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Recognition of Fungal Protease Activities Induces Cellular Activation and Eosinophil-Derived Neurotoxin Release in Human Eosinophils

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Eosinophils are multifunctional leukocytes implicated in the pathogenesis of asthma and in immunity to certain organisms. Associations between exposure to an environmental fungus, such as Alternaria, and asthma have been recognized clinically. Protease-activated receptors (PARs) are G protein-coupled receptors that are cleaved and activated by serine proteases, but their roles in innate immunity remain unknown. We previously found that human eosinophils respond vigorously to Alternaria and to the secretory product(s) of eosinophils releasing their proinflammatory mediators. In this study, we investigated the roles of protease(s) produced by Alternaria and of PARs expressed on eosinophils in their immune responses against fungal organisms. We found that Alternaria produces aspartate protease(s) and that human peripheral blood eosinophils degranulate in response to the cell-free extract of A. alternata. Eosinophils showed an increased intracellular calcium concentration in response to Alternaria that was desensitized by peptide and protease ligands for PAR-2 and inhibited by a PAR-2 antagonist peptide. Alternaria-derived aspartate protease(s) cleaved PAR-2 to expose neo-ligands; these neo-ligands activated eosinophil degranulation in the absence of proteases. Finally, treatment of Alternaria extract with aspartate protease inhibitors, which are conventionally used for HIV-1 and other microbes, attenuated the eosinophils’ responses to Alternaria. Thus, fungal aspartate protease and eosinophil PAR-2 appear critical for the eosinophils’ innate immune response to certain fungi, suggesting a novel mechanism for pathologic inflammation in asthma and for host-pathogen interaction.


Eosinophils are multifunctional leukocytes implicated in the pathogenesis of asthma and in immunity to certain organisms. Associations between exposure to an environmental fungus, such as Alternaria, and asthma have been recognized clinically. Protease-activated receptors (PARs) are G protein-coupled receptors that are cleaved and activated by serine proteases, but their roles in innate immunity remain unknown. We previously found that human eosinophils respond vigorously to Alternaria and to the secretory product(s) of eosinophils releasing their proinflammatory mediators. In this study, we investigated the roles of protease(s) produced by Alternaria and of PARs expressed on eosinophils in their immune responses against fungal organisms. We found that Alternaria produces aspartate protease(s) and that human peripheral blood eosinophils degranulate in response to the cell-free extract of A. alternata. Eosinophils showed an increased intracellular calcium concentration in response to Alternaria that was desensitized by peptide and protease ligands for PAR-2 and inhibited by a PAR-2 antagonistic peptide. Alternaria-derived aspartate protease(s) cleaved PAR-2 to expose neo-ligands; these neo-ligands activated eosinophil degranulation in the absence of proteases. Finally, treatment of Alternaria extract with aspartate protease inhibitors, which are conventionally used for HIV-1 and other microbes, attenuated the eosinophils’ responses to Alternaria. Thus, fungal aspartate protease and eosinophil PAR-2 appear critical for the eosinophils’ innate immune response to certain fungi, suggesting a novel mechanism for pathologic inflammation in asthma and for host-pathogen interaction.

Epidemiological and clinical reports recognize associations between the presence of or exposure to certain airborne fungi, such as Alternaria (in North America) and Cladosporium and Aspergillus (in Europe) (4), and asthma and allergic diseases. For example, increased fungal spore counts and fungal Ag levels correlate with allergic symptoms (5–7). In a large multicountry and cross-sectional study, sensitization to Alternaria or Cladosporium, but not to pollens or cats, is a significant risk factor for severe asthma (odds ratio 2.34) (8), and exposure to Alternaria is a risk factor for respiratory arrest in patients with asthma (9). Thus, a major question remains as to the immunological mechanisms underlying the association between fungal exposure and asthma and allergic diseases.

Drosophila detects fungal infections by recognizing pathogen-associated molecular patterns (PAMPs)5 and by monitoring the effects of fungal virulence factors. Specifically, the receptor Gram-negative binding protein 3 on Drosophila recognizes the fungal cell wall β-(1,3)-glucan, and the secreted fungal virulence factor

5 Abbreviations used in this paper: PAMP, pathogen-associated molecular pattern; Abz-SKGRSLIGKdD, Abz-33Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys41(Dnp)-Asp; APMSF, 4-amidinophenylmethanesulfonyl fluoride hydrochloride; ATBI, alkaloide thermophilic Bacillus aspartate protease inhibitor; [Ca2+]i, intracellular calcium concentration; (DABCYL)-ERNleFLSP(EDANS), DABCYL-Glu-Arg-Nle-Phe-Leu-Ser-Phe-Pro-EDANS; EDN, eosinophil-derived neurotoxin; GLIKVS, Gly-Leu-Ile-Val-Lys-Ser-NH2; IGKVDGI, Ile-Gly-Lys-Val-Asp-Gly-NH2; LC-MS/MS, capillary reverse-phase HPLC with tandem mass spectrometric detection; LIGKVD, Leu-Ile-Gly-Lys-Val-Asp-NH2; LSGIKV, Leu-Ser-Ile-Gly-Lys-Val-NH2; PAR, protease-activated receptor; PRR, pattern-recognition receptor; SLIGKVS, Ser-Leu-Ile-Gly-Lys-Val-NH2;
PR1 protease cleaves *Drosophila* Persephone, activating the downstream immune response to the fungi (10). Because fungi use the PR1 protease to break down the protective cuticle of the insect and allow infection (11), the *Drosophila* Persephone may act as a sensor to monitor the fungal protease activity and integrity of the cuticle. Whether humans have analogous sensor systems to recognize fungal virulence factors remains unknown.

