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

Kota Wada^{a, c} Takao Kobayashi^c Yoshinori Matsuwaki^{b, c} Hiroshi Moriyama^b
Hirohito Kita^c

^aDepartment of Otorhinolaryngology, Toho University, and ^bDepartment of Otorhinolaryngology, The Jikei University School of Medicine, Tokyo, Japan; ^cDepartments of Immunology and Medicine, Mayo Clinic, Rochester, Minn., USA

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.

Copyright © 2013 S. Karger AG, Basel

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 1×10^6 /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 μ g/ml) or poly I:C (25 μ g/ml) or combinations thereof. In separate experiments, we further purified DCs with a magnetic cell separation system (MACS; Miltenyi Biotec, 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°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 immunoglobulin G 2b (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 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 directions.

Quantitative Real-Time PCR

Total RNA was isolated from DCs using RNeasy Mini Kit (Qiagen, Valencia, Calif., USA). The RNA was then transcribed into cDNA using Superscript II (Invitrogen, Grand Island, N.Y., USA). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Calif., USA), according to the manufacturer's 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 min and clarified by centrifugation at 3,000 g at 4°C for 15 min with a microcentrifuge. Ten micrograms 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, N.J., USA). 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 are presented as means ($\pm$ 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 (1×10^6 cells) were incubated with *Alternaria* (0, 25 and 50 μ g/ml) and poly I:C (0 and 25 μ g/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 ef-

Fig. 1. *Alternaria* and poly I:C produced IL-6 in a dose-dependent manner. Mouse BM-derived DCs 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 h, the cell-free supernatants were measured by ELISA. Results show the means $\pm$ SEM from 5 different samples. Alt = *Alternaria*. * $p < 0.05$ and ** $p < 0.01$ were significant differences compared with medium alone.

fects 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.

Fig. 2. *Alternaria* and poly I:C enhance expression of MHC class 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 class II, CD40, CD80 and CD86) or 48 h (OX40L). The expression of MHC class II and costimulatory mol-

ecules was analyzed by FACS. Mean expression levels are shown from 3 (MHC class II and OX40L) and 4 (CD40, CD80 and CD86) independent experiments (mean ± SEM). * $p < 0.05$ and ** $p < 0.01$ compared with medium alone. Alt = *Alternaria*; MFI = mean fluorescence intensity.

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.

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 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 and ** p < 0.01 compared with medium or poly I:C alone. Alt = *Alternaria*.

Characterization of the Inhibitory Factors in *Alternaria* 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* extracts were 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. The fraction at <100 kDa slightly inhibited IL-6 production; however, the effect of poly I:C to stimulate IL-6 production was not apparently affected by *Alternaria* specimens. 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. 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 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 $\pm$ 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 $\pm$ 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*.

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- κ B 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 man-

ner. 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.

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* potentially 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

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.

Acknowledgement

This work was supported by the National Institutes of Health Grants AI49235 and AI71106 and the Mayo Foundation.

Disclosure Statement

The authors have no financial conflict of interest.

