Review

From structure to function – Ligand recognition by myeloid C-type lectin receptors

Swantje Fischer\textsuperscript{a,b,1}, Felix Stegmann\textsuperscript{a,b,1}, Vinayaga Srinivasan Gnanapragassam\textsuperscript{a,b}, Bernd Lepenies\textsuperscript{a,b,*}

\textsuperscript{a} Institute for Immunology, University of Veterinary Medicine Hannover, 30559 Hannover, Germany
\textsuperscript{b} Research Center for Emerging Infections and Zoonoses, University of Veterinary Medicine Hannover, 30559 Hannover, Germany

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ABSTRACT

The relevance of protein-glycan interactions in immunity has long been underestimated. Yet, the immune system possesses numerous classes of glycan-binding proteins, so-called lectins. Of specific interest is the group of myeloid C-type lectin receptors (CLRs) as they are mainly expressed by myeloid cells and play an important role in the initiation of an immune response. Myeloid CLRs represent a major group amongst pattern recognition receptors (PRRs), placing them at the center of the rapidly growing field of glycoimmunology. CLRs have evolved to encompass a wide range of structures and functions and to recognize a large number of glycans and many other ligands from different classes of biopolymers. This review aims at providing the reader with an overview of myeloid CLRs and selected ligands, while highlighting recent insights into CLR-ligand interactions. Subsequently, methodological approaches in CLR-ligand research will be presented. Finally, this review will discuss how CLR-ligand interactions culminate in immunological functions, how glycan mimicry favors immune escape by pathogens, and in which way immune responses can be affected by CLR-ligand interactions in the long term.

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Abbreviations:
BCG, Bacillus Calmette-Guérin; CLR, C-type lectin receptor; CRD, Carbohydrate recognition domain; CTL, C-type lectin; CTLD, C-type lectin-like domain; DAMP, Danger-associated molecular pattern; DAP12, DNAX-activation protein 12; DC, Dendritic cell; DCAR, Dendritic cell immunoactivating receptor; DCIR, Dendritic cell immunoreceptor; DC-SIGN, Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin; Dectin, Dendritic cell-associated C-type lectin receptor; DLS, Dynamic light scattering; DNGR, Dendritic cell natural killer lectin group receptor; ECD, Extracellular domain; ECM, Experimental cerebral malaria; ELISA, Enzyme-linked-immunosorbent-assay; Fc, Fragment crystallizable; Fc\*R, Fragment crystallizable receptor \gamma; FRET, Förster resonance energy transfer; Fuc, Fucose; Gal, Galactose; GalNAc, N-acetylgalactosamine; Glc, Glucose; GlcNAc, N-acetylgalosaminic acid; GTfs, Glycosyltransferases; HIV-1, Human immunodeficiency virus 1; HLA, Human leukocyte antigen; HSPC, Hematopoietic stem and progenitor cells; Ig, Immunoglobulin; IL, Interleukin; ITAM, Immunoreceptor tyrosine-based activating motif; ITC, Isothermal titration calorimetry; ITIM, Immunoreceptor tyrosine-based inhibitory motif; Lact, Lactose (Gal\(1,4\)-Glc); LacNAc, N-acetyllactosamine (Gal\(1,4\)-GlcNAc); Le, Lewis; LPS, Lipopolysaccharide; Man, Mannose; MBP, Mannan-binding protein; MCL, Macrophage C-type lectin; MD, Molecular dynamics; MCL, Myeloid DAP12-associated lectin; MGDC, Monoglycosyl diacylglycerol; MGL, Macrophage galactose-type lectin; MHC, Major histocompatibility complex; Mincle, Macrophage inducible C-terminal Ca\textsuperscript{2+}-dependent lectin receptor; MMR, Macrophage mannose receptor; MNP, Monosodium urate; mTOR, Mammalian target of rapamycin; Mν, Macrophages; NFAT, Nuclear factor of activated T-cells; NF-κB, Nuclear factor kappa B; NK, Natural killer (cells); NMR, Nuclear magnetic resonance; PAMP, Pathogen-associated molecular pattern; PDPN, Podoplanin; PRR, Pattern recognition receptor; QCM, Quartz crystal microbalance; Raf-1, Rapidly accelerated fibrosarcoma 1; RIA, Radioimmunoassays; ROS, Reactive oxygen species; SAMP, Self-associated molecular pattern; SAP130, Spliceosome-associated protein 130; SARS-CoV, SARS coronavirus; SI2, Src-homology 2; SHP, Src homology region 2 domain-containing phosphatase; SIGNR, Specific intracellular adhesion molecule 3-grabbing non-integrin homolog-related; SPR, Surface plasmon resonance; SYK, Spleen tyrosine kinase; TCA, Tricarboxylic acid; TDM, Trehalose-6,6′-dimycolate; TLR, Toll-like receptor; TNF, Tumor necrosis factor; WTA, Wall teichoic acid.

* Corresponding author.

E-mail addresses: swantje.fischer@tiho-hannover.de (S. Fischer), felix.stegmann@tiho-hannover.de (F. Stegmann), vinayaga.srinivasan.gnanapragassam@tiho-hannover.de (V.S. Gnanapragassam), bernd.lepenies@tiho-hannover.de (B. Lepenies).

1 These authors have contributed equally to this article.

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Contents

1. Introduction .......................................................... 5791
   1.1. Lectins ................................................................. 5791
   1.2. C-type lectins ..................................................... 5791
2. Structural overview of myeloid CLRs .......................................................... 5792
   2.1. The C-type lectin-like domain (CTLD) fold .................. 5792
   2.2. Carbohydrate binding domain and CTLD calcium binding sites 5793
   2.3. Neck domain ....................................................... 5794
   2.4. Transmembrane domain ........................................ 5794
   2.5. Intracellular domain ............................................ 5794
   2.6. Computational approaches to identify novel CLRs and their domains 5795
3. Overview of myeloid CLR ligands .................................................. 5795
   3.1. Glycans as myeloid CLR ligands .......................... 5795
   3.1.1. β-Glucans binding to Dectin-1 .......................... 5796
   3.2. (Glyco-)lipids as myeloid CLR ligands ............... 5796
   3.3. (Glyco-)proteins as myeloid CLR ligands .......... 5797
   3.4. Crystalline structures as myeloid CLR ligands .... 5797
4. Methods for CLR-ligand investigations ........................................ 5798
   4.1. In silico methods/predicting novel CLR-glycan interactions using LectinOracle 5800
   4.2. Qualitative/screening methods using CLR-based binding studies 5800
   4.3. Quantitative/affinity methods .............................. 5801
   4.4. Mechanistic methods .......................................... 5802
5. Functional aspects of ligand recognition ........................................... 5802
   5.1. Self- vs non-self-discrimination by molecular cues ........... 5802
   5.2. Intracellular signaling upon CLR engagement ........ 5803
   5.2.1. Dectin-1 engagement and the resulting signaling pathway 5803
   5.3. Signaling flexibility ............................................. 5804
   5.4. Cellular effects upon CLR engagement .................. 5804
6. CLR-mediated immunomodulation ................................................ 5805
   6.1. CLR hijacking and molecular mimicry by pathogens .... 5805
   6.2. CLRs in trained innate immunity: Immunomodulation by Dectin-1-β-glucan interaction 5805
7. Summary and outlook ......................................................... 5806
CRediT authorship contribution statement ........................................ 5807
Declaration of Competing Interest .................................................. 5807
Acknowledgments ...................................................................... 5807
References ............................................................................. 5807

1. Introduction

1.1. Lectins

Lectins comprise a wide range of glycan-binding (glyco-) proteins with high sequence and structural variability [1,2]. Evolutionarily, lectins are ancient and occur in all kingdoms of life with an increasing complexity in higher animals [3]. Alongside their variety, lectins possess a highly diverse repertoire of functions in different species [4]. Pathogens either utilize their own lectins to bind to glycoconjugates on the surface of their host cells or exploit host-associated lectins to facilitate the infection and their persistence in the host [5–8]. In vertebrates, lectins are involved in a variety of physiological processes such as protein folding and protein trafficking [5]. Beyond that, lectins of the immune system have evolved to recognize self [9–11] as well as non-self-glycoconjugates [12–14]. This discrimination between self- and non-self enables the immune system to react to pathogen encounter while leaving healthy host-tissue intact [15]. This concept is based on pathogen-, danger- and self-associated molecular patterns (PAMPs, DAMPs and SAMPs), respectively [16]. Such evolutionarily conserved molecular patterns are recognized by pattern-recognition receptors (PRRs) [17]. Besides the well-characterized Toll-like receptors (TLRs), for example, myeloid C-type lectin receptors (CLRs) belonging to the C-type lectin (CTL) superfamily serve as important PRRs in innate immunity.

1.2. C-type lectins

The designation “CTLs” was given to this superfamilly due to their often Ca2+-dependent ligand binding (the “C” in CTL refers to calcium). However, in the meantime, several CTLs were identified that exhibit Ca2+-independent ligand binding, e.g. dendritic cell-associated C-type lectin receptor-1 (Dectin-1; CLEC7A) [18]. Carbohydrate binding is mediated by the carbohydrate recognition domain (CRD), which is characteristic for many CTLs [19–21]. Later, when non-carbohydrate ligands for CTLs were identified, the more comprehensive term “C-type lectin-like domain” (CTLD) was suggested instead of “C-type lectin domain”. As such, we opted to employ the term CTLD throughout this review (nomenclature reviewed more in depth in [20]).

