Microreview

Molecular view on PRR cross-talk in antifungal immunity

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Summary

The identification of a major class of innate immune receptors, termed pattern recognition receptors (PRRs), has boosted research on innate pathogen recognition. The immune response to a specific pathogen is not restricted to the recognition by one type of PRR or activation of a single cell type, but instead comprises complex collaborations between different receptors, cells and signal mediators. Here we will discuss the cross-talk between PRRs involved in fungal recognition, focusing on the molecular interactions occurring at the plasma membrane.

Introduction

In multicellular organisms, the innate immune system is the first line of defence against invading pathogens. Recognition and uptake of these microbes is crucial for an effective host defence, and is facilitated by phagocytes such as neutrophils, macrophages and dendritic cells (DC). Phagocytosis of microorganisms by neutrophils triggers direct cellular antimicrobial immunity such as production of reactive oxygen species and the fusion of cytoplasmic granules with pathogen containing vacuoles (Segal, 2005). This mechanism is highly effective in killing most bacteria and fungi. Furthermore, recognition of pathogens by phagocytes activates intracellular signalling pathways that result in production of numerous cytokines and chemokines. These mediators attract more phagocytes and activate antigen presenting cells (APC) such as DC, creating an essential foundation for the initiation of adaptive immunity, which protects us from reinfection.

Despite its importance, in the past the innate immune system has received relatively little attention. The identification of the innate immune receptors termed pattern recognition receptors (PRRs) provided a boost for research on innate pathogen recognition. Indeed, proper pathogen recognition is key to adequate immune defence, and is facilitated by PRRs expressed on phagocytes. By definition, these germline-encoded receptors detect pathogen-associated molecular patterns (PAMPs), structures conserved among microbial species. Well-known PAMPs include the bacterial components lipopolysaccharide, peptidoglycans and flagellin, viral nucleic acids and fungal polysaccharides such as mannan and β-glucans. Currently, PRRs are categorized into four classes: (i) Toll-like receptors (TLRs), (ii) C-type lectin receptors (CLRs), (iii) retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) known also as RIG-I helicases (RLHs) and (iv) the NOD-like receptors (NLRs) (Box 1).

In the past, the majority of in vitro studies aiming to unravel the mechanism of pathogen recognition by PRRs used an isolated cell type or a single PAMP. In recent years it has become increasingly apparent that the immune response to a specific pathogen is not restricted to the recognition by one type of PRR or activation of a single cell type, but instead comprises complex collaborations between different receptors, cells and signal mediators. Indeed, in vivo invading pathogens generally contain multiple PAMPs that are recognized by various PRRs on multiple cell types. Additionally, there is redundancy in the recognition of PAMPs between different classes of PRRs. The simultaneous or sequential binding of multiple PRRs to different PAMPs resulting in co-ordinated activation or inhibition of signal transduction is referred to as ‘receptor cross-talk’. It is anticipated that
Box 1. Four classes of pattern recognition receptors.

**TLRs** are the best-characterized PRRs. These type I transmembrane proteins are present on the plasma membrane or within the endosomal compartment. The extracellular domain is involved in the recognition of PAMPs, while the intracellular TIR domain activates signalling pathways leading to the activation of NF-κB. So far, 10 functional TLRs have been identified in man versus 12 in mice. Each TLR detects different PAMPs from viruses, bacteria, fungi and parasites (Akira et al., 2006). Some TLR, such as TLR2, form functional heterodimers, thus further broadening PAMP recognition.

**CLRs** represent the second class of PRRs, mainly recognizing carbohydrate ligands. The CLR family comprises transmembrane- and soluble receptors that share a carbohydrate-recognition domain (CRD) (Osorio and Reis e Sousa, 2011). Not all CLRs effectively function as PRRs. Those which do, can be divided into two classes: ‘self-sufficient’ CLRs that autonomously recognize PAMPs initiating downstream signalling, and CLRs that do recognize PAMPs but are dependent on partnering proteins for intracellular signalling.

