

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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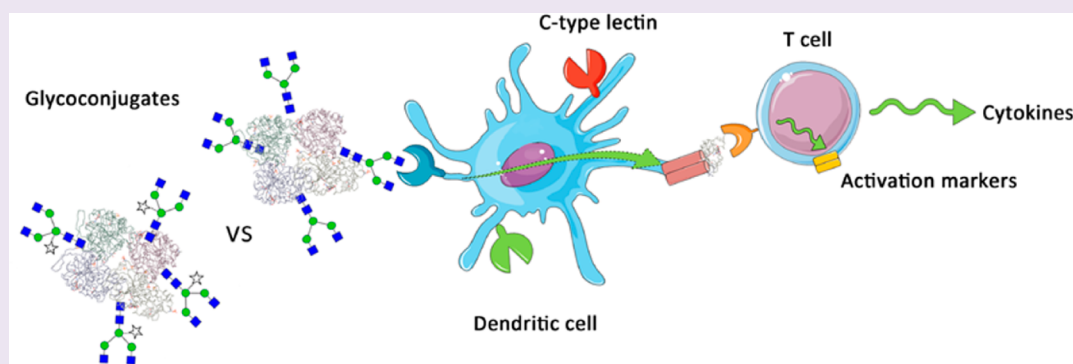
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S Supporting Information



ABSTRACT: Targeting antigens to dendritic cell subsets is a promising strategy to enhance the efficacy of vaccines. C-type lectin receptors (CLRs) expressed by dendritic cells are particularly attractive candidates since CLR engagement may promote cell uptake and may further stimulate antigen presentation and subsequent T cell activation. While most previous approaches have involved antibody-mediated CLR-targeting, glycan-based CLR targeting has become more and more attractive in recent years. In the present study, we show that small structural glycan modifications may markedly influence CLR recognition, dendritic cell targeting, and subsequent T cell activation. A biantennary N-glycan (G0) and its analogous O-2 core xylosylated N-glycan (XG0) were synthesized, covalently conjugated to the model antigen ovalbumin, and analyzed for binding to a set of murine CLR-Fc fusion proteins using lectin microarray. To evaluate whether the differential binding of G0 and XG0 to CLRs impacted dendritic cell targeting, uptake studies using murine dendritic cells were performed. Finally, effects of the ovalbumin glycoconjugates on T cell activation were measured in a dendritic cell/T cell cocultivation assay. Our results highlight the utility of glycan-based dendritic cell targeting and demonstrate that small structural differences may have a major impact on dendritic cell targeting efficacy.

Carbohydrates have multiple functions and play important roles in numerous biological events such as cell proliferation, cell–cell recognition, or the immune response.¹ Glycoproteins together with other glycoconjugates decorate the surface of most cells and form the so-called glycocalyx, an effective physical barrier preventing the unrestricted entrance of pathogens into the underlying tissue.² During host–pathogen coevolution, many pathogens have developed strategies to attach to host cell surface lectins or glycans, e.g., by mimicking host glycosylation or by the expression of lectins recognizing host glycan motifs, thereby facilitating their entrance into the cell.^{3,4} The glycocalyx is formed by membrane bound

glycoproteins presenting sugars that are either attached via serine/threonine (O-glycans) or asparagine residues (N-glycans). The N-glycan biosynthesis in the endoplasmic reticulum (ER) is shared by all species up to a common dolichol-diphosphate linked high mannose type nonasaccharide. However, its further processing by multiple glycosyltransferases and glycosidases in the Golgi apparatus leads to a large variation of the N-glycan repertoire that differs markedly

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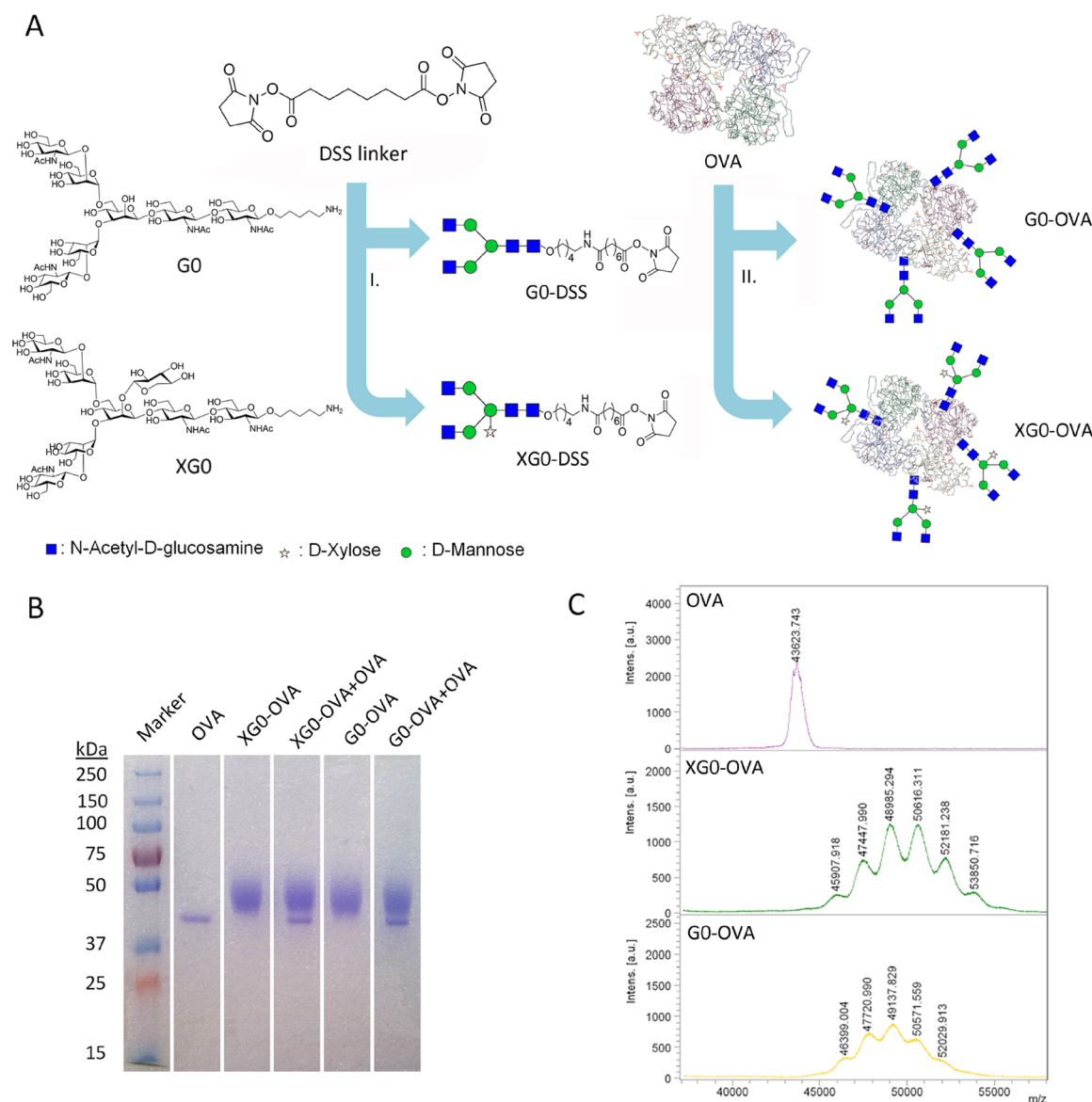