To date, 17 different groups of CTLs were described based on their phylogeny and structures. Most CTLs have a single CTLD while there are also exceptions with multiple CTLDs, e.g., the macrophage mannose receptor (MMR, CD206) [22]. Further, there are both soluble (e.g. collectins) as well as membrane bound CTLs (e.g. selectins). Membrane-bound CTLs are single-pass transmembrane proteins that can be further classified based on their topology into type I (extracellular N-terminus) and type II (intracellular N-terminus) CTLs [23]. While the majority of CTLs are expressed as monomers, some are self-organized into homo- or hetero-multimeric structures, e.g., Langerin (CD207) [24]. By assembling into multimeric structures, CTLs can increase their
avidity [25,26]. Functionally, CTLs play important roles in development, cell-cell interactions, homeostasis, and immunity [27]. For innate immunity, myeloid CLRs that mainly belong to the asialoglycoprotein- (group II), natural-killer (NK) cell- (group V) and multi-CTLD receptor group (group VI) are of major relevance [20,27,28]. Myeloid CLRs are mainly expressed by cells of myeloid origin like macrophages (Mₚ) and dendritic cells (DCs; Fig. 1). Additionally, myeloid CLRs are key modulators of innate immune responses either directly or in combination with other PRRs. CLRs play indispensable roles in innate immune responses by sensing and processing both PAMPs and DAMPs [29–31].

This review aims to address the structural characteristics of myeloid CLRs (section 2) and of selected CLR ligands (section 3) to unravel how CLR structure and functions correlate. Additionally, we will present an overview of cutting-edge methodological approaches to investigate CTL-ligand interactions (section 4). Finally, we will highlight biological functions of CTL-ligand interactions (section 5), with an emphasis on their exploitation by pathogens for immune evasion as well as their role in trained innate immunity (section 6).

2. Structural overview of myeloid CLRs

Structurally, myeloid CLRs are either type I or type II single-pass transmembrane proteins consisting of one or multiple CTLDs and a neck (stalk) region, both of which form the extracellular domain (ECD), followed by a transmembrane domain and the intracellular tail. CLRs of group II are transmembrane proteins consisting of single C-terminal CTLD possessing the carbohydrate and Ca²⁺-binding domain, followed by a neck region and a short transmembrane domain, which ends with an N-terminal cytoplasmic tail. Among the group II CLRs, the length of the extracellular neck region is variable, which influences their ability for oligomerization [32]. Similarly, CLRs of group V are type II transmembrane proteins with a single C-terminal extracellular CTLD, which lacks carbohydrate and Ca²⁺-binding domains, followed by a transmembrane domain and a short N-terminal cytoplasmic tail. In contrast to the previous two groups, CLRs from group VI contain an N-terminal extracellular domain, consisting of a ricin-like domain, a fibronectin type 2 domain, followed by multiple CTLDs (usually 8 to 10), each carrying the carbohydrate and Ca²⁺-binding domain. They also contain a transmembrane region and a short C-terminal cytoplasmic tail [28] (Fig. 1). In summary, the three groups of myeloid CLRs are structurally similar with distinct differences: CLRs of group II and VI contain carbohydrate- and Ca²⁺-binding domains within their CTLD(s), whereas CLRs of group V lack them. While CLRs of group VI possess multiple CTLDs containing additional domains, group II and group V CLRs are restricted to single CTLDs.

2.1. The C-type lectin-like domain (CTLD) fold

The CTLD fold is evolutionarily conserved among metazoan species and has a characteristic double-loop structure. The term loop structure refers to the random coil structure, which connects the protein secondary structures (α-helices and β-sheets). The CTLD fold usually contains both long (greater than 10 amino acids) and short loop regions (<10 amino acids) [33]. The anti-parallel β-sheet is composed of a short loop with N- and C-terminal β-strands (β1-β5) and contains four conserved cysteine residues (C1-C4), which eventually form disulfide bridges at the base of the loops. The α₁-helix is connected to the β5-sheet by cysteine C1 and C4 residues in the whole domain loop, whereas C2 and C3 contribute to connecting β3- and β5-strands within the long loop region. The remaining part of the chain is organized into two α-helices and one β-sheet [20]. The long loop region contains the major Ca²⁺-binding sites and mediates Ca²⁺-dependent ligand interactions. A maximum of four Ca²⁺ motifs (generally referred to as Ca₁ to Ca₄) are present in the CTLD, whose primary role is to coordinate and stabilize the interaction with bound carbohydrate ligands [34,35]. There exist two main types for the CTLD fold:
Therefore, the selective binding of a CRD to a specific glycan is based, in part, on the glycan composition (as recognized by the chirality of their hydroxyl groups), but they also discriminate between different glycosidic linkages (for example, β-1,3- vs β-1,4-), the presence or absence of branching, and other structural aspects within the glycan chains [44]. Additional modification of glycans, e.g. sulfation, may also contribute to binding as observed for the CLR Langerin. Langerin binds to Man and sulfated Gal, where two conserved lysine residues in the CRD are responsible for the interaction with the sulfate group [45]. In summary, the ligand binding preference of myeloid CLRs is strongly influenced by the presence of the tripeptide motifs EPN, EPS and QPD, which may allow for predicting potential CLR ligands.

The Ca\(^{2+}\)-binding site in the CTLD enables complexation of four Ca\(^{2+}\) ions [20]. Crystallographic studies of the CTLDs of the human asialoglycoprotein receptor and the rat mannann-binding protein (MBP)-A proved useful for understanding the occupancy of Ca\(^{2+}\)-binding motifs. Generally, Ca\(^{2+}\) I, II and III are bound at the upper long loop of the CTLD, and Ca\(^{2+}\) IV is located at the corresponding short loop and is involved in ionic bond formation between α2 and β1/β5-sheet [46]. Additional Ca\(^{2+}\)-binding sites can influence the ligand interaction and the rapid transition of CLRs from active to inactive state [47]. Ca\(^{2+}\) binding affinity to the CTLD is often a pH sensitive process. At lower pH, the murine dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin-related (SIGNR) 2, 3 and 7 display reduced affinity for Ca\(^{2+}\) binding, in contrast to SIGNR8 that exhibits increased affinity to Ca\(^{2+}\) at low pH [48]. Similarly, pH sensitivity of MBP-A was observed. The primary Ca\(^{2+}\)-binding site of rat MBP-A is involved in ligand binding, whereas the secondary Ca\(^{2+}\)-binding site acts as a pH sensor, for releasing the bound ligand [49]. In summary, the Ca\(^{2+}\)-binding motif often plays an important role in carbohydrate ligand binding and/or may aid in releasing the bound ligand from the internalized CLR in a pH-dependent manner.

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**Fig. 2.** Structural aspects of the CTLD. (A) The trimeric crystallographic structure of Langerin with a single CTLD highlighted (middle) and the expansion of the CTLD (blue-grey) with the binding motif (EPN in this case) highlighted (red–orange; lower left). The binding motif predominantly determines the binding specificity of a given CTLD (PDB entry: 3KQG). (B) Highlights of the common structural motifs present in the CTLD. On the left, the major secondary structures (α-helices, β-sheets), long loop region (rose) and the disulfide bridges combining the secondary structures (red) are shown. On the right, the major conserved residues involved in binding of a ligand (e.g. mannose; green) are highlighted including a complexed calcium-ion (black; PDB entry: 3P7G). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
2.3. Neck domain

One of the key functions of the neck domain is the assembly into homo- and heterodimers. For instance, the extracellular neck domain of MGL is involved in clustering by forming a trimeric structure, stabilized by coiled-coil interactions [50]. Coiled-coil interactions are also responsible for trimer formation in Langerin, where ligand binding depends on the formation of the trimeric structure [51]. Multimerization of DC-SIGN is mediated by the neck domain carrying variable neck region repeats [52]. A detailed investigation of naturally occurring DC-SIGN variants suggested that disulfide bridges within the CTLD domain support neck investigation of naturally occurring DC-SIGN variants. Structural analysis of the DC-SIGN neck domain revealed an extensive α-helical structure, which potentially promotes its tetramerization independently of the CRD [53]. It was also shown that the multimerization of DC-SIGN neck domain directly contributes to the structural stability of DC-SIGN expressed at the cell surface and thereby influences pathogen recognition as well as pathogen-induced cluster formation [54,55]. This tetramerization process is pH-dependent and may influence ligand binding affinity [50,51]. An experimentally truncated neck domain prevents DC-SIGN tetramerization, which affects surface organization and consequently pathogen recognition of DC-SIGN [56–58]. Overall, multiple studies highlight the role of the neck domain in structural stabilization and oligomerization.

2.4. Transmembrane domain

The transmembrane domain in CLRs plays an important role by anchoring the receptor to the cytoplasmic membrane and also by functionally connecting the extracellular to intracellular domains. Generally, single pass transmembrane proteins contain approximately 25 amino acids in their transmembrane domain [59]. Upon ligand-induced signal transduction, the transmembrane domain may undergo conformational changes. This structural flexibility allows for transmitting signals to the cytoplasmic domain [60,61]. For instance, the transmembrane domain of Mincle contains a conserved single positively charged Arg residue, which mediates the interaction with the negatively charged fragment crystallizable (Fc) receptor γ (FcgRγ) immunoreceptor tyrosine-based activating motif (ITAM). Mutation of this Arg to Ile leads to a complete abrogation of this FcgRγ interaction (Fig. 3). This indicates that the CLR transmembrane domain may contribute significantly to immune signaling [62].

