Chapter 1

Introduction

*Parts of this chapter have been published in:*
HIV and AIDS

Despite considerable progress in the last decade, the Acquired Immunodeficiency Syndrome (AIDS) is still one of the most prevalent and deadly infectious diseases [1]. AIDS was recognized as a disease in 1981 [2] and its causative agent, the human immunodeficiency virus (HIV) was discovered in 1983 [3,4]. HIV infects cells of the immune system, which over time results in severe opportunistic infections and the ultimate death of the infected individual. HIV has infected around 78 million people since 1981 and caused around 39 million deaths (www.who.in/gho/hiv/). Despite great progress in the last decade with combination antiretroviral therapy (cART), there were a reported 1.2 million AIDS-related deaths and 2.0 million new infections in 2014 and 0.8% of all adults worldwide are living with HIV [5].

HIV is a lentivirus that belongs to the family of the Retroviridae. This family of viruses is unique in that they use a reverse transcriptase enzyme (RT) to copy their single stranded RNA genome into DNA [6]. HIV consists of two main groups: the less virulent HIV-2 group, which is largely confined to parts of West Africa, and the HIV-1 group of viruses, which are responsible for the majority of the pandemic. Most research efforts are therefore geared towards HIV-1. Several zoonotic transmissions of simian immunodeficiency virus (SIV) variants from primates to humans took place that gave rise to distinct HIV-1 types (O, N, P and M) [7]. Only group M (“Major”) HIV-1 viruses spread worldwide to cause the pandemic, which started around Kinshasa in the Democratic Republic of Congo, while O, N and P viruses are mostly restricted to small regions in Cameroon [8,9]. Group M can be divided in at least nine subtypes as well as circulating recombinant forms that sometimes can be found in distinct geographical locations, i.e. subtypes A and C are prevalent in Africa, whereas subtype B viruses are endemic in the Americas, Europe and Australia. The sequence diversity between subtypes is around 25 to 30%, while intra-subtype diversity is around 10-15%.

HIV-1 replication cycle

HIV-1 attaches to CD4+ immune cells (mainly T cells, macrophages and dendritic cells) using its envelope glycoprotein (Env) spike on the virion surface. This trimeric protein complex undergoes significant conformational changes leading to the fusion of the viral and target cell membranes [10]. Subsequently, the viral core is released in the cytosol and the RNA genome is reverse transcribed into double stranded DNA (dsDNA) by the RT enzyme [11]. This enzyme is error-prone and makes around ~1.1 mutations per viral genome, which has contributed to the high sequence diversity between HIV-1 isolates [12]. Subsequently, the viral dsDNA is incorporated in the genome of the host cell, thus forming the so-called “provirus”. Subsequently, the provirus is transcribed and this produces numerous viral copies that can infect additional CD4+ T cells. A small population of proviruses remains transcriptionally inactive, resulting in latent infection of a pool of infected cells. However, these latently infected cells cannot be recognized by cells of the immune system, such as CD8+ killer T cells. However, these latent proviruses sometimes become active and cause new infections of healthy CD4+ T cells, exhausting the immune system over time. This eventually leads to a seriously compromised immune system, which is not able to overcome opportunistic infections.
HIV-1 vaccines

Vaccines have been one of the most successful medical interventions in human history. Because of vaccines we have eradicated small pox and we are now close to eradicating poliomyelitis and perhaps mumps [13]. However, human trials with vaccines against HIV have been dramatically less successful. There have been four major clinical phase IIb/III trials with HIV vaccine candidates and most of these vaccines did not confer any protection against infection or, in one trial, only a very modest level of protection [14–16]. Major improvements in vaccine design are probably needed to induce improved immune responses.

A successful HIV-1 vaccine should probably induce neutralizing antibodies (NAbs) that are able to neutralize the virus before it can infect CD4+ T cells. However, the only target for NAbs on the virus particle is Env, which is not well recognized by the humoral immune system and displays a high degree of sequence diversity, making the induction of broadly neutralizing antibodies (bNAbs), i.e. antibodies that can neutralize a wide variety of circulating HIV-1 strain, a serious challenge.

Figure 1. Genomic organization and structure of the HIV-1 Env trimer. A. Linear representations of HIV-1 Env. Left: complete HIV-1 Env: C1-C5: constant regions 1-5; V1-V5: variable regions 1-5; HR1 and 2: heptad repeat 1 and 2; MPER: membrane-proximal region; TM: transmembrane region; CT: cytoplasmic tail. Middle: design of a typical soluble uncleaved Env trimer: red cross: inactivated furin cleavage site; foldon: heterologous trimerization domain. Right: design of SOSIP.664. SOS: inter-subunit disulphide bond; I559P: trimer stabilizing gp41 mutation; R6: hexa-arginine motif for more efficient furin cleavage. SOSIP.664 is truncated before the MPER (position 664). B. Different structural models of the HIV-1 Env trimer. Left: one gp140 heterodimer is highlighted with the gp120 subunit in orange and the gp41 ECTO in dark grey. The inner domain of gp120 facing the central trimer axis in yellow. Middle: the complete trimer in cartoon representation. Highlighted are the bNAb epitope clusters in colored spheres (CD4bs; V1V2 on the trimer apex; V3/OD-glycans; gp120/gp41 interface) and the the MPER (not solved in the crystal structure, depicted with a dotted line). Note that the non-neutralizing V3-loop (magenta) is buried. Right: the glycan shield of the Env trimer. Glycans were modeled using Glyprot (http://www.glycosciences.de/modeling/glyprot/) according to Table 1 and Table S2 of Guttman et al. [176]. Images in Fig. 1 were prepared in PyMol [177] using the crystal structure of BG505 SOSIP.664 (PDB: 4TVP [22]). Figure is from [178].
**HIV-1 Env structure**

