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The Capsular Polysaccharides of Cryptococcus neoformans Activate Normal CD4⁺ T Cells in a Dominant Th2 Pattern

Geisy M. Almeida,* Regis M. Andrade,* and Cleonice A. M. Bento²†

Capsular components of Cryptococcus neoformans induce several deleterious effects on T cells. However, it is unknown how the capsular components act on these lymphocytes. The present study characterized cellular and molecular events involved in immunoregulation of splenic CD4⁺ T cells by C. neoformans capsular polysaccharides (CPSs). The results showed that CPSs induce proliferation of normal splenic CD4⁺ T cells, but not of normal CD8⁺ T or B lymphocytes. Such proliferation depended on physical contact between CPSs and viable splenic adherent cells (SAC) and CD40 ligand-induced intracellular signal transduction. The absence of lymphoproliferation after fixation of SAC with paraformaldehyde has discarded the hypothesis of a superantigen-like activation. The evaluation of a cytokine pattern produced by the responding CD4⁺ T lymphocytes revealed that CPSs induce a dominant Th2 pattern, with high levels of IL-4 and IL-10 production and undetectable inflammatory cytokines, such as TNF-α and IFN-γ. Blockade of CD40 ligand by relevant mAb down-regulated the CPS-induced anti-inflammatory cytokine production and abolished the enhancement of fungus growth in cocultures of SAC and CD4⁺ T lymphocytes. Our findings suggest that CPSs induce proliferation and differentiation of normal CD4⁺ T cells into a Th2 phenotype, which could favor parasite growth and thus important deleterious effects to the host. The Journal of Immunology, 2001, 167: 5845-5851.

Cryptococcus neoformans is an important encapsulated fungal pathogen that causes cryptococcosis in patients with impaired cell-mediated immunity such as those with AIDS (1). The fungus enters the host via inhalation and establishes itself in the lungs, where it can produce either a local subclinical infection or an invasion through various organs, which frequently results in lethal meningitis (2). Several reports have demonstrated the importance of cell-mediated immunity in protection against C. neoformans (3–6). Clinical and murine model studies have indicated that the clearance of pulmonary infection requires the presence of both CD4⁺ and CD8⁺ T cells (7–9). However, splenic CD4⁺ T cells appear to be the main determinant of protection to disseminated cryptococcosis (10, 11). Both lymphocyte subsets may have direct effects against yeast cells or act indirectly by stimulating anticytotoxic activity of effector cells, including cytotoxic and phagocytic cells (NK cells, neutrophils, and macrophages) (12, 13, 17, 18).

The cloned murine helper CD4⁺ T (Th) lymphocytes can be divided into functional subsets on the basis of the immunoregulatory cytokines (14). Thus, Th1 clones are characterized by production of IL-2, IFN-γ, and TNF-β and mediate delayed-type hypersensitivity responses. Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and are largely responsible for B cell maturation and Ig isotype switching (14). Th0 clones, which can produce both Th1- and Th2-type cytokines, have been also identified (15). Th1 T cells, which secrete proinflammatory cytokines, are crucial for effective anticytotoxic response. Several studies have associated the Th1 cytokines IFN-γ and IL-2 with resistance to C. neoformans infection (5, 6, 16). Other essential cytokines for establishment of resistance to C. neoformans include TNF-α, IL-12, and IL-18 (10, 17). The latter two cytokines seem to work synergistically to enhance IFN-γ production by NK cells and facilitate the development of Th1 lymphocytes, which in turn produce more IFN-γ and IL-12 (6, 18).

