Dendritic Cells Discriminate between Yeasts and Hyphae of the Fungus *Candida albicans*: Implications for Initiation of T Helper Cell Immunity In Vitro and In Vivo

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Abstract

The fungus *Candida albicans* behaves as a commensal as well as a true pathogen of areas highly enriched in dendritic cells, such as skin and mucosal surfaces. The ability of the fungus to reversibly switch between unicellular yeast to filamentous forms is thought to be important for virulence. However, whether it is the yeast or the hyphal form that is responsible for pathogenicity is still a matter of debate. Here we show the interaction, and consequences, of different forms of *C. albicans* with dendritic cells. Immature myeloid dendritic cells rapidly and efficiently phagocytosed both yeasts and hyphae of the fungus. Phagocytosis occurred through different phagocytic morphologies and receptors, resulting in phagosome formation. However, hyphae escaped the phagosome and were found lying free in the cytoplasm of the cells. In vitro, ingestion of yeasts activated dendritic cells for interleukin (IL)-12 production and priming of T helper type 1 (Th1) cells, whereas ingestion of hyphae inhibited IL-12 and Th1 priming, and induced IL-4 production. In vivo, generation of antifungal protective immunity was induced upon injection of dendritic cells ex vivo pulsed with *Candida* yeasts but not hyphae. The immunization capacity of yeast-pulsed dendritic cells was lost in the absence of IL-12, whereas that of hypha-pulsed dendritic cells was gained in the absence of IL-4. These results indicate that dendritic cells fulfill the requirement of a cell uniquely capable of sensing the two forms of *C. albicans* in terms of type of immune responses elicited. By the discriminative production of IL-12 and IL-4 in response to the nonvirulent and virulent forms of the fungus, dendritic cells appear to meet the challenge of Th priming and education in *C. albicans* saprophytism and infections.

Key words: *Candida albicans* • yeast • hyphae • dendritic cells • cytokines

Introduction

Candida albicans is the most frequently isolated fungal pathogen in humans (1). In mucosal colonization and systemic infection of mice with the fungus, Th1 cells mediate phagocyte-dependent protection and are the principal mediators of acquired protective immunity. In contrast, production of inhibitory cytokines such as IL-4 and IL-10 by Th2 cells and high levels of IgE are associated with disease progression (2–4). Th2-like reactivity is frequently observed in patients with *Candida*-related pathology, such as in symptomatic infections (5) and allergy (6). Th1-type responses may thus characterize the carriage of saprophytic yeast and the resistance to disease seen in healthy humans, whereas Th2 responses associate predominantly with pathology (7, 8).

In murine candidiasis, Th1 differentiation requires the combined effects of different cytokines, including IL-12, in the relative absence of counterregulatory cytokines, such as IL-4 and IL-10, which are, by themselves, necessary and sufficient to drive Th2 polarization (9). Although deficient IFN-γ, TGF-β, IL-6, and TNF-α responses may each block the induction of protective immunity, none of these cytokines is as correlative of Th1 development as IL-12 (9). This cytokine was both required and prognostic for the de-

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velopment of protective Th1 responses to *Candida* (10, 11), and acted as an adjuvant in response to a *Candida* vaccine (12). The availability of IL-12 at the level of the antigen recognition triad (APC, antigen, $CD4^+$ cells) allows the latter cells to differentiate into Th1 cells and to initiate cell-mediated immunity to the fungus. Therefore, understanding cell sources and fungal antigen(s) for IL-12 release has fundamental implications for immunity, vaccine, and therapy of *C. albicans* infections and pathology.

C. albicans can switch from a unicellular yeast form into various filamentous forms, all of which can be found in infected tissues (13). The ability to reversibly switch between these forms is thought to be important for *Candida*'s virulence (14). Although recent studies have clearly shown that the ability to switch from yeast to filamentous form is required for virulence (15, 16), whether it is the yeast or the hyphal form that is responsible for pathogenicity is still an open question. Other pathogenic fungi appear to proliferate in the host exclusively as yeast form cells (17–19). One possibility is that the filamentous growth form is required to evade the cells of the immune system, whereas the yeast form may be the mode of proliferation in infected tissues. For this to be possible, a cell must exist that finely discriminates between the two forms of the fungus in terms of class of immune response elicited. Recent evidence in mice indicates that neutrophils discriminate between the two forms of the fungus, being able to produce IL-12 in response to *C. albicans* yeasts and IL-10 in response to *C. albicans* hyphae (20, 21).

With the recognition that dendritic cells (DCs)[1] are uniquely able to initiate responses in naive T cells and that DCs also participate in Th cell education (22, 23), the present study was undertaken to understand whether DCs interact with *C. albicans* in its different forms, and to elucidate possible mechanisms and consequences of this interaction. This issue appears to be particularly relevant in candidiasis, considering that the fungus behaves as a commensal as well as a true pathogen of skin and mucosal surfaces (13), known to be highly enriched in DCs. For adaptive immune responses to be mounted against fungi, it would seem necessary that DCs should be phagocytic at some stage in their life cycle. Therefore, we have taken advantage of an immature myeloid DC line established from fetal mouse skin (FSDC [24]), capable of efficiently stimulating T cells in vitro and in vivo, upon cytokine treatment (25). In a system devoid of contaminating cells, we found that FSDCs ingested both yeasts and hyphae of the fungus, apparently through different phagocytic mechanisms. Both forms of the fungus were found inside phagosomes. However, hyphae escaped the phagosome and were lying free in the cytoplasm of the cells. In vitro, ingestion of yeasts activated FSDCs and purified splenic DCs for IL-12 production and priming of Th1 cells, whereas ingestion of hyphae inhibited IL-12 and Th1 priming and induced IL-4 production. In vivo, generation of antifungal protective immunity was observed upon injection of DCs ex vivo pulsed with *C. albicans* yeasts but not hyphae. However, the immunization capacity of yeast-pulsed DCs was lost in the absence of IL-12 and that of hypha-pulsed DCs was gained in the absence of IL-4. Therefore, DCs, by discriminating between the virulent and nonvirulent forms of the fungus, are responsible for Th priming and education in *C. albicans* saprophytism and infection.

