

ORIGINAL ARTICLE

Aspergillus fumigatus regulates mite allergen-pulsed dendritic cells in the development of asthma

S. Fukahori, H. Matsuse, T. Tsuchida, T. Kawano, S. Tomari, C. Fukushima and S. Kohno

Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan

Clinical & Experimental Allergy

Summary

Background The role in allergic asthma development of the immune response against fungi with concomitant exposure to other common aeroallergens has yet to be determined. In particular, there is little understanding of how inhaled fungi affect the host response to mite allergens.

Objective To characterize the *in vitro* and *in vivo* effects of concurrent exposure of *Aspergillus fumigatus* (Af) and *Dermatophagoides farinae* (Derf) on dendritic cells (DCs) in the development of allergic asthma.

Methods Murine bone marrow-derived DCs were pulsed with Derf and/or live or heat-inactivated Af. Cytokine production and the expression of pathogen recognition receptors (PRRs) were determined *in vitro*. Subsequently, these DCs were inoculated into the airway of naïve mice to assess the development of allergic airway inflammation *in vivo*. The effect of antibodies against PRRs was also evaluated.

Results Live Af significantly enhanced IL-10 production and the expression of Toll-like receptor (TLR) 2 and Dectin-1 in Derf-pulsed DCs. Live Af infection significantly attenuated Derf-pulsed DC-induced allergic airway inflammation *in vivo*. Antibodies against either TLR2 or Dectin-1 significantly reversed the inhibitory effects of live Af in the development of Derf-pulsed DC-induced allergic airway inflammation.

Conclusion Concurrent exposure of DCs to fungal antigens has profound influences on the subsequent mite allergen-induced allergic airway inflammation. Live Af could regulate the functions of airway DCs in the development of mite allergen-induced allergic airway inflammation via regulation of their PRRs.

Our results suggest that concurrent exposure to pathogens such as fungi and mite allergens has profound influences on the subsequent allergen-induced allergic airway inflammation. Furthermore, modulating PRR signalling could provide a therapeutic regimen for the development of asthma.

Keywords allergy, dendritic cells, fungal, lung, tolerance

Submitted 6 July 2009; revised 21 March 2010; accepted 22 March 2010

Correspondence:

H. Matsuse, Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
E-mail: hmatsuse@nagasaki-u.ac.jp

Introduction

Respiratory tract infections and mite allergen sensitization represent important risk factors in the development of asthma [1]. In addition, respiratory tract infections are the most frequent cause of asthma exacerbation in both children and adults [2]. Similar to viruses and atypical pathogens, *Aspergillus fumigatus* (Af), an ubiquitous fungus, is considered to be an important risk factor in the development and exacerbation of asthma [3, 4]. Af causes invasive aspergillosis in immunocompromised hosts [5]

and allergic bronchopulmonary aspergillosis in patients with asthma [4, 6]. In an immunocompetent mouse model, respiratory exposure to Af spores resulted in the recruitment of IFN- γ -producing, fungus-specific T-helper type 1 (Th1) CD4⁺ T cells to airways [7, 8], whereas in the asthma mouse model, respiratory exposure to Af spores resulted in the recruitment of Th2 CD4⁺ T cells [9–11]. However, the factors that determine when CD4⁺ T cells are activated in response to Af exposure and whether the responding T cells will be biased to a Th1 or Th2 phenotype remain to be determined. The other unanswered question in

understanding the role of fungal infection in the development of asthma is what effect immunological interaction between different aeroallergens has during asthma development. People are continuously inhaling aeroallergens from the atmosphere and atopic subjects are generally sensitized with multiple allergens. It is likely that atopic subjects are first sensitized to aeroallergens, such as mites, *in utero* or in early life, causing an establishment of allergic airway inflammation, and are then exposed to another allergen, such as *Af*. There is little understanding of how *Af* inhalation affects the host response to mite allergens.

Dendritic cells (DCs) are known to play an important role in primary allergic sensitization to aeroallergens [12, 13]. DCs possess the ability to uptake antigen, present to and activate, naïve Th cells thereby initiating adaptive immune responses in the respiratory mucosa [14–16]. As pulmonary DCs occupy such a key position in the immune response, there has been much interest in the mechanism by which they interact with aeroallergens such as *Af* conidia. Several families of pathogen recognition receptors (PRRs) expressed on DCs including the Toll-like receptors (TLRs) [17–22], C-type lectins (e.g. Dectins) [23, 24] and pentraxins [25, 26] are involved in the innate immune response to *Af* and differentially regulate the type of adaptive immune response [27–31].

Based on these findings, we hypothesized that concomitant exposure of *Af* with mite allergen might regulate DC function in mite allergen sensitization via regulation of PRR expression. To test this hypothesis, we pulsed murine bone marrow-derived DCs with mite allergen and/or live or heat-inactivated *Af* spores, and determined the expression of PRRs and cytokine production *in vitro*. Subsequently, these DCs were inoculated into the airways of naïve mice, and their *in vivo* capacity to initiate allergic airway inflammation was compared.

Materials and methods

Animals

Female BALB/c mice (5- and 10-week-old) were purchased from Charles River (Yokohama, Japan) and housed in a specific pathogen-free facility. Animal procedures were reviewed and approved by the Nagasaki University School of Medicine committee on animal research. All experiments were repeated at least three times.

Aspergillus fumigatus

Af MF-13, isolated from the sputum of a patient with pulmonary aspergilloma, was subcultured on Sabourand dextrose agar (Becton Dickinson, Cockeysville, MD, USA) at 30 °C for 7 days. The conidia were then harvested with sterile saline containing 0.02% Tween 80 (Wako Pure

Chemical Industries, Tokyo, Japan). The suspension was filtered through a 40 µm pore-size cell strainer (Millipore, Molsheim, France) to separate conidia from the contaminating mycelium and verified microscopically (100% resting conidia). The suspension was then counted in a haemocytometer and diluted with sterile saline. For the preparation of heat-inactivated *Af* (HI *Af*), conidial suspensions were autoclaved at 121 °C for 30 min.

