Supporting Information

Influence of Core β-1,2-xylosylation on Glycoprotein Recognition by Murine C-type Lectin Receptors and its Impact on Dendritic Cell Targeting

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MALDI-TOF characterization of glycoconjugates.

For MALDI-Tof measurements, a small volume of both neoglycoconjugates and OVA solutions were subjected to buffer exchange against nanopure water in a 10 kDa Amicon filter devices to avoid signal suppression due to the presence of salts. Protein solutions (1 µL) were deposited on a MTP 384 polished steel MALDI plate (Bruker Daltonics, Bremen, Germany) and dried at room temperature. On top, 2 µL of matrix solution containing sinapic acid, 4 mg/mL in CH_3CN: aqueous TFA 1% (1:1) were deposited and dried at room temperature. MALDI-Tof mass measurements were performed on an Ultraflextreme III time-of-flight mass spectrometer equipped with a pulsed Nd:YAG laser (λ 355 nm) and controlled by FlexControl 3.3 software (Bruker Daltonics, Bremen , Germany). The acquisitions (total of 2000-3000) were carried out in linear positive ion mode with pulsed ion extraction of 450 ns and laser frequency of 1000 Hz. Laser fluence was set up to 60-80 % and the *m/z* range was chosen according to the mass of the sample. The accumulated spectra were processed using the Bruker software FlexAnalysis 3.3.

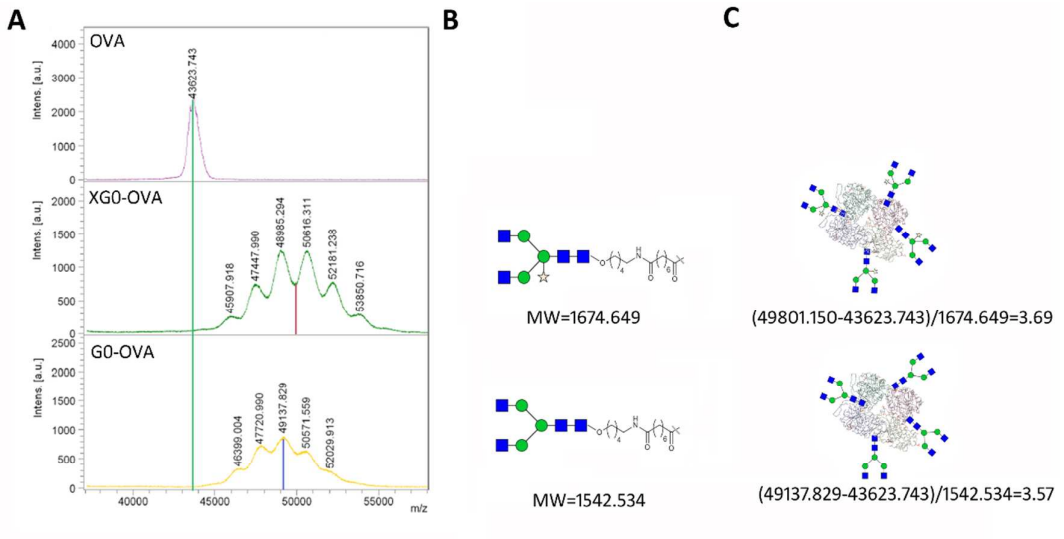

Figure S1. Characterisation of the glycoconjugates G0-OVA and XG0-OVA by MALDI-Tof. The mass shift (**A**) upon conjugation of OVA with activated glycans G0-DSS and XG0-DSS (**B**) indicated (**C**) an average conjugation of 3-4 glycans per OVA molecule.

Calculation of the Alexa Fluor® 647 degree of substitution (DOS).

In order to obtain the same degree of substitution (DOS) of OVA and both glycoconjugates XG0-OVA, G0-OVA with Alexa Fluor® 647 NHS ester, the concentration of proteins and dye were kept unchanged for all three conjugation reactions. After extensive removal of excess of dye by dialysis, DOS was calculated and the ratio between dye/protein was established as an average of 11-12 for all three samples (Table S1).

