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HIV-1 Glycoprotein Immunogenicity

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1. Introduction

The Human Immunodeficiency Virus type 1 (HIV-1) is a C-type enveloped retrovirus (classification based on morphology of retroviruses electron microscopy) of the *Retroviridae* family. It's consisting in a genome of 2 positive stranded RNA molecules and a specific polymerase reverse transcriptase enzyme (RT). The viral RNA is reverse transcribed by RT into a double-stranded DNA molecule which is then integrated into the genome of infected cells (figure 1). The virus is wrapped by a bilayer membrane derived from the host cell. Homotrimers of the viral glycoprotein gp160 are inserted in the envelope (figure 1). Several cellular proteins are also incorporated into the viral membrane with relative abundance, including MHC class I and II molecules and intracellular adhesion molecule. Lentiviruses, to which HIV belongs, cause disease with a 'slow' evolution preceded by a period of clinical latency. Apart from humans, lentiviruses infect several other species of mammals such as

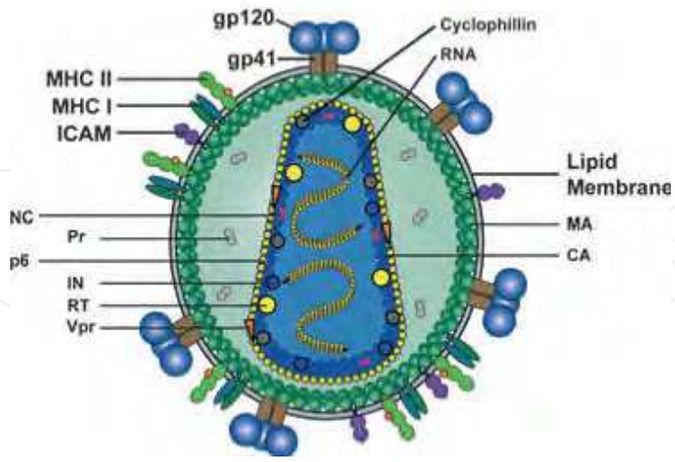

Fig. 1. Schematic representation of the HIV-1 particle. Two copies of the viral positive stranded RNA genome are packed in the capsid. Viral enzymes: reverse transcriptase (RT), integrase (IN), protease (Pr) and structural proteins; capsid (CA), nucleocapsid (NC), matrix (MA) and p6 are inside the particle together with the viral regulatory protein Vpr and the cellular protein cyclophilin (Phogat et al., 2007).

primates (Simian Immunodeficiency Virus, SIV), feline and ovine. Infection by HIV-1 causes a very rapid destruction of the majority of T cells memory $CD4^+/CCR5^+$ and especially those residing in the intestinal mucosa.

Although antiretroviral therapy for HIV infection prevents AIDS-related complications and prolongs life, it does not fully restore health. Long-term treated patients remain at higher than expected risk for a number of complications typically associated with aging, including cardiovascular disease, cancer, osteoporosis, and other end-organ diseases. The potential effect of HIV on health is perhaps most clearly exhibited by a number of immunologic abnormalities that persist despite effective suppression of HIV replication. These changes are consistent with some of the changes to the adaptive immune system that are seen in the very old ("immunosenescence") and that are likely related in part to persistent inflammation. HIV-associated inflammation and immunosenescence have been implicated as causally related to the premature onset of other end-organ diseases.

This review summarizes the importance to generate immune responses against HIV-1 glycoprotein in further vaccinal approaches and detail the complexity of the envelope structure and the relative high HIV-1 glycoprotein immunogenicity.

2. Molecular basis of HIV envelope glycoprotein

2.1 HIV-1 envelope glycoprotein

The gp120 (SU) and gp41 (TM) envelope glycoproteins are encoded by the HIV gene *env*. This gene codes for an Env precursor polyprotein (p90) which undergoes post-transcriptional modifications as glycosylations resulting in the gp160 precursor. It is interesting to note that the HIV cleavage sequence is highly conserved (Veronese et al., 1985) and is included in one of the most conserved domains within gp120 (C5). Gp160 is produced as an inactive precursor in the rough endoplasmic reticulum and subjected to extensive N-glycosylation resulting in high mannose chains linked to Asn residues at either Asn-X-Ser or Asn-X-Thr glycosylation sites. The gp160 are sorted through the constitutive secretory pathway of the infected cells (Moulard and Decroly, 2000). Once transported to the cellular plasma membrane, trimeric gp120–gp41 complexes are incorporated into budding virus for the release of new infectious particles. Before its anchoring on the surface, gp160 is cleaved by a host protease named furine in two sub-units associated by non-covalent interactions (gp120 on the surface and the transmembrane gp 41) (Hallenberger et al., 1992). Envelope glycoproteins form trimeric spicules of gp120-gp41 complexes on the surface of the virus conferring viral tropism (Chan and Kim, 1998). Env is also involved in the interaction, recognition and the fusion of the viral and cellular membranes leading to the introduction of the viral genome into the cytoplasm of the target cells. The Env spikes are thought to be trimeric and structure-based models have been proposed (figures 2&3). Cryoelectronic microscopy tomography shows heterotrimeric complexes (Roux and Taylor, 2007; Briggs et al., 2009). However, despite intensive efforts, the arrangement and orientation of the loop-deleted gp120 core atomic structures within a native Env spike and their association with the gp41 subunits have remained largely speculative.

The entry of virus into the target cell involves the fusion of cell membranes and the viral envelope. Whereas, the recognition of the target cell by the gp120 is not sufficient to directly cause membrane fusion and mediate the injection of viral genome. The adsorption of HIV-1 on cell surface is mediated by interactions between envelope glycoproteins and two types of surface molecules: attachment factors (or adhesion receptors) and coreceptors (figure2).

Coreceptors are actively involved in the penetration of the virus and are selective for the entry of the virion into the host cells depending on their tropism.

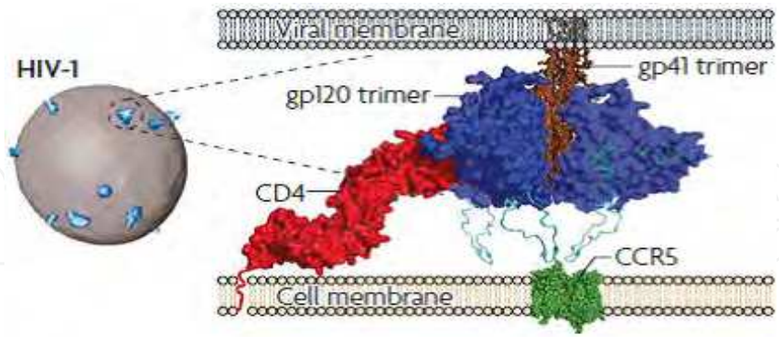

Fig. 2. Schematic representation of the interaction between envelope glycoprotein and viral receptors. On the left, a surface rendered cryo-electron microscopy tomographic image of HIV-1. Viral spikes are blue and the viral surface is grey. On the right is shown a structure-based model of the envelope glycoproteins of HIV. Trimeric HIV‑1 gp120 proteins shown in cyan bind to the primary receptor, CD4. Following conformational changes, the gp120–CD4 complex binds the chemotaxis receptor adapted from (Karlsson Hedestam et al., 2008).

2.2 Gp120 envelope glycoprotein

Gp 120 glycoprotein represents the external part of the envelope glycoprotein of HIV with a molecular weight of 120 kDa. It contains 480aa with 9 disulfide bridges and 20 to 24 N-glycosylation sites. Gp120 is involved in the earlier steps of the infection of target cells. HIV‑1-receptor binding is mediated by external Env gp120, which binds the CD4 primary on target cells. Then, a series of Env conformational changes occur that result in exposure of a transient binding site allowing the virus to interact with its co-receptor, usually the chemokine receptor CCR5 or CXCR4, to initiate another cycle of infection (figure 3A, B). HIV-1 gp120 consists of five conserved (C1-C5) and five extremely variable (V1-V5) domains. The conserved domains contribute to the core of gp120, while the variable domains (and numerous N-linked glycosylation sites) are located near the surface of the molecule. The gp120 is extensively glycosylated with N-linked glycan host derived carbohydrates. The role of these modification is to mask them and their micro-heterogeneities form a perfect immunological shield that allow a decrease of the immune response against viral particles (Pancera et al., 2010) The presence of glycosylation sites in the V3 region seems to affect the viral tropism. Removal of the glycosylation site at position 301 allows the chimeric virus to evolve to X4 phenotype (Pollakis et al., 2001). The removal of three amino acids on different glycosylation sites of gp120 from the laboratory strain X4R5 HIV89.6 tropism results in the loss of tropism for CCR5 (Yang et al., 2010). This phenomenon could be due to the unmasking of neighboring basic amino acids or the loss of negative charges located on the glycoprotein. Moreover, the number of glycosylations present on gp120 influences its secondary structure, which could alter the interactions between the V3 region and neighboring regions (figure 3F). The ability of some strains to be infectious and replicate even in the absence of V3 loop has been described (Cormier & Dragic, 2002)(Lin et al., 2007). The V3 region (35 amino acids) plays a significant role in the recognition and interaction with the cellular receptors of the target cell. This region also

contains at its extremity a highly conserved sequence of amino acids (GPGRAF). This pattern is surrounded by variable amino acids which alter the viral tropism through interaction of gp120 with the cellular chemokine CCR5 and CXCR4. The V1-V5 regions form exposed loops anchored at their bases by disulfide bonds (Teixeira et al., 2011). Crystal structure of gp120 shows that this protein is folded as a connection of four β elements called "bridging sheet"with an inner and an outer domain (Kwong et al., 1998). The internal domain is a near point of contact with gp41 and the N and C-Term of gp120. In the native conformation, trimers are more stable due to the interaction with gp41 and V1-V2 loops. Oligomeric gp120 protein can adopt a conformation called 'closed' as in primary isolates resulting in masking the receptor binding site of CD4 in V1 and V2 regions while gp120 of TCLA viruses preferentially adopt an 'open' shape thereby reducing its affinity for this receptor. Finally the interaction with CD4 gives rise to an open structure enabling the link of viral and cellular membrane and an exposition of the V3 loop (Roux & Taylor, 2007).

