Contact, Collaboration, Conflict: Signal integration of Syk-coupled C-type lectin receptors[1]

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Running title: Signaling cross-talk of Syk-coupled C-type lectin receptors

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Abstract

Several Syk-coupled C-type lectin receptors (CLR) have emerged as important pattern recognition receptors for infectious danger. Since encounter with microbial pathogens leads to the simultaneous ligation of several CLR and TLR, the signals emanating from different PRR have to be integrated to achieve appropriate biological responses. Here, we briefly summarize current knowledge about ligand recognition and core signaling by Syk-coupled CLR. We then address mechanisms of synergistic and antagonistic cross-talk between different CLR and with TLR. Emerging evidence suggests that signal integration occurs through a) direct interaction between receptors, b) regulation of expression levels and localization, c) collaborative or conflicting signaling interference. Thereby, we aim to provide a conceptual framework for the complex and sometimes unexpected outcome of CLR ligation in bacterial and fungal infection.

Keywords: C-type lectin, Toll-like receptor, Dectin-1, Dectin-2, Mincle, Mcl, TLR2, TLR4, TLR9, Syk-Card9, Mycobacteria, Fungi, Inflammation

Innate immune receptors cooperate

The immune system identifies invading microbial pathogens by conserved microbial motifs, known as pathogen-associated molecular patterns (PAMP). For any given pathogen a combination of such PAMP is recognized by pattern recognition receptors (PRR) on innate immune cells. Detection of a pathogen by a combination of receptors ensures redundancy, results in lower likelihood for immune evasion by the pathogen and robustness against genetic diversity in the host. Furthermore, engagement of a pathogen-specific set of receptors allows to tailor the immune response to protect the body against specific infections.

Toll-like receptors (TLR) are the best-studied family of PRR expressed on innate immune cells. 10 functional TLR are known in humans, 12 have been described in mice. Interactions of TLR and cross-talk of TLR signaling has been studied for almost two decades. C-type lectin receptors (CLR) as another major group of PRR have entered the field later, but their investigation has gained much momentum in the last decade. Many studies have been conducted on CLR assigned to the so-called Dectin-1 or Dectin-2 clusters, localized within the NK cell gene cluster on human chromosome 12 or mouse chromosome 6 (1-3). Several excellent reviews on the function of these CLR in anti-microbial defense and homeostasis are available (4, 5). In this review we summarize the current knowledge about signaling downstream of the activating CLR Dectin-1 (Clec7a), Dectin-2 (human Clec6a, mouse Clec4n), Mincle (Clec4e) and Mcl (Clec4d) that is largely dependent on the kinase spleen tyrosine kinase (Syk). Table I provides an overview of defined ligands and microorganisms bound by this group of PRR. In addition to microbial carbohydrate and glycolipid structures acting as PAMP, several CLR bind endogenous ligands such as SAP130 released by dying cells or cholesterol crystals. Thus, these CLR are involved in homeostatic responses and inflammatory conditions (6-9), in addition to host response to pathogens and commensals (10-

12). CLR-induced APC-activation directs T helper cell differentiation (see Geijtenbeek et al. for review (13)), and synthetic ligands for CLR are under development as adjuvants (14, 15). Bacteria and fungi can express more than one CLR ligand, therefore simultaneous engagement of CLR during recognition of microbial pathogens is likely. In addition, concurrent activation of TLR and CLR will occur, leading to synergistic and antagonistic responses with sometimes unexpected outcomes. For a generalized concept of signal integration in innate immunity we refer to a recent publication by Elinav et al. (16). With regard to CLR signaling, there is evidence that Dectin-1, Dectin-2, Mincle and Mcl do not only act as activating PRR, but are particularly important for regulation and tailoring of immune responses. Here we discuss the interactions following simultaneous engagement of several CLR and TLR. Conceptually, we propose that such signal integration can occur on different levels, which will be discussed in this structured review:

- Contact between receptors, with possible consequences for ligand binding, receptor stability or localization.
- Control of receptor expression levels, adjusting the responsiveness.
- Collaborative signaling, leading to synergistic responses.
- Conflicting signaling, tailoring the inflammatory response.

C-type lectin receptors and Toll-like receptors activate distinct inflammatory pathways and gene expression programs

Ligand binding to the (either extracellular or endosomal) ectodomain of TLR leads to dimerization of the cytoplasmic Toll/signaling-1R (TIR) domain. Dimerization can occur as both homo- or heterodimers (TLR1/TLR2 and TLR2/TLR6). Adaptor proteins are subsequently recruited by TIR-TIR interactions. Downstream signaling is induced dependent on MyD88 (engaged by all TLR except TLR3) and/or TIR domain-containing adaptor protein inducing IFN-β (TRIF, engaged by TLR3, TLR4). Activation of nuclear factor-κ B (NFκB) and IFN-regulatory factors (IRFs) are central events downstream of MyD88 and TRIF (17) (Fig. 1). Synergistic responses have been described for combination of MyD88 and TRIF-dependent TLR ligands (18, 19). C-type lectins are a protein superfamily with more than 1000 members belonging to 17 subgroups based on structural and ligand binding features (20). The receptors in the Dectin-1 and Dectin-2 cluster, of which many contribute to innate immunity, belong to the related subgroup II (Asialoglycoprotein and DC receptors, Ca^{2+}-binding) and subgroup V (NK-cell receptors, non-Ca^{2+}-binding). Ligand binding is mediated by the C-type lectin domain (CTLD) (21), often containing a QPD or EPN motif. Dectin-1 ("Dendritic cell-associated C-type lectin 1", Clec7a, CD369, Clecsf12), Dectin-2 (human Clec6a, mouse Clec4n, Clecsf10), Mincle ("Macrophage-inducible C-type lectin", Clec4e, Clecsf9) and Mcl ("Macrophage C-type lectin", Clec4d, CD368, Clecsf8) are activating receptors that share signaling via immunoreceptor tyrosine-based activation motifs (ITAM) and the kinase spleen tyrosine kinase (Syk). Dectin-1 recruits Syk via a hemITAM motif, while Mcl, Mincle and Dectin-2 associate with the ITAM-containing FcRγ chain. Downstream of Syk, activation of the canonical NFκB pathway is dependent on formation of the Card9/Bcl10/Malt1-complex (4) (Fig. 1).

Consistent with the shared activation of NFκB and MAPK by TLR and CLR, ligands for both types of PRR induce an overlapping set of proinflammatory cytokines and chemokines. However, there is also evidence for selective and preferential target gene expression. A limited number of microarray studies has compared stimulation with CLR ligands such as ß-glucans (Dectin-1) or trehalose-dibehenate (Mincle) with TLR ligands such as Pam3 (TLR2), LPS (TLR4), or CpG (TLR9) (22-25). While TLR9 ligation induces strong IL-12 production associated with Th1 generation, the induction of IL-1β, IL-6 and IL-23 after engagement of Syk-Card9 coupled CLR is observed across cell-types and species, promoting the differentiation of IL-17-producing CD4+ T cells (23, 24, 26, 27). Remarkably, to date there is only very limited information about the effects of combined stimulation of CLR and TLR pathways on global gene expression.

