

Review Article

Vaccine progress

JAMES I. ITO*, JOSEPH M. LYONS*, DIANA DIAZ-AREVALO†, TERESA B. HONG† & MARKUS KALKUM†

*Division of Infectious Diseases, and †Department of Immunology, City of Hope National Medical Center and Beckman Research Institute Duarte California USA

Despite the recent development of new anti-mould agents, there remains a significant incidence of invasive aspergillosis in the most immunocompromised hosts and the response to these agents is still dismal. There is a need for a different approach: prevention by vaccination. We have demonstrated that a hyphal sonicate of *Aspergillus fumigatus* was capable of conferring protection against subsequent invasive pulmonary aspergillosis in corticosteroid immunosuppressed mice. Subcutaneous vaccination was superior to nasal vaccination. Mice exposed intranasally to viable conidia were noted to respond serologically to a 19kDa protein. This protein was identified as the allergen Asp f 3 by mass spectrometry. Vaccination with recombinant Asp f 3 was protective. Truncated forms of Asp f 3 that lacked either one of the two known IgE binding sites were cloned and also demonstrated protection against aspergillosis. Although all of these recombinant proteins required an adjuvant (TiterMax) for efficacy, a particulate preparation of rAsp f 3 was also found to be protective without requiring adjuvant. At least two T-cell epitopes (11-mer and 13-mer) have been identified in Asp f 3. There are homologues of Asp f 3 in other *Aspergillus* species as well as in other moulds (*Coccidioides posadasii*, *Penicillium citrinum*) and yeasts (*Candida albicans*, *C. boidinii*, *Saccharomyces cerevisiae*). Asp f 3, truncated non-allergenic versions of Asp f 3, and T-cells epitopes of Asp f 3 are potential candidates for vaccines potentially capable of protecting immunocompromised hosts against invasive aspergillosis.

Keywords *Aspergillus* vaccine, Asp f 3

Despite the recent development of new anti-mould agents, there remains a significant incidence of invasive aspergillosis in our most immunocompromised hosts, the hematopoietic cell transplantation (HCT) recipients. The response to these agents is still dismal at 31% [1] and the mortality rates still high at approximately 60% [2]. There is a need for a different approach, and,

indeed, there have been calls to develop a vaccine against aspergillosis [3].

Until recently, vaccination has been given little attention probably because of the ‘paradox of vaccination in HCT’. That is, since an HCT recipient cannot be adequately immunized (with standard vaccines) until at least a year after transplantation (when immune reconstituted), how would vaccination administered either before or after transplantation be expected to protect while the recipient is still immunosuppressed, i.e., under immunosuppressive therapy and not yet immune reconstituted?

There are also other questions regarding the feasibility of such a vaccine in an immunocompromised host that must be answered: (1) Is there acquired immunity

Received 25 September 2008; Accepted 14 October 2008

Correspondence: James I. Ito, Division of Infectious Diseases, City of Hope National Medical Center, 1500 E. Duarte Road, Duarte, CA 91010-3000, USA. Tel.: +1 626 256 4673; fax: +1 626 301 8954; E-mail: jito@coh.org

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DOI: 10.1080/13693780802552614

Table 1 Mortality from invasive pulmonary aspergillosis after vaccination or infection

Group	No. of mice	Cumulative deaths after infection, days							Mortality, %	<i>P</i> ^a
		1	2	3	4	5	6	7		
Control	8	0	3	8	8	8	8	8	100	—
Prior infection	8	0	1	1	1	1	1	1	12.5	0.001
Sonicate										
Intranasal	7	0	2	2	2	2	2	2	29	0.01
Subcutaneous	8	0	0	0	0	0	0	0	0	0.0002
Filtrate										
Intranasal	8	0	3	5	6	6	6	6	75	0.46
Subcutaneous	8	0	2	2	2	2	2	4	50	0.076

^aFisher's exact test (vs. control).

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to *Aspergillus*?; (2) Can innate or acquired immunity be boosted through infection or vaccination?; (3) Can *Aspergillus* antigens confer immunity as well as live organisms (pre-infection)?; (4) Can these protective antigens be selected and allergens be avoided?; (5) Will adjuvants be required, and, if so, be safe for human use?; (6) Can these selected *Aspergillus* antigens confer cross-protection against other *Aspergillus* species (*A. flavus*, *A. niger*, *A. terreus*) or other moulds?; (7) Can systemic vaccination confer local (sino-pulmonary) protection?; (8) Will immunity attained while immunocompetent provide protection during subsequent immunosuppression (neutropenia, corticosteroid therapy, T-cell immunosuppressive therapy, graft versus host disease)?; and (9) Can this immunity be transferred from donor to recipient under current transplantation conditions?