2.5. Intracellular domain

The intracellular domain plays a critical role for receiving signals from the extracellular domain and initiating downstream signaling events. Four functional classes of myeloid CLRs are described based on the signaling motifs (Fig. 3). These comprise the ITAM-coupled and the hemITAM-bearing CLRs, often considered as “activating” CLRs. In contrast, immunoreceptor tyrosine-based inhibitory motif (ITIM)-bearing CLRs are referred to as “inhibitory” CLRs, whereas a fourth class signals independently of ITAMs/ITIMs [63]. Characteristic for ITAMs are the YxxL tandem repeats (Y = Tyr, x = any amino acid, L = Leu), usually present in CLR-associated adapter proteins. Mincle and Dectin-2 (CLEC6A) are both examples of ITAM-coupled CLRs that associate with the FcgR chain adaptor protein, while the DNAX-activation protein 12 (DAP12) associates with myeloid DAP12-associating lectin (MDL-1; CLEC5A) [62,64,65]. In contrast, hemITAM-bearing CLRs harbor a single YxxL signal motif in their cytoplasmic tail [66]. Both hemITAMs and ITAMs do recruit the spleen tyrosine kinase (SYK) upon phosphorylation of two tyrosine residues, which enables binding of the Src-homology 2 (SH2) domain of SYK. SYK then activates itself by auto-phosphorylation. Notable hemITAM-bearing CLRs are SIGNR3, Dectin-1 and CLEC2 [67–69]. ITIM-bearing CLRs assemble with tyrosine phosphatases like Src homology region 2 domain-containing phosphatase (SHP)-1 or -2. Amongst this group are human DCIR and its two mouse orthologues mDCIR1 (CLEC4A2) and mDCIR2 (CLEC4A4), and the myeloid inhibitory C-type-like lectin (MICL; CLEC12A) [40,70,71]. The last group of CLRs, which com-

Fig. 3. Intracellular structural aspects of the signaling motifs in myeloid CLRs. Characteristic signaling motifs of myeloid CLRs with affiliated CLRs listed. Depending on the signaling motif (in general) activating signals are initiated via ITAM (VYxxL-YxxL)- or hemITAM (VYxxL)-associated CLRs, while inhibitory signals are initiated via ITIM (VYxxL-YxxL)-bearing CLRs. Some CLRs lack intracellular signaling motifs and therefore act independently of ITAMs or ITIMs. Abbrev.: ITAM = Immunoreceptor tyrosine-based activating motif; ITIM = Immunoreceptor tyrosine-based inhibitory motif; FcgRγ = fragment crystallizable receptor γ; SYK = spleen tyrosine kinase; SHP = Src homology region 2 domain-containing phosphatase.
prizes human DC-SIGN, Langerin, and human MGL among others, signal independently of ITAM/ITIM motifs (Fig. 3) [69,72].

2.6. Computational approaches to identify novel CLRs and their domains

Identification of novel myeloid CLRs is critical to elucidate their potential role in innate immunity. Computational tools facilitate the identification of CLRs and their signature domains. This is made possible by emerging machine learning processes. During the machine-learning process, the software continuously creates and adapts an algorithm when applying it to new data. After several iterations, respective models are highly optimized at making realistic predictions and can ultimately outperform humans in solving complex biological questions [73]. In biology, two of the most well-known examples of these technologies are the AlphaFold2 and RoseTTAFold tools [74,75], which merely require primary amino acid sequences to readily predict probabilistic protein structures. For screening CLRs and their respective domains, the amino acid sequence of a given CLR is compared against that of a selected species database, using basic logical alignment tool (BLAST) [76]. Afterwards, the sequences with the highest homology are further analyzed for individual domains and motifs using pairwise sequence alignment tools. For the alignment between two CLR amino acid sequences EMBOSS Needle might be used. For simultaneous alignment of multiple CLRs Clustal Omega might be used. However, the aforementioned tools have certain limitations, as the primary amino acid sequences will not consider the protein structure of the CLRs. Therefore, structure-based homology modeling is a sophisticated approach to screen for the presence of CLRs domains in the target sequence. The three-dimensional structure of the target sequence can be constructed based on experimentally validated related protein structures as templates by using SWISS-MODEL, a homology modeling portal [78,79]. This approach is useful but still has certain limitations, in particular for identifying novel CLRs, when there are no related protein structures available for a given animal species. In this case, phylogenetic analysis by computational simulation of CLRs may represent an alternative strategy, e.g. screening the conserved domains of CLRs across species [80]. Another approach is the primary amino acid sequence based three-dimensional structure prediction, by using Alpha Fold 2 [74] and RoseTTAFold [75].

3. Overview of myeloid CLR ligands

Myeloid CLRs selectively bind to a variety of ligands derived from pathogens and self-antigens, referred to as PAMPs and DAMPs, respectively, via their CTLDs. CLR binding is not restricted to glycans, as CLRs have also been shown to bind non-glycan ligands, such as proteins and lipids [20,28,81]. Additionally, several inorganic crystals were also reported as CLR ligands. Thus, CLR, MGL, was also shown to bind to β-glucan from fun-gal cell walls (Fig. 4) [82]. As previous reviews have described glycan ligands of CLRs in great details (see, for instance, [83]), only a few examples are shown for the recognition of pathogen-derived glycans by myeloid CLRs will be mentioned in the following, of which only β-glucan recognition by Dectin-1 will be more detailed.

Human Langerin binds to a conserved β-1,4-linked GlcNAc that is found on the wall teichoic acid (WTA) of Staphylococcus aureus and thereby contributes to immunomodulation [84]. Another CLR, MGL, was also shown to bind to S. aureus strain ST395 in a GalNAC-dependent fashion [85]. The Gram-negative bacterium Yersinia pestis interacts with Langerin via the core oligosaccharide of its lipopolysaccharide (LPS), thereby facilitating Y. pestis dissemination. Similarly, DC-SIGN has been shown to recognize the core oligosaccharide of Y. pestis [86]. It was also studied that the opportunistic bacterium Haemophilus alvei binds to Dectin-2 via its mannosylated O-antigen of LPS and that the loss of mannosylation inhibits this binding [87].

Glycan structures of viruses are also recognized by myeloid CLRs. For instance, N-linked glycans of numerous viruses, such as Human immunodeficiency virus 1 (HIV-1), SARS coronavirus (SARS-CoV), Ebola virus, West Nile virus, Nipah virus and Newcastle virus are recognized by DC-SIGN [32]. DC-SIGN often recognizes Lewis (Le) antigens in viral glycoproteins, e.g. the Gal-β-1,4-(Fuc-α-1,3)-GlcNAc (Le(x)) trisaccharide; however, binding to
oligomannose and biantennary N-glycans expressing Le(x) was also observed [88]. Additional examples for CLR-virus interactions include the MMR, which binds to the gp120 glycoprotein of HIV via its oligomannose N-glycans, and to Dengue virus [89], and MGL that binds to the GP2 envelope protein of Ebola virus via its Gal and GalNAc residues [90].

CLRs are also famously known to contribute to fungal recognition by interacting with fungal glycans. For instance, the fungal pathogen *Candida albicans* can be recognized by Dectin-1 through β-glucans and by the CLRs Dectin-2, MMR, and DC-SIGN via the mannan structures expressed in its cell wall [91]. A study has revealed that these CLRs recognize cell wall epitopes that are differentially expressed in different growth phases of *C. albicans* [92]. More specifically, MMR and DC-SIGN bind to the terminal Man residues of the N-mannan chains, whereas Dectin-2 binds to the core N-mannan that are present deeper in the cell wall [92]. The ability of Dectin-1 to bind to β-glucans enables it to recognize multiple fungal pathogens, such as *Aspergillus*, *Histoplasma* and *Pneumocystis* species [93–95]. Similarly to Dectin-1, Langerin binds to β-glucan, but also to mannans of *Candida*, *Saccharomyces* and *Malassezia* species [96].

3.1.1. β-Glucans binding to Dectin-1

β-Glucans are homopolymers of β-glucose linked in β-1,3 and are a well-characterized ligand of Dectin-1 due to its presence in fungal cell walls [83]; β-glucans exist in diverse forms, according to their source and type of branching (short or long β-1,6-branching). Many aspects are known to influence its immunomodulatory activity such as its molecular weight, conformations, solubility and particle size (Fig. 4) [97]. More specifically, a minimum length of 10 Glc residues is required for recognition by Dectin-1 [98]. Additionally, Dectin-1 comprises two anti-parallel β-sheets and two α-helices and binds to β-glucans in a Ca²⁺-independent manner [18]. Structural data obtained from X-ray diffraction studies have shown that the two amino acids His223 and Trp221 are critical for ligand binding, which also explains why they are well-conserved across different animal species. Additionally, a hydrophobic amino acid stretch between Trp221 and His223 was predicted by computational graphical rapid analysis to potentiate β-glucan binding. Furthermore, a combination of hydrophobic packing and hydrogen bond formation between the side chains of the amino acids Tyr228 and Gln230 also contributes to the binding of Dectin-1 to β-glucans [99,100].

