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Comparison of the Interleukin-1β-Inducing Potency of Allergenic Spores from Higher Fungi (Basidiomycetes) in a Cryopreserved Human Whole Blood System

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Key Words
Basidiospores · Human whole blood · Interleukin-1β · Proinflammatory potency

Abstract
Background: Spores from basidiomycete fungi (basidiospores) are highly prevalent in the atmosphere of urban and rural settings. Studies have confirmed their potential to affect human health as allergens. Less is known about their potential to serve as stimuli of the innate immune system and induce proinflammatory reactions. ***Methods:*** In this study, we evaluated the proinflammatory potential of spores from 11 allergenic basidiomycete species (gilled: *Pleurotus ostreatus, Oudemansiella radicata, Armillaria tabescens, Coprinus micaceus, Pluteus cervinus,* and *Chlorophyllum molybdites,* and nongilled: *Pisolithus arhizus, Merulius tremellosus, Calvatia cyathiformis, Lycoperdon pyriforme, and Boletus bicolor)* based on their potency to induce the release of the proinflammatory cytokine interleukin (IL)-1β in a cryopreserved human whole blood system. In addition, the roles of morphological features of the spores (surface area, shape, and pigmentation) were examined for their role in the IL-1β-including potency of spores. Peripheral blood from healthy volunteers was collected, pooled, and cryopreserved. After stimulating the cryopreserved pooled blood with 10^6 to 10^3 basidiospores/ml, the concentration of IL-1β in culture supernatants was determined with ELISA. ***Results:*** Basidiospores manifested concentration-dependent IL-1β-inducing potency, which was more marked among basidiospores from gilled basidiomycetes. At higher concentrations of basidiospores, the IL-1β-inducing potency could be differentiated in the cryopreserved human whole blood system. Morphological features did not correlate with the IL-1β-inducing potency of the basidiospores, suggesting that nonmorphological properties modulate the IL-1β-inducing potency. ***Conclusion:*** Our data provide evidence of the proinflammatory potential of basidiospores, and the utility of cryopreserved human whole blood as a human-based in vitro system to study the immune reactivity of allergenic basidiospores.
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Introduction

Fungal spores are an important biological component in the atmosphere of urban and rural settings worldwide [1–3]. The ecology of airborne fungal spores in the atmosphere of urban and rural settings is not limited to those

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of mitosporic micromycetes (e.g. *Cladosporium* spp., *Alternaria* spp., *Aspergillus* spp., and *Penicillium* spp.), but also include those from the basidiomycota group (e.g. *Coprinus* spp. *Pleurotus* spp., *and Chlorophyllum* spp.) [1, 4–6]. In addition to contributing to the biological diversity, spores of basidiomycetes (basidiospores) are also sources of the organic component of airborne particulate matter that interacts with the human respiratory system [2, 7, 8]. More importantly, studies have documented the allergenic potential of basidiospores and their possible link with incidences of chronic proinflammatory respiratory diseases, such as asthma and allergic rhinitis [9–12]. Therefore, more information about the health effects of basidiospores following interaction with the human immune system is warranted.

The aerodynamic size of fungal spores varies between species, but many of them, including those of basidiomycetes, are small enough to penetrate deep into the respiratory tract and interact with cells of the immune system [13–16]. This interaction often leads to the activation of innate immune cells and subsequent release of proinflammatory mediators, such as the cytokine interleukin (IL)-1β. Nevertheless, most studies that have evaluated the innate immune activation potential of fungi have focused on fungal pathogens (e.g. *Candida* spp., *Aspergillus* spp., and *Cryptococcus* spp.). Less is known about basidiospores. Given the potential of basidiospores to interact with cells of the immune system, the potential of basidiospores to activate the innate immune system should be evaluated.

Human whole blood provides a feasible system to evaluate immune function because cells of the immune system react in their natural environment (e.g. cell-to-cell and cell-to-serum component interactions), which is important for proper immune reactivity. This advantage of human whole blood was exploited to detect pyrogenic (proinflammatory) contamination of parenterals, and to evaluate proinflammatory potency of non-lipopolysaccharide (LPS) microbial compounds and the concentration-dependent proinflammatory potential of airborne particulate matter samples [17–22]. This assay has been internationally validated for pyrogenic testing of pharmaceuticals and medical devices [23, 24]. The modification of pooling and cryopreserving the blood allows for high-throughput examination of numerous samples, makes the cryopreserved blood an immediately accessible reagent, and overcomes the artifact of interindividual variability in immune reactivity [23–25]. Because responses of human whole blood have been comparable to that of alveolar macrophages, this assay may also enable the assessment of the potential of a sample to induce lung proinflammatory responses [23]. Human whole blood, therefore, can provide a tool to evaluate the proinflammatory potential of components of airborne particulate matter, such as fungal spores from basidiomycetes, which may pose a health risk to individuals suffering from respiratory diseases (e.g. asthma and allergies).

