Design of neo-glycoconjugates that target Mannose Receptor and enhance TLR-independent cross-presentation

Satwinder Kaur Singh\textsuperscript{1}, Ingeborg Streng-Ouwehand\textsuperscript{1}, Manja Litjens\textsuperscript{1}, Hakan Kalay\textsuperscript{1}, Sven Burgdorf\textsuperscript{2}, Eirikur Saeland\textsuperscript{1}, Christian Kurts\textsuperscript{3}, Wendy W. Unger\textsuperscript{1} and Yvette van Kooyk\textsuperscript{1}

\textsuperscript{1} Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands

\textsuperscript{2} LIMES Institute, Rheinische Friedrich Wilhelms University, Bonn, Germany

\textsuperscript{3} Institutes of Molecular Medicine and Experimental Immunology (IMMEI), Rheinische Friedrich Wilhelms University, Bonn, Germany

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Design of neo-glycoconjugates that target the mannose receptor and enhance TLR-independent cross-presentation and Th1 polarization

Satwinder Kaur Singh, Ingeborg Streng-Ouwehand, Manja Litjens, Hakan Kalay, Sven Burgdorf, Eirikur Saeland, Christian Kurts, Wendy W. Unger* and Yvette van Kooyk*

1 Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands
2 LIMES Institute, Rheinische Friedrich Wilhelms University, Bonn, Germany
3 Institutes of Molecular Medicine and Experimental Immunology (IMMEI), Rheinische Friedrich Wilhelms University, Bonn, Germany

Cross-presentation is an important mechanism by which DCs present exogenous antigens on MHC-I molecules, and activate CD8+ T cells, cells that are crucial for the elimination of tumors. We investigated the feasibility of exploiting the capacity of the mannose receptor (MR) to improve both cross-presentation of tumor antigens and Th polarization, processes that are pivotal for the anti-tumor potency of cytotoxic T cells. To this end, we selected two glycan ligands of the MR, 3-sulfo-Lewis A and tri-GlcNAc (N-acetylglucosamine), to conjugate to the model antigen OVA and assessed in vitro the effect on antigen presentation and Th differentiation. Our results demonstrate that conjugation of either 3-sulfo-Lewis A or tri-GlcNAc specifically directs antigen to the MR. Both neo-glycoconjugates showed, even at low doses, improved uptake as compared with native OVA, resulting in enhanced cross-presentation. Using MR−/− and MyD88-TRIF−/− bone marrow-derived DCs (BMDCs), we show that the cross-presentation of the neo-glycoconjugates is dependent on MR and independent of TLR-mediated signaling. Whereas proliferation of antigen-specific CD4+ T cells was unchanged, stimulation with neo-glycoconjugate-loaded DCs enhanced the generation of IFN-γ-producing T cells. We conclude that modification of antigen with either 3-sulfo-Lewis A or tri-GlcNAc enhances cross-presentation and permits Th1 skewing, through specific targeting of the MR, which may be beneficial for DC-based vaccination strategies to treat cancer.

Keywords: Antigen presentation • Cross-presentation • DC targeting • Glycans • MR

Supporting Information available online

Introduction

Activation of antigen-specific cytotoxic T cells is crucial for the induction of adequate anti-tumor immunity. Since most tumor cells are poorly immunogenic, optimal presentation of tumor-derived antigens in MHC class I molecules on the surface of antigen presenting cells is required. An important mechanism that allows DCs to present exogenous antigens, such as tumor-derived antigens, in MHC class I molecules is cross-presentation [1].

*These authors contributed equally to the work.
At tumor lesions, multiple factors and cells are present that prevent the proper activation of DCs that enter the lesion to sample for antigens [2, 3]. Consequently, these DCs will not be able to properly activate antigen-specific CD8⁺ T cells in the tumor-draining LN. To obtain therapeutic anti-tumor immunity powerful vaccination protocols are required. Current strategies focus either on ex vivo loading of autologous DCs as well as specifically targeting of antigens to DCs in vivo. These new therapies may be combined with a Treg depletion regimen, as these cells have been shown to block anti-tumor immune responses [3–6].

As a classical C-type lectin receptor (CLR), the mannose receptor (MR) binds carbohydrate structures such as mannose, fucose and N-acetylglucosamine (GlcNAc) in a calcium-dependent manner [7, 8]. Besides these carbohydrate structures, the MR has recently also been reported to bind sulfated sugars, such as sulfated oligosaccharides of the blood group antigens Lewisα (Leα) and Lewisβ (Leβ) [8–10]. Binding of these types of ligands occurs via the cysteine-rich (CR) region of the MR and in a calcium-independent manner [8].

