

CD4⁺ T Cells Mediate the Protective Effect of the Recombinant Asp f3-Based Anti-Aspergillosis Vaccine

Diana Diaz-Arevalo, Karine Bagramyan, Teresa B. Hong,
James I. Ito and Markus Kalkum
Infect. Immun. 2011, 79(6):2257. DOI: 10.1128/IAI.01311-10.
Published Ahead of Print 21 March 2011.

Updated information and services can be found at:
<http://iai.asm.org/content/79/6/2257>

These include:

REFERENCES

This article cites 51 articles, 29 of which can be accessed free
at: <http://iai.asm.org/content/79/6/2257#ref-list-1>

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new
articles cite this article), [more»](#)

Information about commercial reprint orders: <http://iai.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>

CD4⁺ T Cells Mediate the Protective Effect of the Recombinant Asp f3-Based Anti-Aspergillosis Vaccine[▽]

Diana Diaz-Arevalo,¹ Karine Bagramyan,¹ Teresa B. Hong,¹ James I. Ito,² and Markus Kalkum^{1*}

City of Hope, Beckman Research Institute, Department of Immunology,¹ Division for Infectious Diseases,² Duarte, California

Received 14 December 2010/Returned for modification 4 January 2011/Accepted 14 March 2011

The mortality and morbidity caused by invasive aspergillosis present a major obstacle to the successful treatment of blood cancers with hematopoietic cell transplants. Patients who receive hematopoietic cell transplants are usually immunosuppressed for extended periods, and infection with the ubiquitous mold *Aspergillus fumigatus* is responsible for most cases of aspergillosis. Previously, we demonstrated that vaccination with recombinant forms of the *A. fumigatus* protein Asp f3 protected cortisone acetate-immunosuppressed mice from experimentally induced pulmonary aspergillosis. Here, we investigated the vaccine's protective mechanism and evaluated in particular the roles of antibodies and T cells. After vaccination, Asp f3-specific preinfection IgG titers did not significantly differ between surviving and nonsurviving mice, and passive transfer of anti-Asp f3 antibodies did not protect immunosuppressed recipients from aspergillosis. We experimentally confirmed Asp f3's predicted peroxisomal localization in *A. fumigatus* hyphae. We found that fungal Asp f3 is inaccessible to antibodies, unless both cell walls and membranes have been permeabilized. Antibody-induced depletion of CD4⁺ T cells reduced the survival of recombinant Asp f3 (rAsp f3)-vaccinated mice to nonimmune levels, and transplantation of purified CD4⁺ T cells from rAsp f3-vaccinated mice into nonimmunized recipients transferred antifungal protection. In addition, residues 60 to 79 and 75 to 94 of Asp f3 contain epitopes that induce proliferation of T cells from vaccinated survivors. Vaccine-primed CD4⁺ T cells are not expected to clear the fungal pathogen directly; however, they may locally activate immunosuppressed phagocytes that elicit the antifungal effect.

Invasive aspergillosis (IA) is presently the leading cause of mortality in patients with hematologic malignancies who have received a hematopoietic cell transplant (HCT) and are undergoing prolonged immunosuppressive treatment (primarily corticosteroids) to control graft-versus-host disease (GVHD) (5, 16, 28, 32, 51). Most cases of IA are caused by *Aspergillus fumigatus*, a ubiquitous saprophytic mold that spreads via airborne spores (conidia). Infection with *A. fumigatus* usually occurs through inhalation of conidia that can reach the distal airways and pulmonary alveoli (29). In immunocompetent hosts, cells of the innate immune system, namely, macrophages and neutrophils, constitute the first line of defense to protect against the pathogen (8, 22, 31, 33, 44). Despite the primacy of the innate immune response in preventing invasive fungal infections in immunocompetent individuals (18, 21, 30, 38), it has become apparent that adaptive immunity can be activated as a second line of defense to protect the immunosuppressed from IA. The development of an antifungal vaccine to enhance the survival chances of high-risk patients, such as HCT recipients, has therefore been proposed as an attractive goal (15, 23–25, 36, 46). Because the vaccine must exert its protection in an immunosuppressive setting, it is crucial to understand its mechanism of action. Thus far, T-cell- and antibody-mediated approaches to antifungal protection have been described (reviewed in reference 47). For example, it was shown that anti-β-glucan antibodies were reactive with the cell walls of *Can-*

didia albicans, *A. fumigatus*, and *Cryptococcus neoformans*; caused fungal growth inhibition *in vitro*; and proved protection against experimental candidiasis and systemic aspergillosis in a mouse model (48, 49). However, human aspergillosis typically starts as an infection of the respiratory tract. Culturing of *Aspergillus* from the blood of aspergillosis patients is usually not successful, hinting at a limited systemic component of the disease (26).

T cells have been recognized as important mediators of protection (6, 50), and Th1-associated responses were deemed to contribute to phagocytic cell-mediated defense against *A. fumigatus*. Stimulation of cultured macrophages with Th1 cytokines enhanced fungicidal activity, while stimulation of similar cultured cells with Th2 cytokines had the opposite effect (41–43). Likewise, the combination of antigen-specific T-cell clones, antigen-presenting cells, and neutrophils produced a significantly higher degree of hyphal damage than each population alone. This was explained by the fact that the antifungal activity of the phagocytes was increased by anti-*Aspergillus* T-cell cytokines, particularly gamma interferon (IFN-γ) (6, 19). Consistently, impaired IFN-γ, interleukin-5 (IL-5), IL-17, and tumor necrosis factor alpha (TNF-α) responses to *A. fumigatus* infection in immunosuppressed mice inhibit Th1 polarization and lead to lack of control of the inflammation, which is associated with high mortality rates (3). Therefore, we concluded that a vaccine that uses an adaptive mechanism to activate anti-*Aspergillus* T cells, which in turn would stimulate phagocytes, would be the most promising approach to restore antifungal immunity in immunosuppressed patients.

Recently, we showed that immunizations with recombinant Asp f3 (rAsp f3) of *A. fumigatus* effectively protected CF-1 mice from invasive fungal infections in a corticosteroid model

* Corresponding author. Mailing address: City of Hope, Beckman Research Institute, Department of Immunology, 1500 East Duarte Road, Duarte, CA 91010-3000. Phone: (626) 301-8301. Fax: (626) 301-8186. E-mail: mkalkum@coh.org.

[▽] Published ahead of print on 21 March 2011.

of immunosuppression (25). Asp f3 is a putative peroxisomal protein and was identified as a potential vaccine candidate by mass spectrometric analysis of antigens that bound to antibodies from immunocompetent mice after pulmonary exposure to nonlethal doses of *A. fumigatus* conidia (25). The Asp f3 protein has also been described as a major allergen. IgE antibodies were detected in the sera of patients with allergic bronchopulmonary aspergillosis (ABPA) (20). However, it was also shown that IgE antibodies from ABPA patients bind to a bipartite conformational epitope composed of the first 12 amino acids at the N terminus and 8 amino acids (143 to 150) at the C terminus of Asp f3 (40). Therefore, previously, we engineered truncated nonallergen versions of rAsp f3 that lacked the IgE-binding epitope and protected immunosuppressed mice against aspergillosis. The rAsp f3 variant that spans residues 15 to 168, Asp f3(15–168), elicited better protection (83%) than full-length rAsp f3(1–168) (25).

Here, we demonstrate that rAsp f3-reactive CD4⁺ T cells are required for rAsp f3 vaccine-mediated protection. We rule out the possibility of a protective role for antibodies that are also generated by rAsp f3 vaccinations. Furthermore, we show that Asp f3 is indeed an intracellular protein and likely localizes to peroxisomes. Natural Asp f3 is inaccessible to antibodies, unless the cell walls and membranes of the fungal pathogen have been permeabilized. We also identify specific T- and B-cell epitopes of Asp f3 that associate with a protective response.

MATERIALS AND METHODS

Animals, strains, and reagents. Reagents were from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated. Female CF-1 mice 6 to 8 weeks of age and one New Zealand White rabbit were purchased from Charles River Laboratories. All animal experiments were conducted in a biosafety level 2 containment facility in compliance with animal care regulations and under care and use protocols approved by the Research Animal Care Committee of the Beckman Research Institute of City of Hope. Animal numbers (*n*) per group are indicated in the text or figure captions.