Env is synthesized as a gp160 precursor protein in the endoplasmic reticulum (Fig. 1A). The gp160 sequence contains numerous potential N-linked glycosylation sites (PNGS) to which N-linked oligomannose glycans are attached cotranslationally. The resulting gp160 glycoprotein subsequently oligomerizes in the endoplasmic reticulum, forming trimers. During transport through the Golgi, further glycan processing takes place, at least on a subset of glycans, and the gp160 glycoprotein trimer is cleaved by furin proteases, resulting in a heavily glycosylated envelope trimer consisting of heterodimers of gp120 and gp41 subunits [17,18] (Fig. 1B). The three gp120s, contributing most to the exposed surface of Env, are linked by weak non-covalent interactions to the membrane-anchored gp41 subunits. Gp120 comprises five conserved regions (C1-C5) and five highly variable surface exposed regions (V1-V5). Gp41 consists of an external domain with two heptad repeat regions, a transmembrane domain and a cytoplasmic tail (Fig. 1A). HIV-1 infection of CD4+ immune cells is facilitated by the Env spike, which initially binds to the CD4 receptor. This is followed by dramatic conformational changes in Env, whereby specific protein patches on gp120 are exposed that enable binding to a chemokine receptor, CCR5, and CXCR4. Additional changes occur in the gp41, which first shoots a hydrophobic fusion peptide into the target membrane and then assumes a six-helix bundle structure that triggers the merger of the viral and cellular membranes, depositing the viral core with the genetic material into the cytosol [10].

**Env as antibody target**

Because Env is the only external viral protein on the virion surface, it is the only target for NAbs. However, HIV-1 has evolved in many ways to avoid recognition by and induction of NAbs [19]. First, the error-prone HIV-1 reverse transcription process leads to an enormous sequence variability in the HIV-1 genome that facilitates escape from NAbs. This not only makes it difficult for the B cells to develop an effective antibody (Ab) response in an individual patient, but it has also resulted in a highly diverse virus population, even when compared to other highly variable viruses, such as influenza [20]. The few epitopes in Env that are conserved, such as the CD4 binding site (CD4bs) and the co-receptor binding site, are mostly protected from Ab recognition since these are located inside the trimeric spike and/or covered by variable loops [21]. Second, the Env trimer is heavily glycosylated and it is estimated that only ~3% of the protein surface is accessible for Abs without interacting with the surrounding glycans [22] (Fig. 1B). Third, the conformation of Env is not fixed, but “breathes” between different conformational states, which hampers an efficient interaction with low-affinity naïve B cell receptors [21,23]. Fourth, the virion only contains a limited number of around 8-12 Env spikes on its surface, which reduces avidity-based binding and activation by B cell receptor cross-linking [24,25]. Fifth, because of the weak interaction between gp41 and gp120, some virions shed gp120 subunits from the Env trimer complex [26], resulting in the exposure of internal non-neutralizing epitopes and the elimination of quaternary structure dependent NAb epitopes [27] (Fig. 1B). During affinity maturation, B cells that target these non-neutralizing epitopes might compete with B cells that target neutralizing epitopes, hampering the induction of bNAbs [28]. Together, these assets make the HIV-1 Env trimer a notoriously hard target for raising bNAbs, in both infection and vaccination.
Abs against Env
Detailed studies in HIV-1 infected patients have shown that the humoral immune system can circumvent the obstacles for bNAb induction listed above, but it appears that bNAb development often is the result of extensive affinity maturation driven by an iterative process of viral escape from NAbs and renewed NAb affinity maturation [29–31]. Unfortunately, these patients do not benefit from their own bNAbs, because by the time they emerge, the viral load and variation facilitate escape even from these bNAbs [32]. However, bNAbs provide protection when used in passive immunization experiments [33–35], and thus would potentially be protective if they could be induced by vaccination. Most bNAbs have several unusual properties. First, many bNAbs, in particular those directed to the V1V2 apex, contain very long heavy complementary determining region 3 (HCDR3) loops, which are very rare in the naïve B cell receptor repertoire [36]. Second, most bNAbs have undergone extensive affinity maturation, which suggests that one has to precisely guide the Ab maturation process by vaccination to raise such bNAbs [37]. Third, many bNAbs are reactive with host proteins and autoreactive B cells are usually deleted during clonal B cell selection [38,39]. Despite these unusual properties, approximately 20-30% of the patients develop potent bNAbs, illustrating that the human humoral immunize system is capable of making them despite all HIV-1’s defenses against them [40,41].

The isolation of bNAbs has fueled hope that an HIV-1 vaccine is possible, and has yielded a wealth of knowledge on the epitopes on Env that may serve as suitable templates for vaccine design [27,37]. Moreover, detailed studies in patients have revealed the affinity maturation pathways that led to bNAbs and provided hints for iterative vaccine design [29,30,42]. This has sparked a whole new branch of HIV-1 vaccine research that aims to mimic this Ab affinity maturation of bNAbs [43]. First, such vaccine strategies involve the design of immunogens that can activate the correct naïve B cell receptor (i.e. the germline precursors that have the potential to become bNAbs) [44,45], followed by sequential vaccinations with other immunogens to steer the Ab development toward neutralization breadth.

