The exploitation of distinct recognition receptors in dendritic cells determines the full range of host immune relationships with Candida albicans

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Abstract

Dendritic cells (DC) sense saprophytic yeast and pathogenic, filamentous forms of Candida albicans in a specific way, resulting in disparate patterns of DC and Th cell activation. Using human and murine DC, such disparate patterns could be traced to the exploitation of distinct recognition receptors. Although usage of mannose receptors led to protective type 1 responses in mice, entry through Fcγ receptors was responsible for suppression of mannose receptor-dependent reactivity, onset of type 2 responses and associated pathology. As the usage of distinct receptors selectively occurred with yeast or hyphal forms of the fungus, these findings suggest that the responsibility for pathogenicity of C. albicans is shared by the organism and DC, with implications for fungal virulence, immunity and vaccine development.

Introduction

Multiple receptors on dendritic cells (DC) participate in the microbial recognition event (1–3). Receptors for antigen recognition and uptake by DC include mannose receptors (MR), the β-glucan receptor Dectin-1, complement receptors (CR), Fcγ receptors (FcγR) and the pattern-recognition receptors (PRR) (4–9). Recognition of pathogen-associated molecular patterns (PAMP) shared by a diverse array of pathogens is considered to be a prerequisite for the innate system to discriminate among pathogens and to instruct the adaptive immune response (9).

Candida albicans is the most frequently isolated fungal pathogen in humans (10). The delicate balance between the host and this otherwise harmless commensal fungus may turn into a parasitic relationship, resulting in the development of severe infections. It is recognized that the nature and extent of the impairment of host defense influence the manifestation and severity of infection (10). However, the fungus is not a mere passive participant in the infectious process and a hypothetical set of virulence factors has been attributed to it (11). C. albicans possesses a variety of PAMP (12), including glucans, mannans, mannoproteins and phospholipomannan, which are capable of initiating phagocytosis and activating pro-inflammatory pathways upon recognition by MR and β-glucan receptors, mannose-binding lectins (MBL) and the Toll-like receptor 2 (8,13–15). In addition, the environmental interaction of the fungus is profoundly affected by antigenic variability, phenotypic switching and dimorphic transition from unicellular yeast to filamentous forms (10,16–18).

Candida handling by DC may be most critical at the host/fungal interface, because the organism behaves as a commensal or as a true pathogen of skin and mucosal surfaces (10). Recent evidence indicates that DC discriminate...
between the different forms of the fungus, resulting in the activation of functionally opposing T<sub>h</sub> cell responses (19,20). Upon ingestion of different fungus morphotypes, the downstream cellular events, ultimately leading to DC activation and maturation, have been found to be considerably different.

The present study was undertaken to determine the impact of fungal recognition via different receptor ligands expressed by DC. We exposed human DC as well as DC from mice lacking Fc<sub>R</sub>I, II and III (Fc<sub>R</sub>I<sup>+</sup>) or CR3 (CR3<sup>+</sup>) to live unopsonized or differentially opsonized Candida yeasts or hyphae in the presence of saturating amounts of receptor-specific F(ab')<sub>2</sub> antibody fragments or receptor-specific ligands. We assessed the relative contributions of MR1, Dectin-1, CR3 (CD11b/CD18) and Fc<sub>R</sub>I to phagocytosis, cytokine production, expression of co-stimulatory molecules and nature of the Candida-specific immune response. Several distinct recognition receptors mediated the uptake of unopsonized yeasts or hyphae; however, opsonization greatly affected receptor engagement and cooperativity. The engagement of different receptors translated into downstream signaling events, ultimately affecting cytokine production and co-stimulation. In vivo studies confirmed that the use of different receptors underlies the disparate patterns of host immune reactivity to the fungus.

**Methods**

**Mice**

Fc<sub>R</sub>I<sup>+</sup>, II- and III-deficient (Fc<sub>R</sub>I<sup>−/−</sup>), CD11b-deficient (CR3<sup>−/−</sup>), and wild-type (+/+ ) mice were generated in the laboratory of S. Verbeek (21). Wild-type mice are characterized by a mixed genetic background (C57BL/6, 129SV and BALB/c strains for Fc<sub>R</sub>I<sup>−/−</sup>), and C57BL6 and 129SV strains for CR3<sup>−/−</sup>), and the C57BL6 and 129SV strains for the production of CR3<sup>−/−</sup> mice were kindly provided by Professor T. Mayadas to S. Verbeek. Procedures involving animals and their care were conducted in conformity with national and international laws and policies. Animals were used at 8–10 weeks of age.

