

Candida albicans Is Phagocytosed, Killed, and Processed for Antigen Presentation by Human Dendritic Cells

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Candida albicans is a component of the normal flora of the alimentary tract and also is found on the mucocutaneous membranes of the healthy host. *Candida* is the leading cause of invasive fungal disease in premature infants, diabetics, and surgical patients, and of oropharyngeal disease in AIDS patients. As the induction of cell-mediated immunity to *Candida* is of critical importance in host defense, we sought to determine whether human dendritic cells (DC) could phagocytose and degrade *Candida* and subsequently present *Candida* antigens to T cells. Immature DC obtained by culture of human monocytes in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4 phagocytosed unopsonized *Candida* in a time-dependent manner, and phagocytosis was not enhanced by opsonization of *Candida* in serum. Like macrophages (M ϕ), DC recognized *Candida* by the mannose-fucose receptor. Upon ingestion, DC killed *Candida* as efficiently as human M ϕ , and fungicidal activity was not enhanced by the presence of fresh serum. Although phagocytosis of *Candida* by DC stimulated the production of superoxide anion, inhibitors of the respiratory burst (or NO production) did not inhibit killing of *Candida*, even when phagocytosis was blocked by preincubation of DC with cytochalasin D. Further, although apparently only modest phagolysosomal fusion occurred upon DC phagocytosis of *Candida*, killing of *Candida* under anaerobic conditions was almost equivalent to killing under aerobic conditions. Finally, DC stimulated *Candida*-specific lymphocyte proliferation in a concentration-dependent manner after phagocytosis of both viable and heat-killed *Candida* cells. These data suggest that, in vivo, such interactions between DC and *C. albicans* may facilitate the induction of cell-mediated immunity.

Candida albicans is a component of the normal microflora of the alimentary tract and mucocutaneous membranes of the healthy host. However, when immune defenses are compromised or the normal microflora balance is disrupted, *Candida* transforms itself into an opportunistic pathogenic killer. Indeed, dissemination of *Candida* is the leading cause of invasive fungal disease in premature infants, diabetics, and surgical patients and of oropharyngeal disease in AIDS patients (2, 9, 15, 20, 28, 34, 45, 61, 79). Despite appropriate therapy, mortality from systemic *Candida* infections in immunocompromised individuals is nearly 30% (82). In human immunodeficiency virus-infected individuals who have not yet developed advanced immunodeficiency, the prevalence of oropharyngeal *Candida* is from 7 to 48% of patients (33). As the immunodeficiency in AIDS patients progresses, the prevalence of oral candidiasis increases to 43 to 93% (59). Furthermore, the development of oral candidiasis in the early stages of human immunodeficiency virus infection is highly predictive of worsening immunodeficiency.

Host resistance against infections with *C. albicans* is mediated predominantly by neutrophils and macrophages (M ϕ). During the induction of cell-mediated immunity (CMI), *Candida* antigens are presented to T cells and stimulate their proliferation with subsequent cytokine synthesis, and in both humans and mice these cytokines enhance the candidicidal functions of the phagocytic cells (3, 18, 26, 46). In systemic

candidiasis in mice, cytokine production has been found to be a function of CD4⁺ T helper (Th) cells. The Th1 response, characterized by the production of interleukin-2 (IL-2), IL-12, gamma interferon (IFN- γ), and tumor necrosis factor alpha (TNF- α), is associated with M ϕ activation and enhanced resistance against reinfection. The Th2 response, which produces IL-4, IL-6, and IL-10, is linked with the development of chronic disease (10, 11, 60, 64, 65, 67). However, while it is generally accepted that production of Th1 cytokines is important for M ϕ activation and clearance of the fungus, recent studies suggest that a critical balance of both Th1 and Th2 cytokines is necessary to develop protective immunity (48, 49, 66). Furthermore, the balance of Th1 and Th2 cytokines produced depends on the model of candidiasis being studied.

Although M ϕ can serve as antigen-presenting cells (APC), dendritic cells (DC) are more potent antigen presenters than M ϕ (70). DC precursors originate in the bone marrow, enter the blood, and seed nonlymphoid tissues. These DC are classified as immature and are specialized in antigen uptake and processing (5). The immature DC differentiate into mature DC as they migrate to tissue-draining secondary lymphoid organs, where they efficiently present antigen to T cells (4, 14, 42, 54, 63, 68). The strategic location of DC in tissues, on mucosal surfaces, and in the skin suggests that these cells are in a prime position to initiate protective immune responses against *Candida*.

For these adaptive immune responses to occur, the DC either would have to take up soluble fungal antigens or phagocytose the fungus themselves. If the latter occurs, it would require not only uptake of the fungus but also killing, degradation, and processing of fungal antigens. Teleologically, it

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would seem to be more efficient if the DC ingested the *Candida* rather than their having to wait for antigens to be shed or regurgitated by other immune cells such as M ϕ . Recently, we demonstrated that immature human DC were able to phagocytose, kill, and degrade the fungal pathogen *Histoplasma capsulatum* (24). Furthermore, the DC were able to process *H. capsulatum* antigens and stimulate T-cell proliferation.

Human and mouse M ϕ bind and internalize *Candida* cells via the mannose-fucose receptor (MFR) (37, 47). As human DC and Langerhans cells also express the MFR on their surface (6, 13), we hypothesized that immature DC might phagocytose *Candida* through the MFR and subsequently kill the organism, process *Candida*-specific antigens, and stimulate lymphocyte proliferation.

Most recently it was reported that mouse DC phagocytose and kill *Candida* and that recognition was through the MFR (19). The data presented herein confirm these observations for human DC and also demonstrate two important differences in the interaction of human and mouse DC with *Candida*. The first is that human and mouse DC apparently kill *Candida* by different mechanisms. The second difference is that *Candida*-infected human DC stimulate T-cell proliferation without the requirement for the addition of exogenous IL-2.

MATERIALS AND METHODS

Reagents. N^G-monomethyl-L-arginine (NMMA) was purchased from Calbiochem, La Jolla, Calif. Fluorescein isothiocyanate (FITC), type III ferricytochrome *c*, N^G-nitro-L-arginine methyl ester (NAME), superoxide dismutase (SOD), catalase, mannitol, cytochalasin D (CD), L-fucose, galactose, N-acetyl-D-glucosamine, D-glucosamine, α -methyl-D-mannoside, mannosylated bovine serum albumin (BSA), and D-mannose were purchased from Sigma, St. Louis, Mo. Diphenylene iodonium bisulfate (DPI) was a gift of Andrew Cross, The Scripps Research Institute, La Jolla, Calif. A 1 mM stock concentration of DPI was prepared in dimethyl sulfoxide (DMSO) and stored at -80°C . DPI was diluted into RPMI 1640 (Gibco-BRL, Gaithersburg, Md.) to the desired concentration just before use. CD was dissolved in DMSO at a concentration of 1 mg/ml, and aliquots were stored at -80°C until use. Cytochrome *c* (1 mM) was dissolved in Krebs-Ringer phosphate buffer containing 0.2% dextrose and stored at -20°C . All other reagents were prepared in RPMI 1640 and sterile filtered. Pooled human serum (PHS) prepared from six to eight individual donors was pooled and stored in 200- μl aliquots at -80°C until use.

