The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus

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Abstract | The ability to elicit broadly neutralizing antibody responses against HIV-1 is a crucial goal for a prophylactic HIV-1 vaccine. Here, we discuss the difficulties of achieving broad HIV-1 neutralization in the context of both the effective annual human influenza virus vaccine and the need to develop a pandemic influenza vaccine. Immunogen-design strategies are underway to target functionally conserved regions of the HIV-1 envelope glycoproteins, and similar strategies might be applicable to pandemic influenza virus vaccine development. Efforts to develop broadly neutralizing vaccines against either HIV-1 or influenza virus might establish a paradigm for future vaccines against highly variable pathogens.

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Vaccination is the prime strategy to combat viral infections in humans; vaccination strategies have resulted in the eradication of smallpox and are the main tools for the eradication of polio and measles. Although live-attenuated virus vaccines, such as those against mumps, measles, polio and rubella viruses, induce cell-mediated and humoral immunity, they are thought to provide protection primarily through their ability to stimulate polyclonal neutralizing antibodies[1,2]. Non-replicating virus vaccines, such as whole-inactivated viruses (used for polio ('Salk')), 'split-virus' (detergent-disrupted virus preparations; used for influenza) or viral subunits (such as the particle vaccine that is used for hepatitis B and human papilloma virus), almost exclusively induce antibody responses, yet are also efficacious. The seasonal inactivated influenza virus vaccine elicits type-specific neutralizing antibodies that are detectable in the serum of vaccinated individuals. This vaccine is highly protective and substantially limits the morbidity and mortality of annual influenza virus epidemics. The subunit vaccine against the chronic hepatitis B virus is also highly protective, but vaccines against other persistent viruses that establish and maintain a chronic infection are less successful. For the chronic and highly variable HIV-1 (FIG. 1; BOX 1), the ability to stimulate broadly neutralizing antibodies through vaccination, as a subset of the polyclonal antibody response, is a long-desired goal. Current models predict that if vaccine-induced immunity to HIV-1 could substantially lower the level of acute viraemia or lower the chronic viral load 'set-point' in an individual, then the transmission rate in the human population would be greatly reduced[3].

In this Review, we compare and contrast the challenge of developing a vaccine that induces broadly neutralizing antibodies against HIV-1 with the challenge of annually developing an efficacious vaccine for influenza virus. We also discuss the challenge that is faced in the development of vaccines for pandemic influenza virus (the so-called avian 'flu), as these vaccines probably need to deal with considerable viral variation if they are to be efficacious (BOX 1). Both the current inactivated split-virus influenza vaccine and the recently licensed live-attenuated influenza vaccine rely on the 'predict and produce' approach, as they are generated from the virus strains that are the most likely to spread across the globe in the upcoming influenza season (see below).

For HIV-1, however, there are already 33 million infected individuals who each harbour a substantial array of HIV-1 quasi-species, which results in an enormous number of variants that are simultaneously seeded and circulating in the human population. Providing protection against this vast array of potentially infectious isolates is a challenge of unprecedented magnitude in vaccine development. Not surprisingly, the classical vaccine approaches of chemical inactivation or live attenuation have not produced a broadly protective or safe HIV-1 vaccine. In some ways, developing a pandemic influenza virus vaccine presents a similar and daunting challenge. Due to the extreme worldwide variability in avian 'flu reservoirs

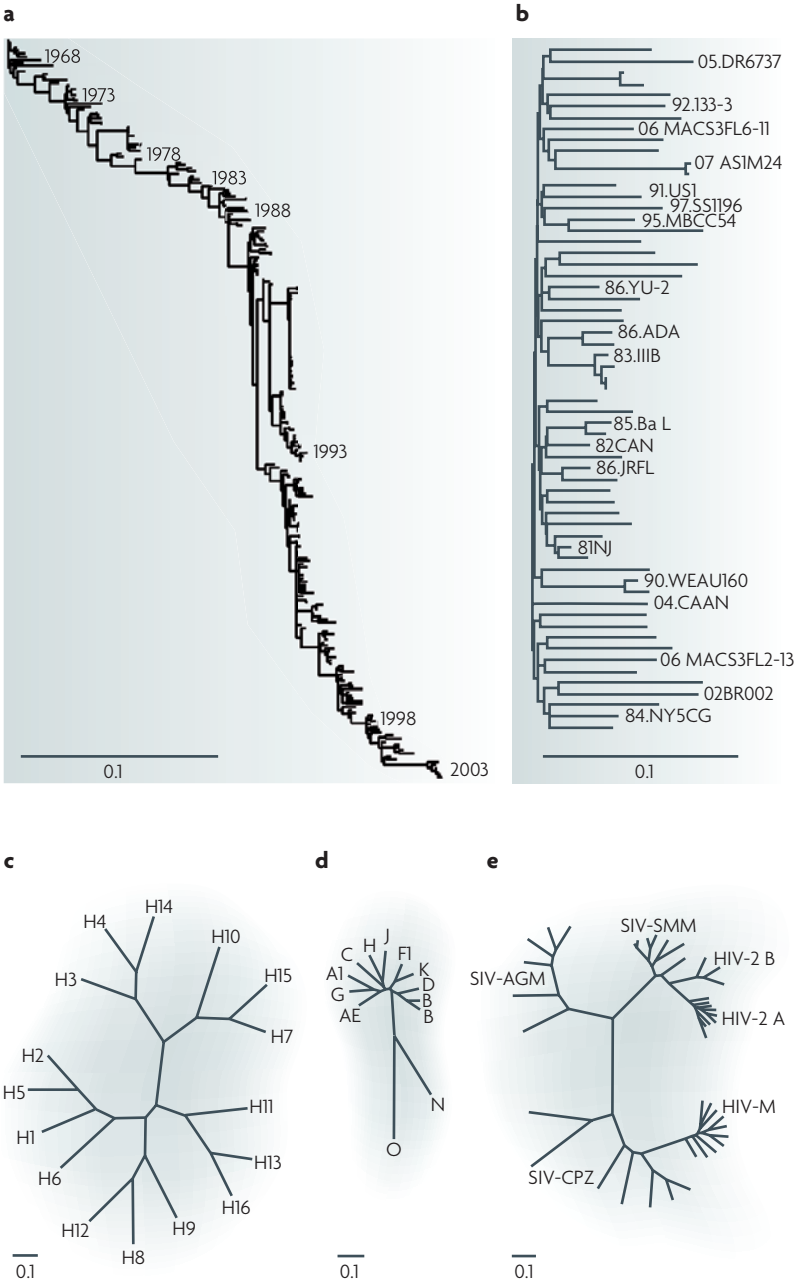

Figure 1 | **Influenza virus, HIV-1/HIV-2/SIV envelope glycoprotein coding sequences.** DNA maximum-likelihood trees were generated using HA0 and gp160 open reading frames. Scale bars are shown for each panel and represent approximately 10% of nucleotide differences between close relatives (note that the bars are not representative for long distances). For ease of comparison, Panels **a** and **b** are on the same scale, as are panels **c**, **d** and **e**. Panel **a** shows H3 subtype sequence diversity derived from human infection over 35 years, whereas panel **b** shows HIV-1 envelope sequence diversity within clade B over 25 years. The strong selection for drift variants of influenza A virus (panel **a**) does not obstruct vaccination, as long as the vaccine is updated every few years. By contrast, owing to the genetic diversification of HIV-1 (clade B is shown), the resulting simultaneous antigenic diversity is extremely challenging to encapsulate in a single vaccine. Panel **c** shows a comparison of worldwide haemagglutinin (HA) diversity from all influenza A virus subtypes in avian reservoirs; panel **d** shows HIV-1 diversity in humans using representative clade envelope glycoprotein (Env) sequences; panel **e** shows the diversity of primate lenti immunodeficiency viruses, including HIV-2 in humans and SIV in simians (sequences from REF. 133). As seen, overall HA diversity (maximum pair-wise distance 1.33; H5 to H14) is greater than that of HIV-1 Env gp160, but is less than that of Env sequences of primate lentiviruses (1.63; HIV-M to HIV-2).

