The interaction of human dendritic cells with yeast and germ-tube forms of *Candida albicans* leads to efficient fungal processing, dendritic cell maturation, and acquisition of a Th1 response-promoting function

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Abstract: T helper cell type 1 (Th1) cell-mediated immunity plays a crical role in protection against the opportunistic pathogen *Candida albicans*. Virulence of the fungus is closely associated with its ability to form germ-tubes (GT), the early phase of the dimorphic transition from the commensal yeast (Y) to the more invasive hyphal (H) form. In this study, we examined the functional outcome of the interaction of Y or GT forms with human dendritic cells (DCs), professional antigen-presenting cells, which are pivotal for initiation and modulation of T cell responses. DCs phagocytosed and killed Y and GT cells with a comparable efficiency, becoming able to trigger strong proliferative responses by *Candida*-specific, autologous T cell clones. Both fungal forms induced DC maturation, as indicated by up-regulation of CD83, CD80, CD86, CD40, and major histocompatibility complex classes I and II surface antigens. Chemokine receptors were also modulated in *Candida*–DCs, which showed increased CCR7/CXCR4 and decreased CCR5 expression. Y- and GT-activated DCs differed in the pattern of cytokine expression. In particular, GT cells, in common with fully differentiated H cells, induced significantly more elevated levels of interleukin (IL)-10 than Y cells. Nevertheless, Y-, GT-, or H-pulsed DCs secreted comparable amounts of IL-12p70. In addition, irrespective of the fungal form triggering DC activation, *Candida*–DCs acquired the ability to prime naive T lymphocytes with a defined Th1 phenotype. Overall, our findings highlight the induction of substantially similar functional patterns in human DCs encountering the different forms of growth of *C. albicans*, both seemingly activating the Th1-type immunity which is characteristic of the healthy human subjects, naturally immunized and protected against the fungus. *J. Leukoc. Biol.* 75: 117–126; 2004.

Key Words: *dimorphism · phagocytosis · APC · antifungal immunity*

INTRODUCTION

Experimental work in animal models strongly suggests that CD4+ T helper cell type 1 (Th1)-mediated immunity is crucial in immunosurveillance and protection against the opportunistic pathogen *Candida albicans* [1, 2]. This notion is also indirectly supported by the observation that almost all healthy human individuals exhibit robust *Candida*-specific Th1-type immune responses and may safely harbor the fungus as a commensal in the gastrointestinal and other mucocutaneous tracts [3, 4]. Consequently, impaired antifungal T cell immunity is a major predisposing factor to severe mucosal candidiasis, such as those occurring in AIDS patients [5]. Conversely, disseminated forms of candidiasis are mostly associated with neutropenia, but T cell immunity clearly regulates defense mechanisms involved in systemic anti-*Candida* protection through phagocyte activation and help for production of protective antibodies [1, 6].

Initiation and maintainance of specific T cell responses strictly rely on the interaction of the fungus with professional antigen-presenting cells (APCs). Among them, dendritic cells (DCs) are particularly efficient in priming and expanding *Candida*-specific T lymphocytes in the inductive sites of the immune system [7, 8]. Immature DCs patrol peripheral tissues at potential sites of pathogen entry. Upon exposure to microbial pathogens and/or to signals from infected tissues, these cells can undergo maturation into potent T cell-stimulatory and migratory effector DCs, capable of activating naïve T cells in draining lymph nodes [7, 8]. During maturation, sentinel DCs are able to decode and integrate pathogen- and environment-derived information, thus acquiring not only the ability of triggering an immune response but also the competence for a fine regulation of the amplitude or the class of the response to be evoked in T cells [7, 9]. In this process, the nature of the DC-interacting pathogen plays an important role. In fact, it is increasingly evident that distinct microbial pathogens (or microbial products) can drive the development of qualitatively

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different T cell-immune responses through directly priming sentinel DCs into effector DCs with diverse functional phenotypes and Th-directing ability [1, 9–12].

Interactions of human DCs with *C. albicans* and their consequences in terms of initiation of T cell immunity against this important human pathogen have been only marginally studied. Newman and Holly [13] reported that in vitro-differentiated human DCs can phagocytose and kill unopsonized yeast (Y) cells of *C. albicans*, becoming able to activate memory T lymphocytes from autologous, *Candida*-sensitized individuals. In this study, however, no attempt was made to assess whether *Candida*-pulsed DCs also acquired the ability to prime naive T cells for a primary response nor to discriminate the responses generated in DCs by the different forms of growth of the fungus. These latter issues appear to be of major relevance, as the ability of *C. albicans* to convert in vivo from the Y to the hyphal (H) habit of growth has long been associated with the transition from commensalism to virulence [14–16]. Y-to-H switch is initiated, in vivo or in vitro, by the emergence of short H protrusions, usually referred to as germ-tubes (GT), which are considered a critical, transitional stage for host invasion by the fungus [15, 16].

Overall, a positive correlation between the ability to develop GT and the experimental pathogenicity of the fungus has been postulated by several authors [14, 16], but the actual role of the morphology switch for *Candida* virulence in humans is still debated, as most of the correlations between form of growth and virulence have been established in experimental models of rodents, which are not, as are humans, persistently colonized by the fungus [15]. Formation of GT/H cells has repeatedly been suggested to contribute to the pathogenesis of *Candida* infections by fostering immune evasion or immune deviation [1, 17–19]. In particular, in a murine model of invasive fungal infection, H-form *Candida* cells were shown to selectively prime a nonprotective host's immunity by means of modulation of DC differentiation and functions [20]. In fact, murine splenic DCs produced interleukin (IL)-12 and induced protective anti-*Candida* Th1-type immunity when activated by the Y form, whereas they produced IL-4 and promoted initiation of inefficacious Th2-type T cell responses when activated by the H form [20].

In human candidiasis, the ability to modulate human underlying T cell immunity through interaction with DCs might be instrumental to the fungus in favoring its persistence at infection sites. Several authors have indeed suggested an association between Th2-biased antifungal responses at a systemic or local level and severe or recurrent *Candida* infections [21–23]. Moreover, *Candida*-induced dysregulation or impairment of specific Th1 reactivity has been proposed to occur in concomitance with severe, disseminated infections such as chronic mucocutaneous candidiasis or *Candida* sepsis [24, 25].

These observations prompted us to examine whether the distinct fungal forms could induce different functional patterns in human DCs. We therefore investigated the functional activities of human monocyte-derived DCs exposed to Y or GT cells of *C. albicans* in an experimental in vitro model, mimicking the encounter of human immature, tissue-resident DCs with the fungus. Our results unambiguously demonstrated that human DCs can efficiently phagocytose and kill Y and GT forms of *Candida*; upon ingestion of either form, DCs undergo a full maturation process and acquire the ability to prime naive T lymphocytes with a defined Th1 phenotype.

