# Both viable and killed *Candida albicans* cells induce *in vitro* production of TNF-α and IFN-γ in murine cells through a TLR2-dependent signalling

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**ABSTRACT. The *in vitro* production of TNF-α and IFN-γ in response to *Candida albicans* was investigated in wild type, TLR2-/- and TLR4-/- murine cells. TLR2-/- resident peritoneal macrophages showed a strong impairment of TNF-α production in response to viable and non-viable (heat-killed, antimycotic-treated and formaldehyde-fixed) yeasts and hyphae (germ tube-bearing cells) of the high virulence *C. albicans* ATCC 26555 strain, as compared with macrophages from wild-type and TLR4-/- mice. The *in vitro* production of IFN-γ was investigated in murine splenocytes obtained three days after intravenous injection with the low virulence, non-germinative *C. albicans* PCA2 strain, and again, TLR2-/- splenocytes showed a strong impairment of the *in vitro* production of IFN-γ in response to non-viable (heat-killed, antimycotic-treated and formaldehyde-fixed) *C. albicans* ATCC 26555 yeasts, as compared with splenocytes of TLR4-/- and wild type mice. These results indicate that the TLR2-mediated recognition of *C. albicans* leading to a proinflammatory Th1 host response appears to be well conserved in killed *C. albicans* cells, regardless of the inactivating treatment employed.**

**Keywords:** *Candida albicans*, Toll-like receptor 2 (TLR2), TLR4, murine macrophage, splenocyte, TNF-α, IFN-γ

Resistance to candidiasis requires the coordinated action of both innate and adaptive host immune responses [1, 2]. Recognition of pathogen-associated molecular patterns (PAMPs) of invading fungi by the innate immune system through pathogen recognition receptors (PRRs) is the first step towards activating a rapid immunological response and ensuring survival after infection; phagocytes can kill the pathogen *via* intracellular and extracellular mechanisms, macrophage activation releasing several key mediators, including proinflammatory cytokines such as TNF-α, which are important for protecting the host against disseminated candidiasis [1-3]. Antifungal CD4+ T helper 1 (Th1)-mediated responses play a central role in anti-*C. albicans* defences, providing control of fungal infectivity through production of IFN-γ. This cytokine is required for optimal activation of phagocytes and suppresses the induction of the Th2 response, which is associated with the susceptibility of mice to systemic *C. albicans* infection and is characterized by the production of anti-inflammatory cytokines, such as IL-10 and IL-4; however, the pro-inflammatory (Th1) host response needs to be counterbalanced through Th2 and Treg cells to ensure an optimal, protective Th1 response [1].

Toll-like receptors (TLRs) constitute a family of PRRs that mediate recognition of microbes through PAMPs, induce subsequent inflammatory responses and also regulate the adaptive responses [3-5]. Our group has shown that TLR2 is essential for murine resistance to invasive candidiasis, and triggers production of proinflammatory cytokines, whereas TLR4 appears not to have a relevant role in these events [6-8]. Other authors have described a role for TLR2 and TLR4 in murine resistance to infection and in the *in vitro* cytokine production in response to *C. albicans* cells [9-11]. TLR2, identified as the receptor for the *C. albicans* cell wall-associated PAMP phospholipomannan, triggers the production of proinflammatory cytokines through the activation of the nuclear factor-kappa B [12, 13]. In addition, recognition by TLR4 of O-linked mannosyl residues present in the *C. albicans* cell wall mannoproteins has been shown to mediate cytokine (TNF-α) induction in murine macrophages, whereas TLR2 has a minor role through recognition of β-glucan by the dectin-1/TLR2 receptor complex [14]. In addition, controversial hypotheses have been proposed concerning the role of *C. albicans* recognition by TLR2 and TLR4 in host protection [8, 9, 15, 16], and two distinct models have been proposed. According to one of these models, TLR2 basically recognizes the *C. albicans* hyphae and confers susceptibility to infection through induction of IL-10 and Treg cells that results in a Th2 response, whereas the yeast form is recognized by both TLR2 and TLR4, leading to a Th1 protective response [11, 15]. In the other model, proposed by our group, both yeasts and hyphae signal through TLR2, and differences in the immune response to both fungal morphologies may involve interaction with other PRRs, such dectin-1 [8, 16]. These inconsistencies point out the complexity of the fungus-host interaction, which may be influenced by a wide range of host- and fungal-related parameters.

