BIOPHYSICAL IMAGING AND COMPUTATIONAL BIOLOGY

Absence of Fungal Spore Internalization by Bronchial Epithelium in Mouse Models Evidenced by a New Bioimaging Approach and Transmission Electronic Microscopy

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Clinical data and experimental studies suggest that bronchial epithelium could serve as a portal of entry for invasive fungal infections. We therefore analyzed the interactions between molds and the bronchial/bronchiolar epithelium at the early steps after inhalation. We developed invasive aspergillosis (*Aspergillus fumigatus*) and mucormycosis (*Lichtheimia corymbifera*) murine models that mimic the main clinical risk factors for these infections. Histopathology studies were completed with a specific computer-assisted morphometric method to quantify bronchial and alveolar spores and with transmission electron microscopy. Morphometric analysis revealed a higher number of bronchial/bronchiolar spores for *A. fumigatus* than *L. corymbifera*. The bronchial/bronchiolar spores decreased between 1 and 18 hours after inoculation for both fungi, except in corticosteroid-treated mice infected with *A. fumigatus*, suggesting an effect of cortisone on bronchial spore clearance. No increase in the number of spores of any species was observed over time at the basal pole of the epithelium, suggesting the lack of transepithelial crossing. Transmission electron microscopy did not show spore internalization by bronchial epithelial cells. Instead, spores were phagocytized by mononuclear cells on the apical pole of epithelial cells. Early epithelial internalization of fungal spores *in vivo* cannot explain the bronchial/bronchiolar epithelium invasion observed in some invasive mold infections. The bioimaging approach provides a useful means to accurately enumerate and localize the fungal spores in the pulmonary tissues. (*Am J Pathol* 2015, 185: 2421–2430; <http://dx.doi.org/10.1016/j.ajpath.2015.04.027>)

Invasive pulmonary mold infections dominated by invasive aspergillosis and mucormycosis are a major threat for immunocompromised patients with a 3-month overall mortality rate >45% and an increasing incidence.^{1–5} Among the different clinical presentations of invasive aspergillosis, the broncho-invasive form is characterized by bronchial and peribronchial hyphal invasion and is reported in 14% to 38% of patients receiving corticosteroids.^{6–8} Mucormycosis can also involve bronchi, either trachea and main bronchi especially in diabetic patients, or small bronchi/bronchioles as suggested by the presence of tree-in-bud opacities on chest tomodensitometry.^{9,10} Altogether these data suggest that

bronchial/bronchiolar epithelium could serve as a portal of entry for *Aspergillus* spp. and *Mucorales*.

Several not exclusive hypotheses for the bronchial invasion by fungi can be raised and were mostly assessed

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through *in vitro* models: i) internalization of fungal spores by host cells (human bronchial epithelial cell lines, and mammal primary tracheal or bronchial epithelial cells^{11–13}); ii) chemical disruption of the epithelial-cell barrier by production of fungal proteases or toxins leading to the cleavage of tight junctions^{13–15}; iii) interactions with professional phagocytic cells such as dendritic cells that could bypass the epithelial barrier¹⁶; and iv) damage of bronchial epithelial cells.^{13,17}

We therefore studied the early events after spore inhalation, knowing that most of the pathogenic fungi can be internalized by nonprofessional phagocytic cells.¹⁸ *Aspergillus fumigatus* was chosen as the leading cause of invasive aspergillosis and by far the most studied mold in the context of invasive fungal disease. We added for comparison *Lichtheimia corymbifera* (formerly *Absidia corymbifera*) which ranks second or third as a cause of mucormycosis.^{4,5} Both molds share common features such as a ubiquitous presence in the environment, a spore size <3 µm in diameter, the ability to grow at >37°C, and the main risk factors (neutropenia and corticosteroid) predisposing to infections.^{1,4} In contrast, however, diabetes mellitus is a more specific risk factor for mucormycosis.¹⁹ We thus developed mouse models of intratracheal inoculation with three immunosuppressive conditions that mimic the risk factors (neutropenia, diabetes mellitus, and corticosteroids). After histopathology analysis of the pulmonary lesions, we developed a computer-assisted method to quantify the spores in the pulmonary tissue and to refine the localization of the spores with transmission electron microscopy (TEM).

