# Chapter 4

## Specific glycan elements determine differential binding of individual egg glycoproteins of the human parasite *Schistosoma mansoni* by host C-type lectin receptors

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# Abstract

During infection with the blood fluke *Schistosoma mansoni*, glycan motifs present on glycoproteins of the parasite's eggs mediate immunomodulatory effects on the host. The recognition of these glycan motifs is primarily mediated by C-type lectin receptors (CLR) on dendritic cells (DC) and other cells of the immune system. So far, it is unknown however which individual glycoproteins interact with the different CLR, and which structural components are involved. Here we investigated the structural basis of the binding of two abundant egg antigens, kappa-5 and IPSE/α1, by the CLR DC-specific ICAM3-grabbing non-integrin (DC-SIGN), macrophage galactose-type lectin (MGL) and mannose receptor (MR). In the natural soluble form, the secretory egg glycoprotein IPSE/α1 interacts with DC mainly via MR. Surprisingly, in plate-based assays MR preferentially bound to mannose conjugates, while in cell-based assays, IPSE/α1 is bound via the fucosylated Galβ1-4(Fucα1-3)GlcNAc (LeX) motif on diantennary N-glycans. Kappa-5, in contrast, is bound by DC via all three CLR studied and for a minor part also via other, non-CLR receptors. Kappa-5 interacts with MGL via the GalNAcβ1-4GlcNAc (LDN) antenna present on its triantennary N-glycans, as well as the GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) antennae present on a minor N-glycan subset. DC-SIGN binding of kappa-5 was mediated via the LDN-F antennae, whereas binding of MR may involve either LDN-F antennae or the fucosylated and xylosylated chitobiose core. This study provides a molecular and structural basis for future studies of the interaction between CLR and other soluble egg antigen (SEA) glycoproteins and their effects on the host immune response.

# Introduction

Schistosomes are parasitic helminths that infect over 200 million people world-wide. The main pathological symptoms of schistosomiasis are caused by the deposition of parasite eggs by adult worms into the organs of its human host and the subsequent immunological consequences, which include the formation of perioval granulomas associated with a pronounced Th2-type immune response [1]. Many aspects of these egg-induced immune processes are thought to be at least partly mediated by protein glycosylation of the soluble egg antigens (SEA) [2]. This requires binding of the glycans to host receptors on antigen presenting cells (APCs) such as macrophages and dendritic cells (DC), leading to internalisation of the glycoprotein and/or activation of signaling pathways.

Previously, Van Liempt et al. demonstrated that the complex *Schistosoma mansoni* SEA mixture when coated on beads, is recognised by monocyte-derived human immature DC (iDCs) primarily through three C-type lectin receptors (CLR): dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN), macrophage galactose-type lectin (MGL) and mannose receptor (MR) [3]. The binding of DC-SIGN to the SEA-coated beads could be partly inhibited using monoclonal antibodies (mAbs) directed against the glycans Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX) and GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), suggesting that these two glycan structures on SEA glycoproteins contribute to the interaction between DC-SIGN and SEA [4]. In contrast, MGL appears to recognise terminal GalNAc moieties in (fucosylated) GalNAcβ1-4GlcNAc (LDN) ligands on solid phase coupled SEA glycoproteins, as concluded from studies using anti-LDN and anti-LDN-F blocking antibodies [5]. MR ligands in SEA have not been investigated yet, but it is known that MR can bind fucose- and mannose-containing glycans [6].

While these studies provide valuable indications for the involvement of specific glycan elements in SEA-CLR interactions, the role of individual glycoprotein components of the complex SEA mixture in these interactions has not yet been assessed. Moreover, binding and uptake of SEA by the CLRs has only been studied using SEA coupled to a solid phase as in coated to beads or to ELISA-plates [3;4]. In the natural *S. mansoni* egg context, however, protein and glycoprotein constituents of SEA are presented to the host as soluble molecules rather than bound to a particle, cell or whole organism. Some SEA components are excreted by the schistosome egg through pores in the egg shell, forming the so-called excretory/secretory fraction (ES) while others come into contact with the host after eggs die and release their soluble contents into the tissue surrounding the egg. Therefore, the biological context may be better reflected by studying the binding between the natural soluble egg components and cell-associated CLR.

Previously, three major soluble *S. mansoni* egg glycoproteins displaying immunogenic activities have been identified: omega-1 [7], which is able to drive DC-mediated Th2 responses both *in vitro* and *in vivo* [8]; IPSE/α1 [9], an inducer of IgE-dependent IL-4-release in human and murine basophils [10;11]; and kappa-5 [12], which has been shown to be a target of IgE in human *S. mansoni* infection sera. Detailed glycosylation analysis showed that the glycans expressed by these glycoproteins make up a major subset of glycans found in total SEA [13;14]. Omega-1 and IPSE/α1 are glycosylated almost identically, both carrying diantennary N-glycans with a difucosylated core and LeX antennae (Figure 1 and [15;16]). Kappa-5, on the other hand, expresses a completely different set of SEA glycans, characterised by the presence of triantennary N-glycans with terminal LDN and, to a lesser extent, LDN-F motifs (Figure 1 and [17]).

In this study, we have examined the interaction of IPSE/α1 and kappa-5 with DC-SIGN, MGL and MR as expressed by DC and single CLR-cell lines. Using exo-glycosidases, we were able to create specific glycoprotein variants and could precisely define the structural elements involved in the binding of the egg glycoproteins by individual cellular CLR. To our knowledge, this is the first report that assesses the binding of individual, native S. mansoni egg glycoproteins to CLR in a cellular context. These data provide a molecular basis for the CLR-mediated interaction of DC with soluble glycoproteins secreted/excreted by schistosome eggs and thereby contribute to the understanding of how individual components of SEA induce CLR-dependent immune responses.

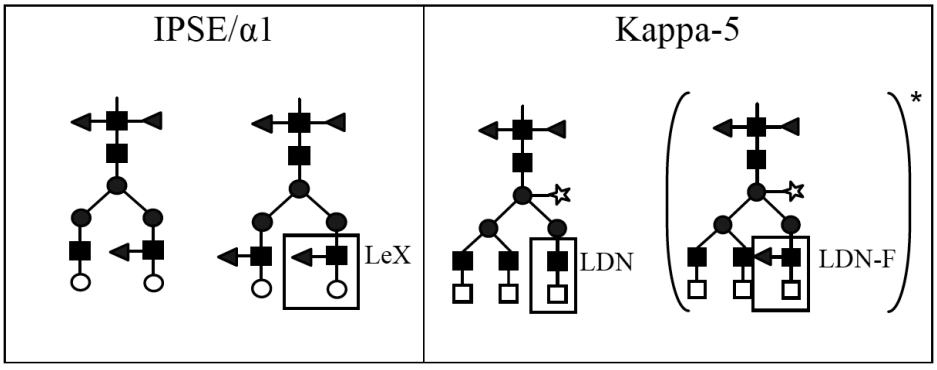


**Figure 1. Major glycan structures of IPSE/α1 and kappa-5.** Triangle, fucose; open square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; open circle, galactose; dark circle, mannose; open star, xylose; LeX, Galβ1-4(Fucα1-3)GlcNAc; LDN, GalNAcβ1-4GlcNAc; LDN-F, GalNAcβ1-4(Fucα1-3)GlcNAc. *Structure is present on a small subset of kappa-5 glycoproteins.

