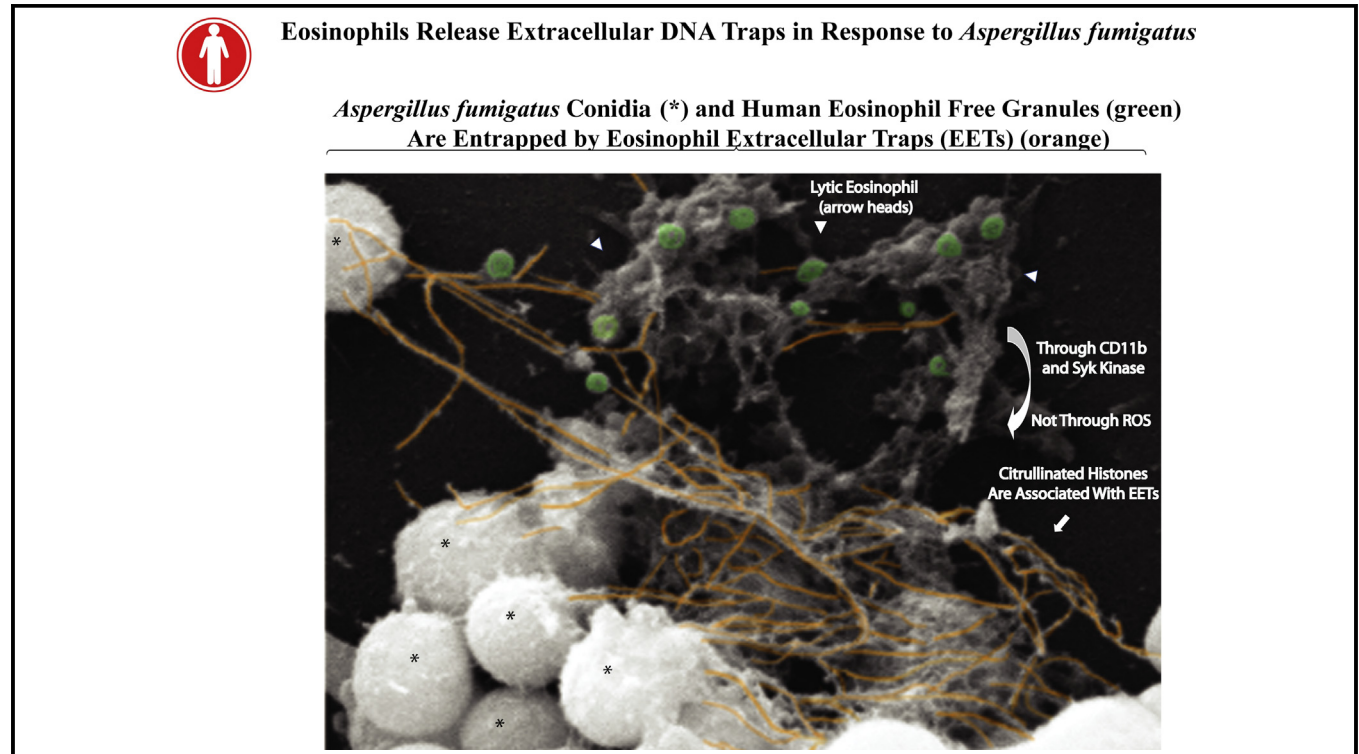


# Eosinophils release extracellular DNA traps in response to *Aspergillus fumigatus*

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## GRAPHICAL ABSTRACT



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**Background:** Eosinophils mediate the immune response in different infectious conditions. The release of extracellular DNA traps (ETs) by leukocytes has been described as an innate immune response mechanism that is relevant in many disorders including fungal diseases. Different stimuli induce the release of human eosinophil ETs (EETs). *Aspergillus fumigatus* is an opportunistic fungus that may cause eosinophilic allergic bronchopulmonary aspergillosis (ABPA). It has been reported that eosinophils are important to the clearance of *A fumigatus* in infected mice lungs. However, the immunological mechanisms that underlie the molecular interactions between *A fumigatus* and eosinophils are poorly understood.

**Objective:** Here, we investigated the presence of EETs in the bronchial mucus plugs of patients with ABPA. We also determined whether *A fumigatus* induced the release of human eosinophil EETs *in vitro*.

**Methods:** Mucus samples of patients with ABPA were analyzed by light and confocal fluorescence microscopy. The release of EETs by human blood eosinophils was evaluated using different pharmacological tools and neutralizing antibodies by fluorescence microscopy and a fluorimetric method.

**Results:** We identified abundant nuclear histone-bearing EETs in the bronchial secretions obtained from patients with ABPA. *In vitro*, we demonstrated that *A fumigatus* induces the release of EETs through a mechanism independent of reactive oxygen species but associated with eosinophil death, histone citrullination, CD11b, and the Syk tyrosine kinase pathway. EETs lack the killing or fungistatic activities against *A fumigatus*.

**Conclusions:** Our findings may contribute to the understanding of how eosinophils recognize and act as immune cells in response to *A fumigatus*, which may lead to novel insights regarding the treatment of patients with ABPA. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

**Key words:** Eosinophil, extracellular DNA traps, allergic bronchopulmonary aspergillosis, inflammation

Eosinophils mediate the immune response in numerous infectious diseases, including parasitic helminth, bacterial, fungal, and viral infections.<sup>1,2</sup> Eosinophils contain an abundant singular population of crystalloid-bearing granules, which are central to their functional responses. Eosinophil granules house preformed stores of cationic proteins, including eosinophil cationic protein, major basic protein (MBP), and eosinophil peroxidase enzymes, and more than 30 cytokines, chemokines, and growth factors.<sup>1-3</sup> Eosinophils may secrete their granule-stored proteins via classic exocytosis, cytolysis (which enables the release of intact, membrane-bound, functional free extracellular granules), and piecemeal degranulation.<sup>3</sup> The association between eosinophilic inflammation and infection or colonization with fungi has been long recognized.<sup>4-6</sup> Allergic bronchopulmonary aspergillosis (ABPA) is characterized by early allergic and late-phase lung injury in response to repeated exposures to *Aspergillus* antigens, which are the consequences of persistent fungal colonization of the lungs.<sup>4</sup> Patients with ABPA present a strong T<sub>H</sub>2 pulmonary response, intense lung eosinophilia, and positive serological tests for *Aspergillus* species. The association between *Aspergillus* species and chronic eosinophilic airway inflammation has also been described in patients with severe asthma and specific types of rhinosinusitis.<sup>5,6</sup>

#### Abbreviations used

|          |                                                                                 |
|----------|---------------------------------------------------------------------------------|
| ABPA:    | Allergic bronchopulmonary aspergillosis                                         |
| DPI:     | Diphenyleneiodonium                                                             |
| EET:     | Eosinophil extracellular DNA trap                                               |
| ET:      | Extracellular DNA trap                                                          |
| ETosis:  | Extracellular trap cell death                                                   |
| EETosis: | Eosinophil extracellular trap cell death                                        |
| LDH:     | Lactate dehydrogenase enzyme                                                    |
| MBP:     | Major basic protein                                                             |
| NADPH:   | Nicotinamide adenine dinucleotide phosphate                                     |
| NET:     | Neutrophil extracellular DNA trap                                               |
| OXSI-2:  | 3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide  |
| PFO:     | Paraformaldehyde                                                                |
| PI:      | Propidium iodide                                                                |
| PMA:     | Phorbol 12-myristate 13-acetate                                                 |
| RT:      | Room temperature                                                                |
| ROS:     | Reactive oxygen species                                                         |
| XTT:     | 2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxyde |

If undiagnosed, ABPA may result in progressive lung damage, pulmonary fibrosis, and death. *Aspergillus fumigatus* is an opportunistic filamentous fungi in which conidia are frequently inhaled into airways and begin to germinate after they reach the lung.<sup>7</sup> Important fungicidal activities have previously been described for eosinophils. Yoon et al<sup>8</sup> have demonstrated that human eosinophils react vigorously to *Alternaria alternata* by releasing their cytotoxic granules and killing the fungus in a contact-dependent manner via the  $\beta_2$ -integrin molecule CD11b, which adheres to  $\beta$ -glucan, a major fungus cell-wall component.<sup>8</sup> In 2014, Lilly et al<sup>9</sup> showed that eosinophils are important contributors to the clearance of *A fumigatus* from the lungs of mice *in vivo*. They also demonstrated that eosinophils generated from mouse bone marrow exhibit a killing activity against *A fumigatus in vitro*, which does not require cell contact.<sup>9</sup>

The process of extracellular release of DNA traps (ETs) by leukocytes has been described as an important mechanism of the innate immune response in different infectious diseases. The finding that neutrophils release filamentous ETs was first described by Brinkmann et al<sup>10</sup> in 2004. Neutrophil ETs (NETs) contain antimicrobial molecules, and they immobilize and may potentially kill pathogens, which thereby prevents microbial spread and contributes to an antimicrobial environment.<sup>10,11</sup> The process of release of ETs has also been described for other cell types, including mast cells and macrophages.<sup>12,13</sup> In neutrophils and other cells, this process is frequently accompanied by cell death and reactive oxygen species (ROS) production; however, controversial findings have been reported.<sup>10,14-18</sup> Purified human eosinophils release ETs (EETs) via different stimuli, including bacteria and cytokines. In 2008, Yousefi et al<sup>14</sup> provided the first evidence that eosinophils rapidly and noncytolytically catapult mitochondrial, but not nuclear, DNA to form EETs in response to bacteria and other stimuli. Moreover, an analysis of colon biopsies from patients with Crohn disease, bacterial gastrointestinal infection, or schistosoma infection indicated multiple extracellular DNA extrusions that were apparently generated via cells that expressed eosinophil

cationic protein, most likely eosinophils.<sup>14</sup> Another study has demonstrated that eosinophils respond to thymic stromal lymphopoietin stimulation, a factor produced by epithelial cells at barrier surfaces, in a cell-adhesion- and ROS-dependent manner, by releasing mitochondrial EETs and toxic granule proteins.<sup>19</sup> Thus, a mechanism of release of EETs associated with eosinophil death was not originally recognized. In a recent study, Ueki et al<sup>20</sup> provided the first evidence that human eosinophils undergo extracellular trap cell death, that is, ETosis. Importantly, this study identified a process by which eosinophils undergo cytolysis to liberate filamentous chromatin fibers concurrently with the release of intact, cell-free, and secretion-competent granules in a nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-dependent manner. During eosinophil ETosis (EETosis), plasma membrane rupture releases both nuclear-derived (histone-positive) EETs and membrane-bound intact eosinophil granules.<sup>20</sup> ETosis-derived EETs in human allergic diseases such as eosinophilic chronic rhinosinusitis and eosinophilic otitis media are also reported.<sup>21,22</sup> However, the occurrence of EETosis or EETs in clinical samples of individuals with ABPA remains to be established. Moreover, there are no reports that indicate that human eosinophils release EETs in response to *A fumigatus*. In 2010, Bruns et al<sup>23</sup> showed that neutrophils release NETs in response to *A fumigatus* *in vitro* and *in vivo* via a ROS-dependent mechanism. However, they did not identify an influence of NET formation on the killing of *A fumigatus* conidia. In a different study, it was reported that neutrophils do not kill *A fumigatus* conidia<sup>24</sup>; in contrast, they only inhibit its germination. In this same study, it was reported that neutrophils kill *A fumigatus* hyphae via a mechanism independent of NETs that requires ROS formation and the myeloperoxidase system.<sup>24</sup> Although the relevance of eosinophils in allergic aspergillosis is under discussion, the immunological mechanisms that underlie the molecular interactions between *Aspergillus* and eosinophils have been poorly understood. Thus, to shed light on this discussion, the goal of this study was to characterize the presence of EETs in clinical bronchial mucus samples of patients with ABPA and investigate whether eosinophils release EETs in response to *A fumigatus* *in vitro* and the mechanisms involved.

## METHODS

### Study approval

All protocols and experimental procedures that involved human blood-isolated eosinophils were approved by the Committee on Human Research at Clementino Fraga Filho Hospital (Federal University of Rio de Janeiro). Bronchoscopic specimens were obtained from patients with ABPA with a positive microbiologic culture for *A fumigatus* according to the diagnostic criteria.<sup>25</sup> Informed consent was obtained under Institutional Review Board-approved protocols—Clementino Fraga Filho Hospital (license number 190/09) and Akita University Hospital (license number 1535).

### Fungal culture and conidial preparation

*A fumigatus* conidia ATCC 46645 (strain NCPF 2109) cryopreserved in liquid nitrogen and maintained in medium that contained 0.9% saline, 0.01% tween, and 30% glycerol were thawed and inoculated by spreading in solid medium that contained potato dextrose agar (Neogen, São Paulo, Brazil) for 5 to 7 days at 37°C. Plates that contained mycelium of *A fumigatus* were scraped with sterile PBS (Lonza) that contained 0.05% tween-20 (Bio Rad), and the conidia were collected via filtration through a sterile nylon mesh with a porosity of 40  $\mu$ m (BD Biosciences). The conidia were precipitated via centrifugation

(3150g, 25°C, 15 minutes), resuspended in RPMI 1640 (phenol red-free) (Sigma), estimated by counting in a Neubauer chamber through an optical microscope (Leica Microsystems, Wetzlar, Germany) with 40 $\times$  magnification, and adjusted for the subsequent stimulation of cells.

### Eosinophil purification

Eosinophils were isolated from the blood of healthy donors using negative selection as previously described.<sup>26</sup> The viability and purity of the freshly isolated eosinophils were more than 99% as analyzed via trypan blue exclusion and panoptic kit staining, respectively.

