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Galactoxylomannan-Mediated Immunological Paralysis Results from Specific B Cell Depletion in the Context of Widespread Immune System Damage[1,2]

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The mechanisms responsible for polysaccharide-induced immunological paralysis have remained unexplained almost a century after this phenomenon was first described. *Cryptococcus neoformans* capsular polysaccharides glucuronoxylomannan and galactoxylomannan (GalXM) elicit little or no Ab responses. This study investigates the immunological and biological effects of GalXM in mice. GalXM immunization elicits a state of immunological paralysis in mice characterized by the disappearance of Ab-producing cells in the spleen. Immunological paralysis and lack of immunogenicity could not be overcome by immunization with GalXM conjugated to a protein carrier, *Bacillus anthracis* protective Ag. Additionally, immunization with GalXM in either complete or IFA was associated with spleen enlargement in BALB/c mice. TUNEL and flow cytometry revealed widespread apoptosis in the spleen after GalXM administration. Administration of a cocktail of caspase-3 inhibitor Z-DEVD-FMK and general caspase inhibitor Z-VAD-FMK or Fas-deficient mice abrogated the complete disappearance of Ab-producing cells. Analysis of spleen cytokine expression in response to GalXM systemic injection revealed that GalXM down-regulated the production of inflammatory cytokines. Hence, we conclude that GalXM-induced immune paralysis is a result of specific B cell depletion mediated by its proapoptotic properties in the context of widespread dysregulation of immune function. *The Journal of Immunology,* 2009, 183: 3885–3894.

The phrase "immunological paralysis" was first coined by Lloyd Felton in the early 20th century in reference to the inability of pneumococcal polysaccharide to elicit Ab responses in mice (1). The phenomenon was studied extensively with pneumococcal polysaccharide in the early to mid-20th century (1). The term "paralysis" was broadly defined as a condition produced in the host by an Ag given in such doses that a subsequent immunization fails to elicit an immune response (1). Unfortunately, no complete satisfactory mechanism was identified, but two important explanations for the phenomenon were proposed, immunological paralysis and immunological unresponsiveness. Immunological paralysis was thought to be a consequence of the suppression of Ab formation at its source, while immunological unresponsiveness was thought to result from neutralization of formed Ab by excess Ag (1). Later studies provided evidence suggesting that T cells were implicated, despite the dogma that polysaccharides were T cell-independent Ags (2). Given that Ab responses to polysaccharide Ags are often protective against encapsulated pathogens, the phenomenon of immunological paralysis has great medical importance. In recent years, the development of polysaccharide-protein conjugate vaccines has emerged as an effective means to bypass the lack of responsiveness often associated with capsular Ags.

Cryptococcus neoformans is an encapsulated fungal pathogen that causes life-threatening infections in humans that usually manifests clinically as meningoencephalitis (3). Before the 1980s, cryptococcosis was relatively uncommon and was associated with the immunosuppression resulting from corticosteroid therapy or with lymphomas (4–6). However, as a result of the AIDS pandemic, the medical importance of this microbe has increased dramatically (7, 8). In addition to those with advanced HIV infection, other groups at risk are patients receiving immunosuppressive therapies for cancer and following transplants. One of the major virulence factors of *C. neoformans* is its capsule. The capsular polysaccharide is known to be composed of glucuronoxylomannan (GXM),[4] galactoxylomannan (GalXM), and mannoproteins (7, 9, 10). The major capsular component on a mass basis is GXM, and this polysaccharide has been shown to have numerous deleterious effects on host immunity, including inhibiting leukocyte migration, inhibiting phagocytosis, inducing the expression of FasL in macrophages and producing a state of immunological unresponsiveness (8, 11–13). GalXM is composed of an α-(1→6)-galactan

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[4] Abbreviations used in this paper: GXM, glucuronoxylomannan; GalXM, galactoxylomannan; IFA, incomplete Freund's adjuvant; PA, protective Ag; scFv, single-chain variable fragment.

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backbone with several branches of α-Man-(1→3)-α-Man(1→4)-β-Gal-trisaccharide with variable amounts of β-(1→2) or β-(1→3)-xylose side groups (14). Light scattering measurements revealed that GalXM was on average ~20-fold smaller than GXM, with an average mass of 1×10^5 Da. The radius of gyration is the average distance between the center of the GalXM molecule to the outer edges and was determined as 95 nm in comparison to GXM, which has a radius of gyration that, depending on the strain, can range from 68 to 208 nm (10). Since GalXM has a smaller molecular mass, the molar concentration of GalXM in polysaccharide that is shed could exceed that for GXM in *C. neoformans* exopolysaccharides (10). Pericolini et al. reported that GalXM inhibited PBMC and T cell proliferation, increased IFN-γ and IL-10 production, and induced apoptosis of T-lymphocytes by DNA fragmentation through the activation of caspase-8. GalXM is able to induce Fas directly, making this a unique property of GalXM and not of GXM (15). GalXM is also able to induce T cell apoptosis through the induction of FasL on APCs such as monocytes/macrophages (15, 16). GalXM-induced apoptosis is mediated by the interaction of GalXM with the glycoreceptors such as CD7, CD43, and CD45, which are expressed on the T cell surface and are normally involved in galectin-induced apoptosis. It was found that CD45 activation correlated with the induction of apoptosis in Jurkat cells, while CD7 and CD43 activation correlated with apoptosis of T cells (17). These results suggest that GalXM has a functional role in virulence by targeting human T cells and impairing specific T cell responses, and that its mechanism may potentially be different from GXM (18). Recently, Villena et al. described that GalXM was more potent than GXM at induction of Fas/FasL expression and apoptosis on macrophages (16). Their findings revealed the induction of FasL-dependent macrophage apoptosis. Although GalXM and GXM induced Fas and FasL and upregulated Fas expression on the macrophage surface, lower doses of GalXM were sufficient to block proliferation in RAW 264.7 cells and induce FasL expression. Neutralizing mAbs specific for FasL were used to restore a proliferative response in vitro. The study also looked at FasL-deficient *gld* mice. The results revealed that GalXM and GXM failed to induce apoptosis in macrophages in vivo.

Early studies using total polysaccharide from *C. neoformans* revealed that this Ag had a propensity for inducing immunological paralysis (19, 20). Murphy et al. showed that polysaccharide had a dose-dependent primary immunological response in mice using the hemolytic plaque assay as indicated by the increase in Ab-producing cells (12). However, after a subsequent challenge with capsular polysaccharide a state of immunological unresponsiveness was induced. There was a diminution of Ab response and the abolition of spleen plaque-forming cells. Herein we investigate the Ab response to GalXM in mice and show that GalXM is a potent immunomodulatory molecule. Like GXM, GalXM causes immunological paralysis in mice. However, unlike GXM, the state of immunological unresponsiveness to GalXM is accompanied by pathological changes and the induction of apoptosis in splenic tissue.

Materials and Methods

Animals

Six to 8-wk-old BALB/c female mice were obtained from the National Cancer Institute. Three to 5-wk-old Fas-deficient mice MRL/MpJ-Fas^{lpr}/J and the genetically matched controls MRL/MpJ were obtained from The Jackson Laboratory.

Immunizations

Mice were used for the i.p. immunization with 0.5. 5, 50, and 500 μg of GalXM in PBS or GalXM-protective Ag (GalXM-PA) conjugate in CFA. The first group of mice was immunized with the above-mentioned doses and sera were collected for ELISA 4 days after immunization. Mice were sacrificed and the spleens were harvested for ELISA spot assay. The second group of mice was also immunized with the above-mentioned doses and subsequently challenged with 0.1 μg of GalXM or conjugate in incomplete Freund's adjuvant (IFA) at day 14. The mice were bled and sacrificed at day 28. MRL/MpJ-Fas^{lpr}/J and the genetically matched controls MRL/MpJ were initially injected with 50 μg of GalXM, and boosting immunization was done at day 7 with 5 μg of GalXM in IFA, and these were then followed to day 14. All animal experiments were conducted in accordance with institutional guidelines.

