

Original article

Vaccination of mice against invasive aspergillosis with recombinant *Aspergillus* proteins and CpG oligodeoxynucleotides as adjuvants

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Abstract

In a murine model of invasive pulmonary aspergillosis, dendritic cells (DCs) pulsed with *Aspergillus* antigens induced the activation of CD4⁺ Th1 cells capable of conferring resistance to the infection. Here we show that the combined, local delivery of unmethylated CpG oligodeoxynucleotides (ODNs) and the Asp f 16 *Aspergillus* allergen resulted in the functional maturation and activation of airway DCs capable of inducing Th1 priming and resistance to the fungus. Therefore, ODNs act as a potent adjuvant for the vaccine-induced protection against the fungus by promoting dominant Th1 response to *Aspergillus* antigens and allergens.

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1. Introduction

Invasive pulmonary aspergillosis (IPA) is the most common manifestation of *Aspergillus fumigatus* infection in immunocompromised patients [1]. Although distinct clinico-pathologic forms of IPA have been described [2], IPA is a fulminant and highly lethal infection with a reported mortality rate of approximately 60% [3]. Aspergilli are respiratory pathogens, and pulmonary infections are usually acquired through the inhalation of conidia [4,5]. With a diameter of about 2.5–3.5 µm, the conidia are able to reach small airways and the alveolar space, where the impaired host defense mechanisms allow hyphal germination of conidia and subsequent tissue invasion. Although epithelial and endothelial cells may internalize conidia [4,5], effector mechanisms of the innate immune system have long been recognized as major host defenses against IPA [6]. Resident alveolar macrophages ingest and kill resting conidia mainly through non-

oxidative mechanisms, while neutrophils use oxygen-dependent mechanisms to attack hyphae germinating from conidia that escape macrophage surveillance [7,8]. More recent studies in mice [9–12] and humans [13,14] have shown that a T helper (Th)1/Th2 dysregulation and a switch to a Th2-type immune response may contribute to developing an unfavorable outcome of IPA.

Antigens and allergens of *A. fumigatus* have been described as being capable of inducing distinct patterns of Th cytokine production [4,15–17]. Clinical and experimental evidence indicates that the production of cytokines from Th2 [18,19] or Th1 [20,21] cells or both [22,23], in response to different *Aspergillus* proteins, contributes to the pathogenesis of allergic and autoimmune diseases upon exposure to the fungus. We have shown that a crude culture filtrate antigen (CCFA) preparation of *A. fumigatus* acted as immunodominant antigen(s) and as possible vaccine in mice with IPA [11]. Dendritic cells (DCs) pulsed with CCFA induced the activation of CD4⁺ Th1 cells capable of conferring resistance upon adoptive transfer into susceptible mice [11]. As DCs were found to be essential in the activation of Th1 response to the fungus, in vivo [24] and in vitro [25], it is

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conceivable that the ability of *Aspergillus* antigens and allergens to activate DCs for priming of disparate Th cell responses may determine the full spectrum of reactivity observed in response to different fungal antigens and allergens.

It is known that through the use of different pattern-recognition receptors, including the Toll-like receptors (TLRs), DCs sense the different pathogen-associated molecular patterns and originate qualitatively distinct Th responses to them [26–28]. The unmethylated CpG oligodeoxynucleotides (ODNs) are pathogen-associated molecular patterns that, upon recognition by TLR9, activate plasmacytoid DCs for Th1 priming [29–32]. Distinct classes of ODNs stimulated different types of DC precursors [33] and acted as adjuvant for Th1-specific vaccination in rodents [34–36] and primates [37]. Interestingly, the mucosal delivery of ODNs converted a Th2 allergic response to a dominant Th1 response in a mouse model of asthma [38,39], and ODNs conjugated to allergens have been proposed as candidates for allergen-specific immunotherapy [40].

Because the specific sensitization to *Aspergillus* proteins in patients with different fungus-related diseases suggests clear differences in the pathways of fungal exposure and immunological recognition [41,42], it is conceivable that the flexibility of the immune recognition of the fungus could be exploited for strategies of immune intervention in fungal diseases. In the present study we have evaluated the ability of different recombinant *Aspergillus* allergens, either alone or in the presence of ODNs, to induce protective Th1 responses and oppose inflammatory pathology in mice with IPA.

2. Materials and methods

2.1. Mice

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

2.2. 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. The microorganism was grown on Sabouraud dextrose agar (Difco Laboratories, Detroit) supplemented with chloramphenicol for 4 days at room temperature. Abundant conidia were produced under these conditions. Conidia were harvested by washing the slant culture with 5 ml of 0.025% Tween-20 in saline and by gently scraping the conidia from the mycelium with a plastic pipette (or by shaking it vigorously). Cell debris were allowed to settle by gravity, and the suspension was decanted into 50-ml plastic conical tubes. After the conidia were extensively

washed with saline, they were counted and were diluted to the desired concentrations. The viability of the resting conidia was > 95%, as determined by serial dilution and plating of the inoculum on Sabouraud dextrose agar. For intranasal (i.n.) infection, mice were lightly anesthetized by inhaled diethyl ether before instillation of a suspension of 2×10^7 conidia/20 µl saline, which was applied slowly to the nostrils using a micropipette with a sterile disposable tip. Animals were held in upright position until the suspension was completely inhaled and normal breathing resumed. This procedure was repeated for 3 consecutive days. Mice dying of fungal challenge routinely underwent necropsy for histopathological confirmation of invasive aspergillosis. For histology, tissues were excised and immediately fixed in formalin. Sections (3–4 µm) of paraffin-embedded tissues were stained using the periodic acid-Schiff procedure.

