# SAPIENZA

UNIVERSITA' DI ROMA

DOTTORATO DI RICERCA IN MEDICINA SPERIMENTALE
XXXI CICLO

# "Integrase Defective Lentiviral Vector as a Vaccine Platform for Delivering HIV-1 Antigens"


DOTTORANDO

Dott.ssa Alessandra Gallinaro

DOCENTE GUIDA

Dott. Andrea Cara


COORDINATORE DEL DOTTORATO
Prof. Maurizio Sorice

ANNO ACCADEMICO 2017-2018

**Index**

**Introduction** ..... **3**

**Chapter 1** ..... **6**

**Human immune-deficiency virus-1 (HIV-1)** ..... **6**

*1.1 HIV-1 structure and genome* ..... 7

*1.2 HIV-1 life cycle* ..... 10

*1.3 HIV-1 pathogenesis and clinical manifestation* ..... 17

*1.4 Treatment of HIV-1 infection* ..... 20

*1.5 HIV-1 vaccination strategies: state of art* ..... 21

**Chapter 2** ..... **24**

**Integrase Defective Lentiviral Vectors (IDLVs)** ..... **24**

*2.1 HIV-based LVs: from disease causing agent to efficient and safe tool for therapy and vaccination* ..... 24

*2.2 First-generation Lentiviral vector system* ..... 25

*2.3 Second-generation Lentiviral vector system* ..... 26

*2.5 Further improvements in LVs design* ..... 29

*2.6 Integrase Defective Lentiviral Vectors (IDLVs)* ..... 29

*2.7 Simian immunodeficiency virus (SIV) based LVs* ..... 32

**Chapter 3** ..... **33**

**Development of Integrase Defective Lentiviral Vector as delivery platform for vaccination against HIV-1** ..... **33**

*3.1 Rationale and aim of the work* ..... 33

*3.2 Materials and methods* ..... 36

*3.2.1 Plasmids construction* ..... 36

*3.2.2 Stages of plasmids construction process* ..... 37

*3.2.3 Production of Integrase Defective Lentiviral Vectors (IDLVs)* ..... 39

*3.2.4 IDLVs titration by Reverse Transcriptase (RT) activity assay* ..... 39

*3.2.5 Cytofluorimetry (FACS) titration of IDLVs* ..... 40

*3.2.6 Confocal laser scanning microscopy (CLSM)* ..... 40

*3.2.7 Western Blot* ..... 41

*3.2.8 Animal immunization protocols* ..... 41

*3.2.9 Interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay* ..... 42

*3.2.10 Enzyme-linked immunosorbent (ELISA) assay* ..... 43

*3.2.11 Neutralization assay* ..... 44

**Chapter 4** ..... **45**

**Results** ..... **45**

*4.1 Production of IDLV expressing HTI immunogen* ..... 45

*4.2 Mice immunization with IDLVs expressing HTI* ..... 48

*4.3 Generation of SIV-based IDLVs expressing ConSOSL.UFO antigens* ..... 50

*4.4 Mice immunization with IDLV-UFO.664 and IDLV-UFO.750* ..... 53

*4.5 Non-human primates immunization with IDLV-UFO.750* ..... 55

**Chapter 5** ..... **57**

**Discussion** ..... **57**

**Bibliography** ..... **64**

# Introduction

It has been 35 years since HIV-1 was first identified as the causative agent for AIDS (*Barre-Sinoussi et al., 1983; Popovic et al., 1984*). However, despite improvements in the therapy of HIV infection allow for controlling AIDS pandemic spread, to date no effective preventive HIV-1 vaccine is available. Indeed, almost 37 million people worldwide were still living with HIV/AIDS and about 1 million people worldwide died of HIV-related illnesses at the end of 2017 (*World Health Organization*). Therefore, the development of a prophylactic vaccine able to prevent HIV infection remains an urgent need.

To be effective, a global HIV-vaccine will have to elicit potent and durable immune responses against the enormous diversity of HIV variants (*Picker et al., 2012*). All the vaccine strategies tested so far in clinical trials have been unable to induce complete protection from HIV infection, although they were able to elicit various degrees of immunological responses (*O'Connel et al., 2012*). Two main obstacles had hampered the development of an effective HIV-1 vaccine: virus variability and the imperfect characterization of correlates of protection against the virus. High HIV-1 mutation rate leads to a continuous viral evolution both among populations and within the individual hosts, enabling the virus to evade immune responses. Indeed, it is well known that in the early stages of infection robust cellular and humoral immune responses are developed against the virus, but fail to control viral replication in the majority of the infected individuals.

Initial vaccine candidates were designed with the aim of inducing protective neutralising antibodies (NAbs) by immunizing with recombinant viral envelope (Env) glycoprotein (*Pitisuttithum et al., 2006*). This classical strategy failed in different clinical trials and researchers' efforts begun focusing on the development of an HIV vaccine able to induce strong HIV-1 specific CD8 T cells, an approach called "T-cell vaccination" (*Korber et al., 2009*). While elicitation of anti-Env antibodies seems to be essential to protect against HIV acquisition, induction of specific cellular immune responses towards HIV-1 structural proteins like Gag is essential to control viral replication, thus impacting on epidemic spread. Several studies have shown that T-cell mediated immune responses can control viral load in SIV-infected macaques as well as in HIV-infected individuals (*Janes et al., 2013; Chakraborty et al., 2014*). Therefore, a successful HIV vaccine should elicit both specific neutralizing antibodies and T-cell. The RV144 clinical trial was the only vaccine strategy that

showed some efficacy (*Rerks-Ngarmet al., 2009*). The trial combined a prime with vector-delivered structural genes and a boost with Env glycoprotein and provided evidence of a direct correlation between protection and anti-V1/V2 non-neutralizing antibodies (Abs), antibody-dependent cellular cytotoxicity (ADCC) and an inverse correlation with anti-Env IgA response in plasma. Unfortunately, Abs titres decreased by 6 months post-boost.

Importantly, vaccine delivery systems play a pivotal role for a successful vaccine. Live-attenuated or whole inactivated virus have been used for vaccination of macaques, but their use in humans was impeded due to safety issues (*Gibbs et al., 1994*). In order to improve the safety of vaccine formulation, many different types of vehicles have been developed: plasmids, liposomes, bacterial and viral vectors. Immunization with DNA plasmid represents the easiest technology in terms of manufacturing; DNA is very stable and after injection elicits strong B and T cell responses in mice. However, DNA vector immunogenicity is lower in more complex animal models and in humans, although it can be greatly improved by electroporation.

Among viral vectors, poxvirus- and adenovirus-based vectors have been the most used so far, due to their ability to express at high levels the immunogen(s). On the other hand, they can induce anti-vector immunity as well as they use can be hampered by pre-existing anti-vector immunity.

Lentiviral vectors (LVs) represent an attractive tool for gene delivery into target cells, both in vitro and in vivo, and can be used for gene therapy as well as vaccine applications. LVs derive from wild-type lentivirus (*Retroviridae* family) and have been engineered by removing all unnecessary gene products in order to prevent the reconstitution of pathogenic replication competent virus in vivo. LVs have several advantageous features: (i) large coding capacity, (ii) high transduction efficiency, (iii) stable and long-term transgene expression due to the integration into the genome of transduced cells, (iv) possibility to be easily pseudotyped with different envelope proteins to widen or specifically select target cells; (v) low pre-existing anti-vector immunity. Moreover, in contrast with all other retroviral vectors that depend on cell division to replicate, LVs are able to transduce quiescent cells, a crucial issue for gene transfer in terminally differentiated cells like neurons, myocytes, retinal cells and hepatocytes, and an important characteristic for immunization purposes. Indeed, LVs can transduce macrophages and dendritic cells, the

main antigen presenting cells (APC), becoming reservoirs for antigen presentation to T-cells.

In the context of HIV-1 vaccination, HIV-derived LVs expressing either HIV-1 or SIV antigens have been used in several pre-clinical studies in which have induced high titres of specific cytotoxic T-cells and Abs in the mouse and NHP models (*Beignon et al., 2009; Buffa et al., 2006*).

However, by inserting into host genome in unpredictable sites, LVs may cause insertional mutagenesis, disruption of host gene expression or functionality and malignant transformation of cells. This is what happened during a clinical trial in which Moloney-virus-based gamma retroviral vectors were used for SCID-X1 gene therapy, whereas several leukaemia cases were observed (*Hacein-Bey-Abina et al., 2003*). Enhancing sequence of MoMLV integrated in proximity of a proto-oncogene determining its activation and thereby causing tumorigenicity. Although it has been showed that LVs did not cause increase in cancer incidence in mice compared to gamma retroviral vectors (*Cesana et al., 2014*), their use in clinical applications raises several safety concerns. In order to improve the safety of LVs, Integrase Defective Lentiviral Vectors (IDLVs) have been developed by impairing the functionality of Integrase (IN) protein thereby avoiding the risk of insertional oncogenesis (reviewed in *Negri et al., 2011)*. In absence of a functional IN, IDLV genome accumulates in circular DNA forms, called E-DNAs that are transcriptionally active and persistent in non-dividing cells allowing for efficient expression of the target gene. IDLV expressing HIV gp120 Env protein or Gag gene have been shown to be immunogenic and capable to induce humoral and cellular immune responses both in mouse and NHP animal models (*Negri et al., 2007; Buffa et al., 2006*). In particular, immunization of rhesus macaques with IDLV-Env (*Negri et al., 2016)* elicited specific and persistent Abs that were detectable up to one year after the injection.

# Chapter 1

## Human immune-deficiency virus-1 (HIV-1)

HIV-1 belongs to *Retroviridae* virus family which main feature is the reverse flow of genetic information from RNA to DNA. Retroviruses have a diploid RNA genome, that is reverse-transcribed into cDNA by the viral Reverse Transcriptase (RT) after virus entry into the target cell. Viral cDNA integrates into the host chromosomal DNA by using the viral encoded enzyme Integrase (IN), thus becoming a template for the production of viral RNAs and proteins that are assembled in the infected cells to form the new virions. Retroviruses have been classified in different genera: "simple" retroviruses like alpharetroviruses, betaretroviruses, and gammaretroviruses codifying only for Gag, Pro, Pol, and Env proteins; and "complex" retroviruses like deltaretroviruses, epsilonretroviruses, lentiviruses, and spumaviruses that codify also for regulatory proteins (**Table 1**). Lentiviruses such as HIV-1 cause slow and chronic diseases and target monocytes/macrophages and lymphocytes. Lentiviruses differ from other Retroviruses since they are able to propagate in non-proliferating cells as they evolved a strategy to import cDNA into the nucleus independently of cell division.

| | RETROVIRUS GENERA | |
|---|---|---|
| SIMPLE RETROVIRUSES | ALPHARETROVIRUS | Avian leukosis virus (ALV) |
| | | Rous sarcoma virus |
| | BETARETROVIRUS | Mouse mammary tumor virus (MMTV) |
| | | Mason-Pfizer monkey virus (M-PMV) |
| | | Jaagsiekte sheep retrovirus |
| | GAMMARETROVIRUS | Murine leukemia virus (MuLV) |
| | | Feline leukemia virus (FeLV) |
| | | Gibbon ape leukemia virus (GaLV) |
| | | Reticuloendotheliosis virus (RevT) |
| COMPLEX RETROVIRUSES | DELTARETROVIRUS | Human T-lymphotropic virus type 1,2 |
| | | Bovine leukemia virus (BLV) |
| | | Simian T-lymphotropic virus type 1,2,3 |
| | EPSILONRETROVIRUS | Walleye dermal sarcoma virus |
| | | Walleye epidermal hyperplasia virus 1 |
| | LENTIVIRUS | Human immunodeficiency virus type 1 (HIV-1) |
| | | Human immunodeficiency virus type 2 (HIV-2) |
| | | Simian immunodeficiency virus (SIV) |
| | | Equine infectious anemia virus (EIAV) |
| | | Feline immunodeficiency virus (FIV) |
| | | Caprine arthritis encephalitis virus (CAEV) |
| | | Visna/maedi virus |
| | SPUMAVIRUS | Human foamy virus |

**Table 1.** Taxonomic classification of Retroviruses.

Two HIV serotypes are known: HIV-1, mostly diffused in Europe, America and Central Africa, and HIV-2, spread in West Africa and Asia and responsible for a less severe disease. Phylogenetic analysis of HIV-1 isolates allows to classify the different subtypes into four groups: M (main), O (outlier), N (non-M, non-O), and P, that have different geographical distribution (**Figure 1**). While N and O groups are more closely related to chimpanzee simian immunodeficiency virus ($SIV_{cpz}$), P group is more similar to gorilla SIV ($SIV_{gor}$) (*Plantier JC et al., 2009*). Viruses belonging to M group are the main responsible of global HIV-1 infection and consist of nine clades or subgroups (A, B, C, D, F, G, H, I, and K) and 49 circulating recombinant forms (CRFs). CRFs develop during super-infection of a single cell by two different virus subtypes that recombine their genomes. This remarkable genetic variability is due to the error-prone viral reverse-transcriptase (RT) that introduces 1 to 2 x $10^{-5}$ mutations/nucleotide during each replication cycle. High recombination frequencies, high levels of virus production *in vivo* and high number of infected individuals, increase the genetic heterogeneity of HIV-1. While mutations are distributed throughout the whole HIV-1 genome, the most consistent have been detected in the env gene.

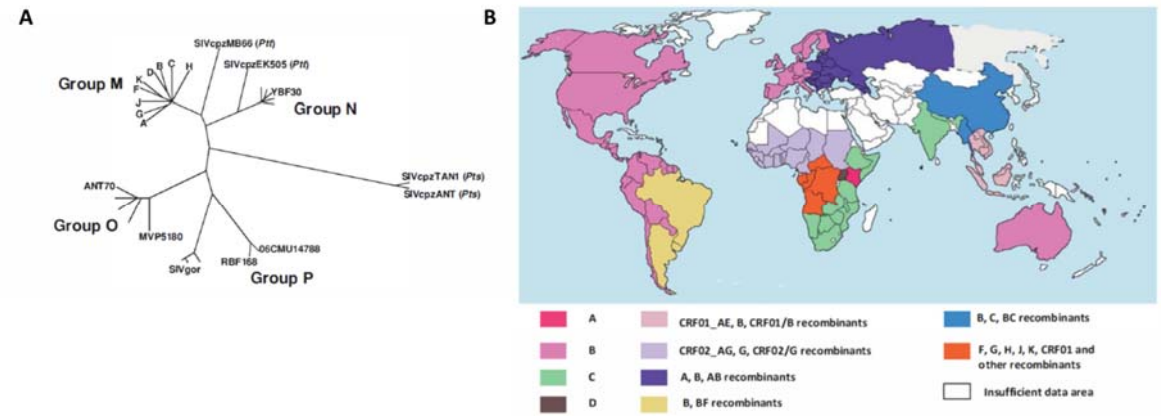



**Figure 1. A.** Phylogenetic relationships of HIV-1 groups M, N, O, and P with different simian immunodeficiency virus (SIV) isolates. **B.** Geographic distribution and prevalence of HIV-1 clades and circulating recombinant forms (CRFs). (Adapted from *Foster et al., 2011*)

## ***1.1 HIV-1 structure and genome***

HIV-1 is an enveloped spherical virus with about 80-120 nm diameter. The envelope consists of a lipid membrane bilayer acquired during the budding from the infected cell. gp120 and gp41 glycoproteins are anchored in the envelope and form trimeric spikes on

the virus surface. Under the membrane envelope the p17 or matrix (MA) protein increases the particle stability (*Ono et al., 2000*), whereas the p24 protein forms a conical nucleocapsid within which viral RNA genome and proteins are hold (viral core). Proteins inside the virion are essential for the early stage of HIV-1 infection: reverse transcriptase (RT) produces the cDNA using genomic RNA as template; integrase (IN) is responsible for pro-viral DNA insertion into host genome; protease (PR) cleaves polyprotein (POL) precursors in functional proteins. The viral genome consists of two linear positive-sense single stranded RNA molecules of approximately 9.7 kb and encodes for nine genes that have been classified in three groups: structural, regulatory and accessory (**Figure 2**).

Structural genes are transcribed and translated as polyproteins and later cleaved by viral and cellular proteases to give rise to individual proteins. In particular:

- pr55-Gag is cleaved into the virion structural components: matrix (p17); the capsid (p24); the nucleocapsid (p7), having two zinger finger domains for binding and packaging of viral RNA genome into particles; p6, essential for the release of new-born virions form the infected cell; and two spacer polypeptides, SP1 and SP2 (*Bukrinskaya, 2007*)
- $pr160^{gag\text{-}pol}$ precursor is cleaved to produce viral enzymes: reverse transcriptase (RT) with ribonuclease H (RNaseH), protease (PR), and integrase (IN)
- glycosylated gp160-Env precursor is cleaved by cellular proteases in surface glycoprotein gp120 (SU), responsible for the binding to the receptor, and transmembrane protein gp41 (TM), involved in the fusion of viral Env with the membrane of the target cell (*Bolinger & Boris-Lawrie, 2009*). Mature and functional Env is a metastable trimer of heterodimers, in which gp120 and gp41 are held together by non-covalent interactions.

Regulatory genes encode for:

- Tat (trans-activating) protein that binds the trans-activation response element (TAR) and increases RNA polymerase efficiency thereby promoting viral RNA transcription
- Rev (regulator of viral expression) protein that binds the rev response element (RRE) in the unspliced viral RNAs facilitating their export from the nucleus into the cytoplasm

Accessory genes codify for:

- Nef (negative regulatory factor) is multifunctional cytosolic protein important for replication *in vivo*: it acts by downregulating the CD4 receptor on the infected cell membrane allowing for virus release. It also downregulates MHC class I levels in order to avoid lysis of HIV-1 infected cells by T cells
- Vpr (viral protein R): facilitates nuclear import of viral cDNA and pre-integration complex in the early phase of infection, enabling HIV to replicates regardless to cell division
- Vif (viral infectivity factor): enhances infectivity by degrading or sequestering the APOBEC-3G protein, a cytidine deaminase that binds to Gag protein becoming part of the new virions. In the next round of infection APOBEC-3G adds mutation to viral DNA during reverse transcription leading to the block of viral replication (*Durand et al., 2011*)
- Vpu (viral protein u) is a membrane protein present only in HIV-1 genome while absent in other Retroviruses. Vpu promotes the degradation of CD4 receptor and enhances virion production by sequestering tetherin into the cytoplasm and preventing virus internalization mediated by tetherin (*Stuart et al., 2008*).

Sequences essential for viral genome transcription and integration are clustered in Long Terminal Repeats (LTRs) at both viral RNA termini. LTRs consist of: 3' unique elements (U3, 453bp), repeat elements (R, 98bp), and 5' unique elements (U5, 83bp). LTRs contain important cis-acting elements: *att* repeats, located at both 5' and 3' end, are required for proviral DNA integration into cellular genome; enhancer/promoter sequences; transactivation response element (TAR); and polyadenylation signal (polyA).

Other *cis*-acting sequences are: the primer binding site (PBS), necessary for starting the synthesis of minus strand DNA; the viral packaging signal (ψ), essential for viral RNA packaging into viral particles; viral RNA dimerization signal (DIS) that holds together the two RNA copies; the major splice donor site (SD) for the formation of subgenomic mRNAs; central polypurine tract (cPPT) and the central termination sequence (CTS); the RRE described above; and the polypurine tract (PPT), the initiation site of plus strand DNA synthesis (*Srinivasakumar et al., 2001*).

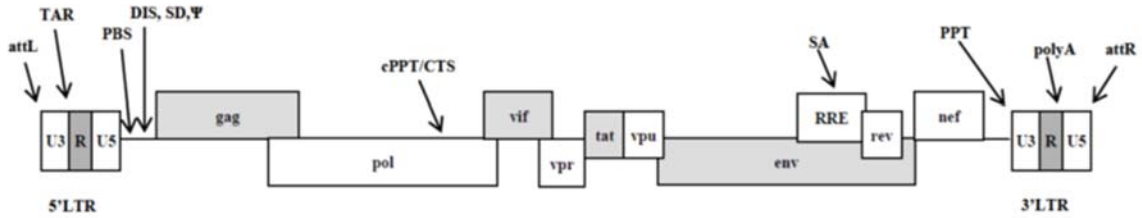



**Figure 2.** Organization of HIV-1 genome. Genes and LTRs are shown as boxes; c*is*-acting elements are indicated by arrows.

## ***1.2 HIV-1 life cycle***

HIV-1 replicates through a complex life cycle starting with viral envelope glycoprotein gp120 binding with CD4+ receptor, a 55-kD member of the immunoglobulin (lg) superfamily expressed on the surface of helper T lymphocytes, monocytes/macrophages, dendritic cells (DCs) and on certain cells in the brain (*Dalgleish et al., 1984*; *Maddon et al., 1986*). While CD4 is necessary for virus binding to target cell surface, it is not sufficient to mediate virus entry. Indeed, members of the chemokine receptor family, among which CRR5 and CXCR4 are the most important, have been identified as coreceptors (*Feng et al., 1996; Edinger et al., 1998*). The selective usage of one of the two coreceptors determines the tropism of HIV-1 isolates (*Bergeret al., 1998;* **Table 2**). In particular, the viruses that use CXCR4 coreceptor are defined X4- or T-tropic and their principal target are helper T-cell; whereas viruses that bind CCR5 are defined R5- or M-tropic and infect preferentially monocytes/macrophages and immature DCs. Moreover, some HIV-1 strains are able to use both coreceptors for infecting the target cells and they are classified as R5X4- or dual-tropic.

| TROPISM AND BIOLOGICAL PROPERTIES OF HIV-1 ISOLATES | | | |
|---|---|---|---|
| Co-receptor used | T-cell line replication | Macrophage replication | Syncitium-inducing phenotype |
| X4 | + | - | ++ |
| R5 | - | + | - |
| X4/R5 | + | + | + |

**Table 2.** Co-receptor usage of HIV-1 isolates

gp120 binding to CD4 receptor induces drastic conformational changes in the envelope protein, promoting the binding to coreceptors. After the formation of a ternary CD4-co-receptor-gp120 complex, conformational changes in gp41 occur that induce the exposure of the fusion peptide, a hydrophobic region at the N-terminus of gp41 consisting of two amphipathic heptad repeat (HR) domains. Interactions between HR1 and HR2 generate a six-helix bundle that brings the viral envelope closer to the cellular membrane, triggering the fusion and the entry of the virion core inside the cell (**Figure 3**). Here, during the uncoating, viral RNAs and proteins are released into the cytoplasm and reverse transcription begins.