Preparation of dendritic cells

Murine bone marrow-derived myeloid DCs (mDCs) were generated from the femurs and tibiae of female 10-week-old BALB/c mice by culturing the marrow for 10 days with recombinant murine GM-CSF (R&D Systems, Oxford, UK) as described previously [32, 33]. Six groups of mDCs namely, medium-pulsed control DC (Control-DC), *Derf*-pulsed DC (Df-DC), live *Af*-infected DC (Live *Af*-DC), HI *Af*-infected DC (HI *Af*-DC), *Derf*-pulsed plus live *Af*-infected DC (Live *Af*-Df-DC) and *Derf*-pulsed plus HI *Af*-infected DC (HI *Af*-Df-DC), were prepared as follows: On Day 9 of culture, 0.1 mg/mL of *Derf* allergen (Cosmo Bio, Tokyo, Japan) was added to the culture medium of Df-DC, Live *Af*-Df-DC and HI *Af*-Df-DC. Concomitantly, either live *Af* or HI *Af* was inoculated into the culture medium of Live *Af*-DC and Live *Af*-Df-DC or HI *Af*-DC and HI *Af*-Df-DC, respectively. Co-culture with DC and *Af* conidia was performed as described previously [34]. Briefly, DC and *Af* conidia were co-cultured at a density of 2×10^6 each per dish. This ratio was determined from our preliminary experiments in which a higher ratio of *Af* to DC significantly reduced the viability of DC and a lower ratio of *Af* to DC failed to develop an immunological response. Amphotericin B (0.5 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) was further added to the cell cultures of Live *Af*-DC and Live *Af*-Df-DC 6 h after *Af* inoculation to prevent fungal overgrowth and death of the cultured cells. Also, it is known that Amphotericin B has no indirect effect on DC maturation or function [34]. For all experiments, incubation was conducted in a humidified 5% CO₂ atmosphere at 37 °C. On Day 10, the culture supernatants were assayed for cytokine levels (IL-12, IL-10 and IL-23) using an ELISA (Endogen, Woburn, MA, USA) and the methods described by the manufacturer. Total RNA was also isolated from each group of DC with TRIzol (Life Technologies, Gaithersburg, MD, USA) using the method recommended by the supplier.

Analysis of the expression of Toll-like receptors 2 and 4 and Dectin-1 on dendritic cells

The expression of TLR2, TLR4 and Dectin-1 on mDC was determined by real-time RT-PCR and flow cytometry. A High-Capacity cDNA Archive kit (Applied Biosystems, Tokyo, Japan.) was used to synthesize cDNA from 2 µg of

total RNA, and 200 ng of cDNA was amplified with primers complementary to the published sequences of murine TLR2, TLR4, Dectin-1 and control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative real-time PCR was performed using an ABI 7500 (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems). Probes (IDT) labelled with 5' FAM (6-carboxyfluorescein) and 3' TAMRA (6-carboxytetramethylrhodamine) were used at a final concentration of 0.9 mM, and primers were used at 0.2 mM (GIBCO BRL, Grand Island, NY, USA). The PCR programme was as follows: 50 °C for 2 min and 95 °C for 10 min, then 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Specific signals were normalized to the signal from the constitutively expressed GAPDH. For flow cytometric analysis, DCs were stained with FITC- and PE-conjugated monoclonal antibodies (mAbs). DCs were analysed for the expression of TLR2, TLR4 and Dectin-1 using a FACScan II flow cytometer (Becton-Dickinson) as described previously [35]. The following mAbs were used for immunofluorescent staining: FITC-anti-mouse Dectin-1 (2A11; Cell Science, Canton, MA, USA), FITC-anti-mouse TLR2 (eBioscience Inc., San Diego, CA, USA), FITC-anti-mouse TLR4 (eBioscience Inc.) and PE-anti-mouse CD11c (BD Pharmingen, San Diego, CA, USA). The cells were stained with mAbs against CD11c and TLR2, TLR4 or Dectin-1 for 30 min at 4 °C (1:100). Staining with the appropriate isotype control mAb (eBioscience Inc.) was also performed. In each case, 10 000 cells were acquired and analysed using Cell Quest (Becton-Dickinson). The mean fluorescent index (MFI) of the control antibody was subtracted from the MFI obtained for each antibody used. The results are expressed as the delta MFI of TLR2, TLR4 and Dectin-1 on CD11c-positive cells and the data represent the mean of at least three values $\pm$ SEM.

Immunization with Ag-pulsed dendritic cells and secondary exposure to Ag

On Day 11, 1×10^5 cells/50 μ L of each group (Control-DC, Df-DC, Live Af-DC, HI Af-DC, Live Af-Df-DC and HI Af-Df-DC) were intranasally instilled into a second group of naïve BALB/c mice. Thereafter, these mice were intranasally challenged with 50 μ g/50 μ L of *Derf* once a day for five consecutive days (Days 11–15). Mice were lightly anaesthetized using diethyl ether (Wako, Osaka, Japan) during the intranasal instillation of the DC and *Derf* challenge. Mice were killed 24 h after the final *Derf* challenge, and lung tissues were obtained for either histopathological examination or mononuclear cell (MNC) isolation.

Bronchoalveolar lavage examination

Bronchoalveolar lavage (BAL) was conducted in the immediate post-mortem period. Differential cell counts

were performed on cytocentrifuged BAL stained with May-Grunwald-Giemsa.

Histopathological examination

Paraformaldehyde-PBS fixative (4%) was gently infused through the lavage catheter. Resected lungs were fixed for an additional 24 h, embedded in paraffin and sliced into 4 μ m sections. Staining was performed using haematoxylin and eosin and periodic acid-Schiff.

Assay of lung mononuclear cell cytokines

Lung MNCs were prepared from the six groups of mice as described previously [36]. MNCs (2×10^5 /200 μ L/well) were cultured in the absence or presence of 100 mg/mL of *Derf* in 96-well plates for 48 h. The concentration of IFN- γ and IL-5 in the supernatants was determined by ELISA (Endogen).

Antibody blocking experiments

In a different set of experiments, the Live Af-Df-DC group was subjected to blocking studies both *in vitro* and *in vivo*, and the Df-DC and Live Af-DC groups were subjected to a blocking study *in vitro*, in which antibodies directed against TLR2, TLR4 and Dectin-1 were used to block their respective signal transduction in DCs. Thus, DCs were pre-incubated for 30 min with either anti-Dectin-1 IgG_{2B} (R&D Systems), anti-TLR2 IgG or anti-TLR4 IgG antibodies (Imgenex, San Diego, CA, USA/Biomol, BIOMOL International, L.P., Plymouth Meeting, PA, USA) at a concentration of 1 μ g/mL, as recommended by the manufacturer. In all experiments, the appropriate isotype control (normal mouse IgG_{2B} or normal rabbit IgG; R&D Systems) was used. Thereafter, each group of DC was co-cultured with *Derf* and/or live or HI Af and analysed *in vitro* and *in vivo* as described above.

