1. INTRODUCTION
Much of vaccine development seeks to determine and then to mimic the protective immune response generated during natural infection by a pathogen. For example, polio and influenza vaccines each generate specific antibodies to viral surface proteins that can inactivate the invading virus during the earliest stages of infection, thereby preventing severe forms of disease [1]. During HIV infection, most individuals develop a strong antibody response to the viral envelope (Env), but most of these antibodies are unable to neutralize or to inactivate naturally circulating strains of the virus [2,3]. The known monoclonal antibodies against HIV have been studied with the goal of discovering responses that inform vaccine design. Few naturally occurring antibodies display the combination of potent neutralization and breadth of reactivity [2,4,5] needed for vaccines that could be deployed for public health purposes. Additionally, in the past, the limited natural examples of broad HIV-neutralizing antibodies have made it difficult to understand how an HIV vaccine might generate an effective antibody response [6]. Moreover, initial attempts to develop HIV vaccines focused on recombinant forms of glycoprotein (gp120), a surface protein that is part of the HIV-1 virus envelope. When tested in clinical trials, these protein vaccines did not provide protection against HIV infection or delay HIV-1 disease progression [7,8]. Importantly, a prime-boost regime in a phase III trial did, however, provide ~30% efficacy, though significant neutralizing antibody or CD8 T-cell responses were not observed [9]. While these gp120 vaccines induced vaccine-specific antibody responses, the low level of vaccine efficacy was probably related to their failure to elicit broadly neutralizing antibodies [10–12]; understanding the molecular and immunological bases for the poor to weak protection offered by these prior vaccine failures may facilitate the development of more effective HIV-1 vaccines.

We have used a number of approaches to ascertain information about HIV-1 and the human immune response that could provide new insights for the development of an effective HIV-1 vaccine. Structural and computational biology represent technologies that provide an understanding of the structure of the HIV Env and that help to define the structural regions necessary for viral entry into host cells [13] (figure 1). At the same time, this information is useful in designing immunogens that elicit targeted immune responses and antibodies capable of neutralizing HIV-1. These structural and informatics-based approaches seek to integrate information about virus–antibody interactions and results from antigenic and immunogenicity studies.

2. CHALLENGES TO HIV-1 VACCINE DESIGN
HIV-1 is an enveloped virus, and the only viral protein that protrudes through the host-derived lipid membrane that surrounds and protects the viral core structural proteins is HIV Env, composed of the gp120-envelope and the gp41-transmembrane glycoproteins that form the viral spike (figure 1a). These glycoproteins are known targets of neutralizing antibodies and are necessary for HIV-1 infection, as they mediate attachment of the virus to the cell via the cellular CD4 receptor. Neutralizing antibodies to these viral proteins act by either binding to the spike and preventing attachment to the cell or receptor, or binding to the spike and preventing conformational changes that are required for virus entry.

The gp120 protein core in its CD4-bound state contains an inner domain, an outer domain (OD), and a four-stranded bridging sheet minidomain, the latter of which is composed of two β-hairpins which extend from the inner domain (β2–β3) and OD (β20–β21), respectively [15,16] (figure 1b). The OD is extensively glycosylated, and previous studies involving antigenic
analysis and fitting into the viral spike reveal that the glycan surface covers most of the exposed surface of the spike, making it immunologically silent [17]. Thus, while it would seem straightforward to induce antibodies to the spike proteins that would be able to neutralize the virus, HIV-1 has developed multiple mechanisms of immune evasion involving the molecular structure of the virus, particularly the viral spike, including glycan shielding, variable loop divergence and extensive conformational flexibility, all of which succeed in preventing either the elicitation or the binding of most antibodies.

