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Spleen deposition of *Cryptococcus neoformans* capsular Glucuronoxylomannan in rodents occurs in red pulp macrophages and not marginal zone macrophages expressing the C-type lectin SIGN-R1

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Summary

The fate of microbial polysaccharides in host tissues is an important consideration because these compounds are often immune modulators. Splenic marginal zone macrophages that express the C-type lectin receptor SIGN-R1, take up neutral polysaccharides such as dextran and the capsular polysaccharide of *Streptococcus pneumoniae*. Given that the major component of *Cryptococcus neoformans* capsular polysaccharide, glucuronoxylomannan (GXM), localizes in the spleen when injected intravenously, we investigated whether GXM uptake was mediated by splenic macrophages expressing the SIGN-R1 receptor in mice. No significant differences in the amount and location of GXM deposition were detected in the spleens of mice treated with a SIGN-R1 blocking antibody when compared to controls. Similarly, a blocking antibody to Dectin-1, a co-receptor of -SIGN-R1, had no effects on GXM distribution within the spleen. Histological examination of spleens from mice and rats injected with FITC-Dextran and GXM revealed no significant co-localization, with Dextran and GXM being found in marginal and red pulp macrophages, respectively. Hence we conclude that GXM was not deposited in marginal zone macrophages. However, GXM deposition was found in the red pulp. These results indicate that there is a selective localization of these polysaccharides to different receptors such as SIGN-R1 for FITC dextran in marginal zone and a to-be-identified receptor selectively expressed by red pulp macrophages for GXM.

Keywords

SIGN-R1; Marginal Zone Macrophages; Red Pulp; Cryptococcus neoformans; GXM

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Introduction

The encapsulated yeast *Cryptococcus neoformans*, is a relatively frequent cause of meningoencephalitis in immunocompromised individuals [1,2]. Patients with advanced HIV infection are particularly vulnerable to cryptococcosis and antifungal therapy often fails to eradicate the pathogen [3]. *C. neoformans* is unusual among eukaryotic microbes because it contains a polysaccharide capsule with functional similarities to those of encapsulated bacteria such as, *Streptococcus pneumoniae* and *Haemophilus influenzae* [4,5]. In fact, cryptococcal polysaccharide is known to share some antigenic determinants with certain pneumococcal polysaccharides [6,7]. The *C. neoformans* capsule is composed of glucuronoxylomannan (GXM), galactoxylomannan (GalXM), and mannoprotein (MP), of which the major component on a mass basis is GXM [8,9]. GXM can interfere with many aspects of immune function, including leukocyte migration and phagocytosis [3,10–12]. The ability for GXM to interfere with immune function is believed to contribute to *C. neoformans* virulence by allowing the fungal cells to evade the immune system [11,13]. GXM is also attributed to causing immunological paralysis, reducing the number of antibody producing cells in the spleen [10].

The spleen is intimately involved with the effective clearance of pathogens by the immune system [14,15]. The major compartments of the spleen are the white and red pulp which are separated by the marginal zone (MZ) The MZ contains highly phagocytic macrophages that play a critical role in the spleen since it is the location where antigens that come into the marginal sinuses from the bloodstream are initially screened [16,17]. Marginal zone macrophages (MZM) also have been found to exclusively uptake neutral polysaccharides such as Dextrans and Ficoll [16,18]. In contrast, acidic polysaccharides localize primarily to the red pulp of the spleen [15,16,19]. The efficiency of this filtering system also results from the vascular structure of the red pulp through which blood is slowed down, enabling macrophages to phagocytose polysaccharide antigens [20].

The C-type lectin SIGN-related 1 (SIGN-R1) receptor is expressed on the macrophages of the marginal zone of the spleen and on medullary and subcapsular macrophages in lymph nodes [4,21,22]. SIGN-R1 is a mouse homologue of the DC-SIGN receptor found in dendritic cells. DC-SIGN is functionally associated with several, critically important biological processes including the ability to be hijacked by the HIV-1 virus, presentation and facilitating transmission of the virus to $CD4^+$ T-cells [14,21]. Recently it has been shown that SIGN-R1 can bind the complement C1 component C1q and assemble a C3 convertase against *S. pneumoniae* infection [23]. Furthermore, the SIGN-R1 receptor in the marginal zone macrophages can bind and internalize the capsular polysaccharide of *Streptococcus pneumoniae* and Dextrans [4,22]. This phenomenon was demonstrated by using a transient SIGN-R1 receptor knock out system (TKO), whereby the monoclonal antibody (mAb) 22D1 is used to selectively block receptor function [4,21,23].

