Microbial polysaccharide: new insights for treating autoimmune diseases

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1. ABSTRACT

The immunosuppressive properties of Glucuronoxylomannnan (GXM), a purified capsular polysaccharide of the opportunistic fungus *Cryptococcus neoformans*, have been extensively elucidated. GXM can inhibit the function of cells belonging to both the innate and adaptive immune systems, leading to inhibition of proinflammatory responses as well as of autoimmune inflammation. This review focuses on the role of GXM as a novel anti-inflammatory agent and puts in perspective the direct implications of its potential destination to clinical trials.

2. INTRODUCTION

Cryptococcus neoformans is unique in that it is the only fungus pathogenic to humans endowed with an external polysaccharide capsule. In fact, all clinically relevant strains of *C. neoformans* have a capsule that can be considered an invariant requirement of all wildtype pathogenic strains. The primary function of this structure is related to survival in the environment, since it provides substantial protection against desiccation (1). Moreover, it is essential for the formation of the cryptoccocal biofilm, which is an assemblage of microbial cells enclosed in a
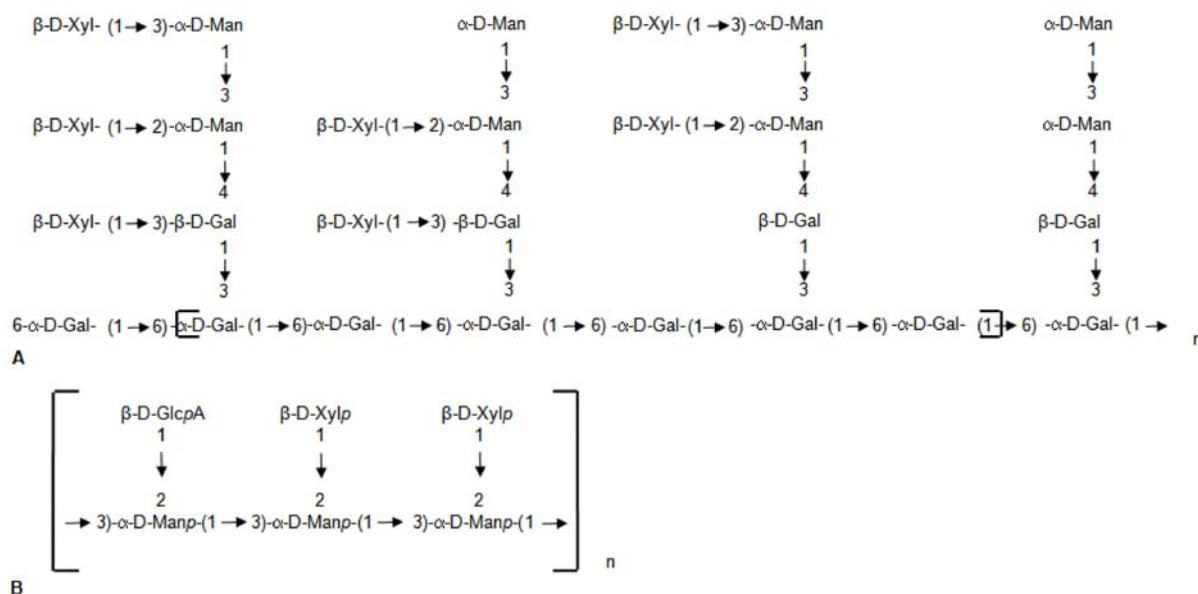
GXM as novel anti-inflammatory agent

Figure 1. Structure of *C. neoformans's* capsule polysaccharides. A) Chemical structure of GalXM B) Chemical structure of GXM.

