Journal of Biomolecular Screening

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Journal:	*Journal of Biomolecular Screening*
Manuscript ID	JBSC-15-0181
Manuscript Type:	Original Research
Date Submitted by the Author:	02-Dec-2015
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Keywords:	surface plasmon resonance, Immuno assays, mannose binding lectin, inhibitor's screening, glycodendrimers
Abstract:	Mannose-binding lectin (MBL) is a circulating protein that acts as a soluble pattern recognition molecule of the innate immunity. It binds to carbohydrate patterns on the surface of pathogens or of "altered cells", with activation of the lectin pathway of the complement system. Recent evidence indicates that MBL contributes to the pathophysiology of ischemia-reperfusion injury and other conditions. Thus, MBL inhibitors offer promising therapeutic strategies, since they prevent preventing the interaction of MBL with its target sugar arrays. We developed and characterized a novel assay based on surface plasmon resonance for *in vitro* screening of these compounds, which may be useful before the more expensive and time-consuming *in vivo* studies. The assay measures the inhibitor's ability to interfere with the binding of murine MBL-A or MBL-C, or of human recombinant MBL, to mannose residues immobilized on the sensor chip surface. We have applied the assay to measure the IC_{50} of synthetic glycodendrimers, two of them with neuroprotective properties in animal models of MBL-mediated injuries.

A new surface plasmon resonance assay for *in vitro* screening of mannose-binding lectin inhibitors

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Keywords: surface plasmon resonance; immunoassay; mannose binding lectin; inhibitor's screening; glycodendrimers.

ABSTRACT

Mannose-binding lectin (MBL) is a circulating protein that acts as a soluble pattern recognition molecule of the innate immunity. It binds to carbohydrate patterns on the surface of pathogens or of "altered cells", with activation of the lectin pathway of the complement system. Recent evidence indicates that MBL contributes to the pathophysiology of ischemia-reperfusion injury and other conditions. Thus, MBL inhibitors offer promising therapeutic strategies, since they prevent preventing the interaction of MBL with its target sugar arrays. We developed and characterized a novel assay based on surface plasmon resonance for *in vitro* screening of these compounds, which may be useful before the more expensive and time-consuming *in vivo* studies. The assay measures the inhibitor's ability to interfere with the binding of murine MBL-A or MBL-C, or of human recombinant MBL, to mannose residues immobilized on the sensor chip surface. We have applied the assay to measure the IC_{50} of synthetic glycodendrimers, two of them with neuroprotective properties in animal models of MBL-mediated injuries.

Introduction

Mannose binding lectin (MBL) is a calcium-dependent (C-type) lectin, belonging to the collectin family, with an important role in innate immunity [1]. MBL circulates in blood complexed with MBL-serine proteases (MASPs) and acts as a soluble pattern recognition receptor, binding specific carbohydrate patterns exposed on the surface of pathogens or of "altered-self" cells through its carbohydrate-recognition domain (CRD). On binding the target cells, MASPs are activated, initiating the lectin pathway (LP) of the complement system.

MBL has been implicated in experimental models of myocardial [2], renal [3] and gastrointestinal injury [4], cerebral ischemia/reperfusion injury [5, 6] and traumatic brain injury [7, 8] as well as pre-eclampsia [9]. In all these conditions, MBL appears to contribute to tissue injury and its deletion or inhibition is protective. MBL inhibitors might therefore be as promising therapeutic strategies, and reliable *in vitro* assays are needed to identify them, before more expensive and time-consuming *in vivo* studies.

MBL-binding properties of compounds can be investigated by a direct surface plasmon resonance (SPR) assay in which unlabeled putative MBL ligands are flowed onto recombinant MBL immobilized on the sensor chip [5]. Binding is revealed in real time as a change of mass at the surface, and the interaction can be characterized in terms of on and off rates (kinetics) and binding strength [10]. Although this approach is fairly straightforward and useful for initial screening purposes, it suffers some limitations: MBL can be bound on a site different from CRD, so it may not result in inhibitory activity; binding affinities can be overestimated due to multivalent interactions; immobilization procedures can alter the binding properties of MBL. We experienced difficulties in immobilizing recombinant murine MBL-C (mMBL-C, one of the two murine isoforms, together with mMBL-A [11]). Then, last

but not least, immobilization of MBL on a surface makes this configuration different from the physiological condition.

We have now developed and characterized a novel SPR-based assay that allows *in vitro* identification of MBL inhibitors, with a design that closely mimics real conditions, since the compound's effects are measured by their ability to inhibit MBL binding to mannose residues exposed on the (sensor) surface. Thus, this approach can assess the inhibition of a property of MBL which is relevant to its functional activity. The assay is suited to study the inhibition of native plasmatic mMBL-A and mMBLC, and of recombinant human MBL (rhMBL). We used it to measure the inhibitory effects of synthetic polymannosylated glycodendrimers, two of them – Polyman2 and Polyman9 – with protective properties in animal models of MBL-mediated injuries [5, 8, 9].

Materials and Methods

Animals

Plasma was obtained from eight-week-old C57Bl/6 mice (wild-type, WT, 20-25g; Harlan Laboratories) and from C57Bl/6 mice with a target mutation of both MBL-A and MBL-C genes (MBL-/-, 20-25g, purchased from Jackson Laboratories-USA and colonized at the Mario Negri Institute). Briefly, blood samples were collected from the vena cava in 10 mM ethylendiaminetetracetic acid and 0.125% polybrene (Sigma-Aldrich). Plasma was separated by centrifugation at 2000 g at 4°C for 15 min, immediately stored and kept at -80°C until use. The IRCCS-Istituto di Ricerche Farmacologiche Mario Negri adheres to the principles set out in the following laws, regulations, and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014; Authorisation n.19/2008-A issued March 6, 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing

internal authorisation for those conducting animal experiments (Quality Management System Certificate – UNI EN ISO 9001:2008 – Reg. No. 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition) and EU directives and guidelines (EEC Council Directive 2010/63/UE). The Statement of Compliance (Assurance) with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals has been recently reviewed (9/9/2014) and will expire on September 30, 2019 (Animal Welfare Assurance #A5023-01). The studies reported here were within a project specifically authorized by the Italian Ministry of Health (Decree n° 161/2014B).

SPR-based assay

SPR is a powerful label-free method widely used to study binding between two macromolecules [12]. Typically, one of the interacting partners is immobilized on a sensor chip surface and the other is flowed through a microfluidic system in contact with the chip surface. Binding is revealed in real time as a change of mass at the surface. Although the most common application of SPR instruments is to determine affinity parameters for biomolecular interactions [10], its versatility permits many other uses, including label-free immunoassays [13-15].