In this study, we used the fungus, *Alternaria*, as a model microbe relevant to human asthma, to investigate the molecular mechanisms involved in the immune recognition of ubiquitous environmental allergen(s). Human eosinophils are activated by live *Alternaria alternata* organisms, release their granule proteins, and kill the fungi (12). Eosinophils, but not neutrophils, responded to secreted products from *A. alternata* (13). We found that eosinophils are equipped with innate cellular activation machinery that responds to the extracellular aspartate protease activity secreted by *Alternaria*. A novel mechanism to activate protease-activated receptor (PAR)-2, as compared with serine protease activation of PAR-2, is most likely involved. Thus, human eosinophils may recognize certain danger signals or virulence factors produced by fungi and respond with inflammatory reactions against these organisms. Dysregulation of such an innate immune mechanism may play roles in the pathophysiology of human diseases, such as asthma.

**Materials and Methods**

**Materials**

Culture extracts from a. *alternata* are derived from the fungi’s growth media; as fungi grow, they excrete proteins into the medium; the medium liquid is dialyzed and lyophilized (Greer Laboratories). EGTA, trypsin, chymostatin, 4-amidinophenylmethanesulfonyl fluoride hydrochloride (APMSF), and trans-epoxysuccinyl-t-leucylamide (4-guanidino) butane were from Sigma-Aldrich. Ionomycin, alkalo-thermophilic Bacillus aspartate protease inhibitor (ATBI), Indo-1/AM, and PMA were from Calbiochem. Platelet-activating factor (PAF) was from BIOMOL. Agarose beads conjugated with the aspartate protease inhibitor, pepstatin A, and control agarose beads were from Pierce. Ritonavir, an HIV aspartate protease inhibitor, was provided by A. Badley (Mayo Clinic, Rochester, MN). The 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride was from Sigma-Aldrich. Tethered ligand peptides specific for human PAR-2 were made at Mayo Clinic, as follows: PAR-2 agonistic peptide, Ser-Leu-Ile-Gly-Lys-Val-NH$_2$ (SLIGKV); PAR-2 antagonist peptide, Leu-Ser-Ile-Gly-Lys-Val-NH$_2$ (LSIGKV); and control PAR-2 scrambled peptide, Gly-Leu-Ile-Val-Lys-Ser-Asp-Arg-Ser-NH$_2$ (GLIVKS) (14, 15). The PAR-2 peptides, which represent novel N-terminal sequences generated by trypsin cleavage site (i.e., 36Arg-37Ser) of PAR-2, was from JPT Peptides. The 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride was from Sigma-Aldrich. A. alternata extract was separated by DEAE anion-exchange chromatography (GE HealthSciences; buffer A, 20 mM Tris (pH 7.5); buffer B, 20 mM Tris, 1 M NaCl (pH 7.5), and 45 1-ml fractions were collected; 2 μl/fraction was tested for eosinophil degranulation.

**Measurement of intracellular calcium concentration ([Ca$^{2+}$]$_i$)**

Real-time changes in [Ca$^{2+}$], were measured by flow cytometry (18) using the calcium indicator, Indo-1/AM (19). To load the eosinophils with Indo-1, a 1-mL suspension (1 × 10$^6$ cells/ml) was incubated with 3 mM Indo-1/AM in phenol red-free HBSS with 10% α calf serum and 10 mM HEPES for 30 min at 37°C. After washing, cells were suspended in HBSS with 0.1% human serum albumin, 10 mM HEPES, and 1.2 mM calcium. To measure [Ca$^{2+}$], cells were stimulated with agonists, including *Alternaria* extract, PAF, and ionomycin, and fluorescence was analyzed by a FACs analyzer with an ion-argos laser (BD Biosciences). [Ca$^{2+}$], was monitored for 600 s based on the ratio of the fluorescence of the calcium-bound Indo-1/AM emission (401 nm) and the free Indo-1/AM emission (475 nm). To examine the dependency of the eosinophil [Ca$^{2+}$], response on extracellular calcium, cells were preincubated with 3 mM EGTA for 15 min at 37°C before stimulation. To investigate the roles of PAR-2 in the eosinophils’ [Ca$^{2+}$], response to *Alternaria*, we used both desensitization and PAR-2 antagonist approaches. Eosinophils, loaded with Indo-1 as above, were first incubated with 1 μM trypsin, 100 μM PAR-2 agonist peptide (SLIGKV), or 100 μM control peptide (GLIVKS) at 20 s and then stimulated with 50 μg/ml *Alternaria* extract or 1 μM ionomycin. Alternatively, eosinophils were preincubated with PAR-2 antagonist peptide (LSIGKV) or control peptide (GLIVKS), and then stimulated with *Alternaria* extract.