Materials and Methods

Mice. BALB/c (*H-2*^{d}), and (C57BL/6 × DBA/2)F1 (B6D2F1), *H-2*^{b}/*H-2*^{d} mice, 8–10 wk old of both sexes, were purchased from Charles River. IL-4–deficient and IL-12 p40–deficient (knockout [KO]) mice on BALB/c background (provided by Dr. Manfred Kopf, Basel Institute for Immunology, Basel, Switzerland; and Dr. Luciano Adorini, Roche Milan, Milan, Italy, respectively [26]) and wild-type (WT) mice were bred under specific pathogen-free conditions in the Animal Facility of Perugia University. Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

C. albicans Strains and Culture Conditions. The origin and characteristics of the *C. albicans* highly virulent CA-6 strain and the live vaccine strain, PCA-2, used in this study have already been described in detail (10, 11). Because the CA-6 strain is capable of undergoing yeast to hyphal transition in vitro (27), whereas the PCA-2 strain is not, the CA-6 and PCA-2 strains were used as sources of hyphae and yeasts, respectively. For phagocytosis of hyphae, the CA-6 cells were allowed to germinate by culture at 37°C, 5% CO_2, for 2 h in RPMI 1640 medium with 10% heat-inactivated FCS (by that time, >98% of cells had germinated) (27). The hyphae were then harvested, counted, and resuspended in IMDM (GIBCO BRL) containing 5% filtered heat-inactivated FCS and 50 μg/ml gentamicin (GIBCO BRL). For phagocytosis of yeasts, the PCA-2 cells were harvested at the end of the exponential phase of growth, centrifuged, and resuspended in the above medium. For yeast opsonization, the PCA-2 yeasts were exposed to RPMI 1640 medium with 10% heat-inactivated FCS for 2 h at 37°C, 5% CO_2. Systemic infection of BALB/c and B6D2F1 mice with CA-6 resulted in a progressive, Th2-associated disease, whereas infection with PCA-2 resulted in a Th1-mediated, self-limiting infection (28).

Propagation of FSDCs. Immature FSDCs were generated by retroviral immortalization as described previously (24, 25). Cells were cultured in IMDM containing 5% filtered FCS, 50 μM 2-ME, 2 mM l-glutamine, and 50 μg/ml gentamicin (complete medium). For routine passaging, the cells were detached from tissue culture flasks (Falcon Labware) with 2 mM EDTA in PBS.

Phagocytic Assay. FSDCs (5×10^5 cells/100 μl of complete medium) were incubated at 37°C with *C. albicans* yeasts or hyphae (2.5×10^5 cells/100 μl of complete medium) in suspension culture dishes (Falcon Labware) for 60 min. For mannan inhibition, cells were first incubated with different concentrations of mannan from *Saccharomyces cerevisiae* (Sigma-Aldrich) for 3 min, before addition of fungal cells, as described (25). Phagocytic cells were separated from nonphagocytosed *C. albicans* cells by centrifugation on a fetal bovine serum gradient, and a 0.1-ml sample of the harvested phagocytic cells was used for cytospin preparation. After Diff-Quik staining, the fungal cell internalization was ex-

[1]*Abbreviations used in this paper:* DC, dendritic cell; ELISPOT, enzyme-linked immunospot assay; HPRT, hypoxanthine-guanine phosphoribosyltransferase; KO, knockout; NO, nitric oxide; RT, reverse transcriptase; TEM, transmission electron microscopy; WT, wild-type.

pressed according to the following formula: percentage of internalization = number of cells containing one or more fungal cells/100 cells counted.

Transmission Electron Microscopy. For transmission electron microscopy (TEM), FSDCs were incubated in suspension culture dishes with *C. albicans* yeasts or hyphae, as in the phagocytic assay, for times varying between 15 min and 4 h. A total of 5×10^5 cells were pelleted at 1,200 rpm for 5 min, washed twice with PBS, and fixed in cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate/1% sucrose buffer for 2 h. The cells were postfixed in 1% osmium tetroxide (50 min), encapsulated in 1% agar, stained with uranyl acetate and phosphotungstic acid, and dehydrated in a series of graded ethanolic solutions finishing with propylene oxide before finally being embedded in Epon 812-Araldite mixture. Ultrathin sections (50 nm) were cut on an ultramicrotome (LKB Wallac) and placed under 200-mesh standard copper grids, contrasted with uranyl acetate and lead citrate, and examined with a Philips TEM 400 transmission electron microscope.

Candidacidal Assay and Nitric Oxide Production. For the candidacidal assay, 5×10^5 FSDCs (100 μl/well) were plated in 96-well flat-bottomed microtiter plates (Costar) and incubated with different numbers of *C. albicans* yeasts or hyphae for 2 and 4 h, respectively, as described (29). Triton X-100 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 CFU was determined after 18 h of incubation at 37°C, and the percentage of CFU inhibition (mean ± SE) was determined as follows: percentage of colony-forming inhibition = 100 − (CFU experimental group/CFU control cultures) × 100. Control cultures consisted of *C. albicans* cells incubated without effector cells. For nitrite determination, a measure of nitric oxide (NO) synthesis, FSDCs were cultured as above or in the presence of IFN-γ (400 U/ml) and LPS (40 ng/ml) for 18 h before assaying NO production in the culture supernatants, by a standard Griess reaction, as described previously (29).

Purification and Candida Pulsing of DCs. DCs were purified from spleens by magnetic cell sorting with MicroBeads conjugated to hamster anti–mouse CD11c mAbs (clone N418), according to the manufacturer's instructions (Miltenyi Biotec). In brief, after overnight plastic adherence to remove macrophages, 10^8 collagenase D (Sigma-Aldrich)–treated nonadherent spleen cells were reacted with 100 μl of CD11c MicroBeads before magnetic separation. Positively selected DCs (at ~1% yield recovery) routinely contained >90% $N418^{high}$ cells. In accordance with previous reports (30–32), $CD11^+$ purified DCs express high levels of MHC class II antigen and comprise both CD8α and DEC-205, high and low populations, on FACS® analysis. FSDCs or purified DCs were pulsed with live yeasts or hyphae, as detailed in the phagocytic assay, for 2 h before addition of 2.5 μg/ml of Amphotericin B (Sigma-Aldrich) to prevent *Candida* overgrowth. Cells and supernatants, to be assessed for cytokine gene expression and cytokine content, were harvested either immediately (2 h exposure) or at 6, 24, and 48 h of culture. Control experiments indicated that Amphotericin B alone did not modify patterns of cytokine gene expression and production by DCs.

In Vitro Activation of $CD4^+$ T Cells by Candida-pulsed DCs. Purified (>90% pure on FACS® analysis) $CD4^+$ T splenocytes were obtained by positive selection as described (10, 11). Irradiated (2,000 rads), 18 h–pulsed FSDCs or purified DCs were cultured (5×10^5/ml) with 5×10^6/ml $CD4^+$ T cells, in 24-well tissue culture plates (Falcon Labware) for 5 d in complete medium. In cocultures of hypha-pulsed FSDCs or DCs, 50 U/ml of human IL-2 (Hoffmann-La Roche) were added. Subsequently, cells were washed and restimulated (5×10^6/ml) with heat-inactivated *Candida* cells (5×10^5/ml) in the presence of irradiated, T-depleted splenocytes (APCs; 5×10^6/ml) and IL-2 for 72 h before cytokine determination in culture supernatants. This procedure allowed for successful priming of IL-4–producing $CD4^+$ T cells in vitro (33). For lymphoproliferation, 5×10^6 primed $CD4^+$ T cells were cultured in 200 μl complete medium, in flat-bottomed 96-well microtiter plates (Falcon Labware) in the presence of APCs, heat-inactivated *Candida* cells, and IL-2 for 4 d at 37°C under 5% CO_2. 8 h before harvesting, cells were pulsed with 0.5 μCi of [^{3}H]thymidine per well. Incorporation into cellular DNA was measured by liquid scintillation counting. The results are expressed as mean cpm of stimulated cells, after subtraction of cpm of unstimulated cells.

