On the interactions between carbohydrates and immune cells

Steuten, K.

Chapter 1

General introduction

1.1 Glycan–lectin interactions on myeloid cells

One of the main mechanisms by which cells sense and respond to their surroundings is via binding of ligands with their surface receptors. Most such interactions involve apolar molecular surfaces that exclude water upon engagement, yielding binding free energy via solvent entropy gain.[1] Glycan ligands, however, are different. They are polymers of polyols with many hydrogen bond donors, and represent a class of hydrophilic biomolecules that deviate from this paradigm.[2] The interaction between a glycan and a lectin relies on glycan binding domains that themselves are mostly of hydrophilic nature.[3,4] Rather than primarily depending on hydrophobic forces and water exclusion, glycan-lectin binding involves hydrogen bonding, metal ion coordination, van der Waals forces, and ionic interactions as illustrated for the mannose receptor in **Figure 1.1**. Furthermore, water molecules near the binding sites and the conformation of glycans significantly influence the interaction. Additionally, lectins often engage multiple glycan epitopes simultaneously, which enhances the overall binding avidity despite the weaker interactions of individual glycan-lectin contacts.[2]

The physicochemical properties of carbohydrates inherently bias their interactions with lectins toward fast and reversible binding events. This characteristic makes them ideal motifs for immune recognition. Myeloid cells must continuously scan their environment for the presence of pathogens which they achieve using pattern recognition receptors (PRRs). These PRRs recognize conserved pathogen-associated or danger or damage associated molecular patterns (PAMPs or DAMPs) and initiate innate immune responses. One key class in this repertoire of PRRs is the immune cell lectins. These receptors encompass the C-type, I-type and S-type (galectin) lectin superfamilies.[2] Typically, these lectins can distinguish between foreign and self-carbohydrates and even between healthy self-associated molecular patterns (SAMPs) and unhealthy DAMP-associated-carbohydrates. Despite their weak binding affinities, they can initiate appropriate immune responses only upon detecting the correct patterns.[5–7] In this chapter, the principles underpinning the functions of **C-type** lectins and **I-type** lectins, two major lectin families that shape immune responses, will be discussed.

1.1.1 C-type lectins and antigen presentation

C-type lectin receptors (CLRs) are key pattern-recognition receptors on myeloid cells that engage in Ca^{2+}-dependent binding to mannose-, fucose-, and GlcNAc-terminated glycans.[9] The general role of CLRs is to initiate pro-inflammatory signaling in the myeloid cell, leading to activation of innate components of the immune system. The CLRs DC-SIGN, Langerin, Mannose Receptor (MR), DNGR-1 and DEC-205 also play a role in the initiation of the adaptive immune response. Aside from their role as archetypal PRRs, these particular lectins are also able to capture and route antigen towards MHC-I or MHC-II-loading pathways, which in turn can initiate T cell

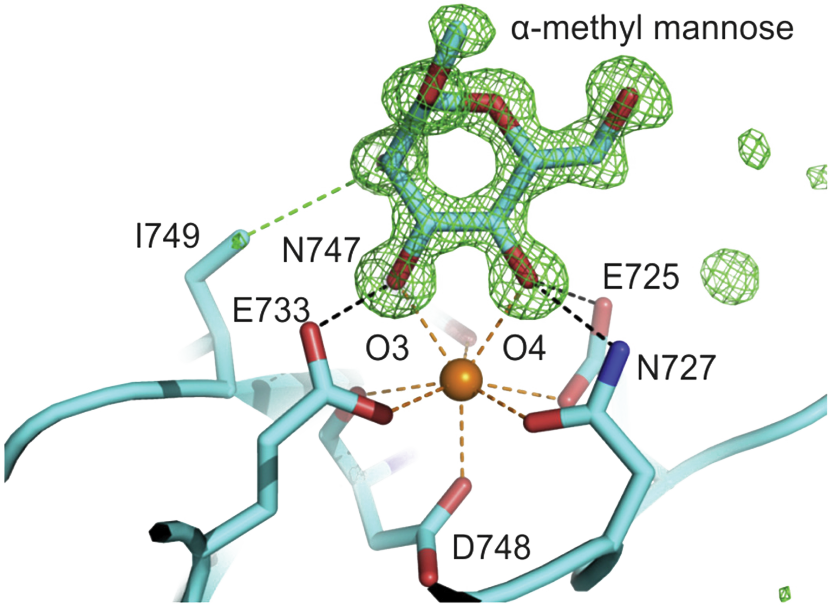

Figure 1.1: Structural features of carbohydrate recognition. Crystal structure of carbohydrate recognition domain 4 (CRD4) of the mannose receptor in complex with mannose. Ligand is shown in green mesh, Ca^{2+} in orange, protein backbone in cyan, oxygen atoms in red, nitrogen in blue. Black dashed lines indicate hydrogen bonds, orange dashed lines indicate metal coordination and green dashed lines indicate nonpolar contacts. PDB: 1EGG, figure is reproduced from figure 5A in Feinberg *et al.* under a CC-BY 4.0 license.[8]

