

Asthma-Related Environmental Fungus, *Alternaria*, Activates Dendritic Cells and Produces Potent Th2 Adjuvant Activity¹

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Asthma is thought to result from dysregulated Th2-like airway inflammatory responses to the environment. Although the etiology of asthma is not fully understood in humans, clinical and epidemiological evidence suggest a potential link between exposure to environmental fungi, such as *Alternaria*, and development and/or exacerbation of asthma. The goal of this project was to investigate the mechanisms of airway Th2 responses by using *Alternaria* as a clinically relevant model for environmental exposure. Airway exposure of naive animals to an experimental Ag, OVA, or a common allergen, short ragweed pollen, induced no or minimal immune responses to these Ags. In contrast, mice developed strong Th2-like immune responses when they were exposed to these Ags in the presence of *Alternaria* extract. Extracts of other fungi, such as *Aspergillus* and *Candida*, showed similar Th2 adjuvant effects, albeit not as potently. *Alternaria* stimulated bone marrow-derived dendritic cells (DCs) to express MHC class II and costimulatory molecules, including OX40 ligand, in vitro. Importantly, *Alternaria* inhibited IL-12 production by activated DCs, and DCs exposed to *Alternaria* enhanced Th2 polarization of CD4⁺ T cells. Furthermore, adoptive airway transfer of DCs, which had been pulsed with OVA in the presence of *Alternaria*, showed that the recipient mice had enhanced IgE Ab production and Th2-like airway responses to OVA. Thus, the asthma-related environmental fungus *Alternaria* produces potent Th2-like adjuvant effects in the airways. Such immunogenic properties of certain environmental fungi may explain their strong relationships with human asthma and allergic diseases. *The Journal of Immunology*, 2009, 182: 2502–2510.

Asthma is common in industrialized, developed countries and, over the past two decades, is increasing in its incidence, prevalence, and severity. Allergic asthma probably results from dysregulated airway inflammatory responses to ubiquitous environmental Ags; these responses are mediated by CD4⁺ T cells polarized to a Th2 cell phenotype (1). Th2 cytokines, such as IL-4, IL-5, and IL-13, drive the pathological features of asthma, including airway eosinophilia, elevated IgE, airway hyperresponsiveness, excessive production of mucus, and airway remodeling (2, 3). Currently, little is known regarding how asthma patients develop such dysregulated Th2 immune responses to environmental Ags; generally, respiratory exposure to an innocuous Ag is considered a tolerogenic event (4, 5). These pathologic Th2 immune responses may mirror the beneficial Th2 immune responses to helminth infection. More specifically, asthma may represent the exaggerated immune responses to chitin-containing or-

ganisms, including house-dust mite, cockroaches, and fungi (6–8). However, the immunological mechanisms linking the exposure to chitin-containing organisms and the development of pathologic Th2 immune responses are not fully understood.

The etiology of human asthma is complex and multifactorial; it likely involves the interactions between genetic factors and environmental stimuli. Among various environmental stimuli, an association between fungal exposure and asthma has been recognized clinically and epidemiologically for three centuries (9). In particular, much evidence suggests an association between a common environmental fungus, *Alternaria*, and asthma (9, 10). *Alternaria* is ubiquitous both outdoors and indoors (11), and it is known for its high rates of spore germination and Ag release (12). Exposure to *Alternaria* is a risk factor for asthma and allergic rhinitis (13–15). Sensitization to *Alternaria* has been associated with asthma in various countries and in regions of the U.S (16, 17). Moreover, severe asthma and potentially life-threatening acute exacerbations of asthma have been associated with *Alternaria* sensitivity or increased airborne concentrations of *Alternaria* spores (17–21).

To investigate the immunological mechanisms involved in pathologic airway Th2 responses, we used *Alternaria* to model environmental exposure relevant to human asthma. *Alternaria* possessed potent Th2 adjuvant effects in vivo and induced airway sensitization to an experimental Ag, OVA, and a common allergen, short ragweed pollen (SRW),⁴ in vivo. When dendritic cells (DCs) were exposed to *Alternaria* in vitro, they demonstrated distinct inflammatory responses. These *Alternaria*-exposed DCs facilitated

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⁴ Abbreviations used in this paper: SRW, short ragweed pollen; BAL, bronchoalveolar lavage; BM, bone marrow; DC, dendritic cell; MHC-II, MHC class II; MLN, mediastinal lymph node; TSLP, thymic stromal lymphopoietin; L, ligand.

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Th2-skewed humoral and cellular immune responses when they were transferred into the airways of naive animals. The unique and potent Th2 adjuvant characteristics of *Alternaria*, which are partially mediated through DCs, may explain the strong association between *Alternaria* and human allergic airway disorders.

Materials and Methods

Mice

BALB/cJ (BALB/c), C57BL/6J (B6), and C57BL/6-Tg(Tcr α Tcr β)425Cbn/J (OTII) mice were from The Jackson Laboratory. All mice were 7–9 wk-old females. The procedures and handling of the mice were reviewed and approved by the Mayo Institutional Animal Care and Use Committee, Mayo Clinic.

Fungal extracts, Alt a 1, SRW extract, and OVA

Culture filtrate fungal extracts (called fungal extract subsequently), including *Alternaria alternata*, *Aspergillus versicolor*, or *Candida albicans*, were from Greer Laboratories. The *Alternaria* extract contained minimal endotoxin activity (11.7 EU/mg extract) by a *limulus* lysate assay (WAKO). The endotoxin in *Alternaria* extract at the dosage used (50 μ g) was equivalent to ~0.019 ng of LPS, which previously did not induce airway sensitization to inhaled Ag (22, 23). Recombinant Alt a 1 was from Indoor Biotechnologies. Sterile, freeze-dried SRW was from Greer Laboratories. OVA, grade VI, which contained a minimal amount of endotoxin (i.e., equivalent to 0.3 ng LPS/mg dry weight, 0.03 ng LPS/exposure), was from Sigma-Aldrich.

Mouse airway sensitization and challenge model

On days 0 and 7, BALB/c mice were lightly anesthetized with avertin and intranasally administered with 100 μ g of OVA or SRW extract in the presence or absence of fungal extract (50 μ g) in 50 μ l of PBS (24) (see Fig. 1, vide infra); on day 14, plasma was collected for analysis of specific Abs; on days 21, 22, and 23, mice were challenged intranasally with 100 μ g of OVA or SRW in 50 μ l of PBS; on day 24, mice were killed by an overdose of pentobarbital. The trachea was cannulated, and the lungs were lavaged three times with HBSS (0.5, 0.25, and 0.25 ml). Cell numbers were counted using a hemocytometer; differentials were determined in cytospin preparations stained with Wright-Giemsa; ≥ 200 cells were counted using conventional morphologic criteria. The supernatants of bronchoalveolar lavage (BAL) fluid were collected and stored at -20°C for cytokine assays. Whole lungs were also collected and homogenized in 1.0 ml of PBS. The homogenates were centrifuged at $10,000 \times g$ at 4°C for 15 min, and the protein concentrations of the supernatants were quantified with the DC Protein Assay kit (Bio-Rad). IL-13 concentrations in BAL supernatants and lung homogenate supernatants were analyzed by ELISA, following the manufacturer's procedure (R&D Systems); sensitivity for IL-13 was 4 pg/ml.