The fungal capsule is clearly the major virulence factor of C. neoformans (19–21). It is well established that the circulating levels of cryptococcal capsular Ags directly correlate with disease progression in patients and in experimental animals with disseminated cryptococcosis (1). Gadesbusch (22) described an “immunological paralysis”-like phenomenon after injection of a large dose of C. neoformans capsular polysaccharides (CPSs) in mice. Murphy and Cozad (23) found that low doses of cryptococcal CPSs injected i.v. induce a cascade of suppressor splenic T cells that secrete factors that down-regulate humoral and cellular anticytotoxic response. C. neoformans-derived CPSs reduce the production of proinflammatory cytokines and elicit the production of other cytokines that have been associated with inhibitory effects on the anticytotoxic response. These glycans inhibit TNF-α secretion from LPS-stimulated human monocytes (21) while inducing IL-10 production by these cells in vitro (21, 25). This anti-inflammatory cytokine, when added to a mixture of infected human monocytes and normal T lymphocytes, inhibits lymphoproliferation. Added to macrophage cultures, it affects IL-12 secretion and expression of class II MHC molecules (6, 21, 25, 26). In this study, we characterized the cellular and molecular events involved in the activation of the murine splenocytes induced by C. neoformans-derived CPSs.

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Abbreviations used in this paper: CPS, capsular polysaccharide; SAC, splenic adherent cell; GXM, glucuronoxylomannan; SEB, Staphylococcus aureus enterotoxin B; SAg, superantigen; CD40L, CD40 ligand.
Materials and Methods

**Mice**
Male BALB/c mice, 6 to 8 wk of age, were obtained from Oswaldo Cruz Institute (Instituto Oswaldo Cruz-Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) animal facility and used as a source of normal splenocytes.

**C. neoformans strains**

The encapsulated form of *C. neoformans* (strain 444) of serotype A, obtained from an AIDS patient with cryptococcal meningitis, and the non-encapsulated mutant *Cap 67* (54 of serotype D) were kindly provided by Dr. L. Mendonça-Previato (Instituto de Microbiologia Paulo de Göes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil).

**CPS purification and biochemical treatments**

*C. neoformans* wild-type strain was maintained on Sabouraud dextrose agar at 4°C, and yeast cells were grown for use for 5 days in a chemically defined liquid medium (27) at 37°C in a shaker at 100 rpm. The cells were then collected by centrifugation (7000 × g, 14 min, 4°C) and washed three times with cold 150 mM NaCl (pH 7.0). To purify the CPSs, washed yeasts (250 g wet weight) were suspended in 250 ml 10 mM citrate buffer (pH 7.0), and the suspension was autoclaved for 90 min at 121°C. Aftercooling, the supernatant was recovered by centrifugation at 6000 × g for 15 min at 4°C. The resulting pellet was suspended in 400 ml 20 mM citrate buffer, and the extraction procedure was performed again. The CPSs were isolated from vacuum-concentrated combined supernatants by precipitation with 2 volumes of ethanol at 4°C. The pellet was dissolved in water, some insoluble material was removed by centrifugation (6000 × g for 15 min at 4°C), and the supernatant (CPSs) was lyophilized. The CPSs were dissolved in HBSS, irradiated (10,000 rad) and stored at −20°C.

To evaluate the possible influence of nonpolysaccharide contaminants of CPSs on the activation of CD4<sup>+</sup> T lymphocytes, our CPS samples were solubilized with 1% (w/v) Tris-HCl buffer (pH 7.5) and digested exhaustively by Pronase E from *Streptomyces griseus* (Merck, Darmstadt, Germany) for 3 h at 37°C; by DNase 1 (Sigma, St. Louis, MO) in 20 mM Tris-HCl (pH 7.5), containing 1 mM MgCl₂ and 1 mM MnCl₂ for 3 h; or by RNase A from Sigma (previously heated at 80°C for 30 min to inactivate DNase) in 150 mM NaCl (pH 7.0) at 37°C for 3 h.

Finally, the contribution of the polysaccharide part in our system was evaluated by successive sodium periodate (IO₄<sup>−</sup>) oxidation and sodium borohydride reduction of CPSs, as follows. The CPSs were treated with an excess of aqueous sodium periodate overnight at 4°C, in dark and the reaction was stopped by the addition of ethylene glycol. The product was reduced with sodium borohydride, and after 1 h the solution dialyzed.

