[Frontiers in Bioscience 14, 570-582, January 1, 2009]

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 that induce the activation of the innate immune responses as well as the subsequent development of adaptive immune responses. It is well established that TLRs, mainly TLR2 and TLR4, are involved in the host interaction with *Candida albicans* and play a significant role in the development of host immune responses during candidiasis. Recognition of *C. albicans* by TLRs on the phagocytic cells activates intracellular signaling pathways that trigger production of proinflammatory cytokines that are critical for innate host defence and orchestrate the adaptive response. T helper (Th) cell reactivity plays a central role in regulating immune responses to *C. albicans*: Th1-response provides control of fungal infectivity, although this proinflammatory (Th1) host response needs to be counterbalanced through Th2 and regulatory T (Treg) cells to ensure an optimal, protective Th1 response. Recently, a new subset of Th cells, Th17, has been shown to play a role in antifungal immunity, and TLRs may also contribute to the polarization towards a proinflammatory Th17 response. Interaction of *C. albicans* with TLRs is a complex process as (i) TLR2 may function as an homodimer or as TLR2/TLR1 or TLR2/TLR6 heterodimers and may collaborate with other non-TLR PRRs in recognizing fungal ligands or in triggering intracellular signalling pathways, and in addition (ii) expression of fungal ligands is different at the surface of fungal cells, depending of the morphotype (yeast cells or hyphae), a phenomenon that influences the type of the induced host immune response.

2. INTRODUCTION

Candida albicans is the major fungal pathogen for the immunocompromised host. This species is present as a commensal organism in healthy individuals, but when the normal host defense is 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. Several factors contribute to the significant mortality rates associated to disseminated candidiasis: (i) the lack of early and accurate diagnostic procedures, as well as (ii) the limited antifungal agents available, which in addition show considerable side effects on patients, and the emergence of resistances that parallels their clinical use (1-4). 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 (1,5). Next sections of the Introduction summarize both aspects, fungal virulence factors and host immune response.

2.1. *C. 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), 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 (1,5).

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 (5-7). 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. The interactions between these components give rise to the mature cell wall structure, and consequently determine the fungal morphology; in fact, significant differences in cell wall organization and composition have been described between budding yeasts and hyphae (5,7,8). In addition, mannoproteins are known to play a key role in these processes allowing the cell surface to be adapted and remodeled constantly to cope with environmental changes (9,10). Numerous cell wall mannoproteins play a major role in host-fungus relationship, participating in the yeast-to-hypha transition, host tissues adhesion and invasion, immune response modulation, etc., and therefore are considered as fungal virulence factors. The *C. albicans* cell wall is a multilayered structure: the microfibrillar polymers, glucan and chitin, form a skeleton that accounts for the rigidity and morphology of the cell wall. Glucans are the main polysaccharide constituent, whereas chitin is a minor component located in the internal layers of the cell wall, close to the plasma membrane. Mannan, a complex structure composed by polymers of mannose, is found in covalent association with proteins (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 (5,7,8). Lipids are present in the phospholipomannan complex (PLM), a type of extensively glycosylated glyscosphingolipid with hydrophilic properties that plays a relevant role in host interactions (11,12).

2.2. Host immune response and Toll-like receptors

Resistance to candidiasis requires the coordinated action of both innate and adaptive host immune response (13,14). 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. Host defense 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 (13-15). Antifungal T helper (Th) 1-mediated responses play a central role in anti-*C. albicans* defenses, 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 (16). 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 (17, 18). 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 (19,20). 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 favors Th2 cell priming (21-23). It has been also reported that interaction of human dendritic cells with yeast and germ-tubes forms of *C. albicans* leads to efficient fungal processing, dendritic cell maturation and acquisition of a Th1 response-promoting function, although germ-tubes induced significantly more elevated levels of IL-10 than yeast cells (24). However, it has been shown that germ-tubes of *C. albicans* cause defective induction of IL-12 in human monocytes (25) 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 (26).

