

# Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells

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**Keywords:** carbohydrate recognition profile, C-type lectins, GalNAc, glycan array

## Abstract

**Dendritic cells (DCs) are key to the maintenance of peripheral tolerance to self-antigens and the orchestration of an immune reaction to foreign antigens. C-type lectins, expressed by DCs, recognize carbohydrate moieties on antigens that can be internalized for processing and presentation. Little is known about the exact glycan structures on self-antigens and pathogens that are specifically recognized by the different C-type lectins and how this interaction influences DC function. We have analyzed the carbohydrate specificity of the human C-type lectin macrophage galactose-type lectin (MGL) using glycan microarray profiling and identified an exclusive specificity for terminal  $\alpha$ - and  $\beta$ -linked GalNAc residues that naturally occur as parts of glycoproteins or glycosphingolipids. Specific glycan structures containing terminal GalNAc moieties, expressed by the human helminth parasite *Schistosoma mansoni* as well as tumor antigens and a subset of gangliosides, were identified as ligands for MGL. Our results indicate an endogenous function for DC-expressed MGL in the clearance and tolerance to self-gangliosides, and in the pattern recognition of tumor antigens and foreign glycoproteins derived from helminth parasites.**

## Introduction

Antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages (M $\emptyset$ ), are the key players in the initiation and control of innate and adaptive immune responses. In order to perform their function, both DCs and M $\emptyset$  are equipped with a full array of specialized receptors, including adhesion receptors, co-stimulatory molecules and several pattern recognition receptors, such as C-type lectins and Toll-like receptors (TLR) (1).

Within the last few years several C-type lectins, which recognize specific carbohydrate structures in a Ca<sup>2+</sup>-de-

pendent manner, C-type lectin-like molecules and selectins have been identified on both DCs and M $\emptyset$ , including DC-SIGN (2), mannose receptor, langerin, DEC205 and L-selectin (3). Specific glycosylation patterns regulate leukocyte-homing and -trafficking processes within the immune system (4). Changes in glycosylation can similarly control the interaction of DCs with other cell types, thereby modulating migration and immune responses (5). Most C-type lectins, with the exception of DC-SIGN and mannose receptor, have been poorly characterized with respect to their carbohydrate specificity

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Transmitting editor: J. Borst

Received 12 October 2004, accepted 22 February 2005

Advance Access publication 31 March 2005

and function within the immune system. It has been postulated that C-type lectins function in cell–cell adhesion, antigen recognition and serve as signaling molecules influencing the balance between tolerance and immunity (6). C-type lectin stimulation can either enhance or inhibit TLR signaling, thereby modulating DC phenotype and outcome of immune responses (7, 8). The cytoplasmic tail of C-type lectins often contains signaling motifs or internalization motifs for processing of antigens (9).

Predictions on carbohydrate specificities of C-type lectins for either galactose-type or mannose-type glycans can be made based on the primary amino acid sequence. However, knowledge on the exact carbohydrate recognition profile is essential to understand the importance of these receptors in immune-related functions. Studies on carbohydrate recognition have long been hampered due to the complexity of glycan synthesis and the limited availability of isolated or synthesized glycans.

Recognition of mannose and fucose structures by DC-SIGN and mannose receptor on DCs has been widely investigated. However, studies on galactose or GalNAc recognition by DCs are limited. One galactose-type C-type lectin has been reported to be expressed by human DCs, namely the macrophage galactose-type lectin [MGL, also called DC-asialoglyco protein receptor (DC-ASGP-R) or human macrophage lectin (HML)] (10–12). MGL is a member of the type II family of C-type lectins. MGL is expressed on human and mouse immature DCs and M $\phi$  in skin and lymph node (13). No natural ligand or function for MGL has been established yet (14). Mice contain two functional copies of the MGL gene, mMGL1 and mMGL2, whereas in humans only one MGL gene is found. mMGL1 and mMGL2 have different carbohydrate specificities, respectively, for Lewis X and  $\alpha/\beta$ -GalNAc structures (15). Earlier studies on COS-1 transfectants of MGL suggested a specificity for the monosaccharides, galactose and GalNAc (12). In contrast, recombinant MGL produced in a bacterial expression system displayed restricted binding to GalNAc (16). The recognition of more complex oligosaccharides by human MGL has not been thoroughly investigated yet.

To gain more insight in the function and carbohydrate specificity of MGL and galactose/GalNAc recognition by human DCs we set out to identify the carbohydrate recognition profile of human MGL using glycan microarray screening. The glycan array was developed by the Consortium for Functional Glycomics (<http://web.mit.edu/glycomics/consortium>) and consists of >100 synthetic and natural glycan structures. The use of glycan arrays for the elucidation of carbohydrate recognition profiles of individual C-type lectins has been applied to Selectins, langerin and DC-SIGN homologues (17–19).

Using an MGL-Fc chimeric protein, we identified oligosaccharides containing terminal  $\alpha$ - or  $\beta$ -linked GalNAc residues as high-affinity ligands for MGL. Such terminal GalNAc residues can be part of protein N- or O-linked glycans, or glycosphingolipids. Identification of this specificity led to the discovery of glycan antigens within egg glycoproteins of the pathogenic helminth *Schistosoma mansoni* as counter-structures for MGL. In addition, MGL strongly interacted with tumor cells in a GalNAc-specific manner. Our results strongly implicate a role for MGL in recognition of self-gangliosides, tumor antigens and pathogenic helminths by DCs.

## Methods

### Cells

The adenocarcinoma cell lines SW948, SKBR3 and ZR75-1, CHO and CHO-MGL cells and the melanoma cell lines BLM, FM3.29, FM6, SK23mel, 90.07 and 00.09 were maintained in RPMI or DMEM medium (Invitrogen, Carlsbad, CA, USA) containing 8–10% FCS.