The novel assay described here involves several steps. Fig. 1 summarizes the four steps required for studies with murine plasma (two mMBL isoforms); studies with rhMBL are limited to the first two steps. The first step is the covalent immobilization of mannosylated bovine serum albumin (Man-BSA, Dextra Laboratories, Reading, UK) on the sensor chip (GLC, Bio-Rad) using amine coupling chemistry [16]. Thus, Man-BSA molecules are bound to the sensor surface through the protein moieties leaving the mannose residues exposed. The ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) used

for these SPR studies, has six parallel flow channels that can immobilize up to six ligands on the same sensor chip. In parallel to Man-BSA we always immobilized BSA (Sigma) as reference. Immobilization levels were about 3000 and 4000 resonance units (RU, 1 RU 1 pg protein/mm2). After the first immobilization step, the ProteOn XPR36 fluidic system is rotated 90° [17] so that up to six different solutions can be injected simultaneously over the immobilized ligands (Fig. 1). In this second step, we injected MBL-containing solutions, preincubated with or without the inhibitors (see below). We used mouse and human plasma, diluted (as indicated) in 10 mM running buffer (TRIS buffer containing 150 mM NaCl and 0.005% Tween 20, pH 7.4 , plus 1.2 mM $CaCl_2$); or running buffer with rhMBL added (R&D Systems, Minneapolis, MN). This second step aims to capture MBL (mMBL isoforms or rhMBL) on the Man-BSA surface. The mannose-dependent SPR binding signal is obtained by subtracting the mannose-independent nonspecific response on the reference surface (BSA only). The following steps are carried out after further rotation of the fluidic system (Fig. 1) and regards the sequential injections of anti-MBL antibodies. After injecting murine plasma we injected 10 μg/mL anti-mMBL-A and 10 μg/mL anti-mMBL-C antibodies (Hycult Biotech, The Netherlands) to specifically recognize and quantify the isoform captured during the second step (Fig. 1). Two anti-hMBL antibodies were tested after injection of human plasma: 10 μg/mL anti-hMBL antibody 3E7 (Hycult Biotech, The Netherlands) and 10 μg/ml anti-hMBL antibody 3B6 (Abcam, Cambridge, UK). All SPR assays were run at 25°C. The sensorgrams (time course of the SPR signal in RU) were normalized to a base-line of 0.

For the analysis of putative MBL inhibitors, diluted plasma or running buffer spiked with rhMBL were preincubated for 30 min at 25°C, with or without glycodendrimers at different concentrations and then flowed onto immobilized Man-BSA and BSA (Fig 1, step 2). The six parallel flow channels meant we could analyze the effect of up to four concentrations

simultaneously, leaving one for mouse plasma preincubated with vehicle alone (total binding, TB) and one channel for MBL-/- mouse plasma (non-specific binding, NSB). The SPR binding signal at t=100 s, in the presence of each drug concentration (BD), was used to calculate the percentage inhibition of specific binding (%INH-SB) as follows:

$$\%INH\text{-}SB = ((TB - BD) / (TB\text{-}NSB)) * 100$$

The aim of these studies was to identify compounds that prevent the MBL interaction with mannose residues and to determine their potency, i.e. the concentration inhibiting MBL binding by 50% (IC_{50}).

Synthetic polymannosylated glycodendrimers

The glycodendrimers we tested as MBL inhibitors are shown in Fig. 2. They are Polyman2 (also referred to as Dendron 12 [18] which is a tetravalent glycodendron carrying a pseudo-trimannoside headgroup; Polyman9, a hexavalent glycodendrimer with a flexible core, carrying a pseudo-dimannoside headgroup (also referred to as glycodendrimer 13.3 [19]) and Polyman31, a hexavalent glycodendron carrying the same pseudo-dimannoside headgroup on a rigid-core architecture [20]. The synthesis and characterization of the compounds we used has been described previously [18-20].

Results

Characterization of the SPR assay for mMBL-A and mMBL-C

Diluted plasma from WT mice injected over a sensor surface coated with Man-BSA resulted in a marked SPR signal (2550 and 1500 RU, on average, at plasma dilutions of 1:50 and

1:100 respectively), much higher than in a parallel reference surface coated with BSA only (800 RU and 530 RU) (representative sensorgrams in Fig. S1A). This indicated the presence of a mannose-dependent binding signal, highlighted in sensorgrams obtained by subtracting the signal in the reference surface (Fig. S1B and Fig. 3A). Most of this specific signal was due to mMBL, since plasma from MBL-/- mice gave a much lower specific signal, only 20±11 % of the WT plasma value (mean±SD, n=18) (Fig. 3A, Fig. S1B).

The binding of plasmatic mMBL to immobilized mannose residues was very stable as shown by the low dissociation rates after the injections (Fig. 3A) thus allowing sequential injection of the antibodies against mMBL-A and mMBL-C. These injections resulted in clear SPR binding signals (Fig. 3B-C) indicating that both murine isoforms were actually captured during the plasma injection; much lower signals were observed in the surfaces where plasma from MBL-/- had been flowed (23±16 % and 11±13 % with antibodies against mMBL-A and mMBL-C, mean±SD, n=18), thus confirming the specificity of the measured signal. The SPR signal with the anti-mMBL-A antibody was linear during the injection period whereas a plateau was reached during the injection of the anti-mMBL-C antibody. This indicates a higher association rate constant of the anti-mMBL-C then the anti-mMBL-A antibody, and that less mMBL-C than mMBL-A was captured by mannose.

As expected, the binding signals due to both mMBL-A and mMBL-C correlated with plasma dilution (Fig. S2A-B), although there was a tendency to saturation with the highest concentrations (1:50 and 1:25). The IC_{50} was determined using plasma diluted at either 1:50 or 1:100, with no significant differences in the results.

Preincubation of plasma with different concentrations of mannose resulted in concentration-dependent inhibition of both mMBL-A and mMBL-C binding to immobilized

mannose, as indicated by the subsequent injections of the corresponding antibodies. The IC_{50} was similar for the two isoforms, respectively 5.4 and 5.2 mM for mMBL-A and mMBL-C.

Characterization of the assay for hMBL

Injection of diluted human plasma over immobilized Man-BSA resulted in a marked SPR signal (400 RU on average, at plasma dilutions of 1:50), much higher than that observed in a parallel reference surface coated with BSA only (50 RU) (representative sensorgrams in Fig. S1C). This indicated the presence of a mannose-dependent binding signal (i.e. obtained by subtraction of the signal in the reference surface), although this was much lower than with murine plasma (Fig. S1D vs Fig. S1B).

Despite the presence of a mannose-dependent binding signal, we were not able to confirm the capture of hMBL with anti-hMBL antibodies. Results were negative with two different anti-hMBL antibodies, at concentrations up to 10 µg/mL, even when plasma was only diluted 1:10 (data not shown). To overcome this problem, we developed a different approach testing the compounds for their ability to inhibit the binding of rhMBL to immobilized mannose. Different concentrations of rhMBL (Fig S2C), diluted in running buffer, resulted in a concentration-dependent binding signal, linear in the 0.5-8 nM range (Fig. S2D). Global fitting of these sensorgrams according to the simplest 1:1 interaction model (Langmuir model) gave an estimated K_D of 1 nM.

Inhibitory effects of polymannosylated glycodendrimers

Polyman2 inhibited the binding of mMBL-A and mMBL-C to immobilized mannose with similar IC_{50} values (93 and 84 µM, Fig. 4A-B) whereas Polyman31 was highly selective for

the two isoforms, inhibiting mMBL-C with a very low IC_{50} (0.5 µM) but being much less active on mMBL-A (IC_{50}>200 µM) (Fig. 4A-B). Polyman9 also had a lower IC_{50} on mMBL-C (65 µM) than on mMBL-A (IC_{50}>200 µM) (Fig. 4A-B [8]).