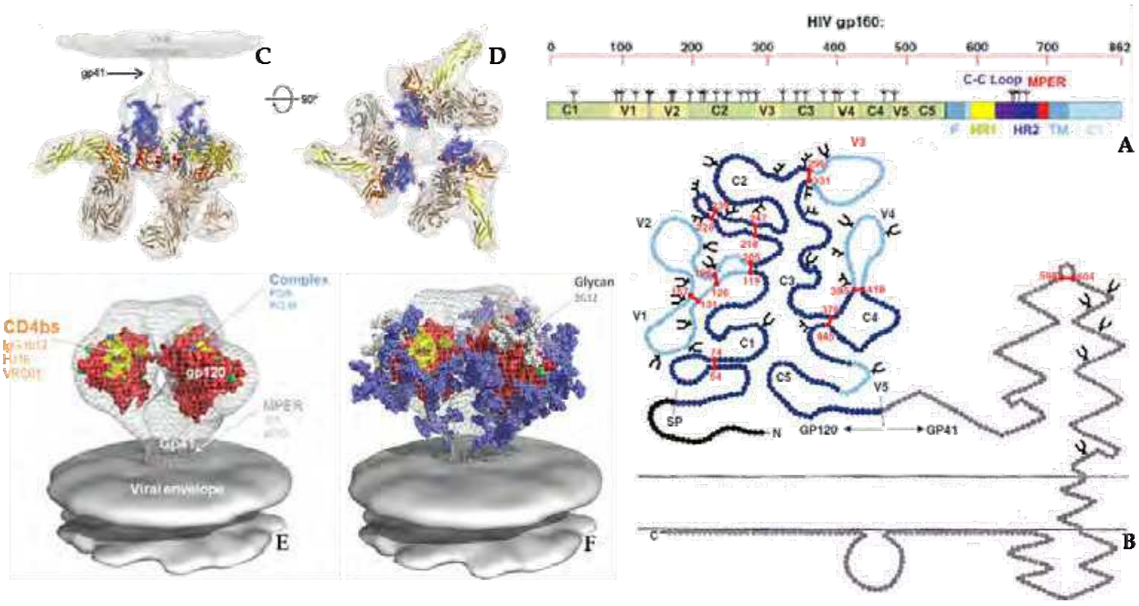

Fig. 3. Schematic representations of HIV-1 envelope glycoprotein A) Glycosylations sites present on gp160 glycoprotein B) Structure of the gp120-gp41 glycoprotein envelope of HIV (Pancera, 2005). C, D, E, F are obtained by cryo-electron tomography C) Structure of an HIV-1 gp120 core with intact gp41-interactive region D) 90° rotation of C placement of the gp120-CD4 complex in the electron density map (light gray), residues involved in gp41 interactions are colored with blue (Pancera et al., 2010) E) Schematic representation of HIV-1 native viral spike. The glycoprotein is shown as a transparent framework and represents three gp120 molecules non-covalently linked to three gp41. Epitopes of broadly neutralizing monoclonal antibodies on gp120 (red) or gp41 are labelled with arrows. The IgG1b12-binding surface is labelled yellow, and the bases of the variable regions (missing in this structure) are labelled blue, green, and pink. (F) The same model with the gp120 glycans shown in blue reveals the extensive glycosylation present as an antibody evasion mechanism on Env, and glycans implicated in BNMAb 2G12 binding are highlighted in white and labelled with an arrow. HIV-1, human immunodeficiency virus type-1; MPER membrane-proximal external region patterns obtained from the works of (Sattentau & McMichael, 2010).

CD4 receptor binding site

The main receptor of HIV is the CD4 membrane glycoprotein. Binding to CD4 initiates the process of infection. Different models forecast the exact configuration of some regions of the gp120 protein whereas they all show clearly the important structural differences in the organization of the trimer between the free and the associated form with CD4. The CD4 molecule binds with high affinity to gp120 protein (Klatzmann et al., 1984). The CD4 binding site is composed of relatively conserved regions located between the two domains of gp120, and the β inter-domains sheet. The contact areas are discontinuous in the native three-dimensional structure of protein. The CD4 binding domain is located in the D1 domain of the CDR2 loop (Complementarity Determining Region 2), on the opposite side of HLA type II binding domain (Fleury et al., 1991). In this area, two residues, Phe43 and Arg59, are particularly important in establishing interactions with gp120 residues of the CD4 binding site (including Glu^{370}, Trp^{427} and Asp^{368}) (Tachibana et al., 2000). The affinity of CD4 to the envelope glycoprotein of HIV is highly variable depending on its quaternary structure (monomeric or oligomeric) and tropism. In addition, gp120 is conformationally flexible, the site of initial CD4 attachment is conformationally inert (Zanetti et al., 2006; Zhu et al., 2006; Roux & Taylor, 2007).

Co-receptors of HIV

The two main HIV co-receptors are the chemokine receptors CXCR4 and CCR5 (figure7). CXCR4 and CCR5 are ubiquitously expressed in immune cells. CXCR4 and CCR5 belong to the superfamily of receptors linked to G proteins and are characterized by a structure comprising an extracellular N-Term segment, seven transmembrane domains in helices, three extracellular loops or ECL, three intracytoplasmic loops or ICL (intracellular loop) and an intracytoplasmic C-Term segment. The N-Term segment and extracellular loops are responsible for receptor affinity for its ligand (Allen et al., 2007).
The natural ligand for CXCR4 is the SDF-1 molecule (Stromal Derived Factor-1), a chemokine α of CXC type (presence of two cysteines separated by one amino acid). As a ligand, it induces internalization of CXCR4 by a process of vesicle clathrin-dependent endocytosis, resulting to its degradation. CCR5 is the receptor of three chemokine β of CC-type (with two adjacent cysteines), all produced by CD8 T cells: RANTES (Regulated upon Activation, Normal T-cell Expressed and secreted), MIP-1α and β (Macrophage Inflammatory Protein-1). RANTES induces CCR5 internalization and is recycled to the cell membrane without being degraded. According to the coreceptor involved in HIV entry into the target cell, the virus is classified depending on its tropism. Thus, viruses use the CCR5 receptor (expressed by monocytes, macrophages, activated T cells or memories and DC) are called 'CCR5-tropic' (or virus type 'R5') and those using the CXCR4 coreceptor (expressed by T cells, monocytes and DCs) are appointed, CXCR4-tropic '(or virus 'X4'). CXCR4 is the coreceptor identified as responsible for the entry of HIV in the lymphoid lineages adapted X4 viruses are also called 'T-tropic' or 'lymphocytotropic', whereas R5 viruses are called 'M-tropic' or 'monocytotropic'. Some isolates are capable of using both CXCR4 and CCR5 coreceptor and are referred as' X4R5' or ‘dual-tropic'. This classification has replaced an old classification based on the ability of the virus or not to induce syncytium formation (a reaction resulting in the cytopathic membrane fusion of several adjacent cells leading to the formation of a 'giant cell' multinucleate) at a T cell line (MT-2). It is now accepted that viruses using the CXCR4 coreceptor to enter target cells induce the formation of syncytia (SI virus like 'Syncytium Inducing virus') in contrast to viruses using exclusively CCR5 (NSI viruses for 'No Syncytium Inducing virus').

2.3 Alternative receptors

However, cells which not express the CD4 molecule on their surface can also be infected, at least *in vitro*. The CD4 is not the only molecule favoring infection by HIV-1.

2.3.1 Interaction with Galactosyl-ceramide (α-GalCer)

Surface molecules such as the α-GalCer glycolipid or lactosylceramide sulfate, present on different tissues (brain, intestine, epithelium and cervix) are capable of binding to gp160 protein V3 loop. These molecules can be considered as alternative receptors for HIV. The α-GalCer is a glycosphingolipid (GSL) most commonly involved in the interactions between cells lacking CD4 and HIV. It binds to the V3 region of gp120 (Harouse et al., 1995). These sequences form a conformational site exposed on the gp120 surface which interact with the galactose residue of GalCer. The affinity of gp120 to GalCer was higher than for other GSL (Long et al., 1994). Saturating the V3 area of gp120 with GalCer analogs can prevent the infection of $CD4^+$ T cells and $CD4^-$ by HIV (Fantini et al., 1997). GalCer is also capable of binding to gp41 domain located in a 35 amino acids region containing the ELDKWA epitope (Alfsen and Bomsel, 2002). This interaction takes place only after fixation on gp120 and GalCer stabilization. The α-GalCer/gp120 or gp41 interaction is also essential for viral particles transmission during endocytosis or transcytosis. The GSL are constituents of the lipid bilayer of the cell membrane and are associated with glycophospholipides (LPG) and cholesterol. This association form within the membrane microdomains distinct called “lipid rafts” (or 'rafts') (Simons and Ikonen, 1997). These lipid rafts traffic areas are preferred across the cell membrane for various pathogens or their toxins that use protein or lipid components of these micro-domains like receptors (Van der Goot & Harder, 2001). Lipid rafts are involved in the infectious cycle of HIV, particularly for the entry into target cells (Campbell et al., 2001). In the case of CD4-dependent infection and after fixation of the gp120 on CD4, an interaction between the central portion of the V3 region and the GSL would be essential to the establishment of the association between gp120 and coreceptors (Nehete et al., 2002). GSL present in lipid rafts can stabilize the attachment of the virus to the cell surface and to ensure migration of gp120-CD4 complex to a co-receptor. Rafts can move within the membrane and facilitate conformational change of gp120 leading to the insertion of the fusion peptide.

2.3.2 Interaction with C-type lectins

C-type lectins (CLR, C-type Lectin Receptor) are proteins that attach, with a calcium-dependent way, the carbohydrated residues through their CRD domain (Carbohydrate Recognition Domain). They are particularly expressed on dendritic cells where they play a key role in capturing pathogens by binding carbohydrates present on micro-organisms (Weis et al., 1998). There are two categories of CLR: CLR's type I which exposed their N-Term part outside the cell and the type II CLR with intracellular N-Term domain. The majority of CLR binds D-mannose, D-glucose or D-galactose and their derivatives. Four CLR type lectins have the ability to bind to gp120: MMR, MBL, DC-SIGN and Langerin.

Lectin MMR (Macrophage Mannose Receptor, or CD206) is highly expressed by dendritic cells generated *in vitro* from monocytes but not expressed by those of blood. Macrophages are capable of transferring HIV to cells expressing CD4 using this receptor (Nguyen and Hildreth, 2003).

MBL (Mannose Binding Lectin) is a CLR type I present in serum as a soluble form where it can bind to pathogens and initiate their attack by the complement system. It is capable of

binding to HIV through highly mannosylated oligosaccharides residues contains in the gp120 (Saifuddin et al., 2000).
The CLR type II DC-SIGN (Dendritic Cell-Specific ICAM-3 Grabbing Non-integrin, or CD209), is expressed as tetramers in lipid rafts of immature dendritic cells (Cambi et al., 2004). Its natural ligand is ICAM-3 (intercellular adhesion molecule-3 or CD50) also present on T cells which allow its interaction with dendritic cells (Geijtenbeek et al., 2000a; Geijtenbeek et al., 2000b). Like MBL, DC-SIGN binds mannose residues embedded into more internal patterns and more complex than MMR. DC-SIGN binds to Gp120 from many strains of HIV and SIV which harbor many oligosaccharides rich in mannose residues (Geijtenbeek et al., 2000b). Finally, Langerin (or CD207) is a CLR type receptor specifically expressed by Langerhans cells. Langerin is responsible for the formation of Birbeck granules (Valladeau et al., 2000) and contains a specific region which interacts with gp120 (Turville et al., 2002).

2.3.3 Interaction with the α4β7 integrin

It was recently demonstrated that gp120 activated form is also able to bind α4β7 integrin expressed on gut-homing T and B lymphocytes and $CD4^+$ NK cells (figure4). A tri-peptide loop (Leu-Asp-Val) at the V2 loop, mimicking the binding motif of the natural ligands of α4β7, is involved in this interaction. This binding enables the formation of viral synapse through the activation of LFA-1 (Arthos et al., 2008). By this way, HIV-1 induces a massive depletion of gut $CD4^+$ T cells which participate to the immune dysfunction in HIV patients.

