CARBOHYDRATES: THE YET TO BE TASTED SWEET SPOT OF IMMUNITY

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CARBOHYDRATES: THE YET TO BE TASTED SWEET SPOT OF IMMUNITY

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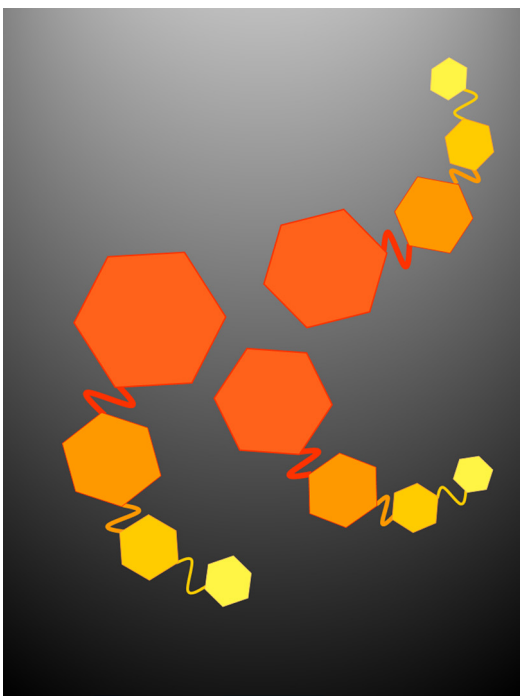

Stylized representation of the core of three heparan sulfate fragments.

Image by Deirdre R Coombe.

Carbohydrates are extremely abundant bio-molecules; they are on all mammalian cell surfaces as well as on bacterial cell surfaces. In mammals most secreted proteins are glycosylated, with the glycan component comprising a significant amount by mass of the glycoprotein. Although, many years ago carbohydrate-protein recognition events were demonstrated as involved in invertebrate self-non self recognition, the contribution of carbohydrate-protein binding events to the mechanisms of the mammalian immune response was not embraced with the same enthusiasm. Adaptive immunity and the contribution of antibodies, T cells and T-lymphocyte sub-sets and protein antigen presentation dominated immunological theory. Unlike protein structures, carbohydrate structures are not template driven yet the numerous enzymes involved in carbohydrate biosynthesis and modification are encoded by a major component of the genome, and the expression of these enzymes is tightly regulated. As a consequence carbohydrate structures are also regulated, with different structures appearing according to the stage of cell differentiation and according to the age or health of the individual. The advent of technologies that have allowed carbohydrate structures and

carbohydrate-protein binding events to be more easily interrogated has resulted in these types of interactions taking their place in modern immunology. We now know that glycans and their ligands (or lectins) are involved in numerous immunological pathways of both the innate and adaptive systems. However, it is clear that our understanding is still in its infancy, as more and more examples where carbohydrate structures contribute to aspects of the immune response are being recognised. The goal of this research topic is to explore the variety of roles undertaken by glycans and lectins in all aspects of the immune response. The particular focus is how the interactions of glycans with their ligands contribute to the mechanism of immune responses.

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EDITORIAL
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Editorial: Carbohydrates: the yet to be tasted sweet spot of immunity

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Keywords: Siglec, sialic acid, glycan, galectin, glycosaminoglycan, hyaluronan, heparan sulfate, heparanase

Glycobiology is an expanding discipline. Nowhere is this more apparent than in our understanding of the immune response. Perhaps the title of this focused research topic should be: "Carbohydrates: *now* the sweet spot of immunity!" The revolution in thinking to embrace the "glyco" component of glycoproteins and glycolipids has been accompanied by the development of new technologies that allow the structure of many different glycans to be determined. The article by van Kooyk et al. provides an introduction to glycan analytical tools (1). These range from technically simple analyses using plant lectins combined with flow cytometry or ELISA methods to obtain clues of glycan structures, to more complex sequencing methodologies for detailed structural characterizations. Nevertheless, determining the structure of some glycans, and particularly the glycosaminoglycans (GAGs), is still extremely difficult. However, good progress is being made in this area (2).

Cell surface glycosylation is a characteristic of all living cells (3, 4), thus it is logical that glycan structures are involved in self or non-self recognition. Nevertheless, glycans have been excluded from the thinking of most immunologists. Probably a lack of appreciation of the specificity of carbohydrate–protein interactions and the diversity of glycan structures led to this outcome. Yet, it is glycan diversity that has been harnessed by microbes to coat their surfaces, and most immunogens on microbes are glycans. As pathogens developed their glycan coats their vertebrate and invertebrate hosts similarly developed molecules to recognize these structures. The idea that invertebrate lectins can recognize glycan structures on microbes, thereby facilitating microbe phagocytosis, was accepted decades ago (5), but the fundamental contribution of glycan–protein interactions to mammalian immunity was accepted only recently. Numerous molecules involved in invertebrate host defense that recognize a spectrum of glycan structures on bacteria, fungi, and other pathogens are clearly related to similarly acting proteins in modern mammals (6). The lectin pathway of complement, toll-like receptors, the pentraxin pattern recognition receptors, and the galectins all probably arose initially in invertebrates ancestors and had roles in self or non-self recognition. We now know glycans and their binding proteins contribute to all aspects of immunology. It was argued that the essential role glycan–protein binding events play in host defense and infection is the driver of glycan diversity (3, 4). The evolutionary selection pressures imposed by the need of pathogens to avoid recognition by the proteins of their host's immune system, and for hosts to rapidly evolve glycan structures that are not sites for pathogen adhesion and infection, it was proposed, led to the conservation of glycan structural diversity (3, 4).

An appreciation of carbohydrate structural diversity is obtained when the number of genes involved in glycan biosynthesis is appreciated. van Kooyk et al. revealed that if all the genes involved in glycan biosynthesis are considered they would comprise around 3–4% of the genome (1). Although they primarily encode enzymes, co-factors, transporters, and activated sugar donors are also involved. Regulation of the expression of these genes, regulation of the activity of the different glycosyltransferases through a diverse collection of mechanisms, coupled with regulation of the expression of core proteins adds an extra dimension to glycoconjugate structural diversity (1).

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Given glycan biosynthetic processes, it is not surprising that glycan structures are altered in response to physiological and pathological cues, and these different structures affect the immunological outcomes of the process in which they are involved.

Dendritic cell (DC) sialic acids illustrate how glycan structures can influence the adaptive immune response. Glycans, both *N*- and *O*-linked, on glycoproteins terminate in sialic acid, and glycolipid gangliosides contain one or more sialic acids. Sialic acids shield host cells from pathogens, prevent the deposition of complement components on DCs, and interact with receptors of the Siglec and Selectin families (7). As explained by Crespo et al., the concentration of sialic acids on DCs is very high, most Siglecs binding to sialic acids on the same DC (i.e., *cis* interactions) (7). Sialidase activity releases these Siglecs allowing them to engage in interactions with sialic acids on pathogens. The balance between cell surface sialic acid and sialidases may regulate key DC functions like phagocytosis, micropinocytosis, migration, and DC–T cell interactions (7). Not all leukocytes can have these high-sialic acid levels, nor can their Siglecs signal via sialic acid in *cis* interactions. As the binding of Siglecs on eosinophils and neutrophils to antibodies or multimeric glycan ligands triggers cell death (8), yet in certain inflammatory conditions, these cell types abound; this could not happen if these cells have high-sialic acid levels that bind Siglecs in *cis* to trigger cell death. Nevertheless, inhibitory intracellular signals upon Siglec binding sialylated antigens are common, because most Siglecs have inhibitory ITIM signaling motifs and DCs may become tolerogenic if their Siglecs recognize sialylated carbohydrate antigens in tumors (7).

Dendritic cell immunogenicity is also regulated by other carbohydrate–protein interactions; the interaction of galectin-1 with DCs encourages a tolerogenic phenotype (9). Galectins are a family of β-galactoside binding lectins. Various galectin family members have been described as "regulators of immune homeostasis," as "pattern recognition receptors," and as "receptors for microbial adhesion and infection" (10). Often there is evidence for the same galectin having opposing functions, the question is how? Baum et al. examined the opposing roles of galectins in microbe–host interactions (11). They described how galectins can bridge specific glycans on viral and bacterial pathogens with glycans on target cell plasma membranes, to increase pathogen attachment. The outcome of galectin–pathogen interactions is not always infection; rather there are numerous examples of galectins contributing to innate and adaptive immune responses to pathogens, and some galectins have direct microbicidal activity (11). The response is dependent on the galectin, the pathogen and the host cell, with factors such as glycan density, glycan clustering, and the glycoprotein or glycolipid upon which the glycan is presented, all contributing to the context-specific outcome. Differences in the *N*-glycans of resting and activated cytolytic T lymphocytes (CTLs), with more galectin-3 ligands being present on activated CTLs, is an example where the density of a glycan structure regulates CTL function. In a galectin-3 rich milieu (e.g., a tumor), reduced motility of galectin-3 cross-linked glycoproteins on activated infiltrating CTLs could explain the decreased CTL activity within tumors (12).

Involvement in the immune response is also in the functional realm of GAGs. Simon Davis and Parish highlight the number of proteins that have heparin/heparan sulfate (HS) binding motifs within their sequences (13). Many of the possible new HS–protein interactions that they discovered may act in immune responses but this is unconfirmed. Other confirmed HS–protein interactions have clear implications for immunity; described are examples of HS–protein interactions contributing to (1) cell adhesion and migration, (2) the regulation of cytokine and chemokine functions, and (3) the sensing of tissue injury (13). The regulation, by HS, of complement pathway triggered inflammation is emphasized by two articles. Perkins et al. used molecular modeling and affinity coefficient data to develop a bivalent, co-operative model of complement factor H (CFH) binding to HS (14). They argued, mutations in either of the CFH HS binding regions that weaken binding, alters the orientation of CFH on the cell surface disrupting C3b binding and the regulation of C3b activity, with the result being inflammatory damage, whereas, Clark et al. offered the opinion that different HS structures (or "postcodes") in the glycomatrix of different tissues determine the levels of immobilized CFH. Probably, both explanations apply and collectively they explain the disease association of CFH polymorphisms (15).

The association of GAGs with inflammation extends beyond complement pathway regulation. Chemokine–HS interactions are known to establish chemokine gradients to direct leukocytes to inflammatory sites (16); but the contribution of the HS enzyme, heparanase (Hpse), to inflammatory disease is under appreciated. Heparanase assists leukocyte migration across basement membranes by acting as a "path-maker"; however, in type 1 diabetes Hpse activity actually drives the disease process (17). Simeonovic et al. describe how within pancreatic islets there are extraordinarily high levels of HS; this HS is essential for beta-cell survival. If active Hpse degrades HS in the islet basement membrane, inflammatory mononuclear cells can enter the islet; Hpse from these cells destroys intra-islet HS, triggering beta-cell death, and destructive insulitis. The ubiquitous non-sulfated GAG, hyaluronan (HA) is also involved in inflammation. Normally, it has a very high-average molecular weight, but at sites of inflammation and tissue injury HA polymers of overlapping length and function occur. As explained by Petrey and de la Motte, HA can promote and suppress inflammation, functions that depend upon polymer length and the activities of HA-binding proteins (18). The ability of hyaluronidases to degrade HA depends on the conformation of HA chains, which is influenced by the degree and hierarchy of protein–HA interactions, both of which depend on the HA-binding proteins in the microenvironment (18). The tissue microenvironment, its carbohydrates and their binding proteins, underpins the regulation of inflammation by HA and HS in a range of diseases including type 1 diabetes (18, 19).

The contribution of HS to human immunodeficiency virus (HIV-1) infection has come of age. Connell and Lortat-Jacob indicate how the elegant design of a potential drug developed through an appreciation of the molecular events involved in HIV-1 infection of CD4+ leukocytes (20). Although the surface exposed V3 loop of the virus protein, gp120, is involve in HS binding, prior CD4 binding was found to induce a HS binding site that is also involved in binding to HIV-1's co-receptors, CXCR4 or CCR5. The glycoconjugate drug candidate was designed to block HIV from

binding to cell surface HS, its co-receptor and CD4. It is composed of a small CD4 mimetic linked to a chemically synthesized HS dodecamer (20). This glycoconjugate had strong anti-viral activity against HIV-1 regardless of its co-receptor usage, which is a major advance.

These articles highlight the contribution of glycans to different aspects of the immune response, yet this is a "taster plate" of their total contribution. Contrary to the often held view, glycan structures frequently bind proteins with quite exquisite specificity; our lack of understanding of their binding modes and the nature of the protein conformations that are recognized cause the miss-interpretation. Reductionist thinking and analyses, although useful, in isolation are unlikely to reveal the truth. Repeatedly, it is the "context," whether the presentation of glycan motifs, or the molecules (proteins and carbohydrates) of the surrounding microenvironment, which determines the outcome of glycan–protein interactions. It is fitting that the concluding article in this series (20) describes the development of a glycan inspired potential therapeutic, because this is an area of drug discovery currently under exploited. Advances in technologies of glycan structure determination and syntheses, coupled with a more holistic approach to understanding glycan interactions with their binding partners will lead to more glycan inspired therapeutics to treat immunological diseases.

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Analytical tools for the study of cellular glycosylation in the immune system

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It is becoming increasingly clear that glycosylation plays important role in intercellular communication within the immune system. Glycosylation-dependent interactions are crucial for the innate and adaptive immune system and regulate immune cell trafficking, synapse formation, activation, and survival. These functions take place by the *cis* or *trans* interaction of lectins with glycans. Classical immunological and biochemical methods have been used for the study of lectin function; however, the investigation of their counterparts, glycans, requires very specialized methodologies that have been extensively developed in the past decade within the Glycobiology scientific community. This mini-review intends to summarize the available technology for the study of glycan biosynthesis, its regulation and characterization for their application to the study of glycans in immunology.

Keywords: glycan analysis, glycosyltransferases, glycans, lectins, immune cells

INTRODUCTION

Glycosylation is the most common post-translational modification of proteins. It is often estimated that more than 50% of all mammalian cellular and membrane-bound proteins are glycosylated, implicating an essential role in protein and cell function for carbohydrates. Indeed, carbohydrates play multiple roles in glycoprotein function: they participate in folding and maturation, contribute to the structural properties of glycoproteins, provide charge and hydrophilicity, and mediate interactions. In particular, carbohydrate-mediated interactions are specially crucial for the immune system (1). Glycans have been involved in the generation and loading of antigenic peptides into MHC-I (2), immune cell trafficking (3), T cell receptor signaling and apoptosis (4), B-cell receptor signaling (5), antibody function (6), immune cell differentiation (7), pathogen recognition (8), and immune homeostasis (9). Therefore, determining glycan structure, their biosynthetic regulation, their aglycon, and their binding partners is a fundamental step toward understanding the role of glycosylation in the immune system.

Glycans are often defined as assemblies of carbohydrates that include monosaccharides, oligosaccharides, polysaccharides, and their conjugates (glycoproteins, glycolipids, and proteoglycans). The structural diversity of glycans depends on several factors, namely differences in monosaccharide composition, anomeric state, glycosidic linkage, branching, the presence of non-carbohydrate substituted components (phosphorylation, sulfation, acetylation, etc.) and linkage to their aglycones (peptide, lipid, etc.) (10). Each of these structural factors is ultimately determined during glycan biosynthesis by the relative composition of the glycosylation machinery. The term "*glycosylation machinery*" refers to the set of, mainly enzymes, but also co-factors, transporters, and activated sugar donors that are necessary for the natural biosynthesis of glycans. It has been estimated that approximately 1% of the genome is dedicated to glycosyltransferases (11) and, if all genes involved in the glycosylation machinery are considered, this figure would probably rise to approximately 3–4%, thus a significant proportion. The glycosylation machinery is not localized to a single specific organelle within the cell and should be envisioned as a virtual engine (**Figure 1**) which involves mainly the Golgi apparatus, but also several other organelles and intracellular compartments, such as the nucleus (sialic acid biosynthesis), the endoplasmic reticulum (initial steps of *N*-glycosylation), lysosomes (monosaccharide recycling), or the cytoplasm (sugar donor and *N*-glycan precursor biosynthesis). With such a widespread localization and the involvement of so many factors it is no surprise that several levels of regulation have been described that affect the glycosylation process. Central to the glycosylation process, many glycosyltransferases have been shown to be regulated through transcriptional (12), post-transcriptional (13, 14), and post-translational (15) mechanisms. In addition, the activity of some glycosyltransferases may also be regulated through the interaction with chaperons (16, 17), competition for substrate with other glycosyltransferases (18), the availability of sugar donors (19), the pH at the Golgi (20), cleavage of their transmembrane domain (21), or even relocation to different organelles (22). Also, the regulation of the expression of glycoproteins as well as their modification by glycosidases (23) once on the cell membrane or the extracellular space contribute to the regulation of glycosylation. These mechanisms may operate in response to physiological (24–26) or pathological (27–29) cues and often have a biological correlate that is dependent on changes in the interaction with glycan-binding proteins (30). Thus, glycosylation is a highly regulated process that is extremely sensitive to both intracellular and extracellular stimuli. Moreover, due to the nature of the glycosylation process, the resulting glycoproteins exist as a mix of the same peptide backbone with a variety of different glycans. The diversity of these glycans depends

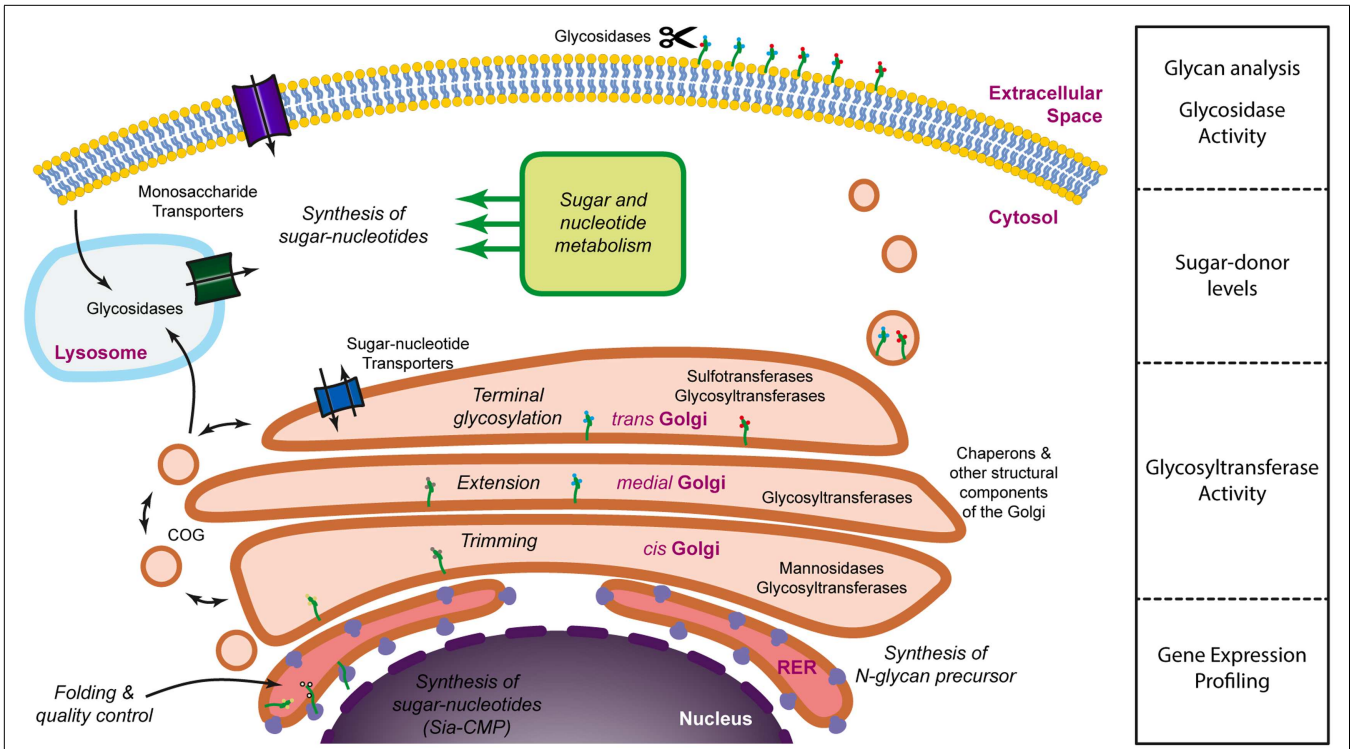

FIGURE 1 | Dissecting the glycosylation machinery. Glycosylation is a complex process that involves a large number of molecules and organelles. The glycosylation machinery can be defined as the set of enzymes, chaperones, transporters, sugar donors, and accessory molecules necessary for the modification of proteins or lipids with carbohydrates. Since many of these molecules are subjected to regulation, glycosylation is a highly dynamic process and it is, therefore, interesting to address not only the array of glycans present on the cell surface or the secretome, but also the activity and the expression levels of the molecules involved in glycan biosynthesis.

on the relative composition of the glycosyltransferases expressed and the interplay of all the regulatory stimuli that operate at a particular moment. This can affect both the number of glycans attached per glycoprotein, a type of variation that is referred to as macroheterogeneity, as well as the nature of these glycan chains (known as microheterogeneity). Thus, glycoproteins usually exist as complex mixtures of glycosylated variants or glycoforms. As an example, the human erythrocyte molecule CD59 consists of more than 120 different glycoforms, despite having a single *N*-linked glycosylation site and a couple of potential *O*-linked glycosylation sites (31).

Unfortunately, we still lack a systems biology approach to allow the modeling of the glycosylation machinery. Such a model would be extremely useful to predict how changes in the relative expression of different components of the glycosylation machinery would lead to alterations in the glycan profile of cells or secreted proteins. Accumulating evidence demonstrates, nevertheless, that there is a good correlation between changes in the transcript levels of glycosyltransferases and differences in the glycosylation pattern, suggesting that the modeling of the glycosylation machinery could be a possibility in the future. Until then, a comprehensive analysis of cellular glycosylation should incorporate different types of methodologies that provide information on the expression of the different components of the glycosylation machinery, their activity, as well as the characterization of the secreted or membrane-bound glycome (**Figure 1**).

Considering the different regulatory checkpoints of the glycosylation machinery, the most logical and accessible assays to address the glycosylation of cells would be the gene/protein expression profile of key components of the glycosylation machinery, their activity, and the glycosylation profile. We will now discuss the different methodological approaches to each of these types of assays, especially in the context of the study of the glycosylation of immune cells.

GENE-EXPRESSION ANALYSIS

The majority of the human and mouse glycosyltransferases known to date were cloned and characterized between the late 80s and the early decade of this century. The development of gene-expression technologies such as microarray technology and real-time PCR coincided with the completion of the list of existing glycosyltransferases and it is, therefore, no surprise that efforts were made to specifically develop gene-expression microarray-based methods to adequately address the glycosylation-related transcriptome. One of the most extensively used microarrays has been the glycogene-chip developed by the Consortium for Functional Glycomics. The last version of this microarray contained probes for more than 1200 human and mouse glycosylation-related genes, including glycosyltransferases (256), glycan-binding

proteins (146), glycosidases (88), nucleotide-sugar synthesizing enzymes and transporters (77), and conserved oligomeric Golgi (COG) complex proteins. In addition, several immune-related molecules such as interleukins, chemokines, and growth factors with their respective receptors were included, making this microarray extremely interesting for the analysis of the transcriptome of different immune subpopulations. In order to enhance specificity, this microarray consisted of 25 probes per gene. Unfortunately, due to the conclusion of the 10-year Glue Grant from the National Institute of General Medical Sciences (NIGMS), production of this microarray has been discontinued, although the data remains publicly available at the website of the Consortium for Functional Glycomics (http://www.functionalglycomics.org/glycomics/publicdata/microarray.jsp). Alternatives to the use of this microarray are genome-wide microarrays (Illumina microarrays also provide quantification based on 20–30 probes per gene) and real-time PCR of selected genes. Some currently available microarray platforms, like Illumina, provide genome-wide microarrays with also a high number of probes per gene. Analysis of the expression of genes encoding for glycosylation-related enzymes on data generated using this type of microarrays should be able to provide information to predict what type of glycans are to be expected on the cell of interest or what kind of glycosylation changes may operate under the treatment of study. In addition, since the whole genome is covered, these microarrays may be helpful in addressing the molecular mechanisms responsible for the regulation of the glycosylation-related gene-expression changes. Still, the use of low-density screening methods, such as real-time PCR (32–34) can be quite informative depending on the research question. The advent of next-generation sequencing technologies (35) will surely provide additional possibilities for quantification of glycosylation-related gene expression, with the advantage to identify mutations/splice variants and epigenetic variation associated with the glycosylation-related genes, potentially leading to the identification of susceptibility markers and inherited disease traits, a concept that has previously been suggested for autoimmunity (36, 37).

GLYCOSYLTRANSFERASE AND GLYCOSIDASE ACTIVITY ASSAYS

As already mentioned, glycosyltransferases may be regulated at the expression level, but also, since they are enzymes, in their catalytic activity. Several factors may contribute to this, including pH, substrate availability, interaction with co-factors or chaperons, and post-translational modifications affecting activity. Thus, determining the activity of glycosyltransferases and glycosidases *in vitro* provides a new layer of information to the study of their regulation and also facilitates the identification of specific inhibitors. However, glycosyltransferase assays (38, 39) are complicated by the fact that all Leloir-type glycosyltransferases (sugar-nucleotide dependent glycosyltransferases) that transfer the same sugar use the same sugar-nucleotide donor, but can differ in their acceptor specificity, and in the regio- and stereochemistry of the transfer reaction. In addition, glycosyltransferases can be rather promiscuous in their acceptor specificity (40). In general, the activity of glycosyltransferases can be monitored by following either the depletion of the sugar donor and the substrate(s) or the formation of the reaction products, whereas glycosidase activity is detected by following the loss of substrate. In order to allow the monitoring many assays make use of radiochemically- or fluorescently-tagged donor or acceptor analogs. Then, chromatographic, radiochemical, spectrophotometric, or immunological techniques are used to separate and/or detect one or more of the reaction species. Although glycosyltransferase activity assays have helped enormously in the characterization of glycosyltransferases and the identification of glycosyltransferase inhibitors, their contribution to understanding the regulation of glycosylation is limited. This limitation depends on the fact that many of the glycosyltransferase assays are based in reagents that are not able to cross membranes and, therefore, cannot be used in living cells or organisms. Alternatively, metabolic labeling approaches have been developed that allow the tagging of newly synthesized glycoproteins with radiochemically labeled glycans. Most recently, the use of bioorthogonal chemical reporters has allowed metabolic glycan labeling even *in vivo* (41). Importantly, the reporter must be non-toxic and small enough to not interfere with the transport of the monosaccharide into the cell, its incorporation into a sugar donor and the glycosyltransferase reaction. This is the case of azido or alkynyl monosaccharide derivatives, which have been used for the labeling of most glycan subtypes, except for glycosaminoglycans and glycosylphosphatidylinositol anchors (41). Unfortunately, monitoring of specific glycosyltransferases is not possible using this technology, but it can still be very useful to address the effect of multiple biological stimuli on specific glycan subtypes (e.g., sialylation, fucosylation, *O*-glycans, etc).

GLYCAN ANALYSIS

The complete characterization of the glycans from cell membranes or purified glycoproteins is a process that involves dedicated Analytical Chemistry technology and requires the integration of different analytical approaches. However, it is not always necessary to perform a comprehensive glycan sequencing and, depending on the type of experimental set up and evidence required, fast and simple approaches such as lectin binding assays may be sufficient. The availability of a large set of plant lectins with defined specificity has allowed the development of simple assays for the high-throughput gross characterization of the glycosylation of cells or purified glycoproteins (42). Small scale screening using selected lectins can easily be set up as flow cytometry or ELISA assays. On the other hand, lectin microarrays are becoming increasingly popular, specially in the development of disease-related biomarkers in cancer (43, 44). Unfortunately, most lectins have basic preferences to a broad set of carbohydrate structures or epitopes and a certain level of cross-reactivity is often observed. Therefore, lectins are not very practical when a detailed glycan characterization is needed. In this case, glycans can be sequenced by several different but complementary approaches. The most extended methodology is based in the purification of glycans after chemical or enzymatic released from their aglycon. This is considerably easier for *N*-linked glycans, which can be enzymatically released from mammalian glycoproteins using an amidase (PNGase F) (45). Unfortunately, only one enzyme has been described so far to be able to cleave the core 1 *O*-glycan, endo-α-*N*-acetylgalactosaminidase (*O*-glycanase), but

not its extended variants or any of the seven remaining *O*-glycan core structures (46, 47). Alternatively, chemical methods such as hydrazinolysis (48), deglycosylation by anhydrous trifluoromethanesulphonic acid (49), or non-reductive alkaline β-elimination (50) can be used instead, although these reactions require careful optimization to prevent glycan degradation (51). Regardless of the method used, released glycans can then be purified and analyzed by chromatographic and/or mass spectrometric methods. Small glycans can directly be analyzed by means of high performance anion-exchange chromatography with pulsed amperometric detection in stand-alone mode (52) or online-coupled to mass spectrometry through a desalter unit (53). High-performance anion-exchange chromatography with pulsed amperometric detection can also be used for monosaccharide analysis of purified glycans (54), which can be useful as an aid for further characterization, but requires high concentrations of experimental sample. Most often, glycans purified after deglycosylation are derivatized at their reducing end with a fluorochrome (55) and then resolved by hydrophilic interaction chromatography with a fluorescence detector. Further characterization is achieved by sequential deglycosylation using exoglycosidases (56), which specifically cleave glycosidic bonds of individual monosaccharide units from the terminal residue. Exoglycosidase digestion results in a shift in the glucose-unit value allowing detailed structural assignments with linkage information (56). Robotic systems and ultra-performance liquid chromatography in combination with sub 2 μm stationary phase capillary columns is allowing the implementation of very promising high-throughput glycan analysis projects that will certainly have an important impact in biomarker discovery (57, 58). In addition, the incorporation of an online mass spectrometer after the fluorescence detector facilitates glycan characterization without the need of extensive exoglycosidase reactions (55, 59). Alternatively, glycans can also be analyzed by porous graphitized carbon LC-MS/MS (60–63).

Derivatization of glycans with 9-aminopyrene-1,3,6-trisulfonic acid (APTS) or 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) provides glycans with electrophoretic mobility and fluorescence detection, allowing their separation by capillary electrophoresis coupled to a laser-induced fluorescence detector (64). The main advantages of this technology are its sensitivity (10^{-15} to 10^{-18} mol of oligosaccharide samples), short separation time (<20 min), and high-throughput potential and, when combined with mass spectrometry, this method provides simultaneous glycan characterization (65).

Glycans can also be directly analyzed by mass spectrometry, with the advantage of providing a link between mass and composition. In order to perform mass spectrometric analysis of glycans it is necessary to derivatize them, since the ionization efficiency of glycans (especially those carrying terminal sialic acids) is generally low. Typical derivatization methods include permethylation (66), methyl-esterification of sialic acids (67), or the above-described fluorescent tagging of the reducing end. Often, rapid profiling is achieved through matrix-assisted laser desorption ionization time-of-flight mass spectrometry because it is fast, simple, and requires only a small amount of sample. Ion fragmentation through electrospray ionization mass spectrometry, collision-induced dissociation and MS/MS help in achieving structural characterization. More recently developed fragmenting technologies such as electron capture dissociation and electron transfer dissociation have created huge expectative for the implementation of top-down proteomics (68) and their application to glycomics and glycoproteomics. Approaches based on this technology would be ideal for the sequencing of *N*- and *O*-linked glycans together with their peptide assignment. Intact *N*- and *O*-glycopeptides from purified glycoproteins have already been successfully analyzed using this approach (61, 69–73), but methods for more complex samples such as cell lysates remain to be implemented. Importantly, the development of analysis software and glycan databases for the direct assignment of glycan structures to specific masses in different platforms is pushing the field forward by facilitating reporting and data mining (74–76).

CONCLUDING REMARKS

Although the glycome of several immune cell populations has already been profiled (25, 26, 77) and accumulating evidence highlights the importance glycosylation regulation in multiple aspects of immune biology (78–81) we still need a better understanding of how glycosylation is regulated in different immune cell subpopulations. A better integration of glycobiological methodology in the immunological community is a pre-requisite, for which we hope this primer will be a useful first step.

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Dendritic cells: a spot on sialic acid

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Glycans decorating cell surface and secreted proteins and lipids occupy the juncture where critical host–host and host-pathogen interactions occur. The role of glycan epitopes in cell–cell and cell-pathogen adhesive events is already well-established, and cell surface glycan structures change rapidly in response to stimulus and inflammatory cues. Despite the wide acceptance that glycans are centrally implicated in immunity, exactly how glycans and their changes contribute to the overall immune response remains poorly defined. Sialic acids are unique sugars that usually occupy the terminal position of the glycan chains and may be modified by external factors, such as pathogens, or upon specific physiological cellular events. At cell surface, sialic acid-modified structures form the key fundamental determinants for a number of receptors with known involvement in cellular adhesiveness and cell trafficking, such as the Selectins and the Siglec families of carbohydrate recognizing receptors. Dendritic cells (DCs) preside over the transition from innate to the adaptive immune repertoires, and no other cell has such relevant role in antigen screening, uptake, and its presentation to lymphocytes, ultimately triggering the adaptive immune response. Interestingly, sialic acid-modified structures are involved in all DC functions, such as antigen uptake, DC migration, and capacity to prime T cell responses. Sialic acid content changes along DC differentiation and activation and, while, not yet fully understood, these changes have important implications in DC functions. This review focuses on the developmental regulation of DC surface sialic acids and how manipulation of DC surface sialic acids can affect immune-critical DC functions by altering antigen endocytosis, pathogen and tumor cell recognition, cell recruitment, and capacity for T cell priming. The existing evidence points to a potential of DC surface sialylation as a therapeutic target to improve and diversify DC-based therapies.

Keywords: dendritic cell, sialic acid, sialylation, lectins, host-pathogen interaction

INTRODUCTION

Immunological studies, nowadays, imply researchers have at least basic knowledge of glycobiology since, at some point of their study, researchers are faced with glycosylation-related features. Glycosylation is a post-translational modification of basically all the secreted and cell surface proteins, as well as of lipids. Thus, all contacts between cell surface and/or serum molecules are continuously accompanied by glycosylation. The immune response lays on innumerous contacts between cells and molecules, a good example being the case of immunological synapses, a junction that forms between T cells and specialized cells and the antigen-antibody interactions. All the immune encounters have, with great probability, glycans occupying, and influencing the juncture. Thus, all self-asserted immunologist should consider to be (at least partially) glycobiologists.

Among the several cell types that constitute the immune system, dendritic cells (DCs) are key players. DCs survey the microenvironment where they are positioned in order to help correctly classify collected antigen information, in a "self" or "'foreign" category, and to respond accordingly. They carry antigen information from the infection site to the secondary lymphatic organs, presenting them to T cells, strongly potentiating a specific immune response against pathogens (**Figure 1**). The immune response is thus tremendously dependent on DCs and impairment of DC functions, as studied using animal models deficient for DC function-related molecules, or absence of DC populations, have been associated with infection or, oppositely, to a wide range of autoimmune diseases (1). DCs also play an important role in anti-tumoral immunity, whereupon specific cytotoxic T cells may be primed by DCs to respond against tumor cells. Investigating the underlying mechanisms of DC-pathogen or DC-host and -tumor cell interactions may help us to better comprehend the immune response in physiological and pathological events and to identify new targets for therapeutic intervention.

Dendritic cells show specific glycan patterns at cell surface, which are modulated during cell differentiation and respond to stimuli such as inflammatory cytokines and pathogens (2–4). Sialic acid is a sugar that frequently terminates glycan structures. Due to its terminal position and properties, sialic acid can mediate many immune processes such as host-pathogen recognition, migration, and antigen presentation, among other non-immune related processes. The addition of this sugar is mediated by a number of enzymes, the sialyltransferases, mainly located in the Golgi apparatus. Sialyltransferase expression is

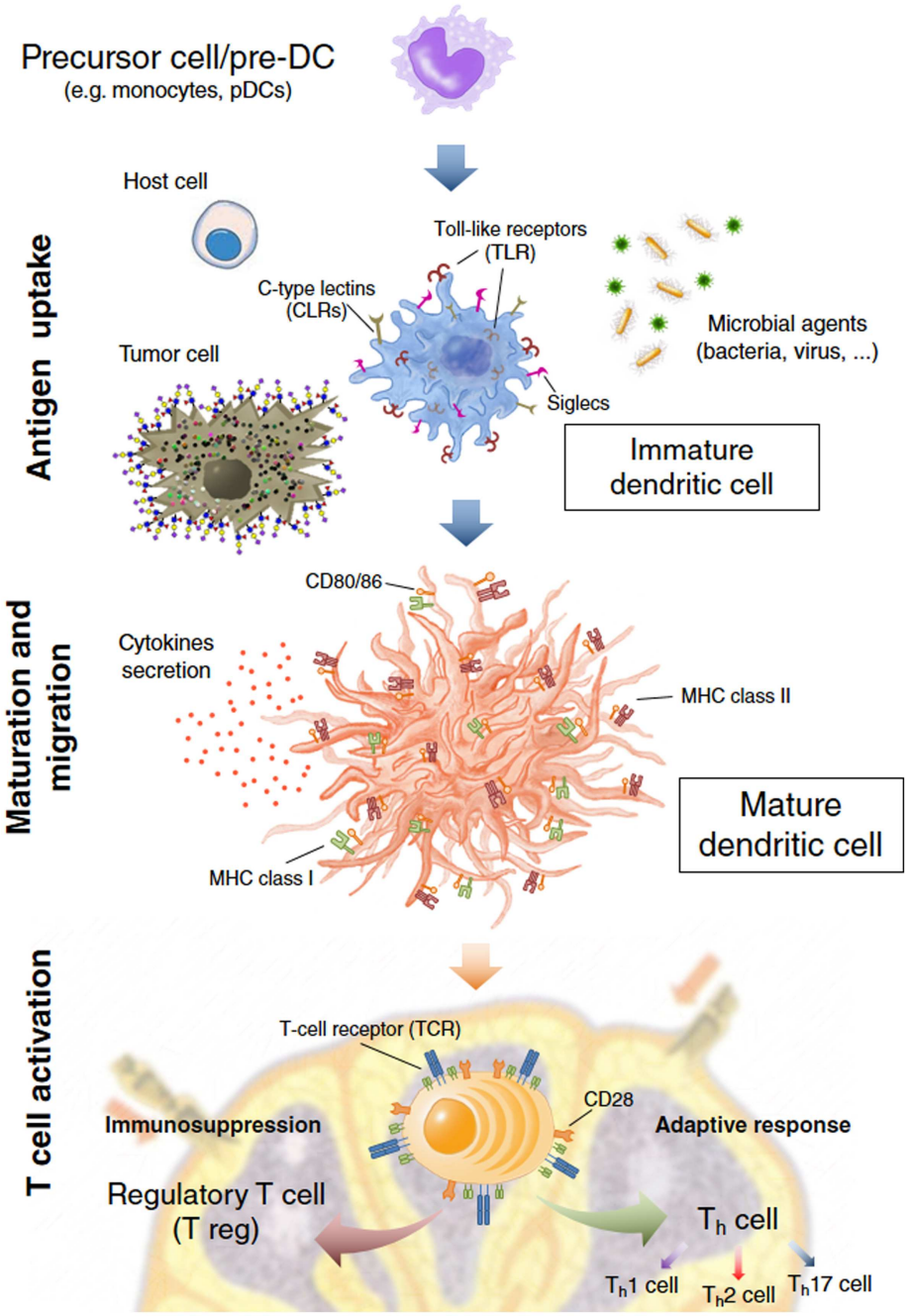

FIGURE 1 | Dendritic cell (DC) immune functions. DCs act on three main events: the *antigen capture* after interaction with host cells, microbial agents, and tumor cells by recognizing Pathogen-Associated Molecular Patterns (PAMPs) and self molecules through Pathogen Recognition Receptors (PRRs) and other cell surface receptors like Siglecs or C-type Lectins (CLRs); *maturation and migration* toward the secondary lymphoid organs; *T cell activation* where DCs present the processed antigens to T cells eliciting a specific and enduring response or tolerance from T cells.

finely regulated during DC differentiation and maturation, concurring with the expression of sialylated structures (3, 4). In diverse immune events, the sialylated glycans will be recognized by lectins, i.e., carbohydrate-binding proteins that are expressed in other cells or by DCs. While promoting cell recognition by some lectins, the presence of specific sialic acids can actually switch off recognition by other lectins specific for asialylated glycans. Thus, glycan recognition by DC lectins may impact the DC immunobiological functions. Thus, a deeper understanding of sialic acid's influence in the DC immunobiology potentially leads to a better understanding of the immune mechanisms mediated by DCs.

This review will focus on DC's glycoimmune processes, with special attention to the sialic acid-mediated ones and how they modulate the different DC functions. It includes an introduction of DCs' function and glycan recognition receptors, following a description of processes known to be mediated by sialic acid such as endocytosis, migration, priming of adaptive immune response, and pathogen/tumoral recognition.

DENDRITIC CELLS

Dendritic cells are part of the innate response and are essential to boost and/or regulate the adaptive immune response. They capture antigens in an earlier phase, process them "on the go" while migrating toward secondary lymphoid organs, such as lymph nodes, where they present, via major histocompatibility complex (MHC), the processed antigens to T cells and thus enacting an adaptive immune response. DCs can also present antigens to B cells, although by non-classical (non-MHC) mechanisms (5–7). Phenotypically, DCs are a heterogeneous population with different cell subsets, populating various organs. They can be broadly classified according the inflammatory status and differentiation state. Accordingly, conventional DCs are seen in a steady-state, that is, in the absence of infection and inflammation, and they can functionally be divided in two major types: migratory and non-migratory (lymphoid-tissue-resident) DCs [reviewed by Shortman and Naik (8)]. A good example of the former are dermal DCs and Langerhans cells that mainly reside in skin tissues and after antigen contact, they mature and migrate to the draining lymph nodes – hence the "migratory" classification. Conventional, non-migratory DCs (like spleen DCs) reside in secondary lymphoid organs, where they constantly screen blood or lymph for pathogens. The variety of DCs inside both these groups is significant and adapted toward the tissue where they reside in the immature state. Regarding DC differentiation, both canonical myeloid and lymphoid hematopoietic progenitors contribute to the steady-state DC pool and, actually, DCs use unique and flexible developmental programs that cannot be categorized into the conventional myeloid or lymphoid pathway. The expression of the Fms-like tyrosine kinase 3 (Flt-3) molecules is characteristic of DC precursors, regardless of the myeloid or lymphoid lineage and DCs development is driven by Flt3-ligand (Flt3L) (8–14). Much interestingly, it was recently reported that conventional DCs are marked by the exclusive expression of the DNGR-1 (15).