GalXM isolation

GalXM was isolated from the culture supernatant using a modified method from previously described protocols (10, 14). Briefly, a 400-ml culture of *C. neoformans* acapsular mutant of strain cap67 was grown in peptone supplemented with 2% galactose for 7 days. The culture supernatant was separated from the cells by centrifugation at $1500 \times g$ for 15 min at room temperature, and then concentrated using a 10,000 MW cut-off Amicon centrifugal filter (Millipore). The material was then dialyzed for 1 wk against distilled water, and the 10-kDa retentate, containing GalXM and mannoproteins, was passed through a 0.2-μm filter. The retentate was lyophilized and stored at room temperature. To separate GalXM from the mannoproteins, the freeze-dried mixture was dissolved in 25 ml of start buffer (0.01 M Tris base and 0.5 M NaCl solution (pH 7.2), to which $CaCl_2$ and $Mn(II)Cl_2$ were sequentially added at final concentrations of 1 mM each). The GalXM and mannoprotein solution was then continuously passed through a Con A-Sepharose 4B column (2.5×10 cm) (Sigma-Aldrich) for 16 h at 4°C using a peristaltic pump with a flow rate of 16 ml/h. The material was eluted with five column washes of start buffer and collected as 20-ml fractions. GalXM-containing fractions were identified by the phenol-sulfuric acid assay (21). The fractions were combined, ultraconcentrated as described before, and dialyzed against water for 7 days. GalXM was then recovered by lyophilization. Compositional analysis of GalXM was confirmed by combined gas chromatography/mass spectrometry of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. Composition analysis revealed the mole percentages for xylose (17%), mannose (28%), and galactose (55%). These numbers closely approximate the mass composition described by Vaishnav et al. (14): xylose, 22%; mannose, 29%; and galactose, 50%. The possibility of Con A contamination of GalXM preparations was evaluated by immunoblot analysis of purified GalXM with an anti-Con A Ab (Vector Laboratories). No Con A was detected in the GalXM preparations by Western blot analysis (data not shown). Lyophilized GalXM was reconstituted in 1× PBS and dialyzed in Endosafe LPS-free water for 3 wk until the dialysate was negative by the *Limulus* amebocyte assay (Cambrex).

Synthesis of GalXM-Bacillus anthracis *protective Ag conjugate*

GalXM hydroxyl groups were activated using cyanogen bromide (Sigma-Aldrich). The activated GalXM was then derivatized with the bifunctional linker adipic acid dihydrazide (Sigma-Aldrich). GalXM (5 mg/ml in 0.2 M NaCl) was activated with 5 mg/ml cyanogen bromide with continuous pH monitoring such that the pH was maintained between 10.5 and 11.0 for 6 min at 4°C. Subsequently, an equal volume of 0.5 M $NaHCO_3$ at pH 8.5 containing 0.5 M adipic acid dihydrazide was added. The reaction mixture was tumbled at 4°C for 18 h and dialyzed against 0.2 M NaCl. The reaction mixture containing GalXM-AH was then mixed with 5.0 mg/ml PA from *Bacillus anthracis* (Wadsworth Laboratories) and brought to pH 5.6 with 0.05 N HCl and 0.05 M of the water-soluble carbodiimidine, 1-ethyl-3(3-dimethylamino-propyl)-carboiimide (EDAC; Sigma-Aldrich). The pH was continuously maintained at 5.6 for 1 h at 4°C. The reaction mixture was dialyzed against 0.2 M NaCl for 24 h at 4°C (22). Bio-Rad Quick Start protein assay was used according to the manufacturer's instructions to detect protein in the conjugate. In some experiments the conjugate was further purified by capture with the PA-binding mAb 10F4 (23) conjugated to agarose beads by AminoLink Plus coupling resin kit (Pierce) according to the manufacturer's instructions.

HPLC

To establish that the GalXM-PA conjugate contained both carbohydrate and protein, we tested for the presence of carbohydrates and protein using

the phenol-sulfuric assay and the Bradford protein colorimetric assay, respectively. The analysis revealed that the conjugate was positive for both protein and carbohydrate (not shown). Additionally, we used HPLC to detect the conjugate by comparing differences in elution time between GalXM and PA alone and the conjugate. GalXM-PA, GalXM, and PA were analyzed in an Alltech Prevail Carbohydrate ES column (250 × 4.6 mm) using a Waters 600 liquid chromatography system. The mobile phase consisted of 75% acetonitrile in water. Sample volumes were 20 μl at a concentration of 1 mg/ml and were detected at 280 nm with a flow rate of 1.0 ml/min with a Waters 486 detector. The column was maintained at room temperature and the samples were stored at 4°C after injection. The major elution peak for PA is 11.44 min, GalXM 5.50 min, and 6.57 min for the GalXM-PA conjugate. The conjugate preparation did not contain any material eluting at 11.44 and 5.50 min. Capture ELISA using anti-PA Ab 7.5 G and hyperimmune sera tested positive for both GalXM and PA (data not shown) (23).

Serum Abs

Blood was collected from preimmune and immunized mice for ELISA. Costar plates were coated with 50 μg of GalXM, the plates were blocked with 2% BSA, and a 1/100 dilution of serum was serially diluted in a 1:3 ratio along the plate. A cocktail (1 μg/ml) of IgM, IgA, IgG (H+L)-alkaline phosphatase conjugated (SouthernBiotec) was used as the secondary Ab. *p*-Nitrophenyl phosphate was used to reveal the presence of the alkaline phosphatase. Absorbance was measured at 405 nm using a microplate reader (Multiskan; Labsystems). BSA-coated plates were used as a negative control.

Immunofluorescence

C. neoformans strains cap67, B3501, 24067, and H99 were grown in Sabouraud dextrose broth (Difco Laboratories) for 3 days at 30°C. The cells were washed three times with sterile PBS (pH 7.4) and then counted using a hemocytometer. Strains were normalized to a suspension of 2×10^6 cells/ml and incubated with a 1/100 dilution of GalXM-PA preimmune serum or serum not stimulated by the Ag as a control in 2% BSA and 0.05% goat serum. Cells were washed three times with 2% BSA and 0.05% goat serum and incubated with 4 μg/ml goat anti-mouse IgM-FITC.