Adoptive Immunization, Fungal Challenge, and Assessment of Protection. According to preliminary experiments performed to evaluate route, dose, and schedule of DC administration, splenic DCs, either unpulsed or after 18 h pulsing with yeasts or hyphae, were subcutaneously injected, a week apart, at a concentration of 5×10^5 cells/mouse, in 20 μl of PBS. 7 d after the last immunization, the priming of Th cells in vivo was assessed by evaluating the intracellular content of IFN-γ and IL-4 in $CD4^+$ T splenocytes (see below). For infection, mice were intravenously infected with the virulent CA-6 *C. albicans* strain, 3×10^5 cells/0.5 ml of PBS. Protection was assessed by quantifying the number of CFU (mean ± SE) recovered from kidneys, and by parameters of adaptive Th immunity such as the pattern of antigen-specific cytokine production and proliferation. For patterns of cytokine production, 10^6 splenocytes were stimulated with 10^5 heat-inactivated *C. albicans* cells for 48 h. Lymphoproliferation of purified $CD4^+$ T cells from spleens was done as above.

Cytokine Assays. The levels of IFN-γ, IL-4, and IL-10 in culture supernatants were determined by means of cytokine-specific ELISA using pairs of anticytokine mAbs, as described (10, 11). The antibody pairs used were as follows, listed by capture/biotinylated detection: IFN-γ, R4-6A2/XMG1.2; IL-4, BVD4-1D11/BVD6-24G2.3; IL-10, JES5-2A5/SXC-1 (BD PharMingen). IL-12 p70 was determined by the DuoSet ELISA (R&D Systems). Cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant cytokines (from BD PharMingen, or Genetics Institute [for IL-12]).

Intracellular Cytokine Determination. $CD4^+$ T cells were purified from spleens of mice injected with unpulsed or *Candida*-pulsed DCs, as described above. Cells (5×10^6/ml) were cultured in vitro with heat-inactivated *Candida* (5×10^5/ml) cells, APCs (5×10^6/ml), and 50 U/ ml IL-2 in 24-well tissue culture plates. After 72 h of culture, living cells separated on Ficoll gradient were restimulated with PMA (1 μg/ml; Sigma-Aldrich) and ionomycin (50 ng/ml; Sigma-Aldrich) for 4 h at 37°C, with 10 μg/ml brefeldin A added for the last 2 h to promote intracellular accumulation of secreted proteins. Cells were stained for intracellular IFN-γ and IL-4 by using the Cytofix/Cytoperm kits (BD PharMingen), as per the manufacturer's instructions. Analysis was performed with a FACScan™ flow cytometer (Becton Dickinson) equipped with CELLQuest™ software, and was based on 2×10^5 events live-gated according to forward and side scatter characteristics.

Enzyme-linked Immunospot Assay. IL-4–producing FSDCs were enumerated by enzyme-linked immunospot (ELISPOT) assay, as described (21). In brief, FSDCs were exposed to *C. albicans* yeasts or hyphae for 2 h, before addition of amphotericin B to prevent fungal overgrowth. Subsequently, cells were extensively

washed and cultured (10^2–10^4 cells/well) in complete medium for 18 h in 96-well plates previously coated with BVD4-1D11 mAb. Biotinylated BVD6-24G2 mAb was used as the detecting reagent, the enzyme was avidin-alkaline phosphatase conjugate (Vector Laboratories), and the substrate was 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine salt (Life Technologies). Results were expressed as the mean number of IL-4–producing cells (± SE) per 10^4 cells, calculated using replicates of serial twofold dilutions of FSDCs.

Reverse Transcriptase PCR. RNA extraction and amplification of synthesized cDNA from FSDCs were done as described (10, 11). For hypoxanthine-guanine phosphoribosyltransferase (HPRT) and cytokines, the primers, positive controls, cycles, and temperature were as described elsewhere (10, 11). The HPRT primers were used as a control for both reverse transcription and the PCR reaction itself, and also for comparing the amount of products from samples obtained with the same primer. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples for each experiment.

Statistical Analysis. Student's *t* test was used to determine significance of values among experimental groups (significance was defined as $P < 0.05$). In vivo groups consisted of six to eight animals. The data reported were pooled from three to five experiments.

Results

FSDCs Internalize Both Yeasts and Hyphae of C. albicans. Neutrophils and macrophages are recognized as the prototypical phagocytic cells of *C. albicans* (34, 35). To determine whether DCs also phagocytose *C. albicans*, FSDCs were exposed to either yeasts or hyphae of the fungus, and internalization of fungal cells was evaluated at different times after exposure, by light microscopy and TEM. Light microscopic observations suggested that FSDCs ingest both yeasts and hyphae of *C. albicans* in a time-dependent manner, with optimum phagocytosis between 20 and 30 min. Opsonization of the yeasts in serum did not enhance phago-