### Fluorimetric assay for the quantification of EETs

Purified human eosinophils ( $2 \times 10^5/200 \mu$ L) were resuspended in RPMI 1640 (phenol red-free) supplemented with 0.1% FCS (Life Technologies), 1% of L-glutamine (Life Technologies), and antibiotics (penicillin and streptomycin) (Lonza). The eosinophils were initially stimulated in 96-well tissue culture plates with *A fumigatus* conidia at cell:fungus *A fumigatus* conidia ratios of 1:1, 1:10, 1:50, and 1:100 for 3, 6, and 9 hours at 37°C to examine the kinetics of the eosinophil response. At the end of each time period, Sytox Green (5  $\mu$ M, Life Technologies), an extracellular DNA probe impermeable to viable cells, was added for 10 minutes. The samples were analyzed in a Flexstation plate reader (Molecular Devices) with a wavelength combination of excitation at 485 nm and emission at 538 nm. The values were expressed in relative fluorescence units. The cell:fungus ratio of 1:10 in the time of 6 hours was subsequently used in all experiments. Eosinophils stimulated with phorbol 12-myristate 13-acetate (PMA, Sigma), a protein kinase C activator, at a concentration of 50 nM for 2 hours were used as positive controls. To evaluate the participation of the Syk kinase signaling pathway in *A fumigatus*-induced release of EETs, eosinophils were pretreated for 30 minutes before the fungus stimulation with piceatannol (40  $\mu$ M, Caymann) and 3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide (OXSI-2) (2  $\mu$ M, Merck Millipore) (both pharmacologically distinct inhibitors for Syk tyrosine kinase) or their vehicles (ethanol and dimethyl sulfoxide, respectively, in corresponding dilutions). To evaluate the participation of ROS, eosinophils were pretreated for 30 minutes with diphenyleneiodonium (DPI, 30  $\mu$ M, Sigma), a nonselective inhibitor of enzymes involved in the production of ROS, or its vehicle (dimethyl sulfoxide, in a corresponding dilution), as well as MitoTempo (500  $\mu$ M, Sigma), an inhibitor of mitochondrial ROS. In a specific set of experiments, DNase (Fermentas) was added to the samples 15 minutes before the end of the incubation time at concentrations of 10 and 40 IU/mL, diluted in RPMI 1640 medium (phenol red-free) supplemented with MgCl<sub>2</sub> and CaCl<sub>2</sub> (50 mM). To assess the involvement of the dectin-1 receptor and CD11b in the release of EETs, eosinophils were pretreated for 30 minutes with anti-dectin-1 (clone 259931, 10  $\mu$ g/mL, R&D Systems) or anti-CD11b (clone 2LPM19c, 20  $\mu$ g/mL, Santa Cruz Biotechnology) antibodies. Control antibodies in the corresponding dilutions were also used.

### Determination of cell viability

Purified human eosinophils were plated in 96-well plates at a density of  $2 \times 10^5$  cells/well. Following stimulation with *A fumigatus* conidia (cell:fungus ratio of 1:10, 6 hours), the samples were centrifuged. The supernatants (50  $\mu$ L) were mixed in a 96-well plate with 100  $\mu$ L of lactate dehydrogenase enzyme (LDH) test solution, prepared according to the manufacturer's instructions (Sigma). The samples were maintained in the dark at room temperature (RT) for 20 minutes. HCl (15  $\mu$ L, 1 mol/L) was added to all wells to terminate the reaction. The absorbance was read in a 96-well plate reader at 490 nm. Triton X-100 (0.1%) was used to determine the total LDH (100%) released/condition. The results were expressed as a percentage of release in relation to the Triton X-100 condition.

### Fungal metabolic activity measurement

Fresh or fixed 10% paraformaldehyde (PFO) human eosinophils ( $2 \times 10^5$  cells/well) or fresh or fixed *A fumigatus* conidia (cell:fungus ratio

of 1:10) were plated in a 96-well plate. After 6 hours of incubation, the plate was centrifuged at 290g for 5 minutes. The supernatant was subsequently discarded, and 200  $\mu$ L of RPMI was added to each condition. Next, 54  $\mu$ L of 2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl]-2H-tetrazolium-hydroxyde (XTT)-menadione (Sigma) was added, followed by incubation for 2 hours at 37°C. The reaction of XTT-menadione indicates the cell metabolic activity. The mitochondrial dehydrogenase enzyme present in mitochondria is capable of reducing the XTT-menadione to formazan, which produces an orange color, measured at 490 nm. The XTT solution was prepared by mixing 1 mg/mL XTT salt in PBS and stored at -20°C. Before use, the menadione solution was prepared in acetone and added to XTT to obtain a final concentration of 25  $\mu$ M.<sup>27</sup>

### Scanning electron microscopy

Purified human eosinophils ( $2 \times 10^5/200 \mu$ L) were placed in a 24-well plate (Costar) that contained coverslips pretreated with poly-L-lysine (0.001%) (Sigma). *A. fumigatus* conidia were added at a cell:fungus ratio of 1:10, and the samples were maintained at 37°C with 5% CO<sub>2</sub>. After 6 hours of incubation, the culture medium was removed and adhered cells in the coverslips were fixed with 2.5% glutaraldehyde, 4% formaldehyde (0.1 mol/L), and sodium cacodylate buffer for 2 hours at RT. After 3 washes in sodium cacodylate buffer (0.1 mol/L), the samples were postfixed with an osmium tetroxide solution (1%) and a 0.8% potassium ferrocyanide solution in sodium cacodylate buffer for 30 minutes. The samples were again washed 3 times in sodium cacodylate buffer, followed by dehydration in graded ethanol: 30%, 50%, 70%, 90%, and 3  $\times$  100% for 15 minutes each. The critical point technique with CO<sub>2</sub> was subsequently performed, followed by mounting on a metallic support with carbon tape. The samples were then covered with a thin layer of 20 nm gold (metallization), followed by examination under a conventional QUANTA 250 FEI scanning electron microscope (ThermoFisher Scientific).

### Confocal microscopy

Purified human eosinophils ( $2 \times 10^5/200 \mu$ L) were placed in a 24-well plate (Costar) that contained coverslips pretreated with poly-L-lysine (0.001%) (Sigma). The cells were subsequently stimulated with *A. fumigatus* conidia at a cell:fungus ratio of 1:10 and were maintained at 37°C with 5% CO<sub>2</sub> for 6 hours. In specific sets of experiments, eosinophils were pretreated for 30 minutes with pharmacological inhibitors before the fungus stimulation. At the end of the incubation time, the culture medium was removed and the adhered cells were fixed with 4% PFO for 15 minutes at RT. The cells were washed with sterile PBS 3 times. Sytox Green (5  $\mu$ M) or Hoechst (1:1000, Life Technologies) was subsequently added for 10 minutes for DNA labeling. After 3 additional PBS washes, calcofluor white (100  $\mu$ g/mL, Sigma, a fungus cell-wall marker) was added and incubated with the preparations for 1 hour at RT. The medium was subsequently removed, and the cells were washed with PBS. For MBP labeling, a mouse anti-human MBP mAb (clone BMK-13, 2.5  $\mu$ g/mL, Merck Millipore) diluted in PBS solution that contained 5% human serum and 0.1% saponin (Sigma) was used. After 1 hour of incubation, the cells were washed and incubated for 1 hour with a rabbit anti-mouse IgG phycoerythrin-conjugated antibody (1:500, Jackson ImmunoResearch). For histone labeling, the samples were fixed with PFO 4% and permeabilized with a PBS buffer containing 1% triton x-100 and 2% NP40. Then, a mouse anti-human citrullinated histone H3 antibody (0.8  $\mu$ g/mL, Abcam) was diluted in PBS solution that contained 0.05% tween-20, 2% BSA, and 5% human serum. Following an overnight incubation at 4°C protected from light, the cells were washed and incubated for 1 hour with a rabbit anti-mouse IgG fluorescein isothiocyanate-conjugated antibody (1:1000, Jackson ImmunoResearch). The wells were subsequently washed again 3 times, followed by the addition of the mounting medium Aqua-Poly/Mount. Images were acquired using a fluorescence confocal microscope Leica TCS SP5 (Leica Microsystems) and were analyzed with the LAS-AF Software and the Image J program (Fiji).

Regarding the bronchial sample analysis, eosinophilic mucus plugs were fixed in 10% formalin and embedded in paraffin. Deparaffinized sections were stained using Grocott methods. For histone staining, the antigen retrieval of the sample slides was performed via 30 minutes of incubation in Tris-EDTA buffer in a microwave oven, followed by blocking with 10% BSA and 1%

saponin containing PBS for 30 minutes. The slides were subsequently incubated with primary rabbit anticitrullinated histone H3 mAb (10  $\mu$ g/mL, Abcam, 90 minutes at RT) or mouse anti-histone H1 mAb (5  $\mu$ g/mL, Abcam, 90 minutes at RT). Alexa-488-conjugated antibodies (Life Technologies, 30 minutes at RT) were used for the secondary incubation. Isotype-matched control antibodies and Hoechst 33342 (Life Technologies) were used for each experiment. Samples were mounted using Prolong Diamond (Life Technologies) and the images were obtained with a Carl Zeiss LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany). Coverslips were removed and further stained with the hematoxylin-eosin method.

### Conidia viability evaluation

Fresh human eosinophils ( $2 \times 10^5$  cells/well) and *A. fumigatus* conidia (cell:fungus ratio of 1:10) were plated in a 96-well plate. After 6 hours of incubation, propidium iodide (PI) (1  $\mu$ g/mL) was added to the samples. Images were immediately acquired using a fluorescence confocal microscope Leica TCS SP5 (Leica Microsystems) and were analyzed with the LAS-AF Software and the Image J program (Fiji).

### Fungal germination evaluation

Fresh human eosinophils ( $2 \times 10^5$  cells/well) and *A. fumigatus* conidia (cell:fungus ratio of 1:10) were plated in a 24-well plate. After 24 hours of incubation, calcofluor white (100  $\mu$ g/mL, Sigma, a fungus cell-wall marker) was added and incubated with the preparations for 1 hour at RT. The medium was subsequently removed, and the preparations washed 4 times followed by the addition of the mounting medium Aqua-Poly/Mount. Images were acquired using a fluorescence confocal microscope Leica TCS SP5 (Leica Microsystems) and were analyzed with the LAS-AF Software and the Image J program (Fiji).

### ROS quantification

Purified human eosinophils were plated in 96-well plates at a density of  $2 \times 10^5$  cells/well and incubated with 1 mM of dihydrorhodamine (Sigma) for 30 minutes. The cells were subsequently stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) or PMA (50 nM) as a positive control. To test the effectiveness of DPI in blocking PMA-induced ROS production, 30  $\mu$ M DPI was added 30 minutes before cell stimulation. The ROS generation was measured at different time points via a Flexstation plate reader (Molecular Devices) with a wavelength excitation of 500 nm and emission of 536 nm.

### Flow cytometry analysis

To examine the expression of dectin-1 in eosinophils, cells ( $2.5 \times 10^5/250 \mu$ L) were fixed (PFO 4%) and incubated with labeling buffer (PBS, 5% human serum and 0.1% saponin) containing a mouse anti-human dectin-1 antibody (clone 259931, 0.5  $\mu$ g/mL, R&D Systems) or its isotype control for 1 hour of incubation at 4°C. The samples were subsequently incubated with the secondary rabbit anti-mouse IgG fluorescein isothiocyanate-conjugated antibody (1: 500, Jackson ImmunoResearch) for 45 minutes at 4°C. The samples were centrifuged, resuspended in 300  $\mu$ L of PFO (4%), and analyzed using an ACCURI flow cytometer (BD Biosciences). Neutrophils were used as a dectin-1 antibody positive control.

### Statistical analysis

The data obtained in the experiments were analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, Calif) and are presented as the mean  $\pm$  SEM. The results were analyzed by 1-way ANOVA, followed by the Newman-Keuls test. *P* values of less than .05 were considered significant (2-tailed test).

## RESULTS

### ETosis-derived EETs are abundant in bronchial mucus samples of patients with ABPA

ETosis-derived EETs have been previously reported in human allergic diseases such as eosinophilic chronic rhinosinusitis and