As an example of raw data, Fig. S3A shows representative sensorgrams obtained injecting of plasma preincubated or not with 3 µM Polyman31, in parallel with plasma from MBL-/- mice. At this concentration, Polyman31 had no effect on MBL-A binding (Fig. S3B), but it reduced MBL-C binding by 85% (Fig. S3C). The 20% decrease during the plasma injection (Fig. S3A) indicated that most (~ 77%) of the MBL captured by mannose was accounted for by MBL-A.

Preincubation of rhMBL (8 nM) with different concentrations of dendrimers resulted in dose-dependent inhibition of rhMBL binding to immobilized mannose (Fig. 4C). In this assay Polyman31 was the most potent inhibitor (IC-50 62 µM), followed by Polyman9 (~ 136 µM) and Polyman2 (~ 270 µM).

Discussion

We report the characterization of a novel SPR assay for *in vitro* screening of MBL inhibitors, analyzed for their ability to interfere with MBL binding to carbohydrate patterns. This was achieved by flowing MBL-containing solutions, with or without inhibitors, over a sensor chip exposing mannose residues.

The mannose-dependent SPR signal with diluted murine plasma is mostly (80% on average) due to mMBL, as indicated by the results with plasma of $MBL^{-/-}$ mice. This binding has a very low dissociation rate, as expected for multivalent binding, so permitting subsequent injections of antibodies against mMBL-A and mMBL-C to specifically recognize and quantify the isoform captured by mannose. In our conditions 70-80% of the mMBL-

dependent SPR signal is accounted for by mMBL-A, although its serum levels in C57Bl/6 mice are 4-5 times lower than those of mMBL-C [21, 22]. Since the molecular mass of the two isoforms is comparable [21], this suggests that mMBL-A has greater affinity than mMBL-C for immobilized mannose. However, the smaller proportion of mMBL-C captured by immobilized mannose is compensated by the higher affinity of the corresponding antibody. Thus, the SPR signal 100 sec after injection of the anti-mMBL-C antibody was similar, on average, to that seen 100 sec after injection of the anti-mMBL-A antibody.

The SPR data obtained injecting diluted human plasma indicated a mannose-dependent binding signal which, however could not be definitely associated to the capture of hMBL. In fact, there was no SPR signal on subsequent injection of anti-hMBL antibodies. Possibly the "mannose-dependent" binding signal after injection of human plasma is either: i) not due to hMBL capture or ii) actually due to hMBL, but the two anti-hMBL antibodies tested did not bind it efficiently enough for detection in our set-up.

The mannose-dependent binding signal with human plasma was much lower (20%) than the signal with murine plasma, in agreement with the lower hMBL concentration (1-1.5 µg/mL) [23, 24] than mMBL-A (8-20 µg/mL) or mMBL-C (30-100 µg/mL) [21, 22]. Insufficient capture of hMBL on the sensor chip might thus contribute to the lack of measurable binding with anti-hMBL antibodies, although results were also negative using plasma or serum ten-fold only diluted. Other factors required for an efficient SPR assay, and possibly not met with native hMBL, are: i) adequate affinity of hMBL for the immobilized mannose residues, particularly an association rate constant permitting the capture of enough hMBL during the short association phase (few minutes), and/or ii) suitable affinity of the anti-hMBL antibodies employed for these studies. In this regard, successful detection of plasmatic mMBL-A is very likely due to its high affinity for immobilized mannose (higher than that of mMBL-C),

whereas successful detection of mMBL-C is probably likely due to the high serum concentrations and the high affinity of the anti-mMBL-C antibody (higher than that of anti-mMBL-A antibody).

To evaluate the effects of potential inhibitors on hMBL, we developed an SPR assay using rhMBL. A suitable SPR binding signal was obtained by flowing 8 nM rhMBL, dissolved in buffer, over immobilized mannose residues (about 7 µg/mL, i.e. much higher than the concentration present in diluted human plasma), while lower concentrations gave proportionally lower SPR signals.

We used these assays to study the inhibitory effects of synthetic polymannosylated glycodendrimers. Native MBL is found as oligomers of a trimeric building block, with multiple CRD domains [25] which efficiently bind to carbohydrate patterns through multivalent protein-sugar interactions. Thus, to interfere with these, multivalent inhibitors such as glycodendrimers have been designed and studied over the past ten years as potential antagonists [26, 27]. Polyman2 [18] is a tetravalent pseudo-trimannoside dendron which showed potent protective activity in mouse models of cerebral ischemia [5] or pre-eclampsia [9] where MBL significantly contributed to the injury. Polyman9 is a hexavalent pseudo-dimannoside dendrimer based on a flexible scaffold [19] which showed neuroprotective effects in a murine model of traumatic brain injury [8]. Polyman31 is a new hexavalent pseudo-dimannoside dendrimer based on a rigid-core architecture [20]. In our assay, Polyman2 showed no selectivity between mMBL-A and mMBL-C (IC_{50} 93 and 84 µM) whereas Polyman-31 had a completely different interaction profile, being very potent on MBL-C (IC_{50} 0.5 µM) but almost inactive on mMBL-A up to 200 µM. Polyman9 had a similar profile, inhibiting mMBL-C (IC_{50} 65 µM) but not MBL-A (IC_{50} >>200 µM) [8]. These molecules also inhibited rhMBL binding of to mannose residues with IC_{50} from 62-270 µM.

The inhibitor concentrations we used (μM range) greatly exceed those of MBL (nM range, in diluted murine plasma and rhMBL). Thus, the percentage inhibition of MBL binding to mannose is indicative of the occupancy of MBL proteins and the IC_{50} is a measure of the inhibitor's affinity for MBL. The affinities of Polyman2 for mMBL-A and rhMBL (IC_{50} 93 and ~270 μM) are higher than those measured by direct SPR assay, i.e. directly flowing the dendrimer over immobilized recombinant mMBL-A (K_D 4.7 μM, data not shown), or rhMBL (K_D 6 μM, [5]). A similar difference was reported for polymeric inhibitors of the lectin DC-SIGN using analogous SPR approaches [15] and is possibly due to a lower impact of avidity effects when interactions occur in solution.

A mannan immunoassay has been described in which murine plasma was incubated on mannan-coated microtiter wells and murine MBL isoforms were then recognized by biotinylated anti-MBL antibodies and europium-labeled streptavidin [21]. This method was used to quantify mMBL-A and mMBL-C concentrations in mouse sera, but to the best of our knowledge it has never been used to screen MBL inhibitors. In comparison with our SPR assay, immunoassays in microtiter wells are probably more sensitive, due to signal amplification. However, the SPR assay has other worthwhile advantages: i) it does not need any labeled reagent and takes less of the antibodies than for microtiter wells; ii) the amount of bound anti-MBL antibody - and thus MBL - is immediately determined by SPR whereas further steps are required when using microtiter wells, e.g. incubation with europium-labeled streptavidin for immunofluorometric assay [21]; iii) the long incubation of plasma in mannan-coated microtiter wells may reduce the binding between the inhibitor and plasmatic MBL, due to the equilibrium shift induced by the high-affinity binding of free MBL to immobilized sugars; this potential artifact, leading to underestimation of the compound's inhibitory effect,

is probably absent during the very short flow of plasma over the mannose-coated SPR surface.