**RLRs** are present in the cytosol and involved in sensing intracellular pathogens. RLRs bind mainly viral nucleic acids. So far, three members of the RLR family have been identified: RIG-I, MDA-5 and LGP2. RIG-I and MDA-5 recognize a wide range of viruses and initiate IFN responses, whereas LGP2 is considered to regulate responses initiated by RIG-I and MDA-5 (Loo and Gale, 2011).

**NLRs** are also expressed intracellularly. Members of the NLR family have a common domain architecture that includes a nucleotide-binding domain (NBD) and a leucine-rich repeat (LRR) domain. The best-characterized NLRs are NOD1 and NOD2. They sense different building blocks of peptidoglycans (PGN). Some NLRs can function as crucial components in multiprotein complexes termed ‘inflammasomes’ that are important for activation of inflammatory caspases and cytokines of the IL-1 family (Elinav et al., 2011).

The presence of PRRs in multi-receptor complexes in the plasma membrane facilitates this cross-talk. As a consequence, separate or shared signalling pathways are activated that ultimately determine the type and magnitude of the immune responses directed against the pathogen.

The outcome of PRR cross-talk may be quantitative when receptors act synergistically or antagonistically. Synergistic responses may be particularly important under conditions of low ligand concentrations, when recognition by more than one receptor can enhance the immune response. A classical example of the synergistic response is the production of IL-12p70 cytokine by DCs, which increases greatly when multiple PRRs are triggered (Tada et al., 2005). On the other hand, collaboration of PRRs is also important to dampen unwarranted host responses. For example triggering of the CLR, DCIR, selectively inhibits TLR8-mediated IL-12 and TNF-α production (Meyer-Wentrup et al., 2009).

Cross-talk between distinct PRRs can also have a qualitative effect. For instance, recognition of *Salmonella typhimurium* by TLR4 and TLR5 activates transcription of proinflammatory cytokines including IL-1β. Subsequent release of functional IL-1β from the cells, however, requires processing of pro-IL-1β by Caspase-1, which is critically dependent on activation of NLRP3 and NLRC4 in the inflammasome (Miao and Rajan, 2011).

Hence, collaboration between PRRs enhances the specificity of recognition, broadens their signalling capacity and enables the host to detect and respond to almost any type of infection. In this review we will focus on the molecular basis of PRR cross-talk important in detection of the fungal pathogen *Candida albicans*.

**Fungal recognition by the innate immune system**

The last decade has yielded significant advances in the identification and functional characterization of a variety of PRRs that sense fungi. *C. albicans* represents an opportunistic fungus that asymptptomatically colonizes the mucosa of most healthy individuals. However, mucosal and/or systemic infections caused by *C. albicans* are regularly observed in immunocompromised patients, emphasizing the importance of the immune system in clearing fungal pathogens. The severity of such infections is exemplified by the high mortality rate among patients with invasive candidiasis, despite the availability of novel effective antifungal drug classes such as the azoles and echinocandins.

The cell wall of *C. albicans* is comprised of different sugar polymers (chitin, β-glucans, mannan) and proteins that can function as PAMPs for CLRs, TLRs and NLRs (Netea et al., 2008a; Bourgeois et al., 2010). In addition, members of the scavenger receptor (SR) family and certain integrins also possess intrinsic fungal recognition capacity although these are not classified as classical PRRs. Many PRRs that are involved in *C. albicans* recognition belong to the CLR family [reviewed in: (Willment and Brown, 2008)] and can directly activate signalling pathways, or indirectly through interaction with other signalling adaptors or receptors, such as the FcRγ chain or TLRs (Geijtenbeek and Gringhuis, 2009). The importance of CLR in antifungal immune responses has been validated in different murine knock-out models [reviewed in (Netea et al., 2008a; Willment and Brown, 2008)].

