Direct Inhibition of T-Cell Responses by the *Cryptococcus* Capsular Polysaccharide Glucuronoxylomannan

Lauren E. Yauch[¤a], Jennifer S. Lam[¤b], Stuart M. Levitz[¤c*]

Departments of Medicine and Microbiology, Boston University School of Medicine, Boston, Massachusetts, United States of America

The major virulence factor of the pathogenic fungi *Cryptococcus neoformans* and *C. gattii* is the capsule. Glucuronoxylomannan (GXM), the major component of the capsule, is a high-molecular-weight polysaccharide that is shed during cryptococcosis and can persist in patients after successful antifungal therapy. Due to the importance of T cells in the anticryptococcal response, we studied the effect of GXM on the ability of dendritic cells (DCs) to initiate a T-cell response. GXM inhibited the activation of cryptococcal mannoprotein–specific hybridoma T cells and the proliferation of OVA-specific OT-II T cells when murine bone marrow–derived DCs were used as antigen-presenting cells. Inhibition of OT-II T-cell proliferation was observed when either OVA protein or $OVA_{323–339}$ peptide was used as antigen, indicating GXM did not merely prevent antigen uptake or processing. We found that DCs internalize GXM progressively over time; however, the suppressive effect did not require DCs, as GXM directly inhibited T-cell proliferation induced by anti-CD3 antibody, concanavalin A, or phorbol-12-myristate-13-acetate/ionomycin. Analysis of T-cell viability revealed that the reduced proliferation in the presence of GXM was not the result of increased cell death. GXM isolated from each of the four major cryptococcal serotypes inhibited the proliferation of human peripheral blood mononuclear cells stimulated with tetanus toxoid. Thus, we have defined a new mechanism by which GXM can impart virulence: direct inhibition of T-cell proliferation. In patients with cryptococcosis, this could impair optimal cell-mediated immune responses, thereby contributing to the persistence of cryptococcal infections.

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Introduction

Cryptococcosis is an invasive fungal infection that is caused by species of *Cryptococcus*, most commonly *Cryptococcus neoformans* or *C. gattii*. Following inhalation of the organism, the immune response is initiated in the lungs by alveolar macrophages and dendritic cells (DCs) [1,2]. Clinical and experimental data have established that T cells are required for resistance to *C. neoformans*. Disease occurs mainly in those with impaired cell-mediated immunity (CMI), including persons with AIDS, although infections can occur in immunocompetent persons as well [3].

The cryptococcal capsule is composed primarily of the polysaccharide glucuronoxylomannan (GXM), and also contains galactoxylomannan (GalXM). It is the structure of GXM that imparts serotype specificity to the organism. *C. neoformans* is composed of two varieties: *C. neoformans* var. *grubii* (serotype A isolates) and var. *neoformans* (serotype D isolates), while *C. gattii* includes serotypes B and C. GXM is a large polysaccharide; a study of GXM from four cryptococcal strains concluded that the molecular size ranged from 1,700 to 7,000 kDa [4]. A second polysaccharide, galactoxylomannan (GalXM), is also present but at approximately 10% of the mass of GXM [5].

Capsule is thought to contribute to the virulence of the yeast both by inhibiting phagocytosis and by being shed. In patients with cryptococcosis, GXM circulates in the blood and cerebrospinal fluid (CSF) at high concentrations [6] and can often be detected in body fluids for months to years after successful antifungal therapy ([7] and unpublished data). GXM has a number of immunomodulatory properties, including downregulating proinflammatory cytokine secretion from human monocytes and inhibiting leukocyte migration [8].

GXM in the brain of patients with cryptococcal meningitis is associated with macrophages/microglial cells, and it is possible that these cells serve as a reservoir for the polysaccharide once the organism is cleared [9,10]. Supporting a role for macrophages in uptake of GXM in vivo, studies

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Abbreviations: APC, antigen-presenting cell; BMDC, bone marrow–derived dendritic cell; BMMϕ, bone marrow–derived macrophages; CMI, cell-mediated immunity; Con A, concanavalin A; CSF, cerebrospinal fluid; DC, dendritic cell; GalXM, galactoxylomannan; GXM, glucuronoxylomannan; HDC, human monocyte–derived dendritic cell; IFN, interferon; IL, interleukin; MDM, monocyte-derived macrophage; MP, mannoprotein; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PI, propidium iodide; PMA, phorbol-12-myristate-13-acetate

* To whom correspondence should be addressed. E-mail: Stuart.Levitz@Umassmed.edu

¤a Current address: La Jolla Institute for Allergy and Immunology, La Jolla, California, United States of America

¤b Current address: Department of Medicine, University of California San Francisco, San Francisco, California, United States of America

¤c Current address: University of Massachusetts Medical School, Worcester, Massachusetts, United States of America

Synopsis

Infections due to the pathogenic yeast *Cryptococcus* are a significant cause of morbidity and mortality in persons with impaired T-cell functions, particularly those with AIDS. The major virulence factor of *Cryptococcus* is its capsule, which is composed primarily of the polysaccharide glucuronoxylomannan (GXM). The capsule not only surrounds the organism but also is shed during cryptococcosis. GXM is taken up by macrophages in vitro and in vivo; however, little is known about the interaction between GXM and dendritic cells, which are the most potent cells capable of activating T cells. Because of the importance of T cells in the anticryptococcal response, the authors investigated the effect of GXM on the ability of dendritic cells to initiate a T-cell response. They found the polysaccharide was internalized by dendritic cells and inhibited antigen-specific T-cell responses. Furthermore, GXM had a direct, inhibitory effect on T-cell proliferation, independent of the effect on dendritic cells. These findings may help explain the persistence of cryptococcal infections and suggest that GXM could be therapeutic in situations where suppression of T-cell responses is desired.

in mice have found injected GXM localized primarily to marginal zone macrophages in the spleen and Kupffer cells in the liver [11,12]. In vitro, human monocytes and neutrophils and murine peritoneal macrophages internalize GXM, with GXM accumulating in the human monocyte–derived macrophages (MDMs) for up to 1 wk in culture [13,14]. The functional consequences of GXM internalization on phagocyte function include decreased human neutrophil anticryptococcal activity [13] and modulation of MHC class II and costimulatory molecule expression on MDMs [15].