Statistical analysis

The results are expressed as the mean $\pm$ SEM. Differences between groups were examined for statistical significance using repeated-measures analysis of variance with a Bonferroni multiple comparison test. A value of $P < 0.05$ was considered statistically significant.

Results

Live Aspergillus fumigatus conidia alter the cytokine response of dendritic cells to Dermatophagoides farinae in vitro

The effect of infection with Af conidia on cytokine production in Df-pulsed DCs was assayed by comparing the cytokine production of six groups of DCs that were pulsed with Live Af, HI Af, Df or medium control either singly or in various combinations. Cell viability of all six DC groups

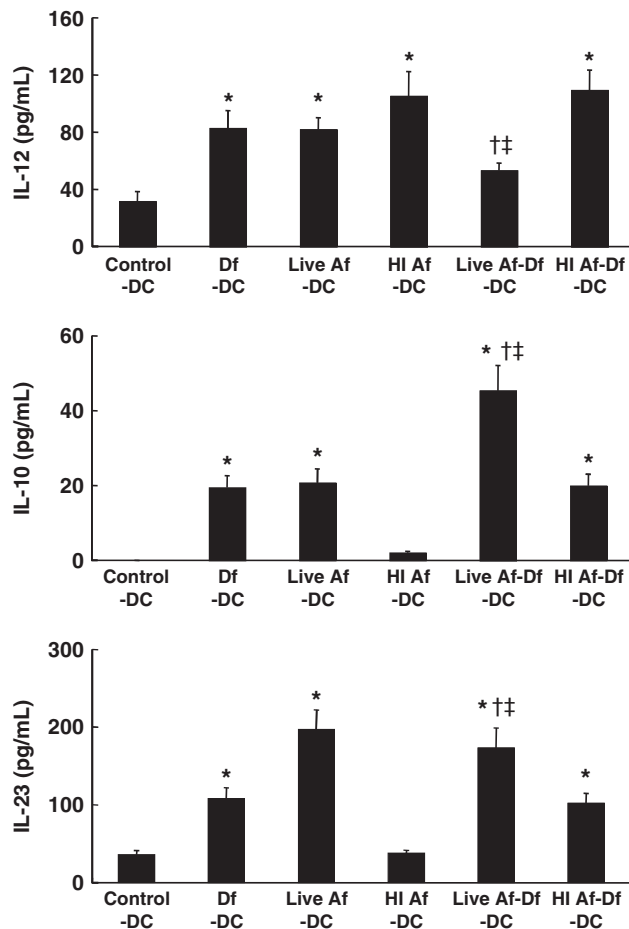


Fig. 1. Cytokine production by dendritic cells (DCs). The concentration of IL-12, IL-10 and IL-23 in culture supernatants of the indicated six groups of DCs was determined by ELISA. The bars represent the mean ($n=8$ for each group) $\pm$ SEM. * $P<0.01$ vs. Control-DC; † $P<0.05$ vs. Df-DC; ‡ $P<0.05$ vs. HI Af-DC. Df-DC, *Derf*-pulsed DC; HI Af-DC, heat-inactivated *Af*-infected DC.

was >90% irrespective of the treatment. The level of the representative immunoregulatory cytokines, IL-12, IL-10 and IL-23, in the culture supernatants of all six groups of DC was determined using ELISA (Fig. 1). The Df-DC group produced significantly higher amounts of IL-12, IL-10 and IL-23 than the Control-DC group. The Live Af-Df-DC group produced significantly higher amounts of IL-10 and IL-23 and significantly lower amounts of IL-12 than the Df-DC group. IL-12, IL-10 and IL-23 production was comparable between Df-DC and HI Af-Df-DC. These results indicate that *Af* alters the cytokine response of DCs to *Derf* and that the effect of *Af* on DCs is dependent on the viability of *Af*.

Differential regulation of Toll-like receptor and Dectin-1 expression in dendritic cells by *Aspergillus fumigatus* and/or *Dermatophagoides farinae*

It has been reported that microbial stimuli affect the expression of their cognate TLR [37–40]. We therefore

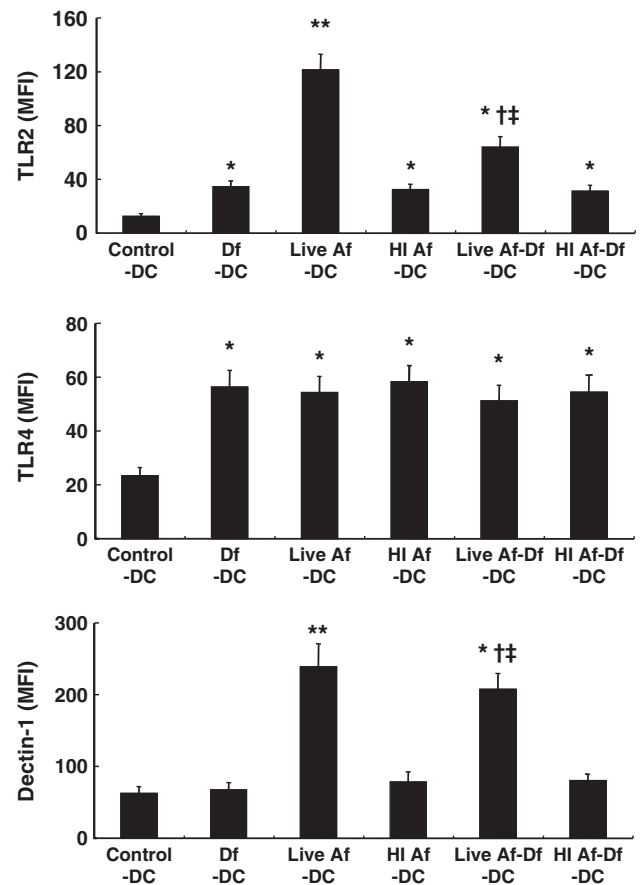


Fig. 2. Quantitative analysis of the Toll-like receptor (TLR)2, TLR4 and Dectin-1 expression on dendritic cells (DCs) by flow cytometry. The expression of TLR2, TLR4 and Dectin-1 on DCs was determined by flow cytometry using specific antibodies. All DCs were also stained for CD11c. Similar results were obtained using real-time RT-PCR analysis. Data from experiments with cells from each of the six groups are summarized and presented as mean ($n=8$ for each group) values $\pm$ SEM. ** $P<0.01$ and * $P<0.05$ vs. Control-DC; † $P<0.01$ vs. Df-DC; ‡ $P<0.01$ vs. Live Af-DC. Df-DC, *Derf*-pulsed DC; Live Af-DC, live *Af*-infected DC.