Several studies of GXM sequestration and tissue localization revealed that although GXM is rapidly cleared from the circulation with a half life of 1.1–2.7 days this is then followed by a plateau with little elimination [24]. Intravenous administration of GXM in rats leads to the deposition in tissue macrophages [24]. However little is known about the mechanism by which GXM binds to macrophages or the fate of bound GXM [24]. In rats, GXM was found to localize to the outer areas of the marginal zone by 5 hours after (i.v.) injection [1,3]. GXM deposition in the spleen is accompanied by a rapid and intense burst of cytokine and chemokine expression [3,24] Multiple receptors such as CD18, TLR4, CD14 and FCγRII may be involved in the cellular uptake of GXM [25–27]. Since the SIGN-R1 receptor binds the *Streptococcus pneumoniae* capsular polysaccharide we investigated whether it is also involved in binding to *C. neoformans* GXM. Our results indicate that red pulp, but not marginal zone, macrophages

are responsible for uptake of GXM by the spleen in a process whereby that SIGN-R1 is dispensable.

Materials and Methods

Animals

C57BL/6, 6–8 week old, female mice were obtained from The Jackson Laboratories. Mice were used for the intravenous (i.v.) injections with glucuronoxylomannan (GXM), FITC-Dextran and PBS. Sprague Dawley (200–250 grams) rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana). Rats were used for the intravenous (i.v.) injections with glucuronoxylomannan (GXM) and saline. Animals were sacrificed by asphyxiation with CO_2. All animal experiments were done according to institutional guidelines.

Antibodies

Monoclonal antibody (MAb) 22D1 was used for the transient knock out (TKO) of marginal zone splenic macrophages possessing the SIGN-R1 receptor [21]. ChromePure Syrian Hamster IgG from the Jackson Immunoresearch laboratories (Bar Harbor, Maine) was used as a control.

Polysaccharides

Fluorescein isothiocyanate- Dextran (FITC-Dextran) of MW 2,000 kDa was obtained from Sigma-Aldrich (St Louis, MO). To study the uptake of the Dextran 100 μg (1mg/ml in sterile PBS) of the polysaccharide was injected intravenous (i.v.) in mice. *C. neoformans* strain 24067 (serotype D) was obtained from the American Type Culture Collection (Rockville M.D.). *Cryptococcus neoformans* polysaccharide (GXM) preparation was done as described previously [28,29].

Administration of polysaccharide and mAb to mice

C57BL/6 mice were injected (i.v.) with 100 μg of either the MAb 22D1 or the control Syrian Hamster IgG antibody in PBS, 24 hours prior to GXM or FITC-Dextran administration. An amount of 100 ug of GXM or FITC-Dextran diluted in sterile PBS, was injected (i.v.) to each mouse. Approximately 24 hours after polysaccharide infusion, the mice were sacrificed and the spleens and livers were removed for analysis of polysaccharide content.

Immunofluorescence with FITC-Dextran

Organs from mice injected with FITC-Dextran were fixed with 10% buffered formalin and embedded in paraffin. Tissues were sectioned into 4 um slices and placed on slides, that were subsequently deparafinized and coated with 0.05M N-propyl gallate to preserve fluorescence. Slides were maintained in the dark and at 4°C until ready for use. All slides were viewed using an Olympus AX 70 microscope (Olympus America, Inc., Melville N.Y). Images were captured with a QImaging Retiga 1300 digital camera using the QCapture Suite V2.46 software (QImaging, Burnaby BC, Canada). Images were merged or contrast adjusted using Adobe Photoshop 7.0 (San Jose, California).

Tissue Immunohistochemistry

Immunohistochemistry was used to localize GXM in splenic tissues using protocols that have been described elsewhere [30]. Briefly, organs were fixed in 10% buffered formalin and embedded in paraffin. Tissues were sectioned into 4 μm slices and placed on slides. Tissues were then deparaffized using 100% xylene and an ethanol gradient of (90, 70, and 50 %). Slides were first incubated in a 1:10 dilution of 30% H_2O_2 to extinguish all endogenous peroxidase activity. Tissue was then blocked for an hour with a (1:20 dilution) of goat or horse serum followed by incubation with a 1.94 μg /ml of MAb 18B7 for 1 hour at room temperature. Slides

were washed 3 times with PBS for 15 minutes. As a secondary antibody, 4 μg /ml of goat anti mouse IgG1- (HRP) conjugate (Southern Biotechnology, Birmingham, AL) was incubated for 1.5 hours. Unbound antibody was washed with PBS for 15 minutes. Diaminobenzidine (DAB Chromogen) (Dako Corporation, Carpinteria, CA) was used as the substrate for the enzyme conjugated to the secondary antibody to develop the reaction, brown color seen in tissue. The tissues were then counterstained with hematoxylin, (Sigma Diagnostics, St.Louis, MO), to facilitate contrast in splenic tissue. Two mice were not injected with GXM and were used as controls (n = 3 mice/rats per group).

Temporal localization of GXM or FITC-Dextran in spleen

Mice were injected intravenously with 100 μg of GXM, FITC-Dextran or PBS. Spleens were collected at 1, 3, 5, 12, and 24 hours after injected and processed for GXM concentration and localization.