**Preparation of murine cells**

Splenocytes obtained from normal mice were depleted of RBC by treatment of whole splenocytes (1.5 × 10⁶/ml) obtained from columns were treated with a saturating dose of anti-Rat Ig mAb MAR 18.5 (1% ascites) plus 10% Low Tox-M rabbit complement for 30 min at 4°C, washed, and treated with anti-rat Ig mAb MAR 18.5 (1% ascites) plus 10% Low Tox-M rabbit complement for 30 min at 37°C. Viable cells were recovered after centrifugation and counted by trypan blue exclusion for each individual well in hemocytometer. Mean viable cell recovery in control unstimulated cultures was taken as reference. By flow cytometry, 92–97% of the resulting T cells were CD4<sup>+</sup>, and 1–3% were CD8<sup>+</sup>.

**Determination of fungus burden**

At the indicated days of culture infection with *C. neoformans* (Cap 67 strain), the whole content of each individual well (500 µl) was gently pipetted up and down and transferred to a synthetic fungus-specific medium (CSC-2500). After growth for 10 days at 37°C, viable fungi were identified in the counting chamber. This CDC-2500 medium is composed by glucose (40 g/L), KH₂PO₄·H₂O (1.36 g/L), urea (1.29 g/L), sodium glutamate (1 g/L), MgSO₄·7H₂O (0.3 g/L), thiamine-HCl (2 mg/L), and biotin (10 µg/mL). Results are presented as mean ± SEM of Cap 67 cell number in triplicate cultures.

**Cell proliferation assay**

Primarily, whole splenocytes (1.5 × 10⁶/ml) were cultured in the presence or absence of crescent doses of the CPSs (0.3 to 60 µg/ml) in 0.2 ml complete medium. In other experiments, CD4<sup>+</sup> T cell (1.5 × 10⁶/ml), in the presence or absence of B lymphocytes (1.5 × 10⁶/ml), were set up with or without 30 µg/ml CPSs. All cultures were established in 96-well flat-bottom microtiter (Corning Glass), incubated for 3 days at 37°C and 7% CO₂ in a humid incubator. The cocultures containing CD4<sup>+</sup> T cells (1.5 × 10⁶/ml) with SAC (5.0 × 10⁵/ml) were kept under the same conditions in the presence of 30 µg/ml CPSs, biochemically treated or not, in 48-well vessels (500 µl). To evaluate whether CPSs work as a superantigen (SAG), SAC monolayers were fixed with 1% (v/v) paraformaldehyde solution, and, as positive control, we used an optimal dose (2 µg/ml) of *Staphylococcus aureus* enterotoxin B (SEB), a classical SAg (Sigma). For blocking experiments, murine cells were cultured in the presence of saturating doses of various mAbs. Anti-CD8 (30% v/v of supernatants of 53.6.7 clone) and anti-CD4 (4 µg/ml) were added to each well. In some wells, to evaluate the effect of costimulator molecules in proliferation assay, saturating doses (10 µg/ml) of the anti-B7.1 and/or anti-B7.2, anti-CD40 ligand (CD40L) (rat IgG2a) or hamster (IgG) control isotypes were used. The cultures were pulsed with 0.5 µCi/well (1012 HJTdR (Sigma); 16 h), and the cells were harvested using a cell-harvesting device. The amount of [3H]TdTdR incorporated into DNA was assessed by liquid scintillation spectroscopy. All cultures were done in triplicate. The SEM were 10% of the mean cpm values. Results are presented as the difference between CPS-treated and control (medium alone) mean cpm values.

**Abs and other reagents**

Anti-CD8 mAb 53.6.7 was purchased from BD Pharmingen (La Jolla, CA). Anti-CD4 mAb GK 1.5 and anti-rat Ig κ-chain mAb MAR 18.5 were gifts from Dr. Ethan Shevach (National Institutes of Health, Bethesda, MD). Anti-TCRγ mAb H57.597 (33) was a gift from Dr. M. Bélio (Instituto de Microbiologia Paulo de Göes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil). All mAbs used in culture were obtained from BD Pharmingen, as follows: anti-B7.1 (CD80) mAb 1G10, anti-B7.2 (CD86) mAb GL1, anti-CD40L (CD154) mAb MR1, hamster IgG (4B3), or rat IgG1 (R3-34) mAbs as control isotypes. Primary and biotinylated secondary mAb from BD Pharmingen were also used in cytokine assay.