A. fumigatus strain AF001, isolated from a patient with invasive pulmonary aspergillosis at City of Hope National Medical Center (Duarte, CA), was used for infection and antigen preparations as previously described (23, 25). Resting conidia were prepared by collecting the spores from 5-day-old *A. fumigatus* cultures on potato dextrose (PD) agar (BD/Difco, Franklin Lakes, NJ) (25). The suspension was washed twice with and then kept in Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS; Mediatech, Inc., Manassas, VA) with Tween 80 (0.1%). Resting conidia were counted with a hemocytometer, and their viability was measured through agar plating of serial dilutions. The resting conidia were cultured in potato-dextrose liquid medium for 6, 12, and 72 h to produce swollen conidia, germlings, or hyphae, respectively.

Synthetic peptides. Ten consecutive 20-mer peptides that spanned the entire sequence of Asp f3(15–168) and overlapped by 5 amino acids each, were synthesized by Peptide 2.0, Inc. (Chantilly, VA) (Table 1). Crude peptides were purified using a Sep-Pak C₁₈ column (Waters Corporation, Milford, MA) and dissolved in dimethyl sulfoxide (DMSO; 100%) to obtain a stock solution (2 mg/ml), which was stored at –20°C.

Anti-Asp f3 polyclonal antibody production. One rabbit was immunized at weeks 0, 2, 4, 8, and 24 with full-length rAsp f3 (350 µg). The protein was administered in incomplete Freund's adjuvant by subcutaneous (s.c.) injections. Serum samples were obtained 2 weeks after the second and fifth immunizations. The antibody was purified with Melon gel (Thermo Fisher Scientific, Rockford, IL). Texas Red conjugation of the antibody for immunohistochemistry (IHC) was performed with the FluorReporter Texas Red-X protein labeling kit (Invitrogen, Carlsbad, CA).

Monoclonal anti-mouse CD4 IgG production. Monoclonal anti-CD4 IgG (GK1.5 monoclonal antibody [MAb]) for depletion experiments was generated from a 1-liter culture of GK1.5 hybridoma cells (TIB-207; ATCC, Manassas, VA) in serum-free MAb medium using a CELLline disposable bioreactor (BD Bio-

TABLE 1. Characteristics of the synthetic Asp f3(15–168) peptides used in this study

Peptide no.	Asp f3 residue positions	Peptide sequence ^a	Titer of polyclonal anti-Asp f3 rabbit antibody
P1	15–34	VFSYIPWSEDKGEITACGIP	1:320,000
P2	30–49	ACGIPINYNASKEWADKKVI	<1:10,000
P3	45–64	DKKVILFALPGAFTPVCSAR	1:160,000
P4	60–79	VCSARHVPEYIEKLPEIRAK	1:320,000
P5	75–94	EIRAKGVDVVAVLAYNDAYV	<1:10,000
P6	90–109	NDAYVMSAWGKANQVTGDDI	<1:10,000
P7	105–124	TGDDILFLSDPDARFSSKISG	1:320,000
P8	120–139	SKSIGWADEEGRTKRYALVI	1:20,000
P9	135–154	YALVIDHKGITYAALEPAKN	1:160,000
P10	150–168	EPAKNHLEFSSAETVLKHL	1:320,000

^a Residues of overlapping sequences are underlined.

sciences, San Jose, CA) and purified with a HiTrap protein G column (GE Healthcare Biosciences, Pittsburgh, PA).

Immunohistochemistry, microscopy, and Asp f3 localization studies. To permeabilize their rigid cell walls, resting conidia were preconditioned prior to IHC. Hypo-osmotic stress was induced by incubation (1 h) of resting conidia in sucrose (0.8 M), followed by transfer into a hypo-osmotic medium containing morpholineethanesulfonic acid (MES)-Tris (1.0 mM, pH 5.2) and calcium sulfate (1.0 mM). The cells were then harvested by centrifugation and resuspended in PBS. Swollen conidia and germlings were passed through a 21-gauge syringe needle up to 25 times to disaggregate clumps. Pretreated resting conidia, swollen conidia, and germlings were washed three times with PBS and fixed for 1 h with formaldehyde (8%) in PBS (pH 7.0) containing EDTA (25 mM), magnesium sulfate (5 mM), and DMSO (5%), and then placed onto poly-L-lysine (0.1%)-treated microscope slides. Cells were permeabilized (22°C, 40 min) on slides using Driselase (10 mg/ml) and β-D-glucanase (16 mg/ml) in sodium citrate (50 mM, pH 4.5), followed by treatment with lyticase (81 U/ml) for 30 min (37). Slides were then washed three times for 10 min in PBS with EDTA (25 mM, pH 6.7), immersed in ice-cold methanol-acetone (1:1), and placed at –20°C for 10 min. Antibody-based IHC staining was performed in a wet chamber with 100-µl volumes of primary (3 h of incubation) and secondary (1 h of incubation) antibodies in PBS with Nonidet P-40 (0.5%). Asp f3 was IHC stained with our primary polyclonal rabbit anti-Asp f3 antibody (0.5 µg/ml) and a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Invitrogen) (0.4 µg/ml), when used in combination with the mitochondrial stain MitoTracker deep red 633 (250 nM). For colocalization of Asp f3 with peroxisomal PMP70, we used the Alexa Fluor 488 peroxisomal labeling kit (Invitrogen), containing an anti-PMP70 primary rabbit IgG (1.25 µg/ml) and Alexa Fluor 488 secondary goat anti-rabbit IgG, highly cross-adsorbed antibody conjugate (0.4 µg/ml), and Texas Red-labeled rabbit anti-Asp f3 antibody (0.5 µg/ml). The Texas Red-labeled anti-Asp f3 antibody was also used in combination with LysoTracker green DND-26 (50 nM; Invitrogen) to evaluate colocalization of Asp f3 with lysosomes. Cells were counterstained with 3 µM DAPI (4',6-diamidino-2-phenylindole) and washed two times in PBS. The antifading solution polyvinyl alcohol–1,4-diazabicyclo[2.2.2]octane (PVA-DABCO; 75 µl [2.5%]), was added to each sample before microscopy. Slides were examined on an Olympus AX70 microscope with a 60× PlanApo oil immersion objective, at City of Hope's Light Microscopy Digital Imaging Core facility. Image processing and Pearson correlation analysis of triplicate samples were performed with Image-Pro Plus v6.3.

Vaccinations, immunosuppression, and challenge. CF-1 mice were vaccinated twice, 2 weeks apart, with s.c. injections of the rAsp f3-based vaccine. Each vaccine injection contained 15 µg N-terminally truncated rAsp f3, encompassing residues 15 to 168, produced and purified from *Escherichia coli*, and suspended in TiterMax (TM) (TiterMax, Inc., Norcross, GA) essentially as previously described (25). This nonallergenic form of rAsp f3 elicited better protection (83%) than full-length rAsp f3 (residues 1 to 168) (25). As controls, mock immunizations were carried out with PBS plus TM. Five weeks after the second immunization, mice were immunosuppressed, beginning with daily s.c. injections of cortisone acetate (CA; 2.5 mg) (Tokyo Chemical Industry, Tokyo, Japan) in suspension with methylcellulose (0.5%) and Tween 80 (0.1%) for 10 consecutive days prior to challenge. To prevent bacterial infections, acidified water contain-

ing sulfamethoxazole (0.8 mg/ml) and trimethoprim (0.16 mg/ml) (Hi-Tech Pharmaceutical Co., Inc., Amityville, NY) was provided during the immunosuppression and infection period. Mice were anesthetized with ketamine-xylazine and intranasally inoculated with 3 million viable conidia in suspension in PBS (30 μ l).

Evaluation of *A. fumigatus* infection. Mice were observed every 2 h during the day for 10 days after *A. fumigatus* challenge. The weight and body temperature of the animals were monitored in the mornings and evenings as described previously (25). Time of death was registered and analyzed in Kaplan-Meier survival curves. Statistical analysis was performed by Fisher's exact test. Disease pathology and assessment of fungal distribution within the lung parenchyma were performed as described previously (25). Briefly, lungs were formalin fixed and embedded with paraffin. Sections of tissue were stained with standard procedures (7) using Accustain silver stain (modified Gomori methenamine silver, Sigma-Aldrich) or with Texas Red-labeled polyclonal anti-Asp f3 antibody.

Fungal burden was assessed by removal and homogenization of lungs, kidneys, and spleens in sterile PBS with Tween 80 (0.1%). Dilution series at 10-, 100-, and 1,000-fold were plated on PD agar plates and incubated at 37°C for up to 2 days, and CFU were determined.