Organ Homogenization

Spleens and livers were homogenized and passed through a 0.20 μm Falcon cell strainer into 2 ml of sterile PBS. Homogenates were spun at 6,000 rpm for 10 min to remove cellular debris and supernatants were digested with 20 μg /ml of Proteinase K prior to overnight incubation at 37° C (Roche Diagnostics, Indianapolis, IN). Organ homogenates that were not used for immediate analysis were stored at −20° C for up to 1 week.

Measurement of GXM in liver and spleen

GXM deposition in the liver and spleen was measured by capture ELISA as described (2). Briefly, microtiter plates were coated with 1 μg /ml goat Ab to mouse IgG1 (Southern Biotechnology, Birmingham, AL). MAb 18B7 to GXM was then added in a solution of 5 μg / ml as the antigen capture antibody (Ab). Organ homogenate supernatant digested with Proteinase-K was incubated at 100°C for 5 minutes to inactivate the enzyme, prior to incubation in microtiter plate. The presence of GXM was then detected by the IgM MAb to GXM 12A1 followed by the addition of alkaline phosphatase conjugated mouse anti-IgM MAb. p-Nitrophenyl Phosphate (PNPP) was used as the substrate for the alkaline phospatase. Absorbance was measured at 405 nm and the amount of GXM in solution was calculated from standard curves.

Conjugation of 18B7 and 2H1 with Alexa Fluor 546 and 488

Alexa Flour 546 and 488 protein labeling kit, Molecular Probes (Carlsbad, California) was used to conjugate MAb 18B7 and 2H1 respectively following manufacturers instructions.

GXM Cellular deposition in spleen

C57BL/6, 6–8 week old, female mice were obtained from the Jackson Laboratories were injected (i.v.) with 100 μg of FITC-Dextran immediately followed by (i.v.) injection with 100 μg of GXM. Twenty-four hours later the spleens were removed, sectioned and processed as described above. Prior to GXM detection, deparifinized slides were treated as described previously [31], and above. To detect GXM in tissue sections 3.9 μg/ml of MAb 18B7 conjugated with Alexa Fluor 546 was incubated at 37°C for 1.5 hours. Unbound antibody was 10 minutes with PBS. Sections were then coated with N-propyl gallate to preserve fluorescence. To detect the F4/80 receptor, 2 μg /mlof MAb F4/80, Serotec (Raleigh, NC) was incubated for 1 hour at 37 ° C in a moist chamber. Sections were washed for 15 minutes with 1% BSA. For studies using the MAb 18B7, mice were injected with either 100 μg of GXM or 100 μg of FITC-Dextran immediately followed by 1.94 μg/ml of the MAb 18B7. Spleens were removed at 5, 12, and 24 hours, each time point was done in duplicate.

Dectin-1 and GXM

C57BL/6, 6–8 week old, female mice were obtained from the Jackson Laboratories were injected i.v. with 65 μg of MAb 2A11 immediately followed by an equal amount of GXM or FITC-Zymozan A from *S .cerevisiae*, Molecular Probes (Eugene, OR). The MAb 2A11 against Dectin-1 was a kind gift of Dr. Gordon Brown, University of Cape Town, South Africa [32]. Twenty-four hours later the spleens were removed and sectioned. Prior to GXM detection, deparrifinized slides were treated as described previously [31] and above. To detect GXM, immunohistochemistry was performed as mentioned above.

Flow Cytometry

C57BL/6 female mice were injected (i.v.) with 100 μg of GXM in PBS or PBS, 24 hours prior to sacrifice. Splenic macrophages were isolated by homogenization in DMEM supplemented with 10% FCS, 10% NTC-109, 1% nonessential amino acids and1% Penicillin-Streptomycin. Cells were spun at 1200 rpm for 5 minutes and red blood cells were lysed by resuspending 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. Cells were fixed with 3.7 % formaldehyde for 5 minutes at room temperature. After fixation, cells were permeabilized with 1% Triton X-100 for 3 minutes at room temperature. Cellular Fc receptors were blocked with mouse Ab to Fc (FcyIII/II anti mouse CD16/CD32 for 30 min in 5% milk with gentle rocking at room temperature. Approximately 5×10^7 cells were incubated for 30 minutes at room temperature with 2.0 ug/ml of MAbs F4/F80-APC (IgG2b) (Serotec, Raleigh, NC), biotinylated ERTR9 (IgM) (BMA Biomedicals, Augst Switzerland) and indirectly labeled with streptavidin anti-IgM-Texas red (Caltag Burlingame, CA). *C. neoformans* monoclonal antibody 2H1 conjugated to Alexafluor-488 or a secondary anti-IgG1- FITC was used to detect GXM. The F4/80 (IgG2b) MAb can recognize the F4/80 molecule in splenic red pulp macrophages [31,33]. Marginal zone macrophages expressing SIGN-R1 are specifically recognized by the MAb ERTR9 [21,34]. Cells were washed 3 times after antibody incubation with staining buffer. Samples were stored at 4°C in the dark until FACS is performed using Cell Quest. The percentage of gated cells represents a population of 10,000 events.

