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ARTICLE

Adoptive immunity mediated by HLA-A*0201 restricted Asp f16 peptides-specific CD8+ T cells against *Aspergillus fumigatus* infection

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Abstract *Aspergillus fumigatus* (*A. fumigatus*) is the most common pathogen of invasive aspergillosis (IA), a life-threatening infection in immunocompromised patients. Recent findings revealed that CD8+ T cells can mediate cytotoxic activity against *A. fumigatus*. Here, we bioinformatically identified three HLA-A*0201-restricted peptides from Asp f16, an *A. fumigatus* antigen which was previously shown to be involved in T cell immunity. Our immunological results demonstrated that these peptides can potently induce cytotoxic T lymphocyte (CTL) response in CD8+ T cells, thus, damaging the conidia and hyphae of *A. fumigatus*. Moreover, the Asp f16 peptides can also raise Th1 cell-like response, as measured by interferon-γ (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT). Furthermore, we established an invasive pulmonary aspergillosis model in HLA-A*0201 transgenic mice. Adoptive transfer of Asp f16 peptides-specific CTL significantly extended the overall survival time in the *A. fumigatus*-infected immunocompromised mice. In conclusion, our results demonstrate that the Asp f16 peptides might provide immunity against invasive *A. fumigatus* infection.

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Introduction

Invasive aspergillosis (IA) is a serious threat to immunocompromised patients, such as acquired immunodeficiency syndrome (AIDS) patients, transplant recipients, and those given immunosuppressive therapy [1–4]. The susceptibility of immunocompromised patients to IA depends mainly on host factors, the most important one of which is neutropenia, a hematological disorder with a hallmark sign of decreased neutrophils. A deficiency of neutrophil function increases the incidence of *Aspergillus* infection. Among these pathogenic *Aspergillus* species, *Aspergillus fumigatus* (*A. fumigatus*), the most frequently isolated causative agent of IA, has been widely studied [2].

Recently, growing evidence from clinical studies indicated that T cell immunity impairment is associated with an increased incidence of *A. fumigatus* infection [5]. Inspired by this finding, multiple strategies that aim to restore or enhance specific T cell immunity against *A. fumigatus* during periods of severe immunosuppression have evolved [6]. Among them, adoptive T cell immunity might be a promising one. The successful induction of adoptive immunity requires the identification of optimal antigens. Most published studies have used crude antigen extracts to induce protective T cell responses to *A. fumigatus*. However, these extracts are not suitable for clinical use because they contain a large number of non-antigenic components and toxins [7]. Hence, novel approaches for developing fungal vaccines have emerged recently [8]. With the advent of the post-genomics era, vaccine development has gained a robust boost from the extensive genomic information. A ground-

breaking study using the complete genome information of *Neisseria meningitidis* identified a series of candidate vaccine antigens against meningococcal meningitis [9]. This inspiring finding brought forward a novel strategy called "reverse vaccinology", in which putative immunogenic antigens are predicted by in silico analysis.

To date, 27 *A. fumigatus*-specific antigens have been well characterized. Among them, Asp f16, a 43-kD allergen encoded by the *crf1* gene, has evoked much attention in recent years. Asp f16 is abundantly expressed on the cell wall of *A. fumigatus* [10]. Asp f16 was proved to act as an immunodominant antigen and confer protective immunity in a murine model of invasive pulmonary aspergillosis [5, 11]. In addition, peptides from Asp f16 have also been shown to induce protective CD4+ and CD8+ T cell responses against *A. fumigatus* in vitro [12, 13]. Therefore, Asp f16 might be a promising vaccine candidate to treat IA.

In this study, we bioinformatically identified three Asp f16-derived peptides restricted by HLA-A*0201, the most common HLA-A allele in Caucasians, and investigated their potential to induce CD8+ T cells-mediated protective immunity against *A. fumigatus* infection in an immunocompromised mice model.

Materials and methods

Mice and fungi

C57BL/6 transgenic mice expressing human MHC-I molecule HLA-A*0201 were purchased from the Jackson Laboratory. All animal work was done according to the requirements of the Laboratory Animal Welfare Ethics Committee, Peking University First Hospital, with ethics approval number J200913. *Aspergillus fumigatus* (BMU 01200), isolated from an IA patient after hematopoietic stem cell transplantation (HSCT), was provided by the Centre of Medical Mycology and Mycoses, the Department of Dermatology and Venereology, Peking University First Hospital [14].