Preparation of human DC and M ϕ . Monocytes were isolated by sequential centrifugation on Ficoll-Hypaque and Percoll gradients (Amersham Pharmacia LKB, Piscataway, N.J.) from buffy coats obtained from the Hoxworth Blood Center, Cincinnati, Ohio, or from blood drawn from normal adult donors in our laboratory (52). To obtain DC, monocytes were cultured in 6-well tissue culture plates (Corning-Costar, Cambridge, Mass.) at $6 \times 10^5/\text{ml}$ in RPMI 1640 containing 200 mM glutamine, 50 μM 2-mercaptoethanol (Sigma), 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL), 50 ng of kanamycin (Sigma)/ml, 1% nonessential amino acids (BioWhittaker, Walkersville, Md.), and 1% pyruvate (BioWhittaker). Human recombinant granulocyte-M ϕ colony-stimulating factor (115 ng/ml; Peprotech, Inc., Rocky Hill, N.J.) and human recombinant IL-4 (rIL-4; 50 ng/ml; Peprotech, Inc.) also were added to each well, and DC were studied after 6 to 8 days of culture.

M ϕ were obtained by culture of monocytes at $1 \times 10^6/\text{ml}$ in Teflon beakers with RPMI 1640 containing 15% PHS, 10 μg of gentamicin (Sigma)/ml, 100 U of penicillin/ml, and 100 μg of streptomycin (Sigma)/ml (52). M ϕ were studied after 5 to 7 days in culture.

Fluorescence-activated cell sorter (FACS) analysis. DC (5×10^5) were incubated with primary monoclonal antibodies (MAbs) at 4°C for 45 min. After two washes in phosphate-buffered saline (PBS) containing 1% BSA (PBS-BSA), the cells either were fixed in 1% paraformaldehyde or were incubated with the fluorochrome-labeled secondary antibody for an additional 45 min at 4°C . The cells then were washed twice with PBS-BSA and fixed overnight with 1% paraformaldehyde before analysis by flow cytometry on a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif.) with standard optics and filter. The acquired data were analyzed with CELLQuest software (Becton Dickinson). Nonspecific

antibody binding was blocked by preincubation of DC with 250 μg of human immunoglobulin G (IgG) (Sigma) per ml for 30 min at 4°C prior to the addition of primary MAb. The following MAbs were used: FITC-labeled CD11a, CD11b, CD11c, CD80, CD18, CD14, CD40, and mouse IgG1, IgG2a, and IgM (Ancell, Bayport, Maine); unconjugated CD19, CD3, CD16, and goat anti-mouse IgG and IgM (Ancell); CD83 (Serotec, Raleigh, N.C.); CD86 (Pharmingen, San Diego, Calif.); HLA-DR (CAL-Tag, South San Francisco, Calif.); CD1a (Bio-source International, Menlo Park, Calif.); and CD56 (Tcell Diagnostics, Inc., Woburn, Mass.). The DC expressed high levels of CD18, CD11b, CD11c, HLA-DR, and VLA-5 and moderate levels of CD11a, CD1a, and CD86. DC were negative for M ϕ , T-cell, B-cell, and NK markers (24).

C. albicans. *Candida* (ATCC 18804) was maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.). Yeast cells were grown for 16 h in Sabouraud dextrose broth at 30°C with orbital shaking at 150 rpm. For binding and phagocytosis assays, yeast cells were harvested by centrifugation, washed three times in 0.01 M phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS) and then heat-killed (HK) at 65°C for 1 h. Yeast cells were stored at 4°C in PBS containing 0.05% sodium azide. To label cells with fluorescein, HK yeast cells were resuspended to $2 \times 10^8/\text{ml}$ in either 0.01 mg of FITC/ml (suspension assays) or 0.1 mg of FITC/ml (M ϕ Terasaki plate binding assay) in 0.05 M carbonate-bicarbonate buffer (pH 9.5). After incubation for 15 min at room temperature in the dark, FITC-labeled *Candida* cells were washed twice with Hanks balanced salt solution containing 0.25% BSA (HBSA) and resuspended to the appropriate concentration in HBSA.

For studies with viable yeast cells, overnight cultures were harvested by centrifugation, washed three times in HBSA, and resuspended to 50 ml in HBSA. The yeast cells were counted on a hemacytometer and standardized to the appropriate concentration according to the assay protocol.

Phagocytosis assay. Phagocytosis of *Candida* by human DC and M ϕ was quantified as described previously for *H. capsulatum* (52). HK *Candida* cells were opsonized in 5% PHS at 2×10^8 yeast cells/ml for 20 min at 37°C . After washing twice in HBSA, opsonized *Candida* cells were resuspended in HBSA. DC were harvested from 6-well plates, and M ϕ were harvested from beakers after 5 to 7 days of culture, washed in HBSA, and standardized to $2 \times 10^6/\text{ml}$. DC or M ϕ (1×10^6) were incubated with opsonized or unopsonized FITC-labeled HK *Candida* cells (5×10^6) in a total volume of 1 ml at 37°C in a water bath with orbital shaking at 150 rpm for various periods of time. At the end of the incubation period, trypan blue (1 mg/ml in PBS) was added for 15 min at 25°C to quench the fluorescence of bound but uningested organisms (52). The cells then were washed with HBSA, cytocentrifuged onto glass slides, and fixed in 1% paraformaldehyde at 4°C . Coverslips were mounted in 90% glycerol in PBS, and phagocytosis was quantified by phase-contrast and fluorescence microscopy. A total of 100 cells were counted per slide and the number of ingested or bound but uningested yeast cells was determined. Results are expressed as the mean \pm standard error of the mean (SEM) of the phagocytic index (PI) (the total number of yeast cells ingested per 100 DC or M ϕ) and as the percent ingestion (the percentage of DC or M ϕ containing one or more yeast cells).

