

***In vitro* adhesion and invasion by *Cladosporium sphaerospermum* in human bronchial epithelial cells (BEAS-2B) and human pulmonary alveolar epithelial cells (HPAEpiC)**

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Abstract. *Cladosporium* spores are ubiquitous in indoor and outdoor environment and may potentially trigger allergic responses upon inhalation. To date, there is limited investigation on the fate of *Cladosporium* spores after being inhaled into the respiratory tract. This study was conducted to investigate the interaction of *Cladosporium sphaerospermum* with Human Bronchial Epithelial Cells (BEAS-2B) and Human Pulmonary Alveolar Epithelial Cells (HPAEpiC). *C. sphaerospermum* conidia were harvested and co-cultured with BEAS-2B or HPAEpiC cells for 72 hours. At each time point (30 minutes, 2, 4, 24, 48 and 72 hours), adherence and invasion of the cells by *C. sphaerospermum* conidia (and hyphae) were investigated by immunofluorescence staining. This study demonstrated the adherence and internalization of *C. sphaerospermum* conidia within these epithelial cells. In addition, the conidia were able to germinate and invade the epithelial cells. The ability of the fungal conidia to adhere, internalize, germinate and invade both the bronchial and alveolar epithelial cells of the respiratory tract *in vitro* might contribute to the understanding of the pathogenesis of *Cladosporium* in respiratory infection and allergy *in vivo*.

INTRODUCTION

Cladosporium species is a member of the phylum Ascomycota. The common species include *C. herbarum*, *C. cladosporioides* and *C. sphaerospermum*. This genus has worldwide distribution. Aerobiological studies reported that majority of fungal spores in outdoor air is from the phyla Ascomycota and Basidiomycota, while *Cladosporium* is one of the most studied allergenic Ascomycetes fungi (Knutsen *et al.*, 2012). *Cladosporium* spores are found abundantly in indoors and outdoors at approximately 18/m³ and 141/m³ respectively (Codina *et al.*, 2008).

As an imperfect dematiaceous fungus, *Cladosporium* species causes opportunistic

infections such as cerebral and cutaneous phaeohyphomycosis usually in immunocompromised patients (Yano *et al.*, 2003; Aldape *et al.*, 1991; Hironaga *et al.*, 1980) and also healthy individual (Vieira *et al.*, 2001). *C. bruhnei* causes allergic lung mycoses (Dugan *et al.*, 2008) while *C. herbarum* as common triggering factor of respiratory allergy (Achatz *et al.*, 1995). There are 14 reported allergens for *C. herbarum* namely mannitol dehydrogenase, vacuolar serine protease, aldehyde dehydrogenase and hydrophobin (Simon-Nobbe *et al.*, 2006; Yew *et al.*, 2016).

Another common species known as *C. sphaerospermum* was found in the sputum which obstructed the basal bronchi and caused intrabronchial lesion in healthy

woman (Yano *et al.*, 2003). Yew *et al.* (2016) reported a total of 28 *C. sphaerospermum* genes was associated with allergy.

There is scarce research on this species despite its high spore density in the environment, and potential allergenicity. This study was conducted to investigate the interaction of *Cladosporium sphaerospermum* with bronchial epithelial and alveolar epithelial cells. These two cell types were selected to represent the microenvironment of the lower respiratory tract because *Cladosporium* conidia are small in size (3-4 microns) and have high probability of attaching and entering the lower airways easily.

MATERIALS AND METHODS

Harvesting of whole fungal elements and spores

The clinical isolates of *C. sphaerospermum* UM843 was obtained from the late Prof Ng Kee Peng's culture collection, Department of Medical Microbiology, University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia, in year 2014. It was used in all experiments. This isolate was isolated from a blood culture. *C. sphaerospermum* was cultured at 27°C on Sabouraud Dextrose agar (Laboratories CONDA, Spain) for 14 days in an incubator (Memmert BE400, Germany). After culturing for 14 days, the fungi were harvested and suspended in sterile phosphate buffered saline (PBS) in the presence of protease inhibitor cocktail (Roche, Switzerland).

Homogenization of fungi

The fungi were homogenized using Microsmash homogenizer (Tomy, Japan) in the presence of 500 µL of PBS with protease inhibitor and glass beads (1.0 mm diameter; Tomy, Japan). The suspension was homogenized at 3,400 rpm for 1 minute, followed by cooling at -80°C for 3 minutes before next homogenization cycle. The homogenate was examined for cell breakage and the process was repeated for 10-15 cycles until 70-90% cell breakage. The crude extracts were collected and centrifuged (Sartorius, Germany) at 12,000 rpm for 15 minutes at

4°C. Supernatant was collected and stored in -80°C. The concentration of the fungal proteins was determined using Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Immunization of rabbit

A New Zealand white rabbit was immunized subcutaneously with 1.25 mL of 0.8 µg/µL crude extract of *C. sphaerospermum* at three-weekly intervals. Pre-immunized serum was collected one day before the immunization. The rabbit was immunized with 1.25 mL of 0.8 µg/µL crude extract emulsified in 1.25 mL of Freund's adjuvant (Sigma-Aldrich Corporation, USA). Subsequently, the booster injections [1.25 mL of 0.8 µg/µL crude extract emulsified in 1.25 mL of incomplete Freund's adjuvant (Sigma-Aldrich Corporation, USA)] were given subcutaneously at two-weekly intervals. Blood samples were collected before each booster injection as post-immunized sera. The antibody levels were determined using enzyme-linked immunosorbent assay (ELISA). The polyclonal antibodies obtained were used for immunostaining.