ELISA spot assay

Costar plates were coated overnight with 50 μg of GalXM or 2% BSA as a control in coating buffer (20 mM K_2HPO_4, 10 mM KH_2PO_4, 1 mM Na-EDTA, 0.8% NaCl, 0.01% NaN_3). Before plating, spleen cells plates were blocked using 2% BSA for 2 h at 37°C. Plates were then washed with 2× DMEM. Spleens were homogenized and passed through a 0.20-μm Falcon cell strainer into 2 ml of DMEM supplemented with 10% FCS, 10% NTC-109, 1% nonessential amino acids, and 1% penicillin-streptomycin. RBCs were lysed by suspending the cell pellet in 0.17 M NH_4Cl buffer for 2 min at room temperature. Cells were placed in a dish for 2 h to allow adherent cells to settle to the bottom. Nonadherent cells were collected by centrifugation and the pellet was resuspended in DMEM media. Viable cells were counted with trypan blue. Nonadherent cells (1×10^6) were added to the first well and 1 log dilutions were plated across. Cells were incubated overnight at 37°C in a 10% CO_2 incubator. Cells were then washed away from the plate with washing buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween 20 (pH 7.2)). A cocktail of IgM, IgA, IgG1, IgG2a, IgG2b, IgG3 biotin-conjugated Abs (SouthernBiotech) was used as the secondary Ab and incubated overnight at 4°C. Thirty minutes before washing the plates a Vectastain ABC streptavidin buffer (Vector Laboratories) was prepared in 1× PBS and 0.1% Tween 20. The plates were incubated with the Vectastain for 30 min at room temperature. Plates were then washed and incubated with 5-bromo-4-chloro-3-indoyl phosphate (BCIP; Sigma-Aldrich) in AMP buffer (203 mg $MgCl_2$-$6H_2O$, 0.1 ml Triton X-405, 95.8 ml of 2-amino-2 methyl-1-propanol (AMP); pH was adjusted to 9.8 with HCl in 1 liter of distilled H_2O) for 3 h at room temperature until a blue color developed. Plates were rapidly washed twice with water and blue spots were counted using a Zeiss inverted light microscope.

In vivo caspase inhibition

BALB/c female mice were injected i.p. with 50 μg of Z-DEVD-FMK, an irreversible and cell-permeable peptide used to inhibit caspase-3 (BD Pharmingen). Mice were subsequently injected i.p. with 50 μg of a general pan-caspase inhibitor, the peptide Z-VAD-FMK (BD Pharmingen). In preliminary experiments we used several controls such 50 μg of Z-FA-FMK, a negative control peptide for caspase inhibitors. Since these peptide inhibitors are only soluble in DMSO (Sigma-Aldrich), and DMSO may cause cellular toxicity, the peptides were then diluted in PBS to dilute the DMSO. As an additional negative control, mice were injected with diluted DMSO in PBS at the same final concentration used for diluting the inhibitors. All inhibitors were injected on a daily basis and within the same time for 23 days. The mice were also injected i.p. with 2 μg of GalXM or saline in CFA at the second day of inhibitor injections. Mice were sacrificed at days 3, 7, 14, and 23 days. Mice sacrificed on day 23 were boosted with 0.1 μg of GalXM. These were then compared with mice that were not given the inhibitors. ELISA spot assay was performed to determine the number of Ab-producing cells.

Capture ELISA

Microtiter plates were coated with 1 μg/ml goat Ab to mouse IgG2b (SouthernBiotech). mAb 7.5G to PA was then added in a solution of 2 μg/ml as the Ag capture Ab. The GalXM-PA conjugate was incubated in the microtiter plate. The presence of GalXM was then detected with hyperimmune sera generated from mice immunized with the GalXM-PA conjugate. These specific Abs to GalXM in the hyperimmune sera were mostly IgM and IgA. Alkaline phosphatase-conjugated mouse anti-IgM mAb was used as the secondary Ab. *p*-Nitrophenyl phosphate was used as the substrate for the enzyme alkaline phosphatase. Absorbance was measured at 405 nm.

Histology

Spleens were harvested and fixed in 10% buffered formalin. Sections were paraffin embedded and processed with H&E, trichrome, and mucicarmine stains. Apoptosis determination was done on paraffin slides using an ApopTag Plus fluorescein in situ apoptosis kit (Chemicon), following the manufacturer's instructions. Slides were viewed using an Olympus AX 70 microscope. Images were captured with a QImaging Retiga 1300 digital camera using the QCapture Suite V2.46 software (QImaging). Brightness and contrast were adjusted using Adobe Photoshop 7.0 in the TUNEL image to show clarity.

SDS-PAGE

A 4.5% stacking and 10% resolving SDS-polyacrylamide gel was made to resolve the conjugate. Protective Ag, GalXM, and the conjugate were analyzed under nondenaturing conditions.

Cytokine measurements

BALB/c 6- to 8-wk-old female mice were treated with different concentrations of GalXM as described above. Spleens were harvested and cell count was normalized, and these were homogenized with 1× cell lysis buffer (RayBiotech) and Complete protease inhibitors (Roche). RayBiotech mouse inflammation cytokine Ab array 1.1 membranes were incubated with spleen cell lysates, washed, and developed according to the manufacturer's instructions. Percentage protein cytokine expression was evaluated by analyzing spot densities with ImageJ software. Background was also subtracted from the spot density. Densities were reported as the percentage from the positive control. The positive control is a set of spots on the array membrane that are colored when the assay is developed and also show that the array is working properly.

B cell isolation

Spleens from BALB/c or Nu/Nu female mice were harvested and splenocytes were isolated by homogenization in DMEM. Cells were spun at 1200 rpm for 5 min and RBC were lysed by resuspending the pellet in 0.17 M ice-cold NH_4Cl buffer for 5 min. B cells were isolated using a Dynal B cell negative isolation kit (Invitrogen) according to the manufacturer's instructions. Isolated B cells were diluted in RPMI 1640 supplemented with 10% FCS, 1% nonessential amino acids, and 1% penicillin-streptomycin and plated in 6-well plates with 5 μg of LPS to stimulate the cells. Cells were treated with GalXM or PBS and incubated for 18 h at 37°C in 10% CO_2.

Flow cytometry analysis

BALB/c female mice were injected i.v. with 0.5, 5, 50, or 500 μg of GalXM or saline in CFA. Mice were subsequently challenged with 0.1 μg of GalXM in IFA at day 14. Splenic macrophages were isolated by homogenization in DMEM supplemented with 10% FCS, 10% NTC-109, 1% nonessential amino acids, and 1% penicillin-streptomycin. Cells were spun at 1200 rpm for 5 min and RBC were lysed by resuspending the pellet in 0.17 M ice-cold NH_4Cl buffer for 5 min. Pellet was resuspended in staining buffer (1% FCS in PBS), and cell viability was determined by trypan blue staining. Cellular Fc receptors were blocked with mouse Ab to Fc (FcyIII/II anti-mouse CD16/CD32; BD Pharmingen) for 30 min in staining buffer (FBS in PBS) with gentle rocking at room temperature. Approximately 5 ×

Table I. *Effect of GalXM and GalXM-PA conjugate on Ab titers*[a]

GalXM (μg) ($n = 3$)	Titers Day 14		Titers Day 28		GalXM-PA Conjugate (μl)	Titers Day 14	Titers Day 28
	6–8 wk	52 wk	6–8 wk	52 wk		6–8 wk	6–8 wk
0.5	1/300	1/900	1/100	1/100	50	1/900	1/8100
5.0	1/900	1/2700	1/100	1/300	50	1/900	1/2700
50	1/900	1/900	1/100	1/100	100	1/2700	1/8100
500	1/900	1/2700	1/100	1/100	CFA	1/100	1/100
CFA	1/100	1/300	1/100	1/100	PBS	1/100	1/100
PBS	1/100	1/300	1/100	1/100	BSA	1/100	1/100
BSA	1/100	1/300	1/100	1/100			

[a] Fourteen days after initial immunization, Ab response was measured using ELISA. After subsequent challenge with 0.1 μg of GalXM or the same initial dose of the conjugate, titers were evaluated at day 28; $n = 3$ mice/group for GalXM-injected mice.