Prediction of HLA-A*0201-restricted epitopes of Asp f16

To identify potential HLA-A*0201-restricted T cell epitopes in Asp f16, we screened its amino acid sequence using HLA peptide binding prediction software BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) and SYFPEITHI (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm).Three potential HLA-A*0201-binding 9-mer peptides were chosen on the basis of their estimated dissociation half-life from MHC class I. At the same time, we selected and synthesized an HLA-A*0201-binding 9-mer peptide sequence CLAVEEVSL from nucleophosmin (NPM1) as a control peptide.

Dendritic cells (DC) and Asp f16 peptides loading

Bone marrow-derived dendritic cells (DC) were generated from HLA-A*0201 transgenic mice and stained with fluorescence-labeled antibodies against mouse MHC-II, CD80, CD86, and CD11c (BioLegend, USA) to monitor their maturation. The mature DC were loaded with the mixture of the three Asp f16 peptides in equal proportion or with the control peptide for 2 h.

Peptides-specific cytotoxic T lymphocytes (CTL)

A mixture of the three Asp f16 peptides (100 μg/mouse) in equal proportions or the control peptide in complete Freund's adjuvant (Sigma, MO) was injected subcutaneously at the base of the tails. After a booster immunization with the same peptides, single-cell suspensions were prepared by draining inguinal lymph nodes with complete media (RPMI 1640 media supplemented with 5 % fetal bovine serum). The cells were cultured together with irradiated DC that had been pre-loaded with either a mixture of the three Asp f16 peptides or the control peptide. After a 5 days of incubation, recombinant mouse interleukin-2 (10 ng/ml, R&D system, USA) was added to the cultures. After two cycles of stimulation, cytotoxic T lymphocytes (CTL) were purified using a CD8+ T Cell Isolation Kit (Miltenyi, Germany). The generated Asp f16 peptides-specific CTL (abbreviated as f16 CTL) and control peptide-specific CTL (abbreviated as control CTL) were cultured in complete media supplemented with 10 ng/ml interleukin-2.

Tetramer assay

In vitro-generated f16 CTL (1×10^6/ml) were stained with phycoerythrin-labeled tetramers (Sanquin, the Netherlands) specific for the three Asp f16 peptides, together with APC-labeled anti-mouse CD8+ monoclonal antibody (Biolegend, USA) at 4 °C for 30 min. The cells were then analyzed using a fluorescence-activated cell sorter (LSR; BD).

Enzyme-linked immunosorbent spot (ELISPOT)

The ELISPOT assay was performed according to the manufacturer's instructions (Dakewe). The peptides-specific CTL (5×10^4 per well) were cultured together with DC (5×10^3 per well) loaded with the appropriate peptides and then subjected to ELISPOT assay. The assay was performed in triplicate wells in three individual experiments and the number of spots in each well was counted using the Bioreader 4000 PRO-X (Bio-Sys).

FUN-1 conidiacidal assay

Aspergillus conidia were incubated with FUN-1 (Molecular Probes, Eugene, USA) and observed under a fluorescence microscope, as described previously [12, 13].

Hyphae damage assay

The hyphae damage assay was performed using a tetrazolium dye XTT as described previously [12, 13]. Briefly, *Aspergillus* conidia were germinated at 45 °C for 16 h to form hyphae. f16 CTL or control CTL were added to hyphae at an effector/target ratio of 3:1 and incubated at 37 °C for 2 h. The percentage of fungal cell damage was determined by: [1 − (A450 of hyphae incubated with CTL − A450 of CTL alone)/A450 of hyphae live)×100 %].

Scanning electron microscopy (SEM)

Aspergillus conidia were incubated at room temperature for 4 h to form swollen conidia, and then these swollen conidia were incubated alone or together with control CTL or Asp CTL for 2 h at 37 °C. The conidia were then harvested and incubated for 20 h in 3 % glutaraldehyde in 0.15 mM sodium cacodylate buffer (pH 7.4) with 0.1 % Alcian blue (Eppelheim, Germany). After fixing with 1 % OsO_4 for 90 min, the samples were coated with gold-palladium and analyzed in a scanning electron microscope (JEOL-JSM5600LV).