Many of these questions were answered by the Romani group at Perugia University seven years ago when they demonstrated that by vaccinating mice prior to immunosuppressive chemotherapy they could protect them from subsequent lethal challenge from inhaled *Aspergillus* conidia during neutropenia [4]. Only one of their three vaccine preparations was effective. This was a crude culture filtrate (CCFA). An Asp f2 (allergen) protein and a heat-inactivated *Aspergillus* conidia preparation were not effective. The route of vaccine administration was intranasal. They also demonstrated that the adoptive transfer of *Aspergillus* antigen-specific CD4⁺ T cells resulted in some protection, but only when using a model of animals injected with conidia *intravenously*. Furthermore, they showed that in the CCFA-vaccinated mice local CD4⁺ cells proliferated in response to CCFA and local IFN- γ

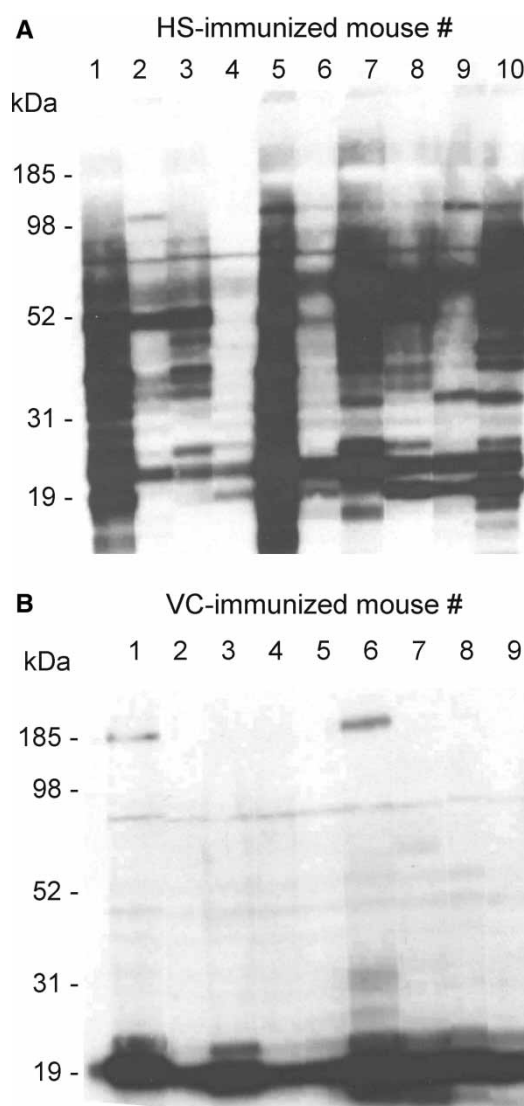


Fig. 1 Nineteen Western blots of hyphal protein extracts probed with sera from individual mice after immunization with either (A) hyphal sonicate (HS) or (B) through naso-pulmonary exposure to viable conidia (VC). Goat anti-mouse IgG-HRP conjugate was used for chemiluminescent detection. (Reproduced in part from reference 11 with permission.)

and IL-2 production increased. They also observed that there was recruitment of lymphocytes and macrophages to the site of infection in vaccinated animals despite a profound systemic neutropenia.

The Romani group subsequently demonstrated that the allergen Asp f 16, but not the allergen Asp f 3, when administered with CpG oligodeoxynucleotides as adjuvants intranasally was protective against invasive pulmonary aspergillosis in neutropenic BALB/c mice [5]. They finally demonstrated that infusion of dendritic cells (DC) pulsed with live *Aspergillus* conidia or