(FIG. 1), it is extremely difficult to predict which avian 'flu strain might acquire the ability to infect and disseminate throughout the human population. Owing to the narrow time frame between the detection of human infection and the generation of an effective influenza vaccine, classical 'predict and produce' methods may not be feasible for the development of a pandemic 'flu vaccine.

The renewed interest in improving the titre and cross-reactivity of vaccine-induced antibody responses to influenza viruses has prompted us to examine parallels between the neutralization of influenza virus and HIV-1, with the goal of promoting crosstalk and collaboration between the two vaccine fields. Cross-fertilization of ideas between the two fields is currently scant, as the annual influenza epidemic generally emanates from a single predominant strain, which renders the classical methodologies of inactivated split-virus vaccine production effective, with little need for alternative vaccine approaches. However, because of multiple lethal infections of humans from avian influenza viruses of the H5 subtype in Asia and elsewhere (BOX 1; REF. 4), interest in developing a broadly protective H5 subtype vaccine has increased[5–8]. The desire to develop pandemic influenza vaccine candidates, and to improve the efficacy of existing seasonal influenza virus vaccines, is motivated by the goal to achieve a more 'universal vaccine' for potential more long-term global coverage.

For an HIV-1 vaccine, the envelope glycoproteins (Envs) gp120 and gp41 are the only virus-encoded determinants that are present on the virus surface. Conserved regions in these proteins are candidate targets for the development of antibodies that can neutralize a wide array of circulating HIV-1 isolates (that is, with 'neutralization breadth'). Besides attempts to develop protein-based subunit vaccine candidates, other approaches that are based on plasmid DNA or recombinant viral vectors that encode HIV-1 Envs are also under development[9], but do not yet induce broadly neutralizing antibodies. Therefore, by necessity, rational structure-based immunogen-design efforts to target HIV-1 Envs are ongoing, with the aim of eliciting broader neutralizing antibody responses. As we describe below, many Env-based design efforts are based on the assumption that it will be necessary to overcome obstacles that are created by the immune-evasion capabilities that are 'built in' to the HIV-1 Envs by host immune-selective pressures[10–13].

So far there has been little focus on structure-based immunogen design of recombinant haemagglutinin (HA) proteins (the major viral neutralizing determinant of influenza) to improve the quality of vaccine-induced antibodies that are directed against influenza virus. So, another goal of this Review is to highlight selected structure-based, rational design efforts that are ongoing in the HIV-1 vaccine field that might also be applicable to increase the neutralization breadth of current influenza virus vaccines. It is possible that selected HIV-1 Env vaccine-design strategies may be more effective when applied to the influenza virus HA, as HA has not evolved as many immune-evasion mechanisms

Box 1 | **HIV-1 and influenza virus — an overview**

HIV-1 is a member of the *Retroviridae* family and belongs to the genus lentiviruses. The *Retroviridae* are enveloped viruses that contain two positive-sense RNA strands. These RNA strands undergo conversion into double stranded DNA by the highly error-prone viral reverse transcriptase enzyme, which generates isolate diversity by point mutation and intergenomic recombination. Based in part on genetic variation of the surface envelope glycoproteins, HIV-1 isolates fall into three groups — M (Major/Main), N (Non-M, Non-O/New) and O (Outlier) — of which group M is the most common. Group M is subdivided into several subtypes or clades (A-D, F-H, J and K), of which B is the most common in the Western world and subtype C is primarily found in India, China and sub-Saharan Africa. The remaining subtypes, as well as HIV-1 variants with characteristics of several different subtypes (so-called circulating recombinant forms), are mainly spread throughout Africa[116]. The genetic diversity of HIV-1 and the related primate immunodeficiency viruses HIV-2 and simian immunodeficiency viruses (SIV) are shown in FIG. 1.

Influenza viruses are negative-strand, segmented RNA viruses that belong to the *Orthomyxoviridae* family. Influenza viruses are classified on the basis of the antigenic properties of their matrix proteins and nucleoproteins into types A, B and C, of which types A and B are the main cause of the seasonal influenza epidemics. Influenza A viruses are further divided into subtypes on the basis of the antigenic properties of their surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). Sixteen HA and nine NA subtypes have been identified in the wild bird reservoir, and various HA–NA combinations can occur. Influenza viruses of subtypes H3N2 and H1N1 are currently endemic in humans. The trimeric influenza virus protein HA is the major target for neutralizing antibodies against influenza virus and is responsible for receptor binding. The amino-acid sequence in each HA subtype varies by up to 20%, whereas the amino-acid-sequence diversity between different subtypes ranges from 30–70%[117,118]. Several comparisons of the genetic variability of the HIV-1 and influenza A virus surface glycoproteins are shown in FIG. 1.

as have HIV-1 Envs to facilitate chronic viral persistence. To illustrate parallels and differences between the HIV-1 and influenza virus Envs, the biosynthesis, architecture and function of Envs are outlined in BOX 2 and are shown in FIGS 2,3,4.

Immune protection against global viruses

Hallmarks of the CD4-tropic HIV-1 retroviral infection are its ability to establish chronic infection in the host and its capacity to generate extraordinary viral genetic variability. The most variable gene products of HIV-1 are the Envs, which, through selection, have evolved many mechanisms to evade host neutralizing antibody responses[10,13]. HIV-1 has only low infectivity by sexual routes, but once it establishes a chronic infection the virus evolves rapidly in the face of potent immune responses in infected individuals[14]. Influenza A virus is a globally persistent virus, and

Box 2 | **Envelope glycoprotein biosynthesis and HIV-1 and influenza virus entry**

The major targets for HIV-1 neutralizing antibodies are the envelope glycoproteins (Envs) gp120 and gp41 (REF. 13). These proteins are generated by proteolytic cleavage of a heavily glycosylated biosynthetic precursor protein (gp160) during transport through the Golgi apparatus. Once transported to the cellular plasma membrane, trimeric gp120–gp41 complexes are incorporated into budding virus for the release of new infectious HIV-1 particles. HIV-1-receptor binding is mediated by the external Env gp120, which binds the primary receptor, CD4, on potential target cells (FIG. 3). Following CD4 binding, a series of conformational changes occur in Envs that result in exposure of a transient binding site that allows the virus to interact with its co-receptor, usually the chemokine receptor CCR5 or CXCR4, to initiate another cycle of infection. The transmembrane Env gp41 carries the trimerization domain and is responsible for membrane fusion, which takes place at the plasma membrane at neutral pH (FIG. 4).

