Cryptococcus neoformans Capsular Glucuronoxylomannan Induces Expression of Fas Ligand in Macrophages[1]

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The major component of capsular material of *Cryptococcus neoformans* is glucuronoxylomannnan (GXM), a polysaccharide that exhibits potent immunosuppressive properties in vitro and in vivo. The results reported here show that 1) soluble purified GXM induces a prompt, long-lasting, and potent up-regulation of Fas ligand (FasL) on macrophages, 2) the up-regulation of FasL is related to induced synthesis and increased mobilization to the cellular surface, 3) this effect is largely mediated by interaction between GXM and TLR4, 4) FasL up-regulation occurs exclusively in GXM-loaded macrophages, 5) macrophages that show up-regulation of FasL induce apoptosis of activated T cells expressing Fas and Jurkat cells that constitutively express Fas, and 6) anti-Fas Abs rescue T cells from apoptosis induced by GXM. Collectively our results reveal novel aspects of the immunoregulatory properties of GXM and suggest that this nontoxic soluble compound could be used to dampen the immune response, to promote or accelerate the death receptor, and to fix FasL expression in a TLR/ligand-dependent manner. In the present study, we delineate potential new therapeutic applications for GXM that exploit death receptors as key molecular targets in regulating cell-mediated cytotoxicity, immune homeostasis, and the immunopathology of diseases. *The Journal of Immunology,* **2005, 174: 3461–3468.**

Macrophages have a central position in both the innate and adaptive immune systems. Their life and function are characterized by significant functional versatility. Macrophages ingest foreign materials, present Ags to T lymphocytes in association with the MHC, and can kill microbes and tumor cells upon activation by cytokines and/or T cells. In addition, they eliminate damaged or apoptotic cells. In contrast, macrophages can also release copious amounts of toxic metabolites that can promote tissue damage during antimicrobial defense responses. Macrophages may also initiate self-destructive autoimmune processes (1). Hence, macrophages are critically important cells for host defense but are also implicated in processes that damage host tissues.

A key function of macrophages is to present foreign Ags (peptides) on their surface to T lymphocytes, thereby acting as APC. APC play an important role in both the initiation of a T cell response and in tolerance. The fate of T cells is determined when they recognize Ags presented by APC. T cells undergo complete activation if the APC express the appropriate costimulatory molecules such as CD80 and CD86, anergy if the APC do not express costimulatory molecules, or apoptosis if the APC express Fas ligand (FasL).[3] The ability of APC to influence the response to allografts is well established (2). It has long been recognized that graft rejection is most intense in tissue that contains many lymphoreticular elements but is relatively less intense in tissue with few lymphoreticular cells such as muscle (3). Removal of APC from graft tissue before transplantation greatly diminishes the rejection response (4, 5).

The importance of peripheral T cell apoptosis in the induction of T cell tolerance has been demonstrated as a means to prolong allograft survival (6, 7). Fas-mediated apoptosis plays an important role in the maintenance of T cell tolerance, such as in the prevention of autoimmune disease. Fas is a type I transmembrane receptor, having a significant role in the immune system, but alternative splicing produces a soluble form, the function of which is unclear (8). Fas expression can be boosted by cytokines such as IFN-γ and TNF but also by the activation of lymphocytes (9, 10).

Fas-mediated apoptosis is triggered by its natural ligand, FasL, a type II membrane protein belonging to the TNF family (11–13). Unlike Fas (14), which is expressed on the surface of diverse cell populations (15–17), the surface expression of FasL appears to be more restricted and often requires cellular activation (16, 18–20). Apoptosis of activated T cells by autocrine interaction of Fas and FasL is a critical process for the maintenance of peripheral T cell tolerance (21).

Modulation of the mechanisms that lead to the induction of apoptosis offers potential strategies to promote graft survival through the deletion of alloreactive T cells. Recently, polysaccharides of microbial origin were described as potent immunomodulators that may target both T cells and APC, such as monocytes and macrophages (22). Glucuronoxylomannan (GXM), the major polysaccharide component of *Cryptococcus neoformans*, is found bound to the fungal cell in the form of a capsule or shed in soluble form as an exopolysaccharide during growth in vivo and in culture.

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[3] Abbreviations used in this paper: FasL, Fas ligand; GXM, glucuronoxylomannan; FLUOS, 5-carboxyfluorescein-*N*-hydroxysuccinimide ester; MDM, monocyte-derived macrophage; PB, permeabilization buffer; FB, fluorescence buffer; PI, propidium iodide.

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GXM is associated with a variety of immunomodulatory effects; in particular, it 1) inhibits the production of proinflammatory cytokines from human monocytes (23), 2) induces inhibitory factors such as IL-10 (24), 3) inhibits activation and maturation of dendritic cells (25), 4) suppresses T cell proliferation in the presence of APC (26, 27), 5) dampens Th1 response and delayed-type hypersensitivity response (26–28), 6) limits MHC class II expression on APC (26), 7) reduces killing (29, 30) and chemotactic activity of natural effector cells (31), and 8) induces apoptosis in splenic mononuclear cells from normal rats (32). These effects are believed to contribute to the pathogenesis of *C. neoformans* infections, which are notoriously persistent and difficult to eradicate.

Recently, we observed that GXM binds to macrophages via multiple receptors, including CD14, TLR4, CD18, and FcγRII, and that it accumulates inside cells (29). GXM can persist in monocytes/macrophages for extended periods of time and can regulate the subsequent T cell response. GXM-mediated suppression of T cell activation is most likely transmitted via APC because direct interaction between T cells and GXM has not been demonstrated. In consideration of these results, this study was designed to investigate the ability of GXM-loaded macrophages to regulate apoptosis in T lymphocytes.