Opposed to the conventional DCs, some populations are inflammatory or infection-derived DCs. These populations include the plasmacytoid DC (pDC) population, a first line of defense against microbial invasion. Functionally specialized in the detection of viral infections, pDCs, develop a fully differentiated DC phenotype after infection and secretion of type 1 interferon (16, 17). Other inflammatory DCs include the monocyte-derived DCs (moDCs), comprising the TNF-α, inducible nitrous oxide synthase-producing DCs (Tip-DCs), a pathogenic subpopulation generated in an infection context (non-steady-state) [reviewed in Ref. (18)].

Dendritic cells constitutively uptake antigens in its surroundings as a surveillance measure (typical of the steady-state), fundamental to rapidly trigger the adaptive response against pathogens (inflammation) (19). DCs are, thus, naturally equipped with distinct means to uptake antigens, including: (1) receptor-mediated endocytosis, on which particles are endocytosed after cell surface receptor recognition; (2) macropinocytosis, or the non-selective endocytosis of solutes, a process constitutive in DCs and the major source of antigens for DC presentation (20); and (3) phagocytosis, the uptake of large molecules or cells, including virus, bacteria, protein clusters, apoptotic, and necrotic cells, which also involves specific membrane receptors. The uptake of foreign antigens usually trigger activation signals that will lead DCs to a mature phenotype, on which all the potential for antigen presentation and stimulation of the adaptive response immune cells is maximized.

Endocytosis is also fundamental in the maintenance of the self-tolerance mechanisms since, at steady-state, self-antigens are normally endocytosed and posteriorly presented by DCs. Endocytosis of self-antigens does not usually induces significant maturation changes (21), thus contributing to turn DCs tolerogenic and promoting regulatory but not effector T cells. Nevertheless, it has been suggested that the presence of very small, time-persistent concentrations of foreign and more common antigens are responsible for the induction of tolerance to those same antigens. These tolerance-inducing antigens are expressed by microorganisms present during the development of the immune system, such as commensal bacteria, flora members, and helminthes. The knowledge about these mechanisms raised the hypothesis that common microorganisms are able to regulate the immune system, the "old friends" hypothesis (22–24). These time-persistent antigens, thus shape our immune system to its present state, being presently not only tolerated but, in fact, needed in order to maintain the general tolerance balance. The "old friend" hypothesis complements the "hygiene" hypothesis stating, in brief, that the lack of immune challengers due to excessive hygiene is related to the growing number of autoimmune and hypersensitivity diseases that is observed in the developed countries, and not in the developing ones (24). Due to its key role in antigen uptake and presentation, DCs too may be involved in this mechanism of tolerance-induction toward these "old friends."

Dendritic cell maturation is the sum of all the phenotypical and functional changes occurring upon encounter with immune stimuli (i.e., antigens, cytokines, etc.) and it is crucial to enable DCs to effectively activate T cells. It is characterized by rapid downregulation of the antigen uptake process, acidification of lysosomal compartments, higher expression of MHC II molecules and of CD80 and CD86 co-stimulatory molecules, *de novo* or upregulated synthesis of DC-specific inflammatory cytokines (25). All these maturation and migration-changes are necessary hallmarks to enable DCs to perform antigen presentation and boosting T and B cell responses (26). It is also known that the molecular nature of uptaken antigens, as well as the cytokines to which DCs are exposed during the uptake process, are responsible for the modulation of the maturation process. This ultimately influences the differentiation of the DC-pulsed T cells into functionally distinct subtypes, namely, T helper type 1 or 2 (Th1 or Th2), T helper 17 (Th17), or regulatory (T_{reg}) cells, actively shaping a future active or tolerance response.

The migration (or homing) of conventional or inflammatory DCs loaded with antigens to T cell niches (normally, secondary

lymphatic organs) is a crucial step for the setting of effective immune responses. This process is characterized by chemokine-mediated cell recruitment to the lymphoid target site and, activation of the surrounding tissues (27–29). Tissue activation helps to increase the cell adhesion to the endothelium, by inducing the expression of several adhesion molecules, of which integrins and selectins and its ligands are the most relevant elements.

From all the above observations, it is, thus, clear and generally accepted that DC functions rely on a complex set of mechanisms that involve DC differentiation, ontogeny, maturation, and permanent contacts with other cells and pathogens.

PATHOGEN RECOGNITION BY DENDRITIC CELLS

Pathogen recognition by DCs depends on the identification of distinct microbial patterns, not present in mammalian cells, but shared by most of the pathogenic microbial, known as "pathogen-associated molecular patterns" (PAMPs) (30, 31). They include bacterial and viral unmethylated CpG DNA, bacterial flagellin, peptides containing *N*-formylmethionine residues, lipoteichoic acids, and double-stranded and single-stranded viral RNA. A substantial part of PAMPs are glycan-containing ones, such as lipopolysaccharide (LPS), *N*-acetylglucosamine, peptidoglycan, and terminal fructose- and mannose-containing glycans, and glucan-containing cell walls from fungi.

Pathogen-associated molecular patterns are recognized by specific receptors named "pattern recognition receptors" (PRRs), with functions aggregating endocytosis and intracellular signaling. Examples of PRRs expressed by DCs include Scavenger receptors, Nod-like receptors, and C-type lectins (CLRs). However, perhaps, the most widely studied are the Toll-like receptors (TLRs), a growing family of 12 evolutionary conserved PRRs consisting of type 1 integral membrane glycoprotein with relevant role in the microbial response. The outcome of TLR recognition is the induction of intracellular signaling and consequent expression of antigen presentation molecules (MHC II molecules), co-stimulatory molecules (CD80/86, CD40), inflammatory and/or antiviral cytokines (such as TNF-α, IL-12, IL-23, IFNα/β), chemokines (i.e., IL-8, RANTES) (32, 33), thus enacting a powerful response against pathogenic microbes.

C-type lectins are another very relevant family of PRRs expressed by DCs (34). Being lectins, their main function is to recognize glycan structures and, in immunological context, they recognize pathogen-associated glycans or glycosylated self-antigens. In DCs, some CLRs of note include the DC-Specific Intracellular adhesion molecule-3 Grabbing Non-integrin (DC-SIGN), CD207/Langerin, the Selectin family (discussed below), the Macrophage Galactose/*N*-acetylgalactosamine-specific Lectin (MGL-1), Mannose Receptor (MR), DEC205, the Blood DC antigens 2 (BDCA 2), the Dendritic Cell Immunoreceptor (DCIR), the Dendritic Cell Immunoactivating receptor (DCAR), and Dectin-1/2/3. In contrast to TLRs, all of these CLRs functionally bind glycan structures expressed by mammalian cells (except for Dectin-1/2/3 that apparently only recognizes fungal and/or mycobacterial glycans), a fact demonstrating its potential role in both host and pathogen recognition (35). CLRs can also recognize and internalize pathogens for presentation without inducing DCs' maturation. In fact, the CLR-mediated antigen uptake doesn't necessarily elicit a factual immune response, and may instead contribute to induce immunological tolerance (36). A downside of these phenomena is the potential immune escape of pathogens recognized via CLRs (35, 37–39).

Like CLRs, the Sialic acid-binding immunoglobulin-like lectins (Siglecs) can also recognize pathogens' glycoproteins and glycolipids thus also contributing to the host's innate immune responses. Siglecs specifically recognize sialic acid-containing glycans and as mentioned below they also play a relevant role in self recognition (40–43). The biological and immunological relevance of CLR and Siglec receptors will be discussed in detail in later sections.

Dendritic cells can also recognize and internalize microbes and its derivate particles by receptors that bind to opsonins in opsonized ("coated") microbes. Opsonization of microbes can occur in two forms: by coating with complement proteins or by binding of antibodies to antigens expressed on their surface. DC recognition of opsonized microbes is thus mainly mediated by complement receptors and Fc receptors and assures the capture of pathogens that might otherwise evade recognition by other DC receptors (44, 45).

Summarizing, DCs can interact in different ways with microbes, as well as with the host antigens, through panoply of receptors. This recognition initiate mechanisms that will induce or suppress a specific immune response. The DC recognition is thus considered to be of great relevance for the development of a suitable, specific immune outcome, dictating the balance tolerance/reactivity of the developing host-pathogen response.

DENDRITIC CELLS-BASED THERAPY

The current knowledge of DC immunobiology allowed several biotechnological and pharmaceutical companies to develop DC-based immunotherapies. Applications for DC-based therapy include a plethora of pathologies ranging from infectious and hypersensitivity diseases to malignancies. One strategy is the *ex vivo* upload of DCs with the antigen to turn them able to efficiently develop an efficient response against the antigen bearer (46–50). The best example of this strategy is the vaccination of cancer patients with DCs loaded with tumor antigens.

Other approaches include the use of specific antibodies targeting DC endocytic receptors that are used to force the upload of specific antigens toward that receptor. Antibodies are also used to block specific receptor-ligand interaction and consequent downstream signaling, counteracting for instance the negative immunomodulatory cues of the tumor microenvironment.

Dendritic cells have also been studied as targets of DNA vaccines encoding for antigens (51). Viral transduction not only targets antigens to DCs, but also induces intracellular pathways to modulate the immune response (52).

All these relatively recent drug-niche that exploits DC unique immune potential is proof of reconnaissance of DCs' cornerstone role in the immune system. Nevertheless, the DC-based therapies still face several hindrances to full application, mostly derived from the lack of full knowledge regarding pathogenesis/tolerance balance mechanisms, an area where glycosylation has been shown to have a relevant role.

GLYCOSYLATION AND SIALYLATION

Glycosylation is the most frequent modification of proteins and lipids. The majority of glycans exist as membrane-bound or soluble glycoconjugates. One consequence of this fact is that all cells present at their surface a glycocalyx, that is, the full surface-complex of glycans, glycoproteins, and glycosylated lipids. The three main classes of glycoconjugates are glycoproteins, proteoglycans, and glycolipids and their synthesis occurs mainly in (but not limited to) the lumen of the endoplasmic reticulum and in the Golgi apparatus. In glycoproteins, the sugar chain is classified as *N*- or *O*-linked, depending if the glycosidic moiety is linked to an asparagine (Asn) residue in the protein moiety or to a serine/threonine (Ser/Thr) residue, respectively.

The cell glycocalyx is the result of many factors. The most relevant one is probably the expression of the set of enzymes responsible for the synthesis and/or transfer of glycosylated structures, i.e., the glycosyltransferases. Also critical is the expression of enzymes responsible for the removal of glycans or entire structures from glycosylated molecules, i.e., the glycosidases. These two sets of enzymes work in a finely controlled balance both during the glycoconjugate synthesis at the Golgi apparatus. Both enzyme types can also be present in plasma membrane or soluble forms, with potentially relevant biological roles as we shall see in sections below (53–56).

Sialic acids are a large family of negatively charged, nine-carbon monosaccharides that are normally found at glycan terminal positions. They include *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and 9-*O*-acetyl-*N*-acetylneuraminic acid (9-*O*-Ac-NeuAc). Human cells can only synthesize Neu5Ac. However, Neu5Gc can also be found in some tumor cells (57). Interestingly, some pathogens may express Neu5Ac, but Neu5Gc has never been reported to be synthesized by any pathogenic bacteria (58). This review will focus mainly on Neu5Ac and, for the sake of simplification, and we will strictly refer to Neu5Ac when using the term "sialic acids."

Sialyltransferases are a family of twenty glycosyltransferases that catalyze the addition of sialic acids to terminal non-reducing position of the oligosaccharide, transferring the sialic acid from the activated sugar donor CMP-Neu5Ac to different sugar acceptors (**Table 1**). Sialyltransferases normally locate at the Golgi apparatus as integral membrane proteins adding sialic acids to glycoconjugates during their synthesis. However, some sialyltransferases are also expressed as soluble enzymes (59) and sialyltransferase activity at plasma membrane has been reported in immune cells (54). Each sialyltransferase presents high selectivity toward its acceptor substrate. *In vivo*, competition between other sialyltransferases and glycosyltransferases' common substrates is observed and, as a result, the cell's sialylation status is the dynamic sum of transferase activities, Golgi localization, and concentration of activated sugar donors. Sialyltransferases depending on their specificities, can establish α2,3-, α2,6-, α2,8-linkages and can be organized in four families depending on linkage specificity and acceptor substrate: the ST3Gal family, catalyzing the addition of sialic acid to a terminal galactose of *O*-linked glycans and glycolipids in an α2,3-linkage; the ST6Gal family, α2,6-linking sialic acids to galactose residues of *N*-glycans; the ST8Sia family, the only known sialyltransferases promoting the linkage to another sialic acid residue in *N*- or *O*-glycans, in a α2,8-bond; and, finally, the ST6GalNAc family, adding sialic acid to terminal *N*-acetylgalactosamine (GalNAc) residues of glycoproteins and glycolipids, in an α2,6-linkage (60). Thus, on the cell surface, sialic acid residues can be present in *N*- and *O*-glycans in glycoproteins, as well as in gangliosides, i.e., a glycolipid containing one or more residues of sialic acid.

The overall sialic acid content of a cell is also regulated by the removal of sialic acid residues, catalyzed by the sialidase enzymes. Four known enzymes fit into this family, also known as the Neuraminidase family: Neu1, Neu2, Neu3, and Neu4. These sialidases are variedly distributed, with Neu1 located at the lysosomes and also expressed on the surface of diverse types of cells, Neu2 at the cytosol, Neu3 integrated in the cell membrane, and Neu4 being an intracellular protein. They are all exoglycosidases, i.e., they cleave terminal sialic acids, but have different substrate specificities: Neu1, Neu2, and Neu4 remove sialic acid residues from glycoproteins, Neu2 and Neu4 also cleaves sialic acids from glycolipids, and Neu3 preferentially hydrolyzes gangliosides. A list of human sialyltransferases and sialidases, their expression patterns in DCs, and their preferred acceptor and donor substrates, is shown in **Table 1**.

SIALYLATION AND MODULATION OF THE IMMUNE RESPONSE

The terminal position occupied by sialic acids on membrane and extracellular glycans puts them on the frontline during leukocyte communication and overall immune response. Sialic acids, on an immune perspective, can function in two (seemingly contradictory) major ways: as biological masks and as recognizable cell patterns (63). In the former way, sialic acid helps shield host cells from pathogen recognition. It also prevents autoimmune responses, by preventing complement deposition over cell surface. Furthermore, it was reported that, during acute phase inflammation, both soluble and cell surface sialic acid is increased, as a consequence of the increase in soluble and circulatory forms of sialyltransferases. Higher sialic acid is thus part of the acute phase response and it seems to protect cells against pathogens, and also helping the immune system distinguishing "self" from "non-self" antigens (64). ST6Gal-I is an example of sialyltransferase whose soluble expression is upregulated during inflammation and its expression has been used by some authors as a serological clinical marker for inflammation (65–67). Non-sialylated glycans are recognized by specific lectins, and the addition of sialic acid to its terminal position may blocklectin binding. As an example, the presence of α2,6-linked sialic acids on *N*-glycans blocks recognition by galectins (68), a family of β-galactoside-binding lectins that regulate diverse cell behaviors, such as cell adhesion, migration, proliferation, differentiation, transformation, apoptosis, angiogenesis, and immune responses (69–73). However, sialic acid masking can also be used by pathogens, as a mimicry tactic in order to evade the immune system. This is the case of some *Trypanosoma* spp. that have mutated ST3Gal sialyltransferases that act as trans-sialidases, transferring the host's sialic acid to coat themselves in order to evade host recognition (74, 75).

Opposed to the asialylated-glycan recognition, sialic acids can be recognized by several cell surface receptors, such as the

Table 1 | Human sialyltransferases and sialidases.

	Preferred saccharide substrate	Glycan specificity	Dendritic cell expression (cell status)
SIALYLTRANSFERASE			
ST3Gal-I	Galβ1,3GalNAc	*O*-glycan	Yes
ST3Gal-II	Galβ1,3GalNAc	*O*-glycan	Yes (mature)
ST3Gal-III	Galβ1,3(4)GlcNAc	*O*-glycan, *N*-glycan	Yes (mature)
ST3Gal-IV	Galβ1,4(3)GlcNAc	*N*-glycan, *O*-glycan	Yes (mature)
ST3Gal-V	Galβ1,4Glc-ceramide	Glycolipid	Yes
ST3Gal-VI	Galβ1,4GlcNAc	*N*-glycan	Yes
ST6Gal-I	Galβ1,4GlcNAc	*N*-glycan	Yes
ST6Gal-II	Galβ1,4GlcNAc	*N*-glycan	No
ST6GalNAc-I	GalNAcα1,*O*-Ser/Thr	*O*-glycan	No
	Galβ1,3GalNAcα1, *O*-Ser/Thr		
ST6GalNAc-II	Galβ1,3GalNAcα1, *O*-Ser/Thr	*O*-glycans	Yes
ST6GalNAc-III	Siaα2,3Galβ1,3GalNAc	*O*-glycan	Yes (?)
ST6GalNAc-IV	Siaα2,3Galβ1,3GalNAc	*O*-glycan	Yes
ST6GalNAc-V	GM1b	Glycolipid	No
ST6GalNAc-VI	All α-series gangliosides	Glycolipid	Yes
ST8Sia-I	Siaα2,3Galβ1,4Glc-ceramide	Glycolipid	No
ST8Sia-II	Siaα2,3Galβ1,4GlcNAc	*N*-glycan on NCAM	No
ST8Sia-III	Siaα2,3Galβ1,4GlcNAc	*N*-glycan on NCAM	No
ST8Sia-IV	$(Sialα2,8)_n$Siaα2,3Galβ1-R	*N*-glycan on NCAM	Yes
ST8Sia-V	GM1b, GT1b, GD1a, GD3	Glycolipid	No
ST8Sia-VI	Siaα2,3(6)Gal	Sialic acid on *O*-glycan	Unknown
SIALIDASES			
Neu1	Siaα2,3	Oligosaccharides	Yes
	Siaα2,6	Glycopeptides	
Neu2	Siaα2,3	Oligosaccharides	No
	Siaα2,6	Glycopeptides	
		Gangliosides	
Neu3	Siaα2,3	Gangliosides	Yes (mature)
	Siaα2,6		
Neu4	Siaα2,3	Oligosaccharides	Yes
	Siaα2,6	Glycopeptides including mucins	
		Gangliosides	

Preferred substrates for each enzyme and expression pattern in human dendritic cells are indicated. Data was based on (2, 4, 61, 62).
? stands for "unknown" regarding the cell status (whether mature or immature).

previously mentioned CLRs and Siglecs (41, 76). Siglecs are sialic acid-recognizing proteins that, albeit structurally similar, are commonly organized in two categories: (i) one comprises the CD22 family [including CD22 (or Siglec-2), sialoadhesin (or Siglec-1), myelin-associated glycoprotein (MAG or Siglec-4), and Siglec-15]; and (ii) the CD33-related family comprising CD33 (or Siglec 3), Siglec-5, -11, -14, and -16 in humans, all chiefly expressed in myeloid and lymphoid cells (63, 77). Siglecs recognize and bind ligands present not only in other cells (viz., in *trans*) but also on the same cell (in *cis*). Many Siglecs present one or two intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), classically described as being involved in signaling to regulation-inducing pathways, or intracellular tyrosine-based activation motifs (ITAMs), involved in the initiation of activation signaling pathways. Hence, Siglecs have a decisive role in regulating, positively or negatively, immune responses such as inflammation or tissue damage by actively discriminating between self-associated molecular patterns (SAMPs) and PAMPs (41, 63, 78, 79).

Studies using mice deficient for selected α2,3- and α2,6-sialyltransferases have provided evidence confirming the importance of sialic acid in immune processes (80–82). ST6Gal-I KO mice were reported as presenting impaired humoral immune response, namely, by reduced concentration levels of circulating and surface IgM, impaired B cell proliferation in response to various activation signals and impaired antibody production following contact with antigens (80). CD22, one of the first described Siglecs (83), was later shown to recognize ST6Gal-I-mediated glycans, functionally regulating several B cell functions and survival mechanisms (84). Other ST6Gal-I KO mice studies have also revealed that soluble forms of ST6Gal-I have a relevant role in myelopoiesis during acute inflammation, namely, by limiting it, thus avoiding uncontrolled excessive neutrophilic and eosinophilic inflammatory responses (59, 85, 86). Using ST3Gal-I KO mice, on the other hand, it has been shown that α2,3-sialylated *O*-glycans are required for $CD8^+$ T cell homeostasis and survival (82).

These are only few examples on how sialic acids influence immune-relevant processes. Other examples include roles in host-pathogen interactions, regulation/modulation of the acute phase response and influence in the progression and differentiation of human malignancies.

DENDRITIC CELLS AND SIALIC ACID

As above mentioned, DCs play a role of enormous relevance in the immune system. Ever since Dr. Steinman and co-workers first described these cells (87–91), there has been an effort to fully characterize their immunobiology, and as part of those efforts, the relevance that glycosylation may have on it. The characterization of the DC's "glycome" ("sialome" included) and its functional impact on the DCs immunobiology and, of course, on the immune system has been a work in progress. There are many questions still open, with many potential clinical applications.

SIALYLATION IN DENDRITIC CELLS

In human DCs, the sialylation profile of inflammatory DCs has been the most studied. This comes as the result of two factors: first, they are the most frequent population of DCs and, second, in more practical terms, they are the easiest subset to obtain *in vitro* with human moDCs being a widely used human conventional migratory and inflammatory DC model. Other vertebrate DC models rely on the obtainment of DCs by differentiation of bone marrow extracts or, more specifically, $CD34^+$ hematopoietic precursors myeloid lineage (92).

Immature moDCs present a high sialylation content, namely α2,3- and α2,6-sialylated glycoproteins, when compared to its monocyte precursors (4, 93). This has been reported by different teams that used plant lectins from *Sambucus nigra* and *Maackia amurensis*, preferably recognizing α2,6-linked sialic acid linked to lactosamine (Neu5Acα2,6Galβ1,4GlcNAc-) in *N*-glycans and α2,3-linked sialic acid linked to lactosamine (Neu5Acα2,6Galβ1,4GlcNAc-), respectively.

Quantitative Real-Time PCR and microarray analysis has shown that both sialyltransferases and sialidases undergo significant gene expression variation during differentiation and maturation (4, 62, 93, 94). In particular, a significant upregulation of the ST3Gal-I and ST6Gal-I genes occurs during moDCs' differentiation that correlates with an increase of enzymatic activity by these two enzymes. Increased phenotypic change in α2,3- and α2,6-sialylation (4) during myeloid lineage-committed differentiation indicates these two sialyltransferases as the major contributors to the biosynthesis of α2,3- and α2,6-linked sialic acid-containing glycan structures specific for moDCs, with potential functional relevance. There are, however, other potentially relevant sialyltransferases that should not be discarded, such as ST3Gal-IV and -VI, being described as essential for the synthesis of the adhesion-related sialyl-Lewis x (sLe^x) antigens. Regarding sialidases, modulation during moDCs' differentiation is similarly observed, with Neu1 and Neu3 being significantly upregulated during this process (62). Maturation of moDCs leads to an increase of α2,3-sialylation and a decrease of α2,6-sialylation (2, 4, 93) although the reported variations are stimulus-dependent processes, and correlated with the sialyltransferases and sialidases activity.

While the functional impact of these observed sialic acid changes has to be further elucidated, there is already some evidence that these variations are biologically relevant, as it will be discussed further on in this review.

SIALIC ACID-RECOGNIZING DENDRITIC CELL RECEPTORS

Sialic acid-containing glycans expressed by DCs are the target of receptors, such as Siglecs, being the largest represented family. Recognition of DC sialylated glycans has functional implications: examples include a recognition mechanism of high α2,6-sialic acid content of immature and tolerogenic DCs by inhibitory Siglecs expressed by effector T cells as a host-tolerance-inducing mechanism (93), or the observed increased binding of Siglecs-1, -2, and -7 correlating with the higher sialic acid content of mature DCs (2). All this gathered evidence point to an even more promising, relevant role of Siglec-mediated immunobiological processes involving DCs and other leukocytes, but still to be unraveled and requiring, thus, further studies.

Besides being recognized by Siglecs through their expression of glycans, DCs express themselves Siglecs enabling them to also recognize sialylated structures. MoDCs and blood-circulating DCs [namely pDCs, $CD1a^+$, and $CD141^+$ DCs (95)] express Siglec-1, -2, -3, -5, -7, -9, -10, -14, and -15 (2, 43, 78, 96), while pDCs have a more restricted pattern and apparently only express Siglec-5 (43). Siglecs, with the exception of Siglec-14 and -15, expressed by DCs present ITIM motifs in their cytosolic portion and are therefore mainly involved in inhibiting activation signals and have an immunoregulatory function (40, 41).

The concentration of sialic acids on surfaces of human cells is very high; for example, Stamatos and colleagues estimated that DCs had 8.9 nM per 5×10^6 cells, which correspond to nearly 10^{18} sialic acid molecules per cell (62).

Therefore, it is possible that the majority of Siglecs expressed at DC surface bind *in cis*, i.e., to sialic acids at their own cell surface. The *cis* interaction will have primacy over the *trans* interactions, the only exception being sialoadhesin, which has an extended structure, projecting their binding site away from plasma membrane and being therefore involved in *trans* interactions (97).

Siglec interactions *in cis* can be released by sialidase activity, either extrinsic for instance from pathogens or intrinsically due to the activity of endogenous sialidases (40, 98). Since DCs ultimate function is to immunomodulate T cells and (to some extent) B cells, Siglecs potentially play a largely relevant role in host-tolerance mechanisms (2, 43, 99). Chen and co-workers reported Siglec-10 as involved in helping distinguish TLR-recognized danger-associated molecular patterns (DAMPs) – generated during cell/tissue damage or even regular cell lifecycle – from PAMPs, thus controlling inflammation (100). There are known examples of T cell activation where DC Siglecs have a relevant role in inducing Th1 and Th2 responses, as is the case of DC Siglecs-1 and -7 *trans* recognition of α2,3-sialic acids and α2,8-polisialic acids, respectively, in mimicked GM1a and GD1a (Siglec-1 recognized) and GD1c (Siglec-7 recognition) gangliosides included in *Campylobacter jejuni*'s LPSs (101).

CD33-related Siglecs can function as endocytic receptors that are important in the clearance of sialylated antigens. On the other hand, many pathogens are able to express appropriate sialic acids

themselves (102–105). Pathogen's sialic acids may interfere with DC functions such as endocytosis (43, 106) thus helping DCs to internalize and further present pathogen's antigens. This, however, may also open an opportunity window for pathogens to modulate DCs' immune functions (by binding to immunoregulating Siglecs) or even use DCs as vectors (i.e., "Trojan horses") for infection of other immune system cells, such as HIV using Siglec-1 as a gateway-receptor for DC entry and posterior transmission to $CD4^+$ T cells (107). However, a safety mechanism may be present: Siglec-15 can act as an activation receptor balancing the negative signaling triggered after recognition of sialylated pathogens (viz., enveloped viruses) through inhibitory Siglecs (78).

DENDRITIC CELL SIALYLATION AND ENDOCYTOSIS

The sialic acid's role on endocytosis has long been studied on the perspective of the pathogen. Besides the already referred trans-sialidase bearing *T. cruzi* parasite, it is also known that several bacteria developed sialic acid-masking mechanisms in order to escape immune surveillance and/or response (108). Recent discoveries, however, hinted that sialic acid's role in these immune processes goes far beyond than "just" being an antigen, with a functional impact on the innate immune phase cells as well, like DCs.

As previously mentioned DCs are functionally well prepared to endocytose pathogens, in order to process and present them to the adaptive immune response cells (20, 109, 110). Using two different approaches – sialidase treatment of moDCs and bone marrow-derived DCs (BMDCs) from sialyltransferase KO mice, it has been determined the functional impact of sialic acid on macropinocytosis and phagocytosis. Asialylated DCs presented significantly reduced ovalbumin-macropinocytosis but increased phagocytosis levels (111). Similar results having been obtained using BMDCs from ST6Gal-I and ST3Gal-I sialyltransferase-deficient mice (3).

Sialic acid removal (or absence in BMDCs) has a positive impact over the DC maturation process, leading to higher expression of maturation markers. Hence, this effect should account for the observed reduction of macropinocytosis levels, since matured DCs tend to have decreased endocytosis ability (112). The observed increase in phagocytosis in asialylated immature and mature DCs (111) seems, therefore, to oppose the endocytosis reduction induced by maturation. It is documented, however, that mature DCs may continuously uptake antigens by phagocytosis and receptor-mediated endocytosis, even if always described in lower levels than immature DCs (110, 113). As no studies have been performed from a sialic acid point of view, this can account for novel, groundbreaking evidence adding to the well-established concepts of endocytosis. Another piece of this apparent puzzle lies in the DC cytoskeleton, which has to be adjusted to perform cellular extensions needed for phagocytosis. After sialidase treatment of DCs, a cytoskeleton disorganization is observed. In addition, the activity of two Rho GTPases – Rac1 and Cdc42 – that regulate, among other processes, the actin-dependent events of macropinocytosis and phagocytosis (19, 112, 114–116) are downregulated, after sialidase treatment. This may justify the cytoskeleton disorganization and decreased macropinocytosis.

Hence, the fact that sialidase treatment accounts for the significant *E. coli* phagocytosis enhancement, in both sialidase-treated immature and mature DCs, is a process unrelated to maturation. Interestingly, the effect on phagocytosis seems to depend on the presence, in *trans*, of bacterial sialic acid (111), adding a potential involvement of Siglecs. Hence, sialidase treatment would release DCs' Siglecs from in *cis* ligands, making them available to bind to ligands in *trans*, such as sialic acid-containing glycans present in the *E. coli* cell wall. However further investigations are still needed to elucidate the underlying mechanisms.

Sialidase-induced activation of receptors is not a novel phenomenon. Receptors like TLR-4 are known to depend on the activity of membrane sialidase prior to LPS-induced activation: in mice, DC phagocytosis is activated by desialylation of surface receptors (62, 117, 118). This evidence is in line with the already mentioned increased expression of sialidases, such as Neu1 during DCs differentiation and maturation (117). Furthermore, physiologically, cell surface sialic acid content is not exclusively cleaved by endogenous sialidases, since exogenous sialidase sources are also released by pathogenic bacteria or virus during the course of an infection. In the mouse model, it was reported that Neu1-induced desialylation activates phagocytosis by macrophages and DCs (118). Also, cell surface desialylation by influenza virus sialidase stimulates the internalization of target virus by infected mouse macrophages (119).

Siglecs and TLRs fit perfectly in the recent model presented by Cabral and collaborators showing that sialidase treatment of DCs favors phagocytosis (111). Since they are receptors with both strong activating- and suppressive-inducing properties, with known roles in regulating immune responses and with the potential of becoming active after sialic acid removal by sialidases, they are also likely to account for the observed upregulation of both pro- and anti-inflammatory cytokines (111). Nevertheless, the referred receptor families may not be the only receptors affected by sialidase treatment in DCs as novel glycan-protein interactions are continuously being revealed, but further studies are in order to better elucidate the role of sialic acid in endocytosis.

SIALYLATION AND DENDRITIC CELL MIGRATION

Dendritic cell migration includes both DC recruitment to non-lymphoid tissue and homing to lymphoid organs.

When located within tissues, DCs may respond to pro-inflammatory cytokines and pathogens, which trigger maturation and DCs then migrate to lymphoid tissues via afferent lymphatic vessels, wherein they activate antigen-responsive T cells. Immature and mature DCs may also enter the blood and from there disseminate to non-lymphoid and lymphoid organs, thereafter returning to blood, thus undergoing cycles of recirculation. Therefore DCs have complex trafficking routes, allowing for dynamic reassortment of DCs, making the most of their capacity to uptake antigens and to encounter T cells to present antigens and activate them.

While, generally, the migratory processes are based upon mechanisms like adhesion and chemotaxis, some processes still show their own particularities. The extravasation of blood DCs to any tissue involves DC adhesion to endothelium and is dependent of selectin interactions with sialofucosylated glycans. The role of certain sialylated glycans as selectin ligands is one of the most recognized functions of sialic acid in the context of leukocyte recruitment (120).

Selectins are CLRs expressed by platelets, endothelium, or leukocytes, hence taking their name: P-, E-, or L-selectins, although endothelial cells also express P-selectins.

All selectins recognize the sialic acid and fucose (Fuc) containing tetrasaccharide, where sLe^x (Neu5Acα2,3Galβ1,4[Fucα1,3] GlcNAc-) is the major prototype. Selectin ligands are expressed in most circulating immune cells and some endothelial cells during inflammation. They mediates essentially the rolling and tethering phase of cell transmigration over the endothelial cell surface (121). sLe^x expression is well characterized in neutrophils and lymphocytes (76, 122) but only relatively approached in DC (123–126). Recently, it was found that moDCs also express functional selectin ligands, based on their observations of moDC tethering and rolling over a P-, E-, and L-selectin immobilized surface (126). They observed decreasing tethering affinities (by decreasing order) toward P-, E-, and L-selectin, with similar lower rolling velocity on P- and E-selectins and the largest rolling velocity observed over L-selectins. These findings were in line with other studies using blood DCs and $CD34^+$-derived DCs (125, 127). Furthermore, the use of anti-sLe^x antibodies on the rolling studies resulted in a significant binding inhibition, definitely proving that sLe^x mediates the moDCs-selectin binding (126).

In order to properly function as a selectin ligand, sLe^x must be expressed in carrier glycoproteins or glycolipids (128, 129). The only described sLe^x carrier-protein described in moDCs is the P-selectin glycoprotein ligand-1 (PSGL-1) (123), a mucin-like glycoprotein, present in the microvilli of most leukocytes (130, 131) (**Figure 2**, "Cell adhesion" feature). In DCs, sLe^x-decorating PSGL-1 is the solely ligand for P-selectin, with significant less affinity toward L-selectin and being indifferent for E-selectin binding (126).

Nevertheless, sialic acids also participate in the chemokine receptor-mediated firm arrest, as well as in β1 integrins function (132–134). There is also evidence concerning the chemokine-mediated migration to the lymph nodes. It was recently reported that ST8Sia-4-dependent polysialylation of neuropilin-2 seems to be relevant for chemokine-driven migration toward lymph nodes (135). Other report claims that ST3Gal-IV is not relevant for chemokine-dependent DC homing, in the mouse model (120), but, interestingly, our team's preliminary studies using ST6Gal-I-deficient mice have shown impaired DC migration toward draining lymph nodes, suggesting a previously unknown role for α2,6-sialylated *N*-glycans in DC homing.

Dendritic cell mobility is a crucial step still needing to be better elucidated. Most of the clinically efficacy of DC immunotherapy relies on the migration ability of these cells. In *ex vivo* generated DC vaccines, it is estimated that only 1–2% of total administered DCs reach secondary lymphatic organs (136). Therefore the majority of *ex vivo* generated DCs are inefficient because they do not meet T cells. Thus, understanding DC migration should be regarded as important to find means to improve DC immunotherapy.

SIALIC ACID IN DENDRITIC CELL-T CELL INTERACTIONS

The ultimate function of DC immunobiology is the DC-T cell interaction, whereupon DCs present the uptaken, processed antigens to T cells, thus eliciting a specific, long-lasting immune response. Since immunological synapses between these two cells involve glycoprotein receptor-mediated process, it is, thusly, potentially influenced by sialic acid.

Dendritic cells' sialic acid-containing glycans have been shown to negatively influence T cell priming, most likely by interference on MHC-mediated antigen presentation and co-stimulation (137, 138). In line with these findings, sialidase-treated moDCs were able to prime T cells and induce proliferation more efficiently than fully sialylated moDCs (3, 111). This effect could be attributable to the increased maturation by sialidase-treated moDCs (3). However, one should not discard a synergistic effect with enhanced protein–protein interaction due to the absence of the negatively charged sialic acid (137), leading to enhanced inter cellular interactions. The verified upregulation of a set of pro-inflammatory, Th1 profile-inducing cytokine expression (viz., IL-1α, -6, -12, and TNF-α) in sialidase-treated moDCs (with subsequent IFN-γ secretion) could also account for the observed increased priming.

Reinforcing these results, others have observed that endogenous sialidase activity also promotes cytokine production by moDCs and this has been attributable to the action of Neu3 upregulation during moDC differentiation (62). Interestingly, tolerogenic, immature moDCs present high sialic acid content, as well as regulatory T cells. Thus it has been hypothesized that, host-tolerance induction by DCs could be a Siglec-mediated process (93).

Taken together, this evidence reminds that DC sialylation has implications in the T cell interaction and it is likely to twist the immunogenic/tolerogenic balance. Thus sialylation should be considered to fine tune DC-based therapy either pathology-treating or tolerance-inducing.

DENDRITIC CELL GLYCAN RECOGNITION OF TUMORS

Dendritic cells functions also include specific identification of tumor cells and presentation of tumor antigens to T cells. One of the mechanisms for tumor cells recognition is through the binding of cells surface receptors to tumor-specific antigen (TSA), with an almost exclusive tumor expression and tumor-associated antigens (TAAs), normally expressed on the cells but of aberrant expression on tumor cells (139). Upon recognition, these antigens normally elicit a maturation response on DCs but the immune potency depends on many factors, including the antigen. Tumors have, however, several evasion strategies from immune responses, achieving this by creating a tolerance-inducing microenvironment, secretion of inhibitory factors, and activation of immunosuppressant intracellular pathways in the immune cells (140–142). DCs present certain flaws in their antigen-presenting strategy that tumor cells take advantage of in order to create defective T cell responses, thus creating problems in generating effective anti-tumoral solutions (142, 143).

Aberrant glycosylation is a hallmark of cancer cells and aberrant glycosylated proteins can be shed into the body fluids of the patients (serum, urine, pleural effusions, etc.). This altered glycosylation pattern in tumor cells includes either a loss or a gain of expression of certain glycan structures, the appearance of truncated structures, as well as of novel structures. Upregulation and/or downregulation of specific glycosyltransferases is often responsible for these changes. Tumor-associated carbohydrate (TAC) structures allow tumor cells to invade and metastasize

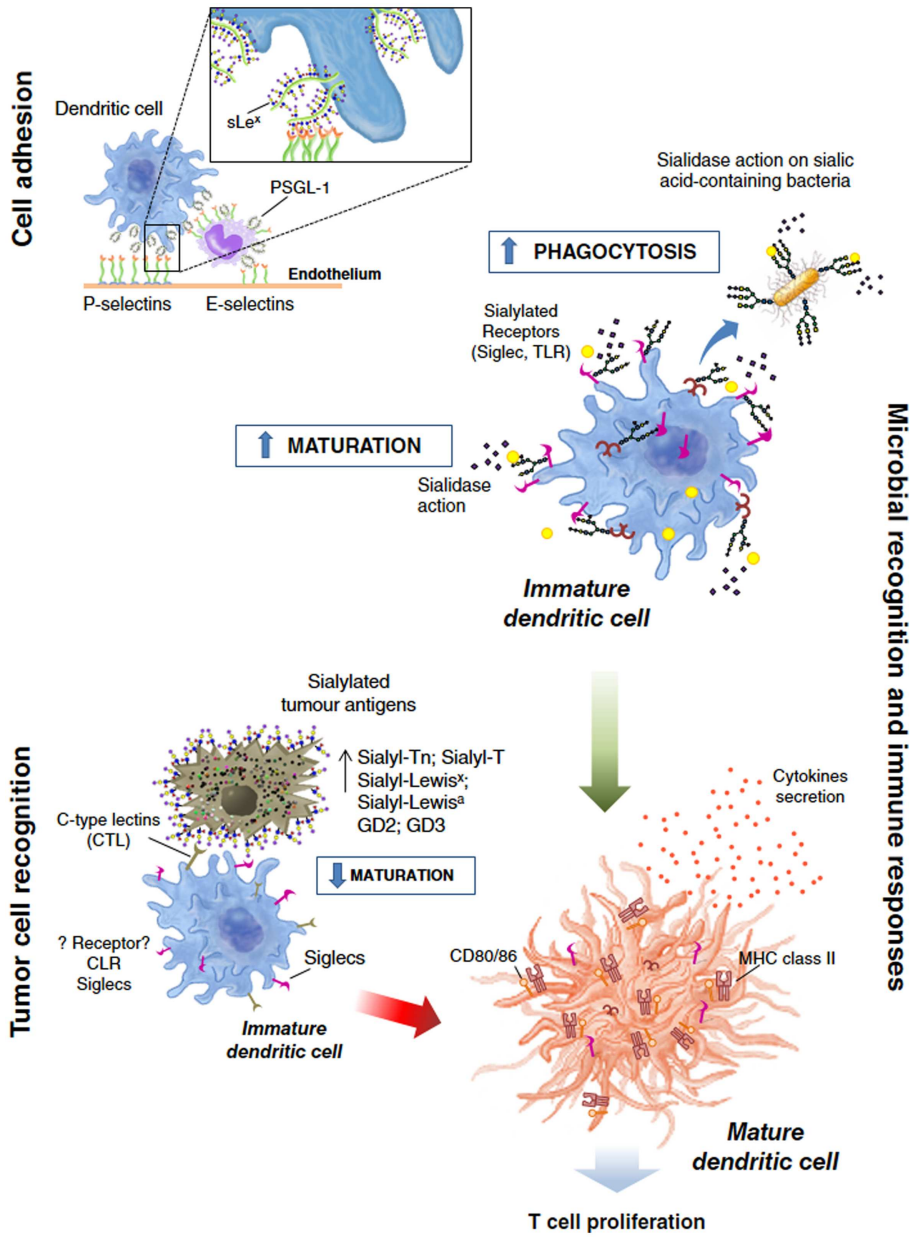

FIGURE 2 | General overview of the dendritic cell functions modulated by sialylation. Sialic acid-containing glycans actively participate and modulate processes like: *cell adhesion* during migration and homing; or in "de facto" immune processes such as *tumor cell recognition* and *microbial recognition*, overall modulating the immune response/tolerance balance.

or to evade the immune system. Immature and/or tolerogenic DCs can migrate to the rapidly growing tumor microenvironment, thus eliciting immune tolerance in several ways, such as T cell deletion, anergy, and T_{reg} activation (142–145). The tolerogenic profile depends on the DCs recognition and binding to TAC. However, how DCs recognize the tumor cells and in particular the TAC are not fully disclosed. The few available studies point, so far, to the CLRs, MGL-1, and DC-SIGN receptors as being relevant in tumor recognition and undesired tolerance induction (37, 146): the former is highly expressed in immature, tolerogenic DCs, and shown to interact with the tumor-associated Tn antigen-bearing forms of MUC1 (147); the latter is also expressed by immature DCs and recognizes Le^x and Lewis Y (Le^y) glycoantigens in a carcinoembryonic antigen-context expressed in colorectal carcinoma. Besides these receptors, the observed involvement of DCs' Siglecs (such as Siglec-3 and -9) could help justifying the frequent tolerance-induction mechanisms: by recognizing overexpressed sialylated antigens at the tumor microenvironment (e.g., sialyl T and sLe^a expressed on mucins), these receptors could send inhibitory intracellular

signals from their ITIM motifs thus preventing DCs from differentiating (by inducing apoptosis of their precursors) or maturating, keeping them in a tolerance-inducing state with concomitant upregulated anti-inflammatory cytokine expression, downregulated pro-inflammatory cytokine expression, and reduced antigen-presenting capability (148–150).

