



# Stimulation of *Cryptococcus neoformans* isolated from skin lesion of AIDS patient matures dendritic cells and promotes HIV-1 *trans*-infection

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## ABSTRACT

Dendritic cells (DCs) play a pivotal role in host defense against invaded pathogens including fungi, while DCs are targeted by fungi for deleterious regulation of the host immune response. A few studies have reported fungal modulation of DC function in these immunocompromised AIDS patients. *Cryptococcus neoformans* (*C. neoformans*) is referred as one of the opportunistic fungi of AIDS. Here, we isolated native *C. neoformans* from an AIDS patient and investigated its effects on DC activation and function. Stimulation of *C. neoformans* matured DCs, and enhanced DC-mediated HIV-1 *trans*-infection; moreover, *C. neoformans*-stimulated DCs promoted the activation of resting T cells and provided more susceptible targets for HIV-1 infection. Microbial translocation has been proposed as the cause of systemic immune activation in chronic HIV-1 infection. Understanding the potential effects of pathogens on HIV-1-DC interactions could help elucidate viral pathogenesis and provide a new insight for against the spread of HIV.

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## 1. Introduction

*Cryptococcus neoformans* (*C. neoformans*) is an encapsulated yeast that particularly infects immunocompromised hosts. In AIDS patients, its infection often causes meningo-encephalitis. Inhalation of infectious particles is the major pathway for establishing infection [1]. Once inside the lung parenchyma, the basidiospores or desiccated yeast of *C. neoformans* encounter the host immune cells such as dendritic cells (DCs) or macrophages [2].

The resident pulmonary DCs and alveolar macrophages play pivotal roles in regulating the innate immune response following pulmonary *C. neoformans* infection [3]. *C. neoformans* is an intracellular pathogen [4], it enters into endosomal and lysosomal pathways following DC phagocytosis and can be killed by lysosomal components [5]. Fc receptor II and multiple lectin receptors can be used for capture of capsular polysaccharide [6,7]. The endocyto-

sed whole yeast or glycoantigens can be degraded for antigen presentation to initiate effective T helper 1 lymphocytes-based protective immunity [8–13]. Cryptococcal DNAs also has been shown to activate mouse bone-marrow-derived myeloid DCs (BM-DCs) via a Toll-like receptor 9-dependent pathway, which contributes to the antifungal inflammatory responses [14–16].

However, DC maturation and function have been modulated by *C. neoformans* for deleterious regulation of host antifungal immune responses. As the predominant virulence factor, polysaccharide capsule shields cell-wall components from interactive DCs, thus hampering DC maturation and avoiding induction of an efficient T-cell response [17]. However, Siegemund et al. have reported that both encapsulated and acapsular strains of *C. neoformans* can induce the up-regulation of MHC-II and CD86 from mouse BM-DCs, but the acapsular mutant shows a better capacity for stimulation [18]. Understanding the modulation of *C. neoformans* on DC activation and function, especially in immunocompromised patients, could benefit mechanistic elucidation of the dysfunction of the host antifungal response and accelerated disease progression during pathogenic co-infections.

Here, we isolated native *C. neoformans* from an AIDS patient and investigated its effects on DC activation and function. Stimulation of *C. neoformans* matured DCs, and enhanced DC-mediated HIV-1 *trans*-infection; moreover, *C. neoformans*-stimulated DCs promoted the activation of resting T cells and provided more susceptible targets for HIV-1 infection.

**Abbreviations:** *C. neoformans*, *Cryptococcus neoformans*; CSF, cerebrospinal fluid; DCs, dendritic cells; H&E stain, hematoxylin and eosin stain; MDDCs, monocytes-derived DCs; PAS, periodic acid-Schiff; PLT, platelets; RBC, red blood cell; VLP, virus like particle; WBC, white blood cell.

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## 2. Materials and methods

### 2.1. AIDS patient and ethics statement

A hospitalized AIDS patient with fungal co-infection was recruited from the First Affiliated Hospital of Kunming Medical College, China. This study was reviewed and approved by the Medical Ethics Review Committee of Yunnan Province, Kunming, China. Written informed consent was provided by his legal guardians. The patient had a decreased whole blood cell count (white blood cells,  $7.1 \times 10^8/L$ ; red blood cells:  $1.49 \times 10^{12}/L$ ; platelets:  $2.5 \times 10^{10}/L$ ). His  $CD4^+$  T lymphocyte count was  $6/\mu l$  and  $CD8^+$  T lymphocyte count was  $38/\mu l$ .

