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FULL PAPER

Dendritic cells transduced with an adenovirus vector encoding interleukin-12 are a potent vaccine for invasive pulmonary aspergillosis

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Invasive pulmonary aspergillosis (IPA) is a common and devastating pneumonia. We developed a novel antiinfective vaccine that couples the potent Ag-presenting capacity of dendritic cells (DCs) with paracrine delivery of interleukin-12 (IL-12) to local immune response sites. Our results showed that DCs engulfed Aspergillus conidia through coiling phagocytosis. Transfection of DCs with adenovirus encoding the cDNA of IL-12 did not affect their morphology and capacity to engulf conidia. The transduced DCs secreted IL-12, which was biologically active, to induce the production of gamma interferon (IFN-γ) from spleen cells. Adoptive transfer of DCs pulsed with heat-inactivated Aspergillus fumigatus (HAF) to naïve mice induced the Ag-specific production of IFN-γ; the transduced HAF-pulsed DCs augmented this immune response further. Animals receiving HAF-pulsed DCs had lower fungal burdens, a more than three-fold higher survival rate at day 3. This protection was associated with a pronounced enhancement in the Aspergillus-specific IFN-γ response. IL-12-engineered DCs augmented this protection strikingly as judged by a higher survival, and almost no Aspergillus could be detected in the lung of mice that had received IL-12-transduced HAF-pulsed DCs. These results suggest that antigen-pulsed DCs and IL-12 gene therapy could be used as adjunct therapy for aspergillosis.
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Introduction

Aspergillus fumigatus (*Af*) is a ubiquitous and opportunistic fungal pathogen of humans and animals. It is associated with a wide spectrum of diseases, ranging from benign colonization of the lung and allergy to life-threatening diseases such as invasive pulmonary aspergillosis (IPA).[1] IPA, characterized by hyphal invasion and destruction of pulmonary tissue, is the most common manifestation of *Aspergillus* infection and a leading cause of attributable mortality among immunocompromised patients. Although the release of fungal metabolites with immunosuppressive activity may contribute to the pathogenesis of aspergillosis,[2] local cellular defects are major predisposing factors of the host to IPA. Cell-mediated immunity plays a major role in host defense against *Aspergillus*. T helper (Th)1/Th2 dysregulation and a switch to a Th2-type immune response may contribute to a poor outcome of IPA. Resistance to infection was associated with TNF-α production,[3–5] interleukin-12 (IL-12) production and responsiveness, and the detection of interstitial lung lymphocytes producing IFN-γ.[3,6,7] In contrast, production of IL-4 and IL-10 by interstitial $CD4^+$ Th2 lymphocytes was associated with disease progression. Given its high incidence and mortality, new strategies for the prevention and treatment of IPA are urgently needed. As *Af* rarely causes diseases among immunocompetent hosts, improving anti-*Aspergillus* immunity of immunocompromised hosts may be an effective measure for the prevention and treatment of IPA.

Dendritic cells (DCs) exhibit the unique ability to stimulate both naïve and memory T-lymphocytes.[8] DCs can activate $CD4^+$ T-lymphocytes through cytokines secreted from them and costimulatory molecules on their surface. The clonal $CD4^+$ T cells then expand and regulate antigen-specific immune responses via two paths, Th1 or Th2 subsets.[9–11] These attributes make DCs ideal vehicles for the delivery of such immunoregulatory molecules. The cognate recognition of DCs and T cells provides the theoretic opportunity of these immunomodulatory molecules to influence the immune response in an Ag-specific manner. DCs were found to produce a large amount of IL-12 in both a CD40 ligand-dependent and -independent manners,[12,13] and have been successfully used to induce antigen-specific immune responses and protective immunity against infectious diseases. DCs are essential in the activation

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of Th1 response to the fungus. Immature myeloid DCs are unique among phagocytes, in that they phagocytosed not only conidia of *Aspergillus* but also hyphae.[14] Thus, DCs not only possess the ability to prime an antigen-specific response but also are able to direct a protective immune response against infections.

IL-12, a heterodimeric Th1-promoting cytokine consisting of two subunits of 35 and 40 kDa, which act as a key link between the innate and cell-mediated Ag-specific immune responses,[15] has been shown to have potent immunotherapeutic effects against a wide range of microbial pathogens.[16–18] This cytokine has pleiotropic effects, including activation of macrophages, augmentation of the cytolytic activity of NK and T cells and induction of the Th1-associated cytokines, notably IFN-γ production. Previous studies have confirmed that IL-12 augments oxidative antifungal activities of monocytes via an IFN-γ-independent route.[19] Gene transfer approaches have been explored in an effort to potentiate the adjuvant properties of DCs. Recombinant adenovirus (rAd) has been demonstrated to be one of the most effective vehicles to deliver foreign DNA into DCs.[20–23] It has been further demonstrated that genetically modified DCs, including adenovirus encoding the cDNA of IL-12 (AdmIL-12)-transduced DCs, can be successfully applied in infection immunotherapy.[20,21,24]

Although several studies have explored the *in vivo* efficacy of DC-mediated vaccination including microbial Ag-pulsed DCs in infectious disease settings[25] and efficacy of adoptive transfer of tumor Ag-pulsed DCs in cancer treatment,[26] to our knowledge, there are no studies that have evaluated the role of AdmIL-12-transduced DCs in aspergillosis. The focus of the present study is to assess a new paradigm in the development of a vaccine to protect against *Aspergillus* infection, using heat-inactivated *Af* (HAF)-pulsed genetically modified dendritic cells, DCs engineered to secrete IL-12, as the immunizing biologic agent. The data presented here demonstrate that murine bone marrow-derived DCs can engulf and damage *Aspergillus* conidia. DCs engineered to secrete IL-12 pulsed with *Af* administered to syngeneic mice lead to induction of a T-cell proliferative response, Ag-specific Th1 response *in vivo*. It was effective in reducing the fungal burden, prolonging survival following a lethal intrapulmonary challenge with *Af*.

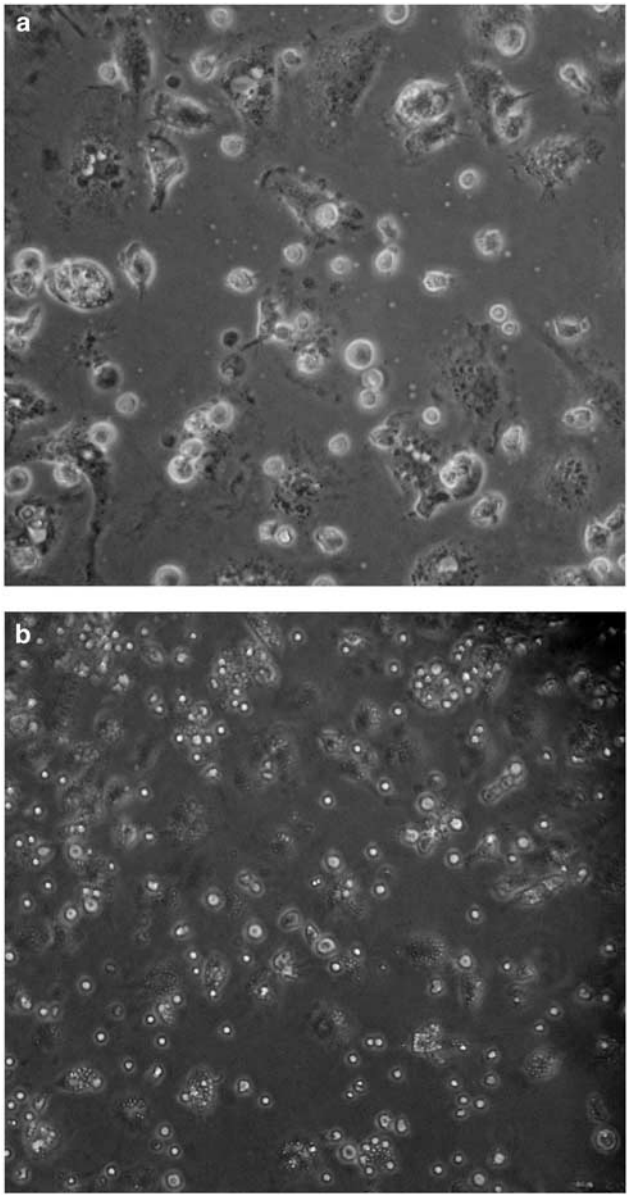

Figure 1 DCs cultured for 5 days display DCs morphology. Bone marrow cells cultured with GM-CSF and IL-4 for 4 days were cocultured with HAF for 24 h and then visualized by light microscopy (magnification ×400). (**a**) Cells cultured in medium alone. (**b**) Cells cultured with HAF for 24 h.