In this study, we determined the proinflammatory potency of spores from 11 species of fungi from the basidiomycota group with documented allergenic potential, based on the release of the proinflammatory cytokine IL-1β from cells in cryopreserved human whole blood. Given that the morphology of spores is highly diverse throughout the fungus kingdom, we evaluated the role of morphological features, such as surface area, shape, and spore pigmentation, in the IL-1β-inducing potency of the basidiospores.

Methods

All chemicals, reagents, and materials used throughout the experiments listed in this section were free of pyrogen.

Basidiospores

Fruiting bodies of basidiomycetes (table 1) were collected from a recreational area in Baltimore (Md., USA); others were collected and shipped from Tulsa (Okla., USA) and Atlanta (Ga., USA) by collaborators in this study. They were brought into the laboratory in clean paper bags. The stipe of the fruiting bodies was removed and the basidocarp placed overnight on depyrogenized aluminum foil to collect basidiospore deposits. The basidiospore deposits were aseptically transferred into microcentrifuge tubes (ThermoFisher Scientific, Waltham, Mass., USA) and stored in a desiccator until analyzed. Loopfuls of basidiospore deposits were transferred into microcentrifuge tubes containing 1 ml of water. The concentration of basidiospores (spores/ml) for each species was determined with a hemocytometer, and dilutions (1.0×10^6, 10^5, 10^4, and 10^3 spores/ml) were prepared for each species. These basidiospore dilutions were later tested in the cryopreserved human whole blood system.

Collection and Cryopreservation of Human Whole Blood

With prior approval of the Johns Hopkins School of Public Health Institutional Review Board, healthy volunteers were recruited from the Johns Hopkins School of Public Health staff and they orally consented to participate in the study. Blood (30 ml) was collected by venipuncture into heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, N.J., USA). Incubations using fresh whole blood must be started within 4 h of blood sampling, as reactivity was significantly lower when challenged with pyrogens at later time points [24, 25]. To mitigate this limitation, whole blood was collected from multiple donors, pooled, and cryopreserved as previously described with minor modifications [25]. Vacutainer tubes were placed in ice before pooling the blood into 50-ml Falcon® tubes (Becton Dickinson). The blood was gently pooled into

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Table 1. Species of basidiomycetes tested and corresponding morphological measurements

Basidiomycete species	Macroscopical description	Surface area, pixels^2	Circularity[a], R	Pigmentation[b]	LPS contamination, ng/10^6 spores
P. arhizus	puffball[c]	3,811	0.685	*37,233*	*0.032*
P. ostreatus	gilled mushroom[d]	3,538	0.673	141,739	0.154
O. radicata	gilled mushroom	*3,859*	*0.801*	116,635	0.161
A. tabescens	gilled mushroom	3,382	0.653	96,399	0.049
M. tremellosus	polypore[e]	3,196	0.635	*143,335*	0.141
C. cyathiformis	puffball	*3,070*	*0.544*	42,412	<LOD
C. micaceus	gilled mushroom	3,223	0.601	52,075	*0.163*
L. pyriforme	puffball	3,108	0.576	92,790	0.032
P. cervinus	gilled mushroom	3,398	0.646	97,402	<LOD
C. molybdites	gilled mushroom	3,398	0.646	90,258	<LOD
B. bicolor	polypore	3,357	0.642	105,889	<LOD

Italic numbers correspond to the highest and lowest values within each column.

