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Dendritic Cells Transport Conidia and Hyphae of *Aspergillus fumigatus* from the Airways to the Draining Lymph Nodes and Initiate Disparate Th Responses to the Fungus[1]

Silvia Bozza,* Roberta Gaziano,‡ Antonio Spreca,† Angela Bacci,* Claudia Montagnoli,* Paolo di Francesco,‡ and Luigina Romani[2]*

Aspergilli are respiratory pathogens and pulmonary infections are usually acquired through the inhalation of conidia, able to reach small airways and the alveolar space where the impaired host defense mechanisms allow hyphal germination and subsequent tissue invasion. The invasive pulmonary aspergillosis is the most common manifestation of *Aspergillus fumigatus* infection in immunocompromised patients and is characterized by hyphal invasion and destruction of pulmonary tissue. A Th1/Th2 dysregulation and a switch to a Th2 immune response may contribute to the development and unfavorable outcome of invasive pulmonary aspergillosis. Dendritic cells (DC) have a primary role in surveillance for pathogens at the mucosal surfaces and are recognized as the initiators of immune responses to them. In the present study, we assessed the functional activity of pulmonary DC in response to *A. fumigatus* conidia and hyphae, both in vitro and in vivo. We analyzed mechanisms and receptors for phagocytosis by DC as well as DC migration, maturation, and Th priming in vivo upon exposure to either form of the fungus. We found a remarkable functional plasticity of DC in response to the different forms of the fungus, as pulmonary DC were able to: 1) internalize conidia and hyphae of *A. fumigatus* through distinct phagocytic mechanisms and recognition receptors; 2) discriminate between the different forms in terms of cytokine production; 3) undergo functional maturation upon migration to the draining lymph nodes and spleens; and 4) instruct local and peripheral Th cell reactivity to the fungus. *The Journal of Immunology*, 2002, 168: 1362–1371.

Aspergillus species are the most common causes of invasive infections in allogeneic bone marrow transplant recipients (1). Prophylactic antifungal therapy had little effect on disease incidence in these patients (1, 2). Invasive pulmonary aspergillosis (IPA)[3] is the most common manifestation of *Aspergillus fumigatus* infection in immunocompromised patients and is characterized by hyphal invasion and destruction of pulmonary tissue (1).

Aspergilli are respiratory pathogens and pulmonary infections are usually acquired through the inhalation of conidia (3). With a diameter of ~2.5–3.5 μm, the conidia are able to reach small airways and the alveolar spaces, where germination of conidia and subsequent tissue invasion occurs in immunologically impaired individuals. Although epithelial and endothelial cells may internalize conidia (3), effector mechanisms of the innate immune system have long been recognized as major host defenses against IPA (4). Resident alveolar macrophages ingest and kill resting conidia, mainly through nonoxidative mechanisms, while neutrophils use oxygen-dependent mechanisms to attack hyphae germinating from conidia that escape macrophage surveillance (3–5). More recent studies in mice (6–8) and humans (9, 10) have shown that a Th1/Th2 dysregulation and a switch to a Th2 immune response may contribute to the development of an unfavorable outcome of IPA.

Dendritic cells (DC) have a primary role in surveillance for pathogens at the mucosal surfaces and are recognized as the initiators of immune responses to them (11–13). A dense network of DC has been described in the respiratory tracts (14–16). In the resting state, respiratory tract DC are specialized for uptake and processing, but not for presentation of Ag, the latter requiring cytokine maturation signals that presumably occur after migration to regional lymph nodes (17–19). The evidence that pulmonary DC, through production of IL-10, mediate unresponsiveness to respiratory Ags (20, 21) indicates that local production of immunoregulatory cytokines may affect the ability of DC to instruct the appropriate T cell responses to the invading pathogens. This is particularly relevant in the case of responses to fungi, as DC appeared to be uniquely able to discriminate among the different forms of them (22). Through the use of different pattern recognition receptors (PRRs), receptors for a number of components of the complement system (CR), and FcR (FcγR), DC finely discriminated between yeast and hyphae of *Candida albicans* and were responsible for Th priming and education in *C. albicans* saprophytism and infection (22). In the present study, we assessed the functional activity of pulmonary DC in response to *A. fumigatus* conidia and hyphae, both in vitro, and in infection in vivo. We analyzed mechanisms and receptors for phagocytosis of fungi by DC as well as DC migration, maturation, and Th priming in vivo upon exposure to either form of the fungus. We found a remarkable functional

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[3] Abbreviations used in this paper: IPA, invasive pulmonary aspergillosis; DC, dendritic cells; PRR, pattern recognition receptor; CR, complement receptor; i.t., intratracheal; FSDC, fetal skin-derived DC; TEM, transmission electron microscopy; PI, phagocytic index; MR, mannose receptor; MFI, median fluorescence intensity.

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plasticity of DC in response to the different forms of the fungus, as pulmonary DC were able to discriminate between the different forms in terms of maturation, cytokine production, and induction of local and peripheral Th cell reactivity.

Materials and Methods

Mice

BALB/c (H-2^d) mice, 8- to 10-wk old, were purchased from Charles River Breeding Laboratories, Calco, Italy. BALB/c mice were bred under specific pathogen-free conditions in the animal facility of the University of Perugia, Perugia, Italy. Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

Microorganism, culture conditions, and infection

The strain of *A. fumigatus* was obtained from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of the University of Perugia (6–8). The microorganism was grown on Sabouraud dextrose agar (Difco, Detroit, MI) supplemented with chloramphenicol for 4 days at room temperature. Abundant conidia were elaborated under these conditions. Conidia were harvested by washing the slant culture with 5 ml of 0.025% Tween 20 in saline and gently scraping the conidia from the mycelium with a plastic pipette (or shaking it vigorously). Cell debris was allowed to settle by gravity and the suspension was decanted into 50-ml plastic conical tubes. After extensive washing with saline, conidia were counted and diluted to the desired concentrations. Swollen conidia were prepared by incubating the resting conidia in Sabouraud's broth until the spores swelled to almost twice their resting diameter but had not germinated (~4 h at 37°C). For generation of hyphae, resting or swollen conidia were allowed to germinate (>98% germination) by further incubation in Sabouraud's broth (~20 and 6 h, respectively). The viability of resting and swollen conidia was >95%, as determined by serial dilution and plating of the inoculum on Sabouraud dextrose agar. For intratracheal (i.t.) injection, immunocompetent mice were anesthetized by i.p. injection of 2.5% avertin (Sigma-Aldrich, St. Louis, MO); a volume of 80-μl saline containing 2×10^8 conidia or 5×10^5 hyphae was injected under direct visualization through the opening vocal cords using a 25-gauge metal catheter connected to the outlet of a micropipette. In the case of infection with FITC-labeled fungal cells, control mice were injected with 12 μg/20 μl FITC (3 mg/ml, F-4274; Sigma-Aldrich) diluted in DMSO. Mice succumbing to fungal challenge were routinely necropsied for histopathological confirmation of invasive aspergillosis.

