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Review

Evolution of Mincle in vertebrates: adaptation to environmental stresses

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Innate immune receptors serve as the first line of defense against pathogens. C-type lectin receptors (CLRs) are a group of innate immune receptors that recognize a variety of ligands, including glycans, glycolipids, proteins, and insoluble crystals. One such CLR is Mincle (Macrophage-inducible C-type lectin), which can recognize foreign or normally sequestered endogenous glycolipids. Therefore, Mincle is both an essential receptor for recognizing pathogens and a sensor for endogenous molecules. While the functions of Mincle have been characterized, how Mincle may have evolved, and which ligands are recognized at each evolutionary stage remain unknown. In mammals, Mincle is known to be conserved, especially in the domains that bind the sugar moieties of glycolipid ligands. Recently, the sequence and structure of a putative Mincle in fish were investigated relative to mammalian Mincle in order to gain an understanding of changes in the binding domains that occurred as vertebrates adapted to terrestrial environments. In this review, we will first describe the identity and binding mode of glycolipid ligands for mammalian Mincle. Then their similarities and differences between putative fish Mincle will be discussed to get an insight into how the scope of recognized ligands changed, and which pressures drove the evolution of this receptor.

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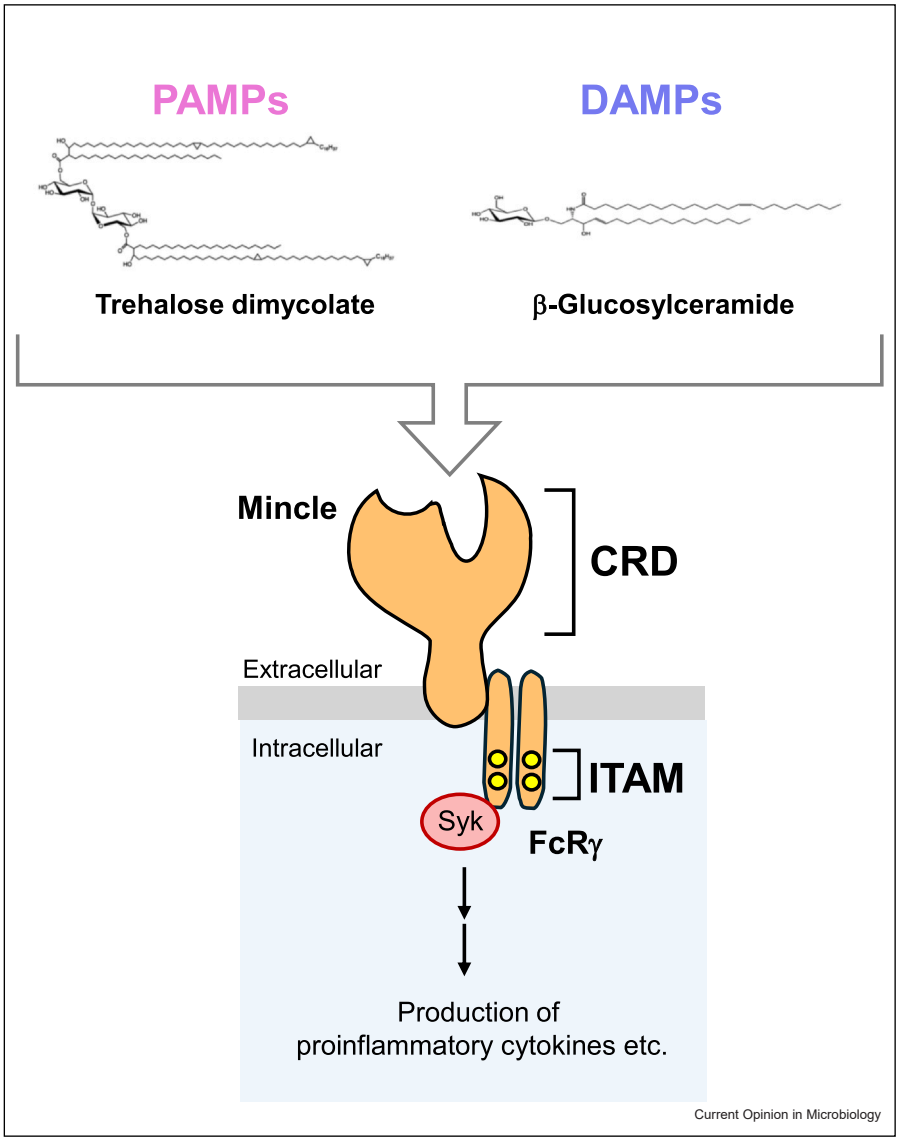
Introduction