### 2.2. *Neoformans*

Facial skin lesion tissues were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). *C. neoformans* was isolated and cultured on Sabouraud agar plates and further identified with API 20C AUX yeast identification system and Christensen urea agar. The cerebrospinal fluid (CSF) was stained with India ink. *C. neoformans* was subcultured for amplification, harvested and killed by boiling for 1 h. Fungal cell counts were determined by light microscopy, diluted at  $1 \times 10^8$  cell/ml in PBS.

### 2.3. Cells, virus stock and virus like particle

Human peripheral blood mononuclear cells (PBMCs) from healthy donors were purchased from Shanghai Blood Center (Shanghai, China).  $CD14^+$  monocytes or  $CD4^+$  T lymphocytes were further purified from PBMCs by using anti- $CD14$  or anti- $CD4$  antibodies-coated microbeads (Miltenyi Biotec), respectively. Monocytes-derived DCs (MDDCs) were generated from  $CD14^+$  monocytes cultured in presence of 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 6 days. MDDCs were stimulated with fungi for 48 h at a 1:10 ratio of cells and observed under light microscope. Human embryonic kidney 293 T cells (HEK293T) and  $CD4^+$  T cell line Hut/CCR5 are kind gifts from Dr. Li Wu (the Ohio State University, USA).

Single-cycle infectious HIV stocks were generated by calcium phosphate cotransfection of HEK293T cells with pLai- $\Delta$ -env-Luc and the expression plasmid for HIV-1 envelope protein (Env) of NL4-3 (X4-tropic) or JRFL (R5-tropic) as previously described [19,20]. Virus like particle (VLP), HIV-1-Gag-GFP/JRFL, was generated by cotransfection of HEK293T cells with a plasmid encoding HIV-Gag-GFP and with JRFL plasmid. The plasmids were kind gifts from Dr. Li Wu (the Ohio State University, USA). Virus stocks and VLP were quantified using p24<sup>gag</sup>-capture enzyme-linked immunosorbent assay and stored at  $-80^\circ C$ .

### 2.4. Flow cytometry

Cells were stained with specific monoclonal antibodies (mAbs) or isotype-matched IgG controls. mAbs against the following human molecules were used (clone numbers and resources are given): PE-CD83 (HB15e; eBioscience), PE-CD86 (IT2.2; eBioscience), PE-CD69 (FN50; eBioscience), APC-cy7-HLA-DR (LN3; eBioscience), PerCP-cy5.5-CD3 (OKT3, eBioscience), APC-Alexa Fluor750-CD11c (B-ly6; BD Pharmingen). Stained cells were detected with an LSRII flow cytometer (BD Pharmingen) and analyzed with FlowJo 7.6.1 software.

### 2.5. HIV-1 infection and cell-mediated viral trans-infection

MDDCs or MDDCs-stimulated primary T cells were pulsed with 5 ng p24<sup>gag</sup> amounts of HIV-luc/JRFL or HIV-luc/HXB2 for 2 h, and

cells were washed for culture for indicated time. HIV-1 infection was monitored by measuring the luciferase activity from cell lysates with a commercially available kit (Promega). For the assay of DC-mediated HIV-1 trans-infection, a cell co-culture system was adopted as described previously [19,20]. Briefly, pseudotyped HIV-luc/JRFL (5 ng p24<sup>gag</sup>)-loaded MDDCs were washed off cell-free viruses and co-cultured with Hut/CCR5 for 3 days, and viral infection was measured by detecting luciferase activity from cell lysates.

### 2.6. HIV-1 binding and internalization

HIV-1 binding and internalization were quantified by flow cytometry using the VLP (HIV-Gag-GFP/JRFL). MDDCs were incubated with 40 ng p24<sup>gag</sup> amounts of VLP for 2 h at  $37^\circ C$  and washed. Some cells were treated with 0.25% trypsin (without EDTA) for 5 min at  $37^\circ C$  to remove surface-bound VLP. The amounts of VLP associated with MDDCs were quantified by flow cytometry.