Labeling of conidia and hyphae with FITC

Live conidia and hyphae were suspended in 0.1 M of carbonate buffer (pH 9.3) at 2×10^8/ml and added to 200 μl of FITC in DMSO, as described (23). After incubation for 2 h at room temperature protected from light, the suspensions were diluted and washed twice in PBS (pH 7.2) to remove all detectable free-FITC as determined by fluorescence measurement of the supernatants compared with PBS alone. The conidia and hyphae pellets were then counted and diluted in PBS to the desired concentration. FITC labeling did not affect the viability of cells.

Propagation of fetal skin-derived DC (FSDC)

Immature FSDC were generated by retroviral immortalization, as described (24). Cells were cultured in IMDM (Life Technologies Italia, Milan, Italy) containing 5% filtered FCS, 50 mM of 2-ME, 2 mM of L-glutamine, and 50 μg/ml gentamicin (complete medium). For routine passaging, the cells were detached from tissue culture flasks (Falcon; BD Labware, Franklin Lakes, NJ) with 2 mM of EDTA in PBS.

Transmission electron microscopy (TEM)

For TEM, FSDC were incubated in suspension culture dishes with *A. fumigatus* conidia or hyphae, as in the phagocytic assay for 1 and 3 h. A total of 5×10^6 cells were pelleted at $8 \times g$ for 5 min and washed twice with PBS. Cells and thin sections of the lungs were fixed in cold 2.5% glutaraldehyde in 0.1 M of sodium cacodylate/1% sucrose buffer for 4 h. The cells were postfixed in 1% osmium tetroxide (50 min), encapsulated in 1% agar, stained with uranyl acetate and phosphotungstic acid, and dehydrated in a series of graded ethanolic solutions finishing with propylene oxide before finally being embedded in Epon 812-Araldite mixture. Ultrathin sections (50 nm) were cut on a LKB ultramicrotome and placed under 200 mesh standard copper grids, contrasted with uranyl acetate and lead citrate, and examined with a Philips TEM 400 transmission electron microscope (Eindhoven, The Netherlands).

DC isolation and culture

DC were isolated from lungs as described by others (25), with minor modifications. After perfusion of the pulmonary vasculature with 5 ml of PBS containing 100 U/ml heparin, the lungs were minced and incubated for 90 min at 37°C in digestion buffer containing 0.7 μg/ml collagenase IV (C-5138, Sigma-Aldrich) and 30 μg/ml type IV bovine pancreatic DNase I (D-4263, Sigma-Aldrich). Large particulate matter was removed by passing the cell suspension through a small loose nylon wool plug. After overnight plastic adherence to remove macrophages, DC were reacted with 100 μl of MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) conjugated to hamster anti-mouse CD11c mAbs (clone N-418), as described (22). Positively selected DC (at ~10–15% yield recovery) routinely contained >90% CD11c^{high} and were not expressing CD8α, in line with previous findings (14, 18, 25). DC were purified from thoracic (paratracheal and parathymic) lymph nodes and spleens by magnetic cell sorting. After overnight plastic adherence to remove macrophages, 10^8 collagenase D-treated (Sigma-Aldrich) nonadherent cells were reacted with 100 μl of CD11c MicroBeads before magnetic separation. Consistent with previous reports (26–28), spleen and lymph node DC (at ~1–2% yield recovery) routinely express high levels of CD11c integrin and between 25 and 30% stained positive for CD8α (data not shown). For cytokine determination, purified DC were pulsed with live conidia or hyphae, as detailed in the phagocytic assay, for 2 h before the addition of 2.5 μg/ml amphotericin B (Sigma-Aldrich) to prevent fungal overgrowth. Supernatants were harvested at 24 h. Control experiments indicated that amphotericin B alone did not modify patterns of cytokine production by DC.

Phagocytosis and inhibition studies

For phagocytosis, FSDC and purified pulmonary DC (2×10^5cells/200 μl) were incubated for different times at 37°C with 10^6 conidia or 2×10^5 hyphae in 6-ml polypropylene tubes (N. 2063, Falcon; BD Biosciences), in 200 μl of IMDM containing 5 μg/ml polymixin B (Sigma-Aldrich) and 50 μg/ml gentamicin but no FCS to avoid nonspecific activation by serum components and endotoxin. Phagocytic cells were separated from nonphagocytosed *A. fumigatus* cells by centrifugation on a fetal serum gradient and a 0.1-ml sample of the harvested phagocytic cells was used for cytospin preparation. After Diff-Quik staining, 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. The phagocytic index (PI) indicates the number of cells containing one (PI = 1) or more (PI > 1) fungal elements per 100 cells. For inhibition studies, fungal internalization at 2 h was assessed in the presence of the following reagents (Sigma-Aldrich, unless otherwise specified): cytochalasin D, 20 μg/ml, added to the cultures, or pre-exposure of cells at 37°C, before addition of fungi, to 20 μg/ml nocodazole for 15 min, 50 mM of EDTA (Life Technologies) for 60 min, 10 μg/ml α-mannan from *Saccharomyces cerevisiae* or 250 μg/ml β-glucan from barley for 10 min, 50 μg/ml laminarin for 60 min, and 0.9 μg/ml galactomannan polysaccharide from seeds of *Ceratonia siliqua* for 60 min. For inhibition of the mannose receptors (MR), CR3 and FcγRII and III, by specific mAbs, cells were preincubated for 30 min at 37°C with the saturating concentration of 20 μg/ml F(ab′)$_2$ of the following purified mAbs: mouse IgG1 (PAM-1) capable of inhibiting MR uptake of human monocyte-derived DC (29), purified from ascites of the corresponding hybridoma and rat IgG M1/70 and 2.4G2, which detect CD11b and FcγRII and III, respectively, from BD PharMingen (Palo Alto, CA). Rat IgG2a mAb (NLDC-145, recognizing the multilectin receptor on DC, DEC-205; Ref. 30) was obtained from BMA Biomedical AG (Rheinstrosse, Switzerland). F(ab′)$_2$ of Abs were generated by pepsin digestion with the ImmunoPure F(ab′)$_2$ preparation KIT (Pierce, Rockford, IL), following the manufacturer's instructions. Isotype-matched control Ab would not affect internalization (data not shown).

Phenotypic analysis

The cell surface phenotype of purified pulmonary, lymph node, and spleen DC was assessed by reacting cells with the following FITC-conjugated mAb reagents: rat IgG directed to I-A^{db} (3-5-34), CD80 (1G10), and CD86 (GL1), all from BD PharMingen. For DC labeling in vivo, lungs, lymph nodes, and spleens were reacted with PE-conjugated anti-CD11c (N418). Before all labeling experiments, FcR blocking was performed by incubating cells with 5% normal mouse serum. Unrelated isotype-matched mAbs were used as control. Analysis was performed on a FACScan (BD Biosciences). Data were evaluated both as the percentage of positive cells and as the median fluorescence intensity (MFI).