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Therefore, *in vitro* assays of cytokine production in response to *C. albicans* can be influenced by the nature of the stimuli used. In this work, we have studied the *in vitro* production of the Th1-cytokines TNF-α and IFN-γ, key molecules involved in the host protective response to *C. albicans*, in murine cells from wild type, TLR2-/- and TLR4-/- knockout mice, challenged with viable and non-viable (heat-killed, paraformaldehyde-fixed, and antimycotic-treated) *C. albicans* cells in order to reveal possible differences in the induced host response due to alterations of the fungal cell surface that may affect exposure of PAMPs due to inactivating treatments.

## MATERIALS AND METHODS

### *Mice and yeast strains*

TLR2-/- and TLR4-/- knockout mice (C57BL/6 genetic background) were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan) [17] and wild type C57BL/6 mice were obtained from Harlan Ibérica (Barcelona, Spain). Eight-to-ten-week-old mice were used for all experimental assays. All assays involving mice were approved by the Institutional Animal Care and Use Committee. The low virulence, non-germinative *C. albicans* PCA2 strain and the high virulence, *C. albicans* ATCC 26555 strain were used in this study [18, 19].

### *Preparation of fungal stimuli for in vitro assays*

Yeasts and hyphae (germ tube-bearing yeast cells) from the high virulence *C. albicans* ATCC 26555 were obtained following incubation of starved yeast cells for 3 h at 28 °C (budding yeast) or 37 °C (germ-tubes) in a minimal synthetic medium, as previously described [19]. Cells were collected by centrifugation and washed with phosphate-buffered saline (PBS) prior to inactivating treatments: (1) heat-killing was performed by incubating the cells (20 x $10^6$ cells/mL in PBS) at 100 °C for 1 h, as reported elsewhere [6, 7]; (2) fixed cells were obtained by incubating the cells (20 x $10^6$ cells/mL) in 4% paraformaldehyde (fixation buffer, eBioscience, San Diego, CA, USA) as previously described [20], followed by extensive washing with PBS to remove the fixing agent, and (3) antimycotic-treated cells we obtained by incubating the cells (20 x $10^6$ cells/mL in PBS) in the presence of 3 μg/mL amphotericin B (Gifco, Barcelona, Spain) for 72 h at room temperature, followed by extensive washing to remove the antimycotic, as previously described [20].

After treatments, inactivation of cells was checked by the absence of growth following incubation of samples (2 x $10^6$ cells) on Sabouraud-dextrose agar plates for 48 h at 28 °C. *C. albicans* PCA2 and ATCC 26555 yeast cells, collected from exponentially growing cultures in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 28 °C, were used as viable fungal stimuli: PCA2 cells as a yeast stimuli, since this low virulence, non-germinative strain is unable to develop hyphal forms [18], and ATCC 26555 cells as hyphal stimuli, as these cells exhibit the hyphal pattern of growth when incubated at 37 °C in complete culture medium for murine cells (RPMI 1640 medium supplemented with 5% heat-inactivated FBS and 1% penicillin-streptomycin, Gifco, Barcelona, Spain) in a 5% $CO_2$ atmosphere. All procedures were performed under conditions designed to minimize endotoxin contamination as described elsewhere [6, 7, 20-22].