**Reagents**

Rat IgG mAb antibodies M1/70 detecting CD11b on murine CR3 and 2.4G2 recognizing murine Fc<sub>R</sub>I were generated by pepsin digestion with the ImmunoPure F(ab')<sub>2</sub> preparation kit (Pierce, Rockford, IL). Mouse IgG1 mAb (PAM-1), capable of inhibiting MR-dependent uptake in human monocyte-derived DC (22), was purified from ascites of the corresponding hybridoma. Mouse IgG1 mAb (ICRF44) to human CD11b and mouse IgG2a to CD14 (M5E2) were from PharMingen. F(ab')<sub>2</sub> fragments of murine IgG1 mAb to human Fc<sub>R</sub>I (CD64, clone 10.1), Fc<sub>R</sub>I (CD32, clone 7.3) and Fc<sub>R</sub>III (CD16, clone 3G8) were from Ancell (Vinci-Biochem, Florence, Italy). Monomeric IgG2a mAb targeting murine Fc<sub>R</sub>I, isotype-matched control antibodies and laminarin (L 9259) were from Sigma (St Louis, MO).

**Candida strains**

The origin and characteristics of the C. albicans highly virulent hyphal strain and the low-virulence yeast strain used in this study have already been described (19). As the hyphal strain is capable of yeast-to-hyphal transition in vitro, whereas the yeast strain is not, the two strains were used as sources of hyphae and yeasts respectively (19). In selected experiments, strains of C. albicans with deletion of the genes coding for the putative two-component histidine kinase (CHK1, chk1<sup>−/−</sup> mutant) or for the response regulator (CSSK1, cssk<sub>−/−</sub> mutant) were used together with the corresponding gene-reconstituted strains (CHK23 and CSSK23 respectively). The isogenic mutant strains (chk1<sup>−/−</sup> and cssk<sub>−/−</sub>) are defective in morphogenesis under certain growth conditions and are avirulent in vivo, as opposed to the revertants (CHK23 and CSSK23) that show hyphal formation in vitro (23,24). Isogenic strains of C. albicans, obtained by mutagenesis in vitro and capable (Vir<sup>−</sup>) or not (Vir<sup>+</sup>) of yeast-to-hyphal transition, as assessed by the germ-tube formation in vitro, were also used (25). For hyphae, cells were allowed to germinate by culture at 37 °C, in 5% CO<sub>2</sub>, for 2 h in RPMI 1640 medium (by that time, >98% of cells had germinated). The hyphae were then harvested, counted and resuspended in IMDM (Gibco Life Technologies, Milan, Italy) containing 5 µg/ml polymyxin B (Sigma) and 50 µg/ml gentamycin. For yeasts, the cells were harvested at the end of the exponential phase of growth, centrifuged and resuspended in the above medium. The different Candida spp. were isolated and identified in our laboratory from clinical specimens.

**DC isolation and pulsing with Candida**

Blood CD11c<sup>+</sup> immature DC and mature DC were isolated as described (3,26). Positively selected CD14<sup>+</sup> monocytes (MiniMACs; Miltenyi Biotec, Bologna, Italy) were cultured for 5 days in IMDM, containing 10% filtered human serum, 50 µM 2-mercaptoethanol, 2 mM L-glutamine, and 50 µg/ml gentamycin in the presence of 50 ng/ml recombinant human granulocyte macrophage colony stimulating factor (Schering-Plough, Milan, Italy) and 200 U/ml recombinant human IL-4 (Peprotech, Inalco, Milan, Italy). The resulting monocyte-derived immature DC were washed and cultured for 24 h with 1000 ng/ml trimeric human CD40 ligand–leucine zipper fusion protein (Immunex, Seattle, WA) to obtain mature DC. Light microscopy confirmed the morphological differences between immature and mature DC (Figs 1 and 6 respectively), with more cytoplasmic projections in the latter cells. FACS analysis revealed that immature DC and mature DC were CD1a<sup>−</sup>, CD11c<sup>+</sup>, CD11b<sup>+</sup>, CD14<sup>−/−</sup>, CD4<sup>−</sup> and CD8<sup>+</sup>. The expression of HLA class II, CD80 and CD86 was high in immature DC, and slightly increased in mature DC (data not shown). Murine DC were purified from spleens by magnetic cell sorting with MicroBeads (Miltenyi Biotec) conjugated to hamster anti-mouse CD11c mAb (19). Positively selected DC contained >90% N418<sup>high</sup> cells and <20% CD8<sup>+</sup> cells. Fractionated CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>−</sup> and CD4<sup>−</sup>CD8<sup>−</sup> DC subsets (>95% pure on FACS analysis) were obtained as described (61). DC were pulsed with Candida yeasts or hyphae, as detailed in the phagocytic assay, for 2 h before the addition of 2.5 µg/ml of amphotericin B (Sigma) to prevent Candida overgrowth. Supernatants, to be assessed for cytokine content, were harvested 22 h later.
Phagocytosis, opsonization and inhibitory studies