The recent finding that human Dectin-1 deficiency causes an increased susceptibility to mucocutaneous fungal infections emphasizes the importance of specific CLR in vivo (Ferwerda et al., 2009). Furthermore, individuals with mutations in the CLR adaptor molecule CARD9 are more susceptible to both mucosal and systemic fungal infections (Glocker et al., 2009). Dectin-1 is expressed by DCs, macrophages and monocytes, where ligation with its ligand β-1,3 glucan induces phagocytosis and an oxidative burst, as well as the production of eicosanoids, inflammatory cytokines and chemokines. Albeit β-1,3 glucans in de cell wall of *C. albicans* are only
exposed in bud scars or after heat inactivation of the yeast, Dectin-1-knock-out mice were documented to have increased susceptibility for *C. albicans* infections (Taylor et al., 2007). However, it must be noted that different results were obtained between the mice models BALB/c and 129/C57BL/6, most likely due to background differences in inherent T-cell polarization (Schofield et al., 2005; Saijo et al., 2007). In addition, differences can arise between systemic versus mucosal *C. albicans* infections. Dectin-1 is mainly involved in mucosal candidiasis, which is evidenced by the study of Ferwerda et al. (2009). Interestingly, Dectin-1 molecules in myeloid cells have recently been reported to cluster in a ‘phagocytic synapse’ crucial for triggering phagocytosis and full antifungal activity (Goodridge et al., 2011). Responses following Dectin-1 ligation are variable depending on cell type and microenvironment; however, prevailing evidence suggests that Dectin-1 signalling preferential directs Th17 polarization (Goodridge et al., 2009). Similar Th17-polarizing effects have been described for other CLRIs such as dectin-2 and MR (van de Veerdonk et al., 2009; Saijo et al., 2010).

Fungal recognition by the TLR family can be mediated by TLR2/6, TLR4 and TLR9 sensing phospholipomannan, O-linked mannann and phagocytosed fungal DNA respectively (Netea et al., 2008a). The latter, however, seems to have a redundant role, as TLR9-deficient mice do not show increased susceptibility to *C. albicans* infection (van de Veerdonk et al., 2008). The role of the individual receptors has not been fully elucidated and divergent results have been published; however, it is generally accepted that TLR-mediated antifungal immunity acts through the induction of inflammatory cytokines. Deletion of the intracellular TLR-adaptor protein MyD88 clearly increases susceptibility to fungal infections [reviewed in (Netea et al., 2008a)]. Moreover, TLR4 polymorphisms in humans have been found to associate with an increased risk of invasive fungal infections (Van der Graaf et al., 2006; Pamer, 2008). In contrast, TLR2-deficient mice are more resistant to disseminated candidiasis, showing enhanced Th1 responses and decreased Treg proliferation (Netea et al., 2004; Suttmuller et al., 2006). A similar, although weaker effect, was shown in TLR6-deficient mice, suggesting both TLR2 and TLR6 are involved in *C. albicans* detection (Netea et al., 2008b).

Recent studies have demonstrated an important role for the NLRP3 inflammasome in antifungal immunity (Joly and Sutterwala, 2010). *C. albicans* can induce caspase-1-mediated IL-1β secretion in a NLRP3-dependent manner in APC, most likely through Dectin-1/Syk kinase/CARD9 signalling (Gross et al., 2009; Hise et al., 2009). Interestingly, a critical role for the inflammasome in regulating Th17/Th1 responses during *C. albicans* infection was recently reported (van de Veerdonk et al., 2011). Other receptors involved in *C. albicans* detection include the family of scavenger receptors (SR), integrins and Fc receptors (FcR). SR represent structurally unrelated proteins that recognize multiple ligands including lipoproteins, β-glucan motifs, microbial antigens, and modified or endogenous molecules derived from the host (Mukhopadhyay et al., 2004). Integrins on the other hand can act as opsonic receptors that recognize fungal particles coated with complement factors, or possess intrinsic microbial recognition. Fc receptors (FcR) recognize antibody-opsonized *C. albicans* and can also efficiently induce phagocytosis, cytotoxicity and/or antigen presentation (van Spriel et al., 1999; Nimmerjahn and Ravetch, 2006).

Recognition of *C. albicans* by these many different PRRs expressed by innate immune cells is a highly complex and dynamic process. Evidence is accumulating that the clustering of receptors, including PRR, into organized membrane complexes, such as lipid rafts or tetraspanin microdomains, is important to regulate ligand binding and subsequent signal transduction (Cambi et al., 2004; Figdor and van Spriel, 2010; Fessler and Parks, 2011). This may allow for cross-talk between the different PRRs that facilitates integration of different incoming signals leading to a potent antifungal response.

