# Human monocyte scavenger receptors are pattern recognition receptors for (1→3)-β-D-glucans

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**Abstract: Glucans are cell wall constituents of fungi and bacteria that bind to pattern recognition receptors and modulate innate immunity, in part, by macrophage activation. We used surface plasmon resonance to examine the binding of glucans, differing in fine structure and charge density, to scavenger receptors on membranes isolated from human monocyte U937 cells. Experiments were performed at 25°C using a biosensor surface with immobilized acetylated low density lipoprotein (AcLDL). Inhibition of the binding by polyinosinic acid, but not polycytidylic acid, confirmed the interaction of scavenger receptors. Competition studies showed that there are at least two AcLDL binding sites on human U937 cells. Glucan phosphate interacts with all sites, and the CM-glucans and laminarin interact with a subset of sites. Polymer charge has a dramatic effect on the affinity of glucans with macrophage scavenger receptors. However, it is also clear that human monocyte scavenger receptors recognize the basic glucan structure independent of charge. *J. Leukoc. Biol.* 72: 140–146; 2002.**

***Key Words:*** *macrophage · acetylated LDL · binding · surface plasmon resonance*

## INTRODUCTION

An important distinction between innate and adaptive immunity includes the receptors that are responsible for immune recognition [1–4]. Adaptive immunity uses somatically generated receptors that recognize antigenic patterns to which the host has been previously exposed. In contrast, innate immunity relies on genetically predetermined pattern recognition receptors (PRRs) that recognize carbohydrates, lipids, and proteins that are unique to microorganisms and are not produced by the host [1–7]. These macromolecular structures, usually found in the cell wall, are referred to as pathogen-associated molecular patterns (PAMPs).

Glucans are (1→3)-β-D-linked polymers of glucose, which are found in the cell wall of fungi and certain bacteria [8–10]. Glucans are known to modulate innate immunity by binding to specific receptors on monocyte/macrophages, neutrophils, and natural killer (NK) cells [9–19]. Of greater significance, clinical studies have shown that glucans are released from fungal cell walls into the systemic circulation of patients with mycoses [20–23]. It has been postulated that glucans may be fungal recognition molecules (PAMPs) for the innate-immune system [9, 19, 24].

Evidence suggests that there are multiple glucan-binding sites on macrophages; however, the nature of the glucan receptors and which receptors are responsible for modulating innate host defenses are the subject of controversy. Ross and colleagues [12, 13] have shown that the type 3 complement receptor (CR3) binds glucans. Zimmerman et al. [17] have reported that lactosylceramide is a glucan-binding moiety on immunocytes. We [10, 19] and others [17, 25, 26] have reported the existence of non-CR3 glucan-binding sites on human monocyte/macrophages and human dermal fibroblasts [27]. The non-CR3 glucan receptors have been tentatively identified as Dectin-1 [26]. Dushkin et al. [28] and Vereschagin and colleagues [29] have reported that a water-soluble, polyanionic, carboxymethylated (CM) glucan binds to mouse peritoneal macrophages via scavenger receptors (SRs) [28, 29]. Vereschagin et al. [29] extended this observation by demonstrating that glucan interaction with macrophage SRs protects against endotoxic shock. This suggests that macrophage SRs may be involved in glucan recognition and signaling. In support of this concept, Pearson et al. [30] have shown that the *Drosophila* CI, (class C) SR can bind laminarin, a low molecular weight glucan, and other microbial cell wall components.

The acetylated low density lipoprotein (AcLDL) receptor (SR) is responsible for the uptake of modified LDL and accumulation of cholesteryl esters in macrophages [31–34]. This receptor also binds negatively charged, high molecular weight polysaccharides, including dextran sulfate, carrageenan, lipopolysaccharide (LPS), lipoteichoic acid, and polyphosphates [31, 35]. SRs have also been shown to bind bacteria [36]. Several recent studies have speculated that SRs may function

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as PRRs for the innate immune response [3–7, 36, 37]. Consequently, there is a growing awareness and interest in the role of SRs in innate immunity [3–7, 36, 37].

Although Dushkin et al. [28], Vereschagin et al. [29], and Pearson and co-workers [30] reported that glucans are recognized by SRs, several important questions remain to be answered. First, these studies examined binding in a non-human system and did not establish if human SRs recognize and bind glucans. Second, because only charged CM-glucan was used, these studies could not determine whether the SRs recognize and bind glucans or whether the interaction they observed was solely because of polymer charge. Third, several compounds tested by Dushkin et al. [28] and Vereschagin and co-workers [29] were not water soluble and so could not be used to critically evaluate binding interactions. Thus, several important issues remain to be determined. In this study, we compared and contrasted the binding of several glucan and non-glucan polymers to scavenger receptors on a human monocyte/macrophage cell. The polymers differed in structure, purity, degree of branching, molecular weight, the presence or absence of anionic charge, and polymer charge density. Our results show that charge and structure contribute to the interaction of glucans with SRs on human promonocytic cells.

## MATERIALS AND METHODS

### Carbohydrate polymers used as competitor ligands

Ligands used in this study are listed in **Table 1**. Water-soluble (1→3)-β-D-glucan phosphate was prepared in our laboratory as described previously [38]. We evaluated two CM-glucan preparations. One CM-glucan was provided by Dr. Grigorij Kogan, Institute of Chemistry, Slovak Academy of Sciences (Bratislava) [39]. This is the same CM-glucan that was used by Dushkin et al. [28] and Vereschagin and co-workers [29]. A second CM-glucan was a gift from Mibelle AG Biochemistry (Switzerland). [For future reference, the CM-glucans will be described as CM-glucan (Slovak) and CM-glucan (Mibelle).] The degree of CM was comparable for the two glucans: 0.78 and 0.75, respectively [40]. CM-cellulose was prepared and characterized by Dr. Grigorij Kogan. It was used as a (1–4)-β-linked glucose polymer control. Schizophyllan, a branched, neutral glucan was obtained from Ajinomoto Corp. (Tokyo). Laminarin, a low molecular weight, neutral glucan polymer was obtained from Sigma Chemical Co. (St. Louis, MO). Each of the polymers was characterized according to methods previously described by our laboratories [41–45]. Each of the polymers was filter sterilized (0.45 μm) before use. We only evaluated water-soluble polymers. Dissolution of the CM-derivatized polymers in aqueous buffer resulted in a small amount of colloidal material. In each case, the colloid was removed by filtration prior to the binding studies.