2.3. Chitin assay

For the quantification of fungal growth in the lungs, the chitin assay was used as described elsewhere [9]. Results are expressed as micrograms of glucosamine per organ. The positive control included serially diluted D(+)-glucosamine (Sigma). The glucosamine content of lungs from uninfected mice was used as a negative control (range, 0.80 and 2.25 µg glucosamine/organ).

2.4. ODNs and *A. fumigatus* recombinant proteins

The ODN sequences representing the proven immunostimulatory sequence 1668 (TCC–ATG–ACG–TTC–CTG–ATG–CT) were as described [43]. The CCFA [11] and the recombinant proteins Asp f 2, Asp f 3, Asp f 12 and Asp f 16 were obtained as described [17,44–46]. All proteins bind to IgE from patients with proven allergic sensitization to the fungus [17,44–46]. Heat-inactivated *A. fumigatus* (HAF) was obtained by autoclaving conidia at 121 °C for 15 min.

2.5. In vivo treatments

Mice were immunosuppressed with 150 mg/kg of cyclophosphamide (Sigma), injected intraperitoneally, 1 day before challenge. For immunization, ODNs, 10 nM/20 µl saline, were administered i.n. 14 days before infection alone or together with 2×10^7 HAF/20 µl saline or 10 µg/20 µl saline (according to preliminary experiments) of each antigen preparation, given i.n. 14, 7 and 3 days before the first *A. fumigatus* inoculation.

2.6. DC isolation and culture

DCs were isolated from para-thracheal and para-thymic lymph nodes by magnetic cell sorting, as described [24]. Adherent cell-depleted lymph node DCs were reacted with 100 µl of MicroBeads (Miltenyi Biotec, Germany) conjugated to hamster anti-mouse CD11c monoclonal antibodies

(MAbs, clone N-418) [47,48]. In line with previous findings [24], positively selected lymph node DCs (at about 1–2% yield recovery) routinely express high levels of CD11c integrin, and between 25 and 30% stained positive for CD8 α . For functional maturation and cytokine determination, purified DCs were resuspended in RPMI containing 10% fetal calf serum, 2-mercaptoethanol (50 mM), sodium pyruvate (1 mM), HEPES (10 mM), and gentamycin (50 μ g/ml) (complete medium), plated at a concentration of 1×10^7 /ml and pulsed with Asp f 16 (5 μ g/ml) and/or ODNs (5 nM) for 24 h before harvesting cells for phenotypic analysis, and supernatants for cytokine production.

2.7. Phenotypic analysis

Cell surface phenotype of purified DCs was assessed by reacting cells with the following FITC-conjugated MAb reagents: rat IgG directed to I-A^{db} (34-5-3), CD80 (1G10) and CD86 (GL1), all from PharMingen (San Diego). 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 (Becton Dickinson, Mountain View, CA). Data were evaluated as median fluorescence intensity (MFI).

2.8. Phagocytic and conidiocidal assays

Macrophages from the bronchoalveolar lavage (BAL) fluid and neutrophils from the peritoneal cavity of thioglycollate-injected mice were obtained as described [9,12]. For the conidiocidal activity, 1×10^6 cells were incubated with 10^5 conidia for 4 h in 96-well flat-bottomed microtiter plates and the number of colony-forming units (CFU) determined as described [9,12]. The percentage of CFU inhibition (mean $\pm$ SE), referred to as conidiocidal activity, was determined as follows: % colony formation inhibition = $[100 - (\text{CFU experimental group}/\text{CFU control cultures})] \times 100$. For phagocytosis, equal volumes of effector cells and a suspension of unopsonized live conidia, at an effector-to-conidia ratio of 1/1, were incubated for 4 h at 37 °C in 6 ml polypropylene tubes (Falcon), in 200 μ l of Iscove medium containing 5 μ g/ml polymixin B (Sigma) and 50 μ g/ml gentamycin but no FCS to avoid non-specific activation by serum components and endotoxin. Phagocytic cells were separated from non-phagocytosed 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 a 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.

