Glucuronoxylomannan, a Microbial Compound, Regulates Expression of Costimulatory Molecules and Production of Cytokines in Macrophages

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Glucuronoxylomannan (GXM) is a microbial compound that can modulate the immune response. We investigated (1) the receptors involved in uptake of GXM on monocyte-derived macrophages (MDMs) from healthy donors, (2) the effects of GXM on expression of specific receptors, (3) the effects of GXM mediated by pattern-recognition receptors, and (4) GXM modulation of MDM accessory and secretory functions. Cellular receptors involved in uptake of GXM included FcγRII, CD18, Toll-like receptor (TLR) 4, and CD14. Some biological functions of MDMs were profoundly affected by treatment with GXM, resulting in (1) increased expression of CD40 and CD86 via perturbation of TLR4, (2) decreased expression of major histocompatibility complex class II, (3) induction of interleukin-10 but not of tumor necrosis factor–α, and (4) decreased lipopolysaccharide (LPS)–induced production of cytokines. GXM represents an attractive compound to limit inflammatory processes and induce an LPS-tolerant state.

Glucuronoxylomannan (GXM) is the major polysaccharide component in the capsule of *Cryptococcus neoformans*. *C. neoformans* causes life-threatening infections in immunocompromised patients, including those with AIDS [1]. GXM is found in the capsule and the extracellular space, in soluble form, during growth both in vivo and in vitro. Recently, it was suggested that GXM interacts with CD14/Toll-like receptor (TLR) 4 to elicit a cell response [2]. Effects ascribed to GXM include inhibition of humoral response [3], T cell proliferation [4], development of Th1 [5], delayed-type hypersensitivity response [6], chemotactic activity [7], production of cytokines, and induction of inhibitory factors such as interleukin (IL)–10 [8].

Recently, we observed that GXM is processed differently by monocytes and by neutrophils. Monocytes provide a long-lasting reservoir for GXM, whereas GXM ingested by neutrophils is rapidly degraded and/or expelled [9]. Immunostaining studies have shown that GXM persists in rat alveolar macrophages for months after pulmonary cryptococcal infection [10]. Given that neutrophils have a limited half-life and that GXM can persist in monocytes/macrophages for extended periods, it is likely that monocytes/macrophages have the primary responsibility in binding GXM and initiating the disparate immunological events associated with this molecule. In the present study, we report a detailed analysis of GXM interference in molecular signals and give particular attention to the dissection of the signaling effects transmitted via pattern-recognition receptors.

MATERIALS AND METHODS

Reagents and media. RPMI 1640 medium plus glutamine and fetal calf serum (FCS) was obtained from

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GIBCO BRL. Phycoerythrin (PE)–conjugated mouse monoclonal antibodies (MAbs) to human CD14 (IgG2ak), CD40 (IgG1), major histocompatibility complex class II (MHC-II) (IgG1), CD16 (IgG1), FcγRII (IgG1), CD64 (IgG1), CD80 (IgG1), and CD86 (IgG1)—together with unlabeled mouse MAbs to human CD14 (IgG2ak), CD18 (IgG2a), CD16 (IgG1), FcγRII (IgG1), CD64 (IgG1), mannose receptor (IgG1), CD80 (IgG1), and CD86 (IgG1)—were purchased from Ancell. Mouse isotype controls IgG1, IgG1k, IgG2a, and IgG2ak, $F(ab')_2$ fragments of PE-conjugated goat anti–rabbit IgG (whole molecule), and $F(ab')_2$ fragments of PE-conjugated sheep anti–mouse IgG (whole molecule) were purchased from Sigma; rabbit polyclonal antibody to human TLR4 (IgG) was purchased from Santa Cruz Biotechnology. Anti–IL-10 MAb was purchased from Genzyme. Phospho–NF-kB p65 (Ser536) and phospho–IkB-α (Ser32) antibodies were purchased from Cell Signaling Technology. Glucan from baker's yeasts was purchased from Sigma.

The characteristics of the MAb to GXM, 18B7, have been described elsewhere [11, 12]. GXM was isolated from the culture supernatant of a serotype A strain (CN 6) grown in liquid synthetic medium [13, 14]. All reagents and media and the GXM used in the present study were negative for endotoxin, as determined by use of a *Limulus amebocyte* lysate assay (Sigma), which had a sensitivity of ~0.05–0.1 ng of *Escherichia coli* lipopolysaccharide (LPS)/mL.

Preparation of monocyte-derived macrophages (MDMs) from peripheral blood monocytes (PBMs) and T lymphocytes. Heparinized venous blood was obtained from buffy coats. Mononuclear and adherent cells were separated, as described elsewhere [9]. MDMs were obtained by incubation of PBMs in RPMI 1640 medium plus 5% FCS (cRPMI) containing 50 ng/mL macrophage colony-stimulating factor (PeproTech EC), for 5 days [15]. Nonadherent cells were E-rosetted, and a lymphoproliferation assay was performed, as described elsewhere [16, 17].

Preparation of fluorescein-labeled MAb 18B7 (carboxyfluorescein diacetate succinimidyl ester [CFSE]–18B7). GXM MAb 18B7 and MAb IgG1k (isotype control) were labeled with CFSE by use of a fluorescein protein labeling kit (Boehringer Mannheim Biochimica), in accordance with the manufacturer's directions.

Flow-cytometric analysis of uptake of GXM by MDMs. Uptake of GXM by MDMs was quantified by flow-cytometric analysis. MDMs were incubated with GXM (50 μg/mL), in RPMI 1640 medium plus 10% FCS, for either 2 h, 2 days, or 5 days at 37°C with 5% CO_2. After incubation, the cells were collected, stained with MAb to GXM, and analyzed by use of a FACScan cytometer (Becton Dickinson), as described elsewhere [9].

Inhibition of GXM binding by antibodies to mannose receptor, CD14, TLR4, CD18, CD16, FcγRII, CD64, CD80, CD86, and CD40. Freshly isolated MDMs (1×10^6) were incubated with GXM (50 μg/mL), in 1 mL of RPMI 1640 medium plus

