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Biological importance of the two Toll-like receptors, TLR2 and TLR4, in macrophage response to infection with *Candida albicans*

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Abstract

The aim of this study was to assess the role of TLR2, TLR4 and MyD88 accessory molecule in the effector and secretory response of macrophages to viable microbial agents. Using TLR-deleted macrophage cell lines generated from the bone marrow of genetically engineered mice (TLR4 gene-deficient, MyD88- and TLR2-knockout mice) and wild-type control mice, we found that TLR2-deleted macrophages exhibit increased ability to contain *Candida albicans* infection compared to TLR2+/+ counterpart. In contrast, both MyD88−/− and TLR4−/− macrophages retain levels of functional activity comparable to that of the respective wild-type MyD88+/+ and TLR4+/+ controls. The difference in anticandidal effector functions observed between TLR2−/− and TLR2+/+ macrophages is abrogated upon opsonization of the fungal target and interestingly is not observed when using other microbial targets, such as *Streptococcus pneumoniae* and *Helicobacter pylori*. When tested for secretory response to *C. albicans*, TLR2-deleted macrophages show a pattern of cytokine production similar to that of TLR2+/+ controls. Finally, flow cytometry analysis reveals that TLR2-deleted macrophages express only TLR4, while, as expected, TLR2+/+ macrophages are both TLR2 and TLR4 positive; in no cases, modulation of such markers occurs in macrophages exposed to *C. albicans* infection. In conclusion, these data indicate that TLR2 and TLR4 have different biological relevance, in which TLR2 but not TLR4, is involved in the accomplishment of macrophage-mediated anticandidal activity, while the secretory response to *C. albicans* appears to be TLR4 but not TLR2-dependent.

Keywords: Toll-receptors; Antimicrobial activity; Macrophages; *Candida albicans*

1. Introduction

Macrophages initiate the innate immune response by recognizing pathogens, phagocytosing them and secreting inflammatory mediators. An effective immune response requires that macrophages recognize pathogen-associated molecular patterns (PAMPs) that distinguish the infectious agents from "self" and in addition discriminate among pathogens [1]. A novel family of receptors, the Toll-like receptors (TLR), has been demonstrated to participate in this process [2–6]. Among several members, TLR2 recognizes the largest number of microbial ligands, including various fungal, gram-positive and mycobacterial components

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such as peptidoglycans [7–9], lipoarabinomannan [10] and bacterial lipoproteins [11,12]. TLR4 binds to lipopolysaccharide (LPS) [13,14], mycobacterial components [10], *Aspergillus* hyphae [15], cryptococcal capsule [16] and lipoteichoic acid [8]. TLR5 and TLR9 interact with flagellin [17] and bacterial DNA [18], respectively.

Upon interaction with the cognate PAMP, TLR transmit the signal from the ectodomain to the cytoplasm, through adapter molecules such as the myeloid differentiation factor 88 (MyD88), Toll/interleukin-1 receptor adapter protein (TIRAP) and Toll/interleukin-1 receptor domain-containing adapter inducing IFN-β (TRIF) [19–21]; consecutively, tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin 1 receptor-associated kinase (IRAK) are involved in the activation of two distinct signalling pathways, activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) [2–4]. Via such pathways, many genes, whose products are involved in inflammatory events, like interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, inducible nitric oxide (NO) synthase, etc. are induced [3,5,12].

Numerous reports describe the potential role of TLR in antimicrobial defences both in vivo and in vitro [3,12]. Nevertheless, the precise role of TLRs remains to be elucidated in various infections since some contradictory findings are found in the literature. For example, it has been demonstrated that TLR2 and TLR4 are implicated in elicitation of immune response against fungal pathogens [3,22]. In particular, Villamon et al. [23] have shown that TLR2 is essential in murine defences against candidal infection, whereas Netea et al. [24] have demonstrated that TLR2 is able to suppress immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. Under the latter experimental conditions, TLR2−/− macrophages have increased capacity to migrate towards the infection site and augmented their killing activity against fungal cells [24]. In the other model, TLR2−/− macrophages have impaired macrophage inflammatory protein (MIP)-2 and TNF-α production, while their phagocytic activity remains unaffected [23]. Furthermore, Bellocchio et al. [25] investigated the levels of anticandidal activity by polymorphonuclear cells and showed that TLR2−/− mice are comparable to wild-type (wt) controls in terms of susceptibility to primary *C. albicans* infection.

Another PAMP receptor, TLR4, might also be involved in antifungal defences, although there is no general consensus regarding its specific role in the response to fungal infections. It is known that TLR4-defective mice have enhanced susceptibility to *C. albicans* infection [22,25]. TLR4 has been shown to trigger intracellular signalling in macrophages upon exposure to *Cryptococcus neoformans* glucoronoxylomannan [16], and induction of KC and MIP-1 chemokine production [22,25,26]. Moreover, by means of double mutant TLR models, evidence has been provided that heterodimerization may occur between TLR, further improving the degree of ligand–receptor specificity [27–30]. Thus, the variety of TLR repertoire together with the possibility of concerted action(s) between distinct TLR seem to allow identification of non-self PAMP from self and to facilitate discrimination between subtle differences in microbial components.

