Fc-conjugated C-type lectin receptors: Tools for understanding host–pathogen interactions

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Funding information
Medical Research Council, Grant/Award Number: MR/N006364/2

Abstract
The use of soluble fusion proteins of pattern recognition receptors (PRRs) used in the detection of exogenous and endogenous ligands has helped resolve the roles of PRRs in the innate immune response to pathogens, how they shape the adaptive immune response, and function in maintaining homeostasis. Using the immunoglobulin (Ig) crystallizable fragment (Fc) domain as a fusion partner, the PRR fusion proteins are soluble, stable, easily purified, have increased affinity due to the Fc homodimerization properties, and consequently have been used in a wide range of applications such as flow cytometry, screening of protein and glycan arrays, and immunofluorescent microscopy. This review will predominantly focus on the recognition of pathogens by the cell membrane-expressed glycan-binding proteins of the C-type lectin receptor (CLR) subgroup of PRRs. PRRs bind to conserved pathogen-associated molecular patterns (PAMPs), such as glycans, usually located within or on the outer surface of the pathogen. Significantly, many glycans structures are identical on both host and pathogen (e.g. the Lewis (Le)X glycan), allowing the use of Fc CLR fusion proteins with known endogenous and/or exogenous ligands as tools to identify pathogen structures that are able to interact with the immune system. Screens of highly purified pathogen-derived cell wall components have enabled identification of many unique PAMP structures recognized by CLRs. This review highlights studies using Fc CLR fusion proteins, with emphasis on the PAMPs found in fungi, bacteria, viruses, and parasites. The structure and unique features of the different CLR families is presented using examples from a broad range of microbes whenever possible.

KEYWORDS
detection, glycans, immunology, microbial-cell interaction, microbial structure

1 Fc CLR STRUCTURES

1.1 Fc domain

For ease of purification and a number of other beneficial factors, which will be discussed below, the C-type lectin receptor’s (CLR’s) ligand-binding domain is generally fused to the hinge region of the human immunoglobulin G1 (IgG1) Fc portion containing the constant heavy (CH) 1 and CH2 domains (Figure 1), which depending on the final application, can have modifications altering the Fc proteins’ in vivo effector functions, for example, reduced activation of complement C1q (P331S), decreased binding to mammalian cell
Fc receptors, or altering the in vivo half-life by changing the pH-dependent affinity of binding to the neonatal receptor FcRn (Boesch et al., 2014; Strohl, 2009).

Producing Fc fusion proteins requires the transfection of the purified plasmid containing the gene of interest into a recipient cell line (See Mayer et al., 2018 for an overview). Numerous mammalian cells lines such as human embryonic kidney (HEK) 239T and Chinese hamster ovary (CHO) cells are routinely used to generate stable or transiently expressed fusion proteins due to their ability to glycosylate, both the Fc portion and the fused ligand-binding domain, although with slight differences between these cell lines which have ramifications for purification and function (Alain et al., 2008; Sheeley et al., 1997). The levels of glycosylation, especially with sialic acid residues not generally common on the Fc portion, can affect the structure and the pl of the fusion protein, furthermore it may impact on its use as a therapeutic agent and on its ability to interact with ligands (e.g. Dendritic cell [DC] immunoreceptor [DCIR] (Bloem et al., 2014) and as reviewed by Alain et al., 2008 and Strohl, 2009). A further consideration is potential glycosylation of the fusion partner itself influencing expression, purification, and function of the fusion protein. Glycosylation of the CLR increases the amount of high molecular weight (HMW) complexes. These complexes can in turn have impact on the function and consequently the accuracy of the Fc CLR-binding properties. HMW proteins in general have less O-linked oligosaccharides, are less sialylated, have more high-mannose N-linked oligosaccharides, and are often non-native in structure impacting on

**FIGURE 1** (a) Cartoon representation of types of Fc CLRs: (a) IgG Fc fusion protein with a N-terminal type II Group V C-type lectin receptor for example Dectin-1 (yellow CRD) with (blue spiral) stalk and two predicted glycosylation sites (green circles), the hlgG Fc CH2 domain (light purple), and CH3 domain (dark purple) showing the glycosylated region (position N297 in unmodified IgG) (green circle), linker hinge region (black lines), and the disulfide bridge (double line). (b) The Mannose receptor Fc fusion protein contains multiple CRD domains (CRD4-7) cloned in tandem. (c) Heterodimeric Fc fusion proteins for example a MCL (green CRD) and Mincle (grey CRD) containing protein could be used to examine function of proteins normally associated in complexes on the cell surface. (d) Pre-complex Fc fusion proteins using a fluorescently tagged (red star) anti-Fc antibody. (e) A modified IgG Fc fusion protein containing the IgM tail piece, microtp (µtp) (red lines), and modified CH2-CH3 domains (yellow ovals) generates multivalent Fc protein complexes (modified from Czajkowsky et al., 2012)
ligand-binding efficiency and precision (Strand et al., 2013), for example Fc DCIR expressed in CHO Lec8 cells, has reduced sialylation and glycosylation levels, resulting in altered ligand-binding properties (Bloem et al., 2013).

The CLR fusion protein is generally expressed with an in frame Ig signal peptide sequence for secretion through the co-translational translocation pathway where the nacent peptide is cleaved as it crosses the ER membrane and the protein is then appropriately folded in the ER lumen. The efficiency of the signal sequence for both high titres and cleavage specificity can vary depending on the combination of cell type expressing the fusion protein and the precise amino acid sequence of the signal (Haryadi et al., 2015). Numerous non-adherent cells expression systems based on HEK293T cells have been optimized for enhanced protein yields and expression in media without serum proteins, which allows for higher purity preparations free from contaminating bovine IgG, and consequently a more accurate assessment of concentration. Purification of the Fc fusion protein requires centrifugation of the culture supernatant containing the secreted soluble protein to remove cellular components, filtration to remove debris, enrichment over a Protein A resin affinity column, a low pH elution step, and re-equilibration in an appropriate buffer (Flanagan et al., 2007; Mayer et al., 2018). A secondary purification step post protein A purification, such as those used to purify alternatively tagged (e.g. His or Flag) versions of fusion protein, further improves purity (Rodrigues et al., 2020).

The necessity for multimeric structures for full function of CLR must be considered when designing screening strategies using FC fusion proteins. If the interaction between the PRR and the glycan is of low affinity, a property shared by many of the CLRs, FC fusion structures with multiple CRDs will have a higher binding affinity (Mnich et al., 2020) for example the Macrophage Mannose Receptor (MMR) Fc contains four ligand-binding domains (CRD4-7) (Figure 1). Multivalent Fc fusion complexes with increased affinity can be prepared in numerous ways; coated on protein-A/G microbeads, engineered as hexameric Fc platforms based on the IgM-T cell microtrop (µtp) sequence, or the use of secondary antibodies recognizing the Fc portion to pre-complex the proteins (Cao et al., 2009; Czajkowsky et al., 2012). Multivalent complexes would more closely mimic the cellular context of the CLR where often multiple CRDs from the same receptor are simultaneously present, such as the tetrameric DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) receptor complexes located in lipid raft clusters, simultaneously binds multiple sites on ligands, thereby increasing affinity. Screening pathogenic ligands displayed at a high glycan density can improve Fc CLR affinity and binding kinetics, either due to the increased glycan length allowing some ligand slippage but maintaining overall binding, or if multiple CRDs are present then switching of the ligand between the various CRDs may occur maintaining the overall association (Mnich et al., 2020). The structure of the CLR ligand-binding domain(s) and how they interact with their ligands (viral, bacterial, fungal and parasite) will be discussed more fully below.

1.2 | The CLR-binding domain

A diverse range of ligand-binding domains have been utilized as Fc fusion proteins to probe pathogens and detect endogenous ligands. This review will discuss one of the major types of glycan-binding domains, the C-type lectins, grouped according to their distinct carbohydrate recognition domain (CRD), which generally binds in a Ca2+-dependent manner. The other three major glycan-binding domain structures, the Siglecs with an immunoglobulin fold, galectins with a β sandwich fold, and those with a Ricin (R)-type-binding domain, will not be addressed (see Taylor & Drickamer, 2019 for a review of mammalian glycan receptors). There are comprehensive online resources which describe and annotate lectins from a variety of kingdoms: plant, viral, fungal, algal, amphibian, bacterial, and mammalian (https://www.unilectin.eu/unilectin3D/search) (Bonnardel et al., 2018).

1.2.1 | C-type lectin receptors

The CLRs are a large superfamily of proteins that have one or more carbohydrate recognition domain (CRD) binding in a Ca2+-dependent manner, the C-type lectin domain. This superfamily of receptors has 17 groups (Group I–XVII) based on their domain organization and phylogeny (see Brown et al., 2018; Taylor & Drickamer, 2019 for recent reviews and https://www.imperial.ac.uk/research/animallectins/ctld/mammals/humanmousedata.html for a database of human and mouse proteins.) The CLRs’ roles in modulation of the immune response, shaped by their expression, cellular context, and signaling capacity, are covered by numerous reviews (Brown et al., 2018; Brubaker et al., 2015; Cao, 2016; Iborra & Sancho, 2015).

The secondary structure of the CLR CRD is maintained through six conserved cysteine residues forming disulfide bonds, creating a hydrophobic core. Conserved amino acid motifs such as EPX (Glu-Pro-X = Asn, Ser or Asp) found in DC-SIGN, Langerin (all Group II), and the MR (group VI) preferentially allow binding to glycans with equatorial 3- and 4-hydroxyl groups such as mannose, fucose, glucose, and N-acetyl-D-glucosamine (GlcNAc) residues. CRDs with a QPD (Gln-Pro-Asp) motif, such as human mannose galactose lectin (MGL) (Group II), preferentially bind glycans with axial 4-hydroxyl groups such as galactose and N-acetyl-galactosamine (GalNAc) residues (See Foster et al., 2016; Mnich et al., 2020 and for reviews). High Ca2+ concentrations are important for glycan binding by receptors with multiple Ca2+-binding sites, such as SIGNR1 (Group II), while receptors containing only one Ca2+-binding site (e.g. DCIR-2) have a basic amino acid present in the secondary site (Drickamer & Taylor, 2015). Not all CRDs bind their ligands in a Ca2+-dependent manner and are termed the C-type Lectin-like domains (CTLD) an example of which is Dectin-1 (Group V) (See Table 1). Some CLRs have additional cysteine residues in the stalk region, located between the transmembrane (TM) region and the CRD, which are necessary for homo-dimerization (e.g. Lox-1), or a positively charged residue, e.g. Arg in Dectin-2, within the TM facilitating hetero-dimerization.
<table>
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<tr>
<th>PRR (aliases)</th>
<th>Pathogen</th>
<th>PRR ligand(s)</th>
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<td>Terminal gal⁺ gluc⁺ GlcNAc⁺ PIM₂⁺ PIM₃⁺</td>
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**Group II CLRs**

