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Role of Toll-like receptors in systemic *Candida albicans* infections

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1. ABSTRACT

Toll-like receptors (TLRs) constitute a family of pattern-recognition receptors (PRRs) that recognize molecular signatures of microbial pathogens and function as sensors for infection. Recognition of *Candida albicans* by TLRs on mature immune cells, such as phagocytic cells, activates intracellular signalling pathways that trigger production of proinflammatory cytokines which are critical for innate host defence and orchestrate the adaptive response. TLR2, and TLR4 in a minor extent, recognize cell wall-associated ligands; endosomal TLR9 and TLR7 recognize DNA and RNA respectively. Interaction of *C. albicans* with TLRs is a complex process, as TLRs may collaborate with other PRRs and expression of surface-associated fungal ligands depends on the strain and the morphotype (yeasts or hyphae), thus defining the final induced adaptive response (Th1/Th2/Th17). TLRs are also expressed on hematopoietic stem and progenitor cells (HSPCs) where they may play a role in modulating hematopoiesis; engagement of TLR2 induces, upon recognition of *C. albicans*, the differentiation of HSPCs towards specific subsets of mature myeloid cells. This has opened a new perspective for anti-*Candida* immunointervention.

2. INTRODUCTION

Candida albicans is the most common fungal pathogen for the immunocompromised host. This species colonizes, as a commensal organism, the mucosal surfaces of the body of approximately 50% of healthy individuals at any given moment. However, when the normal host defences are impaired, the delicate balance between the host and this otherwise normal commensal fungus may turn into a parasitic relationship in which *C. albicans* acts as a serious agent of infection. The nature and extent of the impairment of host immune responses influence the manifestation and severity of candidiasis, a term which includes superficial mucocutaneous infections as well as severe, often fatal, disseminated infections with mortality rates that can reach 40%. Several factors contribute to this mortality rates associated to disseminated candidiasis: the lack of early and accurate diagnostic procedures, as well as the limited antifungal agents available, which in addition show considerable side effects on patients, and the emergence of resistances that parallels their clinical use (1–6). In addition to the host status, the pathogenicity of the fungus also depends on a complex set of fungal attributes that are considered as putative virulence factors, whose expression varies among strains and is often environmentally regulated (1–4,7). Next sections of the Introduction summarize both aspects, fungal virulence factors and host immune response, including host receptors for *C. albicans*.

2.1. *Candida albicans:* cell wall and virulence factors

C. albicans does not act as a passive element during the infectious process but actively participates

in the establishment and progress of the infection by expressing a set of putative virulence factors. These fungal attributes include the yeast-to-hypha transition (morphogenetic conversion from budding yeast to the filamentous growth form or hypha), the secretion of hydrolytic enzymes (such as aspartyl proteases and phospholipases, among others), phenotypic switching (ability to switch between different cell phenotypes), antigenic variability, adhesion to inert (plastic) materials and host ligands and tissues, and immunomodulation of host responses (1,7).

The fungal cell wall, as the outermost cellular structure, plays a major role in the interactions between the microorganism and the environment, including the host, and therefore in the pathogenicity of the fungus (7–9). The *C. albicans* cell wall is a complex and dynamic structure composed by a network of β-1,3 and β-1,6 glucans, chitin, and mannoproteins. This cell wall is a multilayered structure: chitin forms the rigid, inner layer of the cell wall and is covalently attached to β-1,3-glucan, which also contributes to rigidity and is itself attached to an outer layer of branched β-1,6-glucan. These microfibrillar polymers form a skeleton that accounts for its rigidity and morphology. The interactions between these components (chitin, glucans and mannoproteins) give rise to the mature cell wall structure, and consequently determine the fungal morphology. Significant differences in cell wall organization and composition have been described between budding yeasts and hyphae (7,9–11). There are differences between the content of chitin between yeast and hyphal cells; whereas chitin compromises approximately 2% of the cell wall dry weight in yeast cells, in hyphal cells the chitin content increases 3-5 times (11). Recently, it has been shown that there are differences between yeast and hyphal cells in the β-glucan content, linkages and also in its structure, as hyphal β-glucan contains a unique cyclical structure that is not present in yeast glucan. This hyphal glucan has been proven to elicit a stronger proinflammatory cytokine production than yeast glucan, which can have implications for the differential innate immune response of *C. albicans* yeasts versus hyphae (12).

Cell wall proteins from the outer layer are attached to it predominantly through a glycosylphosphatidylinositol (GPI) anchor. Mannan, a complex structure composed by polymers of mannose, is found in covalent association with these cell wall proteins through *N*- or *O*-linkages (mannoproteins), that expand the entire cell wall structure, from the periplasm to the external surface where they are dominant, and some are secreted to the extracellular medium (7,9,10). The cell wall mannan of *C. albicans* has been shown to consist of α-1,2-, α-1,3-, α-1,6-, and β-1,2-linked mannopyranose units with few phosphate groups (13). Mannoproteins are known to play a key role in cell wall structure allowing the cell surface to be adapted and remodeled constantly to cope with environmental changes (14–16). Numerous cell wall mannoproteins play a major role in host-fungus relationships, participating in the yeast-to-hypha transition, host tissue adhesion and invasion, biofilm formation, modulation of immune responses, etc., and therefore are considered as fungal virulence factors. Different studies using mutants involved in the biosynthesis of *N*- or *O*- mannans have shown how this mannosilation is important in the adhesion and virulence of *C. albicans* (16–20). In addition, differences in cell wall composition and organization between yeasts and hyphae may cause differential recognition by receptors on immune cells leading to the induction of distinct types of immune responses (7,12). Finally, lipids are also present in the phospholipomannan complex (PLM), a type of extensively glycosylated glycosphingolipid with hydrophilic properties that plays a relevant role in host interactions (8,16,21,22).

As additional putative virulence factors, *C. albicans* produce a number of secreted proteins that can damage host cell structures, as phospholipases, hemolytic factor, acid phosphatase, esterase, lipase, chondroitin sulfatase and metallopeptidase (7). The best characterized are the secreted aspartyl proteinases (SAPs). The SAP family includes 10 proteinases, SAPs 1-10, and are involved in adherence, tissue damage, invasion, hyphae formation and induction of apoptosis in epithelial cells (23–26). Moreover, some cytosolic proteins, including glycolytic enzymes, such as enolase and glyceraldehyde-3-phosphate dehydrogenase, as well as members of the heat shock protein family (hsp70 and hsp90) have been found in the *C. albicans* cell wall, probably secreted by a non-classical pathway; these proteins can play a role in host interactions (immunomodulation, adhesion) (7).

2.2. Host immune response to *Candida albicans*

Resistance to candidiasis requires the coordinated action of both innate and adaptive host immune response (27,28). Recognition of pathogen associated molecular patterns (PAMPs) of invading fungi by the innate immune system through pathogen recognition receptors (PRRs) is the first step in activating a rapid immunological response and ensuring survival after infection. Resident macrophages have a predominant role in innate immune control of disseminated candidiasis as they function as sentinels for the early detection and control of systemic infections (29). Host defence against systemic candidiasis depends mainly on the ingestion and killing of fungal cells by phagocytes (neutrophils, monocytes and macrophages). Phagocytes can kill the pathogen via intracellular and extracellular mechanisms, and macrophage activation releases several key mediators, including proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, which are important for protecting the host against disseminated candidiasis (27,28,30).

Antifungal T helper (Th) 1-mediated responses play a central role in anti-*C. albicans* defences, providing control of fungal infectivity through production of interferon (IFN)-γ; this cytokine is required for optimal activation of phagocytes and suppresses the induction of the Th2 response (31,32). Th2 response is associated with the susceptibility to systemic *C. albicans* infection and is characterized by the production of anti-inflammatory cytokines, such as interleukin (IL)-10 and IL-4 (33,34). Although protective immunity to *C. albicans* is mediated by Th1 cells, the proinflammatory Th1 host response needs to be counterbalanced through secretion of some Th2 cytokines and Treg cells to ensure an optimal, balanced non-deleterious protective Th1 response (27,35,36). Phagocytosis of the yeast form of the fungus induces murine dendritic cells (DCs) to produce IL-12 and to prime Th1 lymphocytes, whereas ingestion of the hyphal form results in IL-4 production, which favours Th2 cell priming (37–39), consequently, hyphae may fail to properly induce the production of the Th1 cytokine IFN-γ (32,40). It has been also reported that interaction of human DCs with yeast and germ-tubes forms of *C. albicans* leads to efficient fungal processing, DCs maturation and acquisition of a Th1 response-promoting function, although germ-tubes induced significantly more elevated levels of IL-10 than yeast cells (41). However, it has been shown that germ-tubes of *C. albicans* cause defective induction of IL-12 in human monocytes (42) and that phagocytosis of yeast and germ-tubes forms has profound and distinct effects on the differentiation pathway of human monocytes, indicating that differentiation of human monocytes into DCs appears to be tunable and exploitable by *C. albicans* hyphae to elude immune surveillance (43).

