Tumor-associated Neu5Ac-Tn and Neu5Gc-Tn antigens bind to C-type lectin CLEC10A (CD301, MGL)

Introduction
CLEC10A (CD301, macrophage galactose-type lectin), a type II transmembrane receptor, is a member of the C-type lectin family. Suzuki et al. (1996) first identified CLEC10A in human macrophages. Later, Valladeau et al. (2001) showed that immature dendritic cells express the lectin. CLEC10A specifically binds glycans with terminal N-acetylgalactosamine (GalNAc) residues such as Tn antigen (GalNAcα-serine/threonine), 6-sulfo-Tn, Lac-di-NAc (Galβ1,4-GlcNAc) as well as core 5 (GalNAcα1-3GalNAcβ-) and core 6 (GlcNAcβ1-6GalNAcβ-) (van Vliet et al. 2005) in a Ca²⁺-dependent manner (Drickamer 1988; Suzuki et al. 1996). Historically, the Tn antigen has been defined by antibodies (Gunson et al. 1970; Issitt et al. 1972) and its structure has been clarified by Dahr et al. (1975). The Tn antigen gained considerable attention, since the antigen has been found to be associated with human tumors (Tollefsen and Kornfeld 1983; Ju et al. 2008, 2011). Other tumor-associated structures are, for example, Neu5Ac-Tn (Marcos et al. 2004; Julien et al. 2006; Ju et al. 2008; Taylor and Drickamer 2011) and Neu5Gc-Tn (Nguyen et al. 2005; Padler-Karovani et al. 2012), which are generated by α2,6 glycosidic linkage of N-acetylneuraminic acid (Neu5Ac) or N-glycolylneuraminic acid (Neu5Gc) to the Tn antigen. Neu5Gc differs from Neu5Ac by one oxygen atom and can be synthesized in most mammals, e.g. cattle, pig and lamb but not in humans (Schauer 1982). Dietary Neu5Gc can replace Neu5Ac resulting in metabolic incorporation into human glycoproteins (Tangvoramuntakul et al. 2003). It has been suggested that human xeno-antibodies against Neu5Gc-Tn cause chronic inflammation facilitating tumor growth (Varki 2011; Padler-Karovani et al. 2012). Our glycan profiling of recombinant, soluble C-type lectin CLEC10A reveals two novel specificities to tumor-associated Neu5Acα2,6-Tn and Neu5Gcα2,6-Tn antigens. These results were further verified by surface plasmon resonance (SPR). To gain more insight into the binding epitope of 6-sialylated-Tn antigens to CLEC10A, saturation transfer difference (STD) NMR was performed in the case of Neu5Acα2,6-Tn. In addition to these in vitro analyses, both glycans bound to chinese hamster ovary (CHO) cell expressing CLEC10A transmembrane protein. Since Neu5Acα2,6-Tn and Neu5Gcα2,6-Tn are expressed in human tumors, the interaction with CLEC10A by macrophages and dendritic cells may be of functional significance in tumor progression. Furthermore, these glycan structures may be detected in tumor sections by recombinant CLEC10A constructs as recently described (Nollau et al. 2013).

Keywords: carbohydrate–protein interactions / carbohydrate recognition domain (CRD) / lectin / sialyl-Tn / tumor-associated glycans
Results

Binding of recombinant CLEC10A to Neu5Acα2,6-Tn and Neu5Gcα2,6-Tn

The extracellular part of CLEC10A was expressed in HEKT293 cells as soluble recombinant protein with an N-terminal myc tag. The cell culture supernatant containing CLEC10A was tested on a glycan array. As shown in Figure 1 and reported previously (van Vliet et al. 2005), recombinant CLEC10A bound terminal GalNAc residues in α-configuration, e.g. Tn antigen (GalNAcα-) and core 5 (GalNAcα1-3GalNAcα-), and in β-configuration, e.g. Lac-di-Nac (GalNAcβ1-4GlcNAc-). Substitution in the 6th position by N-acetylglucosamine (GlcNAc; core 6) or sulfate did not affect binding. In contrast, no binding was observed when GalNAc was mono-substituted in the 3rd position by Neu5Ac (3-sialyl Tn), by galactose (TF antigen/core 1) or by GlcNAc (core 3). When the 3rd position was substituted, binding remained negative with additional substitutions in the 6th position (core 2, core 4). In addition to the binding specificities reported so far, we observed binding of CLEC10A to Neu5Acα2,6-Tn and Neu5Gcα2,6-Tn antigens (see arrows in Figure 1).