### *Isolation of mouse peritoneal macrophages and in vitro production of TNF-α*

Resident peritoneal macrophages were obtained as previously described [6, 7]. Macrophages (2.3 x $10^5$ cells in 200 μL of complete medium per well, in a 96-well tissue culture plate) were challenged for 24 h with *Escherichia coli* O111: B4 LPS (250 ng/mL, Sigma, Spain), zymosan (7.5 x $10^6$ particles/mL, Molecular Probes, Invitrogen, Spain) and equivalent amounts of killed budding yeast or hyphal (germ-tube) cells (150 μg dry weight/mL), or for 6 h with viable fungal cells (250 000 cells per well) either of PCA2 or ATCC 26555 strains (see above). Culture supernatants were tested using a commercial ELISA kit for TNF-α (eBioscience, San Diego, CA, USA). Assays in the absence of exogenous stimuli were performed as negative controls to check background activation. Student's two tailed *t*-test was used to compare cytokine production by TLR2-/- and TLR4-/- cells to control C57BL/6 cells; data are expressed as mean ± SD and significance was accepted at $*p < 0.05$.

### *Isolation of splenocytes and in vitro production of IFN-γ*

Splenocytes from mice intravenously (i.v.) infected with *C. albicans* PCA2 cells (400 000 cells/mouse) were obtained at day 3 post-infection as described elsewhere [7, 21, 22]. Splenocytes were resuspended ($10^7$ cells/mL) in 1 mL of complete medium per well in a 24-well tissue culture plate, in the presence of 2.5 μg/mL amphotericin B to avoid fungal growth, and challenged for 48 h with LPS (500 ng/mL), zymosan (7.5 x $10^6$ particles/mL) and killed *C. albicans* ATCC 26555 yeast cells (30 μg dry weight/mL) in a 5% $CO_2$ atmosphere. Culture supernatants were tested using a commercial ELISA kit for IFN-γ (eBioscience, San Diego, CA, USA). Assays in the absence of exogenous stimuli were used as negative controls to check background activation. Student's two tailed *t*-test was used to compare cytokine production by TLR2-/- and TLR4-/- cells to control C57BL/6 cells; data are expressed as mean ± SD and significance was accepted at $*p < 0.05$ and $**p < 0.01$.

## RESULTS

### In vitro *production of TNF-α by macrophages in response to C. albicans stimuli*

TNF-α production by macrophages in response to fungal stimuli was triggered through TLR2 in all cases *(figure 1)*. TLR2-/- macrophages showed a significant reduction in TNF-α production (65-78% inhibition) in response to all three types of killed, budding yeast cells, as well as in response to killed, germ-tube cells (70-88% inhibition), whereas no reduction was observed in TLR4-/- macrophages, as compared to wild type cells *(figure 1)*.

