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

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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 yet 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 naïve 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.

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*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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were coated with 10 ng/ml of 0.05% Tween 20. The reaction was stopped with 1 M HCl after 20 min. The absorbance at 450 nm was measured by adding HRP substrate (TMB substrate kit; Pierce) was added, and the reaction was incubated for 30 min at room temperature. After the final washing, the absorbance at 492 nm was measured by enhanced microplate reader (Perkin Elmer). Sensitivity for IL-5 and 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 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 × 10^7/ml of 75 ml of RPMI 1640 medium (Invitrogen Life Technologies), were added to the wells at a 1:5 DC:T cell ratio and cultured for 6 days; the purity of CD11c⁺ DCs was ~95%. Day 6 DCs were stimulated for 24–48 h with Alternaria extract (25–100 μg/ml) or E. coli (O127:B8) LPS (1 μg/ml; Sigma-Aldrich) or PBS alone. DCs were stimulated with 200 ml of RPMI 1640 and were seeded in round-bottom wells of 96-well culture plates at 2 × 10^4 cells/well. 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-A, 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 HL-3) and FITC-conjugated anti-MHC-II-I-A (AMS-32.1), anti-CD40 (HM40-3), anti-CD80 (16-10A1), and anti-CD86 (GL1) or biotinylated anti-CD40 (R&D Systems); sensitivity for 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.

Ag-specific IgE, IgG1, and IgG2a
To quantitate OVA-, SRW-, and Alt a 1-specific IgE, ELISA plates were coated with 50 μg/ml of OVA or SRW extract in the presence or absence of 50 μg/ml of 0.05% Tween 20 (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 μg of OVA or SRW in 50 μl of PBS; on day 24, mice were killed by an overdose of ketamine and xylazine. The sera 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 × g at 4°C for 15 min, and the protein concentrations of the supernatants were quantitated 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.

Mouse airway sensitization and challenge model
On days 0 and 7, BALB/c mice were lightly anesthetized with avertin and intranasally administered with 100 μg of OVA or SRW extract in the presence or absence of 50 μg/ml 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 μg of OVA or SRW in 50 μl of PBS; on day 24, mice were killed by an overdose of ketamine and xylazine. The sera 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 × g at 4°C for 15 min, and the protein concentrations of the supernatants were quantitated 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.

Cytokine production by mediastinal lymph node (MLN) cells and splenocytes
On day 24 of the airway sensitization and challenge model (vida 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 μM 2-ME, 100 units/ml streptomycin, and 100 μg/ml penicillin (complete RPMI 1640) at 4 × 10^6 cells/ml; 100 μl of cell suspension was dispensed into each well of 96-well tissue culture plates (Corning Costar). The cells were stimulated by adding 100 μl of 200 μg/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.

Materials and Methods

**Mice**
BALB/c (BALB/c), C57BL/6 (B6), and C57BL/6-Tg(TcraTcrb)25Cbn/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 μg) 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 μg of OVA or SRW extract in the presence or absence of 50 μg/ml 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 μg of OVA or SRW in 50 μl of PBS; on day 24, mice were killed by an overdose of ketamine and xylazine. The sera 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 × g at 4°C for 15 min, and the protein concentrations of the supernatants were quantitated 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 were coated with 50 μg/ml of OVA or SRW extract in the presence or absence of 50 μg/ml 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 μg of OVA or SRW in 50 μl of PBS; on day 24, mice were killed by an overdose of ketamine and xylazine. The sera 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 × g at 4°C for 15 min, and the protein concentrations of the supernatants were quantitated 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.

**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 μg/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/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 CD4⁺: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 (Biocomat T-Cell Activation Plates; BD Pharmingen) and soluble anti-CD28 (2 μg/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 FACSscan 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 μg/ml endotoxin-free OVA with or without 75 μg/ml Alternaria extract or 1 μg/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 x 10^5 cells/10 μl 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 μg/ml Alternaria extract for 18 h. DCs were washed three times with PBS and transferred intranasally (0.5 x 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 ± 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., Ig subclass driven by Th1 cytokines) decreased (p < 0.05). Minimal levels of IgE Abs to an Alternaria Ag, Alt a 1, were detectible 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 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 x 10^4 eosinophils) (data not shown). In mice previously exposed to OVA...
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). When cultured with OVA, these MLN cells produced large quantities of IL-5 and IL-13 (Fig. 2A). When cultured with BSA, these MLN cells did not produce detectable IL-5 or IL-13 (Fig. 2A). In contrast, when cultured with medium alone or BSA, these MLN cells did not produce detectable IL-5 or IL-13 (data not shown).

To examine 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). When cultured with BSA, these MLN cells did not produce detectable IL-5 or IL-13 (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 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
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 naïve 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 μg/ml *Alternaria* extract were roughly comparable to the levels after DCs were cultured with 1 μg/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 4. *Alternaria* enhances expression of MHC-II and costimulatory molecules by BM-derived DCs.

BM-derived DCs were incubated with medium alone (Med), 100 μg/ml *Alternaria* extract (Alt), or 1 μg/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 ± SEM). *p < 0.05; **p < 0.01 compared with medium alone. MFI; mean fluorescence intensity.

### FIGURE 5. Cytokine production by DCs stimulated with *Alternaria* extract, LPS, or both.

**A**, BM-derived DCs were incubated with medium alone, 100 μg/ml *Alternaria* extract (Alt), or 1 μg/ml LPS for indicated times. IL-6 and IL-12p70 levels in the supernatants were measured by ELISA. Results are mean ± SEM (n = 3–4). **B**, BM-derived DCs were incubated with medium alone, 100 μg/ml *Alternaria* extract (Alt), 1 μg/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 ± 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 μg/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 ± 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 decreased 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.
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 intransal 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/µg, ~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 potently 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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References