Results

Phagocytosis of *Af* by DCs

The DCs incubated with *Aspergillus* for 24 h were examined by electron microscopy as described in the Materials and methods. Using both light and scanning electron microscopy (SEM), as shown in Figures 1 and 2, a typical DCs morphology with characteristic veils, multiply elongated cytoplasmic processes, a kidney-shaped nucleus or lobulated nuclei and a diameter of 15–20 μm were observed. Some *Aspergillus* conidia were internalized into DCs predominately by coiling phagocytosis. Under transmission electron microscopy, as shown in Figure 2, conidia were observed within membrane-bound phagosomes 24 h after the infection, characterized by the presence of overlapping bilateral pseudopods, which led to a pseudopodal stack before transforming into phagosome wall (Figure 2e and f). Multivesicular organelles containing cell membrane-damaged bacteria were observed in some DCs (Figure 2f). The fungi were frequently found in multilaminar and multivesicular vacuoles (Figure 2e). DCs engineered to secrete IL-12 (DCs-IL-12) had no change in morphology. The ability to engulf *Af* conidia had not changed as the concentration of HAF in the supernatants of DCs-IL-12 ($2.37 \pm 0.185 \times 10^6$) is similar to that of DCs ($2.59 \pm 0.164 \times 10^6$) ($P > 0.05$). To analyze whether incubation with *Aspergillus* leads to activation of the DCs, the surface expression of the costimulatory molecules relevant to the interaction of DCs with T cells (CD40, CD80 and CD86) was evaluated. The results showed that DCs incubated with HAF demonstrated an enhanced expression of the costimulatory molecules CD40, CD86 and CD80 (Figure 3). Transfection with IL-12 or control vector

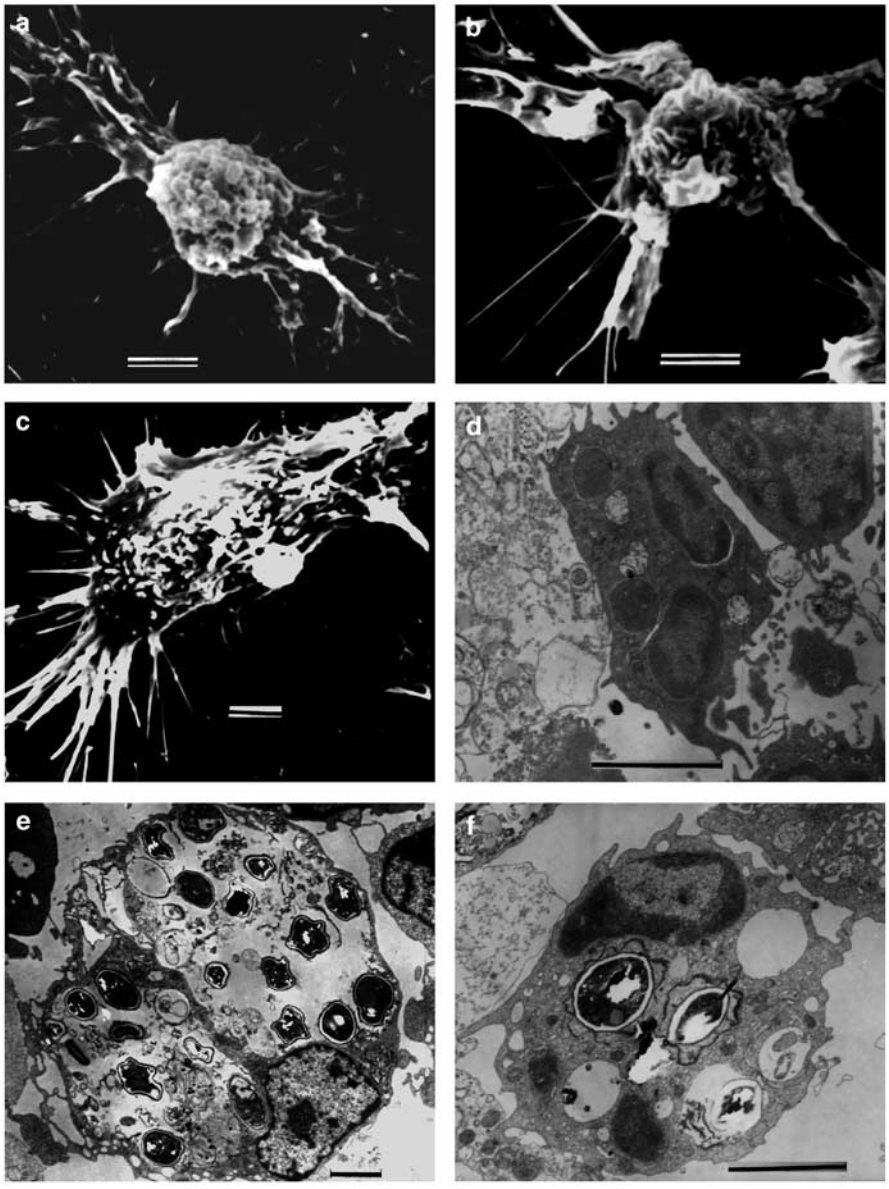

Figure 2 DCs morphology, phagocytosis of *Af* conidia by DCs using SEM (**a**–**c**, bar = 4 μm) and TEM (**d**–**f**, bar = 2 μm). (**a**, **d**) DCs cultured in medium only for 5 days. (**b** and **e**) DCs of 4-day culture were cocultured with HAF at a 1:10 DC:*Af* ratio for 24 h. (**c**, **f**) IL-12 gene-modified DCs were pulsed with HAF for 24 h. Mouse bone marrow-derived DCs cultured for 5 days displayed typical DCs morphology: many long and thin (**a**), either sheet-like (**b**) or spiny (**c**) processes, kidney-shaped or lobulated nuclei (**d**–**f**). DCs cultured with HAF showed that a number of *Af* were internalized within DCs, some multivesicular vacuoles contained a large number of *Af*. Membrane-bound phogosomes containing *Af* were found too. Some DCs illustrated some membrane-damaged conidia in multivesicular phagocytic vacuoles (**f**).

did not affect the expression level of cell surface molecules except CD86. The expression of CD86 was significantly higher in the AdmIL-12-transduced DCs (Figure 3). These findings suggest that *Af* induces the maturation of DCs, and DCs take up heat-inactivated *Aspergillus* conidia through coiling phagocytosis. Transfection of DCs with AdmIL-12 had no effect on their ability to engulf conidia.