3.2. (Glyco-)lipids as myeloid CLR ligands

Trehalose-6,6-dimycolate (TDM; also known as cord factor) is a mycobacterial glycolipid derived from the cell wall of *Mycobacteria (M. tuberculosis)*, and was identified as a Mincle ligand (Fig. 5) [101]. TDM binding to Mincle is a Ca²⁺-dependent interaction and depends on both the trehalose residue and the lipid tail of TDM [102,103]. The trehalose moiety binds to Mincle via its EPN motif and this interaction is stabilized by two Ca²⁺ ions. The EPN-adjacent amino acids Asn and Asp are conserved, and the asparagine residue is responsible for Ca²⁺ coordination. Hydrophobic amino acid stretches have been identified around the Ca²⁺ and glycan binding sites of Mincle (Val195, Thr196, Phe198, Leu199, Tyr201, and Phe202). Site-directed mutagenesis, where the Phe198 and Leu199 amino acids were substituted with Ala, led to a significant loss of binding to TDM. To further demonstrate the importance of the binding site for the lipid moiety, the hydrophobic residues (195 to 202) were substituted by the respective amino acids from Dectin-2 (residues 192 to 199). Reporter cell-based assays also showed a significant loss of binding after introducing a R183V mutation, thus identifying Arg183 as the key amino acid involved in lipid binding [47]. TDM derivatives containing different acyl chains (C8, C10, C12) were synthesized and studied for Mincle binding by surface plasmon resonance. It was shown that a minimal carbon chain length of 10 was required for binding to Mincle [47]. Another glycolipid recognized by Mincle is monogluco
disacylglycerol (MGDG), the anchor of lipoteichoic acid expressed by Group A streptococcus [104]. Another component from lipoteichoic acid, digluco
disacylglycerol (DGDC), was also shown to bind to Mincle. However, while the interaction of MGDG with Mincle activates its signaling pathway, DGDC acts as a Mincle antagonist by blocking the MGDG-mediated activation of Mincle [104].

3.3. (Glyco-)proteins as myeloid CLR ligands

The CLR CLEC2 (CLEC1B) was described to be mainly expressed in platelets as well as in monocytes and DCs and is involved in platelet activation [105]. CLEC2 is expressed as a monomer in humans and mice and its CTLD has two antiparallel β-sheets with two addi-
The mucin-type transmembrane glycoprotein podoplanin (PDPN) was identified as a ligand for platelet-expressed CLEC2 and binds to CLEC2 in a glycan-dependent manner (Fig. 6). PDPN contains multiple O-glycosylation sites with a significant number of O-glycans. PDPN is expressed by various organs and cells, including kidney, lungs, osteoblasts, and brain. So far, PDPN is the only known endogenous CLEC2 ligand (in addition to the rhodocytin from snake venom, which is an exogenous ligand for CLEC2) [106]. X-ray crystallographic studies of CLEC2 complexed with PDPN provided insights into their binding. CLEC2 lacks the canonical Ca²⁺-binding site for carbohydrate recognition and binds the O-glycosylated peptide ligand on the side face (α1, α2, β1, and β2) with no calcium assistance [107]. Characteristic of the ligand-binding site on CLEC2 is a clustering of Arg residues. Receptor specificity critically depends on a two-locus interaction involving both the polypeptide and the carbohydrate of PDPN, thus illustrating how a common O-glycan can be recognized as unique due to a specific adjacent peptide motif. Thus, the CLEC2-PDPN interaction serves as a great example of a dual binding mode that may well be a fundamental principle for the specific recognition of different O-glycosylated glycoproteins by a lectin [107].

### 3.4. Crystalline structures as myeloid CLR ligands

MICL is an ITIM-containing CLR that displays a strong affinity towards damaged/dead cells. Monosodium urate (MSU) crystals were identified as a ligand for MICL, as blocking of MSU crystal formation by allopurinol treatment and protein digestion prevented the binding of MICL to the treated cells (Fig. 7) [108]. MSU crystals are formed due to the high amount of circulating uric acid in the blood, and in the presence of endogenous cations, urate crystals form and deposit in tissues, thereby leading to gout disease [109]. Upon MICL binding to MSU crystals, innate responses are downregulated by dampening the SYK-mediated production of reactive oxygen species (ROS) [108]. Another crystal, hemozoin (which is a crystal composed of hematin, a waste product from Plasmodium infections, the causal agent of malaria), was recently identified as ligand of MICL (Fig. 7) [71]. Deposition of crystalline hemozoin is a means for the parasite to detoxify its environment...
by neutralizing the free heme groups produced by its feeding upon haemoglobin \[110,111\]. As such, Plasmodium-derived hemozoin was shown to bind to and activate MICAL, as this interaction is critical for the induction of cerebral malaria \[71\]. Another example of a crystalline ligand for a CLR is that of cholesterol sulfate crystals that were shown to bind to human Mincle, but not to mouse or rat Mincle. Further investigation confirmed that the binding of cholesterol was mediated by a cholesterol recognition amino acid consensus motif \((L/V)x1–5Yx1–5(R/K))\), which is present only in human Mincle \((LxY^{125}xxxxxR)\). Deletion of this consensus sequence prevented Mincle binding and the activation of Mincle reporter cells \[112\].

4. Methods for CLR-ligand investigations

Unraveling CLR-ligand interactions with regards to their specificity, affinity and biological functions is crucial for understanding CLR-ligand interactions and evaluating their clinical relevance. Therapeutic interventions may include the modulation of pathological processes by specifically targeting selected CLRs (further reviewed in \[113\]). While some CLR-ligand interactions are well characterized (e.g. Dectin-1 with β-glucans (see section 3)), the majority of CLR-ligand repertoires remains to be uncovered. Approaches to investigate CLR-ligand interactions include (1) bioinformatic approaches predicting CLR-glycan interactions guided by machine learning ("in silico methods"), (2) methods focusing on CLR-ligand screening ("qualitative methods"), (3) measuring the affinity between CLRs and their respective ligand(s) ("quantitative methods") and (4) methods to investigate functional roles of CLR-ligand interactions ("mechanistic methods"). Each method possesses its characteristic advantages and drawbacks that should be considered and are laid out below and summarized in Table 1. While each method can be employed at each stage of a given project, a pre-screening of potential interactions, followed

| Method | Advantages | Drawbacks |
|--------|------------|-----------|
| LectinOracle | - Ideal in narrowing down the experimental search space | - Purely in silico |
| ELISA | - Fast and relatively inexpensive | - Only able to give very generalized output |
| Glycan Microarray | - Fast and high-throughput screening | - Difficulties in making predictions for lectins with multiple binding sites with different specificities |
| Immuno-blot/ Immuno-TLC | - Ideal for heterogeneous mixtures containing potential ligand(s), due to separation by size or polarity before staining | - Protein aggregation on the plate can lead to false-positive results |
| Flow cytometry | - Semi-quantitative comparisons possible | - Only limited information is obtained about the ligand |
| Fluorescence microscopy | - Visualization of CLR interactions with single bacteria | - Relatively expensive |
| FRET | - Relatively rapid measurement | - Influence of image acquisition scanner and the fluorophore as a determinant of sensitivity |
| ITC | - Label free | - Different running and optimized conditions required for resolving and detecting a variety of ligands |
| QCM | - Label free | - Time-consuming |
| DLS | - High sensitivity | - Often limited to ligands present on the surface of pathogens |
| SPR | - Real-time measurement | - Time consuming |
| MD simulations | - Predictions of molecular dynamics over time | - Requires advanced staining protocols |
| Radioactive assays | - Highly sensitive assay | - Sensitive to fluctuations in the temperature |
| Reporter cell assays | - Allows investigation of intracellular signaling | - Sensitivity to aggregation |
| Knock-out biological models | - Important insights into biological functions | - Sensitive to fluctuations in the temperature |

Table 1 Overview of advantages and drawbacks of selected methods to investigate CLR-ligand interactions.
LectinOracle

Protein data
e.g. from „ESM-1b“

Glycan data
e.g. from „SweetNet“

Continuous improvement

Quick screening of CLR-glycan interactions

AI suggested candidates

Organism of interest
e.g. pathogens or host cells

CLR-Fc fusion proteins

ECD + Fc part of Ig = Dimeric fusion protein

Detection:

Potential ligand

Anti-Fc antibody

ELISA

Fast & relatively inexpensive

Ideal for initial pre-screenings

Glycan microarray

Fast & high-throughput screening

Control over orientation and density of ligand

Fluorescence Microscopy

Visualizes localization of CLR/cell interactions

Allows for subcellular localization of CLR-ligand

Flow cytometry

Single-cell analysis

Gating: exclude unwanted events & include relevant ones

Immunoblot

Prior separation by size via SDS-PAGE of heterogeneous mixture

Immuno-TLC

Prior separation by polarity/hydrophobicity via TLC of heterogeneous mixture

Reporter cell assays

Allows to identify agonistic CLR ligands as intracellular signaling is coupled to a reporter gene

KO assays

Provides insight into the biological functions of certain CLR-ligand interactions
by a more in-depth analysis of a few of those interactions, is usually recommended.

4.1. In silico methods/predicting novel CLR-glycan interactions using LectinOracle

Even though experimental screening methods are continuously advancing, it is difficult to comprehensively screen CLRs for glycan motif specificity only by using wet-lab techniques. However, advancing computational power helps to confine candidate ligands to later validate them experimentally. Machine-learning software can help to identify novel CLRs from sequencing data (see 2.6). Similar concepts can also be applied to identify novel CLR-glycan interactions. In the field of glycobiology, SweetNet serves as a deep learning algorithm using graph convolutional neural networks [114] especially optimized for the branching structure of glycans [115]. Using this data, Lundstrom et al. developed LectinOracle [116], which is a deep learning tool to predict interactions of glycans and lectins. By combining glycan structure data (from SweetNet) and amino acid sequence-based protein data (from Evolutionary Scale Modeling 1b [ESM-1b]) as input, LectinOracle is able to output increasingly accurate predictions about the interaction and binding between the two pairs (Fig. 8). By employing a curated data set of individual protein-glycan interactions, Lundstrom et al. confirmed that LectinOracle predictions match literature-annotated specificities for a broad variety of lectins. While the software gives highly accurate predictions for lectins with single binding sites, lectins with multiple binding sites like DC-SIGN may still pose a problem [116]. Ultimately, machine learning guided predictions of CLR-ligand interactions are a helpful tool for pre-selection of candidate interactions. However, careful curation of the predictions by experienced personnel and subsequent experimental screening approaches are further required.

