Conference Report: “Functional Glycomics in HIV Type 1 Vaccine Design” Workshop Report, Bethesda, Maryland, April 30–May 1, 2012

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Abstract

A vital part of the renewed hope for a vaccine against the human immunodeficiency virus (HIV-1) is based on recent studies that have highlighted major sites of HIV-1 vulnerability that could be effectively targeted by a preventive vaccine. One of these potential vulnerabilities includes the dense cluster of carbohydrates surrounding HIV-1's envelope glycoproteins gp120 and gp41, typically referred to as the “glycan shield.” Recent data from several laboratories have shown that glycans on the HIV-1 envelope form key epitopes for broadly neutralizing antibodies (bNAb). Moreover, HIV-1 envelope glycans play an important role in viral transmission, antigenicity, and immunogenicity. The recent availability of novel tools and technologies has now allowed investigators to leverage glycomic structure–function relationships in the design of candidate HIV-1 vaccines. Additionally, glycans modulate the immune response, playing an essential role in Fc receptor and complement activity. To promote cross-disciplinary collaboration and promote synergistic HIV-1 glycomics research, the National Institutes of Health (NIH) cosponsored and convened a 1.5-day workshop entitled “Functional Glycomics in HIV-1 Vaccine Design.” The meeting focused on the role of glycan interactions with neutralizing antibodies, the influence of immunoglobulin G (IgG) Fc receptor glycosylation, newly available glycomics technologies, and how new information on the role of glycans could be applied in HIV-1 immunogen design strategies. This report summarizes the discussions of this workshop.

Introduction

THE VACCINE RESEARCH PROGRAM of the Division of AIDS (DAIDS) of the National Institutes of Health cosponsored a workshop entitled “Functional Glycomics in HIV-1 Vaccine Design.” The workshop objectives were to (1) gather investigators and relevant stakeholders to share the latest information on newly available tools and novel approaches in HIV-1 glycomics research, (2) discuss opportunities for collaboration, and (3) promote multidisciplinary cross-talk between glycomics researchers and HIV-1 vaccine researchers. The meeting, chaired by Anne Dell and Galit Alter, brought together glycobiochemists, virologists, immunologists, clinicians, carbohydrate chemists, structural biologists, funding agencies, and other relevant experts, with over 300 registrants.

Importantly, this workshop sought to leverage the investments of the National Institute of General Medical Sciences (NIGMS) Consortium for Functional Glycomics (CFG), as well as other NIH Institutes and Centers, in the development of novel tools and technologies to advance glycomics research. A recent National Academy of Sciences report highlights some of the innovative achievements in glycoscience providing a blueprint for the United States to maintain global preeminence in the decades to come.¹

Glycans, along with nucleic acids, proteins, and lipids, comprise one of the four fundamental classes of macromolecules of biological systems. Glycosylation is the most common posttranslational modification, a nontemplate process resulting from a progression of biochemical steps occurring in the endoplasmic reticulum and the Golgi apparatus.^{2,3} A wide

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array of enzymes called glycosyltransferases and glycosidases create complex glycoconjugates with structural profiles that significantly differ according to cell type, stage of cellular development, and tissue expression.³ While this contributes to functional diversity, it also makes analysis of glycan patterns difficult. It is now possible, with available tools that probe glycan structure and function, to begin to incorporate glycomic approaches into broader studies that interrogate the role of glycans in biological processes, such as innate and adaptive immune responses to HIV-1. The investigators at the meeting (Table 1) outlined several studies where collaborative approaches have led to fundamental shifts in our understanding of HIV-1 biology.

Interfacing Functional Glycomics with Immunobiology of Infections

Various techniques and methods were discussed for application in HIV-1 vaccine design studies including semisynthetic glycoconjugates, nuclear magnetic resonance (NMR), and mass spectrometry (Table 2). Dr. Cummings discussed the specific molecular recognition of defined glycoconjugates and the contributions of carbohydrate structures to biology, highlighting the implementation of glycan arrays to identify and characterize glycan binding proteins (GBPs). The NIGMS

funded CFG was pivotal in the development and uptake of glycan arrays by a broad range of laboratories, including work by Dr. Wilson's laboratory to identify glycan epitopes recognized by broadly neutralizing antibodies (bNAbs).⁴ Cumming's laboratory developed a microarray method, called "shotgun glycomics" where total glycans are released from glycoconjugates extracted from cells, and free glycans can be derivatized with bifunctional fluorescent dyes.⁵ The tagged glycans are purified into a defined library, quantified, and covalently printed on glass slides for screening with GBPs of interest.⁶

