THE ROLE OF GLYCANS IN IMMUNE CELL FUNCTIONS

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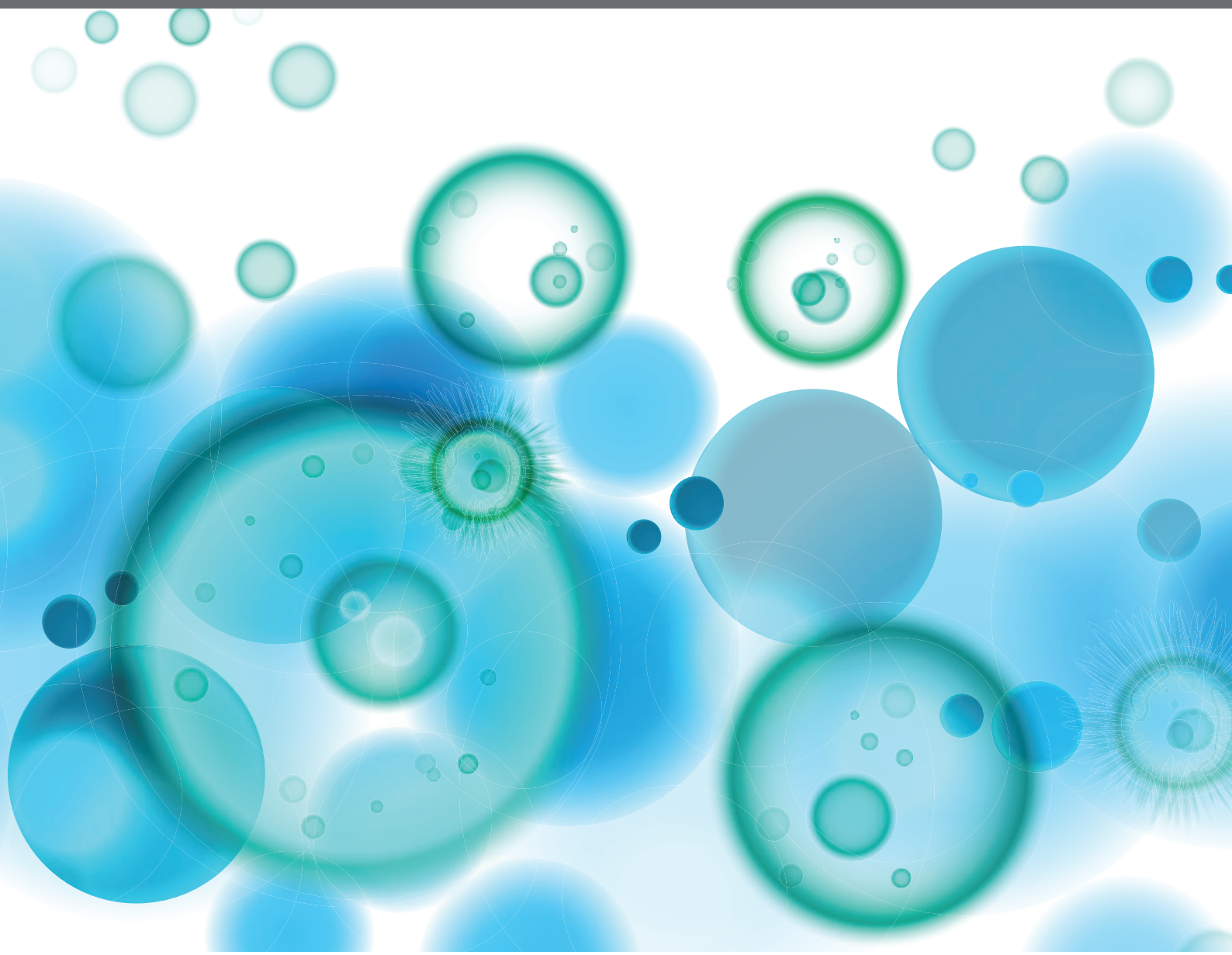

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THE ROLE OF GLYCANS IN IMMUNE CELL FUNCTIONS

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Glycans represent a major constituency of post-translational modifications that occur on most, if not all, proteins. Whether on mammalian or invertebrate cell surfaces, they exist as sugar chain moieties designed from the exquisite and coordinated activity of cell-specific glycosylation. Some of the more common glycan structures are linked to cell surface polypeptides via an asparagine (N)-linked residue or a serine/threonine (O)-linked residue, along with a notable contingent found linked to ceramides in the lipid bilayer known as glycosphingolipids. These glycans can associate with complementary glycan-binding proteins (GBP) or lectins to mediate and translate this carbohydrate recognition to cell function.

In immunity, there is increasing evidence that precise immune cell glycans are recognized by corresponding GBPs in a cell-intrinsic or -extrinsic manner. Unique carbohydrate recognition domains within GBPs are comprised of precisely spaced amino acid functional groups that allow for selective engagement of a particular glycan target. This structure-function relationship is present in immune signaling pathways, whereby glycans and GBPs on the surface of immune cells (and non-immune cells) help control processes such as immune cell activation, recognition of pathogens, suppression and tissue-specific migration. The diversity of glycan structures and glycosylation among individual immune cell subsets is controlled by the expression of genes involved in glycan biosynthesis including glycosyltransferases, glycosidases, glycan-precursor biosynthetic enzymes and nucleotide-sugar transporters. These genes represent more than 3% of the human genome, and cell-specific expression of these genes dictates a cell's glycan repertoire, ultimately influencing its molecular interactions with GBPs. Altogether, these emerging lines of investigation highlight the regulatory capacity of glycans in immune health and disease, which in turn, pave the way for novel diagnostic, prognostic, and therapeutic strategies.

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MINI REVIEW
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Lectin-Glycan Interactions in Corneal Infection and Inflammation

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The cornea is an extraordinary component of vision that functions as the principal barrier to pathogens in the eye while allowing light transmission into the retina. Understanding the cellular and molecular mechanisms that maintain homeostasis in this tissue is the subject of intense scientific study given the high prevalence of corneal disease. Over the past decade, the interactions between lectins and glycans on plasma membranes have emerged as important regulatory factors in corneal biology. In particular, members of the galectin family have been shown to bind multiple β-galactoside-containing receptors to regulate immunopathological processes associated with viral and bacterial infection, transplantation, wound healing, dry eye, angiogenesis, and lymphangiogenesis. In this review, we describe the current understanding of how these surface interactions intersect with different pathways to activate unique cellular responses in cornea as well as their potential therapeutic implications.

Keywords: cornea, galectin, glycosylation, infection, inflammation

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INTRODUCTION

Lectins are proteins widely distributed among the animal kingdom that specifically recognize carbohydrates. Traditionally, they have been classified based on their ability to recognize specific carbohydrate sequences but, with the advent of new molecular biology methods, novel classes have been defined based on the presence of unique structural domains within their amino acid sequences. This novel classification stems from the presence of highly conserved carbohydrate-recognition domains (CRDs) that appear to have evolved from shared ancestral genes (1). Examples of major families of animal lectins include C-type (e.g., selectins, dectins), I-type (e.g., siglecs), P-type (mannose-6-phosphate receptors), and S-type (galectins). Among the different classes of lectins described so far, galectins have been the most extensively characterized in cornea and are the major focus of this review.

Galectins are expressed by different cell types, including epithelial, stromal, endothelial, and immune cells and typically bind β-galactose-containing glycoconjugates. They are grouped into three categories based on structure: (1) prototypical, with a single CRD that may associate to form homodimers, (2) chimeric, with a single CRD and a large amino-terminal domain that contributes to self-aggregation and, (3) tandem-repeat, with at least two CRDs occurring within a single polypeptide (2). Members of these different categories have been reported in humans and include galectins-1,−2,−7,−10,−13, and−14 (prototypical), galectin-3 (chimeric) and galectins-4,−8,−9, and−12 (tandem-repeat). Each galectin CRD recognizes distinctive carbohydrate structures in a manner that is influenced by the oligomeric state of the lectin and the multivalency of the glycan ligand (2). Galectins are exceptional in that they are synthesized on free ribosomes, exhibit no signal sequence and are secreted through a non-classical pathway that bypasses the Golgi (3). Only

a few amino acids within the canonical CRD of galectins make direct contact with carbohydrate ligands, although binding sites for non-carbohydrate ligands, such as those found in the cytosol and nucleus, have also been described on the CRD. The presence of these binding domains ensures that galectins have both intracellular and extracellular activities. On the cell surface, galectins function by forming multivalent complexes with glycosylated receptors to control multiple biological events, such as receptor turnover, cell signaling, host–pathogen interactions and immune cell activation and homeostasis (4).

Other lectins that mediate biological events in cornea include selectins and dectins. Selectins are cell adhesion molecules expressed on platelets, endothelial cells, and leukocytes. They contain a single transmembrane domain and a CRD at the amino terminus with affinity toward sialylated, fucosylated structures (e.g., sialyl Lewis x) (5). Dectins are transmembrane proteins important in fungal defense expressed mainly in dendritic cells and macrophages (6). The two members of this family, dectin-1 and dectin-2, recognize β-glucans, and α-mannans, respectively.

STRUCTURE OF THE CORNEA

The cornea is a clear, curved surface covering the anterior segment of the eye. It is responsible for refracting light onto the lens and retina in addition to resisting infection and damage. The lack of lymphatic and blood vessels is essential to maintaining the transparency of the cornea. Injury resulting from infection, transplantation, autoimmune conditions, and other pathologies can lead to the abnormal growth of vessels and loss of vision (7).

Structurally, the cornea consists primarily of the epithelial, stromal and endothelial compartments (**Figure 1**). The epithelial compartment is the outermost surface and it is composed of a stratified, non-keratinized epithelium along with intraepithelial nerve terminals and dendritic cells. The stromal compartment is a dense connective tissue of significant regularity and represents the structural axis of the cornea. It is populated with keratocytes that synthesize extracellular matrix components and bone marrow derived cells that are recruited in response to injury and infection. The endothelial compartment is a simple low cuboidal epithelium that enables the exchange of ions and fluid between the stroma and the interior of the eye. The cornea is encircled by the corneoscleral limbus, which serves as a reservoir for the adult stem cell population that continuously replenishes the tissue. The use of histochemical techniques has evidenced that the cornea is rich in galectins and galectin-binding sites (8). In normal corneas, galectin-1 is present mainly in the stroma, galectin-3 localizes mainly in the epithelium, and galectins-7,−8, and−9 are present in both corneal epithelium and stroma (9).

LECTIN-GLYCAN INTERACTIONS IN CORNEAL PHYSIOLOGY

The apical surface of the corneal epithelium constitutes an exceptional barrier against foreign particles and microorganisms that attempt to penetrate the eye. Highly glycosylated transmembrane mucins emanating from ridge-like folds of the plasma membrane are an essential component of this protective layer. They have single membrane-spanning regions with large extracellular domains that form rod-like structures, which extend over 100 nm from the cell surface, far above other glycoconjugates in the glycocalyx (10).

Research over the past decade has defined a mechanism by which transmembrane mucins contribute to the physiological protection of the corneal epithelium by interacting with galectins. Microarray analyses have revealed that the mucins MUC1 and MUC16 together with galectin-3 are among the most highly expressed glycogenes at the ocular surface (11). They localize primarily on apical membranes within the superficial stratified squamous epithelia, and the two mucins bind galectin-3 in a carbohydrate-dependent manner. Importantly, the mucin-galectin interaction is necessary to maintain galectin-3 anchored to the cell surface and to preserve transcellular barrier function in corneal epithelial cells (12). The association between transmembrane mucins and galectin-3 further functions to mask viral entry mediators on the corneal epithelial glycocalyx (13). Mechanistically, this protective function of galectin-3 is dependent on its large amino-terminal domain and the ability to form surface lattices in the epithelial glycocalyx (14).

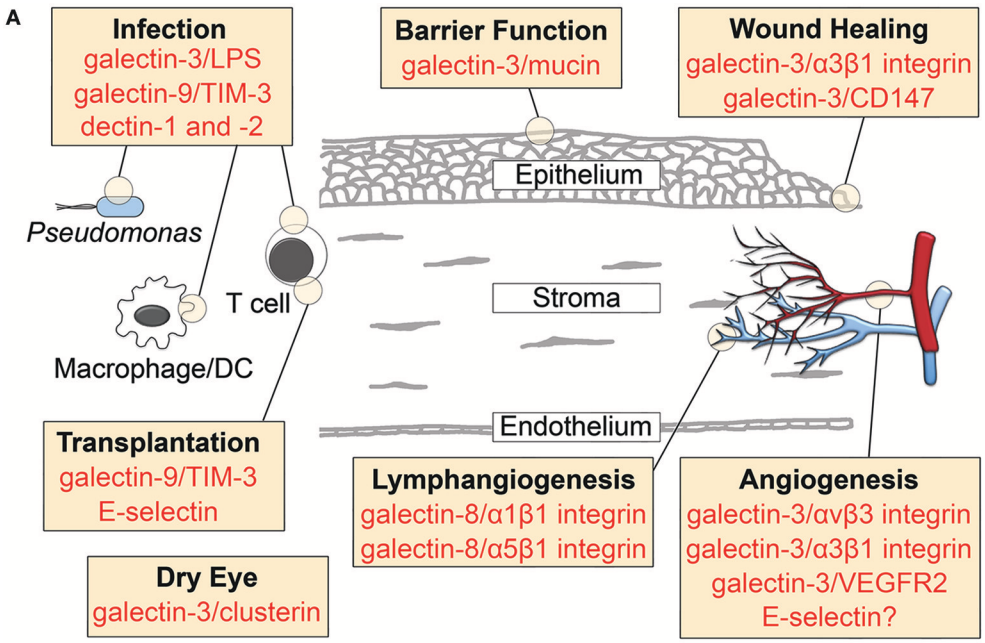
Core 1 O-glycans are major components on transmembrane mucins at the ocular surface (15). Initial experiments targeting c1galt1, a critical galactosyltransferase required for the synthesis of core 1 O-glycans, evidenced the contribution of this modification to promoting surface retention of galectin-3 and maintaining barrier function (12). Yet, use of synthetic glycan microarrays has shown that galectin-3 displays maximum binding affinity toward N-glycans compared to O-glycans (16), implying a role for mucin N-glycans in the stabilization of the epithelial glycocalyx despite having a much lower abundance than O-glycans. Recent evidence supports this hypothesis. Structural data indicate that mucin N-glycans in cornea are rich in complex-type structures that bind galectin-3 and promote barrier integrity (17). Deciphering the relative contributions and biological significance of the different classes of mucin glycans when interacting with galectins should be an important goal of future research on mucosal surfaces.

LECTIN-GLYCAN INTERACTIONS IN CORNEAL PATHOLOGY

Ocular Infection

Microbial colonization of the eye due to viral, bacterial, or fungal pathogens remains an important cause of blindness worldwide. Several findings provide strong evidence that lectin-glycan interactions play an important role in the pathogenesis and immune response to ocular infection.

Primary or recurrent episodes of herpes simplex virus (HSV) infection result in viral replication and destruction of the infected cells. This process triggers non-specific innate host defenses that contribute to infection control but also adaptive responses when dendritic cells leave the site and carry viral antigens to draining lymph nodes (18). A large number of activated T cells in ocular HSV lesions express the inhibitory molecule TIM-3 needed



B

Lectin	Example of preferred glycan ligand
Galectin-3	Repeating LacNAc core
Galectin-8 N-terminal	α2-3-Sialylated β-galactoside
Galectin-8 C-terminal	Blood group A on LacNAc
Galectin-9 N-terminal	Forssman pentasaccharide
Galectin-9 C-terminal	Repeating LacNAc core
Dectin-1	β-Glucans
Dectin-2	α-Mannans
E-selectin	Sialyl Lewis x/a

FIGURE 1 | Lectin-glycan interactions reported in cornea. **(A)** Schematic diagram illustrating the involvement of lectins in cornea. Specific binding partners are indicated for galectins. **(B)** Examples of preferred glycan ligands for lectins shown in **(A)**. DC, dendritic cell; LacNAc, N-acetyllactosamine; LPS, lipopolysaccharide; TIM-3, T cell immunoglobulin- and mucin-domain-containing molecule-3.

to control the lesion. Addition of excess galectin-9, a natural ligand of TIM-3, has been shown to diminish the severity of the lesions by inducing apoptosis of pathogenic effector Th1 cells but also increasing the representation of anti-inflammatory Tregs and decreasing neovascularization (19). Subsequent studies have shown that the interaction of galectin-9 with TIM-3 functions to constrain the response of effector and memory $CD8^+$ T cells to infection (20). Other galectins, such as galectin-1, can also lessen the severity of the HSV lesion by reducing the number of IFN-γ- and IL-17-producing $CD4^+$ T cells and the recruitment of neutrophils into the cornea (21).

It was the Hazlett laboratory that first reported in 1997 the presence of a member of the galectin family in cornea and its potential pathogenic contribution to bacterial infection. Using binding inhibition assays, this group found that adhesion of *Pseudomonas aeruginosa* to corneal epithelial cells could be blocked by an antibody targeting galectin-3, a binding receptor for bacterial lipopolysaccharides (22). Further work established the pattern of expression of galectins in mouse corneas under normal and infective conditions. Exposure to *P. aeruginosa* resulted in overall downregulation of galectin-3 and upregulation of galectins-8 and−9 (9). Galectin-1 within the corneal stroma appeared to limit *P. aeruginosa*-mediated inflammation by impairing the infiltration of neutrophils and $CD4^+$ T cells, particularly proinflammatory Th17 cells (23).

Fungal infection is a major cause of corneal ulceration in developing countries and tropical regions commonly associated with severe inflammation. Evidence suggests that the C-type lectin receptors dectin-1 and dectin-2 play important roles in regulating disease severity and survival. Dectin-1 on corneal macrophages can be activated by β-glucans on *Aspergillus fumigatus* to promote recruitment of neutrophils into the corneal stroma and trigger fungal killing (24). Interestingly, to promote survival, *A. fumigatus* spores express RodA hydrophobin, a

surface protein that confers hydrophobicity and covers cell wall components that would otherwise activate dectin-1 and dectin-2 (25). Dectin-1 also plays a critical role in cornea by controlling *Candida albicans* (26) and *Fusarium solani* (27) infections.

Corneal Transplantation

Corneas are among the most common and successful transplanted tissue worldwide. They express factors that contribute to immune privilege by inhibiting the induction and function of alloimmune T cells among others (28). Recent investigations looking at the repertoire of galectins expressed in accepted murine corneal allografts have demonstrated increased levels of galectins-1,−3,−7,−8, and−9 compared to controls (29). Interestingly, when the corneas were rejected, the levels of galectin-8 were markedly higher, whereas those corresponding to galectin-9 were substantially lower, compared to the accepted corneas. The latter complements initial observations showing that constitutive expression of galectin-9 and its ligand TIM-3 play an immunosuppressive role in corneal allografts, in particular by preventing the destruction of corneal endothelial cells by alloreactive T cells (30).

E-selectin is a carbohydrate-binding protein commonly expressed during corneal inflammatory disease (31). It localizes to vascular endothelial cells in the stroma of rejected corneal allografts, within areas with high T cell and macrophage content (32). Because of its crucial role in leukocyte extravasation and migration, E-selectin has been proposed as a therapeutic target in preventing transplant rejection. Recent data indicate that E-selectin mediates T cell recruitment in corneal transplantation and support a role for E-selectin neutralization in reducing the frequency of mature antigen-presenting cells in the draining lymphoid tissue (33). In these experiments, however, the long-term graft survival was limited, which has been attributed to the overlapping function of factors mediating leukocyte adhesion.

Corneal Injury and Wound Healing

Almost 40 years ago Gipson and Anderson reported the requirement of carbohydrate moieties on cell surface glycoproteins and basement membrane to promote epithelial cell migration during the healing of corneal abrasions (34). This initial work pointed to the presence of glucosamine residues on N-glycans that were upregulated as the stratified corneal epithelium became migratory (35, 36). It was not until two decades later than the Panjwani laboratory radicalized the field by implicating galectins in the re-epithelialization of corneal wounds, particularly galectins-3 and−7 (37). The molecular basis by which galectin-3 modulated epithelial migratory events included the promotion of lamellipodia formation by interacting with complex N-glycans on α3β1 integrin, and the initiation of cell-cell disassembly by inducing matrix metalloproteinase expression in a manner that was dependent on the clustering of the matrix metalloproteinase inducer CD147 (38, 39). More recently, the successful use of recombinant galectin-3 in promoting epithelial migration in non-human primate corneas has emphasized the potential of galectins as a novel therapeutic modality in wound healing (40).

It is now clear that not all kinds of injury lead to a similar expression pattern of galectins in cornea. The expression of galectin-3 is downregulated in mouse corneas following bacterial infection and chemical burn (9). Yet, galectins-7,−8, and−9 are upregulated in the epithelium following infection but not cauterization. It also appears that the changes in galectin expression during injury are species-dependent. Whereas tissue damage in mice leads to reduced galectin-3 expression, injured tissue in patients with active corneal ulceration show a greater galectin-3 immunoreactivity compared to normal subjects (41). It is possible to speculate that the inflammatory environment following injury likely influences the differential responses in galectin expression in cornea.

Dry Eye Disease

Disruption of barrier function at the ocular surface is associated with a wide range of inflammatory disorders that includes dry eye, an age-related disease affecting millions of people worldwide, and whose pharmacological treatment remains unresolved. Both N- and O-glycosylation are altered in the ocular surface epithelia of dry eye patients (42), which has led to question whether there are accompanying changes in galectin expression or localization. Several studies have found that epithelial dysfunction in dry eye correlates with the release of cellular galectin-3 into tears (43, 44). This increase in extracellular galectin-3 appears to have pathological implications, since the lectin can interact with the plasma membrane of corneal epithelial cells to exacerbate the proinflammatory activities of IL-1β (45). Of particular interest are recent findings indicating galectin-3 binds to the homeostatic protein clusterin, one of the most abundant transcript in the human corneal epithelium (46). Preserving the nature of this interaction may provide therapeutic value in a variety of drying conditions at the ocular surface (47).

Corneal Angiogenesis

Corneal angiogenesis represents a major public health problem affecting 1.4 million individuals each year in the United States alone (48). The growth of new vessels occurs within the anterior corneal stroma when pro-angiogenic factors overcome anti-angiogenic stimuli. The subject of how glycosylation and galectin-3 impact vascular endothelial cells and influences corneal angiogenesis was reviewed in 2014 (49); therefore, we present a brief overview and highlight additional findings. An important breakthrough in VEGF- and bFGF-mediated angiogenesis was the discovery that galectin-3 plays a pro-angiogenic role in cornea by clustering N-glycans on αvβ3 integrin and activating focal adhesion kinase (50). This function of galectin-3 has been supported by additional data indicating that galectin-3 can activate VEGFR2 in endothelial cells (51) and form a complex with pericyte-derived NG2 proteoglycan and α3β1 integrin to promote endothelial cell motility (52). Examples of ways in which regulation of galectin-3 can have therapeutic applications have been recently described. Strategies to block galectin-3 with small-molecule inhibitors have proven efficacious in experimental models of corneal neovascularization and fibrosis (53).

In addition to galectin-3, other lectins have been implicated in corneal angiogenesis. Galectin-1 and−9 have been shown to possess anti-angiogenic activity in a mouse model of herpetic keratitis, where they decrease the production of proinflammatory cytokines and molecules involved in the formation of new vessels (19, 21). C-type lectins also appear to be critical to the process of corneal angiogenesis. Human soluble E-selectin is known to induce chemotaxis of human endothelial cells and to be angiogenic in rat cornea (54). These contributions, however, remain controversial (55). Additional experiments using corneal micropocket assays have demonstrated a role for the E-selectin cytoplasmic domain in facilitating the antiangiogenic activity of endostatin, a collagen derivative that inhibits endothelial cell migration by binding to α5β1 integrin (56). These findings evidence that formation of new vessels in cornea depends on a delicate balance of lectin-receptor interactions that can either promote or inhibit angiogenic stimuli.

Corneal Lymphangiogenesis

The lymphatic vasculature plays an important role in coordinating antigen transport and immune-cell trafficking from peripheral tissues to secondary lymphoid organs. At the ocular surface and under inflammatory conditions, lymphatics in the limbal region can give rise to new vessels that extend pathologically into the cornea (57). There is scarce information on the role of lectin-glycan interactions in corneal lymphangiogenesis, with just one report implicating galectin-8 (58). Here, the authors demonstrated that galectin-8 is markedly upregulated in inflamed corneas and can promote corneal lymphangiogenesis. Mechanistically, they found that in the absence of VEGFC or VEGFR3, the CRDs of galectin-8 crosslink integrins α1β1/α5β1 and heavily O-glycosylated podoplanin to activate lymphangiogenic signaling. These interactions can potentiate the VEGFC/VEGFR3 axis when present, and further increase the magnitude of the lymphangiogenic response.

CONCLUDING REMARKS

Progress has been made in providing mechanistic insights into the role of lectin-glycan interactions in cornea (**Figure 1**). Manipulating these signals represents a useful approach to control or cure ocular diseases, yet the therapeutic translation of this knowledge faces numerous challenges. For galectins, these stem from their ability to recognize a myriad of receptors on any given cell, each receptor with a unique binding affinity, in a process that is heavily influenced by the metabolic state of the cell and the cellular environment. The extent to which inhibition or activation of specific galectin signaling pathways affect others remains to be better defined, as this knowledge will be critical to produce comprehensive physiological responses. In this regard, any modulation of galectin activity will need to take into consideration the glycosylation state of the cellular receptors to achieve success. We anticipate that a better understanding of the coordinated function of lectins and glycans in cornea will unlock novel therapeutic approaches for pathological states.

AUTHOR CONTRIBUTIONS

DA and PA reviewed the literature and wrote the article.

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Improving Immunotherapy Through Glycodesign

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Immunotherapy is revolutionizing health care, with the majority of high impact "drugs" approved in the past decade falling into this category of therapy. Despite considerable success, glycosylation—a key design parameter that ensures safety, optimizes biological response, and influences the pharmacokinetic properties of an immunotherapeutic—has slowed the development of this class of drugs in the past and remains challenging at present. This article describes how optimizing glycosylation through a variety of glycoengineering strategies provides enticing opportunities to not only avoid past pitfalls, but also to substantially improve immunotherapies including antibodies and recombinant proteins, and cell-based therapies. We cover design principles important for early stage pre-clinical development and also discuss how various glycoengineering strategies can augment the biomanufacturing process to ensure the overall effectiveness of immunotherapeutics.

Keywords: immunotherapy, glycosylation, antibody-drug conjugates (ADCs), monoclonal antibodies, antibody-dependent cell cytotoxicity (ADCC), glycoengineering, metabolic glycoengineering

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INTRODUCTION

Over the past 30 years immunotherapy, a term that encompasses any strategy that induces, enhances, or suppresses the body's natural immune system to treat disease, has emerged as today's preeminent approach to new drug development. In reality immunotherapy is a centuries-old technology, dating from Edward Jenner's discovery in 1796 that inoculation with fluid from cowpox lesions could protect against smallpox. Over the next ∼200 years immunotherapy largely involved vaccine development until the advent of recombinant DNA technology in the 1970s and 1980s opened the door to today's impressive repertoire of immunotherapeutics, which include hormones, cytokines, antibodies, enzymes, and immune cells (1–6). The value of immunotherapeutics reached $107 billion (U.S. dollars) in 2017 with market projections soaring to $180 billion by 2025 (7); this strong projected growth indicates that many new immunotherapies are anticipated in the near future. This article describes how glycosylation is critical for the ongoing success of this important segment of today's burgeoning "biologics" drug market (**Figure 1**) by ensuring the safety and improving the function, activity, efficacy, physicochemical, and pharmacokinetic properties of immunotherapeutics (9–14).

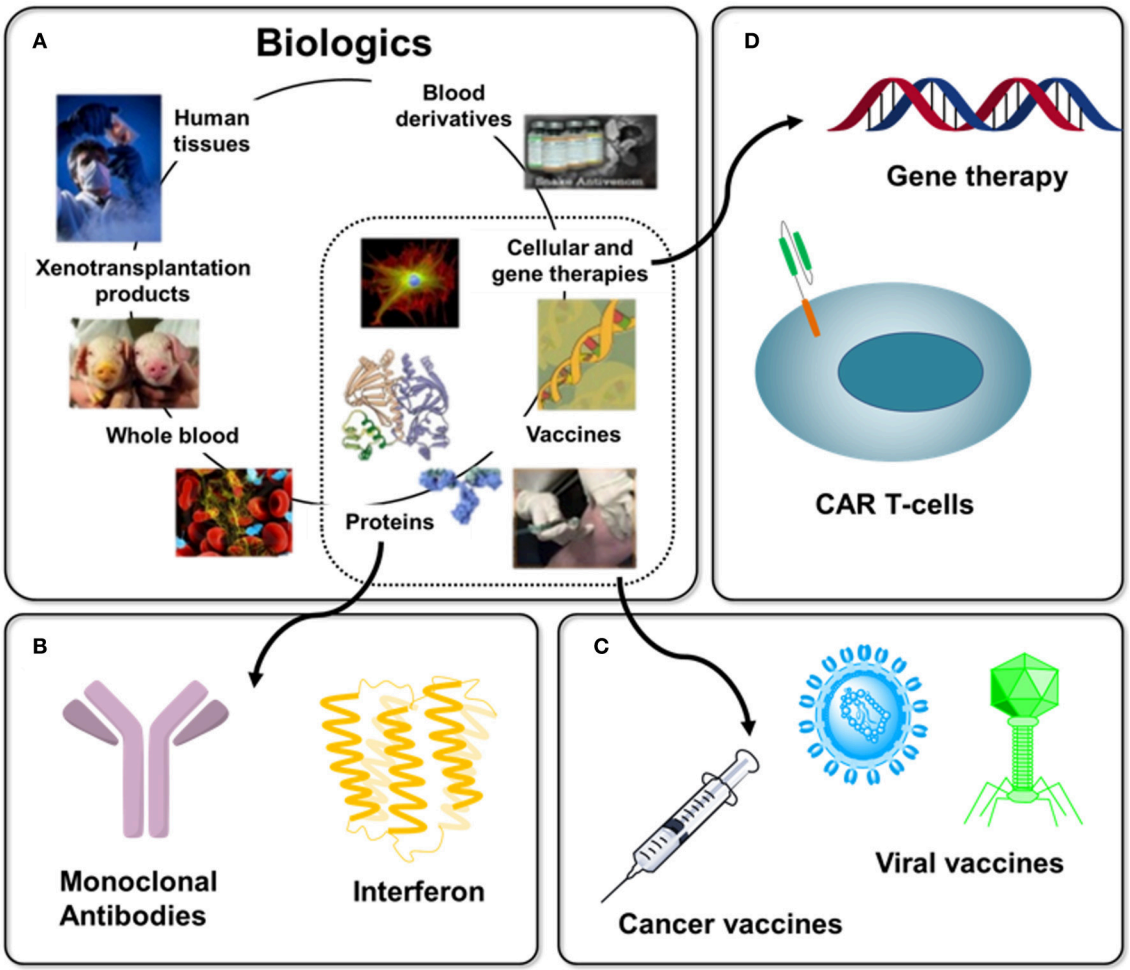

FIGURE 1 | Overview of Biologics with immunotherapy-related examples. **(A)** "Biologics" is a broad term that refers to any therapy created using material derived from a living system, several examples are shown [as adapted from Chhina (8)]. **(B)** Protein-based biologics dominate today's commercial products with examples discussed in this article including monoclonal antibodies (section Antibodies) and interferon (section Blocking Antibodies). **(C)** Until a few decades ago, vaccines dominated immunotherapy, a 200-year old endeavor (section Vaccines), with cancer vaccines (section O-Glycans in Immunotherapy and 3.3) representing one example of this trend today. **(D)**. The extraordinarily diverse nature of immunotherapy is illustrated by emerging cell-based (e.g., CAR T-cell, section Chimeric Antigen Receptor (CAR) T-cell Therapy) and gene therapies.

Abbreviations: ADC, antibody-drug conjugates; ADCC, antibody-dependent cell cytotoxicity (also referred to as antibody-dependent cellular cytotoxicity or antibody-dependent cell-mediated cytotoxicity); ASGP, asialoglycoprotein; CAR, chimeric antigen receptor; CDC, complement dependent cytotoxicity; CHO, Chinese hamster ovary; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/targeted Cas endonuclease; CSF, colony stimulating factor; EMA, European Medicines Agency; EPO, erythropoietin; ER, endoplasmic reticulum; Fab, fragment antigen-binding; Fcγ, (fragment crystallizable γ); FDA, Food and Drug Administration; FRET, Förster resonance energy transfer; Fuc, fucose; Fut/FUT, fucosyltransferase; Gal, galactose; GalCer, galactosylceramide; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcCer, glucosylceramide; GlcNAc, N-acetylglucosamine; GSL, glycosphingolipid; HEK293, human embryonic kidney 293 cells; HSPC, hematopoietic stem and progenitor cell; IgG, immunoglobin G; IL-2, interleukin-2; IVIG, intravenous immunoglobin; LacNAc, Galβ(1-4)GlcNAc; LLO, lipid-linked oligosaccharide ($Glc_3Man_9GlcNAc_2$-P-P-dolichol); mAb, monoclonal antibody; Man, mannose; Mgat, N-acetylglucosaminyltransferase; MSC, mesenchymal stem (or stromal) cell; MUC1, mucin 1; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; NMR, nuclear magnetic resonance; PD1, programmed cell death protein-1; PDL1, programmed death ligand-1; PEG, polyethylene glycol; PTM, post-translational modifications; scFv, single chain variable fragment; SIGN-R1 or DC-SIGN, specific intracellular adhesion molecule-grabbing nonintegrin R1; sLe^x, sialyl Lewis x; TALEN, transcription activator-like effector nuclease; α-Gal, galactose-α(1,3)-galactose.

To begin this article (next, in section The Role of Glycosylation in Immunotherapy), we provide an overview of mammalian glycosylation—with a focus on N-glycosylation—and highlight how specific glycans impact human immunity and then in section Glycodesign of Immunotherapeutics provide illustrative examples of how glycans modulate various types of immunotherapies. The sheer complexity and vast diversity of glycosylation makes quality control during the manufacturing of biologics a daunting task (15); we are confident, however, that various "glycoengineering" strategies, as outlined

in section Design Considerations and Biomanufacturing, hold great promise for improving existing, and developing novel, immunotherapeutics.

THE ROLE OF GLYCOSYLATION IN IMMUNOTHERAPY

Historically, the central dogma of biochemistry was based on the belief that the flow of information from a DNA template to RNA to protein could unlock and predict underlying functional and evolutionary relationships in biology. In recent years this paradigm has shifted dramatically by emphasizing upstream epigenetic factors that control gene expression as well as downstream post-translational modifications (PTMs). This article focuses on glycosylation, a ubiquitous PTM in all three domains of life (archaea, bacteria, and eukarya); in mammals, carbohydrates can be divided into three primary types: N-linked glycans, O-linked glycans, and glycolipids (16). With the emergence of glycobiology in 1980s (17) and the realization that glycans modulate almost all aspects of human biology—especially the immune system [exemplified by the role of glycans in modulating the function of IgG antibodies (18), a topic discussed throughout this article]—the stage was set to apply lessons learned to the burgeoning field of immunotherapy. Here, in section The Role of Glycosylation in Immunotherapy, we briefly review mammalian glycosylation and its impact on immunotherapy; this focus stems from emerging dominance of mammalian systems as the predominant production platform for immunotherapeutics (6).

N-Glycans

N-Glycans are oligosaccharides covalently linked to the amide nitrogen of asparagine; they constitute one of the most common and almost certainly the most complex type of PTM (19, 20). Here we provide an overview of mammalian N-glycan biosynthesis [for more thorough information, see (19–22)] along with illustrative examples of how various N-glycans modulate immunity. In the next sub-sections we describe N-glycan biosynthesis in a step-by-step manner and highly salient features relevant to immunotherapy. This information provides a foundation for optimizing drugs—mostly biologics—used in immunotherapy (this class of drugs is referred to as "immunotherapeutics" in this paper).

Early Steps in N-Glycan Biosynthesis

N-Glycan biosynthesis occurs in two distinct stages in the endoplasmic reticulum (ER) and the Golgi apparatus, respectively (19, 23). N-Glycan biosynthesis begins in the ER with the synthesis of the lipid-linked oligosaccharide (LLO) structure. Dolichol is an isoprenoid lipid that functions as an oligosaccharide carrier during early LLO synthesis on the cytosolic face of the ER membrane (19, 24, 25) where $Man_5GlcNAc_2$-P-P-dolichol is formed. This glycolipid is translocated into the ER lumen by a flippase (26, 27) where it is further elaborated to the final 14-mer LLO structure ($Glc_3Man_9GlcNAc_2$-P-P-dolichol), which is transferred by an oligosaccharyltransferase to an asparagine residue in the consensus motif Asn-X-Ser/Thr of a nascent polypeptide chain during its translation across the ER membrane (28, 29).

N-Glycan Processing and Structural Diversification

The second phase of N-glycan biosynthesis encompasses the processing of LLOs (as outlined in **Figure 2**) into three general categories (high mannose, hybrid, and complex) decorated with thousands of potential structural motifs (31–33) after transport of the host protein from the ER to the Golgi. This diversification of N-glycans—being a non-template based process—results in numerous and difficult-to-predict glycoforms. As described below, the sequential modification of mannose, GlcNAc, galactose, fucose, and sialic acid modulates many aspects of biology, including most aspects of immunotherapy (20).

Mannose

In the Golgi, a proportion of the $Man_{8/9}GlcNAc_2$ structures avoid further modification (beyond the cleavage of mannose residues to form $Man_{5-9}GlcNAc_2$) resulting in high mannose type N- glycans (19) that affect glycoprotein secretion, folding, and stability (34). For example, high mannose N-glycans can increase serum clearance and immunogenicity of IgG antibodies (35–37) although this is not always the case (38). High mannose N-glycans are associated with enhanced IgG monoclonal antibody (mAb) binding to FcγRIIIa and concomitant higher antibody-dependent cell cytotoxicity (ADCC) activity [ADCC is discussed in more detail in section Antibody-dependent Cell Cytotoxicity (ADCC)]. This effect was observed across the range of five to nine mannose residues (36, 37, 39–41) suggesting that enhanced activity could be due to a lack of core fucosylation (discussed below in section Fucose) and not the presence (or absence) of mannose *per se*. High mannose glycans with more than five mannose resides also lessen C1q (a vital receptor for complement dependent cytotoxicity [CDC]) binding, yielding diminished CDC activity (36, 39, 42).

Branching (Mgat1,2,4,5)

In most cases, high mannose type N-glycans are further processed in the Golgi resulting in hybrid- and complex-type N-glycans (**Figure 2**). The process of N-glycan branching and elongation begins in the *medial*-Golgi with the transfer of GlcNAc to the $Man_5GlcNAc_2$ structure by N-acetylglucosaminyltransferase, Mgat1 (43). For hybrid N-glycans, the high mannose branch remains unaltered while the branch ending in GlcNAc is usually further elongated with galactose and GlcNAc or capped with sialic acid, or fucose, as described below. Complex type N-glycans have two additional mannose residues cleaved by α-mannosidases (Man2a1 or Man2a2) to produce $GlcNAcMan_3GlcNAc_2$ (44), which is elaborated with bi- (and sometimes tri-, and tetra-) antennary branches by the sequential addition of GlcNAc residues via Mgat2, Mgat4, and Mgat5. The GlcNAc transferases have decreasing affinity (higher K_m values) for

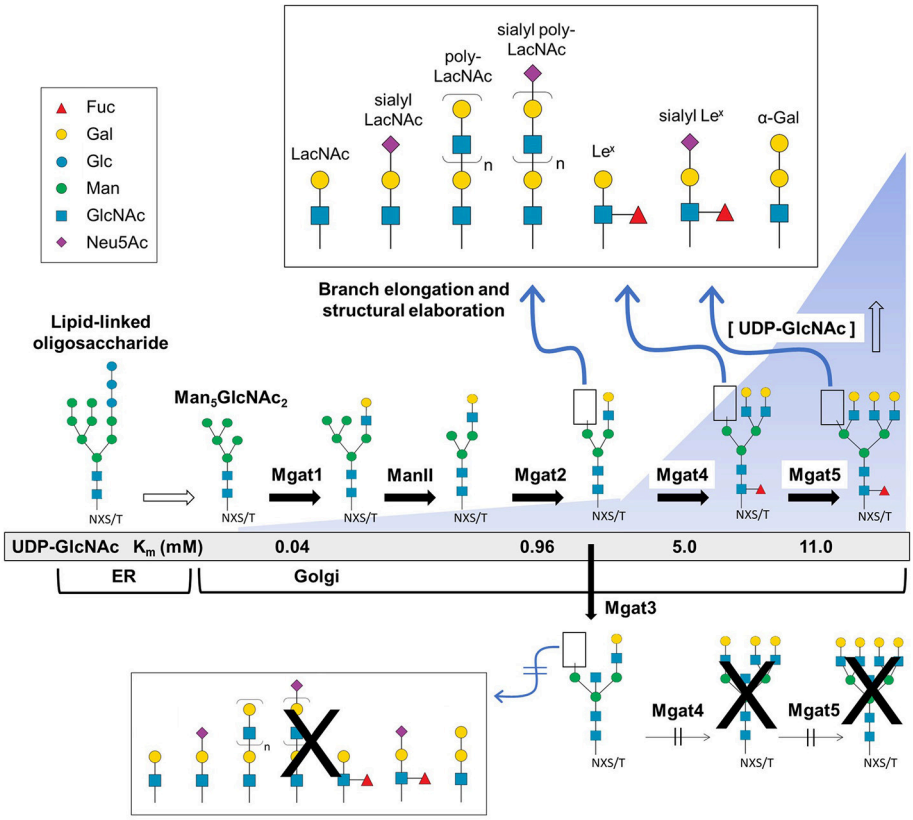

FIGURE 2 | Branch elongation and structural diversity of N-glycans. The $Glc_3Man_9GlcNAc_2$-P-P-dolichol LLO structure is synthesized in the ER where it is further processed and transferred to the Golgi resulting in high mannose (e.g., $Man_5GlcNAc_2$), hybrid, and complex type N-glycans that undergo branching via Mgat1, 2, 4, and 5 GlcNAc transferase activity that respectively creates di-, tri-, or tetra-antennary structures. Following the initial branching step, the glycan structure may be fucosylated or undergo additional elongation and capping modifications (Top panel). Alternatively, Mgat3 may add a bisecting GlcNAc residue which blocks Mgat4 and 5 activity thereby preventing tri- and tetra-antennary and further terminal diversification (bottom). The presence of a bisecting GlcNAc also hinders core fucosylation (red triangle) and reduces the capacity for downstream elongation and capping. [All glycan symbol structures in this figure and throughout this document were made using software from Cheng and coauthors (30)].

the substrate UDP-GlcNAc creating an ultrasensitive cascade (**Figure 2**) that usually limits branching to bi-antennary structures (e.g., as shown in **Figure 3** for a typical IgG mAb) (43, 50).

N-Glycan branching plays numerous roles in regulating the immune system ranging from T-cell activation (38, 51), autoimmunity (38, 51), cytokine production (52), cancer metastasis (53), to cell proliferation and differentiation (54). From an immunotherapy perspective, N-glycan branching influences the physicochemical properties and the metabolic turnover of immunotherapeutics by modulating the overall charge, isoelectric point, size, and valence of these molecules; more specifically increased branching provides more sites for sialylation giving the glycoprotein a higher negative charge (55) that impacts physicochemical properties (see section Design Considerations and Biomanufacturing). The serum half-life of immunotherapeutics also is influenced by terminal sialylation, which masks the penultimate galactose moiety from the hepatocyte asialoglycoprotein (ASGP) receptor (**Figure 3C**), reducing glomeruli clearance in the kidneys (56, 57).

Bisecting GlcNAc (Mgat3)

The discerning reader may have noted the curious omission of Mgat3 from the previous paragraph; the reason is that this enzyme is an outlier that counteracts several aspects of N-glycan diversification and elongation. Specifically, Mgat3-catalyzed addition of GlcNAc to the β-mannose of an N-glycan in a bisecting orientation (53, 58) inhibits the activity of Mgat4 and Mgat5 negating tri- and tetra-antennary branching (and subsequent elongation of the resultant antennary branches) and also reduces core fucosylation (**Figure 2**) (41, 43, 59). Although only a single monosaccharide, the ability of bisecting GlcNAc to block subsequent branching and core fucosylation has a disproportional impact on overall N-glycan structure and bioactivity [e.g., in cancer metastasis (60–63), apolipoprotein B function (64) and the epithelial-mesenchymal transition (65, 66)].

The potent ability of bisecting GlcNAc to modulate biological activity makes this monosaccharide a crucial design parameter in immunotherapy. For example, bisecting GlcNAc blocks tri- and tetra-antennary N-glycan branching, which limits the number of

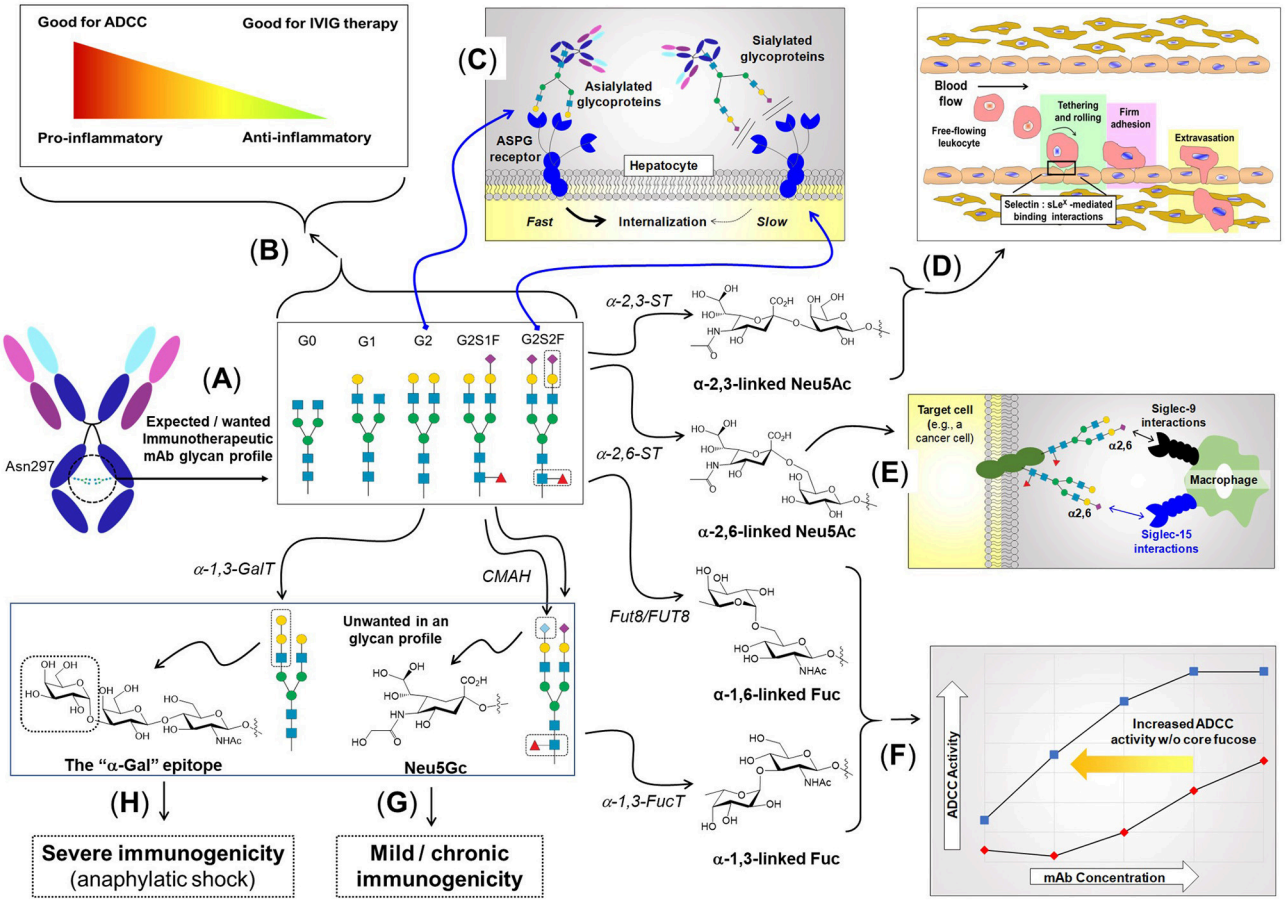

FIGURE 3 | The role of N-linked glycosylation in mAb function and other aspects of immunity. **(A)** IgG type antibodies have two N-linked glycosylation sites at Asn297 of the Fc region that usually bear biantennary complex type N-glycans elongated with zero (G0), one (G1), or two (G2) galactose residues. **(B)** The presence of fucose and sialic acid inhibits FcγRIIIa binding resulting in lower ADCC activity; conversely, the anti-inflammatory character of sialic acid makes its presence desirable for IVIG therapy. **(C)** The presence (or absence) of sialic acid affects binding to the ASGP receptor, resulting in quick recycling of asialylated therapeutic proteins, which reduces serum half-life. By contrast, sialylation block ASGP receptor-mediated recycling, improving pharmacokinetic properties. **(D)** Neu5Ac added to galactose in an α2,3-linkage elicits a certain set of biological responses, one of which is—as part of the sLe^X epitope (shown in **Figure 4**)—to facilitate immune cell trafficking throughout the body by enabling "tethering and rolling" steps of leukocyte extravasation from the vascular system. **(E)** Neu5Ac in an α2,6-linkage elicits a distinct set of response, including binding to Siglec receptors (45), where in the example shown, adapted from Büll et al. (46), this moiety modulates macrophage activity. **(F)** Core fucose, in particular in the α1,6-linkage, inhibits ADCC requiring higher mAb antibodies compared to defucosylation drug [adapted from GlycoWord (47)]. Glycans can also result in unwanted immunogenicity ranging from mild, chronic responses emanating from Neu5Gc **(G)** (48), to life-threatening, anaphylactic responses from α-Gal **(H)** (49).

potential sites for sialylation on a glycoprotein thereby reducing serum half-life and altering the physicochemical properties (sialylation is further discussed in section Sialic Acid). Similarly, limiting N-glycan branching alters the overall structure and composition of glycoproteins which has numerous implications for surface charge, hydrophobicity and colloidal/conformation stability, which is discussed further in section Physicochemical Properties Mgat3 inhibits α(2,3)-sialylation, which can reduce terminal sialylation or alternately, enhance α(2,6)-sialylation (67) (**Figure 3**). The presence of a bisecting GlcNAc in Fc region N-glycans in IgG antibodies increases binding affinity to FcγRIIIa leading to a 10-20 fold increase in antibody dependent cell cytotoxicity (68); which is consistent with the loss of core fucosylation that can increase ADCC activity by up to ∼100-fold (69–71). Finally, Mgat3 impedes synthesis of galactose-α(1,3)-galactose (α-Gal, **Figure 3**), an epitope that can elicit severely-deleterious immunogenic responses (49, 72).

Galactose

After GlcNAc has been added to a nascent N-glycan to form hybrid or complex structures, this moiety is commonly elongated with galactose by a β(1,4)-galactosyltransferase, which creates the Galβ(1-4)GlcNAc unit known as "LacNAc" (73, 74). Additional galactose residues may be added by β(1,4)- or α(1,3)-galactosyltransferases, either consecutively or interspersed with other monosaccharides (e.g., GlcNAc) to create a variety of N-glycan structures (**Figure 2**). Although terminal galactose has minimal influence on ADCC activity or the pharmacological

properties of recombinant IgGs (75, 76), it can nonetheless impact the efficacy of various therapeutic mAbs (41, 77); for example, increases in heavy chain galactose content can increase CDC in rituximab (78) and alemtuzumab (79). Although generally modest, galactose-dependent CDC has led regulatory bodies to require strict monitoring of galactosylation patterns of immunotherapeutics (and other biologics) with terminal galactose groups (G0, G1, or G2, **Figure 3**) now a major quality control parameter in the biomanufacturing industry (77, 80, 81).

Galactose linked to an underlying galactose via an α(1,3)-linkage constitutes the α-Gal epitope, which can have widespread ramifications for the safety, efficacy, and pharmacokinetic properties of immunotherapeutics. The α-Gal epitope is common in non-primate mammals but is absent in humans; as a result people have circulating antibodies against this antigen, which led to severe immunogenic responses, and even patient deaths, in early immunotherapy trials in 2004 (49, 82, 83). Sequential addition of GlcNAc in conjunction with galactose produces LacNAc units that often are added preferentially to a specific N-glycan branch resulting in structural asymmetry that impacts function and biological recognitionthat, in one example, affects the immunomodulatory properties of milk oligosaccharides through tuning interactions with both pathogens and glycan binding proteins such as galectin (84).

Fucose

Hybrid and complex type N-glycan branches often end with GlcNAc or galactose but can also be decorated with fucose (this section) or terminally capped with sialic acids, meaning that typically once these sugars are added, the oligosaccharide chain cannot be further elongated (section Sialic Acid, below). Fucose is a prevalent modification of the complex type N-glycans; in humans fucosyltransferases add this sugar in an α(1,2) (FUT1,2), α(1,3/4) (FUT3-7,9), or α(1,6) (FUT8) orientation; in mammals, Fut8 adds a fucose residue exclusively to the innermost Asn-linked GlcNAc group (*a.k.a.*, "core" fucosylation). Fucose can also be added as a capping moiety to an outermost galactose by Fut1,2 forming Lewis and blood group antigens (85, 86) (see **Figure 4**).

Core α(1-6) fucose has widespread biological activity ranging from modulating growth factors (87–89) and to affecting the incidence and progression of cancer (90–94) while *Fut8*-null mice display multiple phenotypes including semi-lethality, the development of emphysema, brain dysfunction, and impaired immunity (58). Based on the many biological and physiological roles of core fucosylation, it is not surprising that this sugar plays integral roles in immunotherapy; for example, core fucosylation inhibits IgG binding to FcγRIIIa thereby decreasing ADCC activity (41, 70, 71, 95–105). Conversely, defucosylation of clinically-used mAbs including rituximab, trastuzumab, and pertuzumab can increase ADCC activity up to two-fold (70, 71, 101, 105). Another wrinkle of core fucosylation is that α(1,3)-fucosylation—which is prevalent in plant cells including those under consideration for biomanufacturing (106)—can impact mammalian immunity [e.g., through Fc receptor interactions (107)]; as a result, the use of plant hosts for biomanufacturing is proceeding cautiously.

Sialic acid

Sialic acids – a family α-keto acids comprised of a nine carbon backbone with over 50 different variants—ubiquitously cap glycans (19, 20, 108). N-Acetylneuraminic acid (Neu5Ac) is the predominant sialic acid in humans and is typically found at the termini of N-glycan branches where it is added to the penultimate galactose via α(2,3)-, α(2,6)-, or less commonly, α(2,8)-sialyltransferases (109, 110). Depending on its linkage [e.g., α(2,3)- vs. α(2,6)-] sialic acid exhibits numerous biological functions in nervous system embryogenesis, cancer metastasis, immune responses, and protein bioactivity and stability (110, 111).

Relevant to therapeutics, sialic acid increases the serum half-life of numerous recombinant glycoproteins including erythropoietin (EPO), interferon γ, interferon α, IgG antibodies, and serum albumin (12) by masking the terminal galactose and GlcNAc residues from the hepatocyte ASGP receptor and thus preventing endocytosis to prolong circulatory lifetime (12, 57, 112). Furthermore, the negative charge of sialic acid reduces proteolytic degradation and kidney clearance (12, 113, 114) due to its impact on physicochemical properties. Finally, sialylation (along with fucose) can tune the immunogenicity of antibodies (**Figure 3**) resulting in contrasting effects illustrated by ADCC and intravenous immunoglobin (IVIG) therapy. Sialylation of IgG interferes with FcγRIIIa binding reducing ADCC activity in mouse hybridoma lines (41, 76); conversely, this immunosuppressive activity is critical for IVIG therapy (see section Intravenous Immunoglobulin (IVIG) Therapy). Mechanistically, suppression of inflammation is linked to the C-type lectin receptor-specific intracellular adhesion molecule-grabbing nonintegrin R1 (SIGN-R1 or DC-SIGN in humans), which requires IgG ligands with sialylated Fc glycans (115–117).

Another example of a coordinated function of sialic acid and fucose is provided by sialyl Lewis x (sLe^x) (86) where both sugars are required for selectin-mediated immune cell trafficking (section Mesenchymal Stem Cell (MSC) Homing). The mechanism for homing relies on the selectin family comprised of E-selectin (CD62E), L-selctin (CD62L), and P-selectin (CD62P) which bind to a sialofucosylated epitope, namely sLe^x, in a Ca^{+2}-dependent manner. The sLe^x epitope is vital for both naïve T-cell and activated T effector cell homing to various tissues (118).

O-Glycans

O-Glycans are monosaccharides or oligosaccharides covalently linked to serine or threonine. Similar to N-glycans, O-glycan synthesis is not template-based and is defined by a vast array of possible structural permutations that play many biological and pathological roles including: protein stability, structure, folding, activity, metabolism, cell signaling, cell-cell interactions, and oncogenesis (119–122). This section focuses on mucin type O-glycans and how this category of O-linked glycosylation impacts immunotherapeutics.

Mucin Type O-Glycans

Although there are several types of O-glycans including O-linked GlcNAc, O-linked glucose, and O-linked fucose (120,

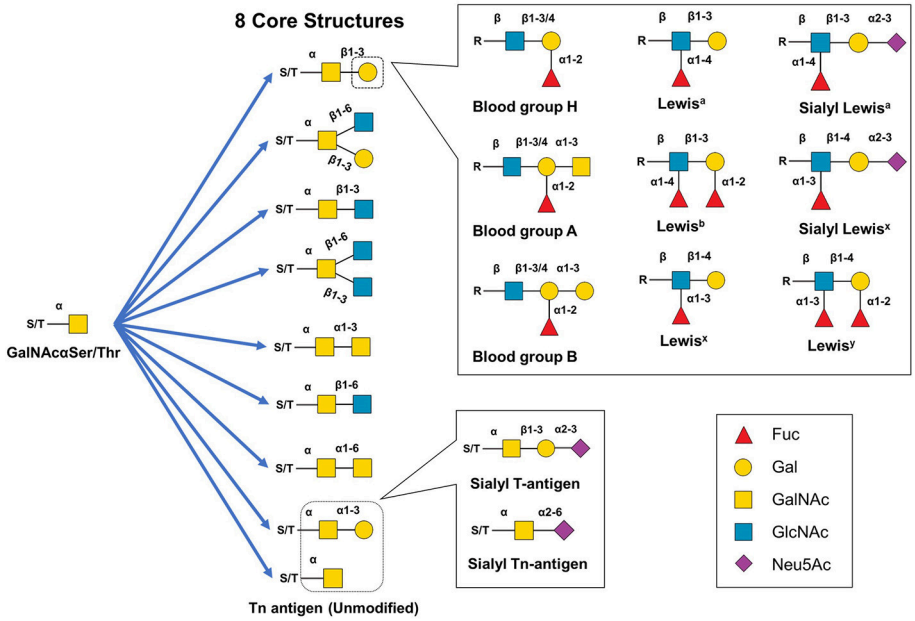

FIGURE 4 | Structural diversity of mucin-type O-glycans. Mucin type O-glycan biosynthesis begins with the transfer of GalNAc to serine or threonine. The GalNAc monosaccharaide can be left unmodified but is typically extended to create eight different core structures that can be further modified with single monosaccharides, Lewis structural epitopes, blood group antigen groups, or other glycan epitopes (e.g., the cancer-related sT or sTn antigens).

122) this article focuses on mucin-type O-glycans because of their relevance to immunotherapeutics. Mucin-type O-glycans, so named because of their abundance in mucins (and their initial isolation and characterization from mucus), are defined by having a GalNAc at the reducing terminus (119). Biosynthesis of mucin-type O-glycans begins in the Golgi with the transfer of GalNAc to a Ser or Thr residue by one of ~22 GalNAc transferases (123–125). While possible, a single unextended GalNAc (Tn antigen) is uncommon, instead various glycosyltransferases generate one of eight core structures (121, 122) (**Figure 4A**). These core structures can be further elongated and capped (generally with GlcNAc, Gal, sialic acid, fucose) to create numerous motifs such as the Lewis antigens (e.g., Le^y, Le^x, sLe^x, Le^a, sLe^a, Le^b) thereby substantially increasing structural diversity (119, 122, 126). Mucin-type O-glycans are involved in many biological functions including fertilization, signal transduction, cell structure, adhesion, homing, glycoprotein clearance, stability, and of course, immunity (119, 122).

O-Glycans in Immunotherapy

An early example of O-glycosylation in immunotherapy is provided by mucin 1 (MUC1), a transmembrane glycoprotein overexpressed and abnormally glycosylated with Tn and sialyl Tn antigen in adenocarcinomas, squamous cell carcinomas, and myelomas making it a broad based cancer biomarker (127–129). Astonishingly, in 1999 it was estimated that cancers with aberrant MUC1 expression accounted for 72% of new cases and 66% of deaths in all cancers (130). The widespread occurrence of MUC1 across multiple types of cancer has made it a popular immunotherapy target with 16 new trials initiated in 2017 alone (127). Interest in MUC1-based cancer immunotherapy stems from this marker's aberrant glycosylation in tumor cells due to truncated, highly sialylated O-glycans that occur at up to five potential sites on each of MUC1's 20 amino acid tandem repeat sequence (**Figure 5A**). MUC1-targeting immunotherapies fall into three general categories vaccines, mAbs, and adoptive cell therapies. First, vaccines based on several different MUC1 antigens, such as synthetic peptides or MUC1 endogenously expressed by plasmid, synthetic mRNA, or viral vectors are now being tested (127–129, 131). An especially intriguing "cancer vaccine" approach to MUC1 employs metabolic glycoengineering strategies (a technology described in more detail in section Metabolic Glycoengineering) that incorporate non-natural sialic acids into glycan structures that increase their immunogenicity [as shown in **Figure 5B** and described in a series of papers primarily from the Guo group (132–135)]. In another approach, murine anti-MUC1 antibodies (muHMFG-1, mAB-AR20.5) and humanized anti-MUC1 antibodies (hPAM4, AS1402) are being evaluated in clinical trials (128, 136). Finally, autologous dendritic cells engineered to contain MUC1 as a peptide, mRNA or fused tumor cells have been designed to elicit immune-based antitumoral cytotoxicity (137–139) and most recently, chimeric antigen receptor (CAR) T-cells have been engineered to target MUC1 and the Tn antigen with 10 current phase I/II trials targeting MUC1 (127, 140–143).

In contrast to robust efforts to exploit O-glycans in immunotherapy, as just illustrated by MUC1, O-glycans largely have been overlooked as a design parameter in the biomanufacturing of immunotherapeutics; indeed, until a few years ago human IgGs—the largest class of

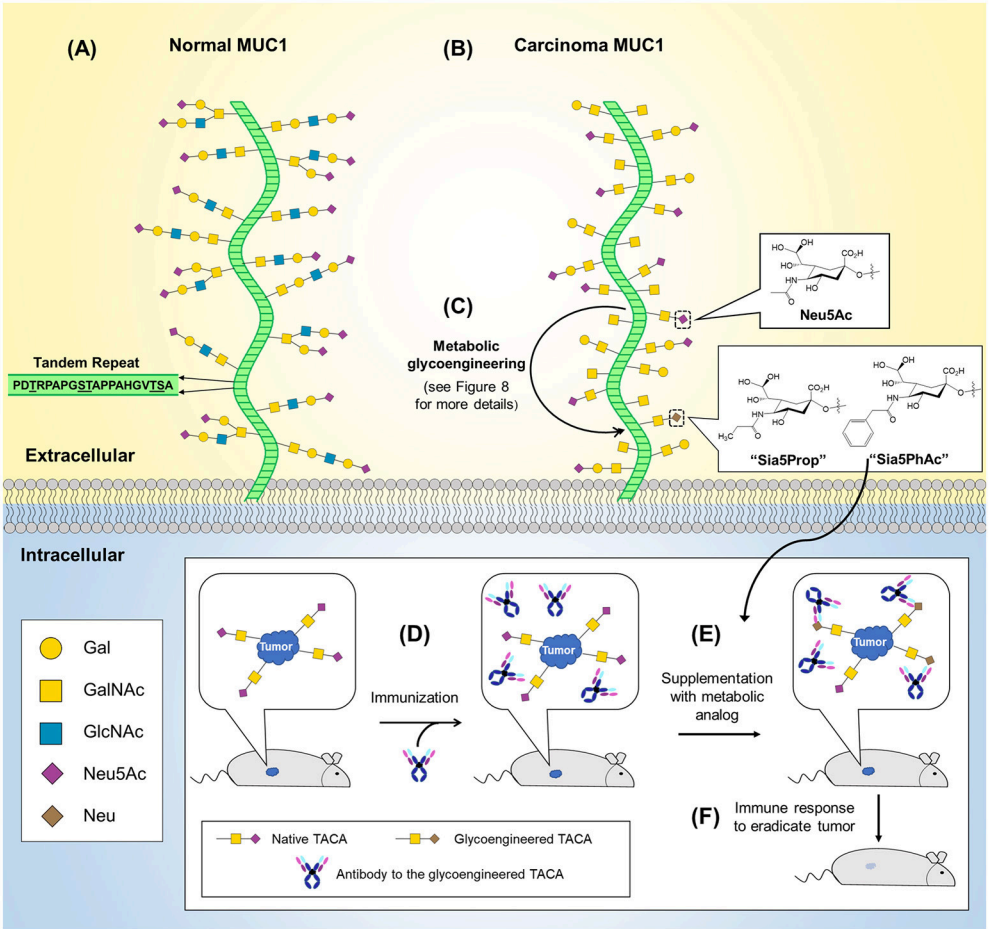

FIGURE 5 | O-Glycans in normal and cancerous MUC1 and MUC1-based cancer vaccine development. **(A)** The MUC1 protein core (green) is composed of a 20 amino acid tandem repeat with each unit having five potential O-glycosylation sites. **(B)** MUC1 is overexpressed in numerous cancers (not shown) and is characterized by truncated O-glycans (shown). **(C)** MGE can be used to introduce non-natural chemical moieties (e.g., Sia5Prop and Sia5PhAc) to enhance the immunogenicity of tumor-associated cancer antigens (TACAs). As shown in the inset (bottom), antibodies can be developed to the glycoengineered TACAs and used to immunize a tumor-bearing animal **(D)**. **(E)** Supplementation with the MGE analog induces expression of the non-natural version of the TACA, resulting in tumor-selective binding and stimulation of the immune system to recognize and eradicate the tumor **(F)**.

immunotherapeutics—were not thought to contain O-glycans. It is now known, however, that the hinge region of several classes of human immunoglobins including IgA (144–146), IgD (147, 148), as well as IgG (149, 150) have potential O-glycosylation sites. Specifically, IgA1 has nine potential O-glycosylation sites with three to five typically occupied (146, 150); IgD has six potential sites (148, 151); and human IgG has three potential sites with occupancies between 10 and 13% for IgG3 (150). Although relatively little is known about how O-glycosylation modulates the activity, specificity, or stability of mAbs it has been shown O-glycosylation plays an important role in Fc-fusion protein serum longevity. Notably, increased sialylation of the O-glycans of etanercept (tumor necrosis factor α receptor II-Fc-fusion) and BR3-Fc fusion enhance serum half-life (152, 153). Similar to N-glycans, this effect is attributed to sialic acid's ability to mask galactose from ASGP receptors preventing degradation in the liver (41). In the future, as the biological implications of mAb O-linked glycosylation are uncovered, the biomanufacturing industry (section Design Considerations and Biomanufacturing) likely will focus additional effort on controlling mucin-type O-glycosylation. At present O-glycans nevertheless provide an attractive "chemical handle" for conjugation reactions to improve glycoprotein pharmacokinetics. For example, GalNAc-transferases have been used to modify recombinantly-produced proteins with polyethylene glycol (PEG), a technology termed GlycoPEGylation (154). Covalently attaching PEG to recombinant proteins can augment serum half-life, pharmacokinetic and pharmacodynamic properties. Typically, recombinant proteins are PEGylated through amino acid residues, however it is vital to avoid conjugating PEG to amino acids in or near an active site or, for mAbs, near the antigen recognition domain (155). This issue can be circumvented by targeting O-glycans, which are usually located away from an active site (156, 157). GlycoPEGylation is predominantly used

for recombinant therapeutic proteins expressed in *Escherichia coli* that lack endogenous mucin-type O-glycosylation and occurs in two general steps: (i) GalNAc-transferase adds a GalNAc to a Ser/Thr residue and (ii) CMP-Neu5Ac with covalently-attached PEG is added by a sialyltransferase. This technology has been employed for two clinically approved biologics: granulocyte/macrophage colony stimulating factor, and interferon-α2b (154, 158).

Glycolipids

Glycolipids—a third major class of glycans—are perhaps an unlikely candidate for immunotherapy considering their longstanding role in provoking severe, detrimental immune responses (e.g., sepsis) that remains an increasing source of mortality in American hospitals (159). Sepsis is triggered by highly-immunogenic, microbe-derived Lipid-A-linked oligo- or polysaccharides that typically contain non-mammalian monosaccharides (**Figure 6**) (163). Interestingly, in 2009 Piazza and coworkers were able to rationally design glyco- and a benzylammonium-modified lipids that function as lipid-A antagonists and inhibit lipopolysaccharide-induced septic shock *in vivo* (162). This class of molecules provides a "small molecule" example of an immunotherapeutic that mimics IgG antibodies in that the compound's inherent immunomodulatory ability can be tuned up or down by chemical structural modifications. Since then, "immunopharmacy" efforts have continued to develop lipid A variants for vaccines and other therapies, as summarized by Wang and coauthors (164).

Mammalian glycosphingolipids (GSLs), comprised of a sphingolipid, fatty acid, and carbohydrate (**Figure 7**) provide another example of immunotherapy. GSLs are part of the cell membrane with various biological functions including cellular adhesion, cell-cell interactions, signal transduction, oncogenesis, ontogenesis, and immunogenicity (165–167). To date, efforts to exploit GSLs in immunotherapy have focused on cancer; these molecules are aberrantly expressed in a variety of cancers including breast, lung, colorectal, melanoma, prostate, ovarian, leukemia, renal, bladder, and gastric thereby constituting attractive broad-based diagnostic biomarkers and providing potential targets for cancer immunotherapy (168). Notably, multiple antibodies are in preclinical and clinical trials that target GSLs including GD2 (169), GM2 (170), Neu5GcGM3 (171), Gb3, Gb4, and Globo H (172). Another GSL, α-GalCer, has potential anti-tumor activity and is currently in phase 1 clinical trials in high risk melanoma patients (173).

Finally, from the perspective of the production of immunotherapeutic products, inhibition of GSL biosynthesis in Chinese hamster ovary (CHO) cells can enhance sialylation; for example, repressing the GSL biosynthetic enzyme UDP-glucose ceramide glucosyltranferase increased recombinant EPO sialylation. Interestingly, GSL inhibition did not change CMP-Neu5Ac levels in the Golgi or cytoplasm, suggesting that CMP-Neu5Ac was diverted to EPO sialylation as part of a dynamic equilibrium between GSL and N-glycan biosynthesis (174). Overall, this study provides an option for modulating GSL biosynthesis as a glycoengineering strategy to produce glycoproteins with favorable glycoforms.

GLYCODESIGN OF IMMUNOTHERAPEUTICS

Over the past 30 years immunotherapy has moved from a focus on vaccines to encompass a diverse array of treatments with glycosylation now firmly established as a key parameter in the design, development, and production of virtually all types of immunotherapeutics. Here, we describe specific examples of how glycosylation impacts and modulates the efficacy of antibody-, recombinant protein-, and cell-based therapies while highlighting glycoengineering techniques that can ameliorate problems (e.g., safety) and enhance bioactivity and pharmacokinetics during the development and manufacturing of immunotherapeutics.

Antibodies

Antibodies' ligand-specific targeting and their ability to elicit downstream effector functions (175) have established them as one of the largest classes of biologics overall and as the dominant commercial immunotherapeutic. As described in the following sub-sections, these versatile immunotherapeutics fall into several—often overlapping but sometimes very distinct—categories; several of these categories are summarized with a focus on the role of glycosylation.

Blocking Antibodies

Blocking antibodies, as their name implies, are designed to bind to a biological target and by doing so, diminish its activity; for example, Cetuximab (a.k.a., Erbitux)—a pioneering cancer immunotherapeutic from ∼20 years ago—blocks epidermal growth factor receptor activation and downstream oncogenic signaling (176–178). Interestingly, this early immunotherapeutic alerted the biomedical community to the importance of glycans when several patients suffered severe immune reactions to the α-Gal epitope (**Figure 3**) (49). As an aside, this unfortunate incident provided impetus for the subsequent transition of almost all recombinant mAb production to CHO cells (discussed in more detail in section Chinese Hamster Ovary (CHO) Cells) (6, 179, 180). Despite these early setbacks, interest in blocking antibodies remains strong with the programmed death ligand-1 (PDL1) providing a recent high-profile example. PDL1 is a transmembrane protein [which is glycosylated itself (181)] that binds to the programmed cell death protein-1 (PD1) thereby inhibiting T lymphocyte proliferation and cytolytic activity, immune suppression, and cytokine production (181). PDL1-blocking antibodies alleviate these inhibitory PDL1/PD1 interactions and reactivate T-cells to fight cancer (181, 182) with promising results against both leukemias and solid tumors (183). One recent study developed a mAb targeting glycosylated PDL1 in triple negative breast cancer cells which blocks PDL1/PD1 interactions and enhances PDL1 internalization and degradation. Furthermore, conjugating the anti-mitotic drug monomethyl auristatin E to this mAb resulted in significant cytotoxicity to cancer cells expressing glycosylated PDL1 with limited host toxicity (184).

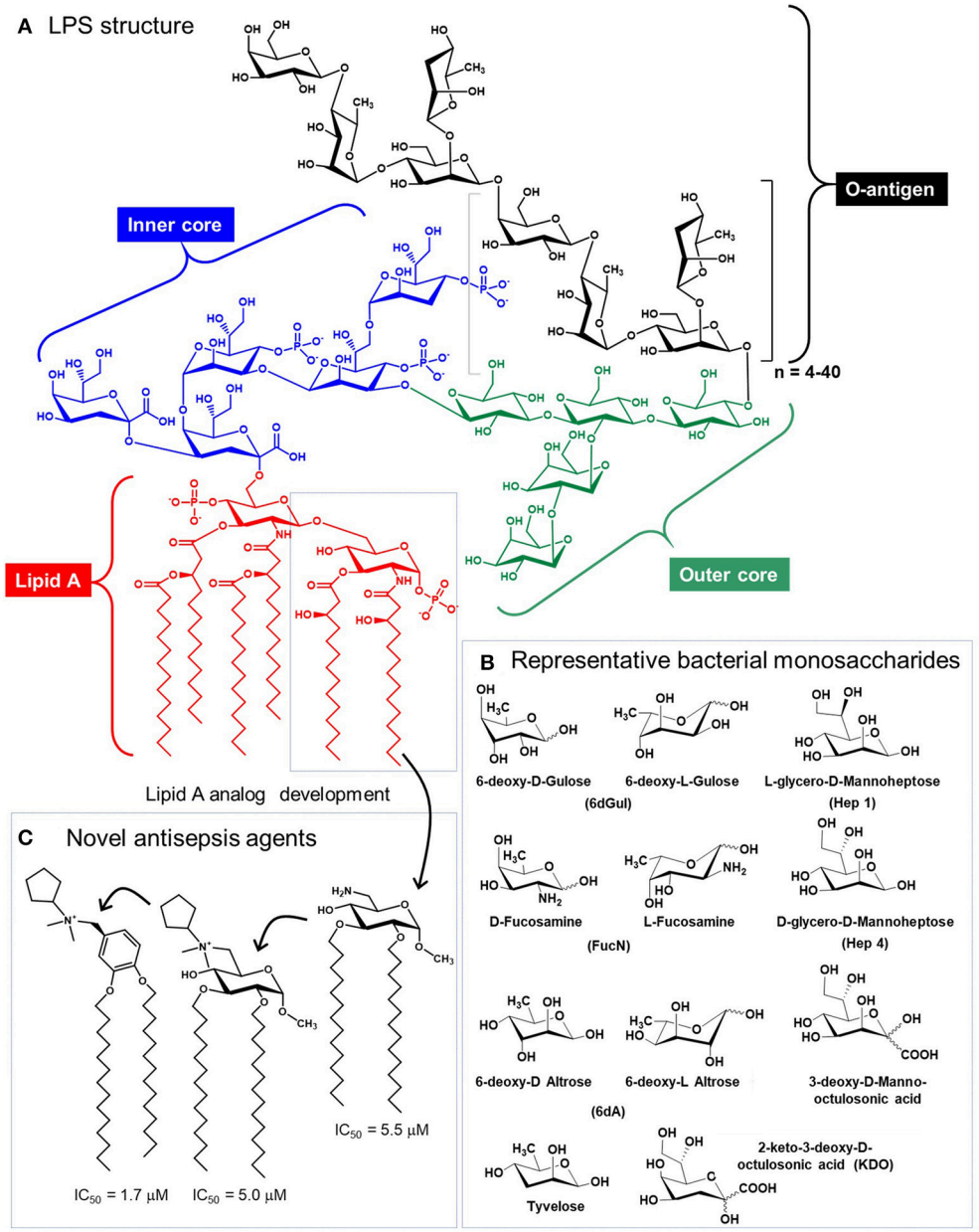

FIGURE 6 | Structure of lipopolysaccharide (LPS). **(A)** Glycolipids, exemplified by bacterial structures such as LPS contain the Lipid A, and inner core, an outer core, and the O-antigen, which varies based on species and strain [*Salmonella enterica* Serotype Typhi is show (160)]. **(B)** LPS glycans contains a variety of non-mammalian monosaccharides, which contributes to their immunogenicity and provokes sepsis [**(A,B)** are adapted from Saeui et al. (161)]. **(C)** Medicinal chemistry efforts have exploited the Lipid A structure to create anti-inflammatory analogs [three are shown, from Piazza et al. (162)] that are promising anti-sepsis agents.

Antibody-Dependent Cell Cytotoxicity (ADCC)

ADCC is a cell-mediated immune defense where effector cells (typically natural killer cells but also macrophages, neutrophils, and eosinophils) actively lyse a target cell whose membrane-surface antigens have been bound by specific antibodies (185). In immunotherapy, antibodies are designed to selectively coat cancer cells, targeting them for eradication by Fc receptor effector cells (186). ADCC can be improved (or hindered) by glycosylation as illustrated by the glycosylation profiles of anti-HIV monoclonal antibodies (187) and the role of fucose and sialic acid in ADCC, as outlined by Ravetch and coauthors (101, 102, 188, 189); the "take home" message is that sialylation and core fucosylation generally inhibit ADCC, positioning simpler N-glycans that lack sialic acid, and especially fucose (e.g., as shown in **Figure 3**) as ideal glycoforms for antibodies designed to elicit ADCC. Interestingly, certain mAbs intended to block biological activity (section Blocking Antibodies) also elicit ADCC thus doubly benefitting cancer immunotherapy;

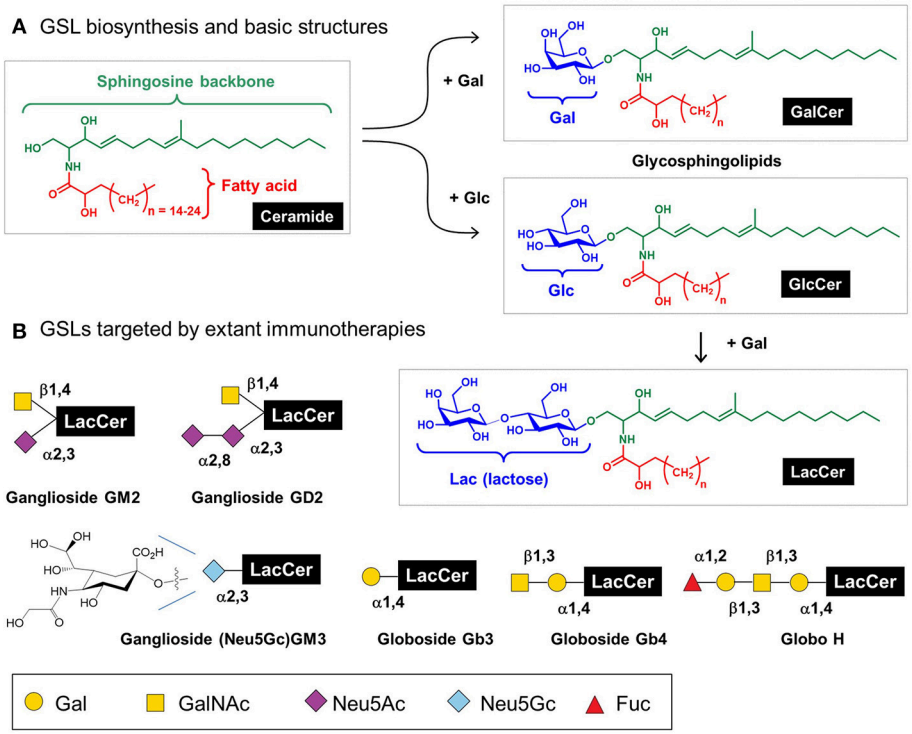

FIGURE 7 | Glycosphingolipids (GSL) structures and role in immunotherapy. **(A)** Human GSLs are derived from ceramide upon addition of galactose (to form "GalCer") or, more commonly, addition of glucose (to form "GlcCer"); a fraction of GlcCer is further elaborated with galactose to form "LacCer," which is the building block for lacto(neo)series, globosides, and gangliosides as cataloged elsewhere (21); here [in **(B)**] we show several GSLs currently targeted by immunotherapy.

indeed, the pioneering drug Cetuximab fits this criteria (190, 191).

Intravenous Immunoglobulin (IVIG) Therapy

In contrast to ADCC where sialic acid is unwanted, this sugar is critical for immunosuppression as illustrated by IVIG therapy, which is used to treat a wide range of autoimmune, infectious, and inflammatory diseases (115, 188, 192–194). In IVIG therapy, patients are dosed with concentrated IgG collected from pooled plasma (195). Although sialylation is not the sole determinant of the anti-inflammatory response underlying IVIG therapy (194), efficacy is enhanced by sialic acid (188). Because only ∼10% of IgG Fc glycans are sialylated (with just 1–3% disialylated), very high doses (e.g., 1–2 g/kg) of IgG are required for IVIG therapy (9, 188, 196). A study by Washburn et al. where tetra-Fc sialylation of recombinant human IgG1 was achieved by the enzymatic addition of sialic acid showed up to ∼10-fold higher anti-inflammatory activity than unsialylated IVIG across multiple animal models (18, 194).

Antibody Drug Conjugates (ADCs)

ADCs are an emerging class of therapeutics that leverage the specificity of mAbs to minimize off-target effects of small molecule drugs (197, 198). Historically, conjugation of drugs to antibodies typically utilized amino acids such as lysine and cysteine. However, with ∼30 surface-exposed lysines and 8 hinge cysteines this strategy yields a heterogenous ADC mixture with a wide distribution of drug antibody ratios resulting in suboptimal pharmacokinetic properties, lower efficacy, and reduced specificity (197, 199, 200). An alternative approach to attach a drug to an antibody is to exploit the glycans located at Asn-279 in the IgG domain as a "chemical handle"—for example, mild oxidation of the terminal sialic acid creates an aldehyde capable of drug conjugation via oxime or hydrazone ligation (201, 202). One pitfall in this approach is that IgG Fc-region glycans are poorly sialylated (<10%) (9) but efforts are underway to increase sialylation or incorporate non-natural sialic acid groups through metabolic glycoengineering (203) (**Figure 8A**). Alternative strategies include utilizing IgG antibodies with fragment antigen-binding (Fab) glycosylation or targeting fucose instead of sialic acid, a strategy that has been demonstrated with 6-thiofucose (204). Once the glycan moieties of an antibody have been chemically remodeled, a variety of chemoenzymatic ligation methods are available to attach a drug including copper catalyzed or strain-promoted alkyne:azide "click" reactions (197, 205–207) (**Figure 8B**). Interestingly, ADCs can evoke multiple facets of activity, for example drug-conjugated gPD-L1 antibody (which is the PDL1 blocking antibody mentioned in section Blocking Antibodies) induces a potent cell-killing effect as well as a bystander-killing effect on adjacent cancer cells lacking PD-L1 expression (184, 208).

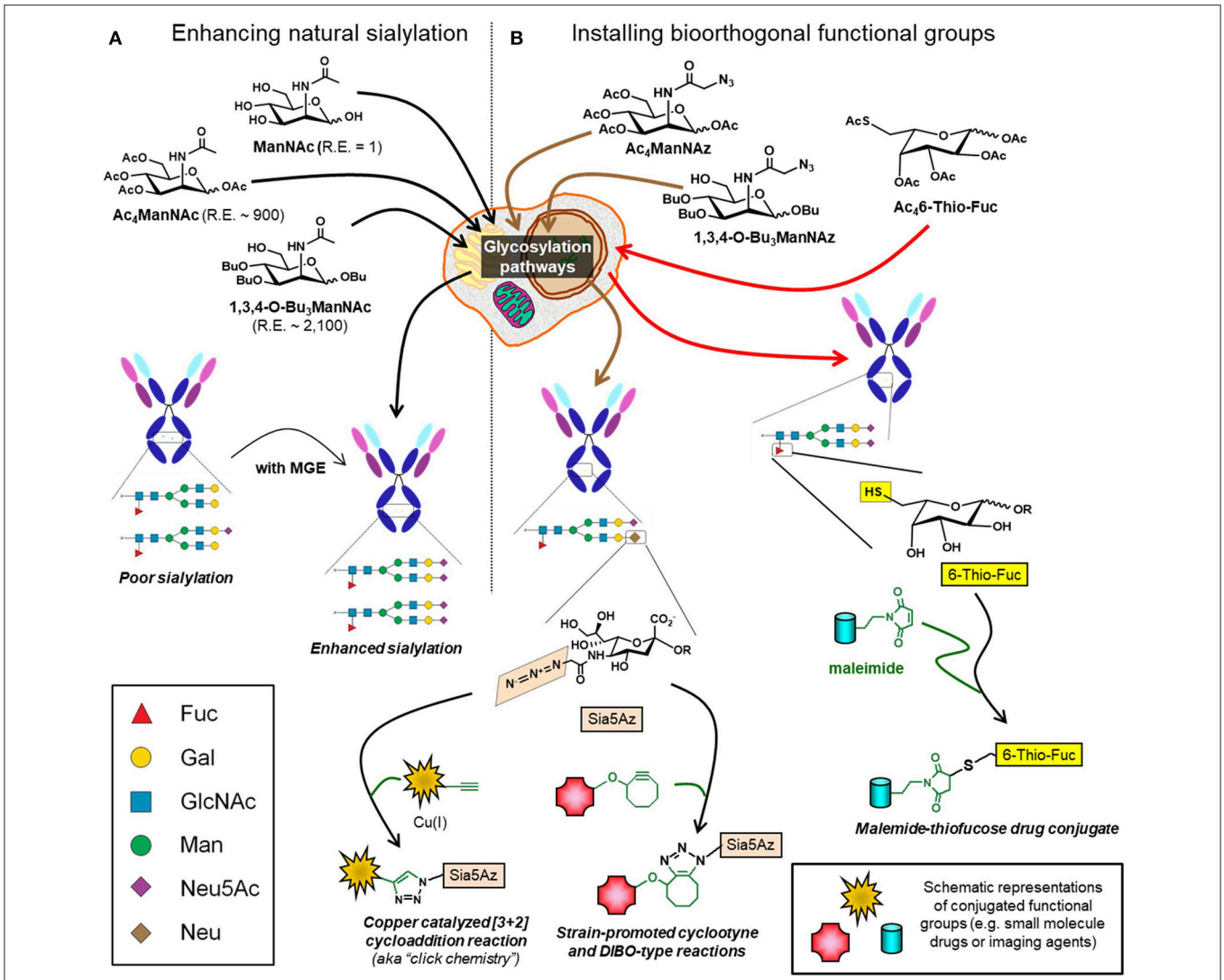

FIGURE 8 | Glycoengineering mAbs for enhanced sialylation and glycan-targeted ADC production. **(A)** Cells can be supplemented with ManNAc or analogs (e.g., Ac_4ManNAc or 1,3,4-O-Bu_3ManNAc), which intercept and increase flux through the sialic acid biosynthetic pathway with the indicated relative efficiencies ("R.E." values) increasing sialylation of recombinant glycoproteins, such as mAbs. **(B)** Alternatively, cells can be supplemented with analogs containing non-natural chemical moieties (e.g., Ac_4ManNAz or 1,3,4-O-Bu_3ManNAz to install azide groups or $Ac_4$6-Thio-Fuc to install thiols). These functional groups, which do not naturally occur in glycans, constitute chemical handles for conjugation to small molecules including drugs, toxins, or imaging agents.

Single Domain Antibodies and Nanobodies

Canonical antibodies are complex, glycosylated molecules comprised of Fab domains linked to a constant Fc region via a flexible hinge region; furthermore, many antibodies are linked to proteins, toxins, small molecule drugs, or radionuclides that increases their size and complexity (209–211). These properties can lead to incorrect domain association and aggregation (6, 212). To circumvent these pitfalls, efforts have been made to engineer mAbs with smaller sizes and fewer domains. This idea was galvanized in the 1990s by the discovery that *Camelidae* (camels) produce fully functional antibodies devoid of light chains (213). This breakthrough has escalated the development of monovalent (Fab, single chain variable fragment (scFv), single variable V_H and V_L domains) and bivalent (Fab'_2, dibodies, minibodies) antibody-derived fragments now generally termed single domain antibodies or nanobodies. Single domain antibodies and nanobodies are advantageous due to their small size, high solubility, thermal stability, versatility, refolding capacities, reduced aggregation, high tissue penetration, lack of requirement for PTMs, and ability to be produced in nonmammalian cells (212, 214–216). These properties make single domain antibodies and nanobodies especially attractive for imaging, blocking, and neutralization applications (212, 215).

Although the non-essentiality of PTMs has been a "selling point" for single domain antibodies and nanobodies, glycosites can nevertheless ameliorate and expand the utility of this class

of antibodies. For example, PEG conjugated to the N-glycan of scFv increased serum half-life ∼10-fold (217). Another study showed that fusing a single domain antibody with N-linked glycosylation to one lacking glycans improved the construct's ability to neutralize foot-and-mouth disease virus 4-fold (218). Interestingly, shark and camel single domain antibodies can naturally contain sites of glycosylation; although the functional importance these glycans is currently unknown (215). These studies suggest that glycosylation can be used to augment the efficacy of single domain antibodies and nanobodies at least in part through physicochemical considerations (section Design Considerations and Biomanufacturing).

Additional Immunomodulatory Glycoproteins

In addition to antibodies, the largest category of today's immunotherapeutics (219), many other glycoproteins modulate immunity. Three of these (interferons, interleukins, and colony-stimulating factor) that have already achieved clinical translation are summarized below.

Interferons

Interferons are a subclass of cytokines naturally produced by the body. These signaling proteins are grouped into three subclasses (α, β, and γ) according to their cell of origin and inducing agent. Upon binding to their cognate receptors, interferons activate signaling networks that provide antiviral, immunomodulatory, and antiproliferative activity (220). Given their ability to regulate the immune system, these cytokines have been exploited for therapeutic purposes. For example, interferon β–a naturally glycosylated protein—slows the progression of multiple sclerosis, a chronic autoimmune disease resulting in demyelination of nerve sheaths of the central nervous system (221–223). The hyperglycosylation of interferon β enhances its biophysical and pharmacokinetics properties by improving its physicochemical properties (224, 225). Although non-glycosylated interferon β is available, superior versions of glycosylated recombinant interferon β now in clinical use include Avonex® and Rebif® (226, 227).

Interleukin-2

Interleukin-2 (IL-2) is a naturally-occurring cytokine and an early example of an immunotherapeutic protein. Recombinant IL-2 is Food and Drug Administration (FDA) approved for treatment of metastatic renal cell carcinoma and metastatic melanoma with clinical trials underway for several additional diseases (228–231). The importance of glycosylation, usually a critical factor in the efficacy of a biologics drug, remains ambiguous for IL-2; the World Health Organization initially established glycosylated IL-2 as the standard for human use. Subsequent screening of glycosylated and non-glycosylated IL-2, however, showed similar bioactivity (232, 233) although glycosylated IL-2 produced in Jurkat cells had superior thermal stability. Nevertheless, T-cell-derived recombinant IL-2 is no longer in use as a therapeutic (234). Instead, today's FDA-approved recombinant IL-2 (e.g., Proleukin [also known as Aldesleukin] and other variants) is produced using *E. coli*, a species that lacks protein glycosylation (235). All in all, IL-2 provides an interesting example of a biologics drug where the role of glycosylation remains ambiguous although, based on overwhelming evidence from other products, we would not be surprised if superior forms of glycosylated IL-2 are developed in the future.

Colony Stimulating Factor

Colony stimulating factors (CSFs) are potent activators of the innate immune system that modulate the activity and populations of granulocytes and macrophages (236), which are critical hematopoietic cells involved in fighting bacterial, viral, and fungal infections. Given this function, CSFs have been explored to activate the immune system; in particular granulocyte-CSF is commonly used to stimulate the bone marrow to increase neutrophil production to treat neutropenia (237). Presently, five types of granulocyte-CSF have been produced using various expression systems including aglycosylated variants in *E. coli* (molgramostim and filgrastim), an O-glycosylated type in yeast (sargramostim), and versions with mammalian-type glycosylation in CHO cells (regramostim and lenograstim) (238, 239). A comparison of these various forms of granulocyte-CSF suggests that glycosylation prolongs serum half-life without significantly affecting biological activity (240).

Vaccines

As mentioned earlier, vaccines pioneered the field of immunotherapy two centuries ago (241) and remain highly relevant today, as cancer vaccines provide another example (as introduced for MUC1 in section O-Glycans in Immunotherapy and outlined in **Figure 5**). In the modern era, glycans have become an integral part of vaccine development with polysaccharide-directed vaccines such as PCV13 and PPSV23 constituting a critical defense against pneumococcal infections (242, 243) illustrating how glycoconjugates have emerged as some of the safest and most efficacious vaccines (244). Today, vaccine development almost always requires cognizance of glycosylation with firmly established roles ranging from well established, intensely studied viruses such as HIV (245, 246) and influenza (247, 248) to sporadic and emerging threats such as the ebola (249) and zika viruses.

Cell-Based Immunotherapy

Cell-based immunotherapy is rapidly emerging strategy that utilizes living cells such as T-cells, dendritic cells, and mesenchymal stem cells (MSCs) to harness the body's natural immune system to fight disease. In this section we review how glycosylation impacts the efficacy and development of two pioneering cell-based immunotherapies based on CAR T-cells and MSCs.

