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REVIEW

SYK kinase signaling and the NLRP3 inflammasome in antifungal immunity

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Abstract Host protection against fungi depends on intact innate and adaptive immune responses. Consistently, fungal infections can cause systemic life-threatening diseases in immunocomprimised individuals, suffering e.g. from cancer or AIDS. Recent work has uncovered essential roles for the spleen tyrosine kinase (SYK) and the cytosolic NLRP3 inflammasome for Interleukin-1β (IL-1β) production in innate antifungal immunity. Upon fungal infection, SYK is activated by several C-type lectin pattern recognition receptors on myeloid cells. Subsequently, SYK signals for the production of reactive oxygen species and for gene transcription to induce pro-inflammatory factors, including pro-IL-1β to initiate antifungal responses. Mature IL-1β production additionally requires cleavage of the pro-IL-1β precursor protein by the inflammatory caspase-1 which is controlled within the NLRP3 inflammasome. Here, we discuss how SYK signaling cooperates with the NLRP3 inflammasome for IL-1β production in antifungal immunity.

Keywords ITAM receptors · SYK · CARD9 · Inflammasome · NLRP3 · IL-1β

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Introduction

Infections with yeast (e.g., *Candida* spp.) or filamentous fungi (e.g., *Aspergillus*) can lead to life-threatening diseases including severe pneumonia or disseminated disease in patients with innate or acquired immune dysfunctions associated with cancer or AIDS [1–3]. *Candida* spp. can also cause severe mucocutaneous infections, thrush, and onychomycosis in susceptible hosts such as patients with diabetes mellitus, autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy or Job's (hyper-IgE) syndrome [3, 4]. Thus, the understanding of the immune response to fungi is critical for the development of future strategies to treat or prevent fungal infections.

Significant progress has recently been made in deciphering how the innate immune system senses fungi and how this sensing triggers antifungal defenses. In general, the innate immune system depends on germline encoded pattern recognition receptors (PRRs) that bind conserved molecular patterns on pathogens (pathogen-associated molecular patterns (PAMPs)). These PRRs include the transmembrane Toll-like receptors (TLRs) [5] and C-type lectin receptors (CLRs) [6] as well as intracellular sensors such as members of the NOD-like receptor (NLR) family [7], RIG-I like helicases [5], and others. Particularly, CLRs are important for fungal detection [8–10]. Several CLRs engage the spleen tyrosine kinase SYK for signal transduction which is a key enzyme for antifungal immunity. Fungal PAMP recognition by CLRs triggers phagocytosis, the generation of reactive oxygen species (ROS), and via de novo gene transcription the production of cytokines and chemokines that orchestrate the immune response against the pathogen. CLRs can also cooperate with TLRs (like TLR2) for cytokine induction [11].

Earlier experiments have demonstrated that the highly active pro-inflammatory cytokine IL-1β plays an essential

role in antifungal immunity [12]. The production of IL-1β is controlled by transcriptional and posttranscriptional signals. NF–κB-mediated gene transcription is essential for the synthesis of the IL-1β precursor pro-IL-1β (signal 1). In addition, a second stimulus (signal 2) triggers proteolytic processing of pro-IL-1β into mature bioactive IL-1β by caspase-1 containing intracellular signaling complexes termed inflammasomes [7, 13]. Work over the last year has revealed an important role for the NLRP3 inflammasome in IL-1β generation upon fungal infection and subsequent host defense. Here, we discuss how SYK tyrosine kinase signaling and NLRP3 cooperate for IL-1β production and antifungal immunity.

SYK signaling upon fungal recognition

Several SYK coupled transmembrane CLRs operate as fungal PRRs [7, 13]. Best characterized among these is Dectin-1 [6], which is expressed on monocytes, myeloid dendritic cells (myeloid DCs), and B cells [6, 14]. Dectin-1 contains a ligand binding ectodomain and an intracellular signaling tail with an atypical immunoreceptor tyrosine-based activation motif (ITAM) with a single YxxL motif. Dectin-1 recognizes β-glucans which are present in the cell wall of *Candida albicans*, *Pneumocystis carinii*, *Aspergillus fumigatus*, and other fungi [14]. Upon ligand binding, the cytoplasmic signaling domains of Dectin-1 are phosphorylated by SRC tyrosine kinases. These events lead to a recruitment and activation of the kinase SYK, eventually resulting in cell activation. Studies with SYK-deficient cells have established a key function of SYK in the generation of ROS upon Dectin-1 signaling [15]. Via SYK, Dectin-1 activates several transcription factors including NF–κB for the production of cytokines like TNF-α, IL-6, IL-2, IL-10, and also of pro-IL-1β [16–18]. Essential for Dectin-1/SYK-mediated cytokine production via NF–κB is the caspase recruitment domain containing adapter protein CARD9. CARD9 in turn cooperates with the adapter BCL10 to assemble a complex that induces the activation of the canonical IκB kinase-dependent NF–κB pathway in a cell-type-specific manner [16, 19, 20] (Fig. 1). In addition, CARD9 and BCL10 are also required for NF–κB activation downstream of other ITAM-associated myeloid cell receptors [21, 22]. Dectin-1 can also activate the NF–κB pathway via the kinase RAF1 in a SYK-independent manner [23]. It is possible that the SYK- and RAF-1-dependent pathways complement each other in Dectin-1-induced activation of