**Quantiﬁcation of PAR-2 cleavage activity and aspartate protease activity**

The enzymatic activities of proteases were measured using synthetic, ﬂuorogenic peptide substrates where cleavage of the internal sequence of the peptide substrate generates ﬂuorescence by ﬂuorescence resonance energy transfer (20). An N-terminal peptide from human PAR-2, Abz-SKGRS-LiGKVd, and the substrate for malaria aspartate protease, (DABCYL)-ERNleFLSFP(EDANS) (21), were used to measure protease activities. *Alternaria* extract (50 and 100 μg/ml) and 1 μM trypsin in HBSS with 10 mM HEPES and 0.01% gelatin (pH 7.4) were mixed with 10 mM substrate. The time course of enzymatic activity was measured spectrofluorometrically ($\lambda_{	ext{exitation}} = 360 \text{ nm}; \lambda_{	ext{emission}} = 460 \text{ nm}$) on a CytoFluo MultiWell Plate Reader (PerSeptive Biosystems).

**Role of aspartate protease in PAR-2 cleavage and eosinophil activation**

To investigate the role(s) of aspartate protease in PAR-2 cleavage and eosinophil activation, several approaches were taken. First, *Alternaria* extract was treated with a half volume of peptatin A gel (Pierce; 0020215), Reactivex (Pierce; 0020260 as control agarose); for 15 min. After centrifugation at 1000 × g for 5 min, the supernatants were assayed for EDN release, PAR-2 cleavage activity, and [Ca$^{2+}$], response. As controls, trypsin, PMA, or PAF was treated similarly to the *Alternaria* extract. The final concentrations were as follows: *Alternaria* extract, 50 μg/ml; trypsin, 6 μM; PMA, 1 ng/ml; and PAF, 1 μM. Second, to investigate other protease inhibitors, stimuli were treated with ATBI, ritonavir, APMSF, or medium alone for 30 min at room temperature. These mixtures were incubated with eosinophils, and EDN release, PAR-2 cleavage, and [Ca$^{2+}$], were measured.
Production of PAR-2-cleaving protease(s) by Alternaria cultured with mucin

The effects of Alternaria’s growth and production of PAR-2-activating enzyme(s) were examined. *A. alternata* (ATCC11680) was purchased from ATCC and cultured on potato agarose media (Difco). The fungi were tagged with GFP gene, as previously described (22). Spores of GFP-transformed *A. alternata* (1000 spore/well) were suspended in HBSS medium supplemented with or without bovine submaxillary gland mucin (Sigma-Aldrich). After 24 or 48 h at 30°C, fungal growth was measured by the GFP fluorescence intensities using a CytoFluor MultiWell Plate Reader. Supernatants of fungal cultures were collected, and the PAR-2-activating proteases in the supernatants were measured by PAR-2 fluorogenic peptide substrates, Abz-SKGRSLIGKdD, as described above.

Capillary reverse-phase HPLC with tandem mass spectrometric detection (LC-MS/MS) analysis of the PAR-2 cleavage site(s) by Alternaria extract

To identify the PAR-2 cleavage site(s) used by *Alternaria* extract, we analyzed cleavage products of the peptide substrate, Abz-SKGRSLIGKdD, which contains a sequence near the N terminus of human PAR-2. The peptide substrate was incubated with *Alternaria* extract (50 and 100 μg/ml) or 1 nM trypsin (as a positive control) for 30 min. To examine specificity, the proteases were also preincubated with pepstatin A gel, control gel, or APMSF, as described above, before incubation with the peptide substrate. The peptide products were subjected to capillary reverse-phase liquid chromatography using a Cap liquid chromatography system (Waters). A total of 10 μl from each incubation was injected and trapped on a Targa C18 cartridge column (5 μM, 2.5 × 0.5 mm; Higgins Analytical) before separation on a Targa C18 column (5 μM, 50 × 0.150 mm; Higgins Analytical). Peptides were separated using a gradient starting at 95% mobile phase A (98% water:1% acetonitrile:1% n-propanol:0.2% formic acid, v/v) and going to 55% B (10% water:80% acetonitrile:10% n-propanol:0.2% formic acid, v/v) over 35 min. The flow rate was 12 μl/min and was split precolumn, allowing ~400 nl/min into the nano-electrospray ionization source on a Micromass Q-TOF API-US quadrupole time-of-flight mass spectrometer (Waters). MS and MS/MS spectra were collected in positive mode using a precursor ion scan range of 100-1800 m/z. To identify the peptides, the experimental peptide masses were compared with the expected masses from the known peptide substrate sequences.

Analysis of a fraction from DEAE anion-exchange chromatography

An active fraction from DEAE anion-exchange chromatography, namely fraction 18, was further characterized by SDS-PAGE and by proteomic analysis. Briefly, fraction 18 was purified by hydroxyapatite chromatography (Bio-Rad) and the DuoFlow Fast Performance Liquid Chromatography system (Bio-Rad: buffer A, 50 mM phosphate (pH 6.8); buffer B, 500 mM phosphate (pH 6.8)). *Alternaria* crude extract and partially purified fraction 18 were electrophoresed with SDS-PAGE and stained with silver stain. Fraction 18 was also tryptic digested, and the resulting peptides were analyzed by LC-MS/MS, as described above. The experimental peptide masses were Blasted/searched against several databases (GenBank NR, a set of predicted proteins derived from the *Alternaria brassicicola* whole genome sequence, and *Alternaria* expressed sequence tags) to identify fungal genes encoding potential immunostimulatory proteins.