2.9. Spot enzyme-linked immunosorbent (ELISPOT) assay

CD4⁺ T cells (> 95% pure on FACS analysis) were isolated from lungs by using anti-mouse CD4-conjugated magnetic MicroBeads, as described [24]. Briefly, the organs were removed aseptically, minced, and incubated for 90 min at 37 °C in digestion buffer containing 0.7 μ g/ml collagenase (Sigma) and 30 μ g/ml type IV bovine pancreatic DNase I (Sigma). After removal of large particulate matter, 1×10^7 total cells were incubated with 10 μ l of MACS CD4⁺ MicroBeads for 15 min at 6 °C, washed and magnetically separated with a positive selection column, according to the manufacturer's instructions. For enumeration of IFN- γ , IL-10- and IL-4-producing CD4⁺ T cells, an ELISPOT assay was used [24]. Freshly harvested cells were cultured (1×10^5 cells/well) in complete medium for 18 h in 96-well plates previously coated with rat anti-murine R4-6A-2 (for IFN- γ), BVD4-1D11 (for IL-4) or JES5-2A5 (for IL-10) MAbs. Biotinylated AN-18.17.24 (for IFN- γ), BVD6-24G2 (for IL-4) or SXC-1 (for IL-10) MAbs were used as the detecting reagents (PharMingen). The enzyme was avidin-alkaline phosphatase conjugate (Vector Laboratories), and the substrate was 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine salt (Life Technologies). Results are expressed as the mean number of cytokine-producing cells ($\pm$ SE) per 10^5 cells, calculated using replicates of serial twofold dilutions of cells.

2.10. Cytokine assays

The levels of IL-12 p70 in BAL fluids and culture supernatants were determined by Kit ELISA (R&D Systems). The detection limit of the assay was <16 pg/ml.

2.11. Proliferation assay by flow cytometric analysis

Lung CD4⁺ T lymphocytes (1×10^6 /ml) were cultured with 5×10^5 /ml purified lymph node DCs in the presence of ODNs (5 nM) or Asp f 16 (5 μ g/ml), either alone or in combination, or concanavalin A (10 μ g/ml). A 5-mM stock solution of CFSE 5(6)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes), in DMSO (stored desiccated at -20 °C) was added to a final concentration of 10 mM. After incubation at 37 °C for 5 days, cells were washed and analyzed by flow cytometry with logarithmic detection of green fluorescence (CFSE). Data were evaluated as MFI.

2.12. Delayed-type hypersensitivity (DTH) assay

A direct assay system for measuring the DTH response to cell surface antigens was employed in which HAF (2×10^6 /0.04 ml of pyrogen-free saline) was inoculated into the footpads of either naive or immunocompetent mice exposed to *Aspergillus* conidia, as described [9,10]. The DTH reaction was recorded 24 h later by weighing the footpad as a measure of swelling, and the results were expressed as the

increase in footpad weight (right hind) over that of the saline-injected (left hind) counterpart. Data are the means $\pm$ SE of six mice per group.

2.13. Statistical analysis

Survival data were analyzed using the Mann-Whitney U test. Student's *t*-test was used to determine the significance of values in the experimental group (significance was defined as $P < 0.05$). In vivo groups consisted of 6–10 animals. The data reported were pooled from three to five experiments with similar results, unless otherwise specified.

3. Results

3.1. Course and outcome of IPA in mice treated with different *Aspergillus* allergens alone or together with ODNs

Multiple i.n. inoculations of viable *Aspergillus* conidia into immunocompetent BALB/c mice caused a transient, self-limiting infection that conferred immune resistance to subsequent infection [9]. Here we show that recombinant proteins of the fungus given i.n. differ in the ability to induce resistance to infection. In line with previous findings [11], HAF or the Asp f 2 antigen failed to induce protection, as opposed to the protective effect observed with the CCFA (Table 1). The Asp f 3, Asp f 12 and Asp f 16 allergens also failed to induce resistance to infection. However, the combined treatment with ODNs, locally delivered, greatly increased the immunization potential of Asp f 16, as indicated by the increased survival (almost 100% of mice surviving the

infection) and the reduced fungal burden in the lungs. No such effect was observed upon treatment with ODNs alone or combined with HAF or with the other proteins (Table 1). Histological examination of lung sections from infected mice, treated either with ODNs alone or with Asp f 16+ODNs, revealed patterns of lesions comparable to those observed in mice susceptible or resistant to IPA [9,10]. Lung sections from infected, ODN-treated mice showed signs of bronchial wall damage and infiltrates of predominantly polymorphonuclear cells scattered throughout the lung parenchyma along with numerous fungal cells (Fig. 1A, C). These features were not observed in Asp f 16+ODN-treated mice, whose lungs were characterized by few healing infiltrates of mononuclear cells and no evidence of parenchymal destruction or fungal growth (Fig. 1B, D). The analysis of cellular infiltrate into the lungs substantially confirmed the histological findings by showing a significant recruitment of phagocytes, predominantly neutrophils, in the lungs of mice succumbing to the infection (data not shown).