Preparation of BEAS-2B and HPAEpiC cells

BEAS-2B and HPAEpiC cell lines were purchased from American Type Culture Collection (ATCC) and ScienCell Research Laboratories, United States respectively. Each well of a 24-well plate was seeded with 5×10^4 of either BEAS-2B or HPAEpiC cells in 1 mL of Dulbecco's Modified Eagle's and Ham's F-12 (DMEM-F12) medium and Alveolar Epithelial Cell Medium (AEpiCM) respectively. The plates were incubated overnight in a CO₂ incubator at 37°C for cell attachment.

Culture and harvest of *C. sphaerospermum* conidia

C. sphaerospermum was cultured at 27°C on Sabouraud Dextrose agar for 14 days. The conidia were harvested by rinsing the fungi with PBS containing 0.01% Triton X-100 (Bio-Rad Laboratories, USA). The suspension was centrifuged at 4,000 rpm for 7 minutes. The

pellet containing the conidia was re-suspended in DMEM-F12 or AEpiCM medium, counted using haemocytometer and adjusted to a concentration of 5×10^4 conidia/mL.

Co-incubation of *C. sphaerospermum* conidia with BEAS-2B or HPAEpiC cells

After removing the medium in 24-well plates containing BEAS-2B or HPAEpiC cells, 1 mL of conidia suspension was added into each well for co-incubation. The plates were incubated at 32°C in a 5% CO₂ incubator. After co-incubation at each time point (30 minutes, 2, 4, 24, 48 and 72 hours), the plate was removed from the incubator respectively. The medium was discarded and non-adherent conidia in the wells were removed by washing twice with 1 mL of PBS. Next, 200 µL of 3% paraformaldehyde were added into each well for 15 minutes in order to fix the cells. After removing the paraformaldehyde, each well was washed with 1 mL of PBS. Then, 1 mL of PBS was added into each well and the plates were stored in 4°C for immunostaining later.

Immunofluorescence staining

PBS in each well was discarded and the cells were stained according to the protocol

(Table 1). After the last step, 500 µL of PBS were added into each well to prevent drying up. The adherence and invasion of *C. sphaerospermum* to epithelial cells were examined and photographed under a Nikon Eclipse Ti-U inverted microscope with camera attachment (Nikon Corporation, Tokyo, Japan). The number of adhered and internalized conidia was determined by counting 10 fields per well at 200 x magnifications. Three images were taken at each field with different filters: bright field (DIC), FITC filter (for Alexa 488) and TRITC filter (for Alexa 568). Alexa 568 conjugated anti-rabbit immunoglobulin (red) can only bind to the conidia or hyphae outside of the cell while Alexa 488 conjugated anti-rabbit immunoglobulin (green) can bind to the conidia or hyphae in the cell. Hence, those that fluoresces in green are counted as internalized or invaded into the cell. Each condition was conducted in triplicates, and four separate experiments were performed.

Transmission electron microscopy

Two 25 cm³ flasks were seeded with either 7×10^5 BEAS-2B cells or HPAEpiC cells in 5 mL of DMEM-F12 medium and AEpiCM medium respectively. The flasks were incubated overnight in a CO₂ incubator at

Table 1. Immunofluorescence staining protocol

Step	Solution	Volume (µL)	Temperature (°C)	Duration (minutes)
1	Rabbit polyclonal antibody as primary antibody (1: 10 dilution)	200	37	120
2	PBS	500	25	10
3	Alexa 568 conjugated anti-rabbit immunoglobulin (1: 500 dilution)	200	37	60
4	PBS	500	25	5
5	PBS	500	25	5
6	0.2% Triton X-100 in PBS	200	25	5
7	PBS	500	25	10
8	Rabbit polyclonal antibody	200	4	Overnight
9	PBS	500	25	10
10	Alexa 488 conjugated anti-rabbit immunoglobulin (1: 500 dilution)	200	37	60
11	PBS	500	25	5
12	Ultrapure water	500	25	5

PBS, Phosphate buffered saline.

37°C for cell attachment. Then, the medium in each flask was removed and 5 mL of DMEM-F12 medium or AEpiCM medium containing 7×10^5 conidia were added into each flask. The flasks were then incubated at 32°C in a 5% CO₂ incubator for 48 hours. The monolayers were washed with phosphate buffered saline and fixed with 4% glutaraldehyde overnight. The cell monolayers were scrapped using a cell scraper, transferred into 1.5 mL tubes, and centrifuged at 1500 rpm. The pellets were washed three times with cacodylate buffer and fixed with 1% osmium tetroxide for 2 hours. The samples were then dehydrated through a graded series of ethanol. Next, the samples were embedded in Epon epoxy resin (Hexion Ohio, USA), cut into ultrathin

sections and stained with 4% uranyl acetate and lead citrate before the examination under a transmission electron microscope at 80 kV equipped with camera (LEO-Libra 120 transmission electron microscope, Carl Zeiss, Germany) at University of Malaya, Malaysia.

RESULTS

At 30 minutes to 4 hours of incubation, there was no germination of conidia seen in all the samples except one which co-incubated with HPAEpiC cells. At 24 hours post incubation, some conidia germinated or formed hyphae. At 48 hours, more germination of conidia was observed (Figs. 1 and 2). The majority of