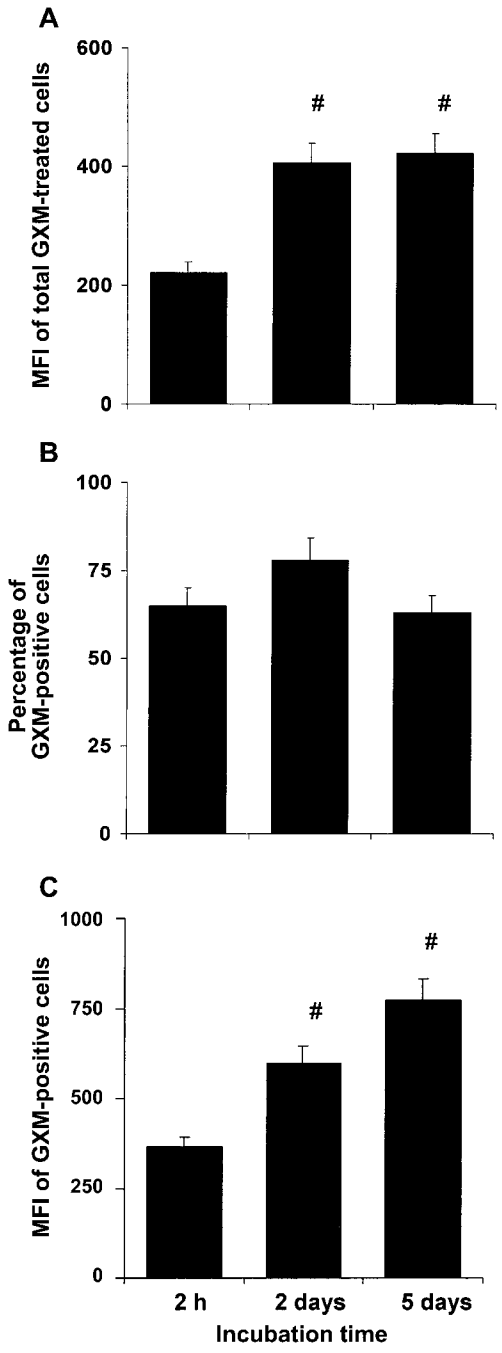

Figure 1. Time course for accumulation of glucuronoxylomannan (GXM) by monocyte-derived macrophages (MDMs). MDMs (1×10^6 cells/mL) were treated with GXM (50 μg/mL) for either 2 h, 2 days, or 5 days at 37°C. After incubation, the cells were stained for GXM with carboxyfluorescein diacetate succinimidyl ester–18B7 and analyzed by flow cytometry. *A,* Mean fluorescence intensity (MFI) of total expression of GXM-treated cells; *B,* percentage of GXM-positive cells; *C,* MFI of GXM-positive cells. The MFI of GXM-negative cells was similar to that of cells treated with isotype control antibody. The results are the mean ± SEM of 10 separate experiments using MDMs from different donors. #$P < .05$ (MFI at 2 days and 5 days vs. MFI at 2 h).

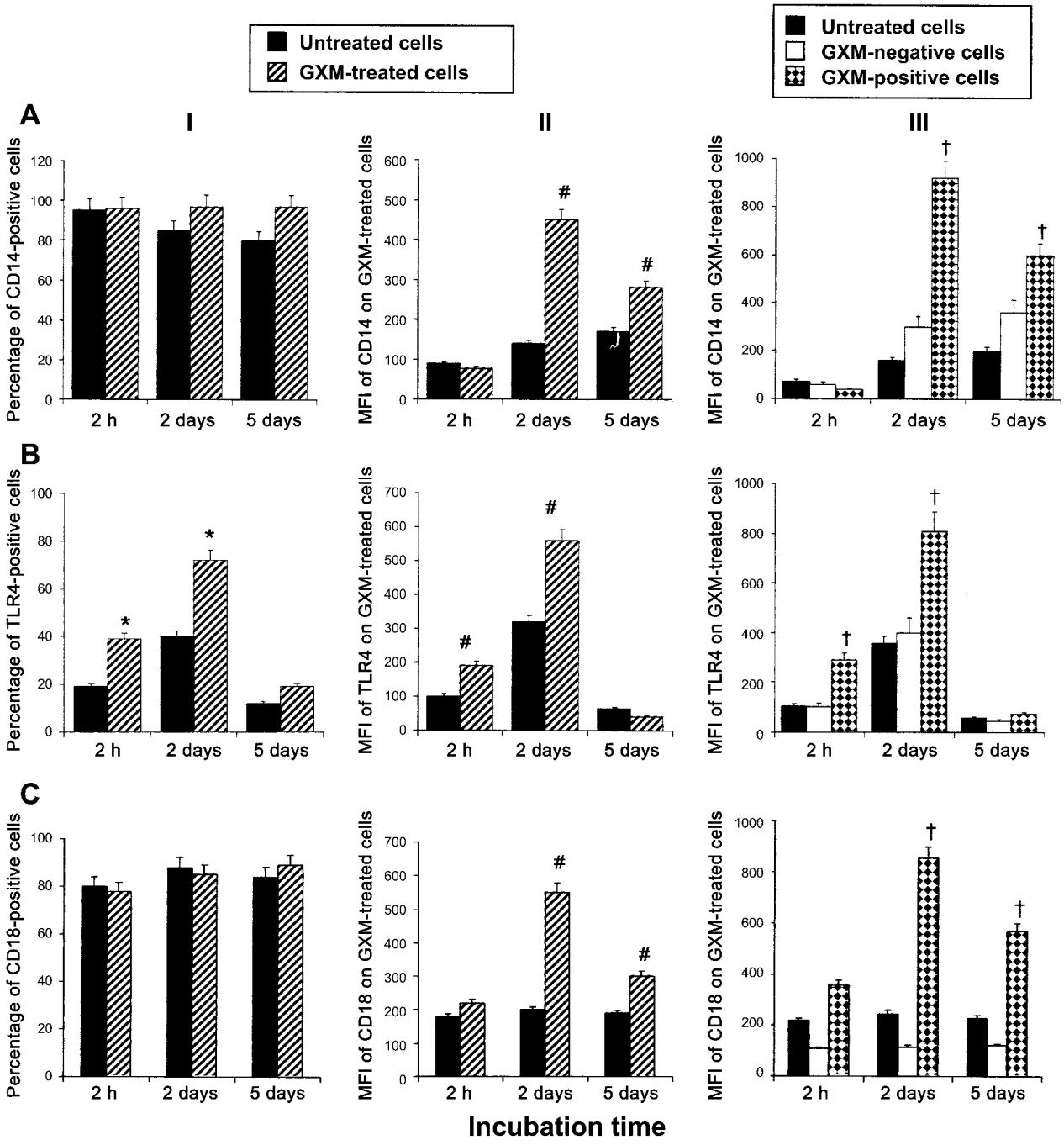

Figure 2. Effect of treatment with glucuronoxylomannan (GXM) on the kinetics of expression of CD14, Toll-like receptor (TLR) 4, and CD18 by monocyte-derived macrophages (MDMs). MDMs (1×10^6 cells/mL) were incubated with or without GXM (50 μg/mL) for either 2 h, 2 days, or 5 days at 37°C. After incubation, the cells were stained for GXM with carboxyfluorescein diacetate succinimidyl ester–18B7 followed by phycoerythrin (PE)–anti-CD14 *(A),* anti-TLR4 followed by PE-conjugated antibody *(B),* or anti-CD18 followed by PE-conjugated antibody *(C),* and then were analyzed by flow cytometry. *I,* Percentage of CD14- *(A),* TLR4- *(B),* or CD18-positive *(C)* cells; *II,* mean fluorescence intensity (MFI) of surface expression of CD14 *(A),* TLR4 *(B),* or CD18 *(C)* by MDMs; *III,* MFI of surface expression of CD14 *(A),* TLR4 *(B),* or CD18 *(C)* by GXM-treated cells gated into GXM-positive and GXM-negative cells. The MFI of GXM-negative cells was similar to that of cells treated with isotype control antibody. The results are the mean ± SEM of 5 separate experiments using MDMs from different donors. *$P < .05$ (percentage of positive cells, GXM-treated vs. untreated cells); #$P < .05$ (GXM-treated vs. untreated cells); †$P < .05$ (GXM-positive vs. GXM-negative cells).

10% FCS, for 2 h at 37°C with 5% CO_2, in the presence or absence of anti-CD14 (5 μg/mL), anti-TLR4 (2.5 μg/mL), anti-CD18 (10 μg/mL), anti–mannose receptor (10 μg/mL), anti-CD16 (5 μg/mL), anti-FcγRII (10 μg/mL), anti-CD64 (10 μg/mL), anti-CD80 (5 μg/mL), anti-CD86 (5 μg/mL), or anti-CD40 (5 μg/mL). After incubation, the cells were collected, stained with MAb to GXM, and analyzed by use of a FACScan cytometer, as described elsewhere [9].

