

Distinct CD4⁺-T-Cell Responses to Live and Heat-Inactivated *Aspergillus fumigatus* Conidia

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Aspergillus fumigatus is an important fungal pathogen that causes invasive pulmonary disease in immuno-compromised hosts. Respiratory exposure to *A. fumigatus* spores also causes allergic bronchopulmonary aspergillosis, a Th2 CD4⁺-T-cell-mediated disease that accompanies asthma. The microbial factors that influence the differentiation of *A. fumigatus*-specific CD4⁺ T lymphocytes into Th1 versus Th2 cells remain incompletely defined. We therefore examined CD4⁺-T-cell responses of immunologically intact mice to intra-tracheal challenge with live or heat-inactivated *A. fumigatus* spores. Live but not heat-inactivated fungal spores resulted in recruitment of gamma interferon (IFN- γ)-producing, fungus-specific CD4⁺ T cells to lung airways, achieving *A. fumigatus*-specific frequencies exceeding 5% of total CD4⁺ T cells. While heat-inactivated spores did not induce detectable levels of IFN- γ -producing, *A. fumigatus*-specific CD4⁺ T cells in the airways, they did prime CD4⁺ T-cell responses in draining lymph nodes that produced greater amounts of interleukin 4 (IL-4) and IL-13 than T cells responding to live conidia. While immunization with live fungal spores induced antibody responses, we found a marked decrease in isotype-switched, *A. fumigatus*-specific antibodies in sera of mice following immunization with heat-inactivated spores. Our studies demonstrate that robust Th1 T-cell and humoral responses are restricted to challenge with fungal spores that have the potential to germinate and cause invasive infection. How the adaptive immune system distinguishes between metabolically active and inactive fungal spores remains an important question.

Aspergillus fumigatus is a ubiquitous mold that produces small, 2- to 3- μ m spores (aka conidia) that are inhaled into lung alveoli. The hydrophobic nature of the spores and their small size allow them to remain airborne for long periods (30, 31). *A. fumigatus* spores are ubiquitous, and it is estimated that humans inhale several hundred conidia per day (31). In the lung airways, conidia are phagocytosed by alveolar macrophages, where they swell (the first stage of germination), thereby triggering the production of reactive oxygen intermediates (ROI) by the macrophage, a lethal event for the spore (30, 31, 34, 41, 44). Conidia that escape killing by alveolar macrophages germinate, eventually forming hyphae that invade tissues and blood vessels. Neutrophils are recruited to sites of fungal invasion, where they adhere to the hyphal surface and release ROI as well as hydrolytic enzymes that damage the fungal cell wall (22, 30, 33, 34, 44).

Administration of immunosuppressive medications increases the incidence of invasive aspergillosis (IA) (31) and is responsible for the current status of *Aspergillus fumigatus* as one of the most prevalent airborne fungal pathogens (30, 31). The contributions of innate and adaptive immunity to protection of the immuno-competent host from invasive *A. fumigatus* infections are complex and incompletely defined. Clinical experience and studies in animal models implicate neutrophils and macrophages and their products, such as ROI and pentraxin 3, in innate immune

defense against *A. fumigatus* (19, 22, 30, 31, 33, 34, 44). T cells are increasingly recognized as important mediators of protection from IA (53). Vaccination studies using dendritic cells pulsed with fungal antigens and adoptive transfer studies with T cells from *A. fumigatus* immune mice suggest that T cells can protect mice from invasive fungal infection (5, 6). Likewise, studies with cyclophosphamide-treated mice or with normal mice intravenously infected with conidia indicate that CD4⁺ T helper subsets influence the outcome of *A. fumigatus* infection (8–11). Inhibition of gamma interferon (IFN- γ) results in enhanced invasive disease after *A. fumigatus* challenge, suggesting that Th1 T cells mediate protection (8, 35). On the other hand, defense against IA is impaired by interleukin 4 (IL-4), and mice lacking this cytokine are more resistant to fungal invasion, suggesting that Th2 CD4⁺-T-cell responses are detrimental (7, 28, 37).

A. fumigatus also causes allergic bronchopulmonary aspergillosis (ABPA), a disease that occurs in patients with asthma and exacerbates airway hyperactivity, peribronchial fibrosis, immunoglobulin E (IgE) production, and eosinophilia (15, 20, 31). *A. fumigatus* expresses a variety of allergens, several of which have been cloned by screening expression libraries with the sera of ABPA patients (17, 18). Most patients have been found to react to the ribotoxin Asp f I, the peroxisome-like protein Asp f 3, the manganese superoxide dismutase Asp f 6, and the allergen Asp f 2 (17, 18). The presence of *A. fumigatus* allergen-specific antibodies in the sera of ABPA patients is an important diagnostic criterion for this disease and may play a pathogenic role (15, 20, 31). A mouse model that recapitulates the hallmarks of human ABPA has been used to dissect which

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components of the immune response contribute to pathogenesis (15, 20). A central role for CD4⁺ T cells in promoting the pathogenesis of ABPA has been demonstrated (12–14, 25, 29), with the Th2 cytokines IL-4, IL-5, and IL-13 contributing to pulmonary pathology (3, 4, 23, 27, 28, 38). The factors that determine when CD4⁺ T cells are activated in response to *A. fumigatus* exposure and whether the responding T cells will be biased to a Th1 or Th2 phenotype are unknown.

In this study we assessed whether the metabolic state of the *A. fumigatus* spore influences CD4⁺-T-cell activation and differentiation by comparing responses to intratracheal challenge with live conidia or heat-inactivated conidia (HIC). We found disparate cytokine profiles in the two groups of mice, with Th1 type cytokines predominating upon exposure to live conidia while production of Th2 cytokines was more prominent following immunization with HIC. Although CD4⁺ T cells in draining mediastinal lymph nodes (MLN) proliferated in response to *A. fumigatus* antigens following immunization with live or heat-inactivated conidia, IFN- γ -producing CD4⁺ T cells specific for hyphae were present only in the airways of mice infected with live conidia. Humoral immune responses to *A. fumigatus* antigens were mounted in mice infected with live but not inactivated fungus. These results indicate that the immune system discriminates between inactivated and metabolically active spores, restricting optimal Th1 CD4⁺-T-cell responses and antibody generation for in vivo challenge with viable fungal spores.

MATERIALS AND METHODS

Mice. Inbred C57BL/6J (B6) female mice, 6 to 8 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, Maine) and were maintained under specific-pathogen-free conditions prior to any antigenic challenge.

