

Airborne Fungi Induce Nasal Polyp Epithelial Cell Activation and Toll-Like Receptor Expression

Seung-Heon Shin Young-Ho Lee

Department of Otorhinolaryngology, School of Medicine, Catholic University of Daegu, Daegu, South Korea

Key Words

Fungus · Epithelial cell · Protease · Toll-like receptor

Abstract

Background: The nasal epithelium is the first barrier encountered by airborne allergens and is an active participant in airway inflammation. Fungi have been increasingly recognized as important pathogens in sinusitis and airway diseases. The aim of the study was to evaluate fungal protease activity during cytokine production in nasal polyp epithelial cells and to determine the expression of Toll-like receptor (TLR) mRNA by fungi. **Methods:** Nasal polyp epithelial cells were obtained from patients and stimulated with *Alternaria* and *Aspergillus*. Interleukin-8 (IL-8) and granulocyte macrophage colony-stimulating factor (GM-CSF) were measured to determine the activation of epithelial cells. Reverse transcriptase polymerase chain reaction for the TLR mRNA expression of the nasal epithelial cells was performed. Cytokine production was inhibited with protease inhibitors and anti-human TLR antibodies. **Results:** The fungi enhanced the production of IL-8 and GM-CSF from nasal epithelial cells. When nasal epithelial cells were activated by the fungi, TLR2, TLR3 and TLR4 mRNAs were more strongly expressed than in the nonactivated cells. Cytokine production was inhibited by protease inhibitors and anti-human TLR4 antibodies. **Conclusions:** The results of this study showed that fungi interacted with nasal epithelial cells and enhanced the

production of cytokines and TLR mRNA expression. The cytokine production was related to the protease in fungi and TLR4.

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Introduction

Chronic rhinosinusitis (CRS) is a chronic inflammatory disease of the nasal and paranasal sinus mucosa; it is defined by persistence of symptoms for more than 12 weeks. Although the cause of CRS remains unclear, non-infectious inflammation plays a key role in the pathogenesis of CRS. Nasal polyps are edematous projections that originate from the nasal mucosa and the inflammation is a prominent feature of nasal polyps. However, the events that trigger nasal polyp formation have not been fully described. A variety of microorganisms and microbial products have been proposed, but no single agent has been proven to play a causative role [1, 2]. Fungal disease of the sinuses has been recognized since 1981, and was once considered rare [3]. However, the number of diagnosed cases has increased over the last decade. Fungi are being increasingly recognized as important pathogens in patients with sinusitis, but their role in the pathogenesis of CRS remains controversial. Fungi are ubiquitous in nature. The number of fungal species probably exceeds 50,000 but relatively few species are implicated in human

disease, some of which are commonly associated with airway inflammatory disease, that is, *Alternaria*, *Cladosporium*, *Aspergillus*, *Penicillium*, and *Candida*. Fungal elements, such as proteins and enzymes, induce immune and inflammatory responses. The protease of fungal extracts may facilitate antigen access to target tissue and cause direct activation of cells [4].

Toll-like receptors (TLRs) have recently been recognized as playing a key role in pathogen recognition as well as in the induction and regulation of the innate and adaptive immune response. TLRs are expressed on macrophages, dendritic cells, endothelial cells and epithelial cells. Eleven TLR members have been identified in humans, they are triggered by conserved molecular structures expressed by bacteria, viruses and fungi [5]. The pattern of TLR expression has been studied in many different tissues and cell types. TLR2 and TLR4 mRNA have been found to be expressed in the nasal epithelial cells, and these two genes have been reported to be significantly higher in a chronic rhinosinusitis [6]. When comparing nasal polyps with and without cystic fibrosis, TLR2 mRNA expression was significantly elevated in the cystic fibrosis group, but TLR4 mRNA expression was not significantly different [7].

Fungi have often been associated with bronchial asthma and sinusitis, their enzymatic activity leads to the production of pro-inflammatory cytokines by direct activation of epithelial cells. However, the exact mechanism underlying epithelial activation has not been fully described. Therefore, the aim of this study was to evaluate fungal protease activity during cytokine production in nasal polyp epithelial cells, and to determine the expression of TLR mRNA by fungi and the role of TLRs in the production of chemical mediators.

