

Aspergillus fumigatus Induces Immunoglobulin E–Independent Mast Cell Degranulation

Mirjam Urb, Philippe Pouliot, Fabrice N. Gravelat, Martin Olivier, and Donald C. Sheppard

Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada

Background. Pulmonary colonization by *Aspergillus fumigatus* in chronic lung disease is associated with progressive decline in lung function even in the absence of specific allergic response. We hypothesized that *A. fumigatus* contributes to this decline by inducing pulmonary mast cell degranulation even in the absence of antigen-specific immunoglobulin E (IgE). Therefore, we investigated whether *A. fumigatus* can induce mast cell degranulation independently of IgE.

Methods. We studied the interactions of *Aspergillus* species with mast cells in the absence of IgE in vitro with use of scanning electron microscopy. The extent of mast cell degranulation was quantified by measuring the release of β -hexosaminidase.

Results. Mature *A. fumigatus* hyphae induced mast cell degranulation in the absence of IgE. Hyphae of *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus nidulans* induced much less mast cell degranulation. Mast cell degranulation required direct contact with mature *A. fumigatus* hyphae, and was not induced by conidia or immature hyphae. Killed hyphae induced significant degranulation, whereas live hyphae from mutants deficient in the fungal development regulators StuA and MedA induced very little degranulation.

Conclusions. Factors expressed on the surface of mature *A. fumigatus* hyphae that are controlled by StuA and MedA induce mast cell degranulation in the absence of IgE.

Aspergillus fumigatus is a ubiquitous fungus with distinct developmental stages. The life cycle begins when airborne conidia are deposited on organic matter, where they germinate and mature into filamentous hyphae. Mature hyphae then produce conidiophores, which in turn produce new conidia. It has been estimated that a person inhales and eliminates a mean of >100 conidia of *A. fumigatus* daily [1, 2]. In immunocompetent patients with preexisting lung disease, such as cystic fibrosis, bronchiectasis, and asthma, chronic airway inflammation and damage lead to an inability to eliminate

conidia. These conidia then germinate into hyphae that colonize the airways without significant tissue invasion [3]. A minority of these patients develop allergic bronchopulmonary aspergillosis, a severe allergic response to *A. fumigatus* allergens, which is characterized by elevated total and *A. fumigatus*-specific immunoglobulin E (IgE) levels [4]. Although the majority of patients with airway colonization by *A. fumigatus* do not develop allergic bronchopulmonary aspergillosis, they still manifest a progressive decline in lung function with more frequent hospitalizations [5]. Treatment of these patients with antifungal drugs can improve lung function [6]; this suggests that the presence of *A. fumigatus* contributes to the progressive decline in pulmonary function. However, the mechanism by which *A. fumigatus* colonization accelerates this progression of obstructive and inflammatory lung disease is poorly understood.

One possible mechanism whereby *A. fumigatus* may directly mediate airway disease is through interaction with pulmonary mast cells. Mast cells are key mediators of the inflammatory response in obstructive airways disease. On activation, mature mast cells undergo degranulation and quickly release a variety of presyn-

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Reprints or correspondence: Dr. Donald C. Sheppard, Duff Medical Bldg., Rm. 511, McGill University, 3775 University St., Montréal, PQ H3A 2B4, Canada (donald.sheppard@mcgill.ca).

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thesized enzymes and bioactive substances, such as histamine and tryptase, that mediate pulmonary inflammation and airway constriction [7]. Classically, degranulation is induced in response to antigen-specific IgE, which in the presence of antigen, cross links FcεRI on mast cell membranes [8]. This aggregation leads to the activation of various signaling pathways, which results in mast cell degranulation. However, a growing body of evidence suggests that mast cells can degranulate in an IgE-independent manner in response to pathogens [9, 10] or other environmental factors [11]. The mechanisms underlying IgE-independent degranulation remain uncharacterized.

We hypothesized that *A. fumigatus* hyphae can directly stimulate mast cells in the absence of IgE. Using an in vitro model system to investigate this hypothesis, we found that developmentally mature hyphae of *A. fumigatus* induce mast cell degranulation in a contact-dependent, IgE-independent manner.

METHODS

Fungal strains and growth conditions. Fungal strains [12–14] are listed in table 1; *A. fumigatus* wild-type strain Af293 was used in the majority of the experiments. Strains were grown on yeast peptone dextrose agar (Difco) plates at 37°C for all *A. fumigatus* strains and at 30°C for *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus nidulans*. Conidia were harvested on day 6 with phosphate-buffered saline (PBS) plus 0.1% Tween 80 and were washed before experiments.

Mast cells. Rat basophilic leukemia (RBL–2H3) cells were grown in Royal Park Memorial Institute 1640 medium (RPMI-1640; Wisent) supplemented with 10% fetal bovine serum (FBS; HyClone) and penicillin-streptomycin (Wisent) at 37°C in 5% carbon dioxide. Bone marrow-derived mast cells (BMMCs)

were generated by flushing the femurs and tibias of 4–6-week-old male 129/SvJ mice. The progenitor cells were cultured in RPMI-1640 medium (Wisent) supplemented with 10% FBS (Wisent), 4 mmol/L L-glutamine (Gibco), 25 mmol/L HEPES (BioShop) in PBS, 1 mmol/L sodium pyruvate (Invitrogen), 1× nonessential amino acids (Invitrogen), 50 μmol/L 2-mercaptoethanol (BioShop), 10 ng/mL recombinant murine interleukin-13 (Biosource), and penicillin-streptomycin. After 4 weeks, the population was composed of >95% mature mast cells, as measured by morphology with 0.1% toluidine blue staining and by flow cytometric detection of the surface expression of c-kit with phycoerythrin-conjugated anti-CD117 (Caltag; data not shown).