Figure 1. (A) Synthesis of OVA glycoconjugates employing disuccinimidyl suberate ester (DSS) as a linker: (I) monosubstituted NHS-activated suberate ester, (II) addition of activated N-glycan to OVA solution. Evaluation of the glycoconjugate synthesis. (B) SDS-PAGE (10% acrylamide) stained with Coomassie Blue G-250. (C) MALDI-ToF spectra of OVA (violet), XGO-OVA (green), and GO-OVA (yellow).

between organisms. For instance, yeasts and some parasites such as *Trypanosoma brucei* typically express high-mannose structures, while vertebrate N-glycans are mainly of the complex type,^{5,6} often decorated with core α -1,6 fucose. Plants and some parasites add β 1,2-linked xylose or α -1,3 fucose modifications, which are readily recognized as nonself glycan motifs by the human immune system.^{7–9}

Dendritic cells (DCs) are highly specialized antigen-presenting cells (APCs) constantly sampling their environment in search for foreign molecules.¹⁰ Once DCs encounter an antigen, they are able to capture it, further process it, and present antigen fragments to T cells. Since DCs stimulate T cell responses but are also capable of inducing tolerance, they are an attractive target for immunotherapy¹¹ and have been exploited to enhance vaccine efficacy.^{12–14}

Some of the DC-targeting vaccines are generated *ex vivo* using antigen-loaded monocyte-derived DCs that are further readministered to the patient.^{15,16} More direct methods involve the *in vivo* targeting of specific DC receptors, including C-type

lectin receptors (CLRs). CLRs represent a large family of pattern recognition receptors specialized in the recognition of carbohydrates on pathogens and self-antigens through their extracellular carbohydrate recognition domain (CRD).^{17–19} Since CLRs exhibit a differential expression on DC subsets and a number of them act as endocytic receptors, they are attractive candidates for an efficient antigen targeting to DCs.¹⁸ Still, most of the efforts toward CLR targeting utilize monoclonal antibodies²⁰ (e.g., anti-DEC205,²¹ antimannose receptor mAbs), and far fewer examples are available for glycan-based CLR targeting.²² However, glycan-based CLR targeting offers some advantages compared to antibody-mediated targeting, such as a low immunogenicity and the possibility to target several CLRs simultaneously. Furthermore, targeting CLRs with glycans exploits natural CLR functionality and allows modulating the protein–glycan binding strength by adjusting ligand density and spatial orientation.²³ In addition, glycans or glycomimetics are discrete molecular entities that can be prepared in a relatively straightforward manner by chemo-