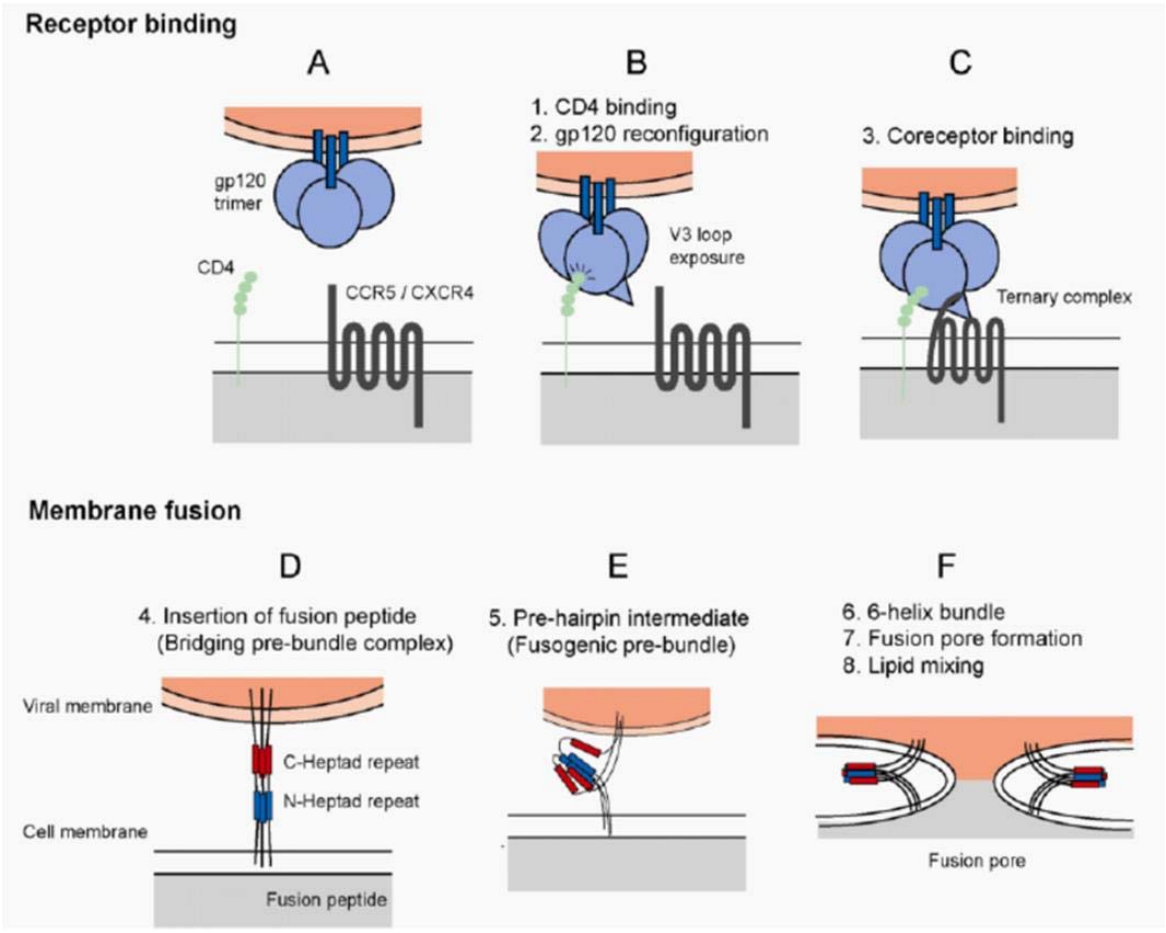



**Figure 3.** Schematic representation of membrane fusion process. gp120 binding to CD4 receptor (B) induces conformational changes in Env protein that facilitates binding to coreceptor (C). Then, gp41 changes conformation allowing for the exposure of fusion peptide and its insertion in cellular membrane (D). Interactions between gp41 HR1 and HR2 (E) lead to the formation of the "six-helix bundle", that puts viral and cellular membranes in contact, triggering to their fusion (F). (Adapted from *Lobritz et al., 2010*)

The process of reverse transcription generates a linear double-strand DNA via a complex series of steps (**Figure 4**). DNA synthesis is mediated by viral Reverse Transcriptase that has two distinct enzymatic activities: DNA Polymerase, able to use both DNA and RNA as template, and ribonuclease H (RNaseH), that specifically digests RNA in RNA:DNA hybrid. Synthesis of minus-strand DNA starts from the 3'-OH of viral $tRNA^{Lys}$ annealed to PBS sequence on genomic RNA acting as primer. DNA synthesis then proceeds to the 5' end of the genome generating a minus-strand strong-stop DNA (-sssDNA) that is relatively short since PBS is close to the 5' end of viral RNA. RNaseH degrades the RNA portion of the newly formed RNA:DNA hybrid and allows transfer of the -sssDNA to the 3' end of the genome, where it hybridizes to R region. Minus-strand DNA synthesis restarts while RNaseH digests the RNA in the resulting RNA-DNA hybrid with the exception of cPPT and PPT sequences used as primers for the synthesis of plus-strand DNA. The degradation of tRNA by RNaseH exposes the PBS at the 3' end of the plus-strand DNA, allowing annealing with the PBS region at the 3' end of the minus-strand DNA (second-strand transfer). Plus- and minus-strand syntheses proceed to completion and the proviral double-strand DNA is formed. Proviral DNA associates with the pre-integration complex (PIC), which is composed of both viral and cellular proteins, like viral MA and IN and cellular α-importins (*Maillot et al., 2013; Ao et al., 2010*). Although the molecular mechanism by which HIV-1 PIC crosses the nuclear envelope in non-dividing cells is still poorly understood, it is clear that the PIC enters into the nucleus by passing through the nuclear pore complex (NPC). Indeed, PIC estimated dimensions of about 56 nm in diameters exclude the possibility of a passive diffusion through the much tighter nuclear pores (about 9 nm). The unique ability of Lentiviruses to cross actively the nuclear membrane to reach the host cell genome allows for virus replication in non-dividing cells arrested in the G1 phase of cell cycle. This distinguished feature makes lentiviruses attractive candidate for the development of lentiviral gene vectors.

Within the nucleus IN protein catalyzes the integration of the viral DNA into the chromosomal DNA of the infected cell, an essential step for infectious particle production. Soon after association with viral DNA into the PIC, IN cleaves the 3' termini of both DNA strands generating a pre-integration substrate with 3' recessed ends.

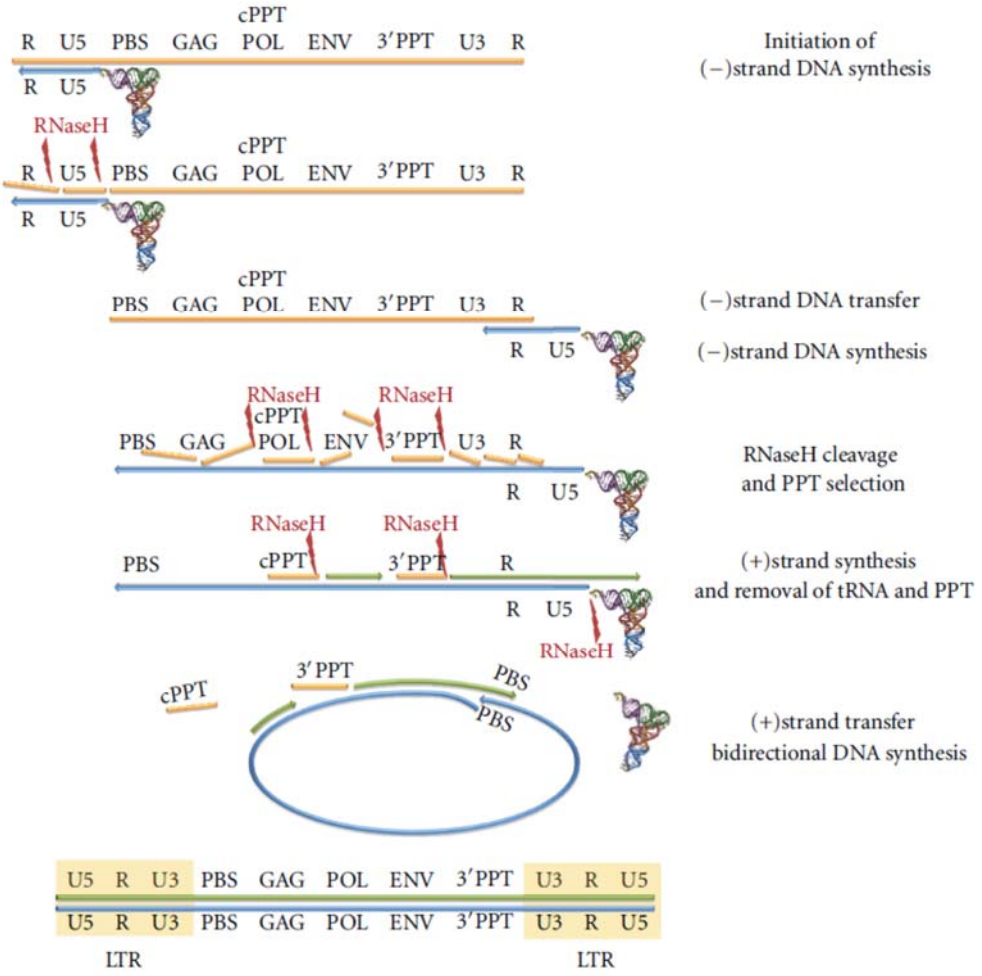



**Figure 4.** Reverse transcription of HIV-1 genome. Negative strand DNA (-sDNA, indicated in blue) synthesis starts when $tRNA^{Lys}$ binds to the priming binding site (PBS) near the 5'-end of the (+)strand RNA genome (orange). Viral reverse transcriptase (RT) catalyzes the DNA synthesis till to the 5'-end of the RNA genome. RNase H degrades the RNA in the RNA:DNA hybrid. The short new synthetized DNA is transferred to the 3'end of viral RNA where hybridizes with R region in 3' LTR. The synthesis of –sDNA proceeds, while RNase H function hydrolizes the whole RNA strand of the RNA:DNA duplex with the exception of cPPT and PPT regions, that are used as primers for the synthesis of (+)strand DNA (green). RNase H degrades PPT segments and $tRNA^{Lys}$, freeing the PBS sequence of the (+)strand DNA that anneals with PBS sequence on the –sDNA (second strand transfer). DNA synthesis then continues producing a linear dsDNA with long terminal repeats (LTRs) at both ends. (Adapted from *Esposito et al., 2012*).

Upon entry in the nucleus, IN cleaves the cellular DNA and the 3' recessed ends of viral DNA are joined to the 5' staggered termini of the host DNA (*Brown et al., 1989*). The cellular repair machinery fills the gap and proviral DNA is integrated into the infected cell.

In addition to the linear integrated viral DNA, two types of non-integrated circular DNAs (E-DNAs) can be formed in the nucleus, if the provirus is not integrated in the host's genome: 1-LTR circles, formed by homologous recombination between the two LTRs of viral DNAs (*Farnet et al., 1991*), and 2-LTR circles, formed after head to tail junction of the two LTR

ends of the fully reverse transcribed DNA. Although none of these circular forms serve as IVprecursor to the integrated provirus, it has been shown that circular E-DNAs persist in non-dividing cells in vivo (*Cara et al., 2002; Saenz et al., 2004*) and are transcriptionally active, albeit at lower levels than their integrated counterpart (*Wu et al., 2003*).

In infected and activated T-cells, transcription of integrated viral DNA marks the beginning of late phase of HIV-1 life cycle. Cellular RNA Polymerase II is responsible for viral RNA synthesis and is recruited by 5' LTR. In particular, transcriptional promoter and multiple regulatory elements, like binding sites for nuclear factor kappa-B (NF-kB) and nuclear factor of activated T cells (NFAT), are located in the U3 region of 5' LTR. However, basal activity of 5'LTR promoter is low and ineffective for complete viral DNA transcription so that only short and uncompleted RNAs are produced. A low percentage of these transcripts is spliced by the cellular splicing machinery in small mRNAs that are exported into the cytoplasm. Here, Tat, Rev and Nef proteins are synthetized and then enter into the nucleus. Tat is a very potent activator of HIV-1 gene expression and binds to TAR element, an RNA stem-loop structure in the R region of the LTR; therefore, transcription of TAR is required before Tat can enhance HIV transcription (*Barboric et al., 2005*). Tat interacts also with cellular transcriptional factor (such as NF-kB), ensuring the production of high levels of viral RNA. RNA transcription begins at the first nucleotide in R region in 5'LTR, whereas it terminates at the last nucleotide of R region in 3'LTR, where RNA is polyadenylated.

In addition to full-length genomic mRNA (about 9.2 kb in length, encoding for Gag and Pol proteins), several different mRNAs are produced exploiting the cellular splicing machinery. Viral mRNAs can be classified in two classes: early fully spliced transcripts encoding for Tat, Rev and Nef, and late partially spliced mRNAs that codifies for accessory proteins Vif, Vpr, Vpu as well as Env. RRE element, placed in the Env coding region, is present in all unspliced and partially spliced RNAs (*Pond et al., 2009*) and is bound by Rev protein. Having both a nuclear localization signal (NLS) and a nuclear export signal (NES), Rev mediates transcripts translocation from the nucleus to the cytoplasm.

gp160 Env precursor is translated in the endoplasmic reticulum (ER) and is co-translationally inserted in its membrane. During the passage through the cellular secretory pathway, gp160 is glycosylated, assembled into trimeric complexes and cleaved by cellular protease furin in the gp41 and gp120 subunits. Vesicular transport guarantees Env

translocation at plasma membrane level. On the contrary, the Gag and Gag-Pol polyproteins are synthesized on free cytoplasmic ribosomes. The Gag and Pol coding sequences are in different reading frames and a frameshift is necessary to produce the Pol polyprotein from the Gag-Pol precursor. It has been estimated that one Gag-Pol is produced for every twenty Gag molecules.

Once the Gag, Gag-Pol, and Env polyproteins are produced, they associate together and along with two copies of viral genomic RNA and $tRNA^{Lys}$ primer, and condense at the plasma membrane for particle assembly and budding. Gag plays a central role in assembly, having the ability to direct the budding of virus particles from infected cells. Because of these properties, Gag has been described as the "orchestrator of HIV-1 assembly" (**Figure 5.** *Cimarelli et al., 2002*). Gag protein consists of different subdomains: matrix (MA), containing myristoylation sites; capsid (CA), containing oligomerization domains responsible for Gag-Gag interactions (*Dorfam et al., 1994; Alfadhliet al., 2005*); nucleocapsid (p7, NC), having two zinc-finger domains for viral RNA binding (*Cimarelli et al., 2000*); p6 region, containing binding sites for several viral and cellular proteins; and two small spacer peptides SP1 and SP2, which help to regulate the conformational changes that accompany viral maturation. In the first steps of assembly, Gag proteins form dimers and adopt a compact shape (*Kutluay and Bieniasz, 2010*) in which NC domain associates with viral genomic RNAs (*Jouvenet et al., 2009*) and with several cellular factors. Myristoylation of MA domain is essential for Gag recruitment to the cellular membrane (*Bryant et al., 1990; Zhou et al., 1994*) where Gag molecules multimerize via CA domain. Multimerization induces the formation of a curvature in the plasma membrane and immature spherical particles start to bud. In order to be released from infected cells, HIV-1 usurps cellular endosomal sorting complexes required for transport (ESCRT) (*Bieniasz, 2009*; *Hurley and Hanson; 2010*) recruited by Gag p6 domain (*Usami et al., 2009; Morita et al., 2009*).

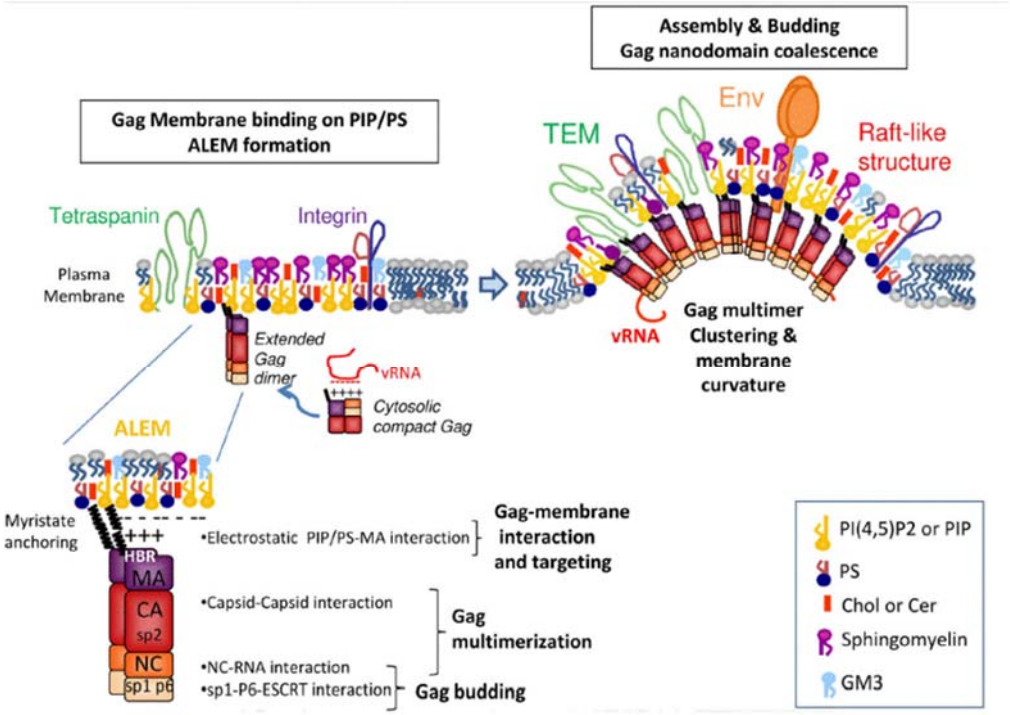



**Figure 5.** Role of Gag protein in the HIV-1 assembly. All Gag domains are involved in the assembly and budding of immature HIV-1 particle. In the first step of assembly, the NC domain of Gag dimers selectively bind the viral genome whereas the myristyolated N-term MA anchors to inner side of cellular plasma-membrane. Then several Gag proteins multimerize via CA domain and high ordered microdomains of Gag are formed under the plasma membrane causing a curvature of cellular membrane. p6 domain at Gag C-term interacts with cellular ESCRT complex in order to facilitate virus budding. (Adapted from *Mariani et al., 2014*)

HIV-1 replication cycle is completed after budding from infected cells when viral protease (PR) cleaves Gag precursor in its functional domains. Proteolysis is essential for the formation of mature infectious virions and triggers important morphological changes in the virus structure by which virions acquire the conical core made up of CA proteins (*Briggs et al., 2004*). Therefore, during maturation, immature virions are converted into infectious particles able to enter and replicate in new target cells. Steps of HIV-1 replicative cycle are schematically summarized in **Figure 6.**

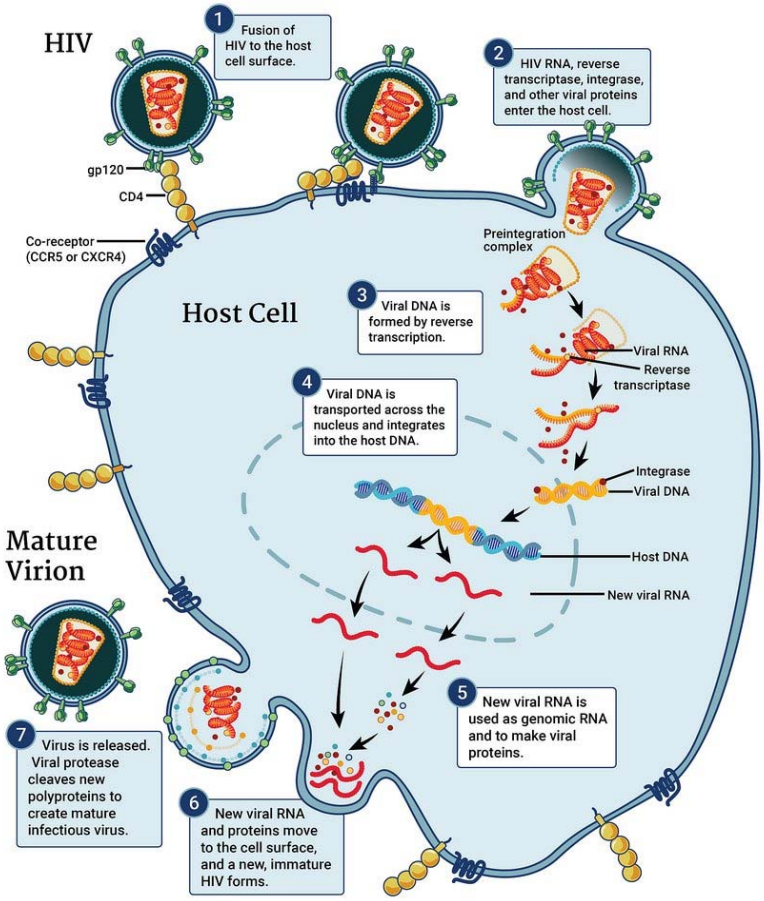



**Figure 6.** HIV-1 life cycle. Replication cycle of HIV-1 begins with the binding of viral gp120 to CD4 receptor and CCR5 or CXCR4 co-receptors on the target cell. To this follows the fusion with plasma membrane and the entry of viral genome and proteins into the cell. Viral RNA is reverse transcribed in cDNA that is translocated into the nucleus where IN protein mediates its insertion in cellular genome. The host's transcription machinery transcribes the DNA in several copies of RNA: some of these are translated to produce viral proteins, whereas other copies of RNA become the genomes of new virions. Genomic RNA and viral proteins move to the plasma-membrane where immature virions are formed. After the budding from the infected cells, viral protease cleaves the polyproteins in their subunits, creating the mature and infectious virus. Details of each step are described in the text. (Adapted from *National Institute of Allergy and Infectious Diseases* website, www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle)

### ***1.3 HIV-1 pathogenesis and clinical manifestation***

As for other Lentiviruses, HIV-1 infection is characterized by long and variable incubation periods, persistent viral replication, neurological manifestations, and depletion of specific hematologic and immunological cells. Since HIV-1 uses cellular CD4 molecule as receptor to infect target cells, HIV-1 infection determines a progressive and dramatic decline in the

number of CD4+ lymphocytes leading to a profound immune-suppression thereby increasing the probability to develop opportunistic infections and cancers.

HIV-1 infection can occur via three routes: sexual, parenteral and mother to infant transmission. Sexual transmission is responsible for most cases of HIV-1 infection worldwide; whereas transmission mediated by contaminated blood transfusion drastically decreased thanks to routine blood screening. Maternal transmission represents the 90% of all HIV-1 infections in infants and children and can occur when the maternal blood enters in the foetal circulation, during birth or via breastfeeding. Transmission rate depends on the size of the inoculum but there are other factors that can increase the risk to acquire the infection, including co-infections (including malaria, tuberculosis or other sexually-transmitted diseases), and host genetic factors such as the amount of CCR5 co-receptors on mucosal cells. Indeed, individuals with a spontaneous deletion of CCR5 gene, that impedes its expression on cell surface, are resistant to infection by R5-tropic HIV-1 strains (*Dean et al., 1996*). However, they are not protected in case of infection mediated by X4- or dual-tropic virus isolates (*Biti at el., 1997*).

Regardless of the infection route, the course of disease varies enormously among infected persons. While for some individuals the progression to AIDS disease can occur within six months from infection, other HIV-1 positive individuals (defined long term non-progressors) have been infected for more than 25 years without evidence of immune deficiency (*Migueles and Connors, 2010*).

The natural history of HIV-1 disease is classified in three stages, depending on clinical symptoms, viral load and degree of immunosuppression measured by CD4+ lymphocytes count: primary or acute infection; asymptomatic phase; advanced disease and AIDS (**Figure 7**). Primary HIV infection is also called the seroconversion illness and represents the stage after the acquisition of the virus during which antibodies are developing. During this stage, approximately 3-6 weeks after infection, infected individuals present mild symptoms ranging from glandular fever to encephalopathy. Moreover, there is a burst of viral replication, and a concomitant decline of CD4+ T cells in the peripheral blood. Initial sites in which virus replication occurs are lymph nodes, spleen, bone marrow and gut-associated lymphoid tissue (GALT). The viremia peak is probably due to the infection of GALT CD4+ lymphocytes, the most of which are T helper cells that produce interleukin 17 (IL- 17). Since

these cells are crucial for the integrity of the mucosal barrier, their depletion triggers the loss of integrity of gut mucosa, allowing microbial translocation into the systemic circulation and thereby establishing a state of chronic systemic immune activation (*Brenchleyet al., 2004; Mehandru et al., 2004; Brenchley et al., 2006*). Therefore, approximately 3-6 weeks after infection, specific antiviral immune responses are developed and determine a decline in plasma viremia, resolution of clinical symptoms, and stabilization of CD4+ cell count. Both humoral and cellular immune responses are involved in infection containment. In particular, natural killer (NK) cells number increases during the acute infection in conjunction with decreased viremia. Importantly, about the 20-30% of HIV+ individuals produce neutralizing antibodies (Nabs) able to block viral infection by binding the Env protein on viral particle or by preventing the viral fusion after gp120-CD4 binding (*Hraber et al., 2014*). In addition, viral proteins presented by MHC-I molecules on infected cells surface induce the expansion of cytolitic CD8+ T cells (*Rosenberget al., 2000*). This partial control of viral load marks the transition from the acute infection to the asymptomatic phase that is a relatively long period (8-12 years post-infection) during which a virologic *quasi-steady state* is established and patients have few or no clinical manifestations. This clinical latency does not imply viral latency: although patients are almost asymptomatic, CD4+ lymphocytes count constantly declines and virus continues to replicate (*Ho et al., 1995*). Indeed, since during its replication HIV-1 integrates the viral genome into the host genome, a stable reservoir of resting memory CD4+ cells carrying replication-competent integrated provirus has been established in the acute infection (*Finzi et al., 1997; Chun et al., 1997*). Because these latent proviruses are not transcriptionally active, no viral proteins are produced and these cells are protected from CD8+ cells that consequently are not able to eradicate the virus.

HIV-1 is able to escape immune system pressure and virus population *in vivo* is rapidly replaced by a multitude of mutant viruses that are no more recognized by NAbs and CD8+ specific cells (*Richman et al., 2003; Wei et al., 2003*). When viral diversity becomes too huge to be controlled by immune system, HIV-1 completely eludes the immune control and plasma viremia increases notably. Consequently, CD4+ cells count rapidly decreases and several opportunistic infections as well as neoplasms occur determining the state of AIDS. Pneumonia caused by Pneumocystis carinii is the most common infection in AIDS patients and is one of the main causes of death (*Masur et al., 1989*). Cancers associated with AIDS

are generally related to other viral infections, as Kaposi sarcoma (KS) due to Human Herpes Virus 8 (HHV8); lymphomas caused by Epstein-Barr Virus (EBV); and cervical and anal carcinoma associated with Human Papilloma Virus (HPV) infection (*Shiels et al., 2011*).

