**Binding of glucuronoxylomannan to CD14 receptor in human**

**A549 alveolar cells induces IL-8**

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**Abstract**

Glucuronoxylomannan (GXM) is the major capsular polysaccharide of *Cryptococcus neoformans*. GXM receptors have been characterized in phagocytes and endothelial cells, but epithelial molecules recognizing the polysaccharide remain unknown. In the current study, we demonstrate that GXM binds to CD14 receptor in human type II alveolar epithelial cells, resulting in the production of the pro-inflammatory chemokine IL-8.

**Text.**

*Cryptococcus neoformans* is an encapsulated fungal pathogen mainly infecting immunosuppressed patients. Infections are acquired by inhalation of desiccated cells, which are available in the environment as basidiospores or poorly encapsulated yeasts. Inhaled cells are deposited in the alveolar space, where they are ingested by macrophages (24), and interact with epithelial cells (2, 7, 8). An effective interaction of *C. neoformans* with epithelial alveolar cells is probably essential for the establishment of pulmonary infection. Microscopic studies of pulmonary cryptococcal infection reveal that yeast cells are in close apposition to lung epithelial cells (7). Once *C. neoformans* establishes in the lung it proliferates locally and causes a primary lesion that is usually contained by granuloma formation (10, 24). Two components of *C. neoformans* that are important for adhesion to human type II alveolar epithelial cells are phospholipase B (8) and glucuronoxylomannan (GXM) (2).

GXM is a virulence factor (4) that represents a potential vaccine component and is the target of therapeutic antibodies (3, 11, 17, 19). As the major constituent of the capsule it is the primary component of a structure that is antiphagocytic and thus protects the fungal cell from immune cells. However, cryptococcal infections are also associated with the release of large amounts of GXM into host tissues where they have many deleterious effects on the host immune response by multiple mechanisms (15). GXM has been reported to interact with numerous cellular receptors. Receptor for GXM in macrophages, neutrophils and endothelial cells include Toll-like receptors (TLR) 2 and 4, CD14 and CD18 (12, 13, 16, 25). The receptors for GXM in epithelial cells, however, remain to be characterized.

GXM was purified from strain $T_1$-444 of *C. neoformans* (serotype A) following

standard methods (5) and incubated with A549 cells (100 μg/ml) for 1 h at $37^o$C (2).

Binding of GXM to host cells was confirmed by immunofluorescence with the GXM-

binding monoclonal antibody (mAb) 18B7 (3) (Figure 1, inset). Lysates were obtained as

described previously (21) and immunoprecipitated by sequential incubation with mAb

18B7 (3) and Sepharose-bound protein G. SDS-PAGE of the immunoprecipitated

material indicated the presence of a major band with a molecular mass corresponding to

55 kDa (Figure 1A). The same mixture analyzed by SDS-PAGE was boiled for 5 min to

disintegrate protein-polysaccharide conjugates. GXM was then removed from this

preparation by ultrafiltration (cutoff 100 kDa) and the filtrate was incubated with

Sepharose-bound protein G for antibody depletion. The purified fraction containing the

GXM-binding protein, but no polysaccharide or antibodies, was concentrated to dryness

and used to coat polystyrene 96-well plates (5 μg/ml, 100 μl/well). After addition of

GXM in serial dilutions, the plates were sequentially incubated with mAb 12A1, a mouse

IgM mAb with specificity for GXM (6) and a goat anti-mouse antibody with specificity

for IgM for 1 h. Reactions were developed after the addition of *p*-nitrophenyl phosphate,

followed by reading at 405 nm. As demonstrated in Figure 1B, the purified fraction binds

GXM in a dose-dependent fashion.