# Results

*Binding of CLR-Fc constructs to SEA-associated glycoconjugates*

SEA is a largely uncharacterised mixture of proteins and glycoproteins that interacts with DC-SIGN, MR and MGL on cells of the innate immune system [3]. We have previously studied the glycosylation of two proteins abundantly present in SEA; IPSE/α1 carries diantennary glycans with one or two LeX antennae ([15] and Figure 1), while kappa-5 glycosylation is characterised by the presence of triantennary N-glycans with terminal LDN motifs with a minor subset of kappa-5 glycans carrying terminal LDN-F motifs ([17] and Figure 1). To test and confirm that DC-SIGN, MR and MGL can bind to glycan elements present on kappa-5 and IPSE/α1, binding of Fc constructs of these CLR to a glycoconjugate microarray containing several schistosome-related synthetic glycoconjugates was analysed (Figure 2).

The majority of fucose-containing conjugates on the array, including LeX and LDN-F, were bound by DC-SIGN-Fc in a concentration-dependent manner (Figure 2A). This is in line with previous data showing that DC-SIGN has a broad specificity for α1-3 and α1-4 fucosylated motifs [18;19]. Relatively weak binding of DC-SIGN was observed to conjugates terminating with



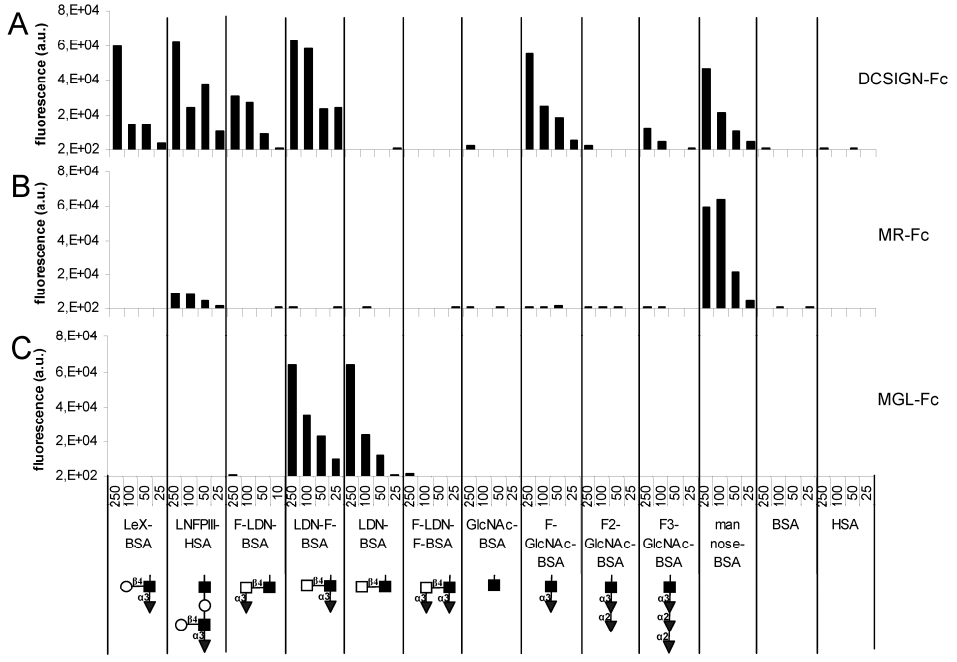


**Figure 2.** Binding of Fc-constructs of DC-SIGN, MGL and MR to an extensive set of schistosome-related, synthetic glycoconjugates. Glycan-binding specificities of human DC-SIGN (A), murine MR (B) and human MGL (C) were tested by glycan array screening. The glycan array was probed with 2 μg ml$^{-1}$ DC-SIGN-Fc or 5 μg ml$^{-1}$ MGL-Fc and MR-Fc. Results of one out of two independent experiments are shown. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose.

Fucα1-2Fucα1- (see F2-GlcNAc and F3-GlcNAc in Figure 1A), a sequence that has so far only been found in schistosomes [13;20]. F-LDN-F is not bound by DC-SIGN-Fc (Figure 2A), even though it contains α1-3-linked fucoses, which may be explained by spatial interference due to close proximity of the two fucoses. MR, like DC-SIGN, is considered to be a mannose- and fucose-recognising receptor [6;18]. On the glycoconjugate array, MR-Fc showed a relatively high affinity only for mannose-BSA (Figure 2B), while binding of DC-SIGN-Fc to mannose-BSA was similar to the binding to fucosylated ligands such as LeX (Figure 2A). MGL-Fc selectively bound to LDN and LDN-F on our array (Figure 2C), which is in accordance with the reported affinity of MGL to terminal, unsubstituted GalNAc residues [5].

Conventional ELISA confirms that DC-SIGN-Fc binds to LeX-expressing IPSE/α1 (Figure 3A). Surprisingly, DC-SIGN-Fc bound kappa-5 to a similar extent as IPSE/α1 (Figure 3A), which is remarkable as the only known DC-SIGN-ligand present on kappa-5, LDN-F (Figure 2A and [18]), is present on only a very minor subset of kappa-5 glycoproteins ([17] and Figure

