Interleukin-12 Counterbalances the Deleterious Effect of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein gp120 on the Immune Response to *Cryptococcus neoformans*

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The mechanism involved in the envelope glycoprotein gp120–induced Th2 response to *Cryptococcus neoformans* was investigated. Peripheral blood mononuclear cells (PBMC) from healthy donors were treated with human immunodeficiency virus gp120 and an encapsulated or acapsular strain of *C. neoformans* in the presence or absence of glucuronoxylomannan, the major capsular polysaccharide. gp120 inhibited early and late production of interleukin (IL)–12 by PBMC. This reduction paralleled IL-10 induction and inhibited translocation of CD40 to the surface of monocytes. Flow cytometric analysis revealed that gp120 down-regulated the expression of IL-12 receptor β2 subunit on T cells responding to *C. neoformans*. Because the IL-12/IL-12 receptor β2 subunit pathway is critical for the Th1 differentiation process, underexpression demonstrates that gp120 contributes to Th2 bias. Exogenous IL-12 added simultaneously with gp120 up-regulated interferon-γ secretion and limited IL-4 production. These results suggest that gp120 limits the Th1 response to *C. neoformans* and that exogenous IL-12 could offset this effect.

*Cryptococcus neoformans* is a pathogenic yeast that is responsible for invasive and potentially fatal infections in patients with impaired cell-mediated immunity, such as patients with AIDS [1]. Both cellular and humoral immunity play important roles in the control of infection [2]; however, many of the fine details of the host immune response to *C. neoformans* remain to be determined. *C. neoformans* is not an obligate intracellular pathogen but is able to enter into human macrophages via complement receptors [3] or via specific antibody [4] and survive in the acidic phagolysosome [5].

Impaired anticyptococcal activity has been observed in monocytes from patients with AIDS [6, 7]. One potential mechanism for the decreased anticyptococcal activity is envelope glycoprotein gp120–induced inhibition of monocyte/macrophage function against *C. neoformans* [8]. Importantly, gp120 has been detected in the serum of patients with AIDS [9], and the participation of free gp120 in AIDS-related immunologic disorders, including a suppressive effect on the T cell response to recall antigens, has been reported elsewhere [10].

Recently, we demonstrated that gp120 exerts an antiproliferative effect on T cells responding to *C. neoformans* and promotes the emergence of a Th2-dominant response [11]. In particular, T cells cocultured with cryptococci-laden monocytes in the presence of gp120 displayed enhanced interleukin (IL)–4 secretion and almost complete abrogation of interferon (IFN)–γ production relative to the levels of cytokine secretion observed in the absence of gp120 [11]. Analysis of the intracellular levels of these cytokines revealed that gp120 regulates the synthesis of IL-4 and IFN-γ [11].

Studies have shown that polarization of human T cells to Th1 or Th2 is regulated primarily by IL-12 production by antigen-presenting cells and IL-4 production by T cells [12]. In addition, a defect in IL-12 production during human immunodeficiency virus (HIV) infection [13] and involvement of gp120 as an inhibitor of IL-12 secretion have been observed [14].

In a previous study, we demonstrated a biphasic pattern of IL-12 production by human monocytes exposed to *C. neoformans*. During the first phase, IL-12 secretion is dependent on the direct effect of fungal insult; the second phase is characterized by secretion of IL-12 that is dependent on the presence of IFN-γ and ligation of CD40 molecules on monocytes, with CD40 ligand expressed on T cells [15].

The purpose of this study was to examine the influence of gp120 on *C. neoformans*–induced IL-12 production, the influence of gp120 on early- and late-phase IL-12 secretion in response to *C. neoformans*, the mechanisms involved in gp120-induced IL-12 production, and whether and to what extent the addition of recombinant human IL-12 is able to reverse the gp120-induced Th2-dominant response to *C. neoformans*. 