Phagocytosis and culture assays were performed in IMDM with polymyxin, but no FCS, to avoid non-specific activation by serum components and endotoxin. For phagocytosis, DC (2 × 10⁵ cells/200μl) were incubated at 37°C with 10⁶ C. albicans yeasts or 6 × 10⁵ hyphae (in 200 ml Iscove’s medium) in 6-ml polypropylene tubes (Falcon, Becton Dickinson, Meylan, France) for 15–30 min. For opsonization, yeasts or hyphae were exposed to 5 μg/ml MBL (kindly provided by Professor M. W. Turner), 40% normal mouse serum or 40% human serum from healthy donors, for 2 h at 37°C, 5% CO₂, in conditions known to efficiently opsonize the fungus (27,28). FACS analysis of yeasts and hyphae revealed effective binding of MBL (MBL opsonization), binding of IgG and C3 deposition (upon exposure to human serum, C3 + IgG opsonization), and C3 deposition without IgG binding (upon exposure to normal mouse serum, C3 opsonization) (data not shown). CR3-dependent phagocytosis was triggered without phorbol myristate acetate pretreatment (29). For inhibition of the MR, CR3 or FcγRI, II and III by the relevant antibodies, cells were pre-incubated for 30 min at 37°C with F(ab′)₂ fragments of the respective antibodies, at the saturating concentration of 20 μg/ml (both human and murine). Isotype-matched control antibody would not affect internalization (data not shown). For Dectin-1 inhibition, DC were incubated with 100 μg/ml laminarin for 30 min at 37°C before pulsing. Phagocytic cells were separated from non-phagocytosed C. albicans cells as described (19). After a DiffQuik staining, aliquots of cells were

![Fig. 1. Phagocytosis of unopsonized C. albicans yeasts and hyphae by DC occurs through different receptors. Monocyte-derived human DC (A) and murine splenic DC (B) were exposed to unopsonized yeasts or hyphae in the presence of 20 μg/ml F(ab′)₂ fragments of antibodies to the MR, CD11b on CR3, FcγRI, II and III, or monomeric IgG2a targeting FcγRI for murine DC and 100 μg/ml laminarin to block Dectin-1. DC from FcγR+/+, FcγR±/± or CR3 ±/± mice were also assessed. The numbers refer to the percent internalization visualized by light microscopy. The data are the means ± SE of several independent experiments. *P < 0.05, fungal internalization under conditions of receptor blockade or deficiency versus controls (i.e. wild-type DC reacted with yeasts or hyphae alone). The percentages of yeast and hypha internalization in CR3±/± mice were 69 ± 7 and 61 ± 9 respectively.](https://academic.oup.com/intimm/article-abstract/16/1/149/721747)
Dendritic cell recognition of Candida albicans

spin down on slides on a cytocentrifuge and mounted in buffered glycerol to be examined by light microscopy by at least three independent, blinded observers. For each experiments, at least five fields in each slide were counted and at least 200 DC were analyzed in each well. All conditions were tested in triplicates. Data are representative of three to five experiments. Fungal cell internalization was expressed according to the following formula: percentage of internalization = number of cells containing one or more fungal cells/100 cells counted. Photographs were taken using a high-resolution microscopy color Camera AxioCam Color, using AxioVision software release 3.0 (Carl Zeiss, Milan, Italy). For the assessment of the intracellular killing, 5 × 10^5 human DC were incubated with 10^6 unopsonized Candida yeasts for 2 h at 37°C before the microbiological assessment of yeast viability on phagocytic cells separated from non-phagocytosed yeasts as described (19).

Flow cytometry
FITC-conjugated rat IgG directed to murine I-A^b (clone 34-5-3), CD80 (clone 1G10) and CD86 (clone GL1) were from PharMingen. FITC-conjugated mouse IgM directed to human CD80 (BB1) and to CD86 (BU63), and mouse IgG1 to HLA-DR, -DQ and -DR (TDR31.1) were from Ancell. FcR blocking was performed by incubating cells with FcR blocking reagents (Miltenyi Biotec). Unrelated isotype-matched mAb were used as a control. Analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA). Histograms are representative of one out of four independent experiments.

In vivo studies
Mice were infected i.v. with 10^8 Candida yeasts or hyphae in 0.5 ml PBS (19). Resistance to infection was assessed by quantifying the number of c.f.u. (mean ± SE) recovered from kidneys, and the frequency of IL-12-, IL-4- or IL-10-producing cells in fractionated splenic DC and that of IFN-γ or IL-4-producing cells in positively selected CD4^+ T splenocytes (19). For the MR inhibition, mice were given i.p. 0.5 mg F(ab')_2 fragments of PAM-1 antibody, 2 h before and after, and at the time of challenge. The data reported are pooled from three experiments. In vivo groups consisted of six mice.