It has also been described that immune recognition of *C. albicans* induces the differentiation of proinflammatory Th17 cells and that this T-helper effector subset is involved in antifungal defence in mucosal surfaces. Th17 cells are maintained in the presence of IL-23, whereas IL-17 induces chemokine production at sites of infection and causes recruitment of neutrophils. A number of studies, using defective mice in different Th17 cytokines/receptors or patients with Th17-related disorders, support the role of Th17 responses in *Candida* infections (44). *In vitro* development of Th17 response requires Treg cells, TGF-b, IL-6 and IL-23 and is inhibited by IL-12. Production of IL-12 and IL-23 appear to be dissociated during fungal recognition: *C. albicans* yeast cells are able to induce more IL-12 than IL-23, and therefore a strong Th1 response, whereas hyphae induce mainly IL-23 and a strong Th17 response (45–47).

Recently, it has been demonstrated that the activation of inflammasomes play an important role for anti-*Candida* host defence. The inflammasomes are a set of multiprotein intracellular complexes which participate in the detection of a variety of stimuli, including microbes, and that activates the highly pro-inflammatory cytokines IL-1β and IL-18. Different studies have demonstrated that the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome is activated in macrophages in response to *C. albicans* hyphae and that NLRP3 would have an important role in anti-*Candida* defences (44,48–51).

2.3. Host receptors for *Candida albicans* sensing: toll-like receptors

C. albicans cells are sensed directly by host cells through various PRRs, such as mannose receptor (MR), dectin-1, dectin-2, galectin-3, DC-SIGN, TLRs and by complement and immunoglobulin Fc receptors (CRs, FcRs) following opsonization, which trigger immune responses (phagocytosis, production of cytokines) (8,30,52–59). TLRs constitute a family of PRRs that mediate recognition of microbes through PAMPs, induce subsequent inflammatory responses and also regulate the adaptive responses (60–65). TLRs are type I membrane proteins characterized by an ectodomain containing leucine-rich repeats that are responsible for recognition of PAMPs and a cytoplasmic domain homologous to the cytoplasmic region of the IL-1 receptor (TIR-domain) which is required for downstream signalling.

Thirteen and ten TLRs have been identified to date in mouse and human respectively. TLR 1-10 are conserved between human and mice, but murine TLR10 in not functional because the gene is disrupted by the insertion of retrovirus. TLR11-13 are deleted in the human genome (65). TLRs can be classified into two groups based on subcellular localization. One group, which includes TLR1, 2, 4, 5 and 6, are located at the plasma membrane, whereas the second group, that includes TLR3, 7, 8 and 9, localize to intracellular compartments such as endosomes (or endolysosomes). The cell-surface TLRs mainly recognize components located on the surface of different microorganisms, whereas intracellular TLRs sense microbial and viral nucleic acids that are released following pathogen endocytosis and degradation in late endosomes or lysosomes (61, 63–66).

Some TLRs, such as TLR2, are able to detect a variety of microbial ligands that have nothing in common in terms of their structure, suggesting the involvement of accessory proteins, most of them yet to be characterized, in ligand sensing by TLRs (67). Formation of heterodimers of particular TLRs with other TLRs or non-TLR PRRs may also serve for ligand discrimination. TLR1/TLR2 and TLR2/TLR6 heterodimers recognize several microbial products and discriminate different bacterial lipopeptides (68,69); other TLR2 ligands do not require TLR1 or TLR6 for signalling, suggesting that TLR2 may recognize some ligands as homodimers or heterodimers with other non-TLR molecules (61,64). Upon ligand recognition, TLRs activate intracellular signalling pathways leading to

the induction of inflammatory cytokine genes, such as TNF-α, IL-1β, IL-6 and IL-12. Signal transduction starts with the recruitment of a set of intracellular TIR-domain-containing adaptors (MyD88, TIRAP, TRIF, TRAM) that interact with the cytoplasmic TIR domain of the TLRs. MyD88 (myeloid differentiation factor 88) is the universal adaptor molecule, shared by all TLRs except TLR3, that triggers inflammatory pathways through activation of the transcription factors NF-κB and AP-1, which in turn induce the expression of inflammatory cytokine genes. TRIF is critical in the induction of type I interferon by TLR3 and TLR4, through the activation of the transcription factor IRF3. TIRAP and TRAM participate in the MyD88- and TRIF-dependent signalling pathways, respectively (61,63–66).

TLRs on DCs (a specialized family of antigen presenting cells that link innate recognition of invading pathogens to the generation of appropriate types of adaptive responses) elicit the secretion of immunomodulatory cytokines (IL-4, IL-10, IL-12), as well as the upregulation of co-stimulatory molecules, an essential step in the induction of pathogen-specific adaptive immune responses (70,71). In addition, negative regulation of TLR-mediated signalling is required to limit a deleterious excessive inflammatory response, and several negative regulators of TLR signalling have been identified; basically these molecules either downregulate TLR expression or, alternatively, negatively regulate TLR-mediated signalling (61,63,71).

Extensive information concerning fungal recognition has been achieved since early reports showed the capacity of TLR2 and TLR4 to sense zymosan (a cell wall particle of the yeast *Saccharomyces cerevisiae*) and fungal species such as *Cryptococcus neoformans*, *Aspergillus fumigatus* and *C. albicans* (11,27,72–77). In this review we will focus on the role of recognition of *C. albicans* by TLRs, and its consequences on the host immune responses.

3. TOLL-LIKE RECEPTORS AND HOST PROTECTION AGAINST SYSTEMIC CANDIDIASIS

Most of the information about the involvement of the TLR-mediated signalling pathways in host defences against *C. albicans* infections has been obtained following *in vivo* studies using murine models of infection in knockout mice for various genes, as well as *in vitro* assays using different cell lines or immune cells from knockout mice challenged with fungal stimuli to determine the induced responses (production of cytokines, phagocytosis and killing of fungal cells, etc.). First we will deal with the murine models of infection, and next with the innate and adaptive immune responses elicited by *C. albicans*.

3.1. Susceptibility to infection in mouse models

A global role for TLRs in the host defence against disseminated candidiasis was demonstrated by the increased susceptibility of MyD88$^{-/-}$ mice to *C. albicans* infection, determined both as survival curves and fungal burden in kidney. The extremely high susceptibility of these mice to infection, compared to control C57BL/6 mice, even with a low virulence *C. albicans* strain, indicates an essential role of one or more TLRs in host protection against candidiasis (78,79).

Early studies demonstrated a role for TLR2 in the recognition of zymosan, as well as a role for TLR4 in recognizing fungal species, such as *A. fumigatus* and *C. neoformans* (72–74). Therefore the first studies on *Candida*-host interactions focused on these TLRs.

Heterogeneous results have been reported regarding the susceptibility of TLR2$^{-/-}$ mice to disseminated candidiasis. Our group showed that TLR2 knockout mice experimentally infected with a high virulence strain of *C. albicans* have a significant impaired survival following primary infection, as compared with control mice (80). Other authors have shown that TLR2$^{-/-}$ are less susceptible to hematogenously disseminated primary infection, suggesting that *Candida* recognition through TLR2 constitutes a novel strategy for immune evasion (81); however, this observation has not been further confirmed. A third study showed that TLR2$^{-/-}$ mice are resistant, as are the control mice, to infection with a low virulence *C. albicans* strain, whereas both mouse types showed similar susceptibility to primary infection with a high virulence strain (78); these results indicate that TLR2$^{-/-}$ mice are not more resistant than control mice, and do not preclude the possibility that the TLR2$^{-/-}$ mice could show increased susceptibility using lower doses of *C. albicans* cells leading to a longer survival of control mice. Interestingly, two independent studies showed that, following primary infection with a low-virulence *C. albicans* strain, TLR2$^{-/-}$ mice were significantly protected against secondary reinfection with a high virulence strain, although to a lower extent than control mice (78,82). As TLR2 dimerizes with TLR1 or TLR6, the role of these two receptors has been also investigated. Although both TLR1 and TLR6 knockout mice did not show increased susceptibility to an *in vivo* infection, TLR6 deficient mice displayed a defective production in IL-10 and IFN-γ, suggesting a minor role for TLR6 during *C. albicans* infections (83). Overall these results indicate that TLR2 plays a significant role in the protection of mice against primary disseminated *C. albicans* infections.