Infection, culture conditions, and histology. *Aspergillus fumigatus* strain 293 is a clinical isolate and was provided by Michael Anderson (University of Manchester, Manchester, United Kingdom). The fungus was grown on Sabouraud dextrose agar slants (Becton Dickinson) for 7 to 10 days at 37°C. A suspension containing conidia at 20×10^8 spores/ml was prepared as previously described (51). For heat inactivation, conidial suspensions were autoclaved at 121°C for 30 min. Live and inactivated spores were imaged in an upright Leica TCS SP2 AOBS confocal microscopy system using transmitted light to determine the structural integrity of the preparations. For the infections, mice were lightly anesthetized with inhaled isoflurane (2 min/mouse) and injected with 50 μ l of a conidial suspension (10^8 spores/mouse) by direct intratracheal inoculation using a blunt-ended 20-gauge needle. Mock-infected animals were given 50 μ l of phosphate-buffered saline (PBS)–0.025% Tween 20. To deplete neutrophils, the monoclonal antibody RB6/8C5 (100 μ g/mouse) was injected intraperitoneally 24 h prior to infection (49). At various times postinfection, mice were sacrificed, and their lungs, MLN, bronchoalveolar lavage (BAL) cells, and sera were collected for further analysis. For histological analysis, lungs were excised, immersed in 10% buffered formalin, paraffin embedded, and stained with modified Grocott's methanamine silver (GMS) stain according to the manufacturer's instructions (Richard-Allan Scientific, Kalamazoo, MI). Lung tissues were processed and analyzed at the Genetically Engineered Mouse Phenotyping Core Facility (Cornell University Medical College, New York, NY).

Conidial-homogenate preparation and Western blot analysis. To obtain conidial proteins, 2×10^9 live or heat-inactivated conidia were resuspended in lysis buffer (100 mM Tris, pH 7.5, 1 mM EDTA, and protease inhibitor cocktail [Roche, Indianapolis, IN]) and mixed with an equivalent volume of 0.5-mm glass beads (Biospec, Bartlesville, OK). The conidial suspensions were then subjected to mechanical disruption by vigorous agitation in a Bead Beater homogenizer (Biospec, Bartlesville, OK) with three cycles of 1-min agitation at 4,800 rpm and 1-min incubations on ice in between. The conidial homogenates were removed from the glass beads and centrifuged at 10,000 rpm for 5 min to separate the protein extracts from cell wall fragments and intact conidia. Equal volumes of live or heat-inactivated conidial homogenates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide)

and Western blot analysis using standard techniques. The nitrocellulose membranes were immunoblotted with polyclonal rabbit anti-*A. fumigatus* sera (1:800 dilution) as the primary antibody and with peroxidase-labeled donkey anti-rabbit antibodies (1:2,000 dilution; Amersham Bioscience, Piscataway, NJ) as the secondary antibody. The presence of reactive bands was detected by chemiluminescence utilizing an ECL detection kit from Amersham Bioscience.

Antibodies and fluorescence-activated cell sorting. All the antibodies were obtained from PharMingen (San Diego, CA). Stained cells were analyzed by flow cytometry using a BD-LSR flow cytometer (Becton Dickinson, Mountain View, CA).

Cloning, expression, and purification of recombinant proteins. To obtain *Aspergillus fumigatus* recombinant allergens Asp f I, Asp f 2, Asp f 3, and Asp f 6, the following primers were utilized to amplify the coding sequences from an *A. fumigatus* 293 cDNA library (Stratagene, La Jolla, CA): Asp f I forward (CCATATGACCTGGACATGCATCAACCAAC), Asp f I-reverse (CGAATT CGGATGAGAACACAGTCTCAAGTC), Asp f 2-forward (AAGGATCCG GACGCTGGCGCGGTGACCTCG), Asp f 2-reverse (ACCAAGCTTAGT GCAATGAAGCTTGTCACC), Asp f 3-forward (CGAATTCATATGTC TGGACTCAAGGCCGGTGAC), Asp f 3-reverse (CGAATTCGGCAGGTG CTTGAGGACGGTCTC), Asp f 6-forward (CACCATGCATATGCAATACAC GCTCCACCCCTC), and Asp f 6-reverse (AGAATTCGGCAGCTTCAT GAATGGGTGTCC). For Asp f I, a previously characterized inactivating mutation of His to Leu was introduced at position 136 (54) by site-directed mutagenesis with the following primers: sense, GCGGCATTTGGCCCTTCAGC GGGGG; antisense, CCCCCGCTGAAGGGCCACAATGCCGC. All coding sequences were amplified by PCR utilizing a high-fidelity *Pfu* I polymerase with annealing temperatures varying according to the primers. PCR products were first cloned into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced by the automated DNA sequencing facility of the Memorial Sloan-Kettering Cancer Center. Fragments with the correct sequence were then cloned into the bacterial expression vector pET27b (Novagen, San Diego, CA) and transformed into *Escherichia coli* BL21(DE3) (Stratagene, La Jolla, CA). Protein expression was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 4 to 5 h. The presence of the recombinant protein was confirmed by comparing uninduced and induced samples by 15% SDS-PAGE and staining with Coomassie blue according to standard procedures. His-tagged recombinant proteins were purified under denaturing conditions, with a nickel-nitrilotriacetic acid agarose suspension (QIAGEN, Valencia, CA), and eluted according to the manufacturer's instructions. The relative purity of the recombinant proteins was checked by SDS-PAGE. Purified proteins were dialyzed against PBS and their concentrations determined with a bicinchoninic acid kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

T-cell proliferation. A total-lymphocyte suspension was prepared from the MLN by utilizing the frosted ends of a glass slide. Cells were resuspended at 4×10^6 /ml in RPMI containing 10% fetal calf serum (FCS) and plated in flat-bottom 96-well plates at 4×10^5 cells/well. The fungal growth inhibitor voriconazole was added at a final concentration of 0.5 μ g/ml. Antigens were added to the corresponding wells at the following concentrations: hyphal fragments at 1:50 to 1:1,000 dilutions by volume, live or heat-inactivated conidia at a conidium-to-cell ratio of 2:1 or 4:1, recombinant antigens at 50 μ g/ml. For assays using purified CD4⁺ T cells, a negative MACS sorting kit was utilized (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of the final cell suspension was confirmed by cell surface staining and flow cytometric analysis and found to be 94 to 98%. A total of 2×10^5 purified CD4⁺ T cells were cocultured with 3×10^5 antigen-presenting cells (APCs). To prepare the APCs, naïve syngeneic splenocytes were depleted of T cells by incubation of the cell suspension with anti-Thy1.2 antibodies and rabbit complement (Low Tox; Cedarlane Labs, Hornby, Ontario, Canada) at 37°C for 45 min, and the remaining cells were irradiated (3,000 rads). Cultures were incubated at 37°C with 5% CO₂ for a total of 84 to 90 h. [³H]thymidine (1 μ Ci/well) was added for the last 16 h of culture. Samples were harvested onto glass fiber filters and thymidine incorporation assessed by liquid scintillation utilizing a TopCount liquid scintillation counter (Packard Instruments, Meriden, CT).

