Indinavir-treated *Cryptococcus neoformans* promotes an efficient antifungal immune response in immunosuppressed hosts

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A previous paper demonstrated that indinavir affects the virulence of *Cryptococcus neoformans*, thereby rendering the fungus more susceptible to killing by natural effector cells. This study demonstrates that inoculation of immunosuppressed mice with *C. neoformans* previously exposed to indinavir, in comparison to untreated *C. neoformans*, results in: (i) a more pronounced secretion of interleukin-12 by splenic dendritic cells; (ii) reduction of CD14 and FcγRs expression on splenic dendritic cells, and upregulation of CD86 and CD40 molecules; (iii) enhancement of interferon-γ and interleukin-2 production by splenic T cells and increase of their proliferation in response to fungal antigens; and (iv) survival from an otherwise lethal challenge, correlated with a drastic decrease in colony forming units from brain and liver. In conclusion, these data indicate that indinavir interaction with *C. neoformans* could be beneficial because of its direct influence on fungal virulence and blunting of the deleterious effects exerted by *C. neoformans* on host immune response. Thus, indinavir could be crucial in addressing the outcome of cryptococcosis in immunocompromised hosts.

**Keywords** *Cryptococcus neoformans*, indinavir, immune response, fungi, fungal virulence, immunoregulation

**Introduction**

*Cryptococcus neoformans* is the cause of life-threatening opportunistic infections in immunocompromised subjects including AIDS patients. The host defense against this pathogen is mainly mediated by cellular immunity [1], and cytokines secreted by type-1 Th cells play a central role in the establishment of protective immunity [2–5]. These mechanisms usually succeed in restricting the infection within the lung by preventing the pathogenic organism from disseminating to the central nervous system, whereas functional abnormalities such as defective interleukin (IL)-12 synthesis facilitate the spread of infection [5].

To date, amphotericin B and fluconazole represent the gold standard therapy for cryptococcosis, but this approach has important clinical limitations including toxic side-effects and, for fluconazole, development of resistance after prolonged therapy [6]. Thus, the search for powerful new drugs against cryptococcosis remains a crucial issue. The use of antiretroviral protease inhibitors (PI) as part of highly active antiretroviral therapy (HAART) in AIDS substantially increases the number of circulating CD4+ T cells [7,8]. In addition, the combined use of indinavir (IDV), an aspartyl-protease inhibitor, and nucleoside analog reverse transcriptase inhibitors in HAART has been shown to produce a strong decrease in viral RNA in plasma [9–11]. Furthermore, observational studies clearly indicate a beneficial effect of antiretroviral PI on certain...
opportunistic infections, including cryptococcosis, in AIDS [12,13]. With specific regard to cryptococcosis, HAART has been shown to induce clinical and microbiologic improvement. Based on the above evidence, we investigated the effects of IDV on C. neoformans and, in a previous paper, demonstrated that IDV decreased selected C. neoformans virulence factors rendering the fungus more susceptible to killing by natural effector cells [14].

Here we demonstrate that inoculation into immunocompromised hosts of C. neoformans previously exposed to IDV (IDV-Cn) results in greater secretion of IL-12 by splenic dendritic cells (DC), modulation of activatory, and costimulatory (CS) molecules on splenic DC, enhancement of IFN-γ, and IL-2 production by splenic T cells, and increase in their proliferation in response to the fungus. Moreover, mice challenged with IDV-Cn showed prolonged survival and reduced fungal burden in target organs in respect to mice infected with untreated C. neoformans (Cn).

Materials and methods

Reagents and media

Indinavir was obtained from Antonio Cassone (Istituto Superiore di Sanità, Rome, Italy). RPMI 1640 with L-glutamine and FCS were obtained from Gibco BRL (Paisley, Scotland). Human serum (HS) was purchased from Sigma (St. Louis, Missouri, USA). All reagents and media were negative for endotoxin, as assessed by Limulus ameocyte lysate assay (Sigma).

Microorganism

A thinly encapsulated strain of C. neoformans var. grubii (N° 6995 = NIH 37) was obtained from Central Bureau voor Schimmel (CBS) Cultures (Delft, The Netherlands). The cultures were maintained by serial passages on Sabouraud agar (BioMérieux, Lyon, France). The cells were harvested by suspending a single colony in saline, washed twice, counted in a hemocytometer and adjusted to the desired concentration. For in vitro treatment, 1 × 10⁹/ml C. neoformans were incubated for 48h in RPMI in the presence of 10 or 25 µg/ml IDV, then washed, resuspended in saline, and used for challenge. Heating at 60°C for 30 min was used to kill C. neoformans cells (HI-Cn).