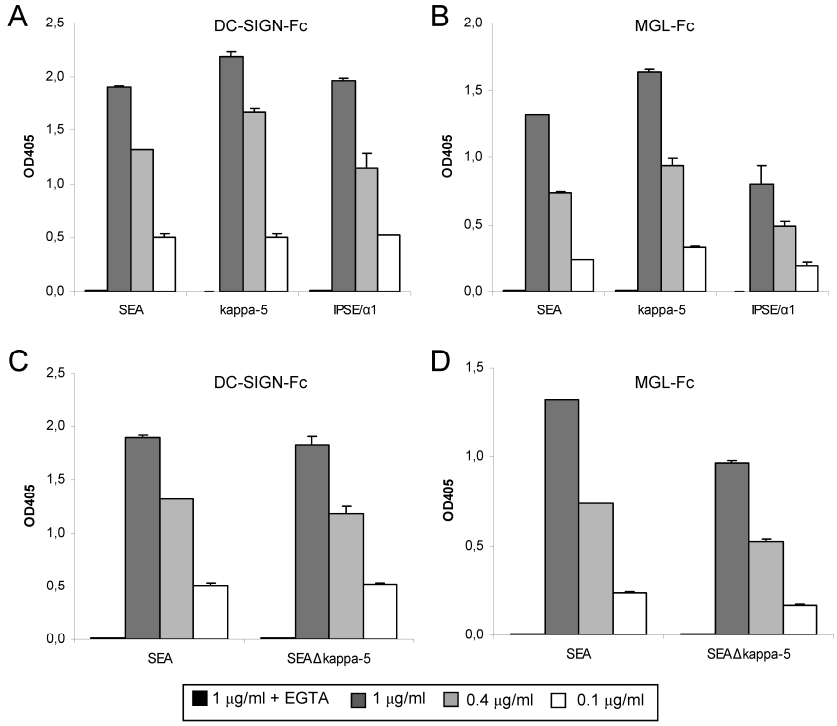


**Figure 3. Interaction of IPSE/α1 and kappa-5 with DC-SIGN-Fc and MGL-Fc constructs.** Binding of DC-SIGN-Fc (A, C) and MGL-Fc (B, D) to IPSE/α1 and kappa-5 (A,B) and SEA depleted of kappa-5 (SEAΔkappa-5) (C,D) was determined by ELISA in the presence (black bars) or absence (grey and white bars) of EGTA. Various concentrations for each Fc construct were used. Results are representative for three independent experiments.

1). In ELISA, MGL-Fc binds relatively intensely to kappa-5 as compared to IPSE/α1 (Figure 3B), likely because LDN-motifs are abundantly expressed on kappa-5 ([17] and Figure 1). Kappa-5 has been demonstrated to be the major LDN-containing glycoprotein in SEA [17]. In line with this observation, MGL-Fc binding to SEA depleted of kappa-5 (SEAΔkappa-5) is decreased compared to that of total SEA (Figure 3D), but significant other MGL ligands are still present. The affinity of DC-SIGN-Fc for SEA upon kappa-5 depletion remained high (Figure 3C).

*Human iDCs bind the differentially glycosylated kappa-5 and IPSE/α1 through different CLR*

The array and ELISA data using Fc-constructs of CLRs indicate that MGL and DC-SIGN are candidate receptors for kappa-5 and IPSE/α1. As a more accurate reflection of the natural situation in which soluble egg glycoproteins are bound by cell surface expressed CLRs, we next incubated fluorescently labeled kappa-5 and IPSE/α1 *in vitro* with human iDCs. The iDCs expressed each of the CLR DC-SIGN, MGL and MR (Supplementary Figure 1). The contribution of each single CLR in binding of kappa-5 and IPSE by DCs was assessed by addition of specific CLR blocking agents (Figure 4).

The observation that binding of IPSE/α1 to iDCs was strongly inhibited by addition of the calcium chelator EGTA indicates that this binding is CLR mediated (Figure 4A). Moreover, mannan, a natural ligand that competes for binding to DC-SIGN and MR, significantly reduced IPSE/α1-binding by DCs. More specifically, preincubation with blocking antibodies against DC-SIGN, MGL and MR showed that MR is the major receptor for IPSE/α1 on iDCs, while MGL and DC-SIGN both appeared to play only a minor role (Figure 4A).

In contrast, for kappa-5, preincubation with the specific CLR antibodies as well as with EGTA and mannan suggested the involvement of all three CLRs in kappa-5 binding by iDCs without one or the other in a dominant role (Figure 4B). Inhibition induced by mannan evidently exceeded the inhibition by the specific antibodies against DC-SIGN or MR, strongly supporting a role for both MR and DC-SIGN in the binding of kappa-5 by iDCs. Mannan inhibited the binding of kappa-5 by iDCs to a lesser extent as compared to IPSE/α1-iDC binding, in line with the larger role of MR for the binding of IPSE/α1. In addition, after preincubation of the iDCs with EGTA, still 24% of the initial kappa-5 binding could be detected (Figure 4B), indicating a role for other, $Ca^{2+}$-independent receptors in the interaction of kappa-5 with DCs.

In summary, iDCs bound IPSE/α1 preferentially via MR, while kappa-5 was recognised through multiple CLRs including DC-SIGN, MGL and MR, as well as via other, $Ca^{2+}$-independent receptor(s).

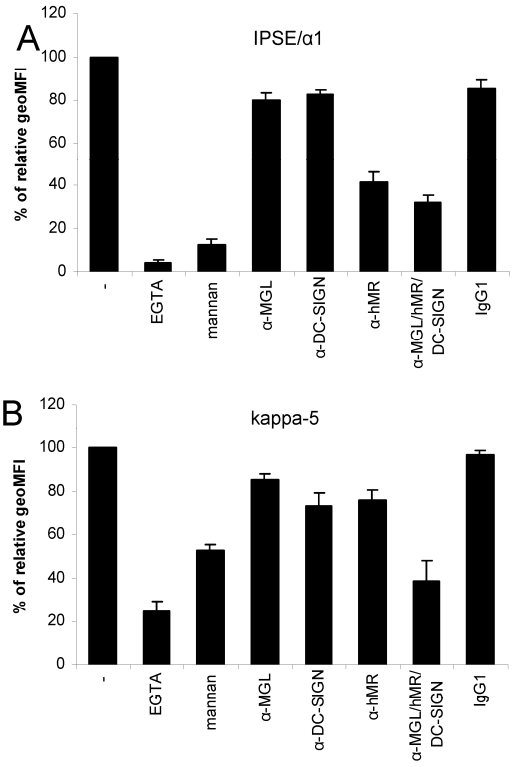


**Figure 4. Interaction of IPSE/α1 and kappa-5 with CLR on immature DCs**. Binding of fluorescently-labeled IPSE/α1 (A) and kappa-5 (B) to monocyte-derived human iDCs was assessed in the absence or presence of EGTA (blocking all CLR), mannan (blocking DC-SIGN and MR) or blocking mAbs directed against DC-SIGN, MGL, MR or a control antibody (IgG1). Unblocked conditions ( - ) were set to 100 %. The presented results represent three combined, independent experiments. Error bars represent standard error of the mean. * $p < 0.05$, ** $p<0.01$, for significant differences compared to the unblocked conditions ( - ), based on a two-sided paired *t*-test. # n=2, no statistical analysis was performed. MFI; mean fluorescence intensity.