It is now evident that TAC and in particular, sialic acid expression influences tumor progression. DCs become tolerogenic after recognition of TAC (including glycan-bearing/glycosylated TAC), favoring tumor progression and being generally associated with bad prognosis. The collected evidence regarding the glycan influence on anti-carcinogenic immune processes should be, therefore, seriously considered whenever DC-based immunotherapies against specific malignancies are available.

CONCLUDING REMARKS

The weight of glycosylation and, in particular, sialic acid in biological processes is being increasingly acknowledged. Being at the terminal position of many glycans, it plays an essential role in modulating many of the DC functions. In human DCs, the majority of studies to date have focused on moDCs and only scattered and very scarce data was reported regarding other subsets. It would be extremely important to study these and other immune mechanisms from the newly identified subsets' perspective and to complement those studies using mouse DCs other than the traditionally (myeloid) BMDCs. Being known that different subsets of DCs have different functions/affinities toward different pathogens/tissues (and elicit different responses) it should not come as a surprise that different subsets could express different glycans and glycan-recognizing receptors, having thus different underlying mechanisms and eliciting different immune responses. The discovery and accessibility of new, faster, and more precise glycobiology-related techniques may allow a better understanding of the role of sialylation and glycosylation in DCs. The problem that poses glycobiologists, and immunologists in particular, is trying to add a new perspective and knowledge, in the same magnitude, to the amount of knowledge that proteomics and genetics have gathered the last 30 or 40 years, in a short amount of time. That premise is getting growingly important every time a relevant role for glyco-based phenomenon is identified. Our hope, with this review is that we contributed a little bit more to put the spotlight on Glycoimmunology and encourage further investigations on this subject.

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Microbe–host interactions are positively and negatively regulated by galectin–glycan interactions

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Microbe–host interactions are complex processes that are directly and indirectly regulated by a variety of factors, including microbe presentation of specific molecular signatures on the microbial surface, as well as host cell presentation of receptors that recognize these pathogen signatures. Cell surface glycans are one important class of microbial signatures that are recognized by a variety of host cell lectins. Host cell lectins that recognize microbial glycans include members of the galectin family of lectins that recognize specific glycan ligands on viruses, bacteria, fungi, and parasites. In this review, we will discuss the ways that the interactions of microbial glycans with host cell galectins positively and negatively regulate pathogen attachment, invasion, and survival, as well as regulate host responses that mitigate microbial pathogenesis.

Keywords: galectin, cell surface glycans, microbial pathogen, virus, bacteria

INTRODUCTION

Lectins are proteins that recognize glycan ligands. In mammalian hosts, different types of endogenous lectins can bind to self glycans, e.g., selectins that tether circulating leukocytes to the endothelium or siglecs that regulates signaling in leukocytes by binding to cell surface glycoprotein receptors (1–4). Also in mammalian hosts, various types of lectins can recognize glycans that are typically found on pathogens, but not on host cells, such as mannose binding protein and dectin-1 that bind high mannose and β-glucan ligands, respectively, on yeast (5–8). Thus, these types of lectins have been grouped in the category of pattern recognition receptors (PRRs) that bind pathogen-associated molecular patterns (PAMPs) expressed exclusively on microbes (9, 10).

However, it is becoming increasingly clear that there are several lectins that cannot be exclusively segregated into either category of lectins that only recognize self or lectins that only recognize non-self. These include some C-type lectins, such as DC-SIGN, DCIR, and the macrophage mannose receptor, that can recognize both host and microbial glycans (11–13). Similarly, several members of the galectin family can recognize both host and microbial glycans (14–17). Many C-type lectins and galectins are considered to be components of the innate immune response to pathogens, because binding of these lectins to microbial glycans can either directly or indirectly promote host defense, by triggering leukocyte activation, phagocytosis, complement fixation, and cytokine production (7, 8, 14). However, some of these same lectins can also enhance microbial infection of different hosts, an effect that would seem to be counter-intuitive if lectins are considered to act solely as host defense molecules (7, 17). Thus, the effects of specific lectins as pro-microbe or pro-host must always be considered in a context-dependent manner.

This review will examine roles of galectin family members in both promoting microbial infection and enhancing host resistance to infection. The galectins are a family of lectins with a common carbohydrate recognition domain (CRD), and are found in all multicellular organisms, including fungi, nematodes, insects, and vertebrates. Importantly, all galectins are either bivalent or can multimerize into dimers, pentamers, and higher order oligomers; the multivalency of galectins allows these molecules to bind multiple glycan ligands on either the same cell or on opposing cells, or, in some cases, on microbes and host cells (14, 15, 18).

In many organisms, galectins have been described as participating in recognition and defense against microbial pathogens. Several recent reviews have addressed the indirect effects of galectins on microbial pathogenesis through regulation of innate and adaptive immunity, e.g., promoting dendritic cell maturation and migration, enhancing cytokine production, or initiating release of intracellular mediators such as histamine (14, 15, 19–21). In contrast, we will specifically address mechanisms by which galectins directly interact with microbial pathogens to effect three distinct outcomes – enhancement of microbial infection, blockade of microbial infection, or microbicidal activity (**Figure 1**).

GALECTINS PROMOTE MICROBIAL INFECTION

The best-described role for galectins in microbial infections is to promote pathogen attachment to or entry into host cells. As galectins are typically multivalent, galectins can bind to glycan ligands on both the microbial pathogen and the host plasma membrane to "bridge" the microbe to the target cell. This effect has been described for viruses, bacteria, and parasites.

Sato and colleagues demonstrated that galectin-1 can promote HIV-1 infection of human T cells and macrophages by promoting viral adsorption to the target cells (22, 23). This effect was specific for galectin-1, as galectin-3 did not enhance HIV-1 infection of T cells or macrophages. Moreover, galectin-1 promoted viral attachment, but did not affect fusion, supporting the "bridging" function

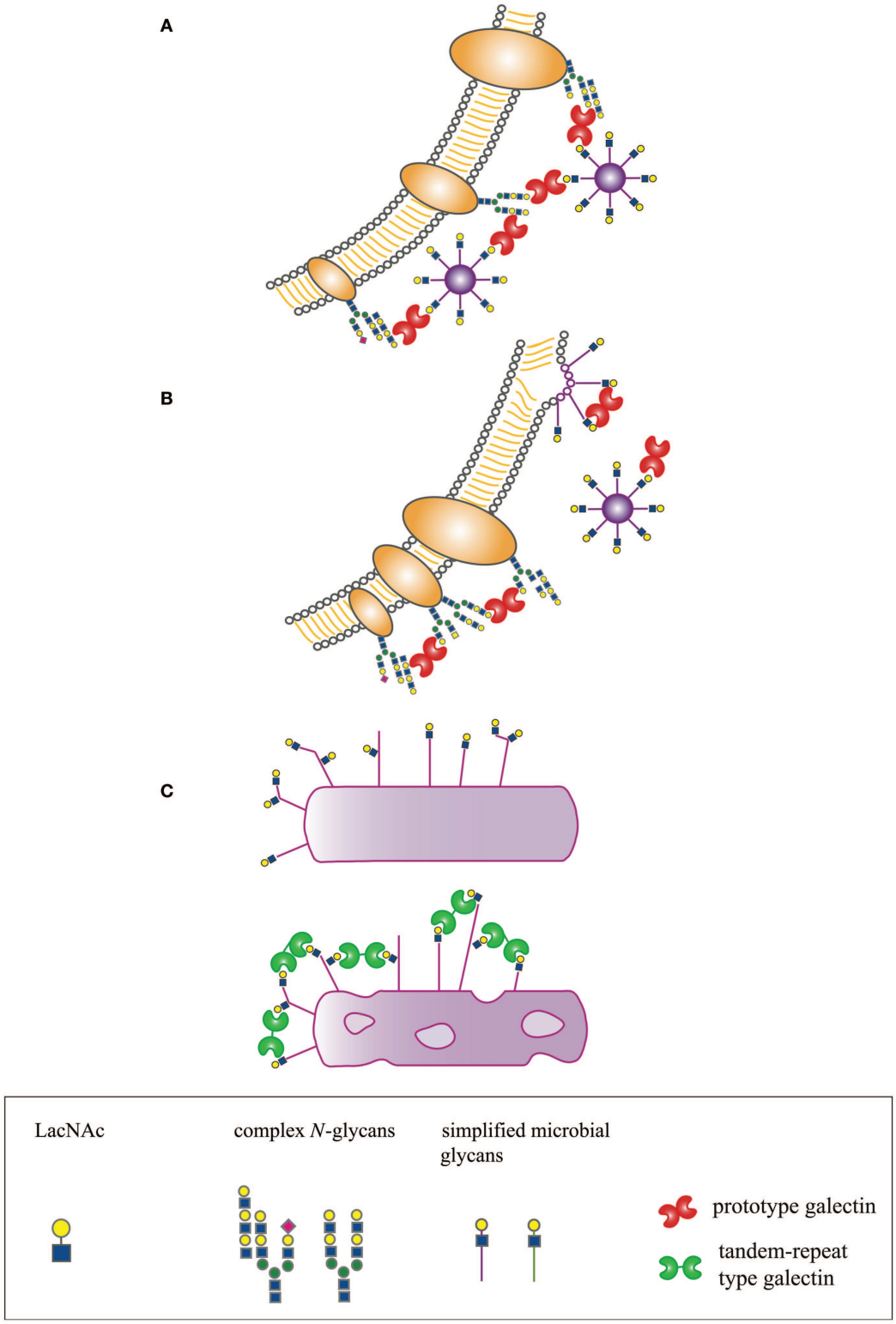

FIGURE 1 | Host-derived galectins can positively and negatively regulate microbe–host interactions. **(A)** Galectins can promote microbial attachment to and infection of host cells by bridging pathogen glycans to host cell surface glycans. **(B)** Galectins can inhibit microbial attachment and infection by binding to host or microbial glycans and blocking molecular interactions essential for microbial binding and entry, or by binding to glycans on nascent microbes, such as budding viruses, and preventing proper structural assembly. **(C)** Galectins can be directly microbicidal, an effect regulated by expression of specific glycan ligands on the pathogen surface.

of galectin-1 in this context. Glycoprotein receptors for galectin-1 have been proposed to be gp120 on the virus and CD4 on the host cells. However, galectin-1 can also bind to CD43 and CD45 on T cells (24, 25); these large glycoproteins are very abundant on the plasma membrane of these cells, and may also contribute to adsorption of virus to target cells. In addition, it is known that budding viral particles can incorporate host cell glycoproteins in the viral envelope, raising the possibility that host cell glycoproteins such as CD45 on the viral envelope may also contribute to galectin-1 binding (26). A similar mechanism of galectin-1 bridging virus to host target cells has recently been observed by our labs for the Nipah virus. Galectin-1 binds to specific glycans on viral envelope glycoproteins and on the target cell plasma membrane to increase adsorption of virus to target cells, resulting in increased efficiency of infection of target cells (Garner et al., in preparation).

Our labs also found that HIV infection of target cells was promoted by another galectin family member, galectin-9 (27). However, in this case, enhanced infection was not achieved by direct "bridging" of the virus to target T cells. Rather, galectin-9 regulated the redox environment at the T cell surface by retaining the thioreductase protein disulfide isomerase (PDI) on the cell surface; as reduced forms of both the viral Env glycoprotein gp120 and the T cell counterreceptor CD4 promote viral entry, the enhancing effect was likely due to the reducing environment created by galectin-9 retention of PDI.

Both galectin-1 and -3 have been implicated in increasing target cell infection with human T cell leukemia virus type 1 (HTLV-1) (28). As described for HIV, galectin-1 was found to stabilize attachment of HTLV-1 to human T cells, resulting in increased efficiency of infection as well as increased fusion of HTLV-1 infected cells. Galectin-3 was also found to increase T cell infection by HTLV-1, although the proposed mechanism differs from the direct bridging of virus to host cell that has been proposed for galectin-1 and HTLV-1. HTLV-1 forms extracellular viral assemblies, and the attachment of these viral assemblies to host T cells is promoted by extracellular matrix proteins such as agrin and collagen, as well as by cell surface proteins including galectin-3. Unlike the HIV envelope glycoprotein, the HTLV-1 env protein is poorly glycosylated, so that galectin-3 may bind to these viral assemblies via recognition of glycans on T cell-derived glycoproteins in these supramolecular aggregates (29). However, in both cases, the galectin-mediated interaction results in increased infection of human T cells with HTLV-1.

A similar bridging function has been proposed for galectin-3 in promoting infection of human corneal epithelial cells by herpes simplex virus (HSV)-1 (30). Galectin-3 specifically bound to HSV-1 in a carbohydrate-dependent manner, but did not bind the highly related HSV-2, indicating that specific glycans on viral envelope glycoproteins are determinants of galectin-3 recognition. Reduction of galectin-3 expression on corneal keratinocytes reduced HSV-1 binding and infection of the cells, indicating that galectin-3 can directly promote HSV-1 infection of target cells.

Direct bridging of pathogen to host target cells has also been described for enhancement of bacterial adhesion to host cells by galectins. Galectin-3 specifically increased binding of *Neisseria meningitidis* to human monocytes and macrophages, but not to nasopharyngeal epithelial cells (31). Galectin-3 bound *N. meningitidis* lipopolysaccharide (LPS) that bears terminal LacNAc sequences, a preferred glycan ligand for galectin-3. Full-length galectin-3 was required for this effect; as proteolytic removal of the N-terminal multimerization domain abrogated the enhanced binding, this implied that galectin-3 must multimerize for enhancement to occur. In addition, galectin-3 null mice demonstrated reduced levels of bacteremia, compared to wildtype mice, after challenge with live *N. meningitidis*, further implicating galectin-3 in promoting bacterial infection of host cells. Similarly, LPS from *Pseudomonas aeruginosa* also bound galectin-3; galectin-3 is produced by human corneal epithelial cells, a target of *P. aeruginosa* infection. Antibodies specific for either the outer core region of *P. aeruginosa* LPS or for galectin-3 blocked binding of bacteria to cultured human corneal epithelial cells, implicating galectin-3 in corneal infection and development of bacterial keratitis (32).

Numerous studies have also found roles for galectins in bridging parasite pathogens to host cells. *Leishmania major* can bind both galectin-3 and -9 (33, 34). Galectin-9 binding to polygalactose epitopes on *L. major* lipophosphoglycans promotes binding of the parasite to macrophages; specific galectin-9 receptors on the target cells were not identified, although the authors proposed that other macrophage lectins, such as the macrophage mannose receptor, may be involved. In contrast to galectin-9, galectin-3 binds *L. major* but this resulted in proteolytic cleavage of galectin-3 and did not promote binding of *L. major* to macrophages. As the N-terminal domain of galectin-3 regulates multimerization and the C-terminal domain of galectin-3 contains the CRD (15), cleaved galectin-3 would be unable to multimerize; thus, galectin-3 could not tether the parasite to host cells, because the bridging effect relies on the multivalency of galectins.

Intriguingly, galectin-mediated bridging of *L. major* to host cells is also important at another point in the parasite life cycle. Valenzuela and co-workers found that a galectin homolog in the midgut of the sand fly *Phlebotomus papatasi* participates in binding of *L. major* at the procyclic phase to gut epithelial cells during infection of this obligate insect host (35). The *L. major* parasite replicates in the sandfly midgut and differentiates into the metacyclic phase that is highly infectious to mammalian hosts and is transmitted during insect bites. Differentiation into the metacyclic phase coincides with alterations in the parasite lipophosphoglycans that reduce binding to the sandfly galectin and allow release of the parasite from the insect gut epithelial cells. Thus, changes in the parasite surface glycoconjugates can promote or reduce binding to various types of galectins in both insect and human hosts at different points during the *L. major* life cycle.

Galectin-3 has also been implicated in enhancing infection of human host cells by the parasite *Trypanosoma cruzi*. Galectin-3 bound to the surface of *T. cruzi* trypomastigotes and exogenous galectin-3 enhanced binding of the parasite to human coronary artery smooth muscle cells. In addition, reducing expression of endogenous galectin-3 by these cells dramatically reduced *T. cruzi* adhesion (36). Galectin-3 also promoted binding of *T. cruzi* to extracellular matrix proteins such as laminin (37). As

T. cruzi infection increases expression of ECM components such as laminin by host cells, the parasite may use the bridging function of galectin-3 to accumulate in the basement membranes surrounding host target cells such as cardiac myoblasts, thus increasing the likelihood of infection (38).

Most work on the roles of galectins in promoting microbial infection has focused on galectins-1, -3, and -9, that are highly expressed by cells of the immune system as well as endothelial cells and many types of epithelial cells. Other galectins have a more restricted expression pattern; for example, galectin-7 is expressed by squamous epithelial cells. Okumura et al. investigated attachment factors that would promote infection of cervical epithelial cells by *Trichomonas vaginalis* (39). This group found that galectin-1, but not galectin-7, bound to *T. vaginalis*, although both galectins were expressed by cervical epithelial cells. Addition of exogenous galectin-1, but not galectin-7, also enhanced parasite binding to cervical epithelial cells, while reducing expression of endogenous galectin-1 in these cells via siRNA decreased parasite binding. Thus, as has been found for several of the pathogens described above, there is specific binding of one or more distinct galectin family members to different microbial pathogens, demonstrating the unique functions of different galectins at discrete points during infection.

GALECTINS BLOCK MICROBIAL INFECTION

While it is clear that several galectins can bind to microbial glycans to bridge the microbes to host target cells, galectin binding to microbial glycans does not always result in a pro-microbe effect; a microbial glycoprotein that may be available for host attachment may, at another point in the microbial lifecycle, be a liability when galectin binding prevents microbial dissemination. An example of this dichotomy is the interaction of galectin-1 with the NiV–F fusion protein. As described above, binding of galectin-1 to Nipah virus envelope glycoproteins can enhance infection of target endothelial cells by bridging the virus to host plasma membrane glycoproteins (Garner et al., in preparation). However, post-infection, the fusion-promoting activity of the NiV–F protein has a pathogenic effect beyond facilitating viral entry. Endothelial cells infected with Nipah virus and thus expressing NiV–F at the plasma membrane fuse with one another to form giant syncytia; *in vivo*, this process leads to endothelial cell disruption and death and is a primary cause of the hemorrhagic diathesis seen in Nipah virus infection (40–42).

Galectin-1 binding to NiV–F expressed at the plasma membrane prevented cell syncytia formation by three distinct mechanisms. First, NiV–F initially appears at the plasma membrane as an immature precursor that must be endocytosed and proteolytically cleaved intracellularly; the cleaved, mature form recycles to the plasma membrane and is fusion-competent. However, galectin-1 binding to the immature NiV–F protein retards this essential endocytosis step and thus results in decreased production of fusion-competent NiV–F. Second, optimal membrane fusion requires lateral movement of NiV–F on the plasma membrane; however, galectin-1 binding to NiV–F reduces this lateral movement and thus reduces the extent of syncytia formation. Third, NiV–F must undergo a conformational change to effect the membrane mixing required for fusion, and galectin-1 binding inhibits this conformational change of NiV–F. While the NiV–F glycoprotein has five *N*-glycan attachment sites, one particular glycan, the F3 glycan, on NiV–F contributed significantly to all three of these inhibitory effects, i.e., the inhibitory effects of galectin-1 on NiV–F maturation, movement, and conformational changes were reduced when the F3 site was mutagenized to remove the *N*-glycan (40, 42). As the NiV–F3 glycan was also important for the bridging effect that enhances NiV entry into endothelial cells (Garner et al., in preparation), this demonstrates that the same galectin–glycan interaction that can promote viral entry at an early time point during infection can reduce the pathophysiological effect of viral infection at a later time point.

Finally, the presence of galectin-1 during viral budding reduced the production of Nipah virus particles from infected endothelial cells (42). While it is not yet clear whether this effect resulted specifically and directly from binding of galectin-1 to viral envelope glycoproteins NiV–F or NiV–G, or whether binding of host plasma membrane glycoproteins by galectin-1 disrupts efficient viral budding, this observation reinforces the point that the same lectin, galectin-1, can have a pro-viral or anti-viral effect, depending on the timing and context of galectin binding to viral glycoproteins.

Another example of a galectin directly blocking microbial infection is described in the study by Shiau and colleagues (43); this group found that galectin-1 can bind directly to envelope glycoproteins of influenza virus, an effect that impaired virus infection of target cells *in vitro*. In addition to demonstrating this protective effect of galectin-1 *in vitro*, the authors described increased expression of galectin-1 in airway epithelia *in vivo* in a murine model of influenza virus infection. Moreover, intranasal administration of recombinant galectin-1 during influenza virus infection reduced viral load and accompanying inflammation, tissue damage, and mortality in the murine model, and galectin-1 null mice were more susceptible to influenza infection than wildtype mice.

While not directly blocking viral entry, the Panjwani and Argüeso labs found that galectin-3 can bind to ocular mucins to contribute to the barrier function of ocular mucins in preventing infection. Thus, while galectin-3 can bind to HSV-1 to promote viral entry into epithelial cells, as described above (30), galectin-3 also contributes to reducing HSV-1 entry by organizing cell surface mucins to maintain mucosal barrier function in the eye (30, 44), providing another example of a galectin having both infection-promoting and infection-reducing effects for a particular microbial pathogen.

Numerous studies have implicated galectins in promoting a protective innate or adaptive immune response to pathogens, although many of the studies have described effects that do not directly involve binding of the pathogen to a galectin. However, Schwarz and colleagues have demonstrated that the glycophosphatidylinositol (GPIs) on the surface of *Toxoplasma gondii* can bind directly to galectin-3 on the surface of macrophages (45). The binding of these parasite GPIs to cell surface galectin-3, which associates with TLR2, is essential for TLR2-mediated production of tumor necrosis factor (TNF) α by the macrophages. As TNFα is a critical cytokine that promotes parasite clearance, the direct

interaction of galectin-3 and *T. gondii* GPIs is an important initial step to reduce the extent of infection.

Finally, several studies have proposed that galectins in the gastrointestinal tract can participate in the organization of mucins into a protective layer that impedes access of microbial pathogens to the host epithelium, implying an indirect role for galectins in reducing microbial invasion. Galectin-4, a galectin that is highly expressed in gut epithelial cells and has been shown to organize epithelial plasma membrane domains by binding to specific glycoproteins and glycolipids, can bind directly to epithelial glycolipids that are receptors for pathogens such as *Bordetella pertussis* and *Helicobacter pylori*. This observation gave rise to the hypothesis that galectin-4 may directly impede the ability of bacterial pathogens to attach to glycolipid receptors and infect host epithelium (46, 47). Experimentally, such a role has been shown for the *Caenorhabditis elegans* galectin LEC8, which has significant homology to mammalian galectins and, like galectin-4, binds glycolipids. Ideo et al. found that LEC8 is expressed in the *C. elegans* digestive tract, and that LEC8 directly blocked infection of the worms with *Escherichia coli*. Moreover, LEC8-deficient animals were more susceptible to *E. coli* infection compared to wildtype worms (48). Thus, depending on the availability of specific microbial glycoprotein receptors for galectins, the anatomic localization of galectin expression, and the timing of galectin expression during infection, galectins may block pathogen binding to and entry into target cells, rather than enhance binding as described in the previous section.

GALECTINS HAVE ANTI-MICROBIAL ACTIVITY

Galectins are evolutionarily ancient molecules found in multicellular fungi, nematodes, and insects as well as vertebrates. Thus, in addition to interfering with pathogen attachment or entry into host cells, as described above, galectins can act as "danger receptors" by either enhancing cytokine production or phagocytic clearance of pathogens by cells of the innate immune system, or by having direct microbicidal activity.

Early work described galectins associating with microbial pathogens in phagocytic vesicles, such as the association of galectin-3 with *Mycobacterium tuberculosis* in macrophage vesicles (49). While a direct anti-microbial mechanism was not determined in this study, galectin-3 null mice had a reduced capacity to clear *M. tuberculosis* late in infection, suggesting that galectin-3 had anti-microbial activity.

A more specific role for galectins in phagocytic clearance of pathogens was recently described for galectin-8 in defense against *Salmonella typhimurium*. *Salmonella* bacteria invade epithelial cells and initially reside in a specialized vacuole. The bacteria generate pores in these vacuoles, damaging the integrity of the vesicle membrane, which allows the bacteria to access the cytosol but also releases "danger signals" that initiate autophagy by the cells to contain the spread of infection. Galectins-1, -3, -8, and -9 are all recruited to vesicles damaged by endocytosed bacteria, and have all been proposed to participate as "danger-sensing" molecules; however, specific actions of galectin-1, -3, and -9 in controlling bacterial damage have not been elucidated. However, Randow and colleagues found that cytosolic galectin-8 is a specific and critical component for activation of autophagy in cells infected with *S. typhimurium* (50). This group has proposed that cytosolic galectin-8 is recruited to the sites of damaged vacuoles by binding to glycan ligands on glycoproteins displayed on the interior face of the vacuoles, although this remains to be definitively demonstrated. Cells lacking galectin-8 failed to recruit other molecular components to the damaged vesicles to initiate autophagy, demonstrating a role for galectin-8 in initiating this critical anti-microbial defense. Randow and colleagues also found that intracellular galectin-8 was recruited to damaged endosomes and lysosomes during infection with *Listeria monocytogenes* or *Shigella flexneri*, as well as during sterile vesicular damage, indicating that galectin-8 is a general component in the autophagy-initiating machinery.

As mentioned above, other galectins have been found associated with endocytosed or phagocytosed pathogens. A direct role for galectin-3 in the phagocytosis of helminths such as *Schistosoma mansoni* has been proposed (51). Galectin-3 can bind GalNAc–β1,4-GlcNAc (LacdiNAc) sequences found on the surface of helminths, and can also bind LacdiNAc ligands that are components of *S. mansoni* soluble egg antigen. Addition of recombinant galectin-3 enhanced recognition and phagocytosis of LacdiNAc-coated beads by rat macrophages, and galectin-3 was abundantly expressed *in vivo* in granulomata containing *Schistosoma* eggs in infected hamsters. Thus, galectin-3 has been proposed to play an important role in the uptake and immunologic "containment" of parasites within granulomata.

A role for galectin-3 in phagocytosis of yeast has also been shown. The yeasts *Candida albicans* and *Candida parapsilosis* can be phagocytosed by macrophages and neutrophils. Bliss and colleagues found that galectin-3 secreted by neutrophils was important for effective phagocytosis of yeast by neutrophils; adding blocking antibody to galectin-3 reduced phagocytosis, while addition of exogenous galectin-3 increased phagocytosis (52). Thus, in neutrophils, galectin-3 appears to act at an early point in the yeast recognition and engulfment process to enhance uptake of fungal pathogens. In contrast, an indirect role for galectin-3 in anti-fungal immunity has also been proposed, as galectin-3 has been found to be important for production of the cytokine TNF-α by macrophages exposed to *C. albicans*. Using a murine macrophage cell line, Fink and colleagues found that galectin-3 was not essential for fungal uptake, but that galectin-3 associated with Dectin-1, a C-type lectin that specifically recognizes fungal β1,3-glucan in the fungal wall and directly mediated fungal uptake; loss of galectin-3 expression in these cells did not reduce fungal uptake, but reduced TNF-α production (53). Similarly, Poulain and colleagues found that, while galectin-3 can bind specific β1,2-mannosides on *C. albicans* mannoproteins, *C. albicans* uptake by human monocyte-derived macrophages required TLR2 but not galectin-3; however, galectin-3 associated with TLR2 in macrophages that had phagocytosed yeast, and galectin-3 expression by the macrophages was essential for optimal TNF-α production after yeast phagocytosis (54).

In addition to promoting phagocytic uptake or autophagic destruction of bacteria, fungi, and parasites, galectins have been shown to have direct microbicidal activity. As mentioned above, galectin-3 can recognize specific fungal mannosides on *C. albicans*. In examining the binding of galectin-3 to intact, live *C.*

albicans, Kohatsu et al. observed that galectin-3 binding was directly fungicidal (55). The effect was dependent on galectin-3 binding to specific glycan ligands on the yeast, and the fungicidal effect was only seen with *Candida* species that expressed specific β1,2-oligomannans. Galectin-3 binding to susceptible yeast resulted in morphologic changes, including cell shrinkage and increased intracellular granularity. Cell death was confirmed by a number of assays, and direct fungicidal, rather than fungistatic, activity was confirmed. While the mechanism of fungal death has not been precisely determined, galectin-3 binding appeared to directly damage the integrity of the cell membrane, given the observed cell shrinkage and uptake of a fluorescent dye that would be excluded by intact cells.

A direct microbicidal effect was also demonstrated for galectin-4 and -8 in the killing of *E. coli* that express the blood group B glycans on the cell surface (56). Unlike the autophagy-promoting activity of intracellular galectin-8 described above (50), addition of either galectin-4 or -8 to susceptible *E. coli* strains in the absence of any host cells resulted in rapid killing of the bacteria, as demonstrated by loss of membrane integrity and failure of the bacteria to divide. The complement-independent, rapid, and direct damage to the bacterial membrane is reminiscent of the activity of galectin-3 on susceptible *Candida* species, described above. As with *Candida*, the mechanism of bacterial death triggered by galectin binding has not been determined; however, it is tempting to speculate that the cross-linking of numerous glycoprotein or glycolipids on the pathogen surface by galectin binding results in physical changes or mechanical stresses to the surface or interior of the pathogen that compromise cellular integrity. Stowell and colleagues propose that the ability of galectins-4 and -8 to directly kill bacteria is part of an innate immune function of galectins, providing an important line of defense against gastrointestinal pathogens.

While not a mammalian defense against microbial pathogens, another form of microbicidal activity by galectins has been described for the conger eel (57). This organism expresses two galectin family members, congerin I and II, in the digestive tract. In eels infected with parasitic *Cucullanus* nematodes, these galectins mediate adhesion of nematodes to peritoneal cells that encapsulate and sequester the parasites. This effect may be similar to the effect of mammalian galectin-3 sequestering *Schistosoma* eggs in granulomata in mammalian hosts; while not directly microbicidal, the effect of encapsulation or sequestering of microbial pathogens prevents pathogen replication and protects the host.

CONCLUSION

Galectins have been described as PRRs that can discriminate microbial glycans from host glycans, and as danger receptors that can participate in innate immune defense. These are certainly functions of the galectins, but it is becoming increasingly clear that galectins can bind both host and microbial glycans; thus, assuming that there is a non-overlapping dichotomy of pathogen vs. host glycans is not valid. Rather, galectins bind a variety of glycans in a context-dependent manner on both microbes and on mammalian cells (58). Moreover, the outcome of binding can be quite variable, both promoting infection and blocking infection, and both directly killing microbes and initiating immune responses; as pathogens evolve in this environment of host glycans and host galectins, microbes may adapt by exploiting these host features to enhance infection or evade an immune response (59–62). This broad array of functional outcomes does not reflect a lack of specificity of the galectins, but rather emphasizes that specific responses are dependent on both the pathogen and the host cell and are determined by factors such as glycan density, glycan clustering, glycan presentation on specific glycoproteins and glycolipids, and interactions with other cell surface molecules, all of which are features that define unique cellular landscapes in which the galectins act. Thus, as more examples of galectin–microbe interactions are discovered, it may be possible to begin to define features of the galectins and glycans that would predict the outcome of a particular binding event. As galectins are evolutionarily ancient molecules, predating vertebrate innate and adaptive immunity, it is not surprising that these lectins have evolved into a large family with a wide range of context-specific functions.

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Heparan sulfate: a ubiquitous glycosaminoglycan with multiple roles in immunity

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Heparan sulfate (HS) is a highly acidic linear polysaccharide with a very variable structure. It is ubiquitously expressed on cell surfaces and in the extracellular matrix and basement membrane of mammalian tissues. Synthesized attached to various core proteins to form HS-proteoglycans, HS is capable of interacting with various polypeptides and exerting diverse functions. In fact, a bioinformatics analysis of mammalian proteins that express a heparin/HS-binding motif and are associated with the immune system identified 235 candidate proteins, the majority having an intracellular location. This simple analysis suggests that HS may, in fact, interact with many more components of the immune system than previously realized. Numerous studies have also directly demonstrated that HS plays multiple prominent functional roles in the immune system that are briefly reviewed in this article. In particular, the molecule has been shown to regulate leukocyte development, leukocyte migration, immune activation, and inflammatory processes.

Keywords: glycosaminoglycan, heparan sulfate, heparanase, hematopoiesis, homing, inflammation

INTRODUCTION

Heparan sulfate (HS) is a glycosaminoglycan (GAG) that is ubiquitously expressed on cell surfaces and in the extracellular matrix (ECM) and basement membrane (BM). Each HS molecule is a linear polysaccharide composed of repeating disaccharides of hexuronic acid and D-glucosamine that can exhibit immense structural diversity due to substitution to varying extents with sulfate groups and epimerization of glucuronic acid to iduronic acid, with areas of high sulfation and glucuronic acid epimerization being co-located in "hot spots" throughout the molecule (**Figure 1**). HS is structurally related to heparin, an extremely highly sulfated form of HS that is restricted to mast cells. The biosynthesis and modification of HS chains is thought to take place within the endoplasmic reticulum, Golgi apparatus, and trans Golgi network, which in the end produce unique HS chains that are covalently attached to a range of core proteins to form HS-proteoglycans (HSPG) (**Figure 1**) (1, 2). After synthesis HS chains can be modified by the endoglycosidase, heparanase (3), and endosulfatases, Sulf1 and Sulf2 (4–6), to regulate HS availability and function. Although the core proteins can function independently of the HS chains they carry (7), HS predominantly dictates the ligand-binding capability and therefore the biological roles of HSPG (8). Furthermore, while different cell types may express similar core proteins, the HS chains these core proteins carry can be markedly distinctive, resulting in HSPG with highly diverse yet specialized roles in mammalian physiology (8, 9). In this mini-review, we will discuss some of the contributions of HS to the functioning of the immune system, notably leukocyte development, leukocyte migration, immune activation, and inflammatory processes.

DIFFERENT CELLULAR LOCATIONS OF HS CHAINS

In general, cell surface HSPGs includes members of the transmembrane syndecans (syndecan-1-4) and glycosylphosphatidylinositol (GPI)-linked glypicans (glypican 1-6). ECM/BM associated HSPGs are comprised of perlecan, collagen type XVIII and agrin. These HSPGs are collectively termed "full-time" HSPGs. "Part-time" HSPG include cell surface CD44 (isoform 3 is HS-linked) and extracellular betaglycan, testican, and neuropilin (8, 10). Secretory vesicle-associated serglycin is a HSPG that is exclusively expressed intracellularly, particularly in mast cells (11). Furthermore, HSPG can also be localized in the nucleus where they potentially regulate gene transcription (12–16).

PREVALENCE OF HS-BINDING PROTEINS IN THE MAMMALIAN IMMUNE SYSTEM

Due to the structural similarities between HS and heparin, the latter is often used as an experimental model for biochemical studies of HS-protein interactions and predicting potential HS-binding partners. Several heparin-binding proteins are known to carry the "consensus" heparin/HS-binding motifs XBBXBX or XBBBXXBX (B being the basic amino acids arginine, lysine, or histidine and X being one of a range of aliphatic/aromatic amino acids) (17). If correctly displayed in the secondary structure and optimally positioned within the three-dimensional conformation of polypeptides, these sequences are hypothetically capable of facilitating strong ionic interactions with negatively charged GAGs (17, 18). Based on this simple amino acid sequence criterion, we screened for protein sequences matching selected G0 terms in the Ensembl database (release 72) with a custom Python script for murine gene products that carry these motifs and are listed on the

UniProt database (www.uniprot.org) as being reported to have immunological functions. We identified a total of 235 HS-binding proteins in the mouse genome (**Table 1**), a list that includes known HS-binding proteins and many potential new ligands for HS. An intriguing feature of this analysis was that 66% of the molecules that potentially bind HS are expressed intracellularly, with only 18% being exclusively expressed on the cell surface and only 10% being in the extracellular compartment. Remarkably, only one HS-binding protein, the HS degrading enzyme heparanase, was identified that can be expressed in intracellular and extracellular compartments as well as being able to associate with plasma membranes. This finding is consistent with the multiple functional roles of the enzyme.

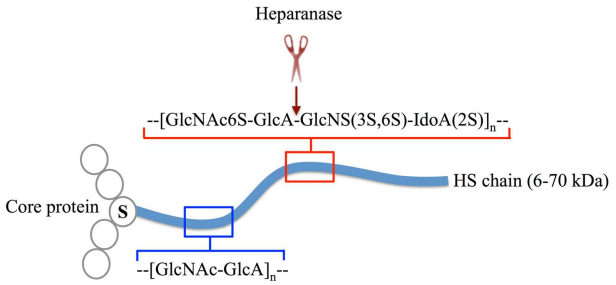

FIGURE 1 | The structure of HSPG. HS chains (blue line) are linear polysaccharides composed of repeating disaccharide subunits, which in their unmodified form are D-glucosamine and D-glucuronic acid (blue box). During synthesis, HS chains are covalently attached to core proteins (open circles) at serine (S) residues. A single HSPG molecule may carry multiple HS chains or contain other glycosaminoglycans other than HS (not shown). HS modifications include various degrees of O and N-sulfation and epimerization of D-glucuronic acid to D-iduronic acid by HS-modifying enzymes (red box) (1). The modifications occur in regions (hot spots) along the polysaccharide chain, these hot spots being separated by regions of low sulfation. Post-synthesis structural alterations are primarily mediated by the endo-β-glucuronidase, heparanase, which cleaves HS chains within highly sulfated regions (cleavage site indicated by red scissors and arrow) (3).

Despite earlier reports claiming that HS negatively regulates gene transcription primarily by repressing the activity of p300 and pCAF histone acetyltransferase (14, 15), the bioinformatics screen implies that intracellular HS plays a more elaborate role in dictating cellular responses to various stimuli. Thus, it is predicted that HS interacts with several regulators of histone-modifying enzymes, such as Jarid2 (motif: MKRRHI), Kat6a (LHHLRM, KKVKK, RRVRK), and Mll1 (LRRFRA, IKKLRA, LKKAKA, VHRIRV, KKVKR, RHLKK) alongside key molecules that are involved in signal transduction and regulation of gene transcription, notably Vav1 (VKHIKI), STAT5A (KRIKR), STAT6 (KKIKR), Bcl6 (WKKYKF), Bcl11a (KHMKK), Ciita (LKRLKL), PTBP3 (VHRVKI, HRFKK), Lck (VKHYKI), IRAK1 (RRAKK), IRAK4 (HHIHR), Foxj1 (FKKRRL), Syk (RKAHH), ITK/TSK (IKHYHI), Card11 (KRFRK), Zap70 (KKLFLKR), Jak3 (IHKLKA, AKKLKF, RRIRR), and Cblb (RHFHH) and some components of the NF-κβ signaling pathway including NFKB2 (YHKMKI),

Table 1 | Hypothetical HS-interacting proteins.

Cellular location	Total	Protein
Intracellular	154	Nlrp3, Nkap, Lrp5, Lyst, Lck, Irak4, Sla2, Mx2, Syk, Oasl2, Tcf3, Myo1e, Atp7a, Cplx2, Tusc2, Ak7, Vav3, Blnk, Spta1, Skap1, Fgr, Pmaip1, Aim2, Shb, Ddx60, Dapk3, Nfkb2, Apobec3, Ripk2, Nod1, Sptb, Oas1d, Mapk14, Ptbp3, March8, Dapk1, Zfp385a, Bcl2, Mecom, Chd7, Gpam, Ap3d1, Lcp2, Arid4a, Polr3a, Tnfaip1, Kat6a, Ung, Myo9b, Bcl6, Eprs, Sp3, Bag6, Plcg1, Nbn, Pml, Klf1, Rb1, Sfxn1, Bcl11a, Farp2, Jarid2, Xrcc6, Itk, Myo1f, Nlrc5, Cyp27b1, Ikzf1, Ddx58, Pip4k2a, Ciita, Foxj1, Rnf168, Prkdc, Pms2, Mll1, Stk3, Mef2c, Ahctf1, Prkx, Rag1, Batf3, Map3k14, Ilf2, Herc6, Card11, Card9, Dlg1, Itch, Dyrk3, Tnip3, Cdk6, Irgm1, Rnf31, Apc, Unc13d, Tlr13, Tlr8, Nedd4, Msh6, Pcid2, Sh2b2, Aicda, Myh9, Pik3cd, Zap70, Vav1, Stat5a, Tgtp1, Mx1, Enpp2, Dock2, Pgm3, Unc93b1, Plcg2, Stxbp2, Ifi44l, Zfp35, Inpp5d, Oas3, Cblb, Ostm1, Bcl11b, Eps8, Prkd1, Ctnnbl1, Polr3b, Samhd1, Nlrc4, Tec, Tet2, Map3k5, Pou1f1, Ncaph2, Stat6, Smarca4, Fnip1, Jak3, Cactin, Dicer1, Atm, Ikbkg, Satb1, Eif2ak2, Stap1, Msh3, Sgpl1, Cdk13, Foxe1, Zc3h8, Spib, Maea, March1, Ank1, Mink1
Plasma membrane	42	Mpzl2, Adam10, Ntrk1, Icosl, Cxcr5, Cd97, Tlr6, Pde2a, Adam9, Tlr1, Ccr3, Treml2, Tril, Tek, Lrrc8a, Il2ra, Selp, Fas, Hfe, Cd83, Cd22, Ccbp2, Ctla4, Tlr2, Klre1, Gpr183, Ccr7, Abcc9, Hoxb4, H2-M5, Thsd1, Dcstamp, Il7r, Procr, Amica1, Chrnb2, Tnfrsf13c, Csf1r, Tlr4, Tyr, Cd93, Eda, Cd40lg
Extracellular	24	Masp1, Ccl25, Osm, Il9, Bmp4, Inhba, Pdgfb, Scg2, Hc, Fam20c, Wnt2b, Lrrc17, Gas6, C7, Il1a, Wnt5a, Cxcl12, AI182371, C4b, Cxcl5, C8b, Ccl17, Serping1, Ccl28
Intracellular, plasma membrane, and extracellular	1	Hpse
Intracellular and plasma membrane	3	Blk, Flt1, Flt3
Intracellular and extracellular	2	Isg15, Prg4
Plasma membrane and extracellular	8	Tgfb1, Vegfa, Enpp3, Ctsg, C8a, Ptpro, Adam17, Enpp1

List of murine proteins that carry "consensus" heparin/HS-binding motifs. The motifs adapted for bioinformatic screens are the standard motifs, which are primarily XBBBXXBX or XBBXBX (17) (http://gduserv.anu.edu.au/~cameron/protein.html). Short motifs are truncated versions of the standard motifs XBBBBX, BBXBB, or BBXXXBB (B = basic amino acids R, K, H; X = A, V, I, L, M, F, Y, W). Only proteins in the UniProt database (www.uniprot.org) that are known to play immunological roles are listed.