Flow-cytometric analysis of CD14, TLR4, CD18, FcγRII, MHC-II, CD40, and CD86. MDMs (1×10^6) were incubated in the presence or absence of GXM (50 μg/mL), in RPMI 1640 medium plus 10% FCS, for either 2 h, 2 days, or 5 days at 37°C with 5% CO_2. After incubation, the cells were collected, stained as described elsewhere [9], washed twice with fluorescence buffer (FB), and stained either with PE-conjugated mouse MAb specific for CD14 (5 μg/mL), MHC-II (5 μg/mL), CD40 (5 μg/mL), CD86 (10 μg/mL), CD18 (10 μg/mL), or FcγRII (10 μg/mL) or with rabbit anti-TLR4 (2.5 μg/mL) in FB for 20 min on ice. Then, MDMs were washed again in FB, and 5000 events were analyzed by flow cytometry. Cells incubated with MAb to CD18 or MAb to FcγRII were later stained with PE-conjugated sheep anti–mouse IgG (whole molecule), whereas cells incubated with rabbit anti-TLR4 were stained with PE-conjugated goat anti–rabbit IgG (1:20) in FB for 20 min on ice.

To determine the total (intracellular plus surface) expression of CD14, TLR4, CD18, and FcγRII on GXM-treated MDMs, after 2 days of incubation, the cells were washed, fixed, and permeabilized, as described above, and were incubated with specific antibodies, in labeling buffer (LB), at the above-mentioned concentrations. After incubation, MDMs were washed with permeabilization buffer, incubated with a conjugated, specific antibody, in LB, and analyzed by use of a FACScan cytometer.

To analyze the expression of the above-mentioned receptors on GXM-positive or GXM-negative MDMs, the cells were stained with the CFSE-labeled MAb to GXM, and discriminatory gates were placed around the GXM-positive (stained green) and GXM-negative (unstained) MDMs. Specific fluorescence was assessed by comparison with results from the mouse isotype control CFSE-IgG1k.

Viability of MDMs treated with GXM. The viability of MDMs treated for 5 days with GXM was measured, by use of a colorimetric reaction, on the basis of the capacity of the mitochondrial dehydrogenase in living cells to reduce levels of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Aldrich Chemical), as described elsewhere [18].

Production of MDM culture supernatant. MDMs (1×10^6) were incubated with or without GXM (50 μg/mL), in the presence or absence of MAb to TLR4 (2.5 μg/mL), in RPMI 1640 medium plus 10% FCS, for 2 h at 37°C with 5% CO_2. After incubation, the cells were washed and incubated (5×10^6 cells/mL) with or without LPS (10 μg/mL), in RPMI 1640 medium plus 10% FCS, for 18 h at 37°C with 5% CO_2. Culture supernatants were harvested and stored at −20°C until assayed.

Determination of cytokines. Levels of cytokines in culture supernatant were measured by ELISA, by use of kits for human IL-10 (Cytopass) and tumor necrosis factor (TNF)–α (BD Biosciences Pharmigen).

Determination of IkB-α and NF-κB. MDMs (1×10^6) were incubated with or without GXM (50 μg/mL), in RPMI 1640 medium plus 10% FCS, for 1 h at 37°C with 5% CO_2, were washed, and were incubated (5×10^6 cells/mL) with or without LPS (10 μg/mL), in RPMI 1640 medium plus 10% FCS, either for 30 min (for analysis of NF-kB) or for 30 min, 60 min, and 24 h (for analysis of IkB-α) at 37°C with 5% CO_2.

After coculture, the cells were washed and treated with 200 μL of mammalian protein extract reagent (Pierce), in the presence of protease inhibitors (Pierce), and lysates were collected by centrifugation for 10 min at 12,000 *g*. Extracted 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 incubated for 1 h at room temperature in a blocking buffer containing 0.1% Tween in Tris-HCl buffered solution and 5% nonfat dried milk

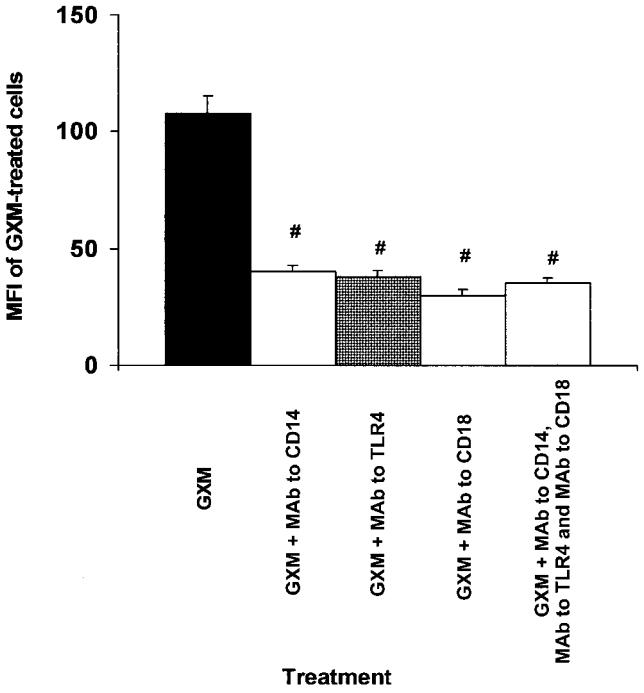

Figure 3. Effect of monoclonal antibodies (MAbs) to CD14, Toll-like receptor (TLR) 4, and CD18 on accumulation of glucuronoxylomannan (GXM) by monocyte-derived macrophages (MDMs). MDMs (1×10^6 cells/mL) were incubated with GXM (50 μg/mL), in the presence or absence of MAb to CD14, TLR4, and CD18 or in the presence of all antibodies together, for 2 h at 37°C. The results are the mean ± SEM of 3 separate experiments using MDMs from different donors. #$P < .05$ (GXM plus antibody–treated vs. GXM-treated cells). MFI, mean fluorescence intensity.