Binding assays. DC and M ϕ were harvested after 5 to 7 days of culture, washed, and resuspended to $4 \times 10^6/\text{ml}$ in HBSA. Fifty microliters of DC were incubated in 12- by 75-mm polypropylene tubes for 30 min at 37°C with various sugars or HBSA only. Fifty microliters of FITC-labeled *Candida* cells ($2 \times 10^7/\text{ml}$) then were added to each tube and incubated for 20 min at 37°C in a water bath with orbital shaking at 150 rpm. Ten microliters of sample then was mounted on a clean glass slide and coverslipped for immediate quantitation via phase-contrast and fluorescence microscopy. One hundred DC were counted per slide, and the results are expressed as the mean \pm SEM of the attachment index (the total number of organisms bound per 100 cells).

For binding assays with M ϕ , the cells (2.5×10^3) were allowed to adhere for 1 h at 37°C in 5% CO_2 -95% air in the wells of a Terasaki tissue culture plate (Miles Scientific Division, Naperville, Ill.) that previously had been coated with 1% human serum albumin. The cells were washed twice with HBSA and then incubated with various sugars or HBSA for 30 min at 37°C . Five microliters of FITC-labeled HK *Candida* cells ($2 \times 10^7/\text{ml}$) then was added to each well and the mixture was incubated for 20 min at 37°C . Unbound organisms were removed by washing with HBSA and the monolayers were fixed with 1% paraformaldehyde. Attachment of the yeast cells was quantified via fluorescence microscopy on an inverted microscope (Diaphot; Nikon Inc. Instrument Group, Melville, N.Y.) by counting 100 M ϕ per well. Results are expressed as mean \pm SEM of the attachment index (52).

Quantitation of DC and M ϕ fungicidal activity. DC and M ϕ (5×10^5) were incubated with *Candida* cells (5×10^4) in the presence or absence of 10% PHS in polypropylene tubes for 1, 2, or 4 h at 37°C in a water bath with orbital shaking at 150 rpm. At each time point, the cells and yeast cells were centrifuged, the

supernatant was aspirated, and the mixtures were resuspended in 1 ml of sterile water. The contents then were vortexed vigorously prior to serial dilution and plating on Sabouraud dextrose agar plates. Inspection of the lysate by light microscopy confirmed that the yeast cells were not clumped. After incubation at 30°C for 48 h, CFU were counted and the percent *Candida* cells that were killed was calculated by comparison with the CFU obtained from the original inoculum. Some experiments were performed under anaerobic conditions in a Forma Scientific (model 1024; Marietta, Ohio) anaerobic chamber.

Quantitation of PL fusion. DC (1×10^6) were incubated for 2 h at 37°C in M199 (Gibco-BRL) containing 10% human serum, 10 µg of gentamicin/ml, and 18-nm-diameter colloidal gold beads stabilized with horseradish peroxidase (HRP: Au18) (53). The DC were washed three times with medium and then incubated an additional 2 h at 37°C to ensure that the HRP: Au18 entered the lysosomal compartments. After the second incubation, 1×10^6 viable *Candida* cells were added and phagocytosis was allowed to proceed for 1 h at 37°C. After 1 h the DC were washed three times with ice-cold 0.1 M sodium cacodylate buffer (pH 7.4) and then processed for electron microscopy. DC phagolysosomal fusion (PL fusion) was quantified by counting the number of 18-nm gold particles in phagosomes containing *Candida* (53). The data are expressed as the mean \pm SEM of the number of gold particles per yeast-containing phagosome. *H. capsulatum* and *Saccharomyces cerevisiae* also were studied for comparison.

Quantitation of superoxide anion (O_2^-) production. O_2^- generation by DC and M ϕ was quantified as the SOD-inhibitable reduction of ferricytochrome *c* (type III; Sigma) as described previously (69). DC and M ϕ (2×10^6) were incubated for 1 h at 37°C with opsonized or unopsonized *Candida* cells (1×10^7) in Krebs-Ringer phosphate buffer with 0.2% dextrose containing 80 µM cytochrome *c*. Control wells contained cytochrome *c* without *Candida* (resting cells) or the *Candida* plus 40 µg of SOD/ml. At the end of the incubation, the supernates were collected by centrifugation at 4°C. The absorbance of the supernates at 550 nm was measured in a Spectronic Genesys 5 (Milton Roy, Rochester, N.Y.), and the background absorbance in control tubes containing only buffer and cytochrome *c* was subtracted. All experiments were performed in triplicate and results were calculated as nanomoles of O_2^- using $E_{550} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Results are expressed as nanomoles of cytochrome *c* reduced per 2×10^6 cells per hour.

T-cell isolation. At day 6 of the DC culture, blood was obtained from the same donor, and mixed mononuclear cells were isolated on Ficoll-Hypaque gradients (52). The mononuclear cells were standardized to 7.5×10^7 /ml in RPMI 1640 containing 5% FCS and 10 µg of gentamicin/ml, warmed to 37°C, and passed over a nylon wool column at 37°C. After 1 h of incubation on the column, the first 15 ml of column flowthrough was collected and cultured at 2×10^6 /ml overnight in a T flask (Corning-Costar). After 24 h of culture, the nonadherent cells were collected, washed in Dulbecco's PBS (DPBS) containing 2% FCS, and incubated for 1 h on ice with mouse MAbs to human CD56 (Becton Dickinson) and human CD16 (Medarex, Annandale, Calif.) to eliminate any remaining NK cells. The cells then were washed in DPBS containing 2% FCS and incubated for 1 h at 4°C on a rocking shaker (Thermolyne, Dubuque, Iowa) with goat anti-mouse IgG magnetic beads (Perseptive Biosystems, Framingham, Mass.) at 10 beads per cell. Purified T cells were obtained after incubation of the cell-bead suspension on a Dynal MPC-1 magnet (Oslo, Norway) for 10 min. The T cells remaining in suspension were collected and used in the antigen presentation assays as described below. T cells were 98.5% CD3⁺ by FACS analysis (24).

Antigen presentation assays. Antigen presentation by DC to T cells was quantified by the incorporation of [³H]thymidine (24). DC were incubated with either HK or viable *Candida* cells for 1 h at 37°C in a 96-well plate to allow for phagocytosis of the yeast cells. Autologous T cells then were added to each well and the plate was cultured at 37°C for 7 days. In these experiments, the media contained 10% autologous serum rather than PHS. In wells containing viable *Candida* cells, amphotericin B (1.25 µg/ml) was added after the first 48 h of culture to prevent overgrowth of the yeast cells. On day 7 of culture, 1.0 µCi of [³H]thymidine (specific activity, 6.7 Ci/mmol; Dupont-New England Nuclear) in RPMI 1640 was added to each well. After further incubation for 24 h at 37°C, the contents of the wells were harvested onto glass fiber filters and counts per minute were determined in a liquid scintillation counter. The results are expressed as the mean \pm SEM of the counts per minute incorporated by T cells in the presence of various amounts of DC and *Candida*. DC and T cells were greater than 95% viable on all days of culture, as determined by trypan blue dye exclusion.

