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Influence of glycosylation on the immunogenicity and antigenicity of viral immunogens

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ABSTRACT

A key aspect of successful viral vaccine design is the elicitation of neutralizing antibodies targeting viral attachment and fusion glycoproteins that embellish viral particles. This observation has catalyzed the development of numerous viral glycoprotein mimetics as vaccines. Glycans can dominate the surface of viral glycoproteins and as such, the viral glycome can influence the antigenicity and immunogenicity of a candidate vaccine. In one extreme, glycans can form an integral part of epitopes targeted by neutralizing antibodies and are therefore considered to be an important feature of key immunogens within an immunization regimen. In the other extreme, the existence of peptide and bacterially expressed protein vaccines shows that viral glycosylation can be dispensable in some cases. However, native-like glycosylation can indicate native-like protein folding and the presence of conformational epitopes. Furthermore, going beyond native glycan mimicry, in either occupancy of glycosylation sites or the glycan processing state, may offer opportunities for enhancing the immunogenicity and associated protection elicited by an immunogen. Here, we review key determinants of viral glycosylation and how recombinant immunogens can recapitulate these signatures across a range of enveloped viruses, including HIV-1, Ebola virus, SARS-CoV-2, Influenza and Lassa virus. The emerging understanding of immunogen glycosylation and its control will help guide the development of future vaccines in both recombinant protein- and nucleic acid-based vaccine technologies.

1. Introduction

Enveloped viruses are the etiological agent of a multitude of acute and chronic diseases. Effective vaccines can work by eliciting a sterilizing immune response capable of neutralizing the virus before infection is established. In addition, vaccines can enhance the cellular immune response to augment viral clearance. The advent of recombinant protein technologies has enabled a focus on immunogens based on viral spike attachment and fusion proteins (Caradonna and Schmidt, 2021). This approach is supported by the observation that highly potent neutralizing antibodies (nAbs) target these glycoprotein complexes (Murin et al., 2019).

Viruses are completely dependent on the molecular machinery of the host to replicate. This includes, but is not limited to, the translational machinery, post-translational modifications and protein folding chaperones. Asparagine (N)-linked glycosylation plays key roles in both the physicochemical stabilization of protein folds and in facilitating the interaction of a nascent protein with disulphide bond isomerases and

Abbreviations: bnAb, broadly neutralizing antibody; CDR, complementarity determining region; CHO, Chinese ovary cells; CNX, calnexin; CRT, calreticulin; DENV, Dengue virus; E, Envelope protein; EBOV, Ebola virus; EMPEM, electron microscopy polyclonal epitope mapping; Env, Envelope glycoprotein; ER, endoplasmic reticulum; ERAD, ER-associated degradation; FP, fusion peptide; Fuc, fucose; FUT8, Fucosyltransferase 8; Gal, galactose; GC, Germinal center; GL, germline precursor; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GnTI, *N*-acetylglucosaminyltransferase; GPC, glycoprotein complex; GPI, glycosyl phosphatidylinositol; GT, germline targeting; HA, haemagglutinin; HCV, Hepatitis C virus; HIV-1, Human immuno deficiency virus 1; HLA, Human leukocyte antigen; HR, heptad repeat; IAV, Influenza A virus; IMP, intrinsic mannose patch; KO, knock-out; LAMP-1, lysosomal-associated membrane protein 1; LASV, Lassa virus; LRT, lower respiratory tract; Man, mannose; MBL, mannose binding lectin; MD, molecular dynamics; MLD, mucin-like domain; MPER, membrane-proximal external region; nAb, neutralizing antibody; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; NFL, natively flexibly linked; NP, nanoparticle; OST, oligosaccharyltransferase; PNGS, potential N-linked glycosylation site; RBD, receptor binding domain; S, spike glycoprotein; SHM, somatic hyper mutation; SP, signal peptide; TAMP, trimer associated mannose patch; TF, transmitted founder; Tfh, T follicular helper; TMD, transmembrane domain; UDP, Uridine diphosphate; UFO, uncleaved prefusion-optimized; UGG7, UDP-glucose:glycoprotein glucosyltransferase; URT, upper respiratory tract; V loop, variable loop; VLP, virus-like particle; ZIKV, Zika virus.

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chaperones (Lamriben et al., 2016; Mitra et al., 2006; Shental-Bechor and Levy, 2008; Tannous et al., 2015; Wormald and Dwek, 1999). For this reason, whether in the context of a viral glycoprotein or a recombinant mimetic, glycosylation is usually an important feature for native-like folding.

In many cases, the role of host-derived glycans on viral glycoproteins extends past the glycan-mediated folding and physicochemical stabilization of native protein architecture (Bagdonaite and Wandall, 2018; Watanabe et al., 2019). The limited immunogenic nature of host-derived glycans underpins their role in viral protein epitope masking (Scanlan et al., 2007). Under selective pressure from the host's humoral immune system, many viruses genetically encode N-linked glycan sites to thwart B- and T-cell mediated neutralizing responses (Fischer et al., 2021; Zhang, 2004). By restricting the proteinaceous area that can be readily targeted by nAbs, the ability of the immune system to recognize the viral spike becomes restricted (Wei et al., 2003). In order to overcome epitope shielding by N-linked glycans, B-cells undergo extensive rounds of somatic hypermutation (SHM) to either entirely avoid, directly bind or accommodate glycans as part of a larger glycoprotein epitope (Bonsignori et al., 2017; Crispin et al., 2018; Saunders et al., 2019; Sok et al., 2013). Additionally, glycopeptides containing N-linked glycan attachment sites have been shown to be presented on MHC HLA class II molecules (Parker et al., 2021; Sun et al., 2020). The impact of glycosylation both in contributing to MHC epitopes and presumably, in many cases, impeding peptide presentation underscores the role of glycosylation in shaping T-cell mediated immunity.

Changes in viral glycan shield density often come hand in hand with changes in nAb sensitivity (Wei et al., 2003). Viral glycan shield composition can therefore be a consideration for rational immunogen design with the goal of inducing focused, protective neutralizing B- and T-cell responses. For example, efforts to design an effective HIV-1 vaccine have proved extremely challenging in part due to the extensive glycan network that shields the viral envelope (Env) glycoprotein (Deimel et al., 2022; Seabright et al., 2019; Stewart-Jones et al., 2016). The importance of N-linked glycans can also be observed on Influenza haemagglutinin (HA), whereby selective pressure from the long-term endemic circulation of H3N2 has caused the accumulation of new glycan sites (Altman et al., 2019; Lee et al., 2021b; Skehel et al., 1984). Here, we discuss viral glycosylation in the context of vaccine design and review the importance of maintaining native antigen mimicry and discuss incidences where mimicry is not required, and where it can be beneficial to go beyond mimicry alone.

1.1. Overview of mammalian N-linked glycosylation

As obligate parasites, viruses frequently exploit the host glycosylation pathway for a number of functions that extend across the viral life-cycle, from virion assembly, infectivity, and modulating the interaction with host innate and adaptive immune responses (Bagdonaite and Wandall, 2018; Watanabe et al., 2019). Here, we principally consider N-linked glycosylation that occurs on asparagine residues within the consensus Asn-X-Ser/Thr-X (N-X-S/T-X), where X ≠ Pro (P) (Nita-Lazar et al., 2005). The resultant glycans project into the solvent but can also exhibit interactions with the protein surface and thereby impart thermodynamic stability and solubility (Petrescu, 2003; Solá and Griebenow, 2009; Wormald and Dwek, 1999). Whether a glycosylation site is occupied by a glycan or not, and how the glycan is enzymatically matured during glycoprotein secretion, is an important factor in understanding how the glycoprotein interacts and stimulates the host immune system both in the context of natural infection and in the context of immunization (Bagdonaite and Wandall, 2018; Loke et al., 2016; Watanabe et al., 2019).

The process of N-linked glycosylation begins with the co-translational attachment of a preformed glycan precursor, $Glc_3Man_9GlcNAc_2$, by oligosaccharyltransferase (OST) in the endoplasmic reticulum (ER) lumen (Kornfeld and Kornfeld, 1985). Accumulating evidence has deduced the mechanistically distinct functions of OST isoforms, STT3A and STT3B, and the importance of isoform synergy in maximizing N-linked glycosylation efficiency (Harada et al., 2019). Nascent polypeptide folding begins almost immediately as it enters the ER lumen during protein translation. For N-linked glycosylation sequons to become efficiently glycosylated, STT3A associates with the translocon to facilitate the co-translational attachment of N-glycan precursors as these sites become available (Gemmer et al., 2023; Lu et al., 2018), while STT3B acts in a post-translational manner (Sato et al., 2012). However, in regions containing a high density of potential N-linked glycosylation sites (PNGS) (*e.g.*, N-X-S/T-N-X-S/T), STT3A displays a bias towards particular glycan attachment sites which often leads to sequon skipping (Malaby and Kobertz, 2014; Nita-Lazar et al., 2005). This preferential glycosylation efficiency is influenced by the chemical characteristics of second position (X) and consensus site hydroxyl (Ser/Thr) residues (Gavel and von Heijne, 1990; Kasturi et al., 1995; Malaby and Kobertz, 2014). Large hydrophobic and negatively charged residues at the X position comparably reduce glycosylation efficiency relative to small polar residues. This vulnerability is significantly pronounced within N-X-S sequons, which also complements claims that the presence of an additional methyl group in N-X-T contributes to higher glycosylation efficiency compared with N-X-S (Gavel and von Heijne, 1990).

Sequon skipping has been observed on the SARS-CoV-2 spike (S) glycoprotein (Newby et al., 2023; Pegg et al., 2023; Sabyasachi Baboo et al., 2023; Shajahan et al., 2023; Wang et al., 2023). The N17 site, which is conserved across most viral lineages and displays high glycan occupancy, is not preferentially glycosylated on the Gamma S protein. A novel PNGS is introduced by a T20N mutation is shown to be favorably glycosylated over N17, which may be attributed to the nature of the side chain of the second amino acid residue within the N-X-S/T glycan sequon (Newby et al., 2023).

Following the attachment of $Glc_3Man_9GlcNAc_2$ by OST, the terminal glucose residues can be hydrolyzed by α-glucosidase I and II. Misfolded glycoproteins are a substrate for UDP-glucose glycoprotein glucosyltransferase (UGG1) which generates a monoglucosylated structure targeted by the chaperones, calnexin (CNX) and calreticulin (CRT) (Lamriben et al., 2016; Modenutti et al., 2021). The CNX-CRT cycle consequently acts as a folding checkpoint retaining and retrieving misfolding glycoproteins within the ER for further folding or eventual ER-associated degradation (ERAD) (Hauri et al., 2000; Moremen and Molinari, 2006; Parodi, 2000). Liberation from this folding checkpoint is triggered following glucose hydrolysis, and when correct disulphide bond formation means that reglucosylation is not triggered (Kozlov and Gehring, 2020). Since native viral protein folding is essential for receptor binding, and that viral glycoproteins extensively utilize the CNX-CRT folding pathways, certain sugar mimetics (namely iminosugars) have been reviewed for their antiviral activity (Stohrer and Hunter, 1979). By impeding ER α-glucosidase activity, iminosugars are capable of generating misfolded, dysfunctional viral glycoproteins, which exhibit reduced infectivity (Alonzi et al., 2017; Miller et al., 2018; Warfield et al., 2020). Iminosugars continue to be investigated as antivirals and they may find utility particularly in the immediate response to emerging pathogens (Dwek et al., 2022; Robinson et al., 2022).

In parallel to protein folding, a single mannose residue is cleaved from $Man_9GlcNAc_2$ by ER α-mannosidase I to yield $Man_8GlcNAc_2$. The resultant glycoprotein, when not displaying glucosylated structures, is shuttled into the *cis*-Golgi apparatus using COPII-mediated anterograde transport, and Golgi-resident mannosidases commence sequential mannose trimming (Brandizzi and Barlowe, 2013). The product of this trimming sequence, $Man_5GlcNAc_2$, is then subjected to *N*-acetylglucosaminyltransferase (GnTI), generating the $GlcNAc_1Man_5GlcNAc_2$ intermediate, which facilitates the heterogenous diversification of glycan processing. The dynamic and competitive nature of *trans*-Golgi glycan processing enzymes gives rise to a diverse pool of glycoforms at a single site, each varying in functional characteristics, and can be grouped into

hybrid- or complex-type glycans. Complex-type N-glycans are generated through the action of α-mannosidase II, followed by a range of enzymes which can include galactosyl-, sialyl-, *N*-acetylglucosaminyl- and fucosyl-transferases, which produce a diverse assortment of glycans. Hybrid-type N-glycans are so-called as they contain an underlying oligomannose-type structure (typically $Man_5GlcNAc_2$), arising through the absence of α-mannosidase II trimming, together with 'complex-type' processing following modification by GnTI and potentially others, and may contain galactose, sialic acid and/or fucose. The exact final processing state of an N-linked glycan is dictated by many factors, including the expression level of glycosyltransferases, the metabolome of the cell, disease state, and steric accessibility of a glycan site to the glycan processing enzymes (Alley et al., 2012; Briggs et al., 2019; Fisher et al., 2019; Freeze and Sharma, 2010; Grigorian et al., 2007; Hossler et al., 2009; Hossler et al., 2007; Rudd et al., 2022; Rudd and Dwek, 1997).