**Cytokine determination**

Supernatant fluids (500 µl/well) were collected for cytokine determination from cultures containing SAC (5.0 × 10⁶/ml) with or without CD4<sup>+</sup> T splenocytes (1.5 × 10⁶/ml) that were submitted to different treatments. The supernatants were collected 72 h after the treatments and submitted to evaluations of cytokine contents by sandwich ELISA, according to the protocol provided by the manufacturer, using two cytokine-specific mAbs for each cytokine (rabbit anti-mouse TNF-α and rat anti-mouse IFN-γ, IL-4, and IL-10), which was biotinylated (BD Pharmingen). The reaction was revealed with alkaline phosphatase-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL), using p-nitrophenyl phosphate (Sigma) as substrate. Recombinant murine IL-4, IFN-γ, TNF-α, and IL-10 (BD Pharmingen), ranging from 1 to 50 ng/ml were used to construct standard curves.

**Statistical analysis**

The Student t test for paired means was applied to all data reported in this article. The experimental means were compared with control means for each experiment.
Results

C. neoforms CPSs induce proliferative response of normal murine CD4+ T cells by a mechanism dependent on splenic adherent cells

C. neoforms CPSs induced maximum proliferative response of normal murine splenocytes at 30 μg/ml (Fig. 1A). To analyze the cellular mechanism involved in this immunostimulatory effect, splenic T subsets were blocked with neutralizing mAb against CD4+ and/or CD8+ T cells. Functional blockade of the CD4+ T subset reduced, in ~60%, the uptake of tritiated thymidine, when compared with the control (medium alone) (Fig. 1B). These results suggest immunoregulatory effects by CPSs on CD4+ T cells. However, the addition of CPSs to CD4+ T cell-enriched cultures, with or without splenic B cells, did not induce proliferation (Fig. 1C). Nevertheless, the ability to induce proliferative response of CD4+ T cells was acquired when these lymphocytes were cocultured with SAC monolayers (Fig. 1C). In all subsequent experiments, cocultures containing CD4+ T cells were standardized with SAC to evaluate the events triggered by CPSs under the conditions used here. To verify whether the proliferative effect induced by C. neoforms CPSs on the murine CD4+ T cells was indeed mediated by glycan components, the CPS preparation was submitted to different biochemical pretreatments. As indicated in the Fig. 1D, only the treatment with periodate ablated CPSs induced in vitro proliferative response of murine CD4+ T cells.

The role of SAC monolayers in the events induced by CPSs on normal CD4+ T cells

To characterize the molecular and cellular mechanisms involved in the immunoregulation by SAC, these adherent cells were pretreated with CPSs for 18 h, and supernatant was harvested and added in various dilutions to CD4+ T cells (Fig. 2A). Additionally, the SAC monolayers were washed exhaustively and cocultured with CD4+ T lymphocytes (Fig. 2B). It was observed that supernatants collected from the adherent cells were unable to induce the in vitro proliferative response of CD4+ T cells (Fig. 2A). However, the direct contact between splenocytes provided mitogenic activity of CPSs on CD4+ T cells (Fig. 2B). Our results showed that mitogenic activity of CPSs is dependent on contact with APC. Next, we tried to determine whether CD4+ T cell activation depended on TCR cross-link by a SAg-like mechanism. As demonstrated in Fig. 2C, paraformaldehyde-fixed SAC did not stimulate CD4+ T cells to proliferate in the presence of CPSs. These results suggest the need of induced events on viable APC to activate CD4+ T cell in response to CPSs. The next step was to analyze the contribution of costimulatory molecules expressed by the APC and CD4+ T cells that could participate in CPS-induced lymphoproliferation. For this purpose, we added neutralizing anti-B7.1, anti-B7.2, or anti-CD40L mAbs to the cultures. Effective blockade of the CPS-induced lymphoproliferation was achieved only by anti-CD40L mAbs (Fig. 3). Neither anti-B7.1 nor anti-B7.2 modified the level of [3H]thymidine uptake on these in the presence of CPSs.