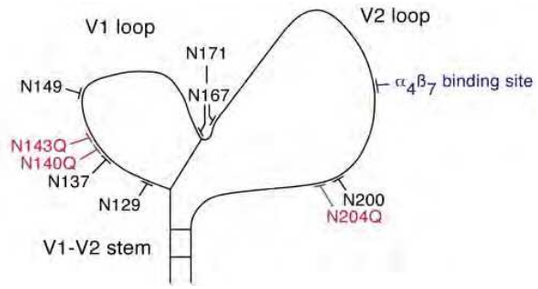

Fig. 4. Positions of all potential N-Glycosylation and α4β7 binding sites located in the V1/V2 loop gp120 (Nawaz et al., 2011).

2.4 Gp41 envelope glycoprotein

The binding of gp 120 to CD4 and coreceptors permits its conformational changes and triggers the establishment of the fusion complex compounds by trimers of gp41 (figure6). (Salzwedel et al., 1999, Munoz-Barroso et al., 1999, Dwyer et al., 2003). Gp41 is the transmembrane domain of the entire envelope glycoprotein which anchors the viral spikes in the bilayer lipid membrane of viral particles and plays an important role in membrane fusion and cell entry. Gp41 is composed by 345 amino acids (512 to 856 of HIV-1 HXB2 strain) with a molecular mass of about 41KDa. Gp41 presents an extracellular domain or ectodomain, a transmembrane domain or membrane spanning domain, and an intracytoplasmic domain (figure5). Gp41 sequences are clearly more conserved than gp120 and also contain only four N-glycosylation sites on its ectodomain. These glycosylation sites are highly conserved and appeared to contribute to optimal viral replication efficiency (Johnson et al., 2001).

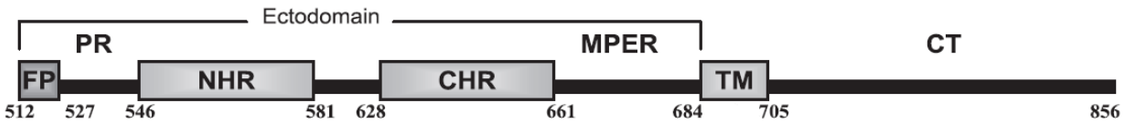

Fig. 5. Schematic representation of gp41 domains. Gp41 is divided in 3 regions. The ectodomain contains the fusion peptide (FP), a polar region (PR), N and C-terminal heptad repeat regions separated by an immunodominant region (NHR & CHR), a rich tryptophane highly conserved region (MPER), a transmenbrane region (TM) and an intracytoplasmic tail (CT) (Montero et al., 2008).

Gp41 contains different domains involved in the fusion process of viral and host cell membrane. The N-Term hydrophobic region consist in the fusion peptide (512–527), a polar region (525-543) called the Fusion Peptide Proximal Region (FPPR) or Polar Region (PR), the N-Term heptad repeat (NHR or HR1) (546– 581) and C-Term heptad repeat (CHR or HR2). These regions are folded as α-helix and linked by a loop called immunodominant loop (598-604) containing a disulfide bridge. A highly conserved Tryptophan Rich Membrane Proximal Ectodomain/External Region (MPER) (660-683) is also present (Chan et al., 1997). Finally, the membrane spanning domain or transmembrane (MSD/TM) and the intracytoplasmic tail or C-tail are two other hydrophobic regions present at the C-Term portion of gp41 (figure5). A complete description of the whole molecule gp41 is not clearly defined. A crystallized intact trimer would be structurally definitive, but it's seems that large regions appear to be in constant motion as part of the conformational masking defence of potential epitopes from Abs. It is widely assumed that the failure of Env-based vaccine candidates to date relates, in part, to the difficulty in generating soluble versions of Env proteins that faithfully mimic key structural features of native *in situ* Env.

2.4.1 Fusion peptide (FP)

The FP corresponds to the first N-Term domain of the gp41 ectodomain (figure5). FP is highly conserved among HIV-1 clades and other viruses. For example, GFLG is a pattern found among fusion peptides of different retroviruses such as HIV-1, HIV-2, SIV and HBV (Durell et al., 1997). A mutation in the GFLG alters the fusogenicity of the FP (Pritsker et al., 1999). Other studies of HIV with truncated or mutated FP showed that it's crucial to the fusion process and viral entry into host cells (Qiang et al., 2009).

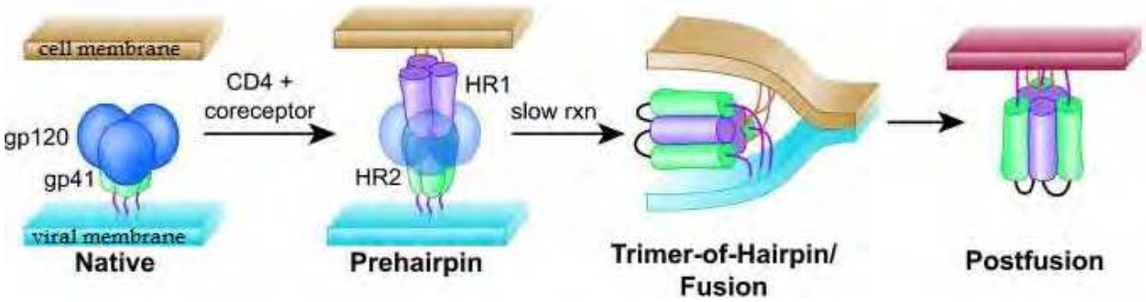

Fig. 6. Model of HIV entry pathway and gp41 conformational intermediates. Gp41 HR1 and HR2 regions are depicted in magenta and green, respectively (Miller et al., 2005).

FP is mostly hydrophobic (Del Angel et al., 2002). The substitution of glycine residues results in a fusogenicity decrease (Vanlandschoot et al., 1998). It has been suggested that the succession of FP glycines is involved in the oligomerization of fusion peptides, in the balance of amphipathicity necessary for membrane fusion and/or orientation of fusion peptides in the membrane (Delahunty et al., 1996). Recent studies showed that FP can also change its conformation according to the biochemical environment ((Buzon et al., 2005; Gordon et al., 2008). Solid-state nuclear magnetic resonance (MNR) spectroscopy (Zheng et al., 2006) confirmed that the FP oscillates from an α-helical state in low concentration of cholesterol to the β-strand form what reveals a high playing a crucial role during the viral fusion process. There are also controversies over the functional structure and the size of the FP it could have a different length and by the way a different sequence as 16 aa (Kamath and Wong, 2002), 23 aa (Delahunty et al., 1996; Gordon et al., 2002) or 33 aa (Pritsker et al., 1999) . To date the native structure in prefusogenic and fusogenic state remains unravelled.

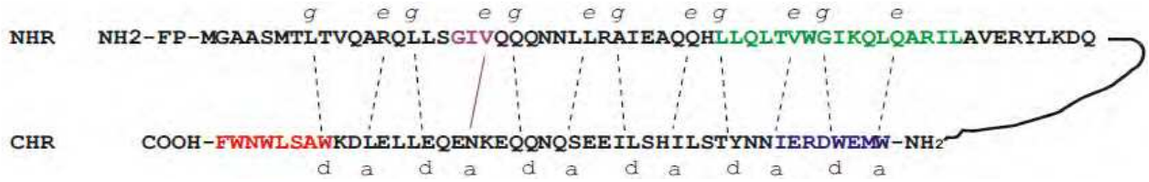

Fig. 7. Interaction between critical sequences in NHR and CHR regions of gp41 fusion intermediate. Dashed lines between the NHR and CHR domains indicate the interaction between the residues located at the e, g and a, d positions in the helical spiral of NHR and CHR domains, respectively. The critical sequences in NHR and CHR include: GIV (residues 547–549, *red*) is a determinant of resistance to T-20 in NHR region, LLQLTVWGIKQLQARIL (residues 565–581, *green*) is the cavity-forming sequence in NHR region, WMEWDREI (residues 628–635, *orange*) is the cavity binding sequence in CHR region; and WASLWNWF (residues 666–673, *pink*) is a partial tryptophan-rich sequence (Pan et al., 2010).

2.4.2 N-heptad repeat (NHR) / C-heptad repeat (CHR) regions

Adjacent to the FP is the first of two HR's (N and C-HR, respectively) that play critical roles in the fusion activity of gp41 (figures5, 6). HR motifs contain a characteristic repeating pattern of seven residues (abcdefg). Amino acid sequence of these segments is composed of seven AA which occupy seven positions on the fusogenic structure forming a pattern. This pattern repeats 7 times hence the name seven-repeat (heptad repeat). In the CHR, AA in position "a" and "d" are generally apolar or hydrophobic and are crucial for stabilizing trimers (figures7, 8). The "e" and "g" residues in NHR frequently interact with residues in the "a" and "d" positions. Characteristic packing of the hydrophobic side chains as the HR in a helical configuration stabilizes the coiled-coil structures N-HR (aa 541-581). Two binding sites have been characterized which bind the CHR region (aa 610-683) through the residues "g" and "e" or the endogenous homotrimeric NHR region through the residues "a" and "d" (Bewley et al., 2002).

A five-amino acid hydrophilic loop containing two cysteine residues with an intramolecular bisulfure bridge links the two HR regions together. These cysteines are highly conserved among retroviruses. It has been proposed that this loop creates a 'knob-like' protrusion in the TM subunit that permits packing in a cavity 'socket' in the surface subunit (figure6). This region is disordered in high-resolution studies of the gp41 ectodomain and has been

reported to lead to aggregation of the protein (Caffrey et al., 2000). Structures of pre-fusion forms of gp41 are undetermined. Nevertheless the crystal structure of gp41 and X-ray and NMR structures determines its 6Helix Bundle and final states this studies revealed that three NHR are folded into a central triple-stranded coiled coil of α-helices, and three CHR are packed, anti-parallel, as α-helix into the three channels of the coiled-coil (figures6, 7, 8A) (Chan et al., 1997). The inner-helical NHR trimer in the 6HB structures has been used to model the receptor-activated state of gp41 and to design several mimetics of the NHR coiled-coil that can potently inhibit fusion like the T20 or N36 peptides. This 6HB is thermally stable and can confidently be considered a final structure. The formation of 6HBs was originally accepted as being the process that brings the viral and the cellular membranes together and allows the aggregation of several activated Env complexes to form a pore, leading to the entry of the nucleocapsid into the cell (Markosyan et al., 2009).

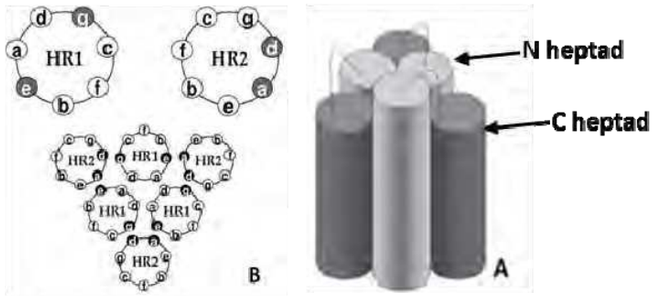

Fig. 8. Schematic representations of the six-helix bundle (6HB) in gp41 at the fusogenic state. A) Description of the six helix bundle: describes the N-(bright) and C-(dusky) heptad repeats that fold in an anti-parallel shape (hairpin). Three monomers of NHR form a α-helix trimer in the central triple-stranded coiled coil and the C-HR region comes to bind the grooves of this structure to form the final state of 6HB. The link between the two heptad repeat regions is involved in the aggregation of this complex. (Weiss, 2003) B) Overview of depiction of a 6 helix bundle. Three NHR from each gp41 monomer form grooves and to complete the bundle three HR2 dock in them (Markosyan et al., 2009).