Although there are natural examples of HIV-neutralizing antibodies, they have been limited in number and the assays for neutralization have previously been time- and resource-consuming, making it difficult to determine how to design an HIV vaccine that would generate an effective antibody response [6]. However,
the availability of new high throughput assays has improved the ability to test large panels of sera for HIV neutralization, and these analyses have led to the recognition that about 10–25% of HIV-infected individuals do in fact make relatively broadly reactive neutralizing antibodies during the course of HIV infection [18]. This important new insight suggests that humans, even those with immune systems damaged by HIV-1 infection, have the ability to generate neutralizing responses following HIV-1 infection. By studying the sera and the antibody secreting B-cells from infected donors, we can better understand how such antibodies arise during natural HIV infection. This type of knowledge can then be used to design HIV vaccines and vaccination strategies that may make it possible to elicit antibodies with similar neutralization specificities.

3. USE OF INFORMATICS FOR UNDERSTANDING THE IMMUNE RESPONSE TO HIV

The functional HIV-1 viral spike is a trimer that consists of three gp120s, which associate non-covalently with the ectodomains of three gp41s. Despite extensive efforts by several groups worldwide, the trimeric spike has thus far resisted atomic-level determination. However, low resolution cryoelectron microscopy studies have provided important vaccine-relevant information about gp120–gp41 arrangements [14,19], including structures of the viral spike prior to receptor encounter, intermediate states of the virus during entry and post-fusion states. This type of information can be used to assess antibody functionality on a molecular level that may be predictive of biological activity in more complex in vitro and in vivo assays of antibody activity. Additionally, we and others have obtained atomic-level structural information on individual gp120 and gp41 components. For gp41, only post-fusion structures have been determined. For gp120, the crystal structures of a number of states for a conserved core have been determined, including antibody-bound conformations, although the best characterization comes from the CD4-bound state.

Thus, structural biology has provided important information about the three-dimensional organization and chemical structure of the HIV-1 glycoproteins. This information and in particular an understanding of atomic-level structure can be used rationally to design proteins that have specific biological properties and functions that would be important in vaccine design, such as incorporation of conserved sequences, which are generally associated with functions that are essential to the virus, and properties of virus proteins, such as the ability to stimulate specific protective immune responses.

This concept is being applied to HIV-1 to provide a structural definition of the functional viral spike (figure 1a), which is used by the virus to enter host cells and is the target of all known virus-directed neutralizing antibodies. The ability to conduct atomic-level analysis of the spike facilitates immunogen designs that stabilize and help to present potential sites of neutralization more optimally to the immune system. Unfortunately, the same virus strategies noted above that allow the virus spike to evade an effective immune response also hinder structural analysis, and the entire HIV-1 spike has resisted and continues to resist atomic-level characterization.

Nevertheless, structural analysis and molecular modelling have facilitated the understanding of the antibody response against HIV. This knowledge includes (i) the capability to dissect the types of antibodies in sera and to ascertain what regions of the HIV Env are targeted [20], (ii) the ability to isolate neutralizing antibodies from individual B-cells [21,22], and (iii) the capacity to determine the atomic-level structure of neutralizing antibodies bound to the HIV Env [23–25]. We have used this knowledge of the structure of the HIV Env to design protein probes that expose various regions of the HIV Env in specific conformations (figure 2). The concept underlying these probes is that one can use molecular biological tools and structural and functional knowledge to design and generate a molecule that preserves the antigenic structure of the viral surface that one wishes to study, but does not contain irrelevant

Figure 2. Resurfaced stabilized core proteins derived from HIV-1 Env can serve as probes to define antibodies to the CD4-binding site and as prototype immunogens. By modifying surface exposed residues of the HIV-1 Env (left) and replacing them with alternative amino acids from SIV Env or other acceptable substitutions (red), it is possible to develop a defined protein (right) that can be used both for analysis of sera and as prototype immunogens.