In conclusion, we have extensively characterized a novel assay, convenient for *in vitro* screening of putative MBL inhibitors, to select the appropriate compounds for the more expensive and time-consuming *in vivo* studies. This approach has been successfully used to identify a selective mMBL-C inhibitor, Polyman9, which conferred neurobehavioral protection when given shortly after traumatic brain injury [8]. We also found another glycodendrimer, Polyman31, with even higher affinity and selectivity for mMBL-C.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

Financial support including a fellowship to D. De Blasio was provided by Fondazione Cariplo (grant number 2012-0590).

References

1. Endo, Y.; Matsushita, M.; Fujita, T. New insights into the role of ficolins in the lectin pathway of innate immunity. *Int Rev Cell Mol Biol* **2015,** *316*, 49-110.
2. Walsh, M. C.; Bourcier, T.; Takahashi, K.; et al. Mannose-binding lectin is a regulator of inflammation that accompanies myocardial ischemia and reperfusion injury. *J Immunol* **2005,** *175* (1), 541-6.
3. de Vries, B.; Walter, S. J.; Peutz-Kootstra, C. J.; et al. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am J Pathol* **2004,** *165* (5), 1677-88.
4. Hart, M. L.; Ceonzo, K. A.; Shaffer, L. A.; et al. Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q. *J Immunol* **2005,** *174* (10), 6373-80.
5. Orsini, F.; Villa, P.; Parrella, S.; et al. Targeting mannose-binding lectin confers long-lasting protection with a surprisingly wide therapeutic window in cerebral ischemia. *Circulation* **2012,** *126* (12), 1484-94.
6. Gesuete, R.; Storini, C.; Fantin, A.; et al. Recombinant C1 inhibitor in brain ischemic injury. *Ann Neurol* **2009,** *66* (3), 332-42.
7. Longhi, L.; Orsini, F.; De Blasio, D.; et al. Mannose-binding lectin is expressed after clinical and experimental traumatic brain injury and its deletion is protective. *Crit Care Med* **2014,** *42* (8), 1910-8.
8. De Blasio, D.; Fumagalli, S.; Longhi, L.; et al. Pharmacological inhibition of mannose binding lectin ameliorates neurobehavioral dysfunction following experimental traumatic brain injury. *J. Cereb. Blood Flow Metab* **2015,** *submitted*.
9. Petitbarat, M.; Durigutto, P.; Macor, P.; et al. Critical Role and Therapeutic Control of the Lectin Pathway of Complement Activation in an Abortion-Prone Mouse Mating. *J Immunol* **2015,** *Epub, doi:10.4049/jimmunol.1501361*.
10. Cooper, M. A. Optical biosensors in drug discovery. *Nat Rev Drug Discov* **2002,** *1* (7), 515-28.
11. Hansen, S.; Thiel, S.; Willis, A.; et al. Purification and characterization of two mannan-binding lectins from mouse serum. *J Immunol* **2000,** *164* (5), 2610-8.
12. Rich, R. L.; Myszka, D. G. Higher-throughput, label-free, real-time molecular interaction analysis. *Anal Biochem* **2007,** *361* (1), 1-6.
13. Mullett, W. M.; Lai, E. P.; Yeung, J. M. Surface plasmon resonance-based immunoassays. *Methods* **2000,** *22* (1), 77-91.
14. Canovi, M.; Lucchetti, J.; Stravalaci, M.; et al. A new surface plasmon resonance-based immunoassay for rapid, reproducible and sensitive quantification of pentraxin-3 in human plasma. *Sensors (Basel)* **2014,** *14* (6), 10864-75.
15. Zhang, Q.; Collins, J.; Anastasaki, A.; et al. Sequence-controlled multi-block glycopolymers to inhibit DC-SIGN-gp120 binding. *Angew Chem Int Ed Engl* **2013,** *52* (16), 4435-9.
16. Gjelstrup, L. C.; Kaspersen, J. D.; Behrens, M. A.; et al. The role of nanometer-scaled ligand patterns in polyvalent binding by large mannan-binding lectin oligomers. *J Immunol* **2012,** *188* (3), 1292-306.
17. Bravman, T.; Bronner, V.; Lavie, K.; et al. Exploring "one-shot" kinetics and small molecule analysis using the ProteOn XPR36 array biosensor. *Anal Biochem* **2006,** *358* (2), 281-8.
18. Sattin, S.; Daghetti, A.; Thepaut, M.; et al. Inhibition of DC-SIGN-mediated HIV infection by a linear trimannoside mimic in a tetravalent presentation. *ACS Chem Biol* **2010,** *5* (3), 301-12.
19. Varga, N.; Sutkeviciute, I.; Ribeiro-Viana, R.; et al. A multivalent inhibitor of the DC-SIGN dependent uptake of HIV-1 and Dengue virus. *Biomaterials* **2014,** *35* (13), 4175-84.

20. Ordanini, S.; Varga, N.; Porkolab, V.; et al. Designing nanomolar antagonists of DC-SIGN-mediated HIV infection: ligand presentation using molecular rods. *Chem Commun (Camb)* **2015,** *51* (18), 3816-9.
21. Liu, H.; Jensen, L.; Hansen, S.; et al. Characterization and quantification of mouse mannan-binding lectins (MBL-A and MBL-C) and study of acute phase responses. *Scand J Immunol* **2001,** *53* (5), 489-97.
22. Shushimita, S.; van der Pol, P.; R, W. F. d. B.; et al. Mannan-Binding Lectin Is Involved in the Protection against Renal Ischemia/Reperfusion Injury by Dietary Restriction. *PLoS One* **2015,** *10* (9), e0137795.
23. Zanier, E. R.; Zangari, R.; Munthe-Fog, L.; et al. Ficolin-3-mediated lectin complement pathway activation in patients with subarachnoid hemorrhage. *Neurology* **2014,** *82* (2), 126-34.
24. Osthoff, M.; Katan, M.; Fluri, F.; et al. Mannose-binding lectin deficiency is associated with smaller infarction size and favorable outcome in ischemic stroke patients. *PLoS One* **2011,** *6* (6), e21338.
25. Drickamer, K.; Dordal, M. S.; Reynolds, L. Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tails. Complete primary structures and homology with pulmonary surfactant apoprotein. *J Biol Chem* **1986,** *261* (15), 6878-87.
26. Bernardi, A.; Jimenez-Barbero, J.; Casnati, A.; et al. Multivalent glycoconjugates as anti-pathogenic agents. *Chem Soc Rev* **2013,** *42* (11), 4709-27.
27. Imberty, A.; Chabre, Y. M.; Roy, R. Glycomimetics and glycodendrimers as high affinity microbial anti-adhesins. *Chemistry* **2008,** *14* (25), 7490-9.

Figure Legends

Figure 1. Schematic summary of the surface plasmon resonance assay for inhibitors of murine MBL isoforms.

Figure 2. Structures of the glycodendrons used in this study: Polyman2, Polyman9 and Polyman31.