Dr. Prestegard discussed NMR spectroscopy and strategies that have demonstrated accessibility and dynamics of protein glycosylation. NMR usually begins with recombinant proteins that are isotopically labeled, or with isotopically labeled sugar residues, which are then remodeled on proteins. One of the available techniques in NMR for studying protein-carbohydrate interactions in solution is saturation transfer difference nuclear magnetic spectroscopy, or STD-NMR. STD-NMR is a robust method, but limited, as it detects fragments of carbohydrates. It does not require isotopic labeling, and small amounts of protein are sufficient for analysis. A complementary source of structural information on a bound ligand can also be obtained by transferred NOE (trNOE).⁷

Dr. Prestegard presented NMR work from his laboratory with dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN), a membrane protein best known for its recognition of mannose residues on pathogens,⁸ and with IgG Fc.⁹

Dr. Desaire presented work on the structural characterization of glycoproteins using mass spectrometry (MS) to identify N- and O-linked glycans.¹⁰ The majority of the work in Dr. Desaire's laboratory is on N-linked glycans, but using the example of gp120, nine different isoforms were reproducibly detected at a single O-linked site, and were confirmed by collision-induced dissociation (CID) and electron transfer dissociation (ETD) approaches.^{11,12} For 25 different sites in a protein, her team typically analyzed over 300 unique glycoforms per peptide. The workflow with glycans typically entails cleaving the carbohydrates from the protein and studying the two entities separately. In contrast, with glycopeptide interrogation, the protein itself is digested, yielding peptides with the glycans still intact, permitting the determination of glycan heterogeneity at individual sites.

An ongoing concern is that MS studies have used recombinant gp120 as opposed to gp120 from virus-infected cells due to the large amount of protein required (~100 µg). The limitations of this approach may prove crucial in vaccine and immunogen design studies, since the remarkable heterogeneity seen with recombinant proteins may be greater than that seen with native glycoproteins. Dr. Desaire presented data suggesting that glycosylation site occupancy was very similar between Chinese hamster ovary (CHO) and 293T cell-derived transmitted/founder gp120; however, minimal differences in glycan profiles may critically impact envelope immunogenicity.¹²

Glycan Sites of Vulnerability in the HIV-1 Envelope Trimer

This session focused on conserved glycan sites that serve as targets for the immune response and/or are critically important for viral transmission.

TABLE 1. MEETING INVESTIGATORS

<i>Speakers</i>	<i>Affiliations</i>
Alter, Galit	Ragon Institute
Arthos, James	National Institutes of Health
Barnett, Susan	Novartis Vaccine and Diagnostics, Inc.
Berman, Phillip	Baskin School of Engineering
Bewley, Carole	National Institutes of Health
Burton, Dennis	Scripps Research Institute
Cardozo, Timothy	NYU School of Medicine
Cobb, Brian	Case Western Reserve University
Cummings, Richard	Emory University
Dell, Anne	Imperial College of London
Desaire, Heather	University of Kansas
Desrosiers, Ronald	Harvard Medical School
Feizi, Ten	Imperial College of London
Hioe, Catarina	NYU Langone School of Medicine
Hu, Shiu-Lok	University of Washington
Kwong, Peter	National Institutes of Health
Lu, Shan	University of Massachusetts Medical School
Magnani, John	GlycoMimetics, Inc.
Moore, Penny	National Health Laboratory Service, RSA
Nabel, Gary	Sanofi Pasteur
Nimmerjahn, Falk	University of Erlangen-Nürnberg, Germany
Paulson, James	Scripps Research Institute
Pierce, Michael	University of Georgia
Prestegard, James	University of Georgia
Sodroski, Joseph	Dana-Farber Cancer Institute
Wang, Denong	Stanford Research Institute
Wang, Lai-Xi	University of Maryland
Wilson, Ian	Scripps Research Institute
Woods, Robert	University of Georgia

TABLE 2. TECHNIQUES AND METHODS

<i>Technique</i>	<i>Application</i>	<i>Example</i>
Cryo-EM	Cryo-electron microscopy: allows observation of biological specimens at cryogenic temperatures in their native state	Subunit organization of the membrane-bound HIV-1 envelope glycoprotein trimer ³⁴
STD-NMR	Saturation-transfer difference nuclear magnetic resonance: allows study of protein–ligand interactions	Structure of the HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9 ²⁷
trNOE	Transferred Nuclear Overhauser Effect: allows for recognition of ligands for a given target protein in a mixture of compounds, or the identification of pairs of molecules that bind to a protein simultaneously on adjacent sites; can be used to determine bioactive conformation of peptide-ligands	NMR structural characterization of substrates bound to <i>N</i> -acetylglucosaminyltransferase V ⁷
MS	Mass spectrometry: allows determination of the mass of a molecule, such as a carbohydrate or peptide, by measuring the mass to charge ratio (<i>m/z</i>) of its ion. Glycopeptide analysis identifies glycosylation characteristics at individual sites of glycan attachment	Characterization of glycosylation profiles of HIV-1 transmitted/founder envelopes by mass spectrometry ¹²
Glycan arrays	Arrays that allow analysis of glycan binding specificity of suspected glycan binding proteins (GBPs) such as lectins, antibodies, etc. Natural and synthetic glycans are typically printed onto slides	A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield ⁵