Our body is constantly exposed to diverse microorganisms. Innate immune receptors, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), and Rig-I-like receptors (RLRs), are the first line of defense the immune system uses to distinguish harmless commensals from pathogens. These receptors detect pathogen-associated molecular patterns (PAMPs) and rapidly induce immune responses to clear pathogens and damaged self to regain homeostasis [1,2].

CLRs are a well-conserved family of innate immune receptors known to recognize diverse ligands, including glycans, glycolipids, glycoproteins, lipoproteins, proteins, and crystalline metabolites [3]. Due to their broad ligand recognition spectrum, CLRs recognize various infectious agents like fungi, bacteria, and acid-fast bacilli, including *Mycobacterium tuberculosis* (Mtb). To the current day, Mtb remains a global threat, with an estimated 400,000 people infected with tuberculosis and 150,000 deaths per year worldwide [4]. The cell wall of mycobacteria is composed of a thick layer of various glycolipids such as trehalose dimycolate (TDM) and lipoarabinomannan (LAM), which can be recognized by CLRs, activating a protective immune response [5].

CLRs form two gene clusters named the Dectin (dendritic cell-associated C-type lectin)-1 cluster and Dectin-2 cluster, suggesting that they expanded their members through gene duplication [6–10]. Many CLR members,

Figure 1



Recognition of glycolipids by Mincle. Both PAMPs, such as trehalose dimycolate, and DAMPs, such as β-glucosylceramide, are recognized by the C-type lectin receptor Mincle. The CRD of Mincle binds these ligands and transduces signals via ITAM-bearing adaptor protein FcRγ. The intracellular signaling cascade involves Syk activation. Yellow circles in FcRγ indicate tyrosine residues that are phosphorylated upon signal transduction.

especially members of the Dectin-2 cluster, Dectin-2, Mincle (macrophage-inducible C-type lectin), MCL (macrophage C-type lectin), MICL (myeloid inhibitory C-type lectin receptor), and DCAR (dendritic cell immunoactivating receptor), recognize mycobacterial glycolipids, suggesting that the expansion of members within this cluster, at least in part, is a result of the arms race against mycobacteria [11–16].

Mincle is a CLR that is important for the induction of immune responses against mycobacteria [12,17,18]. Mincle contains a single extracellular carbohydrate recognition domain (CRD) and associates with FcRγ, an adaptor molecule for intracellular signaling through an immunoreceptor tyrosine-based activation motif (ITAM) (Figure 1) [19]. Upon recognition of a ligand such as TDM or LAM by other CLRs such as MCL or Dectin-2, the surface expression of Mincle is induced [20–22]. After induction, Mincle initiates downstream signaling cascades through FcRγ, leading to the production of proinflammatory cytokines [12,23], but this expression pattern is different from that of constitutively expressed receptors, which also associate with FcRγ, such as Dectin-2 [22]. Also, several studies show that Mincle and MCL, a CLR that cooperates with Mincle, are necessary for the induction of Th1 and Th17 responses [24–27].

Some studies also report the release of an anti-inflammatory cytokine [28–30]. Beyond recognizing exogenous PAMPs [12], Mincle also recognizes endogenous damage-associated molecular patterns (DAMPs), including β-glucosylceramide, which is always present within cells, but is released from damaged cells [31]. This dual recognition capability and inducible nature position Mincle as both an antimicrobial receptor and a stress sensor, which is upregulated only when ligands are present, thereby contributing to tissue homeostasis by recognizing unfamiliar glycolipids while limiting excessive inflammation [22].