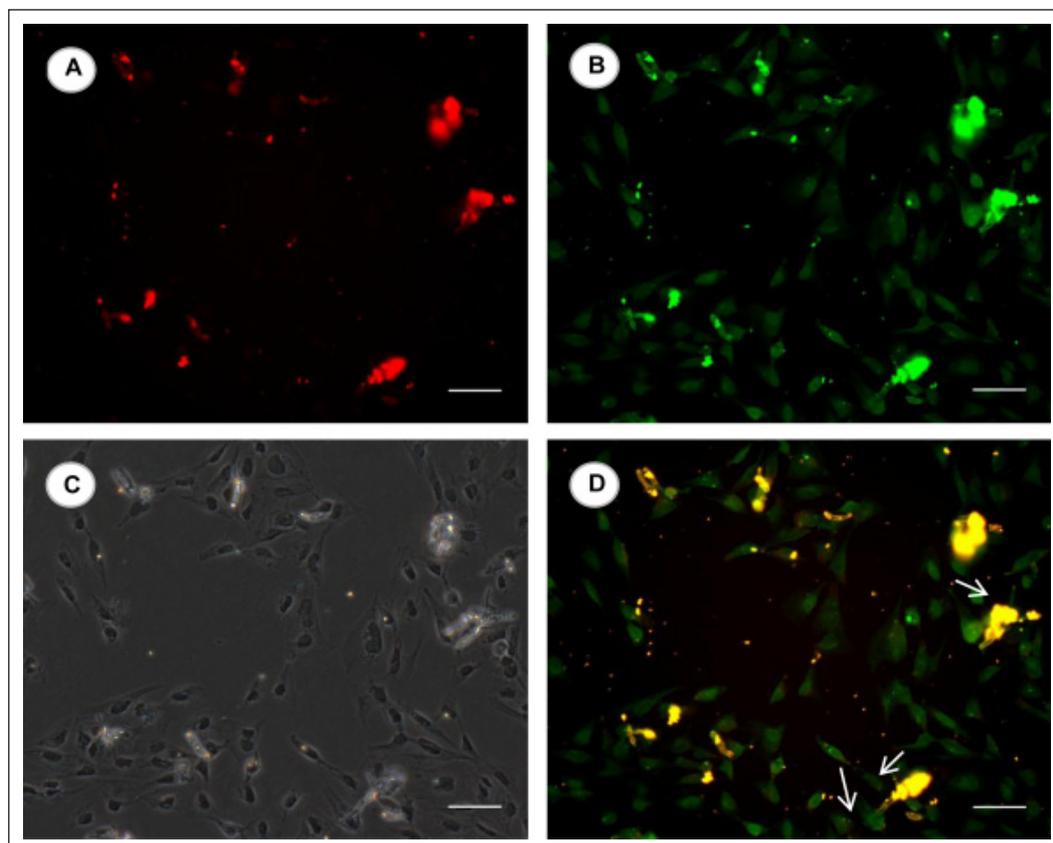


Figure 1. Immunofluorescence images showed invasion of *Cladosporium sphaerospermum* conidia into BEAS-2B cells after co-incubation for 48 hours. (A) Fluorescence image of the red channel (extracellular conidia and hyphae). (B) Fluorescence image of the green channel (total adhered and internalized conidia and hyphae). (C) Differential interference contrast (DIC) image. (D) Merged overlay of images A and B using NIS-Elements microscope imaging software. Note the hyphae invaded into the BEAS-2B cells (arrows). Scale bars correspond to 20 μ m.

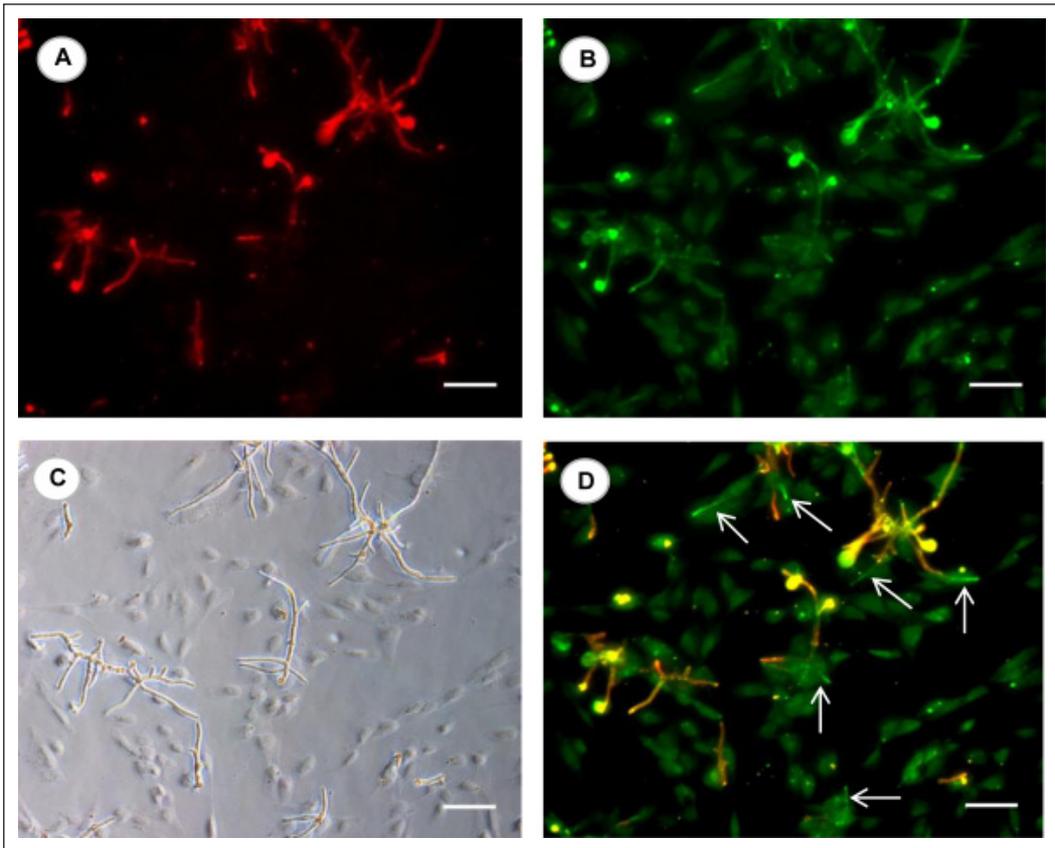


Figure 2. Immunofluorescence images showed invasion of *Cladosporium sphaerospermum* conidia into BEAS-2B cells after co-incubation for 72 hours. (A) Fluorescence image of the red channel (extracellular conidia and hyphae). (B) Fluorescence image of the green channel (total adhered and internalized conidia and hyphae). (C) Differential interference contrast (DIC) image. (D) Merged overlay of images A and B using NIS-Elements microscope imaging software. Note the hyphae invaded into the BEAS-2B cells (arrows). Scale bars correspond to 20 μm .

the conidia germinated at 72 hours and most of the hyphae invaded into BEAS-2B or HPAEpiC cells (Figs. 3 and 4).