ELISAs and ICCS. To determine the cytokine profile of the *A. fumigatus*-specific CD4⁺ T cells, cells were purified and cultured as described in the preceding section. Culture supernatants were collected 72 h post-culture initiation and analyzed for the presence of IFN- γ , tumor necrosis factor alpha (TNF- α), IL-4, IL-5, IL-6, IL-10 (BD OptEIA sandwich ELISA kits; BD Biosciences, San Diego, CA), and IL-13 (Duo Set; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. To assess the presence of intracellular IFN- γ , splenic APCs were prepared as for T-cell proliferation assays and allowed to process *A. fumigatus* antigens overnight. MLN or BAL cells were pooled from infected mice and cocultured with antigen-pulsed APCs in round-bottom 96-well

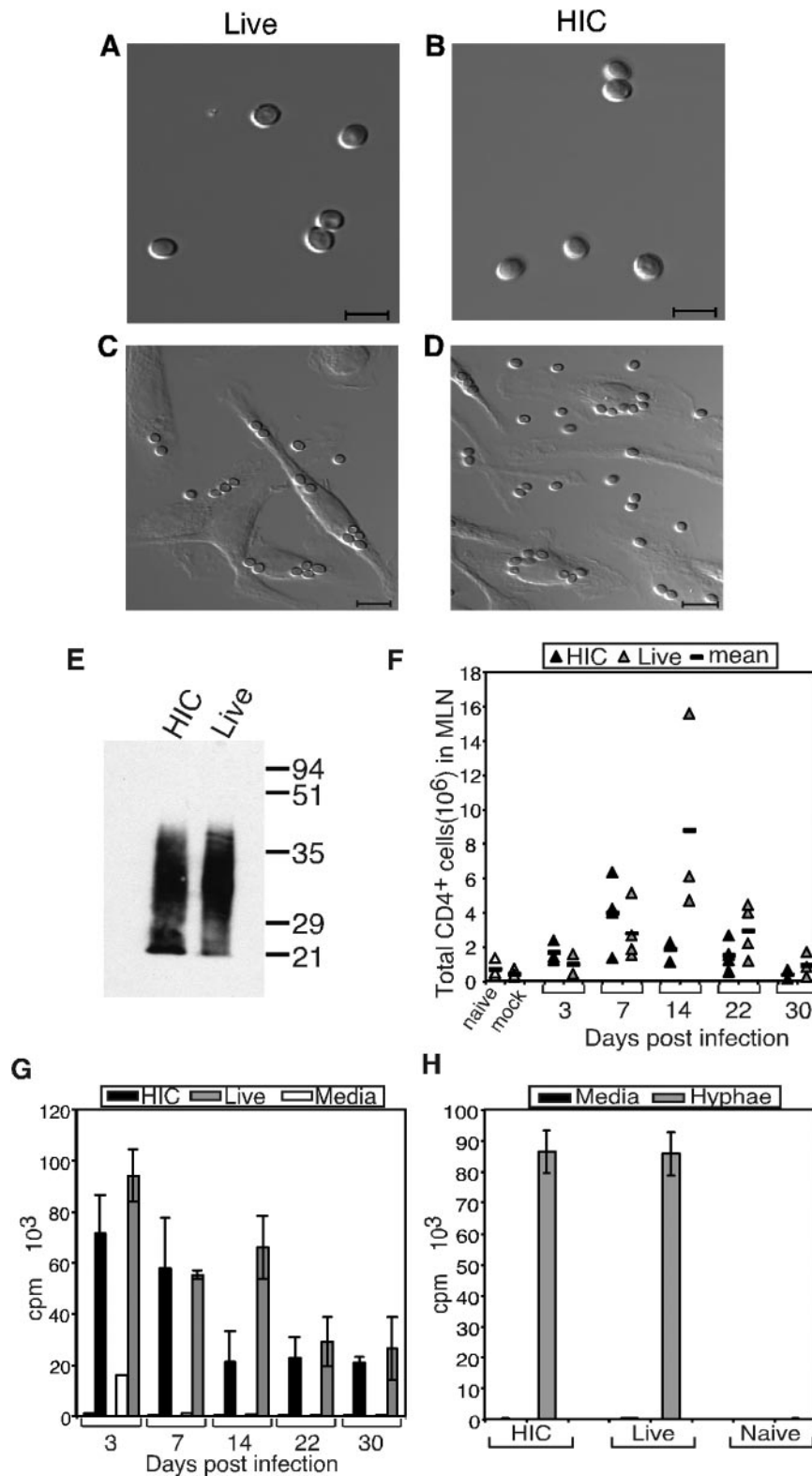


FIG. 1. Similar CD4⁺-T-cell recruitment and proliferation in the MLN of mice challenged with live or heat-inactivated conidia. (A and B) Representative confocal images of resting, live *A. fumigatus* conidia (A) or of heat-inactivated spores (B). Bars, 5 μ m. (C and D) Bone marrow-derived macrophages cultured with live (C) or heat-inactivated (D) *A. fumigatus* conidia. Bars, 10 μ m. (E) Live or HIC homogenates were analyzed by 10% SDS-PAGE and immunoblotting with anti-*A. fumigatus* polyclonal antibodies. (F) Mice were infected with 10^8 live *A. fumigatus* spores or with a similar dose of heat-inactivated conidia, and the numbers of CD4⁺ T lymphocytes recruited to the MLN were analyzed at different times postchallenge. Each symbol represents one mouse. Horizontal black lines indicate the mean value per group. Results are representative of two separate experiments. (G) T-cell proliferation by total MLN cells obtained from mice challenged with heat-inactivated conidia (black bars)

plates for 5 h in the presence of the protein transport inhibitor GolgiPlug (PharMingen, San Diego, CA). Cell surface and intracellular cytokine staining (ICCS) were performed following standard procedures. To measure *A. fumigatus*-specific antibodies in mouse serum, enzyme-linked immunosorbent assay (ELISA) plates were coated with either a 1:100 hyphal fragment suspension or recombinant proteins at 10 µg/ml in 0.1 M bicarbonate buffer (pH 9.5) overnight at 4°C. Plates were then blocked with 10% FCS-PBS solution for 2 h at room temperature. Twofold dilutions of the serum samples in 10% FCS-PBS were performed starting at a 1:50 dilution. Serum samples were incubated for 2 h at room temperature. A panel of goat anti-mouse antibodies specific for total IgG, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE was obtained from Southern Biotechnology Associates (Birmingham, AL) and utilized according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed by Student's *t* test utilizing Microsoft (Redmond, WA) Excel software.