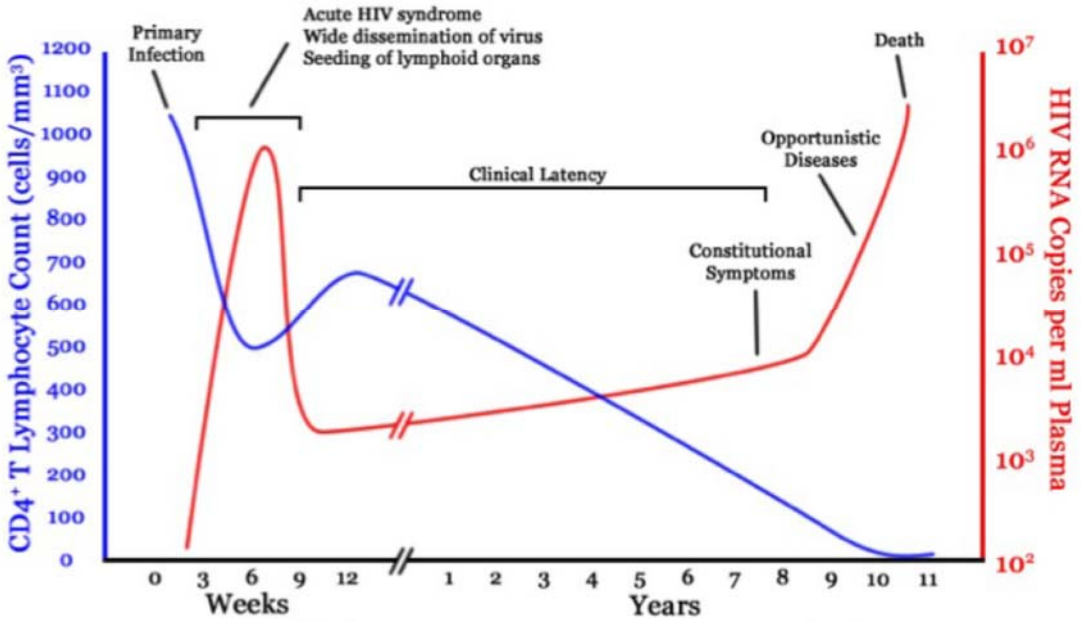



**Figure 7.** Natural history of HIV-1 infection. HIV-1 primarily infects CD4+ lymphocytes. One to three weeks after contagion, the acute phase begins: high titres of virus (red line) are detectable in the blood whereas CD4+ cell number (blue line) declines. Cellular and humoral immune responses are activated and can control viral replication. Plasma viral load decreases and a long phase of clinical latency begins, during which only weak symptoms are detectable. However, CD4+ cells number slowly decreases. When the count drops below 200-400 cells/ul, state of immunodeficiency (AIDS) is established, facilitating the development of opportunistic infections and cancer that will lead to death. (Adapted from *Kogan and Rappaport, 2011*).

### ***1.4 Treatment of HIV-1 infection***

Since *highly active antiretroviral therapy* (HAART) has been introduced in 1995 for the treatment of HIV-1 infection, AIDS-related mortality worldwide has decreased by more than 80% (*Egger et al., 2002; Porter et al., 2003*). Besides reducing plasma viral load, HAART therapy helps also in restoring CD4+ T cells count (*Hoggs et al., 2001*).

Standard HAART treatment involves the use of three or more drugs belonging to two different classes of inhibitors (*Robbins et al., 2003*; *Shafer et al., 2003*; *Yeni et al., 2004*). Indeed, antiretroviral drugs have been classified in four classes depending on their molecular targets: fusion inhibitors; reverse transcriptase inhibitors; protease inhibitors; and integrase inhibitors.

Fusion inhibitors act in the initial phase of HIV-1 replicative cycle because they inhibit viral entry into the target cell. Among these, some drugs prevent the binding of viral gp120 with either CD4 receptor (Ibalizumab) or CCR5 coreceptor (Maraviroc), whereas others (T20) alter the kinetic of fusion between viral envelope and cellular plasma membrane (*Gulick et al., 2008; Lalezari et al., 2003*). Inhibitors of HIV-1 protease (Tipranavir and Indinavir) differ from fusion inhibitors as they do not prevent cell from been infected by HIV-1 but determine the release of non-infective HIV-1 particles: they inhibit the cleavage of Gag and Gag-Pol polyprotein precursors into their mature components, affecting viral structure and enzyme functionality (*Flexner, 1998*). Inhibition of reverse transcriptase (RT) can be obtained by using either substrate analogous (nucleoside and nucleotide RT inhibitors, NRTIs and NtRTI) or by non-competitive inhibitors (non-nucleoside RT inhibitors, NNRTIs). The formers need to be phosphorylated by cellular kinases and then compete with nucleotides during the reverse transcription: since they lack the OH group at their 3' end, once incorporated into the cDNA, they act as chain terminators. On the contrary, the latter are small molecules that bind the catalytic domain of viral RT inducing conformational changes in the enzyme, that trigger to its inactivation (*Spence et al., 1995*). Integrase inhibitors (Raltegravir) prevent viral DNA integration into cellular genome, blocking HIV-1 replicative cycle.

Use of combined therapy rather than monotherapy has the advantage to limit development and selection of new drug-resistant viral quasi-species. However, HAART is not able to completely eradicate HIV-1 infection. HIV-1 therapy is defined successful when viral load is controlled under a certain threshold allowing infected people have a reasonable life quality, even if the therapeutic regimen must be maintained for the lifetime.

### ***1.5 HIV-1 vaccination strategies: state of art***

Despite advances over the course of the past decades, an effective vaccine able to prevent HIV-1 infection is still unavailable. Many obstacles have hampered its design**.** One of the most difficult one to overcome is the natural high genetic variability of the virus due to RT protein. This enzyme lacks the proof-reading activity and, hence, determines the generation of a great number of mutated viral subtypes, some of which escape the host immune control. Another mechanism evolved by HIV-1 to evade the immune responses is

the downregulation of the MHC-I molecules, in order to minimize virus recognition by cytotoxic T-lymphocytes (CTLs). Moreover, in the early phase of HIV-1 infection, integrated proviral DNA in host genome determines the establishment of reservoirs that can be latent for the whole life of the patient, impeding virus eradication (*Chun et al., 1997*). To date, correlates of protection that could prevent HIV-1 infection or significantly delay disease progression are still poorly understood (*Pantaleo and Koup, 2004*): during HIV-1 infection both antibodies and CTLs are produced but they fail to eradicate the virus.

Another significant hurdle in the HIV-1 vaccine development is the lack of a small and appropriate animal model. The only animals available to study HIV-1 infection are non-human primates (NHPs), that can be infected with SIV or SIV/HIV hybrid viruses (SHIVs) to mimic the HIV infection in humans. However, the dose of virus needed in challenge experiments to study the efficacy of vaccine formulations is very high and does not correspond to the dose of HIV-1 at which humans could be naturally exposed (*Vernazza et al., 1999; Chakraborty et al., 2001*).

Also, the choice of the formulation used to induce immune responses is important in determining the success of the vaccine. Although it has been observed that *nef*-defective SIV mutants could protect rhesus macaques from infection with pathogenic SIV (*Daniel et al., 1992; Wyand et al., 1999*), the use of live attenuated vaccines in humans has been precluded for safety concerns. In the same way, safety issues have obstructed the development of whole inactivated HIV-1 vaccines. All these considerations have targeted the efforts of researchers toward innovative strategies that employ recombinant protein subunits, viral vectors or naked DNA.

Initial HIV-1 vaccine strategies have used recombinant envelope proteins to stimulate the production of antibodies with the aim to block infection. Recombinant gp120 (rGp120) has been used in Vax003 and Vax004 clinical trials in which induced the production of Env-specific binding antibodies that waned rapidly and were not associated with protection against HIV infection. Moreover, neutralizing antibodies (NAbs) isolated from vaccinated individuals were active against a subset of tier 1 viruses (highly sensitive to antibody-mediated neutralization), but only weak responses were seen against tier 2 viruses (*Pitisuttithum et al., 2006; Flynn et al., 2005; Gilbert et al., 2005*).

T-cell vaccination is a different strategy that aims at eliciting specific cellular immune responses and production of CD8+ CTLs that affect viral replication by killing infected cells. The goal of this approach is to lower viral load and delay disease progression, rather than preventing initial infection. In the Step and Phambili phase IIb clinical trials, HIV-1 negative individuals at high risk of HIV-1 acquisition were vaccinated with recombinant adenoviral vector type 5 (rAD5) expressing HIV-1 Gag, Pol and Nef proteins. These antigens were selected because they are commonly recognized during natural infection and are relatively conserved across different clades of HIV-1. Unfortunately, vaccine recipient showed an increased risk to acquire infection compared to individuals receiving the placebo and the studies have been interrupted (*Buchbinder et al., 2008; Gray et al., 2011*).

RV144 trial was the first clinical trial of a vaccine against HIV-1 to show any degree of efficiency (*Rerks-Ngarmet al., 2009*). The study was a randomized, multicentre, double blind trial started in Thailand in 2003 among more than 16.000 healthy participants that were vaccinated with a combination of two immunogens in a prime-boost protocol: four vaccinations with ALVAC canarypox expressing Gag, Pol and Env, followed by two boosts with AIDSVAX expressing the gp120 protein form clade B/E. Follow-up of RV144 trial showed that vaccine induced IgG Abs specific for V1V2 loop of gp120 correlated with reduced risk of HIV-1 acquisition. These binding Abs were not neutralising and their effectiveness was due to the induction of ADCC. On the contrary, Env specific IgA were associated with increased risk to become infected. Furthermore, in 20% of non-infected vaccinated volunteers that completed all the immunizations, anti-Gag or anti-Env cellular immune responses were detectable. Although RV144 lowered the rate of HIV-1 infection acquisition by 61% after 1 year from the time of last vaccination, its protective effect waned overtime: at 3 years of follow-up the infection acquisition rate was lowered by 31.2% (*Haynes et al., 2012*).

Therefore, while RV144 is an important step ahead in the design of an effective vaccine against the HIV-1, further vaccination strategies need to be designed to increase the efficacy and the persistence of effective immune responses.

# Chapter 2

## Integrase Defective Lentiviral Vectors (IDLVs)

### *2.1 HIV-based LVs: from disease causing agent to efficient and safe tool for therapy and vaccination*

Because of their ability of efficiently infect and deliver their genome into target cells, Lentiviruses have been modified to construct Lentiviral Vectors (LVs). To date, LVs have been developed both from primate lentiviruses (HIV-1, HIV-2 or SIV) and non-primate lentiviruses (FIV, EIAV, CAEV, VV, JDV) (*Wiznerowicz* et al., 2005; *Negre and Cosset, 2002; Barraza and Poeschla, 2008; Molina et al., 2002; Mselli-Lakhal et al., 2006*).

LVs have attracted much attention for their use as vehicle to deliver transgenes and antigens for both gene therapy and vaccination purposes. Indeed, they have several highly advantageous features: large packaging capacity, being able to delivery transgene cassette of more than 10kb and reduced anti-vector immunogenicity compared to others viral vectors, such as adenoviral vectors (*Kumar et al., 2001; Abordo-Adesida et al., 2005; Chirmule et al., 1996; Murphy et al., 2009*). Moreover, LVs can be easy pseudotyped with different envelope proteins in order to achieve broad or specific tropism (*Cronin et al., 2005; Watson et al., 2002*). Importantly, since LVs use the pre-integration complex (PIC) to cross the nuclear membrane and transfer cDNA from the cytoplasm to the nucleus, they can proficiently transduce quiescent cells. This is an essential property for gene transfer in terminally differentiated cells as neurons, retinal cells and hepatocytes (*Naldini et al., 1996*). The ability to integrate the transgene in the host genome allows for long-term expression, but on the other side represents an important safety issue in case of detrimental insertional mutagenesis (*Hacein-Bey-Abina et al., 2003*). Furthermore, additional safety concerns are due to the potential for horizontal and cross-species transmission of recombined chimeric lentiviruses.

Because of their well-known pathogenicity, HIV and SIV derived LVs have been studied the most. Several efforts have been made to design LVs with high safety features in order to prevent their replication in vivo. In the LV genome all *cis*-acting elements necessary for genome packaging into vector particles (Ψ), reverse transcription (PBS), nuclear import (cPPT) and integration (att, LTRs) have been retained, whereas all open reading frames

(ORFs) of unnecessary viral genes and responsible for virulence have been removed. In addition, the viral genome has been split into several parts so that viral structural proteins and enzymes are provided *in trans* from different plasmids or helper packaging cells. The partition of the genome further increases their safety since minimizes the risk of recombination events that could lead to the formation of replication-competent Lentivirus (RCL) (*Chong et al., 1998*). Indeed, for the construction of LVs at least three plasmids are typically used so that the probability that RCLs form is very low as two recombination events among three different plasmids would be necessary. Hence, LVs have a high safety profile, allowing for the delivery of a gene of interest in the target cell, without expressing viral proteins inside the transduced cells.

### ***2.2 First-generation Lentiviral vector system***

The first-generation LVs have been developed in 1996 and were produced by using three plasmids (**Figure 8**; *Naldini et al., 1996a and 1996b*).

The *transfer vector* plasmid expresses the vector genome and contains the transgene of interest, transcribed by a heterologous promoter, such as the ubiquitous CMV or PGK. The *transfer vector* contains also all *cis*-acting elements that are essential for packaging, reverse transcription, nuclear import and integration into the host genomic DNA. Importantly, the *transfer vector* does not express any ORFs for HIV-1 protein. Since both LTRs are intact, Tat protein is needed for LV genome transcription. Rev protein is also necessary for RNA export from the nucleus since Gag elements, present in the vector and necessary for packaging in the vector particles, contain nuclear retention sequences.

The *packaging plasmid* codifies for all structural, regulatory and accessory HIV-1 proteins (*Gag, Pol, Tat, Rev, Nef, Vif, Vpr, Vpu)* necessary for particle formation. Ψ and LTRs sequences have been deleted. In fact, 5'-LTR has been substituted by Cytomegalovirus (CMV) or Rous sarcoma Virus (RSV) promoter to drive RNA synthesis; while 3'-LTR has been replaced by heterologous polyadenilation signal (polyA) from SV40 or insulin. On the contrary, RRE and the 5' SD site have not been changed, allowing mRNA splicing and Rev-mediated export from the nucleus. Importantly, this plasmid lacks *cis*-acting elements for packaging, reverse-transcription and integration.

Since HIV-1 *env* gene has been removed from the *packaging* plasmid, the gene for a membrane glycoprotein is provided by a third plasmid called *envelope vector*. The use of a heterologous glycoprotein allows for the pseudotyping of LVs, thus providing the enormous advantage of modifying the tropism of LVs, using cell-specific or broadly infecting Envelopes. The glycoprotein G from vesicular stomatitis virus (VSV.G) is the most used glycoprotein to widen LVs tropism. As VSV.G binds the LDL receptor (LDLR) family, it is able to mediate transduction of almost all mammalian cell types via the endocytic pathway at high efficiency (*Finkelshteinet al., 2013; Aiken, 1997*). Importantly, it has been shown that VSV.G increases the stability of LVs particles. The presence of a heterologous Env also has an important implication for the safety due the absence of HIV-1 homologous sequences.

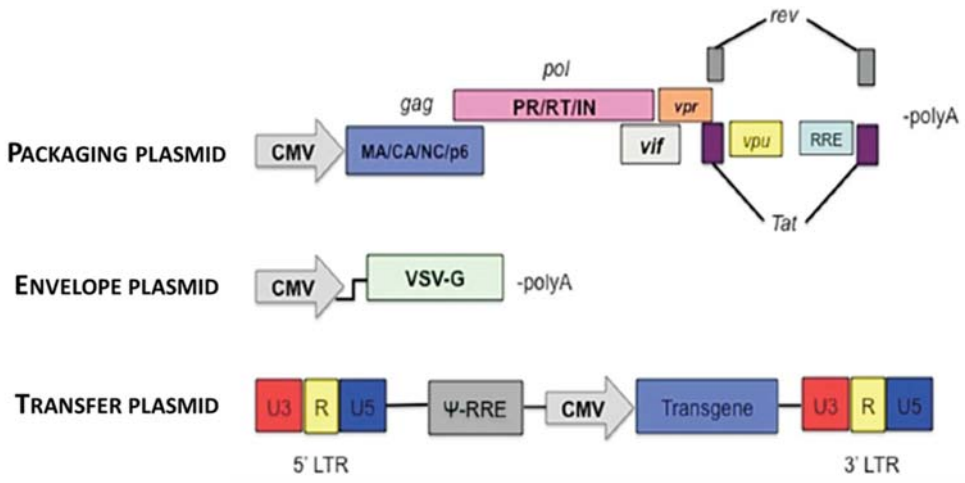



**Figure 8.** First-generation system for production of LVs. Packaging plasmid codifies for structural, enzymatic and accessory proteins necessary the production of vector particle. The envelope plasmid encodes for the surface glycoprotein G of VSV to widen the spectrum of target cells. Transfer plasmid carries the vector genome and the transgene of interest under the control of CMV promoter. It contains all the cis-acting elements necessary for genome packaging, reverse transcription and integration, without carrying viral ORFs. (Adapted from *Pietrangeli et al., 2014*).

## ***2.3 Second-generation Lentiviral vector system***

In order to further decrease the risk of RCLs formation, second-generation LVs have been developed by removing accessory genes from the packaging vector (*Zuffrey et al., 1997; Kim et al., 1998*). In this system *packaging plasmid* expresses only Rev and Tat proteins together with the Gag and Gag-Pol polyproteins (**Figure 9**). Therefore, even in case of recombination events among the plasmids, wild type viral features will not be restored.

Removal of *vif*, *vpr*, *vpu* and *nef* genes does not affect LVs production since these proteins are necessary for the pathogenicity of HIV-1, but not for its infectivity.

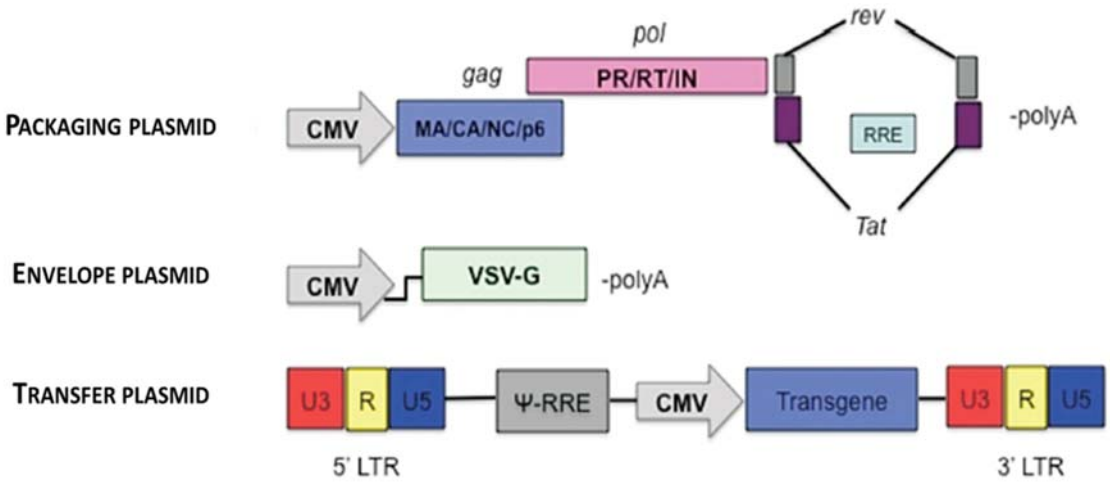



**Figure 9**. Plasmid used for production of second-generation LVs. The packaging plasmid does not codify for accessory proteins Vif, Vpr, Vpu and Nef. This approach prevents the formation of replication competent Lentiviruses. The other plasmids are the same used in the 1st generation system. (Adapted from *Pietrangeli et al., 2014*).

## ***2.4 Third-generation Lentiviral vector system: self-inactivating LVs***

Third-generation system for LVs production involves the use of a packaging plasmid expressing only Gag and Gag-Pol polyproteins (**Figure 10**; *Dull et al., 1998*). Rev is expressed by a fourth plasmid whereas Tat has been completely removed.

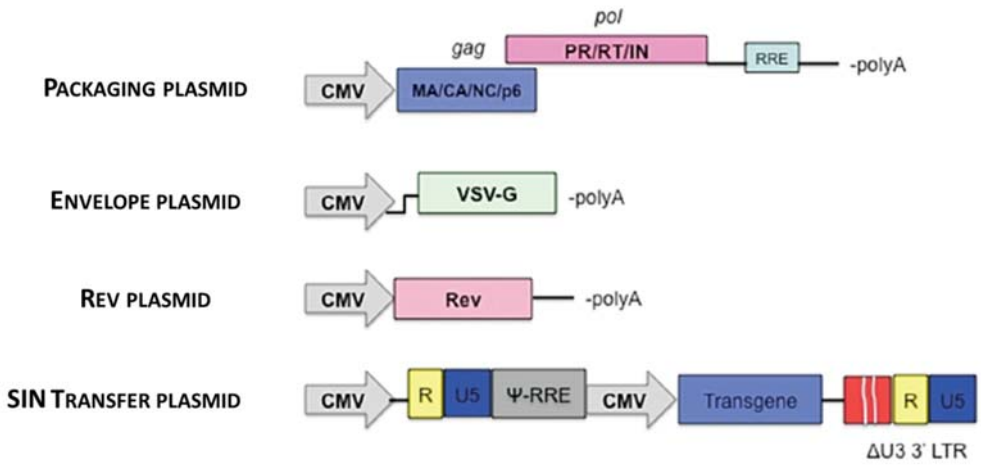



**Figure 10.** Schematics of plasmids used for the production of third-generation LVs. To increase the safety of LVs, all the genes for accessory and regulatory proteins have been removed from the packaging plasmid. Rev is provided by a fourth plasmid, whereas Tat is no more necessary since the

Tat-dependent promoter in the 5'LTR have been replaced by heterologous CMV promoter. The transfer plasmid is self-inactivating due to the deletion of U3 at 3' LTR (see figure 11). (Adapted from *Pietrangeli et al., 2014*).

To overcome the lack of Tat, that is necessary for RNA transcription from LTRs, the transfer plasmid has been modified: U3 region at 5' LTR has been replaced by heterologous promoters, usually CMV; whereas enhancer sequences in the U3 of 3'LTR have been deleted (*Iwakuma et al., 1999*). This kind of transfer vector is called self-inactivating (SIN) since during reverse-transcription deleted U3 is used as template for the 5'LTR, generating proviral DNA with both LTRs inactivated (**Figure 11**).

Third-generation system notably increases the safety of LVs: prevents the activation of cellular genes after integration into cellular genome since LTRs are inactivated; minimizes the risk of recombination as wild-type homologous sequences have been removed; decreases the probability of vector mobilization in case of superinfection with HIV-1 as full-length transcripts are not produced (*Bukovsky et al., 1999*).

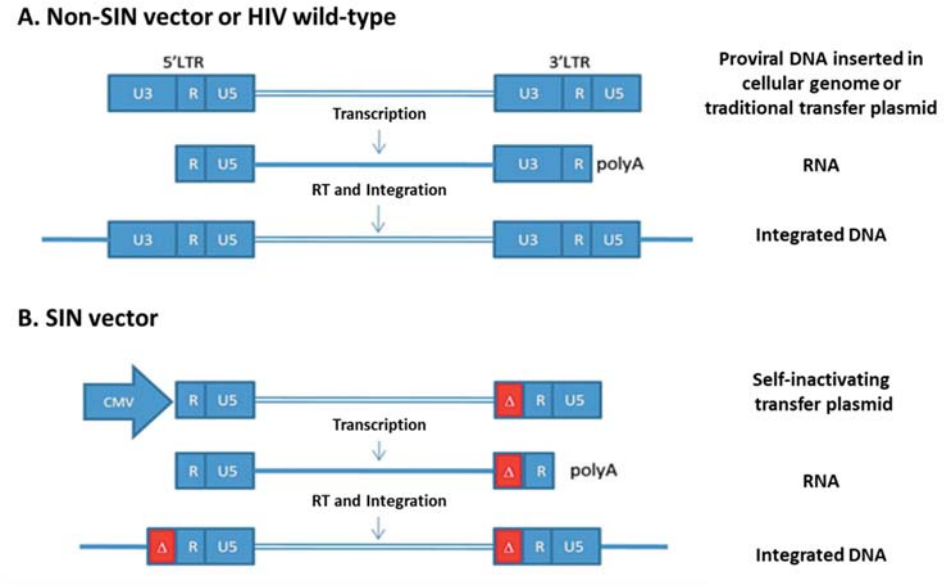



**Figure 11.** Comparison of transcription, reverse transcription and integration processes between traditional vector or HIV-1 wild-type virus (**A**) and self-inactivating (SIN) vector (**B**). During reverse transcription of SIN vector, the deletion in U3 region of 3' LTR (ΔU3) is duplicated in the 5'LTR. The resulting integrated cDNA lacks the inner promoter at 5' LTR, avoiding the risk of insertional mutagenesis. (Adapted from *Pietrangeli et al., 2014*).

## *2.5 Further improvements in LVs design*

Lentiviral vectors production system has been further modified in order to improve the efficiency of gene delivery and the expression of the transgene in the target cells (**Figure 12**). This latter aim was achieved with the insertion of the Woodchuck Hepatitis Virus Post-Transcriptional Regulatory Element (WPRE) placed downstream of the transgene in the *transfer vector*. WPRE increases mRNA transcription termination and export of unspliced mRNAs from the nucleus to the cytoplasm, leading to higher vector titres and transgene expression (*Higashimoto et al., 2007; Oh et al., 2007; Zuffrey et al., 1999*). The use of WPRE may rise safety issues since it contains a truncated form of WHV X gene, that has been associated with hepatocellular carcinomas in animals (*Kingsman et al., 2005*). In later configurations of the vector, this sequence has been removed from WPRE without affecting its functionality (*Zanta-Boussif et al., 2009; Schambach et al., 2006*).