Scanning electron microscopy (SEM). Cell culture–treated cover slips in 24-well tissue culture plates were inoculated with 6×10^4 *A. fumigatus* conidia in serum-free RPMI-1640 medium without phenol red (SF-RPMI; Wisent) and were incubated at 37°C for 24 h. Next, 8×10^5 RBL-2H3 cells were added, and the cells were incubated for 30 or 90 min. The specimens were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer. Samples were then washed 3 times with water and dehydrated with increasing concentrations of ethanol, followed by critical point drying with liquid carbon dioxide (Ladd Research SPDryer). Dried cover slips were mounted on platforms with double sticky tape and were coated with gold for 3 min in Hummer VI Sputter Coater before viewing in a Hitachi S-3000N scanning electron microscope. Electronic images were cropped for size but, otherwise, were not electronically manipulated.

β-hexosaminidase release assay. Conidia (5×10^4) of *Aspergillus* species in 50 μL SF-RPMI were germinated in 96-well

Table 1. Fungal strains used in this study.

Strain	Source or reference
<i>Aspergillus fumigatus</i> (Af293)	Nierman et al. [12]
c1 ^a	MUHC
c2 ^a	MUHC
Δ <i>stuA</i> ^b	Sheppard et al. [13]
Δ <i>stuA::stuA</i> ^b	Sheppard et al. [13]
Δ <i>medA</i> ^b	F. N. Gravelat, D. E. Ejzykiewicz, L. Y. Chang, J. C. Chabot, M. Urb, K. D. MacDonald, N. Al-Bader, S. G. Filler, D. C. Sheppard, unpublished observation
Δ <i>medA::medA</i> ^b	F. N. Gravelat, D. E. Ejzykiewicz, L. Y. Chang, J. C. Chabot, M. Urb, K. D. MacDonald, N. Al-Bader, S. G. Filler, D. C. Sheppard, unpublished observation
Δ <i>gliP</i> ^b	Spikes et al. [14]
Δ <i>gliP::gliP</i> ^b	Spikes et al. [14]
<i>Aspergillus flavus</i>	MUHC
<i>Aspergillus niger</i>	MUHC
<i>Aspergillus nidulans</i> (A26)	FGSC

NOTE. FGSC, Fungal Genetics Stock Center (University of Missouri, Kansas City, MO); MUHC, McGill University Health Centre Microbiology Laboratory (Montréal, Québec, Canada).

^a Clinical isolates from sputum samples from patients with cystic fibrosis.

^b Mutants and complemented strain derived from Af293.

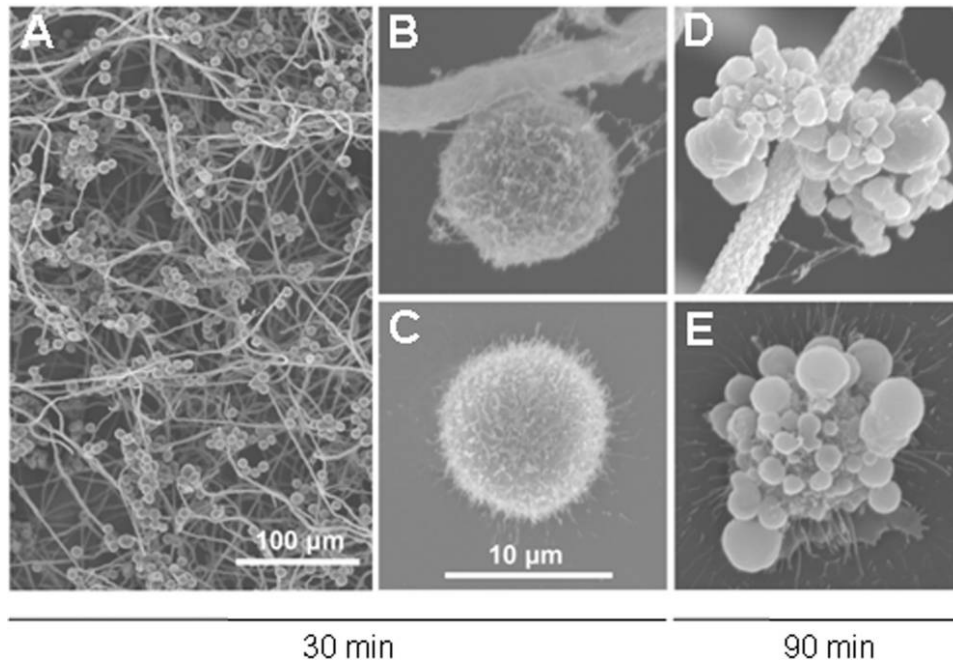


Figure 1. Adherence of mast cells to *Aspergillus fumigatus* hyphae results in mast cell degranulation in the absence of immunoglobulin E. Scanning electron micrographs of rat basophilic leukemia (RBL-2H3) cells in contact with hyphae of *A. fumigatus* Af293 (A); mast cells adherent to hyphae at 30 min of coculture (B); an unstimulated mast cell (C); mast cells adherent to hyphae, showing morphological changes similar to degranulation at 90 min of coculture (D); and mast cell degranulation in response to Ca^{2+} -ionophore A23187 at 90 min of coculture (E).

flat bottom tissue culture plates at 37°C for 0, 8, or 24 h, after which 1×10^5 RBL-2H3 cells in 50 μL SF-RPMI were added to the resulting hyphal mat and were incubated for an additional 3 h. In some experiments, hyphae were killed by incubating them with 0.02% thimerosal in PBS overnight at 4°C,

followed by extensive rinsing in PBS. Killing was verified by overnight culture. In other experiments, *A. fumigatus* culture supernatants were prepared from the above mentioned 24-h cultures by filter sterilizing the conditioned medium. This conditioned medium was added to the wells in place of the or-