### 2.7. Confocal microscopy

The formation of virological synapses was observed by confocal microscopy. MDDCs were pulsed with HIV-1 VLP (40 ng p24<sup>gag</sup> of HIV-Gag-GFP/JRFL) and cocultured with Hut/CCR5 or PHA-p-activated primary  $CD4^+$  T cells for 30 min. Cells were seeded on the poly-L-lysine coated microscope slides and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 1 h at  $4^\circ C$ . Cells were immunostained with anti- $\beta$ -actin antibodies (AC-15, Sigma), followed by staining with Alexa-Fluor 555-labeled goat anti-mouse IgG (Invitrogen). Nuclei were indicated with DAPI. Slides were mounted with Fluorescent Mounting Medium (Dako) and observed using a laser scanning confocal microscope (Leica SP5).

### 2.8. T-cells activation and viral infection

Fungi-stimulated MDDCs or heat-killed fungi were used to coculture with or to treat allergenic resting  $CD4^+$  T cells for 48 h at the same ratio of cells. The T cells were gated based on CD3-positive populations, and the activation was monitored by detecting the transient surface expression of CD69 by flow cytometry. For viral infection, the activated T cells from DC-T cell co-cultures were purified by anti-CD3 antibody-coated magnetic beads and then challenged with 5 ng p24<sup>gag</sup> amounts of HIV-1/HXB2 for 2 h. After washing, the cells were further cultured for 5 days, and HIV-1 infection was detected with luciferase activity assay as mentioned above.

### 2.9. Statistical analysis

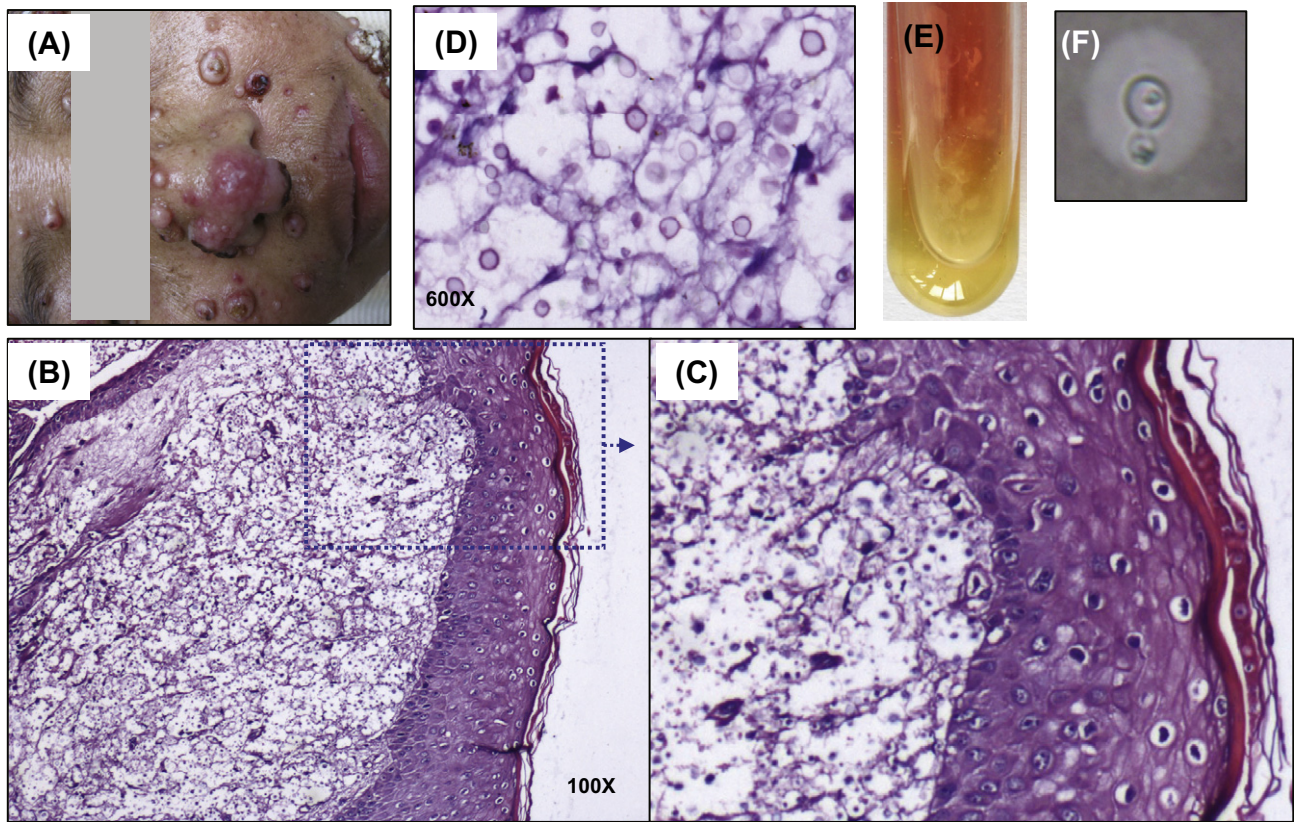
Statistical analysis was performed using paired *t* test with the SigmaStat 2.0 Software.