Purification of recombinant CLEC10A

For SPR and STD NMR experiments, recombinant soluble CLEC10A was purified from culture supernatant by an anti-c-myc immunosorbent. The myc-tagged protein eluted from the immunosorbent was identified in western blot using a monoclonal anti-c-myc antibody (Figure 2A). The calculated theoretical molecular weight of the extracellular domain of recombinant CLEC10A is ~30 kDa. CLEC10A carries two potential N-glycosylation motives in the coiled region. As reported recently, the two bands detected in the molecular weight range of 38 and 50 kDa correspond to glycosylated variants of CLEC10A (Nollau et al. 2013). The purity of the preparation was analyzed by a Coomassie stained gel. As shown in Figure 2B, the two bands with a molecular weight of 38 and 50 kDa were identified as CLEC10A by a tandem mass spectroscopy. The third band with a molecular weight of 66 kDa was identified as an albumin contaminant.

STD NMR analysis of CLEC10A binding to Neu5Ac-Tn and Tn antigens

The STD NMR spectroscopy is well suited for the determination of binding constants of small molecules to proteins and the elucidation of the binding epitope of the ligand (Mayer and Meyer 1999, 2001; Meyer and Peters 2003; Wilhelm et al. 2012). We characterized the interaction of Tn and sialyl-Tn antigens (Neu5Accα2,6-Tn antigen) to CLEC10A C-type lectin, which was complexed to an anti-c-myc antibody and streptavidin. NMR spectra and fits of the build-up of the STD effect as a function of ligand concentration to a
one-site binding model are shown in Figure 3A and C. The dissociation constants for the two molecules were 103 µM for the Tn antigen and 179 µM for the Neu5Acα2,6-Tn antigen.

This result indicates that the affinity of Neu5Ac-Tn and Neu5Ge-Tn antigens to CLEC10A is mainly due to the GalNAc moiety. This is in accordance with our data from SPR (cf. below). The Tn antigen binds mainly via the H-2, H-3 and H-4 regions of the GalNAc residue with the N-acetate and the serine also contributing to binding to the carbohydrate recognition domain (CRD) of CLEC10A. At the same time, the “back” of GalNAc with H-5 and H-6a,b shows no major interactions with CLEC10A. This fact indicates the possibility that substituents at C-6 might be tolerated. At elevated concentrations >1 mM of the Tn antigen, non-specific interactions were observed. The group epitope of the sialylated Tn antigen exhibits a similar binding mode with major contributions of the N-acetate group and the H-2, H-3 and H-4 protons of the GalNAc residue with a slight shift

Fig. 3. STD NMR group epitope mapping and binding curves of Tn (GalNAc α1-O-serine) and Neu5Acα2,6-Tn (Neu5Acα2,6-GalNAc α1-O-serine) antigens. STD NMR and reference 1D 1H NMR spectra of Tn and Neu5Acα2,6-Tn antigens (A). The top traces show in each case the STD NMR and the bottom traces the normal 1D 1H NMR spectrum. Molecular structures of Tn and Neu5Acα2,6-Tn antigens with protons that are in close contact to the CRD of CLEC10A highlighted (B). Binding curves of Tn and Neu5Acα2,6-Tn antigens from STD NMR titrations (C). Data points in gray were not used for the fit of the data to the one-site binding model and are likely due to unspecific interactions. The crossed eye plot of Neu5Acα2,6-Tn in the proposed binding mode (D). Protons that show a close distance to the CLEC10A are space filled and highlighted in magenta. The lectin has one dominant binding site for the Tn antigen (thick black line) and a secondary weak binding site for interactions with sialic acid (thin gray line).
toward a higher contribution of the N-acetate. Even though the Neu5Aca2,6-Tn antigen binds less strongly by about a factor of 2 than the Tn antigen, the sialyl residue shows close contacts to CLEC10A. Especially, the axially directed H-3 proton of the neuraminic acid has lipophilic contributions, whereas the equatorial positioned H-3 proton shows a larger distance to CLEC10A. The signal of the anomeric proton of GalNAc (4.806 ppm) could not be analyzed in the STD spectra due to its close distance to the HDO signal.