Engineering DCs to constitutively produce biologically active IL-12

Transfection of DCs with the AdmIL-12 resulted in high levels of IL-12 gene expression. However, there was no expression of IL-12 gene in the empty control vector group, as determined by RT-PCR and IL-12 ELISA. The results obtained by RT-PCR assays are shown in Figure 4. DCs transduced with AdmIL-12 expressed mRNAs for cytokine IL-12, while mRNA for IL-12 could not be detected in AdNull-transduced DCs (Figure 4). IL-12 was also detected in the supernatants from DCs-IL-12 by ELISA assay, with a mean level of 1110 pg from 10^6 cells in 24 h (Figure 5a), significantly higher than that detected from nontransduced murine DCs ($P < 0.01$). Pulsing AdmIL-12-transduced DCs with HAF did not alter their ability to produce high levels of IL-12 (Figure 5a). Even after pulsing with HAF, the amount of IL-12 produced by nontransduced DCs never reached the level produced by AdmIL-12-transduced DCs. AdNull transduction cannot enhance the ability of DCs to produce IL-12, but HAF-pulsed DCs secreted more IL-12 than that by no-pulsed

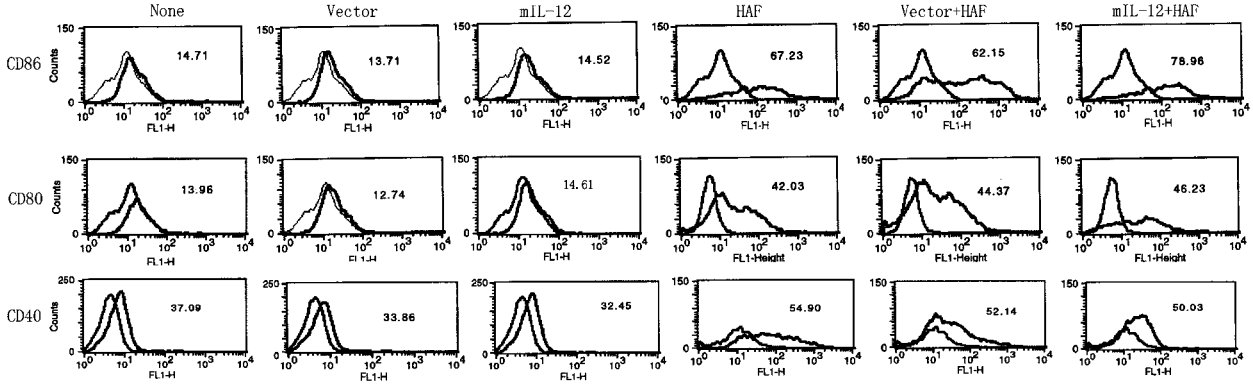

Figure 3 Phenotype of HAF-pulsed and/or AdmIL-12-transduced DCs. DCs were transfected with AdmIL-12 (DCs-IL-12) or AdNull (DCs-Vector) for 24 h and incubated or not with HAF at 10 CFU/cell for 24 h and then CD40, CD86 and CD80 costimulatory molecules expression was assessed by immunofluorescence analysis with anti-B7.2-PE (CD86), anti-B7.1-PE (CD80) and anti-CD40-PE. Analysis was performed on a FACScan.

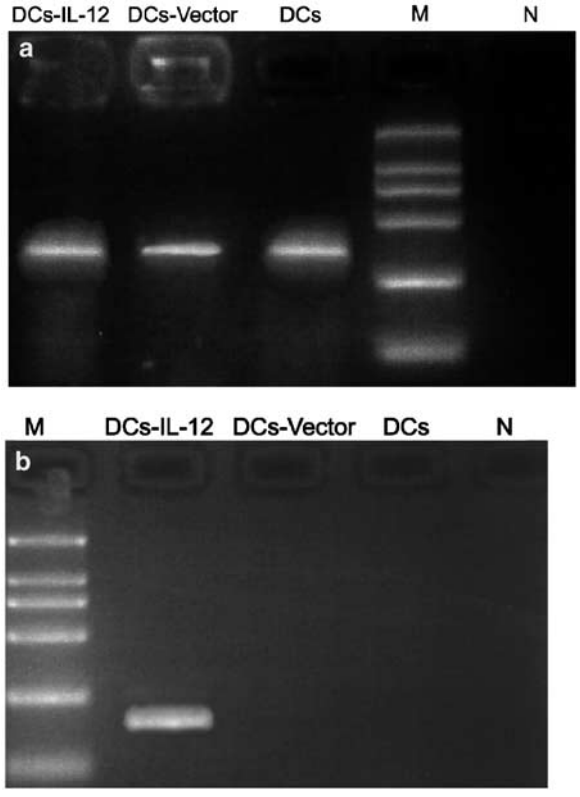

Figure 4 Expression of mRNA transcripts for β-actin- (**a**) and mIL-12- (**b**) in AdmIL-12-transduced DCs. Lanes 1–3 depict the results obtained with cellular RNA obtained from DCs transduced with the AdmIL-12 construct, DCs transduced with the AdNull and nontransduced DCs, respectively. Lanes N: no DNA was added to the amplification mixture during PCR. M, marker: DL2000.

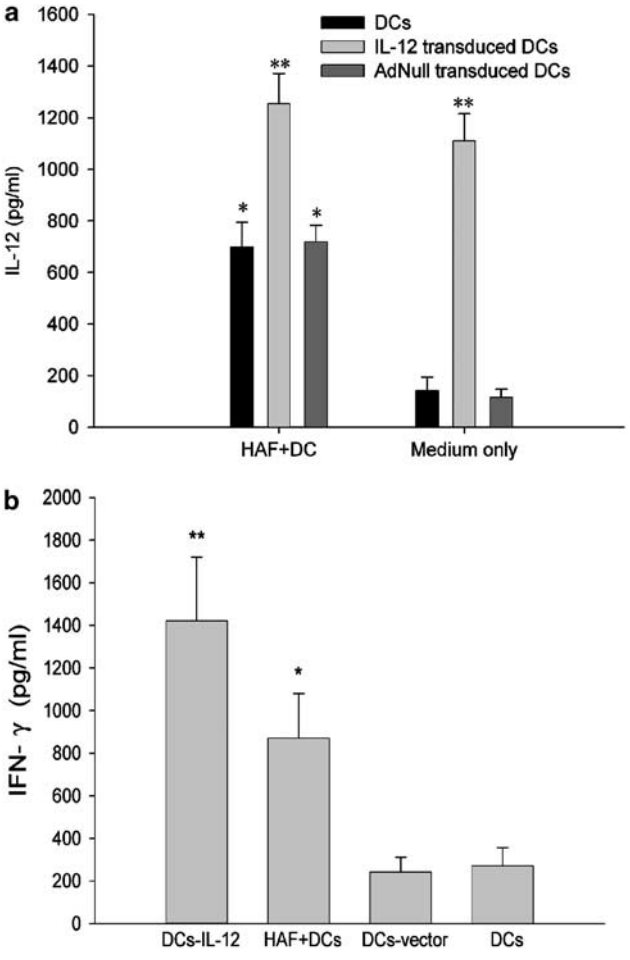

Figure 5 Secretion of bioactive IL-12 from HAF-pulsed and/or AdmIL-12-transduced DCs (DCs-IL-12). Supernatants were collected from *in vitro* cultures of 10^6 DCs-IL-12 with (HAF + DC) or without (medium only) HAF stimulation for 24 h as described in Materials and methods. Nontransduced and AdNull-transduced DCs (DCs-Vector) were taken as negative controls. The supernatants were assayed for IL-12 by ELISA (**a**) and for bioactive IL-12 (**b**) as measured by the induction of IFN-γ production in spleen cells (2×10^6) from normal BALB/c mice. Results are representative of those obtained in at least two separate experiments. Statistical significance: $^*P < 0.01$ compared with non AdmIL-12-transduced DCs groups without stimulation; $^{**}P < 0.01$ compared with non-AdmIL-12-transduced DCs groups.

ones (Figure 5a). The secreted IL-12 was bioactive, as evidenced by the induction of 1421 pg of IFN-γ in resting spleen cells from the supernatants of AdmIL-12-transduced DCs (Figure 5b).

Aspergillus-specific T-cell proliferation following *in vivo* immunization with HAF-pulsed and/or AdmIL-12-transduced DCs

To analyze whether DCs pulsed with *Aspergillus* were capable of eliciting an *Aspergillus*-specific immune

response or not, T cells harvested from spleens of mice 7 days after immunization with DCs were coincubated with *Aspergillus*-pulsed, mitomycin-treated syngeneic DCs. Spleen-derived T cells from mice immunized with HAF-pulsed DCs showed increased proliferation compared to T cells from naïve mice or mice that had received naïve DCs (Figure 6). However, the T cells of all groups incubated with syngeneic unstimulated DCs showed no proliferation. When T cells derived from mice immunized with DCs pulsed with HAF were exposed to mitomycin-treated, LPS-pulsed DCs or T cells derived from mice immunized with DCs pulsed with LPS were exposed to mitomycin-treated, HAF-pulsed DCs, no levels of proliferation were shown either. Transduction of DCs with AdmIL-12 augumented the increase of T-cell proliferation (Figure 6). These results demonstrate that immunization with HAF-pulsed DCs leads to induction of a T-cell proliferative response against DCs presenting *Aspergillus* antigen(s), and transduction of DCs with AdmIL-12 enhanced the induction.