Syk-coupled CLR : Structure, ligands and signaling

Dectin-1 and the Dectin-1 subfamily

Dectin-1 is the best-studied receptor in the Dectin-1 family; signaling events downstream of Dectin-1 ligation are often regarded as prototypic for Syk-coupled CLR (28, 29). Dectin-1 recognizes β-glucans in fungal and mycobacterial cell walls in a Ca^{2+}-independent manner (30-33) (see Table I). β-glucans bind to Dectin-1 homodimers and ligand binding has been suggested to induce oligomerization (34). Whereas particulate ligands result in formation of a ”phagocytic synapse”, stimulation with a soluble ligand does not induce a response (35). Dectin-1 signals via its internal hemITAM motif (single YxxL/I motif) (36) which is phosphorylated upon ligand binding. Recruitment of Syk to the phosphorylated hemITAM is pivotal for Dectin-1 responses (37) and requires a phosphatase-independent chaperone function of SHP-2 (38). Lipid raft formation has been shown to be important for Syk recruitment (39, 40). Downstream of Syk, canonical NFκB signaling is dependent on the activation of PLCγ2 (39), phosphorylation of PKCδ (41, 42) and formation of the Card9/Bcl10/Malt-1 (CMB) complex (43, 44), which involves the ubiquitin ligase Trim62 (45). Different NFκB subunits are activated following Dectin-1 ligation through Syk and Raf-1 as reviewed in detail by Geijtenbeek and Gringhuis (46). So far, Syk-independent signaling via Raf-1 has only been described after Dectin-1 ligation in human DC (47-49). IRF1 (50) and IRF5 (51) are further transcription factors induced. Phosphorylation of the MAPK p38 and JNK appears to be partially Syk-independent (26, 38, 52), in contrast, phosphorylation of the MAPK ERK requires Syk and is mediated by Card9 and H-Ras (53, 54). Activation of ERK is critical for ROS production, which has been linked to induction of autophagy (55), and to assembly of the NLRP3 inflammasome (56-58). Assembly of a non-canonical Malt1-Caspase-8-ASC inflammasome triggered by Dectin-1 has as well been described (59-61). In addition to its requirement for assembly of the CMB complex, PLCγ2 induces Ca^{2+} flux triggering the classical calcineurin/NFAT pathway which directly induces Egr1 expression (39, 62) and is required for anti-fungal defense (63).

Dectin-2 cluster: Dectin-2, Mcl, Mincle

The genes encoding these receptors are localized adjacent to each other in the Dectin-2 cluster on human chromosome 12/ mouse chromosome 6 (3, 64-66). Dectin-2 and Mcl likely arose from gene duplication of Mincle (64, 67). Dectin-2, Mincle and Mcl do not contain a cytoplasmic signaling motif, but instead they associate with the ITAM (YxxL/I,YxxL/I)-containing adaptor FcRγ chain (6, 67-69) (Fig. 1).

While ligands of Dectin-2 and Mincle are diverse and not always structurally characterized, Dectin-2, Mincle and Mcl all recognize ligands on fungi and mycobacteria in a Ca^{2+}-dependent manner (see Table I). Several studies have addressed the structural requirements for interaction of Mincle with the mycobacterial cord factor Trehalose-dimycolate (TDM) or synthetic Trehalose-esters (70-74), which have recently been summarized in excellent reviews

(75-77). Mincle binds the trehalose part of the cord factor with its Ca^{2+}-dependent sugar binding pocket and its structure revealed a hydrophobic groove that likely accommodates the lipid component of TDM or TDB. The recent interest in Mincle ligands for adjuvant development (78-80) has engendered the chemical synthesis of multiple glycolipids, which help to determine the requirements for receptor binding and macrophage activation (81-84). Different from cord factor binding, recognition of the nucleoprotein SAP130 is Ca^{2+}-independent (6). Human and murine Mincle have divergent ligand specificities e.g. for glycerol mono-mycolates (82, 85) or cholesterol crystals (86). The CTLD of Mcl is much less conserved among species than the Mincle CTLD, in consequence Mcl appears to be a functional TDM receptor in mice (67), but not e.g. in guinea pigs (87), suggesting divergent physiological roles of Mcl between species. Mincle- or Mcl-deficient mice showed mostly moderate phenotypes in mycobacterial (88-91) or fungal infection models (92-94) compared to knockouts of the downstream Card9 (43, 95), indicating receptor redundancy. It is quite possible that double-deficient mice will show more severe phenotypes. Whereas both Dectin-2 and Mcl have phagocytic properties (66, 68), Mincle was described to be dispensable for glycolipid uptake (96), although required for cytokine production after glycolipid stimulation (97, 98). Ligand binding to Dectin-2 and Mincle leads to engagement of the FcRγ–Syk–PLCγ2–PKCδ–Card9 axis and activation of the canonical NfκB pathway similar to Dectin-1 (6, 42, 98-100) (Fig. 1). Gringhuis et al. described that Dectin-2 engagement specifically activates c-REL controlled by Malt1, in contrast to induction of all NFκB subunits by Dectin-1 stimulation (101). Engagement of Dectin-2 and Mincle furthermore leads to activation of the MAPK p38, ERK and JNK (99, 102-105). ERK phosphorylation after stimulation of Dectin-2 with *Candida albicans* is dependent on Syk and PLCγ2 but not Card9 (100, 103). Activation of PKB (synonym Akt) is found downstream of Mincle and Dectin-2, dependent on PI3K (50, 106). Both Dectin-2 and Mincle ligation can lead to production of reactive oxygen species (ROS) and inflammasome activation (107-112).

Cooperation by contact: Heteromerization of CLR

Activation of TLRs can not only result from homodimer formation but also from heterodimerization. TLR2 can pair with either TLR1 or TLR6, resulting in an increased ligand spectrum (113-115). Homodimeric forms of human and mouse Mincle have been described quite early (116), and recently heterodimerization of Mcl has been described with Mincle (117) and with Dectin-2 (93).

