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The Contribution of the Toll-Like/IL-1 Receptor Superfamily to Innate and Adaptive Immunity to Fungal Pathogens In Vivo[1]

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In vitro studies have indicated the importance of Toll-like receptor (TLR) signaling in response to the fungal pathogens *Candida albicans* and *Aspergillus fumigatus*. However, the functional consequences of the complex interplay between fungal morphogenesis and TLR signaling in vivo remain largely undefined. In this study we evaluate the impact of the IL-1R/TLR/myeloid differentiation primary response gene 88 (MyD88)-dependent signaling pathway on the innate and adaptive Th immunities to *C. albicans* and *A. fumigatus* in vivo. It was found that 1) the MyD88-dependent pathway is required for resistance to both fungi; 2) the involvement of the MyD88 adapter may occur through signaling by distinct members of the IL-1R/TLR superfamily, including IL-1R, TLR2, TLR4, and TLR9, with the proportional role of the individual receptors varying depending on fungal species, fungal morphotypes, and route of infection; 3) individual TLRs and IL-1R activate specialized antifungal effector functions on neutrophils, which correlates with susceptibility to infection; and 4) MyD88-dependent signaling on dendritic cells is crucial for priming antifungal Th1 responses. Thus, the finding that the innate and adaptive immunities to *C. albicans* and *A. fumigatus* require the coordinated action of distinct members of the IL-1R/TLR superfamily acting through MyD88 makes TLR manipulation amenable to the induction of host resistance to fungi. *The Journal of Immunology*, 2004, 172: 3059–3069.

Innate and acquired immunities are both essentially required for the development of optimal resistance to pathogenic fungi (1). The host immune system is a major determinant of which particular form of disease will develop after exposure to the ubiquitous *Aspergillus fumigatus* or whether transition from saprophytism to infection will occur with the human commensal *Candida albicans* (2–4). These fungi are sensed by the innate immune system in a morphotype-specific fashion; that is, different effector mechanisms of immunity are elicited by the different fungal morphotypes (5–7). Dendritic cells (DCs)[3] showed a remarkable functional plasticity in response to the different forms of fungi, being able to discriminate between the different forms in terms of maturation, cytokine production, and induction of Th cell reactivity in vitro and in vivo (5, 6, 8, 9). Different receptors on DCs were found to participate in the fungal recognition event, either independently or through receptor cooperativity (4, 5, 6, 10).

Toll-like receptors (TLRs) are a family of conserved, mammalian cellular receptors that mediate cellular responses to structurally conserved pathogen-associated microbial products (11, 12). All TLRs activate a core set of stereotyped responses, such as inflammation (13). However, individual TLRs can also induce specific programs in cells of the innate immune system that are tailored for the particular pathogen (12–14). TLRs bear homology to the IL-1R type 1 (IL-1RI) and share a similar signaling cascade culminating in activation of NF-κB and mitogen-activated protein kinases (15, 16). This process facilitates the transcription of genes regulating the inflammatory and adaptive immune responses. The common signal pathways used by IL-1RI and TLRs involve recruitment of the adapter protein myeloid differentiation primary response gene 88 (MyD88) through the homophilic interaction of the Toll/IL-1R homology domain (17). MyD88, in turn, activates a series of IL-1R-associated kinases that are crucially involved in innate immunity. MyD88-deficient mice do not make TNF-α in response to activators of TLR2, TLR3, TLR4, or TLR9, and IL-1R-associated kinase-deficient mice are impaired in their response to LPS and IL-1 (18). However, in the case of TLR4-dependent LPS signaling, another protein (Mal/TIRAP) may also serve as an adapter molecule with or in place of MyD88 (19, 20). It is recognized that the intricacies of how TLRs signal will ultimately provide an explanation for the molecular basis of how cells involved in innate immunity dictate the processes of host defense specific to the provoking pathogen (14).

In the case of fungi, in vitro studies have shown that *Cryptococcus neoformans*, *C. albicans*, and *A. fumigatus* may signal through TLRs, particularly TLR2, TLR4, and TLR9, in a morphotype-specific fashion (21–28). This will not come as a surprise, given the initial discovery of the Toll pathway in *Drosophila* as an essential mechanism of antifungal resistance (29). However, because of the complexity of TLR activation and function, including TLR cooperativity (30), the need for coreceptor (24), the sharing of common signal transduction pathways (31), and the dynamic

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[3] Abbreviations used in this paper: DC, dendritic cell; i.g., intragastrically; IL-1RI, IL-1R type 1; i.n., intranasally; LTA, lipotheicoic acid; MLN, mesenteric lymph node; MyD88, myeloid differentiation primary response gene 88; PMN, polymorphonuclear neutrophil; ROI, reactive oxygen intermediate; SOD, superoxide dismutase; TLN, thoracic lymph node; TLR, Toll-like receptor; Zym, zymosan.

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regulation of TLR expression in vivo (32), the in vitro studies may not be sufficient to predict the impact of the total repertoire of TLRs on infections in vivo. In addition, the use of inactivated fungi in the in vitro assays cannot anticipate the impact on TLR functioning of fungal morphogenesis, which is known to be activated in vivo by a wide range of tissue-specific signals (33) and to affect host responses (4, 34).

In the present study we have taken a comprehensive approach to define the role of the IL-1R/TLR/MyD88-dependent signaling pathway in response to *C. albicans* and *A. fumigatus*. We evaluate the course and outcome of the infections together with parameters of innate and adaptive antifungal immunity in IL-1RI-, TLR2-, TLR4-, TLR9-, and MyD88-deficient mice with experimental models of infection in which the contributions of innate and adaptive immunities are well established (35). We found that the IL-1R/TLR/MyD88 system plays a strategic role at the interface between the host and fungi, having an impact on the generation of both host antifungal immunity and fungal infectivity.

Materials and Methods

Animals

Female, 8- to 10-wk-old C57BL6 mice were obtained from Charles River Breeding Laboratories (Calco, Italy) or The Jackson Laboratory (Bar Harbor, ME). Breeding pairs of homozygous TLR2- (36), TLR4- (37), TLR9- (38), IL-1RI- (39), and MyD88- (40) deficient mice, raised on the C57BL6 background, were bred under specific pathogen-free conditions at the breeding facilities of University of Perugia (Perugia, Italy) or Boston University Medical Center. All in vivo studies were performed in compliance with national, Perugia University, and Boston University animal care and use committee guidelines.

Microorganisms, culture conditions, and infections

Isogenic strains of *C. albicans*, obtained by mutagenesis in vitro and capable (Vir^{-}13), or not (Vir^{-}3), of yeast-to-hyphal transition, as assessed by germ-tube formation in vitro, were used (41). The *A. fumigatus* strain was obtained from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of University of Perugia (41). Yeasts were harvested at the end of the exponential phase of growth and resuspended in IMDM (Life Technologies Italia, Milan, Italy) containing 5 μg/ml polymyxin B (Sigma-Aldrich, St. Louis, MO) and 50 μg/ml gentamicin. Conidia were harvested by extensive washing of the slant culture (on Sabouraud dextrose agar (Difco, Detroit, MI) supplemented with chloramphenicol for 4 days at room temperature) with 5 ml of 0.025% Tween 20 in saline. For generation of hyphae, *Candida* cells were allowed to germinate by culture at 37°C in 5% CO_2 for 2 h in RPMI 1640 medium (by that time, >98% of cells had germinated). For *Aspergillus* infection, conidia were given intranasally (i.n.) for 3 consecutive days (2×10^7/20 μl saline/each injection) as previously described (6). Mice were anesthetized by i.p. injection of 2.5% avertin (Sigma-Aldrich). For primary disseminated candidiasis, mice were injected i.v. with 10^6/0.5 ml of *C. albicans* yeasts or hyphae; for reinfection, mice surviving the primary infection were injected i.v. with 10^6/0.5 ml of *C. albicans* hyphae 14 days later (41). For the gastrointestinal infection, 10^8 *Candida* hyphae were injected intragastrically (i.g.), via an 18-gauge, 4-cm-long plastic catheter in a volume of 0.2 ml saline/mouse as previously described (35). Cyclophosphamide (Sigma-Aldrich) was given at 150 mg/kg i.p. 1 day before challenge. Quantification of fungal growth in the organs of infected mice was performed by plating serial dilutions of homogenized organs in Sabouraud dextrose agar, and results (mean ± SE) were expressed as CFU per organ.