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<td>Viral</td>
<td>Terminal GalNAc</td>
<td>Terminal Gal</td>
<td>Tn-antigen</td>
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<td>mMGL2</td>
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<tr>
<td>ASGPR</td>
<td>Hepatitis E virus</td>
<td>Gal</td>
<td></td>
<td></td>
<td>Singh et al. (2009)</td>
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<td>Group VI CLR</td>
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<tr>
<td>Mannose receptor (MR)</td>
<td>Candida spp., S. cerevisiae</td>
<td>High man</td>
<td>Sulfated sugars</td>
<td>Terminal man</td>
<td>Fusion</td>
<td>Zamze et al. (2002); Linehan et al. (2003); McGreal et al. (2006); Chen et al. (2008); Miller et al. (2008); Hsu et al. (2009); Lee et al. (2011); Vendele et al. (2020)</td>
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**Abbreviations:** Ac, acylated; CPS, capsular polysaccharide; DHN, 1,8-dihydroxynaphthalene; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GAS, group A Streptococcus; GBS, group B Streptococcus; Glc, glucose; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; GLPS-F3, Medicinal fungus Ganoderma lucidum polysaccharide extract; GXM, glucuronoxylomannans; H antigen, Galβ4GalNAcβ3; HIV-1, Human immunodeficiency virus type 1; HMW, high molecular weight; HSV, Herpes simplex virus; IPM2: inositol-monophosphate dimannose; Lac, lactose Galβ1-4Glc; LacdiNAc, Galβ1-4GlcNAcβ3; LacNAc, Galβ1-4GlcNAc; LDN, GalNAcβ1-4GlcNAc; LDNF, fucosylated LDN [GalNAcβ1-4(Fucα1-3)GlcNAc]; Lewis (Le)<sup>x</sup>, Galβ1-3(Fucα1-4)GlcNAc; Le<sup>x</sup>, Fucα1-2Galβ1-4(Fucα1-3)GlcNAc; LOS, lipo oligosaccharide; Man, mannose; Man3, α1-3,α1-6-mannotriose; ManLam, mannose-capped lipooligosaccharide; MurNAc, N-acetylmuramic acid; NeuAc, N-acetylneuraminic acid or sialic acid; PIM, phosphatidyl-myoinositol mannosides; PRRSV, porcine reproductive and respiratory syndrome virus; SARS-CoV, severe acute respiratory syndrome coronavirus; sialyl Le<sup>x</sup>, NeuAcα2-3Galβ1-3(Fucα1-4)GlcNAc; sialyl Le<sup>x</sup>, NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc; TDB, trehalose-6,6′-dibehenate; TDM, trehalose-6,6′-dimycolate; Tn, GalNAc α-O-linked to serine or threonine;

<sup>3</sup> Fc staining detected but no cell membrane expressed receptor binding.
<sup>4</sup> As identified by cell membrane expressed receptor binding assays.
<sup>4</sup> Moderate to weak binding.
<sup>4</sup> Using bacterial expressed CLR.
with the signaling molecule FcRgamma (Ohki et al., 2005; Sato et al., 2006). The presence of these residues and any glycosylation sites, within the extracellular regions, can impact on the expression and function of the glycan-binding Fc fusion proteins (see below). A brief overview of selected Group II, V, and VI receptors is given highlighting their use as Fc CLR proteins.

**The Group V CLRs**

The Group V CLRs are type II proteins, with a single extracellular CTLD C-terminal domain generally separated from the membrane by a stalk/neck linking the TM region to the N-terminal intracellular region. Members of the Dectin-1 sub-cluster, include Lox-1, MelLec, CLEC12A, CLEC12B, CLEC-2, and CLEC9A (See Tone et al., 2019 for a recent review). These receptors as well as a further Group V member, CLEC5A which associates with the signaling molecule DAP12, have been expressed as fusion proteins to probe pathogens and their use as Fc CLRs are discussed in more detail (See Foster et al., 2016 for a review and see Table 1). The majority of these CLRs are cloned such that their C-terminal end is fused to the Fc region (e.g. the Fc Dectin-1 fusion protein includes the stalk region (Graham et al., 2006)) (See Figure 1). This allows two CRDs to be simultaneously presented as a dimeric complex for ligand binding, which is not always sufficient to detect low-affinity binding without further pre complexing, for example Fc Lox-1 (Cao et al., 2009).

**Dectin-1.** The β-glucan-binding receptor Dectin-1, with high specificity for β 1,3-linked glucans in a Ca²⁺ independent manner, was first identified in a screen for receptors binding to the Saccharomyces cerevisiae-derived particle zymosan. The receptor has been used as an Fc fusion screening tool in numerous studies involving fungi, bacteria, parasites, and endogenous ligands (Table 1). The receptor is widely expressed as a monomer on a range of cell types and becomes dimerized to induce appropriate downstream signals (see Patin et al., 2019 for a review). The detection of pM concentrations of soluble β-glucan can be achieved using Fc Dectin-1 with the sensitivity of detection influenced by the glucan backbone chain length and degree of β-1,6-linked side chains present. The use of arrays based on a wide range of purified carbohydrates, oligosaccharide probes, and neoglycolipids (NGL) defined the narrow glycan ligand repertoire of Dectin-1 to β-1,3-linked glucans with a minimum of unit length of 11 units (see Li & Feizi, 2018 for a review of these assays). Despite the specificity for β-1, 3 glucans, unidentified PAMPs and some endogenous proteins interact with Dectin-1 via the same binding site. Examples of these PAMPs are demonstrated by Fc Dectin-1-binding Nontypeable Haemophilus influenzae (NTHi) strain 2019 and live M. tuberculosis in a β-glucan inhabitable manner, yet neither pathogen contains β-1,3-glucans (Cheng et al., 2017; Heyl et al., 2014; Rothfuchs et al., 2007). The endogenous ligands such as the proteins, vimentin, and galectin-9 are recognized by Dectin-1 in a β-glucan inhabitable manner (Daley et al., 2017; Thiagarajan et al., 2013). A second ligand-binding site on Dectin-1 exists and is able to interact with the endogenous ligands IgG and Annexins in a β-glucan independent manner. Dectin-1 binds IgG at a core fucose linked to an N-terminal asparagine with an adjacent aromatic amino acid, extending the range of known ligand structures (Bode et al., 2019; Manabe et al., 2019).

Fungi are a rich source of β-glucan (see below and Erwig & Gow, 2016 for a review) consequently Fc Dectin-1, due to its high affinity for β 1,3 glucan, has been used to probe the fungal cell wall (e.g. Candida albicans [See Figure 2]) for alterations in exposure due to growth conditions, by flow cytometry, EM, and immunofluorescent microscopy (Lenardon et al., 2020; Pradhan et al., 2019; Vendele et al., 2020). Comparisons of Fc Dectin-1 binding to a variety of Candida sp. show the following trends in β-glucan exposure with binding to C. glabrata < C. tropicalis equivalent to C. parapsilosis < C. albicans, when all cultured in a similar manner (Thompson, Griffiths, et al., 2019). Detection of exposed β-glucan in fungi such as A. fumigatus and P. carinii sp. Muris has been exploited as an antifungal therapy in murine infection models, and in vitro assays binding of Fc Dectin-1 prevents Coccidioides posadasii from eliciting a pro-inflammatory response (Mattila et al., 2008; Del Pilar Jimenez et al., 2008) (See Section 2).

**Lox-1.** Lox-1, a member of the scavenger receptor family, is a high affinity ligand–receptor for oxidized low-density lipoprotein (oxLDL) due to its oligomerization state at the cell surface. On the cell membrane, it forms a functional homodimer via disulfide bonds and through non-covalent interactions of the neck domain. These dimeric properties have impact on the function of the Fc fusion protein (Cao et al., 2009). The positively charged terminal CRD region, which when dimerized forms a basic spine, is essential for ligand binding. A variety of negatively charged ligands, such as oxLDL, anionic structures (polyinosinic acid and carrageenan), bacteria, anionic phospholipids (phosphatidylerine and phosphatidylinositol), and proteins such as Heat shock protein (HSP) 70 and C-reactive protein are bound by Lox-1 (See Foster et al., 2016 for a review). Fully functional Fc fusion proteins of human Lox-1 require oligomerization, either through polyclonal antibody complexing of the Fc portion of the single CRD fusion protein (Figure 1) or enhanced by the cross linking of multimeric CRD fusion proteins with two tandem CRDs separated by a short linker (Cao et al., 2009). There are limited data available for Fc Lox-1 fusion proteins as tools for binding microbial pathogens, potentially due to the need for pre-complexing; however, in vitro cell-expressed receptors have shown interactions with S. aureus and E. coli (Shimaoka et al., 2001). Significantly, using a double CRD Lox-1 fc fusion protein, anionic oligoglycerol dendronized sulfates targeting the dimeric basic spine region were detected, and these compounds inhibit binding to E.coli, an endogenous ligand on red blood cells, and to oxLDL (Kumari et al., 2019).

**CLEC-2.** CLEC-2 (CLEC1B) is a platelet-expressed Ca²⁺ independent binding homodimeric C-type lectin receptor. Its ligands are the non-glycosylated snake venom toxin rhodocytin/aggrein, the endogenous protein podoplanin, and fucoidan a seaweed-derived sulfated polysaccharide (See Rayes et al., 2019; Suzuki-Imoue et al., 2011 for reviews). CLEC-2 binds to a core O-glycan (NeuAca2-
3Galβ1-3[NeuAcα2-6]GalNAc1) linked to either a threonine or serine, preceded with a EDXX motif on podoplanin (Nagae et al., 2014). Its role in HIV uptake, possibly through binding a cellular factor incorporated in the viral envelope, and induction of the release of extracellular vesicles to both Dengue virus (DV) and type A influenza virus H5N1 are less well understood (see Recognition of viral PAMPs) (Chaipan et al., 2006; Nagae et al., 2014). CLEC-2, when cell membrane expressed, may require other CLRs such as DC-SIGN for full function as it binds ligands (e.g. DV) with low affinity (Sung et al., 2019). The Fc CLEC-2 protein would need to be pre-complexed or expressed as a heterodimeric receptor to fully explore its PRR ligand-binding functions (see Figure 1). Nevertheless, a

**FIGURE 2** Legend on next page
homodimeric Fc CLEC-2 used in two glycan array screens bound a limited range of glycans some of which are present on both host and pathogen surfaces (see Table 1). Binding of Fc CLEC2 to agarose-limited range of glycans some of which are present on both host and homodimeric Fc CLEC-2 used in two glycan array screens bound a unique site. A number of critical arginine residues in these sites were identified using mutated Fc CLEC-2 Fc-fusion proteins in surface plasmon resonance (SPR) assays. A large-scale slot blot method using an Fc CLEC-2 fusion protein was validated using small molecule inhibitors, such as hematoporphin, which disrupt the binding to cellular expressed podoplanin. This Fc CLEC2 screen demonstrated that therapeutic inhibitor compounds targeting protein–protein interactions, such as those involved in cancer and thrombosis could be identified (Rayes et al., 2019; Watanabe et al., 2019).

