Tumour-associated glycan-modifications of antigen enhance MGL2-dependent uptake and MHC-class I restricted proliferation

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Dendritic cells (DCs) are specialised antigen presenting cells (APC) well known for their capacity to induce MHC class I and MHC class II antigen presentation by stimulating antigen specific CD8 and CD4 T cells, respectively. Distinct classes of receptors are involved in processes that regulate recognition and uptake of antigen while others receptors induce DC maturation upon antigen recognition. The most commonly studied pattern (pathogen) recognition receptors (PRR) on DCs are the Toll like receptors (TLR) and C-type lectin receptors (CLR). TLR recognise microbial and endogenously conserved molecular patterns resulting in induction of intra-cellular signaling cascade. Activation of TLR signalling cascade results in DC maturation and production of pro-inflammatory cytokines. On the other hand, CLR are endocytic receptors that are expressed on CD8\(^+\) DCs, whereas DCIR2 and dectin-1 are present in the cytoplasmic tail of CLRs. Second, different DC subsets express distinct patterns of CLRs. Therefore, different DC subsets express distinct patterns of CLRs. A study demonstrated that CD8\(^+\) DCs, whereas DCIR2 and dectin-1 are

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expressed on CD8\(^+\) DCs.\(^6\) In contrast to mice, DEC-205 is widely expressed on other cell types including myeloid blood DC and several lymphocyte subpopulations,\(^1\) indicating that DEC-205 targeting may not be DC specific.

To date, little is known on the targeting potency of the CLR, macrophage galactose-type lectin (MGL) that is expressed on macrophages and dendritic cells both in humans and mice.\(^2\) In human and rats only 1 copy of the MGL gene is found, whereas in mice 2 distinct genes mmgl1 and mmgl2 are expressed.\(^3\) In particular, MGL2 is the murine homologue of human MGL based on its glycan specificity and specific recognition of tumours cells.\(^4\) The glycan specificity of human MGL has been well documented for terminal GalNAc (Tn antigen), core 5 and 6 O-glycan structures.\(^5\) We and others have reported that the murine MGL2 has overlapping glycan specificity for Gal/GalNac residues.\(^6\) MGL2 is expressed in the dermis of skin, small intestines and lymph nodes.\(^7\) The human homologue of MGL2, hMGL, has been reported to bind tumour cell lines.\(^8\) Furthermore, it recognises and binds tumour antigen MUC1.\(^9\) The epithelial mucin MUC1 is highly glycosylated and frequently upregulated in adenocarcinoma. Tumour-associated MUC1 contains O-linked glycans such as Tn-antigen, Thomsen-Friedenreich antigen (TF-antigen) and their sialylated counterparts that are significantly shorter than on normal MUC1.\(^10\) DCs have been shown to internalise MUC1-Tn glycopeptides in a MGL dependent manner, where they are delivered to endosomal and lysosomal compartments in DC. The MUC1-Tn glycoprotein is only found in MHC class II compartments but does not enter MHC class I pathway.\(^11\) Similar to hMGL, MGL2 also strongly interacts with tumour-associated glycans on murine adenocarcinoma cell-lines C57MG and PancO2 as the MGL2 interaction with these cells correlates with presence of Tn/TF antigens present on these cells, suggesting a role of MGL2 in tumour immunity\(^3\); however, it is still unknown whether MGL2 interaction with (tumour) Tn-antigens induces CD4 and CD8 T cell responses.

Recent studies have shown that mannose glycans exposed on the model antigen OVA, targets to mannose receptor resulting in induction of CD8 T cell responses.\(^6\) In the current study, we generated neo-glycoconjugates by chemical modification of native OVA with tumour-associated GalNAc glycan structures, which may target MGL2 and investigated the role of tumour-associated glycan on the induction of T cell responses by DCs. We show that tumour-associated GalNAc enhanced IFN-\(\gamma\) producing CD4 T cell proliferation and facilitates cross-presentation of antigen to CD8 T cells. These results indicate that tumour-associated GalNAc modified antigens may lead to better cross-presentation providing future implications for designing tumour vaccines.

**Material and methods**

**Mice**

C57BL/6 mice 8–12 weeks of age were purchased from Charles River (L’Arbresle, France). OT-I mice and OT-II mice bred in our animal facility under pathogen-free conditions express a transgenic V\(\alpha\)2 V\(\beta\)5.1/5.2 T cell receptor (TCR) specific for the OVA peptides presented on H2-Kb (amino acids 257–264; SIINFEKL) or on I-Ab (amino acids 323–339; ISQAVHAAHAEINEAGR) respectively. All experiments were performed under specific pathogen-free conditions and according to institutional, state and federal guidelines.