Figure 3. Capture of plasmatic mMBL-A and mMBL-C by immobilized mannose residues. A: representative sensorgrams (i.e. time course of SPR signal in resonance units, RU) with plasma (1:50) from wild-type (WT) and MBL-/- (KO) mice injected over chip surfaces coated with mannosylated-BSA (Man-BSA). These sensorgrams were obtained after subtraction of the non-specific signal measured on a parallel surface coated with BSA (see also Fig S1). Plasma was injected for 10 min (bars). B-C: representative sensorgrams with sequential injections of anti-MBL-A or anti-MBL-C antibodies shortly after the injection of plasma.

Figure 4. Inhibitory effect of dendrimeric compounds on mMBL-A (A), mMBL-C (B) or rhMBL (C) binding to mannose residues. Polyman31 is indicated in blu, Polyman2 in red, Polyman9 in green (data of Polyman9 from ref. 8). Each value is the mean±SD of 2-3 independent SPR sessions.

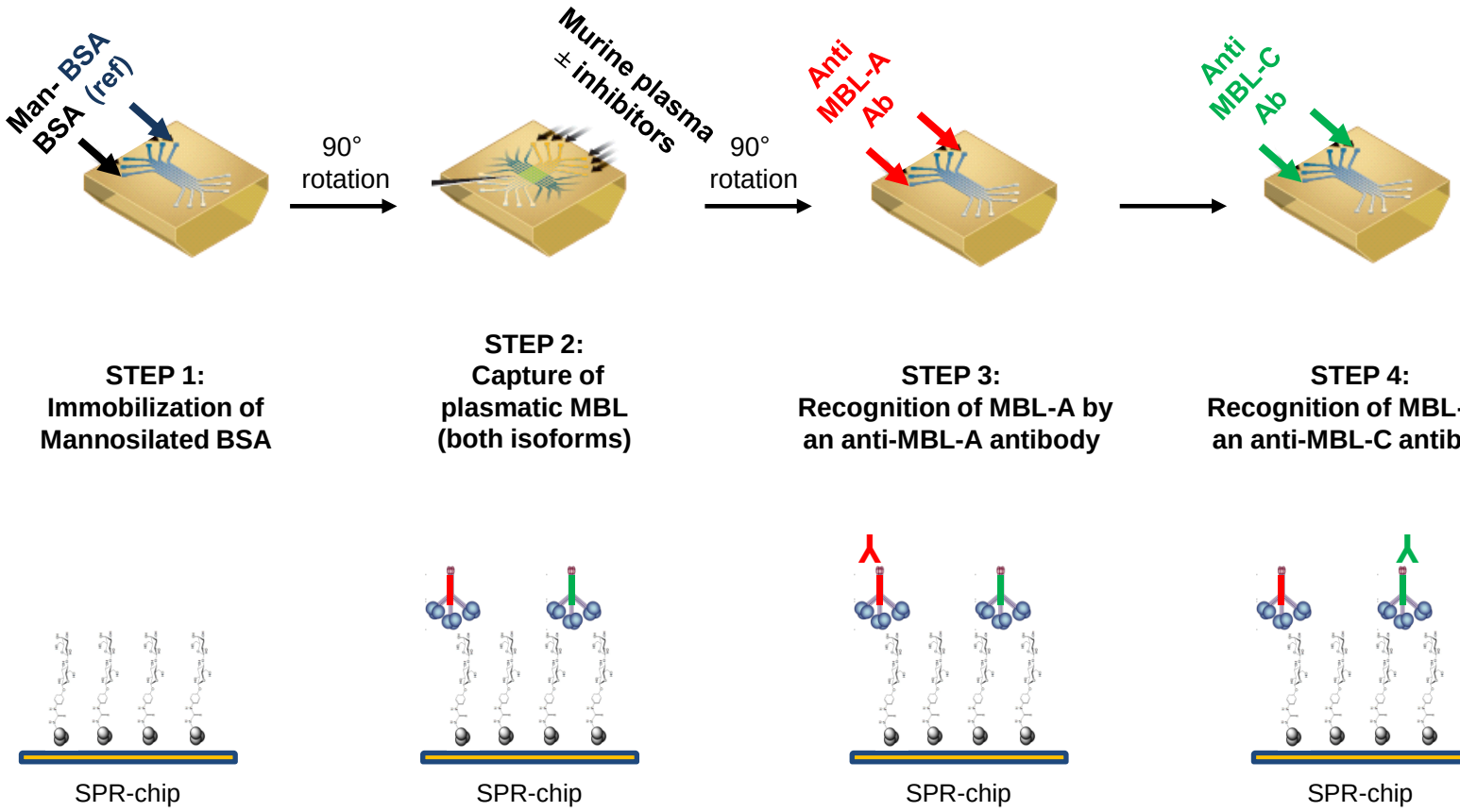
Man- BSA
BSA (ref)
90°
rotation
Murine plasma
± inhibitors
90°
rotation
Anti
MBL-A
Ab
Anti
MBL-C
Ab
STEP 1:
Immobilization of
Mannosilated BSA
STEP 2:
Capture of
plasmatic MBL
(both isoforms)
STEP 3:
Recognition of MBL-A by
an anti-MBL-A antibody
STEP 4:
Recognition of MBL-C by
an anti-MBL-C antibody
SPR-chip
SPR-chip
SPR-chip
SPR-chip