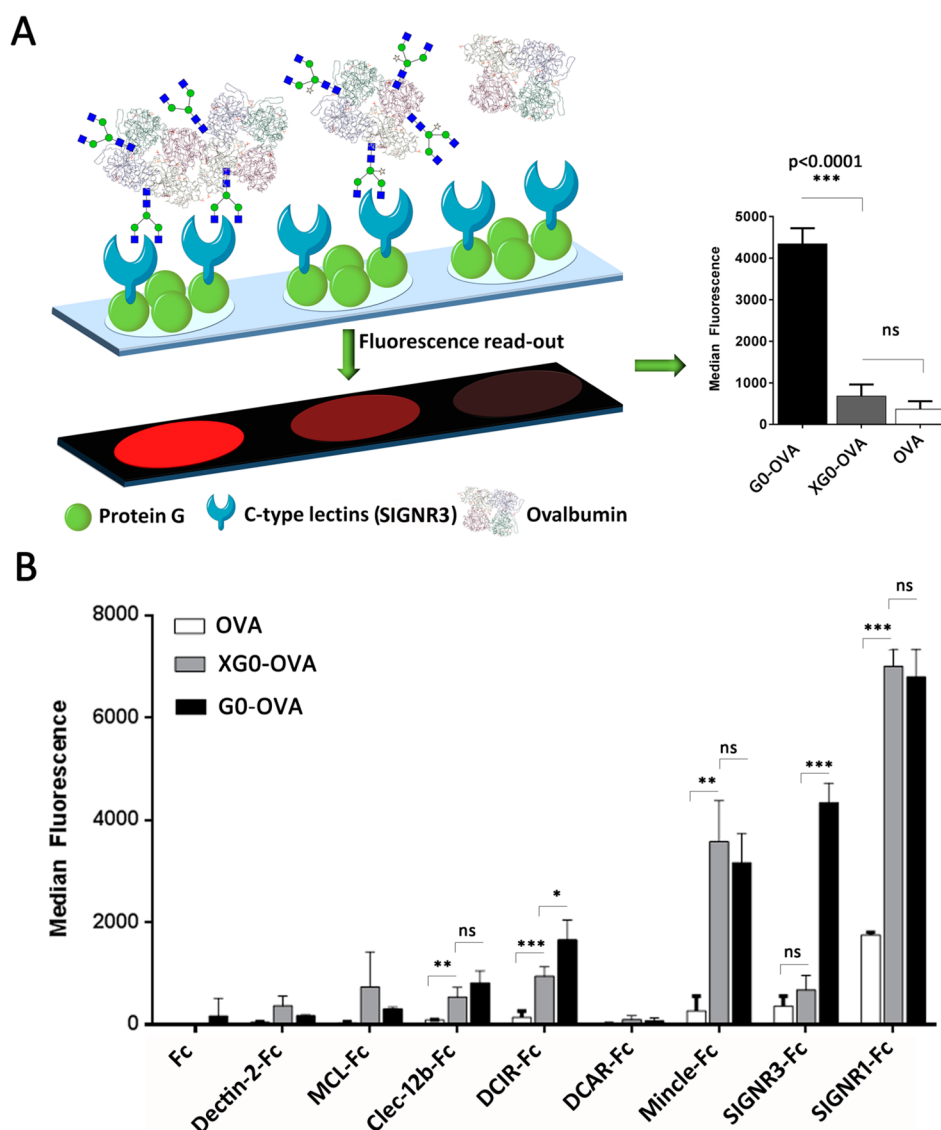


Figure 2. (A) Schematic representation of the lectin array-assisted binding studies with glycoconjugates and unconjugated OVA showing SIGNR3 as an example. (B) C-type lectin microarray-based analysis of binding of CLR-Fc fusion proteins to glycoconjugates XG0-OVA (gray) and G0-OVA (black) compared to unmodified OVA (white). Statistical analysis was performed with an unpaired student's *t* test, *** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, ns: not significant.

enzymatic methods^{24,25} and show a potentially more favorable pharmacokinetic profile when compared to large protein drugs.²⁶ The inherently weak interactions between single carbohydrates and proteins can be substantially increased by a multivalent glycan presentation. To this end, nanoparticles, liposomes, and dendrimers have been used as different display systems for multivalent CLR targeting.¹⁷ Notably, the Kiessling group recently discovered potent nonglycan inhibitors of DC-SIGN with up to 1000-fold enhanced binding over the monosaccharide ligand in a high-throughput screen of commercial small molecule libraries that show great potential as tools for studying DC-SIGN function.^{27,28}

Here, we show that glycans can be used for an efficient targeting of CLRs expressed by DCs and, importantly, that small structural modifications can markedly influence glycan recognition by CLRs and subsequent T cell activation. In a recent study, we described that the presence of core xylose disrupted the interaction of several glycans with the mannose binding plant lectins, *Galanthus nivalis* agglutinin (GNA) and

Concanavalin A (ConA). Moreover, the presence of the core xylose on the biantennary N-glycan XG0 (Figure 1A) completely abolished binding to the human Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN),²⁴ a CLR involved in the recognition of pathogens such as HIV, *Schistosoma mansoni*, or the Ebola virus.^{29–31}

In this study, we further analyzed the differential recognition of G0 and XG0 (Figure 1A) by CLRs and evaluated their potential for DC-specific targeting. To this end, both N-glycans were chemically cross-linked to the model antigen ovalbumin (OVA) and glycoconjugate recognition by CLRs was analyzed by lectin microarray using a library of CLR-Fc fusion proteins. To evaluate the DC-targeting capability of the glycoconjugates, their uptake into murine DCs and the subsequent activation of antigen-specific T cells were measured. Our results indicate that already small structural differences may have a major impact on glycan-based CLR targeting, thus CLR targeting can be exploited to shape immune responses.