**Cells**

**Bone marrow derived DCs.** Bone marrow derived DCs (BM-DCs) were cultured as previously described.\(^12\) The femur and tibia of mice were removed, both ends were cut and marrow was flushed with Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco, CA, USA) using a syringe with 0.45-mm diameter needle. The bone marrow suspension was vigorously re-suspended and passed over a 100-\(\mu\)m gauge to obtain a single cell suspension. After washing, cells were seeded 2 \(\times\) 10\(^6\) cells per 100 mm petridish (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) in 10 ml IMDM, supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin and 50 \(\mu\)M \(\beta\)-mercaptoethanol (Merck, Damstadt, Germany) and 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 DC were harvested and used for subsequent experiments. DCs were purified by positive selection with anti-CD11c MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s protocol.

Frozen bone-marrow of MyD88-TRIF/\(-/-\) were a kind gift Dr. T Sparwasser (Hannover, Germany) and were cultured using the above-mentioned protocol.

**Splenic DC isolation.** Spleens from 3 to 5 mice were cut into small pieces and digested by stirring at 37\(\degree\)C for 45 min in IMDM containing 1 WU/ml Liberase RI (Roche, Basel, Switzerland) and 50 \(\mu\)g/ml DNase I (Roche, Basel, Switzerland). EDTA was added to a final concentration of 10 mM, and the cell suspension was incubated for an additional 10 min at 4\(\degree\)C. IMDM with 10% FCS/10 mM EDTA/20 mM Hepes (IMDM/HE) was added, and the cells were pelleted. Red blood cells were lysed with ACK lysis buffer. Cells were washed once with IMDM/HE and undigested material was removed by filtration through a 100-\(\mu\)m cell strainer.

CD11c\(^+\) DCs were purified using anti-CD11c microbeads.

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**Reagents and antibodies.** All reagents were of the highest quality available and used as per manufacturer’s guidelines.
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Monocyte-derived dendritic cells. Monocytes obtained from buffy coats of healthy donors (Sanquin, Amsterdam) were cultured for 4–7 days in the presence of IL-4 (500 U/ml) and GM-CSF (800 U/ml) to generate immature monocyte-derived DC (moDCs). The immature phenotype of the cultured DCs was analysed by flow cytometry.

Antigen specific T cell isolation. OVA specific CD4+ and CD8+ T cells were isolated from spleen and lymph nodes cell suspensions from OT-II and OT-I mice, respectively. Lymph nodes and spleen were crushed and cell suspensions were pelleted. Erythrocytes were lysed, and the cells were passed through a cell strainer. CD4 and CD8 T cells were isolated from the suspensions using Dynal mouse CD4/CD8 negative isolation kit (Invitrogen, CA, USA) according to the manufacturer’s protocol.

CHO cell lines. Chinese hamster ovary cells (CHO) cells were transfected with MUC1 (7 tandem repeats) stable clones generated under neomycin (G418) selection. Non-transfected CHO cells were used as control.

Antigens

The following antibodies were used: anti CD11c-APC, anti-IFN-γ APC, anti-IL-4 PE and anti-IL-17 PE were used (ebioscience, San Diego, CA), anti-MGL (ER-MP23; a kind gift from Dr. P. Leenen, Rotterdam, The Netherlands), anti-OVA antibody (Sigma Aldrich, St. Louis, MO, USA) and MPBH (1:1 molar equivalent) in DMSO-acetic acid (17:3 v/v). A mixture of sodium cyanoborohydride (Sigma-Aldrich, St. Louis, MO, USA) and MPBH (1:1 molar equivalent) in DMSO-acetic acid (17:3 v/v) was freshly prepared and added to the carbohydrate mixtures (10:1 molar equivalent). After 2 hr incubation at 70°C, the mixtures were cooled down to room temperature. One millilitre of ice-cold isopropanol (HPLC grade; Riedel de Haan, Seelze, Germany) was added and further incubated at −20°C for 1 hr. The precipitated derivatised carbohydrates were pelleted by centrifugation (5 min at 14,000 rpm) and washed twice with ice-cold isopropanol. Pelleted carbohydrates were dissolved in 1 mM HCl (at this pH the maleimide moiety will remain stable). A sample was also dissolved in 50% acetonitrile and spiked with 0.1% acetic acid for offline analysis using offline needles (Promega) on Iontrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA). Ovalbumin (Calbiochem, Darmstadt, Germany) in PBS was added to derivatised carbohydrates of interest (10:1 molar equivalent carbohydrate/OVA) and conjugation was performed overnight at 4°C. Neo-glycoconjugates were separated from reaction reductants using PD-10 desalting columns (Pierce, Rockford, USA).