Cell purification

Polymorphonuclear neutrophils (PMNs) were isolated from the blood and peritoneal cavity of mice as previously described (35). Briefly, peritoneal PMNs were obtained 8 h after the i.p. injection of 1 ml of endotoxin-free 10% thioglycolate solution (Difco). Endotoxin was depleted from all solutions with Detoxi-gel (Pierce, Rockford, IL). To purify Gr-1$^+$ PMN, 10^7 cells were incubated with biotin-conjugated anti-mouse Gr-1 mAbs (clone RB6-8C5; BD PharMingen, San Diego, CA) for 30 min at 4°C and then with avidin-conjugated magnetic MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 15 min at 6°C, and magnetically separated with a positive selection column (Miltenyi Biotech) according to the manufacturer's instructions. On FACS analysis, Gr-1$^+$ PMN were >98% pure and stained positively for the CD11b myeloid marker. Cytospin analysis confirmed that the population consisted of polymorphonuclear cells (>98%). Murine CD11c$^+$ DCs were isolated from spleens and lungs, and CD4$^+$ T cells were isolated from mesenteric lymph nodes (MLN), thoracic lymph nodes (TLN), and spleens by magnetic cell sorting as previously described (5, 6, 8, 9).

Phagocytosis, cultures, and antifungal effector activity

For phagocytosis, peritoneal neutrophils from infected mice were incubated at 37°C with unopsonized *Candida* yeasts or *Aspergillus* conidia for 30 and 60 min, respectively, at an effector to fungal cells ratio of 1:3. After incubation, phagocytic cells were separated from nonphagocytosed fungal cells by centrifugation on an FBS gradient, and a 0.1-ml sample of the harvested phagocytic cells was used for cytospin preparation. The percentage of internalization was calculated on Giemsa-stained preparations as previously described (5, 6). For staining of degranulated PMNs, the cytospin preparations were subjected to methylene blue (for azurophil granules) or eosin (for nonazurophil granules) staining following standard procedures. For myeloperoxidase detection, the liquid diaminobenzidene substrate Pack Kit (BioGenex, Menarini Diagnostics, Florence, Italy) was used according to the manufacturer's instructions on ematoxilin-counterstained preparations. For the fungicidal activity against *Candida* yeasts or *Aspergillus* conidia, PMNs were incubated with unopsonized fungal cells (at an effector to fungal cell ratio of 1:5) for 60 min (for yeasts) or 120 min (for conidia) at 37°C. Triton X was then added to the wells, and serial dilutions from each well were made in distilled water. Pour plates (four to six replicate samples) were made by spreading each sample on Sabouraud glucose agar. The number of CFUs and the percentage of CFU inhibition (mean ± SE), referred to as candidacidal or conidiocidal activity, were determined as previously described (5, 25). For the fungicidal activity against hyphae, a colorimetric MTT assay was used as previously described (42, 43). Briefly, graded numbers of PMNs (10^6, 5×10^5, and 10^5), either unexposed or pre-exposed for 2 h at 37°C to 10 ng/ml rTNF-α (R&D Systems, Space Import-Export, Milan, Italy), were added to hyphae obtained from 10^5 yeasts in 96-well, flat-bottom microtiter plates. After 2 h at 37°C with occasional shaking (by that time, >98% of cells had germinated), the supernatants were aspirated, effector cells were lysed by adding sodium deoxycholate (0.5%), and hyphal viability was determined by MTT staining. Antifungal activity was calculated according to the following formula: percentage of hyphal damage $[(1 - x)/C] \times 100$, where x represents the OD of test wells, and C represents the OD of control wells with hyphae only. Each set of conditions was tested in duplicate, and the results were averaged. PMN production of reactive oxygen intermediates (ROI) was performed by quantifying the superoxide anion (O_2^-) production by measuring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* as previously described (44). Peripheral PMNs (2×10^6) were incubated in the presence of 80 μM cytochrome *c* with 2×10^7 unopsonized yeasts or conidia for 60 or 120 min, respectively, at 37°C. A_{550} was measured in the culture supernatants in a Microplate Reader 550 (Bio-Rad, Rome, Italy), and the background absorbance in control tubes containing only buffer and cytochrome *c* was subtracted. Experiments were performed in triplicate, and the results were expressed as nanomoles of O_2^-. The effects of TLR stimulation on fungicidal activity, respiratory burst, and degranulation were assessed by preincubating PMNs for 120 min with the different TLR ligands (zymosan (10 μg/ml) and lipoteichoic acid (LTA; 1 μg/ml) from *Staphylococcus aureus* and LPS (10 μg/ml) from *Salmonella minnesota* Re 595 (all from Sigma-Aldrich) and unmethylated CpG oligonucleotide 1826 (2 μM) of proven immunostimulatory sequence (25)) before the addition of unopsonized yeasts or resting conidia. Photographs were taken using a high resolution microscopy color camera (AxioCam Color), using the AxioVision software Rel. 3.0 (Carl Zeiss, Milan, Italy). For cytokine determination, purified DCs were resuspended in IMDM with no serum, but with polymixin B, to avoid nonspecific activation by serum components and endotoxin and were pulsed with unopsonized *Candida* yeasts and hyphae or *Aspergillus* resting conidia for 2 h before the addition of amphotericin B to prevent fungal overgrowth as previously described (5, 6). Cytokine measurement was performed after an additional 22 h of coculture. The levels of cytokine production by DCs added with amphotericin B alone were below the detection limits of the assays (data not shown).

Proliferation assay by flow cytometric analysis

Proliferation of 1×10^6 splenic CD4$^+$ T lymphocytes stimulated with 5×10^5 Ag-pulsed splenic DCs for 5 days at 37°C was assessed by labeling with CFSE (Molecular Probes, Eugene, OR) as previously described (9).

Cytokine and ELISPOT assays

The levels of cytokines in culture supernatants, lung, stomach, and kidney homogenates from infected mice were determined by ELISA kits (R&D Systems). The detection limits of the assays were <32 pg/ml for TNF-α and <15 pg/ml for IL-10 and IL-12 p70. For enumeration of cytokine-producing $CD4^+$ T cells, an ELISPOT assay was used on purified $CD4^+$ T cells as previously described (5, 6, 9). Results are expressed as the mean number of cytokine-producing cells (±SE) per 10^5 cells, calculated using replicates of serial 2-fold dilutions of cells.

Statistical analysis

The log-rank test was used for paired data analysis of the Kaplan-Meier survival curves. Student's *t* test or ANOVA and Bonferroni's test were used to determine the statistical significance of differences in organ clearance and in vitro assays, as indicated in the figure legends Significance was defined as $p < 0.05$. In vivo groups consisted of four to six animals. The data reported were pooled from three to five experiments unless otherwise specified.

