Broadly Neutralizing Antibodies to Highly Antigenically Variable Viruses as Templates for Vaccine Design

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Abstract Development of vaccines to highly variable viruses such as Human Immunodeficiency Virus and influenza A viruses faces multiple challenges. In this article, these challenges are described and reverse vaccinology approaches to generate universal vaccines against both pathogens are laid out and compared.

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Current Topics in Microbiology and Immunology (2020) 428: 31–87
https://doi.org/10.1007/82_2020_221
© Springer Nature Switzerland AG 2020
Published Online: 10 July 2020

1 Introduction: Broadly Neutralizing Antibodies—Challenges and Solutions

1.1 Requirements for Successful Vaccines to Viral Pathogens

Successful vaccines to viral pathogens largely rely on the induction of functional antibody responses to effectively protect the host from infection (Plotkin 2010, 2013). While antibodies can aid host protection through a multitude of Fc-mediated functions, e.g., via antibody-dependent cellular cytotoxicity (ADCC), the most important correlate of antibody-mediated protection from viral pathogens is the ability to inhibit productive infection of host cells, or direct viral neutralization. Neutralization is typically measured by incubating antibodies or polyclonal sera of interest with live or pseudo-typed virus before transfer onto an appropriate reporter cell line to indicate inhibition of infection (Li et al. 2005). In case of influenza, although neutralization assays have become more widely used, hemagglutination inhibition assays (HAI) are still quite common to determine antibody-mediated protection in humans; HAI titers of or above 1:40 are considered as protective (Coudeville et al. 2010). However, HAI assays only detect inhibition of receptor binding and will not quantify neutralizing antibodies that prevent host cell infection by means other than blocking receptor binding (Bachmann et al. 1999; Wyrzucki et al. 2014; Maurer et al. 2018). Direct neutralization is considered a consequence of high-affinity binding of neutralizing antibodies (nAbs) to an exposed epitope on the infectious virus particle. While the exact mechanism(s) of neutralization are frequently unknown for a given nAb, the most common and best understood mechanisms of direct neutralization are (i) steric obstruction of viral entry (attachment or fusion) into the host cell and (ii) allosteric mechanisms, e.g., by hindering conformational changes in the viral receptor protein and thus preventing the formation of the viral fusion spike (Julien et al. 2013b). Passive transfer studies of HIV nAbs with intact or impaired Fc-effector domains in vivo further solidified that direct neutralization is sufficient for host protection, but can be enhanced by Fc-mediated immune responses to both free virus or infected cells (e.g., ADCC) (Hessell et al. 2007).

To achieve sterilizing immunity from infection, vaccines need to be able to generate memory responses of potent nAbs upon immunization, comprised of both circulating levels of nAbs, which are continuously produced by long-lived plasma cells in the bone marrow and corresponding memory B cells, which can rapidly morph into plasmablasts and secrete nAbs into the bloodstream upon antigen contact. It is important to note that different vaccines may rely on different memory components for success—e.g., to prevent HIV infection, readily available, circulating nAbs are likely required as infection of a single, or small number of cells is deemed sufficient to establish latent infection. Although antibodies are of importance, e.g., by blunting viremia, clearance of acute viral infections is often the result of the cytotoxic T lymphocytes (CTLs) (Borrow et al. 1994). However, preexisting

antibodies are the only means to prevent (re-)infection and can provide immunity by inactivating pathogens before they are able to infect target cells, also referred to as sterilizing immunity. Moreover, maternal antibodies can extend maternal immunity to the offspring.

Traditional vaccination approaches with live-attenuated or inactivated whole virus particles, as well as multimerized protein subunits, so-called virus like particles (VLPs), are in fact very effective at inducing long-lasting, protective immune responses. Advances in vaccine research enabled the global eradication of smallpox (Fenner 1993), the near-complete eradication of polio (Greene et al. 2019), as well as sharp reductions in the incidence of many common viral diseases, such as measles, mumps, rubella and hepatitis A and B in most countries with vaccination rates high enough to sustain herd immunity.

1.2 Highly Antigenically Variable Viruses

However, a subset of pathogens defy traditional vaccination approaches due to their extraordinarily high rates of genetic variability paired with a variety of immune-evasive measures. These pathogens include bacteria (e.g., Streptococcus pneumoniae), protozoa like Plasmodium falciparum and Trypanosoma brucei, the causative agents of malaria and sleeping sickness, respectively, and most notably RNA viruses such as HIV, influenza virus and hepatitis C virus (HCV) (Burton et al. 2012).

The driving force behind the enormous genetic variability of RNA viruses is the low fidelity of viral RNA-dependent polymerases. Reported mutation rates for riboviruses (and some single-stranded DNA viruses) are in the range of 10^{-4}–10^{-6} per nucleotide site per infection cycle, which is strongly elevated compared to DNA viruses that range from 10^{-6} to 10^{-8} errors per nucleotide per infection cycle (Sanjuán et al. 2010). Retroviruses have comparable mutation rates to RNA viruses, averaging $\sim 2.0 \times 10^{-5}$ errors per nucleotide per infection cycle. By comparison, the human genome was estimated to replicate with only $\sim 1.1 \times 10^{-8}$ errors per nucleotide per haploid genome (Roach et al. 2010). Given the vast proliferative capacity of RNA viruses in natural infection paired with their small genome size (typically 5–15 kbp), the high error rate could theoretically generate every possible point mutation and many double mutations during each round of replication in a given host (Lauring et al. 2013). However, it is important to note that high genetic variability also causes a substantial amount of fitness-reducing and lethal mutations. For Vesicular Stomatitis Virus (VSV), for example, 70% of random mutations were found to be either deleterious or lethal, while only 5–10% showed fitness enhancement, the remainder being neutral (Sanjuán et al. 2004). Purifying selection thus reduces the antigenic diversity presented to the host immune system, which is typically derived from viral quasi-species with highly competitive replicative fitness (Wood et al. 2009). In the case of HIV, high selection pressures associated with sexual transmission further reduce viral diversity immediately following infection,

often to a single viral isolate, so-called transmitted founder viruses (Keele et al. 2008; Parrish et al. 2015).

Because of the relatively long response time of approximately 7 days that the humoral immune system requires to mount an antibody response to a novel target, the rapid mutation rates and large intra-host population sizes of RNA viruses pose a tremendous threat. Strain-specific antibodies targeting variable epitopes on surface-exposed viral proteins do apply selection pressure (Laver et al. 1979a, b; Both et al. 1983), at best selecting for escape mutants with reduced replicative fitness, but inevitably becoming obsolete upon viral escape. However, by necessity, all viruses also comprise epitopes that are essential to their survival and therefore generally cannot be altered without some fitness penalty. These essential, conserved epitopes commonly include receptor-binding sites, required for host cell tethering and binding, as well as fusion proteins, enabling membrane fusion with the host cell. Thus, high antigenic variability is not necessarily problematic, as long as conserved epitopes exist that can be readily targeted by the immune system. A great example for this is measles virus, which is highly genetically diversified into eight clades and numerous subtypes and has a mutation rate of 4.4×10^{-5} errors per nucleotide per replication cycle, which is an order of magnitude higher than influenza A virus (Sanjuán et al. 2010), and comparable to the average mutation rate per replication cycle measured for HIV ($\sim 3.6 \times 10^{-5}$) (Rawson et al. 2016). However, unlike HIV and influenza virus, measles virus has a single serotype and is reliably neutralized by vaccine-induced nAbs targeting epitopes surrounding its conserved receptor-binding site on measles hemagglutinin (Tahara et al. 2012).

Consequently, a productive way to think about the level of difficulty associated with vaccine development for a given viral pathogen is to ask how immunodominant its conserved, life-cycle-dependent epitopes are. As discussed in-depth in Chap. 4, immunodominance can be defined as the likelihood of a successful immune response against a given epitope. This in turn is a measure of the affinity of the epitope to the closest germline B cell receptor (BCR), the abundance of appropriate precursor B cells and the relative frequency of the target epitope on the viral surface, which affects binding avidity and as a consequence BCR cross-linking and activation. It follows that prominent viral evasion strategies aim at lowering the immunodominance of essential epitopes. A common strategy deployed by both influenza virus and HIV is the camouflaging of conserved epitopes with N-linked glycans (Sok et al. 2016b; Wu and Wilson 2017). N-linked glycans are sterically obstructive, typically associated with immunologic self (Scanlan et al. 2007) and are easily added and removed by mutations of the N×T/N×S glycosylation signals, where x represents any amino acid other than proline.

In this context, two classes of viruses can be discerned, based on their requirement to persist in the presence of immune countermeasures: evasion strong and evasion lite viruses (Hangartner et al. 2006; Burton 2017). While some evasion lite viruses display high antigenic diversity, their life cycle allows them to move on to a new host quickly, thus reducing the fitness advantage of sophisticated and

burdening measures of immune evasion (e.g., measles and polio). On the other hand, viruses that need to thrive in antibody-rich environments during their infection cycle, e.g., HIV, influenza virus and HCV, have developed refined strategies of immune evasion, including camouflaging and obstructing essential epitopes, which poses a substantial problem for vaccine design. As traditional vaccination approaches consistently failed to create universal vaccines for these pathogens, entirely new and more focused strategies are needed to tackle evasion strong viruses, most prominently HIV and influenza virus, on which we will focus for the remainder of this review.

1.3 Broadly Neutralizing Antibodies and Reverse Vaccinology

In the case of HIV, a small subset of 10–30% of long-term infected individuals manage to circumvent viral evasion measures and develop some level of nAbs that mostly derive their binding energy from contacts with relatively conserved surface residues (McCoy and McKnight 2017). Some of these antibodies very potently neutralize a wide range of HIV isolates (Sok et al. 2014b), as high as 98% of global isolates (Huang et al. 2016), or, in the case of influenza A, potently neutralize all hemagglutinin (HA) subtypes across both serologic groups (Corti et al. 2011; Dreyfus et al. 2012; Wyrzucki et al. 2015). These antibodies have been dubbed broadly neutralizing antibodies (bnAbs), due to their extraordinary breadth in neutralization, as compared to strain-specific nAbs that target variable, non-essential epitopes.

To overcome the structural restrains that HIV and influenza virus have evolved over time, bnAbs against these pathogens show a variety of unusual genetic features that have made their re-elicitation through vaccination challenging, although tremendous progress has been made in recent years (Burton and Hangartner 2016; Sautto et al. 2018). The discovery and distinct features of bnAbs will be discussed in the following section, followed by a brief review of correlates of protection from HIV infection that have been identified in experimental HIV infection models and clinical vaccine trials.

There are several vaccination strategies that seek to induce bnAbs, which will be briefly discussed at the end of this chapter. The basic concept underlying all of these strategies is a detailed molecular understanding of the broadly neutralizing epitopes and their corresponding bnAbs, which is than leveraged using protein design to develop rationally designed immunogens. This concept (Fig. 1) is referred to as ‘reverse vaccinology 2.0’ as it starts with the neutralizing antibody and aims to reverse engineer a vaccine candidate that is complementary in shape, in a reverse of the traditional flow of vaccine design (Burton 2002, 2017). The following sections

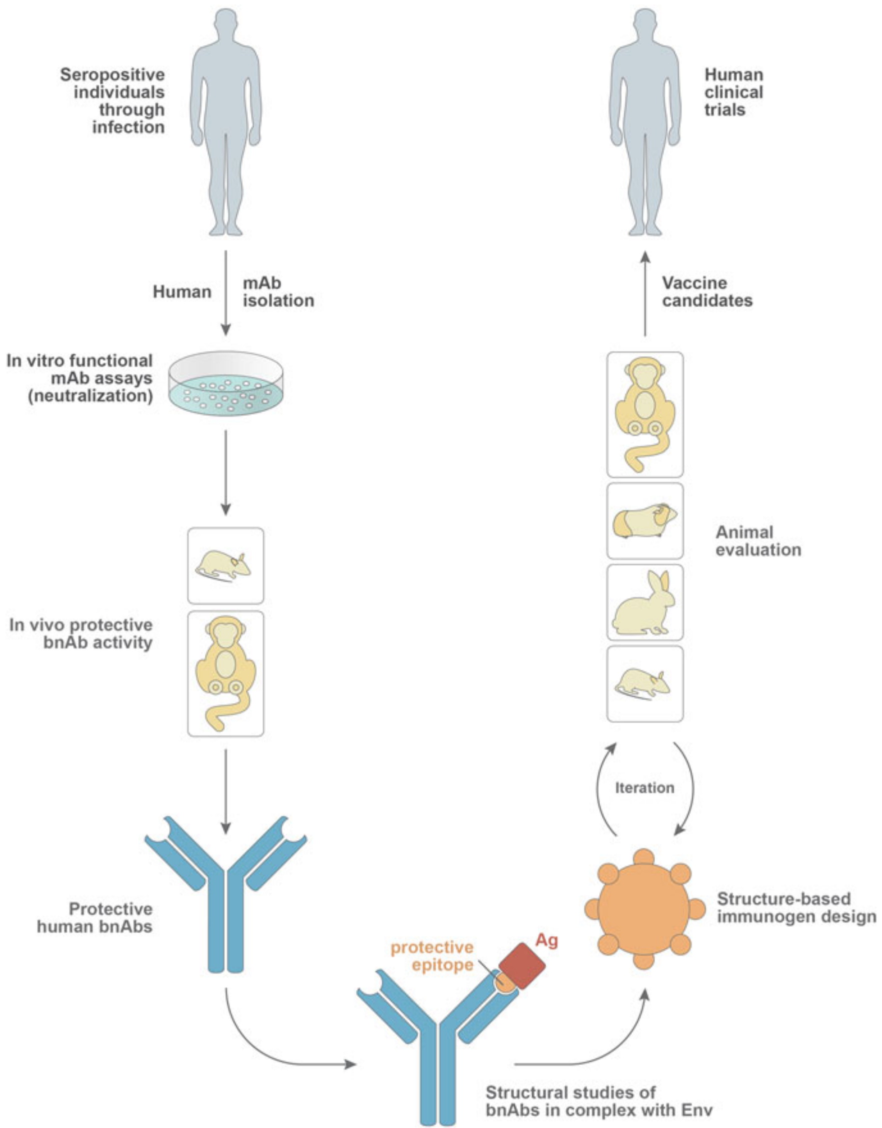


Fig. 1 The reverse vaccinology 2.0 workflow from mAb isolation to iterative vaccine design and clinical trials

will summarize the known details of bnAbs targeting HIV and influenza virus, respectively, followed by a short discussion of how these findings are currently being utilized to inform vaccine design.