Neuraminidase treatment. To remove sialic acid, cells were treated with Neuraminidase from Vibrio cholerae (Roche Diagnostics, Mannheim, Germany) at 37°C for 1 hr. The cells were washed with Tris-sodium buffer pH 7.4 with 0.5% bovine serum albumin.

Flow cytometry

5 × 10⁴ cells were incubated with differentially conjugated antibodies for 30’ at 4°C. After incubation cells were washed with PBS +0.5% BSA. The surface marker expression was analysed by flow cytometry (FACScanlibur, BD Pharmingen, San Diego, CA, USA).

Specific primers for mouse MGL2 were designed by using the computer software Primer Express 2.0 (Applied Biosystems, CA, USA) and synthesized at Invitrogen (CA, USA).

Real time PCR reactions were performed using the SYBR Green method in an ABI 7900HT sequence detection system (Applied Biosystems, USA).

Generation of neo-glycoconjugates

α-0.6-N-acetylgalactosaminyl 1-3 galactose (Dextra labs, UK) was conjugated to Ovalbumin creating the GalNAc modified antigen (OVA-GalNAc), using a bi-functional cross-linker (4-N-Maleimidophenyl) butyric acid hydrazide (MPBH; Pierce, Rockford, USA). This cross-linker has a hydrazide moiety and a maleimide moiety that is reactive towards thiols at neutral pH. Via reductive amination the hydrazide moiety of the linker was covalently linked to the reducing end of the carbohydrate, and the maleimide moiety of the linker was later used for coupling the carbohydrate structures to ovalbumin. Briefly, the carbohydrates were dissolved in 100 μl dimethyl sulfoxide (DMSO)-acetic acid (17:3 v/v). A mixture of sodium cyanoborohydride (Sigma-Aldrich, St. Louis, MO, USA) and MPBH (1:1 molar equivalent) in DMSO-acetic acid (17:3 v/v) was freshly prepared and added to the carbohydrate mixtures (10:1 molar equivalent). After 2 hr incubation at 70°C, the mixtures were cooled down to room temperature. One millilitre of ice-cold isopropanol (HPLC grade; Riedel de Haan, Seelze, Germany) was added and further incubated at −20°C for 1 hr. The precipitated derivatised carbohydrates were pelleted by centrifugation (5 min at 14,000 rpm) and washed twice with ice-cold isopropanol. Pelleted carbohydrates were dissolved in 1 mM HCl (at this pH the maleimide moiety will remain stable). A sample was also dissolved in 50% acetonitrile and spiked with 0.1% acetic acid for offline analysis using offline needles (Promega) on Iontrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA). Ovalbumin (Calbiochem, Darmstadt, Germany) in PBS was added to derivatised carbohydrates of interest (10:1 molar equivalent carbohydrate/OVA) and conjugation was performed overnight at 4°C. Neo-glycoconjugates were separated from reaction reductants using PD-10 desalting columns (Pierce, Rockford, USA).

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Targeting of MGL2 results in cross-presentation and Th1-skewing

Probes, Eugene, OR, USA) or FITC-labelled goat anti-human Fc (Jackson Laboratories, West Grove, PA, USA) for 30 min, before analysis by flow cytometry. BM-DCs were incubated with labelled OVA for 30' and 60' at 37°C. Binding/uptake was analysed by flow cytometry.

Enzyme-linked immunosorbent assay
Glycoconjugates (PAA-biotinylated glycans or OVA neo-glycoconjugates) were coated to NUNC maxisorb plates (Roskilde, Denmark) overnight at room temperature. Plates were blocked with 1% BSA in PBS for non-specific binding. After extensive washing conjugates were incubated either with MGL2-Fc,23 biotinylated Helix Pomatia Agglutinin (HPA; Sigma Aldrich, St. Louis, USA) or anti-OVA antibodies for 1.5 hr at room temperature. Binding was detected using peroxidase-labelled F(ab')2 fragment goat anti-human IgG-Fc fragment specific antibody, peroxidase labelled streptavidin or peroxidase-labelled goat anti-mouse IgG-Fc fragment specific antibody, respectively. The reaction was developed with TMB substrate and optical density measured by a spectrophotometer.

T cell proliferation assay
Irradiated DCs were pulsed with different concentrations of neo-glycoconjugates in round bottom 96-wells plate and washed 3 times. Purified T cells either CD4 T cells from OT-II mice or CD8 T cells from OT-I mice were added to each well and co-cultured with antigen-pulsed BM-DC for 48 hr. After 48 hr, [3H]-thymidine (1 μCi/well; Amersham Biosciences, NJ, USA) was added to detect incorporation into DNA of proliferating T cells. Cells were harvested onto filters and [3H]-thymidine incorporation was assessed using a beta counter.