Materials and Methods

Reagents and media

RPMI 1640 medium with L-glutamine was obtained from Invitrogen Life Technologies. FCS and penicillin-streptomycin solution were obtained from Sigma-Aldrich. Mouse mAb to human TLR4 (IgG2a) and to human TLR2 (IgG2a) were purchased from HyCult Biotechnology. Mouse mAb to human CD178 (FasL, IgG1), mouse mAb to human CD18 (IgG2a), mouse mAb to human FcγRII (IgG1), and mouse blocking mAb to human Fas (APO-1, IgG2b) were purchased from Ancell; FITC-conjugated mouse mAb to human Fas (IgG1) and mouse mAb to human CD14 (IgG2a) were purchased from Immunological Sciences. Mouse isotype control IgG1 and IgG2a, $F(ab')_2$ of PE-conjugated sheep anti-mouse IgG (whole molecule), cytochalasin D, cycloheximide, and ionomycin were purchased from Sigma-Aldrich. All media used for cell culture were negative for endotoxin as detected by *Limulus amebocyte* lysate assay (Sigma-Aldrich), which had a sensitivity of ~0.05–0.1 ng of *Escherichia coli* LPS/ml.

The characteristics of mAb 18B7 (IgG1,k), specific for GXM, have been described previously (33, 34). mAb 18B7 was purified from culture supernatant fluid by protein G affinity chromatography (Pierce), and the Ab concentration was determined by ELISA relative to isotype-matched standards of known concentrations. GXM mAb 18B7 and mAb IgG1k (isotype control) were labeled with 5-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) by use of a labeling kit according to the manufacturer's directions (Boehringer Mannheim).

Microorganisms and cryptococcal polysaccharide

An acapsular strain of *C. neoformans* var. *neoformans* (CBS no. 7698 = National Institutes of Health B-4131) was obtained from Central Bureau Schimmel Cultures. The cultures were maintained by serial passage on Sabouraud agar (bioMerieux). Log-phase yeasts were harvested by suspending a single colony in RPMI 1640 medium, washed twice with saline, counted on a hemocytometer, and adjusted to the desired density in RPMI 1640 medium (23). *Candida albicans* (strain PCA-2) was supplied by D. Kerridge (University of Cambridge, Cambridge, U.K.). This is an agerminative strain that grows as a yeast form in vitro at 28°C or 37°C in conventional media. Yeasts were heat killed at 60°C for 30 min before use.

GXM was isolated from the culture supernatant fluid of serotype A strain (CN 6) grown in liquid synthetic medium (35) in a gyratory shaker for 4 days at 30°C. GXM was isolated by differential precipitation with ethanol and hexadecyltrimethyl ammonium bromide (Sigma-Aldrich) (35), and the procedure has been previously described in detail (36). Soluble GXM isolated by the above procedure contained <125 pg LPS/mg of GXM as detected by *Limulus amebocyte* lysate assay (QCL-1000; BioWhittaker).

Jurkat cells

The human Jurkat T cell leukemia cell line was obtained from the American Type Culture Collection. Cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and were cultivated in a humid atmosphere containing 5% CO_2.

Preparation of monocyte-derived macrophages (MDM) and T lymphocytes

Heparinized venous blood was obtained from healthy donors and diluted with RPMI 1640 medium plus 5% FCS (complete RPMI 1640). The mononuclear cells were separated by density gradient centrifugation on Ficoll-Hypaque, washed twice in RPMI 1640 medium, and incubated for 2 h at a density of 2×10^6 to 3×10^6/ml in cell culture petri dishes (Nunc). After 2 h in culture medium, nonadherent cells were aspirated, and fresh complete RPMI 1640 containing 50 ng/ml M-CSF (PeproTech) was added. The remaining adherent cells were >98% viable as evaluated by trypan blue dye exclusion. After 5 days of incubation, the MDM were recovered and suspended at the desired density (37). The MDM cultures used in each experiment were from single subjects. Nonadherent cells were E-rosetted as described previously (38). The cells recovered were T lymphocyte $T(E^+)$ cells and >98% $CD3^+$ as evaluated by flow cytometry analysis.

Flow cytometry analysis of FasL molecules on MDM

MDM (1×10^6) were incubated in the presence or absence of GXM (50 μg/ml) in RPMI 1640 medium plus 10% FCS for 2 h or 2 or 5 days at 37°C with 5% CO_2. After incubation, cells were collected by centrifugation, fixed in 1% paraformaldehyde in PBS, permeabilized for 10 min at room temperature with PBS containing 0.1% saponin (Sigma-Aldrich) (permeabilization buffer (PB)), washed twice with PB, and stained with FLUOS-18B7 (1:100) in PB plus 5% FCS (labeling buffer) for 20 min on ice. After incubation, the cells were washed twice with PB, mixed with mAb to FasL (CD178, CD95L) (1:50) in PBS containing 0.5% BSA and 0.4% sodium azide (fluorescence buffer (FB)) for 20 min on ice, washed in FB, stained with PE-conjugated mAb anti-mouse IgG, washed twice more in FB, then 5000 events were analyzed by flow cytometry.