ELISPOT

IFN-γ- and IL-4-producing $CD4^+$ T cells were enumerated by ELISPOT assay, as described (22). Purified $CD4^+$ T splenocytes (>90% pure on FACS analysis) were obtained by positive selection as described (22). Cells were cultured (1×10^5 cell/well) in complete medium (RPMI 1640 with 10% FCS, 50 mM of 2-ME, and 50 μg/ml gentamicin sulfate) for 18 h in 96-well plates previously coated with rat anti-murine R4-6A-2 (for IFN-γ) or BVD4-1D11 (for IL-4) mAbs. Biotinylated AN-18.17.24 (for IFN-γ) or BVD6-24G2 (for IL-4) were used as the detecting reagents; the enzyme used was avidin-alkaline-phosphatase conjugate (Vector Laboratories, Burlingame, CA) and the substrate was 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine salt (Life Technologies). Results are expressed as the mean number of cytokine-producing cells (±SE) per 10^4 cells, calculated using replicates of serial twofold dilutions of cells.

Cytokine assays

The levels of TNF-α, IL-4, IL-10, and IL-12p70 in culture supernatants were determined by kit ELISA (R&D Systems, Milan, Italy). The detection limits of the assays were <32 pg/ml for TNF-α, <3 pg/ml for IL-4, <4 pg/ml for IL-10, and <16 pg/ml for IL-12p70.

Statistical analysis

The Student *t* test was used to determine the significance of values in experimental groups (significance was defined as $p < 0.05$). In vivo groups consisted of six to eight animals. The data reported were pooled from three to five experiments.

Results

DC internalize both conidia and hyphae of A. fumigatus

Resident alveolar macrophages ingest and kill resting conidia, while neutrophils attack extracellular hyphae germinating from conidia that escape macrophage surveillance (3, 4). To determine whether DC phagocytose conidia and hyphae of *Aspergillus*, FSDC were exposed to either conidia or hyphae of the fungus and internalization of fungal cells was evaluated at different times after exposure, by light microscopy and TEM. As swelling of conidia is a prerequisite step for hyphal development (3), the internalization of swollen conidia was also assessed. FSDC ingested conidia, swollen conidia, and hyphae of *A. fumigatus* in a time-dependent manner, with optimum phagocytosis at ~2 h (Fig. 1, *A–C*). DC internalized swollen conidia to an extent similar to that of hyphae. In terms of the number of fungal elements internalized by DC, swollen conidia and hyphae were only internalized as single elements while conidia could be internalized as either single or multiple elements (see also TEM). For conidia, the number of cells with PI > 1 was a function both of time and multiplicity of infection (Fig. 1, *D* and *E*). TEM revealed that the uptake of fungal cells occurred through different forms of phagocytosis. Internalization of conidia occurred predominantly by coiling phagocytosis (Fig. 2*A*), characterized by the presence of overlapping bilateral pseudopods, that led to a pseudopodal stack before transforming into a phagosome wall. In contrast, entry of hyphae occurred by a more conventional zipper-type phagocytosis, characterized by the presence of symmetrical pseudopods, which strictly followed the contour of the hyphae before fusion (Fig. 2*E*). Three hours later, conidia (Fig. 2*B*) and hyphae (Fig. 2*F*) were present inside the cells, but only the latter could be found in partially degraded forms (Fig. 2*G*). In contrast, conidia were found to be still alive inside the cells, and, interestingly, in close association with mitochondria (Fig. 2, *C* and *D*). Studies in vivo suggested that cells with DC-like morphology and topology (15) had the ability to internalize *Aspergillus* conidia and to migrate between epithelial cells. Soon after the infection, conidia were found to be internalized by phagocytic cells with the characteristics of DC morphology, as judged by numerous cytoplasmatic extensions and an abundant cytoplasm, present in the alveolar spaces (Figs. 3, *A* and *B*). DC were also found to emit pseudopods which engulf conidia and, eventually, make contact with epithelial cells (Fig. 3*B*). At the site of contact, the epithelial cells appear to invaginate (Fig. 3*C*), thus allowing DC as well free conidia to translocate to the space below, within the alveolar septal wall (Fig. 3*D*). Although macrophages are competing for the ability to phagocytose conidia (3), and the close proximity between macrophages and DC in the airways (15) may impede a clear distinction between them, the extent to which phagocytosis of conidia by bronchoalveolar macrophages is occurring in vivo is

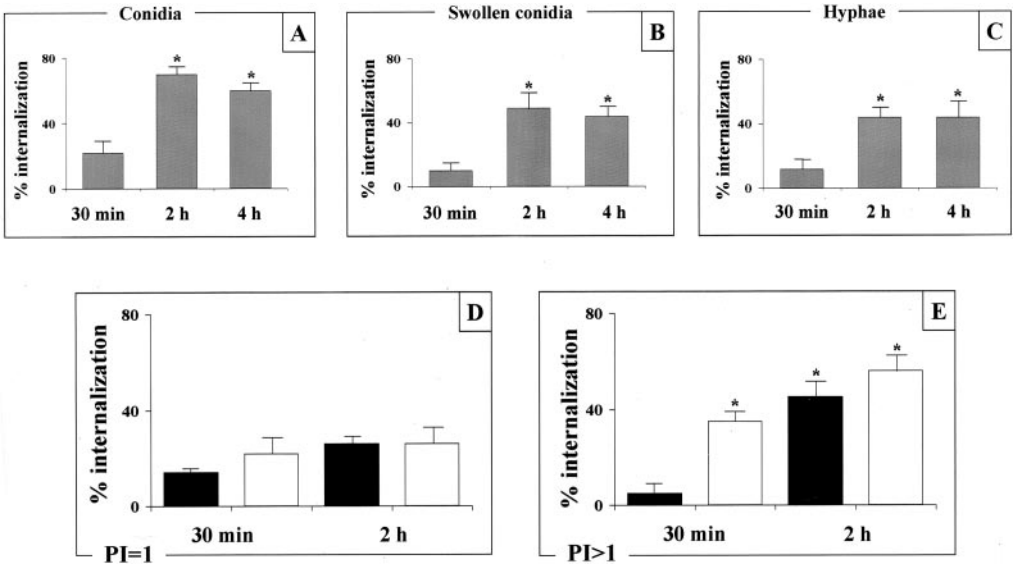

FIGURE 1. DC internalize conidia, swollen conidia, and hyphae of *A. fumigatus*. Immature myeloid immortalized DC were exposed to conidia (*A*), swollen conidia (*B*), and hyphae (*C*) at a DC-fungi ratio of 1:5 (for conidia and swollen conidia) and 1:1 (for hyphae) for different times before being evaluated for internalization, visualized by light microscopy, and expressed as described in *Materials and Methods*. *D* and *E*, Internalization of conidia by FSDC at a DC-fungi ratio of 1:1 (■) and 1:5 (□). The PI indicates the number of cells containing one (PI = 1) or more (PI > 1) fungal elements per 100 cells. ∗, $p < 0.05$; 2 or 4 h vs 30 min internalization.