IKBKG (MRKRHV), and Nkap (RRAKK, KKAKK, KKYKK). Interestingly, in the Rag1 protein, the presence of a HS-binding motif adjacent to a site critical for DNA binding (560D, UniProt) (AKRFRY), and overlapping the site that is essential for DNA hairpin formation [971F and 972R, UniProt (19)] (RRFRK), may imply a role for HS in regulating V(D)J recombination.

It is not surprising that cytokines including IL-1α (LKKRRL), IL9 (HRVKR), TGF-β1 (VKRKRI), and chemokines such as CCL17 (KHVKK), CCL19 (RRLKK), CCL25 (ARKRLVHM), CCL28 (VKRRRI), CXCL5 (KKAKR), and CXCL12 (VKHLKI) also carry HS-binding motifs, presumably allowing HS to act as their atypical receptors. Moreover, HS-binding motifs are also present on the cognate receptors for soluble factors such as the cytokine receptors IL-2Rα (HRWRK) and IL7R (KKVKH) and the chemokine receptors CCR3 (WKFFHA), CCR7 (AHRHRA), and CXCR5 (YRRRRL), indicating new roles for HS in regulating leukocyte homeostasis and trafficking. In addition, HS may regulate the availability of crucial components in the immunological synapse as indicated by the presence of HS-binding motifs in the leader sequences of ICOSL (WKKLHV) and CTLA4 (LRRYKA). The presence of HS-binding motifs in CD22 (KKARR) and CD40L (KKLKR) also suggests additional roles for HS in cell-cell communication and T cell costimulation.

Examination of the innate immune system reveals that, although HS is already known to interact with TLR-4 (20), it appears that HS may also be recognized by other TLRs, including cell surface TLR-1, TLR-2, and TLR-6, and endosomal TLR-8 and TLR13. To be more precise, the HS-binding motifs appear to be in the extracellular domains of TLR-4 (RHIFWRR) and TLR-2 (IRRLHA) and in the cytoplasmic domains of TLR-1 (HRARH) and TLR-6 (YHKLRA and HRARH). For the endosomal TLRs, both motifs (LKKLHL and LKKKHF) are facing luminal for TLR-8, and cytoplasmic (HRLRK) and luminal (LKRLKI) for TLR13. It is possible that HS is involved in regulating downstream signaling when the motifs are present in the TLR cytoplasmic domains. In contrast, when these motifs are facing the luminal or extracellular space, HS may be a ligand or a regulatory component that modulates the interaction between a TLR and its cognate ligands. Furthermore, HS-binding motifs are also present on inflammasome components such as Nlrp3 (LKKFKM), Nlrc4 (LKKMRL, RHIHR), and Aim2 (LKRFKY), implicating a role for HS in regulating the activation of inflammatory caspases. In another aspect of innate immunity, several studies have reported that heparin and HS are able to interact with several components of the complements system, including C1 (21), C1q, C1 inhibitor, C2, C4, C4b, C4bp, C6, C8, C9, Factor B, Factor D, Factor H (22), MASP1, MASP-2 (23), and complement receptors CR3 (CD11b/CD18) (24) and CR4 (CD11c/CD18) (25). In support of these observations, we have identified the presence of HS-binding motifs in complement proteins such as C4b (FRKFHL), Hc/complement C5 (FHKYKV), C7 (KRLYLKR), C8a (WRKLRY), and C8b (KRYRH) as well as regulatory components of the complement machinery including MASP-1 (KHWRR), Serping1 (HKIRK), and CD93/complement component C1q receptor (YHKRRA), further highlighting the role of HS as a major modulator of the complement system (26).

However, it should be noted that more in-depth analyses are required to assess the validity of these predicted interactions, in particular the demonstration that the predicted HS-binding sites are correctly presented within the secondary structure and final three-dimensional conformation of the putative HS-binding proteins. Also, there are known HS-binding proteins that lack the heparin/HS-binding motifs used in this analysis, suggesting that the list of binding proteins identified in this screen may, in fact, be an under estimate. Thus, at face value, the data set implies that a wide range of HS-binding proteins participate in the immune system.

FUNCTIONAL ROLES OF HS IN IMMUNITY

Despite the previous section suggesting that there are many unknown HS-protein interactions that may control the immune system, there are a number of well-established functional roles for HS in immunity. Indeed HSPGs, through their HS chains, are involved in a broad spectrum of biological processes, profoundly influencing development (27), homeostasis (28), and the progression of many diseases (29). In the case of the immune system, HSPGs are fundamentally involved in regulating cell adhesion, cytokine and chemokine function, sensing tissue injury, and mediating inflammatory reactions. Each of these functional roles will be briefly discussed below, with specific examples given which highlight each function.

REGULATOR OF CELL ADHESION

Cell adhesion molecules are important to facilitate and regulate cell-cell signaling, migration and activation of leukocytes during development, homing, and recruitment to inflammatory sites. For example, of particular relevance to leukocytes development in the bone marrow is the receptor complex on hematopoietic stem cells (HSCs) comprised of CD45 and Mac-1 (CD11b/CD18) that has been shown to bind to surface HS on bone marrow-derived stromal cells and facilitate strong adhesion (30). In a related study, the HSPG glypican-3 enhances the antagonizing effect of tissue factor pathway-inhibitor (TFPI) on CD26, the stromal-bound ectopeptidase that is involved in cleaving surface CXCL12, a typical ligand for CXCR4 on HSC. As a result, glypican-3 indirectly supports the directional homing of grafted HSC toward, and their retention in, the bone marrow (31). Similarly, in the thymus a particular subset of cortical epithelial cells known as thymic nurse cells are reported to express high levels of highly sulfated HS that is thought to aid thymocyte adhesion and facilitate T cell development (32–35).

During an inflammatory response, HS positively regulates the recruitment of inflammatory cells at three different stages based on the following observations. First, endothelial surface HS can reduce neutrophil rolling velocity via L-selectin-mediated cell adhesion (36). Second, once attached, the HS-mediated Mac-1-CD44v3 interaction enhances the binding of leukocytes to the endothelial surface to drive extravasation (37). Finally, within the endothelial ECM/BM, collagen type XVIII promotes leukocytes infiltration in again an L-selectin-dependent manner (38, 39). However, the role of HS in the adhesion of leukocytes to the endothelium can occur paradoxically, in a biphasic manner. Under physiological conditions, glycocalyx HSPGs of pulmonary endothelial cells are known to impede neutrophil adhesion (40).

Following the induction of an experimental sepsis model of acute lung injury, the localized production of TNF-α activates endothelial cells to produce heparanase, which in turn catalyzes the partial degradation of HS constituents of the glycocalyx. The loss of HS results in a significant increase in neutrophils binding (40), presumably via neutrophils L-selectin binding to residual endothelial HS and via cytokine-induced endothelial P- and E-selectin (41). Additionally, reduction (in wild-type mice) or deletion (in knockout mice) of syndecan-1 from murine endothelial cells strongly accentuates antigen-specific lymphocytes infiltration into inflammatory sites during a delayed-type hypersensitivity reaction (42).

MODULATOR OF CYTOKINE AND CHEMOKINE FUNCTION

Soluble factors, such as cytokines and chemokines, are crucial to support growth, maintain homeostasis, and orchestrate immune cell trafficking across various locations. However, some of these molecules are inactive or susceptible to degradation in their native, soluble form. Furthermore, these factors need to be timely presented at the right site to exert their anticipated functions. HSPGs have been implicated in modulating various aspects of cytokine and chemokine function (43, 44).

HS-proteoglycans interact with various cytokines primarily on target cells or act as atypical cytokine receptors on cytokine presenting cell. The former situation enables HPSGs to regulate the availability and influence the interaction between cytokines and their cognate receptors on target cells. For example, the binding of cell surface HS to cytokines such as IL-7 and IFN-γ proved critical to protect them against proteolysis (45, 46). Furthermore, the lack of HSPG expression on the mouse pro-B cell surface severely impairs IL-7-dependent maturation toward pre-B cells suggesting that in this situation HS acts as a primary IL-7 receptor. In addition, HSPG can contribute to IL-7 biological activity by presenting IL-7 on stromal cells to promote lymphopoiesis in the bone marrow (47). In the thymus, however, the ability of stromal cell HSPGs to bind IL-7 and aid thymocytes development is dispensable (48). Depending on the degree of sulfation, cell surface HSPG has also been shown to potentiate the IFN-γ-IFN-γ-receptor interaction (49). Importantly, HSPG also facilitate cytokine localization in specific niches, forming depots where they can be made available to target cells. For instance, perlecan binds IL-2, sequestering it from the circulation and subsequently depositing the cytokine in the marginal zone and red pulp of the murine spleen to modulate murine T cells homeostasis (50). HSPGs also facilitate the storing of IL-2 within the vascular smooth muscle wall where cytokine availability is regulated through heparanase-mediated ECM degradation (51).

The ability to bind and present a chemokine to target cells is insufficient to drive cell migration, the hallmark of chemokine function. HSPGs are not only capable of binding and assisting in inducing conformational changes in bound chemokines (52, 53), but also contribute to the establishment of immobilized (haptotactic) chemokine gradients in tissues (54). For example, HSPG facilitate the oligomerization of bound RANTES/CCL5, CXCL8, MCP-1, and MIP-1, thereby allowing better recognition by their cognate G-protein-linked transmembrane receptors (55). In addition to the sequestration of CXCL2 (56), HSPG also mediate transcytosis of CXCL8 across the endothelium, presenting both chemokines on the luminal side and establishing haptotactic gradients that aid neutrophils recruitment during inflammation (36). In a separate study, the migration of tissue dendritic cells (DC) to regional lymph nodes and the local positioning of DC within lymph nodes was also found to be mediated by a HS-dependent haptotactic gradient of CCL21 (57). A similar interaction is believed to facilitate lymphocytes homing through the high endothelial venules into peripheral lymphoid organs (58, 59), although it is unclear if HS also influences local positioning in specific niches. Also, shedding of HSPG, such as syndecan-1 (60) and removal of glucosamine 6-*O*-sulfate by the endosulfatase, Sulf2 (6) has been implicated in regulating the interaction between HS and various chemokines. Furthermore, the inactivation of HS-modifying enzymes can modify neutrophil binding to the endothelium, Ndst1 (HS *N*-deacetylase/*N*-sulfotransferase) deletion severely impairing (36), while Hs2st (HS 2-*O*-sulfotransferase) deletion significantly augmenting (61), neutrophil binding. These studies support the concept of regulating HS function by altering the availability of enzymes that are involved in HS biosynthesis.

A SENSOR OF TISSUE INJURY

Tissue injury may induce cell necrosis, an event that is often associated with the release of various endogenous damage-associated molecular pattern (DAMP)-containing molecules that are potent inducers of inflammatory responses and initiators of tissue repair mechanisms (62). Both surface bound HSPG and soluble HS participate in sensing tissue injury and also in repair mechanisms. For example, endothelial cell surface HS mediates the oligomerization of the receptor for advanced glycation endproducts (RAGE) (63) and together form a receptor complex that efficiently recognizes the chromatin protein, high-mobility group protein B1 (HMGB1) released from necrotic cells (64). As part of the tissue repair mechanism, HS on the surface of professional phagocytes also assists in the clearance of necrotic cells (65). In fact, soluble HS itself can also function as a DAMP (66) by interacting with TLR-4 on leukocytes (20). This interaction has been shown to modulate the release of pro-inflammatory cytokines by macrophages (67) and markedly induce the maturation of DC, as indicated by the up-regulation of MHC-II, CD40, ICAM-1, CD80, CD86, and reduced antigen uptake, a typical phenotype of a professional antigen presenting cells (68). Although this is beneficial in triggering immune activation following an insult (20), it is also implicated in the underlying mechanism of disease progression which can occur in experimental pancreatitis (69), sepsis-like syndrome (70), hyperacute rejection in graft-versus-host disease (GvHD) (71), and cardiac injury (72).

PHYSICAL BARRIER TO LEUKOCYTE MIGRATION

The ECM/BM associated HS is crucial to form a temporary depot of HS-binding soluble factors and to form a physical barrier that supports tissue integrity. In order to migrate, particularly through blood vessel walls, leukocytes need to break down the ECM/BM barrier and heparanase is primarily involved in this process (10, 73). For example, tissue DCs increase the availability of cell surface heparanase to aid ECM degradation before migrating into lymphatic vessels leading toward regional lymph nodes where they

induce antigen-specific responses (74). Subsequent studies have suggested that the matrix metalloproteinase, MMP-14, cooperatively works with heparanase to more efficiently degrade ECM/BM barriers (75). During inflammation, infiltrating monocytes and neutrophils also exhibit similar modes of degrading ECM/BM barriers to aid their extravasation (76, 77). It has also been demonstrated that heparanase derived from infiltrating leukocytes is primarily responsible for the destruction of the pancreatic islet β-cells that produce insulin, thereby providing a novel explanation for the underlying immunopathology of autoimmune Type 1 diabetes (78). In a separate study, leukocytes were shown to also use endogenous myeloperoxidase to produce oxidants that degrade the core protein of perlecan, releasing soluble factors and allowing leukocyte migration across ECM/BM barriers (79). Although HS can also be degraded by nitric oxide (80, 81) and reactive oxygen species (82–84), their direct relevance in the degradation of ECM/BM-associated HSPGs and therefore their contribution to leukocytes extravasation is yet to be elucidated.

CONCLUDING REMARKS

The evidence presented in this mini-review further corroborates the fundamental importance of HS in the mammalian immune system. HSPGs, primarily through their HS side chains, regulate various aspects of the immune system ranging from hematopoiesis to homing of leukocytes to peripheral tissues and, most importantly, regulating the elicitation of immune responses. Perturbing HS function or availability has been proven to results in various abnormal immune phenotypes. Furthermore, based on a simple bioinformatics screen presented in this review, it is suggested that HS may in fact interact with many more components of the immune system than previously realized. A better understanding of HS function across various systems is fundamental to exploit its potential in boosting beneficial immune responses and also in finding treatments for related immunopathologies.

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Molecular interactions between complement factor H and its heparin and heparan sulfate ligands

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Complement factor H (CFH) is the major regulator of the central complement protein C3b in the alternative pathway of complement activation. A molecular view of the CFH interaction with native heparan sulfate (HS) is central for understanding the mechanism of how surface-bound CFH interacts with C3b bound to host cell surfaces. HS is composed of sulfated heparin-like S-regions that alternate with desulfated NA-regions. Solution structural studies of heparin (equivalent to the S-regions) and desulfated HS (the NA-regions) by scattering and ultracentrifugation showed that each structure was mostly extended and partially bent, but with greater bending and flexibility in the NA-regions compared to the S-regions. Their solution structures have been deposited in the Protein Data Bank. The largest HS oligosaccharides showed more bent and flexible structures than those for heparin. A folded-back domain structure for the solution structure of the 20 domains in CFH was determined likewise. CFH binds to the S-regions but less so to the NA-regions of HS. The bivalent interaction of CFH–heparin was observed by ultracentrifugation, and binding studies of CFH fragments with heparin-coated sensor chips. In common with other CFH interactions with its physiological and pathophysiological ligands, the CFH–heparin and CFH–C3b interactions have moderate micromolar dissociation constants K_D, meaning that these complexes do not fully form *in vivo*. The combination of the solution structures and binding studies indicated a two-site interaction model of CFH with heparin at cell surfaces. By this, the bivalent binding of CFH to a cell surface is co-operative. Defective interactions at either of the two independent CFH–heparin sites reduce the CFH interaction with surface-bound C3b and lead to immune disorders.

Keywords: analytical ultracentrifugation, surface plasmon resonance, X-ray scattering, complement factor H, heparin, heparan sulfate

INTRODUCTION

Complement is a major defense and clearance system of the innate immune system (1, 2). Pathogens activate complement C3 through one of three pathways, the classical, lectin, or alternative pathways. Unactivated C3 consists of 13 domains, namely eight macroglobulin domains, a linker domain, an anaphylatoxin domain (C3a) a complement C1r/CIS–UEGF–BMP1 domain (CUB), a C345 domain, and a thioester domain (TED, also known as C3d). These three pathways lead to the removal of the small C3a domain from C3, the main complement protein, to convert this to C3b. Active C3b is rapidly generated through a cascade and becomes covalently attached to cell surfaces through its TED domain (**Figure 1A**). This triggers the assembly of the membrane attack complex that lyses pathogen cells and the clearance of C3b-opsonized cells by phagocytosis. C3b formation needs regulation, because too much C3b will damage host cells, while too little C3b means that the host becomes immuno-compromised. Recent reviews refer to this balance as a "double-edged sword" (3, 4). C3u is formed by the spontaneous hydrolysis of the thioester bridge in C3, but is no longer able to bind to cell surfaces. The main C3b regulator is complement factor H (CFH; **Figure 1A**), and CFH also binds to C3u. Both C3 and CFH are relatively abundant complement proteins in plasma. The most abundant plasma proteins are human serum albumin (30–50 mg/ml) and the immunoglobulins (10–15 mg/ml). In comparison, C3 is typically found at 1.0–1.6 mg/ml (5.3–8.5 μM), while CFH is almost equimolar to C3 at around 0.116–0.81 mg/ml (0.8–5.3 μM). The large amounts of these complement proteins compared to others, such as, for example, the main coagulation proteins present at 0.1–10 μg/ml (0.1–0.3 nM), are attributable to the need for abundant reagents to combat infections. The uncontrolled release of C3b is regulated by CFH. Firstly, CFH blocks the binding of complement Factor B to C3b and that of its activated form Bb to C3b, the binding of which produces the C3 convertase that cleaves C3 to produce even more C3b. Secondly, CFH acts as a cofactor of the protease Factor I that cleaves C3b into the inert fragments C3d and C3c. CFH functions both in plasma and by binding to host cell surfaces through interactions with anionic oligosaccharides bearing clusters of negative charges.

Here, we summarize recent progress in our molecular understanding of how CFH binds to heparin and heparan sulfate (HS), thereby protecting host cell surfaces. Firstly, we describe recent

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; CFH, complement factor H; CUB, complement C1r/CIS–UEGF–BMP1 domain of C3; K_D, dissociation constant; SCR, short complement regulator; TED, thioester domain of C3.

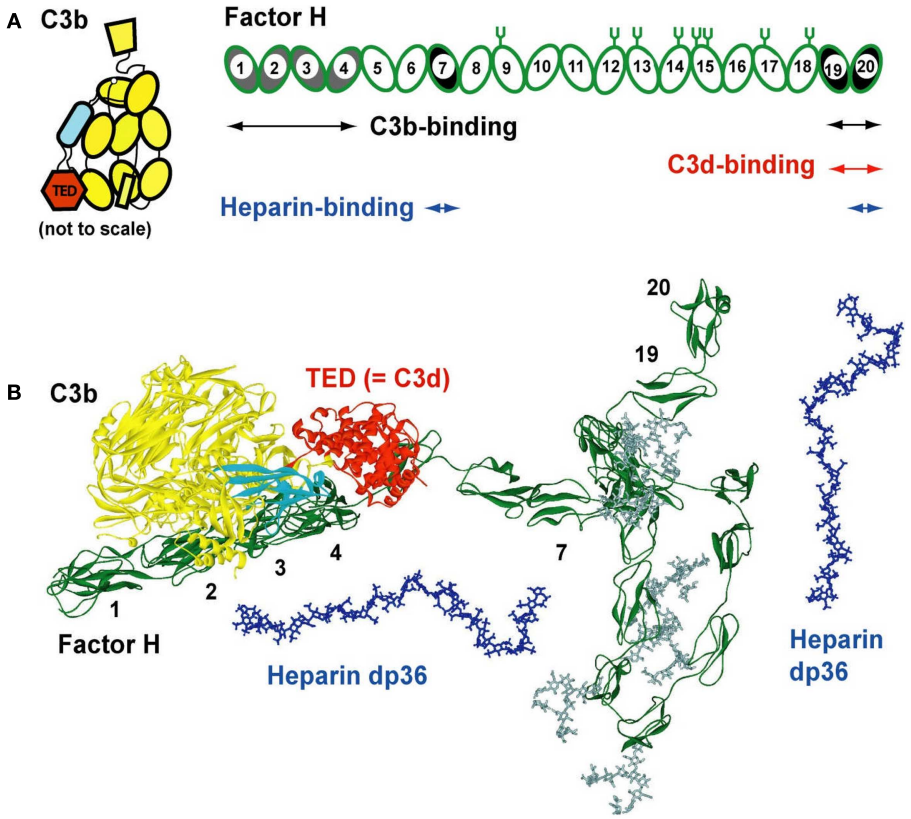

FIGURE 1 | Domain structure of factor H and its C3b and heparin ligands. **(A)** Schematic view of the 20-SCR domains of CFH. The positions of two C3b binding sites (SCR-1/4 with C3b decay acceleration and factor I activity, and at SCR-19/20), two heparin-binding sites at SCR-7 and SCR-20, and two C3d-binding sites on each of SCR-19 and SCR-20 are shown schematically. The location of eight *N*-linked glycosylation sites is shown by symbols; a ninth site at SCR-4 is not occupied. The 12 domains of C3b are shown to the left, with the TED domain ("thioester domain"; equivalent to C3d) in red and the CUB domain ("complement C1r/CIS–UEGF–BMP1") in cyan. **(B)** Comparison of molecular views for CFH with structures for its C3b and heparin dp36 ligands. All are on the same scale. A folded-back domain arrangement of a best-fit CFH structural model is shown with its longest length running from left to right (SCR-1–SCR-20). The CFH structure is from two recent studies (27, 54). The eight CFH oligosaccharides are shown in gray. The CUB and TED domains are shown in cyan and red, respectively. The SCR-1/4 domains are shown bound to C3b at the left (28). The folded-back central region of CFH with the shorter and glycosylated SCR domains is shown to the bottom right. Two solution structures of dp36 (blue) are shown proximate to SCR-7 and SCR-19/20.

three-dimensional molecular structures for heparin and HS. Their original structures came from a ^{1}H NMR study of heparin and the crystallography of small oligosaccharides bound to proteins. These first structures have now been supplemented by the use of a powerful new method based on the modeling of X-ray scattering curves and sedimentation coefficients that produced new structures for the larger fragments of heparin and HS. Secondly, we summarize the molecular structure for CFH. Intact CFH is comprised of 20 short complement regulator (SCR) domains, each of length about 61 residues, and joined together by linkers of lengths 3–8 residues (**Figure 1A**). Crystal and/or NMR structures are available for over half the CFH SCR domains. Intact CFH has not been crystallized to date, this being attributed to its comparatively large size, its ability to dimerize in at least two sites, its sizeable glycosylation in the center of CFH, and the potential flexibility of the inter-domain linkers. Instead, X-ray scattering and sedimentation coefficient modeling gave the first molecular structures for full-length CFH (**Figure 1B**). Thirdly, we describe the complexes formed between CFH and heparin. Again no crystal structures for CFH–heparin complexes are known to date. The application of analytical ultracentrifugation to the heparin complexes with CFH revealed the existence of bivalent CFH–heparin complexes. Binding experiments by surface plasmon resonance confirmed the existence of these bivalent complexes, and importantly showed that these complexes are formed co-operatively.

These structural advances have transformed our understanding of the molecular basis of the interactions of CFH with heparin and HS. They also provide new insight on the molecular mechanisms that lead to immune diseases such as age-related macular degeneration (AMD) and atypical hemolytic uremic syndrome (aHUS).

BIOPHYSICAL METHODS

We summarize for immunologists the biophysical methods used for these studies. The joint use of three independent (or "orthogonal") biophysical methods is a powerful means of characterizing

the molecular interactions of heparin and HS with CFH. This combination reduces the experimental uncertainties inherent in the use of one method alone. For example, the dissociation constants K_D of an interaction can be obtained from all three methods as a quantitative test of consistency. In addition, each method has unique strengths.

(a) In analytical ultracentrifugation, sedimentation velocity experiments subject the samples to high rotor speeds. These, sediment to the bottom of the rotor cells at rates that depend on the macromolecular shape and mass according to the Svedberg equation. Modern PCs record as many as several hundred sedimentation boundaries during an experiment. The resulting sedimentation coefficient distribution $c(s)$ produces peak(s) that correspond to the macromolecule(s) present in the sample (see below). When complexes are studied, the unbound and complexed species are revealed by separate peaks (if slow exchange conditions are satisfied) that provide the individual sedimentation coefficients $s_{20,w}$ (after correction to 20°C and the density of water) and their molecular masses. The K_D values are obtained from integration of the $c(s)$ peak areas (5). The $s_{20,w}$ values report on the shape and can be compared directly with molecular models. The main advantage of this method is the ability to resolve distinct species.

(b) X-ray scattering measures the diffraction from macromolecules in random orientations in solution, from which the overall dimensions of the solution structure can be determined. Dimensions are measured using the radius of gyration R_G values from Guinier plots and the lengths from distance distribution $P(r)$ analyses. If atomic structures are available for modeling, such as the domains of a large multidomain protein, a molecular structure can be determined by constrained modeling methods. By this, the known domain structure is rearranged into thousands of possible allowed conformations, then the best-fit molecular structure is identified by curve-fitting. This is the main advantage of scattering. The resulting structure is deposited in the Protein Data Bank as a permanent record. If scattering is used for determining the K_D value, this requires knowledge of the scattering curves for the unbound structures as well as for their complex (5). The ratio of the unbound and complexed species is determined by scattering curve fits measured at different concentrations, from which the K_D value is calculated.

(c) Surface plasmon resonance uses ligands or macromolecules that are immobilized on a sensor chip. The on-rate and off-rate of a soluble "analyte" binding to the immobilized interaction partner (the "ligand") are determined. If these rates are relatively slow, the ratio of the off-rate/on-rate gives the K_D value. If these rates are relatively rapid, the overall intensity change of the response when the analyte is bound to the ligand as a function of its concentration given an alternative determination of the K_D value (6). No shape information is available by this method. However, a different sensor chip technology termed dual polarization interferometry (data not shown) provides both the K_D value and the dimensions of the bound analyte molecule.

SOLUTION STRUCTURES OF HEPARIN dp6–dp36

The disaccharide subunit (dp2) of heparin contains two residues of uronic acid and D-glucosamine linked by a (1 → 4) glycosidic bond (**Figure 2A**). The uronic acid can be either α-L-iduronic acid (α-IdoA), which accounts for up to 90% of heparin, or β-D-glucuronic acid (β-GlcA), which accounts for up to 10% of heparin. A heparin disaccharide most often contains three sulfate groups, one located on the 2-OH group of α-IdoA, and two at the 2-NH_2 group and the 6-OH group of D-glucosamine (α-GlcNS), namely [→4)-α-L-iduronic acid-(1 → 4)–α-D-glucosamine (2,6-disulfate)-(1→]. This is abbreviated as IdoA2S–GlcNS6S. In fact, heparin is most heterogenous (7). While the main structure is the repeating tri-sulfated disaccharide IdoA2S–GlcNS6S, there are four possible uronic acids in heparin, namely GlcA, GlcA2S, IdoA, and IdoA2S, with the last one being the most common. There are seven possible glucosamines, namely GlcNS, GlcNS6S, GlcNS3S, GlcNS3S6S, GlcNAc, GlcNAc6S, and rarely glycosamine with a free amine. The most common is GlcNS6S. The other disaccharide in heparin is [→4)-β-D-glucuronic acid-(1 → 4)–α-D-*N*-acetyl glucosamine-(1→], which is abbreviated as GlcA–GlcNAc. The high degree of sulfation and carboxylation makes heparin

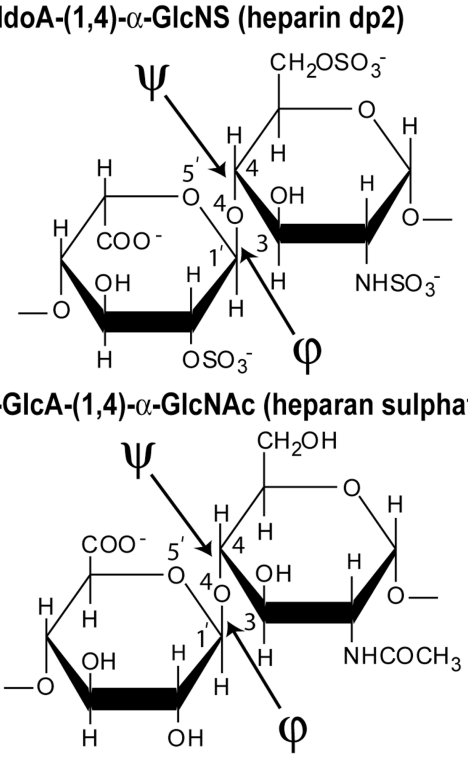

FIGURE 2 | Chemical structures of the two disaccharide repeats of heparin and HS. (A) The major repeating disaccharide unit in heparin (α-iduronic acid-2-sulfate → α-glucosamine-2,6-disulfate; α-IdoA2S and α-GlcNS6S). The molecular mass of this trisulfated disaccharide is 573 Da. The φ angle between the rings is determined from the O5′–C1′–O4–C4 atoms and the ψ angle is determined from the C1′–O4–C4–C3 atoms, indicated by two arrows. **(B)** The major repeating non-sulfated disaccharide unit of HS (β-glucuronic acid → α-*N*-acetylglucosamine; β-GlcA and α-GlcNAc). The molecular mass of this averaged disaccharide is 378 Da.

the most negatively charged macromolecule known in biology. For experimental studies, the six fragments of heparin dp6–dp36 were prepared from bovine lung heparin, starting from heparinase-I-digested heparin, followed by Biogel P-10 preparative gel permeation chromatography to separate the individual oligosaccharides (8).

Our ultracentrifugation and scattering fit procedure gave molecular structures for the six heparin fragments dp6–dp36. Conformational information on the structure of heparin had previously come from NMR studies of heparin dp12 and crystallography studies of proteins co-crystallized with small heparin fragments with sizes close to dp5. Our new structural analyses (8) proceeded in three stages:

(a) First, using analytical ultracentrifugation, sedimentation velocity runs were performed on each of the six heparin fragments dp6–dp36. Single clean peaks were seen in the $c(s)$ analyses, indicating that the purifications gave satisfactorily homogenous preparations. The six experimental sedimentation coefficients increased with size, ranging from 1.09 S for dp6 to 1.84 S for dp36. Linear molecular models were created for heparin dp10–dp40 starting from the NMR molecular structure for heparin dp12. The sedimentation coefficients $s_{20,w}$ calculated from these linear models agreed well with the experimental values.

(b) Second, X-ray scattering curves with very good signal–noise ratios were measured at the ESRF synchrotron, even for the smallest heparin, dp6. This resulted because of the high beam intensity, low scattering backgrounds, and improved detector technology. Good linear Guinier analyses and distance distribution curves resulted in R_G values that increased from 1.03–1.33 nm for heparin dp6 to 3.12–3.52 nm for heparin dp36. The comparison with the linear heparin models showed that the modeled R_G values increased linearly with heparin size, while the experimentally measured R_G values for dp18–dp36 did not increase in proportion. Therefore, the experimental R_G values showed sensitivity to bending in the heparin structures with increase in size. Other scattering parameters (i.e., the cross-sectional R_G values and the maximum dimensions) also indicated that the larger heparin structures displayed bending.

(c) Third, constrained atomistic modeling revealed the molecular structures of heparin dp6–dp36 that best fitted the X-ray scattering curves. The major conformational determinant of heparin are the two torsion angles φ and ψ of the glycosidic linkage (**Figure 2A**), defined by the O5′–C1′–O4–C4 atoms and the C1′–O4–C4–C3 atoms, respectively. Both torsion angles were randomized in steps of up to ±45°. This process generated 5,000 randomized heparin structures, starting from a linear structure. Each randomized model was compared with the experimental X-ray data by calculating the R_G value of each model and the goodness-of-fit R-factor of each curve fit. The best-fit polysaccharide structures were determined from V-shaped graphs of R-factors vs. R_G values by identifying the points with the lowest R-factors that showed the best agreement with the experimental R_G values (red circles; **Figure 3**).

In conclusion, the best-fit molecular models for heparin showed that their bending increased with heparin size (**Figure 4**). This outcome extends the earliest NMR and crystallographic results for the small heparin structures that suggested that they were mostly linear. Our heparin dp6 and dp12 solution structures are mostly extended, but those for heparin dp18, dp24, dp30, and dp36 are reduced in lengths by 16–29% compared to their linear structures (**Figure 5A**). These bent models agree with the ultracentrifugation $s_{20,w}$ values although the $s_{20,w}$ values are not sensitive to conformational bending. The original models (8) have been updated by removing minor steric overlaps between individual atoms using a constant force field termed the DREIDING minimization (9). They are available in the Protein Data Bank at http://www.rcsb.org/pdb/ or after 2009 in PDB-formatted files in the Supplementary Material of our publications. As far as is known, the heparin study was the first successful application of atomistic scattering modeling to oligosaccharides, this method having previously been used for multidomain proteins.

The best-fit models from varying only the φ and ψ angles between the sugar rings were readily identified at the minima in **Figure 3**. The quality of the dp18–dp36 scattering curve fits is better than those found with many multidomain proteins (**Figure 4**). From the fits, the solution φ and ψ values were around −60° and 130°, respectively, for the IdoA2S–GlcNS6S bond, and around 100° and 85°, respectively, for the GlcNS6S–IdoA2S bond (**Figures 6A,B**). Interestingly, comparisons with 19 crystal structures of heparin dp4–dp10 in protein complexes revealed similar φ and ψ angles (**Figures 6A,B**). When the outliers were removed, the crystallographic φ and ψ values were −79° and 132°, respectively, for the IdoA2S–GlcNS6S bond, and 84° and 100°, respectively, for the GlcNS6S–IdoA2S bond (**Figures 6A,B**). Given standard deviations of typically ±20°, these φ and ψ values suggest that the free heparin solution structures for dp18–dp36 is essentially unchanged in conformation from small heparin fragments bound to proteins in crystal structures. Thus, heparin in both its complexes or free in solution has a semi-rigid and extended conformation that is optimal for binding to proteins without major conformational changes.

The repulsion of the sulfate and carboxylate groups in heparin may influence these extended structures. The anionic sulfate and carboxylate groups of heparin form mostly ionic contacts with basic amino acids on protein surfaces. The axial orientations of these groups in heparin are thus crucial, and these repeat themselves after every four oligosaccharide rings. The dp4 fragment possesses at least six sulfate groups and two carboxylate groups. In dp4, two sulfate groups are located in opposed axial orientations in GlcNS6S, while the third sulfate and the carboxylate group are located in opposed axial orientations in IdoA2S residues (8). When crystal structures of dp3 fragments are viewed centered on either IdoA2S or GlcNS6S, these axial orientations are largely preserved and aligned with each other between IdoA2S and GlcNS6S residues (8). Our solution structures of heparin dp18–dp36 suggest that these extended arrangements and orientations seen in crystal structures are mostly preserved.

The larger heparin fragments show more pronounced bending, even though the mean φ and ψ angles are unaffected. The scattering modeling represented this bending as occasional kinks

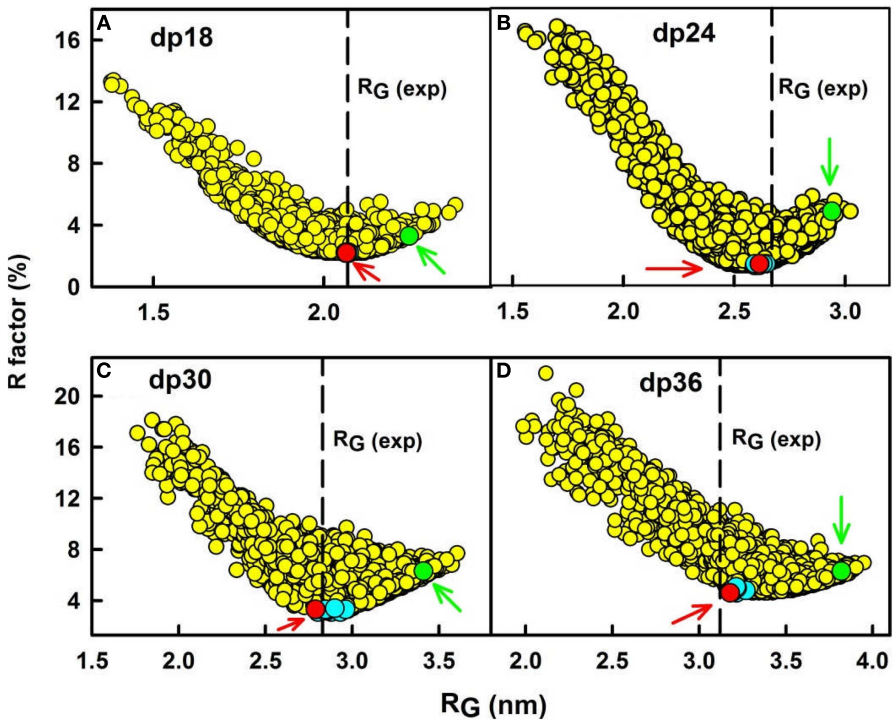

FIGURE 3 | Constrained modeling analyses of heparin dp18–dp36. **(A)** The *R*-factor values from the curve fits for 5,000 trial randomized models of heparin dp18 are compared with their R_G values. The vertical dashed line corresponds to the observed experimental R_G value. The red circle (arrowed) denotes the best-fit model, and the green circle (arrowed) denotes the linear model for heparin. Other best-fit models are shown in cyan close to the *R*-factor minimum. Here and in **Figures 4–6**, the heparin structures are corrected for minor steric overlaps (9). **(B–D)** The corresponding analyses for each of the 5,000 trial models for heparin dp24, dp30, and dp36 are shown in that order.

in the heparin structure. These bends may arise from the occasional occurrence of GlcA–GlcNAc residues in the dominant IdoA2S–GlcNS6S structure. It is more likely that kinks occur naturally within the IdoA2S–GlcNS6S structures, these having been observed in several protein–heparin crystal structures (8). Thus the observation of bending indicates that heparin is not completely rigid, and that limited flexibility in heparin is permitted.

The heparin structures clarify three different scenarios for heparin–protein interactions. As far as is known, heparin binding sites are generally found at surface-exposed positions in proteins. One scenario involves two independent and different heparin binding sites on the same protein, such as that in CFH. Because heparin is semi-rigid and extended, it is unlikely that a single large heparin molecule can undergo large conformational changes in order to bind both CFH–heparin sites simultaneously to form a 1:1 complex, thus these two sites remain independent (10). In a second scenario, heparin will mediate conformational changes in proteins in order to induce functional activity. The best-known case is that of antithrombin, in which the allosteric activation of its β-sheet structure by heparin lead to the inhibition and regulation of the blood coagulation protein Factor IXa, Factor Xa, and thrombin (11). A third scenario is the ability of heparin to enable functionally active protein dimers to form through heparin binding to single sites on each protein monomer [for example, the fibroblast growth factor family and their receptors (12, 13)]. All three scenarios are facilitated by the semi-rigid and extended structures of heparin.

SOLUTION STRUCTURES OF HEPARAN SULFATE dp6–dp24

Native HS plays key roles in the regulation of physiological and pathophysiological processes. Native HS is comprised of the same two alternating residues of uronic acid and D-glucosamine residues as in heparin, but with a reduced degree of sulfation (**Figure 2B**). HS contains a higher proportion of the GlcA–GlcNAc disaccharide compared to the sulfated IdoA2S–GlcNS6S disaccharide in heparin considered above.