Infection model and adoptive transfer of CTL

Eight-week-old C57BL/6 transgenic mice were intraperitoneally injected with cyclophosphamide (Hengrui Medicine Co. Ltd., China) on day −4 (150 mg/kg) and day −1 (75 mg/kg). On day 0, the mice were inoculated intranasally with 30 μl of sterile PBS containing 6×10^6 conidia. On day 1, either f16 CTL or control CTL (10^5 cells /mouse) were injected intravenously.

Tissue staining

Periodic acid-Schiff (PAS) and hematoxylin–eosin (HE) staining were used to evaluate the lung tissue architecture and *Aspergillus* incidence.

Pulmonary fungal burden

Fungal burdens in the lungs of mice were determined by colony-forming units (CFU). Briefly, lung samples were homogenized in sterile saline supplemented with gentamicin and chloramphenicol. Serial dilutions of the homogenates were plated in triplicate onto potato dextrose agar at 37 °C. After 24 h of incubation, colonies were counted and CFU per gram of lung tissue for each mouse were calculated.

Pulmonary fungal burden was also assessed by real-time polymerase chain reaction (PCR) using a previously described method [15]. The conidial equivalent (CE) of each sample was interpolated from a six-point standard curve generated by spiking uninfected mouse lungs with known amounts of conidia (10^2 to 10^7).

Statistical analysis

An independent samples *t*-test (SPSS 16.0) was used to evaluate the statistical difference in this study. Significance was defined as $p<0.05$. The Kaplan–Meier method was used to estimate the overall survival. The log-rank test was used for univariate comparisons.

Results

Preparation of short peptides of Asp f16 with potential immunogenicity

Previous studies using overlapping pentadecapeptides spanning the entire 427 aa sequence of the Asp f16 allergen have reported their ability to induce CTL responses [12]. In order to more specifically study the immunoactivity of Asp f16, an epitope-specific study was performed. To this end, we synthesized three potential HLA-A*0201-restricted T cell epitopes in Asp f16 with the help of bioinformatics. These peptides have a theoretically optimal size for binding to HLA-A*0201 molecules, and for processing and presentation by mature DC to prime-specific CD8+ T cell immunity. The results of the bioinformatic prediction of these three peptides are indicated in Table 1. A HLA-A*0201-binding 9-mer peptide from nucleophosmin was also prepared to serve as a control.

CD8+ T cell response to Asp f16 peptide-pulsed DC

First, we evaluated if the Asp f16 peptides we synthesized have the immunoactivity to induce a CD8+ T cell response. Bone marrow-derived DC were collected and treated with TNF-α. After treatment, flow cytometry was used to determine the expression of MHC class II antigens, CD80, CD86, and CD11c. As shown in Fig. 1a, b, TNF-α-treated DC showed a significant increase of these molecules, an

Table 1 Bioinformatic identification of HLA-restricted Asp f16 peptides

Peptides	Subsequence	BIMAS[a]	SYFPEITHI[b]
Asp1	ALWCSAPSL	177.308	26
Asp2	HLLGQLWLL	46.357	28
Asp3	YTAAALAAV	10.220	25