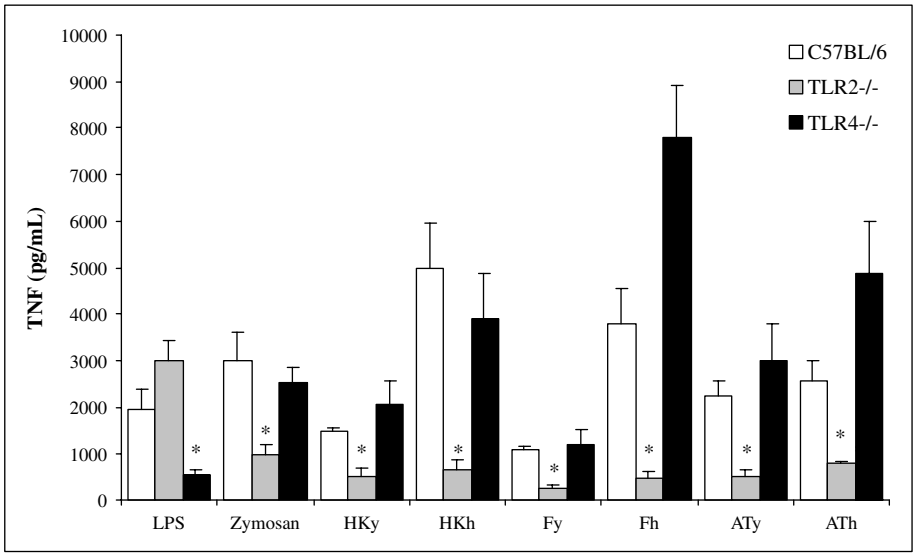


**Figure 1**

***In vitro* TNF-α production by resident peritoneal macrophages in response to non-viable *C. albicans* ATCC 26555 cells.** Resident peritoneal macrophages from C57BL/6, TLR2-/- and TLR4-/- mice were challenged with LPS, zymosan, heat-killed *C. albicans* yeasts and hyphae (HKy and HKh, respectively), fixed *C. albicans* yeasts and hyphae (Fy and Fh, repectively), and antimycotic-treated *C. albicans* yeasts and hyphae (ATy and ATh, respectively). Cytokine levels in the absence of exogenous stimuli (negative controls) were below the detection limit (20 pg/mL). Depicted are means ± SD of duplicates from one representative experiment of four.
(*) $p < 0.05$ compared to the control sample (C57BL/6).

The expected results were obtained with control stimuli: (1) the TNF-α production in response to LPS (a pure agonist of TLR4) showed a 73% reduction in TLR4-/- cells (the retained response suggests the presence of some contamination in the LPS preparation), and (2) TNF-α production in response to zymosan (a TLR2 agonist) showed a 70% reduction in TLR2-/- cells. Therefore, the different inactivating cell treatments used did not result in significant changes in the macrophage response against *C. albicans* mediated through TLR2 and TLR4. To compare these results with the response to viable fungal cells, we performed *in vitro* assays using viable cells of *C. albicans* PCA2 and ATCC 26555 strains as stimuli, and a shorter incubation period to avoid fungal overgrowth *(figure 2)*. Results showed that after six hours of the challenge, there were detectable levels of TNF-α, and again TLR2-/- macrophages showed an impaired production of TNF-α in response to both yeast (PCA2; 79% inhibition) and hyphae (ATCC 26555; 75% inhibition) as compared with wild type macrophages, whereas no significant differences were found between wild type and TLR4-/- cells *(figure 2)*. Control stimuli (zymosan, and heat-killed *C. albicans* cells) produced the expected results according to the results shown in *figure 1*. This observation indicates that killed and viable cells share common PAMPs that are able to trigger TNF-α production by murine macrophages in response to *C. albicans,* through a TLR2-dependent signalling.