Glycan modulation of the HIV-1 Env antibody response

For many licensed vaccines, neutralizing antibodies represent the best correlate of protection.^{13,14} Consequently, the development of an effective HIV-1 vaccine will likely require the design of immunogens that elicit bNAbs to overcome the high sequence variation in HIV-1 Envs.¹⁵ However, bNAbs against the HIV-1 Env glycoproteins have been difficult to elicit by immunization. Dr. Nabel discussed reverse vaccinology approaches at the Vaccine Research Center (VRC) and the central role glycans have played in these efforts. Information on the structural basis of the interaction of bNAbs with HIV-1 Env may be useful in the development of candidate immunogens, as well as to probe the efficacy of elicited antibodies.

Recent work has demonstrated that several human monoclonal antibodies have been isolated from HIV-1-infected patients that neutralize HIV-1 potently.^{16–25} Specifically, many of these new bNAbs recognize conserved glycopeptide epitopes.^{4,18,19}

At the VRC, structure-based vaccine design approaches have largely focused on eliciting Abs against the conserved CD4 binding site (CD4bs) that is vulnerable to several known bNAbs. The efforts include the use of various forms of the HIV-1 Env such as monomers, trimers, and multimerized outer domains.²⁶ The artificial introduction of glycans to mask immunodominant surfaces was required in some of the immunogens to restrict the specificity of the immune response *in vivo*. This served as a proof of concept that in general, glycan masking can be used to focus the immune response. Neutralization profiles of the antibodies generated by these immunogens were directed only against Tier 1 viruses, but not Tier 2 or Tier 3 viruses.

To begin to determine the mechanisms or structures responsible for the varying neutralization patterns elicited by

different HIV-1 constructs, glycan modifications were introduced that left intact the binding of high neutralizing Abs such as VRC01 and VRC-PG04, while engineering out reactivity with weaker or nonneutralizing Abs such as b12 and b13. By enforcing an angle of approach to the CD4bs similar to that of the bNAbs, it may be possible to generate successful VRC01-like responses. However, there is no “magic bullet” on how to address lowering the immunogenicity of unwanted epitopes in a construct by adding glycans while retaining overall immunogenicity. Moreover, the effects of trimer modifications have been difficult to reproduce from strain to strain in part because of the heterogeneity in glycosylation and variable regions. Importantly, structures identify targets, but do not predict if an immunogen will license B cells to mature and generate protective Abs.

In addition to the CD4bs, Dr. Nabel discussed another site of vulnerability identified by PG9-like antibodies that require N-linked glycans. Dr. McLellan and colleagues showed that the bNAb PG9 intimately binds two Man₅GlcNAc₂ attached to Asn-160 in the V1/V2 fold of HIV-1 gp120.²⁷ The glycan recognition residues define conserved motifs that may be critical in immunogen design, and have also been implicated in immune evasion.²⁸ Transplants of the minimal V1/V2 epitope onto protein scaffolds, which retain binding to PG9, have been created and used as probes for screening patient sera and as immunofocusing immunogens to elicit V1/V2 antibodies. These structures have been expressed in a single protein, or self-assembling particles such as ferritin, to gain multimeric expression and will be tested in animal models.

Antibody recognition of the HIV-1 Env glycan shield

Dr. Wilson discussed the recognition of HIV-1 Env glycans by a new family of MAbs, the PGT antibody family.¹⁹ These

bNAbs are very potent, suggesting that they may provide protection at relatively low serum concentrations. The epitopes recognized by several members of the PGT family identified a new conserved V3-glycan site of vulnerability. It appears that antibodies from this family all recognize glycans attached at position N301 or N322 with a diverse mode of recognition and with a range of affinities. The conformational flexibility of carbohydrate attachment might favor immunogen design by providing a broader, more promiscuous vaccine target.