4.2. Qualitative/screening methods using CLR-based binding studies

To identify and characterize agonistic ligands, several in vitro assays are available. Libraries of CLRs in different formats expressed with protein-tags such as His-tag, Biotin-tag or Fc-tag are successfully used for routine ligand screening studies [117,118]. Among those, CLR-Fc fusion protein-based binding studies were performed to identify novel CLR-ligand interactions [41,92,119–124]. These libraries include different species, in particular human [125], murine [126] and ovine CLRs [127], as well as invertebrate CLRs [128]. Structurally, these constructs often consist of the Fc part of immunoglobulin (lg) molecules (usually human IgG1 [hFc]) fused to the ECD of the corresponding CLR. The common Fc part allows for an easy one-step purification by affinity chromatography and for standardized detection when using secondary anti-Fc antibodies, conjugated to either a fluorescent dye or an enzyme. Most CLR-Fc fusion proteins are designed to form a soluble, dimeric protein once expressed. Its dimeric form allows for bivalent presentation of the ECD, which increases its avidity and allows for detection in various assays (Fig. 8). Moreover, adaptations in the cloning strategies would also allow for the generation of either C- or N-terminal fusion proteins and the construction of mono- or polymeric fusion proteins [123,129] which could more accurately reflect the multivalent binding of some CLRs, e.g. the tetrameric DC-SIGN [130]. The construction of and screening using CLR-Fc fusion proteins was previously described [125,131]. Some of these assays include ELISA-, microarray-, immunoblot-/TLC-, flow cytometry- and fluorescence microscopy-based binding studies.

ELISA - Enzyme-linked immunosorbent assay (ELISA)-based binding studies to screen for novel CLR ligands usually work by immobilizing the potential ligand in a plate-based format. Subsequently, binding of the CLR-Fc fusion proteins to the ligand is visualized using an appropriate secondary antibody, often followed by a colorimetric reaction with the intensity being a linear parameter for the binding kinetics [132]. As ELISA-based studies are relatively cheap, fast and high-throughput, they may be used for pre-screening studies of CLR-ligand interactions (Fig. 8). In many cases, success of the screening is dependent on the presentation of the biomolecules to be analyzed, as insufficient accessibility of the ECD to the potential ligand site might lead to false-negative results (e.g. micelle formation for glycolipid molecules) while extensive protein aggregation on the plate could lead to false-positive results (e.g. unspecific interaction with certain kinds of micro plastics) [125].

Glycan Microarray - In glycan microarrays, potential glycoconjugate ligands are immobilized on a glass slide or a wafer. Similar to ELISAs, the samples are subsequently incubated with soluble fusion proteins followed by the detection using secondary antibodies tagged with a fluorophore. The glycan microarray technology allows for fast and high-throughput screening [133] with typically large numbers of different glycoconjugate ligands to be screened for CLR interactions. Printing of the glycans in a spatially controlled fashion allows for the adjustment of orientation and density of the ligands (Fig. 8). Advancements in glycan synthesis in recent years allow for more targeted holistic approaches: For instance, microbe glycan arrays make use of large libraries of natural or synthetic pathogen-focused glycans. In a recent study, binding specificities of CLRs could be screened against a whole array of synthetic bacterial glycan structures [134]. Shotgun microarrays focus more specifically on a single organism, cell or tissue by separating glycosphingolipids using multidimensional chromatography followed by printing them on glass slides. This way, the whole glycome of a given organism can be fractioned and exposed for binding assessment [135]. The neoglycolipid technology links oligosaccharides to an amino-phospholipid tag to construct artificial glycolipids. The presentation of oligosaccharides in this manner allows for increased flexibility of the molecules to more closely represent the display of glycans on cell surfaces [136]. Based on the neoglycolipid technology, the Beam Search Array approach is a process to

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*Fig. 8.* Overview of selected methods to investigate CLR-ligand interactions. To identify and characterize CLR-ligands, several assays are available which are exemplified using a model organism. In silico machine-learning guided mechanisms (such as LectinOracle) can be used to pre-screen known glycans present in the model organism for their potential interactions with CLRs to identify AI suggested candidates (top). CLR-Fc fusion proteins, which consist of the ECD of the respective CLR and the Fc part of IgG, can be used in several in vitro assays (middle box). ELISA and glycan-microarrays may be used for broader pre-screenings. Immunoblot and TLC assays are attractive approaches to separate molecules from lysates by size (Blot) or polarity/hydrophobicity (TLC) before staining. Fluorescence microscopy can be used to visualize CLR-cell interactions, thereby locating the position of potential ligands. Flow cytometry can be used for single cell analysis while gating allows to exclude unwanted events. Mechanistic studies (bottom) are useful to investigate downstream effects of CLR-ligand interactions. Reporter cells enables the identification of agonistic CLR ligands as their intracellular signaling is linked to an expression cassette (e.g. GFP-signal). Knockouts of respective CLRs in mice provide important insights onto the biological functions of certain CLR-ligand interactions. Abbrev.: ESM-1b = Evolutionary Scale Modeling 1b; Al = artificial intelligence; ECD = extracellular domain; Fc = fragment crystallizable; Ig = Immunoglobulin; ELISA = enzyme-linked immunosorbent-assay; TLC = thin-layer chromatography; KO = knockout; GFP = green fluorescent protein; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis. All depicted glycans are following the symbol nomenclature for glycans guidelines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
characterize glycan ligands from natural sources [137] similar to Shotgun microarrays. However, while Shotgun microarrays focus more on a holistic approach to analyze all individual ligands of a glycome, the Beam Search approach is an iterative, ligand-guided process focusing on the most biologically relevant hits [138].

**Immunoblot/Immunol-TLC** - Immuno-blotting and immuno-TLC are assays that can be used to identify (glyco-)proteins and (glyco-)lipid ligand candidates, respectively. Immuno-blotting usually involves the separation of a mixture of molecules (usually proteins) according to their size by SDS-PAGE or native-PAGE before the transfer to a membrane, while immuno-TLC involves the separation of a mixture of molecules (usually lipids according to polarity/hydrophobicity) by thin-layer chromatography using silica-coated plates [139,140]. Once the separated molecules have been immobilized, both approaches detect potential ligand interactions by incubation with the fusion proteins of interest and an appropriate conjugate (Fig. 8). As both techniques involve a separation procedure of the sample before detection, they are attractive methods when working with heterogeneous mixture (e.g. lysates of pathogens or tissue samples) [141].

**Flow cytometry** - Flow cytometry-based binding studies are usually used to investigate CLR-cell interactions, e.g. to determine CLR binding to a potential ligand on the surface of a pathogen. To this end, cells of interest are incubated with the CLR-Fc fusion protein, followed by detection with a secondary, fluorescently labeled antibody in solution, thereby avoiding the problem of protein aggregation as sometimes seen in ELISA-based assays. Afterwards, the samples are directed through a set of lasers which allows for a rapid, single-cell analysis based on light scattering- and fluorescent characteristics (Fig. 8). Additionally, the possibility of using viability dyes and associated gating allows to exclude unspecific signals that are caused by cell debris [125].

**Fluorescence microscopy** - CLR-Fc fusion protein-based binding studies can also be done by fluorescence microscopy. Analysis is performed following basic immunofluorescence staining procedures: fixation of the sample, binding of the CLR-Fc fusion protein and secondary antibody, coating on a cover slip, embedding and analysis using a fluorescence microscope. This makes fluorescence microscopy-based binding studies a valuable approach to visualize localization of CLR-cell interactions by directly observing the sub-cellular localization of the ligand (Fig. 8) [71,141,142].

### 4.3. Quantitative/affinity methods

Interactions of single glycans with CLRs are usually weak, thus often requiring multimeric display in natural settings. Therefore, measuring CLR-ligand affinity is a means to quantitatively assess a CLR-ligand interaction. For affinity measurements, several methods are available, of which only a few will be briefly mentioned below. Many of these methods permit the investigation of multivalency effects by using scaffold material for ligands to present multiple copies of potential ligand candidates (for a more in-depth review see [143]).

**FRET** - Förster resonance energy transfer (FRET) describes the transfer of energy between an excited donor fluorophore and an acceptor fluorophore by a non-radiative process. As this process is highly dependent on the distance between both (with a maximum of 1 to 10 nm), it can be used to measure the affinity between two molecules [144]. A putative ligand is associated to fluorescently tagged or inherently luminescent material (donor) and put into proximity of a fluorescently labeled CLR (acceptor). Increasing affinity is therefore measured with increasing fluorescent signal from the acceptor.

**Fluorescence quenching** - Fluorescence quenching describes the ability of certain materials (like gold) to quench fluorescent signals [145]. By this mechanism, potential ligands can be associated to gold-particles and put into proximity with fluorescently labeled CLRs. In this case, higher affinity is visualized by decreased fluorescent signal as it will be increasingly quenched by the gold particle the closer the CLR comes to its ligand.

