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***Alternaria* Inhibits Double-stranded RNA-Induced Cytokines Productions through TLR3**

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Abstract

Background—Fungi may be involved in asthma and chronic rhinosinusitis (CRS). PBMCs from CRS patients produce IL-5, IL-13 and INF- γ by *Alternaria*. In addition, *Alternaria* produces potent Th2-like adjuvant effects in the airway. Therefore, we hypothesized that *Alternaria* may inhibit Th1-type defense mechanisms against virus infection.

Methods—Dendritic cells (DCs) were generated from mouse bone marrow. The functional responses were assessed by expression of cell surface molecules by FACS (MHC Class II, CD40, CD80, CD86 and OX40L. Production of IL-6, IP-10, I-TAC and IFN- β were measured by ELISA. TLR3 mRNA and protein expression were detected by quantitative Real time-PCR and Western blot.

Results—*Alternaria* and poly I:C enhanced cell surface expression of MHC Class II, CD40, CD80, CD86 and OX40L, and IL-6 production in a concentration-dependent manner. However, *Alternaria* significantly inhibited IP-10, I-TAC and IFN- β production induced by viral double-stranded RNA (dsRNA)-mimic poly I:C. TLR3 mRNA expression and protein production by poly I:C were significantly inhibited by *Alternaria*. These reactions are likely caused by heat-stable factor(s) in *Alternaria* extract with >100 kDa molecular mass.

Conclusion—These findings suggest that fungus, *Alternaria* may inhibit production of IFN- β and other cytokines by DCs by suppressing TLR3 expression. These results indicate that *Alternaria* may inhibit host innate immunity against virus infection.

Keywords

Alternaria; dsRNA; Poly I:C; dendritic cell; toll-like receptor-3; IP-10; I-TAC; IFN- β

INTRODUCTION

The prevalence of asthma has markedly increased over the past three decades (1). The etiology of asthma is not fully understood. It is considered that gene-environmental interactions likely play a pivotal role. Specifically, asthma may represent the exaggerated immune responses to chitin-containing organisms, including house-dust mite, cockroaches,

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Disclosures

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and fungi (2–4). Among various environmental stimuli, an association between fungal exposure and asthma has been clinically and epidemiologically recognized (5). In particular, exposure to *Alternaria*, which is ubiquitous both outdoors and indoors (6), is one of the major risk factors for developing asthma (7, 8). Viral infection is also associated with acute exacerbations for asthma. Respiratory tract viruses have emerged as the most frequent triggers for exacerbations in both children and adults (9, 10). However, the immunological mechanisms underlying these medical problems are poorly understood. In adults who are sensitized to allergens, exposure to sensitizing allergens and respiratory tract viral infection acted in a synergistic manner to increase significantly the risk of hospitalization with acute asthma (11).

Chronic rhinosinusitis (CRS) is one of the common chronic diseases. CRS is defined by the task force of the American Academy of Otolaryngology–Head Neck Surgery based on clinical symptoms and clinical diagnostic criteria (12). CRS has been treated by medications (13) and endoscopic sinus surgery (14). The pathogenesis and etiology of CRS are not fully understood. An association between fungi and chronic eosinophilic airway inflammation has been described in patients with severe asthma and certain types of CRS, such as allergic fungal rhinosinusitis (15). CRS is frequently refractory to treatment. Especially CRS patients with bronchial asthma or with aspirin-induced asthma sometime require long-term treatment with corticosteroids and repeated sinus surgery. CRS patients often experience development or worsening of their symptoms after a viral infection (16). Viral infection also has been shown to cause obstruction of sinus ostia (17), production of inflammatory mediators by nasal epithelial cells (18), and damage to epithelial cells and cilia (19).

Viruses and viral components are recognized by TLRs, mainly by endosomally located TLR3, TLR7, and TLR8. TLR3 detects dsRNA, which appears during viral replication processes and is expressed in placenta, pancreas, lung, liver, and heart (20), and especially in myeloid DCs and epithelial cells (21). Induction of immune responses by respiratory viruses involves TLR as well as the RNA helicases RIG-I and MDA-5 (22, 23). Both TLR3 and RNA helicases recognize double-stranded RNA (dsRNA) produced during viral infections as well as poly I:C, a synthetic ligand mimicking viral dsRNA (24–28). TLR3 induces type I IFN responses by activating IFN-regulatory factor (IRF) family, IRF3. Importantly, even low levels of autocrine or paracrine IFN trigger amplification of the antiviral response (29, 30). IFN- β is detected by IFN-receptor and forms IFN-stimulated gene factor, which translocates to the nucleus and induces the transcription of effector molecules. These effector molecules directly influence protein synthesis such as IP-10 and I-TAC.