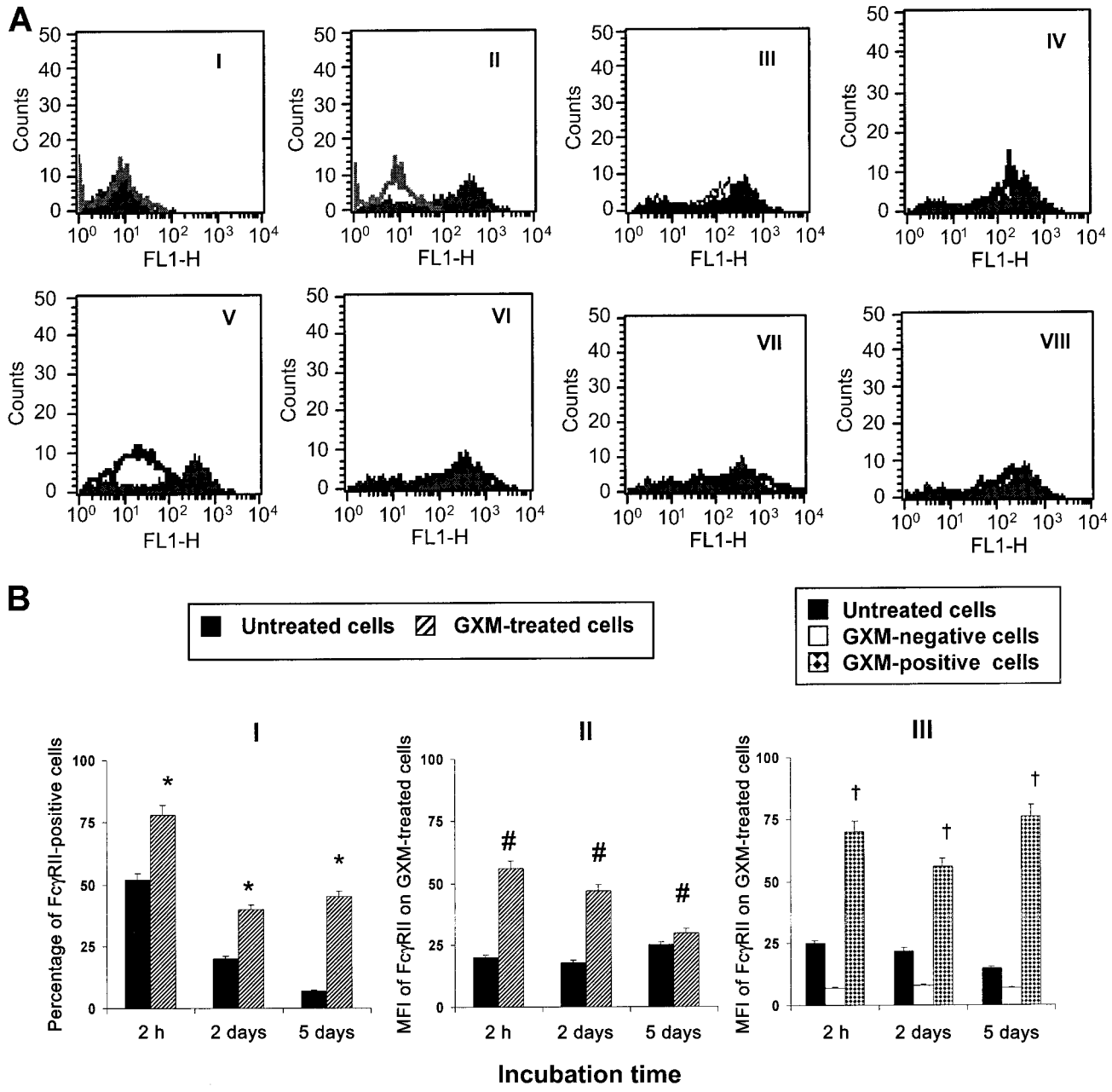

Figure 4. Effect of monoclonal antibodies (MAbs) to CD16, FcγRII, CD64, CD80, and CD86 on accumulation of glucuronoxylomannan (GXM) by monocyte-derived macrophages (MDMs) *(A)* and effect of treatment with GXM on expression of FcγRII by MDMs *(B). A,* MDMs (1×10^6 cells/mL) were incubated with or without GXM (50 μg/mL) for 2 h at 37°C in the presence or absence of various antibodies. After incubation, the cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE)–18B7. *A: I,* MDMs incubated alone *(black)* or with MAb isotype control CFSE-IgG1k *(gray); II,* MDMs incubated with GXM (50 μg/mL) and stained with CFSE-18B7 *(gray shading)* or with MAb isotype control CFSE-IgG1k *(gray lines); III,* MDMs incubated with GXM (50 μg/mL), in the presence or absence *(gray shading)* of MAb isotype control IgG1 (5 μg/mL) *(black lines),* and stained for GXM with CFSE-18B7; *IV–VIII,* MDMs incubated with GXM (50 μg/mL), in the presence *(thick black lines)* or absence *(gray shading)* of MAbs to CD16 (5 μg/mL) *(IV),* FcγRII (10 μg/mL) *(V),* CD64 (10 μg/mL) *(VI),* CD80 (5 μg/mL) *(VII),* or CD86 (5 μg/mL) *(VIII),* and stained for GXM with CFSE-18B7. Results are from 1 experiment representative of 4 experiments, all of which produced similar results. Counts, number of cells analyzed; FL1-H, fluorescent channel 1 height. *B,* MDMs (1×10^6 cells/mL) were incubated alone or with GXM (50 μg/mL) for either 2 h, 2 days, or 5 days at 37°C. After incubation, the cells were stained for GXM with CFSE-18B7, followed by incubation with anti-FcγRII and staining with phycoerythrin-conjugated antibody, and then were analyzed by flow cytometry. *B: I,* Percentage of FcγRII-positive cells; *II,* mean fluorescence intensity (MFI) of surface expression of FcγRII by MDMs; *III,* MFI of surface expression of FcγRII by GXM-treated cells gated into GXM-positive and GXM-negative cells. The MFI of GXM-negative cells was similar to that of cells treated with isotype control antibody. The results are the mean ± SEM of 5 separate experiments using MDMs from different donors. *$P < .05$ (percentage of positive GXM-treated vs. untreated cells); #$P < .05$ (GXM-treated vs. untreated cells); †$P < .05$ (GXM-positive vs. GXM-negative cells).