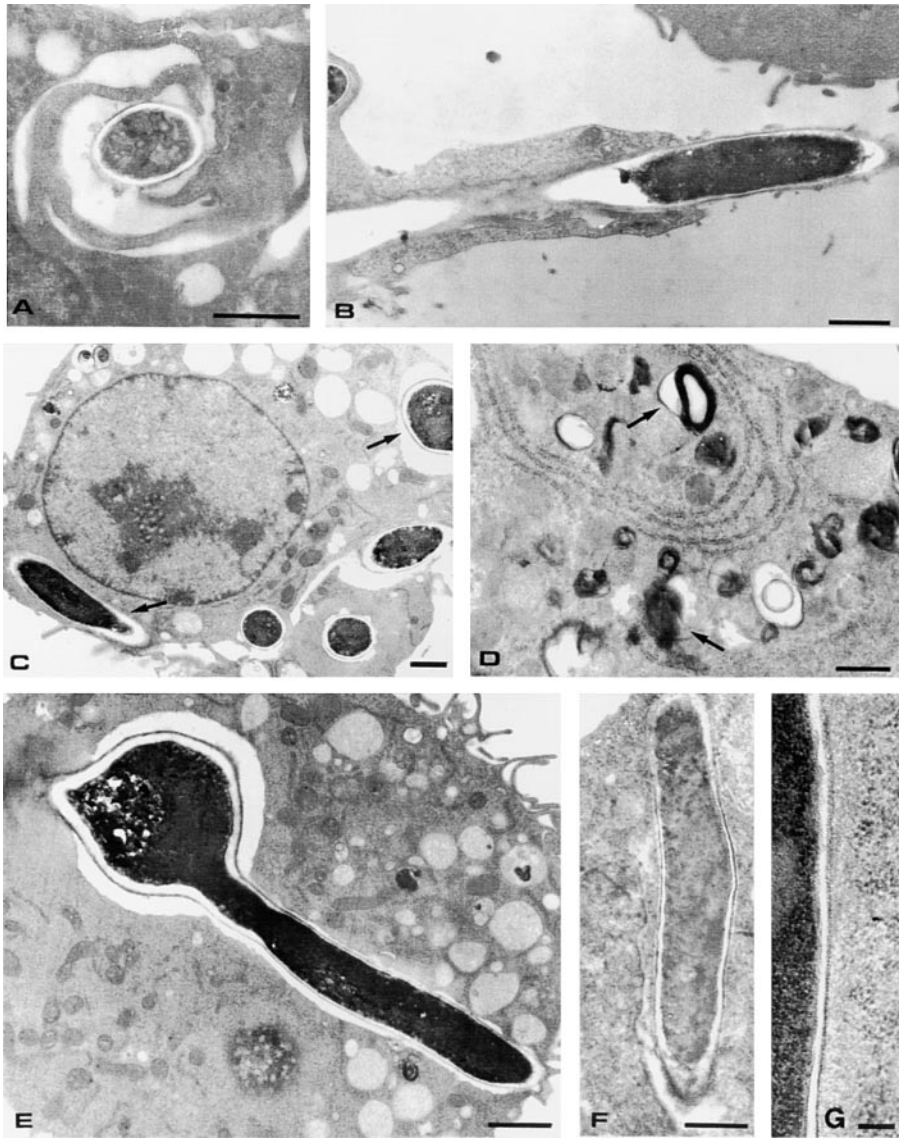

Figure 1. TEM of phagocytosis of *C. albicans* by DCs. FSDCs were incubated with *C. albicans* yeasts (A, C, and D) or hyphae (B, E, F, and G) at an FSDC/*C. albicans* ratio of 2:1, for 15 min (A and B), 1 h (E–G), 2 h (C), and 4 h (D) before processing for TEM. (A) Yeast engulfment through coiling phagocytosis and (B) hypha uptake through zipper-type phagocytosis, at 15 min after infection. (C) Fungal elements inside phagolysosomes at 2 h after exposure (arrows), and (D) in partially degraded forms at 4 h after exposure (arrow). (E) Hyphae escaping the phagosome at 2 h, and (F and G) lying free in the cytoplasm. Original magnifications: (A) ×17,000; (B) ×12,000; (C) ×7,000; (D) ×22,000; (E) ×12,000; (F) ×12,000; and (G) ×70,000. Bar, (A–C, E, F) 1 μm; (D) 0.5 μm; and (G) 0.1 μm.

cytosis. Apparently, FSDCs phagocytosed more yeasts than did neutrophils (data not shown). TEM revealed that just 15 min after infection, yeasts (Fig. 1 A) and hyphae (Fig. 1 B) had already been engulfed by FSDCs. Interestingly, the uptake of fungal cells occurred through different forms of phagocytosis. Internalization of yeasts occurred predominantly by coiling phagocytosis (Fig. 1 A), characterized by the presence of overlapping bilateral pseudopods, that led to a pseudopodal stack before transforming into a phagosome wall. In contrast, entry of hyphae occurred by a more conventional, zipper-type phagocytosis, characterized by the presence of symmetrical pseudopods, which strictly followed the contour of the hyphae before fusion (Fig. 1 B). 2 h later, numerous yeast cells were found inside phagosomes (Fig. 1 C), and were partially degraded at 4 h (Fig. 1 D). Interestingly, as early as 1 h after infection, hyphae appeared to escape the phagosome (Fig. 1 E) and were lying free in the cytoplasm of the cells (Fig. 1 F), as clearly magnified in Fig. 1 G. These results show that DCs are endowed with the ability to phagocytose both forms of the fungus, and, importantly, each fungal form appears to be internalized by different phagocytic mechanisms and to reside in different cell compartments.

As uptake of macromolecules, including zymosan, by immature DCs and FSDCs could be blocked by mannan (25, 36, 37), and mannose receptors were sufficient to mediate macrophage phagocytosis of *C. albicans* (35, 38), we performed uptake experiments in the presence of mannan. At concentrations of mannan known to inhibit the mannose receptor uptake by immature DCs (37), we observed >80% inhibition of phagocytosis of yeast cells, and <50% inhibition of that of hyphae (Fig. 2).

FSDCs Behave as Antifungal Effector Cells. To assess whether phagocytosis of *C. albicans* results in the activation of the antifungal effector machinery in DCs, FSDCs were assessed for the ability to inhibit the fungal growth and to release NO upon exposure to *Candida* yeasts and hyphae. The results (Fig. 3 A) showed that, as early as 2 h after exposure, FSDCs inhibited the growth of yeasts and hyphae. Both activities were higher against yeasts than hyphae, and this correlated with the higher production of NO (Fig. 3 B) and expression of the inducible NO synthase (iNOS) gene upon yeast exposure (not shown). Therefore, DCs exhibit antifungal effector activities upon phagocytosis of fungal cells.

Production of IL-12 and IL-4 by FSDCs and Purified DCs Exposed to C. albicans Yeasts or Hyphae. The outcome of encounters between antigen-bearing APCs and naive T cells depends, in part, on the nature of the cytokines released locally by APCs. This is particularly relevant in can-

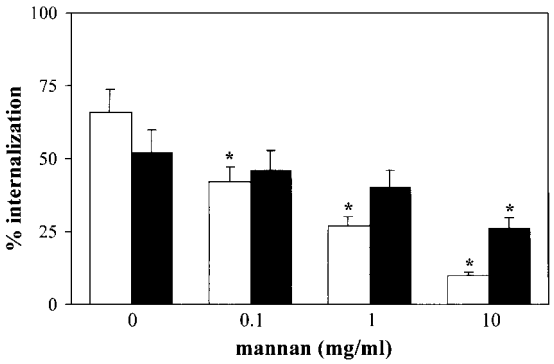

Figure 2. Effect of mannan on internalization of *C. albicans* by DCs. FSDCs were incubated with mannan at different concentrations for 10 min before the addition of *C. albicans* yeasts (white bars) or hyphae (black bars) at a ratio of 2:1 (FSDC/*Candida*). Internalized fungal cells were visualized by light microscopy after 30 min of incubation, as described in Materials and Methods. *$P < 0.05$, yeast or hypha internalization in the presence of mannan vs. yeast or hypha internalization in the absence of mannan.

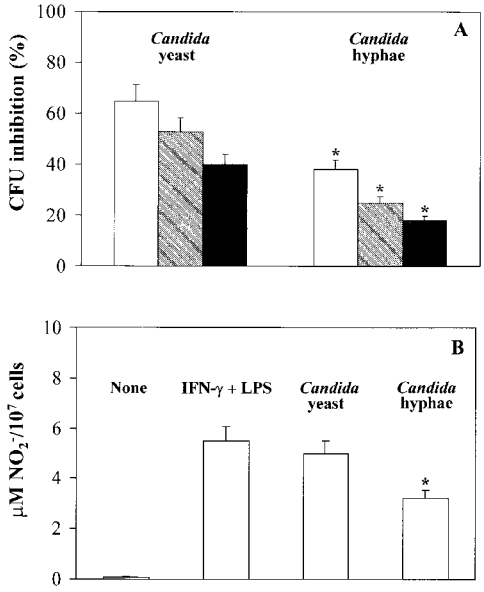

Figure 3. Antifungal activities of DCs. (A) FSDCs were exposed to *C. albicans* yeasts or hyphae (FSDC/*Candida* ratios of 10:1, white bars; 5:1, hatched bars; and 2.5:1, black bars) for 2 h before determination of CFU inhibition, as described in Materials and Methods. (B) NO production by DCs unexposed (None), or upon exposure to IFN-γ (400 U/ml) and LPS (40 ng/ml) or *Candida* yeasts or hyphae. *$P < 0.05$, hypha-pulsed vs. yeast-pulsed DCs.