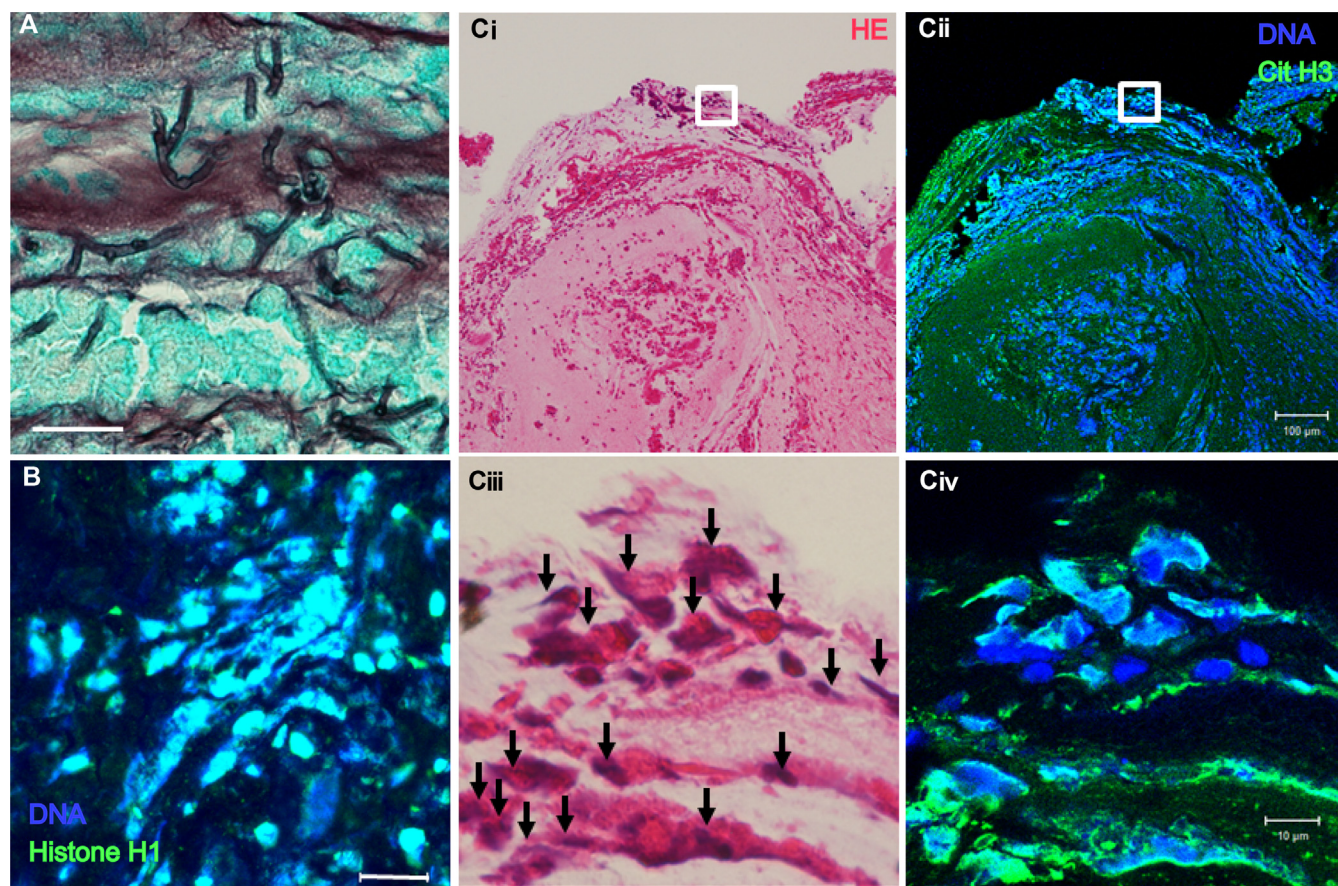


**FIG 1.** Presence of *A fumigatus* and abundant EETosis-derived EETs in bronchial mucus plugs obtained from a patient with ABPA (case 7, Table E1). **A**, Light microscopic images were obtained from Grocott-stained sections of a mucus plug (scale bar: 20  $\mu$ m). Branching fungal hyphae in the mucus was evident. **B**, Serial section of mucus plug stained for histone H1 (green) and DNA (blue) and analyzed using a laser scanning confocal microscope (scale bar: 20  $\mu$ m). The mesh-like EETs colocalized with histone H1 spread throughout the mucus. **C**, Identification of ETotic eosinophils. Section of mucus plug was immunostained for citrullinated histone H3 (citH3, green) and DNA (blue). After fluorescence imaging (ii and iv), coverslips were removed and the same section was further stained with the HE method (i and iii). Lower panels ( $\times 100$  objective, scale bar: 10  $\mu$ m) are the boxed area in upper panels ( $\times 10$  objective, scale bar: 100  $\mu$ m) presented at a higher magnification. The mucus contains a massive infiltration of eosinophils (>96% of nucleated cells) with negligible numbers of other cells. Lytic eosinophils with decondensed nuclei and ETs (iii, arrows) were positive for citrullinated histone H3 (iv). HE, Hematoxylin-eosin.

eosinophilic otitis.<sup>21,22</sup> We wondered whether EETosis or EETs were present in clinical samples of patients with ABPA. Thus, we investigated the microscopic morphology of *A fumigatus* and eosinophils in highly viscous bronchial secretions obtained from a series of patients with ABPA with a positive microbiologic culture for *A fumigatus* (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Grocott staining indicates that the fungal hyphae were scattered throughout the mucus plug (Fig 1, A). Hoechst 33342–positive DNA was arranged in fibrous structures with morphological ET characteristics and was abundantly present in the mucus plug (Fig 1, B). The DNA was colocalized with histone H1 (Fig 1, B), indicating nuclear-derived ETs. We next stained the mucus plug for citrullinated histone H3 because histone citrullination is considered to play an essential role in the formation of ETs.<sup>28</sup> After immunostaining, the same sample was additionally stained by hematoxylin-eosin to identify the eosinophils (Fig 1, C). At low magnification, a massive infiltration of eosinophils and abundant citrullinated histone H3 were evident in the mucus

plug (Fig 1, Ci, ii). High magnification image indicated that significant numbers of eosinophils exhibited defining nuclear characteristics of EETosis, including decondensed nuclei, loss of lobulation, and/or disrupted nuclei (Fig 1, Ciii, arrows). The ETotic eosinophils and ETs were stained with citrullinated histone H3, although nuclei of intact cells were not (Fig 1, Cii). Similar results were obtained in other 6 patients with ABPA (see Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). These findings indicated the saprophytic growth of *A fumigatus* and the presence of EETosis-derived EETs in mucus plugs.

#### ***A fumigatus*–induced human eosinophil release of EETs**

As we have characterized the presence of the massive infiltration of eosinophils that exhibited the nuclear characteristics of EETosis in association with EETs in bronchial mucus plugs obtained from patients with ABPA, we next investigated whether

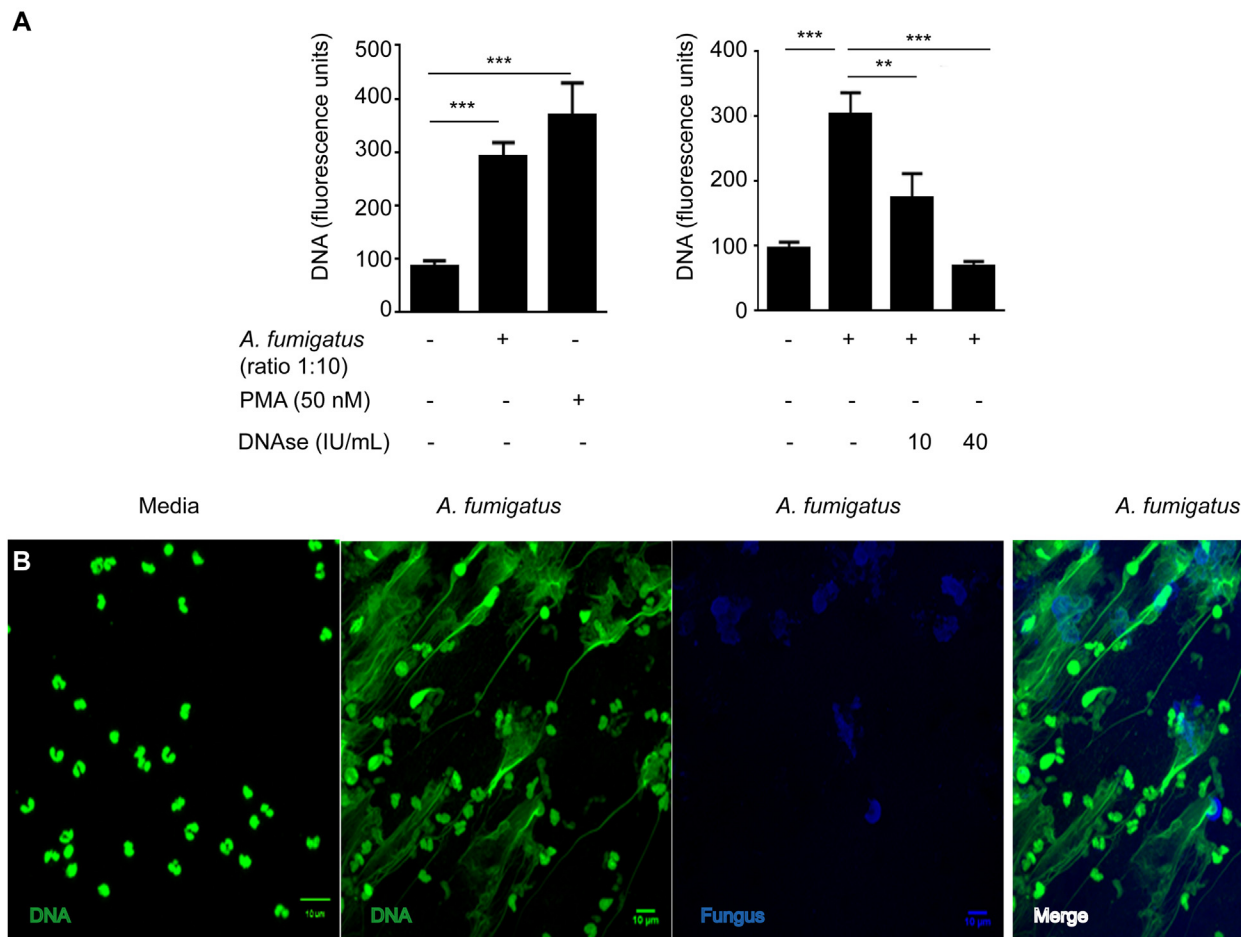


**FIG 2.** *A. fumigatus*-induced human blood eosinophil EETs release. **A**, Human eosinophils were stimulated with *A. fumigatus* conidia for 6 hours (cell:fungus ratio of 1:10) ( $n = 16$ ) or PMA for 2 hours ( $n = 12$ ) (left panel). DNase added 15 minutes before the end of the incubation time (6 hours) dose dependently degraded EETs ( $n = 4$ ) (right panel). EETs were quantified with Sytox Green (5  $\mu$ M) in a fluorescence microplate reader. The graph represents the mean  $\pm$  SEM of independent experiments from different donors. \*\*\* $P < .001$ , \*\* $P < .01$ , 1-way ANOVA followed by the Newman-Keuls test. **B**, Confocal fluorescence microscopy of human eosinophils with or without *A. fumigatus* stimulation (cell:fungus ratio of 1:10, 6 hours) after staining for DNA (Sytox Green, green) and fungus (calcofluor, blue). Representative images of 4 independent experiments ( $n = 4$ ).

human eosinophils release EETs in response to *A. fumigatus* *in vitro*. To ascertain whether *A. fumigatus* induced the production of EETs, isolated human eosinophils were incubated with *A. fumigatus* conidia (strain NCPF 2109) at different ratios of cell:fungus for different time periods. Using a fluorimetric method, the formation of EETs was detectable at 3 hours and increased during the subsequent hours of incubation. We determined that fresh human eosinophils stimulated with *A. fumigatus* start releasing EETs at cell:fungus ratios of 1:10, 1:50, and 1:100 within 3 hours. After 6 hours of stimulation, the amount of EETs detected also increased for the ratio 1:1. For 1:10, 1:50, and 1:100, the amount of EETs detected after 6 hours of stimulation was approximately 4 times higher than the control. We also monitored the release of EETs after 9 hours of stimulation (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). For further studies, we chose the ratio 1:10 and 6 hours as the time point of stimulation. Human eosinophils were stimulated with *A. fumigatus* conidia for 6 hours

(cell:fungus ratio of 1:10) or PMA for 2 hours (Fig 2, A, left panel). PMA was used as the EETs release positive control. To further confirm that EETs produced against *A. fumigatus* consisted of extracellular DNA, we added DNase to eosinophil-*Aspergillus* cocultures 15 minutes before the end of the incubation time (6 hours). DNase addition concentration-dependently degraded the EETs (Fig 2, A, right panel). Within 15 minutes of the addition of DNase, the EETs were completely disrupted, which indicates that EETs detected in this system were composed of DNA. We also conclude that the fluorescence was a result of extracellular DNA because the addition of DNase abolished it. *A. fumigatus*-induced release of EETs also occurs with other *A. fumigatus* strains such as NCPF 2140 (data not shown), which indicates that the EETs released are not limited to the strain used. Confocal images of cultures stained with Sytox Green (green; a DNA marker that is nonpermeable to viable cells) and calcofluor white (blue; a fungal wall marker) indicate that human eosinophils produced EETs. Eosinophils alone without fungi