**SPR analysis of CLEC10A binding to Neu5Ac-Tn, Neu5Gc-Tn and Tn antigens**

SPR is a very sensitive technique for the elucidation of binding events. Modern instruments, e.g. Biacore T100, allow even analyzing the interaction of small molecules with proteins. For the determination of dissociation constants of Tn and sialylated Tn antigen (Neu5Aca2,6-Tn and Neu5Gcα2,6-Tn) recombinant, purified CLEC10A was immobilized on a carboxymethylated dextran chip (CM5). The immobilization of the protein proved to be challenging due to the acidic nature of the construct with a pI of well below 4.8 due to the acidic myc tag and the presence of several neuraminic acids at the glycosylation sites at N22 and N89 (data not shown). The effect of neuraminic acids on the overall pI of proteins was shown to be −0.1 to 0.3 per residue (Barrabés et al. 2010). However, covalent immobilization requires a pH between 3.5 and the pI of the immobilized molecule. Thus, the direct covalent immobilization of CLEC10A failed. Successful immobilization was achieved via an anti-c-myc antibody that in turn had been immobilized covalently. The data of the SPR binding analyses are in accordance with the STD NMR data. The affinity of the antigens proved to be mainly due to the GalNAc moiety and have values of the same range (Figure 3C). The Tn antigen shows the highest affinity of the three analyzed molecules of K_D = 63 μM. Substitution at the 6th position of GalNAc by Neu5Gc results in a slightly decreased affinity with K_D = 78 μM. A stronger decrease in the affinity was observed for Neu5Ac with K_D = 297 μM. A biotinylated spacer molecule served as a negative control, which showed no significant binding (K_D > 3 mM). It seems that the difference in binding affinity between

the Neu5Ac and Neu5Gc structures arises from a slower on-rate of the Neu5Ac structure as is clearly visible in Figure 4 from the slower rise of the curves in the sensorgrams. A fit of the k_on and k_off rates was not possible across the whole data sets. The consistency of the results from dissociation constant determinations shown here by STD NMR and SPR is good. However, differences are observed that arise from the completely different experimental set up. Differences in the K_D values are known to arise from the fact that SPR is a flow system with immobilized protein, whereas STD NMR results are obtained in a non-flow homogeneous set up.

**Binding of Tn, Neu5Ac-Tn and Neu5Gc-Tn antigens to CLEC10A transfectomas**

In order to test whether the Tn, Neu5Aca2,6-Tn and Neu5Gcα2,6-Tn antigens are bound by the transmembrane form of CLEC10A, a full-length clone of CLEC10A cDNA was transfected into CHO cells. As reported previously, CHO cells do not express CLEC10A (van Vliet et al. 2005). The expression of CLEC10A was shown by a specific antibody (Figure 5). The respective biotin-conjugated polymeric glycans were complexed with cyanine 3-labelled streptavidin. When Tn, Neu5Aca2,6-Tn or Neu5Gcα2,6-Tn antigens were added to the CLEC10A transfectomas, a co-localization of the respective glycans was observed (Figure 5A–C). As control, no significant binding of the TF antigen was observed (Figure 5D). No binding was detected when the labeled Neu5Aca2,6-Tn antigen was added to non-transfected CHO cells (Figure 5E).