Chimeric Antigen Receptor (CAR) T-cell Therapy

In 1989 Eshhar and coworkers developed a novel CAR that combined a scFv with a transmembrane domain and an intracellular signaling unit, CD3 ζ chain, enabling targeting to specific epitopes and concurrent activation of T-cells without

dependence on the major histocompatibility complex molecules (250–252). Subsequent efforts enhanced CAR specificity, reduced off target effects, integrated costimulatory receptors, and increased T-cell proliferation capacity (252, 253). Current CAR T-cell preparation involves six steps: (i) harvesting white blood cells from the patient through leukapheresis, (ii) activating the cells using antibody coated beads, (iii) reprogramming the T-cells utilizing retroviruses to express CARs, (iv) expanding the CAR T-cells *ex vivo*, (v) placing the patient in an immunocompromised state via lymphodepleting chemotherapy, and (vi) transfusing the patient with the engineered CAR T-cells (254, 255).

CAR T-cells have been engineered to target glycan epitopes of glycolipids and glycoproteins aberrantly expressed in cancer including TAG72 (the sialyl Tn O-glycan epitope), the Lewis y antigen (Le^y), the disialoganglioside GD2, and Tn MUC1 (256, 257). An early CAR T-cell therapy targeting TAG72 failed to elicit a clinical response possibly due to the CARs murine origin, lack of T-cell co-stimulation, or the affinity of the CC49 anti-sialyl Tn mAb (256, 258). A subsequent CAR T-cell therapy against Le^y was more successful (259) showing therapeutic potential in a phase I clinical trial (260). The ganglioside GD2, which is commonly overexpressed in neural crest-derived tumors, has been targeted in separate CAR T-cell studies. The first was safe and induced tumor necrosis *in vivo* and provided complete response in three out of eleven patients (261, 262). A subsequent GD2-targeting test conducted in conjunction with lymphodepletion resulted in improved CAR T-cell expansion in patients but failed to significantly improve patient antitumor response and survival time (263). Finally, the Tn and sialyl Tn MUC1 epitopes have been targeted by CAR T-cells using a humanized version of the 5E5 antibody (264). Although glycan-targeting CAR T-cell therapy has yet to achieve FDA approval, prospects are bright with 10 active phase I and II CAR T-cell trials targeting MUC1 glycoforms alone (127, 256).

Mesenchymal Stem Cell (MSC) Homing

MSCs, which display potent immunosuppressive properties including inhibiting proliferation and activity of T-cells, inhibiting production of pro-inflammatory cytokines, mediating differentiation of B cells, and inducing macrophages *in vitro* (265, 266), are an emerging type of immunotherapy. Delivery of MSCs *in vivo*, however, typically suffers from inefficient homing and migration of MSCs to the target tissue (267). This pitfall has spurred research in several laboratories to improve MSC homing with efforts largely converging on exploiting selectin-mediated cell trafficking to direct systemically-delivered MSCs to sites of inflammation (or other desired locations, such as the bone marrow) in the body (267).

Selectin-mediated cell trafficking critically depends on the fucose-containing tetrasaccharide sLe^x [Neu5Ac-α(2,3)-Gal-β(1,4)-[Fuc-α(1,3)]-GlcNAc-R, **Figure 3**]. MSCs lack expression of the fucosyltransferases (Fut3-7) required for sLe^X synthesis (268, 269); without sLe^x MSCs have poor homing ability, which limits their immunotherapeutic potential. This pitfall is being overcome through a variety of strategies where MSCs are endowed with the requisite fucosyltransferase activities needed to create sLe^x motif and efficiently home to specific locations in the body (270–274). For example, glycoengineering via glycosyltransferse-programmed stereosubstitution and transfection with modified mRNA has been used to drive over expression of sLe^x to augment the homing capacity of numerous cell types including hematopoietic and progenitor stem cells (HSPCs) (275), MSCs (270), neural stem cells (276), and lymphocytes (118, 277).

DESIGN CONSIDERATIONS AND BIOMANUFACTURING

We begin this section by discussing how the physicochemical properties of glycans—which have been alluded to several times already, mostly in the context of pharmacokinetics—impact immunotherapeutics in section Physicochemical Properties. We then discuss, in section Cell-based Production Options, how the selection of the appropriate host cell as a biomanufacturing platform is crucial for endowing an immunotherapeutic drug with appropriate glycoforms to optimize not only physicochemical properties but also to maintain safety and improve bioactivity. Finally, in section Glycoengineering Approaches to Improve Immunotherapeutics we provide an overview of "glycoengineering" strategies—that typically complement and are fully compatible with cell-based production platforms that are being developed to enhance future immunotherapeutics.

Physicochemical Properties

Physicochemical considerations are critically important during the optimization of virtually all biologics, including immunotherapeutics. Even when the biological properties of a potential drug are tuned for optimal efficacy during early discovery phases, intractable "developability" issues often crop up later related to the physicochemical nature of the candidate. Physicochemical problems that can thwart drug development include difficulties in formulating a biologic for appropriate dosing, absorption to surfaces that causes large variance in delivery, protein aggregation or stability during storage, and solubility. Commonly employed strategies to improve physicochemical properties, such as PEGylation (which is mentioned above, e.g., in section O-Glycans in Immunotherapy), can affect immunity in sometimes unpredictable ways and also adversely impact safety (278–280). The *Guidance for Industry: Immunogenicity Assessment for Therapeutic Protein Products* published by the FDA states that

"For proteins that are normally glycosylated, use of a cell substrate production system and appropriate manufacturing methods that glycosylate the therapeutic protein product in a non-immunogenic manner is recommended (281)."

Consequently, although initially easier to implement than undertaking efforts to optimize pharmacokinetic properties through glycosylation, PEGylation of glycoproteins may require more work in the end because anti-drug antibody assays need to be developed to detect both the anti-protein antibody as well as antibodies against PEGylated epitopes

found on the protein. A somewhat underappreciated role for glycosylation is the profound impact that it can have on the physicochemical properties of proteins, many of which are important for the developability of a lead biologic drug candidate. The fact that glycosylation can be viewed as "more natural" by the body (considering human types of glycosylation only), is another advantage that promotes the need for optimized glycoengineering strategies described in this review. Finally, as noted above, glycosylation often tunes biological activity (e.g., Fc effector function) in ways not accessible through PEGylation. Below, we discuss the impact that glycosylation has on the physicochemical properties of therapeutic proteins and the development of biologic drug candidates.

Protein Aggregation

Many amino acids are electrically charged, are basic or acidic, or contain a thiol; the peptide backbone of a biologic is therefore typically vulnerable to unwanted and difficult-to-control chemical reactivity, and problems such as protein aggregation are often encountered during development. Aggregate bodies can elicit immunogenicity that ultimately leads to the intolerance and rejection of drug candidates (282). It has long been observed, however, that glycosylation can significantly improve the aggregation properties of proteins. For example, O-linked glycosylation can suppress the polymerization of an immunomodulating protein like human granulocyte-CSF (283). Crystallographic analysis of glycosylated interferon β marketed by Pfizer (Rebif®) revealed this drug was 10 times more potent than its unglycosylated counterpart due to the prevention of the formation of large, soluble aggregates (224, 284). In fact, interferon β produced in *E. coli* that is unglycosylated quantitatively contains about 60% aggregates that elicit antibodies in a high portion of patients while the glycosylated form contains only ∼2% aggregates and is far less immunogenic (285).

From a production perspective, prevention of aggregate formation is important for improving yields of useable drug product (286). Aglycosylation—a strategy typically employed to simplify the production of antibodies—can increase aggregation (287). The prevention of protein aggregation by glycosylation is a complex physicochemical phenomenon that is not easily rationalized simply by the attachment of a hydrophilic constituent to a protein because glycans theoretically interact less favorably with water than the peptide backbone (286). Nevertheless, in theory, glycosylation slows aggregation by increasing the molecular solvent accessible surface area of a protein. In one study, increased glycosylation changed the surface area of the glycoconjugates from ∼9,000 Å to ∼16,000 Å, and the exposed surface area of the protein concomitantly decreased (from ∼9,000 Å to ∼5,000 Å), which influenced the internal electrostatic and biophysical properties of proteins through a steric diaelectric effect (288). Glycoengineering and optimization of production platform glycosylation stands to improve both the biomanufacturing process and biological drug properties of immunotherapeutics.

Colloidal Stability

Another important physicochemical parameter that influences aggregation is colloidal stability. Proteins have intrinsic colloidal properties and most, if not all, biologics are administered and stored as solutions; therefore, improving the colloidal stability of protein therapeutics is critically important for shelf-life. Høberg-Nielsen and co-workers, for example, demonstrated that glycosylation promoted colloidal stability of aggregation-prone forms of the phytase enzyme from *Pheniophoria lycii* (286). In addition to the influence that N-glycans have on Fc receptor binding, these glycans stabilize the Fc C_H2 regions of mAbs by protecting against aggregation through colloidal properties (289). Interestingly, previous studies have shown that under conditions of high temperature and high concentration (60°C and 20 mg/mL) aggregation in the model protein α-chymotrypsin could not be inhibited by a small glycan, but two or more larger glycans improved colloidal stability and abrogated aggregation (290). Based on this precedent, and others, the glycoengineering of immunotherapeutics is expected to improve shelf-life and ameliorate formulation issues by modulating of the colloidal properties of these proteins.

Conformational Stability

Over the last 30 years nuclear magnetic resonance (NMR), circular dichroism, Förster resonance energy transfer (FRET), and powerful *in silico* techniques have provided important insights into how glycosylation influences the secondary structure and conformational dynamics of a protein (291). Complementary NMR-FRET studies have shown that β-turns followed by a surface loop transition, a common motif for sites of N-linked glycosylation, have a more compact peptide secondary structure when glycosylated with a chitobiosyl disaccharide group. These regions adopt an open and extended Asn-turn conformation when aglycosylated while the introduction of a glycan results in a compact type I β-turn structure, illustrating how glycosylation can serve as a "conformational switch" for proteins (291, 292). These observations also correlated with the *in silico* statistical calculations performed by Petrescu et al. who surveyed 506 glycoproteins and found that N-glycans alter the distribution of torsion angles within the protein to possibly reduce overall flexibility (293). Similarly, earlier elegant work revealed that oligosaccharides enhanced global dynamic stability and the unfolding equilibrium of RNaseB, and furthermore, this effect could be observed as far as 30 Å away from the site of glycosylation (294). The take home message is that glycosylation can serve to alter the equilibrium states between folded and unfolded proteins and can help select for small populations of conformers that have defined, stable, and precise structure (e.g., proteins with N-glycan proximal to their β-loops). Ultimately, this increased glycan-mediated stability complements glycan-mediated benefits related to aggregation and the colloidal properties of glycoproteins as discussed above.

Protection of Proteins From Oxidation

Another physicochemical feature of biologics tuned by glycosylation is susceptibility to oxidative insult. Because extracellular space is an oxidizing environment, the half-life,

distribution, and efficacy of immunotherapeutics could be enhanced by resistance to oxidative stresses ubiquitous inside of a living organism. Again, glycosylation is beneficial because it can protect the polypeptide backbones of proteins from free-radical damage (295); protection was linked to the total degree of glycosylation and not any specific glycan or sugar moiety, indicating that "highly branched" glycans would be broadly protective. In the model protein EPO, oxidative damage to tryptophan that led to loss of biological activity, was thwarted by glycosylation (296). Related to immunotherapy, oxidation of methionine and tryptophan triggers the degradation of monoclonal antibodies (297, 298) and interferons are also susceptible to oxidation (299–301). In general, oxidized proteins also are immunogenic, an unwanted attribute of immunotherapeutic drugs; interestingly, despite earlier examples where glycans were the source of immunogenicity (e.g., for α-Gal or Neu5Gc, **Figure 3**) the examples provided in this paragraph illustrate how glycans can instead be protective by minimizing oxidative damage.

Physicochemical Conclusions

Although the impact of glycosylation on immunotherapeutics is often focused on biological function, glycans also have a powerful ability to tailor physicochemical features critical for clinical translation and commercial developability. Specifically, glycosylation can optimize physicochemical considerations of biologics to improve features such as shelf-life, colloidal stability, resistance to oxidation, and the avoidance of unwanted immunogenicity. Although synthetic techniques such as PEGylation have been extensively used to improve physicochemical properties, control of glycosylation—achieved through appropriate selection of cell line for production (section Cell-based Production Options) or through glycoengineering methods (section Glycoengineering Approaches to Improve Immunotherapeutics)—can potentially provide superior results because glycosylation has been developed by nature over hundreds of millions of years to finely regulate the biology of proteins.

Cell-Based Production Options

Early generations of immunotherapeutics, such as vaccines, largely were produced in embryonated eggs or collected from animal products and human blood donations (5, 302). Today's immunotherapeutics, however, exploit recombinant DNA technology to produce proteins in cell-based manufacturing platforms (whereas certain immunotherapies, as discussed above [section Cell-based Immunotherapy], consist of the cells themselves). Cell-based biomanufacturing efforts have explored a wide range of expression systems including non-mammalian (bacteria, yeast, plant, and insect) and mammalian (human, hamster, and mouse) cells (179) to optimize product yield and install appropriate PTMs. From 2004 to 2013 biopharmaceuticals approved by the FDA and European Medicines Agency (EMA) were predominantly obtained from mammalian cells (56%), *E. scoli* (24%), *Saccharomyces cerevisiae* (13%), insect cells (4%), and transgenic animals and plants (3%) (303). The majority of products, obtained from mammalian cells, includes virtually all recent therapeutic proteins (including immunotherapeutics) where PTMs, especially glycosylation, can be optimized for safety, biological activity, function, stability, physicochemical properties, and pharmacokinetics (2, 111, 304). For this reason—after providing a brief synopsis of non-mammalian options (section Non-mammalian Cell lines)—we focus on the selection of mammalian expression systems used in biomanufacturing beginning with the use of human (section Human Cell Lines) and murine (section Murine Cell Lines) cell lines used in the early production of modern immunotherapeutics (i.e., mAbs). As discussed below, each of these cell lines had substantial pitfalls, leading to today's consolidation of production in CHO cells (section Chinese Hamster Ovary (CHO) Cells).

Non-mammalian Cell Lines

Insulin, the earliest recombinant human protein, was produced in *E. coli*, which benefits from low cost and high productivity (303, 305). Although a few biologics are still produced in *E. coli* (e.g., IL-2, as described in section Interleukin-2), the lack of N-glycans that ensure quality control during folding (306) makes prokaryotic production untenable for most glycoproteins including mAbs. Yeast (*S. cerevisiae* and *Pichia pastoris*) provide another high productivity, low cost production platform (307, 308) and—being eukaryotic cells—do have N-glycans; yeast glycans, however, tend to be highly mannosylated which reduces serum longevity thus compromising pharmacokinetics and also impacting downstream effector functions (309). Even though efforts have been made to "humanize" yeast glycosylation, these cells have not become a widely-accepted biomanufacturing platform (309). Finally, insect (e.g., *Trichoplusia and Drosophila*) cells have been investigated for recombinant glycoprotein production, but despite efforts to humanize glycosylation (310–312), these cells also have substantial pitfalls for biomanufacturing including minimal sialylation ability (311, 313).

Human Cell Lines

The inability of the initial bacterial, yeast, and insect production platforms to produce properly glycosylated human proteins led to production efforts in human cells. The first immortalized human cell line, HeLa, was derived from cervical cancer in 1951 (314) and paved the way for the development of other immortalized human cell lines, notably human embryonic kidney 293 (HEK293) and fibrosarcoma HT-1080 cells used to produce viral vaccines (106, 180, 315). However, it wasn't until ∼2001 that the first therapeutic glycoprotein produced in human cells (HEK293), Drotecogin alfa, was approved by the FDA and EMA; since then several glycoprotein immunotherapeutics have been produced in human cells primarily in the HEK293 and HT-1080 lines (179).

Human cells offer important advantages over other production platforms including the ability to closely mimic PTMs, particularly glycosylation, naturally found in people. For example, human cells lines express Mgat3, α(1,3/4)-fucosyl transferase, and α(2,6)-sialyltransferse which are silent or missing in CHO cells. Furthermore, human cell lines do not produce immunogenic structures, such as α-Gal and

N-glycolylneuraminic acid (Neu5Gc), thus minimizing safety and compatibility concerns. These factors reduce the need to genetically engineer cells and limit the cost of downstream processing (106, 180, 316). Although human cells have these attractive features as production platform, they also have substantial limitations and drawbacks. For example, human lines suffer from low growth rates, production capacities, and protein yields making them impractical for the production of many therapeutic proteins including mAbs. Furthermore, the absence of a species barrier makes human cell lines a significant safety risk due to the potential for contamination and transmission of human pathogens. In theory, these disadvantages can be overcome with advances in technology and adherence to stringent good manufacturing practices (106, 180, 316); in practice, most immunotherapeutics are now produced in rodent cells, as described next.

Murine Cell Lines

Murine myeloma cells, predominantly NS0 and Sp2/0, are another cell platform that is periodically used for the production of recombinant glycoproteins. Both the NS0 and Sp2/0 cell lines were developed from tumors and subsequently genetically engineered to stop producing their native immunoglobins yet retain the cellular machinery to secrete recombinant proteins at high levels (317, 318). Accordingly these lines have been used to produce of the commercial mAbs Cetuximab, Palivizumab, Dinutuximab, Necitumumab, and Elotuzumab (179, 180, 319). A downside of murine cells is their ability to incorporate α-Gal and Neu5Gc into glycans, thereby presenting a considerable risk of immunogenicity (49, 320, 321). Thus, murine cells used for therapeutic protein production must be thoroughly screened for clones lacking these immunogenic epitopes while producing desirable glycan profiles.

Chinese Hamster Ovary (CHO) Cells

In 1986 tissue plasminogen became the first FDA-approved recombinant biopharmaceutical to be produced in CHO cells (180, 316, 322); since then these cells have become the predominant manufacturing platform for biologics producing an estimated 70% of recombinant biopharmaceutical proteins (2, 323, 324). Furthermore, over 90% of commercial antibodies are now produced in CHO cells (6, 179, 180). The success of CHO cells in commercial biomanufacturing stems from several key advantages. First, CHO cells can be grown in large bioreactors as a cell suspension in serum-free, chemically-defined media while maintaining high production rates. From a safety perspective, many viral entry genes are not expressed in CHO cells and there is a species barrier that minimizes risk of transferring infectious agents to humans (325, 326). Furthermore, over the past three decades the extensive documentation that CHO cells are safe hosts aids in facilitating regulatory approval to bring immunotherapeutics to the market (316, 322). Perhaps most importantly, CHO cells produce recombinant glycoproteins with compatible glycoforms that are bioactive in humans (179, 180, 322, 327).

Despite the advantages of CHO cell production platforms, shortcomings exist. CHO cells (as with most mammalian cell lines) retain the ability to produce glycans not found in humans including α-Gal and Neu5Gc (320, 328). Humans inherently express antibodies against these immunogenic epitopes that can lead to severe, potentially fatal immunogenic responses and/or negate the effects of immunotherapeutics (49, 320, 321). However, the levels of α-Gal and Neu5Gc are relatively low (<2% Neu5Gc and <0.2% α-Gal) in CHO cells, meaning this issue can be circumvented by selecting clones lacking these non-human epitopes (179, 320). CHO cells also lack certain types of glycosylation found in humans, such as α(2,6)-sialylation, α(1,3/4) -fucosylation, and bisecting GlcNAc (329–332). Overcoming these differences by "humanizing" CHO cell glycosylation is, at least in theory, possible through genetic and metabolic "glycoengineering" approaches, as discussed next in section Glycoengineering Approaches to Improve Immunotherapeutics.

Glycoengineering Approaches to Improve Immunotherapeutics

Various approaches to modulate glycans in living cells—i.e., "glycoengineering" methods—have developed over the past ∼3 decades during the same time as the importance of glycosylation in immunity has been unraveled. Today, these parallel developments have set the stage to employ the various glycoengineering strategies now available to generate recombinant proteins (or even entire cells) with desirable glycan profiles (12, 333, 334) during immunotherapeutic design and manufacturing. Glycoengineering falls into two main approaches: genetic and metabolic; we will discuss specific examples of both approaches while describing general strengths and drawbacks to each approach. Although glycoengineering strategies are being developed for many production platforms [bacteria (161), yeast (335), plants (336), insects (337)], we will focus our discussion on mammalian cells used to produce the vast majority of today's immunotherapeutics.

Genetic Approaches to Glycoengineering

Many genetic approaches have been used to target glycosylation pathways and enzymes via gene knockdown, knockout, overexpression, knockin, and selective nucleotide mutation. These "genetic engineering" strategies have been used to reduce or silence undesirable glycosyltransferase activities, enhance glycosyltransferase activities, activate endogenously silent genes, introduce new glycosites, mimic hypomorphic disease mutations, and insert foreign genes (334). In recent years, genetic glycoengineering has been galvanized by the discovery and development of zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/targeted Cas endonuclease (CRISPR/Cas) technology (334, 338, 339). A strength of genetic approaches is their versatility and ability to make permanent cellular modifications; however, genetic approaches have limitations such as off-target effects, inefficient *in vivo* delivery systems, confounding epigenetic regulation of glycosylation pathways, and unpredictable alterations to cellular physiology (334, 340).

Sialic acid is one of the most frequently targeted monosaccharides for glycoengineering due to its manifold impact on the pharmacokinetics of recombinant glycoproteins in general and its specific impact on bioactivity in ADCC, IVIG, and ADCs. Genetic manipulation of sialyltransferases constitutes a common approach to glycoengineer sialic acid; in particular β-galactoside α(2,6)-sialyltransferases (usually ST6GAL1) in CHO cells enables the production of glycoproteins with both α(2,3)-sialic acids (from the cells' endogenous STs) and α(2,6)-linked sialic acids (from the newly-expressed ST6GAL1), similar to glycoproteins produced in humans (339, 341–343). In addition, overexpression of ST6GAL1 (or other sialyltransferases) increases the overall sialylation of therapeutic glycoproteins including EPO (343–345), tissue plasminogen activator (342, 346), interferon γ (347, 348), and IgG (346, 349, 350). Other studies have targeted the preceding step, the addition of galactose, to enhance terminal sialylation levels. Multiple studies have demonstrated that concomitant over-expression of β(1,4)-galactosyltranferase and α(2,3)-sialyltranferase in CHO cells yielded increased sialylation and galactosylation in EPO, IgG, and tissue plasminogen activiator (344, 346). Another strategy is to overexpress Mgat4 and 5 to increase tri- and tetra-antennary branched N-glycans, thereby creating more sites for terminal sialylation; this strategy has been employed in EPO (345), albumin EPO (351), and interferon γ (352, 353).

Another strategy for improving sialylation targets enzymes and transporters in the sialic acid biosynthetic pathway to increase CMP-Neu5Ac levels. One approach recapitulated point mutations in the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE) associated with sialuria (354, 355), a congenital disease that leads to excessive synthesis of sialic acid due to the absence of feedback regulation (356), which led to increases in intracellular CMP-sialic acid levels and EPO sialylation (357, 358). Although increasing intracellular CMP-Neu5Ac levels can increase glycoprotein sialylation there may be a saturation point due to the inefficiency of the CMP-sialic acid transporter responsible for transporting CMP-Neu5Ac to the Golgi. To overcome this barrier one study overexpressed CMP-sialic acid transporter in CHO cells, but only saw modest increases (4–16%) in interferon γ sialylation (359). Inhibiting or eliminating sialidases (or neuraminidases) is a complementary strategy for enhancing glycoprotein sialylation; these enzymes are glycosidases that catalyze the hydrolytic removal of sialic acid from glycoproteins, glycolipids, and polysaccharides (360). One study utilized short interfering RNA and short-hairpin RNA to lower expression of the *Neu1* and *Neu3* sialidase in CHO cells, which increased recombinant interferon γ sialylation by up to 33% (361).

In another approach, genetic glycoengineering can be utilized to introduce new glycosites into glycoproteins through creation of the Asn-X-Ser/Thr consensus sequence for N-glycosylation. This approach is illustrated by darbepoetin alfa, a genetically modified form of EPO that has five (instead of three) N-glycan sites (362); this enhanced level of glycosylation improved serum longevity ∼3-fold (362) but was accompanied by adverse effects such as increased risk of stroke (363). (As a caveat, there is no evidence from carefully controlled studies that increased risk is a general feature of over-glycosylated therapeutic proteins beyond darbepoeitin alfa or a direct consequence of the newly-installed glycans). Another interesting example of "building in" N-glycosites is provided by Ibalizumab, where the strategic addition of an N-glycan to this mAb improves its HIV-neutralizing activity (364). In the future, installation of glycans on various immunotherapeutics, e.g., *Camelidae* antibodies (section Single Domain Antibodies and Nanobodies), may prove enhance the physicochemical properties and translational potential of these emerging drugs.

Metabolic Glycoengineering

The second major strategy to control glycosylation is metabolic glycoengineering (MGE), where living cells or entire organisms are supplemented with monosaccharide precursors that either increase natural flux through a biosynthetic pathway (**Figure 8A**) or increasingly, substitute natural metabolites with their non-natural counterparts (**Figure 8B**). The exogenously-supplied synthetic monosaccharides are processed by the biosynthetic pathway, ultimately yielding glycans with enhanced glycoforms (e.g., improved sialylation) or non-natural chemical groups (203, 365, 366). Historically the sialic acid biosynthetic pathway has been the premier target of MGE due to this pathway's tolerance for non-natural variants of mannosamine or sialic acid (203). One advantage of MGE is its simplicity, where an analog can be directly added cell culture medium to exploit the intrinsic cellular machinery without any need to genetically manipulate the host cell, thus averting off-target complications. However, MGE can be non-trivial because of the need for custom synthesis of the required monosaccharides and expensive to implement on an industrial scale because their concentration in culture media must be maintained to obtain a desired glycan profile (203, 365).

One application of MGE relevant to immunotherapy involves increasing therapeutic glycoprotein sialylation through supplementation with ManNAc (367), this strategy, outlined in **Figure 8A** in the context of IgG antibodies has the potential to increase the physicochemical and pharmacokinetic properties of these antibodies, endow them with anti-inflammatory activity needed for IVIg therapy, or provide sialic acids required for ADC production [Neu5Ac can be oxidized to contain an aldehyde group allowing for drug conjugation via oxime ligation (201)]. A pitfall for ManNAc supplementation is that millimolar concentrations of ManNAc are required, which increase intracellular CMP-sialic acid levels up to 12-fold but only produces moderate gains in protein sialylation (368–370); the requirement for large concentrations of ManNAc (e.g., 20–50 mM) to achieve modest improvements are impractical from a biomanufacturing perspective due to the cost of ManNAc ($20 / g or higher). Efforts have long been underway to improve the efficiency of monosaccharide analogs intended as metabolic supplements ranging from fluorinated ManNAc analogs in the early 1980s (371) to disaccharides in the mid-1990s (372) to non-natural ManNAc analogs used in MGE in the late 1990s (372) through peracetylation (e.g., as illustrated by Ac_4ManNAc, **Figure 8A**). Despite improving efficiency by ∼900-fold (373), growth inhibition and cytotoxicity (374, 375) limit

the application of per-acetylated analogs in a biomanufacturing setting. To circumvent these issues our group has developed (376, 377) and characterized (378) butyrated ManNAc analogs that can be applied to culture medium in micromolar concentrations. The analog's butyrate groups enhance cellular uptake by ∼2,100-fold and are subsequently cleaved by nonspecific esterases allowing the ManNAc to intercept and increase flux through the sialic acid biosynthetic pathway (379). Supplementation of CHO cells with the "high-flux" ManNAc analog (1,3,4-O-Bu_3ManNAc, **Figure 8A**) improves EPO and IgG sialylation (**Figure 8**) (380, 381) and in theory, could be used to augment the pharmacokinetic and physicochemical properties of any recombinant immunotherapeutic.

In a second MGE-based approach, ManNAc analogs can be used to install non-natural chemical moieties into glycans (**Figure 8B**), in essence creating a chemical handle for bioorthogonal conjugation of small molecules including toxins, drugs, genes, imaging agents, and polymers (203). This strategy has been used to incorporate numerous non-natural functional groups such as ketones (382–384), azides (377, 385), alkynes (386), diazirines (387), aryl azides (388), and thiols (389) into glycans for subsequent conjugation via click chemistry. A sialic acid-based MGE approach can be used to introduce conjugation sites restricted to the Fc region of mAbs for developing ADCs (373, 390, 391); similarly, the fucose-replacing analog 6-thiofucose can introduce thiol moieties into 70% of IgG heavy chains with 90% conjugation efficiency to small molecule drugs via maleimide chemistry (204). As superior metabolic analogs [e.g., butyrated ManNAz [1,3,4-O-Bu_3ManNAz], **Figure 8B** (377)] and conjugation chemistries [e.g., strain-promoted alkyne:azide cycloaddition (376, 377)] are developed we anticipate a bright future for MGE-based ADCs.

Combined Genetic and Metabolic Engineering Approaches

The field of MGE has often been regarded as a genetically "silent" method to label glycans based on the assumption that the "glycosylation machinery" of a cell is not substantially perturbed while processing the exogenously-supplied sugars required for this methodology. While this premise is basically accurate, our group (and others) have described how metabolic flux engendered by MGE monosaccharide analogs (and even natural sugars) can on occasion affect the expression of "glycogenes" with this effect most well studied for the sialic acid biosynthetic pathway (392–395). The ability of MGE analogs to affect gene expression and cell physiology extends beyond glycogenes *per se* and can have a profound impact on cellular processes such as cell differentiation (396–398). We briefly mention these effects both to caution researchers to the complex interplay between metabolic, genetics, and cell fate that can occur during MGE interventions but also to highlight the opportunities to use this technology to tune biological activity, which we fully anticipate will facilitate future generations of immunotherapy.

CONCLUSION AND FUTURE PERSPECTIVES

Over the past 30 years immunotherapy has become the most promising approach for developing new medicines and treating disease. In order to maintain the rapid advancement of immunotherapies it is critical to not only optimize glycosylation for maximal efficacy but also exploit these macromolecules to ameliorate existing treatments. To reach these goals it is vital to better understand the underlying biology of glycosylation which requires the ongoing development of novel tools for studying glycosylation and continued improvement of carbohydrate chemistry methods. Moving forward areas of glycobiology not typical associated with immunotherapy, such as O-linked glycosylation [both mucin-type and other forms, such as the intracellular "O-GlcNAc" PTM now being linked to immunity (399–402)] and glycolipids, are sure to offer new opportunities for creating biotherapeutics. Finally, although immunotherapy has already achieved substantial success in treating disease we are only scratching the surface, therefore we foresee glycosylation a key to helping immunotherapy realize its full potential in the future.

AUTHOR CONTRIBUTIONS

KJY and MJB: Writing, editing, figures; CTS: Writing, editing; SRS, RA: Writing.

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REVIEW
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Glycans as Key Checkpoints of T Cell Activity and Function

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The immune system is highly controlled and fine-tuned by glycosylation, through the addition of a diversity of carbohydrates structures (glycans) to virtually all immune cell receptors. Despite a relative backlog in understanding the importance of glycans in the immune system, due to its inherent complexity, remarkable findings have been highlighting the essential contributions of glycosylation in the regulation of both innate and adaptive immune responses with important implications in the pathogenesis of major diseases such as autoimmunity and cancer. Glycans are implicated in fundamental cellular and molecular processes that regulate both stimulatory and inhibitory immune pathways. Besides being actively involved in pathogen recognition through interaction with glycan-binding proteins (such as C-type lectins), glycans have been also shown to regulate key pathophysiological steps within T cell biology such as T cell development and thymocyte selection; T cell activity and signaling as well as T cell differentiation and proliferation. These effects of glycans in T cells functions highlight their importance as determinants of either self-tolerance or T cell hyper-responsiveness which ultimately might be implicated in the creation of tolerogenic pathways in cancer or loss of immunological tolerance in autoimmunity. This review discusses how specific glycans (with a focus on *N*-linked glycans) act as regulators of T cell biology and their implications in disease.

Keywords: *N*-glycosylation, glycans, T cells, immune response, autoimmunity, self-tolerance

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INTRODUCTION

The immune system is highly regulated by a series of stimulatory and inhibitory pathways that are crucial to maintain a healthy and balanced system. Disruption of the control of this immunological balance can result in abnormal stimulatory signals associated with the loss of immune tolerance in autoimmunity or in the creation of aberrant immunosuppressive networks that occur in cancer. Accumulating evidences have been demonstrating the importance of glycans and glycans binding proteins [including galectins (1, 2), C-type lectins (3), and sialic acid-binding immunoglobulin-type lectins (siglecs) (4, 5)] in the regulation of both innate and adaptive immune responses. In fact, all cells are covered with a dense coat of glycans that constitute a major molecular interface between cells and their environment. The diversity of glycans presentation at cell surface is enormous, encoding a myriad of important biological information that remains to be fully characterized. Glycosylation is the enzymatic process responsible for the attachment of glycans (carbohydrates) to

proteins or lipids (predominantly via nitrogen (*N*) and oxygen (*O*) linkages), a process that occurs in the Endoplasmic Reticulum/Golgi compartment of essentially all cells being mediated by the coordinated action of a portfolio of different glycosyltransferases and glycosidases enzymes (6). The proper development and function of the immune system relies both on the dynamic regulation of the expression of glycan-structures and glycan-binding proteins, and the interactions between them (7). This review discusses the role of glycans (with a focus on *N*-linked glycans) on T cells biology and function, including T cell development, activation, differentiation, and signaling. This dynamic interplay between glycans and T cells activity controlling both auto-reactivity and self-tolerance will be presented and discussed (**Figure 1**).

GLYCANS IN T CELL DEVELOPMENT AND THYMUS SELECTION

T cells are developed in the thymus where a microenvironment is set, which enables the selection of T cell receptors (TCRs) to generate a diverse repertoire of potential antigen recognition (8). Lymphoid progenitors from the bone marrow enter into the cortical tissue of the thymus, where they start to expand and develop (9, 10). Despite the fact that the role of glycosylation in T cell development and thymus selections still remains to be fully understood, some important findings highlight the relevance of glycans in this process (**Figure 2**).

Role of Glycans in Thymus Seeding and T Cell Lineage Commitment

The initial step of T cell development, the trafficking of thymus-seeding progenitors (TSPs) to the thymus, is an active process that relies on the expression of P-selectin in the thymic epithelium and its partner, P-selectin glycoprotein ligand-1 (PSGL-1), expressed by circulating TSPs-derived from the bone marrow (11). The expression and post-translational modifications of PSGL-1 are regulated in bone marrow progenitors. The deficiency of α1,3 fucosylation on PSGL-1, required for its binding to P-selectin, was shown to be associated with the impairment of TSPs homing into the thymus (12). Once TSPs enter the thymus, they develop into early thymocyte progenitors (ETPs), a subset of the $CD4^{-}CD8^{-}$ double negative 1 (DN1) population, which give rise to multiple lymphoid lineages (8). The conserved Notch signaling pathway is responsible for the commitment of DN1 thymocytes to the T cell lineage (13). The glycosylation profile of Notch receptors (and ligands) was shown to regulate Notch-dependent intracellular signal transduction. The lunatic, manic, and radical Fringe are the glycosyltransferases that modify Notch receptors by transferring *N*-acetylglucosamine (GlcNAc) to *O*-linked fucose glycans of epidermal growth factor-like (EGF-like) repeats, present in the extracellular domain of Notch, and described to regulate its cell-surface signaling and function (14, 15). Loss of the three Fringe glycosyltransferases leads to a reduced binding of Notch to Delta-like ligands (DLL), namely DLL4, altering the frequencies of several T cell subsets in the thymus (16). The first indication that Fringe-mediated Notch glycosylation was involved in T cell development was shown when the lunatic Fringe gene, *Lfng*, was misexpressed under a *lck*-proximal promoter (17). This alteration of the Notch glycosylation profile (lack of GlcNAc in the EGF-like repeats) resulted in a large B cell population developed from lymphoid progenitors in the thymus. In fact, further work showed that *Lfng* is poorly expressed in $CD4^{+}CD8^{+}$ double positive (DP) thymocytes, but when ectopically expressed in that population (under *lck*-proximal promoter), led to an increased binding of Notch to its ligands on stromal cells, blocking DN development, and enabling B cell differentiation (18). These studies also revealed that changes in the glycosylation of Notch across T cell development also impacts on its signaling pathway. At DN stages, the reactions that drive development are dependent on Notch interactions with DLLs, which exist at functionally limiting concentrations. The high levels of *Lfng* expression in DNs facilitate Notch interactions with DLLs and the dramatic downregulation of *Lfng* in DPs coincides with Notch-independent reactions of T cell development. The final commitment to the T cell lineage occurs at the DN3 stage, where a recombination-activating genes (RAG)-mediated productive rearrangement of the *Tcrb* leads to the expression of the ß chain of the TCR (TCRß) and the formation of a pre-TCR signaling complex (13, 19).

Role of Glycans in Thymocyte ß Selection

Together with Notch and Interleukin (IL)-7, the pre-TCR signaling initiates ß-selection, by inducing the downregulation of the RAG complex expression (*Rag1* and *Rag2*) in quiescent DN3 (DN3a), becoming large cycling DN3 thymocytes (DN3b), which differentiate into DN4 cells. A deficient pre-TCR signaling in *lck*-null cells is rescued by *Lfng* overexpression, but not in a $Rag2^{-/-}$ background, indicating a pre-TCR dependency for development (20). Upon ß-selection, it was recently demonstrated that DN4 cells upregulate glucose and glutamine metabolites that enter into the hexosamine pathway, increasing the production of UDP-GlcNAc, which is needed to undergo clonal expansion (8, 21). The UDP-GlcNAc is also the substrate of the *O*-GlcNAc transferase (OGT) in the process of *O*-GlcNAcylation of intracellular proteins on serine and threonine residues (22). Recent evidences showed that *O*-GlcNAcylation regulates the process of T cell development (23). Using a conditional knockout mouse model of OGT in the DN stage, it was shown a reduced population of DPs, indicating either a deficiency on ß-selection or in clonal expansion of DN4s. The absence of OGT appeared not to impact self-renewal of DNs, or their differentiation into DPs, but to promote the failure of the clonal expansion of DN4, in response to Notch ligands. A feedback mechanism was proposed in which the metabolic changes (the shift to glycolysis) that support the DN-to-DP stage of thymocyte differentiation, controlled by Notch, induces c-Myc expression, which in turn controls the rate of T cell nutrient uptake as well as the expression of OGT and consequently the abundance of *O*-GlcNAc (15). The *O*-GlcNAcylation of c-Myc was also shown to increase its stability (24), further contributing to the feedback loop.

In the stage of post-β selected DN4 thymocytes, it was seen a 10-fold increase in expression of ST6 β-Galactoside

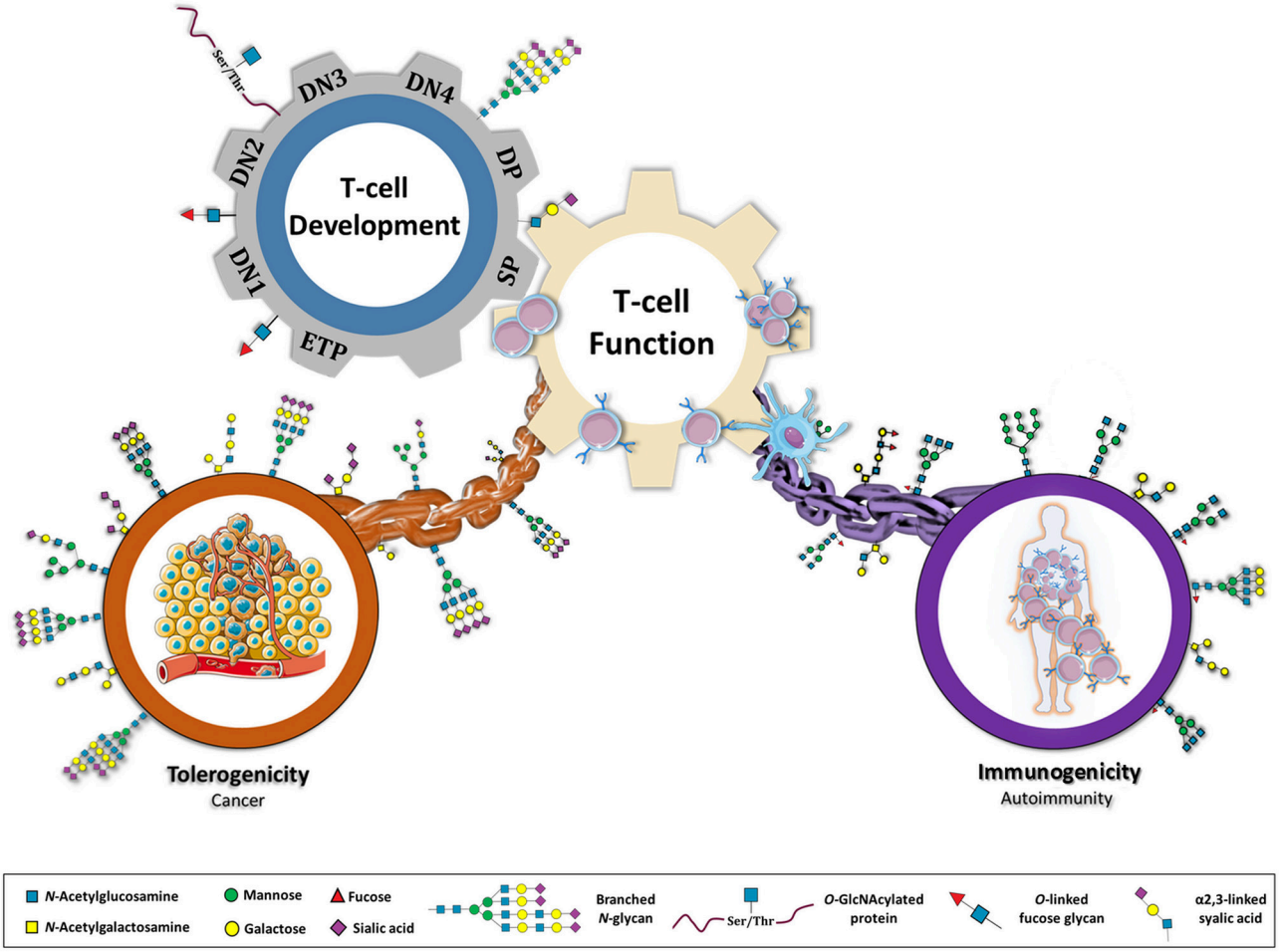

FIGURE 1 | Glycans as a major connective chain that controls T cell response in either a tolerogenic or immunostimulatory scenario. Glycosylation appears to be central in regulating several steps of a T cell's life. During T cell development, different population of T cells (ETP, early thymocyte progenitor; DN1, 2, 3, and 4, double negative; DP, double positive; SP, single positive) display specific glycosylation patterns. The normal glycosylation of SP population results in an educated T cell function. However, by genetic, environmental or metabolic constrains, T cell glycosylation can be compromised re-directing immune system toward an immunostimulatory or tolerogenic response. Glycans are proposed here as key players in immune-unbalanced diseases, such as autoimmunity and cancer.

α2,6-Sialyltransferase 1 (ST6Gal I) when comparing to the DN3 population, which resulted in an increase in α2,6-linked sialic acid (25). Accordingly, in *ST6Gal1* deficient mice, the DN populations were decreased, beginning at the DN1 subset. Microarray data showed a downregulation of CD96 (receptor molecule of nectin-1, that plays a putative role in cell migration) in the DN2 and DN3 populations in the *ST6Gal1* deficiency background, and a disruption of thymopoiesis in these mice was proposed. Moreover, ST3 β-Galactoside α2,3-Sialyltransferase 1 (ST3Gal I) expression is decreased in most DN and in all DP, only increasing in single-positive (SP) thymocytes (26). In *ST3Gal1*$^{-/-}$ mice, the TCR repertoire was significantly altered, indicating a role for sialylation in thymocyte selection (27).

Role of Glycans in Positive and Negative Selection in the Thymus

The ß-selected DN4 cells undergo rapid self-renewal, giving rise to a clonally expanded population, that differentiate into DP $CD4^+CD8^+$ thymocytes (8). In this developmental stage, mature TCRαß receptors are formed (28) and the expression of the co-receptors CD4 and CD8 confer a MHC class II and class I restriction of TCR activation, respectively. The newly formed mature TCRs are then screened by thymic epithelial cells (TECs) by the specificity and binding strength for the MHC ligands presented. The next developmental process is named positive selection, where the DP population is enriched for cells that express an immunocompetent TCR (8, 29). The selected DPs then commit to the SP $CD4^+$ or $CD8^+$ lineage and go through a process called negative selection, which eliminates autoreactive T cells (8, 29). The affinity of the correctly assembled TCRαβ for the MHC-antigen complexes determines cell survival and differentiation. Glycosylation modifications of the TCR may provide an alternative mechanism to control positive and negative selection by directly affecting the TCR-MHC-antigen binding, TCR interaction with its co-receptors and the threshold of activation (30), an issue that is far from being fully elucidated.

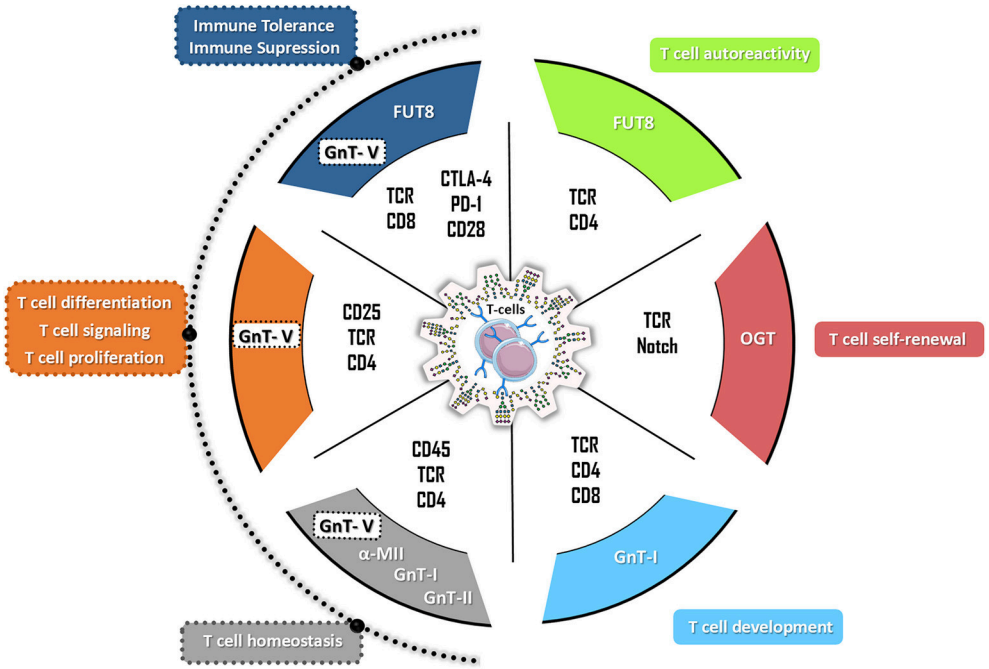

FIGURE 2 | The hallmarks of glycans in T cell biology. *N*-glycans have a broad effect on the multiple T cell functions with impact both in autoreactivity and in immune tolerance. Particularly, the complex branched *N*-glycans catalyzed by beta 1,6-*N*-acetylglucosaminyltransferase V (GnT-V) (encoded by *MGAT5* gene) have been demonstrated to control different T cells functions by targeting different T cells receptors (such as TCR, CD25, and CD4) and therefore regulating T cell proliferation, T cell differentiation, T cell signaling as well as the production of inflammatory cytokines. Alterations on GnT-V activity but also in alpha-mannosidase II (α-MII) as well as in *N*-acetylglucosaminyltransferase I (GnT-I, *MGAT1* gene) and II (GnT-II, *MGAT2* gene) activity were shown to compromise T cell homeostasis being associated with the development of several autoimmune disorders in humans and mouse models (such as EAE, IBD, SLE, TID). The FUT8-mediated core fucosylation of TCR was associated with hyperactivation of $CD4^+$ T cells (T cells autoreactivity) whereas the modification of the co-inhibitory receptors (CTLA-4 and PD-1) by FUT8-mediated core fucose results in immune tolerance. The T cell development and T cell self-renewal are controlled by GnT-I-mediated glycosylation and by *O*-GlcNAcylation through OGT (*O*-GlcNAc transferase), respectively.

The subunits of the TCRαβ contain at least 7 potential sites for *N*-linked glycosylation and the TCR-CD3 complex is estimated to have 12 *N*-glycan addition sites that contribute to TCR folding and functions (31, 32). Indeed, selective removal of conserved *N*-glycosylation sites of the constant regions of the TCR, enhanced its functional avidity (the sensitivity of the T cell response to other cell which carries the respective MHC-peptide) (32). However, whether *N*-glycosylation in the variable regions of the TCR affect its selection remains to be addressed. Moreover, low levels of sialylation in DPs are associated with binding to Major Histocompatibility Complex (MHC) class I (common to all nucleated cells) and the increased expression of sialic-acid linkages on differentiated SP $CD8^+$ thymic T cells was shown to decrease the binding avidity of CD8 for MHC class I molecules, which acts as a regulation for a TCR affinity dependent negative selection (33).

Furthermore the deficiency of the *Mgat5* gene, that encodes for a Golgi branching enzyme *N*-acetylglucosaminyltransferase V (GnT-V) was shown to markedly increases TCR clustering and signaling at the immune synapse, resulting in a lower T cell activation threshold and increased incidence of autoimmune disease *in vivo* and in human (30). In a model of positive selection, it was demonstrated that branching *N*-glycosylation dynamically expands the affinity spectrum of positive selection by differentially controlling both the lower and upper limits of positively selected TCR-MHC-antigen interactions (34). The intracellular domains of CD4 and CD8 co-receptors bind Lck, enhancing TCR responses to low-affinity MHC-antigen complexes when coupled to the TCR (35). Both co-receptors have *N*-glycosylation sites and it was shown that the branching deficiency in $Mgat1^{f/f}$Lck-Cre^+ T cells resulted in decreased surface expression of CD4 and CD8 receptors (34). The lack of branched *N*-glycans in the same genetic background also decreased TCR threshold signaling (30). These evidences supported that branching *N*-glycans display an important role in the maturation of DN cells and/or TCR selection.

Changes in the expression of *O*-linked glycans also impact T cell development by modulating galectin binding. Galectin-1 was shown to induce apoptosis of immature thymocytes through binding to core 2 *O*-glycans expressed in CD43 and CD45 (36). In contrast, CD45 on mature thymocytes bears core 1 *O*-glycans as well as *N*-glycans capped with α2,6-linked sialic acid, which inhibits galectin-1 binding (36).

Overall, glycosylation appears to play a critical role in the different stages of thymocyte development and in the generation of an efficient immune system. Nevertheless, further research is

needed in order to understand how glycans control each stage of thymocytes development, differentiation and selection, which might reveal novels insights on the influence of the glycome in major diseases, such as autoimmunity and cancer.

GLYCANS IN THE REGULATION OF T CELL ACTIVITY AND FUNCTIONS

The proper function of T lymphocytes is highly dependent on their surface receptors, which in turn are highly mediated by glycosylation. Although *O*-glycan structures have been shown to play important roles on immune-associated molecules (37), the prominent role of *N*-linked glycans is emphasized in this section (**Figure 2**).

As previously mentioned, MHC I is expressed by almost all nucleated cells and interacts with TCRs on $CD8^+$ T cells; in turn, MHC II is expressed by professional antigen presenting cells (APCs) (dendritic cells - DC, macrophages, B cells and TECs) and is recognized by $CD4^+$ T cells (7, 38). More than 3 decades ago, it was demonstrated that blocking MHC1a *N*-glycosylation, through acceptor site mutation, results in significant increases in intracellular misfolded protein along with decreases in cell surface expression (39). MHC II is assembled by two glycoproteins, α and β chains. The α chain contains *N*-linked high-mannose and complex glycans whereas the β chain is only constituted by complex *N*-glycans (40). In contrast to the role of MHC I, MHC II glycosylation was shown to have a particular impact on the effective antigen binding, as well as in the presentation of microbial carbohydrate antigens, which consequently influences downstream T cell responses. This was demonstrated by the depletion of the *Mgat2* gene, which compromises *N*-glycan branching, decreasing carbohydrate antigen presentation by MHC class II and leading to loss of T cell stimulatory activity (41).

During TCR signal transduction, glycans play a key role in stabilizing individual molecules in the complexes at the immunological synapse and by protecting them from the action of proteases during T cell engagement (31). Additionally, glycans can also restrict nonspecific protein-protein interactions, like aggregation of TCRs on the membrane, helping to orient the interactions of the proteins in the central clusters (31). Demetriou et al. demonstrated that β1,6-GlcNAc branched *N*-glycans structures (catalyzed by GnT-V) regulate T cell activity, namely in $CD4^+$ T cells by increasing the threshold of T cell activation, suppressing T cell growth and signaling (30, 42). Moreover, core-fucosylation, which refers to fucose attached to the innermost *N*-acetylglucosamine of *N*-linked glycans, catalyzed by α1-6 fucosyltransferase (FUT8), was also shown to affect T cell activity in immune mediated disorders (42, 43).

The T cell activity is also dependent on glycosylation of co-receptors, such as the complex formation between TCR and CD45. Galectin-3 is a key mediator of this complex, by establishing a molecular lattice through binding to polylactosamine structures in branched *N*-glycans. Consequently, CD45 phosphatase activity induces downregulation of T cell signaling, preventing T cell activation (44). Furthermore, CD45 is alternatively spliced into five different isoforms on human leukocytes (CD45ABC, CD45AB, CD45BC, CD45B, and CD45RO) (45–47), all decorated with up to 11 *N*-glycans in the membrane proximal region. Importantly, all isoforms present different glycosylation profiles (48, 49), that change during T cell differentiation and activation (50, 51), as reviewed in (36). CD28 is another T cell surface glycoprotein acting as a secondary signaling molecule of T cell activation. Interestingly, nearly 50% of the molecular mass of CD28 is constituted by *N*-glycans (52). Previous studies reported that *N*-glycosylation of human CD28 can negatively regulate CD28-mediated T cell adhesion and co-stimulation, namely the interaction between CD28/CD80. Mutation of all potential *N*-linked glycosylation sites of CD28 as well as treatment of Jurkat cells with inhibitors of *N*-glycosylation pathway resulted in a defective CD28 glycosylation with enhancement of the binding to CD80 expressed on APCs (52). The branching *N*-glycosylation of CD25 receptor also modulates its cell surface retention controlling T differentiation with impact in immune tolerance. Recently, it was demonstrated that a decreased UDP-GlcNAc and complex branching *N*-glycosylation induces a decreased cell surface retention of CD25 and IL-2 signaling, promoting a T helper (T_H) 17 over induced regulatory T cell (iTreg) differentiation (53) (**Figure 2**).

Importantly, the co-inhibitory receptors are likewise modulated by *N*-glycosylation. One of the major negative regulators of T cell response is the cytotoxic T-lymphocyte protein 4 (CTLA-4), that comprises two *N*-glycosylation sites described to modulate its cell surface retention on T cells and thereby its affinity for CD80/CD28 on APCs (54–56). The impact of *N*-glycosylation in the modulation of the inhibitory functions of CTLA-4 and programmed cell death protein-1 (PD-1) is discussed in more detail in section "Glycans in tolerogenic/immunosuppressive responses". Nonetheless, other co-inhibitory receptors like Lymphocyte-activation gene 3 (Lag-3), mucin-domain-containing molecule-3 (Tim-3), and T cell immunoreceptor with Ig and ITIM domains (TIGIT) may also undergo glycans-mediated regulation, as they exhibit *N*-glycan-binding sites, however the role of glycans on these molecules remains to be explored (57).

Taken together, *N*-glycosylation plays an instrumental role in the regulation of T cell activation and functions by targeting not only TCR but also its co- receptors (**Figure 2**).

GLYCANS AS MODULATORS OF HYPER-REACTIVE/AUTOIMMUNE RESPONSES

Autoimmunity is characterized by the loss of self-tolerance and development of an autoreactive immune response toward the individual's own organism. Glycan motifs play a crucial role in the determination of self/non-self antigens. Specific glycan structures, expressed by microbial pathogens, are commonly responsible for the primary activation of the innate immune system; however, the mechanisms involved in the self/non-self discrimination, mediated by glycans are far from being fully

elucidated. Abnormal levels of branched *N*-glycans have been associated with exacerbated immune responses in murine models (58). Particularly, the dysregulation of the *N*-glycosylation pathway has been associated with autoimmune-like phenotypes. The inability to synthetize β1,6-GlcNAc antennae, in *Mgat5*$^{-/-}$ mice has been associated with an increased susceptibility to immune-mediated disorders such as an increased delayed-type hypersensitivity responses, as well as increased susceptibility to develop experimental autoimmune encephalomyelitis (EAE) (30, 59) and severe forms of colitis (60). The lack of β1,6 branching *N*-glycans favors TCR clustering, leading to a decrease of the TCR threshold and consequently increased T cell activation (30) associated with the hyperimmune response observed in these mice (**Figure 2**). This hyperimmune phenotype is also due to an abnormal formation of lattices between TCR-branched glycosylation and galectins (61, 62). Accordingly, β3 GnT2-deficient mice show T cell hypersensitivity due to the reduction of polylactosamine on the *N*-glycans (ligands of galectins), similarly to what is observed in *Mgat5* deficient mice (30, 61). Furthermore, absence of α-mannosidase II (which catalyses the last hydrolysis of the α-mannose), was shown to result in signs of glomerulonephritis, deposits of glomerular IgM immunocomplexes and complement component 3 as well as high levels of anti-nuclear antibodies (63, 64), which is consistent with a Lupus-like syndrome (**Figure 2**). Taken together, these evidences support the role of *N*-glycosylation in the perspective of T cell biology.

The role of *N*-glycans in antigen presentation and recognition is still elusive, and in fact abnormal glycoantigen presentation might also impact T cell activity. Abnormal accumulation of high-mannose, paucimannose, and agalactosyl bi-antennary glycans, have been detected in kidney tissue from MRL-lpr mouse (a well-stablished murine model of SLE) (65). Moreover, evidences have been showing that *Mgat1*$^{f/f}$Syn1-Cre mice, with *Mgat1* deletion at the Synapsin I-*expressing cells* (abundant in neural tissues), presented neurological defects, with high levels of neuronal apoptosis and caspase 3 activation (66). These high levels of apoptosis are observed in several autoimmune diseases, which results in activation of immune system (67) (**Figure 2**). Although highly unexplored, rare autoimmune diseases are also associated with *N*-glycosylation dysfunctions. As example, idiopathic inflammatory myopathies (IIM) are a group of rare diseases of autoimmune nature, whose etiopathogenesis is far from being totally understood (68). Muscle cells surface is enriched with glycoproteins and several lines of evidence provide support for a fundamental role of glycosylation in muscle homeostasis and function (69, 70). Glucosamine (UDP-*N*-Acetyl)-2-Epimerase/*N*-Acetylmannosamine Kinase (GNE) genetic mutations (a gene that encodes *N*-acetylmannosamine (ManNAc) kinase enzyme, responsible for the biosynthesis of *N*-acetylneuraminic acid) results in hypo-sialylation of muscle glycoproteins; the prophylactic supplementation with sialic acid precursor (ManNAc) was shown to prevent the muscle phenotype in mice with gene mutations that cause hereditary inclusion-body myositis (hIBM), a muscle phenotype that resembles one type of IIM (71). Altogether, these findings highlight the importance of further studies addressing the role of *N*-glycosylation in the perspective of *neoautoantigens*, since autoantigens contain a significant amount of glycoantigens due to the increased number of *N*-glycosylation sites comparing with other proteins (72).

The Glycan binding proteins (GBPs) are expressed in the APCs being characterized by a carbohydrate recognition domain which specifically recognizes glycan structures present at the cell surface receptors. This glycan-GBPs engagement results in either an anti- or pro-inflammatory response (73). C-type lectins, siglecs, and galectins are examples of GBPs, that are instructors of immune responses (5, 73). As example, SIGN1R (expressed by APCs and the analogous of the human dendritic cell-specific ICAM-grabbing non-integrin - DC-SIGN) signaling was shown to result in the expansion of IL-10-secreting Treg cells, preventing the development of autoimmune diseases such as EAE and type 1 diabetes (T1D) (74). Galectin-1 also plays an important immune-regulatory role in EAE (75) as mice deficient in galectin-1 (*Lgals1*$^{-/-}$) have increased T_H1 and T_H17 responses being more susceptible to EAE when compared with wild type mice (76). More recently, Galectin-1 was shown to modulate the cytolytic activity of $CD8^+$ T cell. The interaction of Galectin-1 and Fas ligand seems to be responsible for the retention of this glycoprotein at the surface of cytotoxic T lymphocytes hampering the cytolytic ability of these cells (77). Overall, GBPs-glycoprotein interaction is essential to instruct a T cell—mediated immune response.

Notably, one of the first evidences addressing the relationship between the dysregulation of *N*-glycosylation and human autoimmunity was observed in multiple sclerosis (MS) patients. During active, relapse or in very early stages of remission, peripheral blood mononuclear cells from MS patients display a significant decrease of the enzymatic activity of Golgi β1,6 *N*-acetylglucosaminyltransferase (core 2 GlcNAc-T), compared to healthy subjects (78). Moreover, *MGAT5* polymorphisms were associated with MS severity (79) together with *MGAT1*, *IL2R,* and *IL7R* Single Nucleotide Polymorphisms (80–82). Additionally, in Inflammatory Bowel Disease (IBD), it was also demonstrated that *lamina propria* T lymphocytes from ulcerative colitis (UC) patients exhibited a deficiency in β1,6-GlcNAc branching *N*-glycans due to decreased levels of *MGAT5* gene expression (83). Importantly, low levels of branched *N*-glycans in lamina propria early at diagnosis were shown to predict UC patients that will fail the response to standard therapy, thus displaying a bad disease course (84). The supplementation of intestinal T cells from UC patients and mouse models with colitis with GlcNAc promoted the enhancement of β1,6 branching *N*-glycans on T cells, suppressing TCR signaling and reducing the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). Pre-clinical studies both in IBD and MS demonstrated the immunomodulatory properties of *N*-glycans in the control of T cell-mediated immune response (60, 85), paving the way for the development of human clinical trials, that are currently on going (53, 60). Less explored but of utmost importance is the study of *N*-glycosylation profile in rare autoimmune disorders, since its etiopathogenesis is still very elusive. Glycosylation changes in muscle-associated human disease have focused in muscular dystrophies (86) and congenital

disorders of glycosylation (87). Recent studies have shown that muscle cell surface glycosylation is finely regulated and subjected to alterations under inflammatory conditions (88), pointing to a possible interaction between muscle glycocalyx and the extracellular milieu, which is particularly enriched in immune cells and antibodies in IIM patients (89).

Overall, glycans are critical determinants in autoreactive responses both by directly regulating T cell activity and also through the creation of abnormal glycoantigens that may unleash an autoreactive immune response.

GLYCANS IN TOLEROGENIC/IMMUNOSUPPRESSIVE RESPONSES

Recent studies have been highlighted that alterations on the glycosylation pattern of T cells' receptors, as well as the alterations of the glycosylation profile of tumor cells (tumor glyco-code), are implicated in the modulation of the immune response leading to immunosuppressive pathways, known to occur in the tumor microenvironment associated with tumor immunoescape (90).

Role of Glycans in the Modulation of Inhibitory T Cell Receptors

PD-1, as already introduced, is a cell surface inhibitory T cell receptor responsible for immune-inhibitory responses associated with the so-called "T cell exhaustion" (91). The expression of this cell surface receptor, as well as Tim-3, was described to be positively regulated by the core fucosylation pathway, catalyzed by FUT8 enzyme (92). The inhibition of core fucosylation in PD-1 was demonstrated to lead to an anti-tumor immune response mediated by T cells activation, being a new attractive target for enhancing anti-tumor immunity in future clinical settings (**Figure 2**). This was a pioneer study that supported the importance of PD-1 post-translational modifications by glycosylation on T cell-mediated immunosuppression (92). Additionally, the glycosylation of programmed death ligand-1 (PD-L1), a PD-1 ligand, was described to have an important role in its cellular stabilization. The interaction of non-glycosylated PD-L1 with glycogen synthase kinase 3β (GSK3β), a key enzyme on glycogenesis, leads to the degradation of this molecule (93). In triple-negative breast cancer cells, it was further shown that the β1,3-*N*-acetylglucosaminyl transferase (B3GNT3), involved in the biosynthesis of poly-*N*-acetyllactosamine chains, is important for the interaction between PD-1 and its ligand PD-L1 (94). The use of an antibody targeting the glycosylated form of PD-L1 resulted in its degradation and internalization, with the blockage of PD-L1/PD-1 interaction and consequently the inducement of anti-tumor activity in triple-negative breast cancer *in vitro* and *in vivo* models (94). In accordance, Tregs from healthy humans and mice were shown to display an increased variability on its *N*-glycosylation pattern when compared with $CD4^+$ T cells. The levels of the complex branched *N*-glycans were shown to be correlated with the expression of proteins involved in Treg suppressive functions, including PD-1, PD-L1, and also other negative regulators of T cell response, namely CTLA-4 (95). In fact, the CTLA-4 protein, comprises multiple *N*- and *O*-glycosylation sites known to modulate its retention at T cell surface and consequently affecting its function (56). The TCR activation is associated with an increased β1,6-GlcNAc branched *N*-glycosylation of CTLA-4, which enhances CTLA-4 retention at the T cell surface and thereby suppresses T cell activation promoting immune tolerance (96) (**Figure 2**). Accordingly, the presence of Thr17Ala polymorphism in human *CTLA-4* was shown to result in the reduction of the *N*-glycosylation sites from one to two sites, which limited CTLA-4 retention at T cell surface (80). Supplementations with GlcNAc and Vitamin D promoted an enhancement of *N*-glycans branching expression, increasing the cell surface retention of CTLA-4, culminating in immunosuppression (80).

Glycans as Instructors of Immunosuppressive Responses

Tumor cells aberrantly express different types of glycans structures when compared with normal counterparts, such as an increased sialylation, an expression of truncated glycans and an overexpression of branched *N*-glycans (97). This alteration in the cellular glycosylation profile governs several steps of tumor development and progression, such as tumor cell dissociation, proliferation, invasion, metastasis, angiogenesis, with recent evidences pointing toward its effects in tumor immunoediting and immunosurveillance (98). GBPs expressed on immune cells are able to recognize altered glycan structures expressed at tumor cell surfaces instructing either immunostimulatory or immunoinhibitory responses.

The expression of sialylated glycans, such as Tn antigen and Lewis antigens, aberrantly expressed in tumor cells, were described to be recognized by DC-SIGN, expressed by macrophages and immature DCs, which lead to immunosuppression (99). The fucose residues present in Lewis structures (Lewis x and Lewis y), attached to carcinoembryonic antigen (CEA) (100), were described to trigger the upregulation of the anti-inflammatory cytokines IL-10 and IL-27 by APCs and the induction of T_H2, follicular (T_Hf), and Treg immune responses (101, 102). Besides, antigen-containing liposomes modified with DC-SIGN-binding Lewis b and x resulted in glycans recognition and internalization through DCs with consequent activation of $CD4^+$ and $CD8^+$ T cells (103). Furthermore, macrophage galactose binding lectin (MGL) was found to be able to recognize Tn antigen and *N*-acetylgalactosamine (GalNAc) residues, resulting in an increased recognition by Toll-like receptor 2, ultimately resulting in the secretion of cytokines (IL-10 and TNF-α). (104). Its interaction with terminal GalNAc residues on CD45 glycoprotein negatively regulates TCR signaling, with consequent decrease of T cell proliferation and increased T cell death (105). Moreover, by blocking the tumor-infiltrated macrophages (responsible for the high levels of IL-10), it was observed an effective $CD8^+$ T cells response, highlighting the importance of combining anti-tumor immune therapy with conventional chemotherapy (106). Furthermore, it was recently demonstrated in chronic infection that IL-10 induces the upregulation of the *Mgat5*

gene increasing branched *N*-glycans on $CD8^+$ T cells, which in turn decreases T cell activity and allows viral persistence (107). Despite the different context in which this hypothesis was studied, *Mgat5*-mediated branching glycosylation can constitute a potential mechanism by which IL-10 is suppressing $CD8^+$T cells in cancer.

In addition, sialylated glycans also play a role in immunosuppression, mediated by siglecs, a family of lectin receptors that predominantly exhibit immune-inhibitory functions. In *in vitro* and *in vivo* studies, the binding to sialylated antigens by siglec-E expressed on DCs promoted an increase of antigen-specific Treg response and a reduced numbers of antigen-specific Teff cell response, associated with tumor growth (108, 109). Indeed, the sialylated tumor antigens, such as Sialyl-Tn (sTn) and Sialyl-T (sT) expressed in mucins, namely MUC1, were associated with tumor immune tolerance. The recognition of MUC1-ST by siglec-9 on tumor-infiltrating macrophages was shown to initiate inhibitory immune pathways mediated by MEK-ERK signaling (110). Moreover, siglec-binding to sTn-expressing mucins, led to the maturation of DCs and DC-mediated induction of $FOXP3^+$ Treg cells and reduced INFγ-producing T cells (111, 112). A recent study also demonstrates that siglec-9 expressed by $CD8^+$ tumor infiltrating lymphocytes (TILs) in non-small cell lung cancer (NSCLC) patients was associated with reduced survival. Accordingly, siglec-9 polymorphisms were associated with the risk of developing lung and colorectal cancer. Additionally, the characterization of siglec-9^+ $CD8^+$ TILs revealed that these cells concomitantly express several inhibitory receptors, including PD-1, TIM-3, Lag3, and others. In addition, the same study further reveals that lack of sialic acid-containing glycans in tumor cells led to a delay of tumor growth and an increased infiltration of $CD3^+$ and $CD8^+$ T cells (113).

Another important GBP that have been pointed out as a crucial checkpoint in T cell viability and activity are galectins. Galectin-1, 3, and 9 were predominantly described in T cell immunosuppression. Galectin-1, was demonstrated to be expressed by tolerogenic DCs (75) and $CD4^+CD25^+$ T cells (114), triggering T cell apoptosis through binding to *N*-glycans and *O*-glycans on CD45, CD43, and CD7 or by sensitizing resting T cells to FAS-induced death (115, 116). The T_H1 and T_H17 activated cells are susceptible to galectin-1-induced cell death once these cells express the repertoire of glycans required for galectin-1 binding, while T_H2 cells are protected via α2,6-sialylation on cell surface glycoproteins, which was described to preclude galectin-1 recognition and binding (76). In addition, several tumors have the capacity to secrete galectin-1 in order to promote immunosuppression, through a mechanism that involves a bias toward a T_H2 cytokine profile and activation of tolerogenic circuits mediated by IL-27-producing DCs and IL-10-producing type 1 Treg cells (117). On other hand, galectin-3 has an ambiguous role in T cell viability: when it is localized at intracellular level, this protein presents a protective role through a cell death inhibition pathway that involves B-cell lymphoma 2 (Bcl-2) (118), whereas extracellular galectin-3 induces cell death in activated T cells, by binding to glycosylated receptors of T cells through a distinct way than galectin-1 (115). Moreover, galectin-3 has the capacity to bind to *N*-glycans on CTLA-4 prolonging the inhibitory signals (119), as well as to Lag-3 on the surface of $CD8^+$ T cells, suppressing its function (120). Finally, galectin-9 abrogates T_H1, T_H17, and $CD8^+$ T cells through glycosylation-dependent binding to Tim-3 (121–123), whereas may regulate pro-inflammatory cytokine production by binding with other receptors (124).

Altogether, these findings support the relevance of glycans on T cells-mediated immunosuppressive/tolerogenic pathways which have relevant implications in tumor progression. Targeting the abnormal glycosylation pattern of cancer cells constitutes a promising strategy to instruct an effective anti-tumor immune response, an issue that needs to be further explored.

GLYCANS AS METABOLIC REGULATORS OF T CELL FUNCTION

The impact of glycosylation on T cell development and functions is enormous, as revealed by the critical roles of glycans in the development and progression of major diseases such as auto-immunity and cancer, as described herein. In order to accompany the bioenergetic and biosynthetic demands required for T cell proliferation and activation, a shift in the T cell metabolism is required. While naïve T cells are in a metabolic quiescent state, mainly using oxidative phosphorylation to maximize ATP production, T cells under clonal expansion or under differentiation, reprogram their metabolic status to aerobic glycolysis and glutaminolysis in order to increase the availability of glycolytic precursors for the biosynthesis of nucleotides, amino acids and lipids (125–127). During T cell activation, the hexosamine biosynthetic pathway (HBP—a branch of the glucose metabolism) is upregulated in order to generate the nucleotide sugar-donor substrate UDP-GlcNAc, required for *N*-glycosylation, O-GlcNAcylation, and glycosaminoglycans production that are needed for a proper T cell function (128).

Mediators from the glycolytic pathway such as glucose (Glc), glutamine (Gln), acetyl CoA are known to interfere with the availability of the UDP-GlcNAc in the cell (129–131). Together Glc and Gln were shown to increase UDP-GlcNAc in nutrient-starved T cells. In the same setup, the supplementation of both Glc and glucosamine (GlcN–a metabolite of the HBP) further increased the UDP-GlcNAc cellular content, demonstrating the sensitivity of the HBP to nutrients that enter directly (GlcN) or through a precursor pathway (Glc in glycolysis) (130). Despite the general use of the UDP-GlcNAc as a substrate donor of HBP, there are some glycosyltransferases that are more susceptible to nutrient changing than others, such as the case of OGT (132). In fact, the supply with Glc and Gln are crucial for protein O-GlcNAcylation, that is important during T cell development, being associated with T cell malignant transformation (23). Among the *N*-acetylglucosaminyltransferases (GnTs) that participate in the HBP, the less sensitive to nutrient changing (and thus substrate availability) are GnT1, GnT2, and GnT3, due to lower *Michaelis Constant* (K_m) levels, meaning that these enzymes require low

levels of the substrate to synthetize the specific glycans. In contrast, GnT4 and GnT5 present higher K_m and therefore their activity is highly dependent on the availability of the UDP-GlcNAc substrate (119, 133). Therefore, these two enzymes are sensitive to alterations in glucose and HBP metabolism (as the GlcN or *N*-acetyl glucosamine–GlcNAc) (62), which ultimately will interfere in the *N*-glycan branching biosynthesis on T cells with impact in their activity, as detailed in section "Glycans in the regulation of T cell activity and functions" (60). In fact, supplementation with Glc, Gln, and GlcNAc increases branching *N*-glycans on Jurkat cells and resting T cells from mice (85, 119, 130). Moreover, $CD4^+$ T cells from $MGAT5^{+/+}$ or $MGAT5^{+/--}$ mice supplemented with oral GlcNAc also results in up to 40% increase of branching *N*-glycans, detected by L-PHA (130). This enhancement of branching *N*-glycosylation upon GlcNAc supplementation was shown to functionally impact on T cells activity by reducing T cell activation, decreasing T_H1 differentiation, and increasing retention of the growth inhibitory receptor CTLA-4 at T cell surface (85, 130).

Importantly, evidences suggest that the glycolysis and glutaminolysis compete with HBP pathway for the same metabolites. Recently, Araujo et al showed that, during T_H17 differentiation the existence of common mediators shared between HBP, glycolysis (fructose-6-phosphate) and glutaminolysis (Gln) results in a starvation of the HBP mediators, translated in a reduction of *N*-glycan branching due to the limitation on the UDP-GlcNAc availability (53). Fueling HBP with GlcNAc switched the cell fate from T_H17 to iTreg differentiation, through stimulation of IL2-Rα signaling (53). This interplay between metabolic pathways was further demonstrated by the increase on Glc, Gln, fatty-acids uptake, and lipid storage upon stimulation of the HBP with GlcNAc supplementation, suggesting a reprogramming of the cellular metabolism upon GlcNAc flux (53, 134).

The impact of glycans as metabolic regulators of T cells is also testified by its effects in *ex vivo* and *in vivo* models of autoimmune diseases. The metabolic supplementation with GlcNAc in *ex vivo* human colonic T cells from IBD patients resulted in an enhancement of the branching *N*-glycosylation pathway that was accompanied by a significant reduction of T cell proliferation, supression of T_H1/T_H17 immune response (through decreased production of IFN-γ and IL-17A pro-inflammatory cytokines) and decreased TCR signaling (60). Accordingly, the GlcNAc supplementation of mice models with auto-immune diseases such as EAE, TID, and IBD results in inhibition of T_H1, T_H17 immune response concomitantly with a significant improvement of the clinical symptoms (60, 85). Treatment with GlcNAc after disease onset also demonstrate inhibitory effects on the development of the EAE, by reducing the secretion of INF-γ, TNF-α, IL-17, and IL-22 (85). Interestingly, a dual role of GlcN (the precursor of GlcNAc) on the progression of autoimmune disorders was shown, by demonstrating its impact in preventing T_H1-mediated Type I diabetes (through the reduction of IFN-γ producing $CD4^+$ T cells), but also the GlcN effects in exacerbating T_H1/T_H17–mediated EAE symptoms (trough stimulation of T_H17 response) (135). In contrast, another study showed that GlcN suppresses acute EAE through the blockage of T_H1 and induction of T_H2 response (136). GlcN supplementation was further shown to mediate T cell activation by decreasing the *N*-glycosylation of CD25 (IL-2Rα) from $CD4^+$ T cell (135). This down-regulation of *N*-glycosylation might be explained by the competition between GlcN and Glc for the same glucose transporter which might impact in the reduction of the GlcNAc concentration.

Altogether, alterations on the glucose metabolism and partially changes in the metabolic flux of HBP have a direct impact on T cells *N*-glycosylation profile with major consequences in their function and activity. Ultimately, the modulation of the HBP constitutes an important metabolic target able to control both autoreactive and immunosuppressive responses known to occur, respectively, in autoimmunity and cancer.

CONCLUDING REMARKS

The contribution of the glycome as a major regulator of the immune system is clear. Glycans actively participate in the cellular and molecular mechanisms underlying the genesis of the loss of immunological tolerance associated with (auto)immunity, from one hand, participating also in the creation of tolerogenic pathways associated with cancer progression, from the other. The importance of glycans in immune response spans from its role in the modulation the T cell development; their importance as a source of glycoantigens presentation; as well as their role as fine tuners of T cell response. In this context, glycans can exert a dual role, acting either as immune inhibitory checkpoints or as immune stimulatory signals. Understanding in depth the influence of glycans in the immune regulatory circuits that mediate the pathophysiology of autoimmunity and cancer will generate a platform with extraordinary potential to illuminate the identification of novel biomarkers and targets for the development of efficient immunomodulatory strategies with applications in the clinical setting.

AUTHOR CONTRIBUTIONS

All the authors wrote the manuscript. AD and NP created the figures. SP performed the critical review of the manuscript.

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Hyaluronan and Its Interactions With Immune Cells in the Healthy and Inflamed Lung

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Hyaluronan is a hygroscopic glycosaminoglycan that contributes to both extracellular and pericellular matrices. While the production of hyaluronan is essential for mammalian development, less is known about its interaction and function with immune cells. Here we review what is known about hyaluronan in the lung and how it impacts immune cells, both at homeostasis and during lung inflammation and fibrosis. In the healthy lung, alveolar macrophages provide the first line of defense and play important roles in immunosurveillance and lipid surfactant homeostasis. Alveolar macrophages are surrounded by a coat of hyaluronan that is bound by CD44, a major hyaluronan receptor on immune cells, and this interaction contributes to their survival and the maintenance of normal alveolar macrophage numbers. Alveolar macrophages are conditioned by the alveolar environment to be immunosuppressive, and can phagocytose particulates without alerting an immune response. However, during acute lung infection or injury, an inflammatory immune response is triggered. Hyaluronan levels in the lung are rapidly increased and peak with maximum leukocyte infiltration, suggesting a role for hyaluronan in facilitating leukocyte access to the injury site. Hyaluronan can also be bound by hyaladherins (hyaluronan binding proteins), which create a provisional matrix to facilitate tissue repair. During the subsequent remodeling process hyaluronan concentrations decline and levels return to baseline as homeostasis is restored. In chronic lung diseases, the inflammatory and/or repair phases persist, leading to sustained high levels of hyaluronan, accumulation of associated immune cells and an inability to resolve the inflammatory response.

Keywords: hyaluronan, inflammation, fibrosis, macrophages, lung, wound healing, extracellular matrix

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HYALURONAN IN THE HEALTHY LUNG

Hyaluronan (HA) in Lung Development

HA is a high molecular mass glycosaminoglycan (>1 MDa) composed of repeating disaccharide units of D-glucuronic acid and N-acetyl glucosamine (1). During fetal development of the lung, HA is present in the interstitium (2) and the alveolar space is filled with amniotic fluid that is rich in HA and hyaladherins that possess anti-inflammatory and wound healing properties (3, 4). During this time, fetal monocytes populate the mouse lung where they differentiate into CD11b^{+} CD11c^{lo} Siglec F^{lo} pre-alveolar macrophages (pre-AMs) (5–7). At birth, air fills the lungs, and pre-AMs develop into functional AMs (CD11c^{+} Siglec F^{hi} CD11b^{-}), coinciding with the decrease in HA levels in the lung (7). AMs express high levels of CD44, a cell surface receptor for HA that is required for HA uptake *in vitro* (8, 9), and AMs are responsible for reducing HA levels *in vivo* (2).

HA Expression in Healthy Lung Tissue

In the uninflamed lung, HA, detected by biotinylated HA binding protein (HABP), is bound to the surface of AMs in the alveolar space [**Figure 1** and (10–12)]. HA is also in the basement membrane region of bronchial and bronchiolar epithelium, and in the perivascular region (prominent in the adventitia) of large blood vessels [**Figure 2** and (2, 10–12)]. In lung sections, HA is not apparent in the alveolar interstitium or on alveolar epithelium (10). CD44 is present on the basolateral surface of bronchial epithelium (10), consistent with the localization of HA to the basement membrane.

HA Turnover in the Alveolar Space

In general, HA turns over very rapidly compared to other extracellular matrix components: approximately a third of the body's HA turns over daily (15). HA is produced by HA synthases (HAS1-3) at the plasma membrane that extrude HA into the extracellular space (16). Type II alveolar epithelial cells (AECs) express HAS2 and surface HA (17). HA is loosely attached and shed from the apical surface of primary AEC cultures, and can be observed above the airway epithelium (18), although this is not always the case (10, 12). This suggests that HA can be released into the alveolar fluid above the AECs. Since HA levels are low in bronchoalveolar lavage fluid (BALF) from healthy animals (19), HA must be turned over, possibly by AMs, which take-up and degrade HA *in vitro* (8, 9) and bind HA *in vivo* (12). Clearance of HA involves its degradation into smaller fragments by hyaluronidases such as Hyal 2 (20, 21), TMEM2 (22), and possibly KIAA1199 (23) at the cell surface. These fragments are then internalized by receptors such as CD44 and HARE/Stabilin-2 and taken to the lysosome where they are degraded by Hyal 1 (16, 24, 25).

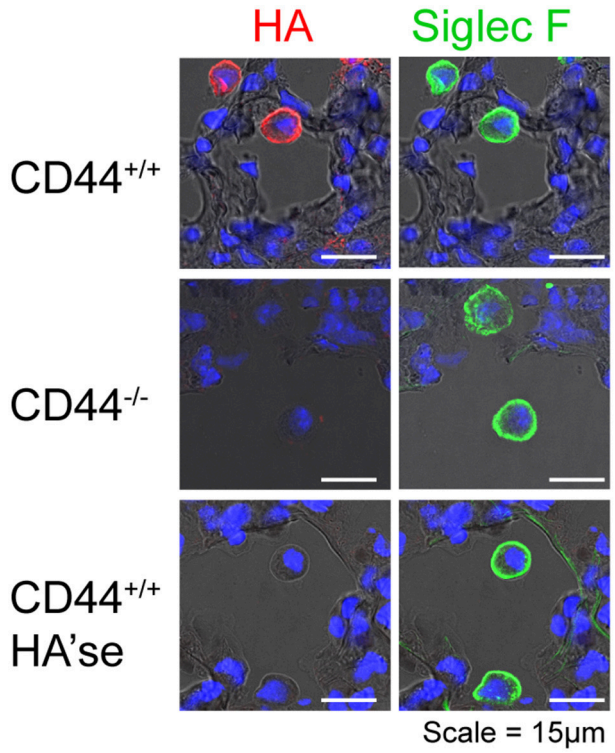

FIGURE 1 | $CD44^{+/+}$ AMs, but not $CD44^{-/-}$ AMs, have a HA coat. Frozen lung sections from $CD44^{+/+}$ and $CD44^{-/-}$ mice were obtained and labeled with HABP to detect HA (red), and Siglec F, a marker of AMs (green), and cell nuclei identified by DAPI (blue). In the lower panel, the sections were treated with bovine testicular hyaluronidase (HA'se). Top and bottom row are lung sections from $CD44^{+/+}$ mice, and the middle row is from $CD44^{-/-}$ mice. Images were captured on a Leica SP5 scanning laser confocal microscope, under identical acquisition settings. Images are representative of those published in Dong et al. (12).

HA Binding to AMs Promotes Their Survival and Maintenance

The ability of CD44 to bind HA is highly regulated in cells (26–30). AMs express a form of CD44 that constitutively binds HA (8, 12, 31). In contrast, CD44 on unactivated monocytes, macrophage colony stimulating factor-derived macrophages, and peritoneal macrophages, do not bind fluoresceinated HA (FL-HA) (31, 32). However, when peritoneal macrophages are introduced into the lung airways, they gain the ability to bind FL-HA (12), highlighting the influence of the alveolar environment on HA binding. Granulocyte-macrophage colony stimulating factor (GM-CSF or CSF-2) and PPARγ are both important in the alveolar space for AM development and maintenance (6, 33) and treatment of bone marrow-derived macrophages with GM-CSF and a PPARγ agonist, rosiglitazone, induces CD44-dependent HA binding (12), implicating these factors in regulating HA binding by AMs in the alveolar space.

AMs possess a HA coat that is anchored to its surface by CD44, and is absent in $CD44^{-/-}$ AMs, **Figure 1** and (12). The HA coat was unexpected, given the AMs ability to take-up and degrade HA (8, 9). Although high molecular mass HA (HMW-HA, >1 MDa) predominates in uninflamed lung tissue (19), the size and origin of HA in the AM coat is not known. What is known is that this HA coat promotes the survival of AMs, and its removal by hyaluronidases induces apoptosis (12). $CD44^{-/-}$ AMs are more susceptible to apoptosis and mice lacking CD44 have reduced numbers of AMs in the lung (12). The engagement of HA by CD44 is required for optimal AM survival *in vivo*, as its disruption with an HA blocking CD44 antibody leads to reduced numbers of AMs (12).

Effect of Type II AEC Generated HA

AMs reside in the alveolar space, above the AEC layer, in the fluid surfactant layer, where some AMs closely associate with AECs (10, 34). They form intimate connexin-43-dependent gap-junction interactions which can modulate inflammation (34). CD200-CD200R and αvβ6-tumor growth factor beta (TGFβ)-TGFβR interactions further support an association between these cells, which acts to limit AM activation (13, 35). Although type II AECs express HAS2 (17), it is unclear if type I AECs, which form the majority of the alveolar epithelial surface, also synthesize HA. At homeostasis, HA produced by type II AECs may be bound and/or taken up by AMs, keeping HA levels low in the surfactant layer. Alternatively, HA binding by AMs may strengthen their immunosuppressive connection with AECs, or

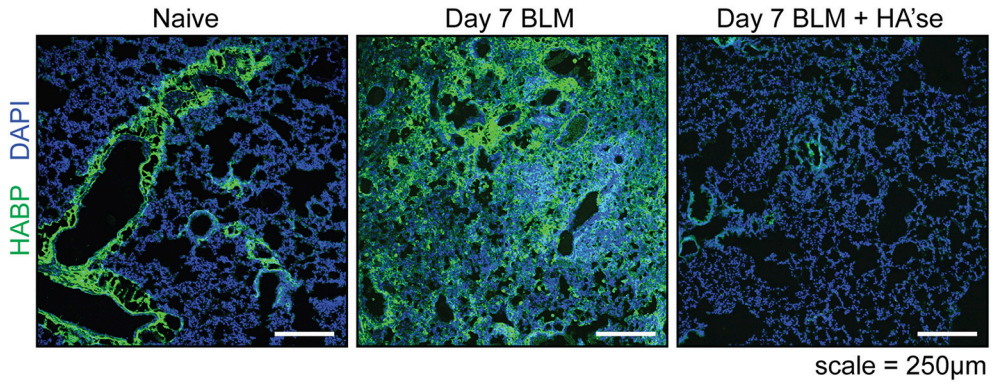

FIGURE 2 | Hyaluronan in the healthy and inflamed mouse lung. Frozen lung sections from CD44$^{+/+}$ mice were labeled with HABP (green) to detect HA, and stained with DAPI (blue) to label cell nuclei. On the left is a representative image of the healthy (naïve) lung where HA is present on the major bronchioles and blood vessels, with little labeling in the interstitium. The middle panel shows HA present in the lung 7 days after bleomycin induced lung inflammation (Day 7 BLM). The panel on the right is a control, showing a lung section from day 7 BLM, after treatment with hyaluronidase. All images were captured using an Olympus FV1000 scanning laser confocal microscope under identical acquisition settings. These images are similar to data described in Cheng et al. (11), Hussell and Bell (13), and Sahu and Lynn (14).

independently promote immunosuppressive behavior, as HMW-HA has been shown to limit activation in other cells (36, 37). HAS2 overexpression in type II AECs protects these cells against bleomycin-induced apoptosis, as does HMW-HA (38). Conversely, loss of HAS2 expression leads to their decreased renewal capacity *in vitro* (17) supporting the idea that, like AMs, HA promotes the survival/self-renewal ability of type II AECs. HMW-HA also supports the survival/ self-renewal capacity of stem cells [reviewed in (39–41)], suggesting a common function for HMW-HA in promoting the survival of cells capable of self-renewal.

HA IN THE INFLAMED LUNG

HA Levels Are Elevated in Lung Disease

HA is upregulated during tissue inflammation in many diseases and across many tissues (42, 43). The upregulation of HA is a general characteristic of inflammation, occurring in a broad repertoire of inflammatory and infectious conditions irrespective of the type of stimuli or the type of immune response generated (inflammatory: type 1, or allergic, fibrotic: type 2), and has been the subject of many excellent reviews (43–47).

In the lung, the concentration of HA is elevated in the BALF of patients suffering from asthma (14), chronic obstructive pulmonary disease (48), interstitial pulmonary fibrosis (49), and other lung diseases [reviewed in (43, 44)]. In animal models, HA is upregulated in the bleomycin model of sterile injury (19, 50), asthma (ova and cockroach allergen) (11, 51), ozone-induced airway hyperreactivity (52), LPS-induced acute lung injury, and *Escherichia coli* (53), *Klebsiella pneumoniae* (54), or Influenza infection (55).

Hyaladherin Expression Is Increased Upon Inflammation

In addition to HA, several hyaladherins are upregulated upon lung inflammation [reviewed in (46, 56–60)]. These include versican, the heavy chain (HC) of the inter-alpha-trypsin inhibitor (IαI), link protein, tumor necrosis factor stimulated gene 6 (TSG-6), pentraxin-3, and aggrecan, which can bind to, and modify, the HA glycocalyx. TSG-6 is an enzyme that catalyzes the covalent transfer of the HC of IαI to HA (59). TSG-6 can also independently bind and crosslink HA to form a more compact matrix (61) that has increased binding to CD44 (62). In intestinal inflammation, smooth muscle cells generate distinctive HA cables that are modified by HC and adhesive for platelets, key cells in the wound healing process (63). Activated platelets express Hyal 2 (64) and can degrade HA cables down to 20 kDa fragments, but lack Hyal 1 which would allow complete digestion (65).

HA Levels Correlate With Inflammatory Infiltrate

Animal models of lung infection, injury, and inflammation allow a closer analysis of the changes that occur to HA during the inflammatory response. In models of acute and chronic asthma, HAS1 and 2 are rapidly upregulated in the lung only a few hours after re-exposure to allergen, while Hyal 1 and 2 decrease over time, leading to the accumulation of HA that is maximal after 6 days and is maintained with continued chronic stimulation (11). Inflammatory stimuli also induce TSG-6 expression, which maintains HA deposition and eosinophil recruitment (66). Eventually, HA levels return to baseline after about 8 weeks (11).

In a model of acute sterile lung inflammation, a single dose of bleomycin induces HA expression in the lung tissue (67) and **Figure 2** (Day 7 BLM), which, together with leukocyte infiltration, peaks at day 7 (19, 50). In inflammation, HA has a smaller average molecular mass of 0.5 MDa compared to 1.5 MDa in naïve lungs (19). Detection of HA decreases as collagen deposition increases in the remodeling phase (day 14 to past 21) (19, 47). Final resolution of the response involves the return of HA to baseline levels which occurs around 5 weeks (50),

together with the removal of collagen, myofibroblasts, fibrotic macrophages, and the restoration of lung epithelium by type II AECs (24).

In the absence of CD44, HA levels continue to increase after bleomycin treatment and the severity of inflammation increases. HA sizes become smaller and more heterogeneous, ranging from 0.02 to 2 MDa (19). HA levels and the leukocyte infiltrate are reduced if $CD44^{+/+}$ bone marrow cells are transplanted into irradiated $CD44^{-/-}$ mice, implicating $CD44^{+/+}$ leukocytes (potentially macrophages) in the uptake and clearance of HA.

HMMR/RHAMM has been described as a receptor for HA-mediated motility and as a intracellular centrosomal protein involved in spindle orientation and integrity that is upregulated during the cell cycle (68). Genetic deletion of HMMR at exon 2 is lethal in mice (69) whereas deletion of exons 8 or 10 leads to their survival (70, 71). The exon 8 targeted mice have reduced HA and lung inflammation and less inflammatory macrophages in response to bleomycin, whereas mice overexpressing RHAMM in scavenger receptor A positive macrophages show the opposite (72). Thus the effects of RHAMM are distinct from that of CD44. RHAMM affects both macrophage proliferation and motility *in vitro* (72), but further work is required to determine its mechanism in lung inflammation.

Influenza virus causes severe damage in the lung that is repaired for months after the virus has been cleared (35). During this recovery period, the lung is more susceptible to bacterial infections. Recent work found that HA levels remain high in the lung tissue due to elevated HAS2 expression in epithelial, endothelial, and fibroblast cells (55). TSG-6 levels are also elevated and this generates a HC-modified HA matrix (55). Interestingly, a single dose of hyaluronidase at day 6 after influenza infection reduces the HA content in the lung, reduces the number of $F4/80^{+}CD11b^{+}CD11c^{lo}$ macrophages and improves lung function at day 16 (55).