Material and Methods

Nasal Polyp Epithelial Cell Culture and Activation with Fungi

Nasal polyps were obtained from 8 patients undergoing endoscopic sinus surgery. The diagnosis of CRS with polyp was based on the endoscopic and computed tomography findings. Patients were excluded if they had a history of allergy, asthma, aspirin intolerance, systemic diseases or had received systemic or topical steroid or antibiotics during the 4 weeks preceding the study. The study was approved by the institutional review board of Daegu Catholic University Medical Center and each patient signed a consent form that outlined the objectives of the research and experiments. Isolation of epithelial cells from nasal polyp was done according to the method previously described [8]. The specimens were placed in Ham's F12 medium supplemented with 100 international units penicillin, 100 µg/ml streptomycin and 2 µg/ml amphotericin B, and transported to the laboratory. Nasal polyp

epithelial cells were isolated using protease digestion methods. The polyps were rinsed with Ham's F12 medium supplemented with antibiotics and incubated with Dispase (Roche Diagnostics, Mannheim, Germany) in Ham's F12 medium for 16 h at 4°C. After incubation, the nasal epithelial cells were isolated by gentle agitation. Cell suspensions were filtered through a No. 60 mesh cell dissociation sieve and centrifuged at 1,000 rpm for 5 min at room temperature. The cell pellet was then resuspended in Ham's F12 medium supplemented with antibiotics, 150 µg/ml glutamine, 5 µg/ml transferring, 25 ng/ml epithelial growth factor, 15 µg/ml endothelial cell growth supplement, insulin 5 IU/ml 200 pM triiodothyronin, 100 nM hydrocortisone and 15% fetal calf serum. Cell suspensions (10⁶ cells/ml) were plated in 6-well culture plates and placed in a 5% CO₂ humidified incubator at 37°C. The culture medium was changed after 24 h and every 2 days thereafter. When the epithelial cell cultures reached confluence, Ham's F12 medium was changed to RPMI-1640 medium supplemented with 10% fetal calf serum and 50 µg/ml gentamycin. Nasal epithelial cells were activated with endotoxin-removed *Alternaria alternata*, *Aspergillus versicolor* 10, 25, 50 and 100 µg/ml (Greer Lab, Lenoir, N.C., USA). After 48 h of incubation the supernatants and epithelial cells were harvested and stored at -70°C until assayed. Cytokine production (interleukin-8, IL-8; granulocyte macrophage colony-stimulating factor, GM-CSF) was quantified by using commercially available ELISA kits (R&D Systems, Minneapolis, Minn., USA).

TLRs mRNA Expression of Nasal Epithelial Cells

After stimulation with fungi, the cell pellets were placed in cryotubes and 1 ml Trizole reagent was added. RNA was extracted according to the manufacturer's instructions (Roche Diagnostics). The RNA purity and concentration were measured using a spectrophotometer (Beckman, Mountain View, Calif., USA). One microliter of RNA was used for the reverse transcription polymerase chain reaction amplifications and they were performed in a Perkin Elmer thermal cycler (Norwalk, Conn., USA). The primers used in this study were as follows: TLR2 (sense, CCA GCA AAT TAC CTG TGT G; antisense, CTG AGC CTC GTC CAT GGG CCA CTC C; 637 bp), TLR3 (sense, CGG GCC AGC TTT CAG GAA CCT G; antisense, GGC ATG AAT TAT ATA TGC TGC; 400 bp), TLR4 (sense, TGC AAT GGA TCA AGG ACC AGA GGC; antisense, GTG CTG GGA CAC CAC AAC AAT CAC C; 449 bp), TLR5 (sense, CCT CAT GAC CAT CCT CAC AGT CAC; antisense, GGC TTC AAG GCA CCA GCC ATC TC; 355 bp), TLR9 (sense, GCG AGA TGA GGA TGC CCT GCC CTA CG; antisense, TTC GGC CGT GGG TCC CTG GCA GAA G; 510 bp) and β-actin (sense, CTG GCC GGG ACC TGA CT; antisense, GCA GCC GTG GCC ATC TC; 268 bp). The annealing temperature was 60°C for TLR2, 52°C for TLR3, 61°C for TLR4, 58°C for TLR5 and 68°C for TLR9. Samples were visualized using agarose gel electrophoresis and the band densities were measured using the multi Gauge v2.02 (Fujifilm, Tokyo, Japan). The band intensities were expressed as a percentage of treated versus untreated cells.