**Discussion**

Human tumors express glycan structures that differ from the structures present in the corresponding normal tissues. One of these structures is the Tn antigen, defined as a GalNAc residue bound to serine or threonine by an α-glycosidic linkage (Dahr et al. 1975). The GalNAc residue is the first carbohydrate moiety in core structures 1–8 of O-linked glycans of glycoproteins (Brockhausen et al. 2009). The Tn structure has been described as a tumor-associated antigen in various human tumor entities (Ju et al. 2011). Besides the Tn antigen, a sialylated Tn structure (Neu5Aca2,6-GalNAca-Ser/Thr) is expressed in human tumors presumably because of the up-regulation of ST6GalNAc-I transferase (Brockhausen 1999; Julien et al. 2006). The Tn antigen is bound by the human glycoreceptor CLEC10A (CD301, macrophage galactose-type lectin), which is expressed by intermediate monocytes, dendritic cells and macrophages (van Vliet et al. 2008). Macrophages, known as tumor-associated macrophages (TAMs), may be present in the microenvironment of malignant tumors. In human carcinomas of a different origin, the presence of TAMs has been correlated with an adverse prognosis of the patients (Qian and Pollard 2010). A particular subpopulation of TAMs, the activated, trophic or M2 macrophages appears to be causally involved in tumor progression (Mantovani and Sica 2010; Qian and Pollard 2010). These macrophages are recruited by cytokines such as macrophage colony-stimulating factor 1 and activated by interleukin-4 (IL-4) and IL-13 (Qian and Pollard 2010). In vitro monocytes can be differentiated into M2 macrophages by addition of the conditioned tumor cell medium. Interestingly,
monocytes stimulated in this way express CLEC10A (Solinas et al. 2010). Human M2 macrophages express CLEC10A also in vivo, as shown in granuloma tissues of patients suffering sarcoidosis (Prokop et al. 2011). Though not formally proven, the interaction of CLEC10A, expressed in M2 macrophages, with Tn and sialyl-Tn, exposed by tumor cells, may modulate the TAM phenotype and/or activity, in this way affecting the progression of human tumors. Since sialyl-Tn is the most tumor-specific Tn-related structure, it may be of particular significance in the interaction with TAMs of the M2 phenotype.

Humans are not able to synthesize Neu5Gc, since they lack cytidine-5′-monophosphate-Neu5Ac-hydroxylase. It has been demonstrated that ingested Neu5Gc can be incorporated into glycan structures of human tumors (Nguyen et al. 2005). Since this glycan is non-self, it may induce an immune response (Nguyen et al. 2005; Padler-Karavani et al. 2012). Thus, the interaction of macrophages and possibly dendritic cells with Neu5Gcα2,6-GalNAc via CLEC10A may play an important role in the regulation of the immune response to Neu5Gc expressed by human tumor cells.

The detailed analysis of the specificity of CLEC10A (van Vliet et al. 2005) has previously revealed that CLEC10A binds to a variety of glycans in addition to the Tn antigen. Generally, the substitution of GalNAc in the 3rd position hinders the binding of CLEC10A. Interestingly, substitutions in the 6th position by GlcNAc (core 6) or sulfate (6-sulfo-Tn) did not affect binding, whereas no significant binding was observed when sialic acid was attached in the 6th position (Neu5Acoα2,6-Tn; van Vliet et al. 2005).

In the present study, the glycan specificities of CLEC10A have been analyzed by ELISA in glycan arrays, by SPR and by STD NMR. In addition, binding studies with CLEC10A transfectedomas were performed. We report on two novel specificities not observed so far. Our binding studies extend the specificity previously known (van Vliet et al. 2005) to 6-sialyl substituted GalNAc residues. As initially shown by ELISA, CLEC10A binds to Neu5Acoα2,6-Tn and Neu5Gcα2,6-Tn antigens.

