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Complementing the sugar code: role of GAGs and sialic acid in complement regulation

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Sugar molecules play a vital role on both microbial and mammalian cells, where they are involved in cellular communication, govern microbial virulence, and modulate host immunity and inflammatory responses. The complement cascade, as part of a host's innate immune system, is a potent weapon against invading bacteria but has to be tightly regulated to prevent inappropriate attack and damage to host tissues. A number of complement regulators, such as factor H and properdin, interact with sugar molecules, such as glycosaminoglycans (GAGs) and sialic acid, on host and pathogen membranes and direct the appropriate complement response by either promoting the binding of complement activators or inhibitors. The binding of these complement regulators to sugar molecules can vary from location to location, due to their different specificities and because distinct structural and functional subpopulations of sugars are found in different human organs, such as the brain, kidney, and eye. This review will cover recent studies that have provided important new insights into the role of GAGs and sialic acid in complement regulation and how sugar recognition may be compromised in disease.

Keywords: sialic acid, heparan sulfate, glycosaminoglycan, complement factor H, properdin, innate immunity, tissue specificity, complement regulation

INTRODUCTION

The complement system plays a vital role in the protection of a host from invading bacteria and other microorganisms. However, this potent immunological weapon must be tightly regulated, or there is a risk of attack of host tissues leading to damage via an inappropriate inflammatory response (1). Sugar molecules provide a diverse and complex means by which the complement system can not only identify bacteria and other invading pathogens as a threat but also identify host surfaces that require protection (2). With three activating pathways of complement, it is the alternative and lectin pathways that utilize sugar molecules the most (1). The lectin pathway is activated by the recognition of carbohydrate moieties, such as mannose or glucose, on the surface of bacteria, by the mannose-binding lectin or ficolins (3, 4). On the other hand, the alternative pathway of complement is modulated in host tissues by glycans such as sialic acid [the predominant form being *N*-acetylneuraminic acid (Neu5Ac)] or the glycosaminoglycan (GAG) chains of proteoglycans. The presentation of specific sialic acid or GAG structures on the surface of a cell, or within the extracellular matrix, can dictate whether positive or negative regulation of an immune response occurs, including complement activation. This is because the sugar compositions of both GAGs and sialic acid can vary greatly from one organ to another and even between different regions/microenvironments within the same tissue (2, 5, 6).

Glycosaminoglycans and sialic acid play an important role in the recruitment and control of a wide range of innate/cellular immune system regulatory proteins, as well as proteins involved in tissue remodeling following an inflammatory response (7, 8). For example, GAGs are key regulators of pulmonary inflammation during lung infection through their binding of cytokines, chemokines, and growth factors, which leads to leukocyte adhesion and accumulation (9). Interestingly, the protein tumor necrosis factor-stimulated gene-6, which plays a role in protecting tissues from the damaging effects of inflammation, has recently been found to antagonize the interaction of the chemokine CXCL8 with the GAG heparan sulfate (HS) on the surface of endothelial cells and thereby inhibit neutrophil extravasation (10). In this mini-review, we will concentrate on the role of sulfated GAGs (particularly HS) and sialic acid on the recruitment and regulation of components of the complement cascade.

Abbreviations: AMD, age-related macular degeneration; aHUS, atypical hemolytic uremic syndrome; BM, Bruch's membrane; CCP, complement control protein; DS, dermatan sulfate; FH, factor H; FHL-1, factor H-like protein 1; HS, heparan sulfate; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcN, *N*-glucosamine; IdoA, iduronic acid; Neu5Ac, *N*-acetylneuraminic acid.