Immature monocyte-derived DCs were cultured for 5–7 days from monocytes obtained from buffy coats of healthy donors (Sanquin, Amsterdam, The Netherlands) in the presence of IL-4 (500 U ml<sup>-1</sup>) and granulocyte macrophage colony-stimulating factor (800 U ml<sup>-1</sup>).

### Antibodies and reagents

The following mAbs were used: MLD-1 [anti-MGL (10)], AZN-D1 (anti-DC-SIGN), SMLDN1.1 and SMFG4.1 [anti-Lac-di-NAc (LDN) (20) and anti-Fuc $\alpha$ 3-Lac-di-NAc (anti-LDNF), respectively, provided by A. Nyame and R. Cummings, (University of Oklahoma Health Sciences Center, OK, USA) and 6H3 (anti-Lewis X). Biotinylated polyacrylamide (PAA)-coupled glycoconjugates were obtained from Lectinity (~20% substitution, Lappeenranta, Finland). Crude *S. mansoni* soluble egg antigen (SEA) extract was prepared as previously described (provided by F. Lewis) (21). Forssman glycolipid was provided by R. Geyer (University of Giessen, Germany). DC-SIGN-Fc has been described previously (22).

The peroxidase-labeled or biotinylated lectins Con A (*Canavalia ensiformis*), *Helix pomatia* agglutinin (HPA) (*Helix pomatia*), *Maackia amurensis* agglutinin (MAA) (*Maackia amurensis*), peanut agglutinin (*Arachis hypogaea*), soybean agglutinin (SBA) (*Glycin max*), *Sambucus nigra* bark agglutinin (SNA) (*Sambucus nigra*) and Wheat germ agglutinin (WGA) (*Triticum vulgare*) were obtained from Sigma–Aldrich (St Louis, MO, USA).

### Isolation and expression of the cDNA-encoding MGL and MGL-Fc

The cDNA-encoding human MGL (12) was amplified on total RNA from immature DCs, cloned into expression vector pRc/CMV and confirmed by sequence analysis. Stable CHO transfectants were generated using lipofectamin (Invitrogen). MGL-positive cells were sorted using the MoFlo (DAKOcytation, Glostrup, Denmark).

The extracellular part of MGL (amino acids 61–289) was amplified on pRc/CMV-MGL with PCR, confirmed by sequence analysis and fused at the C-terminus to human IgG1-Fc in the Sig-plgG1-Fc vector. MGL-Fc was produced by transient transfection of CHO cells. MGL-Fc concentrations were determined by ELISA.

### Glycan array (Consortium for Functional Glycomics)

Biotinylated synthetic or natural glycan structures were coated at saturating densities to streptavidin-coated high binding capacity black plates (Pierce, Rockford, IL, USA) and probed with MGL-Fc (2.5  $\mu$ g ml<sup>-1</sup>). Bound MGL-Fc was detected using a FITC-labeled anti-human IgG-Fc antibody. Plates were read at 485–535 nm on a Wallac Victor<sup>2</sup> 1420

multi-label counter (Perkin Elmer, Wellesley, MA, USA). Standard procedures for glycan array testing are available at <http://web.mit.edu/glycomics/consortium>. The repertoire of glycan structures probed are described elsewhere (23, 24).

#### MGL-Fc adhesion assay

*Schistosoma mansoni* SEAs and biotinylated PAA-coupled glycoconjugates were coated ( $5 \mu\text{g ml}^{-1}$  or as indicated) on streptavidin-coated plates (Pierce) or NUNC maxisorb plates (Roskilde, Denmark) overnight at room temperature. Plates were blocked with 1% BSA and MGL-Fc was added ( $1 \mu\text{g ml}^{-1}$ ) for 2 h at room temperature in the presence or absence of 10 mM EGTA or  $20 \mu\text{g ml}^{-1}$  mAbs. Binding was detected using a peroxidase-labeled anti-human IgG-Fc antibody (Jackson, West grove, PA, USA).

To identify the carbohydrate nature of the MGL ligands, NUNC maxisorb plates were coated with goat anti-human Fc antibody ( $4 \mu\text{g ml}^{-1}$ , Jackson), followed by a 1% BSA-blocking step (30 min at  $37^\circ\text{C}$ ) and MGL-Fc ( $1 \mu\text{g ml}^{-1}$  for 1 h at  $37^\circ\text{C}$ ). MGL-Fc-coated plates were incubated overnight at  $4^\circ\text{C}$  with tumor cell lysates ( $10 \cdot 10^6$  cells  $\text{ml}^{-1}$ ). After extensive washing,  $1 \mu\text{g ml}^{-1}$  biotinylated or peroxidase-labeled lectins (Sigma-Aldrich) were added for 2 h at room temperature. Binding of biotinylated lectins was detected using peroxidase-labeled avidin (Vector Laboratories, Burlingame, CA, USA).

#### Flow cytometry and cellular adhesion assays

Cells were incubated with primary antibody ( $5 \mu\text{g ml}^{-1}$ ), followed by staining with a secondary FITC-labeled anti-mouse antibody (Zymed, San Francisco, CA, USA) and analyzed on FACSCalibur (BD Pharmingen, San Diego, CA, USA).

Streptavidin-coated fluorescent beads (488/645 nm, Molecular Probes, Eugene, OR, USA) were incubated with  $1 \mu\text{g}$  of the PAA-coupled glycoconjugates or biotinylated SEA. Fluorescent bead adhesion assay was performed as previously described (2) and analyzed on FACSCalibur and presented as the percentage of cells which have bound the fluorescent beads.