Statistics

Data from ≥3 experiments from different donors were summarized and presented as mean ± SEM. A one-way ANOVA with repeated measures, Student’s *t* test, or Mann-Whitney *U* test were used to analyze statistical significance. Significance was established at the *p* < 0.05 level.

**Results**

**Alternaria induces eosinophil degranulation that depends on 
**[Ca2+]i**

To examine the effects of *Alternaria* exposure on eosinophil activation and degranulation, eosinophils were incubated with increasing concentrations of *A. alternata* extract in vitro. Alternaria extract induced degranulation (as measured by EDN release) of human eosinophils in a concentration-dependent manner (Fig. 1A). The effects reached a plateau at 50 μg/ml Alternaria. This degranulation increased with time (results not shown), and after 3-h incubation, *Alternaria* (50 μg/ml) induced maximal EDN release, ~30% of total cellular EDN. To investigate whether eosinophils recognize *Alternaria* products through TLR, we used blocking Abs, including anti-TLR2 and anti-TLR4, and observed minimal (~10%) inhibition of *Alternaria*-induced degranulation (Fig. 1B).

The molecular mechanisms for eosinophil degranulation are incompletely understood, but increased [Ca2+]i plays a pivotal role (2, 23). Therefore, we examined whether exposure to *Alternaria* extract induces increased [Ca2+]i. Eosinophils incubated with 75 μg/ml *Alternaria* extract showed gradual increases in [Ca2+]i, but not on TLRs. Consequently, we examined whether exposure to *Alternaria* extract induces increased [Ca2+]i. Eosinophils loaded with the calcium-sensitive fluorescent dye, Indo-1/AM, and stimulated after 20 s (denoted by arrows) with 75 μg/ml *Alternaria* extract or 1 μM PAF in the presence or absence of 3 mM EGTA. [Ca2+]i, was analyzed by FACS. *C.* Eosinophils were loaded with the calcium-sensitive fluorescent dye, Indo-1/AM, and stimulated after 20 s (denoted by arrows) with 75 μg/ml *Alternaria* extract or 1 μM PAF in the presence or absence of 3 mM EGTA. [Ca2+]i, was analyzed by FACS. *D.* Eosinophils were incubated with 50 μg/ml *Alternaria* extract or 1 μM PAF in the presence or absence of 3 mM EGTA. [Ca2+]i, was analyzed by FACS. *E.* Significant differences compared with medium alone (*p* < 0.05). *F.* Significant differences compared with medium alone (*p* < 0.05).
response. In contrast, an authentic lipid agonist for eosinophils, which stimulates a seven-transmembrane G protein-coupled PAF receptor (24), rapidly increased [Ca\(^{2+}\)]_i by 50 s, followed by gradual decrease for 250 s. EGTA modestly affected the rapid phase, but abolished the plateau phase, suggesting that PAF induces the initial release of calcium ion from the intracellular stores, followed by influx from extracellular milieu. EGTA also inhibited the Alternaria- and PAF-induced EDN release by 97% (p < 0.01; n = 4) and 85% (p < 0.01; n = 4), respectively (Fig. 1D). Thus, eosinophils exposed to Alternaria extract show a robust [Ca\(^{2+}\)]_i response and degranulate, and the [Ca\(^{2+}\)]_i response plays a critical role in degranulation.

**Eosinophil response to Alternaria involves PAR-2**

Fungi produce large quantities of proteases (4). Heat treatment of Alternaria extract at 56°C for 30 min destroys its ability to induce eosinophil degranulation (13), suggesting protease-like activity. A four-member family of seven-transmembrane G protein-coupled receptors, PARs, is activated by proteases, in particular serine proteases (25, 26); in general, PAR-1, PAR-3, and PAR-4 respond to thrombin, and PAR-2 responds to trypsin and trypsin-like serine proteases. With human eosinophils, trypsin induces cellular activation and triggers degranulation through PAR-2 (27, 28); other PARs are probably not involved in eosinophil activation. Therefore, we suspected a role for PAR-2 in the Alternaria-induced [Ca\(^{2+}\)]_i response and subsequent degranulation of human eosinophils. To test the hypothesis, we examined whether an authentic enzymatic agonist for PAR-2 (i.e., trypsin) would desensitize the eosinophils’ [Ca\(^{2+}\)]_i response to Alternaria extract. Eosinophils were exposed to trypsin at 20 s and then stimulated with agonists, Alternaria extract, or ionomycin (a negative control) at 210 s. Trypsin partially decreased the Alternaria-induced [Ca\(^{2+}\)]_i response (Fig. 2A). The ionomycin-induced [Ca\(^{2+}\)]_i response was not affected by trypsin pretreatment, suggesting specificity for trypsin’s desensitizing effects.

Trypsin cleaves the extracellular N terminus of PAR-2 between the R\(^{36}\) and S\(^{37}\) and exposes a tethered neo-ligand (i.e., S\(^{37}\)LIGKV-) that, in turn, binds intramolecularly to PAR-2 and triggers receptor activation (25, 26). Synthetic peptides, such as SLIGKV, corresponding to the sequence of the tethered neo-ligand, can also bind to uncleaved PARs; these peptides are valuable tools to study the consequences of ligand binding to PAR-2 without exogenous proteases. Similarly to desensitization with trypsin, the incubation of eosinophils with PAR-2 peptide agonist, SLIGKV, desensitized eosinophil PAR-2 and markedly decreased the Alternaria-induced [Ca\(^{2+}\)]_i response (Fig. 2B); incubation with a scrambled peptide, GLIVKS, did not desensitize the PAR-2.