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Materials and Methods

Reagents and media. RPMI 1640 with glutamine and fetal calf serum (FCS) were obtained from Gibco BRL. Human serum type AB was purchased from Sigma. HIV-1 recombinant gp120 (strain LAI) was obtained from a mammalian (CHO) expression system (Intracel) and had been used previously in studies that provided the background for the present set of experiments [11, 16]. gp41 was purchased from DuPont NEN Research Products. Mouse monoclonal anti-human CD40 (IgG1; catalog no. 189.020), R-phycocerythrin-conjugated mouse monoclonal anti-human CD14 (IgG2; catalog no. 163-050), and mouse monoclonal anti-human CD4 (IgG1; catalog no. 147.020) were obtained from Ancell. Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (Fab'); catalog no. F2838 and an irrelevant mouse monoclonal antibody (IgG1; catalog no. M5284) were purchased from Sigma. Glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans*, was isolated from culture supernatant fluid of serotype A strain CN6 by differential precipitation with ethanol and cetyl-trimethyl-ammonium bromide [17]. Human recombinant IL-12 was from the Genetic Institute. All media and buffers used in this study were tested for endotoxin contamination by limulus amebocyte lysate assay (Sigma), which has a sensitivity of >0.05–0.1 ng/mL *Escherichia coli* lipopolysaccharide. All tested media and buffers were negative.

Isolation and culture of peripheral blood monocytes. Heparinized venous blood was obtained from healthy volunteers and was diluted with RPMI 1640. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation over Ficoll-Paque Plus (Pharmacia Biotech) [18]. PBMC were recovered, were washed twice in RPMI 1640 supplemented with 5% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL), were plated into cell culture petri dishes (Nunc), and were incubated for 1 h at a concentration of 2–3×10⁶/mL. Nonadherent cells were removed by washing the dishes 3–5 times with prewarmed RPMI 1640 medium. Adherent cells (peripheral blood monocytes) were recovered by means of a cell scraper (Falcon), were washed twice, and were suspended in RPMI 1640 supplemented with 10% human serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). The harvested cells were 95%–98% esterase positive and >98% viable, as evaluated by trypan blue dye exclusion test. Nonadherent cells were E-rosetted as described elsewhere [19]. The cells recovered were T lymphocytes, >98% CD3⁺, as evaluated by flow cytometry analysis.

Microorganisms. *C. neoformans* 6995 is a thinly encapsulated strain of serotype A (obtained from Central Bureau Schimmel [CBS] Cultures [CBS 6995 = NIH 37]). *C. neoformans* 7698 is an acapsular mutant from CBS (CBS 7698 = NIH B-4131) The B-4131 strain, a well-characterized capsule-deficient mutant, was originally derived from serotype D strain B-3501 [20, 21]. *C. neoformans* 3168 is a heavily encapsulated serotype A strain (obtained from National Collection of Pathogenic Fungi [NCPF]; NCPF 3168). The cultures were maintained by serial passage on Sabouraud agar (BioMérieux). Log-phase yeasts were harvested by suspending a single colony in RPMI 1640, were washed twice with saline, were counted on a hemocytometer (Sigma), and were adjusted to the desired concentration in RPMI. All yeasts were killed before use by heating at 60°C for 30 min.