These models of lung inflammation show that HA levels increase with inflammation, suggesting a role for HA in supporting the leukocyte infiltrate. HA is hygroscopic and has been linked with edema formation (73), which would allow easier movement of leukocytes in the damaged tissue. In the bleomycin model, HA levels decrease after the peak of inflammation, whereas in the asthma and influenza models, increased HA levels persist. Only after remodeling and completion of the repair process do HA levels return to baseline levels.

HA Fragments in Inflammation: Present Challenges

It is important to keep in mind that both pericellular and extracellular HA matrices are thought to turnover frequently. This means that HA is continually synthesized and degraded, and during inflammation, increased synthesis leads to the accumulation of HA. The size of this HA is more heterogenous and HA fragments (varying from small oligosaccharides to 0.5 MDa) are considered damage-associated molecular patterns that stimulate inflammatory responses [reviewed in (26), (36), (38), (42), (43)]. However, this has recently been challenged by studies showing that some HA and hyaluronidase preparations are contaminated with endotoxin (74, 75). This, together with the absence of evidence showing direct binding of HA fragments to TLRs, has questioned whether HA fragments directly promote inflammation. An alternative explanation is that smaller sized HA fragments displace HMW-HA bound to CD44, and disrupt its protective, immunosuppressive effect (76). In some cells, HMW-HA inhibits NF-κb signaling (77), and so its displacement by HA fragments would result in a proinflammatory NF-κb response. Another explanation for the variation in results seen with different sized HA fragments may arise from the use of polydisperse HA where a range of HA sizes within a single preparation compete for receptors to initiate the inflammatory signal (78). There is some evidence that HARE/Stabilin-2 responds to specific sizes of HA (79). Why would HA fragments as large as 200 kDa be seen differently from 1 MDa HA, when HA receptors, such as CD44, recognize just a few sugar units (80)? The answer may lie in the ability of different forms of HA to cluster HA receptors and thereby influence the signal delivered. Recent work shows that HA undergoes a transition from a random coil to a rod shape at around 200 kDa, suggesting that these forms could differentially impact HA receptor clustering and signaling (81). In support of this idea, TSG-6-crosslinked HA creates a more compact matrix (61) that is more efficiently recognized by the HA receptors, CD44 (62) and Lyve-1 (82). HC-modified HA forms cables (83) that also alter how HA is perceived by the cell (63). Clearly, more work is needed to understand the contribution of HA fragments and hyaladherins in the inflammatory response.

HA Binding Immune Cells in Lung Inflammation and Repair

In a type I inflammatory response, neutrophils and inflammatory monocytes are recruited to the site of infection, where they contribute to the proinflammatory environment and respond to the threat. Neither of these cell types bind appreciable levels of FL-HA (26). In a type 2 allergic response, eosinophils are recruited (11) and these cells bind low levels of FL-HA (84). In animal models of acute and chronic asthma, eosinophils are present in HA-rich areas of the inflamed lung (11). Once in the inflamed lung, inflammatory monocytes differentiate into macrophages and become $F4/80^{+}$ $CD11b^{+}$ $CD11c^{+}$ Siglec F^{lo} during the repair phase (12, 85). These macrophages bind FL-HA (12), produce TGFβ and drive bleomycin-induced fibrosis (85). In a mouse model of allergic asthma, $F4/80^{+}$ macrophages are found with HA and versican in the subepithelial region of the lung (86). Thus, HA binding may provide one possible means of bringing immune cells such as eosinophils and fibrotic macrophages into close proximity with the HA-producing myofibroblasts involved in repair.

HA Producing Myofibroblasts in Lung Repair

During the course of lung inflammation, the cells responsible for the increase in HA synthesis have not been clearly defined. However, individually, fibroblasts, myofibroblasts, endothelial cells, smooth muscle cells, and type II AECs can all produce

pericellular HA coats in response to inflammatory or reparative stimuli (24, 42, 46). Myofibroblasts are major HA-producing cells that have key roles in wound repair, collagen deposition, and fibrosis (24). TGFβ induces the differentiation of fibroblasts into smooth muscle actin positive myofibroblasts and enhances their production of pericellular HA (87). HA further promotes their differentiation and maintenance (85, 88, 89). TGFβ induces HAS1 and 2 expression in fibroblasts (90, 91) and reduces Hyal 1 and 2 expression (92), and this HA is required for TGFβ-induced fibroblast proliferation by providing a late pERK signal (93, 94). TGFβ also induces TSG-6 which generates HC modified HA cables that facilitate myofibroblast differentiation (88, 89, 95). Overexpression of HAS2 in lung myofibroblasts leads to a severe fibrotic response and invasive fibroblast phenotype (96), while the deletion of HAS2 in fibroblasts increases cellular senescence in a mouse model of pulmonary fibrosis (97). Thus, pericellular HA is intimately linked to the fibrotic/repair function of myofibroblasts.

Type II AECs in Lung Inflammation and Repair

In the repair phase, damaged type I AECs are replaced by the differentiation of type II AECs, which have stem cell-like properties (98). Both the loss and overexpression of HAS2 in type II AECs have significant effects on epithelial cell repair in response to bleomycin, with HA protecting against epithelial damage and apoptosis, and the loss of HA impairing AEC renewal and leading to severe fibrosis (17, 38). Likewise, type II AECs isolated from patients with severe pulmonary fibrosis have reduced levels of surface HA (17). Thus, HA has a protective effect on type II AECs.

AMs in Lung Inflammation and Repair

Lung inflammation results in the depletion of tissue resident, fetal monocyte-derived AMs, with the extent of their loss proportional to the severity of the insult [(12, 33, 85, 99, 100) and Dong et al., unpublished data]. The cause of this loss is not understood, but it has been suggested that macrophage necrosis triggers the ensuing inflammatory response (101–103). Maximal loss of AMs occurs at the peak of leukocyte infiltration and HA accumulation, after which the AMs increase in numbers, due in large part to self-renewal (12, 33, 100). Since AMs are implicated in HA uptake and degradation (8, 9), it is possible that their loss could contribute to the increased levels of HA observed upon inflammation. However, this remains to be determined. Whether the change in size of HA during inflammation also impacts AM survival, or its uptake by AMs, is also not known. During inflammation, AMs gain CD11b, but still retain high levels of CD11c and Siglec F, as well as their ability to bind HA, and are distinguishable from the newly differentiated monocyte-derived macrophages ($CD11c^+$, $CD11b^+$, Siglec F^{lo}) that play a critical role in driving repair/fibrosis (12). The recovery of AM numbers during the repair phase suggests a function in the later stages of the response, perhaps in helping the return to homeostasis. With the resolution of inflammation and repair, monocyte-derived macrophages become phenotypically identical to tissue resident AMs, but they may not be functionally identical (104, 105).

In summary, HA levels are low in the alveolar space in the healthy lung, and HA bound to AMs promotes their survival. In the lung tissue, HA is present in the basement membranes of bronchioles and in the perivascular area. HA levels dramatically increase upon lung inflammation, perhaps enabling leukocytes to access the site of injury. Hyaladherins are also produced in response to inflammation and their interactions with HA can influence its physical properties and increase immune cell interactions. After repair and remodeling, HA levels eventually return to baseline, as inflammation is resolved. In situations of chronic disease, the persistence of HA is associated with increased inflammatory and fibrotic responses that are not resolved.

ETHICS STATEMENT

The figures were taken from work carried out in accordance with the guidelines for ethical animal research from the Canadian Council of Animal Care with protocols approved by the University of British Columbia Animal Care Committee.

AUTHOR CONTRIBUTIONS

PJ wrote the initial draft which was then worked on by all authors. AA provided the figures.

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MINI REVIEW
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Galectin-Glycan Interactions as Regulators of B Cell Immunity

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Cell surface glycans and their glycan-binding partners (lectins) have generally been recognized as adhesive assemblies with neighbor cells or matrix scaffolds in organs and the blood stream. However, our understanding of the roles for glycan-lectin interactions in immunity has expanded substantially to include regulation of nearly every stage of an immune response, from pathogen sensing to immune contraction. In this Mini-Review, we discuss the role of the ß-galactoside-binding lectins known as galectins specifically in the regulation of B-lymphocyte (B cell) development, activation, and differentiation. In particular, we highlight several recent studies revealing new roles for galectin (Gal)-9 in the modulation of B cell receptor-mediated signaling and activation in mouse and man. The roles for cell surface glycosylation, especially I-branching of N-glycans synthesized by the glycosyltransferase GCNT2, in the regulation of Gal-9 binding activity are also detailed. Finally, we consider how dysregulation of these factors may contribute to aberrant immune activation and autoimmune disease.

Keywords: B cells, B cell activation, B cell receptor, galectin, I-branch, I-antigen, GCNT2

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OVERVIEW: GALECTIN-GLYCAN INTERACTIONS IN IMMUNE FUNCTION

Galectins are a family of evolutionarily conserved glycan binding proteins (lectins) widely expressed in both stromal and immune tissues (1). In immunity, extensive research has established galectins as important regulators of immune homeostasis (2), inflammation (3), malignancy (4–6), and autoimmune disease (7). In the innate immune system, galectins are known to regulate granulocyte chemotaxis, dendritic cell maturation, mast cell activation, and many other activities (3). However, galectins are perhaps most widely recognized for their effects on T lymphocyte function, where galectins (Gal)-1,-3, and -9 have been shown to differentially modulate development, activation, differentiation, and effector function (3, 8, 9). The roles of galectins in innate and cell-mediated adaptive immunity have been reviewed at length elsewhere (1–4, 7, 8). Yet, while significant progress has been made in deciphering roles of galectins in innate immune cell and T cell biology, the roles for galectins in B cells have only recently begun to be deciphered. Here, we review the state of galectin literature in the B cell compartment, particularly with regard to B cell development, activation, differentiation, and effector function. We also discuss how differential glycosylation in B cells serves to regulate galectin function during different stages of B cell maturation. Finally, we conclude with emerging roles of galectins in B cell-mediated immune disease, particularly autoimmune disease.

GALECTINS: STRUCTURE AND FUNCTION

The glycan binding functions of galectins are mediated by highly conserved carbohydrate recognition domains (CRDs), which favor binding to β-galactoside-containing glycans, especially N-acetyllactosamines and their derivatives (1). To date, 15 galectins have been identified in mammals, classified based on their structure as either prototype, chimera-type, or tandem-repeat type (1). Prototype galectins, which includes galectins (Gal)-1,-2,-5,-7,-10,-11,-13,-14, and -15, possess one CRD and typically form homodimers by non-covalent association(1, 10–12). Chimera-type galectins, of which Gal-3 is the only family member identified to date, possess a single CRD connected to a collagen-like oligomerization domain that facilitates formation of higher order pentamers (13). Tandem-repeat type galectins include Gal-4,-8,-9, and -12 and contain two distinct CRDs that are covalently joined by a variable linker region (1, 14). The precise specificity of individual galectins varies somewhat between family members, and each galectin generally shows preference for a restricted set of glycoconjugates (1). Although galectins can function intracellularly, galectins predominantly operate at the cell surface and in endosomal compartments through interaction with membrane glycoconjugates (1, 15). Paradoxically, galectins lack a canonical secretion signal and therefore how galectins transit to the cell surface remains a major unresolved question in the field (1). Regardless of galectin structure and specificity, a unifying property is their capacity for bivalent or multivalent binding, which permits formation of galectin-glycoprotein networks called "lattices" that regulate glycoconjugate membrane dynamics (16–18). By tuning glycoprotein compartmentalization, diffusion speed, internalization, and lateral association with other glycoconjugates, galectins impact many critical cellular processes, especially signal transduction (16, 17). In some cases, the outcomes of galectin binding can be contradictory due to the vast diversity of galectin ligands, the binding of which can be modulated by the cell's metabolic, transcriptional, or glycosylation state (1). Therefore, the physiological functions of galectins are highly contextual, and consequently represent a dynamic mechanism to regulate immune cell activation and function.

GALECTINS IN B CELL DEVELOPMENT

Galectins are recognized as regulators of thymic T cell development, with Gal-1,-3,-8, and -9 each reported to regulate thymocyte apoptosis and selection (19–25). However, a significant body of research has also amassed implicating galectins in early B cell development, particularly at the pre-BI to large pre-BII transition, when productively rearranged heavy chains pair with "surrogate" light chain (26) and the signaling chains CD79a/b (Igα/Igβ) form the pre-B cell receptor (pre-BCR) (26, 27). Signaling through the pre-BCR serves as a developmental checkpoint critical for pre-B cell expansion and development. However, whether signaling occurs by ligand-independent or ligand-dependent mechanisms has been a matter of debate (26).

Accumulating evidence suggests Gal-1 may serve ligand-like properties, albeit non-essential, in pre-BCR signaling. Using a combination of pulldown assays with recombinant surrogate light chain and surface plasmon resonance, Gauthier et al. identified that Gal-1 binds to the λ5 component of the surrogate light chain (28). In this and subsequent functional studies, Gal-1 was found to be produced by specialized bone marrow stromal cells that interact with pre-B cells, augmenting pre-BCR signaling by enhancing pre-BCR clustering at the pre-B cell/stromal cell synapse (28–33). Unusually, while Gal-1-mediated clustering of pre-BCR unequivocally depends on interactions between Gal-1 and several glycosylated pre-B cell integrins, binding of Gal-1 to surrogate light chain is not glycan-dependent (28, 31, 33). Instead, Gal-1 was found to interact with the "unique region" of λ5 via non-glycan-mediated hydrophobic interactions (34). Taken together, a model has emerged in which bone marrow stromal cell-secreted Gal-1 binds pre-B cell glycans expressed on integrins and facilitates pre-B cell / stromal cell synapse formation, while non-CRD-mediated interactions between Gal-1 and surrogate light chain subsequently promote pre-BCR clustering and signaling. However, it should be noted that the overall significance of Gal-1 to B cell development *in vivo* remains somewhat unresolved, as B cell development is minimally impaired in Gal-1-deficient mice (26, 30). How Gal-1 may overlap with other regulators of pre-BCR signaling, including heparan sulfates (35, 36), as well as with ligand-independent mechanisms of pre-BCR signaling, remains to be conclusively determined. Current paradigms suggest that both Gal-1-dependent and Gal-1-independent mechanisms jointly contribute to efficient pre-BCR signaling, and may exert compensatory activity (26).

Besides Gal-1, Gal-3 has also been implicated as a potential regulator of bone marrow B cell development. *LGALS3-/-* mice exhibit abnormal levels of several developing B cell subsets, including CD19+ B220+ c-Kit+ IL-7R+ pro-B cells (37). Accordingly, Gal-3-deficiency also correlated with dramatically augmented production of IL-7 transcript and increased levels of Notch ligands Jagged-1 and Delta-like 1 by bone marrow stroma in *LGALS3-/-* mice (37). While the precise mechanism was not investigated, these data suggest Gal-3 may act on bone marrow stroma to shape B cell development.

GALECTINS IN B CELL SIGNALING AND ACTIVATION

In addition to the growing body of literature implicating a role for galectins in B cell development, emerging evidence suggests that galectins play important roles in the regulation of B cell signaling and activation. To date, Gal-1,-3, and-9 have each been implicated as both positive and/or negative regulators of B cell signaling.

In a recent study, Tsai et al. found that Gal-1 induces stimulatory signaling in murine B cells that bears hallmarks of antigen-receptor signaling through the BCR. They found that Gal-1 induces calcium flux, upregulation of B cell activation markers CD69 and CD86, and proliferation (38). Furthermore,

using a phospho-proteomic approach, the authors observed that activation by Gal-1 leads to similar phosphorylation circuits as stimulation through IgM. Studies analyzing the role of Gal-1 *in vivo* revealed impaired proliferation of Gal-1-deficient B cells in response to antigenic challenge. Interestingly, Gal-1 from non-B cell sources was required for optimal B cell activation, as Gal-1 sufficient B cells in Gal-1 deficient hosts also showed reduced proliferation *in vivo*. Importantly, however, several groups have also reported that although Gal-1 is not highly expressed in resting mature B cells, it is highly upregulated with B cell activation, making the relevant contribution of B cell-intrinsic vs. B cell-extrinsic Gal-1 uncertain (39–42). In studies of B cell chronic lymphocytic leukemia (B-CLL) which depend on BCR signaling for survival and proliferation, Croci et al. observed that specialized tumor-supporting monocytes, so called "nurse-like" cells, enhanced BCR signaling and survival through the production of Gal-1 (43). Specifically, the authors found that Gal-1 bound B-CLL cells in a glycan-dependent manner and lowered the threshold of productive BCR signaling. Gal-1 also simultaneously promoted B-CLL survival through Gal-1-mediated enhancement of BAFF and APRIL expression by nurse-like cells. Collectively, these findings suggest a model where exogenous, and possibly B cell-intrinsic Gal-1, promote B cell activation through a BCR-dependent mechanism.

Paradoxically, however, in a few studies, Gal-1 has also been implicated as a negative regulator of B cell activation. In a study by Tabrizi et al. Gal-1 was highly expressed by resting and especially activated IgM+ memory B cells, inhibited Akt signaling, and promoted B cell death (40). Another study of human BL36 Burkitt lymphoma cells found that Gal-1 directly bound CD45 and inhibited its phosphatase activity (44). In mammalian two hybrid studies from the Roeder laboratory, Gal-1 was also found to bind (in a non-glycan-dependent mechanism) the B cell transcriptional co-activator and promoter of BCR signaling Oca-B, which the authors hypothesized inhibited cytoplasmic Gal-1 secretion and prevented Gal-1 induced suppression of CD45 phosphatase activity (41). Thus, the physiological functions for Gal-1 in B cells may be diverse, complex, and context dependent (44).

Besides Gal-1, many studies have implicated Gal-3 in the regulation of B cell activation. In a recent study by Beccaria et al. Gal-3 was also found to modulate B cell activation and germinal center (GC) immune responses. Specifically, the authors observed that Gal-3 was expressed in resting splenic B cells at steady state, and loss of Gal-3 in *LGALS3-/-* mice resulted in heightened activation (measured by CD80 and CD86 expression), spontaneous GC formation, augmented antibody secreting cell numbers, and increased circulating IgG2c and IgG3 (45). This phenotype was B cell-intrinsic, as adoptive transfer of *LGALS3-/-* B cells into B-cell deficient (but otherwise Gal-3-sufficient) mice showed similar results, as well as in other corroborating studies with *LGALS3-/-* B cells *in vitro.* Although the effects of Gal-3 were B cell-intrinsic, interplay between GC B cells and follicular T helper cells was postulated to be important, and IFNγ (produced most prominently by T cells but also B cells) was essential for spontaneous B cell GC formation. Additionally, data from several other studies of *LGALS3-/-* mice seem to support the overall conclusions of Beccaria et al., with *LGALS3-/-* showing overall improved antibody responses in several models of parasite infection, including *Plasmodium yoelii* (46) and *Schistosoma mansoni* infection models (37, 45, 47–50), but not *Plasmodium berghei* and *Plasmodium chabaudi* infection (46). Although a clear understanding of the molecular mechanisms involved is still lacking, studies of the role of Gal-3 in human diffuse large B cell lymphoma cell lines have shown that Gal-3 binds CD45, dampens its phosphatase activity, and promotes lymphoma cell survival (51). Interestingly, Gal-3 is known to be downregulated in primary human GC B cells (52), suggesting that loss of Gal-3 may be important for altering CD45 signaling activity within GCs, where CD45 is known to be essential for GC persistence (53). Additional studies will be required to decipher the molecular mechanisms operating that may restrict B cell activation.

In addition to Gal-3, Gal-9 has recently emerged as a negative regulator of BCR signaling and activation. Gal-9 was first implicated in the regulation of B cell activation in studies analyzing Gal-9-deficient mice, where Sharma et al. observed that mice lacking Gal-9 have increased viral-specific IgM, IgG, and IgA titers as well as enhanced formation of antibody secreting cells in response to influenza A challenge (54). These initial data were further supported by studies in human B cells, which demonstrated that recombinant and mesenchymal stem cell-derived Gal-9 antagonizes B cell proliferation and antibody-secreting cell formation in a dose dependent manner, and that treatment of mice with recombinant Gal-9 *in vivo* resulted in diminished antigen specific serum titers in response to immunization (55).

Recently, our groups independently investigated the molecular mechanisms for Gal-9 mediated regulation of B cell activation (56, 57). We found that Gal-9 is detectable on the surface of primary naïve B cells in both mice and humans and could act in a B cell-intrinsic manner to negatively regulate BCR signaling. Mechanistically, Gal-9 antagonized BCR signal transduction by similar but slightly different mechanisms. In human B cells, we found that a major Gal-9 receptor was CD45 (57). Binding of CD45 by Gal-9 triggered a negative signaling cascade through Lyn, CD22, and SHP-1 that dampened BCR-triggered calcium flux and inhibited activation of calcium-sensitive transcription factors, including NFAT-1 and NF-κB. In an analogous but distinct manner, in murine B cells, we observed that Gal-9 bound not only CD45 but also IgM-BCR (56). Functionally, murine Gal-9 regulated BCR-antigen microclustering and downstream signaling, both of which were enhanced in Gal-9-deficient murine B cells. However, rather than altering calcium signaling, murine Gal-9 mitigated activation of CD19 and ERK1/2 downstream of BCR ligation. We hypothesize that this impaired signaling response is due to Gal-9's ability to prevent exclusion of inhibitory receptors from the signalosome, as we found CD45 and CD22 are specifically enriched within Gal-9 lattices and showed enhanced colocalization with IgM-BCR. Moreover, using dual-color super-resolution microscopy, we observed that association of IgM-BCR with CD22 is reduced in resting Gal-9-deficient B cells, and propose that this provides a plausible mechanism for enhanced BCR signaling in the absence

of Gal-9 (56). Taken together these data suggest that Gal-9 acts to attenuate BCR signaling through facilitating interactions with endogenous regulatory networks (**Figure 1**). These findings provide exciting potential for therapeutic development targeting steady-state B cell signaling networks.

GALECTINS AS MODULATORS OF B CELL DIFFERENTIATION AND CELL FATE

An accumulating body of evidence suggests that galectins can also influence cell fate decisions in mature B cells, particularly in regulating the balance of B cell differentiation to memory or plasma cells.

Acosta-Rodriguez et al. examined the role of Gal-3 in B cells both *in vitro* and *in vivo* following *Trypanosoma cruzi* infection (58). The authors found that Gal-3 was upregulated in response to IL-4 or CD40-mediated stimulation and in B cells during ongoing parasite infection *in vivo*. Silencing of Gal-3 by RNA interference *in vitro* and *in vivo* prevented IL-4-induced downregulation of Blimp-1, a transcription factor critical for plasma cell development, and enhanced plasma cell differentiation. A mechanism was proposed in which Gal-3 works in concert with IL-4 to disfavor plasma cell differentiation and promote differentiation to memory B cells (58). Indeed, this hypothesis has since been supported by numerous studies demonstrating increased antibody-secreting cell numbers and antibody titers at steady state and in response to parasite infection in *LGALS3*–/– mice (37, 45–50). Interestingly, B-lymphopenia, significantly disrupted follicular architecture in lymph nodes and spleen, increased spontaneous GC numbers, and lupus-like pathology have also been reported for *LGALS3*–/– mice (45, 47, 48, 50).

In contrast to Gal-3, data suggests that Gal-1 and Gal-8 favor plasma cell fate decisions. Studies examining Gal-1 expression in murine and human B cells have noted that Gal-1 is significantly upregulated with B cell differentiation and is directly induced by Blimp-1 (39, 59). Through a combination of *in vitro* approaches that included ectopic expression, genetic knockdown, synthetic galectin inhibitors, and use of galectin-deficient mice, Tsai et al. demonstrated Gal-1 is sufficient to positively regulate plasma cell differentiation *in vitro* (39, 59). However, the authors propose that Gal-1 is not strictly required, as Gal-8 was found to be able to functionally compensate for loss of Gal-1 (39). In a separate study, however, Anginot et al. demonstrate that, at least *in vivo*, Gal-1 is required for optimal plasma cell responses and may not be fully compensated by Gal-8 (60). Specifically, Gal-1-deficient mice exhibited impaired antibody secreting cell number and diminished IgM and IgG titers in response to immunization, particularly in response to the T-dependent antigens (60). Interestingly, both groups report that Gal-1 is produced by (39, 59, 60) and binds (39, 59) only early plasma cells and not fully differentiated plasma cells, suggesting that Gal-1 and -8 drive the earliest stages of plasma cell differentiation. While the specific mechanism of action remains unresolved, Gal-1 and Gal-8 expression and/or treatment were associated with enhanced expression of XBP-1 (Gal-1 and Gal-8), Blimp-1 (Gal-8), IL-10 (Gal-1), and IL-6 (Gal-8) (39). In addition, Gal-1 also appears to have pro-survival roles in plasma cells (60). By contrast, Gal-1 has been reported to be expressed by IgM+ memory B cells, in which it was shown to inhibit Akt signaling compared to Gal-1^{lo} naïve B cells and promote BCR-induced apoptosis (40). Thus, Gal-1/-8 and Gal-3 appear to have opposing roles in skewing the outcome of B cell differentiation.

GALECTINS IN B CELL EFFECTOR FUNCTION

Galectins have also been reported to have roles in the regulation of B cell effector function. As secreted molecules, galectins can exert cytokine-like activity. A documented example is Gal-1, which is upregulated with B cell activation and secreted into the B cell milieu, where it has been shown to induce apoptosis of inflammatory T cells (42). Besides serving as effector cytokines, galectins have also been reported to augment the immunoglobulin secretion capacity of plasma cells. Although Gal-1 and Gal-8 facilitate plasma cell differentiation (described earlier), they also have been shown to directly enhance secretion of antibody by augmenting expression of XBP-1s and by increasing the ratio of secreted / membrane IgM transcripts (39, 59). Once secreted, antibodies themselves can be bound by galectins. Both Gal-3 and Gal-9 have been shown to bind IgE bound to mast cell FcεR and prevent clustering-induced degranulation of inflammatory mediators (61–63).

GLYCOSYLATION IN THE REGULATION OF GALECTIN ACTIVITY

As lectins, extracellular galectin activity is often highly dependent on the favorable glycosylation of target receptors (1). Receptor glycosylation is regulated by a host of factors, including cell metabolism, ER and Golgi nucleotide sugar-donor transporters, and the rate of glycoprotein flux through the Golgi (64). Frequently, however, receptor glycosylation is dictated by the expression and activity of relevant ER- and Golgi-resident glycosyltransferases, glycosidases, and glycan-modifying enzymes.

Recently, our laboratory analyzed the global N-glycan repertoire of human tonsillar naïve, GC, and memory B cells by whole glycome mass spectrometry (MS) and plant lectin based flow cytometry (57). We found that all three B cell subsets expressed tri- and tetra-antennary complex-type N-glycans replete with poly-N-acetyllactosamine (poly-LacNAc), which are repeating units of the disaccharide N-acetyllactosamine (Gal-GlcNAc) that canonically serve as high affinity binding determinants for many galectins. Indeed, poly-LacNAc expression by naïve and memory B cells corresponded with robust binding to Gal-1 and, to our surprise, Gal-9, which had previously not been reported to bind B cells. However, whereas Gal-1 showed similarly strong binding to GC B cells as non-GC cell types, Gal-9 binding was starkly reduced in GC B cells. Closer examination of the N-glycomic profile of naïve, GC, and memory B cells by tandem MS revealed that

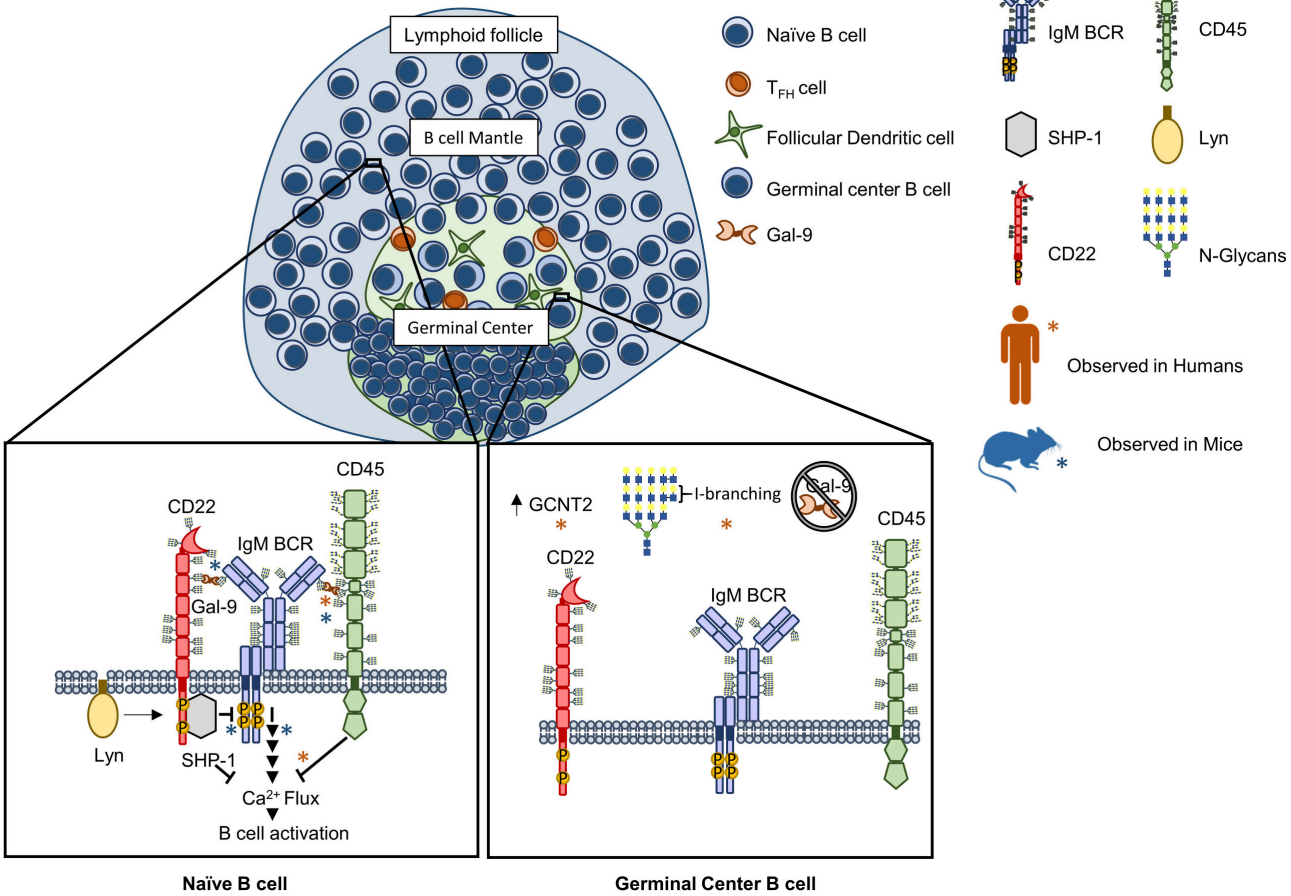

FIGURE 1 | Galectin-9 regulates B cell receptor signaling in human and murine B cells through analogous but distinct mechanisms**.** Naïve B cell activation is antagonized by Gal-9 through binding to the receptor tyrosine phosphatase CD45 on human B cells that activates a Lyn-CD22-SHP-1 dependent circuit and inhibits calcium accumulation downstream of the BCR [Left, orange asterisks (*)]. In murine B cells, Gal-9 similarly regulates BCR signaling by altering the nanoscale organization of signaling molecules. Specifically, Gal-9 has been found to bind CD45 but also IgM BCR, preventing exclusion of CD45 and CD22 upon B cell activation and leading to impaired signal transduction following BCR ligation [Left, blue asterisks (*)]. In humans, Gal-9 binding activity is differentially regulated between naïve and germinal center (GC) B cells via concerted alterations in N-glycosylation. Specifically, Gal-9 binding has been shown to be greatly diminished in germinal center (GC) B cells via upregulation of the glycosyltransferase GCNT2, which catalyzes I-branch formation on glycan ligands of Gal-9 (poly-LacNAcs), and attenuates Gal-9 binding (56, 57).

many poly-LacNAcs in GC B cells were modified with internal β1,6 GlcNAc- and galactose-containing disaccharides, termed "I-branches" or I-blood group antigen, that were not present in naïve and memory B cells (57). I-branch expression at the GC stage corresponded with upregulated expression of the I-branching enzyme, GCNT2, and genetic studies in B cell lines demonstrated that GCNT2/I-branches were both necessary and sufficient to inhibit Gal-9 binding (57), as well as Gal-3 binding [(65) and unpublished observations] (**Figure 1**). Interestingly, Gal-1 binding was largely unaffected by I-branches, suggesting that I-branches may preferentially target Gal-3 and Gal-9 glycan binding motifs, whereas terminal modifications such as α2,6-sialylation by the sialyltransferase ST6Gal1 may more selectively target Gal-1 (1, 8). Therefore, selective glycosyltransferase expression may be a mechanism of disparately regulating the activity of different galectin family members in B cells.

Beyond I-branches, regulation of Core 2 poly-LacNAc expression on O-glycans by GCNT1 was shown by Clark et al. to be a major factor controlling Gal-3 binding in B cell lines (51). Whereas B cell lines expressing GCNT1 showed robust binding and cell surface localization of Gal-3, B cells with inherently low GCNT1 or GCNT1 knockdown did not (51). Although the expression pattern of GCNT1 in native B cell populations was not determined, studies in our laboratory (66) indicate that naïve and GC B cells robustly express GCNT1/Core 2 poly-LacNAcs, whereas more differentiated B cells (memory B cells and plasmablasts) downregulate GCNT1/Core 2 poly-LacNAcs. The significance of this glycan expression pattern to Gal-3 binding activity is currently under investigation by our laboratory.

It is important to emphasize that while glycosylation can significantly contribute to the regulation of galectin activity, not

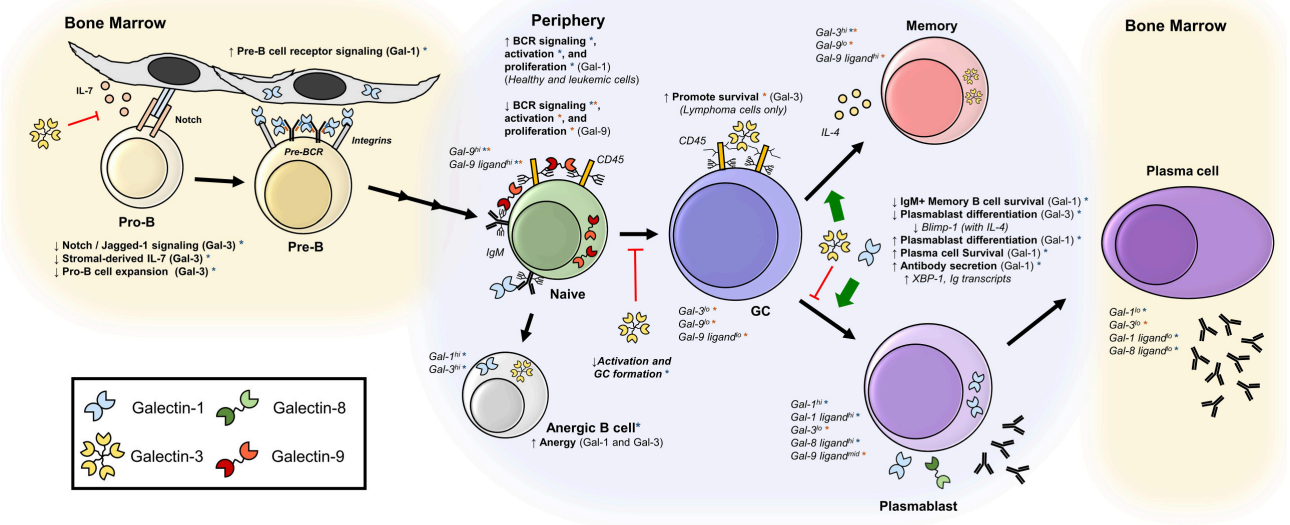

FIGURE 2 | Galectins regulate B cell development, activation, and differentiation. Depicted are published roles of galectins at various stages of differentiation, as well as reported expression of galectins and galectin-binding glycans. Orange asterisks (*) indicate findings described in human B cells, and blue asterisks (*) indicate findings observed in mice. Bone marrow B cells (28–34, 37, 60); naïve B cells (38, 43, 45, 56, 57); anergic B cells (37, 68); GC B cells (45, 51); memory B cells (37, 40, 45–50, 58); plasmablasts and plasma cells (37, 39, 59, 60).

all galectin functionality is exclusively glycan-dependent. In a study by Bonzi et al., it was demonstrated that non-glycosylation dependent interactions between Gal-1 and pre-BCR induce conformational changes in the Gal-1 CRD in a manner that alter its glycan-binding preferences, perhaps allowing Gal-1 to disengage from glycosylated integrin ligands and promote non-glycosylation dependent pre-BCR clustering. Moreover, galectins have been shown to exhibit roles intracellularly in mitochondrial, cytoplasmic, and nuclear compartments (15).

As novel functions emerge for galectins in B cells, identifying the factors regulating their activity, especially expression of favorable glycan ligands, will remain crucial to understanding their physiological role *in vivo*, and how galectin-glycan interactions may be exploited therapeutically.

GALECTINS IN B CELL AUTOIMMUNITY

The increasingly apparent roles for galectins in modulating B cell activation and cell fate suggests that galectins may serve important roles as regulators of B cell tolerance. While clearly a budding and ongoing area of investigation, a few studies have suggested potential (albeit complex) roles for Gal-1, Gal-3, and Gal-9 in B cell-mediated autoimmune disease (67). Recent evidence suggests that Gal-3 deficient mice develop systemic autoimmunity with lupus-like features, including spontaneous GC formation, elevated levels of anti-nuclear antibodies, and kidney pathology (45). This lupus-like pathology became increasingly pronounced with age, and was found to be absolutely dependent on B cell-intrinsic Gal-3 as well as IFNγ, the production of which was increased in both B cells and T cells (68). Of note, studies by Clark et al. also noted autoantibody development in *LGALS3*–/– mice when crossed to LamH transgenic mice, which express an antibody heavy chain reactive against the self-antigen laminin (69). Interestingly, the numbers of autoreactive B cells that escape tolerance mechanisms are increased further in mice doubly deficient for both Gal-3 and Gal-1 (69). While specific mechanisms linking Gal-3, and possibly Gal-1, to maintaining B cell tolerance have yet to be fully elucidated, it should be noted that both Gal-3 and Gal-1 have been shown to be more highly expressed in anergic murine B cells (68).

Besides Gal-1 and Gal-3, Gal-9 has also been implicated in the development of lupus-like disease in several models of SLE. In a recent study, Panda *et al.* demonstrated that treatment of BXSB/MpJ and (NZB × NZW)F1 lupus-prone mice with Gal-9 before symptoms manifests diminishes the probability of developing pathology, including tissue inflammation and splenomegaly associated with disease onset (70). Mechanistically, the authors present evidence that Gal-9 antagonizes TLR7- and TLR9-dependent activation of plasmacytoid dendritic cells (pDCs) and B cells, as well as type I Interferon production by pDCs. In a second study from Moritoki et al., Gal-9 was found to ameliorate pathology in a MRL/lpr model of lupus, apparently by inducing plasma cell apoptosis, although a direct link was not firmly established (71). Interestingly, these two studies are seemingly opposite to findings from Zeggar et al., who using a pristane-induced lupus model, observed that *LGALS9*–/– mice exhibited reduced disease burden and unaltered TLR7-type I interferon signaling (72). The reasons underlying these disparate results are unclear, but may reflect the different model systems used, including spontaneous *vs.* inducible models of lupus and disparate genetic backgrounds (73). Future studies will

be required to parse the precise contribution of Gal-9, Gal-3, and Gal-1 to B cell tolerance, and to better determine a possible role for these lectins (or relevant glycans) in the development of autoimmune disease in humans.

CONCLUSIONS AND OUTLOOK

Here, we have reviewed the emerging roles of galectins in B cell immunobiology. Over the past two decades, studies have revealed a complex network of positive and negative regulatory roles for galectins acting throughout B cell development, activation, differentiation, and antibody responses (**Figure 2**). Recent studies in particular have highlighted novel roles for Gal-9 and glycosylation in the regulation of BCR signaling and activation. Moving forward, studies investigating the precise mechanisms of galectin function in B cells, and concomitant regulation of galectin activity by B cell glycosylation, will be of great interest. Furthermore, how galectins contribute to B cell-mediated disease, including autoimmune disease, will remain a critical area of future research that will likely yield important insights into disease etiology and/or novel therapeutic approaches targeting galectin-glycan interactions. Undoubtedly, the continued investigation of the multitudinous and complex roles of galectins in B cell biology will be an exciting pursuit in the years ahead.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human B Cell Differentiation Is Characterized by Progressive Remodeling of O-Linked Glycans

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Germinal centers (GC) are microanatomical niches where B cells proliferate, undergo antibody affinity maturation, and differentiate to long-lived memory B cells and antibody-secreting plasma cells. For decades, GC B cells have been defined by their reactivity to the plant lectin peanut agglutinin (PNA), which binds serine/threonine (O-linked) glycans containing the asialylated disaccharide Gal-β1,3-GalNAc-Ser/Thr (also called T-antigen). In T cells, acquisition of PNA binding by activated T cells and thymocytes has been linked with altered tissue homing patterns, cell signaling, and survival. Yet, in GC B cells, the glycobiological basis and significance of PNA binding remains surprisingly unresolved. Here, we investigated the basis for PNA reactivity of GC B cells. We found that GC B cell binding to PNA is associated with downregulation of the α2,3 sialyltransferase, *ST3GAL1* (ST3Gal1), and overexpression of ST3Gal1 was sufficient to reverse PNA binding in B cell lines. Moreover, we found that the primary scaffold for PNA-reactive O-glycans in B cells is the B cell receptor-associated receptor-type tyrosine phosphatase CD45, suggesting a role for altered O-glycosylation in antigen receptor signaling. Consistent with similar reports in T cells, ST3Gal1 overexpression in B cells *in vitro* induced drastic shortening in O-glycans, which we confirmed by both antibody staining and mass spectrometric O-glycomic analysis. Unexpectedly, ST3Gal1-induced changes in O-glycan length also correlated with altered binding of two glycosylation-sensitive CD45 antibodies, RA3-6B2 (more commonly called B220) and MEM55, which (in humans) have previously been reported to favor binding to naïve/GC subsets and memory/plasmablast subsets, respectively. Analysis of primary B cell binding to B220, MEM55, and several plant lectins suggested that B cell differentiation is accompanied by significant loss of O-glycan complexity, including loss of extended Core 2 O-glycans. To our surprise, decreased O-glycan length from naïve to post-GC fates best correlated not with ST3Gal1, but rather downregulation of the Core 2 branching

enzyme GCNT1. Thus, our data suggest that O-glycan remodeling is a feature of B cell differentiation, dually regulated by ST3Gal1 and GCNT1, that ultimately results in expression of distinct O-glycosylation states/CD45 glycoforms at each stage of B cell differentiation.

Keywords: glycosylation, glycan, B cell, CD45, peanut lectin, PNA, ST3Gal1, GCNT1

INTRODUCTION

B lymphocytes are essential mediators of prophylactic immunity, conferring durable immune protection through the secretion of soluble antigen-binding receptors called antibodies. The most effective B cell responses arise from the germinal center (GC) reaction, named for the transient microanatomical structures that appear in B cell follicles during B cell immune responses (1). The GC reaction is initiated by B cell activation by cognate T cells at the T-B follicular border, which leads to upregulation of the GC transcriptional program and T-B cell co-migration back into the B cell follicle. Within GCs, GC B cells undergo massive clonal expansion, somatically mutate their antibody binding sites, and undergo Darwinian-like selection for the highest affinity clones (1). After several rounds of proliferation and selection, GC B cells differentiate and exit the GC as either long-lived memory B cells or antibody secreting cells, both of which mediate pathogen clearance and provide durable prophylactic immunity against secondary antigenic encounter. However, this process is not infallible, and can result in poorly neutralizing antibodies, aberrant self-directed antibodies, or malignant transformation (1). Therefore, the continued unraveling of the mechanisms guiding GC responses remains a high priority for developing therapeutics that enhance or quell B cell responses in a variety of clinical settings, including generation of more potent vaccines.

A longstanding but still poorly understood aspect of GC B cells is GC reactivity with peanut agglutinin (PNA) (2, 3). PNA is a plant-derived glycan-binding protein (lectin) that exhibits strong binding to the serine/threonine (O)-linked disaccharide Gal β1,3-GalNAc-Ser/Thr, often referred to as Thomsen Friendenreich antigen or T-antigen, as well as extended O-glycan structures containing T-antigen (4–6). Typically, T-antigen is not exposed on healthy cells due to the further elaboration by other Golgi-resident glycosylation enzymes (glycosyltransferases) that modify the core T-antigen structure (7, 8). Indeed, T-antigen expression and associated PNA binding is a feature of malignant transformation (7, 8). Nonetheless, under rare circumstances, the T-antigen moiety can become transiently exposed in healthy cells. In T cells, T-antigen is expressed at specific stages of thymic development and after mature T cell activation and differentiation (2, 9–16). Functionally, altered T-antigen expression on the T cell coreceptor CD8 induces conformational changes that regulate CD8 affinity for MHC Class I, an interaction central to thymocyte positive selection (17, 18). Additionally, T-antigen expression on thymocytes and activated T cells is associated with synthesis of Core 2 poly-N-acetyllactosamines (poly-LacNAcs) (9–11, 19–25). These poly-LacNAcs regulate binding of immunoregulatory lectins known as galectins, which modulate thymocyte survival, mature T cell differentiation, and T cell effector function (25, 26). Simultaneously, Core 2 poly-LacNAcs also serve as scaffolds for synthesis of glycan functional groups such as sialyl lewis X, which drives selectin-mediated trafficking of T cells to tissues (27). Thus, altered PNA binding often heralds alterations to glycosylation that have important physiological consequences. Yet, despite being first reported almost 40 years ago (2, 3), the mechanisms and functional significance of PNA ligand exposure in GC B cells has remained unclear.

Here, we investigated the mechanisms underlying PNA binding, and attempted to generate insight into the function of this glycobiological change by identifying the scaffolds bearing PNA-reactive glycans. We present evidence that strongly implicates the α2,3-sialyltransferase *ST3GAL1* (ST3Gal1) in regulating the PNA phenotype of human GC B cells, particularly through modification of O-glycans on CD45. In the course of this investigation, we unexpectedly discovered that O-glycan remodeling is in fact not restricted to B cells at the GC stage, but rather a more general feature of B cell differentiation. Specifically, we observed that B cell differentiation to memory and plasmablast fates is associated with truncation of O-glycan chains, particularly of Core 2 O-glycans. Loss of Core 2 O-glycans toggled binding between the glycoform-specific CD45 antibodies B220 and MEM55, suggesting that this glycosylation switch occurs to a significant extent on CD45. Interestingly, although ectopic expression of ST3Gal1 was sufficient to truncate O-glycans *in vitro*, we found that expression of the Core 2 O-glycan branching enzyme GCNT1 best correlated with O-glycan length in primary B cells. Therefore, considering both T-antigen expression in GC B cells and O-glycan truncation with B cell differentiation, we conclude that global O-glycan remodeling is a general feature of B cell differentiation that drives expression of discrete CD45 glycoforms among distinct B cell populations.

Abbreviations: GC, germinal center; PNA, peanut agglutinin; MAL-II, *Maackia amurensis* lectin II; SNA, *Sambucus nigra* agglutinin; STA, *Solanum tuberosum* agglutinin; HPA, *Helix pomatia* agglutinin; PHA-L, *Phaseolus vulgaris* leuco-agglutinin; OE, overexpressing; DLBCL, diffuse large B cell lymphoma; IP, immunoprecipitation; MS, mass spectrometry; CORA, Cellular O-glycome Reporter Amplification; Gal, galactose; GalNAc, N-acetylgalactosamine; poly-LacNAc, poly-N-acetyllactosamine; mAb, monoclonal antibody.

RESULTS

GC B Cells Downregulate Expression of the Core 1 O-Glycan Sialyltransferase *ST3GAL1*

Palatine tonsils are sentinel lymphoid tissues continually exposed to oral microbes, and therefore represent a valuable and

accessible site for study of human B cells. Using tonsil tissue discarded from routine tonsillectomies, we analyzed PNA binding to several B cell subsets *ex vivo*, including naïve, GC, memory, and plasmablast B cells **(Figures 1A,B)**. As expected, GC B cells showed exceedingly strong binding to PNA that was >10-fold higher than naïve or memory B cells, indicating strong expression of O-glycans containing the asialylated Core 1 O-glycan moiety (T-antigen). Surprisingly, however, we found that plasmablasts also demonstrated strong binding to PNA that equaled that of GC B cells, suggesting that PNA reactivity may more accurately reflect B cell activation rather than be part of a GC program *per se*.

We reasoned that expression of T antigen or T-antigen-containing O-glycans (collectively, "PNA-reactive O-glycans") in B cells may arise from one of several possibilities **(Figure 1C)**. First, and most plausibly, PNA-reactive O-glycans may be expressed due to downregulation of sialyltransferases, which normally obstruct PNA binding by capping the galactosyl moiety of T-antigen with sialic acid. In this regard, the α2,3 sialyltransferase ST3Gal1 was the most plausible candidate due to its well-documented Core 1 O-glycan specificity and reported modulation of PNA binding in thymocytes and T cells **(Figure 1C)** (5, 12, 13, 19, 21, 28, 29). Second, expression and/or activity of sialic acid cleaving enzymes (sialidases) could also contribute to increased PNA binding by revealing T-antigen moieties. Third, augmented expression of PNA-reactive O-glycans in GC B cells may arise from increased expression of the T antigen-synthase glycosyltransferase, C1GALT1. Finally, an overall increased level of O-glycosylation could also potentially explain enhanced binding of PNA lectin **(Figure 1C)**.

To narrow down which of these possibilities most likely accounted for increased expression of PNA-reactive O-glycans in GC B cells, we analyzed expression of O-glycosylation related genes among human naïve, GC, and memory B cells using publicly available expression array data (GSE12195) (30, 31). Analysis of O-glycosylation initiating enzymes, polypeptide N-acetylgalactosamine transferases (*GALNT*s) revealed no general upregulation of O-glycosylation in GC B cells that could account for increased T-antigen expression **(Supplementary Figure 1)**. With the notable exception of *GALNT12* and *GALNT14,* expression of the vast majority of *GALNTs* were markedly downregulated in GC B cells, including *GALNT1, GALNT3, GALNT10, GALNT11, GALNT6* (compared to naïve), and *GALNT7* (compared to memory). Moreover, although T-antigen synthase (*C1GALT1)* and its essential chaperone Cosmc (*C1GALT1C1)* showed divergent expression, downregulation of *C1GALT1* in GC B cells suggests augmented Core 1 O-glycan synthesis is unlikely to account for increased T-antigen expression **(Supplementary Figure 1)**. When sialidase expression was examined, we found that no endogenous sialidase genes (*NEU1-4)* were significantly upregulated in GC B cells compared to naïve or memory B cells. On the other hand, two sialyltransferase genes showed significantly decreased expression in GC B cells: *ST3GAL5* and *ST3GAL1*. Because *ST3GAL5* (also known as GM3 synthase) has been reported to predominantly act on lipids (32), *ST3GAL1* emerged as the most likely regulator of the PNA^{hi} phenotype of GC B cells.

Given that our preliminary microarray analysis implicated ST3Gal1, we next sought to validate this finding by quantitative real-time reverse transcription PCR (qRT-PCR). Indeed, flow cytometric sorting of primary tonsillar B cell subsets and qRT-PCR analysis revealed strikingly diminished *ST3GAL1* transcript levels in GC B cells and plasmablasts compared to naïve and memory B cells, in a manner reciprocal to PNA binding **(Figure 1D).** Therefore, these data supported diminished ST3Gal1 activity and loss of sialylation on Core 1 O-glycans as a major factor in expression of PNA-reactive O-glycans in primary GC B cells.

ST3Gal1 Directly Modulates Expression of PNA-Reactive O-glycans in GC B Cells

To more directly test the hypothesis that ST3Gal1 regulates expression of PNA reactive O-glycans in B cells, we ectopically expressed ST3Gal1 in a PNA^{hi} GC-derived B cell line, Ramos (**Figure 2A**) (33), and analyzed the effect on PNA binding. Consistent with Core 1 O-glycan activity, ST3Gal1 overexpression (ST3Gal1OE) virtually ablated PNA binding entirely (**Figure 2B**) while augmenting binding to another plant lectin that preferentially binds α2,3-sialylated T-antigen, *Maackia amurensis* lectin-II (MAL-II) (**Figure 2C**) (34). By contrast, ST3Gal1OE had no significant effect on either complex N-glycan levels or α2,6-linked sialic acids, as measured by binding of *Phaseolus vulgaris* leucoagglutinin (PHA-L) and *Sambucus nigra* agglutinin (SNA), respectively (**Supplementary Figure 2A**). To validate whether this effect was specific to sialic acid and not due to off-target effects, we treated whole cells with *Arthrobacter ureafaciens* sialidase to remove sialic acids, and assessed the impact on PNA and MAL-II binding. As expected, sialidase treatment restored PNA binding to ST3Gal1OE B cells (**Figure 2D**). Sialidase treatment also augmented binding of PNA to control cells (empty vector transduced), suggesting that some sialylated Core 1 O-glycans were present even in PNA^{hi} Ramos cells. In all cases, sialidase treatment abolished binding of MAL-II lectin, consistent with the specificity of MAL-II for α2,3-sialylated Core 1 O-glycans (**Figure 2E**) (34). Taken together, these data strongly suggest that decreased levels of ST3Gal1 in primary GC B cells is a significant factor contributing to expression of PNA-reactive O-glycans in GC B cells.

CD45 Is Major Scaffold Bearing PNA-Reactive Glycans in GC B Cells

In T cells, downregulation of ST3Gal1 and exposure of T-antigen or other PNA-reactive O-glycans is known to be correlated with activation, proliferation, enhanced T cell trafficking, increased susceptibility to cell death, and altered thymocyte selection (9, 12, 13, 17–20, 23, 35). In part, the diverse functions associated with PNA reactive glycans arise from both lectin-independent and lectin-dependent effects on multiple glycoprotein scaffolds, including CD8, CD43, and CD45 (9, 17–19, 21–23, 25, 36–38). Therefore, in order to better understand the functional significance of PNA-reactive O-glycans on B cells, we sought to identify the glycoproteins bearing these O-glycans. To this end, we immunoprecipitated PNA-binding glycoproteins from

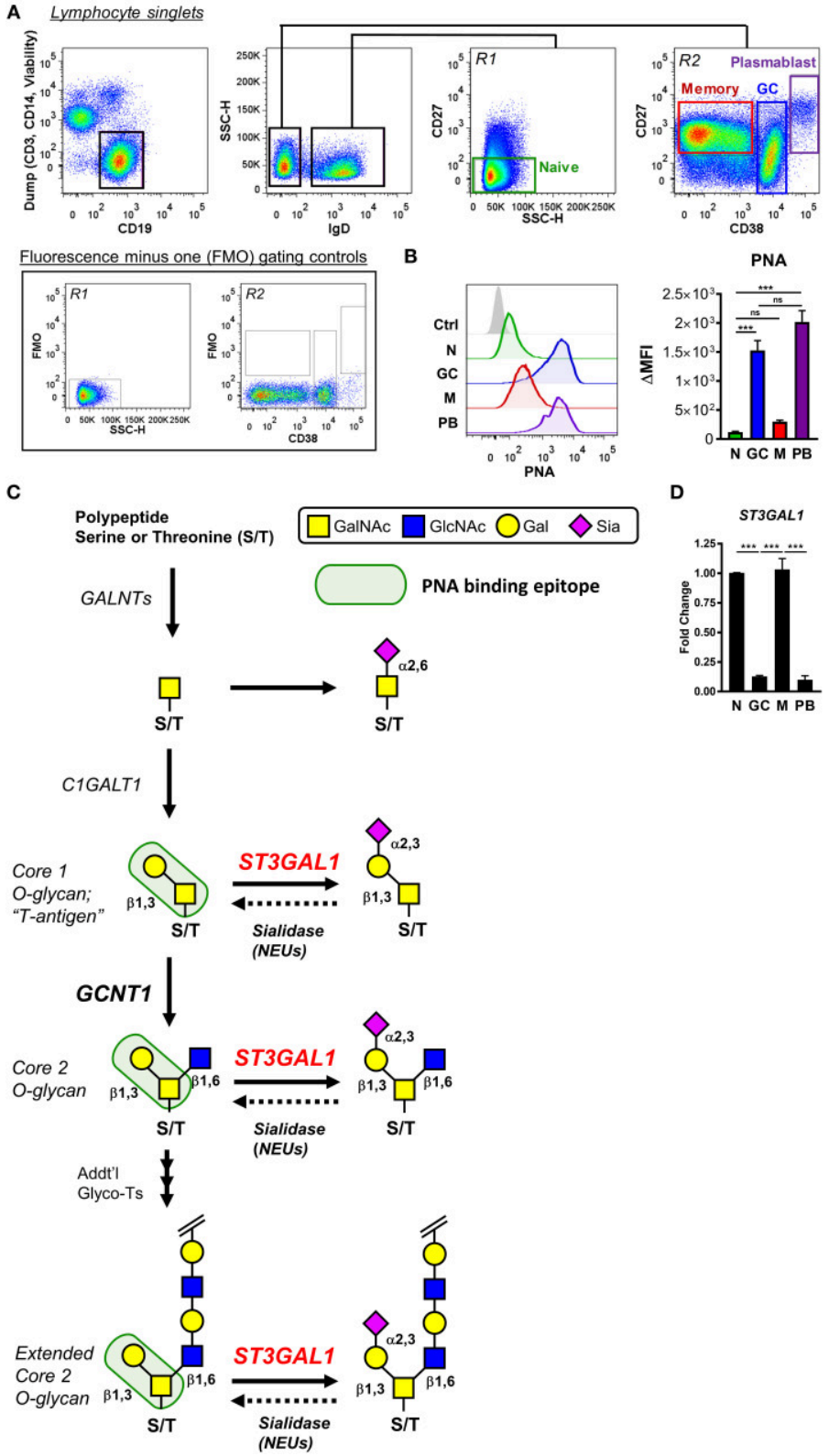

FIGURE 1 | Expression of PNA-reactive glycans by germinal center B cells is correlated with downregulation of the α2,3-sialyltransferase ST3Gal1. **(A)** Gating strategy for analyzing tonsillar naïve (N), germinal center (GC), memory (M), and plasmablast (PB) B cells by flow cytometry. Representative fluorescence minus one (FMO)

(Continued)

FIGURE 1 | controls used for gating of CD27 are shown. **(B)** Analysis of peanut lectin binding to human tonsil B cells. Representative histograms of results are shown (*left*) as well as quantification of geometric mean fluorescence intensities (MFI) (*right*). **(C)** Schematic of synthesis of potential PNA-reactive O-linked glycans on B cells. O-glycan synthesis is initiated in the Golgi apparatus by polypeptide N-acetylgalactosamine transferases (*GALNTs*), which transfer a single GalNAc to select serine/threonine residues of a polypeptide backbone. The initiating GalNAc can be terminally sialylated, or further extended by C1GalT1 (*C1GALT1*) to form the simplest PNA-reactive epitope, a Core 1 O-glycan termed "T-antigen." This core T-antigen moiety can be branched and elongated by other glycosyltransferases to form extended Core 2 O-glycans, which retain binding to PNA, or modified with sialic acid by the α2,3-sialyltransferase ST3Gal1 (*ST3GAL1*), which destroys PNA reactivity. Endogenous (or exogenous) sialidases may remove sialic acids and restore PNA binding. **(D)** Analysis of *ST3GAL1* expression in tonsillar B cells by quantitative real-time reverse transcription PCR (qRT-PCR), sorted as in **(A)**. Data are normalized to the housekeeping gene *VCP* and presented relative to naïve B cells. Data are representative of eight **(B)** or three **(D)** distinct tonsil specimens pooled from two **(B)** or three **(D)** independent experiments. Statistics were calculated using a Kruskal–Wallis test with Dunn's multiple comparisons test **(B)** or One-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. Throughout, bars and error bars depict the mean and SEM, respectively. ns = not significant, $^{***}p \leq 0.001$. ΔMFI, background subtracted geometric mean fluorescence intensity; GalNAc, N-acetylgalactosamine; Gal, galactose; Sia, sialic acid.

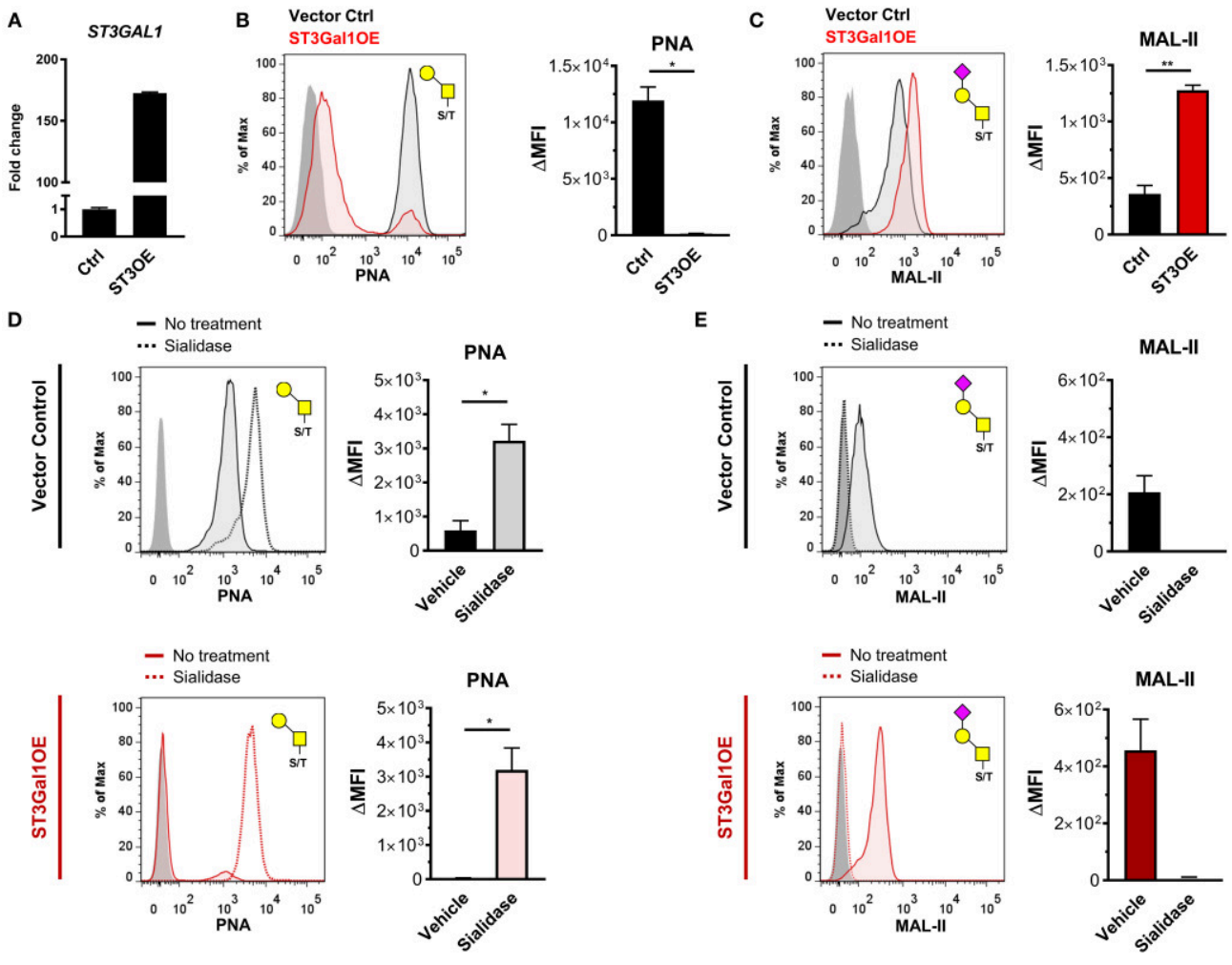

FIGURE 2 | ST3Gal1 regulates PNA binding in B cells by sialylating Core 1 O-glycans. **(A)** Validation of *ST3GAL1* overexpression in Ramos B cells by qRT-PCR. Data were normalized to housekeeping control *VCP* and presented relative to vector control. **(B)** Representative histogram (*left*) and quantification (*right*) of flow cytometric analysis of PNA binding to vector control and ST3Gal1OE Ramos B cells. The Core 1 O-glycan/ T-antigen specificity of PNA is depicted at top right. **(C)** Representative histogram (*left*) and quantification (*right*) of flow cytometric analysis of MAL-II plant lectin binding to vector control and ST3Gal1OE Ramos B cells. The α2,3-sialylated Core 1 O-glycan/sialylated T-antigen glycan favored by MAL-II lectin is depicted at top right. **(D)** Representative histogram (*left*) and quantification (*right*) of PNA binding to vector control (*top*) or ST3Gal1OE Ramos B cells (*bottom)* before and after removal of sialic acids by intact cell treatment with *Arthrobacter ureafaciens* sialidase. **(E)** Representative histogram (*left*) and quantification (*right*) of MAL-II binding to vector control (*top*) or ST3Gal1OE (*bottom)* Ramos B cells before and after sialidase treatment, as in **(D)**. Data in **(B–E)** are from three independent experiments with three biological replicates in total. Statistics were calculated using Welch's unpaired, two-tailed *t*-test **(B–E)**. Throughout, bars and error bars depict the mean and SEM, respectively. ns = not significant, $^{*}p \leq 0.05$, $^{**}p < 0.01$, ΔMFI, background subtracted geometric mean fluorescence intensity; Ctrl, vector control. ST3OE; ST3Gal1 overexpression.

lysates of two PNA^{hi} B cell lines of purported GC origin, Ramos and Raji B cells, using PNA-agarose beads (33). Subsequent immunoblot with PNA (to maximize sensitivity) revealed several candidate bands, including a prominent ∼260 kDa band in both Ramos and Raji lysates that was absent in negative control IP conditions (PNA-IP in the presence of lactose, a competitive inhibitor of PNA binding) **(Figures 3A,B)**. Based on previous studies, we postulated that the 260 kDa band might correspond with CD45, which on B cells is expressed as a full-length isoform ("CD45RABC") containing approximately 60 predicted O-glycosylation sites [NetOGlyc 4.0, http://www.cbs.dtu.dk/services/NetOGlyc/ (39)] predominantly clustered in exons 4, 5, and 6 (corresponding with A, B, and C isoforms) (25). Indeed, blotting with CD45 antibody revealed CD45 in PNA immunoprecipitates of both cell lines **(Figures 3A,B)**. Notably, several other candidate bands at ∼130 and 95 kDa were also revealed by PNA blot in Raji and Ramos B cells, although the identity of these bands was not determined. Preliminary mass spectrometric analysis of a gel fragment containing immunoprecipitated proteins >37 kDa revealed many other potential candidates besides CD45, including CD43 (∼130 kDa when decorated with Core 2 O-glycans) and transferrin receptor (CD71, ∼95 kDa in fully glycosylated form) **(Supplementary Information 1)**, both of which have been reported to be modified with T-antigen in other cell types (25, 40). Because Ramos and Raji are both of Burkitt's lymphoma origin, we confirmed CD45/PNA co-immunoprecipitation in a third cell line, SUDHL-4, which is derived from a GC-type diffuse large B cell lymphoma **(Supplementary Figure 2B)** (33)**.** Additionally, to rule out potential contribution of glycolipids to PNA binding, we analyzed PNA binding in Ramos B cells treated with the glucosylceramide synthase inhibitor D, 1-threo-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) (a gift from Dr. Ronald Schnaar, Johns Hopkins University). As expected, PPPP treatment showed very little effect on PNA binding, despite significant loss of the GC-enriched glycolipid Gb3 (CD77) **(Supplementary Figure 2C)**.

Next, to test whether CD45 is a direct target of ST3Gal1, we analyzed PNA binding by lectin blot of ST3Gal1OE and control Ramos B cell lysates. Whereas control B cell lysates showed robust binding to PNA, overexpression of ST3Gal1 in Ramos B cells resulted in significantly diminished PNA binding, particularly of a band co-migrating with CD45 **(Figure 3C)**. By contrast, the reverse binding pattern was observed in MAL-II lectin blots **(Figure 3D)**.

To extend our analysis to primary B cells, we also examined potential PNA binding proteins present in tonsillar naïve and GC B cells. To this end, we magnetically enriched tonsillar naïve and GC B cells to >85% purity by positive selection with anti-IgD and anti-CD77 antigens, respectively, and then performed SDS-PAGE and PNA lectin blotting. In a similar manner to our findings in B cell lines, PNA blot revealed an analogous ∼260 kDa band in GC lysates that was only faintly visible in naïve B cells **(Figure 3E)**. Sequential probing with anti-CD45 antibody in a separate fluorescence channel revealed considerable co-migration between the ∼260 kDa PNA-reactive band and CD45 **(Figure 3E)**. Interestingly, the ∼95 and ∼130 kDa PNA-reactive bands observed in Ramos and Raji B cells were not apparent in either primary GC B cells or SUDHL-4 cells **(Supplementary Figure 2B)**, suggesting that these proteins may bear PNA-reactive O-glycans in Burkitt lymphoma cells only. Thus, these data strongly suggest that CD45 is decorated by PNA-reactive O-glycans in GC B cells, and that the PNA-reactive T-antigen epitopes in these O-glycans are normally masked by ST3Gal1-mediated sialylation in naïve B cells.

Ectopic Expression of ST3Gal1 Toggles Reactivity Between Glycoform-Specific CD45 Antibodies in a Manner Not Reversible by Sialidase

Our data suggested that non-GC and GC B cells express different CD45 glycoforms containing sialylated vs. asialylated Core 1 epitopes. Previous reports examining CD45 monoclonal antibody (mAb) binding between disparate B cell subsets have identified two CD45 mAb clones, RA3-6B2 (more commonly referred to as "B220") and MEM55, that are sensitive to CD45 O-glycosylation and sialylation (41–44). (Note: While clone B220 is largely pan-reactive for B cells in mouse, it shows a more restricted binding within the human B cell pool). In particular, B220 binding has been shown to be enhanced by loss of sialic acid, whereas MEM55 binding has been shown to be absolutely dependent on sialic acid (41, 42, 44). We therefore reasoned that ST3Gal1-driven alterations to Core 1 sialylation on CD45 O-glycans might toggle expression of B220- and MEM55-reactive glycoforms.

To test this, we assayed binding of B220 and MEM55 CD45 mAbs to vector control and ST3Gal1OE Ramos B cells by flow cytometry. Strikingly, whereas control-transduced PNA^{hi} Ramos B cells displayed strong binding to B220 but not MEM55 mAb, overexpression of ST3Gal1 induced a reversal of mAb binding **(Figure 4A)**. Western blot analysis of MEM55 binding to vector control and ST3Gal1OE Ramos B cells showed similar results and confirmed the CD45 specificity of this mAb **(Figure 4B)**. Importantly, overexpression of ST3Gal1 did not affect binding of a glycosylation-insensitive CD45 mAb (HI30) **(Figures 4A,B)**.

We next sought to test whether altered binding between B220 and MEM55 mAbs in ST3Gal1OE B cells was absolutely dependent on sialic acid. To test this, we treated B cells with *A. ureafaciens* sialidase to reverse ST3Gal1-mediated sialylation at the cell surface. Paradoxically, whereas MEM55 binding was completely dependent on sialic acid, as expected, cleavage of sialic acids in ST3Gal1OE B cells failed to restore binding of B220 to control levels **(Figure 4C)**. Surprisingly, in contrast to previous reports, we did not observe enhanced binding of B220 in sialidase-treated Ramos vector control or ST3Gal1OE Ramos B cells. This result was unexpected and clearly did not fit a model in which sialylation was the only factor regulating binding between B220- and MEM55-reactive CD45 glycoforms. Thus, while ST3Gal1OE regulates binding of glycosylation-sensitive CD45 mAbs, loss of B220 binding could not be explained solely by the addition of a sialic acid moiety by ST3Gal1.

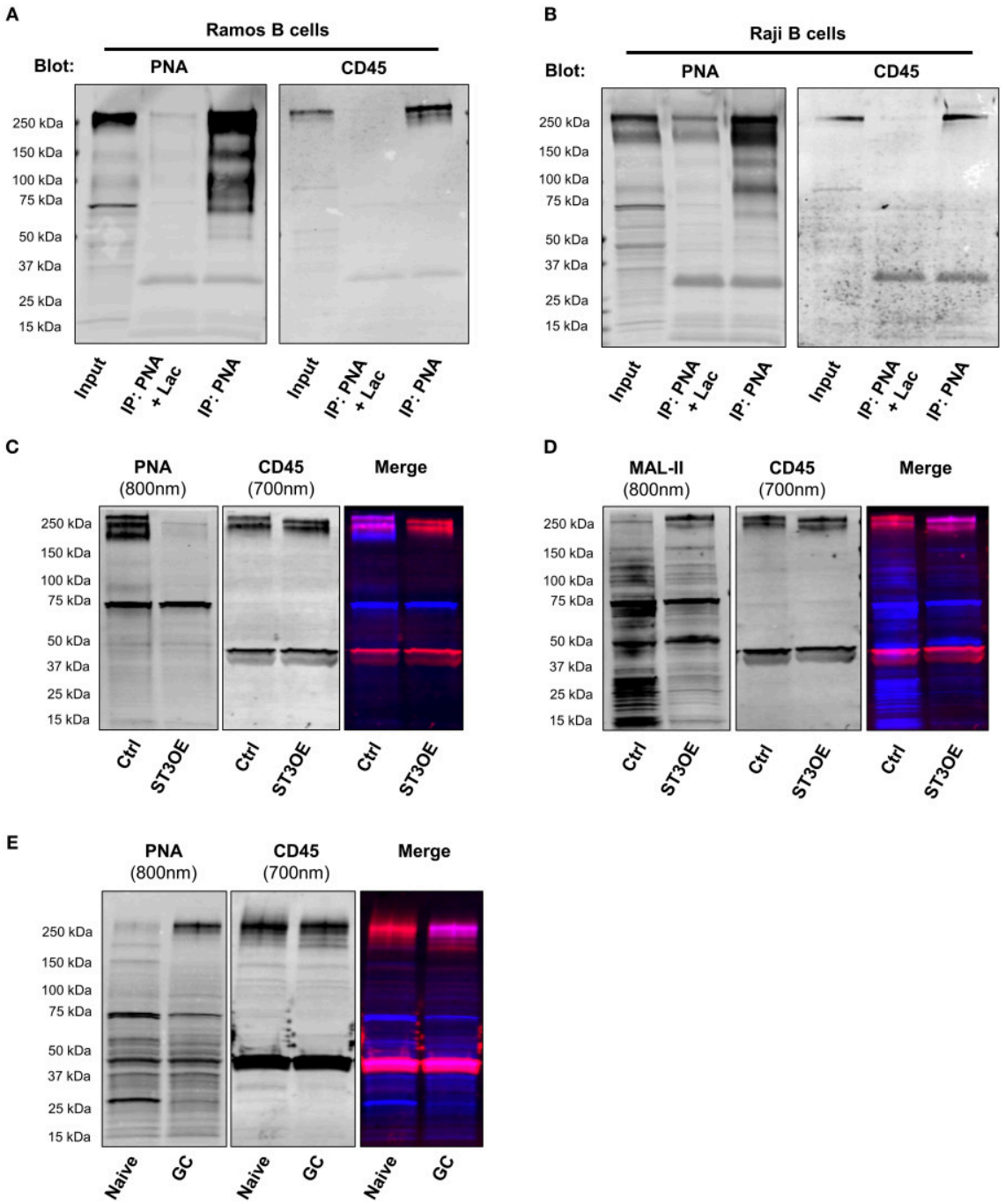

FIGURE 3 | CD45 is a major glycoprotein bearing PNA-reactive O-glycans on B cells. **(A)** Immunoprecipitation (IP) with PNA-agarose beads from lysates of a GC-derived Burkitt lymphoma B cell line (Ramos), followed by SDS-PAGE and immunoblot with either PNA (*left*) or CD45 antibody (*right*). As a negative control, IP was also performed in the presence of a sugar inhibitor, lactose (Lac; middle lane). **(B)** IP and immunoblot of PNA-binding proteins of lysates from a second GC-derived Burkitt lymphoma B cell line (Raji), as in **(A)**. **(C)** Western blot analysis of PNA binding to Ramos vector control and ST3Gal1OE lysates (*left;* 800 nm fluorescence channel*)* followed by immunoblot with CD45 antibody (*middle,* 700 nm fluorescence channel). *Right,* merged. **(D)** Western blot analysis of staining of Ramos vector control and ST3Gal1OE lysates with MAL-II lectin (*left;* 800 nm fluorescence channel*)* followed by CD45 antibody (*middle,* 700 nm fluorescence channel). *Right,* merged. **(E)** Immunoblot of lysates from magnetically-enriched naïve and GC B cells with PNA (*left;* 800 nm fluorescence channel*)* followed by CD45 antibody (*middle,* 700 nm fluorescence channel). *Right,* merged. Data from **(A,B)** are from one experiment each showing similar results using three different B cell lines (Ramos, Raji, and SUDHL4; see also **Supplementary Figure 2**). Data in **(C–E)** are representative of three independent experiments with distinct cell aliquots or tonsil specimens. Ctrl, vector control; ST3OE, ST3Gal1OE.

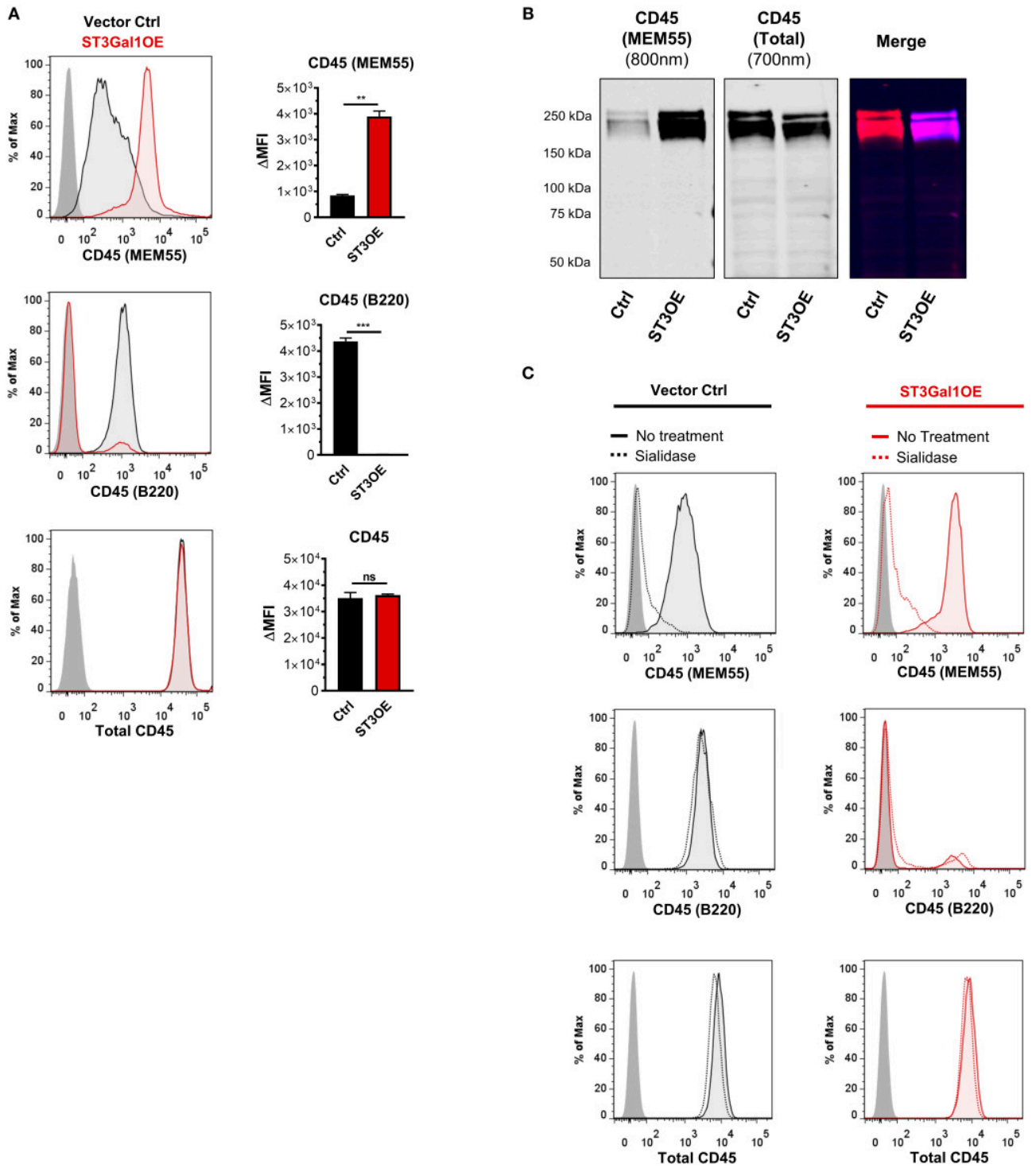

FIGURE 4 | Overexpression of ST3Gal1 in B cells modulates binding of glycosylation sensitive CD45 antibodies. **(A)** Representative histogram (*left*) and quantification (*right*) of binding of two glycosylation-sensitive CD45 antibodies (MEM55 and B220) and one glycosylation-insensitive antibody (total CD45, clone HI30) to vector control and ST3Gal1OE Ramos B cells by flow cytometry. **(B)** Western blot analysis of binding of CD45 mAb MEM55 (800 nm fluorescence channel) and CD45 mAb HI30 (700 nm fluorescence channel) to Ramos vector control and ST3Gal1OE lysates. **(C)** Representative histograms depicting flow cytometric analysis of CD45 antibody binding to vector control and ST3Gal1OE Ramos B cells, before and after treatment with *Arthrobacter ureafaciens* sialidase. Data in **(A,C)** are from three independent experiments with three biological replicates. Data in **(B,C)** are representative of similar results from three **(B)** or two **(C)** independent experiments. Statistics in **(A)** were calculated using Welch's unpaired, two-tailed *t*-test. Throughout, bars and error bars depict the mean and SEM, respectively. ns, not significant, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, ΔMFI, background subtracted geometric mean fluorescence intensity; Ctrl, vector control; ST3OE, ST3Gal1OE.

ST3Gal1 Overexpression Blocks Formation of Core 2 O-glycans *in vitro*

The ability of ectopically expressed ST3Gal1 to regulate B220- and MEM55-associated CD45 glycoform expression in a non-reversible manner was surprising, and did not comport with a purely sialic acid-dependent mechanism of action. Rather, the non-reversible nature of this effect implied other structural changes to CD45 O-glycans that could not be reversed at the cell surface by treatment with exogenous sialidase. Besides preferred sialylation of T-antigen, ST3Gal1 has also been reported to block Core 2 O-glycan formation by competing with the Core 2-branching enzyme GCNT1 for the T-antigen precursor **(Figure 1C)** (19–21). Additionally, it has previously been reported that B220 binding correlates with expression of Core 2 O-glycans (44). We therefore reasoned that loss of Core 2 O-glycans due to competition with GCNT1 may account for impaired B220 binding (and resulting gain of MEM55 binding) in ST3Gal1OE B cells. To test this, we analyzed binding of two Core 2 O-glycan-reactive reagents: *Solanum tuberosum* agglutinin (STA), which binds poly-LacNAc on Core 2 O-glycans (and also poly-LacNAc on N-glycans); and the CD43 mAb 1D4, which specifically binds CD43 modified with elongated Core 2 O-glycans (45). Consistent with a competitive role for ST3Gal1, binding of STA and 1D4 were both drastically reduced in ST3Gal1OE B cells **(Figures 5A,B)**, suggesting that high expression of ST3Gal1 is sufficient to block Core 2 O-glycan formation by GCNT1 in B cells.

To more precisely define the B cell glycan repertoire associated with B220 and MEM55 binding, we analyzed the N- and O-linked glycomes of untransduced, vector control transduced, and ST3Gal1OE Ramos B cells by mass spectrometry (MS). For analysis of O-glycomes, we utilized both conventional O-glycomics MS techniques as well a recently developed highly sensitive technique known as Cellular O-glycome Reporter Amplification (CORA) (46, 47). Using both approaches, we observed significant structural alterations between control and ST3Gal1OE B cells, including an expected increase in the ratio of sialylated to asialylated Core 1 O-glycan structures in ST3Gal1OE B cells **(Figure 5C**; **Supplementary Figure 3)**. However, consistent with a competitive relationship between ST3Gal1 and GCNT1 (at least when ST3Gal1 is expressed at very high levels), ST3Gal1OE B cells exhibited a striking reduction in Core 2 O-glycans compared to controls **(Figure 5C**; **Supplementary Figure 3)**. Intriguingly, we also noted I blood group antigen ("I-branch") expression on Core 2 O-glycan poly-LacNAcs in untransduced and vector control Ramos B cells that was particularly apparent in samples prepared by the CORA technique (**Figure 5C**). I-branch expression on N-glycan poly-LacNAcs has recently been shown by our laboratory to be a feature of GC B cells (48), and because Ramos B cells are believed to have arisen from a lymphoma of GC origin (Burkitt's lymphoma) (33), these data suggested that GC B cells may also express I-branches on poly-LacNAcs of O-glycans.

Subsequent analysis of the N-glycomes of untransduced, control, and ST3Gal1OE Ramos B cells revealed largely similar results in all three groups, suggesting that ST3Gal1 predominantly acts on O-glycans, as reported. Specifically, we found that all three groups uniformly expressed high levels of multi-antennary complex N-glycans modified with I-branched poly-LacNAcs **(Supplementary Figure 4)**, in a manner highly concordant with the N-glycomic phenotype of primary GC B cells (48). However, while the N-glycomes were mostly unperturbed by ST3Gal1, we did note slightly reduced quantities of I-branches on N-glycans in ST3Gal1OE B cells, possibly resulting from the increased metabolic demand imposed by ST3Gal1 overexpression (**Supplementary Figure 4**).

Taken together, these data suggest that B220 mAb binding to CD45 is associated with B cell expression of elongated Core 2 O-glycans, whereas MEM55 binding to CD45 is associated with B cell expression of predominantly truncated Core 1 O-glycans. Moreover, whereas untransduced and control-transduced Ramos B cells natively express $B220^{hi}$ and PNA^{hi} CD45 glycoforms, overexpression of ST3Gal1 *in vitro* is sufficient to convert CD45 to $MEM55^{hi}$ PNA^{lo} glycoforms.

B Cell Differentiation Is Associated With Progressive Loss of O-glycan Complexity

In previous studies, naïve and GC B cells were found to preferentially express B220-reactive glycoforms of CD45, whereas memory and plasmablast subsets preferentially expressed MEM55-reactive glycoforms (41–43). In the present study, we observed that B220 mAb binding corresponded with global expression of elongated Core 2 O-glycans, whereas MEM55 mAb binding corresponded with expression of sialylated, truncated O-glycans. Therefore, we reasoned that differences in binding between B220- and MEM55-reactive primary B cells may thus also correspond with differences in O-glycosylation.

To test this, we first examined binding of B220 and MEM55 to tonsillar and peripheral blood B cells by flow cytometry. As reported, B220 showed preferential binding to naïve and GC B cells (42, 43), whereas MEM55 exhibited superior binding to memory B cells and plasmablasts **(Figures 6A,B)** (41). We noted significant heterogeneity in binding, particularly among the memory B cell population. Therefore, to better dissect expression of B220 and MEM55 among B cell populations, we implemented a dual B220/MEM55 staining approach to assess whether expression of B220/MEM55 glycoforms was mutually exclusive or able to be co-expressed. Intriguingly, we found that while memory and plasmablasts showed clear bias toward MEM55 expression over B220 expression, a significant portion of memory and plasmablasts appeared to be in transition and exhibited binding to both antibodies **(Figure 6C).** Dual mAb binding was less apparent in peripheral blood B cells **(Figure 6D)**, suggesting that CD45 glycosylation may be actively remodeled during ongoing immune responses (such as in tonsil) but may be more stable in resting B cell populations (such as in peripheral blood of healthy donors). Whereas we did not note enhancement of B220 binding in sialidase-treated Ramos B cell lines **(Figure 4C)**, treatment of primary tonsillar B cells with *A. ureafaciens* sialidase did slightly enhance B220 binding; MEM55 binding, by contrast, was

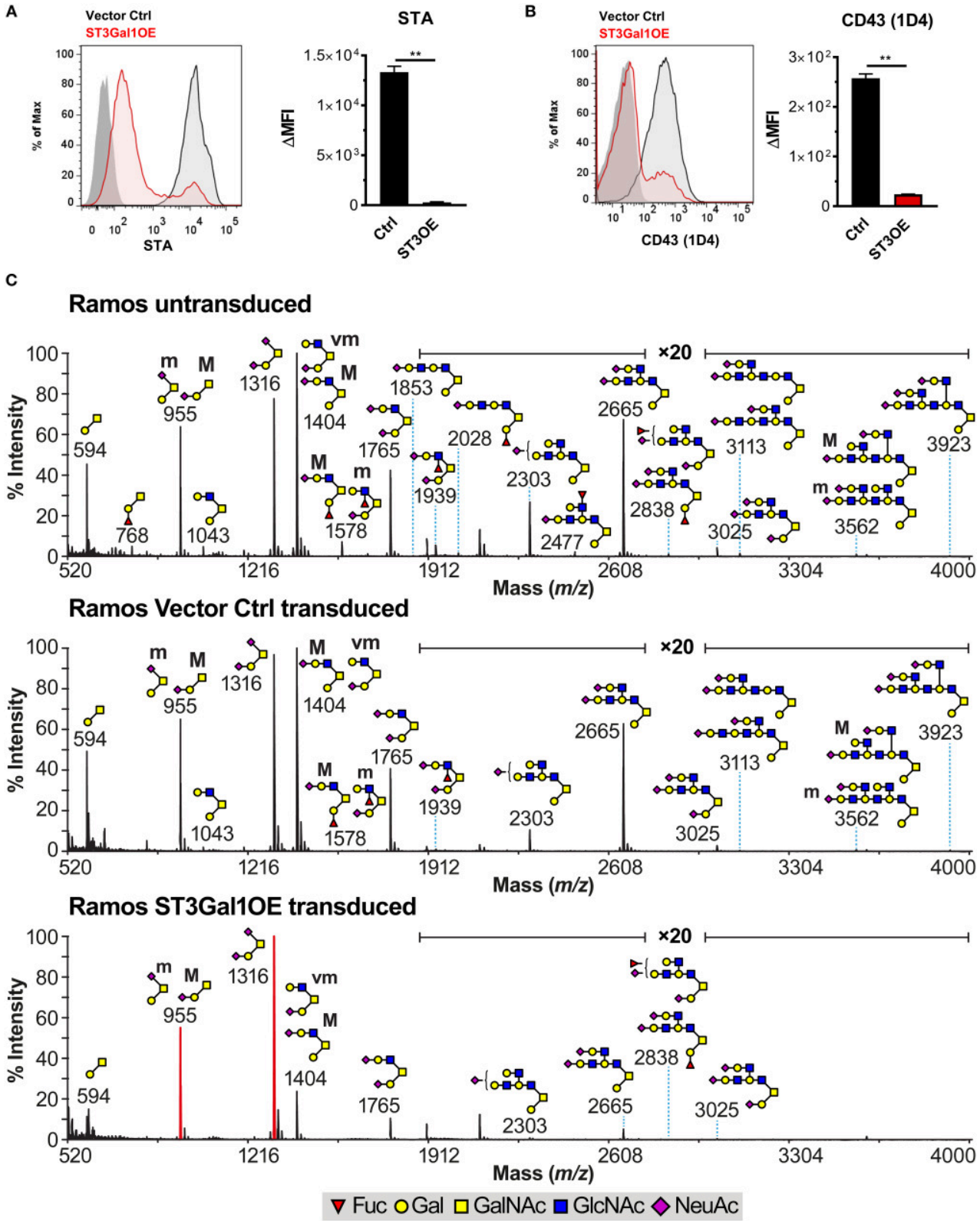

FIGURE 5 | Overexpression of ST3Gal1 truncates O-glycans in B cells. **(A)** Representative histograms (*left*) and quantification (*right*) of binding of the Core 2 poly-LacNAc binding lectin *Solanum tuberosum* agglutinin (STA) to vector control and ST3Gal1OE Ramos B cells by flow cytometry. **(B)** Representative histograms

(Continued)

FIGURE 5 | (*left*) and quantification (*right*) of binding of the CD43 mAb 1D4 (Core 2 O-glycan-specific glycoform) to vector control and ST3Gal1OE Ramos B cells by flow cytometry. **(C)** Cellular O-glycome Reporter/Analysis (CORA) of untransduced Ramos, Ramos vector control, and Ramos ST3Gal1OE B cells. Depicted are MALDI-TOF MS spectra of peracetylated Benzyl-α-GalNAc-linked O-glycans. Structures above a bracket have not been unequivocally defined. Indicated areas in the spectra have a 20-fold magnification. "M" and "m" designations indicate major and minor abundances, respectively. Cartoon structures were drawn according to http://www.functionalglycomics.org guidelines and are representative from repeat experiments on two different biological replicates. Structure assignments are based on composition, tandem mass spectrometry, and biosynthetic knowledge. Full methods for MS analysis can be found in Materials and Methods. Data depict results from three **(A,B)** or two **(C)** biological replicates. Statistics in **(A)** and **(B)** were calculated using Welch's unpaired, two-tailed *t*-test. Throughout, bars and error bars depict the mean and SEM, respectively. $^{**}p \leq 0.01$, ΔMFI, background subtracted geometric mean fluorescence intensity. Ctrl, vector control; ST3OE, ST3Gal1OE; Fuc, fucose; Man, mannose; Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid (sialic acid).

almost completely abolished by sialidase treatment, as previously reported **(Supplementary Figure 5B)** (41, 42, 44). Although GC B cells did display a slightly higher amount of CD45, significant differences in total CD45 levels were insufficient to explain differences in binding of either antibody between B cell subsets **(Supplementary Figure 5A**).