Internalisation and confocal laser scanning microscopy
The native and GalNAc-modified antigen (OVA and OVA-GalNAc) were labelled with DyLight 594 conjugate according to the manufacturer’s protocol (Thermo Fisher Scientific, Rockford, USA). BM-DCs were incubated for 2 hr at 37°C with DyLight 594 conjugated-OVA/OVA-GalNAc (30 μg/ml). The labelled cells were fixed and permeabilised with PBS/0.1% saponine for 20 min at 37°C, and stained in phosphate-buffered saline containing 0.5% bovine serum albumin and 0.1% saponin with antibodies against LAMP-1 (lysosomes) or EEA-1 (early endosomes). Cells were allowed to adhere to poly-L-lysine-coated glass slides, followed by mounting with anti-bleach reagent and analysis by confocal microscopy. A Leica AOBSP2 confocal laser scanning microscope (CLSM) system containing a DM-IRE2 microscope with glycerol objective lens (PL APO 63×/NA1.30) was used; images were acquired using Leica confocal software (version 2.61).

In vitro T helper assay
1 × 10^4 BM-DCs were pulsed with 30 μg/ml neo-glycoconjugates for 4 hr in round bottom 96-wells plate and washed 3 times. 5 × 10^4 purified naïve CD4 T cells from OT-II mice were added to each well and co-cultured with antigen-pulsed BM-DC. After 2 days, 10 U/ml recombinant mouse IL-2 was added to each well. On day 7, the cells were re-stimulated with 30 ng/ml PMA (Sigma Aldrich, St. Louis, USA) and 500 ng/ml ionomycin (Sigma Aldrich, St. Louis, USA) for 6 hr, including 5 μg/ml brefeldin A (Sigma Aldrich, St. Louis, USA) was added for the last 4 hr. Intra-cellular production of IFN-γ, IL-4 and IL-17 were analysed by flow cytometry.

Results
MGL is expressed on dendritic cells in vitro and in vivo
To analyse the expression of MGL on dendritic cells, we analysed DCs cultured from bone marrow of C57BL/6 mice and ex vivo isolated CD11c<sup>+</sup> splenic DCs. MGL expression was analysed using monoclonal antibody ER-MP23 recognising both MGL1 and MGL2. As previously demonstrated MGL was expressed both on CD11c<sup>+</sup> BM-DCs and splenic DCs (Fig. 1a).23 However, to distinguish the expression of MGL2 from MGL1 we verified the expression of MGL2 on mRNA level. MGL2 was expressed on BM-DCs and also on splenic DCs, although at lower levels (Fig. 1b).23 Moreover, we also confirmed the expression of MGL on CD11c<sup>+</sup> DCs in the dermis of skin (Fig. 1c).

MGL2 recognises neo-glycoconjugate OVA-GalNAc and MUC1
As earlier reported, MGL2 specifically recognises GalNAc while MGL1 prefers Lewis X binding.22,23 Indeed, MGL2 and not MGL1 specifically bind biotinylated PAA-GalNAc (Fig. 2a). GalNAc has been demonstrated to be exposed on MUC1 and indeed hMGL binds and internalises MUC1.20 Indeed MGL2, the murine homologue of hMGL binds tumour-associated glycans on tumour cell lines.23 We here confirm that also MGL2 binds MUC1 expressing CHO cells (CHO-MUC1) that express GalNAc upon neuraminidase treatment (Fig. 2b). Plant lectins, such as Helix pomatia agglutinin (HPA), that specifically recognises GalNAc,23 binds to neuraminidase treated CHO-MUC1 cells, confirming the exposure of GalNAc epitopes (Fig. 2c). Binding can be inhibited by EGTA illustrating that C-type lectin-glycan interactions are involved. To investigate whether we could target MGL2 using its specific natural ligand GalNAc, the free cysteines of model antigen OVA were chemically conjugated to reduced GalNAc to create neo-glycoconjugate GalNAc modified antigen. While, MGL2 did not bind native glycosylated antigen, specific binding to GalNAc modified antigen was observed when probed with MGL2-Fc (Fig. 2d). Additionally, the binding activity of HPA verified the presence of α-GalNAc mOieties conjugated to the model antigen OVA. The absence of interaction between either HPA or MGL2 to native antigen provide additional evidence that the native antigen does not contain GalNAc moiesities (Fig. 2e). Moreover, both native antigen and GalNAc-modified antigen were equally recognised by specific anti-OVA antibody indicating that labelling

did not destroy the natural conformation of OVA antigen (Fig. 2f). Titration of native antigen and the neo-glycoconjugates indicated similar antigen concentration in the preparations as detected by the anti-OVA antibodies (data not shown). Moreover, using human DC that express hMGL, we indeed could confirm that the GalNAc-modified antigen can be used to specifically target MGL2 in mice.