Recently, it has been shown that immune recognition of *C. albicans* induces the differentiation of proinflammatory Th17 cells and that this T-helper effector subset is involved in antifungal defense. 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. *In vitro* development of Th17 response requires Treg cells, TGF-β, 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 (27-29).

C. albicans cells are sensed directly by host cells through various PRRs, such as mannose receptor, dectin-1, dectin-2, galectin-3, DC-SIGN, Toll-like receptors (TLRs) and by complement and immunoglobulin Fc receptors (CRs, FcRs) following opsonization, which trigger immune responses (phagocytosis, production of cytokines, etc.) (6,15,30-34). TLRs constitute a family of PRRs that mediate recognition of microbes through PAMPs, induce subsequent inflammatory responses and also regulate the adaptive responses (35-39). 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 signaling. Twelve and ten functional TLRs have been identified to date in mouse an human respectively. 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. Intracellular TLRs sense microbial and viral nucleic acids that are released following pathogen endocytosis and degradation in late endosomes or lysosomes (36, 38-40). 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 (41). 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 (42,43); other TLR2 ligands do not require TLR1 or TLR6 for signaling, suggesting that TLR2 may recognize some ligands as homodimers or heterodimers with other non-TLR molecules (36,39). Upon ligand recognition, TLRs activate intracellular signaling 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 factor NF-κB that induces the expression of inflammatory cytokine genes (36,38-40). 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 (44). In addition, negative regulation of TLR-mediated signaling is required to limit a deleterious excessive inflammatory response, and several negative regulators of TLR signaling have been identified; basically these molecules either downregulate TLR expression or, alternatively, negatively regulate TLR-mediated signaling (36,38).

Extensive information concerning fungal recognition and its the consequences on the host immune responses, has been achieved since early reports showing the capacity of TLR2 and TLR4 to sense zymosan (a cell wall particle of the yeast *Saccharomyces cerevisiae*) as well as fungal species such as *Cryptococcus neoformans*, *Aspergillus fumigatus* and *C. albicans* (45-48). Therefore, in this review we focus on the recognition of *C. albicans* by TLRs, mainly TLR2 and TLR4.

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

Most of the information about the involvement of the TLR-mediated signaling pathways in host defenses against *C. albicans* infections has been obtained following (i) *in vivo* studies using murine models of infection in knockout mice for various genes, as well as (ii) *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 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 the TLRs in the host defense 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 (49,50).

Early studies demonstrated a role of TLR2 in the recognition of zymosan, as well as a role of TLR4 in recognizing fungal species, such as *A. fumigatus* and *C. neoformans* (45-47). Therefore 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 (51). Other authors have shown that TLR2-/- are less susceptible to hematogenously disseminated primary infection (52). 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 (49); 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-/- were significantly protected against secondary reinfection with a high virulence strain, although to a lower extent than control mice (49,53). 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 (48). In a second study, TLR4-/-, 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 (49). 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 (49). 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 (54). Overall these results suggest a minor role of TLR4 in host protection against hematogenously disseminated candidiasis.

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 (49). These observations suggest that TLR9 is not required for resistance to infection.

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, mannose receptor) and receptors for opsonins (FcRs and CRs), to internalize microbes (55). This internalization is accompanied by inflammatory responses elicited by TLRs that are recruited to phagosomes. Therefore, phagocytosis and TLR signaling may be functionally linked: signaling by TLRs can modulate phagocytosis, and signaling by phagocytic receptors can modulate TLR signaling through a crosstalk between both types of receptors (45,56,57). Despite the functional overlap between TLRs and phagocytic signaling, current data indicate that TLRs do not function directly as phagocytic receptors and there is no direct evidence showing that TLR-signaling modulates the efficiency of internalization, although TLR-mediated signaling activates transcription of a large number of genes, and many of these gene products are known to participate in phagocytosis (57). Phagocytic cells from MyD88-/- mice showed impaired phagocytosis and intracellular killing of *C. albicans* (49,58); 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-α in response to zymosan (45,58,59). In addition, macrophages and neutrophils from TLR2-/- and TLR4-/- mice did not show an impaired ability to internalize and kill *C. albicans* cells (49,51). 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 signaling and/or through impairment of cellular transcription of genes involved in phagocytosis (57,58).