In this study, we examined the effects of fungal products on innate immunity, in particular antiviral immunity produced by dendritic cells (DCs). We found that production of IFN- β and chemokines by DCs stimulated with viral RNA was profoundly inhibited by product(s) of fungus, *Alternaria*. These observations may explain diminished antiviral immunity in those patients with asthma or CRS.

MATERIALS AND METHODS

Mice

BALB/CJ mice were from Jackson Laboratory (Bar Harbor, ME). All mice were 7–9 week-old females. The procedures and the handling of those mice had been reviewed and approved by the Mayo Institutional Animal Care and Use Committee, Mayo Clinic Rochester, MN.

***Alternaria* extract**

Alternaria alternata culture filtrate fungal extract was from Greer Laboratories (Lenoir, NC). This extract was derived from media in which *Alternaria alternata* was grown. The culture filtrate was concentrated, dialyzed and lyophilized.

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

DCs were generated from mouse BM using an established protocol (31) with minor modifications (32). 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 six-well tissue culture plates, and cultured for 6 days; the purity of CD11c⁺ DCs was about 85%. Day 6 DCs were stimulated for 24–48 hours with *Alternaria* extract (25–50 µg/ml), or poly I:C (25 µg/ml) or combinations thereof. In separate experiments, we further purified DCs with a magnetic cell separation system (MACS; Miltenyi Biotec, Auburn, CA) 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 this study. The functional responses of DCs to *Alternaria* extract and poly I:C were assessed by expression of cell surface molecules by FACS, quantitative real-time PCR and Western blotting. Cytokine production was analyzed by ELISA. For FACS analysis, after stimulation for 24 h (MHC Class II, CD40, CD80, CD86) or 48 h (OX40L), DCs were preincubated with Fc-receptor blockers (anti-CD16/CD32) for 30 min at 4°C and stained with PE-conjugated anti-CD11c (clone HL3) and FITC-conjugated anti-MHC Class 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, Mountain View, CA) by gating on a CD11c-positive forward scatter-high population. For cytokine analysis, cell-free supernatants were collected after 24 h stimulation; concentrations of IL-6, IP-10, I-TAC and IFN-β were measured by ELISA kits (R&D Systems) according to the manufacturer's directions.

Quantitative Real-Time PCR

Total RNA was isolated from DCs using RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA was then transcribed into cDNA using Superscript II (Invitrogen, Grand Island, NY). Quantitative Real-Time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), according to the manufacturers' instructions. Mouse *TLR3* mRNA transcripts were quantified by quantitative real-time PCR with Taqman Gene Expression Array primer-probe sets (Applied Biosystems) and iCycler with iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). Transcription was normalized to the 18S rRNA transcription in each sample.

Western blot

DCs were lysed with Nupage sample buffer (Invitrogen). Samples were boiled for 10 minutes and clarified by centrifugation at 3000g at 4°C for 15 minutes with a microcentrifuge. Ten µg of cell lysate per lane was applied to SDS-PAGE and transferred electrically onto polyvinylidene difluoride transfer membranes. The membranes were blocked with PBS containing 0.1% Tween-20 and 5% skim milk, probed with the anti-

TLR3 antibodies, and visualized with an enhanced chemiluminescence system (Amersham, Piscataway, NJ). Densitometric analysis was carried out with National Institutes of Health image software. The increased densities were compared with the density of samples preincubated with medium alone; they are reported as ratios.

Statistical analysis

Data presented as means ($\pm$ SEM). One-way repeated measures ANOVA were used to assess significant differences, with post hoc analysis using Dunnett's or Tukey's tests. Differences were considered statistically significant at $P < 0.05$.