Based on our O-glycomic analysis of B cells expressing B220- and MEM55-reactive CD45 glycoforms *in vitro,* we hypothesized that transition of B220 to MEM55 binding in primary B cells would be associated with truncation of O-glycans. To test this, we analyzed binding of several O-glycan-reactive plant lectins, including STA, Jacalin, MAL-II, and *Helix pomatia* (HPA) lectins, to primary B cells. The binding preferences of each lectin is graphically depicted in **Figure 7A** (5). Consistent with our results *in vitro*, both tonsillar and peripheral blood B cells that acquired MEM55 reactivity showed significantly reduced binding to STA lectin, consistent with reduced expression of Core 2 O-glycan poly-LacNAcs **(Figures 7B–E)**. Consistent with a recent study by our laboratory, we also observed much higher binding of STA to GC B cells compared to naïve B cells, attributable to differences in I-branching of N-glycans between naïve and GC B cells rather than differences in Core 2 O-glycan expression (48). Besides STA, Jacalin lectin [which binds sialylated and asialylated T-antigen, but not in the presence of Core 2 O-glycans (49)] and HPA lectin [which binds terminal GalNAc, especially truncated O-glycans consisting of a single GalNAc moiety (5, 50)] both showed dramatically enhanced binding to more differentiated B cells compared to naïve and GC B cells, suggesting a progressive decrease in O-glycan length with differentiation (**Figure 7F).** MAL-II lectin also showed starkly increased binding to more differentiated B cells compared to naïve and GC B cells, possibly reflecting an inability of this lectin to bind sialylated T-antigen modified by Core 2 O-glycans **(Supplementary Figure 5C,** *top*). By contrast, the N-glycan specific lectin PHA-L did not show similar trends as Jacalin or HPA lectins, suggesting that increased binding of these lectins was not simply due to increased cell size **(Supplementary Figure 5C,** *bottom*). Thus, these data suggest that B cell differentiation to memory B cell and plasmablast fates is associated with a general loss in O-glycan complexity. Moreover, when considered together with our O-glycomic analysis of B220 and MEM55-reactive Ramos B cells, our data suggest that the B220 to MEM55 conversion reflects a transition in CD45 glycoform expression from elongated, Core 2-containing (naïve and GC) to truncated and sialylated (memory and plasmablast).

Reduced O-glycan Complexity With B Cell Differentiation Correlates With Decreased Expression of GCNT1

In our *in vitro* studies, we observed that overexpression of ST3Gal1 induced O-glycan truncation that converted B cells from B220- to MEM55-reactive **(Figures 4**, **5).** However, in primary cells, expression of ST3Gal1 did not readily correlate with O-glycan chain length (**Figures 1D**, **6**, **7**). Indeed, naïve and memory B cells possessed similar transcript levels of *ST3GAL1*, despite exhibiting significant differences in O-glycan length. Therefore, these data suggest that truncation of O-glycans by ST3Gal1 may be highly dependent on level of expression, and (at least in B cells) may inhibit formation of Core 2 O-glycans only when expressed to a very high degree. In this regard, we reasoned that, in parallel with ST3Gal1-mediated sialylation of T-antigen, a second mechanism may be operating to regulate differences in O-glycan chain length with B cell differentiation.

One possible explanation for the observed loss in O-glycan complexity with B cell differentiation is downregulation of the Core 2 branching enzyme GCNT1. As described earlier, GCNT1 initiates formation of Core 2 poly-LacNAc chains by transferring a GlcNAc moiety to a Core 1 O-glycan precursor (51, 52). Indeed, analysis of *GCNT1* expression in a publicly available dataset (53) revealed that naïve B cells, which are $B220^{hi}$ **(Figure 6)**, expressed the highest mean transcript levels of *GCNT1* of all hematopoietic subsets, whereas memory B cells, which are $B220^{lo}$ and $MEM55^{hi}$ **(Figure 6)**, possessed among the lowest *GCNT1* transcript levels **(Figure 8A).** Subsequent sorting and qRT-PCR analysis of *GCNT1* expression in primary naïve, GC, memory, and plasmablast B cells confirmed that *GCNT1* was robustly expressed in naïve B cells but steadily declined with B cell differentiation **(Figure 8B,** *left*). Similar findings were observed with naïve and memory B cells from peripheral blood **(Figure 8B**, *right*). Therefore, these data strongly suggest that Core 2 O-glycans are expressed by less differentiated naïve and GC B cells due to high levels of GCNT1, but are strongly downregulated in more differentiated B cell subsets, including memory B cells and plasmablasts, due to diminished GCNT1 expression.

Taken together, our data support a model in which two O-glycosylation enzymes, ST3Gal1 and GCNT1, separately regulate two glycosylation features during B cell differentiation: α2,3-sialylation of Core 1 O-glycans and formation/extension of Core 2 O-glycans, respectively. Whereas naïve B cells exhibit sialylated, elongated O-glycans, our data suggest that these O-glycans become transiently unsialylated and then progressively

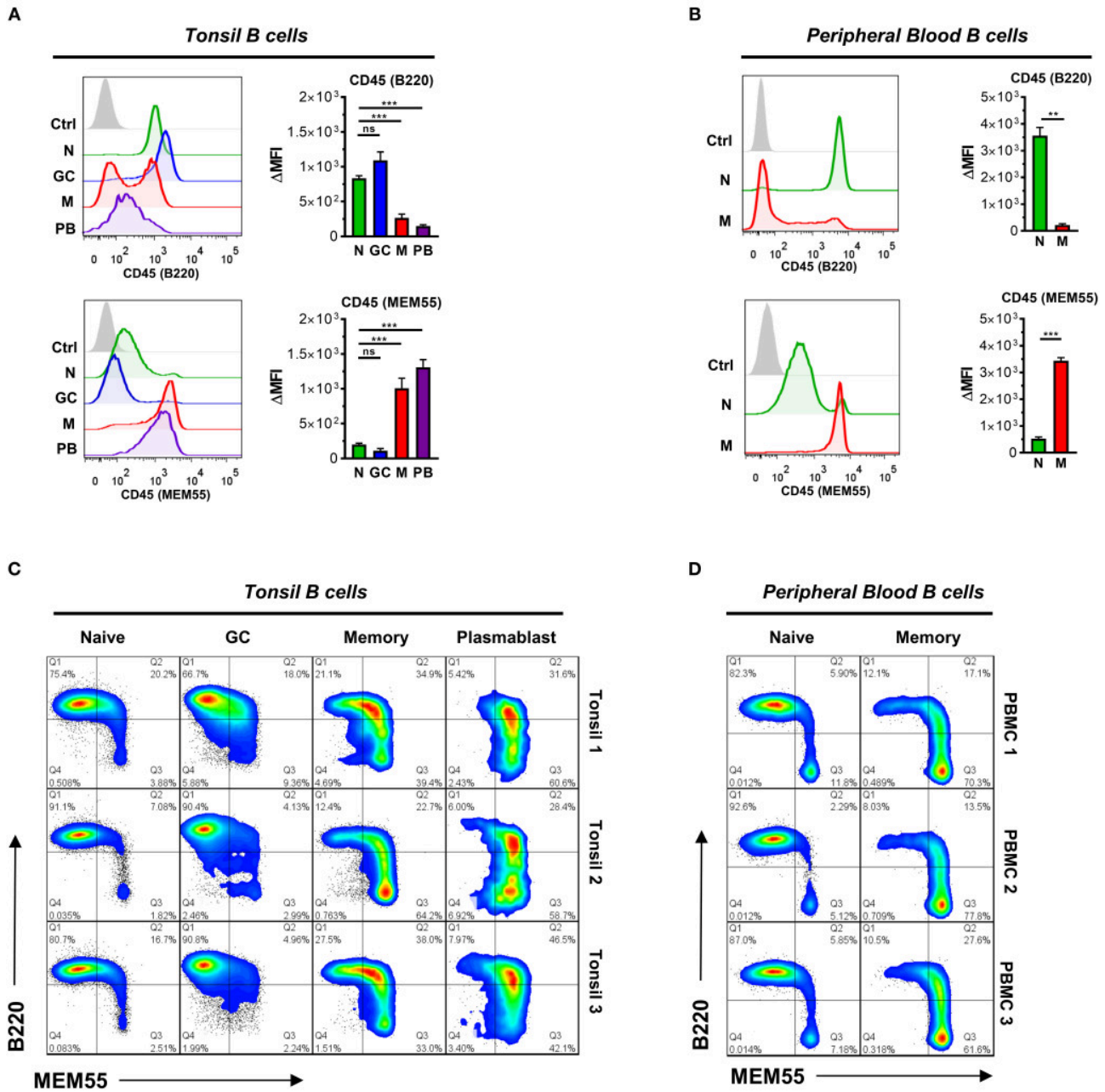

FIGURE 6 | B cells transition from B220- to MEM55-reactive CD45 glycoforms during B cell differentiation. **(A)** Representative histograms (*left*) and quantification (*right*) of binding of glycosylation-sensitive CD45 antibodies B220 and MEM55 to the indicated tonsillar B cell subset. Subsets are gated as in **Figure 1A**. **(B)** Representative histograms (*left*) and quantification (*right*) of binding of B220 and MEM55 to peripheral blood naïve (CD19+ IgD+ CD27-) and memory (CD19+ IgD- CD27+) B cells. **(C)** Pseudocolored smoothed dot plots depicting binding of MEM55 and B220 to tonsillar naïve, GC, memory, and plasmablast B cells in three distinct tonsil specimens. **(D)** Pseudocolored smoothed dot plots depicting binding of MEM55 and B220 to peripheral blood naïve and memory B cells in three distinct peripheral blood donor specimens. For **(A,B)**, $n = 5$ and $n = 3$, respectively. For **(C,D)**, data depict three separate tonsil and peripheral blood specimens, respectively. Statistics were calculated using a Kruskal–Wallis test with Dunn's multiple comparisons test **(A)** or an unpaired, two-tailed Welch's *t*-test **(B)**. Throughout, bars and error bars depict the mean and SEM, respectively. ns, not significant, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. ΔMFI, background subtracted geometric mean fluorescence intensity.

shortened with GC- and post-GC differentiation, respectively. Additionally, in combination with results examining binding of CD45 mAbs B220 and MEM55, we further propose that these global alterations in glycosylation drive expression of distinct CD45 glycoforms at each stage of B cell differentiation (summarized in **Figure 9**).

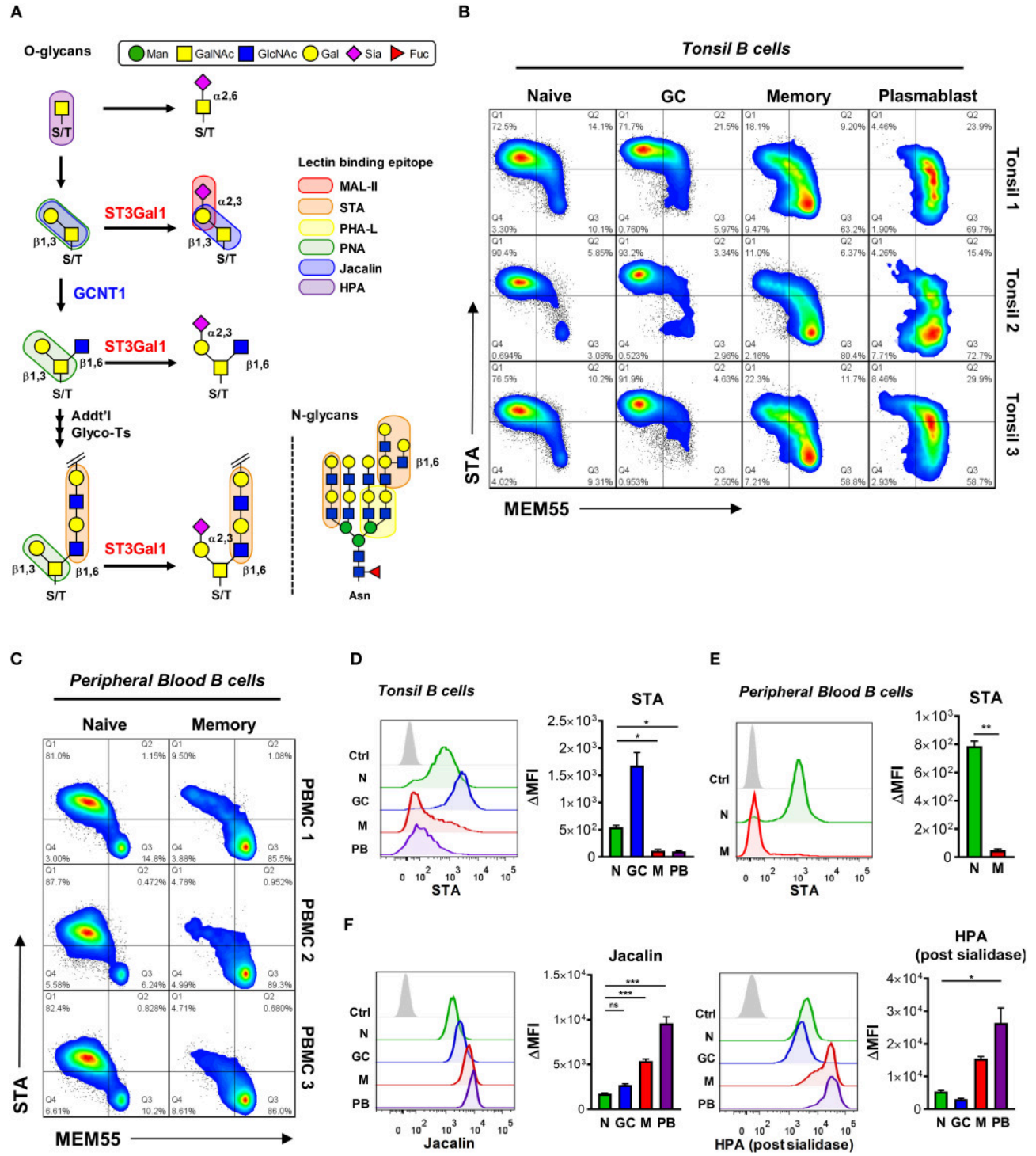

FIGURE 7 | Primary B cell conversion from B220- to MEM55-reactive CD45 glycoforms is associated with truncation of O-glycans. **(A)** Schematic of O-glycan biosynthesis and corresponding plant lectin binding epitopes. A representative N-glycan is also shown to illustrate N-glycan specificities, where applicable. **(B)** Pseudocolored dot plots depicting dual staining of MEM55 mAb and STA plant lectin in tonsillar B cell subsets from three distinct tonsil specimens. **(C)** Pseudocolored dot plots depicting dual staining of MEM55 mAb and STA plant lectin in peripheral blood naïve (CD19+ IgD+ CD27−) and memory (CD19+ IgD− CD27+) B cell subsets from three distinct healthy donors. **(D)** Representative histograms *(left)* and quantification *(right)* of binding of STA plant lectin to tonsillar B cells by flow cytometry. **(E)** Representative histograms *(left)* and quantification *(right)* of binding of STA lectin to peripheral blood naïve and memory B cells by flow cytometry. **(F)** Representative histograms *(left)* and quantification *(right)* of binding of Jacalin and HPA plant lectins to primary tonsillar B cells by flow cytometry. For HPA staining,

(Continued)

FIGURE 7 | cells were first treated with Arthrobacter ureafaciens sialidase. Data depict three **(B,C,E)**, eight **(D)**, nine (**F**, Jacalin), or six (**F**, HPA) distinct tonsil specimens. Statistics were calculated using a Kruskal–Wallis test with Dunn's multiple comparisons test **(D,F)** or Welch's unpaired two-tailed T-test (E). Throughout, bars and error bars depict the mean and SEM, respectively. ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. MFI, background subtracted geometric mean fluorescence intensity; N, naïve; GC, germinal center; M, memory; PB, plasmablast; Man, mannose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Gal, galactose; Sia, sialic acid; Fuc, fucose.

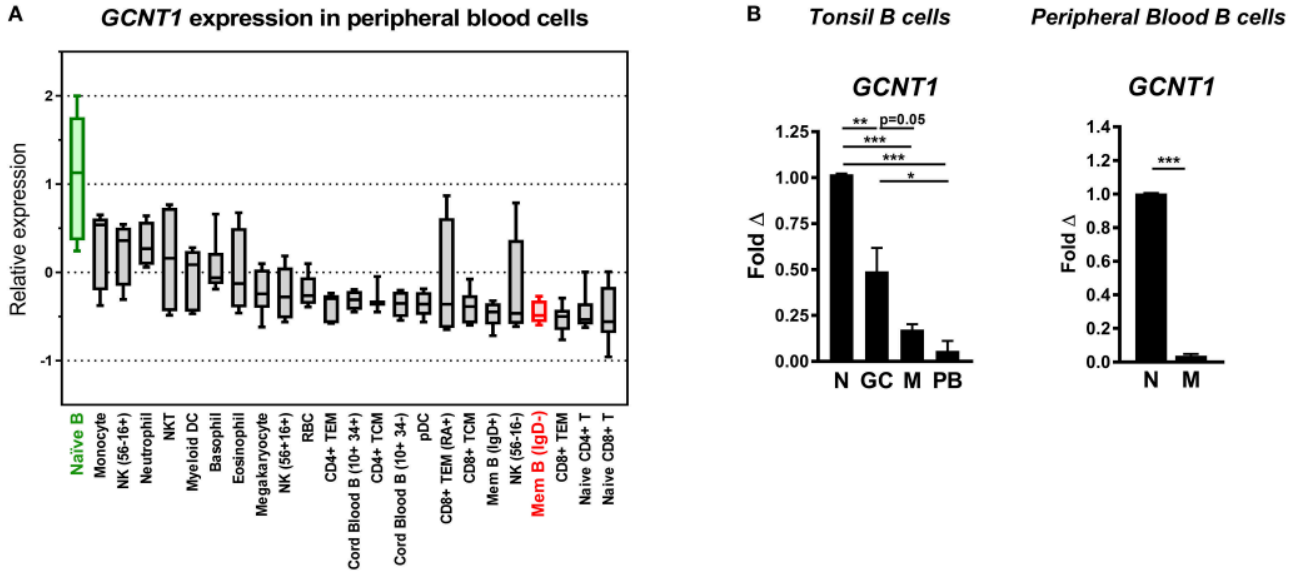

FIGURE 8 | Loss of O-glycan complexity with differentiation is associated with downregulation of the Core 2 branching enzyme GCNT1. **(A)** Relative mean *GCNT1* expression in peripheral blood cells, analyzed from the publicly available dataset *GSE24759*. Box and whiskers depict minimum value, maximum value, median, and interquartile range. **(B)** qRT-PCR analysis of transcript levels of *GCNT1* in primary tonsil and peripheral blood B cell subsets. Data were normalized to the housekeeping gene *VCP* and are depicted relative to naïve B cells. For **(B)**, $n = 3$ distinct tonsil or peripheral blood specimens. Bars and error bars in **(B)** depict mean and SEM. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. N, naïve; GC, germinal center; M, memory; PB, plasmablast.

DISCUSSION

The PNA binding phenotype of GC B cells was first described by Rose and colleagues nearly 40 years ago (2, 3). Surprisingly, however, the glycobiological mechanisms driving PNA reactivity, and its physiological significance, have remained unclear. Here, we investigated the glycobiological basis for PNA reactivity of GC B cells. We found that the most plausible explanation for PNA-reactivity of GC B is downregulation of ST3Gal1, a sialyltransferase with a reported preference for Core 1 O-glycans (5, 12, 13, 19, 21, 28, 29). Overexpression of ST3Gal1 was sufficient to ablate PNA binding to a GC-derived cell line, Ramos. Functionally, CD45, a central regulator of BCR signaling, emerged as a plausible scaffold for PNA-reactive glycans in primary GC B cells. We further found that ST3Gal1 overexpression unexpectedly toggled reactivity between two glycosylation-sensitive CD45 mAbs, B220 and MEM55, by truncating Core 2 O-glycans. Analysis of B220 and MEM55 binding in primary B cells, in conjugation with several O-glycan binding plant lectins, revealed a gradual transition during B cell differentiation from expression of extended Core 2 poly-LacNAc-containing O-glycans to shorter, sialylated O-glycans. In contrast to *in vitro* studies, ST3Gal1 in primary B cells did not readily correlate with O-glycan length but rather expression of the Core 2 branching enzyme GCNT1. Therefore, in the course of investigating PNA reactivity of GC B cells, we uncovered two distinct differentiation-associated alterations in O-glycosylation, both of which occur at least in part on the glycoprotein CD45 and are regulated in parallel by the O-glycosylation enzymes ST3Gal1 and GCNT1.

Our finding that ST3Gal1 modulates the PNA phenotype of GC B cells is not entirely unexpected, given similar reports for ST3Gal1 in modulating T-antigen expression (and related O-glycans) in thymocytes and mature T cells (2, 9–16). However, our study now provides evidence for a similar ST3Gal1-mediated mechanism in GC B cells that was perhaps suspected, but to our knowledge, not rigorously investigated. In our analysis of PNA binding, we also observed that plasmablasts were also strongly reactive with PNA and similarly downregulated ST3Gal1, suggesting that exposure of PNA-reactive O-glycans is a general feature of activated B cells and not part of a GC-specific program. However, somewhat puzzlingly, plasmablasts strongly bind both PNA (reactive with glycans containing asialylated T-antigen) and MAL-II lectins (reactive with sialylated T-antigen) compared to naïve B cells. This disparity might be explained if MAL-II lectin binding is inhibited by the presence of Core 2

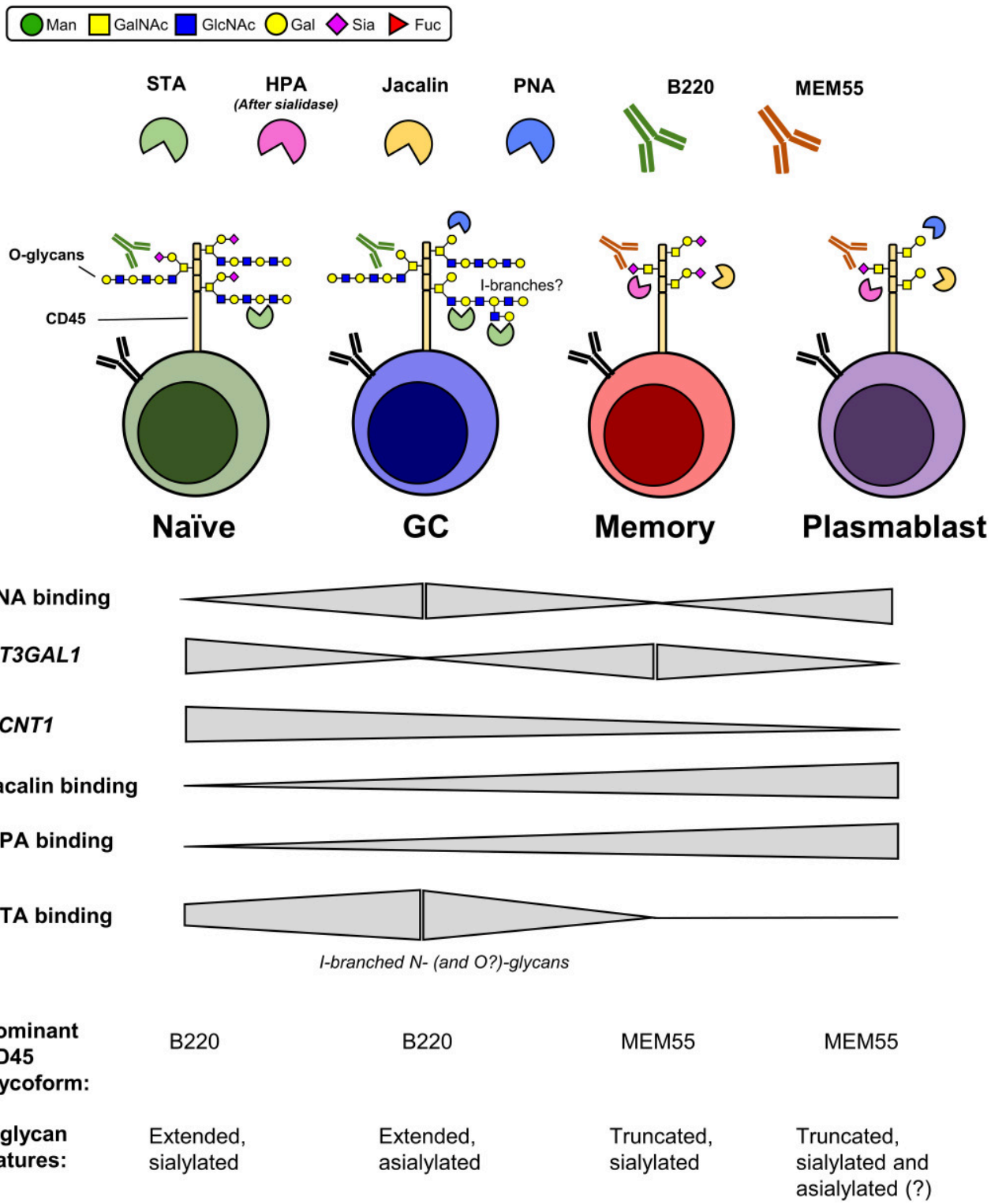

FIGURE 9 | Proposed O-glycosylation phenotype of human B cell subsets and expression of associated CD45 glycoforms. Summary of relative plant lectin binding, glycosyltransferase gene expression, and expression of CD45 mAb-reactive glycoforms for each B cell subset, as determined in the present study. Glycan features that are inconclusive are marked with a "?". Man, mannose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Gal, galactose; Sia, sialic acid; Fuc, fucose.

O-glycosylation. Based on these data, the precise sialylation status of Core 1 O-glycans in plasmablasts is difficult to define.

In addition to PNA ligand exposure on GC B cells, several glycosylation-sensitive CD45 mAbs had previously been reported to differentially bind disparate B cell subsets (41–43). These data suggested that CD45 may transition through several glycoforms during B cell differentiation. However, the nature of these glycoforms have remained largely undefined, because the glycans associated with antibody binding had not been extensively analyzed. Our O-glycomic analysis of B220-reactive and MEM55-reactive B cells suggests that naïve and GC B cells (B220-reactive) express bulky glycoforms of CD45 containing Core 2 O-glycan poly-LacNAcs, whereas memory and plasmablast B cells (MEM55-reactive) express shorter and more sialylated glycoforms of CD45. Expression of Core 2 O-glycans primarily by naïve and GC B cells is supported by the higher levels of GCNT1 expressed by these subsets compared to memory and plasmablasts. Notably, this model is also supported by a recent O-glycomic study by Macauley and colleagues, in which the authors were able to detect Core 2 poly-LacNAcs on bulk tonsil B cells, which are composed primarily of naïve and GC B cells (54). The expression of Core 2 O-glycans and high levels of GCNT1 by naïve B cells is surprising and is opposite of from the expression pattern of T cells. Whereas naïve T cells express shorter O-glycans and upregulate Core 2-containing O-glycans with activation, B cells appear to exhibit the reverse behavior (9, 25). The functional significance of this difference will be an important area of future investigation.

An interesting observation arising from the disparate sialylation and O-glycosylation status of naïve, GC, memory, and plasmablasts is that each subset expresses a distinct CD45 glycoform at each stage of B cell differentiation (**Figure 9**). What might be the physiological significance of these distinct glycoforms? Ostensibly, modular glycosylation between B cell subsets might serve as an analogous mechanism to CD45 isoform switching on human T cells (25). In a study by Xu and Weiss, sialylation and O-glycosylation of CD45 were found to inhibit homodimerization-induced inactivation, thereby enhancing CD45 activity (55). By this model, differential sialylation and O-glycosylation of CD45 may serve to intrinsically tune CD45 signaling at different stages of B cell maturation. Besides intrinsic CD45 signaling, differential CD45 O-glycosylation may regulate interaction with endogenous lectins. Indeed, in studies using DLBCL B cells, Clark and colleagues found that Core 2 O-glycans, regulated by GCNT1, were required for optimal CD45 binding of galectin-3, which upon binding dampened CD45 phosphatase activity and promoted B cell survival (56). Moreover, studies assessing N-glycosylation of CD45 have also noted critical roles for differential N-glycosylation between B cell subsets in the regulation of galectin binding. For instance, our lab has recently reported that differential I-branching of N-glycans between resting and GC B cells is a major regulator of binding of the inhibitory lectin galectin-9, which in B cells dampens BCR calcium signaling (48). Besides galectins, the sialic acid-binding inhibitory receptor CD22 has also recently been shown to be regulated by glycan-dependent interactions with CD45 (57, 58), as well as altered GlcNAc sulfation between naïve/memory and GC B cells (54). Thus, alterations in O-glycosylation may serve analogous functions in the regulation of lectin binding in *cis* or in *trans.* Finally, alterations in glycosylation on CD45 may also serve to regulate intercellular communication, either through intrinsic properties (such as the negative charge of sialic acid) or through lectin-mediated interactions in *trans*. The expression of unique glycoforms of CD45 in different B cell subsets may therefore serve not only to differentially regulate CD45 activity, but also to dictate the strength and/or repertoire of lectin binding in *cis* and *trans*.

Taken together, our data suggest that B cells undergo extensive alterations in O-glycosylation with B cell differentiation that drive expression of distinct CD45 glycoforms. These findings add to a growing body of evidence indicating that lymphocytes undergo glycan remodeling in order to acquire or discard specific functionality at discrete stages of differentiation.

MATERIALS AND METHODS

Contact for Reagent and Resource Sharing

Requests for reagents or additional information should be directed to corresponding author, Charles J. Dimitroff (cdimitroff@bwh.harvard.edu).

Oligonucleotide Sequences

Primers and other oligonucleotide sequences used in this study can be found in **Supplementary Table 1**.

Antibodies and Reagents

A full list of antibodies and reagents used in this study can be found in **Supplementary Table 2**.

Cell Lines

Ramos and Raji cells were generously provided by Dr. Shiv Pillai (Ragon Institute of MGH, MIT, and Harvard). SUDHL-4 B cells were a gracious gift from Dr. Alan Epstein (USC Keck School of Medicine). All B cell lines were maintained at 0.5×10^6-2.0×10^6 cells mL^{-1} in complete RPMI medium [RPMI 1640 + 10% (v/v) FBS + 25 mM HEPES + 1% (v/v) Penicillin/Streptomycin]. Media was renewed every 2–3 days (Ramos, Raji) or every 3–5 days (SUDHL-4). For each cell line, aliquots were frozen in cell culture media supplemented with 10% FBS and stored in the vapor phase of a liquid nitrogen freezer for later use.

To generate ST3Gal1 overexpression Ramos B cells, human ST3Gal1 cDNA (Origene #SC111017) was amplified by PCR and then subcloned into pLVX-EF1α-IRES-ZsGreen1 (Clontech #631982), a bicistronic lentiviral expression vector allowing for simultaneous co-expression of ST3Gal1 and ZsGreen1 from a single mRNA transcript. The ST3Gal1 insert was sequenced and was found to match the NCBI reference sequence NM_173344.2 for ST3Gal1 transcript variant 2, except for one synonymous mutation at base 261 (C->T) of the coding sequence. Lentivirus

containing the ST3Gal1 construct was produced by co-transfection of HEK293T cells with the helper plasmids pMD2.G-VSV-G and psPAX2-Δ8.9 using Lipofectamine 2000 (Thermo #11668-027). Forty-eight hours later, 1×10^6 Ramos B cells were resuspended in 1 mL of viral supernatant, plated in 24 well flat-bottom tissue culture plates, and spinfected at 1,000 × g for 90 min at room temperature, followed by culture in fresh media for 24 h. Successfully transduced cells were sorted to >99% purity on ZsGreen1-fluorescent cells by flow cytometric sorting on a BD FACSAria at the Harvard Division of Immunology's Flow Cytometry Core. Sorted ST3Gal1-expressing (ZsGreen1+) cells were expanded and frozen for subsequent use.

Expression Array Analysis

Raw Affymetrix HG-U133plus2 CEL file data for sorted tonsillar B cell subsets were downloaded from NCBI GEO (GSE12195) and dChip (59) was used to normalize probe hybridization intensities across arrays, followed by extracting gene signals using custom probe set information; HG-U133plus2_customV10.CDF (60). Resulting signal intensities were then analyzed across sample groups to calculate average fold intensity differences and significance using unpaired two-tailed *t*-test analyses with resultant ranking for FDR *q*-values using Morpheus software (Broad Institute, https://software.broadinstitute.org/morpheus). For analysis of peripheral blood cell subsets, gene expression data was directly downloaded from the Differentiation Map Portal (Broad Institute, http://www.broadinstitute.org/dmap/; also available from GEO database, GSE24759) (53).

Tonsil and Blood Processing, Cryopreservation, and Thawing

Discarded, anonymized tonsil specimens were obtained from routine tonsillectomies performed at Children's Hospital Boston, in accordance with the Partners Institutional Review Board (IRB), which deemed the research as not meeting the definition of human subjects research. Tonsils were briefly (<1 h) stored on ice in isotonic saline solution before being transferred to Hank's Balanced Salt Solution (HBSS) for processing. Tonsils were subsequently minced in HBSS, mashed with a 5 mL syringe plunger into a 70 um nylon mesh, and removed to a conical tube stored on ice. Mononuclear cells were isolated from the interface following density gradient centrifugation through Histopaque 1077 (Sigma) at 1,000 × g in an Allegra X-12R centrifuge, without the brake. The cells were then washed 3x with cold HBSS and frozen in 90% FBS/10% DMSO freezing media in a Mr. Frosty at −80°C, before being transferred to liquid nitrogen storage. As needed, cryopreserved tonsil mononuclear cells were rapidly thawed by standard procedures. Viability was routinely >80%.

Peripheral blood mononuclear cells were isolated from de-identified leukopacks acquired from the Children's Hospital Boston Blood Donor center. Buffy coats were removed following density gradient centrifugation, washed, and frozen for later use, as described above for tonsil.

Flow Cytometry Sorting for Gene Expression Analysis

Tonsil mononuclear cells were thawed, washed, and counted as described above. To exclude apoptotic and necrotic cells, cells were first stained with Zombie NIR fixable viability dye (Biolegend) for 15 min at room temperature in PBS. Cells were then washed and stained with a cocktail of surface stain antibodies, including anti-IgD, anti-CD27, anti-CD38, anti-CD19, anti-CD3, and anti-CD14 (all from Biolegend), and incubated for 45 min on ice. Subsequently, cells were washed two times, passed through a 35 μm nylon mesh and sorted on a BD FACS Aria II at the Harvard Division of Immunology Flow Cytometry Core. After electronically gating on lymphocytes by forward and side scatter properties and eliminating cell doublets, B cells was gated as in **Figure 1A**. For peripheral blood B cells, naïve B cells were gated as follows: CD19+ CD3- CD14- IgD+ CD27- cells (naïve), CD19+ CD3- CD14- IgD- CD27+ (memory). In both cases, the CD27 gate was set using a fluorescence minus one (FMO) gating control. Sorted cells were pelleted, washed 2x with PBS, then lysed for RNA extraction in Buffer RLT (Qiagen).

Quantitative Real-Time Reverse Transcription PCR (qRT-PCR)

For gene expression analysis of tonsil or peripheral blood B cells by qRT-PCR, B cell subsets were flow cytometrically sorted to >95% purity, washed, and lysed in Buffer RLT (as described above) before RNA isolation using the RNeasy Mini (naïve, GC, memory) or Micro (plasmablast) isolation kit (Qiagen), according to the manufacturer's instructions. For cell lines, cells were isolated during log phase of growth. RNA concentration and purity were checked using a BioDrop μLITE, and 0.25 μg RNA per reaction was subsequently converted to cDNA using the SuperScript VILO cDNA synthesis kit (ThermoFisher), per the manufacturer's instructions. Samples were assayed using Fast SYBR Green Master Mix (Applied Biosystems), and kinetic PCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems). Samples were assayed in triplicate. Data was normalized to the housekeeping gene Valosin-containing protein (*VCP*). Relative transcript levels were analyzed using the $2^{(-\Delta\Delta Ct)}$ method (61). Primer sequences used can be found in **Supplementary Table 1**.

Magnetic Enrichment of Naive B Cells and GC B Cells for Western Blot

Tonsil mononuclear cells were labeled for 10 min on ice with anti-CD77-FITC and anti-IgD-biotin antibody (Biolegend) in MACS buffer (PBS + 0.5% BSA + 2 mM EDTA), followed by washing and labeling in anti-biotin microbeads (Miltenyi) for 20 min on ice, per manufacturer's instructions. Cells were washed, resuspended in MACS buffer and fractionated on LS columns to collect labeled population (naïve-enriched). The unlabeled population was subsequently labeled with anti-FITC microbeads (Miltenyi) for 20 min on ice, washed, and loaded onto LS columns to isolate the GC-enriched fraction. Post-sort B cell purity was confirmed on a FACS Canto I using the flow

cytometry staining procedures described above, and were at least 85% pure, but typically >90%. Naïve- and GC-enriched fractions were washed 3x in PBS before lysis in 2% NP-40 buffer / Buffer A (150 mM NaCl, 0.5 mM Tris, 1 mM EDTA) supplemented with protease/phosphatase inhibitors (Protease/Phosphatase Inhibitor Mini tablets, Thermo).

Plant Lectin and CD45 Glycoform Staining by Flow Cytometry

Tonsil mononuclear cells were thawed, washed, and counted as described above. For cell lines, cells were grown as described above and harvested in log phase of growth. Fresh media was consistently added 1 day before the experiment to ensure adequate nutrients for proper glycosylation. Dead cells were stained by Zombie NIR fixable viability dye (Biolegend) in PBS for 15 min at room temperature (for tonsil cells only), followed by washing and staining in one of several biotinylated or FITC-conjugated plant lectins: *Arachis hypogaea* (peanut) agglutinin (PNA, Sigma), *Maackia amurensis* lectin-II (MAL-II), *Solanum tuberosum* agglutinin (STA), Jacalin lectin, *Helix pomatia* agglutinin (HPA), or *Phaselous vulgaris* leucoagglutinin (PHA-L) (all from Vector) for 45 min on ice in 1% bovine serum albumin (BSA) in PBS. For biotinylated lectins, cells were washed and subsequently incubated in Streptavidin-fluorophore conjugate for 30 min in 1% BSA in PBS on ice. Alternatively, cells were incubated in biotinylated anti-CD45 antibody (B220 (BD) or MEM55 clone (Thermo) followed by detection with Streptavidin-fluorophore conjugate, or directly assayed with FITC-conjugated MEM55 (Thermo). For analysis of total CD45 levels, APC-conjugated CD45 mAb (HI30 clone, Biolegend) was used. For tonsil cells, cells were subsequently washed and stained using a panel of surface stain lineage antibodies, including anti-IgD-FITC (or APC), anti-CD19-PerCP, anti-CD3-APC/Cy7, anti-CD14-APC/Cy7, anti-CD27-PE/Cy7, and anti-CD38-PE (all from Biolegend). For dual MEM55 and B220 or MEM55 and STA stains, cells were jointly incubated with MEM55-FITC (Thermo) and biotinylated B220 (BD) or STA (Vector), followed by detection with Streptavidin-APC conjugate, and surface stain using anti-IgD-PE, anti-CD19-APC Fire 750, anti-CD27-PE/Cy7, anti-CD38-PerCP/Cy5.5 (all from Biolegend). After staining, cells were immediately acquired on a BD FACSCanto I. Analysis was performed using FlowJo software (TreeStar). Cells were gated electronically for lymphocytes and doublet discrimination, followed by gating on B cells as shown in **Figure 1A**. For CD27 stains, a PE/Cy7 FMO gating control was employed. The geometric mean was used for calculation of mean fluorescence intensities (MFI) unless otherwise indicated.

Near-Infrared Western and Lectin Blots

For sample preparation for lectin and protein immunoblots, B cell lines or magnetically-enriched primary naïve/GC B cells were washed 3x before lysis in ice-cold 2% NP-40 buffer/Buffer A (150 mM NaCl, 0.5 mM Tris, 1 mM EDTA) supplemented with protease/phosphatase inhibitors (Protease/Phosphatase Inhibitor Mini tablets, Thermo). Debris was pelleted by centrifugation and samples were quantitated by BCA assay (Thermo) to ensure equal loading. Lysates were boiled for 10 min in Laemlli reducing sample buffer. Equivalent amounts of lysate (10–30 μg per lane) were resolved on 4–20% Criterion Tris-HCl polyacrylamide gels (BioRad), followed by transfer to 0.2 μm pore-size nitrocellulose membranes for immunoblot. Membrane blocking was performed in Odyssey Blocking buffer (Li-cor) for at least 1 h (or overnight) at room temperature. For primary antibody or lectin stain, blots were incubated in antibody / lectin stain overnight at 4°C. Staining reagents were diluted in Tris-buffered saline (pH 7.4) + Tween 20 (0.1%), diluted 1:1 in Odyssey Blocking buffer (Li-Cor). Primary reagents were detected using anti-mouse IgG (H+L), anti-rabbit IgG (H+L), or Streptavidin IR-Dye 680 or 800 CW conjugates. Blots were scanned and recorded using an Odyssey CLx Near-infrared Imaging System (Li-Cor). For dual stains, the blot was first probed and recorded with the lectin (PNA or MAL-II) in the 800 nm channel, and then subsequently re-probed and scanned with anti-CD45 antibody (Biolegend) in the 680 nm channel.

PNA Immunoprecipitation

For PNA immunoprecipitation experiments, 30 μL of PNA-agarose beads (4.4 mg/mL PNA) were pre-blocked in 0.1% BSA, washed, and mixed with 100 μg Raji, Ramos, or SUDHL-4 lysate generated as described above. PNA-reactive glycoprotein-bound beads were immunoprecipitated overnight at 4°C, on a rotator, washed 3x with lysis buffer, then eluted by boiling in Laemmli reducing sample buffer. As a control, where indicated, immunoprecipitations were performed in the presence of 0.1 M lactose. Equal volumes of immunoprecipitated material were subsequently subjected to SDS-PAGE and Western/lectin blot with either PNA-biotin (Sigma) or mouse anti-human CD45 (Biolegend), followed by fluorophore conjugated secondary reagents (Li-Cor).

Enzymatic Removal of Cell Surface Sialic Acids

Cleavage of cell surface sialic acids was performed on live tonsil mononuclear cells or B cell lines using *A. ureafaciens* sialidase (Roche, [final] = 125 mU mL^{-1}) in serum-free RPMI for 1 h at room temperature. Cells were pelleted and washed 2x before proceeding with flow cytometric staining. Effective removal of sialic acid removal was confirmed by flow cytometric staining with *Sambucus nigra agglutinin* and *Maackia amurensis agglutinin*-II.

Cellular O-glycome Reporter Analysis

Cells and glycans were prepared for Cellular O-glycome Reporter Analysis as previously described (46). Briefly, Ramos untransduced, vector control-transduced, or ST3Gal1OE-transduced B cells were seeded at 0.3×10^6 mL^{-1} in tissue culture medium (with 5% FBS) in six well tissue culture plates. Peracetylated Benzyl α-D-GalNAc (Ac3GalNAc-α-Bn) was added to each well to a final concentration of 50 μM. Cells were grown for 72 h, followed by cell pelleting and collection of media.

To purify glycans from media, media was filtered through a 10-kDa centrifugal filter (Amicon Ultra 4, Millipore) for 30 min at 2,465 × g. Bn-containing O-glycans were purified using a Sep-Pak-3-cc C18 cartridge (Waters). To equilibrate the

column, 2 mL acetonitrile was applied two times followed by four washes with 2 mL 0.1% (v/v) trifluoroacetic acid (TFA). Glycan-containing media was then added to the column, followed by four washes with 2 mL 0.1% (v/v) TFA. To elute Bn-containing O-glycans, 1.5 mL 50% (v/v) acetonitrile, 0.1% (v/v) TFA was applied to the column two times. Organic solvents were evaporated by SpeedVac, and the samples were lyophilized prior to MS analysis.

Preparation of Cells for N- and O-glycomic Analysis

Ramos untransduced, vector control-transduced, and ST3Gal1OE-transduced B cells were harvested in the log phase of growth ($0.75–1.25 \times 10^6$ cells mL^{-1}), pelleted, washed in excess PBS two times, and media completely aspirated. Cell pellets (20×10^6 cells per condition) were snap frozen in a dry ice / isopropanol slurry for 5 min and immediately stored at −80°C prior to MS analysis.

Glycomics Analysis of Ramos B Cells

For N- and conventional O-glycan structural analysis of untransduced, control and ST3Gal1OE Ramos B cells were treated as described previously (48, 62). Briefly, cell pellets were subjected to sonication in the presence of detergent (CHAPS), reduced in 4 M guanidine-HCl (Pierce), carboxymethylated, and digested with porcine trypsin (Sigma). The digested glycoproteins were then purified by C_{18}-Sep-Pak (Waters Corp., Hertfordshire, UK). N-glycans were released by peptide N-glycosidase F (E.C. 3.5.1.52; Roche Applied Science) digestion, whereas O-glycans were released by reductive elimination. Released N- and O-glycans were permethylated using the sodium hydroxide procedure and purified by C_{18}-Sep-Pak. Purified permethylated N- and O-glycans were found on the 50% acetonitrile fraction. The results shown are representative of two independent cell glycan preparations.

For CORA O-glycan structural analysis of untransduced, empty vector control and ST3Gal1OE Ramos B cells were treated as described previously (46). Isolated Bn-O-glycans were permethylated using the sodium hydroxide procedure and purified by C_{18}-Sep-Pak as described above for the conventional O-glycan structural analysis. Purified permethylated Bn-O-glycans were found on the 50% acetonitrile fraction. The results shown are representative of two independent cell glycan preparations.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF/TOF MS/MS were employed to analyze the structure of all above permethylated released glycans. MS and MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems Sciex) mass spectrometer. Permethylated samples were dissolved in 10 μl of methanol, and 1 μl of dissolved sample was premixed with 1 μl of matrix (10 mg/ml 3,4-diaminobenzophenone in 75% (v/v) aqueous acetonitrile), spotted onto a target plate, and dried under vacuum. For the MS/MS studies, the collision energy was set to 1 kV, and argon was used as collision gas. The 4,700 Calibration standard kit, calmix (Applied Biosystems Sciex), was used as the external calibrant for the MS mode, and [Glu1] fibrinopeptide B human (Sigma) was used as an external calibrant for the MS/MS mode. The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The processed spectra were subjected to manual assignment and annotation with the aid of a glycobioinformatics tool, GlycoWorkBench (63). The proposed assignments for the selected peaks were based on ^{12}C isotopic composition together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS and linkage analysis experiments.

Statistical Analysis

Statistical analyses were performed using Prism 7.0 software (GraphPad). For tests involving two groups, hypothesis testing was carried out using Welch's unpaired two-tailed *t*-test. For hypothesis testing of groups of three or more samples, and when variance was found to be not significantly different by *F*-test, a one-way analysis of variance (ANOVA) test was used with Tukey's correction for multiple comparisons. Where variances were unequal, a Kruskal–Wallis test was used instead with Dunn's correction for multiple comparisons. Bars and errors bars always depict the mean or standard error of the mean (SEM) from biological replicates, respectively, unless otherwise indicated. *P*-values < 0.05 were considered statistically significant.

DATA AVAILABILITY STATEMENT

The following datasets analyzed in this study are available on the Gene Expression Omnibus website (https://www.ncbi.nlm.nih.gov/geo/) under the following identifiers: GSE12195 (tonsil B cell expression analysis) (30); GSE24759 (hematopoietic cell expression analysis) (53). Hematopoietic cell expression data is also accessible at the following link: http://www.broadinstitute.org/dmap/home.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Partners Institutional Review Board, which deemed the work as not meeting the definition of human subjects research.

AUTHOR CONTRIBUTIONS

NG and CD conceived the study. NG and AA performed the experiments and analyzed the data. NG, SB, and HW generated the ST3Gal1 cell lines. MK and RC provided technical assistance and expertise with CORA O-glycomics analysis. AA and SH performed O-glycomic analyses. GL assisted with tonsil tissue acquisition. NG, AA, JL, JG, SK, AD, SB, HW, SH, and CD contributed intellectually to the study. SH and AD supervised MS glycomics assessments. CD supervised the entire study. NG, AA, SH, and CD wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02857/full#supplementary-material

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Clinical Relevance of Galectin-1 and Galectin-3 in Rheumatoid Arthritis Patients: Differential Regulation and Correlation With Disease Activity

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Galectins, a family of animal lectins, play central roles in immune system regulation, shaping both innate and adaptive responses in physiological and pathological processes. These include rheumatoid arthritis (RA), a chronic multifactorial autoimmune disease characterized by inflammatory responses that affects both articular and extra-articular tissues. Galectins have been reported to play central roles in RA and its experimental animal models. In this perspective article we present new data highlighting the regulated expression of galectin-1 (Gal-1) and galectin-3 (Gal-3) in sera from RA patients under disease-modifying anti-rheumatic drugs (DMARDs) and/or corticoid treatment in the context of a more comprehensive discussion that summarizes the roles of galectins in joint inflammation. We found that Gal-1 levels markedly increase in sera from RA patients and positively correlate with erythrocyte sedimentation rate (ERS) and disease activity score 28 (DAS-28) parameters. On the other hand, Gal-3 is downregulated in RA patients, but positively correlates with health assessment questionnaire parameter (HAQ). Finally, by generating receiver-operator characteristic (ROC) curves, we found that Gal-1 and Gal-3 serum levels constitute good parameters to discriminate patients with RA from healthy individuals. Our findings uncover a differential regulation of Gal-1 and Gal-3 which might contribute to the anti-inflammatory effects elicited by DMARDs and corticoid treatment in RA patients.

Keywords: rheumatoid arthritis, galectin-1, galectin-3, inflammation, autoimmune disease

INTRODUCTION

Rheumatoid arthritis (RA) is a highly prevalent chronic disease with multifactorial etiology. It is characterized by generalized inflammation in multiple joints, leading to cartilage and bone erosion and articular deformation. The disease comprises a complex interaction between genetic susceptibility and environmental stimuli, including epigenetic modifications (1). Galectins have emerged as master regulators of immune system homeostasis, playing key roles in the amplification and/or resolution of inflammatory processes, including RA (2, 3).

GALECTINS IN INFLAMMATION

Galectins are soluble lectins defined by their affinity toward galactose-β1-4-*N*-acetylglucosamine (*N*-acetyl-lactosamine, LacNAc)-enriched glycoconjugates present on the cell surface or extracellular matrix. Until now, 15 galectins have been described in vertebrates and classified into three groups according to their molecular architecture: (1) "proto-type" galectins (e.g., Gal-1), consisting of only one carbohydrate recognition domain (CRD) which can homodimerize; (2) "tandem-repeat" galectins (e.g., Gal-8 and−9), which present two different CRDs in tandem connected by a short peptide; and (3) the "chimera-type" galectin, Gal-3, consisting of one CRD connected to a non-lectin N-terminal region that supports oligomerization (4, 5). The glycan-binding specificities of individual members of the galectin family have been extensively discussed recently (4).

Although some galectins exhibit a broad tissue localization (e.g., Gal-1 and Gal-3), others show a selective distribution pattern (2). Whereas some members of the galectin family trigger anti-inflammatory responses and serve as pro-resolving mediators, others display pro-inflammatory activity enhancing innate and adaptive immunity (6). Thus, the functional outcome of galectin signaling may differ greatly, depending on the particular galectin involved, the number and branching of specific glycans serving as potential ligands and the biochemical nature of these multivalent interactions (4, 7). In this regard, inflammation induces changes in the glycosylation signature of both immune cells and inflamed tissue, leading to either masking or unmasking of galectin-specific glycoepitopes (4, 8). Particularly, LacNAc residues recognized by Gal-1, which are present on the branches of *N*- or *O*-linked glycans, are created by the concerted action of specific glycosyltransferases including the N-acetylglucosaminyl transferase 5 (MGAT5), an enzyme that generates β1-6-N-acetylglucosamine branches in complex *N*-glycans, and the core 2 β1-6-N-acetylglucosaminyl transferase 1 (C2GNT1), which acts on asialo-galactose-β1-3-N-acetylgalactosamine core 1 *O*-glycans to synthetize the core 2 branching structure (4). Since Gal-1 and Gal-3 are ubiquitously expressed and display context-dependent functional roles, their immunomodulatory effects have been described in several inflammatory microenvironments (2).

Given the prominent expression of Gal-1 in tumors and immune privileged sites and its up-regulation during the recovery phase of autoimmune inflammation (9–13), this lectin has been proposed to play key roles in suppression of antitumor responses, maintenance of immune tolerance and resolution of chronic inflammation, acting as a novel regulatory checkpoint that links innate and adaptive responses (14). Gal-1 shapes immune responses by selectively deleting Th1 and Th17 effector cells (15), promoting a tolerogenic and pro-migratory dendritic cell (DC) phenotype (13, 16), fostering expansion of regulatory T cells (Tregs) (10, 17–19) and fine-tuning the function of neutrophils, monocytes and macrophages (20, 21). These broad immunoregulatory effects have been validated in several experimental models of autoimmunity, allergy, infection, and cancer (2, 7, 22–24).

On the other hand, Gal-3 has controversial pro- or anti-inflammatory activities depending on various factors including its intracellular or extracellular localization and the target cell implicated in these processes (25). Although it may contribute to resolution of inflammation by clearing apoptotic neutrophils (26), this lectin displays mostly pro-inflammatory effects by reinforcing activation of macrophages, DCs, mast cells, and natural killer cells, as well as T and B lymphocytes (27).

GALECTINS IN RHEUMATOID ARTHRITIS

Heritability of RA is calculated to be around 65%, with more than 100 RA-risk-associated genomic loci (28). A few polymorphisms in individual galectins that could be associated to progression or severity of RA have been described. *LGALS3 +292C*, a polymorphism in the gene encoding Gal-3, is more common in RA patients (29). Moreover, a polymorphism in the gene encoding Gal-8 (rs2737713), generated by a missense mutation that changes a phenylalanine for tyrosine (F19Y), exhibits a strong association with RA in a Caucasian population (30). This mutation seemed to have no major effect on carbohydrate binding at least in solid-phase assays. Furthermore, a C3279T polymorphism in *LGALS2 gene* (encoding Gal-2), has been associated with diastolic blood pressure in RA patients at increased risk for hypertension (31).

A common feature of RA is the altered hyper-activated state of the stromal tissue and the immune system (1). Changes in both innate and adaptive immune pathways are common findings in RA patients (32). Gal-3 has been identified as a pro-inflammatory mediator both in RA patients and animal models of the disease. Gal-3 mRNA and protein were detected at the synovial membrane, while Gal-3-binding protein was found to be predominantly expressed at sites of bone destruction (33). Interestingly, expression of Gal-1 was not found at sites of synovial fibroblast invasion in RA (33). Synovial fibroblasts from RA patients expressed higher levels of CD51 and CD61 integrins, which individually, or by forming the $\alpha_V\beta_3$ complex (vitronectin receptor), binds to cartilage oligomeric matrix protein and induces secretion of Gal-3 (34). Externalization of this lectin influences the shape and persistence of joint inflammation by inducing local fibroblasts to secrete pro-inflammatory cytokines including IL-6, GM-CSF and MMP-3 and chemokines such as CCL2, CXCL8, CCL3, and CCL5 (35). Stimulation of IL-6 secretion by Gal-3 is mediated by Toll-like receptor-2,−3, and−4 in human synovial fibroblasts (36), contributing to amplification of pro-inflammatory responses (**Figure 1**).

Before the clinical onset of the disease, a "pre-RA" condition arises, which displays both immunologic and metabolic alterations (37). Follow-up studies in undifferentiated arthritis (UA) patients, naïve for both disease-modifying anti-rheumatic drugs (DMARDs) and corticosteroids, showed that serum Gal-3 levels are high in those patients that progress to RA after 1 year. Although serum Gal-3 was a poor prognostic marker itself, the combination with anti-cyclic citrullinated peptide (CCP) levels or bone marrow edema score could help categorize UA subsets at early phases (38). Moreover, in another study, serum Gal-3 levels

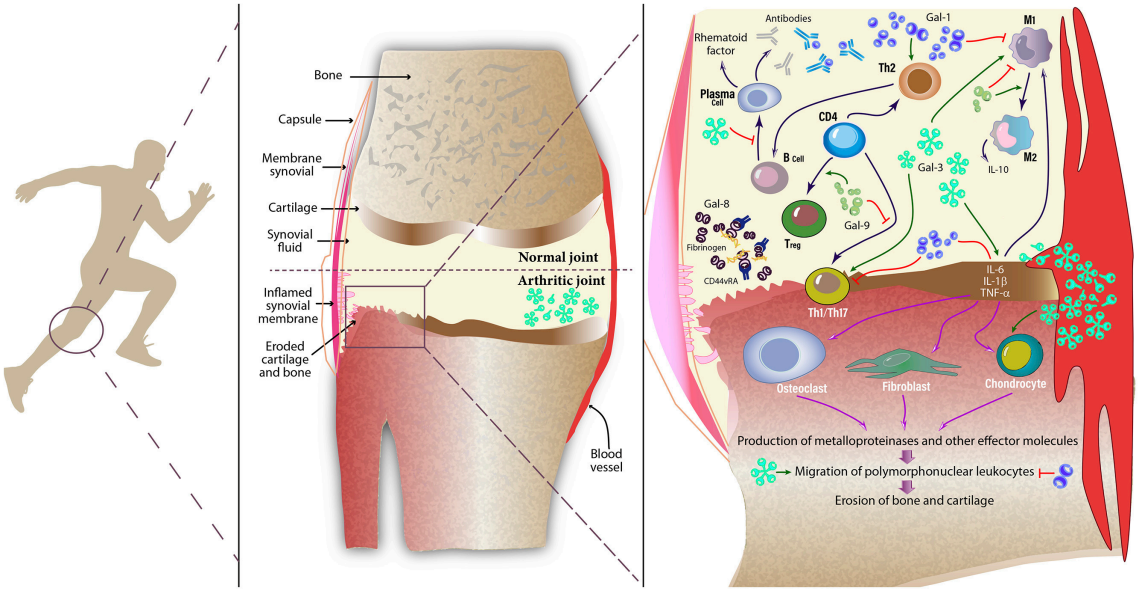

FIGURE 1 | Role of galectins in inflamed synovial tissue. Galectins are expressed by a number of inflammatory cells (both innate and adaptive immune cells), endothelial cells, stromal cells, and synovial fibroblasts. These glycan-binding proteins influence a variety of cellular programs that control amplification and resolution of inflammatory responses. Galectins can behave as pro- or anti-inflammatory mediators by modulating the physiology of immune cells, including monocytes, macrophages, synovial fibroblasts, Th1, Th2, and Th17 cells, regulatory T (Treg) cells, B cells, neutrophils and mast cells. By positively or negatively regulating inflammation, galectins may directly or indirectly influence the clinical course of RA. While Gal-1 enhances a Th2-Treg response profile, polarizes macrophages toward an M2 phenotype and induces apoptosis of Th1 and Th17 cells, Gal-3 activates fibroblasts and induces secretion of pro-inflammatory cytokines. Circulating autoantibodies reduce effective Gal-1 concentrations in synovial fluid of patients with RA. On the other hand, Gal-9 controls $CD4^+$ T cell functions through binding to $TIM\text{-}3^+$ cells. Moreover, Gal-8 has pro-apoptotic and anti-inflammatory activity in the inflamed joint; however a soluble form of CD44 reduces availability of this tandem-repeat galectin by forming complexes with fibrinogen. Gal, Galectin; TNF, Tumor necrosis factor; IL, Interleukin; Th, T helper cell; Treg, regulatory T cells; M1, pro-inflammatory macrophage; M2, anti-inflammatory macrophage.

showed no differences compared to controls in DMARDs- and corticosteroid-naïve patients with <6 months of RA diagnosis, but were significantly elevated in anti-CCP positive vs. anti-CCP negative patients and healthy subjects (39). Furthermore, in a cohort of 20 RA patients serum Gal-3 levels positively correlated with those found in synovial fluid (33), suggesting possible association between systemic and local galectins.

Notably, autoantibodies that could reduce or block biological activities of galectins have been found in different settings. Xibillé-Friedmann and colleagues reported reduced Gal-1 levels in synovial fluid of RA patients due to the presence of anti-Gal-1 autoantibodies (40), a similar effect as that found in uveitis patients (41) (**Figure 1**). Moreover, autoantibodies against Gal-8 and Gal-9 have also been detected in RA patients (42, 43).

In a model of antigen-induced arthritis, Forsman and colleagues found that joint inflammation and bone erosion were attenuated, antigen-specific IgG and pro-inflammatory cytokines TNF-α and IL-6 were decreased, and the number of Th17 cells was significantly reduced in *Lgals3*$^{-/-}$ vs. WT mice, suggesting a pathogenic role for this lectin in the development and progression of RA (44). In contrast, *Lgals1*$^{-/-}$ mice developed a more severe inflammatory response in a model of collagen-induced arthritis (CIA) with higher penetrance and an accelerated clinical onset (45). In this regard, in early studies, we demonstrated the therapeutic potential of Gal-1 in the CIA model. Injection of syngeneic fibroblasts genetically engineered to secrete Gal-1, or daily administration of recombinant Gal-1 suppressed clinical and histopathological manifestations of arthritis and promoted a shift toward a Th2-mediated anti-inflammatory response (46). These findings were integrated by Wang et al. who successfully treated rats using lentiviral vectors aimed at overexpressing Gal-1 or silencing Gal-3, revealing broad anti-inflammatory responses characterized by improved radiographic and histological scores (47). Additionally, downregulation of Gal-1 and upregulation of Gal-3 expression were found in synovial tissue from patients with juvenile idiopathic arthritis (48, 49).

Interestingly, Eshkar-Sebban et al. found that a CD44 variant expressed in synovial fluid of RA patients -CD44vRA- sequesters Gal-8 by forming a soluble complex with fibrinogen, thus reducing the availability of this lectin in the inflamed joint (50). Furthermore, elevated levels of Gal-9 were detected in synovial fluid from patients, an effect that was accompanied by a higher percentage of Gal-9-positive cells in synovial tissue (51). By using a stable mutant protein resistant to proteolysis, Seki et al. showed that Gal-9, but not Gal-1,−3, or−8, induced apoptosis of fibroblast-like synoviocytes (51). Later, the authors found that Gal-9 suppressed clinical manifestations of CIA by reducing the synthesis of pro-inflammatory cytokines IL-17, IL-12, and IFN-γ in the joints and lowering the number of $CD4^+$ T cells expressing T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) in peripheral blood (52). Nonetheless, this effect was impaired in RA patients due to reduced TIM-3 expression (53). Furthermore, Gal-9 also reduced the severity

of immune complexes-induced arthritis by downregulating FcγRIII and upregulating FcγRIIb in macrophages, an effect that ultimately led to IL-10 secretion and inhibition of TNF-α and IL-1β production (54). Mechanistically, Gal-9 acted by inducing the *in vitro* differentiation of Tregs, while suppressed polarization toward a Th17 phenotype (52). In contrast, a recent study suggested a rather pro-inflammatory role of Gal-9, as intra-articular injection of this lectin facilitated mononuclear cell migration and favored arthritogenic responses (55). Thus, the coordinated action and differential regulation of individual members of the galectin family will finally dictate clinical responses in RA patients (**Figure 1**).

CLINICAL RELEVANCE OF GAL-1 AND GAL-3 IN PATIENTS WITH RHEUMATOID ARTHRITIS

Based on its broad anti-inflammatory activity, we evaluated Gal-1 serum levels in patients with established RA (defined by the American College of Rheumatology 2010 classification criteria). We recruited 32 patients and 19 sex- and age-matched healthy volunteers from Hospital de Clínicas "José de San Martín" (Buenos Aires, Argentina) (**Table 1**). Patients ranged from 1 to 28 years since RA was first diagnosed and were all under treatment with at least one DMARD, mainly methotrexate. Determination of Gal-1 was performed using an in-house ELISA as described (56). Detailed description of Materials and methods is shown as **Supplementary Data**.

Analysis of circulating Gal-1 showed significantly higher levels of this lectin in serum obtained from RA patients compared to control individuals (**Figure 2A**). To further validate these findings and given the lack of differences reported in another study (40), we recruited a second, independent and larger cohort of patients from Hospital "José Bernardo Iturraspe" (Santa Fe, Argentina). Twenty nine healthy volunteers and 48 RA patients under DMARD treatment were enrolled in the study. Cohort 2 validated our previous observation, as RA patients again showed significantly higher levels of serum Gal-1 compared to controls (**Figure 2B**).

Next, we explored the potential associations of Gal-1 with clinical parameters of disease activity. For this purpose, and to gain statistical robustness, we pooled data from both cohorts. Regardless of differences in the median Gal-1 serum levels between RA patients from cohort 1 (median = 68.77 ng/ml) and cohort 2 (median = 95.63 ng/ml), analysis of pooled data from both cohorts revealed, as expected, elevated Gal-1 levels in sera from RA patients compared to controls (**Figure 2C**). Based on this finding, we regrouped RA patients based on their functional status classification, and found that, compared to controls, serum Gal-1 levels were significantly increased in all functional classes; yet revealing no statistical differences (**Figure 2D**).

Next, we analyzed whether Gal-1 serum levels may correlate with quantitative parameters of disease activity derived from patients' questionnaires, such as VAS (Visual Analog Scale) and physical function such as HAQ (Health Assessment Questionnaire). As shown in **Figures 2E**,**F**, neither VAS nor HAQ parameters showed correlation with circulating Gal-1 levels ($r = 0.17$, $p = 0.15$; $r = 0.04$, $p = 0.72$, respectively). We then explored whether Gal-1 serum levels correlate with the Erythrocyte Sedimentation Rate (ESR). Notably, we found a very strong positive correlation between Gal-1 serum levels and ESR (**Figure 2G**, $r = 0.039$, $p = 0.0006$), a blood parameter that indicates the extent of systemic inflammation. Moreover, we also found a positive correlation between Gal-1 serum levels and DAS28 (Disease Activity Score 28) (**Figure 2H**, $r = 0.25$, $p = 0.029$).

Since RA is a chronic inflammatory disease that aggravates gradually, we also explored whether Gal-1 serum levels could

TABLE 1 | Demographic, clinical, and laboratory characteristics of patients with RA.

	Cohort 1 (n:32)	Cohort 2 (n:48)
Gender		
Female	29	46
Male	3	2
Age, median years (range)	41 (24–64)	48 (30–67)
RA duration, mean years (range)	7.8 (1–28)	9.1 (1–28)
Disease activity parameters		
Functional Class		
Class I	4/32 (12.6%)	20/48 (41.7%)
Class II	14/32 (43.7%)	21/48 (43.7%)
Class III	11/32 (34.3%)	5/48 (10.4%)
N/A	3/32 (9.4%)	2 (4.2%)
DAS-28, mean (range)	4.4 (1.75–8)	4.4 (1.96–6.28)
HAQ-A, mean (range)	1.30 (0.25–2.25)	1.27 (0–4.12)
VAS, mean (range)	41.4 mm (0–100)	37.1 mm (0–100)
ESR, mean (range)	27.7 mm (10–91)	32.6 mm (5–68)
Serology		
RF		
Positive	28	38
Negative	0	7
N/A	4	3
Anti-CCP		
Positive	18	14
Negative	1	0
N/A	12	34
Treatment		
Methotrexate	26/32 (81.3%)	40/48 (83.3%)
Corticosteroids	19/32 (59.4%)	43/48 (89.6%)
HCQ/CQ	9/32 (28.1%)	19/48 (39.6%)
Sulfasalazine	1/32 (3.1%)	4/48 (8.3%)
Leflunomide	1/32 (3.1%)	11/48 (23%)
Anti-TNFα	6/32 (18.8%)	2/48 (4.2%)
Other biologicals (rituximab, abatacept)	2/32 (6.3%)	0/48 (0%)
NSAIDs	9/32 (28.1%)	33/48 (68.8%)
Other	9/32 (28.1%)	4/48 (8.3%)
N/A	3/32 (9.4%)	3/48 (6.2%)

N/A, Not Available; RF, Rheumatoid Factor; anti-CCP, anti-cyclic citrullinated peptide; NSAIDs, non-steroidal anti-inflammatory drugs. Others: folic acid, VitD3, risedronate, calcium.

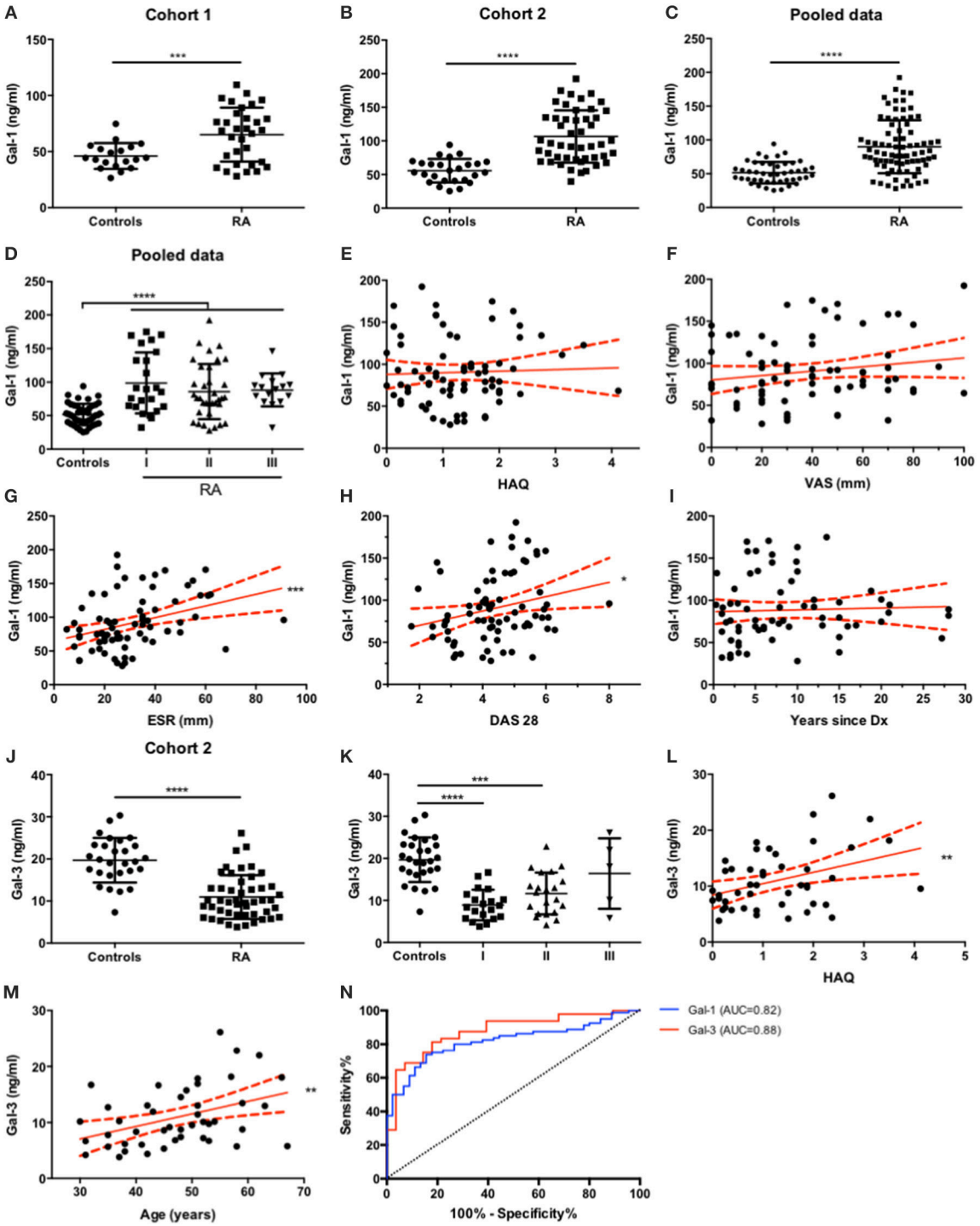

FIGURE 2 | Serum Gal-1 and Gal-3 expression levels discriminate RA patients from healthy individuals. **(A-C)**. Determination of serum Gal-1 levels (ELISA) in controls and RA patients from cohort 1 **(A)**, cohort 2 **(B)** and pooled data **(C)**. **(D)**. Gal-1 serum levels from all patients **(C)** classified by functional status. **(E-I)**. Correlation analysis of Gal-1 serum levels of all patients with HAQ **(E)**, VAS **(F)**, ESR **(G)**, DAS-28 **(H)** and RA duration **(I)**. **(J)**. Determination of serum Gal-3 levels (ELISA) in controls and RA patients from cohort 2. **(K)**. Gal-3 serum levels of RA patients from cohort 2 **(J)** classified by functional status. **(L-M)**. Correlation analysis of Gal-3 serum levels of RA patients from cohort 2 with HAQ **(L)** and age **(M)**. **(N)**. ROC curve analysis to assess Gal-1 (blue) and Gal-3 (red) capacity to discriminate between RA patients and healthy individuals. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $^{****}p < 0.0001$. All variables analyzed were tested for Gaussian distribution with D'Agostino and Pearson omnibus normality test. For comparisons between two groups, unpaired *t* test with Welch's correction or Mann-Whitney tests were applied as appropriate. For comparisons between more than two groups, Kruskal-Wallis test was applied. For correlation analysis, Pearson or Spearman correlation tests were applied as appropriate. To determine the capability of Gal-1 and Gal-3 serum level measurements to discriminate between RA patients and controls, ROC curves were generated.

change over time. We found no significant correlation between serum Gal-1 and disease duration (**Figure 2I**, $r = 0.18$, $p = 0.15$). Additionally, no correlation was found between Gal-1 serum concentrations and patients' age ($r = 0.03$, $p = 0.80$, graph not shown).

In order to broaden our study and given the different roles of Gal-3 in the arthritogenic process, we then examined serum levels of this chimera-type lectin in this patient cohort using a human Gal-3 ELISA kit (R&D Systems; DY1154). Interestingly, RA patients showed significantly lower levels of Gal-3 in circulation compared to control subjects (**Figure 2J**). Similar to our previous analysis, we categorized RA patients according to their functional status classification and found that serum Gal-3 levels were significantly diminished in functional class I and II compared to controls, but found no statistical differences between controls and class III RA patients (**Figure 2K**). Moreover, a positive linear trend was found, showing that Gal-3 serum concentrations tended to be higher in classes with higher disease activity ($r = 0.18$, $p = 0.0037$) (**Figure 2K**). Then, the same correlation analysis applied to Gal-1 and clinical parameters of disease was performed for Gal-3. Although we found no correlation between Gal-3 serum concentrations and VAS ($r = -0.08$, $p = 0.60$), ESR ($r = -0.15$, $p = 0.31$) or DAS28 ($r = -0.06$, $p = 0.69$), a significant positive correlation was detected between Gal-3 levels and HAQ score (**Figure 2L**, $r = 0.38$, $p = 0.0098$). Furthermore, though circulating Gal-3 levels did not correlate with RA duration ($r = 0.23$, $p = 0.16$), we found a positive correlation with patients' age (**Figure 2M**, $r = 0.40$, $p = 0.0062$).

Finally, we generated Receiver-Operator Characteristic (ROC) curves in order to assess the ability of Gal-1 and Gal-3 serum levels to discriminate between RA patients and healthy controls. Both Gal-1 and Gal-3 serum levels proved to be good parameters to distinguish patients with established RA from controls, as the area under the ROC curve (AUC) for both parameters was above 0.8 (Gal-1 AUC = 0.82, Gal-3 AUC = 0.88; both $p < 0.0001$) (**Figure 2N**). Serum Gal-1 concentrations above 60.94 ng/ml (sensitivity = 80.0% and specificity = 73.3%) and serum Gal-3 concentrations below 16.82 ng/ml (sensitivity = 85.42% and specificity = 71.43%) successfully differentiated RA patients from controls.

CONCLUSIONS

Galectins have emerged as amplifiers or silencers of inflammatory responses, capable of orchestrating complex regulatory circuits in innate and adaptive immune cells, as well as in synovial fibroblasts. In this perspective article we summarize relevant data pinpointing the contribution of galectins to the pathogenesis of RA (**Figure 1**) and report new clinical observations, highlighting the differential regulation of Gal-1 and Gal-3 at the systemic level in RA patients and their association with disease activity (**Figure 2**).

In two independent cohorts we found increased concentrations of Gal-1 in sera from RA patients compared to control individuals. Elevated levels of this lectin were found in all functional classes of patients and were independent of age and disease duration. To our knowledge, only one study has evaluated circulating Gal-1 levels in RA patients. Xibillé-Friedmann et al described in a cohort of 60 patients that plasma Gal-1 levels were similar in patients and controls; however Gal-1 concentrations were reduced in synovial fluid of patients and correlated with the presence of anti-Gal-1 autoantibodies (40). Although both studies recruited patients under DMARD treatment, differences between them could be related to distinct DMARD used, genetic background and/or environmental factors influencing concentrations of this immunoregulatory lectin. Of note, control subjects from that study exhibited considerably higher levels of Gal-1 in serum (low μg/ml range) compared to our controls and data published by other groups (often ranging in the low ng/ml range) (57–62).

Interestingly, we found a strong correlation between Gal-1 concentrations and ESR, an indicator of systemic inflammation. Similarly, in a previous study, Gal-1 serum levels were significantly increased in classical Hodgkin lymphoma patients who also showed an elevated ERS (57). Accordingly, we observed a positive correlation between serum Gal-1 and DAS-28, a composite score of disease activity derived from examination of 28 joints (number of swollen joints and tender joints) combined with ESR and VAS measurements. Like PD-1, CTLA-4 and other immune checkpoints, Gal-1 expression is upregulated in response to severe inflammatory conditions, acting as an homeostatic mechanism to counterbalance exuberant inflammation (13, 63). Interestingly, nuclear factor (NF)-κB, a transcription factor associated with induction of pro-inflammatory genes, also controls expression of immune inhibitory programs including those involving PD-1 and Gal-1 on T cells (64, 65). Thus, during the peak of inflammation, similar transcriptional mechanisms may operate to activate homeostatic programs that contribute to resolution of inflammatory responses.

The pathogenic role of IL-6 in RA has been widely studied, showing correlation between systemic levels of this cytokine and disease activity (66). Recently, we found that systemic upregulation of IL-6 mobilizes myeloid-derived suppressor cells (MDSCs) which drive Gal-1 production by γδ-T cells (67). In this regard, expansion of MDSCs correlated with disease severity (DAS-28) in RA patients (68, 69). As serum Gal-1 levels positively correlate with inflammation and DAS-28, activation of an "IL-6-MDSCs-Gal-1" axis could also take place in RA. Additional studies should be conducted to verify this hypothesis. On the other hand, a Gal-1-mediated pro-inflammatory signature has been observed in chondrocytes from osteoarthritic patients, suggesting context-dependent regulatory effects of this lectin (70).

Remarkably, Gal-1 and Gal-3 act by cross-linking *N*- and *O*-glycans on the surface of immune cells (15, 71). Since glycosylation is considerably altered in rheumatologic disorders (72), further studies are warranted to examine the relevance of cell surface glycans on immune cells, particularly those implicated in galectin-glycan interactions (complex *N*-glycans, core-2 *O*-glycans and absence of α2,6-sialylated structures) during the evolution of the arthritogenic process in RA patients. In this regard, low levels of galactosylation and sialylation of autoantibodies are associated with disease severity in RA patients (73). Moreover, Pfeifle and colleagues showed that IL-23-activated Th17 cells suppress α2,6-sialylation of

IgG through downregulation of the α2,6-sialyltransferase-1 in antibody-producing plasma cells, skewing the balance from anti-inflammatory toward pro-inflammatory responses (74). Further studies aimed at exploring glycosylation patterns of pathogenic cells in RA will contribute to fully elucidate the role of galectins in this pathology.

Finally, we and others observed a positive correlation between Gal-3 levels and HAQ (39). Interestingly, we found lower concentrations of Gal-3 in RA patients compared to controls. In contrast, Issa and colleagues reported augmented Gal-3 serum levels mainly in untreated patients (38, 39). Such discrepancies could be probably due to DMARD and/or corticosteroid treatment in our patient cohorts. Supporting this assumption, glucocorticoid treatment inhibited lipopolysacharides-induced upregulation of Gal-3 in monocytic THP-1 cells (75). Moreover, a significant increase in IgG galactosylation and sialylation was detected in RA patients after initiation of methotrexate therapy, showing reversion to physiologic conditions (76, 77). Thus, low serum Gal-3 levels in combination with augmented Gal-1 expression could influence activation of tolerogenic circuits during RA remission states. Future studies involving treated and untreated RA patients will shed light on how different treatments affect both the glycosylation patterns of inflammatory cells as well as the expression pattern of pro- and anti-inflammatory galectins, leading to activation or de-activation of immune signaling programs.

ETHICS STATEMENT

Patients and controls were informed in detail about the study, and written consent was obtained in accordance with the Declaration of Helsinki. The protocol was approved by Ethics and Research Committees of Hospital de Clínicas José de San Martín, Hospital José Bernardo Iturraspe and Instituto de Biología y Medicina Experimental (IBYME).

AUTHOR CONTRIBUTIONS

SPM-H acquired data, analyzed, and interpreted data, supervised the study and wrote the manuscript. PFH interpreted data, and wrote and revised the manuscript. JCS acquired data and revised the manuscript. LGM analyzed and interpreted data, and revised the manuscript. NAP and SMM analyzed data and wrote the manuscript. AMB, JAC, JLM, and GGN managed patients and revised the manuscript. GAR conceived, designed, and supervised the study, interpreted data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03057/full#supplementary-material

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The First Step in Adoptive Cell Immunotherapeutics: Assuring Cell Delivery via Glycoengineering

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Despite decades of intensive attention directed to creation of genetically altered cells (e.g., as in development of chimeric antigen receptor (CAR) T-cells) and/or to achieve requisite *in vitro* accumulation of desired immunologic effectors (e.g., elaboration of virus-specific T cells, expansion of NK cells, differentiation of dendritic cells, isolation, and propagation of Tregs, etc.), there has been essentially no interest in the most fundamental of all hurdles: assuring tissue-specific delivery of administered therapeutic cells to sites where they are needed. With regards to use of CAR T-cells, the absence of information on the efficacy of cell delivery is striking, especially in light of the clear association between administered cell dose and adverse events, and the obvious fact that pertinent cell acquisition/expansion costs would be dramatically curtailed with more efficient delivery of the administered cell bolus. Herein, based on information garnered from studies of human leukocytes and adult stem cells, the logic underlying the use of cell surface glycoengineering to enforce E-selectin ligand expression will be conveyed in the context of how this approach offers strategies to enhance delivery of CAR T-cells to marrow and to tumor beds. This application of glycoscience principles and techniques with intention to optimize cell therapeutics is a prime example of the emerging field of "translational glycobiology."

Keywords: E-selectin ligand, adoptive cell therapy, CAR T cell, GPS, sialyl Lewis X, sLeX, fucosyltransferase, translational glycobiology

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INTRODUCTION

Imagine that a product manufacturer (or vendor of the item) must make multiple shipments of the same item to a given recipient because the delivery system is neither accurate nor efficient, i.e., the physical transfer of that product to the intended arrival destination is imprecise. Such transit-related loss of goods would require that far more product be manufactured than would be needed. The faulty transport would thus be a key driver of excessive production expenses, let alone recipient costs.

Cancer treatment has entered an era whereby tumor-specific immunocytes can be created and expanded *ex vivo*, and can thereafter be administered to patients. The development of chimeric antigen receptor (CAR) T-cells is a salient example of this approach, and these antigen-specific cells have the immense advantage of achieving MHC-independent cytotoxicity of tumor targets. Once cell numbers sufficient for treatment are generated, the cells are infused into patients and serve as living drugs. To date, this approach has shown great promise in the treatment of hematologic

malignancies (particularly, malignancies of B-cell origin) and is gaining applicability in solid malignancies. Yet, remarkably, in the development of such cell-based immunotherapeutics, an essential prerequisite has been uniformly overlooked: tumor regression is critically dependent on the ability of infused effector cells to enter the tumor parenchyma (1–4).

Fundamentally, it is important to draw a distinction between tissue-specific recruitment of administered cells (homing) vs. retention of administered cells at a target site. The former reflects explicit migration of cells to the intended site, whereas the latter reflects the entrapment of cells. In the case of CAR-T cells, entrapment occurs when cells that have entered a site non-specifically become retained/lodged within that tissue upon encountering their cognate antigen. Operationally, killing of malignant cells by tissue-resident T cells would ensue regardless of whether administered cells have homed to lesional sites or have entrapped there. However, entrapment is a stochastic process, and treatment efficacy could be much improved if cells were capable of homing to the affected site. For the case of CD19-directed CAR-T cells, especially for their application in acute lymphoblastic leukemia, it would be desired for administered cells to preferentially home to bone marrow. However, to date, no preclinical nor clinical studies have evaluated the extent to which administered CD19-directed CAR T-cells migrate to marrow. Instead, all past and current applications of CAR T-cells have focused on administrating sufficient quantities of cells in order to achieve the anticipated cancer treatment effect(s), with no attention to the overt waste of such cells within unaffected sites and/or the biologic consequence(s) related to off-target distribution. The inefficiency of intra-tumoral cell delivery, apart from simply requiring an exceedingly abundant cell expansion *ex vivo*, results in accumulations of cells in non-lesional sites/unaffected tissues resulting in significant treatment-related toxicities. As such, particularly for the case of CAR T-cell therapeutics, the impact of "loss of goods" should not be considered simply in terms of production expenditures, it must be factored with highest attention to the incidence of toxicities and significant patient suffering that further compound treatment-related costs. Ideally, the infused cells should not result in serious complications or, worse, mortality, but life-threatening toxicities are routine with current CAR T-cell therapy and their severities correlate with the infused cell dose (5–10).

CIRCULATING LYMPHOCYTE COUNTS, THE CAR T-CELL DOSE RANGE, AND ADVERSE EVENTS ASSOCIATED WITH CAR T-CELL ADMINISTRATION

In humans, total blood volume averages 8% of total body weight (e.g., a 50 kg person has ∼4 L of blood volume). The usual lymphocyte count in humans under steady-state (healthy) conditions ranges from 1×10^9 to 3×10^9 cells/L. In clinical trials to date, the infusion dose of CAR T-cells has typically ranged from upwards of 2×10^6-2×10^7 cells/kg of recipient body weight (e.g., reflecting a dose range of 10^8 cells to 10^9 cells for a 50 kg person). Because this cell bolus is distributed within the total blood volume, the intravascular T-cell count immediately post-infusion ranges from 25×10^6/L to 250×10^6/L (please note that the conversion factor for cell dose in cells/kg into cells/L of blood volume is 12.5). Importantly, all patients that receive CAR T-cells are given lymphodepleting chemotherapy prior to the cell infusion. In essence, then, the overwhelming majority of circulating lymphocytes post-infusion are CAR T-cells, and the resulting cell count reflects as much as one-fourth the number of lymphocytes that would natively be present in the blood of a healthy person (i.e., 0.25×10^9 lymphocytes/L, where normal count is 10^9 lymphocytes/L). There is no precedent in any physiologic immune response for a circulating lymphocyte pool that is comprised predominantly (if not solely) of cells with mono-specificity for a given antigen, especially encompassing lymphocytes bearing receptors and costimulatory motifs that uniformly trigger cell activation upon encountering the cognate antigen.

The most frequent clinical adverse event associated with CAR T-cell infusions is a condition known as "cytokine release syndrome" (CRS), which is consequent to T cell activation. CRS encompasses a spectrum of clinical features including fevers, third-spacing of fluid, hypotension, and hypoxia. This constellation of physical changes is incited by release of inflammatory cytokines such as IL-6 and γ-interferon, and it can be managed by agents that block IL-6 (e.g., tocilizumab, an antibody directed to the interleukin-6 receptor), and, if necessary, steroids (6, 11). Though infrequent, CRS can progress to frank respiratory failure and other severe organ toxicities (e.g., cardiac failure, hepatitis, renal failure), requiring intensive care support (e.g., intubation/ventilatory care, vasopressors, hemodialysis), sometimes culminating in death due to organ failure. In addition to CRS, neurotoxicity known as "CAR-related encephalopathy syndrome" (CRES) can ensue, characterized by mental status changes (somnolence and/or agitation with confusion/disorientation), which can progress to increased intracranial pressures, seizures, motor weakness, and coma. As in the case of severe CRS, steroids are utilized in therapy for management of life-threatening CRES but blockade of IL-6 is ineffective in treatment of CRES, perhaps because this entity is driven by CNS infiltration of CAR T-cells (11, 12). In this regard, the potency of steroids may reflect the ability of these agents to interrupt lymphocyte trafficking (13). Importantly, though steroids yield beneficial anti-inflammatory effects, these agents can also dampen the effectiveness of the CAR T-cell assault on tumor cells.

The severity of CRS and CRES correlates principally with the dose of CAR T-cells administered, but is also related to the tempo of the *in vivo* expansion of the CAR T-cells and the extent of CAR T-cell expansion, processes that each reflect both the initial cell dose and the tumor burden of the recipient. In any case, since the localization of CAR T-cells in off-target tissues contributes to the observed organ toxicities (5, 11, 12), it is reasonable to speculate that improving the specificity of CAR T-cell infiltration within tumor sites would lessen the onset and severity of both CRS and CRES. There is strong evidence in support of this notion, as the presence of CAR T-cells in cerebrospinal fluid is correlated with the severity of CRES (12).

Moreover, in preclinical studies (14–16) and in a clinical trial (17), administration of CAR T-cells directly into cancer sites has yielded marked anti-tumor effects. Importantly, in preclinical studies, the efficacy of CAR T-cells directly injected into tumor sites is much greater than that of intravenous injection (14–16), with as much as 10-fold greater cells needed intravenously to obtain equivalent anti-tumor effects (16). In the clinical trial of CAR T-cell regional administration, high doses (10^7 cells) were administered locally without manifestations of severe systemic toxicities (17). Thus, to optimize the therapeutic window of intravascularly systemically administered CAR T-cells, it is first necessary to develop strategies to program a more precise delivery of systemically administered CAR T-cells to the relevant tumor site(s).

THE MOLECULAR BASIS OF CELL TRAFFICKING

Host defense critically depends on the capacity to ensure rapid and precise delivery of leukocytes to inflammatory sites. To this end, circulating leukocytes express a highly specific set of molecular effectors that engage endothelial cells within sites of tissue injury/inflammation. The first hurdle in all transmigration events involves the initial tethering and then rolling attachment of circulating cells to target endothelium with sufficient strength to overcome the prevailing forces of hemodynamic shear (18). This "Step 1" braking interaction is principally mediated by selectins (E-, P-, and L-selectin; known as CD62E, CD62P, and CD62L, respectively) and their ligands. Following this initial endothelial engagement, a cascade of events occur whereby cells undergo chemokine-mediated activation of integrin adhesiveness (Step 2), followed by integrin-mediated firm adherence to the endothelium (Step 3), finally resulting in transmigration (Step 4) (18).

As indicated by their nomenclature, the selectins are "lectins," i.e., proteins that bind to carbohydrates. This family of lectins require Ca^{++} to bind their target (i.e., the selectins are Ca^{++}-dependent lectins). The prototypical carbohydrate binding determinant for all selectins is a terminal sialofucosylated lactosaminyl glycan known as "sialyl Lewis X" (CD15s) (**Figure 1**). This tetrasaccharide consists of a "core" disaccharide composed of the monosaccharides galactose (Gal) and N-acetylglucosamine (GlcNAc), which are joined in β(1,4)-linkage [this disaccharide is called a "Type 2" lactosamine unit (LacNAc)] (see **Figure 1**). The sLe^X determinant contains sialic acid [also known as "neuraminic acid (Neu5Ac)] that is α(2,3)-linked to the Gal, and fucose (Fuc) that is α(1,3)-linked to the GlcNAc: Neu5Ac-α(2,3)-Gal-β(1,4)-[Fuc-α(1,3)-]GlcNAcβ1-R (18). This glycan is created by step-wise addition of sialic acid and then fucose onto the terminal type 2 lactosamine core structure by respective glycosyltransferases (see **Figure 1**), and it is recognized by a variety of monoclonal antibodies (mAbs), including the mAb known as "CSLEX-1" and another mAb known as "HECA452." Compared to HECA452, the CSLEX-1 mAb has a more restricted specificity in that it recognizes only sLe^X, whereas HECA452 recognizes both sLe^X and the isomeric sialofucosylated type 1 lactosaminyl glycan known as sialylated Lewis A (sLe^A). These mAb do not react with the unsialylated glycans known as "Lewis X" (Le^X) and "Lewis A" (Le^A) even though they share a common trisaccharide core structure with sLe^X and sLe^A, respectively. Notably, the Le^X determinant is best known by its CD designation ("CD15"), and it is a key marker of human myeloid cells (see **Figure 1**).

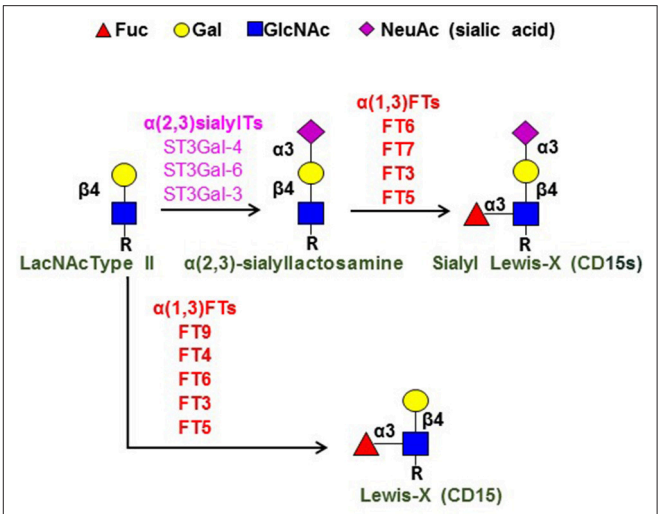

FIGURE 1 | Terminal lactosamine structures. Depicted are the structures for terminal sialylated Type 2 lactosamine (LacNAc), sialylated Lewis X (sLe^X; CD15s), and Lewis X (Le^X; C15). Component monosaccharides are shown using colored symbol nomenclature (key is at top of figure). Shown at left is the Type 2 lactosamine unit (LacNAc Type II), a disaccharide comprised of Gal β(1,4)-linked to GlcNAc. "R" refers to the reducing end glycans, which are typically comprised of polylactosamine chains (i.e., repeating units of Type 2 lactosamines). The key enzymes in creation of sLe^X [the α(2,3)-sialyltransferases (α(2,3)sialylTs) and α(1,3)-fucosyltransferases (α(1,3)FTs) are as shown, as are the α(1,3)FTs that create Le^X; these enzymes are ordered (top to bottom, high-low) to depict the relative activity of each enzyme in creating the pertinent structure [see reference (19) for details].

E- and P-selectin are expressed on vascular endothelium (P-selectin also on platelets), and L-selectin is expressed on circulating leukocytes (18). E- and P-selectin are typically inducible endothelial membrane molecules that are prominently expressed at sites of tissue injury and inflammation. However, the microvasculature of bone marrow and skin constitutively expresses these selectins, and they play a key role in steady-state recruitment of blood-borne cells to these sites (20). Importantly, within all inflammatory sites and sites of tissue injury/damage in primates (but not rodents), E-selectin is the principal vascular selectin mediating cell recruitment, as the promoter element responsive to the inflammatory cytokines TNF and IL-1 has been deleted from the primate P-selectin gene. Thus, at all inflammatory sites of humans (including tumor endothelial beds), vascular E-selectin expression is more pronounced than that of P-selectin, and E-selectin also has higher baseline expression than P-selectin in human marrow and skin (18, 20).