Results

Impact of the IL-1R/TLR/MyD88-dependent signaling pathway to susceptibility to candidiasis and aspergillosis

To assess the role of the IL-1R/TLR/MyD88-dependent pathway in the course and outcome of candidiasis and aspergillosis, TLR2-, TLR4-, TLR9-, IL-1RI-, and MyD88-deficient mice were infected with *Candida* yeasts or hyphae i.v., *Candida* hyphae i.g., or *Aspergillus* conidia i.n. Mice were then assessed for survival and fungal growth in the kidneys, stomach, and lungs, respectively. Similar to wild-type mice, TLR2-, TLR4-, and TLR9-deficient mice survived the infection with low virulence *Candida* yeasts, in contrast to IL-1RI- and MyD88-deficient mice, which succumbed to it (Fig. 1*A*). Susceptibility to virulent *Candida* hyphae given i.v. was not different among the different types of mice, in that all succumbed to the infection, although TLR4- and TLR9-deficient mice survived significantly longer (Fig. 1*B*). For aspergillosis, although each type of intact mouse survived the infection (data not shown), upon immunosuppression with cyclophosphamide there were important differences among strains, as TLR-4- and MyD88-deficient mice died sooner, and IL-1RI-deficient mice died later than the other strains (Fig. 1*C*). We also assessed the susceptibility to reinfection with *Candida* hyphae of mice surviving the primary infection with *Candida* yeasts and found that TLR-9-deficient and wild-type mice survived reinfection, whereas the majority of the TLR2- and TLR4-deficient mice succumbed (Fig. 1*D*). Resistance to reinfection of intact mice surviving the *Aspergillus* primary infection was also reduced in TLR2-deficient mice (data not shown).

The assessment of the actual fungal growth in the relevant target organs correlated with the pattern of survival. The fungal burden was significantly higher in IL-1R1- and MyD88-deficient mice highly susceptible to *Candida* yeasts compared with the other types of mice, although it was significantly lower in resistant TLR-9- and TLR-2-deficient mice (Fig. 2*A*). Fungal growth in the kidneys of mice infected with *Candida* hyphae i.v. was not different among the different types of mice, except for the significant reduction in TLR9- and TLR2-deficient mice (Fig. 2*B*); however, it was different in the stomach after i.g. infection, as shown in Fig. 2*C*. The fungal burden was significantly lower in resistant TLR9-deficient mice and was significantly higher in TLR2- and TLR4-deficient mice, particularly in IL-1RI- and MyD88-deficient mice. No signs of dissemination to visceral organs, such as esophagus, small intestine, liver, and kidneys, were observed. Moreover, the fungal burden continued to be elevated 2 wk after the infection, but started to decline at 4 wk, and all mice eventually survived the infection (data not shown). For *Aspergillus*, the growth of the fungus was significantly lower in the lungs of TLR9- and IL-1RI-deficient mice, but was higher in TLR2-, TLR4-, and MyD88-deficient mice compared with wild-type mice (Fig. 2*D*). Histological analysis of infected organs revealed the presence of numerous fungal elements in the relative absence of signs of inflammatory pathology in IL-1RI-, TLR4-, and MyD88-deficient mice (data not shown). Together, these results show that the expression of host resistance to fungi may occur through common as well as divergent patterns of TLR activation depending on fungal species and morphotype as well as the route of the infection. IL-1RI and TLR4 were required for resistance to the primary infection with *Candida* and

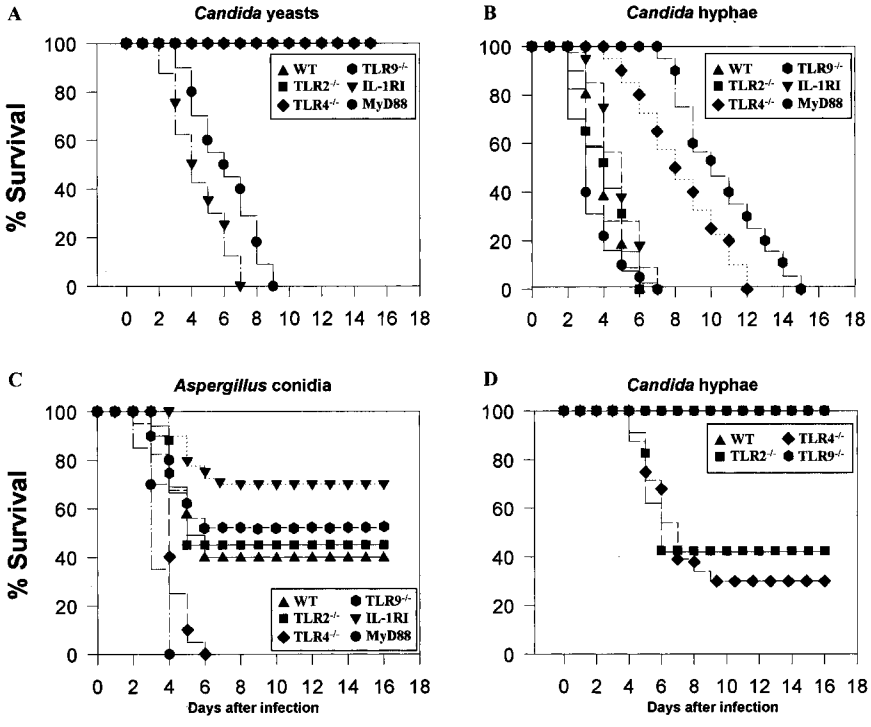

FIGURE 1. Survival of TLR2-, TLR4-, TLR9-, IL1-RI-, and MyD88-deficient and wild-type mice after infection with candidiasis or aspergillosis. Mice (a total of 20/experimental group) were injected i.v. with *Candida* yeasts (*A*) or *Candida* hyphae (*B*), or i.n. with *Aspergillus* conidia (*C*). Mice surviving the primary infection with yeasts were reinfected i.v. with *Candida* hyphae 2 wk later (*D*). *C*, Mice were immunosuppressed with 150 mg/kg i.p. cyclophosphamide 1 day before the infection. The median survival times of TLR4- and TLR9-deficient mice (*B*) and that of IL-1RI-deficient mice (*C*) were significantly different (*, $p < 0.05$, by the log-rank test) from that of wild-type mice.