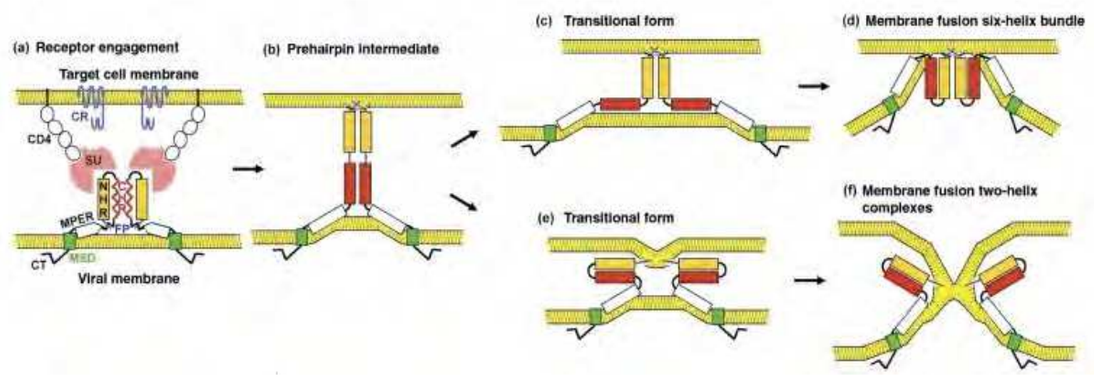

Fig. 9. Schematic speculative representation of topological questions related to single-spike-mediated fusion mechanisms. a,b) gp120 interacts with CD4 then with a co-receptor CR. The binding of the fusion peptide segment allows a changing of conformation and the HR regions form pre-hairpin shapes. c, d) Model where conformational changes undergo easily but require membrane breaks to form the 6HB. E, f), Second model where the membranes remain intact during the folding of gp41. CHR and NHR helix bind together and form the fusion pore. NHR, gold; CHR, red (Roux & Taylor, 2007).

2.4.3 The Membrane Proximal Ectodomain/external Region (MPER)

The MPER segment or Membrane-Proximal External Region is bordered by HR2 region and TM segment of gp41 (figures5, 9). This region is rich in tryptophan residues and interacts with cholesterol in the lipid bilayer cell membrane playing an important role in the viral fusion process and infectivity of HIV-1 (Salzwedel et al., 1999). MPER seems to be involved in the early steps of viral fusion and syncytium formation (Munoz-Barroso et al., 1999).
Mutations in MPER induce a decrease of the virus ability to cause syncytium formation in infected cells due to the inability to form the fusion complex (Zwick et al., 2001; Veiga and Castanho, 2007). MPER is highly conserved and represents a major target for therapeutic approaches. MPER sequence (^{663}ELDKWASLWNWFNITNWLWYIK^{683}) is the same in most HIV-1 strains and includes epitopes of 3 well described human broadly neutralizing monoclonal Abs as (ELDKWA) for the 2F5 and WF(N/D)IT for 4E10 and Z13 (figure12)(Ofek et al., 2004; Zwick and Burton, 2007). MPER has also been described as α-helix or as an extenteded β-turn. Recent studies have revealed that MPER adopts a helical form that is kinked and somewhat L-shaped and the segment that contains the epitope of 4E10 is embedded within the bilayer lipid membrane (Sun et al., 2008; Franquelim et al., 2011). The structure of the MPER in the context of lipids is well known, but the question of its native structure(s) on the cell surface in the context of gp41 remains open.

2.4.4 Transmembrane (TM) or Membrane spanning domain (MSD)

The TM region or MSD contains 25aa (aa681–705) is surrounded at its N-Term side by MPER and by the cytoplasmic domain at its C-Term tail (figures5, 9). This region is encrusted in the viral bilayer lipid membrane and anchors the gp41 into the membrane. This highly conserved region is composed mostly of neutral and hydrophobic AA (figure10). Two models have been proposed to explain the conformation assumed by MSD: a stable sloping α-helical conformation and a metastable kinked curved conformation and the MSD seem to switches from one to another while conformational changes of the gp41 (Gangupomu and Abrams, 2010).

Fig. 10. AA sequence of HIV-1 membrane spanning domains: The consensus sequence of the HIV-1 MSD was generated by the alignment of the Env MSD sequences of all M and N groups HIV-1 isolates from the Los Alamos HIV database. The graphic shows the percentage of conserved residues among isolates from (Shang et al., 2008).

The MSD contains two common features of lentiviral MSD, a basic charged residue Arg^{694} buried in bilayer lipid membrane (Shang et al., 2008) and a very conserved motif ^{688}GXXXG ^{694} . The loss of this feature doesn't affect the formation of glycoprotein Env trimers during the transport from the endoplasmic reticulum to the Golgi apparatus. However, it alters the membrane fusion and viral infectivity such as the loss of Lys^{681}, Arg ^{694} or Arg^{705} (Shang et

al., 2008). The MSD or TM plays a very important role in viral fusion (figure9) (Miyauchi et al., 2010). Truncation studies on HIV-1 Env revealed that only 17 amino acid residues (Lys^{681}-Ala^{697}) are needed for a stable anchoring of the gp41 in the membrane and mediating cell to cell fusion (Yue et al., 2009). Small deletions (three amino acid residues) in the region between Arg^{694} and Arg^{705} showed normal cell to cell fusion, while larger deletions were more deleterious, suggesting that, the length of this region is more important than its AA sequence (Owens et al., 1994). Substitution of all leucine residues or two other highly conserved residues, Phe^{683} with Val^{687} AA residues in the helical core of MSD is critical for the fusogenicity of Env complexes and infectivity (Shang et al., 2008).

2.4.5 The C-Term tail / intra cytoplasmic tail (CT/ICS)

Lentiviruses, including HIV-1, have TM envelope (Env) glycoproteins with cytoplasmic tails (CT) that are quite long compared with those of other retroviruses. The gp41 CT is remarkably long and follows the MSD. CT is a hydrophobic region of 150 AA inserted into the plasma membrane (figure9A, 11) (Viard et al., 2008). The CT contains three regions called lentiviral lytic peptide and form amphipathic helices. The LLP1 and LLP2 regions have high hydrophobic properties. It have been admitted that the CT is entirely contained inside the cell or virion (Gallaher, 1987). Whereas it have been revealed that there are Abs directed to an epitope in the CT and some of them present neutralization properties on HIV virions (Cleveland et al., 2003; McInerney et al., 1999; McLain et al., 2001; Reading et al., 2003). Then, since antibodies do not traverse the membrane and infectious virus are by definition intact, this suggests that part of the tail is exposed on the virion surface allowing antibody binding and neutralization, thus contrasting with the traditional intracytoplasmic location of the entire C-Term sequence of gp41 (Hollier & Dimmock, 2005). Studies have attempted to address this divergence between the old model of an exclusively intracytoplasmic tail and an alternative model with external segments of the CT, as the Kennedy peptide (aa731-752) (Kennedy et al., 1986) containing three patterns a conserved one ^{741}EEEGGE^{746} and two others ^{747}QDRDRS^{752} or ^{731}PRGPDRPGRI^{740}. At the early steps of the viral entry, CT is implicated in the regulating of the kinetics of fusion and in the ability of Env to promote syncytia (Edwards et al., 2002; Wyss et al., 2005). Mutational studies have revealed that long CT interacts with domains of HIV matrix like the p55 Gag protein during the budding and this interaction is required for the envelope incorporation into the virion (Dubay et al., 1992; Yu et al., 1993).

3. Envelope glycoprotein immunogenicity

HIV -1 patients generate strong Ab responses to the viral envelope glycoprotein. However, these antibodies are generally strain-specific or non-neutralizing what disable the immune system to install a protective or a preventive immune response (Kwong et al., 2002; Wei et al., 2003). Most of the monoclonal antibodies raised against the env glycoprotein seems to be shaped by 1F7 (IgM) idiotypic dominance (Zhou et al., 2010). Evidence suggests that anti-HIV broadly neutralizing Abs requires high levels of somatic hypermutation and polyreactivity. However it is unclear if Abs without 1F7 idiotype also requires these characteristics to neutralize a broad spectrum of viral variants. It is also unknown whether BnAbs not expressing the 1F7 idiotype are any easier, or even possible, to induce by vaccination than idiotypic BnAbs, if the 1F7 idotype is suppressed (Parsons,M.S. et al., 2011). Genetic sequence variability created by its error prone reverse transcriptase and by host immune pressure lead

to the evolution of the HIV-1 into multiple subtypes or clades with circulating recombinant forms. A fundamental barrier to HIV-1 vaccine development lies with the unique properties of the virus to predominantly enter through mucosal surfaces, to target preferentially human $CD4^+$ T cells, and to establish quickly a persistent reservoir of latently infected cells. Properties of transmitted (founder) viruses from mucosal transmission indicate that in 70–80% of cases, a single virus or virus-infected cell establishes productive clinical infection (Fischer et al., 2010). Such viruses typically exhibit C-C chemokine receptor type 5 (CCR5)-dependence. Mask functional envelope trimers needed to trigger efficient antibody response, and undergo rapid mutation as productive infection ensues (Goonetilleke et al., 2009; Keele et al., 2008). These viral properties narrow innate and adaptive immune pathways that can efficiently defend against HIV-1 entry and productive infection. Because of this global diversity (up to 35% in envelope gp120), it may impossible to design a single vaccine candidate that can induce potent effector immunity to multiple key antigenic determinants among worldwide circulating infecting HIV-1 strains. Although innate immune mechanisms contribute to HIV-1 control (Alter et al., 2007; Alter & Moody, 2010), it remains unclear whether recapitulating these responses with a vaccine will enhance protection against HIV-1 acquisition.

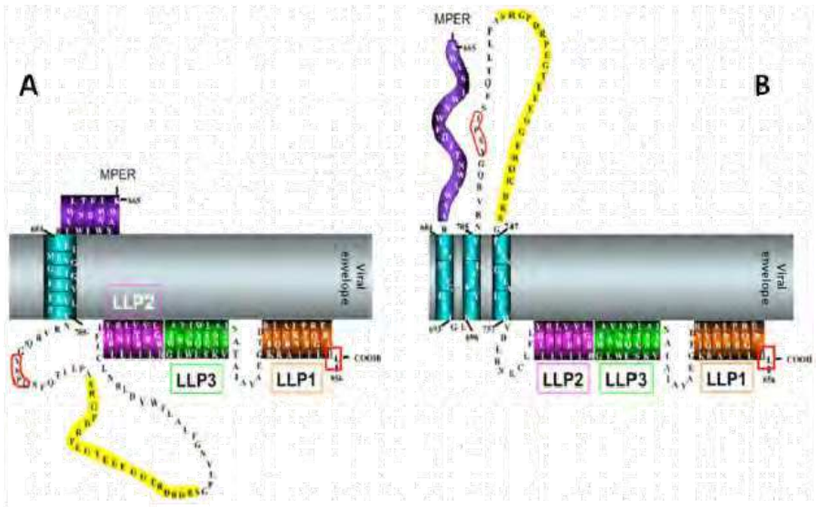

Fig. 11. Schematic models of the HIV-1 CT. A) A total intracytoplasmic localization of the CT with one membrane-spanning α-helix sequence (Traditional model). B) Alternative model with 3 MSD β-sheets and an extracellular segment that bears the Kennedy peptide Adapted from (Steckbeck et al., 2010).