### Determination of carbohydrate polymer charge

Carbohydrates were dissolved (1 mg/ml) in 18 Mohm water and brought to pH 4.0 by addition of 10 mM HCl. Titration curves were produced by addition of 10 mM NaOH to each carbohydrate solution and measurement of pH. The amount of NaOH added was normalized to the concentration of carbohydrates and expressed as the number of moles of NaOH required per carbohydrate subunit (glucose) to adjust the carbohydrate solution to 7.4 pH. This relationship was used to establish the charge present on each polymer. Pullulan and dextran were used for charge comparison and were not used in competition studies.

### Isolation and chemical modification of human LDL

LDL were isolated from human ethylenediaminetetraacetate (EDTA) plasma at a density of 1.006–1.063 g/ml by preparative ultracentrifugation as described by Kelley et al. [46]. Isolated LDL was dialyzed overnight at 4°C against phosphate-buffered saline (PBS), pH 7.4, to remove EDTA and KBr added during the isolation procedure. AcLDL was prepared from native LDL by acetylation with acetic anhydride as described by Brown et al. [47]. AcLDL exhibited increased electrophoretic mobility as measured by agarose electrophoresis compared with native LDL.

### Cell culture

U937 monocyte-like cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 media (Mediatech, Washington, DC) containing 9% heat-inactivated calf serum, 1% heat-inactivated fetal calf serum (HyClone, Corp., Logan, UT), and 0.2% (v/v) penicillin-streptomycin (Sigma Chemical Co.) at 37°C and 5% $CO_2$ and 95% humidity.

### Isolation of U937 membranes

Cells were harvested during the logarithmic phase of growth, centrifuged at 2000 rpm for 10 min, counted, centrifuged again at 2000 rpm (10 min), and frozen at −80°C. Cells were thawed in PBS solution in the presence of 10 μl protease-inhibitor cocktail (Sigma P-8340) per $10^6$ cells. The solution was maintained at 4°C and sonicated at 35% power for 30 s (3×; Sonic Dismembrator, Fisher Scientific, Atlanta, GA). The samples were centrifuged at 650 g for 10 min at 4°C to spin out nuclei. The pellet was resuspended, sonicated, and centrifuged. The combined supernatants were centrifuged at 68,000 rpm for 30 min at 4°C in a Beckman Type 80 rotor. The pellet containing U937 membranes was suspended in Hanks' balanced salt solution at a concentration of 1 mg/ml. Aliquots of the membranes were stored in liquid nitrogen for later use.

### Surface plasmon resonance (SPR) studies

Binding assays were performed using a BIACORE 2000 (Biacore, Piscataway, NJ) SPR instrument. Samples were maintained at 4°C using an ISOTEMP circulating bath (Fisher Scientific).

TABLE 1. Monosaccharide Composition, Predominant Intrachain Glycosidic Linkages, Molecular Weight Distribution, and Degree of Substitution of Polysaccharides Evaluated as Competitors of U937 Membrane Binding to AcLDL

| Carbohydrate | Monosaccharide | Type of linkage | $M_w$[a] | Degree of substitution[b] |
|---|---|---|---|---|
| Glucan phosphate | Glucose | β-(1–3) | 156,000 | 0.10–0.14 |
| CM glucan (Mibelle) | Glucose | β-(1–3), (1–6)[c] | 356,000 | 0.75 |
| CM glucan (Slovak) | Glucose | β-(1–3), (1–6)[c] | 100,000 | 0.78 |
| CM cellulose | Glucose | β-(1–4) | 456,000 | 0.76 |
| Schizophyllan | Glucose | β-(1–3), (1–6)[c] | 306,000 | Neutral[d] |
| Laminarin | Glucose | β-(1–3), (1–6)[e] | 7700 | Neutral[d] |

[a] Data are expressed as g/mol. [b] Indicates the degree of substitution of phosphate or CM groups. Larger numbers indicate greater charge density. [c] Indicates the presence of (1–6)-β glycosidic side-chain branches. Degree of branching 1:3. [d] The polymer is not charged. [e] Indicates the presence of (1–6)-β glycosidic side-chain branches. Degree of branching 1:10.

## Immobilization of AcLDL to biosensor chip

Experiments were performed at 25°C using a running buffer containing 150 mM NaCl and 10 mM HEPES. AcLDL was immobilized on a BIACORE L1 sensor chip derivatized with C16–C18 alkyl moieties at a flow rate of 10 μl per min. The sensor surface was first activated by sequential 60-s exposures to octyl glucoside (40 mM) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (20 mM). The surface was exposed to 0.3 mg/ml AcLDL (2×20 min). Following immobilization of AcLDL, the flow rate was increased to 20 μl/min, and the surface was stabilized by exposure to 50 mM NaOH. We performed saturation studies by exposing the AcLDL surface to varying protein concentrations. Competition studies were performed by alternately exposing the surface to a fixed concentration of U937 membranes (10 μg/mL) in the absence and presence of competitors. The concentrations that were studied were limited to those at which solubility or viscosity of the carbohydrates would not affect receptor interactions. Samples were incubated for at least 30 min (4°C) and passed over the AcLDL surface at 20 μl/min for 150 s with intervening dissociation periods of at least 30 min. Separate experiments on a BIACORE CM5 carboxymethyl dextran surface without attached alkyl moieties or AcLDL were used to assess the nonspecific binding. Results are expressed as the mean ± SEM of more than four replicate experiments using three to four flow cells.

## Immobilization of glucan phosphate to biosensor chip

Experiments were performed at 37°C using a running buffer containing 150 mM NaCl, 10 mM HEPES, 3 mM EDTA, and 0.005% polysorbate 20 (Biacore). Glucan phosphate was immobilized on three flow cells of a BIACORE CM5 sensor chip derivatized by exposure to N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysuccinimide as demonstrated by Kougias et al. [27]. The fourth flow cell served as a control surface without attached glucan phosphate. Competition studies using the immobilized glucan-phosphate surface were performed at a flow rate of 20 μl/min. Samples were passed over the glucan surface for 300 s and were regenerated by sequential 60-s exposures to 0.3% Triton X-100 and 3M guanidine HCl. Results are expressed as the mean ± SEM of at least four replicate experiments using three flow cells.