## 3. Results

### 3.1. Isolation of *C. neoformans* from AIDS patient

We investigated an AIDS patient with *C. neoformans* infection. The papules and nodules were distributed over the face, trunk and limbs. Some of the surface lesions displayed ulceration and blood scabs, and some papule surfaces had a wax-like luster (Fig. 1A). The H&E staining showed mucoid infiltration and many round, thick-walled spores in the dermis (Fig. 1B and C). Glycogen detection with PAS staining showed an abundance of round, oval fungal spores in the dermis (Fig. 1D). Fungal species were isolated and cultured on Sabouraud agar plates, and further identified by API 20CAUX yeast identification system. *C. neoformans* decomposes urea in Christensen urea agar to turn the medium red (Fig. 1E). CSF pressure was





**Fig. 1.** Characterization of *C. neoformans* isolated from an AIDS patient. (A) AIDS patient with *C. neoformans* infection. Facial skin lesion are shown. (B and C) H&E staining of tissue from facial skin lesions. Mucoid infiltration and many round, thick-walled spores with thick capsules were observed in the dermis. (D) PAS staining showed the fungal glycogen located in the dermis. (E) *C. neoformans* decomposed urea in Christensen urea agar to turn the medium red. (F) *C. neoformans* isolated from CSF showed the characteristics of encapsulated yeast-like cells with India ink staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

>330 mm H<sub>2</sub>O, and the round, thick-walled spores with translucent capsule were stained with India ink staining (Fig. 1F). These fungi were subcultured for amplification, counted using a hemocytometer, and heat-inactivated at 100 °C for 1 h.

### 3.2. *C. neoformans* matures MDDCs and impairs HIV-1 infection

To investigate DC activation by *C. neoformans*, MDDCs were co-cultured with fungi at a ratio of 1:10, and cell morphology was examined by light microscopy after 48 h incubation. *C. neoformans*-stimulated MDDCs were stretched on the culture plate compared with medium-treated control cells (Fig. 2A). MDDCs phenotype was also monitored by immunostaining of cell surface markers. The co-stimulatory molecules of CD83 and CD86 showed enhanced expression, increasing from 0.7% to 42.4% and 63% to 97.6%, respectively. The surface expression of HLA-DR also increased (Fig. 2B).

To examine the potential effects of *C. neoformans* stimulation on HIV-1 infection, *C. neoformans*-stimulated and medium-treated MDDCs were infected by single-cycle infectious virus HIV-luc/JFRL. Viral infection was assessed by measuring luciferase activity. HIV-1 infection was dramatically impaired in MDDCs after *C. neoformans* stimulation (Fig. 2C). These data indicated that the stimulation of *C. neoformans* can mature DCs and block HIV-1 infection.

### 3.3. *C. neoformans*-stimulated MDDCs activate resting-CD4<sup>+</sup> T cells for fueling HIV-1 infection

DCs can prime naïve T cells to differentiate into different subsets for bridging adaptive immunity. To address whether *C. neoformans*-