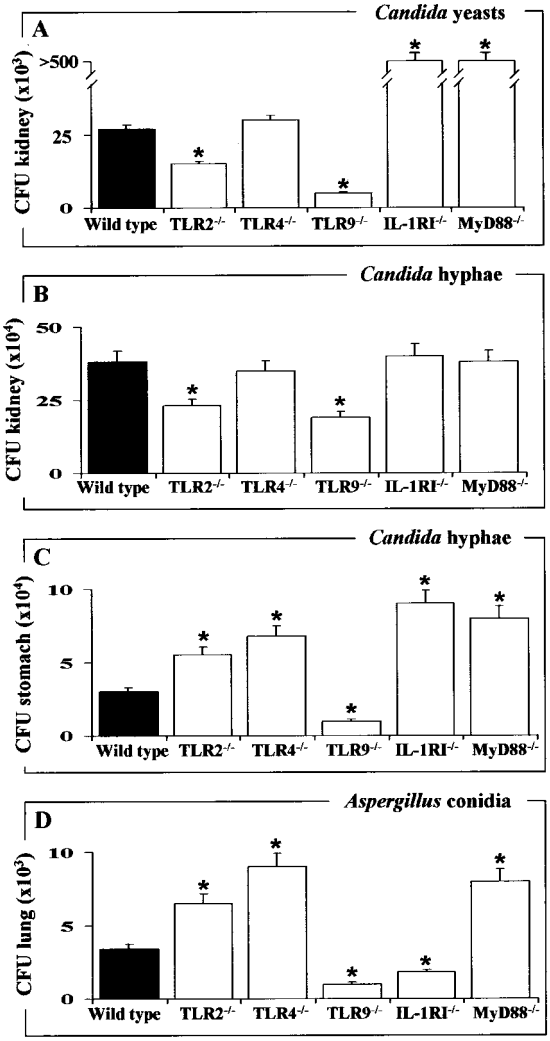

FIGURE 2. Fungal growth in TLR2-, TLR4-, TLR9-, IL1-RI-, and MyD88-deficient and wild-type mice with candidiasis or aspergillosis. Intact mice were injected with *Candida* yeasts i.v. (*A*), with *Candida* hyphae i.v. (*B*) or i.g. (*C*), and with *Aspergillus* conidia i.n. (*D*). Fungal growth in the relevant target organs was assessed 3 days (IL-1RI- and MyD88-deficeint mice) or 6 days (TLR2-, TLR4-, and TLR9-deficient and wild-type mice) after the infection in *A*, 3 days after the infection in *B*, 1 wk after the infection in *C*, and 3 days after the last conidia inoculation in *D*. Quantification of fungal growth in the organs (mean ± SE) is expressed as CFU per organ. ∗, $p < 0.05$, mutant vs wild-type mice (by ANOVA and Bonferroni's test).

Aspergillus, respectively, and the MyD88-dependent pathway was required for resistance to both. TLR2 and TLR4 were required for resistance to reinfection; the requirement of TLR4 for resistance to *Candida* was dependent on the type of infection (mucosal vs disseminated candidiasis). Finally, TLR9 was not required for resistance to either fungus. In addition, the ability of the mutant mice to survive the *Aspergillus* infection if intact suggests that resistance to the fungus may also occur through compensatory TLR-independent mechanisms.

Impact of the IL-1R/TLR/MyD88-dependent signaling pathway on the innate antifungal response

To correlate the above findings with patterns of innate antifungal resistance, we measured the levels of TNF-α production early in the course of infection in kidneys and lungs of mice with candidiasis and aspergillosis, respectively, because this cytokine is required for resistance to both fungi (35) and is prototypically released through TLR-dependent pathways (12, 13, 17). We also assessed the antifungal effector activities of PMNs, as they are essential components of the innate resistance to fungi (45, 46). The levels of TNF-α were particularly elevated in TLR2-deficient mice, similar to control mice in TLR9-deficient mice, and were ablated in MyD88-deficient mice infected with either fungus (Fig. 3). However, fungal species-specific differences did emerge, as TNF-α levels were lower in TLR4- and IL-1RI-deficient mice infected with *Candida*, but not *Aspergillus*. Therefore, TNF-α production was not predictive of resistance or susceptibility to infections, as neither was associated with a specific TLR repertoire. However, TNF-α production was crucially dependent on the MyD88 pathway.

To evaluate PMNs, we assessed their ability to phagocytose and kill fungi, the expression of TLR upon exposure to fungi, and the functional responses to known TLR ligands. As the recruitment of PMNs at the sites of the infection, i.e., the kidneys, stomach, and lungs, was slightly decreased in TLR2- and TLR4-deficient mice only (as judged by cytospin analysis; data not shown), we assessed PMNs from infected and uninfected mice for functional antifungal activity, such as the ability to internalize and kill both fungi. Similar to what we observed with PMNs from uninfected mice, differences were found in the ability to handle fungi by PMNs from the different types of mice upon infection. The fungicidal activity

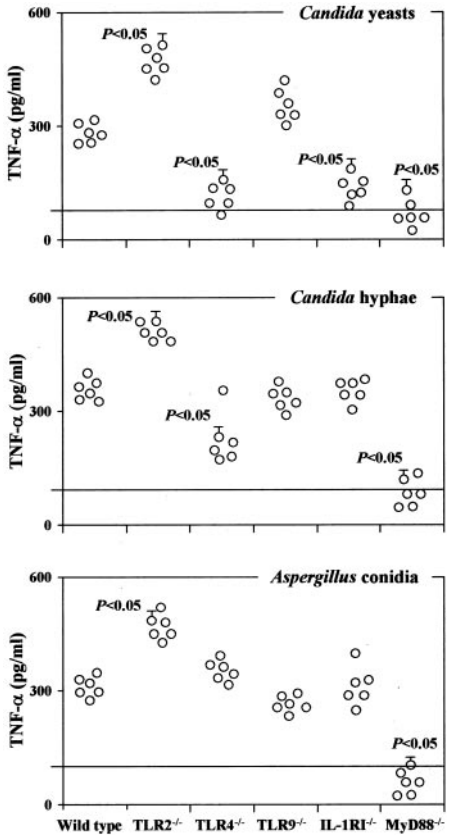

FIGURE 3. TNF-α production in TLR2-, TLR4-, TLR9-, IL1-RI-, and MyD88-deficient mice with candidiasis or aspergillosis. Intact mice were infected as described in Fig. 2, and the levels of TNF-α production were determined in the homogenates from kidneys (*Candida* i.v.), stomach (*Candida* i.g.), or lungs (*Aspergillus*) 3 and 7 days after i.v. or i.g. *Candida* infection, respectively, or 1 day after the last conidia inoculation. ∗, $p < 0.05$, mutant vs wild-type mice (by ANOVA and Bonferroni's test). TNF-α was measured by specific ELISA. The levels of the cytokine in uninfected mice were below the detection limit of the assay.

determined in vitro mirrored the in vivo susceptibility to the primary infections (Fig. 4*A*). Phagocytosis was slightly impaired, and killing activity was virtually abolished in PMNs from MyD88- and IL-1R1-deficient mice against *Candida* yeasts; in the case of *Aspergillus* conidia, phagocytosis and killing were impaired in MyD88-deficient and particularly in TLR4-deficient PMNs. In contrast, TLR2 and TLR9 deficiencies did not affect the ability to internalize and kill either fungus, except for the slight impairment of the conidiocidal activity observed in TLR2-deficient PMNs. In the case of *Candida* hyphae, the antifungal effector activity of PMNs from uninfected mice was significantly increased in TLR4- and TLR9-deficient mice and was impaired in MyD88- and IL-1RI-deficient mice. Because cytokine production during the infection may have an impact on the antifungal functions of PMNs, and TNF-α is known to selectively impair the activity of PMNs against *Candida* hyphae (43), the effector function of PMNs was also assessed in the presence of TNF-α. The ability of hyphal damage was greatly reduced by TNF-α in TLR4-deficient PMNs and was slightly reduced in PMNs from TLR9-deficient or wild-type mice. No effect was observed on PMNs from the other types of mice, including MyD88-deficient mice which, similar to TLR4-deficient mice, showed defective TNF-α production during the infection (Fig. 4*A*). Therefore, additional mechanisms, besides TNF-α production, might regulate the expression of the antifungal effector functions of PMNs at the site of infection.