DCs are powerful antigen-presenting cells (APCs) that recognize a wide variety of microbes and microbial products via their pattern-recognition receptors and subsequently initiate and direct T-cell responses [16]. Because of the importance of DCs in orchestrating immune responses, numerous pathogens have evolved ways of interfering with their functions [17]. Few studies have examined the effects of GXM on DCs. While acapsular *C. neoformans* cells induced the activation and maturation of human monocyte–derived DCs (HDCs), encapsulated organisms did not. In addition, soluble GXM did not affect the expression of MHC class I, MHC class II, CD40, or CD86 on HDCs [18].

Given the importance of the CMI response in the defense against *Cryptococcus*, we hypothesized that the organism's major virulence factor, GXM, would interfere with the ability of DCs to activate T cells. We found that DCs internalize GXM and the presence of GXM impairs antigen-specific T-cell responses. Surprisingly, the dominant mechanism responsible for this effect was a direct, inhibitory effect of GXM on T-cell proliferation.

Results

The ability of DCs to internalize GXM was measured by flow cytometry using an anti-GXM antibody. We found GXM is taken up by both murine bone marrow–derived DC (BMDCs) and HDCs (Figure 1). GXM internalization increased over time, up to at least 2 d in culture. At all time points, the vast majority of the GXM was intracellular, as opposed to surface-bound (unpublished data).

Having demonstrated that GXM is internalized by DCs, we

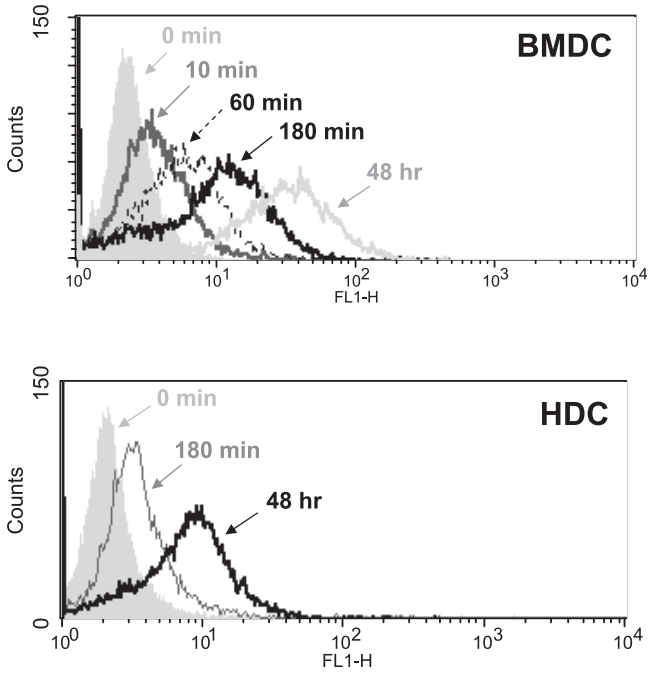

Figure 1. GXM Internalization by DCs

BMDCs or HDCs were incubated with GXM for the indicated lengths of time. Cells were harvested, washed, fixed, permeabilized, stained with an anti-GXM antibody, followed by a Cy2-conjugated secondary antibody, and analyzed by flow cytometry. The experiment was done twice with similar results.

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next studied the effects of GXM on the ability of DCs to activate antigen-specific T cells. In the first set of experiments, BMDCs were incubated with varying concentrations of GXM in the presence of cryptococcal mannoprotein (MP) and MP-specific hybridoma T cells. Activation of the T cells was determined by assaying interleukin (IL)-2 production. GXM inhibited the activation of T cells in a dose-dependent manner, with GXM at the highest concentration tested inhibiting activation by approximately 45% (Figure 2).

We next utilized the OT-II system to examine whether GXM also inhibited the response of primary antigen-specific T cells. BMDCs were pretreated with varying concentrations of GXM before the addition of antigen, either whole OVA (contained in endotoxin-free egg white) or $OVA_{323–339}$ peptide. Naïve OVA-specific $CD4^+$ T cells, purified from OT-II transgenic mice, were added and proliferation was measured. GXM at 30, 100, and 300 μg/ml significantly inhibited T-cell proliferation induced by BMDC plus egg white, with GXM at 300 μg/ml inhibiting proliferation by almost 50% (Figure 3). Similar results were obtained when OVA contaminated with endotoxin was used as antigen (unpublished data). To determine if GXM was affecting antigen uptake and/or processing, we activated OT-II T cells with BMDC plus $OVA_{323–339}$ peptide. This peptide directly binds to MHC class II, thus bypassing the need for antigen uptake and processing [19]. GXM inhibited proliferation induced by the peptide to a similar extent as observed with egg white, indicating the mechanism of inhibition is not interference with antigen uptake or processing (Figure 3). In support of this, inhibition of proliferation was similar whether GXM was added 2 h before or 2 h after egg white

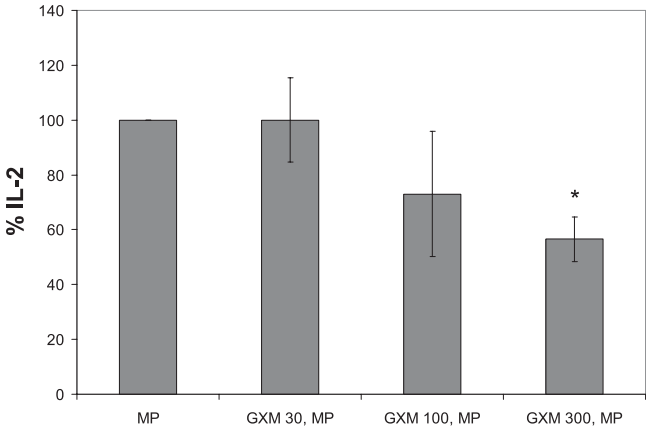

Figure 2. GXM Inhibits the Activation of Cryptococcal MP-Specific T Cells

BMDCs were pretreated with varying concentrations of GXM (30, 100, or 300 μg/ml) before the sequential addition of cryptococcal MP and MP-specific hybridoma T cells. Following 24-h incubation, IL-2 levels in the supernatants, a measure of T-cell activation, were measured by bioassay. The data are expressed as percent of IL-2 produced compared with MP alone (set at 100%) and are the average ± SEM of three to five independent experiments done in triplicate. Mean IL-2 in the MP-alone group was 68 U/ml. *$p < 0.001$.