## Polyinosinic and polycytidylic acid competition

Brown and colleagues [48] showed that polyinosinic, but not polycytidylic acid, will competitively inhibit SR interactions with AcLDL. To confirm that we were studying SR binding, we performed competition studies with polyinosinic and polycytidylic acid. Polyinosinic and polycytidylic acid were purchased from Sigma Chemical Co. Samples containing a fixed concentration of U937 membranes (10 μg/ml) in the absence and presence of competitor were injected alternately. U937 membranes were mixed with competing polyinosinic acid or polycytidylic acid for at least 1 h prior to injection on the BIACORE instrument.

## Analysis of binding data

Data were normalized to the baseline established at the start of the experiment and analyzed by unweighted, nonlinear regression using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). We found that the bulk shift occurred over about 15 s and therefore estimated the amount of membrane protein bound to the surface by measuring the increase in resonance units (RUs) at 30 s after changing from membrane to buffer exposure. For competition experiments in which the immobilized surface was exposed to a fixed concentration of membrane protein in the absence or presence of competitor, RU values for each competitor concentration were adjusted for nonspecific binding of the carbohydrate and normalized to binding in the absence of competitor (100%). Results were analyzed using models for competitive displacement at a single binding site, a single binding site plus nonspecific binding, and/or a two binding site model. The best model was chosen statistically using the sequential F-test.

# RESULTS

## Charge density of the carbohydrate polymers used as competitor ligands

Using a titration method, we found that the polymers varied in charge. As expected, CM-glucan and CM-cellulose were the most charged with one charge per 2.5–3 glucose subunits. Glucan phosphate (1 charge/13 glucose), schizophyllan (1 charge/22 glucose), and dextran sulfate (1 charge/24 glucose) were charged intermediately. Laminarin (<1 charge/40 glucose) and pullulan (<1 charge/46 glucose) were essentially uncharged, as was dextrose.

## Saturable binding of U937 membranes to immobilized AcLDL surface

Binding U937 membranes to an immobilized AcLDL surface was saturable (**Fig. 1**) and indicated a single binding site with a $B_{MAX}$ of 770 ± 43 RU (pg/mm$^2$) and a dissociation constant ($K_d$) of 91 μg protein/mL (95% CI, 70–110 μg protein/mL).

## Polyinosinic acid, but not polycytidylic acid, inhibits binding of U937 membranes to AcLDL

Brown et al. [48] reported that poly I, but not poly C, inhibits the binding of SRs to AcLDL competitively. To confirm that we were examining monocyte SRs, we performed competition studies with polyinosinic or polycytidylic acid. Polyinosinic acid inhibited binding of U937 membranes completely to the AcLDL surface with characteristics of a single binding site (**Fig. 2**) and a $K_d$ of 0.35 μg/mL (95% CI, 0.22–0.63 μg/mL). Polycytidylic acid (10 μg/mL) produced no inhibition (98±3%). These results are quantitatively similar to those observed by Brown et al. [48] using $^{125}$I-AcLDL in an in vivo macrophage cell culture model and confirm the ability of SPR technology to measure binding interactions between AcLDL and SRs.

## CM-glucans partially inhibited the binding of U937 membranes to AcLDL at high affinity with characteristics of a single binding site

Both CM-glucans partially inhibited binding of U937 membranes to immobilized AcLDL with characteristics of a single binding site (**Fig. 3**). The $K_d$ for CM-glucan (Slovak) was 22 nM (95% CI, 4.9–99 nM; **Table 2**). The CM-glucan (Slovak) competed for 67 ± 8% of binding. The $K_d$ for CM-glucan

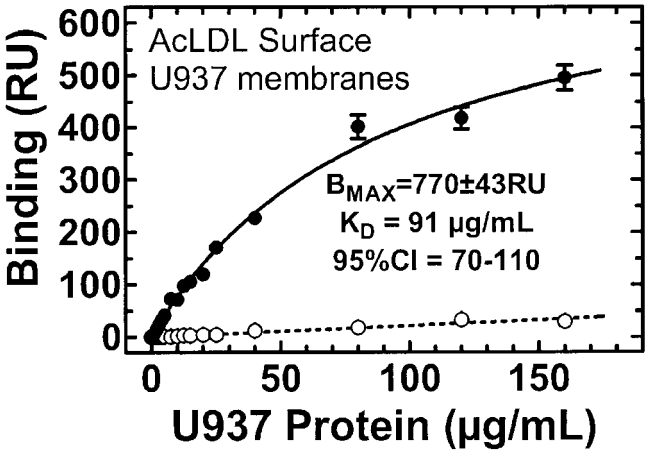


**Fig. 1.** U937 membrane interactions with immobilized AcLDL (●, solid line) are characteristic of a single binding site with a with a $B_{MAX}$ of 770 ± 43 RU (pg/mm$^2$) and a $K_d$ of 91 μg protein/mL (95% CI, 70–110 μg protein/mL). Nonspecific binding to a dextran surface is also shown (○, dashed line). The data represent eight replicates measured in two experiments.

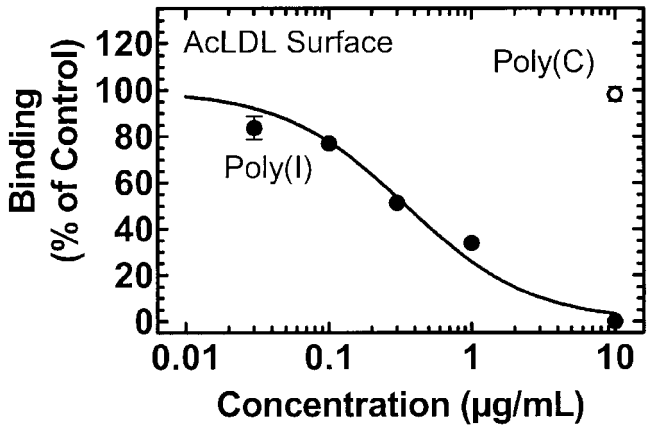


**Fig. 2.** Polyinosinic acid but not polycytidylic acid inhibited the binding of human U937 membranes to an AcLDL biosensor surface. Inhibition by polyinosinic acid (●, solid line; $K_d$=0.35 μg/mL) but not polycytidylic acid (○; $K_d$>>10 μg/mL) confirms the interaction of immobilized AcLDL with SRs ($n$=4–5/data point).