2 Broadly Neutralizing Antibodies to HIV

2.1 The Envelope Trimer, the Sole Target of bnAbs to HIV

Over the last two decades, the isolation of broadly HIV neutralizing antibodies has grown exponentially, which has paralleled a gradual shift in the HIV vaccine field from a strong focus on $CD8^+$ T cell induction to strategies aimed at the elicitation of bnAbs (Burton and Hangartner 2016). The sole target of neutralizing antibodies to HIV is the exposed envelope (Env) protein, which forms the so-called envelope spike above the viral membrane. Envelope's function is to bind CD4 as well as CCR5 or CXCR4 as co-receptors on the surface of immune cells, which then initiates membrane fusion and viral entry (Dalgleish et al. 1984). Membrane-coated HIV virions are approximately 110 nm in diameter, but display an average of only 8–14 Env spikes on the virion surface (Zhu et al. 2006; Zanetti et al. 2006), which represents a rather low spike density compared to other enveloped RNA viruses, such as influenza virus (Fig. 2). Interestingly, the surface spike density is regulated by the intracellular C-terminal (Ct) domain of Env, deletion of which increases the spike density by almost an order of magnitude to 73–88 per virion (Zanetti et al. 2006). A recent study suggests that only 2–3 spikes per virion need to be engaged

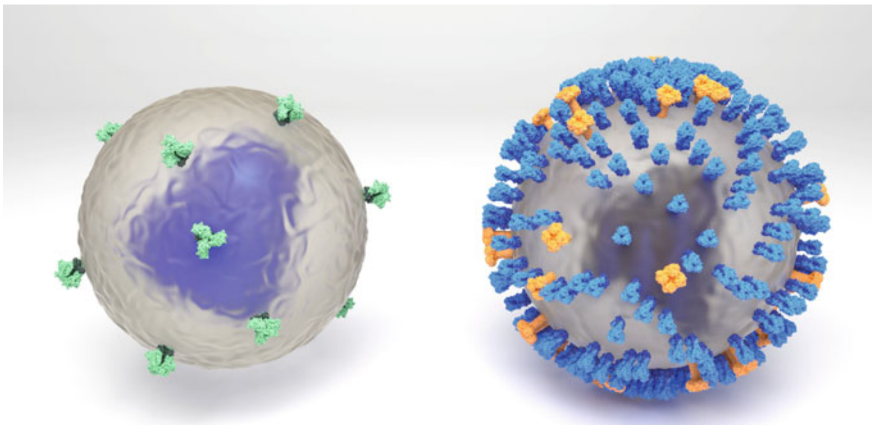


Fig. 2 Comparison of the virion structures of HIV-1 (left) and influenza A (right). The virions of HIV and influenza A virus differ considerably in the number and density of viral spikes. While only around 14 HIV envelope spikes can be found on HIV (Zhu et al. 2006), there are an estimated 290–350 HA, and 35–50 NA spikes present on influenza A virions (Harris et al. 2006). These different spike densities are likely to influenza epitope accessibility: While HIV trimers are fully accessible from all sides, access to lateral and basal epitopes on influenza A virions may be restricted due to the dense packing. Virion models were constructed to scale in Blender 2.78 using the surface meshes for HIV Env (PDB 2HTY), and H1 HA trimers (PDB 1RD89). The NA tetramer was modeled using the structure for the globular N1 head (PDB 2HTY) and stalk modeled from known dimensions

to facilitate viral entry; the exact number was found to be strain dependent and varied from 1 to 7 infectious spikes per virion (Brandenberg et al. 2015).

Envelope is translated as a single heavily glycosylated protein, named gp160. As gp160 is translated in the ER and shuttled through the Golgi apparatus on its way to the plasma membrane, two important post-translational modifications take place that are important for the overall structure and immunogen design considerations. Firstly, gp160 is cleaved into its major building blocks gp120, which is comprised of five variable loops (V1–V5) flanked by constant regions (C1–C5) and contains the receptor-binding site, as well as gp41, which contains the fusion machinery. The cleavage is mediated by the host protease furin, which recognizes a conserved sequence of 4–6 lysines in the gp160 peptide sequence (Decroly et al. 1994). On the cell surface, gp120 and gp41 then form what is commonly described as a trimer of hetero-dimers, with three subunits of membrane-anchored gp41 forming the trimeric pedestal upon which a second, non-covalently attached trimer of gp120 subunits rests. Secondly, the $\sim$25 potential N-linked glycosylation sites (PNGS) per gp120 and 4 PNGS sites per gp41 subunit (Korber et al. 2001) become glycosylated during their interactions with glycosyltransferases and glycosidases in the ER and Golgi apparatus (Doores et al. 2010). Recent studies using novel mass spectrometry approaches (Cao et al. 2018a) have shown that PNGS on recombinant gp120 (Struwe et al. 2017) as well as stabilized gp140 trimers (Cao et al. 2017), are in fact almost fully occupied. While similar data sets for virion-derived gp120 existed (Panico et al. 2016; Pritchard et al. 2015a), glycan occupancy analysis was lacking. However, recent data now shows that PNGS on live- and pseudo-virus-derived Env are occupied to a similar or higher degree than recombinant gp140 trimers (Cao et al. 2018b). This makes HIV Env the most densely glycosylated protein described to date.

2.2 Immune-Evasive Features of the Native Env Trimer

There are three main features of the HIV Env spike that deter the immune system from efficiently mounting neutralizing Ab responses to conserved epitopes cluster on Env.

Firstly, the low density of native Env spikes on the virion surface (Zhu et al. 2006) is a major immune-evasive mechanism of envelope, as the scarce spacing of spikes disfavors B cell receptor cross-linking and reduces binding avidity and consequently B cell activation (Dintzis et al. 1976).

Secondly, many conserved, broadly neutralizing epitopes are conformational and only displayed correctly in the context of the native Env trimer. The non-covalent attachment of gp120 promoters to the gp41 base, however, renders the Env spike inherently labile or meta-stable. Consequently, non-native Env trimers with missing or splayed-open gp120 crowns, as well as shed gp120, are presented to the immune system alongside native trimers, thus presenting a multitude of non-native, non-neutralizing epitopes that dilute the frequency of desirable (broadly)

neutralizing epitopes (Poignard et al. 2003; Moore et al. 2006; Crooks et al. 2011). Another immune-evasive aspect of the trimeric spike is the steric occlusion of essential epitopes, e.g., the CD4 binding site, in poorly accessible locations between promoters, proximal to the C3 symmetry axis of the trimer (Julien et al. 2013a; Lyumkis et al. 2013; Pancera et al. 2014).

The third, and likely most significant immune counter-measure that HIV commands, is the dense layer of oligomannose, hybrid- and complex-type glycans covering the protein surface, which is commonly referred to as the glycan shield (Wei et al. 2003). Reasons for the shielding property include: (i) due to the hydrophilic nature of glycans, protein–glycan-binding interactions yield lower free-binding energy per buried interface area than protein–protein interactions (Liang et al. 2007). This energy difference means that antibodies targeting glycan or proteoglycan epitopes must bury large amounts of surface area, i.e., increasing their binding footprint, to accumulate enough free-binding energy needed for high-affinity interactions. A larger binding footprint, in turn, renders nAbs more susceptible to viral escape, especially when conserved epitopes are flanked by highly variable areas. Most glycan-binding bnAbs to HIV also make additional contacts with the underlying peptide surface, which often requires unusual genetic features like very long HCDR3s to reach through the dense glycan layer. The requirement of these features decreases the frequency of such bnAbs naturally occurring, as they typically require extensive somatic hypermutation (SHM) during affinity maturation in germinal center (GC) reactions to evolve. (ii) The viral genome has evolved to facilitate shifts or complete loss and addition of PNGS with extraordinary ease (Coss et al. 2016). In many cases, single point mutations suffice to alter the glycan shield composition, thus enabling rapid viral escape from nAbs, either by shifting or deleting bnAb-bound glycans (MacLeod et al. 2016) (Moore et al. 2012) or by obscuring bnAb epitopes via glycan addition (Sok et al. 2016b). Interestingly, glycan-site deletion in response to nAb-pressure sometimes increases the accessibility of otherwise concealed essential epitopes, like the CD4 binding site or the V3-glycan area, paving the way for subsequent bnAb development (Moore et al. 2012; Gao et al. 2014). (iii) Finally, N-linked glycosylation is a common post-translational modification of mammalian proteins and as such associated with immunologic self. This, at least theoretically, reduces the pool of suitable germline BCRs that are not counter-selected during B cell maturation. However, the frequency and density of N- and O-linked glycosylation sites in the human proteome is very small compared to viral receptor proteins, which has important implications for glycan processing. Of over 20,500 structurally characterized proteins in the PDB, less than 10% are predicted to have N-linked glycosylation sites, and of those proteins bearing PNGS sites, more than 80% only comprise a single PNGS site (Li et al. 2016). Mass spectrometry analysis of the human sperm proteome similarly found only 10% of glycosylated proteins to bear more than three glycan sites (Wang et al. 2013). Low glycan density and comparably slow physiological protein production rates increase glycan accessibility and interaction with the ER and Golgi glycosylation machinery and thus allow for complete processing from oligomannose-type toward complex-type glycans on most human proteins. In the

case of Env, however, rapid parallel expression of gp160, extraordinary density of glycosylation sites and structural constraints arising from the trimer structure diminish access and exposure to the glycosylation machinery, thus strongly elevating high-mannose-type glycan frequencies (Doores et al. 2010; Bonomelli et al. 2011; Pritchard et al. 2015a, b). High-mannose-type glycans especially cluster in a relatively conserved area of gp120 dubbed the high-mannose patch, surrounding the glycan at asparagine 332 (N332), as well as the structurally inaccessible areas that result from trimerization of gp120 and gp41 (Doores et al. 2010; Pritchard et al. 2015b). It follows that production cell line, Env transfection ratios, efficient gp160 cleavage and especially monomeric as compared to trimeric protein designs have a notable impact on the glycoforms composition of recombinantly generated immunogens and viruses (Bonomelli et al. 2011; Cao et al. 2017, 2018b).

In summary, although dense glycosylation of Env is a strong deterrent to effective immune responses to HIV, the unusually high density of oligomannose-type glycans on Env also provides the immunological basis for bnAb recognition of glycan-dependent epitopes, largely in the absence of autoreactivity to N-glycosylated human proteins.

2.3 Discovery and Isolation of bnAbs to HIV

The discovery of novel HIV bnAbs happened in two distinct waves, which reflected the technical possibilities at the time, as well as access to well characterized and large cohorts of long-term infected individuals. The first bnAbs to HIV, b12 and 2F5 were isolated in the early 1990s in the laboratories of Dennis Burton and Hermann Katinger. b12 was isolated using phage display, which allowed for the screening of large libraries but did not maintain the original heavy and light chain pairing (Burton et al. 1991, 1994; Barbas et al. 1992). 2F5 was isolated by immortalizing B cells through hybridoma fusion, which was fairly inefficient and limited in throughput (Buchacher et al. 1994; Conley et al. 1994). In the coming years, two more important early bnAbs were described, 2G12 and 4E10 (Buchacher et al. 1994; Zwick et al. 2001; Stiegler et al. 2001). These antibodies showed neutralization breadth of 32–98% on global isolates, respectively, albeit with low median IC_{50}s of above 1 μg/ml (Binley et al. 2004; Walker et al. 2009). However, these antibodies were shown to protect in passive transfer studies in nonhuman primates (NHPs), which generated new hope for antibody-based HIV vaccines (Parren et al. 2001; Hessell et al. 2009, 2010).

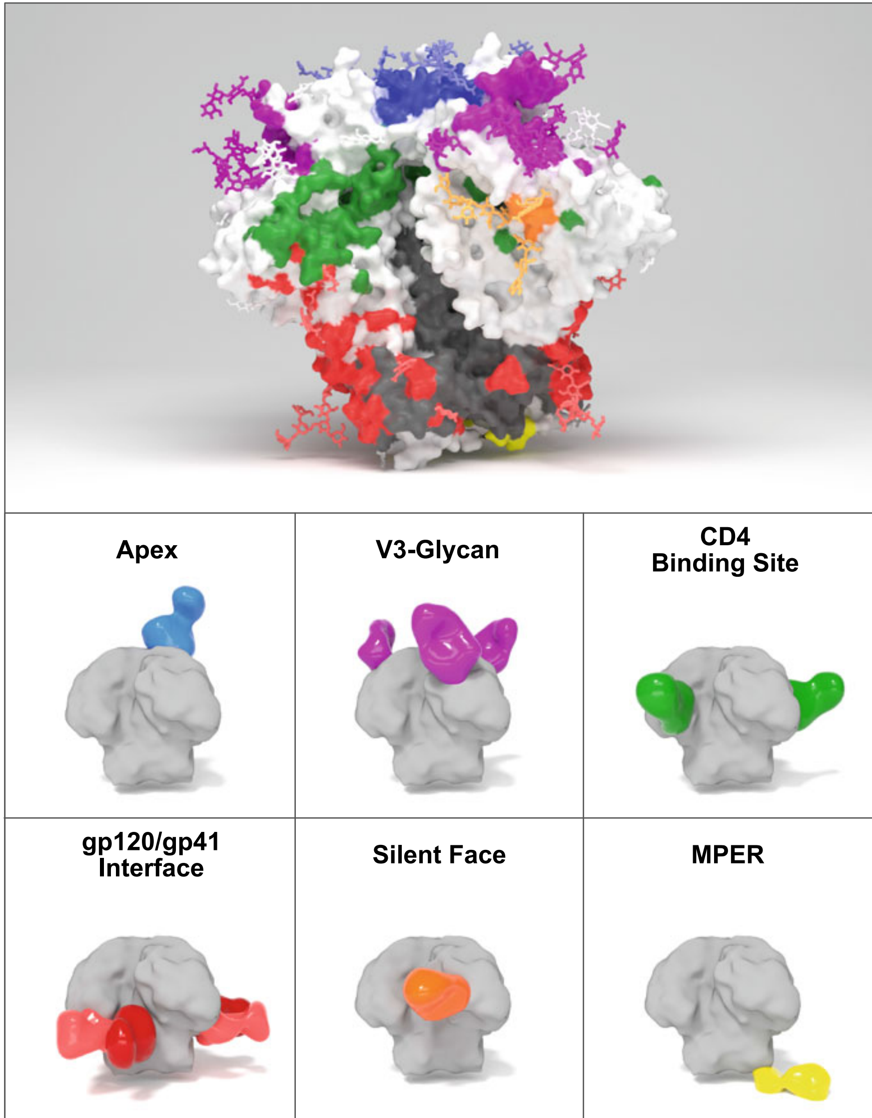
The advent of the second generation of HIV bnAbs began with the isolation of PG9 and PG16 by direct neutralization screening of the culture supernatants of 30,000 B cells, derived from a long-term infected donor of the IAVI Protocol G cohort (Walker et al. 2009). What enabled the isolation of these bnAbs was both a rigorous screening and ranking of 1800 Protocol G samples on a cross-clade 6-virus panel (Simek et al. 2009), paired with direct B cell stimulation and culture as a new high-throughput screening technology. B cell cultures supernatants were both tested

for binding to HIV Env subunits gp120 and gp41 and for neutralization against isolates SF162 and JR-CSF. Remarkably, PG9 and PG16 were later shown to target a quaternary apex epitope of the trimeric HIV Env spike (Walker et al. 2009; Julien et al. 2013c) with weak or no binding to monomeric gp120 subunits from most HIV strains. Given the absence of soluble native-like trimer proteins at the time, their isolation was only possible by including neutralization of intact HIV pseudo-virions as readout. PG9 and PG16 showed good neutralization breadth of ~75% on a 162-virus panel, but did so with previously unseen potency of ~0.2 μg/ml, which was an order of magnitude higher than the first-generation bnAbs (Walker et al. 2009). In the following years, this approach was expanded and directly led to the isolation of the PGT121, PGT128, PGT135, PGT145 and PGT151 bNAb families from multiple Protocol G donors (Walker et al. 2011; Falkowska et al. 2014). PGT121 and PGT128 again pushed the limit of neutralization potency by an order of magnitude to ~0.02 μg/ml, while maintaining neutralization breadth of at least 70% on a panel of 162 global isolates (Walker et al. 2011). Based on the low serum concentrations of PGT121 that were sufficient to protect 3/5 rhesus monkeys in a passive transfer study (mean titer 1:285 or 0.18 μg/ml PGT121) (Moldt et al. 2012), this class of bnAbs reinvigorated the hope for globally effective bNAb-based HIV vaccines (Burton et al. 2012).