It has been well documented that the TLR accessory molecule MyD88 plays a critical role on host susceptibility to infections mediated by TLR. MyD88-deficient mice show reduced resistance to both fungal [25] and bacterial [31] infections. Moreover, in vitro data document the differential role of MyD88 in regulating macrophage response to the pathogenic yeast *C. albicans* and the mould *Aspergillus fumigatus* [32]. The plethora of information available on TLR and lack of consensus regarding the role of a particular TLR in the regulation of responses especially to fungal pathogens, most likely results from differences in the experimental models that employ genetically different and/or non-homogeneous host cells and/or diverse pathogen strains. The possibility of systematic studies within in vitro systems, as simplified prototypes of host–pathogen interaction, may aid in the effort of dissecting and better understanding discrete steps in the TLR-PAMP interplay. In line with this idea, we generated macrophage cell lines from bone marrow of mice that do not express TLR4 gene (deletion of the entire locus) [13] and from TLR2 and MyD88 knockout mice, genetically engineered to abrogate the gene expression via homologous recombination using neomycin selection cassette [33]. The genetically targeted mice were backcrossed several times to C57BL/6 genetic background. Macrophage cell lines generated from bone marrow of C57BL/6 mice were used as wt controls in our experiments [34 and unpublished data]. We performed comparative studies aimed at assessing the role of TLR2, TLR4 and MyD88 accessory molecule in the secretory and effector responses of macrophages to in vitro infection with *C. albicans*. The responses of macrophages to infection with *Streptococcus pneumoniae* and *Helicobacter pylori* have also been monitored as representative of gram-positive and gram-negative bacteria, respectively.

2. Materials and methods

2.1. Culture medium and macrophage cell lines

RPMI 1640 medium (Hyclone Europe Ltd, Cramlington, UK) was supplemented with 10% heat-inactivated fetal bovine serum defined (Hyclone), gentamycin (50 μg ml^{-1}) (Sigma), and L-glutamine (2 mM) (Sigma) (complete medium). The complete medium was used in all the experiments.

The macrophage cell lines employed have recently been generated following a previously established protocol [35]. Briefly, bone marrow-derived macrophage cell lines were established by a recombinant retroviral J2 construct as previously described [35]. The bone marrow cells were obtained from tlr2−/− [33] and MyD88 gene knockout mice [36]. The TLR4−/− cell line was generated from C57BL/10ScCr mice with a complete deletion of the tlr4 gene [13]. As wt control for TLR4−/− macrophages, we used a macrophage cell line generated from C57BL/10. A mice (B10.A), hereafter referred as TLR4+/+. As wt counterpart for TLR2−/− cell line, we used a macrophage cell line derived from bone marrow of C57BL/6J mice that shared the same genetic background as the TLR knockout mice. These wt control cell lines had previously been characterized in detail [34] and are, hereafter, referred as TLR2+/+. The MyD88+/+ cell line was derived from bone marrow of MyD88+/+ mice [36].

The TLR-deleted cell lines were genotyped and characterized using specific macrophage markers. All macrophage cell lines were F4/80-positive, Mac2-positive, B7.1- and B7.2-positive, FcRI II- and III-positive, surface Ig-negative, B220-negative, CD3-negative, constitutive MHC class II-negative, MHC class II-positive following 24–48 h treatment with IFN-γ. All macrophage cell lines were able to ingest latex beads and produced nitric oxide in response to appropriate stimulation. All the cell lines were maintained in a complete medium. Every two weeks, cells were detached by vigorous shaking and diluted in fresh complete medium.

2.2. Genotyping analysis of the generated macrophage cell lines

The presence or absence of neomycin (neo) cassette was confirmed using genomic DNA prepared from the cell line by salting-out procedure [33,36,37] and using the neo primers that recognize the mutated allele (insertion of neo cassette into a gene of interest during generation of knockout mice). The following two primers were used to confirm the presence of the neo allele in the TLR2−/− and MyD88−/− cell lines: Neo 1, 5′CTTGGGTGGAGAGGCTATATC3′ and Neo 2, 5′AGGTGAGATGACAGGAGATC3′. The PCR conditions used to amplify the neo product were as follows: 94 °C for 4 min, followed by 30 cycles at 94 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min and final extension at 72 °C for 10 min. A 200-bp product was then analysed by electrophoresis and stained with ethidium bromide.The presence or absence of the TLR4 mRNA, TLR2 mRNA and MyD88 mRNA was confirmed using reverse transcription PCR (RT-PCR), the corresponding specific set of primers (tlr4, tlr2 and MyD88) and template cDNA generated from each cell line. cDNA was prepared using 1 μg of total RNA extracted from each cell line and the RT-PCR kit from Roche (Indianapolis, IN). Briefly, reverse transcription reaction was performed using the Superscript II reverse transcriptase at 45 °C for 50 min in the presence of 1 U of RNAse inhibitor. Following reverse transcription, the enzyme was heat inactivated at 70 °C for 10 min. The following primers, designed from the MyD88 gene Accession No. BC005591, were used to amplify a MyD88 mRNA fragment: forward (115–134), 5′TCCCTGGACTCCTTCATGTT3′ and reverse (811–830), 5′TTCTGTTGGACACCTGGAGA3′. The PCR conditions used to amplify the MyD88 product were: 30 cycles at 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min for 30 cycles and subsequently an extension at 72 °C for 10 min. Hot start at 84 °C was performed using the Taq polymerase. A 716-bp product was analysed by electrophoresis and staining with ethidium bromide.