Dr. Wilson described an example from the PGT125–130 family, PGT128.⁴ Using glycan arrays and the crystal structure, his laboratory showed that PGT128 penetrates the glycan shield and recognizes two conserved glycans (Man_{8/9}GlcNAc₂), attached to N332 and N301, as well as a short β -strand segment of the gp120 V3 loop, accounting for its high binding affinity and broad specificity. Although glycan flexibility exists, from the alpha(1–6) linkages, the glycan recognition site appears structurally conserved and a promising potential mimic in immunogen design. In this regard Dr. Cardozo briefly presented ongoing studies from his laboratory that precisely identified hidden, conserved structures on the variable loops that could be considered as potential targets for immunogen design.^{29,30}

Another member of the PGT family was described, PGT135, which shows another mode of interaction to N332 that involves a complex interplay of glycans. The bNAb 2G12, a naturally occurring dimer resulting from domain exchange,³¹ also recognizes glycans in the same region attached at N332. However, the epitope is unique and is formed by a cluster of N-linked high-mannose glycan groups. These data suggest that bNAbs, like PGT128, PGT135, and 2G12, while recognizing distinct epitopes, also share a common target, N332.³² N332 has been linked to immune escape in work by Dr. Moore that shows the influence of autologous strain-specific and broadly neutralizing antibodies on Env glycan evolution.³³

A role for an O-glycosylated threonine in HIV-1, SIV, and influenza

Dr. Desrosiers discussed the role of O-linked carbohydrates in envelope glycoproteins as potential vulnerable sites. Dr. Desrosiers' laboratory has discovered a highly conserved C-terminal threonine as a target of O-glycosylation in HIV-1 and SIV gp120; this site is critical for virus infectivity. Surface glycoproteins from other families of enveloped viruses, such as influenza A, have a similarly placed, highly conserved C-terminal threonine that they have either demonstrated is O-glycosylated or is predicted to be O-glycosylated. The O-linked glycan appears to be essential in stabilizing gp120 on the surface of cells and the surface of virions. This finding opens up the possibility of a better understanding of the mechanism of association between gp120 and gp41 with the potential to identify a new conserved target for antiviral drug development.

Architecture of the HIV-1 envelope glycoprotein trimer

Dr. Sodroski discussed the architecture and function of the HIV-1 envelope glycoprotein trimer.³⁴ In HIV-1-infected cells, envelope glycoproteins are synthesized as a heavily glycosylated trimeric gp160 precursor, which is cleaved into three gp120 exterior Env glycoproteins and three gp41 transmembrane glycoproteins. This is followed by trimer movement to

the cell surface and incorporation into virion particles. Dr. Sodroski's laboratory employed single-particle cryo-EM to reconstruct the trimer three-dimensional structure from a large number of images of purified protein complexes.³⁴

The architecture of the the unliganded HIV-1 Env trimer is significant because it (1) explains the metastable nature and helps researchers understand receptor triggering of virus entry, (2) provides information about neutralizing antibody epitopes, and (3) reveals viral defenses against the binding of neutralizing antibodies (quaternary constraints, glycans). The unliganded trimer architecture seems to be held in place by three main areas of contact between Env monomers: the transmembrane region of gp41, the gp41 and gp120 inner domain interaction, and the trimer association domain (TAD) formed by the variable loops. The carbohydrate profile of the TAD contributes to structural stability.

The presence of glycans, while not directly bound to the receptor binding site, can influence the accessibility of antibodies to epitopes on the trimer surface. This work shows that while the epitope is important, so is the angle of approach.³⁴ Potent neutralizing antibodies bind the HIV-1 envelope glycoprotein trimer with minimal steric clashes with adjacent protomers.

Dr. Hanover, who joined the panel discussion, asked several relevant questions. (1) What controls the extent of glycan modification in a cell that is already stressed by infection? (2) What does viral infection do to the Golgi and to the N-glycosylation pathway?

HIV-1 Immunogen Design

The session on HIV-1 Immunogen Design discussed strategies to generating protective antibodies.

Insight on elicitation from naturally occurring N160-directed broadly neutralizing HIV-1 antibodies

Dr. Kwong discussed how structural information of the bNAb-bound epitope, combined with next generation sequencing and bioinformatics, contributes to our understanding of the B cell ontogeny of a protective glycan-specific neutralizing antibody response.

Dr. Kwong's laboratory sought to define the epitopes of the two antibodies PG9 and PG16, which are somatic variants.¹⁸ Both antibodies bind a glycosylated epitope in V1/V2 and preferentially bind the trimer over the monomer. Structural differences in the antibodies were suggested because V3 mutations greatly affected PG16 over PG9, translating into dissimilarities in quaternary specificity.³⁵

Structure–function analysis of the crystal structure of PG16 indicated that affinity maturation along with long heavy chain-third complementarity-determining region (CDR H3) were necessary for effective neutralization.^{35–37} They demonstrated that PG16 has developed a second glycan interaction site probably to compensate for weaker binding with the protein. The ability of somatic variants to recognize different N-linked glycoforms may increase the breadth of HIV-1 Env recognition illustrating the importance of a polyclonal response against differential glycosylation exposed on the HIV-1 Env.