The overall structure of native HS shows a distinct three-domain organization that is comprised of short S-domains with IdoA2S–GlcNS6S disaccharides, long NA-domains with GlcA–GlcNAc disaccharides, and mixed domain regions at the junctions between the S-domains and NA-domains that includes IdoA2S–GlcNS6S disaccharides. The S-domains and mixed domain regions occur as "hypervariable" regions that lead to different HS functions when HS is purified from different cell types. The S-domains correspond closely to heparin. For experimental studies of the NA-domains, the non-sulfated HS oligosaccharide fragments dp6–dp24 were prepared by digests, and this product is termed the HS fragments below, unless specified otherwise. First, exhaustive heparinase I digestion was used to minimize the content of fully sulfated heparin sequences in a crude glycosaminoglycan

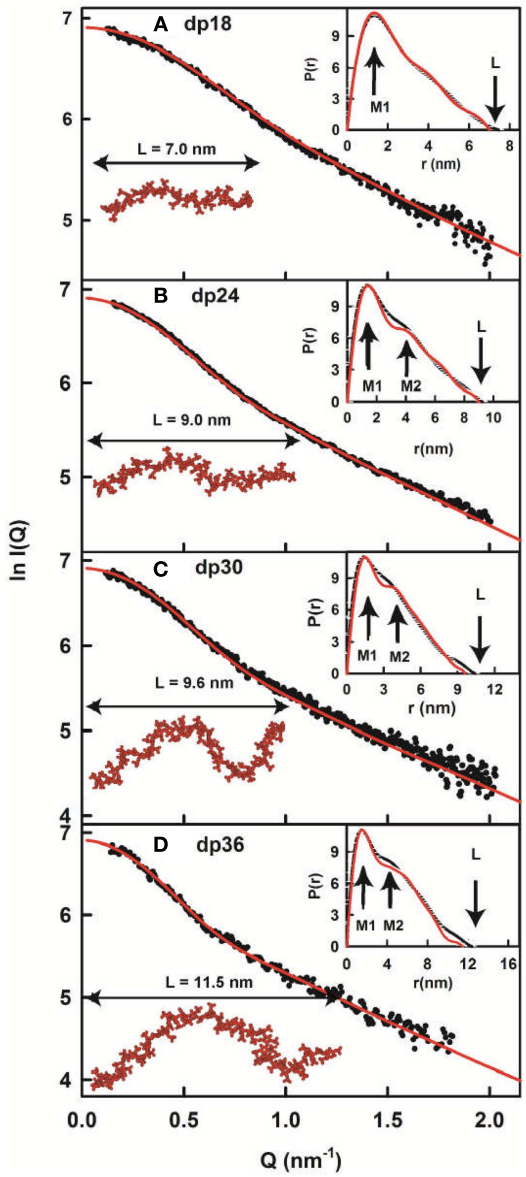

FIGURE 4 | X-ray modeling curve fits for best-fit heparin dp18–dp36 models. (A–D) Correspond to the structural fits for heparin dp18, dp24, dp30 and dp36. The experimental $I(Q)$ and $P(r)$ X-ray scattering data are represented by black circles or lines, respectively. The red lines and models correspond to the best-fit dp18–dp36 models from the searches (**Figure 3**). The maximum lengths of the models are shown for comparison with their L values in the $P(r)$ curves.

mixture. This generated the NA-regions dp6-dp24 that remained following the removal of sulphated oligosaccharides, then Biogel P-10 preparative gel permeation chromatography was performed to separate these individual GlcA–GlcNAc oligosaccharides. The HS fragments dp6–dp24 were submitted to the same ultracentrifugation–scattering-modeling strategy used above for heparin.

Analytical ultracentrifugation on each of the HS fragments dp6–dp24 revealed homogenous preparations from the single peaks seen in the $c(s)$ analyses. The experimental sedimentation coefficients $s_{20,w}$ increased with HS size from 0.82–1.05 S for dp6 to 1.26–1.35 S for dp24 as expected. This time, their values were generally reduced compared to the $s_{20,w}$ values for heparin dp6–dp36. Most of this reduction comes from the lower masses of HS dp6–dp24 compared to heparin dp6–dp24 (**Figure 2**). The remaining reduction is attributable to a more compact solution structure for the HS fragments compared to heparin, i.e., HS is not as extended as heparin. Linear molecular models were created for HS dp6–dp30 starting from a HS dp4 crystal structure. Again the experimental $s_{20,w}$ values were lower than the predicted values from the linear HS models, showing that HS does not have an extended structure (14).

X-ray scattering curves for the HS fragments dp6–dp24 gave R_G values that increased from 0.98–1.03 nm for HS dp6 to 2.82–3.00 nm for HS dp24. The comparison with the linear HS models showed that the predicted R_G values increased linearly with HS size, while the experimental R_G values starting from dp18 did not increase by as much. Thus the experimental R_G values also revealed bending in the larger HS structures. Bending was also indicated from the cross-sectional R_G values and the distance distribution curves $P(r)$ (9, 14).

The constrained scattering modeling identified molecular structures for the eight HS dp6–dp24 fragments. Totals of 5,000–12,000 conformationally randomized structures were generated by variations of the two torsion angles φ and ψ. Each model was compared one-by-one against the X-ray curve. For HS dp6–dp16, good curve fits were obtained with almost linear structures with slight bending (cf: **Figure 4**; not shown). Interestingly, the two largest HS fragments dp18 and dp24 showed a different outcome. The R-factor vs. R_G graphs (cf: **Figure 3**; not shown) for dp18 and dp24 showed that the modeled R_G value at the minimum R-factor was different from the experimental R_G value. The smaller modeled R_G value of 2.13 nm (dp18) and 2.47 nm (dp24) suggested that the HS structures were noticeably bent, while the larger experimental R_G value of 2.34 nm (dp18) and 2.82 nm (dp24) suggested that the HS structures were mostly extended. The modeled $s_{20,w}$ values for both the bent and extended structures were the same within error. This outcome suggested that conformational heterogeneity was present in HS, and that each of HS dp18 and dp24 exhibited both bent and extended structures simultaneously in solution (**Figure 5B**).

The organization of sulfated heparin-like S-domains and unsulfated NA-domains in the HS structure has been clarified by our work. The comparison of our heparin and HS structures becomes essentially that between S-domains and NA-domains (**Figure 5**). The greater bending and flexibility of HS compared to heparin is attributable to the GlcA–GlcNAc disaccharides in HS and the IdoA2S–GlcNS6S disaccharides in heparin. The available crystal structures show that the separations between the rings in GlcA–GlcNAc and IdoA2S–GlcNS6S are similar. Thus the different heparin and HS conformations must result from altered φ and ψ angles. The φ and ψ angles for heparin dp18–dp36 in solution are within 18° of those in 19 crystal structures (**Figures 6A,B**). The φ and ψ angles for HS dp6–dp24 in solution were all within 5° of those in the HS dp4 crystal structure (**Figures 6C,D**). While not significantly larger than the standard deviations, the largest

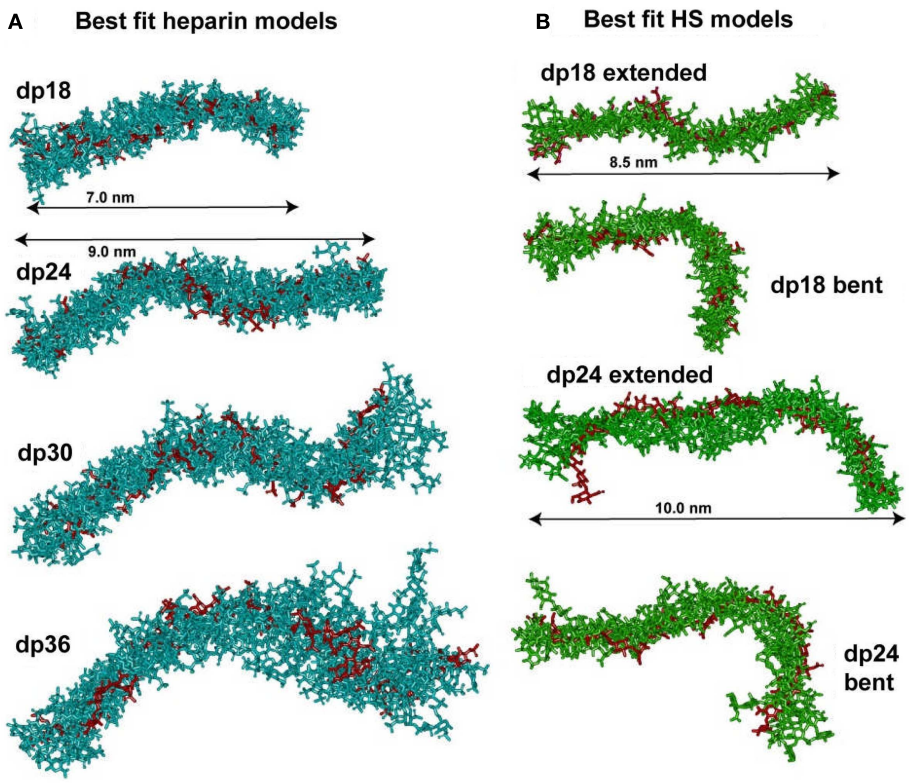

FIGURE 5 | Superimposition of the best-fit models for heparin and HS. **(A)** The nine best-fit models for each of the four heparin fragments dp18–dp36 were superimposed. Only the non-hydrogen atoms are displayed. The best-fit model is shown in red, while the others are shown in cyan. The overall lengths of heparin dp18 and dp24 are shown as 7.0 and 9.0 nm, as arrowed. **(B)** Each set of eight best-fit models for the two HS fragments dp18 and dp24 were superimposed. The best-fit model is shown in red, while the others are shown in green. The overall lengths of the extended HS dp18 and dp24 models were shown as 8.5 and 10.0 nm, as arrowed. The extended best-fit HS structures were obtained primarily from filtering on the R_G values, while the bent best-fit structures were identified by filtering on the *R*-factor values only.

differences between heparin and HS involve the two φ angles rather than the two ψ angles. The physical basis for this is most likely to arise from the IdoA2S–GlcNS6S sequences in heparin compared to the GlcA–GlcNAc sequences in HS. Unlike HS, heparin will be influenced by greater repulsions between pairs of sulfate–sulfate, sulfate–carboxylate, and carboxylate–carboxylate groups. Thus, the combination of the NA- and S-domains within native HS suggests that different native HS structures with greater or lesser bending may arise through variations of the sizes of the NA-domains and S-domains. For example, a three-part native HS structure comprised of a NA-region at its center and flanked by two S-regions would show conformational flexibility in its central region and influence its immune reactivity.

An important caveat in these molecular studies should be noted. When we first published our HS best-fit structures (15), the anomeric configurations of GlcA and GlcNAc in our HS structures should have alternated between α and β. Unfortunately, the anomeric configurations were all β in our original HS models, thus our original study was withdrawn. The HS structures were remodeled with the correct anomers and republished (14). Although we take full responsibility for our mistake, the error was traced back to a misunderstanding of the starting HS dp4 structure in the Protein Data Bank. That HS dp4 structure was written out with its reducing end at the left, when it is more conventional to write this at the right end. In addition, terminological inconsistencies exist in the Protein Data Bank that contributed to our misunderstanding. For example, the α-GlcNAc and β-GlcA anomers of HS should have been written as NDG and BDP, respectively, in the HS dp4 crystal structure, and not as NAG and GCU (PDB code 3E7J; dated 18 August 2008). Related discrepancies in carbohydrate structures have been reported by others (16, 17). It appears essential to check carbohydrate structures from the Protein Data Bank before using these in molecular modeling.

LIGAND INTERACTIONS OF COMPLEMENT FACTOR H

We first summarize the immune functions of CFH. The 20-SCR domains of CFH perform several functions by binding to various ligands. The major N-terminal and C-terminal binding sites for complement C3b in CFH are located at SCR-1/4 and SCR-19/20, and the C3d fragment of C3b binds to SCR-19/20 (**Figure 1A**) (18). A third C3b binding site specific for C3c has been proposed in the central part of CFH (18–20). These interactions lead to the regulatory breakdown of C3b by CFH. The two heparin binding sites of CFH are located close to SCR-6/8 and SCR-19/20 (18). These sites enable the binding of CFH to host cell surfaces, but not to the surfaces of pathogens, thus leading to the complement

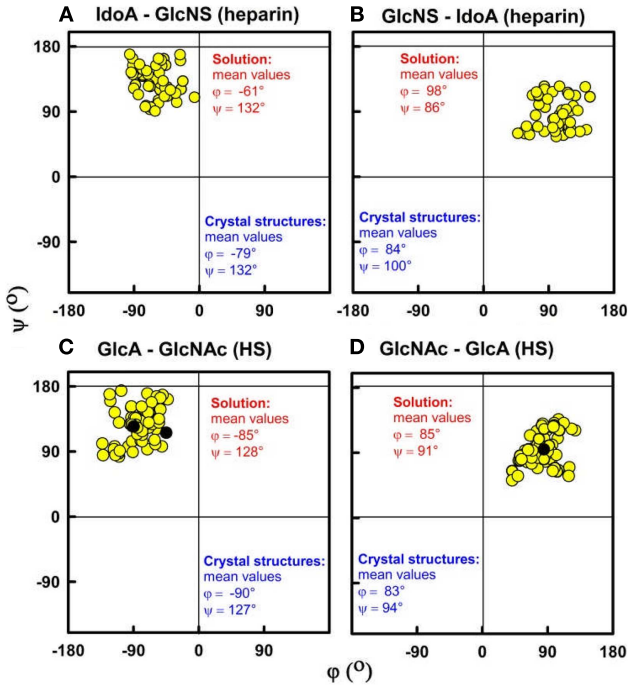

FIGURE 6 | Phi (φ) and psi (ψ) dihedral angles for heparin and HS. The four atoms that define the φ and ψ dihedral angles are shown in **Figure 2**. **(A,B)** For heparin, the IdoA2S–GlcNS6S and GlcNS6S–IdoA2S φ–ψ angle pairs from the best-fit solution structure models are shown as filled circles for each of dp18, dp24, dp30, and dp36. Their mean values are shown in red (9). The φ–ψ pairs from the corresponding crystal structures are shown in blue. **(C,D)** For HS, the GlcA–GlcNAc and GlcNAc–GlcA φ–ψ angles in the best-fit models for each fragment are shown. These correspond to structures for dp6 to dp24, and their mean values are shown in red (14).

regulatory protection of host cells. CFH also binds C-reactive protein at SCR-6/8 and SCR-16/20 (6). The binding of C-reactive protein to damaged host cells will lead to C3b regulation following CFH binding to C-reactive protein. Weak zinc binding sites are primarily located within SCR-6/8 (21); these sites lead to the precipitation of CFH–C3b complexes in the pathophysiological concentrations of zinc found in the retina (22). In addition to these ligand interactions, CFH self-associates with itself to form dimers and higher oligomers (23). Although the physiological role of CFH oligomers is not yet clear, except perhaps in facilitating the development of drusen deposits in the retina at the onset of AMD (23), CFH oligomer formation is a significant factor in the design of experiments with CFH.

For immune function, the four most notable features of CFH–ligand interactions are their moderate binding strengths, their multivalency, their dependence on ionic strength, and CFH self-association:

(i) The strength of the CFH–ligand interaction is central for complement regulation. For most macromolecular interactions, the dissociation constant K_D is similar to the physiological concentration. The K_D corresponds to the concentration at which a given complex is 50% dissociated. Given that C3 and CFH occur at 2–7 μM levels in plasma, the first compilation of all the CFH–ligand K_D values (24) unsurprisingly showed that most K_D values are also micromolar. These micromolar values mean that only partial complexes of CFH with C3b and heparin are formed during normal CFH regulatory function. In addition, these micromolar affinities mean that these CFH–ligand interactions can be easily misinterpreted in biochemical assays for reason of incomplete binding.

(ii) Complement factor H undergoes multivalent interactions with its major C3b, C3d, heparin, and CRP ligands. Multivalency means that analyses based on simple 1:1 interactions may not be adequate for accurate quantitative studies of the CFH–ligand interaction. This issue is best resolved by performing CFH fragments–ligand studies alongside studies based on full-length CFH, such as those of CFH with heparin to characterize its co-operative binding (10).

(iii) Many CFH–ligand interactions involve opposing ionic interactions between CFH and its ligands. The buffer in plasma corresponds to 137 mM NaCl/11 mM phosphate (24). Experiments that use low salt will promote these interactions, while high salt will inhibit these interactions. While the use of low (50 mM NaCl) and high (250 mM NaCl) salt levels can be useful, they may lead to undesired side-effects. For example, C3d is monomeric in 137 mM NaCl, yet forms dimers and trimer/tetramer in 50 mM NaCl buffer (25). A related case is 2 mM calcium that stabilizes C-reactive protein, thus the use of 2 mM calcium is important for its binding studies with CFH (6).

(iv) Full-length CFH is well-characterized to undergo 5–14% self-association in 137 mM NaCl/11 mM phosphate buffer. The presence of CFH oligomers will complicate experiments that require high protein concentrations.

BIVALENT AND CO-OPERATIVE BINDING OF CFH–HS

The immune function of CFH is determined by its major functional activities at its N-terminal and C-terminal SCR domains (**Figure 1**). The middle SCR domains possess shorter sequences, longer inter-domain links, and higher glycosylation levels, suggesting that these middle domains act as conformational spacers. Because there is no crystal structure for full-length CFH, the combination of X-ray and neutron scattering and sedimentation coefficient modeling produced the first molecular structures for full-length CFH (26, 27). The CFH structure determination approach is similar to those for heparin and HS fragments above. The scattering modeling was based on molecular structures for all 20-SCR domains. These were taken directly from crystal or NMR structures for small CFH fragments, or from predicted SCR structures by homology modeling based on the closest match with known crystal or NMR structures. The inter-SCR linkers are variable and not predictable in their conformation. For the scattering modeling, these linkers were conformationally randomized and used to assemble 2,000 SCR models for CFH in randomized orientations. The scattering curve fits showed that only folded-back domain structures with an overall length of 40 nm (**Figure 1B**) fitted the CFH scattering data. Its length is much reduced compared to a hypothetical linear structure for CFH, which would be 73 nm

in length. Two distinct models for CFH have been computed, which give similar and indistinguishable scattering fits. In the first one, the SCR-1/7 domains are extended in shape and SCR-8/20 are looped back (**Figure 1**) (24). In the second one, the SCR-13/20 domains are extended and SCR-1/12 are looped back (27). The first model is more easily docked with the C3b–CFH crystal structure, because this requires SCR-1/4 to be extended in shape (28).

The CFH–HS interaction forms the basis for the protection of the native HS-coated host cell surfaces via the S-domains of HS from attack by the innate immune system, directing this immune response instead against pathogenic bacteria, which lack a polyanionic oligosaccharide coating and are therefore unprotected by CFH. Polyanionic molecules such as HS and others such as the sialic acids on host cell surfaces enhance the regulatory effectiveness of CFH by 10-fold through its inhibition of complement activation (29–31). Two independent heparin binding sites are located at the SCR-6/8 and SCR-19/20 domains in CFH (**Figure 1A**). Notably, SCR-7 and SCR-20 have the two most basic charge densities in CFH (27), these basic charges being optimal for interactions with anionic S-domains. The availability of molecular structures for CFH, heparin, and HS fragments (above), and the combined application of ultracentrifugation, scattering, molecular modeling, and surface plasmon resonance (below) to study CFH–heparin complexes provided the first molecular picture of CFH binding to heparin.

The ultracentrifugation experiments of CFH mixtures with heparin dp6–dp36 (equivalent to S-domains) identified multiple different CFH–heparin complexes in the size distribution analyses $c(s)$ (10). Unbound monomeric CFH shows a peak close to 5.5 S. The peaks for heparin dp6–dp36 are between 1.1 and 1.8 S and do not overlap with that for CFH. Even though unbound CFH showed small oligomer peaks between 8 and 16 S that make up 15% of the total peak intensity, the marked differences in these peaks after adding heparin provided unequivocal evidence of complex formation (**Figure 7**). The CFH mixtures with dp6 and dp12 showed small decreases in these peaks between 8 and 16 S (not shown). However the CFH mixtures with dp18–dp36 showed large peak intensity increases of up to 63% for dp36. If CFH and heparin dp30/dp36 formed a 1:1 complex, the increased peak sizes corresponded to a K_D value of about 0.5 μM. Even with 63% CFH–heparin oligomer formation, the CFH monomer peak continued to be visible, showing that complex formation is incomplete. Ultracentrifugation also showed that heparin dp10 bound tightly to SCR-6/8 and this is consistent with a micromolar affinity (32). In addition, the small CFH oligomer peaks shifted to lower S values after adding heparin (**Figure 7**). This showed that a new type of CFH oligomer structure with more extended structures had formed with heparin. As controls, experiments with native HS material (containing both S-domains and NA-domains) showed similar peak changes, while the use of NA-domains alone showed much reduced interactions with CFH.

The scattering experiments were complementary to ultracentrifugation because of their sensitivity to aggregate formation that may be missed in the ultracentrifuge. In agreement with ultracentrifugation, little changes were seen by scattering for CFH mixtures with heparin dp6 and dp12 (10). However, large increases in both molecular weights and R_G values were seen for CFH mixtures with heparin dp18–dp36. The distance distribution analyses $P(r)$ showed that the maximum dimension of CFH increased from 34 to 40 nm in the presence of heparin dp36. Because no indefinitely sized aggregates were seen by scattering, these results agree with the formation of large CFH–heparin oligomers with specific sizes.

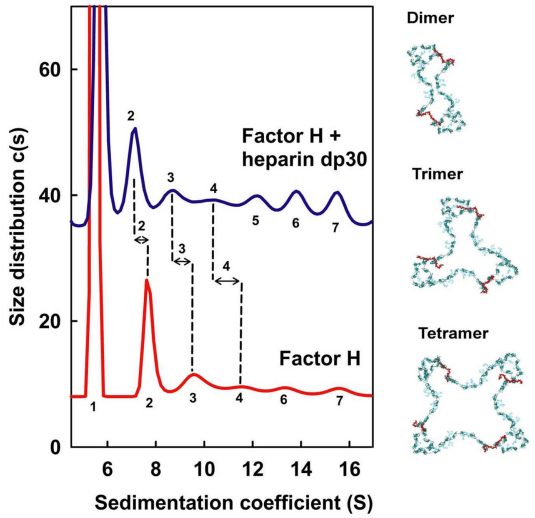

FIGURE 7 | Sedimentation velocity analyses of CFH mixtures with heparin dp30. CFH was studied alone (red) and with a 1:1 molar ratio of dp30 (blue) by sedimentation velocity in the analytical ultracentrifuge. The CFH monomer $s_{20,w}$ peak is labeled as 1, and the CFH oligomer $s_{20,w}$ peaks for the dimer, trimer, and tetramer onward are denoted by 2, 3, 4, and upwards. Peaks 2, 3, and 4 are better defined and the change in the sedimentation coefficients between CFH and CFH–dp30 is shown by the vertical dashed lines. On the right, dimer, trimer, and tetramer models for CFH are shown in blue–green, with the heparin cross-links shown in red. The dp30 heparin sizes are sufficiently long enough to cross-link the SCR-7 and SCR-20 domains between different CFH molecules, leading to the formation of dimer, trimer, and tetramer as ring-like molecular structures. Redrawn from Khan et al. (10).

To clarify the ultracentrifugation and scattering results, surface plasmon resonance experiments were performed with both full-length CFH and recombinant SCR-6/8 and SCR-16/20 in solution with heparin immobilized on the sensor chip. The binding data showed mostly fast on-rates and off-rates with the heparin surface. Curve fits of the binding responses gave K_D values of 1–3 μM for full-length CFH, and similar but significantly weaker K_D values of 4 μM for the SCR-6/8 fragment and 20 μM for the SCR-16/20 fragment (**Figure 8**). These K_D values confirm cooperativity between the two different heparin binding sites in CFH. As controls, the use of immobilized unfractionated HS containing both NA-domains and S-domains showed a K_D value of 2 μM with full-length CFH, while use of immobilized HS fragments (i.e., NA-domains) showed much weaker CFH binding. These experiments confirmed the importance of the heparin-like S-domains for this interaction.

These methods show that the immune function of CFH at host cell surfaces is well-described by a bivalent and co-operative model of CFH binding to the S-domains of HS. To visualize this model, molecular modeling of the ultracentrifugation $s_{20,w}$ data

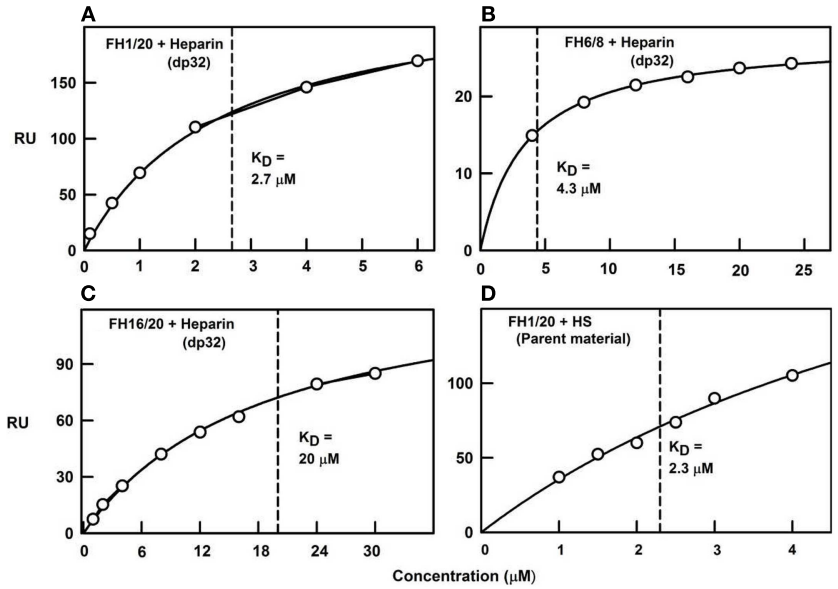

FIGURE 8 | Surface plasmon resonance of the CFH interaction with heparin and HS. The binding curve fits used to determine the K_D values are shown for four experiments using immobilized heparin over which the CFH samples were flowed. Redrawn from Khan et al. (10). **(A)** Biotinylated heparin dp32 was immobilized on a streptavidin chip, and studied using plasma CFH to give a K_D value of 2.7 μM. **(B)** CFH SCR-16/20 was used with the same dp32 chip, from which the K_D value was determined as 20 μM. **(C)** CFH SCR-6/8 was used with the same dp32 chip, from which the K_D value was determined as 4.3 μM. **(D)** Biotinylated unfractionated HS was immobilized on a streptavidin chip, and studied using plasma CFH to give a K_D value of 2.3 μM.

was performed. In the folded-back CFH solution scattering model (**Figure 1B**), the heparin binding SCR-7 and SCR-20 domains were separated by 26 nm. In contrast, the best-fit slightly bent heparin dp36 model is only 12 nm long. Thus, heparin dp36 is not long enough to cross-link the two heparin binding SCR domains in a single CFH molecule. If however the SCR-7 and SCR-20 domains in different CFH molecules were daisy-chained in alternation with heparin dp36, ring-like models for dimers, trimers, tetramers, and pentamers of CFH with heparin could be created (**Figure 7**). These CFH–heparin models explained the experimental $s_{20,w}$ values for the oligomer peaks (10) within an acceptable error of ±0.2 S (blue; **Figure 7**). Either of the two distinct models for CFH (see above) may be used to create these ring-like models. The modeling therefore explained the changes in the peak positions after adding heparin dp36. The modeling also explained why dp6 and dp12 did not form oligomers with CFH, i.e., because they were too short to cross-link CFH.

Complement factor H flexibility at its central domains is unlikely to play a role in heparin binding. Our experimental data for the heparin/HS fragments and CFH indicate that neither possesses sufficient flexibility to form one-to-one complexes. No increased $s_{20,w}$ value for the CFH monomer was seen that would indicate that CFH underwent a significant compaction in the presence of heparin (apart from relatively low changes caused by the increased mass of the CFH–heparin complex). This lack of CFH flexibility concurs with the little conformational variation seen in CFH with change in salt or pH (27). It is likely that the heavily glycosylated and smaller SCR-12/15 domains at the center of CFH maintain the structural independence of its N-terminal and C-terminal ends. The presence of these two different heparin sites at opposite sides of the CFH central region enables CFH to bind selectively, bivalently, and co-operatively to host cells showing a sufficient density of polyanions at their surfaces.

Flexibility in native HS is defined by the presence of semi-rigid S-domains and flexible NA-domains that were deduced from the scattering analyses. Our binding studies show that CFH binds to heparin-like S-domains, but less so to the non-sulfated NA-domains. If the parent HS structure shows flexibility at the NA-domains, this would enable the individual S-domains to reorientate themselves to optimize their stronger contacts with CFH. Thus CFH interacts in the same way with native HS as many other proteins do that bind to native HS through the S-domains (33).

The quantification of the CFH–HS interaction permits critical comparisons with other CFH–ligand affinities. The first full set of K_D values for CFH–ligand complexes was compiled recently (24). This summary (**Table 1**) currently shows that the strongest CFH interactions involve heparin and C3b/C3u. The affinity of CFH (concentrations of 2–5 μM in plasma) for heparin is around 1–2 μM, with the CFH binding for heparin being bivalent. These micromolar K_D values mean that the CFH–heparin complexes will not be fully formed in plasma, and the free and bound forms will be in equilibrium with each other (footnote, **Table 1**). The larger K_D values of 4 and 20 μM for the separate SCR-6/8 and SCR-16/20 fragments correspond to weaker binding to heparin at each of two independent binding sites (10). In comparison with the K_D value for intact CFH, this shows co-operative binding. The CFH interactions with C3b and C3u have low K_D values of around 1 μM. Since C3 is 5 μM in plasma, these complexes (**Figure 9A**)

Table 1 | Selected dissociation constants, K_D, for the CFH interactions with its ligands.

Interaction	K_D (μM)[a]	Method[b]	Buffer (abbreviated)[c]	Reference
CFH–HEPARIN				
CFH–heparin dp32/dp36	0.5, 2.7	SV, SPR	10 mM HEPES with 137 mM NaCl, pH 7.4	(10)
SCR-6/8–heparin dp32	4.3	SPR	10 mM HEPES with 137 mM NaCl, pH 7.4	(10)
SCR-16/20–heparin dp32	20	SPR	10 mM HEPES with 137 mM NaCl, pH 7.4	(10)
CFH–C3b OR CFH–C3u				
CFH–C3b	0.59–1.6	SPR	10 mM HEPES with 150 mM NaCl, pH 7.4	(18)
CFH–C3u	0.59	SV	PBS with 137 mM NaCl, pH 7.4	(34)
SCR-1/4–C3b	11	SPR	PBS with 150 mM NaCl, pH 7.4	(28)
SCR-1/4–C3b	9.8–13.5	SPR	10 mM HEPES with 150 mM NaCl, pH 7.4	(18)
SCR-19/20–C3b	5.4	SPR	PBS with 140 mM NaCl, pH 7.3	(20)
SCR-19/20–C3b	3.5–4.5	SPR	10 mM HEPES with 150 mM NaCl, pH 7.4	(18)
SCR-19/20–C3b	0.54	SPR	10 mM HEPES with 150 mM NaCl, pH 7.2	(35)
CFH–CRP				
CFH–CRP	4.2	SPR	10 mM Tris, 137 mM NaCl, 2 mM $CaCl_2$, pH 7.4	(6)
CFH SELF-ASSOCIATION				
CFH–CFH	28	SE	10 mM HEPES with 137 mM NaCl and EDTA, pH 7.4	(23)
CFH–ZINC				
CFH–zinc	~10	SAXS	10 mM HEPES, 137 mM NaCl, pH 7.4	(21, 23)
C3b–zinc	~100	SAXS	10 mM HEPES, 137 mM NaCl, pH 7.4	(22)

[a] *If the interacting species are both at 5 μM and the K_D value is 1 μM, 64% of the complex will be formed. If the K_D value is 10-fold weaker at 10 μM, 27% of the complex will be formed.*

[b] *ED, equilibrium dialysis; SAXS, small-angle X-ray scattering; SE, sedimentation equilibrium; SPR, surface plasmon resonance; SV, sedimentation velocity.*

[c] *Buffer additives are common, especially for SPR studies. These are reported in the more detailed survey of Perkins et al. (24).*

will again be only partially formed in plasma. The K_D values of around 10 and around 4 μM for the separate SCR-1/4 and SCR-19/20 sites for C3b, respectively, are also weaker (18). The reason why the latter two K_D values are weaker compared to intact CFH is not clear, because it has not been shown experimentally that CFH binds bivalently to one molecule of C3b or C3u. A CFH–C3b complex formed through SCR-1/4 binding (**Figure 1B**) may also bind to a second molecule of C3b at its C3d (TED) domain, hence decreasing the observed K_D value (**Figure 1A**). Overall, however, the similar binding affinities of CFH for C3b/C3u and heparin indicate that these values are optimal for the effective immune role of CFH as a host cell surface regulator of C3b activity.

The other CFH–ligand interactions are weaker than those of CFH–heparin. C3d (K_D of 3–8 μM) and C-reactive protein (K_D of 4 μM) (6, 36) show weaker affinities for CFH (**Table 1**), suggesting that these interactions only become important during excess inflammation (acute phase response) when high local concentrations of C3d or C-reactive protein may occur. When either of these two ligands is bound to host cell surfaces, additional CFH binding sites are potentially available to reinforce host cell protection, similar to that suggested for CFH–heparin (**Figure 9B**). The monomer–dimer K_D value of 28 μM for CFH self-association (23) and the K_D value of about 10 μM for CFH–zinc binding and about 100 μM for C3b–zinc binding indicate even weaker affinities (22). The latter interactions are more relevant to drusen formation (retinal deposits of aggregated proteins and oxidized lipids associated with AMD) (37) than to the CFH–heparin interaction at host cell surfaces.

Other groups have reported K_D values for the CFH–heparin interaction. That of 9.2 nM was reported for CFH binding to unfractionated heparin in 50 mM Na phosphate, 100 mM NaCl buffer, pH 7.2, while that of 9 μM was reported for CFH SCR-19/20 binding to heparin dp4 in 20 mM acetate, 200 mM NaCl buffer, pH 4 (38, 39). The differences from the values in **Table 1** are attributable to the less physiological ionic strengths used in these experiments, these buffers being rather different from that of Dulbecco's phosphate buffer (137 mM NaCl and 11 mM phosphate, pH 7.4).

CONCLUSION AND FUTURE CONSIDERATIONS

The immune significance of the CFH–HS interactions has been clarified by our molecular solution structures for heparin, HS, and CFH. We provide a first molecular model of the CFH–HS interaction, i.e., a bivalent and co-operative CFH binding mechanism to heparin and HS exists that clarifies how CFH binds to host cell surfaces. At the cell surface, native HS is comprised of sulfated heparin-like S-domains interspaced by desulfated NA-domains of HS. The heparin solution structures possess semi-rigid extended conformations with notably less flexibility than those found in the flexible extended or bent desulfated HS structures. The solution structure of CFH revealed a folded-back 20-SCR domain structure in which the functional N-terminal and C-terminal domains are extended outwards from a compact core of shorter glycosylated SCR domains with longer inter-SCR linkers. Unlike the NA-domains of HS, there is no evidence for significant flexibility in full-length CFH. Consequently, the combination of

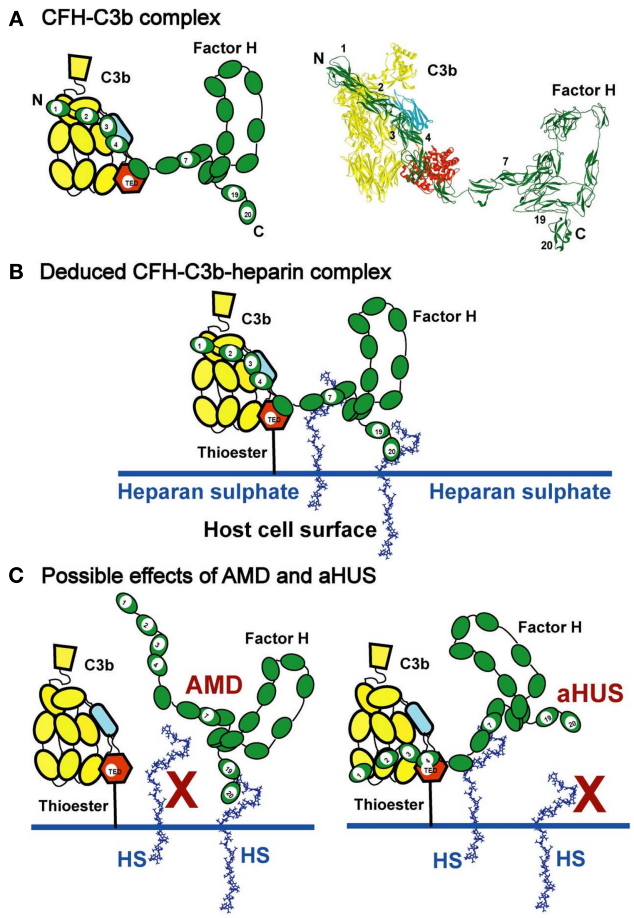

FIGURE 9 | Schematic views of CFH binding to a host cell surface. **(A)** CFH is represented by a cartoon showing the 20-SCR domains, and bound to C3b in the cleft formed between the CUB/TED domains (blue–red) and the macroglobulin, C345, and linker domains (yellow). A molecular view of this interaction is shown on the right. **(B)** CFH binds bivalently at its SCR-7 and SCR-20 domains to anionic charges on the HS-coated host cell surface (denoted as HS and represented here by heparin dp36). For simplicity, the C-terminal interaction of CFH with C3d (**Figure 1A**) is not shown. This bivalent binding positions the SCR-1/4 domains in a conformation that binds readily to C3b when C3b is covalently bound to host cell surfaces through the thioester bond. The C3b interaction with CFH results in the Factor I-mediated degradation of C3b to form C3d (the TED domain) and C3c, thereby regulating complement activation at host cell surfaces. **(C)** The potential effect of polymorphisms and mutations on C3b regulation is schematically indicated. In AMD, the association of the Tyr402His polymorphism in SCR-7 is presumed to weaken the interaction between HS and SCR-7, so the proximity of CFH in relation to SCR-1/4 of surface-bound C3b is reduced. In aHUS, mutations in SCR-19 and SCR-20 are presumed to weaken the interaction between HS and SCR-19/20, while CFH remains bound to HS through SCR-7. In both AMD and aHUS, the effect is that CFH is no longer held in an optimal position on the host cell surface to interact with C3b at the SCR-1/4 domains.

the non-flexible CFH and heparin dp18–dp36 structures *in vitro* leads to the formation of ring-like models for their complexes (**Figure 7**). *In vivo*, the views of **Figure 7** are readily transformed to suggest how a CFH monomer binds to two different S-domains on a host cell surface (**Figure 9B**). The flexibility of the NA-domains may facilitate optimal binding of the CFH and S-domains at the cell surface.

Such a bivalent CFH binding mechanism to HS has implications for immune function. In theory, the combination of two separate weak binding events with micromolar affinities becomes a much strengthened interaction if both weakly bound CFH sites bind simultaneously to HS at a cell surface. This prediction was confirmed by surface plasmon resonance studies of full-length CFH and its functional fragments to immobilized heparin on sensor chips. Such a binding interaction may position CFH SCR-1/4 away from the cell surface to facilitate their binding to surface-bound C3b for its regulation. The binding of CFH at SCR-19/20 to the cell surface may orientate CFH into a position that is optimal for its SCR-1/4 domains to bind to C3b (**Figure 9B**).

For reason of co-operativity, CFH is envisaged to bind preferentially to surfaces showing the right spatial density of anionic oligosaccharides. This implies that the moderately strong binding of CFH to host cell surfaces may differ between different cell types or degrees of sulfation. If HS binding is reduced at either SCR-7 or SCR-20 in CFH, co-operativity implies that there will be a disproportionate effect on CFH regulatory function. In CFH-associated genetic diseases such as AMD and aHUS, the CFH–heparin interaction may be affected by polymorphisms or mutations. Disease-risk polymorphisms will exert their effect over a period of decades, while disease-causing mutations will show a much earlier effect during a lifespan. The AMD-risk CFH polymorphism Y402H occurs in 33% of individuals. AMD occurs primarily in the aged population, this being responsible for over 50% of blindness in the elderly in the Western world (40). Mutations leading to aHUS occur mostly in the C-terminal SCR-19/20 domains of CFH, and aHUS is a common cause of renal failure in young children (41).

The biochemical mechanism of CFH-associated genetic disease may involve either the facilitation of CFH aggregation to form pathogenic deposits, or the biochemical loss of CFH regulatory control (42). Present evidence for either mechanism is not definitive. In terms of an aggregation mechanism, CFH has been found in drusen that are a hall-mark of AMD (43). Glycosaminoglycans have also been identified within Bruch's membrane, although their size and structure in drusen is not yet known (44). The availability of free S-domains in glycosaminoglycans with size dp18 or more may lead to the formation of ring-like CFH–heparin aggregates, e.g., if these S-domains are released from the cell surface during inflammatory attack. Polyanions also cause CFH to aggregate (45). In terms of an alternative mechanism based on reduced inflammatory regulation that may lead to disease, CFH disease-causing mutations have been summarized on the web (46). These affect each of the two CFH–heparin binding sites:

(i) Three studies of the Tyr402His polymorphism in SCR-7 show that CFH His402 binds more weakly to heparin than CFH Tyr402 (47–49). If so, the weaker binding of CFH His402 to the heparin-like S-domains of HS would compromise both the CFH interaction with C3b and the bivalent binding of CFH to cell surfaces (**Figure 9C**). This scenario is similar to that proposed for the CFH–CRP interaction (6) in that the weaker binding of CFH His402 to host cell surfaces would

predispose toward greater inflammatory damage. However, other studies reported variable outcomes depending on the heparin preparation in use and its degree of sulfation (50, 51), while another study reported that no significant difference was observable between the Tyr402 and His402 allotypes (52). In opposition to these results, the crystal structure of SCR-6/8 His402 bound to a heparin analog suggests that the S-domains bind more strongly to the His402 allotype than to the Tyr402 allotype (53).

(ii) For C-terminal CFH mutations in SCR-19/20 that lead to aHUS, these occur mostly in young individuals, often being triggered by an immune insult to the kidney such as a bacterial infection (41). aHUS is primarily caused by mutations within SCR-19/20, often those affecting heparin binding or C3d-binding properties (46). The C-terminal mutations may affect CFH function by perturbing the orientation of SCR-1/4 relative to C3b when CFH is bound to the host cell surface (**Figure 9C**).

It is not clear at present why a polymorphism at SCR-7 in CFH leads to one immune disease, while mutations at SCR-19/20 lead to a different disease altogether. Further developments to elucidate the molecular mechanism for CFH binding to native HS may lead to new therapeutic approaches for diseases such as AMD or aHUS.

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The proteoglycan glycomatrix: a sugar microenvironment essential for complement regulation

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Proteoglycans (PGs) are major components of all mammalian tissues, being present ubiquitously on cell surfaces and within extracellular matrices (ECM). They play vital roles in mammalian physiology and have been implicated in many disease processes. PGs comprise a single core protein with one or more glycosaminoglycan (GAG) chains attached where these un-branched polysaccharides are composed of repeating disaccharide units that show considerable diversity in their disaccharide composition, glycosidic linkages, and levels/positions of sulfation (1, 2). GAGs, therefore, contain huge numbers of structural permutations (even in the same chain), representing a vast possible array of diverse structures that can determine the fate of local environments (3): i.e., through their modulation of protein-binding and activity. Thus, PGs constitute a tissue and region-specific microenvironment of sugar molecules, both within the ECM and at the cell-matrix interface, which acts as a local regulator of tissue function and homeostasis. As described below, it is our opinion that this proteoglycan "glycomatrix" plays a key role in the regulation of the immune system by acting as a molecular postcode that controls local immune function (4). Here we will illustrate this with examples of the effects of PGs/GAGs on the immune system in the eye, heart, kidney, and lung. In particular, we will focus on recent evidence that GAGs can positively and negatively regulate the alternative pathway of complement and suggest how the dysregulation of this aspect of innate immunity may contribute to disease processes in a tissue-specific manner.