The critical involvement of TLR-mediated signaling in inducing cytokine production by myeloid cells in response to *C. albicans* has been well established using MyD88 deficient mice (49,50,58). 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 (32,33,48,51-54,60-65). 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-β1production 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, which possess a non-functional TLR4, was similar to control cells, although production of chemokines (KC and MIP-2) was impaired (48). 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 (32,51,65); 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 (32,54,65). Further observations confirmed the relevant role of TLR2 in triggering cytokine production in response to *C. albicans*, as well as a role for TLR4 (33,60,61,66-68). 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 (68).

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 (C57BL/6 background), in agreement with the impaired cytokine production observed in these mice (51,54). However, also 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* (48).

Similarly to TLR2-/- and TLR4-/- 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 (49).

3.3. Adaptive responses

Protective immunity to *C. albicans* is mediated by Th1 cells, although some Th2 and Treg cells are required for the maintenance of a balanced non-deleterious proinflammatory Th1 response (13,14,17,18). Therefore, *in vitro* production of Th1 cytokines and quantification of IFN-γ producing cells has been used as a parameter to determine Th1 response in mouse models of infection. The *in vitro* production of Th1 cytokines was determined in splenocytes from mice infected with the low virulence PCA2 strain which induces the development of a Th1-protective immunity in mice (17,18). MyD88-/- cells showed a fully impaired ability to produce TNF-α, IL-12p70 and IFN-γ, indicating the critical role of the TLR-mediated signaling in the development of a protective Th1 response (50). TLR2-/-speloncytes showed a significant impairment of Th1 cytokine production, whereas TLR4 splenocytes showed similar levels than control cells (32,53,54).

Splenocytes from MyD88-/- infected with a low virulence *C. albicans* strain showed an strong impairment of the frequency of IFN-γ producing-CD4 T lymphocytes upon *in vitro* challenge with *C. albicans*, thus confirming that TLR signaling pathways are essential to generate a Th1 response (50). The frequency of IFN-γ 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 (32,54). In a different study, the frequency of IFN-γ producing CD4 T cells were found to be impaired in both TLR2-/- and TLR4-/- mice following intragastric infection with *C. albicans*, and this effect was accompanied by and increase in the frequency of IL-4 producing CD4+ T cells (49).

Dendritic cells 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 infections (44). Differential response to *C. albicans* yeasts and hyphae occurs following phagocytosis of fungal cells by dendritic cells, as yeasts induce the production of IL-12 and prime Th1 lymphocytes, whereas ingestion of the hyphal form results in IL-4 production which favors Th2 cell priming (22). Production of IL-12p70 and IL10 in response to *C. albicans* by purified dendritic cells has been studied in MyD88 and TLRs knockout mice. Dendritic cells from mutant 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 (49). These observations confirm the essential role of the MyD88-dependent signaling on dendritic cells for antifungal Th1 priming, and indicated that IL-10 production does not require signaling through MyD88. Interestingly, TLR2-/- dendritic cells 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 -/- dendritic cells in response to both yeasts and hyphae, whereas IL-12 production was not significantly affected (49).

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 are able to suppress both CD4+ and CD8+ lymphocytes and hinder the induction of immune responses against pathogens (69-71). Therefore, Treg cells play a central and dynamic role in the immune responses to pathogens, including *C. albicans* (13,14,71-73). 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 (44). The release from Treg-mediated suppression *in vivo* requires TLR MyD88-activation on DCs, and this signaling 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 (44,74-76).