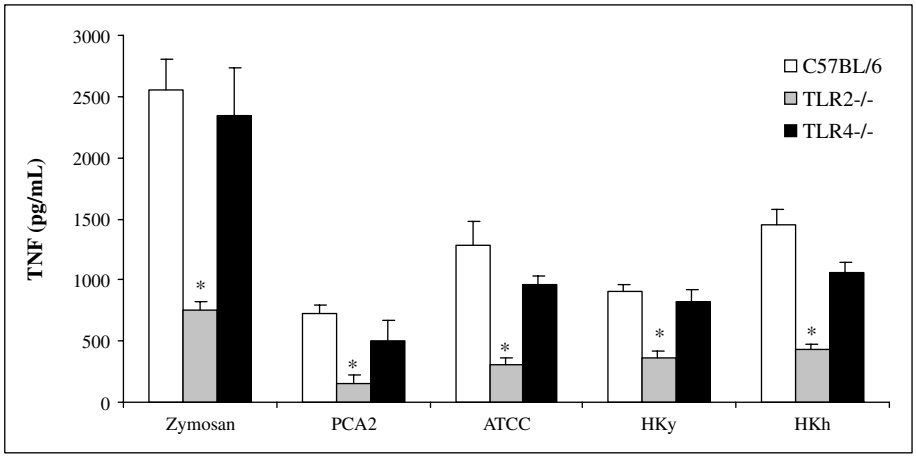


**Figure 2**

***In vitro* TNF-α production by resident peritoneal macrophages in response to viable *C. albicans* cells.** Resident peritoneal macrophages from C57BL/6, TLR2-/- and TLR4-/- mice were challenged with zymosan, viable cells of *C. albicans* ATCC 26555 (hyphae) and PCA2 (yeast) strains (see Materials and methods section for further details), and heat-killed *C. albicans* ATCC 26555 yeast and hyphae (HKy and HKh, respectively). Cytokine levels in the absence of exogenous stimuli (negative controls) were below the detection limit (20 pg/mL). Depicted are means ± SD of duplicates from one representative experiment of three.
(*) $p < 0.05$ compared to the control sample (C57BL/6).

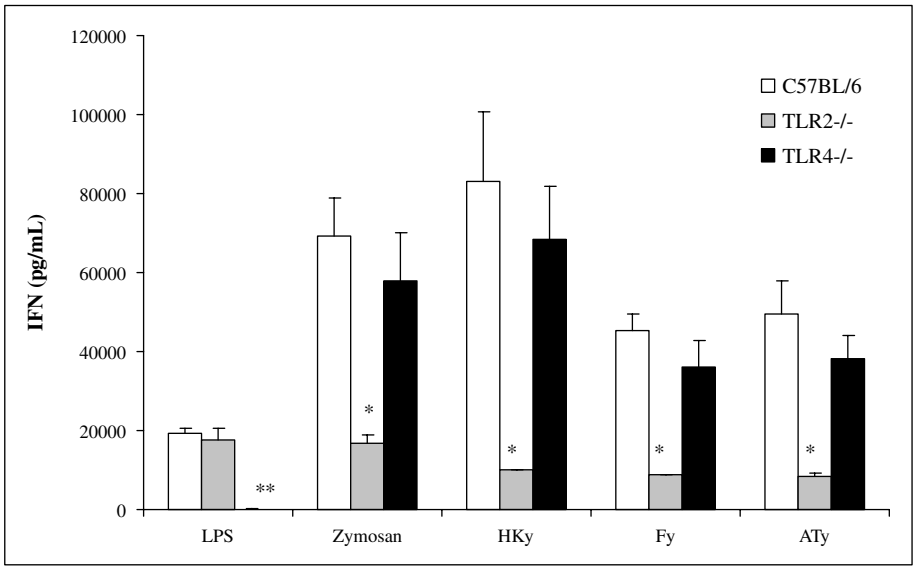


**Figure 3**

***In vitro* IFN-γ production by splenocytes of *C. albicans* PCA2-infected mice in response to *C. albicans* ATCC 26555.** Splenocytes of C57BL/6, TLR2-/- and TLR4-/- mice were isolated at day three post-infection with the *C. albicans* PCA2 strain, and *in vitro* challenged with LPS, zymosan, heat-killed yeasts (HKy), fixed yeasts (Fy) or antimycotic-treated yeasts (ATy) of the *C. albicans* ATCC 26555 strain (see Materials and methods for further details). Cytokine levels in the absence of exogenous stimuli (negative controls) were below the detection limit (20 pg/mL). Depicted are means ± SD of duplicates from one representative experiment of three.
(*) $p < 0.05$, (**) $p < 0.01$ compared to the control sample (C57BL/6).

Moreover, zymosan as well as inactivated and viable fungal stimuli still retained TNF-α-inducing capacity (roughly 20-30%) in TLR2-/- macrophages, which indicates that other receptors also participate in triggering TNF-α production upon recognition of these stimuli.