A first study using C3H/HeJ mice, which possess a non-functional TLR4, suggested that these mice are more susceptible to primary *C. albicans* infection than the control C3H/HeN strain, based on fungal outgrowth in kidneys (75). In a second study, TLR4$^{-/-}$ mice, in a

C57BL/6 background, survived similarly to wild type mice when intravenously infected with a low virulence *C. albicans* strain, and susceptibility to virulent *C. albicans* infection did not increase in TLR4$^{-/-}$ mice, which even survived significantly longer than the C57BL/6 mice, although all succumbed to infection; in addition, no differences were found concerning fungal outgrowth in kidneys (78). Interestingly, following primary infection with a low virulence strain, TLR4$^{-/-}$ mice showed an increased susceptibility to reinfection with virulent fungal cells as compared to control strain, although TLR4$^{-/-}$ mice were also significantly protected, as compared with survival to primary infection (78). A third study using both TLR4$^{-/-}$ and C3H/HeJ mice has shown that the overall host resistance to systemic candidiasis in TLR4 defective mice is not different to that of control mice (84). An explanation for these differing results could be that the TLR4 involvement seems to be largely dependent on the *C. albicans* strain used, as it will be discussed below. On the whole, all the results suggest a predominant role for TLR2 and a minor role for TLR4 in host protection against hematogenously disseminated candidiasis (85).

Additionally, the role of other TLRs during candidiasis has been studied. Susceptibility to disseminated candidiasis, determined as survival of intravenously infected mice, was found not to increase in TLR9 knockout animals compared to control mice. TLR9$^{-/-}$ mice survived longer than controls to primary infection and fungal burden in kidneys of infected animals was diminished in these mice. In addition, TLR9$^{-/-}$ mice were fully protected against reinfection following primary infection with a low-virulence *C. albicans* strain (78). Accordingly, it has been reported that TLR9 signalling would be redundant in a model of disseminated candidiasis (86). All these observations suggest that TLR9 is not required for resistance to infection. Also, the involvement of TLR7 in anti-*Candida* defence has been suggested, as TLR7 deficient mice show increased susceptibility to systemic infection (87).

3.2. Innate responses

Effector and secretory responses of phagocytes elicited by *C. albicans* are critical for the development of a protective host response. Macrophages orchestrate innate immunity by phagocytosing fungal cells and coordinating inflammatory responses. Phagocytes use a variety of surface receptors, such as PRRs (dectin-1, DC-SIGN, MR) and receptors for opsonins (FcRs and CRs), to internalize microbes (88). This internalization is accompanied by inflammatory responses elicited by TLRs that are recruited to phagosomes. Therefore, phagocytosis and TLR signalling may be functionally linked: signalling by TLRs can modulate phagocytosis, and signalling by phagocytic receptors can modulate TLR signalling through a crosstalk between both types of receptors (72,89,90). Despite the functional overlap between TLRs and phagocytic signalling, current data indicate that TLRs do not function directly as phagocytic receptors and there is no direct evidence showing that TLR-signalling modulates the efficiency of internalization, although TLR-mediated signalling activates transcription of a large number of genes, and many of these gene products are known to participate in phagocytosis (90). Phagocytic cells from MyD88$^{-/-}$ mice showed impaired phagocytosis and intracellular killing of *C. albicans* (78,91); however, targeted deletion of MyD88 or TLR2 had no effect on the ability to internalize zymosan, and expression of dominant negative forms of MyD88 and TLR2 did not affect phagocytosis whereas inhibited production of TNF-a in response to zymosan (72,91,92). In addition, macrophages and neutrophils from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice did not show an impaired ability to internalize and kill *C. albicans* cells (78, 80). Moreover, it has been recently reported that galectin-3 would be a PRR involved in the phagocytosis of *C. albicans* hyphae (93). These results indicate that TLR2 and TLR4 are not directly involved in the phagocytosis of *C. albicans*, and that the defect in phagocytosis observed in MyD88$^{-/-}$ cells may be an indirect effect associated with impaired inflammatory signalling and/or through impairment of cellular transcription of genes involved in phagocytosis (90,91).

The critical involvement of TLR-mediated signalling in inducing cytokine production by myeloid cells in response to *C. albicans* has been well established using MyD88 deficient mice (78,79,91). However, the role of individual TLRs in cytokine production upon recognition of yeasts and hyphae of *C. albicans* is not as clear as in the case of the MyD88 adaptor molecule (54,55,75,80–82,84,94–99). An initial study showed that the *in vitro* induction of proinflammatory cytokines by *C. albicans* is partially mediated by TLR2, as blocking anti-TLR2 antibodies caused a reduction of TNF-α and IL-1β production by human peripheral blood mononuclear cells, whereas blocking anti-TLR4 antibodies did not influence the production of proinflammatory cytokines; similarly, *in vitro* production of proinflammatory cytokines by macrophages from C3H/HeJ mice, which possess a non-functional TLR4, was similar to control cells, although production of chemokines (KC and MIP-2) was impaired (75). Our group has demonstrated that *in vitro* production of proinflammatory cytokines, such as TNF-α and IL-12p70, by macrophages in response to inactivated and viable *C. albicans* yeasts and hyphal cells is partly mediated by TLR2, as TLR2$^{-/-}$ cells showed a diminished cytokine production elicited by fungal cells (54,80,99); no defect in cytokine production was observed in TLR4$^{-/-}$ and C3H/HeJ macrophages, suggesting that this receptor plays a secondary role in *C. albicans* recognition (54,84,99). Further observations confirmed the relevant role of TLR2 in triggering cytokine production in response to *C. albicans*, as well as a role for TLR4 (40,55,94,95,100,101). Both receptors have been shown to mediate TNF-α production by phagocytic cells

in response to yeasts and hyphae, although differential levels of cytokines are mediated through TLR4- and TLR2-recognition: yeasts recognition by TLR4 directs high levels of TNF-α and low levels of IL-10, leading to a high proinflammatory response and production of high levels of IFN-γ, whereas TLR2 mediated recognition of both yeasts and hyphae leads to a decreased proinflammatory response through a limited production of TNF-α and an increased production of IL-10 (40). In agreement with the reports showing that TLR9 deficient mice are more resistant to candidiasis (78, 86), it has been also shown that *C. albicans* induces phagosomal recruitment of TLR9, although this receptor would negatively modulate macrophage antifungal immunity, as TLR9 deficiency increased macrophage TNF-α production, activation and microbicidal activity against *C. albicans* (102).

It should be noted that more recently a significant role for type I IFN in host defence against *C. albicans* has been demonstrated (32). Smeekens *et al* (103) have reported that type I IFN plays a crucial role in anti-*Candida* host defence in humans and that modulates Th1/Th17 cytokine profiles, increasing IFN-γ and decreasing IL-17 production. Other authors have described that conventional DCs are able to mount a type I IFN response against *C. albicans* that requires TLR7-mediated signalling (104), although it has been also reported that IFN-β production by DCs is largely dependent on dectin-1 and is crucial for immunity to *C. albicans* (105)

Neutrophils are major effector cells of innate immunity, phagocytosing and killing fungal cells. Neutrophils predominantly phagocyte non-opsonised *C. albicans* via TLRs and C-type lectins. Intra- and extracellular killing of *C. albicans* cells occur via oxidative and nitrosative mechanisms (106). Recruitment of neutrophils at the site of infection following intraperitoneal injection of inactivated *C. albicans* cells was found to be impaired in TLR2$^{-/-}$ but not in TLR4$^{-/-}$ mice, in agreement with the impaired cytokine production observed in these mice (80,84). However, an impaired recruitment of neutrophils was found in C3H/HeJ mice, in agreement with the impaired chemokine production described in this mouse strain in response to *C. albicans* (75). Supporting the role of TLR2 in neutrophil activation during *C. albicans* infection, Tessarolli *et al* demonstrated that TLR2 deficiency causes a decrease in neutrophil recruitment to the site of infection and chemokine production, as well as an impaired phagocytic activity of neutrophils and macrophages, nitric oxide production and myeloperoxidase activity (107). Unlike TLR2 knockout cells, antifungal activity of neutrophils from TLR9-deficient mice was either not affected (against *C. albicans* yeasts) or even increased (against hyphae) as compared to control neutrophils. TNF-α production in kidneys from infected mice were similar in TLR9-deficient and control mice, and as above cited, TLR9$^{-/-}$ mice were particularly efficient in restricting fungal growth upon primary candidiasis (78,102).