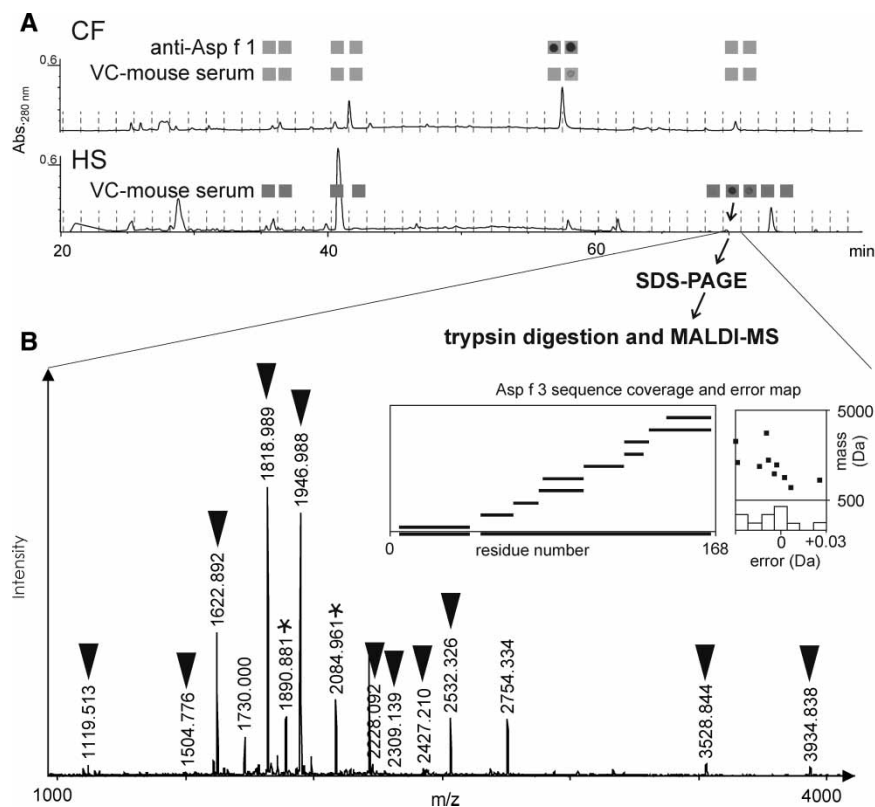


Fig. 2 (A) Reversed phase HPLC separation and dot-blot analysis of culture filtrate (CF) and pre-fractionated hyphal sonicate (HS). The input was the 10-to-30-kDa HS fraction from ultrafiltration. The 70-min HPLC fraction of HS reacts positively with serum from VC immunized mice. (B) MALDI-MS analysis of a 19-kDa band of this fraction identifies *A. fumigatus* Asp f 3 as the major protein component; black triangles mark assigned peptide ions that match the Asp f 3 sequence. Stars mark two peptide ions from coflin, a minor protein component of the same fraction. (Reproduced from reference 11 with permission.)

transfected with conidial RNA protected mice against invasive pulmonary aspergillosis while undergoing allogeneic HCT, and that protection was superior to adoptive transfer of *Aspergillus*-specific T-cells [6]. Shao *et al.* showed that infusion of DC transduced

with an adenoviral vector encoding for IL-12 and pulsed with heat-inactivated *Aspergillus* also protected mice against invasive pulmonary aspergillosis [7].

Regarding the cross-protective potential of fungal vaccines, Torosantucci *et al.* reported that laminarin