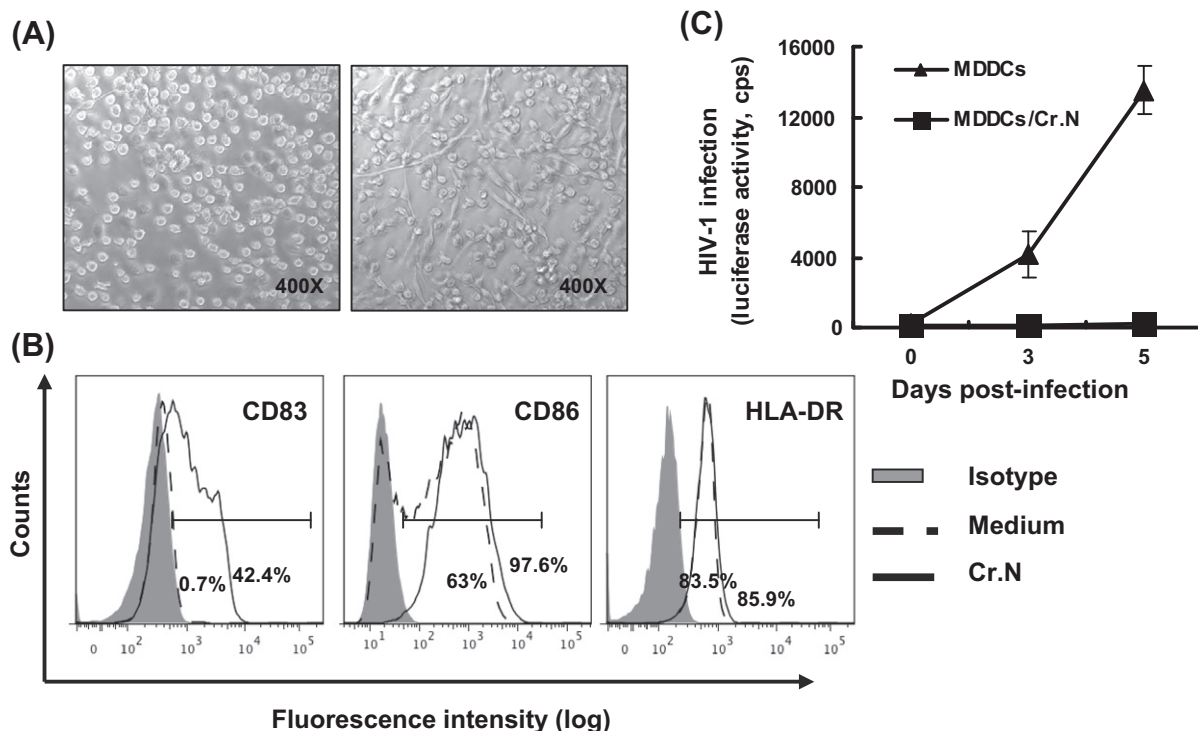
stimulated MDDCs can facilitate T-cell activation and provide more susceptible target cells for HIV-1 infection, purified resting CD4<sup>+</sup> T cells were co-cultured with *C. neoformans*- or medium-stimulated MDDCs for 48 h, and transient expression of CD69 on the cell surface was measured to evaluate cell activation. The CD4<sup>+</sup> T cells primed by *C. neoformans*-stimulated MDDCs enhanced CD69 expression (Fig. 3A), indicating that more T cells were activated.

To measure whether the activated T cells promoted susceptibility to HIV-1 infection, the activated T cells from co-culture were purified by anti-CD3 antibody-coated magnetic microbeads, and the susceptibility to HIV-1 infection was examined. The T cells activated by *C. neoformans*-stimulated MDDCs significantly promoted HIV-luc/HXB2 infection (Fig. 3B).

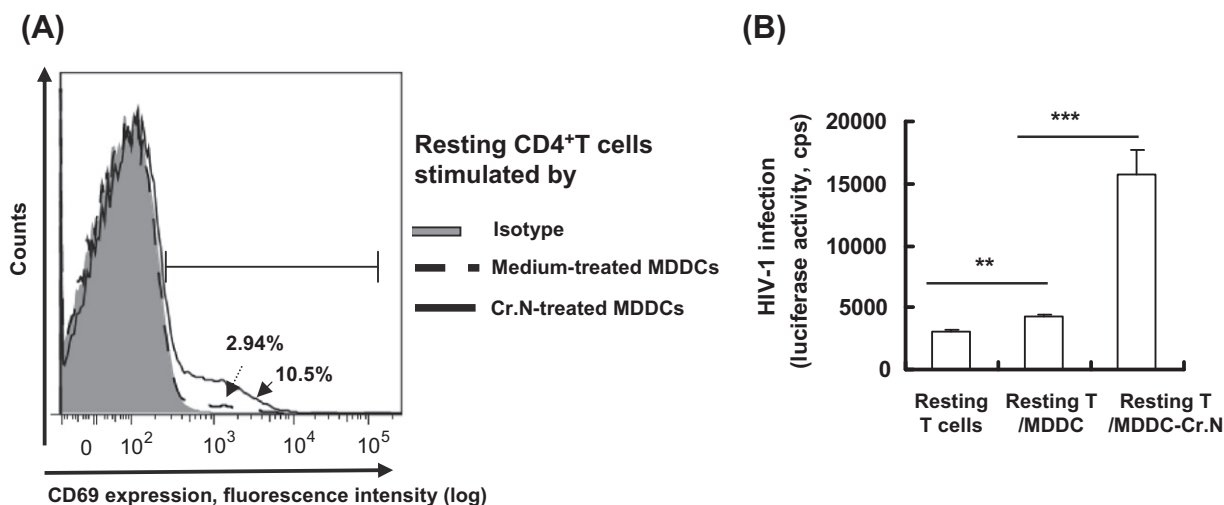
Taken together, these data demonstrate that *C. neoformans*-stimulated MDDCs activate resting-CD4<sup>+</sup> T cells and provide susceptible targets for HIV-1 infection.