CLEC12A and CLEC12B. These two receptors are understudied in the context of glycan binding, but have been used as Fc-fusion proteins in the screening of microbial ligands often used as baseline binding control proteins due to their limited interactions with pathogens detected to date (Mayer et al., 2018; Stappers et al., 2018). CLEC12A (myeloid inhibitory C-type lectin MCL, DCAL-2, KLRL-1, and CLL) and CLEC12B (Mah) are both inhibitory receptors, due to the presence of immunoreceptor tyrosine inhibitory motifs within their cytoplasmic signaling tail. CLEC12A plays a role in homeostasis, as it binds dead cells and uric acid crystals, and limits inflammation in rheumatoid arthritis (Neumann et al., 2014; Redelinguys et al., 2016). In microarray screens mild-to-weak binding for both Fc CLEC12A and Fc CLEC12B was observed to a limit range of glycans (see Table 1 for a list of these glycan structures). More recently CLEC12A has been found to bind hemozoin, an insoluble crystal derived from Plasmodium (Rauff et al., 2019), again indicating that crystal structures could be recognized by CLEC12A. Interestingly, Fc CLEC12A has unidentified ligand(s) on both T. gondii oocytes and C. jejuni (Fabian et al., 2021; Mayer et al., 2018).

CLEC1 (MelLec) and CLEC9A (DNGR). MelLec (CLEC1a) has been recently identified as the first CLR able to bind melanin, recognizing the naphthalene-diol unit of 1,8-dihydroxynaphthalene (DHN) synthesized by A. fumigatus (See Figure 2) but not melanin synthesized from fungi using the alternative L-Dopa pathway (Stappers et al., 2018). The Fc fusion protein binds to related structures by ELISA and has been used for fluorescent microscopy and flow cytometry assays probing melanin content of fungi. The heptaketide naphthopyrone YWA1, an intermediate of the melanin biosynthesis pathway, was able to block binding of the Fc MelLec to conidial spores (Stappers et al., 2018). No other MelLec microbial ligands have been identified to date, but further structures may still be identified using complexed Fc fusion proteins to enhance binding affinity. Significantly, a human MelLec fusion protein bound with high affinity to a mammalian histidine-rich glycoprotein (HRG), but the nature of the interaction is unresolved (Gao et al., 2020).

DNGR (CLEC9a), a dimeric receptor, is able to recognize the DAMP ligand F-actin. An F-actin and myosin II complex binds with higher affinity, whereas purified myosin II does not bind at all (Schulz et al., 2018). Limited evidence of DNGR-binding microbial ligands exists with M. tuberculosis Beijing strain, M. bovis BCG and T. gondii oocytes recognized by the Fc protein (Table 1).

CLECSa (MDL-1). CLECSa (or Myeloid DAP12-associating lectin, MDL) binds Dengue virus, Japanese encephalitis virus, and type A influenza virus via mannose and fucose residues on the viral envelope proteins (Table 1). The dimeric C-type lectin CLECSA associates with the signaling molecules DAP12 and DAP10 and can form a complex with DC-SIGN when engaged with viral ligands. Blocking of CLECSA attenuates these viral diseases by inhibiting downstream signaling and inflammation (see Brown et al., 2018 for a review). Unlike DC-SIGN and MR, which bind to Dengue virus in a Ca$^{2+}$-manner, CLECSA binds via a fucose moiety in a Ca$^{2+}$-independent manner, and is a much lower affinity receptor (See Sung et al., 2020).
for a review). Further CLEC5A ligands, identified using Fc fusion proteins to screen bacterial glycan arrays derived from *S. pneumoniae*, *P. aeruginosa*, *S. flexneri*, and *E.coli*, were identified (Table 1). CLEC5A binds N-acetylglucosamine (GlcNAC), N-acetylmuramic acid (MurNAc) disaccharides, and the *L. monocytogenes* cell wall teichoic acid (TA) (Figure 2), regardless of the terminal sugar moiety. As a cell membrane expressed receptor CLEC5A's binding to *L. monocytogenes* is enhanced when the receptor is complexed with TLR2 (Chen et al., 2017). CLEC5A is further an example of a CLR's full binding function dependent on the presence other receptors.

**The Group II CLRs**

The Group II family of C-type lectin receptors are similar in structure to the Group V cluster, they are type II proteins with a TM region, stalk, and a single C-type lectin-like domain, and have a short cytoplasmic tail. The Dectin-2 sub cluster of receptors: blood DC antigen 2 (BDCA-2), DC immunoactivating receptor (DCAR), DC immunoreceptor (DCIR), Macroage C-type lectin (MCL), and Mincle; the DC-SIGN-related molecules: DC-SIGN, DC-SIGNL, DC-SIGN-related protein (SIGNR)1 and SIGNR3 and further Group II CLRs with PRR glycan-binding properties discussed are Langerin, CD23, Asialoglycoprotein receptor (ASGPR), and macrophage galactose lectin (MGL) (See Table 1 and Dambuza & Brown, 2015; Drickamer & Taylor, 2015; Foster et al., 2016 for reviews).

**Mincle, Dectin-2, and MCL.** There is an interdependent relationship between these three myeloid expressed receptors with their surface expression and signaling reliant on the presence of the signaling γ subunit of the immunoglobulin Fc receptor (FcγR) and each other for a full immune response. This co-dependent regulation of function highlights their combined role in the coordinated sensing and responding to a wide range of pathogens, in particular to mycobacteria and fungi (see Table 1 for PAMP ligands). The mycobacterial cell wall glycolipid TDM binds to Mincle with higher affinity than MCL, with the former having a CRD EPN motif and the latter an unusual EPD motif. These motifs are essential for their binding functions as determined using Fc fusion proteins of the human Mincle and human MCL to recognize TDM (Furukawa et al., 2013). TDM inhibits binding of Fc Mincle to a proteinaceous ligand from a *L. major* soluble extract (SLA) found in all parasitic stages (Iborra et al., 2016). Apart from endogenous DAMPs such as SAP130, further microbial-derived ligands detected by Fc Mincle fusion proteins are *S. pneumoniae*-derived α-glucosyl-diacylglycerol (αGlcDGAG), glycerol monomycolate (for human Mincle only) and β-gentiobiosyl diacylglycerides from mycobacteria, and a fungal ligand from the *Malassezia* sp. (see Table 1). Verification of Mincle fungal binding is required, due to the contradictory evidence of Mincle binding as an Fc fusion protein, but the receptor is unable to bind when cell membrane is expressed (Patin et al., 2017). The effective removal of any contaminating host proteins, such as viral host proteins, when screening for ligands is required as many Fc PRRs, including Mincle, bind released DAMPs. Taking this into consideration using a highly pure La Crosse virus (LACV) preparation, with host cell-derived proteins and DNA removed, significantly binding by Fc Mincle, Fc Dectin-2, and FcDectin-1 could be detected (Monteiro et al., 2019).

Dectin-2, due to its EPN motif, recognizes terminal α-1,2-mannose in fungi such as *C. albicans*, internal Manα1-2Man in mannan, and other polysaccharides, mannos capped LAM from *M. tuberculosis* and mannosylated O-antigens with a terminal mannosone residue found in *M. furfur* and *M. sympodialis*. (Table 1). Dectin-2, as demonstrated using a biotin-tagged tetrameric protein, binds to structures with mannosone α1-2 and α1,4-linked disaccharides, with higher affinity relative to mannosone, due to the reducing end mannosone occupying the primary binding site and the non-reducing terminal mannosone residue binding in an extended binding site (Feinberg et al., 2017). Other ligands identified using Fc Dectin-2 are the endogenous N-glycosylated ligand β-glucuronidase, requiring the intact EPN motif (Mori et al., 2017), the *Blastomyces dermatitidis* glycoprotein Eng2, the mannoprotein (MP98) from acapsular *C. neoformans* Cap67, and bacterial capsular polysaccharides from *S. pneumoniae* (Table 1).

MCL (Dectin-3, CLECFS8, CLEC4D) has a role in the immune response to mycobacteria with knockout mice displaying a phenotype and Fc MCL binding the cell wall-derived TDM (Furukawa et al., 2013; Miyake et al., 2015; Wilson et al., 2015). Fc MCL binds to *C. neoformans* serotype AD and *C. gattii* serotype B, interacting with the cell wall glucuronoxymannolins (GMX) (Figure 2), furthermore a role for the cell membrane expressed receptor has been demonstrated (Huang et al., 2018). No binding by Fc MCL was detected on a range of glycan arrays (Graham et al., 2012; Maglino et al., 2014) and although a MCL bacterial recombinant protein recognizes α-mannans on *C. albicans* hyphae, the role for this receptor in anti *C. albicans* immunity in vivo is contradictory (Wilson et al., 2015; Zhu et al., 2013).

**Langerin & BDCA-2.** Langerin and BDCA-2 are receptors with restricted expression profiles expressed in Langerhans cells and in human plasmacytoid DCs, respectively. Both contain the EPN motif required for binding mannosone, glucose, or GlcNAC. While very little is known about the oligomeric state of BDCA-2, Langerin is a trimeric CLR, due to the coiled neck region, with a distinct glycan-binding pattern mostly resolved using bacterial expressed tagged proteins (Drickamer & Taylor, 2015). The most recent study has demonstrated binding to bifunctional mannosylated oligosaccharides when presented in increasing copy number on a peptide scaffold (Li et al., 2020). Bacterial glycan arrays of LPS O-chains, polysaccharides, CPS and LPS, and *M. tuberculosis* structures have been assessed and ligands identified (see Campanero-Rhodes et al. (2019) for a review and Table 1). Mannose and β-glucan structures from a broad range of fungi, *Candida* sp., *M. furfur*, and Saccharomyces, but not Cryptococcus, are recognized and bound by both soluble and cell membrane-expressed Langerin (de Jong et al., 2010). A few studies have compared Langerin Fc-fusion protein binding with other Fc PRRs (e.g. Galustian et al., 2004; Hsu et al., 2009; Lee et al., 2011). These studies show that Langerin specifically binds a
6′-sulfated galactose found in sulfated LeX-related sequences, high mannose, GlcNAcβGal/GalNAc, and fucose-containing antigens, but in contrast to the de Jong et al. (2010) study no binding, in a β-glucan-dependent manner, was detected. Binding to live fungi and to a glycan microarray, using pre-complexed Fc fusion protein, demonstrated that specific binding to 6′-sulfo-LacNAc, but not to the isomer 6-sulfo-LacNAc or the unsulfated form of Galβ1-4GlcNAc, occurs in the same binding site for mannosylated glycans (Tateno et al., 2010). Pre-complexing Fc Langerin prior to ELISA-enhanced binding tenfold when compared with a dimeric HIS tagged version demonstrates the importance of presenting the receptor in a multimeric form for ligand binding assays (Lee et al., 2011).