Statistics. Statistical analysis of the data was performed using Sigma Stat (Jandel Scientific, San Rafael, Calif.). Student's *t* test or the Mann-Whitney rank sum test was used to analyze the data for statistical significance, and results were considered significant at a *P* level of <0.05.

RESULTS

Phagocytosis and killing of *C. albicans* by human DC. Previously, we have demonstrated that immature human DC phagocytose and kill *H. capsulatum* (24), and others have shown that immature human DC can phagocytose some bacteria and protozoa (1, 21, 32, 78). Therefore, we first examined the ability of human DC to phagocytose *Candida* and compared their phagocytic activity to human monocyte-derived M ϕ . DC and M ϕ were incubated in suspension with unopsonized FITC-labeled HK *Candida* for various periods of time, and phagocytosis was quantified as described in Materials and Methods. Figure 1 shows that DC ingested *Candida* in a time-dependent manner. Phagocytosis was evident as early as 10 min, and after 1 h almost 60% of DC had ingested at least 1 *Candida* yeast cell, with an average of over 5 yeast cells per DC. Opsonization of the yeast cells did not enhance DC phagocytosis of *Candida* (Table 1) nor did it affect the percentage of DC-associated yeast cells that were ingested (61% for unopsonized yeast cells and 58% for opsonized yeast cells).

To determine if ingested *Candida* cells were killed by DC, DC and M ϕ were incubated with viable *Candida* cells in the presence or absence of 10% PHS for 1, 2, and 4 h at 37°C. The data in Fig. 2 demonstrate that over the 4-h incubation period, both DC and M ϕ were equally efficient at killing *Candida*, and fungicidal activities were equivalent regardless of whether or not PHS was present to opsonize the yeast cells.

Binding of *Candida* cells to human DC is mediated by the MFR. Our original rationale for these experiments was based on the fact that DC contain high levels of the MFR on their surface (6, 13). As M ϕ utilize the MFR to recognize and phagocytose unopsonized *Candida* cells (37, 47), we hypothesized that human DC MFR might perform the same function. To test this hypothesis, DC and M ϕ were preincubated with various sugars and their subsequent capacity to bind FITC-labeled HK *Candida* cells was quantified. Because M ϕ bound *Candida* poorly in suspension, they were studied under adherent conditions in Terasaki plates. DC or M ϕ were preincubated with various sugars for 30 min at 37°C and then incubated with FITC-labeled *Candida* cells for an additional 20 min. Preincubation of DC or M ϕ with mannose, fucose, or mannosylated BSA, but not galactose, significantly inhibited the binding of *Candida*, strongly suggesting that binding was mediated through the MFR (Table 2).

Mechanism of DC fungicidal activity against *C. albicans*. As it has long been known that polymorphonuclear cells kill *Candida* predominantly via the production of toxic oxygen metabolites (16, 77, 81), we next sought to determine if incubation of DC with *Candida* would stimulate a respiratory burst. Therefore, DC and M ϕ were incubated for 1 h with opsonized or unopsonized *C. albicans* cells, and superoxide anion production was quantified by the SOD-inhibitable reduction of cytochrome *c*. Figure 3 shows that both unopsonized and opsonized *Candida* stimulated the release of O_2^- to levels roughly half that produced by M ϕ . However, preincubation of DC or M ϕ with either SOD (200 µg/ml), catalase (100 µg/ml), or mannitol (0.04 M) did not result in inhibition of fungicidal activity by either cell type (data not shown).

Because lack of inhibition by respiratory burst inhibitors may be caused by an inability of these agents to gain entrance

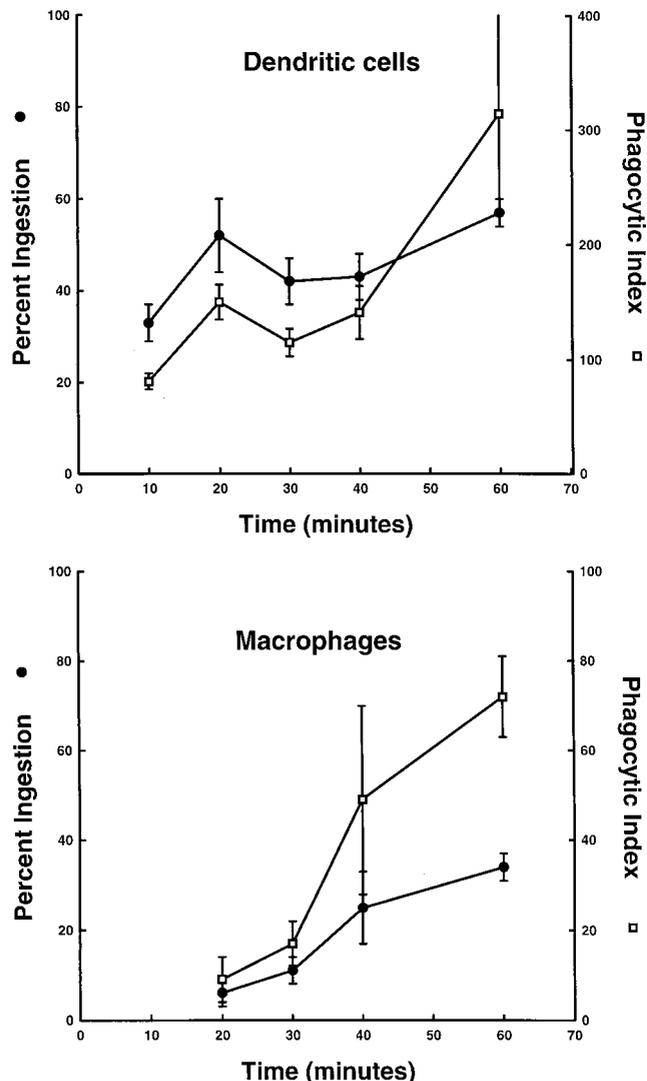


FIG. 1. Human DC phagocytose *C. albicans* in a time-dependent manner. DC or M ϕ (2×10^6) were incubated with unopsonized FITC-labeled *Candida* (1×10^7) for various periods of time. At the end of each time period 1 mg of trypan blue/ml was added to each sample for 15 min to quench the fluorescence of uningested organisms. An aliquot of cells was cytocentrifuged onto glass slides and phagocytosis was quantified by phase and fluorescence microscopy. The data are presented as the percent ingesting (percent DC or M ϕ containing at least one ingested organism) and the phagocytic index (the total number of organisms ingested/100 DC or M ϕ). The data are the mean \pm SEM of four experiments with different donors.