CD14 is a 55 kDa glycosylphosphatidylinositol (GPI)-anchored membrane

protein mainly found on cells derived from the monocyte/macrophage lineage, as well as

neutrophils and B lymphocytes (8, 18). This molecule, which is a member of the

heteromeric lipopolysaccharide (LPS) receptor complex that also contains TLR4 and

MD2 (17), is a GXM receptor in endocytic cells (12, 16, 25). This information and the

molecular mass of the purified GXM-binding protein presented in Figure 1A led us to investigate whether CD14 could be the GXM receptor expressed by A549 cells. Human cells were detached from culture plates using 1 mM EDTA and fixed in 4% paraformaldehyde. After blocking with TBS-BSA, A549 cells were incubated with a phycoerythrin (PE)-labeled mouse mAb to CD14 (BD Biosciences, San Jose, CA). Analysis in a FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer revealed that A549 cells express CD14 (Figure 2A), confirming previous reports (20, 23). Control cells, incubated with an irrelevant PE-labeled antibody, presented low levels of fluorescence.

Given our recent observations that *C. neoformans* efficiently infects A549 cells (2) we evaluated if a CD14-binding molecule would interfere with this process. Untreated human cells or an A549 population that was pretreated with LPS (10 μg/ml) for 1 h were incubated with *C. neoformans* under previously established conditions (2). The index of association between cryptococci and alveolar cells was measured as the reactivity of A549 cells with mAb 18B7 in flow cytometry assays (2), which showed that the efficacy of the interaction of cryptococci with untreated cells was higher than that observed with LPS-treated epithelia (Figure 2B). Controls consisted of similar preparations that were not infected with *C. neoformans*. Pre-treatment of A549 cells with anti-CD14 antibodies also resulted in a lower association between *C. neoformans* and host cells (data not shown).

To confirm that CD14 is indeed a GXM-binding molecule in human alveolar cells, a modification of classical immunoprecipitation methods was used. Lysates of GXM-treated cells, presumably containing polysaccharide-CD14 complexes, were added

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to the wells of a 96-well plate previously coated with the anti-GXM IgM 12A1. After successive blocking and washing, the plate was incubated with a mouse mAb (IgG) to CD14 (BD Biosciences, San Jose, California) and then with a phosphatase-labeled goat antibody with specificity for mouse IgG. CD14 was co-precipitated with GXM, as suggested by the dose-dependent recognition of complexes by the anti-CD14 antibody (Figure 3). Controls consisted of similar assays in which lysates from GXM-treated cells were replaced by extracts from untreated alveolar cells. Additional controls included ELISA tests in which A549 lysates (positive control) and protein extracts from Chinese hamster ovarium (CHO) cells (negative control) were used to coat the plates, followed by blocking and sequential incubation with the anti-CD14 mAb and phosphatase-labeled secondary antibodies.

Association of microbial compounds with CD14 in alveolar cells can elicit the secretion of pro-inflammatory cytokines (20). We therefore speculated whether A549 cells could produce a cytokine response activated by GXM. For cytokine determinations, the culture medium was replaced by fresh medium containing no serum, but supplemented with 10 μg/ml GXM. After 4h at 37°C in a 5% $CO_2$ atmosphere, culture supernatants were collected and assayed for cytokines using a RayBio® Human Cytokine Antibody Array (RayBiotech, Inc). Procedures followed the manufacture's protocol. Cytokine production was quantified by using the Scion Image software (2000 Scion Corporation, NIH). Twenty cytokines were assayed and, in our experimental conditions, the production of IL-8 by GXM-treated cells was considered to be significantly different from that detected in non-stimulated supernatants (Figure 4). GXM induced the secretion

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of GRO and IL-3 by human cells (data not shown), although the results obtained with control and GXM-treated cells were not significantly different.

Since the CD14-LPS association resulted in IL-8 production by epithelial cells in previous reports (20), we investigated whether binding of GXM to CD14 molecules would have the same effect. Supernatants of non-stimulated A549 cells or of human cells after treatment for 1 h with LPS (positive control, 10 μg/ml), *C. neoformans* (2), or GXM (100 μg/ml) were collected and the presence of IL-8 assayed by ELISA-based techniques (Human CXCL8/IL-8 detection kit, R&D Systems, Minneapolis, MN). In any the conditions used for chemokine determination the viability of the A549 population was significantly affected (data not shown). A significant increase of IL-8 production was observed when fungal cells, GXM or LPS were incubated with A549 cells (Table 1). The levels of IL-8 produced in our experimental conditions were very similar to those observed in a previous study using LPS-treated A549 cells (14). Supplementation of the polysaccharide solution with polymyxin B produced similar ($P = 0.18$) levels of IL-8 (data not shown), indicating that the results obtained were not due to LPS contamination. When human cells were previously incubated with antibodies to CD14, however, GXM-treated and non-stimulated cells expressed similar levels of IL-8 (Table 1).