**GalNAc-modified antigen targeted to MGL2 expressed on BM-DC induces CD4 T cell proliferation**

Previous studies have shown that DCs loaded with native glycosylated OVA antigen that contains mannose moieties, targets antigen to mannose receptor to induce cross-presentation. We questioned whether GalNAc modification of this native antigen, creating MGL2 targeting structures, would influence skewing of MHC class II mediated CD4 T cell proliferation. To determine the potency of the GalNAc-modified antigen to target MGL2 on APC and to boost antigen presentation and T cell proliferation, we incubated BM-DCs obtained from C57BL/6 mice with different concentrations of native antigen and the neo-glycoconjugate, the GalNAc-modified antigen. Antigen-pulsed immature BM-DCs were further cultured with antigen specific CD4+ OT-II T cells. GalNAc modified antigen targeted to DC enhanced antigen presentation by DC compared with native antigen, resulting in 3-fold higher CD4 T cell proliferation (Fig. 3a). Surprisingly, no increased CD4 T cell proliferation was observed when CD11c+ splenic DCs were targeted by GalNAc-modified antigen, this can be due to low expression of MGL2 on splenic DCs or by the fact that the nature of antigen can result into different responses in different DC subsets (Fig. 3b).

**GalNAc-modified antigen targeted to MGL2 on BM-DCs induces IFN-γ producing CD4 T cells**

As we have observed that GalNAc-modified antigen that directed to MGL2 on BM-DC resulted in CD4 T cell proliferation, we further studied the effect of GalNAc-modified antigen and T cell polarisation. DCs loaded with native or GalNAc-modified antigen were incubated with CD4 T cells and intra-cellular cytokines were measured in proliferating T cells. We observed that when GalNAc-antigen-loaded DCs were used to prime CD4 T cells, the number of IFN-γ producing CD4 T cells was significantly increased compared with DCs loaded with native antigen (Fig. 4). Furthermore, DCs loaded with GalNAc-modified antigen that were simultaneously activated with TLR9 ligand CpG also showed similar induction of IFN-γ producing CD4 T cells as when DCs were triggered with GalNAc-modified antigen in the absence of CpG, indicating that IFN-γ producing Th1 cell induction is specifically glycan mediated. These data imply that glycosylated antigens targeted to MGL2 on DCs results in induction of IFN-γ producing effector T cells.

**GalNAc-modified antigen is routed both through early endosomal and lysosomal compartments to MHC class I and MHC class II, respectively**

Since we observed that GalNAc-modified antigen targeting MGL2 results in enhanced MHC class II restricted CD4 T
Figure 2. MGL2 specifically binds MUC1 and GalNAc modified antigen. (a) Binding of MGL1Fc and MGL2Fc to PAA-GalNAc glycans was determined by ELISA. (b) and (c) Binding of MGL2 and HPA to MUC1 expressing CHO cells respectively. (d) Binding of MGL2 to GalNAc modified antigen neo-glycoconjugates was determined using MGL2-Fc ELISA. (e) The plant lectin HPA recognises GalNAc moieties conjugated to OVA antigen. (f) Anti-OVA antibody recognises both native antigen and GalNAc modified antigens. One representative experiment out of 3 is shown. (g) Rapid internalisation of GalNAc modified OVA was detected into human monocyte-derived dendritic cells by flowcytometry.
cell proliferation in BM-DC, we investigated intra-cellular routing of both native and GalNAc-modified antigen into BM-DC. Fluorescent-labelled antigen and GalNAc-modified antigen were incubated with antigen and GalNAc-modified antigen for 4 hr, washed and co-cultured with antigen specific CD4 T cells (OT-I). T cells were re-stimulated with PMA and ionomycin, and intra-cellular IL-4 (Th2) and IFN-γ (Th1) were analysed on a single cell basis by flow cytometry. Representative results of 3 different experiments are shown.

This increase was also seen at earlier time points (data not shown). Additionally, we also observed increased co-localisation of internalised GalNAc-modified antigen with endosomal marker EEA-1 (yellow), compared to native antigen as previously reported by Burgdorf and co-workers. These data correlate with our antigen presentation and T cell proliferation data demonstrating that GalNAc-modified antigen targeted to MGL2 shows increased MHC class II presentation. Moreover, the high co-localisation with endosomal compartment prompted us to investigate whether GalNAc modified antigen might potentially induce CD8 T cell priming through cross-presentation, as previously demonstrated for mannose receptor that routes native OVA to endosomes for efficient cross-presentation.