Materials and Methods

Reagents

All of the reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) unless otherwise specified.

Mold Strains

The *A. fumigatus* reference strain Af293 (CBS 101355; Centraalbureau voor Schimmels Cultures, Utrecht, the Netherlands) was selected.²⁰ In the absence of a consensual reference strain for *L. corymbifera*, a clinical strain (CNRMA 11.1047; Institut Pasteur, Paris, France) isolated from lung lesions of a neutropenic patient with proven invasive mucormycosis was used. Endospores of *L. corymbifera* and conidia of *A. fumigatus* (thereafter designated spores) were stored frozen in milk at –80°C and subcultured on potato dextrose agar. Spores were harvested by scraping 6-day subcultures on potato dextrose agar slants at 30°C. Spore suspensions in sterile phosphate-buffered saline (PBS) that contained 0.05% Tween 80 were filtered through a nylon filter (11-µm pore size) to remove hyphae and agglutinated spores and were enumerated with a hemacytometer.

Appropriate dilutions were then made in PBS. Spore suspension was kept at >4°C for <24 hours until use.

Animals

Outbred male OF1 mice (8 weeks; Charles River, L'Arbresle, France) were housed seven per cage in our animal facilities and given food and water *ad libitum*. This study was conducted in accordance with the European Union guidelines for animal care (Directive 2010/63/UE; September 22, 2010), and experimental protocols were approved by the Institut Pasteur Animal Experimentation Ethics Committee (CETEA 2013-0135).

Experimental Infection

Each immunosuppressive drug was administered via the intraperitoneal route to groups of three mice each. Corticosteroid-induced immunosuppression was achieved by administration of cortisone acetate (10 mg suspended in PBS that contained 1% Tween 20) 72 and 24 hours before inoculation.²¹ Administration of cyclophosphamide (100 mg/kg in PBS), 96 and 24 hours before inoculation,²² produced neutropenia as assessed by leukocyte counts with the use of an automated hematology analyzer (scil vet ABC, Altorf, France) on the day of inoculation. Type 1 diabetes was induced by the administration of streptozocin (200 mg/kg in 0.2 mL of ice-cold citrate buffer, pH 4.5).²³ Because OF1 mice reacted differently to streptozocin than C57BL/6 or BALB/c mice, diabetes developed in 2 days instead of 7 days (with death from diabetes occurring between 3 and 10 days after injection of streptozocin). Glycemia was assessed with a glucometer (Accu Check Aviva; Roche Diagnostics, Meylan, France). Only OF1 mice with a glucose concentration >16.6 mmol/L at day 2 after streptozocin injection (ie, 85% of the mice injected with streptozocin) were inoculated.

Intratracheal inoculation of 50 µL of PBS with 0.05% Tween 80 that contained 1×10^7 spores [spore viability (means ± SD), 98.6% ± 2.7%]²⁴ was performed with a blunt-end, 24-gauge needle after isoflurane (Iso-Vet; Piramal Healthcare, London, United Kingdom) anesthesia. The mice were sacrificed by carbon dioxide inhalation at 1, 6, and 18 hours after inoculation (hpi), and the lungs were harvested. Because it was impossible to do all of the experimentations at the same time, the same batch of mice was used for a given underlying condition, and all of the experiments were performed over a 3-month period.

Lung Histopathology

The lungs were fixed in 10% neutral-buffered formalin and embedded in paraffin. Four-micrometer sections were cut in the middle of the paraffin block and stained with hematoxylin and eosin to describe histologic lesions

