**Alternaria** Inhibits Double-Stranded RNA-Induced Cytokine Production through Toll-Like Receptor 3

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**Key Words**

*Alternaria* · Polyinosinic-polycytidylic acid · Dendritic cells · Toll-like receptor 3 expression · Host innate immunity

**Abstract**

*Background:* Fungi may be involved in asthma and chronic rhinosinusitis (CRS). Peripheral blood mononuclear cells from CRS patients produce interleukin (IL)-5, IL-13 and interferon (IFN)-γ in the presence of *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, chemokine CXCL10 (IP-10), chemokine CXCL11 (I-TAC) and IFN-β was measured by ELISA. Toll-like receptor 3 (TLR3) mRNA and protein expression was detected by quantitative real-time PCR and Western blot. **Results:** *Alternaria* and polyinosinic-polycytidylic acid (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 production of IP-10, I-TAC and IFN-β, 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 the 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.

**Introduction**

The prevalence of asthma has markedly increased over the past 3 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 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 of 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 significantly increase the risk of hospitalization for acute asthma [11].

Chronic rhinosinusitis (CRS) is a common chronic disease. CRS has been defined by the Task Force of the American Academy of Otolaryngology-Head Neck Surgery based on clinical symptoms and clinical diagnostic criteria [12]. It is treated by medication [13] and endoscopic sinus surgery [14]. Its pathogenesis and etiology 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]. It is frequently refractory to treatment. CRS patients, particularly those with bronchial asthma or with aspirin-induced asthma, sometimes 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 has also been shown to cause obstruction of the 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 Toll-like receptors (TLRs), mainly by endosomally located TLR3, TLR7 and TLR8. TLR3 detects double-stranded RNA (dsRNA), which appears during viral replication processes and is expressed in the placenta, pancreas, lung, liver and heart [20], and especially in myeloid dendritic cells (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 dsRNA produced during viral infections as well as polyinosinic-polycytidylic acid (poly I:C), a synthetic ligand mimicking viral dsRNA [24–28]. TLR3 induces type I interferon (IFN) responses by activating IFN regulatory factor (IRF) family, IRF-3. 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 that of chemokines CXCL10 (IP-10) and CXCL11 (I-TAC).

In this study, we examined the effects of fungal products on innate immunity, in particular antiviral immunity produced by 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 patients with asthma or CRS.

Materials and Methods

Mice

BALB/CJ mice were from Jackson Laboratory (Bar Harbor, Me., USA). All mice were 7- to 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, Minn., USA.

Alternaria Extract

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

Generation and Stimulation of Mouse Bone Marrow-Derived DCs

DCs were generated from mouse bone marrow (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 × 10⁷/ml in RPMI 1640 with 10% FBS, 10 ng/ml murine GM-CSF and 1 ng/ml murine interleukin (IL)-4. Cells were plated into wells of 6-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 h 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 Biotech, Auburn, Calif., USA) and anti-CD11c (N418) immunomagnetic beads (Miltenyi Biotec). These highly purified DCs (>97% purity) behaved similarly to those without MACS purification. We used BM-derived DCs without MACS purification in this study because these extra manipulations might have affected DC functions. 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 and CD86) or 48 h (OX40L), DCs were preincubated with Fc-receptor blockers (anti-CD16/CD32) for 30 min at 4 ºC and stained with PE-conjugated anti-CD40 (HM40-3), anti-CD80 (16-10A1) and anti-CD86 (GL1) or biotinylated 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-CD11c (AMS-32.1) and FITC-conjugated streptavidin for 30 min at 4 ºC. FITC-conjugated mouse immunoglobulin G2b (IgG2b), rat IgG2a and rat IgG2a and biotinylated Armenian hamster IgM were used as isotype controls. All Abs used for DC FACS analysis were used as isotype controls. All Abs used for DC FACS analysis were used as isotype controls. All Abs used for DC FACS analysis...
were obtained from BD Pharmingen. After washing, DCs were re-suspended 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, Calif., USA) 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 instructions. Mouse BM-derived DCs were incubated with different concentrations of Alternaria (0–50 μg/ml) and poly I:C (0–25 μg/ml) or medium alone at 37°C. To monitor IL-6 production after 24 h, the cell-free supernatants were measured by ELISA. Results show the means ± SEM from 5 different samples. Alt = Alternaria.