2. Factors shaping viral glycosylation

Many viruses utilize the N-linked glycosylation pathway for a diverse number of applications, including, but not limited to, enhancing native protein folding, protein epitope shielding, and receptor binding (Bagdonaite and Wandall, 2018; Watanabe et al., 2019). However, distinct characteristics of viral glycans that differ to those observed on healthy mammalian proteins can support their use as nAb epitopes, which may be important to recapitulate on viral immunogens (Scanlan et al., 2007). Many studies have detailed the upregulation of oligomannose-type glycans on both soluble- and viral surface glycoproteins relative to serum and cell-surface glycoproteins (Paulson, 1989; Taylor and Drickamer, 2019). To this end, primitive oligomannose glycans can act as ligands for endogenous lectins implicated in pro-inflammatory innate immune responses (Helenius and Aebi, 2001; Marth and Grewal, 2008; Pinho et al., 2023; Tokatlian et al., 2019). As such, Golgi-mediated maturation of these underprocessed glycans on healthy mammalian glycoproteins is favorable in order to subvert such harmful immune responses. Further, the presence of $Man_8GlcNAc_2$ glycans can allude to an emergence of misfolded protein and acts as a signal for ERAD (Liu et al., 2016; Patel et al., 2020; Szathmary et al., 2005). The divergence of viral glycan processing can be exemplified by the fact that viral glycoproteins are often decorated in underprocessed oligomannose glycans, which can serve to enhance cellular entry mediated by lectin recognition and establish viral infection (Brown et al., 2010; Chang et al., 2010; Fuchs et al., 2010; Miller et al., 2021; Spear et al., 2000).

An additional consideration during immunogen design may be the conservation or removal of glycan signatures that diverge from human glycan processing. Animal-borne viruses can present carbohydrate moieties that deviate from those within the human glycan repertoire, while insect-borne viruses can present pauci-mannosidic structures (Broszeit et al., 2019; Crispin et al., 2014; Hacker et al., 2009; Hasan et al., 2018; Routhu et al., 2019; Spruit et al., 2022; Suzuki et al., 1997). Non-human monosaccharides, including *N*-glycolylneuraminic acid (Neu5Gc), and non-human linkages, such as α1–3-galactose (Gal) and α1–3-fucose (Fuc), can be highly immunogenic and may therefore be disadvantageous if abundant on vaccine candidates (Crispell et al., 2019; Mastrangeli et al., 2021; Rendić et al., 2007).

Despite this, considerable research and development efforts have supported the use of non-human mammalian cell lines in biopharmaceutical contexts, namely Chinese hamster ovary (CHO) cells, for the production of human biologics. CHO cells are the predominant producer cell line for the generation of ~70% of human biologics, including viral vaccines and monoclonal antibodies, and incorporate non-human monosaccharides and corresponding linkages at very low levels (<2%) (Bosques et al., 2010; Lalonde and Durocher, 2017; Tihanyi and Nyitray, 2020). In contrast, there has been a substantial lag in the utilization of humanized insect cell lines for the same purpose despite the ability to express high levels of recombinant protein at low cost, with favorable immunogenicity profiles and wider epitope recognition in some cases (Kong et al., 2010). Popular insect cell lines, including S2, Sf9, High Five, and others, encode α1–3 fucosyltransferases that generate immunogenic core fucose linkages (Palomares et al., 2018; Yee et al., 2018). Genetic ablation of these fucosyltransferases will likely favorably influence the immunogenicity of recombinant material expressed using this system.

In contrast, the incorporation of MERS-CoV S protein and EBOV GP into virus-like particles (VLP) expressed in Sf9 cells have been shown to induce robust, protective nAb and cell-mediated responses, comparable with mammalian cell-expressed material, despite the differences in glycosylation. A Sf9-expressed subunit nanoparticle (NP) vaccine against SARS-CoV-2 (NVX-CoV2373) was licensed by the FDA in July 2021, which displayed 90.4% efficacy against the Wuhan-hu-1 strain in a phase 3 clinical trial (Dunkle et al., 2022; Tian et al., 2021). This highlights the potential of insect cell lines to be utilized for the manufacture of viral vaccines. Although, the exploitation of engineered insect cell lines for the production of viral immunogens currently falls behind the widespread utilization of mammalian cell lines. Perhaps the balance between overall cost of production, protein yields and induction of favorable post-translational modifications supports the use of mammalian cell lines over those of insect origin.

2.1. Structural inhibition of glycan processing

The processing state of N-linked glycans is interdependent on the local 3D protein architecture (Rudd and Dwek, 1997; Wormald and Dwek, 1999). The fine structure of glycans can therefore be used as an indicator of native-like protein folding and can be sensitive to oligomeric protein state (Behrens et al., 2017) and even the presence of neighboring glycans (Seabright et al., 2020). Oligomannose-type glycans are not commonly presented on mammalian glycoproteins (Clerc et al., 2016; Crispin et al., 2004; Kawahara et al., 2021; Moremen and Molinari, 2006; Paulson, 1989). However, these underprocessed glycoforms can become elevated in states of cellular stress, disease states, limited substrate availability and through steric hinderance (Briggs et al., 2019; Kuribara and Totani, 2022; Loke et al., 2016; Mittal et al., 2021; Radcliffe et al., 2007). The influence of steric hinderance to viral glycan processing is broadly observed across many different trimeric viral glycoproteins, such as SARS-CoV-2, HIV-1, Influenza HA and LASV GPC. As ER α-mannosidase I is sensitive to local 3D protein architecture, the extent of glycan processing can serve as a native protein folding indicator during immunogen design (Fig. 1A) (Lee et al., 2021b; Pritchard et al., 2015b; Watanabe et al., 2020; Watanabe et al., 2018).

The effect on glycan processing by a neighboring protomer is exemplified in the case of SARS-CoV-2. Trimeric association of SARS-CoV-2 S induces a quaternary-structure-dependent oligomannose signature at N234 (Allen et al., 2021; Wang et al., 2020). Molecular dynamics (MD) simulations have attributed the limited glycan processing at this site to low accessible surface area through the association of protomers (Allen et al., 2021). Revealed by mass spectrometry analysis, monomeric expression of the S1 subunit enhances N234 glycan processing to predominantly complex-type, thereby corroborating this quaternary structural influence on glycan processing (Wang et al., 2020). The impact of quaternary architecture on glycan processing is further illustrated by the differential glycosylation of receptor binding domain (RBD) expressed in isolation *versus* in the context of trimeric S protein. In isolation, RBD domains exhibit enhanced glycan processing and even show the presence of O-linked glycosylation which is of very low abundance, or even absent, when the RBD is expressed in a more native, trimeric context (Eldrid et al., 2021). This observation of elevated aberrant O-glycosylation parallels that seen in the recombinant expression of HIV-1 gp120 (Behrens et al., 2017).

Similarly, the clustering of glycans can impact their processing state. One such example is the micro clustering of glycans on the outer domain of HIV-1 Env (Seabright et al., 2020). By thwarting α-mannosidase catalysis, neighboring glycans can be stalled at the oligomannose-type

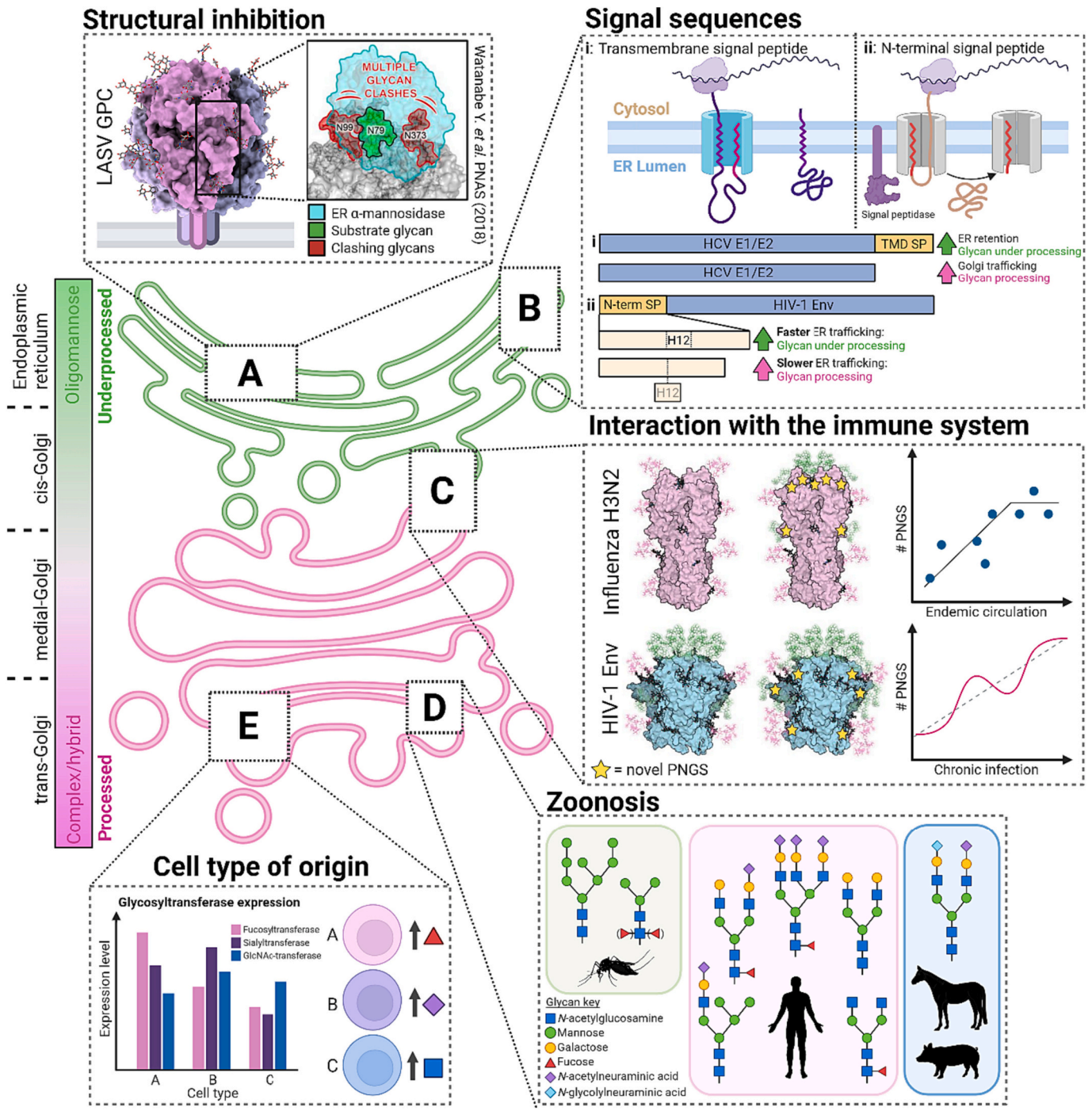

Fig. 1. Factors shaping viral glycosylation. A) Structural inhibition of glycan processing can induce elevated levels of oligomannose-type glycans. B) The rate of ER-Golgi trafficking and localization of viral glycoproteins can be impacted by N- or C-terminal signal sequences. C) The number of glycan sites across viral glycoproteins can change in response to consistent exposure to the host immune system. D) Viral glycan processing and composition can differ depending on the viral host. E) The differential glycosyltransferase expression in different cell types can result in differential glycan processing. Schematics of the factors influencing viral glycosylation are arranged across the ER/Golgi trafficking pathway according to the extent of glycan processing that they are each most closely associated with, across a scale of least- to most-processed (oligomannose-to-complex).