MODULATION OF COMPLEMENT BY SULFATED GAGs

There are four different types of sulfated GAGs that are found ubiquitously in human tissues – namely chondroitin sulfate, dermatan sulfate (DS), HS, and keratan sulfate – all of which are attached to proteoglycan core proteins and have considerable diversity in their "sequence" of sugars (11). Of these, HS is the most structurally diverse and plays a vital role in cell differentiation, signaling, and immune homeostasis (12–16). The HS chain comprises repeating disaccharide units of a glucuronic acid (GlcA) or iduronic acid (IdoA) linked to *N*-glucosamine (GlcN) (17, 18). As shown in **Figure 1A**, each disaccharide has four positions that can be variably modified with sulfation (or acetylation in the case of the *N* position of GlcN) and, along with the epimerization of some GlcA sugars to IdoA, this allows for immense structural diversity of HS chains that are typically 50–200 disaccharides in length. This diversity is made more complex by the subdivision of HS chains into *N*-sulfated (NS) regions and *N*-acetylated (NA) regions (of variable length) separated by small "transition" (NS/NA) zones (see **Figure 1B**). Overall, it is this complexity that provides a broad range of structures that can be recognized differentially by proteins, such that the biosynthesis of distinct "sequences" at particular tissue sites can promote/regulate their binding within a particular microenvironment (2, 19). For example, the complement regulatory proteins factor H (FH) and factor H-like protein 1 (FHL-1), a truncated version of FH generated through alternative splicing [that has 7 rather than 20 complement control protein

