HIV-1 envelope trimer fusion proteins and their applications
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Chapter 10

General discussion

Parts of this chapter have been published in:
Towards a vaccine against HIV/AIDS

AIDS remains one of the deadliest infectious diseases in the world and to prevent new infections or perhaps eradicate this disease an effective vaccine against HIV-1 is highly desirable. Such a vaccine should probably induce broadly neutralizing antibodies (bNAbs) to attain sterilizing protection against different strains of the virus [1,2]. However, the only target for bNAbs, the viral envelope glycoprotein spike (Env), is a master of disguise. Glycans and variable loops shield conserved epitopes and the overall conformational flexibility hampers proper B cell recognition [3–5]. Moreover, there is massive variability between Env sequences of different virus isolates and inducing bNAbs that can recognize Env from different isolates or even subtypes is very difficult [6]. Numerous immunogens have been designed to induce Ab responses that can neutralize HIV-1, as discussed in the Introduction to this thesis. Thus far, this has not led to a vaccine that can raise protective Ab responses.

The development of vaccines against hepatitis B virus and human papillomavirus and other studies have taught us that three properties are critical for an effective subunit vaccine [7]. First, the antigen used in the vaccine should be a close mimic of the functional antigen on the virus. Second, this vaccine antigen should be presented in a geometrically repetitive manner, e.g. on a virus-like particle (VLP) or nanoparticle, to induce a potent antibody response. Third, a suitable adjuvant is needed to induce an appropriate immune response. However, an effective HIV-1 vaccine should protect against many different viral isolates and thus an additional fourth characteristic is key: the huge sequence diversity of HIV-1 needs to be overcome.

In this thesis, we employed soluble Env trimer mimics to study binding of bNAbs.

Step 1: soluble Env trimers as antigenic mimics of the viral Env spike

The recent development of a native-like soluble mimic of the viral Env spike, termed BG505 SOSIP.664 [8], provided important insights into the structure of the HIV-1 Env trimer [9–13]. Moreover, the immunogenicity studies with the clade A BG505 SOSIP.664 and the clade B B41 SOSIP.664 trimers confirmed that proper Env mimics are likely needed to induce NAbs against neutralization-resistant viruses [14,15].

PGT151 binding to the gp120 and gp41 interface stabilizes the gp120-gp41 non-covalent KS 20160508.indd   151
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interaction, which enabled the extraction of native viral Env trimer complexes from the cell-surface membrane. This technique was used to purify and solve the first high-resolution cryo-EM structure of a viral native Env trimer [21]. This structure was highly similar to that of BG505 SOSIP664 [9–11,13,22], proving that well-folded soluble SOSIP.664 native-like Env trimers indeed resemble the conformation of the functional virion-associated Env trimer. Moreover, stable Env-PGT151 complexes are interesting immunogens because the PGT151 Fab stabilizes the viral Env trimer in its native conformation, while the Fc-tail of PGT151 could increase immunogenicity by engaging a range of Fc-receptor functions in immune cells [23]. Furthermore, these Fc effector functions can be tuned by changing the type of Fc tail on PGT151 to increase immunogenicity [24].

Hiding non-neutralizing epitopes on SOSIP.664 trimers for improved immunogenicity

Preferably, soluble native-like Env trimer immunogens should not induce non-NAbs, since these might interfere with the induction of NAbs [25]. Recently, structure-based improvements have yielded soluble Env trimers that bind less to the non-neutralizing epitope in the V3-loop and also have a reduced tendency to undergo the CD4 induced conformational changes [15,26,22].

Another potential non-neutralizing epitope is located at the bottom of soluble Env trimers [27]. Computer modeling suggested that this area is probably the largest epitope that is not shielded by glycans [27]. In order to hide this bottom epitope, we generated a native-like trimer, based on BG505 SOSIP.664, containing polyethylene glycol (PEG)-like amino acid chains (PAS) that is considered to be non-immunogenic (chapter 7). BG505-SOSIP.664-PAS retained most of its preferential characteristics compared to non-PASylated BG505 SOSIP.664. PASylated Env trimers could have an improved immunogenicity since the bottom epitope is hidden by the PAS moiety. Moreover, PAS-conjugated pharmaceuticals have a significantly increased half-life in circulation [28], which might bode well for the half-life of PASylated Env trimers. Consequently, these immunogens could also have an increased chance of encountering the correct B cell in vivo and thereby induce better Ab responses. Of course, this is assuming that the PAS-tail is non-immunogenic when used in a vaccine. Moreover, PASylated Env trimers can be used to determine if vaccination with a particular soluble Env trimer induced Abs that target the trimeric bottom.