To correlate these findings with the possible functional activity of TLRs on PMNs, purified PMNs from uninfected wild-type mice were exposed to *Candida* yeasts and hyphae or *Aspergillus* conidia and hyphae and were assessed for TLR2, TLR4, and TLR9 expression by RT-PCR. In addition, as the ability of PMNs to kill fungi occurs through both oxidative and nonoxidative mechanisms (47), we also assessed the antifungal effector activity of PMNs upon exposure to the relevant TLR ligands in terms of ROI production and degranulation. We found that the expression of both TLR2 and TLR9 was induced upon exposure to viable unopsonized yeasts or conidia, but not to hyphae; however, TLR4 expression was induced upon exposure to each fungal morphotype (data not shown). In terms of the functional consequences of stimulation of the relevant TLRs on PMNs, CpG did not significantly modify the antifungal effector activity against both yeasts and conidia; LTA completely inhibited the fungicidal activity of PMNs, whereas LPS and zymosan increased it (Fig. 4*B*). Activation of the respiratory burst leading to the generation of ROI was observed in response to yeasts and, to a lesser extent, in response to resting conidia. Similarly, degranulation, as observed upon eosin staining of nonazurophil granules (pink staining) or methylene blue staining of azurophil granules (blue staining), was minimal upon stimulation with resting conidia compared with that of yeasts. Stimulation with zymosan and LTA maximally stimulated the production of ROI against both fungi, but LPS and CpG did not (Fig. 4*C*). In contrast, LPS induced the extracellular secretion of azurophil granules in response to both fungi to an extent superior to that in wild-type mice, either unstimulated or stimulated with zymosan, LTA, or CpG. Interestingly, PMN stimulated with LPS also stained positively with myeloperoxidase (Fig. 4*C*, *inset*). Together, these data indicate that the expression of the innate immune response to fungi occurs through the involvement of distinct members of the IL-1R1/TLR superfamily, each probably activating specialized antifungal effector functions on PMNs and implicating the MyD88-dependent signaling pathway. Furthermore, they confirm that oxidant generation by PMNs may not always be sufficient to mediate fungal killing without complimentary nonoxidative mechanisms, as previously suggested (46–48).

Impact of the IL-1R/TLR/MyD88-dependent signaling pathway on antifungal adaptive immunity

To define the impact of the IL-1R/TLR/MyD88-dependent signaling pathway on the quality of the adaptive immunity to fungi, we determined the frequencies of IFN-γ- or IL-4-producing $CD4^+$T lymphocytes in uninfected mice or mice with gastrointestinal candidiasis or pulmonary aspergillosis. To this purpose, mice were infected i.g. with *Candida* hyphae or i.n. with *Aspergillus* conidia, and the number of Th1 or Th2 cytokine-producing cells was assessed in the MLN and TLN, respectively, 1 wk after the infection. No differences were observed in the frequencies of cytokine-producing cells among the different types of uninfected mice. However, the number of cells producing IFN-γ was significantly reduced and that of cells producing IL-4 was significantly increased in MyD88-deficient mice with either infection. A similar pattern of Th1/Th2 reactivity was observed in TLR9-deficient mice despite their remarkable antifungal resistance. Th2 cells predominated over Th1 cells in IL-1R-deficient mice with candidiasis, but not with aspergillosis. Finally, evidence of activation of both Th1 and Th2 cells was found in TLR2- or TLR4-deficient mice with candidiasis and aspergillosis, as the number of IL-4-producing cells was increased, and the number of cells producing IFN-γ was either decreased, in candidiasis, or remained unchanged, in aspergillosis, compared with that in control mice (Fig. 5).

As DCs are instrumental in shaping the adaptive Th response to fungi (4, 9, 49), we assessed IL-12 p70/IL-10 production by purified DCs from each type of mouse in response to yeasts, conidia and hyphae. The ability to phagocytose either fungus was not significantly different between DCs from mutant mice and those from wild-type mice (data not shown); however, their abilities to produce cytokines were different. The production of IL-12 p70 was virtually ablated in DCs from MyD88-deficient mice against each fungal morphotype, and this was associated with a significantly increased production of IL-10. A similar cytokine pattern was observed with IL-1RI-deficient DCs in response to *Candida* yeasts and in TLR-4-deficient DCs in response to *Aspergillus* conidia. An opposing pattern was observed in TLR2- and TLR9-deficient mice. The production of IL-12 p70 was increased and that of IL-10 was decreased in TLR2-deficient DCs in response to yeasts and conidia, and the reverse was true with TLR9-deficient DCs. It is of interest that although the production of IL-12 p70 in response to hyphae was only reduced in MyD88-deficient DCs, the production of IL-10 was significantly increased in MyD88-deficient mice, but, interestingly, was significantly decreased in TLR2-, TLR4-, and TLR9-deficient DCs (Fig. 6*A*). Together these results suggest that the production of cytokines by DCs in response to fungal morphotypes is dependent on the type of receptor involved, even though signaling through MyD88 is essentially required for IL-12 p70, but, interestingly, not for IL-10 production. Criss-cross experiments in which the proliferative response of $CD4^+$ T cells from MyD88-deficient or wild-type uninfected mice was assessed in the presence of fungus-pulsed DCs from MyD88-deficient or wild-type mice confirmed the crucial role of MyD88-dependent signaling on DCs for antifungal Th1 priming. Lymphoproliferation was observed in cultures of lymphocytes with DCs from wild-type mice, but not from MyD88-deficient mice (Fig. 6*B*). However, the proliferative capacity of lymphocytes from wild-type mice was lost, and that of lymphocytes from MyD88-deficient mice was gained in the presence of DCs from mutant or wild-type mice, respectively (Fig. 6*B*). High levels of IFN-γ production and no IL-4 were detected in culture supernatants of proliferating cells (data not shown).