quantified the effect of 24-h stimulation of DCs with *Derf* and/or *Af* on the expression of TLR2, TLR4 and Dectin-1 by real-time PCR and flow cytometry. Because both analyses showed similar trends, only the results of flow cytometry are shown in Fig. 2. The Df-DC group expressed significantly higher levels of TLR2 and TLR4 than Control-DC, whereas the level of Dectin-1 was relatively unchanged. Live Af-DC expressed significantly higher levels of TLR2, TLR4 and Dectin-1 than Control-DC. In contrast, HI Af-DC significantly enhanced only TLR2 and TLR4 mRNA and protein. When *Af*-infected Df-DCs were compared with Df-DCs, the Live Af-Df-DCs expressed significantly higher levels of TLR2 and Dectin-1, whereas the level of TLR4 was comparable for the two groups. Compared with Live Af-DC, Live Af-Df-DC showed a significantly decreased expression of TLR2 and Dectin-1. In contrast, the HI Af-Df-DC group failed to significantly

alter the expression of TLR2, TLR4 or Dectin-1 compared with Df-DC. These results indicate that live *Af* enhances the Df-DC expression of TLR2 and Dectin-1 and that this effect is dependent on the viability of *Af*.

Live Aspergillus fumigatus infection attenuates Dermatophagoides farinae-dendritic cell-induced allergic airway inflammation in vivo

To determine if the functional changes observed in the six different DC groups *in vitro* were actually translated *in vivo* and to investigate the effects of prior exposure of DCs to a combination of *Af* and *Derf* *in vitro* on subsequent mite-allergen challenge *in vivo*, the six groups of DC were intranasally inoculated into naïve mice. These mice were then challenged intranasally with 50 µg/50 µL of *Derf* once a day for five consecutive days. At 24 h after the final *Derf* challenge, lung pathology was assayed by histochemical staining. Representative photographs of lung pathology are shown in Fig. 3. Lung tissues of mice harbouring Live Af-DC and HI Af-DC showed normal histology that was comparable with that of mice harbouring the control DC. In contrast, lung tissues of mice inoculated with Df-DC exhibited peribronchovascular cellular infiltration with lymphocytes and eosinophils as well as goblet cell hyperplasia. However, mice inoculated with Live Af-Df-DC showed a marked attenuation of Df-DC-induced allergic airway inflammation. In contrast, mice inoculated with HI Af-Df-DC did not show any significant differences in lung pathology compared with mice inoculated with Df-DC. These pathological changes were evaluated in a quantitative manner by BAL analysis, and the results confirmed the lung pathology (Fig. 3). As a first step toward determining the immunological mechanism underlying the observed lung pathology, cytokine production of lung MNCs from the six groups of mice was compared (Fig. 4). Inoculation of Df-DC resulted in significant increases in IFN- γ and IL-5 compared with the inoculation of Control-DC. In contrast, inoculation of either Live Af-DC or HI Af-DC failed to cause significant cytokine production. Mice inoculated with Live Af-Df-DC showed a significantly reduced production of both IFN- γ and IL-5 compared with Df-DC and the level of both IFN- γ and IL-5 was comparable with the Control-DC groups. In contrast, HI Af-Df-DC resulted in significant increases in IFN- γ and IL-5 compared with the inoculation of Control-DC. Nevertheless, HI Af-Df-DC-induced IFN- γ and IL-5 production were significantly decreased compared with that induced by Df-DC. This result is probably due to the incomplete prevention of *Af* germination by heat inactivation treatment. Thus, concomitant exposure of DCs to a combination of live, but not dead *Af*, and *Derf* inhibits the development of *Derf*-induced allergic airway inflammation, which is associated with the inhibition of Th1 and Th2 cytokine production.

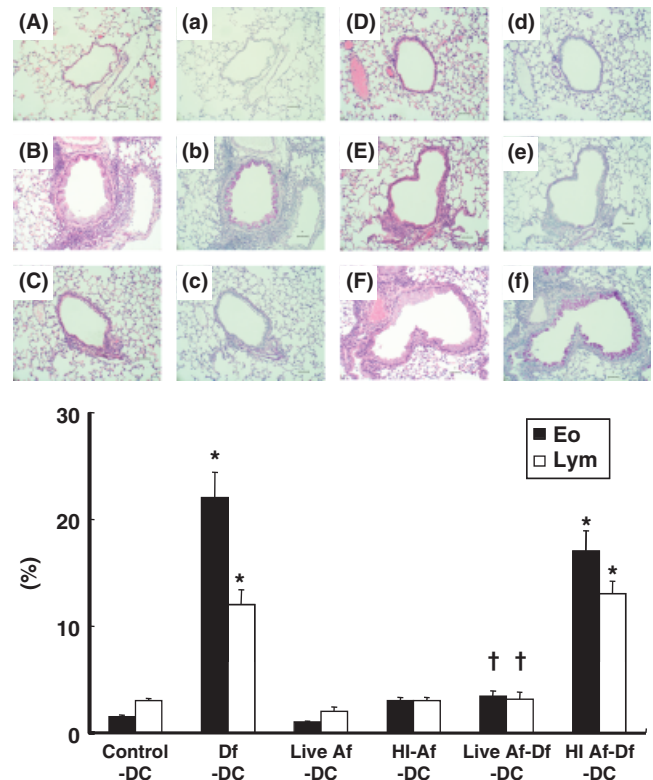


Fig. 3. Histopathological changes in the lungs of mice intranasally inoculated with dendritic cells (DCs). (upper) Lung tissues were obtained from mice intranasally inoculated with DCs and stained with haematoxylin and eosin (a–f) and periodic acid-Schiff (A–F). Representative microphotographs from each group are shown. (A and a) Control-DC, (B and b) Df-DC, (C and c) Live Af-DC, (D and d) HI Af-DC, (E and e) Live Af-Df-DC and (F and f) HI Af-Df-DC. Original magnification $\times 400$. Percentages of eosinophils (Eo) and lymphocytes (Lym) in BALF are shown (bottom). BAL was performed 24 h after the final exposure to *Dermatophagoides farinae* allergen. Differential cell counts are based on May–Grunwald–Giemsa staining. Results are expressed as the mean $\pm$ SEM ($n = 8$ for each group). * $P < 0.01$ vs. Control-DC and † $P < 0.01$ vs. Df-DC. Df-DC, *Derf*-pulsed DC; Live Af-DC, live *Af*-infected DC; HI Af-DC, heat-inactivated *Af*-infected DC; Live Af-Df-DC, *Derf*-pulsed plus live *Af*-infected DC; HI Af-Df-DC, *Derf*-pulsed plus heat-inactivated *Af*-infected DC.