10^7 cells were incubated for 30 min at room temperature with the following Abs: F4/80$^+$ (Serotec), CD4$^+$ (BD Pharmingen), CD8$^+$ (BD Pharmingen), neutrophils (Serotec), CD11c$^+$ (BD Pharmingen), CD19$^+$ (Serotec), CD5$^+$ (Ly1; eBioscience), and CD21$^+$ (eBioscience). Cells were washed three times after Ab incubation with staining buffer. Fifteen minutes before analysis, apoptosis probes annexin V-Alexa 350 and 7-aminoactinomycin D were added to samples in annexin binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$ (pH 7.4)). Flow cytometry was performed in a FACSAria cytometer (BD Biosciences) and analyzed with FloJo version 8.7.

Recombinant single-chain variable fragment (scFv) using GalXM hybridomas

We extracted the mRNA from three hybridoma cultures before they became unstable and cloned their cDNA in bacterial vectors. We obtained 18 valid sequences for the variable heavy (V_H) and variable light (V_L) regions from these three cultures. Despite not being clonal, we were able to isolate a common κ L chain and two common H chain sequences, one of the γ isotype and the other from the μ isotype, from them. The variable gene usage was very similar to previously described anti-carbohydrate Abs (24). The H chain variable region of the γ isotype used VH5.1 and JH2 gene elements linked by an unknown D gene, being the same that were found in the anti-GXM mAb 18B7 (24). The μ-chain used VH3, DH2.1, and JH4 genes. The γ H chain had several mutations when compared with the germline, indicating considerable affinity maturation. The sequences were deposited in GenBank (accession nos. FJ233886–FJ233904). Using genetic engineering, we produced two scFv Abs using either the IgG or the IgM variable region linked to the variable L chain. These recombinant Abs were able to bind GalXM (data not shown).

Statistical analysis

Statistical analysis was done by the Kruskal-Wallis test and Microsoft Excel.

Results

Serum Ab responses to GalXM and GalXM-PA conjugate

This study began with an attempt to generate mAbs to GalXM for capsule analysis. However, the problems encountered with making Abs to GalXM led us to investigate the immunologic mechanisms responsible for the poor immunogenicity of this polysaccharide. To identify mice suitable for spleen harvest, mice were injected with GalXM or the GalXM-PA conjugate and serum titers to GalXM were examined. GalXM immunization elicited modest amounts (titer 1/900) of serum Abs reactive to GalXM regardless of immunization dose. Older mice had a slightly better response, with serum titers of 1/2700. Additionally, mice were also immunized with LPS-free GalXM and the results were identical with both young and older mice (data not shown). We also consistently noted that after subsequent challenge at day 14 with GalXM, Ab titers were reduced to levels that were no longer measurable (Table I). To confirm that these effects were different from GXM, mice were injected with the GXM polysaccharide and a modest dose-dependent response was observed with the highest titer (1/2700) at 0.5 μg and the lowest response (1/100) at 500 μg (data not shown). The GXM results confirm earlier work done by Murphy and Cozad (12). To evaluate whether the poor immunogenicity of GalXM could be overcome by protein conjugation, the polysaccharide was conjugated with the 83,000-Da PA protein from *B. anthracis*. Immunization studies with the conjugate revealed that although the primary Ab response to the conjugate was better than with GalXM alone, it was still modest (1/900), but a booster immunization at day 14 elicited a significantly increased Ab response (1/8100) (Table I). Hybridomas with both GalXM and GalXM-PA yielded unstable clones that ceased to produce or grow during soft agar cloning, but were suitable donors for Ig genes that recognized GalXM (see below). However, a screen done in parallel for PA yielded several positive Abs in which one was protective in mice (data not shown), suggesting that the B cell unresponsiveness was limited to cells producing mAb to GalXM.

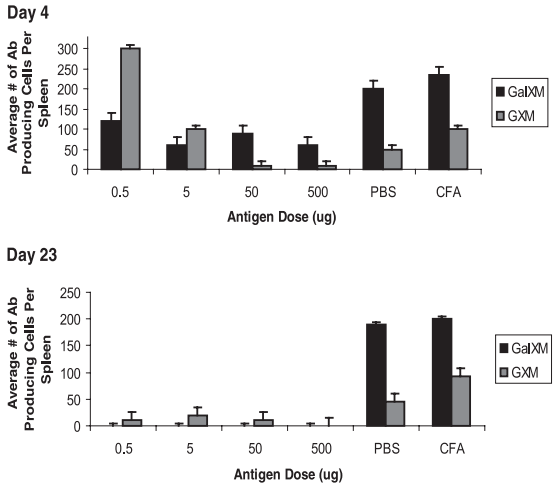

FIGURE 1. ELISA spot Assay for spleen Ab-producing cells to GalXM. Ab-producing cells were detected as blue spots on ELISA plates. Black bars are spots detected for GalXM, and gray bars are spots detected for GXM. *Upper panel*, The numbers of Ab-producing cells decrease in a dose-dependent manner for both Ags at day 4. *Lower panel*, After an immune challenge, Ab-producing cells are completely abolished for GalXM-treated mice by day 23. Although there is a reduction for Ab-producing cells against GXM, these are still detectable; $n = 5$ mice/group.

Hyperimmune serum from mice immunized with the conjugate was used for immunofluorescence on *C. neoformans* strains cap67, B3501, 24067 (serotype D), and H99 (serotype A). The polyclonal Ab bound near the cell wall in acapsular strains (data not shown), a pattern consistent with prior reports that GalXM is mostly cell wall-associated (25). In encapsulated serotype D strains the serum bound all around outer edge in a punctuate pattern. In H99 cells the fluorescence pattern was scattered and punctate (26). We also generated recombinant scFv region Ab fragments derived from the mRNA of GalXM-PA hybridoma cell lines that secreted anti-GalXM Abs before becoming unstable. Preliminary results with these scFvs showed a punctate fluorescence pattern as for the GalXM-PA serum (data not shown) (14, 25). The recovery of Ig genes from unstable hybridomas producing Ab to GalXM that reacted with GalXM when assembled into scFv suggested that the problem in recovering hybridomas was with the cells and not the Ab molecules.

Spleen plasma cell response to GalXM immunization

Given our inability to recover stable hybridomas, we proceeded to investigate the B cell response to GalXM immunization, and we enumerated the Ab-producing cells in the spleen following GalXM immunization by ELISA spot (Fig. 1). The lowest immunizing

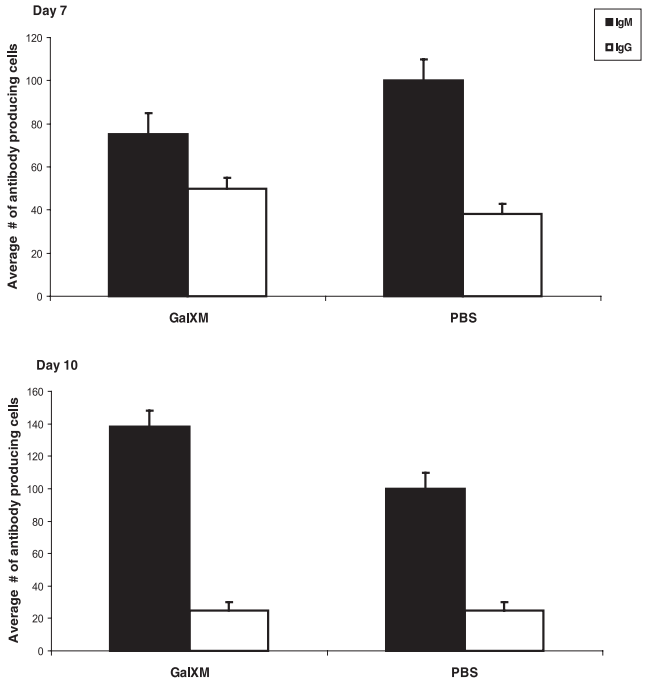

FIGURE 2. Splenocytes in BALB/c mice are able to produce nonspecific Abs after GalXM immunization. ELISA spot assay shows the average number of Ab-producing cells against IgM or IgG in BALB/c after injection of 20 μg of GalXM and PBS. The results are after 10 days of treatment. The mice were boosted with 0.5 μg of GalXM at day 8; $n = 5$ mice/group.

