A Blueprint for HIV Vaccine Discovery

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A BLUEPRINT FOR HIV VACCINE DISCOVERY

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Abstract

Despite numerous attempts over many years to develop an HIV vaccine based on classical strategies, none has convincingly succeeded to date. A number of approaches are being pursued in the field, including building upon possible efficacy indicated by the recent RV144 clinical trial, which combined two HIV vaccines. Here, we argue for an approach based, in part, on understanding the HIV envelope spike and its interaction with broadly neutralizing antibodies (bnAbs) at the molecular level and using this understanding to design immunogens as possible
vaccines. BnAbs can protect against virus challenge in animal models and many such antibodies have been isolated recently. We further propose that studies focused on how best to provide T cell help to B cells that produce bnAbs are crucial for optimal immunization strategies. The synthesis of rational immunogen design and immunization strategies, together with iterative improvements, offers great promise for advancing toward an HIV vaccine.

THE HIV VACCINE PROBLEM: WHY HAVE PAST APPROACHES TO DEVELOP AN HIV VACCINE FAILED?

It is nearly 30 years since HIV was identified as the causative agent of AIDS and yet no vaccine is approaching licensure. This is intensely disappointing. It is partly due to the fact that the process of vaccine development typically takes a long time period, partly to the failure of classical viral vaccination strategies when applied to HIV and partly to the many barriers to immune recognition evolved by the virus. For acute viruses such as smallpox and polio, natural infection leads to a large fraction of individuals who are immune to re-infection. Hence, vaccination strategies to mimic natural infection without adverse sequelae can be designed. For persistent viruses, immunity to re-infection cannot be readily determined but nevertheless, in some cases such as human papillomavirus (HPV), a relatively straightforward approach in which vaccines elicit neutralizing IgG is effective (Day et al., 2010). However, for the highly variable retrovirus HIV, the classical vaccination strategies have failed and it is worth considering why this is the case.

Immunogen design strategies have failed to elicit broadly neutralizing antibody (bnAb) responses to circulating HIV

As for other viruses, animal model studies, including the macaque, provide an abundance of evidence for protection against HIV or SHIV (chimeric HIV/SIV with envelope (Env) of HIV) challenge by neutralizing antibodies. However, to protect against the huge diversity of global circulating HIVs, the neutralizing antibody response should be broad, and traditional vaccine approaches have failed to induce such a response. We note that, although there are reports of some degree of antibody protection in the absence of serum neutralizing antibodies, including the RV144 trial, the protection is generally not particularly strong and so we focus here on neutralizing antibodies.

Live attenuated and killed viruses: direct pathogen mimicry—Many highly successful vaccines rely on direct mimicry of the pathogen. Live-attenuated viruses (e.g. measles, mumps, yellow fever), inactivated viruses (e.g. poliovirus) or virus-like particles (e.g. HPV) have been used to imitate natural infection and imprint immunological memory. However, for many reasons these approaches have been unsuccessful for HIV vaccine development. (Burton et al., 2005; Kong and Sattentau, 2012; Kwong et al., 2011; Overbaugh and Morris, 2012; Pantophlet and Burton, 2006; Verkoczy et al., 2011; Wyatt and Sodroski, 1998). The challenges in this approach are largely related to the properties of the HIV envelope (Env) spike, which is a heterodimer of the glycoproteins gp120 and gp41 that forms timers on the virion surface and mediates entry by binding to CD4 and CCR5 or CXCR4 on the target cell surface. First, the variable regions of the functional HIV Env trimer spike (Figure 1), the sole target of neutralizing antibodies, are typically immunodominant relative to conserved regions of the spike and, thus, the neutralizing antibody response against HIV is generally highly strain-specific. Second, the extraordinary variability in antigenic regions of the Env spike means that the number of circulating HIV strains is extremely high and conventional concepts of viral serotypes are rendered irrelevant. Third, because of the instability of the HIV spike, most viral particle-based vaccines tend to express immunodominant, non-functional forms of Env on the virion
surface (Poignard et al., 2003), which favor the induction of non-neutralizing antibodies (Crooks et al., 2007). Fourth, there is a relatively low copy number of Env molecules on HIV particles, leading to expectations of rather poor activation of B cells as compared to viruses with dense Env coating, such as influenza. Fifth, other complicating factors, such as the induction of antibodies against cellular proteins, have contributed to difficulties with viral particle-based immunogens (Chan et al., 1992; Crooks et al., 2007; Hammonds et al., 2005). Although a number of strategies to overcome these hurdles have been employed, including pseudotyping HIV with heterologous envelopes (Kuate et al., 2006; Marsac et al., 2002) and generating VLPs with cleavage-defective or disulfide-shackled Env to prevent gp120-gp41 dissociation (Crooks et al., 2007), none of these approaches has yet induced potent heterologous antibody responses in non-human primate (NHP) models. Finally, it should be noted that, because of the risk of mutation and reversion to a pathogenic form, the use of live attenuated HIV vaccines in humans raises formidable safety and liability issues.