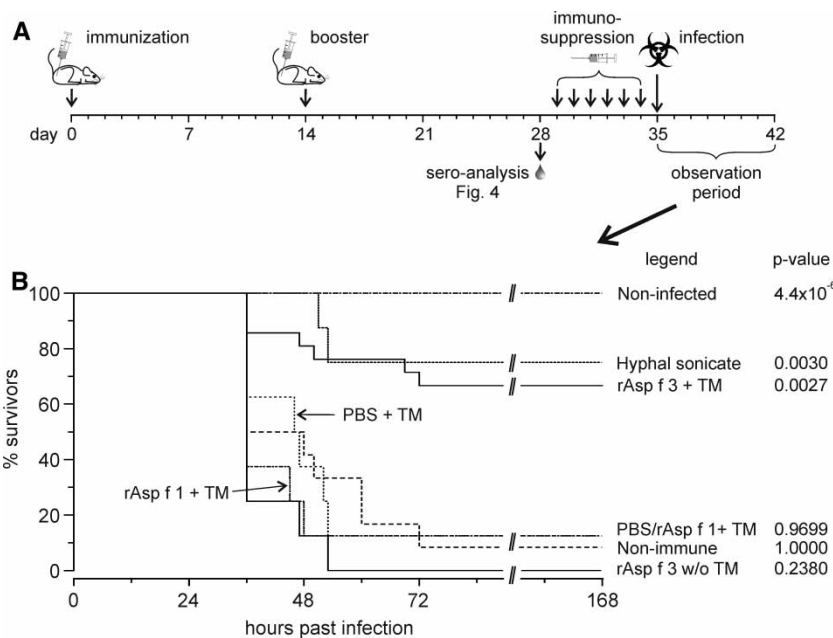
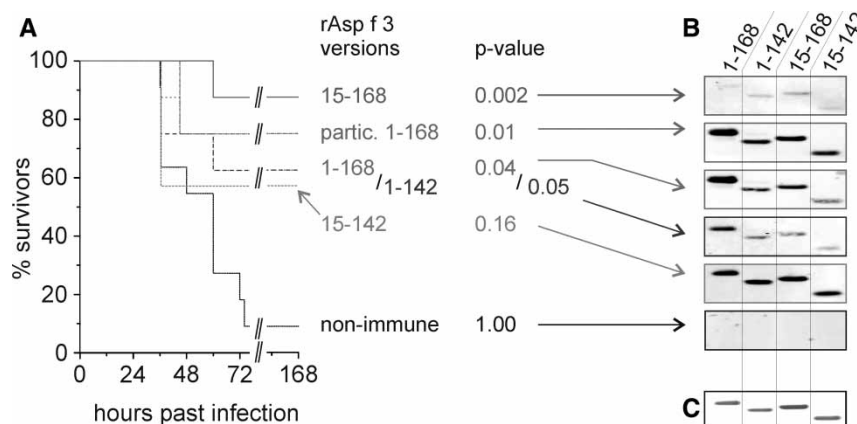


Fig. 3 (A) Experimental scheme of immunization, immunosuppression and challenge. (B) Survival curves recorded during the observation period. Number of animals per group were: 16 non-infected controls, 8 hyphal sonicate, 21 rAsp f 3+TiterMax adjuvant (TM), 8 PBS+TM, 8 rAsp f 1+TM, and 12 non-immune controls that received PBS injections instead of antigens or adjuvant. *P* values were calculated with the Mann-Whitney U test with reference to the non-immune group.

Fig. 4 (A) Survival curves for mice vaccinated with truncated versions of Asp f 3 with TM adjuvant (as indicated by each first and last amino acid residue number) and particulate, adjuvant-free full-length rAsp f 3 (partic. 1-168). Eight animals were used in each group, except for Asp f 3 (1-142) with seven and the control group with 11 mice. (B) Serological analysis of pooled plasma from these mice by Western blot analysis (around 19 kDa). (C) The Coomassie-stained gel indicates comparable loading amounts of the of the rAsp f 3 versions (250 ng/band). P values were calculated with the Mann-Whitney U test with reference to the non-immune group.

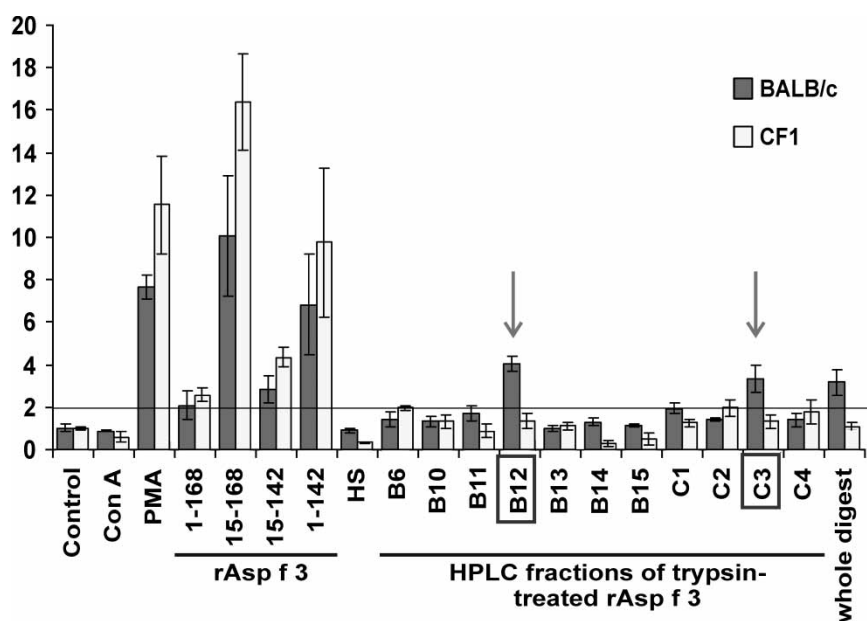


(a glucan from the alga *Laminaria digitata*) conjugated with the diphtheria toxoid CRM197 was protective against both disseminated candidiasis and also an intravenous challenge of *Aspergillus* [8]. And, finally, the Stevens group of Stanford University demonstrated that subcutaneous vaccination with killed or live *Saccharomyces* protected against an intravenous challenge of *Aspergillus* conidia [9]. Efficacy of these two fungal vaccines against an inhalational challenge of *Aspergillus* conidia has yet to be demonstrated.