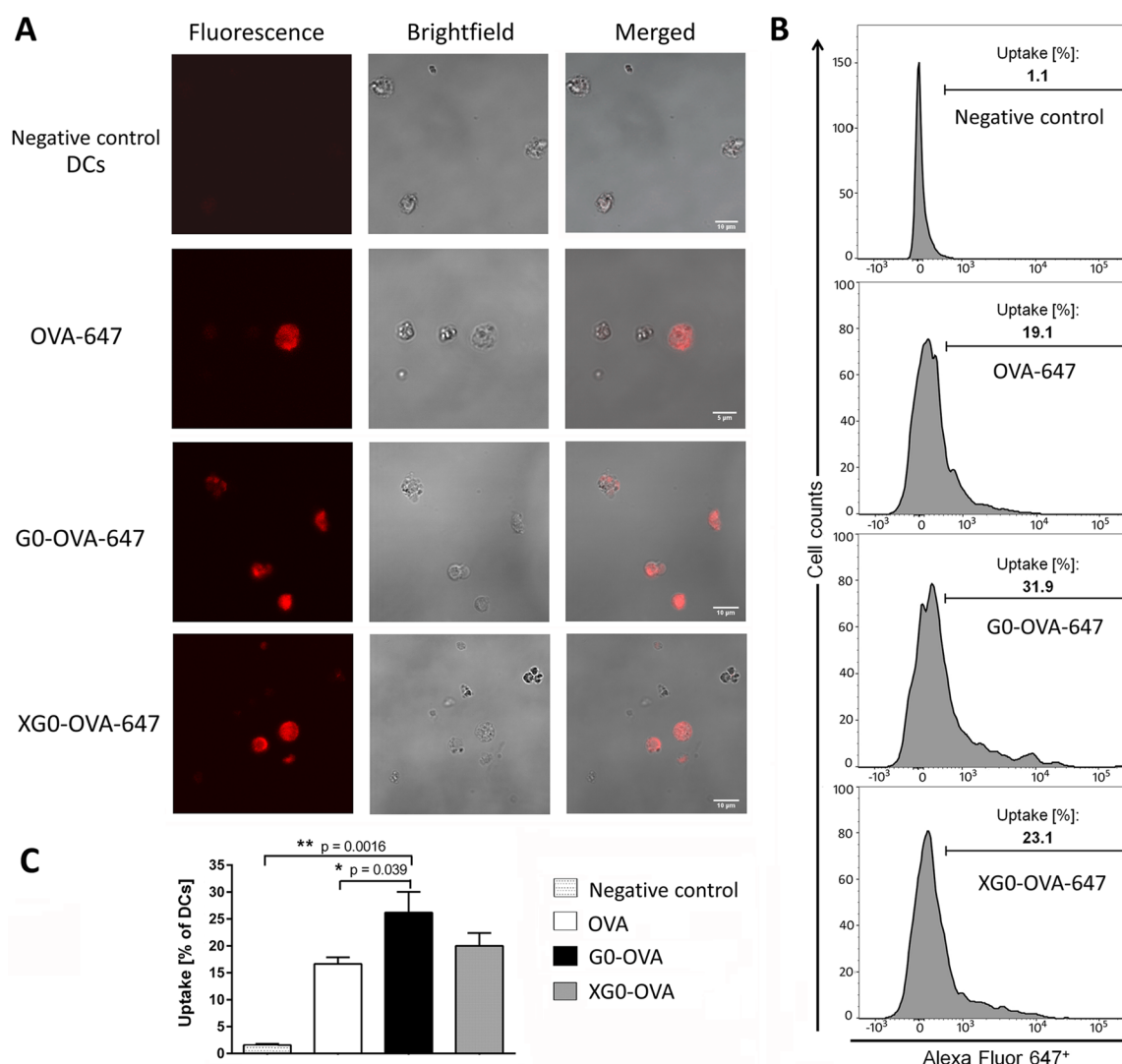


Figure 3. Uptake of the Alexa Fluor-647 labeled glycoconjugates XG0-OVA and G0-OVA and OVA by murine DCs. (A) Representative images showing internalization of compounds as measured by confocal fluorescence microscopy. (B) Uptake of glycoconjugates and OVA analyzed by flow cytometry (data are representative for three independent experiments). (C) Statistical analysis of data obtained from three independent flow cytometry experiments.

RESULTS AND DISCUSSION

Generation of Ovalbumin Glycoconjugates (XG0-OVA and G0-OVA). N-glycan structures (G0 and XG0) equipped with a C5 amino linker for further conjugation were chemically synthesized as reported recently.^{24,32} The corresponding OVA conjugates were generated by first activating the glycan-amino linker with disuccinimidyl suberate ester (DSS; Figure 1) and then cross-linking accessible OVA lysine residues in a second step.³³ The glycoconjugates G0-OVA and XG0-OVA were characterized by gel electrophoresis (Figure 1B) and by MALDI-ToF mass spectrometry (Figure 1C). The mass shift upon conjugation of OVA with activated glycans G0-DSS and XG0-DSS (Figure 1C) indicated an average conjugation of three to four glycans per OVA molecule (see Figure S1).

To evaluate the influence of core xylose on the recognition by CLRs and DC targeting, a similar glycan valence was required for both, XG0-OVA and G0-OVA. By carefully adjusting the glycan/OVA ratio and the protein concentration during the conjugation reaction, we were able to produce both

OVA glycoconjugates displaying equal numbers of glycan moieties.

Binding of OVA Neoglycoconjugates to CLRs. To identify CLRs involved in the recognition of the glycoconjugates G0-OVA and XG0-OVA by murine DCs, we performed binding studies using the lectin microarray technology. To this end, a library of CLR-Fc fusion proteins consisting of the extracellular part of the respective CLR and the Fc part of human IgG1 molecules was used.³⁴ We analyzed eight recombinant murine CLRs for their binding to G0-OVA and XG0-OVA: DC immunoactivating receptor (DCAR), DC immunoreceptor (DCIR), Dectin-2, Macrophage inducible C-type lectin (Mincle), Macrophage-restricted C-type lectin (MCL), Clec12b, and the murine DC-SIGN homologues SIGNR1 and SIGNR3.

To achieve a uniformly oriented presentation of the CLR extracellular CRDs on the microarray surface, slides were first coated with protein G before the CLR-Fc fusion proteins were subsequently spotted on top (Figure 2A). Protein G binds to the Fc region of the CLR-Fc fusion proteins thus improving the orientation of the CLR on the microarray surface.³⁵ Human Fc

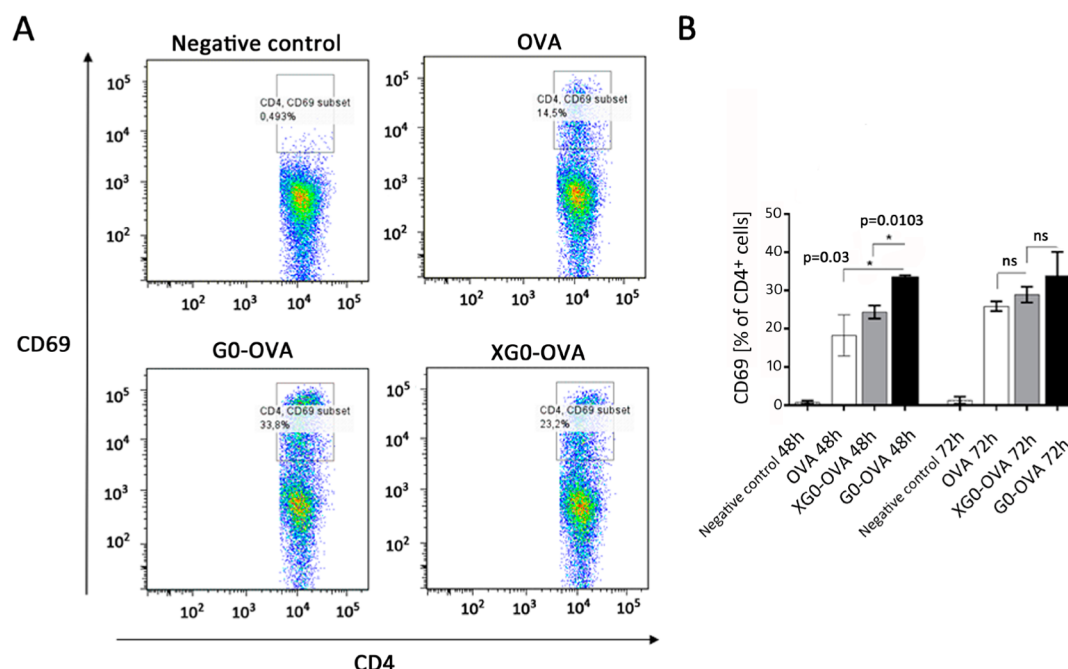


Figure 4. Frequencies of OT-II CD4⁺ T cells expressing the early activation marker CD69 upon stimulation with glycoconjugates (G0-OVA, XG0-OVA) and OVA. (A) Representative dot plots after 48 h. (B) Statistical analysis for the incubation over 48 and 72 h (data are representative for three independent experiments). Statistical analysis was performed with an unpaired student's *t* test, **p* < 0.05, ns: not significant.