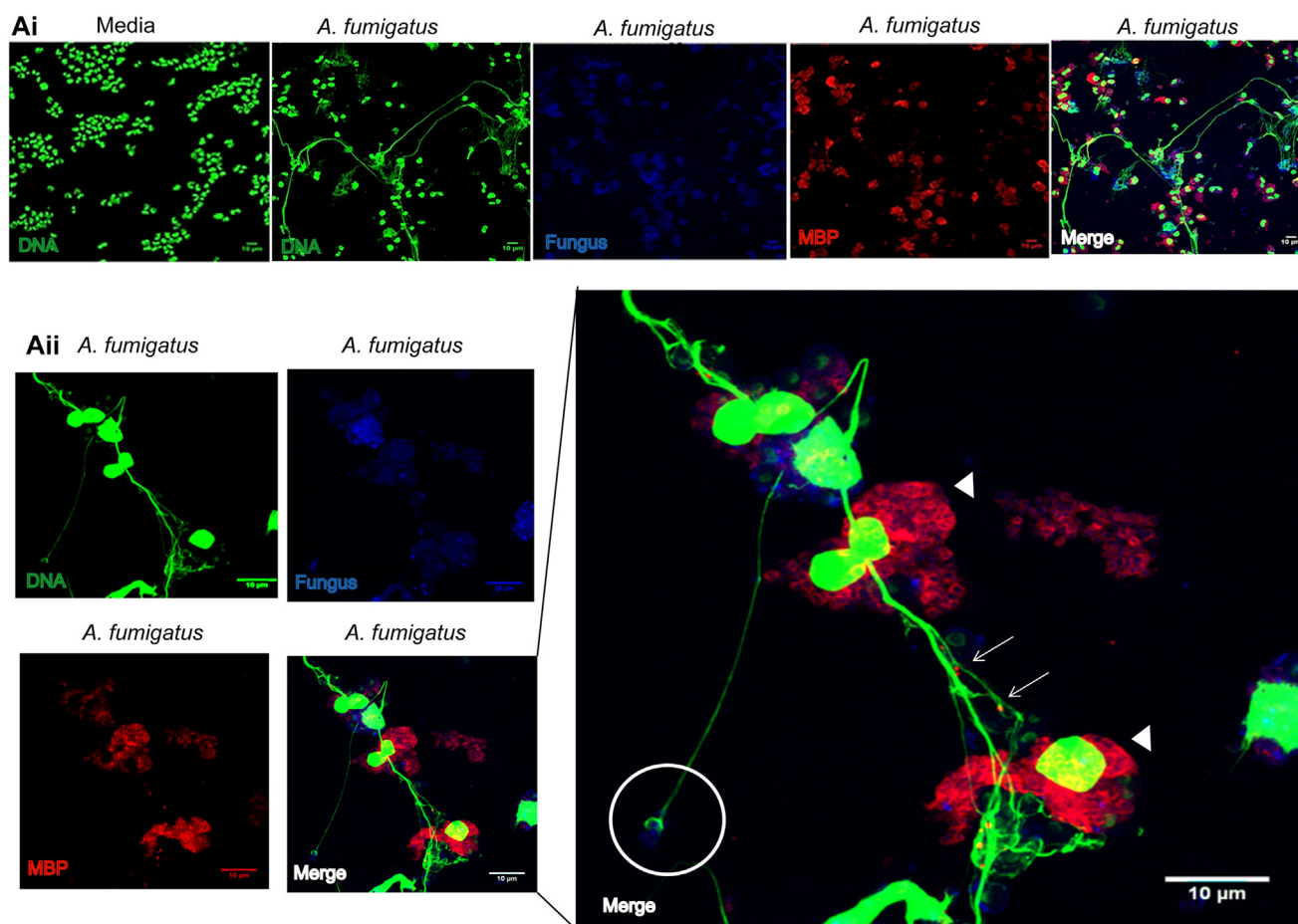


**FIG 3.** EETs induced by *A fumigatus* are predominantly not associated with free granule MBP. **Ai**, Eosinophils were or were not stimulated with *A fumigatus* conidia (cell:fungus ratio of 1:10) for 6 hours, and confocal fluorescence microscopy was performed. **Aii**, Different sets of images in higher magnification. Fungus labeling is mostly associated with cells or EETs (circle). Released EETs did not exhibit free MBP protein labeling; instead, they exhibited punctate (arrows) or cell-associated staining (arrowhead). The samples were stained for DNA (Sytox Green, green), MBP (antibody anti-human MBP, red), and fungus (calcofluor, blue). Representative images of 3 independent experiments ( $n = 3$ ).

did not produce EETs (Fig 2, B). It has previously been reported that neutrophil and eosinophil granule-derived proteins, many with antimicrobial activities, have been recognized to be bound as free proteins to EETs.<sup>14,29</sup> To assess whether specific eosinophil granule proteins were released free or were otherwise bound to EETs, *A fumigatus*-stimulated eosinophils were stained with Sytox Green (green) in combination with calcofluor (blue) and an anti-MBP mAb (red) (Fig 3, Ai). Notably, we determined that EETs lacked linear immunostaining for free MBP, which suggests that EETs and free MBP are not colocalized (Fig 3, Ai). Otherwise, we noted an MBP immunostaining localized to smaller punctuate foci (Fig 3, Aii, arrows) or cell-associated domains (Fig 3, Aii, arrowhead). The fungus labeling is predominately merged with apparently lytic cells or EETs (Fig 3, Aii, circle). Unstimulated eosinophils demonstrated no formation of EETs. High-resolution scanning electron microscopy subsequently confirmed that human eosinophils release EETs in response to *A fumigatus* (Fig 4). Interestingly, scanning electron microscopy revealed that EETs were released from lytic eosinophils (arrowheads, Fig 4, A) and that these EETs (highlighted in orange in Fig 4, Ai and Bi) exhibited

intimate contact with conidia (asterisks, Fig 4, A and B) and some free eosinophil secretory granules (highlighted in green in Fig 4, Ai).

### EETs release induced by *A fumigatus* is ROS independent

Our experiments indicated that *A fumigatus* induces the release of EETs in isolated blood eosinophils. Thus, because there is evidence that ROS are involved in extracellular DNA release in both neutrophils and eosinophils depending on the stimuli, we subsequently investigated whether the release of EETs following *A fumigatus* stimulation also exhibits ROS dependence. The inhibition of ROS production with DPI (30  $\mu$ M, an NADPH oxidase inhibitor) before *A fumigatus* stimulation does not block the release of EETs (Fig 5, A). Confocal fluorescence microscopy studies in which the samples were stained for DNA (Sytox Green, green) and fungus (calcofluor, blue) confirmed these findings (Fig 5, C). Mitochondrial ROS have been described as important for DNA extracellular release in neutrophils depending on the stimuli. To investigate this issue, human eosinophils were

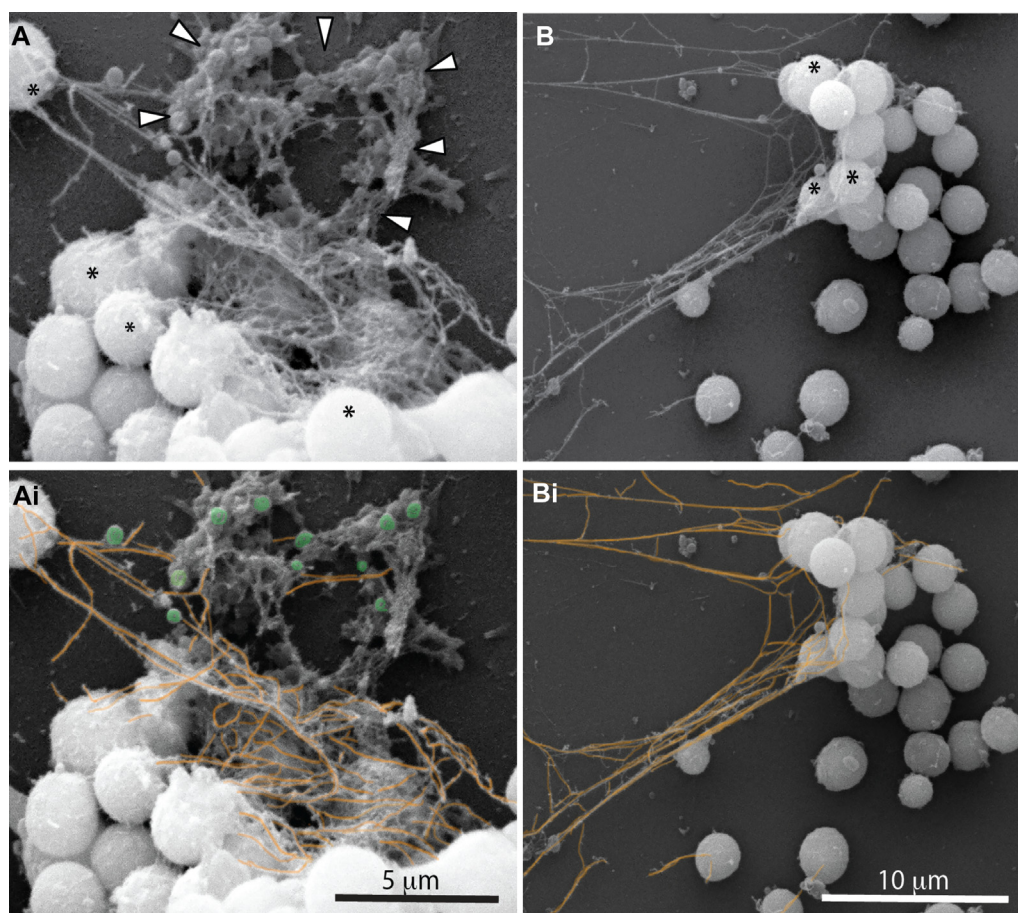


**FIG 4.** *A. fumigatus* conidia and free secretory granules are entrapped by web-like EETs. **A**, Scanning electron microscopy (SEM) shows web-like DNA traps released from a lytic eosinophil (arrowheads) entrapping *A. fumigatus* conidia (\*). **A** and **B**, Note the close association of DNA traps with conidia (\*). EETs were highlighted in orange (Fig 4, **Ai** and **Bi**) while secretory granules were marked in green (Fig 4, **Ai**). Eosinophils were stimulated with *A. fumigatus* conidia for 6 hours (cell:fungus ratio of 1:10) and processed for SEM.

pretreated with MitoTEMPO (500  $\mu$ M) (a specific mitochondrial ROS inhibitor) or DPI plus MitoTEMPO for 30 minutes and were subsequently stimulated with *A. fumigatus* conidia. In both conditions, no inhibition of release of EETs was identified (Fig 5, **A**). Furthermore, as indicated in Figure 5, **B**, a time point quantification of ROS production by dihydrorhodamine 123 (an ROS fluorescent probe) demonstrated that ROS production in eosinophils following *A. fumigatus* stimulation is comparable to the resting eosinophil values. PMA was used as a positive control for the release of EETs and ROS production, as well as to determine the effectiveness of DPI.

#### ***A. fumigatus*-induced release of EETs affects eosinophil viability and is associated with histone citrullination**

The release of NETs is dependent on ROS and involves cell death in a process referred to as neutrophil extracellular trap cell death.<sup>10,11</sup> To determine whether cell death was involved in the release of EETs, eosinophils were stimulated with *A. fumigatus* for 6 hours, and LDH, an established cytoplasmic protein, activity was measured in the supernatants. An increase in LDH activity levels in the supernatant is indicative of the loss of cell membrane

integrity. As indicated in Fig 6, **A**, the LDH activity levels were higher in the supernatants of cells stimulated with *A. fumigatus* for 6 hours compared with the control. To evaluate whether citrullinated histones were associated with EETs, confocal fluorescence microscopy of human eosinophils stimulated with *A. fumigatus* (cell:fungus ratio of 1:10, 6 hours) after staining for DNA (4'-6-diamidino-2-phenylindole, dihydrochloride, blue) and citrullinated histones (anti-human Cit H3 antibody, green) was performed (Fig 6, **B**). We identified colocalizations of DNA and citrullinated histone H3 (Fig 6, right panel). The control isotype antibody for the anticitrullinated histone H3 yielded no staining (data not shown). Altogether, these findings indicate that *A. fumigatus* induces eosinophil death (ie, EETosis) and the release of EETs with citrullinated histones, as observed in bronchial mucus samples obtained from patients with ABPA.

#### ***A. fumigatus*-induced release of EETs does not impair the fungal metabolic activity, viability, and germination**

Despite the clear induction of the formation of EETs by human eosinophils, we did not identify an influence of the formation of EETs on the killing of *A. fumigatus in vitro* as assessed by a

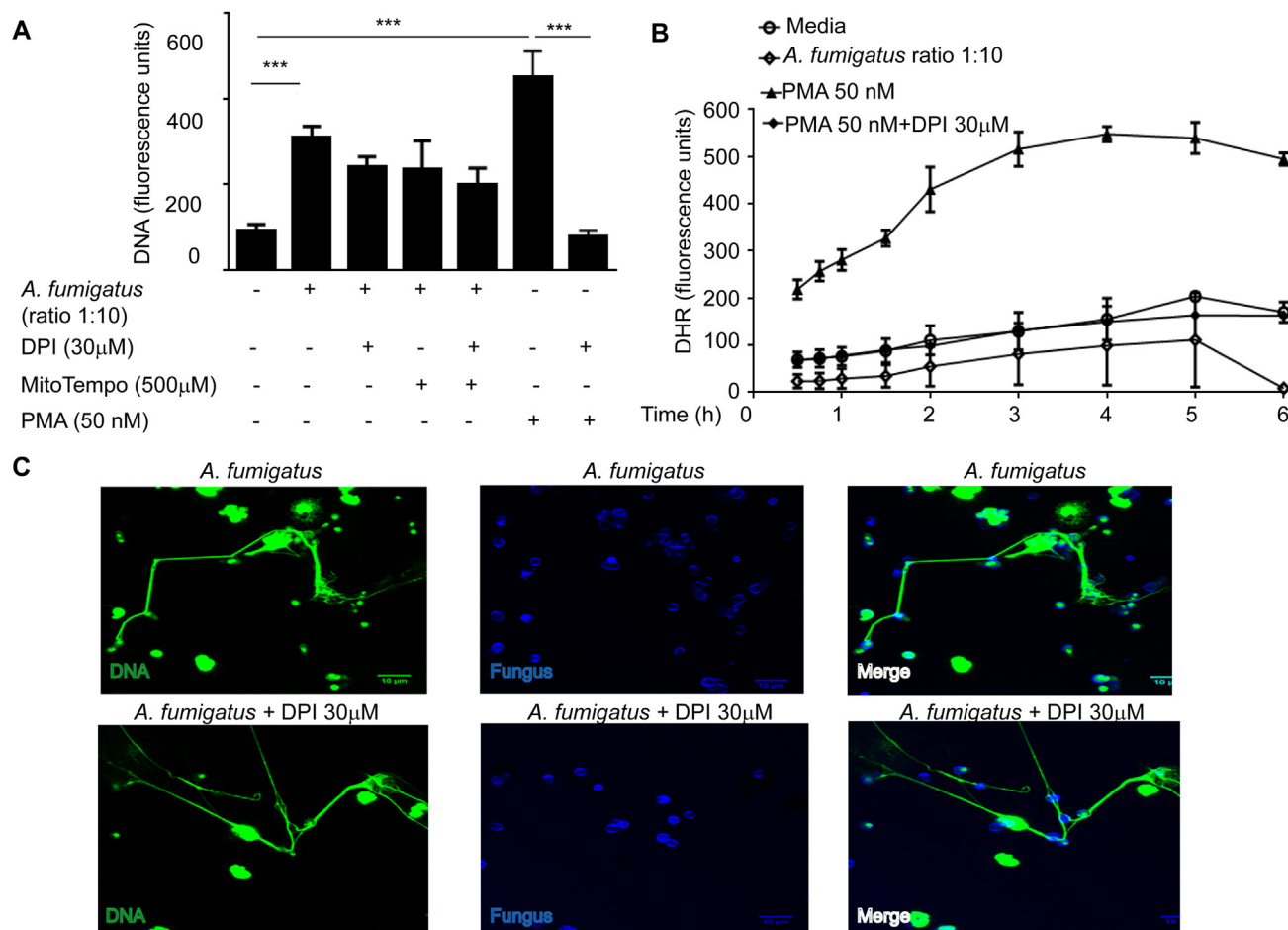


**FIG 5.** *A. fumigatus*-induced release of EETs is independent of ROS. **A**, Human eosinophils were pretreated with DPI (30  $\mu$ M) (n = 10), MitoTEMPO (500  $\mu$ M) (n = 3), or both compounds (n = 3) for 30 minutes and were subsequently stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) for 6 hours. EETs were quantified with Sytox Green (5  $\mu$ M) in a fluorescence microplate reader. PMA was used as a positive control. The graph represents the mean  $\pm$  SEM of independent experiments from different donors. \*\*\* $P$  < .001, 1-way ANOVA followed by the Newman-Keuls test. **B**, Human eosinophils were or were not stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) for 6 hours in the presence of dihydrorhodamine 123. Fluorescence was measured in a fluorescence microplate reader. PMA was used as a positive control. The graph represents the mean  $\pm$  SEM of 3 independent experiments from different donors (n = 3). **C**, Confocal fluorescence microscopy of human eosinophils pretreated with DPI (30  $\mu$ M) and subsequently stimulated with *A. fumigatus* (cell:fungus ratio of 1:10, 6 hours) after staining for DNA (Sytox Green, green) and fungus (calcofluor, blue). Representative images of 3 independent experiments (n = 3).