matrix of primarily polysaccharidic material (2). The cryptococcal capsule is mainly composed of two different polysaccharides: Galactoxylomann (GalXM) and Glucuronoxylomannan (GXM). The chemical composition of both GXM and GalXM is reported in (Figure 1) (3, 4). GalXM, an alpha1-6 linked galactan containing different types of mannosyl and xylosyl substitutions, is a minor component of the capsular polysaccharidic material (5, 6), and it is shed during infection and is found in the body fluids of infected hosts (7). It is not covalently bound to the cell wall (8), and its association with other yeast-cell components remains unknown. GXM, on the other hand, is a high-molecular weight polysaccharide representing about 90% of the fungus' capsular material (6). The basic structural unit of GXM is a tri-mannose repeat with a glucuronic acid residue in the first mannose. This structure is further modified in individual strains by the addition of xylose substitutions on the mannose backbone which can be *O*-acetylated at the carbon 6 of some of mannosyl units (3). Cherniak and collaborators defined six triads known as M1-M2 that are found in various proportions in GXM from its various serotypes (9). M2 is the most common triad in serotype A GXM, the most common clinical isolate. Recently, Nakouzi *et al.* (10) have synthesized a heptasaccharide oligosaccharide representing M2, have conjugated it with human serum albumin (HAS), and came to the conclusion that the M2 motif elicits non-protective responses (10).

In contrast to capsular components of bacteria, GXM is synthesized in the cytoplasm (11-13) and transferred by the means of vesicles to the extracellular space (13, 14), where it is connected to the cell wall and used for distal capsular enlargement (15). Recent data point out that the method used for purifying GXM can significantly influence its structural and antigenic

properties, and consequently its impact on immune cells (16). Most studies, including ours, have been performed with GXM isolated through the classical protocol involving precipitation in supernatants with ethanol followed by selective precipitation by hexadecyltrimethyl ammonium bromide (17, 18). By using this protocol, Cherniak *et al.* have demonstrated that GXM was obtained in its pure form (18) Besides, the GXM used in our experiments was endotoxin-free to less than 003 endotoxin U/ml, as determined by the *Limulus* amebocyte lysate assay.

In addition to coating the surface of *C. neoformans*, GXM is shed from the fungus similarly to GalXM. In fact, just like experimental animals infected with *C. neoformans*, patients with cryptococcosis display high GXM levels in their serum and/or cerebrospinal fluid at concentrations well into the micrograms per milliliter range; also, it is likely that in infected tissues such as spleen and liver (19, 20) local concentrations of GXM in the milligram per milliliter range are achieved (21). While relatively little work has been done with GalXM, the biological effects of GXM have been extensively studied (22, 23): in fact, GXM generates a myriad of suppressive effects, affecting both innate and adaptive immune responses. Specifically, this polysaccharide can reduce MHC class II expression on antigen presenting cells (APC) (24, 25), inhibit activation and maturation of dendritic cells (26), reduce T cell proliferation in the presence of APC (27, 28), dampen Th1 response (28, 29), induce apoptosis of T cells in the presence of monocytes/macrophages via the Fas ligand (FasL)/Fas system (30), induce macrophage apoptosis via FasL (31), inhibit the production of proinflammatory cytokines by monocytes/macrophages (32), and induce IL-10 production by monocytes/macrophages (33, 34). At present, little is known about the GXM epitopes responsible for

immunosuppression; however, it is reported that GXM presents several distinct epitopes that can bind different types of protective and non-protective antibodies (35, 36). Moreover, it has been observed that *O*-acetylation and xylosilation of the (1→3)-alpha-D-mannan backbone of GXM contribute to the biological activities of this polysaccharide (17, 37-39). Indeed, it has been demonstrated that the 6-*O*-acetylated mannose of GXM is a crucial motive for the inhibition of neutrophil recruitment (40), and that the xylose plays a role in complement deposition on *cryptococci* and accumulation of GXM in the spleen (38, 41). Neither *O*-acetyl nor xylose appear to play a role in the inhibition of phagocytosis of GXM *in vitro* (38, 42). Recently, it has been demonstrated that a C-type lectin (Ha-lectin) has a wide sugar binding spectrum. It has two different carbohydrate recognition domains (CRDs): CRD1 and CRD2 arranged in tandem, which can bind different sugars including xylose (43). Consistently with this current literature, we can suppose that the xylose residues of GXM could be recognized by the CRD2 domain of a C-type lectin.

In this review, we focus on the effects of GXM on innate and T cell-mediated immune responses, describe the regulatory mechanisms involved in its immunosuppressive action and examine the potential immunotherapeutic use of this non-toxic compound.