To amplify the TLR2 mRNA fragment, we used primers previously designed by Wang et al. [37]. As a template, both cDNA or genomic DNA could be used. The following primers, designed based on the TLR2 gene Accession No. AF124741, were used: forward (1970–1994), 5′ACAGCTACCTGTGTGACTCTCCGCC3′ and reverse (2547–2571), 5′GGTCTTGGTGTTCATTATCTTGCGC3′. The PCR conditions were as follows: 30 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Hot start at 84 °C was performed using the Taq polymerase. A 602-bp product was analysed by electrophoresis and staining with ethidium bromide.

Finally, to amplify the TLR4 mRNA fragment, we also used primers previously designed by Wang et al. [37]. The designed primers based on the TLR4 gene Accession No. AF095353, were: forward (1414–1433), 5′GACCTCAGCTTCAATGGTGC3′ and reverse (2131–2154), 5′TATCAGAAATGCTACAGTGGATACC 3′. The PCR conditions used to amplify the TLR4 product were: 30 cycles at 95 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 10 min. Hot start at 84 °C was performed using the Taq polymerase. A 741-bp product was then analysed by electrophoresis and staining with ethidium bromide.

2.3. Microorganisms

Two strains of *C. albicans* were employed, the agerminative avirulent strain PCA2 and the virulent strain CA6 described elsewhere [38]. The fungal strains were maintained by daily passages on Sabouraud dextrose agar plates. PCA2 yeast cells were used in the phagocytosis and antifungal assays. CA6 was employed in the assay for cytokine production. Yeast cells were harvested from agar plates, washed twice in saline solution by low-speed centrifugation, counted and diluted to the

appropriate concentration in complete medium before use. Hyphal forms were obtained after 3 h incubation of CA6 yeast cells in complete medium.

Streptococcus pneumoniae, encapsulated strain HB565, serotype 3 (kindly provided by Prof. Gianni Pozzi, University of Siena, Italy) was cultured in Tryptic Soy Broth (TSB) supplemented with 3% defibrinated horse blood (Oxoid). Bacteria were grown to mid-exponential phase, centrifuged for 20 min at 1500*g*, resuspended in fresh TSB containing glycerol (10%) and stored in aliquots at −80 °C. Numbers of bacteria were determined by viable counting of serial dilutions in sterile phosphate-buffered saline and plating onto blood agar plates. Before use, bacteria were thawed, washed and harvested by centrifugation and suspended at the desired concentrations for the in vitro assays.

Helicobacter pylori, type I strain SPM326, was also used and bacteria were grown at 37 °C for 24 h on Columbia agar plates as described in [39].

2.4. Phagocytosis assay

The phagocytosis assay was performed as described previously [38]. Effector cells were exposed to *C. albicans* at macrophage:yeast ratio of 1:10 at 37 °C for 2 h. The excess of microorganisms was removed by centrifugation of the cell suspension on a Ficoll cushion at 300*g* for 10 min. The cells at the interface were recovered and washed. Fungal uptake was directly evaluated in Giemsa-stained cytospin preparations. A minimum of 200 macrophages were scored and any cells containing one or more yeasts were counted as phagocytic. The phagocytosis index was calculated as the total number of phagocytosed yeasts/total number of phagocytic cells.

2.5. Measurement of antifungal and antibacterial activities

Antifungal and antibacterial activities were assessed as described previously [38–40]. Briefly, effector cells were infected with *C. albicans*, *H. pylori* or *S. pneumoniae* at various macrophage:microbial cell ratios and for different times. Subsequently, Triton X-100 (0.1% final concentration) was added to the wells and appropriate dilutions of the cell lysates were plated on: Sabouraud dextrose agar (if infected with *C. albicans*), Columbia agar (if infected with *H. pylori*) or blood agar (if infected with *S. pneumoniae*). The colonies were counted after 24–72 h incubation time at 37 °C. Control cultures consisted of microbial cells incubated without effector cells. Results were expressed as percentage of colony-forming units (CFU) inhibition according to the following formula: % CFU inhibition = 100 − (CFU experimental groups/CFU control cultures) × 100.

2.6. Nitrite determination

The concentration of nitrite (NO_2^-) in culture supernatants of stimulated and unstimulated cells was measured using the Griess reagent and was taken to reflect the amount of nitric oxide (NO) generated. A standard curve was established using NO_2^- (Sigma) in a range between 3 and 200 μM. Griess reagent consisted of equal volumes of 0,1% naphthylethylene diamine dihydrochloride in distilled water and a mixture of 1% sulfaminilamide plus 5% H_3PO_4. Following a 10-min reaction at room temperature, a mixture of equal volumes of standard or supernatant samples and Griess reagent was monitored using an ELISA reader at 550 nm.

2.7. Cytokine production and flow cytometryc analysis

The levels of MIP-2, TNFα and IL-6 produced by the TLR-deleted and control macrophage cell lines, were evaluated in cell-free supernatants by ELISA, according to the manufacturer's instructions (BioSource).