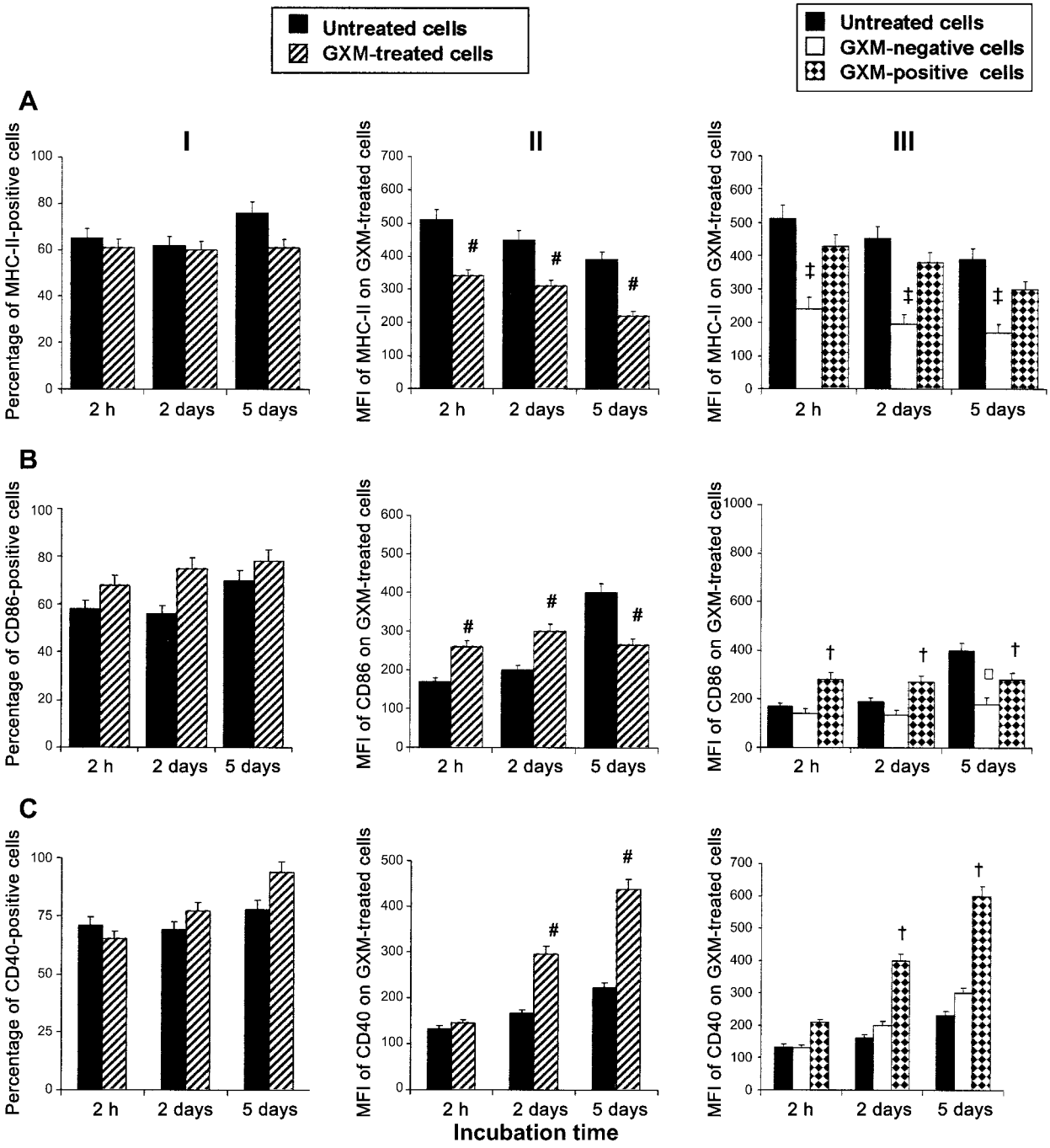

Figure 5. Effect of treatment with glucuronoxylomannan (GXM) on expression of major histocompatibility complex class II (MHC-II), CD86, or CD40 by monocyte-derived macrophages (MDMs). MDMs (1×10^6 cells/mL) were incubated with or without GXM (50 μg/mL) for either 2 h, 2 days, or 5 days at 37°C. After incubation, the cells were stained with carboxyfluorescein diacetate succinimidyl ester–18B7, followed by staining with phycoerythrin–anti–MHC-II *(A)*, anti-CD86 *(B)*, or anti-CD40 *(C)*, and then were analyzed by flow cytometry. *I*, Percentage of MHC-II– *(A)*, CD86- *(B)*, or CD40-positive *(C)* cells; *II*, mean fluorescence intensity (MFI) of surface expression of MHC-II *(A)*, CD86 *(B)*, or CD40 *(C)* by MDMs; *III*, MFI of surface expression of MHC-II *(A)*, CD86 *(B)*, or CD40 *(C)* by untreated and GXM-treated cells gated into GXM-positive and GXM-negative cells. The MFI of GXM-negative cells was similar to that of cells treated with isotype control antibody. The results are the mean ± SEM of 5 separate experiments using MDMs from different donors. #$P < .05$ (GXM-treated vs. untreated cells); ‡$P < .05$ (GXM-negative vs. GXM-positive cells); †$P < .05$ (GXM-positive vs. GXM-negative cells).

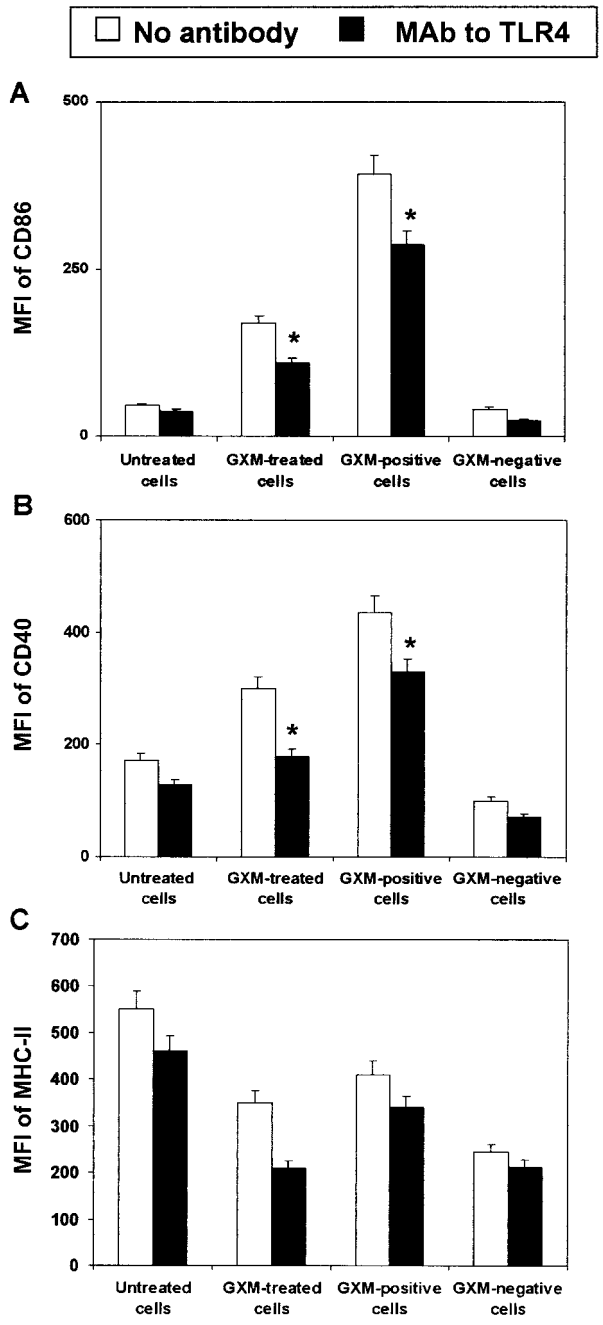

Figure 6. Effect of blockade of Toll-like receptor (TLR) 4 on expression of CD86 *(A),* CD40 *(B),* and major histocompatibility complex class II (MHC-II) *(C)* by untreated cells and by glucuronoxylomannan (GXM)–treated monocyte-derived macrophages (MDMs) gated into GXM-positive and GXM-negative cells. MDMs (1×10^6 cells/mL) were incubated with or without GXM (50 μg/mL), in the presence or absence of monoclonal antibody (MAb) to TLR4 (2.5 μg/mL), for 2 days at 37°C. After incubation, the cells were stained with carboxyfluorescein diacetate succinimidyl ester–18B7, followed by staining with phycoerythrin–anti–mouse IgG, and then were analyzed by flow cytometry. The mean fluorescence intensity (MFI) of GXM-negative cells was similar to that of cells treated with isotype control antibody. The results are the mean ± SEM of 5 separate experiments using MDMs from different donors. *$P < .05$ (antibody-treated vs. untreated cells).

and then were incubated with rabbit antibody (diluted 1:1000) specific for either phospho–NF-kB p65 (Ser536) or phospho–IkB-α (Ser32) (Cell Signaling), in blocking buffer, overnight at 4°C. The membranes were stained by use of a labeling kit (WesternBreeze Chemiluminescent Western Blot Immunodetection Kit; Invitrogen), in accordance with the manufacturer's directions, and immunoreactive bands were visualized and quantified by use of the Chemidoc Instrument (BioRad).