In selected experiments, the MDM (10^6/ml), treated or without GXM (50 μg/ml), were incubated with heat-killed acapsular *C. neoformans* 7698 (E:T ratio = 1:2) or with *C. albicans* (E:T ratio = 1:2) for 2 h at 37°C and 5% of CO_2, then analyzed for FasL surface expression.

To determine the amount of total and intracellular FasL expression, the cells were stained with anti-CD178 (FasL) (1:50) in labeling buffer for 20 min on ice, washed with PB, stained with PE-conjugated mAb anti-mouse IgG, washed with FB, and 2000 events were analyzed by flow cytometry. The intracellular pool of FasL was calculated by subtracting the surface pool from the total pool of FasL.

To evaluate the role of TLR4 in FasL surface expression, MDM (1×10^6) were incubated in the presence or absence of GXM (50 μg/ml) and in the presence or absence of mAb to TLR4 (2.5 μg/ml) in RPMI 1640 medium plus 10% FCS for 2 days at 37°C with 5% CO_2. After incubation, the cells were collected, stained with anti-CD178 (FasL) as described above, and analyzed by FACScan (BD Biosciences).

To analyze the expression of this receptor on GXM-loaded or GXM-unloaded MDM, the cells were stained for GXM binding as described above and analyzed by flow cytometry. The cells were distinguished on the basis of forward light scatter and green fluorescence intensity, and a discriminatory gate was placed around the GXM-positive MDM and GXM-negative MDM. Specific fluorescence was assessed by comparison with results from a mouse isotype control FLUOS- IgG1k.

Cycloheximide and actinomycin D treatment of MDM

Cycloheximide and actinomycin D isolated from *Streptomyces* species were obtained from Sigma-Aldrich. Cycloheximide (10 μg/ml) or actinomycin D (1 μg/ml) was added to MDM 30 min before the GXM challenge. Cell viability was >98% after treatment with cycloheximide or actinomycin D. Viability of the cells was measured with a colorimetric MTT viability assay (Sigma-Aldrich) (39).

Analysis of FasL release from MDM

The levels of soluble FasL (sFasL) in MDM supernatant fluids were determined, according to the manufacturer's directions, by FasL ELISA kit (Oncogene Research Products). Cellular viability was assessed by the trypan blue exclusion test.

Flow cytometry analysis of Fas molecule on T cells

Fresh T cells (5×10^6/ml) were either stimulated or not with PHA (5 μg/ml) for 2 days at 37°C with 5% CO_2 in RPMI 1640 medium containing 10% FCS. After incubation, T cells were stained with FITC-conjugated

mouse mAb to human CD95 (Fas) in FB for 20 min on ice, washed with FB, and 5000 events were analyzed by flow cytometry.

Apoptosis

Isolated T cells (10^5/ml), preactivated or not with PHA (5 μg/ml) for 2 days at 37°C, were added to MDM, preincubated or not with GXM for 48 h, in RPMI 1640 medium at 10% of FCS (E:T ratio = 10:1). In selected experiments, Jurkat cells were added to MDM, instead of PBL, at various ratios (E:T ratios = 1:10, 1:5, or 1:1). The percentage of PBL undergoing apoptosis was quantified after 3 or 4 days of incubation by staining with propidium iodide (PI) (50 μg/ml; Sigma-Aldrich). Briefly, cells were centrifuged, resuspended in hypotonic PI solution, and kept for 1 h at room temperature. Apoptosis was evaluated as described previously (40). Alternatively, FITC-annexin V binding was used to follow the expression of phosphatidylserine on early apoptotic PBL using a commercially available kit (Annexin-V-FLUOS Staining kit; Roche Diagnostic Systems). The percentage of Jurkat cells undergoing apoptosis was quantified after 3 h by the methods described above (41). Cellular viability was assessed by trypan blue exclusion.

FasL and MyD88 determination

MDM (5×10^6) were incubated in the presence or absence of GXM (50 μg/ml) in RPMI 1640 medium plus 10% FCS for 48 h at 37°C with 5% CO_2 in the presence or absence of mAb to TLR4 (2.5 μg/ml).

After coculture, the cells were washed, treated with 250 μl of Mammalian Protein Extraction Reagent in the presence of protease inhibitors (Pierce), and the lysates were collected by removing particulate material through centrifugation for 10 min at 12,000 $\times$ *g*. Lysate proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Pierce) for 1 h at 100 V in a blotting system (Bio-Rad) for Western blot analysis. Membranes were then placed in blocking buffer, incubated overnight at 4°C with rabbit polyclonal Ab to FasL (1 μg/ml) (Calbiochem) or to MyD88 (Apotech) in blocking buffer, and stained according to the manufacturer's directions (WesternBreeze Chemiluminescent Western Blot Immunodetection kit; Invitrogen Life Technologies). Immunoreactive bands were visualized and quantified by a Chemidoc Instrument (Bio-Rad).

Statistical analysis

Data are reported as the mean ± SD from replicate experiments. Data were evaluated by one-way ANOVA. Post hoc comparisons were done with Bonferroni's test.