Cells (10^6/50 μl) were incubated with appropriate dilutions of anti-mouse TRL2 polyclonal antibody (eBioscience) for 45 min and secondary FITC anti-rabbit antibody (eBioscience) for additional 45 min or with phycoerythrin (PE) conjugated anti-TLR4 monoclonal antibody (eBioscience) for 45 min; subsequently, cells were washed, resuspended in PBS and analysed using a Cyflow ML (Partec GmbH, Muenster, Germany), equipped with an air-cooled argon ion laser (488 nm, 200 mW, kept at 150 mW) and a UV Mercury lamp HBO (100 long life, 100 W), a red diode laser (635 nm, 25 mW), and a CCD camera. FITC (FL1) and PE (FL2) were collected with a 520/30 bandpass filter and by a 590/30 bandpass filter, respectively. Data were analysed using the Partec Flomax 3.0 and WinMDI softwares.

2.8. Statistical analysis

Significance of the data was evaluated using the non-parametric Mann–Whitney test. Data depicted in Figs. 1 and 4 are from a representative experiment performed twice. Results depicted in the rest of the figures and in the tables are means ± standard deviation of 3–6 independent experiments.

3. Results

In the initial set of experiments, the TLR-deleted and control macrophage cell lines were analysed for the presence/absence of a neo allele and TLR specific mRNA. Fig. 1 shows the results of the PCR analysis using as templates either genomic DNA (neo primers) or cDNA prepared using the reverse transcriptase reaction (TLR4, TLR2 and MyD88 primers). cDNAs were prepared

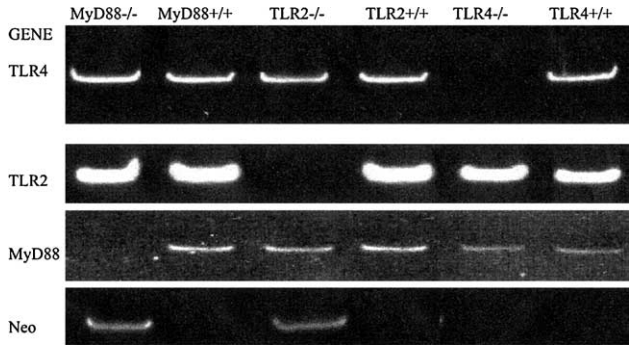

Fig. 1. Genotype analysis of TLR2−/−, MyD88−/−, TLR4−/− and control wt cell lines. PCR amplification of a fragment of neomycin cassette was performed using genomic DNA as a template. PCR amplification of TLR2, TLR4 and MyD88 specific fragments was performed using cDNA templates prepared from total RNA extracted from each of the cell lines. cDNAs were prepared using total RNA extracted using Trizol reagent, according to manufacturer instructions. Reverse transcription reaction was performed using Superscript II reverse transcriptase at 45 °C for 50 min in the presence of 1U of RNAse inhibitor. Following reverse transcription, the enzyme was heat-inactivated at 70 °C for 10 min. Results from a representative experiment performed twice are illustrated. Sequences of primers are listed under Section 2.

from total RNA extracted from each cell line (MyD88−/−, MyD88+/+, TLR2−/−, TLR2+/+, TLR4−/− and TLR4+/+). As shown in Fig. 1, it was confirmed that TLR2−/−, TLR4−/− and MyD88−/− lacked the TLR2, TLR4 and MyD88 mRNA, whereas the controls retained normal transcriptional activity for the tested genes, as predicted. The presence of the neomycin cassette was also confirmed in TLR2−/− and MyD88−/− macrophage cell lines.

In functional studies, we compared the TLR2−/−, MyD88−/− and TLR4−/− macrophages to the respective counterparts. Such pairs were assessed for phagocytic and antifungal activity against *C. albicans*. In particular, macrophages, exposed to the yeast cells (E:T = 1:10) for 2 h, were evaluated for percentage of phagocytic cells and phagocytosis index. As shown in Fig. 2(a), all the macrophage populations were phagocytic, but to a different extent. Particularly, MyD88−/− and TLR4−/− macrophages showed levels of activity comparable to each other and similar to the levels observed for their respective MyD88+/+ and TLR4+/+ controls. In contrast, TLR2−/− macrophages exhibited significantly higher activity than wt counterpart, the percentage of phagocytosis being 79 versus 48; the phagocytosis index was also higher in TLR2−/− than in TLR2+/+ control macrophages (4.6 versus 3.2).

To assess antifungal activity, each of the macrophage populations was infected with *C. albicans* (E:T = 10:1). After 4 h of incubation, the CFU inhibition assay was performed. The results presented in Fig. 2(b) show that TLR2−/− macrophages expressed levels of anticandidal activity significantly higher than those of TLR2+/+