Mice and infection

Female, 8–10 weeks old, inbred BALB/c mice were obtained from Harlan Nossan Laboratories (Milan, Italy) and housed at the Animal Facilities of the University of Perugia, Perugia, Italy. Procedures involving animals and their care were conducted in conformity with national and international laws and policies. To induce systemic infection the mice, treated with 150 mg/kg cyclophosphamide intraperitoneally 3 days before the challenge, were injected intravenously with 1 × 10⁸ C. neoformans cells untreated or previously exposed to 10 or 25 µg/ml IDV. Infected animals were monitored for organ clearance and survival. Quantification of fungal growth, at different times after infection, was assessed by plating serial dilutions of brain and liver homogenates onto Sabouraud agar. For histology, 5 days after infection brains and livers were excised and immediately fixed in formalin. Sections (3–4 µm) of paraffin-embedded tissues were stained using the periodic acid-Schiff procedure.

Cell separation

DC or CD4⁺ T lymphocytes were separated from spleens using N-418 or L3T4 mAbs-conjugated MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by magnetic separation according to the manufacturer’s instructions.

Cytokine and NO determination

DC (4 × 10⁵) were incubated overnight in RPMI 1640 +10% FCS in a 96 well plate at 37°C and 5% CO₂ alone or in the presence of HI-Cn (1.6 × 10⁶) in a final volume of 200 µl. After incubation, culture supernatants were collected and the level of IL-12 p70 was determined by a commercially available specific ELISA kit (Biosource, Camarillo, CA, USA); NO was evaluated in supernatants by the Griess method [15]. In selected experiments, CD4⁺ T lymphocytes (1 × 10⁹) co-cultured with DC (1 × 10⁶) overnight in RPMI +10% HS in a 96 well plate at 37°C and 5% CO₂ in the presence or absence of HI-Cn (1 × 10⁶) in a final volume of 200 µl. After incubation, culture supernatants were collected and the levels of IL-2 and IFN-γ were determined by commercially available specific ELISA kits (Biosource). Cytokine titers were calculated by reference to standard curves, constructed with known amounts of recombinant cytokines.

Flow cytometry analysis

For analysis of activatory or costimulatory molecule surface expression, purified DC were incubated for 40 min with labeled monoclonal antibodies to CD14 (1 µg/10⁶ cells), MHC-I (1 µg/10⁶ cells), MHC-II (1 µg/10⁶ cells) (all from BD Biosciences, San Jose, CA,
USA), CD16/32 (10 μl per test), CD86 (10 μl per test), CD40 (10 μl per test) (all from Chemicon Int., Temecula, CA, USA), washed twice with fluorescence buffer (FB), fixed with formalin 10% for 10 min at room temperature, resuspended with FB, and analysed using a FACSscan flow cytofluorometer (BD Biosciences, Franklin Lakes, NJ, USA). Purified DC were also incubated with antibodies to SIGN-R1 (1 μg/ml) (R&D Systems, Minneapolis, MN, USA) as above described, followed by labeled Cy3 conjugated affinity purified secondary antibody (dil. 1/100) (Chemicon Int.) and analysed using a FACSscan flow cytofluorometer. Non-viable cells were excluded from analysis by accepted procedures involving propidium iodide and narrow forward-angle light scatter gating. Control staining of cells with irrelevant antibody was used to obtain background fluorescence values. Data are expressed as mean fluorescence intensity (MFI) of labeled cells.

**Proliferation assay**

DC or CD4+ T lymphocytes were purified from spleens of mice infected with Cn or IDV-Cn as described above. As positive control 1 × 10^5 DC plus 1 × 10^6 CD4+ T lymphocytes were stimulated with 5 μg/ml of PHA (Sigma) or HI-Cn (1 × 10^5) in round-bottomed 96-well plates (final volume 200 μl). Cells were cultured for 4 days in RPMI+10% HS at 37°C, 5% CO₂. Eighteen hours before harvesting, cells were pulsed with 0.5 μCi of [3H] thymidine per well. Incorporation into cellular DNA was measured by liquid scintillation counting. The results are expressed as mean cpm ± SE of triplicate cultures.

**Statistical analysis**

Statistical analysis was performed with the Primer of Biostatistics software program. Data are reported as the mean ± standard error from replicate experiments. Data were evaluated by one-way analysis of variance (ANOVA). Post-hoc comparisons were done with Bonferroni’s test. A value of p < 0.05 was taken as significant. Differences in mortality were evaluated according to the Mann Whitney U Test.