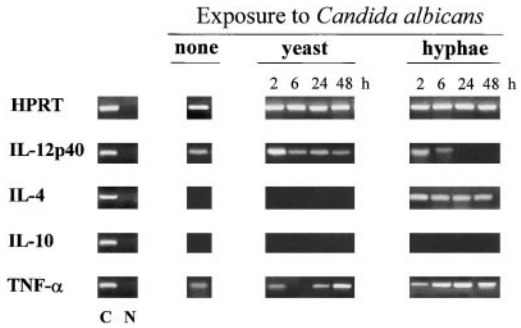

Figure 4. Cytokine gene expression by DCs after exposure of *C. albicans* yeasts or hyphae. FSDCs were either unexposed (none) or exposed to fungal cells at a ratio of 2:1 for different time periods before analysis by RT-PCR. C, HPRT- or cytokine-specific control; N, no DNA added to the amplification mix during PCR.

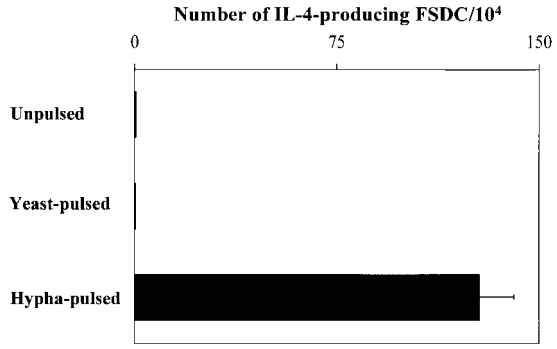

Figure 5. Frequency of IL-4–producing cells in DCs exposed to *C. albicans*. FSDCs were exposed to yeasts or hyphae, at a ratio of 2:1, for 18 h before ELISPOT assay.

didiasis because development of Th1-predominant protective immunity is clearly dependent on production of IL-12, in the relative absence of IL-4 (39). Therefore, we determined the pattern of cytokine production in FSDCs and purified DCs exposed to *C. albicans* yeasts or hyphae, by evaluating cytokine gene expression and production by reverse transcriptase (RT)-PCR, cytokine-specific ELISA, and ELISPOT assay. Cells and supernatants were harvested at 2, 6, 24, and 48 h of culture. Results of RT-PCR revealed that the IL-12 p40 mRNA was continuously detected upon incubation of FSDCs with yeasts, as opposed to the IL-4 and IL-10 mRNA that were not detected at any time (Fig. 4). The TNF-α message was maximally evident at 48 h. In contrast, the IL-12 p40 message was progressively disappearing upon exposure to hyphae, being reduced at 6 h after exposure and completely undetectable at 48 h. Instead, the IL-4 message was detected in hypha-exposed FSDCs, being evident at 2 h after exposure. Similar to yeast exposure, the IL-10 message was never detected. The TNF-α message was continuously present. In terms of protein production, IL-12 p70 was produced upon exposure to yeasts, but not upon exposure to hyphae. In contrast, IL-4 was promptly detected upon phagocytosis of hyphae but not yeasts (Table I), and this correlated with an elevated frequency of IL-4–producing FSDCs upon exposure to hyphae (Fig. 5). According to cytokine gene expression, IL-10 was never detected (Table I), whereas comparable levels of TNF-α (523 vs. 476 pg/ml) were measured at 48 h exposure to yeasts or hyphae. Levels of TGF-β were never detectable in either type of culture (data not shown). Similar to what was observed in the phagocytic assay, serum opsonization of the yeasts did not change the pattern of cytokine production, as similar levels of IL-12 p70 and no IL-4 or IL-10 were observed upon exposure to opsonized yeasts (Table I). On measuring levels of cytokine production by purified splenic DCs upon exposure to yeasts or hyphae, a pattern similar to that observed with FSDCs was found, in that production of IL-12 p70 occurred in response to yeasts and production of IL-4 in response to hyphae, and no IL-10 was detected (Table I). These results indicate that, upon exposure to *C. albicans* yeasts or hyphae, FSDCs and, importantly, purified splenic DCs differentially produce IL-12 and IL-4.

Candida-exposed DCs Prime CD4⁺ T Lymphocytes In Vitro. To assess the ability of DCs exposed to *C. albicans* to activate naive $CD4^+$ T cells in vitro, $CD4^+$ T splenocytes from unprimed mice were cocultured with yeast- or hypha-pulsed FSDCs or purified splenic DCs. After 5 d, the primed cells were restimulated with APCs and *Candida* antigens for an additional 72 h before measurement of cytokines in secondary culture supernatants and for 96 h before assessment of lymphoproliferation. The results (Fig. 6) show that yeast-exposed FSDCs or yeast-exposed DCs induced the production of IFN-γ, but not IL-4 or IL-10. In contrast, FSDCs or DCs exposed to hyphae induced low

Table I. *Production of IL-12 p70, IL-4, and IL-10 by FSDCs and Purified DCs upon Phagocytosis of C. albicans Yeasts or Hyphae*

		IL-12 p70*		IL-4*		IL-10*	
Cells‡	Exposure to:	6 h	48 h	6 h	48 h	6 h	48 h
		ng/ml		*ng/ml*		*ng/ml*	
FSDCs	None	<0.01	<0.01	<2	<2	<2	<2
FSDCs	*Candida* yeasts	2.4 ± 0.7	3.1 ± 0.7	<2	<2	<2	<2
FSDCs	*Candida* yeasts§	2.0 ± 0.8	3.5 ± 0.5	<2	<2	<2	<2
FSDCs	*Candida* hyphae	<0.01	<0.01	29 ± 6	25 ± 5	<2	<2
DCs	None	<0.01	<0.01	<2	<2	<2	<2
DCs	*Candida* yeasts	2.1 ± 0.8	2.7 ± 0.3	<2	<2	<2	<2
DCs	*Candida* hyphae	<0.01	<0.01	16 ± 3	23 ± 4	<2	<2

Values indicated by "<" were below the limit of the assays, as per the manufacturer's indications (see Materials and Methods).
*Cytokine levels (mean ± SE) as determined by cytokine-specific ELISA.
‡FSDCs or purified splenic DCs were exposed to yeasts or hyphae at a cell to fungi ratio of 2:1 for 6 and 48 h before assessment of cytokine content in culture supernatants.
§*Candida* yeasts were opsonized by exposure to RPMI 1640 with 10% heat-inactivated FCS for 2 h at 37°C, 5% CO_2.