It has been controversial whether Mcl interacts directly with the adapter protein FcRγ. Mcl lacks the conserved arginine residue in the stalk region that is required for interaction of murine Mincle with FcRγ (6), and Graham et al. could not find association of human Mcl with FcRγ, DAP10 or DAP12 (92). In contrast, Miyake et al. demonstrated that murine Mcl co-immunoprecipitates with FcRγ in absence of Mincle, uniquely utilizing a hydrophilic threonine residue rather than arginine (67). Direct association with FcRγ was likewise found for guinea pig Mcl, which similar to human Mcl has a serine at position 38 (87). Lobato-Pascual et al. showed formation of disulfide-linked Mincle-Mcl heterodimers and suggested that rat Mcl interacts with FcRγ in an indirect fashion via heterotrimer formation with Mincle (117). Two independent studies demonstrated that the surface expression of Mincle and Mcl on myeloid cells is interdependently stabilized by their heterodimerization (118, 119). In consequence, Mcl-deficient mice have reduced Mincle surface expression but Mcl-transgenic mice show enhanced responsiveness to TDM stimulation, and Mcl surface levels are strongly reduced in Mincle-deficient cells. The interaction of murine Mincle and Mcl requires four hydrophobic residues in the stalk region of Mincle (118). In contrast, Zhao et al. neither found co-immunoprecipitation of human Mincle and Mcl co-expressed in RAW264.7 cells, nor did they observe synergistic responses (120). Previously, the authors had described the dimerization of human and murine Mcl with Dectin-2 and demonstrated a synergistic role of Dectin-2 and Mcl for protection in a murine *C. albicans* infection model (93). A phenotype in *C. albicans* infection had not been observed in an earlier study (92), neither was co-regulation of Dectin-2 and Mcl in mice confirmed in two other reports (94, 119). Overall there is strong evidence that Mcl is able to dimerize with related CLR, notwithstanding some disagreement in the literature. Further studies are needed to investigate if discrepant results can be attributed to different cell types or receptors originating from different species. Several roles for Mcl in these interactions have been suggested and are depicted in Fig. 2: 1) Transcriptional regulation of Mincle expression (further discussed below), 2) Post-transcriptional regulation by interdependent stabilization of Mincle surface expression (118, 119), 3) Mincle could benefit of phagocytic capacity of Mcl (117, 121), 4) Enhanced ligand binding by heterodimerization with Dectin-2 or Mincle, leading to an increased response (93, 122), 5) Alteration of ligand specificity (121). It can be expected that molecular dynamics simulations based on existing crystal structures of Mincle and Mcl, and further structural work will be instrumental in answering which of these models is correct.

Control of expression levels and localization of receptors

Expression of PRR is a prerequisite for recognition of a microbial ligand. However, expression of PRR is not uniform among different innate immune cell types and can be massively regulated by cytokines and microbial stimuli. Hence, cross-regulation of expression levels is in principle a logical mechanism for cross-talk between different PRR and their signaling pathways. Specifically, Syk-coupled CLR show large differences in expression between different cell types and activation states. Dectin-1 mRNA can be induced by GM-CSF and IL-4, but is downregulated by LPS, IFNγ and IL-10 (123). Dectin-2 protein in monocytes increases under inflammatory conditions (124). Similarly, Mincle mRNA expression is low in resting murine macrophages and DC but strongly inducible upon stimulation with inflammatory stimuli (64, 67, 104). Matsumoto et al. identified Mincle ("Macrophage inducible C-type lectin") originally in a screen for target genes of the transcription factor C/EBPβ following LPS/IFNγ-stimulation (64). Mincle expression is also upregulated by its ligand TDM in a feed forward loop through Mincle itself (98, 104), or through Mcl acting as constitutively expressed low-affinity receptor for TDM in mice (67, 120) (Fig. 2A, Fig. 3C). It is currently unclear whether such transcriptional regulation of Mincle expression is conserved in other species which express higher constitutive levels of Mincle mRNA (50, 87, 122, 125).

In addition to the sequential control of Mincle mRNA expression, Mcl also controls the surface expression of Mincle protein. As described above, Mcl was recently identified to interact with Mincle via its stalk region and to be essential for surface expression of Mincle (118, 119) (Fig. 2B). While the molecular and kinetic details of the Mincle-Mcl interaction are not yet fully understood, it becomes evident that protein interactions and protein localization are a means to control the responsiveness beyond the transcriptional level.

Collaborative signaling: Synergistic responses of CLR

Many fungi and bacteria contain several different CLR ligands (see Table I) which will lead to the concurrent triggering of more than one CLR in phagocytes and DC upon making contact with the microbes. Furthermore, scavenger receptors like CD36, complement receptors, TLR and cytosolic nucleic acid sensors are engaged upon pathogen contact. Receptor crosstalk can result in synergistic or conflicting signaling, thereby modulating the immune response. Examples for experimental ligands binding to both CLR and TLR are non-depleted zymosan (Dectin-1 – TLR2) or mannosylated O-antigens (Dectin-2 – TLR4) (126, 127). Dectin-1 – TLR2 crosstalk is the most extensively studied example of CLR – TLR crosstalk, mostly but not exclusively leading to synergistic responses (Fig. 3).

Dectin-1 and complement receptor 3 (CR3 or CD11b/CD18, encoded by *Itgam* and *Itgb2*) both recognize β-glucans (Fig. 3A). CR3 was described as zymosan receptor in neutrophils (128, 129) and as receptor for soluble β-glucan in mononuclear cells (130). The idea of Dectin-1 – CR3 crosstalk is further promoted by the observation that the receptors co-localized on lipid rafts after *Histoplasma capsulatum* stimulation. Collaborative TNF and IL-6 responses were dependent on Syk and JNK but not NFκB (40). CD11b can itself recruit Syk and was shown to negatively regulate TLR-mediated inflammatory responses via the E3 ubiquitin ligase Cbl-b (131, 132). Thus, CR3-Syk appears to synergize with Dectin-1 signaling, but downregulates TLR-induced responses. Very recently, Cbl-b-mediated ubiquitination and degradation of Dectin-1, Dectin-2 and Syk were demonstrated, revealing a broader role for this ubiquitin ligase in regulation of TLR and CLR signaling (133-135).

Dectin-1 and TLR2 are both required to obtain strong production of TNF and IL-12 and NFκB activation in murine macrophages and DC after zymosan stimulation; co-localization was observed upon stimulation (126, 136) (Fig. 3B). Results were similar after stimulation with particulate β-glucans followed by ligands for TLR2, TLR3, TLR4, TLR5, TLR7 or TLR9 (137, 138). Prolonged IκB degradation and enhanced NFκB translocation resulted in more-than-additive production of TNF, IL-23, IL-6 and IL-10, but reduced production of IL-12 (137, 139). Syk and Card9 were required for the synergistic response (137, 140), which was similarly detected in human monocytes and macrophages (141). Of note, the synergistic signaling via Dectin-1 and TLR2 does not only result in proinflammatory cytokine production, but also in augmented secretion of anti-inflammatory IL-10 (Fig. 3B). Secretion of IL-10 is controlled by the MAPK ERK and p38, phosphorylation of mitogen-and-stress-activated protein kinase 1/2 (MSK1/2) and engagement of the transcription factor CREB, consistent with induction of a regulatory phenotype and reduced activation of T cells (53, 142-144).

Synergistic TNF and IL-10, but reduced IL-12 secretion has similarly been described for simultaneous engagement of Mincle and TLR ligands (145, 146) (Fig. 3C). IL-10 can itself regulate IL-12 production in an autocrine manner as observed after co-stimulation of TLR2 and Mincle by synthetic ligands and mycobacteria (146). As mentioned above, TLR-derived signaling increases Mincle expression and can thereby enhance responsiveness to TDM (104, 147). This mechanism may also contribute to the beneficial effect of TLR ligands in *Fonsecaea pedrosoi* infection, a model for human chromoblastomycosis (145) (Fig. 3C).