T-cell proliferation assay. Splenocytes (10^5 cells per well of a 96-well plate) of immunized survivors and nonimmunized controls were left nonstimulated or were stimulated by coculture in the presence of truncated versions of Asp f3 (5 μ g/ml) or synthetic peptides (10 μ g/ml) or with phorbol myristate acetate (PMA) as a positive control for 3 days. T cells were purified by CD4/CD8 magnetic bead cell separation in microtiter plates (Miltenyi Biotec, Inc., Bergisch Gladbach, Germany) and analyzed for ATP content with the CellTiter-Glo luminescent cell viability assay (Promega Corporation, Madison, WI). Stimulation indices were determined by dividing the mean of relative luminescent units of stimulated cultures by that of relevant nonstimulated controls.

Measurement of immunogenicity. Antibodies specific for rAsp f3(15–168) were analyzed 14 days after the second immunization by enzyme-linked immunosorbent assay (ELISA) using BD Falcon 96-well ELISA plates (BD Biosciences, San Jose, CA) coated with either rAsp f3(15–168) (0.5 μ g/well) or Asp f3 synthetic peptides (1.0 μ g/well). The plates were incubated (2 h at 22°C) with various dilutions of mouse or rabbit sera diluted in PBS with skim milk (5.0%). Bound antibodies were detected using either horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a (Alpha Diagnostic Intl., Inc., Santa Monica, CA), or horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences, GE Healthcare Biosciences, Pittsburgh, PA) as secondary antibodies, and tetramethylbenzidine (Thermo/Pierce, Rockford, IL) was used as substrate. Prechallenge ELISA data of antibody reactivity for survivors and nonsurvivors to the 10 synthetic peptides (Table 1) were analyzed by the Mann-Whitney U test with Bonferroni correction.

Western blots were performed with cytosolic extracts of resting conidia, swollen conidia, germlings, and hyphae, using reducing 4 to 12% Bis-Tris SDS Nu-PAGE gels (Invitrogen). The total protein loading amounts were 10 μ g/lane for the extracts and 10 ng/lane for rAsp f3. Proteins were transferred to polyvinylidene difluoride membrane (0.22- μ m pore size; Bio-Rad, Life Science Research, Hercules, CA) by using an Xcell II blot module (Invitrogen). Membranes were blocked (4°C overnight) in Odyssey blocking buffer (LI-COR Biotechnology, Lincoln, NE). Rabbit serum was 10,000-fold diluted in Odyssey blocking buffer. IRDye 680 donkey anti-rabbit IgG (H+L) (LI-COR Biotechnology) was used as secondary antibody in 10,000-fold dilution in accordance with the manufacturer's instructions.

Antibody transfer. Blood was collected from rAsp f3-vaccinated mice by cardiac puncture 14 days after the second immunization. Polyclonal antibodies from vaccinated and mock-vaccinated mice were purified by Melon gel (Thermo/Pierce) and quantified by the microbicinchoninic acid (micro-BCA) protein assay (Thermo/Pierce). Twenty-four hours before *A. fumigatus* challenge, CA-immunosuppressed mock-vaccinated mice received a single intravenous injection of 5 mg anti-rAsp f3 IgG or unspecific mouse IgG. The mice were then monitored for 7 days, and survival data were analyzed as described above.

In vivo depletion of CD4⁺ T cells. Vaccinated mice received GK1.5 MAb or rat isotype control antibody (1.0 mg per animal) by intravenous injection into the tail vein on days –2 and –1 prior to fungal challenge (see Fig. 3A). Other controls included a group of mock-vaccinated (PBS) mice and a group of mice vaccinated with rAsp f3(15–168) without T-cell depletion. Depletion was monitored by fluorescence-activated cell sorter (FACS) analysis, using tail vein blood (50 μ l) collected from two animals of each group. The T cells were stained with anti-CD3-phycoerythrin (PE) conjugate and anti-CD4-FITC-labeled antibodies (eBioscience, Inc., San Diego, CA) after ammonium acetate lysis of erythrocytes. The FACS data were analyzed with FlowJo 7.5.

CD4⁺ T-cell transfer. Splenic CD4⁺ T cells from rAsp f3-vaccinated and mock-vaccinated CF-1 mice were purified by negative selection of CD4⁺ T cells

using the AutoMACS magnetic purification system (Miltenyi Biotec). The purity of CD4⁺ T cells was assessed by FACS analysis. On the day before *A. fumigatus* challenge (day –1), 2.5 million enriched CD4⁺ T cells per recipient were transplanted into CA-immunosuppressed CF-1 mice by tail vein injection as described previously (52).

RESULTS

Anti-Asp f3 IgG titers do not correlate with vaccine-induced protection. To elucidate the mechanism by which the rAsp f3-based vaccine provides protection, we studied the role of antibody responses in mice vaccinated with rAsp f3. Because the Asp f3-induced protection was never complete and typically ranged from 70 to 90%, we were able to compare anti-Asp f3 IgG titers at a serum dilution of 1:20,000 from vaccinated surviving mice ($n = 11$), vaccinated nonsurviving mice ($n = 17$), and nonvaccinated mice as controls ($n = 10$), which were immunosuppressed and challenged with conidia, using archived plasma samples from our previous study (25). No statistical difference ($P = 0.98$) was observed for the titers of total anti-Asp f3 IgG between survivors and nonsurvivors (Fig. 1A).

Protection is not transferred with anti-Asp f3 antibodies. Five milligrams of purified IgG antibodies from mice vaccinated with rAsp f3 and the same amount of antibody from mock-vaccinated mice (immunized with TM adjuvant alone, control) were transferred by tail vein injection into recipient immunosuppressed mice. Twenty-four hours later, the recipient mice were exposed to 3.1 million viable conidia, inoculated intranasally. Mice that received IgG from rAsp f3-vaccinated mice did not have improved survival over those that received nonspecific IgG from control animals (Fig. 1B) ($P = 1$, $n = 8$ for each group, Fisher's exact test). ELISA analysis showed that the antibodies from rAsp f3-vaccinated mice, used for transfer, reacted with rAsp f3 in dilutions as low as 1:2 million, corresponding to an ELISA detection limit of 10 ng/ml IgG1 or IgG2a (Fig. 1C).

Localization of Asp f3 in *A. fumigatus*. To evaluate the immunological accessibility of Asp f3 in *A. fumigatus*, we examined its localization and expression *in vitro* in cultured *A. fumigatus*, as well as *in vivo* in lung tissue sections of infected mice. Anti-Asp f3 immunohistochemistry (IHC) staining on lung tissue slices of *A. fumigatus*-infected mice using a rabbit polyclonal anti-Asp f3 antibody demonstrated Asp f3 expression in hyphae that were invading pulmonary tissue (Fig. 2A). Western blot analysis revealed Asp f3 in cytosolic extracts of resting conidia, swollen conidia (6 h of incubation with medium), germlings (12 h of incubation), and branched hyphae (72 h of incubation). Asp f3 expression was notable in conidia and strongest in hyphae (i.e., germlings and branched hyphae) (Fig. 2B). IHC micrographs of cultured growth stages of *A. fumigatus* showed Asp f3 in the cytosol, and its subcellular localization correlated with that of peroxisomal PMP70, having a Pearson correlation coefficient (ρ_p) of 0.90 (Fig. 2C). The localization of Asp f3 did not correlate with that of mitochondria or lysosomes; ρ_p was only 0.44 and 0.03, respectively. Others previously described Asp f3 as a "conidial surface protein" (4); however, in our IHC analyses Asp f3 was not detected on the surface of hyphae or conidia. Indeed, Asp f3-specific IHC staining was only obtained after enzymatic digestion of cell walls and methanol fixation/removal of mem-

