HIV-1 envelope glycoproteins with costimulatory domains for vaccine applications
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CHAPTER 1

General Introduction
AIDS and human immunodeficiency virus (HIV)

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV) that was first identified in 1983 (1, 2). HIV-1 is a lentivirus of the *Retroviridae* family (3). Two virus variants exists, HIV-1 and HIV-2, HIV-1 being the most widespread. HIV-1 is highly variable and this variability leads to the classification of HIV-1 into three main phylogenetic groups: M (main), O (outlier) and N (non-M/O). Group M represents the majority of the HIV-1 epidemic worldwide and is divided into 11 subtypes (or clades); subtype A1, A2, B, C, D, F1, F2, G, H, J and K (4).

HIV-1 can enter the body via various routes upon unprotected sexual intercourse, blood-blood contact or from mother to child during pregnancy, childbirth or breast feeding (5). HIV-1 is believed to be transmitted to the human population at the beginning of the 20th century in Central Africa from the chimpanzee, the natural host of simian immunodeficiency virus (SIV) strains that resemble HIV-1. Since the first cases were reported in 1981, more than 25 million people have died of AIDS worldwide and currently around 35.3 million people are living with HIV/AIDS (UNAIDS report on the global AIDS epidemic 2013). According to the 2012 UNAIDS report on the global AIDS epidemic 2.5 million people acquired HIV infection in 2011. As a consequence HIV has become one of the world’s most serious health problems. There are many campaigns to reduce sexual transmission, to prevent HIV infection among drug users and to eliminate new HIV infections among children. In addition, there is an active search for treatment strategies aimed at the eradication of HIV.

HIV-1 and disease progression

HIV-1 predominantly targets and infects CD4-positive (CD4⁺) T lymphocytes, although monocytes, macrophages and dendritic cells (DCs) are also susceptible to HIV-1 infection. In the early phases of infection, massive CD4⁺ T lymphocyte depletion and viral replication occurs in the peripheral blood (6) and gut-associated lymphoid tissue (7, 8). After this acute phase, a drastic decline in viremia is observed until it reaches a steady state which is known as the viral load setpoint (7, 9). This decline in viral load may be the consequence of limiting numbers of target cells and/or an effective antiviral immune response (10). The viral set point can be maintained for a long period after infection without symptomatic manifestations and the level of the set point is predictive for the clinical course of HIV-1 infection. This asymptomatic period of HIV-1 infection, which eventually develops into AIDS, varies
significantly between individuals and is characterized by an increase in viral load, progressive
decline in CD4+ T lymphocyte levels and exhaustion of other immune components, including
the humoral immune system. AIDS, the final stage of the disease, is defined by CD4+ T
lymphocyte levels below 400 cells/ml, at which the immune system cannot control
opportunistic infections anymore.

**HIV-1 virion**
The HIV-1 viral genome contains two copies of positive single-stranded RNA encoding nine
genes. The protein products of these genes are the three structural proteins: Gag, Pol and
evelope glycoprotein complex (Env); two essential regulatory proteins: Tat and Rev and the
accessory proteins: Vif, Vpr, Vpu and Nef. Gag and Pol are synthesized as polyproteins and
cleaved by the viral protease. Cleavage of Gag results in production of the structural proteins
matrix (MA), capsid (CA), nucleocapsid (NC) and p6 and spacer peptides p1 and p2, whereas
processing of Pol results in generation of the viral enzymes used for replication such as
protease (PR), reverse transcriptase (RT) and integrase (IN) (11, 12). Tat is essential for
activation of viral transcription (13) and Rev is necessary for nuclear transport of unspliced
and partially spliced viral RNAs (12, 14). The accessory proteins Vif, Vpr, Vpu and Nef are
essential for optimal viral replication (15, 16). The entry of the virion into the host cell is
facilitated by Env (17-19). Env attaches to CD4+ cells through the CD4 receptor followed by
binding of one of the chemokine receptors CXCR4 or CCR5, also termed co-receptors, to
start viral entry (20, 21). After entry, the RNA genome of the virus is released into the cytosol
and converted into double-stranded DNA by RT. Subsequently, the viral DNA is transported
to the nucleus of the host cell and integrated into the host genome. Transcription and
translation of new viral, genomic RNAs, mRNAs and proteins by the host cell machinery is
followed by virion assembly, which are then released by budding from the cell surface (22).