was included as negative control. Glycoconjugates and OVA were fluorescently labeled and incubated in the dark on the CLR microarrays at 4 °C overnight. An equal level of labeling of glycoconjugates and OVA (an average of 11–12 dye molecules/protein) was achieved, first, by adjusting the concentration of both proteins and dye used during the conjugation and, second, by strict control of the reaction time (see SI, Table S1, Figure S2). As fluorescence emission spectroscopy showed equal fluorescence for all constructs, no further normalization was applied (Figure S2). The lectin microarray was washed, and the median fluorescence was measured. Binding studies revealed that glycoconjugates bound strongly to three of the eight analyzed CLR-Fc fusion proteins: Mincle-Fc and both DC-SIGN homologues, SIGNR1-Fc and SIGNR3-Fc (Figure 2B). Lower binding was detected for Clec-12b-Fc and DCIR-Fc. While the presence of a core xylose in XG0-OVA had no significant effect on the binding toward Mincle-Fc and SIGNR1-Fc, it strongly reduced the interaction with SIGNR3-Fc compared to the nonxylosylated homologue G0-OVA. Unconjugated OVA was used as a control in all experiments to evaluate the impact of background glycosylation and potential protein–protein interactions on binding. With the exception of SIGNR1-Fc, with a known specificity toward high-mannose structures,³⁶ we did not see any binding to the control protein. Although native chicken OVA contains a single N-linked glycosylation site (Asn-292), which is predominantly substituted with high mannose (Man₅GlcNAc₂/Man₆GlcNAc₂) or less abundant hybrid (Man₅GlcNAc₄/Man₅GlcNAc₃) glycans,³⁷ this sugar modification seems to play a minor role in recognition by CLRs, probably due to the monovalent presentation. Thus, the G0/XG0 N-glycans are exclusively responsible for recognition by the CLR-Fc fusion proteins.

A significantly reduced binding of XG0-OVA compared to G0-OVA was observed for SIGNR3-Fc. We have shown previously that the xylose modification completely abolishes binding of XG0 toward DC-SIGN-Fc.²⁴ The murine DC-SIGN

family consists of several homologous type II transmembrane proteins, each of which displays a single carboxyl terminal CRD.³⁸ Previously, it has been shown that the dominant specificities found for the DC-SIGN homologues SIGNR1 and SIGNR3 are mannose and fucose motifs found on high mannose type N-glycans and in Lewis^{a/b} and Lewis^{x/y} structures.³⁹ The differential carbohydrate specificities of both DC-SIGN homologues were previously exploited for cell targeting using glycoliposomes.⁴⁰ SIGNR3 has been reported to bind to bacterial pathogens such as *Mycobacterium tuberculosis* but also to commensal microbiota.^{41,42} Since SIGNR3 is considered the biochemically closest murine homologue of the human DC-SIGN receptor,^{43,44} it might exhibit a similar glycan binding pattern as DC-SIGN, thus explaining the differential binding of G0 and XG0 by SIGNR3.

DC Binding and Uptake Assay. We then employed confocal microscopy to study the internalization of fluorescently labeled OVA glycoconjugates G0-OVA, XG0-OVA, and unconjugated OVA by murine DCs isolated from C57BL/6 mice. CD11c⁺ splenic DCs were purified by magnetic-activated cell separation (MACS) and seeded on poly-D-lysine-coated glass coverslips overnight. Purified DCs were incubated with fluorescently labeled OVA and glycoconjugates G0-OVA and XG0-OVA, washed to remove unbound proteins, and fixed. While the analysis of confocal images only indicated a rapid internalization of both glycoconjugates and OVA within 10 min (Figure 3A), to quantify potential differences in glycoconjugate uptake, in particular to measure the influence of the core xylose modification on internalization, we analyzed the uptake by flow cytometry (Figure 3B). Purified DCs were pulsed with fluorescently labeled OVA and conjugates G0-OVA and XG0-OVA, incubated, and subsequently washed to remove non-internalized fluorescent conjugates.

We observed that both sugar modifications increased OVA uptake by DCs. After 10 min of incubation, OVA was detected in 16.6 ± 1.2% of murine DCs, while a slight increase was