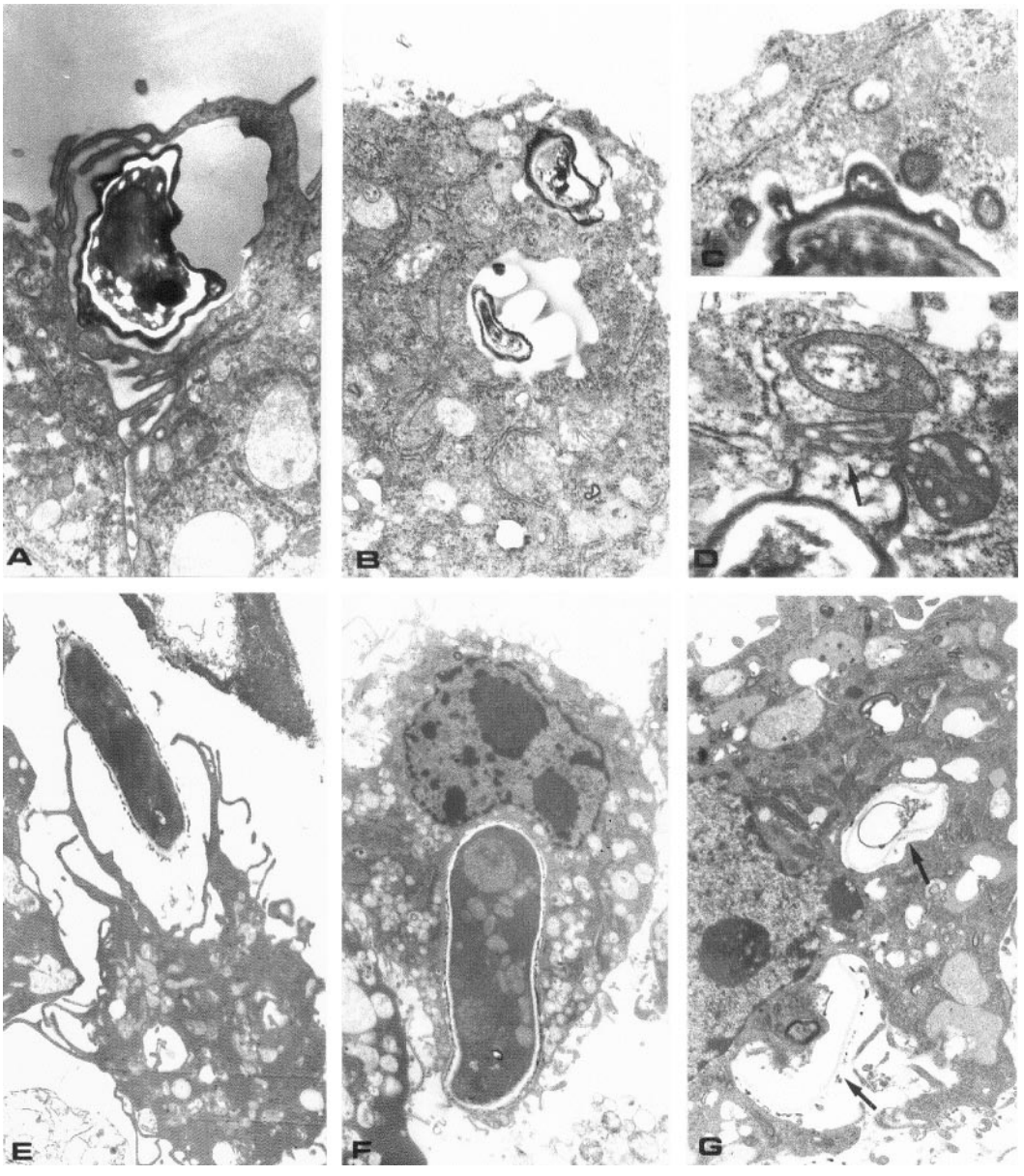

FIGURE 2. TEM of phagocytosis of *A. fumigatus* by DC. FSDC were incubated with *A. fumigatus* conidia (*A–D*) or hyphae (*E–G*) at a FSDC-*Aspergillus* ratio of 1:5 to 1:1, respectively, for 1 h (*A, E*) or 3 h (*B–D, F, G*) before processing for TEM. *A,* Conidia engulfment through coiling phagocytosis (magnification, ×20,000); conidia inside the cells 3 h later (*B;* magnification, ×12,000). *C,* Conidia are emanating thick projections (magnification, ×30,000) through which (*D*) they make contact with mitochondria (arrow; magnification, ×35,000). *E,* Hyphae uptake through zipper-type phagocytosis (magnification, ×8,000) at 1 h after infection and (*F*) inside the cells (magnification, ×8,000). *G,* Hyphae in partially degraded forms at 3 h after exposure (arrows; magnification, ×8,000).

not known at the moment. Our results suggest that both in vitro and in vivo, DC appear to be endowed with the ability to phagocytose both forms of the fungus, and, importantly, each fungal form appears to be internalized by different phagocytic mechanisms and to have different fates once inside the cells.

DC internalize both conidia and hyphae of A. fumigatus *through different recognition receptors*

As DC finely discriminated between the yeast and hyphae of *C. albicans* by using distinct recognition receptors (22)[4], we analyzed the phagocytosis of the different forms of *A. fumigatus* by pulmonary DC in the presence of distinct inhibitors or receptor ligands. Live unopsonized conidia or hyphae of the fungus were both internalized mainly through a phagocytic process, as internalization was inhibited in the presence of cytochalasin D and nocodazole (Fig. 4). The internalization of conidia, but not that of hyphae, was inhibited in the presence of EDTA, a finding suggesting the involvement of a C-type lectin receptor in the recognition of conidia. The inhibition of conidia internalization observed in the presence of the NLDC-145 mAb, known to bind to the lectin-like receptor DEC-205 (30), confirms the involvement of a lectin-like receptor. In a previous study (22), a >80% inhibition of phagocytosis of yeast cells was observed in the presence of α-mannan, a finding confirming that MR is sufficient to mediate phagocytosis of fungal yeast (31, 32). We performed uptake experiments in the presence

[4] C. Montagnoli, A. Bacci, S. Bozza, A. Spreca, P. Allavena, F. Bistoni, P Puccetti, and L Romani. The interaction of dendritic cells with *Candida albicans*: different recognition receptors determine the state of immunity or tolerance to the fungus. *Submitted for publication.*