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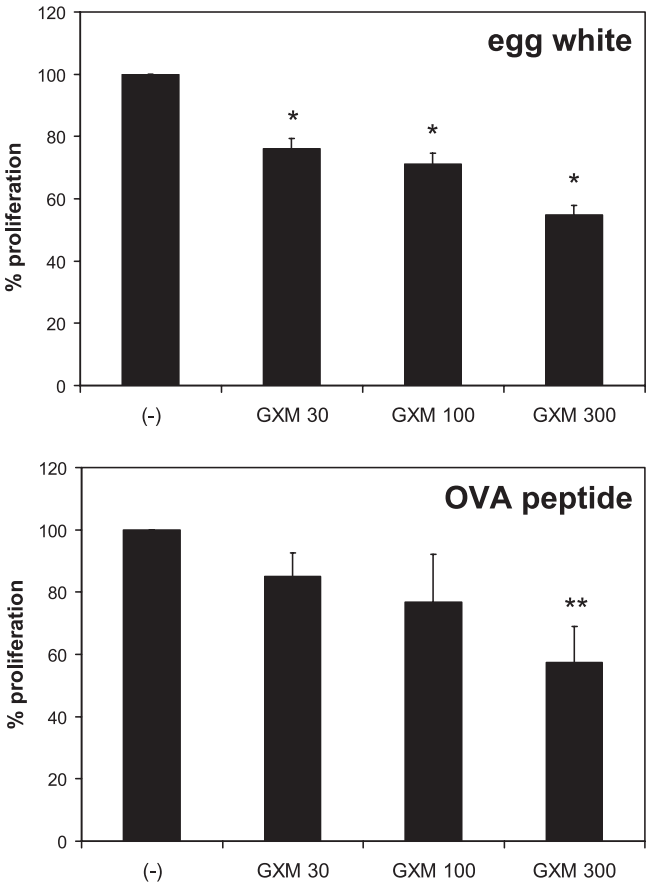

Figure 3. GXM Inhibits the Proliferation of OVA-Specific T Cells

BMDCs were pretreated with GXM (30, 100, or 300 μg/ml) before the addition of egg white or $OVA_{323–339}$ peptide. Purified $CD4^+$ T cells from OT-II mice were then added and proliferation was measured by $[^3H]$thymidine incorporation. The data are expressed as the percent proliferation compared with antigen alone (–) (set at 100%) ±SEM of three independent experiments done in triplicate. Mean CPM for egg white and $OVA_{323–339}$ peptide were 116,830 and 220,560, respectively. *$p < 0.01$; **$p \leq 0.001$.

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(unpublished data). Reduced T-cell proliferation could also be the result of impaired DC maturation, but we found GXM did not inhibit lipopolysaccharide- or tumor necrosis factor α–induced upregulation of MHC class II or CD86 on BMDC (unpublished data), suggesting GXM does not interfere with DC maturation.

After determining that GXM inhibited the proliferation of antigen-specific T cells activated by BMDC, we sought to examine if the effect was specific to BMDCs or would be observed if macrophages were used as the APCs. We first established that bone marrow–derived macrophages (BMMϕ) internalize GXM, with uptake increasing over time (unpublished data), similar to uptake by MDMs [13] and BMDCs (Figure 1). Purified $CD4^+$ OT-II T cells were activated by BMMϕ plus egg white, in the presence or absence of varying concentrations of GXM. While GXM at the lower concentrations tested (30 and 100 μg/ml) had no significant effect on T-cell proliferation, GXM at 300 μg/ml inhibited proliferation by 57 ± 9% ($p < 0.01$, $n = 2$). Thus, GXM-mediated inhibition of T-cell proliferation is observed whether DCs or macrophages present the antigen.

GXM was present for the duration of the coculture in the T-cell proliferation experiments described above. Accordingly, although some GXM was undoubtedly taken up by the APCs, there was likely GXM present extracellularly in contact with the T cells. Therefore, we questioned whether intracellular GXM would be sufficient to impair the ability of BMDCs to activate T cells. To investigate this, BMDCs were incubated with GXM and then washed to remove any GXM not bound or internalized before the addition of antigen and T cells. Pulsing of BMDCs with 100 μg/ml GXM before the addition of egg white had no effect on subsequent T-cell proliferation, and pulsing with 300 μg/ml had a minimal, inhibitory effect on proliferation (unpublished data). Thus, GXM-pulsing of BMDCs slightly reduced T-cell proliferation but not to the extent observed when GXM was added directly to the cocultures, suggesting GXM was having a direct effect on T cells. To investigate direct effects of GXM on $CD4^+$ T cells, we first demonstrated that GXM bound to $CD4^+$ T cells (unpublished data) and then used three stimuli that activate T cells independent of APC: concanavalin A (Con A), plate-bound anti-CD3ε antibody, and phorbol-12-myristate-13acetate (PMA)/ionomycin. GXM at 100 μg/ml added to anti-CD3ε–activated T cells modestly increased proliferation; however, higher concentrations of GXM (300 and 1,000 μg/ml) significantly inhibited proliferation (Figure 4). GXM at 100 μg/ml had no effect on Con A–mediated T-cell proliferation, yet 300 μg/ml GXM inhibited proliferation by 60% (Figure 4). We also found GXM inhibited the proliferation of PMA/ionomycin-activated T cells in a dose-dependent fashion (Figure 4). These data demonstrate that GXM has a direct, inhibitory effect on T-cell proliferation. Moreover, inhibition is observed regardless of whether T cells are activated in a physiologic or pharmacologic manner.