Another area of focus is the evolution or the maturation pathway of bNAbs by 454 pyrosequencing and bioinformatics to define paths by which to obtain antibodies that

effectively neutralize HIV-1.¹⁷ Interestingly, the analysis of phylogenetic trees of PGTs 141–145, which are in the same clonal family and have the same heavy/light chain(s) origin genes,^{19,38} showed overall similarities in antibody evolution and in degree of divergence.

Glycoform heterogeneity in vaccine antigens used in the VAX003 and RV144 trials

Dr. Berman discussed recent characterization of the vaccines used in the VAX003 and RV144 Trials with respect to carbohydrate analysis.³⁹ Both the VAX003 study and the RV144 study used the same vaccine design platform: bivalent AIDSVAX B/E vaccine prepared from gp120s from the MN and A244 strains of HIV-1. Both proteins were made in CHO cells and purified by immunoaffinity chromatography (no selection for a particular type of carbohydrate).

Uniform glycosylation is very difficult to control in vaccine development, especially with a highly glycosylated protein such as gp120. The challenge lies in the ability to replicate the carbohydrate structure of protein antigens used in the RV144/VAX003 trials and in follow-up studies with new envelope proteins. Glycosylation of the protein affects formulation, stability, biodistribution, and antigenicity among other factors. Glycosylation also varies as a function of cell line, culture media, and virus strain. Often, a higher protein expression level results in reduced sialic acid incorporation, which may in turn severely affect protein half-life.

The approach used by Dr. Berman's group to characterize the glycosylation on MN and A244 HIV-1 gp120s was isoelectric focusing, measuring gross differences in net charge. Isoelectric focusing can be coupled with endoglycosidase digestion to determine the extent of high mannose and complex carbohydrates on proteins. N-linked carbohydrates can be divided broadly into the high mannose form, the hybrid form, and the complex form.³ With MN/CHO, 16 different glycoforms were distinguished on gp120; with A244/CHO, 24 different glycoforms were identified. Proteins expressed in HEK 293 cells exhibited less sialic acid incorporated. With A244/293, more than 40 different glycoforms were resolved. Thus, the MN and A244 envelope proteins used in the RV144 trial exhibit far more variation in net charge and glycosylation than previously described. Consideration should be given in generating synthetic and homogeneous HIV-1 Env glycoproteins for vaccine development, since glycosylation patterns may not reflect the glycosylation profiles found in circulating HIV-1 strains or the quasispecies to which humans are exposed. Importantly, the glycosylation state of the HIV-1 envelope *in vivo* is still unknown.^{40–43}

Dr. Berman and his team also examined antibody binding of the gp120 proteins and found that cell line-dependent glycosylation differences affect the binding of neutralizing antibodies to the CD4bs and V2 domain.

Reports in the literature have indicated that the relative positions of glycosylation sites may affect the extent of post-translational modification and trimming.^{44,45} In vaccine development, it would be good to cover as much glycan diversity as possible.

Targeting the glycan shield of HIV-1

Dr. Burton presented immunogen design work based on 2G12.⁴⁶ As noted by Dr. Wilson, 2G12 has a very unusual

domain exchange structure that explains the high affinity and ability to bind closely spaced epitopes.³¹ The 2G12 interactions became clear with the crystal structure of 2G12 in complex with Man₆GlcNAc₂ and the revelation of a novel form of paratope at the V_H–V_H interface.⁴⁷ While several immunogens have been investigated on the 2G12 model, none of the generated antibodies significantly cross-reacted with gp120 or neutralized the virus.^{48–53}

To investigate the minimal requirements for domain exchange, the researchers engineered a germ-line form of 2G12 that behaved like a conventional antibody.⁵⁴ The investigators found that domain exchange can result from a small number of substitutions in key structural areas suggesting that mutations, as part of the evolutionary process of antibody development, could lead to domain exchange. A nondomain exchanged version of 2G12 was generated with substitution of a single critical residue 2G12. Although both forms of 2G12 recognize di-mannose equivalently, only the domain-exchanged form binds the HIV-1 glycan shield.⁵⁵ Domain exchange is irreversible. Importantly, it can occur with great preservation of antigen recognition. These factors suggest a pathway to inducing domain exchange in antibodies.

During the panel discussion Dr. Barnett stated that two concerns in vaccine development related to glycobiology are (1) utilizing glycomics tools to control the heterogeneity in a vaccine product, particularly during scale-up, and (2) generating the methods to optimize elicitation of specific protective antibodies. Additionally, consideration of the roles of delivery systems, along with adjuvants, and the impact of adjuvants on the formulation of candidate vaccines will likely influence the immune response.