[a] Circularity of a sphere, rectangle, and star-shaped object is 1.000, 0.670, and 0.086, respectively. [b] Pigmentation (as a measure of color-integrated density) of a black object and the background of the images is 0 and 184,563, respectively. [c] Puffballs produce basidiospores in an internal fruiting body. [d] Gilled mushrooms have basidiospore-bearing gills. [e] Polypores have basidiospore-bearing tubes in the fruiting body.

a 1-liter Nalgene® storage bottle (Thermo Scientific), and clinical grade dimethyl sulfoxide (DMSO; Gaylord Chemical, Slidell, La., USA) was added slowly into the pooled blood, with gentle mixing, until the concentration of DMSO reached 10% of the final volume. The pooled whole blood with DMSO was aliquoted into precooled 4.5-ml cryovials. The cryovials were kept at 4°C for 20 min, stored at –20°C for an additional 20 min, and were then placed in a Styrofoam box and stored at –80°C. The Styrofoam box was used to control the decrease in temperature of the cryovials once stored at –80°C. Quality control tests of the cryopreserved batches of blood were performed to make sure that it maintained its reactivity against a standard stimulus (0.5 ng/ml of LPS from *Escherichia coli* O113:H10; Associates of Cape Cod, East Falmouth, Mass., USA), and displayed a consistent and minimal baseline reactivity (e.g. ≤5 pg IL-1β/ml) to the culture medium (RPMI 1640; Invitrogen, Carlsbad, Calif., USA). The incubation protocol for the quality control tests included the positive control [180 μl of RPMI, 40 μl of thawed blood, and 20 μl of LPS from *E. coli* O113:H13 (0.5 ng/ml)], and the negative control (200 μl of RPMI and 40 μl of thawed blood). ELISA of the supernatant for IL-1β was performed as described below.

Stimulation of Cryopreserved Human Whole Blood

The stimulation of the thawed cryopreserved human whole blood was performed as previously described with minor modifications. Basidiospore suspensions (final concentrations of 1.0 × 10^6, 10^5, 10^4, and 10^3 spores/ml) were incubated in triplicate in 180 μl of RPMI 1640 in the presence of 25 international units (IU) of the polycationic antibiotic polymyxin B sulfate (Xgen Pharmaceuticals, Big Flats, N.Y., USA) for 1 h at 37°C. This compound has high affinity to negatively charged phosphate groups, such as those present in the LPS from Gram-negative bacteria. Preincubation with 25 IU of polymyxin B sulfate was performed to inhibit any contamination of the basidiospores with LPS, which is a strong stimulus of innate immune cells. The concentration of polymyxin B sulfate used was determined in a preliminary study, in which 25 IU were found to be effective in blocking the IL-1β response of 0.5 ng of LPS of *E. coli* O113:H10 and at the same time was not toxic to the cells in the cryopreserved blood (data not shown). The level of LPS contamination was determined with the kinetic chromogenic *Limulus* amoebocyte lysate assay Pyrochrome® (Associates of Cape Code), following the manufacturer's instructions, prior to performing the whole blood assay (table 1). After preincubation with polymyxin B sulfate, vials of cryopreserved blood were thawed for 15 min at 37°C and 5% CO_2. The thawed blood was then stimulated with the basidiospores suspensions or controls within 15 min to avoid any toxic effects that the DMSO may have on the blood immune cells [25]. Forty microliters of thawed blood were transferred into the wells containing the basidiospore suspension, the plate gently tapped for 30 s, and incubated at 37°C and 5% CO_2 for 18 h. After incubation, the culture supernatants were collected and stored at –80°C until ELISA was performed with capture and biotinylated-detection antibody pairs for the proinflammatory cytokine IL-1β (R&D Systems, Minneapolis, Minn., USA). Serial dilutions of LPS (0.5, 0.25, and 0.1 ng/ml) from *E. coli* O113:H10 and RPMI 1640 alone were included as positive and negative controls, respectively; fig. 2a). Reagent controls, for water and polymyxin B sulfate, were performed and yielded no response (data not shown).

Microscopy and Image Analysis of Spores

Microscopic slides of each species of basidiospores were prepared by placing a loopful of basidiospore dilution on a clean microscope slide and allowing it to air-dry overnight. Slides were examined at a magnification of ×1,000 with a Nikon Eclipse E800 (Nikon, Melville, N.Y., USA) microscope connected to a SPOT RT3 (Diagnostic Instruments, Sterling Heights, Mich., USA) camera synchronized to a computer. Two images/slide were captured with the software SPOT Advanced (Diagnostic Instruments) and