To further investigate the reduced proliferative response, we sought to determine if GXM inhibits the upregulation of T-cell activation markers. Surface expression of CD69, which

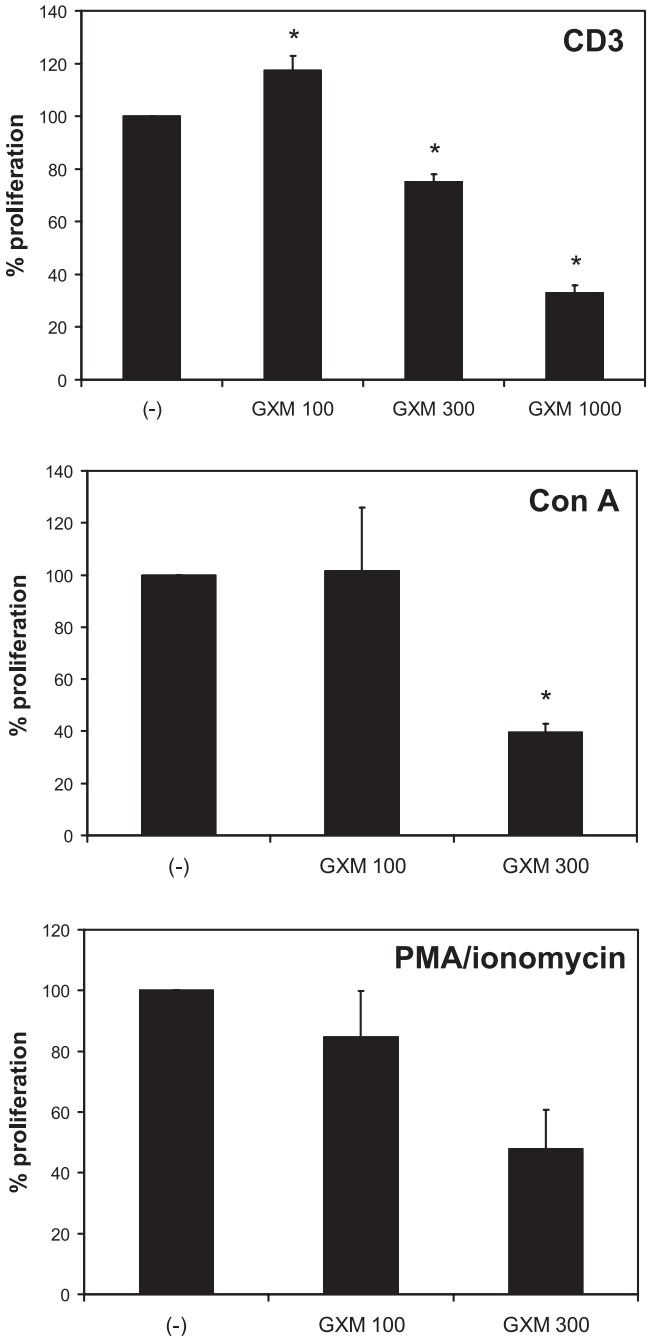

Figure 4. Effect of GXM on T-Cell Proliferation Induced by Anti-CD3, Con A, or PMA/Ionomycin

Purified $CD4^+$ murine T cells were activated with plate-bound anti-CD3ε antibody, Con A, or PMA/ionomycin, in the presence or absence of GXM (100, 300, or 1,000 μg/ml). The data are expressed as the percent proliferation compared with the stimuli alone (–) (set at 100%) ±SEM of two to five independent experiments done in triplicate. The mean CPM for anti-CD3, Con A, and PMA/ionomycin were 224,440, 41,820, and 118,875, respectively. *$p < 0.01$.

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is an early activation antigen [20], was evaluated. GXM did not affect the upregulation of CD69 on T cells activated with anti-CD3ε or BMDCs plus egg white (unpublished data). Similarly, GXM did not inhibit the upregulation of CD25 (unpublished data), which is the α-chain of the high-affinity IL-2 receptor and another activation marker of T cells [21].

The decreased T-cell proliferation observed in the presence of GXM could be due to induction of necrosis or apoptosis. Therefore, we assessed the effect of GXM on T-cell viability by Annexin V/propidium iodide (PI) staining. GXM at 300 μg/ml induced a small decrease in the percentage of live cells and a corresponding increase in the percentage of late apoptotic or dead cells when T cells were activated by anti-CD3ε or BMDCs plus egg white (Figure 5). The addition of 1,000 μg/ml GXM to anti-CD3ε–activated T cells also resulted in a minimal, statistically insignificant increase in the percentage of late apoptotic or dead cells (unpublished data). However, this effect of GXM on T-cell viability is minimal and does not appear to be sufficient to account for the impaired proliferative responses. The effect of GXM on viability was also examined by adding the polysaccharide to cell lines and measuring proliferation. GXM at 300 μg/ml had no significant effect on the incorporation of [^{3}H]thymidine by a number of T cell lines and a B cell line, demonstrating that GXM is not directly cytotoxic (Figure S1). Taken together, these data indicate GXM-mediated inhibition of T-cell proliferation is not primarily due to cell death.

We next sought to determine the effect of GXM on cytokine production by primary T cells. IL-2, IL-10, and interferon (IFN)-γ, but not IL-4, were detected in supernatants of BMDCs/egg white/OT-II T-cell cocultures and T cells directly activated with anti-CD3ε (Figure 6 and unpublished data). The presence of GXM resulted in reduced IL-2 levels when T cells were activated with anti-CD3ε or BMDCs plus egg white (Figure 6). However, GXM had no significant effect on IL-10 or IFN-γ levels (unpublished data). Because IL-2 is a T-cell growth factor and is produced by activated T cells, the effect of GXM on IL-2 production was investigated further. Reduced levels of IL-2 in the presence of GXM could be due to a direct inhibition of IL-2 production or a result of fewer T cells due to an impaired proliferative response. To address the former possibility, we added exogenous IL-2 to T cells activated with anti-CD3ε. IL-2 at concentrations up to 100 U/ml did not restore the proliferative response of cells activated in the presence of 1,000 μg/ml GXM (68 ± 5% inhibition with GXM alone and 62 ± 13% with GXM plus 100 U/ml IL-2; mean ± SEM of two independent experiments performed in triplicate). We also examined the effect of GXM on the amount of IL-2 produced by phytohemagglutinin (PHA)-activated Jurkat T cells and found GXM had no significant effect on IL-2 production (PHA plus 300 μg/ml GXM = 110 ± 24% of IL-2 induced by PHA alone; mean ± SEM of three independent experiments done in triplicate). Together, these data reveal the inhibitory effect of GXM on T-cell proliferation cannot solely be attributed to impaired IL-2 production.