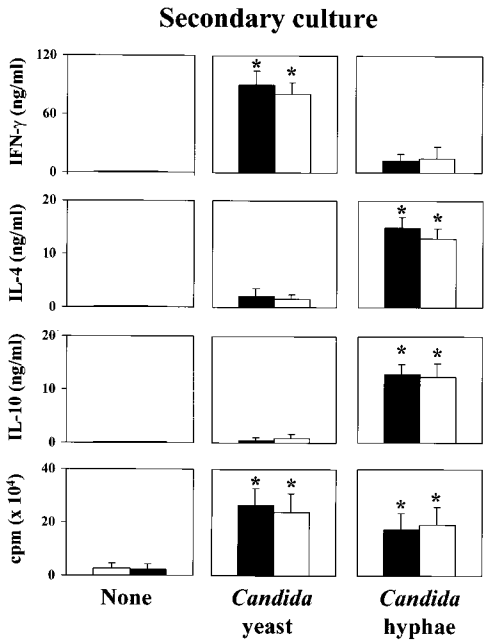

Figure 6. In vitro activation of CD4+ T cells by DCs upon exposure to *C. albicans*. FSDCs (white bars) or purified splenic DCs (black bars), either unexposed (none) or exposed to *Candida* yeasts or hyphae were cocultured with responder CD4+ T cells from naive B6D2F1 (for FSDCs) or BALB/c (for purified splenic DCs) mice (see Materials and Methods for details). After 5 d, cells were restimulated with fresh APCs (irradiated, T-depleted splenocytes) and inactivated *Candida* cells for 72 h (for cytokine determination) or 96 h (for lymphoproliferation). Cytokines were determined by specific ELISA (mean ± SE, ng/ml). Lymphoproliferation was measured by evaluating thymidine incorporation. The results are expressed as mean cpm ± SE of stimulated cells, after subtraction of cpm of unstimulated cells. *$P < 0.05$, *Candida*-pulsed vs. unpulsed FSDCs or DCs.

levels of IFN-γ, but high levels of IL-4 and IL-10. Priming with *Candida*-pulsed FSDCs or DCs also induced CD4+ T cells to proliferate (Fig. 6). The proliferating activity of cells primed by hypha-pulsed FSDCs or DCs was dependent on IL-2, as no proliferation could be detected in the absence of added IL-2. Instead, the high levels of IL-2 being produced in cultures of yeast-pulsed FSDCs or DCs (data not shown) might have allowed the elevated proliferation observed in these cultures (Fig. 6). Together, these results indicate that, upon exposure to *C. albicans* yeasts or hyphae, DCs acquire the ability to prime CD4+ T cells for Th1 or Th2 cytokine production in vitro.

Yeast-exposed DCs Prime CD4+ Th1 Lymphocytes In Vivo and Induce Protective Antifungal Immunity in Infected Mice. To investigate whether *Candida*-pulsed DCs were capable of priming antigen-specific CD4+ Th responses in vivo, purified DCs, either unpulsed or pulsed with yeasts or hyphae, were adoptively transferred into mice by subcutaneous injection. 1 wk after the last injection, CD4+ T cells were purified from spleens, restimulated in vitro, and assessed for cytokine production by intracellular staining. Compared with control mice injected with unpulsed DCs, the number of cells producing IFN-γ was increased, and the number of cells producing IL-4 decreased in mice adoptively transferred with yeast-pulsed DCs as opposed to what was observed in mice receiving hypha-pulsed DCs (Fig. 7). To correlate this finding with the in vivo antifungal resistance, mice received a sublethal dose of *C. albicans* intravenously, 1 wk after the last DC injection. For comparison, a group of mice that was immunized by vaccination with the live PCA-2 vaccine strain was also included. At 7 d after infection, mice were monitored for resistance to infection and parameters of antigen-specific Th cell activation, such as lymphoproliferation and cytokine production. The results (Fig. 8) show that resistance to infection was greatly increased upon transfer of yeast-pulsed DCs, as judged by the reduced number of CFU recovered from kidneys, which was similar to that observed in vaccinated mice. The number of CFU was not reduced upon transfer of unpulsed or hypha-pulsed DCs, being actually increased upon transfer of the latter. The analysis of antigen-specific proliferation and cytokine production by CD4+ T cells and splenocytes revealed that resistance or susceptibility to infection correlated with the activation of Th1 or Th2 cells, respectively. Levels of IFN-γ were higher, and those of IL-4 lower, in mice immunized with yeast-pulsed DCs or PCA-2 compared with mice receiving unpulsed or hypha-pulsed DCs. These results, together with those shown in Fig. 7, indicate that adoptively transferred *Candida*-pulsed DCs are able to prime specific antifungal Th responses in vivo, the quality of which depends on forms of the fungus and the nature of the cytokines. To directly address this issue, purified splenic DCs from IL-12 KO mice were pulsed with yeasts and those from IL-4 KO mice with hyphae,

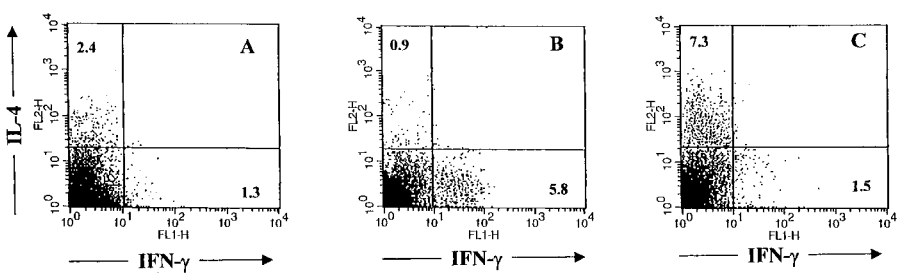

Figure 7. Intracellular cytokine expression in CD4+ T cells from BALB/c mice upon adoptive transfer of *Candida*-pulsed DCs. Splenic DCs, unpulsed (A) or ex vivo pulsed with *Candida* yeasts (B) or hyphae (C), were subcutaneously injected twice, once a week, 7 d before collection of splenocytes. For intracellular staining, purified CD4+ splenocytes were restimulated in vitro with APCs and inactivated *Candida* cells for 72 h. After washing, the cells were further stimulated with PMA and ionomycin in the presence of brefeldin A and then stained for intracellular IFN-γ and IL-4. Percentages reflect cytokine-positive cells.