Results

An initial series of experiments evaluated FasL expression on human MDM (derived from monocytes cultured for 5 days in the presence of 50 ng/ml M-CSF) incubated with GXM (50 μg/ml) for various times (2 h or 2 or 5 days). The results (Fig. 1*A*) show that GXM treatment resulted in significant up-regulation of FasL expression on MDM cell surfaces. Our previous studies showed that MDM preparations contained populations of cells that did or did not accumulate GXM. Gating of MDM to identify cells that did or did not accumulate GXM showed that the increase in FasL occurred only on GXM-loaded MDM (Fig. 1*A*). FasL overexpression was specific to GXM because other microbial stimuli such as *C. albicans* or acapsular *C. neoformans* failed to regulate FasL expression. The failure of the fungal cells to stimulate FasL expression was not due to suppression by these cells because up-regulation was observed when MDM were costimulated by GXM and the fungal cells (Fig. 1*B*). Ionomycin (1 μg/ml) (42, 43) was used as a positive control for inducing FasL, and ionomycin-induced up-regulation of FasL was comparable to that observed for GXM (Fig. 1*A*). In selected experiments, we investigated whether other polysaccharides, such as mannan or glucan, affected FasL induction. Addition of mannan to MDM (50 μg/ml) produced a slight but not significant increase of FasL (Fig. 1*A*). Furthermore, addition of glucan to MDM (50 μg/ml) did not modulate FasL expression (Fig. 1*A*).

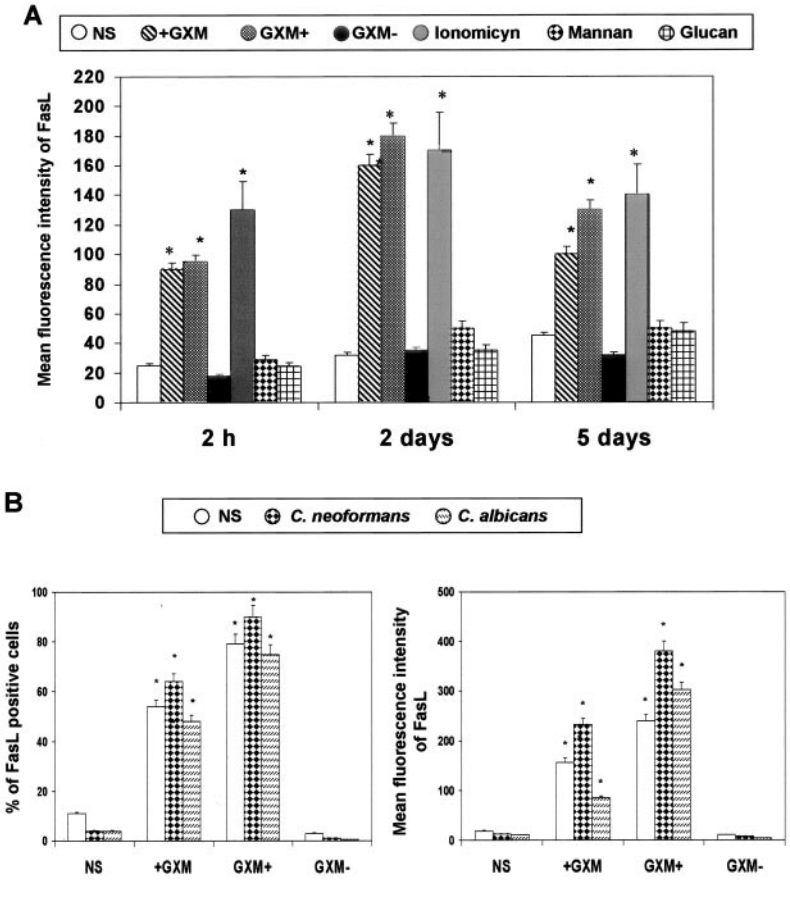

FIGURE 1. FasL surface expression. *A*, MDM (10^6/ml) were incubated with GXM (50 μg/ml) for various times (2 h or 2 or 5 days) or with ionomicyn (1 μg/ml) or with mannan (50 μg/ml) or glucan (50 μg/ml). *B*, MDM (10^6/ml) were incubated with GXM (50 μg/ml) for 2 h and were treated for 2 h with acapsular *C. neoformans* and *C. albicans*. FasL expression was analyzed on nonstimulated cells (NS), GXM-treated MDM (+GXM), and GXM-treated MDM gated to identify cells that accumulated GXM (GXM+) and in cells that did not accumulate GXM (GXM−). Bars represent the mean ± SEM of five experiments using MDM from different donors. *, A value of $p < 0.05$ treated vs untreated cells.

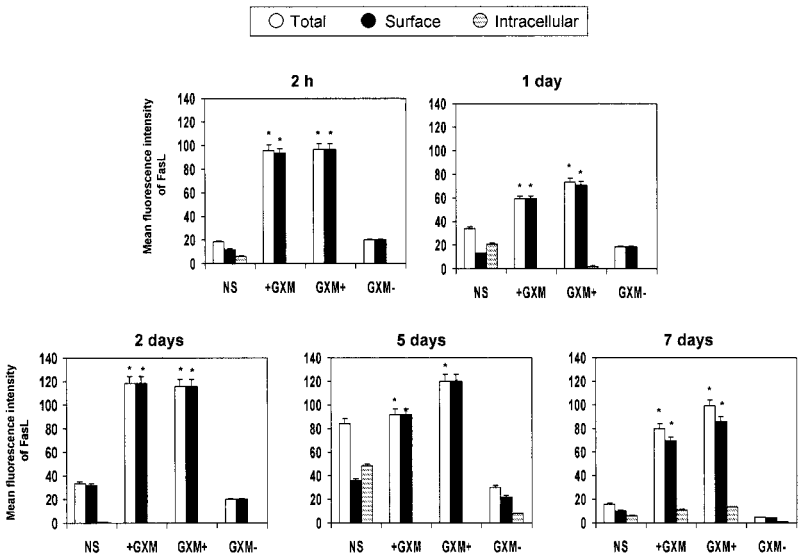

FIGURE 2. Time course of total and surface FasL expression. MDM (10^6/ml) were incubated with GXM (50 μg/ml) for various times (2 h or 1, 2, 5, or 7 days). After incubation, the cells were untreated (surface) or permeabilized with saponin (total). Intracellular pool of FasL was calculated by subtracting the surface pool from the total pool of FasL. FasL expression was analyzed on not stimulated cells (NS), GXM-treated MDM (+GXM), and GXM-treated MDM gated to identify cells that accumulated GXM (GXM+) and cells that did not accumulate GXM (GXM−). Bars represent the mean ± SEM of five experiments using MDM from different donors. *, A value of $p < 0.05$ GXM-treated vs GXM-untreated cells.