Epitope studies have also revealed that many bNAbs target, at least partly, the Env glycan shield, revealing it as a major site of vulnerability [46–55]. Analyses of viral and soluble native-like Env trimers showed that this glycan shield is so densely packed that restricted access to glycans leads to underprocessing by glycosylation enzymes. As a result native Env mostly contains oligomannose glycans [56–58] that are more readily recognized by the immune system as non-self than processed, complex-type, glycans [59,60].

Env immunogens
Over the years, many different variants of Env proteins have been evaluated as vaccines to raise NAbs. We will provide an overview not in historical order, but starting with the smallest Env-derived immunogens – peptides – and concluding with the largest Env-based immunogens: particulate antigens. The different forms of Env antigens are summarized in Figure 2.
### Immunogen

<table>
<thead>
<tr>
<th>EP</th>
<th>Antigenicity</th>
<th>Immunogenicity</th>
<th>References</th>
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<tbody>
<tr>
<td>N.A.</td>
<td>Peptides can contain linear epitopes. But there are no restrictions on the angle of approach for Ab binding.</td>
<td>Peptides induce epitope-specific binding Abs only and Tier 1 NAbs in the case of V3 peptides.</td>
<td>[61-64, 71-73]</td>
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<tr>
<td>N.A.</td>
<td>Epitope scaffolds have been described for MPER, CD4bs, V3-glycan and V3 epitopes.</td>
<td>Scaffolds induce specific Abs directed to the epitope, but NAbs are limited to Tier 1 viruses. The scaffold can also be immunogenic.</td>
<td>[74-77, 79-81, 85]</td>
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<tr>
<td>N.A.</td>
<td>Gp120 OD proteins bind to most non-quaternary gp120 bNAbs, such as CD4bs and sometimes V3-glycan bNAbs. Gp120 OD immunogens bind to non-NAbs directed to the V3 loop and the CD4bs.</td>
<td>Gp120 OD proteins induce binding Abs and Tier 1 NAbs.</td>
<td>[99, 92-95]</td>
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<td>N.A.</td>
<td>Gp120 core proteins bind bNAbs directed to the CD4bs, but lack epitopes that are located in the V1/V2 and V3 loops. Core gp120 proteins usually also bind to non-NAbs directed to the CD4bs.</td>
<td>Gp120 core proteins induce CD4bs-specific Abs and NAbs against Tier 1 viruses.</td>
<td>[84, 98-100]</td>
</tr>
<tr>
<td>N.A.</td>
<td>Gp120 binds to all non-quaternary gp120 bNAbs, such as CD4bs and sometimes V3-glycan bNAbs. Gp120 OD proteins bind to PG9 (a V1/V2 apex bNAb). Monomeric gp120 shows reactivity with a large number of non-NAbs.</td>
<td>Gp120 induces binding Abs and Tier 1 NAbs and, very rarely, Tier 2 NAbs. Gp120 is the only Env immunogen that has been tested in large phase III clinical trials.</td>
<td>[15, 66, 101, 102, 104-106, 111, 113, 114]</td>
</tr>
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<td>N.A.</td>
<td>Gp140 trimer induces increased binding Ab and Tier 1 NAb titers compared to gp120, but no Tier 2 NAbs. Gp140 proteins raise non-NAbs against the CD4bs and trimerization domains used for stabilizing gp140 trimer are immunogenic. Gp140 trimer have been evaluated in small phase I clinical trials.</td>
<td>SOSIPs can induce binding Abs and Tier 1 NAbs and, importantly, induce NAbs against the autologous Tier 2 virus. In mice, SOSIPs seem to induce non-NAbs against an epitope located at the bottom of the trimer.</td>
<td>[66, 104, 106, 110, 112, 115, 117-125, 127-130, chapter 2, chapter 3]</td>
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<tr>
<td>N.A.</td>
<td>SOSIP proteins bind to most bNAbs, including quaternary dependent bNAbs (e.g. PGT151, PGT145, PG16) and bind efficiently to most non-NAbs directed to the V3 loop, CD4bs and gp41.</td>
<td>SOSIPs can induce binding Abs and Tier 1 NAbs and, importantly, induce NAbs against the autologous Tier 2 virus. In mice, SOSIPs seem to induce non-NAbs against an epitope located at the bottom of the trimer.</td>
<td>[22, 66, 104, 116, 124, 125, 131-136, 138-141, 145-150, 151, chapters 4-7]</td>
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<tr>
<td>N.A.</td>
<td>Gp120 induces binding Abs and Tier 1 NAbs and, very rarely, Tier 2 NAbs. Gp120 is the only Env immunogen that has been tested in large phase III clinical trials.</td>
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<td>[44, 96, 169]</td>
</tr>
<tr>
<td>N.A.</td>
<td>Modified OD variants (e.g. eOD-GTR) are designed to bind to CD4bs and their inferred germline versions.</td>
<td>In knock in mice, eOD-GTR can induce CD4bs-directed Abs, but these Abs are mostly non-neutralizing.</td>
<td>[chapter 8, chapter 9]</td>
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**Figure 2.** Overview of the different immunogens that are discussed in this review. The schematic representations in the left column are not drawn to scale. The white scale bar in the EM images is 10 nm. N.A.: not applicable.