In parallel, the notion that only few Ab lineages mediate broad serum reactivity (Walker et al. 2010), together with advances in single-cell sorting and especially single-cell PCR amplification of antibody heavy and light chain genes, enabled a new approach for nAb-isolation based on binding of memory B cells to recombinant Env protein probes (Scheid et al. 2009). The first bnAb isolated using this methodology was VRC01. To isolate HIV-reactive memory B cell from a donor whose serology indicated reactivity against the CD4 binding site, a fluorescently labeled gp120 core protein named RSC3 was developed at the Vaccine Research Center (VRC) and used to sort epitope-specific memory B cells from that donor (Wu et al. 2010). The development of recombinant, soluble HIV trimers, so-called SOSIP trimers named after their two key stabilizing mutations, a disulfide-bond between gp120 and gp41 (SOS) (Binley et al. 2000) and an I595P (IP) mutation (Sanders et al. 2002), enabled the isolation of quaternary bnAbs by single-cell sorting. Using SOSIP trimers of the BG505 isolate that displayed authentic antigenicity resembling the native Env spike (Sanders et al. 2013; Julien et al. 2013a; Lyumkis et al. 2013), Env trimers were used to isolate the trimer-specific PGDM1400 Ab family, which again raised the bar for potency of bnAbs, displaying a median IC_{50} of 0.003 μg/ml on large virus panels (Sok et al. 2014b). Both antigen-specific B cell sorting as well as single-cell culture and stimulation approaches have since greatly enriched the repertoire of HIV bNAb families (McCoy and Burton 2017), which, counting clonally related antibodies, encompasses several hundred bnAbs today.

2.4 *Major Broadly Neutralizing Epitopes on HIV Env*

Although a great variety of bnAbs to HIV has been described in recent years, the vast majority can be classified into five major epitope clusters, which are by definition relatively conserved among global isolates (Fig. 3). From 'top to bottom,' these sites of vulnerability are the trimer apex (V2-apex), the glycan-V3 region, the



◂**Fig. 3** Recognition of HIV envelope trimers by broadly neutralizing antibodies. Top panel: Surface regions and glycans recognized by broadly neutralizing antibodies to the apex (blue), the high-mannose patch (or V3-glycan; purple), the CD4 binding site (green), the silent face (orange), the gp120/gp41 interface (red) and the MPER region (yellow). gp120 is depicted in white, gp41 in gray and N-linked glycans by sticks. The Env trimer coordinates from PDB 4TVP were used. Lower panels: Stoichiometry and angles of approach for representative bnAb densities from all major classes. Most antibody classes bind with a stoichiometry of 3 Abs per trimer, with the exception of apex-specific antibodies that mostly bind with a single, and occasionally two Fabs per trimer, and interface-specific antibodies where a maximal occupancy of 2 has been observed for PGT151. The density maps of PG9 (apex), PGT121 (V3–glycan), VRC01 (CD4 binding site), 8ANC195 (interface, bright red), PGT151 (fusion peptide specific, interface, dark red) are displayed in the respective panels. The density map for VRC-PG05 (orange, silent face) has been generated by aligning the gp120/VRC-PG05 co-crystal structure (PDB 6BF4) onto the BG505 Env trimer coordinates (PDB 5I8H), followed by generation of a density map by a computational low pass filtering of the complex coordinates using the EMAN 1.9 software package. The densities for 10E8 (yellow, MPER) are partially buried since it was shown that binding of this antibody requires Env to be lifted out of the membrane (Lee et al. 2016). Unless indicated differently, the EM 3D class average density maps were kindly provided by Gabriel Ozorowski and fitted into the EMDB 5782 EM Env density map (gray)

CD4 binding site, the gp120-gp41 interface and the membrane-proximal external region (MPER) (Burton and Hangartner 2016). To these five legacy epitope clusters, recently a sixth one has been added, which is comprised of the densely glycosylated ‘silent face’ proximal to the CD4 binding site; it is called such due to the historical absence of neutralizing antibodies targeting this region.

The reasons for conservation for these areas are now largely clear and typically linked to vital functions of the viral life cycle. The CD4 binding site, for instance, is crucially important for target cell tethering and entry (Klatzmann et al. 1984). The MPER region is involved in the membrane fusion process, in which a three-helix bundle within gp41 is formed and inserted into the host cell membrane (Salzwedel et al. 1999; Muñoz-Barroso et al. 1999). Likewise, the gp120/gp41 interface harbors parts of the fusion machinery (Kong et al. 2016b) and additionally needs to be conserved to ensure proper cleavage of pre-fusion Env (Blattner et al. 2014; Falkowska et al. 2014). The V3-glycan region was shown to incorporate parts of the CCR5 co-receptor-binding site at the base of the gp120 V3 loop, which, given the high variability of adjacent residues and glycans, explains the strong conservation among CCR5-tropic isolates (Sok et al. 2016b). Reasons for the conservation of the V2-apex site likely include both trimer-stabilizing interactions of the V2 loops as well as dense packing of basic amino acids to slightly destabilize the apex. While this may seem paradoxical, the resulting delicate meta-stability likely enables structural rearrangements preceding co-receptor-binding and spike opening upon CD4 engagement. In the following sections, we will discuss in detail the corresponding bnAbs to all mentioned epitope clusters.

2.4.1 CD4 Binding Site Epitope Cluster

Antibodies to the CD4 binding site (CD4bs; Fig. 3 green) are commonly induced during natural infection; however, the majority of these antibodies are non-neutralizing or strain-specific (Scheid et al. 2009). Binding-based mapping assays with core gp120 or RSC3 (Wu et al. 2010) detect CD4 binding site reactivity in over 80% of donor sera, but only small fraction of these sera contain nAbs that compete with RSC3 in neutralization assays (Landais et al. 2016; Lynch et al. 2012). This is likely because many Abs evolving against this epitope cluster recognize the CD4 binding site as displayed on g120 or dysfunctional forms of the Env spike. In the context of Env trimer, the CD4bs is much more occluded because of its proximity to the adjacent protomer, which requires an angled binding approach (Lyumkis et al. 2013; Zhou et al. 2015). Additionally, variable loops 2 and 5 as well as glycans surrounding the CD4 binding loop, most notably N197, N276, N362 and N461, were shown to hinder antibody recognition of this epitope cluster (Lyumkis et al. 2013; Crooks et al. 2015; Zhou et al. 2015). This underlines the importance of using native-like trimers or rationally designed immunogens when attempting to elicit bnAbs of this class.

There are two major subgroups of bnAbs targeting the CD4 binding site, the HCDR3 loop dominated binders, with bnAb b12 as its prototype, and VH-gene restricted binders, of which VRC01 is the iconic class member.

HCDR3 loop binders, as the name suggests, make most of their binding contacts to side chain residues of the CD4bs with their CDR loops, with special emphasis on their HCDR3 loop. The HCDR3 loop is elongated to $\sim$20aa for many antibodies of this class, including b12, HJ16, VRC13 and VRC16, with CH103 being the exception with a short 13aa HCDR3 loop (Burton et al. 1994; Liao et al. 2013; Zhou et al. 2015). With the exception of VRC13, HCDR3 loop binders show VH-gene mutation levels of $\sim$15% (nt), which is only half of the very high mutation rates observed for some VH1-2-gene restricted bnAbs like VRC01. Loop binders further do not induce the CD4 bound state (CD4i) on trimeric Env, which exposes a set of epitopes involving the gp120 bridging sheet that is recognized by strain-specific and non-neutralizing antibodies (nnAbs) such as 17b. On the contrary, b12, for example, binds to the relaxed trimer conformation and prevents further exposure of CD4i epitopes (Scheid et al. 2011; Falkowska et al. 2012). VRC13 is currently the most broad and potent member of this class (Zhou et al. 2015).

VH-gene-restricted CD4bs bnAbs, on the other hand, have shorter HCDR3s and engage with the CD4 binding site in a sCD4-like fashion, largely using residues of the HCDR2 loop and HC framework regions to form hydrogen bonds with main-chain atoms of the CD4 binding site (Zhou et al. 2010, 2013, 2015). VH-gene-restricted bnAbs can be sub-divided into VH1-2-derived antibodies, featuring VRC01, 12A12, NIH-45-46, PGV04, 3BNC117 and the more recently isolated N6 (Wu et al. 2010; Scheid et al. 2011; Falkowska et al. 2012; Huang et al. 2016) and bnAbs utilizing the related VH1-46 gene, including 8ANC131, 1B2530, as well as the CH235 lineage (Scheid et al. 2011).

Aside from very high heavy chain SHM levels needed to enable CD4-like binding, VH-gene-restricted bnAbs require modifications to their CDRL1 binding loops to prevent clashes with the conserved N276 glycan, which is part of the 'glycan fence' encircling the CD4 binding site (Zhou et al. 2013). Both VH1-2 and VH1-46 class bnAbs solve this problem by either deleting 3-6 CDRL1 residues, or by mutating 1-3 glycine amino acids into their CDRL1 loops, thus increasing flexibility (Zhou et al. 2013). The only notable exception is CH235.12, the most potent member of the CH235 lineage (Bonsignori et al. 2016), which has a naturally short 6aa CDRL1 derived from the VK3-15 gene. The binding angle of VH1-2-restricted bnAbs further requires unnaturally short CDRL3 loops that are 5aa in length to accommodate and bind loop D residues (Zhou et al. 2013). VH1-46-restricted bnAbs, on the other hand, approach the CD4 binding site slightly rotated compared to VH1-2 group antibodies, thus avoiding CDRL3 length constraints (Zhou et al. 2015). The more recently isolated IOMA bnAb presents an interesting hybrid between both classes; although it is VH1-2 derived, a unique interaction between residue D93 in its 8aa CDRL3 and gp120 N280 (loop D) allows IOMA to wedge its long CDRL3 between the gp120 loop D and V5 loop, mildly displacing the V5 loop in the process (Gristick et al. 2016).

To develop some of the mentioned rare genetic features through serendipitous germline gene re-arrangement and somatic hypermutation (SHM) may take a long time during natural infection (Kong et al. 2016a), which is mirrored by the elevated share of CD4 binding site targeting broadly neutralizing donors in cohorts with longer median infection periods (Simek et al. 2009; Walker et al. 2010). However, VH-restricted CD4bs bnAbs can reach up to 98% breadth on global isolates, with potencies that are on average an order of magnitude higher than HCDR3 loop binders (with the exception of the highly mutated VRC13, which neutralizes 82% of global isolates at 0.09 µg/ml median IC_{50}) (Zhou et al. 2015). Likewise, VH1-2-derived bnAbs are typically broader and significantly more potent than VH1-46-derived bnAbs, the notable exception here being CH256.12, which neutralizes 90% of global isolates at 0.6 µg/ml median IC_{50} (Zhou et al. 2015). Nevertheless, the undisputed new paragon of CD4bs bnAbs, succeeding VRC01, is the VH1-2-derived N6 antibody, which neutralizes 98% of global isolates at the very high potency of 0.04 µg/ml median IC_{50} (Huang et al. 2016). The high neutralization breadth and decent potency of VH1-2-restricted CD4bs bnAbs makes this class of antibodies an attractive target for reverse vaccine design strategies; however, their rare genetic features require rational design approaches to accelerate maturation, which will be discussed in Sect. 4.

2.4.2 Membrane-Proximal External Region (MPER) Epitope Cluster

MPER-directed bnAbs are observed rather infrequently in natural infection (Walker et al. 2010; Landais et al. 2016), although this epitope was found to be targeted in 27–40% of sera by strain-specific nAbs (Huang et al. 2012a; Landais et al. 2016). In a recent analysis of the Protocol C cohort, up to 40% of donors were able to

neutralize HIV-2 chimeric viruses harboring the HIV-1 MPER epitope cluster; however, only 10% of MPER-targeting sera, or 2.5% cohort-wide, developed neutralization breadth (Landais et al. 2016), which is in agreement with previous reports (Binley et al. 2008; Walker et al. 2010; Gray et al. 2011). Reasons for the frequent development of strain-specific nAbs to this epitope cluster in natural infection may relate to the overall high frequency with which the adaptive immune response targets gp41 early in infection and following vaccination (Tomaras et al. 2008). It was proposed that preexisting microbiota-targeting B cell clones facilitate fast development of typically non-neutralizing antibody responses to gp41 in humans and rhesus macaques (Williams et al. 2015; Han et al. 2017), while in the process, however, outcompeting rarer, potentially protective B cell clones.

All MPER bnAbs target overlapping areas on an amphiphatic α-helical peptide sequence at the base of gp41, adjacent to the plasma membrane. This region is involved in regulating the assembly of the fusion protein and thus highly conserved, which explains the extraordinary neutralization breadth of up 98–99% that MPER bnAbs 4E10, 10E8 and DH511.2 display (Huang et al. 2012a). However, the first generation of MPER bnAbs, 4E10, 2F5 and Z13e1 (Buchacher et al. 1994; Zwick et al. 2001), showed fairly limited potency, which raised the question whether high enough serum titers to achieve sterilizing protection could be induced by vaccination. Notably, both 2F5 and 4E10 protected from infection in passive transfer studies against the neutralization sensitive SHIV Ba-L (Hessell et al. 2010). The isolation of the over fivefold more potent (0.35 μg/ml median IC_{50}) 10E8 in 2012, reinvigorated efforts to design MPER-based vaccines (Huang et al. 2012a). The recently isolated DH511.2 shows intermediate potency of 1.0 μg/ml median IC_{50}, thus falling between 10E8 and 4E10 (Williams et al. 2017).

In order to efficiently bind to their epitope, MPER bnAbs use elongated HCDR3s (20–24aa) that are rich in tryptophan and other hydrophobic residues, which allows for hydrophobic packing with both the peptide and the plasma membrane itself. MPER bnAbs were in fact long thought to be able to bind to membrane phospholipids (Alam et al. 2009) in addition to the MPER peptide, which was shown for both 4E10 and 10e8 by x-ray crystallography (Irimia et al. 2016, 2017).

The long hydrophobic HCDR3 loops that enable lipid reactivity, however, also facilitate binding to ubiquitous phospholipids, which renders this class of bnAbs prone to develop poly- or autoreactivity. Indeed, antibodies 4e10 and many members of the DH511 lineage were shown to be polyreactive (Haynes et al. 2005; Mouquet et al. 2010; Liu et al. 2014), and mouse knock-in models of bnAbs 4E10 and 2F5 showed strong depletion of mature B cells, due to negative selection of autoreactive B cell clones in the bone marrow (Doyle-Cooper et al. 2013) (Verkoczy et al. 2010, 2011; Doyle-Cooper et al. 2013). Importantly, bnAbs 10e8 and DH511.2 show no signs of poly- or autoreactivity (Huang et al. 2012a; Liu et al. 2014), which taken together with the increased potency of second-generation MPER bnAbs has made this bnAb class a target for both rational immunogen design and passive immunotherapy (Irimia et al. 2017).