(Behrens et al., 2017). The density of glycans is such that recombinant gp120 exhibits a so-called intrinsic mannose patch (IMP). An elevation in the abundance of oligomannose-type is further observed in trimeric versions of the spike where quaternary restraints induce a trimer associated mannose patch (TAMP), which encompasses glycan sites at the apex and other inter-subunit boundaries (Behrens et al., 2017; Pritchard et al., 2015b). The presentation of conformationally-sensitive glycan epitopes are important indicators of native-like folding and oligomeric association and, as such, have aided the development of native-like viral immunogens and will be further discussed. A similar effect can be observed on the HA region of H3N2 Influenza strains (Lee et al., 2021b). The acquisition of PNGSs is a likely response to sustained immune pressure, which has led to underprocessed oligomannose-type glycan signatures through steric glycan-glycan clashes, of which some are only present in a trimeric form (An et al., 2015; Lee et al., 2021b).

The steric factors shaping viral glycosylation are also well illustrated

by the Lassa virus (LASV) glycoprotein complex (GPC). The extensive glycan network resembles that of HIV-1 Env through oligomannose clustering at the subunit interface (GP1/GP2) (Fig. 1A) (Watanabe et al., 2018). Furthermore, oligomannose signatures at N79, N89, N99, N365 and N373 likely arise due to a steric restriction of glycan processing by ER α-mannosidases as a result of glycan-glycan and glycan-protein clashes, and provide useful insight into native structure formation (Watanabe et al., 2018). The glycan signatures observed in full-length glycoprotein from pseudoviruses (Watanabe et al., 2018) have been shown to be largely recapitulated in recombinant immunogens (Brouwer et al., 2022; Perrett et al., 2022). Monitoring of glycosylation thereby provides a useful measure of native-like folding (Brouwer et al., 2022).

The consequences of the induction of oligomannose-type glycans as a result of steric clashes between glycan processing enzymes and their target likely extend beyond antibody-based recognition (Parker et al., 2021; Sun et al., 2020). Parker et al. investigated the immunopeptidome of the SARS-CoV-2 S protein and demonstrated that glycopeptides are presented by antigen-presenting cells (Parker et al., 2021). When the glycan composition of the glycopeptides being presented were investigated, there was extensive glycan truncation evident, with paucimannose glycans present at many sites. Interestingly, sites that present a high abundance of oligomannose-type glycans on the viral spike, such as N801, appear to be protected from glycan truncation during antigen presentation, and the corresponding glycopeptide contains an identical glycoform to pre-processed S protein. This demonstrates the importance in preserving the steric network of the glycan shield of viral spike glycoproteins beyond B-cell responses alone, and glycopeptide T-cell epitopes should also be considered.

2.2. Zoonosis

Many viruses adopt adaptive mechanisms that allow for interspecies transmission. The ramifications of zoonotic viral endemics are vast, with considerable worldwide mortality burdens and socio-economic implications (Rahman et al., 2020). There are many molecular influences that contribute to viral zoonoses, including interspecies receptor similarity, evasion of pre-existing immunity, and virus-glycan interactions (Bolles et al., 2011; Ciminski et al., 2021). The latter contribution is largely implicated in Influenza A and flaviviruses and will be the focus of this section.

Due to the glycan-binding nature of Influenza A virus (IAV) HA, IAV has a broad zoonotic host repertoire (Ciminski et al., 2021). The capacity for HA to bind to terminal sialic acid (Neu5Ac) on the surface of lung epithelial cells is the key determinant for host specificity and interspecies transfer of IAV from avian, swine, and other animal hosts to humans. Notably, different HA strains display variable preferences for α2–3 and α2–6-linked Neu5Ac moieties (Rogers and Paulson, 1983; Zhao and Pu, 2022). These Neu5Ac linkages are differentially distributed in the human respiratory tract – α2–6-linkages are more prevalent in the upper respiratory tract (URT) whereas α2–3 linkages are the predominant linkage of the lower respiratory tract (LRT) (Kumlin et al., 2008; Nicholls et al., 2007). Additionally, Neu5Gc, which is not expressed in human epithelial cells, is a substrate for IAV neuraminidase, further impacting the host range of IAVs (Fig. 1D) (Broszeit et al., 2019; Nemanichvili et al., 2022; Spruit et al., 2022; Zhao and Pu, 2022).

Dengue (DENV) and Zika (ZIKV) virus are closely related flaviviruses that exploit *Aedes* mosquitoes as vectors for human infection. The mammalian and insect N-linked glycosylation pathways are starkly dissimilar. Displaying overlap exclusively amongst the early mammalian glycan processes in the ER, an abundance of oligomannosidic and paucimannosidic glycoforms are presented on mature insect-derived proteins (Fig. 1D) (Hacker et al., 2009). Containing only two PNGS across the envelope (E) protein of DENV (N67 and N153), and one PNGS in ZIKV (N154), it is clear that these glycan sites do not comprise a glycan shield. Instead, these glycan sites enhance physicochemical stabilization of the native-like protein structure and, crucially, facilitate the process of zoonotic transfer to humans through the recruitment of C-type lectins (Liu et al., 2014; Miller et al., 2008; Navarro-Sanchez et al., 2003). The induction of oligomannose-type structure by protein-directed steric effects will mean that these continue to be presented after infection has been established in the human host. However, in other examples where mannose-terminating structures are only present due to the cellular origin, there will be a switch in glycan-mediated immune interactions once virions are produced by the new host (Crispin et al., 2014). Elucidating the varied presentation of glycans throughout zoonosis will aid in understanding immune responses to viral antigens.

2.3. Cell type of origin

The orchestration of glycosylation involves a complex metabolic system in which the glycan processing state is dependent on donor substrate availability, glycosyltransferase expression and availability, inflammatory state, and many more determinants (Fig. 1E) (Moremen et al., 2012; Scheper et al., 2023; Schjoldager et al., 2020). As such, the resultant glycome varies between individual cells, with more elevated differences observed between different cell types. In addition, viral infection can transiently alter the cellular glycome through influencing cellular metabolism, highlighting the interconnection between the glycome and the metabolome. Rao and Suvas highlight the upregulation of glycolysis as a result of herpes simplex virus infection (Rao and Suvas, 2019). Enhanced glycolysis has been shown to result in increased substrate availability for glycan biosynthesis, which results in the presentation of glycans that display enhanced N-linked glycan branching. As a result of the constant variation in the glycome of the cell, understanding the glycosylation of viruses in the complex context of natural disease has proved analytically challenging. However, an example of the importance of cell-specific glycosylation is given by the differential susceptibility to antibody-mediated neutralization of HIV-1 derived from infected macrophages and primary T-lymphocyte cultures (Willey et al., 1996). Macrophages exhibited extended polylactosamine structures which imparted reduced neutralization sensitivity. Additionally, differential binding of HIV-1^{+} serum IgG was observed against monomeric gp120 expressed in various cell lines (Raska et al., 2010). These observations underscore that glycan variation should be considered when evaluating the immune response to candidate vaccines and may compound differences in neutralization potency between *in vitro* and *in vivo* experiments. These observations also stimulate interest in understanding the diversity of glycosylation amongst virions that lead to infection, for example, those of HIV-1 circulating in blood and those found in semen (Houzet et al., 2014).

Cell type-specific glycosylation is also pertinent when considering the *in vivo* production of immunogen following inoculation with RNA-based and adenoviral-based immunogens (Brun et al., 2021; Ozdilek and Avci, 2022; Watanabe et al., 2021). In one extreme, non-native glycosylation of a bacterial immunogen expressed *via* RNA transfected mammalian cells had reduced immunogenicity due to artifactual modification of N-glycosylation sequons present within the sequence (Ozdilek and Avci, 2022). In the other, natively glycosylated antigen may exhibit diverse glycosylation depending on the precise cell type at the site of RNA or adenovirus administration (Brun et al., 2021; Watanabe et al., 2021). This could emerge of particular importance in HIV-1 immunogen design given the interest in presenting glycan-based bnAb epitopes.

It is challenging for current analytics to measure the glycome of a single cell, but there have been substantial advancements in single cell transcriptomics that allow for a better understanding of the underpinnings that contribute to glycan diversity. Dworkin et al. have proposed a transcriptional atlas of the glycosylation capacity at an organ level and at a single-cell level using screens of multiple human and mouse large intestine and kidney cell types (Dworkin et al., 2022). It is well-known that the relationship between gene transcript expression

and protein expression is non-linear and is extremely diverse between tissues and cell types and adaptable depending on many environmental factors. This transcriptional atlas of glycosylation is a step in the right direction to understand the cell-type determinants of glycan diversity. However, it is likely that parallel advances in understanding the precise mapping of virus production and in glycoproteomics of low abundance material will be important.

A further contribution to cell type specific glycosylation is the intrinsic viral tropism. For example, lung epithelial cells and dendritic cells are associated with high levels of cell surface-expressed terminal a2,3- and a2,6-linked sialic acid, which act as a determinant for Influenza A infection (Cabral et al., 2010), and can facilitate HIV-1 infection (Izquierdo-Useros et al., 2012; Puryear et al., 2012).

2.4. Viral signal peptide sequences

There has been a growing body of work exploring the impact of viral signal sequences on viral protein glycosylation (Ciczora et al., 2010; Cocquerel et al., 1999; Cocquerel et al., 1998; Upadhyay et al., 2020; Yolitz et al., 2018). Like most membrane-bound and secreted proteins, viral attachment proteins possess an N-terminal signal peptide (SP) that directs the nascent polypeptide to the ER. The amino acid composition of the transmembrane region of viral attachment proteins can, in some cases, act as a signal peptide to promote static ER retention, thereby restricting glycan processing and guiding viral budding (Fig. 1B) (Ciczora et al., 2010; Cocquerel et al., 1999; Cocquerel et al., 1998; Hsieh et al., 2008; Mu et al., 2021). Additionally, the genetic basis of these signal peptides can dictate the rate at which the glycoprotein undergoes maturation (Asmal et al., 2011; Eichler et al., 2003; Yolitz et al., 2018). As such, there appears to be an interplay between glycan processing and signal peptide sequence composition that extends beyond canonical ER-Golgi cargo cycling, which may support viral pathogenesis and immune evasion tactics (Hauri et al., 2000; Robinson et al., 2018). As both signal sequences exhibit a strong influence over ER localization of viral glycoproteins, the likelihood of viral glycosylation becoming impacted is high.

An example where the SP appears to influence viral glycosylation is given by hepatitis C virus (HCV). The envelope glycoprotein complex (E1E2) is extensively coated in glycans (5 PNGS in E1 and 12 in E2), which support native protein folding and heterodimer association as well as mask antigenic protein epitopes targeted by nAbs (Falkowska et al., 2007; Goffard et al., 2007; Goffard et al., 2005; Helle et al., 2007; Meunier et al., 1999). Glycosylation analysis of pseudoviral- and soluble recombinant E1E2 has revealed an interesting disparity in glycan processing. Native-like viral and pseudoviral material almost exclusively present underprocessed oligomannose-type glycans at all occupied sites, while a near-complete shift in glycan processing is observed on soluble recombinant material (Sliepen et al., 2022; Torrents de la Peña et al., 2022). These observations corroborate the concept that the transmembrane domains (TMD) of the E1E2 complex may influence ER-Golgi trafficking (Fig. 1B(i)). The mechanism by which this effect occurs can be inferred through the extent of susceptibility of E1E2 glycans to Golgi-resident glycosidases and glycosyltransferases.

To investigate the sequence specificity that dictates ER-Golgi trafficking of membrane-bound E1E2, Cocquerel et al. (1998) exchanged the TMD of HCV E2 for that of CD4, a cell-surface glycoprotein, and glycosylphosphatidylinositol (GPI) (Cocquerel et al., 1998). Using Endo H sensitivity to impart extent of ER-Golgi trafficking, HCV E2 became markedly resistant to Endo H cleavage following TMD replacement, thereby revealing a transition from oligomannose- to complex-type glycans. Conversely, swapping the TMD of CD4 for that of E2 led to ER retention of CD4 and heightened sensitivity to Endo H. A follow-up study by Cocquerel et al. (1999) investigating possible contributions towards ER-Golgi trafficking by the E1 TMD present similar conclusions (Cocquerel et al., 1999). These results show that the stunted processing of HCV glycans can be attributed to the TMD of E1 and E2 subunits, which contribute towards static retention in the ER as opposed to retrieval from the Golgi apparatus and may be important for viral assembly and budding (Ariumi et al., 2011; Corless et al., 2010).

In contrast to HCV, the signal peptide of HIV-1 Env is N-terminal and appears to have a parallel but distinct impact on glycosylation. The SP of HIV-1 Env is comprised of a basic N-terminal region, a hydrophobic core, followed by the cleavage site (Yolitz et al., 2018). Compositional variation within the SP has been evidenced to influence the rate of SP cleavage, ER trafficking and ER retention, with a dependence on the presence of particular basic residues, such as His12 (Fig. 1B(ii)) (Snapp et al., 2017; Yolitz et al., 2018). Frequently absent from chronic phase viral isolates, overrepresentation of His12 is a signature of transmitted/founder (TF) viral isolate SPs (Gnanakaran et al., 2011). Thought to enhance the efficiency of viral replication of TF viruses, the SP is likely a modulator of Env glycan processing despite being cleaved from the native glycoprotein (Gnanakaran et al., 2011).