### 3.4. *C. neoformans*-stimulated MDDCs facilitate HIV-1 transfer to T cells

DCs play double-edged roles during HIV-1 infection. DCs have been demonstrated to be hijacked by HIV-1 to transfer captured viruses to contacted T cells for replication [21]. To establish whether *C. neoformans*-stimulated MDDCs can facilitate HIV-1 trans-infection, the MDDCs stimulated with *C. neoformans* or medium were loaded with HIV-luc/JFRL, and then co-cultured with Hut/CCR5 cells. Viral transfer was measured by testing viral infection in T cells. *C. neoformans*-stimulated MDDCs promoted HIV-1 transfer to T cells (Fig. 4A).



**Fig. 2.** Phenotype of *C. neoformans*-stimulated MDDCs and HIV-1 infection. MDDCs were treated with *C. neoformans* or medium for 48 h, and cells were observed by light microscopy (A) or immunostained for visualization of cell surface markers. Expression of CD83, CD86 and HLA-DR was monitored by flow cytometry (B). (C) *C. neoformans*- or medium-stimulated MDDCs were infected with HIV-luc/JRFL (5 ng p24<sup>gag</sup>); viral infection was measured by detecting luciferase activity from cell lysates. Results of one representative experiment out of three are shown. Data are mean  $\pm$  SD. cps, counts per second. Cr. N, *C. neoformans*.



**Fig. 3.** *C. neoformans*-stimulated MDDCs facilitated activation of resting T cells. (A) Activation of resting CD4<sup>+</sup> T cells. Resting CD4<sup>+</sup> T cells were co-cultured with *C. neoformans*- or medium-stimulated MDDCs for 48 h, then CD3<sup>+</sup> T cells were gated, and the surface expression of CD69 was detected by flow cytometry. (B) Enhanced viral infection in primary CD4<sup>+</sup> T cells activated by *C. neoformans*-stimulated MDDC. Resting CD4<sup>+</sup> T cells were co-cultured with *C. neoformans*- or medium-stimulated MDDCs for 48 h, and the T cells were purified and infected by HIV-luc/HXB2 (5 ng p24<sup>gag</sup>). Three days later, viral infection was measured by detecting luciferase activity. Results of one representative experiment out of three are shown. Data are mean  $\pm$  SD. cps, counts per second. \*\* $P$  < 0.01, and \*\*\* $P$  < 0.001 were considered significant differences in Student's  $t$  test.

DC activation and altered viral intracellular trafficking are associated with enhanced viral spread [20–22]. To elucidate the underlying mechanism for promoting viral transfer mediated by *C. neoformans*-stimulated MDDCs, viral binding and internalization were tested. The non-infectious VLP, HIV-gag-GFP/JRFL, was used. The *C. neoformans*-stimulated MDDCs took up much more VLPs compared with that of medium-treated MDDCs, and the GFP-positive

cells increased from 2.7% to 20.5%, as detected by flow cytometry (Fig. 4B). Notably, most of the VLP could not be removed with trypsin digestion from the cell surface of *C. neoformans*-stimulated MDDCs, suggesting cellular viral internalization. As expected, VLP could be removed with trypsin digestion from cell surface of immature MDDCs (Fig. 4B). It has been demonstrated previously that immature DCs-mediated viral uptake mainly depends on viral surface

binding. The endocytosed viruses have been demonstrated to be sequestered into non-lysosome, non-classical multiple vesicular bodies, probably for escaping cellular proteolysis [20–22].

The formation of DC-T cell conjunction, or so-called virological synapses, at which many intact viral particles and viral receptors can be recruited, appears to be required for efficient viral transfer [20–22]. We hence compared the formation of virological synapses, and both Hut/CCR5 T cells and PHA-p-activated primary CD4<sup>+</sup> T cells were used as recipients. Compared with medium-treated MDDCs, *C. neoformans*-stimulated MDDCs recruited much more VLPs at the site of cell-cell contact, indicating a stronger formation of virological synapses between T cells with *C. neoformans*-stimulated MDDCs (Fig. 4C).