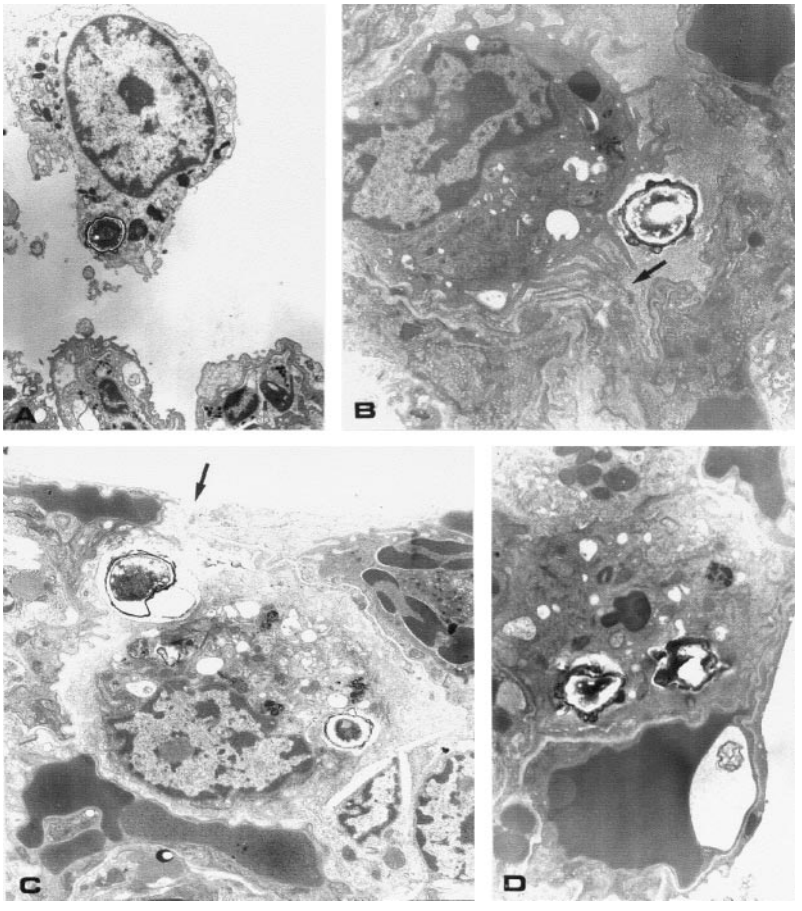

FIGURE 3. TEM of lungs of mice with *A. fumigatus* infection. Mice were i.t. injected with viable *Aspergillus* conidia (2×10^8) 2 h before being processed for TEM. *A,* Conidia are internalized by phagocytic cells with characteristics of DC morphology, as judged by numerous cytoplasmatic extensions and abundant cytoplasm, present in the alveolar spaces (magnification, ×12,000). *B,* Through emission of pseudopods, DC engulf conidia and make contact with the epithelial barrier (arrow; magnification, ×8,000). *C,* DC with engulfed conidia and free conidia migrate through invaginated (arrow) epithelial cells (magnification, ×8,000). *D,* DC with engulfed conidia are present within the alveolar septal wall (magnification, ×12,000).

of concentrations of mannan known to inhibit the MR uptake by immature DC (22), and of saturating concentrations of the MR-reacting mAb, PAM-1. We found that internalization of conidia, but not hyphae, was greatly inhibited by blocking MR with either PAM-1 or α-mannan and, interestingly, galactomannan (Fig. 4). For hyphae, the internalization was greatly inhibited by blocking CR3 with either the M1/70 mAb or β-glucan or laminarin. Interestingly, β-glucan partially inhibited conidia internalization, and this finding, together with the significant, albeit very low, inhibition observed in the presence of the M1/70 Ab, suggested that unopsonized conidia may also exploit CR3 to enter DC. Internalization of hyphae was also inhibited by blocking FcγRII and III with the 2.4G2 mAb, thus implying the involvement of these receptors in the recognition and entry of hyphae in DC. As pulmonary DC are known to express functional MR (16, 33), DEC-205, CR3 (14, 18), and FcγR (34), all together these results indicated that different recognition receptors mediate the entry of unopsonized conidia and hyphae of *A. fumigatus*. MR and C-type lectins of galactomannan specificity appear to mediate the entry of conidia. In contrast, CR3 together with FcγR is sufficient to mediate the entry of unopsonized hyphae.

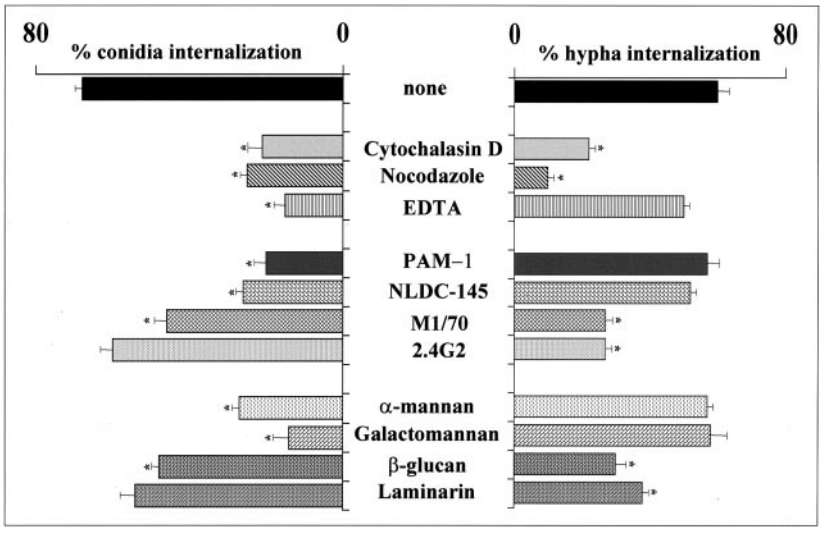

FIGURE 4. Pulmonary DC internalize unopsonized *A. fumigatus* conidia or hyphae through different Ag-recognition receptors. DC were exposed to live conidia or hyphae (DC-fungi ratio, 1:5 and 1:1, respectively) for 2 h, in the presence of cytochalasin D added at 20 μg/ml and saturating concentrations (20 μg/ml) of PAM-1, NLDC-145, M1/70, and 2.4G2 mAbs. Cells were pre-exposed at 37°C to 20 μg/ml nocodazole for 15 min, 50 mM of EDTA for 60 min, 10 μg/ml α-mannan, 250 μg/ml β-glucan, 50 μg/ml laminarin for 60 min, and 0.9 μg/ml galactomannan. Internalization was visualized by light microscopy and expressed as described in *Materials and Methods*. *, $p < 0.05$; fungal internalization in the presence of mAbs or ligands or inhibitors vs fungal internalization alone (none).

Production of cytokines by pulmonary DC exposed to A. fumigatus *conidia or hyphae*

To assess the pattern of cytokine production by DC upon phagocytosis of conidia or hyphae of the fungus, purified DC from lungs were exposed to either form of the fungus before evaluating cytokine levels in supernatants by cytokine-specific ELISA at 24 h. Similar to what was observed with *C. albicans* (22), TNF-α was produced in response to either type of fungal forms. IL-12p70 was produced upon exposure to conidia, but not to hyphae, while IL-4 was detected upon phagocytosis of hyphae, but not conidia (Table I). Different from what was observed with unopsonized *Candida* (22), the internalization of *Aspergillus* hyphae also resulted in IL-10 production which could not be detected in response to conidia. On measuring levels of cytokine production by FSDC upon exposure to conidia or hyphae, a pattern similar to that observed with pulmonary DC was found (data not shown). These results indicate that, upon exposure to *A. fumigatus* conidia or hyphae, pulmonary DC differentially produce IL-12 and IL-4/IL-10.