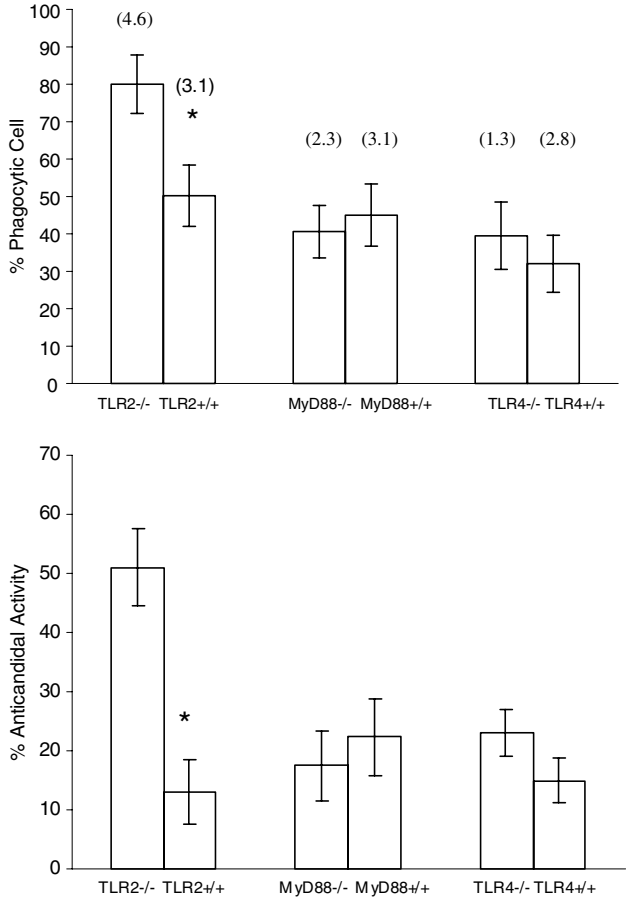

Fig. 2. Phagocytic and anticandidal activity of the TLR2−/−, MyD88−/−, TLR4−/− and control wt cell lines. Phagocytic activity (upper panel) of the different macrophage populations was tested in a 2-h incubation assay employing viable *C. albicans* (E:T = 1:10). Cytospin preparations were stained and evaluated as detailed in Section 2. Anticandidal activity (lower panel) was measured by a CFU inhibition assay after a 4 h incubation with *C. albicans* (E:T = 10:1). Data are means ± SD of 4 independent experiments. $*p < 0.01$: TLR deleted- *versus* wild type- macrophages.

counterpart; on the contrary, little or no differences were observed between MyD88−/− and MyD88+/+ or between TLR4−/− and TLR4+/+ macrophages, whose anticandidal activity remained below 20%. These data suggest that TLR2 deletion results in enhancement of both phagocytic and anticandidal activity by murine macrophages.

To investigate whether the above-described functional pattern might be affected by preopsonization of the fungal target, *C. albicans* yeast cells were pre-exposed to immune serum for 30 min prior to their use for the in vitro infection of TLR2−/−, MyD88−/− and TLR4−/− macrophages. Fig. 3 indicates that the three macrophage cell lines were highly and comparably effective in terms of antifungal activity when infected with pre-opsonized *C. albicans*, whereas, consistently with the results shown in Fig. 2(b), TLR2−/− macrophages were significantly more effective than

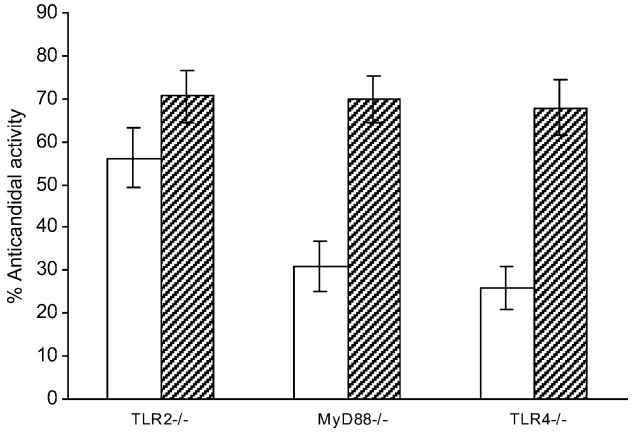

Fig. 3. Anticandidal activity of TLR2−/−, MyD88−/− and TLR4−/− macrophage cell lines against unopsonized and opsonized fungal cells. Anticandidal activity was measured by CFU inhibition assay after 4 h incubation with unopsonized (empty columns) or opsonized (black columns) *C. albicans* (E:T = 10:1). Opsonization consisted in preincubation of *C. albicans* for 30 min in fresh serum. Data are means ± SD of 3 independent experiments.

either MyD88−/− or TLR4−/− counterpart against unopsonized fungal targets. These results indicate that opsonization of the fungal targets allows to overcome the functional differences in anticandidal activity observed between TLR2−/− and the other macrophage cell lines tested.

In order to establish whether the above-described phenomena were exclusively observed when using *C. albicans* or rather shared with other microbial agents, we performed experiments where the three TLR-deleted macrophage cell lines were tested for antimicrobial activity against the gram-positive *S. pneumoniae* or the gram-negative *H. pylori* bacteria. As shown in Table 1, TLR2−/− and TLR2+/+ macrophages, MyD88−/− and MyD88+/+ macrophages, TLR4−/− and TLR4+/+ macrophages exerted comparable levels of antimicrobial activity against *S. pneumoniae*. Similar data were observed at 2 and 6 h. Furthermore, when the TLR-deleted and control macrophages were assessed for antimicrobial activity against *H. pylori*, no differences were observed between TLR2−/− and TLR2+/+ macrophages or between TLR4−/− and TLR4+/+ macrophages. Surprisingly, MyD88−/− macrophages were significantly more effective than the MyD88+/+ counterpart; the phenomenon was observed when unopsonized targets had been employed and occurred at both E:T ratios tested. Once again, all the cell lines were highly and comparably effective against opsonized *H. pylori*. Taken together, the above-described data indicate that the genetic defect on TLR2, TLR4 and MyD88 differently affects macrophage-mediated antimicrobial activity. In fact, TLR2, but not TLR4 or MyD88 deletion, upregulates anticandidal activity, while MyD88 ablation enhances anti-*H. pylori* activity. Under both conditions, microbial opsonization renders TLR-deleted and control macrophages comparably high effective (see Table 2).