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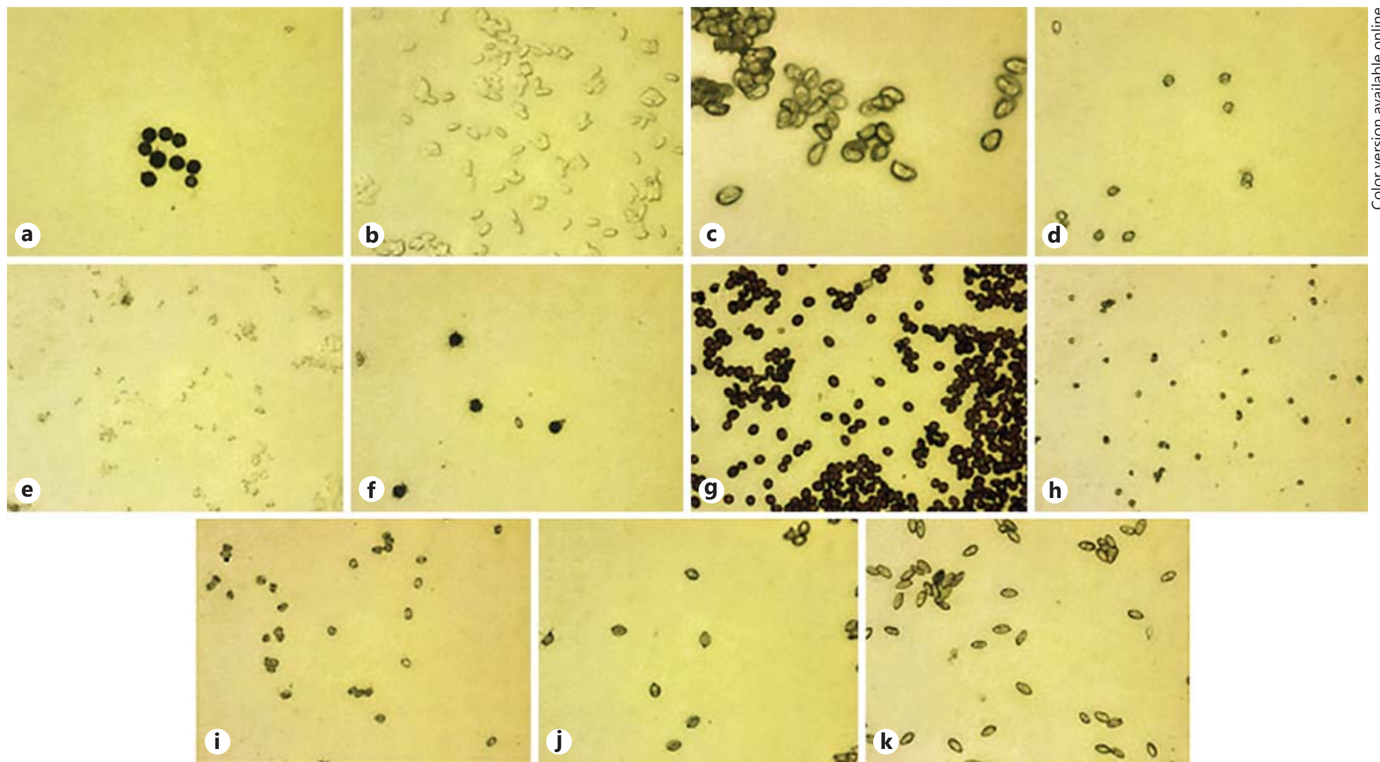

Fig. 1. Images of basidiospores (×1,000). **a** *P. arhizus*. **b** *P. ostreatus*. **c** *O. radicata*. **d** *A. tabescens*. **e** *M. tremellosus*. **f** *C. cyathiformis*. **g** *C. micaceus*. **h** *L. pyriforme*. **i** *P. cervinus*. **j** *C. molybdites*. **k** *B. bicolor*.

analyzed with the image analysis software ImageJ (National Institutes of Health, Bethesda, Md., USA) for surface area (pixel^2), spore pigmentation (integrated density of the basidiospore color), and shape (measure of circumference). Measurements of the above morphological features were performed on 10 spores/slide.

Each image was color segmented to isolate the particle of interest (spores) from the background. After segmentation, measurement options for surface area, integrated density, and circularity were selected. The software automatically calculated the selected measurements, and the results were exported into an Excel™ file. As reference, the circularity ranges were from 0 to 1: a sphere, rectangle, and star-shape object have circularities of 1, 0.6, and 0.08, respectively [26]. For integrated density, a completely black object has an integrated density of 0 and the background of the image of the basidiospores had an integrated density of 184,563. Therefore, the more hyaline or brightly colored a spore, the higher the number and closer to the integrated density of the background.