**Statistical Analysis**

Data are presented as means (±SEM). One-way repeated-measures ANOVA was used to assess significant differences, with post hoc analysis using the Dunnett or Tukey 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 Class 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 × 10⁶ cells) were incubated with Alternaria (0, 25 and 50 μg/ml) and poly I:C (0 and 25 μg/ml) for 24 or 48 h. Alternaria extract induced BM-derived DCs to produce and release IL-6 in a dose-dependent manner (fig. 1). IL-6 production was also induced by poly I:C alone. There were no additive or synergistic effects of Alternaria and poly I:C when they were added together to DCs. In addition, Alternaria increased the expression 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 the expression of MHC class II and costimulatory molecules by DCs.

**Alternaria Inhibits IP-10, I-TAC and IFN-β Production by Stimulation of poly I:C**

To investigate the effects of Alternaria on antiviral innate immune responses, we examined IP-10, I-TAC and IFN-β production by DCs; these chemokines and cytokine suppress viral proliferation and growth. IP-10, I-TAC and IFN-β production from DCs was significantly induced by poly I:C (fig. 3). Alternaria did not induce IP-10 production, but it did induce 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 the 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 the 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 co-cultured with Alternaria and poly I:C, TLR3 mRNA was significantly inhibited compared to DC stimulation by poly I:C alone (fig. 4a). By Western blot, poly I:C increased TLR3 protein expression, but 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 the values of medium alone as 100%. Thus, Alternaria likely inhibits TLR3 expression by DCs stimulated with poly I:C by suppressing mRNA expression.
Characterization of the Inhibitory Factors inAlternaria Extracts

To characterize the molecules in Alternaria extract that are involved in the inhibitory effect 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 extract was exposed to 4, 37 and 56 °C for 30 min. The ability of Alternaria extract to stimulate IL-6 production was not affected by treatment at 4 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 (fig. 5a). In contrast, the capacity of Alternaria to inhibit IP-10 production was not affected by treating the extracts at 4, 37 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) that inhibits poly I:C-induced IP-10 production by DCs is largely heat-stable and has a molecular mass of >100 kDa.

Discussion

We used mouse DCs for our research. DCs play a critical role in vertebrate immunity. Many similarities and differences are reported between mouse and human DCs [33, 34]. We found that Alternaria inhibits the DC 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 production. By characterizing the inhibitory activities, they are likely derived from heat-stable molecules, which is a MW >100 kDa in the case of Alternaria.

Alternaria induces calcium-dependent eosinophil degranulation, surface expression of CD63 and CD11b and

Fig. 3. Alternaria inhibits IP-10, I-TAC and IFN-β production by stimulation of poly I:C. DCs were incubated with medium alone, Alternaria extract (0–50 μg/ml) and poly I:C (0–25 μg/ml) for 24 h. IP-10, I-TAC and IFN-β levels in the supernatants were measured by ELISA. Results are mean ± SEM (n = 5). * p < 0.05 and ** p < 0.01 compared with medium or poly I:C alone. Alt = Alternaria.
**Fig. 4.** *Alternaria* inhibits expression of TLR3 of DCs. DCs were incubated with poly I:C (0, 25 μg/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 ± SEM (n = 5). * p < 0.05 compared with medium or poly I:C alone. b Samples were analyzed by Western blotting through the use of antibody to mouse TLR3 antibody. The figure is representative of 5 experiments showing similar results. M = Medium. 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 ± SEM from 5 separate experiments. * p < 0.05 compared with medium or poly I:C alone. Alt = *Alternaria*.