**Subunit vaccines: recombinant Env**—Using the same paradigm that formed the basis for successful development of a vaccine against hepatitis B, initial attempts to generate a protective vaccine against HIV focused on the elicitation of Env-specific humoral immune responses using gp120 subunit immunogens. Unfortunately, the results of clinical trials indicated that the antibodies elicited by monomeric gp120 failed to neutralize HIV primary isolates, prevent HIV infection, reduce viral loads, or delay disease progression (Flynn et al., 2005; Pitisuttithum et al., 2006). Therefore, over the ensuing years, more attention has been focused on the generation of soluble, recombinant trimers as immunogens to better simulate the structure of the native HIV Env spike. Indeed, given that antibody binding to the trimer is both necessary and sufficient for neutralization (Pantophlet et al., 2009; Parren and Burton, 2001; Roben et al., 1994; Sattentau and Moore, 1995; Wallace and Stamatatos, 2009; Yang et al., 2006), a recombinant native trimer represents an excellent starting point for the elicitation of bnAb responses. However, the instability of the functional HIV spike has presented challenges to the development of recombinant trimers that mimic the structure of the native spike (Phogat and Wyatt, 2007). Various strategies, including the introduction of disulfide bonds to covalently link gp120 and gp41, deletion of the furin cleavage site in gp160 so that furin-mediated proteolysis to yield non-covalently linked gp120 and gp41 subunits does not occur, and incorporation of trimerization motifs into the gp41 ectodomain, have been employed to stabilize recombinant trimers (Phogat and Wyatt, 2007). However, none of the recombinant trimers developed to date display antigenic profiles that truly mimic the native HIV spike and these immunogens have elicited bnAb responses that are only incrementally improved relative to those elicited by monomeric gp120 (Grundner et al., 2005; Kang et al., 2009; Li et al., 2005). As for viral particle-based vaccines, immunization with these constructs has resulted in the elicitation of predominantly non-neutralizing or strain-specific antibody responses (see above).

**Immunogens based on the epitopes recognized by bnAbs**—A significant fraction of HIV-infected individuals develop broadly neutralizing responses over time (Binley et al., 2008; Doria-Rose et al., 2009; Gray et al., 2011; Gray et al., 2009; Li et al., 2006; Sather et al., 2009; Simek et al., 2009; Stamatatos et al., 2009), including some “elite neutralizers”, whom display outstanding serum neutralization potency (Simek et al., 2009). Mapping of serum responses in broad neutralizers shows the targeting of a relatively small number of broadly neutralizing epitopes on Env, such as the CD4 binding site (CD4bs), glycanc-dependent epitopes, quaternary structure dependent epitopes and the membrane proximal external region (MPER) (Gray et al., 2009; Stamatatos et al., 2009; Walker et al., 2010). Broadly neutralizing monoclonal antibodies (bnMAbs) define these epitopes more precisely (Figure 1) and suggest the possibility of incorporating the epitopes into more favorable presentations than current Env preparations, such as engineered Env molecules or scaffolds.
or multivalent glycan presentations (Burton, 2002; Schief et al., 2009). Such approaches have, however, been unsuccessful for a number of reasons that probably include: (a) Until very recently, only a few bnM Abs were available so that, for instance, all attempts to design immunogens based on the CD4bs were guided by the sole anti-CD4bs bnMAb, b12. The remarkable discoveries of many more potent anti-CD4bs bnMAbs in the last 2 years may renew opportunities in this area (Corti et al., 2010; Falkowska et al., 2012; Scheid et al., 2011; Wu et al., 2010; Wu et al., 2011), although there are still concerns that unusually precise directional targeting to the CD4bs epitope may be required (Chen et al., 2009). (b) Targeting the glycan epitope recognized by the bnMAb 2G12 presents problems in terms of the special domain-exchanged configuration of this antibody (Calarese et al., 2003), which was thought necessary for recognition of the glycan shield that surrounds gp120. However, new data bring this requirement into question and may offer new strategies for vaccine targeting either of the glycan shield or of penetrating the shield via a mixed protein-glycan immunogen (Pejchal et al., 2011; Walker et al., 2011a). (c) Continuous epitopes on the MPER of Env gp41 (Buzon et al., 2010; Morris et al., 2011; Muster et al., 1993; Salzwedel et al., 1999; Zhu et al., 2011; Zwick et al., 2001) offer potentially attractive vaccine targets and some success has been achieved in eliciting strong responses to relevant peptides derived from this region in one or more viral conformations (Correia et al., 2011; Correia et al., 2010; Ofek et al., 2010). However, the proximity of the epitopes to the membrane may offer special problems in eliciting MPER antibodies. In addition, MPER antibodies have been associated with autoreactivity (Haynes et al., 2005a; Haynes et al., 2005b) and this argued to be a problem in inducing such antibodies. However, the recent description of a potent MPER bnMAb that shows no autoreactivity (Huang et al., 2012) suggests that autoreactivity may not be the problem envisaged earlier.