To ascertain whether the effect of GXM on murine T-cell proliferation would be replicated using human cells, in the final set of experiments, we investigated whether the polysaccharide affected the proliferation of peripheral blood mononuclear cells (PBMCs) in response to the recall antigen, tetanus toxoid. In addition, as all of the above studies were performed using GXM from serotype A *C. neoformans* strain Cn6, in these experiments we also tested the effects of GXM isolated from other cryptococcal serotypes. Accordingly, PBMCs were activated with tetanus toxoid in the presence

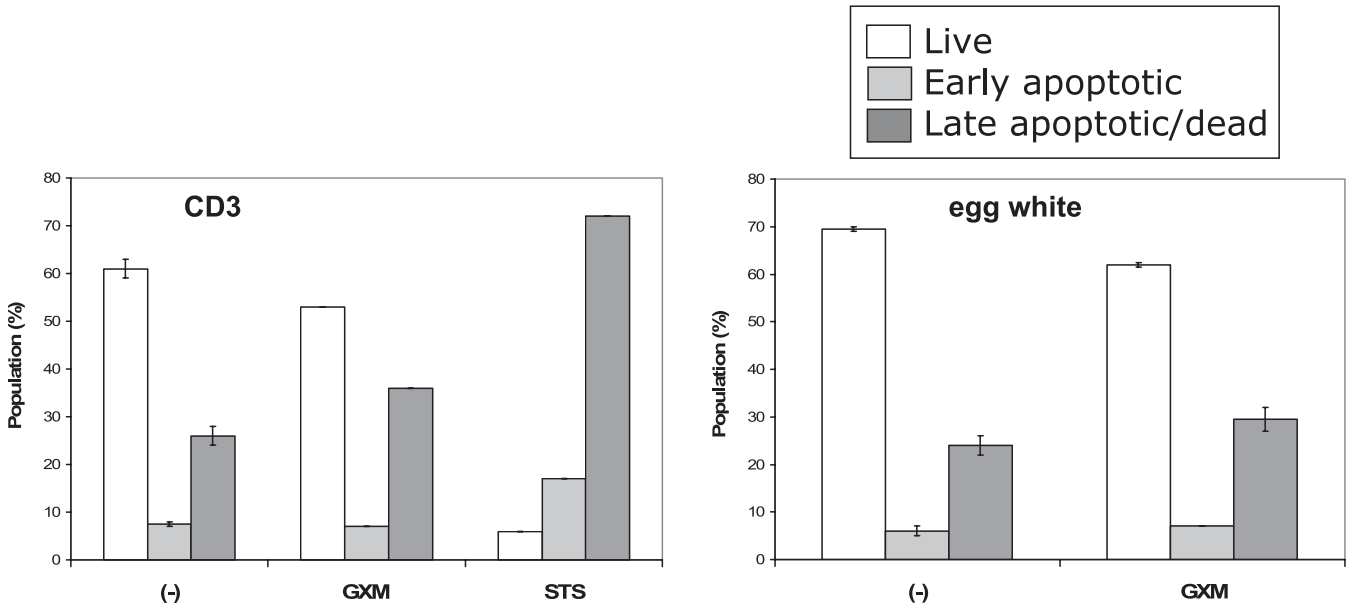

Figure 5. Effect of GXM on T-Cell Viability

Purified $CD4^+$ T cells from OT-II mice were activated with anti-CD3ε antibody or BMDC plus egg white. Cells were left untreated (–) or treated with GXM (300 μg/ml) or staurosporine (STS). At 48 h, T cells were stained with Annexin V/FITC and PI and analyzed by flow cytometry. Annexin–PI– cells are defined as live, Annexin+PI– as early apoptotic, and Annexin+PI+ as late apoptotic or dead. Data are expressed as mean ± SEM of one experiment (representative of three) done in duplicate (except STS).

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or absence of GXM isolated from serotype A *C. neoformans* strain H99, serotype B *C. gattii* strain R265, serotype C *C. gattii* strain Cn18, and serotype D *C. neoformans* strain B3501, as well as the strain Cn6 GXM. GXM from all serotypes significantly inhibited PBMC proliferation, although the GXM preparations varied in their potencies (Figure 7). GXM isolated from the serotype A strains inhibited proliferation to the greatest extent; serotype D GXM was the least potent, and GXM from the serotype B and C strains were intermediate in their ability to suppress proliferation.

Discussion

The data presented herein establish a new mechanism by which the capsular polysaccharide GXM imparts virulence upon *C. neoformans* and *C. gattii:* direct inhibition of T-cell proliferation. While GXM is taken up by DCs, inhibition of T-cell responses occurs in an APC-independent manner. Thus, GXM-mediated inhibition is seen regardless of whether T cells are activated by APC plus antigen or directly by mitogens. Moreover, GXM inhibits both murine and human T-cell responses.

GXM inhibited T-cell proliferation induced by BMDCs presenting the immunodominant cryptococcal MP antigens. Assuming these effects occur in vivo during cryptococcosis, the anticryptococcal CMI response may be dampened once a cryptococcal infection gets firmly established and GXM titers rise. As GXM also impairs humoral immune responses and phagocytic defenses [22], this could lead to a situation where the host is left without effective innate or acquired immunologic defenses. Indeed, prior to the introduction of effective antifungal drugs, cryptococcal meningoencephalitis was nearly always fatal.

In addition to its effect on antigen-specific T-cell responses, GXM had a direct effect on primary, naïve T cells, as demonstrated by its capacity to inhibit the proliferation of T cells activated with anti-CD3ε or Con A. GXM also has been reported to inhibit the proliferation of Con A–activated rat spleen mononuclear cells [23]. In those studies, the impaired proliferation may have been due to increased cell death, as GXM was found to induce apoptosis in these cells [24]. In contrast, the impaired proliferative responses we measured could not be attributed to GXM-mediated cytotoxicity, as the polysaccharide did not significantly affect T-cell viability.

GXM also inhibited the proliferation of T cells activated with the chemical mitogens PMA and ionomycin, indicating GXM interferes with T-cell signaling downstream of T-cell receptor activation. GXM did not inhibit the early activation of T cells, as measured by CD25 and CD69 expression. Similarly, *Salmonella* inhibits T-cell proliferation without inhibiting the upregulation of T-cell activation markers, including CD25 and CD69 [25]. Additionally, HIV has been shown to inhibit $CD4^+$ T-cell cycle progression without affecting the expression of early activation markers [26].