**Env biosynthesis and structure**
The HIV-1 entry process is mediated by Env, which forms trimeric spikes on the surface of
the virions (23, 24). Env is synthesized as a gp160 precursor protein and folded in the
endoplasmic reticulum where its 10 disulfide bonds are formed and ~30 N-linked glycans are
added (25). After proper folding, gp160-trimers are formed and transported to the Golgi
complex. In the Golgi, Env is cleaved into the gp120 surface and gp41 transmembrane
subunits, which then associate non-covalently (26, 27). A subset of the N-linked glycans are further processed in the Golgi. Finally, Env is incorporated into budding virions.

The surface subunit gp120 is composed of five conserved regions (C1-C5) that are interspersed by five variable regions (V1-V5) (28) and can be divided into an inner domain that is formed in the central core by the conserved domains and an outer domain that is highly glycosylated. Variable domains, as the name indicates, can be highly diverse in amino acid composition in viral isolates, even within patients (29). These domains form flexible loops that emanate from the conserved gp120 core and can mask the conserved domains to prevent them from being targeted by broadly neutralizing antibodies (bNAbs).

Gp41 consists of several domains with different functions. The trans-membrane domain (TM) anchors the gp41 subunits to the virus membrane. The N-terminal fusion peptide (FP) is inserted to the target cell membrane during the fusion process, and heptad repeat region 1 (HR1) together with heptad repeat region 2 (HR2) form a six-helix bundle structure that juxtaposes the viral and target cell membranes for the membrane fusion step (reviewed in (30)). Furthermore, gp41 has a highly conserved and hydrophobic region, designated the membrane-proximal external region (MPER).

### HIV-1 Immunity

Despite intensive efforts since the identification of HIV in 1983, no effective HIV-1 vaccine has been developed. Upon HIV infection innate, cellular and humoral immune responses combat the virus; however the end point of all untreated HIV-1 infections is AIDS, indicating that HIV-1 is a master of immune evasion. Dendritic cells (DCs) are one of the first lines of immune defense encountered by pathogens including HIV-1 and they are classified as antigen presenting cells (APC). DCs capture and degrade pathogens and migrate to the lymph node where they present pieces of the pathogen to other immune cells such as CD4+ T-helper lymphocytes which can then activate CD8+ cytotoxic T lymphocytes (CTLs) and B cells (31). CTLs can detect the infected cells by recognizing the pathogen-derived peptides that are presented by major histocompatibility complex class I proteins on the surface of infected cells. HIV-1 specific cellular immune responses are dominated by epitopes on Gag and Nef. Proper functioning of CTLs is dependent on CD4+ lymphocytes that are the main target cells for HIV-1 and that are progressively depleted during HIV-1 infection.
Humoral immunity to HIV-1 Env

Humoral immunity is also activated in HIV-1 infection and the great majority of HIV-1 infected individuals raise detectable Env antibodies (Abs) early in infection. Abs are secreted by B cells and play a major role in the host defense against pathogens. The induction of protective antibodies, including broadly neutralizing Abs (bNAbs), is highly desirable for an HIV-1 vaccine. The binding of neutralizing Abs (NAbs) blocks Env attachment to the cellular receptors and/or or limits the conformational changes required for Env-mediated membrane fusion, thus leading to inhibition of HIV-1 infection of target cells. The first anti-Env Abs that are raised during natural infection generally target gp41 and are non-neutralizing (32). These responses are followed by Abs to immuno-dominant variable gp120 regions that are strain-specific, i.e. recognizing the autologous virus (33). Around 20% of infected individuals induce bNAbs. Unfortunately, these patients do not benefit from these bNAbs because the virus escapes from them too easily. Nevertheless, bNAbs do prevent the individual from virus acquisition and are therefore high on the wish list for an HIV-1 vaccine.

A number of Env properties prevent the efficient induction of bNAbs in natural infection and in a vaccination setting. The variable domains, which differ in sequence and length between viral strains, mask the conserved regions of the Env glycoprotein (34-39). Moreover, Env is highly glycosylated and glycosylation also limits the induction of Abs to the conserved regions. Since these glycans are generated by the host glycosylation machinery, the immune system generally sees them as “self”, making it difficult to raise Abs targeting these glycans. Nevertheless a number of glycan targeting bNAbs have been identified from HIV-1 infected individuals (40-45). Furthermore, it is thought that the humoral response focuses on non-neutralizing decoy epitopes, which are exposed on non-functional Env forms due to the instability and structural dynamics of Env. Such non-neutralizing epitopes can be exposed on the surface of HIV-1 particles, on infected cells or on monomeric gp120 shed from Env trimers (46-49).