MATERIALS AND METHODS

Reagents

Granulocyte macrophage-colony stimulating factor (GM-CSF; Leucomax) was purchased from Sandoz (Basel, Switzerland) and IL-4 from R&D Systems (Minneapolis, MN). Tritiated [^{3}H] thymidine (specific activity, 5 Ci/mmol) and [^{3}H] glucose (specific activity, 50 Ci/mmol) were from Amersham (Little Chalfont, UK). Phytohemagglutinin was obtained from Murex (Dartford, UK). Amphotericin B (Am B) was purchased from Gibco-BRL (Grand Island, NY). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma Chemical Co. (St. Louis, MO).

Media

RPMI 1640 (Euroclone Ltd., UK) was used, supplemented with 100 U/ml kanamycin, 1 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 10% fetal calf serum (FCS; Hyclone, Logan, UT; complete medium). Where indicated, FCS was replaced with 5% human serum (Sigma Chemical Co.).

Fungal cells

Live Y, GT, and H cells were obtained, as described previously [19], from the H conversion-competent strain BP, maintained in the established type collection of the Istituto Superiore di Sanità (Rome, Italy). Briefly, Y cells, grown in Winge medium for 24 h at 28°C, were washed in saline, resuspended at a density of 2×10^6 cells/ml in buffered Lee's medium, and incubated at 28°C or 37°C. At 28°C, fungal cells maintained the Y form, whereas at 37°C, more than 90% Y cells produced, within ~60 min, a short, H protrusion, as long or twice as long as the Y cell of origin (GT), which grew in the following 3 h of incubation, developing into H cells, five to seven times longer than the Y cell of origin. Y, GT, and H cells were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS at the desired concentration.

DC–*C. albicans* cocultures

Peripheral blood mononuclear cells were purified from heparinized blood on a density gradient (Lymphoprep, Nycomed Pharma AS, Oslo, Norway), as described previously [26], and were resuspended in PBS + 10% FCS. Monocytes were positively sorted using anti-CD14-labeled magnetic beads (MACS, Miltenyi, Germany), according to the manufacturer's instructions. Freshly isolated monocytes were resuspended in complete medium + FCS 10%, containing GM-CSF (200 U/ml) and IL-4 (100 U/ml) at 4×10^5 cell/ml, and were cultured in six-well culture plates at 3 ml/well for 5 days. Suspensions of live *C. albicans* cells were then added to the cultures at the DC:*Candida* ratio indicated in single experiments. To prevent *Candida* overgrowth, Am B (0.62 μg/ml) was also added to the cultures, 30 min after *Candida* pulsing. Control DC cultures were treated with Am B only or with Am B plus 0.1 μg/ml lipopolysaccharide (LPS). Phagocytosis of *C. albicans* cells by DCs was evaluated in each experiment by periodic acid-Schiff (PAS) staining of formaldehyde-fixed cytospin preparations from parallel cultures.

Evaluation of phagocytosis and killing of *C. albicans* by DCs

Phagocytosis was measured as inhibition of [^{3}H] glucose uptake by Y or GT cells upon ingestion by DCs, as described previously [17]. Briefly, triplicate cocultures of Y or GT cells with DCs (10^6/ml), at a DC:*Candida* ratio of 1:4, were prepared in complete medium without Am B and incubated 45 min at 37°C, 5% CO_2, to allow phagocytosis of fungal cells by DCs. Uninternalized *Candida* cells were then labeled with [^{3}H] glucose for 1 h. Percent phagocytosis was calculated by comparing the radiolabel incorporation by fungal cells cultured with DCs with the incorporation by control Y or GT cells cultured without DCs.

Killing of the different fungal forms was evaluated in triplicate cocultures, in the same medium as above, at a DC:*Candida* ratio of 10:1 or 5:1, upon a 4-h interaction [21]. DCs were then lysed with 0.2% Triton X-100, and survived fungal cells were enumerated by colony-forming unit (CFU) counts. Percent killing was calculated by comparing CFU counts from *Candida*:DC cocultures with CFU from cultures of Y and GT alone.

Fluorescein-activated cell sorter (FACS) analysis

Cells were stained with the following antibodies: anti-human leukocyte antigen (HLA) class I, HLA class II, CD1a, CD14, CD25, CD40, CD80, CD83, and CD86 and with the antichemokine receptor antibodies anti-CCR5, CXCR4, and CCR7, all purchased from PharMingen (San Diego, CA). Viability of cells was evaluated by propidium iodide (PI) staining (Sigma Chemical Co.). Human-adsorbed, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunogobulin G (IgG) and goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) were used as the secondary antibodies. Staining of intracellular cytokines in T cells following stimulation with DCs was performed using phycoerythrin-conjugated rat anti-human IL-4 or IL-10 and FITC-conjugated mouse anti-human interferon-γ (IFN-γ) or IL-2 in peridinin chlorophyll protein-conjugate $CD3^+$ cells (PharMingen) after fixation and permeabilization of cells with Cytofix/Cytoperm™ (PharMingen), according to the manufacturer's instructions. Stained cells were analyzed by flow cytometry using a FACScan cytometer (Becton Dickinson, Mountain View, CA) using Cellquest Software (Becton Dickinson). PI was used to exclude dead cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

PolyA + RNA was extracted from immature, control DCs or from *Candida*- or LPS-treated cells (1×10^6), using the Micro-FastTrack 2.0 kit (Invitrogen, San Diego, CA), according to the manufacturer's instructions, and reverse-transcribed as described previously [19]. Aliquots of cDNA yielding equivalent amounts of β-actin-amplified band were used for the semiquantitative evaluation of cytokine gene expression. PCR was performed in a 10-μl vol in a Perkin-Elmer 9600 thermal cycler. Cytokine-specific primer pairs were synthesized by Gibco-BRL, according to published sequences [26–31], and amplification conditions were chosen accordingly. The PCR products were visualized by electrophoresis and ethidium-bromide staining and were identified by their expected molecular size.