To investigate the impact of natural SP compositional variation on ER trafficking and glycan processing, Yolitz et al. constructed recombinant gp120 derived from TF and chronic isolates containing either the parental SP or a swapped SP (Yolitz et al., 2018). Using a combination of lectins and Abs that possess differential specificity for α-linked mannose moieties, this study and others suggests that there is a strong interdependence between the presence of N-terminal basic residues within the SP and stunted glycan processing (Asmal et al., 2011; Go et al., 2011; Upadhyay et al., 2020). The presence of His12 within the SP has been shown to positively influence viral fitness by increasing the rate of Env translation, which is perhaps lost in chronic viral isolates due to selective immune pressure (Upadhyay et al., 2018). Together, these examples emphasize the strong interplay between signal peptide sequence and ER trafficking, which in turn can shape the glycosylation of viral glycoproteins.

2.5. Interaction with the host immune system

Successful viral transmission is reliant on efficient cellular attachment and entry, replication, followed by dissemination. In addition, viruses are at an advantage if they subvert host immunity to be able to transmit from cell-to-cell and from host-to-host. Both the evolving immunity of the host and the herd immunity from sustained pandemics contribute to the selection pressures that viruses face. As well as impacting the structure of viral proteins, the presence and processing of viral glycans are influenced by the host's immune system.

Influenza HA exemplifies how viral glycans can be shaped by immune selection pressures resulting from recurrent endemic circulation (Fig. 1C). The influenza A virus, H3N2, appeared in Hong Kong in 1968 (HK68) and caused a global pandemic. Subsequently, the H3N2 virus has re-emerged many times across the world, causing smaller endemic outbreaks and over time the H3N2 HA has mutated through antigenic drift. Some of this antigenic drift is undoubtedly a result of continued exposure to host immunity and one facet of this is the accumulation of N-linked glycan sites over time. The HK68 H3N2 HA contains 7 PNGS whereas the more recent Vic11 H3N2 HA contains 12 PNGS. As host-derived glycans are poorly immunogenic, it is likely that the accumulation of these PNGS is a mechanism to subvert host immunity by shielding the underlying protein epitopes at the expense of viral fitness (Houser et al., 2022). This is further evidenced by the observation that the introduced sites tend to be acquired on the head domain of HA, which is more immunodominant than the stalk (An et al., 2015; Lee et al., 2021b). Not only do more recent isolates of H3N2 HA contain more N-linked glycan sites, but the additional glycan sites modulate the processing of existing sites. The N165 site on HK68 is conserved across many strains, however the accumulation of PNGS around the N165 site restricts glycan processing, giving rise to a higher abundance of underprocessed oligomannose-type glycans. This effect is so pronounced that an anti-HIV-1 antibody, 2G12, which recognizes larger oligomannose-type glycans on HIV-1 Env, can neutralize H3N2 HA from

strains such as Vic11 but is unable to neutralize earlier isolates such as HK68 (Lee et al., 2021b).

In addition to pre-existing immunity, viral infection results in an evolution of the host immune response which viruses must overcome in order to sustain chronic infection. The high mutation rates of HIV-1 Env through an error prone reverse transcriptase allow the virus to constantly adapt to immune responses directed towards the virus. This sustained interaction shapes the glycan shield, with the number and position of PNGS varying throughout an infection (Wei et al., 2003) (Fig. 1C). One study illustrated the dynamic nature of the HIV-1 Env glycan shield during chronic infection by analyzing the glycans present on Env isolates taken multiple week intervals throughout infection (Coss et al., 2016). Early (6 week) isolates contained 27 PNGS on a single HIV-1 Env protomer, which increased to 31 by week 176. In addition, the exact position of these glycan sites changed throughout the infection, with the N332 glycan site occurring in all early isolates but disappearing or shifting to N334 at later timepoints (Coss et al., 2016). It has been well established that the oligomannose-type glycans at N332 contribute to key bnAb epitopes and the remodulation of this site demonstrates how viral glycosylation can be shaped by the host immune response (Krumm et al., 2016; McCoy et al., 2015; Pritchard et al., 2015a; Sok et al., 2014; Wagh et al., 2018).

With there being multiple facets influencing viral glycan processing, the degree of native-like glycan mimicry on viral immunogens that may be required to elicit protective immunity will be further discussed. In some cases, preserving viral glycosylation (whether the processing state alone or glycan site occupancy) in an immunogen context appears important for the generation of protective nAbs. In other cases, the removal of glycan sites or entire domains can instead enhance nAb engagement through enhanced epitope accessibility. The diversity of viral antigens, epitope accessibility and sequence variation all contribute to uncertainties surrounding how viral glycan mimicry can impact the antigenicity of a viral immunogen. As vaccines are produced outside the context of viral infection, the contributing factors that dictate glycosylation must be understood for both the infectious virus, and how this is consistent with, or deviates from, recombinant immunogen production.

3. Importance of recombinant protein technologies

Many class I fusion viral membrane glycoproteins, such as HIV-1 Env, SARS-CoV-1 and SARS-CoV-2 spike, LASV GPC, and Ebola virus (EBOV) GP, form a trimer in their native state. These glycoproteins exist in a pre-fusion conformation prior to receptor attachment and membrane fusion and are the principal target of nAbs. The membrane fusion event for class I fusion glycoproteins involves a substantial conformational change, which induces trimer dissociation and membrane engagement by the fusion peptide (FP) (Harrison, 2015). Since membrane fusion gives rise to a set of intermediate protein conformations that are only momentarily exposed, most epitopes that engage nAbs are present on the pre-fusion conformation. Utilizing inactivated viral formats for many viruses frequently does not provide robust protection, and often a moderate risk is associated with the administration of inactivated viruses. For example, the low safety profile surrounding the use of inactivated HIV-1 and the possible integration events that could occur following administration prevent the use of this vaccine format (Harris, 2022). Hence, recombinant protein technology has unlocked the possibility of vaccine development against previously difficult to vaccinate viruses. Recombinant protein technology has substantially accelerated the advancement of viral vaccinology, allowing for the generation of artificial attachment glycoproteins that capture the native-like pre-fusion conformation (Sanders and Moore, 2021). Additionally, large quantities of clinical grade glycoprotein can be generated (Dey et al., 2018) that can promote the engagement of a focused humoral immune response *in vivo* (Houser et al., 2022).

3.1. Stabilization of the native-like conformation

A challenge faced during the design of an immunogen that elicits a strong protective neutralizing antibody response *in vivo* is generating a stable native-like viral membrane glycoprotein. Most protein-based immunogens capitalize on recombinant protein technology due to the ease of purification and impressive yields relative to membrane-bound counterparts and has been pivotal for vaccine development. However, this development has not been straightforward. With issues surrounding spontaneous shedding and oligomeric dissociation, extensive focus with the aim of generating native-like soluble structures has precipitated the exploitation of foldon domains (Li et al., 2013), disulphide linking (Binley et al., 2000), Gly-Ser linkers (Lu et al., 2014), enhanced furin cleavage sites (Binley et al., 2002), and utilization of proline-stabilization (Sanders et al., 2002). Different combinations of these engineering strategies have emerged as the preferred strategy for recombinant production of glycoproteins from different viruses. Fig. 2 illustrates the range of techniques used, yet it is rare for all approaches to be employed in a single immunogen as some approaches are less effective at stabilizing native-like conformations.

Solubilization of a viral spike ectodomain is achieved by removing the transmembrane region, thereby discouraging oligomeric protomer association. To restore this property, trimerization (foldon) domains

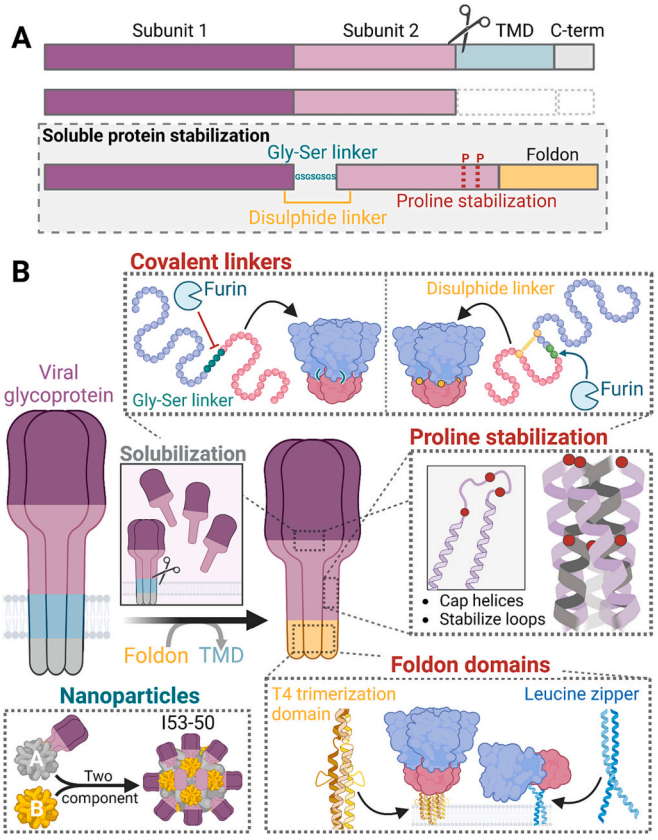

Fig. 2. Methods to stabilize soluble viral glycoproteins. A) Example protein schematic demonstrating commonly used protein engineering approaches to stabilize the non-covalent trimeric conformation following loss of the transmembrane domain (TMD), colored light blue. TMD replacement using foldon domains is shown in yellow. The dashed boxes represent regions of viral glycoproteins where the highlighted engineering approaches can be employed. B) Summary of key innovations around recombinant protein stabilization including covalent linkers, proline stabilization (red circles), the use of foldon domains and the introduction of additional components to promote nanoparticle display. Truncation of the protein sequence upstream of the TMD is represented using scissors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from other systems, such as bacteriophage T4 fibritin (termed in this context, "T4 trimerization domain"), have been employed and conjugated to the carboxy termini of spike mimetics (Fig. 2A). This technology has been exploited in the initial design of trimeric Influenza HA and has since been routinely used in the design and expression of other trimeric spike mimetics, including SARS-CoV-1 S, SARS-CoV-2 S protein, and more (Agnolon et al., 2020; Henderson et al., 2020; Hsieh et al., 2020; Krammer et al., 2012; Lu et al., 2014; Tai et al., 2016; Yang et al., 2002; Zhao et al., 2016). Whilst foldons have received widespread use in these cases, their use has been superseded by the development of the SOSIP format in HIV-1 vaccine research (Sanders et al., 2013), discussed further below.

The recruitment of proline substitutions to rigidify structurally informed regions of a polypeptide chain formed a notable pivot point in the generation of metastable pre-fusion stabilized trimers (Gruenke et al., 2002). Using HIV-1 Env SOS (intermolecular disulphide stabilization) gp140 as a prototype (Binley et al., 2000), Sanders et al. (2002) described a method for heightening trimeric stability. Engineering of the I559P mutation within the α-helical heptad repeat region of gp41, generating trimeric BG505 SOSIP.664, unlocked a route for expediting vaccine design for a multitude of viruses. Further design refinement (Sanders et al., 2015), high-resolution structure determination (Julien et al., 2013; Lyumkis et al., 2013), and immunological testing (Pancera et al., 2014) determined that this mode of stabilization displayed robust antigenicity and stability. The SOSIP mutations have eliminated the reliance of foldon domains to maintain oligomeric association following solubilization of Env, and similar mutations have since been employed in different contexts. A landmark review by Sanders and Moore succinctly details the extensive applications of proline stabilization in other viral immunogens (Sanders and Moore, 2021). Applying recombinant protein technologies in the development of recombinant immunogens has proven critical in establishing native-like glycosylation on soluble immunogens. Prior to the introduction of disulphides and proline substitutions, recombinant Env glycosylation deviated from viral Env. This was most pronounced at the TAMP around N156 and N160, with these sites displaying elevated complex-type glycans on gp120 monomer and uncleaved, poorly assembled Env trimers (Behrens and Crispin, 2017). Subsequent analysis of viral Env demonstrated oligomannose-type glycans at these sites, comparable to that observed on immunogens containing SOSIP modifications (Cao et al., 2018; Struwe et al., 2018). As these glycan sites are key bnAb epitopes, the development and application of recombinant stabilization is interconnected with the reconstitution of native-like glycosylation.