A Tissue inflammation

B Spore phagocytosis

C Spore alteration

D Spore germination

■ untreated ■ corticosteroid ■ diabetes ■ neutropenia

Figure 1 Comparison of early pulmonary events observed after inoculation of *Aspergillus fumigatus* or *Lichtheimia corymbifera* to mice with various underlying conditions. Groups of OF1 mice were either untreated or immunosuppressed by cortisone acetate, streptozocin (diabetes), or cyclophosphamide (neutropenia) and were inoculated intratracheally with 1×10^7 *A. fumigatus* and *L. corymbifera*. Lungs were harvested at 1, 6, and 18 hours after inoculation and were processed for histopathologic examination. The same experimented operator (G.J.) blindly performed a grading of various variables by using a 5-grade scale from 0 to 4 (0, none; 1, minimal; 2, mild; 3, moderate; 4, marked). Each symbol represents a mouse. **A:** Inflammatory infiltrates (arrowheads) caused by *A. fumigatus* are characterized by inflammatory cells surrounding bronchi or alveoli. **B:** Clusters of *L. corymbifera* spores are surrounded by a cell membrane, suggesting indirect evidence of phagocytosis. **C:** Structural alteration of *L. corymbifera* spores (arrowheads). **D:** Ultimate stage of *A. fumigatus* germination is represented by fungal hyphae invading the lung parenchyma. $n = 3$ OF1 mice per group. Hematoxylin and eosin staining (**A**); Grocott methenamine silver staining (**B–D**). Br, bronchi.

(distribution of lesions, inflammation, necrosis) and Grocott methenamine silver for the detection of fungi. One slide that included the six lobes was analyzed for each mouse. The analysis was performed by a single member of the group (G.J.) blinded to the fungal species and the underlying condition. Qualitative histologic changes were recorded and semiquantitatively scored with a 5-grade scale as previously reported (0, none; 1, minimal; 2, mild; 3, moderate; 4, marked).^{25,26} The scoring took into account tissue damage (necrosis and inflammation characterized by infiltration of neutrophils, lymphocytes, and macrophages), fungal changes on the basis of structural characteristics (altered fungal wall, spore germination, presence of hyphae), and indirect evidence for phagocytosis (spore clustering surrounded by a cell membrane). Silver-stained slides were then digitized

(Axioscan Z1; Zeiss, Marly Le Roi, France) for computer-assisted morphometric analysis.

Computer-Assisted Morphometric Analysis Using Icy

Icy is an open community platform for bioimage informatics.²⁷ A specific plugin called mycosis lung quantifier was developed to quantify and analyze the lung distribution of fungal spores. Plugin and its source code are available (http://icy.bioimageanalysis.org/plugin/Mycosis_Lung_Quantifier, last accessed April 8, 2015). Because the differentiation between bronchi and bronchioles were difficult on silver-stained slides, we delineated manually all of the sections surrounded by a cubic epithelium, thereafter designated bronchus. The plugin allocated each spore to the closest bronchus section (Supplemental Figure S1). Red lines tracked the spores inside

Figure 2 *Aspergillus fumigatus* spore count is twofold higher in the alveoli around the bronchi than *Lichtheimia corymbifera* at 1 hour after inoculation of OF1 mice. Four groups of OF1 mice were intratracheally inoculated with 1×10^7 *A. fumigatus* or *L. corymbifera*, and euthanized 1 hour after inoculation for lung histopathologic analysis. One lung section per mouse was analyzed. The mean number of spores per 10^5 bronchial surface units in pixels (mean spores/BSU) was counted by using the mycosis lung quantifier Icy plugin in the alveoli (ie, at a distance >16 and <320 pixels from the apical pole of the bronchi). Each bar represents the fold increase of the mean alveolar spores/bronchial surface units of *A. fumigatus* compared with that of *L. corymbifera*. No difference was found between the underlying conditions ($P = 0.39$, analysis of variance test). $n = 3$ OF1 mice per group.

the bronchus lumen by measuring a negative distance from the apical pole of the bronchial epithelium delineation. Yellow lines measured a positive distance between spores and the apical pole of the bronchial epithelium. Distances are expressed in pixels (px) and are not converted to micrometers for accuracy.

Image Processing

The same person (B.R.) blindly processed all of the slides. The first step was to digitize the lung slides (Axioscan Z1; Zeiss) by using $\times 20$ magnification. The image size was reduced to 40% of the original size by using Zen lite 2012 software version 2.1 64-bit (Zeiss), and the image file was converted into the tiff format. The maximum image size that can be processed by Icy depends on the computer configuration. The minimal required configuration for the computer by using Icy plugin was 16 Mb random access memory and 256 Go of memory to allow processing of images <1 Go. After loading the image on Icy, a preliminary step consisted of bronchial lumen surface delineation and artifact exclusion. With the use of the region of interest area tool on Icy, delineation of each bronchus surface was performed manually on the whole surface of the lung section. Bronchus section was considered as such when cubic epithelium was present, even if the section was not perfectly round. Artifacts such as silver staining of the vascular connective tissue were excluded with the black paintbrush.