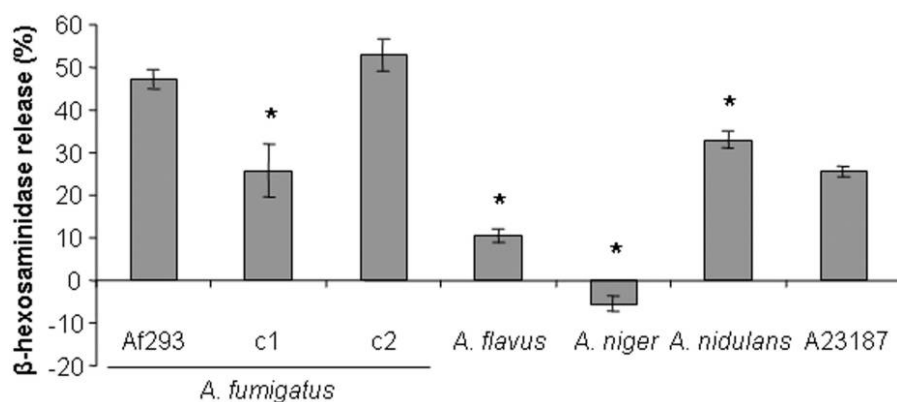


Figure 2. *Aspergillus fumigatus* induces greater mast cell degranulation than do other *Aspergillus* species. Rat basophilic leukemia (RBL-2H3) cell degranulation measured by β -hexosaminidase release on stimulation with the indicated strains and species of *Aspergillus* (*A. fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus nidulans*) or Ca^{2+} -ionophore A23187. Mature hyphae were incubated with RBL-2H3 cells for 3 h, after which β -hexosaminidase release was measured. Because we observed that *A. fumigatus* degraded free β -hexosaminidase, we used a modified estimate of β -hexosaminidase release by measuring the residual β -hexosaminidase within mast cells after degranulation. The percentage of β -hexosaminidase release was defined as 1 minus the amount of lysate β -hexosaminidase divided by the total amount of mast cell β -hexosaminidase, multiplied by 100. The percentage of spontaneous mast cell degranulation was subtracted from all values. Results are mean $\pm$ standard error of 3 independent experiments in triplicate. * $P \leq .001$, in comparison with *A. fumigatus* Af293 by analysis of variance.

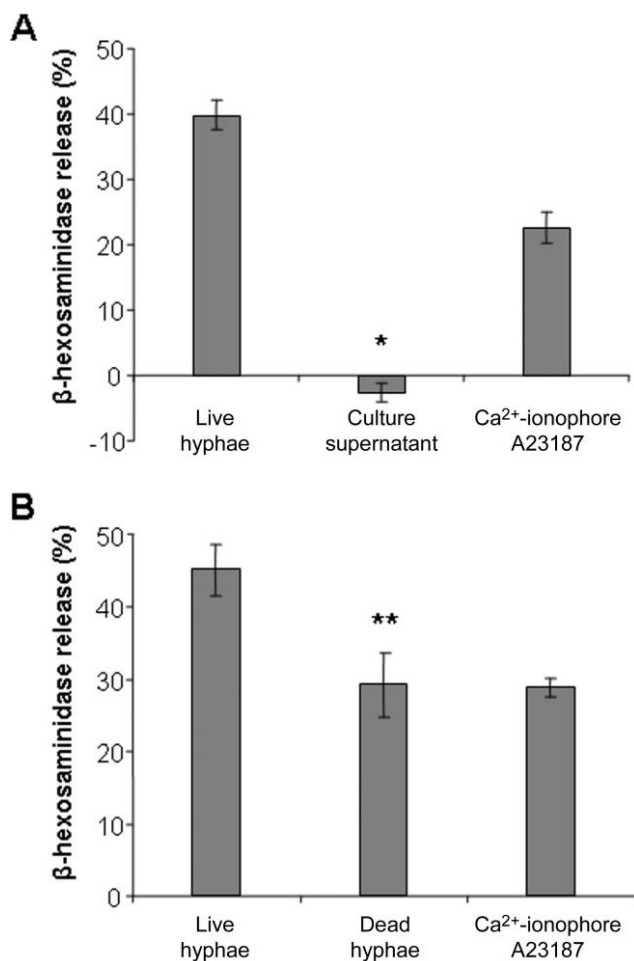


Figure 3. Direct contact, but not fungal viability, is required for *Aspergillus fumigatus* hyphae to trigger mast cell degranulation. Rat basophilic leukemia (RBL-2H3) cells were stimulated with live hyphae of *A. fumigatus* Af293 and age-matched culture supernatants (A), or with thimerosal-killed hyphae (B) for 3 h. Degranulation was measured by β -hexosaminidase release assay. Results are the mean $\pm$ standard error of 3 independent experiments in triplicate. * $P < .001$; ** $P \leq .01$; both are for comparison with live hyphae by analysis of variance.

ganisms. In all experiments, mast cells incubated without *A. fumigatus* were used to control for spontaneous degranulation, and mast cells incubated with $2 \mu\text{mol/L}$ Ca²⁺-ionophore A23187 were used as a positive control. At the end of the incubation period, culture supernatants were collected, and the remaining cells were lysed with $100 \mu\text{L}$ SF-RPMI plus 1% Triton X-100. The degree of mast cell degranulation was determined by measuring the release of β -hexosaminidase from both culture supernatants and lysates by incubating $40 \mu\text{L}$ of samples with $40 \mu\text{L}$ of 4-nitrophenyl N-acetyl- β -D-glucosaminide in 0.05 mol/L of citrate buffer (pH, 4.5) for 1 h at 37°C . The reaction was stopped with $100 \mu\text{L}$ of 0.2 mol/L glycine (pH, 10), and the release of o-nitrophenol was measured by optical density at 405 nm (OD_{405}) [15].

Adherence assay. Hyphal mats were grown on the bottom of a 24-well tissue culture plate by incubating 5×10^5 *A. fumigatus* conidia per well in SF-RPMI at 37°C for 24 h. RBL-2H3 cells (6×10^5) were added to the wells and incubated for 30 min. At this time, the RBL-2H3 cells had adhered to the hyphae but had not yet degranulated. Next, nonadherent mast cells were removed by washing with SF-RPMI. The RBL-2H3 cells that remained adherent to the hyphae were lysed with SF-RPMI plus 1% Triton X-100. The amount of β -hexosaminidase in the lysate was measured as described above and was considered to be proportional to the number of RBL-2H3 cells that remained adherent to the hyphae.