**ITC** - Isothermal titration calorimetry (ITC) is a method to measure heat which is absorbed or released from bio-molecular interactions. By titrating a putative ligand into a solution that contains the CLR of interest, the heat released or absorbed in this process can be measured [146,147]. As ITC requires neither chemical modifications of the ligand nor the receptor, it has the advantage of being a label-free non-destructive technique. However, the measurement is also prone to fluctuations as every source of heat (external or from other reactions within) can impact the readings and thereby complicate their analysis. Additionally, occasional formation of aggregates also negatively impacts the readings on ITC which makes it especially prone to reactions with multivalent ligands [143].

**QCM** - Quartz crystal microbalance (QCM) makes use of physical properties of piezoelectric quartz crystals. When an electric current is applied to piezoelectric crystals, they begin to oscillate. The resonant frequency at which they oscillate is critically determined by their mass. As the frequency directly correlates with the mass, changes due to aggregations can indicate a binding event [146]. Therefore, these crystals can be coated with possible ligands and if a CLR in solution binds, this will change the oscillation which is measured. Similar to ITC, QCM has the advantage of being a label-free method.

**DLS** - Dynamic light scattering (DLS) depends on the physical correlation between the size of a particle with its movement speed following a random movement pattern (Brownian motion), given that the particle is in a dilute solution without any particle–particle interactions. If these requirements are met, DLS can be used to determine CLR-ligand affinities, as interactions of CLRs and potential ligands would lead to agglomerates with an increased particle size [149].

**SPR** - Surface plasmon resonance (SPR)-based assays determine interactions of biomolecules (immobilized on a thin metal film coated on a sensor) and analytes (in solution) based on optical measurements of the refractive index. If interactions occur, this will cause the refractive index to change with binding generally increasing SPR response and dissociation generally decreasing SPR response [150]. This allows for a label-free real-time measurements of kinetics with usually small amounts of sample [151]. As an example, SPR was used to show that Langerin binding affinity increases along with the number of mannosides [152].

**MD simulations** - Molecular dynamics (MD) simulations is one of many techniques to determine macromolecular structures. However, most techniques only allow to take single snapshots of a molecule, thereby often neglecting its flexibility. Instead of cumulating single experimental structures under different conditions (environment, complexation with other macromolecules) in an approach to understand the dynamics, MD simulations may be performed. Using computational simulation, the movement of every atom in a protein or other molecular system may be predicted over time based on interatomic interactions [153,154]. For example, MD simulations were instrumental in understanding why certain glycan moieties bind to a particular CLR, by visualizing the conformational rearrangement in the interaction of Mincle with TDM [155].

**NMR spectroscopy** - Nuclear magnet resonance (NMR) spectroscopy is a technique primarily focused on determining the molecular structure of a molecule. When a molecule is exposed to a powerful magnetic field, the individual nuclei of elemental isotopes will start to spin in a characteristic manner [156]. Analysis of these spins provides a map of how the individual atoms are chemically linked, how close they are in space and how rapidly they
move with respect to each other. Coupled with MD simulations, NMR spectroscopy allowed to visualize the high flexibility of the CRD in MGL upon binding of GalNAc [157].

Radioimmunoassays - For Radioimmunoassays (RIA), a known antigen is radiolabeled and incubated with an analyte molecule to facilitate binding. By introducing an unlabeled version of the antigen (from a sample), competition for the analyte molecule will occur. Increasing amounts of the unlabeled version will lead to a higher dissociation of the radiolabeled variant which can be measured [158] and used to screen for CLR ligands in e.g. serum samples.

4.4. Mechanistic methods

Mechanistic studies are useful to investigate downstream effects, e.g. functional consequences, of CLR-ligand interactions and include methods such as reporter cell-based assays or in vivo experiments.

Reporter cell assays - Reporter cell-based assays use cells engineered to express selected CLRs on their surface. In these reporter systems, intracellular signaling is coupled to an expression cassette with activation of a fluorescent signal (e.g. coupling nuclear factor of activated T cells (NFAT) to GFP in T cell hybridoma cells [62,108]) or secretion of an analyte molecule (e.g. secretion of secreted alkaline phosphatase in HEK293 cells) (Fig. 8) [159]. In addition, cell-based fragment screening assays were developed that allow for a sensitive identification of fragment hits in a physiologically relevant environment [160].

Knockout approaches - Gene knockouts (KO) refer to genetic techniques to render selected genes of an organism inoperative to selectively study the function of the respective gene (or lack thereof). In vivo studies comparing wild-type vs CLR-deficient mice provide important insights into the biological functions of certain CLR-ligand interactions (Fig. 8). However, redundancy in binding specificities of CLRs and other receptors (e.g. recognition of Mycobacterium tuberculosis by TLR2 and Dectin-1) [161] or among CLRs (e.g. recognition of Candida albicans by Dectin-1 and Mincle) [162,163] can pose difficulties to demonstrate the importance of a single CLR in vivo [164]. Additionally, when comparing different species, CLR repertoires can markedly differ (e.g. the DC-SIGN family members) and their expression patterns are not always overlapping (e.g. expression of Langerin on different subsets of dendritic cells [165]). A reverse strategy would involve the knockout or potential (glyco-)ligands instead of CLRs. In that context, glycosyltransferases (GTs) represent the more attractive targets as the role of most GTs in the biosynthesis of specific glycoconjugates and glycan structures is well defined [166,167] and mainly involves the initiation, immediate core extension and/or branching of distinct types of glycoconjugates [168]. Thus, the group of Clausen et al. facilitated the use of the CRISPR/Cas9 gene editing tool to examine the roles of the glycome by genetic dissection of biosynthetic pathways rather than by direct glycan analysis [169], which could also be adaptably used for roles of glycoconjugates in CLR research as well.

In summary, a plethora of methodological approaches are available to help investigate the multitude of CLR-ligand interactions. Usually, a combination of selected assays is necessary to fully unravel the interaction of a CLR with a selected ligand as it is highly dependent on the pathogen or DAMP in question.

5. Functional aspects of ligand recognition

This section will highlight how myeloid CLR engagement by ligands orchestrates and influences immunological responses. Prerequisites for well-balanced immune responses and maintenance of homeostasis comprise the discrimination between self- and non-self-ligands, signal transduction from the extracellular environment to the intracellular compartment, and the crosstalk of CLRs with other classes of PRRs mediated by signal integration within cytosolic pathways.

5.1. Self- vs non-self-discrimination by molecular cues

The immune system discriminates between self- and non-self-(Fig. 9) to enable protective responses against pathogens while leaving the hosts healthy tissue intact [15]. Some exceptions are the microbiota (of non-self origin) that promote well-being and homeostasis for the host and diseases like cancer (“aberrant” self) that are detrimental to the host well-being [170]. Carbohydrates, (glyco-)proteins, and (glyco-)lipids constitute important molecular cues used in discrimination between non-hazardous self (referred to as self-associated molecular patterns or SAMPs) and danger signals for myeloid CLRs [16,171]. Selected examples will follow to highlight the role played by myeloid CLRs in self-/non-self-discrimination by notably comparing mammalian and bacterial glycoconjugates.

Mammals - Mammals express a plethora of glycoconjugates distant from bacteria. An omnipresent group of glycoconjugates in mammals are glycoproteins, of which roughly 90 % of characterized glycoproteins are known to contain N-glycans or both N- and O-glycans, which serves to further illustrate their omnipresence [172]. The core of mammalian N-glycans consists of two GlcNAc followed by three Man residues. Differential trimming, branching and elongating activities result in the formation of different classes of N-glycans, such as oligomannose, hybrid and complex glycans. Regarding O-glycosylation, GalNAc residues are linked to Ser and Thr residues and constitutes the most prevalent and relevant form of O-glycan encountered at the cell surface. O-GalNAc glycans range from single monosaccharides (the Tn antigen) to linear or branched glycans of more than 20 saccharides composed of Gal, Fuc, GlcNAc and/or sialic acid residues. Overall, N- and O-glycans are the two most prominent forms of protein glycosylation in mammals [173].

DCR1-mediated recognition of self asialo-biantennary N-glycans, as an example, was shown to play a crucial role in preventing autoimmune diseases [174]. Aberrant glycosylation patterns are extremely useful for the detection of malignant cells, exemplified by the large number of glycans that are employed as tumor markers [175,176]. Among the best described characteristics of malignant cell-derived glycans are glycan truncation as seen in Tn and T antigens, glycan over-expression, loss of expression, altered branching of N-glycans or peculiar patterns of fucosylation and/or sialylation [177–180]. For example, DC-SIGN recognizes Lea/Leb and Lea/Leb glycans on the carcinoembryonic antigen of colorectal tumors [181,182]. On the other hand, Dectin-1 interaction with Galectin-9 can suppress adaptive immune responses, thus promoting tumor growth in pancreatic ductal adenocarcinoma [183].