Flow cytometry analysis of surface and intracellular CD40. Surface molecule expression was quantified by flow cytometry after various times of culture. Monocytes (1×10⁶) were suspended in supplemented RPMI in polypropylene tubes and stimulated with encapsulated or acapsular strains of *C. neoformans* at an effector:target ratio of 1:2 or with GXM (250 μg/mL) or the acapsular strain plus GXM (250 μg/mL). GXM alone or in combination with the acapsular strain was added at the time of *C. neoformans* treatment, in the presence or absence of gp120 (500 ng/mL), and was incubated for different times. The effector:target ratio of 1:2 was used because our previous experiments found that this ratio was optimal for cytokine response and did not differ appreciably from the 1:15 or 1:10 ratio. In contrast, an inhibitory effect on cytokine production was found at an effector:target ratio >1:20. The dose of 250 μg/mL GXM was chosen on the basis of previous experiments, in which 250 μg/mL gave the best inhibitory effect, which was comparable to that obtained with 500 μg/mL. A lower dose (25 μg/mL) was less effective. As a positive control, peripheral blood monocytes were stimulated with IFN-γ (5 ng/mL) plus lipopolysaccharide (10 μg/mL). After incubation at 37°C in the presence of 5% CO₂, the cells were collected by centrifugation, fixed in 2% paraformaldehyde in PBS, washed twice in PBS containing 0.5% bovine serum albumin and 0.1% sodium azide, and mixed with mouse anti-human CD40. After 30 min of incubation on ice, the cells were washed twice, and the second antibody, FITC-conjugated anti-mouse IgG, was added. Monocytes were labeled with R-phycocerythrin-conjugated mouse monoclonal anti-human CD14, and 2-color analysis was done by use of a flow cytometer (FACScan; Becton Dickinson). The mean fluorescence of labeled samples was determined by use of logarithmic-scale histograms. Auto-fluorescence was assessed with untreated cells. An irrelevant FITC-conjugated isotype-matched antibody was used as a negative control in each experiment.

For determination of the intracellular pool of CD124, after 18 h of incubation, the cells were resuspended, were washed in PBS plus 0.5 m EDTA, and were fixed at room temperature for 10 min with 2% paraformaldehyde. Cells were permeabilized for 10 min with HEPES-buffered PBS containing 0.1% saponin (Sigma) and were stained with mouse anti-human CD40, FITC-conjugated anti-mouse IgG in HEPES-buffered PBS containing 0.1% saponin and 5% FCS, and then phycocerythrin-conjugated mouse anti-human CD14.

Flow cytometry analysis of surface IL-12R β2 receptor. PBMC (1 or 5×10⁶/mL) were stimulated with *C. neoformans* 6995 in the presence or absence of gp120 (500 ng/mL) for 5 days. After incubation, cells (3–5×10⁶) from each sample were incubated with 5 μg/mL rat anti-human IL-12 receptor β2 subunit (IL-12Rβ2; provided by F. Sinigaglia, Roche Milano, Milan, Italy), followed by incubation with FITC-conjugated anti-rat IgG (Sigma). Control samples were stained with the secondary antibody only. After 2 additional washes, cells were analyzed with a FACScan.

Determination of IL-12, IL-10, IL-4, and IFN-γ production. PBMC (5×10⁶) in the case of IL-12, IL-10, and IFN-γ; 2×10⁶ in the case of IL-4) were stimulated with gp120 (500 ng/mL) in the presence or absence of encapsulated or acapsular *C. neoformans* or with GXM (250 μg/mL) or the acapsular strain plus GXM (250 μg/mL). GXM alone or in combination with the acapsular strain was added at the time of *C. neoformans* treatment. Supernatants were
Results

An initial experiment evaluated the effect of gp120 on the early (T cell–independent) and late (T cell–dependent) secretion of IL-12 in response to stimulation with C. neoformans [15]. Addition of gp120 suppressed early (day 2) IL-12 secretion in response to the encapsulated strain and late (day 7) IL-12 secretion in response to the acapsular strain and experimental creation of cells that behave in many ways like encapsulated yeast cells [22]. When GXM and acapsular cryptococci were used in the experimental system, a similar suppressive action on the part of gp120 was observed; however, the magnitude of both the level of IL-12 secretion and the suppressive effect of gp120 was intermediate between those observed for acapsular and encapsulated cells (figure 1).