PROTEOGLYCANS PROVIDE A DIVERSE MOLECULAR POSTCODE FOR PROTEIN REGULATION

Despite the diversity possible in GAG sequences, considerable specificity in chain composition is seen between and within different tissues (5, 6). For example, in the human eye it has been shown that specific GAG structures and PG core proteins are located in defined layers of the retina, resulting in remarkable compartmentalization even within the same organ (6, 7); this has the potential to regulate the binding/function of proteins, such as those that control angiogenesis and innate immunity. Importantly, the glycomatrix can provide a postcode that can be distinguished by different proteins [*via* their abilities to recognize distinct GAG structures (4)], whereby even members of the same protein family [e.g., the interleukin cytokines (8)], can bind differentially, i.e., at "defined" sites.

At present, because of the current limitations in GAG sequencing, there is relatively little detailed information available on the precise structures of glycomatrix postcodes found in different tissues or their protein-binding specificities. What is clear, however, is that GAGs/PGs play an important role in the recruitment and regulation of a wide range of proteins, including modulators of the innate/cellular immune system, as well as those that are involved in tissue remodeling during inflammatory/disease processes. For instance, GAGs have been found to be key elements in regulating pulmonary inflammation during lung infection through their binding of cytokines, chemokines, and growth factors, which promotes leukocyte adhesion and accumulation (9). The binding of cytokines and chemokines to PGs conceals proteolytic cleavage sites: for example, heparan sulfate (HS) limits the proteolytic digestion of interferon-gamma, which increases its activity sixfold (10). Furthermore, GAGs specifically bind matrix metalloproteinases (MMPs) as well as their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). This means that PGs are able to sequester MMPs and TIMPs in specific regions of the lung leading to direct regulation of enzyme activity; e.g., following acute lung injury or infection (9). Other examples include chondroitin 4-sulfate playing a direct role in the presentation of pro-MMP2 to MT3-MMP (where chondroitin 6-sulfate or HS do not do this), thus leading to its activation (11), while on the other hand HS can recruit and inhibit ADAM12 (12).

MODULATION OF THE COMPLEMENT SYSTEM BY PROTEOGLYCANS

The PG glycomatrix can influence the innate immune system *via* recruitment of regulatory factors from the blood; e.g., the positive and negative regulators of the complement system, proderdin, and complement factor H (CFH). Properdin stabilizes the alternative pathway C3 convertase, promoting amplification of the complement cascade, leading to C3b deposition that labels targets for destruction by phagocytosis and allows formation of the membrane attack complex, which can lyse cells; this also leads to the production of pro-inflammatory mediators that attract leukocytes and cause mast cell degranulation. Conversely CFH, once recruited to a surface, can accelerate the decay of the C3 convertase and act as a co-factor for

the proteolytic deactivation of C3b, thus dampening-down a complement response. This fine balance between positive and negative regulation can be greatly influenced by the composition of a tissue's glycomatrix (13). While both properdin and CFH bind HS on renal tubular epithelial cells, they recognize distinct, non-overlapping, sequences within HS GAG chains; i.e., they do not compete for binding sites. It was reported that CFH only recognizes highly sulfated HS, while properdin is able to bind more lowly sulfated HS structures (e.g., those lacking N-sulfation) (14). Thus, this differential recognition of the glycomatrix likely allows both positive and negative regulators of the complement alternative pathway to be present together on the surface of these kidney cells, thus ensuring innate immune homeostasis (13). If this balance breaks down (e.g., due to impairment of protein/GAG interactions) it could lead to kidney damage and may explain the worsening of outcome in proteinuric patients, i.e., due to inappropriate complement activation.

The CFH protein is comprised of 20 complement control protein (CCP) domains where CCPs6–8 and CCPs19–20 mediate GAG-binding [see Ref. (15–17)]. Interestingly, our recent work has provided strong evidence that the HSPGs in the glomerular basement membrane (GBM) of the human kidney recruit CFH solely *via* its CCP19–20 surface recognition domain; this region of CFH recognizes highly sulfated HS structures (17). On the other hand, CCP6s–8 are largely responsible for CFH-binding to sites in the human eye, i.e., the retinal pigment epithelium (RPE) and the underlining Bruch's membrane, a multi-layered ECM. We believe that this is because the GAG-binding specificities of the CCP6–8 and CCP19–20 regions are distinct (15, 17) and can therefore provide tissue specificity through recognition of different GAG structures (postcodes) in different tissue locations (see **Table 1**); i.e., they can distinguish different glycomatrices.

Bruch's membrane separates the RPE and photoreceptor cells in the neurosensory retina from the choroid, a vascular bed posterior to these structures. CFH, being the only secreted regulator of the alternative pathway, is solely responsible for protecting ECM such as Bruch's membrane from complement-mediated damage (i.e., preventing complement amplification in healthy host tissues). We have found previously that CFH-binding sites in Bruch's membrane are comprised mainly of HS, but with dermatan sulfate also playing a minor role (16). Moreover, we discovered that the Y402H polymorphism in the *CFH* gene [that changes a tyrosine to histidine in CCP7 (18)] impairs the ability of CFH to bind to GAG postcodes in Bruch's membrane (16). This is likely to be important since this common polymorphism is strongly associated with the development of Age-related Macular Degeneration (AMD) (19–21), which is the most common form of blindness in the western world; individuals homozygous for the 402H form of CFH have a ∼5-fold increased risk of developing AMD (20). Our studies have demonstrated that the disease-associated 402H variant has a rather restricted specificity, requiring highly sulfated structures, as opposed to the 402Y form which is able to bind a broader range of GAG sequences (15, 22). In the glycomatrix of the Bruch's membrane the binding sites for the 402H variant of CFH are rare relative to those for 402Y (16). On this basis we hypothesize that insufficient binding of 402H within the Bruch's membrane will lead to complement over-activation and local chronic inflammation, and thereby damage the RPE, contributing to the formation of the particulate deposits, called drusen, that are the hallmarks of AMD (16, 23, 24).

As noted above, the two GAG-binding regions in CFH have different specificities where these are likely to differentially regulate the interactions of this protein with sites in the eye and kidney (17). This may explain why mutations/polymorphisms within CCPs19–20 are associated with the kidney disease, atypical Hemolytic-Uremic Syndrome (aHUS), where uncontrolled complement activation is believed to lead to inflammation and the formation of blood clots, whereas, the Y402H polymorphism is linked to AMD. Patients suffering from aHUS do not present with any ocular phenotype and similarly AMD patients rarely have associated kidney problems. This provides a striking example of the tissue-specific nature of the glycomatrix microenvironment (e.g., of the

Table 1 | Comparison of the binding properties of the two GAG-binding regions of CFH.

	CCP6–8		CCP19–20
	402Y	402H	
GAG CHAIN RECOGNITION[a]			
Hyaluronan	×	×	×
Dermatan sulfate	✓	✓	×
Chondroitin 4-sulfate	×	×	×
Chondroitin 6-sulfate	×	×	×
Heparan sulfate	✓	✓	✓
Heparin	✓	✓	✓
HEPARIN SULFATION SPECIFICITY[b]			
2-*O* desulfated	↓	↓↓	↓↓
6-*O* desulfated	↓	↓	↓↓
N-*O* desulfated	↓	↓↓↓	↓↓↓↓
TISSUE SPECIFICITY[c]			
Bruch's membrane	+++ (Broad specificity)	+ (Requires 2- and 6-O sulfation)	+ (Unknown)
RPE	+++ (Broad specificity)	+++ (Requires 2- and 6-O sulfation)	+++ (Unknown)
Kidney GBM	−	−	+++

[a] *Based on direct binding experiments where ✓ means binding and × no binding.*

[b] *Binding to selectively desulfated preparations of heparin where ↓ means small reduction in binding, ↓↓ moderate reduction in binding, ↓↓↓ large reduction in binding, and ↓↓↓↓ means no detectable binding.*

[c] *Based on the binding of fluorescently labeled protein (CCP6–8 and CCP19–20) probes to human tissue where − means no binding, + weak binding, ++ moderate binding, and +++ strong binding.*

Bruch's membrane and GBM) and how this might differentially influence disease processes.

AGE-RELATED EFFECTS ON THE GLYCOMATRIX POSTCODE?

Alterations in the biosynthesis and turnover of PGs are known to occur with age (2), therefore concomitant effects on protein recruitment and tissue function would not be surprising. For example, there is an age-related change in the fine structure of HS that affects the migration of endothelial progenitor cells (25). Here the loss of a specific tri-sulfated disaccharide from their surface HS correlates with a reduction in their migratory response to vascular endothelial growth factor; this impairs the engraftment capacity of these cells, contributing to endothelial dysfunction and age-related vascular pathology. Similarly, human aorta HS is subject to age-related increases in the level of 6-O sulfation (26); this, in turn, leads to increased binding of platelet-derived growth factor resulting in its extracellular accumulation, which is hypothesized to facilitate aberrant smooth muscle cell migration and growth, i.e., in individuals prone to developing atherosclerotic disease. Another example is the recent finding that chondroitin sulfate and keratan sulfate chains of aggrecan, a major PG component of articular cartilage, decrease in both number and length with age, affecting amongst other things the mechanical properties of this tissue (27). Given the importance of GAGs in the regulation of complement (as described above), it is plausible that age-related changes in the glycomatrix of the eye could contribute to AMD pathogenesis, such that this might explain the age-related nature of this disease (15, 24).

PROTEOGLYCANS AS TARGETS FOR THERAPEUTICS?

Given the major role played by PGs and their GAG chains in immune homeostasis, it seems plausible that they might make good therapeutic targets for immunological diseases. However, based on the above information, it will perhaps be prudent to attempt to modulate GAG-protein interactions in a tissue-dependent context. In this regard, it has recently been demonstrated that specific heparinoids, such as *N*- and *O*-sulfated K5 polysaccharides, can inhibit the binding of properdin to HS on renal tubular epithelial cells without affecting CFH, thereby controlling complement activation (13); this has the potential to prevent complement-derived tubular injury in proteinuric kidney diseases. Approaches of this type may be able to selectively inhibit the binding of pro-inflammatory proteins to particular GAG structures in a tissue/organ-specific manner and thus correct immune dysregulation in a wide range of pathological conditions.

CONCLUDING REMARKS

In our opinion, the glycomatrix created by PGs remains an under-appreciated contributor to immune regulation. However, an increasing body of evidence is providing insights into just how important these complicated glycoproteins are in providing the fine control to immunological processes in tissue microenvironments, particularly within the ECM. More work is now needed to fully elucidate the biochemical basis of protein/GAG interactions and determine their roles in pathological processes. Further advances in our knowledge of the proteoglycan glycomatrix should facilitate the development of novel, tissue-specific, therapeutics, e.g., for diseases of the immune system.

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Heparanase and autoimmune diabetes

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Heparanase (Hpse) is the only known mammalian endo-β-D-glucuronidase that degrades the glycosaminoglycan heparan sulfate (HS), found attached to the core proteins of heparan sulfate proteoglycans (HSPGs). Hpse plays a homeostatic role in regulating the turnover of cell-associated HS and also degrades extracellular HS in basement membranes (BMs) and the extracellular matrix (ECM), where HSPGs function as a barrier to cell migration. Secreted Hpse is harnessed by leukocytes to facilitate their migration from the blood to sites of inflammation. In the non-obese diabetic (NOD) model of autoimmune Type 1 diabetes (T1D), Hpse is also used by insulitis leukocytes to solubilize the islet BM to enable intra-islet entry of leukocytes and to degrade intracellular HS, an essential component for the survival of insulin-producing islet beta cells. Treatment of pre-diabetic adult NOD mice with the Hpse inhibitor PI-88 significantly reduced the incidence of T1D by ~50% and preserved islet HS. Hpse therefore acts as a novel immune effector mechanism in T1D. Our studies have identified T1D as a Hpse-dependent disease and Hpse inhibitors as novel therapeutics for preventing T1D progression and possibly the development of T1D vascular complications.

Keywords: heparanase, heparan sulfate, islet, diabetes, inflammation, vascular complications

INTRODUCTION

Heparanase (Hpse) is an endo-β-D-glucuronidase that degrades the glycosaminoglycan heparan sulfate (HS). Cloning studies have identified that catalytically active Hpse is encoded by a single mammalian gene (1–3). Hpse is initially produced as an inactive pre-proenzyme which undergoes post-translational processing to yield a 65 kDa proenzyme for secretion. Proteolytic cleavage of proheparanase by the cysteine protease cathepsin L leads to the formation of catalytically active Hpse, a heterodimer consisting of a 50 kDa (human) or 48 kDa (mouse) polypeptide non-covalently bound to a 8 kDa peptide (1, 2, 4–8). HS is a linear polysaccharide that consists of a repeating disaccharide composed of *N*-acetylated glucosamine (GlcNAc) and uronic acid [glucuronic acid (GlcA) or iduronic acid (IdoA)]. HS biosynthesis occurs in the Golgi compartment of cells, with the assembly of component sugar residues occurring directly onto the core proteins of heparan sulfate proteoglycans (HSPGs) (9–11). During the polymerization of HS chains, selected sugar residues are chemically modified by a suite of enzymes (*N*-deacetylase-*N*-sulfotranferase, C5 epimerase, and 2, 3, and 6-*O*-sulfotransferases), resulting in HS chains with regions that are highly sulfated and other regions of lower or no sulfation (10, 11). The sulfated regions of HS, in particular, bind to a vast array of bioactive ligands that include cytokines, chemokines, growth factors, adhesion molecules, lipases, and proteases (12, 13).

Typically, HSPGs are localized at the cell surface (e.g., syndecans 1–4, glypicans 1–6), in the extracellular matrix (ECM), in basement membranes (BMs) (e.g., perlecan, collagen type XVIII, and agrin) and have been identified in the nucleus of certain cells (14, 15). Secreted proheparanase rapidly interacts with cell surface HSPGs and the proheparanase-HSPG complex subsequently undergoes endocytosis. Similarly, Hpse can be internalized after binding to cell surface lipoprotein receptor-related proteins (LPRs) and mannose-6-phosphate receptors (MPRs) (16). Internalized proheparanase is cleaved by intracellular cathepsin L at acidic pH in late endosomes or lysosomes, to form catalytically active Hpse which can either degrade co-endocytosed HS, thereby regulating the turnover of cell-associated HS, or undergo storage within the lysosomes for subsequent secretion (6, 17–21). Optimal Hpse-mediated cleavage of glycosidic bonds in HS occurs at pH 5.5–6.0 and typically at sites adjacent to *N*- or 6-*O*-sulfated glucosamine (16, 22), e.g., the linkage of glucuronic acid to 6-*O*-sulfated glucosamine (23). HS in BMs and ECM is degraded by Hpse secreted by platelets, endothelial cells, leukocytes, and metastasizing tumor cells (12). In these settings, Hpse activity can result from (i) activation of proheparanase bound to cell surface HSPG or to cation-independent MPRs (CIMPRs) by an extracellular source of cathepsin L, e.g., produced by macrophages (24, 25); (ii) cytokine-, fatty acid-, or nucleotide-stimulated release of an intracellular pool of catalytically active Hpse (26–29) which may be subsequently captured by cell surface receptors such as CIMPRs (25); or (iii) platelet degranulation (30). This regulated release of Hpse in the local microenvironment limits the availability of Hpse activity, preserving the essential and diverse biological functions of HS.

Heparanase also exhibits non-enzymatic functions which impact on cell signaling, adhesion, and migration, as well as on gene expression. Such functions are generally expressed at neutral pH (31–33). Interaction of Hpse with cell surface receptors on

endothelial cells activates intracellular Akt, PI3K, and p38 kinase signaling to stimulate cell migration and Src kinase-mediated upregulation of vascular endothelial growth factor (VEGF) for angiogenesis (6, 18, 34). Hpse lacking catalytic enzyme activity has been shown to increase the expression of certain growth factors (35) and to facilitate cell binding to HS in the ECM and to endothelial cells *in vitro* (32).

Intra-nuclear Hpse modulates intra-nuclear HS/HSPGs and exerts direct effects on gene transcription. Transfer of Hpse to the nucleus occurs via Hsp90 in endothelial cells following fatty acid stimulation (29). Intra-nuclear Hpse decreases the level of the HSPG syndecan-1 in the nucleus of myeloma cells (14) and cleaves nuclear HS which in turn inhibits histone acetyltransferases (36). Recently, active Hpse has been reported to directly mediate epigenetic effects by regulating histone methylation, a process that directly influences the transcription of certain immune response genes involved in T-cell migration and function, e.g., IL-2 and IFN-γ (37). Hpse was also found to bind to the promoters of micro-RNAs involved in T-cell differentiation (37) and to influence the transcription of genes encoding enzymes involved in glucose metabolism (29). Such nuclear roles for Hpse, either with or without HS-degrading activity, would be expected to impact on T cells in inflammatory responses.

HEPARANASE AND INFLAMMATION

Heparan sulfate has several important biological functions which are regulated by Hpse in inflammation. An inflammatory response is generated when leukocytes are rapidly recruited from the blood to sites of tissue injury. In the early stages of inflammation, cell surface HS on cytokine-activated or inflamed endothelial cells functions in presenting lymphocyte-attractant chemokines to leukocytes in the vascular lumen (12, 38). The subsequent immobilization of the leukocytes (e.g., T cells) at the endothelial cell surface is enhanced by the binding of chemokine-activated integrins on the leukocytes to adhesion molecules such as ICAM-1 or VCAM-1 expressed on endothelial cells. Such interactions could potentially be facilitated by the binding of T cell-bound inactive Hpse to HS expressed on the surface of endothelial cells (12, 32, 33). The chemokine-binding role for endothelial cell surface HS may also function in establishing a chemokine gradient to *direct* leukocyte migration across the endothelium (12). Having crossed the blood vessel wall, most probably by passing between endothelial cells, inflammatory leukocytes employ degradative mechanisms to traverse the sub-endothelial BM. In fact BM HS, particularly associated with the HSPG perlecan, helps the BM to act as a barrier to leukocyte migration. This barrier property is attributed to the length of HS chains (up to 400 sugar residues) and to their intrinsic capacity to interact with other BM matrix proteins, forming a cell-impenetrable scaffold (12). To overcome this obstacle, leukocytes including T cells (39, 40), nearby endothelial cells (26) and possibly platelets (40) produce Hpse to degrade BM HS and proteases to destroy BM matrix proteins. The disassembly of the BM matrix components aids the passage of leukocytes across the BM and their entry into the surrounding tissue. Similarly, Hpse is released by inflammatory leukocytes to solubilize HS in the ECM of underlying tissues and to aid their navigation to sites of inflammation (12). During the course of the degradation of extracellular HS, HS-bound cytokines and chemokines can be liberated into the local microenvironment, potentially augmenting cell recruitment and exacerbating the inflammatory response (12).

The role for Hpse as a "path-maker" required by migrating leukocytes is of particular significance for T cell-mediated autoimmune diseases. Indeed, Hpse activity represents a prime target for anti-inflammatory drug development. Experimental autoimmune encephalitis (EAE; an experimental model of multiple sclerosis) in rats was largely prevented by *in vivo* treatment with sulfated polysaccharides. This effect was attributed to the inhibition of Hpse produced by activated T cells, which in turn blocked the solubilization of the sub-endothelial BM (41, 42). In a delayed-type hypersensitivity (DTH) experimental model of inflammation, inhibition of endothelial cell-derived Hpse prevented the degradation of sub-endothelial BM HS and leukocyte migration (27). Hpse, possibly produced by inflammatory cells in rheumatoid arthritis in humans, may release cytokines and/or chemokines from degraded HS in the ECM of rheumatoid joints, promoting joint destruction (43). In ulcerative colitis and Crohn's disease, which represent chronic inflammatory disorders, Hpse is preferentially produced by inflamed gut epithelial cells to drive a local circuit of inflammation (24, 44). A role for Hpse has therefore been established in a broad range of inflammatory conditions.

HEPARANASE AND THE PATHOGENESIS OF TYPE 1 DIABETES

Type 1 diabetes is an autoimmune disease which has been extensively studied in non-obese diabetic (NOD) mice, a recognized preclinical model of T1D in humans. During T1D, the insulin-producing beta cells in the islets of Langerhans in the pancreas are selectively destroyed by a T cell-mediated autoimmune response (45). The priming of autoreactive T cells to their cognate beta cell-specific autoantigens most probably occurs in the draining pancreatic lymph nodes, possibly as a consequence of both the abnormal responsiveness of effector T cells and inadequate control by regulatory T cells (46). Histological studies of NOD female mice at an early age (~6–7 weeks) revealed that leukocytes initially accumulate around the periphery of the islets, forming foci of non-destructive inflammation (insulitis). In adult pre-diabetic mice, the insulitis advances to a destructive phenotype, with peri-islet inflammatory leukocytes invading the islet cell mass (45). However, this pathology does not occur as a synchronized process throughout the pancreas, and the proportion of affected islets as well as the severity of leukocyte invasion progressively increases with time. Clinical symptoms of T1D are observed in 60–80% of female NOD mice from ~100 days of age or older, and are characterized by blood glucose levels exceeding >20 mmol/L (hyperglycemia).

In view of the established role for Hpse in leukocyte migration in other experimental models of inflammation (see above), we predicted that during T1D development, Hpse produced by islet beta cell-specific autoreactive T cells, inflammatory leukocytes, and possibly endothelial cells in the pancreatic vasculature, would be required to degrade HS in the sub-vascular endothelial BM. Thereafter, Hpse-mediated degradation of HS in the underlying pancreatic ECM would allow the inflammatory cells to migrate to individual islets and destroy the islet beta cells. Since T1D

development is a chronic disease process, we furthermore speculated that there would be an on-going need for this degradative activity. Our studies identified, however, that the requirement for Hpse extended far beyond the enzymatic activity necessary for leukocyte migration and the establishment of chronic inflammation. Indeed we discovered a critical role for Hpse at the level of the islets themselves. This local involvement of Hpse stemmed from the exceptionally high levels of HS normally associated with the islets *in situ* (8). Initially we confirmed the presence of a BM at the islet periphery (i.e., peri-islet BM) and revealed the HSPG perlecan as a previously unrecognized constituent (47). This HS +ve islet BM was predicted to act as a barrier to invading cells, analogous to the sub-endothelial BM. On further investigation of the distribution of HS in normal mouse islets *in situ*, we found that HS was expressed not only in the islet BM but at extraordinarily high levels throughout the islet cell mass (8). Immunohistochemical studies demonstrated that insulitis mononuclear cells (MNCs) in NOD mice strongly expressed cell surface Hpse (**Figure 1**; **Figure 2**, Stage 1). Furthermore, Western blotting analyses showed that the insulitis leukocytes expressed high levels of catalytically active Hpse at the time of diabetes onset in NOD mice, in contrast to the expression of inactive Hpse by peri-islet leukocytes in young pre-diabetic mice (8). Intra-islet infiltration by insulitis MNCs correlated with disruption of the islet BM (**Figure 2**, Stage 2), loss of the islet BM matrix proteins including the HSPG perlecan (47), progressive loss of intra-islet HS (**Figure 2**, Stage 3) and beta cell death (**Figure 2**, Stage 4) (8). Our studies have strongly indicated that such processes are mediated by catalytically active Hpse (**Figure 2**, Stages 2–4). This newly unveiled role for Hpse has more recently been validated using a transgenic mouse model of acute T1D and adoptively transferred Hpse-knockout effector T cells (unpublished data).

In vitro studies of beta cells isolated from normal mouse islets revealed both the unique intracellular localization of HS and its function in maintaining the viability of beta cells (8). Loss of intracellular HS correlated with beta cell death and conversely, the restoration of intracellular HS after culture of the beta cells with HS mimetics, correlated with beta cell survival. HS replacement not only preserved beta cell viability but rendered the beta cells resistant to oxidative damage induced by treatment with hydrogen peroxide [a source of reactive oxygen species (ROS)] (8). Collectively these findings led us to speculate that the intrinsic role of intracellular beta cell HS *in situ* in the pancreas is to protect the beta cells from the physiological levels of ROS generated as a consequence of their high metabolic and biosynthetic activity (8). Furthermore, we reasoned that such a mechanism could compensate for the low levels of free radical scavenger enzymes in beta cells (48).

Together, our *in vivo* and *in vitro* studies identified multiple roles for Hpse in T1D, namely promoting the migration of leukocytes from pancreatic blood vessels (i.e., across the sub-endothelial BM and through the pancreatic ECM), aiding the passage of leukocytes across the islet BM and depleting islet beta cells of the intracellular HS needed for their survival (**Figure 2**). In support of this new paradigm, *in vivo* treatment of pre-diabetic adult NOD female mice with the Hpse inhibitor/HS mimetic, PI-88, for 180 days significantly delayed T1D onset and reduced the incidence of diabetes by ~50% (8). Compared to saline-treated control NOD mice, PI-88 treatment significantly increased the proportion of pancreatic islets that were intact, significantly reduced the proportion of islets that showed destructive insulitis and better preserved the HS content of the islets (8). This hallmark study has therefore unveiled T1D disease to be largely Hpse-dependent. The extraordinarily high HS content of the beta cells, which is essential for their survival, renders them particularly vulnerable to Hpse-mediated damage. The localization of HS in the islet BM, which by convention acts as a barrier to impede leukocyte infiltration, has also been confirmed in normal human islets (49). Our studies suggest that intracellular HS maintains beta cell survival at least in part, by acting as a "free radical sink," protecting the beta cells against harmful chemical species generated endogenously. Our findings, which we have subsequently validated in *in vitro* studies of human islets and beta cells (unpublished data), therefore highlight Hpse inhibitors as a new class of therapeutic that can potentially be used to prevent T1D progression in humans.

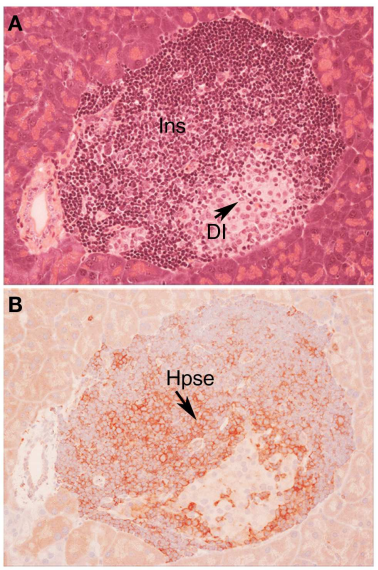

FIGURE 1 | Autoimmune T1D in NOD/Lt mice is characterized by cell surface expression of heparanase on insulitis leukocytes. Histology **(A)** and immunohistochemistry **(B)** of a pancreatic islet in a NOD/Lt female mouse at the time of T1D onset shows islet infiltration by destructive heparanase (Hpse)-expressing insulitis leukocytes **(B)** localized particularly at the insulitis-islet interface, a process which leads to loss of beta cell HS and beta cell death. **(A)** hematoxylin and eosin; **(B)** HP130 anti-heparanase mAb. Ins, insulitis mononuclear cells; DI, damaged islet tissue; Hpse, heparanase.

HEPARANASE AND DIABETIC COMPLICATIONS

The current treatment for T1D is exogenous insulin therapy. While insulin therapy keeps diabetic individuals reasonably healthy, precise control of blood glucose levels invariably fails to be achieved. As a consequence, macrovascular and microvascular diseases develop, resulting in heart disease, nephropathy, retinopathy, and neuropathy. There is compelling evidence that diabetic vascular complications are associated with the accumulation of advanced

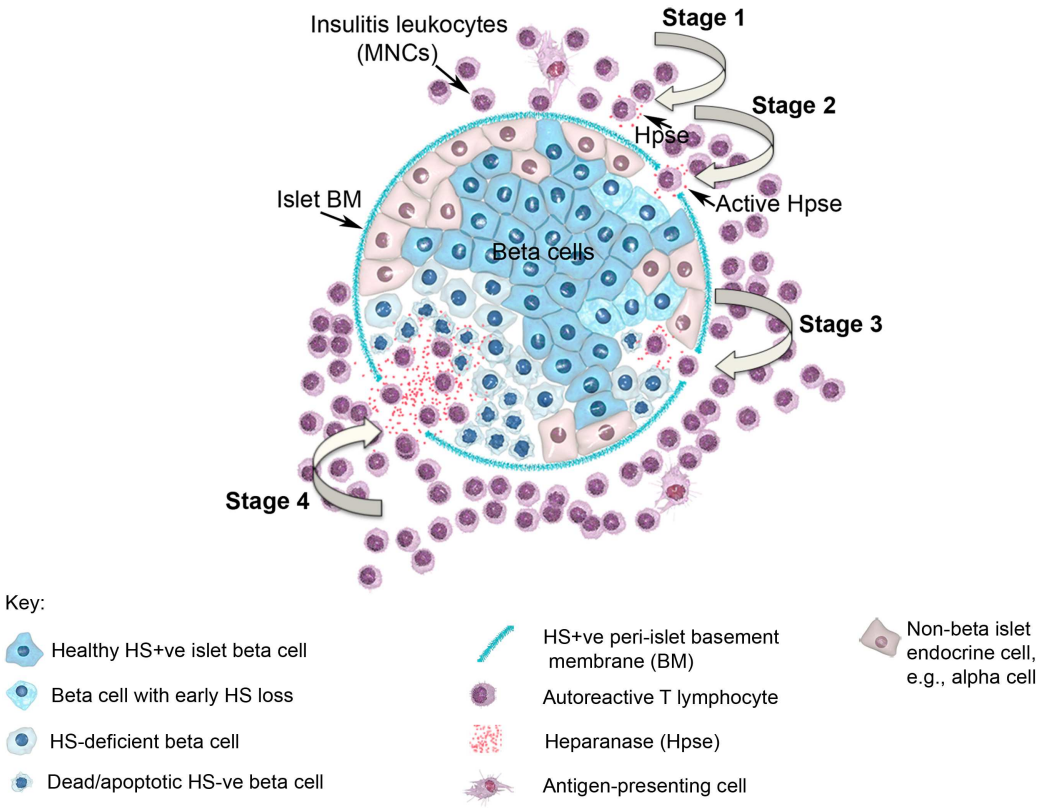

FIGURE 2 | Diagram showing a pancreatic islet and four stages of T1D disease driven by heparanase and loss of intra-islet HS. HS (intense blue color) is shown in normal beta cells and in intact peri-islet BM. In Stage 1 of the disease process, non-destructive insulitis mononuclear cells produce heparanase (red dots). Onset of destructive insulitis occurs when heparanase becomes catalytically active and degrades HS in the islet BM (Stage 2). Damage to the islet BM barrier allows activated autoreactive T cells to enter the islet cell mass where the local production of heparanase leads to degradation of intracellular HS in islet beta cells (paling blue color; Stage 3). Progression of HS depletion throughout the islet beta cell population results in increased beta cell death (palest blue color in "shriveled" beta cells; Stage 4), loss of insulin production and ultimately the development of T1D. MNC, mononuclear cell; BM, basement membrane; Hpse, heparanase.

glycation end products (AGEs) (50). Recent studies have also suggested an important role for Hpse in the development of diabetic nephropathy, a complication pertinent to both Type 1 and Type 2 diabetes. Underpinning this role, involvement of Hpse in proteinuric renal disease has also been established in experimental models of Adriamycin-induced nephropathy and passive Heymann nephritis (22, 51, 52). Diabetic nephropathy is characterized by an increase in the permeability of the glomerular BM (GBM), leading to proteinuria, as well as by tubular and interstitial fibrosis (22, 53). Hyperglycemia has been reported to regulate and, in fact, increase Hpse expression in renal epithelial cells *in vitro* (54). This finding is consistent with the increased expression of Hpse in renal glomeruli (e.g., glomerular podocytes) in human diabetic nephropathy, with significant increased levels of Hpse in the urine of diabetic patients and with a selective decrease in the expression of GBM HS (55–58). Nephropathy resulting from long-term streptozotocin-induced T1D was demonstrated in wildtype mice but not in Hpse-knockout mice (59), supporting a role for Hpse in this condition. The mechanism by which Hpse potentiates diabetic proteinuria may involve altered interactions between glomerular cells and HS-depleted GBM, the release of bioactive molecules from degraded HS, or intracellular signaling in the glomerular cells (22). Hpse is also strongly expressed in renal tubules in diabetic nephropathy and may contribute to tubular fibrosis via effects on FGF-2-signaling (53, 54, 58). An essential role for Hpse in modulating renal tubular morphology was confirmed in diabetic Hpse-knockout mice, which unlike diabetic wildtype controls, were free of histological evidence of tubular fibrosis (59). *In vitro* studies have implicated albumin overload and AGEs, rather than high glucose, in stimulating Hpse expression via the PI3K/Akt pathway in tubular endothelial cells, and in the subsequent loss of cell surface HS (60). Inhibition of both Hpse activity and the expression of tubular fibrosis markers *in vitro* by sulodexide furthermore highlights Hpse as a potential therapeutic target for preventing diabetic renal complications (53, 56).

Heparanase may also play a role in diabetic retinopathy. Increased Hpse expression has been demonstrated in high glucose-treated human retinal endothelial cells and in the retinal vascular endothelium of streptozotocin-induced diabetic rats. *In vitro*, increased levels of active Hpse correlated with enhanced levels of VEGF, a critical angiogenic growth factor required for neovascularization. Upregulation of VEGF in retinal endothelial cells

in vitro and in the retina of diabetic rats was inhibited by PI-88, supporting a regulatory role for Hpse, possibly by Src activation (61). At the level of leukocyte adhesion to rat retinal endothelium, an early marker of diabetic retinopathy, Hpse inhibition correlated with the decreased expression of the adhesion molecule ICAM-1 as well as VEGF, properties that implicate Hpse in both the arrest of leukocytes in the retinal vasculature and the associated local vascular dysfunction (62). Parallel studies have further demonstrated a more general role for Hpse in high glucose-induced vascular injury and have provided suggestive evidence for a role for Hpse in the pathogenesis of diabetic atherosclerosis (63–65).

CONCLUDING REMARKS

The surprising contribution of Hpse to the pathogenesis of T1D in NOD mice, together with the reported involvement of Hpse in the development of vascular complications of diabetes, highlight the potential application of safe and effective Hpse inhibitors for T1D rescue and treatment. Our studies strongly suggest that therapeutic intervention with dual activity Hpse inhibitors/HS mimetics at early stages of the disease could not only prevent the progression of T1D but potentially also fortify the HS content of any remaining beta cells. By preserving the viability and function of residual insulin-producing beta cells, physiological control of glycemia could be maintained without the need for exogenous insulin therapy. Additional benefits of this therapeutic approach would likely extend to preventing the secondary vascular complications of diabetes. Moreover, the studies reviewed here also support the potential treatment of established T1D with Hpse inhibitors to arrest the progression of diabetic vascular diseases, including nephropathy and retinopathy.

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Hyaluronan, a crucial regulator of inflammation

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Hyaluronan (HA), a major component of the extracellular matrix (ECM), plays a key role in regulating inflammation. Inflammation is associated with accumulation and turnover of HA polymers by multiple cell types. Increasingly through the years, HA has become recognized as an active participant in inflammatory, angiogenic, fibrotic, and cancer promoting processes. HA and its binding proteins regulate the expression of inflammatory genes, the recruitment of inflammatory cells, the release of inflammatory cytokines, and can attenuate the course of inflammation, providing protection against tissue damage. A growing body of evidence suggests the cell responses are HA molecular weight dependent. HA fragments generated by multiple mechanisms throughout the course of inflammatory pathologies, elicit cellular responses distinct from intact HA. This review focuses on the role of HA in the promotion and resolution of inflammation.

Keywords: hyaluronan, inflammation, glycobiology, Toll-like receptors, CD44, versican, TSG-6, inter-alpha trypsin inhibitor

INTRODUCTION

The association of increased HA deposition into the extracellular matrix (ECM) after tissue injury and during inflammatory disease has been recognized for over 25 years. Increased accumulation of HA has been demonstrated: in joint tissue of rheumatoid arthritis (RA) patients (1); in lung disease, both in humans (2) and animal experimental models (3–7); in inflammatory liver disease; during vascular disease (8, 9); in rejected kidney transplants (10) as well renal tissue of patients experiencing diabetic nephropathy (11); in the intestine of patients undergoing flares of inflammatory bowel disease (IBDs) (12), and mice with experimental colitis (13). Initially, HA was considered merely an inert space filling substance that had the capacity to surround itself with water molecules thus maintaining structure and preventing dehydration. The accompanying edema that occurs with HA deposition frequently impairs organ function.

Beyond the physical properties of a structural ECM molecule, however, it has become increasingly clear that HA provides cellular cues to regulate inflammation and tissue repair. The mystery is how and why does such a ubiquitous molecule produced in abundance by all vertebrates in every body tissue, foster inflammation under particular circumstances. This review focuses on discussing what is known regarding the mechanisms through which HA recruits and activates leukocytes in pathological inflammatory settings. Discussion will center on: (1) how HA is organized into a leukocyte recruiting matrix; (2) how it is degraded into fragments that are capable of signaling inflammatory responses via specific receptors; and (3) what are the known downstream effects and consequences of such activation. Over the past 15 years specific pathways signaled by HA have been defined and these have shaped the view of HA as a regulator of innate and acquired immunity, providing us with the concept that damaged ECM provides cues to surrounding cells to drive a protective response. In a dysregulated state, for example, the excess production of a pathological matrix, or the overproduction of HA fragments, HA is likely to contribute to chronic inflammatory conditions such as in RA, inflammatory bowels disease, atherosclerosis, and diabetes.

HYALURONAN IS A SIZE-DEPENDENT MEDIATOR OF INFLAMMATION

Data during the past two decades have established the fact that many of the cellular and biochemical processes mediated by HA are dependent upon the distribution of its molecular mass. HA polymers are comprised of repeating disaccharide units of glucuronic acid and *N*-acetyl glucosamine joined by alternating (β-1,3 and β-1,4) glycocidic linkages free of a protein core. An integral component of the ECM, HA polymers are traditionally considered as solvating, structural macromolecules largely responsible for supporting tissue integrity due to substantial viscoelastic properties (14). In normal tissues, HA exists as high-molecular-weight HA (HMW-HA) often with an average molecular weight of ~10^7 Da that is capable of occupying a 1000-fold volume of water (15). HMW-HA is one of the principle components of the glycocalyx and is able to spatially exclude other molecules and cells, functioning as an anti-angiogenic factor. Several studies have shown that HMW-HA inhibits angiogenesis through reducing both the proliferation and migration of endothelial cells (7, 16, 17). At the extracellular surface HMW-HA interacts with surface receptors, prevents immune cell recognition, and blocks phagocytosis by macrophages (18, 19).

In cases of inflammation and tissue injury, HA is significantly more polydisperse and contains a variety of HA polymers with overlapping lengths and functions. Generally speaking, HA with an average molecular mass <500 kDa can be considered a fragment, although the molecular properties of such a size are quite different than those of a 50 kDa fragment. In most studies, the indicated sizes of HA represent the average molecular weight of a polydisperse distribution, and the preparations of HA used are not homogeneous with respect to size. As such, care must be taken when considering the effects of different polymer sizes.

Large HA polymers function as tissue integrity signals and serve to suppress the inflammatory response. As HMW-HA becomes depolymerized in inflammatory conditions such as RA, it loses its lubricant properties as noted in the synovial fluid of RA patients (1, 20). While it is unclear whether the generation of HA fragments is the result of HA catabolic enzymes, reactive oxygen species (ROS), a truncated product of the HA biosynthetic enzymes, or a combination of these mechanisms, it is clear that HA polymers of specific sizes contain distinct biological activities. HA exists as both a pro- and anti-inflammatory molecule *in vivo*, and these contradictory functions depend upon polymer length and which receptors the oligosaccharides engage. HMW-HA elicits protective anti-inflammatory effects that protect lung epithelial cells from apoptosis and is protective against liver injury, acting to reduce pro-inflammatory cytokines in a T-cell mediated injury model (3, 21). In regulatory T-cells, HMW-HA stimulates STAT5 signaling through CD44 crosslinking, promoting their maintenance and thereby inhibiting their proliferation. Conventional T-cell precursors stimulated with HMW-HA produce IL-10, and infusion of these cells attenuates the disease course in a murine model of colitis (22, 23).

The presence of cable-like structures comprised of HA, associated with binding proteins, has been noted in several inflammatory conditions including IBDs, atherosclerosis, diabetic nephropathy, and chronic kidney disease (24–29). HA polymers of indeterminate sizes originate from the surface of multiple cells and coalesce into large HA cables capable of spanning multiple cells and reaching several millimeters in length (30). The presence of HA cables at sites of tissue inflammation can still function as an anti-inflammatory polymer. Monocytes adhere tightly to the HA cables regardless of their activation state, and following adhesion the distribution of CD44 on the monocyte cell surface polarizes to form a "cap" while a portion of the HA cable is internalized (**Figure 1**). HA cables can function as a biological sink for monocytes and platelets, and these interactions likely modulate pro-inflammatory stimuli [see review in Ref. (31)]. The presence of HA cables is not unique to the inflammatory process, and cables are formed in response to ER stress, cycloheximide treatment, and viral infection (24, 32, 33).

HA catabolic hyaluronidase enzymes on the cell surface of platelets are capable of depolymerizing HA at neutral pH, and the subsequent fragments induce monocyte activation (34). HA fragments have also been shown to have a number of pro-inflammatory effects such as activation of macrophages and dendritic cells as well as stimulating transcription of inflammation-related genes including TNF-α, IL-12, IL-1β, and matrix metalloproteinases (35–37). However, it is important to note that some of the pro-inflammatory effects attributed to HA fragments likely result from contaminants in the HA preparations used. Caution should be taken with respect to the source and purity of HA for such studies. Polydisperse HA fragments with an average molecular weight of 200-kDa have been shown to stimulate chemokines, cytokines, growth factors, proteases, and nitric oxide by macrophages (38–44). In some cases, the signaling pathways engaged by HA fragments have been partially defined. Murine macrophages stimulated by HA fragments can activate the inhibitor of nuclear factor-κB pathway, which increases the expression of interleukin-1β and TNF-α (43). In smooth muscle cells, HA fragments promote cell-cycle progression by CD44-dependent activation of the Rac/ERK pathway, while the HMW-HA is inhibitory (45).