Inhibition of Cytokine Production with Protease Inhibitors and TLR Antibodies

The epithelial cells were cultured in 24-well culture dishes until 80–90% confluence was reached. The nasal epithelial cells were pretreated with protease inhibitors; serine protease inhibitor 0.5

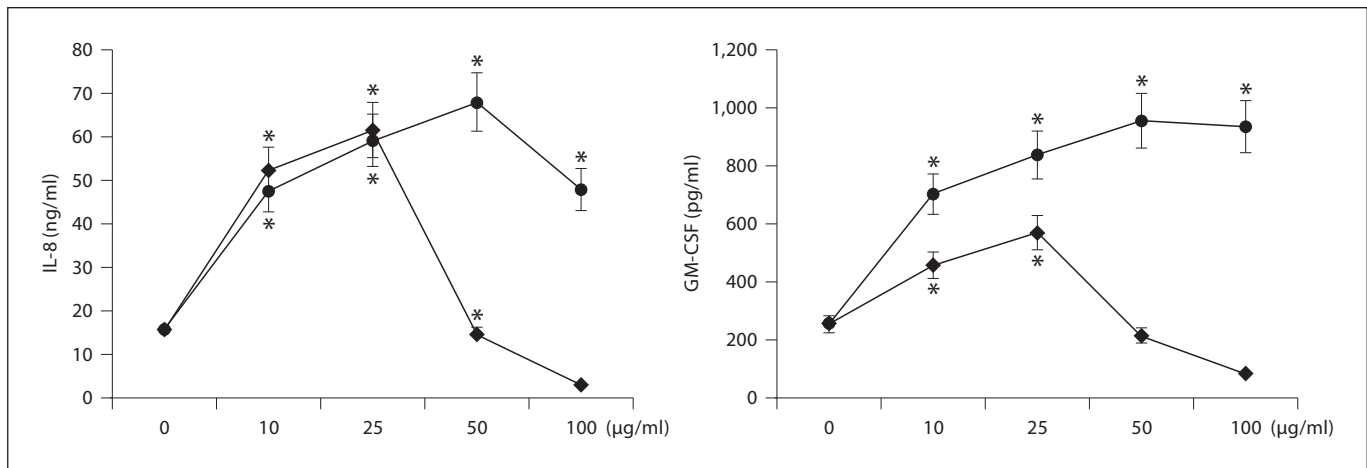


Fig. 1. IL-8 and GM-CSF production by nasal polyp epithelial cell cultures incubated with various concentrations of fungal extract. Circle = *Alternaria alternata*; square = *Aspergillus fumigatus*. Data represent the mean $\pm$ SD of 7 experiments. * Significantly increased production of cytokine. Differences were considered significant if p values were <0.05 .

μM of AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride), cysteine protease inhibitor 5 $\mu\text{g/ml}$ of E-64 (trans-epoxysuccinyl-L-leucylamide-(4-guanidine) butane) and aspartic protease inhibitor 1 μM of pepstatin. All protease inhibitors were obtained from Roche Diagnostics. The protease inhibitors were added to the fungi for 30 min prior to epithelial stimulation. After 48 h, the cultured supernatant and epithelial cells were collected to measure the cytokine and TLR mRNA expression.