The human receptor BDCA-2, which is most closely related to DCIR, binds HIV gp120 and HCV (Hepatitis C virus) glycoprotein E2, but these interactions were discovered using cell expressed receptors (see Kerscher et al., 2013 for a review). Although the BDCA-2 CRD contains the EPN motif a soluble bacterial expressed receptor bound glycans with galactose residues at the non-reducing ends, with the trisaccharide Galβ1-3/4GlcNAcβ1-2Man, often found in mammalian IgGs, as the defined ligand. Similar to DCIR2, extended binding sites are present; the mannose residue binds in the Ca2+-primary-binding site with GlcNAc and galactose residues binding in an adjacent wide shallow groove, necessary for higher affinity binding (Jégouzo et al., 2015). An Fc BDCA-2 protein bound sulfated LacNAc, biotinylated N-glycans, and the Galβ4GlcNAcβ3-H antigen in a CFG glycan array, indicating interactions occur with heavily N-glycosylated cell surface proteins (Hsu et al., 2009). Purified preparations of the soluble HCV glycoprotein E2 binds, in a Ca2+-dependent manner, to both cell membrane expressed DCIR and BDCA-2 (Florentin et al., 2012), indicating that viral PAMPs and possibly other pathogens may express ligands for these receptors and their roles as PRRs are understudied.

**DC-SIGN, L-SIGN, SIGN-R1, and SIGNR3.** The related human receptors DC-SIGN and DC-SIGNR (liver/lymph node-specific ICAM-3 grabbing non-integrin (L-SIGN)) share 77% sequence identity; both have the sugar-binding EPN-motif, but have different cell expression profiles, myeloid and sinusoidal and endothelial cell, respectively. They have distinct functions as DC-SIGN is an endocytic receptor which releases its ligand at low pH and DC-SIGNR is an adhesion receptor (Guo et al., 2004). Both receptors have an extended neck region, which contains seven repeats of highly conserved stretches of amino acids, facilitating homo-oligomerization necessary for high avidity binding to the high mannosylated HIV gp120. In contrast, binding to their endogenous ligand ICAM-3 is of low affinity and independent of oligomerization state (Snyder et al., 2005). The DC-SIGN tandem-neck repeat region is constant in size, whereas the numerous isoforms of DC-SIGNR vary in neck length with consequences for function (Khoo et al., 2008). The full length DC-SIGNR’s neck regions allow the CRDs to be more closely positioned than the DC-SIGN CRDs, when these receptors are membrane expressed. This impacts on the surface area available to interact with ligand barring molecules and pathogens (Feinberg et al., 2021).

The complexed structure of these receptors, in particular DC-SIGN, impacts on their function as soluble glycans-binding molecules. When an interacting ligand contains appropriately distanced glycans, the HMW complexes bind with higher affinity than monomeric structures (Mitchell et al., 2001). In contrast, however, the significantly enhanced binding of a Man9GlcNAc2 oligosaccharide over mannone by both receptors is not facilitated by oligomeric CRD structures implying that multiple binding sites on each individual CRD are needed to interact with the oligosaccharide. Many of these binding sites would not be exposed when the CRDs are clustered into HMW complexes. In general, binding profiles of either DC-SIGN or DC-SIGNR when used as pre-complexed Fc proteins, dimeric Fc protein or bacterial expressed proteins are comparable, with some differences in binding to low-affinity ligands observed (Achilli et al., 2020; Geissner et al., 2019; Tateno et al., 2008).

Fc fusion proteins of DC-SIGN and DC-SIGNR (see Lee et al., 2011 for an example of direct comparisons) have been utilized to resolve function and define ligand-binding profiles in numerous screens using glycan arrays, microbial arrays, and whole pathogen binding assays (Table 1). Bacterial expressed and Fc fusion proteins of these two receptors were used to demonstrate that they bind preferentially to mannone over glucose and GlcNAc, with DC-SIGNR showing greater affinity for mannone than fucose, and it has a higher affinity than DC-SIGN for GlcNAc. The higher affinity for L-fucose than mannone is due to the presence of a Val351 in the primary-binding site of DC-SIGN. This residue interacts with fucose necessary for binding to the LeX and LDNF glycans on the soluble egg antigen (SEA) from S. mansoni (Figure 2), but not for binding of HIVgp120 (Guo et al., 2004; van Die et al., 2003).

Fc DC-SIGN binds a range of α1-3 and α1-4 fucosylated tri- and tetrasaccharides, mannone type N-glycans carrying 5–9 mannose residues, but not α1-6 core fucosylated Man3GlcNAc2, nor ligands with a bulky charged sialic acid residues (Table 1). A soluble non-complexed DC-SIGN binds α1,2-mannose only at the non-reductive end of mannone side chains from *Candida* sp. and does not bind to a mn2 mutant *S. cerevisiae* lacking α1,2-mannose (Takahara et al., 2012). Glycans bound by Fc DC-SIGN in *C. albicans* are localized to the plasma membrane, inner cell wall, and outer fibrils (Vendele et al., 2020), whereas Fc DC-SIGNR binds heat killed *C. albicans*, implying the ligand is not usually surface exposed (Achilli et al., 2020).

Both DC-SIGN and DC-SIGNR, although the latter not confirmed using Fc fusion proteins, bind cyclic β1,2-glucan, derived from *B. abortus* (Li & Feizi, 2018; Zhang, Palma, et al., 2016), furthermore Fc DC-SIGN is able to bind a broad range of short-chain glucose linkages, such as those containing α1,4- linked glucans and the β 1,2-linked glucans (Palma et al., 2015) (see Li & Feizi, 2018 for a review of binding assessed by NGL arrays). Binding of DC-SIGN to HIVgp120 but not to the endogenous ligands ICAM-2 and -3 is inhibited by the α1,4-, β1,4-, and β 1,3- disaccharide-linked glucans confirming a different binding site for the endogenous ligand (Su
et al., 2004). Apart from DC-SIGN binding to viral proteins, the Fc fusion protein has been used to bind Le^X structures found on bacteria, such as *H. pylori*, ManLam from *M. tuberculosis*, ManNAc-terminating oligosaccharides from *S. pneumoniae*, LPS core glycans with terminal L-obligo-D-mannoheptose moieties, and binds the protozoa *L. mexicana* mannose capped lipophosphoglycan (Table 1 & Figure 2).

There are eight mouse homologs of human DC-SIGN, of which SIGNR1 and SIGNR3 are the most extensively studied as glycans-binding Fc CLRs, with subtle differences in ligand-binding profiles detected (Galustian et al., 2004; Lee et al., 2011) (See Table 1). Membrane-expressed SIGNR1 binds the capsular polysaccharide (CPS) of *S. pneumoniae*, *E. coli*, and *S. typhimurium* and binds endogenous ligands, for example α-2,6 sialylated proteins such as the common ligands, for example β chains and binds the exposed ManNAc- M. tuberculosis, ManLam from bacteria, such as Membrane-expressed SIGNR1 binds the capsular polysaccharide detected (Galustian et al., 2004; Lee et al., 2011) (See Table 1). Binding Fc CLRs, with subtle differences in ligand-binding profiles protozoa with terminal L-glycero-D-mannoheptose moieties, and binds the terminating oligosaccharides from *S. pneumoniae*, LPS core glycans (Figure 2).

hcp fusion protein has been used to bind Le^X structures found on circulation. It binds terminal galactose and binds to desialylated glycoproteins and targets them for removal from yet has higher affinity for glycosphingolipids. Both isoforms equally bind GalNAc and sialyl-Tn antigens (Marcelo et al., 2019). Bacterial lipooligosaccharide (LOS) from *N. gonorrhoea*, *C. jejuni*, and *E. coli* are bound by Fc hMGL (See Table 1), but the full range of splice variants have not been fully examined as PRRs. The murine Fc MGL1 binds the Le^X and Le^α antigens, 1,3-lactosamine, and tumor antigens. The Siglec-1 receptor (Sialoadhesin), influenza virus, *T. cruzi* antigen, heat killed Streptococcus sp., and *E. coli* are all ligands; however, these interactions were not performed using Fc fusion proteins (See Denda-Nagai & Irimura, 2016; van Kooyk et al., 2015 for reviews).

**DCIR and DCAR.** There is one single human C-type lectin DC immunoreceptor (DCIR) (CLEC4a) and two distantly related murine proteins with inhibitory cytoplasmic tails: DCIR-1 and -2. Mice express three further related proteins DCIR-3, DCIR-4, both lacking the signaling motif, and a further CLR which associates with FcRγ, the DC immunoactivating receptor (DCAR) also known as DCAR2 or CLEC4B1 (See Toyonaga and Yamasaki (2020) for a review & Table 1). The CRDs of hDCIR, mDCIR1, -3, -4, and DCAR contain an atypical EPS motif, while DCIR2 has a typical EPN motif influencing their ligand binding profiles, although other further elements within the CRDs contribute to specificity and affinity (Lee et al., 2011; Nagae et al., 2016; Omahdi et al., 2020).

hDCIR acts as an attachment factor for HIV, binding via its neck domain containing 23 amino acid tandem repeats; however this binding was not demonstrated using Fc fusion proteins (Lambert et al., 2008). Fc DCIR binds Le^δ, sulfo-Le^δ, Le^α, and Man3 (Figure 2), although with much lower affinity than Fc DC-SIGN and unlike DC-SIGN does not bind *C. albicans*. Interestingly, deglycosylation of the Fc DCIR protein or expression of the fusion protein in CHO Lec8 cells increases ligand affinity, including an enhanced affinity for HIVgp140 over HIVgp120, possibly due to the N-glycosylation site within the CRD affecting binding (Bloom et al., 2013, 2014). Glycan array analysis demonstrated that FcDCIR binds to sulfated LacNAc, Lac, bi-antennary N-glycans, and an O-2 core xylosylated bi-antennary N-glycan, although these studies used glycosylated version of the Fc fusion protein (Brzezicka et al., 2016; Hsu et al., 2009).

*M. tuberculosis* Ac1PIM2 is a DCAR ligand, with the Fc fusion protein binding with higher affinity than either Fc Dectin-2 or Fc Mincle, a mixed linked α-4,6 glucose polymer, is another ligand (See D’Souza & Devarajan, 2015 for a review). Only a few pathogens have been examined for binding to the ASGPR, but an Fc ASGPR fusion protein is able to bind to and inhibit Hepatitis E virus infection, by interacting with the capsid protein (ORF2) (Zhang, Tian, et al., 2016) and assays using cell membrane-expressed receptors have demonstrated binding to Hepatitis A, B, and C viruses.

Human MGL is a trimeric receptor, which exclusively recognizes terminal GalNAc residues (Tn and sialylated Tn antigens) (Figure 2) found on tumor cells, mucins, and CD45. The murine homolog MGL2 (CD301b) is similar in function and binds Tn antigens and galactose (Singh et al., 2009). Splice variants of hMGL display differences in glycan binding assays with the shorter stalkless isoform Fc MGL fusion less able to recognize helminth-derived glycans LDN and LDNF, yet has higher affinity for glycosphingolipids. Both isoforms equally bind GalNAc and sialyl-Tn antigens (Marcelo et al., 2019). Bacterial lipooligosaccharide (LOS) from *N. gonorrhoea*, *C. jejuni*, and *E. coli* are ligands; however, these interactions were not performed using Fc fusion proteins (See Denda-Nagai & Irimura, 2016; van Kooyk et al., 2015 for reviews).