DCs pulsed *ex vivo* with HAF and/or transduced with AdmIL-12 induced a Th1 cell-dominated response

In mice with IPA, resistance to infection correlates with predominant Th1 response, while Th2 responses are associated with susceptibility to the infection.[3] So, we further studied whether HAF-pulsed DCs immunization can selectively induce a Th1-like, IFN-γ-producing response or not. The cytokines produced by spleen T cells from mice immunized with DCs were measured. T cells from HAF-pulsed DC-immunized mice produced higher levels of IFN-γ upon *in vitro* restimulation with *Aspergillus* antigens, compared with T cells from naïve spleen cells (Figure 7a). Although a high level of IFN-γ

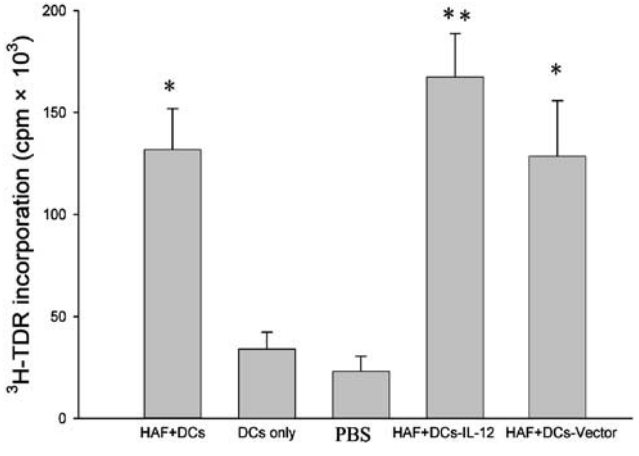

Figure 6 Levels of T-cell proliferation in mice immunized with DCs pulsed with HAF (HAF + DCs), DCs only, PBS, AdmIL-12-transduced DCs (DCs-IL-12), AdNull-transduced DCs (DCs-Vector). BALB/c mice were injected i.v. with 5×10^6 DCs previously pulsed *in vitro* with HAF two times at a weekly interval. At 1 week following the second immunization, T cells were isolated from the spleens and coincubated for 48 h with mitomycin-treated DCs previously pulsed with HAF for 24 h. T-cell proliferation was evaluated by [^{3}H]thymidine incorporation during incubation for 14 h. Experiments were performed in triplicate, and counts per minute (c.p.m.) are expressed as the mean and standard deviation. Statistical significance: *$P < 0.05$ compared with nonpulsed DCs groups; **$P < 0.01$ compared with other respective groups.

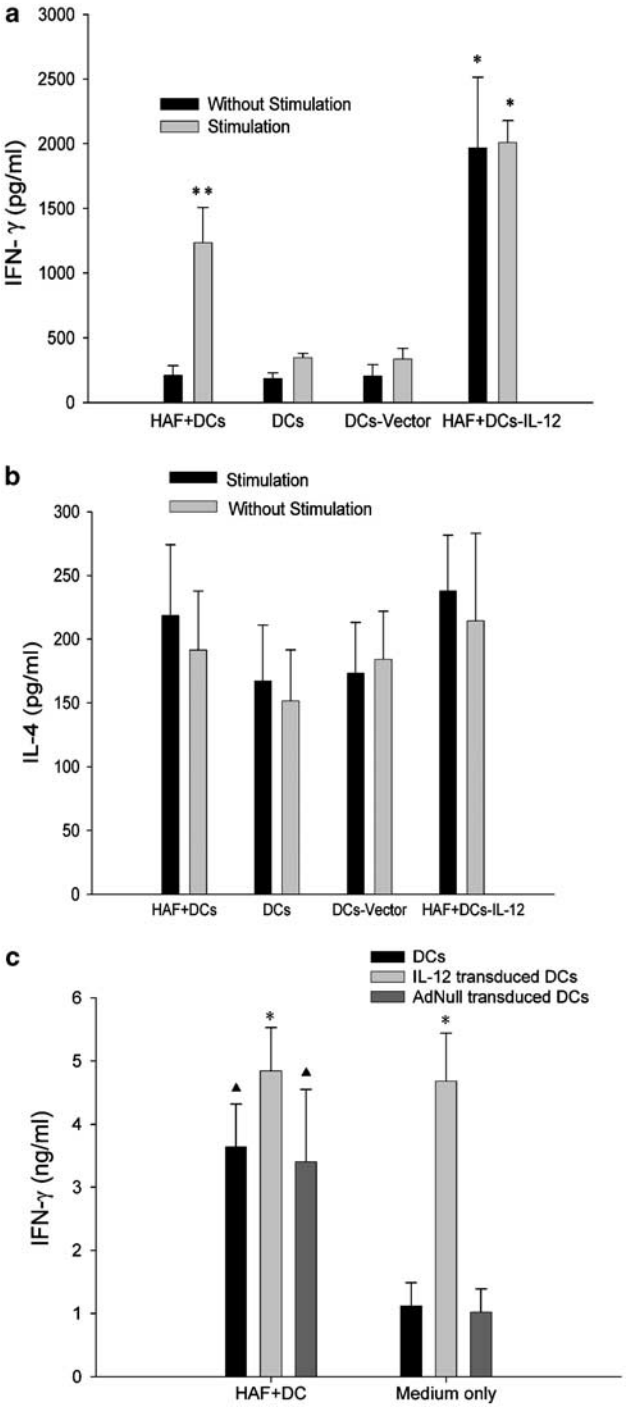

Figure 7 Cytokine responses following adoptive transfer of HAF-pulsed and/or AdmIL-12-transduced DCs. The *ex vivo* Ag-specific IFN-γ and IL-4 production in splenocytes was determined 7 days following administration of adoptive transfer of DCs twice, HAF-pulsed DCs, vector-transduced DCs, IL-12-transduced and/or HAF-pulsed DCs at a weekly interval. Splenocytes were harvested from individual mice ($n = 4$), and spontaneous IFN-γ **(a)** and IL-4 (**b**) productions (mean ± s.e.m.) were determined in the supernatants of splenocyte cultures 24 h later. At 1 week after the second administration of DCs, *Af* was i.n. administered to mice. After 3 days, IFN-γ levels in homogenates of lung tissues from mice were measured (**c**). The data shown are representative of two experiments. Statistical significance: *$P < 0.01$ compared with non-AdmIL-12-transduced DCs groups; **$P < 0.01$ compared with other non-AdmIL-12-transduced DCs groups. ▲$P < 0.01$ compared with non-Ag-pulsed and non-AdmIL-12-transduced DCs groups.

was detected in supernatants of spleen T cells from mice immunized with HAF-pulsed DCs without restimulation, unpulsed DCs or PBS did not induce an Ag-specific IFN-γ response (Figure 7a). In an additional control, spontaneous production or an Ag-induced IFN-γ response was not detected in T cells derived from mice received intravenously (i.v.) HAF alone. In contrast, there was no difference in the level of IL-4 produced by T cells from different group mice.

As IL-12 is a key factor in the initiation of cell-mediated immunity that links innate immunity with Ag-specific adaptive immune responses, we also determined whether delivery of IL-12 via DCs would enhance these Ag-specific responses. The spontaneous release of IFN-γ from spleen T cells derived from mice that received AdmIL-12-transduced DCs was higher than that of nontransduced DCs (Figure 7a). In lung homogenates, a similar trend was also observed. As shown in Figure 7b, the mean IFN-γ level in lung homogenates from mice treated with the AdmIL-12-transduced DCs was 4680 pg/ml, compared to 1020 and 1120 pg/ml in lung tissue from mice treated with the AdNull-transduced DCs or nontransduced DCs, respectively ($P<0.01$).

Together, these observations clearly suggest that DCs pulsed *ex vivo* with HAF can induce an *Aspergillus*-antigen-specific and Th1 cell-dominated response *in vivo*, and this response was augmented significantly by DCs that had been transduced *in vitro* with AdmIL-12.