Although most of the work on low-molecular-weight HA fragments initially illustrated a pro-inflammatory response, a number of studies have shown that HA fragments can also be protective. In a DSS-induced murine model of colitis, intraperitoneal injection of polydisperse HA <750-kDa protects colonic epithelium in a Toll-like receptor (TLR) 4-dependent manner

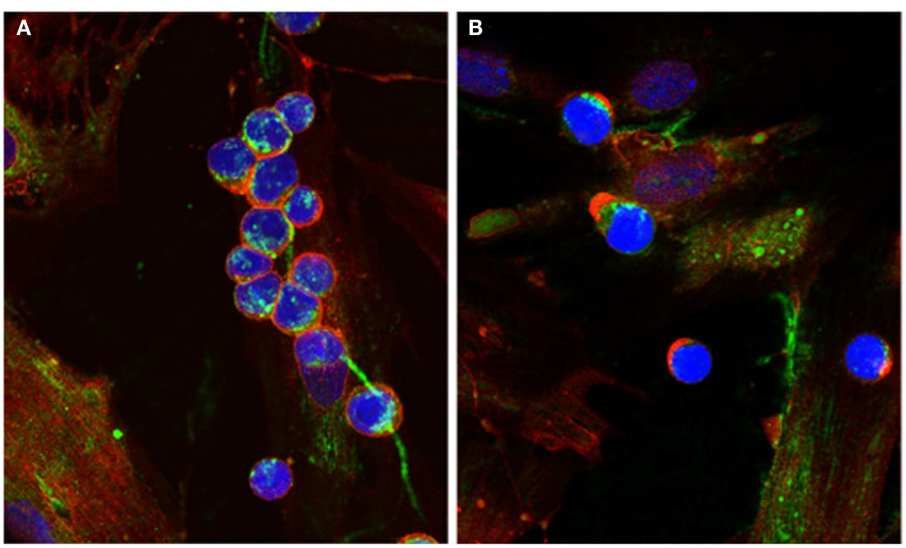

FIGURE 1 | Capping of CD44 on leukocytes. **(A)** Monocytes bind to HA cables (green) produced by M-SMC in response to poly I:C. CD44 (red) is dispersed on the monocyte surface. **(B)** After 15 min incubation at 37°C, CD44 (red) is capped to one pole on the leukocytes, HA cables appear ragged (center panel) and HA is internalized. (Nuclei are blue).

(46). Intermediate-molecular-weight HA <200 kDa stimulates the expression of human β-defensin 2 (HBD2) in human keratinocytes, through TLRs 2 and 4 (47). More recently, our laboratory has shown that HA-dependent induction of HBD2 in intestinal epithelial cells and in the colonic mucosa of mice is highly size specific. The strongest induction was observed with HA of 35-kDa average molecular weight, and was TLR 4 dependent (48).

HYALURONAN-BINDING PROTEINS AND THEIR ROLES IN INFLAMMATION

While many of the HA-binding proteins have been previously reviewed elsewhere (49), several HA-binding proteins are of particular importance with regard to inflammatory processes and will be discussed in the following section. The repeating nature of HA oligosaccharides and the specificity of protein–HA interactions likely dictate many of the mechanisms by which HA functions as either a pro- or anti-inflammatory molecule. The ECM, comprised predominantly of proteoglycans, glycoproteins, and collagens, is increasingly being viewed as having both structural and regulatory roles. Collectively, the ability of HA to function as either a pro- or anti-inflammatory molecule is dependent upon its size, microenvironment, localization, and availability of specific binding partners.

Inter-alpha-trypsin inhibitor (IαI) is one member in a family of serum proteoglycans secreted by the liver that function as serum protease inhibitors. It is reported to circulate in serum at concentrations between 150 and 500 μg/mL, comprises ~5% of total protease inhibitory activity of plasma, and elevated levels reported in inflammatory pathologies (50, 51). This proteoglycan is unusual as a single chondroitin sulfate chain is covalently attached to bikunin, a serine protease inhibitor, and another family of proteins termed heavy chain (HC) (HC1, HC2, HC3) are covalently linked to the chondroitin sulfate chain through an ester bond between C-terminal aspartate and the 6-hydroxyl of an *N*-acetylgalactosamine (52–54). In several inflammatory diseases, such as Crohn's disease, asthma, osteo- and rheumatoid-arthritis (OA, RA), the increase in HA production is commensurate with increased levels of IαI heavy chains covalently attached to HA. In the case of Crohn's disease, heavy chains of IαI are specifically associated with HA cable structures and are highly adhesive for naïve unactivated leukocytes, possibly in competition with inflammatory receptors (12, 55). Further, soluble HA containing HC1 and pentraxin 3 has been shown to induce apoptosis of activated, but not resting, neutrophils and macrophages (56). IαI and heavy chains are present at high levels in the synovial fluid of RA and OA patients, and HC complexes linked to HA have been shown to accumulate within inflamed tissues and correlates with disease severity (57, 58). Recently, the activity of TSG-6 in OA synovial fluid has been demonstrated as a biomarker for OA progression and the need for knee replacement (59). The transfer of HCs to HMW-HA is a reversible and dynamic event influenced by the availability of substrates, and smaller HA fragments (oligosaccharides) can act as irreversible HC acceptors and can disrupt highly adhesive properties of HC–HA (60). Studies of knockout mice lacking bikunin, which are unable to form IαI, have revealed an inhibitory role for IαI heavy chains in complement activation. Lack of IαI in mouse serum led to high levels of complement activation and was reversible upon injection of soluble IαI containing HCs (61). In humans, IαI does not appear to be a potent inhibitor of complement activation, affecting only early stages (62). In chronic inflammatory conditions such as RA, where IαI heavy chains and HA fragments accumulate to high levels and their effects may be more significant.

Tumor necrosis factor-stimulated gene 6 (TSG-6), which is capable of binding to both IαI and HA, catalyzes the transfer of HCs from the CS chain of IαI to HA by a *trans*-esterification reaction to form HC–HA, and has also been well studied in the context of ovulation (63, 64). The expression of TSG-6 is influenced by a number of factors including TNF-α, IL-1β, TGF-β, and LPS, in a highly cell type-dependent manner, and in many cases is coordinated with the synthesis of HA (65). While induced by inflammatory stimuli, TSG-6 has several protective features including enhancing the anti-plasmin activity of IαI and inhibition of MMPs and aggrecanases in cartilage (66, 67). Direct interaction between TSG-6 and the glycosaminoglycan (GAG) binding site of IL-8 has been shown to inhibit neutrophil migration by blocking the interaction of IL-8 with heparan sulfate on endothelial cell surfaces (68). The addition of TSG-6 to HA has been shown to enhance CD44-dependent lymphoid cell adhesion to HA in flow shear experiments (69). Interestingly, the Link module of TSG-6 alone can inhibit neutrophil migration and adhesion via exhibiting anti-inflammatory effects independent of HA and IαI (70). Recent studies have illustrated that: (1) the Link module of TSG-6 has binding sites for both HA and bikunin C4S; (2) TSG-6 is capable of binding non-covalently to HC1, HC2, and HC3 at affinities greater than with HA; and (3) although TSG-6 alone is capable of cross-linking HA into a dense, leukocyte-adhesive matrix, both the degree of condensation and adhesion are reversible by addition of IαI containing HC1 and HC2 (71). In a murine model of asthma, TSG-6 null mice are resistant to airway hyperresponsiveness and exhibit reduced levels of HA deposition, absence of HC–HA complexes and decreased eosinophilic inflammation (72). Some of these findings may be explained by the observation that airway smooth muscle cells stimulated with poly (I:C) and TSG-6 produce increased levels of cell-associated HA cables than treatment with poly (I:C) alone (73).

The adhesive properties of CD44+ cells to HA matrices depend upon the composition of proteins bound to HA. HC–HA complexes isolated from the synovial fluid of RA patients are highly adhesive for CD44+ leukocytes. These HA complexes likely contain several HA-binding proteins including HC3 and pentraxin-3, but were found to be free of TSG-6 (74). However, it has been recently shown that while TSG-6 enhances the interaction of HA and CD44, the *in vitro* addition of IαI, TSG-6, and HCs (HC1 and HC2) appears to counteract the enhanced binding of TSG-6–HA complex alone (69, 71). This is in conflict with prior reports, but suggests that HA-binding proteins themselves may regulate their own interaction with HA (71, 74). As suggested by Day and de la Motte, it is likely that the specific composition or organization of HA–protein complexes dictates the outcome of many cell–HA interactions (31).

Versican is a large CS-rich proteoglycan expressed at high levels by proliferating cells and mesenchymal cells and is deposited into the ECM during tissue remodeling and development. Versican is a member of the lecticans, structurally similar proteins that also

include aggrecan, neurocan, and brevican. Versican consists of an amino-terminal globular domain (G1) and a carboxy-terminal globular domain (G3), separated by several CS attachment sites (GAG-α and GAG-β) between the two globular domains. The G1 domain consists of an immunoglobulin-like fold and a pair of link modules that bind five repeat disaccharides of HA with high affinity. This interaction is further stabilized by link protein itself, which binds both HA and versican (75, 76). Initial studies of versican showed anti-adhesive properties, which now appear to be mediated by the G1 domain (77–79). The G3 domain shares homology with the selectins, containing two epidermal growth factor (EGF) repeats, a C-type lectin domain, and a complement regulatory region. Four alternative splicing isoforms of versican (known as V0, V1, V2, and V3) result in truncation of the number of potential CS attachment sites (V1 and V2), with V3 lacking them. All isoforms retain the G1 and G3 domains and therefore the ability to bind to HA. The modular nature of versican can function as a highly diverse molecular constituent of the ECM capable of binding to a variety of factors involved in inflammatory processes.

Investigation into cancer growth and metastasis has implicated versican as having a central role driven by inflammatory stimuli. Versican, in either intact or fragmented forms containing the G3 domain, can enhance tumor cell migration, growth, and angiogenesis (80–82). In an *in vitro* screen for carcinoma-derived factors capable of activating macrophages, versican was identified as a potent enhancer of metastatic growth through TLR2 and co-receptors, TLR6 and CD14 (80). Versican has been suggested to contribute to HA fragment activation of macrophages, and enhanced cancer metastasis through induction of the hyaluronidases (83, 84). Several inflammation-associated cytokines, including transforming growth factor β1, 2, 3, and platelet-derived growth factor (PDGF), have been shown to increase biosynthetic levels of both versican and HA, while IL-1β, and IFN-γ have been shown to reduce levels of versican (85–91). Leukocyte trafficking and localization to regions of inflammation mediated by interaction with cell-adhesion receptors functions as a critical initiating step in the inflammatory cascade (92). Specific CS chains on versican preferentially bind to chemokines known to attract mononuclear leukocytes (93). Versican itself is capable of binding to a number of cell surface receptors present on leukocytes through interactions also mediated by CS chains, including both L- and P-selectins and CD44 (93–95). Direct binding of P-selectin glycoprotein ligand-1 (PSGL-1) by the G3 domain of versican has also been shown to cause aggregation of leukocytes (82).

Together, these HA-binding proteins contribute to the maintenance of tissue integrity and direct cell–ECM interactions in normal and pathological conditions. Many of the adhesive properties of HA polymers depend upon the presence of HA-binding proteins, and together with IαI, HCs, TSG-6, and versican contribute to a dynamic extracellular environment capable of directing cell adhesion and the production of inflammatory cytokines.

HYALURONAN CATABOLISM AND GENERATION OF HA FRAGMENTS

Enzymatic degradation of HA is initiated by hyaluronidases (hyaluronoglucosaminidases, or HYALs), a family of endoglycosidases that hydrolyze the β-1,4 linkages between *N*-acetyl-hexosamines and glucuronic acid found in GAGs including some activity toward chondroitin 4- and 6-sulfates, and unsulfated chondroitin (96, 97). In humans, genes for six hyaluronidase family members have been identified to date: HYAL1–4, PH-20, and HYALP1. With the exception of the pseudogene HYALP1, each member encodes for protein products, and all but Hyal-3 have been shown to participate in either HA or CS catabolism [for a review see Ref. (98)]. While Hyal-3 does not appear to have a direct role in HA catabolism, it may be involved in an indirect fashion (99, 100). In somatic tissues, Hyal-1 and Hyal-2 function as the major hyaluronidases for HA degradation. In a rat model, exogenous addition of HA indicated that some turnover takes place locally at the site of intravenous injection, but the majority of clearance occurred in the lymph nodes and liver (101). However, other studies have suggested that local turnover is the major route of HA clearance and clearance through the lymphatic system is relatively minor when compared to local turnover by skin or skeletal muscle (102). These discrepancies may exist due to the size of HA used to measure local or lymphatic clearance, as it is likely that low-molecular-weight HA within tissue can be removed from the ECM by lymphatic drainage more easily than HMW–HA. Within cells, HA turnover occurs within the lysosomal compartment by concerted action of the endo-acting hyaluronidases, the lysosomal exoglycosidases, β-glucuronidase, and β-hexosaminidase, in a pH-sensitive manner (103, 104).

Of the six family members, Hyal-1 has been best studied. It is widely expressed and is found in plasma and urine as well as in several tissues including liver, kidney, spleen, and heart and cleaves HA to tetra- and probably also hexasaccharides (105, 106). Hyal1 is a 57-kDa glycoprotein that also occurs as a proteolytically processed 45-kDa form with two chains bound by a disulfide bond. The biological significance of two forms of Hyal-1 is not known, as both are present in tissues and cells while only the 57-kDa form is present in serum (107). Defects in HYAL1 were identified as the genetic lesion in the lysosomal storage disorder mucopolysaccharidosis IX (108). Loss of Hyal-1 activity results in accumulation of HA in serum as well as within the lysosomes of skin fibroblasts and macrophages (109). Once HA is within the lysosome, the lysosomal exoglycosidases appear to share some functional redundancy with Hyal-1 for HA turnover (97). Recombinant Hyal-1 has been reported to have a strict pH optimum near 3.7, with activity decreasing by ~75% ± 0.5 pH (107). However, several proteomics studies of lysosomes have not found Hyal-1 (or Hyal-2) in their analysis. This discrepancy may be due to the method of protein detection as most of these studies rely upon the fact that the majority of lysosomal hydrolases are targeted to the lysosome by the mannose 6-phosphate (M6P) pathway [for more details, see review in Ref. (110)], and therefore have utilized immobilized M6P-receptors as a purification method (111–120). Consequently, with the exception of one study (120), proteins trafficked to the lysosome by M6P-independent mechanisms are missing from these data sets. Other *in vitro* and *in vivo* studies have shown that Hyal-1, either in culture medium or serum, is taken up by endocytosis and does reach the endosomal–lysosomal network. This compartment, however, does not contain the classical late lysosomal hydrolases β-galactosidase or *N*-acetylglucosaminidase, and likely represents a late endosome (121, 122). These data fit

well with the high levels of Hyal-1 observed in plasma and suggest that newly synthesized Hyal-1 reaches lysosomes through a secretion-recapture mechanism. The function of Hyal-1 within serum remains unclear.

Hyal-2 shares many of the features of Hyal-1: it is a 55-kDa glycoprotein widely distributed in a number of tissues, acid active, and found in two forms. Although it is frequently reported to be lysosomal, significant controversy exists around this point (123–125). Hyal-2 contains a glycosylphosphatidylinositol (GPI) linkage that tethers it to membrane surfaces where it can serve as the receptor for the jaagsiekte sheep retrovirus (84, 126, 127). GPI-anchored Hyal-2 has hyaluronidase activity when it is on the surface of human platelets (34). In certain cell types, a soluble, intracellular form lacking the GPI anchor has also been reported (128, 129). Initial studies of soluble Hyal-2 activity indicated that the enzyme is functional only in a narrow pH range around 3.7, similar to Hyal-1. Despite having a high degree of primary sequence homology to Hyal-1, Hyal-2 cleaves HMW–HA to generate 20-kDa fragments (approximately 50 disaccharides) *in vitro* (123, 125). A study of Hyal-2 expressed in HEK293 cells indicates strong membrane co-localization of Hyal-2 and CD44 with a pH optimum of 6.0 for membrane fractionated Hyal-2. Although some HA fragments generated by Hyal-2 were internalized, the majority was released into the medium (121). This is consistent with the finding that in breast cancer cells, Hyal-2 forms a complex with CD44 and the Na^+–H^+ exchanger 1 (NHE1), and HA degradation by Hyal-2 depends upon its interaction with CD44 at the membrane surface. However, controversy exists as to whether CD44 is present on the surface of platelets (130, 131). In culture, the interaction of CD44 and NHE1 leads to extrusion of H^+ ions and a decrease in extracellular pH to 6.6 (132). Possibly, the distribution of Hyal-2 as either membrane-associated or soluble is cell-type or activation-state specific, and insertion into a lipid membrane can affect both the localization and the activity range of Hyal-2.

Degradation of HA by Hyal-1 primarily takes place within cells, and depends upon the ability of CD44 or other HA receptors to internalize HA fragments. Although significant levels of Hyal-1 are present in serum, to date it has not been shown to be functionally active in circulation. Interestingly, MPS IX patients deficient in Hyal-1 have been reported with plasma HA levels at 40 times normal (109). Thus it is unclear whether circulating Hyal-1 could potentially contribute to HA fragmentation. At this point in time, Hyal-1 appears to primarily contribute to intracellular HA turnover.

The ability of the hyaluronidases to depolymerize HA seems to be highly dependent on compartment and contribution of HA-binding proteins. The discrepancies in pH optimum reported in the literature may suggest that in a low pH environment, certain sizes of HA are easily degraded by Hyal-1 and Hyal-2 without the assistance of HA-binding proteins. It has been suggested that HMW-HA exists as a supramolecular structure capable of transitioning between secondary and tertiary structures in *in vitro* solutions (133, 134). However, other studies indicate that these interactions may not occur. Data demonstrating a lack of intra-chain interactions open to competition, and an absence of amide-carboxylate hydrogen bonds required to form a twofold helical HA chain necessary for proposed models of secondary and tertiary HA structures, together suggest that intra-molecular structures are unlikely (135, 136). The structure of HA *in vivo* is likely to be dictated by the specificity, degree, and hierarchy of protein–HA interactions, and the ability of the hyaluronidases to degrade HA probably depends upon the conformation of HA chains. The finding of Hyal-2 in complex with CD44 at the plasma membrane is suggestive that HA-binding proteins can enhance the activity of HA degrading enzymes, and CD44 binding may provide Hyal-2 with a preferable conformation of HA. While Hyal-1 and Hyal-2 have been shown to prefer to cleave HA to tetrasaccharides and 50 disaccharides, respectively, these experiments have been performed *in vitro* for long periods of time and in most cases with exogenous HA free of HA-binding proteins. Given that neither enzyme has been shown to exhibit processivity, the sizes generated by Hyal-2 at the cell surface are more likely to be polydisperse and dependent upon the availability of HA-binding proteins.

While the hyaluronidases discussed above are at present the most plausible sources of HA fragments, they are not likely to be the only contributors. Recently, KIAA1199, a gene of unknown function involved in non-syndromic hearing loss and several forms of cancer has been identified as a HA-binding protein that contributes to HA degradation in cultured skin fibroblasts (137, 138). Interestingly, the authors show that siRNA-mediated knockdown of CD44 or Hyal-2 had no effect on HA degradation. Further, Hyal-1 did not appear to be expressed in Detroit 551 skin fibroblasts, and the majority of the HA depolymerization was lost upon KIAA1199 knockdown. However, it is important to note that although KIAA1199 appears to co-localize and co-precipitate with clathrin heavy chain, the authors were only able to detect depolymerized HA within media and were unable to detect fragments within the cell (137, 138). Future studies will determine whether KIAA1199 truly is a HA degrading enzyme.

Hyaluronan has long been suggested to protect articular tissues by absorbing ROS capable of degrading components of the ECM *in vitro* (139, 140). An additional mechanism suggested in the literature that could lead to HA fragmentation is the accumulation of ROS under conditions of injury or pathological inflammation. Accumulation of ROS in chronic inflammatory conditions has been noted, and direct depolymerization of HA by ROS has been illustrated largely *in vitro*, with some exceptions (141–146). In an epithelial airway culture system, xanthine/xanthine oxidase generation of ROS led to HA degradation and tissue kallikrein-mediated EGF receptor activation (147). However, ROS species themselves are capable of stimulating Hyal-2 gene expression via p38MAPK. In lentiviral-mediated Hyal-2 knockdown of cells exposed to ROS, HA depolymerization was not observed (148). Other species, including $O_2^{\bullet -}$ and $^{\bullet}NO$ are present locally in inflammatory tissues and may recombine to produce peroxynitrite, which specifically degrades the HA, but not heparin/heparan sulfate (149–151). The contribution of ROS species to HA fragment generation will ultimately depend upon the relative ratio of these species, HA, anti-oxidants, and other substrates within the ECM. HA fragments generated directly by ROS species are functionally equivalent to enzymatic products is currently not known.

HYALURONAN SIGNALING RECEPTORS

CD44 is a type I transmembrane glycoprotein and is widely regarded as the major cell-surface HA binding protein (152). Widely studied in several contexts, CD44 interactions with HA have important roles in tumor metastasis, lymphocyte adhesion, T cell signaling, angiogenesis, and inflammation (153–156). CD44 contains a short cytoplasmic tail with multiple phosphorylation sites, a transmembrane domain, an extensively glycosylated variable region, and an amino-terminal HA binding domain containing a Link module (157–159). CD44 exhibits significant protein diversity partially due to variable splicing of exons, each encoding for a segment of the extracellular domain, and over a dozen isoforms have been discovered. Of the variable forms of CD44, the most common is termed hematopoietic or standard CD44 (CD44s, CD44H). The diversity of CD44 is further elaborated by the extent to which it is glycosylated. It contains up to two GAG attachment sites (CS or HS), and extensive *N*- and *O*-linked glycosylation alone can account for the majority of the molecular weight of CD44s. Many of the *N*-linked sites are found within the HA binding domain, and glycosylation of CD44 negatively regulates its ability to bind HA in some cell types (160). CD44 has been shown to require activation for high affinity HA binding, and one mechanism involves the enzymatic removal of terminal sialic acid from two *N*-linked glycans in the HA binding domain (161–163). Crosslinking of HA by binding proteins, such as TSG-6, can significantly alter the way in which HA is structured within the ECM and can alter the affinity of CD44 for HA (69, 158, 164).

CD44 is expressed on many cell types that contribute to inflammation including leukocytes, neutrophils, macrophages, chondrocytes, fibroblasts, epithelial, and endothelial cells. While the GAGs of CD44 can bind to cytokines, growth factors, and ECM proteins such as fibronectin, the majority of the functions of CD44 depend upon its ability to bind to HA (165). Just as the size of HA seems to dictate whether it functions as a pro- or anti-inflammatory molecule, studies have suggested CD44 shares contrasting roles. The molecular mechanisms dictating CD44s function may be driven by its affinity for HA. Several stimuli have been shown to activate or dampen the affinity of CD44 for HA. While if the transition from low- to high-affinity binding state (or vice versa) involves a conformational change has not been directly shown, it has been established that HA oligomers cause a ligand-induced conformational change and HA may have two mutually exclusive modes of binding to CD44 (159). Dimerization of CD44 may lead to activation of HA binding, and binding at the cell surface is the result of multiple weak HA–CD44 interactions, which are influenced by the size of the HA ligand (166–168). Interestingly, stability of HA binding appears to be size dependent and it is possible that stability itself may participate in signaling, which could explain the relationship between molecular weight and signaling (169). T cells are stimulated to bind HA by the cytokines IL-2 and TNF, as well as by chemokines such as MIP-1B, IL-8, and RANTES (170, 171). Monocytes can be stimulated to bind HA by a number of cytokines such as TNF-α, IL-1α, IL-1β, IL-3, IFN-γ, and LPS (163, 171–173). By contrast, some molecules including IL-4 and IL-13 inhibit CD44–HA binding (172, 174, 175). Antibodies to CD44 that interfere in HA binding attenuate inflammation in animal models of IBDs. In particular, CD44v7 has been strongly implicated in murine models of colitis as a potent inflammatory mediator. Disrupting CD44v7 isoforms with blocking antibodies induces apoptosis of mononuclear cells in both murine models of IBD as well as in monocytes isolated from inflamed mucosa of IBD patients (176–178). Mice deficient in all isoforms of CD44 develop normally, suggesting that other receptors can compensate for its loss (179). While CD44 is regarded as the primary cell-surface receptor for HA in many cell types, investigation into the mechanisms underlying HA fragment signaling has demonstrated that HA fragments are capable of signaling independently of CD44 (3, 48, 180, 181).

Toll-like receptors function as surveillance receptors, interacting with a number of microbial-derived molecules and activating the innate immune system in response to pathogen-associated molecular patterns (PAMPs). Increasingly, the TLRs are also shown to sense damage-associated molecular patterns (DAMPs) in response to injury as well (182). The idea that endogenous matrix degradation products act as regulators of cellular processes is not a new one, but with respect to GAG fragments, the role of HA is the best studied (183). HA is a component of the cellular coat on some pathogens including both groups A and C *streptococcus*, some strains of *Escherichia coli*, and *Pasteurella multocida*, and many of these pathogens also express hyaluronidases (184–186). The presence of a HA coat likely assists in evasion by the immune system, while the hyaluronidase enzymes may aid in colonization of the host. HA fragments functioning as DAMPs interact with TLRs, and thereby compete with HMW–HA to initiate receptor signaling. Studies of lysosomal storage disorders, where GAG fragments accumulate within the cell, have suggested that incomplete GAG degradation products are associated with increased activation of TLRs (187–189).

Ten TLR genes have been identified in humans to date, and both TLR4 – the primary signaling receptor for lipopolysaccharides, and TLR2 – a recognition receptor for mycoplasma and gram positive bacteria, are involved in recognition of fragmented HA (3, 35, 43, 48, 83, 190). While an increasing number of studies have shown that TLRs are involved in HA signaling, the underlying mechanisms remain unclear. In dendritic cells, data suggest that TLR4 is required for recognizing HA fragments of 4-, 6-, and 8-sugars, and recognition is independent of CD44, TLR2, or receptor for HA-mediated mobility (RHAMM) (36, 44). However, studies by Noble and colleagues have shown that macrophages isolated from either TLR2 or TLR4 knock out mice are still capable of chemokine gene expression induced by HA fragments, while macrophages from TLR2/4 double knockouts are not. Using a non-infectious bleomycin-induced lung injury model, they also demonstrated that TLR2/4 double knockout mice are protected from acute inflammation due to an impaired ability of macrophages to respond to HA fragments, but at the expense of epithelial cell repair (3).

The data presented by Noble's group showed a requirement for MyD88, a downstream effector molecule shared by the TLRs, and for HA fragment signaling; HA-TLR signaling was abolished in MyD88-deficient macrophages (3). However, a recent study indicates that HA fragments of ~200 kDa are capable of inducing type I interferons by a TLR4 MyD88-independent pathway (191). These differences observed in signaling complex requirements

may depend upon the size of HA used (135- vs. 200-kDa) or the spectrum of receptors present at the cell surface during the experiments. The specific receptor complexes and signaling pathways engaged appear to be highly cell type dependent. Taylor et al. have shown that although CD44, in some contexts, does not appear to be required for TLR4-mediated HA signaling, it can function in an enhancing role. The adapter molecules CD14 and MD-2 are required for TLR4 recognition of LPS, but CD14 is dispensable for HA signaling in cultured cells (35).

Despite a lack of biophysical evidence that TLRs bind directly to HA or other GAG fragments, interplay between these molecules as mediators of the inflammatory process is clear. Although data support CD44-independent TLR signaling by HA fragments in some cell types, it is not clear if there is competition between these receptors for HA fragment ligands or if they work in a co-operative fashion. Future studies are needed to define the signaling pathways activated by different sizes of HA fragments and the receptors utilized.

EVIDENCE FOR *IN VIVO* IMPORTANCE OF HA FRAGMENTS

In vitro studies have defined multiple signaling properties of HA fragments that occur in a cell type-specific manner and are mediated by a complicated arrangement of receptors. It is clear, however, that HA fragments possess important biological roles in innate immunity. A number of animal models have aided our understanding of how HA modulates inflammation in injury and disease. In a non-infectious lung injury model, HA fragments accumulate in lung tissue and require $CD44^+$ macrophages for clearance before the tissue can be repaired (4). HA fragments are cleared as inflammation is resolved in wild-type mice, but fragments accumulate in CD44-deficient animals. The inability to remove HA fragments from the lung epithelium results in gross inflammation and impaired clearance of apoptotic neutrophils (4). At the cell surface, HMW–HA protects lung epithelium from injury through activation of NF-κB and suppression of apoptosis. Disruption of HA–TLR interactions results in loss of the protective effect of HMW–HA, and tissue injury is exacerbated (3).

HA-binding proteins have also been implicated in resolution of lung inflammation. IαI containing HCs expressed in the liver and normally secreted into serum, enter the lung through vascular leakage during inflammation. In bleomycin-challenged mice, IαI and HA normally appear to co-localize within inflamed lungs whereas in mice lacking IαI, increased levels of HA and cellular inflammation were noted. Together this suggests that HA–HC complexes may contribute to the resolution of lung injury (61, 192). Previous studies have shown HA–HC complexes are higher affinity for CD44 (74), and it is plausible that these HA-binding proteins work together to mediate tissue homeostasis within the lung. Some HA–HC preparations do not exhibit enhanced CD44 binding, and it is likely that the properties of HA–HC depend upon its exact composition of HA-binding proteins as well as the background of the cells involved (71). HMW–HA and CD44 interactions support cell survival, and in cases of injury, fragments accumulate and contribute to induction of inflammation. Clearance of HA fragments, the uptake of apoptotic neutrophils by macrophages, and restoration of HMW–HA pro-survival signals all depend upon CD44, and HA homeostasis is required to resolve lung inflammation and initiate tissue repair.

Though not discussed within the scope of this article, inflammatory mediators can promote angiogenesis, which facilitates chronic inflammation. New blood vessels can sustain a chronic inflammatory response by supplying a source of oxygen and nutrients to inflamed tissue and through transport of new inflammatory cells to the site of inflammation. HA polymers regulate the growth of new blood vessels through interactions with endothelial cells. HMW–HA is anti-angiogenic and inhibits endothelial cell proliferation, while fragmented HA is a pro-angiogenic stimulator. Endothelial cell interactions with HA are mediated in concert by CD44 and RHAMM (6, 7). Loss of CD44 disrupts angiogenesis *in vivo*, and this effect is due to the loss of CD44 expression in endothelial cells, as wild-type leukocytes or bone marrow-derived progenitor cells were unable to rescue the diminished angiogenic response. Interestingly, the endothelial cells deficient in CD44 are still capable of binding HA and exhibit normal migration, possibly due to compensation by RHAMM in these animals. In mice treated with an anti-CD44 antibody shown to block HA binding, neovascularization is drastically impaired, and new vessels cannot properly assemble endothelium-lined tubes (5). In tissues where new vessels are formed or become leaky due to injury, IαI from serum is readily available and can alter the adhesive properties of HA. In an *in vivo* matrigel angiogenesis model, mice lacking IαI exhibit drastically reduced vessel growth (192). However, it is worth noting that HA–HC complexes purified from the amniotic membrane appear to be anti-angiogenic, perhaps suggesting a different composition or organization (193). Endothelial cell interactions with a HA matrix depend upon the CD44–HA interactions that can be enhanced by IαI, and other HA-binding proteins, during vessel formation. Disruption of cell–matrix interactions leads to disrupted tube assembly and decreased stability of newly formed vessels.

Fragmentation of HA was recognized as one of the earliest biological markers for RA, and many animal models of RA are induced by ECM components such as type II collagen or aggrecan (14, 194, 195). Large amounts of HA containing HCs from IαI are found in the synovial fluid of RA patients, and HA–HC complexes contained within the synovium are highly adhesive for infiltrating leukocytes (74). Interestingly, mice incapable of forming HA–HC complexes, or treated with an anti-CD44 antibody, are more resistant to arthritis than wild-type littermates (196–198). By contrast, in a proteoglycan-induced arthritis model, mice deficient in CD44 only exhibit moderate to no resistance (199, 200). HA complexes containing TSG-6 and IαI accumulate to high levels at inflammatory sites and may support CD44-mediated leukocyte rolling and adhesion, while potentially having anti-inflammatory effects (69, 155, 201). High levels of TSG-6 have been reported in the secretory granules of mast cells within the inflamed joint tissues of mice, and intra-articular injection or cartilage-specific expression of TSG-6 have significant protective effects (66, 202, 203). It is possible that TSG-6 mediated crosslinking of HA results in a pro-adhesive but anti-inflammatory matrix for CD44+ cells (69). In the absence of CD44, the initial phases of leukocyte recruitment within inflamed synovial vessels are altered, and leukocytes appear to roll rather than adhere (204, 205). Leukocytes deficient in CD44

do not appear to interact as tightly with these HA complexes, suggesting that reduced adhesion contributes to the delayed onset of arthritis in these animals. Importantly, wild-type mice treated with anti-CD44 antibodies cross-link cell surface CD44, which rapidly disables leukocyte rolling. Antibody cross-linking of CD44 was followed by platelet deposition on the surface of granulocytes in the vascular beds of inflamed synovial tissue, and platelet-coated granulocytes were cleared from circulation leading to resolution of inflammation (205). These studies, in which different behaviors are observed when CD44 is either absent or blocked, are indicative of important differences reported in the literature with regards to the effects of HA fragments.

More extensive studies are needed to fully understand the specific molecular interactions between HA, HA-binding proteins, and HA receptors in the initiation and resolution of inflammation. The tissue microenvironment contributes significantly in regulating the inflammatory process. Defining the mechanisms by which inflammatory and protective HA fragments are generated, and how binding proteins influence their effects, will enable us to better understand the progression of inflammatory disease and perhaps reveal new therapeutic targets.

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Human immunodeficiency virus and heparan sulfate: from attachment to entry inhibition

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By targeting cells that provide protection against infection, HIV-1 causes acquired immunodeficiency syndrome. Infection starts when gp120, the viral envelope glycoprotein, binds to CD4 and to a chemokine receptor usually CCR5 or CXCR4. As many microorganisms, HIV-1 also interacts with heparan sulfate (HS), a complex group of cell surface associated anionic polysaccharides. It has been thought that this binding, occurring at a step prior to CD4 recognition, increases infectivity by pre-concentrating the virion particles at the cell surface. Early work, dating from before the identification of CCR5 and CXCR4, showed that a variety of HS mimetics bind to the gp120 V3 loop through electrostatic interactions, compete with cell surface associated HS to bind the virus and consequently, neutralize the infectivity of a number of T-cell line-adapted HIV-1 strains. However, progress made to better understand HIV-1 attachment and entry, coupled with the recent identification of additional gp120 regions mediating HS recognition, have considerably modified this view. Firstly, the V3 loop from CXCR4-using viruses is much more positively charged compared to those using CCR5. HS inhibition of cell attachment is thus restricted to CXCR4-using viruses (such as T-cell line-adapted HIV-1). Secondly, studies aiming at characterizing the gp120/HS complex revealed that HS binding was far more complex than previously thought: in addition to the V3 loop of CXCR4 tropic gp120, HS interacts with several other cryptic areas of the protein, which can be induced upon CD4 binding, and are conserved amongst CCR5 and CXCR4 viruses. In view of these data, this review will detail the present knowledge on HS binding to HIV-1, with regards to attachment and entry processes. It will discuss the perspective of targeting the gp120 co-receptor binding site with HS mimetic compounds, a strategy that recently gave rise to entry inhibitors that work in the low nanomolar range, independently of co-receptor usage.

Keywords: heparan sulfate, glycosaminoglycan, CCR5/CXCR4, gp120, V3 loop, co-receptor binding site, HIV-1, attachment and entry inhibition

INTRODUCTION

HIV-1 is the causative agent of acquired immunodeficiency syndrome (AIDS), a condition in humans in which progressive failure of the immune system leads to the development of severe opportunistic infections and unusual malignant disorders (1). Infection occurs through the transfer of blood, semen, vaginal fluid, or breast milk, in which HIV-1 can be present as both free virus particles and/or within infected cells. The virus infects vital cells of the immune system, including $CD4^+$ T-helper lymphocytes, macrophages, and dendritic cells, all of which are key to the development and orchestration of the immune response (2). This results in the targeted depletion of $CD4^+$ T cells, the main function of which is to promote cytotoxic T-lymphocyte dependant killing of cells expressing foreign antigens and up regulate antibody production by B-lymphocytes. When $CD4^+$ T cells numbers decline below a critical level (400/μl of blood) cell-mediated immunity is compromised and the body becomes progressively more susceptible to opportunistic infections (3). The first step of the HIV-1 replication cycle, attachment and entry into host cells, occurs through specific interactions between gp120, the glycoprotein which constitutes the surface unit of HIV-1 envelope spikes (Env) and the primary cellular receptor, CD4. This promotes further contacts between gp120 and members of the chemokine receptor family, among which CCR5 and CXCR4 are the most physiologically relevant and ultimately lead to the fusion of the viral and the host cell membranes (4). HIV-1 preferentially uses CCR5 during the acute phase of infection but, later in the course of HIV-1 infection progressing to AIDS, HIV-1 variants frequently appear that become adapted to utilize CXCR4.

Before encountering permissive $CD4^+$ cells the virus may interact with several other alternative receptors, often referred as "attachment receptors" (5) such as Galactoside Ceramide (GalCer), present at the surface of epithelial cells, Mannose-Binding Lectin (MBL), Dendritic Cell Specific ICAM-3-Grabbing Non-integrin (DC-SIGN), or Heparan Sulfate Proteoglycans (HSPGs), the latter being present at the surface of virtually all cell types. Although these interactions generally do not permit infection *per se*, they can importantly affect mucosal cells

(the portal through which HIV-1 enters in the body) attachment and transport across epithelial layers, tropism, tissue invasion, or cellular entry (6, 7). This review will discuss some aspects of the HIV-1-HSPG interaction and will describe how the biochemical characterization of this interaction led to the engineering of a new class of potential attachment and entry inhibitors.

HEPARAN SULFATE PROTEOGLYCANS

Heparan sulfate proteoglycans are glycoproteins carrying one or more covalently bound heparan sulfate (HS) chains, a large anionic polysaccharide of the glycosaminoglycan (GAG) family, characterized by astonishing structural diversity and interactive properties. These complex molecules are widely distributed within tissues, and can be found at the cell surface such as the syndecans and glypicans, within the extracellular matrix such as agrin, perlecan, or type XVIII collagen, or intracellularly such as serglycin (8). Being predominantly and ubiquitously present in the extracellular milieu, these macromolecules are unsurprisingly playing essential roles in a vast number of biological processes occurring at the cell–cell and cell–matrix interface.

Over the past two decades, HSPGs have been found indeed to bind to a multitude of protein ligands, including cytokines, chemokines, morphogens, growth factors, adhesion and matrix molecules, receptors, enzymes, plasma proteins, etc. (8, 9). These interactions, which usually involve the HS chains, serve a large number of purposes. Functionally, HS has been known to affect the local concentration, the compartmentalization, the stability, the structure, and/or the activity of its ligands. Protein-HS interactions thus play critical roles, for example, in mediating the formation of chemokine gradients along which cells can migrate directionally (10–12), in providing a scaffold onto which two adjacent proteins such as growth factor-receptor complexes can interact (13), in protecting cytokines against proteolysis (14), in inducing protein conformational changes (15), in controlling or in restricting the diffusion of its ligands (16–18) thereby generating a local concentration of a given protein. As it will be described below, many microbial pathogens hijack HSPGs and take advantage of their interactive properties for their adhesion to host tissues and invasion of host cells. From a structural view point HSPGs' multiple binding activities are believed to be closely related to the extended structural variability of the HS chain. It is a long (20–150 nm) and linear polysaccharide made of a repeating units of a 1 → 4 linked disaccharide motifs, comprising a glucuronic acid (GlcA) or its C-5 epimer, an iduronic acid (IdoA), and a *N*-acetyl- or *N*-sulfated-glucosamine (GlcNac or GlcNS), either or both of which may be *O*-sulfated at different positions. Variation in length, sulfation, and glucuronate/iduronate ratio, which occur in restricted domains of usually three to six disaccharides along the chain (**Figure 1**), generates a very large polydispersity and, as such, provides distinct docking sites for the various ligands of the polysaccharide (19, 20).

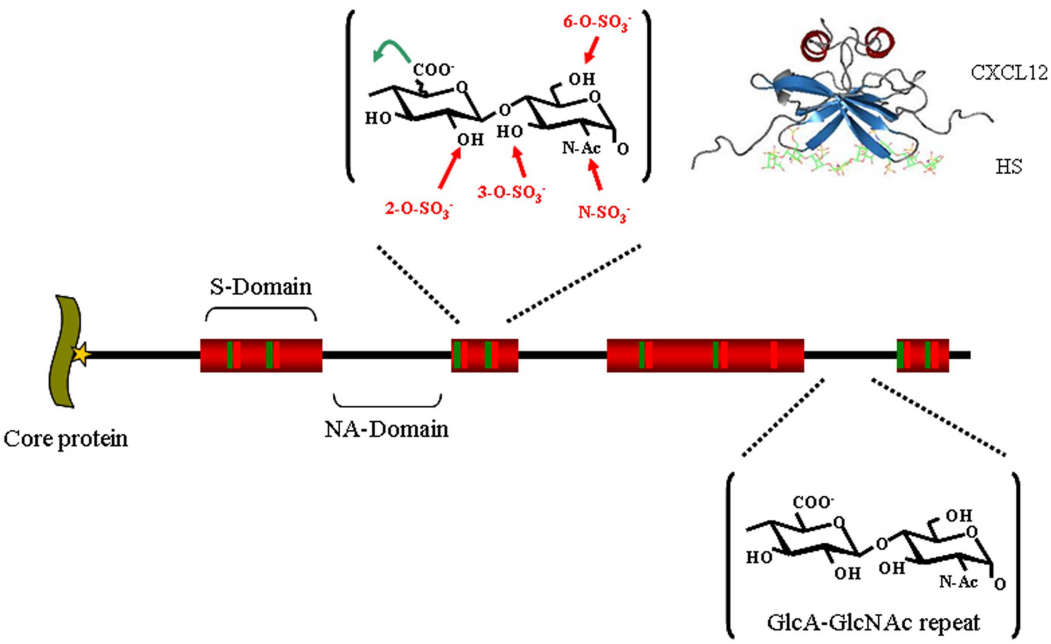

FIGURE 1 | Heparan sulfate structure. HS, whose biosynthesis is initiated by the attachment of xylose (star) to specific serine residues in HSPG core proteins, followed by the formation of a linking tetrasaccharide (xylose-galactose-galactose-glucuronic acid), is initially polymerized by an enzyme complex composed of Ext1 and Ext2 as a GlcA-GlcNAc repeat (black). In restricted regions, called S-domains (shown in red), the chain is extensively modified by a series of enzymatic reactions that remove the acetyl group from GlcNAc residues and substitute the resulting free amino groups with sulfates, epimerizes the adjacent GlcA into L-iduronic acid (IdoA) and adds sulfates on various positions: the C2 of the IdoA (and less frequently that of the GlcA), the C6 of the GlcNS (and less frequently that of the GlcNac), and finally at the C3 of GlcNS or GlcN units. Altogether, these modifications can generate (the theoretical number of) 48 different disaccharides, whose combination within the S-domain gives rise to a large diversity of structures and make up binding sites for protein ligands, as depicted for example with a model of a CXCL12-HS complex [from Ref. (21)].