3.3. Adaptive responses

Protective immunity to *C. albicans* is mediated by Th1 and Th17 cells, although Th2 and Treg cells are also required for the maintenance of a balanced non-deleterious proinflammatory response (27,28,33,34).

In vitro production of Th1 cytokines and quantification of IFN-g producing cells has been classically used as a parameter to determine Th1 response in mouse models of infection. The *in vitro* production of Th1 cytokines has been determined in splenocytes from mice infected with the low virulence *C. albicans* PCA2 strain which induces the development of a Th1-protective immunity in mice (32–34). MyD88$^{-/-}$ cells showed a fully impaired ability to produce TNF-α, IL-12p70 and IFN-γ, indicating the critical role of the TLR-mediated signalling in the development of a protective Th1 response (79). TLR2$^{-/-}$ splenocytes showed a significant impairment of Th1 cytokine production, whereas TLR4$^{-/-}$ splenocytes showed similar levels than control cells (54,82,84). Splenocytes from MyD88$^{-/-}$ mice infected with a low virulence *C. albicans* strain showed a strong impairment of the frequency of IFN-γ producing-CD4 T lymphocytes upon *in vitro* challenge with *C. albicans*, thus confirming that TLR signalling pathways are essential to generate a Th1 response (79). The frequency of IFN-g producing-CD4 T lymphocytes was also significantly diminished in TLR2$^{-/-}$ splenocytes, whereas TLR4$^{-/-}$ showed no differences with control cells, in agreement with the results of the *in vitro* Th1-cytokine production assays (54,84). In a different study, the frequency of IFN-γ producing CD4 T cells was found to be impaired in both TLR2$^{-/-}$ and TLR4$^{-/-}$ mice following intragastric infection with *C. albicans*, and this effect was accompanied by an increase in the frequency of IL-4 producing CD4+ T cells (78).

DCs are crucial in determining the adaptive Th response by sensing and processing microbial information and directing the differentiation of naïve lymphocytes to suitable effector cells against particular types of infection (70). Differential response to *C. albicans* yeasts and hyphae occurs following phagocytosis of fungal cells by DCs, as yeasts induce the production of IL-12 and prime Th1 lymphocytes, whereas ingestion of the hyphal form results in IL-4 production which favours Th2 cell priming (38). Production of IL-12p70 and IL-10 in response to *C. albicans* by purified DCs has been studied in MyD88 and TLRs deficient mice. DCs from knockout mice were able to phagocytose fungal cells in a similar manner than wild type cells; however production of IL-12p70 was ablated in MyD88$^{-/-}$ mice, associated to an increased production of IL-10 in response to both *C. albicans* yeasts and hyphae (78). These observations confirm the essential role of the MyD88-dependent

signalling on DCs for antifungal Th1 priming, and indicated that IL-10 production does not require signalling through MyD88. Interestingly, TLR2$^{-/-}$ DCs showed an increased production of IL-12p70 and a decrease in production of IL-10, particularly in response to yeasts. Production of IL-10 was also decreased in TLR4$^{-/-}$ DCs in response to both yeasts and hyphae, whereas IL-12 production was not significantly affected (78).

A number of studies have focused on the role of endosomic TLRs in *C. albicans* recognition and responses. In 2004, it was shown that despite its resistance to candidiasis, TLR9-deficient mice were incapable of mounting a specific Th1 response; TLR9-deficient mice showed a decrease in IFN-γ-producing Th1 cells and an increase in IL-4-producing Th2 cells following gastric challenge with *C. albicans*. DCs from TLR9$^{-/-}$mice challenged *in vitro* with *C. albicans* yeasts showed an increased production of IL-10 and a diminished production of IL-12, whereas a decreased production of IL-10 was observed against hyphae (78). However, newer studies have reported conflicting results. CpG oligonucleotides, the ligand for TLR9, enhance innate effector and Th1 responses improving host resistance to infection by a variety of microbes, including fungal species such as *A. fumigatus* and *C. neoformans* (108–110). Opposite results have been reported on the protective role of CpG administration against invasive candidiasis, as one report showed increased susceptibility of treated mice (111), whereas other authors have described a protective effect (112). Another study described that *C. albicans* double-stranded DNA participates in the host defence against disseminated candidiasis, providing protection against infection in a murine model (113), thus supporting a protective role for TLR9. It has also been shown that *C. albicans* induces host-protective type I IFN response in murine DCs. This activation, that requires yeast internalization and phagosomal maturation, is MyD88-dependent, and mediated by the recognition of *C. albicans* nucleic acids by TLR7 and TLR9 (104,114). Likewise, *C. albicans* DNA induces IL-12p40 production in bone marrow-derived DCs via TLR9 (115). Therefore, the role of TLR9 during candidiasis is far to be clearly established.

Regulatory T (Treg) cells maintain peripheral tolerance and limit the effector responses to control excessive proinflammatory responses leading to immune-mediated tissue damage. Treg cells, through different mechanisms, are able to control different innate and adaptive cells arms of the immune system and hinder the induction of immune responses against pathogens (116–119). Therefore, Treg cells play a central and dynamic role in the immune responses to pathogens, including *C. albicans* (27,28,118–121). Treg cells function in the maintenance of tolerance to self-antigens but should not interfere with the induction of pathogen-specific protective immune responses. One mechanism that allows activation of pathogen-specific T cells is the block of the suppressive effects of Treg by TLR-activated DCs, which is mediated by IL-6 and other factors produced by DCs in response to TLR activation, and leads to a Th1 response (70). The release from Treg-mediated suppression *in vivo* requires TLR/MyD88-activation on DCs, and this signalling cannot be replaced by DCs activation by inflammatory cytokines; in addition, initial interactions of naïve CD4 cells with TLR/MyD88 activated dendritic cells appears to be required for the *in vivo* generation of memory response, since Th1 cells induced in the absence of both MyD88 and Treg fail to develop into memory cells (70,122–124).

Murine Treg cells express TLRs, including TLR2 and TLR4, and therefore, Treg cells can also sense pathogens directly through TLRs (118,121,125). It has been shown that TLR2 regulates Treg cell expansion and function, and that in the presence of TLR2 ligand, the suppressive phenotype of Treg cells is temporarily abrogated, enabling the enhancement of the immune response *in vitro* and *in vivo* (118,121). These data suggest that TLR2 ligands, provided by a microbial invasion (such as an invasive candidiasis) during acute infection, mediate Treg expansion and abrogation of Treg-mediated suppression, thus allowing the development of a potent proinflammatory (Th1) immune response; after infection, following pathogen clearing and declining of TLR2 ligands, the expanded Treg cells regain their immunosuppressive activity and participate to restore the immune balance (118,121).

However, it was reported that TLR2 suppressed immunity to *C. albicans* infection through induction of IL-10 and Treg cells, and that this represents a novel mechanism of immune evasion (81,126). This hypothesis is far to be unequivocally demonstrated, as (i) TLR2$^{-/-}$ mice appear to be more susceptible to infection according to other studies above cited, (ii) underestimates the role of the TLR2-mediated proinflammatory response to *C. albicans*, and (iii) does not fit with the observation that production of IL-10 by DCs is MyD88-independent. Induction of IL-10 and Treg cells mediated upon recognition of *C. albicans* may simply indicate that TLR2-mediated signalling is also involved in controlling a deleterious exacerbated proinflammatory response. In addition, induction of Treg cells by IL-10-producing DCs is required for long-lasting memory antifungal immunity, and therefore, the Th1 hyporesponsiveness of TLR2-deficient mice may be, at least partly, consequence of the decreased IL-10 production by DCs (78,120). More recently it has been reported that *C. albicans* drives expansion of Treg cell population *in vivo*. This expansion appears to be detrimental to the host, since the augmented number of Treg cells positively correlates with fungal burden in kidney, and the *in vivo* selective depletion Treg cells decreases Th17-cell responses (127). Multiple factors may account for the discrepancies concerning the role of

Treg during candidiasis, such as fungal strain, infective dose and via of infection, that may affect the timing and intensity of Treg response and consequently its impact in the infection fate. Therefore, the role of Treg during systemic candidiasis is has not been unequivocally stablished yet.