Given that a previous study (44) demonstrated the presence of preformed FasL within the cells, we examined the possibility that GXM stimulated rapid mobilization of the molecule from the intracellular pools to the cell periphery. MDM were incubated with GXM for various times (2 h or 1, 2, 5, or 7 days) and analyzed for surface and intracellular expression of FasL. The results (Fig. 2) showed that GXM-treated MDM exhibited up-regulation of the total pool of FasL. The kinetic analysis showed that GXM treatment induced rapid up-regulation of FasL expression that continued for up to 7 days of incubation. When MDM were gated to distinguish between GXM-loaded or -unloaded cells, the results showed that FasL expression was limited to cells that had taken up GXM.

Additional experiments were performed to determine whether protein synthesis was required for increased expression of FasL. We examined the effects of cycloheximide, a potent inhibitor of protein synthesis, and actinomycin D, an inhibitor that prevents DNA-dependent RNA synthesis (45). Untreated MDM or MDM pretreated with cycloheximide (10 μg/ml) or actinomycin D (1 μg/ml) were incubated with GXM (50 μg/ml), and the expression of FasL was examined. The results (Fig. 3) show that treatment with cycloheximide or actinomycin D abrogated FasL up-regulation, indicating a requirement for protein synthesis and de novo gene transcription. To exclude the possibility that blockade of FasL expression was due to direct cytotoxicity of these compounds, MDM viability was assessed after treatment with cycloheximide or actinomycin D. The results showed that viability of MDM was not affected by addition of these compounds under the conditions used in our study.

TLR4 signaling was demonstrated recently to promote apoptosis if NF-κB activation is blocked (46, 47). We previously demonstrated that GXM binds to TLR4 without promoting activation of NF-κB, suggesting that GXM binding via TLR4 might promote apoptosis. Consequently, we investigated the role of this receptor in GXM-mediated up-regulation of FasL. Macrophages were incubated for 2 days with GXM (50 μg/ml) in the presence or absence of mAb to TLR4 and then FasL surface expression was analyzed. The results show that mAb to TLR4 significantly reduced FasL up-regulation (Fig. 4*A*).

It has been reported that GXM uptake occurs also via TLR2 (48), CD14, CD18, and FcγRII (49). To assess the possible contribution of TLR2, CD14, CD18, and FcγRII to FasL induction, we performed blocking-type experiments where Abs to TLR2 (5 μg/ml), CD14 (5 μg/ml), CD18 (10 μg/ml), or FcγRII (5 μg/ml) were incubated alone or in combination with GXM for 2 days. mAb to TLR2 and FcγRII addition did not affect FasL induction (Fig. 4*A*); in contrast, mAb to CD14 or CD18 reduced FasL up-regulation (Fig. 4*A*).

Western blot experiments were performed to confirm up-regulation of FasL and modulation of FasL via TLR4. Western blot analysis revealed that FasL protein was expressed abundantly in GXM-treated MDM and that addition of mAb to TLR4 resulted in a reduction in FasL level (Fig. 4*B*). Given that the TLR4 signal pathway could involve MyD88 (50), we investigated whether GXM stimulated MyD88 via TLR4. To this end, MDM were stimulated with GXM (50 μg/ml), and MyD88 was determined at various times (30 min, 2, 24, and 48 h) by Western blot analysis. In these conditions, GXM did not induce activation of MyD88 (data not shown).

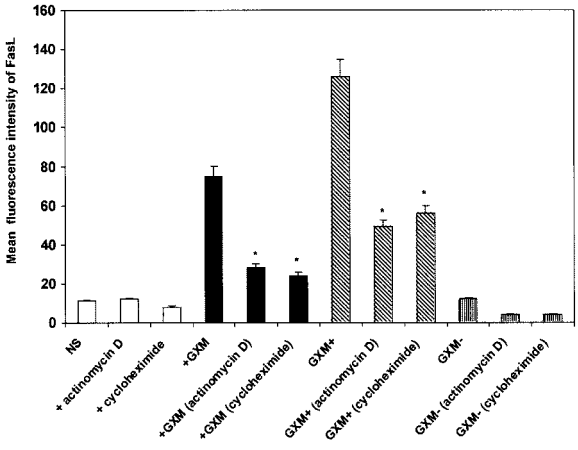

FIGURE 3. Effect of cycloheximide and of actinomycin D on FasL expression. The MDM (10^6/ml), pretreated or not with cycloheximide (10 μg/ml) or actinomycin D (1 μg/ml), were incubated for 2 days in the presence or absence of GXM (50 μg/ml). FasL expression was analyzed on not stimulated cells (NS), GXM-treated MDM (+GXM), and GXM-treated MDM that were gated to identify cells that accumulated GXM (GXM+) and GXM-treated MDM that were gated to identify cells that did not accumulate GXM (GXM−). Bars represent the mean ± SEM of five experiments using MDM from different donors. *, A value of $p < 0.05$ GXM-treated MDM incubate in presence of cycloheximide or actinomycin D vs GXM-treated MDM.