RESULTS

***Alternaria* and Poly I:C induce IL-6 production and enhance expression of costimulatory molecules and MHC-II by DCs**

To investigate the effects of *Alternaria* extract and poly I:C, we examined IL-6 production and expression of cell surface molecules. DCs (1×10^6 cells) were incubated with *Alternaria* (0, 25, 50 $\mu\text{g/ml}$) and poly I:C (0, 25 $\mu\text{g/ml}$) for 24 hrs or 48 hrs. *Alternaria* extract induced BM-derived DCs to produce and release IL-6 in a dose-dependent manner (Fig. 1). Poly I:C alone also induced IL-6 production. There were no additive or synergistic effects of *Alternaria* and poly I:C, when they were added together to DCs. In addition, *Alternaria* increased expressions of MHC class-II, CD40, CD80 as well as OX40L, a costimulatory molecule implicated in Th2 development (Fig. 2). No apparent effects were observed when poly I:C was added to DCs cultured with *Alternaria* extract. These findings suggest that *Alternaria* induces IL-6 production and enhances expression of MHC class II and co-stimulatory molecules by DCs.

***Alternaria* inhibits IP-10, I-TAC and IFN- β productions by stimulation of Poly I:C**

To investigate the effects of *Alternaria* on anti-viral innate immune responses, we examined IP-10, I-TAC and IFN- β production by DCs; these chemokines and cytokine suppress viral proliferation and growth. Poly I:C significantly induced IP-10, I-TAC and IFN- β production from DCs (Fig. 3). *Alternaria* did not induce IP-10 production, however, induced small amounts of I-TAC and IFN- β . However, when *Alternaria* was added to DCs, IP-10 production by poly I:C was completely inhibited (Fig. 3A). Furthermore, I-TAC and IFN- β production by DCs stimulated with poly I:C was significantly inhibited by *Alternaria* in a concentration-dependent manner (Fig. 3B, C). These findings suggest that, while *Alternaria* showed minimal effects to induce production of IP-10, I-TAC and IFN- β , it inhibits production of these cytokines by DCs stimulated with poly I:C.

***Alternaria* inhibits expression of TLR3 of DCs**

To investigate the mechanisms involved in the inhibitory effects of *Alternaria*, we examined expression of TLR3. Quantitative real-time PCR analysis showed that *TLR3* mRNA expression by DCs was significantly enhanced by poly I:C. In contrast, *Alternaria* did not induce *TLR3* mRNA expression. However, when DCs were cocultured with *Alternaria* and poly I:C, *TLR3* mRNA was significantly inhibited as compared to DCs stimulation by poly I:C alone (Fig. 4A). By western blot, poly I:C increased TLR3 protein expression, however, *Alternaria* did not. Furthermore, similarly to mRNA examinations, *Alternaria* inhibited poly I:C-induced TLR3 protein expression (Fig. 4B). These results were confirmed by densitometric analysis (Fig. 4C); data were normalized to value to medium alone as 100%. Thus, *Alternaria* likely inhibits TLR3 expression by DCs stimulated with poly I:C by suppressing expression of mRNA.

Characterization of the inhibitory factors in *Alternaria* extracts

To characterize the molecules in *Alternaria* extract that are involved in the inhibitory effects on DCs, we performed a series of experiments. First, we investigated molecular weight (MW). *Alternaria* extract was subjected to membrane filtration with YM100 Centricom membrane (100-kDa cutoff; Millipore). Second, we investigated the effects of heat-treatment. *Alternaria* extracts were exposed to 4, 37, 56°C for 30 min. The ability of *Alternaria* extract stimulate IL-6 production was not affected by treatment at 4 °C or 37 °C, however, it was significantly inhibited by treatment at 56 °C (Fig. 5A). Furthermore, the stimulatory activity was mainly present in the fraction >100 kDa. The fraction at <100 kDa slightly inhibited IL-6 production, however, the effects of poly I:C to stimulate IL-6 production was not apparently affected by *Alternaria* specimens. In contrast, the effects of *Alternaria* to inhibit IP-10 production were not affected by treating the extracts at 4 °C, 37 °C, or 56 °C (Fig. 5B). Furthermore, the inhibitory activity was mainly present at the fraction with >100 Da MW although the fraction at <100 kDa slightly inhibited IP-10 production. These findings suggest that the *Alternaria* factor(s) to inhibit poly I:C-induced IP-10 production by DCs is largely heat-stable and with molecular mass of >100 kDa.