Dr. Woods noted that advances in molecular biology/protein-engineering, including the routine ability to make point mutations in protein sequences, have allowed greater understanding about how sequence influences structure and function. By contrast, changes in glycan structure cannot be done easily, nor the characterization of protein interactions. However, use of computational simulation has greatly expanded, and three-dimensional structure analysis and dynamics of glycans are commonplace.⁵⁶ Virtual glycan arrays can be screened to highlight the structures that should probably be targeted experimentally.

Dr. Magnani briefly discussed small molecule designs pioneered by GlycoMimetics to stabilize the core structures of glycomimetic compounds and increase binding affinity by reducing entropy costs.⁵⁷ A theory concerning HIV-1 gp120 is that high mannose structures identified by bNAbs are functional carbohydrates. Functional carbohydrates may target the virus particles to a portal of infection, such as DC-SIGN on the dendritic cells, which may in turn control mucosal entry. However, the virus does not control glycosylation as this is a host cell function, although it is conceivable that viral infection has an effect on the host's glycosylation machinery. Alternatively, the virus may modulate the peptide backbone (by mutations), and thus manipulate glycosylation sites.

Dr. Hu's laboratory described his team's focus on vaccine design, immunization strategies, and evaluation of vaccines in animal models. His team determined that modulation of a single glycan at N197 can result in an immunogen that can elicit neutralizing antibody responses in monkeys.⁵⁸ N197 glycan, in a highly conserved region of gp120, modulates Env function, affecting both antigenicity and immunogenicity.

Glycans in Immune Function and Viral Transmission

Impact of antibody glycosylation on IgG Function in vitro and in vivo

Dr. Nimmerjahn discussed the function of IgG glycosylation variants through the recruitment of innate effector cells by Fc receptor (FcR) engagement on those cells. Thirty different IgG glycosylation variants can be identified in human serum in the steady state, and several hundred IgG glycosylation variants exist.⁵⁹ It is unclear why such diversity exists and what the function of the different IgG glycovariants is.

Given that a vaccine elicits polyclonal antibodies, and each of the different subclasses of IgG (IgG1–4) has a specific effector function, glycosylation for some isotypes may be more important than for others. IgG antibodies can be proinflammatory or antiinflammatory; these functions can be modulated through differential glycosylation.⁶⁰

It has been shown that antibodies generated without fucose bind much better to activating Fc gamma receptors, translating into higher antibody activity in an antibody-dependent cell-mediated cytotoxicity (ADCC) assay.^{61–64} In much of the human antibody repertoire, glycosylation has high fucose, so it may not be easy to generate defucosylated Abs during vaccination. Antibodies without fucose actually bind better to only one type of Fc gamma receptor, FcγRIIIA. However, in humans, as opposed to cell lines, more types of Fcγ receptors are available for possible interactions. Dr. Burton's laboratory has shown in a passive protection SHIV model that in the absence of fucose, no enhanced protection occurred in non-human primates (NHP) *in vivo* despite increased IgG binding to FcγRIIIA *in vitro*,⁶⁵ suggesting that other Fcγ receptors may be important for protection from infection.

IgG isotype significantly impacts *in vivo* immunoglobulin activity regardless of glycosylation; however, induction of a-fucosylated HIV-1-specific IgG by vaccination may still be beneficial. Galactose residues do not change IgG activity even though the number of galactose residues on antibodies may decrease during inflammation and sialic acid residues have been shown to be critical for the antiinflammatory activity of IgG.⁶⁶ In HIV-1 vaccine development, the focus should be on all Fcγ receptors. For example, FcγRIIA has a high affinity for human IgG2 while FcγRIIIA has a high affinity for IgG1. Experiments show these factors can affect the inhibition of HIV-1 infection and may be the relevant receptors *in vivo*.⁶⁷ It has also been suggested that FcγRIIIA may be the functional receptor in antibody-dependent cell-mediated viral inhibition (ADCVI) assays whereas FcγRIIA, expressed on neutrophils and macrophages, may be more important for phagocytosis and immune clearance.^{68–70} Several questions remain. (1) What are the functions of the different IgG glycosylation variants? (2) Which cell types and which FcγRs are crucial for anti-HIV-1 IgG activity? (3) How can we generate specific IgG glycovariants by vaccination? While a vaccine has the ability to alter the glycosylation structure of the immune system, where and how these changes occur are open questions.

Sugar coating antibodies to kill HIV-1

The immune system induces natural variation in antibody glycosylation. Dr. Alter discussed how we might manipulate antibody glycans in a targeted manner, such as through vaccination. Many innate immune receptors respond to changes

in glycosylation associated with self signals and nonself signals and Dr. Alter's laboratory is focusing on how glycans on Fc receptors influence the engagement of receptors on innate immune cells such as natural killer (NK) cells.