Ninety-six-well plates (NUNC maxisorb) were coated overnight at room temperature with biotinylated PAA-glycoconjugates ( $5 \mu\text{g ml}^{-1}$ ) or SEA ( $2 \mu\text{g ml}^{-1}$ ) and afterwards blocked with 1% BSA. Calcein AM-labeled DCs (Molecular Probes) were added for 1.5 h at  $37^\circ\text{C}$  in the presence or absence of 10 mM EGTA or  $10 \mu\text{g ml}^{-1}$  mAbs. Non-adherent cells were removed by gentle washing. Adherent cells were lysed and fluorescence was quantified on a Fluorstar spectrofluorimeter (BMG Labtech, Offenburg, Germany).

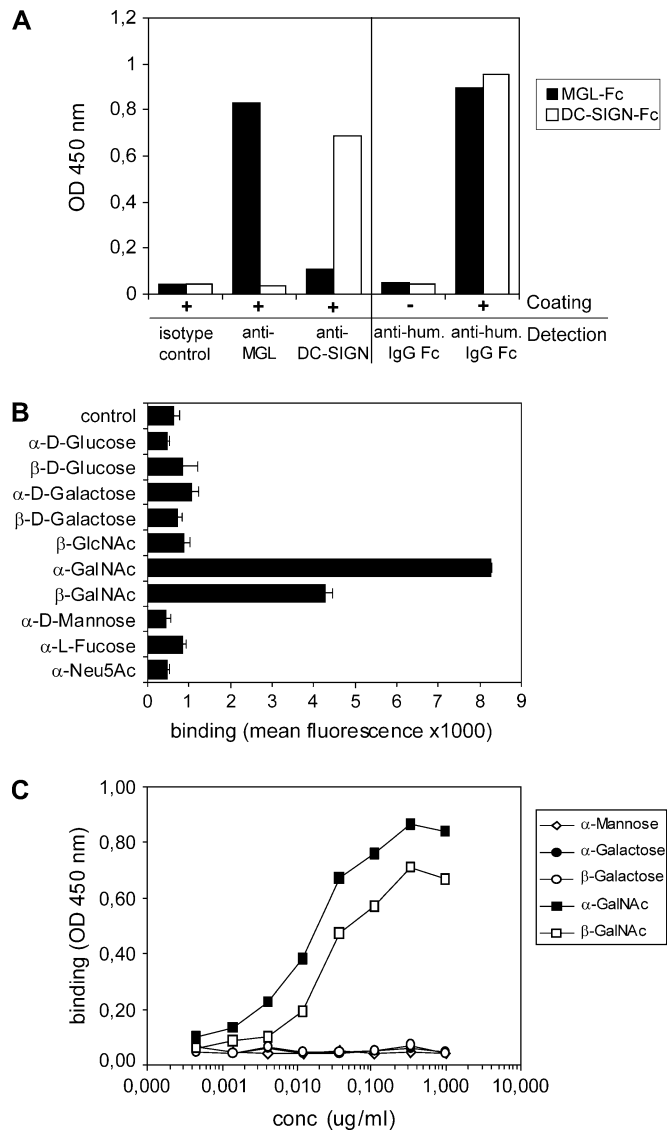
## Results

### MGL specifically recognizes terminal $\alpha$ - or $\beta$ -linked GalNAc residues that naturally occur as part of glycoproteins or glycosphingolipids

To allow efficient screening of multiple potential carbohydrate ligands, we constructed an MGL-Fc chimeric protein, with the extracellular portion of human MGL (amino acids 61–289) fused to a human IgG1-Fc tail. Recombinant MGL-Fc was produced in CHO cells and using an ELISA-based method we

show that MGL-Fc indeed comprises the extracellular domains of MGL fused to the human IgG1 tail (Fig. 1A).

To identify the carbohydrate recognition profile and potential function of MGL, the MGL-Fc chimera was used to screen for carbohydrate ligands on the glycan array of the Consortium for Functional Glycomics (23, 24). MGL-Fc strongly bound the monosaccharides  $\alpha$ -GalNAc and  $\beta$ -GalNAc, whereas no interaction was observed with the related sugar galactose or other monosaccharides tested (Fig. 1B). The exclusive specificity of MGL for GalNAc was confirmed using  $\alpha$ - and



**Fig. 1.** MGL specifically recognizes GalNAc residues. (A) MGL-Fc comprises a human IgG1 tail and the extracellular domains of MGL. MGL-Fc was detected in ELISA by anti-MGL and anti-human IgG-Fc antibodies. DC-SIGN-Fc was used as a control. (B) MGL interacts with GalNAc residues. MGL-Fc binding to monosaccharides coated on the glycan array was detected by FITC-labeled anti-human Fc antibody. (C) MGL recognizes multivalent GalNAc. Streptavidin plates were coated with biotinylated PAA-glycoconjugates. MGL-Fc binding was determined by ELISA. Standard deviation  $<0.02$  OD 450 nm. One representative experiment out of three is shown.

$\beta$ -GalNAc monosaccharides multivalently linked to PAA (Fig. 1C). Our results demonstrate that MGL exclusively recognizes GalNAc, both in  $\alpha$ - or  $\beta$ -linked configuration, whereas no specificity for either  $\alpha$ - or  $\beta$ -galactose is observed.

We next investigated whether MGL recognizes GalNAc moieties present in extended oligosaccharides. During O-glycan synthesis  $\alpha$ -GalNAc is substituted with other monosaccharides to form several O-glycan core structures. Using the glycan array we investigated whether MGL recognizes specific O-glycan core structures (Fig. 2A). MGL specifically interacted with a single  $\alpha$ -GalNAc residue, also known as the Tn-antigen. Sialylation of the  $\alpha$ -GalNAc residue completely abrogated MGL binding, whereas sulfation did not alter the MGL reactivity. Substitution on position 3 of the  $\alpha$ -GalNAc, as found in the core 1–4 structures, abrogated MGL binding. In contrast, addition of another  $\alpha$ -GalNAc-residue to position 3 (core 5) or a  $\beta$ -GlcNAc to position 6 (core 6) did not affect MGL binding activity (Fig. 2A). Thus, MGL-Fc specifically recognizes Tn-antigen and core 5 and 6 O-glycan structures.