In influenza virus, the biosynthesis of the precursor to haemagglutinin (HA), HA0, is similar to that of the HIV-1 Env precursor gp160. Following translocation into the endoplasmic reticulum, HA0 undergoes trimerization and is transported through the Golgi apparatus to the cell surface. In contrast to HIV-1 gp160, which is proteolytically cleaved into gp120 and gp41 in the Golgi apparatus, HA0 in viruses of the H1, H2 and H3 subtypes is cleaved on released virus particles or on the cell surface by a serine protease, yielding HA1 (equivalent to HIV-1 gp120) and HA2 (equivalent to HIV-1 gp41)[119–121]. Cleavage of HA0 into the mature HA primes the protein for fusion. The receptor binding domain of influenza virus is located in a shallow cavity in the membrane-distal globular domain of HA[122]. This site mediates binding to terminal sialic acids of glycoproteins and/or glycolipids[26,123] (FIG. 3). Two types of linkages between sialic acid and galactose, Neu5Acα(2,3)-Gal and Neu5Acα(2,6)-Gal, determine tropism by avian and human influenza viruses, respectively[124]. Only a few amino-acid changes in the binding site on HA determine which receptor will be used[125] and may influence neutralization sensitivity. In contrast with HIV-1 Envs, which mediate fusion by receptor-triggered conformational changes, influenza virus enters an endosomal compartment upon binding to its receptor (FIG. 4). Once in a low pH environment, HA undergoes an irreversible conformational change, which results in the exposure of the N-terminal fusion peptide that is located in HA2.

These different modes of entry of HIV-1 and influenza virus may have implications for the mechanisms by which neutralization can occur. For HIV-1, neutralization can be achieved by antibodies that interfere with either receptor binding or with the ability of Envs to catalyse the membrane-fusion process[10]. By contrast, for influenza virus, most neutralizing antibodies interfere with the ability of the virus to bind its receptor and gain access to low pH compartments, which are required to trigger HA-mediated fusion[41]. A recent study suggests that a single trimer spike is sufficient for HIV-1 entry, whereas as many as 8 or 9 HA trimers are required for entry of influenza virus. This difference might explain in part the relative resistance to neutralization of the two viruses[126].

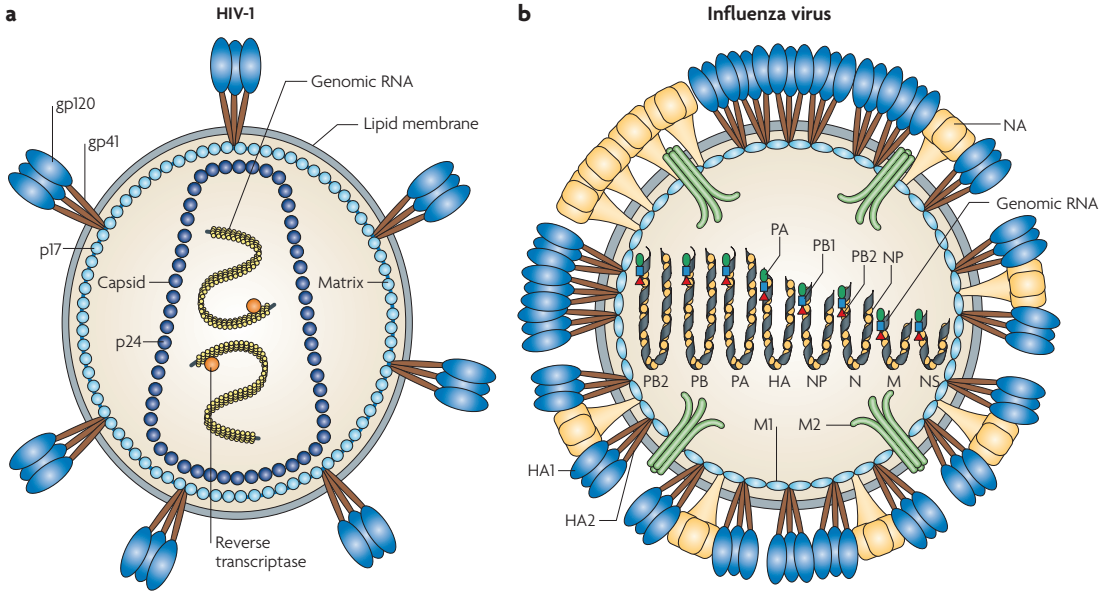

Figure 2 | **Schematic diagram of HIV-1 and influenza A virus.** Both HIV-1 and influenza A virus are approximately 80–120 nm in diameter and are enveloped by a host-derived plasma membrane. **a** | In HIV-1, trimeric gp120–gp41 complexes are embedded in the membrane. The transmembrane glycoprotein gp41 and the external envelope glycoprotein gp120 are depicted in non-covalent association. The cytoplasmic tail of gp41 interacts with the HIV-1 matrix protein p17. The capsid protein, p24, makes up the cone-shaped core, which contains two positive-strand RNA copies of the HIV-1 genome that are surrounded by the nucleocapsid protein (yellow). Reverse transcriptase protein is also packaged into the particle. **b** | In influenza A virus, 3 viral proteins are exposed on the outside of virus particles: haemagglutinin (HA, which forms trimers), neuraminidase (NA) (which forms tetramers) and M2 (which forms tetramers that make up ion-channels). Upon proteolytic cleavage, HA0 (not shown) is processed to HA1 and HA2. The influenza virus matrix protein M1 associates inside the viral membrane, and the viral genome consists of eight negative-strand RNA segments and is packaged into the particle as a ribonucleoprotein in complex with nucleocapsid protein (NP) and the viral polymerases PA, PB1 and PB2. On average, the number of HIV-1 envelope glycoprotein spikes is thought to be considerably lower than the number of HA molecules per influenza A virion[134].

the HA surface glycoprotein displays a high degree of sequence variability (BOX 1; FIG. 1). Interpreted loosely, seasonal influenza virus stays 'one step ahead' of host neutralizing responses by the generation and selection of new variants. So rather than persisting in individually infected hosts, the virus persists in the whole human population by infecting individuals with no or low protective immunity against new variants: this is known as 'antigenic drift'[15,16]. In addition to seasonal influenza viruses in humans, various antigenically variable influenza A viruses circulate in animal reservoirs, and these occasionally re-assort with other influenza genes to cross the species barrier, thereby causing new pandemics in humans by 'antigenic shift'[16]. These reservoirs, largely avian, provide some parallels to the global diversity of HIV-1 (FIG. 1) and represent a major challenge in the development of vaccine candidates against pandemic influenza. Therefore, despite the differences in the biology of the influenza virus (an acute infection) and HIV-1 (a chronic infection), and despite the different selective forces that generate diversity in these viruses, parallels can be drawn between the two viruses and between the challenges of inducing broadly neutralizing antibody responses against them.

Both viruses possess error-prone polymerases, the molecular basis for generating variability. HIV-1 has a highly error prone reverse transcriptase (RT), can tolerate many mutations and can generate fit variants that persist despite diverse host selection pressures. Not only is RT-mediated sequence diversity generated at a high frequency by point mutation or recombination, but HIV-1 also establishes a chronic infection, which includes multiple rounds of viral replication and a continuous generation of immune escape variants. Chronic infections provide the opportunity to follow virus and antibody evolution in infected individuals[14,17,18]. For HIV-1, longitudinal studies in a single host have revealed that the Envs acquire additional glycans (glycan shielding)[17], and expansion or contraction of variable loop lengths over time[19,20]. These changes coincide with the acquisition of neutralization resistance, primarily in antibody epitopes that are located in the variable regions and occasionally in more conserved epitopes that overlap the receptor-binding site[20,21]. The host antibody repertoire that is directed against the virus evolves too, but at a slower rate than does the virus[22].

Thus, for both an HIV-1 vaccine and a pandemic influenza vaccine, a major challenge is the extreme diversity of the Envs, which are the major neutralizing determinants on the surface of the viruses.