Bone marrow-derived DCs pulsed *ex vivo* with HAF and/or transduced with AdmIL-12 induced protection against pulmonary *Aspergillus* infection

HAF-pulsed and/or AdmIL-12-transduced DCs can induce a Th1 cell-dominated response *in vivo* to *Aspergillus*, while Th1 cytokines play a central role in controlling of *Aspergillus* infection.[3] So we explored the ability of antigen-pulsed DCs to induce protective immune responses against pulmonary *Aspergillus* infection in a mouse model. The protection was evaluated by monitoring mouse mortality on a daily basis, and measuring the concentration of infectious *Aspergillus* organism recovered from lung tissues of mouse. All groups of mice had tachypnea, piloerection and prostration 12 h after treatment with cyclophosphamide and aggravated after infection. Mice immunized with HAF-pulsed and/or IL-12-transduced DCs progressively recovered over 2–3 days postinfection, whereas a profound hypodynamic state and respiratory failure developed in other groups. Most of the mice receiving naïve DCs died within 96 h following intrapulmonary challenge with a lethal dose of *Af* (Figure 8a). However, the animals that had previously been i.v. immunized with DCs pulsed with HAF demonstrated prolonged survival time (Figure 8a). Animals who had received HAF without DCs did not survive longer than 96 h following the challenge with *Af*. This observation suggests that immunization with DCs pulsed with HAF greatly improve the overall resistance of the animals to the infection challenge by *Aspergillus*.

To evaluate whether this overall improvement in resistance lies in the reduced severity of *Aspergillus* infection through DCs immunization or not, we further measured the infectious *Aspergillus* organisms recovered from lung tissues of mouse. It can be seen from colony-forming unit (CFU) contents shown in Figure 8b that mice immunized with DCs pulsed with HAF indeed had a significantly lower concentration of infectious *Aspergillus* organisms in their lung tissues than mice treated with DCs or PBS alone. More importantly, immunization with HAF alone was not protective either. These observations together suggest that the DCs pulsed *ex vivo* with HAF can induce a protective response against *Af* infection.

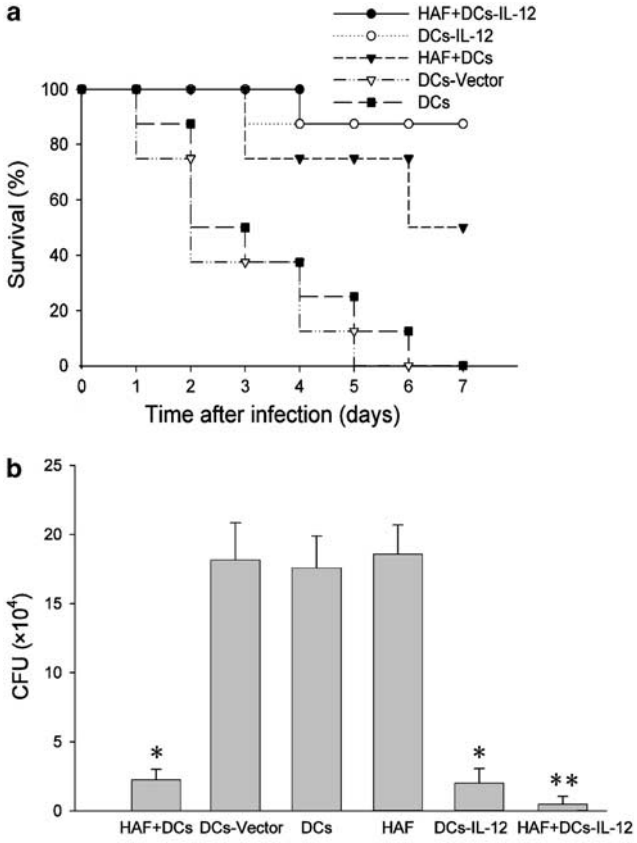

Figure 8 Immunization with HAF-pulsed and/or IL-12-transduced DCs partially protects against subsequent infection with a lethal dose of *Aspergillus* pulmonary infection. Naïve or transduced DCs were incubated with HAF for 24 h, washed and then readministered intravenously to syngeneic mice two times at a weekly interval. After 1 week, *Af* was administered to the animal i.n. and survival was evaluated over time ($n=8$ for all group animals) (**a**). After 3 days after the second time of *Aspergillus* infection challenge, mice were killed for measurement of CFU in lung homogenate. The experiment was performed twice ($n=4$ animals per group) (**b**). Statistical significance: *$P<0.001$ compared to AdNull-transduced DCs, DCs and HAF treated mice; **$P<0.01$ compared to other groups.

Our *in vivo* studies showed that HAF-pulsed, AdmIL-12-transduced DCs enhanced significantly the Ag-specific increases in IFN-γ level in noninfected mice. Therefore, we determined whether the protective effect of HAF-pulsed DCs could be enhanced further by DCs engineered to secrete high levels of IL-12. Almost no *Aspergillus* could be detected in the lung of mice that had received two vaccinations with HAF-pulsed, AdmIL-12-transduced DCs, whereas many *Aspergillus* could be detected in the lung of mice vaccinated with HAF-pulsed DCs and vector-transduced DCs (Figure 8b). A higher survival rate was also found in mice immunized with

HAF-pulsed, AdmIL-12-transduced DCs than that in mice vaccinated with HAF-pulsed DCs, vector-transduced DCs (Figure 8a).

In order to characterize the host response to *Af* in mice that received different DCs, lungs were examined histologically at various time points after intranasal (i.n.) challenge with *Aspergillus*. Lung sections from HAF-treated mice, naïve DCs and AdNull-transduced DCs treated mice (Figure 9c and d) revealed patterns of lesions similar to those observed in untreated mice, characterized by signs of bronchial wall damage, peribronchial necrosis and the presence of numerous infiltrating inflammatory cells (predominantly polymorphonuclear) within both the interstitial and alveolar compartments. Conidia were seen in mice on days 1 and 2, but large numbers of hyphae were shown in these mice on days 3 and 5 after inoculation, which indicated the development of invasive disease. In contrast, these features were not observed in HAF-pulsed and/or IL-12-transduced DC-treated mice whose lungs were characterized by few inflammatory cells, infiltration, and no evidence of parenchymal destruction or fungal growth on day 3 after inoculation (Figure 9a and b).

We also found that mice given HAF-pulsed and/or AdmIL-12-transduced DCs showed marked splenomegaly, with an about two-fold increase in size compared to spleens from mice treated with the vector-transduced DCs. This finding is consistent with a recent report by Kim *et al*[27] that mice given an IL-12 cDNA expression vector showed a level of splenomegaly that was comparable to that observed in mice given recombinant IL-12.

Discussion

IPA is a common and devastating complication of immunosuppression. Host innate immunity is the principal pathway to clear *Af* from the lung. The first and major lines of defense against inhaled *Af* conidia are the pulmonary macrophage and the neutrophil.[1] Cyclophosphamide, an agent in common clinical use as a chemotherapeutic and immunosuppressive agent, can induce a dose-dependent depletion of circulating neutrophils, alterations in the number and function of circulating and pulmonary monocyte/macrophages and various lymphocyte populations. In our model, animals pretreated with cyclophosphamide before inoculation with *Aspergillus* conidia developed an acute, severe pneumonia that histologically resembled IPA becoming complicated in severly immunosuppressed patients.