RESULTS

Rapid recruitment of CD4⁺ T lymphocytes to MLN following pulmonary challenge with live or heat-inactivated *Aspergillus fumigatus* conidia. To assess the potential impact of fungal spore metabolism on CD4⁺-T-cell priming, *A. fumigatus* conidia were inactivated by autoclaving at 121°C for 30 min. Heat-inactivated conidia retained their shape and appeared morphologically indistinguishable from live spores as determined by confocal microscopy performed before and after heat inactivation (Fig. 1A and B). Live and inactivated spores were similarly recognized and phagocytosed by bone marrow-derived macrophages after 15 min of in vitro coculture (Fig. 1C and D). These results indicate that heat inactivation does not alter the morphology and recognition by macrophages of HIC, but they do not rule out the potential loss of intracellular contents. To compare the intracellular composition of live conidia and HIC, conidial homogenates were obtained from both preparations and analyzed by immunoblotting with polyclonal rabbit antisera against *A. fumigatus* conidia. As can be seen in Fig. 1E, anti-*Aspergillus* antibodies recognized multiple proteins in both live and HIC homogenates, indicating that immunoreactive intracellular contents are retained after heat inactivation.

A similar dose of live conidia or HIC was used to challenge B6 female mice intratracheally. The kinetics of CD4⁺-T-cell recruitment to the lung-draining MLN were analyzed at different times postinfection. As shown in Fig. 1F, similar numbers of CD4⁺ T lymphocytes were recruited to MLN following inoculation with either live conidia or HIC. By day 3 postinfection, the number of CD4⁺ lymphocytes present in the MLN of infected mice had doubled relative to the number present in naïve or mock-infected mice (Fig. 1F). The number of CD4⁺ T cells in the MLN continued to increase from day 3 to day 7 and then slowly declined. Some mice infected with live conidia recruited higher numbers of CD4⁺ T cells than HIC-challenged mice, but these differences were not statistically significant.

To determine whether draining MLN contained *A. fumigatus*-specific CD4⁺ T cells following intratracheal infection, we established an in vitro recall T-cell proliferation assay. Total

MLN-derived lymphocytes or purified CD4⁺ T cells together with APCs were cultured for 84 to 90 h in the presence of voriconazole. In previous studies, we demonstrated that voriconazole is a potent fungal growth inhibitor that does not impair T-cell or dendritic-cell activation and proliferation (51). Omission of voriconazole from the cultures resulted in fungal growth in all wells containing lymphocytes derived from mice infected with live fungus. Utilizing this assay, we detected *A. fumigatus*-specific T-cell proliferation in MLN of mice challenged with either live or heat-inactivated conidia. T-cell proliferative responses were detected as early as 3 days following intratracheal challenge and remained detectable 30 days later (Fig. 1G). Thus, CD4⁺-T-cell recruitment to draining lymph nodes in response to pulmonary challenge with *A. fumigatus* spores is rapid and sustained. Assays performed with equivalent numbers of CD4⁺ T cells enriched from MLN of mice challenged with live or heat-inactivated conidia demonstrated similar levels of T-cell proliferation following stimulation with hyphal fragments (Fig. 1H). Because these assays require in vitro restimulation, they do not provide an accurate measure of the magnitude of the in vivo T-cell response. They do demonstrate, however, that both live and heat-inactivated conidia prime CD4⁺ T cells with the ability to undergo in vitro proliferation in response to stimulation with *A. fumigatus* antigens.

To characterize the specificity of the responding *A. fumigatus*-specific T-cell population, we expressed several *A. fumigatus* allergens (Asp f 1, Asp f 2, Asp f 3, and Asp f 6) as recombinant proteins and used these antigens to stimulate CD4⁺ T cells from draining MLN. The functions and molecular weights of the native proteins, as well as the relative purities of the recombinant proteins, are shown in Fig. 2A. The recombinant proteins are larger than their native counterparts due to the addition of an N-terminal periplasmic targeting sequence and a C-terminal histidine tag. To determine whether T cells specific for any of these allergens were primed in vivo after intratracheal infection with live *A. fumigatus* spores, purified CD4⁺ T cells were cocultured with T-cell-depleted APCs pulsed with the recombinant antigens (Fig. 2B). Robust T-cell responses to Asp f 2 and Asp f 3 were consistently detected, while only modest responses to Asp f 1 and Asp f 6 were noted. T-cell proliferative responses to *A. fumigatus* antigens were exclusively due to in vivo-primed CD4⁺ T cells; no proliferative responses were detected in lymphocytes derived from naïve mice or among lymph node cells deprived of CD4⁺ T cells.

Although the proliferative responses of CD4⁺ T cells following immunization with live or heat-inactivated conidia were quantitatively similar (Fig. 1G), proliferative responses to Asp f 2 and Asp f 3 were enhanced three- to sixfold in mice infected with live fungus over those in HIC-challenged mice (Fig. 2C). Heat-inactivated conidia do prime Asp f 2- and Asp f 3-specific CD4⁺-T-cell responses, since naïve mice had negligible proliferative responses to these antigens (Fig. 2C). The greater proliferative responses to Asp f 2 and Asp f 3 of T cells derived

or with a similar dose of live spores (gray bars) at various times postchallenge. Each bar represents the average response of three to four mice per group per time point. Results are representative of two individual experiments. (H) CD4⁺ T cells were purified from the MLN of mice that had been challenged 10 days earlier and restimulated in vitro with APCs pulsed with hyphal fragments. Results are representative of four individual experiments with five mice per group.