**Results**

We previously demonstrated that IDV affects selected virulence factors of *C. neoformans* [14]. In the present study, we questioned whether the effects evidenced in vitro would correlate with a more pronounced clearance of the fungus in vivo. Moreover, we exploited the possibility that treatment with IDV could affect the immune response against the fungus. Given that cryptococcosis occurs in immunocompromised hosts including AIDS patients and that IDV is a usual treatment, we designed an experimental system in which mice were immunodepressed with 150 mg/kg of cyclophosphamide 3 days before the challenge.

Interleukin-12 is critical for induction of protective response against *C. neoformans* [2,16]. We, therefore, tested IL-12 production by splenocytes after intravenous challenge of mice with Cn or IDV-Cn. The results, reported in Fig. 1A, show that when compared with Cn, IDV-Cn induced greater increase of IL-12 production by splenocytes, either not stimulated (NS) or stimulated in vitro with HI-Cn. A similar effect was observed using purified splenic DC (Fig. 1A). Differently from IL-12, IL-10 secretion tested on total splenocytes or purified splenic DC cultures did not show any variation (not shown).

To rule out the possibility that the observed effects could be due to IDV released from fungal cells during infection, we evaluated the presence of the drug in supernatants of *C. neoformans* cultured with IDV for 48 h, washed, counted, resuspended in fresh medium, and cultured for an additional 5 or 7 days at 37°C in...
5% CO₂. Culture supernatants assayed by HPLC showed that IDV is not released by fungal cells, as drug concentration was below the detection limit (<0.1 mg/l) (data not shown). Furthermore, the same culture supernatants inoculated in BALB/c mice did not cause significant modulation in splenic T lymphocytes phenotype (data not shown).

We previously demonstrated that natural effector cells showed more efficient killing against IDV-Cn than Cn [14]. To verify whether this effect was reproduced in vivo during immunosuppression, we tested the ability of unfractonated splenocytes or splenic-purified DC from mice challenged with Cn or IDV-Cn to produce nitric oxide (NO), considered an important factor for anticytotoxic activity [17]. The results show that unfractonated splenocytes as well as DC, either unstimulated or stimulated in vitro with HI-Cn, produced higher NO levels when animals were challenged with IDV-Cn. (Fig. 1B).

Dendritic cells play a critical role in handling pathogens for processing and presentation to T cells, thus eliciting an appropriate immune response [18]. Given that, in our experimental system, DC secretory activity, evaluated in terms of IL-12 production, appeared to be modulated, we analysed the expression of molecules involved in DC maturation and activation. In particular, mature DC express low levels of CD14, Fcγ receptors (FcγR), such as CD16 and CD32, and high levels of CS molecules. The results reported in Fig. 2A show that Cn systemic infection resulted in increased expression of CD14 and CD16/CD32 on splenic DC, whereas IDV-Cn challenge attenuated upregulation. These results suggest that, in immunosuppressed mice, C. neoformans inhibits maturation of DC but the pretreatment of the fungus with IDV leads to a subversion or attenuation of this effect.

Costimulatory molecules and MHC are fundamental in promoting an efficient Ag presentation process, therefore they are highly expressed on mature DC [18]. We evaluated whether treatment with Cn or IDV-Cn could modulate their expression on DC from immunosuppressed mice. To this end, expression of CD40, CD80, CD86, and MHC class I and II on splenic DC was evaluated. The results reported in Fig. 2B and C indicate that Cn downregulated expression of CD40, CD86, MHC class I, and upregulated expression of MHC class I, while CD80 (not shown) was unmodified. IDV-Cn attenuated the inhibitory effect evidenced on CD40 and CD86 (Fig. 2B), but did not modify downregulation of MHC class II (Fig. 2C) or upregulation of MHC class I (Fig. 2C).

Specific ICAM-3-grabbing nonintegrin (SIGN-R1) are considered negative signaling molecules that mediate escape mechanisms for pathogens [19]. It is noteworthy that Cn strongly augments expression of receptors recognizing antibodies to SIGN-R1 on splenic DC, whereas IDV-Cn does not (Fig. 2D).