Involvement of Toll-like receptor 2 and Dectin-1 in the dendritic cell response to concomitant exposure to Aspergillus fumigatus and Dermatophagoides farinae

To confirm that the functional changes observed *in vitro* and *in vivo* with the various groups of DCs were associated with a differential expression pattern of TLR2, TLR4 and Dectin-1, we carried out blocking studies in which specific anti-receptor antibodies were used to block *Derf* and/or *Af* interaction with these receptors. To confirm that these antibodies could block the up-regulation of their mRNA expression induced by *Derf* and/or *Af*, we quantified TLR2, TLR4 and Dectin-1 mRNA after a 24-h culture of Df-DCs, Af-DCs or Af-Df-DCs following pre-treatment with the individual receptor antibodies. None of the

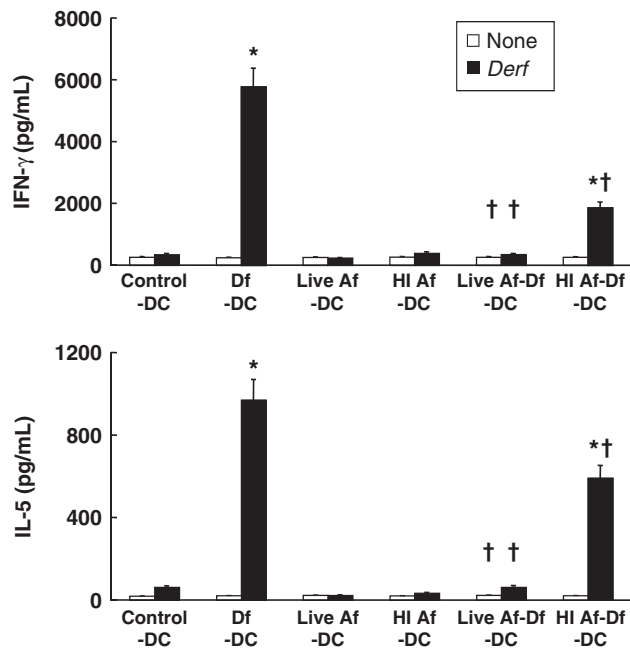


Fig. 4. Cytokine profile of lung mononuclear cells (MNCs) of mice intranasally inoculated with dendritic cells (DCs). MNCs, isolated from the lungs of the six indicated groups of mice, were cultured in the absence or presence of *Dermatophagoides farinae* (*Derf*) allergen for 48 h. The concentration of IL-5 and IFN- γ in culture supernatants was determined by ELISA. Scale bars ($n = 12$ for each) represent the mean SEM. * $P < 0.01$ vs. Control-DCs and † $P < 0.01$ vs. Df-DC. Df-DC, *Derf*-pulsed DC.

antibodies significantly altered the baseline expression of TLR2, TLR4 or Dectin-1. Furthermore, following treatment with each antibody, no significant increase in TLR2, TLR4 or Dectin-1 mRNA expression was observed in DCs that were stimulated by *Derf* and/or *Af* (data not shown). Thus, the enhanced expression of TLR and Dectin-1 mRNA in DCs that was induced by an exposure to *Derf* and/or *Af* was successfully inhibited by antibody treatment. Figure 5 shows the effect of antibody treatment on immunoregulatory cytokine production from the Live Af-Df-DC group. Production of IL-10 and IL-23 from Live Af-Df-DCs was significantly decreased by both anti-TLR2 and anti-Dectin-1 antibodies. In contrast, anti-TLR4 antibody failed to significantly affect IL-10 and IL-23 production. We also analysed the effect of anti-TLR2, -TLR4 and -Dectin-1 antibodies on Df-DC and Live Af-DC. The production of IL-10 and IL-23 from Live Af-DCs was significantly decreased by anti-TLR2 and anti-Dectin-1 antibodies. Production of IL-10 and IL-23 from Df-DCs was significantly decreased by anti-TLR2 antibodies. All of the anti-TLR2, -TLR4 and Dectin-1 antibodies failed to significantly affect IL-12 production from Df-DCs, whereas IL-12 from Live Af-DCs was significantly decreased by anti-TLR4 antibodies. To determine if these antibody effects could be reproduced *in vivo*, Live Af-Df-DCs,

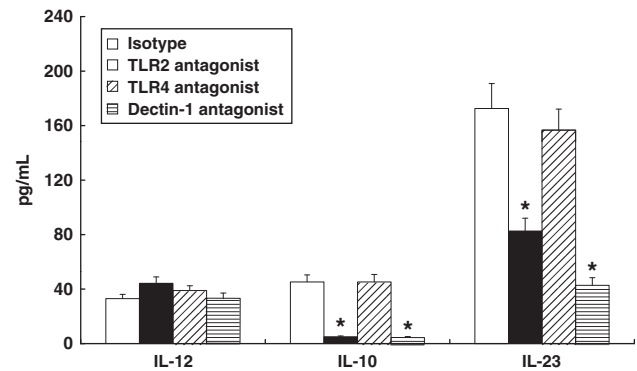


Fig. 5. Cytokine production by antibody-treated dendritic cells (DCs). Live Af-Df-DCs were pretreated with either isotype control or anti-Toll-like receptor (TLR)2, anti-TLR4 or anti-Dectin-1 antibodies before *Dermatophagoides farinae* and *Af* exposure. The concentration of IL-12, IL-10 and IL-23 in culture supernatants was determined by ELISA. Bars ($n = 12$ for each) represent the mean $\pm$ SEM. * $P < 0.01$ vs. isotype control. Af-Df-DC, *Derf*-pulsed plus live *Af*-infected DC.