Ag-specific IgE, IgG1, and IgG2a

To quantitate OVA-, SRW-, and Alt a 1-specific IgE, ELISA plates (Immulon 4 HBX; Thermo Labsystems) were coated with 50 μ l of 5 μ g/ml rat anti-mouse IgE mAb (LO-ME-3; Serotec) in 0.1 M carbonate buffer (pH 9.5) for 2 h at 37°C . The plates were blocked with 200 μ l of PBS containing 1% BSA (Sigma-Aldrich) overnight at 4°C . After blocking, plasma samples, which were diluted with PBS containing 1% BSA and 0.05% Tween 20 (1/20 for anti-OVA IgE or 1/10 for anti-SRW IgE), were added to the plates, and the plates were incubated for 2 h at room temperature. Thereafter, the plates were incubated with 1 μ g/ml OVA, SRW, or Alt a 1, which had been biotinylated using a microbiotinylation kit (Sigma-Aldrich), for 1 h at room temperature, followed by 1/5000 streptavidin-poly- HRP (Pierce) for 30 min at room temperature. After the final washing, peroxidase substrate (TMB substrate kit; Pierce) was added, and the reaction was stopped with 1 M HCl after 20 min. The absorbance at 450 nm was read in a microplate autoreader (Thermomax; Molecular Devices). Between each step, the plates were repeatedly washed with PBS containing 0.05% Tween 20.

To quantitate OVA- and SRW-specific IgG1 or IgG2a, ELISA plates were coated with 10 μ g/ml OVA or SRW, blocked with BSA and incubated with plasma samples diluted in PBS (1/2000 for anti-OVA IgG1, 1/50 for anti-OVA IgG2a, 1/250 for anti-SRW IgG1, and 1/10 for anti-SRW IgG2a), followed by HRP-conjugated anti-mouse IgG1 or IgG2a (1/1000; BD Pharmingen) and peroxidase substrate, as described above. After stopping the reaction with HCl, the absorbance was read in a microplate autoreader. Serial dilutions of plasma in these ELISAs showed linear correlations between Ab concentrations and OD values up to 1.5.

Cytokine production by mediastinal lymph node (MLN) cells and splenocytes

On day 24 of the airway sensitization and challenge model (vide supra), MLNs and spleens were harvested and pooled from each group. Cells were isolated, washed and resuspended in RPMI 1640 medium (Invitrogen Life Technologies) with 10% FBS (HyClone), 10 mM HEPES, 50 μ M 2-ME, 100 μ g/ml streptomycin, and 100 U/ml penicillin (complete RPMI 1640) at 4×10^6 cells/ml; 100 μ l of cell suspension was dispensed into each well of 96-well tissue culture plates (Corning Costar). The cells were stimulated by adding 100 μ l of 200 μ g/ml OVA, *Alternaria* extract, or BSA for 96 h. IL-5 and IL-13 concentrations in the supernatants were measured by ELISA kits (R&D Systems); sensitivity for IL-5 was 8 pg/ml and for IL-13 was 4 pg/ml.

Assessment of airway hyperreactivity

Mice were intranasally exposed to OVA alone, *Alternaria* extract alone, or OVA plus *Alternaria* extract on days 0 and 7 as shown in Fig. 1 and then intranasally challenged with OVA on days 42, 43, and 44. On day 45, airway hyperreactivity was assessed in conscious mice by methacholine-induced airflow obstruction using whole-body plethysmography (Buxco Electronics) as described previously (24). Briefly, mice were exposed for 3 min to aerosolized PBS, followed by incremental doses of aerosolized methacholine (freshly prepared in PBS), and airflow obstruction was measured by enhanced pause (Penh). Results were expressed for PBS (0 methacholine) and each methacholine dose as the percentage of baseline Penh values before PBS exposure.

Generation and stimulation of mouse bone marrow (BM)-derived DCs

DCs were generated from mouse BM using an established protocol (25) with minor modifications (26). Briefly, mouse BM was obtained from the long bones of the hind legs. After erythrocyte lysis, BM cells were suspended at 1×10^6 /ml in RPMI 1640 with 10% FBS, 10 ng/ml murine GM-CSF, and 1 ng/ml murine IL-4. Cells were plated into wells of 6-well tissue culture plates and cultured for 6 days; the purity of CD11c $^{+}$ DCs was ~85%. Day 6 DCs were stimulated for 24–48 h with *Alternaria* extract (25–100 μ g/ml) or *E. coli* (O127:B8) LPS (1 μ g/ml; Sigma-Aldrich) or combinations thereof. In separate experiments, we further purified DCs with a magnetic cell separation system (MACS; Miltenyi Biotec) and anti-CD11c (N418) immunomagnetic beads (Miltenyi Biotec). These highly purified DCs (>97% purity) behaved similarly to those without MACS purification. Because these extra manipulations might have affected DC functions, we used BM-derived DCs without MACS purification in most experiments.

The functional responses of DCs to *Alternaria* extract and LPS were assessed by expression of cell surface molecules and cytokine production by FACS and ELISA, respectively. For FACS analysis, after stimulation for 24 h (MHC class II (MHC-II) I-Ad, CD40, CD80, and CD86) or 48 h (OX40 ligand (L)), DCs were preincubated with FcR blockers (anti-CD16/CD32) for 30 min at 4°C and stained with PE-conjugated anti-CD11c (clone HL3) and FITC-conjugated anti-MHC-II I-Ad (AMS-32.1), anti-CD40 (HM40-3), anti-CD80 (16-10A1), and anti-CD86 (GL1) or biotinylated anti-OX40L (RM134L) plus FITC-conjugated streptavidin for 30 min at 4°C . FITC-conjugated mouse IgG2b, Armenian hamster IgG2, rat IgG2a and rat IgG2a, and biotinylated Armenian hamster IgM were used as isotype controls. All Abs used for DC FACS analysis were obtained from BD Pharmingen. After washing, DCs were resuspended in PBS containing 1% BSA and 0.1% NaN₃, fixed with 1% paraformaldehyde, and analyzed by a FACScan flow cytometer (BD Immunocytometry Systems) by gating on a CD11c $^{+}$ forward scatter-high population. For cytokine analysis, cell-free supernatants were collected after 24 h of stimulation; concentrations of IL-6, IL-12p70, and TNF- α were measured by ELISA kits (R&D Systems); sensitivities for IL-6, IL-12p70, and TNF- α were 4, 4, and 12 pg/ml, respectively.