The MR has been proposed to mediate antigen uptake and presentation by DCs based on the finding that mannosylated proteins are presented more efficiently than non-mannosylated proteins [11, 12]. Fusion of an MR-specific monoclonal antibody to tumor antigens enhanced MHC class I-restricted T-cell responses [13]. Additionally, the glycoprotein ovalbumin (OVA), which contains mannose residues, was reported to be endocytosed through the MR, upon which the antigen was transferred to early endosomes, resulting in strong cross-presentation [14, 15]. By contrast MR-mediated uptake of OVA did not induce CD4⁺ T-cell responses. Processing of native glycosylated OVA in the early endosomes occurs in a TAP-dependent manner. Transport of TAP from ER to the endosomes is mostly, but not entirely, dependent on toll-like receptor-4 (TLR4)/MyD88 signaling [15]. Although these studies report that the MR is an endocytic receptor for mannosylated OVA, in the human setting mannose may simultaneously target other CLR such as DC-SIGN, which shares specificity for mannose [16]. In addition DC-SIGN may preferentially shuttle endocytosed antigens into the MHC class II restricted presentation pathway [17].

Importantly, although no signaling-motif is recognized in the cytoplasmic tail, the MR has been shown to be essential for cytokine production, both pro- and anti-inflammatory. However, the outcome is dependent on TLR co-triggering by pathogens or synthetic ligands. Mannose-capped lipopirinamannans from Mycobacterium tuberculosis inhibited LPS-induced pro-inflammatory cytokine production by DCs [18], whereas Candida albicans-derived mannan triggers IL-17 production [19].

In this study, we exploited the feature of the MR to cross-present antigens, aiming to generate more potent activation of tumor-specific T cells. To this end, we selected two glycan ligands of the MR other than mannose, of which one also has a different binding site than mannose that showed profound binding to bone marrow-derived DCs (BMDCs) and ex vivo purified splenic DCs. These ligands, 3-sulfo-Leα and tri-GlcNAc, were conjugated to the model antigen OVA to examine their potency to enhance antigen presentation in MHC class I and II, as well as Th differentiation.

**Results**

Sulfated and di-GlcNAc glycan moieties bind MR on murine DCs

The glycan-binding specificity of the MR is not solely restricted to mannose. Using purified MR-Fc fusion proteins, also sulfated and GlcNAc glycan moieties were shown to bind [7, 9]. We investigated whether we could use these GlcNAc and sulfated glycan structures to specifically target antigen to the MR.

First, expression of MR on BM-DCs and splenic DCs was confirmed. DCs were either cultured from BM or ex vivo isolated from the spleen of C57BL/6 mice and MR expression was analyzed using flow cytometry. Both, CD11c⁺ BMDCs and splenic DCs expressed significant levels of MR protein on their cell surface (Fig. 1A), herewith confirming previous reports [20, 21].

Subsequently, binding of GlcNAc and sulfated glycan structures was examined by incubating DCs with biotinylated polyacrylamide (PAA)-conjugated glycans at 4°C. Streptavidin-Alexa488 was used to visualize bound glycans. From Fig. 1, it is clear that BMDCs bind GlcNAc and chitobiose (GlcNAcβ1-4GlcNAc) as well as the sulfated blood group antigens 3-sulfo-Leα (HSO₃⁻3Gal b 1-3(Fuc a 1-3)GlcNAc b) and 3-sulfo-Leβ (HSO₃⁻3Gal b 1-3(Fuc b 1-3)GlcNAc) (Fig. 1B). The PAA-conjugated control structure glucitol did not bind to BMDCs. Surprisingly, when purified CD11c⁺ splenic DCs were used, we observed significant binding of PAA-conjugated 3-sulfo-Leα and GlcNAc but not of 3-sulfo-Leβ. This can either be due to low specificity of MR for 3-sulfo-Leα or the involvement of another glycan-binding receptor on BMDCs with specificity for 3-sulfo-Leβ, which is absent on splenic DCs. Together, these results show that sulfated blood group antigens and GlcNAc glycan structures can interact with murine DCs.

Increased binding to DC-expressed MR by the neo-glycoproteins OVA3sulfoLeα and OVA-tri-GlcNAc