The presence of GXM resulted in less IL-2 detected in cultures of T cells activated with BMDC and egg white or anti-CD3ε. Likewise, GXM impaired the response of MP-specific T cells as measured by IL-2 production. However, exogenous IL-2 could not restore the proliferative response of T cells activated in the presence of a high concentration of GXM, and GXM did not inhibit IL-2 release by PHA-activated Jurkat cells. Although it is possible GXM exerts different effects on cell lines and primary T cells, these data indicate GXM impairs T-cell proliferation independently of inhibiting IL-2 production or IL-2 receptor expression. Taken together, our

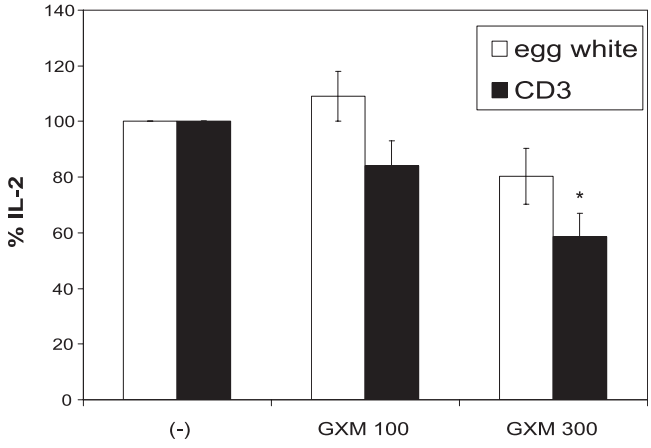

Figure 6. Effect of GXM on IL-2 Production

Purified $CD4^+$ T cells from OT-II mice were activated with anti-CD3ε antibody or BMDCs plus egg white in the presence or absence of GXM (100 or 300 μg/ml) for 48 h. IL-2 levels were determined by ELISA or bioassay. Data are expressed as the percent of IL-2 compared with stimuli alone (–) ±SEM of two to four independent experiments done in triplicate. The mean IL-2 (pg/ml by ELISA) for egg white and anti-CD3 were 210 and 320, respectively. The mean IL-2 (U/ml by bioassay) for egg white was 0.65. $*p < 0.001$.

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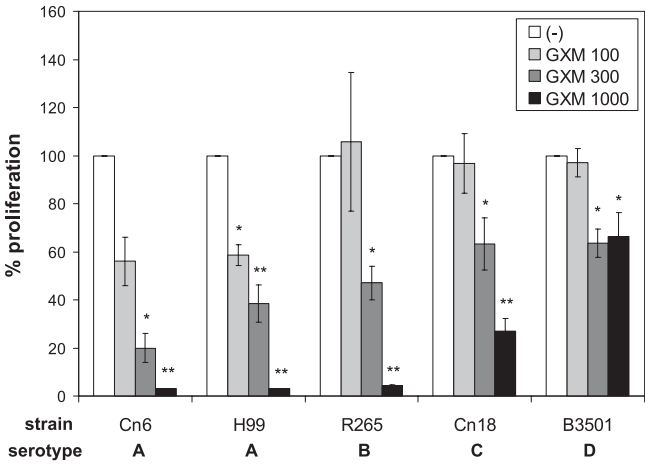

Figure 7. GXM from Four Cryptococcal Serotypes Inhibits Proliferation of Human PBMCs

PBMCs were activated with tetanus toxoid (TT) in the presence or absence of GXM (100, 300, or 1,000 μg/ml) isolated from serotype A strain Cn6, serotype A strain H99, serotype B strain R265, serotype C strain Cn18, or serotype D strain B3501. Proliferation was measured by [^{3}H]thymidine incorporation 6 d later. Data are expressed as the percent proliferation compared with TT alone (–) (set at 100%) and are expressed as the mean ± SEM of four donors (or two donors for strain Cn6 GXM) assayed in triplicate. The mean CPM for TT in the absence of GXM was 63,550. $*p \leq 0.005$; $**p < 0.0001$.

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results suggest that GXM exerts its effect at a later stage of T-cell activation. In fact, GXM may have a direct, inhibitory effect on cell cycle progression in primary T cells, similar to the action of *Helicobacter pylori* vacuolating cytotoxin, VacA, which inhibits IL-2–driven T-cell proliferation [27].

Studies from other laboratories have found that GXM induces the production of the immunosuppressive cytokine, IL-10, from human monocytes [28], and identified a role for IL-10 in GXM-mediated immunosuppression in vitro [23] and in vivo [29,30]. However, we do not observe an induction of IL-10 by human monocytes or BMDCs incubated with GXM (unpublished data). Furthermore, GXM did not enhance IL-10 production from T cells activated with BMDCs plus egg white or anti-CD3ε. Thus, we conclude that GXM-mediated inhibition of T-cell proliferation in our studies likely is IL-10 independent.

In addition to the direct effect of GXM on T cells, we found DCs internalized the polysaccharide continuously over time, yet GXM-pulsed BMDCs were only minimally impaired in their ability to activate T cells. This is not surprising, as GXM treatment does not appear to inhibit BMDC maturation (unpublished data). Similarly, it was recently demonstrated that pneumococcal capsular polysaccharides are internalized by DCs yet do not inhibit LPS-induced maturation [31]. Interference with antigen uptake or processing is also an unlikely mechanism for the inhibitory effect of GXM, as GXM effectively inhibited proliferation when T cells were activated with BMDCs plus $OVA_{323–339}$ peptide.

Importantly, the inhibitory effect of GXM on murine T cells was also observed using human cells. We show that GXM inhibited proliferation of PBMCs activated with tetanus toxoid in a dose-dependent manner. A recent study demonstrated the other capsular component of *Cryptococcus*, GalXM, inhibits human T-cell proliferation by inducing T-cell apoptosis [32]. Consistent with our results, the researchers found a low concentration of GXM did not affect T-cell proliferation induced by PHA or anti-CD3. They did not examine the effects of higher concentrations of GXM, however.

Interestingly, our studies revealed that GXM isolated from all four cryptococcal serotypes inhibited proliferation of tetanus toxoid–activated PBMCs, although not to the same extent. GXM from the serotype D strain B3501 was less inhibitory than GXM from the other strains. Whether this is due to a size or structure difference is unknown. Differences in GXM structure affect virulence, inhibition of neutrophil migration, and tissue accumulation of GXM [33–35]. GXM from B3501 is larger than GXM from H99 [4]; however, the molecular sizes of GXM from strains Cn6, R265, and Cn18 are unknown. Our results suggest differences in virulence between cryptococcal strains may be due in part to the varying capacities of GXM to inhibit T-cell proliferation.