MGL2 expressed on BM-DC and splenic DC specifically cross-present OVA-GalNAc

We determined whether GalNAc-modified antigen targeted to MGL2 could enhance the cross-presentation to CD8 T cells to a level higher than native OVA that targets mannose receptor. BM-DCs and freshly isolated splenic DCs were incubated with antigens and were co-cultured with antigen specific CD8 T cells (OT-1) and were analysed for T cell proliferation using [3H]-thymidine incorporation. Analysis of DCs pulsed with varying dose of native antigen and GalNAc-modified antigen that were co-cultured with antigen specific CD8 T cells showed an enhanced cross-presentation of CD8 T cells of the GalNAc-modified antigen compared to native antigen (Fig. 6a). Surprisingly, the efficacy of cross-presentation of the GalNAc-modified antigen was observed at lower antigen concentration of 30 μg/ml then previously described for native antigen (1 mg/ml). Similarly, splenic DCs pulsed with GalNAc-modified antigen resulted in increased antigen specific OT-1 T cells proliferation, indicating that tumour-associated glycans such as GalNAc greatly facilitate the sensitivity of cross-presentation by targeting antigens to MGL2.
As expected, the concentration of GalNAc-modified antigen required to trigger cross-presentation is higher (30 μg/ml) and exceeds the amount needed to induce MHC class II presentation, which is active even at concentrations of 3 μg/ml (Figs. 6a and 6b). These data suggest that GalNAc-modified antigens directed to MGL2 are efficiently cross-presented via MHC class I molecules to induce antigen-specific CD8 T cell response.

As previously reported, mannose receptor-mediated cross-presentation of native OVA antigen is co-dependent on TLR triggering.34 We therefore investigated the role of TLR triggering on the GalNAc induced cross-presentation. When BM-DCs derived from MyD88-TRIF<sup>−/−</sup> BM-DCs, loaded with either antigen or GalNAc-modified antigen were co-cultured with antigen specific CD8 T cells, GalNAc-modified antigen that targets to MGL2 on DCs still showed enhanced cross-presentation to CD8 T cells. Clearly, no reduction in cross-presentation was observed in MyD88-TRIF<sup>−/−</sup> BM-DC, indicating that no TLR signalling contributes to the GalNAc-associated cross-presentation. This indicates that the enhanced antigen presentation eliciting amplified CD8 T cell response is purely due to glycan modification of antigen (Fig. 6c). We could also observe no reduction of CD4 T cell proliferation by the GalNAc-modified antigen using MyD88-TRIF<sup>−/−</sup> BM-DCs (data not shown). We also analysed our neo-glycoconjugates for the presence of endotoxin using a TLR4-reporter cell line, HEK-TLR4. HEK-TLR4 transfectants were pulsed with native antigen and GalNAc modified antigen and IL-8 production...
was determined by ELISA. As expected, the results obtained indeed showed that both native and the GalNAc-modified antigen were not contaminated by LPS as endotoxin levels were below the detection limit of 2ng/ml (data not shown). Hence, these results show that GalNAc-modified antigens targeted to MGL2 on immature BM-DCs can both be routed to MHC class II and class I pathway to prime both CD4 and CD8 T cells without accompanying TLR signalling.

**Discussion**

In this study, we have demonstrated that the antigen modification with tumour-associated glycan GalNAc specifically targets C-type lectin receptor MGL2 that serves as an antigen uptake receptor on DCs. We have shown that GalNAc-modified antigen binds to MGL2 and is rapidly internalised into DCs for processing in endosomal and lysosomal compartments. BM-DCs showed an enhanced CD4 T cell response through GalNAc-modified antigen, whereas splenic DCs did not, despite the fact that both DCs express MGL2. Furthermore, engagement of GalNAc-modified antigen with MGL2 resulted in differentiation of naïve CD4 T cells into IFN-γ producing Th1 cells. Surprisingly, GalNAc-modified antigen enhanced the induction of antigen specific CD8 T cell responses of both BM-DCs and splenic DCs through cross-presentation at a level much more efficient than earlier reported for native glycosylated OVA antigen that targets to mannose receptor.34 For native glycosylated OVA, it has been demonstrated that mannose receptor mediates antigen uptake and routes antigen to early endosomal compartment for cross-presentation. Additionally, the processing of antigen in endosomes was TAP dependent as TAP was recruited to endosomes by TLR stimuli.34 However, we have observed that GalNAc-modified antigen is routed both to early endosomes and lysosomes.