Cytokine and ELISPOT assays
The levels of tumor necrosis factor (TNF)-α, IL-4, IL-10 and IL-12p70 in culture supernatants were determined by murine ELISA (R & D Systems, Space Import-Export, Milan, Italy) and human ELISA (Endogen, Milan, Italy). The detection limits (pg/ml) of the assays were <16 for IL-12p70, <3 for IL-4, <4 for IL-10 and <15 for TNF-α (murine), and <3 for IL-12p70, <2 for IL-4, <3 for IL-10 and <2 for TNF-α (human). For ELISPOT assay, freshly isolated fractionated DC subsets and purified CD4^+ T splenocytes from infected mice were cultured (1 × 10^6 to 1 × 10^5 cells/well) in RPMI 1640 medium with 10% FCS, 50 μM 2-mercaptoethanol and 50 μg/ml gentamycin for 18 h in 96-well filtration plates (Millipore, Milan, Italy) previously coated with rat anti-murine R4-6A-2 (for IFN-γ), BVD4-1D11 (for IL-4) or JESS-2A5 (for IL-10) mAb. Biotinylated AN-18.17.24 (for IFN-γ), BVD6-24G2 (for IL-4) or SXC-1 (for IL-10) were used as the detecting reagents, the enzyme was avidin–alkaline phosphatase conjugate (Vector, Burlingame, CA) and the substrate was 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine salt (Life Technologies). Results were expressed as the mean number of cytokine-producing cells (± SE) per 10^6 DC or 10^5 CD4^+ T cells, calculated using replicates of serial 2-fold dilutions of cells.

Statistical analysis
Statistical significance was determined using an unimpaired, two-sample Student's t-test.

Results
DC internalize yeasts and hyphae of C. albicans through distinct recognition receptors
To determine the impact of fungal recognition via different recognition receptors expressed by DC, monocyte-derived human DC and freshly harvested splenic DC from FcγR^−/−, CR3^−/− or wild-type mice were exposed to live unopsonized Candida yeasts or hyphae in the presence of saturating amounts of specific F(ab')_2 antibody fragments reacting to MR, CR3, and FcγRII, II and III. Laminarin, a Dectin-1-specific ligand (30), was also tested for possible interference with fungal recognition. Both human and murine DC internalized unopsonized Candida yeasts and hyphae through a phagocytic process (inhibitable by cytochalasin D, EDTA and nocodazole; not shown). Light microscopy showed that externalization of yeasts, but not hyphae, by human DC was inhibited by >85% by blockage of the MR, confirming that the receptor efficiently mediates phagocytosis of the yeasts (31) (Fig. 1A). Laminarin also significantly reduced yeast internalization, whereas blocking CR3 exerted only a limited effect, suggesting that a minority fraction (~10%) of unopsonized yeasts exploits CR3 to enter DC. In contrast, internalization of hyphae was inhibited by >80% by blocking CR3 or FcγRII, II and III, and by >50% by blocking Dectin-1 (Fig. 1A). A similar pattern of receptor engagement was observed with murine DC (Fig. 1B), a noticeable exception being represented by lack of FcγRI involvement. Internalization of yeasts was not blocked or reduced under conditions of FcγR or CR3 deficiency as was that of hyphae (Fig. 1B). This finding suggests an absolute requirement for FcγRI in hypha phagocytosis, and highlights the cooperativity between CR3 and FcγRI (29). The uptake of hyphae, more than yeasts, by murine DC was inhibited by staurosporine and herbimycin A (not shown), indicating that signals from protein kinase C and/or protein tyrosine kinases are required for CR3- and FcγRI-dependent phagocytosis, as demonstrated by other studies (29). Therefore, MR, Dectin-1, CR3 and FcγRI on DC appear to behave as functional PRR for the fungus, and mediate the entry of unopsonized yeasts and hyphae presumably through distinct signaling pathways.

To confirm that unopsonized fungal yeasts reproducibly exploit these recognition receptors on DC, human DC were assessed for phagocytosis of isogenic mutant yeasts derived by gene disruption or chemical mutagenesis as well as of yeasts of different Candida spp. No filamentation, as judged by germ-tube formation under the specific experimental conditions of DC pulsing, was observed for the mutant strains of C. albicans as well as for the different species of Candida (Fig. 2). The internalization of mutant yeasts of C. albicans was
maximally inhibited upon blocking MR, partially upon blocking CR3 and Dectin-1, and was not inhibited at all upon blocking MR, partially upon blocking CR3, FcγR and Dectin-1, although at variable degrees. The internalization of the different filamenting, wild-type strains of the fungus was again inhibited by internalization, visualized by light microscopy. The data are the means of three independent experiments. The SE values, always <8, were omitted. *P < 0.05, fungal internalization under conditions of receptor blockade versus controls (none).