3.2. Resistance to IPA correlates with Th1 cell activation in mice treated with Asp f 16+ODNs

In mice with IPA, resistance to infection correlates with predominant Th1 responses, while Th2 responses are associated with susceptibility to the infection [9–12]. To assess whether parameters of Th1 reactivity occurred in mice treated with Asp f 16+ODNs, the pattern of local cytokine production, the antigen-specific lymphoproliferation and DTH reactivity were evaluated. For cytokine production, the numbers of IFN- γ -, IL-4- and IL-10-producing CD4⁺ T cells in the lungs were determined in mice treated with Asp f 16 or ODNs alone or in combination. The results show that, upon

Table 1

Effect of administration of *Aspergillus* allergens alone or in association with ODNs on the course of invasive pulmonary aspergillosis

Treatment ^a		Median survival time/days	No. of dead mice/no. of animals infected	Chitin ^b content, μ g of glucosamine/organ
–	–	6	18/18	37.7 $\pm$ 4.1
–	HAF	6.5	18/18	29.3 $\pm$ 2.7
–	CCFA	>60 ^c	0/18	15.2 $\pm$ 3.3 ^c
–	Asp f 2	4.5	18/18	n.d.
–	Asp f 3	5	17/18	n.d.
–	Asp f 12	5	18/18	n.d.
–	Asp f 16	7	16/18	28.1 $\pm$ 5.4
ODNs	–	8	18/18	30.2 $\pm$ 3.2
ODNs	HAF	7.5	18/18	27.6 $\pm$ 4.1
ODNs	Asp f 2	6	18/18	32.4 $\pm$ 2.7
ODNs	Asp f 3	5	18/18	30.6 $\pm$ 4.3
ODNs	Asp f 12	6	17/18	26.6 $\pm$ 3.5
ODNs	Asp f 16	>60 ^c	1/18	13.8 $\pm$ 4.0 ^c

For infection, BALB/c mice immunosuppressed with cyclophosphamide (150 mg/kg) a day before, were injected intranasally with 2×10^7 conidia of *A. fumigatus* for 3 consecutive days.

^a Mice were treated intranasally as follows: 2×10^7 of heat-inactivated *A. fumigatus* conidia (HAF), 10 μ g of cell culture filtrate antigen (CCFA) or Asp f 2, Asp f 3, Asp f 12, Asp f 16, administered 14, 7 and 3 days before the infection and CpG oligodeoxynucleotides (ODNs 1668, 10 nM), administered 14 days before the infection.

^b Determined in lungs of infected mice 6 days after the first intranasal injection of conidia.

^c $P < 0.05$ (CCFA-treated vs. untreated mice; ODNs + Asp f 16 vs. ODNs or Asp f 16 alone).

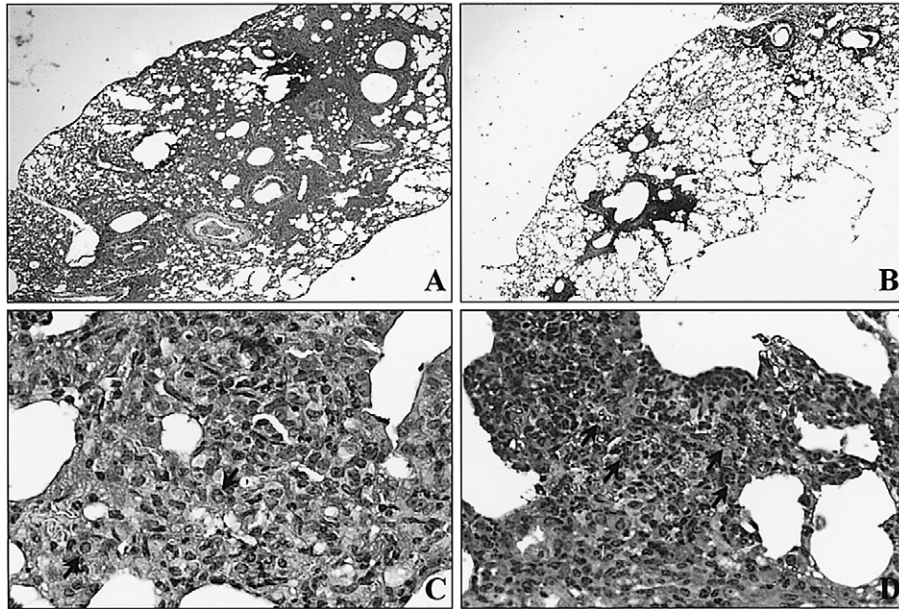


Fig. 1. Lung histology of mice vaccinated with Asp f 16+ODNs and infected with *Aspergillus fumigatus*. Periodic acid-Schiff-stained sections were prepared from lungs of BALB/c mice either treated with ODNs alone (A, C) or together with the recombinant protein Asp f 16 (B, D) and infected intranasally with *Aspergillus* conidia after being immunosuppressed with cyclophosphamide 1 day before the infection. Lungs were taken 6 days after infection. Note the presence of lesions similar to those observed in susceptible mice, characterized by signs of bronchial wall damage, peribronchial necrosis (A) and inflammatory polymorphonuclear cells and fungi (arrows in C), as opposed to the presence of few lesions (B) with infiltration of predominantly inflammatory mononuclear cells in the peribronchial region of resistant mice (arrows in D).

infection, the frequency of IFN- γ -producing cells greatly increased, while those of cells producing IL-4 or IL-10 decreased, in mice treated with Asp f 16+ODNs, compared to mice treated with either agent alone (Fig. 2). The levels of IL-12 p70 in BAL fluids were also significantly higher in mice treated with ODNs and Asp f 16, compared with mice treated with ODNs alone (data not shown). Exposure to Asp f 16+ODNs in vitro significantly increased the proliferation of CD4⁺ T cells over that of cells exposed to either agent alone, as revealed by the decreased MFI value upon CFSE labeling (Fig. 3). Moreover, on assessing the ability of Asp f 16 to elicit DTH reactivity, it was found that a strong DTH reactivity was elicited by Asp f 16 and by the CCFA preparation in both immunized and naive mice (Fig. 4). On comparing the different *Aspergillus* allergens, it was found that none of the proteins was able to elicit DTH reactivity in any type of mouse. Interestingly, HAF elicited DTH reactivity in immunized but not naive mice (Fig. 4), a finding suggesting that different epitopes may be involved in the inductive and expressive phases of DTH reactivity to the fungus.