Whereas, both glycolipids and glycoproteins can be decorated with sLe^X determinants, glycoproteins serve as the primary E-selectin ligands under blood flow conditions since they extend

farther from the surface of the circulating cell than do glycolipids. There are three principal ligands for E-selectin expressed on subsets of human lymphocytes, each consisting of highly sialofucosylated glycoforms of well-recognized glycoproteins: CD162 (PSGL-1), CD43, and CD44. CD44 is a rather ubiquitous cell membrane protein and is best known for binding hyaluronic acid. However, display of sLe^X on CD44 confers new biology, and this specialized CD44 glycovariant, first observed on human hematopoietic stem/progenitor cells (HSPCs), is known as "Hematopoietic Cell E-/L-selectin Ligand" (HCELL) (21–23). As the name indicates, HCELL binds both E-selectin and L-selectin, and *in vitro* assays of E- and L-selectin binding under hemodynamic shear stress indicate that HCELL is the most potent ligand for these molecules expressed on any human cell. Notably, studies using human mesenchymal stem cells have shown that HCELL functions as a bone marrow "homing receptor" (24). Moreover, HCELL is not natively expressed on murine cells, and thus HCELL plays a uniquely prominent role in mediating human, but not mouse, HSPC migration into marrow (25).

E-selectin ligands are natively expressed on a restricted subset of human CD4 and CD8 lymphocytes, and are conspicuously absent on human B cells. However, α(2,3)-sialylated type 2 lactosamines [Neu5Ac-α(2,3)-Gal-β(1,4)-GlcNAcβ1-R] (**Figure 1**) are characteristically displayed on both human CD4 and CD8 cells, and, therefore, assembly of sLe^X on human lymphocytes pivots on α(1,3)-fucosylation of the sialylated LacNAc "acceptor" structure, i.e., the only component missing is α(1,3)-linked fucose modification of N-acetylglucosamine (GlcNAc). Importantly, sLe^X can only be created by fucosylation of sialylated LacNAc, as there is no mammalian sialyltransferase that can place sialic acid in α(2,3)-linkage to Gal in Le^X to create sLe^X. Thus, the terminal, rate-limiting biosynthetic step for assembly of Le^X and sLe^X in each case involves fucose addition to either an unsialylated LacNAc (for Le^X biosynthesis) or to sialylated LacNAc (for sLe^X biosynthesis) (see **Figure 1**). This "terminal" reaction is programmed by glycosyltransferases known as α(1,3)-fucosyltransferases [α(1,3)-FTs]. In humans, there are six α(1,3)-FT isoenzymes (known as FT3, FT4, FT5, FT6, FT7, and FT9), and four of these are specialized to create sLe^X: FT3, FT5, FT6, and FT7 (19). Of these enzymes, FT7 is the one that characteristically drives expression of sLe^X on human leukocytes, including lymphocytes (18, 26).

GLYCOENGINEERING THE EXPRESSION OF E-SELECTIN LIGANDS: IMPLICATIONS FOR ADOPTIVE IMMUNOTHERAPEUTICS

Human T cells typically display high cell surface expression of CD44, CD43, and PSGL-1, the glycoproteins that can serve as scaffolds for decoration with sLe^X (i.e., that function as E-selectin ligands) (27). However, compared to monocytes and neutrophils that uniformly express E-selectin ligands, only a limited fraction of circulating T cells display E-selectin binding activity (27), and their E-selectin binding characteristically drops during culture-expansion in serum-containing medium (26, 28). Importantly, the absence of sLe^X expression on lymphocyte CD44, CD43 and PSGL-1 is solely a function of underfucosylation, as these proteins display copious amounts of terminal sialylated Type 2 LacNAc motifs (27). Indeed, the levels of sialylated LacNAc typically increase during culture-expansion of human T cells and dendritic cells (28, 29). Accordingly, installation of Fuc in α(1,3)-linkage onto GlcNAc completes the creation of sLe^X on the surface of the cultured cells. This cell surface glycoengineering can be achieved by introduction of nucleic acid encoding the relevant α(1,3)-FTs (30), or by exofucosylation of the cell surface using purified recombinant α(1,3)-FTs together with the donor nucleotide sugar GDP-fucose (18, 31). In regards to clinical applications, it may be preferable to employ α(1,3)-exofucosylation rather than enforced intracellular α(1,3)-fucosyltransferase gene ("*FUT*") expression for a variety of reasons, not the least of which is to avoid the potential of alterations in native glycosylation dynamics by introducing a non-physiologic level of the pertinent glycosyltransferase within the Golgi.

The expression of E-selectin ligands controls cellular entry into marrow, skin, and to all inflammatory sites (18). Studies using adoptively transferred regulatory T cells in xenotransplant models of acute graft-vs.-host disease (28, 32) indicate that enforced sLe^X expression via α(1,3)-exofucosylation promotes cellular entry into inflammatory lesions (32) and also into marrow (28). Results of both preclinical and clinical studies using human HSPCs (33, 34), and preclinical studies of human mesenchymal stem cells (24) reveal that exofucosylation potently programs cellular delivery to marrow and, notably, preclinical studies show appropriate distribution within marrow (24, 33), and clinical administration of exofucosylated human HSPCs improves engraftment kinetics without any adverse effects (34). Thus, enforcing E-selectin ligand expression on CD19-specific CAR-T cells would drive marrow delivery of these cells. Given the constitutive E-selectin expression in dermal microvessels, it would be expected that exofucosylated CAR T-cells would migrate to the skin, but immunoreactivity would only be triggered in presence of relevant infiltrating tumor cells. However, more generally, because E-selectin expression is characteristically upregulated in tumor endothelial beds (35–46), higher E-selectin binding would increase the ability of CAR-T cells targeting a pertinent malignant cell type to enter relevant lesional tissue [i.e., for solid malignancies (e.g., breast, colon, and lung) and lymphoid malignancies (lymphomas and Hodgkin's disease)]. Beyond enhancing treatment efficacy, the more efficient influx of infused cells into sites where needed would limit collateral damage by lessening cytotoxic T cell accumulations in non-lesional tissue, would allow for decreasing the amounts of infused cells, and commensurately, would trim production costs by diminishing the numbers of expanded cells required to achieve the intended clinical effect. Thus, glycoscience-based strategies can literally steer the pathways for CAR T-cells, providing a roadmap for achieving improved patient outcomes using these cells and other types of adoptive cell immunotherapeutics.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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The Role of Glycosphingolipids in Immune Cell Functions

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Glycosphingolipids (GSLs) exhibit a variety of functions in cellular differentiation and interaction. Also, they are known to play a role as receptors in pathogen invasion. A less well-explored feature is the role of GSLs in immune cell function which is the subject of this review article. Here we summarize knowledge on GSL expression patterns in different immune cells. We review the changes in GSL expression during immune cell development and differentiation, maturation, and activation. Furthermore, we review how immune cell GSLs impact membrane organization, molecular signaling, and trans-interactions in cellular cross-talk. Another aspect covered is the role of GSLs as targets of antibody-based immunity in cancer. We expect that recent advances in analytical and genome editing technologies will help in the coming years to further our knowledge on the role of GSLs as modulators of immune cell function.

Keywords: glycans, glycolipids, regulation, expression, immunity, differentiation, receptors, cancer

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INTRODUCTION

The surface of cells is covered with glycans (or carbohydrates) that are part of glycoproteins, glycosaminoglycans, or glycosphingolipids (GSLs). GSLs consist of glycans conjugated to a lipid (ceramide) core and comprise a diverse group of over 300 different complex molecules based on variation in the glycan buildup (1–3). The diversity of glycan structures on GSLs is directed by a range of proteins involved in glycan biosynthesis including glycosyltransferases (GTs), glycosidases, enzymes involved in glycan precursor biosynthesis and nucleotide sugar transporters. These proteins are differentially expressed throughout the immune system giving rise to a large variability in GSL expression patterns which suggests a functional role for GSLs in immune cell development or activation (4). GSLs are essential parts of GSL enriched microdomains (GEMs) in the cell membrane, which have an important role in molecular signaling, cellular cross-talk, and cell adhesion (5–7). Consequently, mice deficient in subclasses of GSLs show immunological, reproductive, neuronal, renal, gastrointestinal, and metabolic defects (8). To date, cell surface GSLs have been shown to be involved in diverse immune processes, including differentiation, immune recognition, and transduction of activation signals. In this review, we summarize the literature on GSL expression of various immune cells and highlight the functions that have been attributed to these GSLs.

BIOSYNTHESIS AND EXPRESSION OF GSLS IN NAÏVE AND DIFFERENTIATED IMMUNE CELLS

GSLs are divided into two groups based on the presence of a galactosylated or glucosylated ceramide (Cer) core. The latter group consists of complex structures subdivided into gangliosides, (iso)globosides, and (neo)lacto-series GSLs (**Figure 1A**; **Table S1**). The GTs UDP-glucose ceramide glucosyltransferase (UGCG) and β1,4-galactosyltransferase 5/6 (B4GALT5/6) synthesize glucosylceramide (GlcCer) and lactosylceramide (LacCer) respectively, forming the precursor of GlcCer-based GSLs (8). GSLs are further divided into four major series based on the synthesis pathways (**Figure 1A**). Alpha2,3-sialyltransferase 5 (ST3GAL5) is the key enzyme for the synthesis of GM3, which is the parent structure for *a*-, *b*-, and *c*-series gangliosides. β1,4-*N*-acetylgalactosaminyltransferase 1 (B4GALNT1) catalyzes the generation of asialo GM2 by transferring *N*-acetylgalactosamine (GalNAc) to LacCer, initializing the synthesis of *o*-series gangliosides. Lactotriaosylceramide (Lc3) is the starting structure for synthesis of (neo)lacto-series GSLs, which is synthesized by β1,3-*N*-acetylglucosaminyltransferase 5 (B3GNT5). The (iso)globosides globotriaosylceramide (Gb3) and isoglobotriaosylceramide (isoGb3) are generated by the addition of a galactose to LacCer in α1,4 and α1,3 linkages by α1,4-galactosyltransferase (A4GALT) and α1,3-galactosyltransferase 2 (A3GALT2) respectively (**Figure 1A**). Further extension and modifications of these core structures, including elongation, sulfation, and sialic acid acetylation, contributes to the diversity of the repertoire expressed in (immune) cells (9–13).

The GSL repertoire of different immune cells varies per cell type under physiological conditions (14–16). The expression of some GSLs on immune subsets is well-studied, and antibodies against them have found their way into the cluster of differentiation (CD) marker set established decades ago. At that time, it was not yet known that these antibodies recognized glycan headgroups of GSLs, and therefore they have been assigned a specific CD-number. This group includes CD15, CD17, CD60, CD65, CD75, CD77, CD173, and CD174 (**Figures 1A,B**), some of which are still being used to phenotype and isolate immune cell subpopulations (17). For example, CD77 represents the Gb3 structure, which has been employed to define a B cell subpopulation. Notably, the specific terminal glycan motifs of CD15, CD75, CD173, and CD174, can be carried by GSLs and glycoproteins. In the following sections, we summarize current knowledge on GSL expression patterns in different immune cell subsets (see **Figure 2** and **Table 1** for an overview).

Abbreviations: GSL, glycosphingolipid; GT, glycosyltransferase; GEMs, glycosphingolipid enriched microdomains; Cer, ceramide; UGCG, UDP-glucose ceramide glycosyltransferase; B4GALT, β1,4-galactosyltransferase; GlcCer, glucosylceramide; LacCer, lactosylceramide; ST3GAL, α2,3-sialyltransferase; B4GALNT1, β1,4-*N*-acetylgalactosaminyltransferase 1; GalNAc, *N*-acetylgalactosamine; Lc3, lactotriaosylceramide; Gb3, globotriaosylceramide; isoGb3, isoglobotriaosylceramide; B3GNT, β1,3-*N*-acetylglucosaminyltransferase; A4GALT, α1,4-galactosyltransferase; A3GALT2, α1,3-galactosyltransferase 2; CD, cluster of differentiation; CTB, cholera toxin subunit B; BMMCs, bone marrow culture-derived mast cells; SMCs, serosal mast cells; PMA, phorbol myristate acetate; Le^x, Lewis X structures, Galβ1-4(Fucα1-3)GlcNAcβ1-; S(3)nLc4, Neu5Acα2-3nLc4; S(6)nLc4, Neu5Acα2-6nLc4; S(3)nLc6, Neu5Acα2-3nLc6; moDCs, monocyte-derived dendritic cells; Galα1-3(F(2))ASGM1, Galα1-3(Fucα1-2)asialoGM1; Fo, Forssman glycolipid antigen, GalNAcα1-3Gb4; BMDCs, bone marrow-derived dendritic cells; NKT, natural killer T; NK, natural killer; NeuGc, *N*-glycolylneuraminic acid; LacNAc-GM1, Galβ1-4GlcNAcβ1-3GM1a; Galα1-3LacNAc-GM1, S(3)LacNAc-GM1, Neu5Acα2-3Galβ1-4GlcNAcβ1-3GM1a; IL, interleukin; IFN-α, Interferon-α; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; TCR, T cell receptor; LXR, liver X receptor; ST, shiga toxin; STb, B subunit of ST; PCI, protein-carbohydrate interaction; CCI, carbohydrate-carbohydrate interaction; EGFR, epidermal growth factor receptor; CAR, chimeric antigen receptor; TLR4-MD2, Toll-Like Receptor 4-myeloid differentiation factor 2; EtxB, enterotoxin subunit B; HIV, human immunodeficiency virus; STb, B subunit of ST; NBDNJ, *N*-butyl 1-deoxynojirimycin. Glycan abbreviations and structures are listed in **Table S1**.

Hematopoietic Stem and Progenitor Cells

HSCs are multipotent cells located in bone marrow which can differentiate into myeloid and lymphoid progenitor cells (**Figure 2**). To date, the GSL content of HSCs has hardly been studied, probably due to the low abundance of HSCs in blood and bone marrow and the difficulty to isolate them (75). Some studies suggest the presence of GM1 on HSCs based on binding of Cholera Toxin B (CTB). However, this glycan-binding subunit B of cholera toxin has a broader specificity then just GM1 (discussed in section Organization of Membrane Microdomains) (76–78). Furthermore, Giebel et al. found that GM3 is expressed and localized at the leading edge of polarized $CD34^+$ HSCs, suggesting a role for GM3 GEMs in the polarization of HSCs (18). With respect to neutral GSLs, Gb5 was detected after exposure to fetal calf serum (19), but not on freshly isolated HSCs, even not as a sialylated or fucosylated variant. This finding is supported by a lack of expression of the relevant GTs in HSCs. Thus, environmental factors may change the expression of GTs, which has to be kept in mind when evaluating GSL expression on cultured or stimulated cells. In addition, CD173 and CD174 (**Figure 1B**), which may be carried by GSLs, are found to be specifically expressed on naïve $CD34^+$ HSCs and disappear after differentiation (79).

In human myeloid progenitors—which give rise to mast cells, granulocytes, monocytes, and bone marrow-derived dendritic cells—GlcCer, LacCer, gangliosides (GM2, GM3, and GD3), and globosides (Gb3 and Gb4) are reported (**Figure 2**). In some studies, (neo)lacto-series GSLs (Lc3 and nLc4) were also weakly detected (20, 23). The mouse myeloid progenitor cell line FDC-P1 displays LacCer, gangliosides (GM1, GM2, GM3, GD1a, GD1b, and GD3), and globoside Gb3, while no GlcCer or (neo)lacto-series GSLs were detected (24). This work further revealed that GM1 and GD1a are the two major gangliosides accumulated by FDC-P1 cells. Reports on GSL expression of lymphoid progenitors, the precursors of NK, T and B cells, are absent in literature. We can conclude that gangliosides are expressed in HSCs and progenitor cells, while globosides and (neo)lacto-series GSLs are hardly expressed in HSCs, and at relatively low levels during further differentiation.

Myeloid Cells

Myeloid cells have been studied for decades and express some unique GSLs. The best described myeloid-specific GSL is a fucosylated neolacto-series GSL which is known as the CD65 antigen (**Figure 1B**) (80–82). It is expressed on most myeloid

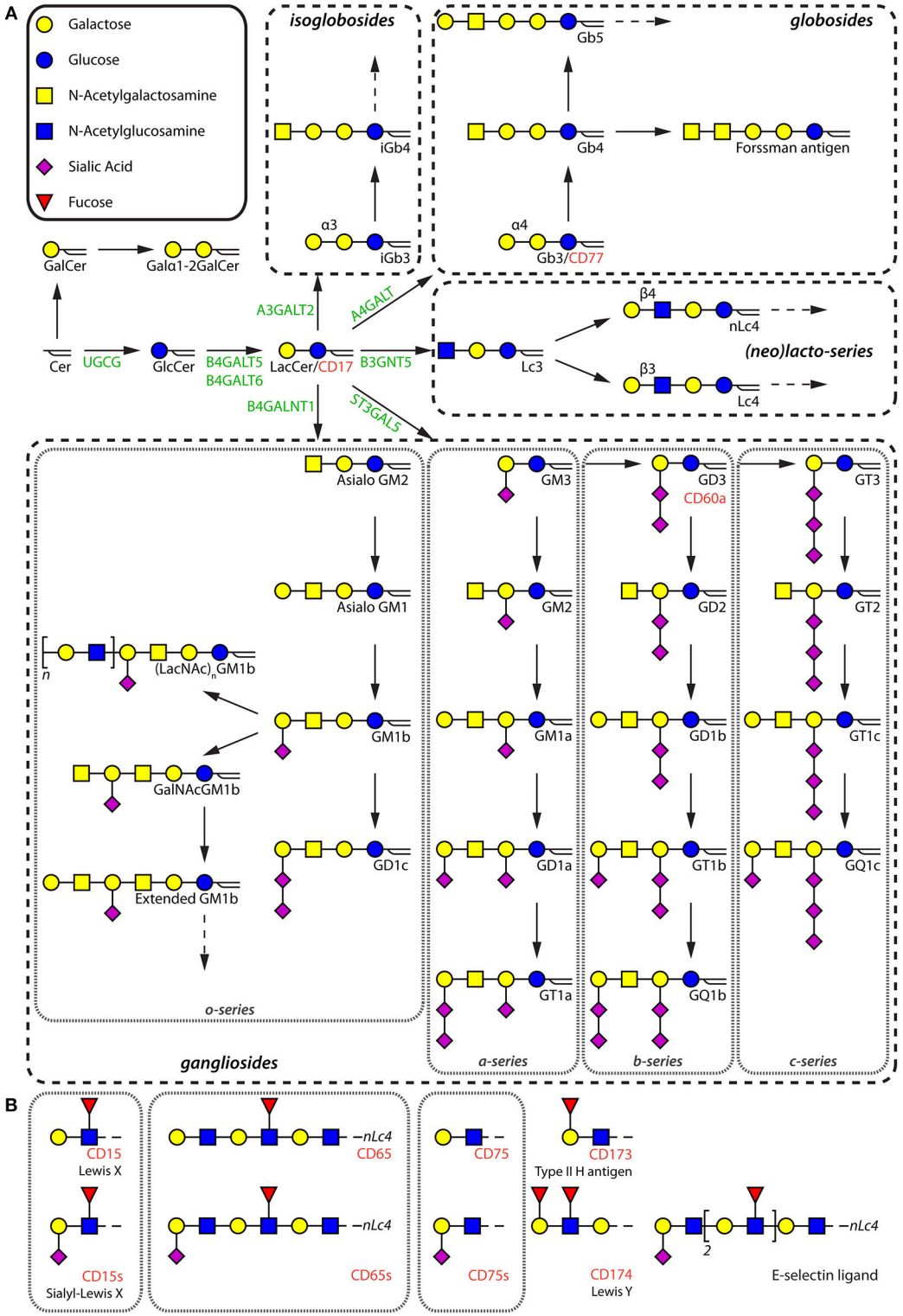

FIGURE 1 | Schematic diagram of the different types of GSLs. **(A)** Major GSLs expressed in immune cells and proposed GSL biosynthetic pathway. The key enzymes are in green. GSLs that have been given a cluster of differentiation (CD) number are annotated in red. **(B)** Terminal glycan motifs that have been given a CD number and the most prominent E-selectin ligand present on human neutrophils.

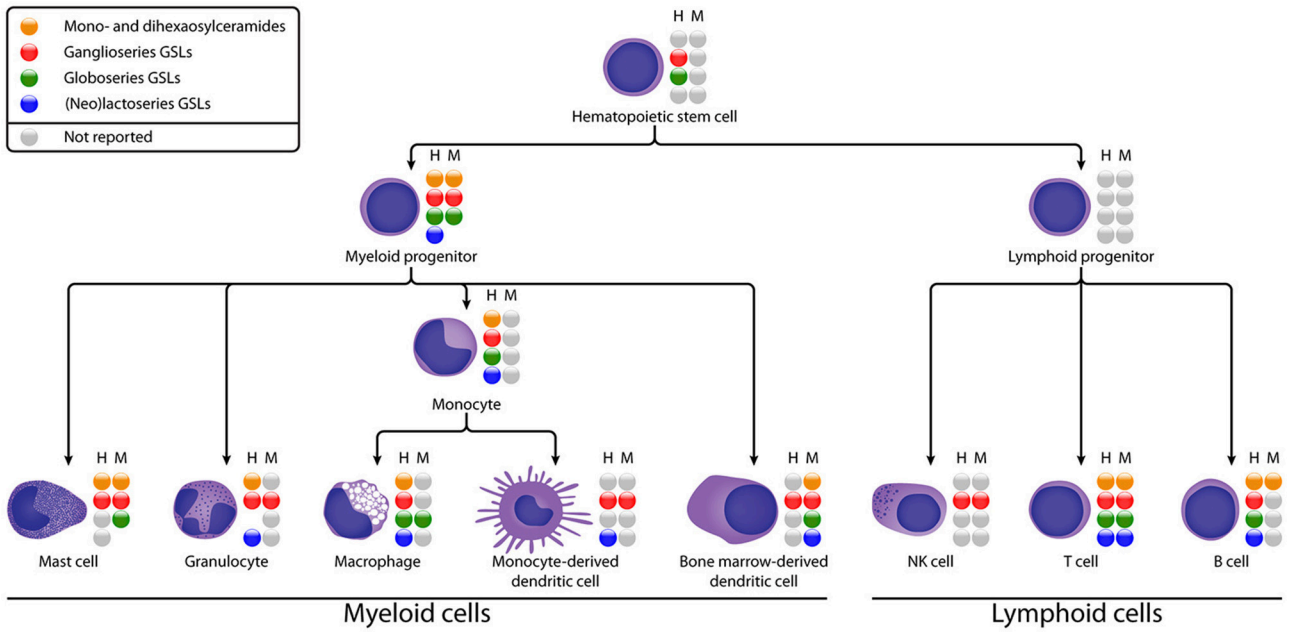

FIGURE 2 | Schematic diagram of GSL expression in different stages of human (H) and murine (M) immune cell differentiation. GSL subsets that have been reported are represented by colored spheres, unreported subsets are represented by gray spheres. The absence of a sphere indicates that the GSL subset could not be detected. See **Table 1** for details on the subset expression.

cells during development, highly on granulocytes and weakly on monocytes in peripheral blood. The sialylated form of CD65 (CD65s) is expressed when the myeloid progenitor antigen CD34 disappears, indicating that CD65s expression marks a turning point in myeloid cell differentiation. In addition to CD65 and CD65s, the expression patterns of other GSLs in mast cells, granulocytes, monocytes, macrophages, and DCs will be discussed in the following sections (**Figure 2**).

Mast Cells

After development from bone marrow-derived progenitor cells, mast cells can circulate as $CD34^{+}$ progenitor cells, or migrate into tissues to differentiate into mature mast cells under the influence of cytokines.

It is well-recognized that GD3 is the most abundantly expressed GSL on the surface of nearly all mast cells (26). Zuberbier et al. studied the alterations of ganglioside expression during maturation of the human mast cell line HMC-1. Upon differentiation, a highly elevated expression of GM3 and GM3-derived *a*-series gangliosides (**Figure 1A**), including GM2, GM1a, and GD1a, were observed as a result of upregulation of the GTs ST3GAL5, B4GALNT1, ST8SIA1, and ST3GAL2 (25). Similarly, mouse serosal mast cells (SMCs) mainly express GM3. The ability to synthesize complex acidic GSLs is possibly lost during mast cell maturation, because *in vitro* differentiated bone marrow-derived mast cells (BMMCs) expressed—next to GM3—GM1, which was lost when matured toward SMC-like cells (29, 30).

Neutral GSLs have not been biochemically analyzed in human mast cells, except for the observation of LacCer in HMC-1 cells (25). For the murine BMMCs, expression of GlcCer, LacCer, asialo GM1, Gb3, and Gb4 has been described, while no (neo)lacto-series GSLs have been reported (27, 28, 83, 84). Interestingly, specifically Gb4 was found to be expressed in secretory granules, where it may have a yet unknown function (28). During *in vitro* activation of BMMCs, surface expression levels of Gb4 increased, which is thought to be the result of the fusion of internal membranes with the plasma membrane (28). Intriguingly, the Forssman glycolipid antigen (Fo), GalNAcα1-3Gb4, is specifically expressed by SMCs and not by BMMCs (27). In contrast to murine cells, only Gb5, but not LacCer, Gb3 or Gb4, was found on rat SMCs (85).

Granulocytes

Neutrophils, eosinophils, and basophils are granulocytes derived from myeloid precursor cells and have similar characteristics and functions in innate immune responses.

Human neutrophils are rich in GSLs, and around 2 mg of GSLs can be extracted from 10^{10} cells. Detailed structural characterization of these GSLs showed neutrophils contain a very complex ganglioside mixture (34, 37, 86, 87). Similar to BMMCs, GM1 and GM3 are the most abundant gangliosides in neutrophils. Compared to other bone marrow-derived cells, mature neutrophils were found to express the highest levels of GM1 (32, 35, 87). Later studies revealed that the presence of GM1 is related to the stage of neutrophil apoptosis, allowing the use of GM1 as an aging marker for neutrophils (40). In contrast to mast cells, neutrophils were not found to express GD3 (34).

With respect to neutral GSLs, human neutrophils express GlcCer, LacCer, and a set of (neo)lacto-series GSLs, but no globoside has been detected (23, 31–33, 35, 39, 88). During differentiation of the promyelocyte cell line HL60 toward

TABLE 1 | GSL expression in human and murine immune cells.

Cell type	Sources	GSL types				
		GlcCer, LacCer, GalCer	Ganglioside	Globosides	(neo)Lacto-series	References
HSCs	Human	N.R.	GM3	Gb5	N.R.	(18, 19)
	Mouse	N.R.	N.R.	N.R.	N.R.	N.R.
Myeloid progenitors	Human	GlcCer, LacCer	GM3[a], GM2, GD3	Gb3, Gb4	Lc3[b], (n)Lc4[b]	(20–23)
	Mouse	LacCer	GM1[a], GD1a[a], GM2, GD3, GM3, GD1b	Gb3[b]	N.D.	(24)
Mast cells	Human	N.R.	GD3[a], GM3	N.R.	N.R.	(25, 26)
	Mouse	GlcCer, LacCer	GM1[a], GM3[a], asialo GM1	Gb3, Gb4, Fo	N.D.	(27–30)
Maturated mast cells	Human	LacCer	GD3[a], GM3, and *a*-series ganglioside (GM2, GM1, GD1a)[c]	N.R.	N.R.	(25)
	Mouse	GlcCer	GM3[a]	N.R.	N.D.	(29, 30)
Neutrophils	Human	GlcCer,LacCer[d], GalCer	GM1[a], GM3[a], complex type, (no GD3)	N.D.	Lc3[d], nLc4, nLc6, S(3)nLc4, S(6)nLc4, S(3)nLc6	(31–40)
	Mouse	N.R.	N.R.	N.R.	N.R.	N.R.
Eosinophils	Human	N.R.	GM1	N.R.	N.R.	(41, 42)
	Mouse	N.R.	N.R.	N.R.	N.R.	N.R.
Basophils	Human	N.R.	N.R.	N.R.	N.R.	N.R.
	Mouse	N.R.	Asialo GM1	N.R.	N.R.	(43)
Monocytes	Human	GlcCer, LacCer	GM3[a]	(iso)Gb3[d], Gb4[d]	Lc3[b],(n)Lc4[b], S(3)nLc4, S(6)nLc4, S(3)nLc6	(36, 44–48)
	Mouse	N.R.	N.R.	N.R.	N.R.	N.R.
Macrophages	Human	GlcCer, LacCer	GM3[a]	Gb3[d], Gb4[d], Gb5	Lc3[b], (n)Lc4[b], S(3)nLc4, S(6)nLc4, S(3)nLc6	(44, 45, 48–52)
	Mouse	N.R.	N.R.	Gb3[d], Gb4[d], Gb5, Fo[c]	N.R.	(53, 54)
moDCs	Human	N.R.	GM3[a]	N.R.	Lc3, nLc4	(55, 56)
	Mouse	N.R.	GM3[a]	N.R.	N.R	(56)
BMDCs	Human	N.R.	GM3[a]	N.R.	N.R.	(56)
	Mouse	LacCer, Galα1-2, GalCer	GM3[a], complex type, (*a*-, *b*- and *o*-series),asialo GM1, asialo GM2	(iso)Gb3, (iso)Gb4, Gb5, Fo	Lc3	(57)
B cells	Human	GalCer, GlcCer, LacCer	GM3[a], complex type (*a*-, *b*- and *o*-series), asialo GM1, asialo GM2, GD3, 7-*O*-GD3 and 9-*O*-GD3	Gb3[d], Gb4[d]	Lc3[b], nLc4[b]	(12, 58–64)
	Mouse	GalCer, GlcCer, LacCer	N.R.	N.R.	N.R.	(65)
T cells	Human	GlcCer, LacCer	GM1[a], GM3[a], complex type (*a*-, *b*- and *o*-series), GD3, 7-*O*-GD3, 7-*O*-GD3	Gb3[d], Gb4	nLc4	(13, 18, 58, 61, 66, 67)
	Mouse	GlcCer, LacCer	GM1[a], GM3[a], complex type (*a*-, *b*- and *o*-series), asialo GM1, extended GM1b (more complex than human)	(iso)Gb3, (iso)Gb4	Lc3	(57, 66, 68–71)
NK cells	Human	N.R.	Asialo GM1, 7-*O*-GD3	N.R.	N.R.	(50, 72)
	Mouse	N.R.	Asialo GM1, GM1	N.R.	N.R.	(69, 73, 74)

N.R., Not reported; N.D., Not detected; [a]Dominant abundance; [b]Low abundance; [c]Specific expression; [d]Dominant abundance among neutral GSLs.

granulocytes using all-trans retinoic acid or phorbol myristate acetate (PMA), the (neo)lacto-series synthase B3GNT5 was upregulated (21, 89). Therefore, Lc3, after LacCer, appeared to be the predominant species accounting for about 10% of the total neutral GSL fraction (38, 90). Notably, the neolacto-series GSLs are the major class in neutrophils, containing Lc3, nLc4, nLc6, and *a*-series of GSLs carrying Le^x (Lewis X structures, Galβ1-4(Fucα1-3)GlcNAcβ1-, also known as CD15 (**Figure 1B**) (35, 38). In addition, sialylated neolacto-series GSLs (S(3)nLc4, S(6)nLc4, and S(3)nLc6) have also been detected (33, 91). The

unique expression of these neolacto-series GSLs by neutrophils in comparison to other immune cells may be required to interact with pathogens or the humoral immune system.

To date, there are hardly any studies on the GSL expression of eosinophil and basophils. Ganglioside GM1 has been detected at the surface of eosinophils, and a stepwise upregulated expression was observed during cell differentiation from the promyelocyte to the eosinophil stage (41, 42). For murine basophils, a high level of asialo GM1 expression has been described (43).

Monocytes, Macrophages, and Dendritic Cells

Monocytes, macrophages, and dendritic cells (DCs) are phagocytic innate immune cells, which drive adaptive immune responses via antigen processing and presentation (92, 93). Monocytes can differentiate *in vitro* into macrophages or monocyte-derived DCs (moDCs) after specific cytokine stimulation. All monocytes, macrophages, and moDCs express high levels of GM3 in both human and mouse (49, 94, 95). Cultured human macrophages yield approximately seven times more GM3 per million cells than *ex vivo* peripheral blood monocytes (2.7 vs. 0.4 μg respectively) (46). Accordingly, such macrophages, but also *in vitro* differentiated moDC express 10-fold higher ST3GAL5 levels compared to freshly isolated monocytes (46, 55, 56, 96). Interestingly, the high expression of acidic GSLs is probably in part also facilitated by a decreased expression of α2,3- and α2,6-sialidases (such as NEU3), which was for example observed in PMA-differentiated THP-1 macrophages (97, 98). Similar to humans and mice, rat abdominal macrophages express GM3 as the predominant acidic GSLs, followed by GM2 (85).

Monocytes and macrophages seem to have a different neutral GSL composition compared to other human myeloid immune cells since they express globosides ((iso)Gb3 and Gb4) as the major neutral GSLs (36, 44, 45, 48, 52). Neolacto-series GSLs such as Lc3 and nLc4 are also detectable and upregulated during differentiation toward moDCs, but are reduced during differentiation toward macrophages as a result of decreased B3GNT5 gene expression (36, 44, 45, 55, 96). Additionally, during macrophage differentiation the expression of Gb5 is upregulated, which—like Gb3—is a target for the human immunodeficiency virus (HIV) gp120 glycoprotein (94, 99). In mouse abdominal macrophages, it has been demonstrated that neutral GSLs are expressed at higher levels than gangliosides. Asialo GM1 was specifically expressed after a 3-day culture, but its expression gradually declined after prolonged cultures. Other neutral GSLs including GlcCer and Gb3 were highly upregulated in macrophage differentiated murine M1 cells (100–102). Fo GSLs are expressed in mature mouse macrophages and increases during the lifetime of the cell. It is used as a differentiation marker and is specifically expressed in defined areas in spleen, lymph nodes, and bone marrow, which suggests it may have a function in lymphoid organ homing or residency (53, 54, 103–105). In addition to the globosides Gb3, Gb4, and Gb5, the specific neutral GSL Galα1-3(F(2))ASGM1 was also found to be highly expressed in rat macrophages (85).

During differentiation of murine bone marrow precursors to bone marrow-derived DCs (BMDCs), no significant change in acidic GSLs nor LacCer or asialo GM1 content was found, even though *a*-series (GM1a, GD1a, and GT1a), *b*-series (GD3, GD1b, and GT1b), and *o*-series (asialo GM1 and GM1b) are generally present in BMDCs (57). However, Lc3, Gb3, Gb4, and Fo GSLs were found to be more abundant on mature BMDCs. Interestingly, Li et al., also described the presence of isoGb3 and isoGb4 to be enhanced in mature BMDC. Though the isoGb3 expression level was very low compared to Gb3, ∼0.8% in both immature and mature DCs. IsoGb3 can be specifically recognized in the context of CD1d by mouse Vα14 and human Vα24 natural killer T (NKT) cells, and plays an important role in regulating NKT cell responses during infections, cancer and autoimmunity (47, 57, 106–108). In addition, a unique Galα1-2GalCer was found in BMDC as well, which can be processed to GalCer for presentation to NKT cells (109). Based on the upregulation of globosides during the differentiation of macrophages, moDCs and BMDCs, globosides function as markers of differentiation (57).

Lymphocytes

Lymphocytes include T cells, B cells, and natural killer (NK) cells (**Figure 2**), which are the main adaptive and innate immune effector cells. GSL expression in B and T cells has been widely studied during differentiation, maturation, and immune responses.

B Cells

After antigen exposure, B cells can differentiate into plasma cells secreting antibodies to clear antigen-bearing entities. Human pre-B cells have a similar GSL-profile to cells of myeloid origin. Human B cells mainly express GM3, but also more complex gangliosides such as GM1, GD1a, GD1b, and GT1 (32, 58, 63). In addition, asialo GM1 and asialo GM2 are expressed in minor amounts (61). Notably, ganglioside GD3 and its *O*-acetylated variants, 7-*O*-GD3 and 9-*O*-GD3 (CD60b and CD60c, respectively), have been described to be expressed on B cells (and also T cells) although the expression levels vary (12, 50, 72). Some of these studies propose an involvement of *O*-acetylated gangliosides in lymphocyte activation processes. Mouse B cells show an even higher expression of the gangliosides GM1 and GM3 and their derivatives compared to human B cells. Interestingly, whereas humans are incapable of synthesizing *N*-glycolylneuraminic acid (NeuGc), gangliosides GM1 and GM3 modified with this sugar are present on mouse B cells. Importantly, the CD22 ligand Neu5Acα2-6Gal-, also known as CD75 (**Figure 1B**), was identified as a major B lymphocyte epitope (95). Additionally, rat B cells lowly expressed Galα1-3(F(2))ASGM1 and some unique extended GM1b structures, which contain the GM1b core extended with LacNAc unit(s), including Galα1-3LacNAc-GM1, Galα1-3$(LacNAc)_2$-GM1, and S(3)LacNAc-GM1 (110).

Both human and murine B cells express GalCer, GlcCer, LacCer, and globosides, but only immature B cells contain (neo)lacto-series GSLs since activated B cells lack expression of the Lc3 synthase B3GNT5 (23, 63, 65, 66). Human peripheral B cells contain relatively large amounts of more complex globosides which are nearly absent in tonsillar B lymphocytes (32, 62).

Importantly, Gb3 (CD77) was initially found to be specifically expressed by germinal center B cells (60, 111). However, it was later identified that not all germinal center B cells express Gb3 (112). In contrast to peripheral and germinal center B cells, GlcCer, and LacCer comprise the largest portion of GSLs in tonsillar B lymphocytes. In addition, Gb3 expression increased 10-fold in a bovine B cell lymphoma cell line after stimulation with different mitogens, suggesting that B cells actively regulate surface expression of Gb3 (113).

Human B cell differentiation and activation are accompanied by sequential regulation of GSL expression via modulation of the corresponding GTs (61, 63, 114). GM3 synthase B4GALNT1 is differentially activated from the pre-B cell stage to the terminally differentiated myeloma (plasma)cells, and GM2 synthase B4GALT has a high activity in lymphoblastoid cell lines and terminally differentiated myeloma cells only. Lc3 synthase B3GNT5 shows a high activity in pro- and pre-B cells, initializing the synthesis of (neo)lacto-series GSLs. But, (neo)lacto-series synthesis is shut down in more differentiated cells. For the expression of globosides, Gb3 synthase A4GALT and Gb4 synthase B3GALNT are only activated in the late stages of B cell differentiation (114). These results explain the stage-dependent expression of GSLs like Gb3, Gb4, GM2, and GM3, suggesting functional roles of GSLs during B cell maturation (63).

T Cells

T cells are the effector cells of adaptive immunity through the production of various cytokines and the activation-induced cell death. Variations in GSL expression have been related to T cell subtype, activation, differentiation, and function (66, 67). Human T cells express both GM1 and GM3, which are clustered in GEMs and thought to be involved in T cell activation (66). Besides these two gangliosides, also minor levels of other gangliosides (GD1a, GD1b, GT1b etc.) have been detected (18, 115, 116). During interleukin-2 (IL-2) stimulation, $CD8^+$ T cells, more than $CD4^+$ T cells, upregulate GM1 expression (117, 118). In contrast, naïve $CD4^+$ T cells stimulated with anti-CD3/CD28 show increased expression of ST8SIA1, driving GD3 expression (119). Similar to B cells, *O*-acetylated variants of the ganglioside GD3 have been described to be expressed by human T cells (10, 12, 13, 50). Desialylation of GSLs was also apparent in T cells, since the sialidases NEU1 and NEU3 are 2- to 3-fold upregulated upon T cell receptor (TCR) ligation of both $CD4^+$ and $CD8^+$ T cells. Interestingly, inhibition of these sialidases resulted in a greater amount of cell surface sialic acids, but also a reduced IFN-γ secretion upon activation of T cells (120, 121). These data indicate that T cell effector function can be modulated by sialic acid bearing GSLs in T cells.

Similar to human T cells, murine T cells express GM3, GM1a, GM1b, GD1b, GD1c, GD3, asialo GM1, and extended GM1b series. Compared to $CD8^+$ T cells, murine $CD4^+$ T cells express higher level of ST3GAL5 to synthesize *a*- and *b*-series gangliosides (GM1a and GD1b). In contrast, $CD8^+$ T cells express more B4GALNT1, resulting in higher levels of *o*-series gangliosides (asialo GM1, GM1b, GalNAcGM1b, and extended-GM1b) (66, 68, 70, 71, 122–126). Although these studies show that stimulation of T cells correlates with elongation of a common GM1b precursor structure, it is as yet unclear how such GSLs contribute to T cell physiology.

The total amount of gangliosides per cell was found to be about 10-fold higher in mature T cells than in thymocytes. This increased level of ganglioside expression mainly resulted from the upregulation of GM1 subclasses and *o*-series gangliosides (GalNAcGM1b and extended-GM1b) in T cells whereas GD1b is downregulated (70, 71). This distinct expression of gangliosides between murine thymocytes and mature T cells suggest a stage and type-dependent expression of gangliosides, similar to B cells (71). Notably, whereas GD1c is highly expressed in both thymocytes and $CD4^+$ T cells, $CD8^+$ T cells downregulate its expression (68, 116, 127). Similarly, GM1a is present on both thymocytes and $CD4^+$ T cells, while only trace amounts are found in $CD8^+$ T cells (70). Compared to the human T cells, activated murine $CD8^+$ T cells also upregulate the sialidase NEU3 and downregulate NEU1 (128). In addition, some unique modified GM1 series, including Galα1-3LacNAc-GM1, Galα1-3$(LacNAc)_2$-GM1, and S(3)LacNAc-GM1 were found in rat thymocytes (110, 129).

With respect to neutral GSLs, both human and murine T cells express GlcCer, LacCer, asialo GM1, globosides, and (neo)lacto-series (57, 58, 67, 71). In murine and rat T cells, quantification of neutral GSLs has revealed that the amount of neutral GSLs was higher in peripheral T cells compared to thymocytes. The major neutral GSLs in thymocytes are globosides while asialo GM1 is the most abundant neutral GSL in mature T cells (58, 69, 130, 131). In addition, some unique neutral GSLs, such as Galα1-3(F(2))ASGM1, have been detected in rat thymocytes (110). The presence of isoGb3 on T cells was recently described, which is recognized by both mouse and human NKT cells when presented by CD1d (57). However, the relevance of this GSL for NKT cells remains to be elucidated since mice that lack the isoGb3 synthesis machinery show a normal phenotype and function (47).

NK Cells

NK cells develop in bone marrow and account for up to 15% of peripheral blood mononuclear cells. NK cell activity is unleashed by a loss of inhibitory signaling of their receptors that recognize MHC class I on a target's cell surface, which often is the case on infected or malignant cells.

To date, the GSL expression on NK cells has not been well-studied. In contrast to NK cell precursors, mature NK cells express asialo GM1 (69, 73, 74, 80). Besides asialo GM1, NK cells in mice have been reported to express GM1 at a relatively high level compared to splenic T cells (69). The ganglioside 7-*O*-acetyl GD3 was found at medium levels in 16% of the $CD16^+$ NK cells (50, 72).

Considerations Concerning GSL Expression Analyses

Many studies have contributed to the current knowledge of GSL expression in immune cells, during development, maturation, or activation. Still, information on GSL subtype expression in several immune cell subsets is incomplete (**Table 1** and **Figure 2**) and in many cases lack structural details, often due to the limitations of the analytical tools employed. Incomplete

structural information poses a challenge in understanding expression, regulation, and function of GSLs in immune cells. Thus, further in depth structural studies are pivotal as a basis for functional investigations.

It is clear though that the subtypes of GSLs are very differentially expressed throughout the immune system, suggesting that GSLs not just constitute a structural requirement for membrane integrity of immune cells but rather play specific roles in their function. For example, (neo)lacto-series GSLs are highly expressed by neutrophils, but not their progenitor cells, suggesting a specific role in neutrophil mediated immunity. This may relate to pathogen recognition through an interaction of neolacto glycans with pathogen-expressed proteins (132). On the other hand, it is curious that the expression of some GSLs by human immune cells significantly differs from their murine counterparts. Does this mean that GSLs are functionally dispensable or at least replaceable? A few functions of GSLs have been identified and will be discussed below. Furthermore, GSL expression alterations in response to cytokines and other modulators have also been observed, suggesting an intricate regulation of synthesis and degradation which will be discussed in the next chapter.

REGULATION OF GSL EXPRESSION IN IMMUNE CELLS

Differentiation and activation of immune cells leads to alterations in the GSL repertoire, likely through modulation of the expression of GTs, glycosidases, glycan precursor synthesizing enzymes, and nucleotide sugar transporters (**Figure 3**) (14–16). Although these processes are well-documented, little information is available on the regulation of GSL expression in immune cells specifically. Nevertheless, the GSL regulation in the context of immune cell differentiation and activation as described in Biosynthesis and Expression of GSLs in Naïve and Differentiated Immune Cells, is often regulated by well-known signals, such as cytokines. We will now further focus on the molecular details of such external signals on the regulation of GSL synthesis and expression in immune cells.

Regulation of GSL Expression by Cytokines

It is yet largely unclear what the intracellular switches and master regulators of GSL expression are. Knowledge of cytokine-induced signaling cascades, whether or not in the context of differentiation or activation, is important to understand GSL regulation and may provide opportunities for the design of intervention strategies. Up to now, regulation of GSL expression on immune cells has mainly been studied by addition of key cytokines such as interleukins, interferon-α (IFN-α), and tumor necrosis factor-α (TNF-α) (**Figure 3**).

IL-4 and especially IL-6 induce expression of Fo GSLs at early stages of mouse BMDM culture, but neither could promote further Fo GSL expression once the intrinsic maximum of these cells had been reached (104, 105, 133). The mechanism of these IL-4 and IL-6 regulated differences in GSL composition is still unclear. One option may be that these interleukins coordinate GSL synthesis through modulation of the nucleotide sugar metabolism. IL-4 and IL-13 have the ability to upregulate the levels of UDP-GlcNAc which is a key nucleotide sugar donor for GSL synthesis. The increased activity of corresponding transcriptional enzymes involved in the production of these intermediates (e.g., Enpp1, Pgm1) was reported for IL-4 activated M2 polarized macrophages as well, and was not observed in IFN-γ and toll-like receptor-induced M1 macrophage polarization (134). An alternative mechanism of GSL regulation was provided by overexpression of IL-3 in mouse NFS60-17 cells, which leads to the specific synthesis of GD1a (114, 123, 135). This change in GSL expression is caused by increased GM3 synthase levels, since other GTs involved in GD1a synthesis were not significantly altered by IL-3 expression. Thus, regulation of GT expression can result in a shift in the GSL repertoire, in this case from *o*-series to *a*- and *o*-series gangliosides (**Figure 3**). IFN-α induces more significant alterations in GSL biosynthesis in mouse B cells compared to other cytokines, including IL-6 and IL-10. In particular, GlcCer, LacCer, and Gb3 are significantly upregulated (65). These changes were attributed to the enhanced expression of UGCG and A4GALT. IFN-α also represses α-galactosidase that catalyzes the degradation of Gb3 further contributing to Gb3 accumulation (65). The effect of TNF-α on GSL expression has been described in several studies. TNF-α binding to its cognate receptor TNFR1 has been reported to enhance ceramide production by upregulating the acidic sphingomyelinase, a ceramide generating enzyme (136, 137). Furthermore, TNF-α increased Gb3, GM2, and GM3 through increased transcription of their specific synthases (134, 138–140). TNF-α also mediated upregulation of GM2 in tumor cells and accelerated tumor-induced T cell apoptosis and immune dysfunction. Furthermore, TNF-α was found to activate sialidases through p38 mitogen-activated protein kinase in lipopolysaccharide(LPS)-stimulated human monocytes, suggesting that TNF-α-induced p38 activation may regulate GSL expression (141).

Regulation of GSL Expression by Other External Signals

Not only cytokines but also other factors have been observed to alter GSL expression. The presence of high-affinity FcεRI is suggested to contribute to the expression of gangliosides. FcεRI positive HMC-1 cells expressed 3-fold higher levels of GM3 compared to the FcεRI negative counterparts. Furthermore, detectable amounts of the gangliosides GM2, GM1, and GD1a were found only in the FcεRI positive HMC-1 cells, with a corresponding increase of mRNA for GalNTs in the presence of the FcεRI. These findings suggest that FcεRI signaling enhances ganglioside production (25). Similarly, TCR stimulation on naïve $CD8^+$ T cells upregulated GM1 expression, which is crucial for responding to self-MHC ligands and IL-2 (117). GM1 levels declined after cell transfer to MHC-I^{low} ($Tap^{-/-}$) mice, indicating that maintenance of GM1 expression required continuous TCR-MHC-I interaction. By contrast, $CD4^+$ T cells

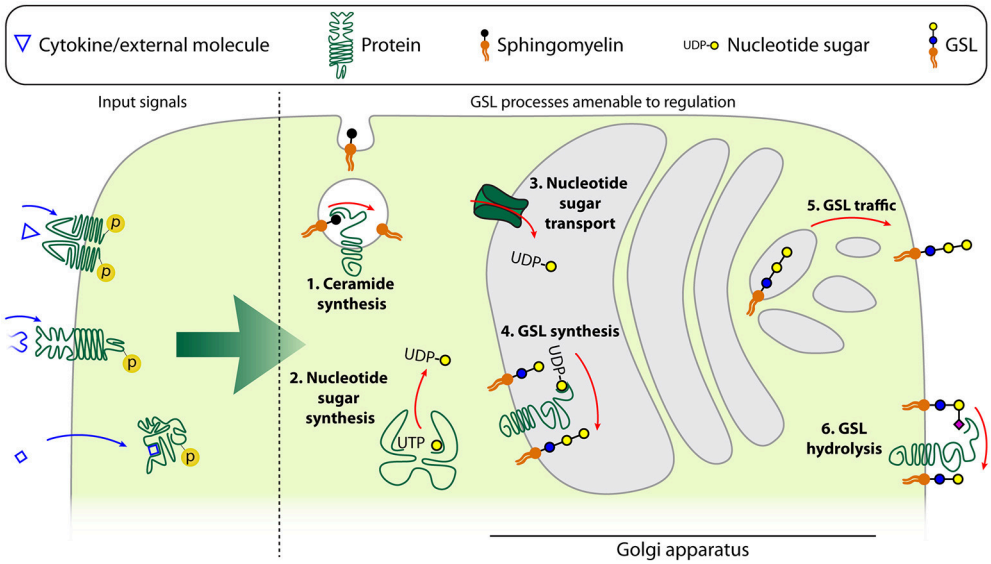

FIGURE 3 | Schematic model of the different levels of GSL regulation. Input signals described to be able to affect the GSL repertoire of a cell are either cytokines, other ligands with membrane-bound receptors or ligands with intracellular receptors. These affect the GSL repertoire by changing the expression or activity of ceramide synthases such as acidic sphingomyelinase (1), nucleotide sugar synthases in the cytoplasm (2), nucleotide sugar transporters which transport the nucleotide sugars into the Golgi apparatus (3), glycosyltransferases (4), trafficking of the GSLs from the Golgi apparatus to the plasma membrane (5), or glycosidases (6).

expressed low amounts of GM1 and were unresponsive to IL-2 (117). In addition, both NEU1 and NEU3 mRNAs were significantly induced in human T cells by TCR stimulation, potentially leading to a decrease of sialylated GSLs (**Figure 3**) (120). Wang et al. further revealed that NEU3 is expressed as a major isoform in activated cells. Transcription of NEU expression in T cells is enhanced by FLI1, whose activity is potentially driven by TCR stimulation. Genetic reduction of FLI1 expression in T cells thus decreased NEU1 and NEU3 levels but also overall GSL expression. However, the mechanism by which FLI1 influences GSL expression is not clear yet (118). GSL levels on $CD4^+$ T cells can also be boosted by stimulation with synthetic liver X receptor (LXR), which signals through the nuclear receptor LXRβ. Stimulation of LXR is known to directly control expression of NPC1 and NPC2 proteins, which regulate cellular GSL transport and recycling (**Figure 3**). Therefore, an elevated LacCer, Gb3, and GM1 expression in $CD4^+$ T cells with highly expressed LXRβ was achieved, which associated with accelerated and sustained GSL internalization and recycling dynamics. Interestingly, this enhanced GSL expression is not correlated with changes in synthase expression but rather associated with the intracellular accumulation and accelerated trafficking of GSLs (67). Yet another GSL modulating stimulus is heparin, which modulates the expression of GSLs in lymphocytes activated by IL-2. Heparin treatment induces downregulation of certain GSLs, including GM1, GD1a, LacCer, asialo GM1, and asialo GM2, whereas globoside and Fo antigen levels are elevated. These changes were attributed to heparin-mediated inhibition of α2-3 sialyltransferase and a β1-3 galactosyltransferase, possibly via heparin-binding domains (142).

Considerations on Regulation of GSL Expression

GSL expression is highly controlled at multiple levels, such as the availability of nucleotide sugars and glycosyltransferases (**Figure 3**). Our understanding of how the GSL synthesis pathway is regulated in specific immune cells needs be improved. The fact that the physiological role of most immune cells is known will then provide opportunities to unravel molecular functions of specific GSLs in these cells. In addition, various laboratories have identified environmental factors that manipulate the GSL repertoire by seizing on components of the GSL synthesis pathway. The limited number of papers describing such regulation of GSL synthesis clearly indicates that this is an underexposed field. Moreover, the available data seems to be biased toward the more well-known soluble proteins. We expect many more GSL regulatory factors to exist that are not yet linked to GSL synthesis. The identification of such GSL modulatory processes may have implications for GSL manipulation in research and potentially even in clinical contexts.

FUNCTIONS OF GSLS ON IMMUNE CELLS

Organization of Membrane Microdomains

GSLs are mainly known for their role in membrane organization which is a dynamic process, especially during activation and differentiation of immune cells. In resting immune cells, GEMs (**Figure 4A**) are suggested to be unstable and small in size. Immune cell activation triggers a change in localization of receptors and signal transducers, in many cases to or from GEMs, which is required to bring receptors and signal

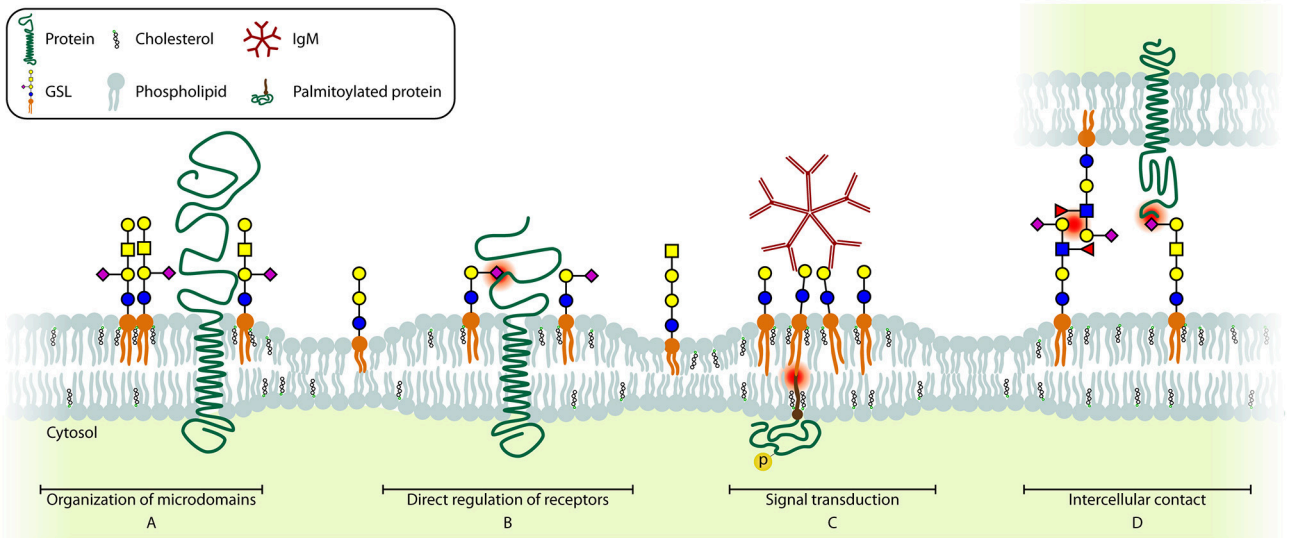

FIGURE 4 | Schematic model of the different GSL functions. Essential glycan-glycan, protein-glycan, and lipid interactions are highlighted (red dot). **(A)** GSLs are involved in including and (not shown) excluding proteins from microdomains. **(B)** Several receptors can be directly regulated by GSLs present in the cell membrane. **(C)** Crosslinking of several GSLs can induce signaling across the membrane. **(D)** GSLs can interact with glycans (CCI, left) or with proteins (PCI, right) on other cells, contributing to cell-cell recognition and adhesion.

transducers in close proximity to enable signaling (143, 144). The best described example in T cells is the activation-induced recruitment of the TCR/CD3 complex to GM1 GEMs together with downstream signaling molecules Lck, SLP-76, and palmitoylated LAT. At the same time, the phosphatase CD45 is excluded from GEMs, further increasing the sensitivity of the TCR (145–151). Additionally, the IL2Rβ is recruited to GM1 GEMs upon stimulation, which is required for its signaling (117). Interestingly, when GM1 GEMs were crosslinked by CTB and anti-CTB antibodies, TCR-like signaling was observed, suggesting that multiple signaling molecules are brought together by crosslinking multiple GM1 GEMs, which indicates a diversity in GM1 GEMs content in different plasma membrane patches (148). General disruption of GEMs in T cells results in a lack of receptor recruitment and exclusion from the immunological synapse, which causes desensitization for ligands and greatly reduced or absent T cell activation. Interestingly, no difference in T cell development has been observed in mice with a T cell specific deletion of UGCG. However, no functional characterization was performed on these T cells other than PMA/ionomycin stimulation, which bypasses signaling from the membrane. In contrast, the development of invariant NKT cells that recognize CD1d-restricted antigens was found to be impaired in these mice (152).

Once B cells encounter an antigen, caveolin-1 recruits the IgM BCR to GM1 GEMs (153–155). The lack of caveolin-1 results in impaired BCR signaling which results in decreased receptor editing and ultimately autoimmune B cells (155). Also MHC-II molecules on B cells require clustering to GM1 GEMs in order to efficiently trigger $CD4^+$ T cell help at low ligand densities (156, 157). Additionally, B cells in $B3GNT5^{-/-}$ mice, which lack (neo)lacto-series GSLs, display alterations in the structure of GM1 GEMs containing BCR, CD19, and Lyn, resulting in increased antigen sensitivity. Consequently these B cells are also more prone to generate autoreactive antibodies (158).

Thus, in both T and B cells, GM1 is part of GEMs that have a crucial role during activation of these cells. The fact that other GSLs expressed by these cells (see section Biosynthesis and Expression of GSLs in Naïve and Differentiated Immune Cells) have not been investigated in this context is likely due to a lack of detection and visualization methods. Importantly, the plasma membrane may contain a large diversity of domains or GEMs with a slightly different (glycosphingo)lipid and protein content as proposed in the revised Fluid-Mosaic Membrane Model (144). Each domain with physically distinct properties may have a specific function, also in immune cells (144). Techniques to visualize these nanometer-domains without introducing artifacts are still scarce. While detection of GM1 by cholera toxin CTB is a broadly accepted method, probes that are considered specific for other GSLs are less well-established, introducing a strong bias toward GM1 GEMs investigations. Therefore, the function of other GSL containing membrane domains and their role in membrane organization is still largely unclear.

Direct Regulation of Immune Receptors

A second function of GSLs is their direct regulation of surface protein function (**Figure 4B**). One of the best-described interactions between GSLs and proteins is the interaction between insulin receptor and GM3. Insulin receptor binds the acidic GM3 through a basic lysine residue (K944) resulting in inhibition of insulin-induced signaling. Thus, upregulation of GM3, for example after TNF-α stimulation, results in insulin resistance (139, 159). Similarly, autophosphorylation of the Epidermal Growth Factor Receptor (EGFR) in the absence of

EGF is prevented by binding of GM3 via a lysine residue (K642) (160).

On immune cells, only few GSL-receptor interactions have been reported, often with limited molecular details. Upon activation of the Toll-Like Receptor 4-Myeloid Differentiation factor 2 (TLR4-MD2) with LPS, Gb4 synthesis is upregulated in mouse endothelial cells. Gb4 can bind the TLR4-MD2 complex to desensitize the LPS-activated signaling pathway thus representing a negative feedback loop (161). Since Gb4 and the TLR4-MD2 complex are also expressed on early human myeloid cells and mature monocytes (23), one may speculate that a similar regulation applies to human immune cells. Interestingly, GlcCer on the membrane of macrophages is essential for efficient LPS-induced TLR4-MD2 signaling since inhibition of GSL-synthesis prior to incubation with LPS significantly reduced cytokine release. *In silico* simulations to explain these observations suggest that GlcCer induces a conformational change of TLR4 thereby enhancing the interaction between TLR4 and the intracellular signaling molecule Mal (52).

In T cells, CD4 interacts with GM1, and additional GM1 incorporation into the membrane results in masking of some CD4 epitopes for antibodies and a subsequent internalization of CD4 molecules, with the underlying mechanisms being unknown. Additionally, GM1 binds PI3K whereas GM3 binds LCK. In order to get successful T cell activation LFA-1 links CD4 and PI3K to LCK by binding both GM1 and GM3 (162, 163).

GSLs are also important for strengthening protein-protein interactions in tetraspanin-rich microdomains. An example of the stabilizing function of GSLs is the enhanced binding of the tetraspanins CD9 and CD82 to integrins in the presence of GM3 and GM1, respectively (164, 165). Thus, GSLs may impact integrin mediated immune cell migration (166). Additionally, loss of functional tetraspanin-rich microdomains results in uncontrolled receptor activity, such as uncontrolled activation of the MET receptor tyrosine kinase and decreased EGFR sensitivity (165, 167). CD82 expression also correlates with increased GM1 and GD1a levels on the cell surface, suggesting an interplay between GSLs and tetraspanin expression either by increasing GSL synthesis or by extending the half-life of GSLs on the plasma membrane (168).

Since CD19 shares amino acid sequences with the Gb3 binding domain of the *E. coli* produced verotoxin, the Daudi B cell line was modified to lack Gb3, which impaired CD19 surface expression. However, the mechanism was not elucidated and since only a subpopulation of germinal center B cells express Gb3 while CD19 is expressed on all B cells, the finding may be an artifact of the cell system that was used (112, 169). Using the same approach MHC-II was identified as another protein that contained a possible Gb3 binding domain, which could be relevant in for example germinal center reactions of B cells, but also for other professional antigen presenting cells such as macrophages and DCs which also express considerable amounts of Gb3 (see section Biosynthesis and Expression of GSLs in Naïve and Differentiated Immune Cells). Unfortunately, no binding data are available for the MHC-II-Gb3 interaction, thus the functionality of these domains is still unclear (170).

Activation of Notch by its ligand Delta-like 1 (Dll1) is dependent on binding of Dll1 to LacCer. Either mutating the LacCer binding site of Dll1 or inhibiting GSL synthesis impairs the capacity of Dll1 to activate Notch (171). This may be relevant during T cell development, where Notch signaling plays a major role (172).

The internalization route of Fas receptor upon ligation with Fas ligand is determined by its interaction with LacCer or Gb3 which results in an endocytotic pathway leading to apoptosis, while the GSL-independent route induces proliferation and differentiation (173). Expression of Gb3 by B cells (112) during the germinal center reaction may support the apoptotic events required for B cell selection.

Besides direct interactions between GSLs and proteins described above, there are also reports on interactions between *N*-glycans and GSLs. The ganglioside GT1b can interact with mannose residues on the *N*-glycan of the α5-integrin, thereby inhibiting integrin-fibronectin interaction (174). Regulation of integrin activity by GT1b may play a role in T cell development, where α5β1 integrin signaling plays a role in T cell selection (175, 176).

GSLs as Signal Transducers

Direct interaction of GSLs with surface receptors may thus have profound impact on signaling events. But GSLs can also transduce signals across the membrane themselves (**Figure 4C**). Crosslinking GSLs by multivalent binders such as bacterial toxins CTB and Shiga Toxin (ST), or alternatively IgM antibodies, has been found to increase intracellular calcium levels that in turn activate Syk (177, 178). This influx of calcium ions upon GM1 crosslinking on the cell surface may be through modulation of L-type calcium channels. Additionally some GSLs regulate intracellular calcium levels by affecting the function of the calcium-dependent messenger protein calmodulin (179, 180). The result of Gb3 crosslinking using ST or anti-Gb3 mAbs in germinal center B cells induces recruitment of Lyn/Syk and the BCR and subsequent internalization of the complex leading to apoptosis (181). Interestingly, the pathways leading to apoptosis differ between ST or anti-Gb3 mediated crosslinking of Gb3 (182–185). Similarly, crosslinking of $GM1^+$ patches in T cells using crosslinked CTB induces LCK-dependent TCR-like signaling (148). Interestingly, crosslinking of $GM1^+$ patches by the *E. coli* heat-labile enterotoxin B induces apoptosis in $CD8^+$ T cells specifically (186). However, there are some doubts on the specificity of these two toxins, which may explain differences in results obtained.

In neutrophils, the kinase Lyn is associated with LacCer enriched microdomains. Crosslinking of these microdomains by anti-LacCer IgM antibodies induces Lyn activation and ultimately leads to superoxide production (39). This signal transduction from LacCer molecules to the palmitoylated form of Lyn is dependent on the length of the fatty acid chain of the GSLs; Lyn is only activated when the fatty acid chain contains 24 carbon atoms and not with shorter fatty acids of 22 or 16 carbon atoms, suggesting that the signal is transmitted within the lipid bilayer relying on specific interactions of the lipid tails (187, 188). Although the length of the fatty acid chain also

influences the general membrane organization and association with proteins which is not addressed yet, a similar association has been described for Lyn and c-Src with photoactivatable GD1b in rat cerebral granule cells (189).

Intercellular GSL Functions

There are two mechanisms by which cells interact with GSLs on other cells; via protein-carbohydrate interaction (PCI), and via carbohydrate-carbohydrate interaction (CCI) (**Figure 4D**). Proteins known to engage in PCI are called lectins, and human lectins may be grouped into three major classes; (1) selectins, that typically bind glycans that are both sialylated and fucosylated, (2) siglecs, which bind sialylated glycans, and (3) galectins, that bind glycans with a terminal galactose. The function of these lectins differ per cell type, with selectins being the major mediators of cell-cell adhesion, particularly between activated endothelial cells and leukocytes. Siglecs specifically interact with sialic acids and are mainly found on hematopoietic cells. Galectins, on the other hand, often bind terminal galactoses and can modulate cell growth, apoptosis, differentiation, and migration (190).

CD83 is an I-type lectin adhesion receptor that is mainly expressed by mature dendritic cells but is also found on activated B and T cells. CD83 interacts with sialic acids on monocytes and activated $CD8^+$ T cells and is required for efficient T cell activation (191). Although the ligand for CD83 was identified as a glycan carried by a glycoprotein on the T cell line HPB-ALL, the authors do not rule out the possibility of ligands carried by GSLs (192).

The sialic acid binding receptor on B cells, CD22 or siglec-2, recognizes α2,6-linked sialic acids that are predominantly expressed in eukaryotes. When the B cell is in an inactive state, CD22 is associated with sialic acids on the B cell surface. However, once the B cell becomes activated, the CD22 is unmasked, and can engage in *trans*-interactions with sialic acids on other cells which induces inhibitory signaling (193, 194). NK cell activation may be controlled by siglec-7 in a similar manner (195, 196). The current hypothesis is that these interactions prevent activation of auto-reactive B and NK cells (197).

Cell-cell interaction in the immune system is critical at sites of inflammation. Inflammation-mediated activation of endothelial cells upregulates selectins like E-selectin in order to recruit immune cells (198). The ligand for E-selectin on neutrophils is a GSL that contains poly-LacNAc repeats with at least two fucose residues and a terminal sialic acid, but E-selectin may also bind GSLs and glycoproteins containing the sialyl-Le^x motif (**Figure 1B**). This interaction is of low affinity and induces typical neutrophil rolling on the endothelium, which is required for transmigration afterwards (199).

CCIs are studied to a lesser extent compared to PCIs. They are involved in early embryogenesis, where the compaction of the embryo is dependent on Le^x structures [for review, see (200)]. Additional reports on CCI describe the interaction between GM3 or Gb4 and asialo GM2 (201). Although a single CCI is generally of very low binding affinity, the carbohydrates may be so prevalent that they may act as a zipper to mediate strong cell-cell adhesion, comparable to CPI or even protein-protein interaction (200, 202).

Although still poorly understood, B cells communicate by forming nanotubes in certain differentiation stages which correlate with expression of GM1 and GM3. The formation of these nanotubes was inhibited by methyl-β-cyclodextrin induced cholesterol depletion, which destroys the integrity of GEMs. Furthermore, only cells with high levels of raftophilic sphingomyelin and phosphatidylcholine generated nanotubes. Thus, the formation of these nanotubes depends on functional GEMs which is possibly related to their GSL contents (203).

Considerations on Molecular Functions of GSLs

GSLs clearly play a role in immunological processes involving cell-cell recognition, adhesion, and communication. However, most of the studies merely provide evidence that certain GSLs are required or sufficient for a particular process, while the exact molecular role of such GSLs remains to be identified for most of these processes. Such mechanistic studies are sparse for a reason, because molecular evidence is often hard to obtain with the current tools. Furthermore, the studies are still limited to a few specific GSLs and do not cover all GSL subtypes. For example, (neo)lacto-series GSLs have largely been neglected in investigations. The relatively recent generation of B3GNT5 knockout cancer cell lines and mice are important initiatives to extend our knowledge on the physiological role of these elusive GSLs (158, 204). Thus, many aspects of GSL functions are still unclear and require further in depth investigations.

RELATIONS BETWEEN GSLS AND IMMUNITY IN DISEASE

Congenital diseases, infections, and cancer showcase aberrant GSL expression, which provides opportunities to gain new insights in (dys)regulation and functions of GSLs. Such knowledge may provide new targets for therapeutic intervention, of which the most recent developments are described in section Targeting GSLs: Opportunities for Treatment.

Gaucher Disease

Patients with Gaucher disease lack the enzyme glucosylceramidase, which is required for the breakdown of GlcCer. Besides neuronal abnormalities this disease is characterized by the presence of large "Gaucher cells" which are macrophages with accumulated GlcCer in lysosomes that concentrate in the spleen and bone marrow. The formation of splenic Gaucher cells is enhanced by rapid splenic clearance of defective red blood cells by macrophages (205). Patients suffering from Gaucher disease are treated either with enzyme replacement therapy or with substrate reduction therapy which consists of the administration of UGCG inhibitors such as *N*-butyl-deoxynojirimycin (Miglustat) (205, 206).

Infection

Various pathogens dysregulate the cellular GSL metabolism, leading to different compositions of the cell surface GSL repertoire. The $p40^{tax}$ protein encoded by the human T cell lymphotropic virus, can induce GD2 expression by upregulating

B4GALNT1, which is normally not expressed in T cells (207). Similarly, it was shown that cytomegalovirus (CMV) induces enhanced synthesis of GSLs, of which specifically (neo)lacto-series remain expressed long after initial infection (208, 209). Additionally, herpes simplex virus alters gene expression of a variety of GTs. The significance of these changes still need to be addressed since the authors could not detect major differences in the profile or total amount of GSLs after infection (210). A potential reason for such dysregulation may be to escape from detection and elimination by the immune system.

Several infectious pathogens and toxins are well-known to use GSLs as cellular entry receptor. Next to CD4, HIV can infect cells through Gb3 and possibly also GM3. *Shigella* bacteria target only activated $CD4^+$ T cells likely through their GM1 and GM3 expression which was inhibited by exogenously added LPS, suggesting a direct interaction between LPS and the gangliosides (211). This would imply that also other gram-negative bacteria may enter host cells through binding of their gangliosides (212).

A variety of bacterial toxins have been described to target GSLs using their binding subunit (B subunit) in order to bring their enzymatically active subunit (A subunit) inside the cell. In 1973, one of the best known toxins, cholera toxin, was described to bind GM1 (213). Although generally used as a marker for GM1, CTB can bind asialo GM1, Fuc-GM1, GD1a, GD1b, GT1b, GM2, GM3, and also to Le^x on glycoproteins although usually with lower affinity. Similarly, it was long thought that enterotoxin B was GM1-specific, until it was shown to cross-react with asialo GM1, GD1b, LacCer, and several galactoproteins (214–216). The B subunit of shiga toxins (STb) and verotoxins associate with Gb3, although all bind Gb3 in a slightly different way (217). Since STb binding to Gb3 induces endocytosis and Gb3 is present on DCs, some research has been devoted to exploiting STb for tumor vaccination (218). However, STb elicited a cytotoxic effect through binding of an *N*-glycan on HeLa cells, suggesting this strategy may have serious side-effects when applied in humans (219). The toxic effects of tetanus toxin and botulinum toxin were greatly reduced in B4GALNT1 (GD2-synthase) deficient mice, suggesting their natural ligands are at least partly complex gangliosides (220). Confirming these findings, type A botulinum progenitor toxin bound asialo GM1, nLc4 and *N*-glycans containing a terminal Galβ1-4GlcNAc (221). Despite these health risks, the physiological function of specific GSL structures was apparently too critical to be efficiently counterselected against during human evolution. Although GSLs are essential during embryonic development, this may also partially be due to the versatile roles of GSLs in immunity.

Finally, several bacteria have the capacity to bind GSLs but it is currently unclear what the pathophysiological reason is for this phenomenon. *Helicobacter pylori*, a microaerophilic organism that can cause severe gastritis, binds to sialic acid-containing GSLs on neutrophils, thereby activating the neutrophil to produce reactive oxygen species (222, 223). Interestingly, neutrophils can phagocytose the bacteria but it seems able to escape the immune cell and cope with the immune response (222, 224). *Neisseria* bacteria, mostly known for their genera *meningitides* and *gonorrhoeae*, are also capable of binding GSLs, although it differs per strain which GSLs they adhere to. *N. subflava* binds sialylated GSLs on erythrocytes by its adhesin Sia1 (225) whereas *N. gonorrhoeae* has an adhesin binding LacCer and asialo GM1 (226). *N. meningitides* binds a wider array of GSLs; LacCer, asialo GM2, asialo GM1, nLc4 but also sialylated nLc6 (227). Additionally, phagocytosis of *N. meningitidis* by neutrophils appears to depend on their expression of (neo)lacto-series GSLs since it is blocked by the LacNAc-Gal-binding antibody 1B2 (228).

The importance of GEMs for the phagocytosis of yeast, such as *Cryptococcus neoformans*, by macrophages has been well-defined since disruption of GEMs using methyl-β-cyclodextrin decreases internalization (229). However, Jimenez-Lucho et al. have shown specific binding of *C. neoformans*, *Candidia albicans,* and other fungi to LacCer, suggesting indeed a role of these GSLs as adhesion receptors for yeast (230). This was confirmed by the identification of an interaction between the bacterial and fungal cell wall polysaccharide β-glucan and LacCer on neutrophils, which triggers superoxide production and CD11b/CD18-mediated phagocytosis of the pathogen (231). These examples indicate potential pathways for different pathogens to be captured by phagocytes, which play an important role in the antimicrobial defense. Moreover, the specific GSL repertoire of neutrophils may allow for improved detection of bacteria, or other pathogens, and possibly contribute to fight infections.

Cancer

Tumors often express high levels of GSLs, which interferes with the killing capacity of the immune system. These high levels of GSLs result, either via an active or passive process, in high concentrations of free GSLs in the tumor microenvironment. For some tumors, such as neuroblastoma, the plasma concentration of tumor-derived GSLs was 50 times elevated as compared to the same patients after treatment or healthy controls (232, 233). Multiple modes of action have been described for the immunosuppressive characteristics of free GSLs.

A portion of T cells isolated from renal cell carcinoma were found to be GM2 positive, while lacking the machinery for GM2 synthesis, suggesting the T cells adopted the GM2 from the tumor microenvironment. These T cells exhibited increased rates of apoptosis compared to their GM2 negative counterparts (234). In addition, *ex-vivo* T cells treated with renal cell carcinoma-derived gangliosides also show a decrease in NFκB signaling (235). T cells incubated with exogenous GD1a lose cytotoxicity since polarization and exocytosis of lytic granules is inhibited, we speculate this may also be due to incorporation of soluble gangliosides in the plasma membrane, disrupting the organization required for proper T cell function (236). Additionally, $CD4^+$ T cells cultured in the presence of GT1b led to a shift from an IFN-γ secreting type-1 phenotype to an IL-4 producing type-2 phenotype (237). Finally, various individual brain-derived gangliosides inhibit T cell proliferation possibly through competing for the IL-2 binding place on the IL-2 receptor or via direct binding to cytokines such as IL-4 and IL-15 (238–241).

Similar to T cells, also DC differentiation and maturation is inhibited by gangliosides through inhibition of NFκB signaling

(242, 243). Besides, brain-derived gangliosides inhibit MHC-II antigen presentation by monocytes (244). GM2 and GM3 shed by melanomas were potent inhibitors of Fc receptor expression on monocytes and macrophages whereas GM1 and GD3 inhibited IL-1β production (245). Similarly, GM2 and GM3 were potent inhibitors of NK cell activity. Since GM2 showed reduced effector-target cell binding and GM3 did not, they are likely to act through different mechanisms (246). IL-3 dependent proliferation of BMMCs was inhibited by GM3, but in contrast to earlier proposed mechanisms, the authors excluded direct GM3-IL-3 interaction. However, it remains unknown whether the mechanism may be through competition with IL-3 for the IL-3 receptor (247). In summary, high concentrations of gangliosides shed by tumors lead to a downregulation of the cellular immune response.

Conversely, microglia downregulate TLR4 while upregulating TLR2 in the presence of free gangliosides, which thus contribute to inflammatory conditions in the brain (248). However, the mechanism by which gangliosides affect the microglial phenotype and whether this actually contributes to an inflammatory state in the brain has yet to be established.

TARGETING GSLS: OPPORTUNITIES FOR TREATMENT

Targeting of GSLs Using Antibodies/CAR T Cells

Since tumors often upregulate GSL expression, as discussed in the previous chapter, the 75 cancer antigen priorities of the National Cancer Institute at Rockville (USA) lists 4 different GSLs (249). The first one on the list is GD2, for which an antibody (dituximab beta; ch14.18/CHO) is currently being tested in phase III trials for patients with neuroblastoma (trial NCT01704716). Additionally, chimeric antigen receptors (CARs) have been designed and overexpressed in T cells to target GD2 overexpressing neuroblastoma (250–252). Next, an anti-GD3 antibody-drug conjugate (PF-06688992) is in a Phase I clinical trial for patients with stage III or IV melanoma (trial NCT03159117). Also for this GSL-target, CARs have been developed (253). Fucosyl-GM1 is being targeted by the antibody BMS-986012 that is currently tested in the preclinical phase with the goal to treat patients with small-cell lung carcinoma (254). The last GSLs on the list is GM3 for which an antibody is undergoing preclinical investigation by Morphotek.

Yet another option is to vaccinate with GSLs or structures that bear GSL antigens in order to induce an antibody response toward the GSLs overexpressed by a patient's tumor. The disadvantage, however, is that vaccinations with carbohydrates require (a lot of) purified carbohydrates and often result in $CD4^+$ T cell independent low affinity IgM responses without long-lived B cell memory (255). To overcome these challenges, either purified carbohydrates or synthetic polymers harboring the epitope can be fused to carrier proteins (e.g., keyhole limpet hemocyanin or tetanus toxoid) that are able to induce $CD4^+$ T cell activation. Since conjugation of carbohydrate epitopes to proteins is hard to control, fully synthetic vaccines are being developed (256).

Inhibition of GSL Synthesis to Active Immune Cells

In 2003 and 2014 the UGCG inhibitors Miglustat [N-butyl 1-deoxynojirimycin (NBDNJ)] and Eliglustat, respectively, received FDA approval for treatment of Gaucher disease in order to prevent accumulation of GlcCer in these patients. Until 1994, NBDNJ was described to inhibit α-glucosidases in the *N*-glycosylation pathway. *In vitro* work on purified proteins shows that the IC_{50} for NBDNJ was 0.57 μM for α-glucosidase I and 20.4 μM for UGCG. However, due to localization of UGCG on the cytoplasmic side and α-glucosidase I on the luminal side of the ER, a 10-fold lower concentration NBDNJ is required to inhibit UGCG compared to α-glucosidase I in intact cells (257–259). For long it has been hypothesized that inhibitors of GSL synthesis like NBDNJ could also be beneficial for other diseases including cancer (260).

In several mouse models it has been shown that inhibition of GSL synthesis decreases tumor load or even cured the mice (261). Moreover, in a multiple myeloma mouse model, inhibition of GSL synthesis decreased osteoclast activation and thereby the osteolytic lesions that are often present in multiple myeloma patients (262). Since it is even suggested that aberrant GSL synthesis by tumors cause drug-resistance (263, 264), inhibiting GSL synthesis would be great for a combination therapy. Apart from drug-resistance, high expression of GSLs by tumors also negatively affects T cell and DC function, so GSL synthesis inhibition could also be beneficial for cancer immunotherapies.

However, in a Phase I trial where NBDNJ was administered to HIV patients it was found that some patients developed borderline or transient leuko- or neutropenia that was unrelated to dosage (265). In addition, GSL inhibitors may have a negative effect on lymphocyte development and maturation *in vivo* (266), In the case of anti-tumor treatment, however, the patient population would only have a temporary inhibition of GSL synthesis and a functional immune system. Additionally, studies in patients suffering from Gaucher disease do not mention any immune-related side-effect of NB-DNJ (267–269). In this review, we discussed several functions of the immune system that rely on GSLs, therefore it is likely that some functions may be impaired by GSL synthesis inhibitors and their off-label use should be well-substantiated.

CONCLUDING REMARKS

It is clear by now that GSLs are important constituents of a functional immune system. GSLs play versatile roles in physiology and pathophysiology. The knowledge on these roles is largely skewed by the limitations of the tools available. Still, investigators have discovered on a molecular level that GSLs are essential for the recruitment of (immune-related) proteins to specific membrane microdomains and that GSLs

can directly interact with surface receptors. Interactions directly with molecules on other cell types further shape the multi-faceted function of GSLs in immunity. We believe that these GSL functions are closely interconnected to control immune cell function through dynamic regulation of GSL composition. As a consequence, various pathologies are highly related to specific GSL repertoires. We therefore also provided a brief summary of the therapeutic opportunities of GSL synthesis dysregulation that are currently being evaluated. New mechanistic insights in the (immunological) functions of GSLs in health and disease will allow to expand the described options and applications. Available state-of-the-art technologies will be of great help to take the field a great leap forward. Specifically, a validated gRNA library to target all known human GTs by CRISPR/Cas9 has been recently constructed (270). Difficulties of introducing the CRISPR/Cas9 machinery into primary immune cells, such as B and T cells, have also been overcome by electroporation protocols and the usage of recombinant gRNA-loaded Cas9 (271, 272). Furthermore, the development and combination of high-sensitive analytical platforms based on mass spectrometry have boosted the detection of less common GSL-species. And the current throughput and analysis efficiency allows for comprehensive profiling, quantification, and structural characterization of GSLs extracted from tissues and cells (48, 273–275). All these advancements allow the community to systemically investigate the role of individual GSLs in immune cells.

AUTHOR CONTRIBUTIONS

TZ and AdW contributed equally to the writing. MW and RS conceived and edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00090/full#supplementary-material

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Recombinant Sialyltransferase Infusion Mitigates Infection-Driven Acute Lung Inflammation

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Inappropriate inflammation exacerbates a vast array of chronic and acute conditions with severe health risks. In certain situations, such as acute sepsis, traditional therapies may be inadequate in preventing severe organ damage or death. We have previously shown cell surface glycan modification by the circulating sialyltransferase ST6Gal-1 regulates *de novo* inflammatory cell production via a novel extrinsic glycosylation pathway. Here, we show that therapeutic administration of recombinant, bioactive ST6Gal-1 (rST6G) mitigates acute inflammation in a murine model mimicking acute exacerbations experienced by patients with chronic obstructive pulmonary disease (COPD). In addition to suppressing proximal neutrophil recruitment at onset of infection-mediated inflammation, rST6G also muted local cytokine production. Histologically, exposure with NTHI, a bacterium associated with COPD exacerbations, in rST6G-treated animals revealed consistent and pronounced reduction of pulmonary inflammation, characterized by smaller inflammatory cuffs around bronchovascular bundles, and fewer inflammatory cells within alveolar walls, alveolar spaces, and on pleural surfaces. Taken together, the data advance the idea that manipulating circulatory ST6Gal-1 levels has potential in managing inflammatory conditions by leveraging the combined approaches of controlling new inflammatory cell production and dampening the inflammation mediator cascade.

Keywords: sialylation, ST6Gal-1, inflammation, infection, airway, extrinsic glycosylation

INTRODUCTION

Acute inflammation is protective and intrinsic to a healing process. However, dysregulated, excessive, or persistent inflammation is detrimental and is often implicated in chronic conditions including cardiovascular, respiratory, and rheumatic diseases, and in extreme cases, systemic inflammatory response syndromes with high risks for mortality. Previously we provided evidence that a glycan-modifying enzyme present in systemic circulation is a potent regulator of inflammatory cell production (1–3). This enzyme, the ST6Gal-1 sialyltransferase, is regarded as a resident of the Golgi-ER secretory network, mediating the attachment of α (2, 4)-linked sialic acid residues to exposed lactosaminyl-bearing nascent glycoproteins during intracellular biosynthetic transit. However, there is a significant pool of extracellular ST6Gal-1, particularly in the blood (5).

It has been known for a number of decades that changes in the level of circulatory ST6Gal-1 and the circulatory sialyl-glycan structures constructed by ST6Gal-1 are associated with a diverse array of clinical conditions including stress (6), atherosclerosis (4, 7), alcoholism (8, 9), as well as certain cancers, particularly colon and breast cancers, and multiple myeloma (10–12). Studies in the 1980's have established that elevated release of ST6Gal-1 into the blood was a component of the hepatic acute phase response (13, 14). Within the last decade, there has been a renewed interest implicating ST6Gal-1 expression in chemoresistance (15), TNF and EGF-mediated signal transduction (16, 17), maintenance of pluripotentency in stem cells (18, 19), and cancer (10, 20, 21). The renewed interest has been based on the assumption of cell-autonomously expressed enzyme, and insight into the functional relevance of ST6Gal-1 released into the blood has remained relatively overlooked.

In a departure from the canonical mode of Golgi-ER glycosylation, which is a cell-autonomous and intracellular process, the extracellular, blood ST6Gal-1 remodels glycans on target cell surfaces in a novel extrinsic mechanism, which is not cell-autonomous (22, 23). Two genetically modified mouse models were used in these studies. The first, *St6gal1*-KO, was globally ST6Gal-1 deficient (24). The other, *St6gal1*-dP1, was deficient only in the liver-derived extracellular pool of ST6Gal-1 in the blood (25). Comparative analysis of these models revealed an overly robust inflammation and exaggerated inflammatory cell production associated with ST6Gal-1 deficiency. Exaggerated inflammation was attributed to deficiency only in the circulating extracellular pool, and not in the intracellular secretory apparatus-bound enzyme (1–3, 5). Lack of circulating ST6Gal-1 resulted in an exaggerated neutrophilic peritonitis upon challenge with *Salmonella typhimurium* or with the sterile eliciting agent, thioglycollate (2, 25). Circulatory ST6Gal-1 deficiency also resulted in more acute Th2 pulmonary inflammation with excessive eosinophil infiltration and elevated inflammatory cytokine release in OVA-sensitized mice (3). Recently, we observed that systemic ST6Gal-1 modifies the Granulocyte-Monocyte Progenitor (GMP) subset of hematopoietic progenitors, attenuating the production of granulocytes by blunting the transition of GMPs into Granulocyte Progenitors (1), thus providing a mechanistic explanation of how insufficiency in the blood-borne pool of ST6Gal-1 promotes a generally pro-inflammatory condition with excessive granulocyte production. We recently showed that subcutaneous implantation of localized B16 melanoma engineered to overexpress ST6Gal-1 could partially alleviate neutrophilic airway inflammation when challenged intratracheally with LPS in mice (1). Extracellular, systemic ST6Gal-1 was identified recently to be a pro-survival factor in transitional B cell development in the marrow, supporting a concept that circulating ST6Gal-1 is a conveyor of systemic cues guiding the development of multiple branches of immune cells (26).

In the present report, we tested the hypothesis that elevating blood ST6Gal-1 activity, by directly infusing a recombinant form of ST6Gal-1 (rST6G), can have therapeutic value in dampening inflammation. Lung diseases such as Chronic Obstructive Pulmonary Disease (COPD), the 4th leading cause of death worldwide, are characterized by episodic bouts of acute inflammation. These acute exacerbations, triggered by bacterial and viral infections, allergens, or other noxious stimuli, lead to an influx of inflammatory immune cells, predominantly granulocytes and macrophages, which drive disease pathology (27, 28). In the most severe forms, these episodes of immune cell recruitment can be directly life threatening, and at best they promote long-term airway destruction leading to permanently diminished airway functions. We used a murine model of acute airway inflammation elicited by NTHI (Non-typable *Haemophilus influenza*), an opportunistic pathogen common in acute exacerbations of COPD (27, 28). Repeated exposure of mice to NTHI recapitulated many of the features of airway damage seen in human COPD including induction and persistence of perivascular lymphocytic infiltrates and tissue destruction where the initial influx of inflammatory cells is thought to contribute centrally to drive organ damage in later stages (29). We observed that animals receiving rST6G 2 h after an NTHI instillation had strikingly less acute inflammation with reduced pathology and less neutrophil infiltration into the lung, when compared to animals receiving only saline. Furthermore, rST6G treated animals had notably blunted local release of inflammatory cytokines. *Ex vivo* treatment of airway macrophages with rST6G resulted in muted NTHI –dependent production of inflammatory mediators. The data point to the value of rST6G administration in alleviating inflammation by suppressing new inflammatory cell production and in mitigating excessive inflammation by blunting the release of inflammatory cytokines.

RESULTS

Reduced Circulatory ST6Gal-1 Is Associated With More Severe Acute Airway Inflammation

To validate that there is an inverse relationship between the naturally occurring ST6Gal-1 in circulation and the need to produce new inflammatory cells during demand granulopoiesis, we subjected naïve, native C57BL/6 mice to a challenge with NTHI directly into the airways. NTHI elicits a Type 1 immune response in the airways that is dominated by neutrophilic infiltration in the initial phase. Circulatory ST6Gal-1 was monitored in these animals by assessing the enzymatic ability to form α2,6-sialyl linkages onto Gal(β1,4)GlcNAc acceptor substrate. NTHI exposure generated a pronounced but transient depression of circulatory ST6Gal-1 activity to ∼30% of baseline levels at 7 h (**Figure 1**, left). In contrast, other sialyltransferase activities in the blood, specifically those forming the α2,3-sialyl structures on Gal(β1,4)GlcNAc and mediated by the sialyltransferases ST3Gal-3,−4, or−6, were not altered upon NTHI exposure (**Figure 1**, right).

We reported previously that insufficient circulatory ST6Gal-1 levels result in accelerated *de novo* granulocyte accumulation (1–3). Here, we validated this observation in the NTHI model of acute airway inflammation. The globally ST6Gal-1 null mouse, *St6gal1*-KO, and the St6gal1-dP1 mouse with deficiency only in

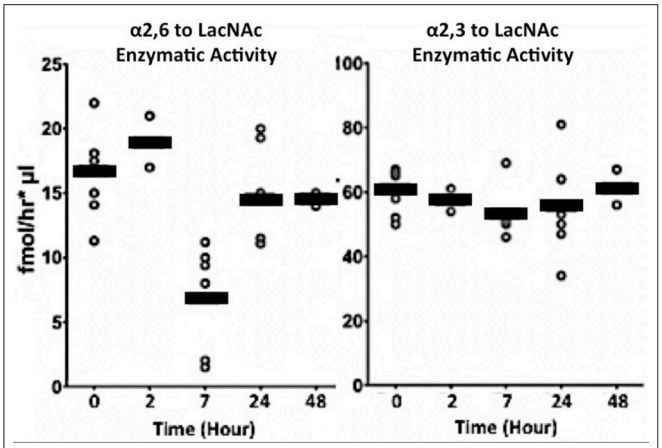

FIGURE 1 | Transient depression of circulatory ST6Gal-1 accompanies acute airway inflammation. Live NTHI bacteria (10^6 CFU / animal) were delivered by oropharengeal instillation. Blood was collected at the times shown after instillation. Sialyltransferase activities in the sera were measured by following the transfer of CMP-[3H]Sia to Galβ1–4GlcNAc-O-Bn (LacNAc). The Siaα2,6 product formed by ST6Gal-1 **(Left)**, was separated from Siaα2,3 product formed by various ST3Gal transferases **(Right)** using SNA-agarose chromatography.

the circulatory pool of extracellular ST6Gal-1 were examined. As summarized in **Figure 2A**, both *St6gal1*-dP1 and *St6gal1*-KO mice had exaggerated neutrophil accumulation in the BALF compared to wild-type animals 18 h after NTHI challenge. Moreover, nearly identical ∼1.5-fold augmented accumulation of neutrophils was observed in both ST6Gal-1 deficit models that were generated independently by different strategies. Neutrophils dominated the inflammatory cell infiltrate into the airway in the acute inflammatory response to NTHI. On average, 0.8 $\times$ 10^6 neutrophils were recoverable in the bronchial alveolar lavage fluid (BALF) at 18 h in native C57BL/6 (wild-type) animal, and neutrophils comprised >75% of the total recovered BALF cells (**Figure 2B**). This result, which is more fully presented in **Supplemental Table 1A**, strongly supports the conclusion that insufficiency in circulatory ST6Gal-1, rather than intracellular Golgi-ER bound ST6Gal-1, was the factor driving excess neutrophil accumulation in the airways in acute inflammation.

Enhanced efficiency in recruitment of granulocytes into the airway of *St6gal1*-dP1 mice compared to wild-type animals did not drive the exaggerated neutrophil accumulation. Neutrophils were isolated from *St6gal1*-dP1 and C57BL/6 mice, differentially labeled with either PKH26 or PKH67 fluorescent dyes, pooled, and intravenously transferred into wild-type recipients. The recruitment of the adoptively infused neutrophils into NTHI-induced acute airway was monitored as ratios of the differentially dyed cells by flow cytometry. **Figure 2C** shows that the ratios of the *St6gal1*-dP1 to wild-type neutrophils recovered in the BALF were not changed from the original input ratios. The data confirm that circulatory ST6Gal-1 deficiency resulted in a more severe acute airway inflammatory response to NTHI challenge, and that the mechanism is more robust granulopoiesis rather than altered efficiencies of cell recruitment to the inflamed lung.