*Antenna fucoses on kappa-5 and IPSE/a1 are involved in binding to DC-SIGN and MR, while terminal GalNAc residues confer binding of kappa-5 to MGL*

Next, we set out to identify the precise glycan elements which mediate kappa-5 and IPSE/α1 recognition by the individual CLRs in a cellular context. Kappa-5 and IPSE/α1 were treated with β-*N*-acetylhexosaminidase (hexnac) and/or α-fucosidase (fuc) to remove specific monosaccharides that form potential CLR ligands. Of note, α-fucosidase from *X. manihotis* was used which specifically removes α1-3-linked antenna fucoses of N-glycopeptides while being unable to cleave off the core-linked fucoses [17]. α-Fucosidase treatment of IPSE/α1 generated a glycoprotein variant mainly carrying diantennary glycans with unsubstituted Galβ1-4GlcNAc (LN) antennae (major set), as well as some LDN antennae (minor fraction) on an N-glycan core

which is substituted with α3- and α6-linked fucoses (IPSE/α1fuc, Supplementary Figure 2 for site N80). The effect of this enzyme was previously established in a mass spectrometric structural study on the egg glycoprotein omega-1, which carries nearly identical glycosylation [16]. Treatment of kappa-5 with β-*N*-acetylhexosaminidase resulted in the complete removal of unsubstituted GlcNAc and GalNAc residues in LDN and LDN-F, as verified by nano-LC-MS analysis of site $N_{251}$ (κ5hexnac, Supplementary Figure 3). In addition, a kappa-5 variant was generated of which fucoses were removed from LDN-F by treatment with α-fucosidase (κ5fuc, Supplementary Figure 3), as well as a variant treated sequentially with α-fucosidase and β-*N*-acetylhexosaminidase (κ5fuc/hexnac, data not shown) leaving intact only the trimannosyl core structure with α3/α6-fucose and β2-xylose core-modifications.

Subsequently, binding of the native and modified glycoprotein variants by cell lines expressing only one of the three human CLR, DC-SIGN, MR or MGL, was tested (Figure 5). Incubation of IPSE/α1 with the cell lines showed that all three CLRs had affinity for IPSE/α1 in a $Ca^{2+}$-dependent manner (Figures 5A, B and C). Interestingly, while MR proved to be the dominant receptor for IPSE/α1 on iDC which express all three CLRs (Figure 4A), IPSE/α1 was bound by the DC-SIGN cell line similarly well as the MR cell line (Figures 5A and B, respectively). Binding of soluble IPSE/α1 by the MGL-expressing cell line appeared minor as compared to binding by the MR- and DC-SIGN cell lines (Figure 5C). In contrast, in the corresponding ELISA (Figure 3B), soluble MGL-Fc showed clear $Ca^{2+}$-dependent binding to plate-bound IPSE/α1, which indicates that significantly different affinities or specificities are observed in these two binding assay types. Furthermore, α-fucosidase treatment of IPSE/α1 revealed the involvement of the antenna fucose, as present in the major antenna motif LeX, in the interaction with both DC-SIGN and MR (Figures 5A and B). As expected, the fucoses did not have any role in the interaction between MGL and IPSE/α1 (Figure 5C).

Incubation of kappa-5 with the cell lines showed strong interactions between kappa-5 and cellular DC-SIGN (Figure 5D) as well as MGL (Figure 5F). Exo-glycosidase treatment of kappa-5 revealed that the terminal GalNAc in LDN(-F) is required for MGL-binding (Figure 5F), while the fucoses of LDN-F appeared essential for the binding of DC-SIGN for kappa-5 (Figure 5D). The latter finding is striking, as an estimated less than 5% of kappa-5 LDN antennae are fucosylated [17], which may indicate a relatively high affinity of DC-SIGN for LDN-F. Binding of kappa-5 by the MR cell line seemed only minor as compared to binding observed for the DC-SIGN and MGL cell lines, and α-fucosidase treatment did reduce kappa-5-MR binding only slightly (Figure 5E). Indeed, the glycoconjugate array data indicated that MR has little to no affinity for LDN-F (Figure 2B). Possibly MR displays affinity for the core-fucoses which remain unaffected by the α-fucosidase treatment (Supplementary Figure 3). Interestingly,

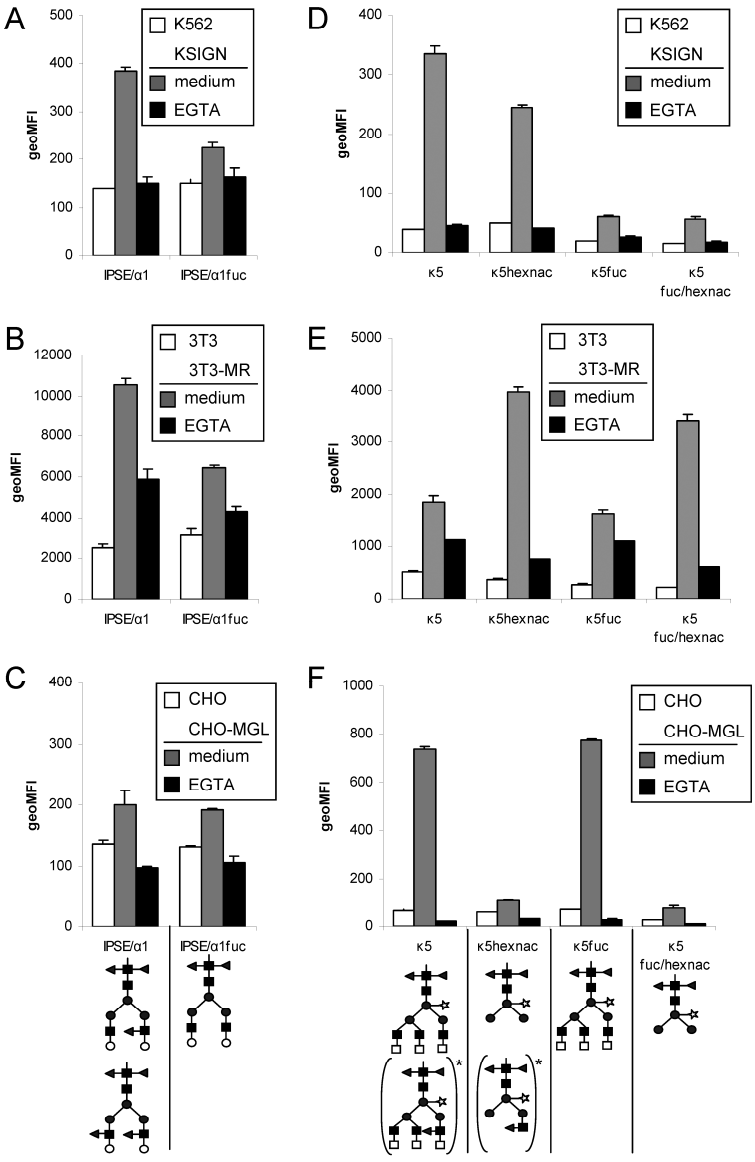


**Figure 5. Structural characteristics of the glycan epitopes on IPSE/α1 and kappa-5 involved in CLR binding.** Binding of untreated and exo-glycosidase-treated fluorescently labeled IPSE/α1 (A,B,C) and kappa-5 (D,E,F) to a DC-SIGN-expressing cell line (A,D), a MR-expressing cell line (B,E) and a MGL-expressing cell line (C,F), was assessed in the absence (grey bars) or presence (black bars) of EGTA. Untransfected or mock-transfected cell lines were used as control (white bars). The given glycan structures represent the major glycans present on the (treated) glycoproteins, while the structures marked with an asterisk (*) are present on a small subset of kappa-5 glycoproteins. The experiments were performed in duplicate and one representative experiment out of two is shown. Error bars represent standard deviation. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose; dark circle, mannose; open star, xylose; κ5, kappa-5; κ5hexnac, kappa-5 treated with β-*N*-acetylhexosaminidase; κ5fuc, kappa-5 treated with α-fucosidase; κ5fuc/hexnac, kappa-5 treated sequentially treated with α-fucosidase and β-*N*-acetylhexosaminidase; IPSE/α1fuc, IPSE/α1 treated with α-fucosidase. MFI; mean fluorescence intensity.