Taken together, these results demonstrate that *C. neoformans*-stimulated MDDCs can endocytose much more viruses and recruit these viruses to the site of cell-cell contact, which are ready for transfer to T cells.

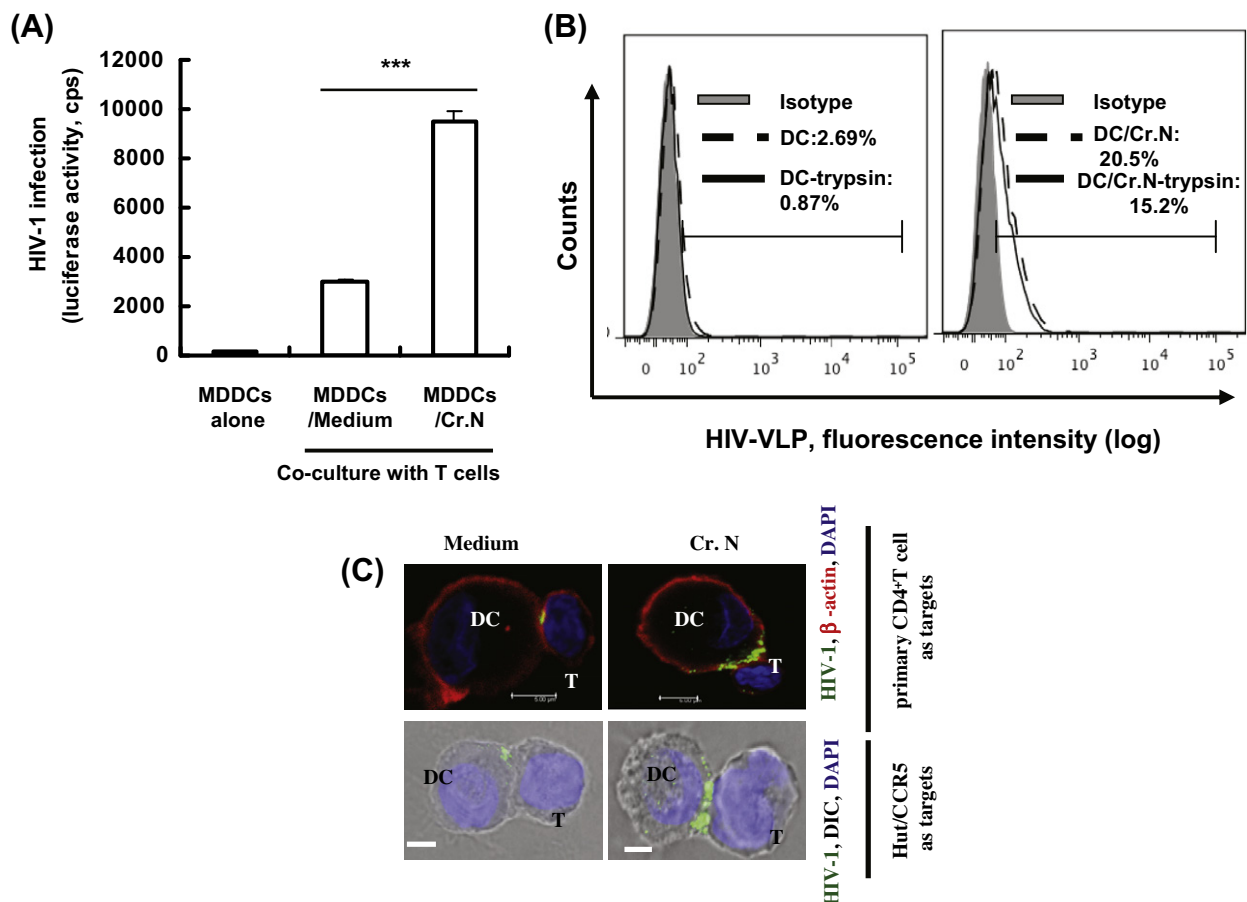
#### 4. Discussion

By using native *C. neoformans* isolated from the skin lesion of an AIDS patient, instead of a previously reported lab-adapted strain, we demonstrated that MDDCs could be activated by *C. neoformans* stimulation. Expression of co-stimulatory molecules of CD83 and CD86 was up-regulated, and the ability of DCs to activate resting T cells was promoted. *C. neoformans*-stimulated MDDCs facilitated

HIV-1 *trans*-infection of T cells, and the activated CD4<sup>+</sup> T cells provided more susceptible targets for HIV-1-amplified infection. Our results emphasized the deleterious modulatory effects of opportunistic pathogens on the compromised host immunity.