In summary, the challenge for epitope-based vaccine design is that only broadly conserved and exposed epitopes are suitable for vaccine targeting but these epitopes, in their natural context, tend to elicit poor antibody responses. When bnAb responses are elicited, the corresponding bnM Abs tend to have unusual features such as high levels of somatic mutations, insertions/deletions, long CDRH3 loops, post-translational modifications, polyreactivity and rare structural motifs such as domain-exchange.

Overall, these observations indicate that successful vaccination may require considerable ingenuity in immunogen design and immunization protocols that go far beyond current norms.

**Sterilizing immunity, or close to it, will likely be necessary for protective immunity against HIV**

The ultimate goal of vaccination is to provide protective immunity against disease. For most successful vaccines (e.g. measles, polio, smallpox, etc.), protection against disease can be achieved in the absence of sterilizing immunity. Indeed, under most circumstances, immunization does not induce sufficiently high and persistent titers of antibodies to prevent infection (Plotkin, 2008). For example, in the case of measles, very high antibody titers of $\geq 1000$ mIU/ml are generally required to protect against infection, but titers of $\geq 200$ mIU/ml are sufficient to protect against disease (Chen et al., 1990). In contrast for HIV, because of the establishment of persistent latent infection in which viral DNA is integrated into host genomic DNA, the window of opportunity to clear virus may close permanently once a pool of latently infected cells is in place (Haase, 2010, 2011), although the results of Picker and colleagues, as noted below, do suggest that, in some cases, a cellular response can control and suppress virus replication to very low levels (Hansen et al., 2011).
Cellular immune responses generally do not prevent acquisition of infection

It is clear from several animal studies that vaccines that induce only CD8+ T cells (also known as Cytotoxic T Lymphocytes (CTLs)), and no virus-specific antibodies, can efficiently control subsequent challenge with a viral pathogen, as shown for lymphocytic choriomeningitis virus (LCMV) (Klavinskis et al., 1989), influenza virus (Ulmer et al., 1993) and respiratory syncytial virus (RSV) (Kulkarni et al., 1995). However, these “CTL-only vaccines” typically prevent severe disease, but not infection itself, and can allow significant viral replication. Indeed, recent studies suggested that cellular responses alone could not block acquisition of SIV infection in the macaque model; the inclusion of Env in the vaccine was required for complete prevention of infection (Barouch et al., 2012). In the context of HIV, CTL vaccines might be expected to permit considerable viral seeding of lymphoid tissue during acute infection and the establishment of a significant pool of latently infected cells with concomitant adverse consequences (Liu et al., 2009). However, recent studies (Hansen et al., 2011) have described a Cytomegalovirus (CMV) vector-based SIV vaccine that appears to function via CD8+ T cell immunity and restricts SIV replication to very low levels in a significant fraction of vaccinated animals. The induction of such a response could contribute to vaccine protection against HIV, and serve as a second line of defense to contain infection at the portal of entry, should humoral responses fail to fully prevent initial infection of target cells.

WHAT GOALS SHOULD BE SET TO MOVE TOWARD A RATIONALLY DESIGNED HIV VACCINE?

Given the challenges identified above, we propose a number of goals that should be targeted in order to move toward an HIV vaccine based on neutralizing antibody, B cell and CD4+ T cell studies.

Fully define the antibodies and epitopes associated with broad neutralization of HIV

NAbS are the best correlate of protection for many viral vaccines (Amanna and Slifka, 2011; Plotkin, 2010) and, for HIV, nAbs have been shown to provide robust protection against mucosal challenge in the macaque model as described above. Therefore, a major goal of HIV vaccine research should be the discovery of immunogens and immunization strategies that can elicit nAbs, or more specifically broadly nAbs (bnAbs), given high sequence variation in HIV Env. As part of this discovery effort, it is important to fully map the landscape of bnAb recognition of the HIV Env spike, the sole target of nAbs, as described above. The recent generation of larger numbers of potent bnMAbs (Figure 2) using single B cell technologies (Burton et al., 2012; Corti et al., 2010; Haynes et al., 2012; Klein et al., 2012; Kong and Sattentau, 2012; Moir et al., 2011; Overbaugh and Morris, 2012; Scheid et al., 2009; Scheid et al., 2011; Tiller et al., 2008; Walker et al., 2011a; Wu et al., 2010; Wu et al., 2011) has begun to reveal new bn epitopes and defined “sites of vulnerability” (Kwong and Wilson, 2009) on the Env spike with much greater accuracy (Figure 1). However, ideally, one would like to generate and characterize enough bnMAbs with enough redundancy in epitope recognition to be confident that HIV Env bnAb space has been fully covered. A full complement of bn epitopes can then be exploited for generating immunogens with optimal precision.