Another way to increase transgene expression is the insertion of the HIV-1 cPPT (central polypurine tract) in the *transfer vector*. cPPT is a short sequence in the wild-type *pol* gene, involved in the nuclear import complex (PIC). Adding cPPT in the LVs increased transduction efficiency of more than ten-fold (*Follenzi et al., 2000*).

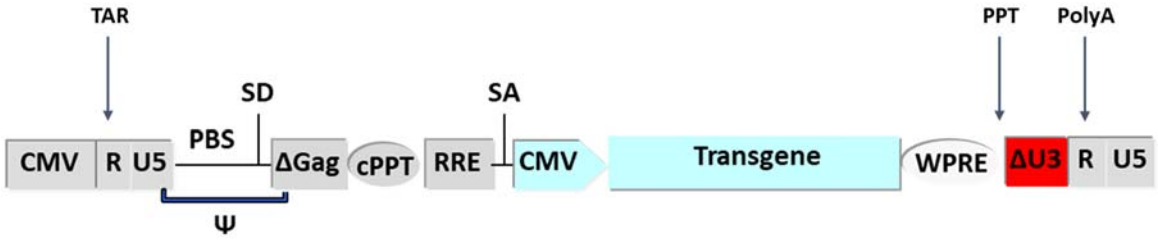



**Figure 12.** Transfer vector used for the production of advanced LVs. CMV at 5' end drives the Tat-independent transcription of vector genomic RNA. cPPT increases vector transduction efficiency and WPRE improves transcription. Heterologous or synthetic polyadenylation signals is inserted into R region of 3' LTR to improve the polyadenylation of transcript.

## *2.6 Integrase Defective Lentiviral Vectors (IDLVs)*

In spite of remarkable improvements in LVs safety, insertional mutagenesis after integration of lentiviral vector genome remains a major safety concern for their use *in vivo*. An approach to avoid this risk is the use of Integrase Defective Lentiviral Vectors (IDLVs), obtained by inactivation of Integrase (IN) protein that catalyzes the genomic insertion of

proviral DNA by a multistep process (**Figure 13**). IN inactivation can be easy achieved by mutating the IN ORF in the packaging plasmid used for LV production. Mutations can affect any of the three IN subdomains: the N-terminal domain with a zinc-finger motif to bind viral DNA, the C-terminal domain that binds non-specific DNA, and the catalytic core containing the amino-acid triad D64, D116 o E152, conserved among Retroviruses (*Engleman and Craige, 1992*). The most commonly used mutations for IDLV production belong to the so-called Class I mutations and target the catalytic triad in which insert a missense aminoacidic residue (*Wanisch and Yanez-Munoz, 2009*). Class I mutations specifically inactivate the IN, without altering other viral processes, such as reverse-transcription and PIC import into the nucleus (*Saenz et al., 2004*). On the contrary, the Class II mutations have pleiotropic negative effects on different stages of the replication cycle and are unsuitable for the design of IDLVs (*Wiskerchen et al., 1995; Engleman, 1999*).

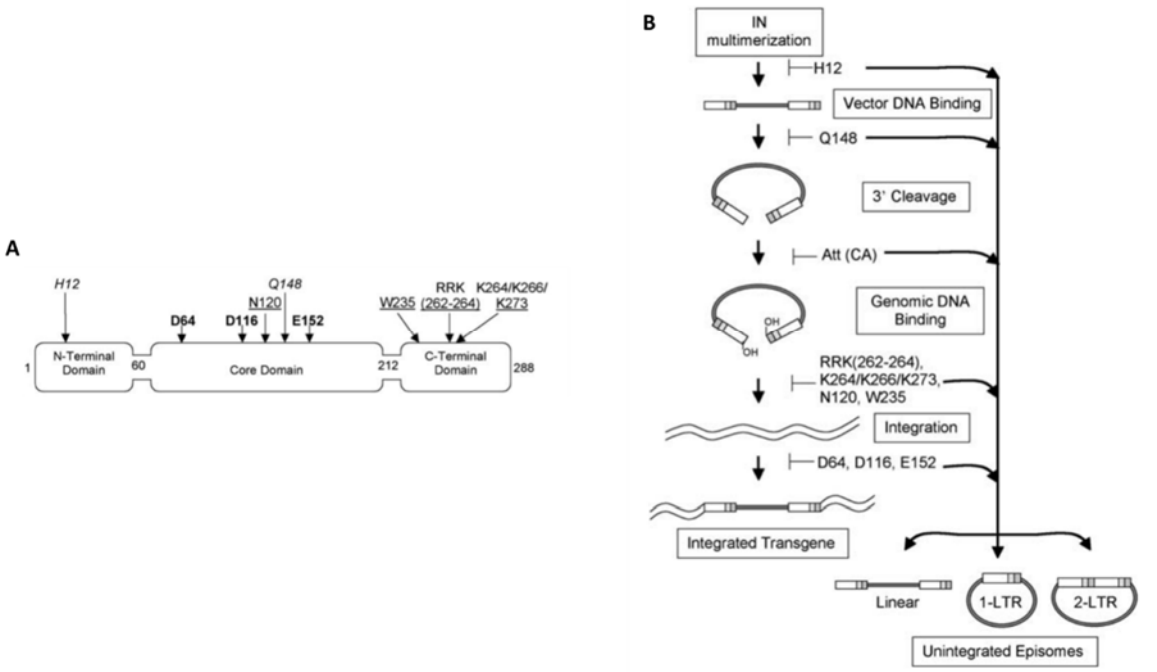



**Figure 13. A.** Schematic representation of HIV-1 integrase (IN) gene composed of three domains: N-terminal domain, catalytic core and C-terminal domain. Mutation used to produce IDLVs are indicated by arrows. **B.** Representation of integration process. In the first step, IN binds to vector DNA; then IN catalyzes the cleavage of 3' LTR generating free hydroxyl groups necessary for integration. IN binds the cellular genomic DNA and catalyzes the insertion of vector DNA. Functionality of IN protein can be impaired by inserting several mutations (indicated below each stage) to block the different steps of integration process. This prevents provirus formation and allows for accumulation of vector DNA as unintegrated episomes. (Adapted from *Banasik and McCray, 2009*).

In detail, LVs carrying D64V or D116N mutations have shown residual integration of about 1000-fold lower than integrated- counterpart, with slightly lower transduction efficiency (*Sarkis et al., 2008; Leavitt et al., 1996*). In addition, integration can be impaired by mutating the *att* sequences in the U3 region of LTRs, in which IN catalyzes the cleavage of 3' ends of viral cDNA creating the substrate for the integration into host genome. Discordant results have been achieved about the efficacy of Δatt in decreasing LV genome integration compared to point mutations in the IN catalytic core. Nevertheless, it has been shown that Δatt and D64V do not have additive effect and probably D64V supersedes Δatt effect (*Apolonia et al., 2007*).

Transgene expression in target cells transduced by IDLVs is due to circular forms of viral DNA, called episomes. These extrachromosomal forms (E-DNAs) were described as by-products of HIV-1 reverse transcription (*Farnet and Haseltine, 1991*): during the natural HIV-1 replicative cycle, sometimes unsuccessful integration reactions occur and unintegrated circular forms are produced. As already described above, two forms of episomes can be generated: 1-LTR and 2-LTR circles. Both are able to express proteins since the transgene remains intact (*Brussel and Sonigo, 2004; Cara and Reitz, 1997*). However, as e-DNAs lack an origin of replication (ORI) their half-life depends on the rate of cell division (*Sharkey et al., 2005*). Therefore, episomes are transient in dividing cells, like T and B lymphocytes or cancer cell, in which E-DNAs last for few days after transduction and are progressively diluted when cells divide. On the contrary, they are stable in quiescent non-dividing cells, such as antigen-presenting cells (APCs), macrophages and DCs, neurons, myocytes and hepatocytes. Transduction of terminally differentiated cells by IDLVs resulted in transgene expression *in vivo*, detectable up to one-year post-injection (*Bayer at al., 2008*).

Importantly, IDLVs should not support production of RCLs since IN protein is essential for viral replication. For these features and for their high safety profile, IDLVs represent proficient vehicles for vaccination purposes. Indeed, it has been shown that a single immunization with IDLV induced specific and persistent immune responses in the mouse model (*Negri et al., 2007*).

### *2.7 Simian immunodeficiency virus (SIV) based LVs*

Since HIV-derived IDLVs do not efficiently transduce primary simian cells, Simian Immunodeficiency Virus (SIV) based IDLVs have been designed in order to evaluate their efficacy as delivery platform for vaccine formulation in non-human primates (NHP) (*Zheng et al., 2008*). This is particularly important in the case of HIV-vaccine strategies, for which NHPs represent the best animal model.

Although the expression of SIV Vpx by HIV-based IDLV improved the transduction efficiency of human and simian DCs, IDLVs derived from HIV are not "fully competent" for transduction of simian DCs (*Negri et al., 2012; Berger et al., 2009*). In particular, SIV Vpx acts on human and simian SAMHD1 protein, a cytosolic deoxynucleoside triphosphate triphosphohydrolase of myeloid cells, which depletes the intracellular dNTP pool, resulting in the inhibition of HIV-1 replication. Vpx induces the degradation of SAMHD1 by addressing it to proteasome (*Laguette et al., 2011; Hrecka et al., 2011*) thereby counteracting the anti-viral effect of SAMHD1. Consequently, reverse transcription of IDLV genome increases, and transgene expression levels become comparable to those achieved in human or simian DCs transduced by integrating LVs. However, overcoming the negative effect of SAMHD1 is not sufficient to ensure that IDLVs derived from HIV can effectively transduce simian DCs. In fact, other species-specific restriction factors could interfere with transduction (*Stremlau et al., 2004; Mariani et al., 2003*). Therefore, SIV-based IDLVs appear to be the best option for evaluating the efficacy of immunogens delivered by IDLV in NHPs. Indeed, SIV-derived IDLVs contain not only the Vpx protein but also SIV proteins needed to counteract specie-specific restriction factors. In particular, *Michelini et al.* (2009) have developed an SIV-based IDLV expressing the enhanced green fluorescent protein (GFP) as model antigen, to test its ability in inducing immune responses in vaccinated BALB/c mice. Analysis of interferon-γ (IFN-γ) production by cells derived from spleen, bone marrow and lymph nodes provided evidence that one immunization with SIV-based IDLV was able to elicit a sustained and specific polyfunctional cellular immune response.
These data paved the way to the use of SIV derived IDLV as vehicle for vaccine formulation in non-human primate model. Indeed, immunization of macaques with SIV-based IDLV expressing Env protein induced specific and functional Abs still detectable after one year from the injection (*Negri et al., 2016*).

# Chapter 3

# Development of Integrase Defective Lentiviral Vector as delivery platform for vaccination against HIV-1

## *3.1 Rationale and aim of the work*

Integrase Defective Lentiviral Vectors (IDLVs) are self-inactivating, non-replicating and non-integrating vectors derived from Lentivirus. High safety and ability to transduce resting cells are IDLVs main properties that have attracted much interest for their use in gene therapy and vaccination strategies. The transgene of interest is expressed from circular extrachromosomal DNA forms (episomes or E-DNAs) that are stable in non-dividing cells and transcriptionally active (*Cara and Reitz, 1997; Cara and Klotman, 2006*). Several studies showed that IDLVs can be used *in vivo* in order to induce specific and persistent immune responses that protects immunized mice from challenge with viruses or cancer cell lines (*Countant et al., 2008; Karwacz et al., 2009; Grasso et al., 2012*). Therefore, IDLVs represent a novel and promising vaccine platform especially against infectious pathogens for which traditional methods are unsafe or ineffective, like human immunodeficiency virus type 1 (HIV-1). In particular, immunization with IDLVs expressing HIV-1 Gag or Env proteins elicited humoral and cellular immune responses in mice (*Negri et al., 2007; Rossi et al., 2014*) and rhesus macaques (*Negri et al., 2016*) that were detectable up to one year after the injection.

Studies on long-term non-progressor and NHPs provided evidence that effective T-cell immune responses against HIV-1 structural proteins Gag/Pol and broadly neutralizing antibodies (bNAbs) against Env protein can prevent HIV-1 infection and limit disease progression (*Baba et al., 2002*). Hence, there is a broad scientific consensus that an effective HIV-1 vaccine should induce both HIV-1-specific T-cell and neutralising antibody responses.

Among all HIV-1 proteins, Gag seems to be the best candidate for a T-cell vaccine since it is highly conserved across different HIV-1 isolates and induces strong immunodominant responses. In particular, anti-Gag CTLs have been associated with reduced viral loads in individuals infected with either clade B or clade C HIV-1 viruses (*Zuñiga et al., 2006; Kiepila*

*et al., 2006*). Nevertheless, immune response directed toward Gag is not always an indicator of antiviral activity. In fact, the use of a wild type Gag-based vaccine could divert immune responses toward epitopes in Gag protein that are highly immunogenic but not protective. For these reasons, immunogens to be included in a T-cell vaccine should be rationally designed. Therefore, in order to induce effective cellular immune responses, *Mothe et al.* (2015) have developed the HIVACAT T-cell Immunogen (HTI), a mosaic of 16 regions in *gag*, *pol*, *vif* and *nef* HIV-1 genes that are relatively conserved among the different strains of HIV-1 (**Figure 14**). These regions include more than 50 CD4+ and CD8+ T-cell epitopes independent of HLA genotypes. Moreover, epitopes included in HTI are defined "beneficial" since they have been identified as target of CTLs in HIV-1 positive patients with low viral load. Immunization of both C57BL/6 mice and Indian rhesus macaques with a DNA plasmid expressing HTI induced broad and balanced T-cell responses to several segments within Gag, Pol, and Vif.

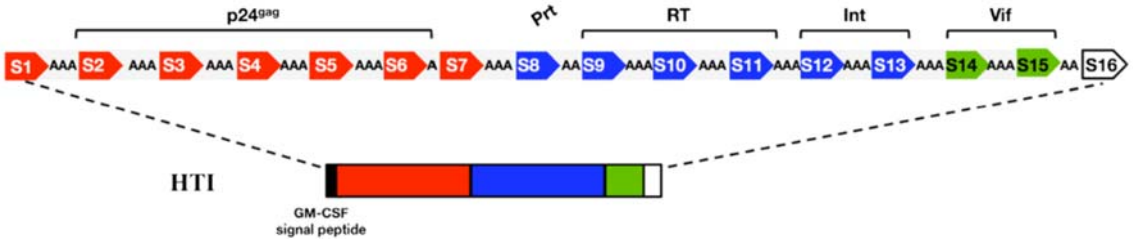



**Figure 14.** Schematic representation of HTI structure. HTI immunogen is composed of 529 aa covering 16 regions in *gag* (red), *pol* (blue), *vif* (green), and *nef* (white) genes of HIV-1. Individual segments are linked via one, two or three alanine amino acid linkers. (Adapted from *Mothe et al., 2015*.)

Concerning the humoral response, bNAbs can be detected in about 20-30% of HIV-1 infected people suggesting that the immune system is able to induce bNAbs against envelope spikes exposed on the virus surface. However, many factors make challenging the development of a vaccine able to induce bNAbs, including the high variability of the highly immunogenic Env V1-V2 loop, the glycosylation of Env proteins and the low stability of Env trimers. The Env precursor gp160 is cleaved by the cellular protease furin into gp120 and gp41, that are not covalently linked, and assemble to create a trimer. The trimer is very unstable and flexible: after the binding of gp120 with CD4 and coreceptors, gp120 dissociates from gp41 and a six-helical bundle with a more favourable energetic status is formed. Moreover, variable domains and glycans on trimer surface create a shield for the

more conserved regions responsible for CD4 binding. The solving of trimer structure in 2013 (*Julien et al., 2013; Lyumkis et al., 2013*) led to rapid progresses in better understanding the trimer metastability, the mechanism by which it drives HIV-1 entry into the cell and the epitopes targeted by bNAbs (*Lee et al., 2015; Huang et al., 2014*). The latter was fundamental for a rational design of novel immunogens to be used as components in a HIV-1 vaccine for the induction of bNAbs. Among them, Uncleaved pre-Fusion Optimized gp140 trimers (ConSOSL.UFO) has been designed to mimic the structure of native trimers (**Figure 15**). In particular, ConSOSL.UFO derives from a consensus sequence of env protein of HIV-1 group M (ConS; *Liao et al., 2006*) and has been modified to increase the trimer metastability by inserting an intramolecular disulfide bond (SOS). A flexible linker was added between gp120 and gp41 and a second one replaced the majority of gp41 HR1 domain improving the yield of well-folded trimers (*Kong et al., 2016*; *Aldon et al., 2018;* reviewed in *Sanders and Moore, 2017*).

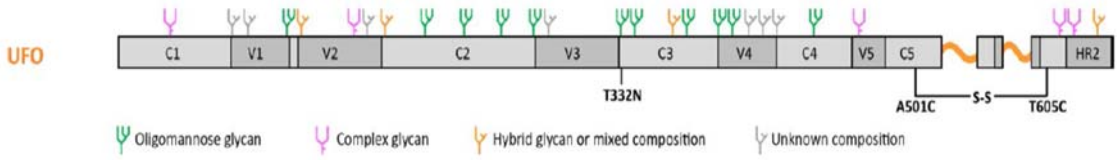



**Figure 15.** Schematic representation of ConSOSL.UFO structure. Conserved (C) and variable (V) domains of gp120 and the heptad repeat 2 (HR2) of gp41 are depicted in gray. Oligomannose (green), complex (purple), or mixed and hybrid (orange) N-linked glycan types and their locations are shown above the construct boxes. The disulphide bond is indicated in black. The flexible linkers between gp120 and gp41 and in the HR1 are indicated by undulating orange lines. (Modified from *Sander and Moore, 2017*.)

In this context, the work described in this thesis aimed to design and implement IDLVs for their use as vehicle for these novel and rationally designed HIV-immunogens. In particular, we have developed either HIV- or SIV-based IDLVs expressing HTI, to induce cellular immune response, or ConSOSL.UFO, for elicitation of bNAbs. Specificity, persistence and functionality of immune responses induced by IDLV-HTI or IDLV-UFO have been analysed in both mouse and NHPs models.

## *3.2 Materials and methods*

### *3.2.1 Plasmids construction*

In order to produce IDLVs expressing either HTI or ConSOLS.UFO immunogens, we constructed four different transfer plasmids: HIV-based pTY2-HTI or SIV-based pGAE-HTI; SIV-based pGAE-UFO.664, expressing the soluble form of Env trimer, or pGAE-UFO.750, expressing the membrane-tethered native-like trimer. Schematic representation of all the transfer vector plasmids used in this work is shown in **Figure 16**. Transfer plasmids expressing green fluorescent protein (GFP), pTY2-GFP and pGAE-GFP (*Negri et al., 2010; Michelini et al., 2009*) were used for the production of IDLV-GFP and used as controls throughout the experimental procedures in vitro and in vivo.

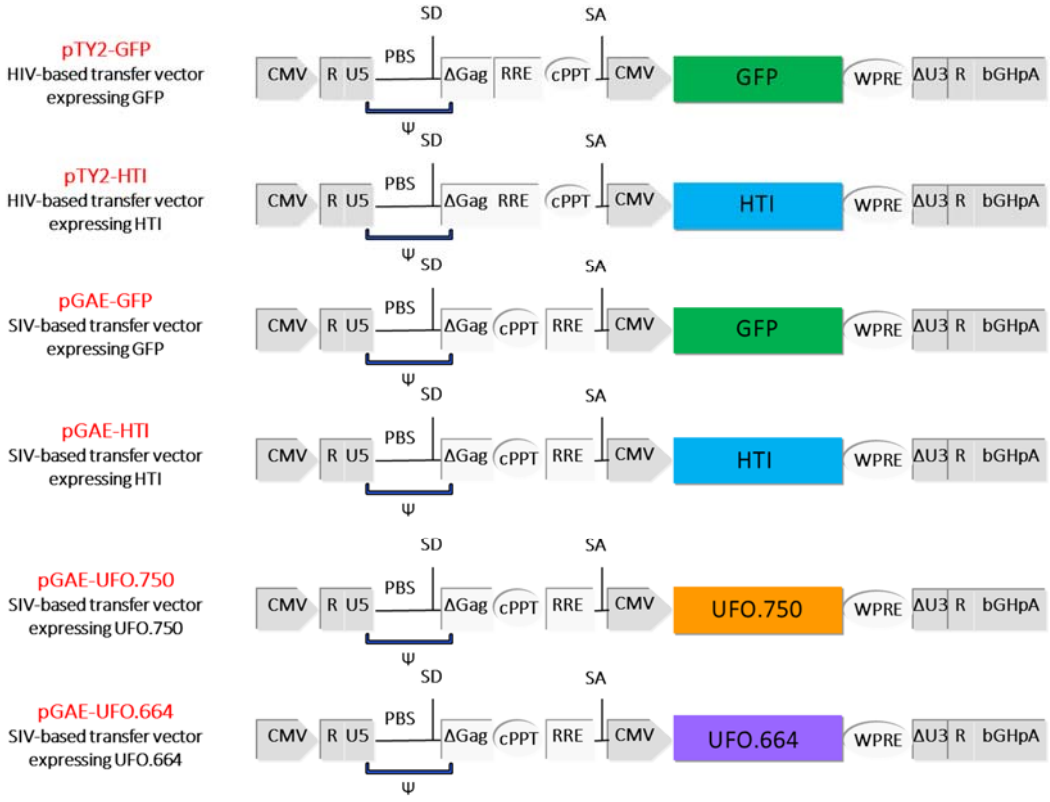



**Figure 16.** Schematic representation of transfer vectors used for the production of IDLVs described in this study.

In detail, to construct pTY2-HTI and pGAE-HTI, HTI ORF and the GM-CSF signal peptide at its N-terminus were excised from plasmid 298hGMSCF-HIVACAT (298-HTI) (*Mothe et al., 2015*) using SnaBI/XbaI or ClaI/SalI and cloned into pTY2-GFP and pGAE-GFP, respectively, by replacing the GFP gene. UFO.664 and UFO.750 ORFs obtained after digestion of

pCDNA3.1-ConSOLS.UFO664 or pCDNA3.1-ConSOLS.UFO750 (kindly provided by Prof. R. Shattock, *Imperial College of London*) with BamHI and XhoI were ligated into SalI/BglII digested pGAE-GFP to obtain pGAE-ConSOSL.UFO.664 (hereafter referred to as pGAE-UFO.664) and pGAE-ConSOSL.UFO.750 (pGAE-UFO.750), respectively.

Fusion plasmids (**Figure 18A**) used for confocal laser scanner microscopy (CLSM) were obtained as follows:

- pHTImCherry: mCherry coding sequence was amplified by Polymerase Chain Reaction (PCR) using Master Mix AmpliTaq Gold Universal (Applied Biosystems) to generate a 711 bp fragment digested with AgeI and XbaI and ligated with SnaBI/AgeI HTI fragment into pCDNA3 digested with XbaI and SnaBI. Primers and PCR protocol that have been used are shown in **Table 3**
- pSIVGagGFP: codon-optimized SIVGag ORF was obtained after pTY2-SIVGagDX (*Buffa et al., 2006*) digestion with NdeI and MscI, ligated with MscI/NotI GFP fragment and inserted into NotI/NdeI digested pCDNA3
- pHIVGagGFP has been previously described by *Hermida-Matsumoto et al.* (*2000*).

| CYCLES | DENATURATION | ANNEALING | ELONGATION |
|---|---|---|---|
| 1 | 95°C – 5 minutes | | |
| 25 | 94°C – 30 seconds | 65°C – 30 seconds | 72°C – 30 seconds |
| 1 | | | 72°C – 10 minutes |
| | **Primers** | | |
| **Forward primer** | 5'-CCACCGGTATGGATCCATTACCACCATGGTGAGCAAGG-3' | | |
| **Reverse primer** | 5'- GCTCTAGATCTCGAGAGTTACTTGTACAGCTCGTCCATG-3' | | |

**Table 3.** PCR protocol used to obtain pHTImCherry plasmid.

### *3.2.2 Stages of plasmids construction process*

Plasmid construction involves several stages: plasmids of origin are digested by restriction enzymes; fragments of interest are excised from agarose gel and ligated together; ligation products are transformed in competent cells; colonies are screened and the positive ones are amplified.

In the first step, 2 µg of DNA were digested with 3-4 units of restriction endonucleases in a specific buffer, to a final volume of 20 µl, for 3 hours at optimal temperature (37°C for most

restriction enzymes). The obtained DNA was purified through electrophoresis on 1% agarose gel. The bands corresponding to the fragments of interest have been excised from the gel and purified following the instructions of Qiagen gel extraction kit. The obtained amount of DNA was evaluated by spectrophotometer, by measuring the absorbance at 260 nm (A260; A260=1 correspond to 50µg/ml of DNA). Vector DNA and insert DNA have been ligated together at 1:3 molar ratio, according to the following formula:

X ng of insert = [(ng of vector X kb of insert)/kb of vector] X molar ratio of vector/insert

Ligation reaction was catalyzed by T4 DNA ligase from T4 bacteriophage that joined the ends of fragments obtained by endonucleases digestion creating phosphodiester bounds between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides. 10X ligation Buffer (300mM Tris-HCl (pH 7.8), 100mM MgCl2, 100mM DTT and 10mM ATP) was added to 1 unit of T4 ligase and DNA fragments in 20ul of final volume. After 15 minutes of incubation at 25°C, ligation reaction was transformed in competent bacterial cells DH10B (Invitrogen). Transformation has been performed following heat shock procedure: 2ul of ligated DNA was incubated in 20ul of DH10B cells for 30 minutes in ice, then for 45 seconds at 42°C and then immediately placed in ice for 2 minutes. After that, 500ul of SOC medium (Invitrogen) have been added, and transformed cells were shaken at 32°C/225 rpm. After 1 hour, cells have been plated on LB (Luria Broth) Agar plates supplied with 100ug/ml of the proper antibiotic, depending on which resistance gene was carried by the vector plasmid.