Table S1. Calculation of the DOS of OVA-647, G0-OVA-647 and XG0-OVA-647

	A_{max}	A_{280}	*[Dye]*	*[OVA]*	*DOS*
OVA	7.98	0.48	6.38e-5	5.28e-6	**12.1**
XG0-OVA	8.52	0.52	6.82e-5	6.12e-6	**11.1**
G0-OVA	8.88	0.54	7.10e-5	6.25e-6	**11.3**

DOS was estimated based on absorbance of dye (ε_{dye}, 250 000 $cm^{-1}M^{-1}$) and protein (ε_{OVA}, 35 900 $cm^{-1}M^{-1}$) and calculated based on the equation:

$DOS = \frac{[Dye]}{[OVA]}$, where

$[Dye] = \frac{(A_{Max} * dilution\ factor)}{\varepsilon_{dye}}$ (molar concentration of Alexa Fluor®-647)

$[OVA] = \frac{(A_{280} - 0.05 * A_{Max}) dilution\ factor}{\varepsilon_{OVA}}$ (molar concentration of OVA or glycoproteins)

Fluorescence emission spectra for three labelled proteins at 20 µg/mL were recorded with a Varioskan Flashmicroplate reader (Thermo Scientific) with excitation wavelength at λ 633 nm.

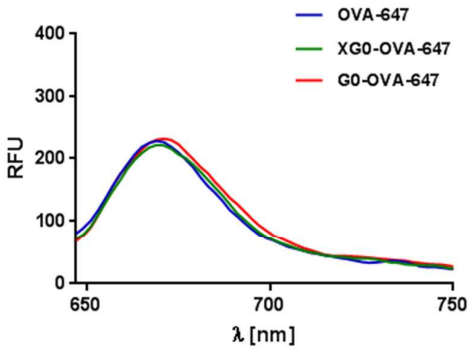

Figure S2. The comparison of fluorescence emission spectra recorded for OVA-647 (blue), XG0-647 (green) and G0-647 (red).

General procedure for the production of the CLR-Fc library

The general procedure for the production of the mouse CLR-Fc fusion protein library has been described previously in detail (Maglinao et al., *J. Control. Release* **2014**, 10, 175, 36-42). In the meantime, the library has been extended by Dectin-2-Fc and SIGNR1-Fc. The following primers were used for PCR amplification: DCAR: 5'-CCATGGAACTTGACAGGTACCATTCATTG-3', 5'-AGATCTTAAGTTTATTTTCTTCATCTGAC-3'; DCIR: 5'-GAATTCGCTACTTCTCCTGCTGCTGG-3', 5'-AGATCTTCCAGTCTTCCAACGGTAAA-3'; Dectin-2: 5'-CCATGGAGAAAACATCATTCCAGCCCC-3', 5'-GAATTCCTGGAGCACCAGTGAGCAGAAC-3'; MCL: 5'-GAATTCTCATTACTTTTTACGCTGGA-3', 5'-AGATCTACAAATCCTTCTCACCTCAAAG-3; Clec-12b: 5'-ACTTTCTCCTAGGATGTCTG-3', 5'-GCATGGGTTTGCAATAGGTC-3'; SIGNR1: 5'-GAATTCGCAGAAGGAACAAGAGAAGATCC-3', 5'-CCATGGGCAACCCCATGCACTGAAGGC-3'; SIGNR3: 5'-GAATTCCATGCAACTGAAGGCTGAAG-3', 5'-AGATCTTTTGGTGGTGCATGATGAGG-3'; Mincle: 5'-CCATGGGGCAGAACTTACAGCCACAT-3', 5'-AGATCTGTCCAGAGGACTTATTTCTG-3'. The cDNA fragments were first cloned into the pDrive cloning vector (Qiagen) and later ligated into the pFuse-hIgG1-Fc expression vector (InvivoGen). The CLR-Fc encoding vectors were then either stably transfected into CHO cells or transiently transfected using the FreeStyle Max CHO-S Expression System (Life Technologies). The CLR-Fc containing cell supernatant was then collected and the CLR-Fc fusion proteins were purified using HiTrap Protein G HP columns (GE Healthcare). SDS-PAGE with subsequent Coomassie stain as well as Western Blot were performed to confirm the identity and purity of the CLR-Fc fusion proteins. CLR-Fc concentrations were determined using Micro BCA Protein Assay Kit (Thermo Scientific).