Antigen-presentation ability and priming of naïve T cells by DCs

Decreasing numbers of immature DCs, LPS-matured DCs, and *Candida*-treated DCs were assayed for their capacity to induce proliferation of autologous, specific, anti-*Candida* T cell clones (TCC; 5×10^4 cells/well) or allogeneic cord blood lymphocytes (CB-T; 5×10^4 cells/well). In some experiments with TCC, DCs pulsed with decreasing numbers of Y or GT cells were compared with DCs loaded with specific fungal antigens and matured by LPS treatment. The assays were performed in complete medium supplemented with 5% human AB serum. Generation and characterization of anti-*Candida* TCC have been reported elsewhere [4]. The proliferative response was measured on day 5 by a 16-h pulse with [^{3}H] thymidine. To examine the functional polarization induced in naive T lymphocyte by immature DCs, LPS-treated DCs, and Y-, GT-, or H-pulsed DCs, these cells were cultured for 7 days with CB-T. Intracellular staining for cytokine production by T cells was performed after stimulation of T cells for 5 h with 10^{-7} M PMA and 0.5 μg/ml ionomycin in the presence of brefeldin at 2 μg/ml. For cytokine determination in the supernatants, parallel cultures were stimulated as described in the absence of brefeldin.

Cytokine determination

Culture supernatants from DC–*Candida* cocultures or from cultures of control or LPS-matured DCs were collected at 7, 18, or 48 h after the administration of fungal cells or LPS. Supernatants of CB-T cells were obtained as described above. Supernatants were frozen until use. Cytokine secretion [IL-10, IL-12p70, and transforming growth factor-β (TGF-β)] was determined using commercially available kits (R&D Systems), according to the manufacturer's instructions, and were expressed in pg/ml.

Statistics

The data were assessed for statistical significance by two-tailed Student's *t*-test or nonparametric Mann-Whitney U-test, as appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

Phagocytosis and killing of *C. albicans* by human DCs

In a first series of experiments, we tested, in comparison, the ability of DCs to phagocytose and kill Y or GT cells of *C. albicans*. We observed that DCs efficiently ingested both fungal forms. In fact, the mean ± SD percent fungal cells ingested by DCs, measured at 2 h in three independent, triplicate experiments at an effector:target (E:T) ratio of 1:4, were 87 ± 16 and 57 ± 12 for Y or GT cells, respectively. Microscope examination of PAS-stained Y– or GT–DC cocultures, at E:T ratios ranging from 1:1 to 1:5, constantly showed that after 18 h of incubation, more than 95% of DCs contained at least one but frequently up to five fungal cells. At lower E:T ratios (1:10), a substantial percentage of fungal cells remained uningested, and further increases of fungal doses were associated to progressively higher DC mortality (data not shown).

As DCs have previously been shown to kill *Candida* cells by intra- and extracellular mechanisms [13], we measured fungal killing by a classical plate-counting assay designed to measure intra- and extracellular killing at the same time. Enumeration of *Candida* CFU after a 4 h-coincubation with DCs, at an E:T ratio of 10:1, indicated that both fungal forms were markedly killed by DCs (56±20 and 32±6 mean percent killing±SD in three independent, triplicate experiments for Y or GT cells, respectively). Small differences between Y and GT cell susceptibility to phagocytosis and killing by DCs were not statistically significant, as assessed by two-tailed Student's *t*-test.

Therefore, these experiments indicated that both fungal forms are efficiently internalized and degraded by human DCs.

Antigen presentation ability by Y– and GT–DCs

To evaluate the ability of Y- or GT-pulsed DCs for activation of memory *Candida*-specific T lymphocytes, we resorted to the use of specific human TCC generated against MP-F2, a purified antigenic preparation containing major immunodominant antigens of the fungus, such as the MP65 mannoprotein [4]. T cells were cocultured with autologous DCs pulsed with increasing concentration of Y or GT cells, and after 5 days of coculture, proliferation was evaluated by [^{3}H] thymidine incorporation. LPS-matured, autologous DCs, pulsed or not with the specific MP-F2 antigen, were used as the positive and the negative control, respectively. As shown in **Figure 1**, these experiments confirmed that Y and GT cells were efficiently processed for antigen presentation by DCs and could comparably activate DCs for stimulation of a robust, memory, proliferative response by MP-F2-specific T cells. TCC proliferation induced by Y- or GT-pulsed DCs was comparable with that induced by DCs which have been loaded with the specific MP-F2 fungal antigen and then induced to mature by LPS treatment, whereas no

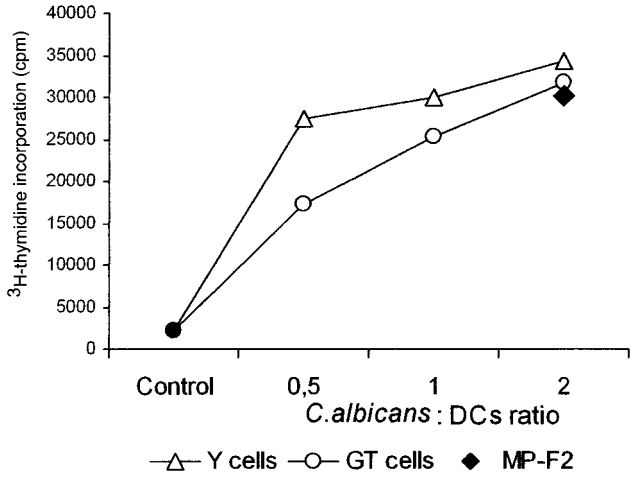

Fig. 1. Y- and GT-pulsed DCs efficiently present *Candida* antigens to *Candida*-specific CD4+ T cells. Autologous, monocyte-derived DCs, treated overnight (o.n.). with an increasing concentration of Y or GT cells, were assayed for their ability to stimulate proliferation of *Candida* antigen-specific, human TCC. Positive and negative control DCs were, respectively, DCs pulsed with the purified fungal antigen (MP-F2, 1 μg/ml) and then matured with LPS (◆) and cells matured by LPS treatment without *Candida* cells or antigen (●). DCs (5000/well) were cocultured with TCC (5×10^4/well) for 5 days. Proliferative response was measured as [^{3}H] thymidine incorporation and is expressed as mean counts per minute (cpm) of triplicate cultures. Data are from one representative experiment out of two performed with similar results.

significant proliferation was triggered by unpulsed, LPS-matured DCs (Fig. 1).

Phenotypic characterization of *Candida*-simulated DCs

We next investigated whether the phagocytosis of *Candida* cells could trigger the process of DC maturation by analyzing DC phenotype after an o.n. cocultivation with Y or GT cells. The interaction with both fungal forms, at a DC:*Candida* ratio of 1:1, induced the expression of the maturation-related surface antigen CD83 at levels comparable with those observed after treatment with bacterial LPS, a potent maturation stimulus for DCs (**Fig. 2A**). However, unlike LPS, neither Y nor GT cells up-regulated CD25, another maturation-related protein (Fig. 2A). Y- and GT-treated DCs showed a similarly increased expression of the costimulatory molecules CD80 (B7.1), CD86 (B7.2), and CD40, as well as of HLA class I and II DR molecules (Fig. 2B). No gross differences in the phenotype displayed by *Candida*-matured DCs were observed using the two different DC:fungal cell ratios of 1:1 or 1:5 or when live fungal cells were replaced with predifferentiated, heat-inactivated (30 min, 80°C) Y or GT cells, in the absence of Am B (data not shown).