Statistical analysis. Statistical significance was determined by use of Student’s paired t test.
FIGURE 2. Effect of envelope glycoprotein gp120 on CD40 expression induced by encapsulated (6995) Cryptococcus neoformans or glucuronoxylomannan (GXM, 250 μg/mL). Monocytes untreated or treated with gp120 (500 ng/mL) in the presence or absence (NS, not stimulated) of stimuli at an effector:target ratio of 1:2 were incubated for 72 h. A, After fixation, cells were stained with IgG control (negative control) or fluorescein isothiocyanate-conjugated monoclonal antibody against CD40 and phycoerythrin-conjugated monoclonal antibody against CD14. Surface CD40 expression (X-axis) on CD14-positive cells (Y-axis) was analyzed by flow cytometry. As positive control, cells were stimulated with interferon-γ (5 ng/mL) plus lipopolysaccharide (10 μg/mL). Results are representative of 3 similar experiments from different donors. B, Percentage of CD40-positive cells. Results represent mean ± SE of 3 separate experiments from different donors. *P < .05 (gp120-treated vs. respective gp120-untreated cells); †P < .05 (GXM- or 6995-treated cells vs. untreated cells).
Figure 3. Effect of envelope glycoprotein gp120 on CD40 expression induced by acapsular (7698) or encapsulated (6995) Cryptococcus neoformans or glucuronoxylomannan (GXM, 250 μg/mL) or acapsular strain plus GXM (250 μg/mL). Monocytes untreated or treated with gp120 (500 ng/mL) in the presence or absence (NS, not stimulated) of stimuli at an effector:target ratio of 1:2 were incubated for 72 h. Surface and total CD40 expression on CD14-positive cells were analyzed by flow cytometry. Intracellular pool was determined by subtracting surface mean fluorescence from total mean fluorescence. Results are expressed as mean fluorescence intensity. Results represent mean ± SD of 4 separate experiments from different donors. * P < .05 (gp120-treated vs. respective untreated cells).

Discussion

In a recent study, we demonstrated that gp120 alters T cell activation and differentiation in response to C. neoformans and promotes a dominant Th2-type response [11]. The available evidence indicates that development of a Th1-type response is critical in promoting a protective response in C. neoformans infection and that IL-12 plays an important role [26]. On the other hand, an inhibitory effect of gp120 on IL-12 production by monocytes stimulated with Staphylococcus aureus has been observed [14]. In this study, we explored the mechanisms involved in gp120 promotion of a Th2 response to C. neoformans and the possibility of blocking this event.

Recently, we demonstrated that human monocytes treated with C. neoformans produce IL-12 in a T cell–independent and –dependent manner. This latter production is dependent on CD40-CD40 ligand interaction [15]. In the present report, we show that gp120 down-regulates early and late IL-12 secretion by monocytes in response to C. neoformans. This phenomenon paralleled down-regulation of CD40 molecule expression on monocytes, IL-10 up-regulation, and changes in IL-12Rβ2 expression on T cells responding to C. neoformans. Furthermore, the ability of gp120 to favor a Th2-type response is blocked by the addition of exogenous recombinant IL-12 such that IFN-γ secretion in increased and IL-4 secretion is decreased.

gp120 from a T cell–tropic HIV strain was used in our study, and different results might have occurred with gp120 from a monotropic HIV strain. However, the ability of gp120 to bind to a coreceptor such as CXCR4 or CCR5 may not be relevant to our studies, because monoclonal antibody to CD4 blocks the effects of gp120.

Recently we observed that gp120 promotes Th2 generation in response to C. neoformans by inhibiting IFN-γ production, as well as by promoting IL-4 [11]. IL-12 plays a key role in induction of the Th1 response [12]. Consequently, the capability of gp120 to inhibit IL-12 secretion may explain, at least in part, the failure of Th1 growth and differentiation.