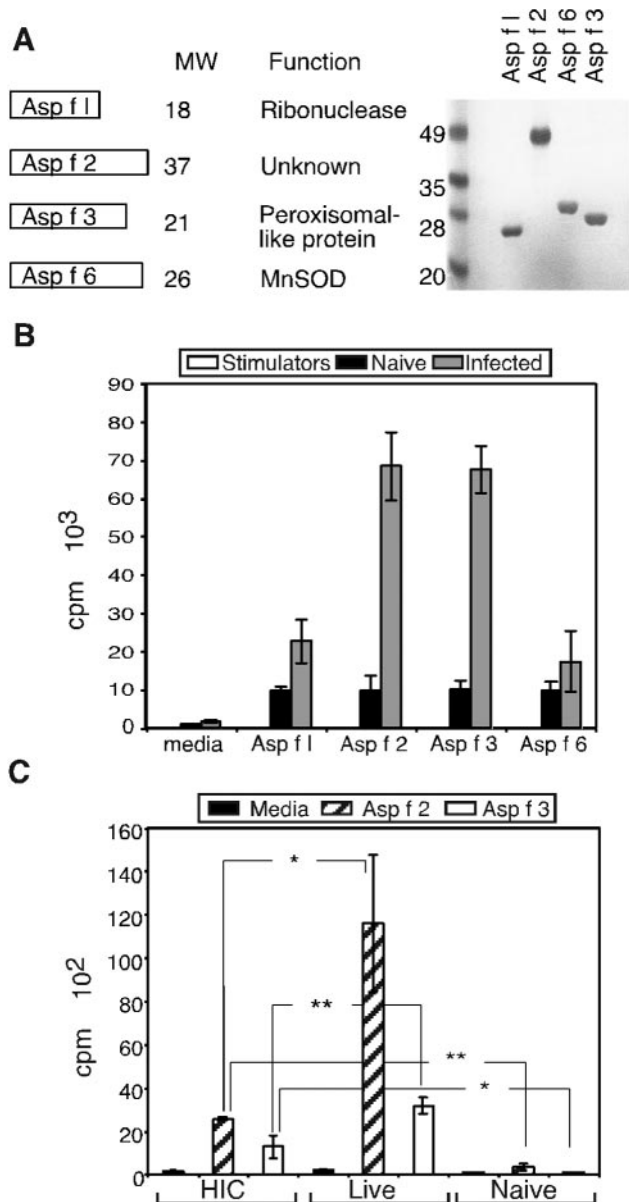


FIG. 2. CD4⁺ T cells specific for various *A. fumigatus* antigens are activated after a single pulmonary challenge with live *A. fumigatus* conidia. (A) (Left) Molecular weights (MW, in thousands) and functions of *A. fumigatus* allergens. (Right) SDS-PAGE of the *A. fumigatus* allergens expressed and purified in *E. coli*. The recombinant proteins depicted in the gel are larger than their native counterparts due to the addition of N-terminal periplasmic targeting sequences and a C-terminal histidine tag. (B) Purified CD4⁺ T cells from the MLN of mice infected 10 days earlier (gray bars) or from the lymph nodes of naive mice (black bars) were stimulated with the indicated *A. fumigatus* antigens or with live conidia. Each bar represents the average proliferation of triplicate wells. Results are representative of three individual experiments. (C) CD4⁺ T cells were purified from the MLN of mice challenged 10 days earlier with heat-inactivated conidia or with live *A. fumigatus* spores. Purified CD4⁺ T cells were stimulated with APCs pulsed with Asp f 2 or Asp f 3. Representative results of two individual experiments are shown. *, $P < 0.05$; **, $P < 0.01$.

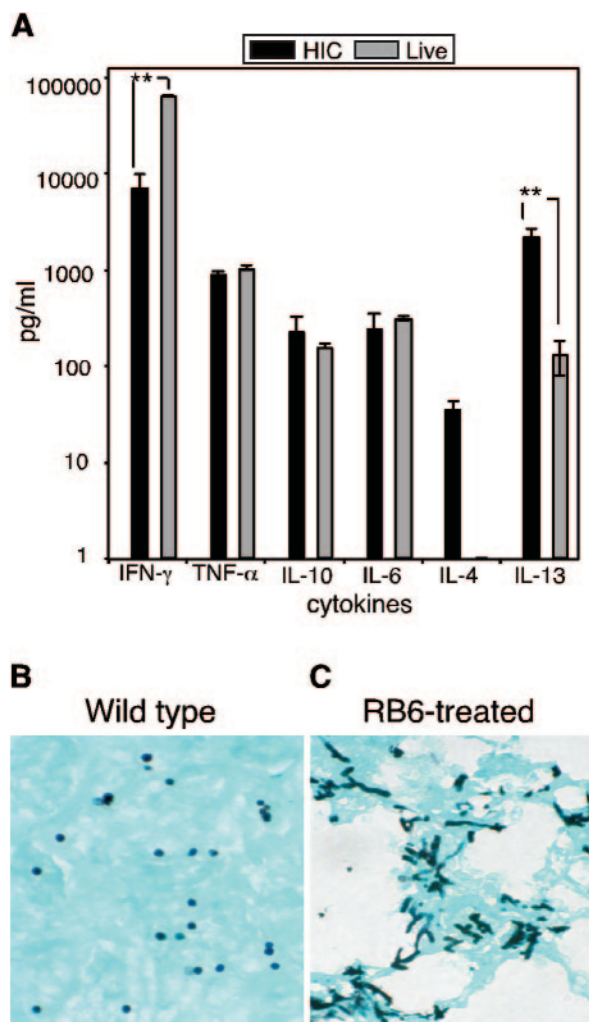
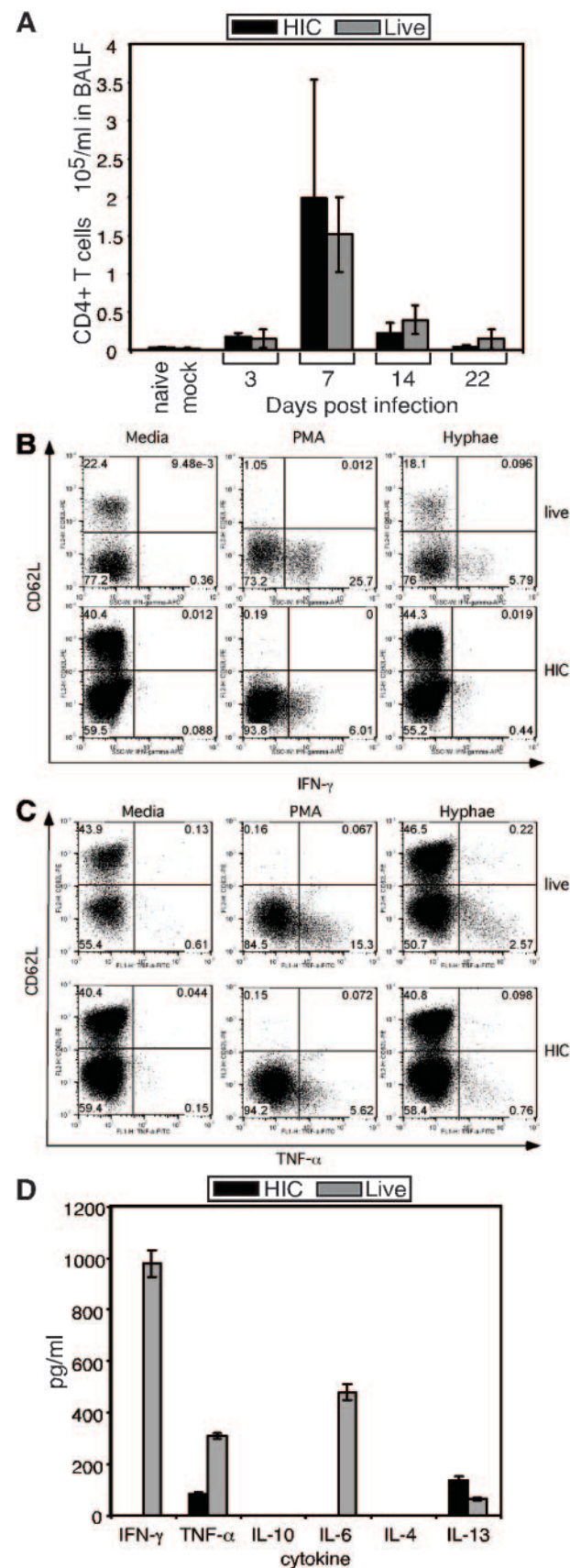