DC transport conidia and hyphae of A. fumigatus *from the airways to the thoracic lymph nodes*

As DC appear to phagocytose conidia and hyphae of *Aspergillus* in vivo (Fig. 3), we address the question of whether pulmonary DC, after phagocytosis of conidia or hyphae of the fungus, would migrate to the draining and peripheral lymphoid organs, such as the thoracic lymph nodes and spleens. For this purpose, FITC-labeled conidia and hyphae were i.t. injected at different times after infection and the number of FITC-positive DC were enumerated in lungs, lymph nodes, and spleens by FACS analysis. In accordance with previous studies (18, 35), DC with low-level autofluorescence are present in the lungs (Fig. 5). However, the number of $CD11c^+FITC^+$ cells greatly increased in the lungs of mice as soon as 3 h after the injection of either FITC-labeled conidia (from 7 to 51%) or FITC-labeled hyphae (from 7 to 44%). At 6 h after the infection, $CD11c^+FITC^+$ cells also appeared in the thoracic lymph nodes (from 7 to 22 and to 19%, for conidia and hyphae, respectively) and in the spleens (from 4 to 12 and to 15%, for conidia and hyphae, respectively). Because no similar increase in the number of $CD11c^+FITC^+$ cells was observed upon injecting the mice with either FITC or DMSO alone (data not shown), passive leakage of FITC from the airway mucosa to the draining lymph nodes did not occur, as already reported (18). Therefore, our results indicate that pulmonary DC transport *Aspergillus* conidia or hyphae to the draining lymph nodes and spleens. Interestingly, no fungal growth could be observed upon assaying lymph nodes and spleens for the presence of viable fungi, 3 days after challenge

Table I. *Cytokine production by lung myeloid DC upon exposure to conidia or hyphae of* A. fumigatus

Exposure to[a]	Cytokine Production[b]			
	TNF-α	IL-12p70	IL-4	IL-10
None	<32	<16	<4	<0.6
Conidia	65 ± 12[c]	41 ± 8[c]	<4	<0.6
Hyphae	117 ± 8[c]	<16	18 ± 2[c]	8 ± 3[c]

[a] Myeloid DC were isolated from lungs of BALB/c mice and exposed to viable conidia or hyphae (at cells, fungi ratios of 1:5 and 1:1, respectively) for 24 h before determination of cytokines in culture supernatants. Amphotericin B was added to the cultures for the last 22 h to prevent fungal overgrowth.

[b] Levels of cytokines (pg/ml; mean ± SE), as determined by specific ELISA; <, below the detection limit of the assay.

[c] $p < 0.05$, pulsed vs unpulsed DC.

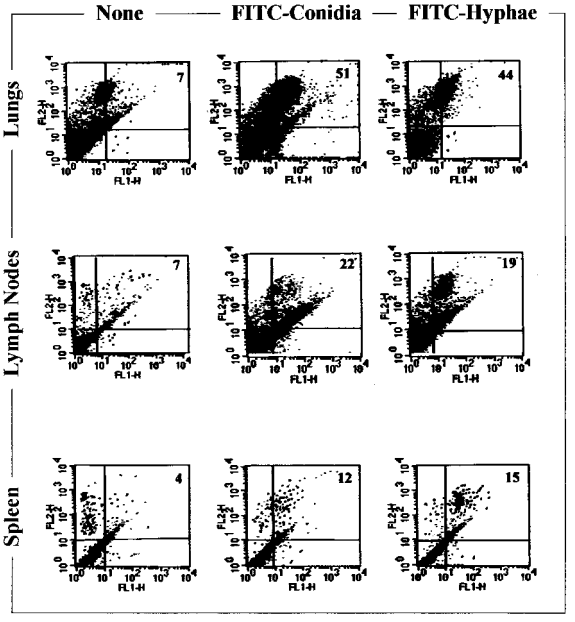

FIGURE 5. Airway DC phagocytose and transport conidia and hyphae of *A. fumigatus* to the draining lymph nodes and spleens. FITC-labeled conidia (2×10^8) or hyphae (5×10^5) of the fungus were i.t. injected into immunocompetent mice (six animals per group). Lungs (3 h later), thoracic lymph nodes, and spleens (6 h later) were removed and labeled with PE-conjugated anti-CD11c rat IgG mAb (N418) and subjected to FACS analysis for FITC positivity. The numbers in the *upper right corner* refer to the percentage of $CD11c^+FITC^+$ cells; none, uninfected control mice.

(data not shown), a finding suggesting that conidia and hyphae may have undergone degradation for efficient Ag presentation by DC, eventually.

DC undergo functional maturation during migration

To assess whether airway DC from mice injected with *Aspergillus* conidia or hyphae undergo functional maturation during migration to the draining lymph nodes and spleens, the levels of MHC class II Ags and CD80 and CD86 costimulatory molecules were assessed in DC purified from lungs, lymph nodes, and spleens at different times after infection. It was found that the exposure to either form of the fungus did not increase the expression of the above activation or costimulatory molecules in pulmonary DC at 3 h after infection, except for the small increase in the MFI for CD80 upon exposure to conidia. In contrast, the expressions of MHC class II Ags and CD80 and CD86 molecules were all greatly increased in DC from spleens, but particularly, lymph nodes of mice infected with either type of the fungus, 6 h previously (Fig. 6). Therefore, pulmonary DC undergo functional maturation upon migration from the airways to the local and peripheral lymphoid organs in mice with aspergillosis.

Th priming in vivo during Aspergillus *infection*

To correlate migration and maturation of pulmonary DC with their ability to induce T cell priming in the lymph nodes and spleens, mice were infected with conidia and hyphae of the fungus and were assessed 3 days later for Th1 or Th2 priming in the thoracic lymph nodes and spleens. To this purpose, the number of IFN-γ- or IL-4-producing $CD4^+$ cells was enumerated by ELISPOT assay. We found that the number of IFN-producing cells greatly increased in both the lymph nodes and spleens of mice injected with *Aspergillus* conidia, while IL-4-producing cells were increased in mice exposed to hyphae (Fig. 7). A small, but significant, lymphoproliferative response was also observed, at this time,