In addition to the structural properties of Env that steer the specificity of Ab responses away from bNAb responses, Env can also restrain the quantity of the Ab response. Env Ab responses generally require multiple booster vaccinations and even then decay with an unusually short half-life (30-60 days) (50, 51). The N-linked oligomannose glycans on Env were shown to actively suppress immune cell functioning (52-55) which might contribute to these phenomena. Consistent with this, it was shown that de-mannosylated gp120 was more
immunogenic than unmodified gp120 in mice (56). Taken together, Env is a poor immunogen due to a large variety of properties.

**An HIV-1 vaccine**

An ideal HIV vaccine should induce both humoral and cellular immune responses. Vaccines focusing on either the T-cell arm or the humoral arm of the immune system have not been protective. For instance, the first two Ab-based vaccines, comprising a subtype B gp120 (VAX004) (57), or a mixture of subtype B and E gp120s (VAX003) (58), both failed, whereas the trial that tested Gag, Pol and Nef B (STEP trial) to induce CTL responses, was stopped prematurely because the vaccine increased the risk of HIV-1 infection (59). A modest success was achieved with a vaccine comprised of a non-replicating recombinant canarypox vector prime (ALVAC-HIV) followed by a recombinant subtype B/E gp120 protein boost (RV144/Thai trial). The RV144 trial has raised hopes that a protective HIV-1 vaccine may be achievable.

The immunogen used in several vaccine trials was monomeric gp120. Although gp120 exposes most of the bNAb epitopes, it acts as an immune decoy because it sheds after binding to CD4 receptor therefore, gp120 can induce binding Abs but these will lack broadly active neutralization capacity. For instance, monomeric gp120 was also included in RV144 trial and no bNAbS were elicited. However, in this trial, a modest protections was observed related to antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated virus inhibition (ADCVI) (60).

A successful HIV-1 vaccine will very likely need to elicit bNAbS. In support of this thought, several studies in non-human primates show that passive immunization with bNAbS can be protective against HIV and SHIV (61-66). Despite the aforementioned structural difficulties that Env evolved to limit the induction of bNAbS, numerous bNAbS have been isolated from HIV-1 infected individuals indicating that such Abs can be made during natural infection. Such bNAbS can neutralize different viruses from various subtypes and target different regions of the Env such as the CD4 binding site (b12, VRC01, VRC-PG04, NIH45-46, 3BNC60)(67-71), the outer domain glycan patch centered around the glycan at N332 (2G12, PGT121, PGT128, PGT135) (40, 43-45, 72, 73), a quaternary structure dependent epitope cluster in the V1V2 domain at the trimer apex (PG9, PG16, PGT145, VRC26, CH01-04) (73-76), or another quaternary structure dependent epitope cluster at the gp120-gp41 interface (PGT151, 35O22) (77-79) and the MPER in gp41 (2F5, 4E10, 10E8) (80-83).
HIV-1 Env as HIV-1 vaccine immunogen

The only relevant target for NAbs is the Env glycoprotein on the HIV-1 virion surface. The enormous diversity of HIV-1 sequence and phenotype, including neutralization sensitivity, created the need for efficient classification of viruses based on their neutralization sensitivity. Accordingly, viruses were ranked into four groups with very high (tier 1A), above-average (tier 1B), moderate (tier 2), or low (tier 3) sensitivity to antibody-mediated neutralization (84). “Real life” primary HIV-1 isolates are usually of tier 2 level neutralization-sensitivity. Vaccines should therefore aim to induce heterologous tier 2 neutralization, but this has not yet been achieved by Env-based immunogens.

Considering the fact that monomeric gp120 exposes multiple decoy epitopes, in order for a subunit HIV-1 vaccine to induce NAbs it might have to include good mimics of the trimeric cleaved Env spike. With this aim in mind our lab has generated a stable Env trimer, SOSIP gp140, which has superior antigenic properties compared to gp120. Stabilized SOSIP gp140 was generated by introducing a disulfide bond between gp120 and gp41, by substitutions A501C and T605C (85), complemented by an I559P substitution to stabilize the trimeric interactions (86), and a hexa-arginine (R6) motif to replace the native furin recognition site in order to enhance protein cleavage (87). The constructs used in chapters 2-7 of this thesis were based on the early generation JR-FL SOSIP.R6 gp140 to which we added a GCN4-based trimerization domain (IZ), as well as costimulatory molecule domains (APRIL or CD40L). The additions at the C-terminus renders JR-FL SOSIP.R6 gp140 largely uncleavable (88, 89). As such these trimers cannot adopt a native-like structure (89, 90) and will be antigenically different than cleaved trimers (91, 92). During the course of the studies presented in this thesis, a next generation SOSIP gp140 became available, BG505 SOSIP.664, based on the subtype A transmitted/founder (T/F) virus BG505 (93), and this trimer was used in chapter 8. In addition to the A501C, T605C, I559P and hexa-arginine substitutions mentioned above, BG505 SOSIP.664 contains two more modifications compared to the natural BG505 sequence: A T332N substitution to restore bNAb epitopes that required the glycan at N332 and the MPER motif was truncated to improve trimer solubility and reduce aggregate formation (94, 95). Detailed characterization of BG505 SOSIP.664 revealed that it was recognized efficiently by bNAbs but not by most non-neutralizing Abs, thus it seems a good mimic of the native spike. BG505 SOSIP.664 is used for several structural studies (96, 97) and immunizations of rabbits with BG505 SOSIP.664 showed that it improved the breadth of tier 1 neutralization and result in autologous tier 2 neutralization (98).
**Improving the Env immunogenicity by fusion of molecular adjuvants**