FIG. 3. CD4⁺ T cells primed with live *A. fumigatus* spores display a predominant Th1 cytokine profile, while Th2 cytokines predominate in the profile of *A. fumigatus*-specific CD4⁺ T cells primed by inactivated fungus. (A) CD4⁺ T cells were purified from the MLN of mice challenged 7 days earlier with live or heat-inactivated *A. fumigatus* spores. Purified CD4⁺ T cells were stimulated with APCs pulsed with hyphal fragments. Culture supernatants were collected 72 h post-culture initiation and assayed for the presence of cytokines by ELISA. Results shown are representative of two individual experiments. **, $P < 0.01$. (B and C) Representative images of lung sections stained with GMS. (B) Immunocompetent B6 mouse infected with live *A. fumigatus* spores 7 days earlier. (C) Neutrophil-depleted B6 mouse 3 days after infection with live *A. fumigatus* conidia.

from mice infected with live fungus may result from the production of these proteins by metabolically active spores.

CD4⁺ T cells primed by live or heat-inactivated conidia are phenotypically distinct. Differentiation of CD4⁺ T cells into Th1 versus Th2 cells following *A. fumigatus* infection has implications for the course of invasive aspergillosis, with Th1 cells and cytokines conferring protection and Th2 cells and cytokines correlating with detrimental outcomes (9–11). To determine the cytokine profile of *A. fumigatus*-specific CD4⁺ T cells primed in vivo by HIC or live conidia, CD4⁺ T cells were purified from MLN 7 days following immunization and stimulated with APCs in the presence of *A. fumigatus* antigens.



Culture supernatants were assayed 72 h following culture initiation for the presence of Th1 and Th2 cytokines (Fig. 3A). CD4⁺ T cells from mice infected with live conidia produced IFN-γ, TNF-α, IL-10, IL-6, and IL-13 after in vitro restimulation with *A. fumigatus* hyphae. We did not detect IL-4 or IL-5 in T-cell culture supernatants following stimulation of lymphocytes derived from mice primed with live fungus. Naïve T cells stimulated with *A. fumigatus* antigens did not produce detectable cytokines, demonstrating that these responses result from in vivo T-cell priming. Similar cytokine profiles were found regardless of the antigen used for in vitro restimulation (data not shown). Although IL-6, IL-13, IL-10, and TNF-α were produced together with the Th1 cytokine IFN-γ, the response appears to be predominantly of the Th1 phenotype, since the amount of IFN-γ produced is 100-fold higher than that of any of the other cytokines. In contrast, CD4⁺ T cells primed by intratracheal challenge with HIC produced 10-fold-lower IFN-γ levels upon in vitro restimulation with *A. fumigatus* hyphae. IL-13 levels, on the other hand, are more than 15-fold higher when CD4⁺ T cells are primed with HIC compared to live conidia. We also detected IL-4 production by CD4⁺ T cells primed with HIC, suggesting that immunization with heat-inactivated conidia promotes Th2 differentiation of responding T cells.

Since it has been shown that antigen doses can influence Th1 versus Th2 differentiation, we wanted to examine to what extent live spores germinated in vivo. The presence of fungal elements in the lungs of infected mice was examined by histological analysis. Sections stained with GMS revealed the presence of abundant conidia in the lung tissues of mice infected 7 days earlier (Fig. 3B). Visible spores retained their spherical shape and did not form hyphae in the lungs of infected immunocompetent mice. On the other hand, extensive hyphal growth could be observed in the lungs of mice depleted of neutrophils by anti-Gr-1 antibody treatment (Fig. 3C). This observation indicates that immunocompetent mice are able to efficiently control conidial germination, inhibiting germ tube formation and hyphal extension. The lack of generation of hyphae by live spores, together with the fact that conidia do not replicate, suggests that mice infected with live *A. fumigatus* conidia are unlikely to be exposed to dramatically different antigenic loads than HIC-challenged mice. These results suggest that the metabolic state of fungal spores can influence the differentiation of the responding cells, with Th1 responses pre-

FIG. 4. *A. fumigatus*-specific CD4⁺ T cells are recruited to the airways of mice challenged with heat-inactivated fungus but are unable to produce IFN-γ. (A) Numbers of CD4⁺ T cells in the BALF of mice challenged with live or heat-inactivated *A. fumigatus* spores at various times postinfection. Results are representative of two separate experiments. (B and C) Mice were challenged with live or inactivated fungus and BALF collected 7 days later. BALF total cells were pooled from five mice per group and stimulated with either hypha-pulsed APCs, APCs alone, or phorbol myristate acetate for 5 h prior to ICCS analysis. Plots depicted are from gated CD4⁺ T cells. Results shown are representative of three individual experiments. (B) IFN-γ production; (C) TNF-α production. (D) Cytokines present in the BALF of mice challenged 7 days earlier with HIC or live fungus. Samples were analyzed by ELISA. Results shown are representative of two individual experiments.