Statistical Analysis

Statistical analysis was performed with Minitab 16.2.2 (Minitab Inc., State College, Pa., USA). The morphological and cytokine data did not show a normal distribution, as determined by an Anderson-Darling normality test. A two-sample Wilcoxon rank sum test was performed to determine significant differences between the highest IL-1β-inducing potency of basidiospores and LPS concentrations of 0.5 and 0.25 ng/ml. Levene's test of equal variance was performed to determine the concentration of basidiospores/ml that induced the highest variability in the IL-1β-inducing potency. The concentration of basidiospores with the highest variability in IL-1β-inducing potency (determined by Levene's test of equal variance) was used to test for any correlation with the morphological features of the basidiospores (surface area, circularity, and color integrated density). Significance was set at $p < 0.05$.

Results

Basidiospores Induced Concentration- and Species-Dependent IL-1β Responses

To compare the IL-1β-inducing potency of spores from the 11 basidiomycete species (table 1; fig. 1), cryopreserved human whole blood was stimulated with different concentrations (10^6–10^3) of basidiospores and the concentration of IL-1β in the culture supernatant was measured with ELISA. Human whole blood was also incubated with 0.5, 0.25, and 0.1 ng/ml of LPS. Any LPS contamination of the basidiospores was blocked with 25 IU of polymyxin B sulfate.

As expected, stimulation of human whole blood with the three concentrations of LPS (fig. 2a) induced a con-

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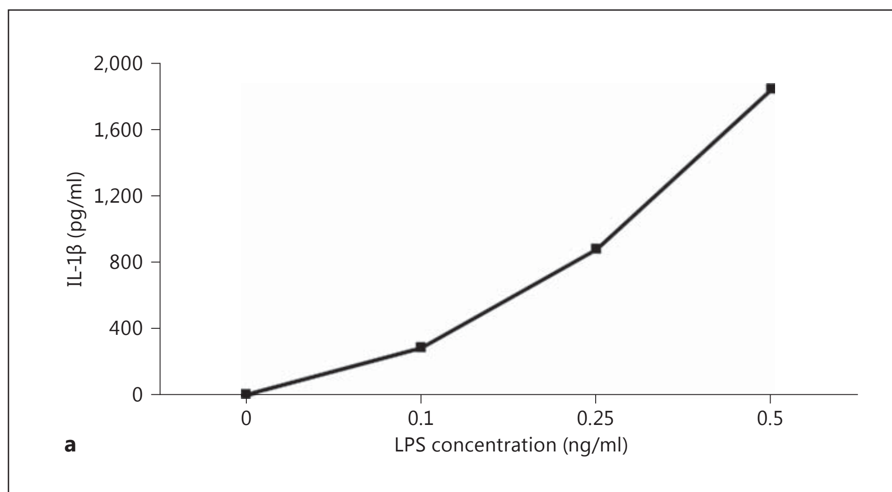