For the screening of positive colonies, single colonies have been picked from the plate, inoculated in 5 ml of LB with the proper antibiotic (100ug/ml), and shaken for 16 hours at 225 rpm (32°C). DNA was extracted according to Qiagen miniprep kit protocol. To verify if the cloning had been successful, enzymatic digestions were performed. Then, 150ul of bacteria from the positive colony have been re-incubated in 150ml of LB medium for 16 hours at 32°C with vigorous shacking at 225 rpm. Qiagen Maxiprep Endofree extraction kit was used to extract higher amount of the newly constructed plasmid. Amount of extracted DNA was measured by spectrophotometer.

*3.2.3 Production of Integrase Defective Lentiviral Vectors (IDLVs)*

IDLVs have been produced by transient transfection of Lenti-X (Clontech) human embryonic kidney 293T cells with transfer vector (described above), packaging plasmid and envelope plasmid. phCMV-VSV.G envelope plasmid produces the vesicular stomatitis virus envelope glycoprotein G (VSV.G) from Indiana to widen IDLVs tropism. pcHELP or pAdSIVD64V have been used as integrase defective packaging plasmids in order to generate HIV- or SIV-derived IDLVs, respectively (*Negri et al., 2010; Negri et al., 2007; Mochizuki et al., 1998; Michelini et al., 2009*). Lenti-X 293T cell line was maintained in Dulbecco's Modified Eagles (DMEM) High glucose 4.5 g/L (Gibco) supplemented with 10% fetal calf serum (FCS, Corning), 100 units/ml penicillin/streptomycin (Gibco). For transfection, $3.5X10^6$ Lenti-X 293T cells were seeded onto 10-cm Petri dishes and, after 24 hours, transiently transfected with 1ml of a calcium phosphate precipitation mixture (Profection Mammalian Transfection kit, Promega) containing the plasmids mixture. Seventeen hours later, the medium was replaced with 8 ml of fresh DMEM 10% FCS. Forty-eight hours post-transfection, the supernatant containing IDLVs was collected, spun at 1500 rpm for 15 min at 4°C and filtered through a 0.45-mm pore size filter (Millipore) to remove cellular debris. IDLVs stocks were stored at -80°C until use.

For *in vivo* immunization protocols, IDLVs have been concentrated by ultracentrifugation. In detail, filtered supernatants were collected and ultracentrifuged on a 20% sucrose gradient at 23,000 rpm at 4°C in sterile Ultra-clear SW28 centrifuge tubes (Beckman) using an SW28 swinging bucket rotor (Beckman). After 2:30 hours, pelleted vector particles were resuspended in 100ul/tube of 1X phosphate-buffered saline (PBS 1x, Gibco) and stored at -80°C until use.

*3.2.4 IDLVs titration by Reverse Transcriptase (RT) activity assay*

Radioactive RT activity assay provides the quantification of the functionally active vector particles produced. 1ml of IDLV preparations was centrifuged at 12.000 rpm for 1 hour at 4°C and the pellets were resuspended in 200ul of Suspension Buffer (TrisCl 50mM pH 7.5, DTT 10nM, KCl 250nm, 0,25% TritonX-100, $H_2O$) followed by freezing and thawing for three cycles. 4ul of lysates have been then mixed to 40ul of the reaction mixture composed of 10ul assay buffer 5x (TrisCl 1M pH 7.5, $MgCl_2$ 1M, TritonX-100 1%, $H_2O$); 2,4ul DTT; 5ul polyA-dT; 1uCi dimethyl 1',2'-3H-thymidine 5' triphosphate (PerkinElmer); and incubated

for 1 hour at 37°C. The mixture was then spread on nitrocellulose membrane filters (Whatman) that have been washed twice with SSC buffer and twice with 99° Ethanol. The filters were dissolved in 5 ml of a scintillation liquid (PerkinElmer) and counted with a β-counter, that measures the amount of incorporated radioactive-labeled nucleotides (counts per minute (cpm)/ml).

*3.2.5 Cytofluorimetry (FACS) titration of IDLVs*

IDLVs preparation have also been tested for their efficiency of transduction by cytofluorimetry (FACS) titration, obtaining the functional titres of the vectors. $5x10^4$ LentiX 293T cells were seeded onto 12-wells plate and 24 hours after were transduced with three dilutions (1:2 – 1:10 – 1:50) of the vector stocks and incubated at 37°C, 5% $CO_2$ for three days. Then, transduced cells were collected, washed in PBS 1X (Gibco) and analysed by FACS Calibur (BD) for the expression of GFP. The titre expressed as transducing units (TU)/ml was calculated as follows:

TU/ml = [dilution factor] x [number of plated cells] x [percentage of transduced cells]

For IDLV-HTI and IDLV-UFO the corresponding transducing units (TU) were calculated by comparing the RT activity to the one of IDLV-GFP virions with known infectious titres (*Berger et al., 2011*).

*3.2.6 Confocal laser scanning microscopy (CLSM)*

For observation of transfected LentiX 293T cells by confocal laser scanning microscopy (CLSM), $2x10^4$ cells were seeded in 24-well plates onto 12-mm cover glasses previously treated with L-polylysine (Sigma). Cells were transiently transfected with fusion plasmids pHIVGagGFP, pSIVGagGFP, and pHTImCherry or plasmids expressing UFO antigens (pCDNA3.1-ConSOSL.UFO.664 and pCDNA3.1-ConSOSL.UFO.750) and ConSgp160 expressing plasmid (pCDNA3-ConSgp160, kindly provided by Prof. R. Shattock, *Imperial College of London*) using calcium phosphate procedure (Promega). Twenty-four hours post-transfection cells were washed, fixed with 4% paraformaldehyde (PFA) for 30' at +4°C or stained with anti-Env 2G12 (#1476, AIDS Reagents Program, NIH), PGDM1400 (kindly provided by Prof. R. Sanders, *University of Amsterdam*), or PGT145 (kindly provided by Dr. D. Katinger, *Polymun Scientific*) Abs and then with AlexaFluor 488 anti-human IgG (Jackson ImmunoResearch). The coverslips were mounted with Vectashield® antifade mounting

medium containing DAPI (Vector Labs) on the microscope slides. CLSM observations were performed on a Leica TCS SP2 AOBS apparatus (Leica Microsystems), using excitation spectral laser lines at 488 and 546 nm (for GFP and AlexaFluor 488) or at 586 and 610 nm (for mCherry), and using the confocal software (Leica) and Photoshop CS5. Signals from different fluorescent probes were taken in sequential scanning mode.

### *3.2.7 Western Blot*

To evaluate UFO antigens expression and UFO.750 presence on IDLV particles, pellets of transfected LentiX 293T cells and IDLV concentrated preparations were resuspended in SDS loading buffer. Lysed cells and virions were separated on 12% SDS polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane (Sartorius Stedim Italy). Filters were saturated for 2 h with 5% nonfat dry milk in PBST (PBS with 0.1% Tween 20) and then incubated with anti-HIV gp120 2G12 Ab or anti-HIV p24 polyclonal Ab (#4250, AIDS Reagent Program, NIH) for 1 h at room temperature followed by incubation for 1 h at room temperature with an anti-human horse radish peroxidase (HRP)-conjugated IgG (Jackson ImmunoResearch). The immunocomplexes were visualized using chemiluminescence ECL detection system (Millipore). ConSOSL.UFO.664 (kindly provided by Prof. R. Shattock, *Imperial College of London*) and SIVmac251 pr55 (#1845, AIDS Reagent Program, NIH) recombinant proteins were used as positive controls, whereas GFP expressing plasmid (pGAE-GFP) and IDLV-GFP were used as negative controls.

### *3.2.8 Animal immunization protocols*

Four- to six-week-old BALB/c female mice (Charles River) and male Mauritius-origin cynomolgus macaques used in these experiments were housed under specific pathogen-free conditions in the animal facility of the Istituto Superiore di Sanita' (ISS, Rome, Italy). All animal procedures have been performed in accordance with European Union guidelines and Italian legislation (Ministry of Health DL 26/2014) for animal care.

In the first experiments (HTI protocol), three mice per group were intramuscularly (i.m.) injected with $1x10^7$ RT units/mice of HIV-based (i) IDLV-GFP or (ii) IDLV-HTI; and SIV-based (iii) IDLV-GFP or (iv) IDLV-HTI. Naive non-immunized mice were kept as negative controls for parallel analysis. Four weeks after the immunization mice were euthanized. At sacrifice single cell suspensions from spleen were prepared by mechanical disruption, followed by

passage through cell strainers and treatment with Ammonium Chloride Potassium (ACK). Splenocytes were used for ELISPOT assay (see below) to evaluate cellular immune responses.

For the second set of experiments (UFO protocol), groups of five mice were immunized once i.m with escalating doses ($4X10^5$ - $2x10^6$ - $1x10^7$) of SIV-based IDLV-UFO.664 or IDLV-UFO.750. Naïve mice were used as negative controls. Blood retro orbital sampling was performed each month with glass Pasteur pipettes and sera were tested for the presence of anti-UFO antibodies by ELISA assay (described below). Mice were sacrificed one-year post-vaccination and splenocytes have been stored in liquid nitrogen for further analysis.

In order to evaluate the immune responses after vaccination with IDLV-UFO.750 in NHP animal model, five cynomolgus macaques were immunized i.m. with $3x10^8$ TU/animal of IDLV-UFO.750 in 1ml injection volume divided into two sites (left and right thighs). Thirty-seven weeks after the prime, animals were boosted with the same vector pseudotyped with a different VSV.G serotype (VSV.G Indiana for the prime, VSV.G Cocal for the boost) to avoid neutralization of IDLV following subsequent immunizations. Peripheral blood cells, sera, plasma, saliva, and rectal swabs were obtained prior to immunization, 2 weeks after each immunization and at monthly intervals throughout the study.

*3.2.9 Interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) assay*

In order to evaluate T-cell immune responses in mice immunized with IDLV-HTI, IFN-*γ*ELISPOT assay was performed following instructions of BD ELISPOT kit (BD Biosciences). Splenocytes were maintained in RPMI medium 10% FCS (Gibco, Lonza), penicillin-streptomycin-glutamine (100 units/mL; Gibco), supplemented with 2-mercaptoethanol 50 mM (Sigma Chemicals), HEPES buffer solution 25mM (Gibco), sodium pyruvate 1mM (Gibco), non-essential amino acids (Gibco). For ELISPOT, $2.5x10^5$/well fresh splenocytes were seeded onto 96 well plates previously coated with 100ul/well of 5ug/ml anti-IFN-γ. Cells were stimulated overnight either with 5ug/ml of the H-2Kd restricted HIV-Gag p24 (AMQMLKETI, #11876 AIDS Reagent Program, NIH) or 2ug/ml of GFP (HYLSTQSAL, UFPeptides, *Gambotto el al., 2000*) epitopes. To assess the immune responses to HTI, 10 pools of 111 peptides of 15 amino acids in length (overlapping by 11 residues) spanning the entire HTI including the linkers regions (*Mothe et al., 2015*) were used at 2ug/ml. Medium alone and concanavalin A (5ug/ml) were used as negative and positive controls,

respectively. Spot-forming cells were counted with an automated ELISPOT reader (A.EL.VIS). Values obtained from the medium-treated cells (background) were subtracted from values obtained from cells stimulated with specific peptides, and results were expressed as IFN-γ secreting cells (spot forming cells, SFC) per million cells. *S*amples were scored positive when a minimum of 50 spots per $10^6$ cells was present and 1.5-fold higher than unstimulated sample.

*3.2.10 Enzyme-linked immunosorbent (ELISA) assay*

Sera from mice vaccinated with either IDLV-UFO.664 or IDLV-UFO.750 and from macaques immunized with IDLV-UFO.750 were tested for the presence of anti-Env binding Abs by ELISA.

For mice sera, ninety-six well Maxisorp plates (Nunc) were coated with ConSOSL.UFO.664 recombinant protein (1ug/ml in PBS 1X) overnight at 4°C. After washing (PBS 1X, 0,05% Tween-20) and blocking with assay buffer (PBS 1X, 1% BSA, 0.05% Tween-20), serial dilutions (1:100, 1:1000; 1:10000) of serum from individual mice were added to wells in triplicate and incubated for 1 h at 37°C. The plates were washed and HRP-conjugated goat anti-mouse IgG (Southern Biotech) was added to the wells for 1 h at 37°C. Then, SureBlue TMB Peroxidase solution (KPL) was added for 5 minutes at room temperature, followed by 50ul/well of TMB stop solution (KPL). Five-fold dilutions of mouse IgG (Southern Biotech) were used to develop standard curves.

For NHP samples, capture ELISA was performed. Maxisorp plates were coated with 2,5ug/ml of mouse anti-human c-Myc 9E10 mAb (produced in house) or goat anti-human IgG (1:2000, Southern Biotech) for the standard. After overnight at 4°C, plates were washed and blocked with casein buffer (Thermo Scientific) at 37°C for 1h. After washing, 1ug/ml of gp140 MycHis Tagged protein (produced in house) was incubated for 1h at 37°C. Three ten-fold dilutions (1:100, 1:1000, 1:10000) of immunized monkey sera or five-fold dilutions of Cynomolgus IgG standard (Molecular Innovations) were added in triplicate and incubated for 1h at 37°C. Then, biotinylated mouse anti-monkey IgG (Southern Biotech) was added 1:50000. After 1h at 37°C, Poly-HRP 40 (1:1000, Fitzgerald) was added for 45 minutes at 37°C and detection was performed with SureBlue TMB Peroxidase solution (KPL) for 5 minutes at room temperature, followed by 50ul/well of TMB stop solution (KPL).

The concentration of both mouse and simian IgG antibodies was calculated relative to the standard using a 5-parameter fit curve (Softmax, Molecular Devices) and results are expressed as ug of HIV-specific antibodies (IgG) and for each group of immunization.

### *3.2.11 Neutralization assay*

In order to evaluate whether immunization with IDLV-UFO.750 had been able to induce broadly neutralizing antibodies (bNAbs), neutralization assay has been performed on sera from vaccinated macaques.

Neutralization of Env-pseudotyped viruses was measured in 96-well culture plates using Tat-regulated firefly luciferase (Luc) reporter gene expression to quantify reductions of virus infection in TZM-bl cells (*Montefiori, 2009*). Test sera and control sera have been complement depleted by heat inactivation for 60 minutes at 56°C and each sample was tested in triplicate. Four four-fold dilutions starting with 1/10 of each serum were incubated with $1{,}5x10^5$ RLU of pseudoviruses for 1h at 37°C. Thereafter, $10^4$ TZM-bl cells were added, the plates were incubated for 48h-72h at 37°C, and then luciferase activity was measured (by using Bright-Glo reagent, Promega). The positive controls were sera with high neutralizing titers, whereas HIV-negative sera were used as negative controls. Six wells were used as cell controls (background) and 6 wells as virus controls. Percent neutralization is determined by calculating the difference in average RLU between test wells (cells + serum sample + virus) and cell control wells (cells only), dividing this result by the difference in average RLU between virus control (cell + virus) and cell control wells. Neutralizing antibody titres are expressed as the reciprocal of the serum dilution required to reduce RLU by 50%.

# Chapter 4

# Results

## *4.1 Production of IDLV expressing HTI immunogen*

For the production of IDLV-HTI, HIV- and SIV-based transfer plasmids expressing HTI have been constructed, as described in Chapter 3, and then were used to transiently co-transfect LentiX 293T cells together with the IN defective packaging plasmid and VSV.G expressing plasmid. IDLV-GFP has been used as control. The recovery of IDLV-HTI, measured as reverse transcriptase (RT) activity, was 1 log lower than parental vector expressing GFP, both in HIV and SIV background (**Figure 17**). This transdominant-negative effect of HTI on IDLV release was expected since HIV Gag mutants interfere with HIV particle assembly and non-myristoylated Gag protein accumulates in cytosolic complex (*Cara et al., 1998; Bryant at al., 1990*). Since Gag in HTI does not have myristoylation site, we hypothesized that HTI could oligomerize with HIV or SIV wild type (wt) Gag expressed by packaging vector.

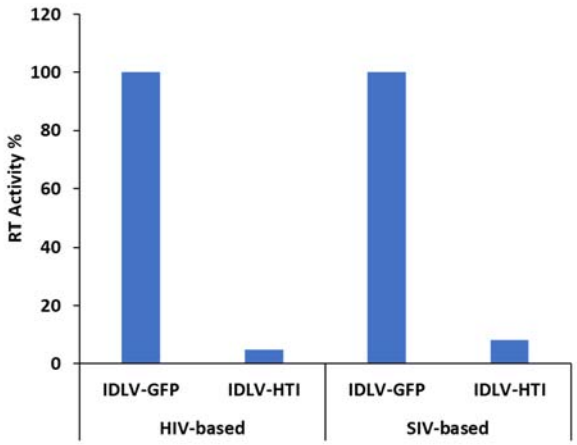



**Figure 17**. Reverse transcriptase (RT) activity of HIV- and SIV-based IDLV-HTI expressed as percentage compared to GFP expressing IDLVs used as positive controls.

To investigate HTI interference in IDLV production, we constructed three fusion plasmids in which HIVGag or SIVGag were fused to GFP, whereas HTI was fused to mCherry fluorescent protein. These plasmids were used to transfected LentiX 293T cells then observed with confocal laser scanning microscope (CLSM) (**Figure 18**). When transfected alone either HIV- and SIV-Gag were membrane-associated, while HTI localized within the

cytoplasm. However, in co-transfection experiments, HTI retained both wtGag proteins into the cytoplasm of transfected cells preventing membrane association of wtGag.

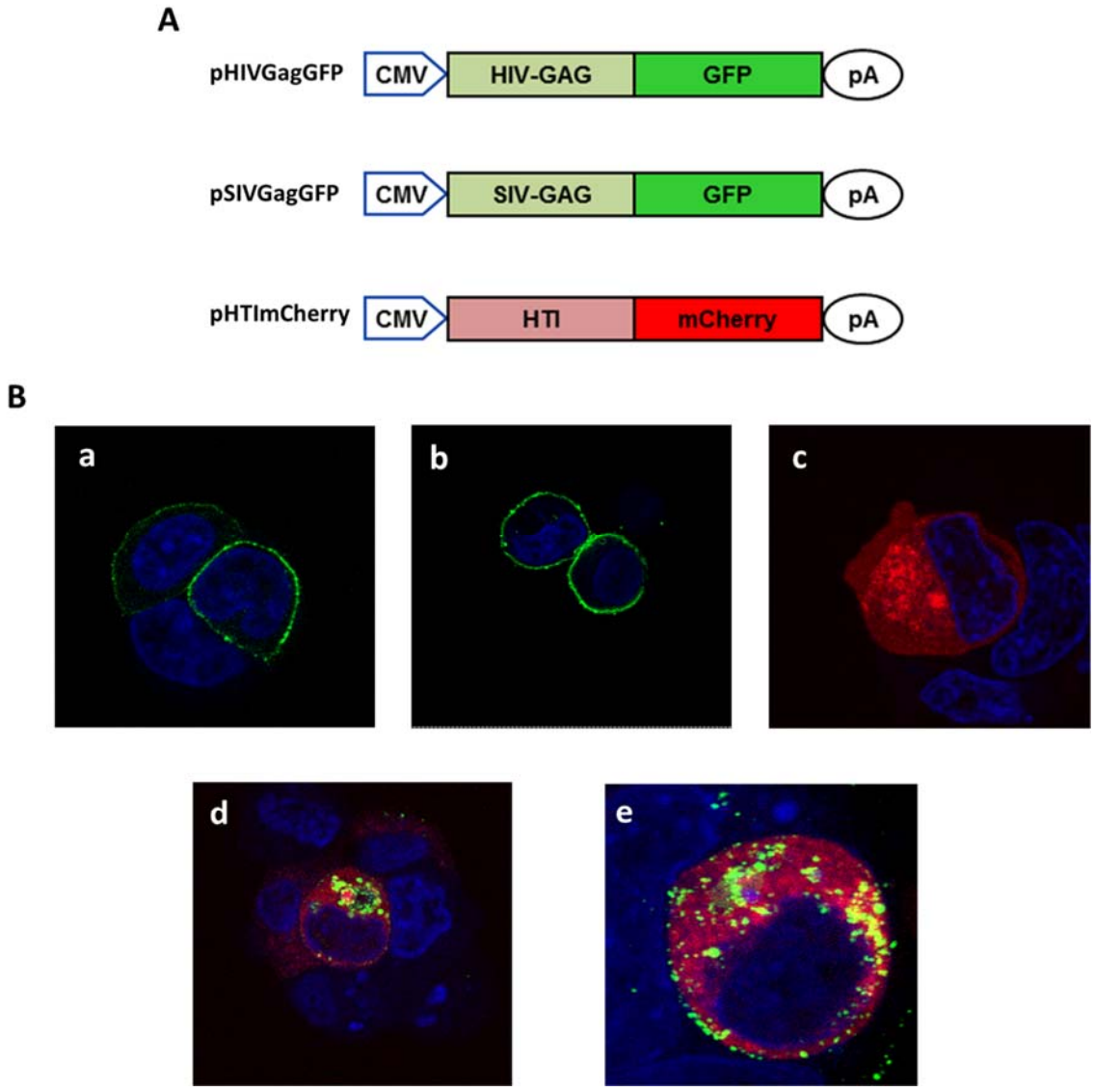



**Figure 18. A.** Schematics of fusion plasmids. **B.** Confocal laser scanning microscopy (CLSM) of 293T transfected with pHIVGag-GFP (a), pSIVGag-GFP (b), pHTI-mCherry (c). Co-transfection of pHTI-mCherry with pHIVGag-GFP or pSIVGag-GFP are shown in (d) and (e), respectively. Nuclei are coloured in blue by DAPI staining.

To mitigate the negative effect of HTI on wtGag, 293T cells were transfected with lower amount of pHTImCherry compared to pHIVGagGFP or pSIVGagGFP. As shown in **Figure 19,** a very low percentage of HIV or SIV Gag proteins was still sequestered by HTI within the cells, while the majority of wtGag localized on the cell membrane.

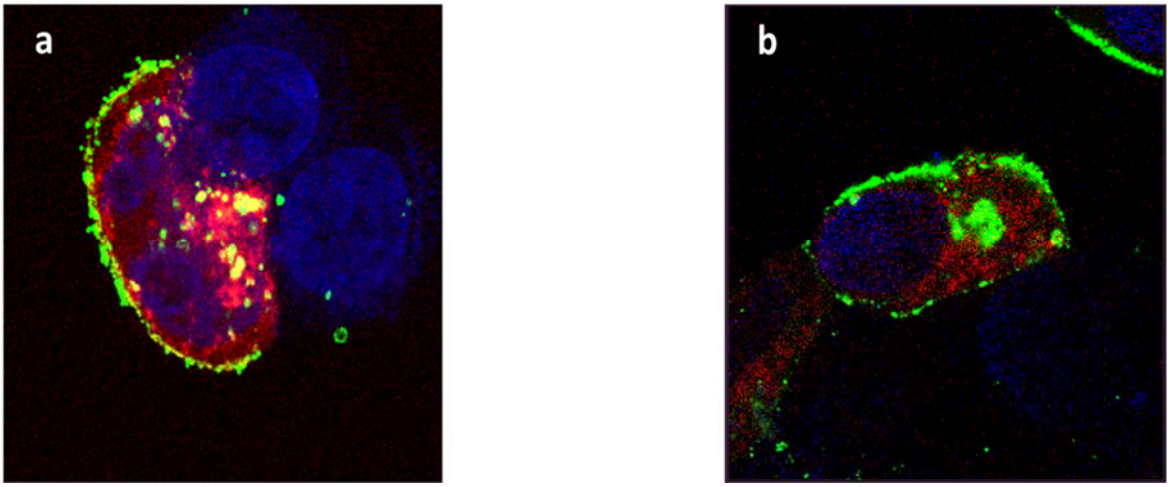



**Figure 19.** CLSM analysis of 293T cells transfected with pHTI-mCherry and pHIVGag-GFP (a) or pSIVGag-GFP (b) using HTI/Gag molar ratio of 0.4. Nuclei are coloured in blue by DAPI staining.

Moreover, production of LV-GFP in presence of decreasing amount of HTI expressing plasmid resulted in increased LV recovery and transducing efficiency, that reached the highest values when HTI/wtGag molar ratio was 0.4, corresponding to 0.5 ug of pHTI (**Figure 20**).