Figure is adapted from [178].
Peptide immunogens

Short Env-derived peptides, containing no or only simple secondary structure, were among the first immunogens tested in the search for an HIV-1 vaccine. Early generation HIV-1 Env peptide vaccines were based on linear epitopes such as the V3 loop in gp120 and the membrane-proximal external region (MPER) in gp41 (Fig. 2). V3 peptides raised NAbs [61,62], but enthusiasm waned quickly when it became apparent that the viruses that were neutralized by these sera were atypically neutralization sensitive and often laboratory-adapted virus strains, i.e. Tier 1 viruses [63]. Moseri and colleagues showed that constraining the V3 peptide with a disulfide bond to better mimic the native V3 conformation resulted in a higher Ab response [64]. However, the major drawback remains that neutralization-resistant (Tier 2) primary virus isolates generally do not expose the V3 (Fig. 1) and are therefore not neutralized by V3-directed Abs [65–67].

The isolation of bNAbs against the well-conserved MPER region, such as 2F5 and 4E10 that recognize apparently linear epitopes [68,69], motivated the design of MPER peptides as immunogens to raise 4E10 or 2F5-like bNAbs [70]. The MPER contains many hydrophobic amino acids and is thought to associate with the viral membrane and therefore MPER immunogens are often formulated with liposomes or grafted onto a heterologous carrier to improve solubility and conformation [70–73]. Immunization with MPER peptides induced Abs against the MPER epitope: however, at best these Abs weakly neutralized Tier 1 viruses, similar to the results obtained with V3 loop peptide immunogens [70,72,73]. The fact that most HIV-1 bNAbs bind to complex conformational epitopes often involving glycans besides protein suggests that vaccination with peptides derived from other bNAb epitopes will probably not yield NAbs.

Epitope-scaffolds

Epitope-scaffold immunogens contain one, or sometimes a few, bNAb epitope(s) grafted onto a heterologous protein scaffold to ensure proper presentation of the conformational epitope(s) [74–77] (Fig. 2). Proof-of-concept for epitope-scaffolds as vaccines was delivered with an epitope-scaffold that presented a NAb epitope of the fusion protein (F) of respiratory syncytial virus (RSV). The epitope-scaffold elicited NAbs that could provide protection against RSV challenge in monkeys [78].

For HIV-1, different epitopes-scaffolds presenting a variety of epitopes have been explored. The earliest scaffolds contained the V3 loop. Compared to V3 peptides, epitope-scaffolds were devised to present a better mimic of the V3 on the native Env spike [79,80]. These scaffolds elicited NAbs against several Tier 1 viruses but not against primary Tier 2 viruses, as might be expected from V3 loop specificities. An interesting take on V3-directed immunogens is the design of a miniprotein expressing the glycan-dependent V3-base epitopes of the bNAb families PGT121–PGT125 and PGT126–PGT128 [81]. This miniprotein was based on an engineered gp120 outer domain (OD) protein [52] (see below), which was further reduced in size, yielding a scaffolded glycosylated mini-V3-loop [81]. However, no immunogenicity experiments have been published yet. Similarly, immunogenicity experiments on a computationally designed epitope-scaffold, presented in the epitope for CD4bs bNAb b12, have yet to be reported [74].

Other epitope-scaffolds were designed computationally to recapitulate the MPER bNAb epitopes 2F5 or 4E10 [75,76]. When these MPER epitope-scaffolds were used
as immunogens in rabbits and guinea pigs, Abs with similar specificities as 2F5 or 4E10 were raised [75,76]. However, none of these showed any neutralizing activity, despite binding to the epitopes with high affinity [75]. Both 4E10 and 2F5 show a high degree of autoreactivity and B cells that express 4E10 or 2F5 in knock-in mice are largely deleted from the B cell pool [82,83]. Thus, Abs with similar specificities and characteristics might be difficult to induce [39]. Moreover, the accessibility to the MPER on virion-associated Env is very restricted [70] and MPER epitope-scaffolds do not recapitulate the constraints on the angles of approach for NAbs to the MPER on the viral spike. As a result, MPER epitope-scaffolds probably induce Abs that are able to bind the exposed MPER, but are not able to access the same epitope on the native Env spike.

In conclusion, no HIV-1 Env epitope-scaffold has yet been able to mount an effective neutralizing response in animals, but the success of an epitope-scaffold in RSV vaccine research has provided a proof-of-concept [78]. To be successful against HIV-1, the restricted angles of approach to bNAb epitopes on the virion surface need to be considered. Epitope-scaffolds can be manipulated, for example by adding glycans to prevent Abs with the wrong approach angles from binding [84]. Alternatively, one could also design scaffolds that carry epitopes that have less restricted approach angles on the trimer, such as the variable regions 1 and 2 (V1V2) [51] (Fig. 1). However, the amino acid sequence of V1V2 is highly variable, and the only effective V1V2 bNAbs bind to complex quaternary epitopes that consist of multiple glycans at the apex of the trimer. Recapitulating such complex epitopes on scaffolds has proven to be difficult thus far [81]. Mimicking the correct glycan composition and configuration is also difficult and a glycan-based epitope-scaffold that mimicked the oligomannose glycan-specific 2G12 epitope did not raise NAbs [85]. Another potential hurdle is that the scaffolds themselves might be immunogenic [79], and if immunodominant, scaffold-specific B cells might compete with B cells that target the NAb epitope [86], thereby potentially interfering with the induction of NAbs.