Statistical Analysis

Statistical analysis was done by the Kruskal Wallis test (Primer; McGraw Hill, New York, NY).

Results

GXM Localization in the Spleen

To determine the location of the marginal zone macrophages and confirm the transient inactivation of the SIGN-R1 receptor using 22D1, we administered FITC-labeled Dextran (i.v). to mice and compared spleen localization in mice given mAb 22D1 or controls (Fig 1a–c) [4, 21]. In normal mice, FITC labeled Dextran localized to marginal zone macrophages, and these cells appeared as rings around the white pulp (1a), when tissue sections are viewed with fluorescent microscopy. In mice injected with the MAb 22D1, the rings of marginal zone macrophages are no longer apparent by fluorescent microscopy because blocking the SIGN-R1 receptor eliminated Dextran uptake (1c). In contrast, GXM did not localize to the marginal zone twenty-four hours post GXM injection. GXM localized to the red pulp of the spleen in the presence and absence of mAb 22D1 (Fig 1d–f).

Given that GXM was not observed in the marginal zone at twenty four hours we evaluated earlier time intervals after (i.v.) infusion since it was possible that intra-splenic transport had occurred by 24 hours [1]. Consequently, we evaluated the location of GXM deposition in the

spleen at 1, 3, 5, 12, and 24 hours after (i.v.) injection. At all time intervals evaluated, GXM was deposited in the red pulp and no deposition was apparent in the marginal zone (Fig 2). As an additional control, we evaluated the tissue localization of FITC-Dextran in the spleen and noted marginal zone deposition at all times evaluated, data not shown.

The amount of GXM deposited in the liver and the spleen was measured by capture ELISA. The SIGN-R1 receptor was transiently blocked with the anti-SIGN-R1, MAb 22D1 and an IgG was used as a control. (Fig 3) [35]. The results reveal that no statistical difference was apparent between livers and spleens of mice injected with MAb 22D1 or the IgG control (p values for 22D1 and IgG were 0.480 and 0.724 for liver and spleen comparisons, respectively, by the Kruskal Wallis statistic).

Dectin-1 is not involved in the binding of GXM

The β-glucan receptor Dectin-1 is a major macrophage receptor for the nonopsonic recognition of yeast and yeast derivatives such as zymosan from *Candida albicans* [32,36]. SIGN-R1 is also involved in the recognition of the yeast-derived particle zymosan. We evaluated the possibility that the β-glucan receptor, Dectin-1 was involved in the binding of GXM since it has been found to be cooperatively involved with SIGN-R1 [36]. Immunofluorescence and immunohistochemistry on splenic sections showed that mAb 2A11 blocked the binding of FITC-zymozan but not GXM (data not shown). GXM localized to the red pulp macrophages in mice with and without blockage of Dectin-1.

GXM and Dextran do not Colocalize in macrophages *in vivo*

In the spleen, FITC-Dextran localized to the marginal zone while GXM was localized to the red pulp. Merged images revealed that each polysaccharide localized to different areas of the spleen (Fig 4 a–d). Additionally, we looked for GXM deposition in rats to investigate if species specific differences were observed. In the rat spleen, FITC-Dextran localized to marginal zone cells while GXM localized to the red pulp as in mice. However, we also noted that in rats GXM and Dextran appeared to be at closer proximities than in mice (Fig 4 e–g). Additionally, immunohistochemistry using the chromagen diaminobenzidine (DAB) revealed that GXM deposition is in the red pulp of the rat spleen however GXM is in closer proximity to the white pulp. This result is in contrast to mice where GXM deposits only in the red pulp (Figure 1d).

F4/80 and GXM Co-localize to the red pulp

Since red pulp macrophages express high levels of the F4/80 antigen we used this marker to more conclusively establish the association between red pulp macrophages and GXM uptake [19,37]. Immunofluorescence studies revealed that the signals from GXM as detected by 18B7-Alexa Fluor 546 and FITC-F4/80 co-localized in spleen (Fig 5). Flow cytommetry was done to quantify how many F4/80 cells bound GXM. The results reveal that only a modest 13% of the cells were F4/80 and GXM double positive when background was subtracted (Figure not shown).

Discussion

GXM is an immunomodulatory molecule that causes immunological paralysis in the spleen, down regulates proinflammatory cytokines and inhibits leukocyte migration [10,38]. For many years, studies have shown that shed GXM remains circulating in the blood after successful antifungal treatment [3,27]. Since GXM has also been found to remain for long periods in the host's organs such as the brain, liver and spleen, studies have attempted to elucidate the possible mechanisms by which macrophages use to sequester the polysaccharide antigen [24,39]. Our results showed that GXM localized to the spleen, is sequestered by the red pulp in both mice and rats. Since GXM did not localize to the marginal zone irrespective of the presence or

absence of mAb to SIGN-R1 we conclude that marginal zone macrophages are not involved in GXM sequestration or processing. In fact, GXM localization was consistently associated with red pulp macrophages. Further validation that GXM does not localize to marginal zone macrophages comes from our ELISA studies where mice immunized with 22D1 and an IgG control antibody reveal identical GXM concentration per organ mass.