responses and thus raise an adaptive immune response.[10,11] It must be noted that although DNGR-1 and DEC-205 have been identified as lectins by sequence homology, no glycan ligands have been identified for these receptors.[12,13] Because of their critical role in the enhancement of antigen presentation, CLRs present attractive targets for the delivery of antigen for T-cell stimulating vaccines.[14] To this end, several groups have conjugated antigenic peptides or proteins to CLR-specific antibodies[15,16] or their native carbohydrate ligands to initiate an immune response. For the carbohydrate ligands, initial work employed the non-specific conjugation of ring-opened monosaccharides to antigenic proteins via lysine- or cysteine-reactive chemistries targeting DC-SIGN or the MR.[17–21] More recently, fully synthetic approaches of defined carbohydrates, linkages and valency have enabled more precise studies into the structure-activity relationship between the glycotype, antigenic cargo and the functional response.[22–25]

Whilst new in class, these studies resulted in non-linear correlations between glycan configuration, multivalency and functional response. For example, the hexavalent

bimannoside B6-gp100-TLR7L self-adjuvanting conjugate in Li and Hogervorst *et al.* showed *reduced* cross-presentation despite being the *strongest* DC-SIGN binder.[24] In a similar approach by Li and Hogervorst *et al.*, a library of mannoside-antigen conjugates was tested for glycan-mediated cross-presentation by the Langerin CTL.[26] Whereas all constructs showed increased binding to Langerin and uptake by dendritic cells, none were more efficiently cross-presented over the control molecule. In a convergent chemo-enzymatic synthesis of $Man_9GlcNAc_2$ glycopeptides, McIntosh *et al.* demonstrated that this modification, when attached at a correct site, leads at best to equal cross-presentation compared to the non-glycosylated control.[23] In an elegant attempt to study the correct site for glycoconjugation to an antigenic peptide, Reintjes *et al.* generated a positional library of C-terminal and N-terminal modified mannose-6-phosphate-, and adjuvant-modified trifunctional glycoconjugates.[25] Again, glycosylation had an inhibitory effect on antigen presentation for all conjugates.

Together, this collection of reports indicates that the design of glycoconjugate peptides as simplified, synthetically accessible, variants of naturally occurring glycosylated biomolecules does not always follow a simple logic of chemical intuition. This calls for an integrative understanding of glycan structure-binding-activity relationships. To obtain meaningful interpretations with potential for therapeutic translation, these studies should ideally be performed on those specific living immune cells that are the key players in antigen presentation biology.

1.1.2 I-type lectins and cis-interactions

I-type lectins bind sialylated glycans through an immunoglobulin-like domain and include the Siglec family which consists of 15 human and 9 murine orthologs.[27] Besides recognizing and internalizing pathogens, Siglecs have cytoplasmic motifs that can engage both in activating and in suppressive immune signaling. For example, binding of sialylated ligands can reduce inflammation by suppressing NF-κB transcription factor activity.[28] Notably, some aspects of Siglec signaling are shaped not only by *trans* ligands on pathogens or neighboring cells but also by *cis* ligands, i.e. sialylated glycans expressed on the same membrane surface.[29,30] These *cis* interactions can mask binding sites, modulate receptor accessibility, and set thresholds for immune activation or suppression.[31–33] Environmental changes in sialic acid expression, enzymatic remodeling, or competitive displacement can unmask specific Siglec domains, enabling *trans* interactions and shifting the balance between tolerance and activation.

Thus far, the effects of*cis*-binding have been studied at a bulk level where cell surface sialylation is perturbed on cells and downstream signaling pathways are subsequently profiled.[29,30,34] While informative from a functional perspective, these approaches do not enable a mechanistic understanding of the dynamic shifting between *cis* and *trans* binding of individual Siglecs and their ligands on the actual cell surface.