XTT damage assay. RBL-2H3-mediated damage of *A. fumigatus* hyphae was determined by a modification of the tetrazolium salt XTT (2,3-bis-[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) assay of Vonk et al. [16]. A hyphal mat from 1×10^5 *A. fumigatus* conidia in SF-RPMI was grown at 37°C for 24 h in a 24-well tissue culture plate, after which 8×10^5 RBL-2H3 cells were added to each well. Wells of hyphae were incubated with medium alone as a negative control and with 0.02% thimerosal as a positive control. After incubation for 1–24 h, the medium above the cells was aspirated, and the RBL-2H3 cells were lysed with cold water. A mixture containing $400 \mu\text{g/mL}$ of XTT (Sigma-Aldrich) and $50 \mu\text{g/mL}$ of menadione (Sigma-Aldrich) in PBS was added to each well. After 1 h of incubation at 37°C , supernatants from each well were removed and the OD_{450} was determined. The percentage of hyphal damage was determined by $\{1 - [(\text{OD hyphae and mast cell} - \text{OD mast cell}) / \text{OD hyphae}]\} \times 100$. 1 minus the difference between the OD of hyphae and mast cells and the OD of mast cells, divided by the OD of hyphae, and multiplied by 100.

Statistics. All experiments were performed in triplicate on at least 3 separate occasions. Differences among experimental conditions were compared by analysis of variance. P values $\leq .05$ were considered to be statistically significant.

RESULTS

***A. fumigatus* hyphae adhere to mast cells and induce mast cell degranulation in an IgE-independent manner.** To investigate the response of mast cells to *A. fumigatus* hyphae, cells of the RBL-2H3 cell line were incubated with mature hyphae in the absence of IgE and were then imaged by SEM. After 30 min of incubation, many RBL-2H3 cells had adhered to hyphae (figure 1A and 1B). The adherent RBL-2H3 cells appeared to be unstimulated, because they were morphologically similar to mast cells incubated in the absence of hyphae (figure 1C). After 90 min of exposure to the hyphae, there was extensive formation of membrane blebs in RBL-2H3 cells (figure 1D). This change in morphology suggests that the RBL-2H3 cells were degranulating, because the change was similar to that of mast

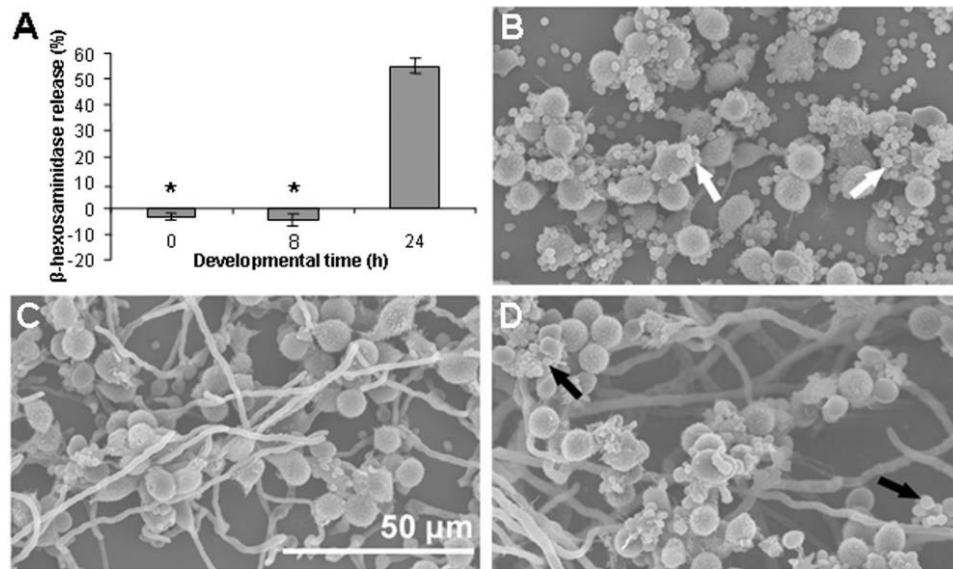


Figure 4. Only mature hyphae trigger mast cell degranulation. *A*, Degranulation of rat basophilic leukemia (RBL-2H3) cells in response to different developmental stages, as measured by β -hexosaminidase assay. Results are the mean $\pm$ standard error of 3 independent experiments in triplicate. Scanning electron micrographs of RBL-2H3 cells in contact with different developmental stages of *Aspergillus fumigatus*, including conidia at 0 h (*B*), immature hyphae at 8 h (*C*), and mature hyphae at 24 h (*D*) after 90 min of incubation. White arrows indicate conidia, and black arrows indicate mast cell granules. * $P < .001$, for comparison with mature hyphae by analysis of variance.

cells stimulated with the Ca^{2+} -ionophore A23187, which is a known inducer of mast cell degranulation (figure 1E).

To verify that degranulation was occurring, we measured the release of β -hexosaminidase, which is a preformed substance that is released by mast cells on degranulation. Contact with mature hyphae induced significant β -hexosaminidase release by RBL-2H3 cells, even in the absence of IgE (figure 2). The degree of β -hexosaminidase release induced by *A. fumigatus* was similar to that induced by A23187. Mast cell degranulation was similar over a range of multiplicity of infection (0.1–2; data not shown). Two other strains of *A. fumigatus* that were isolated from sputum samples from patients with cystic fibrosis induced significant RBL-2H3 cell degranulation, although there was some strain-to-strain variability in the amount of degranulation that was induced (figure 2). Collectively, these results suggest that *A. fumigatus* induces mast cell degranulation in an IgE-independent manner.

Other *Aspergillus* species are found less frequently than *A. fumigatus* in chronic lung disease, despite being commonly found in the environment. Therefore, we examined the ability of *A. flavus*, *A. niger*, and *A. nidulans* to induce mast cell degranulation. *A. flavus* and *A. niger* induced very little degranulation of RBL-2H3 cells (figure 2). *A. nidulans* induced some RBL-2H3 cell degranulation, although less than was seen with *A. fumigatus* Af293. This suggests that mast cell degranulation is induced by a few species of *Aspergillus*.