to the phagosome (36), we next sought to determine if DC would kill *Candida* in the absence of phagocytosis. The rationale was that if killing took place extracellularly, respiratory burst inhibitors might be more effective in inhibiting killing. Therefore, DC and M ϕ were preincubated for 5 min with $2 \mu\text{g}$ of CD/ml to disrupt actin microfilaments and inhibit phagocytosis, and then killing of *Candida* was quantified after incubation with the yeast cells for 1 h at 37°C . The data in Table 3 show that both DC and M ϕ killed *Candida* equally well regardless of whether or not the yeast cells were ingested. Visual inspection of aliquots taken from the tubes containing CD confirmed that yeast cells remained extracellular but were still

TABLE 1. DC phagocytosis of *C. albicans* is not enhanced by serum^a

Treatment	% Ingestion ^b	PI ^c	No. of bound but uningested <i>Candida</i> /100 DC
No serum	57 ± 3	314 ± 87	202 ± 58
5% PHS	60 ± 5	230 ± 70	164 ± 51

^a DC were incubated with *Candida* in the presence or absence of 5% PHS for 1 h at 37°C . Phagocytosis was quantified by phase-contrast and fluorescence microscopy. Data are the mean \pm SEM of three experiments performed in duplicate.

^b Percentage of DC ingesting 1 or more *Candida* cells.

^c Number of *Candida* cells ingested per 100 DC.

bound to DC and M ϕ . Furthermore, in control experiments, CD did not affect the viability of *Candida* or its ability to germinate. In addition, extracellular killing of *C. albicans* occurred over a wide range of pHs. Thus, when the extracellular medium was buffered to pH 4.0, 7.2 (standard assay), 8.0, or 10.0, there was no difference in the amount of fungicidal activity exhibited by either DC or M ϕ (data not shown).

Having demonstrated that DC and M ϕ killed *Candida* yeast cells even when they remained outside the cell, we then sought to determine if inhibitors of the respiratory burst would inhibit killing of *Candida*. Preincubation of DC and M ϕ with SOD, catalase, and mannitol still did not block killing of *Candida* by either DC or M ϕ . We also attempted to block killing using the NO synthase inhibitors DPI ($10 \mu\text{M}$) (74), L-NMMA (1 mM) (56), and L-NAME (1 mM) (41), but these agents also were without effect (data not shown). Finally, we tested DPI and SOD in combination (Table 4), but again there was no inhibition of DC or M ϕ fungicidal activity.

As the production of toxic oxygen radicals did not appear to be the mechanism of DC or M ϕ fungicidal activity, we sought to determine if DC ingesting *Candida* exhibited significant PL fusion. DC were loaded with HRP: Au 18 and incubated with

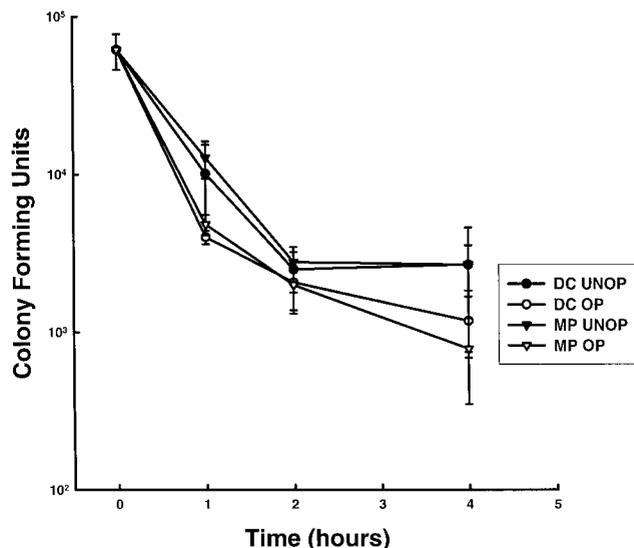


FIG. 2. Human DC kill *C. albicans* as well as M ϕ . Viable *Candida* (5×10^4) were incubated with DC or M ϕ (5×10^5) in the presence or absence of 5% PHS in suspension for 1, 2, and 4 h at 37°C . At each time point, remaining CFU were quantified as described in Materials and Methods. The data are the mean \pm SEM of five experiments.

TABLE 2. Binding of *Candida* to human DC is mediated via the MFR^a

Sugar and concn	DC		Mφ	
	AI ^b (n)	% Inhibition ^c	AI (n)	% Inhibition ^c
None	335 ± 23 (24)		440 ± 38 (19)	
Mannose				
50 mM	31 ± 10 (3)	91*	298 ± 48 (3)	32
100 mM	5 ± 1 (3)	98*	124 ± 45 (3)	72†
Fucose				
50 mM	88 ± 35 (3)	74*	94 ± 21 (4)	79†
100 mM	2 ± 0.3 (3)	99*	42 ± 6 (4)	90†
Galactose				
100 mM	405 ± 15 (3)	0	383 ± 107 (3)	13
Mannosylated BSA				
200 µg/ml	127 ± 41 (4)	62*	160 ± 26 (3)	64†

^a Binding assays with DC were performed in suspension; assays with Mφ were performed in Terasaki trays. All assays were performed in the absence of serum. Sugars were preincubated with the cells for 30 min at 37°C, and then *Candida* cells were added for an additional 20 min. The data are presented as the mean ± SEM.

^b AI = attachment index, which is the number of *Candida* cells bound per 100 DC or Mφ.

^c *, P < 0.005 compared to untreated controls; †, P < 0.01 compared to untreated controls.

Candida, *H. capsulatum*, or *S. cerevisiae* cells for 1 h at 37°C and then processed for electron microscopy. Figure 4 shows that after 1 h of phagocytosis, DC containing *Candida* had an average of almost 4 gold particles per phagosome. In contrast, DC that had phagocytosed *S. cerevisiae* had less than 2 gold particles per phagosome, while DC that had ingested *H. capsulatum* had about 13 gold particles per phagosome.

Our cautious interpretation of these data is that modest PL

TABLE 3. DC and Mφ kill *C. albicans* in the absence of phagocytosis^a

Target	CD	% <i>Candida</i> killed by:	
		DC	Mφ
<i>Candida</i>	–	84 ± 4	90 ± 2
Op <i>Candida</i>	–	86 ± 4	85 ± 3
<i>Candida</i>	+	79 ± 3	90 ± 2
Op <i>Candida</i>	+	80 ± 4	77 ± 4

^a DC and Mφ were incubated with unopsonized *Candida* or *Candida* opsonized in 5% PHS (Op *Candida*) in the presence or absence of 2 µg of CD/ml for 1 h at 37°C. Fungicidal activity then was quantified as described in Materials and Methods. Data are the mean ± SEM of nine experiments.

fusion occurred upon ingestion of *C. albicans* by human DC, even though it was only one-third the amount of PL fusion that occurred when DC phagocytosed *H. capsulatum*. The question that remained unanswered was whether this amount of PL fusion was biologically significant. In other words, was it enough to kill *Candida*?