(Mibelle) was 14 nM (95% CI, 8–26 nM), and it competed for 58 ± 3% of binding (Table 2). There was no statistically significant difference between the $K_d$ values or degree of competitive inhibition for the two CM-glucans.

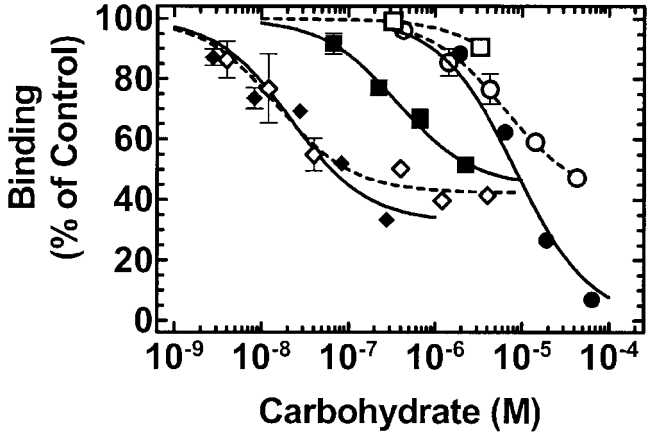


**Fig. 3.** Carbohydrate interactions are dependent on charge and structure. CM-glucans (◆, ◇) partially inhibit the binding of human U937 membranes to immobilized AcLDL. The CM-glucan ligands bound at high affinity ($K_d$=14–22 nM) with characteristics of a single binding site and inhibited binding to the immobilized AcLDL surface by 58–67%, suggesting multiple AcLDL binding sites on human U937 cells. The CM ligands only inhibit binding to a single site. CM-cellulose (■) was used as a CM control polymer. CM-cellulose partially inhibited (56%) binding of U937 membranes to immobilized AcLDL but at lower affinity ($K_d$=360 nM) than did either of the CM-glucans. The overall anionic charge was comparable for the three CM ligands. Glucan phosphate (●) completely inhibited U937 membrane binding to AcLDL but at much lower affinity than did the CM-glucans. The fact that glucan phosphate completely inhibited U937 membrane binding to immobilized AcLDL suggests that this ligand interacts with all available human monocyte SRs. Glucan phosphate has a much lower charge density than CM-glucans and interacts with the U937 SR at lower affinity, suggesting that charge is an important determinant in SR recognition of glucan polymers. Laminarin (○) partially inhibited the binding of U937 membranes to immobilized AcLDL. Coincubation of U937 membranes with increasing concentrations of laminarin inhibited interaction with immobilized AcLDL by 59 ± 4% at a $K_d$ of 6.2 μM. This suggests that laminarin is recognized at low affinity by a subset of human monocyte SRs. Schizophyllan (□), a biologically active neutral glucan polymer, did not compete effectively with AcLDL for binding of U937 membranes ($n$ = 4–5/data point).

TABLE 2. Dissociation Constants of Carbohydrates Used as Competitors of U937 Binding to AcLDL Surface

| Carbohydrate | $K_d$ | 95% Confidence interval | $B_{max}$ (%)[a] |
|---|---|---|---|
| Glucan phosphate | 8.4 μM | 4.0–18 μM | 100/0 |
| CM glucan (Mibelle) | 14 nM | 8.0–26 nM | 58/42 ± 3 |
| CM glucan (Slovak) | 22 nM | 4.9–99 nM | 67/33 ± 8 |
| CM cellulose | 360 nM | 160–870 nM | 56/44 ± 3 |
| Schizophyllan | >10 μM | — | — |
| Laminarin | 6.2 μM | 4.0–9.9 μM | 59/41 ± 4 |

[a] Data are expressed as the % displaced/% remaining.

## CM-cellulose partially inhibited binding of U937 membranes to immobilized AcLDL with characteristics of a single binding site

CM-cellulose partially inhibited the binding of U937 membranes to immobilized AcLDL (Fig. 3). The $K_d$ for CM-cellulose was 360 nM (95% CI, 160–870 nM; Table 2). CM-cellulose inhibited 56 ± 3% of binding (Fig. 3; Table 2).

## Glucan phosphate completely inhibited the binding of U937 membranes to AcLDL with characteristics of a single binding site

Glucan phosphate inhibited binding of U937 membranes to immobilized AcLDL completely (Fig. 3). The $K_d$ for glucan phosphate was 8.4 μM (95% CI, 4–18 μM; Table 2). Glucan phosphate inhibited 100% of U937 membrane binding (Table 2).

## Laminarin partially inhibited binding of U937 membranes to AcLDL, but schizophyllan did not interact with AcLDL binding sites

Pearson et al. [30] have reported that laminarin is recognized by the *Drosophila* CI SR. We show that laminarin is recognized by a subset of glucan receptors on the human U937 cell line [19]. In the present study, we observed that laminarin inhibits the binding of U937 membranes to immobilized AcLDL ($K_d$=6.2 μM, 95% CI, 4–9.9 μM) with characteristics of a single binding site (Fig. 3). However, laminarin only competed for 59 ± 4% of total binding (Table 2). In contrast, SPG, a highly branched, high molecular weight, neutral glucan polymer did not effectively compete with AcLDL for binding of U937 membranes ($K_d$>10 μM; Fig. 3; Table 2).