3. GXM EFFECTS IN *IN VITRO* SYSTEMS

3.1. Negative regulation of monocytes/macrophages function

GXM is recognized, by several natural effector cells such as neutrophils (44), monocytes, macrophages and dendritic cells, but not by T cells (45). The capture and internalization of GXM is predominantly ascribed to monocytes/macrophages (19, 45, 46). These cells show long lasting intracellular loading of the polysaccharide, suggesting that they can function as reservoirs and potential vehicles for spreading this substance to tissues. GXM uptake by macrophages is mediated by multiple cellular receptors, including toll-like receptor TLR4, TLR2, CD14, CD18, and FcgammaRII (44, 47, 48). The prolonged accumulation of GXM inside macrophages is related to the prompt and continuous availability of several cellular receptors carrying GXM inside the cells. Indeed, GXM induces up-regulation of several cellular receptors in order to be bound and internalized. In particular, TLR4 and FcgammaRII were up-regulated early; in contrast, CD14 and CD18 were up-regulated late. Despite the engagement of multiple activating receptors such as TLR4, GXM transduces only suppressive effects (47). Convincing arguments point out that the engagement of TLR4 by microbial compounds triggers activation of NF-kB, leading to the induction of inflammatory and antimicrobial responses (49, 50). This contrasts with findings showing that GXM induced suppressive effects. Indeed, this phenomenon appears to be the direct consequence of the interaction of the polysaccharide with inhibitory immune receptors such as FcgammaRIIB (51). It is well known that multiple FcgammaRs are expressed on phagocytic cells. In particular, macrophages express three classes of

FcgammaRs: FcgammaRI, FcgammaRII (FcgammaRIIA and FcgammaRIIB) and FcgammaRIII. FcgammaRI, FcgammaRIIA and FcgammaRIII are activating receptors associated with immunoreceptor tyrosine-based activation motif (ITAM) (52, 53). Conversely, FcgammaRIIB is considered to be an inhibitory receptor acting via immunoreceptor tyrosine-based inhibitory motif (ITIM) present in its cytoplasmic tail (54) that activates the Src homology 2 domain-containing inositol phosphatase (SHIP) (55, 56). The binding of SHIP to FcgammaRIIB leads to potent inhibition of calcium mobilization and of mitogen-activated protein kinase activation by cleavage of phosphatidilinositol triphosphate (PIP_3) to phosphatidilinositol biphosphate (PIP_2) (57, 58). When both activating and inhibitory receptors are engaged by their ligands, the net outcome is determined by the relative strength of these opposing signals. Our results indicate that the activating signals transmitted by GXM via TLR4 are completely overcome by the suppressive effects following its uptake by the immune inhibitory receptor FcgammaRIIB via SHIP recruitment .It is remarkable that despite its interaction with TLR4, GXM is not able to induce activation of NF-kB (51), a transcription factor that plays a central role in activating inflammatory cytokines (49, 50). This is consistent with the incapacity of GXM to induce TNF-alpha secretion by macrophages. Nevertheless, we recently demonstrated that, by blocking the inhibitory receptor FcgammaRIIB, significant TNF-alpha secretion was observed following GXM stimulation of macrophages (51). As a consequence, one could posit that when FcgammaRIIB is unavailable or absent, the engagement of GXM by receptors such as TLR4 and CD18 could trigger activation signals. This result demonstrates that, bypassing FcgammaRIIB engagement, GXM could exert stimulatory activity, including TNF-alpha induction (Figure 2). Moreover, the addition of GXM to macrophages induces IL-10 secretion (33). It is remarkable that blockade of GXM-FcgammaRIIB interaction completely abrogated the release of IL-10, suggesting that uptake by this receptor is a central event in mediating the suppressive effects of GXM (51) (Figure 2).

Moreover, we demonstrated that engagement of FcgammaRIIB by GXM is also responsible for suppressing lipopolysaccharide (LPS)-induced TNF-alpha release (51). Indeed, when simultaneous stimulation with LPS and GXM occurs in macrophages, this results in potent inhibition of LPS-induced activation. Instead, treatment of macrophages with GXM produced a limited, though significant, downregulation of LPS-induced IL-10; this is in contrast with the dramatic reduction of LPS-induced TNF-alpha secretion, underlying the propensity of GXM for selectively dampening activating cytokines (51).