During the last years, a number of studies have shown the involvement of non-TLR-mediated pattern recognition in the induction and tailoring of adaptive T helper responses. The glucan receptor dectin-1-mediated immune recognition of *C. albicans* induces the differentiation of IL-17-producing T helper cells (Th17) that express chemokine receptors characteristic of mucosal homing (45). IL-23 is critical for generation of Th17, and IL-17 induces chemokine production at the sites of infection and causes recruitment of neutrophils (47); IL-17-deficient mice are more susceptible than control mice to systemic candidiasis, probably due to the decreased influx of neutrophils (128). Curland, a dectin-1 ligand, also induces a Th17 response, indicating that a non-TLR PRR is sufficient to activate adaptive immunity (46). It has been suggested that dectin-2, as well as dectin-1, tend to promote IL-23 secretion and a Th-17 response against hyphae, whereas yeasts, recognized by dectin-1 and TLRs induce a strong Th1 response through production of more IL-12 than IL-23. Signalling through TLRs induces both IL-12 and IL-23 and therefore participates in the generation of Th responses (45–47,62). It has been reported that TLR2- and TLR4-mediated MyD88-dependent signalling participates in IL-23 secretion and development of a Th17 response that promotes inflammation and impair antifungal immune resistance; the IL-23/IL-17 pathway acts as a negative regulator of the Th1-mediated immune resistance to fungi and plays an inflammatory role previously attributed to uncontrolled Th1 cell responses, promoting inflammation and susceptibility in an infectious disease model (129). It should be noted that most knowledge about the Th17 response comes from results obtained in experimental animal models, and findings emerging from humans indicate that the human Th17 differentiation process appears to be different than murine Th17 differentiation (130). The results showing that augmented Th17 and Treg responses are detrimental for the host, contradict the fact that IL-17-deficient mice are more susceptible to systemic candidiasis (128). These differences could arise from the use of knockout IL-17 mice, in which the final balance of Th responses will be deregulated (119).

In summary, it is clear now that although Th17 and Treg cells are reciprocally regulated during T cell differentiation and can act cooperatively against *C. albicans*, the final response will depend on the infection site. In fact the Treg enhancement of Th17 responses results in an increased resistance to infection in oropharyngeal candidiasis, but results in a reduction of resistance in systemic infections (119,127). Therefore, the immune responses to systemic *C. albicans* infections and responses at mucosal sites are different; whereas Th1 responses are predominant during systemic infections, Th17 responses are clearly dominant in mucosal infections (44).

From the reported information above described, which includes some discrepancies, a simplified hypothetical model for Th-type immune responses induced by *C. albicans* during systemic infections is shown in Figure 1. Acquired immune responses to *C. albicans* depend on a delicate balance between differential exposure of fungal ligands at the surface of the yeast and hyphal forms of the fungus and their interaction with PRRs, mainly C-type lectins (see below) and TLRs. Expression of fungal virulence factors as well as host immune status also play a key role in eliciting Th-type immune responses.

While the importance of TLRs in DCs and Treg cells in regulating the immune response to pathogens, including *C. albicans*, has been widely studied, much less is known concerning the importance of direct recognition of pathogens by T- and B-lymphocytes. Different studies have highlighted the importance of TLRs in activation and function of lymphocytes. Expression of several TLRs on B and T lymphocytes has been demonstrated, as well as direct responses to their respective ligands (122,124,131,132). T cell responses can be modulated by TLR ligands by direct co-stimulatory effects on various subsets of T cells, in addition to the modulation of the suppressive activity of regulatory T cells (133). In this context, it has been described that stimulation by TLR2, but not by other TLRs, with pure synthetic ligands, directly triggers Th1 effector functions (134). Whether *C. albicans* cells are able to show a similar TLR2-mediated direct effect on Th1 cells remains to be determined.

3.4. Toll-like receptors and hematopoietic stem cells

The finding in 2006 that hematopoietic stem and progenitor cells (HSPCs) express TLRs, and that its ligation could drive differentiation towards myeloid progenitors, providing a rapid innate immune system replenishment (135), opened an exciting new area in the study of host-pathogen interactions.

During infection there is a need for replenishment of innate immune cells, as they are consumed during the immune response or killed by the invading microbes. This is achieved by “emergency myelopoiesis”, which will result in monopoiesis (production of monocytes and macrophages), granulopoiesis (mainly neutrophil generation), or both, depending on the specific microbe as well as the route and severity of infection (136). Regarding *C. albicans* infection, our group demonstrated

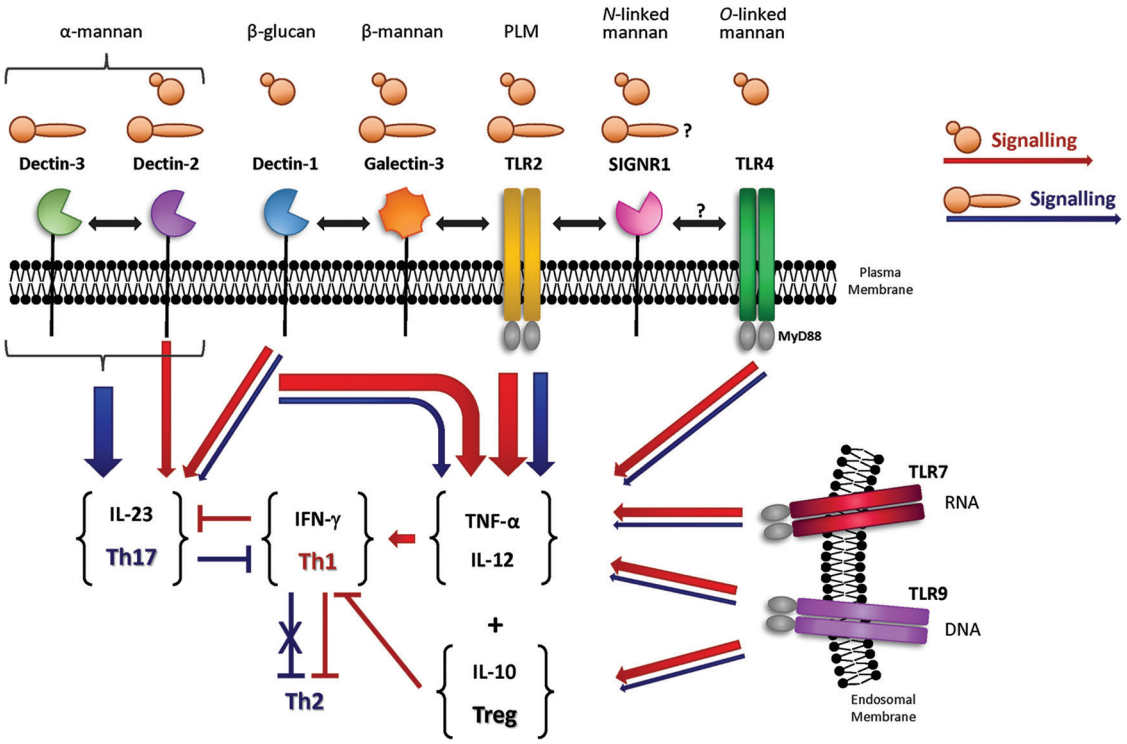

Figure 1. Simplified model for murine immune responses to *Candida albicans* systemic infection mediated upon fungal recognition by TLRs. TLR2 is the main TLR that recognizes both yeast and hyphae, whereas TLR4 appears to recognize preferentially yeast cells. Recognition of yeasts by TLR2 (and TLR4 in a minor extent) induces an early proinflammatory response, mediated by the MyD88-dependent production of TNF-α and IL-12, leading to the development of a Th1 protective response against invasive candidiasis. Other receptors, such as galectin-3, dectin-1, and SIGNR1 may collaborate with TLR2 in the elicitation of this response (physical interactions among receptors are indicated). TLR2 also may function as TLR2/TLR1 or TLR2/TLR6 heterodimers. Dectin-1 and dectin-2 may trigger, upon recognition of yeast cells, a MyD88-independent pathway leading to production of IL-23, a cytokine that generates a Th17 response, which is crucial in protecting against mucosal candidiasis. The balance upon yeasts recognition (red lines) is biased towards a Th1 response, which in turn inhibits Th17 and Th2 development. To avoid a deleterious exacerbate inflammatory effect, the Th1 response needs to be counterbalanced by late production of IL-10, a Th2 cytokine, and Treg cells. TLR2 also mediates recognition of hyphae and induces a proinflammatory response, diminished probably by the fact that other receptors such as dectin-1 and TLR4 preferentially recognize yeasts. In addition, differential recognition of hyphae by other PRRs, such as dectin-2 and dectin-3 (and probably dectin-1 in a minor extent) may favour the development of a Th17 response; therefore the Th1 response is inhibited, which may allow the development of a Th2 response. Consequently, the Th response to hyphae (blue lines) is biased to a non- protective Th2 response or to an enhanced proinflammatory Th17 response. Endosomal TLR7 and TLR9 may also contribute to these differential response, taken into account that phagocytes may have a distinct ability to phagoyctose and kill yeasts or hyphae (see text for further details).