3.1 Gp120 immunogenicity

During HIV-1 infection, gp120-specific Abs are generated and some of them are directed to conserved sites on Env crucial to host receptor recognition or viral fusion (table 2) Conventional insight suggests that constant rather than variable regions of Env should be targeted by the immune system to elicit broad responses against diverse HIV strains. However, these regions were classified on single criteria as the sequence and for only a few virus strains (Zolla-Pazner and Cardozo, 2010). Whereas immunological and 3-D structural studies of Env have shown that these regions were flexible and variable to interact with other surface molecules thus explaining how Abs specific for some variable regions have neutralizing activity against diverse viruses. Although, gp120-specific Abs typically

recognize type-specific neutralizing epitopes located on variable loops (Davis et al., 2009) or recognize dominant non-neutralizing conserved epitopes in gp120 (Palker et al., 1987) or gp41 (Gnann et al., 1987). Inducing neutralizing antibodies that target these epitopes using rationally designed immunogens has, so far, been unsuccessful (Selvarajah et al., 2005; Selvarajah et al., 2008; Wu et al., 2010).

This provides a rational basis for understanding the immunological cross-reactivity of many monoclonal Abs targeting the second (V2) and third (V3) variable loops of gp120, and the quaternary neutralization epitopes (QNEs) formed by V2 and V3. The glycan-rich outer face of gp120 is also the target of 2G12 broadly neutralizing mAb (Calarese et al., 2003; Huskens et al., 2007; Scanlan et al., 2002; Trkola et al., 1995). The V3 and the "bridging sheet" contain the binding site for co-receptor (CCR5 or CXCR4), but Abs against these targets are typically weakly neutralizing due to limited epitope exposure on native Env, and steric and kinetic restrictions post-CD4 engagement (Binley et al., 2004; Hartley et al., 2005; Reeves et al., 2005)

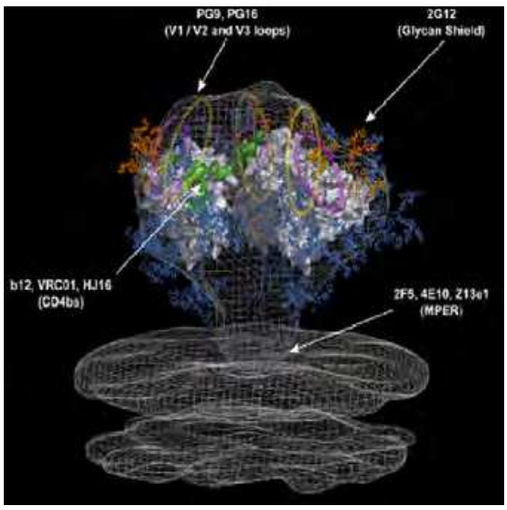

Fig. 12. Epitope modeling of HIV-1 trimer-specific neutralizing antibodies. The above model is adapted from a recent cryoelectron tomographic structure of the HIV-1 trimer (Liu et al., 2008; Schief et al., 2009). The crystal structure of the b12-bound monomeric gp120 core has been fitted into the density map (Zhou et al., 2007). The V1/V2 and V3 loops, which are not resolved in the structure, are represented as yellow and magenta ovals, respectively. The red structure located above the trimer is representative of a human IgG1 molecule. The approximate locations of the epitopes targeted by the existing NAbs are indicated with arrows. Carbohydrate chains are shown in blue, and the oligomannose cluster targeted by mAb 2G12 is shown in orange (Walker and Burton, 2010)

3.1.1 Receptor-binding sites

Binding of gp120 to CD4 results in gp120 conformational changes that expose the binding site for a secondary co-receptor, which is either of the chemokine receptors CCR5 or CXCR4 (Sterjovski et al., 2011). Crystallographic and biochemical studies of gp120 have provided valuable insights into mechanisms involved in CD4-binding and CD4-induced conformational changes that result in formation and exposure of the co-receptor binding site (Huang et al., 2005; Myszka et al., 2000). The unbound gp120 core consists of a highly conserved inner domain, which faces the trimer axis, and a heavily glycosylated, outer domain, which is mostly

exposed on the surface of the trimmer (Kwong et al., 1998; Teixeira et al., 2011; Zolla-Pazner and Cardozo, 2010). CD4 binding site represents the encounter of three separate regions *via* their surface-exposed residues. These regions are distributed over 6 segments of gp120. These regions include the α-helices of the inner domain, the CD4-binding loop of outer domain, and the β20–β21 ribbon, that incorporate the gp120 bridging sheet, which is a structural element of gp120 formed after CD4 binding that is involved in co-receptor binding. Otherwise, thermodynamic and structural analysis of the interaction between gp120–CD4 showed evidence of a structured CD4-binding pocket on the unloaded gp120, and that CD4 binding site elements that influence gp120–CD4 affinity are formed from conformational alterations that occur after gp120 has encountered CD4 (Kwong et al., 1998; Myszka et al., 2000). All these structures occur in the very early steps of the recognition (tropism) and anchorage process and provoke broad immune response. However, Abs remains interesting due to their large capacity to neutralize HIV-1 (table 1). The antibody IgG1b12 recognizes an epitope that overlaps the binding site of gp120 to the CD4 receptor. Several antibodies specific of the CD4 binding site have been described by several teams as the 15th, F105, F91, 1125H, 21H, 654-30D and Fab b6. All these antibodies show responsiveness to monomeric gp120 from different isolates of HIV-1 while this response disappears with trimeric gp120 which is present on the viral envelope spikes. Only the b12 antibody binds these two forms, and neutralizes many strains of HIV-1 (Zwick et al., 2003). Recently, the VRC01 Ab has been identified by screening of sera from infected individuals. It could be detected with high proportion and titers in patients. VRC01 Ab has been mapped to the CD4 binding site of gp120 (Wu et al., 2010; Zhou et al., 2010). In these studies it was observed that among 190 circulating HIV-1 isolates tested for sensitivity to VRC01, 173 were neutralized and only 17 were resistant. A structural analysis of these 17 resistant isolates by threading their sequences onto the gp120 structure showed important variations in the V5 region. However, the low VRC01 resistance frequency suggests that VRC01 use a recognition mechanism that allows binding despite V5 variation. Study of VRC01 interaction with V5 shows that VRC01 recognition of V5 is different than CD4. The V5 loop fits into the gap between heavy and light chains. Interaction with the more conserved residues at the loop base is sufficient for the VRC01 activity independently of variation in the top of the V5 loop.

Neutralizing Abs directed against CD4 binding site have been recently compared (Pietzsch et al., 2010). Around 30% of high titers of broadly HIV-neutralizing Abs expressed by memory B cells in HIV+ patients recognize one or more "core" epitopes that were not defined. Some of them recognize the ^{474}DMR^{476} motif on the gp120 which is conserved in different strains and its mutation alters the viral fusion process. For example, HJ6 exhibit a breadth neutralizing activity comparable to, and could be considered as complementary to b12. HJ16 also showed selective neutralization (Corti et al., 2010).

3.1.2 CD4-induced region

The receptor-binding structures of gp120 are conserved among diverse viral isolates and represent functionally constrained regions that might serve as targets of broadly neutralizing antibodies. However, structural evidence suggests that, within functional spike, the CD4-binding site (CD4bs) is a recessed pocket and the co-receptor-binding site (CD4-induced region) is either not formed or not exposed until gp120 engages CD4 on target cells (Kwong et al., 1998). Numerous Abs were described for their binding to the CD4-induced site and the most interesting by their breadth neutralizing capacities are the 17b, X5, m18, and m14 which all contains long H3s playing a major role in their mechanism of binding

(table 1). The H3s region of X5, m6 and m9 appear to be very flexible and highly potent to neutralize the virus (Wen et al., 2010; Zhu et al., 2006)

3.1.3 V1/V2 regions

The V1/V2 loops are less investigated even if several studies have shown that the V1/V2 domain of the HIV-1 gp120 envelope protein is involved in viral tropism during infection. V1V2 region interfere by masking conserved neutralizing epitopes, in the conformational changes occurring after co-receptor binding.

As V1/V2 domain is highly glycosylated, numerous studies have determined the influence of carbohydrates on neutralizing antibodies production. As an example, mucosal secretory IgA from parotid saliva and also seric IgG from seropositives present differences in their neutralization properties in fuction of the clade and glycosylation state. (Granados-Gonzalez et al., 2009)

Epitope	Monoclonal antibody	Epitope on	Region(s) recognized	Epitope type
CD4-binding site	IgG1b12 HJ16 VRC01 VRC03	Gp120	C2, C3, C4, V5 and C5	Discontinuous
CD4-induced region	17b X5	Gp120	Binding Sheet	Discontinuous
Complex carbohydrate	2G12	Gp120	Carbohydrate moieties in C2, C3 and V4	Discontinuous
V3	447/52-D 2219 3074 HGN194	Gp120	V3 loop	Linear (conformational)
Quaternary neutralizing epitope	2909 PG9 PG16	Gp120 Trimer	V2-V3	Quaternary
Membrane-proximal external region	2F5 4E10 Z13	Gp41	Protein and lipid	Linear (conformational)

Table 1. Main HIV-1 Env-specific neutralizing human monoclonal Abs.

3.1.4 V3 region

The b12 (CD4bs) and 2G12 (glycan) epitopes are presently the most attractive targets for vaccine design owing to their highly conserved nature. Although other sites seems present an interest in the immune response to gp120 as epitopes in the V3 region. This region is recognized by an antibody called 447- 52D (table 1) (Dhillon et al., 2008). Its core epitope is the Gly-Pro-Gly-Arg (GPGR) motif which is located at the center of the V3 region. This recognition is altered with the substitution of AA at the N-Term segment of the V3 region (Rosen et al., 2005). This Ab neutralize both X4 and R5 primary isolates, making it one of the most effective anti-V3 Ab. X-ray crystal structure of 447- 52D in complex with a V3 peptide indicates how this antibody may have the capacity to neutralize more clade B viruses than other anti-V3. Since the binding interaction between the antibody and the peptide is mediated by main chain contacts, which enlarges the ability of the antibody to recognize a variety of V3 sequences (Stanfield et al., 2004). The only side-chain interactions are with the Pro and Arg-residues in the GPGR sequence; the side chains from both residues form extensive interactions with

residues in the Ab combining site. The side chain of the Arg residue in the GPGR sequence is oriented in the opposite direction in the 447-52D complex relative to its orientation in the other complexes with anti-V3 antibodies and V3 (Binley et al., 2004; Rosen et al., 2005). However, exposure of the V3 region during infection seems to occur exclusively in the context of gp120 oligomer on the virus and may be influenced by the presence of glycan moieties. Given these caveats, the V3 region may be a target that only yields antibodies with restricted neutralizing ability. It is worth noting here that a 447-52D equivalent has not been identified so far for non–clade B viruses. A novel human monoclonal Ab HGN194 was isolated from memory B cells of a long-term non-progressor infected with a HIV-1 clade AG circulating recombinant form (CRF). This Ab recognizes a conformational epitope in the V3 and neutralizes all tier 1 viruses which are relatively neutralization-sensitive but only 11% of the tier 2 viruses tested. Tier 2 strains are more difficult to neutralize and reflect the majority of primary HIV-1 isolates. (Watkins et al., 2011) After massive immunization in Rhesus monkeys with the IgG1HGN194, the authors describe the absence of virus reservoirs after HGN194 was cleared. HGN194 seems to be an interesting cross-clade Nabs.