Epithelial TLR2 and TLR4 were inhibited using functional-grade anti-human TLR2 specific neutralizing monoclonal antibodies TL2.1 and TLR4 specific neutralizing monoclonal antibody HTA125 (eBioscience, San Diego, Calif., USA). An IgG2a isotype antibody was used as a control. TLR2 and TLR4 as well as isotype control antibodies were added 1 h prior to fungal stimulation. After 48 h the cultured supernatants were collected to measure the cytokines.

Statistical Analysis

All experiments were performed at least 5 times and revealed comparable results. The results are presented as the mean $\pm$ SD. Statistical significance was determined using the paired Student t test and one-way ANOVA (SPSS 14.0; SPSS Inc., Chicago, Ill., USA). A p value of 0.05 or less was considered significant.

Results

In the presence of fungal extracts, nasal polyp epithelial cells produced larger amounts of GM-CSF and IL-8. A concentration of 50 $\mu\text{g/ml}$ of *Alternaria* and 25 $\mu\text{g/ml}$ of *Aspergillus* induced maximal GM-CSF and IL-8 protein levels (fig. 1). At concentrations over 25 $\mu\text{g/ml}$ of *As-*

pergillus, the production of IL-8 and GM-CSF rapidly declined and reached levels below baseline. To confirm that fungi-induced cytokine production is a protease dependent process, the fungi were incubated for 30 min with the protease inhibitors. As shown in figure 2, *Alternaria* induced IL-8 production at 50 $\mu\text{g/ml}$ and was strongly inhibited by the serine protease inhibitor AEBSF (30% at 0.1 μM , 45% at 0.5 μM and 48% at 5 μM) and cysteine protease inhibitor E-64 (38% at 0.5 $\mu\text{g/ml}$, 44% at 5 $\mu\text{g/ml}$). *Alternaria*-induced GM-CSF production was inhibited only by serine protease inhibitor AEBSF (45% at 0.1 μM , 42% at 0.5 μM and 37% at 5 μM). However, *Aspergillus* induced IL-8 and GM-CSF production at 25 $\mu\text{g/ml}$ was not significantly inhibited by the protease inhibitors.

After stimulation with fungi for 48 h, relatively high values of the TLR mRNA were detected by *Alternaria* and *Aspergillus*. However, *Alternaria* 25 $\mu\text{g/ml}$ was the strongest stimulant of the nasal polyp epithelial cells and TLR2, TLR3 and TLR4 mRNA expression were significantly increased (fig. 3). When *Alternaria* was pretreated with protease inhibitors, only TLR3 mRNA expression was significantly inhibited by the cysteine protease inhibitor, E-64 (39% at 5 $\mu\text{g/ml}$).

To analyze the potential role of TLR 2 and TLR4 in the *Alternaria*-induced production of cytokines, nasal epithelial cells were pretreated with TLR antibodies. *Alternaria* induced IL-8 and GM-CSF production were significantly inhibited by the TLR4 antibody (fig. 4).