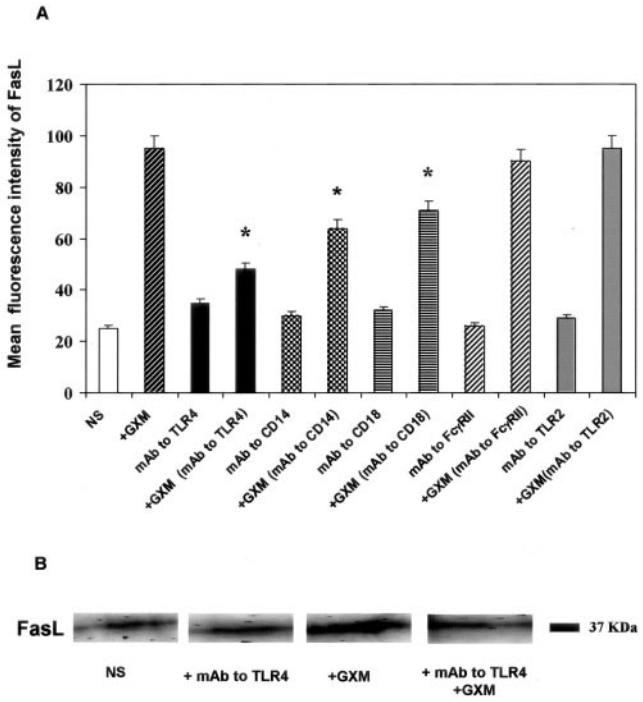

FIGURE 4. Contribution of TLR4, CD14, CD18, FcγRII, or TLR2 to FasL modulation. MDM (10^6/ml) were treated with or without GXM (50 μg/ml) for 2 days in the presence or absence of mAb to TLR4 (2.5 μg/ml) or mAb to CD14 (5 μg/ml), or mAb to CD18 (10 μg/ml) or mAb to FcγRII (10 μg/ml) or mAb to TLR2 (10 μg/ml). *A*, FasL expression on MDM was analyzed on MDM that were not treated with GXM (not stimulated cells (NS)) and GXM-treated MDM (+GXM). The addition of an isotype-matched control Ab did not affect FasL expression. Bars represent the mean ± SEM of eight experiments using MDM from different donors. ∗, A value of $p < 0.05$ MDM GXM-treated (+GXM) vs GXM plus various mAb-treated cells (+GXM plus various mAbs). *B*, Western blot analysis for FasL. MDM (10^6/ml) were treated with or without GXM (50 μg/ml) for 2 days in the presence or absence of mAb to TLR4 (2.5 μg/ml). Cells lysates were subjected to Western blotting. Membranes were incubated with mAb to FasL (1 μg/ml) and stained as described in *Materials and Methods*. Blots are representative of results obtained from three separate experiments.

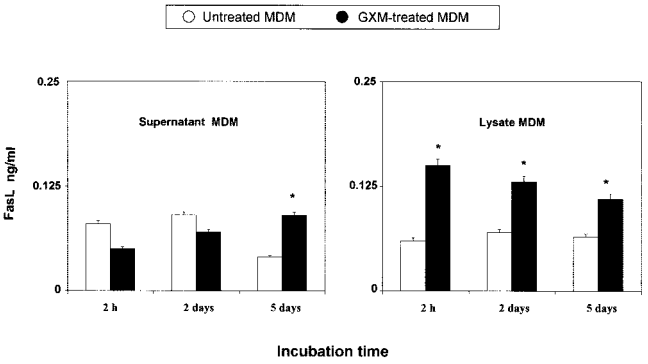

FIGURE 5. Evaluation of FasL in supernatant fluids from MDM. MDM (10^6/ml) were untreated or treated with GXM (50 μg/ml) for 2 h or 2 or 5 days. After incubation, the FasL levels were determined by FasL ELISA kit. Bars represent the mean ± SEM of four experiments using MDM from different donors. ∗, A value of $p < 0.05$ GXM-treated vs GXM-untreated cells.

FasL is found on killer cell-derived vesicles (6, 51) but can also be cleaved from the membrane by a metalloprotease (52–54). Moreover, it has been reported that soluble human FasL can induce apoptosis (18) but conversely soluble mouse FasL cannot (55). Therefore, we investigated the ability of GXM to induce the release of soluble FasL. The MDM were incubated for 2 h or 2 or 5 days with GXM, and cells as well as supernatant fluids were recovered. Lysates were prepared from the macrophages, and FasL concentrations were determined for the supernatant fluids and lysates. The results show that release of FasL from MDM required 5 days of incubation with GXM. In contrast, up-regulation of FasL was quite apparent in lysates prepared from MDM that had been incubated with GXM for only 2 h (Fig. 5). Accumulation of FasL in the supernatant fluid was not due to release by dead cells as demonstrated by determination of cell viability that was >95%.