STD NMR and SPR support these novel binding specificities. STD NMR epitope mapping gave a distinct insight on the binding mode of the Neu5Acoα2,6-Tn antigen to CLEC10A. The proposed binding modes are illustrated in Figure 3B and D. The binding site of the lectin can be subdivided into two areas. The first dominant area binds the GalNAc residue from H-2 to H-4 including the N-acetate as well as Hα and Hβ protons of the serine residue. This result easily explains why GlcNAc, the C-4 epimer of GalNAc, shows no binding to CLEC10A. The second binding region is located next to the primary binding area of GalNAc. It appears that this binding site is less important than the binding area for the GalNAc residue and only minor contributions to the affinity of the molecule to the lectin are due to the sialic acid residue. The affinity of the Neu5Acoα2,6-Tn antigen is by a factor of ~2 lower than that of the Tn antigen. Neuraminic acid alone shows no binding to CLEC10A. The proposed binding modes are illustrated in Figure 3B and D. The binding site of the lectin can be subdivided into two areas. The first dominant area binds the GalNAc residue from H-2 to H-4 including the N-acetate as well as Hα and Hβ protons of the serine residue. This result easily explains why GlcNAc, the C-4 epimer of GalNAc, shows no binding to CLEC10A. The second binding region is located next to the primary binding area of GalNAc. It appears that this binding site is less important than the binding area for the GalNAc residue and only minor contributions to the affinity of the molecule to the lectin are due to the sialic acid residue. The affinity of the Neu5Acoα2,6-Tn antigen is by a factor of ~2 lower than that of the Tn antigen. Neuraminic acid alone shows no binding to CLEC10A (cf. Figure 1). Comparison of the binding epitopes of Tn and Neu5Acoα2,6-Tn antigens explains the reduced affinity of the sialylated Tn structure (Figure 3C). In the Neu5Acoα2,6-Tn antigen, the GalNAc residue has lower relative STD effects than observed for the Tn antigen. This in
turn indicates that the GalNAc residue is somewhat closer to the protein in the complex of CLEC10A with Tn than with Neu5Acα2,6-Tn (H-2, 3 and 4). This fact leads to the conclusion that the GalNAc residue, when carrying the sialic acid residue, cannot dip as deeply into the binding pocket of the CRD of CLEC10A as the terminal un-substituted GalNAc. The loss of affinity is partially compensated by interactions of the sialic acid residue, indicated by the observed STD effects on the sialyl residue. The STD effects of the GalNAc residue in the Tn antigen itself shows no STD effects on the H-6 protons, indicating that there is no contact to the glycan receptor. Therefore, the STD of the Tn antigen alone indicates that a substitution at C-6 of the GalNAc residue is likely tolerated. This hypothesis is further supported by the fact that a GalNAc residue that is substituted in the 6th position by Neu5Gcα2,6-Tn, GlcNAc (core 6) or sulfate (6-O-sulfateGalNAc) also binds the CLEC10A (cf. Figure 1). As the H-3 of GalNAc is in the middle of the binding epitope of the Tn antigen, it is very likely that no substitution is allowed at this position. Indeed, 3-substituted GalNAc residues like Neu5Acα2,3-Tn show no binding (cf. Figure 1) in our assays confirming earlier data (van Vliet et al. 2005). Taken together, it is clear that 6-sialylated Tn antigens have binding affinity to CLEC10A.

The SPR experiments showed that Neu5Gc in the Tn antigen has a higher affinity to CLEC10A than Neu5Ac. A likely
Isolation of CLEC10A

Cell culture supernatant containing recombinant soluble CLEC10A was concentrated 10-fold using Slide-A-Lyzer® Dialysis Cassettes (10 kDa MWCO, Thermo Scientific) and Spectra/Gel Absorbent (Carl Roth). The concentrated sample cell culture supernatant was diluted with an equal volume of PBS (Dulbecco’s phosphate buffer saline, Gibco-Life Technologies, Darmstadt, Germany) and loaded onto the anti-c-myc immunosorbent (monoclonal c-myc 9E10 9E10 Ac agarose, 500 µg/mL, Santa Cruz Biotechnology). After incubation overnight at 4°C, the column was washed with five times with PBS. Bound proteins were eluted with 0.1 M glycine–HCl, pH 2.5, and neutralized with 1 M Tris–HCl, pH 9.0.