## Polyinosinic acid, but not polycytidylic acid, inhibits binding of U937 membranes to immobilized glucan phosphate

Inhibition of U937 interactions at an AcLDL surface by polyinosinic acid and by glucan phosphate suggested that glucan phosphate interacts with SRs. To further assess the contribution of SRs to glucan receptor binding in U937 membranes, polyinosinic acid and polycytidylic acid were used to inhibit U937 interactions at an immobilized glucan-phosphate surface. Polyinosinic acid significantly inhibited the interaction of U937 membranes (**Fig. 4**), with an affinity similar to that seen

at an AcLDL surface, but with a maximum inhibition of 76 ± 4%. This result supports SRs as a significant fraction of glucan interactions in U937 membranes, and the presence of a fraction of receptors insensitive to polyinosinic acid supports our earlier findings of multiple glucan receptor types in U937 membranes.

### Competition by laminarin and CM-glucan is not additive

To examine whether highly charged carbohydrates and uncharged carbohydrates interact at the same binding site, competition studies were performed using U937 in the absence or presence of laminarin (0.3 mg/mL), CM-glucan (0.1 mg/mL), or laminarin and CM-glucan (**Fig. 5**). In these experiments, competition by laminarin alone resulted in a 75 ± 6% decrease in binding to an immobilized AcLDL surface, and competition by CM-glucan alone resulted in a 53 ± 6% decrease in binding. The combination of the two carbohydrates resulted in a similar (63±8%) decrease in binding. These results indicate that CM-glucan and laminarin compete for the same binding site.

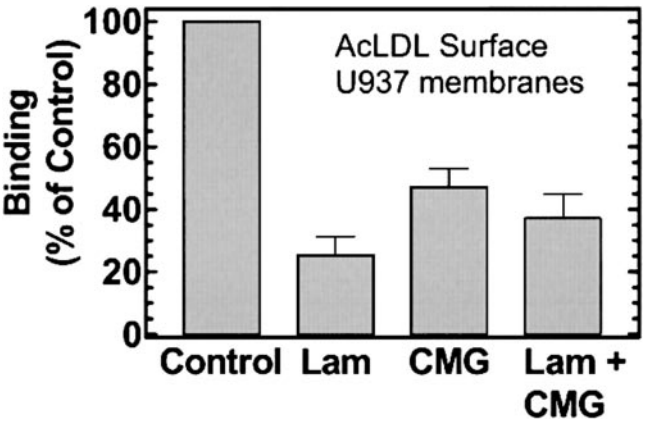


**Fig. 5.** Inhibition by CM-glucan and laminarin is not additive. To examine whether CM-glucan (CMG) and laminarin (Lam) interact at the same binding site, competition studies were performed using U937 in the absence or presence of laminarin (0.3 mg/mL), CM-glucan (0.1 mg/mL), or laminarin and CM-glucan. Similar competition was seen in the presence of both carbohydrates, indicating that CM-glucan and laminarin compete for the same binding site.

## DISCUSSION

Identifying the receptors responsible for recognition and response to pathogens is essential to understanding the innate immune system. Ideally, PRRs would exist on a variety of cell types to intercept pathogens throughout the body and would recognize PAMPs that are present in bacteria and fungi, but not present in higher species. SRs are present on the surface of a variety of cell types and although typically associated with cholesterol metabolism, also recognize and respond to pathogens. Human monocyte/macrophage SRs recognize a variety of PAMPs including LPS and lipoteichoic acid as well as DNA and RNA.

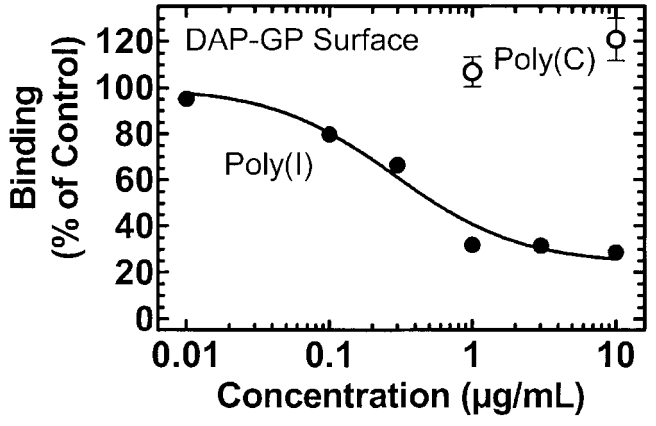


**Fig. 4.** Polyinosinic acid (●) but not polycytidylic acid (○) inhibited the binding of human U937 membranes to an immobilized glucan-phosphate biosensor surface. Polycytidylic acid appears to bind to a site other than the glucan receptor, which increases the apparent membrane mass and gives an SPR increase above control binding. Inhibition by polyinosinic acid but not polycytidylic acid confirms an interaction with SRs, and the presence of polyinosinic acid-insensitive binding supports a subset of non-SR glucan-binding sites ($n$=4–5/data point).

Glucans are (1→3)-β-D-linked polymers of glucose present in the cell walls of fungi and certain bacteria and are released into the systemic circulation during systemic mycoses. Glucans modulate innate immunity through specific receptor interactions on monocyte/macrophages, neutrophils, and NK cells [9–19]. Multiple receptors in immune competent cells recognize glucans, including CR3 [12, 13], lactosylceramide [17], dectin [26], and (1→3)-β-D-glucan receptors [19, 27].

SR interactions with carbohydrate polymers were not only based on charge, but also on structure. Ligands interacting with SRs are typically polyanionic, although many polyanions fail to bind. Not surprisingly, highly charged carbohydrates, such as CM-glucan, compete for AcLDL binding sites with high affinity. Nonglucan CM-cellulose has a similar charge but 16-fold lower affinity, supporting a role for structure as well as charge in determining carbohydrate interactions with AcLDL binding sites. Glucan phosphate, a weakly polyanionic carbohydrate, inhibited AcLDL binding completely with a $K_d$ of 8 μM. Uncharged laminarin had similar affinity, but did not appear to inhibit AcLDL binding completely. It is interesting that schizophyllan, a carbohydrate with intermediate charge density, did not interact with AcLDL binding sites. Schizophyllan is a potent biological response modifier that as been shown to modulate various aspects of innate and acquired immunity [14, 49]. Thus, SRs recognize the basic glucan structure, but the interaction is complex and influenced by charge as well as other structural determinants that remain to be defined.