Overexpression of FasL could facilitate the capacity of MDM to induce apoptosis. To evaluate the capacity of GXM-loaded macrophages to induce apoptosis in T lymphocytes, the MDM were incubated for 2 days with GXM (50 μg/ml), and T cells, untreated or preactivated with PHA (5 μg/ml), were added. T cells stimulated with PHA showed a significant increase ($p < 0.05$) of Fas (mean fluorescence intensity of PHA-stimulated T cells was 68 ± 5 in respect to 15 ± 2 of unstimulated cells). The results (Fig. 6) show a significant increase of annexin-positive T cells (Fig. 6*A*) and T cells with apoptotic nuclei (Fig. 6*B*) after 3 or 4 days of culture with GXM-loaded macrophages. The addition of mAb to Fas (5 μg/ml) to coculture of MDM and T cells, preactivated with PHA, drastically reduced GXM-induced apoptosis. The percentage of annexin-positive T cells, after 3 days of incubation, resulted in 15.6 ± 1 and 39 ± 3% in the presence or in the absence of mAb to Fas, respectively. This effect supports the argument that apoptosis is mediated by FasL overexpression on MDM. Incubation of MDM with ionomycin (1 μg/ml) or with GXM resulted in 44 ± 3 and 40 ± 4% of annexin-positive T cells, respectively. In selected experiments, MDM were incubated for 2 days with GXM (50 μg/ml) in the presence or absence of mAb to TLR4 (2.5 μg/ml) and T cells, preactivated or not with PHA (5 μg/ml), then T cell apoptosis was evaluated after 3 days. The percentage of annexin-positive cells in the presence of mAb to TLR4 was 27 ± 2 in respect to 39 ± 4 in the absence of mAb to TLR4.

To better analyze the apoptotic phenomenon observed after stimulation with GXM-treated macrophages, the macrophages were cocultured with Jurkat cells, which express high levels of Fas (56). The results (Fig. 7) show a significant increase in the percentage of apoptotic Jurkat cells after incubation with GXM-treated MDM compared with Jurkat cells cocultured with GXM-untreated MDM. Moreover, the addition of mAb to Fas blocked the GXM-induced apoptosis, supporting the argument that apoptosis is mediated by FasL overexpression on MDM. Jurkat cells that were incubated for 3 h with GXM (50 μg/ml) alone showed no significant increase in apoptosis, indicating that the observed apoptosis produced by GXM-treated MDM was not due to a direct GXM-Jurkat interaction (data not shown).

Discussion

Signaling through Ag receptors determines whether a cell survives or dies. A subset of TNF receptor family members are responsible for death-transducing signals. One such family member is Fas, which can be regulated by cytokine and leukocyte activation (9, 10). FasL, a type II membrane protein belonging to the tumor necrosis, nerve growth factor family, triggers apoptosis through Fas (13). In this study, we demonstrated that: 1) addition of purified capsular material of *C. neoformans* to macrophages elicited potent overexpression of FasL; 2) among the GXM-treated cell population, only the GXM-loaded cells manifested FasL up-regulation; 3) FasL was up-regulated after 2 h for up to 1 wk; 4) GXM

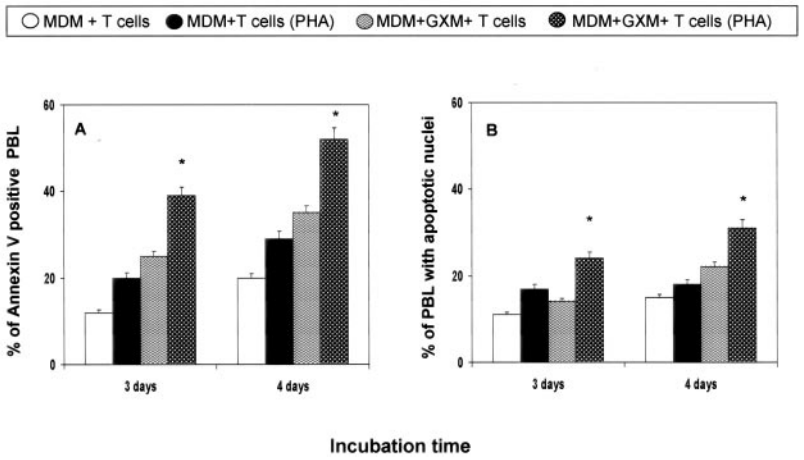

FIGURE 6. Apoptosis of T cells induced by GXM-treated MDM. *A*, Staining of the cell surface with annexin V. *B*, Staining of nuclei with PI. MDM (10^6/ml) treated or not with GXM were incubated with T cells (10^5/ml) preactivated or not with PHA. After 3 and 4 days of incubation, the T cells were recovered and analyzed for annexin V binding and apoptotic nuclei as described in *Materials and Methods*. Bars represent the mean ± SEM of 10 experiments using MDM from different donors. ∗, A value of $p < 0.05$ PBL incubated with GXM-treated MDM vs T cells incubated with MDM.

is associated with enhanced FasL expression in the cellular periphery and induces new FasL synthesis; 5) the induction of FasL occurs in part through GXM-TLR4 interaction; 6) GXM treatment promotes a late cleavage of FasL; and 7) GXM-loaded macrophages induce apoptosis of activated T cells expressing Fas and the Jurkat T cell line.