Neutralization of influenza virus and HIV-1

The structural features of the Envs that comprise the functional trimeric spikes and entry processes of HIV-1 and influenza virus are detailed in BOXES 1,2 and FIGS 2,3,4. In brief, the HIV-1 exterior Env gp120 mediates receptor binding, and the transmembrane Env gp41 mediates viral entry. In an analogous manner, the HA1 subunit of the influenza virus binds sialic acid (the viral receptor) and the HA2 transmembrane protein mediates fusion and entry. The structures of trimeric influenza virus HA in complex with receptor analogues in both the cleaved and uncleaved conformations have been solved[23–25]. HA contacts its receptor through a number of highly conserved residues at the membrane-distal domain of HA1, the sialic acid-binding site. Five antigenic sites (A, B, C, D and E), identified by a combination of *in vivo*-selected and laboratory-selected mutations, have been described for HA[26,27] (FIG. 5b). Three of these sites are close to the receptor-binding site, and one is positioned closer to the membrane. A common feature of these sites is that they are contained in protruding loop-like structures[28]. Amino-acid substitutions in these loops are readily tolerated as the overall framework of HA is not affected and there is no observable fitness cost for the virus to acquire mutations in these loops. This mode of immune evasion is analogous to the HIV-1 gp120 surface-exposed variable loops (FIG. 5b), in which selected mutations are tolerated without any alteration of the capacity of the Envs to bind the viral receptors, CD4 and chemokine receptor CCR5, and mediate entry (FIGS 3,4).

In common with the heavily glycosylated HIV-1 gp120, influenza virus HA1 contains multiple sites for *N*-linked glycosylation. The presence of large *N*-linked glycans on viral surface proteins represents an important means by which viruses shield their functional spikes from neutralizing antibodies[29]. The evolution of the 1968 Hong Kong influenza H3 virus is an illustrative example of the role of glycans to evade immune responses: as many as five new glycosylation sites were acquired in the HA1 glycoprotein between 1968 and 2000 (REF. 30). Similarly, analysis of HIV-1 gp120 also reveals considerable variability in *N*-linked glycosylation sites, especially in the V1 and V2 variable loops[20] and in the gp120 outer domain. Interpreted from the perspective of the gp120 crystal structures, the outer domain of gp120 contains a heavily glycosylated and variable surface that is known as the 'silent face', so named because few antibodies are elicited against this region. Decorated with a high density of shifting host-derived *N*-linked glycans, this exposed surface is probably involved in the shielding of other elements of the spike that are directly under selection pressure from neutralizing antibodies[13,17].

Structural analyses of antibody–antigen complexes have provided significant insights into how neutralizing antibodies interact with viral glycoproteins. The precise contact residues between antibodies and antigens can be analysed by examining the three-dimensional structure of antibody complexes with a range of molecules containing the epitope. However, full appreciation of the epitope and of how its location might affect the mechanisms of neutralization is best defined in the context of the functional receptor-binding Env trimer. For influenza virus, atomic-level structures are available of several antibodies in complex with the receptor-binding HA trimer[31–33]. For HIV-1, binding in the context of the functional spike is less certain as, although models exist[34,35], the crystal structure of the HIV-1 Env trimer has not yet been determined.

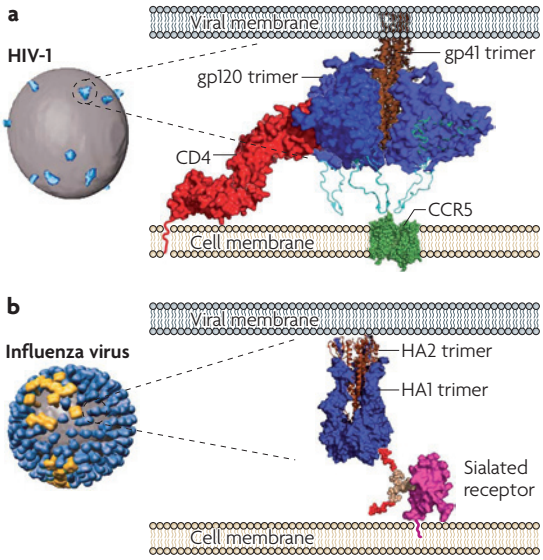

Figure 3 | **Schematics of HIV-1 and influenza A virus and their surface proteins interacting with viral receptors. a** | A surface-rendered cryo-electron microscopy tomographic image of HIV-1 is shown on the left. (Reproduced with permission from REF. 134 © (2006) Macmillan Publishers Ltd.) Viral spikes are blue and the viral surface is grey. On the right is shown a structure-based model of the envelope glycoproteins of HIV-1 and cellular proteins that are involved in binding and entry. Trimeric HIV-1 gp120 proteins (variable loop 3 (V3) loop is shown in cyan) bind to the primary receptor, CD4. (Reproduced with permission from REF. 67 © (2007) Bentham Press.) Following conformational changes, the gp120–CD4 complex binds the chemotaxis receptor CCR5 co-receptor (modelled on bovine rhodopsin), which activates the trimeric gp41 proteins to mediate fusion of the viral membrane to the target cell membrane, leading to viral entry. **b** | A surface-rendered cryo-electron microscopy tomographic image of influenza A virus possesses haemagglutinin (HA) trimers (blue) and neuraminidase (NA) tetramers (yellow). (Reproduced with permission from REF. 135 © (2006) National Academy of Sciences.) On the right is shown a structure-based model of the envelope glycoproteins of influenza A virus and cellular proteins that are involved in binding and entry. Trimeric HA1 proteins bind to sialated glycoproteins or glycolipids. Following receptor interaction and internalization, the trimeric HA2 proteins mediate fusion of the viral membrane and the target cell membrane in low pH endosomal compartments, allowing viral entry. A glycoprotein (magenta), containing terminal galactose and sialic acid (red), that is modelled on the cell surface represents a potential influenza virus receptor.