that inactivated *C. albicans* yeasts and hyphae directly stimulated the proliferation and differentiation of HSPCs towards the myeloid lineage *in vitro*, through a TLR2/MyD88-dependent signalling pathway. The differentiated phagocytes were functional, and were are able to internalize yeasts and secrete pro-inflammatory cytokines (137,138). In a later study, we also showed that after an *in vivo C. albicans* infection, HSPCs were rapidly expanded and new populations of monocyte derived DCs and inflammatory macrophages were generated in the spleen, in a TLR2-dependent manner (139). In the same study the specific myeloid subsets activated following exposure of mouse HSPCs to *C. albicans* was determined: inactivated yeast cells triggered the differentiation of monocyte-derived DCs (moDCs) via TLR2/MyD88- and dectin-1-dependent pathways. Again, these moDCs were able to secrete TNF-α and had fungicidal activity, and therefore could participate in innate immunity against *C. albicans* (139). Nevertheless, transient exposure of HSPCs to a TLR2 ligand prior to their differentiation towards macrophages (by using macrophage colony-stimulating factor) was sufficient to suppress their inflammatory immune response (measured as inflammatory cytokine and reactive oxygen production); however, the phagocytic capacity of these macrophages was not affected, demonstrating a novel mechanism whereby macrophage responses can be programmed by TLR signalling in HSPCs prior to and/or during differentiation (140).

To determine whether TLRs on HSPCs control hematopoiesis during infection by direct recognition of the fungus or their ligands, a new experimental *in vivo* approach was used. By transplanting purified HSPCs from B6Ly5.1. mice (CD45.1. alloantigen) into TLR2$^{-/-}$, TLR4$^{-/-}$ or MyD88$^{-/-}$ mice (CD45.2. alloantigen), and

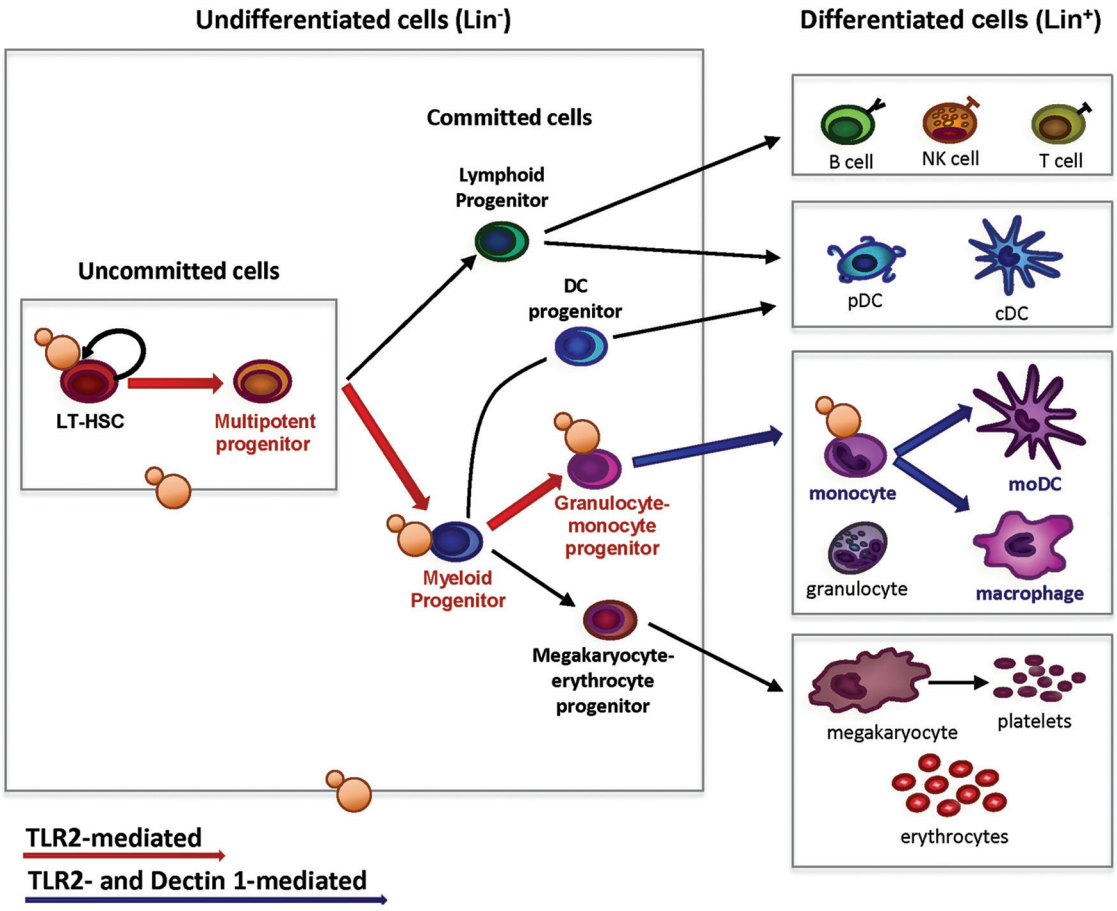

Figure 2. *Candida albicans* systemic infection and myelopoiesis in mouse. Hematopoiesis is initiated in the bone marrow by normally quiescent long-term hematopoietic stem cells (LT-HSC), which have the capacity for self-renewal and give rise to multipotent progenitors (uncommitted cells). These uncommitted cells generate progenitors committed to specific hematopoietic lineages: common lymphoid progenitors, and common myeloid progenitors, that give rise to all lineage types of mature cells (Lin^+). Lin^- cell population contains both uncommitted and commited progenitor cells. *C. albicans* interacts *in vitro* with mouse HSPC from the LT-HSC population to the lineage-commited progenitors, inducing their differentiation towards the myeloid lineage in a TLR2-dependent manner. *C. albicans* also induces TLR2- and dectin-1-dependent production of moDC by Lin^- HSPC *in vitro*, and TLR2-dependent macrophage production by LKS^+ and Lin^- cells upon infection *in vivo*. Dectin-1 activation also promotes monocyte differentiation to macrophages and moDC. Modified from Yañez *et al.* 2013 (136), see text for further details.

injecting soluble TLR ligands (Pam3CSK4, LPS or ODN respectively), we demonstrated that HSPCs can respond directly to TLRs agonists *in vivo*, and that the engagement of these receptors induced macrophage differentiation (141). So next, a similar *in vivo* model of HSPCs transplantation was used to study the effect of *C.albicans* infection. Transplanted (control, $TLR2^{-/-}$ or $TLR4^{-/-}$) cells were detected in the spleen and bone marrow of recipient mice (B6Ly5.1.), and they differentiated preferentially to macrophages in response to both viable and inactivated yeast. The differentiation to macrophage was TLR2-dependent, but TLR4-independent (142). Thus, HSPCs recognize *C.albicans in vivo* and subsequently are directed to produce macrophages by a TLR2-dependent signalling (Figure 2). Taken together, these data strongly support the idea that TLR2-induced myelopoiesis serves to boost anti-*C. albicans* immunity by stimulating myeloid cell production, limiting an exacerbated inflammatory response but preserving the fungicidal activity of the newly generated myeloid cells.