A challenge for the induction of MPER antibodies is that the faithful reproduction of the epitope might require the presence of proximal lipids on the immunogen (Irimia et al. 2016), which may require liposomal delivery or related strategies liked lipid disks. Lastly, MPER bnAbs frequently show incomplete neutralization of HIV isolates (Kim et al. 2014; McCoy et al. 2015), which is likely due to glycan heterogeneity of the four large complex-type glycans present on gp41 (Cao et al. 2017, 2018b) that MPER bnAbs have to accommodate. The importance of incomplete neutralization for in vivo protection is an area of active investigation, but a recent study with 3BNC117 suggests that strong neutralization plateaus of ~70% maximum neutralization percent reached against the challenge virus (using the TZM-bl assay), lessen the protective capacity of passively transferred bnAbs (Julg et al. 2017).

2.4.3 gp120-Gp41 Interface/Fusion Peptide Epitope Cluster

Over the past 4 years, an entirely new family of bnAbs has been described that targets the interface between the gp120 and gp41 subunits (Fig. 3, red). In the Protocol C cohort, ~12–20% of broadly neutralizing sera was shown to target interface or unidentified quaternary epitopes (Landais et al. 2016). Interface targeting bnAbs are unique in that they bind glycans and peptide residues that are spread between gp120 and gp41 and in some instances also between adjacent protomers. Unlike most bnAbs to the CD4 binding site and the trimer apex, bnAbs of this class do not necessarily compete with each other, which is a function of the wide surface area that this site of vulnerability encompasses on the trimer (Burton and Hangartner 2016).

One of the first described members of this class of bnAbs was PGT151, which binds to a complex quaternary epitope comprised of the N611 and N637 glycans of two adjacent gp41 protomers, as well as residues 499-503 on gp120 adjacent to the furin cleavage site (Blattner et al. 2014; Falkowska et al. 2014). PGT151 is thus able to discern between cleaved and uncleaved forms of Env, a unique feature that has since been harnessed to assess cleavage efficiency for recombinant Env trimers. Upon binding of fully cleaved Env, PGT151 stabilizes the trimer in its native, closed conformation, which was recently leveraged to extract and structurally characterize native, full-length Env trimers, only mildly truncated in their C-terminal domain, without further stabilization (Lee et al. 2016). Both PGT151 and another gp41-gp-41 inter-protomer binding bnAb, 3BC315, induce structural rearrangements upon binding that reduced the maximal occupancy to two Fabs bound per trimer (Blattner et al. 2014; Lee et al. 2015). All other described bnAbs of this class can engage the trimer in a 3:1 stoichiometry (Huang et al. 2014; Scharf et al. 2015; Kong et al. 2016b; Wibmer et al. 2017).

Similarly, bNAb 35O22 also stabilizes the trimer upon binding, although its epitope is more central and membrane-proximal compared to PGT151. It binds the gp120 glycans N88, N230 and N241 as well as glycan N625 on gp41, but, unlike PGT151, is not cleavage sensitive (Huang et al. 2014). Both PGT151 and 35O22

show exceptional potency of 0.008 and 0.03 μg/ml median IC_{50} across large virus panels, with decent breadth of ~65%.

A third member of this class, 8ANC195, originally isolated using a gp120 probe (Scheid et al. 2011), was shown to bind to glycans N234, N276 on gp120 and also glycan N637 on gp41 (Scharf et al. 2014), although the latter is not strictly required. It has the ability to bind both open, CD4 bound and closed states of pre-fusion Env, but induces a more closed conformation upon binding, even in the presence of CD4, which likely prevents full opening and fusion of the trimer (Scharf et al. 2015).

Another set of previously isolated bnAbs, 3BNC315 and 3BNC176 (Klein et al. 2012), was shown to target an epitope partially overlapping that of 35O22; however, they were shifted even further toward the base of the trimer (Lee et al. 2015). In contrast to PGT151/35O22, these antibodies destabilize the trimer and accelerate decay as a mechanism of neutralization, which is unique among bnAbs targeting this epitope cluster. They are also unique in that they are the only bnAbs of this class that do not strictly require glycans to bind (Lee et al. 2015).

The latest additions to this evolving family of bnAbs are ACS202 and VRC34, two bnAbs that directly bind the fusion peptide at the N-terminal end of gp41 (aa512–525) as well glycan N88 on gp120 (Kong et al. 2016b). Early comparisons indicate that these bnAbs target remarkably similar epitopes, which are located in between the PGT151 and 35O22/3BNC315 binding sites. Likewise, their neutralization breadth and potency is almost identical at ~0.2 μg/ml and ~45% breadth, which is more potent than 8ANC195 0.81 μg/ml (Kong et al. 2016b), but less potent than PGT151 and 35O22. For VRC34.01, major escape mutations are located in the fusion peptide. Both ACS202 and VRC34, similar to other bnAbs targeting the N-terminal end of gp41, are cleavage-sensitive but, unlike PGT151 and 3BC115, can bind the trimer in a 3:1 stoichiometry (Kong et al. 2016b).

All glycan-binding bnAbs of this class, including the fusion peptide binders, show high incidence of incomplete neutralization on diverse global isolates (McCoy et al. 2015; Wibmer et al. 2017), which is, as for MPER bnAbs, linked to the complex and sometimes incomplete glycosylation of gp41 (Cao et al. 2017, 2018b). PGT151, for example, was shown to bind tri- and tetra-antennary glycans at position N611/N637 (Falkowska et al. 2014) and had the highest proportion of viruses neutralized with less than 80% maximal neutralization among a diverse panel of bnAbs (McCoy et al. 2015). The worst case of a broadly but severely incompletely neutralizing antibody is the recently isolated CAP248-2B antibody, which often times fails to reach 50% maximal neutralization on large virus panels (Wibmer et al. 2017). Its expansive epitope stretches all the way from the plasma membrane/MPER region to the gp120-gp41 interface and is thus prone to be affected by changes in N-glycan glycoforms and variable residues included in its binding footprint.

2.4.4 V3-Glycan Epitope Cluster

The V3-glycan epitope cluster is centered around the N-linked glycan at position N332, which is positioned at the base of the gp120 V3-loop and surrounded by multiple oligomannose-type glycans that collectively form the high-mannose patch (Doores et al. 2010; Pritchard et al. 2015a, b). The core epitope of V3-glycan bnAbs (Fig. 3, purple) is comprised of both glycans, most notably N332 itself, as well as conserved protein residues at the base of the V3-loop which comprises parts of the CCR5 co-receptor-binding site, thereby explaining both the dense glycan shielding and strong conservation among global isolates (Sok et al. 2016b). N332 glycan-dependent broadly neutralizing serum specificities are the most frequently observed serum specificity throughout several cohorts, ranging from 25 to 40% of donors (Walker et al. 2010; Gray et al. 2011; Landais et al. 2016).

The first glycan-dependent bnAb isolated was 2G12 (Trkola et al. 1996), which was shown by mutagenesis to bind strongly to the N295, N332, N339 and N392 glycans (Scanlan et al. 2002). 2G12 is unique in that it is the only known bnAb that exclusively binds to high-mannose glycans (Wang et al. 2008) without making protein contacts. As discussed in the introduction this is no small feat, as low amounts of binding energy derived from protein–glycan interactions generally dictates that glycan-dependent bnAbs must bury large amounts of surface area to gain high affinity, even when additional protein contacts are made (Kong et al. 2013). 2G12, by virtue of a rare genetic set of mutations, shows a domain exchange between its Fab arms, thus allowing 2G12 to gain affinity from binding one large continuous glycan epitope, using both its Fab arms juxtaposed in a parallel orientation (Calarese et al. 2003; Murin et al. 2014). Nevertheless, 2G12 shows fairly weak neutralization potency and breadth (2.4 µg/ml and 32%, respectively) (Walker et al. 2011) as a consequence of its glycan-only binding mode, comprising four core and two alternate glycans, the latter of which are bound in a strain-specific manner (N386 and N448) (Walker et al. 2011; Murin et al. 2014).

When the most prominent bnAb family of the V3-glycan class, PGT121, alongside the PGT128 and PGT135 families, was isolated in 2011, the extraordinary neutralization potency of 0.02 µg/ml and high breadth of 70% of global isolates generated great interest in this epitope cluster for vaccine design (Walker et al. 2011). BnAbs to this epitope cluster typically bind to a core glycan, either N332 or N301, multiple alternate glycans, as well as the 324-GDIR-327 peptide stretch at the V3-loop base (Pejchal et al. 2011; Julien et al. 2013a; Sok et al. 2014a). Alternate glycan sites bound by V3-glycan bnAbs include N295 at the base of the V3-loop, N137 and N156 in the V1/V2-loop, as well as the N386 and N392 glycans in the V4-loop. PGT128, for example, binds the base of the N301 glycan as its core glycan and can utilize glycans N301 and N332 as alternate glycans on most HIV isolates. While the majority of glycans in this epitope cluster are of the high-mannose type, N137 and N301 are at least partially hybrid- or complex-type glycans on virions (Cao et al. 2018b). Some V3-glycan bnAbs can tolerate the removal of an alternate glycan site without loss of neutralization potency, but the removal of their respective core glycan, or the removal of multiple alternate glycans

sites simultaneously, typically causes a decrease or loss of neutralization potency. This plasticity in glycan recognition has been referred to as promiscuous glycan binding (Sok et al. 2014a). Some of the most potent V3-glycan bnAbs, i.e., PGT121 and PGT128, can even tolerate loss of or shift (e.g., N332 to N334) of their core glycan, and thus also neutralize some isolates naturally lacking respective core glycans in a strain-specific manner (Sok et al. 2014a). Medium potency V3-directed bnAbs, like the recently isolated PGDM11 and PGDM21 families or extremely N332-dependent antibodies like 10-1074 and PGT124, both clonal relatives of PGT121, cannot tolerate shifts or loss of core glycans (Mouquet et al. 2012; Shingai et al. 2013; Sok et al. 2014a; Garces et al. 2014). Similarly, there are qualitative differences in the recognition of the 324-GDIR-327 peptide sequence between high- and low-potency V3-glycan bnAbs. For instance, PGT121 makes multiple side-chain contacts with residues D325 and R327 and is not negatively impacted by loss of either single site, while PGDM21 and 11 make contacts with either D325 or R327, respectively, and are thus more prone to viral escape by single amino acid substitutions (Sok et al. 2016b). In summary, V3-glycan bnAbs with higher neutralization potency and breadth have more redundancy in epitope recognition, which is the molecular-level explanation for their ability to broadly neutralize a somewhat variable epitope cluster. Both allosteric inhibition of CD4 binding and accelerated decay of infectious viral particles has been discussed as the mechanism of neutralization for this class of bnAbs (Pejchal et al. 2011; Julien et al. 2013b).

Unlike CD4 binding site directed bnAbs, V3-glycans bnAbs are generated from a heterogeneous set of VH genes, which is mirrored by diverse angles of approach that have been described for this bnAb class (Julien et al. 2013a). The malleability in epitope recognition of the high-mannose patch is likely related to the high incidence of N332-dependent donor sera in long-term HIV infected cohorts and thus attractive to vaccine design (Walker et al. 2010; Landais et al. 2016). Nevertheless, many V3-glycan bnAbs often bear insertions and deletions (indels) which occasionally arise during affinity maturation in germinal centers (Sok et al. 2013; Kepler et al. 2014). Like V2-apex targeting bnAbs, V3-glycan bnAbs utilize long HCDR3 loops, varying from 20 to 26aa in length, to penetrate the glycan shield and reach the underlying peptide surface (Burton and Hangartner 2016).

Two recently isolated V3-glycan bnAbs, BG18 and DH270.6, both show strong median neutralization potencies (0.03 μg/ml and 0.08 μg/ml, respectively) and breadth (64% and 55%, respectively) on large virus panels, that is on par with PGT121 and PGT128 (Freund et al. 2017; Bonsignori et al. 2017a). Unlike the PGT families, however, neither BG18 nor DH270.6 bear indels and both utilize shorter HCDR3 loops that are 21 and 22 aa in length, respectively. DH270.6 displays comparatively low levels of somatic hypermutation in its VH gene ($\sim$12% of nucleotides), reaffirming the suitability of the high-mannose patch epitope cluster for vaccine design. Two studies further detailed the development of V3-glycan bnAbs over multiple years of infection, thus enabling lineage-based immunization approaches to this site of vulnerability on Env (MacLeod et al. 2016; Bonsignori et al. 2017a).

2.4.5 V2-Apex Glycan Epitope Cluster

The trimer apex comprises another quaternary epitope cluster that is conserved among global isolates. However, unlike the gp120/gp41 interface, the core epitope is well defined and comprises glycans of the gp120 V2 loop as well as a stretch of basic amino acids on gp120 strand C (McLellan et al. 2011; Andrabi et al. 2015) at the apex of the trimer. Thus, bnAbs of this class are collectively referred to as V2-apex targeting (Fig. 3, blue). V2-apex bnAbs have been reported in 10–20% of long-term HIV-infected donors throughout multiple cohorts (Walker et al. 2010; Landais et al. 2016).

V2-apex bnAbs strongly overlap in their binding footprint, as they typically bind the trimer close to or along the C3-symetry axis in a 1:1 stoichiometry, but important differences in their exact binding approach can be discerned (McLellan et al. 2011; Julien et al. 2013c; Pancera et al. 2013; Andrabi et al. 2015; Gorman et al. 2016). bnAbs of this class target a relatively conserved epitope that is comprised of the N156 and N160 glycans on the gp120 V2 loop, and a stretch of basic, lysine-rich amino acids, 168-KKQK-171, on gp120 strand C (McLellan et al. 2011; Andrabi et al. 2015; Gorman et al. 2016). The bnAbs bind to this epitope by inserting their HCDR3 loop in between two N160s of adjacent protomers and/or nearby N156/173 glycans of adjacent protomers to form beta-sheet interactions with strand C residues, typically forming numerous hydrogen bonds. In addition, anionic amino acids in the HCDR3 loop, and in some cases 1–2 sulfated tyrosines, form salt bridges with the underlying cationic strand C lysines generating a large amount of binding energy. To penetrate the glycan shield and contact the underlying strand C residues, V2/apex bnAbs rely on the longest HCDR3 loops among all bnAb classes, ranging from 30 to 39aa in length. As is expected, most V2/apex bnAbs are highly selective for trimeric Env, although some bnAbs such as PG9 and VRC38 can also bind to gp120 of select isolates (McLellan et al. 2011; Cale et al. 2017).

The first members of this class, PG9 and PG16, were shown to asymmetrically interact with apex, having a slightly bend angle of approach from the trimer axes (Walker et al. 2009; Julien et al. 2013c). As more antibodies of this class were discovered, most notably the PGT145, CH01 and CAP256-VRC26 families, various angles of approach have been described, with some bnAbs binding straight along the trimer axes, e.g., PGT145 (Walker et al. 2011; Doria-Rose et al. 2014). All members of this class, except CH01, show very high potency ranging from $\sim$0.01 to $\sim$0.06 μg/ml median IC_{50}'s. Two more recently isolated bnAbs from the PGT145 and CAP256 donors, PGDM1400 and CAP256-VRC26.25, respectively, have developed even higher potencies of 0.005 μg/ml (83% breadth) and 0.001 μg/ml (69% breadth) median IC_{50} values, thus representing the most potent bnAbs isolated from human donors (Sok et al. 2014b; Doria-Rose et al. 2015).