Another advantage of recombinant protein technology is the ability to manipulate protein structure through the introduction of artificial flexible linkers. One method includes the replacement of the furin cleavage site of HIV-1 Env with a flexible linker, with the aim that a mature closed state would be achieved without the requirement of proteolytic cleavage, thereby simplifying Env expression (Georgiev et al., 2015; Sharma et al., 2015). Versions of these engineered constructs, including "natively flexibly linked" (NFL) Env, exhibit similar antigenic and structural properties as native-like Envs (Dubrovskaya et al., 2019; Sarkar et al., 2018; Sharma et al., 2015; Yang et al., 2018). This bioengineering approach to produce cleavage-independent Envs will prove particularly valuable for the development of nucleotide-based trimeric immunogens where the potential negative impacts on protein and glycan epitope mimicry by incomplete furin cleavage can be bypassed (Willis et al., 2022).

While the proline-substitution technology has been supportive for the stabilization of many recombinant class I fusion proteins, there is still progress to be made for other viral membrane glycoproteins. For example, Hepatitis C virus (HCV), a class II fusion virus, presents non-covalently linked envelope glycoproteins, E1E2, on the viral membrane. The inherent folding inefficiency of these subunits has severely hindered the elucidation of high-resolution structures of native E1E2 and the production of a homogenous E1E2 population. To tackle this, a recent study co-expressed two HCV bnAbs to stabilize and purify the prefusion conformation of the heterodimer, which allowed for the full structure of native E1E2 to be resolved by cryoEM (Torrents de la Peña et al., 2022). It is hoped that determination of this structure will prove invaluable for future structure-based rational design of HCV immunogens. Recent advancements in native-like recombinant E1E2 design employ carboxy-terminal leucine zippers to promote non-covalent association by mimicking the transmembrane (Fig. 2B) (Guest et al., 2021; Toth et al., 2021). This method displays great promise for the higher yield production of native-like soluble E1E2 and induces a heightened bnAb response relative to membrane-bound material (Wang et al., 2022). Similarly, the employment of cysteine zippers have aided in the stabilization of RSV trimers (Stewart-Jones et al., 2015).

Beyond these approaches, two-component NP formats (I53–50) have recently been employed in the development of permutated HCV E2E1, HIV-1 Env, SARS-CoV-2, respiratory syncytial virus (RSV) immunogens and others (Brouwer et al., 2022; Marcandalli et al., 2019; Sliepen et al., 2022; Ueda et al., 2020; Walls et al., 2020). Displaying heightened potency and breadth relative to the native-like viral glycoprotein counterparts, multivalent NP display may be the key to the design of effective viral vaccines. Perhaps an indirect advantage of multivalent display of viral immunogens is the rapid trafficking of NPs to follicular dendritic cells relative to the monomeric counterparts. The extensive presentation of oligomannose-type glycans presented on the HIV-1 NP, eOD-60mer, triggered mannose binding lectin (MBL) trafficking to follicles, thereby enhancing antibody responses in germinal centers (GC) and heightening T_{fh} cell responses (Read et al., 2022; Tokatlian et al., 2019). Importantly, the level of displayed oligomannose-type glycans correlated with the immune response. These examples together substantiate the powerful impact of different recombinant platforms in the development of viral immunogens and highlighting that one model does not fit all, and that it is plausible to harness innate immune responses to amplify humoral responses against viral envelope glycoproteins.

The capacity of an immunogen to elicit a strong protective nAb response upon natural infection is dependent on several design factors, including conformational flexibility and immunodominant epitope exposure. As discussed above, proline stabilization has been pivotal for the development of pre-fusion stabilized spike glycoproteins, coupled with trimerization domains for those which are oligomeric. Arguably, the degree to which native-like structure mimicry impacts immunogen design is variable depending on the viral spike glycoprotein under investigation. One benefit of presenting a native-like structure, as opposed to a single subunit, domain, or polypeptide, is the heightened opportunity to engage different B-cell precursors through the presentation of many different epitopes. Elicitation of multiple nAbs that target different regions of the viral glycoprotein should provide better protection against natural infection by preventing viral establishment and hastening viral clearance. Additionally, the threat of exposing non-neutralizing distractive epitopes in the case of a non-native protein structure is limited (de Taeye et al., 2015). Aspects that feed into conformational mimicry include 3D protein architecture as well as any associated post-translational modifications. Since many viruses use host-derived glycans as a shielding mechanism, it is important to consider how maintaining glycan occupancy and the glycan composition during the development of an immunogen impacts a focused humoral immune response.

4. Glycan mimicry in recombinant immunogens

The capacity of recombinant immunogens to elicit a nAb response *in vivo* often has a strong interdependence with the degree of glycan shield mimicry (Derking et al., 2021; Pritchard et al., 2015b;Seabright et al., 2019). The host-derived origin of the glycans that decorate viral glycoproteins can hinder the ability of the host immune system to recognize viral protein epitopes upon infection (Scanlan et al., 2007). For this reason, it may be important to consider glycan composition and site

occupancy to ensure that the nAb response generated through immunization is robust. In general, the more of the antigenic protein surface that is shielded by N-linked glycans, the more restricted the nAb footprint. As such, capturing the native-like glycosylation of viruses is a key part of ensuring antigenic mimicry in vaccine design, particularly for heavily glycosylated viral glycoproteins such as from LASV and HIV-1. In contrast, in cases where glycan density is lower, such as in IAV HA and SARS-CoV-2 S, immunogen effectiveness is much less influenced by glycan occupancy and processing. This last point is supported by the clinical success of egg-, plant- and insect-derived immunogens (Rajaram et al., 2020; Rockman et al., 2022; Shinde et al., 2021; Ward et al., 2021). Nevertheless, glycan variants, such as the use of IAV and SARS-CoV-2 glycoproteins with truncated glycans, offer a route to enhanced immunogenicity (Huang et al., 2022; Tseng et al., 2019).

Developments of glycan engineering and glycan variants are emerging as important strategies for eliciting desirable immune responses to the more densely glycosylated viruses. HIV-1 poses a particular challenge in that immunogens are thought to need to stimulate particular desirable B-cell lineages that can evolve into bnAbs (Burton and Hangartner, 2016; Haynes et al., 2023). These challenges have stimulated the exploration of glycosylation variants in the elicitation of desirable immune responses, discussed further below.

4.1. Presenting glycans as antibody epitopes

Comprising part of the adaptive humoral immune response, antiviral nAbs function through high specificity epitope binding to inhibit viral infection (Corti and Lanzavecchia, 2013). Mechanisms by which antibody-mediated neutralization can occur involve blocking receptor binding, preventing conformational changes required for infection, and binding distally to destabilize the pre-fusion state of the viral glycoprotein. Remarkably, glycans can, in rarer cases, serve as nAb epitopes, whether independently or as a clustered network (Balzarini, 2007; New et al., 2020; Umotoy et al., 2019). However, the tolerance to 'self' glycans and, in the case of HIV-1, the general evolution of the key viral components to low immunogenicity, has meant that potent bnAbs have a long and rare mutational journey from the initial lineage and immunogens would seem to need to somehow stimulate this extensive fine-tuning of antibody germline precursors (GL) (Landais and Moore, 2018; Lee et al., 2021a).

Viral glycans have been shown to form epitopes for nAbs against many viral glycoproteins, including HIV-1 Env and LASV GPC. The incidence of these glycan accommodating nAbs is lower relative to protein binding nAbs, which may be attributed to the aforementioned challenges, as well as glycan site microheterogeneity that hinders antibody engagement to particular glycoforms. However, many glycan-binding nAbs have potent and broad reactivity so are attractive targets for induction by immunization. Hence, recapitulating certain glycan structures on viral immunogens can substantially support successful immunogen design.

4.1.1. The importance of glycan epitope mimicry

The ability for antibodies to overcome the shielding mechanism of viral glycans by directly binding to- or accommodating glycans provides hope for eliciting protective immunity against densely shielded viral glycoproteins, exemplified particularly by HIV-1 and LASV. Accordingly, methods for preserving glycan site homogeneity that engage glycan-dependent nAbs are continually being employed in the design of viral immunogens. The objective of maintaining a native-like glycan shield, with particular focus on the processing state of certain sites, is a large consideration of HIV-1 immunogen design due to the fragility of bnAb induction. Owing to the appreciable efforts being made to optimize native-like glycan shield mimicry of HIV-1 Env, considerations to maintain nAb glycan epitopes on Lassa GPC immunogens are surfacing in view of recent discoveries of several potent glycan-binding nAbs (Brouwer et al., 2022; Perrett et al., 2022).

With a median of 25 N-linked glycan sites per protomer, the dense glycan shield of HIV-1 Env contributes to ~50% of its mass. Consequently, much of the antigenic protein surface is shielded by immunologically self-carbohydrate structures thereby limiting the area of exposed protein epitopes. The host's immune system must find ways to adapt to this glycan barrier in order to stay in the arms race of HIV-1 Env evolution. Structural analysis of bnAbs complexed with Env mimetics have elucidated a large number of bnAbs that form contacts with glycans as part of a larger glycopeptide epitope. These glycan-binding bnAbs have been discovered to target regions across the Env trimer, including the outer domain, V1/V2 loops, CD4 binding site, gp120/gp41 interface and membrane-proximal external region (MPER) (Kwong and Mascola, 2012; Seabright et al., 2019). While most anti-Env bnAbs make contacts with glycans to penetrate the glycan shield, only a select few of these bnAbs are dependent on the presence of glycans to achieve heterologous neutralization.

Two examples of antibody epitopes with critical glycan components include those targeted by V2-apex and V3-glycan bnAbs. The V2-apex consists of the V2 loop and highly conserved glycans N156 and N160, of which several potent and broadly cross-reactive bnAbs have been isolated from infected individuals (McCoy and Burton, 2017). These V2-apex bnAbs, including quaternary structure preferring PG9, and quaternary structure dependent PG16 and PGT145, develop uncharacteristically long CDRH3 loops in order to penetrate the glycan shield of Env and access the underlying protein surface (Lee et al., 2017; McLellan et al., 2011; Pancera et al., 2013; Pejchal et al., 2010).

A strong glycan dependence of many V2-apex bnAbs have been reported in which the N160 glycan is required to achieve neutralization (with the exception of CAP256.09, which displays partial dependence for N160 glycans) (Andrabi et al., 2015; Doores and Burton, 2010; Doria-Rose et al., 2014; McLellan et al., 2011). The preference of such antibodies for oligomannose-type glycan structures at N160 has been extensively reported. Differential tolerance of N160 glycans by PG9 and PG16 reveal that a predominance of $Man_5GlcNAc_2$ enables high affinity antibody binding and viral neutralization. Larger oligomannose-type glycans at N160, however, such as $Man_9GlcNAc_2$, have been demonstrated to severely reduce viral neutralization, likely due to an inhibition in CDRH3 access to the apical gp120 peptide epitope. Further, PG9 and PG16 also form hydrogen bond contacts with neighboring glycans at N156, though these interactions are dispensable for viral neutralization. Unlike N160, there is less of a dependence of oligomannose-type glycans occupying N156 for the high affinity binding of PG9 and PG16. This is exemplified by the fact that sialylated hybrid-type glycans at N156 have been evidenced to form supporting contacts for both PG9 and PG16 (Andrabi et al., 2017). The broad reactivity and potency of V2-apex bnAbs make them a very attractive targets to be elicited through vaccination. However, the potential strategy required to achieve mature, protective V2-apex bnAbs likely involves a multi-immunogen approach using structure-based design to guide the maturation of these bnAbs (Andrabi et al., 2015).