An intriguing mechanism of synergistic action of TLR and Mincle signaling acting at the level of translation efficiency was revealed recently: combined stimulation of TLR2 and Mincle induced more-than-additive NO production, particularly at later stages of inflammation (107) (Fig. 3D). Protein expression of inducible nitric oxide synthase (iNOS) was mediated by Mincle-controlled increase in translation, which required p38-dependent hypusination of eIF5A. Importantly, the eIF5A-dependent NO production at later stages of inflammation inhibited Nlrp3-mediated IL-1β production, counteracting the synergistic induction of proIL-1β by TLR2 and Mincle. Blockade of eIF5A or iNOS-deficiency resulted in exacerbating inflammation in TDM-induced lung granulomas and enhanced mortality, identifying Mincle as important regulator of anti-mycobacterial immune responses at later stages of inflammation (107).

In addition to these acute synergistic effects of concurrent stimulation of CLR and TLR, Dectin-1 ligands can prime responses to subsequent stimulation by TLR ligands (49), an effect characterized as “training of innate immunity” by Netea’s group (148). These long-lasting effect of CLR signaling depend on Hif1α and mTOR-dependent metabolic changes and epigenetic programming (149, 150) and are distinct from the collaborative effects described above.

Conflicting signaling of CLR: Negative Regulation

Several mechanisms have been proposed to contribute to the negative regulation of cytokine production after CLR ligation. Eberle et al. demonstrated that SOCS1 is induced after stimulation with depleted zymosan (Dectin-1) and CpG (TLR9) in murine bone-marrow macrophages and DC (52) (Fig. 4A). SOCS1 induction is dependent on Syk, Pyk2 and ERK activation, but Ca^{2+} and NFκB independent. It resulted in decreased and shortened activation of NFκB (p50 and p52) and thus reduced IL-12p40 secretion. In peritoneal macrophages SOCS1 and PIAS1 induction downstream of Dectin-1 has been described in a Ca^{2+}-dependent manner to be dependent on the expression of Wnt5a, induced by the ROS-β-catenin axis. SOCS1 and PIAS1 induction lead to reduced expression of IL-12, IL-1β and TNF and abrogated TLR signaling via degradation of IRAK-1, IRAK-4 and MyD88 (151). Downstream of Dectin-2, but not of Dectin-1, β-catenin stabilization in DC occurs dependent on phosphorylation of LAB and leads to impaired IL-12 production (152).

As mentioned above, Mincle is important for recognition of *Fonsecaea pedrosoi*, but synergistic TLR stimulation and TNF production was required to clear the infection in a mouse model of chromoblastomycosis (145) (Fig. 3C). In contrast, Mincle engagement counter-acted the induction of IL-12 by *Fonsecaea monophora* in human DC (Fig. 4B). *F. monophora* simultaneously engages Dectin-1, leading to activation of IRF1 and IL-12A (IL-12p35) transcription, and Mincle. In a PI3K-PKB-dependent manner, Mincle activates the E3 ubiquitin ligase Mdm2, leading to degradation of Dectin-1 induced IRF1, thus blocking IL-12A transcription. Degradation of TLR-induced IRF1 was similarly observed. The blockade of IL-12A resulted in a shift from a protective Th1 to a detrimental Th2 response in cocultures with T cells in vitro (50). Thus, while sensing of *F. pedrosoi* by Mincle is required for innate protection, the negative effect on IL-12 production may interfere with the development of protective T cell immunity. Along this line, Mincle-deficient mice showed an increased Th17-response in *F. pedrosoi* infection (153).

Finally, Miller and coworkers recently demonstrated an unexpected inhibition of TLR4-dependent inflammatory cytokine expression by the CLR Dectin1 and Mincle (Fig. 4C). First, they observed that Dectin-1-deficient mice showed more hepatic fibrosis in a model of liver inflammation (154). Similarly, Mincle-deficient mice were more susceptible to endotoxic shock than wild type controls, resulting in higher mortality and elevated cytokine levels (155). In both studies, this enhanced susceptibility was attributed to increased levels of the TLR4 co-receptor CD14 in Dectin-1- or Mincle-deficient mice. Blockade of PKC and M-CSF abrogated the elevated CD14 expression in Dectin-1 deficient mice (154). Mincle-deletion lead to enhanced JNK phosphorylation but decreased p38 phosphorylation and subsequent activation of suppressor of cytokine signaling 1 (SOCS1), A20 and ABIN3 which supposedly control CD14 expression, and in addition may induce degradation of Traf6 and MyD88 (155). These findings suggest that control of TLR responses by CLR can not only occur by transcriptional control but also by (indirect) modulation of the levels of components of the TLR signaling machinery. The nature of the ligands for Dectin-1 and Mincle in the hepatic fibrosis and LPS challenge models have not been defined. However, in the case of Mincle, the same group most recently demonstrated evidence that the endogenous Mincle ligand SAP130 (6) triggers Mincle in a mouse pancreatic tumor model, promoting tumor growth through inhibitory effects on T cell responses (156), and in a mouse model of acute liver inflammation, exacerbating disease (157). SAP130 may be induced and released during LPS- or infection-induced inflammation from dying cells and provide the trigger for inhibitory Mincle signaling.

Conclusions

CLR as a group of PRR have gained increasing attention during the last 10 years, with Dectin-1 often regarded as a prototypic receptor. Like Dectin-1, the related receptors Dectin-2, Mincle and Mcl were found to signal dependent on the Syk-Card9 pathway. These CLR have been characterized as receptors not only for various pathogens but also endogenous ligands. Consequently, their roles reach from infection and inflammatory conditions to homeostatic regulation. The number of pathways and signaling events identified downstream of CLR ligation is continuously increasing, providing us with a gradually more precise but also more complex picture of signal transduction and reprogramming triggered in innate immune cells. Further research is needed to clarify which of these pathways are universal, such as the Syk-Card9 axis, and which responses occur in certain species, certain cell-types or for certain receptors or ligands. Pathogens are recognized by multiple PRR simultaneously, therefore it is essential to investigate not only events dependent on a single receptor but also cross-talk between receptors or even classes of receptors. We have reviewed studies investigating the integration of signals derived from CLRs and TLRs, with examples for both synergistic and antagonistic interactions between different CLR or with TLR. While there are many examples of collaborative signaling with strongly boosted responses, e.g. by concurrent stimulation of TLR2 and Dectin-1, accumulating evidence shows that specific CLR signaling can attenuate or abrogate at least certain types of CLR/TLR-induced activation. Another important aspect of CLR research has been the cross-regulation of expression levels at the mRNA and protein level, which can determine the level of responsiveness to the respective microbial ligands. A fascinating question for future research in this area will be to investigate the consequences of direct receptor interaction, such as formation of Mcl – Dectin-2/Mincle-heterodimers, on the avidity and specificity of ligand binding. Thus, signaling crosstalk downstream of CLR specifically modulates immune reactions and can control inflammatory responses. Mapping this complex signaling network will result in a new level of understanding of CLR's role in innate and adaptive immune responses and may open up perspectives to target these receptors for treatment and prevention of infectious and inflammatory conditions.