It has been observed that there were more adhered and internalized germinated conidia in the BEAS-2B cells than in the HPAEpiC cells (Figs. 5 and 6). The interaction of BEAS-2B with the fungal conidia was illustrated under the electron microscope (Fig. 7).

DISCUSSION

Cladosporium species are ubiquitous and found worldwide at different geographical locations. *Cladosporium* spores are small in

size and easily detached from the branched hyphae. The spores are easily disseminated by wind and present in high concentration as airborne particles and hence are significantly associated with allergic rhinitis and other respiratory tract infections (Yew *et al.*, 2016). Furthermore, *C. sphaerospermum* was reported to cause acute meningitis (cerebral phaeohyphomycosis) in an immunocompetent male with well-controlled type 2 diabetes (Chen *et al.*, 2013). The pathogenesis of cerebral phaeohyphomycosis caused by *C. sphaerospermum* remains poorly understood. The hypothetical route of fungal entry could be due to hematogenous spread from the lungs or localized spread from the sinuses or ears. Phaeohyphomycosis

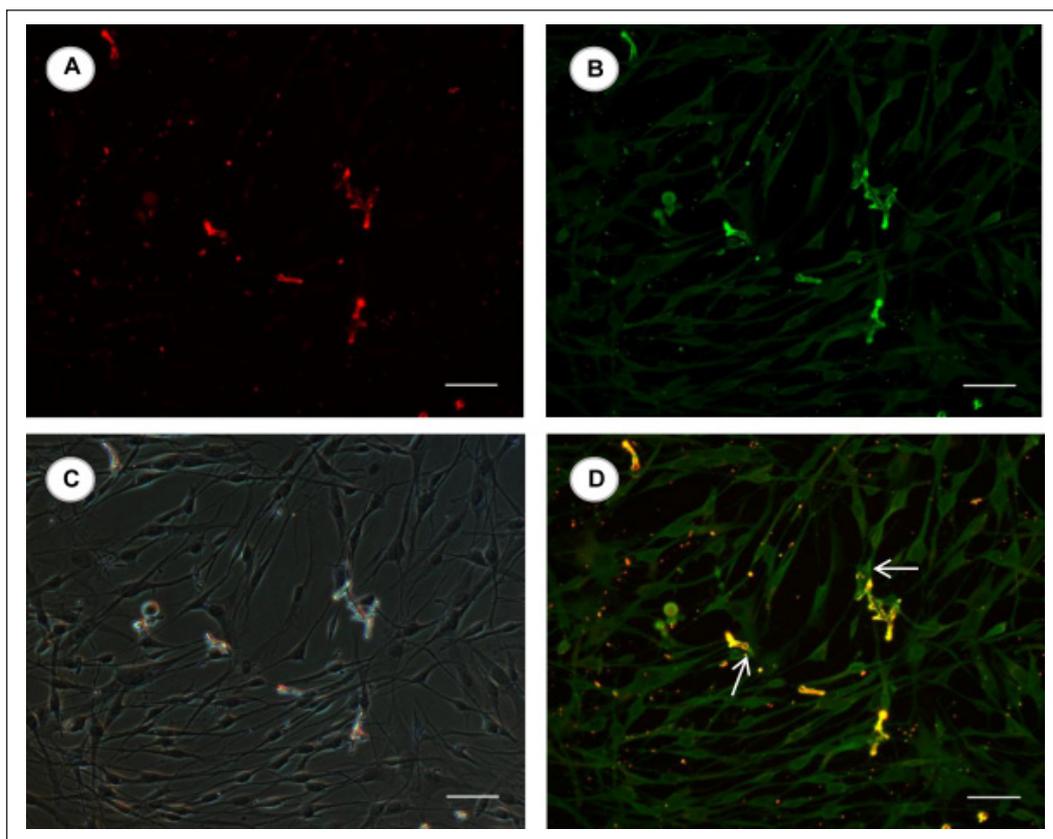


Figure 3. Immunofluorescence images showed invasion of *Cladosporium sphaerospermum* conidia into HPAEpiC cells after co-incubation for 48 hours. (A) Fluorescence image of the red channel (extracellular conidia and hyphae). (B) Fluorescence image of the green channel (total adhered and internalized conidia and hyphae). (C) Differential interference contrast (DIC) image. (D) Merged overlay of images A and B using NIS-Elements microscope imaging software. Note the hyphae invaded into the HPAEpiC cells (arrows). Scale bars correspond to 20 μm .

cases reported in India, Bhutan and Nepal were associated with type 2 diabetes (35% of 20 cases), renal transplantation (30%), long term use of steroids (15%) and human immunodeficiency virus (5%) (Sundaramoorthy *et al.*, 2017). Similarly, another species of *Cladosporium* (*C. cladosporioides*) was reported to cause alveolar haemorrhage (hemorrhagic pneumonia) in a healthy patient (Grava *et al.*, 2016). *C. cladosporioides* was isolated from cerebrospinal fluid and brain biopsy of a healthy 30-year-old Caucasian male with diplopia (Kantarcioglu *et al.*, 2001).