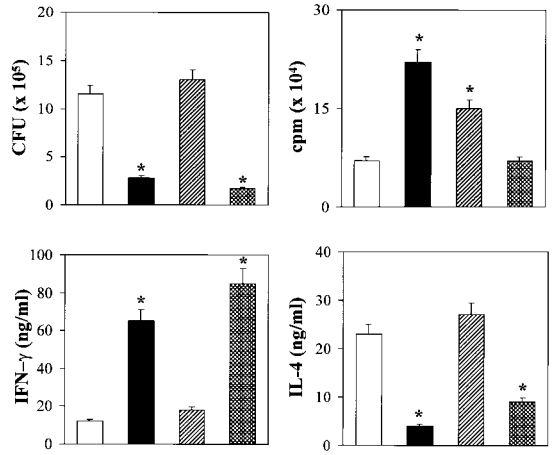

Figure 8. Effect of adoptively transferred DCs on *C. albicans* growth and parameters of Th cell activation in mice with systemic candidiasis. Splenic DCs, unpulsed (white bars) or ex vivo pulsed with *Candida* yeast (black bars) or hyphae (hatched bars) were subcutaneously injected twice, once a week, 7 d before intravenous infection with *C. albicans* virulent strain. 7 d after infection, quantification of fungal growth was done in the kidneys, cytokines were determined by specific ELISA in supernatants of antigen-activated splenocytes, and lymphoproliferation of $CD4^+$ T cells was assessed as in the legend to Fig. 6. Cross-hatched bars, mice vaccinated with the low-virulence *C. albicans* PCA-2, 2 wk before infection with virulent *C. albicans*. $*P < 0.05$, *Candida*-pulsed DCs or vaccinated mice vs. unpulsed DCs.

then assessed for their ability to prime $CD4^+$ T cells in vitro and to elicit antifungal Th responses in vivo upon transfer into WT mice. The results (Fig. 9) show that yeast-pulsed DCs from IL-12 KO mice primed lymphocytes for IL-4 production in vitro and were unable to confer resistance to the infection, as opposed to similarly pulsed DCs from WT mice. In contrast, hypha-pulsed DCs from IL-4 KO mice primed lymphocytes for IFN-γ production and

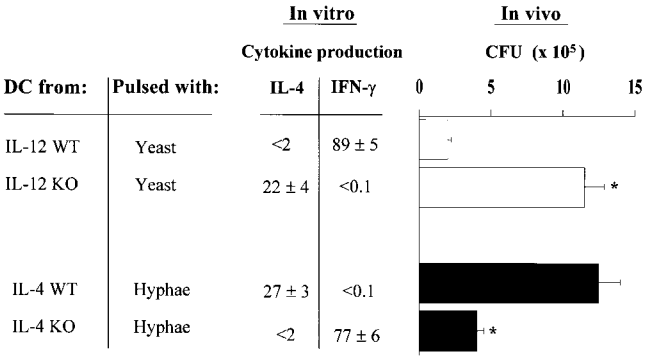

Figure 9. Effect of *C. albicans*–pulsed DCs from IL-12 KO or IL-4 KO mice on Th cell priming in vitro and in vivo. Purified DCs from spleens of mice with IL-12 p40 or IL-4 deficiency were ex vivo pulsed with *Candida* yeasts or hyphae, respectively, and cocultured in vitro with purified $CD4^+$ T splenocytes from WT mice or adoptively transferred into WT recipients. Cocultures and cytokine determination were done as in the legend to Fig. 6. Infection and quantification of fungal growth in vivo were done as in the legend to Fig. 8. $*P < 0.05$, KO vs. WT mice.

were able to confer protection in vivo, as indicated by the significant reduction of CFU in kidneys.

Discussion

The results of this study show that: (a) murine DCs internalize both yeasts and hyphae of *C. albicans*, through distinct phagocytic mechanisms; (b) both forms of the fungus are found inside phagosomes, with signs of degradation, but hyphae escape the phagosome and lie free in the cytoplasm; (c) phagocytosis of yeasts induces IL-12 production, whereas that of hyphae inhibits IL-12 and induces IL-4 production; (c) yeast-pulsed DCs, as opposed to hypha-pulsed DCs, activate Th1 lymphocytes in vitro and in vivo and induce antifungal resistance upon adoptive transfer into recipient mice. Thus, DCs fulfill the requirement of a cell uniquely capable of discriminating between the two forms of the fungus in terms of the type of immune response elicited.

Unlike conventional phagocytic cells, such as neutrophils and macrophages that have had only the phagocytosis of yeasts well documented (27, 34, 35), DCs easily phagocytosed both forms of the fungus, and, upon phagocytosis of either form, produced sets of cytokines with opposing activities in the developing immune response. Although the coiling phagocytosis is now considered to reflect a disturbance of the more conventional zipper-type phagocytosis (40), DCs use both forms of phagocytosis to internalize pathogens (41, 42). Here, we show that FSDCs engulfed yeasts via coiling phagocytosis, as already observed during uptake of yeasts or zymosan particles by phagocytic cells (43). Once internalized, yeasts were detected in phagolysosomes, where different stages of progressive degradation were seen. In contrast, internalization of hyphae appears to occur through a more conventional zipper-type phagocytosis. Once inside the cells, hyphae appeared to promote rupture of the phagosomal membrane and escaped into the cytoplasm. Thus, not only are yeasts and hyphae ingested through different forms of phagocytosis, but, once inside the cells, they reside in distinct cellular compartments. To our knowledge, this is the first demonstration of (a) internalization of *Candida* hyphae through a phagocytic mechanism, and (b) the ability of hyphae to escape and lie free in the cytoplasm.

Yeasts and, to some extent, short filamentous forms of *C. albicans* can be ingested by neutrophils (44, 45) and macrophages (35, 46–48) through a variety of mechanisms and opsonic requirements, ultimately affecting the antifungal effector functions of the cells. Recently, an interesting observation was made on the fate of yeasts and filamentous forms of *C. albicans* upon internalization in mouse macrophages (48). Uptake of yeasts, but not of filamentous forms, had the characteristics of phagocytosis, requiring intact actin filaments and the activity of protein kinase C. Once internalized, both forms of the fungus were found inside phagosomes that rapidly fused with late endosomes and lysosomes (48). Thus, the internalization and the intracellular localization of the different forms of the fungus ap-

pear to occur differently in macrophages and DCs. This may reflect a distinction of labor between different phagocytic cells in the immune response to the fungus. Upon contact with the fungus, effector macrophages and neutrophils rapidly activate oxidative and nonoxidative pathways of killing through phagocytic and nonphagocytic mechanisms (13). In contrast, the antifungal activity of DCs appears to be more tightly regulated, being expressed at higher levels towards yeasts than hyphae. This may ultimately have an impact on fungal antigen presentation by DCs. Indeed, yeast degradation inside phagolysosomes may result in an efficient release of fungal peptides for class II–restricted antigen presentation, whereas hyphae surviving free in the cytosol may eventually intersect the class I–restricted antigen presentation pathway. As both MHC class II– and class I–restricted T cell responses have been detected in mice with candidiasis (26, 49) and, interestingly enough, the activation of $CD8^+$ T lymphocytes occurred in response to the filamentous forms of the fungus (49), our results suggest that DCs are uniquely qualified to serve as APCs in antifungal host immune responses.

The reasons behind the different behavior of the two forms of the fungus in different phagocytic cells are not presently understood. Among other factors, *C. albicans* secretes phospholipase C, a major virulent factor that increases penetration of the fungus into host cell tissues (50). It is interesting that phospholipase C contributes to the perforation of macrophage phagosomes by listeriolysin O (51). It has been postulated that lysosomal fusion and acidification of the vacuole might induce metabolic changes, including enzyme secretion and activation, which may contribute to pathogen infectivity (48). The existence of *C. albicans* strains with deletion of the gene encoding the predominant phospholipase C (50) would allow us to directly assess the role of this virulent factor in *Candida* trafficking inside DCs.