β-*N*-acetylhexosaminidase treatment of kappa-5 increased binding of kappa-5 by MR (Figure 5B), which is probably due to exposure of the xylosylated trimannoside, a structure also frequently observed on native glycoproteins of various *S. mansoni* life stages including eggs [14]. This effect induced by β-*N*-acetylhexosaminidase treatment was not observed in relation to kappa-5-binding by DC-SIGN (Figure 5D). On the contrary, β-*N*-acetylhexosaminidase treatment of kappa-5 reduced the interaction with DC-SIGN, indicating a higher affinity of DC-SIGN for intact LDN-F as compared to the Fucα1-3GlcNAc elements left after treatment. Furthermore, in contrast to MR, DC-SIGN seems not able to bind the xylosylated and fucosylated trimannosyl core (Figure 5D; κ5fuc/hexnac). Finally, ELISA format experiments revealed a recognition pattern of the exo-glycosidase treated glycoproteins by DC-SIGN and MGL (Supplementary Figure 4) analogous to the cell based binding assays. In conclusion, the three CLRs DC-SIGN, MGL, and MR, display differential affinity for the individual glycoproteins which is determined by specific glycan elements.

## Discussion



During *S. mansoni* infection, immunogenic SEA glycoproteins are released by eggs and bound and internalised by DCs and other APCs patrolling the host tissues. We investigated the differential interaction of DC-SIGN, MR and MGL with two abundant soluble *S. mansoni* egg glycoproteins IPSE/α1 and kappa-5 each representing a differently glycosylated group of SEA glycoproteins. A first surprising observation was that on iDCs, MR is the main receptor for IPSE/α1 (Figure 4A) via its LeX glycans, while in the glycan array format LeX-glycoconjugates form relatively weak ligands for MR-Fc (Figure 2 and [21]). Secondly, although we hypothesised that MGL would play a dominant role in kappa-5-iDC interaction due to its capacity to bind to the LDN motifs on kappa-5 [5], all three CLR studied appeared to be involved in the interaction between iDCs and kappa-5 (Figure 4B). In particular the presence of LDN-F motifs on a minor subset of kappa-5 glycans contributed significantly to cellular binding via DC-SIGN.

van Liempt *et al.* previously indicated the importance of the DC-SIGN, MR and MGL, in the interaction of iDCs with SEA [3]. A mixture of blocking antibodies against these CLRs could abrogate binding of iDCs to SEA-coated 1 μm-beads by 90%, which is to the same level as reached with EGTA, a $Ca^{2+}$-chelator which blocks the binding of all CLRs. Also studies using synthetic glycoconjugates have suggested that DC-SIGN and MGL can bind glycan motifs that have been detected in schistosomes [4;5]. However, SEA is a complex mixture of (glyco)proteins [22], and it is yet unknown how many and which SEA glycoproteins actually carry glycan motifs that can be bound by CLRs, possibly leading to modulation of DC function. We believe that the

cellular binding studies using single well-characterised glycoproteins and enzymatically modified glycoforms thereof presented here are required to more precisely define the molecular basis of SEA-DC interactions.

Both the DC-SIGN- and the MR-expressing cell line bound IPSE/α1 in a $Ca^{2+}$-dependent way (Figures 5A and B). While LeX-glycoconjugates form relatively weak ligands for MR but strong ligands for DC-SIGN in the glycan array format (Figure 2 and [18;21]), on iDCs, the LeX-expressing IPSE/α1 is predominately bound by MR (Figure 4A). To be able to bind IPSE/α1, both DC-SIGN and MR require the presence of the antenna fucoses present mainly in the form of LeX (Figures 5A and B). Studies using bead-coupled glycans [4] or glycan arrays (Figure 1A and [4;18;19]) suggest that DC-SIGN has a strong preference for LeX motifs presented on pathogens, particles or a solid surface in a multivalent fashion. It can be hypothesised from our observations that MR plays a dominant role over DC-SIGN in the binding of LeX-containing glycoproteins if presented as soluble ligands. This is in line with a previous report showing a role for MR in the uptake of soluble antigens, but not in that of cell-associated antigens [23]. For DC-SIGN molecules, which bear only one CRD domain, it has been shown that the extracellular domains can aggregate *in vitro* to form DC-SIGN tetramers, which have been suggested to possess enhanced DC-SIGN capacity to bind multivalent ligands [24]. In addition, clustering of DC-SIGN in lipid rafts on the surface of iDCs has been shown to enhance binding and internalisation of viral particles [25].

Although the main glycans present on kappa-5 carry multiple termini of the MGL-ligand LDN [17], MGL did not display a dominant function in kappa-5-iDC interaction (Figure 4B). Clearly, multiple glycan elements present on kappa-5 contribute to the binding to different CLRs. Removal of the terminal GalNAc and GlcNAc units of LDN in kappa-5 by β-*N*-acetylhexosaminidase treatment abolishes MGL binding (Figure 5F), whereas removal of LDN-F associated fucose leads to the loss of DC-SIGN binding (Figure 5D) and the partial loss of MR binding (Figure 5E). These observations suggest that exposure of CLRs or CLR-expressing cells to single glycoproteins can still include multiple putative ligands due to glycan microheterogeneity, the natural variation in protein glycosylation leading to different so-called glycoforms of a single glycoprotein. This underlines that a thorough investigation of all major and minor glycan structures on a specific glycoprotein is imperative for the identification of glycan motifs putatively involved in CLR binding. Moreover, the observation that the LDN-F unit, which is present in only a very minor subset of kappa-5 glycoforms [17], dictates its binding by DC-SIGN (Figure 5D) indicates that small variations in glycosylation may have important biological effects. Consistent with this observation, LDN-F has been shown to be a strong ligand for DC-SIGN by glycan array studies ([18] and Fig. 2).