In microbial infections, a primary function of the airway epithelium is to act as a physical barrier for the exclusion inhaled infectious propagules. However, there is increasing evidence that lung cells can induce a localized immune response through the production of a variety of mediators, including pro-inflammatory cytokines and chemokines (1). These mediators could act, for example, recruitment polymorphonuclear leukocytes (PMNs) from the pulmonary vasculature into the alveolar space. In fungi, it

has been demonstrated that *Aspergillus fumigatus* proteases elicit IL-6 and IL-8

production in lung epithelial cells in vivo and in vitro (26). In cryptococcosis, the

production of these proinflammatory mediators is associated with survival of human

patients (22). Production of such molecules by the airway epithelium could therefore

represent an effective mechanism for the establishment of a local immune response

controlling microbial lung infections.

GXM receptors have been characterized in a number of cells (12, 13, 16, 25). This

polysaccharide mediates the interaction of *C. neoformans* with different host cells and

induces a deleterious effect on the immune system (15). Interactions of cryptococci with

epithelial cells are influenced by GXM (2), but the host receptors involved in this process

are unknown. The identification of CD14 as an epithelial molecule interacting with GXM

with the consequent production of IL-8 brings to light the potential role of epithelial

respiratory cells in the immunity against *C. neoformans*.

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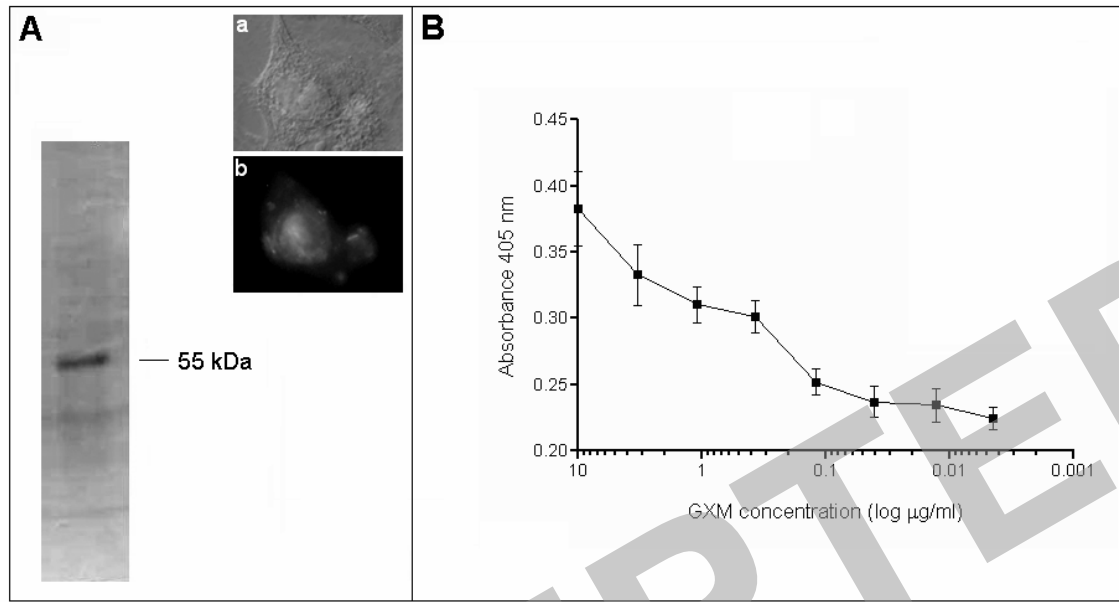
Figure 1. Purification of a GXM-binding molecule from A549 cells by immunoprecipitation. Protein extracts from GXM-treated human cells were sequentially incubated with mAb 18B7 and Sepharose-bound protein G, for further analysis by SDS-PAGE (A). GXM binding to epithelial cells was confirmed by immunofluorescence with mAb 18B7 (inset). An A59 cell observed under differential interferential contrast (a) and fluorescence (b) modes is shown. A single major protein with molecular mass corresponding to 55 kDa was detected. The purified fraction was used in GXM-binding assays in 96 well plates (B). Dose-dependence binding of the 55 kDa protein to GXM detected by IgM mAb to GXM.