Glycans attached on the Fc portion of antibodies have been shown to play a critical role in the recruitment of NK cells; their removal completely abrogates this NK cell activity.^{71,72} During HIV-1 infection, HIV-1-specific glycan production becomes markedly skewed.⁷⁰ Alter's team has observed that a group of individuals who spontaneously control HIV-1 infection, elite controllers, is more prone to elicit antibodies that recruit NK cells, leading to control of viral replication. This occurs in the absence of known protective HLA alleles. Notably, the pool of antibodies made by these same individuals contained fewer fucosylated glycoforms, and a-fucosylation has been shown to allow better ADCC activity.^{61–64} Additionally, (1) the bulk pool of antibodies is less galactosylated (proinflammatory) following HIV-1 infection and (2) HIV-1-specific Abs are less galactosylated, less fucosylated, and less sialylated than bulk Abs in patients who are spontaneously controlling HIV-1 infection.^{70,73} This observation suggests that this altered antibody glycosylation is antigen specific, and as such there are mechanisms by which vaccines may tune this function to optimally induce antibodies with enhanced antiviral activity. By examining glycosyltransferase gene expression in patient B cell populations to query whether this influences the generation of good ADCC recruiting Abs, correlations were found between effector function and glycosyltransferase expression.

N-linked glycosylation in gp120s derived from early-transmitting isolates of HIV-1

Dr. Arthos discussed glycosylation in the mucosal transmission of HIV-1. HIV-1 transmission is highly inefficient; during a transmission event, the quasiespecies in the donor contracts through an extreme genetic bottleneck in the recipient.^{74–76} Consequently, researchers have great interest in the biochemical and antigenic features of early transmitting envelopes that can be exploited in the context of a vaccine immunogen. An important genotypic signature of early-replicating viruses is a relative reduction of potential N-glycosylation sites (PNGs) in V1/V2 and C3 regions of gp120.^{74,75} It is the immune pressure, particularly Ab-mediated pressure, that drives the increase in PNGs.^{77–79} Additionally, a second signature is a basic residue at amino acid position number 12 in the gp160 leader peptide, the part of the protein that does not appear in the mature envelope.⁸⁰

Although reduced glycosylation is associated with transmission, the basis for this increased "transmission fitness" is unknown. Evidence supports the conclusion that removal of PNGs from gp120 can change the affinity for receptors, antigenicity, and neutralization sensitivity.^{81,82}

Dr. Arthos elaborated on the second signature identified where a basic amino acid at position 12 is overrepresented in the leader peptides of early-transmitting Envs.⁸⁰ Early/chronic chimeric gp120s were constructed in order to study the influence of the leader-peptide on glycosylation.

Preliminary and ongoing experiments showed that (1) the leader peptide of an early-transmitting gp120 can modulate glycan addition. (2) Addition of an early-transmitting leader sequence to a chronic gp120 alters glycan processing,

increases reactivity to DC-SIGN, and alters the structure of the mature gp120 subunit. (3) Addition of a chronic leader sequence to an early-transmitting gp120 alters glycan processing.

The underlying mechanism regarding leader changes is being analyzed by the Arthos team and others.^{43,83,84} One possibility is that leader sequences are cleaved at different rates such that longer retention in the ER results in more processing of the gp120 that is ultimately glycosylated.

The panelists discussed how host glycans impact the immune response. Changes in host glycosylation patterns can usually be associated with varying and altered states within the host, and cytokines can alter host glycosylation patterns.⁸⁴ The functional significance of the alterations in glycosylation patterns is often not clear. A whole host of systems and pathways within the immune response is targeted toward recognizing and modulating glycans.^{85–87} HIV-1 envelope glycosylation is very context dependent within the host; the glycosylation of HIV-1 Env may appear different in active viremia versus long-term control of HIV-1. Whether the virus has hijacked cell machinery to skew the glycan patterns, or the differences in glycosyltransferases reflect inflammation, these

studies are interesting and important. Notably, the glycan profiles seen in acute influenza are completely different from those elicited in acute HIV-1 infections.⁸⁸ Importantly, additional data have shown that the vaccination protocol, immunogen, and adjuvant can drastically modulate the Ab glycoprofile.^{89,90} As mentioned above, glycan changes associated with HIV-1 infection also impact Fc antibody activity.⁹¹ The hope of HIV-1 research is to harness the complexity into insights that will improve infectious disease outcome.