Glycan shielding of non-neutralizing epitopes

Non-native-like Env trimers such as those described in chapters 2 and 3 usually contain a heterologous trimerization domain for stability. We showed that two of the most commonly used trimerization domains, GCN4 and foldon, are highly immunogenic (chapter 3). We hypothesized that Abs that are directed to these domains could interfere with the induction of Abs against the Env moiety. Therefore, we added glycans to these trimerization domains and found that rabbits induced significantly lower Ab responses to the glycosylated trimerization domain, while responses to the Env moiety were retained (chapter 3). Thus, dampening the humoral response against trimerization domain epitopes did not redirect the Ab response towards other epitopes on the same immunogen. This corroborates similar findings by others and our own laboratory. For example, removing the foldon trimerization domain of a soluble respiratory syncytial virus fusion glycoprotein (RSV F) immunogen did not increase Ab responses to the RSV F moiety
In our own laboratory we designed Env trimers that showed decreased exposure of unwanted epitopes in the V3-loop. When rabbits were immunized, these immunogens showed decreased V3-directed Ab responses, but this did not lead to increased autologous NAb responses [15]. Importantly, while hiding unwanted non-neutralizing epitopes (e.g. trimer bottom epitopes, V3-loop, trimerization domains) might not directly increase Ab responses to other epitopes, such designs will probably be important in sequential vaccines, since non-NAb expressing B cells that were induced earlier will compete with NAb expressing B cells.

**Step 2: nanoparticle display of Env trimers**

Soluble antigen alone is usually not sufficient to induce potent and long-lasting humoral responses: subunit antigens have to be presented as a repetitive array to induce a potent humoral response [30,31]. The importance of nanoparticle presentation is exemplified by the success of vaccines against human papillomavirus and hepatitis B virus, both based on virus like particles (VLP) [7]. The orderly distribution of antigen on nanoparticles or VLPs increases B cell receptor cross-linking and thereby increase the potency of the Ab response [30]. Additionally, symmetric display of antigen also increases the avidity of an epitope and thus lowers the threshold of B cell receptor activation for an epitope. This may lead to an increase in the number of epitopes that can be targeted by B cells [32,33].

**Presenting native-like Env trimers on ferritin nanoparticles**

The above mentioned studies showed that nanoparticle-display improves the immunogenicity of soluble antigens and we therefore engineered nanoparticles that can display native-like Env trimers ([chapters 8 and 9](#)). Others already showed that ferritin from Helicobacter pylori can be fused to the hemagglutinin spike of influenza virus or gp350 of Epstein-Barr virus, which resulted in well-folded self-assembling nanoparticles [34,35]. In [chapter 8](#) we demonstrated that also the prototypic native-like Env trimer, BG505 SOSIP.664, could be attached to the same ferritin moiety, which yielded nanoparticles with Env spikes. Rabbits that were immunized with this nanoparticle immunogen showed increased immunogenicity compared to BG505 SOSIP.664 alone. In [chapter 9](#) we showed that such particles can also be produced using stabilized Env trimers from other isolates, such as AMC008 (subtype B) and ZM197M (subtype C). These immunogens induced increased autologous responses and weak cross-reactive neutralization against neutralization-resistant (Tier 2) viruses. Thus, ferritin-display of native-like Env trimers is a promising strategy to increase immunogenicity of Env trimers and we argue that the results described in [chapter 8 and 9](#) warrant additional immunization experiments. However, the display of Env trimers on ferritin comes with some caveats and improved nanoparticles for displaying native-like Env trimers are discussed below.