References

- Moorman JE, Rudd RA, Johnson CA, King M, Minor P, Bailey C, et al: National surveillance for asthma – United States, 1980–2004. *MMWR Surveill Summ* 2007;56:1–54.
- Zhu Z, Zheng T, Homer RJ, Kim, YK, Chen NY, Cohn L, Hamid Q, Elias JA: Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 2004;304:1678–1682.
- Reese TA, Liang HE, Tager AM, Luster AD, Van Rooijen N, Voehringer D, Locksley RM: Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 2007;447:92–96.
- Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, He S, Dziura JD, Reed J, Coyle AJ, Kiener P, Cullen M, Grandsaigne M, Dombret MC, Aubier M, Pretolani M, Elias JA: A chitinase-like protein in the lung and circulation of patients with severe asthma. *N Engl J* 2007;357:2016–2027.
- Denning DW, O'Driscoll BR, Hogaboam CM, Bowyer P, Niven RM: The link between fungi and severe asthma: a summary of the evidence. *Eur Respir J* 2006;27:615–626.
- Solomon WR: Assessing fungus prevalence in domestic interiors. *J Allergy Clin Immunol* 1975;56:235–242.
- Crain EF, Walter M, O'Connor GT, Mitchell H, Gruchalla RS, Kattan M, Malindzak GS, Enright P, Evans R 3rd, Morgan W, Stout JW: Home and allergic characteristics of children with asthma in seven US urban communities and design of an environmental intervention: the Inner-City Asthma Study. *Environ Health Perspect* 2002;110:939–945.
- Amr S, Bollinger ME, Myers M, Hamilton RG, Weiss SR, Rossman M, Osborne L, Timmins S, Kimes DS, Levine ER, Blaisdell CJ: Environmental allergens and asthma in urban elementary schools. *Ann Allergy Asthma Immunol* 2003;90:34–40.
- Forno E, Celedón JC: Predicting asthma exacerbations in children. *Curr Opin Pulm Med* 2012;18:63–69.
- Jackson DJ, Johnston SL: The role of viruses in acute exacerbations of asthma. *J Allergy Clin Immunol* 2010;125:1178–1187.
- Green RM, Custovic A, Sanderson G, Hunter J, Johnston SL, Woodcock A: Synergism between allergens and viruses and risk of hospital admission with asthma: case-control study. *BMJ* 2002;324:763.
- Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, Adinoff AD, Bachert C, Borish L, Chinchilli VM, Danzig MR, Ferguson BJ, Fokkens WJ, Jenkins SG, Lund VJ, Mafee MF, Naclerio RM, Pawankar R, Ponikau JU, Schubert MS, Slavin RG, Stewart MG, Togias A, Wald ER, Winther B, Rhinosinusitis Initiative: Rhinosinusitis developing guidance for clinical trials. *J Allergy Clin Immunol* 2006;118:S17–S61.
- Gotfried MH: Macrolides for the treatment of chronic sinusitis, asthma, and COPD. *Chest* 2004;125:52S–60S.
- Moriyama H, Ozawa M, Honda Y: Endoscopic endonasal sinus surgery. Approaches and post-operative evaluation. *Rhinology* 1991;29:93–98.
- Ponikau JU, Sherris DA, Kephart GM, Adolphson C, Kita H: The role of ubiquitous airborne fungi in chronic rhinosinusitis. *Clin Rev Allergy Immunol* 2006;30:187–194.
- Wood AJ, Antoszewska H, Fraser J, Douglas RG: Is chronic rhinosinusitis caused by persistent respiratory virus infection? *Int Forum Allergy Rhinol* 2011;1:95–100.
- Gwaltney JM Jr, Phillips CD, Miller RD, Riker DK: Computed tomographic study of the common cold. *N Engl J Med* 1994;330:25–30.