dose, 0.5 μg of GalXM, yielded the highest number (130 cells) of Ab-producing cells. The effect of GalXM in suppressing Ab-producing cells was dose dependent, such that immunization with the larger amounts significantly reduced the number of cells. However, after a subsequent GalXM immunization at day 14, no Ab-producing cells were detectable in the spleens of immunized mice by day 23. Repeating the experiments with LPS-free GalXM also yielded the same results (data not shown). As a control, we also examined the number of Ab-producing cells in spleen in response to GXM. Although the number of Ab-producing cells to GXM decreased in a dose-dependent manner (300 cells for 0.5 μg of GXM and 10 cells for 500 μg of GXM), Ab-producing cells to GXM did not disappear completely, as was observed with GalXM immunization (Fig. 1). We also examined spleens that were injected with 50 μl of the conjugate in CFA followed by a boost of 10 μl at day 14. The results revealed the presence of 200 Ab-producing cells; however, these results were similar to the CFA control (data not shown). We examined whether the loss of Ab-producing cells was limited to B cells producing GalXM-specific Abs. Mice treated with two immunizations of GalXM continued to have some Ab-producing cells secreting IgM and IgG Abs despite the dearth of cells producing Abs to GalXM (Fig. 2).

GalXM immunization causes spleen enlargement

During spleen harvesting we observed that mice injected with GalXM, and in particular those receiving the 500-μg dose, had a significantly enlarged spleen. The average weight of the spleen (0.320 g) was three times higher than the average of spleen from mice receiving a mock injection with PBS alone (0.111 g) (Fig. 3). At other GalXM immunization doses splenic enlargement was also observed, but the increase was not as dramatic as with 500 μg. Splenic sections using H&E reveal that at doses of 500 μg, splenic follicle definition is lost (Fig. 4). Pathological analysis revealed

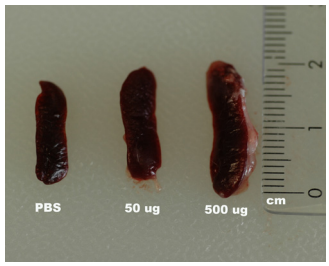

FIGURE 3. GalXM causes splenic enlargement. After two challenges with GalXM, spleen weights tripled in size at a dose of 500 μg (2.0 cm, 0.320 g) as compared with 50 μg (1.5 cm, 0.150 g) of GalXM and the PBS control (1.4 cm, 0.144 g). Spleen sizes are measured in centimeters and spleen weights are measured in grams.

moderate to marked extramedullary hematopoiesis with increased myeloid and megakaryocyte precursors (data not shown). There was also mild lymphoid depletion as compared with PBS control. All of the spleens samples had splenic capsulitis and peritonitis and early fibrosis due to i.p. injection of CFA. A TUNEL assay was used to detect if apoptosis was occurring in the spleen and if the effect was dose dependent. The results revealed that GalXM induced apoptosis in the spleen at all doses administered compared with the PBS control (Fig. 5).

GalXM affects cells in the spleen

Given the observation that GalXM ablated the specific B cell response, we investigated the effect of this polysaccharide on other major cell types in the spleen. Since GalXM is reported to cause apoptosis in T cells and macrophages in vitro (15), we enumerated $CD19^+$ B cells, $F4/80^+$ macrophages, and $CD4^+$ and $CD8^+$ T lymphocytes in the spleen after GalXM immunization. Eight days following the first initial injection of GalXM (10 μg) there was a decrease in the percentage of $CD19^+$ B cells. In parallel, we looked at the percentage of the $CD19^+$ cells that were annexin V positive, a marker of apoptosis. The results also revealed that at 8

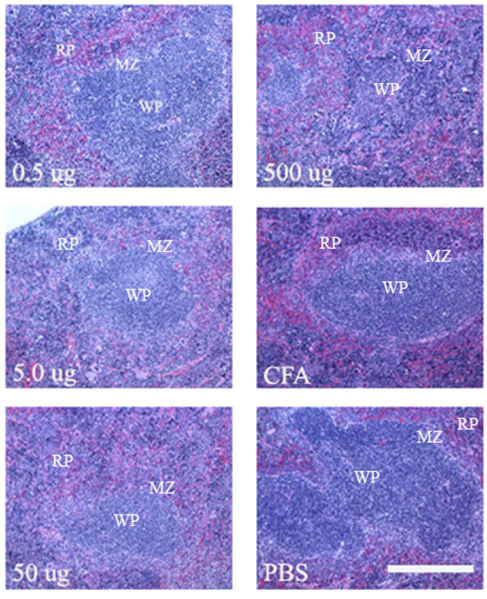

FIGURE 4. Spleen histology after GalXM immunization. Normal splenic architecture with defined red pulp, white pulp, and marginal zones can be seen in most sections. At 500 μg of GalXM, however, follicle definition is lost due to intense extramedullary hematopoiesis. Objective is $\times$40.

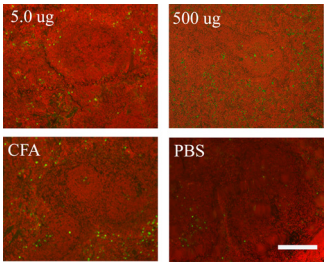

FIGURE 5. TUNEL reveals apoptosis in the spleen of mice immunized with GalXM. Apoptotic bodies (green dots) are present at low doses (5 μg) and high doses (500) μg of GalXM as compared with the PBS control. Scale bar is 110 μm.

days after GalXM injection there was an increase in the percentage of cells that are annexin V-positive. After a second injection of GalXM there was a recovery of $CD19^+$ B cells, as seen on day 23. However, ELISA spot revealed a complete disappearance of GalXM-specific B cells after a second injection. We also tested if $CD5^+$- and $CD21^+$-specific B cells were affected by GalXM but found no differences between spleens from GalXM-treated mice and control mice (data not shown). For $F4/80^+$ macrophages, the initial injection of GalXM caused an increase in the percentage of macrophages by day 8, and a second injection caused an additional modest increase in the percentage of these cells by day 23. Annexin V stain could not be evaluated since it nonspecifically binds to monocytes. For both $CD4^+$and $CD8^+$ T lymphocytes there was a decrease in the percentage of cells after the first injection of GalXM through day 14. Annexin V staining shows an increase in the percentage of cells undergoing apoptosis with the first injection (Fig. 6). The effect of GalXM on isolated splenic B cells was also tested. Isolated B cells were treated with a low dose (0.5 μg) and a high dose (50 μg) of GalXM for 18 h. Flow cytometry analysis for $CD19^+$ and Fas or FasL double-positive showed a modest increase in Fas at a dose of 50 μg, but the differences were not statistically significant ($p = 0.806$) (Fig. 7). Hence, GalXM injection had a qualitative effect in causing a disappearance of GalXM-binding B cells and significant quantitative effects on the numbers and proportions of total B and T lymphocytes and macrophages.