These data suggest that the down-regualtory activity of GXM is not due to an intrinsic immunosuppressive property of this polysaccharide, but rather to its capacity to engage potent immunoinhibitory receptors.

3.2. Negative regulation of T cell response

Though in our experimental system GXM is apparently unable to bind T cells, surprisingly its multiple immunosuppressive effects on T cell responses have been
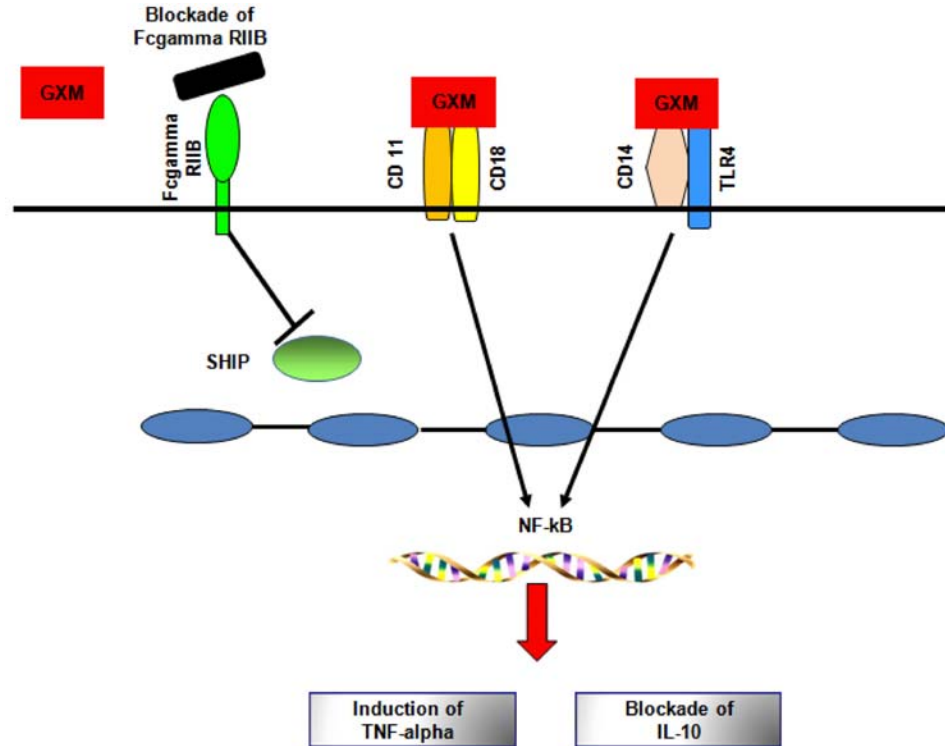
Figure 2. Role of GXM- FcγRIIB interaction on TNF-α and IL-10 production from macrophages. Blockade of FcγRIIB prevents SHIP recruitment. As a consequence, the engagement of GXM by receptors such as TLR4 and CD18 triggers activation of NF-kB, which culminates in the induction of TNF-α production. On the contrary, the blockade of GXM-FcγRIIB interaction completely abrogates the GXM-induced release of IL-10.

documented (59). GXM-mediated inhibition of T cell activation is an indirect effect: indeed, we have demonstrated that it derives from the negative impact of GXM on APC (47). In particular, innate immune cells like monocytes/macrophages, which take up and process GXM through cell-to-cell contact or via release of soluble factors, influence the adaptive response in different ways: i) by reducing T cell proliferation, ii) by dampening Th1 response, iii) by inhibiting DTH response, iv) by inducing T cell apoptosis.