These results are consistent with the observation that marginal zone macrophages (MZM) bind to neutral polysaccharides such as dextrans and pneumococcal polysaccharides, whereas red pulp acid residues that branch from the mannosyl backbone of GXM [40]. Our *in vivo* data suggest that there may be distinct receptors that recognize neutral (Dextrans) and acidic (GXM) polysaccharides.

Our results indicate that either SIGN-R1 is not the cellular receptor for GXM in mice or that there is redundancy in the types of cellular receptors that can interact with this polysaccharide such that cellular uptake does not require SIGN-R1. GXM is known to bind to other receptors such as CD18 and CD14 in neutrophils [41]. Other studies have also suggested that GXM can bind *in vitro* with TLR2 and TLR 4 [26,27]. It has also been shown that GXM can also induce the activation of Fas Ligand in macrophages that in turn induces the apoptosis in T-cells [12]. Currently, there are no studies that suggest that marginal zone macrophages express receptors that are associated with GXM uptake and this study validates that SIGN-R1 is dispensable.

We noted kinetic differences in the macrophage uptake of GXM and FITC-Dextran. GXM localized to the red pulp within 1 h of (i.v.) administration. In contrast, at 1 hour FITC-Dextran was only beginning to localize to the marginal zone. At later times, GXM localized to the red pulp and FITC-Dextran localizes to the marginal zone macrophages. This observation is modestly different than what is seen in rats. It has been reported that in rats GXM is localized primarily to macrophages of the outer marginal zone within 5 hours [1]. We investigated if there were any species specific differences in the localization of Dextran and GXM between mice and rats. In both species, Dextrans localized to the marginal zone and GXM to the red pulp. However, in rats GXM is in closer proximity to the MZ. We also investigated if the beta-glucan receptor Dectin-1 was a possible receptor for GXM since studies suggested that SIGN-R1 cooperates with Dectin-1 in the non-opsonic recognition of zymosan [36]. The results indicate that like SIGN-R1, Dectin-1 is not involved in GXM uptake.

Since the spleen contains a variety of different cell populations that can independently bind GXM and FITC-Dextran we used flow cytommetry to identify cell types that could potentially interact with GXM. The results using the F4/80 a red pulp macrophage marker showed that only a modest 12% of F4/80 macrophages are double positive with GXM. This result implies that GXM may be bound by more than one receptor in the red pulp.

In summary, we report that red pulp but not marginal zone macrophages are responsible for the uptake of the capsular polysaccharide component GXM of *C. neoformans* in a process whereby the SIGN-R1 is dispensable. In light of this evidence, uncovering the location of were GXM localizes in the spleen can allow for the further dissection at a molecular level of other receptor types that are affected in *C. neoformans* pathogenesis.

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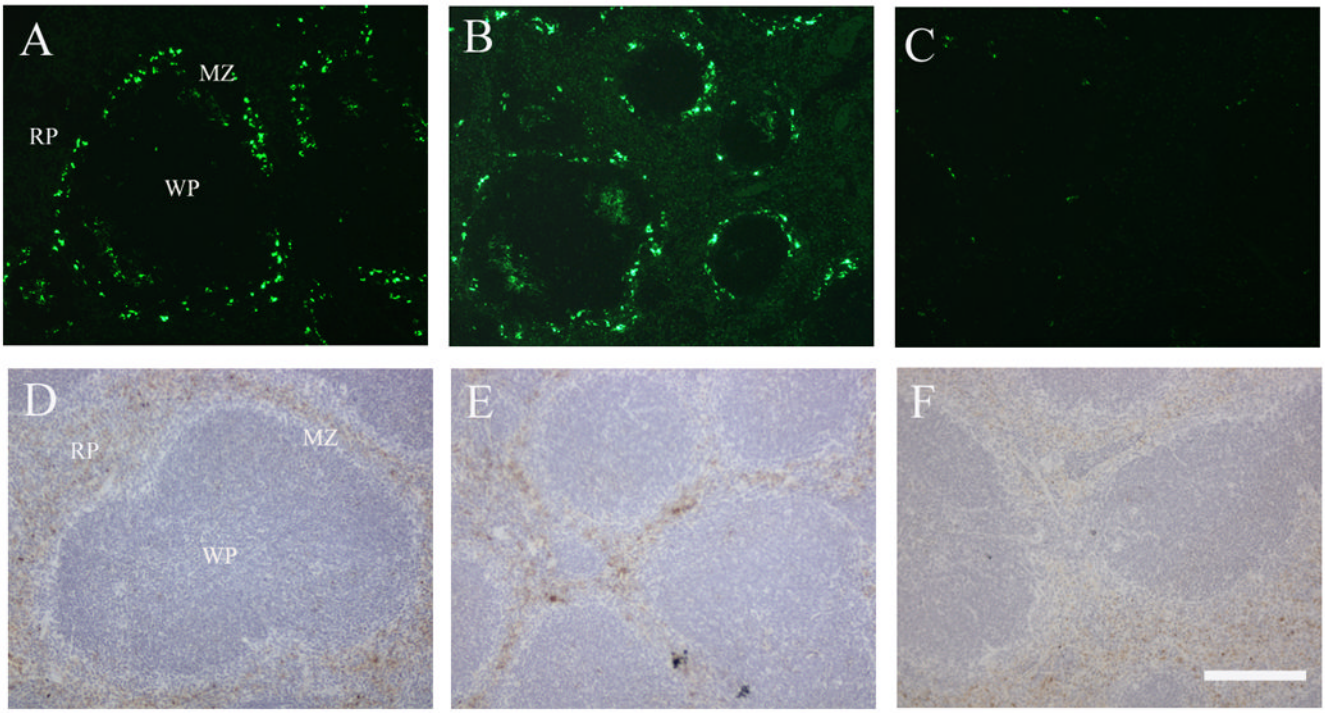