Based on our observation that the PAA-conjugated glycans 3-sulfo-Leα and di-GlcNAc efficiently bind to BMDCs and splenic DCs, we chose to conjugate these glycans to the model antigen OVA to investigate their potential to enhance antigen cross-presentation. Native OVA contains high mannose and bi-antennary type of glycans ([14], and data not shown). We chemically conjugated either activated 3-sulfo-Leα or a polysaccharide of GlcNAc, namely chitotetraose (GlcNAcβ1-4GlcNAc-GlcNAcβ1-4GlcNAc) (hereafter referred to as OVA-tri-GlcNAc, as one of the ring structures needs to be opened to be able to couple it to OVA leaving three GlcNAc glycans are available) to free cysteine residues of native OVA. In this way,
OVA-neo-glycopeptides that additionally contain these specific glycans (OVA-3-sulfo-LeA and OVA-tri-GlcNAc) were created. The presence of 2–3 moieties of either 3-sulfo-LeA or tri-GlcNAc on OVA was confirmed by MALDI mass-spectrometry (Supporting Information Fig. 1). The potential of these newly formed neo-glycopeptides to interact with the MR on DCs was examined as well as the potential difference in binding of glycans conjugated to PAA. We compared the binding of these neo-glycoconjugates with binding of native OVA, which has previously been demonstrated to bind via the MR [21]. Binding of both OVA-3-sulfo-LeA and OVA-tri-GlcNAc to BMDCs was significantly enhanced compared to native OVA (Fig. 2A). In addition, we observed that the binding of the neo-glycoconjugates was slightly increased compared to the number of cells that bound the glycans was increased (Fig. 2B). The binding of these neo-glycoconjugates was indeed MR-dependent as a significant reduction in binding to splenic DCs from MR-deficient mice was observed (Fig. 2B, white bars). However, binding was still increased compared to binding of native OVA to WT or MR-deficient cells. When examining binding of the compounds to freshly isolated CD11c+ DCs we observed increased binding of the neo-glycoconjugates to WT DCs, similar to our observations with BMDCs (Fig. 2C). However, a dramatic reduction in the binding of the neoglycoconjugates was observed upon incubation with splenic DCs from MR-deficient mice (Fig. 2C, black bars). This binding was not significantly different from native OVA to WT or MR-deficient cells. These data indicate a predominant role for the MR in binding of OVA-3-sulfo-LeA and OVA-tri-GlcNAc.

Targeting MR using neo-glycoconjugates improves CD8 T-cell proliferation

To investigate whether MR-targeting of DCs with the neo-glycoconjugates results in increased MHC class I or II presentation, we co-cultured freshly isolated CD11c+ DCs, pulsed with OVA-3-sulfo-LeA or OVA-tri-GlcNAc, for three days with either purified OVA-specific CD8+ or CD4+ T cells, respectively. Before performing these functional assays, the neo-glycoconjugates were analyzed for potential contamination with endotoxins to rule out that increased cross-presentation of the neo-glycoconjugates would be due to TLR4 triggering, which has been shown to be required for cross-presentation of OVA [15]. All three protein-preparations (OVA, OVA-3-sulfo-LeA and OVA-tri-GlcNAc) used in this study tested negative in an LAL-assay, indicating that they are endotoxin-free (Supporting Information Fig. 2A). In addition, equal OVA concentrations were measured in all three protein-preparations by ELISA (Supporting Information Fig. 2B).

Importantly, when titrating the amount of antigen used in these antigen-presentation experiments, we observed that low concentrations (30 μg/mL) of the neo-glycoconjugates were already sufficient to result in potent T-cell proliferation compared to native OVA (i.e. 500 μg/mL [14, 15]), herewith illustrating the strong potential of the neo-glycoconjugates in the activation of T cells. Proliferation of CD4+ T cells activated by DCs pulsed with OVA-3-sulfo-LeA and OVA-tri-GlcNAc was slightly increased compared to T cells primed by native OVA-loaded DCs, despite the presence of mannose on native OVA (Fig. 3A). A much stronger effect of the neo-glycoconjugates was observed on CD8+ T-cell proliferation. OVA-3-sulfo-LeA and OVA-tri-GlcNAc were significantly enhanced cross-presented compared to native OVA, as shown by a tenfold increased proliferation of OVA-specific CD8+ T cells (Fig. 3B). Similar results were obtained when BMDCs were used (Supporting Information Fig. 3). Controls in experiments also included DCs loaded with non-glycan-modified OVA and maltose-modified OVA, which yielded responses that were not significantly different from those generated with native OVA (proliferation measured at highest concentration of antigen was 6.75 × 10^4 ± 749 and 8.55 × 10^4 ± 1093 respectively, for CD8+ T cells and 2.14 × 10^4 ± 632 and 3.33 × 10^4 ± 1093 respectively, for CD4+ T cells (data not shown). Experiments performed with BMDCs derived from MR−/− mice revealed that the uptake and processing route of the neo-glycoconjugates...
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OVA-tri-GlcNAc and OVA-3-sulfo-LeA neo-glycoconjugates were MR-dependent as the proliferation of OVA-specific CD4+ and CD8+ T cells was significantly decreased compared to their response using WT BMDCs (Fig. 3C and D). Although the cross-presentation was greatly reduced using the MR−/− BMDCs, there was still some background presentation of OVA-3-sulfo-LeA and OVA-tri-GlcNAc. As our neo-glycoconjugate preparations did not contain endotoxin above detection level, we conclude that the observed enhanced cross-presentation of OVA-3-sulfo-LeA and OVA-tri-GlcNAc is glycans-mediated and distinct from the previously reported TLR-dependent cross-presentation of native OVA [15]. This was confirmed using MyD88−/− BMDCs; similar to using WT BMDCs, cross-presentation of the neo-glycoconjugates was enhanced in MyD88−/− BMDCs compared to native OVA, indicating that the cross-presentation induced by 3-sulfo-LeA and tri-GlcNAc is independent of TLR-signaling (Fig. 3E). Indeed, addition of LPS improved cross-presentation of native OVA. However, when LPS was mixed with the neo-glycoconjugates, mostly cross-presentation of the lowest antigen doses (e.g. 10 and 3 μg/mL) was affected (Fig. 3F).