Flow cytometric analysis also revealed significant modulations in the expression of distinct chemokine receptors in *Candida*- or LPS-matured DCs as compared with immature cells. All stimulants induced a marked up-regulation of CCR7, in concomitance with down-regulation of CCR5 expression (Fig. 2C). Note that the chemokine receptor CXCR4 was up-regulated only upon *Candida*-induced maturation and only weakly increased or not increased at all upon maturation with LPS.

Cytometric analysis, therefore, indicated that the interaction with *C. albicans* induced DCs to express a full array of phenotypic/functional surface markers typical of mature DCs, with no appreciable differences between Y or GT cells.

Pattern of cytokine expression by *Candida*-matured DCs

Cytokine production is a major functional aspect of DC maturation and differentiation and critically impacts on the ability of DCs to activate and prime T cells. Therefore, we analyzed the profile of cytokine expression induced in DCs by stimulation with Y or GT cells in comparison with LPS.

RT-PCR analysis of cytokine mRNA expression after o.n. stimulation with *Candida* cells (**Fig. 3A**) showed that Y-matured DCs expressed abundant IL-12p40 but only scarce IL-10 mRNA transcripts. In contrast, GT-matured DCs expressed substantial levels of IL-12p40 and IL-10 transcripts. The two fungal forms induced similar TNF-α and low IL-6 expression, whereas GT cells appeared to be more efficient than Y cells in eliciting the chemokines IL-8 and MIP-1α (Fig. 3A). Similar to LPS, both *Candida* forms markedly down-regulated TGF-β1 mRNA expression. No expression of IFN-α or IL-4 mRNAs was observed following stimulation of DCs with Y or GT cells or with LPS (data not shown).

In these experiments, we also observed a sustained transcription of the CCR7 gene in *Candida*- and LPS-matured DCs, thus confirming the results obtained by flow cytometric analysis (Fig. 3A).

Following these results, we compared more in detail Y and GT cells for their ability to stimulate the release of the Th1-related IL-12p70 and of the regulatory IL-10 cytokine. In dose-response experiments (Fig. 3B), the two forms were found almost equally efficient in eliciting a strong IL-12 response by DCs, whereas the IL-10 protein was detected in the supernatants of GT-matured DCs at severalfold higher concentrations than in supernatants of Y-matured DCs, in particular, when a high number of fungal cells were administered to DCs. A similar kinetics of cytokine accumulation in supernatants of *Candida*–DC cocultures was seen after stimulation of DCs with Y or GT *Candida* cells. IL-12 and IL-10 were at low/undetectable levels 8 h after the administration of fungal cells; peak release of both cytokines occurred within 8 and 18 h, and their concentrations only moderately increased in the following 30 h of coincubation (data not shown).

A direct comparison of IL-12 and IL-10 levels following treatment with Y and GT cells in DCs from four different subjects is given in Figure 3C. As Y cells, but not H cells, are able to trigger IL-12 production by purified, murine, splenic DCs [20] and as GT cells are only in the initial phase of Y-to-H transition, we also assayed for IL-12 and IL-10 production by DCs upon activation by fully elongated H cells. Although with donor-dependent, quantitative variations, DCs produced substantial levels of IL-12 in response to any type of fungal form without significant differences among Y, GT, or H cells, as assessed by the two-tailed Mann-Whitney U test (Fig. 3C). Conversely, and similarly to that observed in dose-response experiments, GT and H cells reproducibly triggered, in the DCs from the same donors, an IL-10 production significantly higher than that induced by Y cells (mean pg/ml SEM: 32+15,