On assessing survival of intracellular yeast cells upon internalization by human DC under conditions of MR, CR3 or Dectin-1 blocking, we found that yeast viability was reduced by >80% under conditions of CR3 blocking as compared to the blockade of either MR or Dectin-1 (Table 1). Therefore, in accordance with previous observations on murine DC (32), the use of distinct receptors may determine the fate of the fungus inside human DC, thus affecting fungal ability to survive, which may, in turn, reflect the different release of antifungal effector molecules.

Table 1. The exploitation of the different receptors on DC affects the viability of C. albicans inside the cells

<table>
<thead>
<tr>
<th>Receptor blocked</th>
<th>Yeast viability (%)</th>
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<tr>
<td>None</td>
<td>42.7 ± 8.8</td>
</tr>
<tr>
<td>MR</td>
<td>58.4 ± 12.3</td>
</tr>
<tr>
<td>CR3</td>
<td>7.9 ± 1.7</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>38.7 ± 10.3</td>
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</table>

Yeast viability was assessed on human DC incubated with unopsonized Candida yeasts for 2 h at 37°C, as detailed in Methods. Results are the mean ± SD. P< 0.05 blocked versus unblocked (None) receptor.

Receptor-mediated entry is subverted by fungal opsonization

To evaluate whether fungal opsonization would alter the pattern of receptor-mediated entry into DC, we reacted yeasts and hyphae with MBL or human serum (for C3 + IgG opsonization) under conditions of efficient opsonization (27,28). The opsonized material was added to human DC cultured in the presence of antibodies to the MR, CR3, FcγRI, II and III, and laminarin to block Dectin-1. Assay of germ-tube formation was done in vitro (see Methods). The numbers refer to percent internalization, visualized by light microscopy. The data are the means of three independent experiments. The SE values, always <8, were omitted. *P < 0.05, fungal internalization under conditions of receptor blockade versus controls (none).

The pattern of receptor-mediated entry influences co-stimulatory molecule expression and cytokine production

To define whether the opsonic and non-opsonic receptor-mediated entry of the fungus into DC would result in different activation events, the levels of expression of co-stimulatory (CD80 and CD86) and MHC class II molecules as well as specific cytokine production were assessed in DC exposed to unopsonized or differently opsonized yeasts and hyphae. The expression of CD80, CD86 and class II antigens was increased on both human and murine DC by phagocytosis of either unopsonized or C3 + IgG-opsonized yeasts and hyphae, but reduced below the basal level upon MBL or C3 opsonization (Fig. 4A and B). Therefore, co-stimulatory molecules were up-regulated by both the MR-dependent and FcγR-dependent phagocytosis and down-regulated by CR3-
dependent phagocytosis, as seen upon MBL or C3 opsonization.

Murine DC have been shown to produce IL-12 or IL-4 after exposure to unopsonized yeasts or hyphae respectively, although they will release comparable levels of TNF-α and no IL-10 (19). Human DC also produce IL-12 after exposure to unopsonized yeasts (33). On assaying cytokine production under conditions of selective receptor engagement, as favored by appropriate opsonization, or inhibition, we found that both MBL and C3 + IgG opsonization greatly reduced the production of IL-12 in response to yeasts in both human and murine DC. MBL opsonization also reduced the production of IL-4 in response to hyphae and that of TNF-α in response to both; C3 + IgG opsonization increased the production of both IL-4 and IL-10, particularly as induced by the yeasts (Fig. 5A−D). Receptor inhibition revealed that the production of IL-12 was impaired by the MR blocking and increased by CR3 or FcγRI, II and III. The numbers refer to percent internalization, visualized by light microscopy.

![Fig. 3. The exploitation of the different receptors on DC by C. albicans is subverted by opsonins. Human DC were exposed to Candida yeasts opsonized with MBL (5 μg/ml) or C3 + Ig (40% human serum from healthy donors), for 2 h at 37°C, in the presence of 20 μg/ml F(ab′)_2 fragments of antibodies to the MR, CR3, and to FcγRI, II and III. The numbers refer to percent internalization, visualized by light microscopy.](https://academic.oup.com/intimm/article-abstract/16/1/149/721747)

Table: Receptor blocked

<table>
<thead>
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<th>Opsonization</th>
<th>None</th>
<th>MBL</th>
<th>C3+IgG</th>
</tr>
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<tbody>
<tr>
<td>MR</td>
<td>12±4</td>
<td>52±5</td>
<td>75±6</td>
</tr>
<tr>
<td>CR3</td>
<td>68±3</td>
<td>25±4</td>
<td>10±3</td>
</tr>
<tr>
<td>FcγR I+II+III</td>
<td>80±8</td>
<td>75±6</td>
<td>9±4</td>
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Phenotypically distinct DC subsets interact with yeasts and hyphae of Candida