The multiple suppressive effects of GXM on T cells response have been exhaustively elucidated (59). In this context, GXM-induced apoptosis is one of the most recently observed attributes of this polysaccharide (30). Apoptosis is a fundamental biological mechanism used by nearly all types of tissues and cells. It is essential to embryogenesis, tissue renewal, receptor repertoire selection and immune regulation. The occurrence of either exacerbated or deficient apoptosis is associated with disease. There are three major pathways of apoptosis-associated caspase activation: the mitochondrial/apoptosome pathway; the death receptor pathway; and the cytotoxic T lymphocyte/natural killer-derived granzyme B dependent pathway (60, 61). We demonstrated that GXM induces upregulation of the death receptor FasL in macrophages and this overexpression is prompt and long lasting, occurring exclusively in GXM-loaded macrophages. Additional studies indicated that de

novo protein synthesis was required for FasL over-expression (30). Macrophage apoptosis induced by GXM in a caspase-independent manner has been evidenced in a report by Chiapello *et al.* (62). The enhanced FasL expression in macrophages is instrumental in helping apoptosis of activated T cells via the FasL/Fas pathway: upon Fas engagement, caspase 8 is shortly activated (63). Caspase 8 is an essential component of the death receptor pathway, and upon its activation within the death-inducing signaling complex (DISC), the death signal is propagated by two alternative mechanisms (61). In general, in type I cells caspase 8, via the extrinsic pathway, directly activates effector caspases such as 3, 6 and 7, leading to apoptosis; in type II cells, the death signal has to be amplified by mitochondrial apoptosis, namely the intrinsic pathway (64). In these cells, caspase 8 activation induces the mitochondrial pathway of apoptosis with consequent caspase 9 cleavage. Caspase 9 activates further downstream caspases, and the end result is apoptosis (60).

We recently demonstrated that GXM-induced activation of caspase 8 was prompt, long lasting and still evident after 7 days of incubation. Subsequent caspase 3 cleavage following caspase 8 activation was demonstrated. In particular, apoptosis starts 1 day after GXM treatment. At this time, the death receptor pathway triggers a weak caspase 8 activation, which propagates the death signals through cleavage of effector caspases such as caspase 3. This activation loop is amplified by long-lasting and
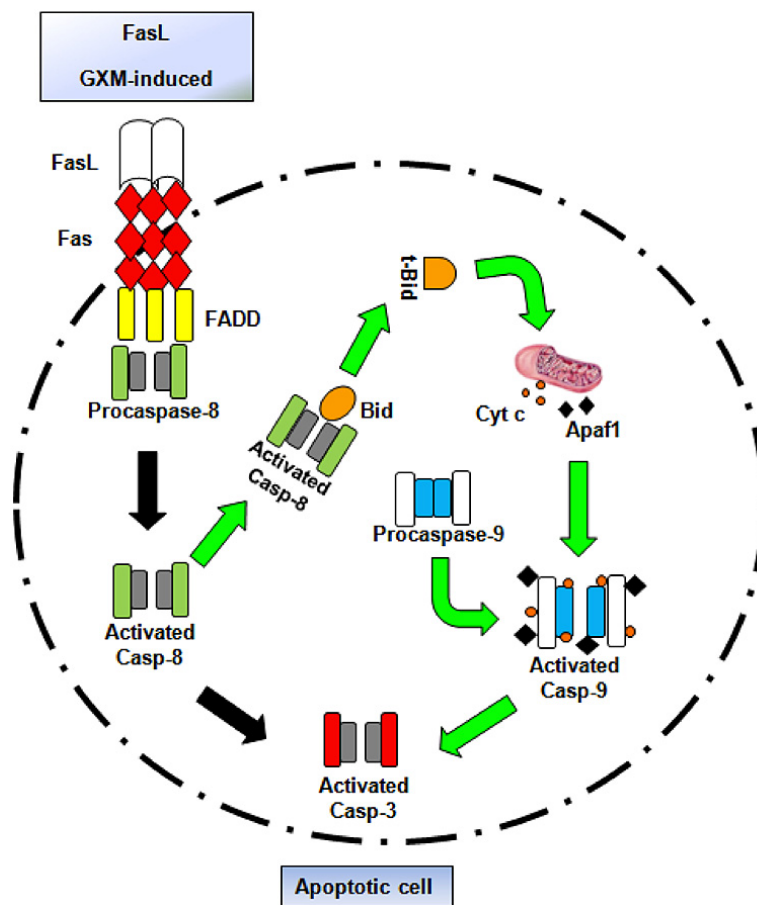
Figure 3. Schematic representation of GXM-induced apoptosis. The FasL (GXM induced)/Fas interaction produces caspase 8 activation, which propagates the death signals through cleavage of effector caspase 3. Caspase 3 activation occurs via two different pathways: directly and indirectly by involvement of the mitochondrial pathway with activation of caspase 9, which cleaves caspase 3 independently of caspase 8. Therefore, in a single cell GXM induces a cross-talk between intrinsic and extrinsic pathways.