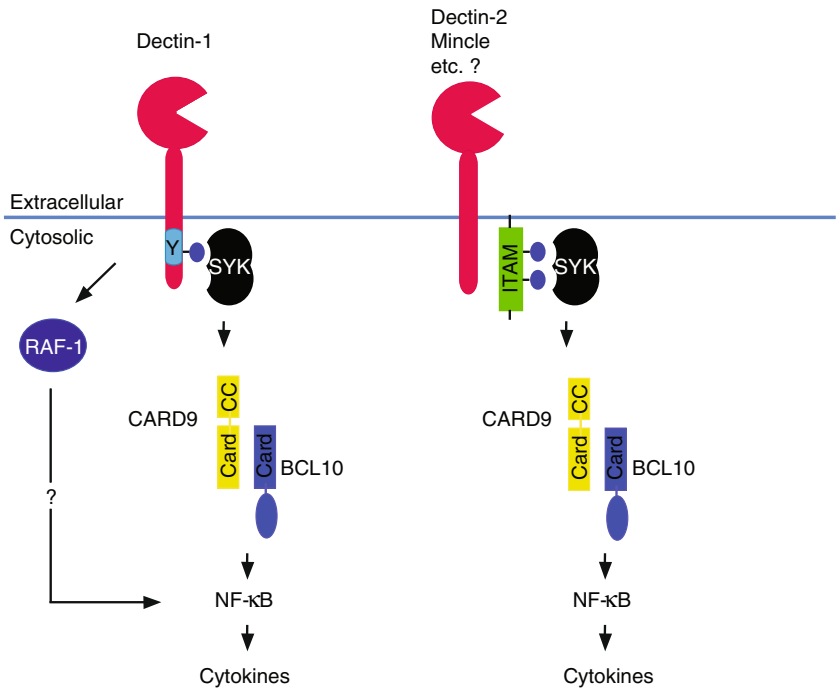

Fig. 1 ITAM receptors and CARD9 signaling. Recognition of fungi by DC-associated C-type lectin 1 (Dectin-1) induces phosphorylation of the intracellular signaling tail with an atypical immunoreceptor tyrosine-based activation motif (ITAM, Y), and subsequently, SYK is recruited. Dectin-2 or Mincle and potentially other CLRs pair with ITAM containing adaptor proteins to recruit SYK for further signal propagation. Via so far unknown mechanisms, a complex is formed containing caspase recruitment domain family member 9 (*CARD9*) and B-cell lymphoma 10 (*BCL10*). This results in activation of *NF–κB*, which then induces the transcription of pro-inflammatory cytokines such as IL-6, TNF-α, or pro-IL-1β. In addition, Dectin-1 can activate the NF–κB pathway via the kinase RAF-1 in a SYK-independent manner. For details, see text

NF–κB, but the contribution of RAF-1 in NF–κB activation by fungi needs to be further investigated for example with the use of gene-deficient mice.

The essential roles of Dectin-1 and CARD9 in antifungal immunity have been demonstrated in knockout mice [16, 24]. Deletion of Dectin-1 results in increased susceptibility to systemic infection with *C. albicans* due to impaired inflammatory responses in macrophages and reduced fungal killing by neutrophils [11, 24]. Likewise, CARD9-deficient mice are also highly susceptible to *C. albicans* infection due to impaired pro-inflammatory responses [16]. Subsequent human genetic studies have described loss of function mutations in Dectin-1 or CARD9 in families that suffer from chronic mucocutaneous candidiasis [25, 26]. Mutations consist of either an early-stop-codon mutation (Tyr238X) in Dectin-1 or a premature termination codon (Q295X) in CARD9. Together, the human and mouse genetic data revealed that the Dectin-1/Syk/CARD9 pathway is an innate signaling pathway that is important for antifungal responses. Importantly, triggering of this pathway in DCs can activate adaptive immunity and induce strong Th17 immune responses which are vital for antifungal defense [18].