CD4 $^{+}$ T cell cytokine production assays

After DCs were stimulated with *Alternaria* extract or LPS, they were incubated with CD4 $^{+}$ T cells, and cytokine production was analyzed by two methods, namely MLR and a TCR-transgenic OTII system. For MLR, DCs from BM of BALB/c mice were stimulated with 75 μ g/ml *Alternaria* extract for 24 h. DCs were washed three times and suspended in complete RPMI 1640 and were seeded in round-bottom wells of 96-well culture plates at 2×10^4 cells/100 μ l/well. CD4 $^{+}$ T cells, which were isolated (purity >90%) from spleens of B6 mice using negative selection (StemCell Technologies), were added to the wells at a 1:5 DC:T cell ratio and cultured for 6 days. Concentrations of IL-4, IL-5, IL-13, and IFN- γ in the cell-free

supernatants were measured by ELISA (R&D Systems); sensitivities for IL-4 and IFN- γ were 4 and 5 pg/ml, respectively. In some experiments, CD4⁺ T cells were restimulated with immobilized anti-CD3 (Biocoat T-Cell Activation Plates; BD Pharmingen) and soluble anti-CD28 (2 μ g/ml; BD Pharmingen) on day 4 for 24 h. Monensin (GolgiStop; BD Pharmingen) was added for the final 10 h. Cells were then incubated with Fc block, stained with PerCP-conjugated anti-CD4 (RM4-5), fixed and permeabilized with Cytofix/Cytoperm kit (BD Pharmingen), and then stained intracellularly with FITC-conjugated anti-IFN- γ (XMG1.2) and PE-conjugated anti-IL-4 (11B11). The stained cells were analyzed by a FACScan flow cytometer.

For a TCR-transgenic OTII system, we used CD4⁺ T cells isolated from OTII mice (B6 background), which express a transgenic TCR that recognizes an OVA peptide presented by MHC-II. DCs were generated from BM of B6 mice and pulsed with 100 μ g/ml endotoxin-free OVA with or without 75 μ g/ml *Alternaria* extract or 1 μ g/ml LPS for 24 h. DCs were washed three times and suspended in complete RPMI 1640 and seeded in round-bottom wells of 96-well culture plates at 1×10^4 cells/100 μ l/well. CD4⁺ T cells from spleens of OTII mice were added to the wells at a 1:10 DC:T cell ratio and cultured for 6 or 10 days. Concentrations of IL-4, IL-13, and IFN- γ (all day 6) and IL-5 (day 10) in the supernatants were measured by ELISA, as described above.

Adoptive airway transfer

To examine the in vivo functions of DCs exposed to *Alternaria* extract in vitro, we adoptively transferred DCs to the airways of recipient mice. BM-derived DCs were obtained from BALB/c mice, as described above, and were pulsed with 1 mg/ml OVA in the presence or absence of 75 μ g/ml *Alternaria* extract for 18 h. DCs were washed three times with PBS and transferred intranasally (0.5×10^6 cells) to lightly anesthetized naive BALB/c mice. To assess the humoral immune responses to OVA, DCs were transferred on days 0 and 7. Plasma was collected on day 14, and the concentrations of OVA-specific IgE, IgG1, and IgG2 were measured by ELISA, as described above. To assess the lung inflammatory responses, DCs were transferred only on day 0. Mice were then challenged by intranasal OVA on days 14, 15, and 16. On day 17, mice were killed, and BAL fluids were analyzed for eosinophils.

Statistical analysis

Data are presented as the mean $\pm$ SEM for the numbers of mice or experiments indicated. The statistical significances of the differences between various treatment groups were assessed with Student's *t* test; $p < 0.05$ was considered significant.

Results

Fungal extracts promote airway Th2-type sensitization to OVA

Respiratory exposure to innocuous Ag, such as endotoxin-free OVA, generally induces immunologic tolerance (4, 5). Therefore, to test the immunostimulatory capacity of fungal extracts, we exposed mouse airways to OVA with and without fungal extracts (Fig. 1A). By day 14, airway exposure to OVA alone produced minimal increases in the plasma levels of anti-OVA IgE, IgG1, and IgG2a (Fig. 1B). In contrast, when animals were exposed intranasally to OVA plus *Alternaria* extract, anti-OVA IgE and anti-OVA IgG1 (i.e., Ig classes driven by Th2 cytokines) increased ($p < 0.05$ and $p < 0.01$, respectively). In mice exposed to OVA plus *Alternaria* extract, anti-OVA IgG2a (i.e., a Ig subclass driven by Th1 cytokines) decreased ($p < 0.05$). Minimal levels of IgE Abs to an *Alternaria* Ag, Alt a 1, were detectable in mice exposed to OVA plus *Alternaria* extract or *Alternaria* extract alone (OD450 < 0.025). When extracts from two other fungus genera, namely *Aspergillus* and *Candida*, were used with intranasal OVA exposure, anti-OVA IgE did not increase. However, OVA plus *Aspergillus* extract increased anti-OVA IgG1 and decreased anti-OVA IgG2a ($p < 0.05$), and OVA plus *Candida* extract also increased anti-OVA IgG1. Thus, when mouse airways are exposed to OVA in the presence of these fungi, *Alternaria* and, to a lesser degree, *Aspergillus* and *Candida* likely promote the development of Th2-type and inhibit Th1-type humoral immune responses to OVA.

To examine the airway recall responses to OVA Ag, these mice were also challenged with OVA intranasally on days 21, 22, and