Discussion

In our study, we used mouse DCs for our research. DCs play a critical role in vertebrate immunity. Many similarities and differences are reported between mouse DCs and human DCs (33, 34). We found that *Alternaria* inhibits DC's defense mechanism for viral infections through TLR3. This conclusion is based on the following observations: *Alternaria* inhibited TLR3 mRNA and protein expression induced by dsRNA. *Alternaria* inhibited dsRNA-induced IFN- β , IP-10, and I-TAC productions. By characterizing the inhibitory activities, they are likely derived from heat-stable molecules, which is MW over 100 kDa in *Alternaria*.

Alternaria induces calcium-dependent eosinophil degranulation, surface expression of CD63 and CD11b, and production of IL-8 (35). Aspartate protease from *Alternaria* induces this eosinophil activation through protease-activated receptor (PAR)-2 (36). This aspartate protease activity also induces IL-6, IL-8, and GM-CSF productions and calcium response in airway epithelium through PAR-2 (37). *Alternaria* also induces TSLP production from airway epithelial cells. IL-4 enhances, however, IFN- γ inhibits *Alternaria*-induced TSLP production (38). In addition, *Alternaria* induces IL-33 production and extracellular ATP release from normal human bronchial epithelial cells (39). Kobayashi et al. reported that strong Th2 responses were observed when mice were exposed to OVA in the presence of *Alternaria* (40). If mice previously exposed OVA and *Alternaria*, the OVA challenge induced marked airway eosinophilia and significantly produced IL-13 in lung homogenates compared with mice exposed OVA alone. When allogenic CD4⁺ T cells from B6 mice incubated with *Alternaria*-stimulated bone marrow-derived DCs from BALB/C mice, Th2 cytokines, such as IL-4, IL-5, and IL-13, were significantly increased compared with no-stimulated DCs, however produced less IFN- γ . Thus, *Alternaria* potently enhances Th2-type immune responses and eosinophilic inflammation in the airways. In this study, *Alternaria* attenuates chemokine production by DCs, suggesting that *Alternaria* likely modulates host's immune responses profoundly.

A question still exists regarding which factors in the *Alternaria* are involved in these responses. Protease activities in *Alternaria* play important roles for activations of eosinophils and epithelial cells, because heat treatment over 56°C abolishes these activities. In our mouse DCs study, carbohydrate structures, such as chitin and glycan may play pivotal roles for these reactions because these activities are not inactivated by heat treatment for 30 min at 56°C and mainly induced by MW over 100 molecules.

Interestingly, respiratory viral infections have been implicated in exacerbations of allergic asthma, characterized by a Th2-biased immune response (41). Contoli showed deficient induction of IFN by rhinovirus in asthmatic primary bronchial epithelial cells and alveolar macrophages, which was highly correlated with severity of rhinovirus-induced asthma exacerbation and virus load in experimentally infected human volunteers (42). Respiratory viruses target airway epithelial cells and dendritic cells (DCs). Torres, et al. reported that intranasal administration of dsRNA in OVA-sensitized wild-type mice significantly increased the airway hyper responsiveness, the lung inflammation, and the OVA-specific Th2 response (28). Reuter, et al. also reported that application of poly I:C induces production of allergen-specific IgE and IgG1, whereas resiquimod (R848) had no effect. Activation of TLR3 in combination with inhaled allergen results in induction of dendritic cell activation and migration (21). Kobayashi et al. show that presence of *Alternaria* leads Th2-like adjuvant effects (40). And we also find that *Alternaria* inhibits TLR3 expression on DC. Further studies will be necessary to elucidate the mechanism involved in inhibition of TLR3 expression by *Alternaria*.

In summary, our findings in this study demonstrate that *Alternaria* inhibits TLR3 expression and productions of IFN- β , I-TAC and IP-10 by DCs stimulated with poly I:C (Fig. 6). We conclude that *Alternaria* inhibits defense mechanism for viral infections. These results suggest the possibility that presence of *Alternaria* exacerbates both upper and lower airway disease such as CRS and bronchial asthma.