Direct Intravenous Infusion of Recombinant ST6Gal-1 Mitigated Acute Airway Inflammation

We observed in murine genetic models of circulatory ST6Gal-1 deficiency more pronounced peritonitis and airway acute inflammation elicited by sterile agents such as LPS (1, 22). Chronic elevation of circulatory ST6Gal-1 by subcutaneous implantation of a B16 melanoma engineered to release ST6Gal-1 partially alleviated the sterile agent induced acute airway (1). Here, we posit that an infection-driven acute inflammation can also be attenuated by raising circulatory ST6Gal-1. We further posit that infusion of pure, recombinant ST6Gal-1 (rST6G), resulting only in temporary elevation of blood ST6Gal-1 activity can be effective against infection-driven inflammation. To explore the potential therapeutic value of rST6G, this and all other following experiments were performed in the wild-type C57BL/6 mouse. A single bolus of rST6G in its present formulation, when infused into wild-type animals at baseline was rapidly cleared from the bloodstream in <1 h (see **Supplemental Figure 1A**). Despite the rapid clearance, a single rST6G infusion resulted in a striking decline in granulopoietic parameters within the bone marrow, with ∼40% decrease in colony forming units in granulocyte (G), Monocyte (M) and GM-CFUs 7 h later (**Figure 3A**). Total marrow cellularity diminished overall by ∼25%, resulting mostly from a >2-fold reduction in marrow neutrophils and a slight reduction in B220-positive cells, the two most-abundant marrow cell populations (**Figure 3B**). Blood differentials revealed an almost 50% reduction in total white cell counts, accountable by the diminution of circulatory lymphocyte numbers that are the major white cell constituents in the blood (**Figure 3C**). Curiously, circulatory neutrophils in the naïve wild-type animals were not altered, although at baseline only a minor (10–12%) percentage of the overall circulating white cells are granulocytes. The complete blood differential counts are presented in **Supplementary Table 1B**.

To assess the anti-inflammatory efficacy of systemically administered rST6G, NTHI-challenged C57BL/6 mice received 2 intravenous infusions of rST6G, the first at 2 h after receiving NTHI, and a booster at 10 h. Two intravenous rST6G infusions were used as a precaution, because we observed rapid clearance of the current formulation of rST6G from the blood (**Supplemental Figure 1A**). The two infusions 5, 8 h apart resulted in circulatory ST6Gal-1 activity that was 2-fold over baseline at 16h after the initial rST6G infusion (**Supplemental Figure 1B**). In cohorts receiving rST6G, BALF neutrophil counts were reduced by ∼50% (**Figure 4B**). Only a slight (10%) reduction in overall BALF leukocyte counts was observed, due to a ∼2.5-fold increase in recruited macrophage. Though the increase in recruited macrophage numbers was unexpected, it is noteworthy that animals with genetic ST6Gal-1 deficit had ∼33% decrease in recruited macrophage numbers in the airway following NTHI exposure (see **Supplemental Table 1A**). Circulating blood counts, monitored at the time BALF was recovered, showed no differences between rST6G and sham animals. This is not unexpected, since circulating neutrophilia was noted to be extremely transient and

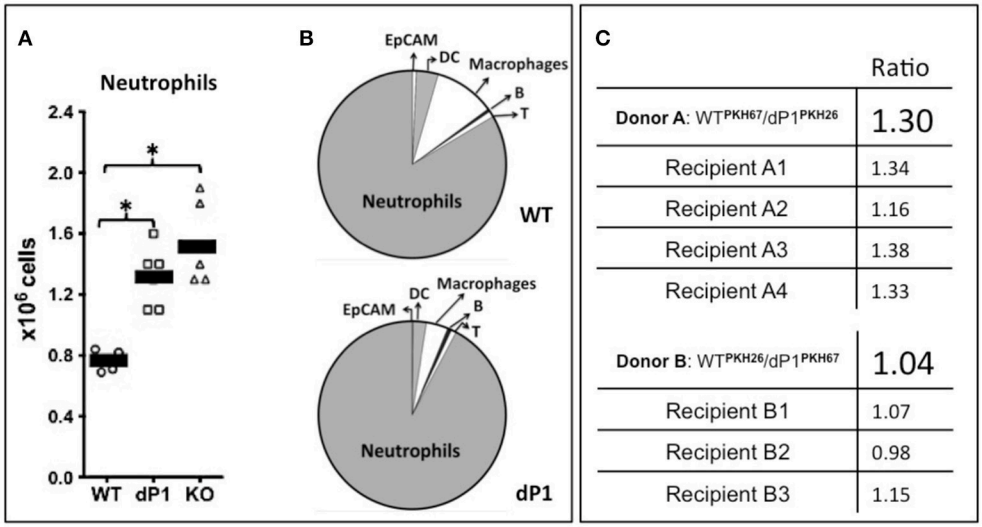

FIGURE 2 | More severe neutrophilic acute airway inflammation in animals with ST6Gal-1 deficiency. Wild type C57BL/6 (WT), *ST6Gal1-dP1* (dP1), and *ST6Gal1-KO* (KO) mice were exposed to 10^6 CFU of live NTHI bacteria by oropharengeal instillation. Eighteen hours later, the bronchoalveolar lavage fluid (BALF) was collected, Leukocyte number was counted, and leukocyte composition was determined by flow cytometry. **(A)** shows the total numbers of neutrophils recovered from the BALF of NTHI-instilled animals, showing greater neutrophilic inflammation in dP1 and KO, compared to WT (1.6 and 2.0-fold, respectively). * $p < 0.05$ for indicated comparisons. **(B)**, top shows the cellular composition of WT BALF, consisting predominantly of neutrophils (83.5%). Macrophage (10.5%), dendritic cells (DC, 3.8%), T- (1%), and B- (0.5%) cells, with a minor constituent of epithelial cells as defined by EpCAM (0.7%). **(B)**, bottom, shows dP1 BALF composition, which was essentially identical to WT BALF in percentage cellular contribution from the assessed cell types. **(C)** Neutrophils from the marrows of WT and dP1 mice were isolated by negative selection. The cells were stained with one of the two distinct membrane dyes (red PKH-26 and green PKH-67), mixed in an ∼1:1 ratio and injected into 3–4 WT recipients 2 h after NTHI challenge. In the top panel, the initial PKH67-labeled WT/ PKH26-labeled dP1 neutrophils ratio was 1.30. In the bottom panel, PKH26-WT/PKH67-dP1 neutrophil donor ratio was 1.04. At 18 h, this neutrophil-fluorescence ratio was again measured in cells obtained from the BALF. No difference in airway recruitment was noted for dP1 neutrophils compared to WT neutrophils.

limited to the first few hours after a peripheral acute challenge, including peritonitis by LPS or thioglycollate (2), airway eosinophilia by OVA to sensitized mice (3), and acute airway inflammation by LPS (30). Blinded histopathologic evaluation disclosed a consistent reduction in pulmonary inflammation among animals treated with rST6G. Compared to animals receiving saline, the rST6G treated group showed smaller inflammatory cuffs around bronchovascular bundles and fewer inflammatory cells within alveolar walls and alveolar spaces (**Figures 4C,D**). The histopathologic scoring is summarized in **Supplemental Table 2**. Most unexpectedly, the rST6G-treated group had strikingly lowered levels of inflammatory cytokines TNF-α, IL-1β, and IL-6 in the BALF. In fact, inflammatory cytokines were close to or below reliable assay detection limits in BALF from animals that received rST6G, when compared to easily quantifiable levels in sham treated animals (**Figure 5**).

To gain mechanistic insight into the blunted inflammatory cytokines released in the airways by rST6G treatment, despite the apparently paradoxical elevation of airway macrophage, one of the principal cell types along with epithelial cells responsible for the release of inflammatory cytokines (31, 32), we examined the response of primary airway macrophages. Airway macrophages isolated from the BALF of resting wild-type C56BL/6 mice were stimulated *ex vivo* with heat-killed NTHI in the presence or absence of rST6G. A reduction of NTHI-dependent release of TNF-α and IL-6 production was observed in the rST6G-treated macrophages (**Figure 6A**). This effect was not unique to airway macrophages, as bone marrow derived macrophages treated with rST6G also had a 3.5-fold reduction in TNF-α (**Figure 6B**), Interestingly, IL-10 production by NTHI stimulated BM macrophages was elevated in the rST6G treated cells, pointing tantalizingly to a possible additional pathway by which rST6G can mitigate acute inflammation. In these *ex vivo* experiments, 0.1 mM CMP-Sia was also included. We have observed that rST6G to have effect on suppressing macrophage activity *ex vivo*, even without added sialic acid donor substrate. Possibly, the leakage of sialic acid donor substrate from neighboring dying cells might be sufficient. However, we also observed that addition of CMP-Sia has the benefit of diminishing variability. *In vivo*, sialic acid donor substrate is believed to be supplied by activating platelets (23, 33).

In an earlier report, we showed that systemic ST6Gal-1 dampens granulopoiesis in the marrow by extrinsic modification of the hematopoietic progenitor cells through the attachment of α2,6-linked sialic acid residues, which can be monitored by the lectin, SNA (*Sambucus nigra* agglutinin) (22). Here, cell surface α2,6-sialylation status of airway macrophage recovered in the BALF of animals challenged *in vivo* with NTHI was assessed for changes in SNA reactivity. The data show a pronounced increase in cell surface SNA reactivity in cells from animals treated with rST6G, compared to saline treated animals (**Figure 6C**). This observation strongly suggests that

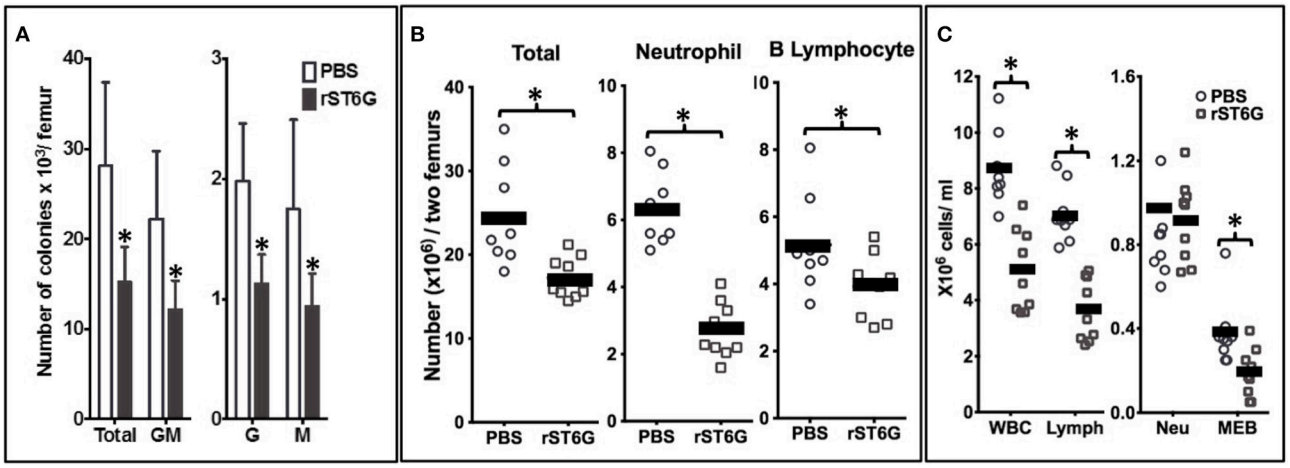

FIGURE 3 | Intravenous rST6G infusion depresses myelopoiesis and alters inflammatory cell availability**.** WT mice receiving either a single 300 μg bolus of recombinant ST6Gal-1 (rST6G) or saline (PBS) were sacrificed 7 h later. Bone marrow cells from hind limbs and peripheral blood were isolated and analyzed as follows. **(A)** presents marrow progenitor clonogenic activity for granulocyte/monocyte (GM), granulocyte (G), or monocyte (M) progenitor colonies. The combined total colony formed is also shown (Total). Saline- (open bars) and rST6G-treated mice ($n = 9$ each group) were used with 4×10^4 marrow cells were plated in Methocult M3534 to promote growth of myeloid progenitors for 10 days. *$p < 0.01$ rST6G compared to PBS. **(B)** summarizes the overall bone marrow cellularity of PBS (round symbols) and rST6G-treated (square symbols) animals, where each symbol denotes one animal. Total bone marrow cellularity (Total), and neutrophil and B cell numbers are shown. *$p < 0.01$. **(C)** Summarizes white cell counts in the blood as total white blood cell (WBC) count and differential count for lymphocyte (Lymph), neutrophil (Neu), monocyte, eosinophil and basophil (MEB). PBS ($n = 8$) or rST6G-treated animals ($n = 9$) were used. *$p < 0.01$.

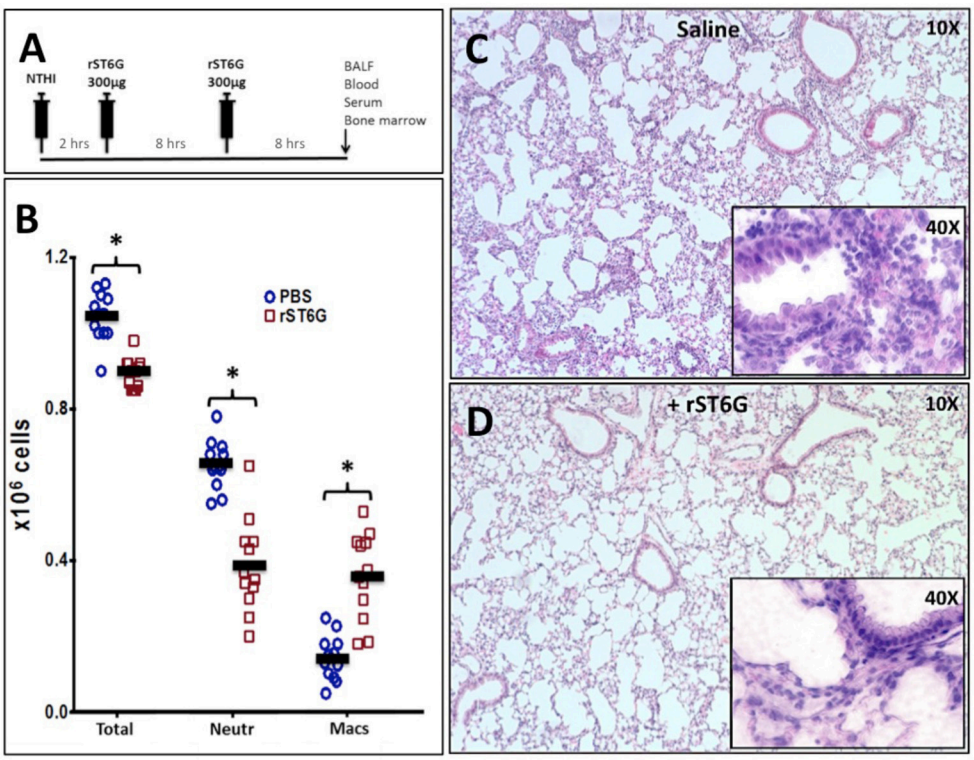

FIGURE 4 | Acute airway inflammation induced by NTHI exposure is mitigated by rST6G infusion. **(A)** shows the intervention protocol where each animal received two 300 μg rST6G or saline/sham injections spaced 8 h apart with the first injection being at 2 h after NTHI challenge. Animals were sacrificed 18 h later and assessed for pulmonary inflammation. **(B)** Inflammatory cell accumulation in the BALF of the rST6G- and sham- (PBS) treated animals showing total BALF cells (Total), neutrophil (Neutr), and macrophage accumulation (Macs). *$p < 0.01$. **(C,D)** Show the lung pathology of saline and rST6G-treated animals, respectively at 18 h.

rST6G introduced into systemic circulation were able to affect the pulmonary macrophages and blunt the release of inflammatory cytokines during an NTHI-elicited acute airway response.

DISCUSSION

It has long been known that changes in the level of circulatory ST6Gal-1 and the circulatory sialyl-glycan structures constructed by ST6Gal-1 are associated with a diverse array of clinical conditions including stress (6), atherosclerosis(4, 34), alcoholism (35, 36), as well as in a certain cancers, particularly colon, breast cancers and multiple myeloma (10–12). Studies in the 1980's have established that elevated release of ST6Gal-1 into the blood was a component of the hepatic acute phase response (37, 38), although insight into the physiologic contribution of blood ST6Gal-1 remained largely elusive. Much is known about the catalytic specificity of this glycan-modifying enzyme, ST6Gal-1, in attachment of α2,6-linked sialic acid residues to exposed lactosaminyl-termini of glycoproteins (39, 40). However, the traditional paradigm of glycosylation was that of an intracellular process, where glycosyltransferases such as ST6Gal-1 reside within the Golgi-ER secretory apparatus and modify the transiting nascent glyco-conjugates in an individual cell-autonomous manner. In contrast, circulatory ST6Gal-1, which is secreted principally by the liver, is extracellular and operates by the novel extrinsic glycosylation mechanism that is non-cell autonomous. Extracellular ST6Gal-1 remodels marrow hematopoietic precursor cells, and in so doing, mutes the ability of the precursors to differentiate and proliferate (22).

Original studies from this laboratory uncovered a link between low circulating ST6Gal-1 and excessive *de novo* inflammatory cell production (2, 3, 5, 22). Later, we also showed that chronically raising blood-borne ST6Gal-1 activity by subcutaneous implantation of a B16 melanoma engineered to overexpress the secretory form of the enzyme was effective in diminishing production of new granulocytes by blunting the transition of GMP to GP in granulopoiesis, and this approach was effective in controlling sterile agent-induced inflammation (1). In the current report, we show that an infection-driven inflammation can also be controlled effectively by direct intravenous infusion of pure recombinant ST6Gal-1 protein, despite using a primitive rST6G formulation that is very rapidly cleared from the blood. We used a murine model of NTHI-elicited acute airway. NTHI commonly colonizes the lower airways of patients with chronic obstructive pulmonary disease (COPD) and frequently contributes to the acute exacerbations driving disease progression (41, 42). In the mouse, airway instillation of NTHI elicits an immediate acute lung inflammatory response characterized by severe neutrophil infiltration into the airway, and repeated exposure to NTHI reproduces and perpetuates many of the pathophysiologic symptoms of COPD (43). Previously we showed that a sterile LPS induced airway inflammation in mice resulted in extrinsic sialylation of pulmonary and circulating leukocytes, and the extrinsic sialylation used sialic acid precursors from activated platelets (33). We showed here that NTHI-elicited acute lung inflammation is more severe in mice with circulatory ST6Gal-1 insufficiency, as characterized by a 1.5-fold exaggeration in the already severe neutrophil infiltration into the airway. In wild-type animals, the onset of NTHI-elicited pulmonary inflammation was coincident with a specific and transient dip in ST6Gal-1 activity in circulation. This observation further supports the idea that depressed circulatory ST6Gal-1 predisposes the host for pro-inflammatory conditions and inflammatory cell production.

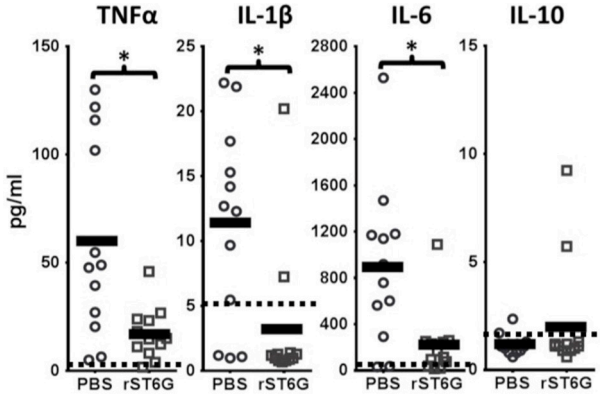

FIGURE 5 | Inflammatory cytokine release during acute airway inflammation is suppressed by rST6G infusion. C57BL/6 wild-type animals were challenged with NTHI and subjected to the rST6G or sham (PBS) treatment protocol as outlined in **Figure 4A**. The bronchial alveolar lavage fluids (BALF) were analyzed for cytokines by Luminex 100 multiplex assays. Dashed lines and the shaded boxed regions represent the reliable lower assay limit of detection for each cytokine. Many of the values obtained, especially for the IL-10 assays and the rST6G-treated cohorts for IL-1β values, were zero. For these, a default low value of "1" was assigned in order to calculate the p value. *$p < 0.001$.

The detailed mechanistic links of how cell surface sialylation affects overall hematopoietic cell behavior remains to be elucidated. However, ST6Gal-1-mediated attachment of α2,6-sialic acids on β1 integrin alters cellular adhesiveness (44, 45) leading to altered cell motility (46), cancer cell differentiation and progression (46). In this study, we show that a recombinant protein corresponding to the soluble form of ST6Gal-1 (rST6G) was effective in mitigating infection-driven acute inflammation. Technical challenges associated with this approach remain. Most notably, suboptimal pharmacokinetic properties and/or enzymatic instability (**Supplemental Figure 1A**) of the present rST6G form resulted in undesirably rapid lost from circulation in the mouse, and rST6G remains difficult to produce in large quantities. Despite these limitations, the data show that systemic rST6G administration reduces overall marrow cellularity, dramatically decreases marrow granulocyte pool, and decreases marrow G-, GM-, and G-CFU clonogenic activities (see **Figure 3**). In response to NTHI challenge in the airway, rST6G intervention after the onset of localized acute inflammatory response resulted in pronounced mitigation of inflammation. While reduced granulocyte accumulation by rST6G administration was predicted by prior results, the muted release of inflammatory cytokines TNFα, IL-1β, and IL-6 in the airways was unexpected. The data showed not only a profound suppression of inflammatory cytokine release *in vivo*, but airway

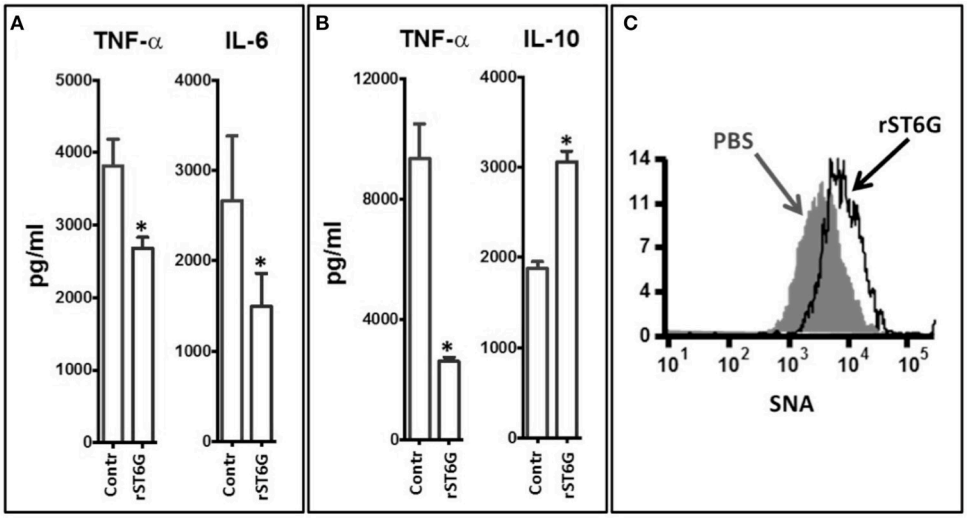

FIGURE 6 | Inflammatory cytokine release by macrophage was attenuated by ST6Gal-1. **(A)** Macrophages were recovered from the BALF of 3 wild-type C57BL/6 mice (at rest). The pooled cells were plated into 5 replicate but identical wells for each determination. Groups of 5 macrophage wells were exposed *ex vivo* to 10^5 CFU/ml heat-killed NTHI either in the absence or presence (20 μg/ml) of rST6G and CMP-Sia (100 μM) for 18 h. TNF-α and IL-6 released into the media was measured next day. *$p < 0.001$. **(B)** Bone marrow–derived macrophages were generated from marrow cells of C57BL/6 WT animals. The identically seeded cells, in groups of 5 wells per treatment, were exposed to heat-killed NTHI in the absence (control) or presence of rST6G (20 μg/ml) and CMP-Sia (100 μM) and incubated overnight (37°C and 5% CO_2). TNF-α and IL-10 released into the media were assessed by ELISA. *$p < 0.001$ based on concentration values from five separate wells for each condition. All data are representative of six separate experiments. **(C)** BALF macrophage was recovered from C57BL/6 WT animals 18 h after oropharyngeal challenge with NTHI using the protocol in **Figure 4A**, either with rST6G (rST6G) or sham (PBS). Cell surface sialylation was measured using the α2,6-sialic acid-specific lectin, FITC conjugated *Sambucus nigra* agglutinin (SNA) by flow cytometry, as shown.

macrophages in culture were less able to release TNF-α and IL-6 upon exposure to heat-killed NTHI. Bone marrow derived macrophages recapitulated not only the suppression of NTHI stimulated release of TNF-α but also the augmented release of the anti-inflammatory IL-10 by rST6G treatment *ex vivo*. The data advance the idea that the ability of circulatory ST6Gal-1 to mitigate inflammation is exerted through the concerted effects of at least two distinct target mechanisms, although additional targets may be likely. The known targets are the control of hematopoietic production of inflammatory cells and suppression of inflammatory cytokines.

Together, the data show that rST6G administration has novel therapeutic potential in the management of inflammatory conditions. This approach leverages the natural function of a natively circulatory glycan-modifying enzyme, the sialyltransferase ST6Gal-1. Intervention by systemic rST6G administration elevates circulating ST6Gal-1 activity, blunting the inflammatory cytokine cascade, and suppressing *de novo* production of inflammatory cells. While not specifically examined here, blunting these components of the initial inflammatory cascade should benefit in mitigating the lasting injury such as airway remodeling and organ injury at later stages of exposure to environmental insults.

MATERIALS AND METHODS

Animals

The *St6gal1*-dP1 and *St6gal1*-KO mice strains were in the C57BL/6J background as described previously (2). Unless otherwise stated, C57BL6/J mice between 7 and 10 weeks of age were used, and both sexes were equally represented. Roswell Park Institute of Animal Care and Use Committee (IACUC) approved maintenance of animals and all procedures used under protocol 1071M. There is no involvement of human subjects or clinical specimens; ethics committee review is not required according to the local and national guidelines.

Recombinant ST6Gal-1 (rST6G) and Sialyltransferase Assays

rST6G is the recombinant secretory form of rat ST6Gal-1 where the catalytic domain was generated as a fusion protein encoding the following: NH_2-signal sequence – 8x His tag – Avi tag – GFP – TEV protease cleavage site – ST6GAL1 catalytic domain – COOH (47). The construct was expressed in HEK293 cells; the recombinant protein was harvested and purified from the medium. The ST6Gal-1 catalytic domain was proteolytically released by TEV protease digestion and further purified (47, 48). Sialyltransferase assays were carried out as described previously (23).

Acute NTHI Exposure and Recombinant ST6Gal-1 (rST6G) Treatment

A frozen glycerol stock of NTHI strain 1479 (clinical isolate from a COPD exacerbation) was streaked on chocolate-agar plates, and single colonies were grown in a liquid culture of brain–heart infusion media supplemented with 10 μg/ml hemin and 10 μg/ml β-NAD (Sigma). After 3–4 h of culture in a 37°C shaking incubator, OD_{600} was determined to dilute the required

number of CFU to 2×10^8 CFU/ml in PBS. Bacteria were pelleted in microcentrifuge tubes at 13,000 × g for 10 min and washed twice in PBS. To initiate acute NTHI-mediated inflammation, mice were anesthetized by isoflurane inhalation, and 50 μl (1×10^6 CFU) live NTHI diluted in PBS was use for oropharyngeal instillation using a 200-μl sterile pipette tip.

rST6G was injected i.v. (750 μg/CC in PBS) 2 and 14 h after NTHI exposure. Same volume of PBS was injected to control mice. After 18 h, mice were sacrificed by injection (i.p.) of two 0.5 ml Avertin (2.5 gr 2,2,2, Tribromethanol, 5 ml 2-methyl-2-butanol in 200 ml distilled water). Bronchoalveolar lavage (BAL) was performed post euthanization by opening the thoracic cavity to expose the trachea, which was cannulated with a 22-gauge i.v. catheter. PBS (750 μL) was injected and withdrawn from the lung two times using a tuberculin syringe. For cytokine assays, BAL fluid (200 μl) or serum (50 μl) was subjected to Luminex 100 multiplex assays using a capture bead system developed by Luminex Corporation (Austin, TX, USA). For pathohistologic evaluations, lungs were excised and fixed in 10% formaldehyde in PBS, paraffin embedded, sectioned, and stained with H&E. Lung pathology was evaluated by a board certified pulmonary pathologist blinded to the identity of the slides.

Flow Cytometry, Cell Differentials, and Bone Marrow Analysis

Flow cytometry was performed using anti-CD45 (hematopoietic cells), anti-Ly6G (neutrophils), anti-B220(B cells), anti-CD3 (T cells), anti-F4/80 (macrophage) anti-CD11c (dendritic cells) antibodies and SNA (*Sambucus nigra* lectin, Vector Laboratories, Peterborough, UK). All reagents were purchased from BioLegend (San Diego, CA). Cells were analyzed using BD LSRII flow cytometer (Becton Dickinson Immunocytometry Systems). For colony forming cell assays, marrow nucleated cells in a volume of 0.1 ml were plated in 0.9 ml of methylcellulose medium (MethoCult 3534, STEMCELL Technologies) in duplicate and placed in humidified incubator with 5% CO_2 at 37°C. Colonies containing at least 50 cells were counted 7 days after incubation.

Ex vivo Labeling of Cells and Transfer Into Recipients

Bone marrow cells were collected from hind-limbs of mice, either *St6gal1-dP1* or C57BL/6 wild-type, re-suspended in RBC lysis buffer (0.8% NH_4Cl, 0.1 mM EDTA buffered with $KHCO_3$ to pH 7.4), washed and re-suspended in phosphate-buffered saline (PBS) with 0.5% BSA or fetal bovine serum and 2 mM EDTA, and then passed through a 100-μm cell strainer (BD Biosciences). Cells were centrifuged and re-suspended in the same buffer (up to 2×10^8 cells/ml), and 50 μl/ml of biotinylated antibodies (anti-cKit. anti-B220, anti-CD3, anti-TER119, anti-CD5) was added to the cell suspension. Partial neutrophil enrichment was accomplished by negative selection using magnetic microparticles according to the manufacturer's protocol (STEMCELL Technologies, Vancouver, British Columbia, Canada). More than 60% of selected cells were neutrophils as verified by flow cytometry. To apply labeling with PKH26 and PKH67 (Sigma Chemical, St Louis, MO), cells were washed in RPMI medium (without serum), and 10^7 cells were re-suspended in 1 mL Diluent C (Sigma) and rapidly added to 1 mL of 4 μM PKH26 or PKH-67. The cells were incubated at 25°C for 5 min, terminated by the addition of 2.5% fetal calf serum. After labeling, the cells were washed twice with cold PBS and counted by hemocytometer. Differentially labeled donor cells were mixed 1:1 immediately before infusion into recipient animals. A small fraction of combined cells was labeled with neutrophil marker (anti-Ly6G antibody) and saved for measuring donor WT/dP1 neutrophil ratios. Each recipient received pooled cells consisting of 10^7 cells intravenously from each labeled group 2 h after NTHI BAL was performed 18 h after NTHI.

Statistics

Testing for differences between mean values was determined using either Students' *T*-Test or two-way ANOVA with post-test comparisons in Graph Pad Prism 6 software (La Jolla, CA). $p < 0.05$ is considered significant.

AUTHOR CONTRIBUTIONS

MN designed the research, performed the experiments, and wrote the paper. AL designed the research and performed the experiments. ZG and KM generated the recombinant enzyme. SN designed the research. PB performed histopathologic evaluations and interpreted information. YT designed the research. JL designed the research, coordinated project activities, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00048/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Theft and Reception of Host Cell's Sialic Acid: Dynamics of *Trypanosoma Cruzi Trans*-sialidases and Mucin-Like Molecules on Chagas' Disease Immunomodulation

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The last decades have produced a plethora of evidence on the role of glycans, from cell adhesion to signaling pathways. Much of that information pertains to their role on the immune system and their importance on the surface of many human pathogens. A clear example of this is the flagellated protozoan *Trypanosoma cruzi,* which displays on its surface a great variety of glycoconjugates, including *O*-glycosylated mucin-like glycoproteins, as well as multiple glycan-binding proteins belonging to the *trans*-sialidase (TS) family. Among the latter, different and concurrently expressed molecules may present or not TS activity, and are accordingly known as active (aTS) and inactive (iTS) members. Over the last thirty years, it has been well described that *T. cruzi* is unable to synthesize sialic acid (SIA) on its own, making use of aTS to steal the host's SIA. Although iTS did not show enzymatic activity, it retains a substrate specificity similar to aTS (α-2,3 SIA-containing glycotopes), displaying lectinic properties. It is accepted that aTS members act as virulence factors in mammals coursing the acute phase of the *T. cruzi* infection. However, recent findings have demonstrated that iTS may also play a pathogenic role during *T. cruzi* infection, since it modulates events related to adhesion and invasion of the parasite into the host cells. Since both aTS and iTS proteins share structural substrate specificity, it might be plausible to speculate that iTS proteins are able to assuage and/or attenuate biological phenomena depending on the catalytic activity displayed by aTS members. Since SIA-containing glycotopes modulate the host immune system, it should not come as any surprise that changes in the sialylation of parasite's mucin-like molecules, as well as host cell glycoconjugates might disrupt critical physiological events, such as the building of effective immune responses. This review

aims to discuss the importance of mucin-like glycoproteins and both aTS and iTS for *T. cruzi* biology, as well as to present a snapshot of how disturbances in both parasite and host cell sialoglycophenotypes may facilitate the persistence of *T. cruzi* in the infected mammalian host.

Keywords: *Trypanosoma cruzi*, *trans*-sialidase, mucin-like molecule, sialic acid, glycan-binding protein, infectious disease, T-cell response

A SNAPSHOT OF THE NATURE OF *TRYPANOSOMA CRUZI* SURFACE COAT

Trypanosoma cruzi presents a complex life cycle spanning two hosts, the hematophagous triatomine, and susceptible mammals (1). Throughout evolution, *T. cruzi* developed the capacity to adapt to hostile environments in both kinds of hosts. An important feature that was certainly decisive for the parasite adaptation to different hosts, as well as different niches within each host, was its ability to remodel its own surface coat (2, 3). It is well established that the cell surface of *T. cruzi* is composed by a wide variety of glycosylphosphatidylinositol (GPI)-anchored glycoconjugates expressed on a developmental stage-specific manner[(4–7).

Regarding the cell coat of the *T. cruzi* forms found in mammals, several studies revealed that it is mainly composed by both glycoinositolphospholipids (GIPLs) and heavily *O*-glycosylated mucin-like molecules (8, 9).

In addition, proteins belonging to *trans*-sialidase (TS) family (10–14); trypomastigote small surface antigen (TSSA) (15–17) and members of a multigenic family identified during the sequencing of the *T. cruzi* CL Brener genome, named mucin-associated surface proteins (MASPs) are found to a lesser extent (18–22).

SIALIC ACID-CONTAINING GLYCANS MODULATE THE ESTABLISHMENT OF *T. CRUZI* INFECTION IN MAMMALS' CELLS

Over the last twenty years, it has been known that simple, as well as complex carbohydrates (glycans) may play major structural, physical and metabolic roles in biological systems (23). Such functions include self/non-self-discrimination, ensuring correct protein folding, cell-to-cell signaling, cell adhesion and even differentiation, among others (24–27). The immune system, akin to the legions protecting the Roman Empire, is poised to defend the body against pathogens and transformed cells alike. One of the most important carbohydrates when it comes to the immune system is sialic acid (SIA) (28–30). More specifically the *N*-acetyl neuraminic acid (Neu5Ac). Immune responses deflagrated against *T. cruzi* are of particular interest, since the parasite is incapable of synthesizing SIA (31, 32). That would put *T. cruzi* squarely in the crosshairs of their mammal hosts' immune systems, since they somewhat rely on SIA to identify pathogens (3, 33, 34). The use of TS provides an elegant mechanism through which *T. cruzi* poaches SIA molecules from the hosts' cells and covers its own surface molecules, effectively creating a molecular ghillie suit to hide from mammalian phagocytes, posing a difficulty for the generation of an effective immune response (35–37). In addition to the enzymatically active members (aTS), which are able to modify the glycophenotype of both parasite and host cells (3, 13, 38, 39), TS also presents an inactive form (iTS), due to the naturally occurring Tyr342→ His substitution, which completely abolishes TS enzymatic activity (40). Despite the lack of catalytic function, it still plays an important role in *T. cruzi*-host cell interaction due to its lectinic activity (41–45) (**Figure 1**). Both extracellular (axenic) amastigote and trypomastigote forms of *T. cruzi* are infective to mammal cells (46–48). Regarding the trypomastigote forms, both iTS and aTS are GPI-anchored surface proteins (49). Recent findings revealed that sialylated mucins are present in lipid-raft-domains far away from TS molecules are found. By using unnatural sugar approach as chemical reporters, the authors demonstrated that the sialylation event is orchestrated by micro-vesicle-associated aTS instead of a membrane-anchored or fully soluble enzyme (34).

The importance of SIA-containing glycans on *T. cruzi*-host cell interplay was suggested over twenty-five years ago, when the authors demonstrated that the parasite's ability to penetrate into SIA-deficient cells was reduced when compared with wild-type cell lines (50). After this finding, many groups began investigating the events triggered by TS *in vitro* and in murine models (3, 37, 51–53).

TRANS-SIALIDASES AS KEY REGULATORS OF THE IMMUNE EVASION

Studies have shown that *T. cruzi* can recapitulate transient thymic aplasia in infected mice. It occurs in an early moment of the infection and aTS was proven responsible for the induction of apoptosis, since recombinant aTS alone can induce the alterations. In other studies, neutralizing anti-TS antibodies and the use of inhibitors prevented these effects (54). Also, an earlier study showed that recombinant iTS was incapable of eliciting these abnormalities (55). A study from Risso and colleagues demonstrated that the level of thymic damage was dependent on the parasite strain. More lethal strains (TcVI: RA, Q501, Cvd, and TcII: Br) present markedly higher levels of TS than their non-lethal counterparts (K-98, Ac and Hc - TcI) (56, 57). A different study showed that aTS does not appear to provoke thymocyte apoptosis directly. Instead, such effect seems to be centered on the thymic nurse cell complex, a region of the thymus cortex that contains mainly double-positive thymocytes, the most affected by TS (58). It is interesting to point out the studies that showed the pro-apoptotic effect

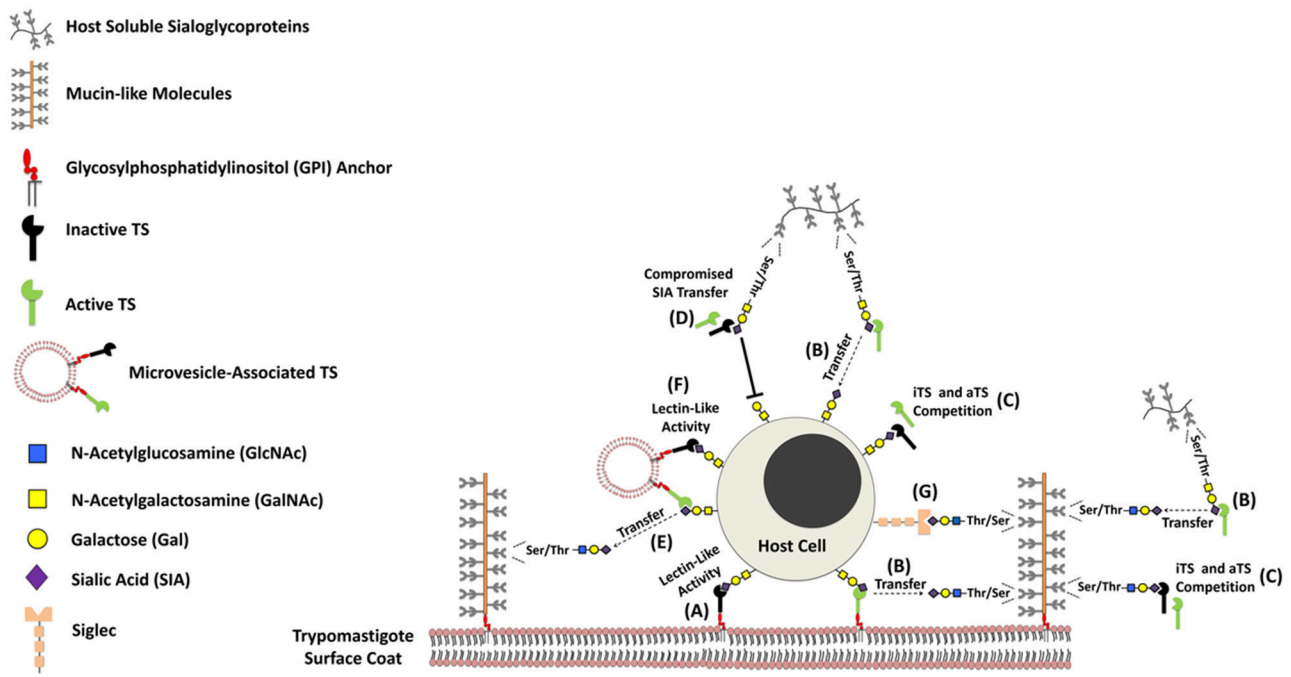

FIGURE 1 | Schematic model showing the presence of *trans*-sialidases and mucin-like molecules on the parasite cell surface. The biological properties of both GPI-anchored proteins (*trans*-sialidases [TS] and mucin-like molecules) have been extensively studied over the last years, and their immunobiological functions have been gradually disclosed. *Trypanosoma cruzi* expresses on its surface both inactive (iTS) and active (aTS) TS proteins, that present similar substrate specificity (α-2,3 SIA). While iTS displays lectinic-like activity **(A)**, aTS shows the ability to modulate the sialoglycophenotype of both parasite and host cell glycans **(B)**. Since both TS proteins compete by α-2,3 sialo-containing glycans **(C)**, it may attenuate and or abrogate the process of SIA transfer mediated by aTS **(D)**. Consequently, it might be able to compromise biological phenomena depend on the catalytic activity displayed by enzymatically active members. In addition, both TS may be found associated to microvisicles, displaying the same properties mediated by both fully soluble enzyme **(E, F)**. The sialylation of glycoproteins found in the parasite cell surface besides to promote protection against soluble factors of the host immune system, may also provide ligand for SIA-binding proteins expressed by host cells, such as Siglecs **(G)**. Since this phenomenon compromises the effective function of immune cells, it may represent an interesting mechanism to guarantee the perpetuation of the parasite in their infected host.

was due to the alteration of the sialylation profile of target cells. By using lactitol, a competitive inhibitor that compromises the transfer of the sialyl residue to endogenous acceptors, but not the hydrolase activity of the enzyme, disallowed *ex vivo* and *in vivo* apoptosis caused by aTS (54). Years later, Lepletier and colleagues proposed that the apoptosis provoked by TS activity might also be capable of provoking an imbalance in the hypothalamus-pituitary-adrenal axis of *T. cruzi*-infected mice, leading to increased release of glucocorticoids, notorious immunossuppressants (59).

Early studies in the 90's already provided evidence of how aTS modulates the host immune system. Chuenkova and Pereira demonstrated that sensitizing mice with TS from conditioned supernatants, as well as recombinant aTS lead to higher parasitemia levels, and increased mortality rates. They also proposed that since animals with severe combined immunodeficiency, which lack functional T and B lymphocytes, were not affected. The logical conclusion was that TS was somehow affecting essential effector components of the adaptive immune system (60).

T lymphocytes must be activated to build up an effective response against invading organisms (61). This process involves loss of SIA residues in α-2,3 bonds from *O*-linked oligosaccharides, exposing free β-1,3 galactose (Gal) residues (62, 63). Such residues can be detected by the use of *Peanut agglutinin* lectin (PNA), which binds to terminal nonreducing Galβ1,3-GalNAc containing-sequences (64). That said *T. cruzi*'s flagship enzyme unique ability to transfer SIA residues springs to mind as the perfect candidate to interfere with this process. Our group demonstrated this by showing that in a TS-free infection, i.e., *Plasmodium berghei*-infected mice, activated $CD8^+$ T cells exhibited a great number of terminal β-Gal residues, while in the presence of aTS, such residues were re-sialylated (37) (**Figure 1**). While further investigation is necessary, it is safe to say that such an effect would be a great help to the parasite, as dampening the cellular response, would help ensure the protozoa's survival within the host. Further evidence of that statement is found in the work of Pereira-Chioccola et al. (65). The authors describe how anti-alpha-Gal antibodies, purified from chronic Chagas disease patients, strongly bind to α-Gal terminals in mucins, causing severe structural perturbations that lead to parasite lysis, while sialylation by TS activity diminishes the damage. The authors proposed that the negative charge provided by SIA

helps stabilizing the *T. cruzi* surface coat by electrostatic repulsion (65).

Although it has been known for more than twenty years that both iTS and aTS have almost identical structures and compete for the same substrate (40, 42, 44), little is known about the biological effects triggered by iTS during *T. cruzi* infection.

In an interesting report, Pascuale et al. (45) demonstrated that the expression of iTS gene in iTS-null parasites was able to improve *T. cruzi* invasion into Vero cells and increased their *in vivo* virulence as shown by histopathologic findings in skeletal muscle and heart tissue of *T. cruzi*-infected mice (45). Although the molecular mechanisms have not been elucidated, the authors claim that iTS might play a different or complementary pathogenic role to aTS (45). Recently, our group demonstrated that mice treated with an elevated (non-physiological) concentration of recombinant iTS showed a compromise of T cells homing to the cardiac tissue during *T. cruzi*-infection (44). Since iTS is capable of recognizing SIA-containing glycans, which are carried by many glycoproteins involved in leukocyte extravasation through activated venular walls (66–68) it would be plausible to speculate that iTS, through its lectinic property, may bind to sialylated peripheral homing receptors, impairing the homing of inflammatory cells to the target tissues. The poor development of genetic tools to directly dissect the biological roles displayed by either iTS or aTS, leads researchers towards alternative approaches for this technical deadlock. The use of both recombinant *T. cruzi*-iTS and aTS, separately or together, may provide a good way for studying the effects triggered by both TS proteins (44). Over the last fifteen years, studies demonstrated that when administered separately, both iTS and aTS elicit similar biological effects (42, 69, 70). However, until recently, there was no published data showing their combined effects. Immunological studies carried out by our group revealed that in *T. cruzi*-infected mice, the intravenous administration of high concentrations of recombinant aTS was able to modulate the expression of inflammatory signals by splenic T cells (44). Nevertheless, when both recombinant iTS and aTS were injected in equivalent amounts, such phenomena were significantly compromised (44). Additional studies are necessary to confirm our previous findings, however, it is plausible to speculate that when present in a soluble form and/or associated to microvesicles (34), iTS may compete with aTS by the same SIA-containing glycotopes and attenuate/abrogate biological events depending of the addition and/or removal of SIA residues.

Another question that needs addressing is the degree to which iTS is able to attenuate or abrogate biological events induced by aTS. In 2010, Freire-de-Lima and colleagues demonstrated that $CD8^+$ T cells from *T. cruzi*-infected mice treated with a high concentration of recombinant iTS, became positive for PNA. These results reinforce the idea that iTS competes with aTS for SIA-containing glycotopes, then compromising an expected re-sialylation phenomenon that naturally happens during *T. cruzi* infection (37).

TRYPANOSOMA CRUZI MUCINS

Trypanosoma cruzi mucins are the parasite's most abundant surface glycoproteins. First described by Alves and Colli in epimastigotes, these highly glycosylated GPI-anchored mucin-like proteins were named A, B, and C glycoproteins (71). These proteins display a great deal of heterogeneity, with the genes responsible for encoding them being divided into two major families (3, 9, 72–74). The *T. cruzi* small mucin gene (TcSMUG) family encodes proteins that are expressed in the insect stages of the parasite's life, being essential to the infectivity on the insect host (75), while the TcMUC family, comprising from five to seven hundred genes, encodes the proteins expressed in the mammalian host. These proteins contain well-conserved N- and C-terminal regions, corresponding to ER and GPI anchor signals, respectively (72, 74, 76). This family can be further divided into three groups: (i) TcMUC I possesses a central domain with tandem repeats, with consensus sequences for *O*-glycosylation sites and it is more expressed in amastigotes (72, 73, 77); TcMUC II, found in trypomastigotes, displays a smaller number of repeats but is rich in serine and threonine residues (9, 72–74). Finally, TcMUCIII refers solely to the expression of a small surface protein, TSSA, or trypomastigote small surface antigen, being expressed only on cell-derived trypomastigotes (15). These mucin-like molecules contain a great number of *O*-linked oligosaccharides that are the main acceptors of SIA in the parasite's surface (**Figure 1**) (78–81). Unlike the classical vertebrate mucins, these oligosaccharides are linked to the protein core through α-GlcNAc residues, instead of α-GalNAc (82). Regardless, they contain a great number of free terminal β-Gal residues, which serve as ideal SIA acceptors (7, 78–81) (**Figure 1**). The *O*-linked oligosaccharides composition and size vary depending both the parasite strain (9, 78–80, 83–85) and its sialylation might promote immunosuppressive properties (please, see below).

The GPI-mucins expressed by *T. cruzi*, also known as sialoglycoproteins, are mucin-like molecules that are highly glycosylated and present a conserved GPI-anchor linked to the parasite cell surface (9, 80–87). All mucin GPI-anchors are constituted by a similar glycan core (Manα1-2Manα1-2Manα1-6Manα1-4GlcN) (9, 80, 85, 87). Except for the cell-derived trypomastigotes, where a branch of Gal residues can modify the GPI anchor (9, 84). The GPI-mucin lipid anchor differs according to the parasite's stage (80, 81, 85). In non-infective insect-derived epimastigotes, they are composed of saturated fatty acids; in metacyclic trypomastigotes, they are mainly inositol-phosphoceramides, and in the cell-derived trypomastigotes, they are composed wholly of alkylacyl-phosphatidylinositol (PI) structures, frequently insaturated (C18:1 or C18:2) (84, 85).

There is abundant data showing that following the early stages of *T. cruzi* infection, the patterns of resistance or susceptibility may be determined before adaptive immunity elements have a chance to respond, with components of the innate immune response playing crucial roles for parasite control (88). *T. cruzi* makes use of an expanded array of molecular strategies to invade an extensive range of host cells, as well as to avoid the host's

immune defense. In the infection site, *T. cruzi* triggers the production of chemokines and pro-inflammatory cytokines, such as interleukin-12 (IL-12) and tumor necrosis factor-a (TNF-α), and the highly reactive oxygen and nitrogen species produced by cells of the Mϕ lineage (84, 85, 89–91). Over the last fifteen years, it has been described that GPI anchors expressed in the surface of *T. cruzi* are determinant in this process (85, 92, 93). In 2006, Bafica and colleagues demonstrated that the activation of innate immune response by *T. cruzi*-derived DNA and GPI anchors from trypomastigote mucins (tGPI-mucins anchors) forms, was able to promote the production of proinflammatory signals (84, 94). The authors revealed that the parasite's DNA stimulates cytokine production by Mϕ in a Toll-Like Receptor-9 (TLR9) dependent mechanism, and synergizes with parasite-derived tGPI-mucins, a TLR2 agonist, in the induction of IL-12 and TNF-α (94). More recently, it has been demonstrated that both living *T. cruzi* trypomastigote forms, as well as tGPI-mucins are able to induce high levels of IL-12 by human monocytes. Additionally, it has been proven that such effect depends on CD40-CD40L interaction and IFN-γ (95). In that work the authors claim that the polarized T1-type cytokine profile observed in *T. cruzi*-infected individuals might be a long-term effect of IL-12 production induced by lifelong exposure to *T. cruzi* tGPI-mucins (95).

It is well accepted that a great array of GPI-mucin genes is responsible for the variability of parasite cell surface (2). In 2004, an interesting work carried out by Buscaglia and collaborators demonstrated that the vast majority of the tGPI-mucin molecules found on the surface of the cell-derived trypomastigotes belong to the TcMUC II group. In this study, for the first time, the authors presented high evidence that multiple products of TcMUC II are concurrently expressed, suggesting that such molecules might represent a sophisticated strategy for the parasite to dampen the host immune response (9).

In 2002, Argibay and co-authors transfected higher eukaryotic cells (Vero cells) with TCMuc-e2 gene, which encodes for a mucin that is expressed in the blood-circulating stage of the parasite. The authors demonstrated that when transfected cells were exposed to human lymphocytes, an event of T cell anergy was observed. In this study, it was also demonstrated that the effect could be reversed by the addition of exogenous IL-2 (35). A different study discussed the effect of the interaction between the *T. cruzi* AgC10, a mucin-like molecule expressed by metacyclic trypomastigotes, as well as on amastigotes (96) and L-selectin in T cell surface. In an event independent of IFN-γ and nitric oxide, it was capable of inhibiting T cell proliferation and IL-2 secretion, as well as impairing IL-2 mRNA expression in response to mitogens. In fact, most genes whose expression is controlled by NFAT (Nuclear Factor of Activated T-cells) were affected and the overexpression of NFAT refuted the effects mediated by the parasite's glycoprotein (97).

The carbohydrate chains of mucin molecules are usually long extended structures (98). Over the last ten years has been demonstrated that the *O*-linked oligosaccharides composition of *T. cruzi* mucin-like molecules might exert direct effect on the host immune system. Since epimastigote forms are easier to be cultured *in vitro*, most of the studies investigating the biological roles triggered by *T. cruzi O*-linked glycans have been performed with non-infective forms for mammal cells. In 2013, Nunes and colleagues showed that a purified preparation of sialylated *T. cruzi* glycoproteins is capable of inhibiting clonal expansion as well as cytokine production by $CD4^+$ lymphocytes. This happens through cell cycle arrest in the G1 phase and cannot be reversed by administration of exogenous IL-2, effectively rendering the cells anergic when stimulated through the T cell receptor (TCR) (99). The authors suggested that the starting point of this effect would be the interaction between the sialylated parasite mucins and Siglecs expressed on the T cell surface (**Figure 1**). An earlier study might substantiate this claim. Erdhmann and co-workers showed that the highly virulent *T. cruzi* Tulahuén strain was able to modulate the functionality of dendritic cells, through the interaction of its sialylated mucins with Siglec-E. The authors also confirmed that the desialylation of the parasite's surface molecules prevents such event (100).

POSSIBLE THERAPEUTIC TARGETS

The mucin-like proteins present in the surface of *T. cruzi* bear a distinct characteristic when compared to mucins or any other *O*-glycosylated protein on the surface of human proteins: the presence of galactofuranose (Gal*f*) residues (79). The flavoenzyme UDP-galactopyranose mutase (UMG) is not found in humans, but is essential to the composition of bacterial and fungal cell walls, as well as an important virulence factor for protozoa (6, 101, 102). A study in the late 80's even managed to show that anti-galactofuranose antibodies lead to a 70% inhibition of cell invasion (103). It should not come as a surprise that some groups treat UMG as an ideal therapeutic target, since the enzyme is not present in humans, and are working towards the development of UMG inhibitors (104–106). One study shows promise in halting the growth of some *Mycobacterium* species (107). It is important to note that this strategy suffers from a fundamental problem in the fact that so far Galf residues have not been found in the mucins expressed in the mammalian host stages'. The presence of Galf residues in metacyclics has been demonstrated (81).

trans-Sialidases also comes off as a potential drug target for the treatment or prevention of Chagas disease, and as such, many groups have been pursuing different strategies focused on TS as a target for either therapeutic or prophylactic methods. Good examples of this are recombinant proteins and DNA vaccines (108–111). Despite early reports showing that immunization with TS inhibits Th1 immune response (70), it was recently demonstrated that such a response can be elicited by the clever use of adjuvants (112). The same group has also shown that using the same model, aTS elicits stronger humoral and cellular responses than other *T. cruzi* antigens (113). Over the last decade, works from many research groups have demonstrated that vaccines candidates based on TS proteins are capable of protecting *T. cruzi*-infected mice (111, 114–118). Groundbreaking studies carried out by Rodrigues and Tarleton groups (119–122) have demonstrated that immunodominant $CD8^+$ T cell immune responses directed to epitopes expressed

by members of the TS family contribute to control *T. cruzi* infection, suggesting that non-antibody mediated cellular immune responses to the antigens expressed in the mammalian forms of *T. cruzi* might be used for the purpose of vaccination. In 2015, Pereira and collaborators started the development of both prophylactic and therapeutic vaccine protocols. The vaccines take advantage of the immunostimulation provided by a replication-defective human Type 5 recombinant adenoviruses (rAd) vector carrying sequences of amastigote surface protein-2 (rAdASP2), and TS (rAdTS). This strategy, rather offers a rational approach for re-programming the host immunity, achieving a more protective profile, leading to interruption of damage and even tissue recovery, particularly when it comes to chronic Chagas heart disease (123).

Another important focus field concerning *T. cruzi* TS is the search for effective inhibitors. A di-sialylated *N*-lactoside compound was shown to promote a 70% inhibition of TS activity through a competition mechanism (124). Sulfasalazine, a first line sulfa drug for rheumatoid arthritis, is also a moderate TS inhibitor. Although it does not lead to a great inhibition of the enzyme activity and it is not particularly toxic to the parasite strains tested by Lara-Ramirez's group, it is a good starting point for the development of new drugs, especially because sulfasalazine has been in use since the early 50s (125).

Several other researches have reported results on promising drugs, from competitive to non-competitive inhibitors, acting through reversible or irreversible mechanisms, some of those reaching up to 50% inhibition in the millimolar range (126–130).

An earlier work from our group has shown that 2-difluoromethyl-4-nitrophenyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid acid (NeuNAcFNP) is able to irreversibly inhibit TS in a time and dose-dependant manner. More importantly, it is able to produce a 90% inhibition of the infection of LLC-MK2 cells by *T. cruzi* Y strain trypomastigotes (131). Although it provides a unique form of inhibition and a chance for less major adverse effects, especially since TS bears no semblance with any human enzyme (132).

CONCLUSION

In this review, we focused on the role of *T. cruzi* glycoconjugates and associated proteins in mediating the relationship between parasite and the human immune system. Throughout the years, several discoveries illustrated how TS, Tc-mucins and SIA are fundamental for the parasite to not only survive, but also thrive in an inhospitable environment like the human body. Mounds of evidence sustain the idea that TS is an important virulence factor, especially during the acute phase of the disease and is pivotal in aiding the parasite in bypassing the immune system. Authors also agree on the fact that mucins are major players in the balance between immune response and parasite survival, especially since it is the primary SIA acceptor in the protozoan membrane.

It is our belief that a better understanding of how *T. cruzi* is able to sabotage the human immune response will provide us with more effective tools to prevent and combat infections. Moreover, the parasite's unique system of handling SIA is almost certainly pivotal, since it involves a one-of-a-kind enzyme and an equally unique group of mucin-like proteins.

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LF, KdC, VC, CF-d-L, AM, LM-P, JP, and LF-d-L participated in the writing of the paper.

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Coalescence of RAGE in Lipid Rafts in Response to Cytolethal Distending Toxin-Induced Inflammation

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The receptor for advanced glycation end products (RAGE) interacts with various molecules in the cell membrane to induce an inflammatory response. The cytolethal distending toxin (CDT) produced by *Campylobacter jejuni* contains three subunits: CdtA, CdtB, and CdtC. Amongst, CdtA and CdtC interact with membrane lipid rafts, by which CdtB enters the nucleus to induce pathogenesis. In this study, we first explored the relationships between RAGE, lipid rafts, and inflammation in gastrointestinal epithelial cells exposed to CDT. Our results showed that CDT activated the expression of RAGE and high mobility group box 1 (HMGB1), followed by the recruitment of RAGE into lipid rafts. In contrast, RAGE antagonist inhibited CDT-induced inflammation via the RAGE-HMGB1 axis. Disruption of lipid rafts decreased CDT-induced downstream signaling, which in turn attenuated the inflammatory response. Furthermore, *in vivo* studies revealed severe inflammation and upregulation of RAGE and IL-1β in the intestinal tissues of CDT-treated mice. These results demonstrate that mobilization of RAGE to lipid rafts plays a crucial role in CDT-induced inflammation.

Keywords: RAGE, HMGB1, cytolethal distending toxin, lipid rafts, inflammation

INTRODUCTION

Campylobacter jejuni is one of the most common causative agents for diarrhea and gastrointestinal diseases in humans (1). CDT produced by *C. jejuni* is composed of three subunits, CdtA, CdtB, and CdtC, which combine to form a holotoxin with cytotoxic activity (2). Among the three toxin components, CdtA and CdtC are pivotal for attachment to the cell membrane, allowing CdtB to enter the cells by endocytosis and to eventually reach the nucleus (3). Nuclear translocation of CdtB, which possesses DNase I activity and induces DNA double-strand breaks (DSB), arrests the cell cycle at the G2/M checkpoint, resulting in cell distention and death (4).

RAGE is a multi-ligand pattern-recognition receptor (PRR), which can interact with advanced glycation end products (AGEs), HMGB1, nucleic acids, and S100 protein family to trigger an inflammatory response (5). Binding of HMGB1 to RAGE activates mitogen-activated protein

kinases (MAPKs) and stimulates nuclear factor kappa B (NF-κB), resulting in the release of several proinflammatory cytokines (6, 7). Clinical studies indicated that RAGE plays a crucial role in the development of inflammatory diseases, such as rheumatoid arthritis (8), diabetes mellitus (9), atherosclerosis (10), and inflammatory bowel disease (11). Importantly, RAGE has been implicated in bacterial diseases that contribute to the severity of disease progression (12–14). Although the interaction of HMGB1 and RAGE is correlated with the inflammatory response (15), the mechanism by which CDT regulates RAGE and HMGB1 expression and triggers pro-inflammatory cytokine production to promote inflammation in epithelial cells remains unknown.

The major components of lipid rafts are cholesterol, glycosphingolipids, and phospholipids, which are insoluble in cold 1% Triton X-100. Thus, lipid rafts are referred to as detergent-resistant membranes (DRMs) (16). Numerous pathogens, including bacteria (17–19), viruses (20–22), and protozoan parasites (23) exploit lipid rafts for internalization by cells. Lipid rafts also allow the binding of bacterial toxins to the cytoplasmic membrane and enhance their efficient delivery into cells (24). Our previous studies demonstrated that *C. jejuni* CDT-induced pathogenesis depends on the coalescence of lipid rafts (25, 26). However, whether CDT relies on lipid rafts to induce RAGE expression to facilitate inflammation is unknown.

HMGB1, a nuclear protein, is released from activated immune cells and binds to TLR4 that in turn activates macrophage tumor necrosis factor (TNF) release (27). A recent study demonstrated that HMGB1 binds to LPS to form a complex that efficiently delivers LPS into the cytoplasm through RAGE-dependent endocytosis, which then reaches the endolysosomes (28). Subsequently, HMGB1 permeabilizes the lysosomes in the acidic environment and allows LPS access to the cytosol and caspase-11, which is crucial for pyroptosis. These findings indicate that HMGB1 and RAGE provide a particular transport pathway to the cytosol, and cargo molecules may avoid destruction by the lysosomes when accompanied by HMGB1 (29). Although the mechanisms underlying HMGB1-mediated intracellular LPS delivery have been elucidated, the interactions between extracellular HMGB1 and CDT, which is transported via RAGE to enable CdtB to gain access into the nucleus, are unclear.

CdtB, a part of the holotoxin, is endocytosed and finally reaches the nucleus where it exhibits DNase I activity (4). The close association of CDT with lipid rafts has been found to be crucial for toxin-mediated pathogenesis (25, 26, 30); however, the specific molecules that contribute to this interaction remain unknown. In this study, we investigated the role of RAGE in the CDT-induced inflammatory response in gastrointestinal epithelial cells. We further explored whether lipid rafts are involved in inducing RAGE expression and the subsequent signaling in response to CDT-induced pathogenesis.

MATERIALS AND METHODS

Preparation of Recombinant CDT

Recombinant His-tagged CDT subunits were cloned by following the standard protocols as described previously (25). *E. coli* BL21-DE3 containing *cdtA*, *cdtB,* or *cdtC* expression plasmids, respectively, were induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C for 4 h. The expression of His-tagged CdtA, CdtB, and CdtC fusion proteins were purified by metal affinity chromatography (Clontech, Palo-Alto, CA) and characterized by SDS-PAGE and western blot analysis.

Cell Culture

AGS cells (ATCC CRL 1739) were cultured in F12 medium (Invitrogen), MKN-45 cells (JCRB0254; RIKEN Cell Bank, Japan) and HT29 cells (ATCC HTB-38; human colorectal adenocarcinoma) were cultured in DMEM (Invitrogen), COLO205 cells (CCL-222; human colon adenocarcinoma cells) were cultured in RPMI 1640 medium (Invitrogen). Cell were cultured in medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and incubated at 37°C in a humid atmosphere containing 5% CO_2.

Cell Cycle Analysis

Each recombinant CDT subunit (100 nM) were added in cell culture medium and incubation at 37°C for 30 min to form a CDT holotoxin (31). After one wash with PBS, AGS cells (1×10^6) were untreated or treated with 100 nM CDT holotoxin for 0, 24, 48, and 72 h. The treated cells were washed and fixed with 70% cold ethanol then incubated at −20°C for 2 h and stained with 20 μg/ml propidium iodine (Sigma-Aldrich, Saint Louis, MO) containing 200 μg/ml RNase A. The stained cells were determined by FACScalibur flow cytometry (Becton-Dickinson, San Jose, CA), and the cell cycle distribution was analyzed by using Cell Quest software WinMDI (Verity Software House, Topsham, ME) as described previously (32).

SDS-PAGE and Western Blot Analysis

Each recombinant CdtA, CdtB, and CdtC was prepared and subjected to 12% SDS-PAGE, respectively. The gel was stained with Coomassie Brilliant Blue R-250 (Amresco, Solon, OH) for further analysis. AGS cells (5×10^5) were exposed to CDT holotoxin with various concentrations for different time durations. The cell lysates were prepared to resolve by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were probed with primary antibodies: RAGE and HMGB1 (Abcam, Cambridge, UK), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Millipore, Temecula, CA). The proteins of interests were detected using the ECL Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ) and visualized by using Azure c400 system and AzureSpot Analysis Software (Azure Biosystems, Dublin, CA).

Immunofluorescence Staining

AGS cells (2×10^5) were seeded on coverslips and treated or untreated with 100 nM CDT holotoxin for 24 h. Cells were then fixed with 4% paraformaldehyde and probed with the primary antibody against RAGE, followed by incubation with Alexa Fluor 488-conjugate goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., Cambridge, UK) and

CTX-B Alexa Fluor 555-conjugate (Invitrogen, Carlsbad, CA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Saint Louis, MO, USA) for 30 min. The stained cells were analyzed using a Zeiss LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany) with a 63 × oil immersion objective (numerical aperture of 1.4).

Reporter Activity Assay

AGS cells were co-transfected with 1 μg *NF-κB* or *IL-8*, and pGL3 luciferase reporters by using jetPEI (Polyplus-transfection, Illkirch, France) according to the manufacturer's instructions. pGL-3 luciferase reporter (Promega, Madison, WI, USA) contains a modified coding region for firefly (*Photinus pyralis*) luciferase that was used to optimize for monitoring transfection efficiency. Reporter lysis buffer (Dual-Luciferase Reporter Assay System; Promega, Madison, WI) was added to each well, and the cells were scraped from the dishes. Equal volumes of luciferase substrate were added to the samples and luminescence was detected using GloMax 20/20 luminometer (Promega), as described previously (33).

Determination of IL-8 Production

IL-8 production was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (34). Briefly, AGS cells were pretreated with 2 μM RAP (Merck Millipore, Billerica, MA), a RAGE antagonist, for 1 h and exposed to 100 nM CDT holotoxin. After incubation for 24 h, the IL-8 concentration was measured by using a sandwich ELISA kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

Co-immunoprecipitation (Co-IP) Assay

The protocol was performed according to the manufacturer's instructions (Immunoprecipitation Kit Dynabeads Protein G, Novex Life Technologies), beginning with the addition of 10 μl of anti-HMGB1 antibody (Abcam, Cambridge, UK) or 10 μl of anti-IgG control (GeneTex, Irvine, CA) to create the co-IP bead-complexes. AGS cells were treated with mock or 100 nM CDT for 24 h at 37°C and cell lysates were prepared. Each sample (50 μg) was added to the anti-HMGB1 antibody or mouse IgG-Dynabeads complexes and incubated for 30 min at 37°C. The bound proteins were eluted and analyzed by western blot assay.

Animal Study

Male BALB/c mice aged 6-weeks-old were purchased from National Laboratory Animal Center (Taipei, Taiwan). Mice were divided into two groups: PBS treated control (n = 3) and 2.5 mg/kg CDT alone (n =3). Each treatment was administered by intragastric gavage once every 2 days for a total of 6 injections. After completing the treatment course, the mice were euthanized and the intestinal tissues were prepared for hematoxylin-eosin (H&E) or immunohistochemistry (IHC) staining. The mice were cared for in accordance with the Laboratory Animal Center of Chang Gung University under a protocol approved by the Institutional Animal Care Use Committee (IACUC Approval No.: CGU16-114).

Statistical Analysis

Statistics analysis comparisons of more than two groups were evaluated using two-way analysis of variance (ANOVA). The P-value for ANOVA had statistically significant difference in those groups, and then used *post hoc* test for ANOVA to analyze the results by Tukey's Honestly Significant Difference Test (Tukey's test). A P-value of <0.05 was considered statistically significant. The statistical software was the SPSS program (version 12.0 for windows, SPSS Inc., Chicago, IL).

RESULTS

CDT Induces RAGE and HMGB1 Expression

Although we previously showed that *C. jejuni* CdtA and CdtC interact with membrane lipid rafts (25), the exact molecules that trigger inflammation are unknown. We therefore established a cell-based assay to determine whether RAGE in lipid rafts contributes to CDT-induced inflammatory signaling. Each His-tagged CDT subunit was purified and validated by SDS-PAGE and western blot analysis (**Figure S1**). We next examined whether CDT induces cell cycle arrest at G2/M in AGS cells, which is a gastrointestinal-derived cell line. As shown in **Figure S2A**, treatment of the cells with 50 nM CDT for 24 h caused G2/M arrest in 79% of cells. The percentage of cells arrested at G2/M approached 90% when the concentration of CDT was increased to 100–500 nM. Remarkable cell distention in CDT-treated cells compared to in the CDT-untreated group was observed by light microscopy (**Figure S2B**). To further examine CDT-induced cell cycle arrest and morphology changes, cells were exposed to CDT holotoxin (100 nM) at 37°C for 0, 24, 48, and 72 h. As shown in **Figure S3**, the number of cells arrested at G2/M gradually increased and cells became distended upon treating with CDT for 24–48 h. We next investigated whether CDT activated RAGE and HMGB1 expression in the cells. AGS cells were treated with CDT (0–500 nM) for different times, and then RAGE and HMGB1 levels were analyzed by western blotting. As shown in **Figures 1A–C**, RAGE and HMGB1 expression gradually increased in cells treated with 50–100 nM CDT and slightly decreased upon treatment with 200–500 nM CDT. Additionally, CDT-induced RAGE and HMGB1 expression was markedly increased after incubation with 100 nM CDT for 3–48 h (**Figures 1D–F**). These results indicate that CDT induced RAGE and HMGB1 expression in dose- and time-dependent manners, and that the optimal conditions were 100 nM CDT and incubation for 24 h. We then investigated whether CDT-induced RAGE and HMGB1 expression in different gastrointestinal-derived cells; four intestinal-derived cell lines (AGS, MKN45, COLO205, and HT29 cells) were employed in this study. Our results showed that the levels of RAGE and HMGB1 were obviously increased in the CDT-treated cells we tested (**Figure S4**).

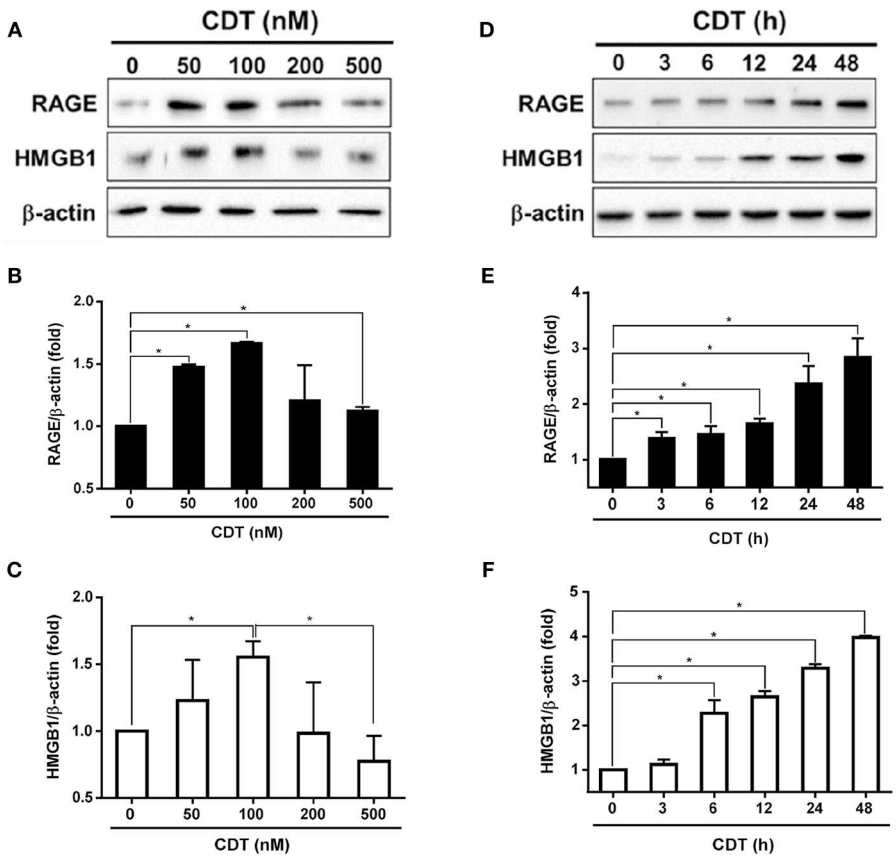

FIGURE 1 | CDT induces RAGE and HMGB1 expression. **(A)** AGS cells were exposed to CDT for 24 h at various concentrations (0–500 nM), and **(B)** treated with 100 nM CDT at different time points (0–48 h). Total cell lysates were prepared to measure the expression of RAGE and HMGB1 by western blotting, and β-actin was used as the protein loading control. Protein expression levels of RAGE and HMGB1 were quantified by densitometric analysis and normalized to β-actin, respectively **(B–F)**. The data are presented as means ± standard deviations for three independent experiments. Statistical analysis was calculated using ANOVA analysis and Tukey's test. *$P < 0.05$ was considered statistically significant.

Blockage of RAGE Signaling Decreases CDT-Mediated Inflammatory Response

The RAGE antagonist RAP, which disrupts the interaction between RAGE and its ligands (35), was employed to investigate whether RAGE is a key factor involved in CDT-mediated inflammation. AGS cells were pretreated with RAP (2 μM) for 2 h prior to treatment with 100 nM CDT, and then the cell lysate was prepared for western blotting. Our results showed that RAP significantly reduced CDT-induced RAGE and HMGB1 expression when compared to CDT treatment alone (**Figures 2A–C**). We therefore analyzed whether blocking RAGE decreased *NF-κB* promoter activity and IL-8 production in CDT-treated cells. AGS cells were co-transfected with *NF-κB* and pGL-3 luciferase reporters prior to treatment with RAGE antagonist followed by exposure to CDT and were then subjected to luciferase reporter assay. In parallel, culture supernatants were prepared to analyze IL-8 production by ELISA. The results showed that both *NF-κB* promoter activity and IL-8 production were significantly increased in CDT-treated cells, while remarkably decreased in cells pretreated with RAGE antagonist (**Figures 2D,E**). These results demonstrate that the CDT-induced inflammatory response was mediated through the RAGE signaling pathway.

CDT Induces the Recruitment of RAGE Into Lipid Rafts

The requirement for lipid rafts to induce RAGE by CDT was evaluated next. As shown in **Figure 3A**, the colocalization of RAGE with CTX-B (which binds to the ganglioside GM1 in rafts) was clearly localized around the membrane lipid rafts in CDT-treated cells (merged in yellow). However, this colocalization was minimal in CDT mock-treated cells (**Figure 3B**). We then examined whether the membrane localization of RAGE was dependent on the presence of cholesterol, which is crucial for the composition of lipid rafts. The cells were pretreated with 5 mM methyl-β-cyclodextrin (MβCD, a cholesterol depletion agent) for 1 h and then exposed to CDT holotoxin. As shown in **Figure 3**, the amount of CDT-induced RAGE that associated CTX-B was visibly reduced upon the cells were pretreated with MβCD. These

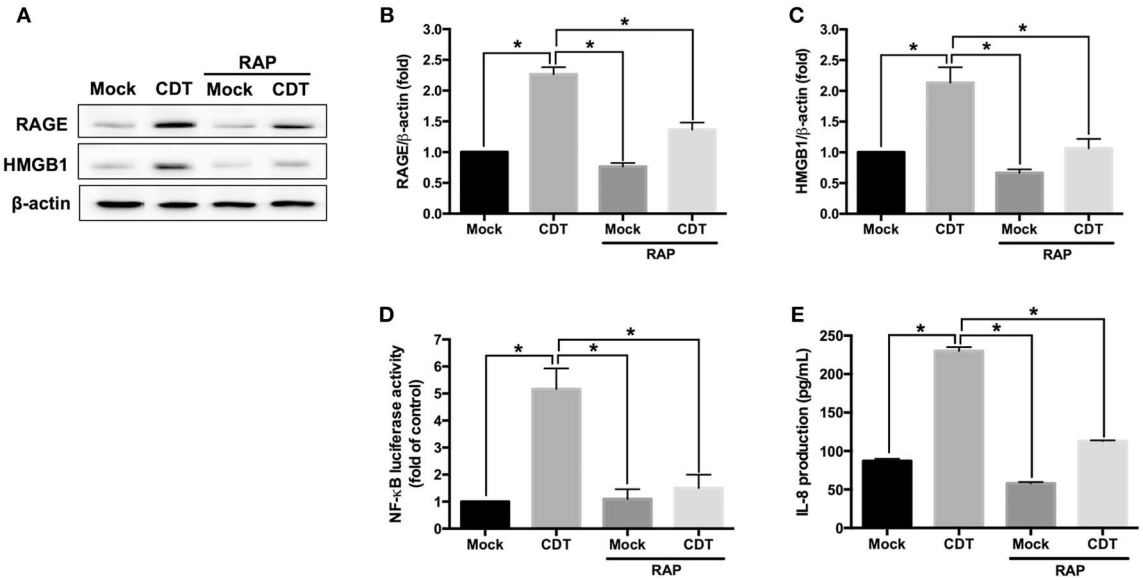

FIGURE 2 | RAGE blockage reduces CDT-mediated inflammatory responses. **(A)** AGS cells were pretreated with RAGE antagonist (2 μM RAP) for 2 h before incubation with 100 nM CDT for 24 h. Cell lysates were analyzed by western blotting with the antibodies against RAGE, HMGB1, and β-actin, respectively. The protein expression of RAGE **(B)** and HMGB1 **(C)** was quantified by densitometric analysis and normalized to β-actin. **(D)** Cells were co-transfected with *NF-κB*- and pGL3-luciferase reporters prior to treatment with the 2 μM RAP followed by exposure to 100 nM CDT for 24 h. pGL3-luciferase reporter was used for monitoring transfection efficiency. *NF-κB* promoter activity was determined and normalized by pGL3 luciferase activity. **(E)** The cell culture supernatant was prepared to evaluate IL-8 production using ELISA. The data are presented as means ± standard deviations for three independent experiments. Statistical analysis was calculated using ANOVA analysis and Tukey's test. $^*P < 0.05$ was considered statistically significant.

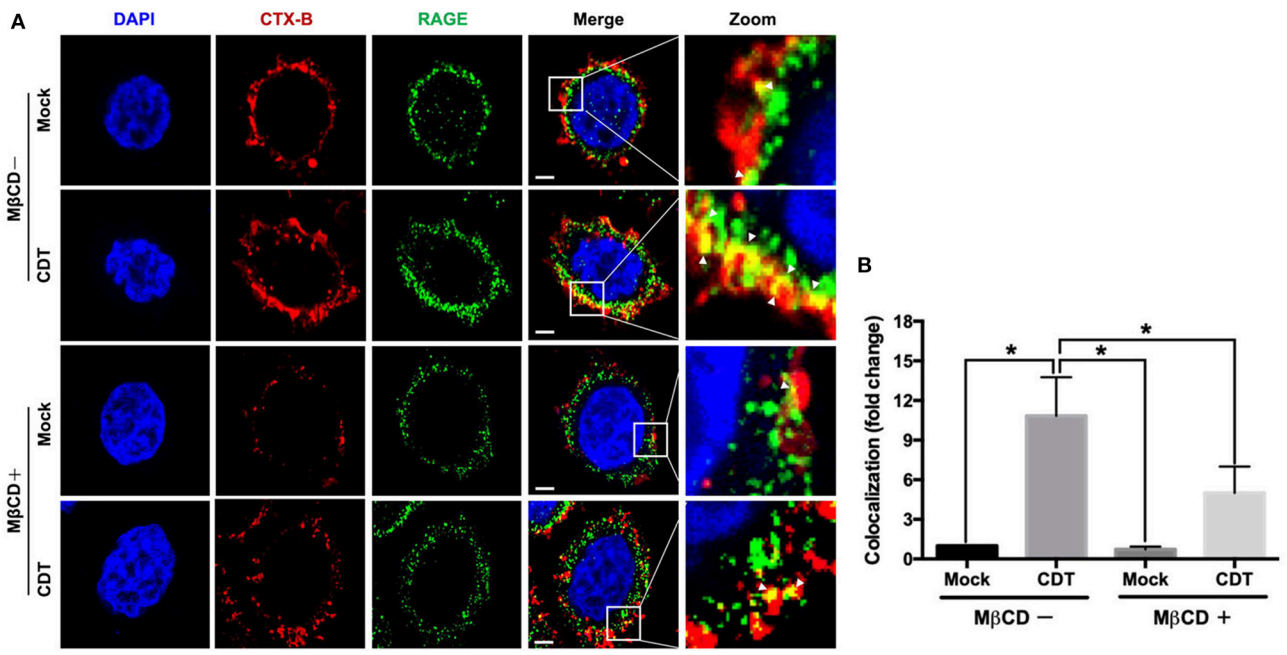

FIGURE 3 | Recruitment of RAGE into lipid rafts by CDT. **(A)** AGS cells were pretreated with or without 5 mM MβCD followed by incubation with 100 nM CDT for 24 h. Cells were fixed and probed with DAPI (blue) to visualize the nucleus, Alexa Fluor 555-conjugated cholera toxin subunit B (CTX-B) to visualize GM1 (red), and an antibody against RAGE (green). Arrows indicated the colocalization (yellow) of CTX-B and RAGE in the overlay. The magnified images were shown in the right panels. Bars, 5 μm. **(B)** The fluorescence intensity of CTX-B and RAGE was analyzed by ZEN software (Carl Zeiss). Colocalized punctate of CTX-B and RAGE were quantified using merged pixels and normalized to those in the mock-control group. Statistical analysis was calculated using ANOVA analysis and Tukey's test. $^*P < 0.05$ was considered statistically significant.

results suggest that the recruitment of RAGE into membrane rafts occurred in response to CDT treatment.

We next investigated whether CDT-induced RAGE expression and inflammation required membrane raft integrity. AGS cells were pretreated with or without 10 μM lovastatin (an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase for cellular cholesterol biosynthesis) and then exposed to CDT. As shown in **Figure 4A**, CDT-induced RAGE and HMGB1 expression were obviously decreased in cells treated with lovastatin. In addition, lovastatin treatment effectively suppressed *NF-κB* promoter activity in CDT-treated cells (**Figure 4B**). Similarly, CDT-induced IL-8 production was significantly reduced when the membrane cholesterol synthesis was inhibited by lovastatin (**Figure 4C**). The amount of secreted HMGB1 was then determined by using ELISA. The results showed that both RAP and lovastatin remarkably reduced the secreted HMGB1 in cells treated with CDT (**Figure S5**). These results demonstrate that depletion of cholesterol inhibited the recruitment of RAGE in lipid rafts and decreased HMGB1 production, which reduced CDT-mediated inflammation.

CDT Induces Intestinal Inflammation in Mice

To further explore the role of RAGE in CDT-mediated inflammation *in vivo*, mice were treated with vehicle-control (PBS) or CDT holotoxin (2.5 mg/kg) through intragastric gavage once every 2 days for a total of six treatments (**Figure 5A**). After completing the treatment course, the mice were euthanized and tissue sections of the small intestine were prepared for histological analysis. As shown in **Figure 5B** (H&E staining), the epithelium was clearly defined without inflammation in the intestinal tissues of the vehicle-control. However, pathological examination revealed disruption of the epithelium and severe inflammatory cell infiltration in the intestinal tissues of CDT-treated mice (**Figure 5**, yellow arrows in the first row). We then examined whether CDT induced the expression of RAGE, HMGB1, IL-1β, TNF-α, and IL-6 in intestinal tissues by IHC. The results revealed stronger expression of RAGE, HMGB1, IL-1β, TNF-α, and IL-6 in the intestinal tissues of CDT-treated mice compared to in the vehicle-control group (**Figure 5B**). Importantly, HMGB1 was translocated from the nucleus to the cytoplasm upon treatment with CDT. These results, together with those from cell-based and animal studies, demonstrate that RAGE is a crucial factor in CDT-mediated inflammation involving lipid rafts.

DISCUSSION

RAGE has been reported to participate in several bacterial diseases (14, 34, 36–38). Although RAGE plays a crucial role in inflammation and is required to control bacterial infections, the effect of RAGE on the immune response to CDT has not been investigated. We found that CDT triggers the RAGE-HMGB1-inflammation axis in lipid rafts. Understanding the role of RAGE in CDT-induced pathogenesis is particularly important, as targeting these critical molecules has been proposed for treating bacterial infectious diseases.

The role of RAGE has been investigated by using animal models infected with different bacterial pathogens (14, 36–38), but showed conflicting results. RAGE was found to elevate the burden of *Streptococcus pneumoniae* in the lungs, which exacerbated pneumonia and increased mortality of WT mice compared to RAGE-deficient mice (37). A recent study also reported that RAGE deficiency increased the survival rates of *Acinetobacter baumannii*-infected mice, which was associated with increased levels of circulating IL-10, an anti-inflammatory cytokine (14). In contrast, RAGE deficiency was found to cause organ failure in a mouse model of *E. coli*-induced sepsis, indicating that a RAGE signaling response is involved in its antibacterial activity (36). RAGE contributes to the defense against *Klebsiella pneumoniae* infection by decreasing the bacterial burden and restraining extrapulmonary dissemination, thereby reducing mortality (38). However, the hyperinflammation was occurred in response to gram-negative bacteria by RAGE signaling and that exacerbated the infection in diabetic mice (12).

Consistent with these findings, our study showed that CDT exploited lipid rafts to induce inflammation through the activation of the RAGE-HMGB1-IL-8 axis, indicating that

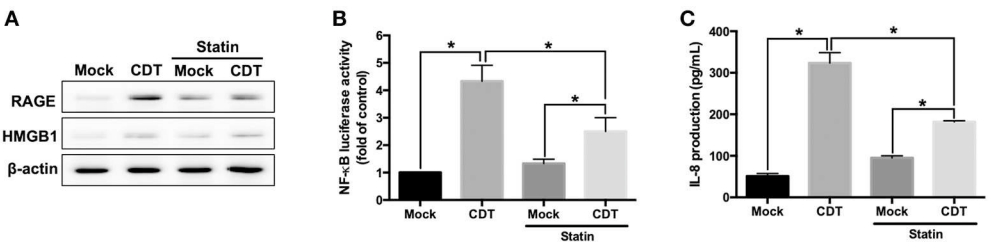

FIGURE 4 | Disruption of lipid rafts decreases CDT-induced inflammatory response. **(A)** AGS cells were pretreated with or without 10 μM lovastatin for 1 h and exposed to 100 nM CDT for 24 h. Cell lysates were analyzed by western blotting with the antibodies against RAGE, HMGB1, and β-actin, respectively. AGS cells were co-transfected with *NF-κB* and pGL3 luciferase reporters in the absence or presence of 10 μM lovastatin before treatment of 100 nM CDT for 24 h. Cell lysates were used to analyze **(B)** *NF-κB* promoter activity and normalized by pGL3 luciferase activity. **(C)** Cell supernatants were subjected to ELISA for the quantification of IL-8 production. The data are presented as means ± standard deviations for three independent experiments. Statistical analysis was calculated using ANOVA analysis and Tukey's test. $^*P < 0.05$ was considered statistically significant.

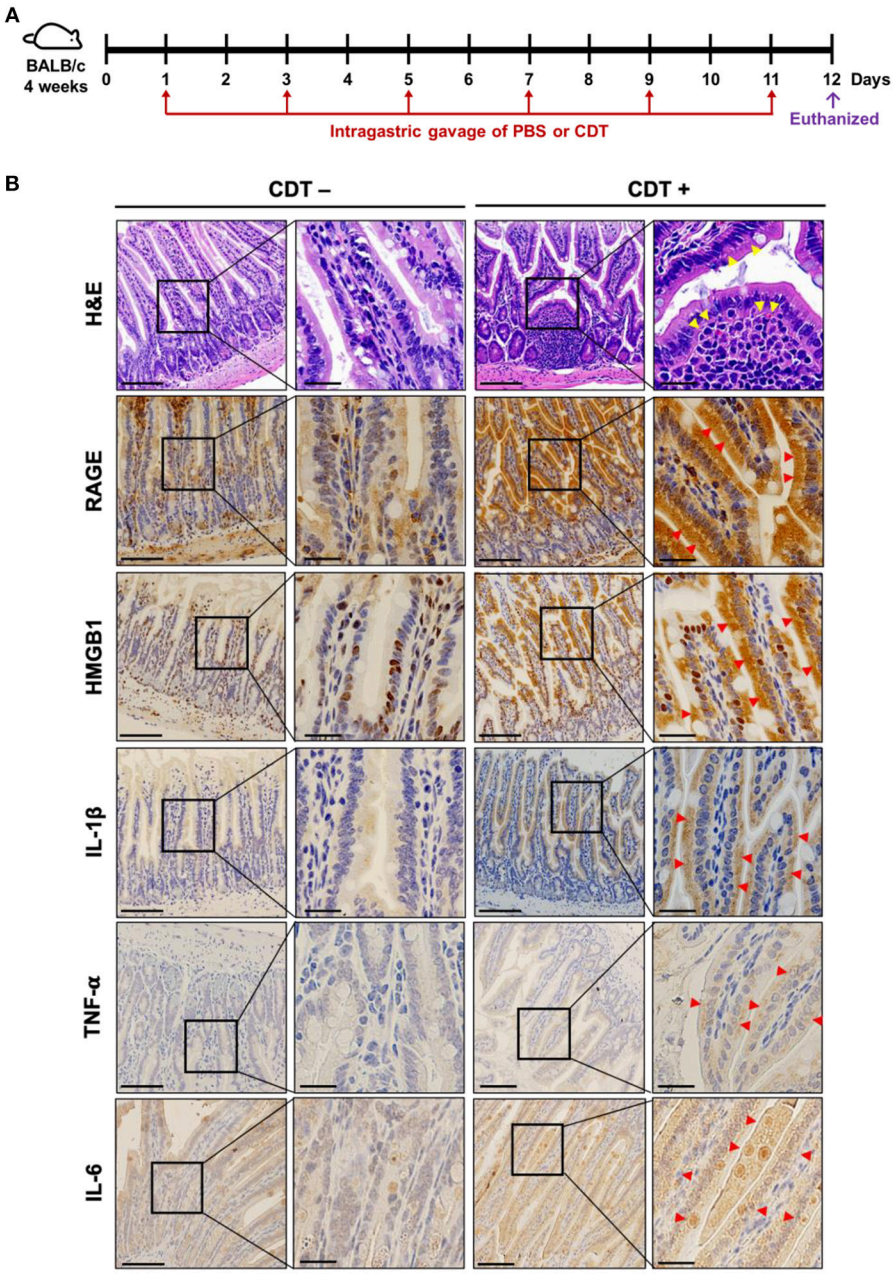

FIGURE 5 | CDT induces RAGE expression and proinflammatory cytokine production in the mouse small intestine. **(A)** Mice were treated with PBS or CDT (2.5 mg/kg) by intragastric gavage once every 2 days for six administrations. Arrows in red indicated the days of CDT administration. **(B)** Tissue sections of the jejunum were prepared and fixed in 4% paraformaldehyde and subjected to hematoxylin-eosin (H&E) or immunohistochemical (IHC) staining with antibodies against RAGE, HMGB1, IL-1β, TNF-α, and IL-6, respectively. The magnified images are shown in the right panel of each cropped area. Arrows in yellow represented severe infiltration of inflammatory cells in the intestinal epithelium with pathological derangement. Pronounced expression of proinflammatory cytokines shown in intestinal tissues were indicated by red arrows. Scale bars in left panels, 20 μm and in magnified right panels, 200 μm.

RAGE is a key factor in this process. Notably, the immune defense against pathogen infection is a double-edged sword that either prevents microbial infections or destroys host cells. Therefore, the exact role of RAGE in the beneficial or deteriorated immune defense against CDT-induced pathogenesis requires further investigation.

Danger-associated molecular pattern (DAMP) proteins, such as HMGB1, S100, IL-1α, and IL-33/ST2, are endogenous danger signals (39–41). DAMP signal activation is mediated by several PRRs, including RAGE and Toll-like receptors (TLRs), which are involved in bacteria-induced inflammation (42–44). Several studies have indicated that DAMPs function as alarmins, forming

immunostimulatory complexes with chemokines and promoting leukocyte migration and inflammatory responses (15, 45, 46), which are correlated with the severity of bacterial infection (47). RAGE is a ligand for DAMP and is involved in activating NF-κB to stimulate the production of pro-inflammatory cytokines (48). Although we demonstrated that RAGE was mediated during CDT-induced inflammation in the intestine, whether pattern recognition receptors other than RAGE are involved in CDT-induced inflammation is unclear. Identifying mechanisms other than the HMGB1-RAGE interaction is critical for improving the understanding of molecular patterns that occur in response to CDT.

We recently demonstrated that *C. jejuni* CdtA and CdtC interact with membrane-associated lipid rafts, enabling CdtB to cross the cell membrane for transport into the nucleus (25, 26, 30, 49). CdtB possesses DNase I activity, which causes DSBs and leads to cell apoptosis (4). Our current study demonstrate that CDT increased the expression of HMGB1. This can occur at the transcriptional and posttranslational levels, although exactly how the expression of HMGB1 was increased remain unknown. Additionally, it was unclear how CDT influenced HMGB1 to affect the repair of DSB. Despite the availability of genetic information and experimental results, the understanding of CDT-induced pathogenesis at the molecular level warrants further investigations.