The cross-presentation of GalNAc-modified antigen to CD8 T cells was prominent even at low concentrations of 30 μg/ml in contrast to previous studies in which high antigen concentrations were needed (1 mg/ml) to achieve cross-presentation.34 Strikingly, both cross-presentation to CD8 T cells and MHC class II presentation to CD4 T cells was

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**Figure 6.** GalNAc-modified antigen targeted to MGL2 on splenic DCs facilitate cross-presentation. (a) and (b) Native antigen and GalNAc-modified antigen targeted to MGL2 on BM-DCs and splenic DCs induces cross-presentation. BM-DCs (a) and splenic DCs (b) were pulsed for 6 hr with neo-glycoconjugates and co-cultured with antigen specific CD8 T cells (OT-I cells) for another 48 hr. Proliferation was determined by [3H]-thymidine incorporation. (c) Enhanced antigen presentation resulted by GalNAc-modified antigens is endotoxin independent. BM-DCs from MyD88−/−/− were incubated with neo-glycoconjugates for 6 hr and co-cultured with antigen specific CD8 T cells (OT-I cells). Proliferation was determined by [3H] thymidine uptake. Data presented are representative of 3 different experiments and is designated as the mean ± SD of triplicates.
Targeting of MGL2 results in cross-presentation and Th1-skewing

Glycan-enhanced cross-presentation

of TLR signalling and was observed in immature DCs. The finding that GalNAc-modified antigen simultaneously enhances cross-presentation of DC, and, to some extent, CD4 T cell responses indicates that GalNAc modification of any antigen may increase the extent of antigen specific CD4 and CD8 T cell responses. Potentially, GalNAc modification of antigen can in the future serve as a tool to target improve anti-tumour responses against any tumour antigen of choice.

Despite the fact that, in mice, 2 functional copies of MGL gene exist (MGL1 and MGL2) and only 1 copy of MGL is found in rats and humans,21,22 the GalNAc specificity of hMGL is conserved in MGL2 and not in MGL1. Given the glycan specificity which is also reflected in a similar specificity to recognise MUC-1 on tumour cells, it is likely that MGL2 is the murine homologue of human MGL. Using human DCs, antigen targeting to hMGL through anti-MGL antibodies, have demonstrated to rapidly internalise antigens and route them to lysosomal compartments for MHC class II loading and subsequently presented to responder CD4 T cells.36 Using GalNAc moieties Napoli et al. reported that hMGL internalises antigen to deliver antigen to both MHC class II and class I compartments; however, the potency of the antigens to induce CD4 or CD8 T cells was not studied.32 Also, here we demonstrate that the GalNAc neo-glycoconjugate that we generate rapidly internalises in human DCs. In mice, MGL has been shown to act as an uptake receptor as it binds α-GalNAc polymers and subsequently internalises to MHC class-II compartments.26

We here demonstrate that, MGL2 the closest homologue of hMGL has specificity for GalNAc and MUC1. Binding of GalNAc-modified antigen enhances internalisation for processing in both endosomal and lysosomal compartment and subsequently elicits enhanced antigen presentation by MHC class II and class I molecules to CD4 and CD8 T cells, respectively. So far, no potential of either hMGL or MGL2 to mediate cross-presentation has been reported. We have demonstrated that GalNAc modified antigen can be cross-presented by both BM-DCs as well as ex-vivo isolated primary splenic DCs thereby eliciting CD8 T cells responses whereas, no enhanced MHC class II presentation was observed by splenic DCs. CD8+ DCs are reported to be specialised in cross-presentation of cell-associated and particulate antigens.10 However, recent studies have also reported the ability of CD8+ DCs to cross-present antigens such as yeast-OVA targeted to dectin-1, which is expressed both on CD8+ and CD8− DCs. Fungal antigens targeted to both DC subsets result in CD4 T cell responses whereas CD8+ DCs specifically cross-present OVA to CD8 T cells.35 Recently, Kumamoto and co-workers have reported that MGL2 is expressed on CD8low DCs in lymph nodes.37 However, further detailed analysis on the expression of MGL2 on different DC subsets is required to address this hypothesis.

