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Emerging glycobiology tools: A renaissance in accessibility

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Abstract

The glycobiology of the immune response is a topic that has garnered increased attention due to a number of key discoveries surrounding IgG function, the specificity of some broadly neutralizing anti-HIV antibodies, cancer immunoregulation by galectin molecules and others. This review is the opening article in a Special Edition of *Cellular Immunology* focused on glycoimmunology, and has the goal of setting the context for these articles by providing a mini-review of how glycans impact immunity. We also focus on some of the technological and methodological advances in the field of glycobiology that are being deployed to lower the barrier of entry into the glycosciences, and to more fully interrogate the glycome and its function.

1. Overview

Glycobiology is the study of carbohydrates, their conjugates, their enzymatic synthesis, modification, transport, and their binding partners. Like nucleic acids, lipids, and proteins, glycans impact the entirety of known biology, from the simplest of single cell organisms and viruses to humans. As a result, asking whether glycobiology impacts any particular biological phenomenon is akin to asking whether proteins are involved with a particular biological phenomenon. The answer is essentially always yes, though the impact could be direct or indirect [1]. However, a number of characteristics, some intrinsic and others extrinsic, have blunted the size and relative impact of glycobiology over the decades.

Intrinsically, glycobiology suffers from reductionism. Carbohydrate synthesis and protein glycosylation are driven by enzymes in a non-templated fashion, making the resulting output heterogeneous and difficult to predict. However, with the recognition that heterogeneous modification of the genome through non-templated and enzymatically-driven epigenetic changes has a powerful and even transmissible impact on our genetics [2], it is becoming appreciated that all biological and experimental systems have important and functional heterogeneity. This is further promoted by the computing power that characterizes the modern technological landscape, which enables the analysis of large and complex datasets. Indeed, the heterogeneity itself is very likely to be a crucial aspect of biology that we are only now beginning to seriously contemplate.

Extrinsically glycobiology has suffered from a paucity of experimental tools suited to anyone outside a small core of expert investigators. This resulted in poor growth of the field,

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a general ignorance among non-experts, and a perceived low impact of glycobiology-based findings. A key turning point in this was the establishment of the National Institute for General Medical Sciences (NIGMS)-funded Consortium for Functional Glycomics (CFG) in 2001 by Jim Paulson and other leaders in the field. The CFG was instrumental in ushering in a new age of glycobiology. Further support for the field comes from a National Institutes of Health (NIH) effort to promote the development and dissemination of approachable tools, informatics platforms, carbohydrate synthesis approaches, and more for the non-expert - all funded through the NIH Common Fund (<https://commonfund.nih.gov/glycoscience>).

Much of the recent attention and impact in glycobiology has centered around glycans in the immune response, including host-pathogen interactions, and in the development and immune evasion of cancer. In this issue of *Cellular Immunology*, experts in these areas provide insight into these discoveries and how they impact both normal biologic function and disease. This opening review is focused on providing some context of glycobiology within the immune system and the recent advances in technology that have fueled its rise.

2. Glycobiology in brief

As a detailed treatment of glycobiology is obviously outside the scope of this review, we refer readers needing details or refreshing to the Third Edition of the *Essentials of Glycobiology* textbook, available in full for free online in the NCBI bookshelf (<https://www.ncbi.nlm.nih.gov/books/NBK310274/>). Ajit Varki's 2017 *Glycobiology* review entitled "Biological Roles of Glycans" is another outstanding resource for those newly approaching the field or in need of refreshing [3].