3.3. ODNs activate DCs for Th1 priming upon exposure to Asp f 16

The activation of local Th1 cell reactivity upon the combined intranasal treatment with Asp f 16+ODNs implies the participation of antigen-presenting cells of the airways in the process. DCs are recognized as the initiators of immune responses to pathogens, including *Aspergillus* [24], in the

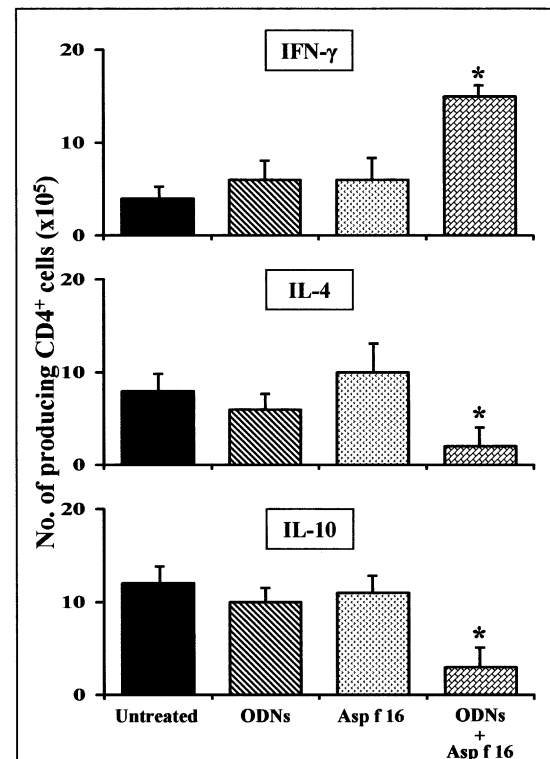


Fig. 2. Effect of treatment with Asp f 16 and ODNs, either alone or in combination, on cytokine production in mice with invasive pulmonary aspergillosis. Number of cytokine producing CD4⁺ T lung lymphocytes from mice treated with Asp f 16 and/or ODNs and infected with *A. fumigatus*. Treatments and infection were done as described in Materials and Methods. The number of cytokine-producing cells were enumerated by ELISPOT assay, 6 days after infection. * $P < 0.05$, ODNs+Asp f 16-treated vs. mice treated with Asp f 16 or ODNs alone, according to Student's *t*-test.

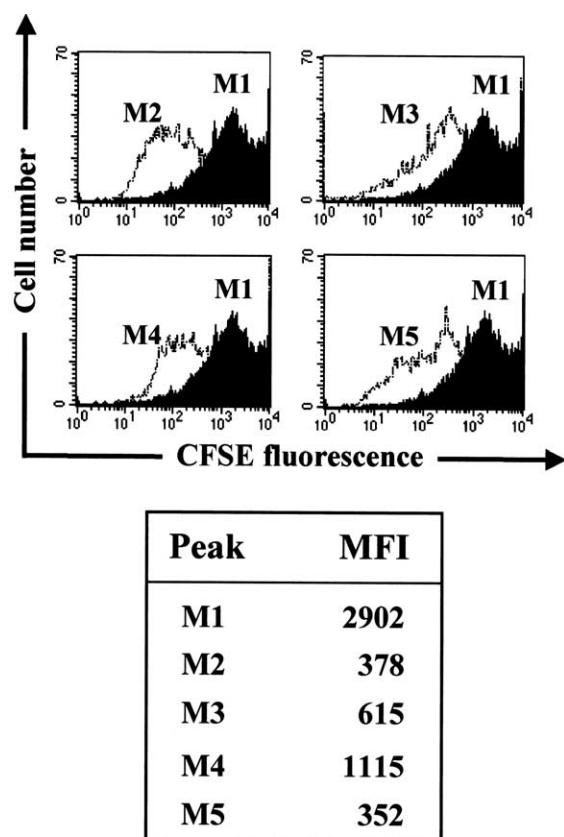


Fig. 3. Proliferative response of lung T cells stimulated with ODNs and/or Asp f 16. Lung T CD4⁺ lymphocytes were coincubated with purified dendritic cells for 5 days at 37 °C and stained with CFSE as described in Materials and Methods. Cells were unstimulated (M1), or stimulated with Con A (M2), ODNs (M3), Asp f 16 (M4) or ODNs+Asp f 16 (M5). Cells were analyzed by flow cytometry. Shown are results from one representative experiment out of three. The numbers refer to the median fluorescence intensity (MFI).