3.1.5 Impact of glycosylations

HIV uses glycans to occlude Ab epitopes on gp120. Around 50% of the molecule is covered with carbohydrates that render the underlying protein surface masked from the immune system and inaccessible (figure12). The virus can also shift the locations of its glycans *in vivo* (Nawaz et al., 2011). These observations gave a non static model called evolving glycan-shield model by which, through the continuous repositioning of its N-glycosylations positons, HIV is able to escape from a specific neutralizing Ab response. Thus, the developed resistance is not a comprehensive one but, instead, a specific adaptation to the particular Ab response in each infected individual (Wei et al., 2003). In addition to being involved in blocking neutralizing Ab responses, glycan repositioning also may compensate for conformational changes in the envelope glycoprotein caused by localized AA changes directly related to virus escape from neutralizing antibody. The works of Trkola led to the characterization of the human monoclonal Ab 2G12 directed against the envelope glycoprotein gp120 of HIV-1. This antibody recognizes clustered α1 → 2-linked mannose residues on the distal ends of oligomannose sugars located on the carbohydrate-covered silent face of the gp120 outer domain. It confers neutralizing activity *in vitro* on T cells infected with HIV-1 primary isolates from clade A or B. This study has demonstrated the ability of 2G12 to induce an immune response as "antibody-dependent cellular cytotoxicity ADCC" directed against infected cells or by activation of the complement system (Trkola et al., 1996). The epitope of 2G12 is described as a dependent structure of N-glycans in the C2, C3, V3, V4 and C4 regions of gp120. Other studies have described that the epitope recognized by monoclonal antibody 2G12 contains mannose type glycans at multiple sites of N-glycosylation (Calarese et al., 2003; Scanlan et al., 2002). This is not aware that the glycosylated epitopes contain microheterogenities at various carbohydrates which can lead to an escape from immune response. Only deletion of N392 glycosylation site is sufficient for decrease drastically 2G12 activies, which may explain the emergence of new strains viral resistance (Huskens et al., 2007; Scanlan et al., 2002).

3.1.6 Quaternary neutralizing epitope

Quaternary epitopes are formed by interactions between proteins during multimerization. It is as a result of such reorganization that many proteins (such as enzymes and the trimeric

gp120 spike of HIV-1) carry out their physiological function. An Ab that reacts with a true quaternary epitope will not interact with the individual monomeric subunits. Monoclonal antibodies mAb2909 and PG16 (figure 14), react with the trimeric form of gp120 on the surface of HIV-1 virions or *env*-transfected cells but not with monomeric gp120 (Gorny et al., 2005; Walker et al., 2009). However, some Abs that react with quaternary epitopes binds preferentialy the trimers rather than monomeric subunits. This seems to be true for the monoclonal antibody PG9. Trimerization can result in changes in quaternary structure within individual subunits or through reorientation of the subunits against each other, so regions contributing to the quaternary epitope can be inter-molecular (*trans*) or intra-molecular (*cis*). Recently, a human mAb (mAb 2909) (figure 14) has been described to bind to a quaternary structure only on virion but not to soluble monomeric gp120 (Changela et al., 2011; Gorny et al., 2005). It has been discovered by immortalization of PBMC from HIV 1 patients asymptomatic and drug-naïve (Gorny et al., 1991). It demonstrates a high neutralizing activity for primary isolate such as SF162 (picomolar concentrations) and specificity for a complex epitope consisting of V2, V3, and the CD4 binding site that is present exclusively on the surfaces of intact virions but not on soluble viral proteins (Gorny et al., 2005). The neutralizing activity of MAb 2909 against pseudovirus SF162 is 750- to 100,000-fold more potent than those of other well-characterized. The occurrence of mAb 2909 suggests the possible existence of additional Abs that are oligomer-specific. Such Abs, in contrast to mAb2909, may possess broader neutralizing activity.

Moreover, two potent broadly neutralizing monoclonal antibodies, PG9 and PG16, have been discovered recently both targeting highly conformational, discontinuous epitopes involving the V2 and V3 loops (Walker et al., 2009). These antibodies have been characterized after a large screening of 1800 sera from HIV- and HIV+ donors from all over the world (Thailand, Australia, and United Kingdom etc). Only donors that exhibit broad and potent neuralizing serum activity were selected. Analysis of the antibody variable genes revealed two pairs of somatic variants, each one contained long, heavy-chain complementaritydetermining region 3 (CDRH3) loops (PG9 and PG16). Long CDRH3 loops have been previously associated with polyreactivity. PG9 and PG16 are somatically related and appear to be derived from the same recombination of heavy and light chains. The both Abs recognize a site on gp120 composed of elements from the V2 and V3 variable regions. Despite the vaunted diversity of the HIV-1 gp120 envelope and the even higher sequence variability in the V2 and V3 regions (Walker et al., 2009), neutralization assays indicate that the recognized epitope is conserved in 70 to 80% of circulating viral isolates. Neutralization by PG9 correlates strongly with that of PG16, indicating that these Abs recognize a common HIV-1 epitope. This suggests that a common surface of the paratope on PG9 and PG16 might be involved in recognition of HIV-1. Substantial differences in sequence are found between PG9 and PG16 (Doria-Rose et al., 2010; Pancera et al., 2010).

3.2 Gp41 immunogenicity

Gp41 is largely occluded by quaternary interactions within native Env (Sougrat et al., 2007; Zanetti et al., 2006; Zhu et al., 2006). During the fusion process, different gp41 regions are exposed and elicit while the changing of conformation due to the intercation with gp120 antibodies. Some of them are very essential and present interesting capacities to block the infection and neutralizing the virus. The neutralizing Abs 2F5, Z13e1 and 4E10 directed against the membrane-proximal external region (MPER) in the C-Term portion of the gp41 ectodomain can bind Env and block a late stage of fusion (table 1) (Nelson et al., 2007;

Stiegler et al., 2001; Dimitrov et al., 2007; Zwick et al., 2005). Despite great efforts, high neutralizing Abs titers against any of the conserved sites on Env have not yet been elicited (Kim et al., 2007; Phogat and Wyatt, 2007).

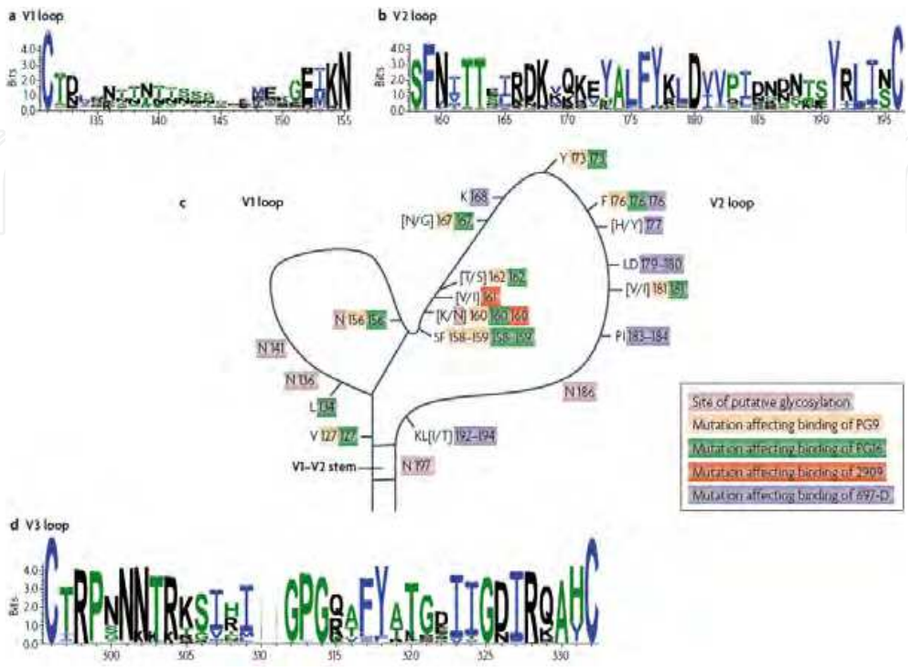

Fig. 14. Conserved and variable residues in the V1, V2 and V3 loops of gp120. A, b, d) Sequence logo describs the AA conservation pattern across a multiple alignment of many V1, V2 and V3 loops. The height of the letter indicates the degree of conservation of the most common amino acid at that position. Data obtained from the Los Alamos National Laboratory, New Mexico, USA. AA are colored according to their chemical properties. C) AA positions and sites of glycosylation that are implicated in binding of the V2-specific monoclonal Ab 697-D and the Quaternay Neutralizing epitopes-specific monoclonal Abs PG9, PG16 and 2909 are mapped onto a schematic illustration. Square brackets indicate more than one commonly occurring AA at a single position. The individual sites associated with binding of each single Ab are distributed throughout the V1–V2 primary sequence linearly but, in 3D space, must group into one or a few overlapping epitopes (adapted from (Zolla-Pazner and Cardozo, 2010)

3.2.1 Polar region (PR)

Only one study described PR-specific Abs in HIV-1+ patients. However, these Abs remains poorly neutralizing (Buchacher et al., 1994).