**Molecular view on PRR cross-talk in fungal recognition**

Collaboration between different classes of PRRs is important for the innate immune responses to *C. albicans*. The underlying molecular mechanisms, however, are still largely unknown. Interaction studies have been performed for some of these PRRs, and suggest physical contact between some of the receptors at the plasma membrane. True insight into the molecular interaction platforms involved in PRR collaboration requires more detailed characterization of these interactions, and should distinguish between direct and indirect binding. Here we will review the current understanding of the molecular interactions among PRRs at the plasma membrane that are involved in fungal recognition.

**TLR-2/TLR-6**

TLR2 is known to form heterodimers with TLR6 in the recognition of fungal PAMPs. This interaction is well characterized at the molecular level with known crystal structure and binding domains. Dimerization of TLR molecules occurs at both the extracellular Leucin-rich repeat domain (LRR) and the intracellular TIR domain. The LRR domain forms the characteristic TLR horseshoe structure, consisting of the 24 amino acids conserved motif XLXXLXXLXXNLXXLPXXXFX. Interestingly, TLR2...
has an aberrant LRR domain that lacks the conserved Asparagine ladder important for structure stability. It has been suggested that this modification allows for variations in structural conformation, permitting the binding of different ligands and receptors. Intracellularly, TIR-TIR interactions depend on the BB-loop, DD-loop and αC-helix, domains that are essential for recruitment of adaptor proteins and domain stability. Indeed, mutations within the BB-loop region have been shown to abrogate TLR signalling. For TLR2/6 complexes, the adaptor proteins MyD88 and Mal directly interact with the TIR domains, mediating downstream signalling leading to NF-κB activation and cytokine production (Carpenter and O’Neill, 2009). In addition, TLR2 can also functionally cooperate with the CD14 receptor, whereby CD14 enhances TLR2-mediated NF-κB activation in response to zymosan (Underhill et al., 1999). Whether a direct interaction of the TLR2 heterodimers with CD14 is essential for this collaboration is unknown.

**Dectin-1**

A molecule well known to functionally synergize with TLR2 is Dectin-1. This CLR family member is composed of an extracellular carbohydrate recognition domain (CRD), a short stalk region, a transmembrane domain and a 40 amino acid long intracellular tail. Alternative splicing generates respectively two and eight isoforms in mice and men, with both species preferentially expressing the ‘stalkless’ Dectin-1 isoform. The extracellular C-type lectin domain is used for β-glucan binding. Ligation of β-glucans triggers phosphorylation of the Dectin-1 intracellular tail, containing an unconventional immunoreceptor tyrosine-based activation motif (ITAM). Conventional ITAM motifs contain two tyrosine phosphorylation sites that are phosphorylated upon receptor activation. Syk family kinases bind to these phosphorylated sites via two Src homology 2 (SH2) domains, forming the basis of the intracellular signalling route. In the Dectin-1 ITAM-like motif, the membrane-distal phosphorylation site is not available for binding to an SH2 domain due to an additional amino acid. However, Syk recruitment and activation requires both SH2 domains to bind phosphorylated tyrosine residues. For Dectin-1 this is only possible by receptor dimerization, providing a binding site for Syk on adjacent clustered Dectin-1 ITAM motifs. Syk subsequently recruits the CARD9/Bcl10/Malt1 complex, leading to cytokine secretion via the activation of Erk, p38 and Jnk MAP kinases, and NF-κB and NFAT transcription factors (Goodridge et al., 2009). Dectin-1 is known to form homodimers and larger clusters in the plasma membrane in order to induce signalling, and has been shown to synergistically collaborate with different TLRs, including TLR2. Despite many attempts, no direct interactions between Dectin-1 and TLR2 have been reported to date.