CPSs induce anti-inflammatory cytokines production

To extend our observations about stimulating effects of CPSs on splenocytes, cytokine secretion by SAC was measured in the presence of CPSs (Table I). Additionally, we also assayed cytokine production by cocultures of SAC with CD4+ T cells in the presence of CPSs (Fig. 4). Clearly, CPSs induced IL-10 secretion by both culture types (Table I and Fig. 4). IL-4 production was detected only on wells containing CD4+ T lymphocytes and was augmented by CPSs (Fig. 4). These results indicate that CPSs mainly induce a Th2-type cytokine pattern. Addition of saturating doses of anti-CD4 mAbs reduces by 80% the ability of CPSs to induce IL-4 and IL-10 secretion. Addition of a polyclonal activator (anti-TCR), however, induced both inflammatory (TNF-α and IFN-γ) and anti-inflammatory (IL-4 and IL-10) cytokines assayed (Fig. 4).

![Graph A](image1.png)

**FIGURE 1.** *C. neoforms*-derived CPSs induce proliferation of CD4+ T cells in a SAC-dependent manner. A, Normal whole splenocytes (1.5 × 10^6/ml; 200 μl/well) were cultured with crescent doses of CPSs of *C. neoforms* (0.3–60 μg/ml); B, Normal whole splenocytes (1.5 × 10^6/ml; 200 μl/well) were cultured with or without saturating doses of blocking anti-CD4 (α-CD4; 5 μg/ml) and/or anti-CD8 (α-CD8; 30% v/v supernatants of clone 53.6.7) mAb, in the presence of the CPSs (30 μg/ml); C, CD4+ T lymphocytes (1.5 × 10^6/ml) were cocultured in the presence or absence of purified B lymphocytes (1.5 × 10^6/ml; 200 μl/well) or SAC (5.0 × 10^6/ml; 500 μl/well); all obtained from normal spleen; D, CD4+ T lymphocytes (1.5 × 10^6/ml) with SAC (5.0 × 10^6/ml; 500 μl/well) were cocultured in the presence of CPSs (30 μg/ml), which had been submitted to different treatments (T) as indicated: In T1, the CPSs preparation was exhaustively dialyzed to release the glucose from culture medium; in T2, the CPSs preparation was treated with Pronase (500 μg/ml); in T3 and T4, the CPSs preparation was treated with DNase or RNase, respectively; and in T5, the CPSs preparation was oxidized with periodate. All the cultures were kept for 3 days, and cell proliferation was assessed by [3H]Tdr uptake (0.5 μCi per well) added during the final 16 h of culture. Results indicate the means ± SEM of triplicate cultures.
Normal SAC (5.0 \times 10^5/ml; 500 \mu l/well) were cocultured with autologous CD4^+ T cells (1.5 \times 10^5/ml) for 3 days in the presence of CPSs (30 \mu g/ml). The role of different signaling pathways in the mitogenic activity was evaluated by addition of saturating doses (10 \mu g/ml) of anti-B7.1 (\alpha-B7.1; IgG1), anti-B7.2 (\alpha-B7.2; GL1) or anti-CD40L (\alpha-CD40L; MR1) mAbs. As control isotype, we used a hamster IgG (\text{hamster IgG}) or rat IgG2a (\text{IgG2a}), both at 10 \mu g/ml. Proliferation was assessed by [3H]TdR uptake (0.5 \mu Ci per well) added during the final 16 h of culture. Results are indicated as means \pm SEM of triplicate cultures.