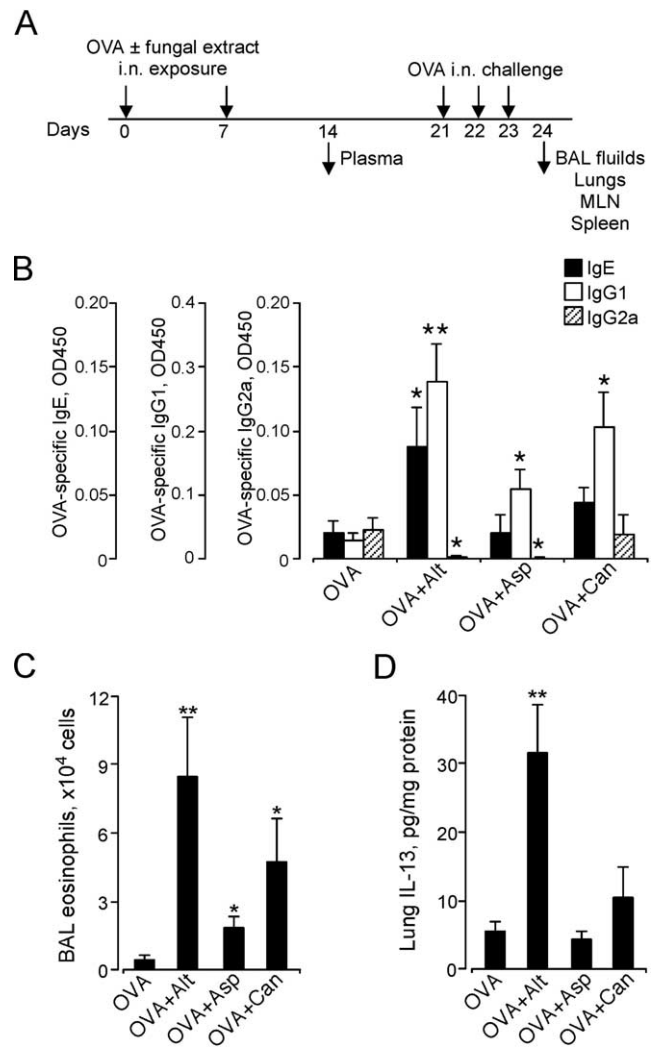


FIGURE 1. Fungi promote Th2-type airway sensitization in a mouse airway sensitization and challenge model. *A*, A schematic representation of a mouse airway sensitization and challenge model. On days 0 and 7, BALB/c mice were intranasally exposed to 100 μ g of OVA with or without 50 μ g of fungal extract. On day 14, plasma was collected for analysis of OVA-specific IgE, IgG1, and IgG2a. On days 21, 22, and 23, mice were challenged by intranasal administration of 100 μ g of OVA. On day 24, BAL fluids, lungs, MLN, and spleen were collected for analyses of inflammation and cytokine responses. *B*, Mice were intranasally exposed to OVA alone or OVA with fungal extracts (Alt; *Alternaria*, Asp; *Aspergillus*, Can; *Candida*). The day 14 levels of OVA-specific Abs, including IgE, IgG1, and IgG2a in the plasma, were measured by ELISA. On day 24, BAL fluids and lungs were collected and analyzed for the number of eosinophils (*C*) and the levels of IL-13 (*D*) in lung homogenates, respectively. Results are the mean $\pm$ SEM ($n = 6-9$ in each group). *, $p < 0.05$; **, $p < 0.01$ compared with mice previously exposed to OVA alone.

23 (Fig. 1A, vide supra). In mice previously exposed to OVA alone, the OVA challenge produced minimal airway eosinophilia (Fig. 1C). In contrast, in mice previously exposed to OVA plus *Alternaria* extract, the OVA challenge produced a marked airway eosinophilia ($p < 0.01$). In mice exposed to OVA plus *Aspergillus* or *Candida* extracts, the OVA challenge also produced airway eosinophilia, although the eosinophilia was not as pronounced as in mice previously exposed to OVA plus *Alternaria* ($p < 0.05$). In mice previously exposed to fungal extracts alone, the OVA challenge showed minimal airway inflammation (i.e., $< 0.8 \times 10^4$ eosinophils) (data not shown). In mice previously exposed to OVA

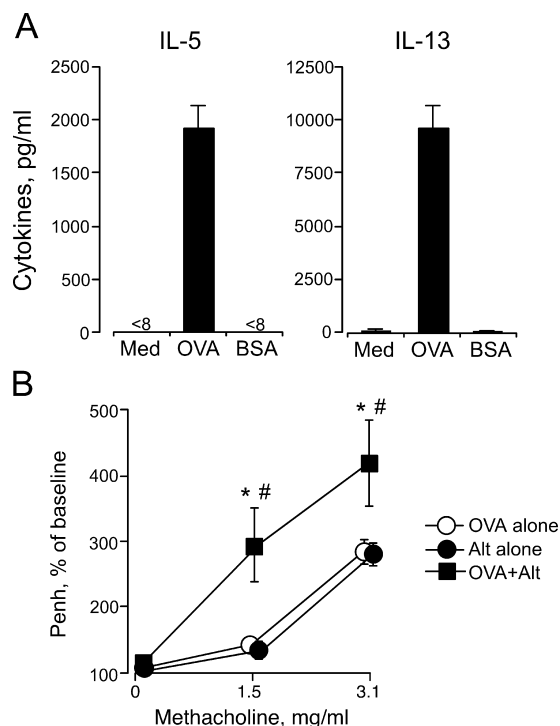


FIGURE 2. Recall response of draining lymph node cells and airway reactivity in mice exposed to OVA and *Alternaria* extract. **A**, Mice were intranasally exposed to OVA plus *Alternaria* extract and challenged intranasally with OVA as in Fig. 1A. On day 24, MLN cells were harvested and cultured with medium alone or with 100 μ g/ml OVA or BSA for 4 days. The levels of IL-5 and IL-13 in the supernatants were measured by ELISA. Results are the mean $\pm$ SD of four separate wells from one of two experiments, which showed similar results. **B**, Mice were intranasally exposed to OVA alone, *Alternaria* extract alone, or OVA plus *Alternaria* extract as shown in Fig. 1A and then challenged with OVA on days 42, 43, and 44. Airway reactivity to methacholine was examined on day 45. Results are a percentage of baseline (before PBS or methacholine challenge) and presented as mean $\pm$ SEM ($n = 5$ mice in each group). *, $p < 0.05$; #, $p < 0.05$ compared with mice exposed to OVA alone or *Alternaria* extract alone, respectively.

and *Alternaria* extract, the OVA challenge produced a marked increase in IL-13 levels in lung homogenates compared with mice previously exposed to OVA alone (Fig. 1D) ($p < 0.01$); no increase in IFN- γ was observed in lung homogenates from those mice previously exposed to OVA and *Alternaria* extract and challenged with OVA (data not shown).

To verify the Ag specificity of the airway recall response, the draining MLN cells from mice exposed previously to OVA plus *Alternaria* extract were cultured with OVA or BSA in vitro. When cultured with OVA, these MLN cells produced large quantities of IL-5 and IL-13 (Fig. 2A). In contrast, when incubated with medium alone or BSA, these MLN cells did not produce detectable IL-5 or produced minimal IL-13. When MLN cells from mice previously exposed to *Alternaria* extract alone were cultured with OVA, no IL-5 or IL-13 could be detected (data not shown).