In contrast to malignant cells, necrotic cell death is characterized by the release of intracellular components into the extracellular space. One example for this is Mincle that recognizes the spliceosome-associated protein 130 (SAP130), which serves as a marker for danger as it is normally absent from the extracellular space (Fig. 9). Upon binding of Mincle to SAP130, pro-inflammatory cytokines enable the recruitment of neutrophils [62]. Similarly, F-actin, an important component of the cytoskeleton that also serves as a signal for cell necrosis, can activate the dendritic cell natural killer lectin group receptor-1 (DNLRG1; CLEC9A) (Fig. 9). While DNLRG-1 does not induce measurable differences in cytokine production, it functions by facilitating antigen cross-presentation [184]. Furthermore, MiCL was described to rec-
The repertoire of surface-exposed glycoconjugates in bacteria is largely dependent on the general classification into Gram-negative and Gram-positive. A common feature of both classes is the cytoplasmic membrane which is covered by peptidoglycan towards the extracellular space and glycosylated surface (S)-layer proteins, which are known ligands for myeloid CLRs [159]. In Gram-negative bacteria, an additional outer membrane that contains LPS covers the peptidoglycan, which is embedded in the periplasm. Mycobacteria share the feature of an outer membrane with Gram-negative bacteria, but their lipid moieties are predominantly composed of mycolic acids linked to the peptidoglycan via arabinogalactan. In contrast, Gram-positive bacteria possess a more pronounced and surface-exposed peptidoglycan layer interfused with WTAs and lipoglycans. Besides glycoproteins, major bacterial glycan structures comprise LPS and teichoic acids [185]. LPS is embedded in the outer membrane and composed of a lipid A core, an oligosaccharide core extended by one of many structurally-diversified O-antigen polysaccharides. Most notably, LPSs are a well-known immuno-stimulating agent and described as a TLR4 ligand [186]. Interestingly, a recent study revealed that the opportunistic pathogen *Hafnia alvei* enhanced TLR4-mediated immune responses due to the recognition by Dectin-2 via its mannosylated O-antigen [87]. Moreover, not only do CLRs interact with pathogen-derived ligands to yield proinflammatory responses, but they can also promote homeostasis after engaging ligands from commensal microorganisms. For instance, it was shown that the engagement of Mincle by gut commensals improved intestinal barrier function [187] or that MCL and DCIR negatively modulate production of pro-inflammatory cytokines in an experimental colitis model [188] (Fig. 9). Furthermore, the interaction of Mincle with *Lactobacillus brevis* led to a balanced production of pro- and anti-inflammatory cytokines that serves to maintain homeostasis [159]. A similar effect was observed for DC-SIGN in response to *Lactobacillus acidophilus* where the interaction led to an increase in interleukin (IL)-10 secretion [189].

5.2. Intracellular signaling upon CLR engagement

Ligand binding to cell surface receptors is transmitted across the cell membrane into the cell via different routes (Fig. 9). CLRs utilize intracellular signaling motifs, which recruit kinases or phosphatases (detailed description provided in section 2). Upon PAMP or DAMP recognition, CLRs often utilize activation signals to enhance immune responses from other PRRs. Notably, the down-regulation of immune responses against commensals or SAMPs and/or the prevention of over-shooting immune responses by CLRs is equally important. Here, we will begin by presenting the signal pathway from Dectin-1 before we further address different aspects of CLR signal modulation.

5.2.1. Dectin-1 engagement and the resulting signaling pathway

A well-described CLR that mediates signal transduction via a hemiITAM is Dectin-1. Upon ligand binding, the intracellular YxxL motif becomes phosphorylated at the Tyr residues (Fig. 9). Phosphorylated Tyr then constitutive an attachment site for SYK that binds via its SH2 domain [68,190]. This interaction between the YxxL motif and SYK induces conformational changes within SYK that lead to its autophosphorylation and activation [191]. Via the activation of F-actin and oxLDL or SAP130 from damaged host cells)

![Diagram of intracellular signaling upon CLR engagement](image-url)
Consequently, these transcription factors induce transcriptional changes that leads to alterations in immune effector functions (Fig. 9). These can include ROS production and the secretion of cytokines such as the pro-inflammatory cytokines tumor necrosis factor (TNF), IL-6 and IL-23 and also anti-inflammatory cytokines such as IL-10 [195,200–202].

5.3. Signaling flexibility

Intracellular CLR signaling motifs can provide the first clues about the different signaling cascades that become activated. Despite the common distinction between “inhibitory” and “activating” CLRs, the signaling motif alone cannot be used to reliably predict the role of a given CLR on shifting immune responses towards either inflammation or tolerance. Instead, canonical responses are complemented by several additional factors throughout the signaling pathways (reviewed in detail in [63,203]). Thereby, signaling flexibility among members of the same (hem-)ITAM, ITIM or ITAM/ITIM-independent class, and even for a single CLR, is possible. When addressing the flexibility of CLR signaling in the following section, the major focuses will be laid on the signaling differences based on ligand nature, receptor attributes and signal integration. Subsequently, downstream effects such as alterations in gene transcription will be discussed.

Numerous examples of CLRs illustrate the great influence of ligand nature on CLR signaling. For instance, engagement of MGL can generate distinct phenotypic profiles of DCs (reviewed by [204]). While the epithelial cancer-associated Tn-MUC1/STR glycoprotein was found to be restricted to the human leukocyte antigen (HLA) II pathway, the smaller Tn-MUC1/STR could also be found within the HLA I pathway for major histocompatibility complex (MHC) class II or MHC class I presentation to CD4+ or CD8+ T cells, respectively [205,206]. Interestingly, the ligand nature influences cargo processing after MGL engagement and endocytosis, and also DC and T cell fates. Thus, ligand size and solubility, but also multivalent CLR-ligand interactions may govern the micromilieu around the receptor. Accordingly, the intensity and duration of intracellular signaling but also the quality of the initiated immune response could be affected (this concept was reviewed by [203]). While high affinity/avidity ligands may induce the classical recruitment of kinases and induce activation, hypophosphorylation can be caused by phosphatase recruitment to the ITAM, which is referred to as “inhibitory ITAM” [191,207]. For some CLR-ligand interactions, the prevalence of a “phagocytic synapse” has been suggested. Generally, CLR ligands associated with large particles seem to induce stronger activation signals. This correlation could be caused by slower phagocytosis and a delayed disassembly of the increasing number of continuously signaling adaptor proteins including kinases [203,208,209].

Besides ligand nature, receptor attributes can have important effects on the observed cellular effects of CLR-ligand interactions. While the focus is often laid on known CLR signaling motifs, several studies indicate that additional motifs can play crucial roles in CLR-mediated signaling. In DNGR-1, an Ile that directly precedes the Tyr from the hemITAM affected cytokine production in response to receptor-ligand interaction. While DNGR-1 is incapable of inducing cytokine release in response to curdlan binding, replacement of the Ile by a Gly residue (to resemble the hemITAM of Dectin-1) abolishes the lack in responsiveness [210]. Previous studies have also highlighted the relevance of the neck region for CLR-mediated signaling. For several CLRs, the expression of different isoforms that vary in length is well known. DNGR-1 long and short isoforms, for example, differ in the presence of 26 additional amino acids residues that confer a functional role for the endocytic pathway by being responsive to changes in pH and ionic strength. Further, more, DNGR-1 long isoform enabled dimerization of the receptor [211]. Similarly, the DC-SIGN neck region is responsible for the tetramerization of the receptor in a pH-dependent manner. Also, the neck region influences the proper display of the carbohydrate recognition domain and spatial arrangement of the receptor which in turn is associated with a more diverse ligand binding capacity [58,212]. Also, the role of the neck region of MICL was addressed recently. The authors concluded that the Cys residues in the MICL neck region were essential for receptor oligomerization, but also signaling properties and expression [213]. Another important structural feature of CLRs is their glycosylation. While glycosylation of the neck region of Dectin-1 is crucial for cell surface expression of the two isoforms A and B, it also plays an important role in the functional response to ligand binding [214]. Finally, the glycosylation of the CRD is equally important, as Bloom and colleagues have demonstrated that changes in the N-glycosylation of the CRD from DCIR affected its ligand affinity and subsequent intracellular signaling [215].

A commonly recognized characteristic of CLRs is their capacity to form homo- and heterodimers [63]. The neck region constitutes a major factor of whether a CLR is presented in either a monomeric or multimeric form, based on the prevalence of Cys residues. Classical examples for homo dimers are the CLRs MGL and Langerin. Another example that was previously mentioned is that of tetrameric DC-SIGN. Typical heterodimer partners are macrophage C-type lectin (MCL; CLEC4D) and Mincle. Their dependency to each other has implications for their expression, ligand uptake and subsequent signaling, for example in response to TDM (Fig. 5). Altogether, spatial co-localization of CLRs expands their ligand binding capacities by elevating binding avidity, but also alters or combines cellular responses of the involved CLRs. Under normal circumstances, different classes of PRRs are simultaneously engaged when encountering a pathogen, which leads to the cross-talk of CLRs with other PRRs, e.g. TLRs. Besides synergistic effects upon ligand-mediated signaling, further interference is mediated by mutual up- or down-regulation of receptor expression and direct contact between CLRs and other PRRs. Such an example was recently discovered for the Dectin-2-TLR4 cross-talk in response to the LPS from H. alvei. While TLR4 is a well-known receptor of LPSs, this study showed that cytokine secretion was elevated in a Dectin-2 dependent manner and that Dectin-2 interacted with the mannosylated O-antigen of H. alvei LPS [87].