It has been reported that TLR2 suppresses immunity to *C. albicans* infection through induction of IL-10 and Treg cells, and that this represent a novel mechanism of immune evasion (52,77). 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 signaling 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 (49,72).

Murine Treg cells express TLRs, including TLR2 and TLR4, and therefore, Treg cells can also sense pathogens directly through TLRs (71,73,78). A crucial role for TLR2 in regulating Treg cell expansion and function has been recently shown: TLR2 participates, combined with IL-2 and T cell receptor (TCR) ligation, in the proliferation of otherwise anergic Treg cells both *in vivo* and *in vitro*, and interestingly, 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 an acute infection model *in vivo*. TCR-triggered Treg cell lines were cultured in the

presence of TLR2 ligands and upon removal of the TLR2 trigger, the *in vitro*-expanded Treg cells fully gained their phenotype and suppressive capabilities (71,73). 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 (71,73).

Despite its resistance to candidiasis, TLR9-deficient mice have been shown to be 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*. Dendritic cells form 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 (49). CpG oligonucleotides, the ligand for TLR9 (see below), enhances 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* (79-81). However, opposite results have been reported on the protective role of CpG administration against invasive candidiasis, as one report showed increased susceptibility of treated mice (82), whereas other authors have described a protective effect (83). Another study also described that *C. albicans* double-stranded DNA participates in the host defense against disseminated candidiasis, providing protection against infection in a murine model (84), thus supporting a protective role for TLR9. Therefore, the role of TLR9 during candidiasis is far to be clearly established.

As above cited (see section 2.2) recent 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 (27). IL-23 is critical for generation of Th17, and IL-17 induces chemokine production at the sites of infection and causes recruitment of neutrophils (29); IL-17-deficient mice are more susceptible than control mice to systemic candidiasis, probably due to the decreased influx of neutrophils (85). Curland, a dectin-1 ligand, also induces a Th17 response, indicating that a non-TLR PRR is sufficient to activate adaptive immunity (28). 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. Signaling through TLRs induces both IL-12 and IL-23 and therefore participates in the generation of Th responses (27-29,37). It has been reported that TLR2- and TLR4-mediated MyD88-dependent signaling 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 (86). 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 mouse Th17 differentiation (87); therefore, the mechanisms addressed for mouse Th17 response might be different in humans.

From the reported information above described, which includes some discrepancies, a simplified hypothetical model for Th-type immune responses induced by *C. albicans* 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 dectins 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 clearly demonstrated, much less is known concerning the importance of direct recognition of pathogens by T- and B-lymphocytes. Recent 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 (74,76,88,89). 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. 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 (90). Whether *C. albicans* cells are able to show a similar TLR2-mediated direct effect on Th1 cells remains to be determined.

4. *C. albicans* LIGANDS FOR TOLL-LIKE RECEPTORS

Despite the wide evidence demonstrating a role of TLRs in recognition of fungal pathogens by the host immune cells, little is known concerning 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 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