To examine physiological changes in mice, we examined their airway reactivity to methacholine by whole-body plethysmography. Mice were intranasally exposed to OVA alone, *Alternaria* extract alone, or OVA plus *Alternaria* extract and then were intranasally challenged with OVA. Mice previously exposed to OVA plus *Alternaria* extract showed increased airway reactivity to methacholine after OVA challenge, compared with those mice previously exposed to OVA alone or *Alternaria* extract alone and then

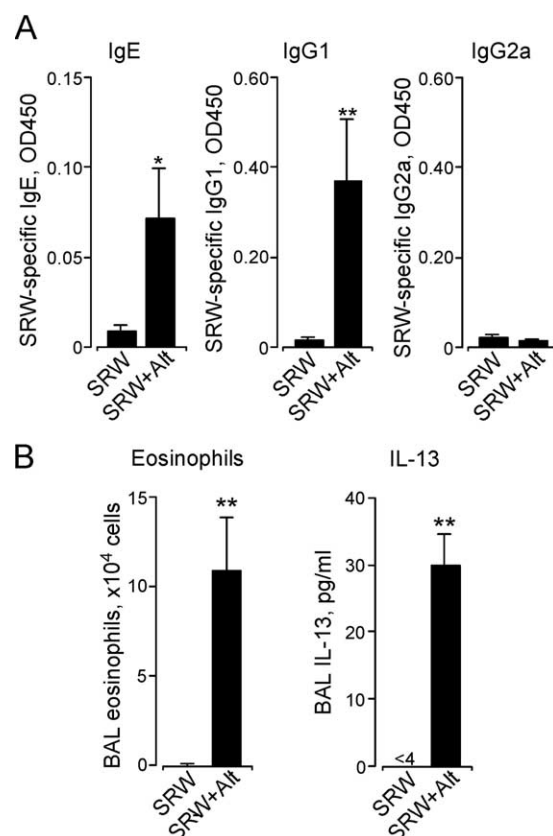


FIGURE 3. *Alternaria* promotes Th2-type airway sensitization to SRW. On days 0 and 7, mice were intranasally exposed to SRW extract alone (SRW) or SRW extract plus *Alternaria* extract (SRW+Alt). **A**, On day 14, plasma was collected, and the levels of SRW-specific Abs were measured by ELISA. On days 21, 22, and 23, mice were then challenged intranasally with SRW alone. **B**, On day 24, BAL fluids were analyzed for airway eosinophilia and IL-13 levels. Results are the mean $\pm$ SEM ($n = 7-8$ in each group). *, $p < 0.05$; **, $p < 0.01$ compared with mice exposed to SRW alone.

challenged with OVA (Fig. 2B). The methacholine dosages needed to increase airway reactivity to 250% of baseline were significantly lower in mice exposed to OVA plus *Alternaria* extract (1.4 ± 0.4 mg/ml) than in mice exposed to OVA alone (2.7 ± 0.3 mg/ml, $p < 0.05$) or *Alternaria* extract alone (2.9 ± 0.2 mg/ml, $p < 0.05$).

To examine whether mice are sensitized systemically, splenocytes from mice previously exposed to OVA plus *Alternaria* extract were tested similarly. When splenocytes were cultured with OVA, they produced IL-5 and IL-13 (228 ± 41 and 1092 ± 54 pg/ml, respectively); when cultured with BSA, splenocytes produced <8 pg/ml IL-5 and 25 ± 39 pg/ml IL-13. When splenocytes from mice previously exposed to OVA alone or *Alternaria* extract alone were cultured with OVA, IL-5 and IL-13 were undetectable (data not shown). Altogether, airway exposure of mice to OVA in the presence of *Alternaria* extract likely promotes local and systemic Th2-type sensitization to OVA, whereas exposure to OVA alone fails to sensitize animals.

Alternaria extract facilitates airway Th2-type sensitization to SRW

To examine whether these Th2-type adjuvant effects of *Alternaria* extract in the airways extend to clinically relevant allergens, we exposed mice intranasally to SRW extract with and without *Alternaria* extract. By day 14, airway exposure to SRW extract alone induced minimal anti-SRW IgE and IgG1; in contrast, exposure to

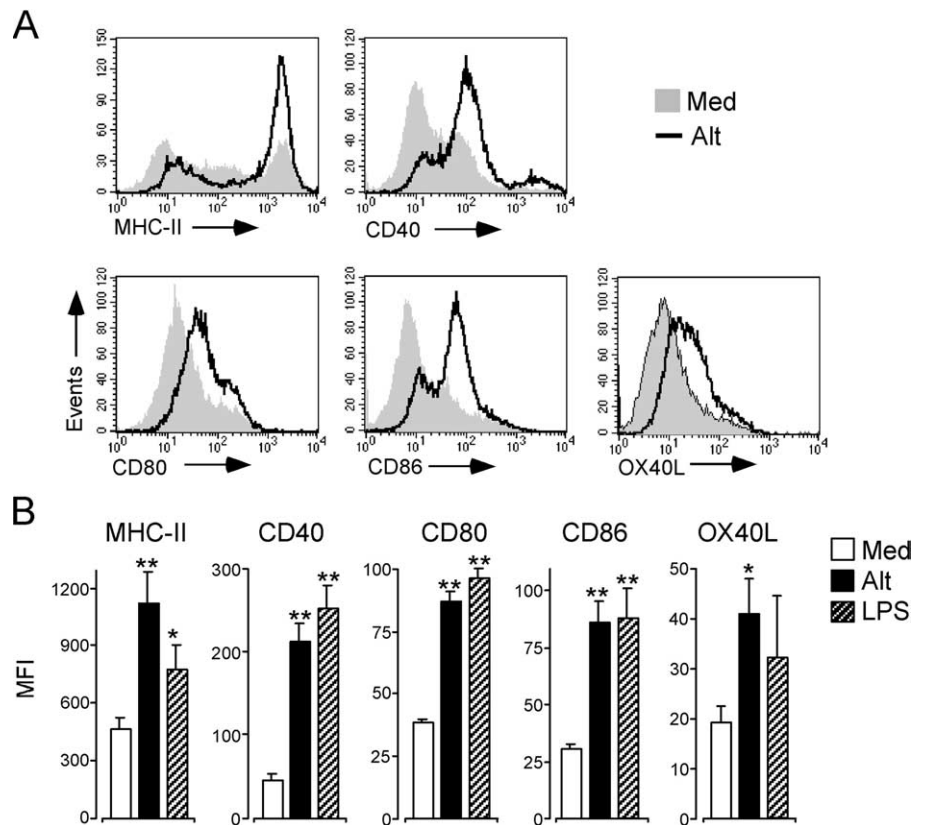


FIGURE 4. *Alternaria* enhances expression of MHC-II and costimulatory molecules by BM-derived DCs. BM-derived DCs were incubated with medium alone (Med), 100 μ g/ml *Alternaria* extract (Alt), or 1 μ g/ml LPS for 24 h (MHC-II, CD40, CD80 and CD86) or 48 h (OX40L). The expression of MHC-II and costimulatory molecules was analyzed by FACS. **A**, Representative histograms show expression on DCs incubated with medium alone or *Alternaria* extract. **B**, Mean expression levels are shown from four independent experiments (mean $\pm$ SEM). *, $p < 0.05$; **, $p < 0.01$ compared with medium alone. MFI, mean fluorescence intensity.

SRW with *Alternaria* extract induced marked increases in anti-SRW IgE and IgG1 but not IgG2 (Fig. 3A). Similar to mice exposed to OVA without *Alternaria*, no airway eosinophilia or increased IL-13 was observed in day 24 specimens from mice exposed to and challenged with SRW alone (Fig. 3B). In contrast, the day 24 specimens from mice previously exposed to SRW in the presence of *Alternaria* extract and challenged with SRW showed marked airway eosinophilia and increased IL-13. Thus, the Th2-type adjuvant activity of *Alternaria* likely extends to clinically relevant, complex allergens.