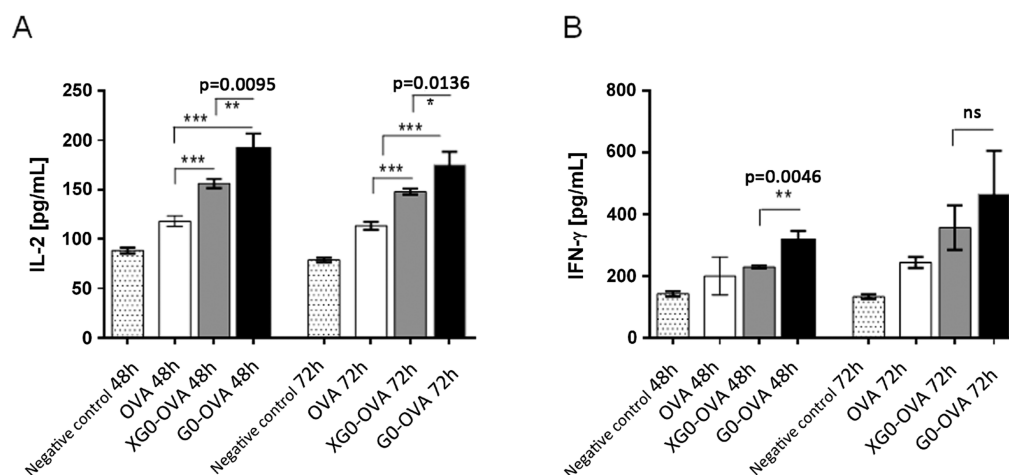


Figure 5. Cytokine production by OT-II CD4⁺ T cells in coc-culture with CD11c⁺ DCs pulsed with OVA, XG0-OVA, and G0-OVA. (A) Interleukin-2 (IL-2) production after 48 and 72 h of incubation. (B) Interferon-γ (IFN-γ) production after 48 and 72 h (data are representative for three independent experiments). Statistical analysis was performed with an unpaired Student's *t* test, **p* < 0.05, ***p* < 0.01, ****p* < 0.0001, ns: not significant.

observed upon incubation with conjugate XG0-OVA ($20.0 \pm 2.4\%$). The finding that unmodified OVA was also taken up to a limited degree indicates that there is a certain level of unspecific endocytosis by DCs. However, incubation with the non-xylosylated glycoconjugate G0-OVA led to significantly increased internalization of glycoconjugate (in $26.2 \pm 3.8\%$ of the murine DCs), indicating that the respective glycan modification indeed impacted DC targeting efficacy. Thus, our results show a specific DC uptake triggered by the nonxylosylated glycoconjugate. Since glycan-modified OVA is a widely accepted model antigen that has been used for DC targeting in previous studies, the increased DC uptake observed with the G0-OVA conjugate substantiates the utility of the DC targeting approach.

Upon internalization into DCs, antigens are processed and peptide fragments are presented on major histocompatibility complex (MHC) molecules to T cells. However, besides activating T cells, DCs may also shape T cell responses, for instance by inducing CD4⁺ T cell differentiation into Th1, Th2, or Th17 cells.⁴⁵ To analyze whether the differential DC targeting capability of the OVA glycoconjugates also impacted subsequent T cell activation, a DC/T cell cocultivation assay was performed. To this end, CD11c⁺ splenic DCs were pulsed with the OVA neoglycoconjugates or OVA alone and were then incubated with purified CD4⁺ T cells from OT-II transgenic mice. This transgenic system allows evaluation of antigen presentation and subsequent T cell activation since OT-II CD4⁺ T cells have a T cell receptor specific for the OVA_{323–339} peptide presented by the MHC-II molecule I-A.⁴⁶

As a read-out, the expression of the early activation marker CD69^{47,48} was measured by flow cytometry. Indeed, we found a higher frequency of OT-II CD4⁺ T cells expressing CD69 on their surface upon stimulation with the OVA glycoconjugates compared to unconjugated OVA (Figure 4A,B). Importantly, a significantly higher frequency of CD4⁺ T cells expressed CD69 when DCs had been pulsed with G0-OVA compared to the xylosylated analogue XG0-OVA (Figure 4A,B). This finding indicates that the differential DC targeting efficiency of the OVA glycoconjugates observed in the DC uptake assay also correlates with subsequent T cell activation.

Besides the expression of activation markers, activated T cells produce cytokines with different effector functions. Thus, as an additional functional read-out, we measured the concentrations of interleukin 2⁴⁹ (IL-2) and interferon gamma⁵⁰ (IFN-γ) produced by the OT-II CD4⁺ T cells. While IL-2 is produced by activated T cells and further promotes T cell proliferation, IFN-γ is an effector cytokine mainly produced by Th1 cells. Indeed, significantly higher levels of IL-2 were produced by T cells stimulated with the OVA glycoconjugates compared to unconjugated OVA (Figure 5A). While both glycoconjugates induced a higher IL-2 production compared to unconjugated OVA, the presence of the xylose modification led to reduced levels of IL-2 when compared to G0-OVA. A similar trend was observed for IFN-γ production (Figure 5B). Thus, the results obtained for glycoconjugate-dependent cytokine production are consistent with the CD69 expression data (Figure 4) and indicate that glycan-based DC targeting may be used to elicit subsequent T cell responses.

Conclusion. The use of antigens alone may not always induce an effective internalization by DCs and subsequent T cell activation. In this context, the use of glycan-modified antigens and their targeting *via* CLRs is a promising strategy to enhance the uptake of the construct. Recent studies indicate that different carbohydrates may even initiate differential signaling processes by engagement of the same CLR, as has been shown for fucose-based DC-SIGN targeting.^{51–53} However, targeting CLRs with glycans is still hampered by incomplete knowledge of CLR binding specificities.⁵⁴ In the present study, we demonstrate the utility of CLR microarrays to identify natural glycan ligands. N-glycans conjugated to the model antigen OVA were targeted to murine DCs *in vitro*. We showed that xylosylated and nonxylosylated biantennary N-glycans enhanced the uptake and presentation of OVA, and more importantly, we could demonstrate that even small structural glycan modifications can have a marked impact on uptake by DCs and subsequent T cell activation. In line with our glycan array based binding study, we found that nonxylosylated G0-OVA glycoconjugate resulted in increased binding to SIGNR3 when compared to XG0-OVA and unconjugated OVA. Consequently, G0-OVA was preferably internalized by murine DCs and led to enhanced T cell

activation as demonstrated by CD69 staining. Finally, the production of IL-2 and IFN- γ measured after 48 h was elevated, further underlining an increase of T cell activation as well as T cell differentiation toward a Th1 phenotype. Ultimately, our study highlights the utility of glycan-based CLR targeting and may open up new possibilities for the specific delivery of vaccine antigens into DCs.