Statistical analysis. Data are reported as the mean ± SEM from replicate experiments and were evaluated by analysis of variance. Post hoc comparisons were performed by Bonferroni's test. $P < .05$ was considered to be significant.

RESULTS

We first evaluated the extent of MDM variation in GXM binding. The mean fluorescence intensity (MFI) of the total cell population showed significant loading of GXM within 2 h of incubation and additional accumulation after 2 and 5 days (figure 1*A*), confirming our earlier findings [9]. Gating analysis of GXM-positive or -negative cells revealed that ~60%–75% of the total population bound GXM, regardless of the incubation time (figure 1*B*), and GXM-positive MDMs showed a gradual and progressive accumulation of GXM over the course of 5 days (figure 1*C*). The viability of MDMs cultured in the presence or absence of GXM was >98% in all determinations performed, indicating that the lack of uptake was not due to cell death.

Subsequently, we examined the kinetics of surface expression of CD14 on MDMs in response to treatment with GXM. Essentially all MDMs expressed a measurable amount of surface expression of CD14, regardless of whether the cells were stimulated with GXM (figure 2*A, I*). However, MFI analysis of CD14 showed a dramatic increase of expression after 2 days (figure 2*A, II*), and only GXM-positive MDMs exhibited increased levels of surface expression of CD14 molecules (figure 2*A, III*). Furthermore, after 2 days, there was a significant increase ($P < .05$) in total expression of CD14 in GXM-treated cells (MFI, 670 ± 43), compared with that in untreated cells (MFI, 180 ± 21), suggesting that this up-regulation of CD14 corresponded to increased synthesis.

Uptake of GXM may also be mediated by TLR4 [2, 19]. Consequently, we analyzed expression of TLR4 in response to treatment with GXM. In contrast to CD14, less than one-half of the MDMs expressed TLR4 in the absence of treatment with GXM (figure 2*B, I*). Treatment with GXM produced strong enhancement of surface expression of TLR4 molecules after 2 days of treatment, as shown by both the percentage of TLR4-positive cells (figure 2*B, I*) and the MFI (figure 2*B, II*). A parallel increase in total expression of TLR4 molecules was observed in GXM-treated cells after 2 days of incubation (data not shown). Gating analysis showed that enhanced expression of TLR4 was limited to GXM-positive MDMs, measured at 2 days of in-

cubation (figure 2*B, III*), after which time there was a considerable loss of expression of TLR4 in all cells, with or without treatment with GXM. Further investigation of cell subpopulations that are receptive or unreceptive to GXM showed that GXM-negative cells manifested very low levels of expression of FcγRII. It has been reported that human neutrophils bind GXM via CD18 [20]. We therefore examined the effects of treatment with GXM on expression of CD18 by MDMs. The results show that 60%–70% of untreated MDMs were CD18 positive and were unchanged by treatment with GXM (figure 2*C, I*). However, MFI analysis of surface expression of CD18 molecules showed a significant increase 2 days after treatment with GXM (figure 2*C, II*), whereas the level of total expression of molecules was largely unchanged (data not shown). Gating analysis showed that enhanced surface expression of CD18 was limited to MDMs that had bound GXM (figure 2*C, III*).

Moreover, treatment of MDMs with a cocktail of MAbs to CD14, TLR4, or CD18 failed to produce complete inhibition of GXM ligation (figure 3). Therefore, we evaluated a series of MAbs to other cellular receptors, such as FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), B7-1 (CD80), and B7-2 (CD86) (figure 4*A, IV–VIII*, respectively). Only FcγRII (figure 4*A, V*) produced a significant ($P < .05$) reduction in uptake of GXM. The binding of GXM to naturally occurring antibody in the serum, with consequent binding to FcγRII, was excluded because the experiments conducted in the absence of serum produced similar results. Further analysis revealed that GXM induced expression of FcγRII (figure 4*B, I–III*). This correlated with a significant ($P < .05$) increase of total expression of FcγRII molecules in GXM-treated cells (MFI, 100 ± 9), compared with that in untreated cells (MFI, 61 ± 5), after 2 days of incubation, suggesting that GXM induces increased synthesis of FcγRII. In addition, gating analysis showed a significant increase of FcγRII in GXM-positive cells (figure 4*B, III*).

Further investigations showed that mannose receptors were not involved in uptake of GXM by MDMs. In fact, the treatment of cells with MAb to mannose receptor (10 μg/mL) did not influence uptake of GXM (data not shown). Similarly, blockade of CD40 by treatment with MAb to CD40 did not influence uptake of GXM (data not shown).

GXM could affect T cell function through effects on antigen-presenting cells (APCs). To investigate this possibility, we examined several molecular signals involved in the antigen (Ag) presentation process. First, we hypothesized that MHC-II could be modulated by treatment with GXM. No differences were observed between treated or untreated cells, in the percentage of positive cells (figure 5*A, I*); however, when we considered the MFI, a significant ($P < .05$) decrease was observed in GXM-treated cells (figure 5*A, II*). Subsequent gating showed that down-regulation of expression of MHC-II occurred primarily in GXM-negative cells (figure 5*A, III*).

In addition, we evaluated the effect of treatment with GXM on expression of costimulatory (CS) molecules by MDMs. Treatment of MDMs with GXM had a negligible effect on the percentage of cells that expressed CD86, compared with that in untreated cells (figure 5*B, I*). However, the MFI of CD86 molecules increased ($P < .05$) during 2 days of treatment with GXM but decreased after 5 days (figure 5*B, II*). The stimulatory effects on CD86 were limited to MDMs that had attached or internalized GXM.

Treatment of MDMs with GXM had no significant effect on the percentage of CD40-positive cells (figure 5*C, I*). However, MFI analysis showed significantly ($P < .05$) enhanced levels of

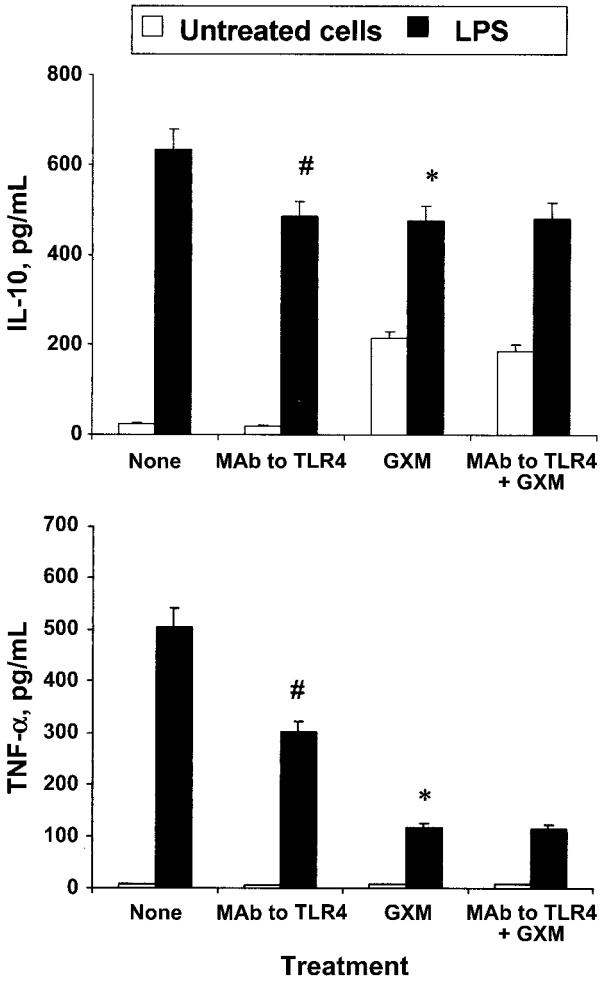

Figure 7. Levels of interleukin (IL)–10 and tumor necrosis factor (TNF)–α in culture supernatants of glucuronoxylomannan (GXM)–treated monocyte-derived macrophages (MDMs). MDMs (5×10^6 cells/mL) were incubated with GXM, in the presence or absence of monoclonal antibody (MAb) to Toll-like receptor (TLR) 4 (2.5 μg/mL), and were either stimulated with lipopolysaccharide (LPS) (10 μg/mL) for 18 h or were not stimulated. Culture supernatants were harvested, and levels of cytokines were determined by ELISA. The results are the mean ± SEM of 5 experiments using MDMs from different donors. LPS, lipopolysaccharide.