[a] BIMAS-predicted dissociation half-life of the peptide/HLA complex

[b] SYFPEITHI-predicted dissociation half-life of the peptide/HLA complex

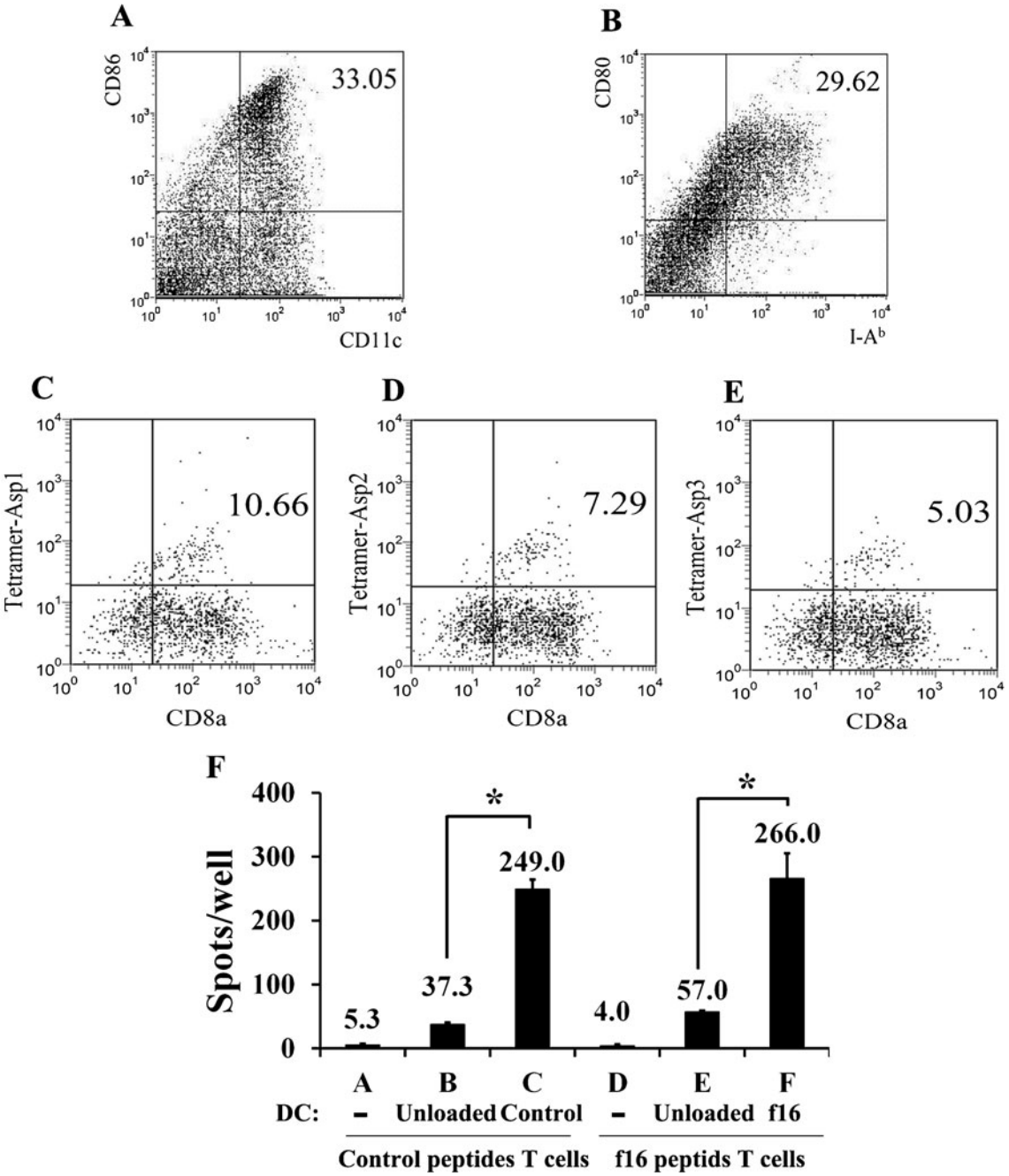

Fig. 1 Cytotoxic T lymphocyte (CTL) responses to peptides loaded dendritic cells (DC). **a**, **b** The maturation of DC derived from murine bone marrow was analyzed by flow cytometry using the antibodies as indicated. **c**–**e** Asp f16 peptides-specific tetramers were used to label the corresponding peptides (indicated in the figure)-specific CTL harvested from the peptides-immunized mice. **f** f16 CTL or control CTL were incubated with unloaded or the corresponding peptides loaded mature DC or not. Enzyme-linked immunosorbent spot (ELISPOT) assay was conducted to detect interferon-γ (IFN-γ) levels. Each value represents the mean ± standard deviation (SD) of triplicate wells performed in three individual experiments. *$p<0.05$, compared to the groups without DC or with peptides-unloaded DC

indication of maturation. The mature DC were then pulsed with Asp f16 peptides mixture or control peptide. The mature peptides-pulsed DC were subsequently used to prime CTL response.