Gp120 outer domain proteins
A number of vaccine approaches involved the outer domain (OD) of gp120, essentially consisting only of the exposed half of the molecule; the inner domain faces the inside of the viral Env trimer, which is normally inaccessible for NAbs [87,88] (Fig. 1). OD constitutes the outside of the Env trimer, containing the variable loops 3-5, its glycan shield and also contains several known bNAb epitopes [89–91]. OD protein immunogens have induced Ab responses, but these were limited to binding Abs and/or NAb responses to Tier 1 viruses [89,92–94]. A minimal OD protein (OD4.2.2) lacking the V3-loop was used to study the epitope of the VRC-PG04 bNAb [95]; however, the immunogenicity of this potential immunogen was not reported. Similar deltaV3 OD proteins did not yield improved Ab responses [89]. Despite the fact that OD proteins efficiently present the bNAb epitopes of the CD4bs, no NAbs against Tier 2 viruses have been raised.

Therefore, a new class of engineered OD (eOD) immunogens have been designed [44]. eOD binds not only to most CD4bs bNAbs, e.g. VRC01, 3BNC60 and PGV04, but also to the inferred precursors of these bNAbs [44]. An improved version of this protein (eOD-GT8) was used to immunize mice carrying the heavy chain (HC) gene of mature CD4bs bNAb 3BNC60 and subsequently induced CD4bs directed Abs, demonstrating the ability
of eOD-GT8 to target and activate specific B cell receptors in vivo [96]. Despite carrying the HC of 3BNC60, these Abs only neutralized viruses that lack the glycan on position 276 [96]. The relatively open eOD-GT8 CD4bs might induce Abs that are incompatible with the approached angles that are allowed in the context of the native Env trimer.

**Gp120 core proteins**

Gp120 core proteins consist of the inner domain and OD of gp120, but lacking the V1, V2, V3, and sometimes the N- and C-termini. Most core gp120 immunogens are engineered to efficiently present the CD4bs, an epitope that can be targeted by different bNAbs from several lineages [97]. Core gp120 proteins have been key for the isolation and characterization of CD4bs-directed bNAbs, such as VRC01 [98]. When used as immunogens, these core gp120 proteins usually did not induce Tier 2 NAb responses efficiently, but sporadic Tier 2 neutralization was observed in one study that used a high-dosage vaccine regimen in rabbits [99]. Different versions of core gp120 proteins have been developed, such as a version that was locked in the CD4-bound state, increasing co-receptor binding site exposure [100]. Furthermore, hyperglycosylated core gp120s have been reported [84], but none of these strategies improved NAb induction.

**Monomeric gp120**

Gp120 is the most surface-exposed protein subunit of the two Env subunits and has been evaluated extensively as a vaccine candidate [14,15,101,102]. Gp120 is composed of an OD that might raise NAb, but also the inner domain that could raise non-neutralizing Abs (non-NAbs) that do not neutralize viruses at all or are only effective against (lab-adapted) neutralization-sensitive viruses (Tier 1), which appear to have a more “open” spike conformation [23,67,103]. Monomeric gp120 proteins bind bNAbs to the CD4bs, OD-glycans and V3-glycans, and (rarely) V1V2-glycan bNAbs, but not bNAbs such as PGT145 or PG16 that are specific for or prefer the trimer [104]. Monomeric gp120s also bind to many non-NAbs that are potential immunological diversions from bNAb epitopes [28,86,104].

When used as immunogens, monomeric gp120 proteins induce Abs that can neutralize Tier 1 viruses, but not the more neutralization-resistant primary (Tier 2) viruses [105–108]. A notable exception is BG505 gp120, which induced some NAb against the autologous Tier 2 virus, although at lower titers compared to a native-like BG505 trimer (discussed later) [66]. The general lack of neutralizing activity against Tier 2 viruses might be explained by the potential immune interference of non-NAb epitopes on gp120, and/or by the inadequate presentation of NAb epitopes. For example, quaternary epitopes such as those for the V1V2-apex bNAb PG16 and PGT145 and the gp120-gp41 interface bNAb PGT151 and 35O22 are not presented by gp120 [47,48,50,104,109]. Furthermore, while bNAb epitopes such as those against the CD4bs are presented by gp120, there are less restrictions on the angles of approach of the CD4bs such as present on the trimer, allowing the induction of non-NAbs against the CD4bs to prevail [104,110] (Fig. 2).

To reduce the exposure of non-NAb epitopes, gp120 has been altered, for example by removing variable loops yielding gp120 core proteins (see above) or by covering unwanted epitopes by attaching N-linked glycans [105,111–114]. One study showed that heavy glycosylation of the V3-region dampened the Ab response to the
glycosylated epitope and increased the Ab response to other parts of gp120 [113]. However, other studies were unable to demonstrate this refocusing effect [105,112]. Together, these studies demonstrated that although engineered monomeric gp120 proteins raise a humoral response, this response is non- or only weakly neutralizing. These results provided the rationale to develop immunogens that mimic the trimeric Env spike more adequately [115].