To address this issue and rule out a role for toxic oxygen radicals, DC and Mφ fungicidal activities were quantified under anaerobic conditions. DC, Mφ, and *Candida* cells were equilibrated in the anaerobic chamber for 30 min prior to initiating the killing assay. The DC and Mφ then were incubated with opsonized and unopsonized *Candida* cells for 1 or 2 h and killing of *Candida* was quantified as described previously. The data in Table 5 show that both DC and Mφ killed *Candida* under anaerobic conditions almost as efficiently as under aerobic conditions.

Antigen presentation by DC. As DC phagocytosed and killed *Candida*, we next tested the ability of DC to process and present *Candida* antigens to T cells. In preliminary experiments, we determined that the optimal concentration of DC was 10⁴/well (data not shown). Next, DC (10⁴) were incubated

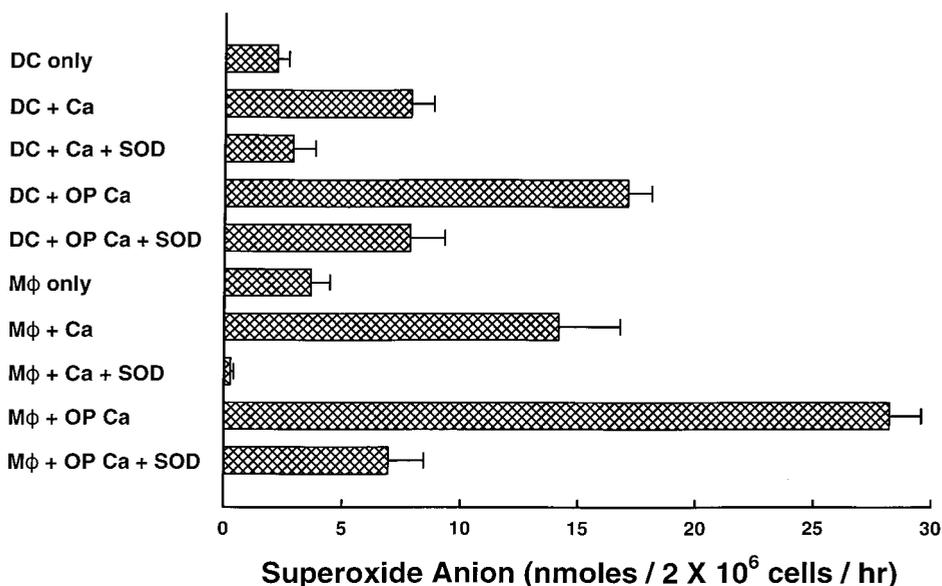


FIG. 3. *C. albicans* (Ca) stimulates human DC to produce superoxide anion. DC and Mφ (2 × 10⁶) were incubated with opsonized (OP) or unopsonized *Candida* (1 × 10⁷) in the absence or presence of SOD (50 µg/ml), and the production of superoxide anion was quantified by the reduction of cytochrome c. The data are the mean ± SEM of eight experiments.

TABLE 4. Inhibitors of the respiratory burst and NO production do not block the killing of *Candida* even in the absence of phagocytosis^a

Target	Inhibitor	% <i>Candida</i> killed by:	
		DC	Mφ
<i>Candida</i>	None	76 ± 2	90 ± 3
Op <i>Candida</i>	None	75 ± 5	74 ± 5
<i>Candida</i>	SOD	80 ± 5	85 ± 8
Op <i>Candida</i>	SOD	76 ± 3	83 ± 2
<i>Candida</i>	DPI	69 ± 5	84 ± 2
Op <i>Candida</i>	DPI	67 ± 3	74 ± 1
<i>Candida</i>	Both	76 ± 1	90 ± 2
Op <i>Candida</i>	Both	71 ± 2	85 ± 0

^a DC and Mφ were incubated with unopsonized *Candida* or *Candida* opsonized in 5% PHS (Op *Candida*) in the presence 2 μg of CD/ml and in the absence or presence of SOD (200 μg/ml), DPI (10 μM), or both SOD and DPI for 1 h at 37°C. Fungicidal activity then was quantified as described in Materials and Methods. Data are the mean ± SEM of two to six experiments.

for 1 h with various concentrations of HK or viable *Candida* cells and then cultured for 1 week with autologous CD3⁺ T cells. Lymphocyte proliferation over the last 24 h was then quantified by measuring the incorporation of [³H]thymidine. As shown in Fig. 5, T-cell proliferation was stimulated by both viable and HK cells in a concentration-dependent manner.

DISCUSSION

DC are the most potent APC of the immune system and are vital for the initiation of primary T-cell-mediated immune responses that are the hallmark of cell-mediated immunity (CMI) (70). As the host defense against *C. albicans* requires

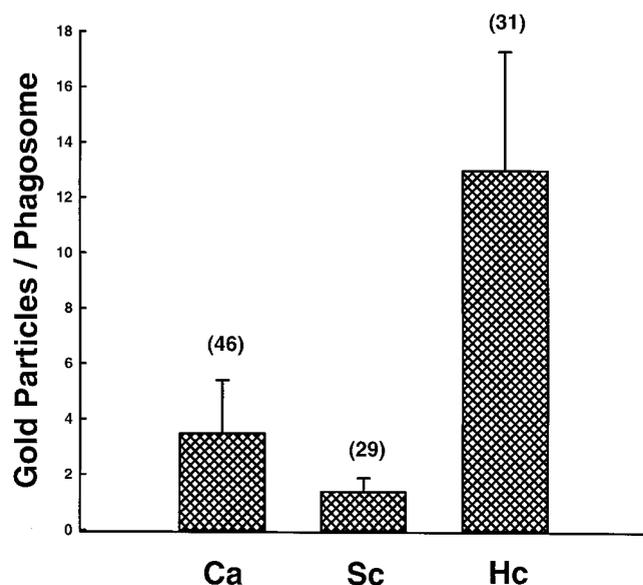


FIG. 4. *C. albicans* (Ca) stimulates PL fusion in human DC. DC were labeled with HRP: Au18 and then incubated with *Candida*, *H. capsulatum* (Hc), or *S. cerevisiae* (Sc) cells for 1 h at 37°C. After fixation and processing for electron microscopy, the number of gold particles in yeast-containing phagosomes was quantified. The data are presented as the mean ± SEM of gold particles per phagosome. The number above each bar denotes the number of phagosomes counted for each fungus.