We then assessed the potential of Asp f16 peptides to induce a T cell response. We prepared Asp f16 peptides-specific or control peptide-specific CTL from HLA-A*0201 mice by the injection of a mixture of the three peptides or control peptide. Tetramer assay was performed to test the proportion of f16 CTL. As shown in Fig. 1c–e, the frequency of the cells stained with the ASP1-, ASP2-, and ASP3-specific tetramers was 10.7, 7.3, and 5.0 %, respectively,

indicating that the CTL have high-affinity T cell receptors directed to HLA-A*0201-Asp f16 peptide complexes. Then, we performed an interferon-γ (IFN-γ) ELISPOT assay to determine the IFN-γ levels. As shown in Fig. 1f (columns A and D), f16 CTL and control CTL both displayed a negligible IFN-γ level under the unstimulated condition. Co-culture with the peptide-unloaded DC moderately increased IFN-γ production (columns B and E), indicating a peptide-nonspecific stimulating effect of DC; however, exposure to the Asp f16 peptides or control peptide-loaded DC significantly increased IFN-γ production (columns C and F) ($p<0.05$). The total number of IFN-γ spots from f16 CTL (266 spots/5×10^4 cells) was comparable to that from control CTL (249 spots/5×10^4 cells). The increased IFN-γ production suggests that Asp f16 peptides can induce CTL response as potently as the control peptide, thus, confirming the immunogenicity of the Asp f16 peptides.

In vitro damage of f16 CTL against *A. fumigatus*

In order to determine whether f16 CTL could exert an anti-*A. fumigatus* effect in vitro, we performed a hyphae damage assay to determine their cytotoxicity against *A. fumigatus* hyphae. *A. fumigatus* hyphae were treated with either f16 CTL or control CTL. As shown in Fig. 2a, f16 CTL treatment resulted in a significantly greater damage (81.5 %) when compared with a moderate non-specific damage induced by control CTL (25.9 %). This indicates the presence of an in vitro hyphal-damaging activity of f16 CTL.

We further investigated whether the culture supernatant of f16 CTL can kill *A. fumigatus* conidia. Accordingly, we performed a FUN-1 staining assay to test this question. In this assay, metabolically active conidia accumulate orange fluorescence in their vacuoles when incubated with FUN-1, a fluorescent stain widely used to stain metabolically active yeast and fungal cells, whereas dead conidia are stained green [12, 13]. As shown in Fig. 2b, c, FUN-1-stained live and dead *Aspergillus* conidia showed a bright orange and green colour, respectively. In contrast, upon treatment of the culture supernatant from f16 CTL, most of the conidia were stained yellow, indicating a loss of metabolic activity, thereby, suggesting the occurrence of damage (Fig. 2d). Some conidia after the above treatment were completely killed, as shown by a clear green staining. These data show that the supernatant can also inhibit the metabolic activity of *A. fumigatus* conidia, suggesting that f16 CTL might secrete some unidentified anti-*A. fumigatus* substances, such as cytokines.

To provide further evidence of damage caused by f16 CTL to *A. fumigatus*, we observed the morphological changes of *A. fumigatus* induced by CTL using SEM. *A. fumigatus* conidia cultured alone or together with control CTL displayed normal morphology with a smooth surface and geminated into long hyphae (Fig. 2e–f). In sharp contrast, co-culture with f16 CTL resulted in increased roughness, wrinkles, and cell leakage (Fig. 2g). In addition, the hyphae were also markedly damaged, with a rough and wrinkled surface. Together, these results provide evidence for the cytotoxicity of f16 CTL against *A. fumigatus* in vitro.

In vivo protection of f16 CTL against *A. fumigatus* infection

We then investigated if Asp f16 CTL can also exert an antifungal activity against *Aspergillus* in vivo. Accordingly,