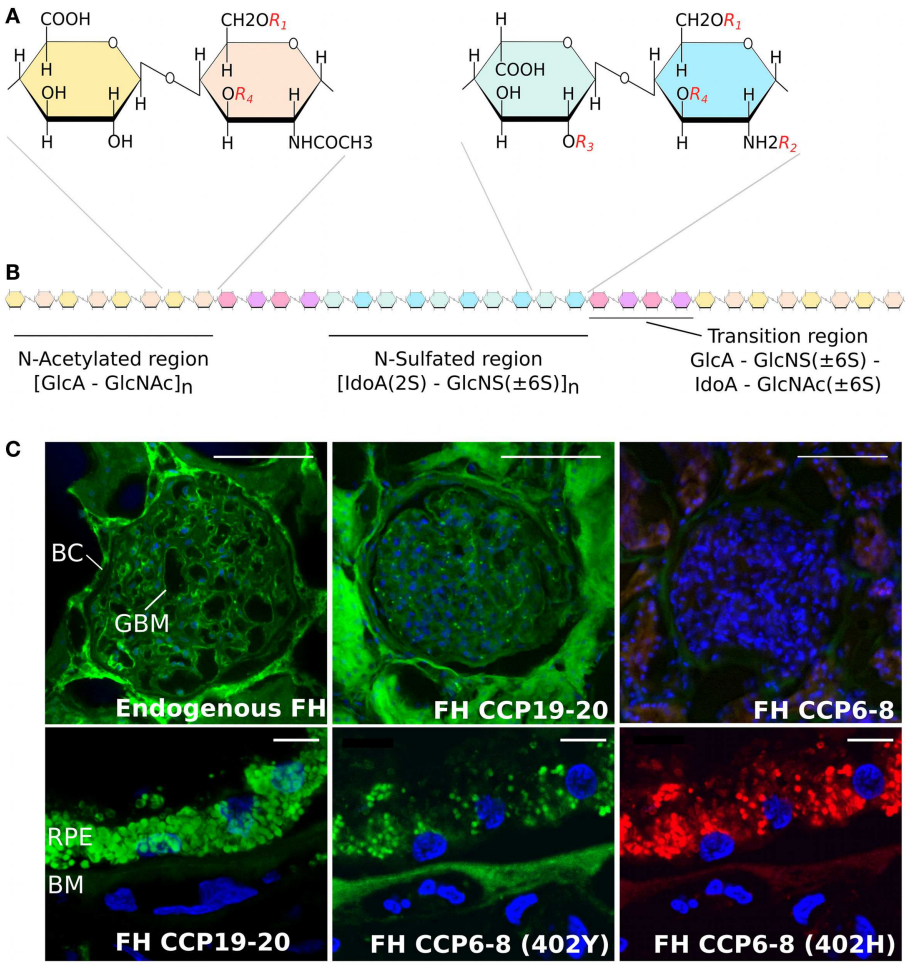

FIGURE 1 | Structure of heparan sulfate and the binding of factor H to human kidney and eye tissue. **(A)** Schematic showing disaccharide structures found in the HS chain. These are comprised of glucuronic acid (GlcA) and *N*-acetylated glucosamine (GlcNAc), found predominately in the *N*-acetylated region, and iduronic acid (IodA) and *N*-sulfated glucosamine (GlcNS) that are found in the *N*-sulfated region. The four possible sulfation positions are listed as: R_1, 6-*O*-sulfation; R_2, *N*-sulfation; R_3, 2-*O*-sulfation; and R_4, 3-*O*-sulfation. **(B)** Diagram demonstrating the distribution of the *N*-acetylated and *N*-sulfated regions of HS and their separation by short transition regions. **(C)** Staining of human kidney glomeruli (top panels) and the macula region of the human eye (lower panels) for endogenous FH and FH CCP6–8 and CCP19–20 binding sites; for full details, see Ref. (21). Endogenous FH (green staining) can be seen in both the Bowman's capsule (BC) and glomeruli basement membrane (GBM) in the human kidney, where this binding is predominately mediated by the CCP19–20 region of the protein. However, the CCP19—20 region of FH binds poorly to the Bruch's membrane (BM) of eye, where the interaction of FH is predominantly mediated by CCP6—8. The Y402H polymorphism, found in CCP7, alters the binding of FH to BM, demonstrated by the lack of red staining in the bottom right hand side panel. Scale bars in the top panels of **(C)** represent 100 μm, and in the lower panels represent 10 μm.

(CCP) repeats], prevent inappropriate alternative pathway activation/amplification in host tissues; in part, this is mediated by their binding to HS (and DS) on cell surfaces and within the surrounding matrix (20–22). One particular variant of FH/FHL-1 (termed 402H; that has a histidine at residue 402 in CCP7) is associated with an increased risk of age-related macular degeneration (AMD), a common cause of blindness in developed nations, and requires a high level of HS sulfation for its binding (23, 24). Because such highly sulfated sequences are rare within the human Bruch's membrane (BM) (an extracellular matrix of the eye), this might be the underlying cause of why complement dysregulation occurs at this site; i.e., due to insufficient FH/FHL-1 binding in 402H individuals (20, 22), leading to local inflammation that drives AMD pathology. FHL-1 has been found to be the major form of FH within BM (22) and unlike FH does not have a second GAG-binding domain (in CCP19–20) to compensate for its impaired tissue recognition; FHL-1 also lacks the sialic acid-binding site in CCP20 (see below). Importantly, the recent finding that the overall amount of HS in BM falls during normal aging (accompanied by a significant reduction in the level of sulfation) might explain the age-related nature of AMD (25); i.e., further impairing binding of the 402H variant of FH/FHL-1. Age-dependent changes in the sulfation patterns of HS have also been reported in tissues such as in the aorta (26) and in outgrowth endothelial cells (27); in the latter, a decrease in the amount of 6-*O*-sulfation with age results in a decrease in the migratory capacity of these cells toward vascular endothelial growth factor and stromal cell-derived factor 1α.

Properdin has an opposing role to FH/FHL-1 in that it is a positive regulator of the complement system (28). Properdin stabilizes the alternative pathway C3 convertase (C3bBb) allowing more conversion of C3 into C3b and thus amplification of complement activation. Because properdin exists as oligomers (dimers, trimmers, and tetramers), which can bind multiple C3b molecules, it can therefore act as a platform for the assembly of additional C3 convertases (29, 30). It has also been demonstrated that properdin can bind to HS and chondroitin sulfate on apoptotic T cells, thereby aiding their clearance by promoting complement-mediated opsonization/phagocytosis (31, 32). Furthermore, it has been shown that properdin and FH bind distinct HS sugars on renal tubular epithelial cells (33, 34) demonstrating the power of GAGs to mediate immune homeostasis on tissues by recruiting both positive and negative regulators of complement through the presentation of different sulfation patterns [reviewed in Ref. (28)].