Figure 1. Immunofluorescence and immunohistochemistry of spleen tissue from mice injected with FITC-dextran or GXM in the presence and absence of mAb to SIGN-R1

Panels show immunofluorescence for splenic sections from mice injected with FITC-dextran alone (A), FITC-dextran and control mAb (B) and FITC-dextran and mAb 22D1 to SIGN-R1 (C). GXM in mouse spleen visualized by immunohistochemistry shows deposition (brown color) outside the marginal zone with (D) or without (E) SIGN-R1 blockage. Panel F shows GXM localization in the red pulp after injection with control IgG. Scale = 296 microns inner diameter of marginal zone.

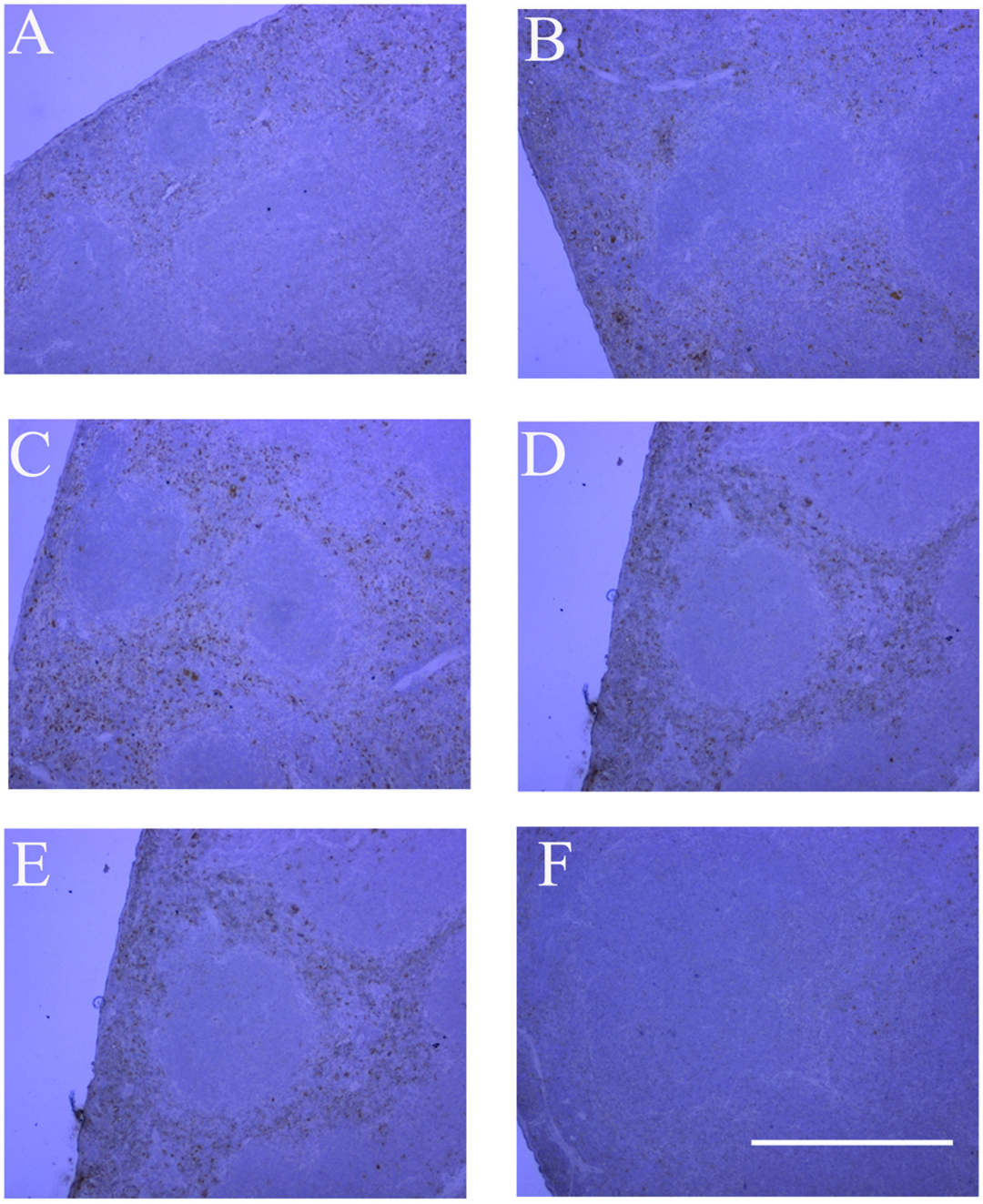
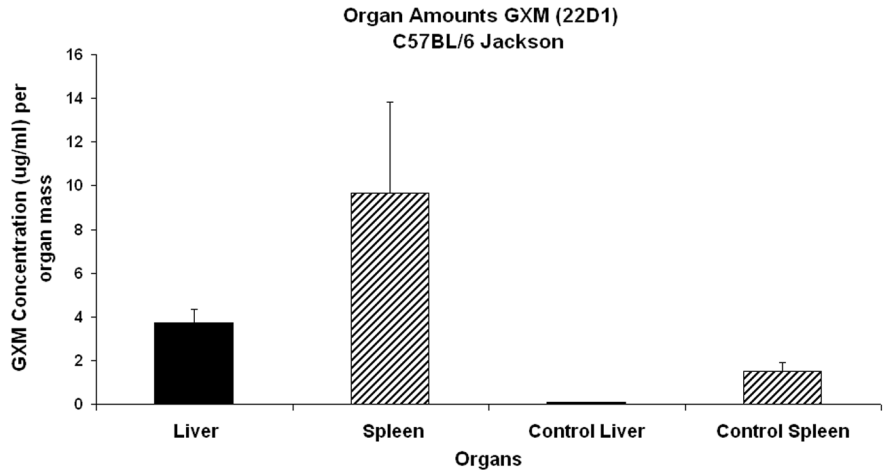