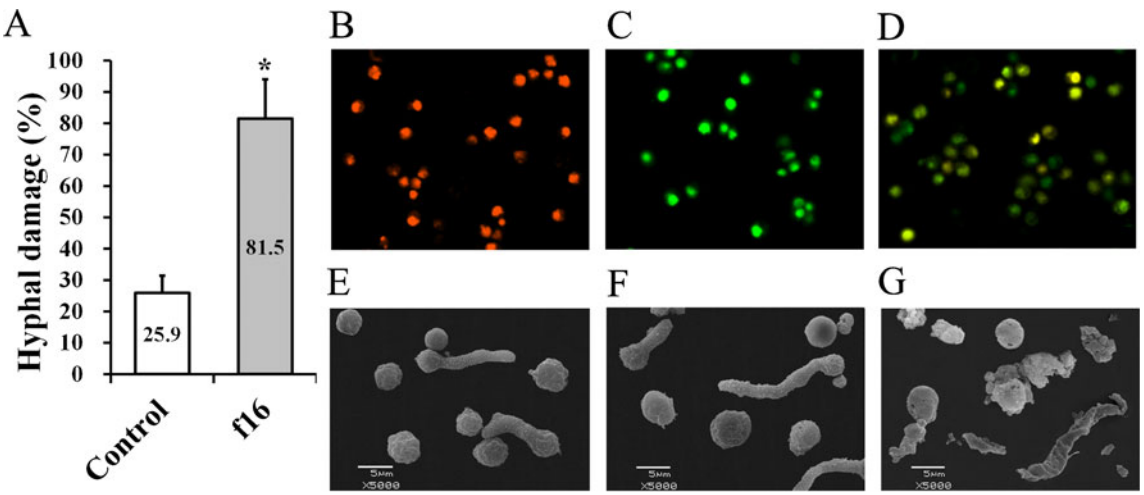

Fig. 2 Antifungal ability of Asp f16 peptides-specific CTL in vitro. **a** *Aspergillus fumigatus* hyphae were incubated with Asp f16 or control CTL and the XTT assay was performed to measure hyphae activity. The column values represent the hyphal damage percentage as described in "Materials and methods". Each value represents the mean ± standard deviation (SD) of data from three individual experiments. *$p<0.05$, compared to control CTL. **b**–**d** *A. fumigatus* conidia were cultured with f16 CTL (**d**) or boiled for 60 min (**c**) or not treated (**b**), and were then subjected to FUN-1 staining. **e**–**g** Scanning electron microscopy (SEM) was performed to observe the microstructure of *A. fumigatus* conidia that had been incubated alone (**e**) or together with control CTL (**f**) or f16 CTL (**g**) for 2 h at 37 °C

we established an invasive pulmonary aspergillosis model in HLA-A*0201 transgenic mice by cyclophosphamide treatment and intranasal administration of *A. fumigatus*. Obviously, the transfer of f16 CTL dramatically reduced the incidence of fungal infections and pulmonary architecture destruction when compared to the mice that received the control CTL or PBS (Fig. 3a). Pulmonary fungal burden was also determined by a CFU counting assay. As shown in Fig. 3b, PBS-treated and control CTL-treated mice showed a comparable CFU count (18.2×10^4/g and 16.8×10^4/g, respectively). In comparison, the mice which received f16 CTL developed significantly less colony formation (7.9×10^4/g, $p<0.05$). Similarly, when assayed by quantitative PCR (qPCR), the lung fungal burden of f16 CTL-treated mice was less severe than that of PBS or control CTL-treated mice (Fig. 3c). These results support an antifungal role of f16 CTL in pulmonary *A. fumigatus* infection.

Next, we analyzed the survival profile of the immunocompromised mice after the adoptive transfer of f16 CTL. Kaplan–Meier curves are shown in Fig. 3d. As shown in the