**CD40L-dependent CD4^+ T cell activation enhances in vitro infection**

As demonstrated above, the ability of CPSs to activate normal CD4^+ T cells cocultured with SAC was CD40L dependent. These activated cocultures secrete IL-10 and IL-4, but not inflammatory cytokines, such as IFN-\gamma and TNF-\alpha (Fig. 4). Therefore, we analyzed the contribution of the CD40L signaling pathway on non-encapsulated C. neoformans growth in normal coculture, in the presence or absence of the CPSs. As indicated in Fig. 5, the blockade of signaling through CD40L, by anti-CD40L mAb, reverted the fungus growth to that observed in CPS-free cocultures. The matching isotype did not change fungus survival. The evaluation of cytokine contents in these cocultures subjected to CD40L blockade demonstrated a marked reduction of IL-10 and IL-4 secretion (Table II). Production of inflammatory cytokines was undetectable either before or after CPS addition (Table II).

**Discussion**

The C. neoformans has many virulence factors such as mannitol, melanin, and extracellular proteinases (33). However, the ability to synthesize CPSs is the most relevant evasion mechanism. The mucine model is very useful for immunopathological studies on C. neoformans infection, because uncontrolled growth of Cryptococcus in the lungs of these animals results in disseminated infection with intense shedding of CPSs (34). Injection of CPSs in experimental animals results in immunosuppression (19, 22, 26, 31, 32, 35–40). These soluble molecules induce a set of immunoregulatory T cells that inhibits the inflammatory response induced by specific and unspecific Ags (31, 32, 40). Similar mechanisms have been suggested for the phenomenon of Ab unresponsiveness to some bacterial polysaccharides (41). Results shown here suggest that C. neoformans-derived CPSs, in the presence of viable SAC, induce in vitro the proliferation of normal CD4^+ T lymphocytes and the production of anti-inflammatory cytokines. The ability to induce CD4^+ T cells activation, in our system, was maintained by SAC pretreated for 4 or 18 h with CPSs (Fig. 1B). We do not believe that dendritic cells are involved because they attach only transitorily to plate surfaces (42). CPSs did not augment the level of [3H]TdR uptake when they were added to cultures containing highly purified splenic B cells (Fig. 1C). This result eliminates the possibility of LPS contamination in the capsular preparation. The involvement of CD8^+ T cells was also discarded by the failure of the relevant neutralizing mAb to alter CPS-induced proliferative response. The failure of total blockade of proliferation in cultures of whole splenocytes treated with anti-CD4 may be explained by the presence of other T cell subsets that may be responsive to CPSs.

![FIGURE 3. Blockade of signaling triggered by the CD40L ablates CD4^+ T cell proliferation induced by CPSs of C. neoformans. Normal SAC (5.0 \times 10^5/ml) were cocultured with autologous CD4^+ T cells (1.5 \times 10^5/ml) for 3 days in the presence of CPSs (30 \mu g/ml).](http://www.jimmunol.org/)

<table>
<thead>
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<th>Treatment</th>
<th>IL-10 (ng/ml)</th>
<th>TNF-\alpha (ng/ml)</th>
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<tr>
<td>Control</td>
<td>8.10 ± 1.60</td>
<td>&lt;1</td>
</tr>
<tr>
<td>CPS</td>
<td>36.70 ± 4.70</td>
<td>&lt;1</td>
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*Normal SAC monolayers (5.0 \times 10^5/ml) were kept in the presence or absence of CPSs (3.0 \mu g/ml). Supernatants were collected (500 \mu g/well) after 72 h culture and assayed for cytokine contents by sandwich ELISA. The concentrations were obtained by comparison with standard curves of respective recombinant cytokines, ranging from 1 to 50 ng/ml. The results are means ± SEM of triplicates.
CPSs of *C. neoformans* induce a Th2-type cytokine pattern in cocultures of CD4⁺ T cells with SAC. CD4⁺ T cell (1.5 × 10⁶/ml) and SAC monolayers (5.0 × 10⁵/ml), both purified from normal mice spleen, were cocultured in the presence of anti-TCR (10% v/v supernatant of H57 clone), CPSs (30 μg/ml), or medium alone (control). Supernatants were collected after 72 h culture and assayed for cytokine contents by sandwich ELISA. The concentrations were obtained by comparison with standard curves of respective recombinant cytokines, ranging from 1 to 50 ng/ml. The results are means ± SEM of triplicate cultures.