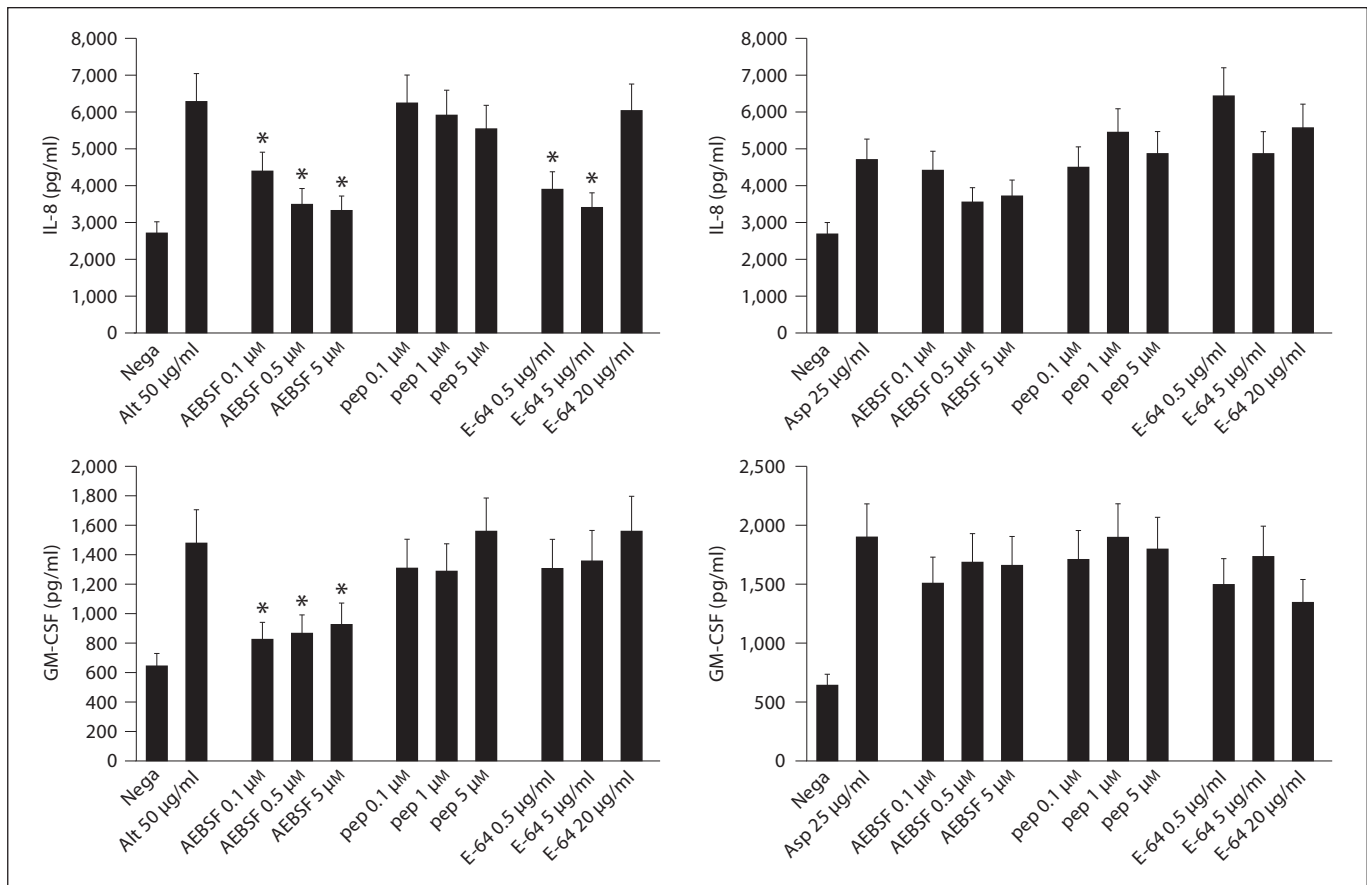
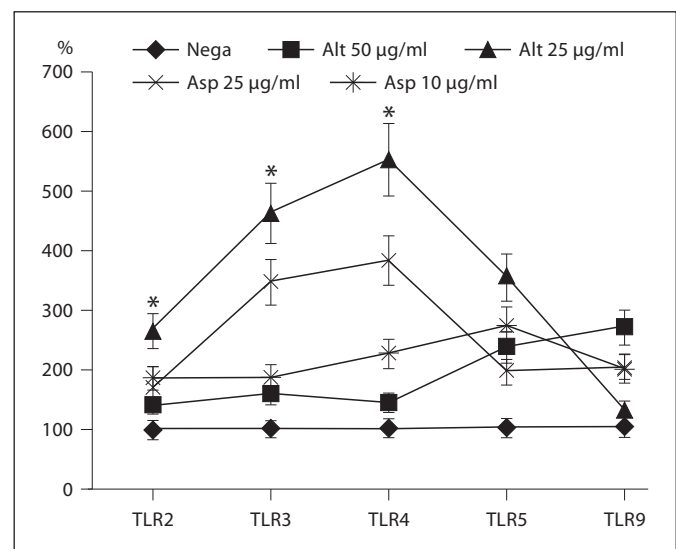


Fig. 2. Inhibition of IL-8 and GM-CSF production by nasal polyp epithelial cells with serine, cysteine and aspartic protease inhibitors. *Alternaria*-induced production of IL-8 was inhibited by serine and aspartic protease inhibitors and GM-CSF production was inhibited by the serine protease inhibitor. Nega = Negative control indicates the cytokine level of spontaneous production; Alt = *Alternaria alternata*; Asp = *Aspergillus fumigatus*; pep = pepstatin. Data represent the mean $\pm$ SD of 5 experiments. * Significantly decreased production of cytokine. Differences were considered significant if p values were <0.05 .