The bnMAbs are, in themselves, valuable tools for guiding vaccine discovery. The isolation of multiple bnMAbs that recognize similar epitopes is revealing the extent to which certain structural features (e.g. long CDRH3s, polyreactivity, domain exchange, high levels of somatic hypermutation, post-translational modifications etc.) are required for recognition. For example, recently described CD4bs-directed antibodies exhibit remarkably broad and potent activity, but unlike b12, do not require a long CDRH3 for gp120 binding (Scheid et
Therefore, the elicitation of bnMAbs against the CD4bs may not necessitate the design of immunization protocols that aim to preferentially select antibodies with long CDRH3s. Also, the isolation of multiple bnMAbs against single antigenic regions helps delineate whether the use of certain germline genes can facilitate epitope recognition. In support of this notion, it has been suggested that the use of two closely related VH germline genes (VH1-2*02 and VH1-46), which encode the variable regions of Abs that contribute to immunogen specificity, allows for a conserved mode of epitope recognition by certain CD4bs-directed bnMAbs (Scheid et al., 2011; West et al., 2012; Wu et al., 2012; Wu et al., 2011; Zhou et al., 2010). The bnMAbs described to date have high levels of somatic hypermutation (Scheid et al., 2011; Walker et al., 2011a; Walker et al., 2009; Wu et al., 2010), a process in which mutations are introduced into the antibody variable regions to generate diversity. This may dictate that immunogens and/or immunization protocols should be designed to increase antibody affinity maturation (e.g. adjuvants, viral vectors, etc.). Alternatively, the high levels of somatic hypermutation may simply reflect the outcome of chronic antigen stimulation resulting from long-term HIV infection. Indeed, anti-Env Abs from chronic infection in general, whether neutralizing or not, tend to have high levels of somatic hypermutation (Barbas et al., 1993; Breden et al., 2011; Scheid et al., 2009). Therefore, it may be possible to generate immunogens and/or immunization protocols that shortcut the route to generation of bnAbs and result in antibodies with much less somatic hypermutation.

### Determine the bnMAbs that provide the best protection against SHIV in the NHP model

Clearly, the property of antibodies that interests most in terms of vaccine discovery is protection in humans. Neutralization is simply a property that can be readily measured in vitro and which has a good pedigree for qualitative prediction of protection against SHIV in the NHP model and against many other viruses in humans. Ideally, we would like to correlate protection in humans with neutralization assays, but this likely requires either large-scale human passive immunization studies or a vaccine that has clear efficacy, although superinfection and mother-to-child transmission studies may also provide useful correlative data (Blish et al., 2008; Chohan et al., 2010; Dickover et al., 2006; Guevara et al., 2002; Lathey et al., 1999; Scarlatti et al., 1993a; Scarlatti et al., 1993b; Smith et al., 2006). The best animal model is probably SHIV infection in macaques and titration of protection for several of the newer bnMAbs in high and low dose SHIV vaginal challenge models is important to better define the relationship between protection and both in vitro neutralization and antibody specificity. Finally, investigation of passive protection in animal models expressing human antibody repertoires, e.g. the humanized BLT (bone marrow/liver/thymus) mouse (Brainard et al., 2009; Wheeler et al., 2011), is highly desirable.

### Design, engineer and produce a pure stable Env preparation that mimics the antigenic profile of the functional Env spike

The functional HIV Env spike is the sole target of neutralizing antibodies as discussed above. Remarkably, because of the instability of the spike, it is likely that no immunization with pure native spikes has yet been carried out; infectious virions express multiple Env species so that even natural infection presents a complex mix of Env molecules to the immune system (Moore et al., 2006; Poignard et al., 2003). A number of strategies to generate a pure stable Env preparation that mimics the antigenic profile of the functional Env spike are ongoing. First, co-crystallization of recombinant Env trimers with a variety of bnMAbs is being attempted and hopefully will eventually generate a high-resolution structure that will allow for rational approaches to stabilizing a recombinant trimer. Second, the resolution and methodologies of cryo-electron microscopy (cryoEM) and cryo-electron tomography are being enhanced and may similarly provide enough molecular detail to allow the rational design of stable trimers (Liu et al., 2008; Mao et al., 2012). Third, various
molecular display and selection strategies, including positive selection with bnMAbs and negative selection with non-neutralizing Abs are being studied. Fourth, gp120/gp41 sector analyses (Dahirel et al., 2011) are being used to identify potential gp120-gp41-cross-linking stabilization sites. It should be noted though that, even if stable trimer immunogens can be designed, engineering strategies will still likely be required to dampen responses to immunodominant variable regions of the Env trimer molecule to favor elicitation of bNAb responses.