The IMP on gp120 comprises of N332, N386 and N392 glycans, which form epitopes for highly potent V3-glycan bnAbs (Kong et al., 2013; Moyo et al., 2020; Nogal et al., 2020b). Similar to V2-apex bnAbs, many V3-glycan bnAbs develop long CDR loops to penetrate the glycan shield to bind protein epitopes and as such form extensive glycan contacts to achieve this. Two V3-glycan bnAbs exhibiting particularly potent and broad neutralization capacity, PGT135 and BG18, display an extensive antibody footprint with extensive contacts made with glycans at N332 and N392 (Barnes et al., 2018). The glycan site dependence of these bnAbs is elucidated in Fig. 3 whereby deletion of these glycan sites abolishes their neutralization sensitivity. Mass spectrometric approaches suggest that strain-specific glycan site microheterogeneity within the IMP potentially contributes towards neutralization sensitivity of PGT135 in which larger oligomannose-type glycans may sterically clash with the CDR loops of PGT135 thereby preventing access to its protein epitope (Pritchard et al., 2015a). The existence of epitopes with

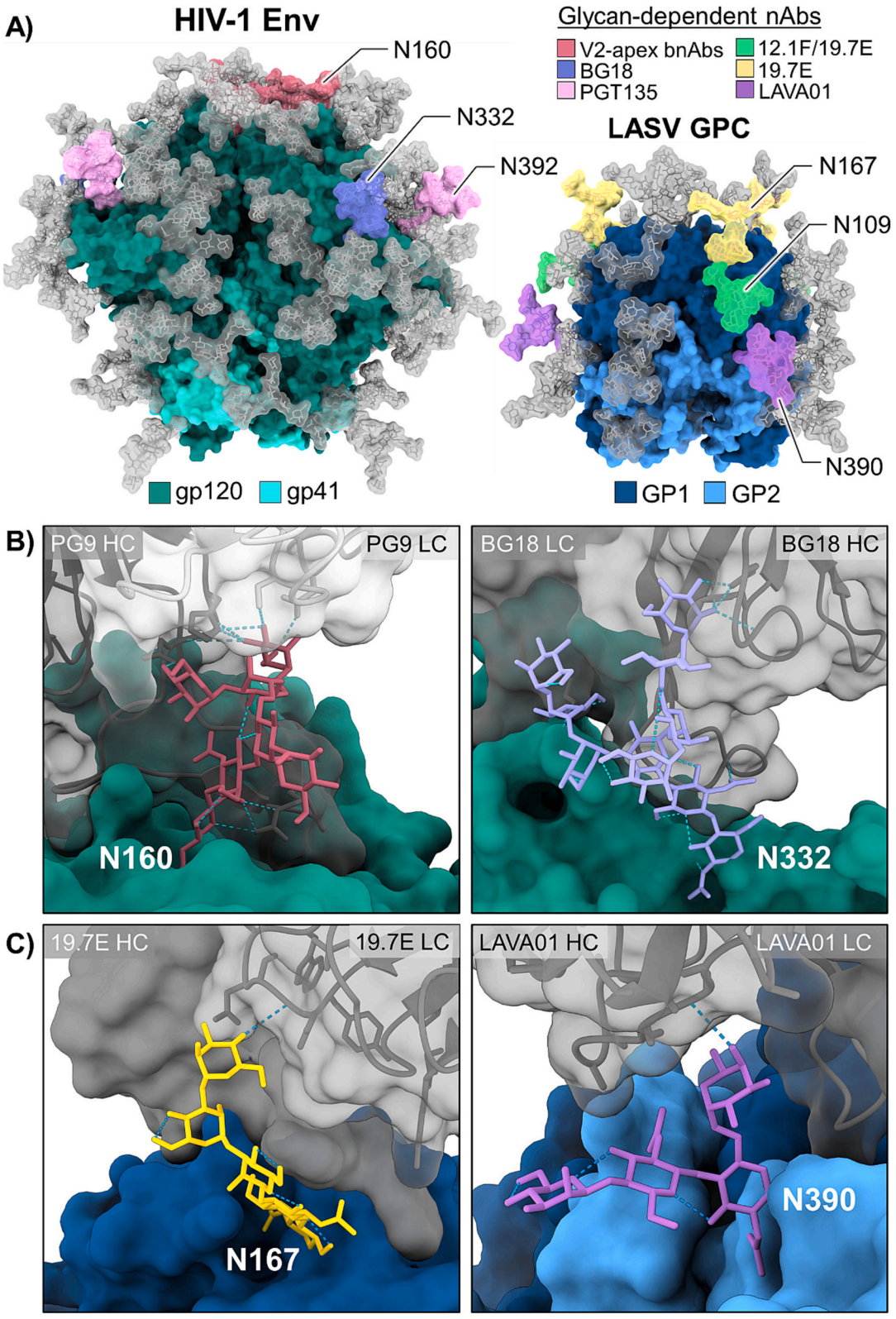

Fig. 3. Glycan-dependent neutralizing antibody epitopes of HIV-1 Env and LASV GPC. A) Examples of complete glycan dependence of glycan-targeting HIV-1 and LASV bnAbs are mapped on the trimers. Glycan sites displaying an integral epitope for glycan-dependent bnAbs are individually colored. Anti-glycan HIV-1 bnAbs include V2-apex bnAbs, BG18, and PGT135. Anti-glycan LASV bnAbs include 12.1F, 19.7E and LAVA01. Hydrogen bond interactions between HIV-1 bnAbs, PG9 and BG18 (B), LASV bnAbs, 19.7E and LAVA01 (C), and N-linked glycans that form part of their composite epitopes. N-linked glycans are colored according to those in panel A. Hydrogen bonds are colored cyan, and nAb Fab regions are colored gray. PDB accession codes: 7T77, 6DFG, 8EJI, 7SGF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a high degree of sensitivity to both the presence and fine processing of N-linked glycans highlight the importance of maintaining native-like glycosylation in immunogen design.

In addition to HIV-1 Env, other densely glycosylated viruses are reliant on the elicitation of anti-carbohydrate nAbs in both infection and vaccination. The GPC is the sole target of nAbs against LASV. Comprised of a trimer of non-covalently associated heterodimers (GP1 and GP2), the GPC trimer is coated in host-derived N-linked glycans with up to 12 PNGS per protomer. The development of an effective vaccine against LASV has been hindered by its antigenic diversity and largely unstable nature in soluble recombinant formats, which severely hinders the engagement of protective nAbs. However, a recombinant LASV GPC NP that captures a high degree of glycopeptide epitope mimicry induces protective immune responses in guinea pigs (Brouwer et al., 2022), and happens to exhibit conserved glycosylation compared to pseudoviral-derived material (Watanabe et al., 2018). The dense glycan shield of LASV GPC also substantially contributes to the virus' immune evasion capacity. Recent cryoEM structures of pre-fusion stabilized trimeric GPCs of several lineages of LASV elucidate the molecular mechanisms underpinning GPC recognition by three nAbs and provide a basis for structure-based immunogen design (Brouwer et al., 2022; Perrett et al., 2022).

Unlike the majority of HIV-1 bnAbs, which have been shown to accommodate glycans (opposed to displaying a glycan dependence), anti-LASV GPC nAbs 12.1F and 19.7E, which neutralize LASV by inhibiting Lysosomal-associated membrane protein 1 (LAMP-1) binding, display substantial glycan dependence (Perrett et al., 2022). A considerable reduction in neutralizing potency of LASV pseudovirus by 12.1F was observed following the removal of the N109 site (S111Q). A similar effect was observed for 19.7E, which requires both N109 and N167 glycans on the GPC to neutralize LASV pseudoviruses. Similarly, the LAVA01 nAb which binds to a conserved region within the LAMP-1 binding site is largely dependent on glycans at N390 (Brouwer et al., 2022). This highlights the importance of maintaining glycan occupancy in the structure-based design of LASV GPC immunogens in order to exploit the glycan shield as an epitope for nAbs. Despite this, pre-clinical studies have demonstrated that non-neutralizing antibody responses against variants of recombinant LASV GPC are sufficient to induce complete protection against lethal LASV challenge through the induction of effective antibody-dependent cellular functions (Abreu-Mota et al., 2018; Ronk et al., 2023). Further studies in non-human primate models may be necessary to predict levels of protection in humans, however.

While several isolated bnAbs display complete glycan dependence against HIV-1 and LASV, which have evolved to extraordinarily overcome the weak immunogenic nature of N-linked glycans, the majority of HIV-1 bnAbs, and some LASV bnAbs, accommodate glycans to some extent. Together, this accentuates the powerful tool that the glycan shield of HIV-1 and LASV can play in the elicitation of potent bnAbs in spite of these viruses using these host-derived modifications to hinder recognition. As such, careful considerations surrounding glycan occupancy and processing may prove to be important for the structure-based rational design of immunogens, employing validation methods such as mass spectrometry and cryoEM to reinforce successful vaccine design (Crispin et al., 2018).

4.1.2. Improving antibody recognition through engineering glycosylation

The magnitude of epitope shielding by host-derived N-linked glycans across many different viral glycoproteins has guided the development of glycan-modified recombinant variants to enhance nAb recognition. The utilization of *in vitro* glycosidase inhibitors, glycosyltransferase knockout (KO) cell lines, and post-production glycosidase treatment are popular techniques to alter glycosylation of viral glycoproteins to successfully improve antigenicity (LaBranche et al., 2018; Tseng et al., 2019; Zhang et al., 2022).

At present, seasonal Influenza vaccines are being depended upon to generate a protective response against circulating strains. If a predictive mismatch occurs in which the HA strain in the vaccine does not match that of the circulating virus, the efficacy of the vaccine is likely be compromised and induce poor levels of protection. Consequently, the development of a pan-influenza vaccine is of extreme interest. Since N-linked glycans effectively mask antigenic protein epitopes, one study sought to investigate the antigenic impact of glycan trimmed H1N1 HA. Perhaps by removing the glycans on HA, a broader nAb response could be elicited following exposure of accessible antigenic protein epitopes. An *in vitro* glycoengineering strategy was utilized by Tseng et al. (2019) in which a small molecule inhibitor of ER α-mannosidase I, kifunensine, followed by Endo H treatment (glycan trimming), were utilized to induce mono-GlcNAcylated glycans, which are much smaller than their WT equivalent (Fig. 4A). Mice immunized with the glycoengineered HA induced greater strain-specific and cross-strain nAb responses relative to un-engineered counterparts, suggesting that a broader nAb response can be elicited by increasing nAb accessibility to glycan-shielded epitopes. Interestingly, a larger proportion of the nAb response was directed to the stem region – a region that is generally less immunodominant, but more conserved, relative to the head region of HA. The capacity for anti-stem bnAb responses to elicited using a glycan trimmed HA (as opposed to using the stem region in isolation) is especially motivating as the HA stem region is an extremely attractive target for the development of an effective pan-influenza vaccine (Nachbagauer et al., 2021). A similar approach was used to elicit enhanced protective responses against SARS-CoV-2 S protein in mouse models (Huang et al., 2022).

The glycan shield of Influenza HA and SARS-CoV-2 S protein is sparse relative to HIV-1 Env and LASV GPC. With increasing glycan density comes reduced accessibility to protein epitopes. Hence, the pressure to maintain glycan occupancy becomes more apparent as the glycan shield of viral glycoproteins increases. The utilization of deglycosylating enzymes in the case of Influenza HA and SARS-CoV-2 S protein has shown to enhance nAb recognition and potency by improving epitope access. In the case of HIV-1 Env and LASV GPC, however, the above glycoengineering strategy would likely unveil distractive, non-native, non-neutralizing epitopes that are incapable of inducing a protective antibody response. There are cases in which the meticulous introduction of glycan holes can in fact enhance bnAb recognition *in vitro* and will be discussed further in section 4.2.2. Developing innovative glycoengineering strategies to tackle nAb inaccessibility problems could thoroughly support the development of protective viral vaccines.