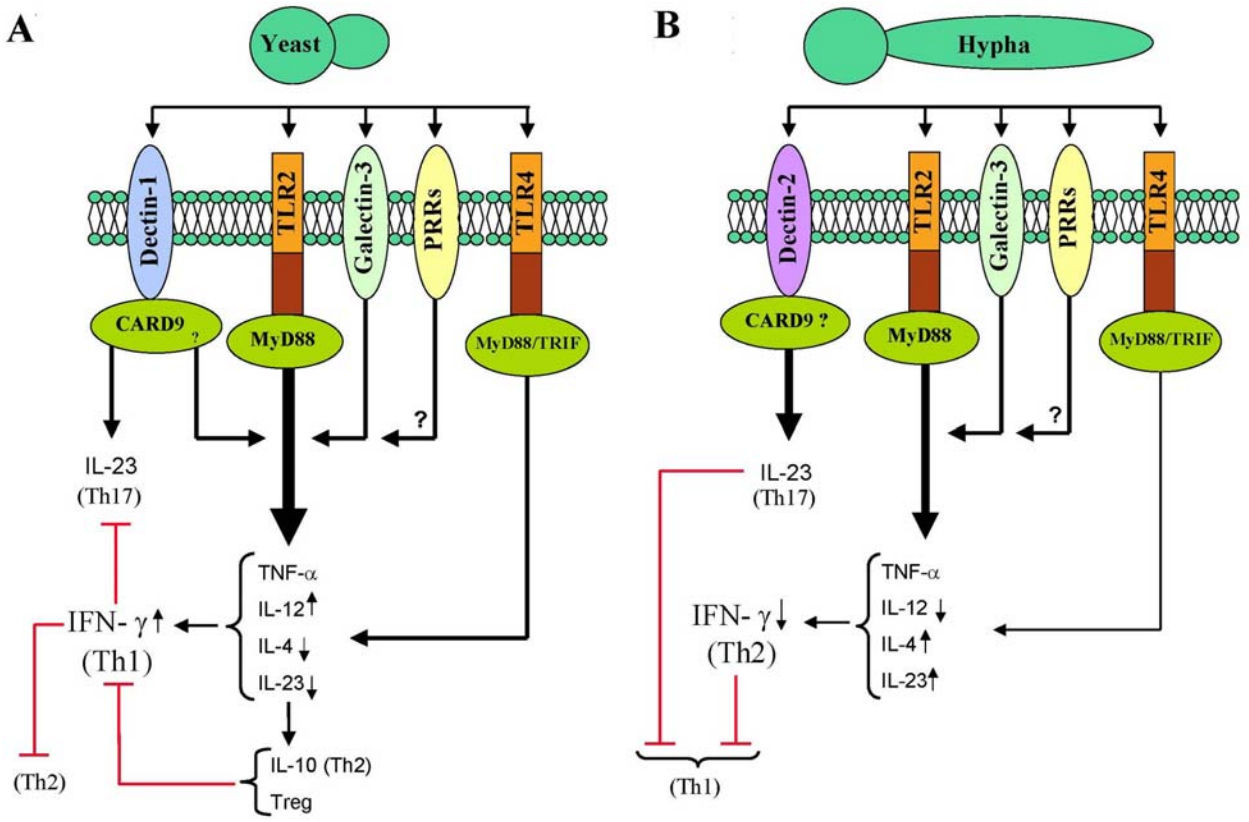

Figure 1. Simplified hypothetical model for Th-type immune responses induced by *Candida albicans* yeasts (A) and hyphae (B). (A) Yeast cells are recognized by TLR2, which induces an early proinflammatory response, mediated by the MyD88-dependent production of IL-12, leading to the development of a protective Th1 response. Other receptors, such as dectin-1, galectin-3 and possibly other PRRs yet to be defined, collaborate with TLR2 in the elicitation of this immune response against yeasts. TLR4 also may contribute to development of this response. Dectin-1 may trigger, upon recognition of yeast cells, a MyD88-independent pathway leading to production of IL-23, a cytokine that generates a Th17 response. The balance is biased towards the Th1 response, which in turns inhibits Th17 and Th2 development. To avoid a deleterious exacerbated inflammatory response, Th1 response needs to be counterbalanced by late secretion of IL-10, a Th2 cytokine, and Treg cells. (B) TLR2 also mediates recognition of hyphae, but in this case induces the development of a non-protective Th2 response, probably due to its collaboration with PRRs that differentially recognize yeasts or hyphae (like dectin-1, that preferentially recognizes yeast cells) and modulate the signaling pathway. TLR4 also may contribute to the response elicited upon hyphae recognition, although with a minor extent than its role in yeast recognition. Dectin-2 preferentially interacts with hyphae and induces IL-23 production leading to a Th17 response. As Th2 response is mediated by a low production of IL-12 and IFN-γ, Th17 development is not inhibited and, in addition, both Th17 and Th2 inhibit Th1 response development. The final balance is the elicitation of Th2 or Th17 responses depending of multiple factors (cytokine environment, site of infection, fungal strain, host status, etc.) not well defined yet; in any case, both responses are detrimental for the host, as anti-inflammatory Th2 response is non-protective, whereas Th17 response results in an enhanced deleterious inflammatory response that confers susceptibility to candidiasis. Modified from Gil and Gozalbo, 2006 (32).