The observation that internalization of yeasts, more than hyphae, was sensitive to mannan inhibition not only suggests that mannose receptors are involved in the entry of *Candida* in FSDCs, but also indicates that additional recognition molecules on DCs may participate in the phagocytosis of hyphae. Receptors that have been identified on immature DCs, including FSDCs (25), include lectins such as the mannose receptor (52, 53) and DEC-205 (54), as well as FcεRI and FcγR (52, 55). Receptors for antigen capture on dendritic and phagocytic cells vary in their ligand and specificity and mode of delivery to antigen-processing compartments (56–58). The mannose receptor–mediated phagocytosis of nonopsonized *C. albicans* resulted in the generation of proinflammatory cytokines (59), and the mannose receptor–mediated phagocytosis of zymosan initiated IL-12 production in phagocytes (60). In contrast, interaction with receptors other than the mannose receptors, including CR3 (61), led to suppression of the immune response to *C. albicans* (46) and other fungi (62). Studies are presently underway to understand whether DCs use different receptors to phagocytose nonopsonized or opsonized fungal elements. In this regard, it is worth mentioning that in our experimental conditions, the exposure of yeasts to serum in order to generate hyphae could have resulted in opsonization of the latter, thus favoring the entry through the FcγR. It has recently been reported that immune complex internalization through the FcγR results in DC maturation and MHC class I–restricted antigen presentation (55). However, although it is an attractive hypothesis, whether this also occurs with opsonized hyphae is not presently known. In the case of yeasts, serum opsonization did not modify the extent of phagocytosis and cytokine production by FSDCs, a finding in line with the observation that the interaction of both opsonized and nonopsonized fungal yeasts with macrophages (35) and neutrophils (45) occurs predominantly through a mannose-specific mechanism.

After phagocytosis of yeasts or hyphae, the downstream cellular events were clearly different. Phagocytosis of yeasts was a potent signal for sustained IL-12 production by both FSDCs and purified DCs. A similar finding was obtained upon exposure of FSDCs to *Leishmania major* amastigotes (63). In contrast, phagocytosis of hyphae resulted in a progressive inhibition of IL-12 production. This inhibition was not due to a generalized deactivation state, as TNF-α production was not affected. Neither was it due to IL-10 or TGF-β production, as neither cytokines were detected, a finding in line with the observation that freshly isolated DCs from spleens do not produce IL-10 (64). Surprisingly, hypha-pulsed FSDCs and purified DCs produced IL-4, detectable at message and protein levels, and in terms of frequency of cytokine-producing cells. No IL-4 was observed in unstimulated DCs, nor upon phagocytosis of yeasts. Production of IL-4 was concomitant with inhibition of IL-12, and although hypha-pulsed DCs from IL-4–deficient mice produced IL-12, no data are presently available favoring the hypothesis of a direct inhibitory effect of IL-4 on IL-12 gene transcription and protein production. Instead, one likely possibility is an indirect effect of IL-4 through the inhibition of release of NO, known to induce IL-12 gene expression (65, 66). That IL-4 inhibits NO production in response to *C. albicans* has already been reported (29). Here, we found that production of NO by FSDCs was lower upon hyphae compared with yeast internalization. Thus, the impaired NO production not only correlates with the decreased antifungal activity of cells toward hyphae, but may also may represent a potent signal regulating IL-12 production in DCs.

One important consequence of phagocytosis of *Candida* yeasts or hyphae by DCs is the ability of the cells to induce different patterns of cytokine production by $CD4^+$ T lymphocytes. Yeast-exposed DCs stimulated, and hypha-exposed DCs inhibited, production of IFN-γ and IL-2 by unprimed $CD4^+$ T lymphocytes both in vitro and in vivo. In contrast, IL-4 and IL-10 were observed in the presence of hypha-exposed, but not yeast-exposed, DCs. Thus, murine DCs, upon phagocytosis of yeasts or hyphae of *C. albicans*, acquire the capacity to induce the differentiation of $CD4^+$ cells towards the Th1 or Th2 phenotype. This correlated in vivo with a different ability to control the infec-

tion in mice receiving yeast-pulsed DCs versus mice receiving hypha-pulsed DCs. The activation of antigen-specific Th1 cells in the former nicely correlated with the fungal burden, because the *C. albicans* growth was highly restricted and similar to that observed in mice vaccinated differently. The ability to induce anticandidal protective Th1 immunity in vivo was impaired upon transfer of DCs exposed to the yeasts in the absence of IL-12, and potentiated upon transfer of DCs exposed to the hyphae in the absence of IL-4. These results suggest that production of IL-12 or IL-4 by DCs may crucially contribute to the induction of protective and nonprotective immune responses in *C. albicans* infection. These observations also ruled out the possibility that the different capacity of yeast- or hypha-pulsed DCs to activate antifungal immune responses is the result of behaviors other than cytokine production, such as homing and cell trafficking in vivo (67). All together, our results would suggest that murine DCs, by producing sets of opposing cytokines upon phagocytosis of yeasts or hyphae, may activate different anticandidal Th cells in vivo.

It is known that murine splenic DCs comprise two major subpopulations of DCs, presumably belonging to distinct lineages, which differentially regulate the development of Th cells in vivo (68–72). Although lymphoid $CD8\alpha^+$ DCs, either directly (68, 71, 72) or indirectly (73), were found to trigger the development of Th1 responses, and myeloid $CD8\alpha^-$ DCs to induce Th2 responses, to what extent, however, each subset contributes to the induction or inhibition of T cell reactivity in vivo is still a matter of debate (74, 75). In our experimental conditions, the purified splenic population of DCs consisted of both $CD8\alpha^+$ and $CD8\alpha^-$ cells. Whether these populations of DCs would respond differently to the different forms of *C. albicans* is an issue that we are presently addressing.

IL-4 has been the missing cytokine in the induction of Th2 cell differentiation by DCs. The recent study by Rissoan et al. (71), in which two types of DCs were clearly shown to be at work in Th1 and Th2 cell differentiation, left open the question of the identity of the molecules responsible for Th2 cell induction by DCs, and factors involved in the generation of functionally distinct types of DCs. Our study, together with a recent paper (76) showing production of IL-4 by DCs upon retroviral exposure, helps clarify these issues by suggesting that exposure to pathogens, together with the ability to discriminate among them, is an important determinant of DC and Th cell differentiation. The capacity to produce IL-4 together with the presence of IL-4 on maturing DCs (77) indicates that autocrine effects of this cytokine may indeed occur.

Accumulating evidence points to a unique role of DCs in infections, as they are regarded as both sentinel for innate recognition and initiator of Th cell differentiation and functional commitment (78). Our study shows that, in doing that, murine DCs are exquisitely sensitive to the different forms of a pathogen, a finding in line with the increasingly recognized importance of pattern recognition receptors in host defense (79, 80). Considering that human DCs also phagocytose (81) and activate T cell responses to *C. albicans* (82), our findings provide important and novel insights into the general mechanisms of immunoregulation in fungal infections. Moreover, as the morphogenesis of *C. albicans* is activated in vivo by a wide range of signals, including stress and metabolic signals (83), DCs may also act as key regulators of Th reactivity in saprophytism.

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