In this review, we will begin by describing Mincle ligands and their binding mechanisms inferred from structural analysis. Then, in the latter part, we are going to discuss the evolution of Mincle based on our recent study of Mincle in lower vertebrates.

Recognition of mycobacterial glycolipids by Mincle

Mincle was originally identified as a transcriptional target of NF-IL6 (nuclear factor for IL-6 or CCAAT/enhancer binding protein β (C/EBPβ)) in macrophages [32]. Further studies discovered that Mincle is a type-II transmembrane molecule with a CRD. After induction, Mincle is expressed on the surface where it, like other CLRs, transduces signals through the ITAM-containing adaptor protein, FcRγ [19,33]. Mincle-specific responses are induced by the timing and strength of ligands binding to Mincle [22]. The first study to report a protective role of Mincle was published in 2008, finding that Mincle plays a role in the host defense against *Candida albicans* [34]. However, the ligand for Mincle in *C. albicans* is still unidentified. Also in 2008, SAP130, a component of small nuclear ribonucleoprotein, was identified as the first endogenous Mincle ligand [35]. Since then, numerous ligands have been identified from both exogenous and endogenous origins, including cell wall components of various organisms, various glycosyl-diacylglycerol compounds, brartemicin, β-glucosylceramide, and cholesterol-containing molecules [12,18,31,36–44].

TDM, one of the major components of complete Freund's adjuvant derived from the cell wall of Mtb, was identified as a Mincle ligand [12,45]. Both sugar and lipid moieties contribute to maximal Mincle binding [12,46,47], which was supported by the structural analysis [48–51]. Additional experiments using Mincle-deficient mice confirmed that Mincle is essential for the recognition of TDM by macrophages and promoting the subsequent immune responses, such as granuloma formation.

Recently, phenolic glycolipid-III (PGL-III) from *Mycobacterium leprae*, the causative agent of leprosy, was also identified as a Mincle ligand [18]. PGL-III potently induced the production of TNF (tumor necrosis factor) and IL-6 from primary macrophages in a Mincle-dependent manner. Mincle-deficient mice on the Rag1-deficient background showed significantly increased *M. leprae* burden compared to the Mincle-sufficient mouse and downregulation of *Nos2*, an effector downstream from Mincle. This suggests that Mincle plays an important role in activating the anti-mycobacterial effectors.