Fig. 3. Reverse transcription polymerase chain reaction analysis of the expression of TLRs in nasal polyp epithelial cells after stimulation with fungi. Band intensities were expressed as a percentage of treated versus untreated cells. TLR2, TLR3 and TLR4 mRNA expression was significantly increased by *Alternaria* 25 µg/ml. Alt = *Alternaria alternata*; Asp = *Aspergillus fumigatus*. Data represent the mean $\pm$ SD of 5 experiments. * Significantly increased expression of TLRs. Differences were considered significant if p values were <0.05 .



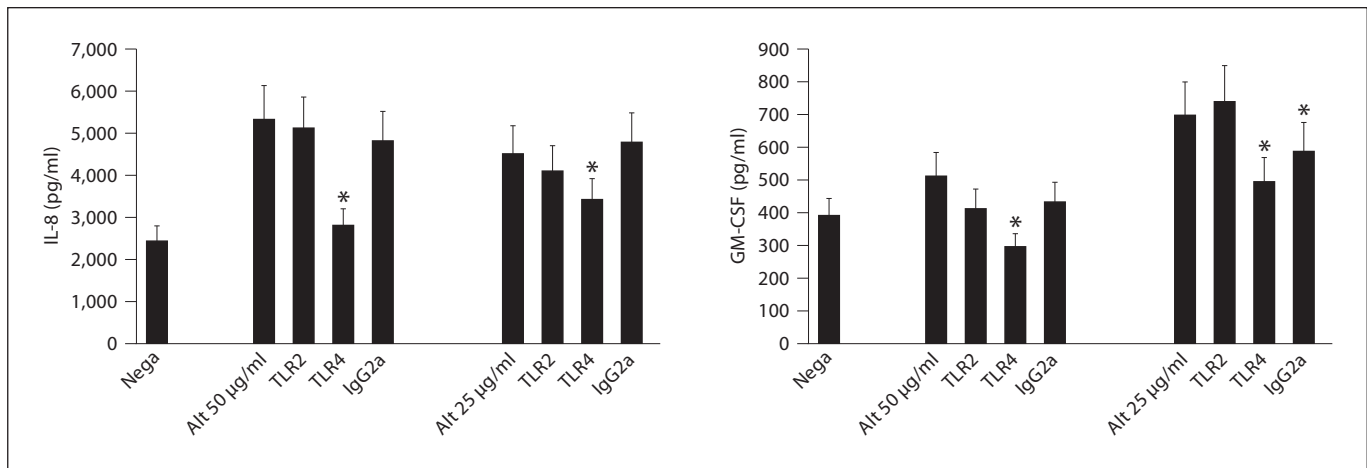


Fig. 4. Inhibition of IL-8 and GM-CSF production by nasal polyp epithelial cells with anti-human TLR2 and TLR4 antibodies. *Alternaria*-induced IL-8 and GM-CSF production was significantly inhibited by TLR4 antibody. Alt = *Alternaria alternata*; Asp = *Aspergillus fumigatus*. Data represent the mean $\pm$ SD of 5 experiments. * Significantly decreased production of cytokine. Differences were considered significant if p values were <0.05 .

Discussion

Respiratory epithelial cells are the first mucosal cells exposed to the environmental stimuli associated with airway inflammation. They defend against allergens and microorganisms through physical barriers such as the tight junction, and produce glycoconjugates, defensive proteins and enzymes that together constitute a protective environment. They also play an active role in inflammation through the production of a variety of lipid mediators, cytokines, chemokines, extracellular matrix and growth factors, in addition to a major source of inflammatory chemical mediators, such as GM-CSF, regulated on activation normal T expressed and secreted (RANTES), eotaxin, IL-6 and IL-8; all are involved in the modulation of the local inflammatory reaction [9].