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Figure legends

Fig. 1: Schematic comparison of CLR and TLR signaling. Transmembrane receptors (green), adapter proteins (blue), kinases (red), transcription factors (orange). Examples of target genes from overlapping and distinct transcriptional responses. The CLR Dectin-1, Dectin-2 and Mincle share canonical signaling via FcRγ, Syk and the CBM complex (left). All TLR except TLR3 recruit the adapter protein MyD88, the adapter TRIF is required for signaling by TLR3 and TLR4 (right).

Fig. 2: Mcl-Mincle cooperation at multiple levels. (A) In the absence of Mincle expression in resting macrophages, binding of TDM to Mcl is sufficient and required to induce Mincle mRNA expression. Mcl-induced Mincle expression establishes a feed-forward loop of TDM responsiveness (67). (B) Mincle and Mcl act as chaperones for each other, increasing the cell surface expression levels via enhanced transport and/or stabilization (117, 118, 147). (C) Heterodimerization of Mcl and Mincle may increase the affinity for ligands via cooperative binding. Depending on the topology of the heterodimers, ligands like TDM may be contacted by Mincle and Mcl forming one heterodimer (left) or may connect two heterodimers together (right). Heterodimer formation could also create specific binding to ligands not recognized by the single receptors.

Fig. 3: Collaborative signaling between CLR and TLR. (A) CR3 (CD11b/CD18) binds zymosan and *Histoplasma capsulatum* dependent on iC3b, triggering Syk activation and cooperates with b-glucan-induced Dectin-1 signaling for robust JNK/AP-1 activation (40). (B) TLR2 and Dectin-1 bind simultaneously to zymosan and synergize in the NFκB activation and production of TNF, IL-23 and IL-6 (126, 136, 137). Enhanced IL-10 production down-

regulates IL-12 expression (144). (C) Macrophage activation in response to *Fonseceae pedrosoi* requires Mincle. Treatment of infected mice with TLR ligands enables the clearance of infection, suggesting that TLR-MyD88 and Mincle-Syk synergize in the upregulation of the cytokines and mediators required for killing of *F. pedrosoi* (145). TLR-MyD88 signals strongly enhance Mincle mRNA and protein expression, and thereby sensitize macrophages for responsiveness to Mincle ligands such as mycobacterial TDM and *F. pedrosoi* (104, 147). Note that TLR7 and TLR9 are localized in the endosome and are shown here as cell surface receptors for reasons of simplicity. (D) TLR-Mincle synergy in protein expression of a subset of inducible genes, most notably iNOS, is mediated by Mincle-controlled increases in translation due to p38-dependent hypusination of eIF5A. While required for robust inflammatory responses, Mincle-signaling contributes to termination and resolution of inflammation by NO-mediated inhibition of the Nlrp3 inflammasome and IL-1 release (107).

Fig. 4: Conflicting signaling: negative regulation by CLR activation. (A) Dectin-1 triggering upregulates Socs1 through Pyk2-ERK activation, which inhibits TLR-induced IL-12 production, associated with inhibition of NFkB activation (52). In a later study, Socs1 induction after Dectin-1 triggering was shown to depend on the β-Catenin-induced secretion of Wnt5a which in turn triggers Pyk2 via Frizzled (151). In this study, Dectin-1-induced Socs1 caused a severe loss of MyD88-IRAK4-TRAF6 proteins and unresponsiveness to TLR ligation. TLR9 is an endosomal receptor shown here in the plasma membrane for reasons of simplicity. (B) The fungal pathogen *F. pedrosoi* triggers both Mincle and Dectin-1 signaling. Wevers et al. showed in human DC selective activation of PI3K-PKB dependent on Mincle, which interferes with Dectin-1induced expression of IL-12 by the targeting of nuclear IRF-1 for degradation through the PKB-mediated activation of the E3 ubiquitin ligase Mdm2. Of note, Mincle activation also inhibited TLR9-induced IL-12 expression through the same

mechanism (50). (C) Mincle and Dectin-1 inhibit responses to LPS by down-regulating the expression of the LPS co-receptor CD14. Mincle$^{-/-}$ and Dectin-1$^{-/-}$ mice are more susceptible to LPS shock due to excessive cytokine production. Macrophages from Dectin-1$^{-/-}$ mice had higher CD14 and TLR4 surface expression (154), whereas in Mincle-deficient macrophages only CD14 was elevated (155). Induction of Socs1, ABIN3 and A20 by LPS, as well as the degration of TRAF6 and Mal, was Mincle-dependent after LPS stimulation. The basis for LPS-induced Mincle/Dectin-1-dependent Syk-activation is at present unknown.

Table I: Overview of microbial and endogenous ligands of Syk-coupled CLR.

Ligand	Comments	Reference
Mcl (Clec4d)		
Klebsiella pneumoniae	protective role in infection model	(158)
Mycobacterium tuberculosis, M. bovis	protective role in infection model	(91)
Trehalose-dimycolate (TDM) from *Mycobacterium spp.*	mouse, human, not guinea-pig	(67, 70, 87, 122)
Blastomyces dermatitidis	mouse	(94)
Candida albicans	controversial role in infection models	(92, 93)
Cryptococcus neoformans	protective role in infection model	(159)
Mincle (Clec4e)		
Cholesterol crystals (endogenous)	CRAC motif, human, not mouse/ rat	(86)
SAP130, dead cells (endogenous)	Ca^{2+}-independent, VEGQ motif	(6-9, 157, 160)
Helicobacter pylori	human	(161)
Klebsiella pneumoniae	protective role in infection model	(162)
Streptococcus pneumoniae	mouse	(163)
Mycobacterium tuberculosis, M. bovis, M. smegmatis	controversial role in infection models	(88-90, 97, 98)
Cyclopropane-fattyacid α-glucosyl diglyceride from *Lactobacillus plantarum*	mouse/ human	(164)
β-gentiobiosyl diaglycerides from *M. tuberculosis* (H37Ra)	mouse, not human	(165)
Trehalose-dimycolate (TDM) from *Mycobacterium spp.*	Ca^{2+}-dependent, mouse/human/guinea pig/cow	(70-72, 87, 97, 98, 105, 122, 166)
Synthetic trehalose-diesters, including trehalose-dibehenate (TDB), corynomycolates	mouse/human	(70-72, 74, 81, 98, 122, 167-169)
Synthetic trehalose-monoesters	mouse	(82)
Glycerol-monomycolate (MMG,GroMM)	human, not mouse	(85)
Brartemicin	mimicks glycolipid binding	(83)
Candida albicans	mouse/ human	(116, 166, 170)
Cladophialophora carrionii	human	(50)
Fonsecaea pedrosoi, F. monophora, F. compacta	mouse/ human	(50, 145, 153)
Malassezia furfur, glycolipid	mouse	(167, 171)
Dectin-2 (Clec4n/Clec6a)		
CD4+ CD25+ T cell ligand (endogenous)	mouse	(172)
Klebsiella pneumoniae, K55 LPS	mouse	(173)
Streptococcus pneumoniae, serotype 3	protective role in infection model	(173, 174)
Mycobacterium spp., mannose-capped lipoarabinomannan (Man-LAM)	mouse	(173, 175)
Aspergillus fumigatus	mouse	(176)