MODULATION OF COMPLEMENT BY SIALIC ACID

Sialic acid also mediates complement interactions and this family of sugars is typically found at the termini of the *N*- and *O*-linked glycans substituting mammalian cell surface and secreted proteins (35, 36). The basic nine-carbon structure can be modified at the 4, 5, 7, 8, and 9 positions to generate a large amount of structural diversity (see **Figure 2**). It is the C2 carbon that forms the glycosidic bond to the neighboring sugar, i.e., at multiple different positions, allowing for variation in its orientation of presentation (35, 37, 38). Like GAGs, sialic acids can also control the activation of complement through binding FH; e.g., on erythrocytes, conferring protection from the spontaneous tick over of the alternative pathway (39). The binding of FH to sialic acid results in an increased affinity for C3b and thus enhances its cofactor and decay accelerating activities. However, FH binding can be influenced by the type and modifications of sialic acid, e.g., 9-*O*-acetylation of sialic acid reduces the affinity for FH (39, 40). In this regard, the molecular mechanism by which FH can attach to surfaces via sialic acid, while simultaneously binding C3b, has recently been elucidated (41); crystal structure analyses identified the amino acid residues in the CCP20 domain of FH that bind the glycerol side chain (C7–C9) and carboxyl group of *N*-acetylneuraminic acid (Neu5Ac). Furthermore, it was shown that there is a high level of specificity in the interaction of FH with Neu5Ac since this is dependent on the type of glycosidic bond present; i.e., FH binds α2–3, but not α2–6 or α2–8 sialic acid linkages. Mutations in the residues in FH that are responsible for recognizing sialic acid are associated with the rare kidney disease atypical hemolytic uremic syndrome (aHUS) (42). These changes perturb the Neu5Ac binding pocket and reduce the affinity of FH for sialic acid, providing a biochemical explanation for poor complement regulation on the glomerular endothelium in aHUS (41).

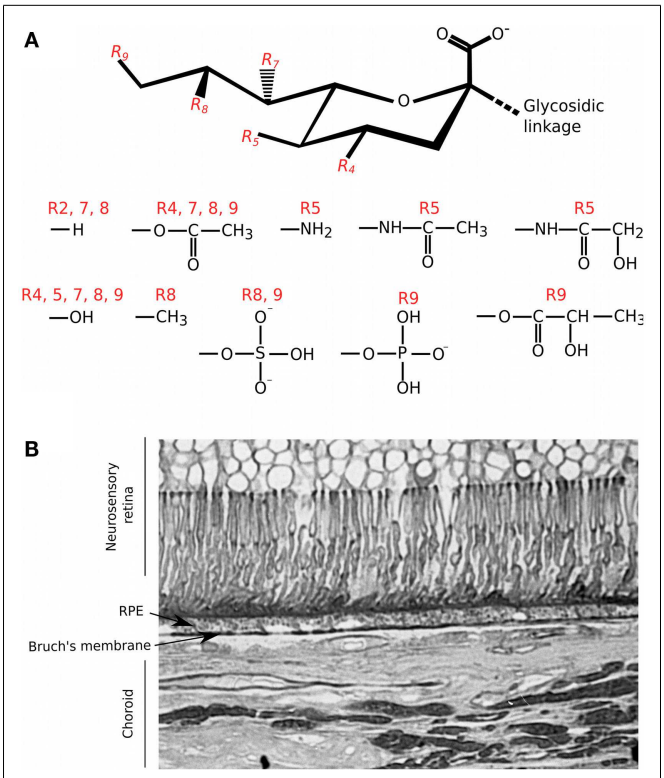

FIGURE 2 | Structural diversity of sialic acids and their distribution in the human eye. **(A)** Schematic of the basic 9 carbon structure of sialic acid and some of the possible substitutions (35); it is the C2 position that forms the glycosidic linkage to other saccharides within *O*- and *N*-linked glycan chains. R4, R5, R7, R8, and R9 groups are variably modified with the chemical groups illustrated. **(B)** Staining of a tissue section of human macula with the *Maackia amurensis* (MAA) lectin was carried out as described in Bishop et al. (5); MAA has high affinity for sialic acid linked α2–3 to galactose (Neu5Acα2–3Gal). Staining with MAA is seen throughout different structures of the eye but with particular intensity on Bruch's membrane.

The CCP19–20 region of FH is also known to bind HS (43), most likely at an interaction surface overlapping that for sialic acid (41); thus there is the possibility that these sugars might compete for binding, e.g., on cell/matrix surfaces where both are present. Although this sialic acid/GAG-binding site in CCP19–20 does not contribute greatly to FH's binding to BM (21), it is known that sialic acid is present throughout the human eye (5); this includes Neu5Acα2–3 (see **Figure 2B**). Therefore, it is possible that sialic acid may contribute to the binding of FH through other, as of yet uncharacterized, sites. Indeed, treatment of eye tissue with HS/DS-degrading enzymes only reduced endogenous FH levels by ~50%, consistent with the possibility that sialic acid could also be mediating binding to structures including BM (20).