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Fig. 1. *Alternaria* and Poly I:C produced IL-6 in dose dependent manner

Mouse bone marrow-derived dendritic cells were incubated with different concentrations of *Alternaria* (0–50 μ g/ml) and poly I:C (0–25 μ g/ml) or medium alone at 37 °C. To monitor IL-6 production after 24 hrs, the cell-free supernatants were measured by ELISA. Results show the means $\pm$ SEM from 5 different samples. *, $P < 0.05$; **, $P < 0.01$ were significant differences compared with medium alone.

Fig. 2. *Alternaria* and Poly I:C enhance expression of MHC-II and costimulatory molecules by DCs

DCs were incubated with medium alone, *Alternaria* extract (0 – 50 µg/ml) and poly I:C (0–25 µg/ml) 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. Mean expression levels are shown from 3 (MHC-II, OX40L) and 4 (CD40, CD80 and CD86) independent experiments (mean ± SEM). *, $P < 0.05$; **, $P < 0.01$ compared with medium alone. MFI; mean fluorescence intensity.

A

B

Fig. 3. *Alternaria* inhibits IP-10, I-TAC and IFN- β productions by stimulation of Poly I:C DCs were incubated with medium alone, *Alternaria* extract (0–50 μ g/ml) and poly I:C (0–25 μ g/ml) for 24 h. IP-10, I-TAC and IFN- β levels in the supernatants were measured by ELISA. Results are mean $\pm$ SEM ($n = 5$). *, $P < 0.05$; **, $P < 0.01$ compared with medium alone or poly I:C alone.

C

Fig. 4. *Alternaria* inhibits expression of TLR3 of DCs

DCs were incubated with poly I:C (0, 25 mg/ml) and *Alternaria* (0, 50 µg/ml). A. *TLR3* mRNA expression from DCs was analyzed by Quantitative Real-Time PCR. Quantitative real-time PCR was performed using specific primer sets for *TLR3* and *18S rRNA*. Results are mean $\pm$ SEM ($n = 5$). *, $P < 0.05$ compared with medium alone or poly I:C alone. B. Samples were analyzed by western blotting through use of antibody to mouse TLR3 antibody. The figure is representative of 5 experiments showing similar results. C. The intensities of the TLR3 bands were quantitated by densitometric analyses. Data were normalized to the values of medium alone as 100%. Data are presented as means $\pm$ SEM from 5 separate experiments. *, $P < 0.05$ compared with medium alone or poly I:C alone.

A

B

Fig. 5. *Alternaria* factor(s) to inhibit poly I:C-induced IP-10 production by DCs is largely heat-stable and with molecular mass of >100 kDa

Molecular weight examinations were investigated. *Alternaria* extract was subjected to membrane filtration with YM100 Centricom membrane. Next, we tried to investigate the effects of temperature on *Alternaria*. After filtration, *Alternaria* extracts were exposed to 4, 37, 56°C for 30 min. Data are presented as means $\pm$ SEM from 4 separate experiments. *, $P < 0.05$; **, $P < 0.01$ significant differences compared with medium/*Alternaria* treated at 4°C and poly I:C/*Alternaria* treated at 4°C.

Fig. 6. TLR3-mediated signaling pathways

TLR3 recognizes a synthetic analog of viral dsRNA, polyinosinic acid-cytidylic acid (poly I:C), and viral dsRNAs derived from dsRNA viruses. TLR3 signaling activates the transcription factors interferon regulatory factor 3 (IRF3). IRF3 translocates to the nucleus, where it forms a transcriptional complex with the transcription co-activator p300/CBP, NF- κ B and Jun, which subsequently binds to interferon responsive elements (IRF-E). Secreted IFNs bind and activate the type I IFN receptor in an autocrine and paracrine manner. This binding leads to the activation of 25 IFN-stimulated gene factor 3 (ISGF3; a heterotrimer of STAT1, STAT2, and IFN regulatory factor 9 (IRF9)), which translocates to the nucleus and induces the transcription of hundreds of effector molecules, called IFN-inducible genes. These effector molecules directly influence protein synthesis such as IP-10 and I-TAC. In summary, *Alternaria* inhibits TLR3 expression and productions of IFN- β , I-TAC and IP-10 by DCs stimulated with poly I:C.