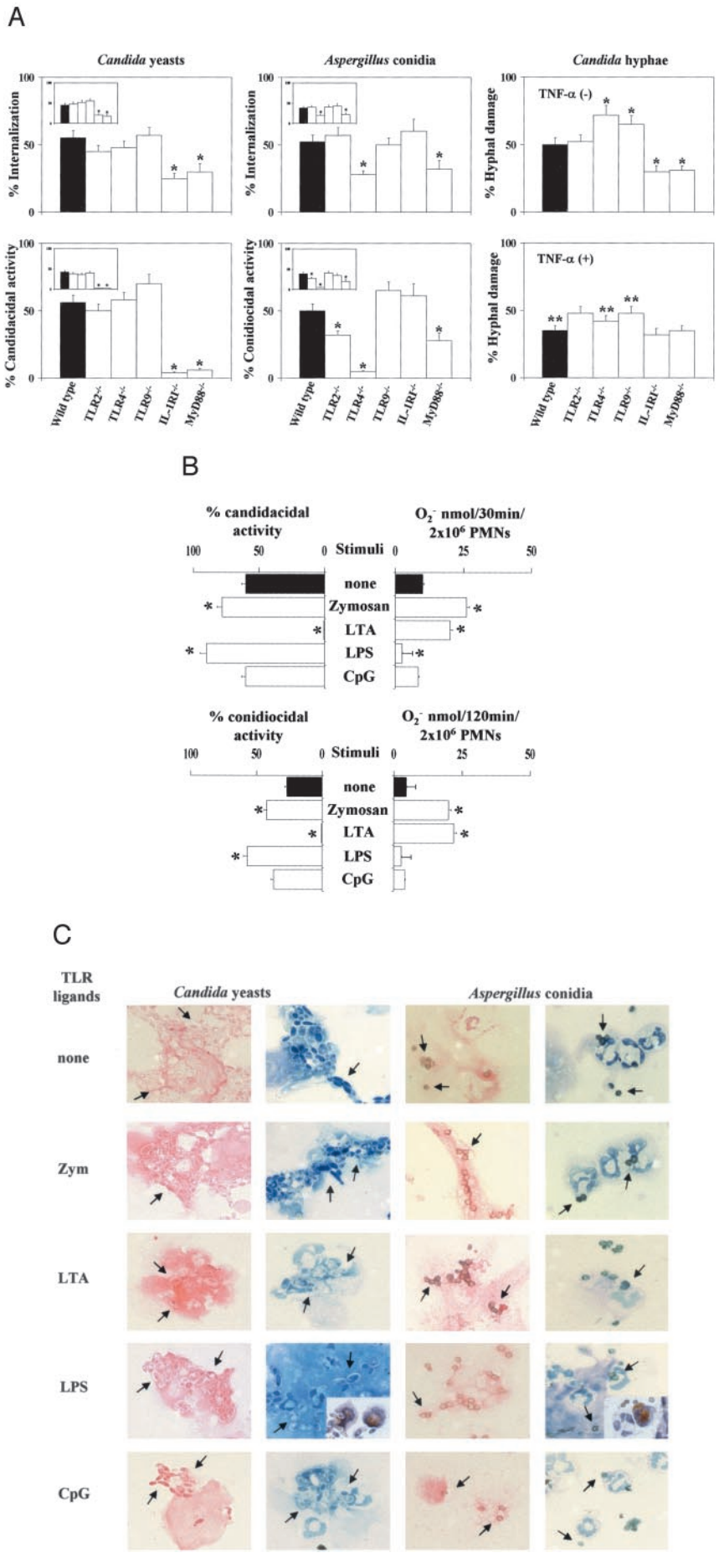

FIGURE 4. Antifungal activities of PMNs from TLR2-, TLR4-, TLR9-, IL1-RI-, and MyD88-deficient and wild-type mice. *A*, Purified PMNs from uninfected (*inset*) or infected mice (at the time of TNF-α assessment) were assessed for phagocytosis and fungicidal activity. For phagocytosis, after Diff-Quick staining, aliquots of cells were spun down on slides on a cytocentrifuge and examined for fungi internalization by light microscopy. All conditions were tested in triplicate. Values are the mean ± SE of samples taken from three experiments. Fungicidal activity was assessed as described in *Materials and Methods*. For hyphal damage activity, uninfected PMNs were either unexposed (−) or pre-exposed to 10 ng/ml TNF-α for 2 h at 37°C. $*$, $p < 0.05$, mutant vs wild-type mice; $**$, $p < 0.05$, TNF-α (−) vs TNF-α (+; by ANOVA and Bonferroni's test). *B*, Peripheral PMNs from uninfected wild-type mice were pretreated with zymosan (Zym; 10 μg/ml), LTA (1 μg/ml), LPS (10 μg/ml), and unmethylated CpG oligonucleotide (2 μM) for 120 min before exposure to unopsonized yeasts or resting conidia for an additional 60 or 120 min, respectively, before assessment of fungicidal activity, O_2^- production, and degranulation (*C*). O_2^- production was quantitated by measuring the SOD-inhibitable reduction of cytochrome *c*, as described in *Materials and Methods*. $*$, $p < 0.05$, TLR ligand stimulation vs no stimulation (none; by ANOVA and Bonferroni's test). *C*, PMN degranulation, as assessed by eosin (pink) and methylene blue (blue) staining. Arrows indicate fungal elements. In the *inset*, myeloperoxidase-positive cells are shown. Photographs were taken using a high resolution microscopy color camera (AxioCam Color).

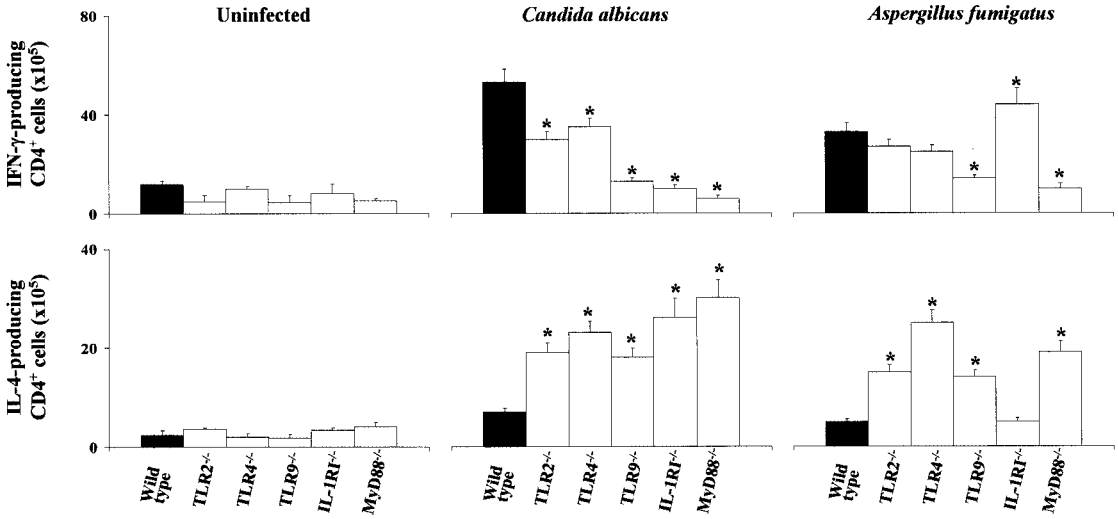

FIGURE 5. Frequencies of Th1/Th2 cells in TLR2-, TLR4-, TLR9-, IL1-RI-, and MyD88-deficient mice with candidiasis or aspergillosis. Intact mice were infected i.g. with *Candida* hyphae or i.n. with *Aspergillus* conidia. $CD4^+$ T cells were purified from the MLN or TLN of mice uninfected or infected with candidiasis or aspergillosis, respectively, 1 wk after the i.g. infection or 3 days after the last conidia inoculation. The frequency of cytokine-producing cells (mean ± SE per 10^5 cells) was calculated by ELISPOT assay. ∗, $p < 0.05$, mutant vs wild-type mice (by ANOVA and Bonferroni's test).

Discussion

This study describes the impact of the IL-1R/TLR/MyD88 system on the host response to *C. albicans* and *A. fumigatus* in vivo. Although in vitro studies have indicated the importance of TLR signaling in response to *Candida* and *Aspergillus* (21–28), the role of TLRs in the generation of innate and adaptive immunity to fungi remains largely undefined. We show in this study that 1) the MyD88-dependent pathway is essentially required for the innate and Th1-mediated resistance to either fungus; 2) the involvement of MyD88 may occur through signaling by distinct TLRs depending upon the fungal species, morphotypes, and site of infection; and 3) TLRs may contribute differently to the occurrence of innate and adaptive Th1 immunities to each fungus, consistent with the ability of each individual TLR to activate specialized antifungal effector functions on PMNs and DCs. However, fungi may also exploit TLRs for fungal growth and survival in vivo, as recently suggested (27).

In line with the idea of the essential role played by MyD88-dependent signaling in the development of Th1 responses (17), the susceptibility of MyD88-deficient mice to candidiasis and aspergillosis was associated with the occurrence of a defective antifungal Th1 response, probably due to defective DC activation. However, as the antifungal effector functions of PMNs were also impaired, MyD88-dependent signaling has a crucial role in the expression of optimal antifungal innate resistance. For *Aspergillus*, the fact that intact MyD88-deficient mice survived the infection suggests that resistance to the fungus may also occur through MyD88-independent mechanisms. Intriguingly, *Drosophila* MyD88, although only needed for antifungal defense, is unable to induce expression of the antifungal peptide drosomycin in the absence of other adapters (50). Preliminary data showing the ability of LPS to activate antifungal effector functions in MyD88-deficient mice support this hypothesis. However, because TLR4 is known to signal through both the MyD88-dependent and -independent pathways (14), but TLR4-deficient mice are not more susceptible to aspergillosis than MyD88-deficient mice, further studies are needed to elucidate the role of MyD88-independent pathways in resistance to *Aspergillus*. It is of interest that the conidiocidal activity, more than the phagocytic ability, was almost completely ablated in TLR4-deficient PMNs, a finding suggesting that internalization of the fungus, in the absence of TLR4 signaling, may contribute to virulence by preventing fungal killing and thus favoring fungal survival.