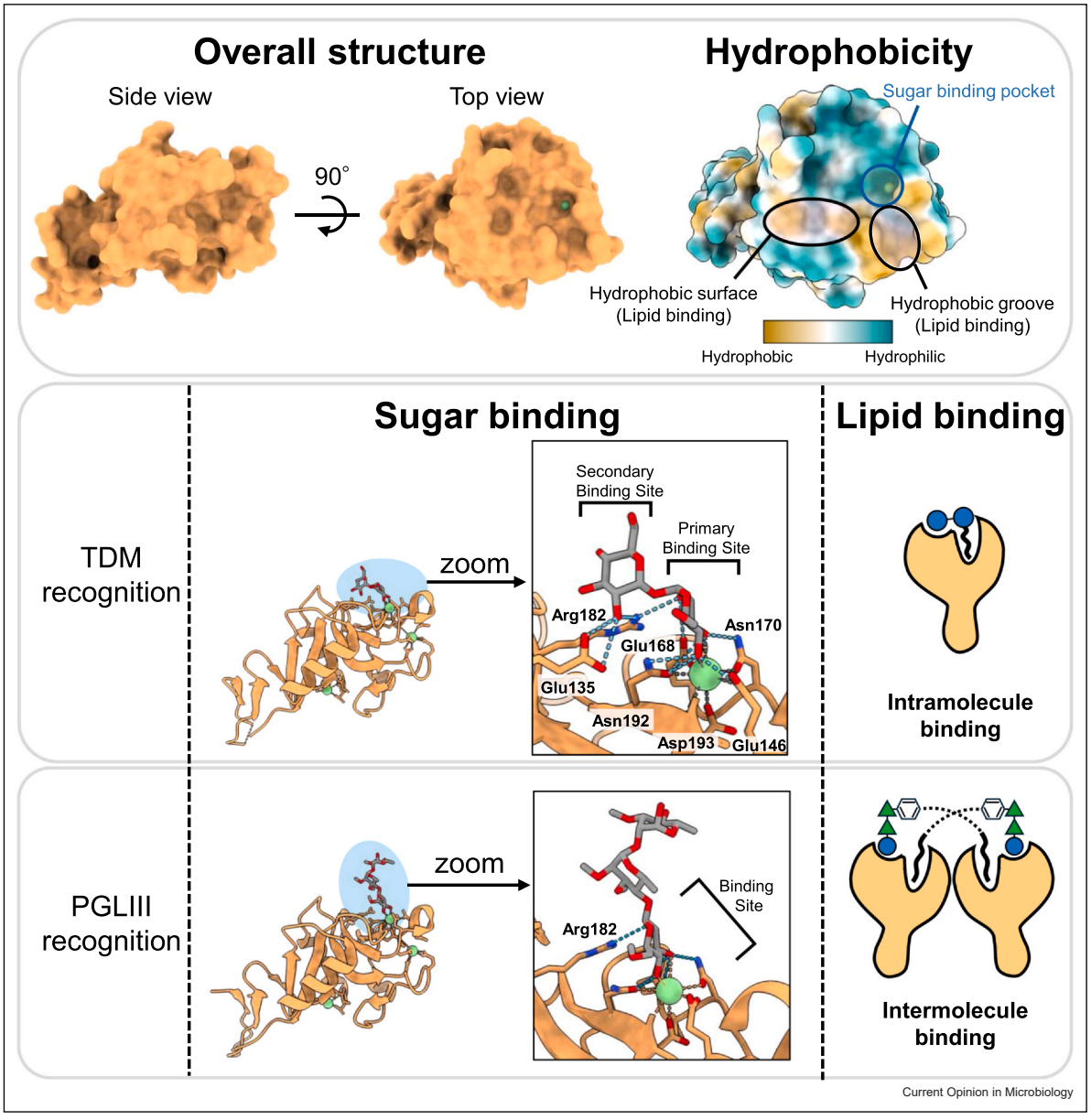
Recognition mechanism of Mincle ligands

The mechanisms of glycolipid ligand recognition by Mincle have been well analyzed. In 2013, the crystal structure of Mincle and its complex with trehalose was elucidated [48,49]. Like other CLRs, the CRD of Mincle contains a calcium ion which forms coordination bonds with amino acids in the carbohydrate-binding pocket (Figure 2) which harbors a motif that determines sugar specificity. Mincle possesses a Glu-Pro-Asn (EPN) motif, which is known to confer specificity for glucose and mannose [50]. One of the glucose moieties constituting the trehalose of TDM binds to Mincle via the CRD. The other glucose moiety interacts with amino acid residues near the pocket (Glu135 and Arg182), thereby contributing to an expanded interaction interface [49]. The larger binding interface compared to that of a single glucose molecule may enable more robust binding. As for the interaction with acyl chains, it was initially proposed that the hydrophobic groove accommodated the lipid chains. Mutational analysis of hydrophobic residues forming the groove supported this hypothesis [49]. Later structural analysis using trehalose derivatives reveals a secondary hydrophobic surface that can also associate with acyl chains [51]. Additional nuclear magnetic resonance analysis of human Mincle and TDM or trehalose C10 also suggested that both the hydrophobic surface and the hydrophobic groove contribute to the lipid binding [52]. Thus, Mincle recognizes glycolipids through three distinct sites: the carbohydrate-binding pocket, the hydrophobic groove, and the hydrophobic surface. Interestingly, both the glucose head and the lipid tail of PGL-III appear to bind to Mincle differently than TDM. Only the terminal glucose of PGL-III interacts with the carbohydrate-binding pocket of Mincle, without additional amino acid binding. Furthermore, the lipid tail appears to bind to adjacent Mincle modules, suggesting multimerization [18].