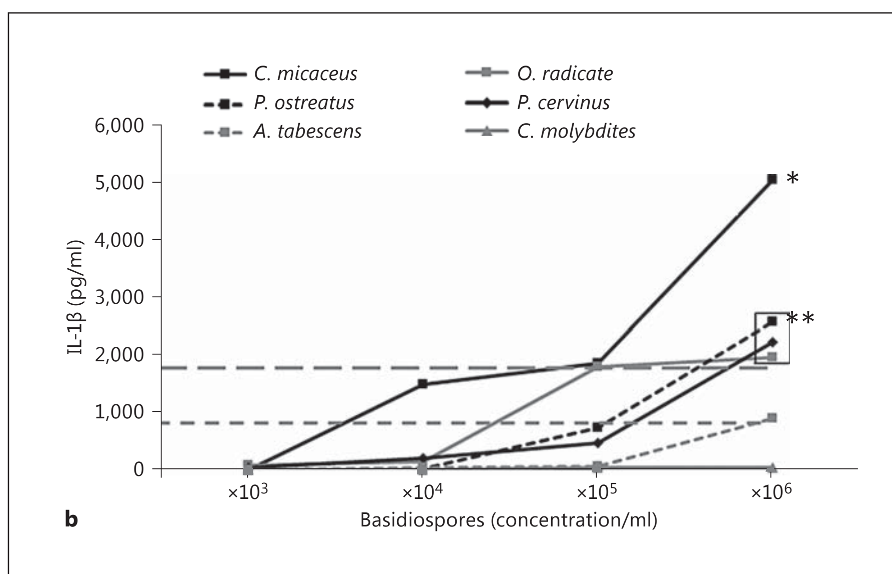

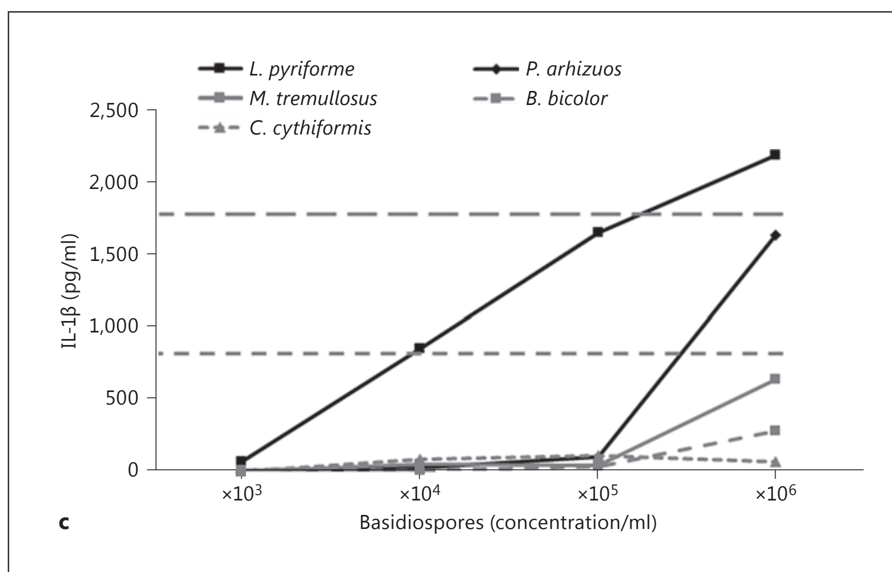

Fig. 2. Concentration-dependent responses of LPS and basidiospores in cryopreserved human whole blood. Cryopreserved human whole blood was stimulated with LPS (**a**), and spores from gilled (**b**), and puffball and polypore basidiomycetes (**c**), and concentrations of the proinflammatory cytokine IL-1β in culture supernatants were determined with ELISA. Long-dash and dash lines represent the IL-1β-inducing potency of 0.5 and 0.25 ng/ml, respectively. Representative data from three independent experiments are shown. ** $p < 0.05$ vs. 0.5/0.25 ng/ml of LPS; * $p < 0.05$ vs. 0.25 ng/ml of LPS.

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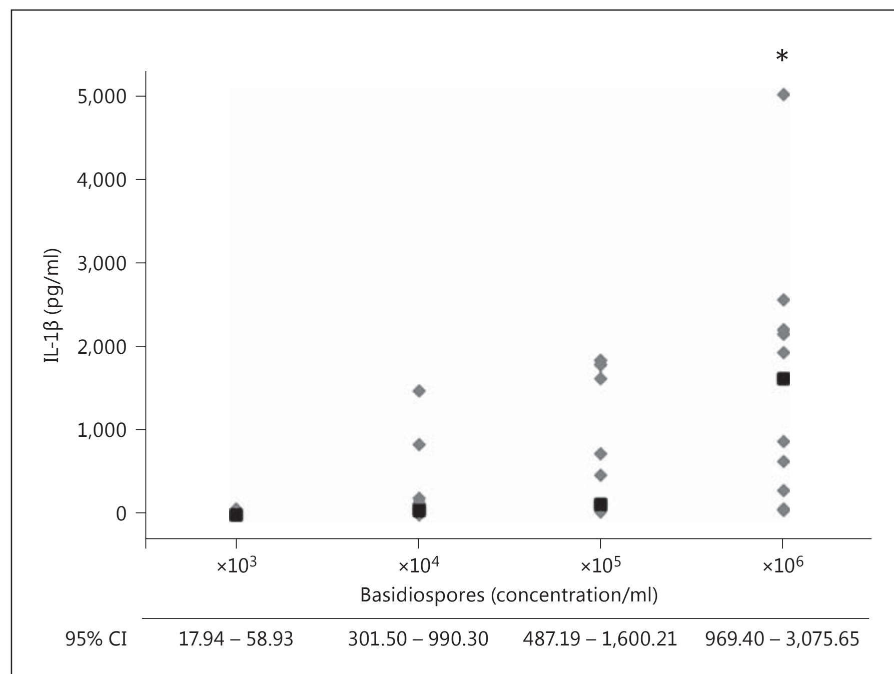