Since the majority of cases of invasive aspergillosis occurs after engraftment, that is, after neutropenia has resolved, the major risk factor in the HCT setting is not neutropenia, but, rather, graft versus host disease (GVHD) and its primary therapy, corticosteroids. Thus, we began to study the feasibility of vaccination against aspergillosis in a corticosteroid

immunosuppressed mouse model [10]. This model consisted of outbred CF-1 mice that were immunosuppressed with cortisone acetate (2.5mg subcutaneously daily $\times$ 6 days prior to infectious challenge). They were challenged with one million viable *Aspergillus fumigatus* conidia intranasally. Two vaccine preparations were used: (1) a freeze-thaw and sonication of hyphal mass (called 'sonicate' or HS) and (2) a filter-sterilized liquid culture supernatant (called 'filtrate' or CF). The results of the vaccine experiments are shown in Table 1. We concluded that: (1) prior intranasal challenge (without immunosuppression) conferred subsequent protection during corticosteroid immunosuppression; (2) both sonicate and filtrate induced immunity that conferred subsequent protection during corticosteroid immunosuppression; (3) sonicate was superior to filtrate as a vaccine; (4) the subcutaneous

Fig. 5 T cell proliferation responses to recombinant Asp f 3 variants and trypsin-derived peptides thereof in immunized mice. The HPLC fractions B12 and C3 contain T cell epitopes recognized by BALB/c mice. Proliferation is represented as a stimulation index derived from ^3H labeling as counts per minute (cpm) of cells with antigen/cpm of cells without antigen. Control means cells cultured without antigens; other antigens are: ConA = concanavalin A; PMA = phorbol myristate acetate; rAsp f 3 in full length (residues 1-168) or in the three truncated forms as indicated; HS = hyphal sonicate; B6 to C4 are HPLC-separated fractions of tryptic digest peptides of rAsp f 3; 'whole digest' is the entire non-separated trypsin digest.