respiratory tracts [49]. However, in the resting state, respiratory tract DCs are specialized for uptake/processing but not for presentation of antigen, the latter requiring cytokine maturation signals that presumably occur after migration to regional lymph nodes [49–51]. We have already shown that DCs from the draining lymph nodes and not from lungs undergo functional maturation upon *Aspergillus* infection in vivo [24]. Therefore, to assess the effect of exposure to Asp f 16+ODNs on the antigen-presenting activity of DCs, we exposed purified lymph node DCs to either agent alone or in combination and assessed the functional maturation of DCs in terms of costimulatory molecule expression and IL-12 p70 production. We found that the exposure to Asp f 16 alone did not affect DC maturation, at least in terms of the above-mentioned criteria (Fig. 5A, B). Exposure to ODNs slightly increased the expression of costimulatory CD80 and CD86 molecules, MHC Class II antigens and the production of IL-12 p70. However, the combined exposure to both further

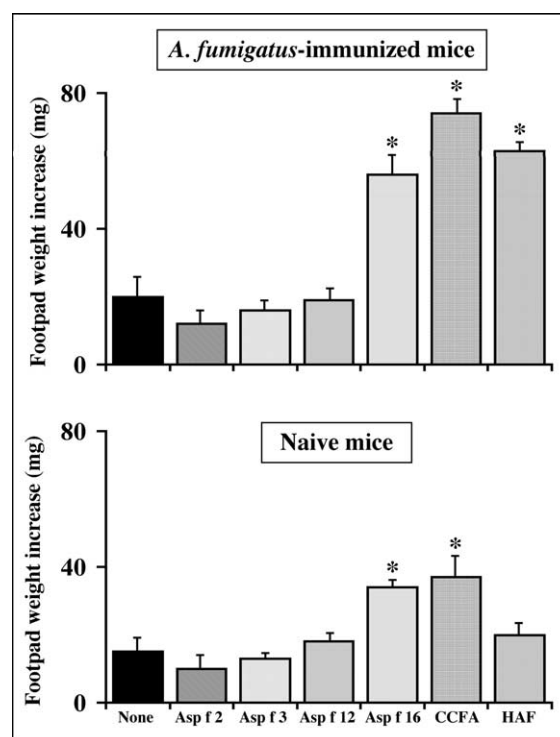


Fig. 4. Ability of *Aspergillus* recombinant proteins to elicit delayed-type hypersensitivity response. Immunocompetent BALB/c mice were infected intranasally with 2×10^7 *Aspergillus* conidia for 3 consecutive days. The recombinant allergens Asp f 3, Asp f 12 or Asp f 16, the heat-inactivated *Aspergillus* (HAF) and the cell culture filtrate antigen (CCFA) were injected intra-footpad into naive or *Aspergillus*-infected mice 14 days after the infection. Footpad weight was measured 24 h after the treatment. Values are the mean $\pm$ SE of 3–5 experiments, each involving 6 animals/group. * $P < 0.05$, Asp f 16 or CCFA vs untreated in naive mice; HAF, CCFA and Asp f 16 vs untreated in immunized mice, according to Student's *t*-test.

increased the expression of costimulatory molecules and the production of IL-12 p70.

3.4. ODNs stimulate the antifungal activity of effector phagocytes

The antifungal activity of effector phagocytes is acknowledged as essential in the resistance to IPA, early in infection [9,10,12]. Moreover, recent evidence indicates that TLRs activate the innate immune system not only to assist the adaptive immune system but also for direct antimicrobial effector activity [52]. Therefore, the possibility exists that ODNs not only promote DC maturation but may also activate local effector cells for prompt phagocytosis and killing of *A. fumigatus*. To verify this hypothesis, alveolar macrophages and peritoneal neutrophils from uninfected mice were assessed for phagocytosis and killing of conidia in vitro in the presence of ODNs. Although macrophages, more than neutrophils, are considered to be effectors against conidia [7], studies by electron microscopy have recently shown that lung neutrophils are also endowed with the ability to ingest and kill resting conidia [53]. The results indicate that the expo-