Interestingly, the more recently isolated VRC38 and BG1 bnAbs (Cale et al. 2017) both developed novel binding approaches to the V2-apex epitope cluster that defy many of the above ‘rules of engagement.’ Both VRC38 and BG1 bind slightly off-center beside the trimer axis with little inter-protomer engagement (Cale et al. 2017). This allows both bnAbs to bind the trimer with a 2:1 stoichiometry, which

was observed by electron microscopy (EM); however, unlike BG1, VRC38 shows an up 170-fold increase in neutralization potency when Fab and IgG neutralization IC_{50} values were compared (Cale et al. 2017). Taken together with EM observations of IgG complexed Env trimers, these findings strongly suggest that VRC38 IgG can cross-link adjacent protomers of the same trimer using its two Fab arms, thus gaining in avidity. Furthermore, as these bnAbs do not have to reach past N160 glycans of adjacent protomers in the apex center, they manage to bind strand C residues by inserting considerably shorter 16-22aa HCDR3 loops between the N156 and N160 glycans of a single gp120 subunit. The novel binding approach limits the core epitope to the N-terminal strand C residues, allowing both VRC38 and BG1 to tolerate anionic residues at positions 168 and 169, respectively, which otherwise completely abrogate neutralization of traditional V2-apex bnAbs (Andrabi et al. 2015; Doria-Rose et al. 2015). Nevertheless, both VRC38 and BG1 show comparably low neutralization potency and breadth (0.54 µg/ml and 0.67 µg/ml with 30% and 37% breadth, respectively), likely due to weaker strand C interactions and increased exposure to variable residues (Cale et al. 2017).

The exceptional neutralization potency and breadth of trimer axis-centered V2-apex bnAbs, paired with comparatively moderate VH mutation frequencies of ~12–16% displayed by many bnAbs of this class, renders the trimer apex especially relevant for vaccine design (Andrabi et al. 2015). At the same time, extraordinarily long HCDR3s are required for broad and potent neutralization of this epitope cluster. HCDR3 length is primarily regulated during germline recombination of V-D-J gene segments, with only certain D-J gene segment combinations enabling HCDR3 loops of 30+ aa length, thus limiting the pool of suitable germline pre-cursor B cells targeting the epitope cluster.

2.4.6 Silent Face Epitope Cluster

This epitope cluster is located on the gp120 outer domain, one of the most densely glycosylated areas on the Env trimer (Fig. 3; orange). As such, this area was for the longest time deemed immunologically silent due to a paucity of neutralizing antibodies targeting the region and was therefore dubbed ‘silent face’ (Wyatt et al. 1998). This only recently changed with the isolation of VRC-PG05 using RSC3 sorting-probes from IAVI donor #74 (Zhou et al. 2018); the same donor from whom the CD4bs bNAb VRC-PG04 antibody had previously been isolated (Falkowska et al. 2012; Zhou et al. 2018).

The VRC-PG05 epitope consists of two main high-mannose glycans, N262 and N488, which comprise about 35% of its binding footprint, respectively, as well as glycan-covered protein residues, specifically E293, and peripheral contacts to the N295 glycan (Zhou et al. 2018). Remarkably, unlike other glycan shield penetrating antibodies of the V3 or apex epitope clusters, this bNAb matured using only mildly mutated heavy and light chain genes (9% and 6% of nucleotides, respectively), and is using an average length HCDR3 loop of 17 amino acids, which raised hopes that this epitope cluster may lend itself to less sophisticated vaccine design approaches.

Unfortunately, the heavy reliance on glycan binding, second only to 2G12, renders VRC-PG05 both lacking in potency and susceptible to escape (which we discuss in detail in Sect. 2.2), which were determined to be 0.8 μg/ml and 27% on a 208-virus panel, respectively.

Interestingly, however, Schoofs and colleagues isolated a new family of somatically related bNAbs to the silent face, which is characterized by its most potent member, SF12 (Schoofs et al. 2019). SF12's neutralization breadth and potency are somewhat higher at 0.2 μg/ml and 62% on a 119-virus panel, respectively; its heavy and light chain are also considerably more mutated however ($\sim$17% of nucleotides) and its HCDR3 loop is 23 amino acids long. Nevertheless, SF12 binds largely the same epitope as VRC-PG05, albeit from a different angle, and is heavily reliant on the presence of glycans N262 and N488 while likewise accommodating glycan N295. Its longer HCDR3 loop, as well as HCDR 1 and 2 loops, forms extensive and distinct binding contacts with the protein surface, which comprises 25% of SF12's binding footprint, as compared to merely 12% of VRC-PG05's—which likely explains the difference in neutralization breadth and potency. Due to the differences in heavy chain contacts, SF12 does not bind E293, but forms ionic bonds to R444 instead, absence of which does halve its neutralization potency. Both VRC-PG05 and SF12 show remarkable neutralization breath against Clade AE viruses, which could make them a valuable addition to therapeutic bNAb cocktails containing V3-glycan bNAbs, which often struggle to neutralize AE viruses due to their shifted N334 glycan site. Indeed, an antibody cocktail containing SF12 was able to control viremia and experimentally infected mice (Schoofs et al. 2019); however mono-therapy is easily escaped from by abrogating or shifting the N448 glycan site (Schoofs et al. 2019), which analogously was observed in donor 74 from which VRC-PG05 was isolated (Zhou et al. 2018). VRC-PG05's mechanism of neutralization is likely interference with conformational changes in the Env trimer that are needed to bind three CD4 subunits, preceding fusion spike formation (Zhou et al. 2018); a similar mechanism is likely for SF12.

3 Heterosubtypic Antibodies to Influenza Virus

3.1 Virion Structure

Electron microscopy and cryoelectron tomography have found that influenza A virions can be divided into the five classes, depending on their internal and external composition. Besides predominantly spherical virions with a diameter of about 120 nm (ranging from 84 to 170 nm), elongated virions can be found as well, which tend to be about 20 nm thinner than the average spherical particle (Calder et al. 2010; Cusack:1985hq; Harris et al. 2006). There are three viral surface proteins present on the virion surface: Hemagglutinin (HA), which is responsible for

viral attachment and entry into the host cell, neuraminidase (NA), which is a receptor-destroying enzyme that has been implicated in the release of progeny viruses, and the extracellular domain of the matrix 2 protein (Zebedee and Lamb 1988) (M2), which is a proton channel responsible for the acidification of the virion interior upon uptake into endosomes. HA and neuraminidase NA spikes are densely arranged on the virion surface, with an average center-to-center spacing of 11 nm. Influenza A virions have been quantified to contain around 300 HA and 38–50 NA spikes (Cusack et al. 1985; Harris et al. 2006), with the latter being found to tend to cluster in patches (Harris et al. 2006). As illustrated in Fig. 1, the surface of IAV is therefore considerably more densely populated with spike proteins than HIV. This dense population might have an impact on accessibility of the membrane-proximal parts of the viral surface proteins, while on HIV virions the Env spikes are so sparse that they should be well accessible from all directions.

Influenza hemagglutinin is the major surface glycoprotein on the virion of influenza viruses and is responsible for virus attachment and entry into the host cell. It is a trimer of dimers consisting of three HA1 subunits forming the globular head that harbors the receptor-binding site and three HA2 subunits forming a coiled-coil stalk-like structure that contains the fusion machinery. The HA protein is expressed as a precursor HA0 protein that is posttranslationally cleft into the N-terminal HA1 and C-terminal HA2 subunits. This proteolytical processing is required to render the virions infectious and has a crucial impact on the pathogenicity of avian influenza viruses. Highly pathogenic avian influenza (HPAI) viruses possess a multibasic cleavage site that can be cut by ubiquitous furin-like proteases. Low pathogenic avian influenza (LPAI) viruses, in contrast, exhibit cleavage sites that rely on trypsin-like proteases for processing. As these are primarily present in the intestinal tract, LPAI viruses primarily cause intestinal infections, while HPAI viruses can spread systematically in birds. Human influenza viruses have LPAI-like cleavage sites depending on trypsin-like proteases that are primarily found in the respiratory tract, such as tryptase Clara which is expressed by Clara cells in bronchiolar epithelium (Kido et al. 1999), or membrane bound proteases, such as transmembrane protease serine S1 member (2TMPRSS2) and human airway trypsin-like protease (HAT) (Böttcher et al. 2006).

Hemagglutinin trimers are responsible for viral attachment to host cells by binding to sialic acid moieties on the host cell surface. While human isolates appear to prefer sialic acids linked in a α-2,6 orientation, avian isolates prefer α-2,3-linked sialic acids, and this linkage preference has been reasoned to be the primary cause for the species preference of different IAV strains (Skehel and Wiley 2000). The receptor-binding site is located on the globular head and consists of a shallow pocket that is formed by the 130-loop, the 150-loop, the 190-helix and the 220-loop of HA1. In contrast to most other viral attachment protein/receptor interactions, hemagglutinin only has a very low affinity for its sialic acid receptor. While HIV gp120 binds CD4 with affinities in the range of 1–10 nM (Ugolini et al. 1999), the binding affinity of hemagglutinin for sialic acid has been determined to be in the 2–4 mM range (Sauter et al. 1989). It is therefore presumed that numerous spike/receptor interactions are required for virus attachment. Also for the formation of the

fusion pore, the most recent model found that at least six hemagglutinin molecules are required, three of which have to undergo a conformational change (Dobay et al. 2011). Following attachment to cells, IAV is internalized into endosomes, and fusion of the virus with the endosomal membrane is induced by the acidification of the endosome (Bullough et al. 1994; Skehel et al. 1982).

Both, the NA and the HA proteins exist in various subtypes that classically have been defined as serotypes. There are currently 18 subtypes described for HA, and 11 for NA. Genetically, subtypes differ in more than 25% of their amino acid sequence from each other but can diverge by more than 50% in their nucleic acid sequence. IAV can infect a broad variety of hosts but aquatic birds are considered as the main reservoir. While avian IAV strains can express most subtypes (H1–H16 and N1–N9), human viruses are restricted to the H1, H2, H3, N1 and N2 subtypes. The recently discovered H17, H18, N10 and N11 subtypes have exclusively been identified in influenza-like viruses isolated from bats (Tong et al. 2012, 2013) and use MHC-II as a cross-species receptor (Karakus et al. 2019). Equine, canine and piniped IAV viruses also only display a limited number of serotypes (Parrish et al. 2015), which makes aquatic birds the genetically most diverse reservoir of influenza A viruses (Fig. 4).

The nomenclature of all influenza viruses starts with the genus, the host species, the site of isolation, the isolate number and the year of isolation, all separated by slashes. The subtype composition is added in brackets to the end of these strings. For human viruses, the host species designation is omitted. Accordingly, A/Chicken/Germany/N'/1949 (H10N7) refers to the virus N' that was isolated from a

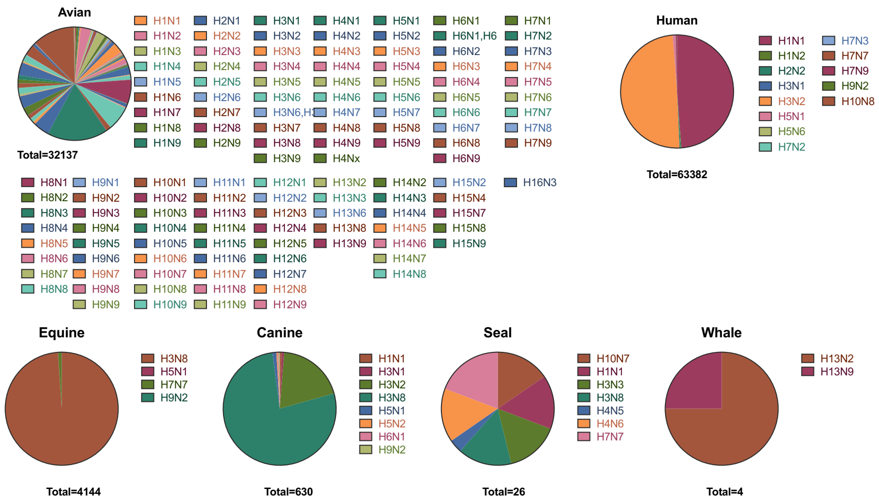


Fig. 4 Subtype distribution in different species. The NCBI Flu database was queried on September 21, 2017, for unique HA and NA sequences from the indicated species and the number of summed up HA and NA sequences were plotted for each indicated host species

chicken in Germany in the year 1949, and that expresses an HA of the H10 and a NA of the N7 subtype.

The segmented genome of IAV resides on eight distinct, negative-sensed RNA molecules. During superinfection of cells, these molecules can be exchanged for each other giving rise to new, reassorted variants. IAV have also a limited ability to be transmitted across species barriers, allowing for the introduction of new subtypes from one species to another. In the case of humans, zoonotic infections from poultry are usually abortive, largely benign and only cause minor symptoms such as conjunctivitis. However, since the late 90s, a rising number of zoonotic infections with HPAI viruses of the H5N1 subtype have been observed. Although these viruses can typically not be transmitted from human to human, their very high lethality rate of over 50% is a major concern (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Also, infections with HPAI viruses of the H7N9 subtype have become more frequent in recent years.

Successful introduction of a new subtype into the human IAV requires species transgression that is follow by the adaption of the transgressed virus to the human host. For instance, a reassorted virus with an avian HA gene has to gain the ability to use the α-2,6-linked sialic acid as a receptor, needs to balance with the remaining genes such as the receptor-destroying neuraminidase gene (Scholtissek et al. 2002; Wagner et al. 2002; Ilyushina et al. 2005) and has to acquire the ability to efficiently transmit between humans. Due to their novel antigenic composition, such reassortant viruses constitute an antigenic shift that typically leads to an influenza pandemic. Since swine express both α-2′,6′ and α-2′,3′-linked glycans in their lungs, they can be infected by both avian and human viruses, and are considered to be the mayor mixing vessel in which avian viruses reassort with human viruses, and gain the ability to use α-2′,6′-linked sialic acid receptors. These viruses can then be transmitted to humans and cause a new pandemic. However, the H1N1 ‘Swine flu’ pandemic indicated that such reassortment is not absolutely required and a porcine virus can directly adapt to the human host. There is considerable concern that zoonotic avian H5N1 HPAI eventually could achieve a similar adaption and gain the ability for efficient human-to-human transmission. Given the incredibly high cumulative lethality rate of over 50% of infected individuals, the effects of such an adaption would be devastating. For comparison, the ‘Spanish Flu’ (see below) had an estimated lethality rate of 1.5% and killed an estimated 50 million people over two years (De Jong et al. 2000).

The twentieth century has seen four major antigenic shifts. The first one, the introduction of H1N1-expressing viruses, caused the devastating ‘Spanish Flu’ in 1918. H1N1 viruses continued to circulate until a virus of the H2N2 serotype replaced them during the ‘Asian Flu’ pandemic of 1957. H2N2 viruses themselves were then replaced by a reassortant H3N2-expressing virus during the ‘Hong Kong Flu’ in 1967. In 1979, viruses that are genetically very closely related to H1N1 viruses circulating in the late 50s caused the ‘Russian Flu’ pandemic (Nakajima et al. 1978). However, these H1N1 viruses failed to replace the circulating H3N2 viruses. It is currently assumed that the ‘Russian Flu’ originated from a failed vaccination experiment using an insufficiently attenuated virus derived from an

isolate circulating in the late 50s. Ever since the introduction of this virus in 1979, both the H1N1 and H3N2 subtypes co-circulate in parallel and evolve independently from each other. This dual subtype circulation was also not resolved during the latest (pseudo-)pandemic caused by a species-transgressed H1N1-expressing porcine virus that caused the 2009 'Swine flu.' However, the novel H1N1 viruses replaced the previously circulating H1 lineage.