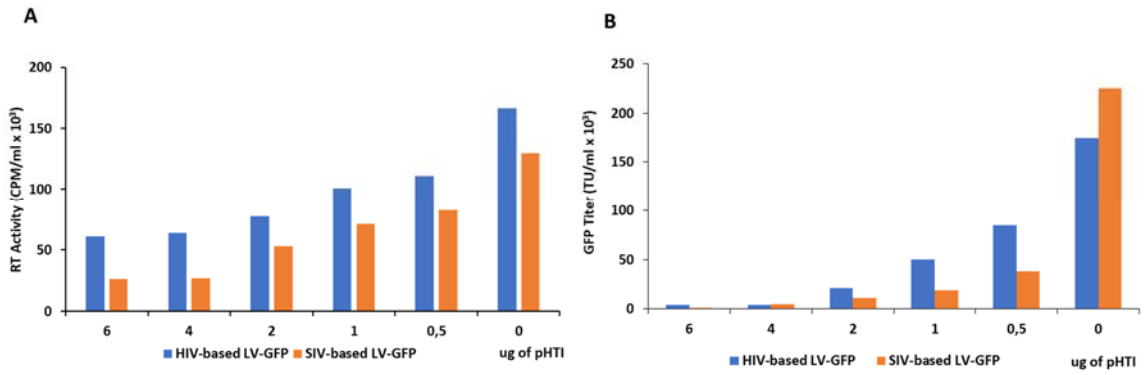



**Figure 20.** RT activity (A) and GFP titer (B) of HIV-based (blue bars) or SIV-based (orange bars) LV-GFP produced in presence of decreasing amount of HTI-expressing plasmid. Results from one representative experiment.

Therefore, we modified IDLV-HTI production protocol, by using higher amount of packaging plasmid compared to transfer vector expressing HTI resulting in HTI/wtGag molar ratio=0.4. The release of IDLV-HTI increased seven-fold for HIV background and five-fold for SIV-based IDLV (**Figure 21**).

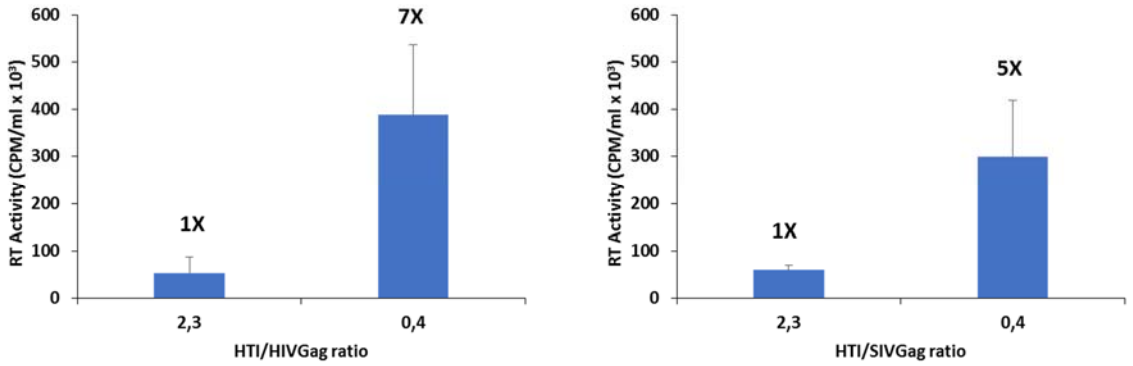



**Figure 21.** RT activity of HIV-based (left panel) or SIV-based (right panel) IDLV-HTI produced with decreasing HTI/wtGAG molar ratio. Data are expressed as the mean results from three independent experiments. The error bars represent the standard errors of the mean.

### *4.2 Mice immunization with IDLVs expressing HTI*

In order to evaluate whether IDLV-HTI is an effective system to induce cellular immune responses against HIV-1 structural antigens, we performed an *in vivo* immunization protocol. BALB/c mice were immunized with a single intramuscular inoculum of HIV-based IDLV-HTI or IDLV-GFP and SIV-based IDLV-HTI or IDLV-GFP, whereas naïve mice were kept as control (**Table 5**). Four weeks from injection, mice were sacrified and T-cell response was assessed in fresh splenocytes by IFN-γ ELISPOT.

| HTI PROTOCOL | | | | | |
|---|---|---|---|---|---|
| GROUP | MICE/GROUP | IMMUNOGEN | LENTIVIRUS OF ORIGIN | DOSE | SACRIFICE |
| A | 3 | HTI | HIV | $10X10^6$ RT units | 4 weeks |
| B | 3 | GFP | HIV | $10X10^6$ RT units | 4 weeks |
| C | 3 | HTI | SIV | $10X10^6$ RT units | 4 weeks |
| D | 3 | GFP | SIV | $10X10^6$ RT units | 4 weeks |
| E | 3 | | None | | 4 weeks |

**Table 4.** Schedule of BALB/c mice immunization with HIV- or SIV-based IDLVs expressing either HTI or GFP.

The H-2d restricted Gag-9mer (AMQMLKETI) and GFP-9mer (HYLSTQSAL, *Gambotto et al., 2000*) peptides and ten overlapping peptide pools spanning the entire HTI sequence (*Mothe et al., 2015*) have been used as stimuli (**Figure 22**).

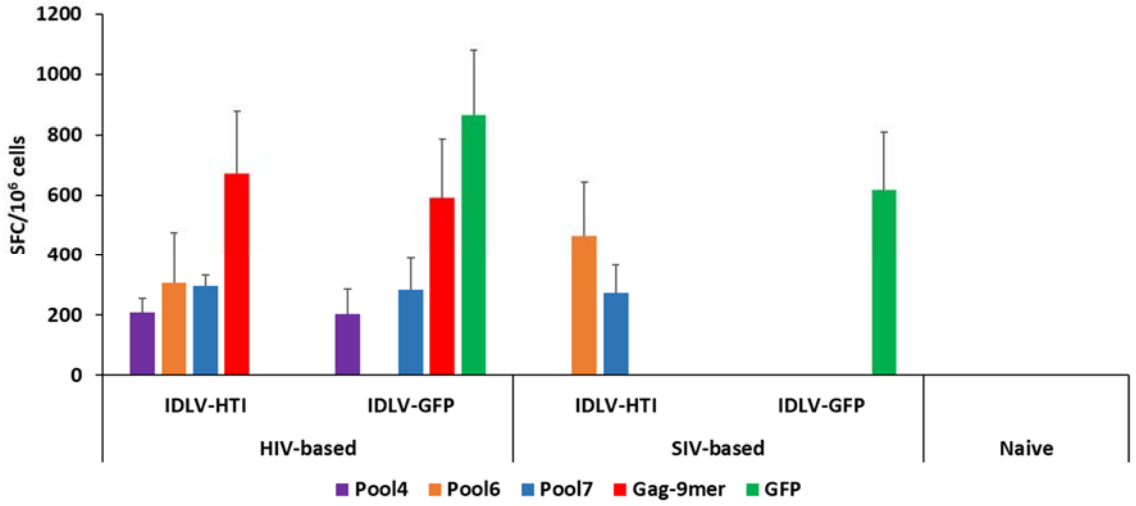



**Figure 22.** IFN-γ ELISPOT assay on splenocytes from BALB/c mice immunized with HIV- or SIV-based IDLV-HTI and IDLV-GFP. IFN-γ producing T cells with frequencies above 50 spots forming cells (SFC) per $10^6$ splenocytes and a 1.5-fold higher than unstimulated sample are shown after background subtraction. Cells were stimulated overnight with the specific GFP-9mer (green bars), Gag-9mer (red bars) and 10 pools of peptides spanning the whole HTI sequence. Error bars represent the standard deviation among animals from the same group.

As expected, GFP-specific response was present on cells derived from mice immunized with either SIV- or HIV-based IDLV-GFP, while a specific response to the immunodominant epitope for HIV Gag in BALB/c was present only in splenocytes from mice immunized with HIV-based IDLVs. Importantly, few peptide pools were reactive with both HTI-expressing IDLVs. In particular, pool 6, including peptides spanning the HTI protease (PR) region, elicited the most specific responses since it was recognized only in the context of immunization with IDLV-HTI although these responses appeared to be subdominant with respect to dominant Gag-9mer peptide in the HIV-based vectors immunized mice. Interestingly, both HIV-based IDLV-GFP and IDLV-HTI induced immune responses to HTI pool 4 (HTI p24) that were absent in mice vaccinated with SIV-based IDLVs. These responses are due to the presence of cross-reactive epitopes present in the structural Gag proteins deriving from HIV-1 in the HIV-based IDLV particles, which are absent in the structural Gag proteins deriving from SIV in the SIV-based IDLV particles. No specific responses were detectable in splenocytes derived from naïve mice.

### *4.3 Generation of SIV-based IDLVs expressing ConSOSL.UFO antigens*

Two UFO immunogens have been selected for this work: ConSOSL.UFO.664 and ConSOSL.UFO.750. Both are based on a consensus sequence of group M HIV-1 Env (ConS) that has been modified to produce native-like env trimers with increased stability and exposure of epitopes able to induce bNAbs. UFO.664 produces high proportion of soluble Env trimers, whereas UFO.750 produces a membrane-tethered version of the same Env trimer (*Aldon et al., 2018*). ORFs codifying for both ConSOSL.UFO.664 and ConSOSL.UFO.750 have been cloned into the SIV-based pGAE transfer vector, obtaining pGAE-UFO.664 and pGAE-UFO.750, as described in detail in Materials and Method (see chapter 3).

To evaluate *in vitro* expression of Env proteins, LentiX 293T cells were transfected with pGAE-UFO.664 or pGAE-UFO.750 and cell lysates were analysed by Western blot (WB). Both UFO.664 and UFO.750 envelopes were efficiently expressed by the transfer vectors (**Figure 23**).

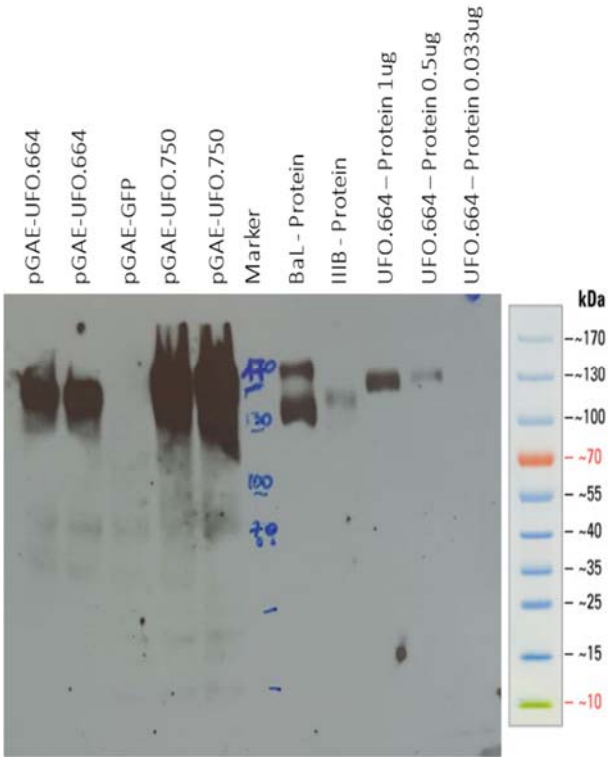



**Figure 23.** Western blot on lysates of LentiX 293T cells transfected with pGAE-UFO.664 or pGAE-UFO.750. Ba-L, IIIB and UFO.664 envelope proteins were used as positive controls; while the transfer vector expressing the GFP (pGAE-GFP) was used as negative controls.

In order to confirm that only UFO.750 was exposed on the cell membrane, transfected cells were also analysed by flow cytometry and CLSM (**Figure 24**). Anti-Env 2G12 antibody was used to detect Env gp120. When cells were stained with 2G12 after membrane permeabilization, both UFO.664 and UFO.750 were detectable. On the contrary, if cells were stained without permeabilizing the membrane, Env expression on the cell surface was detected only in cells transfected with pGAE-UFO.750. Same results were achieved by CLSM observation. Indeed, while UFO.750 was detectable after membrane staining with 2G12 Ab, intracellular staining was necessary to detect UFO.664, thus confirming that UFO.750 is membrane-bound whereas UFO.664 produces soluble trimers.

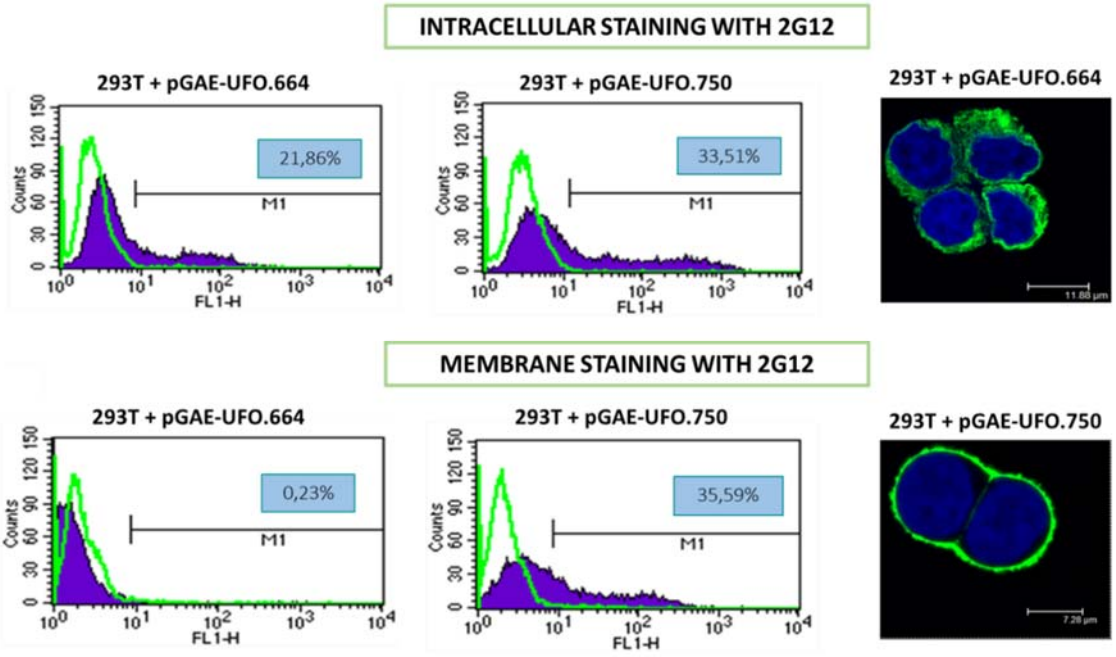



**Figure 24.** Analysis of UFO immunogens expression. Intracellular (top panel) or membrane (bottom panel) staining with 2G12 Ab of LentiX 293T cells transfected with either pGAE-UFO.750 or pGAE-UFO.664 was evaluated by FACS or CLSM.

Furthermore, the expression of Env proteins in the trimeric conformation was evaluated by membrane staining on cells transfected with pGAE-UFO.750 at escalating doses of PGT145 or PGDM1400 bNAbs, that bind specifically and exclusively to the quaternary structure-dependent epitopes at the trimer apex (*Yasmeen et al., 2014; Walker et al., 2011; Sok et al., 2014*). As negative control we used a plasmid expressing the parental ConS gp160, from which UFO antigens are derived, but that is not engineered to increase trimers production.

Flow cytometry and CSLM showed that both PGT145 and PGDM1400 were able to bind UFO.750 on the surface of the transfected cells, confirming that UFO.750 produces stable membrane-bound trimers. Conversely, no signals have been detected when staining was performed on cells transfected with pConSgp160 (**Figure 25**).

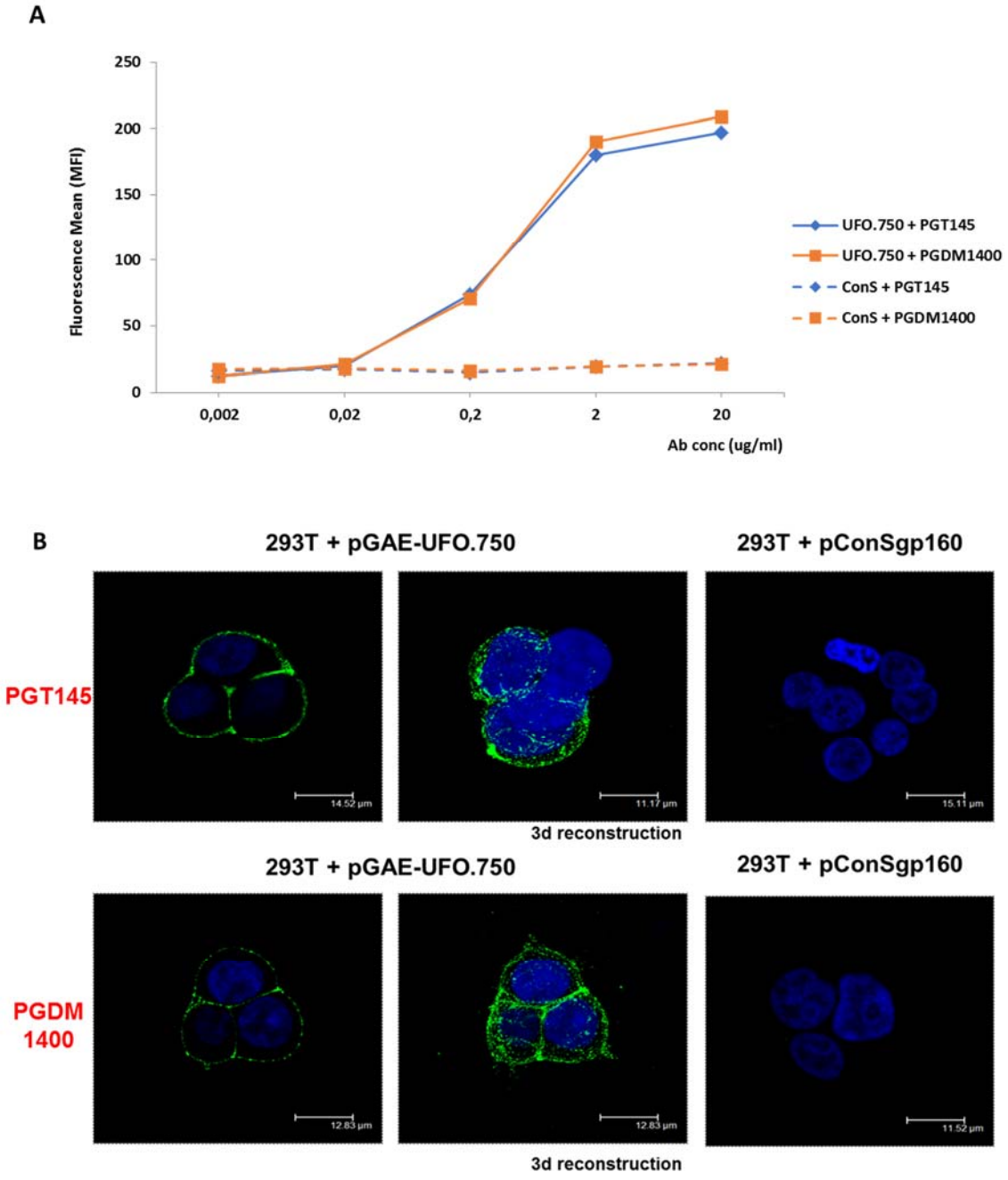



**Figure 25.** Detection of UFO trimers by flow cytometry (**A**) or CLSM (**B**) on LentiX 293T cells transfected with pGAE-UFO.750 or pConSgp160 after membrane staining with either PGT145 or PGDM1400.

We then used the two transfer vectors for the production of SIV-based IDLV-UFO.664 and IDLV-UFO.750. The recovered IDLVs were normalized for RT activity and analysed by WB (**Figure 26**). Filters were probed with anti-Env 2G12 and anti-Gag Abs. As expected, a band corresponding to Gag protein was present in both IDLVs expressing UFO proteins and in GFP expressing IDLV, used as control. Conversely, Env was detected only in the IDLV-UFO.750 preparations, confirming that IDLV-UFO.750 is pseudotyped with UFO.

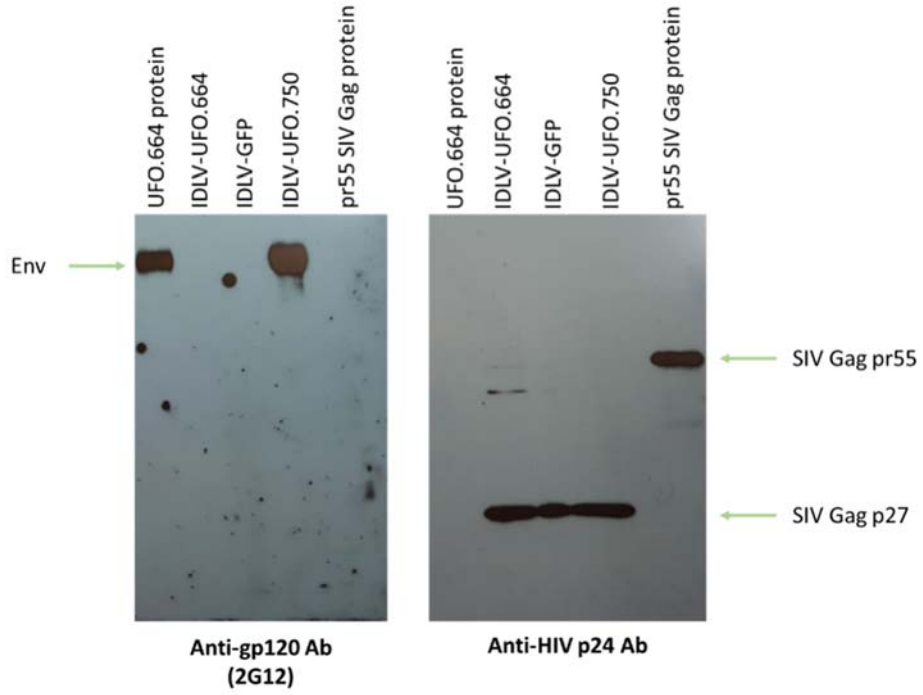



**Figure 26.** WB of lysates from concentrated stocks of IDLV-UFO.664 and IDLV-UFO.750. ConSOSL.UFO.664, pr55 SIVGag proteins and IDLV-GFP were used as controls. Filters were probed with 2G12 anti-gp120 Ab (left) or anti-HIVp24 (right).

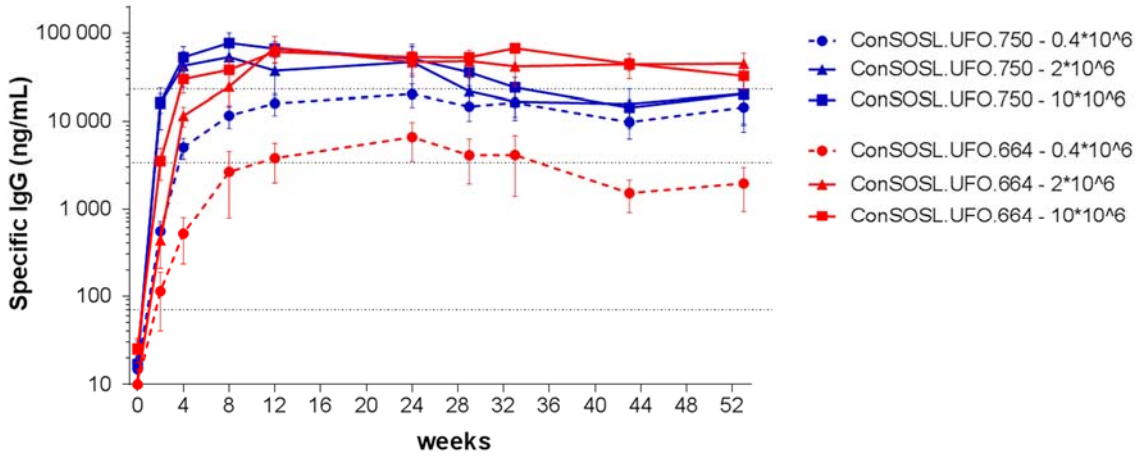
### *4.4 Mice immunization with IDLV-UFO.664 and IDLV-UFO.750*

Since UFO.750 is present on the surface of IDLV particles, we speculated that using IDLV-UFO.750 for immunization would elicit higher humoral immune responses than IDLV-UFO.664 due to the pseudotyping trimeric HIV-Env on the IDLV particles in addition to UFO.750 expressed as transgene from the IDLV genome. Therefore, to compare the immune responses to membrane bound and secreted Envelopes, groups of 5 BALB/c mice were immunized once intramuscularly with scalar doses of either IDLV-UFO.750 or IDLV-UFO.664: $10X10^6$ – $2X10^6$ - $0.4X10^6$ RT units/mouse (**Table 5**). Serum samples have been obtained from blood collected monthly at different time points starting from 2 weeks and until one-year post-inoculum.

| UFO PROTOCOL | | | | | |
|---|---|---|---|---|---|
| GROUP | MICE/GROUP | IMMUNOGEN | LENTIVIRUS OF ORIGIN | DOSE | SACRIFICE |
| A | 5 | UFO.664 | SIV | $10X10^6$ RT units | 1 year |
| B | 5 | UFO.664 | SIV | $2X10^6$ RT units | 1 year |
| C | 5 | UFO.664 | SIV | $0.4X10^6$ RT units | 1 year |
| D | 5 | UFO.750 | SIV | $10X10^6$ RT units | 1 year |
| E | 5 | UF0.750 | SIV | $2X10^6$ RT units | 1 year |
| F | 5 | UFO.750 | SIV | $0.4X10^6$ RT units | 1 year |
| G | 5 | | None | | 1 year |

**Table 5.** Immunization schedule of BALB/c mice vaccinated with decreasing doses of IDLVs expressing either UFO.664 or UFO.750.