Ferritin-display alters trimer presentation to B cells and also affects trimer antigenicity ([chapter 8 and 9](#)). For one, the non-neutralizing epitopes that are possibly exposed at the bottom of soluble Env trimers are hidden on a nanoparticle [27]. On the other hand, bNABs that bind epitopes that are closer to the membrane-proximal side of the trimer had a lower reactivity, while epitopes at the apex of each SOSIP trimer are highly accessible for B cell recognition. Moreover, some SOSIP trimers on ferritin are not completely cleaved, which might have subtle effects on the SOSIP conformation.
that we did not detect using conventional antigenicity studies such as ELISA (chapter 9). Obviously, these differences might affect the specificities of the humoral response induced upon vaccination. Indeed, ferritin-displayed Epstein-Barr virus gp350 induced antibodies with different specificities compared to soluble gp350 in immunized animals [34].

**Improved nanoparticle designs**

A native-like Env trimer conformation is probably key to induce NAbs with Env-based immunogens [14]. The glycosylation profile and antigenicity of ferritin-conjugated SOSIP trimers indicated that probably most trimers on the nanoparticle had a native-like composition. However, it is impossible to verify that each trimer on ferritin has a native-like conformation, which is important to induce proper NAb responses, while not inducing interfering non-NAb responses. Therefore, we argue that improved next-generation Env-carrying nanoparticle should be generated by a two-step process: first purify the Env trimers to select for well-folded Env trimers and subsequently add nanoparticles or another component to induce the formation of Env-carrying nanoparticles in the second step. Such a strategy should yield particles that exclusively carry cleaved and properly folded Env trimers. These nanoparticles could also be tuned to present more Env trimers on their surface (i.e. more than eight trimers as is the case with ferritin), thereby increasing B cell receptor cross-linking and possibly induce more potent humoral responses. Moreover, these new platforms also enable the generation of particles that contain several native-like Env trimers from different isolates or subtypes on the same nanoparticle. These “chimeric” nanoparticles should select more efficiently for NAbs with broader specificities. After all, cross-reactive B cells should have a selective advantage because these cells carry a B cell receptor that binds to all Env trimer variants on a nanoparticle and thus become more efficiently activated than B cells that recognize only a single Env trimer variant. Moreover, the availability of various nanoparticle types that can display Env trimers also allows one to change nanoparticle type for each subsequent vaccine boost in order to avoid that pre-existing anti-nanoparticle Abs interfere with the induction of effective anti-Env trimer Abs. In summary, multimeric display of Env trimers will probably be crucial for inducing enough NAb with good breadth and improvements in nanoparticle designs will be needed to stably present trimers to B cells to induce NAb response. However, such vaccines will further benefit from the correct adjuvant to induce potent Ab responses.

**Step 3: adjuvants in Env trimer vaccines**

Adjuvants are vaccine constituents that are included to provoke an immune response, and this is especially important in the case of subunit vaccines, which lack natural adjuvants that normally are included with inactivated or live attenuated vaccines [36]. Using the correct adjuvant is important to induce the correct immune response and one can skew the responses towards the cellular or humoral side. Adjuvant formulation often needs to be optimized for each type of antigen and animal species. For example, BG505 SOSIP.664 in ISCOMATRIX adjuvant induced potent neutralizing and binding Abs in rabbits, while the same regimen in macaques induced about 5-fold lower antibody responses [14]. One of the possible explanations for this discrepancy is that ISCOMATRIX is a better adjuvant in rabbits compared to macaques. Moreover, for many of the adjuvants that are now in use
in pre-clinical studies we do not know the working mechanism yet. And since adjuvants have systemic effects on the immune response, introducing new adjuvants requires lengthy pre-clinical and clinical assessments to demonstrate their safety. Moreover, when immunized in tissue, most of the adjuvant probably does not end up at the immune cells that also take up the antigen.