Protein glycosylation can take a number of forms, the most widely recognized are the O-linked N-acetyl-galactosamine (GalNAc) and N-linked glycosylation pathways, but O-linked N-acetyl-glucosamine (GlcNAc), O-linked glucose, O-linked mannose, tryptophan mannosylation ("C"-linked mannosylation because of the carbon-carbon linkage), and O-linked fucose are also important contributors to the glycome [4]. O-GalNAc glycans are broken into "core" designations based on the identity and linkage of the branch-initiating monosaccharides (Fig. 1A), and are initiated on serine or threonine residues in the Golgi apparatus and elaborated after a nascent protein is fully synthesized and folded. N-linked glycans (Fig. 1B) are added co-translationally in the endoplasmic reticulum (ER) and assist with protein folding quality control through interactions with ER-localized chaperones like calreticulin. For N-glycans, a large precursor glycan is first transferred onto acceptor asparagine residues within the Asn-X-Ser/Thr consensus sequence [5,6]. Then, terminal monosaccharides are trimmed back systematically to enable the eventual addition of branching points initiated by GlcNAc residues (Fig. 1B) that can then be elaborated into "complex" type N-glycans [7].

O-linked GlcNAc [8] occurs at serine and threonine residues [9] on cytoplasmic and nuclear proteins. It acts as a signaling modality in much the same way, and sometimes in competition with, protein phosphorylation. As such, O-GlcNAc has been reported to be involved with nearly every aspect of cellular function [9]. In contrast, O-linked fucose are

best known for their specific and critical role in Notch signaling [10] and are necessary for proper Notch function [11] and lymphocyte development [12].

Glycosaminoglycans (GAGs), like heparin and heparan sulfate (HS), keratan sulfate (KS), and hyaluronin (HA) are large mammalian polysaccharides that represent a very different component of the glycome. GAGs are generally composed of disaccharide repeating units with varied degrees of modification - the most common of which is sulfation [4]. Molecules like HS are commonly attached to an underlying protein, or proteoglycan, and play critical roles in immunology. For example, GAGs are major components of pre-formed granules in granulocytes where they associate with cationic proteases and chemokines to create a concentration gradient [13] and generate localized inflammation [14].

3. Glycoimmunology

Glycans are nearly always the outermost component of all biological surfaces, collectively called the glycocalyx. From an immunological perspective, this makes them the major point of contact between immune cells and their ligands in the outside world. This is true for epithelial surfaces (*e.g.*, mucins) and the surface of mammalian cells (*e.g.*, glycoproteins, glycolipids, etc.), bacteria (*e.g.*, the O-chain of LPS, peptidoglycan, capsular polysaccharides, etc.), viruses (*e.g.*, capsid glycoproteins), and fungi (*e.g.*, β -glucans). Aside from a fundamental role in barrier integrity, the role of glycans in the immune response, or glycoimmunology, can be further divided into several subdivisions. First, the glycans themselves are important molecular determinants, ligands and antigens. Second, protein glycosylation can have a dramatic impact on the function, interactions and other properties of the underlying protein. Third, glycan binding proteins are a large and diverse family of molecules with wide ranging functions.

Perhaps the most famous glycans in the immune system are the ABO (Fig. 2A) and Lewis blood groups (Fig. 2B). Second to those, the Thomsen-Friedenreich (T) and Tn antigens represent highly truncated forms of O-GalNAc glycans (Fig. 2C) and are known as markers of cancer and have become vaccination targets [15]. Cell surface glycans often play important roles for microbial attachment. Influenza virus hemagglutinin, for example, binds to terminal sialic acids present in surface glycans, and the linkage by which these sialic acids are attached to the underlying glycans (*e.g.*, α 2,3 versus α 2,6) drives viral host tropism [16].

Despite the common misconception that glycans are strictly T cell-independent antigens, MHCII is known to bind and present processed forms of bacterial polysaccharides to CD4⁺ T cells [17] for clonal recognition [18] and activation [19], resulting in potent immunomodulation [20–22]. Likewise, MHCI presents glycopeptides where the responding T cell receptor makes most of its antigen contact with the glycan [23], and CD1 presents glycolipids to natural killer T (NKT) cells such that the glycan moiety is extended outside of the acyl chain binding pockets [24]. Moreover, the glycosylation pattern of the MHCII molecules themselves has even been shown to directly impact antigen binding and presentation [25]. HIV glycans have also become a focal point in vaccination efforts because a number of broadly neutralizing antibodies (bnAbs) characterized are now known to recognize glycans on gp120 [26,27]. For example, the unusual domain-swapped bnAb 2G12

associates with a cluster of three N-linked glycans on gp120 and does not make any molecular contact with the underlying protein [28].