Figure 1

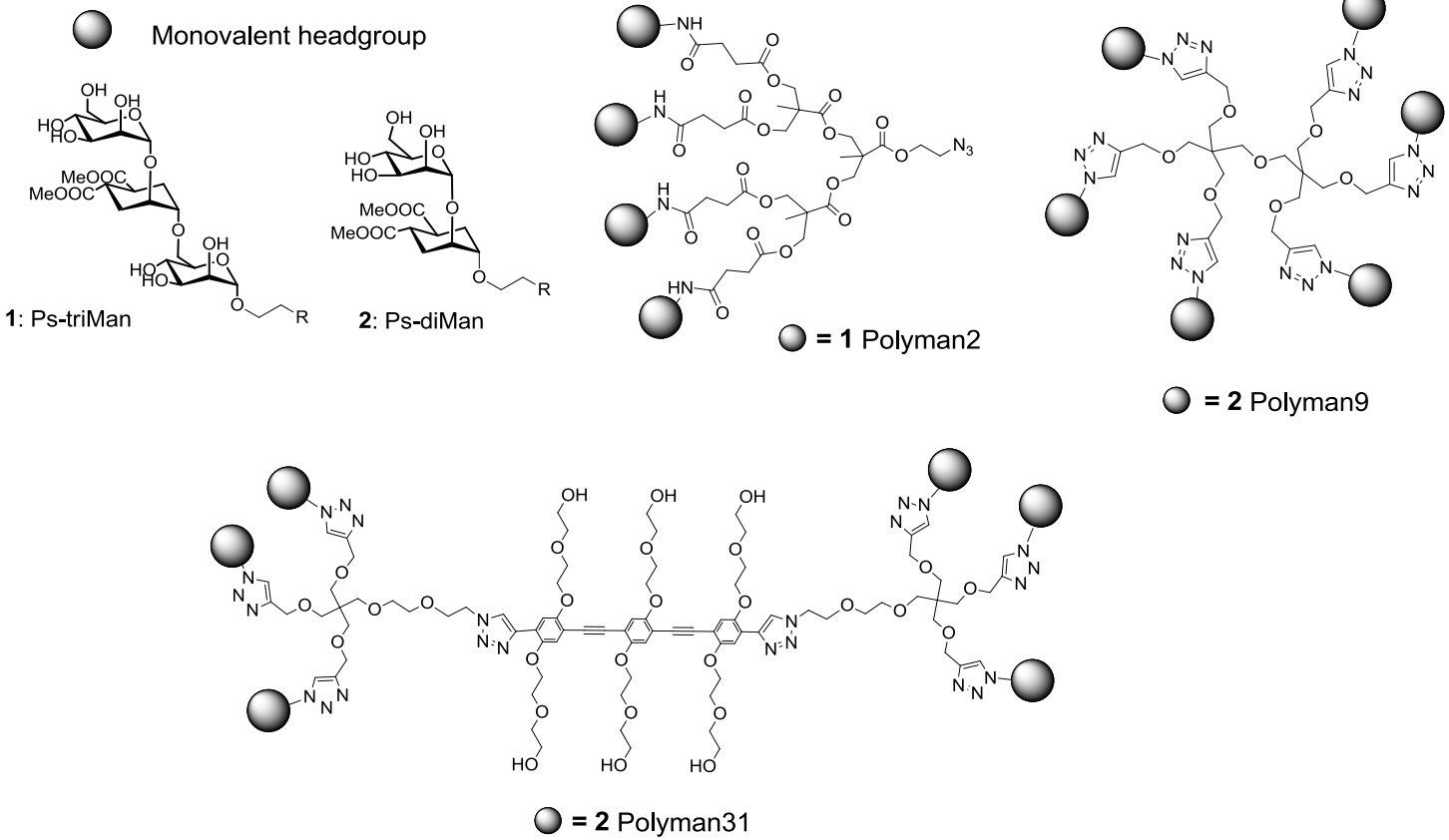
Monovalent headgroup
1: Ps-triMan
2: Ps-diMan
= 1 Polyman2
= 2 Polyman9
= 2 Polyman31

Figure 2

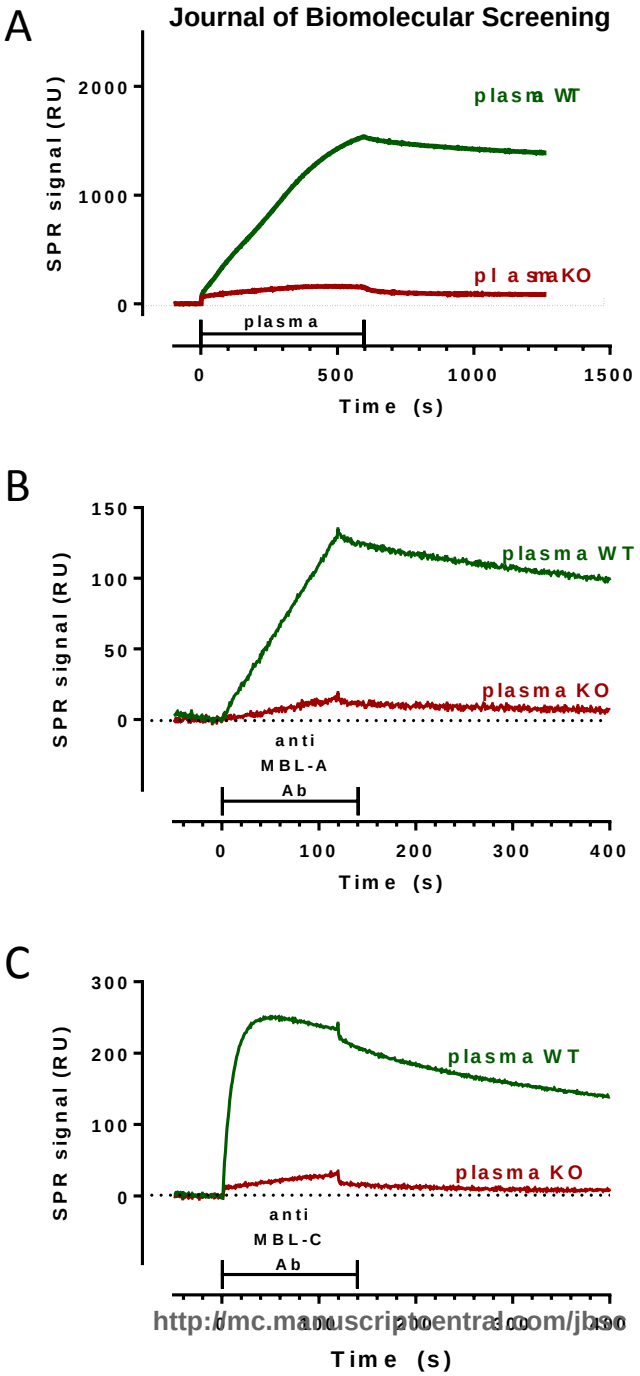

Figure 3

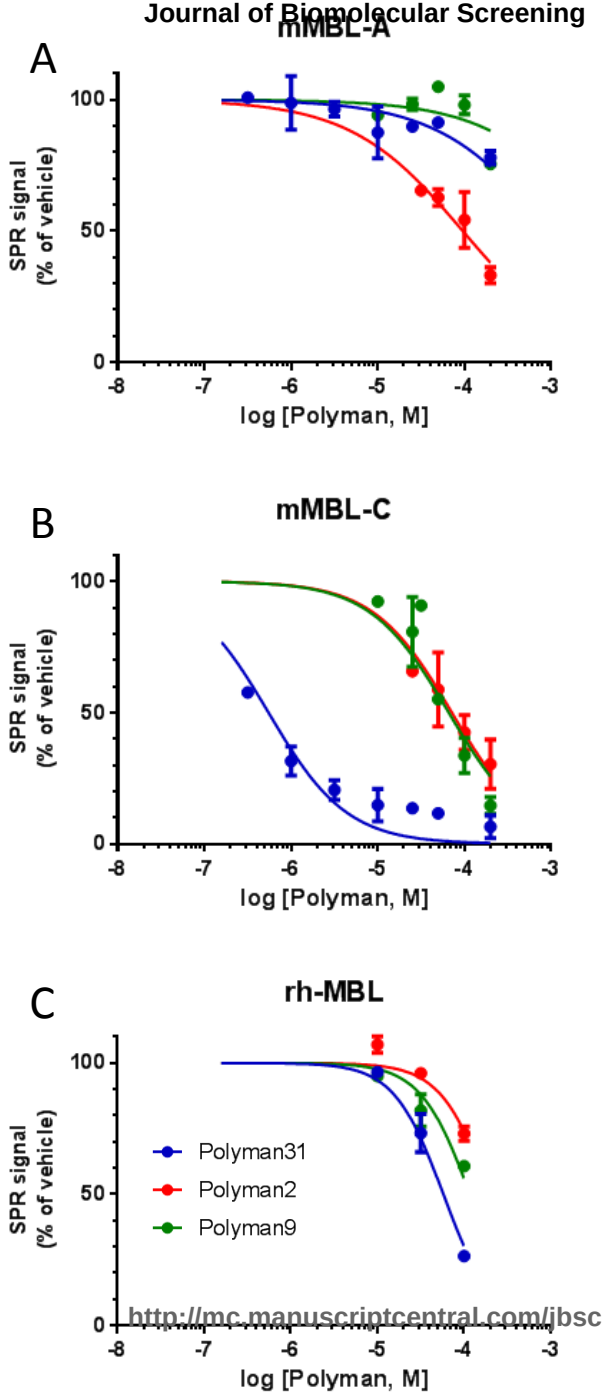

Figure 4

Supporting Information

A new surface plasmon resonance assay for *in vitro* screening of mannose-binding lectin inhibitors