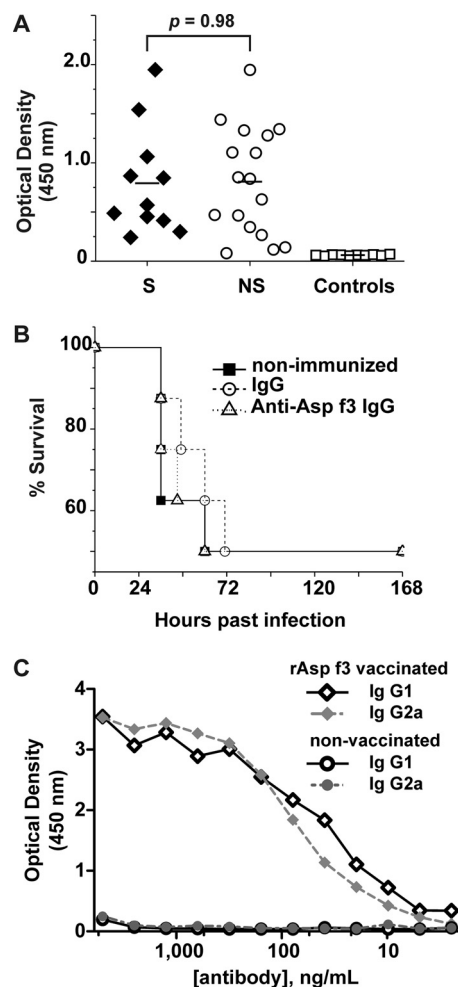


FIG. 1. rAsp f3 vaccine protection is not mediated through Asp f3-specific antibodies. (A) Asp f3-specific antibodies in the prechallenged blood of vaccinated mice that survived (S [solid diamonds]; $n = 11$) or did not survive (NS [open circles]; $n = 17$) pulmonary *A. fumigatus* challenge lie in the same ELISA titer ranges and are not significantly different ($P = 0.98$, Mann-Whitney U test). Antibodies from mock-vaccinated mice (PBS-TM) were used as controls (open squares; $n = 10$). (B) Survival plots of CA-immunosuppressed CF-1 mice challenged intranasally with *A. fumigatus* conidia after they received total IgG from rAsp f3-vaccinated mice (open triangles) or IgG from mock-vaccinated mice (open circles) or those of controls that were not immunized and received no IgG (nonimmunized [solid squares]; $n = 8$ for each group, which are not significantly different from each other). (C) Binding curves of purified anti-Asp f3 IgG1 (open diamonds) and IgG2a (solid diamonds) from Asp f3-immunized mice and IgG1 (open circle) and IgG2a (full circle) from nonvaccinated mice.

branes (Fig. 2D versus E). Because it was possible that our antibody-based IHC staining method could miss certain Asp f3 epitopes, we characterized the epitope recognition of our polyclonal rabbit antibody used for IHC and Western blots by ELISA titration of a set of synthetic overlapping 20-mer peptides (Table 1). These synthetic peptides covered the entire sequence of Asp f3(1–15), and adjacent peptide sequences overlapped with each other with five flanking amino acids. The majority of synthetic peptides exhibited strong affinity and reacted with the antibody, whereas only three peptides

had moderate to low affinity to the antibody (Table 1). Hence, our polyclonal anti-Asp f3 antibody recognizes epitopes throughout the sequence of Asp f3, making it very unlikely that it would not bind to Asp f3 contained in the cell wall if there was any.

CD4⁺ T cells are required for Asp f3 vaccine protection. To determine the role of T cells in Asp f3 vaccine-induced protection, we antibody depleted CD4⁺ T cells in Asp f3-vaccinated mice during cortisone acetate (CA) immunosuppression and then challenged the animals with *A. fumigatus* conidia (Fig. 3A). CD4⁺ T-cell depletion reduced the survival of vaccinated mice to levels comparable to those of nonvaccinated mice ($P = 1$, Fisher's exact test), indicating that the protective effect of the vaccine depends on CD4⁺ T cells (Fig. 3B). Mice vaccinated with rAsp f3(15–168) that received no antibody or only a nonspecific rat antibody showed significantly better survival than nonimmunized mice or T-cell-depleted mice ($P = 0.032$) (Fig. 3B). To ensure the validity of our conclusions, we monitored the antibody depletion of the T cells by FACS analysis using peripheral blood mononuclear cells (PBMCs) from the tail blood of live animals and posthumously with splenocytes (Fig. 3C). CD4⁺ T cells were almost completely absent in mice 24 h after the first injection of anti-CD4 GK1.5 antibody (only 0.99% CD4⁺ T cells remained in the blood). The spleens remained devoid of CD4⁺ T cells 48 h after challenge (only 0.1% CD4⁺ T cells remaining) (Fig. 3C). Mice that received nonspecific rat IgG had no change in their T-cell population (Fig. 3C). Experiments in control mice indicated that T-cell depletion persisted for at least 30 days, after which only ~5% of T cells were regenerated (data not shown). Surviving mice and controls were alive and healthy for at least 2 months after challenge, after which the experiment was terminated.

Adoptive transfer of Asp f3-primed CD4⁺ T cells protects against pulmonary aspergillosis. Enriched splenic CD4⁺ T cells from Asp f3-vaccinated animals, obtained by magnetic bead-negative selection, had ~91% purity (Fig. 4A), and were transplanted into recipient CF-1 mice that had received a 10-day immunosuppressive CA treatment (Fig. 4B). One hundred sixty-two hours after pulmonary *A. fumigatus* infection, animals that received the vaccine-primed CD4⁺ T cells had enhanced survival (46%), which was statistically significant compared to the group that received cells from nonvaccinated mice ($P = 0.013$, Fisher's exact test) (Fig. 4C). There was no detectable fungal burden in the lungs and spleens of animals surviving 10 days after infection at any dilution level tested (74 to 94 mg lung in 1 ml, 10- to 1,000-fold diluted). A near background fungal burden of ~1.4 CFU was detected only in the kidneys of survivors at the highest concentration tested (20 to 40 mg kidney in 1 ml). As expected and compared to the survivors, the nonsurviving animals had significant fungal burdens in the lungs ($P = 0.0039$) and some in the kidneys (not at significant levels) (Fig. 4D), but not in the spleens. The fungal burden in nonsurviving mice that had received the CD4⁺ T cells from Asp f3-vaccinated mice was not significantly different from those that received cells from nonimmunized mice. Histopathological analysis of surviving recipients of CD4⁺ T cells from rAsp f3-vaccinated mice was undistinguishable from that obtained previously with rAsp f3-vaccinated mice (25; data not shown).