Although no small molecule inhibitors for PAR-2 are available, a modified PAR-2-derived peptide, LSIGKV, does interact with a tethered ligand binding site on PAR-2 and inhibits trypsin-induced activation of PAR-2 (14). Thus, eosinophils were exposed at 20 s to LSIGKV, a scrambled peptide (GLIKVS), or medium, and then stimulated with Alternaria at 210 s. LSIGKV, but not GLIKVS, decreased the Alternaria-induced [Ca\(^{2+}\)]_i response (Fig. 2C). Altogether, PAR-2 is most likely involved in the eosinophils’ [Ca\(^{2+}\)]_i response to Alternaria extract.

To examine effects of the modified PAR-2 peptide on eosinophil degranulation, eosinophils were incubated with LSIGKV or a scrambled GLIKVS for 30 min and stimulated with Alternaria extract, PAF, or PMA for 3 h. The LSIGKV peptide partially, but significantly inhibited Alternaria-induced EDN release by 54% (p < 0.01; n = 5; Fig. 2D). The LSIGKV peptide had no effect on PAF- and PMA-induced eosinophil degranulation. In contrast, a control peptide, GLIKVS, showed no effects on Alternaria-induced eosinophil degranulation.

**Aspartate protease activity from Alternaria activates human PAR-2**

To characterize the protease activity in Alternaria extract that cleaves and activates human PAR-2, we used a fluorogenic peptide substrate, Abz-SKRGLSGLKD, which corresponds to the 4 aa on either side of the trypsin cleavage site of human PAR-2 (i.e., \(^{36}\)Arg\(^{37}\)Ser) (29). The Abz group fluoresces only after release of the Lys(Dnp) group, following cleavage of internal peptides. As expected, the PAR-2 peptide was cleaved by trypsin, resulting in a time-dependent increase in the fluorescence intensity (Fig. 3A). Thrombin, an agonist for PAR-1, PAR-3, and PAR-4, did not cleave the peptide (data not shown). Alternaria extract cleaved the PAR-2 peptide in a concentration-dependent manner with kinetics of peptide cleavage similar to trypsin.
Serine proteases, such as trypsin and trypsin-like proteases (e.g., mast cell tryptase, tissue kallikreins, and coagulation factors VIIa and Xa), cleave and activate PAR-2 (25, 26). The ability of cysteine proteases, such as house dust mite Der p 1, to activate PAR-2 and Xa), cleave and activate PAR-2 (25, 26). The ability of cysteine protease activity in Alternaria extract responsible for the PAR-2 cleavage, we used protease inhibitors. As expected, PAR-2 cleavage by trypsin was inhibited by APMSF or medium control for 15 min. Alternatively, Alternaria extract and trypsin were treated with pepstatin A gel (aspartate protease inhibitor) or control gel for 60 min. Activities of the treated proteases to cleave the PAR-2 fluorogenic peptide, Abz-SKGRLIGKdD, were monitored spectrophotometrically for 60 min. B, Alternaria extract (50 μg/ml) and trypsin (6 nM) were treated with 200 μM APMSF or medium control for 15 min. Alternatively, Alternaria extract and trypsin were treated with pepstatin A gel (aspartate protease inhibitor) or control gel for 60 min. Activities of the treated proteases to cleave the PAR-2 fluorogenic peptide, Abz-SKGRLIGKdD, were monitored spectrophotometrically for 60 min.

Aspartate protease activity in Alternaria extract induces [Ca^{2+}]_i response and degranulation of human eosinophils

To characterize the eosinophil-activating aspartate protease activity in Alternaria extract further, we used DEAE anion-exchange column chromatography to fractionate Alternaria extract. The column fractions were tested for their aspartate protease activities by using (DABCYL)-ERNleFLSFP(EDANS) and for their activities to induce eosinophil degranulation by EDN release. The most potent eosinophil degranulation and aspartate protease activities were detected in fractions 17–19 (Fig. 5); other fractions contained minimal or no activity. These active fractions from 17 to 19 also induced the [Ca^{2+}]_i response, similar to the unfractonated Alternaria extract (data not shown). Fraction 18 contained aspartate protease-like protein by proteomic analysis (supplemental figure and table). Thus, the aspartate protease activity in Alternaria extract is a likely candidate to induce eosinophil degranulation.

To verify this finding, we investigated the effects of aspartate protease inhibitors on the Alternaria extract-induced [Ca^{2+}]_i response and degranulation. Treatment of Alternaria extract with pepstatin A gel, but not control gel, inhibited the extract’s [Ca^{2+}]_i response (Fig. 6A). This pepstatin A gel inhibition is most likely specific because neither pepstatin A nor control gel inhibited the

\[ p < 0.01, n = 5 \]

Pepstatin A showed no effects on trypsin-mediated PAR-2 cleavage, demonstrating the inhibitor’s specificity. Furthermore, aspartate protease activity, as examined by using a fluorogenic peptide substrate for malaria aspartate protease, (DABCYL)-ERNleFLSFP(EDANS) (21), was clearly detected in Alternaria extract, but not in trypsin (Fig. 3C). Thus, aspartate protease activity in Alternaria extract, but not serine protease activity, appears to cleave and activate PAR-2.