5.4. Cellular effects upon CLR engagement

When considering the biological significance of CLR-pathogen interactions, the cellular effects after CLR engagement are crucial. One important feature of many CLRs is their ability to mediate endo-/phagocytosis. As such, different cytoplasmic motifs have been associated with this function. For example, MGL contains the YENF (Tyr-Glu-Asn-Phe) motif, while DC-SIGN has a di-Leu motif, and Dec-205 (CD205) possesses the FSSVRY (Phe-Ser-Ser-Val-Arg-Tyr) motif, which are all crucial for endocytic activity of their respective CLR [216–218]. Upon phagocytosis, CLRs impacts important effector functions such as antigen processing by directing the antigen cargo to the lysosomal pathway and promoting the subsequent antigen presentation. Another example is that of the Herpes simplex virus type 1 leads to F-actin release into the extra-cellular space, which in turn is recognized by DNGR-1 and promotes the cross-presentation of the lysosomal cargo to T cells [210,219]. Moreover, myeloid CLRs can also modulate cytokine release, such as Mincle that facilitates pro-inflammatory cytokine secretion such as TNF but also nitric oxide production upon TDM recognition by Mő [101].
6. CLR-mediated immunomodulation

6.1. CLR hijacking and molecular mimicry by pathogens

Pathogens have evolved molecular strategies to hijack CLRs in order to facilitate cell entry or to dampen immune responses in favor of their survival and replication. The situation where pathogens display molecular structures that are also found on host cells was coined molecular mimicry or glycan mimicry (in the case of glycans) [220,221]. Based on the functional aspects of CLRs addressed in this review, pathogens have different possibilities for evading recognition and clearance by respective myeloid cells, such as targeting host-derived CLRs, mimicry of SAMPs by pathogens, secretion of virulence factors by pathogens, and the use of CLRs as attachment factors or entry receptors.

Inhibitory CLRs constitute a target for dampening immune responses. The ITIM-bearing CLR DCIR was shown to play an important role for the development of experimental cerebral malaria (ECM) as DCIR-deficient mice were partially protected from ECM [222]. Interestingly, the ITAM-coupled CLR Mincle can dampen Dectin-1-mediated anti-fungal immune responses elicited by and against Fonsecaea monophora. These findings suggest a role for Mincle in immune evasion enabled by signaling flexibility [223]. Another way for pathogens to evade immunity is through the secretion of virulence factors that remotely activate the immune system while leaving the pathogens intact. Examples for this evasive strategy can be found among parasites. Toxocara roundworms are known to produce excretory-secretory products as well as somatic antigens that are also recognized by CLR members such as MGL-1 and MCL [141]. Additionally, CLRs can be used as attachment factors on host cells or as entry receptors with subsequent inhibition of lysosomal degradation. Prominent examples for this kind of immune evasion can be found among viruses. The interaction between DC-SIGN and the Influenza A virus is known to facilitate cell entry [224]. Similarly, DC-SIGN can also be hijacked by Bunyaviridae (such as the Rift Valley fever virus, Uukuniemi virus and severe fever with thrombocytopenia syndrome virus) during the initial cell infections of dermal DCs after an insect bite and infection [225,226]. Moreover, DC-SIGN was also recently reported as an attachment receptor to facilitate infections by SARS-CoV-2 via the angiotensin-converting enzyme 2 receptor that is used for cell entry [227]. Furthermore, the CLRs DCIR and DC-SIGN were both described to be exploited by HIV-1. While DCIR serves as an attachment factor, DC-SIGN binds to the oligomannose gp120 and mediates cell entry (Fig. 10A) [56,228]. More examples of CLR exploitation by viruses can be found in a recent review [32].

In conclusion, CLRs are not only important receptors in host defense against pathogens but can also become the targets of pathogens. While the targeting of inhibitory CLRs produces a shift towards immune suppression, mimicry of SAMPs also promotes immune tolerance and unhindered colonization of the host. The exploitation of CLRs as attachment factors for cell entry serves as an additional example highlighting the role of CLRs as a “double-edged sword” in innate immunity. Further research is warranted to elucidate the distinct mechanistic roles of CLRs in pathogen recognition and the initiation of immune responses against those.

6.2. CLRs in trained innate immunity: Immunomodulation by Dectin-1-β-glucan interaction

A growing body of literature suggests that the innate immune system is also able to develop an immunological memory. This adaptive behavior is now generally referred to as “trained innate immunity” (reviewed in detail in [229]). Trained innate immunity describes the phenomenon of long-term functional reprogramming of innate immune cells as well as their progenitor cells.

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Fig. 10. CLR-mediated immunomodulation illustrated by evasive pathogen strategies and trained innate immunity. Engagement of CLR-signaling often leads to immunomodulation. (A) Pathogens (e.g. HIV-1) may hijack CLRs for evasion of the immune system and further dissemination. (1) DC-SIGN on the surface of DCs may serve as the entry point of HIV-1 mediated via endocytosis. (2) Once endocytosed, HIV-1 survives inside of reprogrammed non-lysosomal endosomes forming intact virions. (3) These virions can infect CD4+ T cells via the process of trans-infection. (B) Engagement of CLRs may also lead to beneficial immunomodulation, e.g. by binding of complex β-glucans via Dectin-1. This binding event will trigger reprogramming of both metabolic- as well as chromatin profiles in the cells. This establishes the foundation for increased secondary responses to restimulation by different stimuli (e.g. TLR4-β-glucan interaction) known as trained innate immunity. Abbrev.: HIV = human immunodeficiency virus; ICAM3 = intercellular adhesion molecule 3; MHC = major histocompatibility complex; TCR = T cell receptor; TCA = tricarboxylic acid cycle; TLR = Toll-like receptor; LPS = lipopolysaccharide. All depicted glycans are following the symbol nomenclature for glycans guidelines.

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S. Fischer, F. Stegmann, V.S. Gnanapragassam et al. Computational and Structural Biotechnology Journal 20 (2022) 5790–5812
[230]. Reprogramming of these cells is usually triggered by binding of stimuli to PRRs which leads to long-lasting metabolic and transcriptional alterations of the cell. After return to a non-activated state, these alterations are the basis for a modulated reaction towards a second challenge with the same or even a different stimulus [229]. In this field, the interaction of β-glucans with Dectin-1 is among one of the best characterized mechanisms to induce training. β-glucans are usually known to induce states of hyperresponsiveness in monocyctic cells with increased expression of pro-inflammatory cytokines that contribute to an enhanced ability to clear infections (Fig. 10B) [231,232].

**Role of the β-glucan structure** – As previously mentioned in section 3.1, β-glucans from different sources have different modulatory properties [233,234], based on their molecular mass, spatial stereoscopic structure, and degree of branching [100]. In general, oat-derived β-glucans are known to improve metabolic health parameters [235], while yeast-derived β-glucans are often observed to act as immune modulators [236]. In the context of trained innate immunity, most studies focus on β-glucans of high molecular weight and high degree of branching such as those from fungal origin and their distinct immunomodulatory properties [237–240].

**Signaling, metabolism, and transcription** – Intracellularly, Dectin-1 mediated trained immunity is often characterized by activation of a non-canonical Raf-1- as well as mammalian target of rapamycin/protein kinase B/hypoxia inducible factor 1α (mTOR/ AKT/HIF1α) pathways [241,242] which leads to metabolic shifts with increased glucose consumption, lactate production and NAD+/NADH ratio [243]. mTOR is generally considered a sensor of the metabolic environment [244,245] and the master regulator of glycolysis [243]. In turn, the activation of the glycolysis, glutaminolysis and cholesterol synthesis pathways and their crosstalk was shown to be crucial for β-glucan-induced trained immunity. As glycolysis directly feeds into the tricarboxylic acid (TCA) cycle, intermediates from its process (like fumarate and succinate) are accumulated [246]. Fumarate has a direct influence on histone-modifying enzymes which in turn alters transcriptional regulation of the genome by opening or condensation of the chromatin state [246]. Acquisition and consolidation of epigenetic changes are generally considered to be the main mechanism for the memory of innate immune cells upon priming [229] with both H3K4me3 and H3K27ac being the main modifications induced by β-glucan training [243].

**Long term modulation** – Although individual innate immune cells have a very limited lifespan [247], long-term trained innate immunity effects (often several months up to years) have been observed in many instances [248,249]. The general hypothesis is that long-term effects rely on reprogrammed bone marrow progenitor cell that can give rise to primed mature cells [250]. This would imply that inducers of trained immunity gain access to the bone marrow niche. While this has been demonstrated for the anti-tuberculosis vaccine Bacillus Calmette-Guérin (BCG) [250], data regarding β-glucan is still lacking. As Mata-Martinez et al. pointed out in their previous review [68], β-glucan is usually administered through intraperitoneal injection (in contrast to intravenous BCG injection). Following this route, no studies so far have found β-glucan particles in the bone marrow. As hematopoietic stem and progenitor cells (HSPCs) can be differentiated into a trained phenotype by zymosan (an exclusive ligand for Dectin-1), expression of Dectin-1 on HSPCs can at least be expected [251,252], arguing that progenitor cells are in fact equipped to recognize β-glucans. In contrast, orally ingested β-glucans can be absorbed by macrophages in the gut [253,254] to be transported to different organs [254] and fragments of β-glucan were observed to be continuously released over several days in the bone marrow to interact with resident immune cells via the complement recept.
synthesis, isolation, and/or large-scale production of glycoconjugates has been key to gain access to sufficient quantities of glycans for biomedical studies [269,270]. As multivalent ligand presentation is usually needed to exert biological effects through CLR targeting, the mode of ligand display, e.g. backbone used for glycan presentation, spatial orientation, or ligand density are of crucial importance. Innovative carrier systems such as (glyco-) nanoparticles, dendrimers, polymers or liposomes were successfully employed for CLR targeting in vitro and in vivo [271]. Such interdisciplinary approaches in the field of CLR targeting highlight the need for future inter-disciplinary and trans-sectoral research involving (computational) chemistry, nanoscience, immunology, and infection biology as well as human and veterinary medicine.

CRediT authorship contribution statement

Swantje Fischer: Conceptualization, Writing – original draft, Writing – review & editing. Felix Stegmann: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. Vinayaga Srinivasan Gnanapragassam: Writing – original draft, Writing – review & editing. Bernd Lepenies: Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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