Despite the use of recombinant technologies, the induction of bnAbs against HIV-1 *in vivo* has not yet been achieved. The benchmark Env strain, and variants thereof, used in the majority of HIV-1 immunogens is BG505, a clade A T/F viral isolate (Hoffenberg et al., 2013). Following many years of iterative protein engineering, BG505 SOSIP.664 was shown to bind many of the bnAbs isolated from infected patients, which encouraged vaccine design efforts focused around this immunogen (Georgiev et al., 2015; Sanders et al., 2013). However, the native-like BG505 SOSIP.664 trimer does not elicit a strong, focused bnAb response and poorly engages bnAb precursors *in vivo* (Houser et al., 2022; Nogal et al., 2020a; Xiao et al., 2009; Zhao et al., 2020). The glycan shield is a major blockade to germline precursor B-cell engagement, as immature antibodies have not undergone the extensive somatic hypermutation required to bypass the glycan shield. The knowledge that prerequisite antibody germlines are not engaged by BG505 SOSIP.664 has largely shifted the approach of HIV-1 vaccine development in which immunogens are designed to engage the germline precursor (GL) of the desired bnAb lineage (priming immunogen), and the iterative structure-based design of "boost" immunogens will guide the maturation of bnAbs – a process termed germline targeting (GT) (Steichen et al., 2019). As bnAbs possess unique structural features, including unusually long CDRH3 loops (V1/V2 bnAbs, *e.g.*, PGT145), tyrosine sulfation (V1/V2 bnAbs *e.g.* PG9, PG16) and even hydrophobic CDRH3 loops (MPER bnAbs *e.g.* 10E8), extensive rounds of SHM are often required for the development and refinement of these rare features (Irimia et al., 2017;

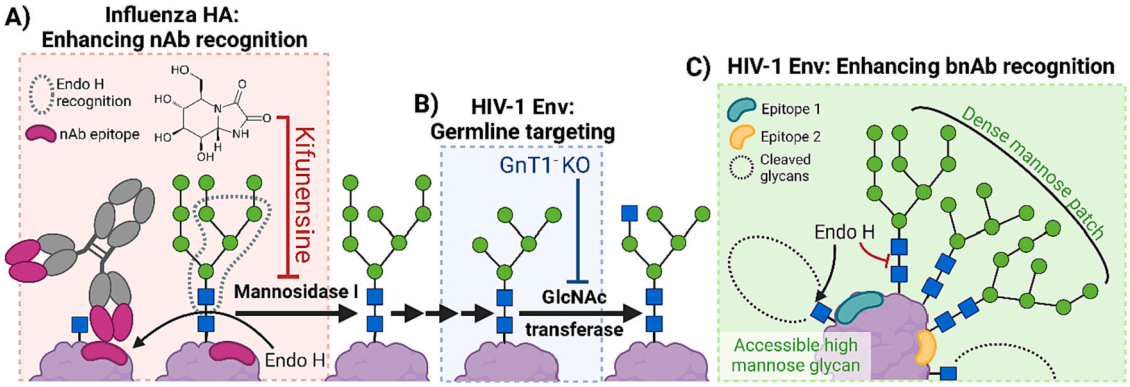

Fig. 4. Glycoengineering techniques to improve nAb engagement by viral immunogens. A) Inhibition of ER α-mannosidase I using kifunensine converts all N-linked glycans on the protein to $Man_9GlcNAc_2$. Treatment with endoglycosidase H (Endo H) cleaves the glycans between the core GlcNAc residues, yielding a single GlcNAc that remains attached to the Asn. B) The generation GnTI KO cell lines truncate glycan processing at $Man_5GlcNAc_2$, thereby inhibiting complex- and hybrid-type glycan processing. C) Endo H treatment of viral glycoproteins with dense glycan shields. Only glycosidase-accessible high mannose glycans will be cleaved, leaving those which are glycosidase-inaccessible intact.

Landais and Moore, 2018; Lee et al., 2017; McLellan et al., 2011; Pancera et al., 2013). As such, vast efforts will need to be invested into the careful design of boosting immunogens so as to guide the introduction of appropriate mutations required for potent and broad antibody neutralization. As of present, the development of priming immunogens remains at the forefront of GT research.

The CD4 binding site (CD4bs) is a key HIV-1 bnAb epitope. The highly conserved nature of this epitope arises due to the dependence on the interaction between Env and its primary receptor, CD4, to mediate membrane fusion. Hence, this epitope remains less variable relative to most other protein nAb epitopes across the trimer. Glycans adjacent to the CD4bs, such as N276, hinder the ability of the host to generate bnAbs targeting this region, however multiple bnAbs targeting the CD4bs have been isolated from infected patients, such as VRC01, 3BNC117, BG24 and others (Barnes et al., 2022; Dam et al., 2022; Scheid et al., 2011; Wu et al., 2010a). A key goal of bnAb-focused vaccine design is to elicit such antibodies using GT to engage precursors by modifying native-like Env immunogens such as 426c, eOD-GT8 60mer and BG505 SOSIP.GT (Jardine et al., 2015; Lin et al., 2020; McGuire et al., 2016; Medina-Ramírez et al., 2017). One approach to germline target CD4bs bnAb precursors through glycoengineering is to produce Env in *N*-acetylglucosaminyltransferase I KO cell lines and subsequently interrogate the binding capacity of VRC01 GL compared with mature VRC01 (LaBranche et al., 2018). The rationale for enhancing Ab recognition by truncating glycan processing to $Man_5GlcNAc_2$ (Fig. 4B) is to boost epitope accessibility to unmutated antibodies with short CDR loops by reducing the size of glycans surrounding the CD4bs, as a $Man_5GlcNAc_2$ glycan is smaller in size than a typical complex-type glycan. This approach has proven successful at engaging CD4bs GL and enhancing viral neutralization potency *in vitro* with the condition that N276 is deleted (LaBranche et al., 2018). As such, glycoengineering of HIV-1 Env immunogens can prove to be an effective method to enhance precursor engagement for a subset of bnAbs but may not be appropriate for the engagement of bnAb precursors for which complex-type glycans form part of their epitope (*e.g.* PGT151 and PGT152) (Falkowska et al., 2014).

Building on the idea of increasing nAb epitope accessibility on Env immunogens using GnT1 KO cell lines, an alternate approach utilizes a mannose-specific glycosidase, Endo H, to cleave only the accessible high mannose (oligomannose- and hybrid-type) glycans on Env recombinantly expressed in normal cellular conditions (*i.e.*, devoid of inhibitors/glycosyltransferase KOs) (Zhang et al., 2022). This method allows for complex-type glycan- and dense oligomannose-type glycan bnAb epitopes to be preserved while increasing bnAb accessibility to epitopes that are typically partially occluded by Endo H-sensitive, solvent accessible glycans (Fig. 4C). Glycan trimming of engineered BG505 uncleaved prefusion-optimized (UFO) NPs induced a better immunological response in mouse models and improved bnAb recognition to several different epitopes across the Env trimer, relative to the untrimmed version, including the CD4bs, FP, gp120-gp41 interface, and V3 base. Enhanced accessibility to these epitopes steered Ab responses away from immunodominant glycan holes, while also eliciting similar responses to epitopes preserved following glycan trimming. This glycan engineering strategy shows promise in the elicitation of multiple desirable bnAb responses by Env immunogens across different vaccine platforms. However, the impact on trimer stability following deglycosylation should be monitored. Berndsen et al. previously demonstrated that enzymatic deglycosylation of BG505 SOSIP.664 imparts modest trimer destabilization (Berndsen et al., 2020), likely an event induced by reduced protein solubility (Solá and Griebenow, 2009).

For viral glycoproteins that are densely glycosylated, such as HIV-1 Env, and those which effectively glycan-mask the most conserved region of the protein, such as Influenza HA, different glycoengineering strategies to improve nAb access have proved fruitful thus far. However, a large emphasis on glycoengineering strategies may not always be necessary. For example, when patient sera were tested for anti-SARS-CoV-2 S Abs, no significant difference in antibody detection was observed between native-like glycosylated S protein, and protein produced with exclusively $Man_9GlcNAc_2$ oligomannose-type glycans. This demonstrates that the antibodies raised towards SARS-CoV-2 infection are generally insensitive to the glycan processing state of the S protein (Chawla et al., 2022). This is presumably due to a high accessibility of nAbs to epitopes across the protein surface. Huang et al. demonstrated that Endo H treatment of kifunensine-expressed spike protein induced better nAb responses *in vivo* (Huang et al., 2022). Together, this suggests that, while further glycan trimming improves the immunogenicity of the antigen by enhancing epitope accessibility, the glycan shield of SARS-CoV-2 S protein does not provide a significant barrier for the robust elicitation of nAbs. These diverse and contradictory examples highlight the complexity of glycans in vaccine design. This suggests that employing native-like glycosylation, or going beyond native-like to improve immunogenicity, must be done on a case-by-case basis, which therefore necessitates the study of viral glycosylation and immune responses that target them.

4.2. *Consequences of glycan occupancy in viral glycoprotein immunogenicity*

One challenge in the development of soluble recombinant viral immunogens is the induction of glycan “holes”. This term describes the

discrepancy in glycan occupancy between viral-derived and soluble mimetics at localized sites, so called "artificial" glycan holes as they are not observed on infectious virions (Cao et al., 2017; Struwe et al., 2018). Glycan site underoccupancy can arise as a direct consequence of solubilization and stabilization of viral glycoprotein mimetics, with C-terminal glycan sites commonly largely impacted (Struwe et al., 2018). These immunodominant non-native glycan holes can induce non-nAb responses, exemplified in the case of densely glycosylated HIV-1 Env, which distract the immune system and prevent the rare somatic hypermutations required to elicit protective bnAbs (Charles et al., 2021; Derking et al., 2021; Klasse et al., 2018; McCoy et al., 2016; Schorcht et al., 2022; Wagh et al., 2020). Bioengineering techniques to increase glycan site occupancy have recently been employed to fill the distractive immunodominant glycan holes in HIV-1 Env immunogens. To a larger extent, the base of these immunogens, which is not accessible on full-length Env, forms a larger target for non-nAbs for which an effective method to silence this region is yet to be developed.

4.2.1. *Glycan holes and undesirable antibody responses*

Electron-microscopy polyclonal epitope mapping (EMPEM) has proven an invaluable tool to highlight how a deviation from native-like glycan occupancy can result in a compromised immune response (Antanasijevic et al., 2021). In a study by Nogal et al., macaques were immunized with BG505 SOSIP.664, and the epitopes recognized by the antibodies elicited were imaged using electron microscopy (Nogal et al., 2020a). Glycan hole epitopes were overwhelmingly targeted in all immunized macaques highlighting the detrimental impact of non-native glycan occupancy, which steers the immunological response away from epitopes which induce bnAbs.

A separate study highlighted non-native glycan holes on recombinant immunogens, and also described methods to bioengineer the immunogens to improve glycan occupancy. Using mass spectrometry and electron microscopy, Derking et al. unveiled non-native glycan occupancy at regions across the trimer, most notably on gp41 at the N611 glycan attachment site (Derking et al., 2021). Antibodies isolated from macaque immunizations targeted the amino acids that would usually be shielded by the N611 glycan, with all of these antibodies unable to neutralize the autologous BG505 virus (Fig. 5A) (Cottrell et al., 2020). Despite the N-linked glycan sequons (N-X-S/T) matching those on the virus, Derking et al. demonstrated that modifying an N-X-S glycan sequon to N-X-T enhanced N-linked glycan attachment at N611 and several other sites. The presence of this glycan diminished the binding of non-neutralizing antibodies to the recombinant protein as a result of steric clashes between the antibody and the glycan (Fig. 5B) (Derking et al., 2021). This approach may provide value for optimizing the glycan occupancy for immunogens against other viruses.

The immunodominant nature of these glycan hole targeting, non-neutralizing Ab responses raise questions surrounding the threshold of glycan site occupancy required to avoid the induction of these distractive immunological responses. Several immunization studies using BG505 SOSIP trimers in different formats display a dominating non-neutralizing response to glycan holes, visualized using EMPEM (Brouwer et al., 2021). Using N611 as an example, as little as 20% site unoccupancy engages a compelling non-neutralizing antibody response.