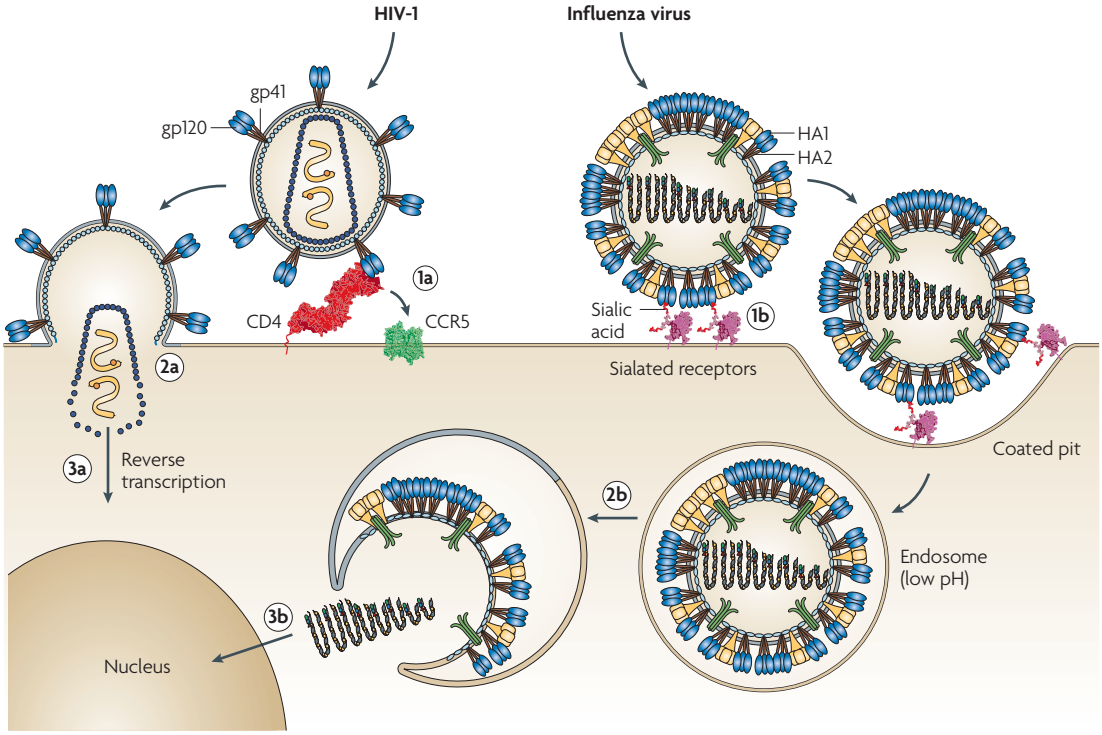

Figure 4 | **Schematic diagrams of HIV-1 and influenza virus fusion and entry processes.** Major conformational changes in the viral envelope glycoproteins occur for fusion of both HIV-1 and influenza virus, but the requirements for fusion differ, as do the viral target cell types (not shown). For HIV-1, gp120 binds sequentially to its primary receptor CD4 and, after an initial conformational change, to co-receptors (chemokine receptor) CCR5 or CXCR4 (not shown) (step **1a**). Co-receptor interaction triggers fusion of the viral and cellular membranes, initiated by the HIV-1 fusion peptide that is located in gp41 (step **2a**). Fusion and entry of the HIV-1 genomic RNA and accompanying viral proteins into the target cell occurs at the cell surface at neutral pH. Following entry, the HIV-1 RNA genomes are transcribed by reverse transcriptase into DNA (step **3a**) and the HIV-1 pre-integration complex is transported to the nucleus for integration into target cell genomic DNA to initiate chronic infection. On different cell types from those affected by HIV-1, influenza virus binds via haemagglutinin 1 (HA1) to terminal sialic acids present on glycoproteins or on glycolipids (step **1b**). The virus is subsequently internalized by receptor-mediated endocytosis into a low pH compartment (endosome), triggering conformational changes that expose the viral fusion peptide that is located in HA2 (step **2b**). Subsequently, the genomic ribonucleoprotein complex is transported to the nucleus to initiate transcription and replication of the viral genome (step **3b**).

There are two major classes of potent neutralizing HIV-1 antibodies that can neutralize at least some circulating primary isolates. The first class of neutralizing antibodies binds to the variable loops of gp120, most notably the variable loop 3 (V3) region[36,37]. Although abundantly elicited during natural infection or vaccination, most isolated anti-V3 antibodies tend to be narrow in their range of neutralization owing to V3 epitope occlusion on the functional spike of most primary isolates[38]. Viral escape by point mutation of the V3 region also occurs to some degree, but the consistent number of residues that comprise HIV-1 V3 probably reflects functional constraints that are related to the role of V3 in co-receptor binding[39]. This is consistent with a report showing that changes in V3-directed neutralization sensitivity are associated with changes in co-receptor usage[40]. Of relevance to this Review, similar alterations in neutralization sensitivity may occur for influenza variants that use alternative sialic acid linkages for entry. The second class of neutralizing HIV-1 antibodies includes rare antibodies that exhibit broader neutralizing activity by recognizing functionally conserved, exposed surfaces in the context of the functional spike on either gp120 or gp41. So far, only four broad HIV-1 neutralizers have been well defined — two against gp120 (b12 and 2G12) and two against gp41 (2F5 and 4E10) (FIG. 5c) — and each of these was elicited by natural infection and will be discussed below. It remains to be seen whether there are other neutralizing specificities to be defined by new and novel monoclonal antibodies.

To understand how influenza virus HA interacts with antibodies in molecular detail, the structures of HA in complex with Fab fragments from three different monoclonal neutralizing antibodies were solved[31–33]. Two of these antibodies, HC63 and HC19, have binding sites that overlap the receptor-binding domain, whereas the third antibody, HC45, binds outside of this region (FIG. 5a). One of the antibodies that binds the receptor site, HC63, also interferes with the ability of HA to undergo the low-pH-induced conformational changes that are required for fusion, an event that occurs after receptor binding. To investigate which activity is

Fab fragment
A fragment of an immunoglobulin that is formed when the molecule is digested with papain. The Fab fragment contains one complete light chain, part of a heavy chain and a single antigen-binding site.

responsible for the neutralizing effect of HC63, the relationship between neutralization of infectivity and inhibition of virus binding to cells was examined[41]. A direct correlation between neutralization and binding was observed, which suggests that HC63, just like HC19, neutralizes infectivity by preventing receptor binding. HC45, which binds at a site that is distant from the receptor-binding site, probably also neutralizes influenza virus by interfering with sialic acid-receptor engagement[41]. One means of viral escape from effective influenza neutralizing responses becomes evident when one observes that most antibody footprints are larger than the sialic acid-binding site. Therefore, escape from neutralizing responses that were elicited in previous years occurs by antigen variation outside of this conserved HA site in the surrounding immunodominant variable loops, altering the overall antibody-binding surface (FIG. 5a, right panel).

Current and future influenza virus vaccines

Seasonal influenza viruses efficiently escape from acquired immunity in the human population through antigenic drift. As a result, a new influenza vaccine must be produced virtually every year to match the predicted predominant circulating strain of the next season as closely as possible. The World Health Organization (WHO) Global Influenza Network is responsible for recommending the antigenic variants that will be included in the coming year's vaccine. The current vaccine is a trivalent vaccine product that consists of two subtypes of influenza A virus (H1N1 and H3N2) and one influenza B virus. The classical annual influenza vaccine is a split-virus vaccine that is produced from detergent-inactivated viruses that are propagated in embryonated hen eggs. These vaccines stimulate high antibody titres in up to 90% of vaccinated individuals and, despite lower responses in frail elderly and immune-compromised individuals, typically protect against morbidity and mortality from the circulating influenza virus strain of a given year[42]. As described above, mutation of a few key amino acids in HA1 variable regions is sufficient to allow viral escape from vaccine-induced antibody responses[28], thereby requiring new virus components to be used in the influenza vaccines in subsequent years.

Recently, a cold-adapted live-attenuated influenza vaccine was licensed for use in the United States of America. This vaccine, which is administered intranasally, is based on virus that is adapted to replicate efficiently at 25°C in the nasal passages, but not at the high temperatures that occur deeper in the respiratory tract[43]. The live-attenuated vaccine induces a higher level of protection than the inactivated seasonal vaccine[44,45], possibly because replicating viruses express antigens for longer periods of time and also stimulate T-cell responses that contribute to protection. Additionally, the nasal route of administration may promote the generation of protective mucosal antibody responses[42]. Although both the inactivated and the live attenuated influenza vaccines are highly protective, they are essentially limited to stimulating homotypic neutralizing antibodies, which do not possess broad neutralizing activity.