Some body tissues, such as eye and brain, that cannot regenerate or repair themselves limit or prevent intense immune response by production of suppressive molecules (57). The eye, an organ that constitutively expresses FasL, is considered the prototype of an immune-privileged site. Expression of the proapoptotic molecule, FasL, is a key component in immune privilege, and it is thought that FasL could be used to control rejection in organ transplantation (58). The fact that a single dose of GXM exerts durable up-regulation of FasL suggests that GXM-loaded macrophages might inhibit the development of inflammatory responses in vivo by killing Fas-positive lymphocytes that reach *C. neoformans*-infected tissues. The elimination of reactive lymphocytes suggests a new mechanism that could contribute to the phenomenon of GXM-mediated immunosuppression (30). Our in vitro experiments were performed using mature macrophage cells that resemble those resident in tissue. Considering that in organs such as the liver the up-regulation of FasL is responsible for inducing death of Fas^+ T cells (57, 59), this mechanism may be responsible for the relative lack of inflammatory-related toxicity observed in organs containing GXM (60–62). Our studies were done using a GXM concentration of 50 μg/ml, which is within the range observed during cryptococcal infection.

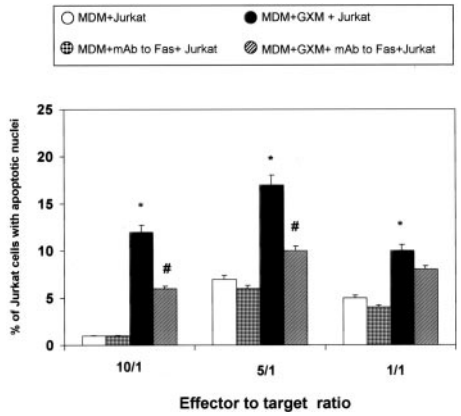

FIGURE 7. Apoptosis of Jurkat cells induced by GXM-treated MDM. MDM (10^6/ml) treated or not with GXM were incubated with Jurkat cells at various E:T ratios in the presence or absence of mAb to Fas. After 24 h of incubation, the cells were recovered and analyzed for apoptotic nuclei with PI as described in *Materials and Methods*. Bars represent the mean ± SEM of four experiments. ∗, A value of $p < 0.05$ Jurkat cells incubated with GXM-treated MDM vs Jurkat cells incubated with MDM. #, A value of $p < 0.05$ Jurkat cells incubated with GXM-treated MDM in presence of mAb to Fas vs Jurkat incubated with GXM-treated MDM in the absence of mAb to Fas.

Following GXM stimulation, macrophages synthesized, displayed, and released FasL. It is noteworthy that FasL release occurred late, up to 5 days of incubation, in contrast with the increase of total FasL that occurred very early. It is possible that the continuous accumulation of FasL on the cell surface could lead to its release when unused, as in our in vitro experimental system, but this may not be the case in vivo when FasL is able to interact with Fas on T cells, thereby producing apoptosis.

Kinetic experiments showed that FasL overexpression is long-lasting and occurs very early after GXM treatment. This prolonged action could be related to the fact that GXM remains internalized in macrophages for >1 mo (60). Hence, the two effects could be related and follow parallel pathways. Indeed, GXM could influence FasL expression in several ways: by inducing the translocation of the molecules on the cellular surface, by reducing recycling of surface FasL, and by promoting new synthesis. Thus, it is possible that a prompt and long-lasting synthesis of FasL could be achieved through GXM treatment. From our observation, a part of macrophages do not interact with GXM, which suggests unavailability or poor availability of receptors involved in GXM engagement.

The induction in FasL was at least mediated in part by GXM binding to TLR4, an observation that suggests activation through TLR4 can promote FasL induction. This observation provides a new insight into the recent observation that TLR4 stimulation can induce apoptotic signals (50). Moreover, we demonstrated that GXM treatment does not activate MyD88, consistent with a recent report of MyD88-independent apoptotic pathways (50).

Recent data show that FasL blocks the growth of excessive blood vessels by inducing apoptosis of recruited leukocytes that secrete TNF and endothelial growth factors (63). Angiogenesis is a process that could be detrimental to the host during inflammatory disease, as well as during tumor progression (64). In diabetic patients, for example, vessel growth causes a variety of problems such as retinal detachment (65). Given that the progression of tumors requires new vessel formation, GXM could exert an angiostatic effect at a local level by inducing up-regulation of FasL and, as a consequence, may reduce tumor invasion.

We have previously reported potent, mostly immunosuppressive, activities exerted by GXM, including induction of IL-10 from

cells of monocyte/macrophage lineage. In addition, GXM suppresses APC function by inhibiting MHC class II and costimulatory molecules (26). GXM-mediated alteration in macrophage function was associated with an inhibition of T cell responses to specific *C. neoformans* stimulation (30). The ability of GXM to stimulate synthesis and surface expression of FasL on macrophages and induction of apoptosis in T cells greatly expands the repertoire of immunosuppressive activities that this secreted molecule can bring to bear in the pathogenesis of cryptococcosis. In this regard, significant apoptosis has been noted in inflammatory cells in the CNS in a rat model of cryptococcosis (66). Importantly, induction of apoptosis has been reported for some viruses or microorganisms such as the HIV virus (67), *Mycobacterium avium* (68), and listeria proteins (69).

In addition to the direct application of these results to our understanding of cryptococcal pathogenesis, the present study also suggests new opportunities for a pharmacological application of this relatively nontoxic compound. The mechanisms described here may be responsible for the previously described anti-inflammatory effects of *C. neoformans* culture filtrate in experimental septic arthritis (70) and immune-mediated glomerulonephritis (71). Additional potential beneficial effects include elimination of self-reactive lymphocytes and production of anti-angiogenic effects. In this regard, anticancer drugs such as FR01228 (72) or carboplatin (73) produce apoptosis, and at least a part of the therapeutic effects of most anticancer drugs against malignancy are believed to include their apoptotic nature (74–76).

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Disclosures

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