The current study, together with the structural data of the glycans of kappa-5 [17] and IPSE/α1 [15], as well as a third SEA glycoprotein omega-1 [16], provides structural background knowledge to several recent functional studies. First, kappa-5 is the major glycoprotein in SEA that carries the granulomogenic LDN motif [17;26], raising the question whether the kappa-5 associated LDN receptor MGL (Figure 5F) plays a role in the induction of the granulomatous immune responses around *S. mansoni* eggs. Second, the observations that MR rather than DC-SIGN is the most relevant CLR involved in the binding of the LeX-containing IPSE to iDCs might indicate an important function for MR during natural infection, as the secreted glycoproteins are generally believed to be the major immunoregulatory molecules of *S. mansoni* eggs [7;8;27-29]. IPSE/α1 is postulated to have a Th2-regulating role during *S. mansoni* infection via the induction of IL-4 production by basophils [10;11], as well as an anti-inflammatory role by reducing cellular infiltration into granulomas [30]. Both immunomodulatory properties are not related to its glycosylation, and thus CLR recognition will not be involved in this process. However, recently it was demonstrated that IPSE/α1 contains a functional C-terminal nuclear localisation signal via which it is transported to the nucleus in monocyte-derived human DCs [31]. Interestingly, this was a $Ca^{2+}$-dependent process, implicating that CLRs are involved in the initial uptake into the cell. Together with our results, it can be hypothesised that MR, which we found to be the primary CLR binding to IPSE/α1 via its antenna fucoses (Figure 4B), can mediate uptake of IPSE/α1 into cells, after which it is translocated to the nucleus to exert a currently unknown function. Interestingly, another major *S. mansoni* egg glycoprotein omega-1, which has RNase activity and Th2-inducing properties [8;32] expresses LeX-carrying glycans similar to IPSE/α1 [16]. In the mouse, a synthetic LeX conjugate has been shown to drive Th2 responses, involving the production of antigen-specific IgE and induction of IL-4 and IL-10 following intranasal immunization [32;33]. However, the SEA glycoprotein associated LeX epitopes alone are not sufficient to confer the Th2-inducing properties initiated by *S. mansoni* eggs, as IPSE/α1 in contrast to omega-1 is not able to induce the strong DC-mediated type 2 responses [8]. Since our current study shows that DCs recognise IPSE/α1 via MR by the same glycans as present on omega-1, it is conceivable that the latter glycoprotein is also bound by MR on DCs. Furthermore, experiments in mice as well as with bone marrow derived macrophages deficient for MR show the involvement of MR in the uptake of cercarial secretions and the downregulation of IFN-γ production in response to *S. mansoni* cercariae [34]. These results demonstrate that soluble schistosome glycoconjugates indeed may depend on recognition by MR on APCs for modulation of the immune response.

In summary, while SEA is bound and internalised by iDCs via three CLRs, DC-SIGN, MGL, and MR, we found that single components in SEA are differentially bound. We

demonstrated that while in plate-or array based assays DC-SIGN but not MR binds relatively strongly to LeX conjugates including the LeX-containing glycoprotein IPSE/α1, MR is the major receptor for IPSE/α1 on iDCs. Furthermore, exo-glycosidase treatment of the two differentially glycosylated egg proteins, kappa-5 and IPSE/α1, revealed the preference of the three CLRs for specific elements in/of the glycan structures: antenna fucoses in LeX for DC-SIGN and MR, antenna fucoses in LDN-F for DC-SIGN, and the GalNAc-termini in LDN(-F) for MGL. This study provides a molecular and structural basis for future studies of the interaction between CLRs and other SEA glycoproteins, and their effects on the host immune response.

# Material and Methods

*Antigens and exo-glycosidase treatments*

SEA and IPSE/α1 were obtained as described previously [10]. Kappa-5 was isolated by soybean agglutinin (SBA) affinity chromatography as described previously [17]. Kappa-5-depleted SEA is defined as the non SBA-binding fraction in the kappa-5 isolation procedure. Kappa-5 and IPSE/α1 were fluorescently labeled with PF-647 using the Promofluor labeling kit according to the manufacturer's recommendations (Promokine, Heidelberg, Germany). Either native or fluorescently labeled kappa-5 was treated with β-*N*-acetylhexosaminidase from *Canavalia ensiformis* (62.5 mU; Sigma, Zwijndrecht, the Netherlands) in 100 mM sodium phosphate buffer, pH 5.0, for 48 h at 37°C. α-Fucosidase treatment of (labeled) kappa-5 and IPSE/α1 was performed with α1-(3,4)-fucosidase from *Xanthomonas manihotis* (1 mU and 0.5 mU, respectively; Sigma) in 100 mM sodium phosphate buffer, pH 5.0, for 48 h at 37°C.

*Glycoconjugate array*

BSA-conjugates were prepared as described previously [35;36]. Lacto-N-fucopentaose III (LNFPIII)-HSA was obtained from Glycotech (Gaithersburg, MD, USA) and mannose-BSA from Sigma. The glycoconjugates were printed and immobilized on epoxide-modified glass slides (Slide E, Schott Nexterion, Jena, Germany) as described previously [37]. Each glycoconjugate microarray was blocked for 60 min with TSM (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) containing 1% (w/v) BSA (Sigma) and 50 mM ethanolamine. After washing, arrays were incubated with either 2 μg $mL^{-1}$ DC-SIGN-Fc (R&D Systems, Minneapolis, MN, USA), 5 μg $mL^{-1}$ MGL-Fc [5], or 5 μg $mL^{-1}$ MR-Fc (a kind gift from L. Martinez-Pomares [38]) in TSM/1% BSA/0.01% (v/v) Tween-20 for one hour. Slides were flushed and subsequently incubated with anti-human-IgG(Fc)-Cy3 (Sigma) for 30 min. After thorough washing and drying, the slides were scanned using the Agilent DNA Microarray Scanner. Data was analysed using GenePix Pro

6.1 (Molecular Devices). Mean fluorescence intensities (MFI) of triplicates were calculated on basis of median spot intensity using circular alignment and local background subtraction [39].

*DC-SIGN- and MGL-Fc ELISA*

96-well Maxi-Sorp plates (NUNC, Roskilde, Denmark) were coated overnight with antigens (1µg $ml^{-1}$) in 0.1M $NaHCO_3$ at 4°C in duplicate. Plates were washed three times with TSM, followed by incubation with TSM/1% BSA for 30 min. After three washes with TSM, various concentrations of DC-SIGN-Fc or MGL-Fc in TSM were added for 2 h, with or without a prior incubation for 15 min with EGTA (10 mM). The plates were washed five times with TSM/0.05% Tween and bound DC-SIGN-Fc or MGL-Fc was detected with alkaline phosphatase-labeled goat anti-human Fc (1:25 000; Sigma) in TSM/0.05% Tween.