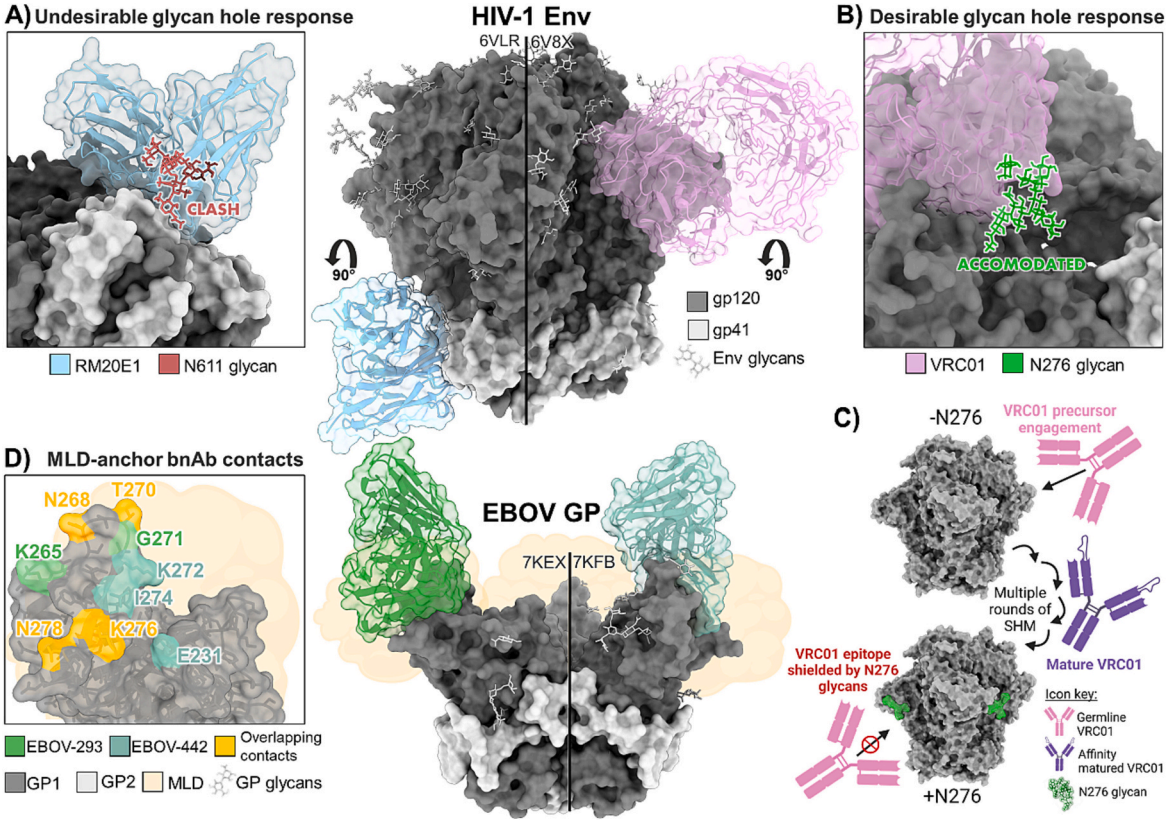

Fig. 5. Antibody responses to glycan holes in HIV-1 Env and Ebola GP. A) An undesirable antibody response to the N611 glycan hole on gp41. RM20E1, a macaque glycan hole non-nAb, binds to the N611 site in the absence of glycans, which dominates mAb responses following immunization with immunogens possessing low N611 glycan occupancy. High glycan occupancy at N611 sterically inhibits RM20E1 binding. B) Mature VRC01-class bnAbs use glycans at N276 as a scaffold to bind the proteinaceous epitopes near the CD4bs. C) A simplified view of the germline-targeting approach using HIV-1 Env mimetics created using BioRender.com. Germline-targeting priming immunogens that engage VRC01-class germlines are devoid of the N276 site to enhance VRC01 precursor access to its epitope. Subsequent guiding immunogens reintroduce the N276 glycan site to further guide VRC01 bnAb maturation. D) Glycan cap bnAbs EBOV-293 and -442 bound to EBOV GP. The CDR contacts of both bnAbs with their epitopes within the MLD-anchor are based upon the data presented in Murin et al. (2021) (Murin et al., 2021). PBD accession codes used: 6VLR; 6V8X; 7KEX; 7KFB.

It is yet to be investigated whether an NXT mutation at this site, which restores glycan occupancy to ~95%, quenches these non-neutralizing responses following immunization.

A response overriding the impact of glycan holes on HIV-1 Env immunogens are those directed to the base of the trimer – a region not exposed on native viral Env. Recent results from the first human clinical trial using the BG505 DS-SOSIP.664 trimer revealed overwhelming non-neutralizing Ab responses to neoepitopes on the base of the trimer (Houser et al., 2022). The outcomes of these clinical trial results, as well as those from earlier pre-clinical models, have prompted further structure-based engineering of BG505 DS-SOSIP.664. Masking the base using protein loops containing additional N-liked glycan sites is a recent approach taken by Kulp et al. to block these undesirable responses (Kulp et al., 2017). The immunological impacts imposed by this so-called "glycan masking" and an evaluation of the glycan occupancy is yet to be interrogated. While this research area is still relatively unexplored, this structure-based design approach provides promise towards quenching these non-neutralizing antibody responses. Further promising engineering approaches involve the presentation of these trimers on nanoparticles, thereby enhancing antibody avidity and hiding the distractive, non-neutralizing base neoepitopes and is discussed further in section 4.2.2.

4.2.2. Glycan holes and desirable antibody responses

While glycan holes in recombinant viral spike mimetics can dilute protective antibody responses, they can also be exploited to induce more focused and potent responses. Methods of introducing glycan holes include mutating the Asn at the site of glycan attachment, the third position Ser or Thr, or removing whole glycan domains entirely. This has been employed in recombinant derivates of HIV-1 Env to promote bnAb induction through GT, and on Ebola GP to achieve broad neutralization.

Beyond simply removing a region of the protein, targeted glycan deletions have been employed to overcome the dense glycan shield of HIV-1 Env. The N276 glycan situated at the CD4bs serves as a significant barrier for the affinity maturation of VRC01-class broadly nAbs (bnAb) (Lynch et al., 2015; Wu et al., 2010b). This region forms an attractive target for epitope-specific germline targeting as this region is well-conserved across HIV-1 group M isolates (Dingens et al., 2019; Nishiyama et al., 2012). The predicted timescale of VRC01-like bnAb maturation is ~5 years (Lynch et al., 2012), and so decreasing the time taken to elicit these highly potent bnAbs through immunization has been a focus of HIV-1 immunogen design. The N276 glycan partially obstructs the CD4bs, and bnAbs that recognize this region must accommodate this glycan through rare antibody architectures that arise from extensive SHM (Umotoy et al., 2019).

As discussed above, the engagement of VRC01-class GL precursors requires an Env immunogen to be devoid of N276 glycans in order to access the CD4bs epitope (Balla-Jhagjhoorsingh et al., 2013). Subsequent introduction of this glycan encourages the evolution and fine tuning of VRC01-class precursor CDR-H and CDR-L genes to accommodate the glycan (Fig. 5C) (Landais and Moore, 2018; Umotoy et al., 2019). The N276 glycan is present on the vast majority of circulating HIV-1 Env strains so the removal of this glycan site represents a deviation from native-like glycan occupancy. Such GT immunogens are already under investigation (Lee et al., 2022). Recent preliminary results from a phase I clinical trial using eOD-GT8 60mer have shown that the removal of conserved glycans can prime VRC01-like B-cell precursors in humans (Venkatesan, 2021), the first HIV-1 immunogen to demonstrate proof-of-concept that germline targeting may be achievable. Pre-clinical studies have revealed that strong germinal center responses can be induced by both protein- and nucleotide-based immunogens, but the nucleotide-based candidate outperforms in terms of inducing antigen-specific GC B cell proliferation (Melo et al., 2019; Tokatlian et al., 2019). This provides a promising trajectory for the delivery of nucleotide-based HIV-1 immunogens.

Furthermore, glycan deletions have been employed in BG505 SOSIP.664 to enhance VRC01-like precursor engagement, referred to as BG505 SOSIP.GT (Caniels et al., 2023; Whitaker et al., 2019). This immunogen lacks a number of glycans that surround the CD4bs including N276, N197 and N462. Pre-clinical studies have demonstrated the success of this approach to prime VRC01-like precursors, and this immunogen is currently moving to phase I clinical trials. These approaches highlight how non-native glycan occupancy can be used to improve the likelihood of inducing a protective antibody response, but these deletions must be introduced at specific locations, otherwise glycan holes can act as highly immunogenic decoys. Therefore, outside of these targeted areas, glycan occupancy must remain native-like.

One example where a whole glycan domain has been omitted from the native viral protein sequence to potentiate desirable nAb responses is in the design of Ebola immunogens. Ebola glycoprotein (EBOV GP) is class I fusion glycoprotein that forms a trimer of GP1/GP2 heterodimers that mediate cell entry and viral pathogenesis and are the main target of nAbs (Lee et al., 2008; Sanchez et al., 1998). Whilst many isolated nAbs target regions within the GP1 core, GP1/2 interface, internal fusion loop and HR2 (Bornholdt et al., 2016; Flyak et al., 2016; Murin et al., 2018; Murin et al., 2014; Saphire et al., 2018; Wec et al., 2017), the high sequence variability between different Ebola species presents a large challenge for the development of an efficacious GP immunogen that elicits broad protection (Jun et al., 2015; Pappalardo et al., 2016). Differing from most other viral class I fusion glycoproteins, EBOV GP possesses a highly O-glycosylated mucin-like domain (MLD) that shields a region of GP1 called the "glycan cap" (Peng et al., 2022; Tran et al., 2014). The glycan cap, which is relatively well-shielded by the MLD in the native pre-fusion state, is an attractive target of nAbs due to its moderately high sequence conservation (Misasi et al., 2016; Murin et al., 2021; Wec et al., 2017). In particular, a site of vulnerability within the glycan cap that engages nAbs with broad and potent neutralizing activity is the "MLD anchor" – a groove within GP1 that fixes the MLD to the glycan cap. Several isolated bnAbs competitively displace this interaction through the use of long CDR loops that make extensive contacts within the MLD anchor (Fig. 4C), including EBOV-293 and EBOV-442 (Murin et al., 2021). Recent studies that aim to generate anti-glycan cap bnAbs have evidenced that the removal of the MLD (Δmuc) in Ebola GP immunogens elicits potent and broad neutralizing responses (Flyak et al., 2016; He et al., 2021; Luczkowiak et al., 2018; Murin et al., 2021; Shedlock et al., 2010). This exemplifies that a deviation from the native-like state can potentiate protective neutralizing responses.

5. Perspectives beyond protein-based recombinant immunogens

Throughout this review we have demonstrated the importance of glycosylation in viral vaccine design, and how recombinant protein technologies can be used to recapitulate viral glycosylation *ex vivo*. Additionally, performing immunogen production *in vitro* provides the possibility of modifying viral glycosylation beyond native-like, to enhance immunogenicity beyond that induced by native-like glycosylation alone. It is clear that a one-size-fits-all approach to glycan engineering is not possible. In some cases, glycans can be critical for antibody recognition, such as in LASV and HIV-1, but in other cases deviation from native-like glycosylation can be employed to improve the breadth and potency of antibody responses, such as for Influenza HA. An intricate knowledge of the factors that govern viral glycosylation, how this differs in recombinant systems, and the implications for antibody elicitation must be understood before deciding whether it is more beneficial to pursue native-like glycosylation, or whether to go beyond. Typically, native-like glycosylation is beneficial for immunogens where an antibody response is readily elicited in infection or by vaccination as this will avoid the introduction of distracting off-target epitopes and maintain the correct protein fold. However, where natural immunity does not prevent infection, most notably in HIV-1 infection, any strategy that can improve recombinant immunogen efficacy should be considered.

Whilst recombinant technologies are capable of reproducing many facets of native-like glycosylation, there is a compromise required to produce glycoproteins *in vitro*. Many of the factors that shape the glycosylation of viruses are difficult to control in a recombinant setting. Recombinant glycoproteins are often produced in clonally selected CHO cells, using recombinant signal peptides such as tissue plasminogen activator (tPa) to achieve the maximum yield possible. In the case of HCV this results in a dramatic shift away from native-like glycosylation (Sliepen et al., 2022), with the majority of glycans on recombinant HCV immunogens populated by complex-type glycans, as opposed to the oligomannose-type glycans present when the native signal peptide is used. The advent of nucleotide-based vaccines provides new opportunities to produce native-like immunogens with glycosylation more reminiscent of a virus in the context of infection. As membrane-bound proteins are difficult to produce in large scale GMP settings, they are typically avoided as immunogens, however the development of nucleotide-based technology has unlocked the possibility of using such immunogens, as the immunogen itself is produced *in vivo*. This enables the use of native signal peptides, transmembrane domains, and the production of viral immunogens within the host. All of these factors will result in immunogens with more native-like glycosylation compared to those that are soluble, protein-based. Likewise, many of the strategies to engineer glycans beyond their native-like state that have been outlined in this review can be enlisted in nucleotide-based vaccines, such as PNGS deletion, and glycan occupancy engineering by N-X-S/T codon modification. This is not feasible across all glycan engineering strategies, most notably the global modification of N-linked glycans through knock-out cell lines, inhibitors, and glycosidase treatment, as these are difficult to currently perform *in vivo* and in human clinical trials. As such, glycan engineering in the era of nucleotide-based immunogens will require further innovation beyond classical approaches and represents a new frontier for glycoengineering.

Overall, the prevalence of glycosylation amongst viral proteins provides both barriers and opportunities in vaccine design. Without the conserved nature of the HIV-1 Env glycan shield, many bnAbs would not be able to bind, which bypasses the hypervariable protein surface. Therefore, the study of viral glycosylation and subsequent development of glycan engineering toolkits is beneficial not only for structural studies of viral proteins, but for the improvement of viral vaccines. It is hoped that innovations in this area will accelerate the development of efficacious vaccines against viruses that have proven challenging using classical vaccination strategies.

CRediT authorship contribution statement

M.L. Newby: Conceptualization, Investigation, Visualization, Writing - original draft, Writing - review & editing. **J.D. Allen:** Conceptualization, Investigation, Supervision, Writing - original draft, Writing - review & editing. **M. Crispin:** Conceptualization, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors for the manuscript titled *Influence of glycosylation on the immunogenicity and antigenicity of viral immunogens* declare no conflicting interests as outlined by Biotechnology Advances.

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