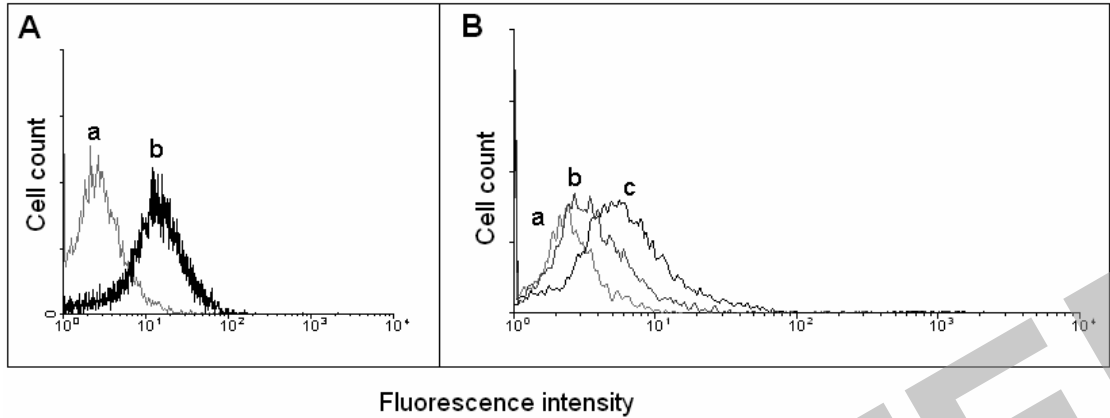
Figure 2. Expression of CD14 by A549 cells. A. Flow cytomoetric analysis of human cells incubated with a PE-labeled mAb to CD14 and demonstrating that the receptor is expressed on the surface of A549 cells. Unstained (a) and antibody-treated (b) cells are shown. B. Pre-treatment of human cells with LPS, a CD14-binding compound, decreases their association with cryptococci. The fluorescence increase after incubation of infected cells with mAb18B7 was used to identify A549 cells in association with cryptococci. Infected cells that were pre-treated with LPS (b) presented a reduced reaction with mAb 18B7, in comparison with untreated cells (c). The fluorescence levels of uninfected cells is shown in a.
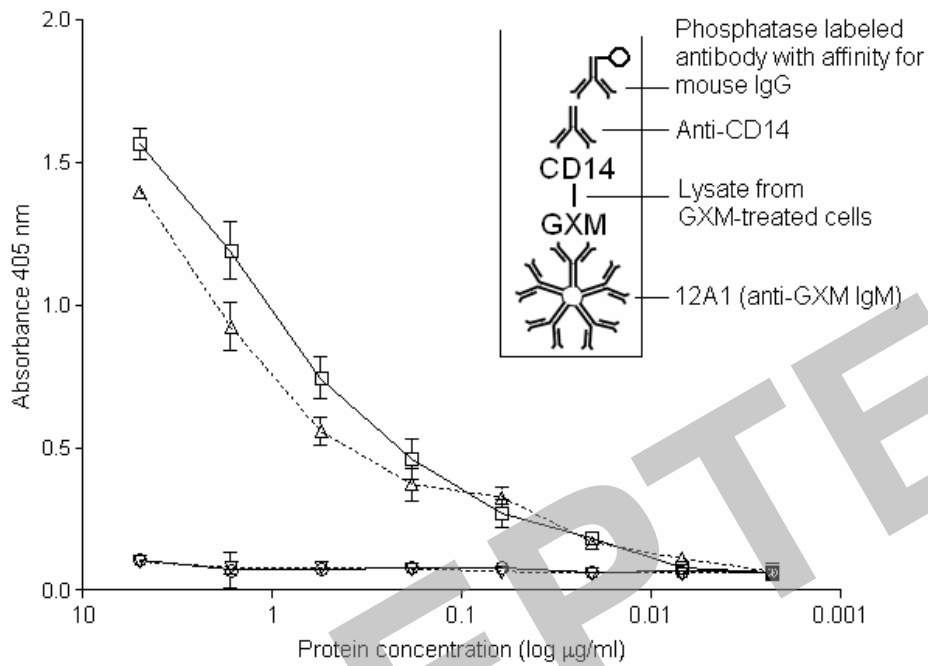
Figure 3. Identification of CD14 as the epithelial receptor for GXM in A549 cells. CD14-GXM complexes (triangles) from polysaccharide-treated cells were captured by IgM antibodies in ELISA. An anti-CD14 was used as the detection probe, as demonstrated in the schematic presentation of the capture ELISA (inset). No significant reactions with the anti-CD14 antibody were observed when protein extracts from untreated cells (inverted triangles) were used. Additional controls included an ELISA in which A549 lysates (positive control, squares) and protein extracts from Chinese hamster ovarium (CHO) cells (negative control, circles), were used to coat the plates, followed by sequential incubation with the anti-CD14 mAb and phosphatase-labeled secondary antibodies.
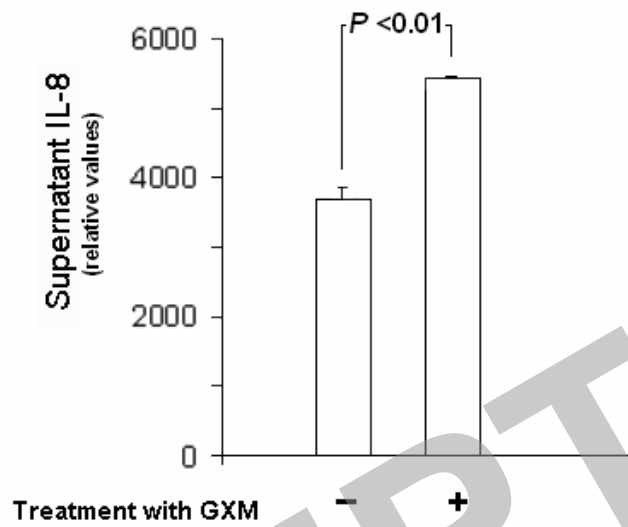
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Figure 4. Production of IL-8 by GXM-treated cells. Supernatant of untreated or polysaccharide-treated cells were collected for cytokine detection. An increased production of IL-8 was observed in supernatants of GXM-treated cells.