In candidiasis, in line with the increased susceptibility to the infection seen in TLR4-defective C3H/HeJ mice (23), our study shows that TLR4-deficient mice mount a defective Th1-protective immunity to fungus in the face of an efficient innate antifungal resistance, particularly against hyphae. However, a more crucial role is played by IL-1R1, as both the innate and acquired Th1 resistances to *Candida* infections were severely impaired in its absence. The finding that, similar to MyD88-deficient mice, IL-1R-deficient mice were highly susceptible to low virulence *Candida* yeasts qualifies the IL-1RI/MyD88-dependent pathway as a major host determinant of fungal virulence in vivo. The killing ability of PMNs against *Candida* was also severely impaired in IL-1R-deficient mice, a finding consistent with the importance of IL-1-dependent (51), but more importantly IL-18-dependent (52), signaling in the activation of PMNs to an antifungal state. However, although anticandidal resistance was decreased in conditions of IL-1 (53) and/or IL-18 (54) deficiencies, neither deficiency was as severe as that of IL-1R1-deficient mice in terms of susceptibility to the infection.

TLR2 signaling by zymosan (55), β-glucan (56), *Candida* phospholipomannan (26), *Aspergillus* conidia (24, 27), and hyphae (27, 28) led to the production of both inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines depending on the cell type. Unopsonized zymosan also stimulated the respiratory burst of phagocytes, although opsonization greatly increased this capacity (57). However, the susceptibility of TLR2-deficient mice to primary candidiasis and aspergillosis was not different from that of control mice, a finding suggesting that the mice are fully competent at the level of innate antifungal resistance, as documented by reduced fungal growth in mice with primary disseminated candidiasis. In contrast, they failed to mount protective Th1 resistance, as evidenced by the high susceptibility to mucosal infection and reinfection, both known to be controlled by Th1 adaptive immunity (35). Both Th1 and Th2 lymphocytes were indeed activated in these mice despite the high level production of IL-12 p70 by DCs.

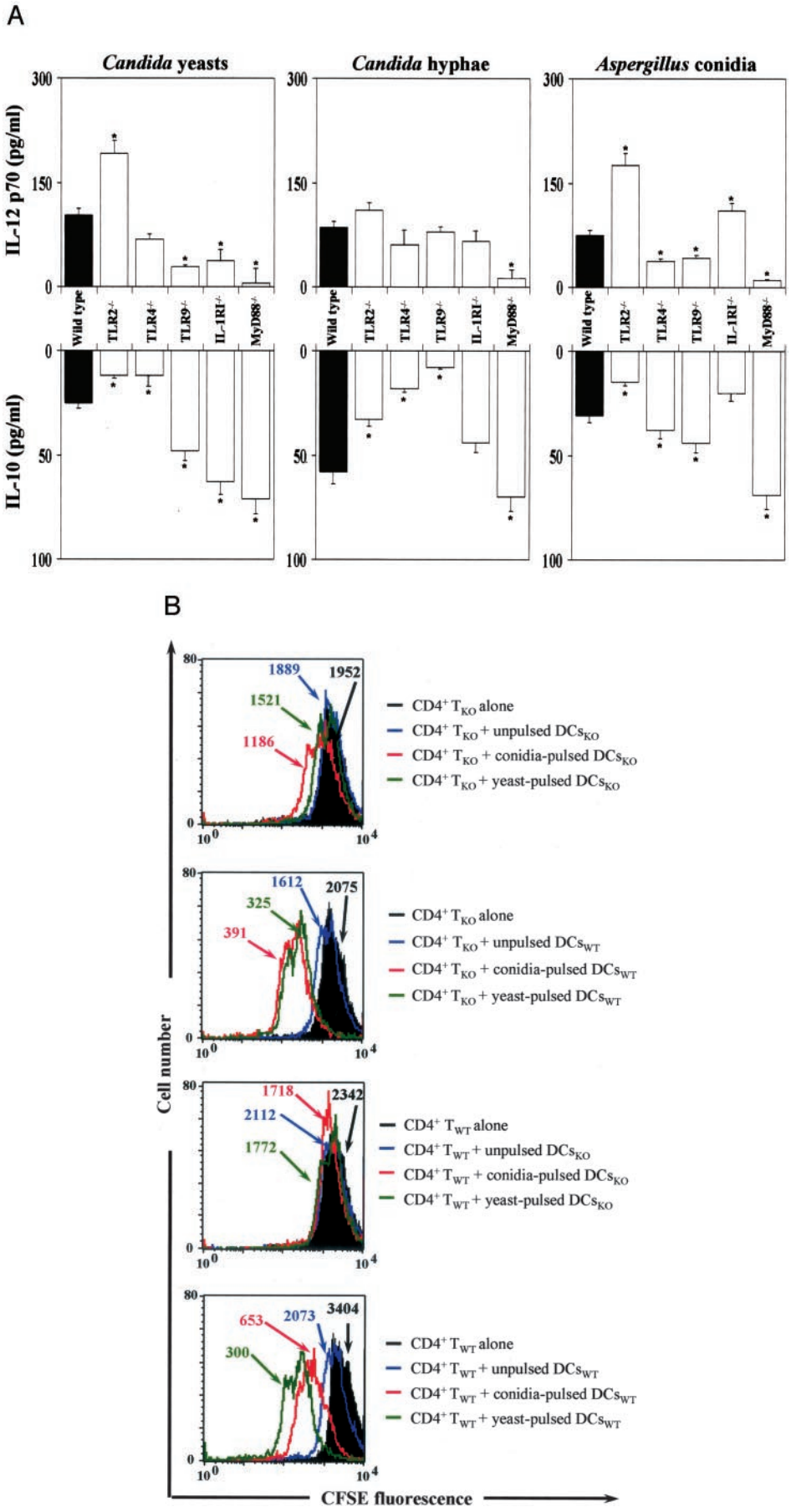

FIGURE 6. Functional activity of DCs from TLR2-, TLR4-, TLR9-, IL1-RI-, and MyD88-deficient mice. *A*, Purified DCs from spleens or lungs were pulsed with unopsonized *Candida* yeasts and hyphae or *Aspergillus* conidia, respectively, for 2 h before the addition of amphotericin B to prevent fungal overgrowth. Cytokine measurement was performed after an additional 22 h of coculture. $*$, $p < 0.05$, mutant vs wild-type mice (by ANOVA and Bonferroni's test). *B*, DCs and $CD4^+$ T cells were purified from the spleens of wild-type (WT) and MyD88-deficient (KO) mice. Yeast- or conidia-pulsed splenic DCs were then cocultured with purified splenic $CD4^+$ T cells for 5 days at 37°C before assessment of lymphoproliferation by CFSE labeling. The numbers refer to the median fluorescence intensity.