Glycan antigens with terminal  $\beta$ -GalNAc residues, such as the LDN (GalNAc $\beta$ 1-4GlcNAc) glycan epitope and its derivative LDNF (GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc), are found in humans, but are much more abundantly expressed by human helminth parasites such as *S. mansoni* (25, 26). In humans complex-type glycans usually contain a lactosamine unit (LacNAc, Gal $\beta$ 1-4GlcNAc) or Lewis X (Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc). In-

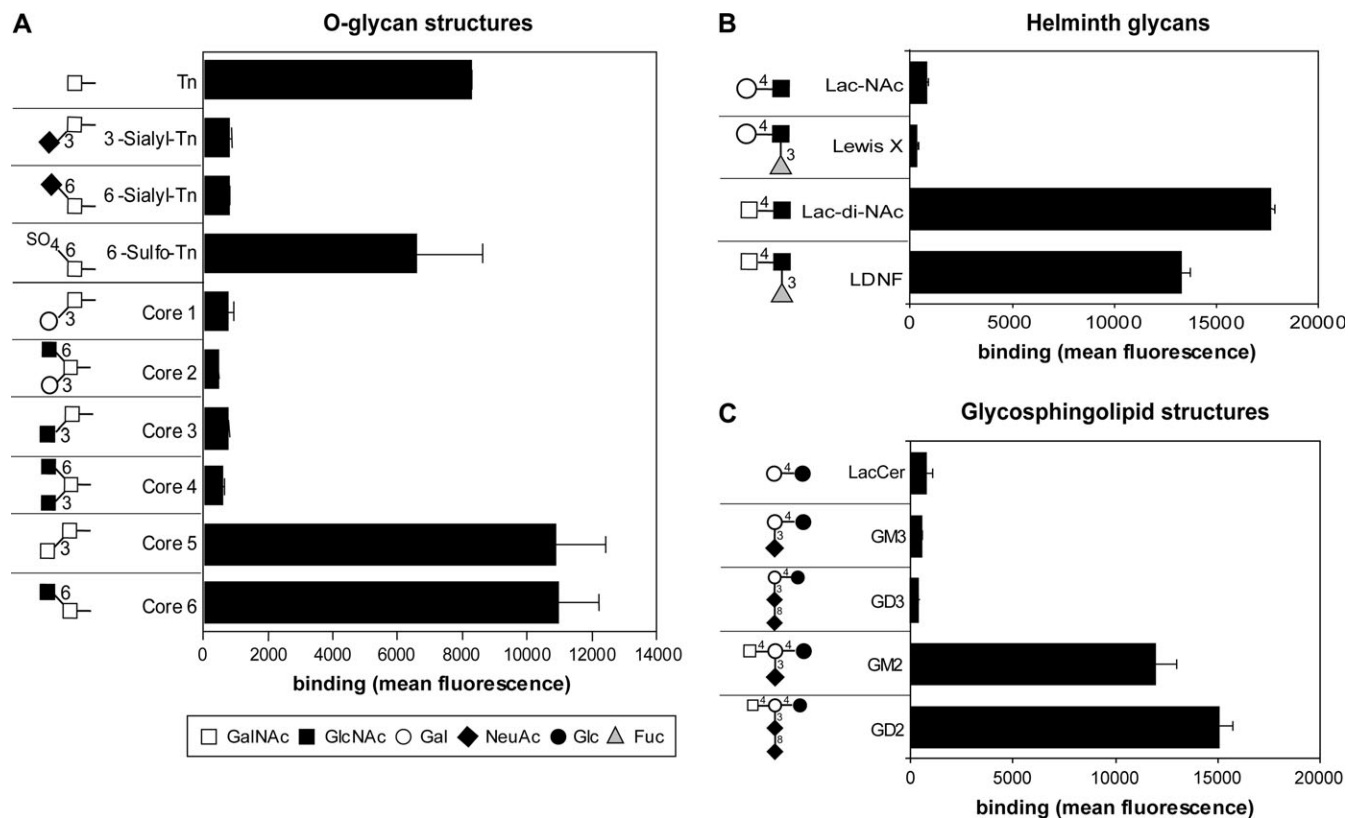
deed MGL recognized both LDN and LDNF oligosaccharides, but not their galactose-containing counterparts Lactosamine or Lewis X (Fig. 2B).

Certain glycosphingolipids contain terminal GalNAc residues, such as the gangliosides GM2, GD2 and the Forssman glycolipid (globopentosylceramide, GalNAc $\alpha$ 1-3GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4GlcCer). MGL strongly interacted with the oligosaccharide component of GM2 and GD2, whereas no binding was observed to oligosaccharides that do not contain a terminal GalNAc, as found in GM3 and GD3 (Fig. 2C). In addition, MGL-Fc recognized the Forssman glycolipid in a glycolipid ELISA (results not shown).

Our results strongly support a MGL recognition profile of terminal  $\alpha$ - and  $\beta$ -linked GalNAc residues (Table 1).

#### MGL is the major GalNAc receptor on human DCs

To confirm the GalNAc specificity of MGL-Fc, MGL-expressing cells were analyzed for their carbohydrate-binding characteristics. CHO-MGL transfectants expressed high levels of MGL on its cell surface (Fig. 3A). Both  $\alpha$ -GalNAc- and  $\beta$ -GalNAc-PAA-coupled beads showed strong binding activity to CHO-MGL, but not to the parental CHO cells. MGL-mediated adhesion is completely inhibited by the Ca<sup>2+</sup>-chelator EGTA or an anti-MGL antibody, but not by an isotype control antibody. Neither  $\alpha$ - nor  $\beta$ -galactose was recognized by CHO-MGL



**Fig. 2.** MGL recognizes terminal GalNAc residues in O-glycans, helminth-associated glycans and glycans that are part of glycosphingolipids. MGL-Fc binding was determined by glycan array analysis of (A) O-glycan core structures, (B) LDN and LDNF glycans and the related oligosaccharides LacNAc and Lewis X and (C) ganglioside glycan structures. Schematic representation of all carbohydrate structures tested is indicated in the figures.