In this study, the interactions of *C. sphaerospermum* with human bronchial epithelial cells (BEAS-2B cells) and

HPAEpiC cells were investigated by co-culturing the conidia with the cells at different time points up to 72 hours. This is the first study that has visualized the pathogenesis of *Cladosporium* sp. by utilizing the immunofluorescence and fluorescence microscopy method. Previous studies mainly focused on allergens. The present study demonstrated that some of the *C. sphaerospermum* conidia adhered to and internalized into these epithelial cells. In addition, *C. sphaerospermum* conidia germinated to form hyphae and penetrated through the bronchial epithelial cells. This may be the mechanism of how *Cladosporium* can invade into the bloodstream in the lungs.

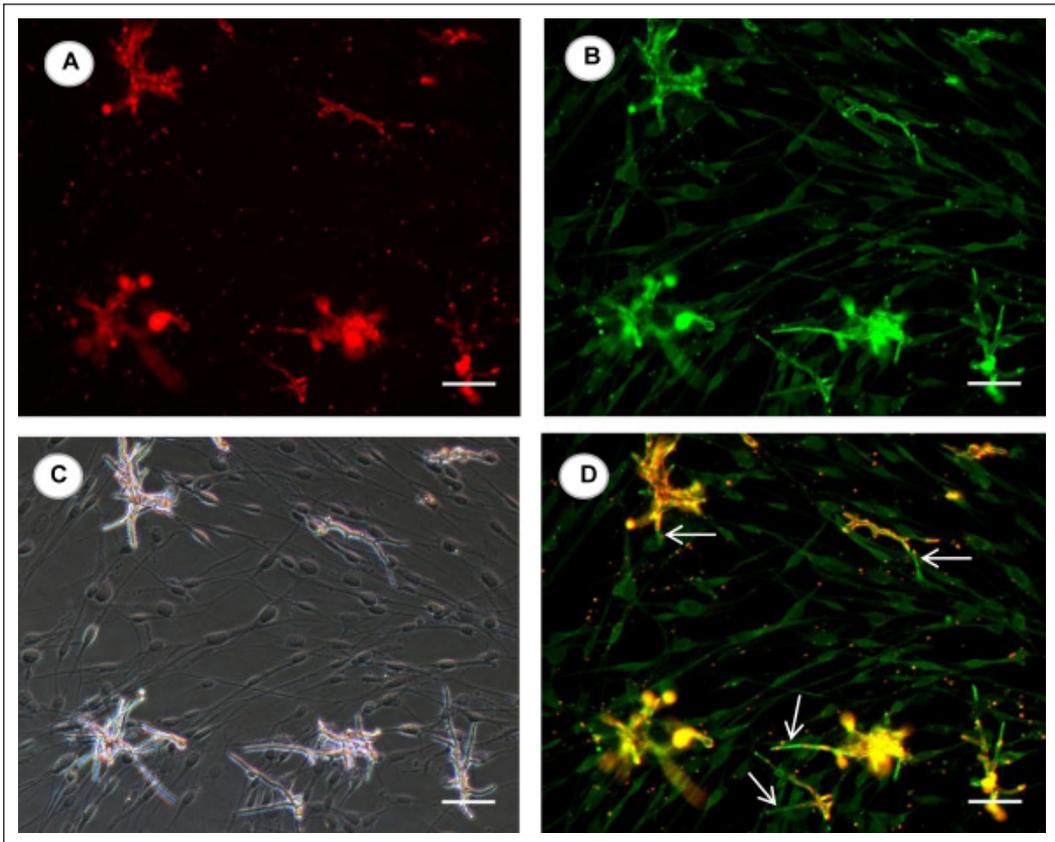


Figure 4. Immunofluorescence images showed invasion of *Cladosporium sphaerospermum* conidia into HPAEpiC cells after co-incubation for 72 hours. (A) Fluorescence image of the red channel (extracellular conidia and hyphae). (B) Fluorescence image of the green channel (total adhered and internalized conidia and hyphae). (C) Differential interference contrast (DIC) image. (D) Merged overlay of images A and B using NIS-Elements microscope imaging software. Note the hyphae invaded into the HPAEpiC cells (arrows). Scale bars correspond to 20 μ m.

Other than *Cladosporium* sp., there are several other fungal genera that invade mammalian host cells *in vitro* and *in vivo* (Wasylnka *et al.*, 2002). *Candida albicans* invades epithelial cells via two distinct mechanisms: induced endocytosis and active penetration (Wachtler *et al.*, 2011). In severe cases, *C. albicans* can penetrate through epithelial layers into deeper tissues, reach the bloodstream and cause life-threatening systemic infections (Wachtler *et al.*, 2011).

Other than *C. albicans*, *Aspergillus fumigatus* also is known to cause invasive pulmonary aspergillosis (IPA). In IPA, inhaled conidia, because of their small size (2–3 microns) enter the alveoli and germinate to establish the infections (Osherov *et al.*, 2012;

Paris *et al.*, 1997; Sheppard, 2011; Tronchin *et al.*, 2008; Pasqualotto *et al.*, 2010). This is similar to *Cladosporium* conidia which are of 3–4 microns. The spores are able to enter to the lower airways easily as well. In immunocompetent individuals, resident alveolar macrophages will ingest and destroy the inhaled conidia (Osherov *et al.*, 2012). However, in immunodeficient individuals, such as those with HIV infection, bone marrow transplantation, the fungi will invade through the alveolar wall into the surrounding blood vessels, obstructing the circulatory system and hence tissue necrosis (Osherov *et al.*, 2012). As the temperature in the airway is slightly lower than the normal body temperature, the co-incubation of the