(Fig. 1B). NK cells, for example, are able to recognize complex polysaccharides (43), and these lymphocytes are not eliminated by the procedure we used to purify CD4⁺ T cells.

When *C. neoformans* CPSs were fractionated by anion exchange chromatography on a Mono Q column, only the bound fraction, containing mannose, xylose, and glucuronic acid residues, was responsible for the mitogenic activity on whole splenocytes and purified CD4⁺ T cells (data not shown), indicating that the ability of CPSs to activate CD4⁺ T cells, in our system, is due to the presence of glucuronoxylomannan (GXM), the major CPS of *C. neoformans* (55). This observation is in agreement with those of others who noted that many deleterious effects induced by the *C. neoformans* capsule are mediated by GXM (24, 25). Other published results also demonstrated structural diversity on GXM molecule from strains of the same serotype (27). Therefore, a detailed molecular analysis, which is being conducted by our group, will permit a closer definition between the function and structure of CPSs.

The assays to characterize molecular events involved in the CD4⁺ T cells activation by CPSs of *C. neoformans* suggest that the proliferation of these lymphocytes is dependent on physical contact with SAC and involves signaling through CD40L. The CD40 expression on CD4⁺ T cells is an early event following activation through the TCR:CD3 complex (47). Furthermore, the ability of activated NK cells to express CD40L strengthens the possibility of these lymphocytes to participate in up-regulating the response to CPSs (48). The capacity of microbial polysaccharides to activate classical T lymphocytes has been described by others. Muzuno et al. (44) reported that α-1→6- and α-1→4-glucans from *Agaricus blazei* induce murine CD4⁺ and CD8⁺ T cells to proliferate. The work done by Matern et al. (45) established a direct correlation between activation of human T lymphocytes and LPS. These authors demonstrated that mitogenic activity on T cells was dependent on physical contact with LPS-treated monocytes and involved signaling through the B7 molecule but was not MHC restricted. More recently, a work by Brubaker et al. (46) reported that CPS A, purified from *Bacteroides fragilis*, induces intra-abdominal abscess in rodents by activating CD4⁺ T cells via a mechanism dependent on physical contact with viable macrophages. Probably, these nonproteic molecules mentioned above can induce by standard activation of T lymphocytes by stimulating expression of costimulatory molecules, such as B7 family (45) and CD40L (46).

In our model, results from cocultures containing paraformaldehyde-fixed SAC monolayers with freshly purified autologous CD4⁺ T cells suggested that 1) intracellular events induced by *C. neoformans* CPSs on SAC are pivotal to assure activation of CD4⁺ T cells and 2) the pathway used by CPSs to activate these lymphocytes do not behave like a SAg-induced activation (Fig. 2C). SAgs, characteristically, are able to activate T lymphocytes by simultaneously associating, without processing, the outside of CD4⁺ T cells (Fig. 1B). However, oxidation of CPSs by periodate abolished the mitogenic activity on CD4⁺ T cells induced by intact CPSs (Fig. 1B). It is possible that CPS-treated APC may achieve the ability to activate autoreactive CD4⁺ T cells by presenting endogenous peptides through molecules of class II MHC. The use of anti-I-E or anti-I-A mAb will reveal the exact contribution of this pathway in CD4⁺ T cell activation in our model.