**Table 1.** Overview of carbohydrate structures recognized by the C-type lectin MGL

Affinity	Glycan structure	Name	S/N ratio <sup>a</sup>	
High	GalNAc $\alpha$ 1-3Gal-	Adi	40.17	
	GalNAc $\beta$ 1-4GlcNAc-	LDN	35.68	
	Neu5Ac $\alpha$ 1-8Neu5Ac $\alpha$ 1-3(GalNAc $\beta$ 1-4)Gal $\beta$ 1-4Glc $\beta$ -	GD2	30.35	
	Bi-LDNF	Biantennary LDNF	28.97	
	GalNAc $\alpha$ 1-3(Fuc $\alpha$ 1-2)Gal $\beta$ 1-4GlcNAc $\beta$ -	A (type 2)	27.50	
	GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc-	LDNF	26.75	
	Neu5Ac $\alpha$ 1-3(GalNAc $\beta$ 1-4)Gal $\beta$ 1-4Glc $\beta$ -	GM2	24.11	
	GlcNAc $\beta$ 1-6GalNAc $\alpha$ -	Core 6	22.06	
	GalNAc $\alpha$ 1-3GalNAc $\alpha$ -	Core 5	21.90	
	$\alpha$ -GalNAc-	$\alpha$ -N-acetyl-D-galactosamine	16.62	
	Bi-LDN	Biantennary LDN	13.32	
	6-Sulfo-GalNAc $\alpha$ -	$\alpha$ -N-acetyl-D-galactosamine-6-sulfate	13.25	
	Medium	GalNAc $\alpha$ 1-3(Fuc $\alpha$ 1-2)Gal $\beta$ -	Atri-long	10.11
		$\beta$ -GalNAc	$\beta$ -N-acetyl-D-galactosamine	8.62
	Low	Gal $\beta$ 1-4GlcNAc $\beta$ 1-6GalNAc $\alpha$ -	6-LacNAc-Tn	6.57

<sup>a</sup>Signal to noise ratio. Signal ratios (S) were determined by dividing the fluorescence value obtained for each carbohydrate by the background fluorescence level (497 fluorescence units). All signal ratios were averaged out to a noise ratio (N) of 4.04. Individual signals (S) were then divided by 4.04 to give the listed signal to noise ratio (S/N). High-affinity S/N > 3  $\times$  N; medium-affinity S/N > 2  $\times$  N, low-affinity S/N > 1  $\times$  N.

(Fig. 3B), indicating that cell surface-expressed MGL exhibits a similar GalNAc specificity as recombinant MGL-Fc.

Human immature DCs naturally express MGL on the cell surface (Fig. 3A). Despite the fact that DCs express multiple C-type lectins, binding of  $\alpha$ - or  $\beta$ -GalNAc to DCs was substantially blocked by anti-MGL antibodies and not by the anti-DC-SIGN antibody (Fig. 3C). DCs displayed only low binding to galactose, which could not be attributed to MGL, as the interaction could not be blocked by anti-MGL antibodies. DC-expressed MGL displayed an exclusive GalNAc specificity and our results clearly indicate a major role for MGL in GalNAc recognition by DCs.

#### MGL functions as a pattern recognition receptor for helminth antigens

The specific recognition of LDN and LDNF by MGL (Fig. 2) prompted us to look at the interaction of MGL with helminth glycan antigens. The SEA of the human parasite *S. mansoni* were selected as a natural source for LDN and LDNF glycans.

Both LDN-PAA- and SEA-coupled beads showed high binding activity to CHO-MGL, but not to parental CHO cells (Fig. 4A). MGL-mediated adhesion was specific, as shown by the complete block with anti-MGL antibodies or EGTA, but not with an isotype control antibody.

Since SEA is composed of several glycoproteins, mAbs against carbohydrate epitopes were used to determine the relative contribution of LDN and LDNF in the binding of MGL to SEA (Fig. 4B). Binding of MGL to SEA was substantially blocked by anti-LDN mAbs (38% reduction) and to a lesser extent by anti-LDNF mAbs (22% reduction), whereas a combination of both anti-LDN and anti-LDNF mAbs further reduced MGL reactivity (46% inhibition), indicating that MGL recognizes both terminal  $\beta$ -GalNAc residues of LDN and LDNF structures present in SEA.

Next, blocking antibodies were used to determine the relative contribution of MGL in relation to other DC-expressed C-type lectins, in the binding of LDN and SEA. MGL mediated 50% of the adhesion of DCs to LDN, as shown by the