Dectin-2 is another SYK-coupled CLR that controls antifungal immunity. Dectin-2 is expressed on myeloid DCs, plasmacytoid DCs, monocytes, macrophages, B cells, and neutrophils [14]. The extracellular CTL domain can bind to *C. albicans*, *Saccharomyces cerevisia*, *Cryptococcus neoformans*, and other fungi. It has specificity for high-mannose structures [27]. In contrast to Dectin-1, Dectin-2 does not contain an intracellular signaling tail but rather associates with the ITAM-containing adaptor molecules Fc receptor γ-chain (FcRγ) [8, 28] to initiate cell activation. Ligand-binding to Dectin-2 (for instance, upon *C. albicans* recognition) triggers phosphorylation of the FcRγ ITAM, recruitment of SYK, and engagement of CARD9 for pro-inflammatory responses [8]. Similar to Dectin-1 ligation, activation of SYK/CARD9 signaling via Dectin-2 activates Th-17 Bitte hier einen space einfügen T cell responses [8].

Mincle is a third myeloid CLR that is able to recognize *C. albicans* and other fungi and induces inflammatory signals [10]. Mincle is expressed on myeloid DCs, monocytes, and macrophages [14]. It detects α-mannosyl expressed by pathogenic fungi like *Malassezia* spp. as well as glycolipids (trehalose-6,6′-dimycolat) present in the cell wall of mycobacteria (such as *M. tuberculosis*) and couples to the ITAM containing FcRγ chain, thereby activating SYK [29, 30]. Thus, Mincle may be able to recognize unique fungal and bacterial structures such as glycolipids to initiate antibacterial and antifungal immunity. Mincle can in addition detect endogenous host ligands like SAP130 (SIN3A-associated protein, 130 kDa), thereby sensing dead cells [31]. Although the precise signaling events downstream of Mincle after fungal infection are not defined, it has been demonstrated that Mincle engages the CARD9 pathway upon SAP130 stimulation [11, 31], and it is well conceivable that Mincle also activates the Syk/CARD9 module upon fungal infection. The fact that Dectin-1-deficient cells can still produce substantial amounts of cytokines upon stimulation with whole fungi indicate receptor redundancy [18], and recently, the scavenger receptors SCARF1 and CD36 were also shown to recognize β-glucans [32]. Yet, both SYK and CARD9 fulfill non-redundant roles in the activation of pro-inflammatory responses, including synthesis of pro-IL-1β [17]. Thus, the SYK/CARD9 axis is a key element for conveying the first signal of fungus-induced IL-1β production. In addition, SYK kinase activity is also required for inflammasome activation and pro-IL-1β cleavage (signal 2) upon fungal infection [17].

Fungi trigger the NLRP3 inflammasome

The term inflammasome refers to intracellular multi-protein complexes that control activation of inflammatory caspases such as caspase-1. Several distinct types of inflammasomes have been discovered that contain specific danger sensors which directly or indirectly couple to pro-caspase-1 to trigger caspase-1 activation [7, 33]. For example, the NLRP1 (also known as NALP1 or DEFCAP) inflammasome mediates IL-1β secretion in response to anthrax lethal toxin [34], while NLRC4 (also known as CARD12 or IPAF) activates caspase-1 upon cytosolic delivery of bacterial flagellin, a component of certain Gram-negative bacteria [35]. Another type of inflammasome is the AIM2 inflammasome [36–39]. AIM2 is a cytoplasmic DNA receptor that activates caspase-1 upon DNA virus infection. Furthermore, the cytosolic RNA helicase RIG-I also triggers caspase-1-dependent inflammasome activation in response to certain RNA viruses [40].

Several studies over the last year have reported that the NLRP3 inflammasome is the essential plattform for caspase-1 activation in response to fungal recognition [17, 41, 42]. Yet, the NLRP3 inflammasome is not specific for fungi. NLRP3 can be activated by multiple distinct exogenous and endogenous triggers which also include crystals (uric acid, silica, and asbestos [43–45]), bacteria (*Staphylococcus aureus*, *Neiseria meningitides* [46]), bacterial pore forming toxins [47], certain DNA (adenovirus) [48], RNA viruses (influenza virus) [49–51], vaccine adjuvants (alum) [52], and fibrillar amyloid-β [53]. At the molecular level, it is still unclear how all these distinct stimuli activate NLRP3.