1.2 Methods to study glycan–lectin interactions

1.2.1 Immobilization-based techniques

The classical method for defining the binding partners of a lectin is either through immunoprecipitation or via glycan array technology. Glycan arrays consist of microwells or printed glass-plates where in each position a unique glycan or glycoconjugate is immobilized by adsorption or covalent linkage chemistry.[35] After incubation of the array with a fluorophore-labeled lectin, binding between the lectin and glycan in each well can be quantified via fluorescence intensity. This method has been instrumental for global profiling of the lectin binding landscapes, but does not yield quantitative binding information. Recently, cell-based versions of these arrays have also been developed, where instead of printed glycans, the glycosylation machinery of cell lines is systematically modified such that their respective glycocalyx serves as binding partner for the array.[36]

Whether traditional or cell-based, glycan arrays typically provide minimal information on the binding strength of a glycan-lectin interaction, but merely result in binary output. To measure the specific binding kinetics of a glycan, the most widely used method is surface plasmon resonance (SPR). This method relies on recombinantly expressed lectins that are immobilized on a sensor whose refractive index changes upon ligand binding. Although SPR can provide precise dissociation constants (K_D)[8,37], its surface-coupled geometry limits its ability to report on the multivalent, orientation-dependent, and spatially heterogeneous interactions that dominate glycan recognition on cells. As a result, important regulatory mechanisms, such as receptor mobility and nanoscale organization of the lectins are not taken into account. The absence of glycocalyx components on the SPR sensor also means that the potential contribution of and competition between *cis* and *trans* ligands remain inaccessible using this technique.

Other kinetic quantification methodologies, such as enzyme-linked immunosorbent assays (ELISAs)[38] or nuclear magnetic resonance (NMR)[39] based approaches can provide similar information as SPR-based assays but share a reliance on recombinant binding partners that are surface immobilized and therefore lack physiological, cellular context. The approximate K_D as derived from binding partner immobilization-based technologies of several C- and I-type lectins and their least complex ligands is summarized in **Table 1.1**.

Given the limitations of current techniques to understand glycan binding kinetics, a key methodological gap that currently remains is that we lack techniques that can measure glycan–lectin binding with single-molecule sensitivity, in live cells and over relevant timescales. Addressing this gap is essential for linking the molecular features of glycan recognition to the plethora of lectin-mediated immunological outcomes.

Table 1.1: Summarized approximate K_D values from immobilization studies for murine C- and I-type lectins and their least complex ligands that are the topic of this thesis.

Lectin	Ligand type	Assay	K_D (approx.)	Ref.
MR (CD206)	α-methyl-monomannose	Radiolabel	1 - 10 mM	[8,40]
	Mannosylated BSA / glycoproteins	Radiolabel	1 - 100 µM	[8,41]
DC-SIGN / SIGNR1 (CD209)	High-mannose N-glycans ($Man_9GlcNAc_2$)	SPR	1–3 µM	[3]
	Multivalent mannose / fucose	Glycan arrays, ELISA	nM–µM (avidity; >100× monomer)	[42]
Siglec-1	α2,3-sialyllactose	SPR	mM	[43]
Siglec-2 (CD22)	α2,6-sialyllactose	SPR	µM - mM	[43,44]
Siglec-4	α2,3-sialyllactose	SPR	µM	[43]
	Monovalent sialyllactose	SPR	100–300 µM	[45]

1.2.2 PAINT imaging

Point Accumulation in Nanoscale Topography (PAINT) is a microscopy method that was discovered by the unique behavior of the organic lipophilic dye Nile red (**Figure 1.2a**). In close proximity to lipid vesicles, this dye rapidly associates and dissociates with the vesicular membrane leading to an intermittent signal (**Figure 1.2b**) for which the k_{off}^{-1} is approximately 150 ms.[46] By acquiring recordings of this process over time using a conventional wide-field microscope, nanometer scale localizations of these sparse binding events could be determined from the point-spread functions of those blinking signals. As such, reconstruction of these localizations over time into a 2D image generates a super-resolution map of the vesicles.

This technology laid the groundwork for DNA-PAINT imaging where the interaction between lipid membranes and Nile red was replaced by semi-complementary DNA strands of which one is attached to an antibody and the other to a fluorochrome.[47] Besides enabling super-resolution imaging of basically any cellular target, time-resolved imaging now allowed for determination of binding kinetics between the interacting partners.[48–51] This technological advance inspired the use of physiological ligands for PAINT-based imaging such as glycans, peptides or proteins, provided that