In view of the functional differences observed in terms of anticandidal activity, TLR2−/− and TLR2+/+ macrophages were investigated for their pattern of secretory response to this microbial insult. According to a previous report showing C. *albicans* as proinflammatory signal for macrophages [38], TLR2−/− and TLR2+/+ macrophage cell lines were incubated 18 h with *C. albicans*, yeast or hyphal form (E:T = 1:10). LPS was also included as positive control [41]. The levels of secretory products were then measured in cell-free supernatants. As detailed in Table 3, both macrophage cell lines were able to respond to LPS; moreover, MIP-2, TNFα, IL6 and NO were produced in both cases to a similar extent. In addition, when tested for ability to respond to *C. albicans*, TLR2−/− and TLR2+/+ macrophages were both responsive, although the former was slightly less effective in terms of MIP-2 and TNFα production. IL6 and NO were little or not detected by each of the two cell

Table 1
Antimicrobial activity of TLR-deleted and control macrophage cell lines against *S. pneumoniae*

Macrophage cell lines	% Antimicrobial activity	
	2 h	6 h
TLR2−/−	45.7 ± 3.1	63.3 ± 5.1
TLR2+/+	48.5 ± 3.4	59.6 ± 6.2
MyD88−/−	44.3 ± 2.0	76.5 ± 8.4
MyD88+/+	46.6 ± 4.2	74.4 ± 6.3
TLR4−/−	34.3 ± 1.8	69.0 ± 2.9
TLR4+/+	31.9 ± 3.0	73.7 ± 5.9

TLR-deleted and control macrophages (5×10^5/ml) were infected with *S. pneumoniae* at the E:T ratio of 10:1. Antimicrobial activity was evaluated at the indicated times as detailed in Section 2. Results shown are means ± SD of three independent experiments.

Table 2
Antimicrobial activity of TLR-deleted and control macrophage cell lines against *H. pylori*

Macrophage cell lines	% Antimicrobial activity		
	Unopsonized		Opsonized
	25:1[a]	5:1	25:1
TLR2−/−	68 ± 3.1	49 ± 2.4	79 ± 5.2
TLR2+/+	72 ± 4.3	48 ± 3.5	83 ± 3.6
MyD88−/−	93 ± 3.9[b]	88 ± 1.7[b]	90 ± 4.1
MyD88+/+	63 ± 6.5	54 ± 6.6	85 ± 5.6
TLR4−/−	57 ± 4.6	51 ± 4.2	84 ± 3.9
TLR4+/+	62 ± 2.9	58 ± 7.7	81 ± 3.0

TLR-deleted and control wt macrophages (5×10^5/ml) were infected with *H. pylori* at the indicated ratios for 2 h. Antimicrobial activity was evaluated against unopsonized and opsonized bacteria as detailed in Section 2. Results shown are means ± SD of three independent experiments.

[a] E:T ratio.

[b] $P < 0.01$: MyD88−/− versus MyD88+/+ macrophages.

Table 3
Secretory response of TLR2−/− and TLR2+/+ macrophage cell lines to *C. albicans*

Treatment	MIP-2[a]		TNF-α[b]		IL-6[c]		NO[d]	
	TLR2−/−	TLR2+/+	TLR2−/−	TLR2+/+	TLR2−/−	TLR2+/+	TLR2−/−	TLR2+/+
Medium	–	–	–	–	–	–	–	–
LPS	12240.2	11430.5	1602.1	1460.0	1629.2	1604.6	25.3	19.7
Ca (Yeast)	137.7	312.5	45.4	226.7	9.2	18.7	–	–
Ca (Hyphae)	251.3	580	236.8	598.4	–	13.4	–	–

TLR2−/− and TLR2+/+ macrophages (10^6/ml) were exposed to LPS (1 μg/ml) or *C. albicans* (E:T = 1:10) for 18 h. Cell-free supernatants were harvested and assayed for cytokine and NO content, as detailed in Section 2.
Ca: *C. albicans*.
[a] The values are expressed as pg/ml; min = 8.
[b] The values are expressed as pg/ml; min = 23.4.
[c] The values are expressed as pg/ml; min = 16.
[d] The values are expressed as μM/ml; min = 3.1.

lines. As expected [38], *C. albicans* hyphal form was a better inducer of MIP-2 and TNFα response in both cell lines. These results indicate that TLR2 ablation has no major impact on macrophage secretory response to *C. albicans*. When MyD88−/− and MyD88+/+ were investigated for secretory response to *H. pylori* no appreciable cytokine production was observed (data not shown), in agreement with the poor stimulatory properties ascribed to this pathogen.

With the aim of investigating the constitutive levels of TLR expression in the macrophage cell lines and the possible modulation of such markers by infection, TLR2−/− and TLR+/+ macrophages were exposed to *C. albicans* and then assessed for TLR2 and TLR4 expression by flow-cytometry. Fig. 4 shows the results of a representative experiment. TLR2−/− macrophages were TLR2 negative and TLR4 positive; moreover, similar profile was detected in *C. albicans* infected cells. Furthermore, TLR2+/+ macrophages were TLR2 positive and TLR4 positive; again, no appreciable differences were observed in macrophages exposed to *C. albicans*. Thus, direct evidence is provided by showing that firstly, TLR2 expression is lost in the TLR2-deleted cell line while it is detectable in the TLR2+/+ counterpart; secondly, TLR4 is detectable in both TLR2−/− and TLR2+/+ cell lines and finally, *C. albicans* infection fails to modulate TLR2 and TLR4 expression on macrophages under our experimental conditions.