***A. fumigatus*-induced mast cell degranulation is contact dependent but does not require live hyphae.** Next, we in-

vestigated whether degranulation could be induced by soluble factors released by *A. fumigatus*. Unlike *A. fumigatus* hyphae, age-matched filter-sterilized culture supernatants did not induce detectable mast cell degranulation (figure 3A). To determine whether fungal viability was required to induce degranulation, we incubated RBL-2H3 cells with *A. fumigatus* hyphae that had been killed with the metabolic poison thimerosal, which preserves the cell wall. Thimerosal-killed hyphae induced significant mast cell degranulation, although ~15% less than live hyphae (figure 3B). Collectively, these results suggest that a factor associated with the hyphal cell surface induces IgE-independent mast cell degranulation.

Normal hyphal maturation is required for *A. fumigatus* induction of mast cell degranulation and adherence. The cell surface of *Aspergillus* species changes significantly as resting conidia first swell and then form precompetent hyphae, which develop into competent hyphae [17]. We therefore investigated the ability of different developmental stages of *A. fumigatus* to induce mast cell degranulation. Conidia and immature hyphae did not induce significant RBL-2H3 cell degranulation (figure 4A), even when RBL-2H3 cells were incubated with 20- or 5-fold excess organisms, respectively, compared with mature hyphae (data not shown). These findings were verified by SEM. Only rare degranulation was seen when RBL-2H3 cells were incubated with either conidia or immature hyphae (figure 4B and 4C).

A. fumigatus development is a complex process that is controlled by several developmental modifiers, including the StuA

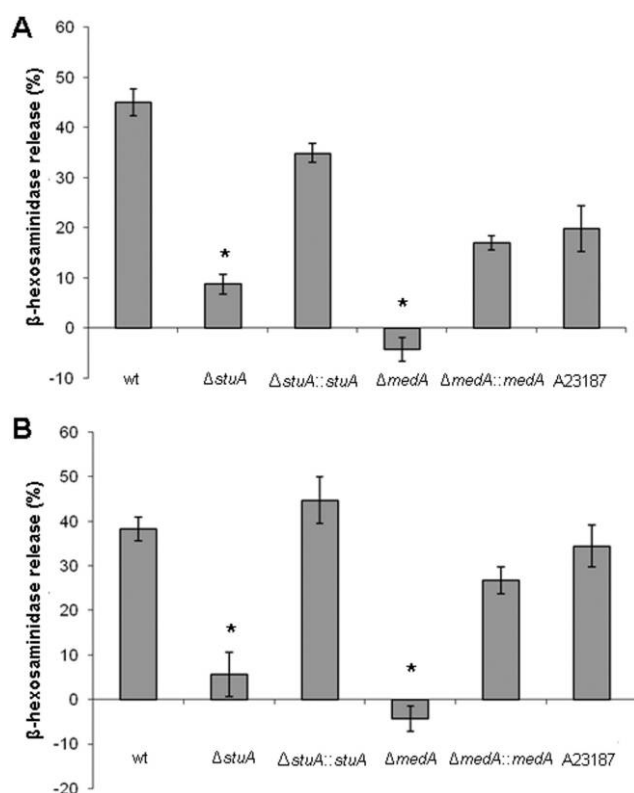


Figure 5. Developmentally deficient mutants are impaired in the ability to induce mast cell degranulation. Rat basophilic leukemia (RBL-2H3) cells (A) and bone marrow-derived mast cells (B) were stimulated with the indicated strains of *Aspergillus fumigatus* mutants and Ca^{2+} -ionophore A23187 for 3 h, after which β -hexosaminidase release was determined. Results are the mean $\pm$ standard error of 3 independent experiments in triplicate. * $P < .001$, for the comparison with the wild-type (wt) strain by analysis of variance.

and MedA proteins. Mutations in these regulatory factors result in abnormal maturation of hyphae (F. N. Gravelat, D. E. Ejzykiewicz, L. Y. Chang, J. C. Chabot, M. Urb, K. D. MacDonald, N. Al-Bader, S. G. Filler, D. C. Sheppard, unpublished observation) [13]. We therefore examined the ability of *A. fumigatus* $\Delta stuA$ and $\Delta medA$ mutants to induce mast cell degranulation. Both $\Delta stuA$ and $\Delta medA$ hyphae induced significantly less mast cell degranulation than did the wild-type parent strain (figure 5A). Complementation of the $\Delta stuA$ and $\Delta medA$ mutants with their respective wild-type allele largely restored their ability to induce mast cell degranulation. These results suggest that mast cell degranulation is mediated by specific *A. fumigatus* factors that are controlled by the developmental regulators StuA and MedA.

Although RBL-2H3 cells are a standard model system for studying mast cell degranulation, they are of leukemic origin. Therefore, to validate the results of these experiments, we tested the ability of *A. fumigatus* strains to induce IgE-independent degranulation in primary BMMCs. We found that wild-type

hyphae of *A. fumigatus* induced significant BMMC degranulation and that both $\Delta stuA$ and $\Delta medA$ hyphae induced much less BMMC degranulation than did wild-type *A. fumigatus* (figure 5B). Collectively, these results validate our use of the RBL-2H3 cells as an appropriate model system for *Aspergillus* mast cell interactions and confirm that developmentally mature hyphae induce significant mast cell degranulation even in the absence of IgE.