3.2 Measures of Immune Evasion (Spacing, Glycosylation, Hidden Epitopes)

3.2.1 Escape Within the Population Versus Within Single Host (Mutation Rates)

In contrast to HIV, which persists within a host and incorporates measures to escape immune pressure exerted by the host immune system, IAV evolves much slower. Their primary selection pressure arises from a dwindling number of non-immune hosts that selects for variants resistant to herd immunity, i.e., the abundance of neutralizing antibodies to circulating variants in the population. This process, referred to as antigenic drift, enables IAV to infect hosts who have previously been infected by the same subtype and ensures continuous circulation of the virus. Antigenic shift, and the short-lived antibody titers, is at the base of the seasonal influenza epidemics and is also responsible for the need to annually revisit the IAV vaccine formulation. Interestingly, antigenic shift is mainly observed in circulating human viruses. In contrast, porcine viruses are antigenically considerably more stable than human IAV (De Jong et al. 2007), and virtually no antigenic drift can be observed in circulating avian IAV (Bean et al. 1992). The difference could be explained by (i) the shorter generation span of both swine and birds which ensures that there is a constant supply of immunologically naïve offspring and (ii) in the case of avian IAV, the many available serotypes make escape from herd immunity less necessary. However, antigenic drift has been observed in H5N1 IAV and is thought to be the result of prolonged circulation in live poultry (Cattoli et al. 2011; Zou et al. 2012).

3.2.2 HA Sites of Vulnerability

Head Region

Using competition experiments with monoclonal antibodies, the classical immunodominant antigenic sites of human IAV HA have been determined. For H1, they are referred to as Ca1, Ca2, Cb, Sa and Sb (Fig. 5 and (Gerhard et al. 1981), for H2 as IA, IB, IC, IIA and IIB (Tsuchiya et al. 2001), for H3 as A, B, C and D (Wiley

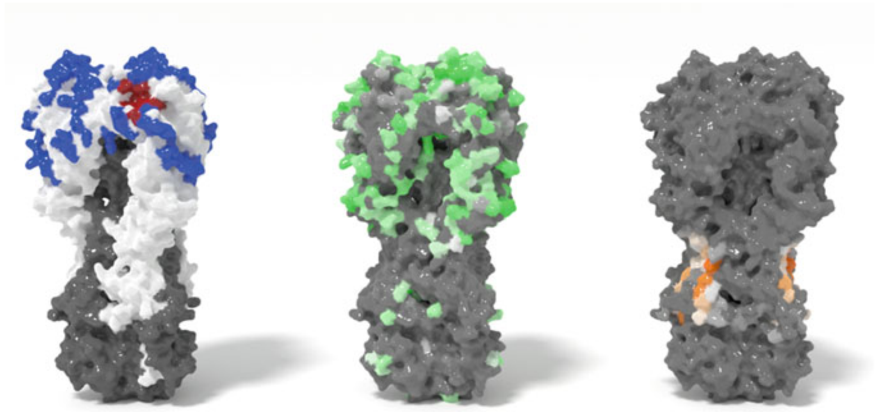


Fig. 5 HA epitope location in relation to sequence variability. Left: the classical antigenic sites, as defined by monoclonal antibodies, are depicted in blue and surround the receptor-binding site (red). Middle: Shannon's Entropy (Variably) for Human H1 between 1918 and 2001. For the variability heat-map, 8219 full-length human H1 protein sequences were downloaded from the NIH Flu database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database) and aligned using the MUSCLE algorithm. Using an aligned FASTA file, Shannon entropy was then calculated with a web-based service provided by the Los Alamos National Laboratories (http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html) and mapped on the surface structure using a custom Python script in MacPyMol. The degree of entropy is indicated by a green (highest entropy, 1.058) to gray (no entropy, 0) gradient. The maximal possible Shannon entropy for standard amino acids is ln(20) or 2.996. Right: residues recognized by stem-specific, heterosubtypic antibodies

et al. 1981), for H5 as I and II (Kaverin et al. 2007), and for H9 as I and II (Ilyushina et al. 2004), or H9-A and H9-B (Peacock et al. 2016). These antigenic sites are all located on the globular head formed by HA1, and surround or slightly overlap the receptor-binding site. In H2 (and probably H9), an additional antigenic site has been found in the stem of the protein (Tsuchiya et al. 2001). A more recent bioinformatic approach extended the definition of the antigenic sites for H1 to include more residues than the classical approach using monoclonal antibody competition (Huang et al. 2012b).

When the Shannon entropy (a mathematical measure for entropy, or unpredictability) is calculated from an alignment of over 8200 unique human H1 HA sequences, it becomes apparent that the greatest variability can be found at the classical antigenic sites, nicely illustrating that the antigenic drift is caused by the selection pressure arising from H1-specific antibodies (Fig. 5). Hemagglutinin is not as heavily glycosylated as the HIV envelope with 6 to 14 N-linked glycosylation sites per protomer. While some of these glycosylation sites are required for proper folding (Roberts et al. 1993), influenza A viruses also primarily use shifting glycosylation to reduce immunogenicity and to evade antibody recognition. Human H3N2 viruses, for instance, have almost doubled the number of N-linked glycosylation sites on hemagglutinin since their introduction into human population in

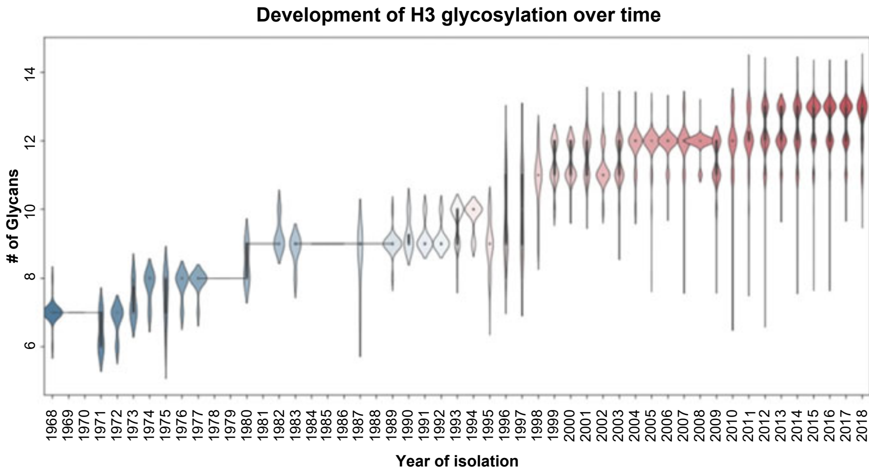


Fig. 6 Acquisition of additional N-linked glycosylation sites on the HA of H3N2 viruses. Since their introduction into humans in 1968, H3N2 viruses have almost doubled the number of N-linked glycosylation sites. Like in HIV-1, this accumulation of glycans is thought to help the virus to escape preexisting antibodies and to lower its immunogenicity. For the generation of the plot, all unique full-length human H3 sequences with known year of isolation were downloaded from https://www.ncbi.nlm.nih.gov/genomes/FLU/ (8/20/2018; 7057 sequences) and potential glycosylation sites counted with a custom Python script and plotted using the Seaborn data visualization library

1968 (Fig. 6), probably in response to pressure for preexisting antibodies in the population. However, for influenza, it has been postulated that introduction of glycans comes at a fitness cost (Das et al. 2011). Moreover, it has been simulated that there is a maximal number of supported glycans for a given configuration, and that after this number is reached, typically after 9–12 years of evolution in the human host, the glycans have to be swapped between different sites, or the hemagglutinin is replaced by a novel pandemic strain (Altman et al. 2017).

3.2.3 Broadly Neutralizing Antibodies to Influenza a Viruses

While the majority of antibodies elicited during infection are highly strain-specific, and only neutralize the eliciting, or closely related viruses, evidence has been accumulating that a small fraction of antibodies is actually capable of recognizing a wide range of viruses within one subtype, or even viruses belonging to different subtypes. The latter are therefore referred to as heterosubtypic antibodies (hnAbs). Heterosubtypic antibodies can be found in a majority of people, albeit at low titers (Li et al. 2012; Kohler et al. 2014). Heterosubtypic antibodies tend to be restricted to phylogenetic group 1 [CR6261 (Ekiert et al. 2009) F10 (Sui et al. 2009), 3.1 (Wyrzucki et al. 2014), PN-SIA49 (De Marco et al. 2012)]. Yet, hnAbs that can

cope with the structural differences between the two phylogenetic groups, and Abs that are able to neutralize viruses from both groups, have been described [CR9114 (Dreyfus et al. 2012), FI6 (Corti et al. 2011), 1.12 (Wyrzucki et al. 2015), FY1/ MEDI8852 (Kallewaard et al. 2016)]. One of these hnAbs, CR9114, can also neutralize viruses of the influenza B virus genus, indicating that even differences between different IAV genera can be overcome. All these antibodies bind to a conserved hydrophobic groove that is formed between HA1 and the A helix of the HA2 subunit, and often also involve contacts to the fusion peptide (FI6, MEDI8852). Stem-specific antibodies do not interfere with receptor binding but prevent the structural rearrangements required for the fusion process (Ekiert et al. 2009). Their binding has also been described to inhibit proteolytical activation of the HA0 precursor protein (Corti et al. 2011). Within the primary stem epitopes, there are several differences, that hnAb must accommodate to neutralize viruses from both phylogenetic groups. These are First, the presence of a larger Asn instead of a Thy residue at position 49 of group 2 HA2. Second, group 1 viruses have a His at position 111 of HA2, while group 2 viruses typically have Thr or Ala at this location. Although these residues are not directly contacted, they determine the orientation of a crucial Trp residues at position 21. And third, the presence of a N-linked glycosylation site at position 38 of group 2 HA2 that constitutes another major difference between the two phylogenetic groups. Accordingly, antibodies need to be able to accommodate these differences to neutralize viruses from both phylogenetic subgroups (Ekiert et al. 2009; Corti et al. 2011; Dreyfus et al. 2012; Laursen and Wilson 2013).

Interestingly, group 1 and pan-IAV heterosubtypic antibodies primarily contact the HA stem groove using residues of the heavy chain (Ekiert et al. 2009; Corti et al. 2011). In the case of pan-IAV hnAb 1.12 that was isolated by phage display, the same heavy chain was found paired with numerous light chains and most heavy-light chain combinations resulted in hnAbs. Also of note is that the broadest pan-IAV antibodies, 1.12 and CR9114, display an unusual stretch of 4–5 tyrosine residues at the tip of their HCDR3 that is involved in antigen recognition. Indeed, antibodies capable of neutralizing both phylogenetic groups have been shown to evolve from antibodies that only neutralize viruses from phylogenetic group 1, and then acquire group 2 specificity during maturation (Kallewaard et al. 2016). Monoclonal antibodies specific only for phylogenetic group 2 have also been isolated, but they are considerably less frequent than those to group 1 [CR8020: (Friesen et al. 2014)] and bind to an epitope located at the base of the stem of the HA trimer (Corti et al. 2011; Friesen et al. 2014). Stem-specific antibodies can be compared to HIV bnAbs that bind to the interface between gp120 and gp41 such as PGT151 (Falkowska et al. 2014), 8ANC195 (Scharf et al. 2014) and 35O22 (Pancera et al. 2014), and have likewise been postulated to interfere with structural rearrangements preventing the formation of the fusion spike.

A second class of heterosubtypic antibodies has been described that mimics sialic acid receptor binding by inserting a complementarity determining region (CDR)-loop into the receptor-binding site. As for HIV, the receptor-binding site of IAV is relatively conserved in order to remain functional, and thus enables

antibodies recognizing key receptor-binding residues to neutralize a diverse range of IAVs (Ekiert et al. 2012). However, in contrast to CD4 binding site-specific bnAbs to HIV, such as VRC01 or 3BNC117, these antibodies usually have a more restricted breadth, yet provide an interesting target for the design of a universal influenza vaccine.

4 Vaccine Design Aspects for HIV and Influenza

4.1 Approaches for Eliciting Broadly Neutralizing/Heterosubtypic Antibodies

It is currently assumed that there are two major obstacles for the elicitation of broadly neutralizing antibodies: First, immunodominance of the variable and strain-specific epitopes is suspected to impede antibody responses to the conserved and clearly less immunogenic h/bnAb epitopes. Second, the scarcity of h/bnAb precursor B cells whose BCR, in case of HIV, often also do not recognize the viral envelope proteins very well (Scheid et al. 2011; Ota et al. 2012; Hoot et al. 2013; Escolano et al. 2016). Moreover, studies of bnAbs to HIV show that a successful vaccine against antigenically highly variable viruses not only needs to induce antibodies that are able to recognize relatively conserved epitopes, but that are also able to tolerate variation within the epitope. This requirement is considerably more difficult to achieve than simply increasing an antibody's affinity. For instance, bnAb b12's affinity for gp120 was improved over 420-fold by in vitro affinity maturation (Barbas et al. 1994; Yang et al. 1995). Yet, despite gaining the ability to neutralize some additional viruses, the overall breadth of the affinity matured antibody decreased, indicating that breadth is a fine-tuned balance of affinity, conservation of the recognized epitope and ability to tolerate variations within the epitope.

There are currently two main immunization strategies by which these obstacles are sought to be overcome (Fig. 7): The first strategy is based on multiple immunogens that are antigenically distinct except for desired epitope(s). The hope in this approach is that the desired epitopes become a common denominator shared between all immunogen variants and will therefore provide an advantage to maturing B cell clones targeting shared over differing epitopes. Such common denominator immunizations can be performed in the form of immunogen cocktails, administering all immunogen variants at once, or in serial immunizations, by using consecutive individual immunogens.