1. probe to isolate B-cells and clone broadly neutralizing antibodies
2. prototype immunogens to elicit antibodies to the highly conserved CD4-binding site
antigenic regions of HIV-1. These kinds of probes can then be used to evaluate the regions of the HIV Env that are targeted by serum neutralizing antibodies. A key virus functional region, the CD4-binding site of gp120, has been studied in this way. Since CD4 is the primary cellular receptor for HIV, antibodies that bind to the CD4-binding site can block HIV infection of CD4+ T cells and thus function as neutralizing antibodies. To define the neutralizing antibodies to the CD4-binding site further, a specific protein probe was designed such that it exposed the CD4-binding site of gp120, while other regions of HIV were altered to be unrecognizable to HIV antibodies, e.g. by substitution with simian immunodeficiency virus (SIV) homologues or other non-HIV residues (figure 2). This epitope-specific probe, along with a knockout mutant was used to identify B-cells making antibodies to the CD4-binding site [25,26]. Following the isolation of these B-cells by flow cytometry, one can then use PCR to amplify the genes encoding the antibody heavy and light chain variable regions (VH and VL) and subsequently express the full IgG monoclonal antibody in tissue culture (figure 3). Having these new informatics-designed monoclonal antibodies in hand, it is possible to verify and to study in detail their ability to neutralize of diverse viral strains.

Another approach to the identification of relevant immunogens seeks to bypass the difficulties of using the entire viral spike and rather focuses on the functionally critical sites that the virus uses for entry. By definition, these sites cannot be altered without hindering spike function. We and others have used this approach to elicit antibodies against the highly conserved site of co-receptor binding [27]. Unfortunately, while it is possible to prepare immunogens that will elicit antibodies to this site, because the site is only revealed after the engagement of the viral and target cell membranes, it is effectively inaccessible to protective antibodies that might be elicited by a vaccine [28]. Thus, in choosing potential vaccine immunogens, one must verify that in addition to functional importance and sequence conservation, the site is vulnerable to the neutralizing antibody.

A third approach to identifying vaccine-relevant immunogens focuses on effective antibody responses [5,29]. The underlying concept is that analysis of monoclonal antibodies selected for their ability to neutralize HIV-1 effectively, will facilitate an understanding of protective immune responses. After identifying the epitope recognized by these monoclonal antibodies, one can work backwards to create molecular mimics of the epitope with the goal of using these mimics to elicit the original template antibody. Unfortunately, many of the identified monoclonal antibodies that effectively neutralize HIV-1 appear to have unusual properties that make it difficult to elicit them. This situation suggests that one
needs information about the frequency and elicitation pathway of the template antibody to succeed with this approach.

4. USING MOLECULAR STRUCTURE AND INFORMATIC TECHNOLOGY TO IMPROVE IMMUNOGEN DESIGN FOR BROADLY NEUTRALIZING HIV-1 ANTIBODIES

The elicitation and generation of a particular antibody response requires at least three steps: antibody gene recombination to form naive B-cell receptors, deletion of autoreactive B-cell clones, and antigen-driven affinity maturation of the antibody response. Surprisingly, despite the presence of substantial quantities of gp120 in HIV-1 infected individuals, the human immune system often does not make antibodies against the CD4-binding site that are effective at neutralizing primary isolates of HIV-1. When CD4-binding site directed neutralizing antibodies do arise, it is only after several years of ongoing HIV-1 infection [20,30]. The reasons for the poor immune response to this important region of HIV-1 Env are unknown. We have analysed the maturation of the antibody VRC01 as a way to provide insight into which of these steps might be responsible for the reduced elicitation of potent CD4-binding site antibodies and perhaps high-affinity neutralizing antibodies in general [25].

Our investigations have shown that HIV-1 recognition by VRC01 primarily involves regions of the antibody derived from VH and variable K light chain precursors, and that its generation does not appear to be dependent on unusual recombination events. Additionally, VRC01 and its genomic precursors do not appear to be autoreactive. An important characteristic of VRC01 is that the level of affinity maturation is approximately 10-fold higher than that of most antibodies in the repertoire. Another important factor is that the putative genomic precursors appear to have low (mM or weaker) affinity for gp120. Thus, a key barrier to eliciting VRC01-like antibodies appears to be the inability of probable genomic precursors of these antibodies to bind the gp120 immunogen. One potential approach to bypassing this barrier might involve creating altered gp120s that are better able to bind to unmutated precursors of VRC01 and thus better drive antibody maturation.