Conservation of the key components for Mincle function

Mincle is broadly conserved among mammals, with the amino acid identity to human ranging from 50% to 99% (Figure 3a). The overall sequence identity among mouse, human, and bovine Mincle is approximately 70%. While amino acid identity varies among mammals, amino acids critical for ligand recognition, including the EPN motif, hydrophobic groove, and hydrophobic surface, are highly conserved (Figure 3b). Interestingly, the hydrophobic

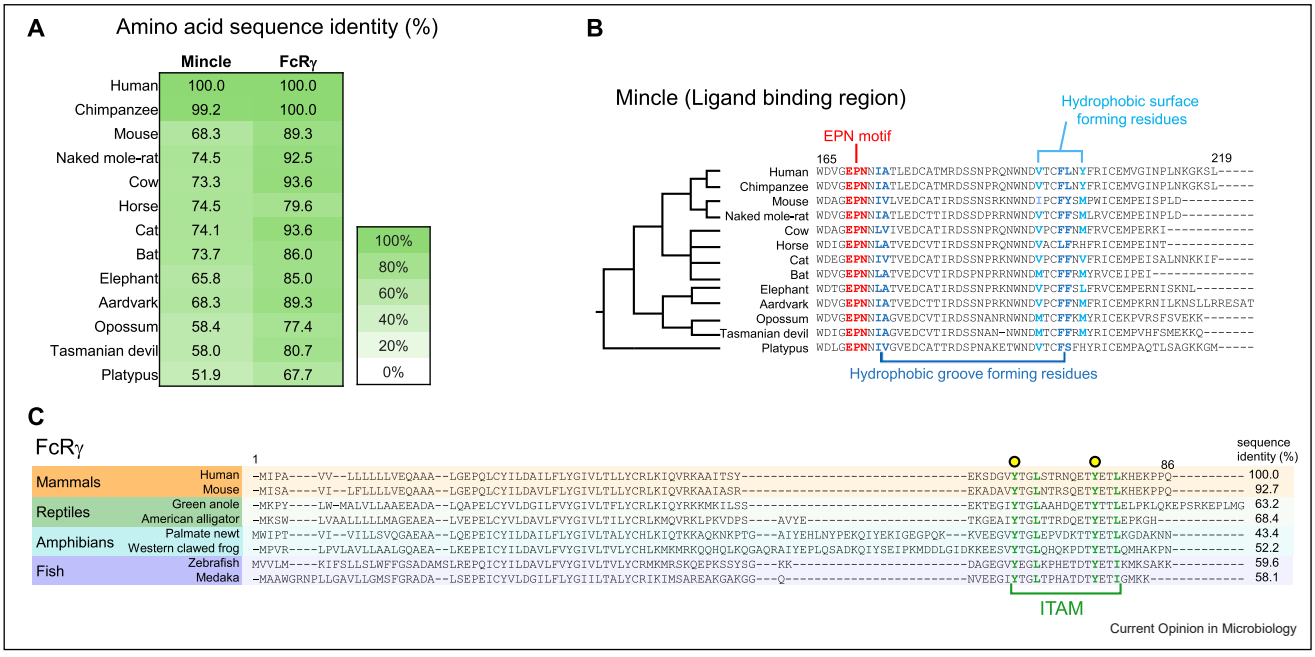
Figure 2



Structural mechanism of mycobacterial glycolipid recognition by Mincle. The overall structure (top left) shows two orientations of the Mincle (90° rotation). The structure in the top left is a top view of Mincle colored by its hydrophobicity. The color scale is indicated below the structure. The sugar-binding pocket, the hydrophobic groove and the hydrophobic surface for lipid binding are indicated with a blue circle and a black circle, respectively. The middle and bottom panels illustrate two distinct modes of ligand recognition. The middle panel shows the recognition mechanism of TDM, and the bottom shows the recognition mechanism of PGL-III. In the right of the middle and bottom panel, schematic of the lipid binding mode is shown. The lipid moiety is shown as solid or dashed black lines, and the sugar moiety is shown as circles or triangles; blue circle: glucose, green triangle: rhamnose. The structures in these panels are shown in cartoon models. Sugars are depicted as a stick model, and the calcium ion is shown as a green sphere. The coordination bonds between the calcium ion and the surrounding residues are shown in gray dashed lines. Hydrogen bonds are shown in blue dashed lines. The PDB IDs of the structures used in this figure are as follows: top, middle; 4ZRW, bottom; 8H4V.