The second strategy employs a sequence of immunogens, each of which is designed to shepherd the antibody response into a desired direction. Some of these strategies involve a priming immunization with an antigen that has been designed to activate rare h/bnAb precursor B cells, which often do not recognize the actual epitope on the pathogen very well. These 'germline targeting' immunogens are then followed by immunizations with immunogens designed to gradually shepherd the B

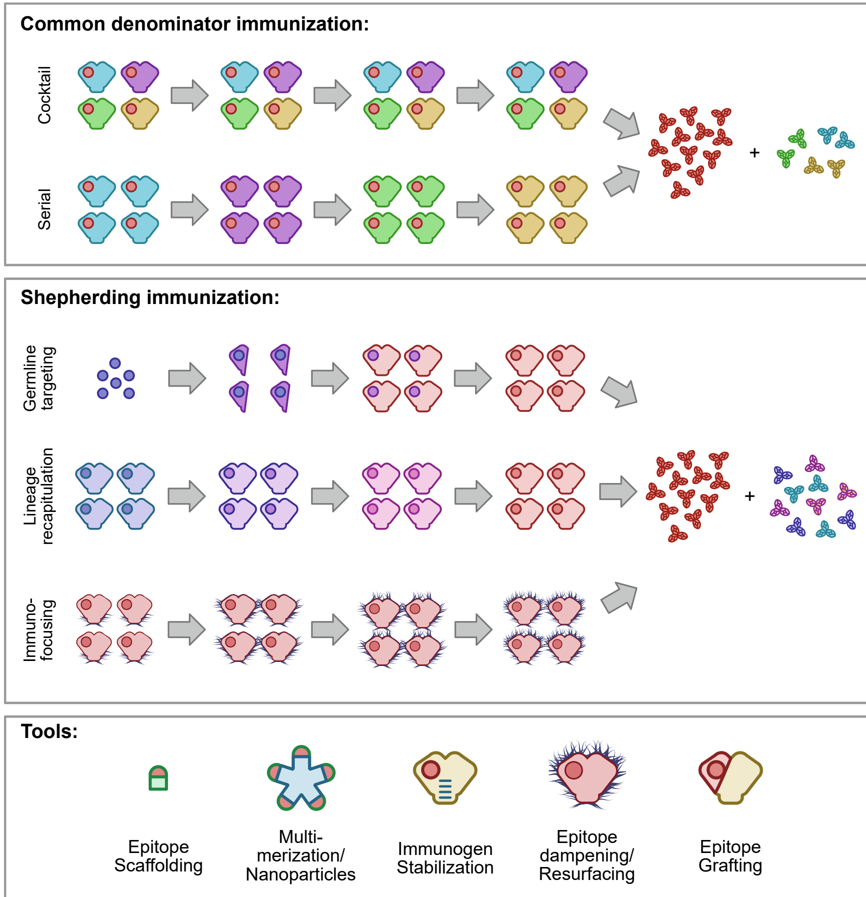


Fig. 7 Strategies to elicit broadly reactive antibodies. The 'common denominator' strategies use immunogens that are antigenically distinct yet share the desired epitope (red circle). This strategy can be pursued using immunogen cocktails, or serial immunization with different individual immunogens. The 'shepherding' strategies start with an immunogen specifically designed to activate B cells expressing the germline of h/bnAbs as BCRs, followed by booster immunizations designed to shepherd the response toward broadly reactive antibody responses recognizing the epitope, as it is present on the pathogen. Alternatively, evolution of h/bnAb lineages is recapitulated by immunizing with immunogens whose succession lead to the selection of h/bnAbs in vivo. Lastly, immunofocusing aims at first eliciting a range of antibody responses from which the desired responses are selected by sequential use of epitope-dampened immunogens, in which only the relevant epitopes are accessible to antibodies (immunofocusing). The tools employed for these approaches include epitope scaffolding, i.e., generation of a small artificial protein displaying the epitope of interest, multimerization to increase immunogenicity, stabilization of meta-stable spike proteins, epitope dampening/resurfacing by modification of the spike surface to reduce immunogenicity of undesirable regions (e.g., by the addition of glycans), and epitope grafting, where regions of interest are transferred to a related but immunologically different spike protein (e.g., the HIV V2 grafted onto SIV Env)

cell response from the artificial ‘germline-recognized’ epitope to the actual h/bnAb epitope present on the pathogen’s surface protein. A second embodiment of the shepherding approach is mainly used for HIV and is attempting to recapitulate the viral/antibody co-evolution as it occurred in individuals who developed bnAbs. Thereby, a sequence of envelope immunogens is applied starting with the transmitter/founder virus envelope that is then followed by variants that triggered substantial shifts toward breadth in the antibody response of the infected individual. This approach requires detailed longitudinal data from h/bnAb donors. The third embodiment aims at triggering numerous antibody responses to the immunogen, but then attempts to focus the response on the desired epitopes during the subsequent immunizations, by using a sequence of gradually more and more restrictive immunogens.

4.2 Challenges and Approaches for the Induction of bnAbs to HIV

Although many vaccine trials using traditional immunization strategies attempted to elicit bnAbs through vaccination, none have been successful thus far. The reasons are many but include the choice of immunogens that did not reflect the antigenicity of the native Env trimer, most prominently soluble gp120 and non-stabilized gp140 proteins. While great progress has been made in developing soluble, trimeric Env proteins displaying native-like antigenicity in recent years (Sanders et al. 2013; Sharma et al. 2015), elicitation of bnAbs in relevant model systems is still outstanding. This is not only due to the unusual genetic features that bnAbs seemingly need to acquire over many cycles of germinal center reactions, but can also be attributed to the immunoquiescence of conserved broadly neutralized epitopes on native Env, which is rooted in the immune-evasive features of the Env spike as discussed in the bnAb section.

In short, as pointed out in the respective epitope cluster sections, bnAbs develop rare genetic features to by-pass the immune-evasive features of Env, which in turn hinders their induction by vaccination. These include (i) high levels of somatic hypermutation, which is problematic for all five major epitope clusters, (ii) insertions and deletions, most prominently among V3-glycan bnAbs, (iii) long CDR-H3 loops, particularly among V2-apex, V3-glycan and some gp120-gp41 interface targeting bnAbs, (iv) restriction to specific VH germline alleles among CD4 binding site bnAbs and (v) potentially limited pools of primary B cells able to engage epitopes that are partially autoreactive, including the glycan and lipid comprising epitopes of V2-apex, V3-glycan and especially MPER-directed bnAbs (Burton and Hangartner 2016).

Furthermore, even native infectious Env spikes on the virion surface present many immunodominant epitopes that are highly variable and thus tend to direct the immune system toward inducing strain-specific nAbs from which the virus will

invariably escape. Therefore, strategies for the guided induction of bnAbs are most likely needed, either by means of engaging germline precursors of mature bnAbs, attempting to guide the immune response along a pre-defined maturation path, or by deploying other immunofocusing strategies, including sequential or cocktail immunizations with native Env trimers (Fig. 7).

4.2.1 Induction of Autologous Tier 2 nAbs by Trimeric Immunogens

The failure of clinical trials to induce tier 2 nAb responses has been associated with differences in the presentation of critical epitopes on the immunogens used, as compared to their presentation on the native Env spike. Therefore, the generation of molecules that more faithfully mimic the spike has opened up new opportunities for the induction of tier 2 nAbs. These trimeric spike mimetics include foremost SOSIP trimers (Binley et al. 2000; Sanders et al. 2002; Sanders et al. 2013), but also native flexible linker (NFL) trimers (Georgiev et al. 2015; Sharma et al. 2015) as well as the more recently described uncleaved prefusion-optimized (UFO) trimers (Kong et al. 2016a). Native-like Env trimers have successfully induced tier 2 nAbs in small animal models (Sanders et al. 2015; McCoy et al. 2016; Torrents de la Peña et al. 2017) and less reproducibly in nonhuman primates (Sanders et al. 2015).

Nonhuman primates (NHPs), and specifically rhesus macaques (RMs), are often argued as the most appropriate pre-clinical model for HIV vaccine studies because of the close genetic relatedness of NHPs to humans. Four RM studies using trimeric Env immunogens reported the induction of autologous Tier 2 nAbs, with two of those studies using SOSIP trimer designs (Sanders et al. 2015; Pauthner et al. 2017) and one using NFL trimers (Martinez-Murillo et al. 2017). However, there have been concerns about the limitations of those results. Tier 2 nAb titers were primarily reported after 6–12 months of immunizations, were relatively low, and washed out rapidly. Most worrisome was the observation that only a fraction of RMs developed nAbs, raising concerns about whether Env trimers will elicit tier 2 nAbs in humans (Havenar-Daughton et al. 2017; Sanders and Moore 2017). To address this problem, an optimized protocol for the reliable induction of tier 2 nAbs in NHPs was recently developed (Pauthner et al. 2017), which enabled the induction of tier 2 nAb titers in all immunized NHPs, and, at high titers, also protected animals from homologous SHIV challenge (Pauthner et al. 2019).

These animal studies have further uncovered that soluble spike mimetics expose highly immunogenic epitopes at the base of the trimer that are not present in the membrane inserted version of the protein (de Taeye et al. 2015; McCoy et al. 2016; Bianchi et al. 2018). Moreover, using a recently developed method to map the epitopes recognized by polyclonal antibodies by electron microscopy (Bianchi et al. 2018), it was found in NHPs that repetitive immunization with soluble proteins induced more antibody specificities than are elicited during experimental SHIV infection. Also, some of the vaccine-induced specificities were found to block access to bnAb epitopes (Nogal et al. 2020).

4.2.2 BnAb Immunization Strategies Based on Mimetics of the Native Env Spike

Because SOSIP and NFL trimers generally have excellent antigenic properties (Sanders et al. 2013; Georgiev et al. 2015; Sharma et al. 2015), meaning that nAb-binding EC_{50} and neutralization IC_{50} are closely correlated, while non-neutralizing antibodies cannot bind, there were high hopes that this class of immunogens would suffice to induce some level of neutralization breadth. However, immunizations with the first generation of native-like trimers failed to induce heterologous tier 2 nAb titers (Sanders et al. 2015; Pauthner et al. 2017). A widely discussed explanation is that native-like trimers still expose a variety of immunodominant variable epitopes that misdirect the immune system away from immunoquiescent, conserved bnAb epitopes (Havenar-Daughton et al. 2017; Pauthner et al. 2017, 2019). The immunological explanation for this phenomenon is that in germinal center (GC) reactions, B cells reactive to easy-to-engage, high-affinity epitopes expand faster than those reactive to hard-to-engage epitopes and thus outcompete the former. As a result, an immunodominance hierarchy arises, which may be further skewed by the abundance of B cells able to engage constrained, immunoquiescent epitopes, which may require rare primary or cross-reactive secondary B cells (Havenar-Daughton et al. 2017; Angeletti et al. 2017). Consequently, multiple strategies have been developed to accelerate and guide bnAb induction.

Despite the overall decent antigenicity of native-like trimers, there is evidence for some flexibility of SOSIP molecules and exposure of regions, such as the V3 loop tip, that are not or infrequently exposed on virion surface trimers (de Taeye et al. 2015). Immunizations with native soluble trimers revealed three principal targets of nnAbs that are frequently targeted: the V3 loop, epitopes exposed due to CD4-induced conformational changes (CD4i), and epitopes at the base of the trimer that are artificially exposed as a result of the truncation at amino acid 664 in the gp41 region, which renders SOSIP trimers soluble (Sanders et al. 2015). To reduce the exposure of these epitopes and thus potentially reduce competition of B cell lineages in GC-reactions, several stabilization approaches have been developed. These include designs to sequester the V3 loop (de Taeye et al. 2015; Ringe et al. 2017; Kulp et al. 2017), as well as strategies to increase the overall thermostability of the trimer to reduce the exposure of CD4i and other non-neutralizing epitopes, e.g., by adding additional disulfide bonds or by chemical cross-linking (de Taeye et al. 2015; Steichen et al. 2016; Ringe et al. 2017; Torrents de la Peña et al. 2017; Kulp et al. 2017). While these designs, as far as they have been tested in vivo, do suppress V3 loop-directed responses to various degrees, thus far none have been able to induce bnAbs or increase the titer of autologous tier 2 nAbs.

Since the biggest deterrent to efficient nAb-binding of broadly neutralizing epitopes is the density of the glycan shield, a novel immunogen design strategy is the deliberate removal of glycans shielding conserved epitopes (Crooks et al. 2017; Dubrovskaya et al. 2017) or the use of isolates with naturally occurring glycan holes (Voss et al. 2017). These immunogens were developed following two studies

reporting that autologous tier 2 nAbs induced by BG505 SOSIP.664 and JRFL NFL immunizations in rabbits, were targeting naturally occurring glycan holes in the respective isolates (Crooks et al. 2015; McCoy et al. 2016). Immunogens with deletions in the glycan fence surrounding the CD4 binding site were able to induce extraordinarily high nAb titers to viruses bearing the same glycan deletions, but induction of nAbs against heterologous wild type isolates has still not been achieved (Dubrovskaya et al. 2017). This will likely require more sophisticated sequential immunization schemes that step-wise re-introduce deleted glycans and finish with wild type trimer boosts; early results using this approach look promising (Dubrovskaya et al. 2017).

Sequential and cocktail immunizations are frequently discussed immunization strategies, which rely on a heterogeneous set of immunogens that only share distinct conserved epitopes (Fig. 7). This approach was computationally predicted to give rise to germinal center dynamics supportive of the bnAb induction (Wang et al. 2015). The principal idea is that by immunizing with a trimer mix that differs in variable domains, GC B cells to conserved epitopes gain a competitive advantage over GC B cells targeting variable domains, especially when specific variable epitopes are removed completely from the immunization sequence. An easy way to achieve this is by immunizing with a heterogenous set of trimers from multiple clades, as, by definition, broadly neutralizing epitopes remain conserved while variable epitopes do not. However, there have been no examples of cross-clade trimer sequential or cocktail immunizations able to induce heterologous nAb titers thus far (Klasse et al. 2016). It is important to point out that germline-targeting approaches generally also use sequential immunization schemes, but instead of excluding variable epitopes, these strategies focus on the evolution of a specific conserved epitope.

4.2.3 bnAb Immunization Strategies Based on Germline-Targeting

Germline-targeting immunization strategies use a selection of specific immunogens to shuttle the immune response along a pre-defined maturation path. Two general approaches can be discerned: (i) so-called lineage-based approaches aim to re-create bnAb evolution as observed in the longitudinal study of infected individuals and (ii) the use of immunogens that engage specific germline versions of known bnAbs, typically by means of epitope scaffolding or rational protein design, followed by appropriate boosting regimens (Stamatatos et al. 2017) (Fig. 7).

The problem underlying both approaches is that most native trimers do not bind germline-reverted bnAb precursors (Xiao et al. 2009). However, the detailed longitudinal study of bnAb and virus co-evolution provides opportunities to identify both germline precursors of mature bnAbs as well as the viral *env* sequences that likely initiated those bnAb lineages (Bonsignori et al. 2017b). Detailed co-evolution studies have been described for bnAbs targeting the CD4 binding site (Liao et al. 2013; Gao et al. 2014), the V2-apex (Doria-Rose et al. 2014), the V3-glycan

(MacLeod et al. 2016; Bonsignori et al. 2017a) and the MPER epitope clusters (Williams et al. 2017). However, early studies using lineage-based immunogens failed to induced heterologous nAb titers in small animals and RMs (Malherbe et al. 2011; Malherbe et al. 2014). Ongoing studies using second-generation stabilized Env trimer immunogens may be more successful, but this remains to be seen.

The second germline-targeting approach is based on rationally designed immunogens that engage specific germline-precursors of mature bnAbs and has been pioneered for CD4 binding site directed bnAbs. This class of bnAbs is a convenient choice, as CD4 binding site directed bnAbs of the VRC01 class are derived from a very limited set of germline VH alleles (VH1-2 and VH1-46) and thus present a precise target for computational design. Two prototypical VRC01-class germline-targeting immunogens have emerged: 426c, developed by Stamatatos and colleagues (McGuire et al. 2013) and eOD GT6–GT8, developed by Schief and colleagues (Jardine et al. 2013). First studies using these immunogens have shown to effectively induce suitable VH1-2/5aa LCDR3 precursors in knock-in and humanized mouse models (Jardine et al. 2015; Sok et al. 2016a). However, sequential immunizations starting with a germline-targeted prime thus far failed to induce nAb titers to WT viruses with N276 glycans present. Immunogens to further guide maturation have been proposed and are underway (Briney et al. 2016). These may involve a new class of hybrid germline-targeting SOSIP trimers that show affinity to germline-precursor bnAbs in the context of a native trimer (Briney et al. 2016; Medina-Ramírez et al. 2017). Importantly, only a fraction of the mutations accumulated over years of affinity maturation is needed to effectively engage the CD4 binding site, underlining the feasibility of a rational vaccine design approach to this epitope cluster (Jardine et al. 2016b). Suitable VRC01-germline precursor B cells in naïve humans have been identified by sorting with eOD GT8 probes (Jardine et al. 2016a).