Considering the fact that an average complex N-glycan can occupy the physical space of an Ig domain [29], it is not difficult to imagine how glycosylation alters many fundamental biochemical properties of immunological proteins. As a point of illustration, consider the crystal structure of IL-13 (PDB: 1IJZ), which was derived from *E. coli* overexpression [30]. IL-13 is a potent cytokine typically associated with T_H2-skewed pro-inflammatory immune responses, such as those in atopic asthma. It contains four N-linked glycosylation consensus sequons. The structure in the PDB, which lacks glycans, is shown in Fig. 3A. Compare this to the same structure with four complex N-glycans modeled onto it using the online portal GLYCAM (Fig. 3B). These glycans represent 7 kDa of the total 17 kDa (> 40% of the mass) the native glycoprotein normally has *in vivo*. The specific functional role for these structures is not known, but they are extremely likely to have at least one role given their sheer molecular mass, differences in hydration, and potential to carry many negatively charged sialic acids. A similar exercise can be done with essentially all secreted proteins, including all chemokines and cytokines, with the same caveat that we lack specific knowledge regarding the impact of glycan composition on structure and function - especially *in vivo*.

In contrast, much is known about glycan function regarding IgG. In 2006, it was discovered that α 2,6-linked sialylation of N-glycans on N297 of the IgG heavy chain acts as a functional switch for IgG [31]. When the glycans are sialylated, the IgG preferentially blunts the immune response, providing an explanation of IVIg therapy's suppressive activity in autoimmune patients [31–33]. Modern understanding states that Fc embedded sialic acid causes a conformational shift [34], altering the affinity of Fc γ R receptor family members for IgG, favoring the inhibitory Fc γ RIIb [35]. Interestingly, it has also been demonstrated that B cell-specific ablation of the sialyltransferase ST6Gall, which is solely responsible for IgG sialylation [36,37], has no impact on IgG sialylation. Rather, IgG sialylation can be controlled outside of the B cell by ST6Gall in circulation [38], meaning that the glycan-driven functional control of IgG is not exclusively dictated by the B cell in mice. Finally, it was recently discovered that the glycosylation of the Fc receptors themselves is also an important attribute that impacts binding affinity between Fc receptor and IgG [39]. Thus, it is critical to recognize that glycan composition (*e.g.*, sialylated or not), not merely presence or absence, can have profound effects on protein function.

Finally, there are numerous families of mammalian lectins, or glycan binding proteins, including C-type lectins (CTLs), Siglecs and Galectins [40]. CTLs are a superfamily of diverse molecules, including a number of pattern recognition receptors like DC-SIGN and Dectin-1, which are familiar to immunologists. Selectins, which drive leukocyte homing through interactions with sialyl-Lewis^x glycans (Fig. 2B), are also members of the CTL superfamily. Siglecs (sialic acid-binding Ig-like lectins) are cell surface glycoproteins with binding selectivity for sialylated glycans that, in general, provide an inhibitory function through ITIM (immunoreceptor-tyrosine based inhibitory motifs) containing cytoplasmic tails that recruit phosphatases like SHP-1 to blunt signal transduction upon recognition [41]. This system is thought to broadly limit autoimmunity because sialic acid is a marker of mammals/self and are typically absent in the microbial world. Finally, galectins are a family

of molecules known to associate with N-acetyl-lactosamine (LacNAc)-containing glycans (Fig. 2D). In immunology, two galectins have been the most widely studied: galectin-1 (gal-1) and galectin-3 (gal-3). Both proteins are involved in a multitude of pathways. Gal-1 is best known for its ability to induce apoptosis, which can drive skewing of the immune response by attrition of unwanted responses. For example, gal-1 targets T_H1 and T_H17 cells for apoptosis, promoting the heavily sialylated T_H2 cell response, since α 2,6-linked sialylation reduces gal-1 affinity and protects from gal-1-induced apoptosis [42]. Gal-1 is also key in the development of cancer, and has become a target for cancer therapies [43]. Gal-3, on the other hand, does not induce apoptosis. Rather, it has been implicated in controlling T cell receptor signaling [44], and is known to promote M2 macrophage development and maintenance downstream of IL-4 signaling [45], among many other functions.