**Cytokine-fusion constructs as Env vaccines**

Therefore, we and others have developed fusion constructs that entail a cytokine, which acts as an adjuvant, attached to an Env trimer (**chapter 2** [37–42]). Such a design assures that both the adjuvant and the antigen end up in the same immune cell and by fusing particular adjuvants one can steer the immune reaction towards the desired response. Moreover, these cytokine-based adjuvants also act as homing molecules that target specific immune cell. We attached A Proliferation Inducing Ligand (APRIL) to an soluble Env trimer (Env-APRIL) to increase Ab responses [40]. APRIL induces B cell proliferation and affinity maturation, which could obviously be beneficial for the induction of improved NAb responses [43]. Covalently attached to a soluble Env trimer, APRIL induced increased AID expression *in vitro* and increased Ab titers *in vivo* (**chapter 2**) [40]. Moreover, Env-APRIL induced no or negligible Abs to the APRIL moiety in two different animal models (**chapter 2**), which is important because Abs against self-APRIL could otherwise provoke serious autoimmune responses. However, we argue that in future experiments one might improve the design of these Env-APRIL immunogens by fusing APRIL to improved native-like Env trimers and/or attach APRIL to nanoparticles that present such trimers. Although these and similar strategies seem promising in itself, additional strategies are undoubtedly needed to generate NAbs that are able to neutralize a wide range of HIV-1 isolates.

**Step 4: sequence diversity of HIV-1 Env**

The need for annual influenza vaccinations demonstrates that vaccines against highly diverse pathogens, even when using native antigens, usually induce NAb responses with narrow specificity [44]. In analogy, an antigenically perfect Env trimer will probably only induce NAbs against the autologous virus and antigenically similar viruses, and thus one immunogen will probably not suffice for a broad spectrum HIV-1 vaccine. Several strategies are being pursued to induce such Abs based on the use of Env trimers.

**Polyvalent vaccination strategies**

To increase the neutralization breadth, one could use native-like trimers from different subtypes or isolates, as a cocktail or sequentially. *In silico* modeling of germinal center reactions and the subsequent Ab reaction of vaccines showed that a polyvalent malaria subunit or polyvalent gp120 vaccine select for cross-reactive B cells more effectively than a monovalent vaccine [45,46]. In the latter study, the authors argue that sequential vaccination of different gp120s is more advantageous than simultaneous vaccination with a cocktail of the same gp120s [46]. This might be different for native-like trimers, since these trimers contain less non-NAb epitopes than gp120 [8,46,47]. However, the annual influenza vaccine also consists of a cocktail and only protects against influenza variants that closely resemble the vaccine strains. Moreover, despite the fact that almost all people have contracted different influenza viruses, they are not always protected
against infection with divergent strains. These considerations indicate that a cocktail or sequential HIV-1 Env vaccine requires careful design and optimization to be effective. In order to induce NAb responses with broader specificities, we boosted rabbits with a cocktail of ferritin-attached subtype B Env trimers and observed NAb responses that showed weak neutralization against some heterologous subtype B Tier 2 viruses (chapter 9). However, many other subtype B Tier 2 viruses were not neutralized by this cocktail vaccine. Considering the wide sequence diversity intra-clade (~15%) and between clades (almost 30%), it seems that inducing NAb responses against cross-neutralizing epitopes with random native-like Env trimers will require many immunization trials in animals to identify the proper combination. Moreover, whether these outcomes can be translated directly to the human context remains to be investigated.

Targeting the germline precursor of bNAbs

An alternative to the empirical evaluation of cocktail and sequential vaccines is the rational design of sequential vaccines that are specifically tailored to guide specific B cell receptor lineages to mature into bNAbs [48]. During natural infection, bNAbs develop through the interplay between the ever-changing virus and the co-evolving Abs [49–52]. In order to induce the same bNAbs, it is probably necessary to mimic these maturation pathways [48].

One strategy that is being pursued is the isolation of longitudinal Env sequences from patients that developed broad neutralization and the generation of soluble native-like Env trimers of these successive sequences. Subsequently, these patient-derived trimers are used for immunization, in monovalent or cocktail formulations, to imitate the evolutionary pathway of the virus [49,53]. Such strategies assume that development of bNAbs depends largely on evolving virus characteristics.