The discriminative response between the two forms of the fungus could be operative at the level of the immune system, but may also represent a pathogen’s property to modulate the reactivity of host DC. To distinguish between these two possibilities, we assessed the relative contributions of different
splenic DC subsets (CD4+CD8−, CD4−CD8+ or CD4+CD8+ DC) and of human mature DC to internalization of unopsonized yeasts or hyphae and the associated discriminative cytokine responses. The three murine DC subsets and human mature DC effectively phagocytosed both fungal morphotypes, even though the CD4+ and CD4− myeloid subsets were more efficient than the CD4−CD8+ lymphoid-related subset (Fig. 6). Human mature DC were less efficient than immature DC. Murine myeloid DC (whether CD4+ or CD4−) produced IL-12 and IL-4 in response to yeasts and hyphae respectively, and CD4−CD8+ DC produced neither cytokine. Human mature DC produced less IL-12 (in response to yeasts) and more IL-10 (in response to hyphae) than immature DC (Fig. 6 compared to Fig. 5), but no IL-4 (not shown). These data suggest that the occurrence of distinct responses by the host immune system is not contingent a priori on either fungal form and yet, within phenotypically distinct DC subsets, murine myeloid and human immature DC do discriminate between yeasts and hyphae of Candida, by producing alternative cytokine responses to distinct stimuli.

The pattern of receptor-mediated entry affects resistance and immunity to infection in vivo

To evaluate the impact of the usage of different recognition receptors on T1 cell education in vivo, wild-type, FcγR−/− and CR3−/− mice, either untreated or treated with antibody (PAM-1) to inhibit MR-dependent entry, were infected i.v. with Candida yeasts or hyphae. Mice were examined for susceptibility to infection in terms of fungal clearance from the organs and patterns of cytokine production by splenic DC and CD4+ T cells. As expected, both types of mutant mice, and particularly the FcγR−/− mice, were more resistant than wild-type mice to infection with virulent hyphae. Resistance to yeasts was increased in both types of mutant mice, but greatly decreased by treatment with PAM-1 antibody (Fig. 7A). Compared to wild-type mice, the frequency of IL-12-producing DC was increased in response to yeasts and that of DC-producing IL-4/IL-10 was decreased in response to hyphae in mutant mice (Fig. 7B).

Importantly, the number of IL-12-producing DC was also increased in response to hyphae and that of DC producing IL-4/10 decreased in response to yeasts (Fig. 7B), a finding suggestive of the possible involvement of different receptors in the recognition of yeasts and hyphae in vivo. Likewise, the frequency of IFN-γ-producing CD4+ T splenocytes was further increased and that of IL-4-producing CD4+ cells further decreased in mutant mice infected with yeasts or hyphae respectively, as compared to wild-type mice (Fig. 7C).

Therefore, multiple receptors are involved in the host recognition of C. albicans in vivo and are responsible for the type of immune reactivity to the fungus.
Fig. 5. The exploitation of the different receptors by C. albicans affects cytokine production by DC. Human and murine DC were cultured with unopsonized (none), MBL- or C3 + IgG-opsonized Candida yeasts or hyphae (A–D), or (E) with unopsonized yeasts (IL-12 and TNF-α) or unopsonized hyphae (IL-4 and IL-10) in the presence of anti-receptor antibodies. For opsonization and anti-receptor antibodies, see legend to Fig. 3. In (E), data with human DC are reported, although similar results were obtained with murine DC. Cytokine determination (pg/ml, except for murine IL-12 expressed as ng/ml) was done by specific ELISA on supernatant of 24-h co-culture. *P < 0.05, cytokine levels under the different culture conditions versus cytokine levels in cultures of DC with unopsonized fungi alone.
Discussion

Our results demonstrate that the exploitation of MR on DC by *C. albicans* invariably leads to the production of bioactive IL-12 and the occurrence of T_h1 cell reactivity. Entry through FcRγRI, II and III either by non-opsonized hyphae or, importantly, by opsonized yeasts was responsible for suppression of MR-dependent type I reactivity, onset of type 2 responses and associated pathology. Opsonic and non-opsonic entry through CR3 by yeasts and hyphae modulated both types of T_h1 responses. Studies *in vivo* in receptor-deficient mice confirmed that the balance between the entry through distinct receptors likely determines the type of immune response elicited. The FcRγRI-dependent phagocytosis of *C. albicans* is responsible for type 2 cytokine production and T_h2 cell activation, an activity counteracted by the T_h1-promoting activity of non-opsonic phagocytosis through the MR. CR3 appears to function as a powerful regulator of both types of T_h1 reactivity, being required for maximal expression of type 2 reactivity and for attenuated expression of type 1 reactivity. Signs of exaggerated inflammatory responses were observed, at sites of infection, in CR3−/− mice (not shown).