4. Technology and tool development

4.1. Glycan microarrays

The Consortium for Functional Glycomics (CFG; <http://www.functionalglycomics.org/>), primarily housed within the National Center for Functional Glycomics (NCFG; <https://ncfg.hms.harvard.edu/>) at Harvard Medical School, spearheaded many of the technological advances that have been at the root of progress in glycobiology. The CFG developed gene chips on an Affymetrix platform that included nearly 900 genes associated with glycobiology, delivered chemically-defined glycans to investigators, organized mouse models, provided mass spectrometry-based glycan structural analyses, and developed glycan arrays to interrogate glycan binding proteins. Recently, the glycan arrays have expanded to include over 600 mammalian glycans and over 300 glycans from microbial sources. These services remain available at the CFG website, while mass spec services can now be found at the NCFG, the Complex Carbohydrate Research Center at the University of Georgia (<https://www.ccrcc.uga.edu/services/>), the Glycotechnology Core at the University of California San Diego (<https://medschool.ucsd.edu/research/GRTC/>), and others.

Simultaneously, a number of other groups have developed a variety of other glycan arrays. Conjugation chemistry (NHS, epoxide, maleimide), glycan density, the presence of an underlying carrier protein (*e.g.*, BSA), and glycan specialization (*e.g.*, only sialylated forms) are some of the distinguishing characteristics of the tools used to interrogate glycan binding activity [46]. Some of these technologies have been directly compared, with interesting results [46,47], and continue to evolve as our understanding of glycan-protein interactions increases. In general, these technologies are employed as a means to understand glycan binding properties of molecules, viruses, and even intact cells. For example, the CFG arrays have been used extensively to reveal surprising influenza virus/hemagglutinin binding specificities and host tropism [48–50].

Glycan arrays can also be used as a substrate platform for enzymatic activity and specificity, or to obtain a detailed accounting of the glycans present in a sample (*e.g.*, glycomics). The CFG mammalian glycan array has been used to characterize the specificity and activity of a secreted sialyltransferase in plasma samples [38]. In addition, ‘shotgun’ glycan arrays are arrays in which all glycans from a biological sample (*e.g.*, cells, molecules, tissues) are

removed, isolated, and spotted onto an array [51–53]. These arrays can then be interrogated with lectins or other glycan binding proteins to explore the function of that sample's glycome as a function of molecule, cell, tissue, and/or disease [54].

Collectively, glycan microarrays are perhaps the most revolutionary addition to the glycobiology tool set over the last decade. They enable a level of detail in probing interactions and activity unmatched by any other approach, and has dramatically increased the throughput by which these biological phenomena are understood.

4.2. Glycoprotein synthesis and mass spectrometry

Glycan microarrays owe their existence to improvements in glycan synthesis and isolation, and the improved ability to make specific complex glycans is also essential in the push to create recombinant glycoproteins with defined glycosylation patterns and structures. There are two general approaches in this area [55,56], either chemical attachment of defined glycans into engineered attachment sites of a protein, or the creation of designer cell lines with a highly defined cohort of glycosyltransferases to directly express a more homogenous glycome. Each approach has its limitations, but the same goal: to better define the impact of specific glycoforms on biological function.

Another area that has seen dramatic improvements is mass spectrometry (MS), as reviewed elsewhere [57–64]. Glycan MS is analogous to nucleic acid sequencing in its importance to the field, thus it is important that both sensitivity and the ability to obtain linkage information have been significantly improved. However, glycan MS largely remains in the purview of the experts. Efforts are being made to 'democratize' glycan MS analysis by developing approaches that can be employed by any MS/proteomic facility, but this has not yet seen broad success, remaining a roadblock for investigators outside the field. Importantly, glycan MS services are available at a number of institutions on a fee-for-service basis, including the UCSD Glycotechnology Core, the CCRC, and the NCFG.