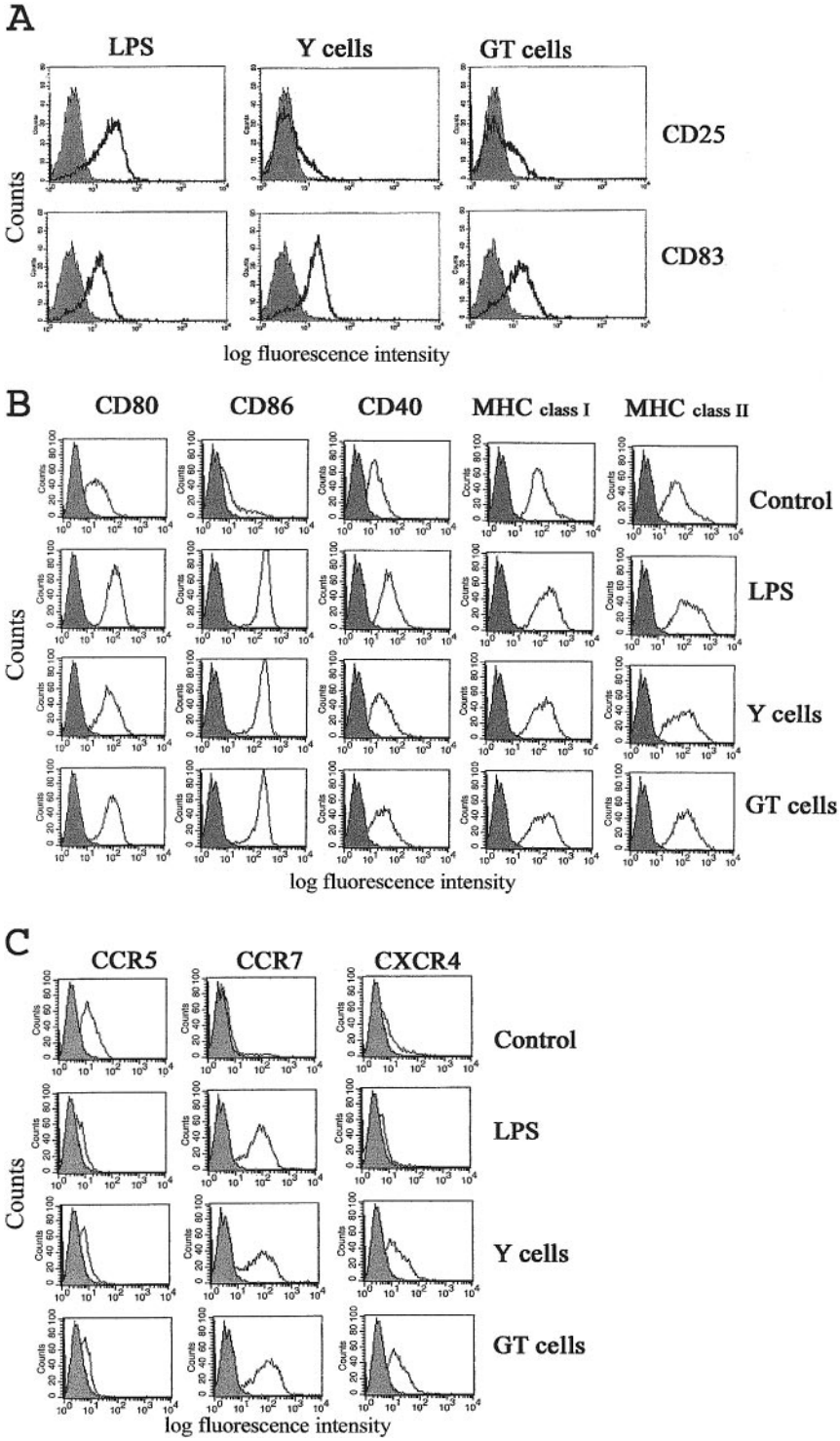

Fig. 2. Interaction with Y or GT cells induces maturation of human DCs. (A) Surface expression of maturation (CD83) and activation (CD25) markers on DCs following stimulation with LPS or with live Y or GT cells. Empty histograms refer to stimulated DCs, whereas filled histograms represent control, unstimulated DCs. (B) Expression of costimulatory [B7.1 (CD80), B7.2 (CD86), CD40] and HLA class I and II molecules in untreated (Control) DCs as compared with LPS-, Y-, or GT-treated DCs. Filled histograms represent staining with an isotype-matched, irrelevant antibody. (C) Modulation of chemokine receptors induced by LPS, Y, or GT treatment. Control shows expression on untreated, immature DCs. Filled histograms represent staining with an isotype-matched, control antibody. FACS analysis of DCs was performed after 18 h of incubation in the presence of *Candida* cells (1:1 ratio with respect to DCs) or LPS (0.1 μg/ml). Control DCs were incubated in medium only. Data are representative of three experiments.

296+109, and 327+132 for Y-, GT- or H-stimulated DCs, respectively; $P<0.05$ comparing Y cells with GT or H cells, Mann-Whitney U test; Fig. 3C).

On the whole, these experiments indicated that Y- and GT- or H-cell-driven DC activation was associated with release/expression of several immunomodulatory cytokines and that the distinct fungal forms, although triggering comparable IL-12 responses by DCs, displayed some differences in the pattern of cytokine induction; in particular, the production of IL-10 was preferentially stimulated by the GT/H forms.

APC functions of *C. albicans*, matured DCs

Functional assays were then performed to evaluate whether Y- and GT-matured DCs were capable of stimulating naive T lymphocytes for a primary response and to characterize the type of functional polarization induced in T cells by these DCs. We again included in these experiments DCs stimulated with H cells, as it has been previously reported that this form, unlike the Y form, selectively primes murine DCs with the ability to trigger Th2-type responses in vivo [20].