surface of platypus Mincle contains a non-hydrophobic amino acid, suggesting evolution within the acyl chain binding moieties in early mammalian species. Additionally, cysteines forming disulfide bonds are well preserved, suggesting similar three-dimensional structures across species [53]. This structural conservation is supported by reports demonstrating that mouse, human, and bovine Mincle all have ligands in common [12,18,37,51], suggesting a shared ligand recognition architecture. Despite the conserved amino acid sequence, structure, and binding to some

Figure 3



Conservation of Mincle and the components across species. **(a)** Amino acid sequence identity (%) of Mincle and FcRγ among 13 mammalian species. The heatmap shows high conservation across species, with chimpanzee showing > 99% identity to human sequences, while more distant species like platypus show lower conservation (52–68%). **(b)** Multiple sequence alignment of the ligand-binding region of Mincle (residues 165–219 in human Mincle). On the left of the alignment of Mincle, a phylogenetic tree indicating the mammalian lineage. Key functional residues are highlighted: EPN motif (red) essential for carbohydrate recognition, hydrophobic groove-forming residues (blue), hydrophobic surface-forming residues (light blue), which are important for lipid binding. **(c)** Multiple sequence alignment of full-length FcRγ (residues 1–86 in human FcRγ) across vertebrates. The ITAM in FcRγ is highlighted in green. Tyrosine residues that are phosphorylated upon signal transduction are highlighted with yellow circles. The sequence identity against human FcRγ is indicated on the right of the alignment. Dashes in the alignments represent gaps.

ligands, there are species-specific differences in ligand recognition [54,55]. For example, human Mincle recognizes glycerol monomycolate from mycobacteria, whereas mouse Mincle does not [55]. Mutational analysis has revealed that this human-specific interaction is attributable to differences in amino acid residues within the hydrophobic groove. Although there are some variations among mammals, the widespread conservation of receptors recognizing some of the same ligands suggests that acid-fast bacteria have posed a common threat across mammalian evolution, necessitating the maintenance of immune sensors against these pathogens.