Define glycosylation on the Env trimer

Approximately half of the molecular mass of gp120 is comprised of N-linked glycans that shield the protein backbone. Considering that these carbohydrate structures are critical for Env folding, binding to lectin receptors that may mediate transmission, antigenicity and immunogenicity, a complete understanding of the identity and heterogeneity of glycans expressed on native trimers may be crucial for the development of HIV vaccine candidates. Although the glycosylation profiles of recombinant Env proteins and native viral Env have been analyzed by mass spectrometry (Bonomelli et al., 2011; Doores et al., 2010; Go et al., 2009; Go et al., 2011; Go et al., 2008; Mizuochi et al., 1988; Zhu et al., 2000), a full definition of site-specific glycosylation on these Envs has not yet been possible. Such an analysis would provide insight into the role of specific glycans in forming or shielding antibody epitopes in the context of gp120 monomers and native HIV Env trimers, which may inform immunogen design efforts. Of note, recent studies suggest that functional Env trimers are substantially resistant to mannosidase, which hydrolyzes mannose glycans, and express a higher abundance of oligomannose glycans that are often found on monomeric gp120 (Bonomelli et al., 2011; Doores et al., 2010; Eggink et al., 2010) (Figure 3). Additionally, even the same recombinant gp120 – when expressed in different, commonly used cell lines- can show widely varying abundances of oligomannose-type glycans as well as exhibiting different varieties of complex-type glycans (Raska et al., 2010). These differences in glycan composition are of direct immunological significance: enzymatic modification of gp120 glycans (Banerjee et al., 2009), or even direct occlusion of gp120 glycans by protective lectin (Banerjee et al., 2012) can dramatically alter the antibody response to gp120. These observations, coupled with the fact that recombinant gp120 has been used extensively in animal studies and human vaccine trials with very limited success, necessitate that consideration be given to the impact of differing glycosylation patterns on the antigenicity and immunogenicity of Env. In contrast to the variation seen between expression systems, the overall glycosylation pattern of native envelope glycoproteins derived from peripheral blood mononuclear cells (PBMCs), even from highly divergent HIV clades, is remarkably well-conserved (Bonomelli et al., 2011).

Determine where, when, how, and which HIV antigens are engaged by B cells

Understanding how the immune system recognizes and processes viral antigens for durable humoral immunity is of fundamental importance for vaccine design (Cyster, 2010). It is now clear that B cells can encounter and respond to antigen through many different mechanisms depending on the nature and size of the antigen itself, as well as on the cellular context and location in which antigen presentation occurs. For example, within the context of the lymph node, small soluble antigens access the B cell follicle, the primary site where B cell activation occurs, through a follicular conduit system (Roozendaal et al., 2009), whereas particulate antigens, such as viruses and large immune complexes, are captured by subcapsular sinus macrophages for presentation to follicular B cells (Gonzalez et al., 2011). In the case of HIV Env, particularly considering the dramatic instability of the native Env spike, it is unclear which Env antigens are presented to B cells and how and where these encounters occur after infection. For example, how often, if ever, do B cells engage functional Env spikes in vivo? Additionally, what are the predominant Env species
presented to B cells: monomeric gp120, gp41, proteolyzed Env fragments and/or uncleaved gp160? Although the above questions concern viral Env in the context of HIV/SIV infection, an equally important issue is how soluble vaccine immunogens are processed and presented to B cells. The answers to these questions will have important implications for the design of improved HIV vaccine candidates. We believe these questions can be addressed in the macaque model and in mice, particularly in bnMAb knock-in mice.