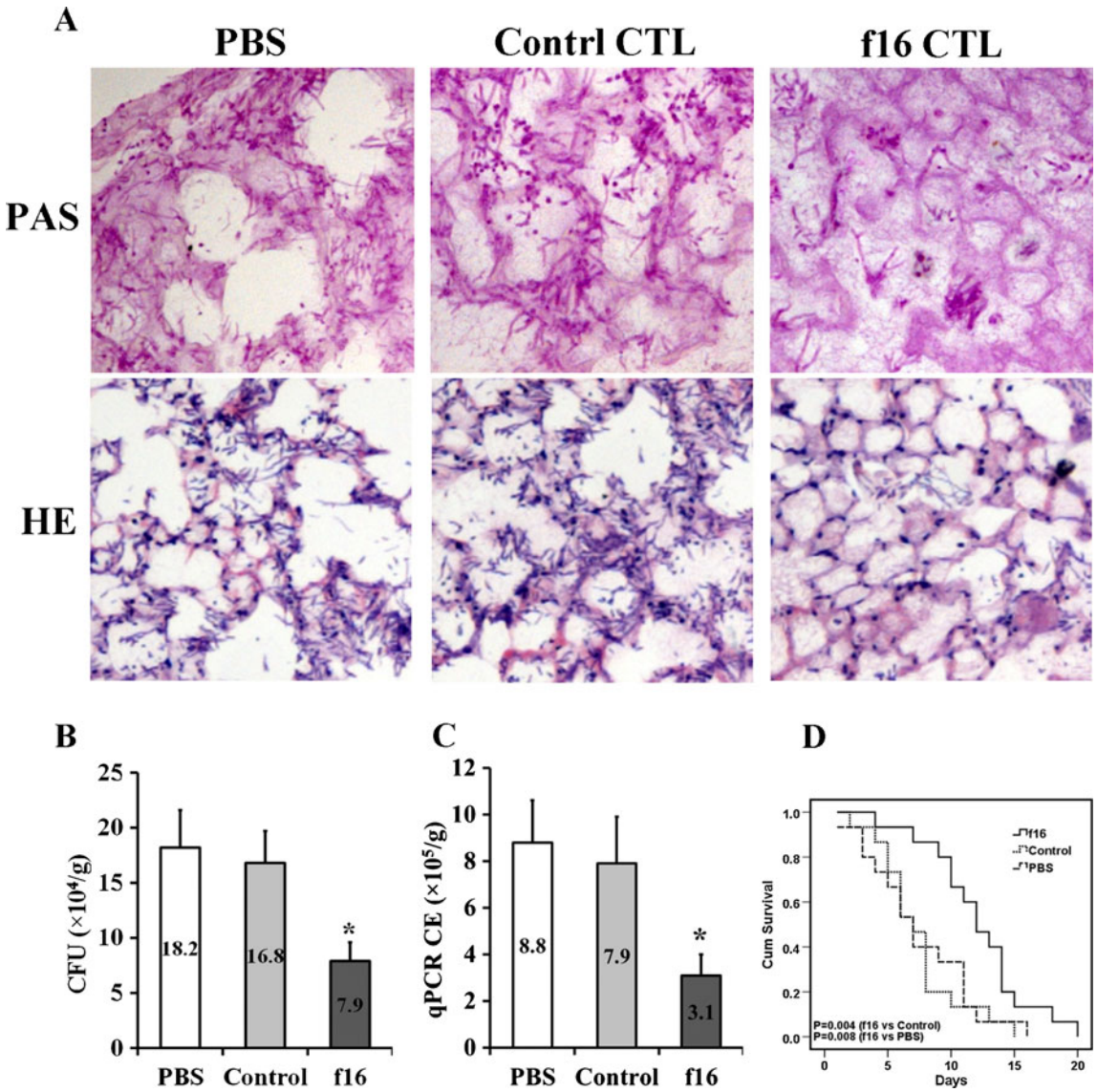

Fig. 3 Protection against *A. fumigatus* infection by the adoptive transfer of f16 CTL in immunocompromised HLA-A*0201 mice. **a** Cyclophosphamide-treated mice infected with *A. fumigatus* conidia were transferred with f16 CTL or control CTL or PBS one day after infection. Two days after CTL transfer, pulmonary fungal incidence was determined by Periodic acid-Schiff (PAS) and hematoxylin–eosin (HE) staining. Representative images are shown. Lung fungal burden was also determined by colony-forming units (CFU) (**b**) and quantitative polymerase chain reaction (qPCR) (**c**) at the same time as for PAS staining assay. The qPCR results were reported as conidia equivalent (CE). Each column represents the mean ± SD from five mice. *$p<0.05$, compared to mice transferred with PBS or control CTL. **d** Kaplan–Meier analysis of the overall survival of the mice (15 mice per group). The log-rank test was used to compare the survival time between groups

curves, the transfer of f16 CTL significantly altered the course of *A. fumigatus* infection. The median overall survival time of the mice transferred with f16 CTL was 12 days, much longer than that of the PBS-transferred (7 days, $p=0.008$) or control CTL-transferred mice (7 days, $p=0.004$). No difference in survival was observed between PBS and control CTL groups ($p>0.05$). All these in vivo data indicate that the transfer of f16 CTL can help protect against the infection of *A. fumigatus* in the immunocompromised mice model.

Discussion

A successful immunoreactive response requires not only normal T cell repertoire, but also efficient processing of an epitope from an entire protein sequence [16]. Accordingly, a novel but increasingly popular vaccination tactic is vaccination with exact MHC-binding peptides derived from the sequence of target antigens. The first peptide vaccine was a peptide encoded by lymphocytic choriomeningitis virus (LCMV) [17]. The peptide, when administered to mice, can induce a strong antiviral CTL response. A bundle of epitope vaccine studies have benefited from this inspiring approach [16, 18, 19]. In this study, following this strategy, we screened Asp f16 epitopes and selected three Asp f16 peptides that might bind to HLA-A*0201. Our results demonstrated that the peptides can elicit a robust CTL response when presented on DC. The activated CTL produced more IFN-γ, which suggests that the peptides can induce a Th1-type T cell-like response. This finding agrees well with other Asp f16 studies [11, 12].