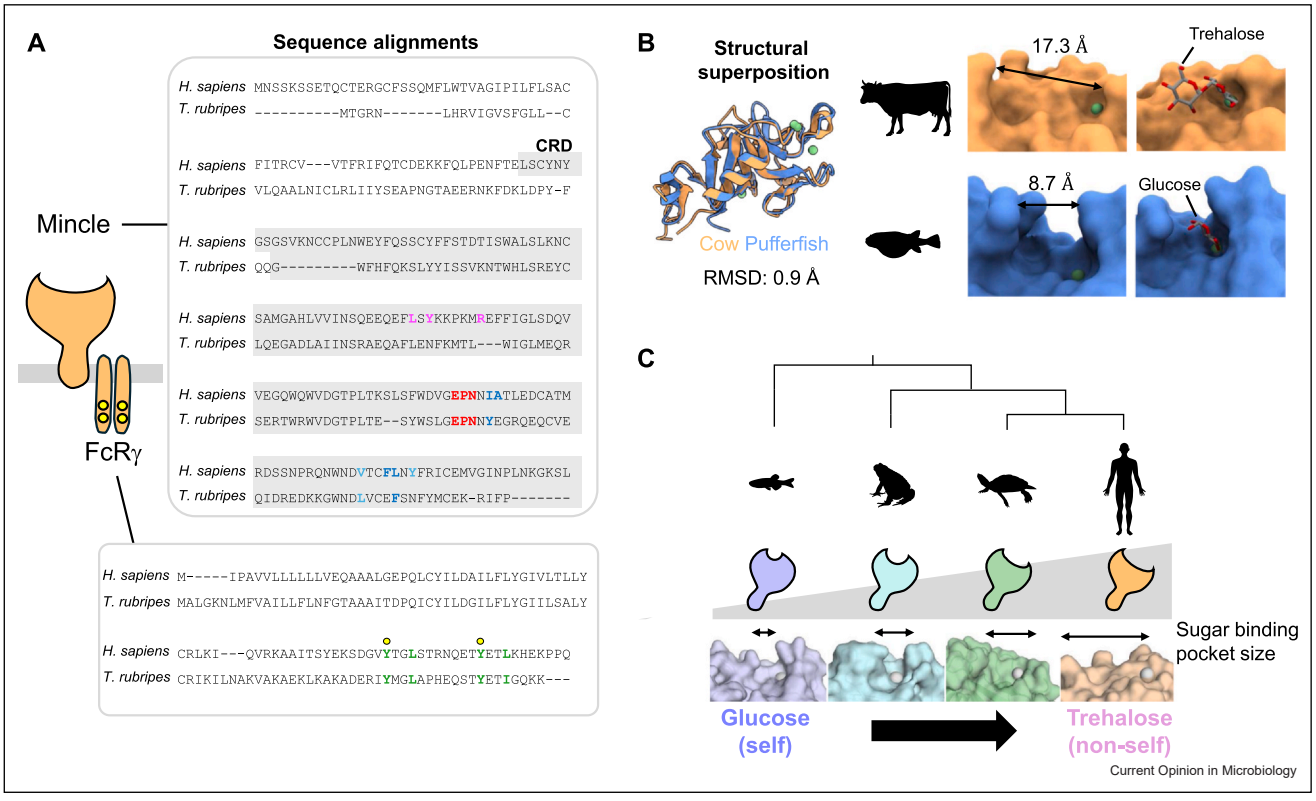
There are reports of putative Mincle in fish, suggesting conservation among vertebrates [56–59]. FcRγ appears to be conserved in other vertebrates (reptiles, amphibians, and fish), with amino acid identity to human FcRγ ranging from 43% to 68% and a conserved ITAM, indicating broad conservation across vertebrates (Figure 3c) [60–62].

Evolution of ligand recognition by Mincle

In addition to recognizing exogenous glycolipids, Mincle and other CLRs can also recognize self-components. Mincle recognizes metabolites, such as β-glucosylceramide or cholesterol crystals, that reside intracellularly but can be bound by Mincle upon cellular damage, thereby initiating a response to restore homeostasis [31,54]. Therefore, it is unclear whether Mincle originally functioned as a receptor for exogenous ligands, such as those from mycobacteria, or endogenous ligands. We recently reported on the evolutionary trajectory of Mincle through analysis of the crystal structure of piscine Mincle and predicted structures of putative amphibian and reptilian Mincle [58].

Pufferfish (*Takifugu rubripes*) Mincle (trMincle) possesses conserved hydrophobic residues for lipid binding, whereas the cholesterol recognition/interaction amino acid consensus (CRAC) motif was not conserved (Figure 4a). FcRγ was also conserved, as previously reported [63]. The crystal structure of trMincle revealed a narrower carbohydrate-binding interface than that of mammalian Mincle (Figure 4b) [58], suggesting an inability to accommodate disaccharide headgroups of glycolipids as observed in mammals. These findings suggest that Mincle originated as a stress receptor recognizing monosaccharide-bearing molecules, possibly from endogenous origin, such as β-glucosylceramide, which is a