The concentrations of GXM we used that consistently inhibited T-cell proliferation (300 and 1,000 μg/ml) are high yet still biologically relevant. One study found 68% of AIDS patients with cryptococcosis had serum GXM titers of at least 1:1,024 (approximately 10 μg/ml), and 21% had titers of 1:10,000 or higher (approximately 100 μg/ml) [6]. In experimental cryptococcosis, serum GXM levels in the mg/ml range have been observed [36]. However, GXM concentrations in infected tissues, particularly at foci of infection, are undoubtedly much higher. Our findings suggest that such high concentrations of GXM could interfere with the generation of T-cell responses. In this regard, while the osmolarity of these high concentrations of GXM has not been determined, hyperosmotic stress has been reported to have direct effects on T-cell signaling pathways [37].

In conclusion, our data reveal a novel inhibitory property of GXM. Inhibition of T-cell proliferation could have severe

consequences during cryptococcosis due to the importance of T cells in the anticryptococcal response. Furthermore, the ability of GXM to persist in patients after successful antifungal therapy underscores how T-cell responses during subsequent infections or neoplasia may be affected as well. Finally, GXM, or products derived from GXM, could prove to be of therapeutic value in situations where suppression of T-cell responses is desired, such as transplantation or autoimmunity.

Materials and Methods

Materials. All chemical reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, United States) unless otherwise specified. All tissue culture media were purchased from Invitrogen Life Technologies (Carlsbad, California, United States) unless otherwise noted.

Isolation and purification of GXM. GXM from serotype A *C. neoformans* strain Cn6 (62066; American Type Culture Collection [ATCC], Manassas, Virginia, United States), serotype A *C. neoformans* strain H99 (208821; ATCC), serotype B *C. gattii* strain R265 (a gift from Dr. J. Heitman, Duke University Medical Center, Durham, North Carolina, United States), serotype C *C. gattii* strain Cn18 (24066; ATCC), and serotype D *C. neoformans* strain B3501 (34873; ATCC) was prepared as described [38]. In brief, cryptococcal culture supernatants were precipitated with ethanol and cetyltrimethylammonium bromide. GXM was dialyzed against 1 M NaCl, followed by dH_2O for 1 wk. The amount of polysaccharide was quantified by the phenol–sulfuric acid method [39]. The strain Cn6 GXM used in Figures 1 through 6 and Figure S1 was in 1× PBS, whereas the GXM preparations used in Figure 7 were lyophilized and resuspended in RPMI 1640. All GXM preparations had undetectable levels (less than 0.03 endotoxin U/ml) of endotoxin as measured by *Limulus* Amebocyte Lysate assay (Associates of Cape Cod, East Falmouth, Massachusetts, United States).

Mice. C57BL/6 and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, United States). OT-II TCR-transgenic mice have $CD4^+$ T cells specific for OVA amino acids 323–339 in the context of MHC class II ($I\text{-}A^b$) [40]. The mouse experiments were approved by the Boston University Institutional Animal Care and Use Committee.

Isolation and culture of Human DCs. Human DCs were obtained as described [41]. Briefly, peripheral blood was obtained by venipuncture from healthy volunteers using a protocol approved by the Boston University Medical Center Institutional Review Board. Blood was anticoagulated with heparin (American Pharmaceutical Partners, Los Angeles, California, United States) and diluted 1:1 with Hanks' balanced salt solution (Cambrex BioScience Walkersville, Walkersville, Maryland, United States). PBMCs were separated by density gradient centrifugation using Lymphoprep (AXIS-SHIELD; PoC AS, Oslo, Norway), and 1 to 3×10^5 PBMCs were added to the wells of a six-well plate. After a 2-h incubation at 37 °C, the wells were washed to remove nonadherent cells. Human DCs were obtained by culturing the adherent cells for 7 d in HDC media (RPMI 1640, 5% heat-inactivated [HI] FBS [Tissue Culture Biologicals, Los Alamitos, California, United States], 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, and 10 mM HEPES [Sigma]) supplemented with 50 ng/ml rhIL-4 (BD PharMingen, San Diego, California, United States) and 150 ng/ml rhGM-CSF (Immunex Corporation, Thousand Oaks, California, United States).

Generation of BMDCs. BMDCs were isolated and cultured according to the protocol of Lutz et al. [42]. Briefly, bone marrow was harvested from the femurs and tibiae of 6- to 14-wk-old C57BL/6 mice and plated at 10^5/ml in 100 × 15 mm Petri dishes (BD Falcon; Becton Dickinson, Franklin Lakes, New Jersey, United States). BMDC media contained RPMI 1640, 10% HI FBS (Tissue Culture Biologicals), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 55 μM 2-mercaptoethanol, and supernatant from GM-CSF–secreting J558L cells [43] at a final concentration of 10%. The cells were fed with fresh supplemented media on days 3, 6, and 8, and the nonadherent BMDCs were harvested and used on day 9 or 10.

Generation of BMMϕ. Bone marrow was harvested as described above, and cells were seeded at 5×10^5 cells/ml in a tissue culture flask. Cells were cultured in RPMI 1640, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% HI FBS (Tissue Culture Biologicals). Media was supplemented with 30% L cell (CCL-1; ATCC)–conditioned medium as a source of M-CSF. On day 2, nonadherent cells were harvested and seeded at 10^5/ml in Petri dishes (BD Falcon) in media containing 30% L cell–conditioned medium. The media was changed on day 6, and macrophages were harvested on day 8 using Versene.

Flow cytometric analysis of GXM binding/internalization. BMDCs, HDCs, BMMϕ, or murine $CD4^+$ T cells (2×10^5) were incubated in their respective media with GXM (40 μg/ml) for varying lengths of time at 37 °C. Cells were harvested, washed two times, fixed with 1% paraformaldehyde, permeabilized with 0.1% saponin, and stained with the anti-GXM monoclonal antibody, 3C2 (a gift from Dr. T. Kozel, University of Nevada School of Medicine, Reno, Nevada, United States), followed by a donkey anti-mouse Cy2-congugated secondary antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, United States). This procedure allowed for detection of total GXM, both surface bound and intracellular. To detect only surface bound GXM, cells were not fixed or permeabilized before staining.