To investigate potential mechanisms for the differences in mast cell degranulation induced by $\Delta stuA$ and $\Delta medA$, the interactions of mast cells with these hyphae were imaged by SEM. BMMCs adhered more avidly to wild-type hyphae, compared with the $\Delta stuA$ and $\Delta medA$ mutant strains (figure 6A). Of interest, clusters of BMMCs were seen to be adherent to wild-type hyphae throughout the culture system, whereas only single adherent cells were observed with each of the mutant strains. To confirm these results and quantify differences in adherence, we used the β -hexosaminidase assay as a surrogate measure for the number of adherent mast cells with use of the remaining RBL-2H3 cells. For these experiments, a 30 min coincubation time was used to ensure that degranulation did not occur. As with BMMCs, the adherence of RBL-2H3 cells to $\Delta stuA$ and $\Delta medA$ hyphae was significantly less than their adherence to wild-type hyphae (figure 6B). However, this reduction in adherence was much less than the decrease in mast cell degranulation observed with these strains. Thus, reduced adherence of mast cells to $\Delta stuA$ and $\Delta medA$ hyphae may only partially explain the reduced mast cell degranulation induced by these mutant strains.

Gliotoxin does not inhibit mast cell degranulation in response to *A. fumigatus*. Gliotoxin is a fungal epipolythiodioxopiperazine toxin produced by *A. fumigatus* that is an important virulence factor for the development of invasive aspergillosis [18]. Previous studies have found that exogenous gliotoxin can inhibit Ca^{2+} -ionophore-induced mast cell degranulation [19]. We therefore tested the ability of a gliotoxin-deficient *A. fumigatus* mutant to induce mast cell degranulation. Similar levels of mast cell degranulation were observed with both the $\Delta gliP$ mutant and the complemented strain in which gliotoxin production was restored by reinsertion of a wild-type allele of *gliP* (data not shown). These results strongly suggest that gliotoxin does not influence mast cell degranulation induced by *A. fumigatus*.

Mast cells cause no damage to *A. fumigatus* hyphae. Although mast cells respond to contact with *A. fumigatus* by degranulation, the effect of mast cells on *A. fumigatus* has not been investigated. We therefore used an XTT metabolic assay to determine whether mast cells can inhibit the growth of *A. fumigatus* hyphae. Incubation of mature *A. fumigatus* hyphae with an 8-fold excess of RBL-2H3 cells for 1–24 h did not cause a detectable reduction in *A. fumigatus* metabolic activity (data

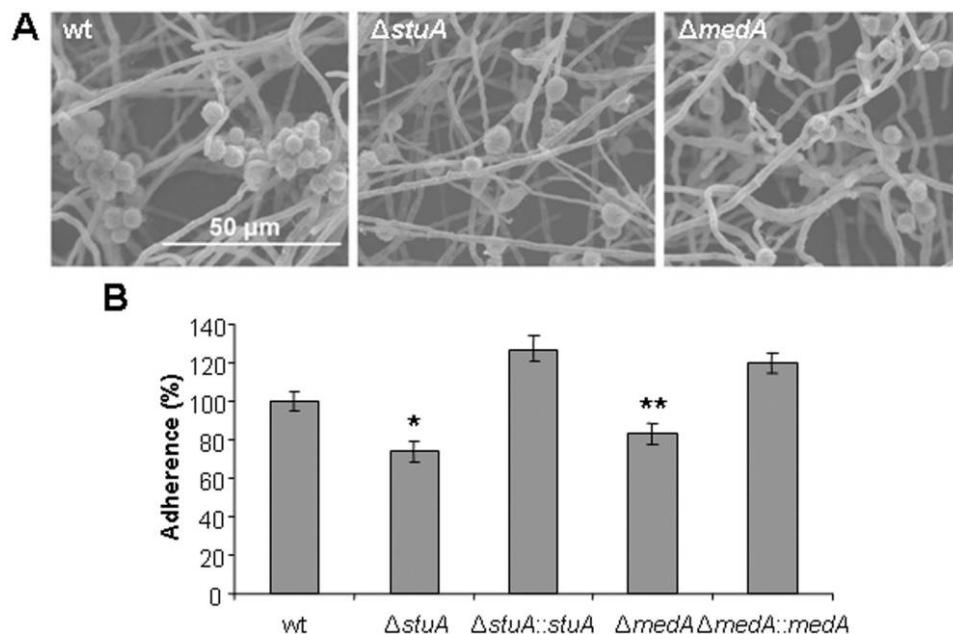


Figure 6. Developmentally deficient mutants are less adherent to mast cells. *A*, Scanning electron micrographs of bone marrow–derived mast cells in contact with wild-type (wt) Af293, $\Delta stuA$, and $\Delta medA$ mutant hyphae after 90 min of coculture. *B*, Adherence of rat basophilic leukemia (RBL–2H3) cells to different *Aspergillus fumigatus* strains, as determined by total β -hexosaminidase content of washed wells after 30 min of interaction. Results are expressed as a percentage of the adherence to wt hyphae and are the mean $\pm$ standard error of 4 independent experiments, each performed in triplicate. * $P \leq .002$; ** $P \leq .03$; both are for comparison with the wt strain Af293 by analysis of variance.

not shown). In fact, 12 h of coincubation the RBL-2H3 cells induced a slight increase (16%) in XTT metabolism, which suggests that mast cells may even enhance the growth of *A. fumigatus*.

DISCUSSION

Most research examining *A. fumigatus* in chronic lung disease has focussed on IgE-mediated allergic bronchopulmonary aspergillosis [20–22]. However, there is evidence that IgE is not necessary for the development of progressive airways disease [23–25]. Indeed, both wild-type and IgE-deficient mice develop similar levels of airway inflammation and bronchial hyper-reactivity after *A. fumigatus* antigen challenge [23]. Similarly, patients with cystic fibrosis who are colonized with *A. fumigatus* and do not develop hyper-IgE still develop worsening lung function [5]. These observations suggest that the interaction between *A. fumigatus* and host cells can induce airway inflammation even when *Aspergillus*-specific IgE titers are low or absent. Although *A. fumigatus* likely interacts with multiple host cells in the airways, it is probable that activation of mast cells, which release multiple types of inflammatory mediators [26], contributes to lung pathology in colonized patients. Recent studies have shown that other microorganisms, such as *Mycoplasma pneumoniae* and *Leishmania* promastigotes, can activate mast cells in the absence of IgE [10, 27]. Here, we show

that *A. fumigatus* hyphae can also induce mast cell degranulation in an IgE-independent manner.