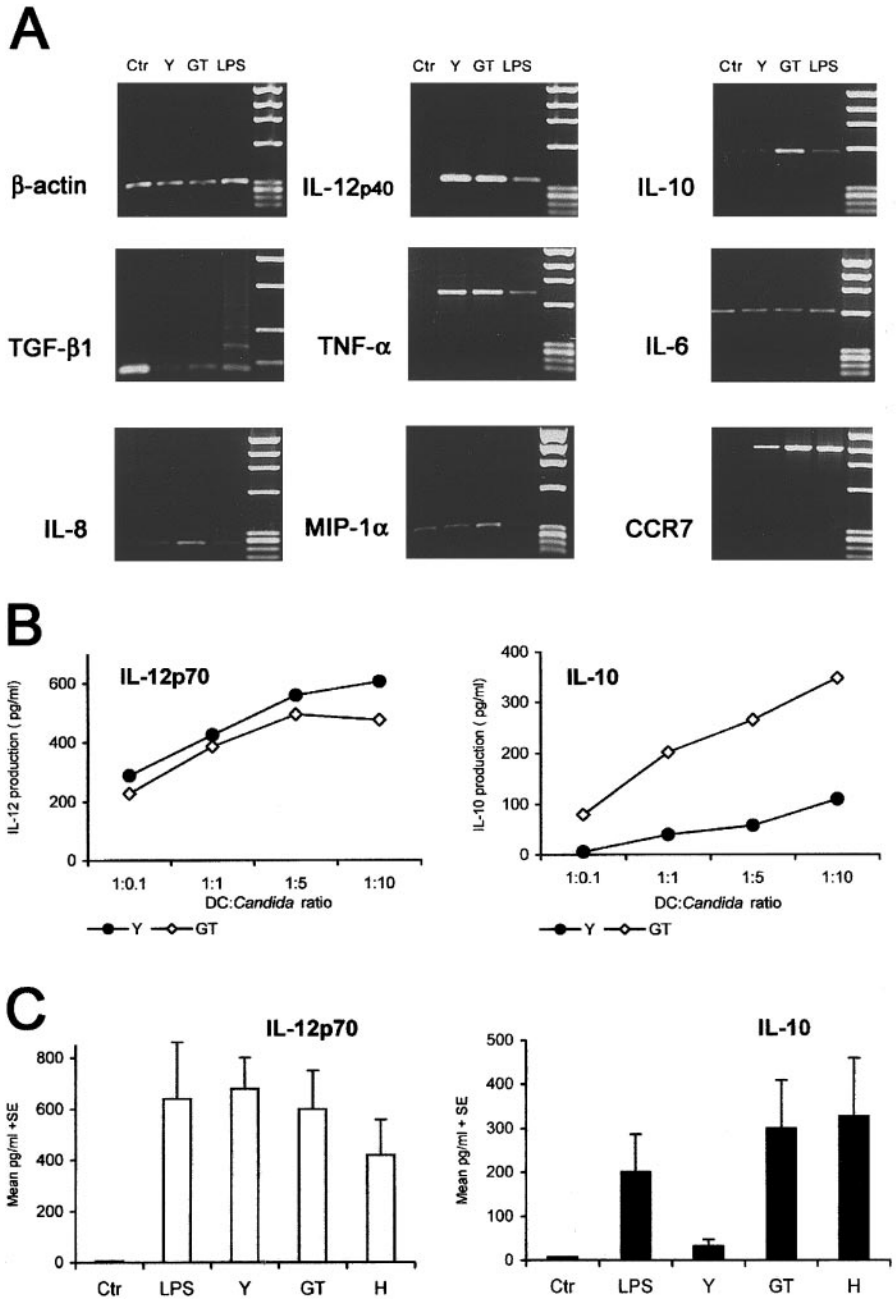

Fig. 3. Pattern of cytokine expression by DCs upon LPS- or *Candida*-induced maturation. (A) Comparative RT-PCR analysis of cytokine mRNAs in Y-, GT-, or LPS-stimulated DCs. DCs were left untreated (Ctr) or stimulated with *Candida* Y or GT cells (DC:fungi ratio, 1:1) or with LPS (0.1 μg/ml) for 18 h before mRNA extraction. Product from the amplification of β-actin cDNA was used to standardize the amount of cDNA template among different samples. One representative experiment out of two independently performed is presented. TNF-α, Tumor necrosis factor α; MIP-1α, macrophage-inflammatory protein-1α. (B) Dose-response IL-12p70 and IL-10 production by DCs stimulated with Y or GT cells. DCs (5×10^5/ml) were cocultured with fungal cells at the indicated ratios. Cytokines released in culture supernatants were measured after 18 h of incubation. (C) IL-12p70 and IL-10 concentration in culture supernatants of DCs following overnight stimulation with LPS (0.1 μg/ml) or with Y, GT, or H forms of *C. albicans* (DC:fungi ratio, 1:1). Control DCs (Ctr) were incubated in medium only. Values in the graph are are mean ± SEM cytokine concentrations measured in four independent experiments with DCs from different subjects.

When allogeneic CB-T were cultured with Y-, GT-, or H-activated DCs, T cell proliferation was significantly stimulated, irrespective of the fungal form used for DC activation (**Fig. 4A**). It is interesting that the proliferative responses induced in CB-T by Y- or GT-matured DCs were always higher than those induced by LPS-matured DCs, as four- to eightfold fewer Y– or GT–DCs were required to trigger the same levels of proliferation stimulated by LPS–DCs (Fig. 4A). In comparison to Y- or GT-activated DCs, H–DCs were found to be less efficient in inducing proliferation by T cells. Nevertheless, the proliferation levels stimulated by these DCs were totally comparable with those induced by LPS-matured DCs (Fig. 4A).

More importantly, flow cytometric analysis of intracellular cytokine accumulation in proliferating CB-T lymphocytes showed that Y- or GT- and even H-matured DCs induced in naïve T cells culture an equivalent number of cells expressing IFN-γ or IL-2 (Fig. 4B). Levels of IFN-γ and IL-2 accumulation in CB-T stimulated by *Candida*–DCs were always comparable with those induced by LPS-matured DCs (Fig. 4B). In addition, no significant percentage of T cells preferentially producing IL-4 or IL-10, suggestive of a Th2-type polarization or T regulatory cell (Treg) induction, was found by FACS analysis (Fig. 4B). In line with this finding, similar, low levels of IL-10 were measured in supernatants from lymphocyte cultures stimulated by *Candida*– or LPS–DCs (66±12, 41±14, 37±14, and 44±9 mean pg/ml±SE in three independent experiments for CB-T stimulated by Y–, GT–, H–, or LPS–DCs, respectively), and no significant production of TGF-β was observed in CB-T cell-culture supernatants, as determined by enzyme-linked immunosorbent assay (data not shown). Lastly, the number of CD4+CD25+ double-positive cells was totally comparable