TISSUE SPECIFICITY

As described above, both GAGs and sialic acid display considerable molecular diversity. However, importantly, there are differences in the populations of structures/sequences of these sugars found within different tissues. For example, HS is thought to play a regulatory role in many physiological processes (13–15) through the tissue-specific (or least tissue-restricted) biosynthesis of particular sulfation patterns as a form of "zip code" [reviewed in Ref. (2, 19)]. Its variations in sequence pattern can even be seen between different regions of the same tissue, as illustrated by the distinct HS epitopes mapped within the human macula (6), within pancreatic islets (44), and in the human kidney (45).

There is also evidence that functional HS "area codes" are different in the human kidney to those found in the human eye (21); i.e., those that mediate FH binding. Like BM, the glomerular basement membrane is an extracellular matrix that protects itself from complement attack by recruiting FH, in this case, through its CCP19–20 domain binding (at least in part) to HS (see **Figure 1C**). It has been shown previously that, while the CCP19–20 region mediates the binding of FH to glomeruli, it is the CCP6–8 region that is mainly responsible for binding to HS (and DS) in BM (20, 21). This demonstrates a level of specificity in the biosynthesis of functional HS sequences in the different tissues, or alternatively, that the binding specificity of these two regions of FH has become tuned to the different "compositions" of HS found in the two locations. This is also consistent with the observation that mutations in the CCP19–20 region of FH (46, 47), which are mainly associated with aHUS, do not present with an ocular phenotype but frequently effect heparin/HS binding [see Ref. (21, 48–50) for further discussion]. Similarly, the Y402H polymorphism in CCP7 of FH/FHL-1, a major risk factor for AMD, does not predispose individuals to kidney disease.

Transgenic mouse studies demonstrate that knocking out expression of FH causes aHUS (51) as well as some features that resemble AMD (52). Furthermore, by expressing a form of murine FH without CCPs16–20, it was demonstrated that this region of FH is important in the development of aHUS (53); this is consistent with the recent findings that the CCP19–20 region of FH likely plays a critical role in self recognition in kidney glomeruli through its binding of HS and/or sialic acid (21, 41). In fact, it has been proposed that FH is held in an inactive "latent" conformation by intramolecular interactions and upon binding to HS or sialic acid the conformation changes to one that has higher affinity for C3b and increased co-factor activity (54, 55). Therefore, the presence of HS or sialic acid on host cells may regulate not only the localization of FH but also the affinity for C3b.

Infection with enterohemorrhagic *E. coli* can also cause typical (or infection-induced) HUS; the shiga toxin produced by the bacteria can bind directly to the CCP6–8 or CCP18–20 regions of FH and impairs cofactor activity on cell surfaces but not in the fluid phase (56). Thus, it seems likely that surface recognition mediated by these regions of FH is inhibited through their binding to shiga toxin, although this requires further investigation. Similarly, the condition dense deposit disease can be caused by systemic loss of FH, normally due to mutations affecting the protein structure or its secretion. The resulting global dysregulation of complement results initially in progressive nephropathy with dense drusen-like deposits in the glomerular basement membrane, and later with drusen formation in BM of the eye (55, 57). However, the Y402H polymorphism in the HS-binding site of FH is associated with increased risk of dense deposit disease (58), so a role for GAGs (or sialic acid) is not an impossibility, but this coding change does also affect other functional activities of FH [see Ref. (12)].

The exciting work from Blaum and co-workers (41) has demonstrated that the CCP20 region of FH mediates considerable specificity for particular sialic acid structures (i.e., for Neu5Acα2–3), where amino acid residues involved in their recognition are associated with complement dysregulation in the kidney (46, 47). This suggests that there may be parallels with FH's tissue specificity for GAG binding (21). In this regard, we know that distinct sialic acid structures are present in different parts of the eye, including within BM (5) and, therefore, it will be interesting to see whether different regions of FH differentially recognize sialic acids in a tissue-specific manner.

The brain is another organ where interactions of complement with host sugars have been found to contribute to immune homeostasis and become dysregulated in disease; in this context, it is believed that complement proteins, including FH, are synthesized locally within brain tissue (59). For example, FH has been shown to associate with the brain lesions of Alzheimer's disease patients through the binding of HS (60), changes in HS structure are associated with disease progression (61). HS has been shown to bind amyloid-β (62) where this is modulated by the level of HS sulfation (63). In fact, it is believed that neurotoxic amyloid-β competes with neuroprotective fibroblast growth factor 2 for a common HS binding site (63). Furthermore, it has been suggested that the presence of amyloid-β prevents the heparanase-mediated turnover of HS chains (64), which could lead to enhanced binding of FH to HS structures within brain lesions, hindering their clearance by complement. HS has also been shown to regulate the processing of the amyloid precursor protein to amyloid-β by the Alzheimer's beta-secretase, BACE-1 (65). This is mediated via direct binding of HS to this enzyme, where the specificity of the interaction, e.g., with regard to sulfation pattern, has allowed the generation of heparin derivatives and HS oligosaccharides with therapeutic potential for Alzheimer's disease (66, 67).