ELISA assay was performed to investigate the induction of humoral immune response against Env (**Figure 27**). Results showed that a single immunization with either IDLVs-UFO elicited anti-Env binding Abs that were still detectable at 53 weeks after the injection. Importantly, IDLV-UFO.750 elicited higher Ab levels than IDLV-UFO.664 in the early time points. At the lowest dose of injected IDLVs, these differences were particularly noticeable and remained stable till the sacrifice. Interestingly, comparing mice immunized with the higher doses of IDLVs, the IgG levels elicited by immunization with IDLV-UFO.750 decreased slowly overtime, while anti-Env Ab titres remained stable in IDLV-UFO.664 immunized mice.



(see figure in previous page)
**Figure 27.** Kinetics of anti-UFO binding antibodies in sera from mice immunized once with escalating doses of either IDLV-UFO.750 (blue lines) or IDLV-UFO.664 (red lines).

### *4.5 Non-human primates immunization with IDLV-UFO.750*

Immunization study in non-human primates (NHPs) with IDLV-UFO.750 has been performed to evaluate whether it is a suitable platform for inducing broadly neutralizing antibodies (bNAbs). Five cynomolgus macaques were vaccinated intramuscularly with 3x10^8 TU/monkey of IDLV expressing UFO.750 (**Figure 28**). ELISA assay on sera collected monthly after vaccination showed that vaccination induced high anti-Env IgG levels in all monkeys (**Figure 29**). The levels of Abs peaked at 2 weeks post-immunization and slowly decreased overtime in three monkeys (AU018, AS304 and AT777) while peaked at 4-6 weeks post-immunization and remained at higher levels in the other two monkeys (AU955 and AU989).

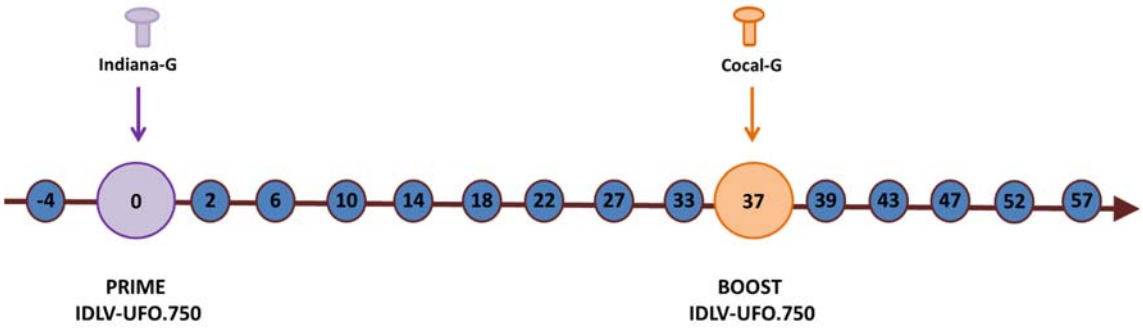



**Figure 28.** NHP immunization schedule. Cynomolgus macaques were primed with IDLV expressing UFO.750 and pseudotyped with VSV.G from Indiana serotype ($VSV.G_{IN}$). Animals were boosted 8 months after the first immunization with the same IDLV-UFO.750 pseudotyped with VSV.G from Cocal serotype ($VSV.G_{CO}$).

Eight months after the prime all animals were boosted with the same vector pseudotyped with VSV.G from non-cross-reactive serotype: VSV.G from Indiana serotype ($VSV.G_{IN}$) was used to pseudotyping IDLV-UFO.750 inoculated for the prime, whereas IDLV-UFO.750 used for the boost was pseudotyped with VSV.G from Cocal serotype ($VSV.G_{CO}$) (see immunization schedule in **Figure 28**). This "VSV.G exchange strategy" was adopted to allow for an efficient boost, thus avoiding neutralizing antibodies directed against $VSV.G_{IN}$ induced by the first immunization that may interfere with the efficacy of the boost. The boost increased anti-Env IgG titres in all monkeys. The peak response was detectable at

two weeks post-boost and then Abs levels slowly waned, but remained stable at higher levels than pre-boost (0,5-1 log) and were detectable until 57 weeks after the first vaccination (last time point tested).

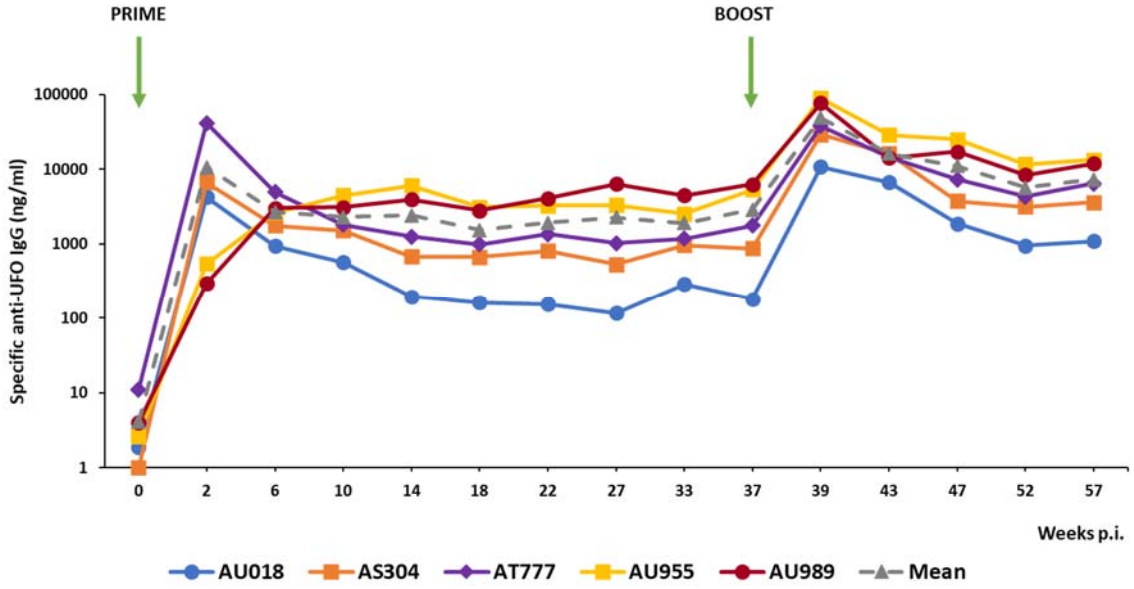



**Figure 29.** Anti-UFO binding antibodies induced by prime-boost immunization of monkeys with IDLV-UFO.750.

Sera from vaccinated monkeys were also tested for the presence of vaccine-induced neutralizing antibodies (NAbs). All monkeys developed NAbs against the MW965.26 virus, a clade C tier 1 HIV-1 isolate, but only after the boost (**Table 6**). No NAbs able to neutralize tier 2 viruses were detected.

| | WEEKS POST-IMMUNIZATION | MONKEYS ID | | | | |
|---|---|---|---|---|---|---|
| | | AU018 | AS304 | AT777 | AU955 | AU989 |
| PRIME | 0 | <50 | <50 | <50 | <50 | <50 |
| | 6 | <50 | <50 | <50 | <50 | <50 |
| BOOST | 37 | <50 | <50 | <50 | <50 | <50 |
| | 43 | 60 | 130 | 528 | 840 | 1155 |
| | 47 | <50 | <50 | 169 | 379 | 109 |

**Table 6.** Neutralization activity against MW965.26 virus.

# Chapter 5

## Discussion

The Thai trial (RV144) has given renewed hope in the field of HIV-1 vaccine by providing the first evidence that vaccination may decrease the risk of acquiring HIV-1 infection (*Rerks-Ngarm et al., 2009*), with vaccine efficacy that positively correlated with IgG directed at HIV Envelope (Env) V1/V2 regions. Unfortunately, the protective effects due to anti-V1/V2 Abs waned after six months from the boost. Therefore, the development of an effective vaccine against HIV-1 remains one of the main global health priorities. The failure of the previous approaches has pushed researchers' efforts toward the development of innovative vaccine formulations in terms of both delivery platform and antigens. A novel approach involves the rational design of immunogens based on the immune reactivity data. Two of these rationally designed HIV-1 antigens have been studied in this work: HIVACAT T-cell Immunogen (HTI) and Uncleaved pre-Fusion Optimized gp140 trimers (ConSOSL.UFO). The former is a polypeptide sequence covering regions in the structural viral proteins that are conserved among the different HIV-1 strains. The design of HTI is based on functional data of more than 1,000 HIV-1 patients, infected with clade B or C virus isolates, with low levels of viral load, in which have been identified the epitopes targeted by T-cells (*Mothe et al., 2015*). Furthermore, immunodominant epitopes for which there is no described beneficial role in control viral replication have been excluded in order to maximize the induction of effective anti-HIV cellular immune response. On the other hand, HIV Envelope antigens with UFO modifications have been designed with the aim to induce broadly neutralizing antibodies (bNAbs). UFOs have been derived from the Group M consensus immunogen (*Liao et al., 2006*) and modified to codify for recombinant HIV-1 envelopes that mimic the virion-associated trimeric spikes, in order to exhibit the so-called trimer-influenced and trimer-dependent bNAbs epitopes in the same way as they appear on native Env spikes. Consequently, including these epitopes in the immunogen design has the chance to increase bNAbs against Env after vaccination. Moreover, since bNAbs epitopes need to be exposed in the appropriate conformational form, UFO sequences have been further modified to increase the stability of Env trimers, otherwise inherently unstable, by covalently linking the gp120 and gp41 domains with an engineered disulfide bond (SOS),

by introducing in the ConS sequence stabilization mutations taken from SOSIP (*de Taye et al., 2015*), and by adding two flexible linkers to replace the gp120/gp41 cleavage site (*Kovacs et al., 2014*), and the region of HR1 domain that undergoes drastic conformational change during viral fusion with host cells (*Kong et al., 2016*). In addition, since the hydrophobic membrane-proximal external region (MPER) at C-terminus of gp140 ectodomain had the tendency to facilitate trimers aggregation, it has been truncated in order to create a more soluble version of trimers, generating the ConSOSL.UFO.664. In addition to soluble ConSOSL.UFO.664, a membrane-tethered version, the ConSOSL.UFO.750, has been evaluated in this study since the truly native form of Env is the one presented on the surface of virus particles (*Aldon et al., 2018*).

In the work described in this thesis, we selected the Integrase Defective Lentiviral Vectors (IDLVs) as vehicle for these novel immunogens since it has been proved that IDLVs represent an effective delivery system able to induce specific and persistent cellular and humoral immune responses in the mouse and NHP models (*Gallinaro et al., 2018; Negri et al. 2016; Michelini et al., 2009; Negri et al., 2007).* IDLVs are derived from the parental integrating lentiviral vectors (LVs), retaining their ability to efficiently transduce non-dividing cells from several lineages (after pseudotyping with VSV.G), but have an inherent increased safety profile which has been achieved by self-inactivation of the LV genome and by impairing the functionality of integrase protein, thus greatly lowering the probability of insertional mutagenesis.

Our results showed that the construction of both HIV- and SIV-based IDLVs expressing HTI has been hampered by the presence of a mutated form of Gag protein in the HTI ORF, that showed a trans-dominant negative effect interfering with the assembly of vector particle, as previously described (*Cara et al., 1998*). Furthermore, the absence of myristoylation domain in the portion of Gag protein codified by HTI caused the accumulation of HTI in the cellular cytoplasm (*Briant and Ratner, 1990*) resulting in the sequestration of wild-type Gag proteins, as shown by confocal laser scanning microscopy observation of cells transfected with wild-type HIV- and SIV-Gag with HTI expressing plasmids. Increasing the amount of DNA molecules (packaging plasmid) expressing either wild-type Gag protein, against the transfer vector expressing HTI, weakened the HTI negative effect and allowed IDLVs-HTI production in amount sufficient to perform immunization of BALB/c mice, using SIV- or HIV-

derived IDLV expressing GFP as control. Analysis of cellular immune responses suggested that SIV-based IDLV is more suitable than HIV-based IDLV as delivery platform to achieve specific immune responses against HIV-1 structural proteins. In particular, while splenocytes from mice immunized with HIV-based IDLV-HTI were reactive against HTI pools covering Gag p24 (pool 4), Protease (pool 6) and RT (pool 7) regions, splenocytes from mice immunized with HIV-based IDLV-GFP shared responses against peptide pools covering Gag p24 (pool 4) and RT (pool 7) but did not react against peptide pool covering Protease (pool 6). This suggests that immunization with HIV-based IDLV-HTI induced a transgene (HTI)-specific response against the Protease region of HTI, while other responses are indicative of the presence of cross-reactive epitopes in the structural proteins present in the HIV-derived vector particle (**Figure 30A**). In addition, HIV-based IDLVs induced a strong cellular response against the immunodominant Gag-9mer epitope (AMQMLKETI) that may be detrimental for the vaccine efficacy since it may divert from the "beneficial" epitopes encoded by HTI. On the contrary, splenocytes from mice immunized with SIV-based IDLV-HTI were reactive against HTI pools covering Protease (pool 6) and RT (pool 7) regions, while splenocytes from mice immunized with SIV-based IDLV-GFP did not react against any HTI pools. Since SIV-Gag protein has only 53% of sequence identity with HIV-Gag (**Figure 30B**), no cross-reactivity with SIV-derived particle was detected, and splenocytes did not react against peptide pool covering Gag p24 (pool 4).

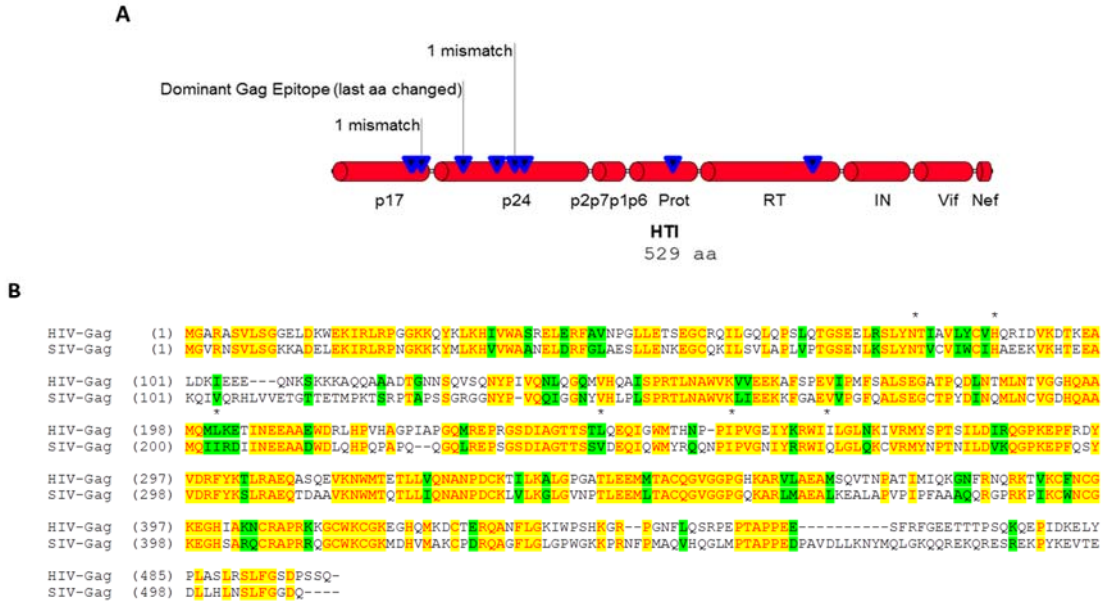



**Figure 30. A.** Schematic representation of HTI protein. **B.** Sequence alignment between HIV and SIV Gag proteins. Triangles (**A**) and stars (**B**) indicate described H2d-restricted epitopes.

The highest HTI-specific response was achieved toward peptide pool 6, covering the protease gene of HTI, since it was recognized only in the context of either IDLVs expressing HTI but not GFP. However, this response was sub-dominant compared to the immunodominant Gag-9mer induced by HIV-based vector particles. These data are consistent with the results published by *Mothe et al.* (2015) in which vaccination of C57BL/6 mice with DNA or MVA expressing HTI had successfully prevented the strong Gag immunodominance induced by full-length protein immunization. In fact, during natural HIV-1 infection, T cell response against immunodominant epitopes develops rapidly but ineffectively, since leads to a rapid selection of escape mutants, whereas more effective responses directed to sub-dominant conserved epitopes arise later (*Goonetilleke et al., 2009*). In this regard, it has been shown that HIV-1 positive individuals with high reactivity against sub-dominant epitopes were able to control viral load compared with individuals with immune response directed only against immunodominant epitopes (*Frahm et al., 2006*). Importantly, *Im* and coworkers demonstrated that inclusion of these epitopes in the design of a T-cell immunogen induced robust immune responses and protection in BALB/c mice against replicating vaccinia virus expressing the HIVA immunogen (WR.HIVA), used as surrogate challenge virus (*Im et al., 2011*).

Avoiding immune response towards “distracting” epitopes and directing it to more effective targets was also the concept underlying the design of UFO immunogens. In this study, SIV-based IDLV-UFO.664 and IDLV-UFO.750 have been evaluated for their ability of inducing anti-Env Abs in animal models. The use of SIV-derived IDLV was deemed necessary for its subsequent use for immunization protocol in monkeys, since SIV-based IDLV has higher transduction and antigen presentation efficiency in simian primary cells due the presence of viral proteins counteracting species-specific restriction factors, among which the presence of Vpx protein that neutralizes the anti-viral effect of cellular SAMHD1 protein (*Laguette et al., 2011*).

First, we performed *in vitro* evaluation of UFO immunogens expression in our system: we demonstrated that UFO.750 was effectively bound to the cellular membrane and that was exposed in trimeric conformation, since it was specifically recognized by PGT145 and PGDM1440 trimer-restricted Abs (FACS and CLSM analysis). Moreover, western blot on purified preparations of IDLV-UFO.750 and IDLV-UFO.664 confirmed that only membrane

tethered UFO.750 was present on IDLV. To compare IDLV expressing soluble trimers with membrane-tethered trimeric Env, BALB/c mice were vaccinated with escalating doses of IDLVs, to better evaluate differences between the two immunogens at high and low dose of injected IDLV. Overall, mice immunized with IDLV-UFO.750 showed higher levels of anti-Env Abs compared to mice vaccinated with IDLV-UFO.664, regardless to the dose received. This was due to the presence of UFO.750 protein on the surface of IDLV particles that, acting as a virus like particle (VLP), induced specific Abs earlier than IDLV-UFO.664, that can express UFO.664 soluble protein only after entry and transcription of vector genome in the target cells. Indeed, Abs titers in mice vaccinated with the highest IDLV-UFO.664 dose reached levels similar to those induced by IDLV-UFO.750 only between eight and twelve weeks post-injection, but remained stable overtime, whereas Abs induced by the highest doses of IDLV-UFO.750 slowly declined overtime. On the contrary, in the group that received the lowest dose of IDLV-UFO.750, Abs titers remained stable and higher compared to IDLV-UFO.664 mice vaccinated with the same dose of vector. These results are in accordance to data described by a recent report in which ConSOSL-UFO.750 induced higher Abs titers than ConSOSL-UFO.664 in mice and guinea pigs after two immunizations with DNA (*Aldon et al*., *2018*). Importantly, anti-Env binding antibodies were still detectable after one-year post-immunization in all groups of our vaccinated mice.

Later, we have performed an immunization protocol in NHPs, in which five cynomolgus macaques were vaccinated once with IDLV-UFO.750. Intramuscular route of administration has been chosen since it has been described that muscle cells can produce Envs exposing quaternary-dependent epitopes targeted by NAbs (*Aldon et al., 2018*). Immunization with IDLV induced specific anti-Env Abs in all vaccinated animals. Average Abs levels slowly declined of less than 0,5 log between week 2 (average peak) and week 37, suggesting that one immunization with IDLV-UFO.750 elicited durable anti-Env Abs. Nevertheless, in order to increase Abs levels, animals were boosted with the same IDLV-UFO.750 pseudotyped with VSV.G from a different serotype. Adopting VSV.G exchange strategy was necessary to avoid prevention of boosting immunization by prime-induced NAbs against VSV.G. In particular, IDLV-UFO.750 used for the first vaccination was pseudotyped with VSV.G from Indiana serotype ($VSV.G_{IN}$), the most commonly used glycoprotein to pseudotype LVs for experimental and clinical applications. On the contrary, VSV.G from Cocal virus ($VSV.G_{CO}$)

has been used for the boost. We selected VSV.$G_{CO}$ over other VSV serotypes since LVs pseudotyped with VSV.$G_{CO}$ are stable, have a broad tropism and can be produced at high titers as well as VSV-$G_{IN}$ LVs (*Trobridge et al., 2010*). Although VSV.$G_{CO}$ sequence shares 71.5% identity with VSV-$G_{IN}$, they are serologically distinct. LVs pseudotyped with VSV.$G_{CO}$ have been only weakly neutralized or not neutralized by cross-reactive anti-VSV.G Abs (*Munis et al., 2018*) and Rhesus macaques immunized with IDLV-Env pseudotyped with VSV.$G_{IN}$ showed low levels of neutralizing Abs against VSV.$G_{CO}$ (*Blasi et al., 2018*), suggesting that VSV.$G_{CO}$ can be used for IDLV pseudotyping in order to achieve effective vaccination with subsequent administrations. Indeed, the boost of NHP with IDLV-UFO.750 pseudotyped with VSV.$G_{CO}$ resulted in 16-fold increase of specific Abs titers 2 weeks post-boost (p.b.). Although, the average magnitude declined by about 7-fold between boost and week 20 p.b. (the last time point tested), Abs levels remained higher than those present at pre-boost levels in all the animals tested. These results suggested that IDLV prime-boost vaccination strategy is useful to maintain higher levels of antibodies.

Moreover, NAbs against clade C tier 1 MW965.26 virus were elicited after the boost, confirming that NAbs induction is related to the overall amount of Abs. Indeed, *Landais et al.* (*2015*) found an association between high titers of anti-Env binding Abs and bNAbs responses in a large cohort of Sub-Saharan HIV-1 primary infected individuals. This correlation seemed to be due to a larger diversity of Abs which exerted cooperative pressure on the virus, limiting the probability of escape and thereby favoring the development of bNAbs, rather than a greater affinity of matured Abs.

Unfortunately, in all our vaccinated animals, no neutralizing activity against relatively neutralization resistant autologous tier 2 ConS virus was detected. Similar results were obtained in previous reports by vaccinations with IDLV, MVA or DNA expressing clade C transmitter/founder 1086 Env that have not been able to induce tier 2 NAbs (*Negri et al., 2016; Fouda et al., 2013*). Our data are in line with results published by *Aldon* and colleagues, that did not induce autologous tier 2 NAbs in rabbits immunized with three administration of ConSOSL.UFO750 expressing DNA and boosted with ConSOSL.UFO664 protein. Hence, modifications in UFO antigens aimed at increasing their stability and immunogenicity were not sufficient for elicitation of NAbs against a broad panel of viral strains in more complex animal model (like NHP) suggesting that further improvements in the design of Env immunogens are still necessary.

Taken together, the findings reported in this thesis provide evidence that IDLVs represent an effective candidate for delivering improved HIV-1 rationally designed antigens, inducing specific and persistent cellular and humoral immune responses, in pre-clinical animal models for the development of HIV-1 vaccines. In particular, SIV-based IDLVs can be exploited to elicit more specific immune response against structural HIV-1 proteins and to evaluate prime/boost vaccine strategies in the NHP model of AIDS in the absence of anti-vector immunity.