Together these data indicate that both OVA-neo-glycoconjugates target the MR and upon uptake are potently cross-presented to CD8+ T cells. The entered cross-presentation pathway is different from native OVA, as the observed cross-presentation occurs independent of TLR-signaling.

**Induction of Th1 cells by MR-targeting neo-glycoconjugates**

The presence of IFNγ-producing CD4+ T cells is crucial for the generation of potent CD8+ cytotoxic T cells as well as their recruitment into the tumor site [22, 23]. We questioned whether targeting DCs with OVA-3-sulfo-LeA or OVA-tri-GlcNAc influenced CD4+ T-cell polarization rather than proliferation. Thereto, naïve OVA-specific CD4+ CD62Lhigh T cells were co-cultured with neo-glycoprotein-pulsed CD11C+ splenic DCs and 1 wk later production of cytokines related to Th1-, Th2 and Th17-differentiation was analyzed using flow cytometry. We compared this with the profile of T cells differentiated by native OVA pulsed CD11C+ splenic DCs. DCs targeted with either neo-glycoconjugate generated significantly higher frequencies of IFNγ-producing CD4+ T cells compared to native OVA-loaded DCs (Fig. 4, left panel). By contrast, OVA-3-sulfo-LeA and OVA-tri-GlcNAc either reduced or did not affect the frequency of IL4 or IL17-producing T cells, respectively (Fig. 4, middle and right panel). These data imply that 3-sulfo-LeA and tri-GlcNAc-glycosylated antigens that target efficiently to the MR on DCs result in induction of IFNγ-producing effector T cells.

**OVA and OVA-3-sulfo-LeA are routed to endosomes in DCs**

As targeting of the MR with OVA-3-sulfo-LeA and OVA-tri-GlcNAc resulted in enhanced cross-presentation to CD8+ T cells, we investigated the intracellular routing of native OVA and OVA-3-sulfo-LeA into BMDCs derived from C57BL/6 and MR−/− mice. To this end, BMDCs were incubated with fluorescent-labeled OVA or OVA-3-sulfo-LeA. Two hours later, cells were washed and co-stained for MR, EEA-1 (endosomal marker) or LAMP-1 (lysosomal marker) and analyzed using confocal microscopy. We observed that OVA and OVA-3-sulfo-LeA (red) that bind to the MR (green, co-localization with OVA appears yellow) co-localized with the endosomal marker EEA-1 (blue, co-localization OVA-MR-EEA-1 = cyan) (Fig. 5A and B). This co-localization is also clearly observed when fluorescence images are converted into histograms (indicated by arrows). Surprisingly, we observed that co-localization...
of the MR-bound OVA-3-sulfo-LeA with EEA-1 was higher compared to native OVA. In addition, we assessed that the internalized OVA-3-sulfo-LeA did not co-localize with the lysosomal marker LAMP-1, but only with the MR (data not shown). The uptake of OVA and OVA-3-sulfo-LeA in BMDCs derived from MR−/− was dramatically decreased (Fig. 5C and D). These data correlate with the data on binding and antigen presentation demonstrating that OVA-3-sulfo-LeA targeted to the MR results in increased internalization of antigen to the endosomal compartment to facilitate loading of antigen to MHC class I molecules leading to enhanced cross-presentation to CD8+ T cells.