colorimetric assay for antifungal susceptibility. Briefly, this assay is based on the reduction of tetrazolium salt XTT in the presence of menadione as an electron-coupling agent, which results in a high production of formazan. The XTT-menadione system provides an assay that enables the quantification of *A. fumigatus* metabolic activity.<sup>27</sup> Thus, *A. fumigatus* conidia were cocultured with freshly isolated eosinophils for 6 hours and subsequently exposed to XTT-menadione for 2 hours. No statistically significant difference was identified in the formazan production between the conditions in which *A. fumigatus* was cultured alone and in the presence of live eosinophils (Fig 7, A). Live or fixed eosinophils and fixed *A. fumigatus* exhibited very low levels of formazan production. Additional studies with PI were performed. *A. fumigatus* conidia were cocultured with freshly isolated eosinophils for 6 hours and subsequently the samples were stained with PI. Cocultures were analyzed by bright-field (Fig 7, Bi) and confocal fluorescence microscopy. Conidia did not stain with PI

(Fig 7, Bii, iii, and iv), indicating that the integrity of the fungal conidia walls has not been compromised (Fig 7, Biv, arrows). In addition, many lytic eosinophils incubated with *A. fumigatus* conidia stained with PI (Fig 7, Bii, iii, iv), confirming our previous findings. Moreover, eosinophils cocultured with *A. fumigatus* conidia for 24 hours did not avoid fungal hyphae formation (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The hyphae organization in the presence of eosinophils seems more aggregated compared with the condition without eosinophils, but equally abundant. We expected that considering the equally clumped state observed for conidia in the presence of EETs.

#### ***A. fumigatus*-induced release of EETs depends on the Syk kinase pathway**

Syk has been shown to mediate cell signaling via different classes of receptors involved in fungus recognition, including

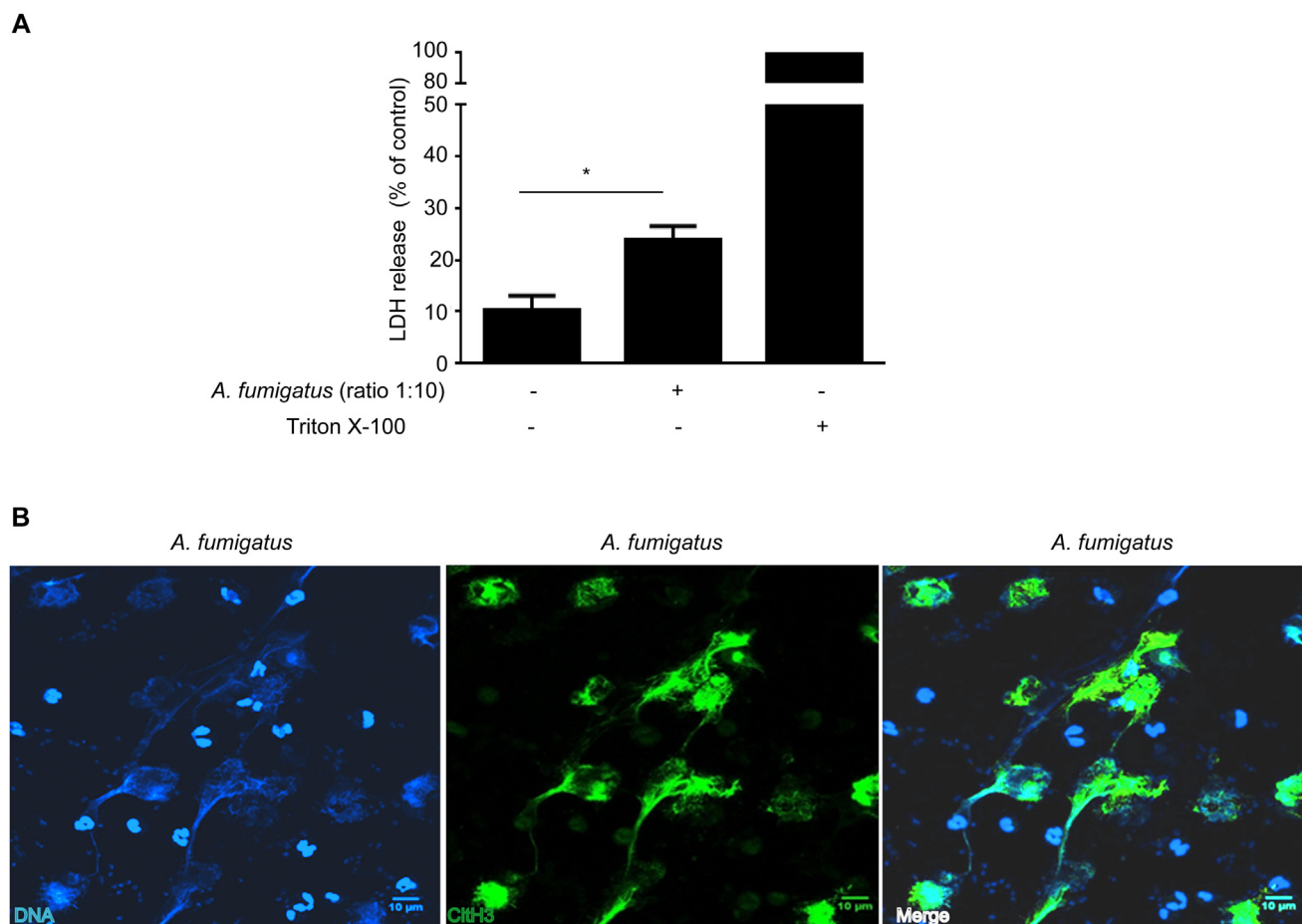


**FIG 6.** *A. fumigatus*-induced release of EETs affects eosinophil viability and is associated with histone citrullination. **A**, Human eosinophils were stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) for 6 hours. Supernatants were recovered, and the lactate dehydrogenase enzyme (LDH) activity was measured. The graph represents the mean  $\pm$  SEM of 6 independent experiments from different donors ( $n = 6$ ).  $*P < .05$ , 1-way ANOVA followed by the Newman-Keuls test. **B**, Confocal fluorescence microscopy of human eosinophils with or without *A. fumigatus* stimulation (cell:fungus ratio of 1:10, 6 hours) after staining for DNA (4'-6-diamidino-2-phenylindole, dihydrochloride, blue) and citrullinated histones (anti-human Cit H3 antibody, 0.8  $\mu$ g/mL, green). Representative images of 3 independent experiments ( $n = 3$ ).

integrins<sup>30</sup> and C-type lectins.<sup>31</sup> To assess the role of Syk signaling in *A. fumigatus*-induced release of EETs, the pharmacological inhibitors piceatannol 40  $\mu$ M and OXSI 2  $\mu$ M were used. Eosinophils pretreated with piceatannol (Fig 8, A) or OXSI (Fig 8, B) for 30 minutes and subsequently stimulated with *A. fumigatus* (cell:fungus ratio of 1:10, 6 hours) prevented the release of EETs. Confocal images of cultures stained with Sytox Green confirmed the fluorimetric findings (Fig 8, C).

#### ***A. fumigatus*-induced release of EETs depends on the CD11b $\beta_2$ -integrin in contrast to dectin-1 receptor**

Following the determination that eosinophils respond to *A. fumigatus* by releasing EETs, we investigated the molecular mechanisms involved in eosinophil-fungus interaction. We suspected that innate receptors, such as dectin-1 and a  $\beta_2$ -integrin (CD11b), which recognize  $\beta$ -glucan, a molecular pattern commonly identified in the cell wall of fungus, play potential roles in this interaction. Adhesion molecules have been shown to play an important role in thymic stromal

lymphopoietin-induced release of EETs,<sup>19</sup> eosinophil activation, intracellular signaling, degranulation, and superoxide production.<sup>8</sup> Thus, we subsequently investigated whether the release of EETs induced by *A. fumigatus* required the participation of CD11b, an adhesion molecule previously recognized to be expressed on eosinophils.<sup>8</sup> Human eosinophils were pretreated with neutralizing antibodies against the integrin CD11b (20  $\mu$ g/mL) and were subsequently stimulated with *A. fumigatus*. The release of EETs was completely abolished (Fig 8, D). We subsequently investigated a potential role for dectin-1. As indicated in Figure 8, E, when eosinophils were pretreated with antibodies that neutralized the dectin-1 (10  $\mu$ g/mL) receptor, the release of EETs following *A. fumigatus* stimulation was not blocked. Furthermore, we confirmed a previously published finding<sup>8</sup> that indicated that human eosinophils do not express the dectin-1 receptor (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

#### **DISCUSSION**

Considering that ABPA is the most significant and prevalent manifestation of allergic aspergillosis that occurs worldwide<sup>32</sup>

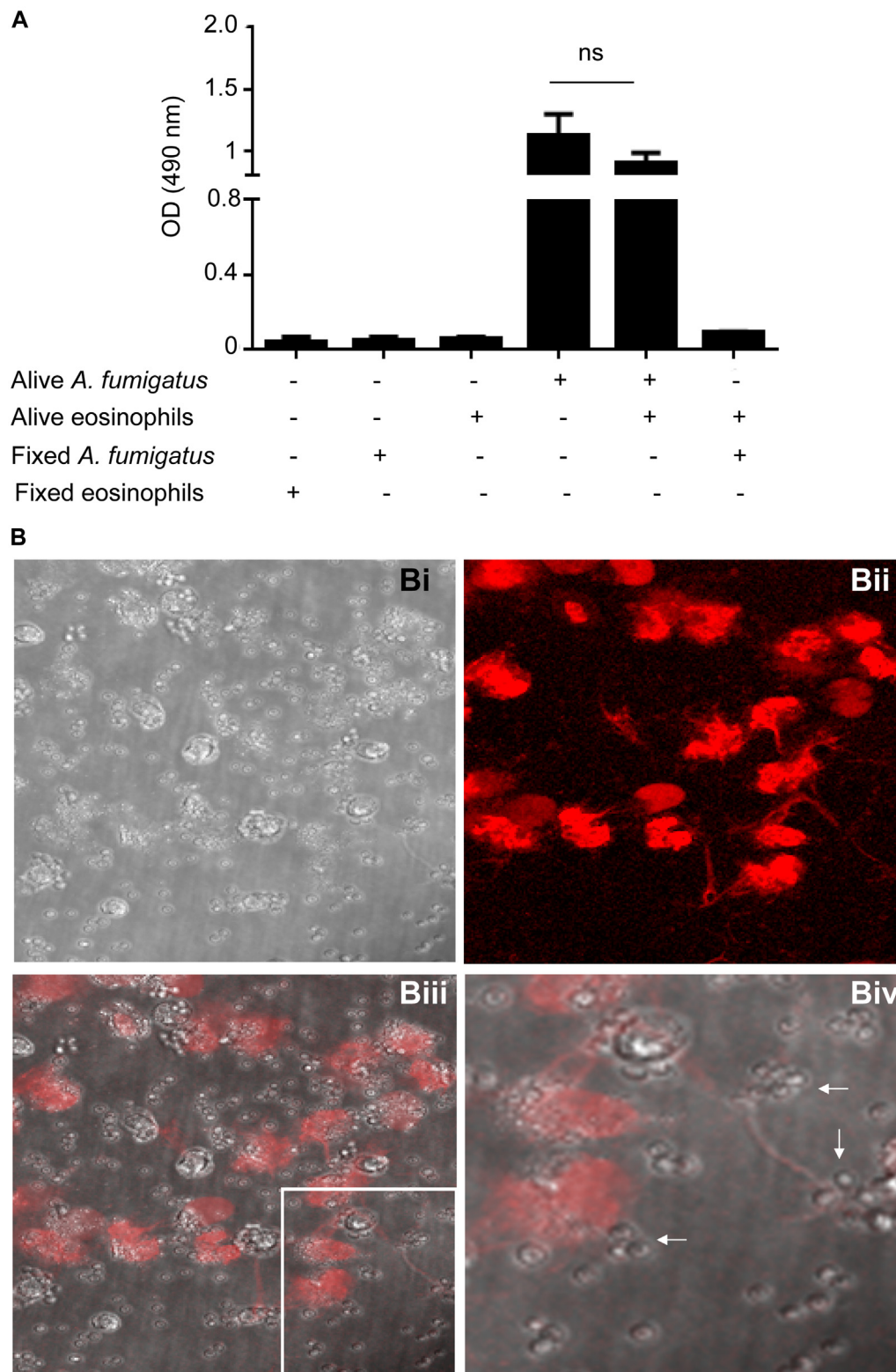


**FIG 7.** *A. fumigatus*-induced release of EETs does not impair the fungal metabolic activity and viability. **A**, Human eosinophils or *A. fumigatus* was incubated for 6 hours under the previously described conditions and were subsequently exposed for 2 hours to XTT-menadione. The graph represents the mean  $\pm$  SEM of 3 independent experiments from different donors ( $n = 3$ ). One-way ANOVA followed by the Newman-Keuls test was the statistical test used. **B**, Human eosinophils were cocultured with *A. fumigatus* (cell:fungus ratio of 1:10) for 6 hours. Images were acquired by bright field (BF) (Fig 7, *Bi*) and confocal fluorescence microscopy (Fig 7, *Bii*) after staining with PI. BF and PI-stained images were overlaid (Fig 7, *Biii*). *Biv* is the boxed area in higher magnification. Arrows point to fungus conidia. Figures are representative of 3 independent experiments ( $n = 3$ ). *ns*, Not significant.