HMGB1 is a sticky molecule that binds several proinflammatory molecules including LPS. The HMGB1-LPS complex is endocytosed via RAGE to reach the endolysosomal compartments, then enables LPS to gain access to the cytosol and induce caspase-11 expression, which induces pyroptosis (28). HMGB1 without co-molecules is a strong inducer for cytokines, but it needs TLR4 rather than RAGE for this induction (27, 50). In contrast, HMGB1 with co-molecules can induce cytokines via RAGE. Our study, by using co-immunoprecipitation assay, showed that CDT binds to extracellular HMGB1 that may be important for endocytosis by RAGE (**Figure S6A**). Although CDT could induce RAGE expression, TLR4 was not involved in this process (**Figure S6B**). In line with previous studies, our results showed that CdtB and HMGB1 form a complex, which may interact with the cell-surface receptor RAGE. However, whether HMGB1 is essential for translocation of CdtB into the cytosol and finally reaching the nucleus through RAGE-mediated endocytosis require to be investigated.

Although the cell-based assay platform has demonstrated that RAGE plays a crucial role in CDT-induced inflammation, some limitations exist in the current studies, including small number of analyzed mice and did not perform this study in the RAGE or HMGB1-knockout mice. In addition, the direct linkage between RAGE/HMGB1 production and inflammatory response needs to be validated by knockdown or knockout approaches. Further investigations *in vivo* are required to fill in the gap in the translational aspect of the study.

In conclusion, our results demonstrate that RAGE played a crucial role in the CDT-induced inflammatory response. Increased levels of RAGE and HMGB1 were observed in cells treated with CDT. In contrast, RAGE antagonists ameliorated CDT-mediated inflammation by inhibiting the RAGE-HMGB1 axis. Furthermore, disruption of lipid rafts reduced the reporter activities of *NF-κB* and *IL-8* in CDT-treated cells, revealing that CDT-induced inflammation was dependent on lipid rafts. Animal studies further showed that the expression of RAGE and HMGB1, and inflammatory cytokines were increased for the intestinal inflammation in response to CDT. Determining the mechanisms of how CDT triggers inflammation may result in the development of new strategies for controlling bacteria-associated pathogenesis.

AUTHOR CONTRIBUTIONS

H-JL, C-HC, and C-HL: conception or design of this work. H-JL, Z-PJ, H-RL, C-LF, C-JC, and C-YY: experimental study. M-ZH, H-YW, Y-AC, and YC: data analysis and interpretation. H-JL, C-HC, and C-HL: writing the manuscript. All authors made final approval.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00109/full#supplementary-material

Figure S1 | Characterization of recombinant CDT subunits derived from *C. jejuni*. **(A)** The locations of *cdtA*, *cdtB*, and *cdtC* on the genome of *C. jejuni* are shown. **(B)** Each CDT subunit (2 μg/mL) was prepared and analyzed using SDS-PAGE. **(C)** Western blot analysis of CDT subunits as detected by antibodies against CdtA, CdtB, or CdtC, respectively. The results represent one of three independent experiments.

Figure S2 | CDT induces cell cycle arrest and cell distension. **(A)** AGS Cells were treated with 0–500 nM CDT and incubated at 37°C for 24 h. The cell cycle distribution was analyzed by flow cytometry. **(B)** Cells were exposed to 100 nM CDT for 24 h, and then the cell distention was observed (right panel). Bars, 100 μm. The results represent one of three independent experiments.

Figure S3 | CDT arrests cell cycle at G2/M and induces cell distension in a time-dependent manner. AGS Cells were untreated (left panels) or treated (right panels) with 100 nM CDT and incubated at 37°C for 0, 24, 48, and 72 h. The cell cycle distribution was analyzed by flow cytometry and cell morphology was

observed by light microscopy. Bars, 100 μm. The results represent one of three independent experiments.

Figure S4 | CDT induces RAGE and HMGB1 expression in gastrointestinal-derived cell lines. AGS, MKN-45, COLO205, and HT29 cells were exposed to CDT (100 nM) for 24 h. Total cell lysates were prepared to determine the expression levels of RAGE and HMGB1 by western blotting. β-actin was used as the protein loading control. The results represent one of three independent experiments.

Figure S5 | Disruption of lipid rafts and inhibition of RAGE decrease CDT-induced HMGB1 secretion. AGS cells were pretreated with **(A)** RAGE antagonist (2 μM RAP) for 2 h or **(B)** 10 μM lovastatin for 1 h, and then incubated with 100 nM CDT for 24 h. Cell supernatants were subjected to ELISA (G-Biosciences, St. Louis, MO, USA) for the quantification of secreted HMGB1. The data are presented as means ± standard deviations for three independent experiments. Statistical analysis was calculated using ANOVA analysis and Tukey's test. $P < 0.05$ was considered statistically significant.

Figure S6 | CdtB binds to extracellular HMGB1 and induces inflammation. **(A)** AGS cells were mock-treated or treated with 100 nM CDT for 24 h and then subjected to co-IP and western blot analysis as described in the Materials and Methods. **(B)** Total cell lysates were prepared to determine the expression levels of TLR4, RAGE, COX-2, and iNOS by western blot assay. β-actin was used as the protein loading control. The results represent one of three independent experiments.

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Selectins and Immune Cells in Acute Myocardial Infarction and Post-infarction Ventricular Remodeling: Pathophysiology and Novel Treatments

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The glycosciences aim to understand the impact of extracellular and intracellular carbohydrate structures on biological function. These glycans primarily fall into three major groups: lipid-linked carbohydrates that are referred to as glycosphingolipids or simply glycolipids; relatively short carbohydrate chains that are often O- or N-linked to proteins yielding common glycoproteins; and extended linear polymeric carbohydrate structures that are referred to as glycosaminoglycans (GAGs). Whereas, the impact of such carbohydrate structures has been extensively examined in cancer biology, their role in acute and chronic heart disease is less studied. In this context, a growing body of evidence indicates that glycans play an important role in immune mediated cell recruitment to damaged heart tissue to initiate wound healing and repair after injury. This is particularly important following ischemia and reperfusion that occurs in the heart in the setting of acute myocardial infarction. Here, immune system-mediated repair of the damaged myocardium plays a critical role in determining post-infarction ventricular remodeling, cardiac function, and patient outcome. Further, alterations in immune cell activity can promote the development of heart failure. The present review summarizes our current understanding of the phases of immune-mediated repair following myocardial infarction. It discusses what is known regarding glycans in mediating the recruitment of circulating immune cells during the early inflammatory stage of post-infarction repair, with focus on the selectin family of adhesion molecules. It offers future directions for research aimed at utilizing our knowledge of mechanisms underlying immune cell recruitment to either modulate leukocyte recruitment to the injured tissue or enhance the targeted delivery of biologic therapeutics such as stem cells in an attempt to promote repair of the damaged heart.

Keywords: glycan, selectin (sE, sL, sP-selectin), leukocyte-endothelial cell adhesion, heart disease, myocardial infarction, post-infarct repair, stem cells, mesenchymal (stromal) stem cells

INTRODUCTION

Cardiovascular diseases, including atherosclerosis, myocardial infarction (MI), and heart failure, represent a primary cause of morbidity and mortality in Western civilization and are rapidly becoming a major epidemic in developing and underdeveloped nations. While the use of lipid-lowering statins, angiotensin-converting-enzyme inhibitors, and medical devices (e.g., coronary stenting, defibrillators, and ventricular assist devices) have reduced the incidence of death, survivors of primary MI are susceptible to secondary heart failure and reinfarction. The factors governing patient outcome are complex, but generally driven by metabolic changes (1), the acute phase response (2), and alterations in leukocyte migration patterns (3). While various basic science studies have examined the putative role of glycosylation in aspects related to cell adhesion and cell signaling, an integrated understanding of their potential impact on the progression of cardiovascular diseases is lacking. The current review addresses this topic with a focus on the impact of selectins in regulating immune system-mediated cardiac repair following myocardial infarction, the current status of anti-selectin therapies directed to the heart, and novel regenerative therapeutic approaches that attempt to exploit naturally occurring cell adhesion processes to improve patient outcome. The discussion suggests that instead of completely abolishing all immune cell interactions following ischemia-reperfusion, a more nuanced approach that finely modulates the relative contributions of different leukocyte populations and exploits glycan-mediated stem cell delivery may be more beneficial.

PATHOPHYSIOLOGY OF ACUTE MYOCARDIAL INFARCTION

Acute MI is typically caused by the abrupt interruption of blood flow through an epicardial coronary artery by plaque rupture and the subsequent formation of an occlusive thrombus, which leads to cardiac myocyte death and compromised heart function. Although the implementation of timely reperfusion strategies has reduced the acute mortality associated with MI, improved patient survival has increased the incidence of chronic heart failure, due in large part to adverse remodeling of the damaged left ventricle (LV) following the initial ischemic event (4). Thus, despite surviving an initial MI, many patients experience a dramatic deterioration in quality of life with the onset of heart failure, a condition for which there is currently a paucity of treatment options that address the fundamental problem of cardiomyocyte loss. This paradigm shift has re-directed translational research efforts toward investigation of the downstream consequences of MI in hopes of identifying novel approaches to reduce adverse LV remodeling and prevent the onset of heart failure.

Work in this area has demonstrated that cardiac repair after MI is characterized by a series of time-dependent events orchestrated by the innate immune system (**Figure 1A**). This begins immediately after the onset of necrotic cell death with intense sterile inflammation and myocardial infiltration of a variety of immune cell subtypes including neutrophils and monocytes during the first several days after MI (**Figure 1B**) (5). Subsequently, there is a transition to a reparative and proliferative phase in which inflammation is resolved, myofibroblasts proliferate, and collagen deposition leads to scar formation. Finally, the scar undergoes a maturation process characterized by extracellular matrix (ECM) cross-linking and quiescence of myofibroblasts. A proper balance and timely resolution of the inflammatory, proliferative, and maturation phases of repair are essential to produce an appropriate wound healing response. For example, an inflammatory phase of excessive magnitude or duration can exacerbate tissue damage, impair scar formation, and perpetuate further cardiac myocyte loss, thereby promoting adverse LV remodeling characterized by infarct expansion, chamber dilatation, and contractile dysfunction (6). Indeed, several experimental (3, 7, 8) and clinical (9–11) studies demonstrate that excessive mobilization and/or recruitment of inflammatory cells impair post-MI healing and is associated with adverse outcomes. Thus, investigation of biological mechanisms underlying the inflammatory, proliferative, and maturation phases of cardiac repair has intensified with the hope that improved understanding of these processes may facilitate the development of therapeutic strategies that optimize healing of the damaged heart following MI. Glycans are an integral part of such studies due to their critical role on leukocyte-endothelial cell adhesion mechanisms and their value as biomarkers of metabolic alteration. The following text discusses in detail the three remodeling phases following acute MI.

Inflammatory Phase of Post-infarction Repair

Although a variety of cell types are involved in mediating post-MI inflammation, circulating leukocytes play a particularly prominent role. Prolonged ischemia and reperfusion injury elicits cardiomyocyte death, primarily via necrosis but also through apoptosis and autophagy (12). Myocyte death, as well as damage to the ECM, prompts the release of danger-associated molecular patterns (DAMPs) that attract circulating immune cells via binding to pattern recognition receptors (PRRs). Besides passive release from necrotic myocytes and damaged ECM, DAMPs and pro-inflammatory cytokines may also be secreted from stressed or reversibly injured myocytes surrounding the infarct core to initiate recruitment of circulating granulocytes and monocytes. These signals also elicit endothelial activation and rapid upregulation of cellular adhesion molecules such as P- and E-selectin that facilitate leukocyte adhesion, endothelial rolling, and, ultimately, extravasation into damaged tissue.

Neutrophils are the first immune cell type to infiltrate the infarcted myocardium and do so via expression of selectin ligands that initiate adhesion to activated endothelial cells. Slow rolling along the endothelial surface allows neutrophils to sense chemokines bound to glycosaminoglycans, subsequently promoting integrin activation, firm adhesion, and transmigration at endothelial junctions. Extravasated neutrophils phagocytize cellular debris, release proteolytic enzymes, and generate reactive oxygen species to degrade extracellular matrix and initiate the

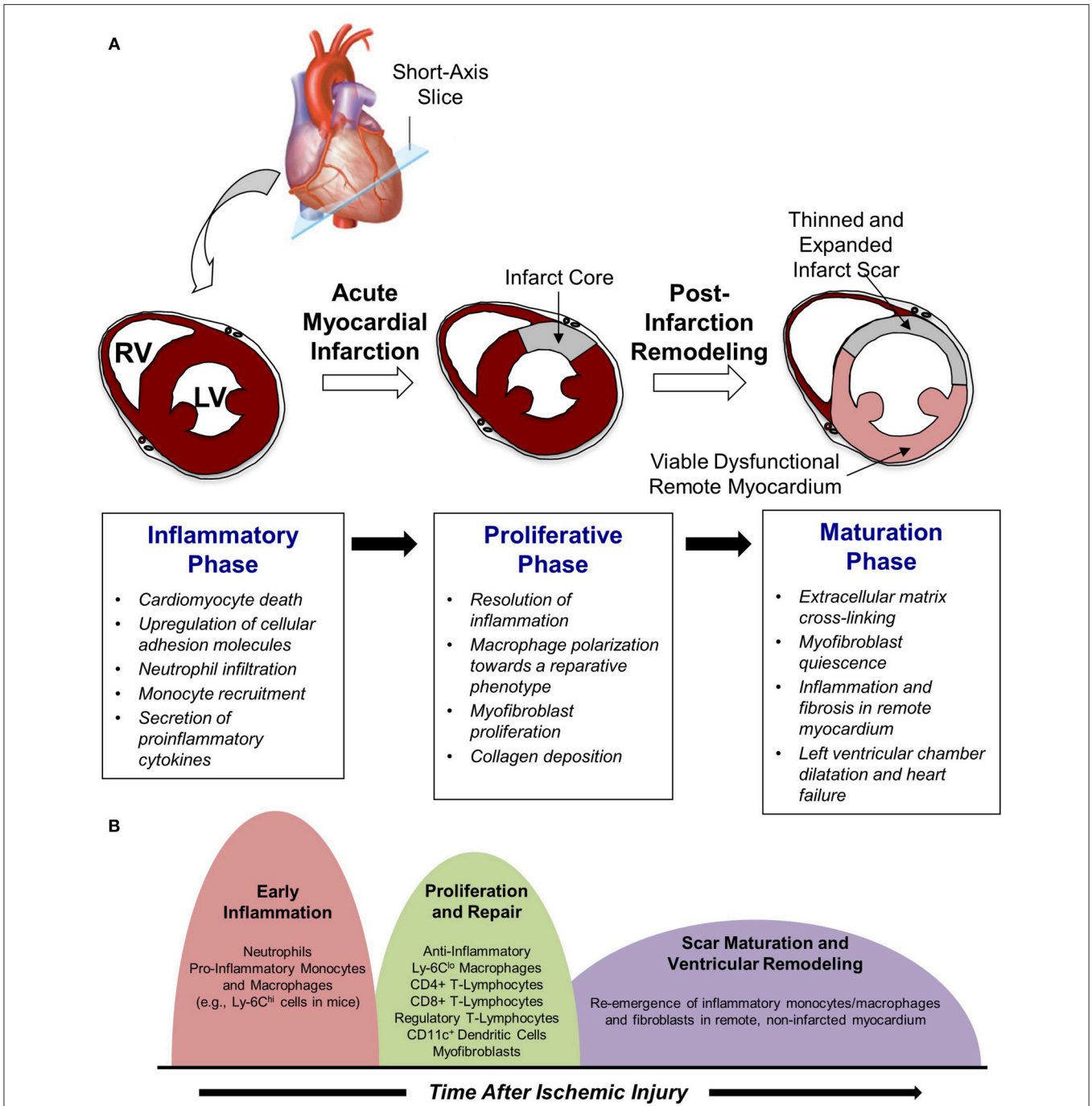

FIGURE 1 | Cardiac repair after myocardial infarction. **(A)** The three phases of repair. **(B)** Temporal changes in leukocyte populations during repair phase.

wound healing response (13). However, these actions perpetuate further inflammation and can exert direct cytotoxic effects on viable myocytes and blood vessels, thereby exacerbating myocardial damage associated with reperfusion injury (14). Excessive intravascular neutrophil accumulation also promotes capillary damage and microvascular plugging that can result in microvascular obstruction and the "no-reflow" phenomenon, thereby compromising the quality of reperfusion and extending the duration of ischemic injury (15). Moreover, neutrophil-mediated endothelial cell injury promotes the development of interstitial edema, which can further impair microvascular perfusion via extravascular compression (16). The adverse effects of neutrophil infiltration can extend beyond the early post-reperfusion period as well, as disproportionate neutrophil

accumulation may interfere with the recruitment of additional leukocyte populations and the transition from the inflammatory to proliferative phase of cardiac repair, an essential component of tissue healing. Nevertheless, experimental neutrophil depletion studies have revealed a critical role for neutrophils in orchestrating post-infarction repair by influencing macrophage polarization toward a reparative phenotype (17), reinforcing the notion that an appropriately tempered inflammatory response is necessary after MI. Indeed blunt abrogation of integrin CD11/CD18 (18) and P-selectin (19), while presenting impressive results in experimental animal studies, failed to convincingly improve outcomes in clinical studies. This reinforces the need to develop a better understanding of MI pathogenesis, particularly in humans.

Shortly after neutrophil infiltration reaches a peak at ∼24-h post-MI, monocytes are recruited to the site of injury and begin to take on a critical role in infarct wound healing and tissue repair (**Figure 1A**). The infiltration of monocytes is facilitated by an increased mobilization of monocytes into the blood from the bone marrow as well as from extramedullary tissue including the spleen, which has recently been recognized to serve as a monocyte reservoir that can be activated following injury (20). Early recruitment of monocytes to infarcted myocardium is primarily regulated via the monocyte chemoattractant protein (MCP)-1/chemokine receptor (CCR)-2 axis, as pro-inflammatory monocytes (e.g., Ly-6C^{hi} cells in mice) expressing high levels of CCR2 are attracted to injured tissue expressing the CCR2 ligand MCP-1 (21). As a result, the initial wave of monocyte infiltration, which peaks ∼3 days after reperfused MI, is characterized by an influx of pro-inflammatory monocytes that differentiate into tissue macrophages and promote removal of necrotic debris and tissue digestion via release of proteolytic enzymes such as matrix metalloproteinases and cathepsins (22). Subsequently, this inflammatory response gives way to a reparative response mediated in large part by a shift in monocyte and macrophage function toward tissue repair through increased expression of anti-inflammatory, pro-fibrotic, and angiogenic growth factors such as interleukin (IL)-10, transforming growth factor (TGF)-beta, and vascular endothelial growth factor (VEGF) (5). Although it was initially proposed that this shift in monocyte/macrophage function was mediated by recruitment of reparative (e.g., Ly-6C^{lo}) monocytes from the blood, recent work in mice has demonstrated that reparative macrophages are also derived from inflammatory (Ly-6C^{hi}) monocytes that are recruited from the blood, undergo a phenotypic switch to anti-inflammatory (Ly-6C^{lo}) macrophages, and proliferate within the infarct to resolve inflammation and promote wound healing (**Figure 1B**) (23).

As with neutrophils, the balanced, timely, and restrained infiltration of monocytes/macrophages is necessary for successful post-infarction healing, as macrophage depletion studies have demonstrated impaired healing and worsened cardiac function after MI (24, 25). However, experimental studies inducing excessive elevations in monocyte and/or macrophage numbers have shown impaired infarct healing and adverse ventricular remodeling as well (26, 27). Thus, therapeutic strategies to limit the supply of inflammatory monocytes after MI continue to receive attention, bolstered by experimental data demonstrating that interventions to reduce monocyte infiltration can reduce infarct size and improve post-infarction cardiac function in rodent models (7, 28). In this regard, while a number of attempts have been made to apply anti-adhesive selectin-ligand targeted therapies to augment post-MI repair and reduce ischemia-reperfusion injury, these have mostly failed to yield favorable results. The above discussion suggests that rather than completely blocking particular or all leukocyte populations, more nuanced strategies that modulate the levels of specific sub-populations in a timed manner may be more beneficial.

Proliferative Phase of Post-infarction Repair

In addition to initiating the inflammatory phase of post-infarction repair, infiltrating leukocytes also play an important role in the timely suppression and spatial containment of inflammation to facilitate transition toward the proliferative phase of healing. This stage typically begins ∼4–7 days after reperfusion and is characterized by resolution of inflammation and proliferation of fibroblasts to initiate the formation of a collagen-rich scar. Neutrophils that had infiltrated the injured area early after reperfusion undergo apoptosis and subsequent phagocytic uptake by macrophages, which induces a phenotypic switch toward a pro-resolving (i.e., "M2") macrophage phenotype characterized by release of anti-inflammatory and pro-fibrotic cytokines including IL-10 and TGF-β (5). Furthermore, apoptotic neutrophils express scavenging chemokine and cytokine receptors that reduce tissue levels of pro-inflammatory mediators, further contributing to a shift toward an anti-inflammatory micro-environment (29, 30).

Beyond the macrophage phenotypic switch elicited by phagocytosis of apoptotic neutrophils, additional leukocyte, and lymphocyte populations contribute to the proliferative phase of repair. For example, CD11c^{+} dendritic cells infiltrate the infarcted myocardium during the proliferative phase of repair and contribute to resolution of inflammation, scar formation, and angiogenesis. These effects appear to be mediated via clearance of pro-inflammatory cell types, as experimental ablation of dendritic cells in a rodent model of MI has been shown to result in sustained expression of inflammatory cytokines, persistent infiltration of pro-inflammatory monocytes and macrophages, and deterioration of left ventricular function (31). Anti-inflammatory T-lymphocyte populations also infiltrate the infarct area during the proliferative phase of repair and facilitate the transition toward maturation. This includes CD4^{+} and CD8^{+} T-cells, regulatory T-cells, and natural killer T-cells that may be activated by as-yet-unknown cardiac autoantigens and limit adverse ventricular remodeling by promoting wound healing, inflammation resolution, and scar development via collagen matrix formation (32). Regulatory T-cells (CD4^{+}Foxp3^{+}) may be particularly important in this context: Weirather et al. recently used a model of genetic regulatory T-cell ablation and an anti-CD25 monoclonal antibody to demonstrate that this population of T-lymphocytes modulates monocyte/macrophage polarization, myofibroblast activation, and collagen expression

within the developing infarct scar to encourage wound healing after MI (33). Furthermore, increasing regulatory T-cell activation with a superagonistic anti-CD28 monoclonal antibody administered 2 days after MI led to improved infarct healing and survival compared with untreated controls, suggesting that therapeutic activation of regulatory T-cells may be a promising approach to boost cardiac repair and limit adverse ventricular remodeling (34).

Fibroblast expansion and conversion to a synthetic myofibroblast phenotype is key component of the proliferative phase of post-infarction repair (**Figure 1A**). In this process, inactivated fibroblasts become activated and develop expression of contractile proteins including α-smooth muscle actin (35). Although myofibroblasts contribute to the inflammatory phase of repair via secretion of pro-inflammatory cytokines and matrix metalloproteinases, they take on a more central role in the proliferative phase by producing anti-inflammatory and pro-angiogenic factors that facilitate the formation of granulation tissue. The source of these cells remains incompletely understood, but it has been suggested that myofibroblasts arise from either resident fibroblasts (36) or circulating bone marrow progenitor cells (37). Additional possible sources of myofibroblasts in the infarct include endothelial cells (via endothelial-mesenchymal transition) and epicardial epithelial cells. Regardless of the source, acquisition of a myofibroblast phenotype leads to proliferative activity and synthesis of extracellular matrix proteins including collagen, fibrin, and fibronectin, all of which contribute to the early phases of scar formation.

The dynamic extracellular matrix changes occurring during the proliferative phase of infarct healing are driven in large part by the induction of matricellular proteins that primarily direct cytokine and growth factor responses rather than provide structural support. These matricellular proteins include thrombospondins, tenascins, periostin, osteopontin, osteoglycin, and proteins from the secreted protein acidic and cysteine-rich (SPARC) and CCN families (38). Along with other proteins from the galectin and syndecan families, these matricellular proteins modulate protease and growth factor activity to provide spatial and temporal regulation of several processes that characterize the transition between initial inflammatory activation and scar formation. For example, structural matrix assembly, angiogenesis, fibrinogenesis, growth factor signaling, and regulation of inflammation have all been demonstrated to be influenced by matricellular proteins (38). Furthermore, it has been suggested that the selective localization of matricellular protein expression in the infarct border zone plays an important role in localizing inflammatory and fibrotic responses to the site of injury, despite diffusion of secreted growth factors and cytokines to remote, non-infarcted tissue (38). As a result, inappropriate induction of matricellular proteins beyond the infarct border may contribute to infarct expansion and adverse ventricular remodeling, thereby representing a possible therapeutic target to enhance post-infarction repair. Similarly, expression of matricellular proteins for an extended period of time during the proliferative phase of healing may lead to excessive fibrosis following injury, as clearance of matricellular proteins is thought to be an important "stop" signal to limit pro-fibrotic signaling. While a vast majority of extracellular matrix proteins are glycosylation, very little is known regarding their role in regulating post-MI cell proliferation and repair.

Maturation Phase of Post-infarction Repair

Following the proliferative phase of repair, the emerging scar undergoes a maturation process in which the extracellular matrix becomes cross-linked and reparative cells including myofibroblasts are deactivated, enter a quiescent state, and may undergo apoptosis (38). The precise mechanisms underlying myofibroblast deactivation and quiescence remain incompletely understood but likely involve withdrawal of pro-fibrotic growth factors and activation of inhibitory "stop" signals via matricellular protein clearance, as mentioned above. In addition, a time-dependent increase in the production of anti-fibrotic factors may diminish matrix synthesis and promote scar maturation. For example, interferon-γ-inducible protein-10 is known to be upregulated after MI and functions to prevent spatial expansion of the pro-fibrotic response beyond the infarct area via proteoglycan-mediated inhibition of fibroblast migration (39, 40).

Beyond scar maturation in the infarct area, late post-infarction remodeling is often characterized by inflammation and fibrosis in viable non-infarcted myocardium in remote areas of the heart (**Figure 1B**). This pattern of remodeling can lead to LV dilatation, global systolic dysfunction, and the onset of heart failure. Clinical studies demonstrate that this series of events is most common in patients with large infarcts as well as in those exhibiting greater initial inflammatory activation (41, 42). Local activation of macrophages and fibroblasts in the remote non-infarcted myocardium as a result of increased wall stress secondary to the loss of contractile activity of the infarct area is thought to occur in this scenario. Moreover, it has been suggested that incomplete or impaired resolution of myocardial inflammation in the late phases of repair may lead to amplification of post-MI injury over time and promote adverse LV remodeling (5). Alternatively, recent data support the intriguing possibility that a second wave of immune activation may occur, due in part to the structural remodeling of the spleen, heightened antigen processing, and trafficking of activated spleen-derived monocytes to the heart to promote apoptosis, fibrosis, and dysfunction (43). Further investigation of this area is necessary and may yield novel understanding of mechanisms underlying immune system-mediated remodeling of the heart in the late phases of post-infarction repair, ultimately leading to new treatments designed to inhibit this process and prevent the development of heart failure.

ROLE OF SELECTINS IN IMMUNE CELL RECRUITMENT FOLLOWING ISCHEMIC INJURY

Emerging evidence supports an important role for glycans in each of the phases of post-infarction repair described above, beginning with recruitment of circulating immune cells early

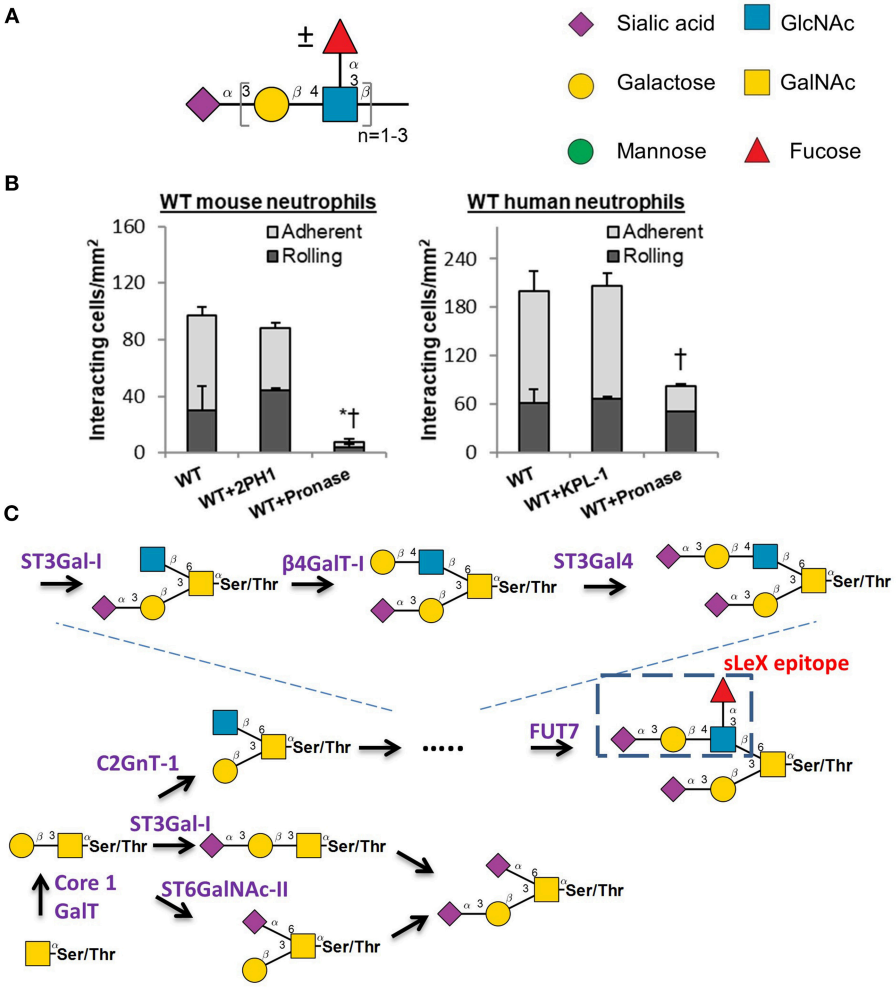

FIGURE 2 | Selectin ligand biosynthesis. **(A)** Sialofucosylated selectin ligands found on leukocytes are composed of at least one α(2,3) sialic acid and one α(1,3) fucose, typically on a Type-II lactosamine chain that may repeat. **(B)** Human leukocyte rolling on HUVEC monolayer is resistant to pronase digestion, but this is not the case for mice. * and †: $P < 0.05$ for rolling and adherent cells, respectively. **(C)** Selectin ligand biosynthesis at the N-terminus of PSGL-1. Competing pathways regulate the biosynthesis of the sialyl Lewis-X (sLe^X) epitope on core-2 based O-glycans. These competing enzymes are the core2 GlcNAc-transferase, ST3Gal-I, and ST6GalNAc enzymes. **(B)** is adapted from Mondal et al. (58) with permission.

after tissue injury. Leukocyte extravasation follows a sequential cascade of steps involving at least three sets of proteins. First, selectins expressed on the inflamed vascular endothelium (E- and P-selectin), leukocytes (L-selectin) and activated platelets (P-selectin). These are type-II transmembrane cell surface proteins with a calcium dependent C-type carbohydrate binding lectin domain that engage a diverse set of glycoproteins and glycolipids on the leukocyte surface. Second, chemokines expressed on the inflamed endothelium that bind their cognate receptors on leukocytes. Chemokine presentation and function is keenly regulated by the surface expression of glycosaminoglycans on the endothelium (44, 45). Third, integrins on the leukocytes that bind members of the immunoglobulin domain proteins (ICAM-1, VCAM-1) among other entities. Importantly, this binding activity is regulated by the N-linked glycosylation status of integrins (46, 47). Overall, leukocyte adhesion interactions to the vascular wall are regulated by all major families of cell surface carbohydrates: O- and N-linked glycans that are common to glycoproteins, carbohydrates attached to glycolipids and the extended glycosaminoglycan (48). While several excellent reviews in the field focus on the latter two aspects (45, 47), the focus of the current discussion is on the role of selectin-glycan interactions since this is a critical step that is necessary for immune cell recruitment.

Selectin-carbohydrate binding interactions are unique in mammalian physiology since the onset of this molecular interaction is rapid (high on-rate) and strong (high tensile bond strength) (49). These unique bond properties enable selectin-ligand binding to facilitate the rapid capture of flowing blood cells to sites of inflammation. The selectins bind sialofucosylated carbohydrate epitopes that typically bear one terminal α(2,3) sialic acid linkage and at least one α(1,3)-linked

fucose attached to a type-II lactosamine chain (Galβ1,4GlcNAc). The prototypic selectin-ligand is called sialyl Lewis-X (sLe^X, Neu5Acα2,3Galβ1,4[Fucα1,3]GlcNAc), though selected ligands can also appear on extended lactosamine chains that carry more than one α(1,3)fucose in a variety of configurations (50–52) (**Figure 2A**). Such selectin-ligands are generated commonly upon the post-translation modification of proteins at Ser/Thr or Asn sites, and to a lesser degree they may be found on specialized leukocyte surface glycolipids that are termed "myeloglycans" (53).

A variety of studies have been performed to identify the precise biosynthetic pathways that yield selectin-ligands and physiological selectin binding glycoproteins. These studies have primarily utilized mouse models (54). While this is generally a beneficial approach, there has been recent criticism that mouse models may have some important limitations with respect to their mimicry of human biology, particularly as it relates to the inflammatory response (55, 56). The development of newer RNA-interference (RNAi) technology and genome editing methods (CRISPR-Cas9) have led to studies that now utilize human leukemic cell lines and also primary human blood cells that are differentiated from $CD34^+$ hematopoietic stem and progenitor cells (hHSPCs) for similar assays (57, 58). It has become evident in these studies that the adhesion molecules and enzyme-regulating selectin-ligand biosynthesis in humans and mouse are potentially organism-specific. This is most notably observed in studies that utilized pronase to cleave glycoproteins on the leukocyte cell surface, since mouse leukocytes fail to interact with stimulated endothelial cells following protease digestion, whereas the human counter-parts display robust cell adhesion under shear (58) (**Figure 2B**). This implies that at least some of the human selectin-ligands are protease insensitive, while this is not the case for mice. It is possible that these differences could in part, account for the failure of previous clinical trials that attempted to design anti-adhesion therapy for humans largely based on observations in murine models.

With regard to binding P-selectin, this adhesion molecule avidly binds an O-linked glycan that is located at the N-terminus of the leukocyte glycoprotein PSGL-1 (P-selectin glycoprotein ligand-1) (59). In humans, this glycan resides at Threonine 57 (T57) at the N-terminus of mature PSGL-1. The extended nature of P-selectin with 9 consensus repeat domains and the position of PSGL-1 at the tip of leukocyte microvilli enhance the probability that P-selectin will interact with its ligand under fluid shear (60). Thus, P-selectin binding to its ligand is often the first step that regulates leukocyte-endothelial cell adhesion interactions. It is now established that the O-glycan at the tip of PSGL-1 that binds selectins is a core-2 glycan with a terminal sLe^X structure (**Figure 2C**). The relative prevalence of this ligand is tightly controlled by the action of three competing enzymes that act to regulate core-2 structure biosynthesis: (i) Core-2 GlcNAc transferase (C2GnT-I) that forms this structure; (ii) ST6GalNAc enzymes that compete to add sialic acid at the 6-position of GalNAc (52), the same location as C2GnT-I; and (iii) The sialyltransferase ST3Gal-I which facilitates core 1 O-glycan sialylation, as its reduction promotes core 2 O-glycan biosynthesis (61) (**Figure 2C**). In this regard, it has been proposed that the balance between ST3Gal-1 and C2GnT-I plays a major role in controlling $CD8^+$ T lymphocyte homeostasis. A dramatic shift from ST6GalNAc dominated α(2,6) sialylated structures to core-2 structures is also observed on T-cells as they transition from resting to activated states (62).

In addition to the above enzymes, studies using transgenic mice suggest additional glycoslytransferases that either partially or fully regulate sLe^X biosynthesis at the PSGL-1 N-terminus. These include polypeptide α-GalNAcT ppGalNAcT-1 (63), core-1 β1,3GalactosylT T-synthase (64), core-2 β1,6GlcNAcT C2GnT-I (65), β1,4GalactosylT β4GalT-I (66), α(2,3)sialylT ST3GalT-IV and VI (67, 68) and α(1,3) fucosyltansferases (FUTs), FUT7 (69), and FUT4 (70). Sulfation of the peptide backbone by tyrosine sulfotransferases is also important for functional selectin ligand biosynthesis on PSGL-1. The molecular players in human leukocytes is likely similar to mice in that FUT4 and FUT7 are the dominant contributors to L- and P-selectin binding under shear (71). ST3Gal-VI may however not be as significant in human leukocytes since knocking out ST3Gal4 (also called ST3Gal-IV) alone is sufficient to abolish cell rolling via both L- and P-selectin (57), both in studies performed with HL-60 cell lines and human neutrophils derived from $CD34^+$ hHSPCs. The exact contributions of the other enzymes to human leukocyte adhesion remains unknown. Additionally, while it is reported that the $CD16^-CD14^+$ classical monocytes express higher sLe^X levels on the cell surface compared to non-classical $CD16^+CD14^{dim}$ (72), the relative contribution of C2GnT-I, ST6GalNAc enzymes in regulating the balance in monocytes is yet to be established, as much of the previous data were derived from neutrophils.

With regards to endothelial selectins, current consensus suggests that E-selectin is the dominant selectin in humans, while P-selectin may be dominant in mice. This is supported by observations that the promoter of the *Selp* (i.e., P-selectin) gene in mice, but not humans, has binding sites for multiple transcription factors including NF-κB and ATF-2 (73). Due to this, P-selectin is both secreted from storage granules and it is transcriptionally upregulated in mice upon stimulation with TNF-α, IL-1β, and LPS. In contrast, whereas P-selectin granule stores exist in humans and rapid exocytosis is noted upon inflammatory stimulus, longer term transcriptional control is absent. In support of this, following inflammatory TNF stimulation, wild-type mice exhibit slow leukocyte rolling and increased cell adhesion unlike transgenic human P-selectin expressing animals that display more rapid rolling and reduced adhesion (73). Overall, the basal and inducible levels of P-selectin differ across species, and its relative contribution to mouse leukocyte adhesion is higher compared to man. Thus, the perceived "central role" of P-selectin during inflammation based on murine studies may not hold in humans where E-selectin likely has a larger role (73, 74).

Besides the above difference in selectin expression patterns, current data suggest that the major E-selectin ligands identified in mice, ESL-1, CD44, and PSGL-1/CD162 (75), may not play a dominant role during human leukocyte rolling on either recombinant E-selectin or IL-1β stimulated human umbilical

vein endothelial cells. In this regard, there is no homolog for ESL-1 in humans (76). PSGL-1 is a relatively minor E-selectin ligand in humans: anti-PSGL-1 blocking antibodies do not block human neutrophil binding to E-selectin (77, 78), and CRISPR-Cas9 knock-out HL-60s lacking PSGL-1 roll robustly on endothelial cells (HUVECs) under shear. Finally, while a specific glycoform of CD44 is known to act as an E-selectin ligand on hHSPCs, it has been previously reported to be absent in mature human leukocytes (75, 79, 80). In recent studies focused on the identification of E-selectin ligands on human monocytes, Silva et al. (72) demonstrate the expression of the E-selectin binding sLe^X epitopes on classical monocytic O-linked glycans expressed on CD43, CD44, and CD162; and possibly also one other yet unidentified 60–70 kDa glycoprotein. Further studies using genetic ablation and blocking mAbs are needed to confirm the potential role of these ligands in human monocyte recruitment to the injured heart. Interestingly, while the O-glycans of CD44 are reported to facilitate monocyte binding in this study, it is the CD44 N-glycans that facilitate E-selectin binding to T-cells. This suggests cell specific differences in glycosylation machinery even within leukocytes sub-populations of a single species.

Besides differences in the protein scaffolds, many studies from our laboratory show that the glycosyltransferases synthesizing E-selectin ligands may differ between humans and mice. Specifically, the α(1,3)fucosyltransferase FUT9 has a more significant role during human leukocyte adhesion (71), compared to FUT7 and FUT4 which are the dominant players in mice (69, 81). Knocking out the α(2,3)sialyltransferase (sialylT) ST3Gal-4 abrogates E-selectin binding (and also other selectins) in humans (57) but this is only partially effective in mice (68, 82). Knocking out glycosphingolipid (GSL) biosynthesis using CRISPR-Cas9 by targeting the enzyme UGCG (UDP-Glucose Ceramide Glucosyltransferase) results in skipping/unstable rolling motion of human myeloid cells on E-selectin (58).

In order to dissect the contributions of N-glycans, O-glycans and glycolipids on human leukocyte cell adhesion, Stolfa et al. (83), made a panel of 7 single, dual and triple knockout cell lines on the human leukemia HL-60 background that bear truncated glycoconjugates (**Figure 3A**). The investigators tested the ability of these cells to be recruited and to roll on recombinant E-selectin substrates and HUVECs. Their studies demonstrated that O- and N-linked glycans, both, control the initial recruitment of neutrophils from flow with N-glycan primarily regulating neutrophil rolling velocity. Whereas, glycolipids did not play a role in the initial recruitment, their proximity to the cell membrane allowed their participation in the slow rolling process, which eventually lead to firm arrest.

Overall, the expression pattern of scaffold proteins bearing the carbohydrate-ligands and the level of cellular glycosyltransferase activity are important parameters that define the E-selectin ligand. More detailed studies are needed to determine if the protein scaffolds and glycan structures identified above for human neutrophils, also contribute to the adhesion patterns of other immune cell types relevant to post-infarction repair. The identification of the E-selectin binding glycoconjugates and related blocking antibodies will greatly simplify this quest.

THERAPEUTIC EFFICACY OF INTERVENTIONS AIMED AT INTERRUPTING SELECTIN-MEDIATED IMMUNE CELL INFILTRATION AFTER MYOCARDIAL INFARCTION

In light of the key role that selectins play in immune cell recruitment, adhesion, and tissue infiltration after ischemic injury, efforts to diminish the detrimental effects of pro-inflammatory immune cells have focused on interrupting selectin-mediated cell adhesion after MI. The earliest approaches centered upon the use of monoclonal antibodies directed against specific selectin molecules. After it was determined that P-, L-, and E-selectin each recognize the common ligand sLe^X, carbohydrate sLe^X analogs were developed and tested as a soluble selectin blocker (84). In addition, discovery of PSGL-1 as a high-affinity ligand for P-selectin encouraged the development of soluble PSGL-1, which has been utilized for functional blockade of selectins *in vivo* (85). The following section summarizes progress that has been made over the past two decades in the investigation of anti-inflammatory therapies designed to interfere with selectin-immune cell interactions after myocardial ischemic injury.

P-selectin antagonism has been attempted using sLe^X and sPSGL-1 analogs in experimental studies of myocardial infarction. Initially, Buerke et al. found a significant, 83% reduction in myocardial infarct size following myocardial ischemia/reperfusion in cats that were treated with CY-1503, a sLe^X oligosaccharide (86). Importantly, the reduction in infarct size elicited by CY-1503 treatment also led to improved cardiac functional performance, based on invasive measurements of cardiac contractility (dP/dt_{max}). Subsequently, Silver et al. tested CY-1503 in a large animal (dog) model and found a nearly 70% reduction in infarct size relative to the ischemic area-at-risk 1-h after reperfusion, with a marked reduction in myeloperoxidase activity compared with controls that was consistent with reduced neutrophil infiltration in CY-1503-treated animals (87). The duration of this benefit was extended to 48-h in a later study by Flynn et al., in which CY-1503 treatment produced ∼55% reductions in both infarct size and neutrophil infiltration in a canine model of myocardial ischemia/reperfusion injury (88). Such studies with classical sLe^X analogs fell out of favor, partly due to the low binding affinity of such entities and limited circulatory half-life (49). Nevertheless, better design of sLe^X synthetic analogs have recently emerged, with compounds like GMI-1070 beginning late-stage clinical trials for sickle cell disease (89). It is possible that success in such orphan disease studies may pave the way for future trials related to myocardial infarction.

Following the discovery of the major P-selectin ligand PSGL-1, attention shifted toward analogs of this glycoprotein since the binding affinity of PSGL-1 for P-selectin is ∼1,000 times that of sLe^X (90, 91), and mouse studies demonstrated an important role for murine PSGL-1 in leukocyte trafficking and neutrophil

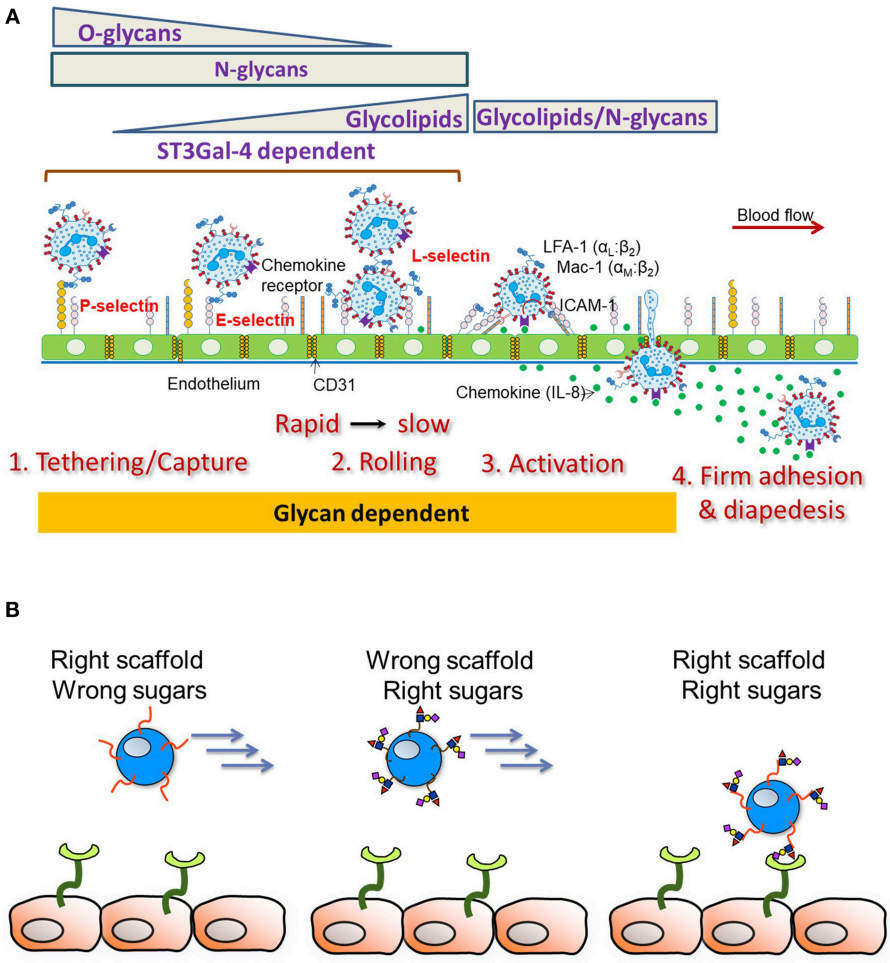

FIGURE 3 | Leukocyte-endothelial cell adhesion cascade. **(A)** Schematic depicts results from studies using human cells. The sialyltransferase ST3Gal4 is indispensable for all aspects of leukocyte adhesion. O-glycans are important for leukocyte capture from flow along with N-glycans. N-glycans largely control leukocyte rolling velocity with glycolipids also contributing to the transition to firm arrest. Besides cell adhesion, emerging evidence points to a role for glycans in also regulating cell activation and the transition to firm arrest. **(B)** Endowing heterologous cell types (like Mesenchymal Stem Cells, MSCs) with selectin scaffold proteins ("right scaffold") along with enzymes facilitating the construction of sialofucosylated glycans ("right sugars") can enable stem cell capture and rolling on the inflamed endothelium.

recruitment following inflammatory injury (92). Following initial positive studies in isolated rat hearts (93) and cats subjected to myocardial ischemia/reperfusion injury (94), efficacy of a recombinant soluble P-selectin glycoprotein ligand-Ig (rPSGL-Ig) was confirmed in a canine model of reperfused MI (95). In this study, dogs were subjected to 90-min of regional myocardial ischemia via balloon occlusion of the left anterior descending coronary artery. Fifteen minutes after reperfusion (achieved by balloon deflation), an intravenous bolus of rPSGL-Ig (1 mg/kg) or saline was administered. Animals were followed for either 2-h or 7-days, at which time infarct size, neutrophil infiltration, and myeloperoxidase activity were assessed. rPSGL-Ig-treated animals exhibited a significant reduction in infarct size relative to the ischemic area-at-risk compared with saline-treated animals at both time points, as well as diminished neutrophil infiltration and myeloperoxidase activity in the ischemic region of the left ventricle (95). These beneficial effects of rPSGL-Ig treatment were associated with an improvement in left ventricular ejection fraction 24-h after reperfusion, although both treatment groups exhibited improvements in ejection fraction over the following week and group differences were no longer statistically significant 7-days after reperfusion.

Besides selectin-ligand analogs, the therapeutic administration of monoclonal antibodies has largely focused on inhibition of P-selectin, based in large part on early experimental studies showing beneficial effects of anti-P-selectin antibodies in animal models of myocardial infarction. For example, administration of anti-P-selectin antibodies resulted in a ∼60% reduction in myocardial infarct size and preserved coronary vascular endothelial integrity in a feline model of myocardial ischemia/reperfusion injury (96), a finding that was subsequently reproduced in a canine model (97). Translation of these

findings to clinical testing eventually led to the completion of the SELECT-ACS trial, in which a highly specific human recombinant monoclonal antibody direct against P-selectin (inclacumab; 5 or 20 mg/kg) was administered to more than 200 patients undergoing percutaneous coronary intervention for non-ST-segment MI (NSTEMI) (19). It is important to note that the rationale for conducting this study was primarily based on the hypothesis that P-selectin antagonism would exert beneficial effects on peri-procedural coronary vascular injury by minimizing platelet adhesion, macrophage accumulation, and neointimal formation at the site of revascularization. Nevertheless, biomarkers of cardiac injury including cardiac troponin I and creatine kinase-myocardial band tended to be lower in patients treated with inclacumab ($p = 0.05$–0.10), suggesting that P-selectin antagonism has some benefit in NSTEMI patients. Interestingly, a follow-up subgroup analysis of the SELECT-ACS trial was performed and demonstrated that the beneficial effects of inclacumab were particularly pronounced in patients that received treatment <3-h before percutaneous coronary intervention (98). This finding reinforces the importance of understanding dynamic time-dependent changes in selectin expression after injury to allow effective therapeutic targeting, particularly when agents with a relatively short half-life are used.

Collectively, these results provide support for the notion that P-selectin blockade may offer therapeutic benefits after myocardial infarction, although strong clinical data are lacking. The reason that the encouraging preclinical results described above have not been rapidly translated to human patients is not immediately clear, although this generally parallels the overall experience to date with anti-inflammatory strategies to treat patients with MI (99). Furthermore, efforts to interrupt neutrophil infiltration after MI via the administration of antibodies against endothelial integrins such as CD18 and CD11 have been unsuccessful in clinical studies, which may have discouraged testing of selectin blockade strategies. Another possible issue relates to the short duration of follow-up that is often employed in preclinical studies. Because infarct size and inflammatory cell infiltration were typically measured in the first several hours after reperfused MI in initial experimental animal studies, the contribution of selectin-mediated immune cell recruitment at later stages of post-infarction repair may have been overlooked. Taken together with information described above indicating species-specific differences in the role of particular selectins in mediating immune cell recruitment and the distinct contributions of different leukocyte sub-types to the injury and repair process, it is possible that E-selectin has been overlooked as a potential therapeutic target. Indeed, early studies testing E-selectin antagonism with monoclonal antibodies did not observe a positive effect on infarct size when measured within the first 4-h after reperfusion, consistent with data demonstrating that coronary vascular expression of E-selectin is minimal during this early post-injury time frame (100). However, the evolution of our understanding of post-infarction healing has revealed that pathological and reparative processes contributing to ventricular remodeling after MI occur at stages beyond the initial hours following reperfusion. Thus, E-selectin antagonism targeted to the acute post-MI inflammatory phase (e.g., 0–24 h after reperfusion) may be an attractive therapeutic strategy as it would diminish neutrophil-mediated reperfusion injury without interfering with the subsequent monocyte-driven repair phase. Furthermore, E-selectin expression by endothelial cells in the bone marrow and spleen has recently been shown to regulate hematopoietic stem cell proliferation, as well as monocyte production and release into the blood after myocardial ischemic injury (101, 102). Further studies are therefore necessary to determine whether novel therapies targeting E-selectin, perhaps in combination with P-selectin blockade, may offer the dual benefit of dampening post-infarction inflammation via a two-pronged approach involving interruption of leukocyte mobilization from the bone marrow and spleen, as well as leukocyte extravasation at the site of myocardial injury. With the appropriate study design that allows for evaluation of efficacy beyond the first several hours after reperfusion, monoclonal antibodies, antisense oligodeoxynucleotides (103), and nanoparticle-based RNA interference-based approaches (104) could each be useful.

ENHANCING THE DELIVERY OF STEM CELL THERAPEUTICS BY MIMICKING NATURAL IMMUNE CELL RECRUITMENT MECHANICS

Because myocyte loss is a fundamental component of ischemic injury and adverse post-infarction remodeling, stem cell-based therapy has emerged as a promising approach to restore cardiac function after MI (105). However, progress in this field has been stymied due, at least in part, to challenges related to cell survival and engraftment after injection. Accordingly, there is interest in learning from the natural homing process of immune cells to inflamed myocardium and glycoengineering stem cells with selectin ligands and other features to enhance retention at sites of cardiac injury. Such cells exhibit low immunogenicity and express multiple bioactive compounds including chemokines and growth factors that may enhance cardiac repair and promote myocardial regeneration. Moving beyond whole cells, stem cell-derived exosomes and microvesicles can also be targeted to deliver microRNA and proteins for therapeutic benefit. These vesicles often contain pro-angiogenic and pro-fibrotic factors that promote endothelial proliferation and TGF-β driven repair processes (106). Such modified cells and exosomes may be administered via a variety of delivery methods including intra-venous (i.v.), intra-arterial (i.a.), or intra-coronary (i.c.) infusion in order to enable their targeting to sites of injury. With respect to the mode of infusion, i.c. injection may be the most suitable approach since i.v. injection results in the delivery of all cells to the right-side of the heart with possible trapping and retention in non-targeted lung alveolar capillaries (107). Arterial injection is also an alternate approach that may result in both directed and passive entrapment within arterial microvasculature. In this regard, stem cell type (e.g., typical mesenchymal stem cells/MSCs and cardiosphere derived stem cells/CDCs) are typically 1.5–2-fold larger than normal blood cells, and they are mechanically

more rigid. The larger size increases the drag force applied on the cells under fluid shear by a factor of 2–4 compared to blood leukocytes, thus making it more challenging to capture these cells in large vessels (108). However, this enhanced size makes them more prone to entrapment in the microcirculation, a region where endothelial cells highly expresses adhesion molecules relevant to inflammation and injury.

An advantage of systemic injection is that unlike localized injection at focal regions of damage that often have reduced nutrient and oxygen levels and suffer from risks of tissue perforation, systemic infusion may allow targeting of the cellular therapeutic in a less invasive manner. Ideally, such cells would be delivered to a well-vascularized, border regions of the heart that is viable, but compromised due to damage to the surrounding tissue. Such regions are often inflamed and they express high levels of selectins, chemokines, and integrins. While early studies suggested that the mesenchymal stem cells (MSCs) may constitutively express selectin and integrin ligands, it is now believed that such expression is not robust, and is highly dependent on the nature of *in-vitro* propagation conditions (109). Additionally, MSCs are not a uniform cell type as surface markers vary between sources and with passage number. Based on this, it is currently thought that the artificial over-expression of selectin-ligand is necessary on stem cells as this may improve targeted delivery by mimicking blood neutrophils that have high tropism for sites of vessel injury. This may then reduce the number of stem cells required for therapy and minimize off-target effects, enhancing retention in the heart above the ∼1–2% of injected dose that is common when the cell surface is unmodified (110).

In support of this concept, Xia et al. (111) demonstrated that human umbilical cord blood $CD34^+$ cells contain reduced amounts of sLe^X expression. The over-expression of this sialofucosylated epitope upon addition of recombinant fucosyltranferase enzyme FUT6 along with GDP-fucose (guanosine diphosphate-fucose) donor enabled the assembly of robust levels of the sLe^X epitope on the cell surface. Enhanced $CD34^+$ stem cell rolling on endothelial monolayers expressing P- and E-selectin ensued and transplantation of these modified cells enhanced blood cell engraftment in mouse models. Similar to this, recent pilot first-in-human trials also suggest that this approach may be feasible clinically as it led to faster neutrophil and platelet engraftment (112). Similar to cord blood, MSCs can also be engineered to overexpress α(1,3)fucosyled epitopes via exofucosylation (109) and also using modified RNA (113) to enhance cellular targeting particularly to the bone, since the marrow constitutively expresses E-selectin. In all these studies, the precise glycoproteins that act as functional selectin-ligands is not fully established though there are suggestions that a sialofucosylated glycoform of CD44 called HCELL may be a key player (109).

An understanding of the precise E-selectin ligand on human leukocytes and also α(1,3)fucose modified stem cells is critical since neither the scaffold protein itself nor the sLe^X epitope alone can mediate robust stem cell recruitment under fluid flow conditions (**Figure 3B**). In agreement with this, when Lo et al. (114) modified MSC and also CDC cell surfaces to express an N-terminus PSGL-1 glycopeptide on a fusion protein scaffold, robust cell rolling interactions on P-selectin was only observed when the PSGL-1 O-linked glycan contained a core-2 sLe^X structure. In the absence of either the PSGL-1 protein scaffold or the sLe^X glycan, stem cell interaction with the selectin substrate was absent. In contrast to site-specific α(1,3)fucosylation on the PSGL-1 glycoprotein, when global α(1,3)fucosylation was performed on all stem cell glycoconjugates, robust leukocyte interactions were only observed on E-selectin substrates, but not P-selectin (115). The combined use of the glycosylated PSGL-1 glycoproteoform along with global α(1,3)fucosylation was needed for the robust binding of stem cells on all selectin substrates and also on endothelial cell monolayers in microfluidics flow studies. Such glycoengineering of stem cells using the combined coupling strategies also enabled short-term retention of stem cells in the left anterior descending artery of the pig heart in a brief ischemia-reperfusion model (115). This study confirmed the safety of the cellular therapy in a pre-clinical large animal model. Besides, glycoengineering approaches which are aimed to recruit stem cells from flow, it has also been demonstrated that decorating hematopoietic stem cells with bispecific antibodies that bind human CD45 and myosin light chain, can enhance cell homing to infarcted myocardium (116). Overall, while there is preliminary data that targeting to the heart is feasible, more investigation is needed in order to determine if this leads to better clinical outcomes.

Besides the selectin-ligands, the overexpression of a variety of chemokines have also been shown to enhance stem cell homing and retention. These methods may enhance the delivery of endogenous chemokine and growth factor receptors to sites of inflammation and injury, thus aiding the activation of cell surface integrins and cellular homing response (110). In this regard, the over-expression of signaling processes via the CXCR4/CXCL12 axis in MSCs has been shown to enhance myocyte preservation in the infarct zone, possibly accompanied by enhanced engraftment (117, 118). In addition, the over-expression of the CCR-1 chemokine receptor on MSCs and direct cardiac injection have has been shown to enhance cardiac engraftment leading to reduced LV remodeling and enhanced recovery of function (119). Besides these, the incorporation of metalloproteinases (MMP-2 and MT1-MMP) during stem cell delivery may also help degrade extracellular matrix components and enhance stem cell migration to sites of injury.

CONCLUSION

At the current time, there is a paucity of therapeutic approaches that target the immune system's response to myocardial ischemic injury to favorably influence cardiac healing and repair. While early studies attempted to address this problem using broad anti-adhesive therapies, often by targeting sLe^X-selectin binding, these were largely met with failure in human clinical trials. The failures may be in part due to an incomplete understanding of the role of leukocytes in cardiac repair, as recent studies show

that this is a complex process orchestrated by numerous sub-families of white blood cells. Moreover, reparative monocytes and macrophages are necessary to improve cardiac function, and thus the blockade of all immune cells using blunt anti-adhesive therapies may have detrimental effects. In addition, recent studies highlight the need for more glycoscience-based investigation, to identify putative human E-selectin ligands in the leukocyte sub-populations and stem cells, as well as to clearly define biosynthetic checkpoints regulating selectin-ligand biosynthesis. There is also clear evidence now that the cellular metabolism which regulates selectin-ligand/glycan biosynthesis may differ between humans and other species like mice. Thus, precise species-specific differences need to be understood and systems-based perturbations are necessary to evaluate the detailed consequences of specific interventions in complex systems, prior to initiation of human trials. Such understanding can help the design of better anti-adhesive therapies, and ultimately reduce the high morbidity and mortality associated with ischemic heart disease by reducing excessive inflammatory injury and/or improving delivery of novel biological therapeutics to the heart.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Sweet-Side of Leukocytes: Galectins as Master Regulators of Neutrophil Function

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Among responders to microbial invasion, neutrophils represent one of the earliest and perhaps most important factors that contribute to initial host defense. Effective neutrophil immunity requires their rapid mobilization to the site of infection, which requires efficient extravasation, activation, chemotaxis, phagocytosis, and eventual killing of potential microbial pathogens. Following pathogen elimination, neutrophils must be eliminated to prevent additional host injury and subsequent exacerbation of the inflammatory response. Galectins, expressed in nearly every tissue and regulated by unique sensitivity to oxidative and proteolytic inactivation, appear to influence nearly every aspect of neutrophil function. In this review, we will examine the impact of galectins on neutrophils, with a particular focus on the unique biochemical traits that allow galectin family members to spatially and temporally regulate neutrophil function.

Keywords: neutrophil, galectin, glycoscience, inflammation, glycans

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INTRODUCTION

A hallmark of effective immunity is the ability to rapidly recognize and respond to invading pathogens while avoiding potential injury to surrounding host tissue. This is especially important during the initial recruitment of neutrophils, one of the earliest and most effective responders to microbial infection (1). Neutrophils express a wide variety of potent antimicrobials, including degradative enzymes and highly reactive free radicals that can neutralize and ultimately kill many different invading pathogens (2–5). Although neutrophils can cooperate with antibodies to focus their effector function toward individual microbes, during the primary exposure to a given microbe, neutrophils rely on less specific mechanisms to recognize and respond to infection (6). Poorly controlled neutrophil infiltration and activation can result in significant tissue injury (3, 7–9). In contrast, inadequate neutrophil mobilization and activation can prevent rapid microbial eradication. In order to effectively defend against invading microbes, while limiting host injury, the localization, activation and eventual removal of neutrophils must be tightly regulated to efficiently eliminate potential pathogens while avoiding additional tissue damage and increased organ dysfunction (3, 7–11).

The importance of appropriately governing early immune effectors is especially apparent in disease states in which neutrophil regulation is compromised. Genetic disease that impair

neutrophil recruitment to sites of infection or reduce neutrophil effector activity leave patients prone to infectious disease (12–14). Similarly, patients who are neutropenic secondary to bone marrow dysfunction or other etiologies are particularly prone to life threatening infection (15). In contrast, excessive neutrophil recruitment and activation often contributes to the pathogenesis of some forms of inflammatory bowel disease, reperfusion injury or unabated infection (7–9). Thus, while neutrophils provide a critical defense mechanism against possible infection, the appropriate regulation and eventual elimination of these early immune effectors is critical if host defense is to be achieved while avoiding additional host injury (10, 11).

While a variety of factors regulate neutrophil recruitment, activation and eventual removal, many studies have demonstrated that a series of carbohydrate binding proteins (CBPs) called galectins play a key role in this process. Galectin family members recognize highly modifiable cell surface carbohydrates to facilitate neutrophil extravasation, activation, microbial killing, and eventual turnover. While there have been many excellent reviews detailing the regulatory roles of galectins in general on immune activity and function (16–19), in this review we will specifically examine the role of galectins in regulating neutrophil function. We will focus on the impact of unique aspects of galectin biochemistry that may contribute to the ability of this CBP family to influence various aspects of neutrophil function.

GALECTINS

Shortly after the identification of the first mammalian CBP, the Ashwell-Morell receptor, now known to govern platelet turnover and production (20–23), several studies sought to determine whether vertebrates possess other CBPs. In 1975, based on the ability of the Ashwell-Morell receptor to recognize terminal galactose residues, Teichberg and colleagues used a similar approach to isolate electrolectin, the ortholog of galectin-1 from the electric organ of the electric eel (24). While other investigators initially failed, the ability of Teichberg and colleagues to isolate and subsequently characterize the first galectin resulted from the inclusion of reducing agents in their isolation buffers (24, 25). Failure to include reducing agents in isolation buffers allowed electrolectin to undergo oxidation, rendering the protein inactive with respect to its carbohydrate binding activity (26, 27). Following the initial isolation and characterization of electrolectin, subsequent studies demonstrated that several other members of the galectin family were also sensitive to oxidative inactivation (28–33). In doing so, these early studies uncovered one of the most distinguishing, yet often overlooked features of galectins, their sensitivity to oxidative inactivation. Given the unique requirement of early galectins for reduced thiols, galectins were initially referred to as S-type lectins to differentiate them from subsequently discovered vertebrate CBPs that required Ca^{2+} to recognize cognate ligand, coined C-type lectins (34).

As not all galectins require reduced sulfhydryls to maintain carbohydrate binding activity (35), yet each appeared to share the ability to recognize β-galactose containing glycans, these CBPs later became distinguished by the name galectin (36). While galectins are unified by their conserved binding affinity for β-galactoside residues, other galactose binding proteins have been described in metazoans. As a result, galectins have been distinguished from these CBPs by the lack of calcium-dependence in glycan binding that is observed in C-type lectins, the presence of a conserved carbohydrate recognition domain (CRD) with highly conserved amino acids required for glycan binding and secretion through a unconventional secretory pathway, which has only recently begun to be characterized (37). In all, over 15 galectins have been described in vertebrates. Typically categorized based on their tertiary and quaternary structure, galectins are often placed into one of three groups: prototypical (e.g., Gal-1, -2, -7, -10), which form homodimers containing one CRD, tandem-repeat (e.g., Gal-4, -8, -9, and -12), which contain two CRD's in tandem joined by a linker region, and chimeric (e.g., Gal-3), which have an N-terminal tail that allows for oligomerization and/or unique protein interactions outside the Gal-3 CRD (38) (**Figure 1**).

GALECTINS REGULATE NEUTROPHIL ACTIVATION

Given the soluble nature of galectins, coupled with their ability to recognize highly modifiable glycan structures, galectins have served as a unique substrate in the evolution of immune regulation. The implication that galectins could influence leukocyte biology, in particular neutrophils, was originally described in studies designed to define interactions between leukocytes and IgE. Previous studies had suggested that IgE could activate neutrophils. However, the mechanism whereby this occurred remained incompletely understood. Surprisingly, biochemical studies seeking to first define the receptor responsible for the impact of IgE on neutrophil function found that rather than expressing conventional IgE-receptors (including Fc epsilon RII/CD23), neutrophils exhibited elevated expression of the S-type lectin Mac-2/Epsilon-bp (i.e., galectin-3) (39), a protein which had previously been shown to bind IgE *in vitro*. Importantly, galectin-3 interactions with IgE on the neutrophil surface resulted in NADPH-oxidase activation and a respiratory burst; neutralizing antibodies against Gal-3 prevented this IgE-mediated effect on neutrophil activation, strongly suggesting that Gal-3 serves as the primary IgE receptor on the surface of neutrophils (40). Gal-3 may therefore regulate neutrophil sensitivity to IgE mediated activation following allergen exposure in at risk patients (41).

The ability of Gal-3 to regulate neutrophil activity through IgE engagement suggested that Gal-3 itself may influence neutrophil function. Subsequent studies demonstrated that Gal-3 can initiate neutrophil oxidative responses. In this setting, recombinant Gal-3 not only binds to neutrophils and stimulates superoxide production, but also directly activates neutrophils completely independent of IgE, in a carbohydrate- and dose-dependent manner (42). Gal-3 oligomerization of potential counterreceptors appears to be required for the induction of ROS production, as the C-terminal domain of Gal-3 (Gal-3C), which has been

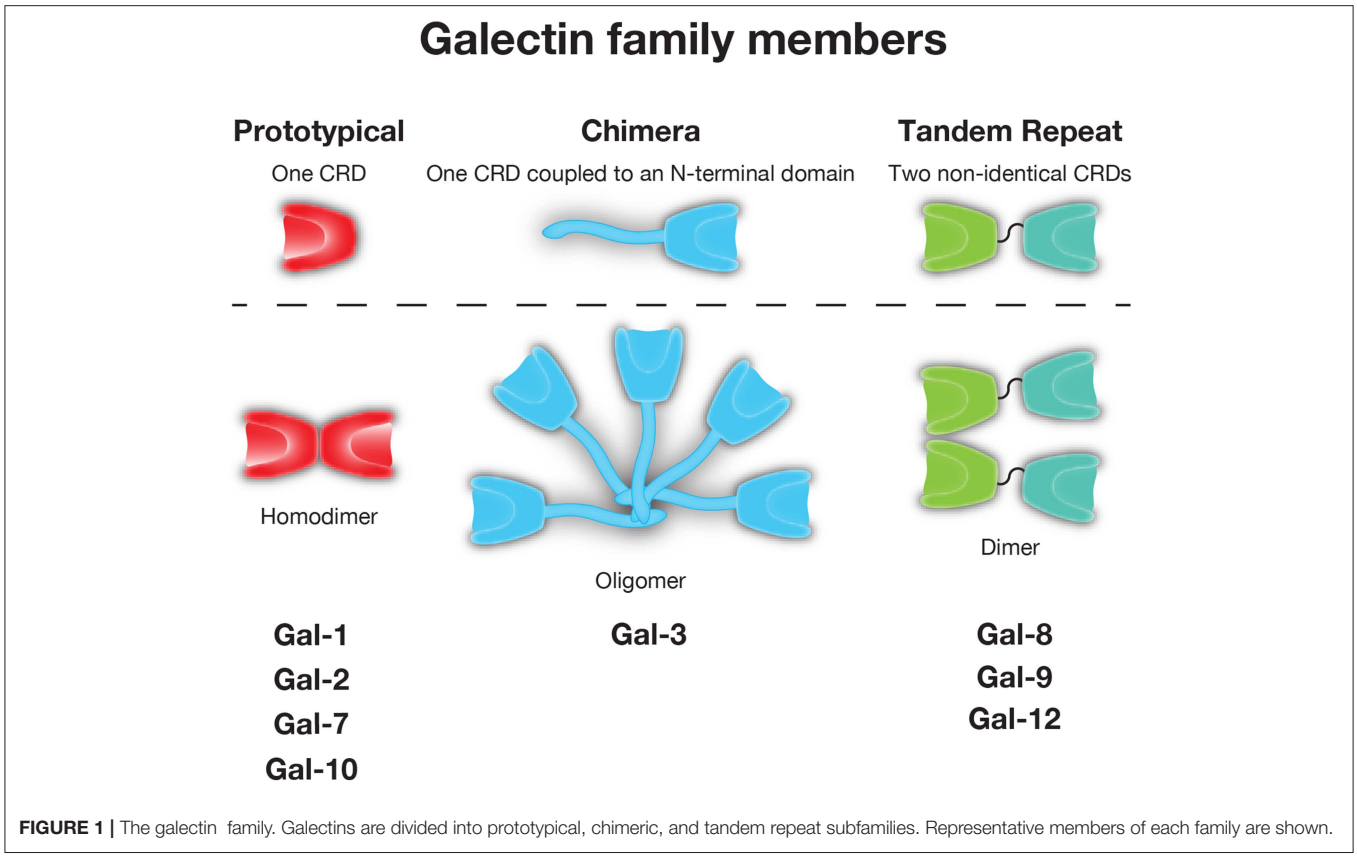

FIGURE 1 | The galectin family. Galectins are divided into prototypical, chimeric, and tandem repeat subfamilies. Representative members of each family are shown.

shown to be defective in oligomerization (43, 44), fails to similarly induce neutrophil ROS (45). Furthermore, antibodies that enhance Gal-3 oligomerization also appear to facilitate Gal-3-induced ROS production (46).

Gal-3 dependent activation of neutrophil NADPH-oxidation occurs preferentially on exudated, but not peripheral (e.g., quiescent) neutrophils, implicating a role for priming events in the sensitization of neutrophils to Gal-3 (47). Priming events that render neutrophils sensitive to Gal-3 are not limited to extravasation, but also include exposure to lipopolysaccharide or lipoarabinomannans from gram negative microbes or mycobacteria, respectively (48–50). Despite the ability of Gal-3 to recognize strain specific carbohydrate O antigen and the lipid A of some forms of LPS (51–53), LPS from a variety of gram negative microbial strains, including *Escherichia coli, Klebsiella pneumoniae*, and *Salmonella minnesota,* possess this priming activity (50), suggesting that this priming event does not likely reflect enhanced Gal-3 binding at the neutrophil surface through LPS carbohydrate engagement. Newcastle disease virus neuraminidase sensitizes neutrophils to Gal-3 (54). As Newcastle disease virus neuraminidase also sensitizes neutrophils to fMLP (54), increased sensitivity may not result from direct exposure of Gal-3 receptors, but instead may reflect general alterations in sensitivity of neutrophils to common activators. In contrast to the extrinsic impact of Gal-3 on neutrophil ROS production primed under various conditions, intrinsic neutrophil Gal-3 appears to attenuate ROS production following *Candida albicans* exposure (55).

In addition to the impact of exposure to distinct microbial products or even microbes themselves on Gal-3 regulation of neutrophil activity, different disease and developmental states may also influence neutrophil sensitivity to Gal-3-induced ROS production. For example, while neonatal immunity is thought to be developmentally immature and less responsive to activating stimuli (56), neutrophils isolated from cord blood are actually more sensitive to Gal-3-induced ROS production than peripheral blood neutrophils isolated from adults (57). Neutrophils isolated from patients with periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) syndrome also experience enhanced ROS response following Gal-3 exposure (58), suggesting that other inflammatory mediators may provide surrogate cues that also prime neutrophils to Gal-3.

In addition to inducing ROS, subsequent studies demonstrated that Gal-3 facilitates neutrophil activation, as evidenced by enhanced L-selectin shedding, increased CD11b expression and IL-8 secretion (59, 60). Gal-3-induced ROS also has consequences on neutrophil sensitivity to additional activators. In conjunction with myeloperoxidase, ROS induced by Gal-3 results in fMLP degradation, which in turn reverses the desensitization neutrophils can experience following exposure to a higher concentration of fMLP (61). As this allows neutrophils to become more sensitive to fMLP, Gal-3-induced ROS provides a positive feedback loop that occurs when both galectins and

bacterial products are present. In this setting, Gal-3 may serve as a damage associated molecular pattern molecule that signals the presence of tissue injury in the setting of microbial invasion (62).

In contrast to facilitating additional activation in the setting of infection, primed neutrophils can cleave Gal-3 into products no longer capable of signaling neutrophil ROS production, suggesting a potential negative feedback loop on Gal-3-mediated neutrophil activation. Importantly, cleaved, but not intact, Gal-3, is also preferentially internalized, raising the possibility that this cleavage event may also enhance Gal-3 removal (60). While Gal-3C can serve as a dominant negative regulator of Gal-3 activity on neutrophils, Gal-3C not only fails to prevent Gal-3 engagement of neutrophil ligands, but actually augments Gal-3 binding (45), making it unclear how Gal-3C modulates Gal-3 signaling. As Gal-3C can be detected on the surface of neutrophils in circulation (45), and extravasation appears to impact cell surface galectin and galectin-ligand levels (63), internalization of Gal-3C may serve as an additional regulator of neutrophil sensitivity to Gal-3 exposure following extravasation.

Early studies also demonstrated that in addition to Gal-3, Gal-1 can induce neutrophil ROS production, with extravasated and not quiescent neutrophils likewise exhibiting the most sensitivity to Gal-1 (64). Subsequent studies confirmed that Gal-1 from a variety of sources could induce ROS production, lysosome release and generalized degranulation (65, 66). Similarly, Gal-8 also stimulates ROS production, signaling primarily through its C-terminal domain (67, 68), while Gal-9 causes neutrophil degranulation and ROS production through a Tim3-dependent pathway (69, 70), suggesting that regulation of neutrophil ROS may be a more generalized galectin phenomenon. Patients with alcohol-induced hepatitis experience elevated levels of Tim3, PD1, PD-L1, and Gal-9 with impaired neutrophil ROS production and phagocytosis, which are reversed with anti-Tim3 and PD1, likewise implicating at least a partial role for Gal-9 *in vivo* in this process (71).

GALECTIN REGULATION OF NEUTROPHIL EXTRAVASATION

In addition to regulating neutrophil NADPH-oxidase activity, galectins have also been implicated in regulating neutrophil extravasation. Early studies observed that injected Gal-1 could reduce phospholipase A2-induced neutrophil accumulation, a process that was inhibited by lactose and anti-Gal-1 antibodies (72). Injection of Gal-1 similarly impairs carrageenan-induced neutrophil extravasation into the peritoneal cavity (73, 74), while Gal-1 likewise attenuates neutrophil infiltration in the setting of ocular inflammation (75). While these reductions could also reflect Gal-1-mediated alterations in neutrophil chemotaxis, pre-incubation of neutrophils with Gal-1 inhibits neutrophil rolling on endothelial cells, while increased neutrophil rolling was noted *in vivo* in response to IL1β in Gal-1 KO mice (63, 76), suggesting a direct effect. However, Gal-1 can limit neutrophil infiltration and Th17 responses following corneal exposure to *Pseudomonas aeruginosa*, suggesting that reductions in Th17 cells may also contribute to decreases in neutrophil infiltration in some settings (77). Similarly, Gal-1 treatment in a model of OVA-induced conjunctivitis reduces both pro-inflammatory cytokine production and neutrophil numbers (78). In contrast, Gal-1 KO mice infected intratracheally with *Histoplasma capsulatum* exhibit an elevated neutrophil pulmonary accumulation that may reflect higher chemokines levels for neutrophils (79).

In contrast to Gal-1, Gal-3 mediates neutrophil adhesion to endothelial cells, suggesting that Gal-3 may positively regulate neutrophil extravasation (80, 81). Consistent with this, Gal-3 injection decreases neutrophil rolling, while increasing adhesion and emigration (82). Gal-3 KO neutrophils exhibit an impaired ability to role on WT endothelium, suggesting a neutrophil intrinsic role for Gal-3 (82). Experimental models of infection appear to confirm a role for Gal-3 in neutrophil extravasation in some settings. Gal-3 KO recipients experience reduced neutrophil broncheoalveolar lavage (BAL) numbers following *Streptococcus pneumoniae* pulmonary infection. Reductions in neutrophil accumulation correlate with increased *S. pneumoniae* burden, an outcome that can be partially reversed by intranasal delivery of recombinant Gal-3 (83). Gal-3 KO mice also exhibit impaired neutrophil recruitment following thioglycolate-induced peritonitis (84) and *Leishmania major* skin infection (85). Although Gal-3 can induce macrophages and other cells to secrete pro-inflammatory cytokines and chemokines (86, 87) and Gal-3 injection can increase IL-1β, TNFα, CCL2, CXCL1, and IL-6 (82), impaired neutrophil recruitment does not appear to reflect a lack of cytokine or chemokine production, as neutrophil mobilization defects observed in Gal-3 KO mice can occur in the presence of increased levels of KC, MIP2, IL-6, and TNFα (60, 83, 85). In contrast, inhibition of Gal-3 can result in reduced TNFα, KC, TGFβ, and MCP-1 levels and neutrophil accumulation, as observed in a pancreatitis model, suggesting that Gal-3 may similarly facilitate extravasation and possibly chemotaxis in this setting (88). Occasionally, reduced neutrophil extravasation may be beneficial to the host. In a model of *Francisella novicida* pulmonary infection, reduced inflammation and neutrophil extravasation in Gal-3 KOs actually correlated with enhanced survival despite no difference in CFU numbers (89).

While the above studies have highlighted a role for Gal-3 in facilitating neutrophil extravasation, several studies suggest that Gal-3 may negatively regulate neutrophil extravasation in certain settings. Gal-3 KO mice actually experience increased neutrophil accumulation and disease severity in several infectious disease models, including neurocysticercosis (90) and polymicrobial sepsis (91). Similarly, a higher number of neutrophils can actually be detected in the BAL of Gal-3 KOs following pulmonary *E. coli,* as opposed to *S. pneumoniae,* infection (83). In contrast, Gal-3 KO mice appear to initially have similar neutrophil numbers in a spinal cord injury model (92) and likewise fail to display significantly different numbers in the setting of dextran sulfate sodium (DSS)-induced colitis (86), suggesting that in some settings Gal-3 may have an redundant role or simply no role in neutrophil recruitment. Finally, in addition to Gal-1 and Gal-3, other galectins have shown an ability to potentially regulate neutrophil extravasation. Gal-8 can also mediate neutrophil adhesion to endothelial cells (93), although the consequences of this interactions *in vivo* remain incompletely studied.

GALECTINS REGULATE NEUTROPHIL CHEMOTAXIS AND PHAGOCYTOSIS

Early studies found that Gal-3 could promote neutrophil cationic-dependent and independent binding to laminin, and at high concentrations could facilitate fibronectin binding (94, 95), suggesting that once neutrophils extravasate, Gal-3 may facilitate attachment and chemotaxis along extracellular matrix (ECM) glycoproteins. Gal-1 also recognizes laminin, fibronectin and neutrophil ligands, although the potential ability of Gal-1 to tether neutrophils to these ECM glycoproteins was never formally tested in these early studies (96, 97). Gal-1 does inhibit neutrophil chemotaxis in response to IL-8 *in vitro* and similarly reduces neutrophil transmigration following IL-1β-induced peritonitis (63). As it is difficult to distinguish extravasation and chemotaxis *in vivo*, alterations in neutrophil accumulation in these models may reflect a role for Gal-1 on neutrophil extravasation, chemotaxis or both (63, 72–78). Different neutrophil responses to Gal-1 may also reflect Gal-1 concentration; lower concentrations of Gal-1 can produce directed neutrophil movement, while higher concentrations appear to induce random motion (98). Intriguingly, whereas Gal-3 promotes extravasation into inflamed tissues (82), Gal-3 can inhibit leukocyte migration in response to IL-8, C5a, and ATP (99).

In addition to Gal-1 and Gal-3, Gal-9 also regulates neutrophil chemotaxis. Following ischemic injury, Gal-9 KO mice experience increased neutrophil infiltration that is partially reversed following injection of recombinant Gal-9 (100). Injected Gal-9 also reduces neutrophil accumulation in a model of emphysema (101), ConA-induced hepatitis (102) and reperfusion liver injury (103). Gal-9 KOs also experience a reduced neutrophil response to *Francisella novicida* pulmonary infection (70). However, as Gal-9 treatment can also reduce IL-6, IL-1β, IFNγ, TNFα, KC, MIP2, GM-CSF, and MMP9 in various models (70, 100, 102–104), alterations in neutrophil accumulation may reflect modulation of neutrophils by regulating either extravasation, chemotaxis, cytokine, and chemokine secretion or a combination of these events. Consistent with this, Gal-9 induces IL-8 production through engagement of Tim-3 on bronchial epithelial cells, resulting in neutrophil recruitment (105). Gal-9 may also regulate neutrophil infiltration by signaling changes in Treg activity, Th17 responses or T cell turnover (104, 106–108). Consistent with a more indirect role for Gal-9 in modulating neutrophil chemotaxis, early studies suggested that Gal-9, originally coined eotaxin, exhibits chemotactic activity toward eosinophils, yet fails to alter neutrophil, monocyte or lymphocyte chemotaxis (109, 110).

In addition to modulating neutrophil extravasation, chemotaxis and activation, galectins may also facilitate neutrophil phagocytosis. Gal-3, for example, facilitates neutrophil phagocytosis of *Streptococcus pneumoniae* (60). Gal-9 can also bind and enhance the phagocytosis of *Pseudomonas aeruginosa* by neutrophils. While galectins have been shown to bind a variety of different bacterial species (51, 111), it is not clear whether this reflects a general phenomenon of galectin-mediated microbial clearance or only occurs following engagement of select microbial strains with unique glycan signatures. Regardless, in the setting of Gal-9, Tim3 appears to be involved (69). In addition to engaging bacterial pathogens, Gal-3 can also facilitate the phagocytosis of *Candida parapsilosis* yeast and *Candida albicans* hyphae, but not *C. albicans* yeast (112). However, Gal-3 may also directly kill *C. albicans* yeast (113). Gal-3 is secreted by neutrophils following exposure to yeast mannans, suggesting a mechanism whereby fungal exposure may trigger Gal-3-mediated removal (112). Gal-3 may also facilitate neutrophil phagocytosis of non-pathogens, such as red blood cells (114).

Galectins may also regulate immunity by inducing alterations in neutrophil function that directly and indirectly impact the immune activity of other cells. For example, neutrophils appear to perform helper activity through enhancing B cell antibody production, a process that requires Gal-3 (115). Neutrophils also produce more IL-17 in Gal-3 KO mice, suggesting that in addition to Gal-3 regulating dendritic cell IL-23, IL1β and TGFβ1 production, the reduction in *Histoplasma capsulatum* infection observed in Gal-3 KO mice may reflect an enhanced neutrophil-mediated Th17 response (116). Gal-9 can modulate neutrophil prostaglandin E2 production, which in turn reduces pro-inflammatory cytokine secretion by macrophages (117). In contrast to directly signaling cytokine responses in neutrophils, crystal forms of galectin-10, originally known as Charcot-Leyden crystal, can drive IL-1β production in macrophages when phagocytosed, which appears to result in neutrophil accumulation (118).

GALECTIN REGULATION OF NEUTROPHIL TURNOVER

While galectins are differentially regulated in models of neutrophil development (119, 120), galectins may also govern neutrophil turnover. Given the role of galectins in regulating T cell viability, early studies similarly evaluated the potential role of galectins on neutrophil turnover. These initial studies investigated the effect of Gal-1 on neutrophils and promyelocytic HL-60 cells viability using Annexin V detection of phosphatidylserine (PS) exposure at the cell surface as a marker of apoptosis (121). Similar to T cells, Gal-1 signaled PS exposure in neutrophils. However, unlike cells undergoing apoptosis, Gal-1-induced PS exposure in neutrophils and HL60 cells occurred in the conspicuous absence of common features of apoptosis, including DNA fragmentation, cytochrome C release, mitochondrial potential changes or caspase activation (121–125). Despite the inability of Gal-1 to induce apoptosis in neutrophils, these cells remained sensitive to phagocytosis by macrophages (121), suggesting that Gal-1 possesses the unique ability to trigger neutrophil removal independent of cell death. Intriguingly, subsequent studies showed that this effect extended to other galectins, notably Gal-2, Gal-3, and Gal-4, which likewise stimulate PS exposure without concatenate apoptosis (124). However, it should be noted that pathways induced by at least Gal-1, Gal-2, and Gal-4 appeared to differ. While Gal-1 and Gal-2 induced an initial intracellular Ca^{2+} flux required

for Gal-1-mediated PS exposure, a similar Ca^{2+} flux following exposure to Gal-4 is not observed (122, 124) (**Figure 2**). It should be noted that there have been conflicting results regarding the consequence of Gal-1 on neutrophil turnover. Additional studies demonstrated that under certain conditions Gal-1 may actually induce neutrophil apoptosis (114), while Gal-3 may indeed delay apoptosis (60). Differences in neutrophil sensitivity to assay conditions may in part account for these differences (126–128).

In addition to directly regulating neutrophil viability and turnover, galectins may also facilitate neutrophil clearance by macrophages. Recombinant Gal-3 enhances macrophage removal of apoptotic neutrophils (129), while Gal-3 KO macrophages have an impaired ability to phagocytose apoptotic neutrophils (60). Impaired Gal-3-mediated removal of neutrophils has also been attributed to worsening of the disease pathogenesis in asthma (130, 131). Gal-9 also co-localizes with corpses of neutrophils following NETosis, suggesting a potential role in the clearance of neutrophils following NETosis induction (132).

GALECTIN NEUTROPHIL LIGANDS

Definitive functional receptors for specific galectin signaling events in neutrophils have largely remained elusive (more than one may likely be involved), though studies strongly indicate CD66a and CD66b are at least in part responsible for ROS induction by Gal-3 (133, 134). IgM-mediated crosslinking of CD66b also induces IL-8 secretion, similar to Gal-3, suggesting that this indeed may be a functional ligand for Gal-3 (135). Despite similarities in ROS induction and overall neutrophil priming requirements for Gal-1 and Gal-3, early studies suggested that different receptors are engaged by Gal-1 and Gal-3 to induce these downstream events (64). The ability of blocking antibodies to CD43, but not CD45RO (another putative Gal-1 ligand) to inhibit Gal-1-induced neutrophil chemotaxis (98) corroborates the notion that Gal-1 may signal neutrophils through a different receptor. Although, it is not known whether CD43 also mediates Gal-1-induced ROS production. In contrast, αM integrin serves as receptor for Gal-8 induced adhesion of neutrophil to tissue culture plates (67), while Tim3 mediates Gal-9 enhancement of neutrophil microbial killing (69), suggesting that a variety of distinct neutrophil receptors may be engaged by different family members.

While many studies have defined galectin counter receptors on the surface of other immune cells, such as CD43 and CD45 on T cells, as the repertoire of glycosyltransferases can fundamentally differ between cell populations, these glycoproteins may or may not be decorated with suitable galectin ligands when expressed on neutrophils (136). As a result, several studies have instead focused primarily on the glycan ligands that support galectin-mediated signaling events in neutrophils (137). For example, several studies demonstrated that Gal-1, Gal-2, Gal-3 and Gal-8 prefer polylactosamine (polyLacNAc) ligands on the surface of HL60 cells. However, the mode of galectin interaction with polyLacNAc HL60 glycan recognition appears to fundamental differ. While Gal-3 and Gal-8 appear to prefer internal LacNAc glycan motifs within a polyLacNAc structure, Gal-1 and Gal-2 preferentially recognize the terminal LacNAc structure (138, 139). These differences have consequences on the sensitivity of HL60 cells to galectin signaling. While sialylation has little effect on Gal-3 binding or signaling of PS exposure, given the preference of Gal-1 and Gal-2 for the terminal LacNAc motif, sialylation can differentially impact Gal-1 and Gal-2 binding. Gal-2 fails to recognize any sialylated polyLacNAc structures, while Gal-1 binding appears to be preferentially inhibited by α2-6, but not α2-3 sialylation (138). Gal-8 glycan recognition is very different than Gal-1, Gal-2, and Gal-3. Unlike Gal-1, Gal-2, and Gal-3, Gal-8 is a tandem repeat galectin with two distinct carbohydrate binding domains. Gal-8 appears to dimerize through association with the N-terminal domain, while C-terminal domain engagement of polyLacNAc structures through internal LacNAc recognition is entirely responsible for Gal-8-induced PS exposure (139). Thus, while galectins can induce PS exposure in HL60 cells, the key features responsible for ligand engagement can differ. Whether similar glycan binding preferences dictate the ability of galectins to modulate neutrophil extravasation, chemotaxis and overall activation remains to be determined.

It should be noted that while a given glycoprotein or glycolipid may serve as the functional receptor for a galectin or several galectin family members, it is certainly possible that galectins signal neutrophils through clustering of several similarly glycosylated receptors to ultimately induce a particular signaling outcome. Consistent with this possibility, Gal-3 clusters neutrophil counter receptors alone and in the context of adhesion to endothelial cells (140). LPS enhances oligomerization of Gal-3 (50), augmenting the ability of Gal-3 to signal neutrophil activation and possibly contributing to the increased lethality observed when Gal-3 is injected intraperitoneally (IP) with LPS when compared to Gal-3 alone (50).

REDOX AS A REGULATOR OF GALECTIN BIOLOGY

Given the ability of galectins to broadly influence neutrophils, and in sometimes opposing manners, one critical challenge to the field is in understanding the dynamics of the regulatory network controlling galectin-glycan interactions to allow for proper control of neutrophil function. Clearly one key element is the expression of glycans on cognate receptors within the extracellular space. However, one often overlooked mechanism may be in the rich and fluctuating redox environment often accompanying inflammation, inflammatory resolution and eventual tissue repair.