**Explain how and why a subset of HIV-infected individuals makes potent bnAb responses**

A series of studies (Sather et al., 2009; Simek et al., 2009; Stamatatos et al., 2009; van Gils et al., 2009) have shown that 10–30% of HIV infected donors develop moderate to potent broadly neutralizing serum responses over time, providing support for the notion that bnAbs can be elicited in humans and providing a window into how to elicit such responses. Although serum-mapping experiments (Binley et al., 2008; Gray et al., 2011; Gray et al., 2009; Li et al., 2007; Li et al., 2009; Mikell et al., 2011; Moore et al., 2011; Stamatatos et al., 2009; Walker et al., 2010) have largely defined the antibody specificities that mediate broad serum neutralization, there is currently limited information on how and why broad responses develop within select individuals. A few longitudinal studies (Euler et al., 2012; Gray et al., 2011; Sather et al., 2009; van Gils et al., 2009) have examined the factors associated with the development of breadth and, although there are some inconsistencies, it has been suggested that broad neutralization correlates with time post-infection, plasma viremia levels, CD4+ T cell count at the viral set-point, and binding avidity to the envelope protein. In a recent longitudinal study (Gray et al., 2011), breadth was found to emerge two years post-infection and plateaued at year four, which corroborates earlier estimates that bnAbs develop after 1–3 years (Gray et al., 2011; Sather et al., 2009; Stamatatos et al., 2009; van Gils et al., 2009). While all of these studies have provided valuable insight into the factors associated with the development of bnAbs, more extensive longitudinal studies (e.g. isolation of mAbs and viral Envs from serial time points) in both humans and NHP models will be required to understand the evolution and maturation of broad responses. A major question is the relative contribution of the virus and the host immune system to the evolution of these responses. Key issues include the influence of the founder virus, the possibility of viral motifs associated with broad neutralization, clinical correlates of broad neutralization, and the roles of B cell dysfunction and CD4+ T cell help in developing bnAb responses. Antibody deep sequencing of donors from whom bnMAbs have been isolated may shed light on how bnAb responses evolve over time (Wu et al., 2011). Of interest, it is expected that deep sequencing methods that maintain correct pairing of the heavy and light chains that comprise antibodies will become available in the near future to allow for functional characterization of antibody responses. The unexpected observation of the development of a highly potent bnAb response in a SHIV-infected macaque only 40 weeks post-infection (Walker et al., 2011b) suggests that the role of the infecting virus and the genetics of the host in bnAb responses, could be readily addressed in NHP studies. Of note, retrospective, nested case controlled studies that utilize systems biological approaches to identify early signatures that correlate with and predict the later development of bnAbs should be useful in providing insights about the mechanisms that control bnAb induction (Querec et al., 2009).

**Develop model systems for immunogen evaluation**

A classical initial approach to immunogen evaluation is to use serum antibody responses in small animals, such as mice and rabbits, as a “gatekeeper” measurement to determine whether to proceed with the immunogen in humans. This approach may be less appropriate for HIV given some of the features of bnAbs described above. An alternate approach is to take advantage of our knowledge of bnMAbs to generate model systems that can be used to evaluate immunogens and immunization strategies for their ability to elicit bnAbs in as high-throughput manner as possible prior to testing in humans. An example of such a strategy is
to create knock-in mice that carry germline forms of the different human bnMAbs. The mice could be used to screen a wide variety of Env antigens for their abilities to induce bnMAb+ murine B cells to enter germinal centers (GCs) where B cells proliferate and develop and to mature into B cells producing fully active antibodies. A comparison of B cells carrying germline and mutated versions of bnMAbs could be used to determine whether an antigen that initiates immunity from “naïve” B cell receptors (BCRs) also stimulates antibody secretion from somatically mutated “mature” BCRs. Further examples of potentially interesting model systems for high-throughput immunogen evaluation include transgenic mice with human repertoires (Legrand et al., 2009; Rathinam et al., 2011) and the mouse BLT model (Brainard et al., 2009; Wheeler et al., 2011) as described above. Another approach is to generate immunogens that bind to NHP germline versions of bnMAbs, as well as the corresponding mature human bnAbs, based on modeling studies and on library selection. Determination of the molecular interactions between germline NHP Abs and immunogen would allow iterative improvement of the immunogens, which could then be investigated as candidate vaccines in NHPs. Whether in knock-in mice or NHPs, it is anticipated that iterative cycles of vaccination followed by analysis of B and CD4+ T cell responses to guide re-design of immunogens and immunization strategies, will be needed to generate optimal vaccines.

**Accurately identify and functionally characterize HIV/SIV-specific T follicular helper cells in humans**

T follicular helper (Tfh) cells are newly appreciated cells, distinct from other CD4+ T cell subsets, that are specialized for B cell help and are required for formation and maintenance of GCs (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009) (Johnston et al., 2009; Rolf et al., 2010; Victora et al., 2010). As such, Tfh cells are almost certainly critical for the development of most neutralizing Abs, GCs, and memory B cell responses (Crotty, 2011) (Figure 4). A substantial population of HIV-specific Tfh cells is present in the lymph nodes of HIV-infected individuals (Lindqvist et al., 2012). Interestingly, new SIV studies have found that Tfh cells are abundant in SIV infected macaques, and Tfh cell abundance correlates with GC B cells frequencies (Hong et al, 2012; Petrovas et al., 2012). Furthermore, Tfh cell frequency positively correlates with both anti-SIV IgG quantity and quality (Petrovas et al., 2012). These findings are consistent with the potential importance of Tfh cells in anti-HIV bnAb development. Therefore, it is critical to be able to identify and track HIV-specific Tfh cells to (1) understand their role in the development of bnAb responses in HIV infected humans, (2) quantify Tfh cells elicited by candidate HIV vaccines, and (3) understand and manipulate Tfh cell functions in HIV-specific immune responses.