**MGL (CLEC10A), ASGPR (CLEC4H).** These closely related receptors are predominantly known as self-glycan-binding receptors with roles in homeostasis, dependent upon their cellular context (Brown et al., 2018). Both receptors have the galactose, GalNAc and glucose-binding QPD motif and although they share homology their carbohydrate-binding specificities differ considerably (See Table 1).

The human ASGPR (asialoglycoprotein receptor) is a two subunit receptor ASGP1 and ASGP2, which forms various combinations of tetrameric complexes. The receptor has a role in homeostasis as it binds to desialylated glycoproteins and targets them for removal from circulation. It binds terminal galactose and N-acetylgalactosamine in a Ca^{2+}-dependent manner and binds tri-antennary over mono-antennary galactose with significantly increased affinity. Positively charged polymers are bound in its anionic-binding site. Pullulan, a carbohydrate-binding specificity differs considerably (See Table 1).

Glycan array analysis demonstrated that FcDCIR binds to sulfated LacNAc, Lac, bi-antennary N-glycans, and an O-2 core xylosylated bi-antennary N-glycan, although these studies used glycosylated version of the Fc fusion protein (Brzezicka et al., 2016; Hsu et al., 2009).
although binding affinity decreases as the number of mannose residues increase (Omahdi et al., 2020; Toyonaga et al., 2016). Using mutated Fc DCAR fusion proteins the role of the EPS motif in binding AcPIM2 was examined, and the requirement for a neutral Ala136 residue surrounding the binding site was identified. A hydrophobic groove leading away from the glycan-binding site contributes to the binding of the phosphate moiety of AcPIMs. Furthermore, exchanging the EPS for an EPN motif reduces affinity for AcPIM2, highlighting the importance of the non-typical sequence (Arai et al., 2020; Omahdi et al., 2020).

**CD23.** CD23 is a low-affinity, pH-sensitive binding receptor, with the EPN motif present in murine and bovine, but not human CD23, explaining some of the differences in ligand-binding profiles between species (Jégouzo et al., 2019). CD23 has an extended neck region allowing receptor trimerization and it is proposed that ligand binding brings two symmetrical trimeric molecules together. Endogenous ligands are recognized by the C-terminal CTLD which binds integrins, and in an N-glycan independent manner binds IgE, the neck region binds MHC-II, and only the human receptor binds CD21 (Jégouzo et al.) (see Sutton & Davies, 2015 for a review). Different splice variants have distinct expression profiles and signaling tails but identical extracellular ligand-binding domains. Shedding of soluble versions of the receptor occurs via cleavage by the membrane metalloproteases and proteases secreted by S. mansoni (Griffith et al., 2015).

Glycan arrays with biotin fusions of the cow CD23 have shown that the disaccharide GlcNAcβ1-2Man, Fucα1-2Gal disaccharide, and several oligomannose structures are ligands, with the murine CD23 having a similar, but a lower affinity, binding profile (Jégouzo et al., 2019). Murine CD23 recognizes α-mannan and β-glucan from C. albicans and A. fumigatus, but not glucuronoxylomannans from C. neoformans (Figure 2); however, these interactions have not been demonstrated using fusion proteins (Guo et al., 2018). There is contradictory evidence regarding the binding of CD23 and DC-SIGN to human Ig molecules (Temming et al., 2019). The necessity for large trimers to ensure sufficient avidity to detect binding may explain the limited use of Fc CD23 fusion proteins in pathogen and glycan array screens.

**GROUP VI CLR: MMR**

This family of receptors are type I TM proteins with multiple CTLDs, a single fibronectin type II (FNII) domain, and cysteine-rich (CR) domain at the N-terminal. Two of the family members, MMR (CD206) and Endo180, have eight CRDs and DEC205 (CD205) has ten. There are limited data available for Endo180 and DEC205 as PRRs; however, the Fc MMR CRD four to seven 7 domains (see Figure 1) binds ligands with high avidity and has been extensively used as a tool to probe pathogens; examples include detecting glycoproteins with high mannose oligosaccharides on the surface of viruses, mannans on yeasts, and mannose-capped LAM on mycobacteria (See Table 1 and Martinez-Pomares, 2012; Taylor et al., 2005 for reviews). CRD4 in co-operation with CRD5 binds high mannose oligosaccharides, GlcNAc, and fucose in a Ca\(^2+\)-dependent manner. CRD4 is the only domain that is able to bind to monosaccharides and structures with terminal Manα1-2Man linkages. A tetrameric biotinylated CRD4 complex displayed decreased affinity for higher glycan structures, possibly due to fewer accessible or less abundant terminal Manα1-2Man linkages on the larger glycans. The presence of non-reducing terminal mannose residues is a shared feature of most of the mycobacterial glycans bound by the MMR. The MMR binds a range of endogenous ligands due to its other domains; the N-terminal CR domain is a Ricin-type domain which binds 4-sulfo-GalNAc residues on the reducing ends of glycans on endogenous ligands, such as luteinizing hormone, and the FN II domain binds to collagen. The CR-Fc protein has been used as a negative/background binding control for detection of fungal glycans by Fc CLRs (Vendele et al., 2020).

## 2 | DETECTION OF PATHOGENS

The immune system has to recognize and differentiate between PAMPs, DAMPs, and self-associated molecular patterns (SAMPs) to maintain the balance between immunity and homeostasis; however, dysregulation of these processes often results in autoimmunity, with many of the glycan-binding receptors highlighted in this review implicated in detecting these changes (See Brown et al., 2018; for a review). A brief overview of the different pathogen types is provided, with examples of the Fc CLRs that have been used to probe their outer exposed glycan content, which can often mimics that of the host SAMPs to avoid recognition (e.g. N. meningitidis) or contains unique glycans (e.g. T. cruzi) resulting in recognition as foreign (See Figure 2).

### 2.1 | Recognition of viral PAMPs

Viruses are obligate parasites dependent on gaining entry to the host’s cytoplasm for delivery of their genome for initiation of replication. The first stage of infection, attachment to the host cell receptors, differs depending on the presence or absence of an envelope surrounding the viral capsid proteins (Figure 2); non-enveloped viruses penetrate directly via binding to receptor molecules, and enveloped viruses bind and fuse with the host cell membrane. These binding and fusion events are defined by specific host receptors, coreceptors, or fusion receptors facilitating entry through recognition of capsid proteins or envelope glycoproteins (see Modis, 2014 for a review). Glycosylation of the viral envelope proteins occurs within the host cell; therefore, these glycan patterns are often recognized as “self,” both allowing escape from recognition by the host’s glycan binding receptors and facilitating attachment during the next round of infection. Although processed by the host’s glycosylation and secretion mechanisms, viruses often have high mannose oligosaccharides (Figure 2) or incompletely processed N-linked glycans with exposed GlcNAcβ1-2Man making them detectable by the immune system (Taylor & Drickamer, 2019). Many mammalian viruses, both capsid and enveloped, directly recognize the host cells’
surface N- and O-linked glycans and glycolipids, for example the influenza hemagglutinin glycoprotein binds the host cells’ sialic acids (see Thompson, de Vries, et al., 2019 for a review). Examples of the different families of viruses that have been bound by Fc CLRs will be highlighted along with the types of techniques used to detect binding.

One of the extensively studied viral families is the Retroviridae, of which the enveloped virus HIV-1 is a member. The HIV viral glycoprotein (gp120) (Figure 2) that mediates viral entry is highly glycosylated containing approximately 30%–40% high mannose-type glycans (Balzarini, 2007). Deglycosylation with PNGasF, but not denaturation, of gp120 destroys binding to Fc DC-SIGN. Furthermore, this binding is pH dependent with loss of function occurring for both recombinant DC-SIGN and Fc DC-SIGNR at low pH, implicating these receptors in viral uptake and processing in endosomal compartments (Snyder et al., 2005).

Other examples of enveloped viruses that interact with Fc CLRs such as DC-SIGN have been identified: a recombinant ( Arenaviridae) Lassa virus (LASV) envelope glycoprotein expressed in a Lymphocytic choriomeningitis virus (LCMV) chimera and the measles virus (strains WTF and MV) ( Paramyxoviridae) bind in a mannann and Ca$^{2+}$-dependent manner (Goncalves et al., 2013; de Witte et al., 2006). Although DC-SIGN, through interaction with high-mannose N-glycans, is the cell entry receptor for viruses of the Bunyaviridae, for example, Rift Valley fever virus (RVFV) and Uukunieni virus (UUKV), these interactions were not identified using Fc fusion proteins but cell-expressed receptors (Lozach et al., 2011). Similar experiments using cell-expressed DC-SIGN and L-SIGN (DC-SIGNR) found that both receptors equally permit Ebola virus infection in a mannann-dependent manner (Alvarez et al., 2002), whereas West Nile virus is preferentially bound by DC-SIGNR. Differences in mannann inhibition of binding were found to be dependent on the source of the virus preparation (mammalian cells vs. insect cells) (Davis et al., 2006) and should be considered for Fc CLR-binding experiments.

SPR assays using non-Fc-fused ectodomains of DC-SIGN, DC-SIGNR, MGL, and Langerin demonstrated strong interactions for all receptors, except Langerin, with immobilized S protein of the Coronavirus severe acute respiratory syndrome (SARS)-CoV-2. The binding of DC-SIGN, while faster than DC-SIGNR, was not dependent on S protein orientation, as the glycosylation pattern is evenly distributed providing numerous binding sites. Multiple binding events were visualized by EM showing numerous orientations of ligand-receptor interactions which might not have been feasible with an Fc-linked fusion protein (Thépaut et al., 2020). Another screen, this time using a range of Fc fusion proteins, including CLRs and Siglecs, found SARS-CoV-2 spike protein binding to the receptors DC-SIGN, and MGL, but not Langerin, Dectin-1, Mincle, and mannose-binding lectin (Chiodo et al., 2020).

All four serotypes of the Dengue virus (DV), the Japanese encephalitis virus and tick-born encephalitis virus (all Flaviviruses), are bound by the Mannose receptor Fc CRD4-7 protein in ELISA-based assays. These assays were verified using soluble Envelope (sE) protein DV antigen, produced both in mosquito and human cells with binding Ca$^{2+}$, mannose, fucose, and galactose dependent. Transfer blots probed with the Fc protein (far eastern blots) confirmed the specificity for binding to the sE glycans (Miller et al., 2008). Fc proteins of CLECSA, DC-SIGN, and DC-SIGNR bind to DV (serotype 2) (Chen et al., 2008).

### 2.2 Recognition of fungal PAMPs

Fungi have long been understood as pathogens considering their considerable burden on human health (Erwig & Gow, 2016). Fungi cause significant disease with more than a million deaths and significantly more life-threatening infections per annum with most fungi-opportunistic pathogens causing disease in patients with compromised immune systems (Bongomin et al., 2017). The most well studied yeasts are S. cerevisiae, C. neoformans, Candida spp., and Malassezia spp. and of the molds Penicillium, Mucor, and Aspergillus. (see Drummond et al., 2014; Garcia-Rubio et al., 2020 for reviews). The outer cell wall (Figure 2) is the first point of contact with the host’s immune system with both the composition of the cell wall and the type of immune cell displaying the binding PRR shaping the immune response. A diverse repertoire of soluble opsonins and membrane bound receptors, such as the CLRs, bind to specific PAMPs on the fungal cell wall (See Erwig & Gow, 2016; Goyal et al., 2018 for reviews).