Discussion

Here, we show that DC-expressed MR is capable of binding sulfated glycans such as 3-sulfo-LeA or GlcNAc besides mannose glycans, present on native OVA. Conjugation of two sulfated glycans to each OVA molecule not only resulted in increased MR-dependent antigen uptake, but also strongly enhanced MHC class I-mediated cross-presentation of OVA to CD8+ T cells. The enhanced cross-presentation was independent of TLR-signaling and inducible at low concentrations of antigen. Furthermore, the addition of 3-sulfo-LeA or tri-GlcNAc to OVA protein enhanced the frequency of IFN-γ-producing CD4+ T cells, illustrating Th1 skewing.
Previous studies showed that the MR specifically binds high mannose, fucose and GlcNAc residues via the carbohydrate recognition domains (CRD) [7, 24]. Of the eight CRDs, CRD-5 are sufficient to generate the affinity of the whole receptor for natural ligands. Moreover, the MR contains an N-terminal CR domain, demonstrated to bind novel sulfated saccharides [9, 25]. In this study, we show that murine DC-expressed MR strongly binds to sulfated blood antigens such as 3-sulfo-LeA and GlcNAc. When these glycans were conjugated to OVA, increased binding and uptake of the neo-glycoconjugates was detected compared to native OVA, which itself is mannosylated. Interestingly, 3-sulfo-LeA and tri-GlcNAc bind to different sites of the MR. Whereas tri-GlcNAc binds to the CRD, 3-sulfo-LeA binds the MR via the CR domain [8–10]. Nevertheless these sulfated glycans exert similar potentiating effects. When chemically conjugated to OVA, these novel MR-specific ligands direct antigen more potently to the MR and enhance cross-presentation of antigens to CD8 T cells when compared to native OVA. This enhancement in cross-presentation is predominantly mediated by the MR as cross-presentation was greatly reduced in MR−/− splenic DCs. The fact that cross-presentation of the neo-glycoconjugates by MR−/− BMDCs was not completely abolished may be explained by binding of these glycans to other receptors, such as SIGNR1 and SIGNR3 [26], although their presence on myeloid DCs has not been formally shown. Although we could exclude the involvement of SIGNR1 since SIGNR1−/− DCs did not show any reduced antigen binding and uptake (data not shown), we cannot completely exclude the involvement of other lectin receptors or processes such as pinocytosis in the uptake of these neo-glycosylated proteins. Thus, we concluded that the MR is predominantly involved in the enhanced induction of antigen presentation, due to this glycan modification.

The potentiating effect of tri-GlcNAc may lie in its higher affinity for the MR than mannose resulting in increased responses [7]. Since 3-sulfo-LeA binds the CR region instead of the CRD, it cannot compete with mannose. However, binding to the CR region might be with stronger affinity than of mannose to the CRD, although to our knowledge a direct comparison between these ligands and regions has not been described. CR-ligand binding may elicit stronger responses than CRD-ligand binding. This is underlined by the fact that the response to OVA-3-sulfo-LeA is stronger than to native OVA. However, as native OVA contains mannoses, it is very likely that our OVA-3-sulfo-LeA conjugate binds both regions, with 3-sulfo-LeA binding to the CR and mannose to the CRD region, respectively.

Both neo-glycoconjugates evoked only a marginal increase in MHC class II restricted presentation via the MR. Our results correlate with previous studies showing that internalization of soluble antigens containing an MR-ligand does not influence presentation to CD4+ T cells [14]. Previously, uptake and presentation of native OVA via MHC class II molecules were shown to be mediated via pinocytosis [14]. We propose a role for pinocytosis in uptake and MHC class II-restricted presentation of our neo-glycoconjugates, despite that we did not observe co-localization of the neo-glycoconjugates with LAMP1 (Fig. 5). However, due to the low concentrations of antigen used in our study it is not possible to visualize pinocytosis using microscopy. In view of the fact that we observed potentiating effects of the glycoconjugates on Th1 development, we also examined proliferation of CD4+ T cells at a later time point (i.e. day 6). We found that at this time point proliferation of CD4+ T cells was significantly enhanced when activated by DCs pulsed with either of the glyco-conjugated proteins compared to T cells primed by native OVA-loaded DCs (data not shown). Although this does not reflect differences in presentation of antigen in MHC class II, it clearly shows that priming of the T cells is affected. This may be due to MR-induced signaling.

Only when accompanied by a TLR4 ligand, native OVA is routed to endosomal compartments for MHC class I loading [15]. In contrast to these findings, we demonstrate here that our novel neo-glycoconjugates mediate enhanced cross-presentation in a strictly TLR-independent manner, as enhanced cross-presentation was observed in the absence of TLR triggering and also present when using MyD88-TRIF-/- DCs. In addition, we could also exclude any endotoxin activity in our neo-glycoconjugates, indicating that this TLR-signaling independent cross-presentation is
strictly mediated by the glycosylation of the antigen. This could be a mechanism that ensures CD8 T-cell tolerance to autoantigens, as cross-presentation of auto-antigens is usually independent of TLR signaling [27, 28].

A clear difference in TLR-dependency of cross-presentation may lay in the antigen dose. In our experiments, cross-presentation of the neo-glycoconjugates was enhanced at a concentration of 30 μg/mL of neo-glycoconjugate, while the TLR-dependent cross-presentation of native OVA was observed at a high antigen dose of 1 mg/mL [14]. Alternatively, the difference in TLR-dependency might be due to the different glycans involved in MR binding. Whereas for native OVA the involvement of mannose structures has been described [14, 15, 21], we here demonstrated the potency of 3-sulfo-LeA and tri-GlcNAc as MR-targeting glycans. The binding of different glycans to CLR has shown to affect different signaling processes that may interfere with TLR signaling [29].