The NLRP3 protein is composed of a C-terminal region with a series of leucin rich repeats (LRRs), a central nucleotide domain termed the NACHT domain (also

referred to as NOD domain), and an N-terminal effector domain (a pyrin domain (PYD)) [7] (Fig. 2). Upon stimulation, NLRP3 presumably undergoes a conformational change to initiate complex formation. The leucine-rich repeats of NLRP3 are supposed to sense putative ligands, thereby leading to the self-oligomerization of the NACHT domain [54] and subsequent inflammasome assembly. For caspase-1 activation, NLRP3 utilizes the adapter protein ASC which contains an N-terminal PYD and a C-terminal CARD domain [7]. Following oligomerization of the NACHT domains, the PYD of NLRP3 is then exposed and forms homotypic interactions with the PYD of ASC. Subsequently, the CARD domain within ASC binds to and recruits caspase-1 via CARD–CARD interaction to finally form the active NLRP3 inflammasome [13] (Fig. 2).

As indicated above, the NLRP3 inflammasome is indispensable for IL-1β production induced by fungi. This observation was confirmed in macrophages or DCs from mice that are deficient for NLRP3, ASC, or caspase-1. They all have defects in caspase-1 activation or IL-1β production upon fungal recognition, although other pro-inflammatory pathways like TNF-α production are intact [17]. Fungi appear to specifically activate the NLRP3 inflammasome as cells from mice lacking NLRC4 (IPAF) produce normal amounts of IL-1β upon *C. albicans* infection [17].

It has been known for some time that IL-1β is critical for host defense against disseminated candidiasis [12, 55]. Consistently, Nlrp3-deficient mice are highly susceptible to systemic *C. albicans* infection [17, 41, 42]. One study reported a particularly relevant mouse model of sustained mucosal *C. albicans* colonization that resembles the clinical situation in human patients. The authors observed increased fungal burdens in the tongues and impaired survival of Nlrp3-deficient mice [41].

The precise molecular details for fungus-induced NLRP3 activation are still not resolved. Yet, several common mechanisms for NLRP3 activation have been identified in other settings. The common triggers of NLRP3 activation include the generation of ROS [43], which may directly be sensed by NLRP3 or alternatively oxidize a cellular factor that binds to and indirectly activates NLRP3. In addition, sub-physiological amounts of intracellular potassium upon signal-induced potassium efflux are commonly required for NLRP3 activation [56]. Moreover, the phagocytic uptake of particles such as amyloid-β or silica can result in lysosomal disruption [44, 53], leading to a release and activation of the lysosomal protease cathepsin B which in turn activates NLRP3 [44]. Finally, full activation of the NLRP3 inflammasome often requires a so called "priming" signal for example through PRRs such as TLRs or NLRs to initiate NF–κB-dependent transcriptional upregulation of NLRP3 [57].

Experiments with ROS inhibitors have indicated important roles for ROS production in *C. albicans*-induced inflammasome activation [17]. Moreover, *C. albicans*-triggered