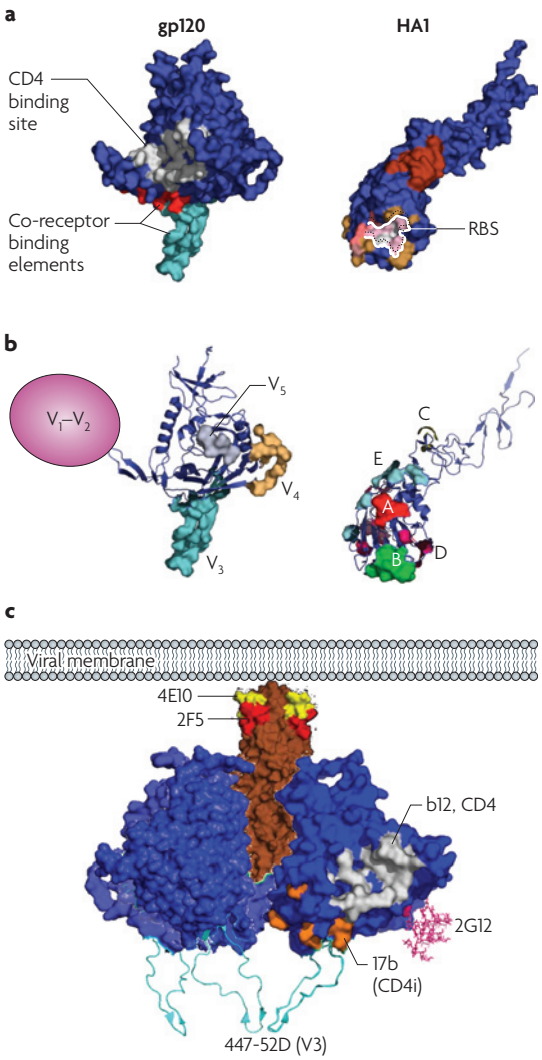

Figure 5 | **The envelope-glycoprotein-receptor-binding domains, variable regions and antigenic surfaces.** **a** | On the left; the monomeric HIV-1 gp120 core surface (blue), possessing the V3 loop (cyan)[39], is shown. The primary receptor CD4-binding site and co-receptor-binding site (which is comprised of elements of V3 and the CD4-induced (CD4i) region (red)) are also shown. On the right, the influenza virus HA1 monomer from strain X31 (A/Hong Kong/1/1968, H3N2) is shown in blue, and the boundaries of the receptor-binding site (RBS; which is comprised of residues 98, 136, 153, 155, 183, 190, 194 and 226) are highlighted. The footprints of selected neutralizing antibodies are shown in pink and white for HC19, brown and white for HC63 and red for HC45. **b** | On the left, the gp120 variable loops V3–V5 are shown, as is the approximate location of variable loops V1–V2. On the right, the proposed antigenic sites on haemagglutinin (HA) are shown as five distinct sites (A–E). **c** | The epitopes for the two broadly neutralizing gp41 antibodies 4E10 and 2F5 that comprise the membrane proximal external region are shown. The two broadly neutralizing gp120 antibodies 2G12, which recognizes a carbohydrate cluster (pink), and b12, which recognizes a surface that overlaps with the CD4-binding site on gp120 (gray) are shown. The epitopes that are recognized by the less potent CD4i-directed antibody 17b and the V3-loop-directed antibody 447-52D are also shown.

Adjuvant
An agent that is mixed with an antigen and increases the immune response to that antigen following immunization.

Although the existing influenza vaccines are effective in inducing systemic antibody responses against the circulating influenza A H1N1 and H3N2 strains as well as influenza B viruses, significant problems are encountered upon vaccination with some of the influenza virus subtypes that are not endemic in humans. This is probably because the vaccine against seasonal influenza primarily only needs to 'boost' pre-existing immune responses, whereas vaccines against novel virus subtypes need to prime immune responses in immunologically naive individuals. Consequently, large amounts of antigen and multiple doses are required to obtain antibody levels that were considered to be protective against infection (reviewed in REFS 4,46). With the continuous threat of a new influenza pandemic arising from avian reservoirs, there is an urgent need to develop technologies to rapidly produce new efficacious influenza vaccines on a global scale[4,46–48]. Ongoing efforts to develop a vaccine against the most prevalent avian subtype H5 are exploring various approaches, including the use of adjuvants to broaden subtype responses to the influenza virus HA across H5 or other viral subtypes[49]. Reverse genetics approaches are also emerging as an alternative strategy to produce virus preparations that contain the HA and neuraminidase (NA) proteins of circulating avian subtypes[50–54]. Other efforts use viral vectors or plasmid DNA to induce immune responses against conserved viral antigens, such as the M2 ion-channel protein or nucleocapsid protein (NP)[55–62] (FIG. 2). The M2 protein has received attention as a broad neutralizing antibody target owing to its conservation. However, its exposure on the surface of influenza viruses may be limited. Alternatively, NA molecules that are shared between human and avian influenza variants may be useful targets to elicit cross-protective responses through DNA vaccination or other means[63]. However, HA remains the most attractive vaccine target, especially if more cross-reactive anti-HA antibodies can be elicited through the use of engineered HA molecules and/or the use of adjuvant.

Variable or conserved neutralizing elements

The annual influenza vaccine, usually consisting of three predominant circulating strains, protects against disease by eliciting homotypic neutralizing antibodies that react with the HA molecules of these strains. Similarly, protection against human papilloma virus is also achieved by including a selected subset of clinically relevant variants in a 'cocktail vaccine'[64]. However, owing to the extreme variability of HIV-1 already present in the human population, a distinctly different vaccine strategy is required to generate a protective vaccine against HIV-1. Strain-restricted protection, or even a feasible collection of such responses, would only account for a small fraction of the complex myriad of HIV-1 variants that circulate simultaneously in the human population. The elicitation of homologous neutralization is sometimes achieved by gp120 vaccination, but the elicitation of neutralization breadth beyond a handful of related isolates is not generally observed.

Logically then, one means to generate an effective neutralizing antibody response against an extremely variable, persistent virus, such as HIV-1, is to target functionally conserved, exposed regions of the Envs. Recent mapping studies of selected broadly neutralizing patient sera determined that neutralization specificity is focused primarily on the conserved CD4-binding site of gp120 (REF. 65). Responses to conserved neutralizing regions are not efficiently elicited following monomeric gp120 vaccination, which may account in part for the failure of monomeric gp120 vaccinations in the first human clinical trials[66]. Owing to the limits of monomeric gp120 as an immunogen, several laboratories have generated soluble mimetics of the trimeric viral spike, but to date these only represent an incremental advance over gp120 monomers (BOX 3).

Fortunately, a handful of sites on the HIV-1 Envs exist that are both conserved and vulnerable to antibody-mediated neutralization. These regions were revealed by their identification as receptor-binding sites, by their identification as the epitopes of rare broadly neutralizing HIV-1 antibodies (FIG. 5c) or by mapping the neutralization specificity in broadly neutralizing patient sera[65]. Other neutralizing determinants may exist, but have not yet been identified. The four rare broadly neutralizing antibodies that have been identified include two with

Box 3 | **Rational HIV-1 envelope glycoprotein immunogen design**

Owing to the failure of gp120 to efficiently elicit broadly neutralizing antibodies, as confirmed by the failure of the first human clinical trial using gp120 as an immunogen[69], much effort has been devoted to engineering soluble versions of the envelope glycoprotein (Env) spike to recapitulate some of the properties of the functional trimer. Soluble versions of the spike, containing full-length gp120 covalently linked to different versions of the gp41 ectodomain, have been produced and are referred to as Env 'gp140 molecules'. Several gp140 molecules have been designed, characterized and tested as immunogens[67], including: C-terminal heterologous trimerization motifs; cysteine-pair linkage of gp120 to gp41; flexible inter gp120–gp41 linkers; variable loop deletion; deletion of the fusion peptide and gp41 immunodominant cysteine loop; solid-phase proteoliposomes and consensus-sequence-derived gp140 soluble spike mimetics. A recent report used a gp140 construct that was derived from the predominant virus of an HIV-1 infected individual, and this construct elicited neutralizing breadth that was greater than has been previously observed[127] and merits further investigation. The spike-mimetic approaches include chemically inactivated virions and virus-like particles[128] and other approaches, such as CD4–gp120 complexes that mimic cryptic transition state Env conformations[129,130]. Although occasional incremental improvements in neutralization breadth and potency have been observed, most of these designs do not generate sufficient neutralization to respond to the diversity of worldwide HIV-1. An important consideration is standardized neutralization assays and HIV-1 neutralization panels, which would facilitate rigorous comparisons of the antibody responses that are elicited in different pre-clinical and clinical vaccine studies[21,131,132].