Dr. Moore reminded the participants that in the context of a natural infection, the virus itself is also changing in addition to host factors. In studies, in patients who develop bNAbs, the glycan targeted by the later crop of bNAbs is often absent on the transmitted founder virus. For example, the glycan at N332 in the C3 domain is absent at transmission but arises as a consequence of neutralization escape.³³ It is known that many viruses have evolved mechanisms to alter antigen processing and avoid detection by the immune system.^{92–94}

Dr. Hioe discussed the effect of glycans on helper T (Th) cell epitopes. Her laboratory has reported that N-glycans in near proximity to CD4 T cell epitopes on HIV-1 gp120 are critical for efficient processing, generation, and recognition of the

TABLE 3. OPPORTUNITIES AND CHALLENGES

Area	Opportunities	Challenges
Functional glycomics	New tools and approaches (Table 2) Determination of glycan composition and positioning Computational simulation of shape and motion of glycans to define protein-Ab bound conformations	Decipher the contribution of glycosylation to biological pathways
Sites of vulnerability	Structural information of the bNAb-bound epitope conformation Conserved structural motifs involving N-glycans Better understanding of the architecture of the HIV-1 envelope glycoprotein trimer Highly conserved O-glycosylated C-terminal threonine in HIV-1/SIV gp120	Unknown glycan composition of the circulating viral strains Glycan heterogeneity Conformational flexibility of the glycans: Mechanisms to stabilize glycans for immunogen design Link glycans to specific functions
Immunogen design	Manipulation of glycans to improve antigenicity Better understanding of the maturation lineages of bNAb by next generation sequencing and bioinformatics Computational simulation to predict 3D structures and dynamics of glycans Glycomimetic compounds directed to exposed vulnerable sites	Manipulation of glycans to improve immunogenicity Cell line-dependent glycosylation of the vaccine immunogens Identification of the best animal model to test immunogens directed to glycans and peptide/glycan structures Autoimmunity
Immune function	Glycosylation is critical for the IgG pro- and antiinflammatory activity Ab effector functions in some HIV controllers correlate with protection from infection Absence of fucose increases IgG binding to FcγRIIIA (however, passive protection against HIV infection is not changed)	Function of the different IgG glycosylation variants Generation and preservation (memory) of Ab glycosylation variants by vaccination
Viral transmission	Better understanding of the evolution of glycan diversity on the viral surface Better understanding of the role of glycans in bNAb development Role of virus leader sequence for glycan processing and glycan addition	Glycan epitopes targeted by some of the bNAbs are often absent on the transmitted founder virus Role of early-transmitting gp120 leader sequence in viral transmission

bNAb, broadly neutralizing antibody; SIV, simian immunodeficiency virus; 3D, three dimensional; FcγRIIIA, Fc gamma receptor.

epitopes by T cells.⁹⁵ MS analyses indicate the glycans help to maintain the surface structure of the downstream epitope. In engineering glycan sites in gp120, the effects on both the B cell epitope and the T cell epitope should be considered.

Dr. Cobb remarked that for microbial polysaccharides, one of the roles of antigen-presenting cells (to CD4⁺ T cells) is cleavage of carbohydrates, which they do with exquisite selectivity.⁹⁶ The pathway is oxidative and dependent on inducible nitrate oxide synthase; this type of nonenzymatic processing may also be effective with glycoproteins. Dr. Hioe added that high mannose glycans tend to be taken up better by dendritic cells.

Conclusions

In light of recent findings illustrating the importance of glycans in tuning antibody effector functions, in the evolution of broadly neutralizing antibody responses as well as their role as potential targets of HIV-1 vulnerability, more efforts should be focused on the exploration of new leads at the intersection of HIV-1 and glycomics research. As highlighted in Table 3, several opportunities and challenges were identified and discussed at the workshop. Advances in these areas could accelerate the development of an HIV-1 vaccine that exploits the modification of glycans in HIV-1-specific protective responses.

With regard to Env glycans, there was consensus among the participants that the elucidation of the function of N- and O-linked glycans on the trimer, beyond the role of a shield against the immune system, will be critical for effective vaccine design. Importantly, we do not yet know the true glycan profile of any *in vivo*-derived HIV-1 Env proteins. Moreover, during the course of HIV-1 infection, the number and distribution of PNGs may change as part of the viral escape strategy.

Given this radical difference in glycan composition, coupled with the fact that recombinant gp120 has been used extensively in animal studies and human vaccine trials, consideration should be given to the impact of differing glycosylation patterns when manufacturing potential HIV-1 vaccine candidates.

The contributions by a range of host cell innate immune lectins that bind HIV-1 glycans in inhibiting or enhancing HIV-1 infection may also provide clues on how to exploit innate immunity in immunogen design.

With regard to the glycans bound to the Fc portion of the antibodies there was consensus during the workshop discussions that the definition of their role in anti-HIV-1 activity will be of utmost importance, both in the setting of vaccination and in passive protection trials.

In conclusion, novel approaches in HIV-1 vaccine design that exploit the modification of glycans in HIV-1-specific protective responses are needed if we are to accelerate the development of an HIV-1 vaccine, although conformational variation and autoimmunity present challenges. The tools are now in hand for glycan characterization and the refinement of structure–function relationships in quantitative and qualitative terms.

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