**Non-native (uncleaved) Env trimers**

Soluble Env trimers are generated by first truncating Env before the transmembrane and cytoplasmic tail to produce soluble gp140 proteins, leaving gp120 and only the ectodomain of gp41 (gp41\_ECTO). However, without the stability provided by the viral membrane, soluble gp140 proteins are extremely unstable and rapidly dissociate into gp120 and gp41ECTO [116]. To create soluble trimeric gp140 these proteins have been stabilized by removing the native furin cleavage site between gp120 and gp41 to prevent shedding of gp120 from gp41\_ECTO, yielding uncleaved gp140 (gp140\_UNC) [115,117-119]. Furthermore, to induce trimerization of gp140\_UNC, many designs included a heterologous trimerization motif at the C-terminus [99,106,120-123].

Most of these immunogens indeed formed trimers, but structural analyses using electron microscopy (EM) show that these trimers do not resemble the native Env spike [66,110,124-126] (Fig. 2). The gp140\_UNC proteins do not assume the compact trimeric propeller-blade structure that is typical for the pre-fusion state of viral Env, but the gp120s suspend from the gp41 and trimerization domain as drooping flowers in a vase (Fig. 2). The EM images also explain that gp140\_UNC trimers expose non-NAb epitopes that are normally occluded on the viral Env spike (CD4 induced epitopes, inner domain of gp41 and the V3-loop), in some respect mimicking the antigenicity of gp120 [104,110,125] (Fig. 2). Biochemical analyses also revealed that many of the gp140\_UNC trimers form aberrant disulfide bonds, indicative of misfolded proteins [127,128]. Finally, the glycan shield of gp140\_UNC trimers markedly differs from that of virion-associated HIV-1 Env, which consists mostly of unprocessed oligomannose-type glycans [56,58]. Because the gp120 subunits are splayed out in gp140\_UNC trimers (Fig. 2), their glycans are more accessible to glycan-processing enzymes yielding processed complex-type glycans [57]. Thus, gp140\_UNC trimers do not recapitulate the native glycan shield, which might be important for their immunogenicity since most bNAbbs interact with glycans, sometimes with high preference for native-like glycan composition [48,60].

None of the tested gp140\_UNC trimers could induce NAbbs against primary Tier 2 viruses [65,106,107,115,129]. In some cases, gp140\_UNC trimers were actually less potent than the sequence-matched monomeric gp120 protein, at least at inducing Tier 2 NAbbs [66]. Detailed analysis of two Abs isolated from gp140\_UNC-immunized macaques revealed that gp140\_UNC trimers induced CD4bs-directed Abs that did not neutralize primary HIV-1 isolates [110]. EM studies showed that the two Abs approached the CD4bs from an angle that is not compatible with binding to the functional Env spike [110]. Together these studies have shown that despite the fact that gp140\_UNC proteins are able to form trimers, these immunogens do not resemble the native spike and thus do not raise Abs that can neutralize primary Tier 2 viruses. When a gp140\_UNC trimer was used in a human clinical trial, none of the human volunteers developed NAbbs against circulating,
primary viruses [119,130], and gp140<sub>unc</sub> trimers based on similar designs are unlikely to induce protective NAb responses in humans.

Native-like Env trimers

Thus far, bNAbs have only been raised in patients and many of them, in particular those that depend on quaternary structure, were probably induced by native Env present on the virus. Therefore, to mimic such responses with vaccination, a soluble mimic of the HIV-1 Env spike is probably necessary. Such immunogens are now becoming available. A collection of native-like Env trimers from different subtypes has recently been described that are based on the “SOSIP” design [66,109,124,131–133] (Fig. 2).

SOSIP proteins were designed iteratively over a period of ~15 years and continue to evolve. It was initially realized that cleaved soluble gp140 proteins readily dissociate into monomeric gp120 and gp41<sub>Ecto</sub> subunits [116,134]. As an alternative to preventing cleavage and to prevent gp120 shedding, a disulfide bond (“SOS”) was introduced that covalently links the gp120 and gp41<sub>Ecto</sub> subunits [116]. Second, trimerization of gp140<sub>SOS</sub> was improved by stabilizing the pre-fusion state of the Env trimer by introducing a helix-breaking Ile-to-Pro mutation (I559P) in the gp41 moiety (“SOSIP”) [134]. Third, to enhance the proteolytic cleavage of gp140 into gp120 and gp41<sub>Ecto</sub>, the natural furin cleavage motif (REKR) was replaced by a more efficient hexa-arginine (R6) motif [135] (Fig. 1A).

The early generations of SOSIP proteins were based on the subtype B clade JRFL and subtype A clade KNH1144 sequences [134–136], of which the latter showed favorable antigenicity and seemed to resemble the overall shape of a trimeric Env spike as viewed by EM [126,137–139]. Rabbits vaccinated with these early-generation SOSIP proteins performed slightly better than rabbits immunized with monomeric gp120, inducing sporadic and weak neutralization against the autologous Tier 2 virus [108,136]. The SOSIP design was further improved by truncation at position 664 (SOSIP.664) to remove the largely hydrophobic MPER region, thereby decreasing aggregate formation [140] (Fig. 1A). EM analysis revealed that these SOSIP.664 trimers very closely resembled the native Env spike at low resolution [141].