3.2.2 N-Heptad Repeat region

Cross-reactive antibodies to the NHR have been detected in HIV-1-positive sera, indicating its immunogenic characteristics (Poumbourios et al., 1992). Moreover, the D5 NAb was generated from a native single-chain variable fragment library. D5 has been shown to bind to the NHR trimer and, like the T-20 peptide, inhibits the assembly of the fusion intermediate *in vitro* (Miller et al., 2005). Those studies indicate the potential of the inner NHR trimer as a target for vaccine design. D5 was derived from B cells of HIV-naive subjects and has not been subject to

extensive somatic hypermutation, with only seven non-complementarity-determining regions (CDR) amino acid changes from germline sequences (four changes in VH and three in VL). Unlike b12, 2F5, and 4E10, D5 does not have an atypically long heavy chain CDR3 region. Unlike 2G12, D5 does not require a ''domain-swapped'' structure for neutralization. Unlike X5, D5 retains antiviral activity against primary HIV isolates when converted to an IgG1 format. Finally, and most importantly, D5 was elected by binding to IZN36 and 5H which are synthetic antigens with well defined structures mimicing the 6 helix bundle structure. In addition, new NAbs with limited potency against the NHR trimer of HIV-1 have been isolated by screening phage-display library (Nelson et al., 2008). Ag-binding and monoclonal Ab-competition experiments using 8K8, DN9 and D5 strongly suggest that the epitopes of 8K8 and DN9 are more closely related to each other than to that of D5. Although, D5 has a preference to bind NHR mimetics in the absence of CHR peptide thus indicating a significant cross-reactivity of D5 with immobilized 6-Helix (Luftig et al., 2006; Miller et al., 2005),. Similarly, C34 HR2 peptide competes efficiently with 8K8 and DN9 binding to immobilized NHR mimetics (e.g. 5-Helix and IZN36), but not with D5. Another antibody, Fab 3764, has been shown to bind to the NHR region with the same efficiency on free (N35CCG-N13) or interacting with CHR peptide in the form of a 6-Helix Bundle (Gustchina et al., 2007; Gustchina et al., 2010). Further more absence of detectable cardiolipin reactivity with 8K8 178 170–183 suggests that autoreactivity, at least to cardiolipin cannot explain the weak titers of 8K8-like Abs in the rabbits (Haynes et al., 2005; Scherer et al., 2007). Three-dimensional structural informations of the mAb-mimetic complexes may be used to better predict the accessibility to the NHR on fusogenic gp41 for prospective next-generation Abs. Recently, the HK20 gp41-specific antibody was obtained from immortalized memory B cells of an HIV-1 infected individual (Corti et al., 2010). This Ab targets the conserved hydrophobic pocket in HR1. Crystal structure of HR1-specific human mAb HK20 in complex with 5-Helix shows that HK20 binds to the same region recognized by D5 but differs significantly in the contact sites and shows a role for somatic mutations in affinity maturation (Luftig et al., 2006). These aspects influence both potency and breadth of neutralization, which are higher for HK20 compared to D5 and depend on somatically mutated residues. In addition, we show that in case of HK20 the scFv is at least 15-fold more potent in neutralization than IgG, consistent with a limited accessibility to the target site. The gp41 footprints of HK20 and D5 and the global structural principles employed by both antibodies are similar.

3.2.3 Immunodominant loop

This region located between the two heptad repeat region N-HR and C-HR was identified during serum-mapping studies (reviewed in Girard et al., 2006; Montero et al., 2008). Even if the response to this region is very frequent most of the Abs elicited do not present neutralization capacities but instead shown enhancement of infection (Robinson et al., 1990). Only one MAb (clone3) neutralize T-cell laboratory adapted viruses from clade B and three primary isolates from group O (Ferrantelli et al., 2004).

3.2.4 C-Heptad repeat region (C-HR)

Considering that MPER is not included in C-HR, no CHR-specific Ab has been described. The poor immunogenicity of this region may be attributed to its lack of exposure on the surface of the native virus and is exposed only if gp120/CD4 interaction occurred. This region is probably masked by gp120 (Nyambi et al., 2000; Pietzsch et al., 2010).

3.2.5 Fusogenic 6 helix-bundle form

The gp41 TM subunit, which anchors the spikes in the viral envelope, maintains their trimeric organization, and plays a major role in fusion of the virus and host cell membrane through its hydrophobic N-Term fusion peptide and a fusion active hairpin structure involving the HR that can fold into a six-helix coiled-coil bundle. This pattern plays an important role in both the early and late stages of the membrane fusion (Chan and Kim, 1998; Johnson et al., 2001; Liu et al., 2005; Lu et al., 1995; Munoz-Barroso et al., 1998). This process can also participate in the formation of the fusion pore through direct protein-membrane interactions (Munoz-Barroso et al., 1998; Shnaper et al., 2004). Different studies have shown the immunogenicity of the fusogenic complex and describe Abs direct against this structure (Gustchina et al., 2010; Opalka et al., 2004; Vincent et al., 2008). Authors were confronted to the difficulty of the construction of a native form of the fusogenic complex with 6 helix-bundle and used a five helix-bundle (Gustchina et al., 2010) or synthetic peptide that mimic the N-HR like (peptide N36) or mimic C-HR (peptides C34 or T20). Abs directed against the complex do not always recognize the HR1 or HR2 individually. The Abs have shown low neutralizing activity excepted in one study with Nabs against primary isolates (Vincent et al., 2008), and the work of Gustchina *et al*., with a monoclonal antibody Fab 3674 that neutralize diverse laboratory-adapted B strains of HIV-1 and primary isolates of subtypes A, B, and C (Gustchina et al., 2007). When gp41 switches from the native conformation to the fusion structure, there are probably several intermediate structures, which can be a target for Abs. Several studies indicate that the prehairpin intermediate state is accessible to Abs and that the access is not restricted (Louis et al., 2003). Therefore, the gp41 6-HB core may serve as an attractive target for development of anti-HIVmolecules.

3.2.6 MPER (membrane-proximal external region)

The Membrane Proximal Ectodomain Region contains the epitopes of three broadly neutralizing Abs, 2F5, 4E10, and Z13 (Buchacher et al., 1994; Zwick et al., 2001) (figure 15). MPER is not highly immunogenic like the N-HR, ID loop or the gp120. Specific anti-MPER Abs present in natural infection are not as broadly neutralizing as 2F5, 4E10 (Braibant et al., 2006; Muhlbacher et al., 1999). Both 2F5 and 4E10 neutralize a broad range of both laboratory adapted and primate isolates of HIV-1 (Mascola et al., 2000; Stiegler et al., 2001). 2F5 is the most potent NAb, whereas 4E10 neutralizes a broader range of HIV-1 isolates, as shown by pseudovirus studies using an extensive panel of Envs derived from primary isolates (Binley et al., 2004). The 2F5 Ab could also be detected at low level in the sera of HIV long term progressors (Braibant et al., 2006). The frequency of seroreactivity to MPER is about 56% in 50 HIV-1-positive subjects. Most of the Abs are specific for the C-Term region of the ELDKWA sequence just preceding the 2F5 epitope. In addition, more than 30% of sera were reactive to the ELDKWA 2F5 epitope. Furthermore, serum reactivity against an MPER peptide (aa 642 to 673) in patients could be correlated to the recognition of infected T cells and to CD4 cell counts (Muhlbacher et al., 1999). There is a possibility that NAbs against MPER are present in low titers or with low affinities in serum that they are not sufficiently neutralizing to inhibit HIV-1 infection and are therefore undetectable in neutralization assays. However, recent studies using chimeric pseudoviruses with epitopes in a context more closely related to the MPER structure (figure 15B) suggest that MPER-specific NABs are relatively rare or absent during natural infection. Moreover, the fact that new NAbs are mapping to a different region in MPER than 2F5 and 4E10 provides hopes by suggesting that vaccine-induced neutralizing antibodies are achievable (Haynes and Montefiori, 2006;

Li et al., 2006). To date, the lack of broadly neutralizing activity shown by MPER binding sera could be due to low titers or absence of NAbs. This issue of quantity *versus* quality has not been fully resolved but has significant implications for vaccine design.

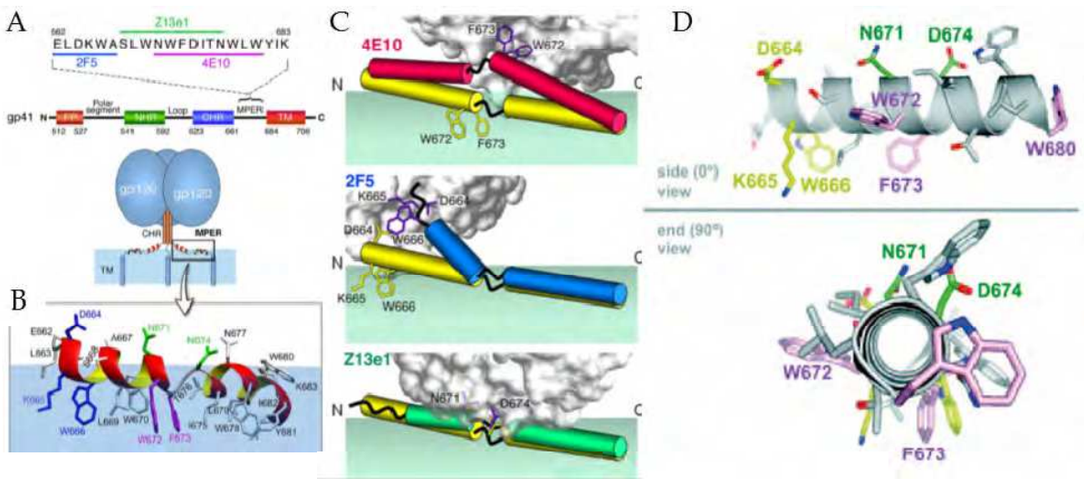

Fig. 15. Different representations of MPER interaction with 2F5 4E10 and Z13 Abs A) Schematic diagram of HIV-1 gp41. FP, fusion peptide region; NHR and CHR, N- and C-terminal-helices of heptad repeat, respectively; and TM, transmembrane domain. The minimal peptide epitopes are indicated for 2F5 (blue), Z13e1 (green), and 4E10 (magenta). B) A simple model of env including MPER and TM based on 3D structural features of HIV-1/SIV-1 trimeric env and the NMR structure of the HxB2 MPER in a virion mimic surface (blue plane). N674 rather than D674 is shown. C) Artistic rendering of MPER orientation changes induced by 4E10, 2F5, and Z13e1. Unbound MPER peptides (yellow tubes) are immersed in the lipid bilayers (light green panel). Red, blue, and green tubes represent the membrane orientation of schematic MPER segments in complex with 4E10, 2F5, and Z13e1 (gray surface area). Several key binding residues positions are indicated before (yellow) and after (purple) antibody binding (Song et al., 2009). D) Hypothetical model of the MPER (DKWASLWNWFDITNWLW) and the 2F5, Z13e and 4E10 cores epitope with the core residues colored in yellow, green and violet, respectively described by (Nelson et al., 2007).

As said before 2F5 Ab has broad neutralizing activity. In animal models it could interrupt the mucosal transmission and confers protection against SHIV infection (Mascola et al., 2000). The 2F5 antibody was isolated from human immortalized B cells. It recognizes an epitope lying between glutamic acid 662 and alanine 667 (ELDKWA). Studies have shown, for expression library and protection against proteases, that this epitope is wider (EQELLELDKWASLWN). By addition of a leucine at each side (LELDKWASL), the affinity for its epitope is increased by more than 2000 times (Ofek et al., 2004; Parker et al., 2001; Tian et al., 2002). In models that study the involvement of the viral membrane using POPC (1-oleyl-2-palmolityl-sn-glycero-3-phosphocholine) and POPC/cholesterol in the recognition and binding to its epitope, it has been shown that 2F5 does not interact with the membrane before fixation (Veiga and Castanho, 2006). In the absence of an hydrophobic environment, 2F5 and 4E10 are unable to bind to MPER (Ofek et al., 2004). This study demonstrate that the recognition of 2F5 and 4E10 epitopes depend on their integrity and conformational context. Recently, it was shown that the binding of 2F5 causes peptide

docking on the membrane and increase the ability of the Mab to be intrusive (Franquelim et al., 2011). The 2F5 induces a confined local disorder in the membrane which can promote the exposure and interaction with gp41. However, the role of the presence of lipids in recognition of epitopes during the membrane fusion has not yet been clearly elucidated. The 2F5 epitope contains a β-turn conformation and antigen which is presented in a similar context may better mimic the epitope and consequently improve the binding of this Ab (Tian et al., 2002). Several difficulties rise to make a stable immunogen, flexible and mimicking faithfully the MPER in a native conformation sported during the steps of rapprochement and viral fusion process. Moreover, the polyspecificity of 2F5 and 4E10 and autoreactivity by binding to cardiolipin could explain the low activity of these antibodies *in vivo* as the immune system deplete all B cells which produce auto-reactive Abs (Haynes et al., 2005).