METHODS

Synthesis of Ovalbumin-Glycoconjugates. G0-OVA. Disuccinimidyl suberate (DSS) linker (6.8 mg, 18.46 μ mol, 13 equiv) was dissolved in 1 mL of DMSO (18.4 mM) and activated with 15 μ L of triethylamine. The biantennary N-glycan⁵⁵ (2 mg, 1.42 μ mol) was dissolved in 0.3 mL of DMSO and added to the solution of linker dropwise, and the reaction was left stirring at RT for 90 min. The product was extracted with 800 μ L of PBS (pH 7.4), washed three times with chloroform, and centrifuged at 3000g to facilitate phase separation. Attachment of DSS linker to N-glycan was confirmed by MALDI-ToF mass spectrometry controlled by FlexControl 3.3 software (Bruker Daltonics). G0-DSS (40 equiv) was added to the 1.25 mg of LPS-free ovalbumin (Hyglos), and the reaction was left stirring overnight at RT. The sample was concentrated in a 10 kDa Amicon filter device and dissolved in sterile PBS.

XG0-OVA. DSS linker (7.4 mg, 20 μ mol, 17 equiv) was dissolved in 1.1 mL of DMSO (22 mM) and activated with 15 μ L of trimethylamine. The xylosylated biantennary N-glycan²⁴ (2.15 mg, 1.2 μ mol) was dissolved in 0.3 mL of DMSO and added to the solution of linker dropwise. The reaction was left stirring at RT for 1 h and 30 min. The product was extracted with 800 μ L of PBS (pH 7.4), washed three times with chloroform, and centrifuged at 3000g. The attachment of DSS linker to N-glycan XG0 was confirmed by MALDI-ToF mass spectrometry. XG0-DSS (40 equiv) was subsequently added to 1.25 mg of LPS-free ovalbumin, and the reaction was left stirring overnight at RT. Sample was concentrated in a 10 kDa Amicon filter device and dissolved in sterile PBS.

Alexa Fluor-647 Labeling of Ovalbumin and Ovalbumin Glycoconjugates. A total of 400 μ L of 10 mg mL⁻¹ solutions of OVA, 70 μ L of 10 mg mL⁻¹ XG0-OVA, and 86 μ L of 10 mg mL⁻¹ G0-OVA in sodium bicarbonate buffer (100 mM, pH = 9) were prepared. Corresponding volumes (50, 12, and 15 μ L) of 10 mg mL⁻¹ Alexa Fluor-647 NHS ester were added to the OVA, XG0-OVA, and G0-OVA solutions individually, and the reactions were gently shaken at RT for 1 h (ovalbumin) or 2 h (OVA-glycoconjugates). The reaction time for labeling of OVA glycoconjugates was increased to 2 h to compensate for the lower number of available free lysines due to partial occupation with glycans G0 and XG0. Excess dye was removed by dialysis (10k Z-lyser) against water (12 h), and glycoconjugates were concentrated by ultracentrifugation with 10 kDa Amicon devices. The amount of attached Alexa Fluor 647 was estimated based on the absorbance of dye (ϵ , 250 000 cm⁻¹ M⁻¹) and protein (ϵ , 35 900 cm⁻¹ M⁻¹). See Table S1. All three constructs showed equal fluorescence emission spectra, making further normalization unnecessary (Figure S2).

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.³⁴ In the meantime, the library has been extended by Dectin-2-Fc and SIGNR1-Fc (see primer list in the SI). cDNA fragments encoding the extracellular part of the respective murine CLRs 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).

Preparation of CLR Microarray. Nexterion-Slide H (NHS-activated) slides were incubated overnight with protein G (20 μ g/mL) in PBS at 4 °C. Each slide was washed three times with PBST (PBS, 0.05% Tween-20), followed by PBS and water and dried in a slide spinner. CLR-Fc solutions were prepared by dilution with printing buffer (PBS, 0.01% Tween-20 and 0.1% glycerol) to a common final concentration of 0.2 mg mL⁻¹ and placed into a 384-well source plate (Sciencion). A total of 0.6 nL of CLR-Fc solutions was spatially arrayed onto protein G functionalized glass slides in five replicate spots employing a sciFLEXARRAYER S11 robotic non-contact spotter (Sciencion). After printing, the slides were placed in a 75% humidity chamber (saturated NaCl solution) at 4 °C for 2 h. To reduce unspecific binding, the slides were passivated with 3% bovine serum albumin (BSA) in PBS for 1 h followed by the standard washing procedure with PBST, PBS, and water. See SI, Figure S3.

Incubation of Microarrays with OVA and OVA Glycoconjugates. CLR microarrays were incubated with solutions of Alexa Fluor-647 labeled OVA and glycoconjugates (XG0-OVA and G0-OVA) at final concentrations of 30 and 50 μ g/mL for both neoglycoconjugates in binding buffer (PBS, 0.01% Tween-20, 5 mM CaCl₂, 5 mM MgCl₂, 1% BSA). Binding was only observed at a glycoconjugate concentration of 50 μ g/mL. After overnight incubation at 4 °C, arrays were washed with PBS, water, and dried in a slide spinner. Fluorescence was measured using an Agilent G265BA microarray scanner system (Agilent Technologies), and quantification was performed with ProScanArray Express software (PerkinElmer) with an adaptive circle quantification method (spot diameter: 50–300 μ m). Histograms represent the average of median RFU values with local background subtraction.

Mice. Dendritic cells isolated from C57BL/6 mice and T cells isolated from OT-II transgenic mice (own breeding at the Federal Institute of Risk Assessment (BfR), Berlin, Germany) were used in all experiments except for the confocal microscopy studies, where dendritic cells isolated from C57BL/6J mice (CIC biomaGUNE, San Sebastian, Spain) were used. Both mouse strains are inbred strains on the C57BL/6 genetic background with the only difference that the former strain was bred by Charles River whereas the latter one was bred by the Jackson Laboratory.

Isolation of Spleen Cells Subset. To isolate splenocytes, spleens were flushed with IMDM medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100- μ g/mL streptomycin, and 10% fetal calf serum (FCS; PAN Biotech). The cell suspension was kept cold and filtered through a 40 μ m cell strainer to remove cell aggregates. After centrifugation (300g, 5 min, 4 °C), cell pellets were resuspended in 5 mL of freshly prepared erythrocyte lysis buffer (10% 100 mM Tris pH 7.5 + 90% 160 mM NH₄Cl), mixed gently, and incubated at RT for 2 min. Cells were washed twice in complete IMDM medium and centrifuged before resuspension in MACS buffer (PBS, 0.5% BSA, 2 mM EDTA).