continuous upregulation of FasL, which reinforces and prolongs the activation of caspase 8. This secondary (late) activation of caspase 8 generates a cross-talk between the extrinsic and intrinsic pathways in the same cell. Therefore, activation of caspase 9 reinforces GXM-mediated apoptosis, which reached its maximum 4 days post GXM treatment; subsequently, the amount of apoptotic cells diminished. However, apoptosis was still present one week after GXM treatment. Here we underline that extrinsic and intrinsic pathways cooperate in an amplification loop, and a new mechanism has been described showing that they both appear activated in one single cell (63) (Figure 3).

The capacity of GXM to induce apoptosis was also observed in an *in vivo* experimental model in which mice were infected with *C. albicans* and subsequently treated with GXM. This polysaccharide induced up-regulation of FasL expression on splenic macrophages and consequently apoptosis of murine T cell splenocytes; this was confirmed by the observed activation of caspase 8 in these cells (63).

4. GXM DAMPENS INFLAMMATORY RESPONSES IN *IN VIVO* EXPERIMENTAL SYSTEMS

4.1. Septic arthritis

Septic arthritis is one of the clinical manifestations in which the inflammatory response plays a critical role (65). The disease is mediated by Group B *Streptococcus* and usually affects neonates (66), however it is often also associated with aging and serious underlying diseases in adults (67, 68). Articular lesions in Group B *Streptococcus* (GBS)-infected mice are similar to those observed in human disease, making the mouse model excellent for studying GBS arthritis (69, 70).

As mentioned above, this type of arthritis is primarily mediated by an inflammatory response with critical involvement of TNF-alpha, IL-1 and IL-6 (69): in fact, these cytokines are known to contribute directly to articular damage. IL-1 beta, together with TNF-alpha, induces the release of tissue damaging enzymes from synovial cells and articular chondrocytes, and it activates osteoclasts (71, 72). IL-6 participates with IL-1 in the

catabolism of connective tissue components at inflammation sites (73, 74) and activates osteoclasts, resulting in joint destruction (75). In mice with arthritis induced by Group B *Streptococcus*, GXM treatment markedly decreased the incidence and severity of articular lesions, clearly providing a consistent therapeutic effect (76). The beneficial effect of GXM was confirmed by histopathological analysis. A fivefold reduction in the magnitude of articular lesions originally classified as severe was observed. In addition, in the joints of GXM-treated animals, a limited cellular influx of inflammatory cells was detected. This diminished cellular recruitment could be ascribed to the previously described capacity of GXM to inhibit the chemotactic process (77) and to suppress the expression of a chemotactic receptor such as C5aR on inflammatory cells (78). Clinical improvement was associated with a significant reduction in IL-6, IL-1beta, macrophage inflammatory protein (MIP) 1alpha, and MIP-2 production at the joint level and with IL-10 increase at the systemic level. It is noteworthy that the observed modification of local and systemic cytokine and chemokine production was not dependent on the number of microorganisms, since a similar bacterial load was detected in the blood, kidneys and joints in both GXM-treated and untreated mice. The beneficial effect of GXM is likely due to multiple suppressive effects, including reduction of macrophage activity, with consequent reduction in proinflammatory cytokine and chemokine production (76).

4.2. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic and debilitating systemic inflammatory disease, characterized by synovial hyperplasia and inflammatory cell recruitment, intra-articular fibrin deposition and, eventually, cartilage and bone destruction. The mechanisms involved in RA pathogenesis are complex and involve both innate and adaptive immunity. Cytokines such as IL-1beta, TNF-alpha, IL-6, TGF-beta, IL-17 are directly implicated in many of the immune processes that are associated with the pathogenesis of rheumatoid arthritis by enhancing production of cytokines, chemokines and degradative enzymes (79, 80). To date, it is clear that a good therapy for rheumatoid arthritis should block more cytokines, since distinct cytokines most likely mediate specific effects at different disease stages (80).