4.3. Plant lectins

A wide variety of plant lectins, or proteins with carbohydrate-binding properties, have been known for many decades. However, with the rise of glycan microarrays, the binding specificity of these molecules have become very well defined. The specificity data coupled with the availability with fluorescent, biotin, and resin conjugates has enabled a reliable use of these molecules in ways that are analogous to the use of antibodies in the laboratory. Lectins can be used for Western blotting, flow cytometric cell sorting/purification [38], ELISA [25], fluorescent immunohistochemistry/confocal microscopy [65], protein purification/enrichment [38], and others. Of all the technologies available to study glycosylation, the use of lectins is easily the most approachable by the broad research community because they can be used so similarly to antibodies, with only minor variations in technique.

Lectins are also being used to develop multiplexed glycosylation assay platforms to determine a glycoform fingerprint of a sample. For example, attachment of lectins in an array setting enables the rapid screening of purified proteins for the specific glycan structures recognized by each lectin [66,67]. Thus, with one small sample, binding by many

lectins can be quantified at once, yielding information on many glycan characteristics (*e.g.*, branching, sialic acid, and fucose levels). The success of such arrays has led to new products with increased throughput and/or sensitivity. For example, multiplexed bead-based lectin arrays with a limit of detection in the low picomolar range [68], and microfluidics to decrease sample size, increase sensitivity, and create a wider linear dynamic range - up to 5 logs [69].

4.4. Chromatography

High pH anion exchange chromatography with pulsed ampero-metric detection (HPAEC-PAD) has been a mainstay of HPLC-based glycan analyses since the initial descriptions in the late 1980s [70–72] and reviewed in 1991 [73]. This method separates oligosaccharides and monosaccharides with a high level of fidelity, thereby enabling quantification of glycan composition. However, the PAD unit is not common, and remains available only on specialized HPLC and UHPLC systems. Although the sensitivity of PAD is highly reproducible, signal varies between carbohydrates, making standardization critical.

More recently, the use of hydrophilic interaction liquid chromatography (HILIC) has become much more widespread in glycobiology. Glycans can be enzymatically removed from cells or proteins, labeled with 2-aminobenzamide (2AB) by reductive amination, and separated on a HILIC column fitted to any HPLC/UHPLC system with a fluorescence monitor. Quantitation of each glycoform is straightforward from a single fluorescent signal, and separation of oligosaccharides is significantly improved over HPAEC-PAD methods. In addition, this method can be applied to any HPLC/UHPLC system fitted with a fluorescence monitor, making it far more accessible. For these reasons, HILIC-based glycomics is rapidly becoming the favored chromatography approach in glycobiology, as seen recently in the HIV [74] and IgG [75] research fields.

4.5. Informatics

Another area in glycobiology that has seen improvement is informatics, although glycomic data remains absent from centralized databases like the NCBI, and so is not linked to the genomic and proteomic information sources, thereby limiting the analytical possibilities and exposure to the broader research community. Despite these limitations, much work has been done to increase the functionality, flexibility and user friendliness of glycomic analyses [76–96]. The details of this progress is far beyond this review, but the efforts range from IT platforms for interrogating MS-specific spectra [76] and other glycomics data to the creation of databases [78,79,81,82,86,88,89] and improved structural annotation [80,83,90,93].

Another area of progress, necessitated by the advancement in array technologies and other big datasets, are IT platforms which enable the identification of motifs [97–100]. For example, when a protein of interest is screened for glycan binding on the CFG array, often a number of glycans show moderate binding. Tools have been developed to search for structural commonalities in those identified glycan structures, identifying a motif to guide future experiments about the true nature of the glycan binding specificity [97–100].

The community has also developed MIRAGE - the Minimum Information Required for a Glycomics Experiment standard [101], a standardized set of criteria required for a dataset to

be included in databases. This was originally founded in 2011 to establish the appropriate guidelines, and has generated standards for glycan microarrays [102], glycomics reporting [103], and MS-based datasets [104].