Several studies have demonstrated the potency of using DC-specific uptake receptors, such as CLR, to enhance antigen specific CD4 and CD8 T cell responses, and are therefore considered as efficient tools for targeting purposes to develop anti-tumour vaccines. Different approaches have been used such as liposomes,38,39 live infectious vectors as lentiviruses40 or antibody conjugates that specifically target DC receptors.41 Targeting antigen to DC receptors such as DEC-205 using specific antibodies-antigen conjugates has been shown to induce high CD8 T cells responses in vivo; however, translating this to vaccination strategies is a challenge. Alternatively, the use of the natural ligands of CLRs such as glycans can be of great advantage and excellent alternative to antibody based therapies in particular as they are not immunogenic. For example, mannosylated OVA engineered in fungi Pichia pastoris targeted to the mannose receptor on murine BM-DC resulted in elevated OVA-specific CD4 T cell proliferation.32 Here we show that, neo-glycosylation of ovalbumin protein as model antigen with tumour-associated glycan GalNAc (Tn-epitope) results in enhanced antigen-specific CD4 and CD8 T cell proliferation. Previous studies on tumour-associated glycoproteins, such as MUC1 that contains many tandem repeats that may potentially bear the tumour-associated Tn-antigen (α-GalNAc-Ser/Thr), have shown that glycosylated soluble MUC1 found in serum of cancer patients, does not elicit T helper responses when administrated exogenously to DC in vitro.43 On the contrary, under-glycosylated MUC1 in the form of long synthetic peptide elicit MHC class I and class II restricted cytolytic activities in primed T cells. The host un-responsiveness to tumour-associated GalNAc exposing MUC1 has been demonstrated to be the result of a blockade of the intra-cellular sorting and processing machinery in DC,44 due to the highly glycosylated state of MUC1, that blocks proper intra-cellular degradation and routing of antigen leading to unresponsiveness due to inefficient MHC peptide loading. To overcome this blockade in degradation, glycosylated MUC1 peptides have been used to elicit cytotoxic T cell responses. Short O-linked glycans, like the Tn or TF-antigens (GalNAc Ser/Thr or Gal-3GlcNAc Ser/Thr), on MUC1 tandem repeats remain intact during DC processing in the MHC class II pathway.45 These glycans control the extent and site specificity of cathepsin L-mediated endosomal proteolysis of MUC1 glycopeptides46 and are able to elicit peptide or mixed glycopeptide specific Th cell responses. In our study, we have shown that proteins such as model antigen OVA can be used to chemically conjugate tumour-associated glycans using the chemical properties of the protein itself. This may help in overcoming the problem of heavy-glycosylation of protein which may potentially block antigen processing and presentation. In our chemical modification strategy, we use cysteine residues present in the antigen to conjugate GalNAc moieties to create a Tn-antigen mimic. The model antigen OVA that we used in our study contains 6 cysteine residues, out of which 3 are required in protein folding.47 The remaining 3 available cysteines were used to conjugate GalNAc, giving rise to 2–3 GalNAc per OVA molecule. The amount of GalNAc is far less than the amount of

Tn-antigen is present on MUC1 protein containing 25–125 tandem repeats (TRs) of 20 amino acids acting as a scaffold for O-glycans such as GalNAc.48 Because of this tandem repeats, the amount of Tn-antigens on MUC1 can exceed up to 100 GalNAc moieties. The 3 GalNAc moieties conjugated to the antigen OVA reflect the similar situation as the glycosylated peptides used by Hiltbold and Stepensky in 2 different studies that show that glycosylated MUC1 peptides are presented in MHC class I and class II fashion.49,50 This indicates that the amount of GalNAc (Tn-antigen) may determine the induction or inhibition of T cell responses. Moreover, to create MUC1-specific immune responses, it is essential to use glycosylated antigens for the induction of anti-tumour responses, as the antibody reactive to mucins are more directed to glycopeptide than to non-glycosylated peptide epitopes.51 To overcome the weak immunogenicity of the importance of DC maturation to facilitate effector T cell responses. In contrast, to these studies, we here show that DC pulsed with glycosylated antigens such GalNAc modified OVA lead to induction of IFN-γ producing CD4 T cells. This induction of effector T cells was independent of TLR-induced DC maturation and strictly glycated CD4 T cells. However, our glycosylated neo-glycoconjugates induced effector T cells without accompanying DC maturation.

In conclusion, we here demonstrate that GalNAc-modified antigen directed to MGL2 on BM-DCs is efficiently internalised and processed in lysosomal and endosomal compartments is loaded on to MHC class II and class I molecules leading to enhanced antigen-specific CD4 and CD8 T cell responses in TLR independent manner. Furthermore pronounced cross-presentation is induced when GalNAc modified antigen is targeted to MGL2 on splenic DCs. It is therefore likely that in vivo targeting of MGL2 will in particular lead to enhanced CD8 T cells induction through cross-presentation. Further in vivo studies are required to address the potential of MGL2 as tool to combat adenocarcinomas.

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