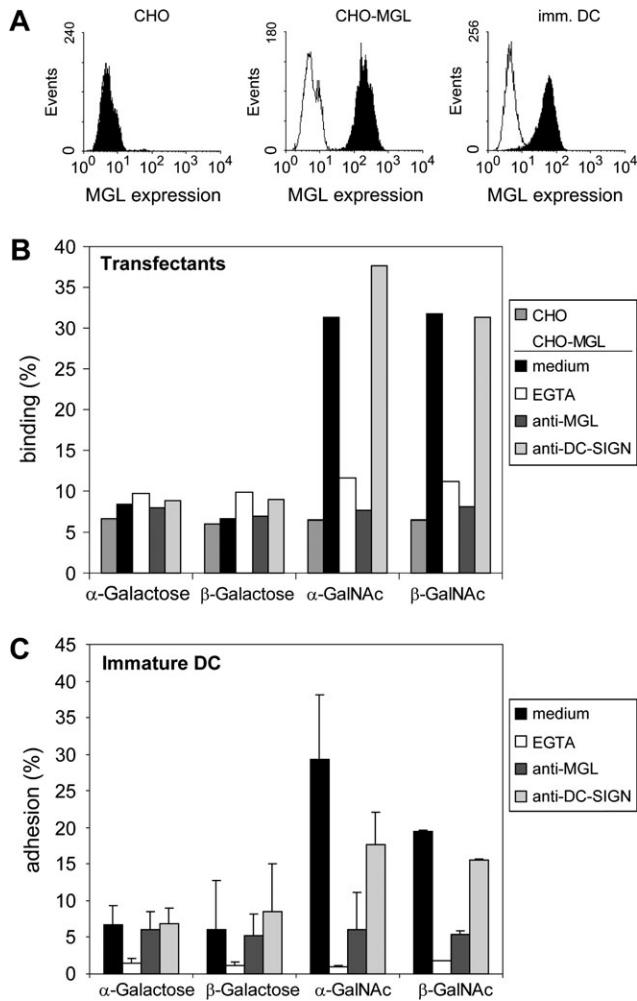
significant reduction in binding with anti-MGL antibodies (Fig. 4C). Since other C-type lectins on DCs, such as DC-SIGN, are reported to be involved in binding SEA through Lewis X structures (20), the interaction of DCs with SEA was further analyzed using blocking anti-MGL and anti-DC-SIGN antibodies. Both MGL and DC-SIGN are responsible for 30% of the DC reactivity, whereas a combination of anti-DC-SIGN and anti-MGL antibodies reduced adhesion by 50% (Fig. 4C). Therefore, DC-expressed MGL functions as a pattern recognition receptor for helminth parasites.

#### The tumor-specific Tn-antigen is bound with high affinity by MGL

Tumorigenicity is associated with an increased degree of sialylation and a reduction in length of the O-glycans expressed (27). Tumor cells, especially of adenocarcinoma origin, are frequently positive for the Tn-antigen (single  $\alpha$ -GalNAc linked to Serine or Threonine). The tumor-associated Tn-antigen was preferentially recognized by MGL (Fig. 2A); therefore, several adenocarcinoma cell lines and melanoma cell lines were analyzed for specific recognition by MGL. All adenocarcinoma cell lines were tested and three out of six melanoma cell lines displayed a strong interaction with MGL-Fc (Fig. 5A).

One high-binding adenocarcinoma cell line, ZR75-1, and one melanoma cell line, 00.09, were selected to investigate the nature of the recognized carbohydrates on tumor glycoproteins. Carbohydrate ligands were captured from tumor cell lysates with MGL-Fc and probed with commercially available plant/invertebrate lectins with known specificities. The lectins, SBA and HPA, which both have specificity for  $\alpha$ -GalNAc residues, showed consistent reaction with glycoproteins, captured by MGL from tumor cells (Fig. 5B), indicating the presence of terminal  $\alpha$ -GalNAc residues on the tumor antigens recognized by MGL.

Since our previous data showed that recombinant and cellular MGL have identical carbohydrate recognition profiles, these results indicate that MGL might function as a DC-specific receptor for tumor-derived cell types.