Whether *C. neoformans* stimulation triggers DC maturation remains controversial [8,18,23,24]. It is believable that the fungal polysaccharide capsule can shroud cell wall components from host cells, thus preventing from maturation and an anti-fungal immune response [17]. When the acapsular and encapsulated strain of *C. neoformans* are compared, the mutant strain shows easier phagocytosis into immature DCs and triggered the expression of many host genes, including those encoding cell surface receptors, cytokines, chemokines, etc. [25]. However, it has also been reported that both encapsulated and acapsular strains of *C. neoformans* can induce the release of IL-12/23 p40 and the upregulation of MHC-II and CD86 from mouse BM-DCs, despite better stimulation being mediated by the acapsular mutant [18]. These discrepancies may attribute to different fungi resources or fungal components were used.

Other opportunistic pathogens, such as *Malaria hemozoin*, *Mycobacterium tuberculosis*, and fungi species including *Candida albicans* and *Penicillium marneffei*, have previously been shown to activate DCs and enhance DC-mediated HIV-1 *trans*-infection [19,26–29]. More intact HIV-1 particles could be endocytosed into fungi-stimulated DCs. The internalized viral particles are sequestered into non-lysosome CD81<sup>++</sup> CD63<sup>+</sup> intracellular vesicles, which poten-



**Fig. 4.** *C. neoformans*-stimulated MDDCs promote HIV-1 transfer to T cells. (A) Increased HIV-1 transfer to T cells mediated by *C. Neoformans*-stimulated MDDCs. HIV-luc/JRFL-loaded *C. neoformans*- or medium-stimulated MDDCs were co-cultured with Hut/CCR5 for 3 days, and HIV-1 transfer was detected by measuring viral infection. Data are mean  $\pm$  SD. cps, counts per second. \*\*\* $P$  < 0.001 was considered a significant difference in Student's  $t$  test. (B) Enhanced viral uptake in *C. neoformans*-stimulated MDDCs. MDDCs were treated with *C. neoformans* or medium as above, and were cultured with HIV-gag-GFP/JRFL (5 ng p24<sup>gag</sup>). MDDCs were digested or not with 0.25% trypsin and GFP level was detected by flow cytometry. Positive cell percentage was labeled. (C) Enhanced virological synapses between T cells with *C. neoformans*-stimulated MDDCs. HIV-gag-GFP/JRFL-loaded MDDCs were co-cultured with Hut/CCR5 or PHA-p-activated CD4<sup>+</sup> T cells for 30 min, and fixed for immunostaining with specific antibodies. Formation of virological synapses was observed under fluorescence microscopy. Scale bar, 5  $\mu$ m.



tially prevents viruses from cellular proteolysis [20–22]. The addition of T cells could trigger the recycling of intact viruses back to the cell surface, which are ready for transmission [21,22]. Our data are consistent with previous reports that opportunistic-pathogen-stimulated DCs can form tighter junction with T cells, and more viruses are recruited at the site of cell–cell contact for transfer.

Inhibition of HIV-1 infection in MDDCs by *C. neoformans* stimulation might be due to stimulation of glucuronoxylomannan and galactoxylomannan, the components of fungal capsular polysaccharide. Lipopolysaccharides from Gram-negative bacteria restricts HIV-1 infection in MDDCs, and HIV-1 displays lack of efficiency for reverse transcription and post-integration events [22,30]. Similarly, we have demonstrated previously that DCs stimulated with other fungal species such as *C. albicans* or *P. marneffei* are less permissive for productive infection [19].

These fungus-stimulated DCs could prime resting T cells to provide susceptible targets for HIV-1 infection. This may explain how co-infecting pathogens accelerate the dramatic increase of viral load at the late stage of HIV-1 infection. Microbial translocation has been proposed as the cause of systemic immune activation in chronic HIV-1 infection [31]. Understanding the potential effects of pathogens on HIV-1-DC interactions could facilitate elucidation of viral pathogenesis and might provide a new insight for intervention against the spread of HIV-1.

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