Plugin Variables Choice

The chosen variables are described below.

Threshold

The sensibility of spore detection depends on the silver staining of each lung section. In fact, if the staining is highly contrasted, the detection threshold has to be lowered to detect only spores. A perfect methenamine silver staining is needed to avoid black artifacts that hamper Icy counting. For each slide, different thresholds were tested, and the optimal threshold was visually determined, ranging from 20 to 110, according to the silver uptake of each slide.

Size

Minimal and maximal sizes were chosen to optimize the detection of each spore at different germination stage. Minimal and maximal sizes were 0 to 20 px and 1000 px, respectively.

Epithelium Width

To enumerate the spores inside the bronchial epithelium, we had to determine the epithelium width. The size of the bronchial epithelium was measured on 10 randomly chosen bronchi from 10 different images, using the ruler helper plugin in Icy (http://icy.bioimageanalysis.org/plugin/Ruler_Helper, last accessed March 25, 2014). The mean epithelium size was 32 px (range, 16 to 49 px). The smallest epithelium width, 16 px, was chosen to avoid unduly counting some spores as intraepithelial.

Maximal Distance

Our purpose was not to quantify all of the spores present in the lungs but to localize spores inside and around bronchi. Furthermore, Grocott methenamine silver stained in black the edges of the lungs, leading to artifacts. The maximal distance from the bronchial lumen was then reduced. We chose 320 px from the apical pole of the attributed bronchus, 10-fold the mean epithelium width, to give a large overview of the distribution of the fungi around bronchi. The maximal number of spores was detected before the limit of 320 px on 12 randomly chosen slides (Supplemental Figure S2A).

Bronchial Surface and Perimeter

Anatomical or experimental variations during lung processing can occur. The number of bronchus sections by slide varies according to the position of the lungs when paraffin-embedded. The mean number of bronchus sections was 66 (range, 22 to 123) in all of the lung slides, and the bronchus surface varied according to their level in the respiratory tract but also according to the cutting section. To standardize bronchi, we decided to exclude smallest and largest bronchi, based on both perimeter and surface measure. The smallest bronchi with a perimeter <300 px and/or a surface <5000 px can widely be open on the alveoli, leading to false-positive detection of bronchial spores, and the bronchial epithelium

Figure 3 Distribution of *Aspergillus fumigatus* and *Lichtheimia corymbifera* spores inside bronchi over time. Four groups of OF1 mice were intratracheally inoculated with 1×10^7 *A. fumigatus* or *L. corymbifera* and were euthanized at 1, 6, or 18 hours after inoculation for lung histopathologic analysis. One lung section per mouse was analyzed. A morphometric analysis was performed with mycosis lung quantifier Icy plugin. Bars represent the mean number of bronchial spores per 10^5 bronchial surface units in pixels. $n = 3$ OF1 mice per group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (Mann-Whitney test) versus bronchial spores from the other fungus compared at each time point. px, pixel.

was too thin to allow reliable counting. The largest bronchi with a perimeter $>10,000$ px and a surface $>200,000$ px were excluded because of an excessive silver uptake by mucus-producing cells, leading to black artifacts. Out-of-range bronchi were colored in red, and spores attributed to these bronchi were also excluded.

The data generated by the plugin were automatically exported as Excel files (Excel version 14.5.2; Microsoft, Redman, WA), reporting for each bronchus analyzed the number of spores, the spore distance in pixels from the bronchi, and chosen variables and the reasons of bronchi exclusion.