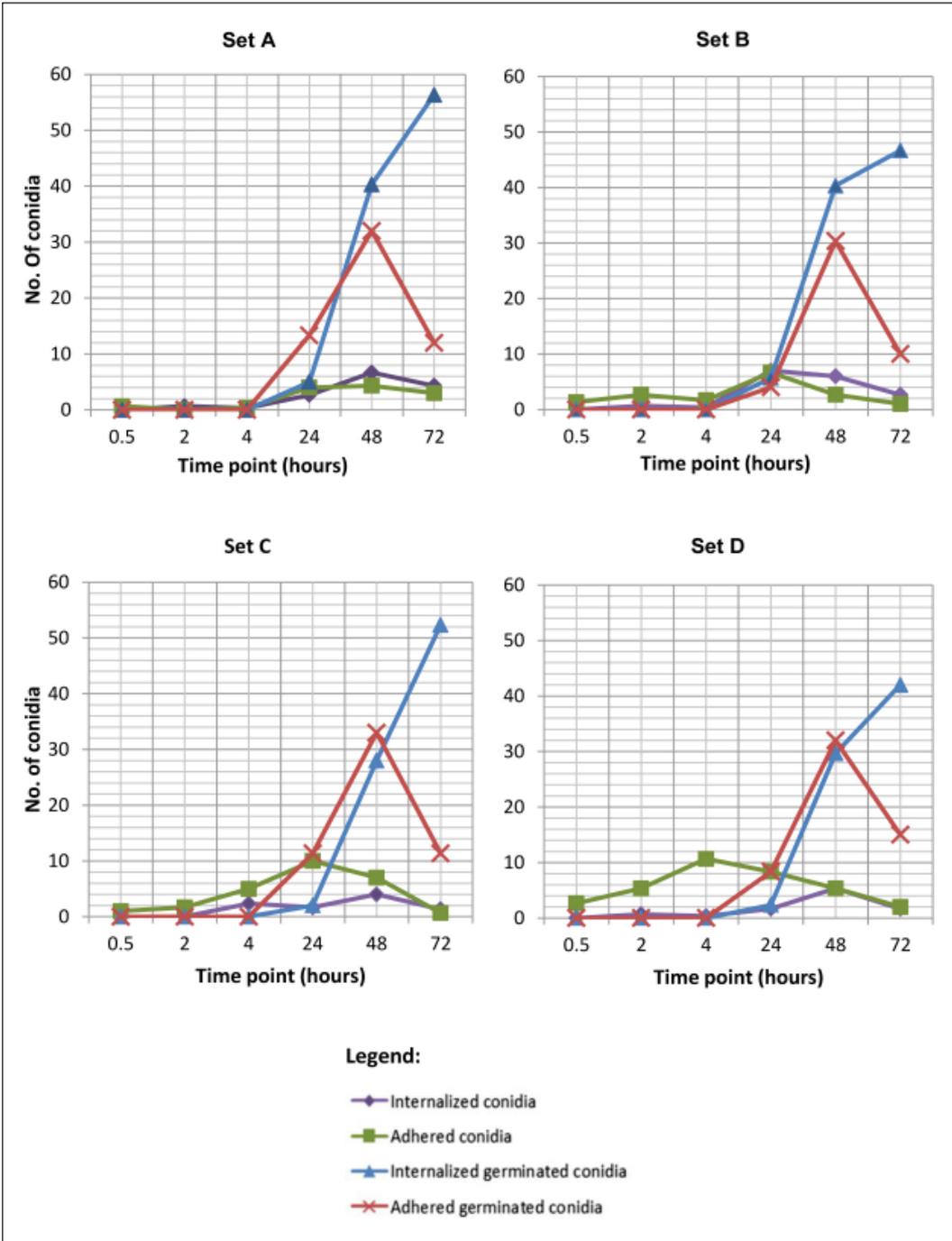


Figure 5. The average number of adhered and internalized *Cladosporium sphaerospermum* conidia and germinated *Cladosporium sphaerospermum* conidia of the three replicates in each set of experiment (Sets A-D). Cell line = BEAS-2B.