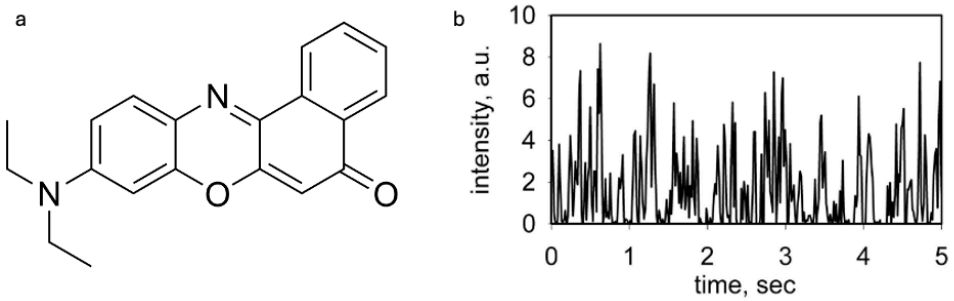

Figure 1.2: Nile red as the first demonstration of the PAINT principle (**a**) Chemical structure of Nile Red. (**b**) The total integrated intensity versus time for a single vesicle probed by Nile red. The intermittency is caused by fluorescence bursts of Nile red molecules undergoing collisions with the lipid bilayer. Figure **b** is reproduced from Figure 1 in Sharonov *et al*.[46] Copyright 2006 National Academy of Sciences.

their binding kinetics are in the correct range.[52–55] A suggested rule of thumb is that the K_D of the interaction must be in the 100 nM - 10 µM range.[56,57] Weaker interactions may lead to too little or too short binding events, whereas stronger binding would make it harder to observe single interactions due to extended binding times.

1.3 Outline of this thesis

In 2021, Riera *et al.* described a PAINT-based method to quantify lectin binding on live cells using their native carbohydrate ligands. This method was dubbed Glyco-PAINT and demonstrated the quantification of on-rates, off-rates and diffusion coefficients for a library of fluorophore labeled mannoside glycans binding to the MR expressed on a CHO cell line (**Figure 1.3**).[55]

In this thesis, the redevelopment of the Glyco-PAINT method by a computational pipeline for automated quantification of glycan binding to live cells is described in **Chapter 2**. This resulted in a Glyco-PAINT-Automated Processing Pipeline (Glyco-PAINT-APP) that resolves averaging artifacts and for the first time enables extraction of single-molecule glycan binding data from primary immune cells. In **Chapter 3**, the Glyco-PAINT-APP is applied to resolve the correlations between mannose binding, antigen cross-presentation, and macrophage polarization with a library of mannosylated model vaccines that are extensively characterized for their biological and immunological functions in dendritic cells and macrophages. In **Chapter 4**, the on-cell kinetics of another lectin class, the Siglecs, are explored. Here, cell surface glycosylation on macrophages is perturbed via two methods to quantify the contribution of *cis* binding glycans to the measured *trans* binding by Glyco-PAINT. **Chapter 5** describes a completely different role of carbohydrates, not as signaling molecules, but

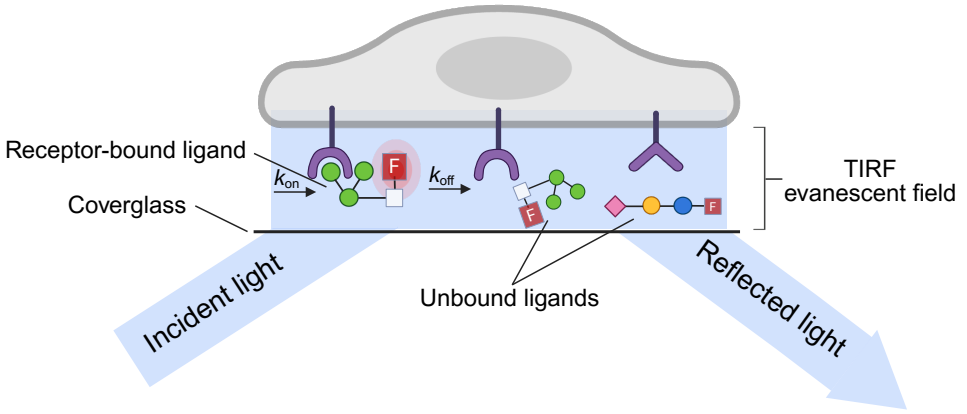

Figure 1.3: Schematic illustration of the principle behind Glyco-PAINT kinetic imaging. Glycan ligands in solution remain dark and undetected whereas ligands that are bound to their receptor are detected by the microscope camera as a blinking signal of which the duration equals the receptor dwell time. From this signal, kinetic and spatial parameters including off-rate, on-rate, diffusion coefficient and receptor clustering can be derived.

as an energy source that is highly partitioned between cancer and immune cells in the tumor-microenvironment. A positional library of alkynyl glucose analogs is screened which leads to the identification of a selective, bioorthogonal, glucose analog that can be multiplexed with an existing glutamine uptake assay. Lastly, in **Chapter 6** an outlook on future receptor–ligand pairs for PAINT studies, a critical reflection on statistical analysis of Glyco-PAINT data, and suggestions for future correlation studies between carbohydrates and immune cells are presented.

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