**Galectin-3**

A molecule possibly linking Dectin-1 and TLR2 molecules is Galectin-3. This S-type lectin receptor belongs to the Galectin family, defined by their conserved elements in the CRD domain. In mammals, 15 members of the Galectin family have been identified, all of which are synthesized and stored in the cytoplasm, separated from their glycan ligands. Upon infection these molecules are released, and function as soluble PRRs or immunomodulators. Galectin-3 contains one CRD and an additional non-CRD domain, which functions in oligomerization. The unbound soluble Galectin-3 is found in monovalent form. Ligand binding induces oligomerization through self-assembly of the N-terminal non-CRD domain, generating pentameric Galectin-3 molecules with multivalent CRDs. Galectins lack a transmembrane domain or signalling motif, but are implicated in direct binding of host glycoproteins, cross-linking receptors and ligands at the cell surface. Indeed, the formation of so-called Galectin-3 lattices has been shown to promote cell-surface retention of cytokine and growth factor receptors, by interfering with endocytosis. This potentially results in prolonged signalling and facilitates receptor collaboration. Importantly, as Galectin-3 binds β-1,2 oligomannans of *C. albicans*, it can also directly cross-link pathogens to this receptor complex (Sato et al., 2009).

Interestingly, Esteban et al. (2011) recently discovered a physical association between Dectin-1 and Galactin-3 in the cell membrane of murine macrophages using co-immunoprecipitation studies. Stimulation with zymosan particles increased the amount of Dectin-1 in the immunoprecipitate (Esteban et al., 2011). In line with this work, cross-talk between TLR2 and Galectin-3 has been reported, which enhanced TLR2-induced TNF-α production. Again, co-immunoprecipitation studies demonstrated an interaction between endogenously expressed TLR2 and Galactin-3 in THP-1 cells, which was critically dependent on prior stimulation with *C. albicans* (Jouault et al., 2006). Unfortunately, secretion of other relevant cytokines such as IL-6, IL-23, IL-10 and IL-12 were not assessed, despite clear effects on TNF-α. This could provide a more complete view on the functional role of Dectin-1-Galectin-3 and TLR2-Galectin-3 collaboration in *C. albicans* detection.

Further insight into the molecular make-up of putative Dectin-1, Galactin-3 and TLR2 complexes requires the identification of the domains involved in the molecular interactions. Both studies use stringent co-immunoprecipitation conditions, suggesting direct interactions. Taken together, these results suggest that
Galectin-3 represents an important mediator in the recognition of *C. albicans* by molecular assembly of TLR2 and Dectin-1 in the plasma membrane at the site of fungal recognition. An important question that remains to be answered is how clustering of these receptors during *C. albicans* detection occurs in time. Possibly, initial binding could be facilitated by coating of fungal material with Galectin-3. This could facilitate ligand binding by Dectin-1 and TLR2/6, as well as recruitment of other cell surface receptors, which will induce intracellular signalling and will stabilize the interaction. Finally, phagocytosis and intracellular degradation may release ligands for intracellular PRRs such as TLR9. Hence, an interesting challenge lies in assessment of the sequential events leading to receptor activation and clustering during *C. albicans* detection.

**Tetraspanins**

Another phenomenon contributing to cross-talk between different PRRs in fungal detection is the occurrence of specialized membrane microdomains controlling signal transduction and cell function. The tetraspanin family of transmembrane-four proteins have the ability to interact *in cis* with specific (immune-) receptors, with each other, and with signalling molecules, whereby they form multimolecular complexes, or ‘tetraspanin microdomains’ (Hemler, 2005; Charrin et al., 2009). There is now convincing evidence that tetraspanins in immune cells control proliferation, antibody production and antigen presentation [reviewed in (Levy and Shoham, 2005; van Spriel, 2011)]. Moreover, tetraspanins are involved in the pathogenesis of infectious diseases (van Spriel et al., 2009; van Spriel and Figdor, 2010). Two independent studies have shown that the tetraspanins CD37 and CD63 interact with Dectin-1 in the cell membrane of human and murine APC (Mantegazza et al., 2004; Meyer-Wentrup et al., 2007). Although the exact binding domains have not been identified, the stalk region present in the larger isoform A of Dectin-1 was not required for the interaction with CD37 as co-immunoprecipitations were successfully performed with isoform B of Dectin-1. CD37 was demonstrated to stabilize Dectin-1 expression at the plasma membrane of macrophages and to inhibit Dectin-mediated signalling leading to IL-6 production. This effect was specific for Dectin-1, because signalling via other PRR (including TLRs) was not affected by CD37 deficiency (Meyer-Wentrup et al., 2007). Thus, tetraspanins can modulate the organization and subsequent downstream signalling of specific PRR by their recruitment into tetraspanin microdomains leading to immune activation or tolerance (Figdor and van Spriel, 2010). It will be intriguing to investigate how the Dectin-1–tetraspanin interaction relates to the reported collaboration between Dectin-1 and TLR2, and moreover whether tetraspanins modulate Syk- or Raf-1-dependent signalling pathways, which are known to be active downstream of Dectin-1.