In addition to these glycomic analyses, it is now possible to model the missing glycans on protein structures within the Protein Databank (PDB) using the GLYCAM portal (www.glycam.org) [105]. This tool models glycans on a known protein structure and provides unique settings to visualize glycans in 3D while using standard symbology to identify monosaccharides. The IL-13 model shown in Fig. 3B was created on the GLYCAM website [105]. The GLYCAM portal also allows the user to calculate glycoprotein interaction interfaces and docking, determining and visualizing structural dynamics and other parameters [106–110].

4.6. Remaining gaps

Despite all of the advances briefly outlined here, the central problem remains linking glycan structure and heterogeneity to biological function. It is well known that changes in glycosylation accompany many biological processes, including disease, yet there are few tools to truly align glycan structure with biological impact. Likewise, the regulation of glycan structure and synthesis by a given cell or tissue is poorly understood. This is a complex problem because the cohort of enzymes in the glycosylation pathway, cellular metabolism, availability of sugar precursors, expression of transporters, nascent glycoprotein structure, concentration, and trafficking, and other parameters all impact the glycome. It is now clear that glycans can even be remodeled after export from a cell, independent from the Golgi apparatus [38,111–115]. In our opinion, new tools to understand glycan regulation *in vivo*, and to align that regulation with disease are needed because these gaps are a central reason that glycobiology remains a niche field.

5. Concluding remarks

Over the last two decades, glycobiology has come of age in a renaissance of approachability and accessibility. The development of array technologies, the increased ability to employ lectins in everyday assays, progress in MS and other analytical approaches, improvements in glycan synthesis, and the associated informatics collectively make glycomics easier to approach for the non-expert. This is not to say our work is finished, since there are many tools that are not in use by general scientific communities and still more to be developed in efforts to transform our field. It is our hope that in the coming era, glycomic data will be as ubiquitous and easy at access as genomic and proteomic data sets at the NCBI, so that the glycome can be fully harnessed to understand biology and disease.

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Abbreviations:

PDB	protein data bank
2AB	2-aminobenzamide
HILIC	hydrophilic interaction liquid chromatography
HPAEC-PAD	high pH anion exchange chromatography with pulsed amperometric detection
MS	mass spectrometry
CFG	Consortium for Functional Glycomics
NCFG	National Center for Functional Glycomics
gal-1	galectin-1
gal-3	galectin-3
LacNAc	N-acetyl-lactosamine
ITIM	immunoreceptor-tyrosine based inhibitory motifs
ITAM	immunoreceptor-tyrosine based activation motifs
Siglec	sialic acid-binding Ig-like lectin
CTL	C-type lectin
bnAbs	broadly neutralizing antibody
T	Thomsen-Friedenreich antigen
HA	hyaluronin
KS	keratan sulfate
GAG	glycosaminoglycan
HS	heparan sulfate
GlcNAc	N-acetyl-glucosamine
GalNAc	N-acetyl-galactosamine

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Fig. 1.

Schematic representations of the O-GalNAc and N-linked glycosylation pathways. The arrows emanating from the specific glycans represent locations where extensive building of complex glycans usually takes place.

Fig. 2.

Examples of important immunologic glycans. (A) ABH(O) blood group glycans. (B) Lewis^x (Le^x) and sialyl-Lewis^x (sLe^x) shown in the context of a complex-type N-glycan. (C) T and Tn antigens. (D) Poly-LacNAc, representing a preferred galectin ligand, shown in the context of O-GalNAc glycan.

Fig. 3. Structural models of recombinant human IL-13 (PDB: 1IJZ), originally crystalized from an *E. coli* overexpression system [30] and modeled with four complex N-glycans to illustrate the size difference between an unnatural de-glycosylated variant compared to one possible glycosylated structure. (A) Ribbon and ball & stick model of the original structure as published. (B) Ribbon and ball & stick visualization of IL-13 with complex N-glycans. The GLYCAM web portal was used to perform energy minimization and to create the visualized glycan symbols.