Blastomyces dermatitidis	mouse	(94, 177)
Candida albicans, C. glabrata, α-mannan, hyphae	mouse	(68, 99, 103, 173, 178)
Coccoides posadasii	mouse	(177)
Cryptococcus neoformans	mouse	(179)
Fonsecaea pedrosoi	mouse	(153)
Histoplasma capsulatum	mouse	(173, 177)
Malassezia furfur, O-linked mannoprotein	mouse	(167)
Microsporum audouinii, hyphae	mouse	(68)
Paracoccoides brasiliensis	mouse	(173)
Trichophyton rubrum, hyphae	mouse	(68, 180)
Saccharomyces cervisiae	mouse	(173)
Schistosoma mansoni	mouse	(108)
Dermatophagoides pteronyssinus, house dust mite	mouse	(181)
Dectin-1 (Clec7a)		
T cell ligand (endogenous)	mouse	(182)
Ligand on parenchymal/ inflammatory cells in liver (endogenous)	mouse	(183)
Vimentin (endogenous)	human	(184)
Haemophilus influenzae, nontypeable	mouse/ human	(185, 186)
Mycobacterium spp.	mouse/ human	(33, 187, 188)
Aspergillus fumigatus, maturing conidia, germ tubes	mouse/ human, protective role in infection model	(189-192)
Candida albicans, β-1,3 glucan, yeast	mouse/ human, protective role in infection model	(31, 68, 124, 193, 194)
Coccidioidis posadasii, C. immitis	mouse	(177, 195)
Histoplasma capsulatum	mouse	(40, 177, 196)
Microsporum audouinii, yeast	mouse	(68)
Penicillium marneffei	mouse	(197)
Pneumocystis carnii	mouse	(198)
Trichophyton rubrum, yeast	mouse/ human	(68, 180, 199)
Trichosporon asahii	mouse	(200)
Saccharomyces cervisiae, glucan	mouse	(198)
Curdlan, particulate β-glucan		(201, 202)
Laminarin, soluble β-glucan	blocking, non-activating	(34, 35, 201, 202)
Zymosan	non-depleted zymosan also binds TLR2	(126, 201-203)
Ju-6, hexavalent lactoside	non-β-glucan, not blocked by Laminarin	(204)