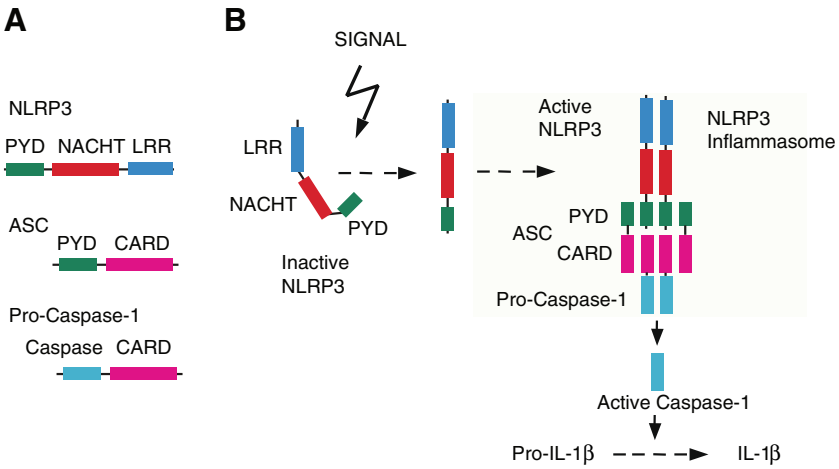

Fig. 2 NLRP3 inflammasome activation. (A) Domain organization of NLRP3, ASC, and caspase-1. NLRP3 consists of three protein domains: the ligand-sensing LRRs, the NACHT domain, and the N-terminal effector domain, a PYD. ASC contains an N-terminal PYD and a C-terminal CARD and is essential for NLRP3 inflammasome formation. Pro-Caspase-1 consists of a CARD domain followed by a caspase domain containing the catalytic residue cysteine. (B) Various exogenous and endogenous signals ("*SIGNAL*") can activate NLRP3 which presumably undergoes a conformational change to initiate complex formation. The leucine-rich repeats of NLRP3 are thought to sense putative ligands, thereby leading to the self-oligomerization of the NACHT domain. This results in homotypic interactions between the PYD and CARD domains found in the inflammasome-forming proteins and ultimately leads to autocatalytic cleavage and activation of caspase-1 and subsequent caspase-1-mediated processing of pro-IL-1β into biologically active IL-1β. *LRRs* leucine-rich repeats; *CARD* caspase recruitment domain; *PYD* pyrin domain; *NACHT* domain conserved in NAIP, CIITA, HET-E, and TP1; *ASC* apoptosis-associated speck-like protein containing a carboxy-terminal CARD

NLRP3 activation requires potassium efflux [17]. In contrast, inhibition of lysosomal acidification in DCs or the use of Cathepsin B-deficient DCs did not affect *C. albicans*-induced IL-1β secretion [17], suggesting that *C. albicans*-induced inflammasome activation can be independent of the lysosomal pathway. In another study, however, the blockage of Cathepsin B with the inhibitor CA-074-Me strongly diminished *C. albicans*-induced IL-1β secretion in macrophages [42]. Thus, Cathepsin-B signaling to NLRP3 could differ among myeloid cell types. Alternatively, CA-074-Me might have off target effects that influence inflammasome activation upon fungal infection.

Intriguingly, NLRP3 inflammasome activation by *C. albicans* requires in addition to ROS production and potassium efflux also SYK kinase activity (Fig. 3). SYK-deficient DCs or cells that were pretreated with a small molecule SYK kinase inhibitor do not produce IL-1β upon fungal infection [17, 58]. This failure is not only caused by defective upregulation of pro-IL1β, which involves the SYK/CARD9 pathway, but also by a failure of caspase-1 activation. Consistently, pre-stimulation of SYK-deficient cells with LPS to induce pro-IL-1β production did not rescue the IL-1β production defect [17]. In contrast, CARD9-deficient cells, which also have a defect in *C. albicans*-induced IL-1β production (due to defective pro-IL-1β synthesis), show regular *C. albicans*-induced caspase-1 activation after pre-stimulation with LPS [17]. Thus, the failure of SYK-deficient cells to activate NLRP3 is not only due to a defect in SYK/CARD9-dependent NF–κB signaling but also involves defective NLRP3 activation. Likewise, production of bioactive IL-1β by Curdlan (a pure β-glucan that is used as a model ligand to study Dectin-1 signaling) requires SYK-mediated synthesis of proactive and bioactive IL-1β [58] further indicating that SYK controls both pro-IL-1β gene expression as well as NLRP3 inflammasome activation. Additionally, RAF-1 inhibition abrogates IL-1β mRNA expression in DCs in response to Curdlan stimulation, suggesting that this pathway may also play a role in fungi induced IL-1β production [23].

As indicated above, SYK signaling upon fungal recognition triggers ROS production [15] and inhibition of SYK kinase activity reduces ROS synthesis [17]. Moreover, ROS inhibition blocks *C. albicans*-induced caspase-1 activation. Thus, SYK-induced ROS synthesis seems to be a prerequisite for *C. albicans*-induced NLRP3 activation. In addition, SYK signaling might also be essential for full transcriptional upregulation of NLRP3 or other factors that are required for inflammasome activation. In this context, it is important to note that SYK triggering by itself with fungal β-glucan preparations (Zymosan) [9, 18] does not activate NLRP3 at shorter stimulation time points (e.g., 2–6 h after stimulation) [42, 58, 59] (and our own unpublished observations). It is also still unclear how much the Dectin-1/SYK signaling axis contributes to ROS production and NLRP3 inflammasome activation upon fungal infection