TABLE 5. Mφ and DC kill *C. albicans* under anaerobic conditions^a

Target	Time (h)	% <i>Candida</i> killed by:	
		Mφ	DC
<i>Candida</i>	1	64 ± 7	50 ± 4
<i>Candida</i>	2	73 ± 8	60 ± 9
Op <i>Candida</i>	1	59 ± 4	59 ± 4
Op <i>Candida</i>	2	73 ± 4	63 ± 11

^a DC and Mφ were incubated with *Candida* in the absence or presence of 10% PHS (Op *Candida*) for 1 or 2 h at 37°C under anaerobic conditions. Fungicidal activity then was quantified as described in Materials and Methods. Data are the mean ± SEM of four individual experiments.

the induction of CMI, we sought to determine a role for human DC in this process. The data presented herein demonstrate that: (i) immature human DC avidly ingest *Candida* and that serum is not required for recognition and phagocytosis; (ii) recognition is via the MFR; (iii) DC kill *Candida* as efficiently as Mφ and do not require the presence of serum; (iv) the mechanism of DC fungicidal activity is oxygen independent; and (v) upon ingestion of viable or HK *Candida* cells, DC stimulate the proliferation of T cells. These data suggest that DC may play an important role in the host response to *Candida* infection by linking innate immunity with CMI.

In our studies of phagocytosis of *Candida*, we found that DC actually were slightly more phagocytic than Mφ. Thus, after only 1 h of incubation, 60% of DC had ingested an average of 5 unopsonized yeast cells/DC, whereas after the same incubation period less than 40% of Mφ had ingested an average of about 2 yeast cells/Mφ. As Mφ are considered professional phagocytes, this counterintuitive result is most likely because of the fact that Mφ function better when adherent to a surface. Indeed, we used adherent Mφ in the binding assay because very few yeast cells bound to Mφ in suspension after 20 min. We observed a similar phenomenon in our studies of human DC and *H. capsulatum* (24).

d'Ostiani et al. (19) recently demonstrated that an immature myeloid DC line established from fetal mouse skin cells (FSDC) ingested both the yeast and hyphal forms of *Candida* via different mechanisms and receptors. Phagocytosis of yeast cells occurred by coiling phagocytosis characterized by the presence of overlapping bilateral pseudopods, whereas ingestion of hyphae was via the classic zipper mechanism with the formation of symmetrical pseudopods. Electron micrographs of human DC with internalized *Candida* cells did not reveal any evidence of coiling phagocytosis, but it was clear that the yeast cells were in membrane-bound phagosomes (data not shown). However, the earliest time point that was observed was 1 h. Not surprisingly, our data and the studies of d'Ostiani et al. (19) demonstrate that *Candida* cells are recognized by the MFR, as has been found for Mφ (37, 47).

Although original reports suggested that DC had weak or no endocytic activity (71, 72, 84), there is now considerable evidence that DC ingest numerous microbial pathogens. Mouse DC and murine-derived DC lines phagocytose bacteria such as *Bordetella bronchiseptica* (29, 31), *Listeria monocytogenes* (30), *Chlamydia trachomatis* (55, 75), BCG mycobacterium (35), *Mycobacterium tuberculosis* (76), and *Salmonella enterica* subsp. *enterica* (23), in addition to the protozoan *Leishmania major* (8, 39, 51, 80, 83). In vivo, murine DC containing internal

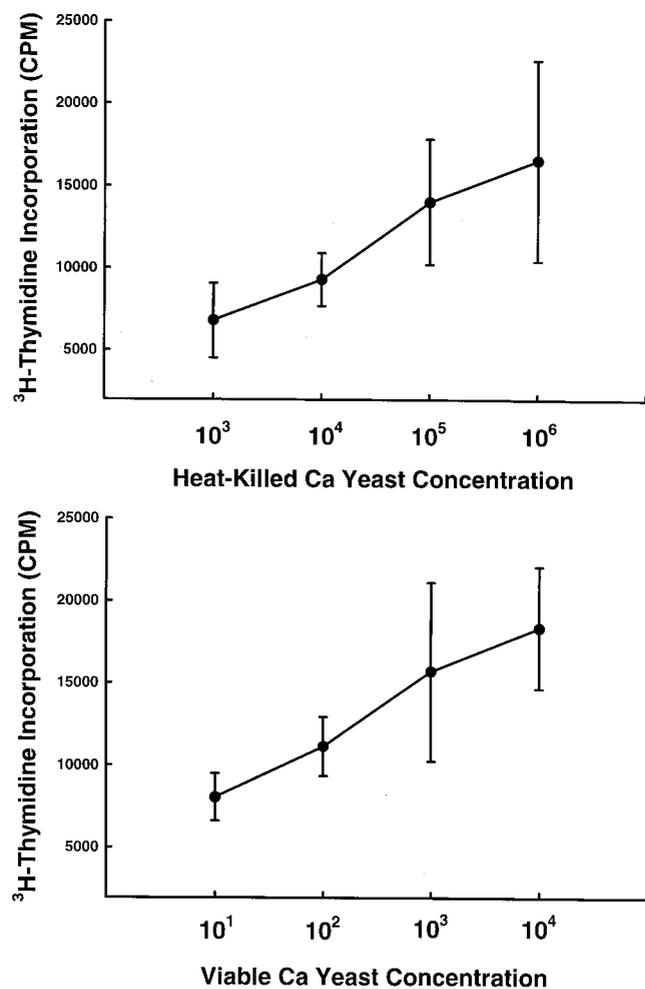


FIG. 5. DC infected with viable and HK *C. albicans* (Ca) cells stimulate T-cell proliferation. Aliquots of 10^4 DC were incubated with various concentrations of either viable or HK *Candida* cells for 1 h at 37°C to allow for phagocytosis. Purified $\text{CD}3^+$ T cells (10^5) then were added to the wells, and the mixtures were cultured for an additional 7 days. On day 7, the wells were pulsed with [^3H]thymidine to quantify T-cell proliferation. The data are presented as the mean \pm SEM of the counts per minute of five experiments performed in triplicate. The counts per minute in control wells containing DC and T cells only was $<2,000$.

Leishmania donovani (25) and *L. major* (50) also have been observed.

Human DC have been reported to phagocytose *Borrelia burgdorferi* (21), *M. tuberculosis* (22, 32, 43), *C. trachomatis* (43), *L. monocytogenes* (38), influenza virus (7), measles virus (27), the protozoans *Trypanosoma cruzi* (78) and *L. donovani* (1), and the fungus *H. capsulatum* (24). Thus, although DC are not considered professional phagocytes, they are capable of considerable phagocytic activity.