C-type lectin microarray fluorescent image and its quantification.

The binding data provided is the result of an optimization process were protein printing conditions, immobilization method and neoglycoprotein concentrations had been evaluated. From the NHS activated and protein G functionalized slides that were tested for immobilization of CLR-Fc fusion proteins, only the protein G functionalized slides provided measurable and specific interaction with the neoglycoprotein constructs. As negative control, Fc-domain was printed alongside the fusion proteins. No binding was seen for the negative control with any of the OVA constructs. The printing concentration for the lectins was adjusted to the lowest CLR-Fc concentration (0.2 mg/mL) which was sufficiently high to observe specific interaction. The printed lectin arrays were incubated at 30 and 50 µg/mL with neoglycoconjugates and OVA solutions, but binding was only observed at the higher concentration. For some CLRs doughnut spot morphology upon drying was observed, a quite common feature in protein arrays. This could not be completely avoided even after the addition of surfactant in printing buffer for improved mixing of buffer and protein or by the addition of glycerol for slowing down the drying process. For the quantification of spots, we employed median instead of mean fluorescence values to account for the variability in spot morphology, as previously suggested in: Wellhausen, R.; Seitz, H. *BioMed Research International* **2012**, *2012*, Article ID 831347, 8 pages.

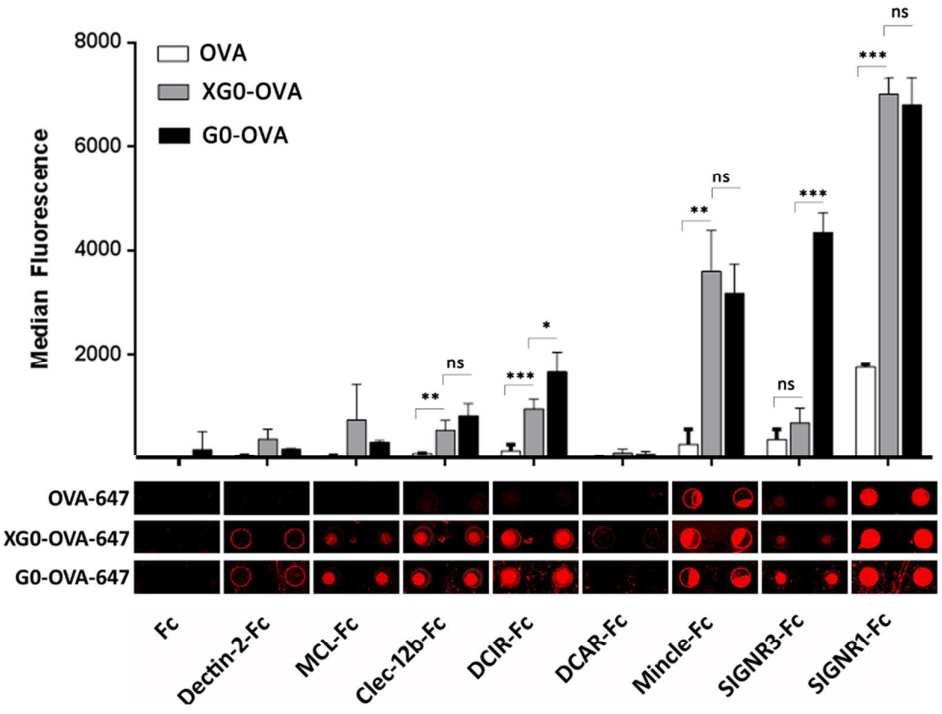

Figure S3. C-type lectin microarray-based analysis of binding of CLR-Fc fusion proteins to glycoconjugates XG0-OVA (grey) and G0-OVA (black) compared to unmodified OVA (white). Below the graph fluorescent image of microarray used for the quantification. Statistical analysis was performed with an unpaired student's t test, ***p<0.0001,**p<0.01, *p<0.05, ns-not significant.

Scatter plots of MACS-purified DCs and OT-II T cells.

Cells were pre-incubated with CD16/32 (Fc-Block, dilution 1:100) blocking antibody in 100 µL FACS buffer (PBS, 0.5% BSA, 2mM EDTA) at 4°C for 10 min. Subsequently, purified DCs and flow through were incubated with an anti-CD11c (APC) (1:100 dilution) for 30 min at 4°C in the dark. Purified T cells were incubated with anti-CD3e (APC) and anti-CD19 (PE) antibodies (1:100 dilution) for 30 min at 4°C in the dark. As a control, unstained samples were also prepared. Cells were washed with 1 mL of FACS buffer, re-suspended in 100 µL FACS buffer and vortexed before each measurement.

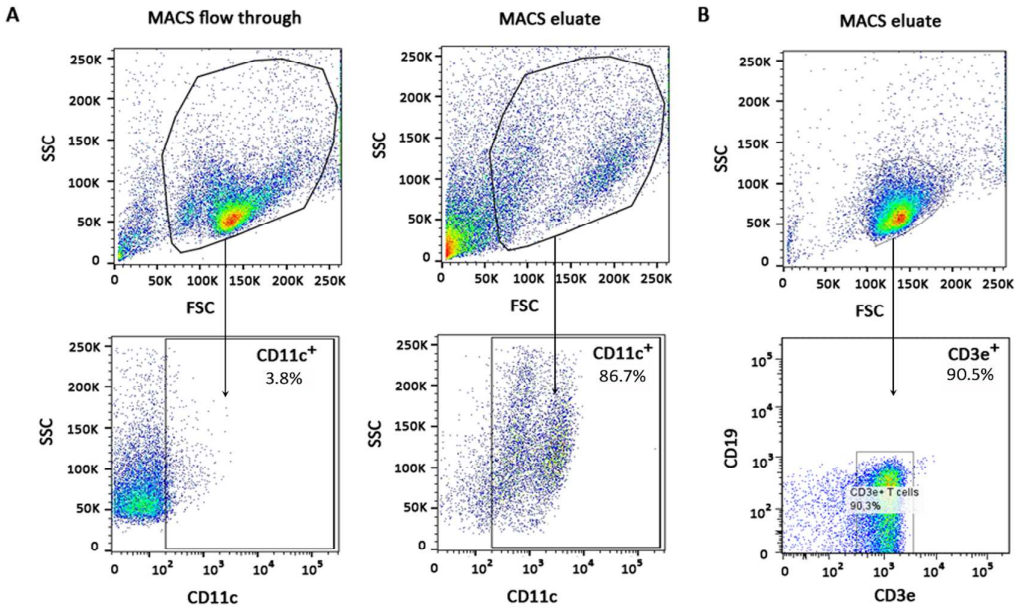

Figure S4. Representative dot plots of MACS-purified DCs and T cells **A.** Splenic CD11c+ cells from wild-type C57BL/6 mice and **B.** splenic T cells from T cell receptor-transgenic OT-II mice. Purity of the DC isolation was analysed by staining with an anti-CD11c antibody, whereas purity of the T cell preparation was confirmed by using an anti-CD3 antibody for staining.