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 (6,36,37,39,41).

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 (12,91,92), 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 (93). 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 signaling induced by *C. albicans* (32,51,60). 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 (12). The precise domain of the PLM molecule recognized by TLR2 as well as the participation of accessory proteins in PLM recognition are not yet characterized.

C. albicans cell wall mannoproteins, and particularly the mannan moieties, are well known important elicitors of cytokine production by host cells (66). Netea *et al.* (33) have demonstrated, using a combination of mannosylation-defective *C. albicans* strains, anti-receptor blocking antibodies and knockout mice, that TLR4 recognizes linear O-linked mannosyl residues, thus identifying this domain as a ligand for TLR4. In the same study, the highly branched N-linked mannosyl chains were identified as ligands for the mannose receptor. 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 β-glucan biosynthesis inhibitor caspofungin, or by mutations in genes involved in biosynthesis of cell wall components, such as mannan and/or mannoproteins (94,95); therefore, results concerning immune sensing of *C. albicans* strains defective in mannosylation should be carefully interpreted (64).

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 signaling through TLRs in response to *C. albicans*. Among all known non-TLR PRRs, fungal ligands for dectin-1 and galectin-3 have been well characterized.

β-glucan is the ligand of the phagocytic receptor dectin-1 (96-98), a receptor that collaborates with TLR2 to elicit a strong inflammatory response (59,97). Moreover, a TLR2-independent function of dectin-1 has been reported (99,100). 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* (101). 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 (102).

Fungal cell surface-associated β-1,2 mannosides bind to a host protein identified as the S-lectin galectin-3 (103). These mannosides are special types of glycans that are expressed by *C. albicans* and are associated with both mannan and PLM (6). It has been shown that specific recognition of C. *albicans* by macrophages requires galectin-3 to discriminate *S. cerevisiae* and needs association with TLR2 for signaling (104), thus suggesting that macrophages differentially sense *C. albicans* and *S. cerevisiae* through a mechanism involving TLR2 and galectin-3 (6,104).

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 signaling pathways leading to activation of inflammatory cytokine genes, type I IFN genes and IFN inducible genes (36, 38, 39). 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 (36, 39, 105). Despite that CpG enhances innate effector and Th1 responses improving host resistance to a variety of microbes, including fungal species as above mentioned (79-81), the role of CpG during invasive candidiasis is not clearly established yet (82,83).

5. PERSPECTIVE

In the last years the study of TLRs has emerged 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. In addition, some of 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, as well as 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, dendritic cells, 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 signaling 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, and therefore, our understanding of the TLR-signaling function in response to *C. albicans* depends also in the advances achieved with other PRRs and their ligands.

6. ACKNOWLEDEGMENTS

This work was supported by Grants PI03/06478, PI04/1472 (Instituto de Salud Carlos III; Ministerio de Sanidad y Consumo, Spain), and ACOMP07/073 (Conselleria d'Empresa Universitat I Ciencia, Generalitat Valenciana).

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Abbreviations: *C. albicans*: *Candida albicans,* IL: interleukine, IFN-gamma: interferon-gammą, TNF-alpha: tumour necrosis factor-alpha, PAMPs: pathogen-associated molecular patterns, TLRs: Toll-like receptors, Treg: T regulatory cells, DCs: dendritic cells, TCR: T cell receptor

Key Words : Toll-like receptors, *Candida albicans*, systemic candidiasis, innate and adaptive immunity

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