In this context, recent reports suggest a cross-talk between gut microbiota and bone marrow. It has been shown that microbiota-mediated signalling promotes the hematopoietic differentiation of myeloid cells in healthy individuals (143), and that the involvement of TLRs is essential for this microbiota-driven myelopoiesis, as systemic recognition of microbiota-derived products by TLRs is necessary to maintain a sufficient pool of bone marrow myeloid cells (144). This regulatory network probably results in the generation of peripheral mononuclear phagocytes that function as sentinels for the early detection and control of systemic infections, including candidiasis (29,143,145).

4. *CANDIDA ALBICANS* LIGANDS FOR TOLL-LIKE RECEPTORS AND CO-RECEPTORS

There is wide evidence demonstrating a role of TLRs in recognition of fungal pathogens by host immune cells and the nature of specific fungal ligands that mediate this recognition. As above mentioned, TLR-mediated recognition of pathogens is a complex process that involves cooperation between TLRs and other PRRs, as well as the participation of accessory proteins that may contribute to the recognition of diverse structural ligands by individual TLRs. The functional significance of some of these proteins, such as CD14 and MD2 in TLR4 signalling is well characterized, but it is likely that additional accessory proteins are involved in ligand sensing by TLRs. In addition, although the initial recognition of fungal cells by phagocytes involves cell wall-associated fungal PAMPs and TLRs located at the plasma membrane, after phagocytosis, intracellular TLRs located at the endosomal membrane may also play a role in triggering host responses by recognizing fungal ligands normally not exposed at the cell surface (8,61,62,64,67).

PLM has been the first described *C. albicans* cell wall-associated ligand for TLRs. Highly purified PLM triggers cytokine production in human and mouse cells (22,146,147), and deletion of the TLR2 gene completely abolishes the secretory response; cells expressing TLR2, but not TLR4 or TLR6, also showed a decreased cytokine production in response to PLM, but to a lower extent (148). Therefore PLM, that triggers production of proinflammatory mediators by cells of the myeloid lineage, has been considered as a fungal ligand for TLR2, a major mediator of the proinflammatory signalling induced by *C. albicans* (54,80,94,149). Recently, it has been described that PLM also activates the production of cytokines in keratinocytes, a non-myeloid cell, through the TLR2/NF-κB and p38 MAPK signalling pathway (150). The structure of PLM consists of hydroxy fatty acid amide linked to phytosphingosine, with a hydrophilic polysaccharide domain composed of a linear chain of β-1,2-linked mannose residues (22). Based in studies using different *C. albicans* serotypes and mutants that present differences in mannosylation, it has been proposed that PLM could be recognized by TLR2/galectin-3 complexes though β-1,2-mannosides (149).

C. albicans cell wall mannoproteins, and particularly the mannan moieties, are well known important elicitors of cytokine production by host cells (100). It has been showed that TLR4 recognize linear *O*-linked mannosyl residues, which are only accessible on the yeast cell surface (as TLR4 fails to recognize hyphae), thus identifying this domain as a ligand for TLR4 (11,40,55). However, more recent studies have shown that the role of TLR4 in *C. albicans* recognition during the infection could be complex. Whereas Wagener *et al* demonstrated that TLR4 is dispensable for cytokine production in epithelial cells (151), other authors have shown that TLR4 is important during an *in vivo* infection (152). A recent study has shed light on these conflicting results: by using 14 different *C. albicans* isolates, the authors demonstrate that its recognition by TLR4 is highly variable and largely dependent on the *Candida* strain used (153). In addition, it should be mentioned here that the cell wall of *C. albicans* is an extremely dynamic structure which may dramatically change in terms of composition and immune recognition as a consequence of defects in cell wall biogenesis induced either by drug treatment, as demonstrated for the b-glucan biosynthesis inhibitor caspofungin, or by mutations in genes involved in biosynthesis of cell wall components, such as mannan and/or mannoproteins (154,155); therefore, results concerning immune sensing of *C. albicans* strains defective in mannosylation and recognition of the fungus by host receptors should be interpreted with caution (98).

TLR9 as well as other members of the TLR family (TLR3, TLR7 and TLR8), localizes to intracellular compartments such as endosomes, and senses viral and microbial nucleic acids following endocytosis and pathogen degradation in late endosomes or lysosomes that causes release of RNA and DNA. TLR9 recognizes DNA containing unmethylated CpG motifs and triggers MyD88-mediated signalling pathways leading to activation of inflammatory cytokine genes, type I IFN genes and IFN inducible genes (61,63,64). The high rate of methylation and low frequency of CpG motifs in mammalian DNA avoids its recognition by TLR, and in addition, as host DNA, unlike microbial DNA, does not usually enter the endosome, restriction of TLR9 to endosomal compartment is critical for discriminating between self and non-self DNA (61,64,156). Despite CpG enhances innate effector and Th1 responses improving host resistance to a variety of microbes, including fungal species as above mentioned (108–110), the role of CpG during invasive *candidiasis* have not been clearly established y*et al*though TLR9 has been implicated in recognition of *C. albicans* purified DNA and induction of proinflammatory cytokines *in vitro*, *in vivo* studies have shown differing results about the role of TLR9 during infection, as discussed above (78,86,102,115). The *C. albicans* TLR7 ligand seems to be the fungal RNA, and this recognition appears to have a non-redundant role in host responses during candidiasis (87).

Other PRRs may collaborate with TLRs in recognition of *C. albicans* yeasts and hyphae by host cells and, therefore, in triggering a differential secretory response to both fungal morphotypes. Hence, ligands for these PRRs may also modulate signalling through TLRs in response to *C. albicans*. Among all known non-TLR PRRs, fungal ligands for C-type lectins (dectin-1, dectin-2, dectin-3, DC-SIGN, MR, Mincle) and galectin-3 have been well characterized, although physical interaction between PRRs have been only confirmed for TLR2 and

galectin-3, galectin-3 and dectin-1, and SIGNR1 and TLR2 and dectin-1 (see below).

C-type lectin receptors play critical roles in the recognition of *C. albicans*. β-glucan is the ligand of the phagocytic receptor dectin-1 (157–159), a receptor that collaborates with TLR2 to elicit a strong inflammatory response (92,158). Moreover, a TLR2-independent function of dectin-1 has been reported (160,161). As β-glucan is exposed at the surface of yeasts cells, whereas surface of hyphal cells do not expose the β-glucan, failure of hyphae recognition by dectin-1 may contribute to an impaired Th1 host response to *C. albicans* (162). In an earlier study, differential chemokine response of human monocytes to yeast and hyphal forms of *C. albicans* was related to the lower surface expression of β-1,6 glucan in hyphae, suggesting that the formation of hyphal filaments might facilitate *C. albicans* escaping from host immunity by minimizing chemokine induction (163). Some reports have described that β-glucan may be also accessible on the hyphal surface and therefore that dectin-1 may also sense hyphae (43,157,164). It has been also shown that dectin-1 promotes Th17 responses and plays a role in balancing Th1 and Th17 cells. Although the role of dectin-1 in fungal host defence, determined as susceptibility to disseminated infection in mouse models remains unclear (165–167), the importance of this receptor is highlighted by the fact that patients possessing a polymorphism in the dectin-1 gene (which impacts on the receptor expression and function) are more susceptible to mucocutaneous candidiasis, and show a defect in the production of cytokines (168). Dectin-2 is the functional receptor for α-mannan, but is not involved in the recognition of β-mannan (58,169). Dectin-2 recognizes *N*-linked mannan residues on the yeast cell wall, although it appears to play a relevant role in hyphae recognition (58,164,170). Dectin-2 deficiency leads to an impairment in cytokine production and in a decrease in survival after a *C. albicans* infection, as well as a dramatic decrease in Th17 cells (169). In a recent publication, a third C-type lectin receptor, dectin-3, has been shown to recognize α-mannan on the surfaces of *C. albicans* hyphae, and its deficiency increases the susceptibility to *C. albicans* infections. This receptor is able to form heterodimers with dectin-2 for bound hyphae α-mannans more effectively (164). Therefore, the simultaneous and differential recognition of mannans from yeasts and hyphae by dectin-2 and dectin-3, as well as different accessibility of glucan to dectin-1 may account for the differential immune responses to the yeast and hyphal forms of *C. albicans* (11,55,58).