Despite the improvements in antigenicity of soluble trimeric Env s, considering the immunesuppressive functions of Env mentioned above, even a perfectly engineered native Env spike mimic might not be sufficiently immunogenic. Several studies attempted to link Env to costimulatory molecules with the aim of targeting Env to specific immune cells and/or trigger the activation of immune cells to improve the response against Env. In contrast to just mixing adjuvant and antigen, the linking strategy ensures that the antigen and the adjuvant will not be separated from each other and that both molecules will reach and activate the same immune cells, generally the APCs (89, 99-107). In previous studies, Env, mostly in the form of monomeric gp120, was fused to proinflammatory chemoattractants such as macrophage inflammatory protein-2 (MIP-2), beta-defensin 2, monocyte chemoattractant protein-3 (MCP-3/CCL7), macrophage derived chemokine (MDC/CCL22) to target the Env to immature DCs (108, 109) and some of these constructs achieved improved Env-mediated Ab responses. Targeting DCs which take up, process, and present the antigen pieces to T cells, contributes in particular to cellular immunity whereas the induction of bNAbs (also) requires naïve B cells to be in contact with intact, i.e. unprocessed, antigen. Therefore targeting intact Env to B cells is a relevant approach for the induction of NAbs.

Molecules such as IFN-γ, TNF-α, Flt-3 ligand and CTLA4 were also fused to Env and improved the Env-binding Ab titers in mice compared to the Env immunized mice (110-113). C3d, which is a proteolytic fragment from complement that is cleaved after antigen binding (114), was fused to gp120 and gp140 from different strains and improved the Env-specific Ab response (115-120). TNF superfamily members CD40 ligand (CD40L), B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) were also fused to the C-terminus of trimeric Env. From these three constructs, Env-CD40L protein activated DCs to produce elevated levels of IL-6, IL-10, IL-12-p70 and TNF-α and DCs pre-stimulated with Env-CD40L primed naïve T lymphocytes to secrete IFN-γ. In immunization studies, Env-CD40L did not significantly increase the gp120-specific Ab responses but the Env-APRIL molecule, which was also functional in in vitro assays, induced stronger anti-Env binding responses in rabbits and had strong neutralization activity of heterologous tier 1 viruses (89, 90).
Scope of this thesis

Various immuno-evasive properties of the HIV-1 Env subunit limit the value of traditional vaccine strategies and urge us to explore different vaccine approaches. We have been working on a vaccine strategy in which we designed dual-functional fusion molecules of trimeric HIV-1 Env with a costimulatory molecule. First, we generated Env-GM-CSF trimers by replacing the V1V2 domain of Env with GM-CSF (Chapter 2). This construct was further optimized in the study described in Chapter 3. Another fusion molecule, Env-IL-21, was generated using a similar design strategy by replacement of the V1V2 domain with IL-21 and its activity was assessed in vitro (Chapter 4). Next we studied both Env-GM-CSF and Env-IL-21 trimers in vivo by immunizing rabbits and mice (Chapter 5). We found that both molecules induced a potent immune response against the cytokine inserts, irrespective of their location in the fusion protein. In Chapter 6 we describe in vitro and in vivo studies on the bioactivity of Env trimers fused to APRIL. We also studied the immunogenicity of the GCN4-based isoleucine zipper (IZ) trimerization domain that is often used in vaccines and therapeutic proteins as well as in our Env-cytokine chimeras (Chapter 7). Furthermore, we describe a strategy to immunosilence this trimerization domain by covering its protein surface with glycans. Finally, in the last research chapter (Chapter 8), we report on the in vitro and in vivo activity of APRIL fused to a next generation native-like Env trimer mimetic, BG505 SOSIP.664 gp140. We note that all our immunization were done with plasmids encoding Env-costimulatory fusion molecules. Different immunization approach may lead to different effects.

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