Dendritic cells (CD11c⁺ cells) were isolated from a suspension of C57BL/6 murine spleen cells by magnetic-activated cell sorting using CD11c⁺ MicroBeads (Miltenyi). Cells incubated with magnetic microbeads were loaded on a MACS column placed in a magnetic field. Unbound cells passed through the column, while remaining CD11c⁺ cells were washed with MACS buffer and eluted from the column. To increase DC purity, the CD11c⁺ cell purification was repeated. The cell suspension was centrifuged, resuspended in IMDM complete medium, and counted.

T cells were isolated from the spleen of OT-II transgenic mice using the Pan T Cell Isolation Kit II (Miltenyi). Nontarget cells were magnetically labeled with a cocktail of biotin-conjugated antibodies against CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, and Ter-119 markers followed by antibiotin monoclonal antibodies conjugated to MicroBeads. Nontargeted cells were retained on the MACS column, while the untouched T cells passed through the column in the first fraction. To increase T cell purity, the T cell purification was repeated. Isolated T cells were subsequently centrifuged, resuspended in IMDM complete medium, and counted. The purity of DCs and T cells isolated by MACS was analyzed by flow cytometry (see Figure S4)

using a FACS Canto II flow cytometry (BD Biosciences). DC purity was analyzed by staining with an anti-CD11c antibody, whereas purity of the isolated T cells was confirmed by staining with an anti-CD3 antibody. Monoclonal antibodies against CD11c (APC), CD3e (APC), CD19 (PE), CD4 (FITC), and CD8 (APC-H7) were purchased from eBioscience.

Uptake of Labeled Glycoconjugates by DCs Followed by Confocal Fluorescence Microscopy. DCs from C57BL/6J mice (2×10^6 cells) were seeded on poly-D-lysine-coated glass coverslips overnight. Alexa Fluor-647 labeled OVA, XG0-OVA, and G0-OVA were added to cells ($10 \mu\text{g/mL}$) and incubated for 10 min at 37°C . After incubation, cells were carefully washed with cold PBS and fixed with 3% paraformaldehyde at RT for 20 min. After washing with PBS and water, the coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories). Fluorescent images were taken using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss) equipped with a red helium–neon (HeNe633) laser and a $63\times$ oil immersion objective.

Uptake of Labeled Glycoconjugates by DCs Followed by Flow Cytometry. Purified DCs were seeded in 96-well round-bottom plates (2×10^4 cells/well) in complete IMDM medium. After 30 min (37°C , 5% CO_2), cells were pulsed with OVA, XG0-OVA, and G0-OVA ($10 \mu\text{g/mL}$). After 10 min of incubation (37°C , 5% CO_2), cells were carefully washed with cold PBS ($2\times$) and resuspended in $100 \mu\text{L}$ of FACS buffer containing CD16/32 (Fc-Block, dilution 1:100) blocking antibody at 4°C for 10 min. Subsequently, a control sample was incubated with CD11c (APC) monoclonal antibody (1:100 dilution) for 30 min at 4°C in the dark. As a control, an unstained sample was also prepared. Cells were washed with 1 mL of FACS buffer, resuspended in $100 \mu\text{L}$ FACS buffer, and vortexed before each measurement. Data were analyzed using FlowJo analysis software (Tree Star). For flow cytometry, the same gating was applied for all experimental conditions within one experiment.

Co-cultivation of OT-II T Cells with OVA-Glycoconjugate Pulsed DCs. Purified DCs were seeded in 96-well round-bottom plates (2×10^4 cells/well) in complete IMDM medium. After 30 min (37°C , 5% CO_2), cells were pulsed with OVA, XG0-OVA, and G0-OVA ($30 \mu\text{g/mL}$). After 1 h of incubation, $60 \mu\text{L}$ of purified T cells (7×10^4 cells/well) were added and incubated at 37°C , 5% CO_2 for 48 h. The expression of the early T cell activation marker CD69 and cytokine levels (IL-2, IFN- γ) in the supernatant was determined after 48 and 72 h of stimulation.

Detection of CD69 by Flow Cytometry. Expression of the T cell activation marker CD69 was analyzed by flow cytometry 48 and 72 h after stimulation using a FACS Canto II flow cytometer (BD Biosciences). Fluorochrome-conjugated monoclonal antibodies against CD69 (PE) and CD4 (Alexa Fluor 700) were purchased from eBioscience and BD Pharmingen (Cowley, UK), respectively. Fc receptors were blocked using anti-CD16/32 (dilution 1:100) in $100 \mu\text{L}$ of FACS buffer at 4°C for 10 min. Subsequently, cells were incubated with anti-CD4-AF700 and anti-CD69-PE (1:100 dilution) for 30 min at 4°C in the dark. Cells were washed with 1 mL of FACS buffer and analyzed in $100 \mu\text{L}$ of FACS buffer. Data were analyzed using FlowJo analysis software. Cells were first gated on live cells using the FSC/SSC plot, then gated on CD4 $^+$ T cells, and then plotted for CD69 $^+$ vs CD4 $^+$ T cells (as shown in Figure 4A).

Enzyme-Linked Immunosorbent Assay (ELISA). Supernatants were collected 48 and 72 h after stimulation. The measurement of the cytokines IL-2 and IFN- γ in the supernatants was performed according to the manufacturer's instructions (PeproTech). Plates were developed with a solution of tetramethyl benzidine (Ultra TMB-Elisa Substrate Solution, Thermo Scientific). After a few minutes, 2 M sulfuric acid solution was added to stop the reaction. Absorbance was measured at 450 nm using an ELISA reader (Tecan).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.6b00265.

MALDI-TOF characterization of glycoconjugates, calculation of the Alexa Fluor 647 degree of substitution, general procedure for the production of the CLR-Fc library, C-type lectin microarray fluorescent image and its quantification, scatter plots of MACS-purified DCs and OT-II T cells (PDF)

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Notes

The authors declare no competing financial interest.

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