Fungi have often been associated with allergic bronchial and sinonasal diseases, their proteolytic activity leads to the production of pro-inflammatory cytokines by direct activation of the epithelial cells. *Alternaria alternata*, *Cladosporium herbarum* and *Aspergillus fumigatus* activated respiratory epithelial cells by serine and cysteine proteases present in the fungal extracts [4, 10]. In this study, *Alternaria* and *Aspergillus* were used to activate the nasal epithelial cells, because these organisms are known to be common pathogens found in nasal secretion and in respiratory tract diseases. Our preliminary experiments with normal nasal epithelial cells showed that

when they were stimulated with fungi the cytokine production was much less than nasal polyp epithelial cells (data not shown). Maximal cytokine production was found at 50 $\mu\text{g/ml}$ of *Alternaria* (IL-8 67.8 ± 13.2 ng/ml, GM-CSF 952.3 ± 345.2 pg/ml) and 25 $\mu\text{g/ml}$ of *Aspergillus* (IL-8 61.6 ± 23.7 ng/ml, GM-CSF 569.7 ± 276.5 pg/ml). We chose 50 and 25 $\mu\text{g/ml}$ of *Alternaria* as well as 25 and 10 $\mu\text{g/ml}$ of *Aspergillus* for TLRs mRNA expression and protease inhibition studies. When the protease activity of fungus was measured at 574 nm with universal protease substrate (Roche Diagnostics), the absorbance of *Alternaria* (100 $\mu\text{g/ml}$) was 0.55 and *Aspergillus* (50 $\mu\text{g/ml}$) was 0.51, similar to 250 ng/ml of trypsin (data not shown). The lower production of cytokines at high concentrations of fungal extract may be due to either inactivation or detachment of the epithelial cells or proteolytic degradation of cytokines.

The recognition of fungi and fungal pathogen-associated molecular patterns, mainly associated with fungal cell walls, leads to the activation of antifungal effector functions, such as the production of pro-inflammatory cytokines, induction of a respiratory burst and degranulation as well as T helper 1 cell differentiation, among immune cells. *Alternaria* and *Aspergillus* induced the production of IL-8 and GM-CSF from the nasal polyp epithelial cells. IL-8 and GM-CSF, as well as other pro-inflammatory cytokines, including IL-1 α , IL-1 β , IL-6 and TNF- α , have been implicated in the development of

protective immunity against fungal infection. *Alternaria*-induced IL-8 production was inhibited by serine and cysteine protease inhibitors and GM-CSF production was inhibited by the serine protease inhibitor. However, *Aspergillus*-induced cytokine production was not inhibited by the protease inhibitors. *Aspergillus*-induced nasal epithelial cell activation may be associated with other proteases, like metalloprotease, or other allergenic components, like robotoxin, heat shock protein and ribosomal protein [11]. Fungal allergens also induce the production of chemokines, like IL-8, RANTES and eotaxin. These CC chemokines produced by nasal epithelial cells induce the migration of eosinophils [12]. Fungal allergens are diverse in their physiological, chemical and immunological characteristics. The protein and enzymatic allergens react with specific receptors of the immune cells in the body. In our study, we used biologically standardized lyophilized fungal allergen extracts from fungal hyphae and spores. However, it is difficult to know whether the fungal extracts had the same immunological characteristics. The commercially available fungal extracts have different fungal components and the allergenic potency could be permanently altered or inactivated by manipulation.