Combining the SOSIP.664 design with the Env sequence of the BG505 strain yielded the BG505 SOSIP.664 trimer [109]. The BG505 sequence was derived from an infant infected with a subtype A virus that developed broad neutralization two years after infection [142–144]. BG505 SOSIP.664 bound all bNAbs that could neutralize the autologous BG505 virus (except the MPER bNAbs because the MPER was deleted), including quaternary dependent Abs that target the trimer apex and the gp120/gp41 interface, while reactivity of non-NAbs was minimal [47,48,109,145]. Ab binding to BG505 SOSIP.664 correlated strongly with neutralization of the parental BG505 virus, indicating that SOSIP.664 was a fairly faithful antigenic mimic of the viral spike [109]. Moreover, EM analysis revealed that BG505 SOSIP.664 appeared to have a closed, trimeric shape forming native-like trimers only [109]. Later studies revealed that the disulfide bond pattern of SOSIP.664 trimers are native-like [124] and that the glycan shield of BG505 SOSIP.664 mostly consisted of underprocessed oligomannose glycans, resembling the glycan composition of the viral Env spike [56–58]. Importantly, BG505 SOSIP.664 provided the first high-resolution views of an Env trimer, using cryo-EM and crystallography [22,146–150] (Fig. 1).
Applying the same SOSIP.664 design to other Env sequences yielded native-like trimers from B41 (subtype B), ZM197M and DU422 (both subtype C), and other clades [124,131–133]. The immunogenicity of only BG505 and B41 SOSIP.664 trimers has been described [66]. Both native-like trimers consistently raised NAbs against the autologous Tier 2 virus. The sequence-matched uncleaved BG505 gp140 trimer did not raise NAbs against the autologous virus, supporting the idea that a native-like conformation is required for raising NAbs [66]. The epitopes targeted by the autologous NAbs differed between animals, suggesting that BG505 SOSIP.664 contains several epitopes that can be targeted by Tier 2 NAbs [66]. However, no NAbs were raised against heterologous Tier 2 viruses and a substantial proportion of the Ab response was directed against the immunodominant non-neutralizing V3 loop [66]. Moreover, recent studies in mice demonstrated that SOSIP.664 might contain a non-neutralizing neo-epitope at the bottom of the trimer [151]. Therefore, despite the success of inducing of Tier 2 NAbs, improvements are needed to eliminate the exposure of immunodominant non-NAb epitopes in favor of subdominant bNAb epitopes.

**Nanoparticle-displayed Env vaccines**

While several Env scaffolds, modified ODs, and native-like trimers present promising new paths toward eliciting bNAbs, classical B cell immunology teaches us that monomeric or trimeric proteins are unlikely to be sufficiently immunogenic to raise potent Ab responses [152–154]. Strikingly, the only two effective viral subunit vaccines, i.e. those against hepatitis B (HBV) and human papillomavirus (HPV), are virus-like particles (VLP), consisting of proteins that self-assemble into small nanoparticles that present multiple copies of the native spike (HBV) or outer capsid protein (HPV) [155]. Both HBV and HPV induce potent and long-lasting protective NAb responses.

Particulate presentation of antigens improves Ab responses by a variety of mechanisms, providing the rationale to consider particulate presentation of Env vaccines. First, nanoparticle displayed immunogens cross-link B cell receptors, which improves B cell activation, leading to increased affinity maturation and the development of long-lived plasma cells [154,156]. Second, natural IgMs that circulate through the periphery are able to bind repetitively displayed antigens through high-avidity interactions, which activates the complement system and decreases the threshold for B cell receptor activation [154]. Third, naive B cell receptors might have a low initial affinity for bNAb epitopes, but a repetitive array of low-affinity epitopes increases avidity, potentially activating the right B cell. This might explain why nanoparticle-displayed antigens often induce Abs against additional epitopes compared to antigen alone, thereby increasing neutralization breadth [81,157–159]. Fourth, human self-antigens usually do not contain highly repetitive structures and recurring epitopes are regarded as non-self by B cells [24]. Some HIV-1 bNAbs are cross-reactive to self-antigens and these B cells are deleted during Ab development however, repetitive display of the same HIV-1 epitopes might rescue these B cells [24,153,154]. Fifth, several studies have shown that repetitive display of antigens also induces a long-lived Ab [156,157]. This is especially relevant for Env vaccines, since monomeric gp120 vaccine as well as trimer-based vaccines usually induce Ab responses that are short-lived [15,66,160,161]. Finally, nanoparticle display of Env trimers should prevent recognition by non-NAbs against the trimer bottom [151] (Fig. 1).
Potential suitable particulate systems to display HIV-1 Env antigens include liposomes, inorganic nanoparticles, VLPs or self-assembling protein-based nanoparticles [162]. Some of these platforms have been successfully used to improve HIV-1 Env immunogenicity. Abs in sera of mice that were immunized with liposomes containing gp140$_{UNC}$ trimers targeted more epitopes than trimer immunized mice [159]. Unfortunately, NAb responses in these mice were not assessed [159]. In a similar experiment, using magnetic beads containing a lipid membrane and chemically cross-linked Env trimers, the nanoparticle displayed vaccines raised slightly higher NAb titers than cross-linked Env trimers [163].