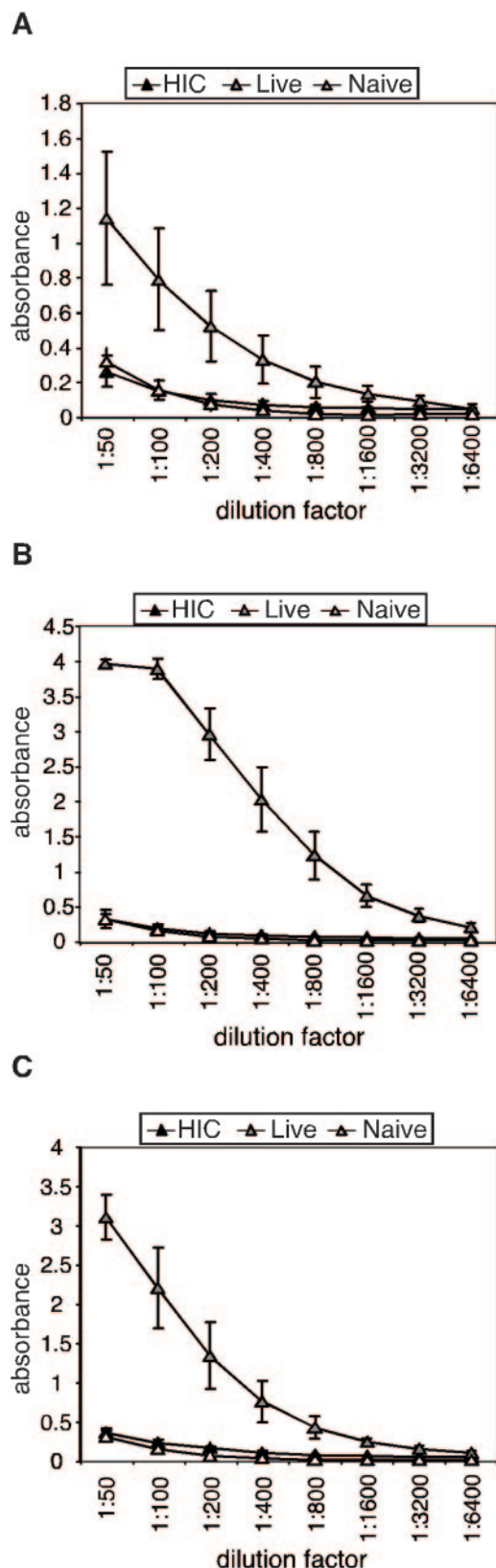


FIG. 5. Absence of *A. fumigatus*-specific IgG antibodies in the sera of mice challenged with inactivated fungus. B6 mice were challenged with an intratracheal dose of heat-inactivated conidia (black triangles) or a similar dose of live *A. fumigatus* spores (gray triangles), and

dominating after a live infection and Th2 responses after HIC challenge.

Activated *A. fumigatus*-specific CD4⁺ T cells are recruited to the airways after challenge with live conidia or HIC, but IFN- γ -secreting cells are present only following live infection. To determine whether activated CD4⁺ T cells are recruited to the airways of infected mice, we analyzed cells in BAL fluid (BALF) at different times postchallenge. Few cells were present in the airways of naïve mice or in mice challenged with vehicle alone (mock) 7 days earlier (Fig. 4A). Recruitment of lymphocytes to the airways was first detected 3 days after infection, increased between days 3 and 7, and declined thereafter. Twenty-two days postchallenge, the number of CD4⁺ T cells present in BALF had decreased sevenfold from the peak number of cells but remained threefold higher than the number in naïve mice. The majority of recruited CD4⁺ T cells were CD62L^{low}, consistent with an activated phenotype (data not shown). Similar numbers of CD4⁺ T cells were found in BALF of mice challenged with live conidia or HIC (Fig. 4A).

To determine the proportion of *A. fumigatus*-specific CD4⁺ T cells present in BALF, we performed ICCS 7 days postinfection. BALF cells were pooled from five mice per group and restimulated in vitro with APCs pulsed with *A. fumigatus* hyphae. In BALF of mice infected with live *A. fumigatus* conidia, approximately 5% of CD4⁺ T cells produced IFN- γ in response to antigen stimulation (Fig. 4B). In contrast, IFN- γ -secreting cells represented less than 0.5% of CD4⁺ T cells recruited to BALF of HIC-challenged mice. Similar results were observed for TNF- α -producing cells (Fig. 4C). Assays of cytokines in BALF demonstrated an absence of IFN- γ in mice immunized with HIC as opposed to live conidia (Fig. 4D). The decreased frequency of IFN- γ -producing T cells in BALF of mice immunized with HIC is consistent with the diminished production of IFN- γ by MLN lymphocytes (Fig. 3A). The quantitative differences in IFN- γ -producing T cells following immunization with live versus inactivated conidia is more dramatic in BALF than in MLN, suggesting that the trafficking of Th1 CD4⁺ T cells from MLN to the airways may be diminished following HIC immunization.

CD4⁺ T cells primed in HIC-challenged mice are unable to provide B-cell help. To further examine disparities in adaptive immune responses to intratracheal challenge with live versus heat-inactivated conidia, we decided to investigate antibody responses. The sera of mice challenged with HIC or live conidia were analyzed for the presence of *A. fumigatus*-specific isotype-switched antibodies at various times postinfection. *A. fumigatus*-specific antibodies were present in the sera of mice infected with live fungus as early as 10 days postinfection (data not shown). IgG antibodies specific for hyphae and for the recombinant antigens Asp f 2 and Asp f 3 were detected in the sera of mice infected with live fungus but not in naïve controls (Fig. 5A to C). Strikingly, HIC-challenged mice failed to produce an isotype-switched humoral response to *A. fumiga-*

hypha-specific (A), Asp f 2-specific (B), and Asp f 3-specific (C) IgG serum antibody titers were determined. The responses depicted here are the values measured at day 22 and are representative of measurements at other time points (day 14 and day 30 postinfection). Values shown are the average responses of four mice per group.