epitopes that are on gp120 and two with epitopes that are on gp41 (REF. 22). To limit the scope of this Review, we will only describe selected HIV neutralizing ligands and their targets, and describe some attempts to use this information to advance rational, structure-based HIV immunogen design. Potential areas that might be applicable to novel HA subunit design are briefly mentioned when applicable. For more extensive treatments of HIV-1 vaccine design and development, readers are referred to several reviews[9,10,67–72].

Rational HIV-1 immunogen design: gp120

The primary receptor CD4-binding site on gp120 was identified by mutagenesis, as were monoclonal antibodies that bind to this general region[13,73–75]. The crystal structure of gp120 in complex with CD4 directly resolved the atomic level contacts and revealed a conserved, recessed footprint that spans the inner and outer domain and the gp120 bridging sheet[76]. Antibody accessibility to this general region was confirmed by the discovery of the broadly neutralizing CD4-binding site (CD4BS) monoclonal antibody b12 (REF. 73). The fact that b12 binds to the CD4BS was demonstrated by both mutagenesis and cross-competition analysis with soluble CD4. Because b12 has now been crystallized in complex with gp120 (REF. 77), the CD4BS is an attractive surface to target for focused immunogen design. Indeed efforts to elicit antibodies against the CD4-binding region are in progress, but limited success has been reported. The crystal structures of gp120 in complex with both CD4 and b12 provide atomic level design pathways to modify gp120, with the goal of eliciting neutralizing, CD4-binding-site-directed antibodies. These include locking gp120 into the single conformation that is recognized by CD4 (REFS 78,79). The approach depends on the fact that gp120 is an extremely flexible molecule, as determined by thermodynamic analysis and by crystallization in the unliganded state[34,80]. Flexibility may present many gp120 decoy conformations to the humoral immune system. By locking gp120 into the entry-relevant, but occluded, conformation that is recognized by CD4, antibodies may be elicited to this region. The CD4-stabilization approach, although not yet fully implemented or successful, did demonstrate that CD4-state immunogens are an incremental improvement compared with the ability of wild-type gp120 to elicit neutralizing antibodies[78]. By analogy, targeting the conserved sialic acid-binding site on influenza HA might present a pathway to simultaneously generate a broadly effective influenza vaccine against multiple HA subtypes, but the elicitation of such antibodies by natural infection or vaccination has not yet been reported.

Design of b12-binding-site immunogens was performed using information from the original CD4–gp120 structure with the aim of focusing the immune response to the antigenically defined b12-binding site. One strategy is to mutagenize gp120 to create additional motifs for the addition of *N*-linked glycans to the already heavily glycosylated protein. Immunodominant gp120 surfaces — such as the V3 loop, the CD4i region or non-neutralizing surfaces — were 'glycan masked' to preferentially expose the b12 epitope[81]. An initial study demonstrated that glycan masking altered the specificity of elicited antibodies, but neutralization breadth was not substantially increased[82]. It is feasible that some or all of the structure-based approaches described in this section need to be implemented in the context of faithful functional spike mimetics to elicit antibodies that can access the conserved neutralization targets on the viral spike; however ideal mimetics are not yet available (BOX 3).

The other gp120-directed broadly neutralizing monoclonal antibody, 2G12, binds to a cluster of conserved glycans on the outer domain of gp120. The gp120 molecule is heavily glycosylated, with *N*-linked carbohydrate accounting for roughly 50% of its mass[83]. Most glycosylation occurs on the outer domain of gp120, and during natural infection such glycans are host-derived, rendering gp120 immunologically silent[84]. The single exception to the paucity of antibodies that bind to the silent face is the broadly neutralizing, glycan-dependent antibody 2G12 (REF. 85). The crystal structure of the Fab fragment of 2G12 in complex with high-mannose glycan[86] shows that 2G12 adopts a highly unusual domain-exchanged dimeric structure that produces an array of proximal antibody combining sites. The 2G12–glycan structure has been used to design novel oligosaccharide-based immunogens[86,87]; however, the elicitation 2G12-like antibodies has not yet been reported.

Other gp120 regions that have been suggested as potential sites for immune targeting include the immunodominant V3[88] and the conserved region that is implicated in binding to the viral co-receptor CCR5. The V3 loop elicits antibodies that are often strain restricted in their recognition or neutralization[38]. One exception is the V3-directed antibody 447-52D[89], which exhibits greater breadth of neutralization[90]. However, the breadth of 447-52D is more limited than is its binding to V3 sequences that are displayed on diverse gp120 proteins. Limitations of V3-directed neutralization is probably a consequence of selective pressures that are exerted by V3 loop antibodies, which are prevalent in most HIV-infected individuals, as these antibodies select for circulating primary isolates that do not expose their V3 loops on the functional viral spike.

Owing to its conservation, the co-receptor binding site on the gp120 core has initial appeal as a broadly crossreactive immune target. This region and V3 comprise the bipartite co-receptor-binding elements of gp120. The epitopes of many antibodies that recognize the conserved co-receptor patch on gp120, which are better presented upon CD4 binding, are known as the CD4-induced antibodies. CD4-induced antibodies neutralize HIV-1 laboratory-adapted isolates owing to exposure of the epitope on these viruses. However, in circulating primary isolates the co-receptor-binding site is not accessible before the engagement of CD4 (REF. 91). Similar to the V3 region, access to the co-receptor-binding site is probably limited in circulating isolates owing to the abundance of antibodies that select for viruses that do not expose the site on the static spike[92]. Access to the site is probably sterically limited following receptor engagement[91], although one study has suggested that CD4-induced elements on gp120 can elicit broad HIV-1 neutralization[93].

Rational HIV-1 immunogen design: gp41

The gp41-directed broadly neutralizing monoclonal antibodies 2F5 and 4E10 bind to a region close to the viral membrane called the membrane proximal external region (MPER)[94,95], which is hydrophobic and highly conserved across clades[96]. One common feature that was appreciated both before and after the structural definition of the four neutralizing antibodies is that they all possess long complementarity determining region (CDR) 3 hypervariable loops, a property that may contribute to their neutralization potency. A cluster of hydrophobic tryptophan residues in the MPER was shown to be important for viral entry, emphasizing the theme of functional conservation[97]. Many attempts to re-elicit 2F5-like or, to a lesser extent, 4E10-like antibodies, using various different contexts have met with limited to no success[98]. The haemagglutinin gp41 equivalent, HA2, does not appear to be a target for neutralizing antibodies, perhaps owing to limitations of exposure of this protein on the viral surface (FIG. 2). Nevertheless, HA2 may represent an interesting target for immunogen design for influenza virus if its potentially poor immunogenicity can be overcome with better adjuvants.