Given that GXM shows anti-inflammatory activity *in vitro* and GXM treatment induces therapeutic benefits in an experimental model of septic arthritis, the possibility that GXM may act as an anti-inflammatory agent in Collagen type II (CII)-induced arthritis (CIA), the most common and reliable animal model for rheumatoid arthritis (81), was explored. In this study, it was demonstrated that GXM treatment is able to suppress CIA progression, and clinical score reduction was noted soon after the first treatment dose with this polysaccharide. The reduction in arthritis severity was also documented by histopathological analysis: in fact, GXM consistently reduced alterations present at the joint level. Both cartilage and bone of untreated mice with CIA suffered significant erosion; conversely, in GXM-treated mice, both cartilage and bone were apparently spared considerable damage. In

addition, GXM induced a significant decrease in the anti-CII antibody response, thereby confirming and supporting the beneficial role of GXM in this model of autoimmune disease (82). The benefits obtained with GXM treatment were comparable and somehow even more efficacious than those obtained with the synthetic glucocorticoid Dexamethasone, often employed as a positive control in animal studies designed to evaluate new candidate drugs for chronic inflammatory diseases. It is very important to note that high doses of GXM (500 μg/ml) are non toxic to immune cells *in vitro,* and that these doses injected in mice do not alter hematological parameters *in vivo* (unpublished results). Moreover, a previous study demonstrated the high tolerability of GXM compared with some steroidal and non-steroidal anti-inflammatory drugs (83).

Our recent data showed that GXM treatment of arthritic mice with this polysaccharide produced down-regulation of TNF-alpha, IL-1beta, IL-6, TGF-beta, IL-17 and an increase in IL-10, which likely contributed to dampening the production of pro-inflammatory cytokines, particularly TNF-alpha. GXM treatment markedly reduced TNF-alpha production in joints as well as in their draining lymph nodes. This down-regulation was appreciable when both protein levels and mRNA copies were tested at these sites. The marked TNF-alpha depression that accompanied GXM treatment assumes particular relevance in the context of the key role played by this molecule in the pathogenesis of RA (80).

The GXM-induced reduction of IL-17 levels in joints and in their draining lymph nodes is a remarkable effect, given that many lines of evidence support the prominent role of T helper cells producing IL-17 in the pathogenesis of human RA (80, 84, 85). In humans, elevated serum levels of IL-17 have been detected in RA patients (86, 87), and elevated levels of IL-17 were measured in their synovial fluid. Also, in these patients, osteoclast formation was inhibited by anti-IL-17 antibody, suggesting an effect on bone resorption (88, 89).