Fig. 3. Higher variability in IL-1β-inducing potency at higher concentrations of basidiospores. Levene's test of equal variances was performed to determine the concentration of basidiospores that, overall, induced the highest variability in the IL-1β-inducing potency after stimulation in the human whole blood. * $p < 0.05$ vs. variance of 10^3, 10^4, 10^5 basidiospores/ml.

centration-dependent IL-1β-inducing potency. Basidiospores from gilled mushrooms (fig. 2b), similar to LPS, stimulated a concentration-dependent IL-1β-inducing potency, with *C. micaceus* inducing the highest response. At 10^6 basidiospores/ml, *C. micaceus* was the only species that induced a response significantly higher than that induced by 0.5 and 0.25 nanograms of endotoxin/ml. IL-1β-inducing potencies of *P. ostreatus, O. radicata*, and *P. cervinus* were significantly higher than that of 0.25 ng/ml of LPS. Among the nongilled basidiomycetes (fig. 2c), only *L. pyriforme* induced a marked concentration-dependent IL-1β response. None of the nongilled basidiomycetes induced a response significantly higher than 0.5 and 0.25 ng/ml of LPS.

To test for variability in the IL-1β-inducing potency between different concentrations of basidiospores, Levene's test of equal variance was performed. As concentration increased, the variability in IL-1β-inducing potency between basidiospores was more evident. The variability of the IL-1β-inducing potency of the basidiospores tested was significantly higher (p, 0.002) at 10^6 basidiospores/ml (fig. 3). Similar to what is shown in figure 2b, c, at 10^3 basidiospores/ml the IL-1β-inducing potency of the basidiospores tested was barely noticeable. Altogether, these data suggest that allergenic basidiospores have a concentration-dependent IL-1β-inducing potency, and that this potency can be different at higher concentrations of basidiospores.

Morphological Features of Basidiospores Did Not Modulate IL-1β-Inducing Potency

To evaluate the role of morphological features of basidiospores in their IL-1β-inducing potency, surface area (pixels^2), circularity (R), and pigmentation (as a measure of color integrated density) of basidiospores were determined with image analysis (table 1). The Spearman rank correlation coefficient was calculated between the morphological features and the IL-1β-inducing potency of the basidiospores at 10^6/ml (the concentration of spores at which the highest variability in IL-1β-inducing potency was detected for the species tested). The morphological measurements varied widely and no correlation was found between morphological features and the IL-1β-inducing potency. This finding suggests that nonmorphological properties of basidiospores modulate their IL-1β-inducing potency in the cryopreserved human whole blood.

Discussion

In this study, we examined species of basidiomycete fungi with documented allergenic potential and determined their respective proinflammatory potency of their spores in a cryopreserved human whole blood system. Our data suggest that the cryopreserved human whole blood provides a useful tool to determine the proinflam-

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Table 2. Spearman's rank correlation coefficient between IL-1β-inducing potency of 10^6 basidiospores/ml and morphological measurements

	Surface area	Circularity	Integrated density
R	0.405	0.378	0.291
p value	0.216	0.252	0.521

matory potency of spores of higher fungi, such as basidiomycetes. More importantly, the study further adds to the potential health effects of basidiospores.

Stimulation of human whole blood with basidiospores induced a defined concentration-dependent proinflammatory potency (fig. 2), mainly in gilled basidiomycetes. This is similar to previous studies in which other non-LPS stimuli, such as peptidoglycan from Gram-positive bacteria, polyinosinic-polycytidylic acid (analog of double-stranded RNA), zymosan (component of the cell wall from fungal spores), and whole spores from mitosporic fungi (e.g. *Cladosporium* spp., *Alternaria* spp., and *Penicillium* spp.) induced concentration-dependent responses of proinflammatory cytokines in human whole blood [27–29]. Similar to the findings of Daneshian [28], polymyxin B sulfate did not affect the IL-1β-inducing potency, at least in the gilled mushrooms (fig. 2b). It remains to be determined if this polycationic compound affects the proinflammatory potency of spores from nongilled basidiomycetes at lower concentrations ($<10^6$ basidiospores/ml; fig. 2c). Despite this, our data suggest that allergenic basidiospores do possess proinflammatory potential. In addition, our study provides evidence that the cryopreserved human whole blood system may be used in conjunction with compounds that can inhibit the action of other proinflammatory microbial components to study the proinflammatory potential of spores from allergenic basidiomycetes.