Moreover, the understanding of the interactions of broadly neutralizing antibodies, particularly studies of the b12 and VRC01 antibodies directed to the CD4-binding site of HIV Env, has provided a conceptual basis for the development of four strategies to elicit antibodies with similar specificities. These strategies use biological and structural information to design immunogens that might enhance the elicitation of effective neutralizing antibodies or that might be used as probes for the analysis of complex mixtures of antisera.

The first strategy is the generation of trimeric forms of the HIV-1 Env by including the gp41 trimerization motifs in the absence of the transmembrane domain. Trimerization sequences from heterologous proteins, such as the fibrinogen protein from phage lambda, can be used to further stabilize this form of the protein. Thus, employing this approach, or by using the natural gp160 trimerization motifs, one can generate stable trimers using site-specific mutations to fix the core structure. The variable domains of these proteins can be deleted because they might otherwise divert immune responses to strain-specific determinants.

In a second strategy, stabilized core Env proteins are further modified using structure-based design [26]. The underlying concept is to generate a molecule that preserves the key surface antigenic structures of HIV-1 involved in neutralization, but that eliminates other antigenic regions. In practice, one uses bioinformatics and computer-assisted design to introduce mutations that eliminate HIV residues on the surface of gp120 and replace these residues with those of a virus that shows minimal serologic cross-reactivity with HIV-1, e.g. SIV Env. By a progressive process of modifying the surface of the constrained Env core protein and subsequently covering the modified region with glycans, one can generate a set of molecules that can function both as probes to analyse complex antisera for the presence of broadly neutralizing antibodies, as well as prototype immunogens to elicit antibodies directed to the highly conserved CD4-binding site (figure 2).

A third approach towards eliciting antibodies with desired specificities is to eliminate immunologic determinants that are not relevant to the desired immune response. As an example of this, we have generated a subdomain of the HIV-1 Env which includes the OD that contains the initial CD4 binding loop, but eliminates most other immunologically irrelevant protein sequences. However, a previous study showed that a soluble form of the OD that contains the b15 loop was not able to bind to the b12 antibody with high affinity. One approach to overcoming this problem involves the inclusion of a transmembrane domain [31]. Others use further site-directed mutagenesis based on the b12/Env and the VRC01/Env structures. These types of methods represent ways to stabilize this interaction and also provide additional hydrophilic surfaces that may improve folding or stabilize additional contacts of the VRC01 antibody. Recently, additional mutations in the OD have been generated that preserve high-affinity binding by decreasing the off-rate in binding as demonstrated by surface plasmon resonance spectroscopy studies. The vectors developed by this approach are currently being evaluated for their ability to elicit broadly neutralizing antibodies and for their usefulness in characterizing these complex antisera.

The fourth approach to immunogen design and development involves the use of scaffolds. These scaffolds are designed to stabilize critical epitopes for antigen presentation. Appropriate conformational structures are identified and critical epitopes, for example the b15 loop, are transplanted onto heterologous scaffolds that are not antigenically related to HIV-1. While several scaffold structures have been created that bind to neutralizing antibodies, they remain of low affinity. This strategy is under continued investigation.

5. ISSUES RELATED TO ELICITING THE DESIRED IMMUNE RESPONSE

In developing strategies to generate a robust neutralizing antibody response to HIV, a number of fundamental aspects of B-cell biology must be considered. These

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The HIV-1 virus replication cycle starts with sequential interactions involving viral envelope glycoproteins, the CD4 molecule on the cell surface and a member of the seven-transmembrane, G-protein-coupled, receptor family (co-receptor), one of which is CCR5.

The b12 antibody is a monoclonal IgG1 antibody that occludes the site of CD4 binding on gp120 and prevents virus attachment to CD4 on target cells [24].

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