The efficiency of splenic DC was evaluated by assaying their capacity to induce specific T cell activation. To this end, DC were co-cultured with CD4⁺ T cells from mice infected with Cn or IDV-Cn. The results
reported in Fig. 3A show that T cell blastogenic response was significantly increased in response to IDV-Cn. In particular, co-cultures of DC and T cells from mice infected with IDV-Cn show a significantly higher specific blastogenic response than co-cultures from mice infected with Cn. This effect mirrored an enhancement of cytokine production by the same co-cultured cells (Fig. 3B). Indeed, analysis of IL-2 and IFN-\(\gamma\) in the same culture settings showed a higher level of production of both cytokines in co-cultures of cells from mice infected with IDV-Cn in respect to Cn. This was particularly evident after in vitro stimulation of cultures with HI-Cn (Fig. 3B).

Finally, we evaluated whether a more efficient response to IDV-Cn could reflect a better fungal clearance during infection. To this end, mice were injected intravenously with a lethal dose of Cn and the same dose of IDV-Cn, then survival and CFU recovery from brain and liver were evaluated. The results in Fig. 4A show that mice infected with Cn died in 3–5 days, while a significant increase in survival of mice treated with IDV-Cn was noted. In particular, the beneficial effect was maximum when C. neoformans was treated with IDV at a dose of 25 \(\mu\)g/ml, giving 80% of survivors (Fig. 4A). Concomitantly, CFU recovery from brain and liver showed a dramatic decrease when IDV-Cn was used (Fig. 4B). Histological examination of the brain (Fig. 5A and B) evidenced that capsule formation was inhibited in the brain of mice treated with IDV-Cn in respect to Cn, panel B and A, respectively. In addition, liver sections (Fig. 5C and D) showed inflammatory foci characterized by few mononuclear cells surrounding C. neoformans. Less heavy colonization and reduced capsule thickness was detected after challenge with IDV-Cn in respect to Cn (panel D vs C).

**Discussion**

In a previous paper we demonstrated that treatment of C. neoformans with IDV resulted in attenuation of some fungal virulence factors [14]. In the present study we demonstrate that the results obtained in vitro are
also applicable in vivo and, remarkably, in the immunocompromised host. Moreover, the results reported here show that C. neoformans exposed to IDV can somehow bypass or attenuate some immunosuppressive effects normally induced by the unexposed fungus.

Indeed, the effects of IDV-Cn were multiple and sometimes unexpected. First, a strong increase of NO production by splenocytes and splenic DC was evidenced. Moreover, systemic challenge with IDV-Cn increased production of IL-12 by splenic DC, while no modulation of IL-10 was observed. This suggests that a more efficient protective response is induced in the immunocompromised host and that DC respond, in terms of IL-12 production, to variations in IL-12 expression by splenocytes and splenic DC was evident. Moreover, CD86 was decreased. It is noteworthy that IDV-Cn partially abrogated the inhibitory activity exerted by Cn on CD40 and CD86. Moreover, CD80 was poorly expressed and did not show any variation after infection with Cn or IDV-Cn. Therefore, IDV seems to attenuate the mainly negative regulation occurring in cryptococcosis. The influence of the drug on specific virulence factors such as proteases, urease and capsule formation [14] could play a key role in quenching the escape mechanisms that C. neoformans uses to survive in the host, thereby promoting more efficient DC function.

Splenic DC from mice infected with IDV-Cn induced a more pronounced T cell blastogenic response, together with increased production of IL-2 and IFN-γ, in respect to mice infected with Cn. This suggests that DC-induced maturation and activation were corroborated by an efficient APC function, instrumental in the development of a proficient specific immune response. Clear evidence for generation of a protective response was provided by the survival rate and prompt clearance of CFU from brain and liver of immunosuppressed mice challenged with IDV-Cn, while animals infected with Cn died in 2–5 days.

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The results of this study support our previous data and add new insights into the interaction between IDV and C. neoformans. The previous in vitro results showed that IDV can attenuate several virulence factors rendering the fungus more susceptible to natural effector cells [14]. However, the in vitro effects could have been non sequitur in infection, considering that in vivo optimal conditions occur for C. neoformans capsule formation and secretion of virulence factors [14]. In addition, the effects exerted by IDV in vitro could have possibly been too early and transient to appreciate the subtle differences in fungal virulence, particularly in immunocompromised hosts presenting several, undefined failures in generating an effective response to the insulting microorganism. The results reported here showing the beneficial effect of IDV in quenching the deleterious effects exerted by C. neoformans on immunity are particularly relevant given the difficulty of immunosuppressed patients to mount a protective response, and the incapacity of available drugs to eradicate the fungus. Thus, the ability of IDV to facilitate and improve antifungal protective response in immunosuppression supports the possibility of exploiting IDV as a potential therapeutic agent in cryptococcosis.

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