A. fumigatus is the most common *Aspergillus* species colonizing the lungs of patients with chronic lung disease [28] and is the species most able to injure airway cells in vitro. For example, although culture filtrates of *A. fumigatus* damaged human nasal ciliated epithelium, culture filtrates from *A. flavus* and *A. niger* only caused minor cellular injury [29]. Here, we observed that *A. flavus* and *A. niger* hyphae induce little or no β -hexosaminidase release from RBL-2H3 cells, compared with *A. fumigatus* hyphae. Only *A. nidulans* hyphae induced mast cell degranulation, albeit to a lesser degree than did *A. fumigatus*. *A. nidulans* is more closely related to *A. fumigatus* [30] and is therefore more likely to express similar factors that induce mast cell degranulation. Although differences in the ability to induce mast cell degranulation may be partially responsible for the predominance of *A. fumigatus* in patients with chronic lung disease, additional species-specific factors likely contribute to pathogenicity, including the degree of environmental exposure, conidial size, and subsequent ability to persist within the respiratory tree.

We found that mature hyphae—but not conidia or immature hyphae—induced degranulation of RBL-2H3 and bone marrow–derived mast cells. This result was not simply a function of hyphal age, because hyphae of the developmental mutants

$\Delta stuA$ and $\Delta medA$ induced significantly less degranulation regardless of the duration of culture. These results are consistent with the observation that daily exposure to conidia does not result in airway disease in the vast majority of individuals, and only chronic colonization with mature hyphae of *A. fumigatus* is associated with worsening of lung disease.

A. fumigatus secretes a wide range of toxins and cytotoxic enzymes, including gliotoxin. Purified gliotoxin and culture filtrates of wild-type, but not gliotoxin-deficient, *A. fumigatus* inhibit ionomycin-dependent degranulation of mast cells [19, 31], suggesting that gliotoxin suppresses mast cell degranulation. However, in our experiments, we found that high levels of mast cell degranulation were induced by the gliotoxin-producing wild-type *A. fumigatus* strain Af293 [14]. Furthermore, the gliotoxin-deficient $\Delta gliP$ mutant did not induce higher levels of degranulation than did the *gliP*-complemented strain in which gliotoxin production had been restored. Collectively, these results suggest that *A. fumigatus* induces mast cell degranulation via a gliotoxin-insensitive mechanism. Studies to further identify the signalling pathways involved in *A. fumigatus*-induced mast cell degranulation are ongoing.

Our results suggest that the specific factors associated with the cell surface of hyphae mediate mast cell degranulation. First, culture supernatants from mature hyphae did not induce mast cell degranulation. Second, killed hyphae largely retained their ability to induce mast cell degranulation. Of interest, thimerosal-killed hyphae of *A. fumigatus* also retained the capacity to damage endothelial cells [32], which suggests similarities between these 2 host cell interactions.

The $\Delta stuA$ and $\Delta medA$ mutants had a marked reduction in both mast cell adherence and degranulation. These results indicate that StuA and MedA influence the composition of the cell surface of *A. fumigatus* hyphae. However, identifying which MedA- and StuA-dependent factors are responsible for mast cell degranulation is likely to prove challenging, because these proteins regulate the expression of hundreds of genes, many of which have not been characterized to any degree (F. N. Gravelat, D. E. Ejzykowicz, L. Y. Chang, J. C. Chabot, M. Urb, K. D. MacDonald, N. Al-Bader, S. G. Filler, D. C. Sheppard, unpublished observation) [13].

Taken together, our results are consistent with those of a model in which mast cell degranulation was a multistep process involving, first, adherence and, then, stimulation of mast cells, leading to degranulation. This process is analogous to other fungal interactions with host cells, such as endothelial cell adherence and invasion by *Candida albicans*. In this case, multiple adhesins have been identified that mediate the adherence of *C. albicans* to epithelial cells, including Als1, Als3, and Hwp1, although only Als3 has been shown to mediate epithelial cell invasion via binding to host E-cadherin [33–35]. It was notable that, although both mutant strains were less adherent to mast

cells, their adherence defects were more modest than their defects in inducing degranulation. It is thus likely that other factors, in addition to adherence, are also involved in inducing mast cell degranulation, some or all of which are controlled by StuA and MedA.

We speculate that *A. fumigatus* may directly worsen chronic lung disease by inducing “frustrated exocytosis” of mast cells. In this model, *A. fumigatus* hyphae grow within the airways of susceptible patients. Mast cells adhere to these hyphae and then degranulate, releasing preformed mediators of inflammation. These pro-inflammatory mediators do not damage the fungi but worsen airway inflammation and obstruction. This hypothesis needs to be tested in vivo, although the lack of an animal model of chronic *A. fumigatus* lung disease limits this approach at present. Finally, because the observations reported in this study were in mast cells of mouse and rat origin, it will be important to verify that similar responses occur in human airways.

References

1. Chazalet V, Debeaupuis JP, Sarfati J, et al. Molecular typing of environmental and patient isolates of *Aspergillus fumigatus* from various hospital settings. *J Clin Microbiol* **1998**; 36:1494–500.
2. Goodley JM, Clayton YM, Hay RJ. Environmental sampling for aspergilli during building construction on a hospital site. *J Hosp Infect* **1994**; 26:27–35.
3. Patterson R, Greenberger PA, Radin RC, Roberts M. Allergic bronchopulmonary aspergillosis: staging as an aid to management. *Ann Intern Med* **1982**; 96:286–91.
4. Mastella G, Rainisio M, Harms HK, et al. Allergic bronchopulmonary aspergillosis in cystic fibrosis: a European epidemiological study. Epidemiologic Registry of Cystic Fibrosis. *Eur Respir J* **2000**; 16:464–71.
5. Stevens DA, Moss RB, Kurup VP, et al. Allergic bronchopulmonary aspergillosis in cystic fibrosis—state of the art: Cystic Fibrosis Foundation Consensus Conference. *Clin Infect Dis* **2003**; 37(Suppl 3): S225–64.
6. Shoseyov D, Brownlee KG, Conway SP, Kerem E. *Aspergillus* bronchitis in cystic fibrosis. *Chest* **2006**; 130:222–6.
7. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* **2006**; 117:1277–84.
8. Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol* **2006**; 6:218–30.
9. Barbuti G, Moschioni M, Censini S, Covacci A, Montecucco C, Montemurro P. *Streptococcus pneumoniae* induces mast cell degranulation. *Int J Med Microbiol* **2006**; 296:325–9.
10. Bidri M, Vouldoukis I, Mossalayi MD, et al. Evidence for direct interaction between mast cells and *Leishmania* parasites. *Parasite Immunol* **1997**; 19:475–83.
11. Suzuki Y, Yoshimaru T, Matsui T, Ra C. Silver activates calcium signals in rat basophilic leukemia-2H3 mast cells by a mechanism that differs from the Fc epsilon RI-activated response. *J Immunol* **2002**; 169:3954–62.
12. Nierman WC, Pain A, Anderson MJ, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **2005**; 438:1151–6.
13. Sheppard DC, Doedt T, Chiang LY, et al. The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. *Mol Biol Cell* **2005**; 16:5866–9.