DC play major roles in host defense against various pathogens as they behave as both sentinel for innate immune recognition and initiator for protective immunity.[28] Cytokines produced by DCs in response to various microorganisms stimulation are key players in Th cell differentiation: IL-12 or IFN-γ ensues differentiation toward a Th1 type, while IL-4 or IL-10 toward Th2 type.[28,29] IL-12 and IFN-γ were found to be important in controlling fungal infection and promoting Th1-type immune response too.[3,30–32] In close agreement with our study, Bozza *et al*[33] found that DCs can engulf *Aspergillus* conidia through coiling phagocytosis. Respiratory tract DCs can internalize and then transport conidia and hyphae of *Af* from the airways to the draining lymph nodes and spleens. Furthermore, some conidia were

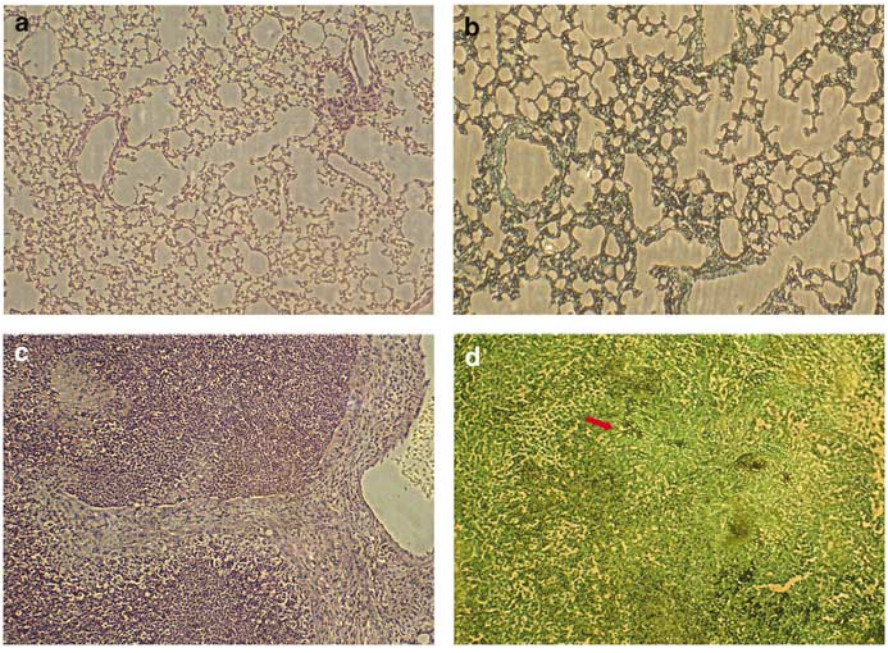

Figure 9 Representative photomicrographs of hematoxylin and eosin (**a** and **c**)- and Gomori methanamine silver (GMS) (**b** and **d**)-stained whole-lung sections from BALB/c mice. These mice were treated with AdmIL-12-transduced, HAF-pulsed DCs (**a**), HAF-pulsed DCs (**b**), AdNull-transduced DCs (**c**) and DCs only (**d**) (5×10^6 cells/mouse) two times at a weekly interval and infected i.n. with *Aspergillus* conidia 4 days after being immunosuppressed with cyclophosphamide. Lungs were taken 3 days after infection (magnification, $\times 200$). (**a** and **b**) A little inflammatory cell infiltration occurred in peribronchial areas, corresponding to the site of conidial deposition. Hyphal forms were not present. In contrast, the sections from the lungs of AdNull-transduced DCs-treated mice (**c**) revealed patterns of lesions similar to those observed in DCs-treated mice (**d**), characterized by signs of bronchial wall damage, peribronchial necrosis and peribronchial inflammatory cell infiltration with branching hyphae (arrow) present.

found being damaged inside DCs in our study. However, no evidence of conidia destruction was observed in Bozza's study. Perhaps, the reason lies in that DCs were incubated with conidia for 24 h in our study instead of 3 h in theirs, and live conidia that release fungal metabolites with immunosuppressive activity were used in their study but HAF in our study. DCs ingest fungi, then secrete IL-12 and initiate fungus-specific immune response. So it is a better strategy to choose DCs as a vaccine against *Aspergillus* infection, which was confirmed in our study.

Recent studies have indicated that *in vitro* antigen-pulsed DCs induce antigen-specific immunity.[34–37] Antigen-pulsed mature DCs express genes that induce DCs migration, activation and recruitment of T cells,[37] and different antigens phagocytosed by DCs induce different patterns of cytokine production by $CD4^+$ T cells, depending on the cytokines produced by the DCs.[34] The availability of DC-derived IL-12 is a requirement for the development of protective immunity.[38,39] So in our study, the efficacy of HAF-pulsed and/or AdmIL-12-transduced DCs for controlling aspergillosis *in vivo* was examined. Firstly, our results showed that HAF-pulsed DCs could induce an *Aspergillus* Ag-specific Th1 response *in vivo*; this immune response can bear the function for protection against *Aspergillus* infection. Furthermore, adoptive transfer of DCs engineered to secrete high levels of bioactive form of IL-12 enhanced the antiinfective immune responses through augmentation of the induction of an *Aspergillus* Ag-specific Th1 response. These results significantly complement and extend the results of previous studies that explored DC-based vaccination strategies for other infectious diseases.[18,20,24,40–42]

Previous work *in vivo* suggested that the mode of delivery of DCs is important in eliciting T-cell responses. DCs given i.v. localized in the reticuloendothelial system and lung, whereas delivery into skin leads to homing of DCs preferentially to draining lymph nodes.[43,44] Kuribayashi *et al*[45] found that the Th1/Th2 cytokine profiles induced by subcutaneous (s.c.) administration of Ag-pulsed DCs were different from those induced by DCs administered i.v. For this reason, we choose to vaccinate mice via the i.v. route. It showed that i.v. injection of DCs induced a dominant Th1 phenotype and that this phenotype is associated with a reduction in fungal burden. However, Bacci *et al*[46] found that Th1 cell activation was observed after s.c. but not i.v. administration of DCs.

In the present study, the whole *Aspergillus* conidia were used to pulse DCs and induced a much stronger protection against *Af* infection. DCs pulsed with conidia activated antigen-specific, IFN-γ-producing T-lymphocytes *in vitro* and *in vivo* on adoptive transfer in mice susceptible to aspergillosis. As the genomes of intracellular microbes are very large, a broad repertoire of microbial Ags is required to induce a potent protective immune response *in vivo*. So the task of identifying protective Ags is arduous. A variety of allergens of *Aspergillus* have been cloned. Clinical and experimental evidence indicated that the different allergens can elicit different immunological reactions.[47] Thus, administration of DCs pulsed with unfractionated pools of microbial Ags to induce a protective polyclonal T-cell response directed against identified Ags should be a better choice. In many studies, DCs were pulsed *ex vivo* with either peptide or whole-protein antigens and delivered *in vivo* to syngeneic hosts to induce a protection correlated with a strong, antigen-specific T-cell response.[34–36,46] In accordance with our study, DCs pulsed with live fungi or transfected with fungal RNA underwent functional maturation, as revealed by the upregulated expression of histocompatibility class II antigen and costimulatory molecules and the production of IL-12 in response to conidia or conidial RNA and of IL-4/IL-10 in response to hyphae or hyphal RNA.[48]

As a key link between the innate and cell-mediated Ag-specific immune responses,[11] IL-12 can induce IFN-γ production from NK cells and T-lymphocytes, and enhances proliferation of Ag-specific T cells. There is clear evidence that IFN-γ production, IL-12 production and IL-12 binding all play critical roles in the control of intracellular infections in humans.[24] In murine studies, recombinant IL-12 has been shown to augment host resistance to several pathogens, including fungus.[22,49] In our data, DCs pulsed with HAF secreted more bioactive IL-12. On adoptive transfer in mice, they activated antigen-specific, IFN-γ-producing T-lymphocytes.

Gene transfer techniques have been used for over a decade to deliver high-level expression of cytokines and other gene products within tumor microenvironment.[50] It has been further demonstrated that genetically modified DCs can be successfully applied in tumor immunotherapy and control of some infection.[20,24,46,51,52] Consistent with previous studies,[22,24] in our study, transfection of DCs with AdmIL-12 did not affect their morphology and ability to phagocytose canidia. Most face molecule expression except CD86, which did not change, suggesting the gene modification has little effect on DCs maturation. Our result showed that it is effective to transduce DCs with adenovirus as vector; about more than 90% DCs were transfected at an MOI of 100 at 24 h. IL-12 gene therapy is a promising approach for inducing efficient immune responses against *Af* infection. Jiang *et al*[20] have demonstrated the feasibility of IL-12 gene therapy for the treatment of coccidioidomycosis by retrovirally transduced J774 cells.