In addition to DC, lectin and β-glucan receptors, CR3 and FcR have all been implicated in the phagocytosis of either live or dead unopsonized *Candida* yeasts by phagocytes (13,30,37,40–42). Less is known about the receptor engagement pattern of hyphae. Although MR were sufficient to mediate phagocytosis of the fungus independently of other known receptors (31), and to produce inflammatory mediators and antifungal effector molecules (31,40), additional newly described receptors may mediate the lectin pathway of fungal entry (7,8,30,42). This and the existence of redundancy in the MR receptor family (43) may account for the ability of MR-deficient mice to resist *C. albicans* infection (44).

As polynuclear as well as mononuclear phagocytes are crucially involved in the control of the candidiasis (10), our *in vivo* results may also suggest an important role for the exploitation of MR, CR3 and FcRγRI on these cells by the fungus under specific conditions. However, because each receptor type on effector phagocytes has been implicated in phagocytosis and killing of the fungus, although to a variable degree and through distinct mechanisms [reviewed in (37)], this is not immediately consistent with the disparate patterns of fungal growth restriction observed *in vivo* in receptor-deficient mice. Therefore, although the involvement of effector phagocytes is beyond dispute, their actual effector function along with receptor exploitation needs to be evaluated in the context of the dysregulated T_h1 reactivity observed in receptor-deficient mice.

Studies with human DC provided further evidence that the entry of the fungus through the MR or FcRγRI is responsible for type 1 or type 2 cytokine production respectively. Murine myeloid and human immature DC were more efficient in terms of phagocytosis and cytokine production as compared to murine lymphoid and human mature DC respectively, a finding possibly reflecting the different expression of receptors for phagocytosis by distinct DC subsets (1,38,39) as well as a different effect of maturation on this expression (4). The engagement of distinct receptors selectively occurred in recognition of yeasts and hyphae of *C. albicans* as well as non-*albicans* species of *Candida*. It is of interest to note that the mutant yeasts of *C. albicans* used in the present study were all avirulent in a mouse model of candidiasis (23–25).

![Fig. 6. *C. albicans* internalization and cytokine production by different DC subsets. Fractionated splenic DC subsets and mature human DC were exposed to unopsonized yeasts (grey bars) and hyphae (black bars) of *C. albicans*, and assessed for phagocytosis and cytokine production. Internalization was visualized by light microscopy, after 15–30 min, and expressed as percent internalization. *P* < 0.05, cytokine levels (at 24 h of co-culture) under different culture conditions versus cytokine levels in cultures of DC alone. Data are the means ± SE of three independent experiments (white bars, unexposed DC).](https://academic.oup.com/intimm/article-abstract/16/1/149/721747)
The exploitation of CR3 for entry into DC by Candida is of interest. It is known that multiple pathways exist leading to CR3 activation for phagocytosis of both opsonized and unopsonized particles (45), likely depending on sugar specificity of ligands and on the existence of several activation states for this receptor (46). The different sugar composition between yeasts and hyphae of C. albicans (12) makes it likely that the interaction of yeasts and hyphae occurs through distinct receptor domains, i.e. the lectin domain for the yeasts and the I domain for the hyphae (13). This may translate in the activation of different transducing events, which may help to explain the alternate cytokine production to either form as well as the effects of MBL opsonization. MBL, members of the collectin family of proteins, bind through multiple sites to various carbohydrate structures on surfaces of C. albicans and promote complement activation through the lectin pathway.

Fig. 7. The exploitation of the different receptors by C. albicans is responsible for the specific type of Th reactivity in infection. (A) Fungal growth (CFU, mean ± SE) in the kidneys of wild-type, FcγR−/− or CR3−/− mice i.v. infected with virulent Candida hyphae or low-virulent yeasts, 3 and 7 days earlier respectively. Mice were treated with the F(ab′)2 fragment of the PAM-1 antibody (anti-MR) or of isotype-matched control antibodies, 2 h before, at the time of challenge and 2 h later. *P < 0.01, mutant versus wild-type mice or PAM-1-treated versus untreated mice. (B and C) Number of IL-12-, IL-4- or IL-10-producing DC (B) and IFN-γ- or IL-4-producing CD4+ T cells (C) from wild-type, FcγR−/− or CR3−/− mice infected with Candida yeasts or hyphae. *P < 0.05, mutant versus wild-type mice. ELISPOT assays were performed at 1 day for DC, and at 3 and 7 days after infection with Candida hyphae and yeasts respectively for T cells.