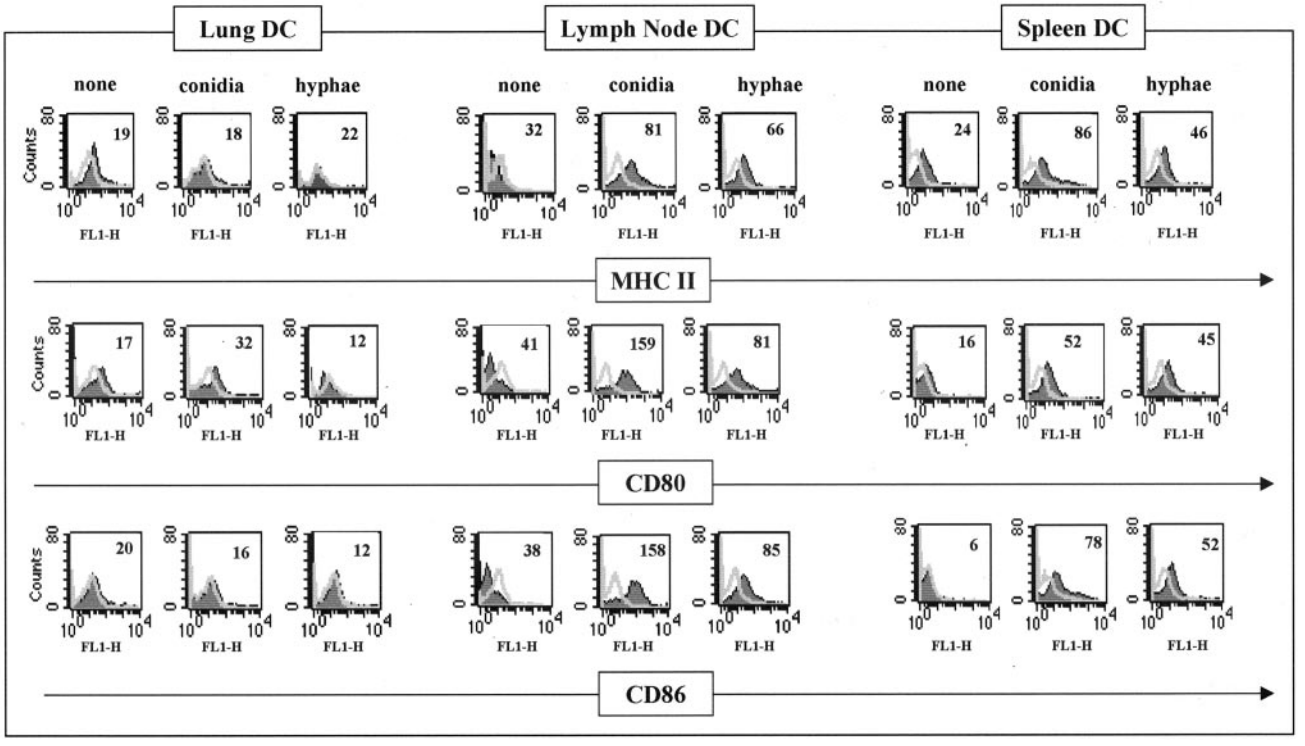

FIGURE 6. Airway DC undergo functional maturation while migrating to the draining lymph nodes or spleens in mice with *A. fumigatus* infection. Mice were injected with conidia (2×10^8) or hyphae (5×10^5) of the fungus i.t. (eight animals per group). Three (for pulmonary DC) or 6 (for DC from lymph nodes or spleens) h after the infection, DC were purified from lungs, thoracic lymph nodes, and spleens and stained with FITC-anti-MHC class II, -anti-CD80, or -anti-CD86 mAbs. The numbers in the *upper right corner* refer to the MFI ($p < 0.05$; MFI of lymph node and spleen DC from infected mice vs control DC); none, uninfected mice.

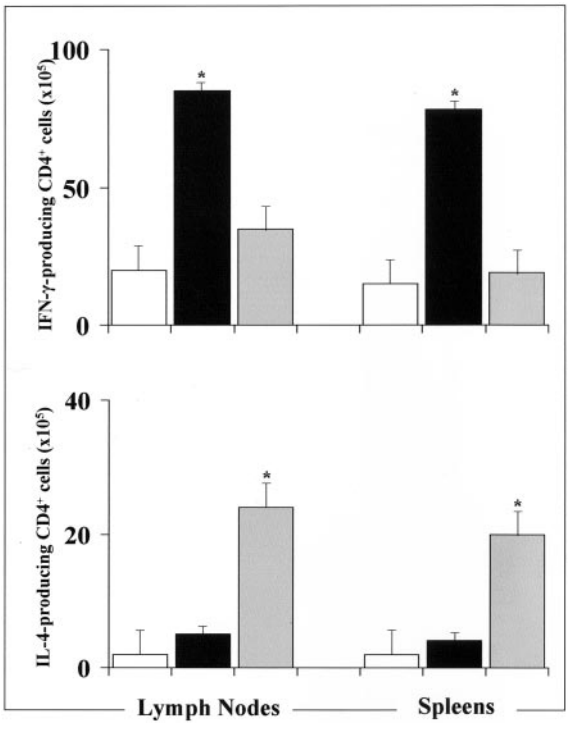

FIGURE 7. Th priming in mice with *A. fumigatus* infection. Mice were injected with conidia (2×10^8; ■) or hyphae (5×10^5; ▨) of the fungus i.t. Three days later, $CD4^+$T cells were purified from thoracic lymph nodes and spleens and the number of IFN-γ- or IL-4-producing cells were enumerated by ELISPOT assay. □, uninfected mice; ∗, $p < 0.05$; infected vs uninfected mice.

in the lymph nodes and spleens of infected mice (data not shown). Together, these data indicate that Th1 and Th2 cell activation occurs in the local and distant lymphoid organs of mice infected with *A. fumigatus*.

Discussion

The results of this study show that: 1) murine pulmonary DC internalize conidia and hyphae of *A. fumigatus* both in vitro, and during infection, in vivo; 2) internalization occurs through distinct phagocytic mechanisms and recognition receptors; 3) phagocytosis of conidia induces IL-12 production, while that of hyphae induces IL-4 and IL-10; and 4) in vivo, pulmonary DC, after phagocytosis of conidia or hyphae, migrate to the draining lymph nodes and spleens, undergo functional maturation, and induce selective Th priming of $CD4^+$ T lymphocytes.

Myeloid DC, unlike conventional phagocytic cells such as macrophages for which phagocytosis of conidia only had been reported (3, 4), phagocytosed both forms of the fungus through different phagocytic mechanisms. Similar to what was observed during uptake of *C. albicans* yeast and hyphae (22), FSDC engulfed conidia, via coiling phagocytosis, and hyphae through a more conventional zipper-type phagocytosis. Although the coiling phagocytosis is considered to reflect a disturbance of the more conventional zipper-type phagocytosis (36), it appears that DC use both forms of phagocytosis to internalize fungi. Once internalized, the fate of conidia and hyphae inside the cells was different. For hyphae, different stages of progressive degradation were seen. In contrast, conidia were apparently still alive 3 h after phagocytosis, a finding confirming the relative resistance of resting conidia to killing by effector phagocytes (5, 37). Interestingly, conidia were found to emit thick protrusions which promote close contact with mitochondria. The significance of this finding is presently under investigation.

Although killing of conidia would seem to be a necessary prerequisite to obtain efficient Ag presentation, it has been demonstrated that infected DC were still capable of stimulating lymphocyte proliferation (38). Therefore, either a small number of conidia are actually degraded by mature DC thus allowing their Ag processing and presentation, or, alternatively, Ags could be processed and regurgitated by other infected phagocytes and then transferred to DC for presentation.

Conidia of *A. fumigatus* are known to be ingested by professional and nonprofessional phagocytes, through a variety of mechanisms and opsonic requirements (3, 39). However, receptors for binding and phagocytosis on the different cell types had not been identified. For monocytes and macrophages, a mannosyl-fucosyl receptor (39) and receptors inhibited by glucan and chitooligosaccharides (40) have been described. Conidial binding to pneumocytes was instead glycosphingolipid-mediated (41).