Figure 2. Localization of GXM at different times

Immunohistochemistry on paraffin sections containing GXM localization (brown color) after sacrificing the mice at 1hr (A) 3hrs (B) 5 hrs (C) 12 hrs (D) and 24 hrs (E). As a control mice injected with PBS (Phosphate Buffered Saline) is also shown (F). Scale = 108 microns from the outermost part of the marginal zone into the red pulp.

A



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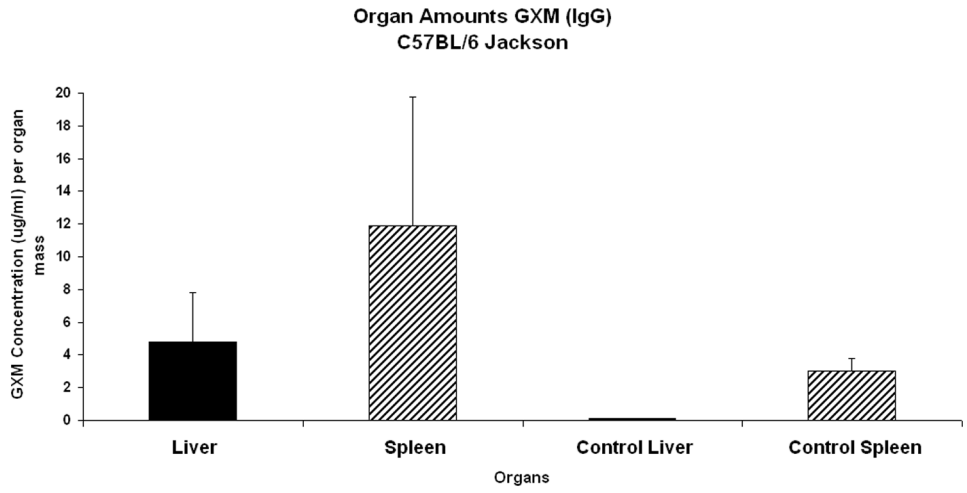

Figure 3. Organ GXM content as measured by capture ELISA

Figure 4A shows GXM deposition in liver (solid bars) and spleen (striated bars) in the presence of MAb 22D1. 4B shows GXM deposition in liver (solid bars) and spleen (striated bars) in the presence of the Armenian hamster MAb IgG (control). n = 4 per group. Control group received saline. Comparison of GXM concentration in liver (3.74 ± 0.61 ug/ml/g) and spleen (9.64 ± 4.2 ug/ml/g) homogenates from mice given mAb 22D1 revealed a significantly greater amount of GXM in the spleen when normalized by organ mass. GXM concentrations in mice that received the IgG control antibody were (4.81 ± 2.98 ug/ml/g in liver and (11.9 ± 7.84 ug/ml/g) in spleen. In mice receiving GXM alone, the results in the liver and spleen were (3.9±1.45 ug/ml/g) and (13.0 ± 4.8 ug/ml/g).