Our results support human monocyte/macrophage SRs as PRRs for fungal (1→3)-β-D-glucans. We observed two AcLDL binding sites on U937 membranes, which interact with glucans. Glucan phosphate interacted with all available AcLDL binding sites, and CM-glucans interacted with higher affinity at a subset of AcLDL binding sites. It is interesting that laminarin, a neutral (1→3)-β-D-glucan, appeared to interact with the same subset of AcLDL binding sites, and schizophyllan, a

(1→3)-β-D-glucan, with intermediate charge density and significant immunomodulatory activity [14, 49], did not interact with AcLDL binding sites. These results suggest that recognition and binding of carbohydrates by human SRs are based on structure as well as polymer charge.

There are at least six classes of SRs [36]. There are multiple class A SRs, including SR-AI, SR-AII, and MARCO [36]. Inhibition by polyinosinic acid, but not polycytidylic acid, is observed in SR-AI and SR-AII and in classes C, E, and F SRs, but is not seen with class B SRs [36]. Several SR classes are found only in *Drosophila* (class C) or endothelial cells (classes E and F). We found complete inhibition of the interaction of U937 membranes with immobilized AcLDL by polyinosinic acid, but not polycytidylic acid. Similar experiments were performed using an immobilized glucan-phosphate surface. A 76% inhibition of the interaction of U937 membranes with immobilized glucan phosphate indicates that glucans have a large binding component to SRs but also confirms the presence of multiple receptors, some of which are insensitive to poly(I). Identification of multiple glucan receptors is consistent with previous studies by Mueller et al. [9, 19], Battle et al. [50], Lowe et al. [51], and Kougias and colleagues [52].

Although this is the first direct biochemical demonstration of the involvement of SRs in the recognition of (1→3)-β-D-glucans, there is much support for the concept that SRs are PRRs for other pathogenic patterns [53]. Macrophage SR-As mediate the recognition of the lipid A domain of LPS [54, 55] and recognize lipoteichoic acid [56].

Our results raise the intriguing possibility that glucans may require the participation of multiple membrane receptors, including SRs, to mediate an immunomodulatory effect. Michalek et al. [25] have demonstrated that activation of macrophages by glucan requires cross-linking of membrane receptors, which are distinct from CR3. We extended this observation by demonstrating that a (1→3)-β-D-linked glucose heptasaccharide (seven subunits) is the minimum binding unit for all available human monocyte glucan receptors but is biologically inactive [51]. We speculate that it is not of sufficient size to cross-link spatially separated glucan receptors. Thus, cross-linking may be a prerequisite for cellular activation, and different glucan structures may favor cross-linking of different membrane receptor combinations, such as CR3, Dectin-1, and SRs. If this proves to be true, it could explain the differences in biological activity and toxicity observed between various glucan preparations.

Recent data from Ahren and colleagues [57] indicate that glucan receptors play a pivotal role in mediating the uptake of nontypeable *Haemophilus influenzae* in human monocytes and epithelial cells. Specifically, these investigators showed that *H. influenzae* entered cells primarily through glucan receptor-mediated endocytosis, which was inhibitable by laminarin [57]. They did not determine whether the receptors in question were SRs, but it is known that SRs can bind bacteria [57]. Consequently, understanding the structural requirements for glucan recognition and binding by SRs may provide important insights into the mechanisms by which the host recognizes and responds to bacterial as well as fungal pathogens.

## CONCLUSIONS

Our data indicate that human monocyte SRs recognize and interact with (1→3)-β-D-glucan polymers. The interaction of glucans and SRs is complex and is influenced by polymer structure, polymer charge, and other as yet undefined parameters. Our studies support the role of SRs as human PRRs for fungal cell wall products in the innate immune response. Future studies are required to determine the structural requirements for glucan interactions with SRs and the role SRs play in activating immunoregulatory and/or proinflammatory signaling pathways in response to ligation with glucans.

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## REFERENCES

1. Modlin, R. L., Brightbill, H. D., Godowski, P. J. (2000) The Toll of innate immunity on microbial pathogens. N. Engl. J. Med. 340, 1834–1835.
2. Bendelac, A., Fearon, D. T. (2000) Innate immunity receptors and effectors of innate immunity. Curr. Opin. Immunol. 12, 11–12.
3. Krieger, M. (1997) The other side of scavenger receptors: pattern recognition for host defense. Curr. Opin. Lipidol. 8, 275–280.
4. Pearson, A. M. (1996) Scavenger receptors in innate immunity. Curr. Opin. Immunol. 8, 20–28.
5. Medzhitov, R., Janeway Jr., C. (2000) Innate immunity. N. Engl. J. Med. 343, 338–344.
6. Peiser, L., Gordon, S. (2001) The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. Microbes Infect. 3, 149–159.
7. Linehan, S. A., Martinez-Pomares, L., Gordon, S. (2000) Mannose receptor and scavenger receptor: two macrophage pattern recognition receptors with diverse functions in tissue homeostatis and host defense. Adv. Exp. Med. Biol. 479, 1–14.
8. Williams, D. L., Pretus, H. A., Browder, I. W. (1992) Application of aqueous gel permeation chromatography with in-line multi-angle laser light scattering and differential viscometry detectors for the characterization of natural product carbohydrate pharmaceuticals. J. Liq. Chromatogr. 15, 2297–2309.
9. Mueller, A., Rice, P. J., Ensley, H. E., Coogan, P. S., Kalbfleisch, J. H., Kelley, J. L., Love, E. J., Portera, C. A., Ha, T., Browder, I. W., Williams, D. L. (1996) Receptor binding and internalization of water-soluble (1→3)-beta-D-glucan biologic response modifier in two monocyte/macrophage cell lines. J. Immunol. 156, 3418–3425.
10. Williams, D. L., Mueller, A., Browder, W. (1996) Glucan-based macrophage stimulators: a review of their anti-infective potential. Clin. Immunother. 5, 392–399.
11. Czop, J. K., Kay, J. (1991) Isolation and characterization of β-glucan receptors on human mononuclear phagocytes. J. Exp. Med. 173, 1511–1520.
12. Thornton, B. P., Vetvicka, V., Pitman, M., Goldman, R. C., Ross, G. D. (1996) Analysis of the sugar specificity and molecular location of the beta-glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). J. Immunol. 156, 1235–1246.
13. Vetvicka, V., Thornton, B. P., Ross, G. D. (1996) Soluble beta-glucan polysaccharide binding to the lectin site of neutrophil or natural killer cell complement receptor type 3 (CD11b/CD18) generates a primed state of the