Cytokine expression in spleen

Since splenic follicle morphology had been drastically altered and apoptosis was observed in the spleen of mice receiving GalXM, we investigated global cytokine expression at 5 and 500 μg of GalXM. Cytokine array revealed that the expression of the inflammatory cytokines decreased with prolonged exposure to GalXM. The cytokine levels of mice that received boosting immunization at day 14 decreased regardless of the GalXM dose (Fig. 8). At day 7, there was a specific decrease in G-CSF, which is produced mostly by macrophages and activates neutrophils and granulocytes; GM-CSF, which is produced mostly by Th2 T cells and activates T lymphocytes, monocytes, neutrophils, and precursors (27); IFN-inducible T cell α chemoattractant, which is produced by neutrophils and is a T cell chemoattractant; keratinocyte chemoattractant, which is produced by keratinocytes and monocytes and is a potent neutrophil chemoattractant (28); leptin, which is expressed in adipose tissue and can modulate the immune response by regulating the proliferation of T cells in monocytes, and it can increase the production of TNF and IL-12 (29); IL-1β, which is produced by many immune cells, such as macrophages, T, B, and NK cells and neutrophils, activates T, B, and NK cells (27); IL-2, which is produced by $CD4^+$ T lymphocytes, promotes T and B cell growth and differentiation (27); IL-3, which is produced by T cells and activated NK cells (27), IL-13, which is produced by T cells and promotes B cell growth and differentiation (27); and IL-17, which is produced by activated T cells and whose function is to further activate T cells (27). At day 14 there is a specific decrease in FasL, a type II transmembrane protein that plays an important role in immune regulation by binding to its receptor Fas (CD95) and inducing apoptosis (30). LPS induces chemokine LIX, an inflammatory chemokine that is strongly induced by bacterial LPS and is able to interact with matrix metalloproteases (31); eotaxin-2,

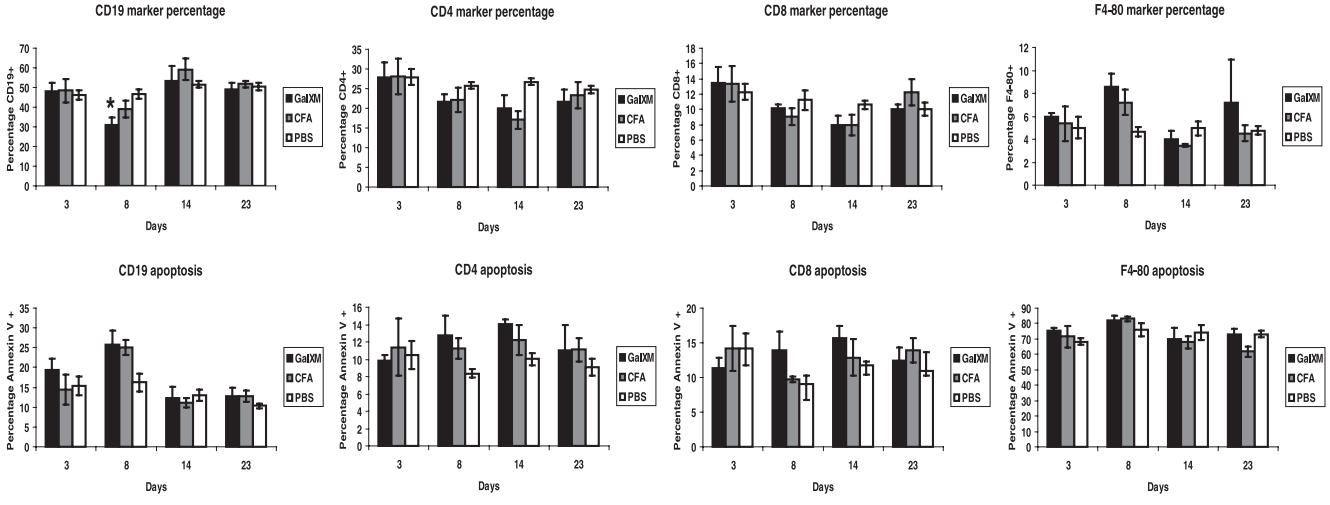

FIGURE 6. GalXM immunization negatively affects several types of splenocytes. *Upper panels*, Percentage of $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells, and $F4/80^+$ macrophages during 3, 8, 14, and 28 days after 10 μg of GalXM injection. BALB/c mice that were followed to day 28 received a GalXM boosting immunization of 0.5 μg of GalXM. For $CD19^+$ B cells injected with GalXM there is a statistically significant decrease (*, $p = 0.006$) when compared with the PBS control. *Lower panels*, Percentage of cells that are double-positive for the marker and annexin V. Representative absolute number of cells in the spleen range as follows: $CD19^+$ B cells: GalXM (3.9×10^6 to 1.69×10^6 cells), PBS (9.7×10^7 to 2.2×10^7 cells), CFA (8.9×10^6 to 1.7×10^7 cells); $CD4^+$ T cells: GalXM (1.4×10^7 to 3.4×10^7 cells), PBS (3.0×10^7 to 4.8×10^7 cells), CFA (2.5×10^7 to 3.5×10^7 cells); and $CD8^+$ T cells: GalXM (6.7×10^6 to 1.8×10^7 cells), PBS (9.0×10^6 to 2.0×10^7 cells), CFA (1.0×10^7 to 1.6×10^7 cells); and $F4/80^+$: GalXM (3.9×10^6 to 7.6×10^7 cells), PBS (3.3×10^6 to 6.9×10^6 cells), CFA (6.8×10^6 to 9.8×10^6 cells); $n = 4$ mice/group.

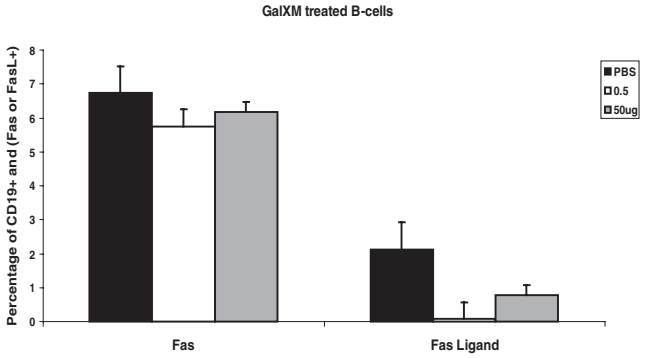

FIGURE 7. Low dose or high dose of GalXM does not increase splenic Fas or FasL in isolated B cells. The percentages of $CD19^+$ and Fas or FasL double-positive B cells, after isolated B cells were treated with 0.5 or 50 μg of GalXM, are shown; $n = 5$ mice/group.

which induces the chemotaxis of eosinophils; and T cell activation-3 (TCA-3), which is produced by T cells. By day 21 a decrease was seen in all other cytokines that affect mostly B and T lymphocytes. The cytokines that were markedly reduced at day 21 are TCA-3, which is produced by T cells after cell activation, and LIX, IL-1β, and IL-12p70, which is produced mainly by B cells and, to a lesser extent, by T cells and stimulates the activation of lymphocytes. Hence, GalXM immunization was associated with global dysregulation of spleen cytokine production.