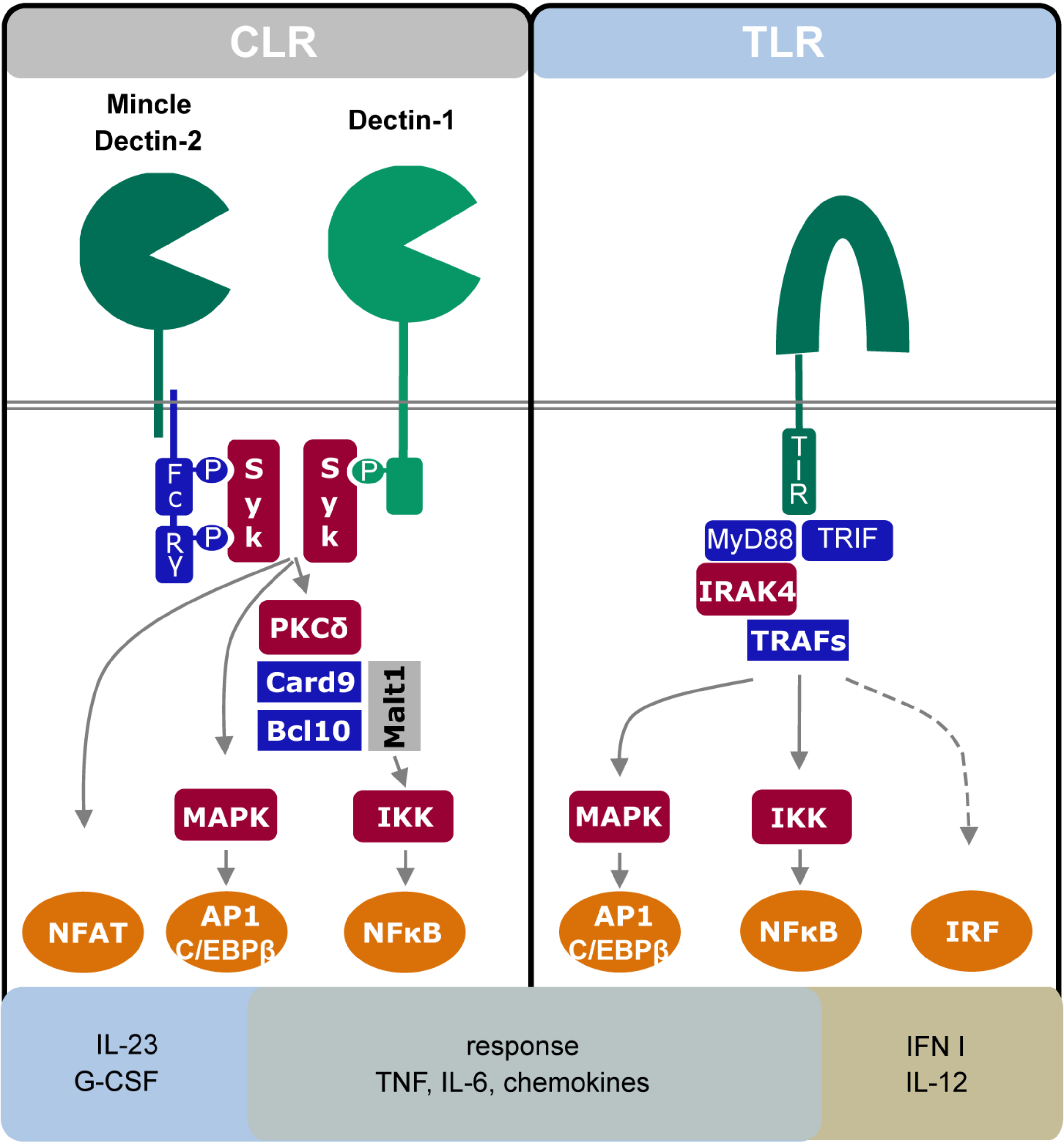
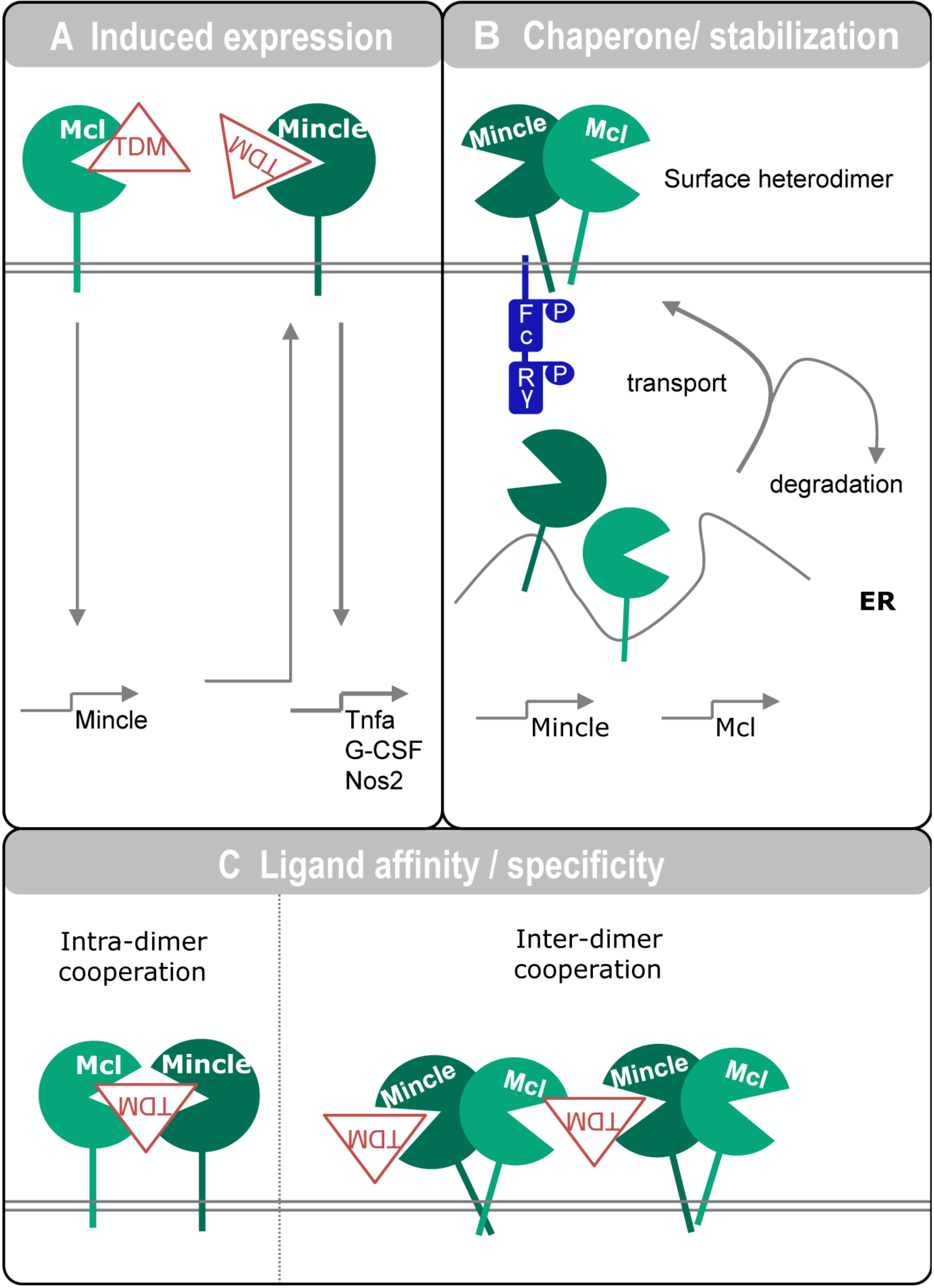
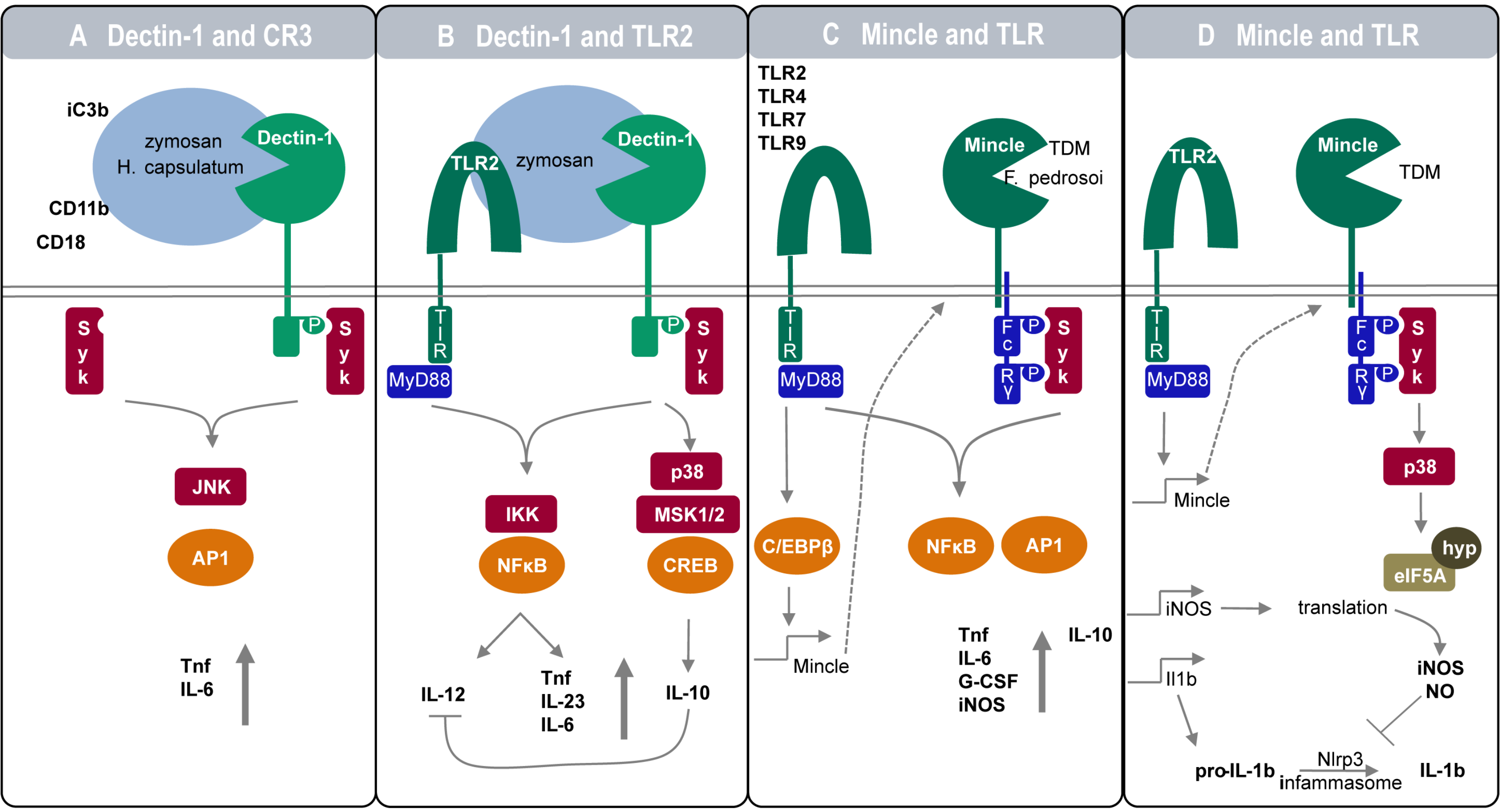