**Describe the mechanisms of Tfh cell generation and identify adjuvants that optimally elicit Tfh cells and potent B cell responses in the context of HIV Env immunization**

The induction of Tfh cell differentiation is a multifactorial process that not only involves the optimal activation of dendritic cells (DCs), in particular expression of the ligand ICOSL that mediates optimal T cell activation by binding to ICOS on T cells (see review by J. Luban in this issue) (Choi et al., 2011; Goenka et al., 2011), but also optimal activation of B cells to maintain Tfh cell responses. Yet, the ‘danger signals’ that are involved in the generation of Tfh cells remain unclear (Crotty, 2012). The identification of these signals and adjuvants may be critically important for rational vaccine design. Therefore, it is crucial to determine the optimal signals for Tfh cell induction in a multistep process, first by determining factors that induce ICOSL and other Tfh signals on DCs and B cells using in vitro screening, and then validate Tfh differentiation adjuvants in the context of candidate vaccines in NHPs.
Determine the relationships between Tfh cell specificity and anatomic distribution for control of HIV/SIV

HIV infection is primarily a mucosally transmitted infection and studies of mucosal tissue immune responses are key. Therefore, it is important to determine the anatomic locations of HIV-specific Tfh cells in the context of natural infection and candidate vaccines to determine their role in mediating systemic humoral immunity and mucosal humoral immunity. An additional issue is one of protein specificity of the CD4 T cell responses. It is likely that the CD4 T cell responses need to be specific for epitopes within gp41 or gp120 to provide appropriate help to Env-specific B cells, given data from other viral infections (Sette et al., 2008). However, it is possible that this is not the case, and responses specific to the HIV structural protein Gag may be sufficient if whole virions are the primary functional antigen. This again goes to the overall issue of it currently being unknown in what form B cells see functional Env spikes. To track HIV-specific Tfh cells requires an improved knowledge base, as HLA class II restricted HIV epitopes are insufficiently well mapped for human (or NHP) population studies involving single cell level analysis via class II tetramers that recognize antigen-specific T cells.

Identify effector mechanisms of HIV-specific CD4+ T cells and define their role in viral control at the portal of entry

After mucosal transmission creates small founder populations of infected cells, local expansion at the portal of entry is necessary to establish infection, which may offer a window of opportunity for cellular immunity to prevent viral dissemination (Haase, 2011; Li et al., 2012). To address the role of early viral containment through cellular adaptive immunity, cross-sectional studies of blood, gut-associated lymphoid tissue (GALT) and vaginal (cervix) biopsies in HIV elite controllers and individuals with progressive disease should be carried out. Longitudinal studies of NHP CD4+ and CD8+ T cell responses in tissue compartments should first define the spatial correlation between these cells and infected cells, and disease stage. Studies of the early kinetics of the T cell responses in previously vaccinated, acutely SIV-infected NHPs will allow determination of whether an initial influx of virus-specific CD4+ T cells precedes robust CTL responses and correlates with early containment (Kumamoto et al., 2011). Alternatively, CD4+ CTL may directly contribute to containment of HIV infection (Soghoian et al., 2012).

BRINGING IT ALL TOGETHER TO DESIGN IMMUNOGENS AND IMMUNIZATION STRATEGIES TO ELICIT BROADLY NEUTRALIZING HIV ANTIBODY RESPONSES

Many immunogens have been designed and are under design based on our current knowledge of the structure of the HIV Env trimer and on our knowledge of the epitopes recognized by bnMAbs (Forsell et al., 2009; Karlsson Hedestam et al., 2008; Kwong et al., 2011, 2012; Mascola and Montefiori, 2010; Schief et al., 2009; Walker and Burton, 2010). These immunogens are being engineered in myriads of ways (Azoitei et al., 2011; Correia et al., 2011; Correia et al., 2010; McLellan et al., 2011; Ofek et al., 2010) and are based not only on immunogen interaction with mature bnMAbs but also on interaction with the bnMAbs in a germ line configuration (Haynes et al., 2012; Xiao et al., 2009). We expect that many of the new tools for analyzing immune responses to immunization, such as systems biology, antibody deep sequencing and single B cell methods for antibody isolation, will be invaluable in providing a rich feedback on the strengths and weaknesses of the immunogen designs and immunization strategies. As the goals described above are realized, we expect the information to be incorporated to further improve the rational design strategy. We see one of the most crucial rate-limiting steps in the whole process to be iterative immunogen
evaluation. Practical considerations appear to rule out the ideal test subjects for such evaluation—humans—and then the best candidates appear to be macaques or small animals expressing human antibody repertoires.