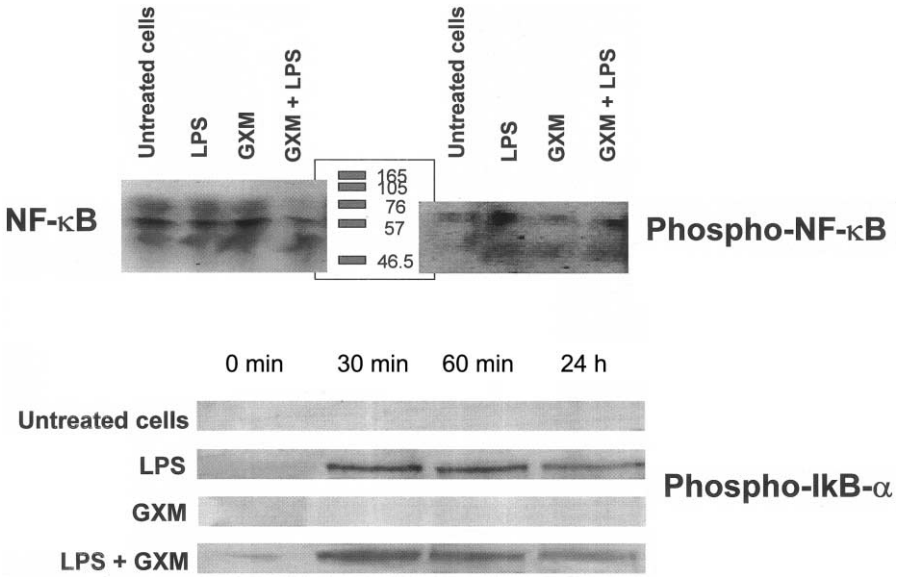

Figure 8. Detection of phosphorylated IkB-α and NF-κB induced by glucuronoxylomannan (GXM) and/or lipopolysaccharide (LPS), in monocyte-derived macrophages (MDMs). MDMs were untreated or treated with GXM for 1 h and then were incubated with LPS for 30 min. Cell lysates were subjected to Western blotting. Membranes were incubated with antibody to phospho–IkB-α and NF-κB or phospho–NF-κB. Blots are representative of results obtained from 3 separate experiments.

expression at 2 and 5 days (figure 5*C, II*). Overexpression of CD40 was limited to GXM-positive cells (figure 5*C, III*).

We analyzed the involvement of TLR4 in the regulatory activity of the CS pathway. MAbs to TLR4 were used to block uptake of GXM, and MHC-II, CD86, and CD40 were analyzed after 2 days. The results show a partial but significant ($P < .05$) blockade of GXM-mediated overexpression of CD86 and CD40 (figure 6*A* and 6*B*, respectively). In contrast, no significant ($P > .05$) effect on expression of MHC-II was found (figure 6*C*).

To verify whether the observed changes influenced the antigen-presentation process, MDMs were challenged with heat-inactivated encapsulated *C. neoformans* and cocultured with autologous T cells (for 7 days). The results showed that GXM-treated MDMs (for 2 days) were less efficient ($P < .01$) at inducing a blastogenic response than were untreated MDMs (3200 ± 120 and 8400 ± 980 cpm, respectively).

The role that TLR4 played in the previously demonstrated GXM induction of IL-10 by monocytes [8] was examined. MAbs to TLR4 had no effect on GXM-induced levels of IL-10. Treatment with GXM produced a limited but significant ($P < .05$) inhibition of LPS-induced secretion of IL-10 (figure 7*A*). In contrast, GXM dramatically reduced (80%) LPS-induced secretion of TNF-α (figure 7*B*). The decrease of secretion of TNF-α was not due to the presence of IL-10, because the neutralization of IL-10 by MAb to IL-10 (5 μg/mL) did not significantly affect the release of TNF-α, compared with that in irrelevant MAb–treated cells (data not shown).

Finally, we investigated whether GXM was able to modulate LPS-induced activation of NF-κB. The results (figure 8) showed that GXM did not stimulate NF-κB or IkB-α phosphorilation but that LPS did. Moreover, treatment with GXM did not affect LPS-induced activation of NF-κB.

DISCUSSION

The present study has examined the effects of treatment with GXM on the biological activities of MDMs. Up- and down-regulatory effects were observed. First, a significant percentage of MDMs failed to bind GXM. Second, GXM promoted expression of TLR4, CD14, and CD18, which have been implicated in binding and/or uptake of GXM by MDMs in previous studies [2, 20]. However, up-regulation of these cellular receptors was limited to cells that accumulated GXM and was associated with increased protein synthesis of CD14 and TLR4 but not of CD18. Third, we identified a potential role for FcγRII in binding and uptake of GXM in an antibody-independent process. As with other potential GXM receptors, expression of FcγRII was up-regulated by treatment with GXM. Fourth, exposure of MDMs to GXM was associated with altered function of APCs, as demonstrated by changes in expression of MHC-II and CS molecules. Fifth, the regulation of CS molecules by GXM involved TLR4. Finally, treatment of MDMs with GXM did not stimulate NF-κB and did not modulate LPS-induced activation of NF-κB. However, treatment with GXM dramatically reduced LPS-induced secretion of TNF-α.

Here, we have identified several receptors involved in uptake of GXM, furnished an explanation for the continuous accumulation of GXM inside the cells, and examined intracellular

signaling pathways and functional activity. MDMs were shown to use multiple receptors for binding and/or uptake of GXM, including CD14, TLR4, and CD18. The inability of a cocktail of MAbs to produce complete inhibition of uptake of GXM could be caused by steric hindrance due to the presence of multiple antibodies and/or the presence of other yet-unrecognized receptors. Furthermore, the expression of each of these receptors increased in GXM-treated cells, with the effect being seen only in GXM-positive cells. However, the kinetics of expression were similar for some receptors and were different for others. In particular, TLR4 and FcγRII exhibited early up-regulation (within 2 h) and returned to baseline levels after 5 days. In contrast, CD14 and CD18 showed late up-regulation (within 2 days), which lasted as long as 5 days.

TLR4 and CD14, which are considered to be key receptors for capturing GXM [2, 19], manifested different kinetics of expression in GXM-treated MDMs. TLR4 was up-regulated earlier and more transiently than was CD14, suggesting that the expression of these receptors could proceed in concert or separately when one is absent or unavailable. Our observations on uptake of GXM via FcγRII and CD18 extend previous studies showing that FcγRII is required for efficient presentation of *C. neoformans* by dendritic cells [21] and that GXM binds to CD18 on human neutrophils [7].