- 18 Wang J, Watanabe S, Matsukura S, Suzuki H: Double-stranded RNA poly (I:C) enhances matrix metalloproteinase mRNA expression in human nasal polyp epithelial cells. *Acta Otolaryngol Suppl* 2009;562:105–109.
- 19 Pedersen M, Sakakura Y, Winther B, Brofeldt S, Mygind N: Nasal mucociliary transport, number of ciliated cells, and beating pattern in naturally acquired common colds. *Eur J Respir Dis Suppl* 1983;128:355–365.
- 20 Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF: A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci USA* 1998;95:588–593.
- 21 Reuter S, Dehzad N, Martin H, Böhm L, Becker M, Buhl R, Stassen M, Taube C: TLR3 but not TLR7/8 ligand induces allergic sensitization to inhaled allergen. *J Immunol* 2012; 188:5123–5131.
- 22 Reynolds HY: Modulating airway defenses against microbes. *Curr Opin Pulm Med* 2002; 8:154–165.
- 23 Gordon S: Pattern recognition receptors: doubling up for the innate immune response. *Cell* 2002;111:927–930.
- 24 Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001;413:732–738.
- 25 Atmar RL, Guy E, Guntupalli KK, Zimmerman JL, Bandi VD, Baxter BD, Greenberg SB: Respiratory tract viral infections in inner-city asthmatic adults. *Arch Intern Med* 1998;158: 2453–2459.
- 26 Doyle SE, O'Connell R, Vaidya SA, Chow EK, Yee K, Cheng G: Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. *J Immunol* 2003;170:3565–3571.
- 27 Jiang Z, Zamanian-Daryoush M, Nie H, Silva AM, Williams BR, Li X: Poly (I:C)-induced Toll-like receptor 3 (TLR3)-mediated activation of NF-kappa B and MAP kinase is through an interleukin-1 receptor-associated kinase (IRAK)-independent pathway employing the signaling components TLR3-TRAF6-TAK1-TAB2-PKR. *J Biol Chem* 2003;278:16713–16719.
- 28 Torres D, Dieudonné A, Ryffel B, Vilain E, Si-Tahar M, Pichavant M, Lassalle P, Trottein F, Gosset P: Double-stranded RNA exacerbates pulmonary allergic reaction through TLR3: implication of airway epithelium and dendritic cells. *J Immunol* 2010;185:451–459.
- 29 Honda K, Takaoka A, Taniguchi T: Immunity. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* 2006;25: 349–360.
- 30 Uematsu S, Akira S: Toll-like receptors and type I interferons. *J Biol Chem* 2007;282: 15319–15323.
- 31 Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM: Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176: 1693–1702.
- 32 Radhakrishnan S, Nguyen LT, Ciric B, Ure DR, Zhou B, Tamada K, Dong H, Tseng SY, Shin T, Pardoll DM, Chen L, Kyle RA, Rodriguez M, Pease LR: Naturally occurring human IgM antibody that binds B7-DC and potentiates T cell stimulation by dendritic cells. *J Immunol* 2003;170:1830–1838.
- 33 Villadangos JA, Shortman K: Found in translation: the human equivalent of mouse CD8+ dendritic cells. *J Exp Med* 2010;207:1131–1134.
- 34 Robbins SH, Walzer T, Dembélé D, Thibault C, Defays A, Bessou G, Xu H, Vivier E, Sellars M, Pierre P, Sharp FR, Chan S, Kastner P, Dalod M: Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol* 2008;9:R17.
- 35 Inoue Y, Matsuwaki Y, Shin SH, Ponikau JU, Kita H: Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. *J Immunol* 2005;175:5439–5447.
- 36 Matsuwaki Y, Wada K, White TA, Benson LM, Charlesworth MC, Checkel JL, Inoue Y, Hotta K, Ponikau JU, Lawrence CB, Kita H: Recognition of fungal protease activities induces cellular activation and eosinophil-derived neurotoxin release in human eosinophils. *J Immunol* 2009;183:6708–6716.
- 37 Matsuwaki Y, Wada K, White T, Moriyama H, Kita H: *Alternaria* fungus induces the production of GM-CSF, interleukin-6 and interleukin-8 and calcium signaling in human airway epithelium through protease-activated receptor 2. *Int Arch Allergy Immunol* 2012; 158:19–29.
- 38 Kouzaki H, O'Grady SM, Lawrence CB, Kita H: Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. *J Immunol* 2009;183:1427–1434.
- 39 Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H: The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. *J Immunol* 2011;186:4375–4387.
- 40 Kobayashi T, Iijima K, Radhakrishnan S, Mehta V, Vassallo R, Lawrence CB, Cyong JC, Pease LR, Oguchi K, Kita H: Asthma-related environmental fungus, *Alternaria*, activates dendritic cells and produces potent Th2 adjuvant activity. *J Immunol* 2009;182:2502–2510.
- 41 Dahl ME, Dabbagh K, Liggitt D, Kim S, Lewis DB: Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol* 2004;5:337–343.
- 42 Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Kebadze T, Mallia P, Stanciu LA, Parker HL, Slater L, Lewis-Antes A, Kon OM, Holgate ST, Davies DE, Kosenko SV, Papi A, Johnston SL: Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006; 12:1023–1026.