4. Discussion

In this study, TLR2−/−, MyD88−/− and TLR4−/− macrophage cell lines and their respective wt counterparts have been tested for effector and secretory responses to viable fungal and bacterial pathogens. Concerning phagocytosis and killing of *C. albicans*, our data show that TLR2−/− macrophages are significantly more effective than any other macrophage cell line. In particular, the percentage of phagocytic cells and the number of ingested *Candida* per macrophage (phagocytosis index) is higher in TLR2−/− than in TLR2+/+, TLR4−/− or MyD88−/− cell lines, whose activities remain comparable to those of their wt controls. These data provide evidence that TLR2 deletion contributes to an enhancement of phagocytic activity, while ablation of TLR4 or MyD88 is irrelevant. Consistently with the present data, it has been reported that TLR4 abrogation does not affect phagocytosis of *Candida* by macrophages [22]. Moreover, by means of a haemagglutinin A-tagged TLR2 transfected mouse macrophage cell line, Underhill et al. [28] provided an elegant demonstration of transient TLR2 recruitment to the phagosome following zymosan binding/internalization; yet, a functional TLR2 appears not required, since both zymosan binding and internalization remain unaffected in macrophages expressing the mutant TLR2 form. Additional studies [23,24] also show that TLR2 ablation does not affect phagocytosis of heat-killed *Candida* by murine macrophages. Our present data argue that TLR2 negatively regulates the ability of macrophages to ingest *C. albicans*. The fact that TLR2−/− macrophages have better phagocytic activity than control macrophages suggests that either such TLR2 receptor is detrimental per se or rather it may interfere with the activity of other receptors, involved in fungal cell recognition/ingestion. Viable and not heat-killed fungal cells have been employed in this study; this may explain the discrepancy between previously published [23,24] and the present report, implying that heat-sensitive PAMPs are involved in macrophage-*Candida* interaction. Results obtained in phagocytosis studies are confirmed and emphasized by analysis of anticandidal activity by TLR-deleted and control macrophages. We show that TLR2−/− macrophages exert levels of anticandidal activity higher than those detected in TLR2+/+ controls and also higher than any other cell line tested, including TLR4−/− and MyD88−/− macrophages. This indicates that, not only ingestion, but also accomplishment of antifungal activity is influenced by

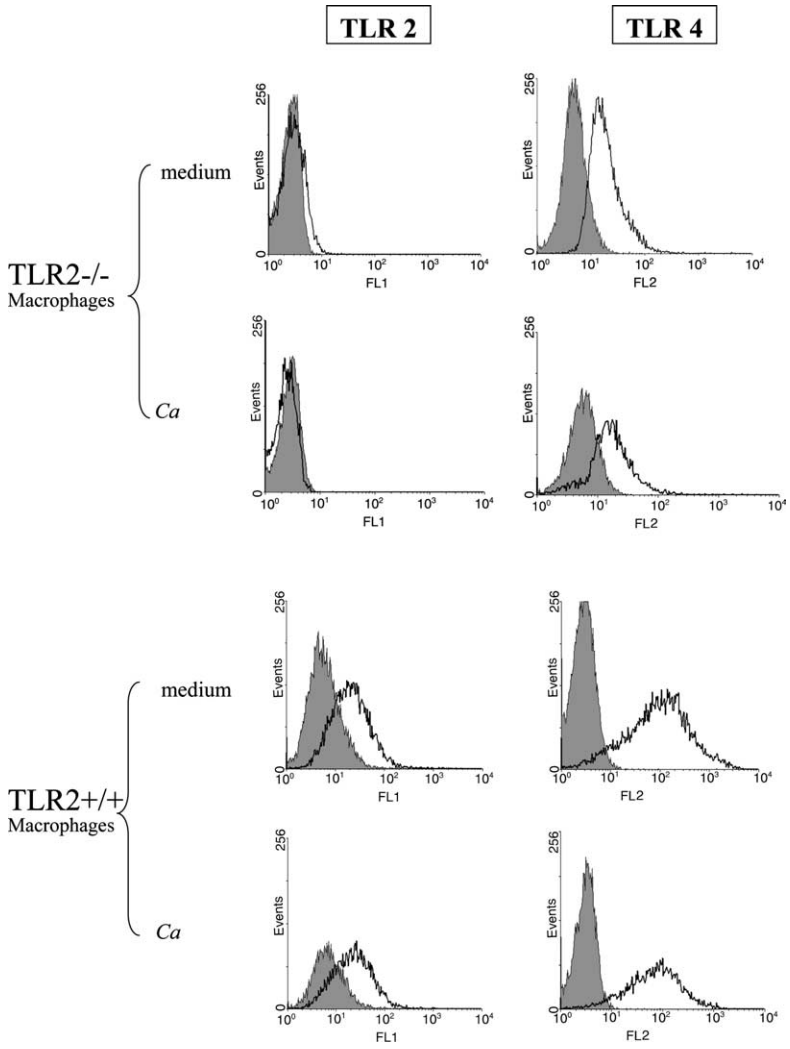

Fig. 4. Profile of TLR expression in TLR2−/− and TLR2+/+ macrophage cell lines exposed or not to fungal cells. Flow-cytometry analysis was performed using cells that had been infected or not with *C. albicans* (E:T = 10:1) for 4 h as detailed in Section 2. Data are from a representative experiment performed twice.