IL-12 is secreted in a biphasic pattern in response to C. neoformans. Early secretion is induced directly by C. neoformans, and late secretion requires the presence of IFN-γ and ligation of CD40 molecules on monocytes [15]. gp120 affects both early and late induction of IL-12. The inhibition of early IL-12 production may be due to gp120 interference with internalization or processing of C. neoformans. This is consistent with previous

Table 1. Effect of interleukin (IL)-12 on IL-10 production from peripheral blood mononuclear cells in response to Cryptococcus neoformans.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No</th>
<th>C. neoformans 7698</th>
<th>C. neoformans 6995</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>3</td>
<td>20 ± 2</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>gp120</td>
<td>5</td>
<td>39 ± 2</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>IL-12</td>
<td>2</td>
<td>9 ± 2*</td>
<td>15 ± 2*</td>
</tr>
</tbody>
</table>

NOTE: Data are mean ± SD of 3 separate experiments from 3 different donors. Peripheral blood mononuclear cells (5 × 10^6/mL) were used at an effector:target ratio of 1:2. IL-10 was determined in supernatants after 7 days of incubation.

* P < .05 (IL-12-treated vs. respective IL-12-untreated cells).
observations showing that gp120 affects killing of fungi by monocytes/macrophages [16, 27]. This mechanism may be involved in regulating CD40 expression, which in turn may be responsible for IFN-γ reduction. This is supported by the observation that the inhibition of CD40 interaction with CD40 ligand greatly regulated IFN-γ production [15]. The increase in CD40 induced by free GXM was unexpected, because GXM induces IL-10 secretion. However, the presence of IL-10 is not necessarily related to CD40 dysregulation on accessory cells [28].

Our results suggest that the decrease in CD40 on the surface of antigen-presenting cells is a consequence of gp120-mediated inhibition of CD40 translocation from the intracellular pool to the cellular periphery rather than inhibition or degradation of mRNA transcripts. One interpretation of these data is that, by regulating CD40, gp120 could act at the antigen-presenting cell level by possibly inhibiting several CD40-mediated positive signals, such as cytokine release and antimicrobial activity [28]. This is supported by our recent observation that CD40 ligation is involved in promoting the antifungal activity of macrophages [29].

At present, IL-10 is the best-studied inhibitor of IL-12 production by phagocytic cells [28]. gp120 has been reported to up-regulate IL-10 in response to S. aureus cowan [14]. We confirm and extend the observed IL-10 up-regulation to C. neoformans. Importantly, we found that gp120 exacerbates the capability of the encapsulated strain to induce IL-10 by monocytes. These data suggest that, in AIDS patients with cryptococcosis, free gp120 may act as an adjunctive immunosuppressive signal.

Expression of IL-12 receptor is necessary to maintain the effect of IL-12 and Th1 lineage commitment [30]. Specific binding of IL-12 to the high-affinity receptor complex composed of 2 subunits, IL-12Rβ1 and IL-12Rβ2, mediates IL-12 responsiveness [31]. During Th2 differentiation there is a selective loss of IL-12Rβ2, suggesting that this receptor may govern the IL-12 biologic response. The underexpression of IL-12Rβ2 contributes to the Th2 bias. In contrast, the capacity to mount a Th1-type immune response appears to be regulated by cells expressing IL-12Rβ2 [25]. Our observation showing that gp120 produces a decrease in IL-12Rβ2 expression appears to be inconsistent with the fact that exogenous IL-12 promotes IFN-γ release. However IL-12 per se is able to up-regulate IL-12Rβ2 on T cells [32]. In our experimental system, the addition of exogenous IL-12 at the time of culture preparation could counterbalance the negative effect of gp120-induced IL-12Rβ2 expression. This is the first suggestion that C. neoformans promotes a differentiation in Th1 cells through promotion of the IL-12Rβ2 chain. This is conceivable, given that the Th1 response has been considered to be protective against C. neoformans [26] and that a protective immune response likely occurs in the immunocompetent host. gp120, in combination with encapsulated C. neoformans, inhibits IL-12Rβ2 expression on T cells responding to C. neoformans, providing additional evi-

Table 2. Effect of interleukin (IL)-12 on interferon (IFN)-γ production in response to Cryptococcus neoformans.