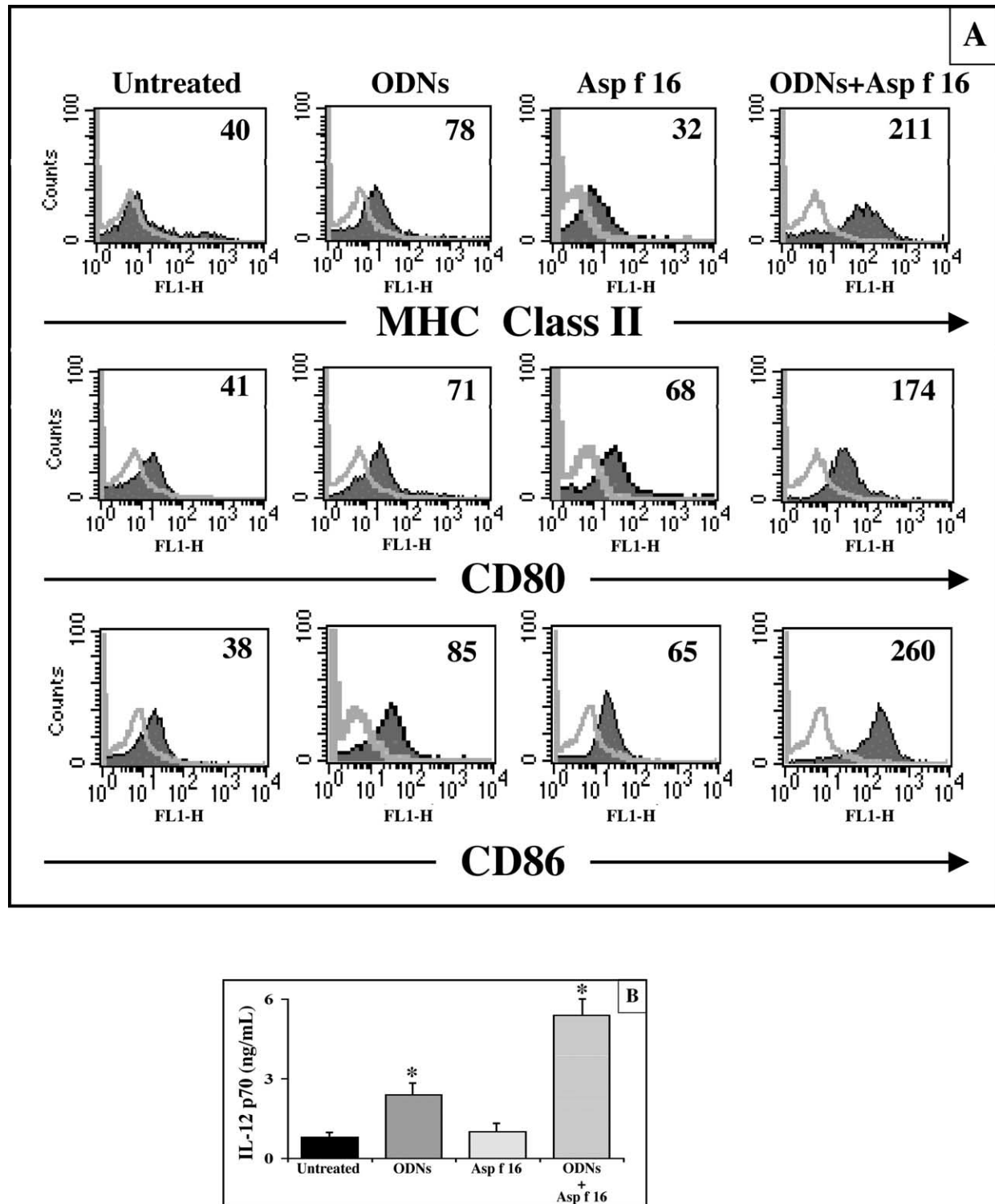


Fig. 5. ODNs activate dendritic cells for Th1 priming upon exposure to Asp f 16. Purified dendritic cells from the thoracic lymph nodes were exposed *in vitro* to ODNs, Asp f 16, or both for 24 h before harvesting cells for phenotypic analysis (A) and supernatants for IL-12 p70 production (B). Cell surface expression of MHC class II antigens and CD80 and CD86 costimulatory molecules was assessed by FACS analysis. Untreated, unexposed cells. The numbers refer to the median fluorescence intensity. Shown are the data from one representative experiment out of three. The production of IL-12 p70 was assessed by specific ELISA. Values are the mean $\pm$ SE of samples from 3–5 experiments. * $P < 0.05$, ODNs+Asp f 16-treated or ODN-treated vs mice treated with Asp f 16 or ODNs alone, according to Student's *t*-test.

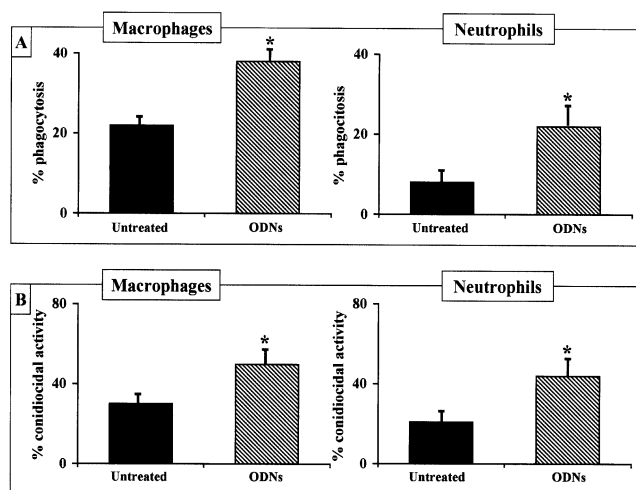


Fig. 6. Effect of ODNs on the phagocytic and conidiocidal activity of effector phagocytes. Bronchoalveolar macrophages and peritoneal neutrophils were incubated with resting conidia in the presence of ODNs and the conidiocidal activity assessed as described in Material and Methods. Values are the mean $\pm$ SE of samples taken from 3–5 experiments, each involving 6 animals/group. Each sample was assayed in triplicate. * $P < 0.05$, neutrophils and macrophages treated with ODNs vs untreated cells, according to Student's t -test.

sure to ODNs significantly increased the phagocytic and conidiocidal activity of macrophages and, to a greater extent, of neutrophils (Fig. 6). Interestingly, the effect appears to be strictly dose dependent, being inhibited at higher concentrations of ODNs (data not shown).