**Table 1:**

**Stimulation of A549 cells with GXM results in CD14-mediated production of IL-8\***

| Treatment of A549 cells [a] | Supernatant IL-8 (ng/mL) | $P$ [b] | Statistical significance |
|---|---|---|---|
| None | $0.82 \pm 0.06$ | -- | -- |
| LPS (10 µg/ml, 1 h) | $1.33 \pm 0.01$ | 0.007 | Yes |
| *C. neoformans* (10 yeast/host cell) | $1.06 \pm 0.01$ | 0.030 | Yes |
| GXM (100 µg/ml, 1 h) [c] | $1.18 \pm 0.03$ | 0.017 | Yes |
| Anti-CD14 (10 µg/ml) then GXM (100 µg/ml) | $0.54 \pm 0.12$ | 0.085 | No |

(\*) Results presented in this Table are representative of three different assays producing similar results. The error bars were generated from multiple trial wells (n = 3) of each single sample.

[a] A549 cells were treated with LPS, fungal cells, GXM or anti-CD14 antibodies for 1 h at $37^{o}$C.

[b] $P$ values were obtained through the use of Student's $t$ test, after comparison with control systems (no stimulation).Differences between stimulated systems and control cells were considered as statistically significant when $P$ values were smaller than 0.05.

[c] Results obtained when A549 cells were treated with GXM alone were statistically compared to the data obtained when alveolar cells were sequentially treated with the antibody to CD14 and GXM. IL-8 levels detected after treatment with GXM alone were

significantly higher ($P < 0.01$) than those obtained when A549 cells were first treated

with the antibody to CD14.