Another strategy focuses on mimicking the evolutionary pathway of the bNAb. To initiate such a pathway, the correct naïve B cell receptors (i.e. the germline precursors that have the potential to become bNAbs) need to be activated, followed by sequential immunogens to steer the Ab development toward neutralization breadth. However, most Env proteins do not bind to inferred germline versions of bNAbs and are thus unable to prime the correct naïve B cells [54,55]. Env proteins can be rationally designed to do so. Examples are the specifically designed epitope-scaffolds eOD-GT6 and eOD-GT8 and gp140\textsubscript{UNC} trimer variants that lack particular glycans surrounding the CD4bs [25,56,57]. When used as an immunogen in knock-in mice that express the inferred germline version of the HC of VRC01 or 3BNC60 as the B cell receptor on B cells, eOD-GT8 was able to specifically prime these B cells \textit{in vivo} [58,59]. The eOD-GT8 immunized gl-VRC01 HC knock-in mice also selected the appropriately short light chain CDR3, a typical characteristic of VRC01-like Abs [58]. Unfortunately, none of the immunized knock-in mice induced NAb responses [58,59], possibly because eOD does not pose the same restrictions on the angles of approach to the CD4bs as the native Env trimer. Together, these knock-in mouse studies imply that immunization with successive immunogens is probably needed to guide Ab responses toward VRC01-like bNAbs. CD4bs bNAbs isolated from humans are usually extensively hypermutated, which could complicate mimicking such development with rationally designed sequential vaccination strategies [60]. The feasibility of correctly directing Ab responses toward bNAbs in the presence of competing Abs against non-NAb epitopes should be studied in other animal models, such as mice containing the human
Native-like trimers, such as those based on the SOSIP design, might also be appropriate germline-targeting immunogens, because they force the correct trimer-compatible angles of approach on Abs and also expose only a limited number of potentially competing non-NAb epitopes \cite{8,25,62}. Moreover, native-like trimers present not just one, but multiple bNAb epitopes, thereby increasing the chance that at least one appropriate Ab lineage is triggered by the immunogen to mature into a bNAb. We and others have shown that the inferred germline versions of CH01, VRC26 and clonal relatives PG9 and PG16, bNAbs that bind to the V1V2 on the trimer apex, and also the gp41-directed bNAb 3BC315, bind to different SOSIP.664 trimers with low affinity (chapter 5) \cite{63,64}. This information can be used for structure-guided immunogen approaches to design germline-targeting native-like trimers that bind with higher affinity. Furthermore, structure-based design and sequence information on viruses that activate the germline version of other bNAbs can be used to design trimers that activate the germline versions of multiple bNAbs. A recent study suggested that multimerization of germline-binding immunogens is necessary to overcome the low affinity and self-reactivity of germline version of 3BNC60 and induce an Ab response from these B cells \cite{65}. The latter finding demonstrates that neither of the strategies stipulated in this thesis and discussed here are mutually exclusive and a combination of approaches is probably necessary to develop an effective Env-based HIV-1 vaccine.

Unresolved questions
The study of humoral responses in HIV-1 infected individuals have reinvigorated the HIV-1 vaccine field. Moreover, the elucidation of high-resolution structures of HIV-1 Env trimer helped to design better immunogens. Yet there are many unresolved fundamental issues and questions regarding Env immunogenicity. For example, what is the influence of the capture of Env immunogens by other immune cells other than B cells in circulation, such as CD4+ T cells or dendritic cells? Does this hamper the immune response or does it actually help to target Env to lymph nodes for more efficient B cell activation? What is the amount of Env that enters the lymph nodes and may induce germinal center reactions? What is the half-life of Env in circulation and what is the influence of vaccination route on protein degradation, immunological processing and the subsequent immune response?

Thus far, these and other basic immunological questions have not been answered sufficiently. The ability to track Env \textit{in vivo} would probably be important for such purposes. A fluorescently tagged version of a soluble native-like Env trimer (described in chapter 6) or other tagged soluble Env trimers could be used to answer some of these questions. Such answers will hopefully further bolster the search for an effective HIV-1 vaccine.

Conclusions
The last decade has been key for HIV-1 research and vaccine research in particular. The isolation of bNAbs from patients and the promise of immunogens and vaccine strategies that might induce such Abs have excited the field. Ongoing studies and future undertakings will teach us what kind of approaches are necessary to generate long-lasting and protective Ab responses. Furthermore, these studies will not only inform HIV-1 vaccine development, but also the development of vaccines against other highly variable...
viruses, such as influenza and hepatitis C virus.

References