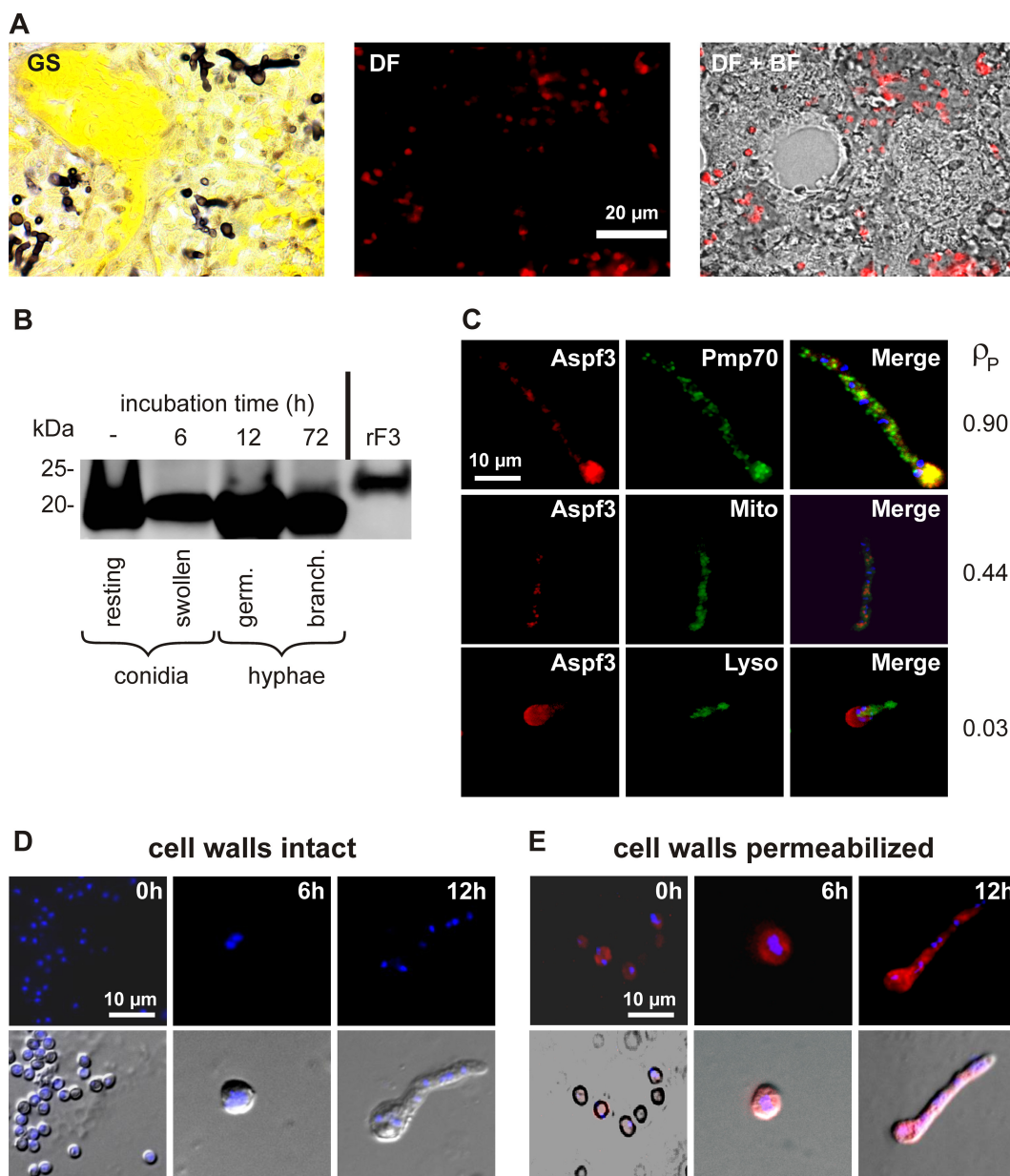


FIG. 2. Intracellular localization of Asp f3. (A) Micrographs of *A. fumigatus*-infected mouse lungs stained with Gomori silver (GS), which darkens the outlines of fungal cell walls, and Texas Red-labeled rabbit anti-Asp f3 antibody (dark field [DF]), and merged DF with bright field (DF + BF). The Asp f3 signal originates from the interior of microtome-cut hyphae that invaded the lungs, visualized with anti-Asp f3 antibody. (B) Western blot of Aspf3 protein expressed by four growth stages of *A. fumigatus*, after the indicated culture incubation times. rAsp f3 is shown as a control (rF3). germ., germling. (C) Colocalization of Asp f3 in 12-h-germinated *A. fumigatus* double stained with anti-Asp f3 antibody (Aspf3) and markers for peroxisomes (Pmp70), mitochondria (Mito), and lysosomes (Lyso). Pearson's correlation coefficients (ρ_p) are given next to each merged image. (D) Asp f3 in resting conidia (0 h), swollen conidia (6 h) and germlings (12 h) cannot be IHC stained with an anti-Asp f3 antibody without permeabilization of cell walls and membranes. (E) Intracellular Asp f3 is IHC stained (red) after cell wall and membrane permeabilization. (C, D, and E) DAPI counterstaining (blue).

T-cell and linear B-cell epitopes of Asp f3(15–168). Next we investigated which T- or linear B-cell epitopes of rAsp f3(15–168) were associated with protective vaccination. Although we could not attribute a protective role to IgG antibodies (recognizing B-cell epitopes), antibody titers to certain rAsp f3 epitopes may associate with protection, which could have utility as a prognostic marker. Previously, we had mapped peptide epitopes of rAsp f3 by measuring immune responses to enzy-

matically derived peptide fragments of rAsp f3 that we identified by mass spectrometry. For such purposes, we had separated peptides of trypsin-digested rAsp f3 by high-performance liquid chromatography (HPLC) and subjected each fraction to mass spectrometric analysis, a T-cell proliferation assay, and ELISA. Initial data from this approach were published in poster form and in a review article on antifungal vaccine development (24). Among several B-cell epitopes, this initial

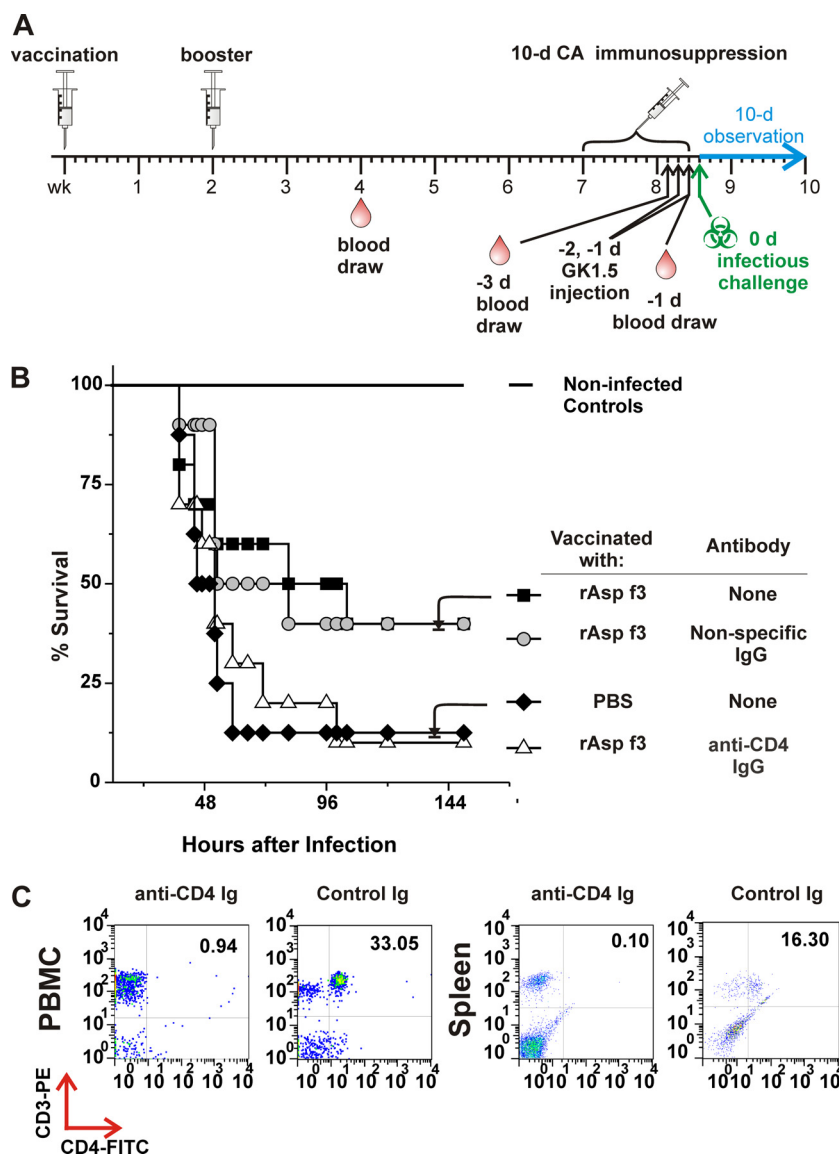


FIG. 3. Effect of CD4⁺ T-cell depletion on Asp f3 vaccine protection. (A) Experimental scheme of immunization, CA immunosuppression, *A. fumigatus* challenge, and T-cell depletion with anti-CD4-IgG (GK1.5). Times of blood draws for antibody titer determination (14 days after second immunization [Fig. 5B]) and FACS analysis are indicated (–3 days and –1 day before *A. fumigatus* challenge). (B) Survival curves of vaccinated and nonvaccinated CF1 mice after *A. fumigatus* challenge. Shown are results from rAsp f3(15–168)-vaccinated mice (rAsp f3), nonimmunized controls that received only the adjuvant in phosphate-buffered saline (PBS), mice that received treatment with nonspecific rat antibody as a control (Non-specific IgG), mice that underwent T-cell depletion through treatment with the GK1.5 (anti-CD4 IgG), or mice that received no treatment. $n = 10$ for each of the four groups. The grouped survival data from rAsp f3-vaccinated mice with no antibody and with nonspecific IgG are significantly different from the grouped survival data from mock-immunized (PBS) and rAsp f3-immunized mice with anti-CD4-IgG treatment ($P = 0.03$, Fisher's exact test). (C) FACS analysis of CD3⁺ CD4⁺ T cells in tail blood (from day –1) and spleens from mice that received rat GK1.5 antibody (anti-CD4 IgG) or nonspecific rat IgG (control IgG). Percentages of CD3⁺ CD4⁺ T cells are indicated in the upper right quadrants. Gates are indicated with solid lines and red arrows.