Plugin Variables Validation

Each plugin variable was validated by using randomized blinded lung sections (B.R.). We first determined whether Icy counting was efficient to identify spores inside bronchi, either in the bronchial lumen or in the bronchial epithelium. We validated the method that enumerated visually all spores present in the lumen or in the epithelium of 60 bronchi (10 bronchi/mouse). Icy count was not significantly different from the visual count (Supplemental Figure S2B). We then checked if the contrast of the lung section could influence the detection of spores by Icy. The detection threshold was visually subjectively chosen. We compared three different thresholds (from 30, highly contrasted slides, to 90, low-contrasted slides) from six different slides (two per threshold) by using visual and Icy counting. The detection threshold did not significantly influence Icy counting (Supplemental Figure S2C). With the use of these same sections, we raised the question of the influence of threshold variations on spore count (Supplemental Figure S2D). Slight variations of the threshold (± 20) did not significantly change the spore count.

Two categories of spores were defined according to their location from the apical pole of the bronchial epithelium as follows: The bronchial spores, including the luminal spores

(harboring a negative distance value and therefore clearly not internalized by the respiratory epithelium), and the epithelial spores located between 0 and 16 px, corresponding to either spores at the apical pole of the epithelium or spores that could be internalized by the epithelium; and the alveolar spores, located between >16 px and <320 px from the apical epithelium pole of the attributed bronchus, knowing that some spores were in the interstitial spaces.

TEM

For ultrastructural studies, corticosteroid treatment and a higher inoculum size (5×10^7 spores) were used. The right lung was cut into 2-mm pieces, fixed for 48 hours at 4°C in 0.2 mol/L sodium cacodylate buffer, pH 7.4, that contained 2.5% glutaraldehyde and 0.5% paraformaldehyde. Specimens were then washed three times in sodium cacodylate buffer (0.2 mol/L, pH 7.4), after being fixed 1 hour with 1% osmium tetroxide in 0.2 mol/L sodium cacodylate buffer (pH 7.4) and washed three times (10 minutes) in sodium cacodylate buffer (0.2 mol/L, pH 7.4). Dehydration (graded ethanol and acetone) and resin infiltration (acetone and epon resin mixture) were performed with automated microwaves (Leica). Specimens were embedded in epoxy resin for 2 hours in automated microwaves. Thin sections of 70 nm were cut with a UC6 ultramicrotome (Leica, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. Specimen sections were observed at 80 kV with a Hitachi H-7650 TEM (Hitachi, Yokohama, Japan). A total of four grids with 200 to 500 spores/grid were observed per mold.

Statistical Analysis

The objective of the computer-assisted morphometry was to analyze spore distribution at the bronchial level. We used

Figure 4 Distribution of spores in the lungs relative to the bronchial epithelium in mice infected by *Aspergillus fumigatus* or *Lichtheimia corymbifera*. Groups of untreated and corticosteroid-treated mice were euthanized, and lungs were collected at 1, 6, and 18 hpi of 10^7 spores of *A. fumigatus* or *L. corymbifera*. Spores located at a maximal distance of 320 px from the apical pole of the bronchial epithelium were enumerated on silver-stained sections by using the mycosis lung quantifier Icy plugin. The mean of spores per 10^5 BSU (in px) located between -64 px and 64 px is represented as a curve. The gray area represents the epithelium width. The mean spores/BSU curve at 1 hpi is superimposed or above the other curves beyond the limit of 16 px, suggesting that spores do not accumulate over time at the basal pole of the epithelium. $n = 3$ mice per group. BSU, bronchial surface unit; hpi, hours after inoculation; px, pixel.

the total bronchial surface of each lung section to perform our analysis. Indeed, each mouse had 65.4 ± 16.2 bronchial sections. Because their shape varied according to the orientation of the lungs when paraffin-embedded, we pooled the bronchial surfaces provided by Icy for each group of three mice after checking that spores counts were similar among mice of each group. We then expressed the Icy counts as a mean number of spores per 10^5 bronchial surface units (BSUs) in pixels (mean spores/BSU) $\pm$ SEM. To compare bronchial surfaces of each group, we used the one-way analysis of variance parametric test after checking the equality of variances. Bronchial distributions of spores according to molds and conditions on Icy analysis were compared with nonparametric tests (Kruskal-Wallis or Mann-Whitney test). For multiple comparisons, Bonferroni correction was applied. For the analysis of the epithelium barrier crossing, the mean spores/BSU located between -64 px and 64 px was analyzed for each condition tested. Curves were smoothed by using eight neighbors' points. All of the statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad Inc., San Diego, CA).