Some strategies that aim targeting antigen to MR involve MR-specific antibody–antigen conjugates. The human anti-MR Ab B11 has been developed as a vehicle to deliver the human tumor Ag pmel17 directly to DCs [13]. Monocyte-derived DCs loaded with the B11-pmel17 fusion protein resulted in antigen-specific CD4+ and CD8+ T-cell proliferation in vitro. Furthermore, injection of the B11-pmel17 conjugate in huMR transgenic mice also resulted in induction of both humoral and cellular antigen-specific immunity [30]. However, the use of MR-specific antibodies for antigen-targeting purposes in humans may induce adverse immune responses due to differences in glycosylation of the antibody with the endogenous MR in humans, which may arise from the cell line used for MR-Ab production. These effects will not appear when using natural ligands of MR to target antigen. The use of natural ligands to target the MR has been successful. Injection of DCs, ex vivo targeted with oxidized mannan-MUC1 conjugates, in mice resulted in the generation of high frequencies of MUC1-specific CTL and protection from tumor challenge [31, 32]. These studies formed the basis of clinical trails using oxidized mannan–tumorantigen conjugates to target MR. In a phase I clinical trial, patients with advanced carcinoma of the breast, colon, stomach and rectum were treated with mannan conjugated to part of MUC1. Although this resulted in antigen-specific humoral responses in half of the patients, and CTL responses in a minority of patients, no apparent clinical responses were detected [33]. A pilot phase III clinical study on oxidized mannan conjugated to MUC1 in stage II breast cancer patients with early disease showed promising results. Evaluation of patients 5 years after the last treatment revealed that all patients receiving immunotherapy were free of tumor recurrences. By contrast, the recurrence rate in patients receiving placebo was 27% [34].

Since the MR shares its specificity for mannose residues with DC-SIGN, vaccination strategies using mannan to target MR are

Figure 5. OVA-neo-glycoconjugates are conveyed to early endosomes. In total, 5 x 10⁴ (A and B) WT and (C and D) MR / BMDC were incubated with 30μg/mL fluorescent-labeled OVA or OVA-3-sulfo-LeA (red) for 2 h at 37°C. After fixation and permeabilization, cells were stained with antibodies specific for MR (green) and EEA-1 (early endosomes, blue). Using confocal microscopy technology multiple cross-sections were made to develop three-dimensional (X, Y and Z) pictures. The left-picture separated by dotted lines shows the X, Y-axis analysis of OVA or OVA-glycoconjugates, MR and EEA-1. The picture right from and beneath the dotted line shows Y, Z- and X, Z-axis analysis, respectively. Upper-right and lower-right pictures show OVA colocalized with EEA-1 or with MR. Graphs represent a quantitative analysis of colocalization; x-axis = length in μm; y-axis = color intensity. From a z-stack, histograms were created for a selected area (indicated by the line in the right part of each panel) using the Leica confocal software. Histograms were created for each fluorochrome and overlays were generated by the program. Arrows in the graphs indicate co-localization of antigen (red line) with EEA1 (blue line) or MR (green line). Graphs shown are representatives of two independent experiments.

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not specific and can involve other CLR, which can severely affect the desired response. Therefore, the urge to develop MR-specific vaccination strategies using other MR-restricted natural ligands is necessary. In this study, we have shown that both 3-sulfo-Le\(^a\) and tri-GlcNAc are potential glycans which can be used to develop MR-specific therapeutic strategies as these two ligands induce enhanced cross-presentation to CD\(^8\) \(^+\) T cells as well as potent Th1 responses. Induction of antigen-specific CD\(^4\) \(^+\) T cells is not only necessary for optimal generation of effector CD\(^8\) \(^+\) T cells, but also play an important role in the maintenance of memory CD\(^8\) \(^+\) T cells [22]. Moreover, the presence of antigen-specific CD\(^4\) T cells has recently been shown to be pivotal for the mobilization of CTLs into the effector site [23].

Together, these findings provide new options for MR-targeting studies to use specific glycans that do not share glycan specificity with other CLRs, and besides showing strong capacity to induce cross-presentation also encompass a Th1 skewing potential.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories and used at 8–12 wk of age. OT-I and OT-II TCR transgenic mice were bred and kept in our animal facility under specific pathogen-free conditions. All experiments were approved by the Animal Experiments Committee of the VUmc.

Antibodies

Unconjugated mouse anti-chicken egg albumin (OVA) antibody (OVA-14) was purchased from Sigma Aldrich; Alexa488-labeled or biotinylated-anti-MR antibody (clone MR5D3) was obtained from Bio-legend; PE-labeled anti-IL-4 (clone 11B11), anti-IL-17 (clone eBioTC11-18H10.1) and APC-labeled anti-CD11c (clone N418) and anti-IFN\(\gamma\) (clone XMG1.2) antibodies were all purchased from e-Bioscience (Belgium).

Secondary antibodies used in this study were peroxidase-labeled goat anti-human IgG and goat anti-mouse IgG (Jackson, West Grove, PA, USA).