Another promising MPER-specific Ab (4E10) was isolated from B cells of HIV patients. It binds a deeper epitope in the MPER and presents neutralizing abilities. The 4E10 is highly protective against infection with primary isolates and laboratory strains of HIV-1. The 4E10 binds to a highly conserved linear epitope on MPER ^{671}NWF (D/N) IT^{676} (Zwick et al., 2001). Crystallographic structures of the epitope have been characterized and form a large helical shape with all the important AA on the same side of the helix (Hager-Braun and Tomer, 2005)(figure 15D). The antigen reacts only with residues of the base and the center of the loop of CDR H3. However much of this loop is not involved in its binding to the antigen. The top of the loop of CDR H3 of 4E10 and this residue is removed from the apolar binding site epitope which suggest a possible interaction with the membrane of the virus. Structural models predict that the formation of the epitope of 2F5 and 4E10 would be highly dependent on the presence of a membrane. The affinity of 4E10 increases in the presence of lipids (Lorizate et al., 2006). The W^{672}, F^{673}, T^{676} residues seems to be crucial for 4E10 binding since their substitution by alanine residues reduce drastically the affinity (Brunel et al., 2006). The sequence ^{671}NWFDITNWLWYIK^{683} is optimal for 4E10 recognition. In resistant strains, the pattern NWF(N/D)IT indicate that the 4E10 epitope is more complex and would be even discontinuous (Hager-Braun and Tomer, 2005). Mass spectrometry studies reveal that 4E10 binds to the N-Term of gp120 and gp41 on the native conformation (Stiegler et al., 2001). It has been suggested that the pattern NWF (N/D)IT forms a cryptic epitope accessible only during the intermediate stages of the merger. It seems that 4E10 has higher affinity to the membrane than 2F5 and that unlike 2F5, 4E10 promotes peptide extraction from the bilayer lipid membrane (Franquelim et al., 2011). The 4E10 Ab has also a polyspecificity for autoantigens as histone or DNA in systemic lupus erythematosus. Several *in vivo* pharmacokinetic studies and phase I/II clinical trials phases I and II with combination of 4E10, 2F5 and 2G12 showed that these three Abs are able to maintain undetectable viral load in patients where the infection has been suppressed by antiviral therapy (Joos et al., 2006). These studies also showed that 4E10 administration is not highly immunogenic with rare IgM against 4E10 but no IgG. Clearance and half-life of 4E10 are identical to those of other classical therapeutic Abs.

One other described MPER-specific Ab is the Z13 Fab. This Ab derived from an expression library recognizes an epitope located on the C-Term of 2F5 epitope and which overlaps 4E10 epitope. The epitope of Z13 is centered around the sequence ^{671}NWFDIT^{676} but this would be dependent on the conformation of this motif and changes such as N-glycosylation of asparagine (D) ^{674} or exposure to a native protein (Zwick et al., 2001).This Ab is able to neutralize weakly several clades such as B, C and D.

3.2.7 The C-Term tail

The external tail loop within the CT of the gp41 TM subunit of the HIV-1 envelope protein comprises approximately 40 AA, and within are 18 AA (^{734}PDRPEGIEEEGGERDRDR ^{751}) including three interesting regions. The antigenicity of this segment of the CT is very complex, and changes according to the biological context of gp41 (Reading et al., 2003). The Kennedy sequence, ^{731}PRGPDRPEGIEEEGGERDRDRS^{752}, is exposed on the outer surface (Kennedy et al., 1986). It was observed that 3 epitopes ^{734}PDRPEG^{739}, ^{740}IEEE^{743}, ^{746}ERDRD^{750} are mostly recognized in the Kennedy sequences (Chen et al., 2001; McLain et al., 2001). Kennedy-specific NAbs could be elicited in different conditions of exposure.

The ^{746}ERDRD^{750} epitope is exposed constitutively and does not require contact with cell receptors or an elevated temperature (Cleveland et al., 2003). ^{734}PDRPEG^{739} is recognised by the non-neutralizing Ab C8, ^{740}IEEE^{743} by the non-neutralizing Ab 1575 (Abacioglu et al., 1994), ^{746}ERDRD^{750} by the non-neutralising Abs 1577 and 1583 (Vella et al., 1993), and the neutralizing EPES (epitope purified and ERDRD specific) and SAR1 antibodies. EPES and SAR1 Abs were both obtained by immunisation of mice with cowpea mosaic virus (CPMV) chimeras expressing short, ERDRD-containing gp41 sequences. EPES is the only Kennedy-specific polyclonal IgG described to have a strong neutralizing activity against primary isolates. It was also shown that some of the IgA from infected patients parotid saliva or sera are able to recognize the linear peptides ^{731}PEGIEEEGGERDRDTSGRLV^{750} or ^{741} RDRDTSGRLVHGFLAIIWVD^{762} (Vincent et al., 2004). IEEE epitope is antigenically dominant , and competes with the binding of ERDRD-specific antibodies. This situation in humans is not known. It is interesting that MAb 1575 appears to have no biological activity other than suppression of the antibody response to ERDRD. Furthermore, the sequence ERDRD is involved in at least three epitopes. These epitopes may be occulted or exposed as a result of conformational changes occurring in another part of gp41, or may be hidden by the associated gp120 and exposed when that gp120 is shed. In addition epitopes may be modulated by conformational changes that affect the ERDRD sequence directly. (Hollier & Dimmock, 2005).

4. Conclusions

The development of an HIV vaccine has proven to be a formidable scientific challenge given the extreme genetic variability of the virus, lack of good animal models, lack of knowledge of all mechanisms involved in immunity or limitations of the technology. However, passive administration of rare human anti-Env monoclonal broadly neutralizing antibodies with titers that can be achieved by immunization can protect against SHIV challenge in rhesus macaques (Hessell et al., 2007; Hessell et al., 2009a; Mascola and Montefiori, 2010; Montefiori and Mascola, 2009). Thus, a major goal of HIV-1 vaccine development is to design highly immunogenic Env antigens capable of inducing Abs that can broadly neutralize HIV-1 (Mascola and Montefiori, 2010; Stamatatos et al., 2009).

According to the clinicaltrials.gov database, more than 543 clinical trials were performed in the field of HIV vaccines. During these last ten years, only one vaccine candidate seems to give objective protection. This prophylactic vaccine from Sanofi Pasteur is composed by an heterologous prime-boost vaccine with a priming with VaxGen gp120 B/E (AIDSVAX B/E) protein and a boost with live recombinant ALVAC-HIV (vCP1521). This trial (RV144) has involved 16,403 individuals and was conducted in Thailand and completed in October 2009,. Vaccination appeared to reduce about 31.2% the rate of acquisition of HIV-1 infection but

had no significant effect on viral loads or on CD4+ T cell counts. This "weak" efficacy is the first indication that effective vaccination against HIV-1 could be reach and give rease for hopes in the developpement of anti-HIV vaccines. The immunological correlates in this trial indicate that induction of Nabs is important for vaccine efficacy but not sufficient. Induction of polyspecific virus-specific CD8+ T cell responses is also an important actor of vaccine efficacy.
So the scope of action that remains for an effective vaccination is restricted to early stages of infection before the virus infects lymphoid organs and mucosal tissue. In addition, HIV has developed during its evolution, an immune system circumvent strategies that really works. The data from human clinical trials demonstrate that the first generation of soluble protein and vectored Env immunogens was safe and immunogenic. However, generated Abs are only effective on highly sensitive strains of HIV-1. One of the major goal in the development of an effective vaccine remain to develop a mimetic immunogen similar to viral proteins as the most accurate meaning can replicate all possible conformations in steps of infection Indeed, conserved Env epitopes targeted by NAbs are poorly immunogenic because they either are masked by glycan similar to host carbohydrates (Binley et al., 2010; Wei et al., 2003), appear transiently (Frey et al., 2008), are sterically hindered (Schief et al., 2009), or must overcome entropy for Ab binding. Another important point is that conserved epitopes (more in the gp41) present homologies with self-proteins which could trigger tolerance mechanisms (Haynes et al., 2005; Verkoczy et al., 2010). Finally, even when Abs can neutralize the infecting strains, their effect is transient due to escape mutations (Richman et al., 2003; Wei et al., 2003). Thus, immunization of non-human primates and humans with HIV-1 Env monomers or trimers has failed to induce broadly NAbs. Induced Abs are mainly effective on easily neutralized strains also called Tier 1 strains but have weak neutralization abilities for Tier 2 and Tier 3 strains (Gilbert et al., 2010; Mascola and Montefiori, 2010). The explanation for the poor cross-reactivity of vaccine-elicited Nabs appears to be related to the restricted repertoire of induced Abs and to the complexity of the native viral spike structure.
NAbs against HIV-1 play an important role in preventing viral infections. Less clear is their role in the containment of viral replication in infected individuals.
However, evidence is accumulating that NAbs may help the cellular arm of the immune response to prevent or delay the progression to AIDS. Detection of NAbs depends on the *in vitro* neutralization assays used, and standardization of the assays is essential in order to be able to compare the magnitude and quality of a NAb response in sera or other fluids from HIV-infected patients, uninfected HIV-1 exposed persons, or vaccinated animals/persons. Viral mechanisms to prevent neutralization include high variability and extensive glycosylation of the Envelope proteins, Envelope trimerization and shedding as well as late exposure of functionally important entry domains by conformational changes induced upon CD4 binding. These are also the difficulties encountered in the design of immunogens able to induce neutralizing antibodies upon vaccination.

5. Acknowledgements

We would like to thank the Cluster 10 of Infectiology of the region Rhône-Alpes and Sidaction to fund our works.

6. References

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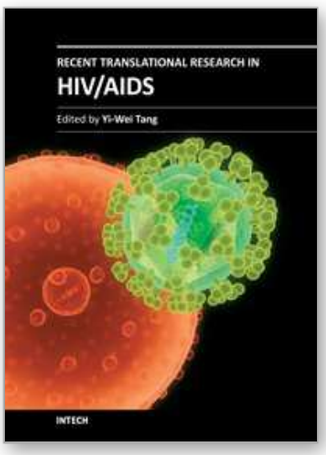

Recent Translational Research in HIV/AIDS
Edited by Prof. Yi-Wei Tang

ISBN 978-953-307-719-2
Hard cover, 564 pages
Publisher InTech
Published online 02, November, 2011
Published in print edition November, 2011

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