Figure 4



Evolutionary trajectory of sugar-binding pocket widening in Mincle. **(a)** Full length amino acid sequence alignments of Mincle and FcRγ between human (*H. sapiens*) and pufferfish (*T. rubripes*). The Mincle alignment shows the CRD with key functional residues highlighted; CRAC motif (magenta) essential for cholesterol binding, calcium ion coordinating residues, including the EPN motif (red), which is essential for carbohydrate recognition, hydrophobic groove-forming residues (blue), and hydrophobic surface-forming residues (light blue). The FcRγ alignment is displayed in the lower panel, with the ITAM indicated by green boxes and highlighted with yellow circles marking critical tyrosine residues that are phosphorylated upon signal transduction. Dashes represent gaps in the alignment. **(b)** Structural superposition of cow (*B. taurus*) (orange) and pufferfish (blue) Mincle CRDs (PDB ID: 4ZRW and 9KPL, respectively) showing high structural conservation with a root mean square deviation (RMSD) of 0.9 Å (among 103 pruned atom pairs), despite sequence divergence. In the right panel, surface representations comparing the sugar-binding pocket in pufferfish (blue, 8.7 Å width) and cow (orange, 17.3 Å width) Mincle are shown. Bound ligands shown as stick models: trehalose in the cow structure and glucose in the pufferfish structure. **(c)** Evolutionary expansion of the sugar-binding pocket correlates with the ability to recognize trehalose-containing ligands. Schematic representation showing the size increase of the Mincle sugar-binding pocket (shown in corresponding colors) from fish to mammals associated with a shift in ligand preference. Structures shown are the surface representations of Mincle and putative Mincle in each species. Fish and mammalian structures are actual crystal structures of pufferfish and cow Mincle. Others are structures predicted with AlphaFold3.

fundamental component of sphingomyelin metabolism used by organisms from ancient times [64,65]. As the presence of released β-glucosylceramide is a sign of danger to the organism, sensors like Mincle were selected to ensure homeostasis is maintained. As the predicted structures of putative Mincle from amphibians and reptiles suggested the progressive enlargement of the sugar-binding pocket (Figure 4c), Mincle may have evolved to recognize ligands with polysaccharide moieties characteristic of the pathogens encountered as organisms adapted to terrestrial life.

We therefore speculate that changes in habitat contributed to the expansion of Mincle's ligand recognition capabilities. Indeed, a recent study showed that the terrestrialization of vertebrates resulted in the gain and expansion of genes related to immune functions suggesting an impact of changing habitat on immunity [66]. Also, changes in CLRs associated with environmental habitat shifts have been analyzed using *in silico* approaches [67]. Comparative analyses of the CLRs MICL, Langerin, and MelLec (melanin sensing C-type lectin receptor) across primates, rodents, and bats revealed accumulation of significantly different mutations, especially in the CRD in each molecule. These differences could be the result of adaptation to distinct pathogen exposures accompanying environmental habitat changes. In TLRs, another family of membrane-bound innate immune receptors, species-specific structures and differences in ligand recognition have been reported, and TLR-pathogen coevolution was proposed [68]. Intriguingly, some mycobacterial species, such as *M. marinum*, cause infection in fish [69,70], and it is possible that a similar protein

in zebrafish mediates *M. marinum* TDM effect in the host [59]. Further studies in a broader range of fish may give additional insights into the selection pressure that determined the size of the sugar-binding pocket of Mincle. Taken together, the magnitude of environmental influence may be substantial enough to generate unique mutations even among orthologous molecules.

Conclusions

While there are many studies that continue to investigate the complex role of Mincle during infection and as a sensor for endogenous molecules, its origin has only begun to be revealed. Comparison of the amino acid sequence and structure of Mincle in fish with that of Mincle from higher vertebrates suggested that Mincle originally recognized monosaccharide-containing glycolipids, which are mainly self-derived. However, adaptation to new environments, especially going from aquatic to terrestrial environments, may have led to the acquisition of mutations that allowed for the binding of the unique ligands, including glycolipids derived from newly encountered, life-threatening microorganisms. Detailed comparative analysis of immune receptors from a broader range of vertebrates and the environmental ligands which they recognize, using *in silico* and *in vitro* approaches, will further clarify their evolution, providing insights into which ligands drive adaptation and/or emergence of new receptors which may have shaped our immune system.

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Data Availability

No data were used for the research described in the article.

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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- • of special interest
- •• of outstanding interest

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