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regarded as absolutely indicative of saprophytism or infection versus infection nor can specific forms of per se scenario, dimorphism response to distinct microbial stimuli (2,37,58,59). In this DC to have the potential to produce alternative cytokines in emerged in microbial pathogenesis (56,57). This would allow receptor interaction in DC, a concept that has recently presented. Rather, the nature of the cell response is strongly compatible with a primitive mechanism of host defense of Candida regulatory T cells which dampen antifungal Th1 reactivity in production of IL-10 and not IL-4. This is consistent with the recent finding that IL-10-producing DC are required to induce regulatory T cells which dampen antifungal T \( \text{h} \)1 response (49) points out the crucial role of CR3 in resistance to fungi (50,51). It is interesting that the attenuation of IL-12 production in human DC upon CR3 engagement was concomitant with the production of IL-10 and not IL-4. This is consistent with the recent finding that IL-10-producing DC are required to induce regulatory T cells which dampen antifungal T \( \text{h} \)1 reactivity in mice with candidiasis and are at the same time absolutely required for long-term memory (52). Thus, usage of CR3 may favor commensalism of the fungus at human mucosal surfaces, including gut and vagina, where a condition of immune tolerance is desirable to the host. As yeast cells appear to survive undigested inside the phagosome once internalized through CR3, as opposed to entry through the MR (32), it follows that yeast cells may exploit the CR3 receptor on DC as a niche to avoid degradation through the multilicate pathway while allowing their own persistence. In so doing, C. \text{albicans} shares with pathogenic bacteria the ability to avert activation of phagocytes by entry through complement receptors that are not accompanied by phagocyte activation (53).

In the case of hyphae, the finding that the entry into DC was inhibited by blocking either CR3 or Fc\( \gamma \)R indicates that ligation of co-receptor is an additional mechanism of CR3 activation. It is known that antibodies and complement synergize in the processes of opsonization and phagocytosis (54). As the phenomenon was observed with hyphae, irrespective of opsonization, this suggests that unopsonized hyphae are endowed with the ability to activate opsonic receptors. In this regard, it is noteworthy that C. \text{albicans} expresses receptors for iC3b, the expression of which depends on environmental growth conditions, is higher in hyphae than yeast cells and correlates with virulence (11). That Fc\( \gamma \)R may interact directly with parasite surface molecules and facilitate their infective capacity has already been hypothesized (55).

The findings of the present study highlight the functional plasticity of DC at the pathogen/immune system interface, which may have important implications for fungal virulence, immunity and vaccines. The selective exploitation of receptor-mediated entry into DC, which may also depend on local levels of opsonization, could explain the full range of the host’s immune relationships with the fungus, including saprophyphism and infection. Thus, the qualitative development of the Th1 response to Candida does not primarily depend on the nature of the antigen (yeast or hypha) being phagocyted and presented. Rather, the nature of the cell response is strongly affected by the type of cell signaling initiated by the ligand–receptor interaction in DC, a concept that has recently emerged in microbial pathogenesis (56,57). This would allow DC to have the potential to produce alternative cytokines in response to distinct microbial stimuli (2,37,58,59). In this scenario, dimorphism per se can no longer be considered as the single most important factor in determining commensalism versus infection nor can specific forms of Candida be regarded as absolutely indicative of saprophyphism or infection at a given mucosal site. Rather, opsonization, in combination with regulatory events that may switch the organism from a benign commensal to an invasive state may greatly affect virulence of yeast cells. However, the nature of these regulatory events and, indeed, their very existence remain speculative. As a matter of fact, most strains that cause disease are commensals from the patient (10). Therefore, it seems that the nature and the extent of fungal opsonization at different body sites will have an important role in determining the biological relevance of receptor usage on DC.

In a time when fungal vaccines are firmly pursued and yeasts provide a powerful activation stimulus to DC (60), our findings call attention to the notion that fungal antigen presentation by DC will not necessarily result in the induction of protective immunity, an event strictly contingent upon the use of an appropriate form of antigen that would engage the appropriate receptor. A corollary to this may be the exploitation of new strategies for fungal/DC-based vaccination that would include specific targeting of the antigen to the appropriate receptor (61,62), an event to which antibodies may significantly contribute (50,63).

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Abbreviations

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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>CR</td>
<td>complement receptor</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<td>Fc( \gamma )R</td>
<td>Fc receptor</td>
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<td>MBL</td>
<td>mannose-binding lectin</td>
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<tr>
<td>MR</td>
<td>mannose receptor</td>
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<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
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<tr>
<td>PRR</td>
<td>pattern-recognition receptor</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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References

Dendritic cell recognition of Candida albicans