Receptors that have been identified on immature DC include lectins such as the MR and DEC-205 as well as FcεRI and FcγR (42–44). Receptors for Ag capture on DC vary in their ligand, specificity, and mode of delivery to Ag-processing compartments (22, 45). In this study, we show that distinct receptors on DC are involved in the internalization of unopsonized conidia or hyphae of the fungus in vitro. Receptor-mediated entry of conidia was greatly inhibited by blocking lectin-like receptors, including MR and the DEC-205. A little but significant inhibition was also observed by blocking the CR3, a finding in line with what was observed with alveolar macrophages (39). Studies done in the presence of different saccharides, known to inhibit the lectin-like attachment on macrophages (39), revealed that pattern of saccharide inhibition was different from that observed for the mannosyl-fucosyl receptor on macrophages (data not shown). We found that conidia internalization was strongly inhibited by galactomannan, whose structure is similar to that reported for galactomannan of *A. fumigatus* (46). Because galactomannans are abundantly secreted during infection (3), it can be hypothesized that secreted galactomannans may compete with conidia for binding to DC, thus preventing DC activation. This will be in line with the observation of high levels of galactomannans in invasive aspergillosis (3, 47).

For hyphae, the internalization by DC was greatly inhibited by blocking CR3 or FcγR II and III with specific-blocking mAbs or specific receptor ligands, such as β-glucan and laminarin for CR3 (48, 49). However, β-glucan and laminarin also bind to a recently discovered PRR for fungal β-1,3-D-glucans, the dectin-1 receptor (50), a finding implicating the possible involvement of additional glucan receptors in host recognition of *Aspergillus*. As the phenomenon was observed with hyphae, irrespective of opsonization, this suggests that unopsonized hyphae are endowed with the ability to activate opsonic receptors. Overall, these results, similar to those obtained in candidiasis (22),[4] suggest that fungi have exploited common pathways of entry into DC, which may include a lectin-like pathway for unicellular forms and opsono-dependent pathways for filamentous fungi.

Whether the engagement of these receptors also occurs in vivo is not known at the moment. However, a number of considerations makes it likely: 1) all of these receptors are known to be expressed on DC in the airways (14, 18, 33) and MR are principally involved in the uptake and transport of macromolecules by these cells (16, 18, 33); 2) activation of a complement occurs in response to *A. fumigatus* (51); and 3) the mannose-binding lectin gene polymorphisms are recognized as a susceptible factor for chronic necrotizing pulmonary aspergillosis (52).

One interesting observation of this study concerns the role of epithelial cells in assisting DC trafficking in the lungs. The activity of epithelial cells includes the formation of invaginations through which DC translocate to the space below. This mechanism is similar to that of caveolae plasma membrane invaginations, known to play an important role in normal transport in the lung (53). However, as chitin derivatives induce transient opening of tight junctions between epithelial cells (54), it is likely that the opening of the tight junctions between epithelial cells by DC may also occur, as recently reported in the gut (55). In addition, as bronchial epithelial cells constitutively produce IL-10 (56) and epithelial cells isolated from lungs produce IL-10 in response to conidia and hyphae of the fungus (data not shown), it is likely that the contribution of epithelial cells to the overall performance of DC in the lung may go beyond the assistance in cell migration to include an effect on cell maturation.

In normal circumstances, a state of tolerance to inhaled Ags is achieved through several mechanisms (57) including IL-10 production by local DC (21). It is known that DC of the respiratory tract are specialized for uptake and processing, but not for Ag presentation, because the latter requires cytokine maturation signals that are encountered after migration to regional lymph nodes (18, 58, 59). Although transfer of Ag between different types of APC in vivo cannot be excluded (60), direct migration of airway DC to the draining lymph nodes has been demonstrated (18, 59). Experiments using i.t. delivery of soluble proteins indicated the appearance of strong Ag-presenting activity in the DC of draining lymph nodes between 6 and 24 h after instillation (18, 58).

In this study, we show that pulmonary DC produce IL-12 in response to conidia and IL-4 and IL-10 in response to hyphae. Different pathways and receptors on DC regulate cytokine production in response to different stimuli (44, 45). It is now recognized that several intracellular pathogens exploit different pathways of IL-12 attenuation (61). IL-12 suppression by these pathways may occur through different mechanisms. The yeast *Histoplasma capsulatum*, for instance, by entry through CR3, down-modulates IL-12 production on phagocytes in a manner dependent on its binding to the β2 integrins (62). The FcγR-mediated inhibition appears to proceed by more than on mechanism, including IL-10 production upon ligation of FcγRI (63). Therefore, the different sets of cytokines produced by DC upon exposure to conidia or hyphae may reflect the selective involvement of distinct recognition receptors.

Pulmonary DC did not up-regulate the expression of MHC class II Ags and costimulatory molecules upon phagocytosis of either form of the fungus, a finding compatible with the features of airway DC. In contrast, the appearance of $CD11c^+FITC^+$ cells in the thoracic lymph nodes and spleens as early as 3 h after the i.t. injection of FITC-labeled conidia or hyphae is consistent with the transport of fungi and migration of airway DC to regional and peripheral lymphoid organs. This phenomenon was associated with the up-regulated expression of activation and costimulatory molecules, particularly for DC with conidia. Moreover, local activation of cytokine-producing $CD4^+$ T cells also occurred, as IFN-γ-producing cells were observed in response to the unicellular form of the fungus and IL-4-producing cells were observed in response to hyphae. Therefore, as with *C. albicans* (64), DC discriminate between conidia and hyphae of *Aspergillus* in terms of the type of Th cell responses elicited.

From a conceptual point of view, it is intriguing that the host has evolved different types of responses toward unicellular (conidia or yeast) or filamentous forms of opportunistic fungi such as *C. albicans* and *A. fumigatus*. In particular, it would appear that the discriminative response toward Th2 is of weak teleological meaning. Although the evolutionary selection pressure on the immune system is such that virulent pathogens to which nonprotective Th2 responses still exist, in the case of fungi, one may argue that the

filamentous, rather than the unicellular, fungal growth is of evolutionary advantage, at least under a selected condition of growth.

Much remains to be learned with regard to factors and mechanisms governing local immune reactivity upon exposure to *Aspergillus* conidia, such as the role and functional activity of the different PRRs, including collectins, pathogen-associated molecular patterns, and opsonins. In this regard, it is worth mentioning that metabolites of *Aspergillus* exist which have the capacity to negatively affect the activity of DC (65). Although circumstantial evidence indicates that a Th1/Th2 dysregulation and a switch to a Th2 immune response may contribute to the development of an unfavorable outcome for IPA (10, 66), little is known about the mechanisms of immunoregulation operating in conditions of continuous exposure to nonfatal low doses of *Aspergillus* conidia, as those which presumably occur in nature. Our finding that the mode of entry of conidia into DC was dependent on time and multiplicity of the infection may suggest that exposure to low or high doses of conidia may impact DC activation and the subsequent immune responses that are elicited differently.

All together, these results point to a unique role of DC in aspergillosis, as they behave as both sentinel for innate immune recognition and initiator of Th cell differentiation and functional commitment. In doing so, murine DC are exquisitely sensitive to the different forms of the fungus, a finding in line with the increasingly recognized importance of PRR in antifungal host defense (67–69). Considering that phagocytosis of inactivated *Aspergillus* conidia induced functional maturation of human DC derived from $CD54^+$ progenitors and that ex vivo-generated DC could partially restore the antifungal effector T cell reactivity in vitro in hemopoietic transplantation (70), our findings provide important and novel insights into the key role of DC in the regulation of antifungal immune reactivity, which may go from immunity to autoimmunity and allergy (71).

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