study indicated the presence of a T-cell epitope in the tryptic peptide HVPEYIEKLPEIR (24). Here, we further refined our search for immunogenic Asp f3 epitopes and confirmed the epitopes previously assigned by mass spectrometry. We performed T-cell proliferation assays with (i) a set of overlapping synthetic 20-mer peptides (Table 1) and (ii) intact rAsp f3(15–168). Proliferation of T cells was determined by luminometric ATP cell titer quantification in positively selected T cells after stimulation. T cells from Asp f3(15–168)-vaccinated but

unchallenged animals as well as vaccinated survivors had proliferative responses to the synthetic peptides P4 (VCSARHVPEYIEKLPEIRAK; residues 60 to 79) and P5 (EIRAKGVVVAVLAYNDAYV; residues 75 to 94) and to intact rAsp f3(15–168) (Fig. 5A and B). T cells from nonvaccinated mice that were nonlethally infected under nonimmunosuppressive conditions described earlier (25) had no T-cell proliferation in response to rAsp f3 or the peptide antigens P4/P5 (Fig. 5C). The synthetic peptide Asp f3(60–79) includes

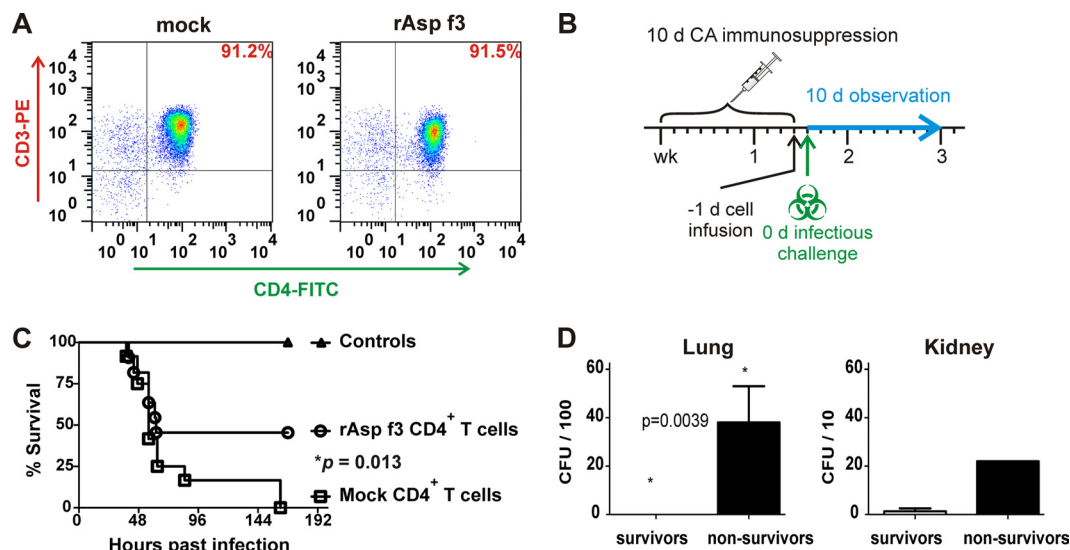


FIG. 4. Adoptive transfer of CD4⁺ T cells from rAsp f3-vaccinated mice provides antifungal protection. (A) Flow cytometry analysis of ~20,000 purified CD3⁺ CD4⁺ T cells from each mock-immunized and rAsp f3-vaccinated mouse used for transplantation. Purity is indicated by the percentage of CD3⁺ CD4⁺ T cells in each upper right quadrant. (B) Experimental scheme of transplantation of enriched CD4⁺ T cells into 10-day CA-immunosuppressed CF-1 mice on day -1 before infectious challenge. (C) Survival curves of pulmonary *A. fumigatus*-infected CD4⁺ T-cell recipients and noninfected controls. Eleven recipients had received purified CD4⁺ T cells from rAsp f3-immunized mice, and 12 had received them from mock-vaccinated mice. Controls ($n = 8$) consisted equally of recipients of cells from vaccinated and mock-vaccinated mice. Recipients with CD4⁺ T cells from vaccinated mice had significantly improved survival compared with recipients of cells from mock-vaccinated mice ($P = 0.013$, Fisher's exact test). (D) Fungal burden in lungs and kidneys of surviving and nonsurviving animals measured as *A. fumigatus* CFU from homogenates in a 100- or 10-fold dilution.

the sequence of a T-cell stimulatory trypsin-derived digest peptide, HVPEYIEKLPEIR (residues 65 to 77), which we previously isolated by HPLC and identified by mass spectrometry (24), suggesting that the deduced common sequence indeed contains a T-cell epitope.

Prechallenge plasma samples from vaccinated survivors of experimental *A. fumigatus* challenge and from nonsurviving mice were analyzed by ELISA. The IgG titers (measured as optical density [OD] in a 1:10,000 serum dilution) to VCSAR HVPEYIEKLPEIRAK were elevated only in the surviving group and were statistically different from the nonsurviving group ($P = 0.004$, Mann-Whitney U test with Bonferroni correction) (Fig. 5D), suggesting that the deduced sequence contains both a B-cell epitope and a T-cell epitope (the latter is underlined).

DISCUSSION

Several studies have suggested a possible protective role for antibodies in immunity against fungal diseases, in particular for yeast infections such as cryptococcosis (35) and candidiasis (11, 27, 35, 39, 48, 49) and a few for aspergillosis, a mold infection (12, 13). Interestingly, most studies were conducted *in vitro* or used animal models of systemic disease, in which fungal spores were intravenously injected, which is quite different from the respiratory route of naturally occurring aspergillosis. Despite this, it has also been suggested that antibodies may play a minor role in supporting opsonization of *A. fumigatus* (34). However, for our rAsp f3 vaccine, passive transfer of IgG from vaccinated animals into naïve recipients did not enhance protection. Furthermore, we demonstrated that in all life stages of

A. fumigatus (resting conidia, swollen conidia, and hyphae), Asp f3 is an intracellular protein. In hyphae, Asp f3 colocalizes with PMP70, a known peroxisomal protein, and without degradation of the cell wall and membrane, Asp f3 is not accessible to IgG antibodies. This also makes it very unlikely that other antibody isotypes are involved in rAsp f3 vaccine-induced protection. Others previously reported that Asp f3 is a cell wall protein or a conidial surface protein, based on indirect methods using enriched cell wall preparations (17) or digestion of conidial cell walls followed by proteomic analysis (4). However, Asp f3 appears to be an abundant, somewhat "sticky" protein and lacks a signal peptide (4). Thus, cell wall preparations and cell wall digestion methods could become biased by contamination with unintentionally liberated Asp f3. We conclude that IHC detection of intracellular Asp f3 is more reliable and direct. That antibodies to surface-displayed fungal antigens (not Asp f3) could provide a vaccine-induced protective effect against aspergillosis remains a possibility and is not excluded by our study.

Although a protective role for anti-Asp f3 antibodies seems unlikely, we found that IgG recognition of a particular B-cell epitope, Asp f3 residues 60 to 79, correlated with vaccine protection. This part of the Asp f3 sequence does not bind IgE from ABPA patients (40) and also contains a T-cell epitope. The T-cell immune response to Asp f3 (or other antigens) was a result of vaccination with Asp f3 and not of fungal infection (Fig. 5). The stringent immunosuppressive treatment and the acuteness of the infection that occurs on a rapid time scale (3 days for most lethality) make the formation of antigen-specific responses unlikely. Although nonprotective, antibody titers against this B-cell epitope could be useful for distinguishing