CLR
TLR
Mincle
Dectin-2
Dectin-1
FcRγ
P
P
Syk
P
Syk
TIR
MyD88
TRIF
IRAK4
TRAFs
PKCδ
Card9
Bcl10
Malt1
MAPK
IKK
MAPK
IKK
NFAT
AP1
C/EBPβ
NFκB
AP1
C/EBPβ
NFκB
IRF
IL-23
G-CSF
response
TNF, IL-6, chemokines
IFN I
IL-12

Figure 1



A Induced expression
Mcl
TDM
Mincle
TDM
Mincle
Tnfa
G-CSF
Nos2
B Chaperone/ stabilization
Mincle
Mcl
Surface heterodimer
F
c
R
γ
P
P
transport
degradation
ER
Mincle
Mcl
C Ligand affinity / specificity
Intra-dimer
cooperation
Inter-dimer
cooperation
Mcl
Mincle
TDM
Mincle
Mcl
TDM
Mincle
Mcl
TDM

Figure 2


A Dectin-1 and CR3
iC3b
zymosan
H. capsulatum
Dectin-1
CD11b
CD18
Syk
P
JNK
AP1
Tnf
IL-6
B Dectin-1 and TLR2
TLR2
zymosan
Dectin-1
TIR
MyD88
P
Syk
IKK
NFκB
p38
MSK1/2
CREB
IL-12
Tnf
IL-23
IL-6
IL-10
C Mincle and TLR
TLR2
TLR4
TLR7
TLR9
Mincle
TDM
F. pedrosoi
TIR
MyD88
FcRγ
P
Syk
C/EBPβ
NFκB
AP1
Mincle
Tnf
IL-6
G-CSF
iNOS
IL-10
D Mincle and TLR
TLR2
Mincle
TDM
TIR
MyD88
FcRγ
P
Syk
p38
Mincle
hyp
eIF5A
iNOS
translation
iNOS
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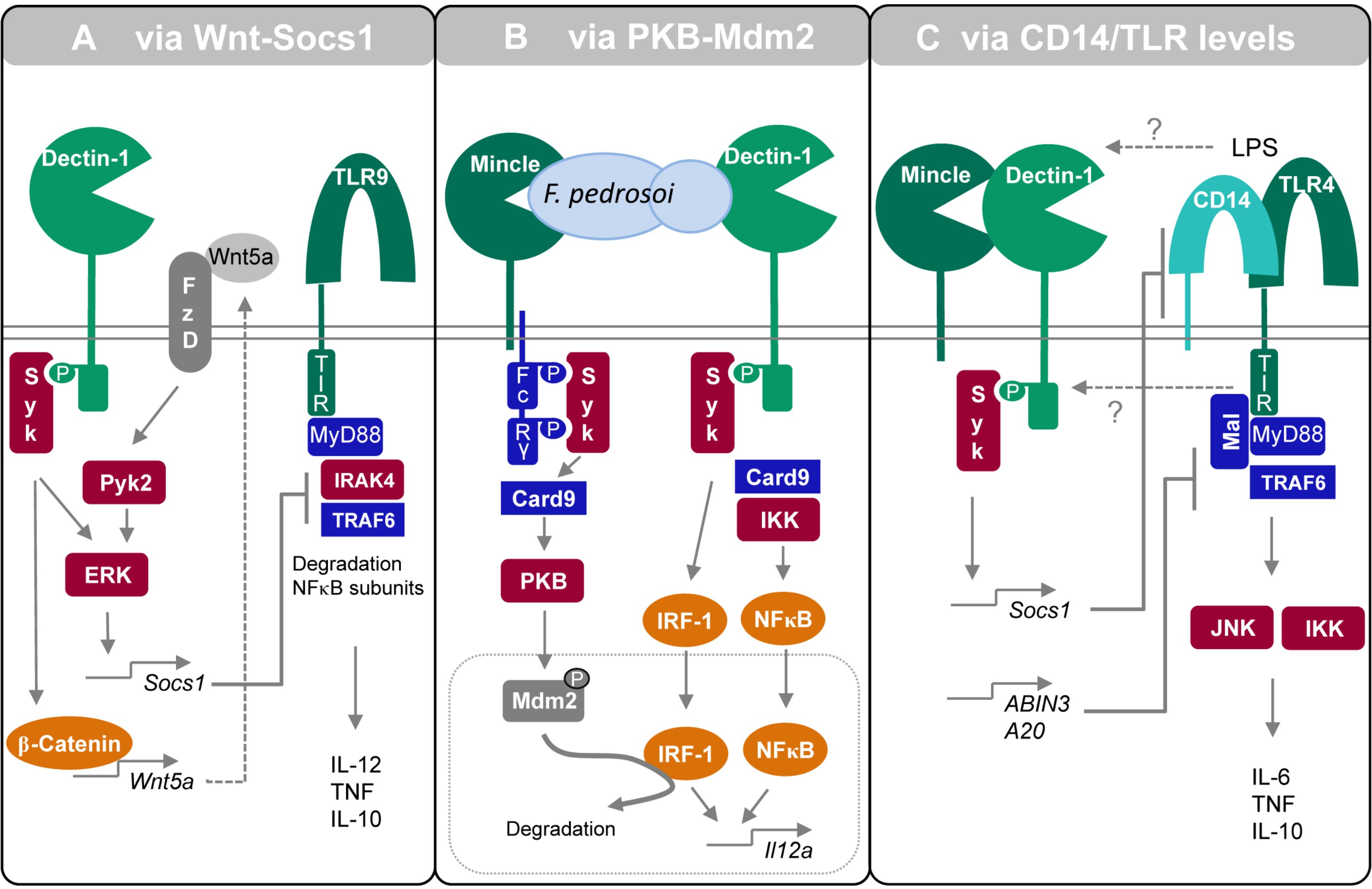
pro-IL-1b
Nlrp3
infammasome
IL-1b

Figure 3



A via Wnt-Socs1
B via PKB-Mdm2
C via CD14/TLR levels
Dectin-1
TLR9
Wnt5a
F z D
S y k
P
T I R
MyD88
IRAK4
TRAF6
Pyk2
ERK
Degradation NFκB subunits
Socs1
β-Catenin
Wnt5a
IL-12
TNF
IL-10
Mincle
F. pedrosoi
Dectin-1
F c R γ
P
S y k
Card9
PKB
Mdm2
IKK
IRF-1
NFκB
Degradation
Il12a
?
LPS
Mincle
Dectin-1
CD14
TLR4
Mal
MyD88
TRAF6
Socs1
ABIN3
A20
JNK
IKK
IL-6
TNF
IL-10

Figure 4