Given that GXM is easily taken up by MDMs, a major question was whether and how GXM could control their biological functions. Here, we have reported an until-now unknown mode of action of GXM that alters accessory (MHC-II and CS), effector, and secretory (production of TNF-α and IL-10) functions. In particular, MDMs became less efficient in stimulating T cell response and in producing proinflammatory cytokines.

Expression of MHC-II and CS molecules is believed to influence the efficiency of Ag presentation and, consequently, the T cell response [22, 23]. The regulation of expression of MHC-II and CS molecules by GXM was unexpected and complex. MHC-II was inhibited in GXM-treated cells, but gating analysis showed that down-regulation occurred only in GXM-negative cells, suggesting that GXM-positive cells could regulate GXM-negative cells via soluble factors or cell-to-cell contact. In contrast, the up-regulation observed in CS molecules was manifested in GXM-positive cells. This increase was, in part, ascribed to GXM/TLR4 interaction, because a significant decrease of CS molecules was observed when TLR4 was blocked.

We have found that GXM quenched the LPS-induced inflammatory response but was unable to interfere with LPS-induced activation of NF-κB. This suggests that the LPS-tolerant state may be due to differences in gene induction by GXM and LPS. Moreover, GXM could induce secretion of IL-10 via an NF-κB–independent pathway regulated by transcription-factor stimulatory protein 1. Furthermore, interaction of GXM with other receptors, such as TLR2, could trigger a cascade that potentially modulates the outcome of TLR4 signaling. With regard to the modest inhibitory effect of anti-TLR4 on LPS-induced IL-10, it is conceivable that the somewhat limited effect is due to simultaneous addition of MAbs to TLR4 and LPS, raising the possibility that LPS is still able to bind some TLR4. The capacity of GXM to quench the LPS-induced effect could be exploited to limit the deleterious response to endotoxin. It is likely that GXM-positive cells play an important role in limiting an inflammatory response; however, we cannot exclude a contribution from the entire cell population, including GXM-negative cells. The inability of GXM to induce activation of NF-κB is in contrast to the results published by Shoham et al. [2]; however, this apparent discrepancy could be related to the different experimental conditions. Shoham et al. used GXM from the J11a strain, at a dose of 250 μg/mL, and either murine RAW 264.7 or human peripheral blood mononuclear cells. Overall, these results show that a microbial polysaccharide can suppress multiple macrophage functions, thereby opening up new perspectives for the role that this microbial compound plays in promoting immunological tolerance.

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References

1. Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans.* Clin Microbiol Rev **1995**; 8:515–48.
2. Shoham S, Huang C, Chen JM, Golenbock DT, Levitz SM. Toll-like receptor 4 mediates intracellular signaling without TNF-α release in response to *Cryptococcus neoformans* polysaccharide capsule. J Immunol **2001**; 166:4620–6.
3. Murphy JW, Cozad GC. Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. Infect Immun **1972**; 5:896–901.
4. Retini C, Vecchiarelli A, Monari C, Bistoni F, Kozel TR. Encapsulation of *Cryptococcus neoformans* with glucuronoxylomannan inhibits the antigen-presenting capacity of monocytes. Infect Immun **1998**; 66:664–9.
5. Syme RM, Bruno TF, Kozel TR, Mody CH. The capsule of *Cryptococcus neoformans* reduces T-lymphocyte proliferation by reducing phagocytosis, which can be restored with anticapsular antibody. Infect Immun **1999**; 67:4620–7.
6. Murphy JW, Cox RA. Induction of antigen-specific suppression by circulating *Cryptococcus neoformans* antigen. Clin Exp Immunol **1988**; 73:174–80.
7. Dong ZM, Murphy JW. Intravascular cryptococcal culture filtrate (CneF) and its major component, glucuronoxylomannan, are potent inhibitors of leukocyte accumulation. Infect Immun **1995**; 63:770–8.
8. Vecchiarelli A, Retini C, Monari C, Tascini C, Bistoni F, Kozel TR. Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. Infect Immun **1996**; 64:2846–9.
9. Monari C, Retini C, Casadevall A, et al. Differences in outcome of the interaction between *Cryptococcus neoformans* glucuronoxylomannan and human monocytes and neutrophils. Eur J Immunol **2003**; 33:1041–51.
10. Goldman D, Lee SC, Casadevall A. Pathogenesis of pulmonary *Cryptococcus neoformans* infection in the rat. Infect Immun **1994**; 62:4755–61.

11. Mukherjee J, Zuckier LS, Scharff MD, Casadevall A. Therapeutic efficacy of monoclonal antibodies to *Cryptococcus neoformans* glucuronoxylomannan alone and in combination with amphotericin B. Antimicrob Agents Chemother **1994**; 38:580–7.
12. Mukherjee J, Feldmesser M, Scharff MD, Casadevall A. Monoclonal antibodies to *Cryptococcus neoformans* glucuronoxylomannan enhance fluconazole efficacy. Antimicrob Agents Chemother **1995**; 39:1398–405.
13. Cherniak R, Reiss E, Slodki ME, Plattner RD, Blumer SO. Structure and antigenic activity of the capsular polysaccharide of *Cryptococcus neoformans* serotype A. Mol Immunol **1980**; 17:1025–32.
14. Houpt DC, Pfrommer GS, Young BJ, Larson TA, Kozel TR. Occurrences, immunoglobulin classes, and biological activities of antibodies in normal human serum that are reactive with *Cryptococcus neoformans* glucuronoxylomannan. Infect Immun **1994**; 62:2857–64.
15. Plesner A, Greenbaum CJ, Lernmark A. Low serum conditions for in vitro generation of human macrophages with macrophage colony stimulating factor. J Immunol Methods **2001**; 249:53–61.
16. Weiner SM, Bianco C, Nussenzweig V. Enhanced binding of neuraminidase-treated sheep erythrocytes to human T lymphocytes. Blood **1973**; 42:939–46.
17. Pietrella D, Monari C, Retini C, Palazzetti B, Bistoni F, Vecchiarelli A. Human immunodeficiency virus type 1 envelope protein gp120 impairs intracellular antifungal mechanisms in human monocytes. J Infect Dis **1998**; 177:347–54.
18. Pauwels R, Balzarini J, Baba M, et al. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J Virol Methods **1988**; 20:309–21.
19. Mansour MK, Levitz SM. Interactions of fungi with phagocytes. Curr Opin Microbiol **2002**; 5:359–65.
20. Dong ZM, Murphy JW. Cryptococcal polysaccharides bind to CD18 on human neutrophils. Infect Immun **1997**; 65:557–63.
21. Syme RM, Spurrell JC, Amankwah EK, Green FH, Mody CH. Primary dendritic cells phagocytose *Cryptococcus neoformans* via mannose receptors and Fcγ receptor II for presentation to T lymphocytes. Infect Immun **2002**; 70:5972–81.
22. Kane LP, Lin J, Weiss A. It's all Rel-ative: NF-κB and CD28 costimulation of T-cell activation. Trends Immunol **2002**; 23:413–20.
23. Oosterwegel MA, Greenwald RJ, Mandelbrot DA, Lorsbach RB, Sharpe AH. CTLA-4 and T cell activation. Curr Opin Immunol **1999**; 11: 294–300.