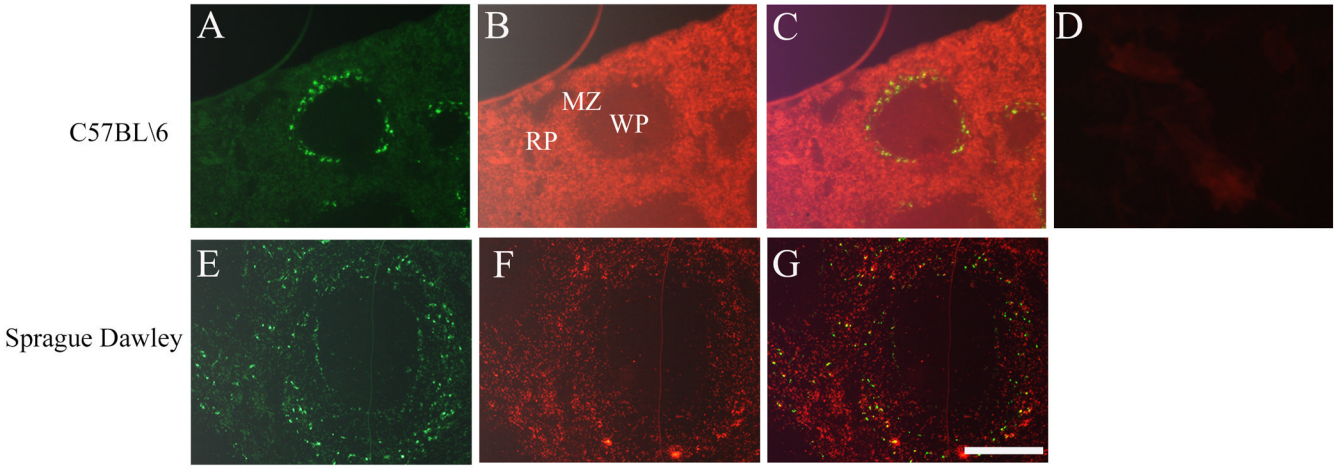

Figure 4. Dextran and GXM binding in splenic macrophages in mice and rats

Immunofluorescence of GXM and FITC-Dextran was observed in the spleen as follows: In C57BL/6 mice FITC-Dextran (A) and GXM detected with MAb 18B7 conjugated with Alexa Fluor 546 (B) in splenic tissue. Panel C shows merging of images from Panels A and B. Panel D is shown as a background control since splenic tissue can autofluoresce if not blocked. In Sprague Dawley rats FITC-Dextran (Panel E) and GXM detected with MAb 18B7 conjugated with Alexa Fluor 546 (Panel F) in splenic tissue. Panel G shows merging of images from Panels A and B. Scale bar = 69 microns.

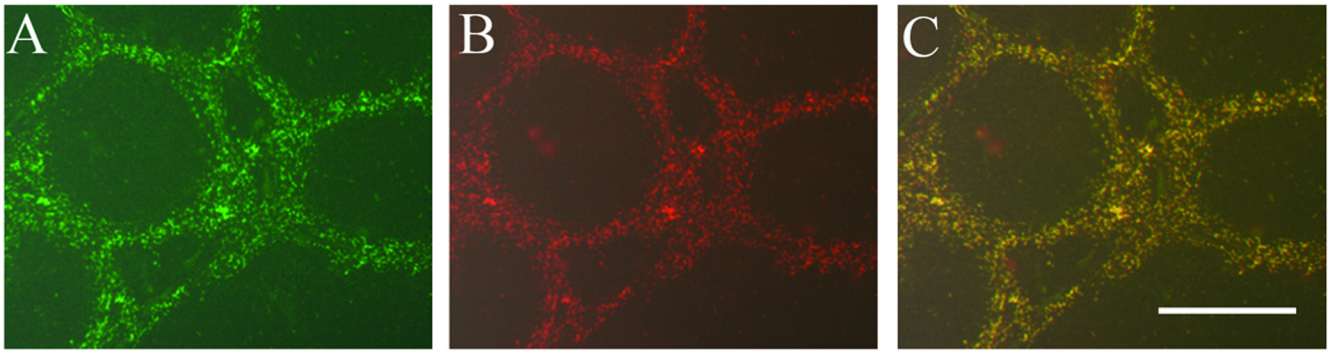

Figure 5. Immunofluorescence of spleen tissue after staining with the F4/80 macrophage marker and GXM

Panel A, the F4/80 cell surface marker is detected by anti-F4/80-FITC MAb (green). Panel B, GXM is detected by 18B7-Alexa Fluor 546 (red). Panel C, is the merged image. Scale = 110 microns.