Alternaria induces distinctive activation of DCs

After encountering Ags, DCs integrate signals from pathologic proteins into a signal that instructs naive T cells (27). Furthermore, DCs critically determine the outcome of T cell differentiation, such as Th1, Th2, and regulatory T cells (28). Therefore, to investigate the underlying mechanisms for the potent Th2-type adjuvant capacity of *Alternaria* in vivo, we studied the effects of *Alternaria* extract on BM-derived DCs in vitro. We used a known TLR4 agonist, *E. coli* LPS, as a positive control. When DCs were incubated for 24 h with *Alternaria* extract, their expression of MHC-II and costimulatory molecules, including CD40, CD80, and CD86, increased dramatically (Fig. 4A). After 48 h, *Alternaria* extract also up-regulated the expression of OX40L, a costimulatory molecule implicated in Th2 development (29–31). The expression levels of costimulatory molecules in DCs cultured with 100 μ g/ml *Alternaria* extract were roughly comparable to the levels after DCs were cultured with 1 μ g/ml LPS (Fig. 4B).

Alternaria extract also induced BM-derived DCs to produce and release IL-6 in a time-dependent manner; however, LPS induced IL-6 levels from DCs ~20- to 50-fold higher (Fig. 5A). Furthermore, *Alternaria* extract did not induce detectable IL-12p70 production up to 48 h, whereas LPS induced IL-12p70 production

within 4 h. Thus, while *Alternaria* extract can induce DCs to produce certain cytokines, its potency appears to be weaker than LPS or it may act differently on DCs compared with LPS. To investigate this question further, we stimulated DCs with LPS with or

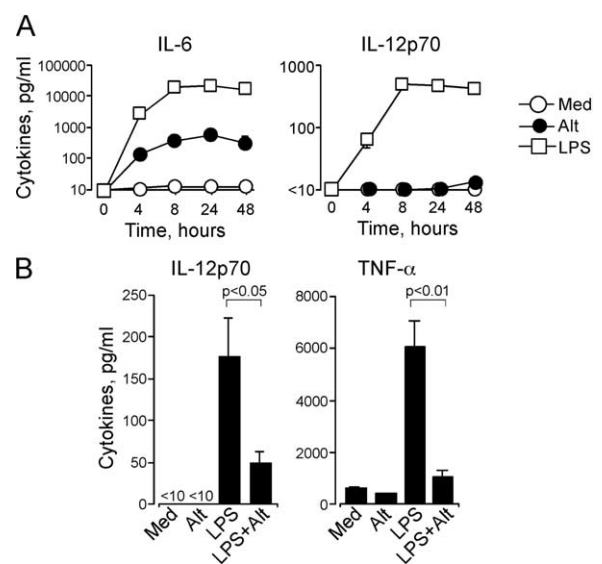


FIGURE 5. Cytokine production by DCs stimulated with *Alternaria* extract, LPS, or both. **A**, BM-derived DCs were incubated with medium alone, 100 μ g/ml *Alternaria* extract (Alt), or 1 μ g/ml LPS for indicated times. IL-6 and IL-12p70 levels in the supernatants were measured by ELISA. Results are mean $\pm$ SEM ($n = 3$ –4). **B**, BM-derived DCs were incubated with medium alone, 100 μ g/ml *Alternaria* extract (Alt), 1 μ g/ml LPS, or a combination of Alt and LPS for 24 h. IL-12p70 and TNF- α levels in the supernatants were measured by ELISA. Results are mean $\pm$ SEM ($n = 3$).

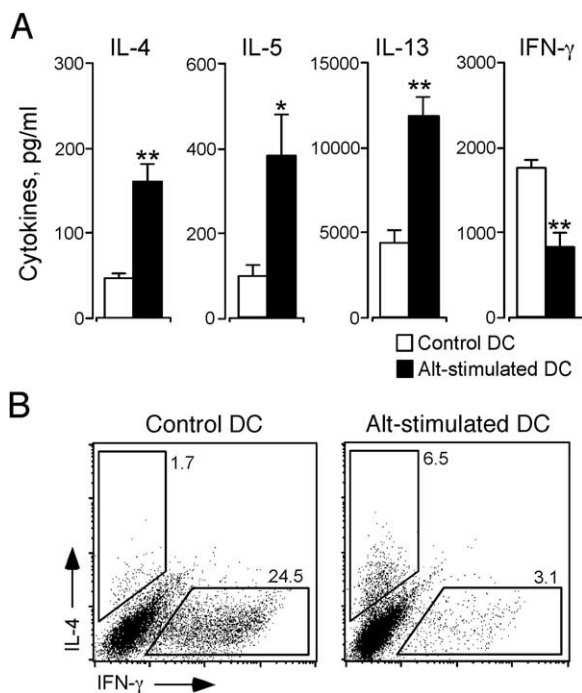


FIGURE 6. DCs stimulated with *Alternaria* extract promote production of Th2 cytokines by allogenic CD4⁺ T cells. BM-derived DCs from BALB/c mice were incubated with medium alone (control DCs) or 75 μ g/ml *Alternaria* extract (Alt-stimulated DCs) for 24 h and washed. **A**, Allogenic CD4⁺ T cells from B6 mice were cultured with these DCs for 6 days, and supernatants were analyzed for cytokines by ELISA. Results show mean $\pm$ SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with control DCs. **B**, On day 4 of coculture, cells were restimulated with anti-CD3/CD28 for 24 h. Intracellular cytokines were stained and analyzed by FACS by gating on CD4⁺ T cells, as described in *Materials and Methods*. Representative data are shown from three reproducible experiments. Numbers indicate percentages of IL-4- and IFN-γ-positive cells.

without *Alternaria* extract. As expected, *Alternaria* alone did not induce IL-12p70 production, but LPS did (Fig. 5B). Interestingly, DCs cultured with *Alternaria* plus LPS showed that *Alternaria* inhibited the IL-12p70 production by 75%. Similarly, DCs incubated with LPS produced TNF- α , but DCs incubated with *Alternaria* did not. When DCs were cultured with *Alternaria* plus LPS, the *Alternaria* markedly inhibited the TNF- α production. Thus, although *Alternaria* extract activates DC expression of MHC-II and costimulatory molecules similarly to LPS, *Alternaria* appears to produce different effects on cytokine production, such as IL-12 and TNF- α .