**Cross-talk between other PRRs involved in *C. albicans* detection**

Different studies have demonstrated that also other CLR, including the mannose receptor (MR), DC-SIGN (SIGNR1 in mouse) and Dectin-2 can collaborate with other fungal sensors during antifungal responses. For example, Dectin-2 was reported to interact with the FcγR chain, which was essential for the detection of *C. albicans* hyphae and the subsequent induction of TNF-α and IL-1 receptor antagonist (IL-1Ra) (Sato et al., 2006). The intracellular domain of Dectin-2 (amino acids 8–14) proximal to the transmembrane domain was required for the association with the FcγR chain. Other studies report functional interactions between Dectin-1 and DC-SIGN/SIGNR1 during *C. albicans* recognition although the underlying molecular mechanisms are ill-defined (Valera et al., 2005; Goodridge et al., 2011). Similarly, TLR4 collaborates with Dectin-1 in APC as has been shown for TLR2 (Ferwerda et al., 2008). Finally, CR3 and also the recently discovered CLR Mincle interacts with Fc receptors in the plasma membrane of APC during *C. albicans* detection (Netea and Marodi, 2010).

Together, clustering of PRRs importantly aids to the formation of the ‘phagocytic synapse’, providing a platform for efficient phagocytosis of the captured antigen (Stuart and Ezekowitz, 2005; Goodridge et al., 2011). However, the amount and type of PRR ligands that are available to immune cells can differ between initial interactions, full blown infections and the type of fungus. Initially, only surface expressed PAMPs on the intact pathogens trigger PRR detection. During ongoing infections, however, more ligands become available through the degradation of fungal material. The quality and quantity of PRRs triggered on immune cells may therefore differ during the development of a fungal infection, possibly resulting in tuning of responses.

**Concluding remarks on the molecular network of fungal recognition**

Based on these studies, one can envisage a dynamic 3D interaction model, in which multiple receptors involved in sensing fungal pathogens can cluster together at the cell surface of innate immune cells (Fig. 1). Receptor compartmentalization provides immune cells with an efficient mechanism for regulated membrane-proximal signalling upon fungal detection. In the context of *C. albicans* recognition, extensive cross-talk between various PRRs has been shown, including the synergistic effects of Dectin-1,
TLR2/6 and Galectin-3 on enhancing NF-κB mediated responses. In addition, negative regulatory circuits are in place, e.g. Dectin-1 interaction with tetraspanin CD37 was demonstrated to inhibit Dectin-1 signalling, possibly by sequestering Dectin-1 molecules away from activating PRR complexes (Meyer-Wentrup et al., 2007). Thus, receptor compartmentalization can act in both stimulatory and inhibitory manners to regulate PRR complexes. Furthermore, we anticipate that PRR complexes are highly dynamic and variable in composition, containing different PRRs and their adapter molecules, as well as non-PRR proteins. Depending on the fungal pathogen, the immune cell type and its activation status, the composition, dynamics and activity of the PRR complexes will be modulated. For example, Dectin-1 ligation with β-glucans reportedly induced NF-κB activation in bone marrow-derived DC, but not in bone marrow-derived macrophages. Furthermore, the Dectin-1/CARD9 signalling pathway is differentially activated in macrophages and DC. In conclusion, PRR cross-talk represents an important regulatory mechanism for the immunological response to pathogens in vivo. Future studies on PRR interactions are therefore necessary to increase further insight in this important process.

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