Matteo Stravalaci, Daiana De Blasio, Franca Orsini, Carlo Perego, Alessandro Palmioli, Giulio Goti, Anna Bernardi, Maria-Grazia De Simoni, and Marco Gobbi

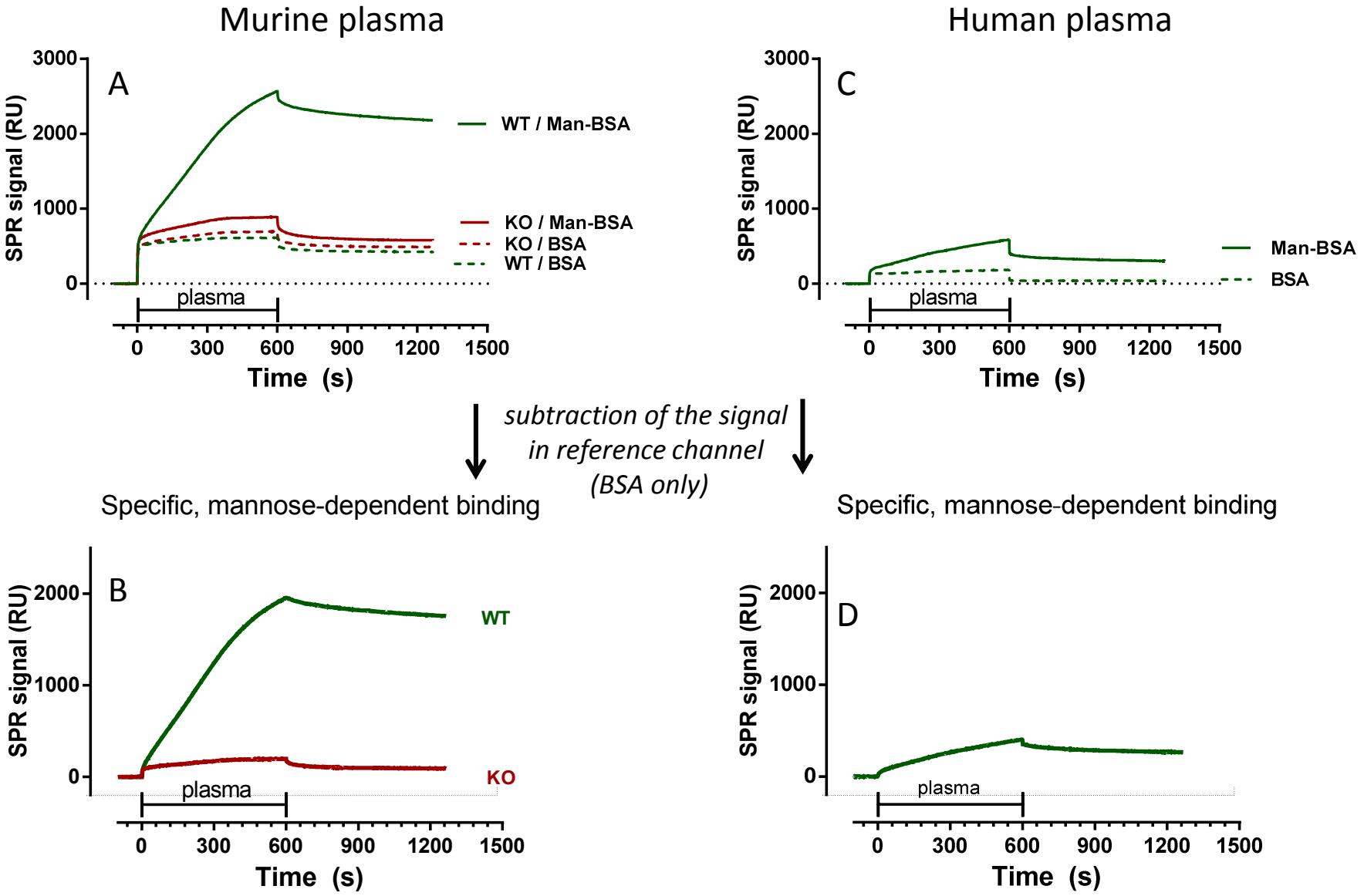

Figure S1

Raw sensorgrams after injection of murine and human plasma, and determination of the mannose-dependent binding signal.

A,C : representative sensorgrams after injection of plasma from wild-type (WT) and MBL-/- (KO) mice, or human plasma over chip surfaces coated with mannosylated-BSA (Man-BSA) or BSA. Plasma (1:50) was injected for 10 min (bars). B,D: normalized sensorgrams obtained after subtraction of the signal observed on the BSA-coated surfaces only. This gives a mannose-dependent SPR binding signal