14. Spikes S, Xu R, Nguyen CK, et al. Gliotoxin production in *Aspergillus fumigatus* contributes to host-specific differences in virulence. *J Infect Dis* **2008**; 197:479–86.
15. Oka T, Hori M, Tanaka A, Matsuda H, Karaki H, Ozaki H. IgE alone-induced actin assembly modifies calcium signaling and degranulation in RBL-2H3 mast cells. *Am J Physiol Cell Physiol* **2004**; 286:C256–63.
16. Vonk AG, Wieland CW, Versteegen M, et al. Influence of endogenous pro-inflammatory cytokines on neutrophil-mediated damage of *Candida albicans* pseudohyphae, quantified in a modified tetrazolium dye assay. *Med Mycol* **2005**; 43:551–7.
17. Adams TH, Wieser JK, Yu JH. Asexual sporulation in *Aspergillus nidulans*. *Microbiol Mol Biol Rev* **1998**; 62:35–54.
18. Gardiner DM, Waring P, Howlett BJ. The epipolythiodioxopiperazine (ETP) class of fungal toxins: distribution, mode of action, functions and biosynthesis. *Microbiology* **2005**; 151:1021–32.
19. Niide O, Suzuki Y, Yoshimaru T, Inoue T, Takayama T, Ra C. Fungal metabolite gliotoxin blocks mast cell activation by a calcium- and superoxide-dependent mechanism: implications for immunosuppressive activities. *Clin Immunol* **2006**; 118:108–16.
20. Kurup VP, Banerjee B, Hemmann S, Greenberger PA, Blaser K, Cramer R. Selected recombinant *Aspergillus fumigatus* allergens bind specifically to IgE in ABPA. *Clin Exp Allergy* **2000**; 30:988–93.
21. Kurup VP, Mauze S, Choi H, Seymour BW, Coffman RL. A murine model of allergic bronchopulmonary aspergillosis with elevated eosinophils and IgE. *J Immunol* **1992**; 148:3783–8.
22. Banerjee B, Greenberger PA, Fink JN, Kurup VP. Immunological characterization of Asp f 2, a major allergen from *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis. *Infect Immun* **1998**; 66:5175–82.
23. Mehlhop PD, van de Rijn M, Goldberg AB, et al. Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc Natl Acad Sci U S A* **1997**; 94:1344–9.
24. Hamelmann E, Takeda K, Schwarze J, Vella AT, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyper-responsiveness requires interleukin-5 but not immunoglobulin E or B lymphocytes. *Am J Respir Cell Mol Biol* **1999**; 21:480–9.
25. Hogan SP, Mould A, Kikutani H, Ramsay AJ, Foster PS. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyper-reactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J Clin Invest* **1997**; 99:1329–39.
26. Marshall JS. Mast-cell responses to pathogens. *Nat Rev Immunol* **2004**; 4:787–99.
27. Hoek KL, Cassell GH, Duffy LB, Atkinson TP. *Mycoplasma pneumoniae*-induced activation and cytokine production in rodent mast cells. *J Allergy Clin Immunol* **2002**; 109:470–6.
28. Gurcan S, Demir M, Altıay G, Tikvesli M, Kilic H, Otkun M. *Aspergillus* spp. Isolations from respiratory tract samples in Trakya University Hospital. *Tuberk Toraks* **2007**; 55:160–6.
29. Amitani R, Murayama T, Nawada R, et al. *Aspergillus* culture filtrates and sputum sols from patients with pulmonary aspergillosis cause damage to human respiratory ciliated epithelium in vitro. *Eur Respir J* **1995**; 8:1681–7.
30. Oshero N, Kontoyiannis DP, Romans A, May GS. Resistance to itraconazole in *Aspergillus nidulans* and *Aspergillus fumigatus* is conferred by extra copies of the *A. nidulans* P-450 14 α -demethylase gene, *pdmA*. *J Antimicrob Chemother* **2001**; 48:75–81.
31. Cramer RA Jr, Gamcsik MP, Brooking RM, et al. Disruption of a nonribosomal peptide synthetase in *Aspergillus fumigatus* eliminates gliotoxin production. *Eukaryot Cell* **2006**; 5:972–80.
32. Lopes Bezerra LM, Filler SG. Interactions of *Aspergillus fumigatus* with endothelial cells: internalization, injury, and stimulation of tissue factor activity. *Blood* **2004**; 103:2143–9.
33. Sheppard DC, Yeaman MR, Welch WH, et al. Functional and structural diversity in the Als protein family of *Candida albicans*. *J Biol Chem* **2004**; 279:30480–9.
34. Phan QT, Fratti RA, Prasad Rao NV, Edwards JE Jr, Filler SG. N-cadherin mediates endocytosis of *Candida albicans* by endothelial cells. *J Biol Chem* **2005**; 280:10455–61.
35. Phan QT, Myers CL, Fu Y, et al. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol* **2007**; 5:e64.