Results

Underlying Conditions Differently Affect Tissue and Fungal Damages in Mice Inoculated with *A. fumigatus* Compared with *L. corymbifera*

In preliminary experiments, we assessed the reproducibility of the results at 6 hpi by repeating the experiments in groups of three mice. Subsequent experiments were thus performed once for ethical reasons. For a given condition, time, and mold, the three mice tested per group displayed tissue damages and fungal changes with <1 grade in difference on the 5-grade scale used. We therefore considered as relevant any difference of >2 grades when comparing the different times and

conditions. Relevant variations were recorded over time per fungi for a given underlying condition, especially comparing 1 hpi with 18 hpi. Few events occurred before 6 hpi. Because the viability of inoculums was close to 100%, the differences observed between fungi and conditions cannot be due to variations of the inoculum.

Albeit moderate, inflammation caused by *A. fumigatus* was higher than that caused by *L. corymbifera* (Figure 1A). When present, inflammatory cells mainly gathered at the bronchus periphery for *A. fumigatus*, whereas for *L. corymbifera* inflammatory cells were sparsely distributed all over the lung parenchyma. Of note, necrosis was present in neutropenic mice instead of inflammation for both molds. Indirect evidence for phagocytosis was significantly more often observed for *L. corymbifera* than for *A. fumigatus* whatever the underlying condition (Figure 1B). Alteration of fungal structures was seen, starting at 6 hpi in untreated and corticosteroid-treated mice and to a lesser extent at 18 hpi in the other underlying conditions (Figure 1C). Fungal alteration was similar for both molds, except in neutropenic mice whereby *L. corymbifera* was more altered at 18 hpi than *A. fumigatus*. Germination of *L. corymbifera* spores was only observed in diabetic and neutropenic mice, whereas germination of *A. fumigatus* spores was observed in all underlying conditions (Figure 1D).

Thus, tissue damages and spore germination increased over time for both fungi. *A. fumigatus* gave more inflammation, germinated more, and was less phagocytized than *L. corymbifera*. In untreated mice, both molds generated tissue damages, indirect evidence of phagocytosis, and higher fungal alteration score. Diabetes provided a similar profile of tissue damage and spore germination with both molds. The corticosteroid model led to more differences between the two fungi than the other underlying conditions. Neutropenia was the condition associated with the best germination and was hardly associated with fungal alteration for both molds.

Figure 5 Interaction between *Aspergillus fumigatus* or *Lichtheimia corymbifera* spores and bronchial cells in TEM at 6 and 18 hours after inoculation of corticosteroids-treated OF1 mice. OF1 mice were immunosuppressed by cortisone acetate. Lungs were harvested at 6 and 18 hours after intratracheal inoculation of 5×10^7 spores/mouse of *A. fumigatus* or *L. corymbifera*. Lungs were then processed for TEM. *A. fumigatus* spores (**A**, arrowheads) and *L. corymbifera* spores (**B**) were not internalized by bronchial epithelial cells but were localized at the junction between serous, ciliated, and/or goblet cells. **C**: Two macrophages (M1 and M2) in an alveolar space. One spore that has started to germinate is seen phagocytized by the M1 macrophage. C, ciliated cell; G, goblet cell; S, serous cell; TEM, transmission electronic microscopy.

Quantification of Bronchial Spores Using a Computer-Assisted Morphometric Analysis

We first assessed that the total bronchial surfaces per group of three mice were not different between the four underlying conditions for *A. fumigatus* and *L. corymbifera* ($P = 0.09$ and $P = 0.12$, respectively). We then looked at the bronchial, epithelial, and alveolar distribution of spores within the limit of 320 px around the bronchi. The proportion of alveolar spores varied from 80% to 95% according to the underlying condition for both fungi (Supplemental Figure S3). Despite the same inoculum, at 1 hpi the mean alveolar spores/BSU of *A. fumigatus* was twofold that of *L. corymbifera* whatever the underlying condition (Figure 2). The mean bronchial spores/BSU of *A. fumigatus* was also higher than that of *L. corymbifera* (Figure 3). The mean bronchial spores/BSU decreased over time for *L. corymbifera* for all of the underlying conditions (Figure 3). Although the general trend for *A. fumigatus* bronchial spores was also a decrease when comparing 1 hpi with 18 hpi for the different underlying conditions (except in corticosteroid-treated mice), an increase was always seen between 6 and 18 hpi. The mean epithelial spores/BSU decreased between 1 and 18 hpi with a 1 hpi:18 hpi ratio of 3.1 in corticosteroid-treated ($P < 0.0001$), 1.6 in diabetic ($P = 0.002$), and 2.9 in neutropenic ($P < 0.0001$) mice for *A. fumigatus*, and 2.9 in control ($P < 0.0001$), 1.6 in corticosteroid-treated ($P = 0.0008$), 2.7 in diabetic ($P = 0.0008$), and 11.2 in neutropenic ($P < 0.0001$) mice for *L. corymbifera*.