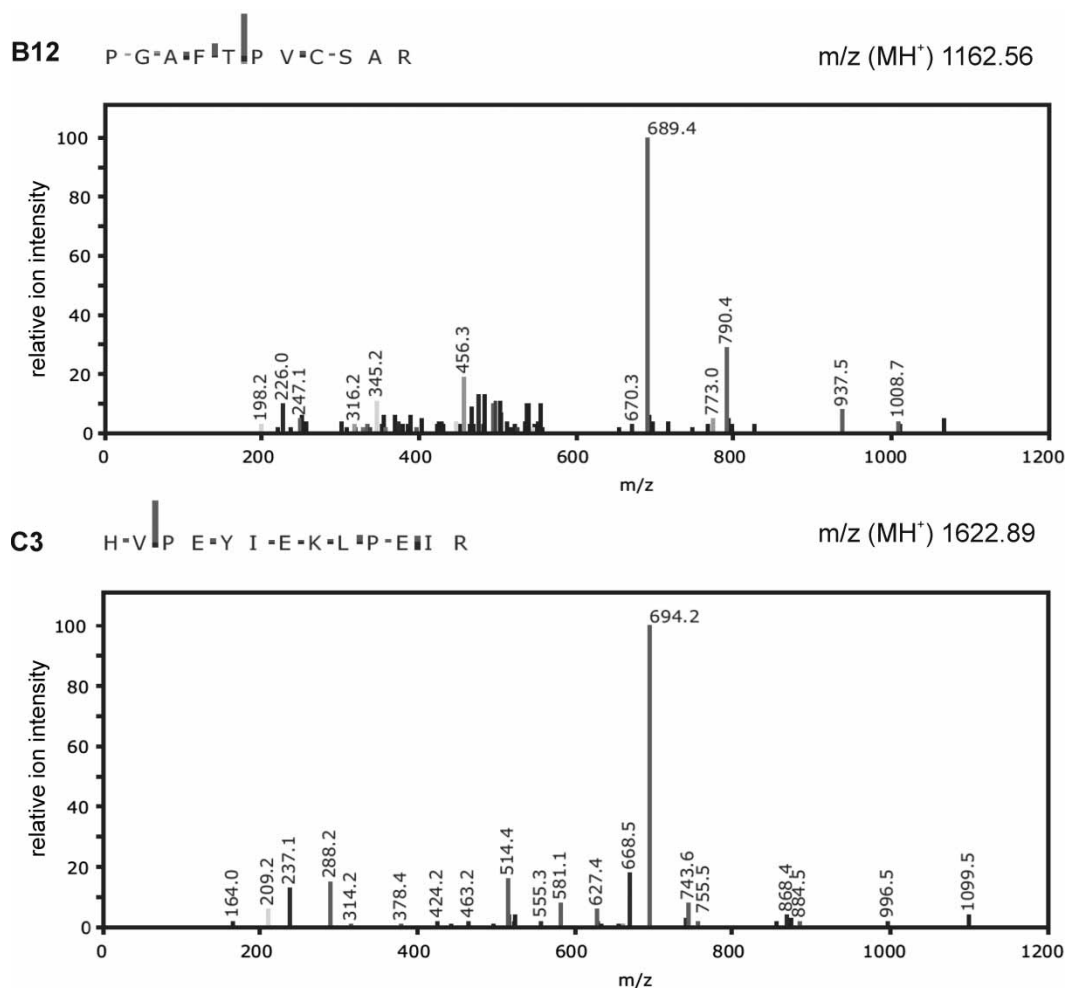


Fig. 6 Mass spectrometric identification of T cell epitopes. MS/MS spectra of rAsp f 3-digest peptides in HPLC fractions B12 and C3 (Fig. 5) were acquired on an LTQ FTICR mass spectrometer (Thermo Scientific) and assigned with *The Global Proteome Machine* (The GPM) search engine. The identified peptide sequences are shown as fragmentograms above the mass spectra. Mass-over-charge values (m/z) of the singly charged and singly protonated parent ions (MH^+) are indicated. Bar colors mark different ion types: dark blue = b-ions, light blue = b-18-ions, red = y-ions, orange = y-17-ions, yellow = a-ions, according to the accepted nomenclature (K. Biemann, 'Nomenclature for peptide fragment ions'. In: McCluskey JA (ed.), *Methods in Enzymology* Vol. 193, Academic, Orlando, FL (1990), pp. 886–888). Briefly, b-ions refer to N-terminal fragments of peptides, whereas y-ions contain C-terminal portions. These ion types can further lose water or ammonia indicated as -18 and -17, respectively.

(or systemic) route of vaccination was superior to the intranasal route. We subsequently attempted to determine what the protective antigen(s) was in this crude vaccine [11].

We first analyzed the sera from individual mice vaccinated with HS and those exposed to viable conidia (VC). These immune sera were tested for reactivity against hyphal protein extracts (HS) by Western blots (Fig. 1A,B). The Western blots of HS-vaccinated mice show a multitude of bands at many molecular weights. In contrast, the Western blot of VC-immunized mice showed bands primarily at 19kDa.

CF and HS were prefractionated by dual-stage ultrafiltration through membranes with nominal cutoffs of 30 and 10 kDa. The CF and HS retentate of the 10 kDa membrane were further separated by HPLC, and individual fractions were subjected to dot blot analysis (Fig. 2A). Asp f 1 was detected by dot blotting with 10-kDa retentate fractions of CF eluting at 57 min by use of a commercially available monoclonal anti-Asp f 1 antibody. The same fraction also showed a weak reaction with sera from VC-exposed mice. A 70 min fraction of the HS retentate reacted strongly with IgG from VC-immune mice. This fraction

was further separated by SDS-PAGE, and the protein content of an 18- to 20-kDa band was reduced, alkylated, trypsin digested, and analyzed by MALDI-Q-time-of-flight and MALDI-Q-ion trap mass spectrometry (Fig. 2B). Peptide mass fingerprinting as well as tandem MS data identified the known allergen Asp f 3 (GenBank accession no. XP_747849) as the major component of the IgG-binding HPLC fraction. Taken together, our data indicate that mice infected with viable conidia produce specific antibodies predominantly against Asp f 3.