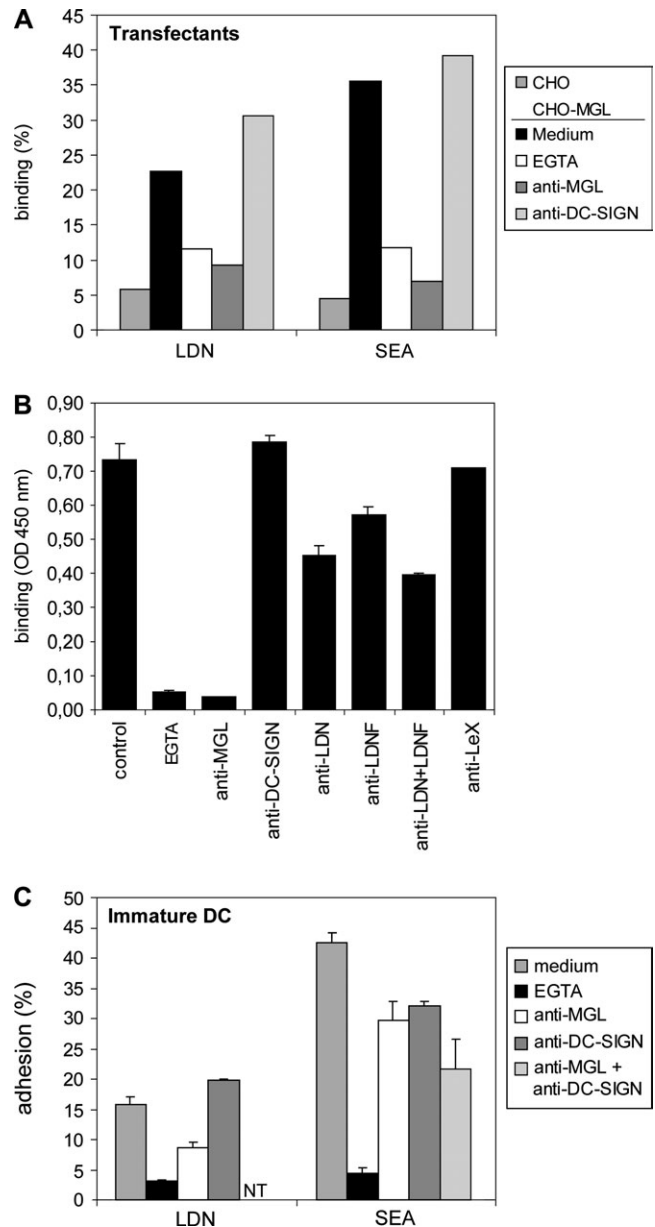


**Fig. 3.** Both cellular and recombinant MGL have identical specificities for  $\alpha$ - and  $\beta$ -linked GalNAc. (A) MGL expression on CHO transfectants and immature DCs. Open histograms represent the isotype control and filled histograms represent the anti-MGL mAb staining. (B) CHO-MGL binds strongly interact with GalNAc but not with galactose in the bead adhesion assay. Specificity was determined in the presence of the  $\text{Ca}^{2+}$ -chelator EGTA, blocking antibodies to MGL or isotype control antibody (anti-DC-SIGN). Standard deviation for the bead adhesion assay is  $<5\%$ . (C) MGL is the major GalNAc receptor on immature DCs. Binding of DCs to PAA-coupled carbohydrates was determined using plate adhesion assay in the presence or absence of EGTA, blocking antibodies to MGL or DC-SIGN. All results are representative for three independent experiments.

## Discussion

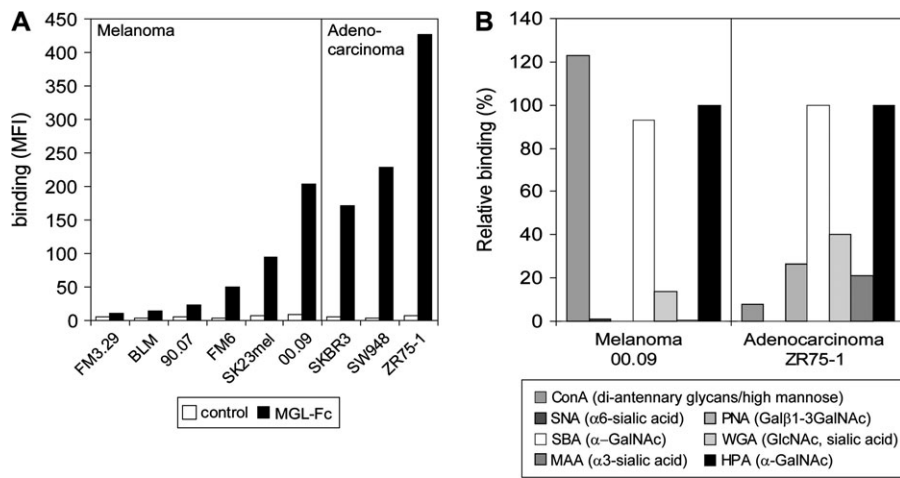
Here we report the carbohydrate recognition profile of the C-type lectin MGL and the implications these results may have for the recognition of self-gangliosides, helminth parasites and tumor cells by DCs.

Using a high-throughput glycan array screening, developed by the Consortium for Functional Glycomics, terminal  $\alpha$ - and  $\beta$ -linked GalNAc residues were identified as the main carbohydrate determinants for MGL recognition. Carbohydrate microarrays for studying protein-carbohydrate interactions are just emerging and most of them work as a proof-of-



**Fig. 4.** MGL strongly interacts with soluble egg glycoproteins of *Schistosoma mansoni*. (A) CHO-MGL binds to LDN glycans and SEA as measured by bead adhesion assay. Specificity was determined in the presence of the  $\text{Ca}^{2+}$ -chelator EGTA, blocking antibodies to MGL or isotype control antibody (anti-DC-SIGN). Standard deviation for the bead adhesion assay is  $<5\%$ . (B) MGL interacts with LDN and LDNF glycans present in the SEA. Binding of MGL-Fc to SEA was determined by ELISA in the presence or absence of EGTA, blocking mAbs to MGL and DC-SIGN (isotype control) or specific anti-glycan mAbs. (C) Binding of SEA to immature DCs is mediated by both MGL and DC-SIGN. Binding of DCs was determined by plate adhesion assay in the presence or absence of EGTA, blocking mAbs to MGL or DC-SIGN; NT, not tested. All results are representative for three independent experiments.

principle, using lectins with known specificities to validate the array system (17). To our knowledge, this is one of the first reports describing the identification of the carbohydrate recognition profile of a single C-type lectin with the use of



**Fig. 5.** MGL recognizes  $\alpha$ -GalNAc residues on tumor cells. (A) MGL recognizes both adenocarcinoma and melanoma tumor cells. Binding of MGL-Fc to tumor cell lines was determined by flow cytometry. (B) MGL recognizes GalNAc residues on tumor glycoproteins. Presence of specific carbohydrate structures on glycoproteins captured by MGL was determined using biotinylated plant/invertebrate lectins with known specificities in an ELISA-based assay. Values were normalized to the HPA reactivity. Standard deviation  $<0.02$  OD 450 nm. All results are representative for three independent experiments.

a carbohydrate array. Carbohydrate recognition profiles of Selectins, Langerin and DC-SIGN homologues have been further refined with the use of glycan arrays (17–19).

Both galactose/GalNAc and GalNAc specificity have been reported for human MGL (12, 16). Initially, the specificity of MGL was evaluated using lysates of MGL transfected into COS-1. Purification on a galactose-sepharose column showed that galactose, GalNAc and even fucose were able to elute MGL from the purification column (12). However, subsequent binding studies with recombinant MGL produced in a bacterial expression system identified a restricted GalNAc specificity (16). Moreover, the MGL specificity was not confirmed for cells naturally expressing MGL. In this article, we define the carbohydrate recognition profile of human MGL-Fc chimeric protein by glycan array and confirm this profile using MGL transfectants and MGL-positive immature DCs.