The interesting finding was that IL-10 production was greatly reduced in DCs from these mice in response to fungi. Because IL-10-producing DCs are required for the expression of long-lasting memory antifungal immunity through the induction of regulatory T cells (58), it is possible that the Th1 hyporesponsiveness of TLR2-deficient mice is a consequence of the decreased IL-10 production by DCs. Whatever the mechanism is, our finding is consistent with the defective response to vaccination with *Borrelia burgdorferi* OspA seen in TLR2-deficient mice (36) as well as with the TLR2 dependency of IL-10 production in response to *Aspergillus* hyphae (27) .

The findings with TLR9-deficient mice are interesting, although largely unexplained. As TLR9 stimulation by CpG enhances innate effector (59) and Th1 responses in experimental models of infection (60), including aspergillosis (25), one would reasonably expect decreased resistance to fungi. We found that TLR9-deficient mice were incapable of mounting an Ag-specific Th1 response, yet they were highly resistant to both mucosal candidiasis and reinfection. TLR9-deficient mice were particularly efficient in restricting fungal growth upon primary infection, particularly with *Candida* hyphae, against which they showed potent antifungal effector activity.

Consistent with their role in immune surveillance, TLRs are expressed at higher levels in tissues exposed to the external environment, such as lung and gastrointestinal tract (32). TLRs have also been shown to be expressed on human peripheral blood PMNs (61) and to mediate antimicrobial responses (59, 62). PMNs can exert antifungal activity by both oxidative and nonoxidative mechanisms (63). The former is characterized by the respiratory burst resulting in the generation of oxidants with potent antimicrobial activity. A multitude of mediators with nonoxidative antifungal activity has been also found in human PMN granules, with the primary (azurophil) granules containing, among others, myeloperoxidase and defensins (64). The quantity and specificity of delivery of these toxic neutrophil products ultimately will determine the relative efficiency of fungicidal activity vs inflammatory cytotoxicity to host cells.

It is well known that the various fungal forms not only elicit differing responses from PMNs and phagocytes, but also differ in their susceptibilities to cellular microbicidal mechanisms (47). We found in this study that murine PMN activation by the different fungal forms occurs through TLR signaling that affected the release of both granule constituents and oxidants more than phagocytosis. This is consistent with previous findings demonstrating roles for zymosan (57), LPS (65), CpG (59), and p38 mitogen-activated kinases (66) in regulating a variety of neutrophil functions, including the production of ROI and degranulation. Consistent with the expression of TLR2, TLR4, and TLR9 on PMNs upon exposure to fungi, zymosan, LPS, and, in part, CpG stimulated the fungicidal activity of PMNs. However, different mechanisms of antifungal activity were elicited. The production of ROI was higher in response to zymosan than LPS or CpG, but degranulation was maximally induced by LPS and less so with zymosan or CpG. Interestingly, azurophilic granules with myeloperoxidase activity were mobilized by LPS more than by zymosan or CpG. Experiments with *Candida* and *Aspergillus* hyphae have suggested that degranulation, more than ROI production, correlated with the maximum antifungal effector function of PMNs, an activity that occurs differently in the different types of mice and in response to the different TLR ligands (S. Bellocchio, C. Montegnoli, S. Bozzo, G. Rossi, and L. Romoni, manuscript in preparation). Together these findings, although highlighting the complexity and multitude of mechanisms underlying the different susceptibilities of TLR-deficient mice to the primary infections with each fungal morphotype, also suggest that different TLRs activate specialized antifungal effector functions on PMNs and support the idea that different PMN functions are mediated by divergent transduction pathways (63). Interestingly, stimulation with LTA abrogated the fungicidal activity of PMNs, and this was associated with stimulation of ROI production, but not with degranulation. This finding, although confirming that ROI stimulation without the concomitant activation of granule proteases is not sufficient to mediate antifungal activity (47, 48), also unmasks the effect of TLR cooperativity at the level of PMN activation.

In line with the idea that TLR2 ligands are recognized by heterodimers formed between TLR2 and other TLRs (30), host recognition of different configurations of lipoprotein/lipopeptides occurs through the heterodimeric association of TLR2 with either TLR1 or TLR6 (67). In this regard it is of interest that the capacity of PMNs to kill *C. albicans* was greatly reduced after exposure to medium, but not long, chain fatty acid-containing lipid emulsions in vitro (68) and in vivo (69). As similar results, in terms of TLR expression and functioning, have been obtained with human PMNs (S. Bellocchio, C. Montegnoli, S. Bozzo, G. Rossi, and L. Romoni, manuscript in preparation), together these data indicate that the expression of the innate response to fungi occurs through the involvement of distinct TLRs, each probably activating specialized antifungal effector functions on PMNs and implicating the MyD88-dependent signaling pathway. Importantly, the finding that TLRs affect the balance between fungicidal oxidative and nonoxidative mechanisms of PMNs suggests that TLR manipulation in vivo may be amenable to the induction of optimal microbicidal activity in the absence of inflammatory cytotoxicity to host cells.

It is still unclear whether pathogen-specific Th2 responses develop by default in the absence of the TLR/MyD88-dependent signal or whether a specialized class of recognition receptors activates Th2 responses upon recognition of Th2-specific pathogens (12, 14). This issue may be particularly relevant for the understanding

Table I. *Synopsis of the impact of the IL-1RI/TLR/MyD88-dependent signaling pathway on resistance to candidiasis and aspergillosis*[a]

	Primary Infection			Reinfection	
Mice	*Candida* yeasts	*Candida* hyphae	*Aspergillus* conidia	*Candida* hyphae	*Aspergillus* conidia
Wild type	R	S	R	R	R
TLR2$^{-/-}$	R	S	R	S	S
TLR4$^{-/-}$	R	S[b]	S	S	
TLR9$^{-/-}$	R	R	R	R	ND
IL-1RI$^{-/-}$	S	S	R		
MyD88$^{-/-}$	S	S	S		

[a] R, resistance, S, susceptibility (as assessed by survival and fungal growth). ND, not done.
[b] Susceptibility varies depending on the route of infection.

of how Th2 immune responses are generated to fungal hyphae. We have recently shown that additional receptors of fungi, such as Fc, complement and lectin receptors, contributed to the generation of Th1/Th2 responses to the different fungal forms (4). The relation between TLRs and these receptors is largely unknown at the moment. The data from the present study show that the TLR repertoire contributes to the functional plasticity of DCs in response to the different fungal morphotypes, including hyphae. MyD88-deficient DCs were clearly impaired in their ability to activate Th1 cells. However, as no obvious correlation could be found between patterns of cytokine production by DCs and type of antifungal Th reactivity, such as in conditions of TLR2 or TLR4 deficiency, these findings indicate the existence of additional dysfunctional activities at the DC level, as previously suggested (70).

In conclusion, this study shows the diversity and complexity of the signaling pathways triggered by TLRs in response to fungi in vivo (summarized in Table I). Members of the TLR/IL-1R superfamily contributed to the generation of host immunity to *C. albicans* and *A. fumigatus* in a manner that is dependent on fungal species and morphotypes as well as the site of infection. Basically, the IL-1R1/MyD88-dependent pathway is essentially required for host resistance to *Candida*, whereas the TLR4/MyD88-dependent pathway is crucially involved in resistance to *Aspergillus*. However, as fungal morphogenesis in vivo is activated by a variety of tissue-dependent stimuli (33), this idea may accommodate the finding that TLRs are differently implicated depending on the site of the infection, as clearly shown in infections with *Candida* hyphae, and suggests that recruitment of multiple TLRs may occur during infection. Finally, the increased antifungal resistance observed under conditions of TLR deficiency not associated with impaired immune resistance suggests that TLRs may be exploited by fungi for survival in vivo.

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