treated with anti-TLR2, anti-TLR4 or anti-Dectin-1 antibodies, were intranasally inoculated into naïve mice following which the mice were challenged intranasally with *Derf*, and lung pathology and cytokine production from lung MNCs were assessed. Representative photographs of lung pathology in these mice are shown in Fig. 6. Pre-treatment with either anti-TLR2 or anti-Dectin-1 antibody restored allergic airway inflammation in Live Af-Df-DC-inoculated mice. In contrast, treatment with the anti-TLR4 antibody failed to restore this inflammation. These pathological changes were quantitatively evaluated by BAL analysis, and the results confirmed the lung pathology data (Fig. 6). Assay of cytokine production in lung MNCs revealed that both anti-TLR2 and anti-Dectin-1 antibodies significantly increased both IL-5 and IFN- γ levels in Live Af-Df-DC-inoculated mice, while the anti-TLR4 antibody failed to do so (Fig. 7). However, anti-TLR2 and anti-Dectin-1 antibodies did not restore either IL-5 or IFN- γ levels. Collectively, these results suggest that TLR2 and Dectin-1 may play a critical role in the inhibitory effect of live *Af* infection on *Derf*-sensitized DC in the development of allergic airway inflammation.

Discussion

The immune response against fungi in an immunocompromised host has been attracting attention as opportunistic fungal infections are considered an important clinical problem for this population. Pathological fungi such as the *Aspergillus* species also cause allergic disease in immunocompetent hosts. However, the role in allergy development of the immune response against fungi in the presence of concomitant exposure to other common aeroallergens has yet to be determined. In particular, there is little understanding of how inhaled fungi interact with

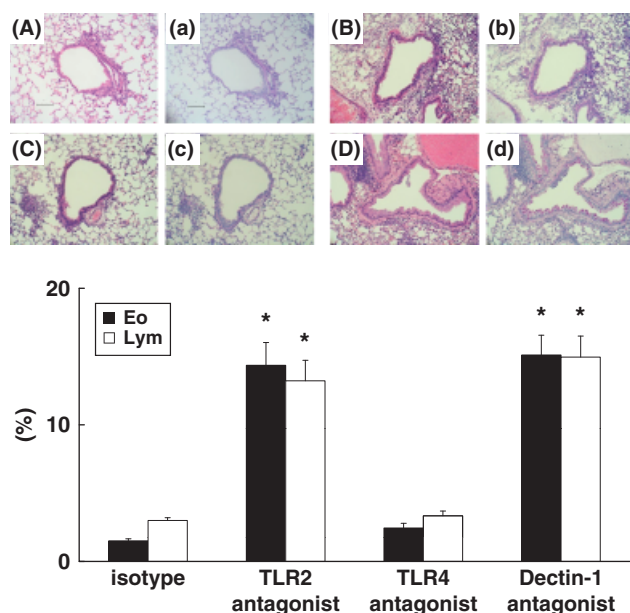


Fig. 6. Histopathological changes in the lungs of mice intranasally inoculated with antibody-treated dendritic cells (DCs). (upper) Lung tissues, obtained from mice intranasally inoculated with antibody-treated Live Af-Df-DCs, were stained with haematoxylin and eosin (a-d) and periodic acid-Schiff (a-d). Representative microphotographs from each group are shown. (A and a) isotype control, (B and b) anti-Toll-like receptor (TLR)2 antibody, (C and c) anti-TLR4 antibody and (D and d) anti-Dectin-1 antibody. Original magnification $\times 400$. Lung pathology was quantitatively analysed by BAL (bottom). Results are expressed as the mean $\pm$ SEM ($n = 8$ for each group). * $P < 0.01$ vs. isotype control. Af-Df-DC, *Derf*-pulsed plus live *Af*-infected DC.

antigen-presenting cells (APCs) in the asthmatic airway. The present study was designed to characterize the effect on DCs, which are specialized airway APCs, of concurrent exposure to *Af* and *Derf* on the development of allergic asthma. For this purpose, two critical parameters of DC function, cytokine production and PRR expression, were examined *in vitro*. The results demonstrated that Live Af-Df-DCs had significantly increased IL-10 and IL-23 production as well as TLR2 and Dectin-1 expression compared with Df-DCs. These characteristics of Live Af-Df-DCs were probably due to the fact that combined exposure to *Af* and *Derf* provide a high level of ligand for TLR2 and β -D glucan surface receptors, which in turn, led to the up-regulation of these receptors on the surface and enhanced signalling. Also, HI Af-Df-DC induced significant decreases in IFN- γ and IL-5 in the present study, compared with those in Df-DC. This result may be due to the fact that heat inactivation could not completely prevent *Af* conidia germination, with the result that the expression of TLR2 ligand and β -glucan on the HI *Af* conidia surface could still slightly enhance signalling via TLR2 and Dectin-1. This potential mechanism was further suggested by the fact that anti-TLR2 and anti-Dectin-1 antibody treatment

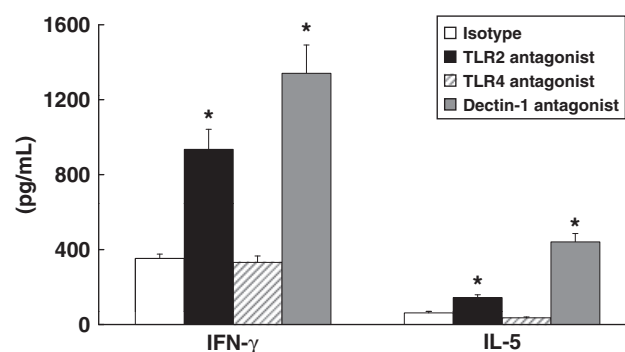


Fig. 7. Cytokine profile of lung mononuclear cells of mice intranasally inoculated with antibody-treated dendritic cells (DCs). Mononuclear cells, obtained from the lung tissue of antibody-treated Live Af-Df-DC-inoculated mice, were cultured in the absence or presence of *Dermatophagoides farinae* allergen for 48 h. The concentration of IL-5 and IFN- γ in the culture supernatants was determined by ELISA. Bars ($n = 12$ for each) represent the mean $\pm$ SEM. * $P < 0.01$ vs. isotype control. Af-Df-DC, *Derf*-pulsed plus live *Af*-infected DC.

significantly inhibited Live Af-Df-DC-induced expression of TLR2 and Dectin-1 and thus lowered the level of IL-10 and IL-23. In contrast, significant differences were not observed between HI Af-Df-DCs and Df-DCs. The lack of effect of HI *Af* conidia with concomitant *Derf* exposure on the DC functions assayed is believed to be due to the fact that metabolically inactive resting conidia cannot induce DCs to express TLR2 and Dectin-1 due to the absence of β -D glucan and TLR2 ligand expression on their surface. These findings are consistent with those of recent studies that different kinds of PRRs are involved in recognition of *Af* conidia and that the immune response to *Af* differs according to whether conidia are in a resting or a mature form [41, 42].