<table>
<thead>
<tr>
<th>Cell type, condition</th>
<th>No stimulus</th>
<th>C. neoformans 7698</th>
<th>GXM 7698 + GXM</th>
<th>C. neoformans 6995</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>2 ± 1</td>
<td>37 ± 2</td>
<td>4 ± 1</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>gp120 only</td>
<td>4 ± 1</td>
<td>25 ± 2*</td>
<td>5 ± 1</td>
<td>7 ± 1*</td>
</tr>
<tr>
<td>IL-12 only</td>
<td>35 ± 2</td>
<td>36 ± 3</td>
<td>30 ± 3</td>
<td>32 ± 2</td>
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<tr>
<td>IL-12 + gp120</td>
<td>32 ± 2</td>
<td>37 ± 4</td>
<td>28 ± 3</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Monocytes + T lymphocytes</td>
<td>3 ± 1</td>
<td>53 ± 4</td>
<td>4 ± 1</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>No treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gp120 only</td>
<td>5 ± 1</td>
<td>27 ± 3*</td>
<td>5 ± 1</td>
<td>14 ± 2*</td>
</tr>
<tr>
<td>IL-12 only</td>
<td>14 ± 2</td>
<td>78 ± 6</td>
<td>18 ± 2</td>
<td>29 ± 2</td>
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<tr>
<td>IL-12 + gp120</td>
<td>16 ± 2</td>
<td>61 ± 7</td>
<td>15 ± 2</td>
<td>31 ± 3</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD of 4 separate experiments from 4 different donors. Peripheral blood mononuclear cells (PBMC) or monocytes plus purified T lymphocytes were treated with different fungi or glucuronoxylomannan (GXM; 250 μg/mL) in the presence or absence of gp120 (500 ng/mL) and/or IL-12 (5 ng/mL) for 7 days. After incubation, supernatants were harvested, and IFN-γ was measured.

a P < 0.05 (gp120-treated cells vs. respective untreated cells).

Table 3. Effect of interleukin (IL)-12 on IL-4 production from peripheral blood mononuclear cells in response to Cryptococcus neoformans.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No stimulus</th>
<th>C. neoformans 7698</th>
<th>GXM 7698 + GXM</th>
<th>C. neoformans 6995</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>12 ± 1</td>
<td>25 ± 3</td>
<td>32 ± 2</td>
<td></td>
</tr>
<tr>
<td>gp120 only</td>
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<tr>
<td>IL-12 only</td>
<td>11 ± 2</td>
<td>8 ± 1</td>
<td>20 ± 1</td>
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</tr>
<tr>
<td>IL-12 + gp120</td>
<td>10 ± 1</td>
<td>8 ± 1</td>
<td>15 ± 1*</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD of 3 separate experiments from 3 different donors. Peripheral blood mononuclear cells (2 × 10^6/mL) were used as an effector:target ratio of 1:2. IL-4 was determined in supernatants after 7 days of incubation. Glucuronoxylomannan (GXM) was used at a concentration of 250 μg/mL.

a P < 0.05 (gp120 plus IL-12-treated vs. respective gp120-treated cells).
It is possible that free gp120 in AIDS patients with cryptococcosis may facilitate the spread of infection by contributing to suppression of a protective immune response. Hence, scavengers of free gp120, as well as immunopotentiating agents able to counterbalance the deleterious effects of gp120, might offer prospects for control of C. neoformans infection in AIDS patients.

Acknowledgments

We thank Eileen Mahoney Zannetti (Microbiology Section, University of Perugia, Perugia, Italy) for excellent and dedicated secretarial and editorial support, the Genetic Institute (Cambridge, MA) for providing recombinant interleukin (IL)-12, and Francesco Sini-gaglia (Roche Milano, Milan, Italy) for providing rat anti-human IL-12 receptor β2 subunit.

References