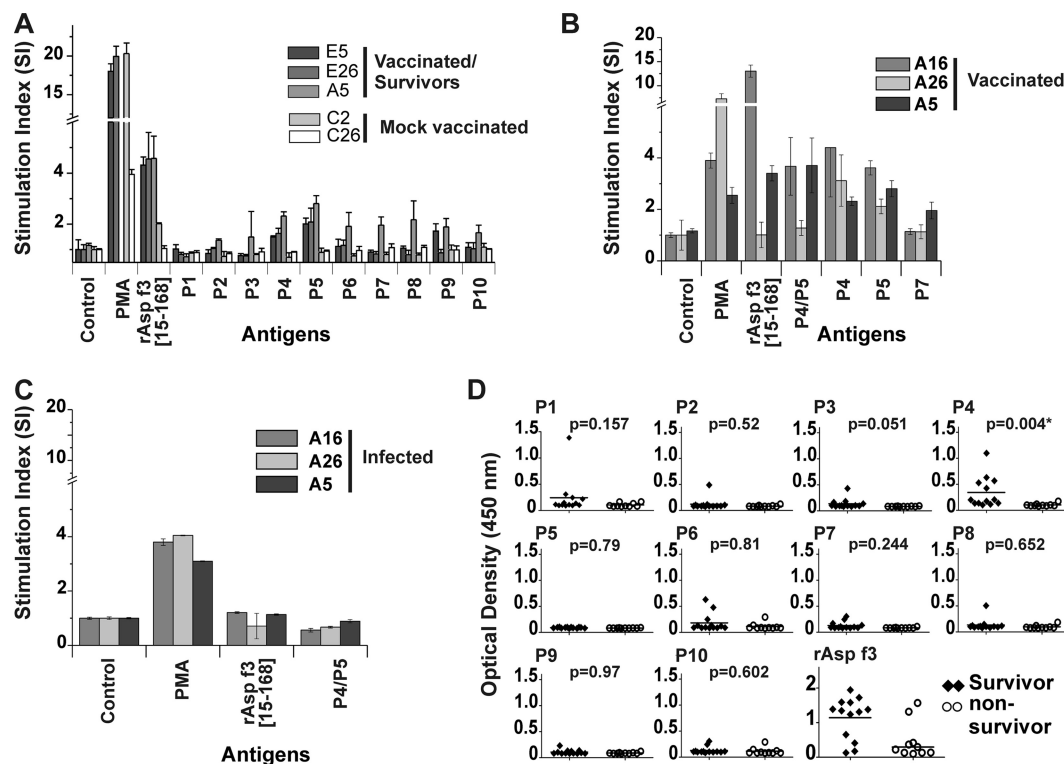


FIG. 5. Identification of T- and linear B-cell epitopes of rAsp f3. (A to C) Lymphoproliferation responses of T cells from individual mice. (A) T cells from rAsp f3-vaccinated survivors of fungal challenge (Vaccinated/Survivors; animals E5, E26, and A5) and nonvaccinated control mice that were mock immunized with PBS and TM adjuvant only and not challenged (Mock; animals C2 and C26); (B) T cells from rAsp f3-vaccinated mice; (C) T cells from sublethally infected, nonimmunosuppressed, nonvaccinated mice. The stimulants were synthetic Asp f3 peptides (P1 to P10) (Table 1) and intact rAsp f3(15–168) (rAsp f3), with PMA as a positive control and RPMI medium as a negative control (Control). Stimulation indices (SIs) in all vaccinated survivors are ≥ 2 for P4, P5, and rAsp f3(15–168). (D) Binding of prechallenge serum IgG to synthetic overlapping Asp f3 peptides (P1 to P10), as measured by ELISA for individual mice. The IgGs were from individual rAsp f3(15–168)-vaccinated mice that survived (solid diamonds; $n = 13$) or did not survive (open circles; $n = 10$) infection with *A. fumigatus*. Statistical significance (*) was defined as $P < 0.05$, calculated by Mann-Whitney U test with Bonferroni correction.

vaccine-protected individuals from the unprotected. Nonvaccinated mice did not mount a T-cell response to Asp f3 epitopes when infected; however, we previously discovered Asp f3 as a vaccine candidate because of the Asp f3-specific antibody response in nonlethally infected mice (25).

CD4⁺ T cells mediate rAsp f3-vaccine protection. Recombinant Asp f3(15–168) vaccine-primed CD4⁺ T cells appear to be essential for the vaccine's mechanism of protection. Depletion of CD4⁺ T cells reduced survival of vaccinated mice to the same level as that of nonvaccinated mice, and the remaining CD8⁺ T cells were not protective. Due to adjusted experimental parameters, the level of vaccine protection in non-CD4⁺ T-cell-depleted control animals was reduced to $\sim 40\%$, which is lower than the commonly observed levels of vaccine protection (typically on the order of 70 to 90%) (25). We attribute this effect to the extensive 10-day regimen of immunosuppression (instead of the previously used 6 days). Despite this reduced initial protection, the data are statistically significant, because CD4⁺ T-cell depletion further reduced survival to $\sim 10\%$, undistinguishable from that of nonvaccinated mice. Furthermore, we confirmed that transfer of splenic CD4⁺ T cells from vaccinated CF-1 mice into CF-1 mice protected the latter under the CA-induced immunosuppressive regimen. The partial protection (46%) against aspergillosis may suggest that either con-

dition for the transfer could be further optimized or that other components such as CD8 T cells could also contribute to protection. Because Asp f3 is an intracellular protein, a CD4⁺ T-cell-dependent mechanism would also require an antigen-presenting cell (APC) for phagocytosis and major histocompatibility complex (MHC) display. Macrophages, dendritic cells, and B cells are professional APCs; although it is not completely clear which cell types are effective APCs under corticosteroid immunosuppressive conditions, dendritic cells were previously shown to be essential to antifungal protection (9, 10).

Conclusions. We conclude that the protective mechanism of the rAsp f3-based vaccine is driven by rAsp f3-primed CD4⁺ T cells. Antibodies do not seem to contribute to the protection induced by this vaccine. Although, it is not clear which effector cells are stimulated by the CD4⁺ T cells, immunosuppressed neutrophils and macrophages are likely candidates. Corticosteroids, such as CA used here, induce the expression of I κ B, the inhibitor of NF- κ B (1, 2). For vaccinated immunosuppressed animals, the inhibited effector cell could receive a strong local stimulus (cytokines) delivered by a fungus-recognizing, vaccine-primed CD4⁺ T cell. Similarly, it was shown that treatment with granulocyte macrophage colony-stimulating factor can reverse the immunosuppressive effect of dexa-

methasone by enhancing degradation of I κ B (14). A T-cell-derived cytokine stimulus such as IFN- γ could likewise lead to activation of I κ B kinases (IKK) in the effector cell (45), to an extent that even increased numbers of corticosteroid-induced I κ B molecules become sufficiently phosphorylated and degraded. Hence, the corticosteroid-induced immunosuppression will be overridden at sites of infection. Further studies will be needed to explore this hypothetical signaling pathway for the Asp f3-vaccine-induced mechanism of protection.

ACKNOWLEDGMENTS

This study was supported in part by the Hermann Foundation, the Tim Nesvig Lymphoma Fellowship and Research Fund, and NIH grant AI075230-01.

We thank Joseph M. Lyons for assistance and advice with the animal models, Dan Trinh for help with the production of rAsp f3, and Jeffrey Longmate for valuable advice on biostatistical data evaluation.