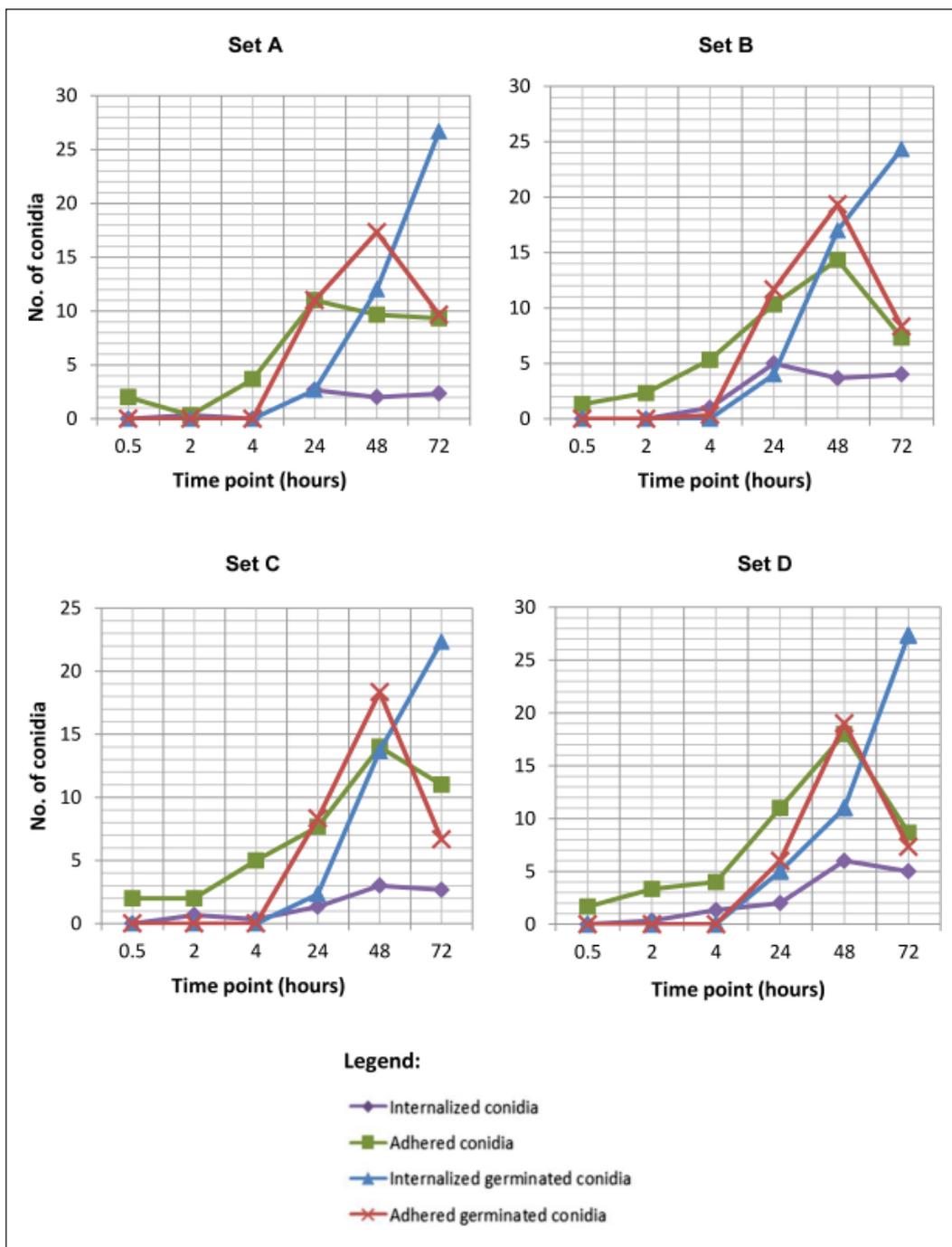


Figure 6. The average number of adhered and internalized *Cladosporium sphaerospermum* conidia and germinated *Cladosporium sphaerospermum* conidia of the three replicates in each set of experiment (Sets A-D). Cell line = HPAEpiC.

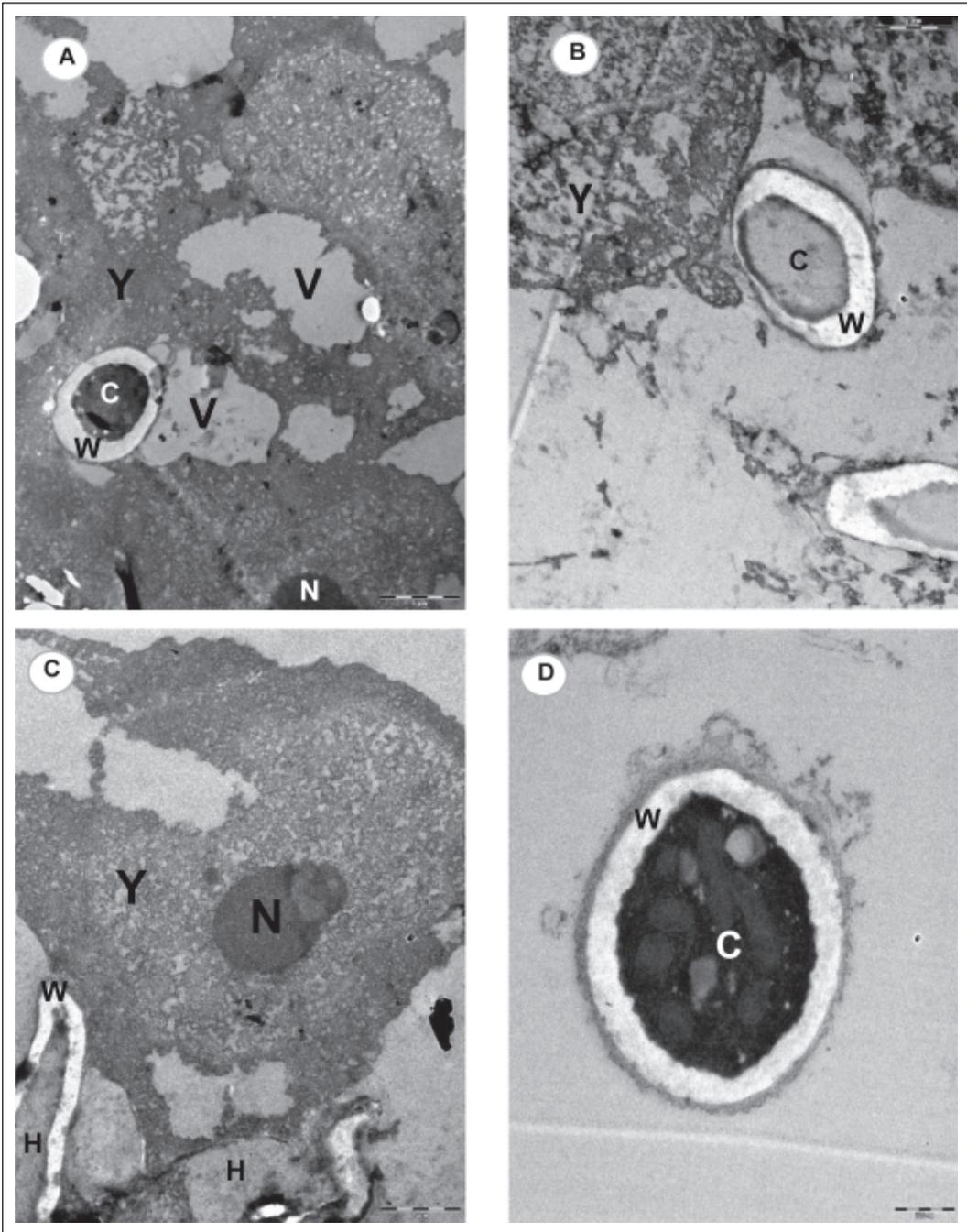


Figure 7. Transmission electron microscopic images showed invasion of *Cladosporium sphaerospermum* conidia into human bronchial epithelial cells (BEAS-2B) after co-incubation for 48 hours. (A) Internalized conidium in BEAS-2B cell. (B) The cell membrane is in contact with the conidium. (C) Cross section of hypha traversing an epithelial cell membrane and cytoplasm. (D) Ultrastructure of a conidium. Note: C: conidium; Y: cytoplasm; V: vacuole; W: cell wall; H: hyphae; N: nucleus. Scale bars correspond to 1 μ m except for (D) as 500 nm.

bronchial and alveolar epithelial cells with *Cladosporium* conidia was conducted at 32°C. At this temperature, the conidia were able to germinate to form hyphae but not at 37°C (data not shown).