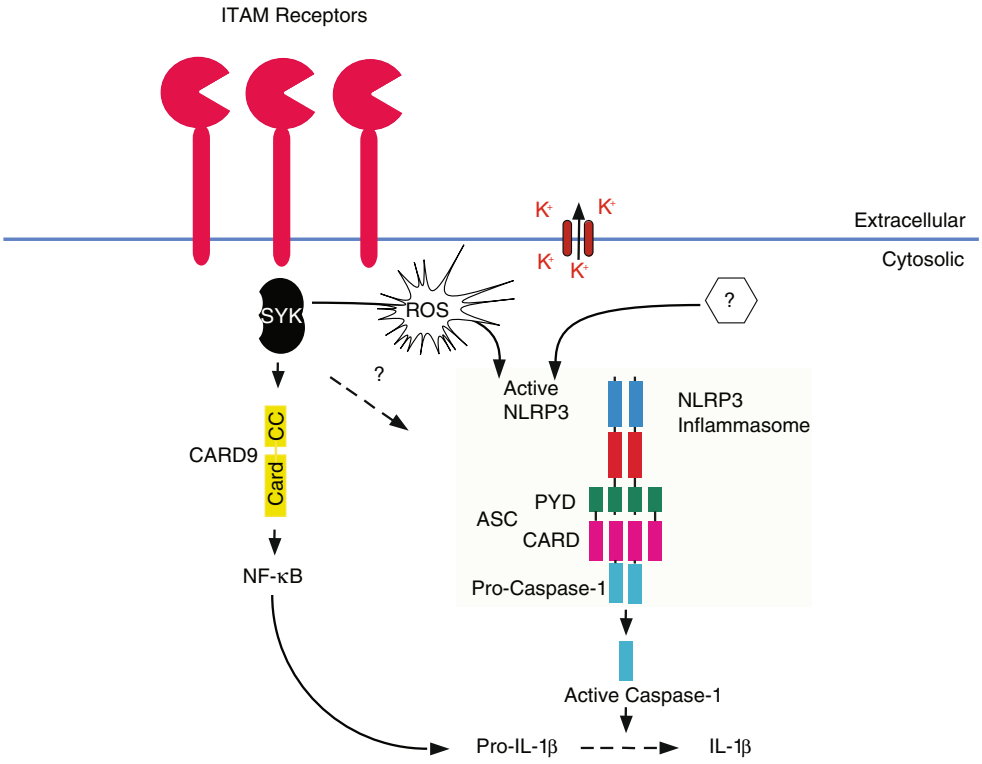

Fig. 3 *C. albicans*-induced inflammasome activation. *C. albicans* induces the synthesis of pro-IL-1β via SYK- and CARD9-dependent pathways. *C. albicans* can also activate the NLRP3 inflammasome through a mechanism that involves SYK-dependent generation of ROS and potassium efflux. C-type lectin receptors (CLRs) that recognize fungi are presumably activating SYK for pro-IL-1β production and NLRP3 activation. In addition, still uncharacterized factors (*?*) from viable yeast are required to cooperate with SYK signaling for NLRP3 activation. For details, see text

in vivo. Moreover, only cell treatment with viable but not with heat-killed or UV-inactivated yeast cells activate NLRP3 [41, 42], and the transition from the yeast to the filamentous phase is important for this effect. Thus, additional still unidentified factors from viable yeast are required to cooperate with SYK for NLRP3 activation. The identification of these factors and (associated) signaling pathways will be an important step in further defining innate antifungal immunity and NLRP3 biology.

Conclusion

The pro-inflammatory cytokine interleukin-1β receives a lot of biomedical attention [60], because it is not only essential for host defense but also responsible for fever, anorexia, tissue damage, and remodeling upon infection. Moreover, excessive IL-1β production is causally associated with a large variety of NLRP3-dependent inflammatory diseases that are triggered by either mutated NLRP3 or NLRP3 activation by environmental or endogenous factors [60]. Over the last year, we learned that SYK and NLRP3 are key regulators of fungus-induced IL-1β production [26, 41, 42, 59]. The identification of this cross talk between SYK and NLRP3 could have broader implications. Pro-inflammatory crystals such as uric acid particles, which are responsible for NLRP3-dependent gout [45], activate SYK by direct lipid membrane binding [61]. Moreover, a recent study reported SYK-dependent NLRP3 inflammasome activation by malarial hemozoin [62]. In addition, SYK-coupled CLRs are emerging as important activators of inflammatory responses and can detect exogenous or endogenous ligands [14]. Thus, it will be interesting to investigate a potential cooperation of SYK signaling and NLRP3 activation in inflammatory conditions.

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