Protein oxidation is one of the strongest regulatory modifications linked to galectins, and is also one of the first identified. In fact, the identification of galectins remained elusive until it was discovered that their purification required reducing conditions (24, 26). In these studies, the authors found that tryptophan oxidation not only inactivated the ability of electrolectin to bind to lactose, but also that this inhibitory

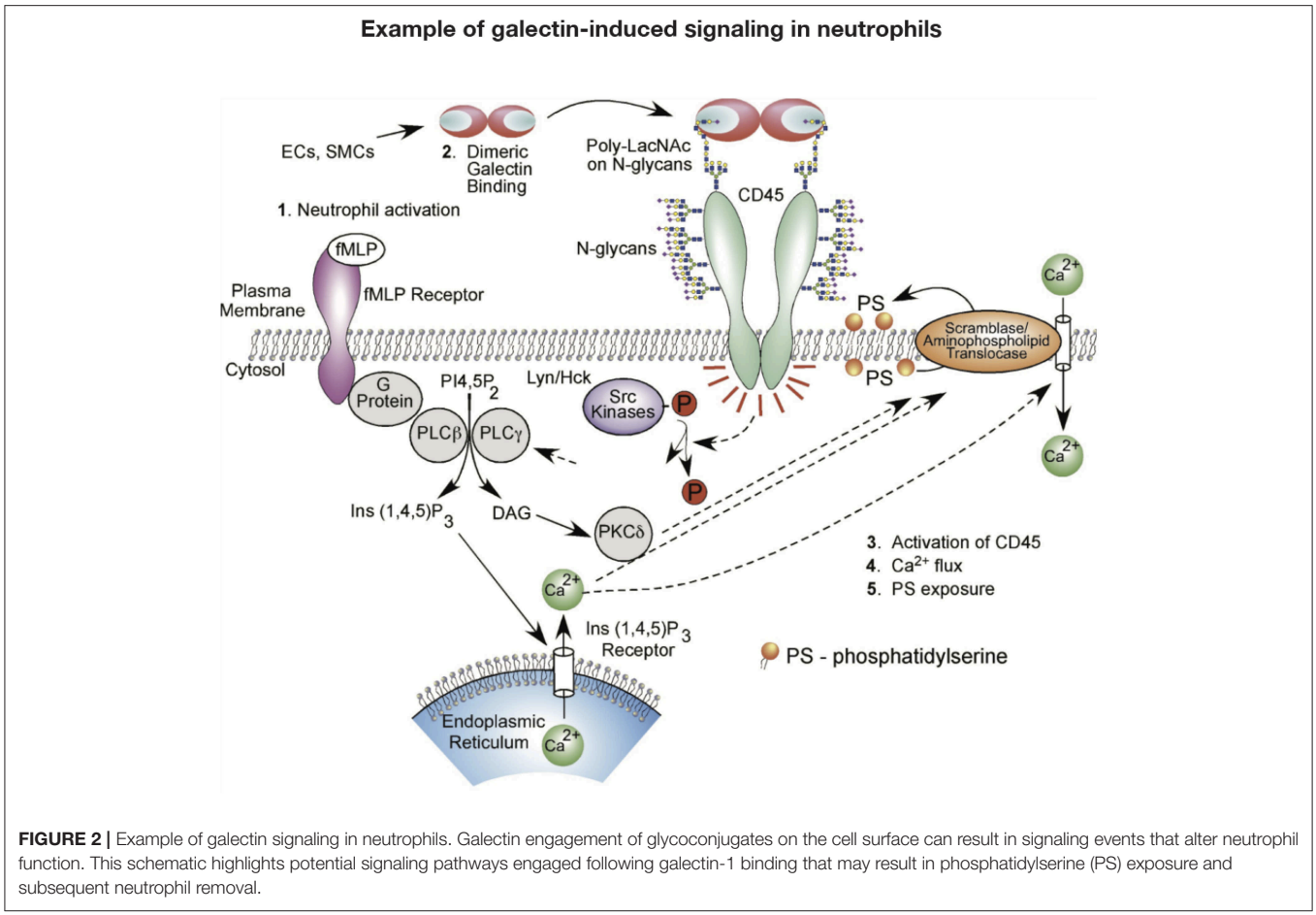

FIGURE 2 | Example of galectin signaling in neutrophils. Galectin engagement of glycoconjugates on the cell surface can result in signaling events that alter neutrophil function. This schematic highlights potential signaling pathways engaged following galectin-1 binding that may result in phosphatidylserine (PS) exposure and subsequent neutrophil removal.

effect was influenced by binding to cognate glycans themselves, as pre-incubation with lactose prevented redox-dependent inactivation. Oxidation also occurred in a pH sensitive manner, with optimal yields only occurring in the setting of a neutral pH. Thus, in the setting of acute injury, which often has high acid and oxidant loads, galectin activity may be titrated toward high affinity interactions.

Subsequent studies performed on vertebrate galectins, in particular Gal-1, have similarly observed sensitivity to oxidation, however these studies found that redox dependent control of Gal-1 appears to impinge on the modification of critical cysteine residues present on the Gal-1 backbone (27, 30–33, 141). As with redox-driven inactivation of other systems, oxidation appears to promote disulfide bond formation (notably on Cys2, Cys16, and Cys88) leading to intramolecular interactions which disrupt the ability of the carbohydrate binding domain to recognize and bind to cognate glycans (33, 142–145) (**Figure 3**). This effect appears to be completely blocked by alkylation and site directed mutagenesis of these cysteine residues also results in stable proteins with sustained binding activity (27, 30). Intriguingly, as was originally observed with electrolectin, redox-driven inactivation of Gal-1 appears to be regulated by the presence of ligand; whereas free Gal-1 monomer is relatively quickly inactivated, ligand-binding by Gal-1, which induces dimerization, increases resistance to inactivation (126, 146, 147). Similar findings have now been documented with Gal-2, another prototypical galectin, where oxidation of Cys57 appears to result in its oligerimization and subsequent inactivation, an effect which can be abrogated by endogenous nitric oxidase in the gastrointestinal tract (148, 149). However, whether this type of regulation occurs with other galectins, or if this affects the ability of galectins to mediate carbohydrate-independent interactions, remains incompletely understood. Intriguingly, though oxidation appears to clearly disrupt Gal-1 glycan binding activity and subsequent dimerization, in certain settings oxidation alters Gal-1 biological function in a manner that appears to be independent from its lectin properties. This observation stems from studies looking at a unique role of Gal-1 in promoting axonal regeneration of peripheral nerves where it was observed that while ectopic oxidized Gal-1 could enhance the rate of axonal growth from transected dorsal root ganglia, alkylated Gal-1 (which prevents redox-dependent conformational changes) could not (33, 150). These results strongly suggest a role for oxidized Gal-1 in tissue regeneration (33, 151–157). Subsequent studies revealed that Gal-1 was not only expressed but secreted from regenerating nerves, and that neutralizing antibodies against Gal-1 could strongly inhibit axon regeneration *in vivo* (158). Studies have now revealed that

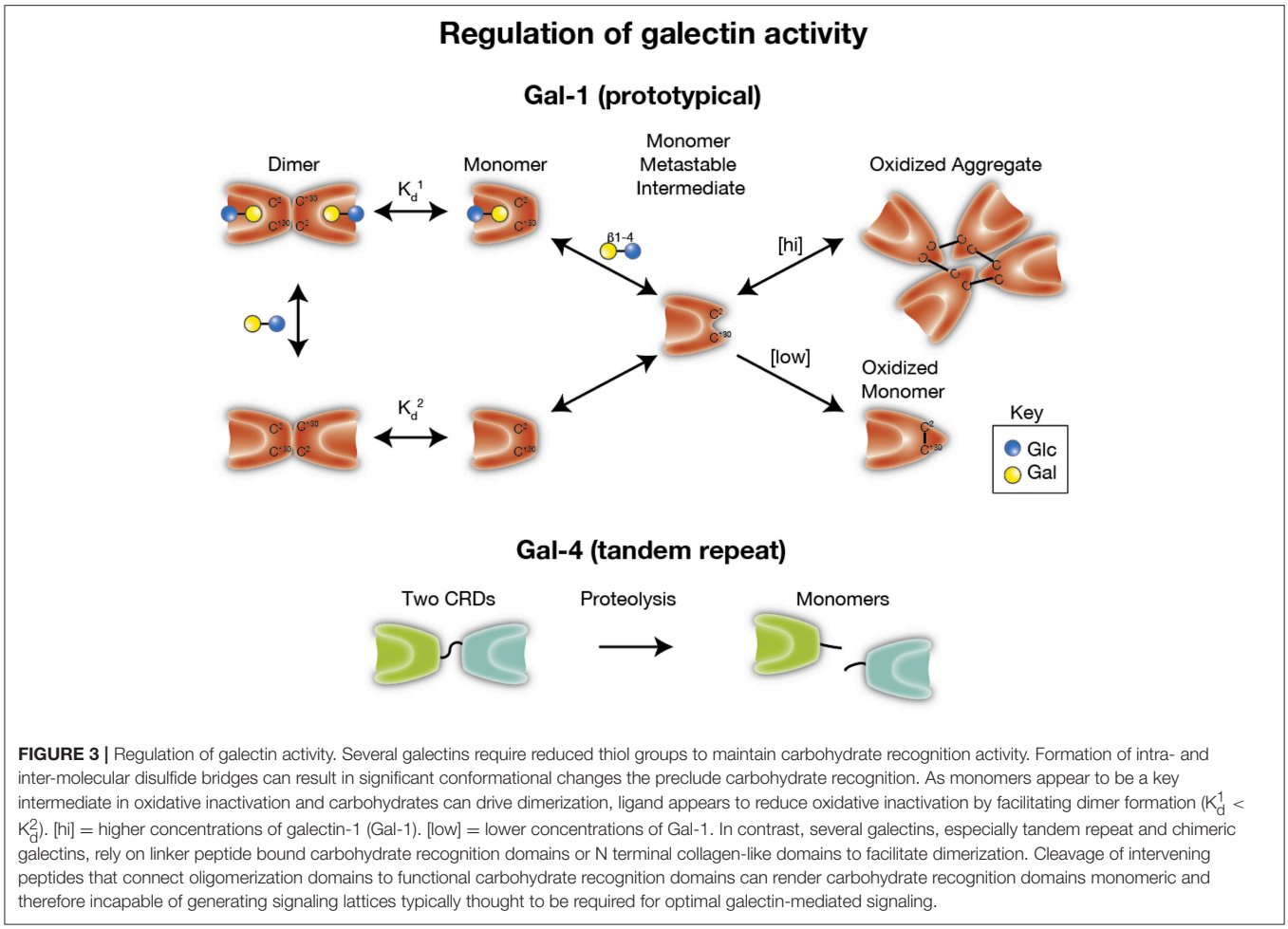

FIGURE 3 | Regulation of galectin activity. Several galectins require reduced thiol groups to maintain carbohydrate recognition activity. Formation of intra- and inter-molecular disulfide bridges can result in significant conformational changes the preclude carbohydrate recognition. As monomers appear to be a key intermediate in oxidative inactivation and carbohydrates can drive dimerization, ligand appears to reduce oxidative inactivation by facilitating dimer formation ($K_d^1 < K_d^2$). [hi] = higher concentrations of galectin-1 (Gal-1). [low] = lower concentrations of Gal-1. In contrast, several galectins, especially tandem repeat and chimeric galectins, rely on linker peptide bound carbohydrate recognition domains or N terminal collagen-like domains to facilitate dimerization. Cleavage of intervening peptides that connect oligomerization domains to functional carbohydrate recognition domains can render carbohydrate recognition domains monomeric and therefore incapable of generating signaling lattices typically thought to be required for optimal galectin-mediated signaling.

oxidized Gal-1 can stimulate macrophages to initiate regenerative responses during axonal injury (156, 159), and through this pathway primes the system for repair.

All these data suggest that Gal-1 appears to act as a morpheein (160), a secreted factor which under distinct conformational conditions adopts certain biologic behaviors; whereas reduced Gal-1 maintains its lectin-binding immune-modulatory activity; cysteine oxidized Gal-1 adopts a new behavior with a tailored regenerative response. The degree to which this effect can be observed with other galectins, including other prototypical galectins remains unknown. Moreover, whether the effect remains specific to regenerating axons, or whether oxidized Gal-1 can stimulate macrophages to promote restitution in other tissues is similarly unknown (**Figure 3**).

PROTEOLYTIC REGULATION OF GALECTINS

While the extracellular environment during acute inflammatory responses is rich in reactive oxygen species and electrophiles which could exert regulatory influences on galectins (as detailed above), it is also well-known that several proteolytic enzymes (such as matrix metalloproteinases or MMPs) are elevated during this time which could also serve to regulate galectin activity. Early studies showed that both MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) can cleave Gal-3 at specific residues within its N-terminal tail (Ala^{62}-Tyr^{63}), leading to reduced cell surface expression in human breast cancer cell lines (161). Subsequent studies confirmed these findings *in vivo* (162), and showed that while this cleavage led to reduced N-terminal self-association/oligomerization of Gal-3, its ability to bind glycans was enhanced (163). Thus, as with oxidation, cleavage appears to act as a switch on galectin function, where certain functions that rely on N-terminal oligomerization (e.g., hemagglutination) are reduced, while others that do not require this function are potentially enhanced.

Regulation of galectin activity by proteases does not appear to be limited to gelatinases (e.g., MMP-2 and - 9); a variety of other MMPs have been implicated in altering Gal-3 expression/activity. MMP-7 (i.e., Matrilysin-1), which is expressed in inflamed tissues often at the leading edge of gastrointestinal ulcers, was shown to cleave Gal-3 at three separate sites (including Asp^{72}-Tyr^{73}) and inhibit Gal-3 driven wound healing activity in T84 cells (162). MMP-13 (i.e., collagenase-3) was shown to cleave Gal-3 at sites identical to MMP-2 and MMP-9. This action was correlated

with altered expression in chondrocytes (164). MMT-MMP (Membrane type 1 matrix metalloproteinase) was similarly found to enhance Gal-3 cleavage, though this effect was presumed to occur through indirect activation of MMP-2 and MMP-9 (165).

In addition to the matrix metalloproteinases, several other classes of proteinases have been implicated in galectin regulation. The serine peptidase PSA (protate specific antigen) was found to cleave Gal-3 in seminal plasma at Tyr^{107}-Gly^{108} and result in a functional monovalent lectin (166), akin to what has been observed with MMPs. Neutrophil elastase can likewise cleave Gal-3 (59). Regulation of galectin cleavage is not limited to endogenous proteases, as *Staphylococcus aureus* and *Leishmania major* possess similar proteolytic activity toward Gal-3 (167, 168). Recent studies have shown that Gal-8 and Gal-9 are susceptible to cleavage by the serine protease thrombin (68). This effect appears to be specific to Gal-8 and Gal-9, as thrombin susceptibility was not observed in galectin-1, -2, -3, -4, -7, -10, and -13. Intriguingly, Gal-8 and Gal-9 cleavage only occurred in long isoforms of these proteins, as short and medium isoforms were either resistant or lacked the site required for cleavage. In both instances, thrombin mediated cleavage abrogated the ability of the long isoform of Gal-8 (Gal-8L) to mediate neutrophil adhesion and Gal-9 eosinophil-chemoattractant activity, respectively. Thus, in the setting of acute inflammatory responses and tissue injury, which are often accompanied by an influx of coagulation proteins including thrombin activation and other proteases (169), this mode of regulation may serve as an additional means to curb galectin activity and prevent excessive tissue damage from inappropriate inflammatory cell activation.

BRINGING IT TOGETHER: GALECTINS AS UNIQUE REGULATORS OF OVERALL NEUTROPHIL FUNCTION

The distinct localization of galectins, their ability to selectively bind cell surface carbohydrates and their sensitivity to oxidative inactivation and proteolytic cleavage likely provided a unique evolutionary substrate to regulate the temporal and spatial activity of neutrophils during inflammation. Galectin expression within vascular endothelial cells and possibly in neutrophils themselves may contribute to extravasation, early activation and even chemotaxis (170). However, unlike most immune regulators, which are either synthesized and released following pathogen exposure, injury and/or selectively expressed by distinct immune cells (171), many galectins are found at high levels in a variety of tissues under baseline conditions (172). Thus, while galectins may interact with neutrophils intravascularly and therefore regulate early events involved in neutrophil extravasation, the expression of galectins in a variety of tissues provides additional opportunities for galectins to regulate neutrophil function (**Figure 4**).

The broad tissue distribution of galectins, coupled with their unique sensitivity to oxidative inactivation and proteolytic cleavage, may provide some insight into the temporal and spatial regulation of neutrophil function. Unlike most cytokines and chemokines, the vast majority of galectins reside in the cytosol, consistent with their lack of a signal peptide and consequential translation on free ribosomes (173). Following tissue injury, total levels of galectin can be upregulated, signaling an active production of these proteins (174). However, various forms of injury can also result in the release of galectins into the extracellular space, a process that may reflect active secretion, but also is likely a consequence of direct cellular injury (174). Initial release from cells requires galectins to transition from a relatively reducing environment largely devoid of proteases that target galectin function, into an environment that is oxidative in nature where proteases abound. While engagement of carbohydrate ligand can inhibit galectin oxidation, saturation of available ligands, coupled with proteolytic cleavage, may render most galectins inactive immediately following an injury event. This relatively rapid loss of galectin activity may aid the inflammatory response by preventing galectins from inhibiting productive chemotaxis and prematurely inducing neutrophil turnover. Furthermore, as significant galectin accumulation in the intravascular space can result in platelet activation, leukocyte aggregation and vascular stasis (175), spatial regulation of galectin activity may also be important in preventing galectin-induced vascular blockage, which would be expected to increase tissue ischemia and prevent additional leukocyte recruitment (176).

As neutrophils effectively remove pathogens and necrotic tissue in the settings of inflammation, these cells can also infringe on surrounding viable tissue (2, 3). In contrast to T cells, NK cells and other immune effectors, once activated, neutrophils do not process clear receptors capable of demarcating self from non-self, especially in the absence of pathogen specific antibodies. As a result, activated neutrophils can cause significant damage to viable tissue (2, 3). Indeed, inappropriate neutrophil activation not only exacerbates inflammatory responses in general, but also underlies the pathogenesis of a variety of disease states (3, 7–11). Galectins may provide some spatial control for neutrophils. As neutrophils encroach on and damage viable tissue surrounding the area of initial injury, intracellular stores of reduced, intact and therefore active galectin are released. Galectin engagement of neutrophils in these peripheral areas may therefore serve to reduce chemotaxis and enhance neutrophil removal (63, 114, 121, 122, 124). This spatial and temporal regulation of galectin activity and consequently neutrophil function may be important in limiting neutrophil-mediated injury while also inducing neutrophil turnover. Galectin-induced ROS production may therefore not only reflect an important early activator of neutrophil function and microbiocidal activity, but may also facilitate complete microbial killing before galectin signaling programs finalize events that mark neutrophils for removal. The ability of recombinant galectin to enhance tissue repair in some models may, in part, reflect the ability of galectins to favorably regulate leukocyte turnover in the setting of ongoing tissue injury and inflammation (177, 178).

While there are a variety of distinct forms of programmed cell death, ranging from apoptosis to necroptosis (179), the ability of galectins to induce PS exposure in the absence of cell death represents a distinct cell removal mechanism that may have uniquely evolved to eliminate neutrophils and perhaps other

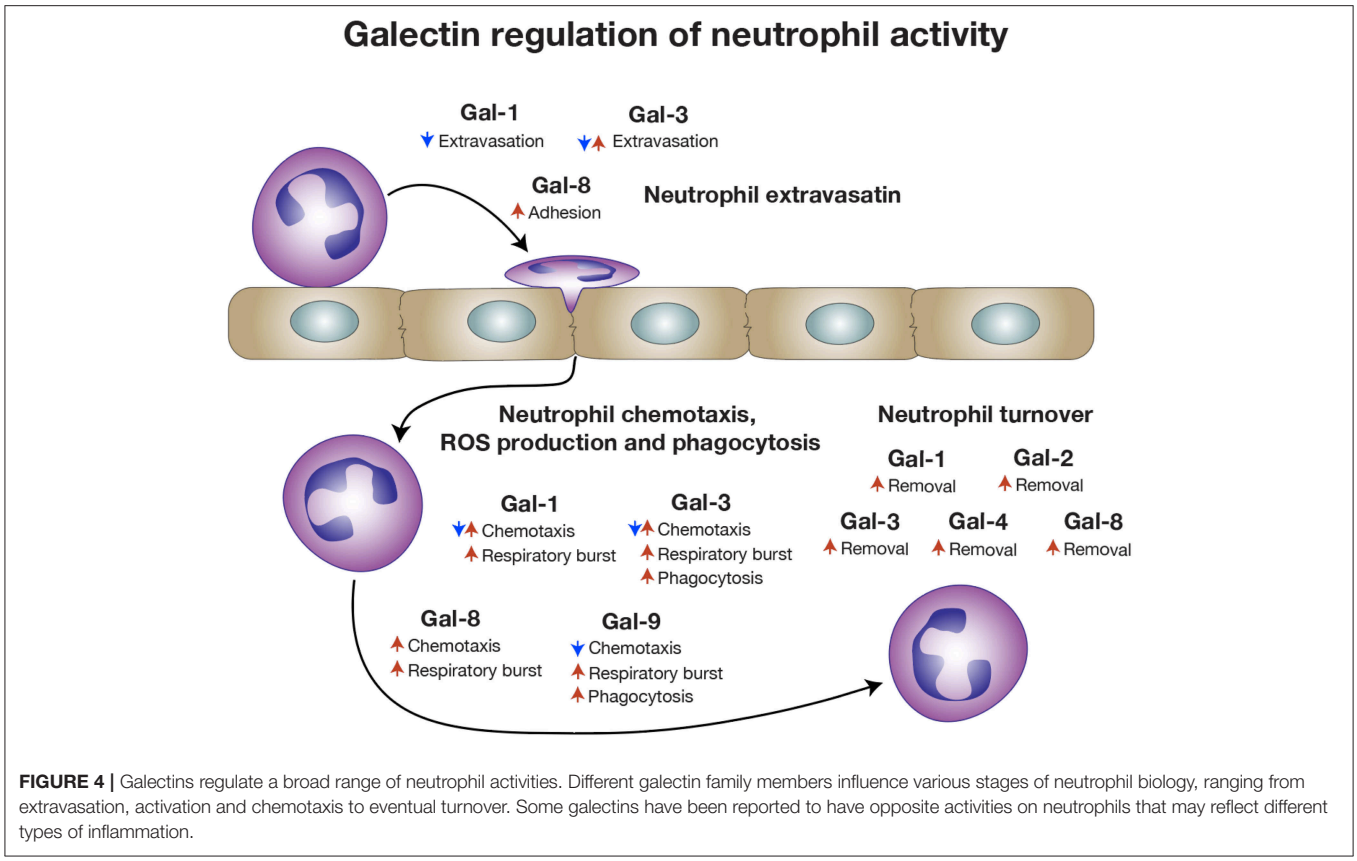

FIGURE 4 | Galectins regulate a broad range of neutrophil activities. Different galectin family members influence various stages of neutrophil biology, ranging from extravasation, activation and chemotaxis to eventual turnover. Some galectins have been reported to have opposite activities on neutrophils that may reflect different types of inflammation.

innate immune cells. As apoptosis typically occurs to prevent inflammation, the ability of galectins to induce PS exposure in the absence of apoptosis may allow neutrophils to maintain membrane integrity in a highly inflammatory and membrane damaging environment until successfully phagocytosed. This is especially important when considering that once neutrophil undergo apoptosis, there is a short window of time before late apoptosis, which is signified by loss of membrane integrity, occurs (180). If apoptotic neutrophils are not quickly phagocytosed, late apoptosis would be predicted to result in unregulated release of neutrophil contents, causing further tissue injury, additional inflammation and impaired inflammatory resolution. Furthermore, as the number of neutrophils often far outweighs the number of macrophages responsible for their removal (3), the ability of galectins to flag neutrophils for removal without inducing actual apoptosis, may likewise allow neutrophils to maintain membrane integrity while awaiting removal. While the residue of galectin released from injured tissue that is not bound to neutrophils, the ECM or other ligands would be predicted to undergo oxidative and/or proteolytic inactivation (126, 147), given the ability of at least oxidized Gal-1 to induce tissue regeneration, oxidized galectin, perhaps in the presence of other tissue factors, such as resolvins (181), may then be uniquely poised to begin the signals necessary for tissue repair as resolution of the inflammatory response occurs (**Figure 5**).

FUTURE DIRECTIONS: CHALLENGES AND OPPORTUNITIES IN HARNESSING GALECTIN REGULATION OF NEUTROPHIL FUNCTION

The ability of galectins to regulate neutrophil function suggests that these proteins may serve as useful pharmacological agents to favorably alter disease states marked by inadequate or exuberant neutrophil function. Consistent with this possibility, the earliest description of galectin-mediated immune regulation occurred following the exogenous delivery of electrolectin in a model of myasthenia gravis. Intriguingly, while the initial hypothesis was that delivery of electrolectin would stabilize the neuromuscular junction, additional experiments demonstrated that electrolectin actually inhibited the immune response required to induce myasthenia gravis (182–184). These results not only provided the first evidence that galectins may regulate immunity, but also suggested that galectin family members may serve as useful pharmacological agents to favorably alter immune function.

Subsequent studies demonstrated improved outcomes could also be achieved following galectin injection in additional models of immune-related pathology, including Concanavalin-A-induced hepatitis, collagen-induced arthritis, experimental autoimmune uveitis and experimental autoimmune encephalitis

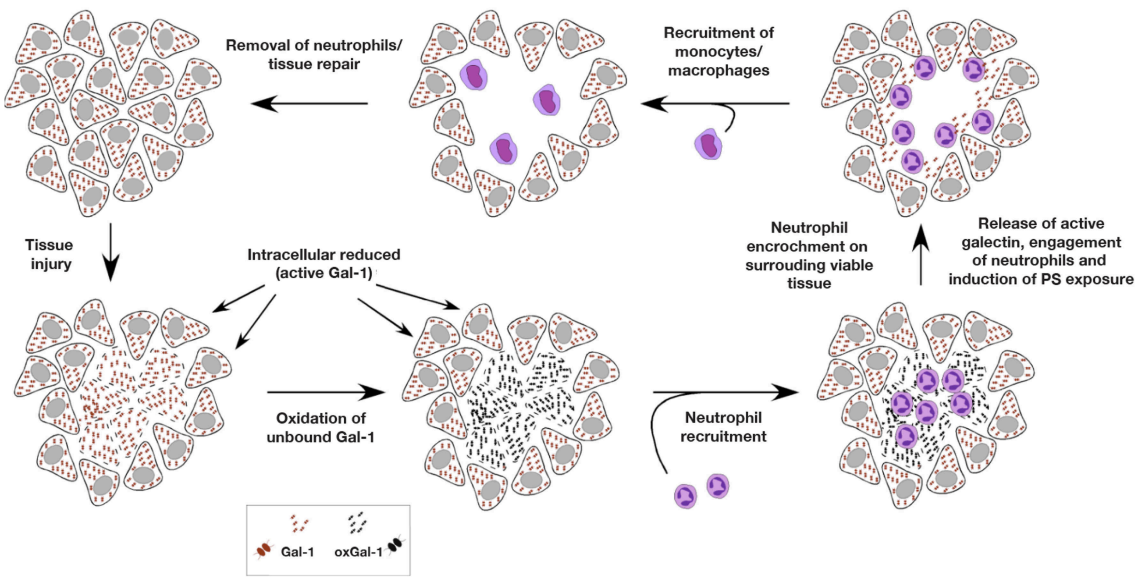

FIGURE 5 | Galectin regulation of neutrophil turnover. As the intracellular environment is reducing, intracellular stores of galectin remain active. However, following cellular injury, intracellular galectin becomes exposed to the extracellular oxidizing environment, where galectin oxidation and inactivation may occur. Rapid infiltration of neutrophils following injury allows for neutralization of potential pathogens and removal of necrotic tissue. As most extracellular galectin may become oxidized following injury prior to significant neutrophil recruitment, the ability of galectins to induce neutrophil turnover would be compromised, preventing galectins from inhibiting a productive inflammatory response. Following removal of necrotic tissue and pathogens, neutrophil encroachment on surrounding viable tissue results in cellular damage and release of reduced and therefore active galectin. Released galectins then engage neutrophils impinging on surrounding viable tissue, induce an oxidative burst that facilitates killing of ingested pathogens and the induction of PS exposure. As galectin-induced PS exposure occurs in the absence of apoptosis, this allows neutrophils to maintain membrane integrity in an otherwise inflammatory environment until successfully phagocytosed by monocyte-differentiated macrophages, which are typically outnumbered by neutrophils and are recruited after significant neutrophil influx. Once neutrophils are removed and inflammation subsides, tissue repair and regeneration ensue.

(185–187). These collective studies, which primarily focused on the outcome of galectin-1 injection, suggested that galectins can inhibit immune-related pathology by reducing the pro-inflammatory activities of macrophages, DCs and T cells (16–19, 185–188). While most of these early studies did not directly examine the impact of galectin injection on neutrophil numbers and function, subsequent studies suggested that recombinant galectin can inhibit neutrophil extravasation, chemotaxis and overall activation (63, 72–78, 82–85, 88, 98, 99, 101–103). Taken together, these results suggest that harnessing the ability of galectins to regulate neutrophil function may have therapeutic potential.

The vast majority of studies that have examined the impact of exogenous delivery of galectin on immune function have employed an intraperitoneal (IP) delivery route, a common approach of introducing substances in small rodent models, but one that is seldomly employed clinically (72, 189). The ability of galectins to bind common glycan motifs present on nearly every cell type is very different from the binding specificity of most naturally occurring or synthetic molecules designed to target immune function. Engagement of glycoconjugates in solution or on the surface of cells within the peritoneal cavity would be predicted to impact the overall biodistribution of galectin following IP delivery. Indeed, it is not clear that galectin injected IP actually arrives at the location of injury, inflammation or immunomodulation. The impact of galectin injection on neutrophil function may therefore reflect indirect effects of galectins that result from general immunosuppression or other types of immunomodulation. As previous studies have suggested that galectins can regulate nearly every immune cell studied (in addition to their ability to alter the activity of many non-immune cells) (16–19, 190), the outcome of galectin injection may reflect a pleotropic effect, where galectins induce alterations in the activities of other cells that converge to influence neutrophil function.

Injection of galectins intravenously (IV) would appear to be a more favorable approach to avoid engagement of intraperitoneal contents and possibly model clinical routes of delivery more accurately. However, unpublished work by numerous labs has demonstrated that IV injection of active galectin-1 results in rapid death, presumably due to immediate galectin-induced hemagglutination and vascular stasis. While galectins have been reported to circulate, galectins detected as serum biomarkers of heart disease and other conditions likely represent inactive galectin as the assays employed in these studies utilize methods of antigen detection that do not directly assess galectin activity (191). As previously discussed, the sensitivity of galectin to oxidation likely provides critical spatial and temporal regulation

that reduces the probability of galectin-mediated vascular stasis observed following an IV bolus of galectin. As the bivalent properties of galectins are not only thought to be responsible for crosslinking counter receptors on the neutrophil surface, but also contribute to hemagglutination, separating the intrinsic biophysical features of galectins that contribute to hemagglutination from their biological activities will likely be difficult (121).

Given the potential challenges of using galectins as modulators of neutrophil function clinically, alternative approaches may be required to fully harness the therapeutic potential of galectins to modify neutrophil function. Several reports have described various synthetic analogs of galectin ligands that appear to specifically inhibit distinct galectin family members (192, 193), providing a potential opportunity to reduce galectin-mediated activation of neutrophils in settings where excessive neutrophil activity may be unfavorable. However, in order to augment a galectin-mediated neutrophil outcome without using recombinant galectin, the actual receptors responsible for mediating the effects of galectins will likely need to be identified and targeted. Identifying galectin ligands on neutrophils that mediate distinct aspects of galectin-dependent regulation not only holds promise in avoiding some of the challenges associated with galectin delivery, but may also provide a more specific approach to dissect different aspects of galectin neutrophil regulation and therefore more deliberately modify neutrophil function in the setting of infection, inflammation or injury. Such an approach may employ antibodies that target protein or glycoprotein epitopes specific to the target receptor, thereby avoiding the potential pleotropic effects that can occur following galectin engagement of more common glycan ligands. However, if the signaling outcome of galectins reflects engagement and clustering of multiple receptors, it may be difficult to recapitulate these activities using a single antibody-based or similar surrogate approach.

Regardless of whether identifying and targeting galectin receptors will provide a suitable substitute for recombinant galectins as a therapeutic strategy, defining functional counter receptors for galectins will allow additional approaches to be used when seeking to further define the roles of galectins on neutrophil activity *in vivo*. As different galectins appear to regulate neutrophil function through similar pathways (16–19), genetic approaches utilizing galectin KOs can be deceiving when only negative results are obtained. As *in vivo* studies have often been driven by initial *in vitro* observations and early *in vitro* data suggest that multiple galectins possess the ability to modulate neutrophil behavior in a similar manner, significant functional redundancy between different galectin family members *in vivo* may reduce the likelihood that a clear phenotype will be observed when using recipients deleted of only one or even several galectin family members. Furthermore, as galectins also regulate other immune populations (16–19), when immunological outcomes are observed following genetic deletion of galectins, it can be difficult to interpret these results, as a particular phenotype observed in a galectin KO may reflect galectin regulation of neutrophil function and/or an indirect outcome of galectin activity on a variety of cell populations, which may in turn influence neutrophil function. Although floxed KO alleles have not be described for any galectin, even this approach, wherein individual galectins can be specifically deleted in neutrophils, may likewise inadequately address this issue, as galectins are expressed by many different cell types, making it virtually impossible to prevent at least extracellular galectin from engaging neutrophils and potentially altering their function. Defining functional galectin receptors on neutrophils will therefore provide additional genetic targets that can be specifically deleted on neutrophils and thus allow an important complementary approach when seeking to examine the potential impact of various galectin family members on neutrophil function *in vivo*.

CONCLUSION

Studies over several decades demonstrate that galectins can regulate a wide variety of neutrophil functions. The ability of galectins to bind a broad range of receptors and similarly be regulated by unique oxidative and proteolytic processes, suggests that evolution selected these unique immune regulators to temporally and spatially shape neutrophil function. In doing so, galectins appear to serve as critical regulators of neutrophil biology. While many *in vivo* studies appear to corroborate galectin activity on neutrophil function, additional studies are needed to formally test many of these hypotheses *in vivo*.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Specific Sialoforms Required for the Immune Suppressive Activity of Human Soluble CD52

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Human CD52 is a small glycopeptide (12 amino acid residues) with one *N*-linked glycosylation site at asparagine 3 (Asn3) and several potential *O*-glycosylation serine/threonine sites. Soluble CD52 is released from the surface of activated T cells and mediates immune suppression via its glycan moiety. In suppressing activated T cells, it first sequesters the pro-inflammatory high mobility group Box 1 (HMGB1) protein, which facilitates its binding to the inhibitory sialic acid-binding immunoglobulin-like lectin-10 (Siglec-10) receptor. We aimed to identify the features of CD52 glycan that underlie its bioactivity. Analysis of native CD52 purified from human spleen revealed extensive heterogeneity in *N*-glycosylation and multi-antennary sialylated *N*-glycans with abundant polyLacNAc extensions, together with mainly di-sialylated *O*-glycosylation type structures. Glycomic (porous graphitized carbon-ESI-MS/MS) and glycopeptide (C8-LC-ESI-MS) analysis of recombinant soluble human CD52-immunoglobulin Fc fusion proteins revealed that CD52 bioactivity was correlated with a high abundance of tetra-antennary α-2,3/6 sialylated *N*-glycans. Removal of α-2,3 sialylation abolished bioactivity, which was restored by re-sialylation with α-2,3 sialyltransferases. When glycoforms of CD52-Fc were fractionated by anion exchange MonoQ-GL chromatography, bioactive fractions displayed mainly tetra-antennary, α-2,3 sialylated *N*-glycan structures and a lower relative abundance of bisecting GlcNAc structures compared to non-bioactive fractions. In addition, *O*-glycan core type-2 di-sialylated structures at Ser12 were more abundant in bioactive CD52 fractions. Understanding the structural features of CD52 glycan required for its bioactivity will aid its development as an immunotherapeutic agent.

Keywords: CD52, immune suppression, glycan structure, analysis, tetra-antennary, α-2,3 sialylation

INTRODUCTION

CD52 is a glycoprotein composed of only 12 amino acid extensively modified by both N-linked and possible *O*-linked glycosylation, anchored by glycosylphosphatidylinositol (GPI) to the surface of leukocytic, and male reproductive cells (1, 2). The conserved CD52 peptide backbone probably functions only as a scaffold for presentation of the large N-linked glycan, which masks the small GPI-anchored peptide and acts as the prime feature of the CD52 antigen with respect to cell-cell contacts (1, 2). This notion is supported by the recent discovery of the immune suppressive role of soluble CD52 *in vitro* and *in vivo* (3–5).

Activated human T cells with high expression of CD52 were found to exhibit immune suppressive activity via phospholipase C-mediated release of soluble CD52, which was shown to bind to the inhibitory sialic acid-binding immunoglobulin (Ig)-like lectin-10 (Siglec-10) receptor on neighboring T cell populations (3). This sialic acid interaction was subsequently shown to require initial binding of soluble CD52 glycan to the damage-associated molecular pattern (DAMP) protein, high-mobility group box 1 (HMGB1). Complexing of soluble CD52 with HMGB1 promoted binding of the CD52 N-glycan, preferentially in α-2,3 sialic acid linkage, to Siglec-10 (4).

In the only previous mass spectrometric analysis, the *N*-glycans on human leukocyte CD52 exhibited extensive heterogeneity with multi-antennary complexes containing core α-1,6 fucosylation, abundant polyLacNAc extensions, and variable sialylation (6). With recent insights into the function of soluble CD52, and its potential as an immunotherapeutic agent, the glycan structure-function determinants of CD52 warrant more detailed investigation. In particular, although the CD52 *N*-glycan is known to be required for bioactivity (3, 4), its structure is not fully elucidated and the glycoforms required for bioactivity have not been identified. In addition, even with a total of six potential serine or threonine attachment sites, *O*-glycosylation of CD52 has not been analyzed. We aimed therefore to identify the structural features of CD52 glycan required for its bioactivity using both purified native human CD52 and recombinant soluble CD52 expressed as a fusion protein with immunoglobulin Fc.

MATERIALS AND METHODS

Human Blood and Spleen Donors

Cells were isolated from human blood buffy coats (Australian Red Cross Blood Service, Melbourne, VIC, Australia) or blood of de-identified healthy volunteers with informed consent through the Volunteer Blood Donor Registry of The Walter and Eliza Hall Institute of Medical Research (WEHI), following approval by WEHI and Melbourne Health Human Ethics Committees. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood on Ficoll/Hypaque (Amersham Pharmacia, Uppsala, Sweden), washed in phosphate-buffered saline (PBS) and re-suspended in Iscove's Modified Dulbecco's medium (IMDM) containing 5% pooled, heat-inactivated human serum (PHS; Australian Red Cross, Melbourne, Australia), 100 mM non-essential amino acids, 2 mM glutamine, and 50 μM 2-mercaptoethanol (IP5 medium).

A cadaveric spleen was obtained via the Australian Islet Transplant Consortium and experienced coordinators of Donate Life from a heart-beating, brain dead previously healthy donor, with informed written consent of next of kin. All studies were approved by WEHI Human Research Ethics Committee (Project 05/12).

Purification of Native CD52 From Human Spleen

Frozen human spleen tissue (10 mg) was homogenized with three volumes of water as per described in Xia et al. (1). In brief, homogenate was mixed with methanol and chloroform 11:5.4 volumes, respectively. Samples were left to stir for 30 min and allowed to stand for 1 h. The upper (aqueous) phase was collected, evaporated, dialyzed, and freeze dried. NHS-activated Sepharose 4 Fast Flow resin was incubated with 1 mg of purified anti-CD52 antibody in 0.5 mL of PBS for 3 h at RT. The mixture was incubated overnight at 4°C and quenched with 1 M ethanolamine. A Bio-Rad 10-mL Poly-Prep column was used for packing and resins were washed with sequential treatment of 5 mL of PBS, 5 mL of pH 11.5 diethylamine, and 5 mL of PBS/0.02% sodium azide. The column was stored at 4°C in 5 mL of PBS/0.02% sodium azide before use. Spleen extracts were solubilized with 2 mL of 2% sodium deoxycholate in PBS, and then added to the packed column and washed with 5 mL of PBS containing 0.5% sodium deoxycholate. The sample was eluted with six times 500 μl of elution buffer (50 mM diethylamine, 500 mM NaCl, pH 11.5) containing 0.5 % sodium deoxycholate. The eluate was collected, neutralized with 50 μl of HCl (0.1 M) and dialyzed against PBS and water.

CD52 Recombinant Proteins

Human CD52-Fc recombinant proteins; CD52-Fc I (Expi293), CD5-Fc II (FreeStyle HEK293F), and CD52-Fc III (Expi293) were produced as described (3). The signal peptide sequences joined to human IgG1 Fc were constructed with polymerase chain reaction (PCR) then digested and ligated into a FTGW lentivirus vector or pCAGGS vector for the transfection of HEK293F and Expi293 cells. The construct included a flexible GGSGG linker, a strep-tag II sequence for purification (7), and a cleavage sites for Factor Xa protease between the signal peptide and Fc molecule. The recombinant proteins were purified from the medium by affinity chromatography on Streptactin resin and eluted with 2.5 mM desthiobiotin (3).

^{3}H-Thymidine Incorporation Assay

PBMCs are primary cells and cannot be cultured for more than one passage under normal conditions. PBMCs (2×10^5 cells/well) in IP5 medium were incubated for up to 3 d at 37°C in 5% CO_2 in 96-well round-bottomed plates with or without the activating antigen, tetanus toxoid (10 Lyons flocculating units per ml), and various concentrations of CD52-Fc or control Fc protein, in a total volume of 200 μL. To measure cell proliferation, the radioactive nucleoside, ^{3}H-thymidine (1 μCi), was added for the last 16 h of incubation. ^{3}H-thymidine is

incorporated into newly-synthesized DNA during mitotic cell division. The cells were collected and radioactivity in DNA measured by scintillation counting.

ELISpot Assay

An IFN-γ ELISpot assay was employed as a further means to demonstrate the immune suppressive activity of CD52-Fc. PBMCs (2×10^5 cells/well) were cultured in 200 μL of IP5 medium in triplicate wells of a 96-well ELISpot plate (PVDF MultiScreen) from Merck Millipore (Bayswater, Australia) containing anti-IFN-γ monoclonal antibody pre-bound (1 μg/mL) at 4°C. Tetanus toxoid (10 Lfu/mL) was added to the wells together with CD52-Fc I, CD5-Fc II or CD52-Fc III (5, 25, and 50 μg/mL). After 24 h, cells were removed by washing and IFN-γ spots, denoting single T cells, were developed by incubation with biotinylated anti-IFN-γ antibody (1 μg/mL) followed by streptavidin-alkaline phosphatase and BCIP/NBT color reagent (Resolving Images, Melbourne, Australia).

Lectin ELISA

We have previously (4) used *Maackia amurensis* and *Sambucus nigra* lectins to distinguish CD52-Fc glycans containing, respectively, sialic acid in α-2,3 and α-2,6 linkage with galactose (8, 9). Here we used *Maackia amurensis* (MAA-I/MAL-I; Vector Laboratories, Burlingame, USA) to identify the α-2,3 linkage. A 96-well flat-bottom plate was coated with 20 μg/mL of MAL-1 overnight at 4°C and subsequently blocked with 200 μl of 1 % BSA for 1 h. After washing with PBS, CD52-Fc I, CD52-Fc II, or CD52-Fc III (20 μg/mL) were added and incubated at RT for 1 h and washed twice with PBS. After washing with PBS, 50 μl of a 1:1,000 dilution of HRP-conjugated antibody to CD52 (Campath H1; 1 μg/mL) was added and incubated at RT for 1 h. 50 μl of 3,3′5,5′-tetramethylbenzidine (TMB) substrate was added and color development stopped by addition of 50 μl of 0.5 M H_2SO_4. Absorbance was measured at 450 nm in a Multiskan Ascent 354 microplate photometer (Thermo Labsystems, San Francisco, USA).

De-sialylation and Re-sialylation of Recombinant CD52-Fc Protein

De-sialylation and re-sialylation of recombinant CD52-Fc III proteins were performed by a modification of the method of Paulson and Rogers (10). Briefly, CD52-Fc (500 μg/each) was incubated with *Clostridium perfringens* type V sialidase (50 mU/mL) for 3 h at 37°C to remove all types of sialic acids. Samples were then passed through a Protein G-Sepharose column, which was washed twice with PBS before the bound protein was eluted with 0.1 M glycine-HCl, pH 2.8 into 1 M Tris-HCl, pH 8.0, followed by dialysis against PBS. Binding to MAL-I lectin was performed to confirm removal of sialic acids. CD52-Fc III from Expi293 cells was then incubated with either of two sialyltransferases, PdST6GalI which restores sialic acid residues in α-2,6 linkage with underlying galactose or CstII which restores sialic acid residues in α-2,3 linkage with galactose, in the presence of 0.46 mM-0.90 mM CMP-N-acetylneuraminic acid sodium salt (Carbosynth, Compton Berkshire, United Kingdom) for 3 h at 37°C. The different CD52-Fc (III) proteins with different linkages (α-2,3 or α-2,6) were passed through Protein G-Sepharose columns, washed twice with PBS and eluted with 0.1 M glycine-HCl, pH 2.8, into 1 M Tris-HCl, pH 8.0, followed by dialysis against PBS. Samples were freeze-dried, re-suspended in PBS at 200 μg/mL and stored at −20°C.

Fc Fragment Removal

CD52-Fc III recombinant protein fractions (50–200 μg) were incubated with 4 μL of Factor Xa protease (purified from bovine plasma, New England Biolabs, Ipswich, USA) in a total volume of 1 mL of cleavage buffer (20 mM Tris-Hcl, pH 8, 100 mM NaCl, 2 mM $CaCl_2$). Samples were incubated overnight at RT. Samples were mixed three times with Protein G-Sepharose beads for 1 h at RT and centrifuged at 10,000 rpm for 15 min. Fc fragment removal was confirmed by Western blot using anti-human IgG (Fc specific produced in goat; Sigma Aldrich, St. Louis, USA) and anti-CD52 (rabbit) antibodies (Santa Cruz Biotechnology, Dallas, USA).

N- and *O*- Linked Glycan Release for Mass Spectrometry Analysis

Mono Q fractionated and whole (non-fractionated) recombinant CD52-Fc III were dot-blotted on a PVDF membrane. Soluble CD52 with the Fc removed was kept in-solution prior to *N*-glycan release by an overnight incubation with 2.5 units of N-glycosidase F (PNGase F from *Elizabethkingia miricola,* Roche, Basel Switzerland) at 37°C followed by a NaBH4 reduction (1 M NaBH4, 50 mM KOH) for 3 h at 50°C. The *O*-glycans were subsequently released by overnight reductive β-elimination using 0.5 M NaBH4, 50 mM KOH at 50°C. The released and reduced *N*- and *O*-glycans were thoroughly desalted prior to the LC-MS/MS as described previously (11).

Mass Spectrometry and Data Analysis of Released Glycans

The separation of glycans was performed by using a porous graphitized carbon (PGC) column (5 μm particle size, 180 μm internal diameter × 10 cm column length; Hypercarb KAPPA Capillary Column (Thermo Scientific, Waltham, USA), operated at a constants flow rate of 4 μl/min using a Dionex Ultimate 3000 LC (Thermo Scientific). The separated glycans were detected online using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) using an LTQ Velos Pro mass spectrometer (Thermo Scientific). The PGC column was equilibrated with 10 mM ammonium bicarbonate (Sigma Aldrich) and samples were separated on a 0–70% (v/v) acetonitrile in 10 mM ammonium bicarbonate gradient over 75 min. The ESI capillary voltage was set at 3.2 kV. The full auto gain control was set to 80,000 kV. MS1 full scans were made between *m/z* 600–2,000. All glycan mass spectra were acquired in negative ion mode. The LTQ mass spectrometer was calibrated with a tune mix (Pierce^{TM} ESI negative ions, Thermo Scientific) for mass accuracy of 0.2 Da. The CID-MS/MS was carried out on the five most abundant precursor ions in each full scan by using 35 normalized collision energy. Possible monosaccharide compositions were provided by GlycoMod (Expasy, http://web.expasy.org/glycomod/) based on the molecular mass of glycan

precursor ions (12). Analysis of MS/MS spectra was performed with Thermo Xcalibur Qual browser software. Possible glycan structures were identified based on diagnostic fragment ions 368 for core fucosylation and others as reported (13), and B/Y- and C/Z-glycan fragments in the CID-MS/MS spectra. A mass tolerance of 0.2 Da was allowed for both the precursor and product ions. The relative abundances of the identified glycans were determined as a percentage of the total peak area from the MS signal strength using area under the curve (AUC) of extracted ion chromatograms of glycan precursor ion (14).

Profiling the *N*- and *O*- Glycans on the CD52 Peptide

MonoQ fractionated and unfractionated CD52 glycoforms without the Fc were desalted on C18 micro-SPE stage tips (Merck-Millipore, Burlington, USA). Elution was performed with 90% acetonitrile (ACN) and samples were dried and redissolved in 0.1% Formic acid (FA). The desalted CD52 glycopeptides were analyzed by ESI-LC-MS in positive ion polarity mode using a Quadrupole-Time-of-flight (Q-TOF) 6538 mass spectrometer (Agilent technologies, Mulgrave, Australia)-HPLC (Agilent 1260 infinity). In parallel experiments, *N*-glycosidase F was used to remove *N*-glycans from some samples of CD52 (with a resulting Asn->Asp conversion i.e., +1 Da) to enable better ionization of the highly heterogeneous and anionic CD52 glycopeptides. The *N*- and *O*-glycan occupancy was (500 ng) were injected onto a C8 column (ProteCol C8, 3 μm particle size, 300 A pore size, 300 nm inner diameter 10 cm length; SGE analytical science). The HPLC gradient was made starting with 0.1% FA with a linear rise to 60% (v/v) ACN 0.1% FA over 30 min. The column was then washed with 99% ACN (v/v) for 10 min before re-equilibration with 0.1% FA for another 10 min. The flow rate was set to 4 μL/min with an optimized fragmentor positive potential of 200 V with the following MS setting: m/z range 400–2,500, nitrogen drying gas flow rate 8 L/min at 300°C, nebulizer pressure was 10 psi, capillary positive potential was 4.3 kV, skimmer potential was 65 V. The mass spectrometer was calibrated with a tune mix (Agilent technologies) to reach a mass accuracy typically better than 0.2 ppm. MassHunter workstation vB.06 (Agilent technologies) was used for analysis and deconvolution of the resulting spectra. The previously determined glycans from the PGC-ESI-MS/MS analysis were used to guide the assignment of glycoforms to deconvoluted CD52 peptides based on the accurate molecular mass.

Mono Q Column Fractionation

CD52-Fc III was diluted into 5 mL 50 mM Tris-HCl, pH 8.3, and applied to a Mono Q column (Mono Q 5/50 GL, GE Lifesciences, Parramatta, Australia). The column was washed with 10 column volumes of 50 mM Tris-HCl, pH 8.3, and then eluted with 50 column volumes of 50 mM Tris-HCl, 500 mM NaCl, pH 8.3 in 0.5 mL fractions. Fractions were then collected and analyzed by isoelectric focusing (IEF).

IEF

Novex pH 3–10 IEF gels (Life Technologies, Carlsbad, USA) were used for pI determination. CD52-Fc fractions were loaded with sample buffer and run at 100 V for 2 h, then at 250 V for 1 h and, finally, the voltage was increased to 500 V for 30 min. After electrophoresis, the gel was carefully transferred to a clean container, washed and fixed with 20% trichloroacetic acid (TCA) for 1 h at RT, rinsed with distilled water, stained with colloidal Coomasie blue (Sigma Aldrich) for 2 h at RT, and thoroughly de-stained with distilled water.

Sequential Sialidase Treatment

N-glycans released from cleaved CD52 (2 μg) were treated with α-2-3-specific sialidase (1 mU, Sigma Aldrich) and broad (α-2-3,6,8 sialidase-reactive) sialidase *V. cholera* (1 mU, Sigma Aldrich). Both reactions were carried out in 50 mM sodium phosphate reaction buffer at 37°C for 3 h. De-sialylated CD52 *N*-glycans were dried and solubilised in water for downstream MS analysis. Fetuin was used as positive control for successful sialic acid removal since, like cleaved CD52, this model glycoprotein carries multi-antennary sialylated *N*-glycans.

EThcD Fragmentation for *O*-Glycan Site Localization on the CD52 Peptide

Fractionated CD52 glycoforms were treated with PNGase F prior to *O*-glycan site localization analysis. CD52 peptides were analyzed using a Dionex 3500RS nanoUHPLC coupled to an Orbitrap Fusion™ Tribrid™ Mass Spectrometer in positive mode with the same LC gradient mentioned in "Profiling the *N*- and *O*- glycans on intact CD52," but with a nano-flow (250 nL/min). The following MS settings were used: spray voltage 2.3 kV, 120 k orbitrap resolution, scan range *m/z* 550–1,500, AGC target 400,000 with one microscan. The HCD-MS/MS used 40% nCE. Precursors that resulted in fragment spectra containing diagnostic oxonium ions for glycopeptides i.e., *m/z* 204.08671, 138.05451, and 366.13961, were selected for a second EThcD (nCE 15%) fragmentation. The analysis of all fragment spectra was carried out using Thermo Xcalibur Qual browser software with the aid of Byonic (v2.16.11, Protein Metrics Inc, Cupertino, USA) using the following parameters: precursor mass tolerance 6 ppm, fragment mass tolerance 1 Da and 10 ppm to respectively, account for possible proton transfer during ETD fragment formation and the MS/MS resolution, deamidated (variable), and two core type 2 *O*-glycans, previously seen in intact mass analysis.

Data are expressed as mean ± standard deviation (SD). The significance of differences between groups was determined by ANOVA, *post-hoc* comparisons of pairs and Bonferroni correction, with Prism software (GraphPad Software). $p < 0.05$ was used throughout as the significance threshold.

RESULTS

Human Spleen-Derived CD52 Exhibits Extensive *N*- and *O*-Glycosylation Heterogeneity

To characterize the natural glycosylation of human CD52, we purified CD52 from human spleen and performed a

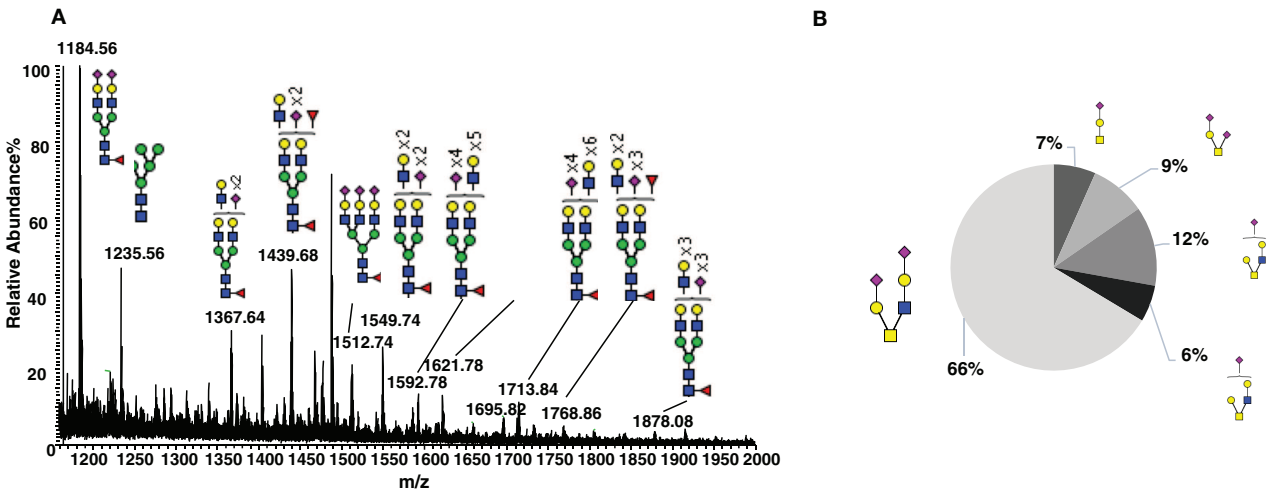


FIGURE 1 | Glycosylation analysis of human spleen CD52. **(A)** Summed MS profile of released *N*-glycans from CD52 purified from human spleen tissue. **(B)** Distribution of *O*-linked glycans released from human spleen CD52. CD52 was purified from one healthy donor spleen.

comprehensive analysis of released *N*- and *O*-glycans by porous-graphitized carbon (PGC)-ESI-MS/MS (**Figures 1A,B**). We confirmed high *N*-glycosylation heterogeneity, expressed as multi-antennary sialylated *N*-glycans with abundant polyLacNAc extensions (**Figure 1A**). Similar *N*- glycans have been previously reported for natural occurring human CD52 (5). The *O*-glycosylation profile was characterized as core type 1 and core type 2 sialylated structures with mainly (66%) di-sialylated core type 2 *O*-glycans (**Figure 1B**). This glycan heterogeneity raises the question whether particular bioactive glycoforms of CD52 exist and whether such heterogeneity is reflected in the recombinant form of human CD52.

The yield of purified native soluble CD52 was insufficient to enable us to pinpoint the bioactive glycoforms on the naturally occurring glycoprotein. Therefore, we engineered human CD52 as a recombinant fusion protein conjugated with an IgG1 Fc fragment as described (3). Previously, we demonstrated the ability of recombinant CD52-Fc, but not its Fc component, to suppress a range of immune functions (3, 4). The two recombinant human CD52-Fc batches we generated for this study recapitulated the previously observed immuno-suppressive bioactivity (**Figure 2A**). However, the Fc has a single *N*-linked glycosylated site at N297 (**Figure 2Ci**), which had to be considered in characterizing and assessing the impact of the *N*-glycosylation of recombinant CD52-Fc. This was addressed in two ways: (i) by analyzing a recombinant form of human CD52-Fc in which Fc contained a N297A mutation, allowing analysis of CD52 *N*-glycosylation profile at the released glycan level without interference from the Fc *N*-glycan (**Figure 2Cii**), and (ii) by removal of the Fc component from CD52-Fc by Factor Xa proteolysis of a cleavage site appropriately incorporated in the CD52-Fc construct, as shown by a Western blot using a specific antibody for CD52 (**Figure 2B**).

Bioactive Recombinant CD52 Glycoforms Displays More Abundant tri- and Tetra-Antennary Sialylated *N*-Glycans

We had noted that the specific bioactivity of recombinant CD52-Fc varied from batch to batch. Therefore, we compared two CD52-Fc variants made in different host cells, here referred to respectively, as CD52-Fc I (from Expi 293 cells) and CD52-Fc II (from HEK 293F cells), which displayed higher and lower immunosuppressive activity (**Figure 3A**).

N-glycans were released *via* in-solution treatment with PNGase F and subsequently analyzed by PGC-ESI-MS/MS (9). *N*-glycans on cleaved CD52 I had greater relative abundances of bi-, tri- and tetra- antennary sialylated glycans compared to CD52 II (**Figure 3B**). Also, CD52 I displayed a significantly higher relative abundance of sialylated structures possibly containing LacNAc moieties (**Figure 3B**). Not only the numbers of antennae, but also their degree of sialylation differed between the two recombinant CD52 glycoforms: tetra-sialylated *N*-glycans were significantly more abundant in CD52 I (6.9 $\pm$ 0.1%) compared to CD52 II (4.2 $\pm$ 0.6; $p < 0.05$). In contrast, CD52 II displayed significantly greater abundance of non-sialylated bi-antennary and bisecting structures (35 and 4% compared to 19 and 2%, respectively; **Figure 3B**).

After the removal of Fc, recombinant CD52 I and CD52 II were then subjected to high-resolution intact peptide analysis using C8-LC-ESI-MS. Both proteins showed *N*-glycosylation profiles similar to those of released glycans. The high resolution of the Q-TOF instrumentation used even in the high m/z range enabled the identification of very elongated sialylated antennary structures including searching for *N*-glycans carrying Lewis-type structures (antenna-type fucosylation). The experimental isotopic distribution of both variants of recombinant CD52 matched the theoretical isotopic distribution of the 90% tri-sialylated (non-Lewis fucosylated) CD52 glycoforms, indicating

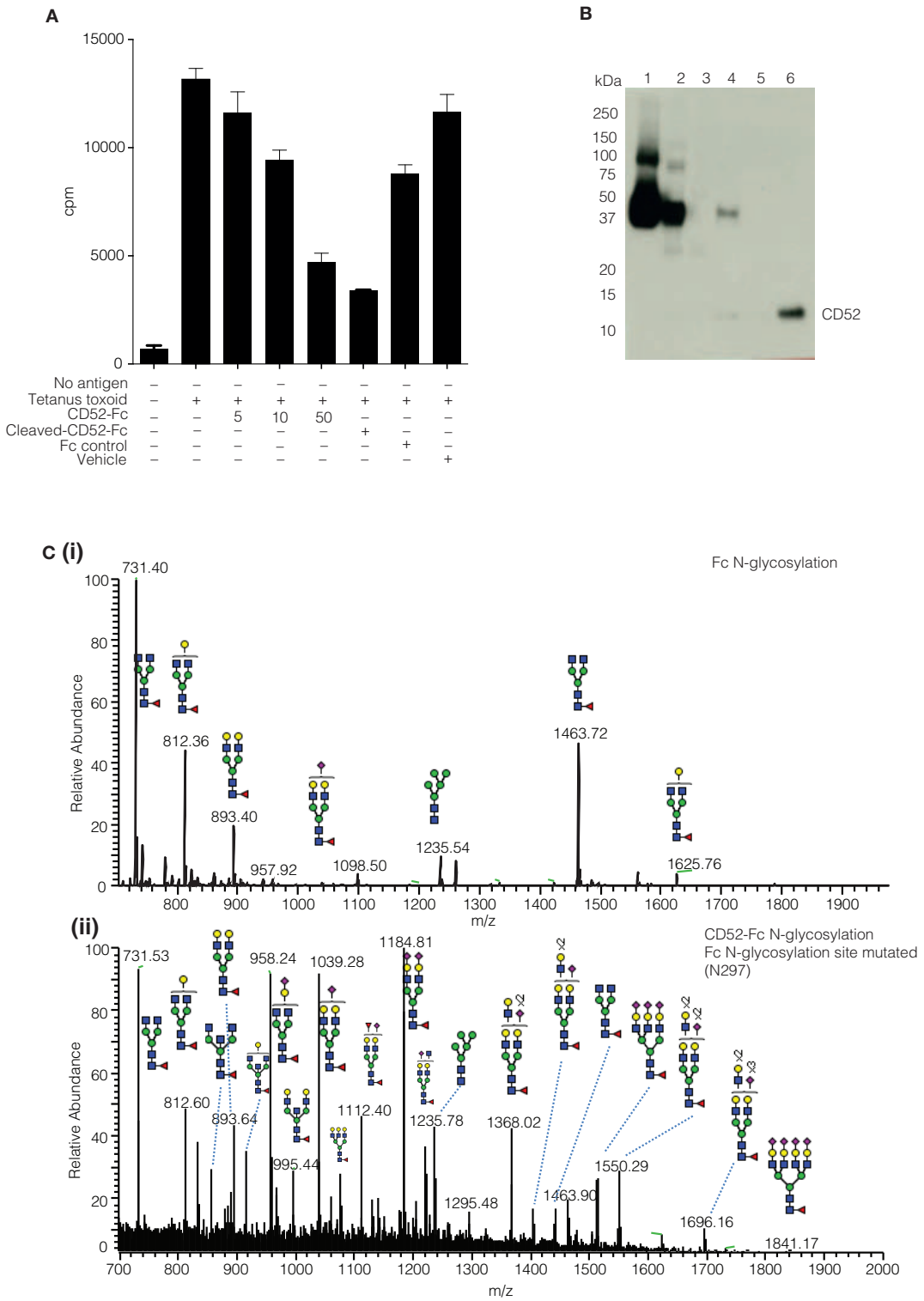


FIGURE 2 | Comparative N-glycoprofiling of recombinant human IgG Fc and CD52. **(A)** Proliferation of human PBMCs (^{3}H thymidine uptake) followed 5 days incubation with tetanus toxoid (10 LfU), histograms show mean ± SD of within-assay triplicates, in the presence of different concentration of proteins (CD52-Fc 5, 10, 50 μg/ml; Cleaved CD52-Fc 50 μg/ml and Fc control 50 μg/ml). The Fc component was cleaved from CD52-Fc with Factor Xa. **(B)** Factor Xa treated-CD52 was analyzed by Western blotting with anti-CD52-HRP antibody (Campath-H1). **(C)** Summed MS profile of N-glycans released from the Fc (I) and CD52 (II); the latter variant was generated by introducing a point mutation (A297N) into the conventional Fc N-glycosylation site.

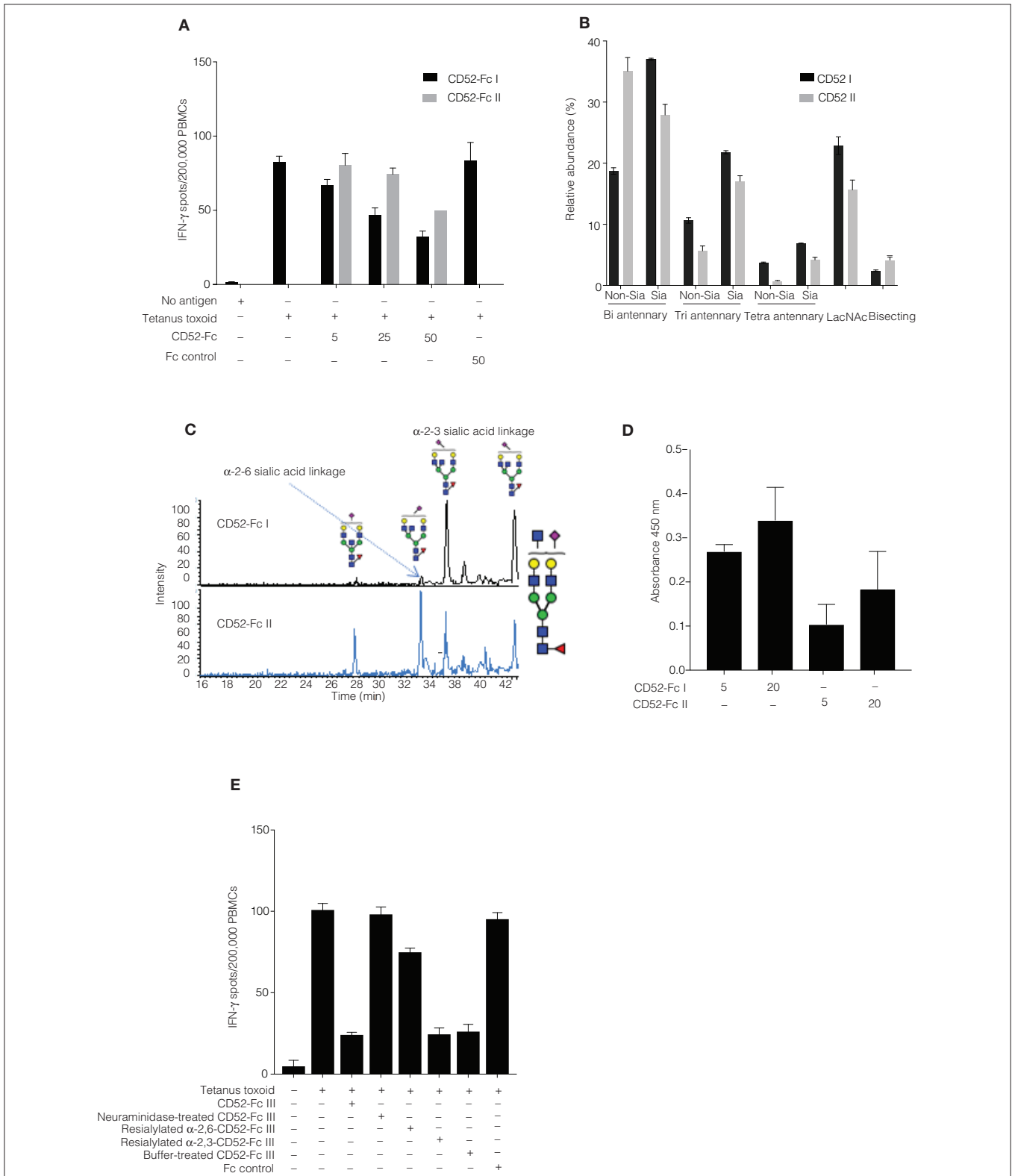


FIGURE 3 | Comparison of recombinant human CD52-Fc variants (I, II, and III) with different immunosuppressive activities. **(A)** IFN–γ production measured by ELISpot assay from human PBMCs (2×10^6) in 200 μL/well. Samples were incubated with no antigen or tetanus toxoid in the presence of two different preparations of CD52-Fc (CD52 I or CD52 II; 5, 25, and 50 μg/ml). **(B)** *N*-linked glycans released from cleaved CD52 I and CD52 II. The abundance of each *N*-glycan class is the sum

(Continued)

FIGURE 3 | of all EICs measured for all glycans in that class relative to the total of all EICs observed for all *N*-glycans. **(C)** EIC of *m/z* 1140.4^{2-} ($GlcNAc_5Man_3Gal_2NeuAc_1$) demonstrating the PGC-based separation of sialo-glycan isomers observed in CD52 I and CD52 II. **(D)** Binding of CD52-Fc I and CD52-Fc II (5 and 20 μg/ml) to the α-2,3 sialic acid recognizing lectin MAL-1. **(E)** ELISpot assay showing activity of CD52-Fc III reconstituted with sialic acid in α2-6, α2-3, and α2-8 linkages with galactose. The data points in **(A,D,E)** are plotted as mean ± SEM of three independent replicate experiments. Data in B and C are mean ± SDs ($n = 3$). ANOVA, *post-hoc* comparisons of pairs and Bonferroni correction were used to test for significant difference between group means.

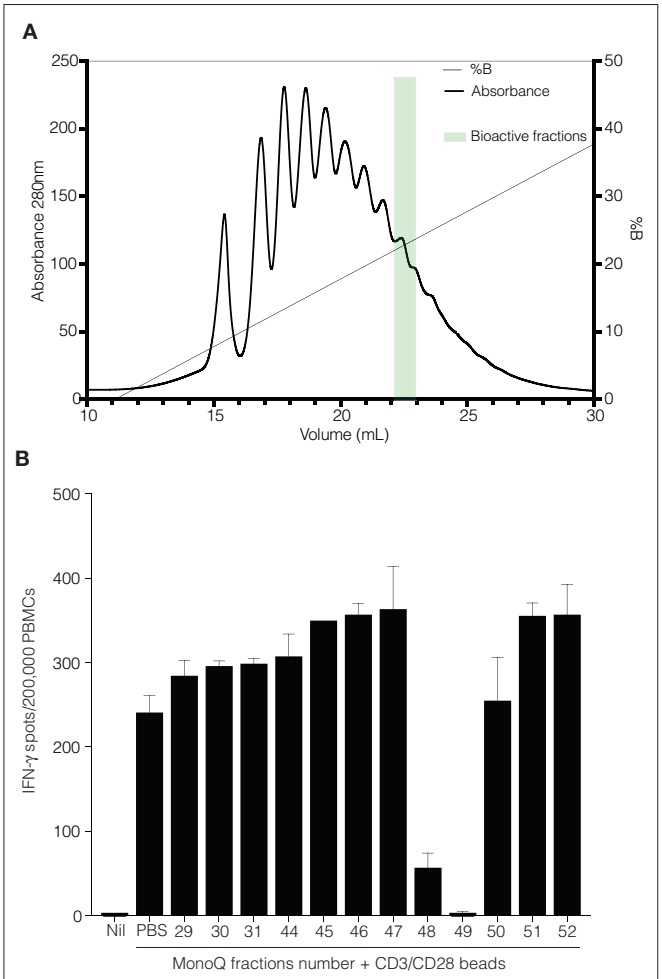


FIGURE 4 | CD52-Fc after fractionation by anion-exchange chromatography. **(A)** Anion exchange chromatography on a MonoQ-GL column fractionated the recombinant human CD52-Fc III into a gradient of anionic glycoforms displaying a spectrum of pI (see **Supplementary Figure 2**). **(B)** IFN–γ ELISpot assay with 2×10^6 PBMCs in 200 μL/well incubated with no antigen or with anti-CD3/CD28 antibody Dynabeads in the presence of recombinant human CD52-Fc fractions (F29–F52; 5 μg/ml).

that the main glycoforms of recombinant CD52 do not carry Lewis-type fucosylation (**Supplementary Figure 1A**). The more bioactive CD52 I displayed a higher level of multi-antennary sialylated and possible LacNAc elongated structures (**Supplementary Figure 1B**).

α-2,3 Sialylated *N*-Glycans Are Indispensable for CD52 Activity

CD52 *N*-glycans displaying α-2,3 sialylation preferentially bind to Siglec-10 (4). PGC-ESI-MS/MS glycan analysis and MAL-I lectin blotting were used to identify any differences in sialic acid linkage between the two variants of recombinant CD52-Fc (CD52-Fc I and CD52-Fc II). MAL-I preferentially recognizes α-2,3 sialic acid linked tri- and tetra-sialylated *N*-glycans (15). Despite the high separation power of PGC for sialoglycans, this technique has difficulty resolving very large multi-antennary sialylated glycans, but can easily discriminate between α-2,3 and α-2,6-sialylation on the more common bi- and tri-antennary *N*-glycans. Several abundant bi-antennary α-2,3 sialoglycans were observed on CD52 I. For one sialylated glycan, *m/z* 1140.4^{2-} ($GlcNAc_5Man_3Gal_2NeuAc_1$), only the α-2,3 sialic acid glycan isomer was observed on CD52 I. On the other hand, the less bioactive CD52 II carried both α-2,3 and α-2,6 sialo-*N*-glycans (**Figure 3C**). This differential sialyl linkage presentation between the two recombinant CD52 variants was supported by MAL-I lectin binding, which was higher for the more bioactive CD52-Fc I (**Figure 3D**). The importance of α-2,3 sialylation for bioactivity of CD52-Fc was confirmed in a parallel experiment in which the immunosuppressive activity of sialidase-treated and re-sialylated CD52-Fc was determined relative to the original recombinant variant. Treatment of CD52-Fc with sialidase completely abolished its immunosuppressive activity, which was fully restored upon re-sialylation with α-2,3, but not α-2,6 (**Figure 3E**). Overall, these findings indicate that the bioactivity of CD52-Fc is associated with the presence of α-2,3-linked tetra-sialylated *N*-glycans found on CD52.

Active CD52 Glycoforms Resolved by Anion Exchange Chromatography

We performed anion exchange chromatography on a MonoQ column in order to separate recombinant CD52-Fc III variants based on their degree of sialylation, with the aim of identifying the most bioactive forms (**Figure 4A**). The increasing degree of sialylation [decreasing isoelectric point [pI]] of CD52-Fc in the collected fractions was confirmed by isoelectric focusing (IEF) (**Supplementary Figure 2**) and mass spectrometry. The released *N*-glycans from fractions 46 to 51 (F46-F51) exhibited a gradual increase in sialic acid content, and structures containing a higher number of antennae (**Table 1**), as shown also from intact glycopeptide analysis (**Supplementary Figure 3**). Released and intact glycan analysis from fraction 30 revealed various GlcNAc and Gal capped structures and a complete absence of sialic acid moieties (**Table 1** and **Supplementary Figure 3**). Remarkably, only two fractions, F48 and F49, with pIs in the 5–6 range, displayed significant immunosuppressive activity (**Figure 4B**). The adjacent fractions were not bioactive, even at higher concentrations of protein (**Supplementary Figures 4A,B**). These late-eluting, uniquely bioactive fractions (F48–49) were highly enriched (60–70%) in tri- and tetra-sialylated glycans.

TABLE 1 | Sialic acid content and antennae distribution of recombinant human CD52 fractions separated by anion chromatography.

	F30 (non-active)	F46 (non-active)	F47 (non-active)	F48 (active)	F49 (active)	F50 (non-active)	F51 (non-active)
A % sialic acid No sialic acid 1 sialic acid 2 sialic acid 3 sialic acid 4 sialic acid	F30	F46 8.7 3.4 48.3 35.9	F47 8.2 32.5 18.2 36.9	F48 11.1 10.6 23.2 51.1	F49 24.4 29.7 20.0 24.4	F50 9.5 32.5 25.9 30.8	F51 29.2 16.5 25.4 27.8
B Glycan antenna forms on CD52 Mono-antenna Bi-antenna Tri-antenna Tetra-antenna	F30 4.5 15.8 20.2 59.5	F46 8.3 3.6 32.1 59.6	F47 21.6 4.2 41.3 37.1	F48 21.9 4.0 41.3 35.9	F49 33.4 27.9 38.7	F50 35.0 1.3 31.9 33.3	F51 1.0 31.0 47.2 21.8

(A) *(upper panel) The total number of sialic acid residues and* ***(B)*** *(lower panel) The antennae distribution identified on CD52 fractions (F30, F46, F47, F49, F50, and F51) using PGC-ESI-MS/MS.*

Active CD52 MonoQ Fractions Are Enriched With α-2,3 Sialylated Structures

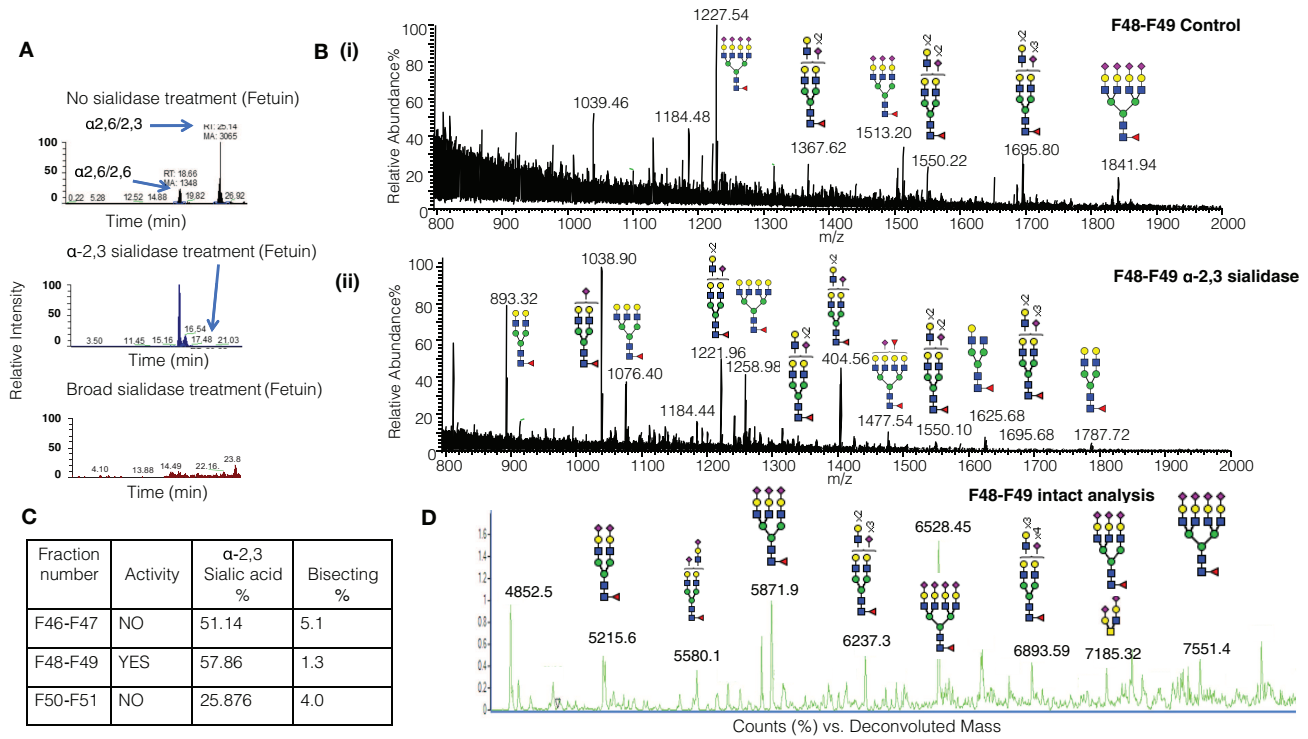
It is challenging to determine the sialylation linkages of large, multi-sialylated *N*-glycans by mass spectrometry. Therefore, differences in sialic acid linkage of active and adjacent non-active MonoQ fractions were probed by α-2,3-specific sialidase treatment. The linkage-specific activity of α-2,3 sialidase was confirmed on bovine fetuin as a control protein; specific removal of α-2,3-linked sialic acid residues from this known bi-antennary sialylated glycan *m/z* 1111.5^{2-} was demonstrated (**Figure 5A**). The glycan products resulting from α-2,3 sialidase treatment of the active fractions of CD52 were determined via PGC-ESI-MS/MS (**Figures 5Bi,ii**). The active MonoQ fractions (F48/F49) had a higher proportion of α-2,3 sialic acid (58%) compared to adjacent earlier (F46, F47) and later (F50, F51) eluting fractions (51 and 25%, respectively) and less bisecting structures than the adjacent non-active fractions (1%, compared to 4 and 5%, respectively; **Figure 5C**). Finally, the profile of the most active CD52 fractions at the intact peptide level supported a predominance of tri- and tetra-antennary sialylated structures (**Figure 5D**).

The Highly Anionic MonoQ Fractions Are Enriched in *O*-Sialylated Glycans

Initially, *O*-glycosylation analysis of de-*N*-glycosylated CD52 at the intact peptide level revealed that both variants of recombinant CD52 (CD52 I and CD52 II) had very low (4%) *O*-glycan occupancy (**Figure 6A**), casting doubt on the relevance of *O*-glycosylation for CD52 activity. Non-deamidated signatures were absent in the spectra for both CD52 I and II, indicating that the CD52 peptides were fully *N*-glycosylated (**Figure 6A**, black symbols). Like human spleen CD52, the recombinant CD52 proteins were found to contain mainly core type 2 *O*-glycans with one or two sialic acid residues (**Figure 6A**, gray and orange symbols, respectively). Sialylated core type 1 *O*-glycans were also identified albeit at very low abundance (<0.5%) (data not shown). Interestingly, the most anionic MonoQ CD52 fractions (F46-F51) had a considerably higher *O*-glycan occupancy (15–20%) compared to the original non-fractionated CD52 (4%). Extracted ion chromatograms (EIC) of the bioactive fractions (F48 and F49) showed an absence of sialo-isomers for the most abundant *O*-glycan structure *m/z* 665.2^{2-} ($GalNAc_1GlcNAc_1Gal_2NeuAc_2$), but not for m/z 1040.4^{1-}($GalNAc_1GlcNAc_1Gal_2NeuAc$) (**Figure 6B**). Finally, *O*-glycan site localization was determined by electron transfer/higher-energy collision dissociation (EThcD), which provided c and z ions, allowing the conclusion that di-sialylated *O*-glycans were conjugated to Ser12, and possibly Ser10, whereas the mono-sialylated *O*-glycans were only found on Thr8 (**Figures 6Ci,ii**).

DISCUSSION

In this study, we determined that CD52 from human spleen and recombinant forms of human CD52-Fc carry *N*-glycans that display complex type core fucosylation, abundant sialylation,



Fraction number	Activity	α-2,3 Sialic acid %	Bisecting %
F46-F47	NO	51.14	5.1
F48-F49	YES	57.86	1.3
F50-F51	NO	25.876	4.0

FIGURE 5 | Sialic linkage analysis of active monoQ active fractions. **(A)** EICs of the di-sialylated *N*-glycan *m/z* 1111.4^{2-} after sequential α-2,3 sialidase treatment of bovine fetuin, known to carry tri-antennary α-2,3-sialylated *N*-glycans. The EICs assess the removal of each of the sialic acid residues. **(B)** Summed MS of all *N*-glycans observed for the active CD52 fractions F48 and F49 before (i) and after treatment with α-2,3-specific sialidase (ii). **(C)** Summary of the degree of α-2,3 sialylation and bisecting GlcNAcylation of late-eluting MonoQ fractions of particular interest. **(D)** High-resolution intact mass analysis of the immune suppressive CD52 fractions (F48/F49).

and LacNAc extensions. These features corroborate a previous report (6) on the *N*-glycan of human spleen CD52, but we extended this in several ways. By comparing two recombinant CD52-Fc glycoproteins that differed in specific bioactivity, made in different host cells, we found that the more bioactive form had a significantly higher abundance of tetra-sialylated *N*-glycan structures with α-2,3 sialic acid linkage. The less bioactive form, on the other hand, exhibited significantly higher bisecting GlcNAc structures. By MonoQ anion exchange chromatography, CD52-Fc was separated into a gradient of anionic glycoforms, which exhibited distinctly different immunosuppressive activities. Again, the most bioactive glycoforms uniquely displayed an abundance of tri- and tetra-sialylated glycans (60–70%), high levels of α-2,3 sialylation (58%), and an absence of bisecting GlcNAcylation. Moreover, the most anionic tri- and tetra-sialylated *N*-glycopeptides had a unique abundance in core type 2 di-sialylated *O*-glycan on Ser 12.

Both glycan- and glycopeptide-based analytical approaches were used to correlate CD52 glycan structure with CD52 bioactivity. The glycan approach depended on the high resolving power of PGC columns to separate glycan isomers and isobaric structures. It was used in conjunction with negative mode ionization to provide fragment ions of certain glycan structural features (11, 14). The glycopeptide-based approach allowed analysis of CD52 glycans directly bound to the peptide backbone with the assurance of no interference by Fc glycan. The two approaches largely corroborated each other, adding confidence in the reported structures. Indeed, we found the same results after CD52-Fc fractionation by anion exchange chromatography, as described. Anion exchange was previously employed to fractionate sialylated glycoforms of the soluble and sperm-associated form of CD52 in the mouse reproductive tract (16), but glycan structure was not analyzed.

We confirmed the importance of the α-2,3 sialic acid linkage for CD52-Fc bioactivity. Previously, we showed that soluble CD52 mediates T-cell suppression by binding to Siglec-10 (3). The diverse family of mostly inhibitory Siglec receptors has evolved to recognize linkage-specific sialic acid residues on host cells and pathogens (17). Siglec-10 is highly expressed on leukocytes (18, 19) and plays significant roles in regulating the innate and adaptive immune response to tissue injury, sepsis and viral invasion (20). Previously, Siglec-10 was reported to have no binding preference for α-2,3 or α-2,6-sialylation (18, 21). However, we recently found that human CD52-Fc binds to Siglec-10 preferentially through the α-2,3 sialic acid linkage (4). In the present study, bioactive CD52-Fc was characterized by a high abundance of the α-2,3 sialic acid linkage, and re-sialylation with α-2,3 restored the bioactivity of sialidase-treated CD52-Fc.

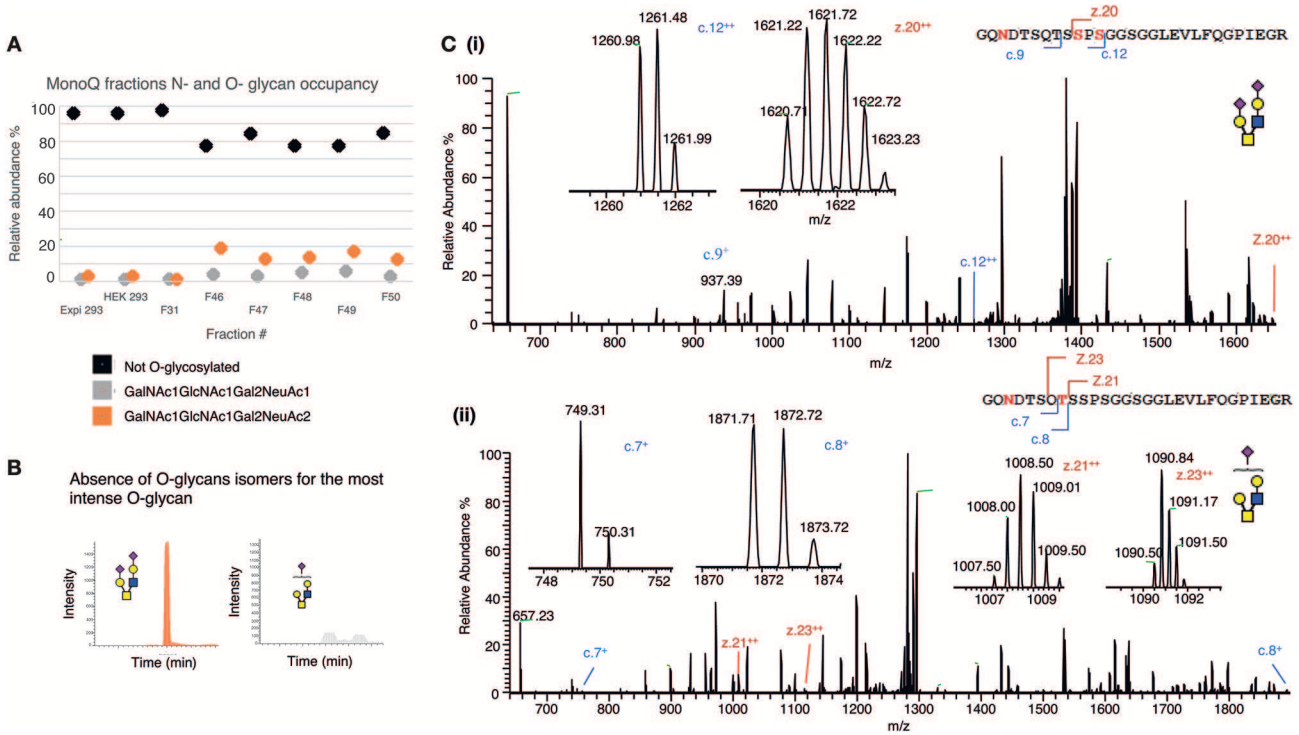


FIGURE 6 | Mapping the *O*-glycosylation of recombinant human CD52. **(A)** *N*- and *O*-glycan occupancy of CD52 I, CD52 II, and selected MonoQ fractions (F31 and F46–F51) measured at the protein level after de-*N*-glycosylation. **(B)** PGC resolution of *O*-glycosylated isomers from active fractions *m/z* 665.2^{2-} ($GalNAc_1GlcNAc_1Gal_2NeuAc_2$) and *m/z* 1040.4^{1-} ($GalNAc_1GlcNAc_1Gal_2NeuAc_1$). **(C)** EThcD-MS/MS based site localization analysis showing the peptide backbone fragments and the ions diagnostic of the amino acid site for both aforementioned *O*-glycans. Fragmentation to c and z ions are shown that indicate that (i) di-sialylated *O*-glycans were conjugated to Ser12, and possibly Ser10, whereas (ii) the mono-sialylated *O*-glycans are found on Thr8.

Regarding CD52 *O*-glycosylation, Ermini et al. (22) deduced the presence of *O*-glycosylation of CD52 by antibody binding, but did not determine the type, occupancy or localization of *O*-glycans. We characterized for the first time the *O*-glycans on human spleen CD52. In addition, recombinant CD52-Fc was found to contain a low abundance (4%) of mainly core type 2 *O*-glycans with one or two sialic acid residues, on Ser 12 and Thr 8, but this increased significantly (to 15–20%) in MonoQ-purified bioactive CD52-Fc. Due to the proximity of the *N*- and *O*-glycosylation sites of CD52 peptide, the low degree of *O*-glycosylation could be due to steric hindrance from the bulky *N*-glycan. Determination of the *O*-glycan sites and occupancies on human spleen CD52 was challenging due to its limited availability. However, with continuing developments in highly sensitive glycoprotemics (20) it should soon be possible to identify the site-specific *O*-glycosylation of CD52 directly from tissues and bodily fluids without prior purification. Our results also indicate that recombinant human CD52 does not require fucosylated *O*-glycans for bioactivity, as found for CD52 of the male reproductive tract (23). The polypeptide of recombinant human CD52 is identical to human spleen CD52 and shares the core type 2 and core type 2 sialylated *O*-glycans with reproductive tract CD52 (24). However, we identified a dramatic enrichment of *O*-glycosylation in the MonoQ active CD52-Fc fractions, strongly implying a role for both *N*- and *O*-glycosylation in the bioactivity of CD52.

Another striking observation was the inverse association between CD52 bioactivity and bisecting GlcNAcylation. Previously, *N*-glycans displaying bisecting GlcNAc were found to correlate with a decrease in tri- and tetra-sialylated structures, since bisecting GlcNAc residues inhibit the activity of GlcNAc-transferases required to generate multi-antennary sialoglycans (25). Furthermore, an increase in bisecting GlcNAcylation has been linked with a decrease in α-2,3 sialylation (26), which we here show is important for CD52 bioactivity. The functions of bisecting GlcNAc are not fully understood, but they have been associated with a decrease in target-cell susceptibility for NK cell-induced lysis (27). Interestingly, CD52 in recombinant human CD52-Fc resembled naturally-occurring CD52 purified from human spleen with respect to *N*- and *O*-glycosylation, except in the degree of polyLacNAc elongation, which was greater in the native form. Although bioactive CD52 was characterized by higher abundance of sialylated structures and polyLacNAcs, the contribution of polyLacNAc units to CD52 activity is yet to be determined.

In conclusion, the comparison of native and recombinant human CD52-Fc, and CD52-Fc variants differing in bioactivity, enabled us to identify glycoform features that underlie the immune suppressive activity of CD52. These can be summarized

as an abundance of tri- and tetra-antennary α-2,3-sialylated *N*-glycans, an absence of bisecting GlcNAcylation and the presence of the di-sialylated type 2 *O*-glycosylation. Further glycomic analysis will be required to detail the length of polyLacNAc extensions and the degree of polyLacNAc branching. The present study extends our knowledge of the glycan structure required for CD52 bioactivity and may assist in the design and production of CD52-Fc as an immunotherapeutic agent.

ETHICS STATEMENT

Cells were isolated from human blood buffy coats (Australian Red Cross Blood Service, Melbourne, VIC, Australia) or blood of de-identified healthy volunteers with informed consent through the Volunteer Blood Donor Registry of The Walter and Eliza Hall Institute of Medical Research (WEHI), following approval by WEHI and Melbourne Health Human Ethics Committees. Healthy human spleen from cadaveric organ donors were obtained from Australian Islet Transplant Consortium and trained coordinators of Donate Life from heart-beating, brain dead donors with informed written consent of next of kin. All studies were approved by WEHI Human Research Ethics Committee (Project 05/12).

AUTHOR CONTRIBUTIONS

LH initiated the study and all authors contributed to its design. EB-S, AS, and AJ performed most of the experiments. AS, EB-S, MT-A, AE-D, NP, and LH analyzed data and drafted the manuscript. EG-B and TA provided advice and technical support. All authors discussed and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01967/full#supplementary-material

Supplementary Figure 1 | Analysis of CD52 I and CD52 II at the intact peptide level. **(A)** The theoretical isotopic distribution of deconvoluted 5871.99 (amu) CD52 glycoform as tri-sialylated ($GlcNAc_5Man_3Gal_3NeuAc_3Fucose_1$) or di-sialylated with two outer fucoses ($GlcNAc_5Man_3Gal_3NeuAc_2Fucose_3$). The bar graph shows the theoretical isotopic envelopes generated when different amount of these two glycans are present. Experimental isotopic distribution values suggest a population of 90–100% tri-sialylated structures. **(B)** High-resolution intact mass analysis of CD52 I (pink) and CD52 II (green).

Supplementary Figure 2 | CD52-Fc III fractions resolved in isoelectric focusing (IEF) gel. Colloidal Coomassie Blue gel showing protein in MonoQ fractions (F29–54). Fractions showed a gradual decrease in isoelectric point (pI) values.

Supplementary Figure 3 | High-resolution intact mass analysis of MonoQ fractions (F30 and F47–50). **(A)** F30 intact mass analysis of the CD52 III part showed absence of sialic acid molecules. **(B)** MonoQ fractionation was able to separate CD52 sialylated structures according to their amount of sialic acid as well as number of antennae. Among fractions F47–50, F49, and F50 contained more of the bigger sialylated structures.

Supplementary Figure 4 | Active MonoQ fractions suppress in a dose-dependent manner. **(A,B)** IFN-γ production measured by ELISpot assay from human PBMCs (2×10^5) incubated in IP5 medium with no antigen or anti-CD3/CD28 antibody Dynabeads. **(A)** Active Mono-Q fractions (F48–49) suppressed in a dose-dependent manner (0.3125, 0.625, 1.25, 2.5, and 5 μg/ml). **(B)** Adjacent fractions (inactive; F46, F47, F50, and F51) do not suppress despite the increase of protein added (5, 10, 20, and 40 μg/ml). The data points in panels **(A,B)** are plotted as mean ± SEM of three independent replicates.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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