BMDCs

BMDCs were cultured as previously described by Lutz et al. [35] with minor modifications. On day 0, the femur and tibia of mice were removed, both ends were cut and the marrow was flushed with Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco, CA, USA) using a syringe with 0.45-mm-diameter needle. The resulting marrow suspension was passed over 100-µm gauze to obtain a single cell suspension. After washing, the cells were seeded at 2 × 10\(^6\) cells per 100-mm dish (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) in 10 mL IMDM, supplemented with 10% FCS; 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin (BioWhitaker, Walkersville, MD, USA) and 50 µM β-mercaptoethanol (Merck, Damstadt, Germany) (1 = IMDMc) and containing 30 ng/mL recombinant murine GM-CSF (rmGM-CSF). At day 2, 10 mL medium containing 30 ng/mL rmGM-CSF was added. At day 5 another 30 ng/mL rmGM-CSF was added to each plate. From day 6 onwards, the non-adherent DCs were harvested and used for subsequent experiments.

BM and spleens derived from MR\(^{-/-}\) mice (bred on the C57BL/6 background) were a kind gift of Dr. C. Kurts and S. Burgdorf (Bonn, Germany). MyD88-TRIF\(^{-/-}\) BM was a kind gift from Dr. T. Sparwasser (Hannover, Germany).

Isolation of splenic DCs

Spleens from 3–5 mice were isolated, cut into small pieces and incubated in medium containing 1 WU/mL Liberase RI (Roche, Basel, Switzerland) and 50 µg/mL DNase I (Roche) at 37°C. After 45 min, EDTA was added to a final concentration of 10 mM, and the cell suspension was incubated for an additional 10 min at RT. The enzymatic digestion was terminated by addition of IMDM supplemented with 10% FCS/20 mM Hepes/10 mM EDTA (IMDM/HE). Red blood cells were lysed with ACK lysis buffer. Undigested material was removed by passing the suspension over 100-µm gauze. From the resulting single cell suspension, CD11c\(^+\) DCs were purified using anti-CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The enriched DC population (~98%) was used for subsequent experiments.

CD4\(^+\) and CD8\(^+\) T-cell isolation

OVA-specific CD4\(^+\) and CD8\(^+\) T cells were isolated from lymphoid tissue of OT-I or OT-II mice, respectively. In brief, LN and spleen were collected and single cell suspensions were obtained by straining the spleens and lymph nodes through a 100-µm gauze. Erythrocytes were depleted by incubation in ACK lysis buffer and CD4\(^+\) or CD8\(^+\) T cells were isolated from the single cell suspensions using the Dynal mouse CD4 or CD8 negative isolation kit (Invitrogen, CA, USA) according to the manufacturer’s protocol.

PAA probe-binding assay

BMDCs (5 × 10\(^4\)/well) were incubated 5 µg/mL with biotinylated PAA conjugated to GlcNAc, GlcNAcβ1-4GlcNAcβ, 3-sulfo-Le\(^a\), 3-sulfo-Le\(^b\), and 1-4GlcNAc (Lectinity, Moscow, Russia) at 37°C in PBS with 0.5% BSA (PBA) for 30 min. Cells were washed and stained with Alexa488-labeled streptavidin for 30 min at RT. Thereafter, cells were co-stained with APC-labeled anti-CD11c for 15 min at RT, and analyzed by flow cytometry (Calibur, BD Biosciences).
Generation of neo-glycoconjugates

For conjugation of the glycans 3-sulfo-LeA (creating OVA-3-sulfo-LeA) and \(N_{\text{GlcNAc}}N_{\text{GlcNAc}}N_{\text{GlcNAc}}N_{\text{GlcNAc}}\) (creating OVA-triacetyl chitotetraose (creating OVA-triacetyl chitotetraose), FACSCalibur. To OVA (Calbiochem, Darmstadt, Germany), a bifunctional cross-linker (4-N-maleimidophenyl butyric acid hydrazide; MPBH; Pierce, Rockford, IL, USA) was used. In short, via reductive amination, the hydrazide moiety of the linker is covalently linked to the reducing end of the carbohydrate. After 2 h incubation at 70°C, the mixtures were cooled down to RT. One milliliter ice-cold isopropanol (HPLC grade; Riedel de Haen, Seelze, Germany) was added and further incubated at −20°C for 1 h. The precipitated derivatized carbohydrates were pelleted and dissolved in 1 mM HCl. OVA dissolved in PBS was added to derivatized carbohydrates of interest (10:1 molar equivalent carbohydrate:OVA) and conjugation was performed o/n at 4°C. Neo-glycoconjugates were separated from reaction-reductants using PD-10 desalting columns (Pierce). The concentration of OVA was determined using the bicinchoninic acid assay (Pierce).