During fungal germination and growth, the production of so-called allergens by fungi increases markedly (33). We used live A. alternata to examine the growth and production of PAR-2 cleavage activity. In HBSS liquid medium supplemented with bovine mucin, A. alternata spores germinated and grew in a mucin-dependent manner with extensive growth between 24 and 48 h (Fig. 4A). Similarly, PAR-2 cleavage activity in the A. alternata supernatants increased with mucin concentration (Fig. 4B). Thus, Alternaria produces and releases PAR-2-activating enzyme(s) extracellularly during germination and active growth.

\[ \text{Aspartate protease activity in Alternaria extract induces } [\text{Ca}^{2+}]_i \text{, response and degranulation of human eosinophils} \]

The online version of this article contains supplemental material.

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Aspartate protease activity and activate eosinophils. Alternaria extract was separated by DEAE anion-exchange chromatography; the elution profile shows absorbance at 280 nm and addition of buffer B. DEAE fractions were analyzed for their aspartate protease activities by using the malaria aspartate protease substrate, (DABCYL)-ERNleFLSFP(EDANS), and for eosinophil degranulation activity by EDN release.

PAF-induced [Ca\(^{2+}\)]\(_i\) response. Two other classes of aspartate protease inhibitors, namely ritonavir and ATBI, which inhibit HIV-1 aspartate protease retropepsin and secreted aspartate proteases of Candida albicans (34–36), respectively, partially inhibited the Alternaria-induced [Ca\(^{2+}\)]\(_i\) response (Fig. 6A). In contrast, these same inhibitors did not inhibit the PAF-induced [Ca\(^{2+}\)]\(_i\) response.

For eosinophil degranulation, a serine protease inhibitor, APMSF, inhibited the trypsin-induced degranulation by ~85%; trypsin treated with pepstatin A gel or control gel retained its ability to induce degranulation (Fig. 6B). In contrast, pepstatin A gel, but not the control gel, inhibited the Alternaria-induced eosinophil degranulation by >90% (p < 0.01, n = 5). APMSF showed no effects on Alternaria-induced degranulation. Neither pepstatin A gel nor APMSF inhibited eosinophil degranulation induced by PMA. Both ATBI and ritonavir partially, but significantly inhibited Alternaria extract-induced EDN release by ~60% (p < 0.01, n = 5) and by ~50% (p < 0.01, n = 5) at optimal concentrations, respectively (Fig. 6C). Thus, the aspartate protease activity in Alternaria extract appears to be involved in the eosinophil’s [Ca\(^{2+}\)]\(_i\) response and degranulation.

**Aspartate protease in Alternaria cleaves human PAR-2 peptide at novel sites**

PAR-2 is cleaved by trypsin or trypsin-like proteases at a specific site, namely between Arg\(^{36}\) and Ser\(^{37}\); however, there are no reports for the effects of aspartate proteases on PAR-2. Therefore, we asked whether the Alternaria aspartate protease(s) cleaves PAR-2 at the same site as trypsin and whether the neo-ligand exposed by this reaction acts as a ligand for PAR-2. We used the peptide substrate, Abz-SKGR\(^{36}\)S\(^{37}\)LIGKdD, which corresponds to the 4 aa on either side of the PAR-2’s trypsin cleavage site. The Abz-SKGRSLIGKdD substrate was incubated with Alternaria extract, with or without pretreatment with protease inhibitor, and peptide fragments from each incubation were analyzed by LC-MS/MS. As expected, trypsin cleaved between Arg\(^{36}\) and Ser\(^{37}\) and produced the SLIGKdD fragment (25, 26) (Table I); trypsin pretreated with APMSF did not produce the SLIGKdD fragment (data not shown).

![Diagram](http://www.jimmunol.org/)

**FIGURE 5.** Partially purified proteins in Alternaria extract show aspartate protease activity and activate eosinophils. Alternaria extract was separated by DEAE anion-exchange chromatography; the elution profile shows absorbance at 280 nm and addition of buffer B. DEAE fractions were analyzed for their aspartate protease activities by using the malaria aspartate protease substrate, (DABCYL)-ERNleFLSFP(EDANS), and for eosinophil degranulation activity by EDN release.

**FIGURE 6.** Aspartate protease inhibitors suppress Alternaria-induced eosinophil [Ca\(^{2+}\)]\(_i\) response and degranulation. A, Alternaria extract, 50 µg/ml, was pretreated with pepstatin A gel and control gel (top) for 60 min or with aspartate protease inhibitors, 20 µM ATBI (middle) and 1 µM ritonavir (bottom), for 15 min. PAF (1 µM), which was treated similarly, was used as a control. Treated Alternaria extract (left panels) or PAF (right panels) was added to eosinophils, and the [Ca\(^{2+}\)]\(_i\) response was measured. B, Trypsin (6 nM), Alternaria extract (50 µg/ml), and PMA (1 ng/ml) were pretreated with pepstatin A gel and control gel for 60 min or with 200 µM APMSF for 15 min. Eosinophils were incubated with treated stimuli for 3 h, and EDN release was measured. Results show the mean ± SEM from five different experiments. * Significant differences compared with no inhibitors (p < 0.01). C, Alternaria extract was pretreated with indicated concentrations of ATBI and ritonavir for 15 min and incubated with eosinophils for 3 h; EDN release was measured by RIA. Results show the mean ± SEM from five different experiments. * Significant differences compared with no inhibitors (p < 0.01).