Caspase inhibition rescues GalXM-binding B cells

Prior in vitro studies had shown that GalXM caused apoptosis in T cells through the activation of caspase-8. Since our results also indicated apoptosis in the spleen, we conducted a pilot study using several caspase inhibitors, including the general caspase inhibitor Z-VAD-FMK, the caspase-8 inhibitor Z-IETD-FMK, the caspase-3 inhibitor Z-DEVD-FMK, and the negative control inhibitor Z-FA-FMK. The negative control inhibitor had no inhibitory effect on apoptosis mediated by caspases, and it only inhibited cysteine proteases when mice were given 0.5, 5, 50, and 500 μg of GalXM. In the first experiment, either the general pan-caspase inhibitor or the caspase-8 inhibitor was administered. The general pan-caspase inhibitor maintained the number of Ab-producing cells >250 cells regardless of GalXM challenge (data not shown). However, the caspase-8 inhibitor alone did not overcome the effects of GalXM since we observed a GalXM dose-dependent decrease in the number of Ab-producing cells enumerated by ELISA spot. In a second experiment we repeated the experiment using a caspase-3 inhibitor instead of caspase-8, since caspase-3 is a downstream effector caspase in the extrinsic apoptotic pathway. The caspase-3 inhibitor maintained the number of Ab-producing cells >400 regardless of GalXM challenge (data not shown). In a subsequent experiment we injected mice with a combination of the caspase-3 and general pan-caspase inhibitor. Mice were injected with 5 μg of GalXM 24 h after the inhibitor cocktail, and then the inhibitors were given every other day and these were followed at days 3, 7, 14, and 23. The mice were also challenged with GalXM on day 14. These mice were then compared with a second set of mice that were not given the inhibitor cocktail and were also challenged on day 14. On days 3 and 7 both groups had ~300–500 cells. On day 14, there was a slight decrease in the number of cells for the group with no inhibitor. However, by day 23 the mice not receiving the inhibitor had only an average of 50–100 cells, whereas those given the inhibitor cocktail had an

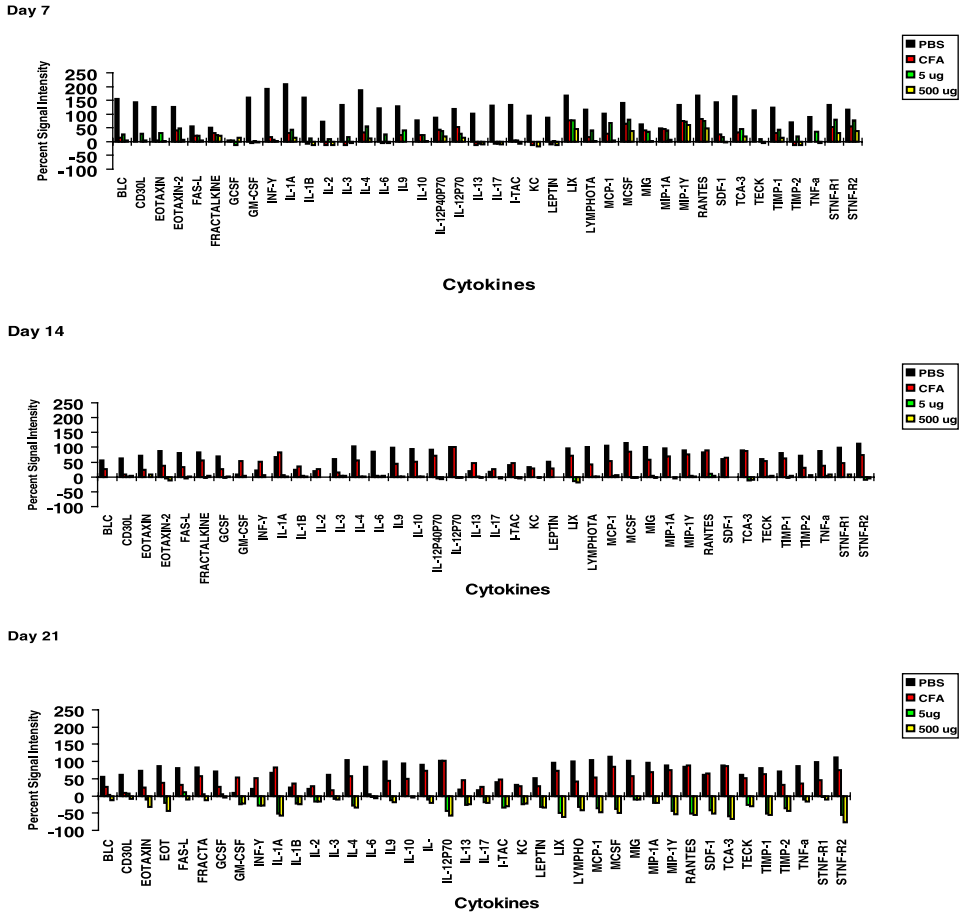

FIGURE 8. Immunization with GalXM results in dose-dependent global reduction of inflammatory cytokine expression. Inflammatory cytokine arrays show the percentage signal intensity of cytokine expression of BALB/c mice injected with PBS (black bars), CFA (red), and 5 μg (green) and 500 μg (yellow) of GalXM in IFA.

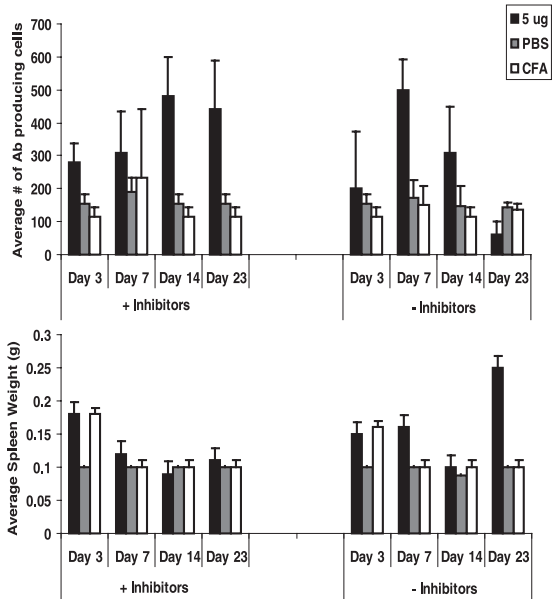

FIGURE 9. Capsase inhibitors prolong the presence of anti-GalXM Ab producing cells. *Upper panel*, ELISA spot assay shows the average number of Ab-producing cells in BALB/c mice with or without caspase inhibitor cocktail after a GalXM injection of 5 μg. Mice followed to day 21 received two doses of GalXM. *Lower panel*, The average spleen weights per treatment groups; $n = 5$ mice/group.

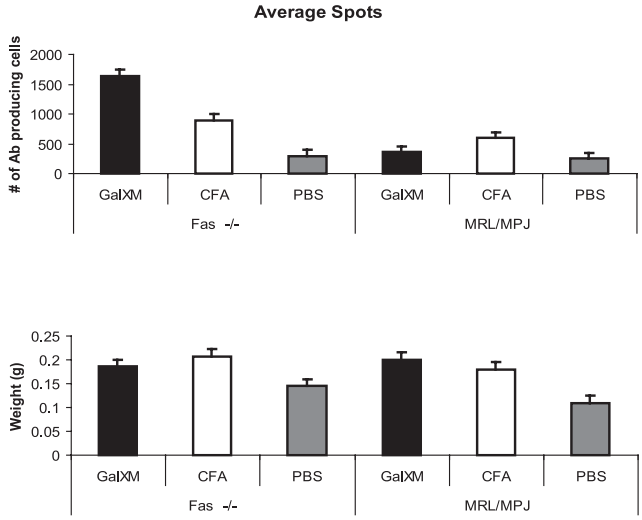

FIGURE 10. Fas-deficient mice sustain GalXM Ab-producing cells. *Upper panel*, ELISA spot assay shows the average number of Ab-producing cells in MRL/MpJ-Fas^{lpr}/J and MRL/MPJ control mice after injection of GalXM, CFA, and PBS. The results are after 14 days of treatment. The mice were boosted with 5 μg of GalXM at day 7. *Lower panel*, The average spleen weights per treatment groups; $n = 5$ mice/group.

average of 450 cells, a difference that is statistically significant ($p = 0.008$) as determined by Kruskal-Wallis test. We also observed that the spleens of mice not receiving the inhibitors were twice as large at 0.2 g (Fig. 9). We attempted to generate Abs from mice treated with the cocktail of inhibitors but none was recovered because the hybridomas were not stable producers.