*Cells*

DCs were generated from monocytes, isolated from venous blood of healthy volunteers by density centrifugation on Ficoll followed by a Percoll gradient as previously described [8;40]. Monocytes were cultured in RPMI medium supplemented with 10% FCS, human rGM-CSF (80 ng $ml^{-1}$, Biosource-Invitrogen, Breda, The Netherlands) and human rIL-4 (250 units $ml^{-1}$, R&D Systems).

The K562 cell line stably expressing DC-SIGN and a mock transfected control (a kind gift from C. Figdor [41]), were maintained in mixed medium consisting of 25% IMDM (Gibco) medium + 5% FCS and 75% RPMI (Gibco) medium + 10% FCS. The CHO cell line stably expressing MGL [41] and an untransfected control were maintained in RPMI (Gibco) containing 10% FCS. NIH3T3 cell lines expressing human MR (3T3.hMR) or the control pFB vector were a kind gift from Joanna Miller and Gordon Brown [38]. 3T3 cell lines were cultured in DMEM (BioWitthaker) medium + 10% FCS. All media were supplemented with penicillin and streptomycin and transfected cell lines were continuously kept under selection of 0.5 mg $ml^{-1}$ geneticin (Gibco).

*Cellular adhesion assay*

Fluorescently labeled kappa-5 and IPSE/α1 were treated or not with various exo-glycosidases, as described in the section "Antigens and exo-glycosidase treatments". Cells (50.000/well) were seeded in a V-bottom 96 well plate. Where indicated cells were pre-incubated with 10mM EGTA, 100 ug $ml^{-1}$ mannan (Sigma-Aldrich), 20 µg $ml^{-1}$ anti-DC-SIGN (clone AZN-D1, azide free, Beckman Coulter, Woerden, the Netherlands), 20 µg $ml^{-1}$ anti-MR (clone 15.2, Biolegend, San Diego, CA, USA) or a combination of two anti-MGL antibodies (20 µg $ml^{-1}$ clone

125A10.03, Dendritics, and 20 μg $ml^{-1}$ 1G6.6 [42]) for 45 min at 37 °C. Subsequently, cells were incubated with 2 μg $ml^{-1}$ PF-647 labeled glycoprotein variants at 37 °C for 1 h and washed in ice cold PBS before analysis using flowcytometry (FACSCalibur, Becton Dickinson).

*Mass spectrometry*

Trypsin (Promega, Leiden, The Netherlands) was added to a sample of IPSE/α1 or kappa-5 variants at a 1:100 trypsin/antigen ratio and incubated overnight at 37 °C. Resulting IPSE/α1 and kappa-5 (glyco)peptides were analysed as described previously using respectively MALDI-TOF-MS [15] and nano-HPLC-ESI-ion trap-MS [16].

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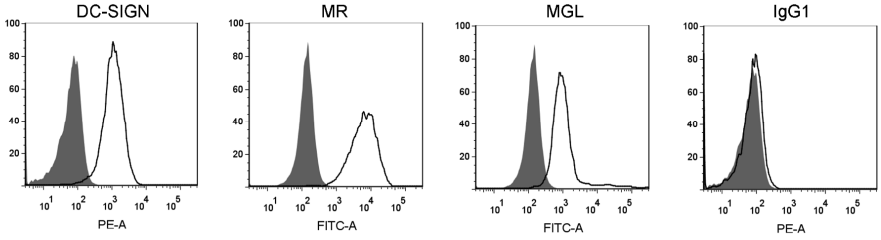
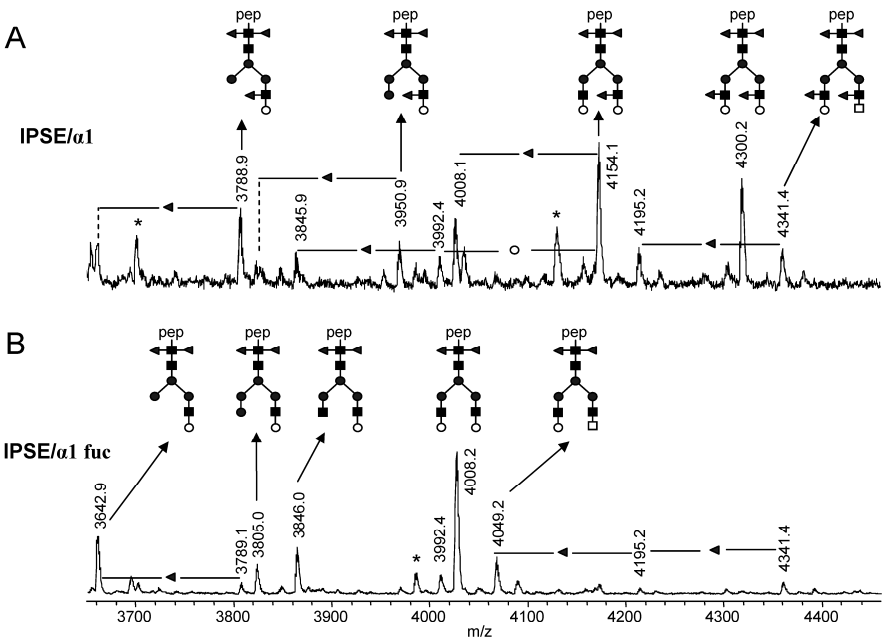
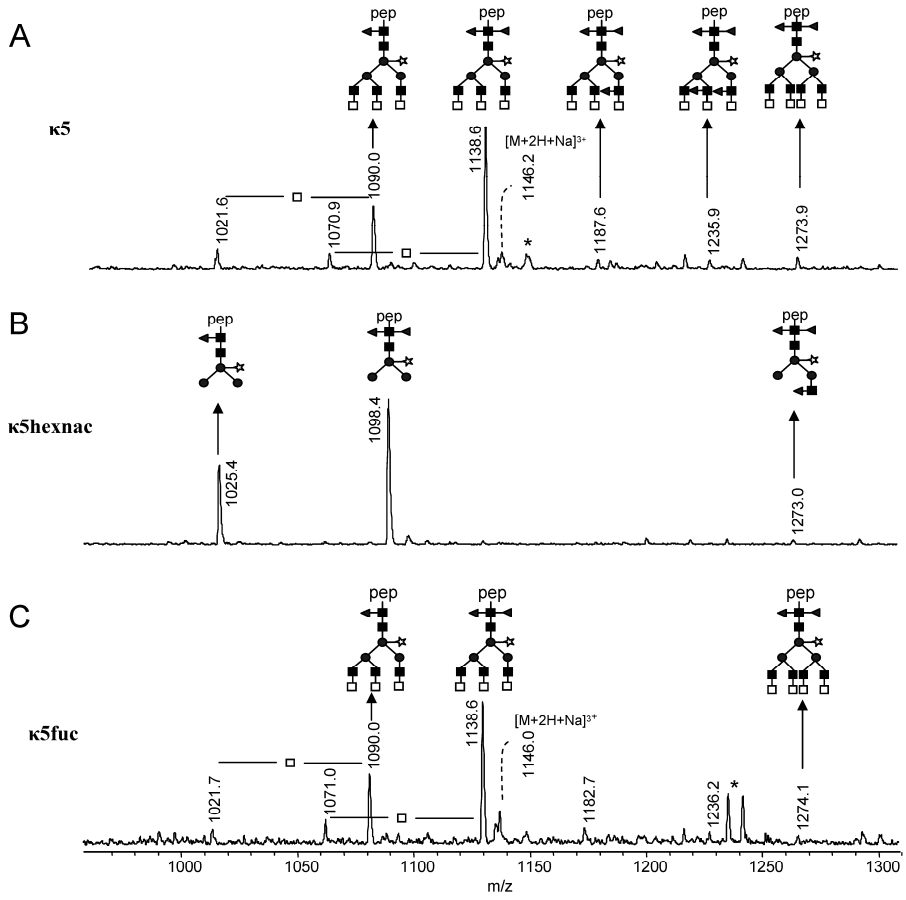
# Supplementary Figures