| Peptide fragments produced from a PAR-2 N-terminal peptide by Alternaria extract* |
|------------------|------------------|------------------|
| Enzymes          | Inhibitor Pretreatment | Generated Peptide and Fragments |
| None             | None             | SKGRSLIGKdD       |
| Trypsin          | None             | -SLIGKdD          |
| Alternaria       | None             | SKGRSLIGKdD, -LIGKdD, -GIGKdD |
| Alternaria APMSF | SKGRSLIGKdD, -LIGKdD, -GIGKdD |
| Alternaria Pepstatin gel | SKGRSLIGKdD |
| Alternaria Control gel | SKGRSLIGKdD, -LIGKdD, -GIGKdD |

* The peptide SKGRSLIGKdD containing a N-terminal sequence of human PAR-2, from Ser\(^{33}\) to Lys\(^{42}\), was incubated with trypsin and Alternaria extract treated with protease inhibitors indicated above for 60 min. The generated peptide fragments were analyzed by LC-MS/MS.
Alternaria/H11001 60 min and analyzed by LC-MS/MS. Chromatograms display ions at 

In contrast, Alternaria cleaved the substrate and produced two new fragments, namely LIGKdD and IGKdD (Table I and Fig. 7A); Alternaria did not cleave the substrate to generate the SLIGKdD fragment. When Alternaria was treated with pepstatin A gel, no new peptide fragments were produced, but neither the control gel nor APMSF inhibited the Alternaria extract's ability to generate new peptide fragments (Table I, Fig. 7A). Thus, compared with trypsin, the aspartate protease(s) in Alternaria cleaves human PAR-2 at unique peptide sequence sites (Ser37/Leu38, Leu38/Ile39).

When cleaved by trypsin, a neo-ligand of human PAR-2, namely S37LIGKV-, serves as a ligand for PAR-2 itself (25, 26). Synthetic peptides, such as SLIGKV, that mimic those produced by aspartate protease(s) from Alternaria, can represent neo-ligands of human PAR-2 and stimulate PAR-2 without proteases. Eosinophils were incubated with the synthetic peptides, L38IGKVD43 and L39GKVDG44, representing neo-ligands produced by the Alternaria extract. The peptide, S37LIGKV42, representing the neo-ligand generated by trypsin, and its scrambled peptide, GLIVKS, served as positive and negative controls, respectively. SLIGKV as well as LIGKVD and IGKVDG induced EDN release from eosinophils (Fig. 7B, p < 0.01, n = 7); IGKVDG induced significantly more EDN release than SLIGKV (at both 50 and 200 μM; p < 0.05, n = 7). A control peptide, GLIVKS, showed no effect. Overall, these results suggest a novel mechanism for PAR-2 activation and cellular activation in human eosinophils stimulated by aspartate protease(s) from Alternaria.

Discussion

In this study, we found that the PAR-2-mediated recognition of aspartate protease activity secreted by the actively growing fungus, Alternaria, triggers human eosinophils to become activated and degranulate. These conclusions are based on the following observations. 1) A PAR-2 agonist enzyme and PAR-2 ligand peptide desensitized the Alternaria-induced [Ca2+]i response, and a modified PAR-2 peptide inhibited the [Ca2+]i response. 2) Aspartate protease activity present in extracts of growing A. alternata, not serine protease activity, cleaved and activated human PAR-2. 3) Various aspartate protease inhibitors, but not serine or cysteine protease inhibitors, reduced the Alternaria-induced PAR-2 activation, [Ca2+]i response, and EDN release in eosinophils. 4) Alternaria aspartate protease(s) cleaved PAR-2 to expose novel neo-ligands (e.g., IGKVDG- and IGKVDG-), which were distinct from neo-ligands generated by trypsin; these neo-ligands activated eosinophil degranulation without proteases. Although we used both biochemical and cell biological methods to demonstrate the role of PAR-2, our study has a potential limitation in that we were unable (for technical reasons) to verify our observations with molecular biological methods, such as small interfering RNA for PAR-2.

Although humans are normally exposed to many airborne proteins and microorganisms, only a small fraction contributes to asthma. How innate immune receptors discriminate between pathogenic and nonpathogenic molecules or microorganisms remains a fundamental immunological question. Because both pathogenic and nonpathogenic organisms most likely have similar PAMPs (37, 38), the recognition of PAMPs by TLRs and other pattern-recognition receptors (PRRs) may not fully explain the discrimination between these organisms. Also, mammalian TLRs and PRRs lack the receptor diversity to match the microbial and environmental diversity (39, 40). Plants respond to infection using a two-branched innate immune system, as follows: one recognizes and responds to molecules common to many microbes, including nonpathogens, and the other one responds to pathogen virulence factors (41). In Drosophila, the detection of fungal infections relies
both on PAMP recognition and on monitoring the effects of virulence factor protease(s) (10). In mammals, the roles for TLRs and other PRRs, such as TLR2, TLR4, TLR9, IL-1R1, and dectin-1, to recognize fungal or yeast infections are well established (42).