Thus, we proceeded to synthesize recombinant Asp f 3 (using a pQE30Xa vector) and test it with the adjuvant TiterMax (TM) in our corticosteroid immunosuppressed mice (Fig. 3). The Asp f 3 sequence was derived by reversed transcription of purified mRNA from *A. fumigatus* as described in Ito *et al.* [11]. TiterMax is a proprietary adjuvant that is made of a synthetic block of copolymer (CRL89-41), squalene, a metabolizable oil, a microparticulate stabilizer, and in contrast to Freund's adjuvant does not contain mycobacterium-derived components. Mice vaccinated with rAsp f 3+TM were protected as were mice vaccinated with the HS (hyphal sonicate). Mice vaccinated with rAsp f 3 without TM or rAsp f1+TM were not protected.

Because Asp f 3 is considered an allergen the possibility exists that it might have the potential to induce an allergic reaction to *Aspergillus* such as allergic bronchopulmonary aspergillosis (ABPA). A vaccine consisting of an allergen would have difficulty winning approval from the FDA. Thus, our work focused on eliminating the allergenic potential of Asp f 3. Since ABPA patients have elevated Asp f 3-specific IgE levels [12], and the IgE-binding epitope is bipartite with both N-terminal and C-terminal binding sites being necessary for binding of one IgE molecule [13], eliminating either one of the two binding sites without losing the 'protective' sites (epitopes) would be the goal of creating a safe, non-allergy inducing, protective vaccine.

Thus, various truncated forms of Asp f 3 were synthesized: (1) 1–142 (C-terminal truncation); (2) 15–168 (N-terminal truncation); (3) 15–142 (bi-truncated). These three truncated versions of Asp f 3 with TM adjuvant along with a 'particulate' form of rAsp f 3 that was adjuvant free (and prepared by precipitation with trichloroacetic acid and resuspended in PBS with 0.5% methylcellulose to stabilize the suspension) were tested in the corticosteroid immunosuppressed model of invasive aspergillosis (Fig. 4). Both the N-terminal (15–168) and C-terminal (1–148) versions of Asp f 3

protected mice. The bi-truncated version (15–142) failed to significantly protect. And, significantly, the adjuvant-free particulate rAsp f 3 vaccine demonstrated significant protection suggesting that adjuvants may not be necessary.

To further delineate the specific protective epitopes in Asp f 3, trypsin-derived peptides of rAsp f 3 were used in lymphoproliferation assays utilizing splenocytes from mice immunized with rAsp f 3 (Fig. 5) [14]. Two fractions (B12 and C3) appear to have stimulated Asp f 3-specific T-cells and are candidates for protective T-cell epitopes. These T-cell epitopes were subjected to mass spectrometric analysis and were identified as a 11-mer (PGAFTPVC SAR) and 13-mer (HVPEYIEKLPEIR) polypeptides that appear to be adjacent polypeptides in Asp f 3 (Fig. 6). These will undergo further testing as protective epitopes.

We have searched for homologues of Asp f 3 in other fungal species and found homology with the following: (1) PMP20 (Asp f 3) from *Aspergillus nidulans*; (2) cDNA from *Aspergillus oryzae*; (3) Pmp1 from *Coccidioides posadasii*; (4) PMPA and PMPB (PMP20) from *Candida boidinii*; (5) alkyl hydroperoxide reductase from *Candida albicans*; (6) Pen c3 from *Penicillium citrinum*; (7) alkyl hydroperoxide peroxidase (Ahp1) from *Saccharomyces cerevisiae*. Asp f 3 may be an antigen common to many fungal pathogens, and suggests the possibility of a universal vaccine that can potentially protect against a variety of invasive fungal infections. We are currently attempting to develop other models of fungal infection with other fungal pathogens in order to test the effectiveness of Asp f 3 and its truncated forms as cross protective vaccines.

In conclusion, the work of others and the work in our laboratory suggest that (1) it is possible to vaccinate animals prior to immunosuppression (neutropenia and corticosteroid therapy) and subsequently protect against invasive aspergillosis while immunosuppressed; (2) Asp f 3 is a protective antigen; (3) the allergenic sites are separate from the protective sites/epitopes and can be removed without losing protective efficacy; (4) adjuvants may not be necessary in the final formulation of a protective *Aspergillus* vaccine; (5) Asp f 3 and its truncated versions as well as other fungal proteins may be cross protective against other *Aspergillus* species and other moulds and other fungi; (6) polypeptide T-cell epitopes have the potential to be effective vaccines.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 26 February 2009.