Materials and methods

Cells

HEKT293 (human embryonic kidney) and CHO (Chinese hamster ovary) cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal calf serum (FCS). (i) Cloning and expression of soluble recombinant CLEC10A: The extracellular part of human C-type glyco-receptor CLEC10A (NCBI ref seq: NM_182906) was amplified from cDNA by polymerase chain reaction and cloned into a pcDNA 3.1/Zeo (+) containing a zeocin resistance marker gene. A murine Igκ-chain leader sequence fused to the N terminus ensures the directed secretion of recombinant glyco-receptors. For precipitation and detection, a c-myc-tag was attached to the N terminus. HEKT293 cells were stably transfected using soluble zeocin. CLEC10A was detected in cell culture supernatant using anti-c-myc western blot analysis. Supernatants were either directly used or stored at 4°C in the presence of 0.01% aprotinin for further analysis. (ii) Expression of full-length CLEC10A: For glycan, fluorescence staining CHO cells were transiently transfected with cDNA encoding full-length CLEC10A (open Biosystems-Thermo Scientific, Rockford, IL) for 48 h using Lipofectamin 2000® (Invitrogen, Karlsruhe, Germany).

Western blotting

Proteins were transferred onto Nitrocellulose membranes (0.45 µM, ThermoScientific Rockford, IL). The membrane was blocked with 3% Bovine serum albumin (BSA) (Carl Roth, Karlsruhe, Germany) in Tris-Buffered Saline and Tween 20 (TBST; 10 mM Tris–HCl, pH 8, 150 mM NaCl, 0.1% Tween) for 1 h at room temperature and hybridized with anti-c-myc (monoclonal c-myc 9E10, 200 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membrane was washed with TBST and incubated with a rabbit anti-mouse HRP conjugated antibody (1: 50,000) for 1 h, washed with TBST and incubated for 1 min with ECL solution (GE Healthcare, Freiburg, Germany). The chemoluminescence was detected on an Amersham Hyperfilm (GE Healthcare).

Carbohydrates

Unless otherwise stated, all carbohydrates were purchased from Lectinity (Moscow, Russia). For ELISA experiments, different carbohydrates were used, which were linked via an aminoglucitol (HOCH2(HOCH)4CH2NH-X) to a poly[N-(2-hydroxyethyl)acrylamide] (glycan-PAA) backbone. Fluorescence staining was performed with the biotin-conjugated variants of these polymeric glycans (glycan-PAA-biotin) were used. Monomeric biotin-conjugated glycans (glycan-biotin) were used for SPR. For STD-NMR, the glyco-amino acids Tn (GalNAc1-O-serine) and Neu5Ac-Tn antigens (Neu5Ac2,6-GalNAc1-O-serine) were purchased from Sigma Aldrich (Germany) and Carbohydrate Synthesis Ltd (Abingdon, UK), respectively.

Glycan-ELISA

Cell culture supernatant containing recombinant soluble CLEC10A and cell culture supernatant from un-transfected HEKT293 cells were dialyzed against 20 mM Tris–HCl, pH 7.4, and 150 mM NaCl using Slide-A-Lyzer® Dialysis Cassettes (10 kDa MWCO, Thermo Scientific). PAA-conjugated glycans (500 ng/well) were coated as quintuplicate to flat-bottom MaxiSorp 96-well plates (Thermo Fisher Scientific/ Nunc, Rockford, IL) in 100 µL of PBS (pH 7.4) overnight at 4°C under constant agitation. Plates were blocked using carbo-free blocking solution (Biozol, Eching, Germany) for 1 h at room temperature. Unbound glycoconjugates were removed. Fifteen microliters of a biotinylated anti-c-myc antibody (monoclonal c-myc 9E10, 200 µg/mL, Santa Cruz Biotechnology) was added to 1.5 mL of dialyzed cell culture supernatant samples for 1 h at 4°C. Three micrograms of the streptavidin-HRP (Thermo Scientific) conjugate was added and incubated for 30 min at room temperature in the dark. Samples were filled up to 10 mL with Tris saline magnesium (TSM) buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 1 mM CaCl2). For binding studies of CLEC10A in a Ca2+-dependent manner, the generated complex was filled up with TSM buffer without Ca2+ and Mg2+. 100 µL/well of each sample was added and incubated overnight at 4°C in the dark. Wells were washed (three times, 300 µL/well) with the same buffer using for the hybridization containing 0.1% Tween-20, ABTS (2,2’-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] dia-mmonium salt) was added as a substrate according to the instructions of the manufacturer (Roche, Mannheim, Germany).
Absorbance was measured at 405 nm on a microplate reader (Tecan, Maennedorf, Switzerland) at 37°C.