For other epitope clusters like the V2-apex and V3-glycan areas, computational design strategies have been harder to implement, partly because of incomplete epitope definition and party due to increased complexity of epitope clusters for which broad recognition involves direct glycan binding as compared to mere glycan accommodation. However, one such study using sequential immunogens designed to engage with and drive germline PGT121 maturation recently reported the induction of broadly neutralizing antibodies in germline–PGT121 knock-in mice, which represents an important proof-of-concept (Escolano et al. 2016; Steichen et al. 2016). Other recent studies repeatedly immunizing with high-mannose glycosylated gp140 or chemically synthesized Man_9-V3-peptides were able to induce V3-glycan directed antibodies in RMs (Saunders et al. 2017). However, like earlier approaches in small animals using high-mannose glycosylated yeast proteins (Zhang et al. 2015), such glycan-reactive antibodies generally fail to neutralize WT isolates that are not produced in the presence of kifunensine, i.e., have forced high-mannose glycosylation (Doores et al. 2010), and it remains unclear how this barrier can be overcome. A similar approach using synthesized strand C peptides bearing Man_5 and Man_3 glycans has been proposed for the elicitation of V2-apex

directed bnAbs (Alam et al. 2013), but more sophisticated immunization strategies using native trimers will likely be necessary for the induction of bnAbs to this quaternary epitope cluster.

4.3 Challenges an Approaches for the Design of Universal Influenza Vaccines

Heterosubtypic antibodies to influenza virus are less hypermutated than bnAbs to HIV and display a skewed VH region gene usage. The VH1-69 germline gene, found in heterosubtypic antibodies CR9114, CR6261, F10 and 1.12, encodes for hydrophobic residues Ile53 and Phe54, which, in the case of CR6261 and CR9114, interact with a hydrophobic pocket adjacent to helix A. The second most predominant class of heterosubtypic antibodies uses the VH3-30 germline gene for the heavy chain in combination with VK4-1 and Vk-1-12 light chains.

As it has been shown for some HIV bnAbs that fail to bind to recombinant Env, but were able to trigger B cell signaling when expressed as IgM on B cells (Ota et al. 2012), also HA-specific germline-reverted VH1-69 antibodies fail to bind to soluble HA at concentrations as high as 100 µg/ml (Lingwood et al. 2012). For VH1-69-encoded antibodies, a triad of residues in the heavy chain are key for breadth: two residues in HCDR2, i.e., a hydrophobic residue at position 53 and Phe54 interacting with one hydrophobic pocket, in combination with a properly positioned Tyr residue at position 98 $\pm$ 1 being inserted into an adjacent hydrophobic pockets. HnAbs can evolve from the VH1-69 germline with as little as one V-region substitution when combined with a properly positioned HCDR3 Tyrosine: Replacement of Ile 53 with a smaller small amino acid, such as Ser, Ala or Gly, allows the germline-encoded Phe 54 and the HCDR3 Tyrosine to be inserted into adjacent hydrophobic pockets and establish the key contacts (Avnir et al. 2014).

In contrast to HIV, development of a pan flu vaccine mostly involves common denominator immunizations (c.f, Fig. 7). As a matter of fact, the seasonal influenza vaccinations to some extent represent both, a cocktail and a sequential immunization strategy. Current influenza vaccines are tri- or quadrivalent and contain two or three IAVs (one H3N3, one or two H1N1) and one influenza B virus. The temporal modifications of the vaccine formulation due to antigenic shift also represents a sequential immunization strategy. While this strategy does not induce protective heterosubtypic immunity, i.e., people still get infected with shifted or drifted strains, there is evidence that (repetitive) vaccination indeed broadens the antibody response (Kohler et al. 2014; Henry Dunand et al. 2015). Experimentally, this approach has further been refined into an immunization strategy in which the relatively conserved stem has been grafted with globular HA heads derived from different IAV subtypes (Fig. 8, right). Using iterations of these grafted antigens, antibodies were induced that could bind different subtypes of HA, yet failed to

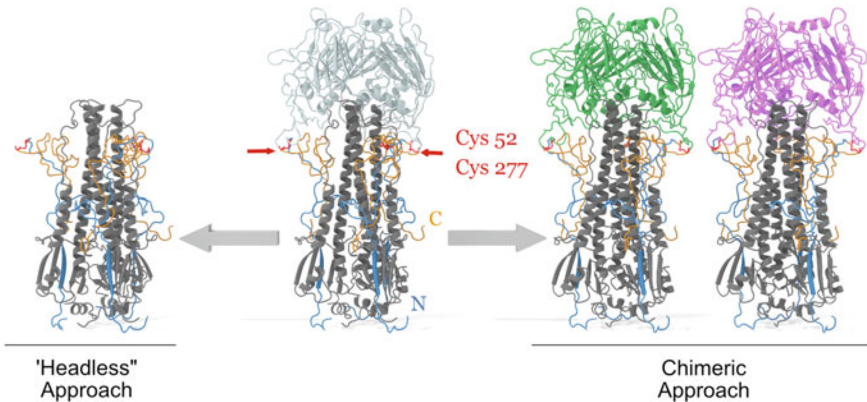


Fig. 8 Immunogen design strategies for eliciting heterosubtypic antibodies. The structure of the H3 HA from A/Aichi/2/1968 H3N2 (PDB 2HMG) is shown with HA2 in gray, and the globular head of HA1 in white. The N-terminal ascending strand of HA1 is depicted in blue and the descending C-terminal end of HA1 in orange, both are linked to each other by the indicated disulfide bridge. The two primary immunogen strategies are (i) 'headless' in which the apical globular portion of HA has been removed and (ii) the chimeric approach in which the globular HA1 head is exchanged with that of different isolates/subtypes (purple and green)

neutralize the corresponding viruses. Despite the lack of heterosubtypic neutralization, some protection could be induced in mice immunized with this strategy (Krammer et al. 2013). However, mice represent a suboptimal animal model for IAV, as protection from lethal infections is relatively easily achieved in mice.

Unlike HIV, where vaccinees are expected to be immunologically naïve, the majority of the recipients of a universal influenza virus vaccine will have preexisting immunity to influenza. This preexisting immunity imposes several challenges: First, there will be plenty of memory B cells specific for the immunodominant epitopes, and it will therefore be more challenging to avoid responses against unwanted immunodominant epitopes. Second, a phenomenon described as Original Antigenic Sin (de St Groth and Webster 1966; Gostic et al. 2016), may contribute another factor that possibly impairs the development of an universal vaccine to influenza virus: It describes the fact that immunization or infection with a new influenza variant rather boosts memory responses to the previous 'original' variants than priming responses to the new antigenic variant. Besides this classical Original Antigenic Sin, it has recently also been described that the first virus encountered will result in a livelong imprinting that favors immune responses to viruses belonging to the same phylogenetic group as the initially encountered virus (Kohler et al. 2014; Gostic et al. 2016). This imprinting, together with cross-reactivity of H3 mAbs to H7 (Henry Dunand et al. 2015), has been postulated to be responsible for the age-related differences in lethality following zoonotic infection with H5N1 and H7N9 HPAI viruses, in that older individuals that have first been exposed to H1N1-expressing viruses cope better with H5N1

infections (both phylogenetic group 1) while younger individuals, more likely to be exposed to H3N2 viruses, cope better with H7N9 zoonotic infections (both phylogenetic group 2) (Gostic et al. 2016).

Although the impact of original antigenic sin on the development of a universal vaccine to influenza is controversial, issues with preexisting immunity can only be avoided if a universal influenza virus vaccine is applied very early in life, and before the virus was encountered for the first time.

4.3.1 hnAb Engineering Approaches to Induce Heterosubtypic Antibodies

There are two major protein-engineering approaches to design immunogens capable of eliciting heterosubtypic antibodies to influenza viruses. These take advantage of a naturally occurring disulfide-bridge formed between Cys 52 and Cys 277, that links the ascending N-terminal with the descending C-terminal end of HA1 (Fig. 8).

The first approach, referred to as 'headless-HA' or 'mini-HA,' replaces the globular head domain with a short linker between these two cysteines. However, to prevent the meta-stable, spring-loaded fusion machinery contained in the stem from folding into the enthalpy-preferred post-fusion six-helix bundle, extensive stabilizing mutations have to be introduced in HA2 to preserve the pre-fusion conformation (Sagawa et al. 1996; Steel et al. 2010; Impagliazzo et al. 2015; van der Lubbe et al. 2018). This approach has the advantage that all of the variable head epitopes are missing but this comes at the expense that epitopes normally buried in the trimer become exposed. In case of HIV BG505 SOSIP.664 trimer immunization, for instance, the neo-epitopes on the bottom of the trimer have proven to be very highly immunogenic (Bianchi et al. 2018), and it will remain to be seen to which extent this will be an issue.

The second approach is a classical 'common denominator' strategy and takes advantage of chimeric proteins in which the stem is kept constant while the HA1 head is exchanged between immunizations (Krammer et al. 2013, 2014; Nachbagauer et al. 2016, 2017; Graham et al. 2019). Such chimeric HA proteins have been shown to display a more open conformation of the head, but this did not affect binding to HA-specific antibodies (Tran et al. 2016).

Both approaches were able to induce heterosubtyic nnAbs that provided protection in mice (Krammer et al. 2013, 2014; Nachbagauer et al. 2016) and ferrets (Nachbagauer et al. 2017) but so far none of these attempts succeeded at inducing heterosubtypic neutralization. The observed protection in mice is most likely mediated by non-neutralizing antibodies via FcγR effector functions. However, it is rather unlikely that similar protection by nnAbs will be achieved in humans, as most humans possess average ELISA titers of 1:1200 to heterologous human and 1:70–100 to nonhuman IAV subtypes (Kohler et al. 2014); yet most people are susceptible to seasonal (or zoonotic) influenza viruses.

4.3.2 Alternative Approaches to Induce Heterosubtypic Antibodies

Besides these two main strategies, several other approaches have been described. These include the removal of glycans (Liu et al. 2016), immunization with mRNA in liposomes (Pardi et al. 2018), antigen dilution (Kanekiyo et al. 2019), and expression of a HA/CD40L fusion protein by recombinant adenoviruses (Fan et al. 2014). The last approach has been the only one to report successful elicitation of heterosubtypic neutralization (Fan et al. 2014). Besides these approaches aiming for the HA surface protein, alternative strategies targeting the relatively conserved M2 protein ectodomain (Jegerlehner et al. 2004; Lo et al. 2008; Rao et al. 2010; Simhadri et al. 2015), and the nucleoprotein have been described (Lo et al. 2008; Rao et al. 2010), albeit with limited success.

Thus, for influenza, a number of strategies have been developed that successfully elicited antibodies that were able to recognize multiple subtypes by ELISA, and that could protect mice from lethal heterologous infections. However, with the exception of a single report, none of the published approaches successfully elicited heterosubtypic neutralization despite detection of stem-specific antibodies. It can be assumed that although these antibodies recognize conserved stem epitopes, their binding does not prevent the structural changes required for neutralizing activity. It is therefore likely that a refinement of the current common denominator strategies for influenza viruses with shepherding approaches, in order to guide the antibody fine-recognition from binding to neutralization, will be successful at eliciting heterosubtypic neutralization. Moreover, passive immunization experiments using stem-specific heterosubtypic antibodies indicate that these antibodies heavily rely on Fc-effector functions for protection, suggesting that the isotype profile of the vaccine response may be of importance for such an IAV vaccine (DiLillo et al. 2014, 2016; Asthagiri Arunkumar et al. 2019; Vanderven and Kent 2020).

In contrast to HIV, where the germline-encoded human antibody repertoire appears to be ill-equipped to recognize many bnAb epitopes, it was found that the presence of the human VH1-69 germline gene in combination with the human D and J elements was sufficient to enable transgenic mice to generate stem-specific antibodies (Sangesland et al. 2019), it therefore might not be necessary to design germline-trargeting immunogens. However, since access to stem-specific epitopes might be restricted on the densely packed surface of influenza virion (Fig. 2), we tested the influence of this possible restraint in an animal experiment (Bianchi, Hangartner, unpublished): in order to expose the conserved stem epitopes more prominently while at the same time occluding access to the variable head, recombinant HA protein was teathered head-down to magnetite beads that were then used for immunization. Two HA proteins were chosen for this approach: first, HA from A/Japan/305/1957 (H2N2), as a representative of phylogenetic group 1, and because of its antigenic properties. Both inverted proteins were very efficient at isolating hnAbs by phage display, including pan-IAV neutralizing mAb 1.12 that was isolated from a normal donor (Wyrzucki et al. 2014, 2015). In contrast to H1N1, where five distinct antigenic sites on the HA globular head have been identified (Gerhard et al. 1981; Caton et al. 1982), and H3N2 IAVs (Wiley et al. 1981; Daniels et al. 1983), H2N2

viruses were shown to have an additional sixth antigenic domain in the stem region (Okuno et al. 1993; Tsuchiya et al. 2001). Therefore, as a second immunogen, HA from A/FPV/Bratislava/1979 (H7N7) was selected, as it belongs to phylogenetic group 2, and also due to its evolutionary distance to the circulating human H3 subtype. Moreover, both H2 and H7 represent a current public health threat. However, although the inverted immunogens readily produced cross-reactive and cross-protective antibodies in mice, they failed to induce heterosubtypic neutralization (Bianchi, Hangartner, unpublished). Hence, although epitope-geography appears to play a role, by itself, it fails to explain immunoquiescence of the hnAb stem epitope.

5 Conclusions

Although immense progress has been made over the past years, universal vaccines to highly variable viruses are still out of reach for now. The primary problems identified are the poor immunogenicity of the shared and conserved epitopes, the immunodominance of undesired variable epitopes, as well as difficulties with constructing stable immunogens that faithfully mimic viral spikes proteins immunologically. Meanwhile, technologies have been developed to not only express stable mimetics of the viral spike proteins, but also to modify them, in order to better engage germline-encoded bnAb/hnAb precursor B cells, or even to generate dedicated antigens designed to best engage germline lineages of broadly neutralizing antibodies. The latter appears to be necessary, as the human germline-encoded B cell repertoire struggles with the recognition of these epitopes. These difficulties reflect the viral evolution to avoid responses to the viral 'Archilles' heel' of the respective surface proteins. The underlying molecular mechanisms for the different immunogenicities of the various epitopes are complex and not yet well understood.

Current research and clinical trials employing both common denominator and sheparding approaches, as well as unmodified spike proteins, however, will provide valuable insight into these issues and will help to inform iterative development of diverse vaccination approaches and immunogens. Almost as a side product, these efforts are also providing profound and fundamental insights into B cell biology and humoral antibody responses more generally along the way.

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