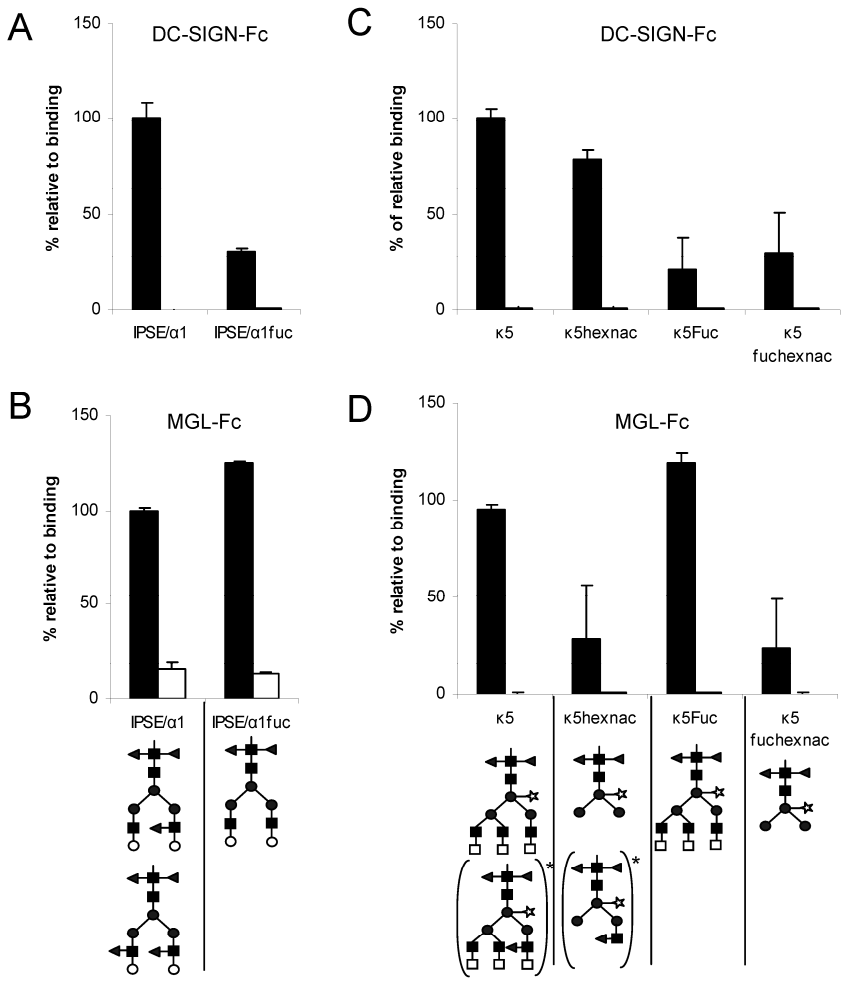

**Supplementary figure 1. Expression of DC-SIGN, MR, MGL and isotype control (IgG1) on monocyte-derived immature DC.** Open histograms represent the mAb staining, filled histrograms represent unstained cells. The presented results are representative of one out of three experiments.




**Supplementary figure 2. Exo-glycosidase treatment of IPSE/α1.** IPSE/α1, either untreated (A) or treated with α-fucosidase (B), was digested with trypsin and the resulting glycopeptides were analyzed by MALDI. Analysis of glycans on glycosylation site $N_{80}$ in peptide $E_{69}$RPYWYLFDNVNYTGR$_{84}$ is given. Signals are labeled with monoisotopic masses and single charged. Non-glycopeptide signals are marked with asterisks (*).Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose; dark circle, mannose; pep, peptide; IPSE/α1fuc, IPSE/α1 treated with α-fucosidase.




**Supplementary Figure 3. Exo-glycosidase treatment of kappa-5.** Kappa-5, either untreated (A) or treated with β-*N*-acetylhexosaminidase (B) and α-fucosidase (C), was digested and the resulting glycopeptides were analyzed by nano-LC-MS. Analysis of glycans on glycosylation site $N_{251}$ in peptide $I_{246}$NENFNK$_{252}$ is given. Signals are labeled with monoisotopic masses. Signals are triple charged (A and C) or double charged (B), unless indicated otherwise. Non-glycopeptide signals are marked with asterisks (*).Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, mannose; open star, xylose; pep, peptide; κ5, kappa-5; κ5hexnac, kappa-5 treated with β-*N*-acetylhexosaminidase; κ5fuc, kappa-5 treated with α-fucosidase.




**Supplementary figure 4. Interactions of exo-glycosidase treated IPSE/α1 and kappa-5 with DC-SIGN-Fc and MGL-Fc.** Binding of 1 μg ml$^{-1}$ of DC-SIGN-Fc (A, C) and MGL-Fc (B, D) to exo-glycosidase-treated IPSE/α1 (A,B) and kappa-5 (C,D) was determined by ELISA in the absence (black bars) or presence (white bars) of EGTA. The given glycan structures represent the major glycans present on the (treated) glycoproteins, while the structures marked with an asterisk (*) are present on a small subset of kappa-5 glycoproteins. Binding of Fc-constructs to untreated kappa-5 or IPSE/α1 was set to 100%. The presented results represent two combined, independent experiments. Triangle, fucose; light square, *N*-acetylgalactosamine; dark square, *N*-acetylglucosamine; light circle, galactose; dark circle, mannose; open star, xylose; κ5, kappa-5; κ5hexnac, kappa-5 treated with β-*N*-acetylhexosaminidase; κ5fuc, kappa-5 treated with α-fucosidase; κ5fuc/hexnac, kappa-5 treated sequentially treated with α-fucosidase and β-*N*-acetylhexosaminidase; IPSE/α1fuc, IPSE/α1 treated with α-fucosidase.