# Bibliography


- Abordo-Adesida E, Follenzi A, Barcia C, et al. Stability of lentiviral vector-mediated transgene expression in the brain in the presence of systemic antivector immune responses. Hum Gene Ther 2005; 16:741–751
- Apolonia L, Waddington SN, Fernandes C, et al. Stable gene transfer to muscle using non-integrating lentiviral vectors. Mol Ther 2007; 15: 1947–1954
- Aiken C. Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. J Virol 1997; 71: 5871–5877
- Aldon Y, McKay PF, Allen J, et al. Rational Design of DNA-Expressed Stabilized Native-Like HIV-1 Envelope Trimers. Cell Rep 2018; 24:3324–3338
- Alfadhli A, Dhenub TC, Still A, et al. Analysis of human immunodeficiency virus type 1 Gag dimerization-induced assembly. J Virol 2005; 79:14498–14506. doi:10.1128/JVI.79.23.14498-14506.2005
- Ao Z, Danappa Jayappa K, Wang B, et al. Importin alpha3 interacts with HIV-1 integrase and contributes to HIV-1 nuclear import and replication. J Virol 2010; 84(17):8650-63
- Baba TW, Liska V, Hofmann-Lehmann R, et al. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat Med 2000; 6:200–206
- Barboric M, Peterlin BM. A new paradigm in eukaryotic biology: HIV Tat and the control of transcriptional elongation. PLoS Biol 2005; 3: e76
- Barraza RA, Poeschla EM. Human gene therapy vectors derived from feline lentiviruses. Vet Immunol Immunopathol 2008; 123:23-31
- Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 1983, 220, 868-871
- Bayer M, Kantor B, Cockrell A, et al. A large U3 deletion causes increased in vivo expression from a non integratin lentiviral vector. Mol Ther 2008; 16:1968-1976
- Beignon AS, Mollier K, Liard C, et al. Lentiviral vector-based prime/boost vaccination against AIDS: pilot study shows protection against Simian immunodeficiency virus SIVmac251 challenge in macaques. J Virol 2009; 83:10963–74
- Berger EA, Doms RW, Fenyo EM, et al. A new classification for HIV- 1. Nature 1998; 391:240
- Berger G, Goujon C, Darlix JL,et al. SIVMAC Vpx improves the transduction of dendritic cells with nonintegrative HIV-1-derived vectors. Gene Ther 2009, 16(1):159–163
- Berger G, Durand S, Goujon C, et al. A simple, versatile and efficient method to genetically modify human monocyte-derived dendritic cells with HIV-1-derived lentiviral vectors. Nat Protoc 2011; 6:806–16

- Bieniasz PD.The cell biology of HIV-1 virion genesis. Cell Host Microbe 2009; 5: 550–558
- Biti R, Franch R, Young J, et al. HIV-1 infection in an individual homozygous for the CCR5 deletion allele. Nat Med 1997; 3:252-253
- Blasi M, Negri D, LaBranche S, et al. IDLV-HIV-1 Env vaccination in non-human primates induces affinity maturation of antigen-specific memory B cells. Comm Biology 2018; DOI: 10.1038/s42003-018-0131-6
- Brenchley JM, Price DA, Schacker Tw, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 2006; 12: 1365-1371.
- Brenchley JM, Schacker Tw, Ruff LE, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. j Exp Med 2004; 200:749-759
- Briggs JA, Simon MN, Gross I, et al. The stoichiometry of Gag protein in HIV-1. Nat Struct Mol Biol 2004; 11:672–675
- Brown PO, Bowerman B, Varmus HE, et al. Retroviral integration: structure of the initial covalent product and its precursor, and a role for the viral IN protein. Proc Natl Acad Sci USA 1989; 86:2525-2529
- Brussel A, Sonigo P. Evidence for gene expression by unintegrated human immunodeficiency virus type 1 DNA species. J Virol 2004; 78: 11263–11271
- Bryant M, and Ratner L. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc Natl Acad Sci USA 1990; 87:523–527. Buchbinder SP, Mehrotra DV, Duerr A, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebocontrolled, test-of-concept trial. Lancet 2008; 372:1881–1893
- Buffa V, Negri DMR, Leone P, et al. Evaluation of a Self-Inactivating Lentiviral Vector Expressing Simian Immunodeficiency Virus Gag for Induction of Specific Immune Responses in Vitro and in Vivo. Viral Immun 2006; 4: 690–701
- Bukovsky AA, Song JP, Naldini L. Interaction of human immunodeficiency virus-derived vectors with wild-type virus in transduced cells. J Virol 1999; 73: 7087–7092
- Cara A, Vargas J Jr, Keller M, et al. Circular viral DNA and anomalous junction sequence in PBMC of HIV-infected individuals with no detectable plasma HIV RNA. Virology 2002; 202:1-5
- Cara A, and Reitz MS Jr. New insight on the role of extrachromosomal retroviral DNA. Leukemia 1997; 11:1395–99
- Cesana D, Ranzani M, Volpin M, et al. Uncovering and Dissecting the Genotoxicity of Self-inactivating Lentiviral Vectors In Vivo. Molecular Therapy 2014; 22(4):774-785.
- Chakraborty H, Sen PK, Helms RW, et al. Viral burden in genital secretions determines male-to-female sexual transimission of HIV-1: a probabilistic empiric model. AIDS 2001; 15(5):621-7
- Chakraborty S, Rahman T, Chakravorty R. Characterization of the Protective HIV-1 CTL Epitopes and the Corresponding HLA Class I Alleles: A Step towards Designing CTL Based HIV-1 Vaccine. Adv Vir 2014

- Cheeseman HM, Olejniczak NJ, Rogers PM, et al. Broadly Neutralising Antibodies Display Superior Potential Over Non-Neutralising Antibodies in Preventing HIV-1 infection of Mucosal Tissue. J Virol 2016; doi:10.1128/JVI.01762-16
- Chirmule N, Propert K, Magosin S, et al. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther 1999; 6:1574–1583
- Chong H, Starkey W, Vile RG. A replication-competent retrovirus arising from a split-function packaging cell line was generated by recombination events between the vector, one of the packaging constructs, and endogenous retroviral sequences. J Virol 1998. 0022-538X/98/04.0010 p.2663–2670
- Chun T, Carruth L, Finzi D, et al. Quantification of latent tissue reservoirs and total body viral load in HIV- 1 infection. Nature 1997; 387: 183-188
- Cimarelli A and Darlix JL. Assembling the human immunodeficiency virus type 1. Cell Mol Life Sci 2002; 59, 1166–1184.doi:10.1007/s00018-002-8495-6
- Cimarelli A, Sandin S, Hoglund S, et al. Basic residues in human immunodeficiency virus type 1 nucleocapsid promote virion assembly via interaction with RNA. J Virol 2000; 74: 3046–3057
- Coutant F, Frenkiel MP, Despres P, et al. Protective antiviral immunity conferred by a nonintegrative lentiviral vector based vaccine. PLoS One 2008; 3:e3973
- Cronin J, Zhang XY, Reiser J. Altering the tropism of lentiviral vectors through pseudotyping. Curr Gene Ther 2005; 5:387-98
- Dalgleish AG, Beverly PCL, Clapham PR, et al. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 1984;3 1 2:763-767
- Daniel MD, Kirchhoff F, CZajak SC, et al. Protective effect of a live attenuated SIV vaccine with a deletion in the nef gene. Science 1992; 258(5090):1938-41
- Dean M, Carrington M, Winkler C, et al. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CCR5 structural gene. Science 1996; 273:1856–1862
- Dorfman T, Bukovsky A, Ohagen A, et al. Functional domains of the capsid protein of human immunodeficiency virus type 1. J Virol 1994; 68:8180–8187
- Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol 1998; 72: 8463–8471
- Edinger AL, Hoffman TL, Sharron M, et al. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. J Virol 1998;72(10): 7934-7940
- Egger M, May M, Chene G, et al. Prognosis of HIV- 1 infected patients starting highly active antiretroviral therapy: a collaborative analysis of prospective studies. Lancet 2002; 360: 119-129
- Engelman A. In vivo analysis of retroviral integrase structure and function. Adv Virus Rev 1999; 52: 411-26

- Esposito F, Corona A, Tramontano E. HIV-1 Reverse Transcriptase Still Remains a New Drug Target: Structure, Function, Classical Inhibitors, and New Inhibitors with Innovative Mechanisms of Actions. Mol Bio Intern 2012; 2012:586401
- Farnet CM, Haseltine WA. Circularization of human immunodeficiency virus type 1 DNA in vitro. J Virol 1991; 65: 6942-52
- Feng Y, Broder CC, Kennedy PE, et al. HIV- 1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 1996;272(5263): 872-877
- Finkelshtein D, Werman A, Novick D, et al. LDL receptor and its family serve as the cellular receptor for vescicular stomatitis virus. Proc Natl Acad Sci USA, 2013; 110:7306-11
- Finzi D, Hermankova M, Pierson T, et al. Identification of a reservoir for HIV- 1 in patients on highly active antiretroviral therapy. Science 1997; 278: 1295-1300
- Flexner C. HIV-Protease Inhibitors (Review Article - Drug Therapy). N Engl Med 1998; 33:1281-1292
- Flynn NM, Forthal DN, Harro CD et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis 2005; 191:654–665
- Foster JL, Denial SJ, Temple BR, et al. Mechanisms of HIV-1 Nef function and intracellular signaling. J Neuroime Pharm 2011; 6:230–246
- Fouda GG, Amos JD, Wilks AB, et al. Mucosal immunization of lactating female rhesus monkeys with a transmitted/founder HIV-1 envelope induces strong Env-specific IgA antibody responses in breast milk. J Virol 2013; 87:6986–6999
- Frahm N, Kiepiela P, Adams S, et al. Control of human immunodeficiency virus replication by cytotoxic T lymphocytes targeting subdominant epitopes. Nat Immunol 2006; 7:173–178
- Gallinaro A, Borghi M, Bona R, et al. Integrase Defective Lentiviral Vector as a Vaccine Platform for Delivering Influenza Antigens. Front Immun 2018; 9:171
- Gambotto A, Dworacki G, Cicinnati V, et al. Immunogenicity of enhanced green fluorescent protein (EGFP) in BALB/c mice: identification of an H2-Kd-restricted CTL epitope. Gene Ther 2000; 7(23):2036–40.
- Gray GE, Allen M, Moodie Z, et al. Safety and efficacy of the HVTN 503/Phambili study of a clade-B-based HIV-1 vaccine in South Africa: a double-blind, randomised, placebo-controlled test-of-concept phase 2b study. Lancet Infect Dis 2011; 11:507–515
- Gibbs JS, Regier DA, Desrosiers RC. Construction and in vitro properties of SIVmac mutants with deletions in nonessential genes. AIDS Res Hum Retroviruses 1994; 10:607–16
- Gilbert PB, Peterson ML, Follmann D et al. Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. J Infect Dis 2005; 191: 666–677
- Goonetilleke N, Liu MK, Salazar-Gonzalez JF, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. J Exp Med 2009; 206:1253–1272

- Grasso F, Negri DMR, Mochi S, et al. Successful therapeutic vaccination with integrase defective lentiviral vector expressing nononcogenic human papillomavirus E7 protein. Int J Cancer 2012; 132: 335–344
- Gulick RM, Lalezari J, Goodrich J, et al. Maraviroc for previously treated patients with R5 HIV-1 infection. N Engl Med 2008; 359:1429-1441
- Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 2003; 348: 255–256
- Haynes BF, Gilbert PB, McElrath MJ, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med 2012; 366: 1275–1286
- Higashimoto T, Urbinati F, Perumbeti A, et al. The woodchuck hepatitis virus post-transcriptional regulatory element reduces readthrough transcription from retroviral vectors. Gene Therapy 2007; 14:1298–1304
- Hraber P, Seaman MS, Bailer RT, et al. Prevalence of broadly neutralizing antibody responses during chronic HIV-1 infection. AIDS. 2014; 28:163–169
- Hrecka K, Hao C, Gierszewska M, et al. Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. Nature 2011, 474(7353):658–661
- Ho DD, Neumann AU, Perelson AS, et al. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 1995; 373(6510):123–126
- Hogg RS, Yip B, Chan KJ, et al. Rates of disease progression by baseline CD4 count and viral load after initiating triple-drug therapy. JAMA 2001; 286:2568-2577
- Huang, J. et al. Broad and potent HIV-1 neutralization by a human antibody that binds the gp41-gp120 interface. Nature 2014; 515: 138–142
- Hurley JH, Hanson PI. Membrane budding and scission by the ESCRT machinery: It's all in the neck. Nat Rev Mol Cell Biol 2010; 11: 556–566
- Im EJ, Hong JP, Roshorm Y, et al. Protective Efficacy of Serially Up-Ranked Subdominant CD8+ T Cell Epitopes against Virus Challenges. PLoS Pathog 2011; 7(5):e1002041
- Iwakuma T, Cui Y, Chang LJ. Self-inactivating lentiviral vectors with U3 and U5 modifications. Virology 1999; 261: 120–132
- Janes H, Friedrich DP, Krambrink A, et al. Vaccine-Induced Gag-Specific T Cells Are Associated With Reduced Viremia After HIV-1 Infection. J Inf Dis 2013; 208:1231-39
- Jouvenet N, Simon SM et al. Imaging the interaction of HIV-1 genomes and Gag during assembly of individual viral particles. Proc Natl Acad Sci USA 2009; 106:19114–19119
- Julien J-P. et al. (2013) Crystal structure of a soluble cleaved HIV-1 envelope trimer. Science 342, 1477–1483
- Karwacz K, Mukherjee S, Apolonia L, et al. Nonintegrating lentivector vaccines stimulate prolonged T-cell and antibody responses and are effective in tumor therapy. J Virol 2009; 83:3094-103
- Kiepiela P, Ngumbela K, Thobakgale C, et al. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 2007; 13:46–53
- Kim VN, Mitrophanous K, Kingsman SM, et al. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. J Virol 1998; 72:811-816

- Kingsman SM, Mitrophanous K and Olsen JC. Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE). Gene Ther 2005; 12:3-4
- Kong L, He L, de Val N, et al. Uncleaved prefusion-optimized gp140 trimers derived from analysis of HIV-1 envelope metastability. Nature Communications 2016; 7:12040
- Korber BT, Letvin NL, Haynes BF. T-cell vaccine strategies for human immunodeficiency virus, the virus with a thousand faces. J Virol 2009; 83: 8300–8314
- Kovacs JM, Noeldeke, E, Ha HJ, et al. Stable, uncleaved HIV-1 envelope glycoprotein gp140 forms a tightly folded trimer with a native-like structure. Proc Natl Acad Sci USA 2014; 111:18542–18547
- Kumar M, Keller B, Makalou N, et al. Systematic determination of the packaging limit of lentiviral vectors. Hum Gene Ther 2001; 12: 1893–1905
- Kutluay SB, Bieniasz PD. Analysis of the initiating events in HIV-1 particle assembly and genome packaging. PLoS Pathog 2010; 6:e1001200
- Laguette N, Sobhian B, Casartelli N, et al. SAMHD1 is the dendritic and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. Nature 2011, 474(7353):654–657
- Lalezari JP, Henry K, O'Hearn M, et al. Enfuvirtide, an HIV- I fusion inhibitor, for drug-resistant HIV infection in North and South America. N Engl Med 2003; 348:2175-2185
- Landais E, Huang X, Havenar-Daughton C, et al. Broadly Neutralizing Antibody Responses in a Large Longitudinal Sub-Saharan HIV Primary Infection Cohort. PLoS Path 2016; 12(1):e1005369
- Leavitt AD, Robles G, Alesandro N, et al. Human immunodeficiency virus type I integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection. J Virol 1996; 70(2): 721-8
- Lee, J.H. et al. Antibodies to a conformational epitope on gp41 neutralize HIV-1 by destabilizing the Env spike. Nat Commun 2015; 6: 8167
- Liao HX, Sutherland LL, Xia SM, et al. A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. Virology 2006; 353:268-82
- Lobritz MA, Ratcliff AN, Arts EJ. HIV-1 Entry, Inhibitors, and Resistance. Viruses 2010; 2, 1069-1105
- Lyumkis, D. et al. Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. Science 2013; 342: 1484–1490
- Maddon PJ, Dalgleish AG, McDougal JS, et al. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell 1986; 47:333-348
- Mariani R, Chen D, Schröfelbauer B, et al. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. Cell 2003, 114(1):21–31
- Maillot B, L.vy N, Eiler S, Crucifix C, et al. Structural and functional role of NI1 and LEDGF in the HIV-1 preintegration complex. PLoS ONE 8(4): e60734
- Masur H, Ognibene FP, Yarchoan R, et al. CD4 counts as predictors of opportunistic pneumonia in human immunodeficiency virus (HIV) infection. Ann Intern Med 1989; 111: 223-231

- Mehandru S, Poles MA, Tenner-Racz K, et al. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. Exp Med 2004; 200:761-770
- Michelini Z, Negri DRM, Baroncelli S, et al. Development and use of SIV-based Integrase defective lentiviral vector for immunization. Vaccine 2009; 27(34): 4622–4629
- Migueles SA, Connors M. Long-term nonprogressive disease among untreated HIV-infected individuals: clinical implications of understanding immune control of HIV. JAMA 2010; 304: 194-201
- Mochizuki H, Schwartz J, Tanaka k, et al. High-Titer Human Immunodeficiency Virus Type 1-Based Vector Systems for Gene Delivery into Nondividing Cells. J of Vir 1998; 72(11): 8873–83
- Molina RP, Matukonis M, Paszkiet B, et al. Mapping of the bovine immunodeficiency virus packaging signal and RRE and incorporation into a minimal gene transfer vector. Virology 2002; 304:10-23
- Montefiori, DC. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol Biol 299; 485: 395–405
- Morita E, Sundquist WI. Retrovirus budding. Annu Rev Cell Dev Biol 2004; 20: 395–425
- Mothe B, Hu X, Llano A, et al. A human immune data-informed vaccine concept elicits strong and broad T-cell specificities associated with HIV-1 control in mice and macaques. J of Transl Med 2015; 13:60
- Mselli-Lakhal L, Guiguen F, Greenland T, et al. Gene transfer system derived from the caprine arthritis-encephalitis lentivirus. J Virol Methods 2006; 136:177-84
- Munis AM, Tijani M, Hassall M, et al. Characterisation of Antibody Interactions with the G Protein of Vesicular Stomatitis Virus Indiana Strain and Other Vesiculovirus G Proteins. J Virol 2018; 00900-18
- Murphy SL, Li H, Mingozzi F, et al. Diverse IgG subclass responses to adeno-associated virus infection and vector administration. J Med Virol 2009; 81: 65–74
- Naldini L, Blomer U, Gage FH,et al. Efficient transfer, integration and sustained longterm expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci USA 1996a; 93: 11382-11388
- Naldini L, Blomer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 1996b; 272: 263–267
- Negre D, Cosset FL. Vectors derived from simian immunodeficiency virus (SIV). Biochimie 2002; 84:1161-71
- Negri DR, Michelini Z, Baroncelli S, et al. Successful immunization with a single injection of non-integrating lentiviral vector. Mol Ther 2007; 15:1716-23
- Negri DRM, Rossi A, Blasi M, et al. Simian immunodeficiency virus-Vpx for improving integrase defective lentiviral vector-based vaccines. Retrovirology 2012, 9:69
- Negri D, Blasi M, LaBranche C, et al. Immunization with an SIV-based IDLV expressing HIV-1 Env 1086 clade C elicits durable humoral and cellular responses in rhesus macaques. Mol Ther 2016

- Negri DRM, Michelini Z, Bona R, et al. Integrase-defective lentiviral vector-based vaccine: a new
  vector for induction of T cell immunity. Expert Opin Biol Ther 2011; 11(6):739-750
- O' Connel RJ, Kim JH, Corey L, et al. Human Immunodeficiency Virus Vaccine Trials. Cold Spring Harb Perspect Med 2012; 2:a007351
- Oh T, Bajwa A, Jia G, Park F. Lentiviral vector design using alternative RNA export elements. Retrovirology 2007; 4: 38
- Pantaleo G, Koup RA. Correlates of immune protection in HIV-1 infection: What we know, what we don't know, what we should know. Nat Med 2004; 10:806–810
- Picker LJ, Hansen SG, Lifson JD. New paradigms for HIV/AIDS vaccine development. Ann Rev Med 2012; 63:95–111
- Pietrangeli B, Fattori E, Biava M, et al. La biosicurezza connessa all'utilizzo di vettori lentivirali nella sperimentazione biotecnologica. INAIL, Settore Ricerca, Certificazione e Verifica, Edizioni Inail, Luglio 2014.
- Pitisuttithum P, Gilbert P, Gurwith M et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J Infect Dis 2006; 194:1661-1671
- Pond SJ, Ridgeway WK, Robertson R, et al. HIV-1 Rev protein assembles on viral RNA one molecule at a time. Proc Natl Acad Sci USA 2009; 106: 1404-1408
- Popovic M, Sarngadharan MG, Read E, et al. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 1984; 224(4648):497-500.
- Porter K, Babiker A, Bhaskaran K, et al. Determinants of survival following HIV-1 seroconversion after the introduction of HAART. Lancet 2003; 362: 1267-1274
- Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 2009; 361: 2209–2220
- Richman DD, Wrin T, Little SJ, et al. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci USA 2003; 100:4144-4149
- Rossi A, Michelini Z, Leone P, et al. Optimization of Mucosal Responses after Intramuscular Immunization with Integrase Defective Lentiviral Vector. PLoS ONE 2014;9(9):e107377
- Rosenberg E, Altfeld M, Poon SH, et al. Immune control of HIV-1 after early treatment of acute infection. Nature 2000; 407:523-526
- Sanders RW, Moore JP. Native-like Env trimers as a platform for HIV-1 vaccine design. Immun Rev 2017; 275: 161–182
- Sarkis C, Philippe S, Mallet J, et al. Non-Integrating lentiviral Vectors. Curr Gene Ther 2008; 8(6): 430-437
- Saenz DT, Loewen N, Peretz M, et al. Unintegrated lentivirus DNA persistence and accessibility to expression in nondividing cells: analysis with class I integrase mutants. J Virol 2004; 78:2906–20
- Schambach A, Bohne J, Baum C, Hermann FG, Egerer L, von Laer D, Giroglou T. Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X

protein and promoter sequences enhances retroviral vector titer and expression. Gene Ther 2006; 13: 641– 645
- Scharf, L. et al. Broadly neutralizing antibody 8ANC195 recognizes closed and open states of HIV-1 Env. Cell 2015; 162: 1379–1390
- Sharkey M, Triques K, Kuritzkes DR, et al. In vivo evidence for instability of episomal human immunodeficiency virus type 1 cDNA. J Virol 2005; 79:5203–5210
- Shiels MS, Pfeiffer RM, Hall HI, et al. Proportions of Kaposi sarcoma, selected non-Hodgkin lymphomas, and cervical cancer in the United States occurring in persons with AIDS, 1980-2007. JAMA 2011; 305:1450-1459
- Sok D, van Gils MJ, Pauthner M, et al. Recombinant HIV envelope trimer selects for quaternary-dependent antibodies targeting the trimer apex. Proc Natl Acad Sci U S A 2014; 111(49):17624-17629
- Spence RA, Kati WM, Anderson KS, et al. Mechanism of inhibition of HIV-I reverse transcriptase by nonnucleoside inhibitors. Science 1995; 267:988-993
- Stremlau M, Owens CM, Perron MJ, et al. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 2004, 427(6977):848–853
- de Taeye SW, Ozorowski G, Torrents de la Peña A, et al. Immunogenicity of stabilized HIV-1 Envelope trimers with reduced exposure of non-neutralizing epitopes. Cell 2015; 163:1702–1715
- Trobridge GD, Wu RA, Hansen M, et al. Cocal-pseudotyped Lentiviral Vectors Resist Inactivation by Human Serum and Efficiently Transduce Primate Hematopoietic Repopulating Cells. Molecular Therapy. 2010;18(4):725-733
- Usami Y, Popov S, Popova E, et al. The ESCRT pathway and HIV-1 budding. Biochem Soc Trans 2009; 37: 181–184
- Vernazza OL, Eron JJ, Fiscus SA, et al. Sexual transmission of HIV: infectiousness and prevention. AIDS 1999; 13(2):155-66
- Walker LM, Huber M, Doores KJ, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature 2011; 477(7365):466-470
- Wanisch K, Yanez-Munoz RJ. Integration-deficient lentiviral vectors: a slow coming of age. Mol Ther 2009; 17(8):1316-32
- Watson DJ, Kobinger GP, Passini MA, et al. Targeted transduction patterns in the mouse brain by lentivirus vectors pseudotyped with VSV, Ebola, Mokola, LCMV, or MuLV envelope proteins. Mol Ther 2002; 5:528-37
- Wei X, Decker JM, Wang S, et al. Antibody neutralization and escape by HIV-1. Nature 2003; 422:307-3 12
- Wiskerchen M, Muesing MA. Human immunodeficiency virus type 1 integrase: effects of mutations on viral ability to integrate, direct viral gene expression from unintegrated viral DNA templates, and sustain viral propagation in primary cells. J Virol 1995; 69: 376–386
- Wiznerowicz M, Trono D. Harnessing HIV for therapy, basic research and biotechnology. Trends Biotec 2005; 23:42-7

- Wu Y, Marsh JW. Early transcription from non-integrated DNA in human immunodeficiency virus infection. J Virol 2003; 77:10376-82
- Wyand MS, Manson K, Montefiori DC, et al. Protection by live, attenuated simian immunodeficiency virus against heterologous challenge. J Virol 1999; 73(10):8356-63
- Yáñez-Muñoz RJ, Balaggan KS, MacNeil A,et al. Effective gene therapy with nonintegrating lentiviral vectors. Nat Med 2006; 12(3): 348-53
- Yasmeen A, Ringe R, Derking R, et al. Differential binding of neutralizing and non-neutralizing antibodies to native-like soluble HIV-1 Env trimers, uncleaved Env proteins, and monomeric subunits. Retrovirology. 2014;11:41.
- Zanta-Boussi MA, Charrier S, Brice-Ouzet A et al., Validation of a mutated PRE sequence allowing high and sustained transgene expression while abrogating WHV-X protein synthesis: application to the gene therapy of WAS. Gene Ther 2009; 16:605-619
- Zhou W, Parent LJ, Wills JW, and el. Identification of a membrane-binding domain within the amino-terminal region of human immunodeficiency virus type 1 Gag protein which interacts with acidic phospholipids. J Virol 1994; 68:2556–2569
- Zuffrey R, Donello JE, Trono D et al. Woodchuck hepatitis virus post-transcriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol 1999; 73: 2886-2892
- Zufferey R, Nagy D, Mandel RJ, et al. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotech 1997; 15:871-875
- Zuñiga R, Lucchetti A, Galvan P, et al. Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J Virol 2006; 80:3122–5