The presence of sialic acid on neuronal cells can prevent the activation of the classical complement pathway by masking the binding sites for C1q (68). The removal of sialic acid results in

C1q binding, activation of the classical pathway, and opsonization of the neuronal cells with C3b; microglial cells in the brain can then recognize C3b via Complement Receptor 3 (CR3) and activate the phagocytosis of these labeled cells. It has been postulated that the presence of sialic acid on the cell surface acts as a marker of cellular health that may be lost/impaired during inflammation and oxidative stress (69).

MODULATION OF THE COMPLEMENT RESPONSE BY PATHOGENS

As described already, FH has two HS-binding regions and at least one site for interaction with sialic acid and with its flexible, modular, structure FH is capable of interacting with several self-ligands on the host surface simultaneously (70), which is believed to enhance its binding avidity. This allows for the recognition of a diverse range of cell and tissue types as well as making it harder for microorganisms to recruit FH to avoid host defense. However, the interplay between host and pathogen is like a constant weapons race. It is therefore not surprising that pathogens have evolved ways to mimic these self-associated molecular patterns (SAMPs) (71). Many human pathogens, including *Pseudomonas aeruginosa* have in common with human cells the sialic acid, Neu5Ac, on their surface (72), which allows them to recruit FH from the blood and thereby prevent a complement-mediated response (73). *Neisseria gonorrhoeae* also have surface sialic acid and this was shown to bind FH in the CCP16–20 region (74), and in light of recent discoveries, the sialic acid is likely to bind CCP20 (41). Bacteria either synthesize the sialic acid *de novo* or acquire it from their host by secreting a sialidase enzyme that cleaves sialic acid from host cells, which can then be taken up and presented via bacterial transporters (75). Currently, no pathogens have developed the ability to create sulfated GAGs (71, 76). However, bacteria have developed proteins that mimic host carbohydrates such as *Neisseria meningitides*, which produces a FH-binding protein that has been shown to bind to the CCP6–8 region of FH (77).

MODULATION OF THE COMPLEMENT RESPONSE BY CANCER

Like pathogens, cancer cells can also protect themselves from complement-mediated immune activation (78). FH and FHL-1 expression is up-regulated in some cancers (79) and inhibition of their expression reduces the growth rate of the cells *in vivo* (80). Cancer cells also commonly up-regulate sialic acid synthesis (81), possibly by up-regulating sialyltransferases (78), to reach a state that has been coined "super-self" (82). It is thought that increased surface levels of sialic acid confer protection against complement by recruiting FH (83) – removing sialic acid from cancer cells enhances their complement-mediated lysis (84) – and contributes to immune evasion from NK and other immune cells by non-complement-mediated mechanisms (78). Interestingly, many breast cancer cells have an increased amount of HS proteoglycans on their surfaces compared to normal mammary cells (85), and therefore, it is tempting to hypothesize that the up-regulation of this SAMP, like sialic acid, confers increased protection of cancer cells to complement by recruiting FH.

CONCLUSION

The structural diversity of GAGs and sialic acids makes a significant contribution to the regulation of immune homeostasis through the formation of "sugar postcodes" in human tissues. In particular, these sugars represent molecular signals capable of specifically recruiting either complement inhibitors, or activators, to a host surface in a tissue-specific fashion. Recent evidence suggests that changes to the GAG/sialic acid "repertoire" in a particular tissue, whether caused by disease or normal aging, can result in an inappropriate complement response and tissue damage. In some circumstances, it may be possible to correct this dysregulation of the innate immune system; e.g., the use of modified GAGs that interfere with the binding of properdin (but not FH) to HS on renal tubular epithelial cells might be of benefit in proteinuric renal disease (33, 34). As such, drugs aimed at modifying complement–sugar interactions in a tissue-specific manner could represent a viable therapeutic option in a number of disease contexts.

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