER and Golgi level. Understanding the interaction between HLA-C and HIV-1 Env might give valuable information for the design of new immunogens and/or compounds that, by reducing viral infectivity, may help controlling HIV-1 infection.

# Rational Design of HIV Vaccines and Microbicides

## Network Annual Conference

## 15-18 November 2010

## Tiara Park Atlantic Hotel

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## Portugal

**Post-entry events of efficient R5 vs. inefficient X4 HIV-1 replication in primary CD4$^+$ T lymphocytes, a transcriptome analysis**

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HIV-1 infects CD4+ cells via interaction with CD4 and either CCR5 or CXCR4. However, only CCR5-using (R5) viruses are efficiently transmitted and sustain the viral pandemics, while CXCR4-using (X4) viruses emerge later in coincidence with the immunodeficiency state and progression to AIDS in about 50% of individuals infected with subtype B HIV-1, but not with other subtypes. Unravelling cellular and molecular correlates of this asymmetric co-receptor use would be relevant to understand HIV pathogenesis as well as for the development of preventive strategies aimed at blocking R5 HIV-1 spreading. We have previously reported that cord blood derived CD4$^+$ T cells (CB4 cells) maintained in a sub-optimally activated state in IL-2 enriched medium for 7-14 days before infection are permissive for R5 and restricted for X4 HIV-1 replication. Of interest, this restriction did not occur at the level of viral entry, but it was rather correlated to a superior capacity of R5 HIV-1 to spread after infection [Vicenzi E *et al.*, 1999]. In the present study, we examined the transcriptomic profile at different time points (8, 24, 48, 72 h) of CB4 cells established from 6 independent donor/infection pairs after infection with isogenic NL4-3 (X4) and NL-AD8 (R5) viruses normalized for MOI. Gene expression was measured using Human Genome U95A chips and analyzed with the DAVID knowledge base software. Approximately 900 and 1,100 genes were selectively mobilized by R5 and X4 HIV-1 infection, respectively, vs. mock-stimulated uninfected control cells. An additional 420 genes were modulated by both viruses vs. controls. R5 HIV-1 induced a rapid mobilization of genes linked to cell proliferation and signal transduction, whereas the X4 virus predominantly modulated the expression of genes associated with cell death and the immune response. Both viruses upregulated the expression of CXCL12/SDF-1$\alpha$, but only X4 downregulated CXCR4 mRNA; CCR5 mRNA was unaffected by either infection at all time points. Other genes previously linked to control of HIV replication that were modulated by R5 and X4 HIV-1 include APOBEC-3G, IFN-$\gamma$, CCL5/RANTES, CCL7/MCP-3 and CCL14/HCC1. We are currently analyzing additional genes discordantly co-modulated by R5 and X4 viruses in the search of host genes associated with the permissive vs. restricted HIV replicative profile in this model system. Thus, both R5 and X4 HIV-1 profoundly affect the transcriptional activity of primary CD4$^+$ T lymphocytes even in the absence of overt replication (as observed in X4 infection).

**HLA-C increases HIV-1 infectivity by interacting with the envelope glycoprotein gp120**

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**Introduction:** Host cell proteins are specifically incorporated into HIV-1 envelope during budding. It has been reported that virionic HLA-C reduces HIV-1 susceptibility to neutralizing antibodies (Cosma et al., 1999). A polymorphism in the 5' region of the HLA-C gene has been associated to individual variation in set point viral loads (Fellay et al., 2007), suggesting a role of HLA-C expression levels in modulating HIV-1 infection.
We have recently reported that HLA-C present in HIV-1 envelope associates to the viral envelope glycoprotein gp120 increasing viral infectivity of both R5 and X4 tropic viruses (Matucci et al., 2008; Baroni et al., 2010).
The purpose of our study is to elucidate this interaction and exploit it for generating new immunogens capable of conferring protective immunity.
**Methods:** Using recombinant HLA-C and Env molecules fused to fluorescent tags, we are studying their association using the bimolecular fluorescence complementation (BiFC) technique. BiFC allows the analysis of the interaction between associated proteins in living cells and to study their co-localization into cellular compartments. Env-deletion mutants and Env swapped domain recombinants are being constructed and tested to identify protein domains involved in their association. HLA-C coded by different alleles are being analyzed for their association with Env to study the influence of HLA-C polymorphisms in increasing HIV-1 infectivity.
**Results:** Preliminary results on Env-HLA-C association and sub-cellular compartmentalisation, using a specific marker for the endoplasmic reticulum (ER), reveal a direct proximity between the two proteins, suggesting an early association at the ER level. Similarly, the analysis of the co-localization between Golgi apparatus and Env-HLA-C complex reveals the presence of the complementation signal between the two proteins in Golgi vesicles.