PBMC purification. Peripheral blood was obtained by venipuncture from healthy volunteers and anticoagulated with heparin. PBMCs were purified by centrifugation on a Histopaque-1077 (Sigma) density gradient, washed, and resuspended in media (RPMI 1640, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% HI FBS).

Activation of cryptococcal MP-specific T cells. BMDCs (10^5) were plated in 96-well flat-bottom plates. Some wells received a 2-h GXM pretreatment before the addition of cryptococcal MP (10 μg/ml). MP was isolated from culture supernatants of *C. neoformans* acapsular strain Cap67 (52817; ATCC) as described [44]. The cryptococcal MP-specific T-cell hybridoma, P1D6, has been described previously [45]. This hybridoma secretes IL-2 when activated with cryptococcal MP in a MHC class II–restricted manner. P1D6 T cells (2×10^5) were added to the BMDC 1 h after MP. Supernatants were collected 24 h later, freeze-thawed, and incubated with the IL-2–dependent cell line CTLL-2 [46]. AlamarBlue (10 μl; BioSource, Camarillo, California, United States) was added 24 h later for 8 h. Absorbance was determined using a plate reader, and the results were compared with an IL-2 standard curve.

Purification of murine $CD4^+$ T cells. Inguinal, brachial, axial, cervical, and mesenteric lymph nodes and spleens were harvested from 6- to 14-week-old C57BL/6 or OT-II mice. Lymph nodes and spleens were disrupted using sterile frosted glass slides, and RBCs were lysed using RBC lysis buffer (eBioscience, San Diego, California, United States). $CD4^+$ T cells were enriched using magnetic bead positive selection ($CD4^+$ [L3T4] beads; Miltenyi Biotec, Auburn, California, United States) according to the manufacturer's instructions.

Proliferation of OVA-specific T cells. Mitomycin C (50 μg/ml)–treated BMDCs were plated in 96-well U-bottom plates, and GXM (30, 100, or 300 μg/ml) was added 2 h before egg white (100 μg/ml) or $OVA_{323–339}$ peptide (0.1 μg/ml; Invitrogen). The endotoxin-free egg white (which contains approximately 54% ovalbumin [47]) was a gift of Dr. T. Singleton, Boston University School of Medicine (Boston, Massachusetts, United States). Naïve purified $CD4^+$ OT-II T cells (10^5) were added to the wells 2 h later. The wells were pulsed with 1 μCi [^{3}H]thymidine (PerkinElmer, Wellesley, Massachusetts, United States) on day 3, and the cells were harvested and freeze-thawed 20 h later. Proliferation was measured on a beta scintillation counter.

Proliferation of Con A–, anti-CD3–, or PMA/ionomycin-activated murine T cells. For anti-CD3 activation, 96-well flat-bottom plates were coated with anti-mouse CD3ε antibody (clone 145–2C11; BD Biosciences PharMingen) diluted in PBS for 2 h at 37 °C. Wells were washed with PBS, and 10^5 $CD4^+$ purified T cells were added to each well. Some wells then received varying concentrations of GXM. In some experiments, IL-2 (Cetus, Emeryville, California, United States) was added to some wells. For Con A or PMA/ionomycin activation, 10^5 $CD4^+$ purified T cells were added to 96-well U-bottom plates and stimulated with Con A (0.5 μg/ml) or PMA (3 ng/ml) and ionomycin (0.15 μM; Calbiochem, La Jolla, California, United States). GXM was then added to some wells. Proliferation was measured by [^{3}H]thymidine incorporation as described above.

Proliferation of PBMCs. Freshly isolated PBMCs (10^5) were added to the wells of a 96-well U-bottom plate. Some wells received varying concentrations of GXM isolated from serotype A strain Cn6, serotype A strain H99, serotype B strain R265, serotype C strain Cn18, or serotype D strain B3501. Tetanus toxoid (TT; Calbiochem) was added for a final concentration of 1 μg/ml. The wells were pulsed with [^{3}H]thymidine on day 6 and harvested 20 h later, and proliferation was determined as described above.

Detection of cytokines. T cells were activated with BMDCs plus egg white or anti-CD3ε as described above. Cell-free supernatants were

harvested and frozen at 48 h. Levels of IL-2, IL-4, IL-10, and IFN-γ were determined by ELISA (eBioscience) according to the manufacturer's instructions; however, in one experiment, IL-2 levels were measured by bioassay using the CTLL-2 cell line as described above. IL-2 produced by PHA (5 μg/ml)-activated Jurkat cells (TIB-152; ATCC) was measured using the CTLL-2 assay.

Analysis of T-cell viability. OT-II $CD4^+$ purified T cells were activated by plate-bound anti-CD3ε antibody or BMDCs plus egg white. Cells were left untreated or treated with GXM (300 μg/ml) for 48 h. At 24 h, staurosporine (0.5 μM) was added to some wells containing anti-CD3ε–activated T cells as a positive control for apoptosis induction. At 48 h, T cells were harvested and stained with Annexin V/FITC and PI (BD PharMingen), and analyzed by flow cytometry. Annexin–PI– cells are defined as live, Annexin+PI– as early apoptotic, and Annexin+PI+ as late apoptotic or dead.

Cell line proliferation. Murine P1D6 T cells, CTLL-2 T cells, J558L B cells, and human Jurkat T cells were plated at 3×10^4 per well in a 96-well flat-bottom plate. GXM (300 μg/ml) was added to some wells. Wells were pulsed immediately with [^{3}H]thymidine, and proliferation was measured 24 h later on a scintillation counter.

Statistical analysis. The mean and SEM values were compared using the Student's t test. Values of $p < 0.05$ were considered significant. Bonferroni's correction was applied when making multiple comparisons.

Supporting Information

Figure S1. Effect of GXM on [^{3}H]Thymidine Incorporation by Cell Lines

Murine P1D6 T cells, CTLL-2 T cells, J558L plasmacytoma B cells, and human Jurkat T cells were incubated in the presence or absence of GXM (300 μg/ml) for 24 h, and proliferation was measured by [^{3}H]thymidine incorporation. Data are expressed as the mean ± SEM of one of two independent experiments with similar results done in triplicate.

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