**Glycan fluorescence staining**

For all steps PBS containing 1 mM CaCl₂ and 2 mM MgCl₂ (0.5 mM/µL) were used. DMEM without FCS (0.5 mM/µL) and 50 mM NH₄HCO₃, 37°C, 16 h. After digestion, the gel – 1 h at 37°C, cells were rinsed once with PBS and 37°C, washed three times with DMEM. After incubation of the CHO cells with the glycan – 30 min. Cells were washed three times with DMEM. After digestion of the CHO cells with the glycan–streptavidin–cyanine 3 complex for 1 h at 37°C, cells were rinsed once with PBS and fixed for 20 min using 4% paraformaldehyde in PBS. After washing with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 3 min, followed by incubation with goat anti-human CLEC10A (1:200) in PBS [containing 1% BSA (biotin-free), 200 µL] for 1 h in the dark. Cells were washed three times with PBS containing 1% BSA (1 mL/µL) and incubated with rabbit anti-goat-APC (100 µL/µL) in PBS containing 1% BSA. Slides were washed with 50 µL PBS, then with 50 µL H₂O in a tray and mounted with Aqua-Polymount (Polysciences, Inc., Warrington, PA). Stained sections were processed with an exponential line broadening of 2 Hz. Absolute STD effects were determined by the integration of resonances in the STD NMR spectrum with respect to the corresponding off the resonance spectrum. Relative STD percentages were calculated with respect to the proton showing the highest STD effect. The dissociation constant was determined by fitting the one-site binding model on the STD amplification factors of the proton that showed the highest STD effect plotted against ligand concentration.

**Surface plasmon resonance**

All experiments were performed using a Biacore T100 instrument (GE Healthcare, Freiburg, Germany). As running buffer, sterile filtered TSM-buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM CaCl₂) was used.

**Mass spectrometry**

The gels were stained with colloidal Coomassie, the bands were cut out, the proteins reduced with DTT (10 mM, 56°C, 30 min), the cysteine residues modified with iodoacetamid (55 mM, ambient temperature, 20 min in the dark) and the protein in-gel digested with trypsin [conditions: 5 ng trypsin/µL (sequencing grade modified trypsin, Promega, Madison, WI) in 50 mM NH₄HCO₃, 37°C, 16 h]. After digestion, the gel pieces were repeatedly extracted (50% acetonitrile/5% formic acid), the combined extracts dried down in a vacuum concentrator, dissolved in 5% methanol/5% formic acid, desalted on a C18 ZipTip (Millipore, Billerica, MA), eluted with 1 µL 60% methanol/5% formic acid and analyzed by nano-electrospray mass spectrometry in a quadrupole time-of-flight (QTOF) II instrument (Micromass, Manchester, UK). Tandem mass spectrometry (MS/MS) spectra obtained by collision induced fragmentation after manual precursor selection were evaluated by the Mascot MS/MS search algorithm version 2.2 (Matrix Sciences, London, UK).

**Immobilization**

In a first step, the anti-c-myc antibody (monoclonal c-myc 9E10, Santa Cruz Biotechnology) was immobilized on a CM5 chip (GE Healthcare) via standard amine coupling. Briefly, the chip was activated by a 5-min pulse of a freshly prepared mixture of 50 mM N-hydroxysuccinimide and 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 10 µL/min on flow cell 1 and flow cell 2. Subsequently, the anti-c-myc antibody, dissolved in 10 mM sodium acetate buffer, pH 5, was immobilized on flow cell 2 yielding 13 kRU (20 min, 10 µL/min). Unreacted carboxyl functions were capped by a 7-min pulse of ethanol amine on both flow cells. The CLEC10A buffered in TSM was captured on flow cell 2 by the immobilized anti-c-myc antibody at a flow rate of 5 µL/min for 28 min. The anti-c-myc binding sites of the immobilized antibody were re-saturated by CLEC10A previous to each cycle by a 1- or 5-min pulse of CLEC10A solution. The non-covalent immobilization yielded 1500–2700 RU (RUmax theoretical: 3500 RU).