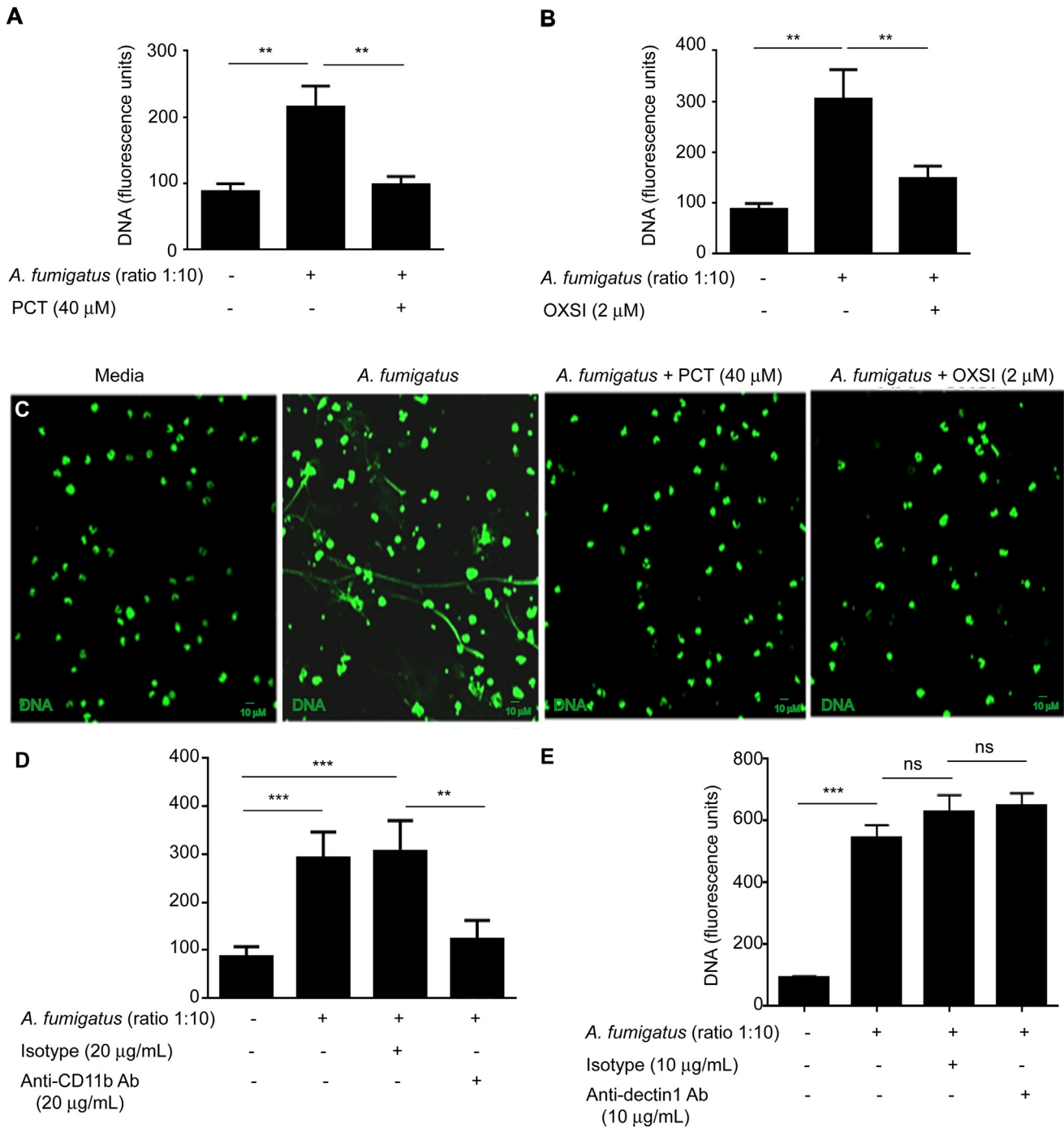


**FIG 8.** Blockage of the Syk kinase pathway and CD11b inhibited *A. fumigatus*-induced release of EETs. **A** and **B**, Human eosinophils were pretreated with PCT (40  $\mu$ M) (n = 6) or OXSI (2  $\mu$ M) (n = 6) for 30 minutes and were subsequently stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) for 6 hours. EETs were quantified with Sytox Green (5  $\mu$ M) in a fluorescence microplate reader. The graph represents the mean  $\pm$  SEM of independent experiments from different donors.  $^{**}P < .01$ , 1-way ANOVA followed by the Newman-Keuls test. **C**, Confocal fluorescence microscopy of human eosinophils pretreated with PCT (40  $\mu$ M) or OXSI (2  $\mu$ M) for 30 minutes and subsequently stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) for 6 hours after staining for DNA (Sytox Green, green). Representative images of 3 independent experiments (n = 3 for each inhibitor). Human eosinophils were pretreated with **(D)** anti-human CD11b antibody (Ab) (20  $\mu$ g/mL) (n = 5) or **(E)** anti-human dectin-1 Ab (n = 3) for 30 minutes and were subsequently stimulated with *A. fumigatus* conidia (cell:fungus ratio of 1:10) for 6 hours. EETs were quantified with Sytox Green (5  $\mu$ M) in a fluorescence microplate reader. The graph represents the mean  $\pm$  SEM of independent experiments from different donors. ns, Not significant; PCT, picetannol.  $^{***}P < .001$ ,  $^{**}P < .01$ , 1-way ANOVA followed by the Newman-Keuls test.

and the lack of direct molecular evidence that mechanistically describes the roles of eosinophils in this pathology, an understanding of how eosinophils recognize and act as immune effector cells in response to *A fumigatus* remains necessary. Here, we initially characterized a unique abundant presence of ETosis-derived EETs in bronchial mucus samples of patients with ABPA. Second, we determined that EETs are released *in vitro* in response to *A fumigatus*. Third, these EETs are released independent of ROS generation and in a process that affects eosinophil viability. Fourth, EETs released in response to *A fumigatus* contain nuclear genome; however, it does not present fungicidal/fungistatic activity. Fifth, by targeting the Syk tyrosine kinase pathway or the adhesion molecule CD11b, EETs formed during *A fumigatus* stimulation were blocked.

This study provides the first evidence for the growth of *A fumigatus* and the presence of ETosis-derived EETs in the mucus plugs of patients with ABPA. Based on this clinical observation, the subsequent step was to investigate whether eosinophils responded to *A fumigatus in vitro* by releasing EETs. It is well established that human neutrophils produce NETs when encountering different *A fumigatus* morphotypes *in vitro*.<sup>23,24</sup> However, the knowledge regarding eosinophils is limited. Using different methods, we have found that fresh blood human eosinophils respond to *A fumigatus* conidia by releasing EETs. Our fluorescent and scanning electron microscopy studies indicated the intimate contact between EETs and conidia, which suggests that EETs provide an adhesive surface for microorganism entrapment. In fact, it has been suggested previously that EETs generated by calcium ionophore-stimulated human eosinophils are capable of binding to different microorganisms including *A fumigatus* via means of passive contact.<sup>21</sup> It has earlier been reported that antimicrobial granule proteins stored in neutrophils and eosinophils are detected bound as free proteins to ETs. In neutrophils, neutrophil elastase and myeloperoxidase translocate to the nucleus and bind to nuclear chromatin before the release of NETs.<sup>29</sup> In eosinophils, it has been reported that for the elicited release of mitochondrial ETs from eosinophils, eosinophil granule proteins were bound to EETs.<sup>14</sup> In our findings, we noted that an MBP immunostaining localized to smaller punctuate foci or cell-associated domains and that EETs lacked linear immunostaining for free MBP. The punctual MBP labeling associated with *A fumigatus*-induced EETs may be indicative of the presence of clusters or individual free secretory eosinophil granules, a hypothesis that was confirmed by our scanning electron microscopy studies. Moreover, our data are at least in part in concert with previous findings that describe an NADPH-oxidase-dependent cytolytic mechanism that leads to EETs and secretion-competent granule release in which EETs are predominantly free of granule protein and mainly associated with clusters of secretion-competent eosinophil granules.<sup>20</sup>

The contribution of ROS to the release of ETs in both neutrophils and eosinophils has been largely demonstrated in the literature.<sup>10,11,14,15,19,20,33-35</sup> Regarding neutrophils, most NET inducers require mitochondrial- or NADPH oxidase-ROS generation<sup>10,11,14,15,33,35</sup>; however, there are reports of ROS-independent release induced by *Staphylococcus aureus*,<sup>16</sup> *Leishmania donovani*,<sup>36</sup> *Candida albicans*,<sup>37</sup> uric

acid,<sup>17</sup> ionomycin,<sup>18</sup> and other stimuli.<sup>38</sup> Neutrophils also produce NETs in response to important fungi, including *Aspergillus* species,<sup>23,39</sup> *Candida albicans*,<sup>11</sup> and *Cryptococcus gattii*<sup>40</sup> in an NADPH oxidase-derived ROS-dependent manner. However, Mejia et al<sup>41</sup> have recently indicated that although the NADPH oxidase inhibitor DPI did not alter the production of NETs against *Paracoccidioides brasiliensis* conidia, it partially suppressed it against yeasts. Taken together, these findings in neutrophils establish that whether the formation of NETs requires ROS production depends on the stimuli or microorganism assayed. In eosinophils, there is evidence that the release of EETs induced by PMA, calcium ionophore, thymic stromal lymphopoietin, C5a, eotaxin, and other factors depends on ROS generation.<sup>14,19,20</sup> Our data using pharmacological tools indicated that *A fumigatus* induces the release of EETs in human eosinophils in an NADPH oxidase- and mitochondrial-ROS-independent manner. Concerning the differences between eosinophils and neutrophils regarding ROS dependency in *A fumigatus*-induced release of ETs, it is possible to speculate that it may be related to the differences in how these 2 immune cells recognize and respond to the fungus. This work is the first report to indicate that human eosinophils release traps in response to *A fumigatus* fungus conidia; thus, additional studies are required to determine whether more than 1 mechanism of the formation of EETs exists in response to different stimuli or fungal morphotypes.

In 2004, Brinkmann et al<sup>10</sup> demonstrated that following stimulation with different stimuli neutrophils release NETs in a process that involves cell death. Subsequent studies have demonstrated that neutrophils may release NETs not requiring neutrophil lysis or breach of the plasma membrane.<sup>14-16,19,42</sup> Ueki et al<sup>20</sup> provided the first evidence that human eosinophils undergo cytolysis to liberate nuclear-derived EETs concurrently with the release of intact, cell-free, and secretion-competent granules. To date, at least for neutrophils and eosinophils, it is not certain that cell death is associated with nuclear or mitochondrial DNA release. The present studies indicate that the release of EETs induced by *A fumigatus* involves eosinophil death. We also identified colocalizations of DNA and citrullinated histone H3, which suggests that the EETs formed contain nuclear-derived genomic DNA. Our data are more in line with the findings of Ueki et al<sup>20</sup>; however, they differ in relation to the NADPH-ROS production dependency. These differential findings may be related to the different stimuli used. It has been reported that ETs may efficiently kill microbial pathogens; however, it is also established that several bacteria may resist the antimicrobial effect of neutrophil trap structures.<sup>43-47</sup> Regarding fungus, it has been described that NETs did not contribute to *A fumigatus*,<sup>24,48</sup> *Cr Gattii*,<sup>40</sup> and *P brasiliensis*<sup>41</sup> killing. However, regarding *Candida albicans*, NETs have been suggested as a mechanism to capture and kill *Candida* hyphae and yeast.<sup>11,49</sup> Using an experimental model of acute fungal asthma, Lilly et al<sup>9</sup> showed that eosinophils are important for the clearance of *A fumigatus in vivo*. Moreover, these authors indicated that bone marrow-derived eosinophils have a cell contact-independent killing activity against *A fumigatus* conidia *in vitro*.<sup>9</sup> Although it remains unclear whether there are differences between mouse and human eosinophils, consistent with most of these studies in neutrophils, we did not identify an

influence of the formation of EETs on the killing or germination of *A fumigatus* *in vitro*. *A fumigatus* resistance to the killing of EETs or fungistatic activities might be an important feature of ABPA pathogenesis, as we observed persistent *A fumigatus* colonization in bronchial mucus even in the presence of abundant ETosis-derived EETs. To investigate the molecular mechanisms involved in eosinophil interactions with the fungus, we searched for potential roles for dectin-1 and a  $\beta_2$ -integrin (CD11b), which recognize  $\beta$ -glucan, a molecular pattern commonly found in the cell wall of fungus. We also investigated the role of the Syk signaling pathway, which has been shown to mediate cell signaling via different classes of receptors involved in fungus recognition, including integrins<sup>30</sup> and C-type lectins (such as dectin-1).<sup>31</sup> We determined that 2 distinct pharmacological Syk signaling blockers and CD11b integrin-inhibiting antibodies prevented the release of EETs induced by *A fumigatus*. Consistent with our findings, adhesion molecules such as CD11b have been shown to play an important role in eosinophil interactions with the fungus *Alternaria alternata*<sup>8</sup> and thymic stromal lymphopoietin-induced release of EETs.<sup>19</sup> In neutrophils, it has been suggested that both the C-type lectin receptor dectin-1 and the  $\beta_2$ -integrin CD11b/CD18 (CR3) have been implicated in the recognition of  $\beta$ -glucans on the cell surface of *A fumigatus*. However, it remains unclear which receptors are important in the recognition and downstream signaling for NET release in response to *A fumigatus*.<sup>24,50,51</sup> Although the expression of the dectin-1 receptor in human eosinophils remains controversial,<sup>8,52</sup> we believe that human blood eosinophils do not require the dectin-1 receptor to induce the release of EETs in response to *A fumigatus*.