The outer cell wall composition of fungi varies considerably between fungal species and even within species, for example C. albicans varies depending on the strain, morphology, growth conditions, and the use of anti-fungal treatments, and these difference can be detected using Fc CLRs (Marakalala et al., 2013; Pradhan et al., 2019; Vendele et al., 2020) (See Table 1). The outer walls of fungi are usually highly glycosylated, may be covered in a hydrophobic layer (e.g. A. fumigatus conidia), or surrounded by a capsule (e.g. C. neoformans). The predominant cell wall components include a range of O- and N-linked mannanns, β-glucans, chitin, melanin, galactomannans, galactosaminogalactans, glucuronoxylomannans, and galactoxylomannans all of which act as PAMPs (See Figure 2) and interact with a range of CLRs (See Table 1) defining the immune response (Erwig & Gow, 2016; Garcia-Rubio et al., 2020).

The distribution of these components within the cell wall can vary between strains, for example Dectin-1 binding to H. capsulatum strain G186A is prevented by a layer of α-glucans, whereas strain G217B or a glucan synthase mutant Δags1 is readily bound by cell-expressed Dectin-1 (Wang et al., 2014). The amount of α-1,3-glucan content can differ for Histoplasma, Blastomyces, and Paracoccidioides during laboratory passage and will consequently impact on the Fc PRR-binding levels. Variations in β-glucan exposure can be influenced by many factors for example the Histoplasma Eng1 glucanase, which is usually highly expressed during the pathogenic phase cleaves exposed β-glucan only allowing detection by Dectin-1 of β-glucan in the septum of budding cells. In contrast, the Eng1 mutant yeast is completely bound by Fc Dectin-1 as observed by immunofluorescent microscopy (Rappleye et al., 2007).
Immunocompromised patients, either through HIV infection or immunosuppressive therapies, are highly susceptible to opportunistic infections with the Ascomycota *P. carinii*. This pathogen has been screened by ELISA and immunofluorescence assays with a CLR library of Fc fusion proteins demonstrating that Mincle, Dectin-2, DC-SIGN, and MCL all bind to the major surface mannoprotein Msg, with more binding observed for Dectin-2 Fc in these assays. Due to the lack of higher mannose glycan structures on *P. carinii*, Fc MR CRD4-7 and Fc DC-SIGN showed significantly more binding to S.c-derived mannan than Msg (Kottom et al., 2019). A novel therapeutic use for a murine IgG Fc Dectin-1 fusion protein was demonstrated with enhanced *P. carinii* uptake and clearance occurring when preopsonized with Fc Dectin-1. Opsonization efficiently targeted the pathogen for Fcγ receptor detection even in immunocompromised SCID mouse model (Rapaka et al., 2007).

Immunocompromised patients are susceptible to cryptococcosis, with 15% of HIV patients worldwide dying from this disease, caused by *C. neoformans* dissemination from the lung to the central nervous system. While a range of innate immune receptors, including the TLRs, have been shown to recognize Cryptococcus; the role of these receptors shows some redundancy. The role of individual CLRs in recognition and clearance of *Cn* is not well defined in animal models of infection, with Dectin-2 KO mice having an exacerbated Th2 response and the MR KO having increased susceptibility (see Campuzano & Wormley, 2018 for a review). The Cryptococcus capsule masks ligands readily recognized by the immune system. The capsule composition varies depending on the fungal morphology, for example the Titan cell which is ten times larger than the yeast cell, is generally composed of glucuronoxylomannan (GXM), galactoxylomannans (GaXM), and mannoproteins (Figure 2). The capsule, linked by α-1,3 glucans to the cell wall membrane, masks a layer of β-1,3 and β-1,6 glucans and mannoproteins, which cover a significant layer of chitin and chitosan. The melamin layer, chitin, chitosan, and GXM are known immune modulators (See Garcia-Rubio et al., 2020 for a review).

Although soluble Fc Dectin-1 binds, presumably to non-out surface exposed β-glucan on Cryptococcus spores, in vitro cell membrane expressed receptor experiments have shown a limited role for Dectin-1 binding and phagocytosis of yeast and spores of *Cn* serotype D. In fact, all CLRs tested, including Dectin-1, Mincle, MCL, except for a twofold response with Dectin-2 showed weak to no binding and limited stimulation in reporter cell-based assays. This is confirmed with bone marrow-derived macrophage cells deficient in either Dectin-1, Dectin-2, Mincle, MR, the signaling molecule Card9 or FcyR, displaying no defect in the immune response to capsulated spores (Walsh et al., 2017). Recently the mannose inhibitable binding of Fc Dectin-2 has confirmed with reporter-based cell assays to lysates derived from an acapsular mutant (Cap67) demonstrated that a mannoprotein (possibly MP98, which is extensively O-glycosylated) but not GaXM is a ligand (Tanno et al., 2019). Binding of Fc MCL to *Cn* serotypes (B, and AD) by flow cytometry and by ELISA to extracted GXM from these same serotypes, as well as evidence from infected KO mice being more susceptible, provides evidence for a ligand for MCL (Huang et al., 2018). These highlighted experiments, using Fc CLRs and relevant controls to probe the glycan ligands on *Cryptococcus*, demonstrate the importance of testing a range of serotypes and that fungal mutants can be utilized to fully probe the fungal cell wall structure.

*C. albicans*, with a large number of well characterized mutants, is a model organism for studying fungal interactions with the immune system and consequently the cell wall structure has been extensively studied. The Candida cell wall (Figure 2) has an inner layer composed of β-glucan interspersed with chitin and outer layer containing fibrils of N-mannans linked to cell wall proteins, O-linked mannans, and phosphomannans (Erwig & Gow, 2016; Garcia-Rubio et al., 2020; Lenardon et al., 2020).

The four Candida species that cause the most hospital acquired infections are *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* with *C. auris* being a more recent growing concern (Bongomin et al., 2017; Chowdhary et al., 2017). These species have been assessed for binding to Fc Dectin-1, detecting the abundant β-1,3-linked glucans (approx. 50%-60% of the dry weight), showing that *C.a* had the least β-glucan exposure (Thompson, Griffiths, et al., 2019). There are 17 closely related *C.a* strain groups (clades), based on multi locus sequence typing, and although they have varying amounts of exposed β-glucan these differences only give a discernible phenotype during in vivo Dectin-1 KO infection models and not in vitro-based assays. Fc Dectin-1 bound more exposed β-glucan on ATCC 18804 (clade 5) than SC5314 (clade 1) hyphae, and in this study both strains increased their surface exposed β-glucan when cultured under hypoxic conditions (Marakalala et al., 2013). In contrast, the amount of exposed β-glucan, as detected by FcDectin-1, of hypoxic SC5314 or iron-depleted yeast cells is reduced, and exposure is increased under low pH, a range of antifungal drug treatments, and during depletion of manganese and zinc micronutrients (Pradhan et al., 2019).

Interestingly SC5314 yeast with higher chitin levels and more exposed β-glucan were less virulent in Dectin-1 KO mice (Marakalala et al., 2013). Although the plant lectin wheat germ agglutinin (WGA) is routinely used to probe chitin content, as it binds β-1,4-GlcNAc-linked residues, it has been utilized as a an antifungal therapeutic Fc fusion protein (Liedke et al., 2017). No Fc PRRs currently recognize chitin, although a few PRRs (e.g. Mannose receptor, NOD2, and TLR-9) have been implicated in shaping the immune response to *C. albicans* derived chitin (Erwig & Gow, 2016).

Mannan, recognized by TLR receptors (e.g TLR2 senses phospholipomannans and in conjunction with galectin-3 responds to β-mannosides (Jouault et al., 2006) and is recognized by a range of CLRs (Table 1). Fc fusion proteins have been used to probe the *C. albicans* cell wall for mannanc content: the MR (CRD4-7) and DC-SIGN fusion proteins detect outer *N*-mannans on the cell wall, Fc Dectin-2 detected inner *N*-mannans. While Fc CRD4-7 strongly binds a range of virulent and attenuated *C. albicans* hyphae, Fc Dectin-2 has vastly different levels of binding, inconsistent with strain virulence, despite its critical role in host defence (Saijo et al., 2010; Vendele et al., 2020). Soluble streptavidin-tagged SIGNR1 binds p1,2-mannose-capped α-
mannotese side chains, whereas DC-SIGN fusions bind α-mannose on non-reducing side chain ends on C. albicans (Takahara et al., 2012).

The use of Fc CLRs to probe the cell wall content and structure of aspergillus currently lags behind that of Candida, despite it being implicated in severe asthma, causes allergic bronchial pulmonary aspergillosis, chronic and invasive aspergillosis. The aspergillus outer cell wall varies considerably between the conidium and hypha (Figure 2). Both morphologies have an innermost chitin layer covered by a layer of β-1,3 glucan, but the conidia then have a layer of α-1,3 glucan, covered by a discrete zone of DHN melanin and an outer hydrophobic rodlet layer. The hyphae have a mixed outer layer comprised of GAG and galactomannans covalently linked to non-reducing ends of α-1,3 glucan side chains (See Erwig & Gow, 2016; Latgé & Chamilos, 2019 for reviews).

Aspergillus exposure of β-glucans bound by a soluble un mutated murine Fc fused with Dectin-1 shows low-binding levels on resting conidia and hyphae, with more uniform binding on swollen conidia and early germcells (Steele et al., 2005). The importance of the exposed β-glucan during antifungal immunity is highlighted from two different assays using Fc Dectin-1. Firstly, intranasally introducing a mutated human Fc region fused to Dectin-1 at the same time as A.f conidia into mice significantly decreases the immune response (CXCL1/KC, TNF-alpha, CCL3/MIP-1, IL-6, and GM-CSF) and results in much higher fungal burdens (Steele et al., 2005). Secondly, the exposed β-glucan bound by a mlG Fc portion fused to Dectin-1 targets the microbe for enhanced clearance via Fc binding receptors, this decreased fungal burden and enhanced survival of the infected mice (Mattila et al., 2008; Rodriguez-de la Noval et al., 2020). The aspergillus GAG layer masks the β-glucan layer as can be observed with the Δuge3 mutant, lacking GalNAc, has increased binding to FcDectin-1 on both swollen conidia and hyphae, and the mutant is less virulent due to the increased detection of the exposed β-glucan by the immune system (Gravelat et al., 2013). There is some inconsistency in the literature regarding the binding of FcDectin-1 to the melanin biosynthesis mutant (Δpksp) with none detected in the study by Valsecchi et al. (2019) and binding observed by Stappers et al. (2018). Interestingly, in the former study not all rodlet mutants bound Fc Dectin-1 as visualized by fluorescent microscopy, implying heterogeneity in the surface β-glucan.