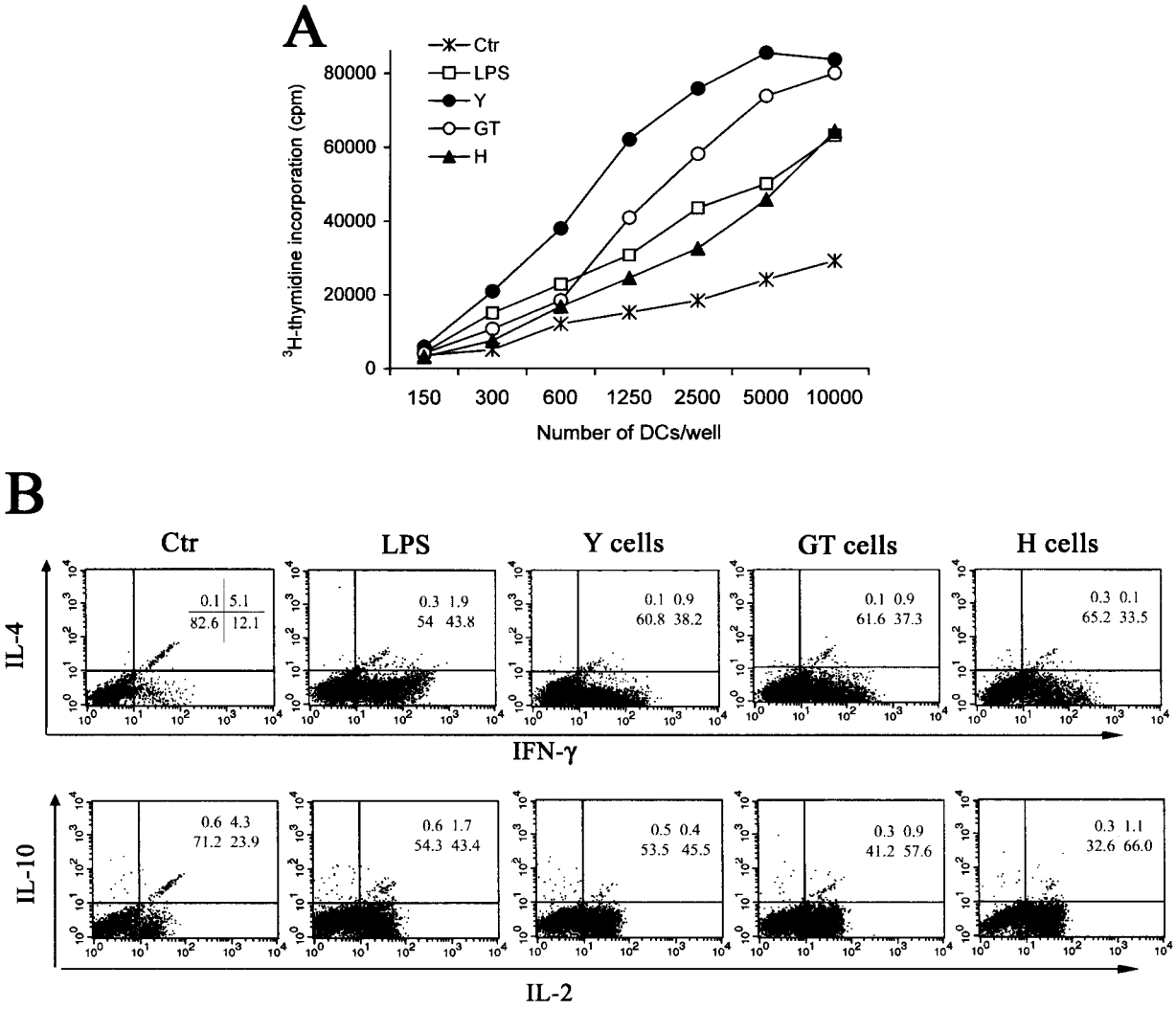

Fig. 4. Maturation induced by Y, GT, or H cells licenses human DCs for efficient priming of naive T lymphocytes with a Th1-type functional polarization. (A) Proliferative response of allogeneic CB-T stimulated with immature DCs (Ctr) or with Y-, GT-, H-, or LPS-matured DCs. DCs were induced to mature by an o.n. cocultivation with fungal cells (1:1 ratio) or by treatment with LPS (0.1 μg/ml). Decreasing numbers of DCs, as indicated, were then cocultured with 5×10^4 CB-T/well. After 5 days, the proliferative response of T cells was evaluated by [^{3}H] thymidine incorporation and expressed as mean cpm of triplicate cultures. Data are from one representative experiment out of two performed with similar results. (B) Flow cytometric analysis of intracellular cytokine accumulation in CB-T primed by Y-, GT-, H-, or LPS-activated, allogeneic DCs. APCs were treated overnight as described in A and cocultured for 6 days with CB-T. Results shown in figure are from one experiment out of two performed.

in lymphocyte cultures activated by Y–, GT–, or H–DCs, as well as by LPS (data not shown).

Altogether, these findings indicated that CB-T lymphocytes stimulated with Y-, GT-, or H-matured DCs undergo a definiteTh1-type polarization.

DISCUSSION

No specific or detailed investigation on the activation of human DCs by the dimorphic fungus *C. albicans* has been addressed, despite the importance of DCs for initiation and maintanance of the protective immunosurveillance, which in the immunocompetent host, hampers tissue invasion by this opportunistic pathogen. In an attempt to fill this gap, we comparatively examined responses generated in DCs upon activation with Y and GT cells of the fungus, in consideration of the supposed, differential role of the two forms in fungal virulence and immunoevasion [14–20].

Initiation of an antifungal response by DCs requires active internalization of fungal cells and/or antigens and processing in vacuolar compartments to allow generation of major histocompatibility complex (MHC)-antigen complexes to be presented to T cells on the surface of DCs [32]. Although DCs are capable of rapidly ingesting numerous microbial pathogens, their ability to kill and degrade microbial cells is extremely variable, depending on the nature of the DC itself and the specific microbial target [33].

Human monocytes/macrophages and neutrophils internalize with different efficiency Y and GT/H cells of *C. albicans*, being the GT/H forms particularly refractory to phagocytosis [19, 34, 35]. Preferential efficacy of different fungicidal mechanisms against the two fungal forms and partial resistance of GT/H cells to phagocyte killing have also been described [35, 36].

Our investigations did not reveal any substantial difference between Y and GT cells in their susceptibility to phagocytosis and killing by DCs, as a comparable percentage of cocultured Y or GT cells was actually ingested and killed by immature DCs. This suggests that DCs can exert an even higher anticandidal activity as compared with other human phagocytic effectors. Overall, our results confirm and extend recent studies showing that human DCs are more potently phagocytic and fungicidal than macrophages against Y cells of *C. albicans* and other important fungal pathogens, such as *Histoplasma capsulatum* [16, 37]. They also indicate that both fungal forms, upon DC encounter, may undergo extensive and comparable antigen processing for MHC II/MHC I presentation.

In analogy with other microorganism [33], interaction with *Candida* cells triggered DC maturation and activation, as indicated by the increased expression of several maturation-associated surface markers and costimulatory molecules involved in antigen presentation and T cell stimulation [7–10]. *Candida*-driven maturation was also accompanied by down-modulation of CCR5 and substantially increased surface expression of CXCR4 and CCR7 chemokine receptors, suggesting that upon *Candida* encounter, DCs acquire the competence for colocalization with T cells in secondary lymphoid organs [38, 39].

We did not observe any gross difference in the phenotypic profiles displayed by Y- or GT-matured DCs. Instead, *Candida*-matured DCs showed some differences from LPS-matured DCs. These differences, however, are not surprising, considering that fungal pathogen-associated molecular patterns (PAMPs) possibly involved in *Candida*-driven DC maturation (mannan/β-glucan) are quite different from LPS, in molecular nature and in receptor specificity [40]. *Candida* cells, but not LPS, up-regulated the surface expression of CXCR4, a chemokine receptor that has been involved in promoting colocalizion of DCs with naive T and B cells in lymphoid tissues [38, 39]. Conversely, the CD25 antigen, a maturation-associated molecule that is up-regulated upon LPS- and bacterial-induced DC activation [41], was not expressed by *Candida*-matured DCs. In murine models, expression of CD25 (α chain of IL-2 receptor) by mature DCs has been suggested to link the functional fate of these cells to the activation of T lymphocytes during antigen presentation [42]. However, possible functional consequences deriving from low expression of this receptor on *Candida*-matured DCs cannot be fully appreciated, as biological activity and functions of CD25 in human DCs are still uncertain.

It is of interest that maturation induced by Y or GT *Candida* cells triggered a different pattern of cytokine expression by DCs. The most remarkable difference was that GT-matured DCs produced significantly more elevated amounts of IL-10 as compared with Y-matured DCs, as observed at gene expression and at protein level, and using several different *Candida*:DC ratios. It is noteworthy that GT cells shared their particular competence for IL-10 induction with fully differentiated H cells. This strengthens the notion that GT forms, although being only the initial phase of H development, are closely similar to H cells, not only in molecular patterns expressed at their surface [43] but also in their interaction with different immunocompetent cells [17–19, 43]. Differential ability for IL-10 induction also suggests that Y and GT/H forms are differently recognized by human DCs and can activate distinct IgG-Fc downstream signaling events in these cells. Recently, this has been reported to occur in murine DC, as in these DCs, the preferential internalization of Y forms through the mannose-fucose receptor (MR) or the prevalent engagement of the IgG-Fc region and CR3 receptors for ingestion of H forms induces different activatory patterns, resulting in priming of APCs with different ability for IL-12 production and T cell polarization [40]. Mannan and β-glucan fungal PAMPs might mediate differential recognition by DCs, as they can engage distinct phagocytic/activatory receptors [40] and are differently expressed in Y and GT/H cells [19, 43].