TABLE 1. Isotypes of *A. fumigatus*-specific serum antibodies 22 days postinfection

Antigen	Presence or absence of the following isotype in serum ^a :						
	IgG	IgG3	IgG1	IgG2b	IgG2a	IgE	IgA
Mice infected with live conidia							
Hyphae	+++	—	+++	+++	+++	—	++
Asp f 2	+++	—	+++	+++	+++	—	+++
Asp f 3	+++	+	+++	+++	++	—	+
HIC-challenged mice							
Hyphae	—	—	—	—	—	—	—
Asp f 2	—	—	—	—	—	—	—
Asp f 3	—	—	—	—	—	—	—

^a +++ , all mice tested had serum antibodies with an absorbance of >1 at a 1:50 dilution; ++ , three out of four mice had serum antibodies with an absorbance of >1 at a 1:50 dilution; + , one out of four mice had serum antibodies with an absorbance of >1 at a 1:50 dilution; — , all mice tested had serum antibodies with an absorbance of <0.2 at a 1:50 dilution.

tus hyphae or to the recombinant antigen Asp f 2 or Asp f 3 (Fig. 5A to C). In mice infected with live fungus, we detected *A. fumigatus*-specific antibodies of the IgA, IgG1, IgG2a, and IgG2b isotypes, with only one mouse producing IgG3 (Table 1). We did not detect IgE antibodies in any of the mice tested. No *Aspergillus*-specific antibodies of any of the tested isotypes were detected in the sera of HIC-challenged mice, again demonstrating that the adaptive immune system distinguishes between metabolically active and inactivated fungal spores.

DISCUSSION

In this study we determined that adaptive immune responses can be shaped by the metabolic state of an airborne pathogen. Intact mice exposed to live *A. fumigatus* spores developed Th1-biased CD4⁺-T-cell responses in the draining lymph nodes and efficiently recruited *A. fumigatus*-specific, IFN- γ -secreting CD4⁺ T cells to the bronchoalveolar space. In these mice, robust Th1 CD4⁺-T-cell responses were accompanied by a humoral response of isotype-switched antibodies against several *A. fumigatus* antigens. In contrast, isotype-switched antibodies were undetectable in the sera of mice challenged with heat-inactivated fungal spores. Furthermore, HIC-challenged mice did not recruit *A. fumigatus*-specific, IFN- γ -secreting CD4⁺ T cells to the airways, and the *A. fumigatus*-specific CD4⁺-T-cell population present in the draining lymph nodes differentiated into a more Th2-biased direction. These results suggest that the adaptive immune system gauges the invasive potential of *A. fumigatus* spores and restricts the differentiation of Th1 responses to exposure to viable fungus but not to the less threatening inactivated spores.

Studies in mouse models of IA have demonstrated that the Th1/Th2 cytokine milieu impacts the progression of invasive disease: Th1 responses can confer protection from invasive disease, while Th2 responses can be detrimental (8–11, 28, 35, 36). The importance that Th1/Th2 subsets can have after an infection has been best studied in a mouse model of leishmaniasis. In this model, resistant mice mount IL-12- and IFN- γ -

driven Th1 responses that activate macrophages for the efficient elimination of *Leishmania major* parasites (2, 39, 45, 48). In contrast, susceptible BALB/c mice mount IL-4-driven Th2 responses that inhibit the development of protective Th1 responses, leading to the development of progressive lesions and systemic disease (26, 46). Extensive studies in this model have identified a variety of factors that determine whether a T cell will differentiate along the Th1 or Th2 lineage, including the nature and amount of the antigen, the site of antigen exposure, and genetic factors (45). In vitro studies have also demonstrated that the amount of antigen influences the extent of T-cell receptor triggering and T-cell differentiation, with low antigen doses directing Th2 responses and high doses directing Th1 responses (16, 21). In our study, the development of distinct Th1/Th2 cytokine profiles after a challenge with live or inactivated spores might be explained by the availability of different doses of antigen. Although mice were challenged with similar numbers of conidia and live spores did not form hyphae in the lungs of infected mice, it is possible that metabolically active spores produce antigens in vivo after inhalation while inactivated spores do not, leading to a lower antigen dose in HIC-challenged mice.

An alternative mechanism to explain the distinct T-cell responses is that live and inactivated spores may trigger different innate inflammatory signals that then direct the activation of adaptive immune responses along a Th1 or Th2 pathway. Recent studies from our laboratory determined that exposure to heat-inactivated spores led to greatly diminished neutrophil recruitment to the BAL of challenged mice (T. M. Hohl et al., submitted for publication). Macrophages exposed to inactivated spores were found to produce reduced levels of TNF- α and MIP-2, while exposure to live conidia triggered Dectin-1- and MyD88-dependent signals that led to abundant production of these cytokines/chemokines (Hohl et al., submitted). The activation of MyD88- and Dectin-1-dependent signaling cascades by viable spores might direct the activation of dendritic cells along a Th1-inducing pathway. Indeed, MyD88-dependent activation of dendritic cells is crucial for CD4⁺-T-cell priming and for induction of Th1/Th2 differentiation of those T cells (40, 42, 47, 50).

Our observation that diminished adaptive immune responses are triggered by inactivated *A. fumigatus* spores is in agreement with similar observations in other mouse models of infection. Immune responses generated to live pathogens have been often found to be quantitatively and qualitatively different from responses elicited by challenge with heat-inactivated pathogens (1, 24, 32, 43). Indeed, heat-inactivated vaccines often result in nonprotective immunity (32, 52). It is possible that the adaptive immune system develops mechanisms that allow it to distinguish among threatening and innocuous particles. This would be particularly important for the mucosal surfaces of the lung airways, which are continuously exposed to airborne microorganisms such as bacteria, viruses, and fungi as well as to innocuous particulate antigens such as pollen and dust. Inefficient responses to pathogens would lead to disease, while exuberant unnecessary responses would lead to tissue damage, potentially compromising organ function. The development of allergic responses to *A. fumigatus* antigens might thus be explained by a constant exposure to inactive spores that trigger a preferential Th2 type CD4⁺-T-cell response.

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