receptor capable of mediating cytotoxicity of iC3b-opsonized target cells. J. Clin. Investig. 98, 50–61.
14. Ohno, N., Miura, N. N., Chiba, N., Adachi, Y., Yadomae, T. (1995) Comparison of the immunopharmacological activities of triple and single-helical schizophyllan in mice. Biol. Pharm. Bull. 18, 1242–1247.
15. Vetvicka, V., Thornton, B. P., Wieman, J., Ross, G. D. (1997) Targeting of natural killer cells to mammary carcinoma via naturally occurring tumor cell-bound iC3b and beta-glucan-primed CR3. J. Immunol. 159, 599–605.
16. Itoh, W. (1997) Augmentation of protective immune responses against viral infection by oral administration of schizophyllan. Mediators Inflamm. 6, 267–269.
17. Zimmerman, J. W., Lindermuth, J., Fish, P. A., Palace, G. P., Stevenson, T. T., DeMong, D. E. (1998) A novel carbohydrate-glycosphingolipid interaction between a beta-(1→3)-glucan immunomodulator, PGG-glucan, and lactosylceramide of human leukocytes. J. Biol. Chem. 273, 22014–22020.
18. Wakshull, E., Brunke-Reese, D., Lindermuth, J., Fisette, L., Nathans, R. S., Crowley, J. J., Tufts, J. C., Zimmerman, J., Mackin, W., Adams, D. S. (1999) PGG-glucan, a soluble beta-(1,3)-glucan, enhances the oxidative burst response, microbicidal activity, and activates an NF-kappaB-like factor in human PMN: evidence for a glycosphingolipid beta-(1,3)-glucan receptor. Immunopharmacology 41, 89–107.
19. Mueller, A., Raptis, J., Rice, P. J., Kalbfleisch, J. H., Stout, R. D., Ensley, H. E., Browder, W., Williams, D. L. (2000) The influence of glucan polymer structure and solution conformation on binding to (1→3)-β-D-glucan receptors in a human monocyte-like cell line. Glycobiology 10, 339–346.
20. Obayashi, T., Yoshida, M., Mori, T., Goto, H., Yasuoka, A., Iwasaki, H., Teshima, H., Kohno, S., Horiuchi, A., Ito, A., et al. (1995) Plasma (1→3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. Lancet 345, 17–20.
21. Miyazaki, T., Kohno, S., Mitsutake, K., Maesaki, S., Tanaka, K., Ishikawa, N., Hara, K. (1995) Plasma (1→3)-beta-D-glucan and fungal antigenemia in patients with candidemia, aspergillosis, and cryptococcosis. J. Clin. Microbiol. 33, 3115–3118.
22. Mori, T., Ikemoto, H., Matsumura, M., Yoshida, M., Inada, K., Endo, S., Ito, A., Watanabe, S., Yamaguchi, H., Mitsuya, M., Kodama, M., Tani, T., Yokota, T., Kobayashi, T., Kambayashi, J., Nakamura, T., Masaoka, T., Teshima, H., Yoshinaga, T., Kohno, S., Hara, K., Miyazaki, S. (1997) Evaluation of plasma (1→3)-beta-D-glucan measurement by the kinetic turbidimetric Limulus test, for the clinical diagnosis of mycotic infections. Eur. J. Clin. Chem. Biochem. 35, 553–560.
23. Hiyoshi, M., Tagawa, S., Hashimoto, S., Sakamoto, C., Tatsumi, N. (1999) Evaluation of a new laboratory test measuring plasma (1→3)-β-D-glucan in the diagnosis of Candida deep mycosis: comparison with a serologic test. Kansenshogaku Zasshi 73, 1–6.
24. Janeway Jr., C. A., Medzhitov, R. (1999) Lipoproteins take their Toll on the host. Curr. Biol. 9, R879–R882.
25. Michalek, M., Melican, D., Brunke-Reese, D., Langevin, M., Lemerise, K., Galbraith, W., Patchen, M., Mackin, W. (1998) Activation of rat macrophages by Betafectin PGG-glucan requires cross-linking of membrane receptors distinct from complement receptor three (CR3). J. Leukoc. Biol. 64, 337–344.
26. Brown, G. D., Gordon, S. (2001) Immune recognition. A new receptor for beta-glucans. Nature 413, 36–37.
27. Kougias, P., Wei, D., Rice, P. J., Ensley, H. E., Kalbfleisch, J., Williams, D. L., Browder, I. W. (2001) Normal human fibroblasts express pattern recognition receptors for fungal (1→3)-β-D-glucans. Infect. Immun. 69, 3933–3938.
28. Dushkin, M. I., Safina, A. F., Vereschagin, E. I., Schwartz, Y. S. (1996) Carboxymethylated beta-1,3-glucan inhibits the binding and degradation of acetylated low density lipoproteins in macrophages in vitro and modulates their plasma clearance in vivo. Cell Biochem. Funct. 14, 209–217.
29. Vereschagin, E. I., Van Lambalgen, A. A., Dushkin, M. I., Schwartz, Y. S., Polyakov, L., Heemskerk, A., Huisman, E., Thijs, L. G., Van den Bos, G. C. (1998) Soluble glucan protects against endotoxin shock in the rat: the role of the scavenger receptor. Shock 9, 193–198.
30. Pearson, A., Lux, A., Kreieger, M. (1995) Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from Drosophila melanogaster. Proc. Natl. Acad. Sci. USA 92, 4056–4060.
31. Goldstein, J. L., Ho, Y. K., Basu, S. K., Brown, M. S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc. Natl. Acad. Sci. USA 76, 333–337.
32. Brown, M. S., Goldstein, J. L. (1983) Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu. Rev. Biochem. 52, 223–261.
33. Dejager, S., Mietus-Snyder, M., Pitas, R. E. (1993) Oxidized low density lipoproteins bind to the scavenger receptor expressed by rabbit smooth muscle cells and macrophages. Arterioscler. Thromb. Vasc. Biol. 13, 371–378.
34. Steinberg, D. (1995) Role of oxidized LDL and antioxidants in atherosclerosis. Adv. Exp. Med. Biol. 369, 39–48.