Nevertheless, differential cytokine expression upon stimulation by the distinct *Candida* forms did not appear to affect DC competence for T cell activation or polarization. In fact, Y- and GT-activated DCs were extremely and equally potent in triggering a prompt expansion of *Candida*-specific or naive T lymphocytes, with no need for additional maturation treatments and in the absence of exogenously added IL-2. No significant difference in efficiency of antigen presentation or T cell activation was noticed between Y- or GT-matured DCs, as shown by an equal capacity to stimulate high-level proliferation in autologous CD4+ T cell clones specific for a major *Candida* antigen, MP-F2 [4]. More importantly, regardless of the different cytokine expression, Y- and GT/H-activated DCs could efficiently prime naive T lymphocytes for an allogeneic response and clearly induced a typical Th1-type polarization in these cells. In fact, CB-T cells, expanded by Y- or GT/H-matured DCs, were able to secrete IFN-γ and IL-2 but not IL-4, IL-10, or TGF-β. The ability of Y- and GT/H-activated DCs to drive T cells into the Th1 phenotype likely reflected their elevated secretion of the Th1-promoting cytokine IL-12.

The different ability by Y and GT/H cells for IL-10 induction is, however, intriguing, as this cytokine can potently modulate DC functions [12, 44]. In particular, *Candida*-activated, IL-10-producing DCs have been shown to expand in vivo Tregs, which down-regulate antifungal Th1 reactivity in mice with gastrointestinal candidiasis [45]. In our in vitro model of T cell priming, we did not find any gross evidence for preferential induction of Treg cells by GT-activated DCs, as indirectly judged from enumeration of T cells preferentially expressing IL-4, IL-10, or TGF-β, i.e., the cytokines typically produced by Treg cells [12, 46]. Nevertheless, it cannot be excluded that GT and H cells, by preferentially inducing IL-10 expression in human DCs, might play a role in triggering anti-*Candida*-regulatory T cell responses in vivo, which in turn, may suppress or attenuate endogenous Th1 immunity. This, however, might not necessarily imply immunoevasion, given the fact that Tregs have been shown to be beneficial rather than detrimental to the host in experimental murine candidiasis [45]. Besides T cell differentiation and activation, differential cytokine secretion by Y- and GT-stimulated DCs may also be involved in other function of DCs, such as the stimulation of B or natural killer lymphocytes [47, 48], and clearly deserves further investigation.

In a recent report, Fè d' Ostiani et al. [20] have suggested that formation of GT and H cells in the course of experimental murine candidiasis may modulate the activation of DCs as to induce an inefficacious, primary, antifungal T cell response. In

fact, murine DCs exposed to Y cells secreted IL-12 and primed protective Th1 responses in vivo, whereas H-exposed DCs produced IL-4 and no IL-12 and primed nonprotective Th2 cells [20]. Our finding that both fungal forms similarly activate human DCs for IL-12 production and Th1-type polarization of naïve T lymphocytes does apparently contrast with this study. It is noteworthy that "myeloid" murine DCs, as the prevalent DC subpopulation in the spleen or as a murine DC line, were prevalently analyzed by Fè dÕOstiani et al. [20, 49, 50]. This murine DC subset is considered a poor IL-12 producer, which preferentially induces Th2-oriented responses in CD4+ T cells [48, 50]. Other murine "nonmyeloid" DC subpopulations have been found to be unable to discriminate between the different fungal forms, in terms of IL-12/IL-4 production [45]. Conversely, monocyte-derived, human DCs are functionally biased for efficient IL-12 production and Th1-type lymphocyte priming, as compared with other known human DC subsets, although are also able, when properly activated, to drive Th2 cell differentiation [49, 50]. Therefore, differences in species and subtype of the DC populations analyzed may explain the discrepancy between the two studies, suggesting that the ultimate nature of DC functional activation by Y or GT/H cells may be predominantly dictated by the specificity of the responder DCs. The specific repertoire of *Candida*-recognizing receptors expressed by distinct DC species and/or subsets might determine the responsiveness of a given DC population to each fungal form. In fact, recognition of *C. albicans* by DCs involves a number of distinct surface receptors, including the MR, the CR3, the DC-specific intercellular adhesion molecule-3-grabbing nonintegrin molecule (DC-SIGN), the recently described β-glucan receptor dectin-1, and the Toll-like receptor 2 [49, 51, 52], whose interplay may differently regulate DC activation in response to the fungus.

Human monocyte-derived DCs are "model" DCs, which cannot be identified with any naturally occurring, human DC population. Nevertheless, for most phenotypic and functional characteristics, these cells are considered representative of immature, dermal, or interstitial antigen-capturing DCs of skin and lymphoid organs [8, 50]. Our study demonstrates that the encounter of these cells with Y or GT cells of *C. albicans* leads to efficient processing of fungal cells for antigen presentation, stimulates the maturation of DCs, and induces DC functional competence for priming a Th1-type polarization in naive T lymphocytes. Although in vitro studies are inevitably reductive and may not reflect DC reactivity in the different in vivo settings, our findings strongly suggest that in vivo, Y- or GT-presenting, human DCs can efficiently recall fungus-specific T cell memory responses and also initiate primary, highly protective CD4+ Th1-type anti-*Candida* immunity.

On their early encounter with the fungus, DCs of unprimed individuals are likely to experience both forms of the fungus, as *C. albicans* rapidly develop GT and H cells when in contact with serum or body fluids [15]. The finding that Y or GT cells can license DCs for Th1-type priming fits well with the observation that natural immunization with *C. albicans* induces in humans the onset of a strongly Th1-polarized T cell immunity. [3, 4, 15, 27]. Our results are also in line with the recent observation that highly polarized, Th1-type, *Candida*-specific vaginal lymphocytes are quite predominating, not only in healthy women but also in patients suffering from recurrent vaginal candidiasis, a pathology that is typically characterized by a massive invasion of vaginal tissue by GT/H-form *Candida* cells [53].

Formation of GT/H cells in vivo is believed to contribute to fungal virulence by suppressing Th1-type host reactivity or by inducing nonprotective Th2-type immunity, a notion mainly founded on experimental work in animal models of disseminated *Candida* infection [1, 2, 17–20]. Data presented here indicate that GT/H formation is insufficient per se to directly modulate DC functional differentiation for weakening protective, antifungal defense in the human host. This, however, might be achieved by other mechanisms, including antigenic variations or expression/induction of immunosuppressive molecules, and by interaction with other immunocompetent cells [1, 17–19, 43].

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