**Saturation transfer difference NMR**

Experiments were obtained at 300 K on a Bruker Avance 700 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with an inverse triple resonance probe. CLEC10A was complexed with a biotinylated anti-c-myc antibody (9E10, Santa Cruz) and streptavidin-HRP (Thermo Scientific) and streptavidin (Neu5Ac2,6-Tn, Neu5Gc2,6-Tn, Tn and TF antigens) in 200 µL of DMEM for 30 min. Cells were washed three times with DMEM. After incubation of the CHO cells with the glycan–streptavidin–cyanine 3 complex for 1 h at 37°C, cells were rinsed once with PBS and fixed for 20 min using 4% paraformaldehyde in PBS. After washing with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 3 min, followed by incubation with goat anti-human CLEC10A (1:200) in PBS [containing 1% BSA (biotin-free), 50 µL/well] for 1 h in the dark. Cells were washed three times with PBS containing 1% BSA (1 mL/well) and incubated with rabbit anti-goat-APC (100 µL/well) in PBS containing 1% BSA. Slides were washed with 50 µL PBS, then with 50 µL H₂O in a tray and mounted with Aqua-Polymount (Polysciences, Inc., Warrington, PA). Stained sections were then examined under a Leica DM5000B microscope.

**Affinity analysis**

Monomeric biotin-conjugated glycans and negative control (biotinylated spacer) dissolved in running buffer were injected with a contact time of 3 min at a flow rate of 30 µL/min in flow cells 1 and 2. The concentrations of the antigens ranged between 15 µM and 1 mM. The dissociations constants were determined by fitting the data with the one-site binding model using OriginPro 8.6G (OriginLab Corporation, Northhampton, MA). Responses from the reference flow cell 1 were subtracted from flow cell 2. In the case of the Tn and NeuGc-Tn antigens, we used absolute RU values at each concentration to determine K₅ values because binding led to previously described (Wilhelm et al. 2012) using the Bruker standard pseudo 2D pulse sequence stddiffesgp. Water suppression was achieved using excitation sculpting. The selective square pulse for solvent suppression was set to 4 ms. For protein saturation, a cascade of 40 Gauss pulses with a length of each 50 ms and an attenuation of 40 db were used. On resonance irradiation was at 0 ppm and off resonance at 40 ppm. Glyco-amino acids were titrated to the CLEC10A solution from 16 mM stock solutions and were analyzed at final concentrations of 158, 390, 689, 1047, 1714 and 2305 µM. The data were processed with an exponential line broadening of 2 Hz. Absolute STD effects were determined by the integration of resonances in the STD NMR spectrum with respect to the corresponding off the resonance spectrum. Relative STD percentages were calculated with respect to the proton showing the highest STD effect. The dissociation constant was determined by fitting the one-site binding model on the STD amplification factors of the proton that showed the highest STD effect plotted against ligand concentration.
negative net responses. The effect is likely due to conformational changes. This phenomenon is often observed for small ligands with weak binding (Gestwicki et al. 2001; Mesch et al. 2010).

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Abbreviations

BSA, bovine serum albumin; CHO, chinese hamster ovary; CLEC10A, C-type lectin domain family 10 member A; CRD, carbohydrate recognition domain; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; HDO, deuterium hydroxide; IL, interleukin; Lac-di-Nac, Galβ1,4-GlcNAc; MS/MS, tandem mass spectrometry; Neu5Ac, N-acetyleneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; NMR, nuclear magnetic resonance; PAA, poly[N-(2-hydroxyethyl)acrylamide]; PBS, phosphate buffer saline; QTOF, quadrupole time-of-flight; SPR, surface plasmon resonance; STD, saturation transfer difference; TAM, tumor associated macrophage; TBST, Tris-Buffered Saline and Tween 20; TSM, Tris saline magnesium.

References