## Conclusions

In summary, we have identified the presence of *A fumigatus* associated with ETosis-derived EETs in the mucus samples of patients with ABPA. Consistent with these findings, we determined that human blood eosinophils release EETs in response to *A fumigatus* *in vitro* in a nonoxidative process dependent on the Syk tyrosine kinase pathway, which involves CD11b  $\beta$  integrin, histone citrullination and cell death. Moreover, EETs lack the killing or fungistatic activities against *A fumigatus*. However, what may be the function of EETs in ABPA? One hypothesis is that excessive release of EETs may contribute to the formation of sticky and viscous mucus, which may trigger breathing difficulty and airway obstruction. Free granules entrapped in EETs may cause long-lasting inflammation leading to epithelial cell damage, which is characteristic of ABPA. Moreover, EETs lack the killing activity against *A fumigatus*, which might explain the persistent fungal colonization observed in individuals with ABPA. Nevertheless, whether the release of EETs by eosinophils is linked with immune protection, host damage, or both factors remains unknown. The identification of the mechanisms that regulate the process of *A fumigatus*-induced release of EETs may be important to improve knowledge regarding ABPA pathogenesis and treatment.

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## Key messages

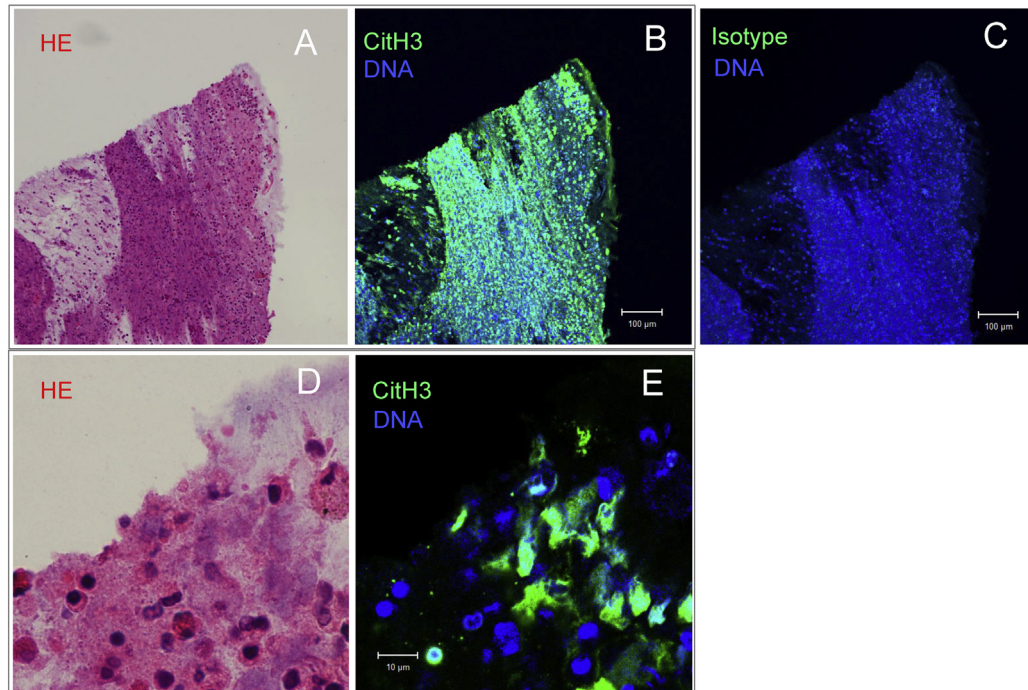
- *A fumigatus* is associated with lytic eosinophil-derived extracellular DNA traps in ABPA.
- *A fumigatus* induced the release of human eosinophil DNA traps *in vitro* in a nonoxidative process dependent on the Syk tyrosine kinase pathway, histone citrullination, CD11b  $\beta$  integrin, and cell death.

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## Case1



## Case 2

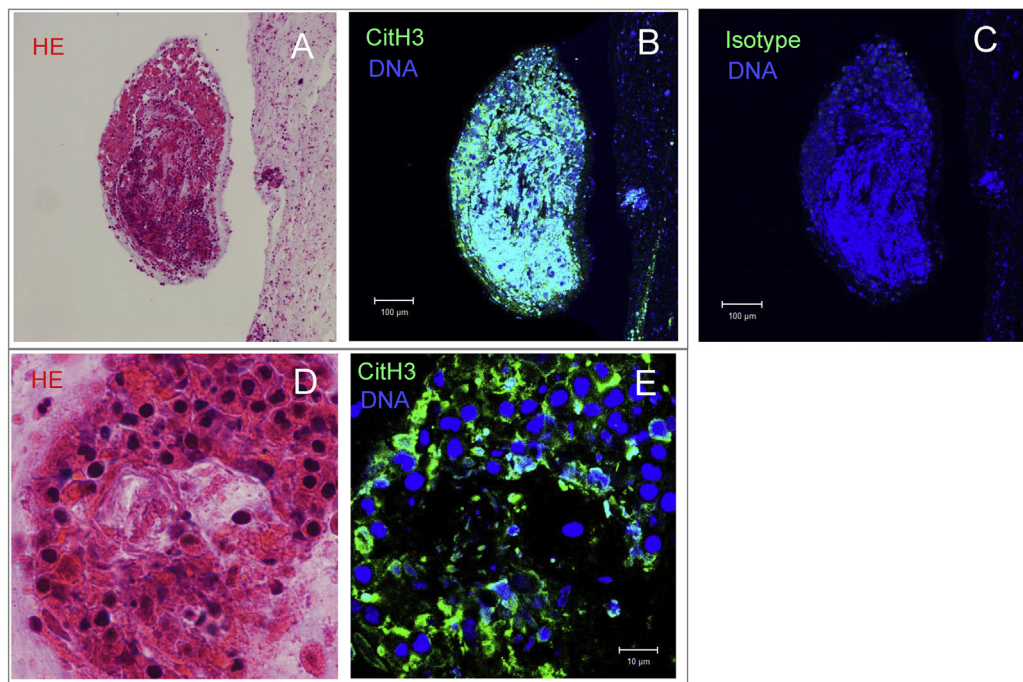
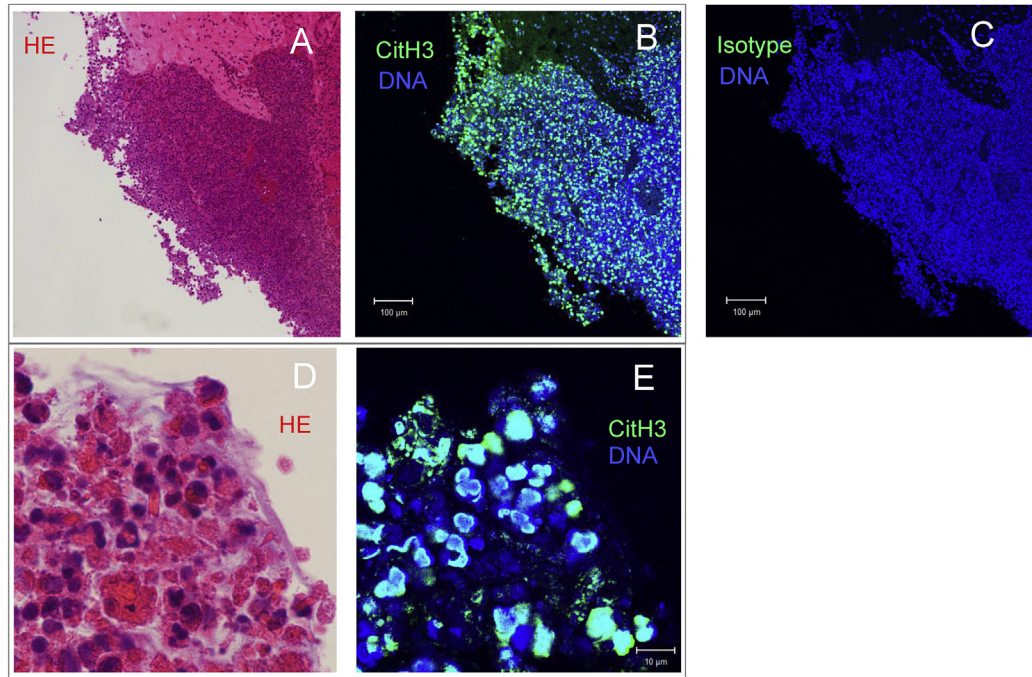


**FIG E1.** ETotic eosinophils and EETs were abundantly present in mucus plugs. Sections of mucus plug stained for citrullinated histone H3 (citH3) (green) and DNA (Hoechst 33342; blue) and analyzed using a laser scanning confocal microscope (**B** and **E**). After removal of coverslips, identical field with hematoxylin-eosin (HE) staining was analyzed using light microscopy (**A** and **D**). Chromatolytic nuclei of lytic eosinophils and EETs were stained with citH3. Scale bar: 100  $\mu$ m for upper panels, 10  $\mu$ m for lower panels. Serial sections with isotype-matched control antibody yielded no staining (**C**).

## Case 3



## Case 4

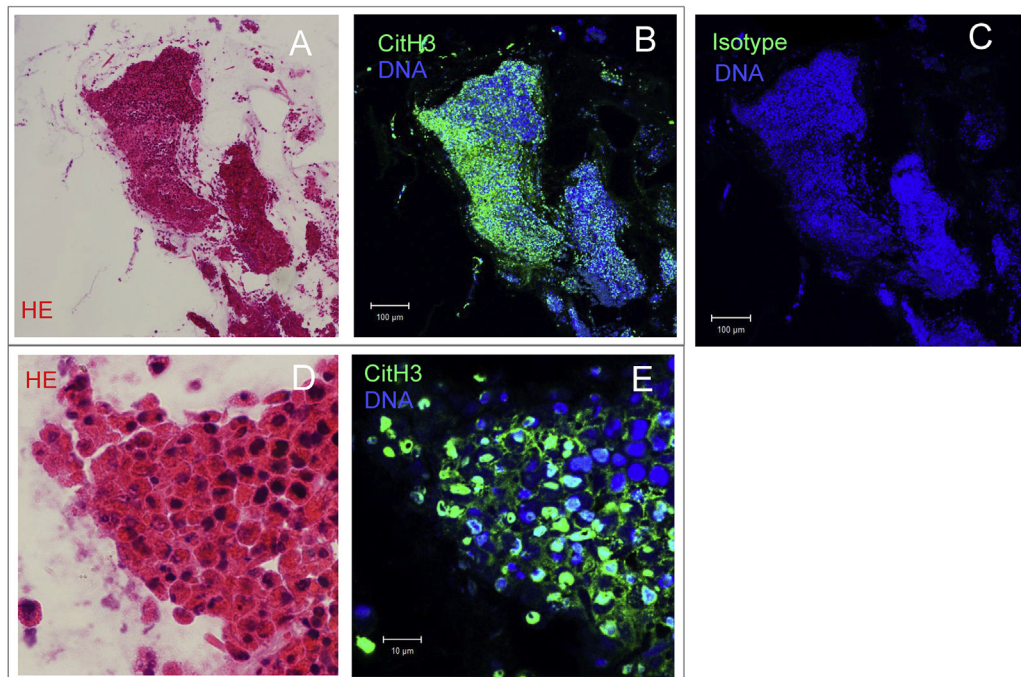
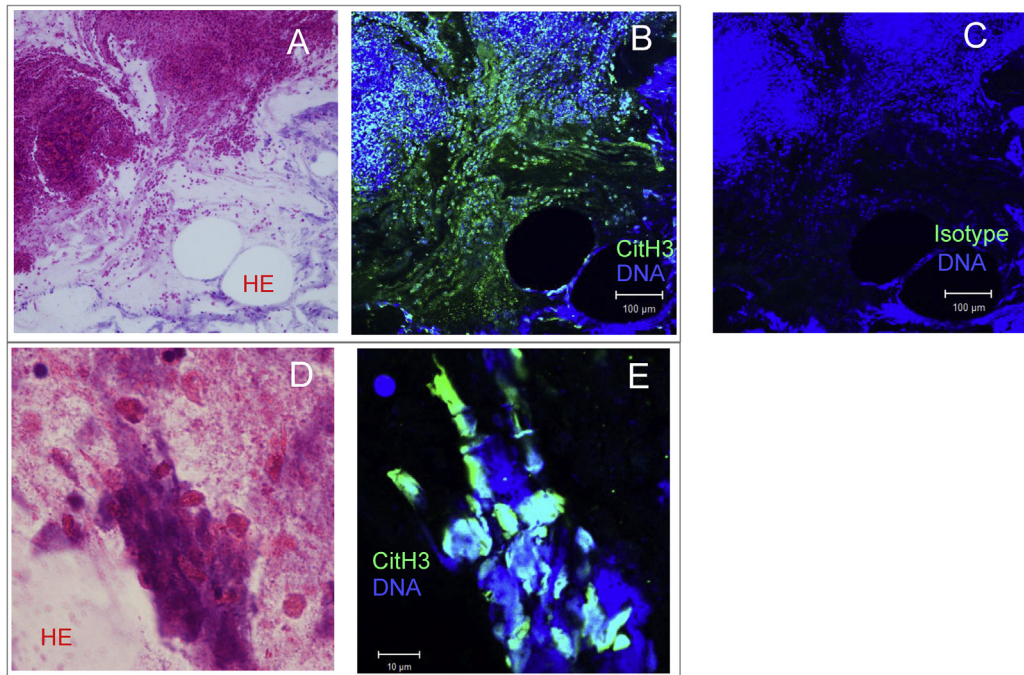


FIG E1. (Continued).