REFERENCES

- Almawi, W. Y., and O. K. Melemedjian. 2002. Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. *J. Leukoc. Biol.* **71**:9–15.
- Almawi, W. Y., and O. K. Melemedjian. 2002. Negative regulation of nuclear factor-kappaB activation and function by glucocorticoids. *J. Mol. Endocrinol.* **28**:69–78.
- Armstrong-James, D. P., et al. 2009. Impaired interferon-gamma responses, increased interleukin-17 expression, and a tumor necrosis factor-alpha transcriptional program in invasive aspergillosis. *J. Infect. Dis.* **200**:1341–1351.
- Asif, A. R., et al. 2006. Proteome of conidial surface associated proteins of *Aspergillus fumigatus* reflecting potential vaccine candidates and allergens. *J. Proteome Res.* **5**:954–962.
- Baddley, J. W., et al. 2010. Factors associated with mortality in transplant patients with invasive aspergillosis. *Clin. Infect. Dis.* **50**:1559–1567.
- Beck, O., et al. 2006. Generation of highly purified and functionally active human TH1 cells against *Aspergillus fumigatus*. *Blood* **107**:2562–2569.
- Beesley, J. E. 1993. Immunocytochemistry: a practical approach. IRL Press at Oxford University Press, Oxford, United Kingdom.
- Bonnett, C. R., E. J. Cornish, A. G. Harmsen, and J. B. Burritt. 2006. Early neutrophil recruitment and aggregation in the murine lung inhibit germination of *Aspergillus fumigatus* conidia. *Infect. Immun.* **74**:6528–6539.
- Bozza, S., et al. 2004. Dendritic cell-based vaccination against opportunistic fungi. *Vaccine* **22**:857–864.
- Bozza, S., et al. 2003. A dendritic cell vaccine against invasive aspergillosis in allogeneic hematopoietic transplantation. *Blood* **102**:3807–3814.
- Bromuro, C., et al. 2010. Beta-glucan-CRM197 conjugates as candidates antifungal vaccines. *Vaccine* **28**:2615–2623.
- Chaturvedi, A. K., A. Kavishwar, G. B. Shiva Keshava, and P. K. Shukla. 2005. Monoclonal immunoglobulin G1 directed against *Aspergillus fumigatus* cell wall glycoprotein protects against experimental murine aspergillosis. *Clin. Diagn. Lab. Immunol.* **12**:1063–1068.
- Chaturvedi, A. K., R. Kumar, A. Kumar, and P. K. Shukla. 2009. A monoclonal IgM directed against immunodominant catalase B of cell wall of *Aspergillus fumigatus* exerts anti-A. fumigatus activities. *Mycoses* **52**:524–533.
- Choi, J. H., E. Brummer, Y. J. Kang, P. P. Jones, and D. A. Stevens. 2006. Inhibitor kappaB and nuclear factor kappaB in granulocyte-macrophage colony-stimulating factor antagonism of dexamethasone suppression of the macrophage response to *Aspergillus fumigatus* conidia. *J. Infect. Dis.* **193**:1023–1028.
- Clemons, K. V., and D. A. Stevens. 2005. The contribution of animal models of aspergillosis to understanding pathogenesis, therapy and virulence. *Med. Mycol.* **43**(Suppl. 1):S101–S110.
- Cordonnier, C., et al. 2006. Prognostic factors for death due to invasive aspergillosis after hematopoietic stem cell transplantation: a 1-year retrospective study of consecutive patients at French transplantation centers. *Clin. Infect. Dis.* **42**:955–963.
- Denikus, N., et al. 2005. Fungal antigens expressed during invasive aspergillosis. *Infect. Immun.* **73**:4704–4713.
- Garlanda, C., et al. 2002. Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* **420**:182–186.
- Hebart, H., et al. 2002. Analysis of T-cell responses to *Aspergillus fumigatus* antigens in healthy individuals and patients with hematologic malignancies. *Blood* **100**:4521–4528.
- Hemmann, S., C. Ismail, K. Blaser, G. Menz, and R. Cramer. 1998. Skin-test reactivity and isotype-specific immune responses to recombinant Asp f3, a major allergen of *Aspergillus fumigatus*. *Clin. Exp. Allergy* **28**:860–867.
- Hohl, T. M., and M. Feldmesser. 2007. *Aspergillus fumigatus*: principles of pathogenesis and host defense. *Eukaryot. Cell* **6**:1953–1963.
- Ibrahim-Granet, O., et al. 2010. In vivo bioluminescence imaging and histopathologic analysis reveal distinct roles for resident and recruited immune effector cells in defense against invasive aspergillosis. *BMC Microbiol.* **10**:105.
- Ito, J. I., and J. M. Lyons. 2002. Vaccination of corticosteroid immunosuppressed mice against invasive pulmonary aspergillosis. *J. Infect. Dis.* **186**:869–871.
- Ito, J. I., J. M. Lyons, D. Diaz-Arevalo, T. B. Hong, and M. Kalkum. 2009. Vaccine progress. *Med. Mycol.* **47**(Suppl. 1):S394–S400.
- Ito, J. I., et al. 2006. Vaccinations with recombinant variants of *Aspergillus fumigatus* allergen Asp f3 protect mice against invasive aspergillosis. *Infect. Immun.* **74**:5075–5084.
- Kami, M., N. Murashige, T. Fujihara, N. Sakagami, and Y. Tanaka. 2005. The mechanism for low yield of blood culture in invasive aspergillosis; the clinical importance of antigen detection tests revisited. *Bone Marrow Transplant.* **36**:85–86.
- Kondori, N., L. Edebo, and I. Mattsby-Baltzer. 2008. A novel monoclonal antibody recognizing beta(1–3) glucans in intact cells of *Candida* and *Cryptococcus*. *APMIS* **116**:867–876.
- Kontoyannis, D. P., et al. 2010. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin. Infect. Dis.* **50**:1091–1100.
- Latge, J. P. 1999. *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* **12**:310–350.
- Latge, J. P. 2001. The pathobiology of *Aspergillus fumigatus*. *Trends Microbiol.* **9**:382–389.
- Levitz, S. M., M. E. Selsted, T. Ganz, R. I. Lehrer, and R. D. Diamond. 1986. In vitro killing of spores and hyphae of *Aspergillus fumigatus* and *Rhizopus oryzae* by rabbit neutrophil cationic peptides and bronchoalveolar macrophages. *J. Infect. Dis.* **154**:483–489.
- Marr, K. A., R. A. Carter, M. Boeckh, P. Martin, and L. Corey. 2002. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. *Blood* **100**:4358–4366.
- Mehrad, B., R. M. Strieter, and T. J. Standiford. 1999. Role of TNF-alpha in pulmonary host defense in murine invasive aspergillosis. *J. Immunol.* **162**:1633–1640.
- Montagnoli, C., et al. 2003. A role for antibodies in the generation of memory antifungal immunity. *Eur. J. Immunol.* **33**:1193–1204.
- Mukherjee, S., S. C. Lee, and A. Casadevall. 1995. Antibodies to *Cryptococcus neoformans* glucuronoxylomannan enhance antifungal activity of murine macrophages. *Infect. Immun.* **63**:573–579.
- Orsborn, K. I., et al. 2006. Protein expression profiling of *Coccidioides posadasii* by two-dimensional differential in-gel electrophoresis and evaluation of a newly recognized peroxisomal matrix protein as a recombinant vaccine candidate. *Infect. Immun.* **74**:1865–1872.
- Ovechkina, Y. Y., R. K. Pettit, Z. A. Cichacz, G. R. Pettit, and B. R. Oakley. 1999. Unusual antimicrotubule activity of the antifungal agent spongistatin 1. *Antimicrob. Agents Chemother.* **43**:1993–1999.
- Philippe, B., et al. 2003. Killing of *Aspergillus fumigatus* by alveolar macrophages is mediated by reactive oxidant intermediates. *Infect. Immun.* **71**:3034–3042.
- Pietrella, D., et al. 2010. A beta-glucan-conjugate vaccine and anti-beta-glucan antibodies are effective against murine vaginal candidiasis as assessed by a novel in vivo imaging technique. *Vaccine* **28**:1717–1725.
- Ramachandran, H., et al. 2002. IgE binding conformational epitopes of Asp f3, a major allergen of *Aspergillus fumigatus*. *Clin. Immunol.* **103**:324–333.
- Roilides, E., A. Dimitriadou-Georgiadou, T. Sein, I. Kadioglou, and T. J. Walsh. 1998. Tumor necrosis factor alpha enhances antifungal activities of polymorphonuclear and mononuclear phagocytes against *Aspergillus fumigatus*. *Infect. Immun.* **66**:5999–6003.
- Roilides, E., et al. 1997. IL-10 exerts suppressive and enhancing effects on antifungal activity of mononuclear phagocytes against *Aspergillus fumigatus*. *J. Immunol.* **158**:322–329.
- Roilides, E., K. Uhlig, D. Venzon, P. A. Pizzo, and T. J. Walsh. 1993. Prevention of corticosteroid-induced suppression of human polymorphonuclear leukocyte-induced damage of *Aspergillus fumigatus* hyphae by granulocyte colony-stimulating factor and gamma interferon. *Infect. Immun.* **61**:4870–4877.
- Schaffner, T., H. U. Keller, M. W. Hess, and H. Cottier. 1982. Macrophage functions in antimicrobial defense. *Klin. Wochenschr.* **60**:720–726.
- Shultz, D. B., M. R. Rani, J. D. Fuller, R. M. Ransohoff, and G. R. Stark. 2009. Roles of IKK-beta, IRF1, and p65 in the activation of chemokine genes by interferon-gamma. *J. Interferon Cytokine Res.* **29**:817–824.
- Stevens, D. A. 2004. Vaccinate against aspergillosis! A call to arms of the immune system. *Clin. Infect. Dis.* **38**:1131–1136.
- Stevens, D. A., K. V. Clemons, and M. Liu. 2011. Developing a vaccine against aspergillosis. *Med. Mycol.* **49**(Suppl. 1):S170–S176.
- Torosantucci, A., et al. 2005. A novel glyco-conjugate vaccine against fungal pathogens. *J. Exp. Med.* **202**:597–606.

49. **Torosantucci, A., et al.** 2009. Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence. *PLoS One* **4**:e5392.
50. **Tramsen, L., et al.** 2009. Clinical-scale generation of human anti-Aspergillus T cells for adoptive immunotherapy. *Bone Marrow Transplant.* **43**:13–19.
51. **Upton, A., K. A. Kirby, P. Carpenter, M. Boeckh, and K. A. Marr.** 2007. Invasive aspergillosis following hematopoietic cell transplantation: outcomes and prognostic factors associated with mortality. *Clin. Infect. Dis.* **44**:531–540.
52. **Yi, T., et al.** 2008. Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. *Blood* **112**:2101–2110.

Editor: G. S. Deepe, Jr.