HSPG AND PATHOGENS

Attachment to host tissues is a critical step for most pathogens' invasion and dissemination. It is therefore not very remarkable that due to its wide expression and large interactive properties, HS is used by many pathogens for that purpose (22–26). These include parasites, for example *Plasmodium falciparum* (27) bacteria, such as *Pseudomonas aeruginosa* (28), *Borrelia burdorferi* (29), or *Mycobacterium tuberculosis* (30), and many viruses, amongst which are found B (31), C (32), delta (33), and E (34) hepatitis viruses, Human Papillomavirus (35), Herpes viruses (36), HTLV-1 (37), or HIV-1 (38). Several lines of evidence have identified HS as an initial receptor for viral infection. Firstly, many capsid or envelope viral proteins bind to HS, secondly, elimination of cell surface HS is usually associated with increased cell resistance to infection that results from a reduction in the virus' ability to bind to the cell surface (39), thirdly, soluble HS or HS like molecules, including heparin, a chemically related GAG and dextran sulfate, inhibit viral attachment and subsequent entry in cell culture experiments (40–42). Finally, it has been described that a number of viruses undergo cell culture adaptation changes resulting in an increased binding to HS (43–46). Together this suggests that selection during cell culture of mutants that bind HS with high affinity confer a selective advantage to the viruses. It has thus been thought that HS could facilitate concentration of the viral particles at the cell surface, restricting their diffusion to the quasi-two-dimensional network of polysaccharides around the cell and as such enhances the probability of access to specific entry receptors. HS can also capture viral particles at the surface of non-permissive cells, and then mediate *in trans* infection by presenting these viruses to attachment and entry receptors on permissive cells (47). HS binding can also go well beyond the simple attachment mechanism and can play a more direct role in cellular entry. This has been demonstrated in particular for HSV, a virus whose entry into epithelial cells involves several glycoproteins of the envelope (48). While the viral envelope glycoproteins gB and gC participate in the initial cell attachment through binding to HS, the gD, which binds to herpes virus entry mediator (HVEM) or to nectin, triggers fusion between host and viral membranes, but can also promote viral entry by interacting with a specific HS motif comprising a 3-*O*-sulfated glucosamine residue (49). Interestingly, a 3-*O*-sulfonated HS octasaccharide, produced by chemical means, was shown to inhibit the HSV-1 host-cell interaction (50), suggesting the use of HS derived molecules as therapeutic tools against viral pathogens.

WHERE DOES HIV-1 MEET HS?

HIV-1 is transmitted by viral exposure at the mucosal surfaces, which can occur in the genital tract (semen, blood) the intestinal tract (semen, blood, breast milk), or through the placenta (maternal blood), or the bloodstream (blood products). Within the context of vaginal or rectal transmission, HIV-1 must first cross a normally protective mucosal epithelium to reach the underlying dendritic cells, macrophages, and T cells which all express the virus primary receptor, CD4 and at least one of its two co-receptors, CCR5 or CXCR4, and are thus the three major cellular targets of HIV-1. In these early events of transmission, before specific cell infection, HS has been shown to play important roles in viral adsorption and dissemination. In the semen first, which is the main vector for HIV-1 dissemination, and which contains both free virions and infected leukocytes, it has been found that spermatozoa can capture HIV-1 in a HS dependant manner. Such spermatozoa-attached viruses are efficiently transmitted to dendritic cells, macrophages, and T cells (51) to which access could be made possible through mucosal microabrasions or through dendritic cell projections that extend to the luminal surface of the mucosa.

The mechanisms used by free or cell associated virions to cross an otherwise healthy mucosal barrier are not well known and might differ depending on the tissue sites (for example vaginal or rectal epithelium) where infection occurs (52). HIV-1 can interact with the epithelial cells and can traverse the epithelium through transcytosis, endocytosis followed by exocytosis or by penetrating the gaps in between cells, gaining access to susceptible leukocytes that will further propagate and spread the infection (53, 54). In this context, it has been well known that epithelial cells express large amount of HSPGs which can sequester HIV particles. For example, attachment of HIV to an ectocervical epithelium-derived cell line can be inhibited both by heparinase (an enzyme that depolymerizes HS and removes it from the cell surface) or by soluble heparin. Interestingly, it has been found that cell surface bound HIV particles remain infectious for at least 6 days, and upon co-culture with $CD4^+$ cells, can be efficiently transmitted to its target cells (55). It has been also reported that HSPG significantly contributes to both attachment to the apical pole of – and transcytosis through – an endometrial epithelium-derived cell line (7). Similarly, cell-free HIV particles have been shown to transcytose (although with low efficiency) through primary genital epithelial cells, a process that was dependant on syndecan, one of the major HSPGs expressed by epithelial cells (56).

Finally, HS was recently found to be indispensable for gp120-mediated induction of TLR signaling in intestinal and genital epithelial cells. In the gp120-TLR-HS complex, HS was critical to activate the intracellular NF-kB pathway which lead to downstream synthesis of proinflammatory cytokines and chemokines, and whose upregulation was associated with tight junction disruption and loss of barrier function (57). Therefore, beyond acting as an ancillary attachment receptor, HS can contribute to barrier loss and initiation of immune activation that could be the first step in the characteristic chronic immune activation of HIV-1 pathogenesis.

Primary infection can also develop from viral exposure in the oral cavity (mother to infant nursing or during oral intercourse), where the palatine tonsil is a replication site for HIV-1. Studies aiming at characterizing the expression pattern for HIV-1 ligands on human palatine tonsils have shown that HS was largely present on both the surface of the stratified squamous epithelium and on the reticulated epithelium lining the tonsillar crypts and where it is likely to provide stable binding for the virus, allowing it to penetrate beneath the luminal surface and encounter $CD4^+$ cells (58). Finally, it has also been shown that HIV-1 infection of trophoblasts is independent of CD4 but, at least partly, relies on HSPG. Mother to child vertical transmission of the virus is a major cause of HIV-1 infection in infants, and direct infection of trophoblasts, the cells that form the placental barrier, may cause this transmission (59).

HIV-1 is also trapped very efficiently by endothelial cells, which usually express large amounts of HSPG. Syndecan-3, for example,

which delineates the contour of endothelial cells in lymphoid tissue high endothelial venules, does not substitute for HIV-1 entry receptors but captures HIV-1 and presents it to passing permissive T cells, thus mediating an *in trans* mechanism of infection. Furthermore, whereas unbound viruses lose infectivity in 1 day, syndecan-attached HIV-1 remains infectious for up to 1 week (60).

Finally, HSPGs also significantly contributes to HIV-1 invasion in the brain and neurological complications that often characterize AIDS patients. Whereas HIV-1 can enter the central nervous system within infected $CD4^+$T-cell and monocytes that traffic across the blood brain barrier, several *in vitro* and *in vivo* reports described that free HIV-1 can be taken up by brain endothelial cells in a HS dependant manner, internalized and exocytosed, as a way to cross the blood brain barrier before infection and replication can occur in central nervous system cells such as microglia and astrocytes (61–63).

Although this review is devoted to HIV-1 attachment and entry, it is worth noting that, in addition to gp120, several other HIV proteins regulating various aspects of the virus life cycle also appeared to bind cellular HS after being released from HIV-infected cells. These protein-HS interactions contribute to trigger a variety of biological effects related to AIDS-associated pathologies. This includes p17, the matrix protein (64) which up-regulates cytokine production thus deregulating the functions of many immune cells; TAT, the transacting activator of transcription (65) which exerts angiogenic, cell proliferation, chemoinvasion activities and induces peripheral neuropathies, immune suppression, and tumorigenesis; and finally Vpr, the Viral protein R (66), which induces cell cycle arrest and apoptosis.

Altogether, regarding attachment and entry, these studies showed that HSPG serves a number of purposes during the early steps of HIV-1 dissemination, from capturing and presenting *in trans* free virions to replicative cells, to permitting the transfer of viral particles across epithelial or endothelial barriers (**Figure 2**). HSPG, when expressed by $CD4^+$ permissive cells may also increase infectivity by favoring *in cis* viral particle concentration at the cell surface (see below).

A number of polyanions have been investigated for their ability to inhibit HIV-1 infection in clinical trials. In addition to suramin, dextran sulfate, and heparin, which were considered for systemic use, this includes many other sulfated/acidic compounds such as carrageenan, cellulose sulfate, polystyrene sulfonate or maleic acid, naphthalene sulfonate, and cellulose acetate phthalate, developed as anti HIV-1 microbicides. Unfortunately, none of these compounds proved to be effective *in vivo* presumably due to poor availability, toxicity, sequestration by plasma proteins, induced reduction in epithelial integrity and concomitant increases in permeability to HIV-1 particles (67, 68). Their variability, in terms of molecular weights and degrees of sulfation also made them difficult to standardize.

THE CELL SURFACE LIGANDS OF gp120 AND THE ENTRY PROCESS

CD4, CCR5, AND CXCR4

Once in contact with permissive cells, i.e., cells that are CD4 and CCR5 and/or CXCR4 positive, the HIV-1 can start its replication cycle. HIV-1 entry into its target cells is initiated by a highly complex series of interactions, which first involve the binding of gp120 to its primary receptor, CD4 (69). This initial step not only enables the viral particles to attach to the cell, but also drives extensive structural alterations that primes the envelope for binding to either CCR5 or CXCR4 (70). This second interaction, which elicits further modifications in Env, triggers the activation of the gp41 fusion peptide whose insertion into the host membrane ultimately leads to the delivery of the viral contents into the host cytoplasm (**Figure 3**). The gp120 thus constitutes the central element for all interactive events occurring during the pre-entry steps and, accordingly, this molecule features several interactive regions and is structurally complex; It consists of five relatively conserved regions (C1–C5), that fold into a "core" structure organized into two distinct regions termed the "inner" and "outer" domains that are connected by a bridging segment, and five surface-exposed variable loops (V1–V5). The CD4 binding site is formed from conserved residues in discontinuous segments

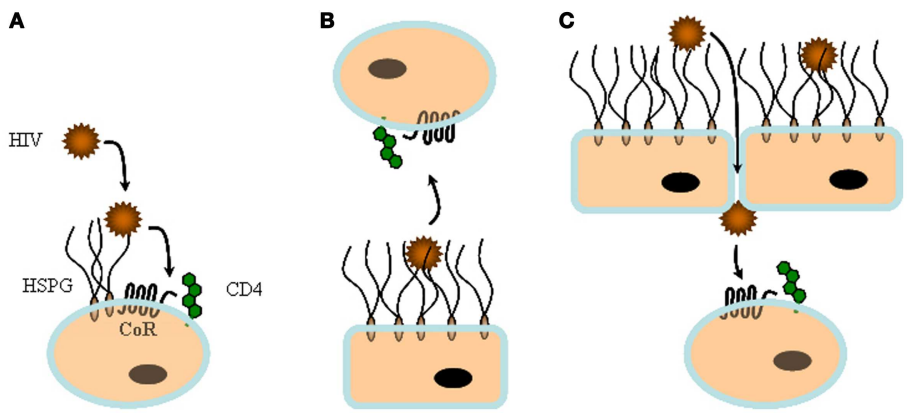

FIGURE 2 | *In cis* and *in trans* capture of HIV-1 by heparan sulfate. HS can play multiple roles during viral infection. **(A)** On top of cells that express large amount of HS, but low CD4, such as macrophages, HS can capture viral particles and facilitate *in cis* subsequent interaction with specific entry receptors. **(B)** HS from non-permissive cells such as endothelia or epithelia can sequester HIV-1 and then mediate *in trans* infection by presenting the virus to permissive cells. **(C)** HS can contribute to both attachment and transcytosis of HIV-1 through epithelia.

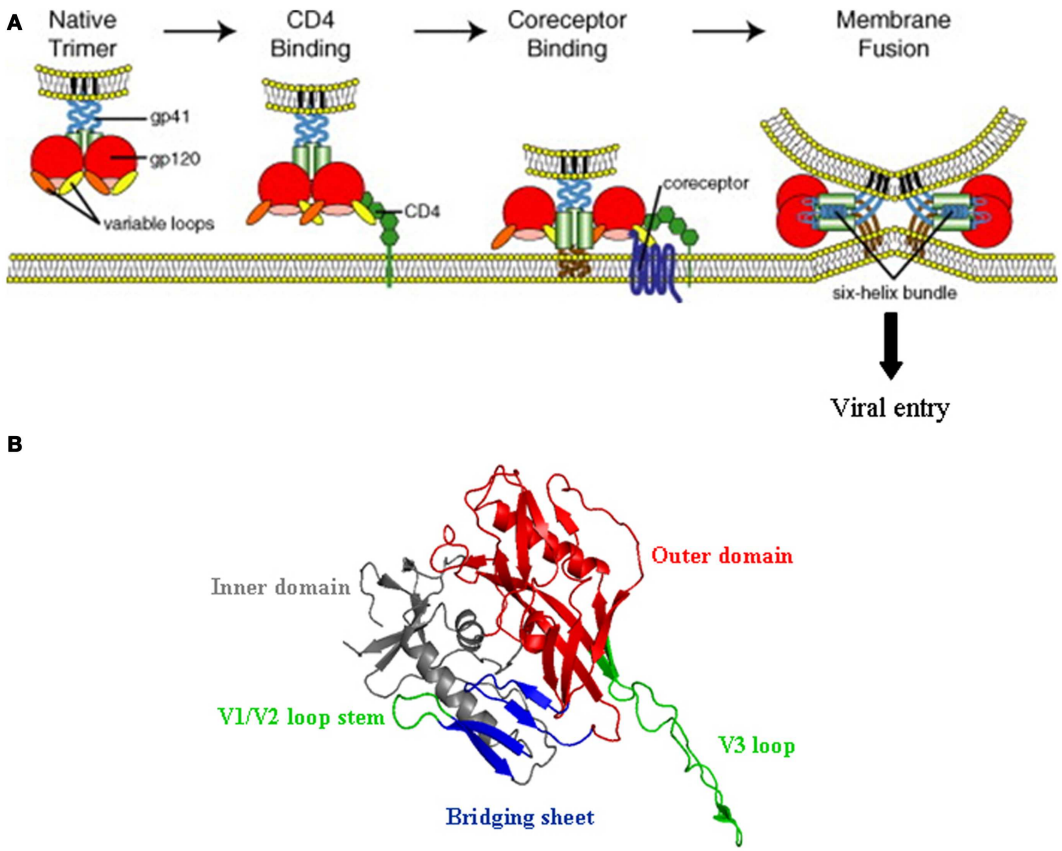

FIGURE 3 | HIV-1 entry mechanism. **(A)** Schematic representation of the multi-step process of HIV-1 entry; from attachment to CD4 (left) to fusion between the viral and the cell membrane (right). The gp120 trimer, upon binding to CD4 (in green), experiences extensive structural changes that open up the variable loops V1/V2 and V3 (orange and yellow), and concomitantly expose and/or fold the so called CD4 induced bridging sheet that will be recognized by the co-receptor (CCR5 and/or CXCR4). This second interaction triggers the insertion of the gp41 fusion peptide into the cell membrane and promotes viral entry (Reprinted from Ref. (102), with permission from Elsevier). **(B)** Three-dimensional structure of gp120 in the CD4-bound conformation (from pdb:2b4c), showing the inner and outer domains, the V1/V2 loop stem, and the four β strands (CD4 induced bridging sheet in blue) that together with the V3 loop (in green) contribute to co-receptor selectivity and interaction.

of the C1, C3, and C4 domains that are brought into proximity in the folded gp120 and located into a depression formed at the interface of the outer and inner domain and the region that connects these two domains (71). Binding to CD4 triggers extensive structural alterations, in particular within the inner domain of the protein. Although X-ray crystallographic analysis have led to atomic models for gp120 on which the V1/V2 and V3 loops were deleted, it seems likely that these structural modifications include both a relocation of the V3 and shifts of the V1/V2 loops, whose base in the inner domain (β2 and β3 strands) is brought into close proximity to a β-hairpin of the outer domain (β20 and β21 strands). This exposes new regions that, partially masked by the V1/V2 and V3 loops, were cryptic in the unliganded gp120, and concomitantly folds a four-stranded β-sheet located within the bridging sheet that connects the inner and the outer domain of the glycoprotein (71, 72). In conjunction with the V3 loop (73), this β-sheet (known as CD4i for CD4 induced epitope) makes up the binding site for either CCR5 or CXCR4 (70, 74, 75). The V3 loop has a major influence on HIV-1 tropism and appears to be a key determinant for co-receptor selectivity, which in turn, affects the overall process of viral pathogenesis. Its sequence is important for defining the extent to which the CD4-bound form of the gp120 interacts with CCR5 or CXCR4, and hence the ability of a particular HIV-1 virus to enter cells using either CCR5 (viruses called R5-tropic) or CXCR4 (X4-tropic viruses). R5X4 or dual tropic strains constitute a third class that can use either of these two co-receptors. It has been reported that in the majority of the infected subjects, the HIV-1 primarily uses CCR5 in order to initiate the infection. However, during the course of infection, the co-receptor usage preference of HIV-1 shifts from CCR5 to CXCR4 in 50% of the infected individuals, a change that is frequently associated with the accelerated $CD4^+$ T-cell decline and the rapid progression toward AIDS. In the context of the interaction between gp120 and HS, it is worth noting that, in general, the R5 to X4 tropism switch is associated with an increase in the net positive charge of the V3 loop (75), which will also determine to which extent gp120 will interact with HS (see below).

HEPARAN SULFATE

Very early in the study of HIV-1, it was found that a number of HS like molecules, including heparin, dextran sulfate, and various heparinoids (such as pentosan polysulfate, fucoidan, curdlan sulfate) inhibit viral replication in cell culture experiments (76–78). This appeared to mostly occur by preventing HIV-1 binding to the cell surface as shown by the ability of heparinase treatment of HIV-1 sensitive lymphoblastic T-cell lines, such as MT-4 and H9, to reduce HIV-1 absorption to these cells (38, 79). Although some studies suggested that heparinoids could bind to CD4 and thus interfere with HIV-1 binding to its primary receptor, several investigations showed that both soluble heparin and cell surface HS interact with gp120 (see below) and target the V3 loop of the viral protein (80, 81). As this domain is not involved in CD4 binding, this excluded that heparinoid works by blocking the gp120-CD4 interaction, which was further confirmed by the observation that (i) HS could be immunoprecipitated from gp120 treated H9 cells with either anti gp120 or anti-CD4 antibodies (82), and (ii) dextran did not interfere with the binding of CD4 to recombinant gp120 at concentrations which effectively prevent HIV-1 replication (83, 84). Interestingly, it was shown that if the polyanionic nature of these compounds were essential for their *in vitro* anti-HIV-1 activities, a number of other sulfated polysaccharides, including for example various chondroitin sulfates (another member of the GAG family), have no such activities. This suggests that specific structural features of the polysaccharide might be important for activity. It thus appears that, in addition to CD4, cell surface HSPG functions as an attachment receptor recognized by the V3 loop of gp120, and therefore suggesting that this interaction allows the virus to scan the cell surface and could assist binding to specific entry receptors. Most of these studies however date back to the discovery of the HIV-1 co-receptors and their importance in tropism. They were performed with T-cell line-adapted HIV-1 that use CXCR4 to enter cells and immortalized T-cell lines that express large amounts of HSPG. The binding of HIV-1 to cell surface HSPG is however likely to depend on the level of expression of this molecule, and it was then reported that if cell surface HSPG facilitates HIV-1 entry into some cell lines it does not into primary T-lymphocytes (85, 86), questioning the physiological relevance of HSPG for capturing viral particles at the surface of $CD4^+$ cells.

Primary T-lymphocytes and macrophages, the two major HIV-1 targets, feature opposite patterns of attachment receptors. In the former, which express high CD4 levels, chondroitin sulfate largely outnumbers HS moieties (87), while macrophages display low levels of CD4 but produce large amount of HS (88). On these cells, HIV-1 attachment is mostly mediated by HSPGs, and this interaction was found necessary for successful infection when a low level of CD4 is expressed at the cell surface (88, 89). Thus, HS may compensate for low level CD4 expression and induce a sufficiently high viral particle concentration for infection that CD4 by itself would not achieve. In contrast, the high levels of CD4 on T-lymphocytes obviate the need for other attachment molecules. Together, this shows that the role of HS in mediating *in cis* HIV-1 infection may depend on the cell surface CD4/HS ratio and their level of regulation. For example, it has been observed that while quiescent primary $CD4^+$ T cells do not express detectable levels of HSPGs, HSPGs are expressed on primary $CD4^+$ T cells following activation by interleukin-2/phytohemagglutinin or anti-CD3/anti-CD28 antibody. This immune activation coincides with binding and entry of HTLV-1, a known HS binding virus (90). Although determining the GAG nature/expression pattern and architecture of primary cells is not trivial, it might be of interest to better characterize the HS status of HIV-1 target cells during the evolution of the pathogenesis. Finally, it is also important to note that HIV-1 susceptibility to HS is dependant on the HIV-1 strain as all do not display high affinity for this GAG (see below).

A BIOCHEMICAL VIEW ON THE gp120-HS INTERACTION

THE V3 LOOP IS THE MAJOR HS BINDING DETERMINANT

Early studies, investigating the mechanism by which polyanions such as heparin or dextran sulfate inhibit HIV-1 replication pointed out that these polysaccharides interact with the gp120 V3 loop. This was essentially based on the observation that polyanions block the binding of a number of monoclonal antibodies, directed against the V3 loop to either recombinant gp120 on ELISA plates or to HIV-1 infected cells expressing gp120 on their surface (80, 82, 84). These results were further confirmed with data showing that HS directly binds to V3 derived peptides (81, 91), whose sequences were characterized by Lys and Arg enriched clusters that are commonly found on protein HS binding sites. However, the V3 loop, a disulfide-bonded structure of approximately 35-residues-long is highly variable and prone to mutation-induced sequence changes (75). Its overall charge may vary from +2 to +10, with that of a CCR5-using HIV-1 strain generally in the range of +3 to +5 and that of a CXCR4-using isolate being from +7 to +10. Therefore, binding to HS may vary according to the gp120 origin and tropism, an evolution toward a more basic structure being linked to adaptation toward CXCR4 usage.

To investigate these points in more detail, the binding of heparin to WT and mutated forms of different gp120s, including MN (X4), HXBc2 (X4), 89.6 (R5X4), W61D (R5X4), Bal (R5), and JRFL (R5) were measured by different means: Surface Plasmon Resonance showed first that the X4-HXBc2 gp120 (whose V3 loop features nine positive charges) strongly binds to heparin, as does the R5X4-89.6 gp120 which has seven positive charges in its V3 loop. The binding of the W61D (another R5X4 gp120 with a V3 loop of +6) was reduced, and finally, the R5-gp120 (whose V3 loop contains only four basic residues) binds relatively weakly (gp120 Bal) or not at all (gp120 JRFL). These results were in agreement with another assay, in which the interaction of [^{35}S]-labeled heparin to the above mentioned gp120s also showed that most of the variation in binding is due to changes in the charge and structure of the V3 loop. It was nevertheless observed that [^{35}S]-labeled heparin binding to HXBc2 gp120 was not entirely suppressed by the V3 loop deletion, suggesting that other regions could contribute, albeit to a lesser extent. Binding of HXBc2 with more substantial deletions, performed to address which other regions of the X4-gp120 might be implicated, showed that deletion of the NH_2- and COOH-termini and the V1/V2 loop structure resulted in a small loss of [^{35}S]-heparin binding. An additional deletion of the V3 loop dramatically reduced [^{35}S]-heparin binding and preincubation of this mutant with the monoclonal antibody 48d further reduced binding to background levels. These data suggest that whereas the V3 loop is the major determinant, the COOH- and NH_2-termini, the

V1/V2 loops and the bridging sheet between the inner and outer domains of gp120 (recognized by the 48d antibody) could contribute to some extent to the binding of polyanions. Consistently with the idea that the V3 loop is the primary high affinity binding site on gp120, at least for the X4 derived Env, molecular modeling of the electrostatic potential of the protein confirmed that the overall charge on the surface is dominated by the V3 loop (92).

The early use of polyanionic compounds such as dextran sulfate as anti- HIV-1 therapeutic agents has not been successful in clinical trials (93). One possible reason for the *in vivo* failure of the molecule (in addition to toxicity and poor bioavailability) could be related to the V3 loop charge differences between R5 and X4 viruses, the former being the phenotype associated with HIV-1 transmission and early infection, while the latter being the only one efficiently targeted by polyanions.

HS BINDING TO OTHER SURFACE EXPOSED REGIONS OF gp120

To obtain unequivocal evidence for a direct interaction between polyanions and regions outside the V3 loops, a new approach designed to identify and simultaneously map potential HS binding sites on a protein surface was developed (94). This method, which uses unmodified native proteins, is based on the formation of cross-linked complexes of the protein of interest with heparin, followed by the proteolytic digestion of these complexes, and the subsequent identification of the heparin bound peptides by N-terminal sequencing using an automated protocol of Edman degradation and HPLC detection of the released amino acids.

Using this approach with HXBc2 gp120, three potential HS binding domains (HBDs) were consistently identified (including the V3 loop that was confirmed by this approach): RGKVQK (HBD 1: residues 166–171) located within the V2 loop, RKRIR (HBD 2: residues 304–308) at the base of the V3 loop and finally, KAKRR (HBD 3: residues 500–504), at the C-terminal domain of the protein (95).

Interestingly, all three HBDs are functionally important. HBD 1 and 2 in particular undergo important structural changes after CD4 binding, resulting in the unmasking of the co-receptor binding site and are thus key to the entry mechanism of the virus. As mentioned above, HBD 2 (within the V3 loop, which determines co-receptor usage) displays essential features for co-receptor binding. In particular, mutation of residues Arg298, Arg306, and Arg308, present in this HBD, strongly decrease the ability of gp120 to interact with CXCR4 (96). It has been reported that heparin enhances the furin cleavage of HIV-1 gp160 into gp120 and gp41, which occurs only three residues downstream HBD 3, but the significance of this observation is not clear (see Ref. (95) for discussion).

Together these data provide a direct demonstration of the existence of additional binding sites and identify some of the residues involved. They are consistent with a kinetic analysis of the gp120-heparin interaction which could be much better described by a complex model than by a single one-to-one binding mode (92) and with the observation that the V3 loop does not fully recapitulate the binding activity of the protein.

A FOURTH HS BINDING SITE IN gp120: THE "CD4 INDUCED" EPITOPE

The gp120 molecules assume several distinct conformations and are characterized by an important intrinsic flexibility, which could likely influence heparin binding. As described above, and as shown by cryo-electron tomography on the native gp120 trimers (including the variable loops that were missing in the X-ray crystallographic analysis), the V1/V2 and V3 loops (comprising the HBD 1 and 2), in particular, are released and move away from the center of the Env spike (97) following binding to CD4. Thus, to further clarify the gp120-HS binding determinant, the unliganded monomeric gp120 (HXBc2 strain) and the gp120 in its CD4-bound conformation, were compared for their ability to interact with heparin. This showed that in its CD4-bound form, gp120 had a substantially increased affinity for the polyanion, suggesting that the CD4-bound conformation could have improved the accessibility of the V1/V2 and V3 loops and/or stabilize them into a structure better recognized by HS.

However, CD4 binding also exposed the "CD4 induced" bridging sheet that was previously masked by the V1/V2 and V3 loops in the unliganded form. Examination of the gp120 electrostatic surface, using the structural data of the CD4-bound core glycoprotein, revealed that the CD4 induced region comprises a cluster of positively charged residues located between the stems of the V1/V2 and V3 loops, organized as a typical HS binding site (**Figure 4**). Using a molecular modeling approach to locate putative HS binding sites further shows that within this domain, amino acids Lys-121, Arg-419, Lys-421, and Lys-432 form a discontinuous surface with a linear shape extending up to 25 Å, which can be predicted by a GRID analysis to be the most favorable anchoring position for an oxygen atom from a sulfate group (98). These molecular modeling predictions could be confirmed by showing that HS and HS derived oligosaccharides strongly inhibited the binding of mAb 17b to the CD4-gp120 complex. The mAb 17b belongs to the CD4i antibody family and recognizes an epitope on the gp120 bridging sheet that is exposed upon CD4 binding. Interestingly, this sterically restricted region, which overlaps the binding site for the co-receptor, is a well conserved element amongst X4, R5, and dual tropic gp120. Mutagenesis then confirmed that of the four amino acids indicated above, Arg-419, Lys-421, and Lys-432 were key to the interaction with HS (95). All together, this defines an additional HS binding domain (HBD 4), located within the gp120 bridging sheet, importantly involved in co-receptor recognition and exposed only after CD4 binding.

DEVELOPMENT OF A CD4-HS GLYCOCONJUGATE TO INHIBIT HIV-1 ATTACHMENT AND ENTRY

These studies thus showed that HS binding to CXCR4 tropic gp120 is constitutive (the V3 loop -which dominates the interaction- is surface exposed), and can be enhanced by CD4 (which, together with V3 loop reorganization, exposes a new HS binding domain), while it is entirely CD4-induced for CCR5 tropic gp120. Interestingly, all these HBDs are located close to each other, at the proximity of- or within- the co-receptor binding site and are collectively involved in the conformational changes induced upon interaction with CD4 and in co-receptor recognition. In particular, mutations of Arg-419, Lys-421, Lys-432 within the bridging sheet, and Arg298, Arg306, Arg308, within the V3 loop, which are targeted by heparin, decrease the ability of gp120 to interact with CXCR4. This strongly suggested that polyanionic compounds, in addition to prevent HIV-1 association to cell surface

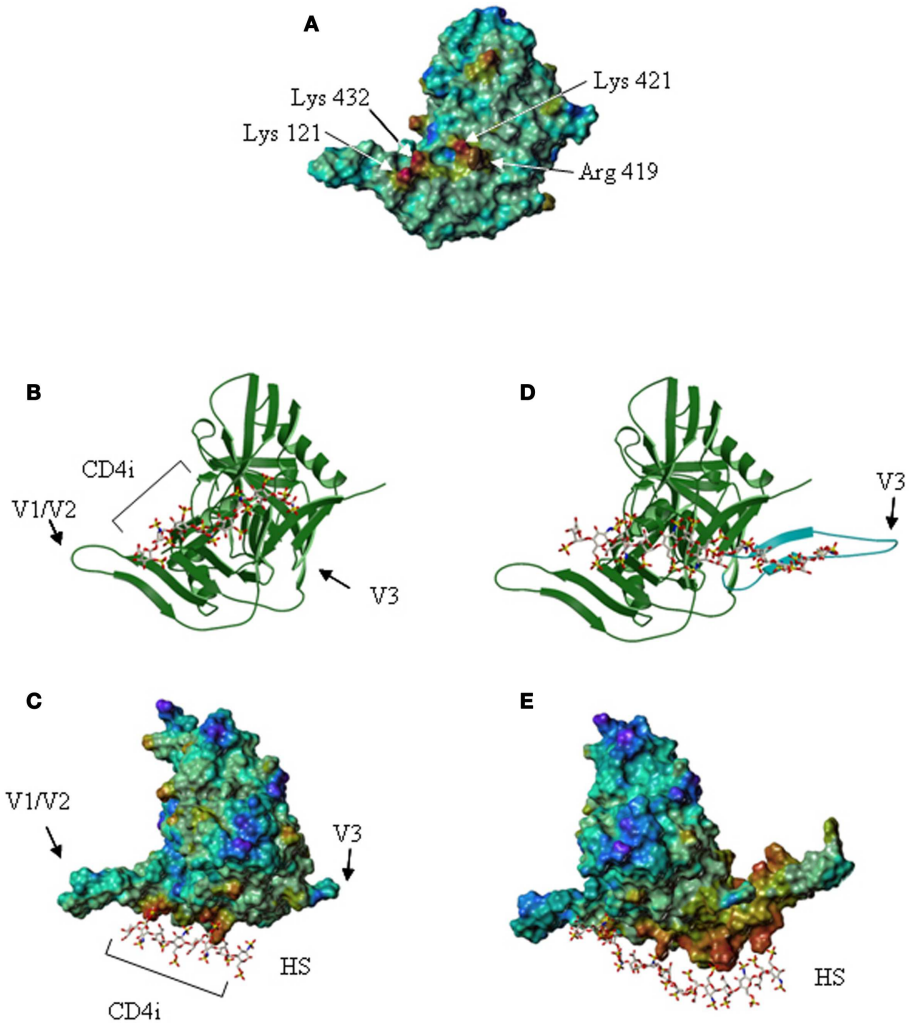

FIGURE 4 | The V3 loop and the co-receptor binding domain of gp120 features HS binding sites. **(A)** The gp120 CD4-induced domain displays a HS binding structure. The Connolly surface of the HXBc2 gp120 core was color-coded according to the electrostatic potential from negative values (blue) to positive values (red). The basic residues of the CD4-induced (CD4i) epitope, which form a HS binding site, are indicated. **(B)** Representation of the lowest energy model of a gp120/HS derived octasaccharide complex. The protein [orientation as in **(A)**] is represented by a ribbon, and the octasaccharide by sticks. **(C)** Lowest energy model of the gp120 (on which the Connolly surface was calculated) in complex with a HS derived octasaccharide. V1/V2 and V3 indicate the stem of the V1/V2 and V3 loops. The location of the CD4i epitope is also indicated. **(D)** Structure of gp120, on which the V3 loop (in blue) was modeled. The HS binding residues of the V3 loop and the CD4-induced epitope are aligned on the surface of the protein and form an extended binding site on which has been docked a HS derived oligosaccharide of appropriate length. **(E)** Connolly surface of gp120, including the V3 loop, in complex with a tetradecasaccharide shown with the same orientation as in **(C)**.

HSPG, could directly block co-receptor binding and thus inhibit entry. However, being cryptic on the HIV-1 associated gp120, the co-receptor binding site needs to be exposed to be efficiently targeted. To this aim, a new molecule composed of CD4 covalently linked to HS has been prepared. To render this molecule potentially druggable it was based on a small CD4 mimetic (rather than recombinant CD4) and a chemically synthesized HS dodecamer (rather than natural derived HS, whose almost infinite structural variety would have made impossible the obtention of a defined compound). This chemically defined glycoconjugate (termed mCD4-HS_{12}), whose size is 6000 Da, was shown to bind to gp120 through its mCD4 moiety and induce the structural modifications necessary to expose the co-receptor binding domain which therefore became available to be blocked by the HS_{12} moiety (**Figure 5**). This compound thus successfully targets two critical and highly conserved domains of gp120; the CD4 and the co-receptor binding domains. From a biochemical point of view, this compound blocks the binding of the prototypic R5-gp120 (YU2), to both CD4 and mAb 17b (directed against the co-receptor binding domain), while it blocks that of the prototypic X4-gp120 (MN) to CD4, HS and mAb 17b, with low nanomolar affinity (99). This molecule also prevents gp120 association to purified native CCR5 and CXCR4 co-receptors (100) and consequently displays a strong antiviral activity against R5- and

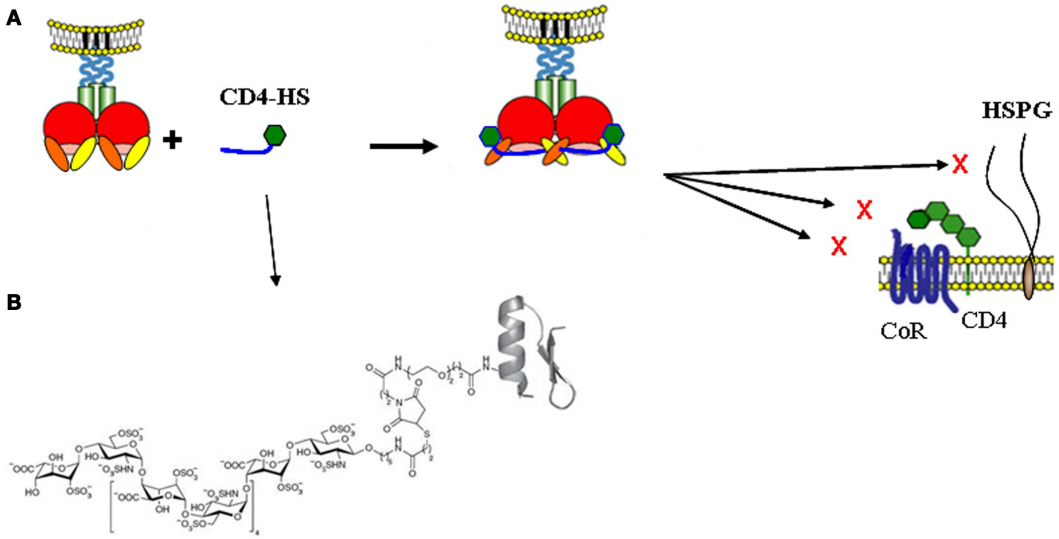

FIGURE 5 | Principe of inhibition of HIV-1 attachment and entry by "CD4-HS." (A) A CD4 mimetic peptide covalently linked to a HS dodecassaccharide (CD4-HS) bind to gp120 through its CD4 moiety and exposes the CD4i epitope, which then becomes available for interaction with the oligosaccharide. Such a bivalent molecule simultaneously binds to the CD4, the HS and the co-receptor binding sites of gp120 and blocks the interaction of the virus with all its principal cell surface ligands, inhibiting both attachment and entry (The gp120 was schematically represented as in **Figure 3**). **(B)** The structure of the mCD4-HS_{12} is also shown [modified from Ref. (99)].

X4- HIV-1 as well as against dual tropic virus with IC_{50} as low as 5 nM.

CONCLUSION AND PERSPECTIVE

Investigation of the gp120 binding to HS has revealed a contrasting situation that is far more complex than previously thought. HIV-1 that uses CXCR4 as an entry co-receptor features up to four HBDs on their gp120, including the V1/V2 and V3 loops and the co-receptor binding site, while those using CCR5 mostly display HS binding activity essentially within the conserved and characteristically basic co-receptor binding domain. In that context, it is worth noting that the N-terminus of CCR5 and CXCR4 contain sulfotyrosine, as do a number of antibodies, directed against the gp120 co-receptor binding domains (101), also indicating that the gp120 CD4 induced region can be liganded by sulfated moieties.

Characterization of these HBDs, in particular within the CD4 induced surface, whose cryptic nature limits its accessibility both temporally and spatially during infection, led to the engineering of a new class of compounds in which HS was covalently linked to mCD4. These compounds, conceptually distinct from any other existing HIV-1 inhibitors, function by simultaneously exposing and blocking the HIV-1 co-receptor binding site, and therefore inhibit binding of gp120 to both CD4 and CCR5/CXCR4. It thus efficiently inhibits viral replication by blocking entry, which is currently considered as a compelling target for controlling viral replication (102). This molecule also inhibits the binding of gp120 to HS and has thus the potential for preventing viral adsorption on mucosa or viral transport through the blood brain barrier. It could therefore be further developed for both prevention and therapy following topical and/or parenteral application. In this regard, it is worth noting that these compounds are much more defined and have a much more specific mode of action than the above described polyanions that have been investigated up to now.

Despite tremendous progresses made in the development of antiviral drugs (103), HIV-1 continues to be a major health concern and remains one of the leading causes of death worldwide, which necessitate the development of new antivirals. With regards to inhibitor development, it is worth noting that mCD4-HS_{12} is bivalent. Multivalency has a number of functional advantages, such as achieving high affinity, and increasing strength and specificity for the binding site. It has been indeed found that the mCD4-HS_{12} is, by far, more active than either its moieties alone, each reciprocally enhancing the blocking activity of the other in a cooperative manner (99). Targeting the gp120 co-receptor binding site, which although is well conserved across various HIV-1 strains exists in a dynamic equilibrium between its unliganded- and CD4-bound conformations, might thus be relatively challenging. From a structural point of view, it is interesting that HS is characterized by considerable internal motion and variation in its local three-dimensional structure. The IdoA, in particular, also exists in a dynamic equilibrium between a chair and a twisted skew-boat form, which may itself represent the average of a rapidly fluctuating ensemble of related structures. The conformation of HS also depends on its local sequence, the presence of poorly sulfated GlcA-GlcNAc domains giving rise to chain flexibility (104). It is thus tempting to suggest that HS is well designed to interact with an ensemble of conformationally dynamic structures such as that of the co-receptor binding domain of gp120, the high specificity of the conjugated bivalent compound being brought by the mCD4 moiety.

Currently, the HS_{12} moiety of the molecule displays a regular and highly sulfated sequence, (2-*O*-sulfated iduronic acid linked

to *N*- and 6-*O*-sulfated glucosamine), so that the exact sulfation pattern recognized by gp120 is hidden in the fully sulfated oligosaccharide. HS synthesis is notoriously difficult, and in view of its huge diversity (see **Figure 1**), the synthesis of a library addressing structure-activity relationships is not realistic. In an effort to pinpoint the sulfate groups that are functionally essential to gp120 binding, the HS was substituted with sulfotyrosine-containing HS mimetic peptide, the synthesis of which is much more straightforward, and more easily amenable to sequence-activity relationship investigation. This molecule compares very well with HS_{12}, and when conjugated to mCD4 broadly inhibits the replication of several HIV-1 strains with an IC_{50} of 1 nM (100), thus, opening the route to future developments.

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