MHC class I and class II-restricted antigen-presentation assay

DCs (2.5 \(\times\) 10^5/well) were incubated with indicated concentrations of antigen in 96-well round bottom plates for 4 h. After washing, either 5 \(\times\) 10^4 purified OVA-specific CD4^+ T cells were added to each well. [3H]-thymidine (1 \(\mu\)Ci/well; Amersham Biosciences, NJ, USA) was added for the last 16 h of a 3-day culture to detect incorporation into DNA of proliferating T cells. Cells were harvested onto filters and [3H]-thymidine incorporation was assessed using a Wallac microbeta counter (Perkin-Elmer, USA).

In vitro CD4^+ T-helper differentiation assay

About 10^6 BMDCs were incubated with 30 \(\mu\)g/mL neo-glycoconjugate for 4 h in 96-wells round bottom plates. After washing, 5 \(\times\) 10^4 purified naive CD4^+ T cells isolated from OT-II mice were added to each well. On day 2, rmIL-2 (10 IU) was added. On day 7, the cells were activated with PMA (100 ng/mL; Sigma) and ionomycin (1 \(\mu\)g/mL; Sigma) for 6 h and brefeldin A (Sigma) was additionally added for the last 4 h. Intracellular production of IFN-\(\gamma\), IL-4 and IL-17 was analyzed using a Wallac microbeta counter (Perkin-Elmer, USA).

Confocal laser scanning microscopy

BMDCs (5 \(\times\) 10^5) were incubated for 2 h at 37°C with DyLight-594 labeled-OVA or -OVA-3-sulfo-LeA (30 \(\mu\)g/mL). Thereafter, cells were fixed, permeabilized and stained with antibodies against LAMP-1 or EEA-1. Subsequently, cells were allowed to adhere to poly-l-lysine-coated glass slides, mounted with anti-bleach reagent and analyzed by confocal microscopy (Leica AOB5 SP2 confocal laser scanning microscope system containing a DMIRE2 microscope with glycerol objective lens (PL APO 63 \(\times\)/NA1.30) was used; images were acquired using Leica confocal software (version 2.61)).

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Enhancing MR-binding ligands enhances TLR-independent cross-presentation

Antigen processing

Lewis(a) and Lewis(x) types in addition to the sulfated N-glycans of lutropin. J. Exp. Med. 2000. 191: 1117-1126.


Abbreviations: BMDC: bone marrow-derived DC  
CLR: C-type lectin receptor  
CR: cysteine-rich  
CDR: carbohydrate recognition domains  
GliNAc: N-acetylglucosamine  
MB: mannose receptor  
PAA: polyacrylamide

Full correspondence: Prof. Yvette van Kooyk, Department of Molecular Cell Biology and Immunology, VU University Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands Fax: +31-20-4448081 e-mail: y.vankooyk@vumc.nl

Additional correspondence: Dr. Wendy W. Unger e-mail: w.unger@vumc.nl

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Supplemental Figure 1. Mass-spectrometric analysis of neo-glyco-conjugates.

MALDI-TOF mass spectrometry measurements were carried out using a 4800 MALDI–TOF/TOF Analyzer (Applied Biosystems, Foster city, CA, USA). Samples were applied using the “dried-droplet” technique using sinapinic acid as a matrix. A 10mg/mL sinapinic acid solution was prepared in 0.1% trifluoroacetic acid solution in 1:1 acetonitrile and protein. Mass spectra were recorded in the range from to 19000 to 155000 m/z in the linear positive ion mode. The data were recorded using 4000 Series Explorer Software and processed with Data Explorer Software version 4.9. A, The MALDI mass spectrum of OVA-Le\textsuperscript{A} (red) shows an increase of 1.2 KDa compared to unconjugated OVA (blue). B, MALDI mass spectrum of OVA-tri-GlcNAc (light-blue) shows an increase of 1.2 KDa compared to unconjugated OVA (blue).
Supplemental Figure 2. Neo-glyco-conjugate preparations are endotoxin-free and contain similar amounts of OVA.

A. Native OVA and OVA-neo-glyco-conjugates were analyzed for possible endotoxin contamination using a LAL assay (Lonza, Cologne, Germany). Numbers indicate OD450 values. B. OVA content was examined using ELISA. Indicated concentrations of native OVA and the OVA-neo-glycoconjugates OVA-3-sulfo-Lea and OVA-tri-GlcNAc were coated onto NUNC maxisorp plates. The next day, OVA was detected using a specific anti-OVA Ab (clone OVA-14) and OD was measured at 450nm.

Supplemental Figure 3: Neo-glycoconjugate targeting to MR on BMDC results in enhanced cross-presentation.

To examine whether MR-targeting neo-glycoconjugates increased MHC class II or I presentation using BMDC, BMDC pulsed with OVA-3-sulfo-Lea or OVA-tri-GlcNAc, were co-cultured with purified OVA-specific A, CD4+ or B, CD8+ T-cells. 3H-thymidine was added to the cultures for the last 16h of a three day culture period. Proliferation is represented as cpm. One representative experiment out of two is shown. P-value <0.05 was considered significantly different from T-cell responses to native OVA. The statistical significance was calculated using Student’s t-test.