In addition to phagocytosis, human DC were found to kill *Candida* as efficiently as human monocyte-derived M ϕ . Fresh complement-preserved serum did not enhance the fungicidal activities of either DC or M ϕ . Presumably, opsonization in serum would lead to recognition and ingestion of *Candida* via additional receptors including the Fc receptor for IgG and complement receptor type 1 (CR1) (40, 85, 86). Thus, engage-

ment of a variety of cell surface receptors by both cell types leads to efficient activation of the cells' microbicidal machinery.

The mechanism of DC and M ϕ killing of *C. albicans* was mainly oxygen independent, probably via lysosomal hydrolases. Thus, killing was almost as efficient under anaerobic conditions as under aerobic conditions. Furthermore, PL fusion did take place in *Candida*-infected DC, albeit only at one-third of that observed after infection with *H. capsulatum*. No evidence was obtained for an oxidative killing mechanism, even though phagocytosis of *Candida* by DC stimulated the production of superoxide anion. Furthermore, there was no evidence that NO was involved. In contrast, murine FSDC do produce NO upon phagocytosis of *Candida* and hyphae, but no definitive evidence was presented to demonstrate that NO actually was responsible for killing the *Candida* cells (19). As our immature DC were derived from peripheral blood monocytes, these data fit in with the general idea that the preponderance of human neutrophil candidacidal activity is mediated via the production of toxic oxygen metabolites (16, 77, 81), whereas killing of *Candida* by human monocytes and M ϕ occurs predominantly through nonoxidative mechanisms (17, 44, 77).

Human DC also have been reported to kill and degrade *L. monocytogenes* (38). In contrast, *T. cruzi* survives and multiplies intracellularly within human DC (78), and *L. monocytogenes* (30), *B. bronchiseptica* (29), and *S. enterica* subsp. *enterica* (23) apparently survive within the murine DC line CB1. Electron microscopy reveals both viable and degraded *Chlamydia* in murine DC (55, 75) and both viable and degraded *B. burgdorferi* in human DC (21). Further, murine DC, collected after in vivo infection with *L. major*, contained viable parasites that caused the development of lesions upon reinjection into BALB/c mice (51).

Although the murine DC line tsDC kills *M. tuberculosis* (76), these bacteria grow more rapidly within human DC than in human M ϕ (22). Most interesting is the fact that IL-10, but not IFN- γ , TNF- α , IFN- γ in combination with TNF- α , IL-4, or transforming growth factor β , significantly suppresses the growth of *M. tuberculosis* in DC but not in M ϕ (22). Thus, it is clear that the interactions of DC with microorganisms vary both with the species of DC and with the particular microorganism under investigation.

Although killing of a microorganism would seem to be a necessary prerequisite to obtain efficient presentation of antigens, Moll and colleagues (51) found that murine DC contained viable, virulent *L. major* parasites, but infected DC still were capable of stimulating lymphocyte proliferation. Because the DC that stimulated proliferation contained viable organisms, possibly a small number of intracellular parasites were actually degraded and antigen was processed and deposited on the surface of the infected DC prior to presentation to lymphocytes. Alternatively, antigens could be processed and regurgitated by other infected phagocytes and then transferred to DC for presentation. However, attempts to demonstrate that mycobacterial antigens could be transferred from infected M ϕ to DC were unsuccessful (58). Whether DC might acquire antigens from other microbial pathogens via this route remains to be determined.

Lastly, we demonstrated that phagocytosis of both viable and HK *Candida* cells by human DC leads to the stimulation of

T-cell proliferation. As we found in our studies of human DC and *H. capsulatum* (24), DC that had phagocytosed viable *Candida* cells actually were more efficient at stimulating T-cell proliferation than DC that had ingested HK cells. Thus, ingestion of 1,000-fold fewer viable yeast cells than HK cells led to equivalent stimulation of lymphocyte proliferation. These results are in agreement with the fact that mice develop better immunity when given a sublethal inoculum of a viable organism than they do with HK organisms. Thus, these data again suggest that the heat-inactivation process may destroy important immunogenic antigens and have implications for the design and use of DC in vaccine strategies.

Although we did not skin test our donors for sensitivity to *Candida* antigens, we presume that the proliferation observed was mostly a secondary response, as *C. albicans* is a commensal organism in humans. Likewise, in our other studies with *H. capsulatum* and human DC (24) we presumed that the T-cell proliferation was a secondary response, as *Histoplasma* is indigenous in Cincinnati. In those studies, we did identify three individuals whose T cells did not proliferate in an in vitro assay that utilized mixed mononuclear cells and HK cells as an in vitro correlate of a skin test. However, *Histoplasma*-infected DC from these three individuals did stimulate T-cell proliferation, demonstrating that human DC could stimulate T cells in a primary response. Further studies with naive (CD45RO-negative) and memory (CD45RO-positive) T cells will be required to determine if human DC stimulate both a primary and a secondary response upon ingestion of *Candida*.

d'Ostiani et al. (19) also reported that *Candida*-infected murine DC (FSDC and splenic DC) stimulated the proliferation of T cells. However, proliferation was not obtained with the mixture of DC, *Candida*, and T cells as reported here. In that system, *Candida*-pulsed DC and T cells first were cultured for 5 days in the presence of IL-2. Subsequently, the primed T cells were restimulated for 4 more days with fresh APC (irradiated, T-cell-depleted splenocytes), heat-inactivated *Candida*, and IL-2 before quantitation of lymphoproliferation.

The most likely explanation for the difference in the results between these two studies is the fact that *C. albicans* is not a commensal organism in mice. Thus, it would appear that in vitro murine DC could not stimulate T-cell proliferation in a primary response even in the presence of IL-2. We hypothesize that after immature human DC phagocytose *Candida* they differentiate into mature DC with potent APC activity. In contrast, at least in vitro, murine FSDC and splenic DC apparently did not differentiate into mature APC. The only other reports that DC stimulated lymphocyte proliferation after phagocytosis of a viable microbial pathogen were with *L. major* (39, 51), *B. burgdorferi* (21), and *H. capsulatum* (24).

There now is considerable evidence that DC play a key role in the host defense against infections by bridging the gap between innate immunity and CMI (12, 57, 62, 73). Our data provide evidence that human DC may act early in *Candida* infection through uptake, processing, and presentation of antigenic material to T cells. A greater understanding of the interaction of DC with pathogenic microorganisms will not only offer insights into pathogenesis but will also provide unique strategies for the future development of immunotherapies.

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