4. Discussion

The present study shows that in a murine model of IPA, the appropriate combination of *Aspergillus* proteins and adjuvants may be useful in vaccination strategies against the fungus. Resistance to infection was not observed after administration of the different allergens or ODNs alone but it was induced upon the combined administration of the Asp f 16 allergen and ODNs. Protection correlated with the activation of DCs for Th1 priming, as suggested by the occurrence of IL-12/IFN- γ production, DTH reactivity and strong lymphoproliferation. However, the exposure to ODNs also potentiated the antifungal activity of effector phagocytes, a finding confirming the benefit of the coordinated activation of the innate and adaptive immune systems in resistance to the fungus [9,10,12].

A variety of allergens of *Aspergillus* have been cloned [15–21,44–46]. Clinical and experimental evidence indicates that the different immunological reactions, elicited by the different *Aspergillus* allergens [42], including those with a degree of sequence identity to the related human proteins [20,21,42,54], may contribute to the pathogenesis of allergic and autoimmune diseases upon exposure to the fungus. However, different IgE allergic responses are induced to the fungus in patients with different fungus-related diseases

[41,42], a finding suggesting that a certain degree of flexibility of the immune recognition pathways to the fungal allergens exists. We found here that the Asp f 16 allergen could be converted into an immunodominant protective antigen by association with ODNs. It is interesting to note that the effect of ODNs could not be observed indiscriminately with the other allergens, a finding suggesting that differences may exist among the different allergens and/or allergen recognition upon which ODNs may work. The Asp f 16 allergen, similarly to the other allergens of the present study, reacted with IgE from patients with *Aspergillus* allergy and induced strong lymphoproliferation [46]. However, at variance with the other allergens, we found here that Asp f 16 was uniquely able to act as immunodominant antigen in both the inductive and expressive phases of the DTH reaction. This would suggest that co-expression of epitopes capable of activating cell-mediated immune reactivity may allow for an *Aspergillus* allergen to behave like a potential protective antigen in infection with the fungus, given the appropriate adjuvant environment.

ODNs fulfilled this requirement, as they primed for local production of IL-12 and IFN- γ in response to the Asp f 16 allergen. ODNs alone also primed for local IL-12 production and induced the functional maturation of DCs, a finding indicating that induction of local Th1-biased response may underlie the adjuvant effect of ODNs in this setting. This would explain the failure of ODNs given intravenously to act as adjuvant in mice exposed to the Asp f 2 allergen in the airways (data not shown). However, as ODNs are specifically recognized by TLR9 [29–32], it is also possible that the efficacy of the site-specific delivery of ODNs may depend on the cellular and site distribution of TLR9. Other studies have shown that the adjuvant effect of ODNs may depend on the route of administration [36], the optimal adjuvant effect being observed upon mucosal delivery [55,56].

Exposure to CpG-DNA is considered to recapitulate some immunological aspects of live bacterial infection, including Th1-biased responses [34–36]. It appears that ODNs of different classes, through the activation of both innate and adaptive immune responses [33,57], act as adjuvant for Th1-specific vaccination in rodents [34–36] and primates [37], are candidates for allergen-specific immunotherapy [38–40], and minimize the mucosal inflammatory response [57]. We found here that the adjuvant activity of ODNs relied on an effect on both DCs and effector phagocytes. A dense network of DCs has been described in the respiratory tracts that mediate the local surveillance of pathogens and initiate immune responses to them [48], including *A. fumigatus* [24]. DCs appeared to be uniquely able to distinguish among the different forms of the fungus, thus showing a remarkable functional plasticity. Through the use of pattern-recognition receptors, DCs finely discriminated between conidia and hyphae and were responsible for the disparate Th responses to them [24].

As recruitment and activation of effector phagocytes into the lung are required for the proper control of IPA and its

inflammatory pathology [12], the finding that ODNs increased the functional activity of alveolar macrophages and, particularly, polymorphonuclear neutrophils against the fungus, is of interest. Neutrophils of ODN-treated mice have already been shown to exhibit an up-regulation of phagocytic receptors, an increased phagocytic activity, and an elevated production of reactive oxygen metabolites [58]. These findings not only shed new light on TLR expression and function on cells of the innate immune system devoted to antigen presentation but also suggest that the exploitation of TLRs on cells of the innate immune system may represent a new option for therapeutic strategies against microbial infections.

In conclusion, the unique nature of the *Aspergillus* infection, a saprophytic fungus colonizing immunocompromised hosts, demands a better understanding of immunological mechanisms required to efficiently oppose fungal infectivity. This study shows that to improve the immune response against *Aspergillus*, the deliberate targeting of cells and pathways of cell-mediated immunity to the fungus may represent a useful strategy in developing effective strategies of vaccination to the fungus. The ultimate challenge will be to design fungal vaccines capable of inducing the optimal class of response by the site-specific delivery in vivo of the appropriate antigen and adjuvant, as already suggested [59,60].

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