Another interesting approach is the use of HIV-1 Env VLPs treated with a cocktail of proteolytic enzymes to remove poorly folded Env trimers [164,165]. Two out of eight rabbits immunized with these VLPs raised potent Tier 2 NAbs against the autologous JR-FL virus naturally lacking the N197 glycan and these sera showed neutralization when assayed against several Tier 2 subtype B viruses lacking the same glycan [166]. These VLPs mimic HIV-1 virions and usually contain a low number of HIV-1 Env spikes, negating some of the typical advantages of nanoparticle display that are listed above. However, the Env content of VLPs can be improved, for example by truncating the cytoplasmic tail of gp41 [167]. Furthermore, membrane-displayed Env trimers are stable, present essentially all bNAb epitopes and force the correct angles of approach on bNAb epitopes, including those in gp41 [168].

Another example is the eOD protein that targets the inferred version of germline bNAbS, which is presented on self-assembling nanoparticles of lumazine synthase from *Aquifex aeolicus* [44]. Nanoparticle-displayed eOD activated B cell lines more potently in vitro [44], but also induced a higher Ab response in vivo compared to trimeric eOD [169]. Native-like trimers can also be displayed on nanoparticles. A promising candidate for this is the ferritin 24-mer from *Helobacter pylori*, a self-assembling nanoparticle that has been used for the presentation of several other viral antigens [158,170,171]. Influenza hemagglutinin (HA) trimers attached to ferritin nanoparticles raised a more potent and broader NAb response than trimeric HA [158]. Comparable results were obtained with gp350 from Epstein-Barr virus (EBV) [170]. Considering that SOSIP.664 and soluble HA trimers are similar in size, we have also used these ferritin particles to present BG505 SOSIP.664 trimers. When used in rabbits, the nanoparticle-displayed trimers showed increased NAb responses compared to BG505 SOSIP.664 trimers alone [172].

Which of these platforms is superior for HIV-1 Env display remains to be investigated and alternative platforms are also being pursued. Several aspects should be taken into account, such as size of the particle to facilitate free draining into lymph nodes (<200 nm) [173] and optimal spacing between epitopes for optimal B cell activation (~10 nm) [154]. The HBV and HPV vaccines teach us that a successful viral subunit vaccine should consist of a native-like representation of the antigen in a multimeric form. Similar designs are probably necessary to induce a potent NAb response with HIV-1 Env vaccines.

Over the years many Env-based immunogens have been evaluated as vaccine candidates and all generated humoral responses without neutralizing activity against primary viruses. However, using stable native-like Env trimers we are now able to induce NABs against primary neutralization-resistant viruses and this represents a significant leap forward in HIV-1 vaccinology. Additional improved trimer designs, nanoparticle scaffolds
and novel vaccination strategies could drive future efforts that hopefully will lead to a vaccine that induces a broad and long-lasting protective humoral response.

Scope of this thesis
In my thesis research I have generated constructs consisting of recombinant HIV-1 Env trimers fused to heterologous molecules with the aim of improving Env trimer immunogenicity, as well as for generating tools that can be used for a variety of research applications. To increase the immunogenicity of Env trimers we fused the APRIL cytokine, a strong activator of B cells, to Env trimers (chapter 2). In chapter 3 we show that the heterologous trimerization domains that we and many other labs used to stabilize soluble viral antigens can be highly immunogenic, and we decreased the immunogenicity of one frequently used trimerization domain by placing glycans on its surface.

The development of a stable and soluble mimic of the viral Env trimer, termed BG505 SOSIP.664, in our laboratory, enabled us to explore a variety of vaccine strategies in the context of this advanced Env spike mimetic. By studying the antigenic structure of BG505 SOSIP.664, we contributed to the characterization of novel bNAbs, in particular the ones that target epitopes at the gp120-gp41 interface [47,48,174,175]. Characterization of the epitope of such a bNAb, PGT151, is described in chapter 4. We also studied the interaction of BG505 SOSIP.664 and native-like SOSIP.664 trimers from two HIV-1 subtypes with predicted precursors of bNAbs and found that several germline precursors were able to interact with native-like SOSIP trimers (chapter 5). Having validated native-like SOSIP proteins as suitable reagents for further work, we use them for the research described in the subsequent chapters.

The high stability of BG505 SOSIP.664 allowed us to fuse different proteins to the C-terminus of this trimer in the absence of heterologous trimerization domains. A fluorescent version of a soluble native-like Env trimer could be a useful tool for various experiments, e.g. to study Env folding, for studying binding kinetics or the isolation of bNAb expressing B cells. We therefore designed and characterized a GFP-fused soluble Env trimer (chapter 6). The surface area of soluble Env trimers is mostly shielded by glycans except for the bottom of the trimer. We attached an amino acid-based polymer to the C-terminus of BG505 SOSIP.664 to hide this potentially distracting non-neutralizing epitope (chapter 7). The breadth and potency of the humoral response can be increased by presenting antigens on ferritin nanoparticles. In chapter 8 we fused ferritin to BG505 SOSIP.664, yielding nanoparticles that present multiple trimers and these immunogens induced more potent NAb responses in rabbits than BG505 SOSIP.664 alone. We also generated ferritin-based nanoparticles containing native-like Env trimers from subtypes B and C intended for cocktail or sequential immunizations. We showed that these nanoparticles were well-formed and able to induce strong NAb responses in rabbits (chapter 9). Finally, in chapter 10 we discuss the contents of this thesis work within the context of the wider HIV-1 vaccine field.
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