Fas deficiency increases spleen plasma cell response to GalXM

Since GalXM is reported to up-regulate the Fas receptor in T cells, we tested the effects of GalXM in vivo by enumerating the number of Ab-producing cells. The results revealed that the number of anti-GalXM Ab-producing cells was significantly larger in Fas-deficient mice (1600 cells) compared with MRL/MpJ control mice (350 cells). The results also reveal that there was no significant difference in spleen size between Fas receptor-deficient mice and the MRL/MPJ control mice (Fig. 10).

Discussion

Many attempts have been made to render the cryptococcal capsular polysaccharides, namely GXM, immunogenic in animals by coupling them to BSA (32), bovine γ-globulin (33), and sheep erythrocytes (34). However, a major success in the field was the conjugation of GXM to tetanus toxoid, which yielded a protective vaccine that was used to generate many of the mAbs used to study *C. neoformans* (22, 35, 36). While attempting to make a mAb against GalXM, we observed that GalXM alone was a poor immunogen since initial titers were modest and the titers dropped to nonimmune levels after boosting with additional GalXM. We attempted to circumvent this problem in several ways. To make GalXM more immunogenic, we immunized mice with GalXM in a CFA emulsion, but this approach yielded only low and transient titers of Abs to GalXM. The second approach was by generating a GalXM-protein conjugate using the protective Ag from *B. anthracis*. Although initial immunization yielded modest titers of GalXM-binding Abs, boosting immunization was also associated with a decrease in GalXM-specific titers. This result suggested that the conjugate helped to overcome initial immunological tolerance in the spleen but that its effect was short-lived, which was seen by the instability of the hybridomas that we generated. Our ability to generate GalXM-binding scFvs from mRNA recovered from dying GalXM-binding hybridomas implies that the problem with unstable hybridomas is likely to reflect a host cell problem rather than loss of Ab production or specificity from ongoing somatic mutation.

The reduction in serum titers to nonimmune levels following GalXM immunization, as well as literature reports showing that GalXM induced apoptosis through the extrinsic apoptotic pathway by promoting the cleavage of caspase-8 in T cells (15, 17), led us to hypothesize that GalXM was producing a state of immunological paralysis (19, 20) through direct induction of apoptosis. Consistent with this notion, GalXM administration was associated with a reduction in the number of specific Ab-producing cells in the spleen, a dose-dependent effect. After subsequent GalXM challenge, Ab-producing cells recognizing GalXM completely disappeared from the spleen. These results are in contrast to GXM, in which not all Ab-producing cells disappear from the spleen (12). We tested whether the GalXM affected Ab production of all B cells. Mice treated with two immunizations of GalXM were still able to make Ab-producing cells that secreted IgM and IgG, suggesting that the GalXM effect was specific to that polysaccharide. Further evidence that the effect was specific was that the immunization with GalXM-PA produced high serum titers to PA, and we recovered several PA-binding hybridomas from the spleens of immunized mice (data not shown). The instability of hybridomas producing Abs against GalXM was observed in four fusions and remains a perplexing observation that raises the possibility that B cell encounters with GalXM in the spleen induce cell damage that lasts through fusion with myeloma cells to produce short-lived hybrid cells.

We were able to avoid the complete depletion of B cells producing Ab to GalXM by administering a cocktail of general pan-caspase and caspase-3 inhibitors. During a pilot study using the inhibitors separately, we found that the caspase-8 inhibitor was

unable to block the reduction of Ab-producing cells, while the caspase-3 and general pan-caspase inhibitors were able to do so. In a subsequent experiment, our results showed that the cocktail of general pan-caspase and caspase-3 inhibitors maintained the number of Ab-producing cells despite increased GalXM concentrations or challenge. However, hybridoma instability was observed in these caspase inhibitor-treated mice. We also show that Fas receptor-deficient mice also maintained the number of GalXM Ab-producing cells. This result suggests that Fas is responsible for GalXM-induced apoptosis. However, when we isolated B cells and treated these with GalXM, only a high dose of the polysaccharide modestly increased the presence of the Fas receptor, but the results were not statistically significant. These results argue against a direct proapoptotic toxicity of GalXM on B cells and suggest that other cell types may be involved in the up-regulation of the Fas receptor in B cells. A preliminary experiment in nude mice showed that GalXM did not affect the up-regulation of Fas in $CD19^+$ B cells, suggesting that T cells may not be the primary cells involved in B cell apoptosis (data not shown).

As for GXM, GalXM causes immunological paralysis in the spleen, as seen in the ELISA spot assay. However, GalXM induces more dramatic changes on immune function than does GXM. For example, massive enlargement of the spleen followed GalXM immunization and was characterized by loss of splenic follicle definition, localized inflammation, and cellular apoptosis. Although CFA caused some splenic enlargement on its own, remarkable splenic enlargement was observed only in mice that received GalXM and CFA. The inflammatory cytokine profile shows that GalXM causes a global reduction in cytokine expression. These changes in cytokine expression are paralleled by dramatic changes in the cellular numbers of B and T lymphocytes and macrophages. Flow cytometry revealed that the percentage of $CD19^+$ B cells decreased after the initial injection of GalXM, but that these cells recovered to initial levels after GalXM boosting. However, GalXM-specific B cells were completely abrogated by a second immunization with GalXM. This observation suggests that the first dose of GalXM affects all general $CD19^+$ B cells, while the second dose affects primarily GalXM-specific B cells. The specific subset of $CD5^+CD21^+$ B cells was not affected by GalXM. $CD4^+$ and $CD8^+$ T cells are affected by single doses of GalXM and these do not recover to initial levels. In contrast, the percentage of $F4/80^+$ macrophages increased modestly in response to GalXM injections, consistent with macrophage infiltration to replenish cells undergoing apoptosis. We observed that after GalXM injection there was a modest increase in annexin V staining. Our in vivo results agree with those reported by Pericolini et al. (15) in T cells and most recently by Villena et al. for macrophages in vivo (16). The large increase in spleen mass associated with GalXM immunization appears to be the result of profuse extramedullary hematopoietic activity that disrupted spleen architecture such that the distinction between marginal zones and pulp was lost. Splenomegaly has been observed during systemic infection by encapsulated pathogens such as *Streptococcus pneumoniae*, and it is due to increased B and T cells and macrophages (37).

In summary, our results show that the mechanism of immunological paralysis after GalXM immunization is the result of apoptotic ablation of B cells producing Abs to GalXM in the context of global spleen immune dysregulation, characterized by a global diminution in cytokine production and derangements in lymphocyte and macrophage prevalence. To our knowledge, this is the first mechanism explaining the classical observation that polysaccharides induce immune paralysis. Although GalXM was shown to induce T cell apoptosis in vitro (15), this mechanism did not extend to B cells, and the specificity of paralysis for the Ab response to GalXM remains unexplained. A comparison of the spleen cytotoxic effects of GalXM and GXM indicate different effects, suggesting that the mechanisms of immunological paralysis are likely to differ depending on the specific polysaccharide. GalXM is a powerful immunomodulatory molecule that could conceivably find applications for the treatment of autoimmune diseases, as was recently reported for GXM in a rheumatoid arthritis model (38). However, GalXM appears to have greater effects on host immune function than does GXM, and effects reported herein provide additional mechanisms for the profound immune suppression that often accompanies cryptococcal infections.

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Disclosures

The authors have no financial conflicts of interest.

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