Melanin, located below the rodlet layer in A. fumigatus conidia, is bound by FcMellLeC in a punctate manner and removal of the RodA protein then shows a uniform melanin layer over the surface of the conidia, which disappears as the conidia swells and germinates (Stappers et al., 2018). This study found melanin on F. pedrosoi and NaOH-treated C. cladosporioides.

### 2.3 | Recognition of bacterial PAMPs

The global burden of bacterial infections is significant, with 1.4 million people dying from TB infections alone in 2019, furthermore there was a 10% rise in antimicrobial resistance, underscoring the importance of combating these pathogens (WHO, 2020). Understanding how bacteria interact with the immune system requires analysis of the first point of contact, the outer cell wall of the bacteria, which can be probed with Fc CLRs to define the exposed glycans (Mayer et al., 2018). A range of bacterial cell wall structures will be highlighted with emphasis on those, such as Mtb which has a complex outer cell wall structure (Figure 2), with known CLR ligands (Table 1).

An inner peptidoglycan (PG) mesh-like layer encases bacteria, and is composed of alternating β-1,4-linked N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) glycan chains connected by short peptides. The single PG layer is covered by an outer lipid membrane in Gram-negative bacteria, in Mycobacteria is separated by an arabinogalactan layer to a unique outer membrane, and is multi-layered in Gram-positive bacteria. Bacterial species vary in the composition of the peptide bridges, the amounts of crosslinking and modifications of the glycan structures, furthermore the PG layer is remodelled in response to stress (e.g. pH, antibiotics) and in some cases (e.g. C. jejuni) is actively altered to avoid recognition by host cells (Frirdich et al., 2019) (See Egan et al., 2020 for a review).

Generally gram positive bacteria have two types of cell wall glycopolymers (CWGs): one attached to the PG layer and the other anchored to the inner lipid membrane layer. The cell wall contains an abundance of secondary CWGs attached to the PG layer; TAs (e.g. S. aureus), which are important for pathogenesis and antibiotic resistance, teichuronic acids (e.g. B. subtilis), cell wall polysaccharides (e.g. B. anthracis), and arabinogalactan (M. tuberculosis). These CWGs are often branched and contain mannos, arabinose, and/or galactose. Examples of cytoplasmic membrane glycolipid anchored CWGs include lipoarabinomannan (LAM) (Mtb) and lipoteichoic acids (LTAs) (S. pneumoniae and S. aureus). Further surface structures such as capsular polysaccharides (CPS), S-layer proteins (B. anthracis), and mycolic acids (M. tuberculosis) are found on the outer surface of bacteria. The CPS, found in species such as N. meningitidis, H. influenza type B and S. pneumoniae are vaccine candidates due to their interaction with the immune system, for example CPS from a range of S. pneumoniae serotypes binds the MMR and SIGNR1, but not Dectin-2, as detected using the Fc fusion proteins (McGreal et al., 2006). Some capsules, such as those from the group B N. meningitidis, are identical (α2-8-linked sialic acid polymers) to host neural motifs and are completely non-immunogenic (See Brown et al., 2013; Varki et al., 2015; Weidenmaier & Peschel, 2008 for reviews).

Streptococcus, Enterococcus, and Lactococcus contain large amounts of L-rhamnose, a sugar not found in humans and therefore could act as a PAMP, as part of their anchored CWPs; however, there are currently no known mammalian PRRs recognizing the rhamnan backbone, which is often decorated by sugars like GlcNAc, GalNAc and glucose to avoid immune recognition (Mistou et al., 2016). CLEC5A, for example, binds to L. monocytogenes independently of the terminal sugars (rhamnose, galactose, glucose) to TA, with inhibition studies suggesting binding occurs to the GlcNAc-MurNAc disaccharide backbone (Chen et al., 2017).

Glycosylation of outer exposed bacterial proteins is a further source of immune recognition and occurs in numerous bacterial species (e.g. mycobacteria, L. monocytogenes, B. anthracis) and although many PRRs recognize these glycoproteins, their full role in detection
of these sugars is not resolved and is a neglected area of research (See Mehaffy et al., 2019; Weidenmaier & Peschel, 2008; Taylor & Drickamer for reviews). Bacterial biofilms are another layer of protection against immune recognition; however, they are also a rich source of carbohydrates such as the mannose-rich P. aeruginosa PSI polymer, recognized by both Fc DC-SIGN and Fc MMR CRD4-7. The PSI polymer interferes with endocytic function of these receptors when cell membrane expressed (Singh et al., 2020).

The unique outer membrane of mycobacteria contains unique molecules (see Figure 2 and Dulberger et al. (2020) for a review on the mycobacterial cell envelope). Some glycans are linked to the mycolic acids such as trehalose monomycolate (TMM) and trehalose dimycolate (TDM) also known as cord factor, the latter recognized by Mincle and MCL (Furukawa et al., 2013). Lipids such as the phosphatidylinositol mannosides (PIMs), which depending on their structure and acylation, are recognized by a range of CLR receptors (e.g. DCAR specifically recognizes the tri and tetra-acylated dimannosyl PIM2 (Toyonaga et al., 2016)). CLRs can recognize multiple mycobacterial-derived ligands, for example the outer membrane lipomannans (LMs) and inner membrane-associated lipoarabinomannans (LAMs) are both recognized by Dectin-2 (Yonekawa et al., 2014) and the mannos-capped lipoarabinomannans (ManLAM) and the larger structure PIMs associate with the MMR and DC-SIGN, with the PIM binding dependent on the terminal carbohydrates and the degrees of acylation (Driessen et al., 2009; Torrelles et al., 2006).

Gram-negative bacteria, such as E.coli, have an outer asymmetrical glycolipid bilayer lipopolysaccharide (LPS), which although structurally diverse between bacteria, consists of three structural domains; lipid A, a core oligosaccharide and O-antigen polysaccharide. Although LPS is predominantly recognized by TLR4 (see Takeuchi & Akira, 2010 for a review), LPS from a range of Klebsiella pneumoniae and Salmonella serotypes is strongly and weakly, respectively, recognized by MMR CRD4-7 fusion protein (Zamze et al., 2002). The recognition of K. pneumoniae LPS (serotype O3 from the K. pneumoniae capsule serotype 55) seems not to be unique to the MMR as it is equally recognized by SIGNR1 and Dectin-2 (McGreal et al., 2006).

2.4 Recognition of parasite PAMPs

Protozoa have complex life cycles, some have both extracellular and intracellular phases, generally have multiple hosts (e.g. insect vectors and mammalian hosts of Trypanosoma cruzi causing Chagas disease), and very diverse mechanisms for cell invasion. In contrast helminths (e.g. the parasitic worms Schistosoma mansoni) live entirely extracellularly avoiding detection. Evading recognition by the immune system while simultaneously binding to receptors to dampen or control the subsequent immune response is a common pathogen survival strategy (e.g. the obligate intracellular parasite Leishmania which replicates within macrophage vacuoles). See Horta et al. (2020) and Veríssimo et al. (2019) for reviews. This review will briefly highlight the glycans detected using Fc CLRs to probe the protozoa Plasmodium, Trypanosoma, Leishmania, and the helminth Schistosoma.

Although mechanisms for eukaryotic protein glycosylation are highly conserved, and many are identical to those of the hosts’ glycans (see Figure 2), parasites display some unusual monosaccharides residues and end caps, uncommon oligosaccharide linkage configurations, and protozoa have glycosylphosphatidylinositol (GPI)-anchored oligosaccharides often differing in composition to the hosts (Veríssimo et al., 2019). An example is T. brucei, which displays high-mannose structures, often decorated with β 1-2-linked xylose or α-1,3-fucose modifications, readily recognized by the host immune system as foreign. Arrays of chemically synthesized core-xylsylated glycans, synthetic N-glycan, and fragments of glycolipids and O-glycans have been probed with Fc CLRs (Brzezicka et al., 2015). The recognition of Fc DC-SIGN and Fc SIGNR3, but not Fc Mincle and Fc SIGNR1, is inhibited by the presence of a core xylose on the GlcNAc terminal oligosaccharides of biantennary N-glycans. Marginal recognition of some non- and xylose glycoconjugates by the Fc CLR fusion proteins, CLEC12B, and DCIR, was detected in this study (Brzezicka et al., 2016).

The Leishmania sp. have a glycolcalyx composed of a variety of GPI-linked carbohydrates, phospholipids, and glycoproteins, phosphoglycans and lipophosphoglycans (LPG), and due to their obligate intracellular lifestyle require receptor-mediated uptake into macrophages without killing to replicate (Horta et al., 2020). A variety of PRRs, including the TLRs, play a role in recognition of Leishmania; the mannose-binding CLRs DC-SIGN, MMR, and SIGNR3 are able to bind Leishmania sp., and while Dectin-1 is known to be required for host defence against L. major, no binding of Fc Dectin-1 to parasites occurs, even though the LPG layer is known to contain branched β-1,3 glucans (Zimara et al., 2018). Uptake of L. donovani and L. major can occur in the absence of MMR, while still being partially inhabitable by MMR ligands, indicating redundancy between the mannose-binding PRRs (Table 1) for initial recognition of the parasite (Alikov et al., 2007; Lefèvre et al., 2013). The MMR does have a role in phagosomal maturation, but this is lost when the parasite is opsonized prior to uptake (Polando et al., 2018), demonstrating the importance of assessing pathogen PAMP exposure in the context it would be presented in vivo. The Max Plank Society parasitic-specific glycan array displayed low levels of DC-SIGN binding to Leishmania LPG containing capping tetrasaccharides, with the presence of a Gal residue with the trimannosides further reduced binding (Geissner et al., 2019). The observation that Fc fusions of the CLRs CLEC9A and MGL both bound promastigote parasites has not been fully explored (Zimara et al., 2018).

The plasmodium-derived crystal, hemozoin, is recognized by the CLEC12A. Using the FcCLEC12A’s specific ability, but not that of a range of Dectin-1 cluster Fc CLRs, to detect a ligand from P. falciparum and P. berghei Antwerpen-Kasapa (ANKA) lysed red blood cells by ELISA, the ligand hemozoin was identified. In vivo experiments using CLEC12A KO mice implicate CLEC12A in experimental cerebral malaria (Rauif et al., 2019). The role of plasmodium glycans
and interaction with CLRs is an understudied area of research (See Gomes et al., 2017 for a review).

Helminths infect more than 1.5 billion people worldwide (WHO, 2012). They display a wide array of glycans, some of which share structural features of their hosts, such as those similar to Leś, an endogenous ligand for DC-SIGN, and the glycans LDLN, LDNF, and Tn-antigen (See Table 1 and Figure 2) (van Die & Cummings, 2009). Other glycans are detected as foreign by PRRs, such as mannosylated proteins from S. mansoni egg-secreted proteins (http://www.functionalglycomics.org/static/index.shtml), mammalian glycans, over 300 microbial glycans and glycan-binding proteins (http://www.functionalglycomics.org/static/index.shtml), and the NGL-based microarray resource (https://glycosciences.med.ic.ac.uk/glycanLibraryIndex.html) contains almost 800 lipid-linked defined glycan probes available to screen (Li & Feizi, 2018). Screening of the early prototype glycan arrays often resulted in no detection of Fc CLR binding; however, using custom-made microbially glycan arrays numerous Fc CLR ligands have been identified (e.g. MCL and Mincle) (Chen et al., 2017; Yamasaki et al., 2009). Synthesis of arrays, based on a series of iterative fractionation and probing using robotic printing of glycans combined with microscale mass spectrometry and immunosequencing methods, in a process termed Beam Search Array, has been deployed to enrich and identify unknown O-glycans from heterogeneous mixtures. An alternative method, ‘Shotgun’ glycomics, uses multidimensional HPLC fractionation to separate glycans prior to printing (as reviewed by Li & Feizi, 2018) are approaches which can be utilized to identify rare glycan ligands for Fc CLRs.