Thus, like plants and Drosophila, our findings suggest that receptors, such as PAR-2, are activated by endogenous and exogenous proteases and may act like sensors to monitor fungal protease activities or putative virulence factors and provoke immune and inflammatory responses. Mammalian TLRs have most likely evolved to survey exogenous products from microorganisms (e.g., LPS) and also the host’s endogenous tissue degradation products (e.g., oligosaccharides of hyaluronan (43) and molecules released from necrotic cell death, such as high-mobility group box 1 protein (44)). Similarly, mammalian PARs may have evolved to survey both exogenous products from microorganisms (e.g., fungal aspartate protease(s)) and endogenous products (e.g., trypsin).

Several reports implicate a role for PAR, especially PAR-2, in airway inflammation and asthma. In mouse airways in vivo, co-administration of PAR-2 agonist peptide and an experimental Ag, OVA, enhanced Th2-type sensitization to OVA, whereas administration of OVA alone induced tolerance (45). In patients with asthma, PAR-2 is overexpressed in airway epithelial cells (46), but natural ligands for PAR-2 in human airways are not fully understood. In human epithelial cells, PAR-2 recognizes serine protease allergens, such as Der p 3, Der p 9, and Pen c 13; this induces production of proinflammatory cytokines and chemokines (31, 47, 48). Arginine-specific (trypsin-like) cysteine proteinases, the gipains, as produced by a periodontopathic bacterium, Porphyromonas gingivalis, also activate PAR-2 (49–51). Furthermore, exogenous chitinase from a bacterium, Streptomyces griseus, cleaves human PAR-2 peptide and induces a PAR-2-dependent [Ca^{2+}] response (52). Thus, PAR-2 can recognize both conventional trypsin-like proteases and perhaps other proteases and glycosidases derived from microbes, fungi, and insects. Further studies will be needed to elucidate whether and how PARs are involved in inflammation and perhaps tissue repair and remodeling in human asthma.

Although the PAR-2 peptides, corresponding to the neo-ligands produced by Alternaria, induced eosinophil degranulation, the amounts of EDN release were smaller than those induced by Alternaria extract (compare Figs. 1 and 7). Perhaps the peptide ligands have lower affinities compared with the natural ligands. However, other explanations are possible. Alternaria extract contains a mixture of various biomolecules, including proteases, glycosidases, and carbohydrates (supplemental figure and table). Human PAR-2 possesses two N-linked glycosylation sequons, and the wild-type molecule is highly glycosylated (53). Deglycosylation of PAR-2 increased its sensitivity to tryptase and decreased its sensitivity to trypsin (53). Moreover, our Alternaria extract contained β-glucan (44 μg/mg extract), which is one of the ligands for eosinophil β2-integrin CD11b, and the interaction between β-glucan and CD11b is implicated in eosinophil activation in response to live Alternaria organisms (12). Furthermore, synergistic molecular interactions between PAMPs and PARs, such as TLR4 and PAR-2, have been recognized (54). Thus, the Alternaria extract’s ability to induce robust EDN release, as compared with PAR-2 peptides, may be explained by the effects of other enzymes in the extract on PAR-2 as well as the presence of carbohydrate molecules, which may act synergistically with PAR-2-activating enzymes.

The Alternaria aspartate protease(s) is most likely sensitive to an authentic aspartate protease inhibitor, pepstatin A, and also to inhibitors for aspartase proteases from other microbes, such as C. albicans and HIV-1 (i.e., ATBI and ritonavir). These inhibitors suppressed PAR-2 cleavage (Fig. 3) and also suppressed the eosinophils’ [Ca^{2+}], response and degranulation (Fig. 6) induced by Alternaria aspartate protease(s). With a molecular mass of ~50–60 kDa (13), the Alternaria aspartate protease(s) is distinct from known Alternaria allergens. Little is known about the biology of Alternaria aspartate protease(s), but aspartase proteases secreted by C. albicans are virulence factors (55–57). These C. albicans-secreted aspartic proteases are most likely critical for infection by breaking down tissue barriers during invasion, destroying host defense molecules, and providing nutrition (57). A novel class of fungal cell wall aspartate proteases, the yapsins, is also implicated in fungal cell wall assembly and integrity (58).

Increased understanding about how Alternaria aspartate protease(s) and other exogenous fungus-derived PAR-activating proteases affect immune cells could explain the interactions between fungi and immune responses and their roles in disease. Recent advances in fungal genomics could facilitate the process (59).

In summary, we discovered that aspartase protease activities secreted by Alternaria induce activation and EDN release from human eosinophils through PAR-2. Thus, a novel communication network may exist involving pathogens, immune cells, proteases, and their putative receptors. A recent study suggests that the lipid-binding property of Der p 2, which mimics MD-2 in the TLR4 signaling complex, provides intrinsic adjuvant activity and makes this molecule highly allergenic (60). Thus, certain allergens could have structural or functional intrinsic characteristics that facilitate interactions with the innate immune system; these characteristics could be pivotal for allergenicity and for the development and exacerbation of allergic diseases. An important, but poorly investigated question, concerns how natural exogenous and endogenous proteases activate PARs on mucosal immune cells under physiological and pathophysiological conditions in humans. A better understanding of airway mucosal immunity and the mechanisms involved in the development of asthma and allergic diseases will create novel strategies to prevent and to treat these diseases.

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References