To confirm that the observed regulation of Df-DC function by concomitant *Af* exposure *in vitro* plays a role in the development of allergic inflammation *in vivo*, these DC were inoculated into the airway of naïve mice followed by intranasal *Derf* challenge. Lambrecht et al. [43] showed that intratracheal injection of CFSE-labelled DCs (1×10^6 cells) was followed by the recovery of these cells in BALF (14.1% of cells), draining mediastinal lymph nodes (0.012%) within 12 h after instillation. Although our mice were instilled with a smaller number of DCs (1×10^5 cells), this number of DCs was sufficient to cause allergic airway inflammation, as shown by the lung pathology (Fig. 3). Mice inoculated with Df-DC developed allergic airway inflammation with an increase in both Th1 and Th2 cytokines in the pulmonary tissue. In contrast, inoculation of Live Af-Df-DC, which produced a significantly higher level of IL-10 compared with Df-DC, significantly attenuated allergic airway inflammation and both Th1 and Th2-like cytokine production. Furthermore, antibody neutralization of either TLR2 or Dectin-1, which

significantly reduced IL-10 production from Live Af-Df-DC, significantly reversed the inhibitory effect of live Af infection on the development of Df-DC-induced allergic airway inflammation. It has been reported that there are striking differences in the conditions leading to the production of cytokines in DCs due to the selective engagement of distinct combinations of PRRs on the surface of DCs. For example, a combination of a Dectin-1 ligand with the TLR2 ligand induces IL-10 production more efficiently in human monocyte-derived DC than either ligand alone [44]. Induction of IL-10 is dependent on TLR2- and Dectin-1-mediated activation of the extracellular-signal-regulated kinase (ERK) mitogen-activated protein kinase via a mechanism independent of the activation protein 1 transcription factor c-Fos [45]. DCs that are activated through TLR2 or Dectin-1 stimulate antigen-specific CD4⁺ T cells poorly due to the presence of IL-10 and the lack of IL-6 [45]. Based on these findings, it is likely that DC infection by live, but not HI, Af amplified TLR2-ERK signalling due to Live Af-induced elevation of TLR2 expression on DCs. This effect of Live Af-Df-DCs would, in turn, lead to a high level of IL-10 and poor stimulation of antigen-specific CD4⁺ T cells, which would then fail to develop allergic airway inflammation *in vivo*. These results agree with previous reports that show that TLR2 ligands inhibit allergen-specific Th2 responses [46, 47].

There is current evidence indicating a close association between fungal exposure and asthma severity [48]. While a number of epidemiological studies have shown that fungal exposure causes acute and severe exacerbation of established asthma [49], the role of fungi as a primary exogenous driver of asthma has been incompletely explored. The present study clearly showed that, instead, concomitant exposure of live Af interferes with mite allergen in the development of asthma at least at the DC level. These results are consistent with the action of another pathogen, respiratory syncytial virus, which also exacerbates established allergic airway inflammation but inhibits myeloid-DC initiation of allergic airway inflammation [33]. The outcome of fungal infection on mite allergen-induced asthma may be dependent on various factors including whether inhaled fungi are alive or dead and whether fungi primarily infect APCs or other constitutive airway cells.

In conclusion, the present study shows that concurrent exposure of DCs to fungal and mite antigens has profound influences on subsequent mite allergen-induced allergic airway inflammation and that Af can modulate both innate and acquired immunity via alteration of the PRR-expression pattern of DC. Our study also suggests the possibility that blocking or stimulating TLRs can modify the Th1/Th2/Th17/Treg balance that affects allergic disorders and thus could provide a therapeutic regimen for infection-induced asthma.

Acknowledgement

This study was supported by a Grant-in-Aid for Scientific Research (no. 17607009) from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

- 1 Sly PD, Boner AL, Björkstén B *et al*. Early identification of atopy in the prediction of persistent asthma in children. *Lancet* 2008; **372**:1100–6.
- 2 Sykes A, Johnston SL. Etiology of asthma exacerbations. *J Allergy Clin Immunol* 2008; **122**:685–8.
- 3 Wark PA, Gibson PG. Asthma exacerbations. 3: pathogenesis. *Thorax* 2006; **61**:909–15.
- 4 Agarwal R. Allergic bronchopulmonary aspergillosis. *Chest* 2009; **135**:805–26.
- 5 Marr KA, Carter RA, Boeckh M, Martin P, Corey L. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. *Blood* 2002; **100**:4358–66.
- 6 Greenberger PA. Allergic bronchopulmonary aspergillosis. *J Allergy Clin Immunol* 2002; **110**:685–92.
- 7 Rivera A, Ro G, Van Epps HL *et al*. Innate immune activation and CD4⁺ T cell priming during respiratory fungal infection. *Immunity* 2006; **25**:665–75.
- 8 Rivera A, Van Epps HL, Hohl TM, Rizzuto G, Pamer EG. Distinct CD4⁺-T-cell responses to live and heat-inactivated *Aspergillus fumigatus* conidia. *Infect Immun* 2005; **73**:7170–9.
- 9 Chauhan B, Hutcheson PS, Slavov RG, Bellone CJ. T-cell receptor bias in patients with allergic bronchopulmonary aspergillosis. *Hum Immunol* 2002; **63**:286–94.
- 10 Chauhan B, Santiago L, Kirschmann DA *et al*. The association of HLA-DR alleles and T cell activation with allergic bronchopulmonary aspergillosis. *J Immunol* 1997; **159**:4072–6.
- 11 Kurup VP, Seymour BW, Choi H, Coffman RL. Particulate *Aspergillus fumigatus* antigens elicit a TH2 response in BALB/c mice. *J Allergy Clin Immunol* 1994; **93**:1013–20.
- 12 van Rijn LS, Lambrecht BN. Role of dendritic cells and Th2 lymphocytes in asthma: lessons from eosinophilic airway inflammation in the mouse. *Microsc Res Tech* 2001; **53**:256–72.
- 13 Vermaelen K, Pauwels R. Pulmonary dendritic cells. *Am J Respir Crit Care Med* 2005; **172**:530–51.
- 14 Werling D, Koss M, Howard CJ, Taylor G, Langhans W, Hope JC. Role of bovine chemokines produced by dendritic cells in respiratory syncytial virus-induced T cell proliferation. *Vet Immunol Immunopathol* 2002; **87**:225–33.
- 15 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**:245–52.
- 16 Moser M, Murphy KM. Dendritic cell regulation of TH1–TH2 development. *Nat Immunol* 2000; **1**:199–205.
- 17 Bozza S, Gaziano R, Spreca A *et al*. Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J Immunol* 2002; **168**:1362–71.
- 18 Netea MG, Ferwerda G, van der Graaf CA, Van der Meer JW, Kullberg BJ. Recognition of fungal pathogens by toll-like receptors. *Curr Pharm Des* 2006; **12**:4195–201.