## Case 5



## Case 6

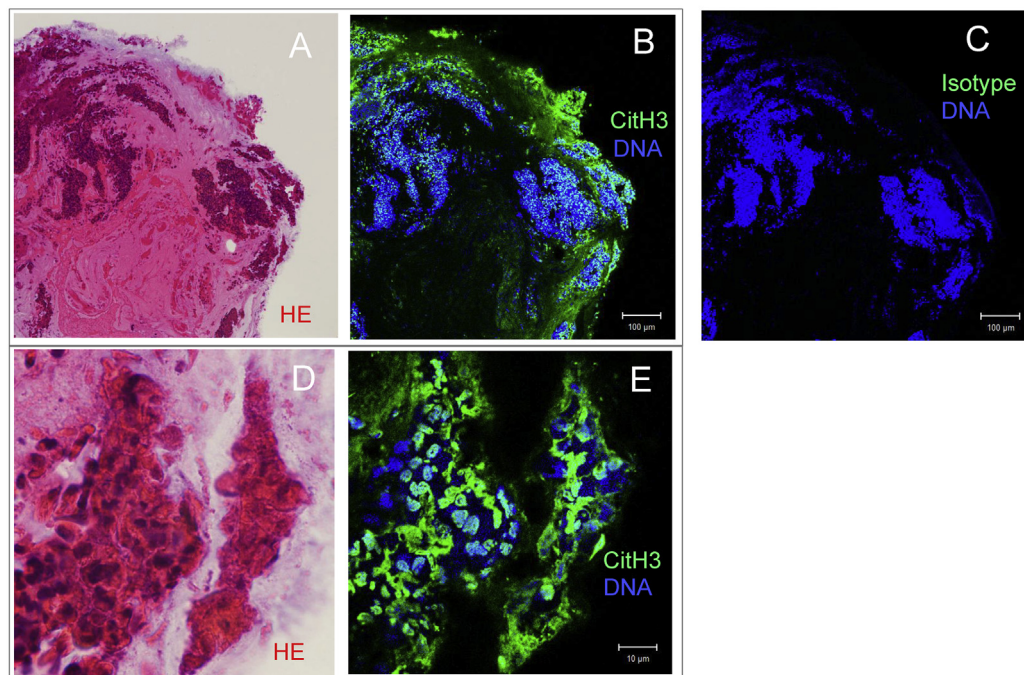


FIG E1. (Continued).

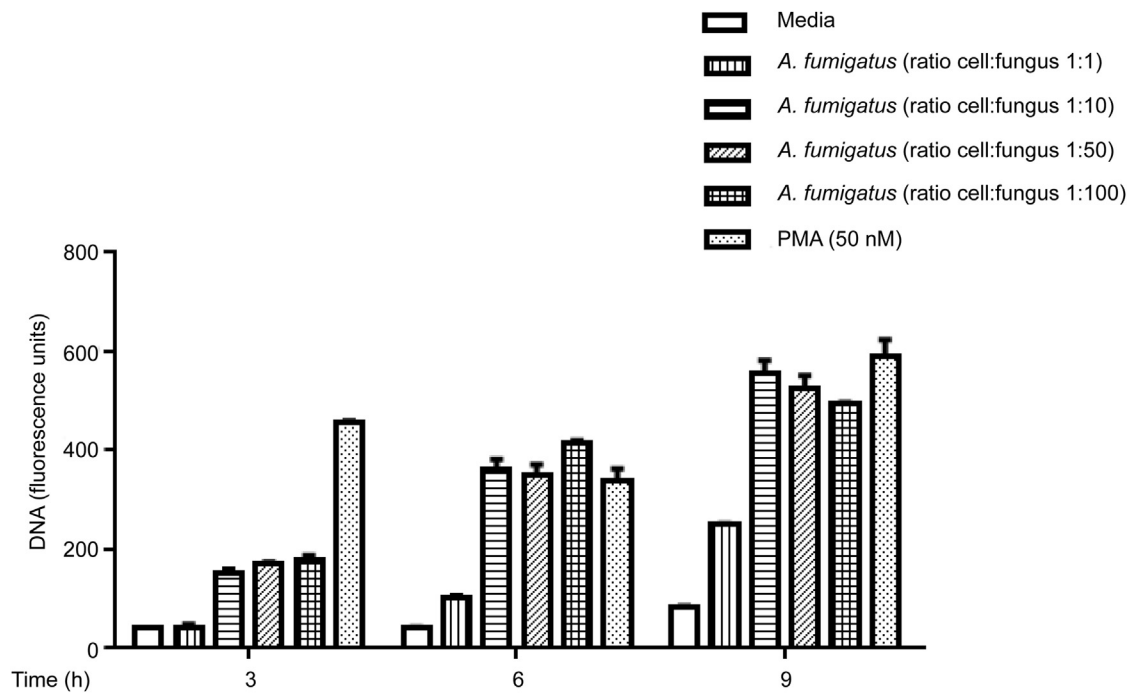


**FIG E2.** *A. fumigatus*-induced human eosinophil release of ETs. Eosinophils stimulated with PMA (50 nM) for 2 hours (positive control) or *A. fumigatus* conidia at different cell:fungus ratios and incubation times. EETs were quantified with Sytox Green (5  $\mu$ M) in a fluorescence microplate reader. The graph represents the mean  $\pm$  SEM (N = 3).

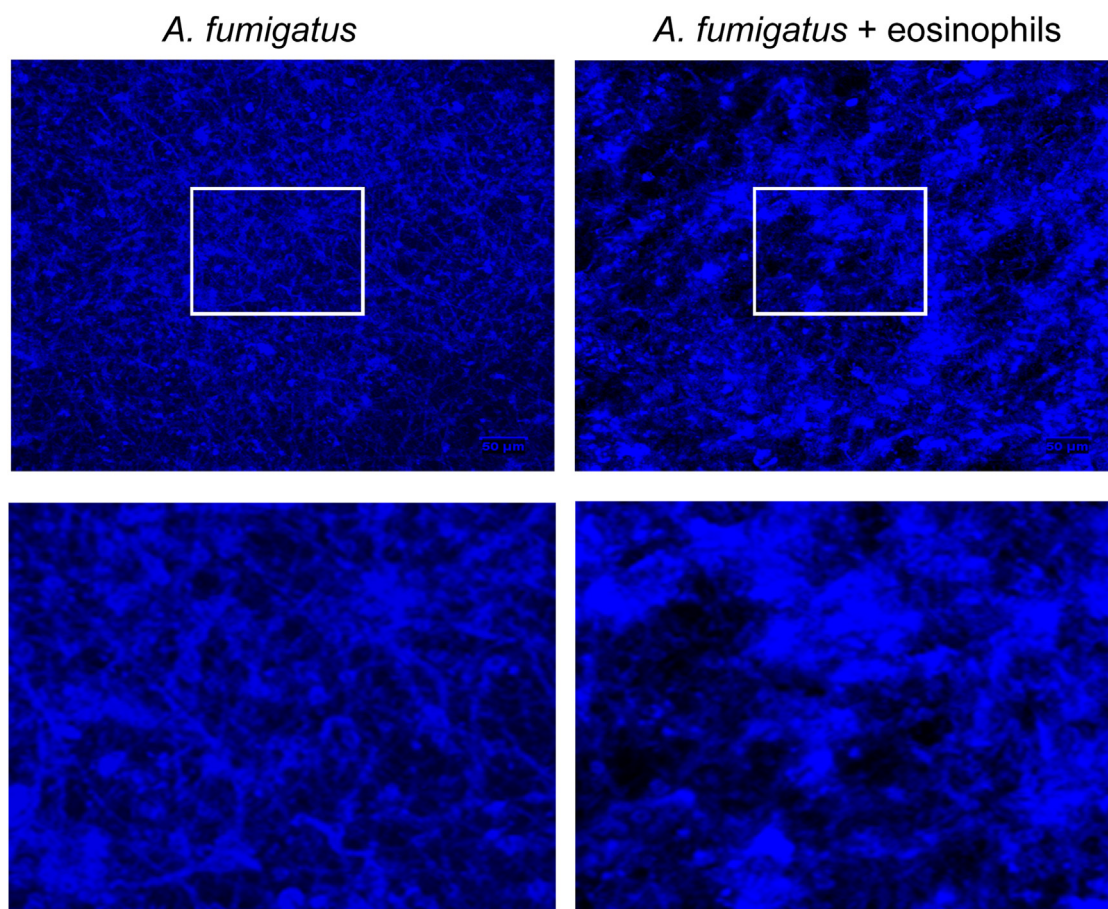


**FIG E3.** Eosinophils did not avoid fungus hyphae formation. Eosinophils were cocultured with *A fumigatus* conidia (fungus:cell ratio of 1:10) for 24 hours. Samples were then stained with calcofluor white (blue; a fungal wall marker). Lower images are boxed areas in higher magnification. Representative images from 3 independent experiments (N = 3).

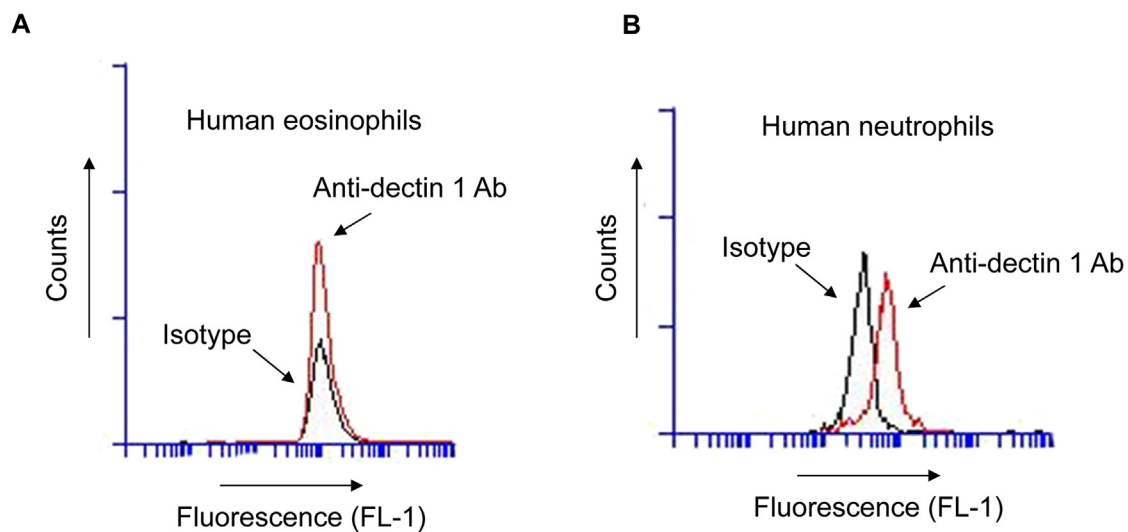


**FIG E4.** Human eosinophils do not express the dectin-1 receptor. Fresh human eosinophils (**A**) and human neutrophils (**B**) were labeled with the anti-human dectin-1 receptor antibody (Ab, 20  $\mu$ g/mL). Cells were analyzed via flow cytometry. Representative histograms from 3 independent experiments (N = 3).

**TABLE E1.** Clinical characteristics of 7 cases of patients with ABPA

| Case | Age (y) | Sex | Material            | Fungus culture     | Past or current asthma | Blood eosinophil count (/mL) | Serum IgE (IU/mL) | <i>Aspergillus</i> IgE | <i>Aspergillus</i> precipitin test | Skin test for <i>Aspergillus</i> (immediate/late reaction) |
|------|---------|-----|---------------------|--------------------|------------------------|------------------------------|-------------------|------------------------|------------------------------------|------------------------------------------------------------|
| 1    | 60      | F   | Sputum              | <i>A fumigatus</i> | +                      | 3,159                        | 12,000            | +                      | +                                  | (+/+)                                                      |
| 2    | 74      | F   | Bronchial secretion | <i>A fumigatus</i> | +                      | 2,190                        | 5,280             | +                      | +                                  | (+/+)                                                      |
| 3    | 40      | F   | Lung biopsy         | <i>A fumigatus</i> | +                      | 744                          | 1,860             | +                      | +                                  | (+/ND)                                                     |
| 4    | 60      | F   | Sputum              | <i>A fumigatus</i> | +                      | 468                          | 3,549             | +                      | +                                  | (+/+)                                                      |
| 5    | 63      | F   | Bronchial secretion | <i>A fumigatus</i> | +                      | 2,925                        | 3,431             | +                      | +                                  | (+/ND)                                                     |
| 6    | 40      | F   | Lung biopsy         | <i>A fumigatus</i> | +                      | 383                          | 1,454             | +                      | +                                  | (-/ND)                                                     |
| 7    | 74      | F   | Lung biopsy         | <i>A fumigatus</i> | —                      | 944                          | 302               | +                      | +                                  | (-/ND)                                                     |

F, Female; M, male; ND, not done.