Because the mean epithelial spores/BSU decreased over time for both fungi, one hypothesis of spore elimination from the bronchi could be the crossing of the epithelium to reach the alveoli/interstitium. Because the epithelium width was considered to be 16 px, any crossing of the epithelium should be evidenced by an accumulation of spores over time beyond the limit of 16 px. We did not find such accumulation of spores irrespective of the fungus and the underlying condition considered (Figure 4).

Fungi Are Not Internalized by Bronchial Epithelial Cells

We performed a TEM analysis to find the precise localization of epithelial spores. We chose the corticosteroid condition because corticosteroid-treated mice showed the highest number of spores associated with the bronchial epithelium. We observed the presence of unbound spores in the lumen, mainly at 6 hpi; the presence of spores in close contact with the bronchial epithelial cells; the presence of numerous spores inside mononuclear phagocytic cells; and the absence of internalization by epithelial cells at 6 and 18 hpi. When the spores were in contact with epithelial cells, they were mainly located at the junction between ciliated and/or goblet cells (Figure 5, A and B). Inside mononuclear phagocytic cells, spores of *A. fumigatus* were seen with germ tubes inside the cytoplasm (Figure 5C), whereas no germination of *L. corymbifera* spores was observed, although they were swollen from 2 to 4 μm . Of note, no spore was observed inside type 2 pneumocytes whatever the time.

Discussion

Few *in vivo* studies have simultaneously compared *A. fumigatus* with *Mucorales* either for pathophysiologic^{28,29} or pharmacologic purposes.³⁰ Because our main interest was the early interactions between spores and bronchial/bronchiolar epithelium to explain the broncho-invasive forms of invasive mold infections, we decided to study these interactions in animal models with the use of classic histopathology and ultrastructural microscopy at selected time points that compared both fungi. To avoid a major limitation in classic histopathology analysis, which is the lack of objective quantification of the observations, we used Icy, an open community platform for bioimage informatics, and designed a specific plugin to quantify and localize fungal spores relative to bronchial structures in the lung of infected mice. This allowed us to evaluate reproducibility and to compare multiple variables, including four underlying conditions, two molds, and three times after inoculation.

We observed more *A. fumigatus* spores around the alveoli and inside the bronchi than *L. corymbifera* spores.

As a corollary, *L. corymbifera* spores were more sparsely distributed all over the lung parenchyma than *A. fumigatus* spores. Although these spores have the same size, we can hypothesize that *A. fumigatus* and *L. corymbifera* probably have different surface adhesins that confer variable binding properties to bronchial epithelial cells. Few data exist on *Lichtheimia* spp. cell wall composition,³¹ whereas another member of the *Mucorales*, *Rhizopus arrhizus* (*oryzae*), is studied more. Chitin and other polysaccharides such as glucans and mannans are major components of the fungal cell wall; their composition varies according to the stage of growth.³² Chitin and glucan synthase genes are also highly different between *R. arrhizus* and *A. fumigatus*.³³ To date, binding factors such as galactosaminogalactan have only been identified in *A. fumigatus*,³⁴ whereas both *R. arrhizus* and *A. fumigatus* have the capacity to bind to laminin and type IV collagen.^{35,36} A comparative analysis of *A. fumigatus* and *L. corymbifera* cell wall could help our understanding of bronchial epithelial cell adhesion. By analogy with the receptor to *Mucorales* found on the endothelial cells in diabetic conditions,³⁷ host factors that have to be determined could also influence the persistence of *A. fumigatus* rather than *L. corymbifera* in the bronchi.