The spatial location of PAMPs can be detected in pathogens by electron microscopy, for example detection of Fc Dectin-2 binding to the inner cell wall of C. albicans (Vendele et al., 2020) and mDectin-1 detection of β-glucan contributed to resolve the molecular structure of the C. albicans cell wall (Lenardon et al., 2020). Direct stochastic optical reconstruction microscopy (dSTORM) imaging using Fc hDectin-1 detected nanoscale exposure of β-glucan in C. albicans and C. glabrata and was able to detect increased size and density in C. a clinical isolates (Graus et al., 2018). These studies have visually demonstrated the spatial and temporal exposure of β-glucan available to interact with immune cell membrane expressed Dectin-1 and this has aided our understanding how pathogens through modification of their outer glycans modulate the downstream immune response (Pradhan et al., 2019).

Fc CLRs, apart from their function in detecting glycans in microbes, have therapeutic and diagnostic uses (Czajkowsky et al., 2012). A murine Fc domain fused to Dectin-1 protects mice against P. carinii infection due to enhanced host recognition and killing of the pathogen by the heightened FcγR-based recognition (Rapaka et al., 2007) and similarly for Aspergillus, growth clearance and survival were all altered when treated with FcDectin-1 (Mattila et al., 2008; Rodríguez-de la Noval et al., 2020). Diagnostic uses include screening for urine and blood stream pathogens by ELISA: an Fc fusion of the serum protein mannose-binding lectin bound to a broad range of bacteria, fungi, viral antigens, and parasites (Seiler et al., 2019). With refinement, future diagnostics could utilize a range of Fc CLRs, in combination with antibodies, to screen and identify pathogens.

The potential to engineer new synthetic, or alter existing lectin-binding domains, and make specific or even alternative probes (e.g. a single-nucleotide polymorphism of Langerin alters its binding properties) provides an exciting future use for Fc CLRs (Hirabayashi & Arai, 2019; Taylor & Drickamer, 2019). The use of intelligent engineering design strategies to modify the Fc fusion ligand domains for use as tailor made binding probes is accelerating existing mainstream protocols such as site-directed mutagenesis (Tinberg et al., 2013). Other strategies based on the heterodimeric nature of some of the CLRs when cell membrane expressed (e.g. MCL and Mincle) could be functionalized into Fc fusions with multiple linked CRDs, similar to the MMR (Fc CRD4-7) (See Figure 1), might be used as multivalent probes to target binding at specific pathogens.

### 4 LIMITATIONS AND CONSIDERATIONS USING Fc CLRs

As evidenced by the CLRs discussed in this review, the binding properties of each CLR must be fully understood before undertaking assays screening pathogens for glycan binding. A number of factors need to be considered; the basic biochemical properties such as Ca2+ dependence for binding, requirement for receptor multimerization to ensure sufficient avidity of binding, species differences in ligand recognition (e.g. CD23), and analysis of the glycosylation state of the CLR and/or fusion protein and how this differs depending on the expression protocol (Taylor & Drickamer, 2019). Glycan-binding results should always be validated by more than one method, ideally...
including the use of soluble inhibitors to verify specificity of the CLR for example MGL, and not the Fc portion of the fusion, specifically recognizes \textit{C. jejuni} LOS as verified by inhibition of binding with an excess of GalNAc, blocking with the anti-MGL antibody, the necessity for divalent cations for binding and the loss of reactivity for bacterial glycosylation mutants (Van Sorge et al., 2009). The mutation of essential amino acids within the glycan-binding site (e.g. the Mincle QPD motif (Furukawa et al., 2013)) is a desirable control for both CLR-binding specificity and the higher background-binding levels inherent in Fc fusion protein assays.

Many bacteria, such as \textit{S. aureus} and \textit{S. pyogenes}, express Fc-binding proteins making the true detection of expressed ligands using Fc fusion proteins unreliable. An alternative approach is to cleave the Fc portion post-purification, for example using a thrombin cleavage or tobacco etch virus cleavage sequence cloned between the receptor and the Fc portion (Guan et al., 2010; Rodrigues et al., 2020). CLRs such as DC-SIGN, Dectin-2, and the MMR which bind oligomannosylated IgG (Boesch et al., 2014), may bind glycosylated antibodies present on the surface of serum blocked pathogens, they could interact with the secondary antibodies used for pre-complexing or interact with the Fc region itself.

Further limitations in using Fc proteins for pathogen ligand screens are not as obvious: for example protein aggregations may give false positive results (Mayer et al., 2018). The proportion of HMW complexes in Fc fusion preparations should be assessed and size-exclusion chromatography used to standardize the Fc CLR production and usage for consistent and comparative binding assays. Differences were observed in glycan array-binding assays using dimeric or multivalent complexes for example Fc DC-SIGN binds glycans from both \textit{C. albicans} and \textit{S. cerevisiae}, whereas tagged probes only recognize those from \textit{C. albicans}. The tagged probes allowed subtle difference in binding between DC-SIGN and mSIGNR1 to be detected, with only the latter receptor able to bind the β-mannose-capped α-mannose side chains found in \textit{C. albicans} (Takahara et al., 2012). In contrast to these data, DC-SIGN binding, tested both as an Fc fusion protein and as a tetrameric soluble protein produced in bacteria, to an array of over 140 glycans-containing bacterial, parasite, mammalian, and plant polysaccharides showed very similar patterns of glycan recognition (Geissner et al., 2019).

Detection of pathogens usually requires two or more different cell surface expressed CLRs to elicit an appropriate immune response, which is shaped by the cell type and its location, and may explain some discrepancies in ligand-binding profiles. Two examples of Fc CLRs binding, but when the CLR is cell membrane expressed no interaction or signaling occurs, are Fc Mincle binds \textit{H. capsulatum}, \textit{C. posadasii}, and \textit{B. dermatitidis} and Fc Dectin-1 binds \textit{B. dermatitidis} and Cryptococcus (Walsh et al., 2017; Wang et al., 2014). In contrast, SIGNR1 binds Dextran when cell membrane expressed but not as an Fc fusion protein due to the polysaccharide being a very low-affinity ligand (Galustian et al., 2004).

Fc CLRs might not detectably bind to ligand-baring pathogens due to a range of factors. Live pathogens may mask or alter their PAMPs in response to cellular growth conditions, or cell wall ligand density is too low by comparison to the high concentrations of purified compounds used in assays (Inoue & Shinohara, 2014; Mnich et al., 2020; Pradhan et al., 2019). Ligand binding may be pH sensitive, with some receptors able to bind and function at a wide range including low pH for example Fc Dectin-1 is able to bind from pH4 to pH8, in contrast glycan binding by Fc CD23 protein is pH sensitive, with optimal binding at pH6.5 (Faro-Trindade et al., 2012; Jégouzo et al., 2019).

A further limitation of Fc CLRs is the inability to simultaneously detect multiple Fc fusion proteins probing unique ligands on the same pathogen. Directly conjugating fluorophores is often problematic as conjugation, using amine labeling chemistry, can render the ligand-binding domain of CLRs non-functional (unpublished data), but has been successfully achieved with DC-SIGN (Zheng et al., 2017). Alternative approaches such as the use of the fusion proteins pre-complexed to fluorescent beads or antibodies, or harnessing systems using variations on fluorescently labeled streptavidin-binding biotin tagging have been successfully used (Achilli et al., 2020) (See Figure 1). These examples highlight some of the challenges using Fc CLRs, instead of cell membrane expressed receptors, as tools for probing ligand content on pathogens.

5 | CONCLUSIONS

The Fc fusion proteins of the CLR family, with their diverse ligand-binding repertoire, are extremely valuable tools for the detection and analysis of the content and structure of pathogen cell walls. Their use reveals the PAMPs exposed for recognition by the host's immune system. The temporal detection of exposed PAMPs has been extrapolated into understanding host-pathogen interactions and how pathogens both evade and engage with host cells.

In the last few years significant progress has been achieved understanding the role of CLRs in immunity and homeostasis; however, there are some outstanding areas of research that still need to be considered. While substantial advances have been made for fungi and viruses, the role of CLRs in the detection and immune response to bacterial, protozoa, and helminth glycans is relatively not very well characterized (Brown et al., 2018; Taylor & Drickamer, 2019; Verissimo et al., 2019). For example, there are currently unknown receptors for rhannose, arabinoferanose, and xyloferanose all expressed by various microorganisms (Zheng et al., 2017). Fc fusion proteins of numerous PPRs have yet to be made (e.g. the CL-P1 collectins) and many of the CLRs reviewed here have not been extensively examined or were screened on the first generation arrays with limited ranges of glycans. Furthermore the ligand binding profiles of many of the Fc CLRs, especially those with low-affinity binding properties, which are usually cell membrane expressed as multimeric structures, should be reassessed using complexed Fc probes in screens with high-density purified glycans (Mnich et al., 2020; Zheng et al., 2017). Recent advances in chemical synthesis and enhanced purification methods of specific glycans should resolve false positive CLR ligand interactions detected due to the impurity of many glycan...
preparations, especially those from bacteria and viruses (Geissner et al., 2019; Zamze et al., 2002). The use of Fc CLRs as well as verification by cellular binding assays, controlled with relevant CLR CRD mutations, glycan inhibition, and/or antibody blocking, should further resolve discrepancies regarding recognition of specific pathogens by CLRs.

Due to our increased understanding of the role that various cell wall structures play in pathogenicity, Fc CLRs have been used as basic diagnostic and therapeutic tools and have the potential to be engineered, modifying sensitivity and specificity, enhancing these functions. Exciting future work using artificial design or strategically modifying binding motifs in CLRs may give rise to probes with unique binding properties able to screen distinct glycans and give rise to a new generation of research, diagnostic, and therapeutic tools.

ACKNOWLEDGMENTS
Funding was provided by the MRC Centre for Medical Mycology (MR/N006364/2) at the University of Exeter, UK.

CONFLICT OF INTEREST
The author has no conflict of interest to declare.

DATA AVAILABILITY STATEMENT
Data sharing is not applicable to this article as no new data were created or analysed in this study.

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The FEBS Journal


How to cite this article: Willment, J.A. (2021) Fc-conjugated C-type lectin receptors: Tools for understanding host-pathogen interactions. Molecular Microbiology, 00, 1–29.
Available from: https://doi.org/10.1111/mmi.14837