In addition to direct cytotoxicity against *A. fumigatus* hyphae, our results also revealed that the supernatant of f16 CTL culture caused metabolic inhibition of conidia, which was also revealed by other studies [12, 13]. Since we did not observe an obvious anti-fungal activity of control CTL which can also secrete IFN-γ, we can conclude that f16 CTL mediates protective immunity against *A. fumigatus* in an IFN-γ independent manner, although a series of cytokines do enhance the killing of *A. fumigatus* [20]. A recent interesting report indicated that granulysin is required for CD8+ T cells-mediated anticryptococcal activity in a perforin-independent way [21]. Given the fact that the granulysin gene is not present in mice, we can rule out the possibility that the antifungal activity of f16 CTL observed in this study is granulysin-dependent. However, we cannot exclude the role of granulysin in Asp f16-induced immunity in human beings. Therefore, further studies should be carried out to confirm the potential role of granulysin in normal human donors. An alternative method is using a granulysin transgenic mouse model, which has been successfully established recently [22]. It should be noted that we did not observe obvious hyphae damage effects of the supernatant (data not shown). This observation was also reported by other studies [12]. This phenomenon suggests that the secreted fungal-killing molecules might target conidia but not hyphae.

Most of the previous *A. fumigatus* vaccine studies have considered adaptive immunotherapy as their ultimate goal. However, most cases of IA are nosocomially acquired. Indeed, lines of evidence revealed that hospital water and air are often contaminated with *A. fumigatus* [23]. This observation indicates that immunotherapy vaccination but not immunoprevention vaccination might be a more promising strategy. Here, our results, for the first time, provide an in vivo evidence for the protection of the Asp f16-specific CTL in pulmonary *A. fumigatus* infection. This agrees well with another recent study that showed an MHC class I-dependent protection of adoptively transferred CD8+ T cells against *Blastomyces dermatitidis* infection in C57BL/6 mice [24]. However, a previous study conducted by Bozza et al. reported that Asp f16 and other *A. fumigatus* allergens can induce resistance to infection only in the presence of adjuncts [11]. This discordance could be explained by the difference of antigen type and vaccination approach used. We chose short peptides of Asp f16-specific CTL as a vaccine, while Bozza et al. selected full-length recombinant Asp f16. Bozza et al. also used BALB/c (H-2d) rather than C57BL/6 mouse strain in their study. An earlier aspergillosis mouse model study demonstrated that the fungal inoculum required for 100 % mortality in C57BL/6 mice was fivefold less than that required in BALB/c mice [25]. In Bozza et al.'s study, 2×10^7 conidia were administered, which is far greater than the 6×10^6 conidia used in our study. This large disparity might be another reason why *A. fumigatus* allergens-induced immunity did not significantly improve the survival course of the infected mice in Bozza et al.'s study, as the "overloaded" fungi in BALB/c (H-2d) mice might depress or cause an inefficient immune response. In addition to Asp f16, Asp f3, another *A. fumigatus* peptide that has been shown to be immunogenic, was also shown to fail to induce resistance to infection alone in Bozza et al.'s report. Therefore, all these disagreements might arise from different experimental procedures.

In summary, we demonstrated in the present study that the epitope-derived Asp f16 peptides can confer CD8+ T cells protection against *A. fumigatus* infection. Our findings strongly support the feasibility of the bioinformatic approach for vaccine design against fungal infections. These peptides might serve as a vaccine against *A. fumigatus* infection in individuals at high risk of IA, particularly in human immunodeficiency virus (HIV)-infected hosts with progressive depletion of CD4+ T lymphocytes. Future studies should be carried out to further examine the effectiveness of these peptides as an anti-*A. fumigatus* vaccine. More importantly, since adoptive T cell transplantation can blindly

attack alloantigens of the recipient, thus, inducing graft-versus-host disease (GVHD), an evaluation of the safety of these peptides-specific CTL should not be ignored.

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Conflict of interest The authors declare that they have no conflict of interest.

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