DCs stimulated with *Alternaria* extract promote CD4⁺ T cell production of Th2-type cytokines

We used MLR and a TCR-transgenic OTII system to examine the integrated effects of the DCs' responses to *Alternaria* extract on the production of cytokines by CD4⁺ T cells. First, for the MLR, BM-derived DCs from BALB/c mice were stimulated with *Alternaria* extract or medium control, washed, and then incubated with allogenic CD4⁺ T cells from B6 mice. Allogenic CD4⁺ T cells incubated with control DCs (i.e., stimulated with medium control) produced IL-4, IL-5, IL-13, and IFN- γ (Fig. 6A). Importantly, allogenic CD4⁺ T cells incubated with *Alternaria*-stimulated DCs produced more IL-4, IL-5, and IL-13 but produced less IFN- γ . Intracellular staining verified that the frequency of IL-4-producing CD4⁺ T cells increased and IFN- γ -producing CD4⁺ T cells de-

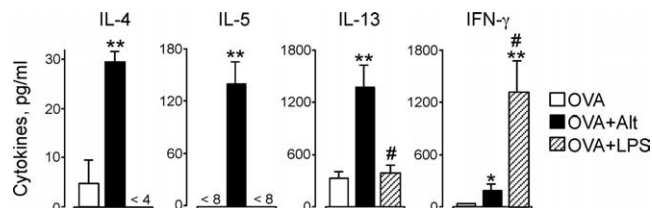


FIGURE 7. DCs stimulated with *Alternaria* and LPS induce distinctive cytokines from Ag-specific CD4⁺ T cells. BM-derived DCs from B6 mice were pulsed with 100 μ g/ml OVA in medium alone (OVA) or in the presence of 75 μ g/ml *Alternaria* extract (OVA+Alt) or 1 μ g/ml LPS (OVA+LPS) for 24 h and washed extensively. CD4⁺ T cells from OTII mice were incubated with these OVA-pulsed DCs. On day 6, supernatants were analyzed for IL-4, IL-13, and IFN- γ and on day 10 for IL-5 by ELISA. Results show mean $\pm$ SEM from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with DCs pulsed with OVA alone. #, $p < 0.05$ compared with DCs pulsed with OVA in the presence of *Alternaria*.

creased when allogenic CD4⁺ cells were incubated with *Alternaria*-stimulated DCs compared with CD4⁺ cells incubated with control DCs (Fig. 6B).

Second, for the TCR-transgenic OTII system, BM-derived DCs from B6 mice were pulsed with OVA in the presence or absence of *Alternaria* extract or LPS. These DCs were washed and incubated with CD4⁺ T cells isolated from spleens of OTII mice; these CD4⁺ T cells express a transgenic TCR that recognizes an OVA peptide presented by MHC-II. The OTII CD4⁺ T cells incubated with DCs pulsed with OVA alone produced IL-13 but minimal or undetectable amounts of IL-4, IL-5, or IFN- γ (Fig. 7). The OTII CD4⁺ T cells incubated with DCs pulsed with OVA in the presence of *Alternaria* extract produced markedly increased levels of IL-4, IL-5, and IL-13 and produced some IFN- γ . In contrast, the OTII CD4⁺ T cells incubated with DCs pulsed with OVA in the presence of LPS produced a marked increase in IFN- γ but did not produce IL-4, IL-5, or IL-13 compared with OVA alone.

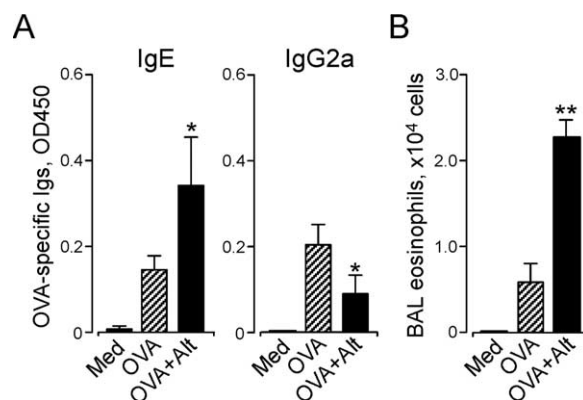


FIGURE 8. Intranasal transfer of DCs pulsed with OVA in the presence of *Alternaria* ex vivo enhances Th2-type humoral immunity and airway inflammation to OVA in vivo. BM-derived DCs were pulsed in vitro with medium alone, 1 mg/ml OVA, or OVA in the presence of 75 μ g/ml *Alternaria* extract for 18 h. DCs were then washed extensively and transferred intranasally (0.5×10^6 cells) into naive mice. **A**, On days 0 and 7, DCs were transferred; on day 14, plasma was collected, and the levels of OVA-specific IgE, IgG1, and IgG2a Abs were measured by ELISA. **B**, DCs were transferred on day 0, and on days 14, 15, and 16, mice were challenged by intranasal administration of OVA. On day 17, BAL fluids were analyzed for the number of eosinophils. Results are the mean $\pm$ SEM ($n = 7-10$ in each group). *, $p < 0.05$; **, $p < 0.01$ compared with DCs pulsed with OVA alone.

In vivo airway transfer of *Alternaria*-activated DCs promote Th2 sensitization

These *in vitro* observations suggest that DCs are activated by *Alternaria* extract and promote CD4⁺ T cell differentiation toward a Th2 type. To clarify the *in vivo* role(s) of the DCs, which have been stimulated with *Alternaria*, and the development of Th2 responses, we used a DC adoptive transfer model. BM-derived DCs were pulsed with OVA in the presence or absence of *Alternaria* extract. After washing, these DCs were transferred intranasally to naive BALB/c mice. On day 14, the transfer of OVA-pulsed DCs induced production of both anti-OVA IgE and anti-OVA IgG2a in naive animals (Fig. 8A). Importantly, the transfer of DCs pulsed with OVA in the presence of *Alternaria* extract induced increased production of anti-OVA IgE and decreased production of anti-OVA IgG2a ($p < 0.05$). Furthermore, in airway OVA challenge experiments, the naive BALB/c mice, which had been transferred intranasally with DCs pulsed with OVA in the presence of *Alternaria* extract, showed increased airway eosinophilia, compared with those mice transferred with DCs pulsed with OVA in the absence of *Alternaria* extract ($p < 0.01$) (Fig. 8B).

Discussion

Because the airways are constantly exposed to environmental Ags and infectious agents, the important feature of the lung's immune system is its ability to tightly regulate immune tolerance and response. In general, soluble nonpathogenic proteins (e.g., OVA without LPS contamination), when delivered through intact mucosal surfaces, do not provoke strong immune responses but instead induce a state of Ag-specific hyporesponsiveness (4, 32). Consistent with previous reports (4, 32), we found that airway exposure to OVA alone induced minimal sensitization to OVA. In contrast, when OVA was administered in the presence of fungal extracts, in particular *Alternaria*, mice developed robust IgE and IgG1 Abs and Th2-type cytokine responses. Similarly, airway exposure to a clinically relevant allergen, SRW, alone did not induce sensitization to SRW, but SRW plus *Alternaria* induced marked Th2 responses to the allergen. Thus, airway exposure to *Alternaria* likely prevents tolerance to innocuous Ags and facilitates Th2-type airway sensitization. We found previously that human eosinophils demonstrate innate immune responses to *Alternaria* (33) and, when exposed to the products of the fungus, eosinophils release proinflammatory mediators (34). Therefore, the potent biological activities of *Alternaria* (and perhaps other fungi) facilitate both the sensitization and effector phases of pathologic Th2 responses. These activities and responses may provide a mechanistic explanation for the well-known association between fungi and the development and exacerbation of asthma and allergic airway diseases in humans (17–19, 35).