The present study demonstrated that genetic engineering of HAF-pulsed DCs to express IL-12 efficiently induced antigen-relevant cellular immune responses with MHC class II-restricted and type 1-dominant $CD4^+$ T-cell help, which resulted in protection of immunized mice against lethal *Af* infection. Administrated *in vivo* for immunization, DCs modified with AdmIL-12 and pulsed with HAF most likely migrate to lymphoid organs and present processed protein antigens of fungal origin on MHC class II molecules. Engineering DCs to secrete IL-12 probably enhanced the rate of encounter of antigen-presenting DCs and antigen-specific $CD4^+$ T cells, thus more efficiently amplifying the relevant antigen-specific immune responses.[40]

In summary, DC-based antiinfective vaccines and therapies comprise a novel approach to a serious disease. *In vitro* antigen-pulsed DCs induce antigen-specific immunity. Autocrine production of IL-12 greatly enhances the ability of DCs to induce primary immune response. Our findings offer encouragement and additional rationale for the development of a DC-based vaccine against *Aspergillus* and perhaps other infectious diseases.

Materials and methods

Mice

Specific pathogen-free BALB/c mice, 8–10 weeks old, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and were bred under specific pathogen-free conditions in the animal facility of the Chinese Academy of Science. Procedures involving animals and their care were conducted in conformity with national and international laws and policies. Immunocompromised mice were made by treatment with 200 mg/kg of cyclophosphamide (Sigma, St Louis, MO, USA), administered intraperitoneally (i.p.) 4 days before i.n. *Af* inoculation. The treatment resulted in peripheral blood neutropenia (absolute neutrophil count, <200 cells/μl) by days 4 and 6 after treatment, with a return of peripheral counts to pretreatment levels by day 10.

Microorganism, culture conditions and infection

The strain of *Af* was obtained from a fatal case of pulmonary aspergillosis at the Infectious Diseases Institute of Huashan Hospital, Fudan University, Shanghai, China. The microorganism was cultured on Sabouraud dextrose agar (Becton Dickinson, Cockeysville, MD, USA) supplemented with chloramphenicol for 4 days at room temperature. The surface of each plate was then washed with 15 ml of sterile 0.1% Tween-80 (Sigma Ultra, St Louis, MO, USA) in normal saline. The resulting suspension was filtered through sterile gauze to remove clumps and hyphal debris, and then washed once and resuspended in 4 ml of 0.1% Tween-80. After extensive washing with saline, the conidia were counted using a particle counter and diluted to the desired concentrations and the concentration was measured again prior to administration. In preliminary experiments, the number of particles determined by the particle counter was in close agreement with the number of viable CFU found by serial dilution and plating of the suspension. The viability of the conidia was $>95\%$, as determined by serially diluting and plating on Sabouraud dextrose agar. HAF was obtained by heating the conidia in a water bath at 100°C for 1 h, washing three times in sterile phosphate-buffered saline (PBS). The efficiency of this killing treatment was verified by the failure of the HAF to grow when cultured for 7 days in Sabouraud dextrose agar. These procedures followed those described in a previous experiment.[53] For i.n. infection, immunosuppressed mice were lightly anesthetized with inhaled diethyl ether before instillation. Then, they were given a daily suspension of 2×10^7 conidia in 20 μl of sterile saline for 2 consecutive days. The suspension was applied to the nostrils slowly by micropipette with a sterile disposable tip. Animals were held in an upright position until the suspension was completely inhaled and normal breathing resumed. Mice succumbing to fungal challenge were routinely necropsied for histopathologic confirmation of IPA. For lung histological analysis, whole lungs were fully inflated with 4% paraformaldehyde and dissected and placed in fresh paraformaldehyde for 24 h. Routine histological techniques were used to paraffin embed the entire lung, and 5 mm sections of the whole lung were stained with hematoxylin and eosin (H&E), and Gomori methanamine silver (GMS).

Adenovirus vectors

The plasmid pLISN containing murine IL-12 (mIL-12) p35 and p40 subunits in a mode of pL-p35-IRES-p40-SN were generously provided by Jinfa Gu (Laboratory of Biotechnology, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). The cDNA of mIL-12 was cut out from pLISN by *Eco*RI and subcloned into pCA13 (pCA13-IL-12). A standard replication-deficient adenovirus containing the cDNA (AdmIL-12) was constructed by standard homologous recombination techniques using the plasmid pCA13-IL-12 and the adenovirus packaging plasmid pBHGE3 (adenovirus packaging plasmid, Microbix Biosystems, Ontario, Canada) in HEK293 cells (human embryonic kidney cells containing the E1A region of adenovirus, Microbix Inc., Ontario, Canada). pCA13-IL-12 (1 μg) was mixed with 0.5 μg pBHGE3 and then cotransfected into HEK293 cells using Effectene Transfection Reagent (Qiagen, Germany). rAd was isolated from a single plaque and expanded in HEK293 cells. The virus was plaque purified, propagated on HEK293 cells and purified by CsCl gradient according to standard techniques. Particle titers of all adenovirus were determined by absorbance measurements at 260 nm, and functional PFU titers were determined by plaque assay on HEK293 cells. The control AdNull vector is structurally similar but contains no transgene. In preliminary experiments using a genetically engineered, identical vector engineered to express green fluorescent protein (Ad-EGFP),[54] we determined that $\geqslant 90\%$ of DCs were transduced at an MOI of 100 at 24 h. All vectors used in this study were free of replication-competent adenovirus.

Generation of bone marrow-derived DCs

A modified procedure described by Inaba *et al*[55] was followed for the generation of bone marrow-derived DCs from mice. Briefly, mouse bone marrow was collected from both tibias and femurs. Erythrocytes were lysed using 0.83% (w/v) ammonium chloride. The cells were plated in six-well culture plates in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS (Gibco Brl), 100 μg/ml streptomycin, 100 U/ml penicillin, 2-mercaptoethanol, MEM sodium pyruvate, L-glutamine and nonessential amino acids, and recombinant murine cytokines (R & D Systems, Minneapolis, MN, USA) GM-CSF (20 ng/ml) and IL-4 (2 ng/ml) at 37°C in 5% CO_2 in air for 5–7 days. At day 2 of culture, floating cells were gently removed and fresh medium was added. Thereafter, the medium was changed with a fresh one every 2 days and cell differentiation was monitored using inverse light microscopy. From days 5 to 7 of culture, nonadherent cells and loosely adherent proliferating DCs aggregates were collected. The DCs fraction contained 80 to 90% DCs based on the culture characteristics, morphology and $CD11c^+$ phenotype. For gene transfection, at day 4 of culture, DCs were genetically modified with AdmIL-12, using AdNull and PBS as controls, at an MOI of 100 for 24 h, washed, and then cultured for 24 h at 37°C or pulsed with HAF as described below.

Ex vivo pulse of DCs with *Aspergillus*

At day 5 of culture, bone marrow-derived DCs were collected and pulsed *ex vivo* with HAF at a 1:10 (DC:*Af*) ratio in the RPMI-1640 culture medium described above.

The cell concentration was kept at 10^6/ml, and the antigen pulse was carried out at 37°C for 24 h. DCs were removed via low speed centrifugation. Concentrations of HAF in the supernatants were tested using a particle counter to value the ability of DCs to engulf *Af*. As a control, 200 ng/ml of LPS (*Escherichia coli* 0111:B4, Detroit, MI, USA) was added to DCs culture for 24 h. The supernatants were collected too, stored at −70°C, and tested for IL-12 at a later time using sandwich ELISA. The cells were washed to remove free *Af* or LPS and resuspended in sterile saline, and then transfered into mice.

Electron microscopy

Infected or uninfected DCs (total of $5–10 \times 10^6$) were washed with PBS and fixed with cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate/1% sucrose buffer (pH 7.2) for 4 h. The cells were postfixed with 1% osmium tetroxide (50 min), encapsulated in 1% agar, stained with uranyl acetate and phosphotungstic acid and dehydrated in a series of graded ethanolic solutions finishing with propylene oxide. Finally, it was embedded in Epon (Agar Scientific, Essex, UK). Thin sections (55 nm) were cut on an ultramicrotome and mounted on 200 carbon-coated grids, contrasted with ethanolic uranyl acetate and lead citrate. Observations were carried out with a JEM 1200 EX transmission electron microscope (JEOL, Japan).