According to the study conducted by Botterel *et al.* (2008), approximately 30–50% of *Aspergillus fumigatus* conidia that were attached to the human bronchial epithelial cell surface were internalized into late phagosomes without undergoing germination. The conidia may remain viable even after being internalized into the phagosomes. Germination of the adhered conidia might damage the cells. Amitani *et al.* (2009) suggested that there might be at least three pathways in which *Aspergillus* invades the bronchial mucosa following the inhalation of conidia: (1) penetration of hyphae through the intercellular spaces in the epithelium; (2) direct penetration of hyphae through epithelial cells; and (3) internalization of conidia within epithelial cells. In the present study, the exact location of *Cladosporium* conidia internalization and invasion could not be pinpointed as it needs the aid of animal model for the localization and comparison of the invasion between *Cladosporium* and *Aspergillus* conidia.

A previous study conducted to investigate the interaction of *Aspergillus fumigatus* conidia and alveolar epithelium using A549 cells reported that the conidia adhered to the cells and initiated rapid cell retraction and loss of focal adhesions. This enables the fungal hyphae to penetrate the basal membrane layer easily and enter the bloodstream. Some of the conidia are endocytosed into the cells while most of them germinated externally on the alveolar surface (Osherov *et al.*, 2012).

There are several other dematiaceous fungi that can also cause pulmonary infection such as *Scedosporium prolificans*, *Cladophialophora bantiana*, *Chaetomium* spp., *Ochroconis gallopava*, *Exophiala* spp., *Alternaria* and *Cladophialophora boppii* (Revankar and Sutton, 2010). An artificial infection of an *ex-vivo* skin model with *Exophiala dermatitidis* was reported by Poyntner *et al.* (2016) and TEM revealed

that *E. dermatitidis* was able to grow and penetrate into the skin fibers (Poyntner *et al.*, 2016). *E. dermatitidis* has been reported to cause fungal pneumonia in a young girl (Kusenbach *et al.*, 1992). For the other dematiaceous fungi, most of the studies reported on experimental therapy or antifungal susceptibility test (Pellon *et al.*, 2017; Al-Abdely *et al.*, 2005; Mariné *et al.*, 2009; Serena *et al.*, 2003; Munchan *et al.*, 2009; Deng *et al.*, 2016).

The findings in this study further proved that *C. sphaerospermum* is able to invade the bronchial and alveolar epithelial cells other than eliciting allergic responses in sensitized individuals. The fact that *C. sphaerospermum* conidia were internalized by these epithelial cells suggested that the internalization might be important in the development of the disease. However, it is unsure why there were more adhered and internalized germinated conidia in the BEAS-2B cells than in the HPAEpiC cells. A study conducted by Schulz *et al.* (2002) also reported that distinct pathways exist for LPS-induced activation of bronchial and alveolar epithelial cells (Schulz *et al.*, 2002). A few studies reported the difference in gene expression between bronchial epithelial cells and alveolar epithelial cells after infection by *A. fumigatus* (Sun *et al.*, 2012; Balloy *et al.*, 2008; Sharon *et al.*, 2011). It is unclear what cellular components caused this discrepancy.

Additional studies are required to elucidate the fate of the intracellular conidia, as well as to determine whether the internalization of *C. sphaerospermum* conidia by the respiratory epithelial cells contributes to the host defence or not. Other than that, the precise mechanism of the internalization and penetration of *C. sphaerospermum* conidia and hyphae remains to be elucidated. One of the limitations of this study is that cell lines are not a complete respiratory epithelium tissue or even an organ. Hence, *in vitro* model of human bronchiole or alveoli with air-mucosal interface which more closely simulates the actual physiological conditions can be used in the future studies for the identification and

characterization of virulence factors which contribute to *Cladosporium* invasion (Amitani *et al.*, 2009).

Even though the present study was limited by the *in vitro* system which cannot accurately represent the *in vivo* microenvironment of the host. Yet, the findings of this study were further supported by Huyan *et al.* (2012) who investigated the pathogenicity of a clinical isolate of *C. sphaerospermum* (2×10^7 CFU/mL) in both immunosuppressed and immunocompetent Balb/c mice. *C. sphaerospermum* is able to establish localized infections with granulomatous dermatitis of scattered hyphae and spores on the inoculated abrasive skin wound and subcutaneous challenge, and cause systemic infections via intravenous challenged route in both immunocompetent and immunocompromised mice. Intravenous inoculation of *C. sphaerospermum* led to 60% mortality for immunocompromised mice by 5 days after inoculation while immunocompetent mice survived for 4-week until the end of the experimentation. Lungs were reported to be most susceptible to *C. sphaerospermum* infection with diffuse patchy necrosis, disruption of alveolar walls and interlobular septae, diffuse interstitial infiltration of lymphohistiocytes and multinucleated giant cells in the presence of aggregated hyphae and spores. There were moderate dilation and congestion of hepatic sinuses, mild swelling and fatty degeneration of hepatocytes, infiltration of Kupffer cells and multinucleated giant cells in the presence of some spores in the liver. Infected kidneys were with marked interstitial congestion, tubular epithelial vacuolation and lymphohistiocytic infiltration and occasionally with some spores.

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