Thanks to the mycosis lung quantifier plugin we also observed the increase of *A. fumigatus* spores in the bronchi at 18 hpi in all of the underlying conditions, whereas *L. corymbifera* spore number decreased over time. Because *A. fumigatus* spores were mainly distributed around the bronchi, we can hypothesize that their elimination through the bronchial tract was enhanced, possibly through mechanical factors such as cough and the mucociliary escalator. Distributed deeper in the lungs, *L. corymbifera* may have been altered on site and eliminated to a lesser extent via the bronchi. Different mechanisms may explain the absence of bronchial spore decrease at 6 hpi in the corticosteroid-treated mice infected with *A. fumigatus*. Mucus composition of bronchial epithelium could have been altered by corticosteroids by impairing the elimination of *A. fumigatus* spores,³⁸ or, as spores were less phagocytized, they may present an earlier stage of germination (ie, earlier changes in their cell wall composition) to unmask different pathogen-associated molecular patterns,³² leading to epithelial cell adhesion.³⁹ Our models allowed us to observe with TEM at least 200 spores in the lung parenchyma. Despite these high numbers, we did not notice *in vivo* internalization of spores by respiratory epithelial cells in the first 18 hours after intratracheal challenge, neither for *A. fumigatus* nor for *L. corymbifera*. This does not definitively rule out this potential initial event in the mold infection because TEM is notoriously poorly sensitive. However, given the high number of spores observed inside the bronchi, we can conclude that if early internalization occurs, this is a rare phenomenon. We cannot exclude either that our experimental conditions, in particular the high spore concentrations used to optimize analysis of structural characteristics,⁴⁰ may have triggered reactions that prevented spore internalization. This lack of internalization contrasts with the easy/frequent internalization of fungal spores by cell lines *in vitro*, either primary epithelial,^{11,41} bronchial, or

alveolar epithelial cell lines.⁴² The discrepancy between *in vitro* and *in vivo* observations may be due to the complex microenvironment present *in vivo* but not *in vitro*. Thus, long pentraxin 3 or mannan-binding lectins may help prevent internalization by epithelial cells while enhancing macrophage phagocytosis.^{43,44} Because addition of Tween 80 is necessary to prevent aggregation of fungal spores during both *in vitro* and *in vivo* experiments even for aerosolized spores,⁴⁵ it cannot explain the absence of *in vivo* internalization but can change the interactions between the spores and the host cells. However, it did not prevent observing differences between both fungi.

Our results are consistent with previous studies about the role of professional phagocytic cells in antifungal defense.^{28,46} Spores were phagocytized in the bronchi by mononuclear phagocytic cells that are difficult to accurately identify with TEM.⁴⁷ Alveolar macrophages are recognized as the main phagocytic cells involved in spore engulfment.^{28,46} They may colonize bronchi, too, but their number in the normal bronchi is difficult to evaluate.⁴⁸ Alveolar macrophages are long-lived cells relatively not affected by radiotherapy or chemotherapy,⁴⁹ but their phagocytic functions can be altered by corticosteroid use and diabetes.^{50,51} Here, we showed that the two underlying conditions in which *L. corymbifera* spores can germinate were diabetes and neutropenia. We already know that alveolar macrophages from diabetic mice are unable to inhibit mucorale germination *in vitro*.²⁸ Because *L. corymbifera* spore phagocytosis in our diabetic mice was comparable with other underlying conditions, we can hypothesize that a killing defect and not altered phagocytosis could explain the development of mucormycosis in diabetic patients.

Conclusion

The systematic comparison of various timings and underlying conditions confirms differences between the two molds in terms of kinetics and extent of tissue lesions induced, phagocytosis, and germination even at the early stages of lung infection. Bioimage informatics provides objective data on the bronchi interaction with fungal spores. *A. fumigatus* seems to better adapt to its host than *L. corymbifera*, partly explaining the higher frequency of aspergillosis in the hematologic setting. Further studies that compare both molds are needed to explore fungal components involved in bronchial adhesion differences between *A. fumigatus* and *L. corymbifera*.

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Supplemental Data

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