For SEM, the DCs were collected and washed after being cocultured with HAF for 24 h. Then, cells were very gently plated onto poly-L-lysine (Sigma)-coated glass coverslips and incubated for 30 min. Cells were prefixed in 2% glutaraldehyde/PBS for 20 min, washed three times, and postfixed for 25 min in 1% osmium tetroxide/PBS. Cells were dehydrated through graded ethanols, critical point dried in CO_2, and gold coated by sputtering. Visualization was performed using a scanning electron microscope S-450 (Hitachi, Japan) at 15 kV.

Flow cytometry analysis

Cell surface markers of the bone marrow-derived DCs were analyzed by antibody staining and flow cytometry. All antibodies were purchased from PharMingen. The following antibodies conjugated with fluorochromes were used: red-phycoerythrin (PE)-labeled anti-mouse CD80 (B7-1), anti-mouse CD86 (B7-2), anti-mouse CD40 and anti-mouse CD11c. After the cell samples were incubated with the appropriate antibodies for 30 to 60 min on ice, the stained cells were analyzed by using a FACScalibur equipped with CellQuest software (BD Bioscience, San Jose, CA, USA). Propidium iodide staining and scatter gating were used to exclude dead cells and debris.

Immunization with HAF-pulsed and/or AdmIL-12-transduced DCs

To evaluate whether HAF-pulsed and/or AdmIL-12-transduced DCs would lead to protective responses against intrapulmonary *Aspergillus* infection, DCs were washed three times with sterile PBS. DCs in 0.3 ml of sterile saline were administered via the lateral tail vein to syngeneic mice (5×10^6 cells/mouse) two times at a weekly interval. Mice receiving saline, naïve DCs, LPS-pulsed DCs i.v. were used as controls. After 7 days, the animals were challenged by an i.n. injection of *Aspergillus*, as described above.

T-cell proliferation following transfer of HAF-pulsed and/or AdmIL-12-transduced DCs

To analyze whether the transfer of DCs pulsed with *Af* would lead to *Aspergillus*-specific proliferation of T cells *in vivo*, the proliferative responses of T cells derived from spleens of mice that were primed with antigen (mice injected with DCs pulsed with HAF) or not primed with antigen (unstimulated mice or mice injected with naïve DC) were compared to those of *Aspergillus*-pulsed, mitomycin-treated DCs. *Aspergillus*-pulsed DCs (2×10^5) were treated with 50 μg/ml of mitomycin C at 37°C for 30 min, washed twice with RPMI and seeded in triplicate in flat-bottom 96-well culture plates for use as stimulator cells. For responder spleen cells, 5×10^6 DCs were injected i.v. into syngeneic mice two times at a weekly interval. At 7 days after the second injection, the spleens were harvested and T cells were isolated by passing the cell suspension through a small, loose, nylon wool plug, and lymphocytes were enriched over a single-step Ficoll gradient. The cells with a final concentration of 2×10^6 cells/well were being cocultured in 96-well plates with *Aspergillus*-pulsed, mitomycin-treated DCs (2×10^5 cell/well) in a total volume of 200 μl of the RPMI-1640, and cultured in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 h, followed by a 14 h pulse with 1 μCi/well of [^{3}H]thymidine, and then collected onto glass fiber filters. Wells containing mitomycin-treated DCs alone and T cells alone were included as controls. [^{3}H]thymidine incorporation was quantified using a Beckman scintillation counter (LS6500, USA).

ELISA and *in vitro* spleen responses

Spleen cells (2×10^6/ml) were cultured in medium alone or stimulated with HAF at an MOI of 10 in RPMI-1640 culture medium described above. Supernatants were harvested at 24 h for analysis of cytokine concentrations by a sandwich ELISA (R&D Systems).

Lung harvest

At designated time points, the mice were killed by CO_2 asphyxiation. The chest cavity was opened aseptically, and the pulmonary vasculature was perfused with PBS via the right ventricle. For histologic examination, lungs were perfused with 1 ml 4% paraformaldehyde in PBS, and then excised *en bloc*. After removal, whole lungs were homogenized in 1.0 ml of PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, ID, USA) by using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA) under a vented hood. Portions of homogenates (10 μl) were inoculated on Sabouraud dextrose agar after serial 1:10 dilutions with PBS or 0.9 N saline (NS) to determine the number of CFU. The homogenates of the lungs were also cultured on chocolate agar plates and blood agar plates to rule out superinfection. The remaining homogenates were incubated on ice for 30 min and then centrifuged at 1400 *g* for 10 min. Supernatants were collected and stored at −70°C for assessment of cytokine levels.

RNA preparation and reverse transcription–polymerase chain reaction

Reverse transcription PCR (RT-PCR) analysis was carried out following Sisto's description.[56] Briefly, total RNA was isolated from 5×10^7 DCs in Trizol reagent (Life Technologies Inc., Grand Island, New York, USA) by

using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA) in accordance with the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed into cDNA using Moloney murine leukemia virus RT (Life Technologies, Gaithersburg, MD, USA). The cDNA was then amplified using specific primers for IL-12 (giving a 220-bp product).[56] Amplifications were performed in 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each deoxynucleotide triphosphate, 1 μM of each primer and 2.5 U of AmpliTaq polymerase (Promega). The β-actin primers were used as controls for the PCR reactions (sense: 5′-CCA GCC ATG TAC GTT GCT ATC CAG-3′; antisense: 5′-GGA ACC GCT CAT TGC CAA TGG TGA-3′, giving a 378-bp product). After an initial incubation at 95°C for 2 min, temperature cycling was begun. The cycles consisted of 20 s of denaturation at 94°C, 30 s of primer annealing at 55°C and 30 s of primer extension at 72°C for 30 cycles. PCR was performed in an automated DNA Thermal Cycler (Perkin-Elmer Cetus). PCR conditions were strictly defined for each cytokine primer pair such that a linear relationship between the input RNA and the final PCR product was obtained. As a control for calibration, an equivalent amount of input cDNA for β-actin was amplified. A negative control was included in each assay to confirm that only cDNA PCR products were detected and that none of the reagents was contaminated with cDNA of previous PCR products. The PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with 0.5 mg/ml ethidium bromide and visualized using a UV transilluminator. The amplifications were repeated at least twice with two separately prepared cDNA samples for each mouse.

Cytokine assays

The levels of IL-12p70, IFN-γ and IL-4 in lung homogenates and culture supernatants of activated cells were determined by a cytokine-specific ELISA kit (R & D Systems, Minneapolis, MN, USA), using pairs of anti-cytokine mAbs as recommended by the manufacturer. The sensitivity range for IFN-γ and IL-12 was 16–1000 pg/ml, and for IL-4 was 15.625–1000 pg/ml. Quantification was made against a standard curve obtained for individual cytokine standards provided by the manufacturer. Samples were correspondingly diluted to obtain values within the linear range of the standards.

The bioactivity of the secreted IL-12 was assayed by measuring its ability to induce IFN-γ production by spleen cells from naïve mice. Spleen cells were obtained from normal BALB/c mice as described above. The spleen cell suspension was treated with isotonic ammonium chloride to lyse erythrocytes. After being washed by centrifugation, the splenocytes were resuspended in RPMI-1640 containing 10% FCS. The cells were dispensed into wells on a microtiter plate at a concentration of 2×10^6 mononuclear cells per well. The cell cultures were incubated in medium alone or in medium containing a 1:10 dilution of the supernatant from different DCs. After a 48 h incubation at 37°C under 5% CO_2, supernatants were collected for assays of IFN-γ protein by a sandwich ELISA (R&D Systems).

Statistics

All data were expressed as means ± s.e. Analysis was performed by SPSS statistical software 10.0. Student's *t*-test was used to determine the statistical significance of values between experimental groups (significance was defined as $P < 0.05$). *In vivo* groups consisted of five to eight animals. The data reported were pooled from at least three experiments.

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