### In vitro *production of IFN-γ by splenocytes in response to C. albicans stimuli*

To assess the antifungal production of IFN-γ upon primary infection, animals were infected i.v. with a low dose of the non-germinative, low virulence *C. albicans* PCA2 strain. This infection does not produce any mortality or any significant symptoms of disease in mice (wild type, TLR2-/- and TLR4-/-) and induces substantial Th1 acquired protection to reinfection with a high virulence *C. albicans* strain [7, 21, 22].

Therefore, this is a suitable experimental approach for inducing the presence, in the spleen, of IFN-γ-producing cells that are immunoresponsive to *C. albicans*. The IFN-γ production was assessed in splenocytes obtained three days after i.v. infection of mice with the *C. albicans* PCA2 strain. These splenocytes were challenged *in vitro* with all three types of killed yeast stimuli obtained from *C. albicans* ATCC 26555 strain *(figure 3)*.

As expected, IFN-γ production in response to LPS was strongly inhibited in TLR4-/- mice (98% inhibition) as well as in response to zymosan in TLR2-/- mice (76% inhibition). Production of IFN-γ was significantly inhibited (80-90% inhibition) in response to all three, non-viable, fungal stimuli in TLR2-/- splenocytes, whereas wild type and TLR4-/- cells showed similar levels of IFN-γ production.

## DISCUSSION

The results reported in this work indicate that TLR2 is a major PRR involved in triggering production of proinflammatory and Th1-cytokines, such as TNF-α and IFN-γ, by murine macrophages and splenocytes, respectively, in response to *C. albicans* stimuli, but do not support a significant role of TLR4 in *C. albicans* recognition, according to our previous observations [6, 7]. In addition, fungal cell, surface-associated ligands (PAMPs) for TLRs appear to be well conserved in viable and all three types of non-viable fungal stimuli assayed (heat-killed, formaldehyde-fixed and antimycotic-treated cells), regardless of the inactivating treatment, which does not affect significantly the exposure of fungal ligands for TLR2 and TLR4.

However, it should be noted that Wheeler and Fink [23] have reported that some inactivating fungal treatments increase β-glucan exposure and lead to high levels of TNF-α elicitation, probably through the dectin-1/TLR2 receptor complex. It has been demonstrated that dectin-1, a phagocytic receptor for the fungal cell wall polymer β-glucan, collaborates with TLR2 in eliciting the inflammatory response to yeast, and that dectin-1 mediates macrophage recognition of *C. albicans* yeasts but not hyphae, as hyphal cells do not expose β-glucan at the cell surface, thus suggesting that failure of hyphae to activate dectin-1 may contribute to an impaired Th1 response [24, 25].

Moreover, dectin-1 can also trigger signalling through a TLR2-independent pathway leading to cytokine production [26, 27].

Although it can not be ruled out that, under the experimental conditions used, differences in the detected levels of cytokine production in response to all three types of inactivated fungal stimuli, may involve modifications of glucan

exposure, our results suggest that glucan exposure is not significantly affected by the inactivating treatments, as reduction of cytokine production in TLR2-/- cells was similar in response to viable and all three types of inactivated fungal stimuli.

Moreover, it has been found that macrophages sense differently *C. albicans* and *S. cerevisiae* through a mechanism involving both TLR2 and galectin-3, which probably associate for the binding of ligands expressing β-1,2 mannosides specific to the *C. albicans* cell wall surface [28]. These observations further support the model proposed by our group of the role of TLRs in host response to *C. albicans* [6-8, 16], indicating that TLR2 is the major PRR involved in the induction of proinflammatory cytokines through a MyD88-dependent signalling pathway in response to both yeasts and hyphae.

However, since zymosan and fungal stimuli still retained some cytokine-inducing capacity in the absence of TLR2, obviously other receptors (such as dectin-1 and others) may be involved in this process. Furthermore, the differences in the macrophage secretory response to *C. albicans* yeasts and hyphae [8, 11] may involve PRRs other than TLR2, such as dectin-1 and galectin-3, that may recognize different PAMPs exposed on the yeast and/or hypha cell surface and collaborate with TLR2.

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