35. Kelley, J. L. (1991) Macrophage scavenger receptor(s) for modified low density lipoproteins. Prog. Lipid Res. 30, 231–235.
36. Gough, P. J., Gordon, S. (2000) The role of scavenger receptors in the innate immune system. Microbes Infect. 2, 305–311.
37. Thomas, C. A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S. C., El Khoury, J. (2000) Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagcytosis. J. Exp. Med. 191, 147–156.
38. Williams, D. L., McNamee, R. B., Jones, E. L., Pretus, H. A., Ensley, H. E., Browder, I. W., Di Luzio, N. R. (1991) A method for the solubilization of a (1→3)-β-D-glucan isolated from Saccharomyces cerevisiae. Carbohydr. Res. 219, 203–213.
39. Machova, E., Kogan, G., Alfoldi, J., Soltes, L., Sandula, J. (1995) Enzymatic and ultrasonic depolymerization of carboxymethylated β-1,3-D-glucans derived from Saccharomyces cerevisiae. J. Appl. Polym. Sci. 55, 699–704.
40. Zülli, F., Saecker, C. (1994) CM-glucan a new yeast polysaccharide for cosmetic use. Cosmetic and Toiletry Manufacturing World, 131–136.
41. Ensley, H. E., Tobias, B., Pretus, H. A., McNamee, R. B., Jones, E. L., Browder, I. W., Williams, D. L. (1994) NMR spectral analysis of a water-insoluble (1→3)-β-D-glucan isolated from *Saccharomyces cerevisiae*. Carbohydr. Res. 258, 307–311.
42. Mueller, A., Pretus, H., McNamee, R., Jones, E., Browder, I., Williams, D. (1995) Comparison of the carbohydrate biological response modifiers Krestin, schizophyllan and glucan phosphate by aqueous size exclusion chromatographty with in-line argon-ion multi-angle laser light scattering photometry and differential viscometry detectors. J. Chromatogr. 666, 283–290.
43. Sandula, J., Kogan, G., Kacurakova, M., Machova, E. (1999) Microbial (1→3)-β-D-glucans, their preparation, physicochemical characterization and immunomodulatory activity. Carbohydr. Polym. 38, 247–253.
44. Kogan, G. (2000) (1-3, 1-6)-β-D-Glucans of yeasts and fungi and their biological activity. In *Studies in Natural Products Chemistry* 23, Part D (A.U. Rahman, ed.), Elsevier Science, Amsterdam, 107–152.
45. Lowman, D., Ensley, H., Williams, D. (1998) Identification of phosphate substitution sites by NMR spectroscopy in a water-soluble phosphorylated (1→3)-β-D-glucan. Carbohydr. Res. 306, 559–562.
46. Kelley, J. L., Rozek, M. M., Suenram, C. A., Schwartz, C. J. (1988) Activation of human peripheral blood monocytes by lipoproteins. Am. J. Pathol. 130, 223–231.
47. Brown, M. S., Goldstein, J. L., Krieger, M., Ho, Y. K., Anderson, R. G. W. (1979) Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. J. Cell Biol. 82, 597–613.
48. Brown, M. S., Basu, S. K., Falck, J. R., Ho, Y. K., Goldstein, J. L. (1980) The scavenger cell pathway for lipoprotein degradation: specificity of the binding site that mediates the uptake of negatively-charged LDL by macrophages. J. Supramol. Struct. 13, 67–81.
49. Sakagami, Y., Mizoguchi, Y., Shin, T., Seki, S., Kobayashi, K., Morisawa, S., Yamamoto, S. (1988) Effects of an anti-tumor polysaccharide, schizophyllan, on interferon-gamma and interleukin 2 production by peripheral blood mononuclear cells. Biochem. Biophys. Res. Commun. 155, 650–655.
50. Battle, J., Ha, T., Li, C., Della Beffa, V., Rice, P., Kalbfleisch, J., Browder, W., Williams, D. (1998) Ligand binding to the (1→3)-β-D-glucan receptor stimulates NF-kappaB activation, but not apoptosis in U937 cells. Biochem. Biophys. Res. Commun. 249, 499–504.
51. Lowe, E., Rice, P., Ha, T., Li, C., Kelley, J., Ensley, H., Lopez-Perez, J., Kalbfleisch, J., Lowman, D., Margl, P., Browder, W., Williams, D. (2001) A (1→3)-β-D-linked heptasaccharide is the unit ligand for glucan pattern recognition receptors on human monocytes. Microbes Infect. 3, 789–797.
52. Kougias, P., Wei, D., Rice, P. J., Ensley, H. E., Kalbfleisch, J., Williams, D. L., Browder, I. W. (2001) Normal human fibroblasts express pattern recognition receptors for fungal (1→3)-β-D-glucans. Infect. Immun. 69, 3933–3938.
53. Peiser, L., Gordon, S. (2001) The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. Microbes Infect. 3, 149–159.
54. Hampton, R. Y., Golenbock, D. T., Penman, M., Krieger, M., Raetz, C. R. (1991) Recognition and plasma clearance of endotoxin by scavenger receptors. Infect. Immun. 64, 3318–3325.
55. Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M., Krieger, M. (1993) Structures and high and low affinity ligand binding properties of murine type I and type II macrophage scavenger receptors. J. Lipid Res. 34, 983–1000.
56. Dunne, D. W., Resnick, D., Greenburg, J., Krieger, M., Joiner, K. A. (1994) The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proc. Natl. Acad. Sci. USA 91, 1863–1867.
57. Ahren, I. L., Williams, D. L., Rice, P. J., Forsgren, A., Riesbeck, K. (2001) The importance of a β-glucan receptor in the nonopsonic entry of nontypeable *Haemophilus influenzae* into human monocytic and epithelial cells. J. Infect. Dis. 184, 150–158.