DCs are one of the key cellular components that regulate immune tolerance and response (4, 36–38). Generally, DCs, which express low levels of MHC-II and costimulatory molecules, induce T cell tolerance or unresponsiveness (5). By producing various soluble or membrane-bound factors, DCs also determine the outcome of T cell responses, such as Th1, Th2, or Treg responses (39). Herein, when DCs were stimulated with *Alternaria* *in vitro*, we observed the following: they up-regulated their expression of MHC-II and costimulatory molecules, including CD40, CD80, CD86, and OX40L; they also produced a proinflammatory cytokine, IL-6, but did not produce IL-12 or TNF- α ; and they facilitated CD4⁺ T cells to produce Th2-type cytokines *in vitro*. Furthermore, when DCs pulsed with OVA in the presence of *Alternaria* extract were adoptively transferred to mouse airways *in vivo*, they stimulated Th2-type airway sensitization to OVA. Thus,

DCs likely respond to the product(s) of *Alternaria*, and these DCs, after exposure to *Alternaria*, likely skew the CD4⁺ T cells' responses to a Th2-type both *in vitro* and *in vivo*.

Although *E. coli* LPS contamination in an OVA preparation was reported to promote airway Th2 sensitization to intranasal OVA (22), LPS contamination in the *Alternaria* extract is unlikely to be responsible for *Alternaria*'s potent Th2 adjuvant activity. The reasons are as follows. 1) The *Alternaria* preparation contained minimal endotoxin activity (11.7 EU/mg, ~0.02 ng LPS/exposure), which is much smaller than that used previously to promote an airway Th2 response to OVA (i.e., 100 ng/exposure (22)). 2) LPS, but not *Alternaria* extract, induced production of IL-12 by DCs. Rather, *Alternaria* extract inhibited LPS-induced IL-12 and TNF- α production (Fig. 5). 3) The LPS-mediated Th2 response was dependent on TLR4 (22); however, the *Alternaria*-mediated Th2 response was independent of TLR4 and TLR2 (40).

What product(s) of *Alternaria* possesses such potent Th2 adjuvant activity? A review of the literature suggests several candidates. For example, certain biological activities of allergens, such as reactive oxygen species and protease activities, may be involved (41–44). Furthermore, carbohydrate structures, such as chitin and a peanut allergen glycan, induce the accumulation of inflammatory cells associated with allergic responses and Th2-skewed responses (7, 45). Therefore, one or more analogous molecules in the *Alternaria* extract may act individually or in concert to induce robust Th2 responses. In pilot studies, we found that these DC-stimulating activities of *Alternaria* extract are highly heat labile, becoming inactivated in 30 min at 56°C, suggesting a proteinaceous nature of the molecule(s) (data not shown). We are continuing efforts to isolate and identify the Th2 adjuvant(s) in the *Alternaria* extract.

Another question remains: how do DCs exposed to *Alternaria* polarize the T cell response to the Th2 type? Because the IL-12-family cytokines are potent inducers of the Th1 response (28), one important condition for DCs to induce Th2 responses is likely the down-regulation of these cytokines. Several studies suggested that Th2 responses likely result from the absence of IL-12 (46, 47) (i.e., a default model). In contrast, other studies show that Th2 cell development requires active participation of Th2-polarizing factors expressed or produced by DCs (48, 49) (i.e., an instruction model). For example, the interaction between OX40L on APC and OX40 on T cells plays roles in the proliferation of CD4⁺ T cells and also contributes to Th2 development (29, 30, 50). A critical role for the OX40L-OX40 interaction in the Th2 response was further supported by a murine model of leishmaniasis (31) and by DCs stimulated with thymic stromal lymphopoietin (TSLP) (49). Nevertheless, the TSLP-induced OX40L on DCs lost its ability to induce Th2 cells in the presence of IL-12 (49), suggesting that the Th1-promoting activity of IL-12 may be dominant. IL-6 may suppress IL-12-mediated T cell polarization and direct Th2 differentiation of naive T cells (51). Therefore, a critical balance and perhaps even a hierarchy likely exist among the Th1- and Th2-polarizing soluble factors and cell surface molecules for optimal development of the Th2 response. Interestingly, we found that *Alternaria* potentially induced the expression of IL-6 and OX40L by DCs (Fig. 4). In addition, *Alternaria* did not induce but rather inhibited IL-12 production by DCs (Fig. 5). Furthermore, microarray studies showed that genes for CCL17, CXCL5, ICOS, and TNFR superfamily member 9 (CD137 or 4-1BB) are highly expressed by DCs incubated with *Alternaria* compared with DCs incubated with LPS (data not shown). Further studies are necessary to pinpoint whether expression of OX40L is sufficient, whether down-regulation of IL-12 is also necessary, and whether any other

molecules, as listed above, are involved in the ability of DCs exposed to *Alternaria* to polarize CD4⁺ T cells robustly to a Th2 type.

In summary, our study demonstrates potent Th2 adjuvant effects in the airways for the asthma-related environmental fungus *Alternaria*. DCs, which respond directly to an *Alternaria*-derived molecule(s), are likely involved in airway sensitization and the Th2-type polarization of CD4⁺ T cells. These observations suggest a mechanistic explanation for the clinical observations that link fungus exposure and allergic airway diseases. In contrast, the immune systems in the airways are likely to be complex, involving not only DCs and T cells but also other airway-resident cells, such as epithelial cells and macrophages. For example, overexpression of TSLP by airway cells activates DCs and promotes Th2-type airway inflammation and IgE production (52). We found that in vitro exposure of human airway epithelial cells to *Alternaria* extract induces production and extracellular release of TSLP (data not shown). Thus, the robust, *Alternaria*-induced Th2 development in the airways in vivo may be due to the synergistic effects of *Alternaria* on DCs and on other airway cells. Another important question is as follows: if *Alternaria* is a potent airway Th2 adjuvant for innocuous Ags, why do only some, but NOT all, individuals or animals develop allergic airway diseases? Further analyses of specific immune and structural airway cells and immunological molecules that are involved in the robust Th2 adjuvant effects of *Alternaria* will better elucidate the mechanisms involved in the development (and prevention) of Th2-type immune responses in the airways. This better understanding will foster the invention of novel strategies to prevent the pathologic immunological pathways and to intervene in the development and/or exacerbation of allergic airway diseases.

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Disclosures

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