- 19 Braedel S, Radsak M, Einsele H *et al.* *Aspergillus fumigatus* antigens activate innate immune cells via Toll-like receptors 2 and 4. *Br J Haematol* 2004; **125**:392–9.
- 20 Netea MG, Warris A, Van der Meer JW *et al.* *Aspergillus fumigatus* evades immune recognition during germination through loss of Toll-like receptor-4-mediated signal transduction. *J Infect Dis* 2003; **188**:320–6.
- 21 Meier A, Kirschning CJ, Nikolaus T, Wagner H, Heesemann J, Ebel F. Toll-like receptor (TLR) 2 and TLR4 are essential for *Aspergillus*-induced activation of murine macrophages. *Cell Microbiol* 2003; **5**:561–70.
- 22 Mambula SS, Sau K, Henneke P, Golenbock DT, Levitz SM. Toll-like receptor (TLR) signaling in response to *Aspergillus fumigatus*. *J Biol Chem* 2002; **277**:39320–6.
- 23 Steele C, Rapaka RR, Metz A *et al.* The beta-glucan receptor Dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathog* 2005; **1**:e42.
- 24 Taylor PR, Tsoni SV, Willment JA *et al.* Dectin-1 is required for β -glucan recognition and control of fungal infection. *Nat Immunol* 2007; **8**:31–8.
- 25 Mezger M, Kneitz S, Wozniok I, Kurzai O, Einsele H, Loeffler J. Proinflammatory response of immature human dendritic cells is mediated by dectin-1 after exposure to *Aspergillus fumigatus* germ tubes. *J Infect Dis* 2008; **197**:924–31.
- 26 Garlanda C, Hirsch E, Bozza S *et al.* Non-redundant role of the long pentraxin PTX3 in anti-fungal innate immune response. *Nature* 2002; **420**:182–6.
- 27 Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2002; **2**:151–61.
- 28 Pasare C, Medzhitov R. Toll-like receptors and acquired immunity. *Semin Immunol* 2004; **16**:23–6.
- 29 Gordon S. Pattern recognition receptors: doubling up for the innate immune response. *Cell* 2002; **111**:927–30.
- 30 Yamamoto M, Sato S, Hemmi H *et al.* Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; **301**:640–3.
- 31 Dillon S, Agrawal A, Van Dyke T *et al.* A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *J Immunol* 2004; **172**:4733–43.
- 32 Machida I, Matsuse H, Kondo Y *et al.* Cysteinyl leukotrienes regulate dendritic cell functions in a murine model of asthma. *J Immunol* 2004; **172**:1833–8.
- 33 Kondo Y, Matsuse H, Machida I *et al.* Regulation of mite allergen-pulsed murine dendritic cells by respiratory syncytial virus. *Am J Respir Crit Care Med* 2004; **169**:494–8.
- 34 Gafa V, Lande R, Gagliardi MC *et al.* Human dendritic cells following *Aspergillus fumigatus* infection express the CCR7 receptor and a differential pattern of interleukin-12 (IL-12), IL-23, and IL-27 cytokines, which lead to a Th1 response. *Infect Immun* 2006; **74**:1480–9.
- 35 Harada T, Miura NN, Adachi Y, Nakajima M, Yadomae T, Ohno N. Highly expressed dectin-1 on bone marrow-derived dendritic cells regulates the sensitivity to beta-glucan in DBA/2 mice. *J Interferon Cytokine Res* 2007; **28**:477–86.
- 36 Kawano T, Matsuse H, Kondo Y *et al.* Cysteinyl leukotrienes induce nuclear factor kappa β activation and RANTES production in a murine model of asthma. *J Allergy Clin Immunol* 2003; **112**:369–74.
- 37 Poltorak A, He X, Smirnova I *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998; **282**:2085–8.
- 38 Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of toll-like receptors in human monocytes and dendritic cells. *J Immunol* 2001; **166**:249–55.
- 39 Muzio M, Bosisio D, Polentarutti N *et al.* Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J Immunol* 2000; **164**:5998–6004.
- 40 Nomura F, Akashi S, Sakao Y *et al.* Endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. *J Immunol* 2000; **164**:3476–9.
- 41 Gersuk GM, Underhill DM, Zhu L, Marr KA. Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* Cellular States. *J Immunol* 2006; **176**:3717–224.
- 42 Bellocchio S, Montagnoli C, Bozza S *et al.* The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J Immunol* 2004; **172**:3059–69.
- 43 Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J. Clin. Invest* 2000; **106**:551–9.
- 44 Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2. *J Exp Med* 2003; **197**:1107–17.
- 45 Dillon S, Agrawal S, Banerjee K *et al.* Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J Clin Invest* 2006; **116**:916–28.
- 46 Talor RC, Richmond P, Upham JW. Toll-like receptor 2 ligands inhibit Th2 responses to mite allergen. *J Allergy Clin Immunol* 2006; **117**:1148–54.
- 47 Codolo G, Mazzi P, Amedei A *et al.* The neutrophil-activating protein of *Helicobacter pylori* down-modulates Th2 inflammation in ovalbumin-induced allergic asthma. *Cell Microbiol* 2008; **10**:2355–63.
- 48 Stark PC, Burge HA, Ryan LM, Milton DK, Gold DR. Fungal levels in the home and lower respiratory tract illnesses in the first year of life. *Am J Respir Crit Care Med* 2003; **168**:232–7.
- 49 Denning DW, O'Driscoll BR, Hogaboam CM, Bowyer P, Niven RM. The link between fungi and severe asthma: a summary of the evidence. *Eur Respir J* 2006; **27**:615–26.