In summary, the central hypotheses that we advocate are that a successful HIV vaccine should elicit protective antibodies, and that the combination of B cell and CD4+ T cell responses is critical for the induction and long-term maintenance of vaccine protection. We believe that it is crucial to define immunogens and immunization regimens that induce protective B cell and CD4+ T cell responses in preclinical models and thereby guide product development strategies for a preventive human HIV vaccine. We note that such a vaccine may well also need to induce HIV-specific CD8+ T cell responses for maximal efficacy as considered elsewhere (Barouch and Korber, 2010; Johnston and Fauci, 2007; McElrath and Haynes, 2010; Walker and Burton, 2008; Watkins, 2008). We propose here integrated efforts focused on two areas: (1) B cell and antibody research to guide the development of immunogens and immunization regimens that elicit protective HIV antibody responses and (2) CD4+ T cell research, taking advantage of key preliminary data to maximize the T cell help offered to B cell responses through immunization, and to harness the direct antiviral activity of CD4+ T cells (Figure 5). Overall, we view this as an extremely exciting period in the field of HIV vaccine research and one that engenders more hope than has been noted for a long time.

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Figure 1. Structure and antibody recognition of the HIV Envelope spike

The molecule is a heterotrimer of composition (gp120)3 (gp41)3. Gp41 is a transmembrane protein and gp120 is the receptor molecule for CD4 and CCR5 (or CXCR4). The model is adapted from a cryo-electron tomographic structure of the HIV trimer (Liu et al., 2008). The crystal structure of the b12-bound monomeric gp120 core (red) has been fitted into the density map (Zhou et al., 2007). Glycans are shown in purple. The CD4 binding site is shown in yellow. The approximate locations of the epitopes targeted by existing bnMAbs are indicated with arrows, and the number of MAbs targeting each epitope is shown in red boxes. A small selection of bnMAbs targeting each epitope is included.
For a long period, MAbs such as b12, 2G12 and 4E10 were the most potent and broad available. MAbs PG9 (Walker et al., 2009) and VRC01 (Wu et al., 2010) were discovered and shown to be broad and about an order of magnitude more potent. Later, MAbs PGT121 and PGT128 (Walker et al., 2011a) were shown to be even more potent. The engineered MAb NIH45-46G54W is still more potent and broad (Diskin et al., 2011). Enhanced neutralization potency, if translated into enhanced protective ability, is important since it reduces the level of antibody that should be induced by a vaccine.
Figure 3. Model for native Env glycosylation

Following removal of terminal α-linked glucose residues in the endoplasmic reticulum (ER), folded glycoproteins contain exclusively oligomannose glycans. During transit through the ER, intermediate compartment and cis-Golgi apparatus, Manα1-2Man termini are removed by mannosidases to yield Man5GlcNAc2. However, the oligomannose cluster intrinsic to monomeric gp120 limits glycan processing on both monomeric and oligomeric gp120. The steric consequences of trimerization further limit Manα1-2Man trimming leading to an additional ‘trimer-associated’ population of Man5–9GlcNAc2. The exposed Man5GlcNAc2 glycans on gp120 that passage through the full extent of the Golgi apparatus and trans Golgi network to the plasma membrane are processed to form complex-type glycans. However, envelope glycoprotein that does not follow this route to the plasma membrane- a notable feature of some pseudoviral production systems- is characterized by an elevated abundance of Man5GlcNAc2 and reduced furin cleavage (Crooks et al., 2011). Thus the intrinsic mannose patch, which includes the 2G12 epitope, persists from the earliest stages of glycan processing whilst other elements of the glycan shield exhibit variably processed glycans depending on oligomerization state and, at least in the case of pseudoviral gp160/gp120, cellular trafficking (adapted from (Bonomelli et al., 2011).
Figure 4. T follicular helper (Tfh) cells are the CD4+ T cells that are required for germinal centers (GC) and control B cell differentiation within the GCs. Tfh cells provide different signals to B cells to control different B cell fates, such as plasma cell (antibody secreting cell) differentiation, memory B cell differentiation, death, or repeated rounds of somatic hypermutation and GC B cell proliferation. Generation of high-affinity neutralizing antibodies is generally a multi-step, iterative process that is dependent on affinity maturation via somatic hypermutation and extensive signaling from Tfh cells (Crotty, 2011).
Figure 5. An approach to HIV vaccine discovery
Molecular data on protective Abs (typically bnAbs), Env and Ab-Env complexes, should facilitate the generation of antigens presenting protective epitopes. These antigens can then be formulated as immunogens and tested in animals and humans. The characteristics of the protective Abs also provide information on how they were generated that can be investigated in B and CD4+ T cell studies, guiding both immunogen design and immunization strategies. Gaps in our current knowledge suggest that iteration will be an important part of the development of vaccine candidates as indicated by the feedback loops shown in the figure.