MR also recognizes highly branched *N*-linked mannosyl chains in the *C. albicans* cell wall and triggers a primary pathway for the generation of a Th17 response (55,165). However, as MR-deficient mice do not show increased susceptibility to disseminated candidiasis, its role in mediating anti-*Candida* immunity appears to be redundant with other lectins (171). Fungal cell components, such as α-mannans, also may induce Th17 responses via mincle, as mincle-deficient mice show increased susceptibility to disseminated candidiasis (172). DC-SIGN in human dendritic cells is involved in the uptake of *C. albicans* via recognition of *N*-linked mannan, although its role in generating a Th17/Th1 response is not well stablished (165, 173, 174).

Fungal cell surface-associated β-1,2 mannosides bind to a host protein identified as the S-lectin galectin-3 (175). These mannosides are special types of glycans that are expressed by *C. albicans* and are associated with both mannan and PLM (8). It has been shown that specific recognition of *C. albicans* by macrophages requires galectin-3 to discriminate *S. cerevisiae* cells and needs association with TLR2 for signalling (176), thus suggesting that macrophages differentially sense *C. albicans* and *S. cerevisiae* through a mechanism involving TLR2 and galectin-3 (8,176). In addition, galectin-3 has been proven to associate with dectin-1 in macrophages, modulating their interaction with yeasts. This association seems to act helping to distinguish between pathogenic and non-pathogenic fungi (177). *In vivo*, galectin-3 has been proven to have an important role in protecting against invasive candidiasis (59).

C. albicans usually carries various cell-surface PAMPs, whose expression varies among strains, conditions and morphotypes. As above mentioned, glucan accessibility is distinct between yeasts and hyphae and mannan structures may also show differential expression on the yeast and hyphal cell wall; consequently *C. albicans* recognition may involve the simultaneous or sequential activation of different PRRs. Therefore, collaboration between receptors in *C. albicans* recognition and/or crosstalk between intracellular signalling pathways may define the final tailored immune response depending of the PAMPs recognized (Figure 1). Signalling pathways for PRRs are well known in some cases, such as TLRs and C-type lectins, and are still poorly defined in others, as for galectin-3 (11,27,76,77). Several carbohydrate-binding proteins have been identified as TLR2 co-receptors: dectin-1, galectin-3 and SIGNR1 (76,178). This collaborative recognition may enhance TLR2-dependent responses or modulate its ligand specificity. Although the collaboration between dectin-1 and TLR2 is well described (see above), no physical interaction between them has been demonstrated so far. However, galectin-3 appears to physically interact with TLR2 as well as with dectin-1, suggesting that galectin-3 may mediate the cooperation between both receptors, TLR2 and dectin-1 (176,177). Dectin-1 has been also reported to synergize with TLR4, although their physical interaction has not been proven (179). SIGNR1 (a member of the murine C-type lectins family, homologue of the human DC-SIGN) recognizes *C. albicans* through a partly distinct

polysaccharide ligand from that of the human DC-SIGN, and has been reported to be also a TLR2 and dectin-1 co-receptor (76,174,180,181). Interestingly, SIGNR1 associates with TLR4 to recognize LPS in gram-negative bacteria, to enhance signal transduction and activate innate responses (182). Therefore, a collaborative co-receptor relationship between SIGNR1 and TLR4 in *C. albicans* recognition should be not discarded.

5. PERSPECTIVES

The study of TLRs has emerged during the last decade as one of the most active areas of research in the field of microbial infections, including fungal infections such as candidiasis. Very important advances have been achieved in our understanding on how host immune cells sense *C. albicans* and trigger mechanisms aimed to control the infectious process, although much still needs to be learned. Sometimes the reported data are conflicting and the nature for these discrepancies need to be defined. The complexity of the experimental model from both sides, the host and the fungus, may partly explain the disparate results reported. Most studies are based on single fungal strains, and the ultrastructure, composition and biological properties of the cell wall may change among strains and also within single strains depending on growth conditions. Therefore, some of the discrepancies between studies could arise from fungal strain differences rather than from host differences.

TLR2 and TLR4, and probably others TLRs (TLR7 and TLR9), appear to play a role in determining host resistance to candidiasis by triggering a balanced proinflammatory host response to *C. albicans* that determines innate and adaptive responses. The ultimate challenge of our understanding of how *C. albicans* stimulate TLR-mediated immune responses is to translate these achievements to the design of therapeutic strategies for treatment and/or prevention of candidiasis in the various groups of at-risk population. However, as the consequences of fungal recognition by TLRs are very complex, further studies will provide valuable information elucidating the contribution of individual TLRs to host protection against candidiasis: (i) TLRs are present in a wide variety of immune cells, including both innate and adaptive immune cells (such as professional phagocytes, DCs, B and T cells), as well as in non-immune cells (epithelial, endothelial, stem cells, etc.), and therefore different mechanisms of protection against infection may be induced through TLRs; (ii) the inflammatory response to candidiasis can be envisaged as a two-edged sword, as excessive unbalanced inflammatory response may result deleterious for host protection, and therefore TLR signalling should be tightly controlled by a number of negative regulators to terminate immune and inflammatory responses and prevent excessive inflammation; (iii) further fungal ligands for TLRs should be identified and more precisely defined to elucidate the molecular domains involved in recognition, as well as their *in vivo* expression in both *C. albicans* morphotypes, yeasts and hyphae, and their contribution to the induction of morphotype-specific immune responses; (iv) collaboration between TLRs and other PRRs, such as dectin-1, galectin-3 and other receptors, as well as interplay between phagocytosis (and phagocytic receptors) and TLRs can modulate the effects on host response. Thus, our understanding of the TLR-signalling function in response to *C. albicans* depends also in the advances achieved with other PRRs and their ligands. Therefore definition at the molecular level of the precise chemical structures of *C. albicans* ligands for TLRs and other PRRs is essential for understanding the mechanisms of pathogenicity and host immune responses, and consequently for development of new antifungal drugs and immunotherapeutic strategies.

It should be stressed that multiple sensing mechanisms of *C. albicans* are involved in host responses, and that collaboration of different receptors (either in ligand recognition, cross-talk by overlapping or independent signal transduction pathways) should be integrated to define host responses to infection (Th1, Th2, Th17). Hence, the fine tuning of immune response depends on a complex balance among signals triggered by multiple *C. albicans* sensing receptors, and probably some of this signals may be redundant. In this context, it should be noted that human MyD88 defects appear not to be particularly prone to *C. albicans* infections, suggesting that the role of TLRs in protection against candidiasis may be at least partially masked by the role of other receptors, such as C-type lectins. This points out that between mice and humans cells there are significant differences in the recognition of *C. albicans*.

Recently, the fact that microbial ligands can be directly recognized by TLRs on HSPCs to boost the immune response against infection by triggering a rapid generation of myeloid cells, has emerged as a novel attractive concept. Activation of HSPCs can occur in response to various stimuli (growth and differentiation factors, inflammatory cytokines, microbial components and endogenous host ligands generated during tissue damage and/or infection). The impact of these stimuli on hematopoiesis may depend on specific physiological conditions (homeostasis, infection, inflammation). Therefore, it is of enormous interest to discern how HSPCs integrate these multiple signals into common/independent or partially overlapping signal transduction pathways to orchestrate an appropriate differentiation during physiologic and pathophysiologic conditions. Besides, there is increasing evidence indicating that properties of myeloid mature cells generated during infection may depend on the PRRs involved as well as the other myelopoietic signals sensed by HSPCs, thus suggesting a fine-tuning modulation of the myelopoyesis in response to specific pathogens. In addition, TLR

ligation on HSPCs also modulates chemokine receptor expression and may favour migration of HSPCs to the focus of the infection, and therefore TLRs may also regulate HSPCs trafficking. Finally, one key question is to determine whether the TLR-mediated transduction pathways triggered in response to *Candida* ligands are similar to those well-known pathways occurring in mature immune cells (macrophages and neutrophils). Two possibilities can be envisaged: (i) TLRs on HSPCs mediate the activation of unique signal transduction pathways, or most probably (ii) TLRs of HSPCs cause activation of the signalling pathways already described in mature cells, resulting in the production of soluble factors (such as cytokines with myelopoietic properites?) that can act in a paracrine manner to influence differentiation of unexposed HSPCs.

The answer to these issues is required to provide new insights into the role of TLRs in host-pathogen interactions during candidiasis (as well as other infections) that hopefully may reveal new strategies for anti-*Candida* immunointervention.

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