The sinonasal cavity is the first point of contact between the airway mucosa and the external environment. Inspired air contains a wide assortment of microbial and nonmicrobial elements, some of which have the potential to harm the host. Because fungi are found everywhere in nature and form airborne spores, it is reasonable to assume that most people have fungal colonization in the nasal cavity. The innate immune response to fungal antigens is important to maintain the physiological condition of the sinonasal cavity. During respiration, the fungal spores enter the nasal cavity, and because of their low aerodynamic diameter the fungi can deposit on the sinonasal mucosa. The pathogenicities of fungi likely depends on the host's genetic background, site of invasion, nature of the local immunity and type of fungal organisms. The immune responsiveness to many microbial pathogens depends on the family of pattern recognition receptors (PRRs). Most fungi are detected and destroyed within hours by innate defense mechanisms mediated by phagocytes and opsonins through the involvement of distinct PRRs. Epithelial cells situated at the interface between the external environment and the mucosal surface of the respiratory, digestive and urogenital tract express TLRs, which are the major innate recognition system of microbial invaders [13].

Activation of the TLRs of epithelial cells allows the primary defensive immune mechanisms to be initiated locally, while initiating the communication of the presence of pathogens to the adaptive immune system. TLRs not only participate in the host defense but also have a more general role in epithelial homeostasis and protection from cell injury [14]. There are 11 known mammalian TLRs, all characterized by the presence of an extracellular domain with leucine-rich repeats and an intracellular signaling domain similar to that of the IL-1 receptor family [15]. TLR2 and TLR4 have specificity for peptidoglycan and lipopolysaccharide, they are important components of Gram-negative bacterial cell walls. TLR3 has specificity for double-stranded RNA, a common product of viral replication. TLR5 recognizes bacterial flagellin and TLR9 recognizes unmethylated CpG containing DNA. The expression and function of TLR in upper airway epithelial cells has not been extensively studied, the contribution of TLR in the nasal epithelial cells to the inflammatory response after fungal exposure requires more detailed investigation.

Prior reports have shown that *A. fumigatus* antigens induced the production of IL-6 and IL-12 from dendritic cells via TLR2 and TLR4. The addition of polymorphonuclear leukocytes to the *Candida albicans* infected oral epithelial cells strongly upregulated cytoplasm and cell surface epithelial TLR4 expression [16]. The majority of previous studies on antifungal immune responses pointed to the critical role of the TLRs, in particular TLR2 and TLR4 in the immune cells, such as epithelial cells, macrophages and dendritic cells. Patients with CRS as a whole, with or without nasal polyps, have demonstrated upregulation of TLR2 and TLR4 [17]. Pitzurra et al. [18] reported that TLR2, TLR3 or TLR4 mRNA were not detected in bacterial- or fungi-colonized nasal polyps. But in this study, fungi enhanced TLR2, TLR3 and TLR4 mRNA expression; this difference might be due to the experiment materials. Pitzurra et al. [18] studied with nasal polyp tissues and we studied with nasal epithelial cells. Although *Aspergillus* was commonly associated with airway diseases, *Alternaria* 25 µg/ml was the strongest stimulant for TLR2, TLR3 and TLR4 mRNA expression, and only TLR3 mRNA expression was inhibited by the cysteine protease inhibitors. *Alternaria*-induced IL-8 and GM-CSF release was dependent on TLR4 function. When nasal polyp epithelial cells were stimulated with TLR agonists, the ligand for TLR3 mediated the strongest pro-inflammatory effects [19]. This suggests that various PRRs might be involved in the complex interaction of fungi and epithelial cells. The use of different TLRs to

differentially induce the release of pro-inflammatory cytokines and chemokines has been described before [20]. The differences in antigen preparations, cytokines analyzed and cell types under study are likely to have a major impact on the results of the study of cell pathogen interaction.

Conclusions

Our findings demonstrated that fungi-induced cytokine production in primary nasal polyp epithelial cells and proteases present in fungal extracts interact with epithelial cells. Nasal polyp epithelial cells expressed high

levels of TLR2, TLR3 and TLR4 by *Alternaria*, and TLR mRNA expression was not closely related to the protease components of the fungi. TLR4 is thought to contribute to the production of IL-8 and GM-CSF. The results of our study suggest that *Alternaria* might have strong immunologic activity in nasal epithelial cells and plays an important role in the pathogenesis of CRS.

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