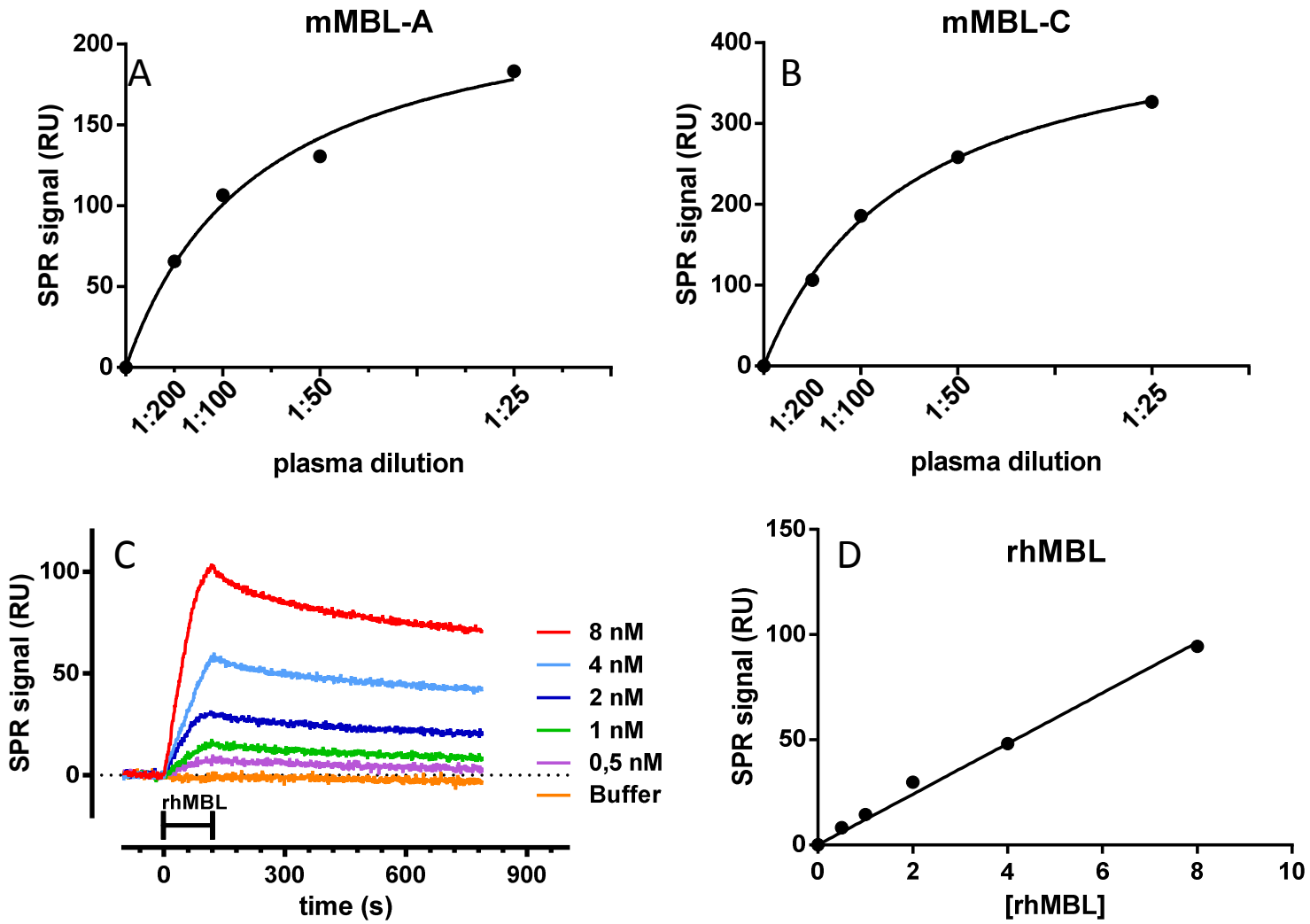

Figure S2

Correlation between MBL concentration and SPR signal.

A-B: the SPR signal due to mMBL-A and mMBL-C correlates with plasma dilution. SPR values i(n resonance units, RU) were measured after injection of anti-mMBL-A or anti-mMBL-C antibodies. Data were fitted with a hyperbola equation. C: Sensorgrams obtained injecting different concentrations of recombinant human MBL (rhMBL) over chip surfaces coated with mannosylated-BSA. rhMBL was injected for 2 min (bar). These sensorgrams were obtained after subtraction of the non-specific signal (even if negligible) measured on a parallel surface coated with BSA. D: linearity between rhMBL concentration and SPR binding signal, measured 100 secs after injection of rhMBL-containing solutions.

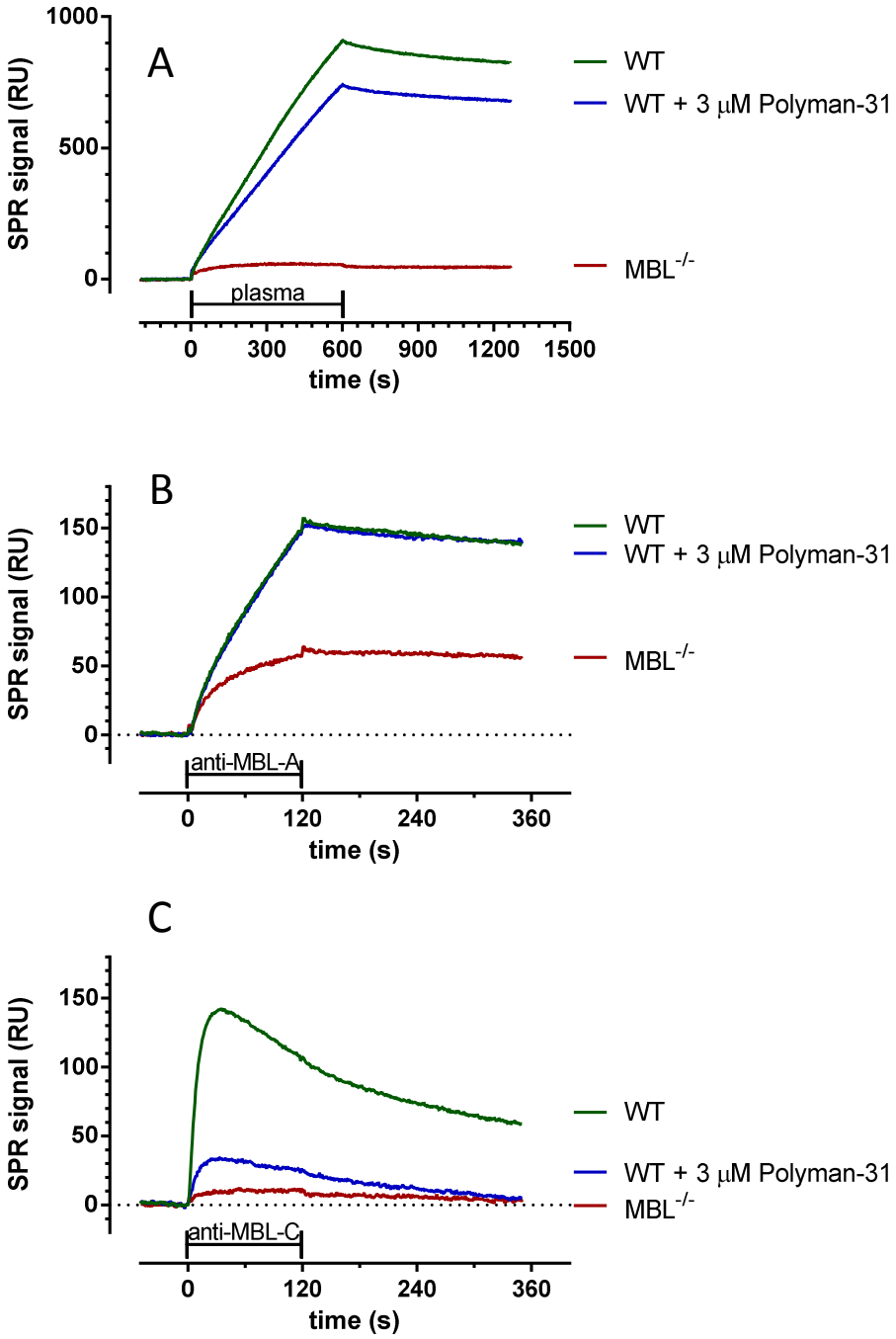

Figure S3

Raw sensorgrams showing the inhibitory effects of 3 µM Polyman31.

A: representative sensorgrams with injection of plasma from wild-type (WT) mice , incubated or not with 3 µM Polyman31, and from MBL$^{-/-}$ (KO) mice, over chip surfaces coated with mannosylated-BSA (Man-BSA). Sensorgrams were obtained after subtraction of the non-specific signal measured on a parallel surface coated with BSA (see also Fig S1). Plasma (1:100) was injected for 10 min (bars). B-C: representative sensorgrams with sequential 2-min injections of anti-MBL-A or anti-MBL-C antibodies shortly after the injection of plasma.