The structures of both 2F5 and 4E10 in complex with their cognate, full-length gp41-derived peptide epitopes are now solved at the atomic level of resolution[98,99]. The 2F5 antibody recognizes an extended loop structure, whereas 4E10 recognizes a helical epitope conformation. Further analysis of the structures revealed hydrophobic residues at the tip of the CDRs of both these antibodies, which suggests that hydrophobicity might be a requirement for efficient recognition of their membrane-proximal epitopes. Binding analyses of gp160-containing liposomes either possessing or lacking a reconstituted lipid bilayer confirmed that the presence of lipid increases 2F5 and 4E10 affinity[98]. Novel approaches are underway to translate the MPER structural information into rational immunogen design by locking the peptide epitopes into distinct conformations (which were revealed in the crystal structures) to potentially elicit MPER-directed antibodies[67,98,100,101].

The role of adjuvants in vaccines

To efficiently stimulate vaccine-induced antibody responses in naive individuals, antigens can be administered with an adjuvant. This may be especially advantageous for antigens such as HIV-1 Env and influenza virus HA that are shielded with *N*-linked glycans and so are poor immunogens. An effective adjuvant may provide dose-sparing effects, an important consideration for any vaccine that needs to be produced in quantities that are sufficient for global distribution. So far, only a few adjuvants have been approved for human use, but this is a an active area of investigation, and pre-clinical studies demonstrate that a diverse set of compounds exhibit adjuvant activity and enhance the quantity or quality of immune responses in immunized animals[102,103].

The most commonly used adjuvant in humans so far is selected aluminium salt particles (Alum) onto which antigen can be adsorbed[103]. Alum is considered safe and effective in terms of enhancing antibody responses; however it elicits a T helper 2 (T_H2)-biased immune response that produces predominantly humoral, but not cellular, immunity. MF59, an oil-in-water squalene emulsion adjuvant, (REF. 104) and AS04, an Alum formulation adjuvant that contains the Toll-like receptor 4 (TLR4) agonist monophosphoryl lipid A (REF. 105), stimulate a more balanced immune response and are also approved for human use. The approval of a vaccine adjuvant that contains a TLR agonist has paved the way for the use of other TLR ligands as novel components of vaccine adjuvants, such as synthetic oligonucleotides that contain CpG motifs (which signal through TLR9) or the small molecule imidazoquinolines (which signal through TLR7 and TLR8). Each of these ligands activate specific subsets of antigen-presenting cells and have been shown to provide adjuvant effects in pre-clinical vaccine studies[106–109]. TLR7/8 and TLR9 agonists stimulate T_H1-biased immune responses and they may therefore be useful to induce both humoral and cellular immunity.

New generation vaccine adjuvants, such as those based on oil-in-water emulsions or liposomes formulated with monophosphoryl lipid A and the saponin fraction QS21, have been developed and are under evaluation in clinical trials[110,111]. Saponins have also been exploited in the generation of immune-stimulating complexes (ISCOMs), which consist of fractions of the *Quillaja* saponin that are formulated with cholesterol and phospholipids to form a matrix into which antigen can be incorporated[112]. ISCOM-based influenza vaccines, which have been licensed for veterinary use, protect mice from lethal infection[50]. Human vaccination trials using this technology are underway[113].

How might adjuvants help stimulate enhanced neutralizing antibody responses against influenza virus or HIV-1? Besides the economic benefit of providing dose-sparing effects, optimal activation of antigen-presenting cells by adjuvants could allow expansion of T cells or generation of B cells with sub-dominant antigen specificities, which otherwise would remain below the threshold for activation or below detection in polyclonal sera. For example, C179 is a murine monoclonal antibody that recognizes a conserved but probably poorly immunogenic epitope in the middle of the HA stem[114]. C179 can neutralize diverse influenza viruses, including subtypes from H1, H2, H5 and H6, but it is currently unknown whether antibodies with this or similar specificity can be re-elicited through vaccination. If the right immunogen–adjuvant combination could be identified, this conserved region is an interesting target for the elicitation of antibodies that can neutralize multiple influenza subtypes. Similarly, carefully selected vaccine regimens, using antigens that drive antibody responses against known (or as-yet undiscovered) crossreactive determinants, combined with an optimal vaccine adjuvant might be beneficial against HIV-1.

Recent studies on an H5N1 influenza vaccine candidate indicated that novel adjuvants had a dose-sparing effect and resulted in antibody responses that were

T_H2-biased immune response
The response that occurs when the cellular immune response is mainly composed of T_H2 cells. There are two effector subsets of $CD4^+$ T cells, T_H1 and T_H2 cells. These are characterized by distinct cytokine profiles and by functional activity. T_H2 cells produce interleukin-4 (IL-4), IL-5, IL-9, IL-10, and IL-13, leading to activation of humoral immune responses. By contrast, T_H1 cells produce interferon-γ, IL-2 and lymphotoxin, which support cell-mediated immunity. Appropriate differentiation of T cells into these subsets is important for mounting immune responses to pathogens, whereas an imbalance between these subsets is associated with diseases.

sufficiently broad to be efficacious against multiple H5N1 variants[49]. Obviously, successful elicitation of more crossreactive antibody responses against avian influenza subtypes would be desirable to potentially control the next influenza virus pandemic. If the mechanism to increase neutralization breadth can be defined and reduced to subunit vaccine design, then perhaps similar approaches could be applied to elicit more crossreactive HIV-1 neutralizing responses as well. A continued discussion between the HIV-1 and influenza fields is therefore encouraged.

Concluding remarks

The most effective viral vaccines are those that target invariant virus-surface antigens or viruses that display limited antigenic variability. Because influenza virus circulates globally as a single predominant variant each year, vaccination against this virus is also effective, despite the relatively restricted neutralizing responses that are generated by 'predict and produce' approaches. By contrast, the extreme variability of HIV-1 presents a challenge of a magnitude not previously encountered in the vaccine field. Vaccine development against pandemic influenza might represent a similar challenge because of the antigenic variability of HA in avian reservoirs[16,115]. By monitoring the viruses that circulate in domestic fowl, rapid reporting of avian influenza virus infections and surveillance to determine if human-to-human infections have occurred, an influenza pandemic might be averted. Alternatively, rapid development of a matched avian influenza virus vaccine, if human-to-human transmission is documented, could be a feasible approach to reduce viral spread in selected populations. However, the development of a more crossprotective 'universal' influenza vaccine is still a desirable goal and would facilitate global readiness for a potential pandemic.

For HIV-1, we have described selected novel immunogen-design approaches (afforded by the availability of Env crystal structures), which have been initiated owing to the failure of classical vaccine approaches to elicit protective antibodies. These efforts represent nascent attempts to elevate vaccine research from an empirical exercise to a scientific discipline — guided by structural information of relevant targets, knowledge of virologic principles and an understanding of basic immunological mechanisms. Such a process might also affect influenza vaccine development, as discussed above. Likewise, the testing of potent adjuvants and other developments that are ongoing in the influenza vaccine field could provide important information relevant to the HIV-1 vaccine field. For both pandemic influenza and HIV-1, structure-based vaccine design is not exclusive of other approaches that are being explored in parallel.

Perhaps a realistic first goal for both a pandemic influenza vaccine and an HIV-1 vaccine is to stimulate sufficient antibody responses that, together with cellular responses, protect the host from disease rather than prevent acquisition of viral infection. Hopefully, overcoming some of the similar obstacles that impede HIV-1 vaccines and pandemic influenza vaccines will establish a new paradigm that will be applicable to other equally challenging vaccine development efforts in the future.

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DATABASES
Entrez Genome: http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome
HIV-1
Entrez Protein: http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein&cmd=&term=&go=Go
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