the expression of TLR2 on macrophages. This is in line with a recent report [24] showing enhanced candidacidal capacity by ex vivo peritoneal TLR2−/− macrophages obtained from *C. albicans* infected mice. In that model, the phenomenon has been interpreted as consequence of an imbalanced production of stimulatory (IFNγ) and inhibitory (IL10) cytokines, occurring upon in vivo infection. In our in vitro model, the enhanced killing observed in TLR2−/− macrophages appears as a direct consequence of the enhanced phagocytosis of *Candida* yeast cells by TLR2−/− macrophages. Furthermore, comparably high levels of activity are observed in all the cell lines upon fungal opsonization; thus, we may conclude that the essential intracellular mechanisms for anti-microbial defence(s) are preserved in the TLR-deleted macrophages, since opsonin-opsonin receptor recognition can fully overcome the functional differences observed in the TLR-deleted and wt control macrophages.

In our experience, the role of MyD88 does not seem to be important for macrophage ability to fulfil *Candida* phagocytosis. This finding is in line with a previous report showing that MyD88 defective macrophages [28] retain unaffected zymosan internalization, while a recent report demonstrates impairment in macrophage-mediated phagocytosis of *Candida* by MyD88 deletion [42]. This dichotomy of results raises the possibility that different patterns of response(s) might occur depending on the microbial strain employed.

The pattern of anticandidal activity by TLR-deleted macrophages happens to be distinct from those observed against bacterial targets. Moreover, other differences have been observed when testing gram-negative versus gram-positive bacteria. In particular, anti-pneumococcal activity appears to be TLR-independent, in agreement with previously published data on the role of TLR2 [43] and TLR4 [44] against *S. pneumoniae* infection. Surprisingly, anti-*H. pylori* activity is better

exerted by MyD88−/− macrophages than any other cell line, including the MyD88+/+ control macrophages. In this infection model, bacterial opsonization also compensates for the differences between TLR-deleted and control cells. Therefore, these data suggest that accomplishment of anti-*H. pylori* activity by macrophages is sensitive to inhibitory signal(s) transduced via MyD88 accessory molecule. The occurrence of both MyD88-dependent and MyD88-independent pathways are known [2] and have recently been described against fungal yeast versus mould [32]. The fulfillment of anti-*H. pylori* and anti-*S. pneumoniae* activities are consistent with these two distinct conditions. In general, our findings on macrophage-mediated antimicrobial effector functions indicate that the macrophage cell lines with TLR2−/−, TLR4−/− and MyD88−/− genotype behave in unique ways depending upon the microbial target encountered. Thus, the specificity of TLR-PAMP recognition seems to dictate the evolution and outcome of the effector cell–pathogen interaction. In this respect, it is known that upon recognition of their cognate ligands [1], TLR induces the expression of a variety of host genes [2–5], including those coding for inflammatory cytokines, chemokines, etc. Here, we show that macrophage response to *C. albicans* is essentially preserved in TLR2−/− macrophages since they still produce MIP-2 and TNFα, though to a slightly lower extent when compared with TLR2+/+ macrophages. Under those experimental conditions, as predictable, TLR2 deletion does not affect macrophage responsiveness to LPS. Thus, these findings imply that macrophage secretory response to *C. albicans* is mainly TLR2-independent, consistently with previous reports showing no involvement of TLR2 in the response to *C. albicans* [22,45] or *C. neoformans* [16]. Furthermore, in parallel experiments we confirmed the expected pattern [3,14,15,46] of defective response by TLR4−/− macrophages; both MIP-2 and TNFα production are not detectable in TLR4−/− macrophages exposed to either LPS, yeast or hyphal forms of *Candida* (data not shown). Taken together, these results indicate that TLR2, known to be not needed for response to LPS [2,12], is also not involved in macrophage–fungal cell interaction, at least in MIP-2 and TNFα production, while TLR4 ablates macrophage response to *C. albicans* and to LPS.

By RT-PCR analysis, Applequist et al. [47] investigated the extent of TLR gene expression in different cell lines representing innate and adaptive immunity. Interestingly, cells of the innate immune system, including macrophages among them, express a broader number of TLR than cells of the adaptive immune system, further supporting the importance of a precise hierarchy within first-line immune cells involved in first-line pathogen recognition. Using flow cytometry analysis, we provide initial evidence that TLR2 and TLR4 are in fact expressed on TLR2+/+ macrophages while only TLR4, but not TLR2, is detectable in TLR2−/− macrophages. This evidence allows us to conclude that genotype and phenotype profiles in our cell lines are congruent. Furthermore, phenotypical data on infected macrophages indicate that the expression of TLR2 and TLR4 is not susceptible of modulation upon *C. alibicans* infection.

In conclusion, this study adds insights on the involvement of TLR in the fulfillment of macrophage-mediated anticandida defences, by arguing on the biological relevance of TLR2 in accomplishment of effector but not secretory response.

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