The results obtained from cytokine dosage showed that CPSs induce IL-10 secretion in SAC monolayers (Table I). This result is in agreement with other data demonstrating in vitro IL-10 production by CPSs such as GXM on murine and human adherent cells (25, 26). The cytokine dosage from cocultures containing SAC with purified CD4⁺ T cells suggested that CPSs induce a Th2 cytokine pattern, with high levels of IL-4 and IL-10 production.
(Fig. 4). The contribution of this T lymphocyte subset for the resulting cytokine pattern was confirmed by treatment with anti-CD4 mAb, which ablated IL-4 secretion and significantly reduced IL-10 secretion (Fig. 4). We believe that the remaining IL-10 production is originated from the SAC monolayers (Table I). As described in the literature, these anti-inflammatory cytokines, besides making macrophages refractory to TNF-α and IFN-γ (50), are involved in induction of humoral immunity (51). The ability of polyclonal activator to induce inflammatory cytokines in our system negated the possibility of a previously established Th2 phenotype. In our system, the functional blockade of signaling through CD40L by mAb significantly diminished the anti-inflammatory cytokines production induced in vitro by CPSs (Table II) and reduced the fungus growth to control levels (Fig. 5). Additionally, the mouse strains most sensitive to cryptococcosis develop exacerbated humoral response (56). Probably, the ability of CPSs to induce IL-4 and IL-10 significantly reduced IL-10 secretion is detrimental for the host by favoring the humoral response. The possible contribution of other nonclassical lymphocytes, such as CD4+NK1+ T cells, may be through recognition of CD1 coupled to polysaccharides and secretion of high levels of IL-4 (52, 53). Preliminary observations by our group on the murine cryptococcosis model revealed that CD1 knockout (CD1−/−) mice are more resistant to cryptococcosis than wild-type strains. It is well established that CD1−/− animals lack CD4+NK1+ T cells, indicating that these nonclassical lymphocytes play an important role in the pathogenesis of cryptococcosis. The procedures used for CD4+ T cells purification in the present work did not eliminate this T cell subset; thus, the involvement of CD4+ NK1+ T lymphocytes on the immunological events induced by CPSs cannot be discarded.

In summary, our results suggest that the ability to induce in vitro CD4+ T cells to proliferate and secrete Th2 cytokines, such as IL-10 and IL-4, makes CPS an important evasion mechanism of Cryptococcus neoformans via inhibition of cellular immune response and thus exacerbation of in vitro infection. We are now investigating the precise mechanism by which CPS-induced CD4+ T cells downregulate protective cellular immune response to fungal infection and their impact on the course of murine cryptococcosis.

Acknowledgments

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References


Table II. Enhancement of anti-inflammatory cytokine production induced by C. neoformans-derived CPS depends on signaling triggered by CD40L

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-10 (ng/ml)</th>
<th>IL-4 (ng/ml)</th>
<th>IFN-γ (ng/ml)</th>
<th>TNF-α (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.10 ± 1.61</td>
<td>2.20 ± 1.25</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cap 67</td>
<td>10.70 ± 2.33</td>
<td>9.70 ± 2.47</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cap 67 + hamster IgG</td>
<td>12.50 ± 1.15</td>
<td>9.60 ± 1.60</td>
<td>&lt;1</td>
<td>3.1 ± 0.67</td>
</tr>
<tr>
<td>Cap 67 + anti-CD40L</td>
<td>12.10 ± 1.60</td>
<td>7.80 ± 1.30</td>
<td>&lt;1</td>
<td>3.8 ± 1.03</td>
</tr>
<tr>
<td>Cap 67 + CPS</td>
<td>36.20 ± 5.60</td>
<td>17.40 ± 2.60</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cap 67 + CPS + hamster IgG</td>
<td>29.40 ± 4.72</td>
<td>17.60 ± 1.13</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cap 67 + CPS + anti-CD40L</td>
<td>12.60 ± 1.35</td>
<td>4.60 ± 0.78</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

"CD4+ T lymphocytes (1.5 × 10^7/ml) were cocultured with SAC monolayers (5.0 × 10^3/ml; 500 µl/well) in the presence of 10^5 Cap 67/64 and with or without CPSs (30 µg/ml). In some wells, we added saturating doses (10 µg/ml) of neutralizing anti-CD40L mAb, or a control hamster isotype (hamster IgG). As control, the cocultures were maintained in the medium alone. After 3 days, the supernatants of cocultures were collected (500 µl/well) and assayed for cytokine contents by sandwich ELISA. The concentrations were obtained by comparison with standard curves of respective recombinant cytokines, ranging from 1 to 50 ng/ml. The results are means ± SEM of triplicates."