Our data clearly demonstrate that both recombinant MGL-Fc and MGL expressed by transfectants and DCs have an identical and exclusive specificity for terminal  $\alpha$ - or  $\beta$ -linked GalNAc residues and no specificity for galactose. Substitution on position 3 or 4 of the GalNAc residue and sialylation, either on position 3 or 6 of the GalNAc, completely eliminate MGL recognition. The effect of the sialylation could be due to the addition of a dominant negative charge, which has been described for other C-type lectins to interfere in binding (22). However, addition of a negative charge in the form of a sulfate group on position 6 does not affect MGL binding (Fig. 2A). The blocking effect of sialic acid on position 6 of the GalNAc is unlikely to be due to steric hindrance, since the addition of GlcNAc on this position does not preclude MGL recognition.

The exclusive GalNAc specificity indicates that human MGL is functionally most closely related to mouse mMGL2, which shares the GalNAc specificity with human MGL (15).

Our finding that anti-MGL antibodies do not completely block binding of  $\alpha$ -GalNAc or LDN to DCs suggests that DCs express next to MGL another receptor with GalNAc specificity. No candidate C-type lectins have been reported to be

expressed by immature DCs, since the well-known galactose/GalNAc-specific lectin ASGP-R (28), abundantly expressed by liver parenchymal cells, is not expressed by DCs (data not shown). Although MGL and ASGP-R have partially overlapping carbohydrate recognition profiles (GalNAc and galactose/GalNAc, respectively), the exclusive expression of MGL on immature DCs and M $\phi$  implicates non-redundant cellular functions for these C-type lectins. Recently, Galectin-3 was shown to be involved in uptake of SEA by M $\phi$  through recognition of LDN (29). Since Galectin-3 is expressed by DCs (30), Galectin-3 might be a promising candidate receptor. Recognition of LDN by DCs could therefore be mediated by both MGL and Galectin-3 simultaneously.

Most C-type lectins contain special motifs within their cytoplasmic tails, which facilitate antigen uptake and thereby enhance antigen processing and presentation. Although C-type lectins do not directly stimulate the immune system, they affect the balance between immunity and tolerance by influencing TLR signaling (8, 31). C-type lectins probably have an important endogenous role in maintaining tolerance towards self-glycoproteins (5).

The specific interaction of MGL with SEA glycoproteins containing LDN and LDNF demonstrates that MGL functions as a pattern recognition receptor for the human helminth parasite *S. mansoni*. SEA are known to skew the immune system towards a T<sub>H</sub>2-type response (32). Recently, Ebola and Marburg filoviruses have been reported as pathogenic ligands for human MGL (33). Interestingly, both SEA and filoviruses target DC-SIGN as well, in a fucose and high-mannose-type manner, respectively (20, 34). In addition, the mouse mannose receptor recognizes SEA in a mannose-dependent fashion (35). The residual binding of DCs to SEA, after complete block of MGL and DC-SIGN, might therefore be mediated by the human mannose receptor. Since several pathogens misuse the tolerogenic pathway induced by C-type lectins for their own survival (36), it will be interesting to pursue how targeting of pathogens to MGL and other cooperating C-type lectins may

modulate DC maturation and the induction of adaptive immune responses.

Furthermore, MGL recognized glycosphingolipids, mainly of the ganglioside subtype. As MGL has the capacity to internalize synthetic glycoconjugates (37), it might internalize glycolipids for loading onto CD1 molecules, similarly as reported for the C-type lectin Langerin (38). Langerin is capable of loading CD1a molecules with foreign glycolipids derived from *Mycobacteria leprae*; however, the gangliosides which bind MGL are normally expressed in the spleen (39). The fact that gangliosides inhibit APC function (40) may hint to a possible function of MGL in antigen presentation and maintenance of tolerance to self-glycolipids.

Our data demonstrate that MGL might be involved in the recognition of tumor cells by DCs. In normal leukocytes and epithelial cells O-glycans are all essentially of core 1 or extended core 2 subtype. Tumorigenicity is associated with increased sialylation and a reduction in length of the O-glycans expressed (27). Tumor cells, especially of adenocarcinoma origin, are frequently positive for the Tn-antigen (single  $\alpha$ -GalNAc) or TF-antigen (Gal $\beta$ 1-3GalNAc). Positivity for the Tn-antigen-specific lectin HPA serves as a diagnostic marker for a wide range of adenocarcinomas and coloncarcinomas and is associated with lymph node metastases, poor prognosis and lower survival rates (41). A high correlation was found between the GalNAc-specific lectins HPA/SBA, and MGL recognition of  $\alpha$ -GalNAc in tumor glycoproteins. Recently, cytotoxic T cells have been described with specificity for the Tn-antigen (42), indicating that it is possible to generate a T cell response against carbohydrate antigens. Targeting of tumor antigens containing the Tn-antigen to MGL on DCs can potentially result in enhanced presentation in MHC I and II. Yet, targeting of antigens to C-type lectins without any additional DC activation may have tolerizing effects and inhibit anti-tumor responses (5, 6). We postulate that the expression of Tn-antigen by tumors might modulate immune responses via targeting to C-type lectins, such as MGL.

Our data show that MGL specifically recognizes terminal  $\alpha$ - and  $\beta$ -linked GalNAc moieties that are present on tumor-derived cell types, pathogens such as helminth parasites and self-antigens such as glycosphingolipids. Future studies should address whether antigen targeting to MGL on APC, such as DCs and M $\phi$ , leads to antigen presentation as well as immune modulation.

### Acknowledgements

This research was supported by the Netherlands Organization for Health Research and Development, Pionier grant 900-02-002.

### Abbreviations

APC	antigen-presenting cell
ASGP-R	asialoglyco protein receptor
DC	dendritic cell
HPA	<i>Helix pomatia</i> agglutinin
LDN	Lac-di-NAc
LDNF	Fuc $\alpha$ 3-Lac-di-NAc
MGL	macrophage galactose-type lectin
M $\phi$	macrophage
PAA	polyacrylamide

SBA	soybean agglutinin
SEA	soluble egg antigen
TLR	Toll-like receptor

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