Basidiomycetes are the most morphologically diverse fungi, and their spores are often morphologically distinct within a particular genus [30]. In addition, many species have small diameters (<2.5 μm), which may allow them to reach lower anatomical sites (e.g. alveoli) of the respiratory tract, which is important in inducing asthmatic episodes [31, 32]. Size is an important parameter for particle uptake by resident antigen-presenting cells (e.g. dendritic cells and macrophages) and the initiation of immune responses in the lower respiratory tract [33]. For these reasons, we proceeded to examine whether morphological features, such as the surface area of the spore, shape (as a measure of circularity), and pigmentation (as a measure of color-integrated density), contribute to the IL-1β-inducing potency. These features often vary between genera and sometimes between species. Interestingly, we did not find any correlation between morphological features and the IL-1β-inducing potency of the basidiospores (table 2). The presence and concentration of different cell wall chemical components such as β-glucan, mannans, chitin (long-chain polymer of acetylglucosamine), and melanin on the surface of fungal spores may explain the variability in basidiospores, as it has been shown using spores of mitosporic micromycetes that these compounds play a role in their interaction and activation of innate immune cells [14, 34–36]. Furthermore, β-glucans with different glycosidic linkages and molecular weights differ in their proinflammatory potency [37]. Therefore, properties of basidiospores beyond morphology may have active roles in the variable proinflammatory potency observed in our study. Nonetheless, the human whole blood system in our study was able to determine the variable proinflammatory responses between spores of basidiomycete species, which opens the opportunity to further explore the role of structural chemical components of basidiospores in their proinflammatory potency.

Limitations of this study include the number of basidiomycete species tested. Because of the difficulty to cultivate them in laboratory conditions, studies with basidiomycetes often rely on collecting fruiting bodies in the field. The limited availability of enough specimens harvested from the field is commonly one of the main reasons studies with basidiomycetes rarely include beyond 10 species [38]. Another limitation is evaluating the proinflammatory potency based solely on IL-1β. We focused our study on this biomarker based on previous validation studies that have shown its reliability as a good proinflammatory end point in the human whole blood system [23, 24]. We intend to further examine the proinflammatory potency of basidiomycetes based on additional biomarkers, such as other cytokines and chemokines that participate in innate immune responses.

Spores from basidiomycete fungi have been shown to significantly contribute to the fungal ecology in the atmosphere of rural and suburban environments [1, 2, 6]. Aerobiological studies have shown that concentrations of basidiospores may reach, depending on the environmental factors, yearly concentrations >100,000 spores/m^3, and genetic analyses of airborne samples have shown species of gilled fungi to be the most predominant among spores

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of basidiomycetes [2, 38]. Similarly, clinical studies have elucidated the potential of airborne basidiospores to exacerbate episodes of respiratory allergies, such as allergic asthma and rhinitis, among susceptible individuals. All fungal species from the basidiomycete group previously studied demonstrated to have allergenic potential [31, 32, 39–42]. However, insights into the priming or proinflammatory properties of basidiospores, as it has been shown with other allergens [43–45], is lacking. In these studies with other allergens (e.g. mite and cat allergens), priming of the innate immune system through proinflammatory cascades adds to the allergenicity of the allergens. These were strong motivating factors for evaluating the proinflammatory potential of allergenic basidiospores in this study. The differences in proinflammatory potency between gilled and nongilled mushrooms may suggest that the allergenic potential of this group of fungi also relies on their proinflammatory potency, and may pose additional respiratory health risks to susceptible individuals. Additional studies are needed to provide more evidence regarding the interface between the proinflammatory and allergenic potencies of spores from basidiomycetes and their role in potential human health effects.

In summary, our data demonstrate the utility of the cryopreserved human whole blood system as a tool to study the proinflammatory potency of allergenic basidiospores. It also provides additional evidence of the potential human health effects, beyond allergies, which basidiospores could have once they interact with the innate immune system. Additional studies are warranted to further characterize the innate immune-activating properties of fungal spores of basidiomycetes in a human-based system.

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Disclosure Statement

The authors have no conflict of interest to disclose.

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