**Fig. 5.** *Alternaria* factor(s) to inhibit poly I:C-induced IP-10 production by DCs is largely heat-stable and with a MW >100 kDa. MW 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, and 56°C for 30 min. Data are presented as means ± SEM from 4 separate experiments. * p < 0.05 and ** p < 0.01 represent significant differences compared with medium/*Alternaria* treated at 4°C and poly I:C/*Alternaria* treated at 4°C. Alt = *Alternaria*.
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 production and calcium response in airway epithelium through PAR-2 [37]. Alternaria also induces TSLP production from airway epithelial cells. IL-4 enhances Alternaria-induced TSLP production, but IFN-γ inhibits it [38]. In addition, Alternaria induces IL-33 production and extracellular ATP release from normal human bronchial epithelial cells [39]. Kobayashi et al. [40] reported that strong Th2 responses were observed when mice were exposed to OVA in the presence of Alternaria. If mice had been exposed to OVA and Alternaria previously, the OVA challenge induced marked airway eosinophilia and significantly produced IL-13 in lung homogenates compared to mice exposed to OVA alone. When allogenic CD4+ T cells from B6 mice incubated with Alternaria-stimulated BM-derived DCs from BALB/C mice, Th2 cytokines such as IL-4, IL-5 and IL-13 were significantly increased compared with nonstimulated DCs; however, they produced less IFN-γ. Thus, Alternaria potently enhances Th2-type immune responses and eosinophilic inflammation in the airways. In this study, Alternaria attenuated chemokine production by DCs, suggesting that Alternaria likely profoundly modulates host immune responses.

A question still exists regarding which factors in the Alternaria are involved in these responses. Protease activities in Alternaria play an important role in the activation of eosinophils and epithelial cells, because heat treatment over 56°C abolishes these activities. In our mouse DC study, carbohydrate structures, such as chitin and glycan may play pivotal roles for these reactions because

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**Fig. 6.** TLR3-mediated signaling pathways. TLR3 recognizes a synthetic analog of viral dsRNA, poly I:C and viral dsRNAs derived from dsRNA viruses. TLR3 signaling activates the transcription factor IRF-3. IRF-3 translocates to the nucleus, where it forms a transcriptional complex with the transcription coactivator p300/CBP, NF-κB and Jun, which subsequently binds to interferon stimulated response elements (ISRE). Secreted IFNs bind and activate the type I IFN receptor (IFNR) in an autocrine and paracrine manner. This binding leads to the activation of IFN-stimulated gene factor 3 (ISGF-3; a heterotrimer of STAT1, STAT2 and IRF-9), 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 production of IFN-β, I-TAC and IP-10 by DCs stimulated with poly I:C.
these activities are not inactivated by heat treatment for 30 min at 56°C and are mainly induced by a MW of >100 molecules.

Interestingly, respiratory viral infections have been implicated in exacerbations of allergic asthma, characterized by a Th2-biased immune response [41]. Contoli et al. [42] showed deficient induction of IFN by rhinovirus in asthmatic primary bronchial epithelial cells and alveolar macrophages, which was highly correlated with the severity of rhinovirus-induced asthma exacerbation and virus load in experimentally infected human volunteers. Respiratory viruses target airway epithelial cells and DCs. Torres et al. [28] reported that intranasal administration of dsRNA in OVA-sensitized wild-type mice significantly increased airway hyperresponsiveness, lung inflammation and OVA-specific Th2 response. Reuter et al. [21] 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 the induction of DC activation and migration. Kobayashi et al. [40] show that the presence of Alternaria leads to Th2-like adjuvant effects. We also found that Alternaria inhibits TLR3 expression on DCs. 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 the defense mechanism for viral infections. These results suggest the possibility that the presence of Alternaria exacerbates both upper and lower airway disease such as CRS and bronchial asthma.

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Disclosure Statement

The authors have no financial conflict of interest.

References


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