Several groups have shown that T helper cells (Th17) differentiation in mice is potently induced by the simultaneous presence of TGF-beta and IL-6 (90, 91). In our experimental system, GXM suppressed these cytokines in joints early after the onset of treatment, however this effect was lost later. This result suggests that the inhibitory effect is tightly related to the relatively short term necessary for Th17 differentiation. Notably, the GXM-mediated suppression of IL-17 is long lasting, suggesting that it could be a consequence of its enduring effects on APC, due to its storage in these cells. We also studied the molecular mechanisms involved in the GXM-mediated inhibition of Th17 differentiation. In the mouse, the transcription factor needed to orchestrate differentiation of Th-17 cells is the retinoic acid-related orphan receptor (RORgammatau), the expression of which is induced by stimulation with TGF-beta and IL-6 and is directly related to the onset of autoimmunity (80, 92, 93). In order to test RORgammatau's involvement in the GXM-mediated down-regulation of IL-17, protein extracts from joints of untreated or GXM-treated mice were analyzed. Our results
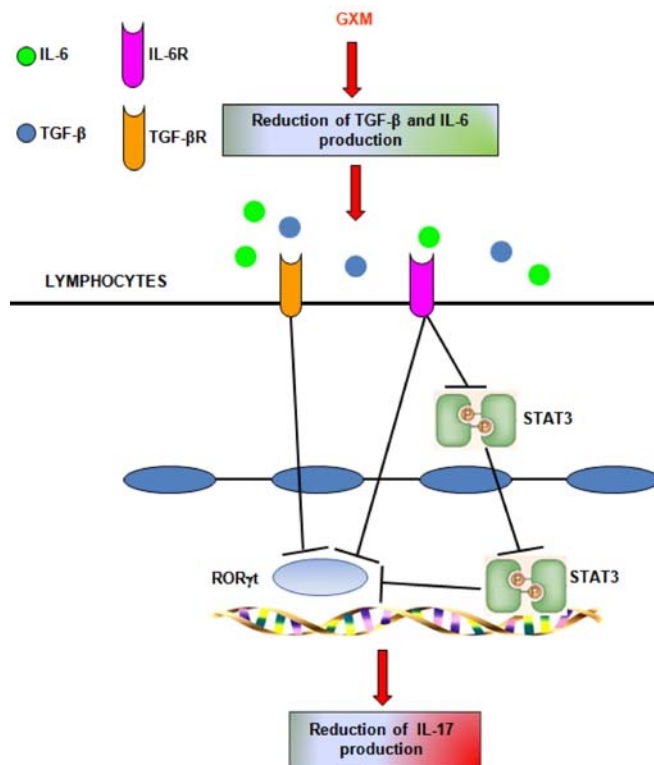
Figure 4. Schematic representation of GXM-induced Th17 suppression. GXM treatment induces a decreased production of TGF-β and IL-6 from macrophages. This results in abatement of STAT3 activation and RORγt synthesis, resulting in down-regulation of IL-17 secretion.

showed that GXM treatment produced significant RORgammatau synthesis suppression at the joint level. Recent studies have shown that levels of RORgammatau are significantly reduced in Signal Transducer and Activator of Transcription 3 (STAT3) deficient T cells, consistently with the significant down-regulation of IL-17 in these cells (94, 95). This suggests that STAT3 can regulate the expression of RORgammatau. Therefore, we tested whether GXM treatment could reduce STAT3 activation. Our recent data demonstrated that GXM reduced STAT3 activation at the joint level, suggesting that both RORgammatau and STAT3 are involved in the GXM-mediated reduction of IL-17 production (Figure 4).

5. PERSPECTIVE

GXM is a polysaccharide which suppresses pro-inflammatory cytokines and induces anti-inflammatory cytokines, inhibits APC function, and greatly decreases T cell response. These effects have been initially observed in an *in vitro* system, and they could be translated to an *in vivo* system as well. Indeed, GXM treatment induces apoptosis of T cells during microbial infection and produces a beneficial therapeutic effect on an autoimmune disease such as rheumatoid arthritis.

In our experimental system of CIA, a clear remission of clinical signs in GXM-treated mice was demonstrated (82). This outcome is due to inhibition of Th17 differentiation with consequent decreased IL-17 production at the local and systemic level. The effect of GXM on Th17 differentiation was related to reduction in STAT3 activation and inhibition of RORgammatau synthesis. The down-regulation of IL-17 production amplifies the spectrum of the immunosuppressive capacities of this polysaccharide. This effect is particularly relevant, given that Th17 cells occupy the center-stage in the pathogenesis of autoimmunity. Indeed, IL-17 emerges as a potential new target in human autoimmune diseases like multiple sclerosis (96, 97) psoriasis (98), rheumatoid arthritis (86, 87, 99) as well as in animals models of autoimmunity (100, 101). The advantages of a novel therapeutic modality employing GXM in the treatment of RA as an alternative to conventional therapies with standard drugs and biological agents such as cytokines or specific antibodies, are that GXM is readily produced in large amounts, it has extended pharmacokinetics, and it shows no overt signs of toxicity *in vivo*. In fact, the dose and timing of GXM administration used for this study seemed to be effective without producing side effects (76). In conclusion, the observation that GXM inhibits Th17 differentiation and reduces the production of several cytokines involved in inducing autoimmune T-cell activation together with potent amelioration of clinical signs and the absence of signs of toxicity opens new scenarios for the therapeutic usage of GXM in the treatment of autoimmune diseases.

6. ACKNOWLEDGMENTS

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