Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Glycopeptides as Targets for Dendritic Cells: Exploring MUC1 Glycopeptides Binding Profile toward Macrophage Galactose-Type Lectin (MGL) Orthologs

Gerard Artigas,[†] João T. Monteiro,[‡] Hiroshi Hinou,[†,§] Shin-Ichiro Nishimura,[†,§] Bernd Lepenies,*[,‡] and Fayna Garcia-Martin*[,†]

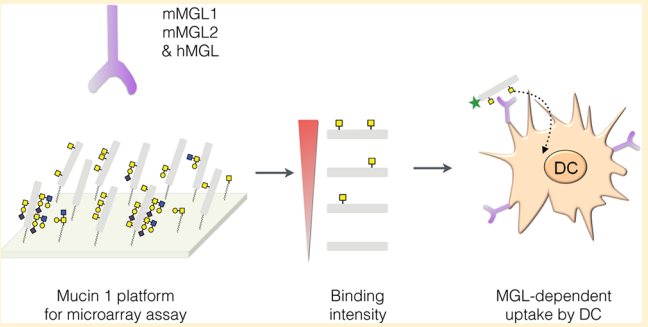
[†]Faculty of Advanced Life Science and Graduate School of Life Science, Hokkaido University, N21, W11, Kita-ku, 001-0021 Sapporo, Japan

[‡]Immunology Unit & Research Center for Emerging Infections and Zoonoses (RIZ), University of Veterinary Medicine Hannover, Bünteweg 17, 30559 Hannover, Germany

[§]Medicinal Chemistry Pharmaceuticals, Co., Ltd. N9W15, Chuo-ku, 060-0009 Sapporo, Japan

Supporting Information

ABSTRACT: The macrophage galactose-type lectin (MGL) recognizes glycan moieties exposed by pathogens and malignant cells. Particularly, mucin-1 (MUC1) glycoprotein presents an altered glycosylation in several cancers. To estimate the ability of distinct MGL orthologs to recognize aberrant glycan cores in mucins, we applied evanescent-field detection to a versatile MUC1-like glycopeptide microarray platform. Here, as binding was sequence-dependent, we demonstrated that not only sugars but also peptide region impact the recognition of murine MGL1 (mMGL1). In addition, we observed for all three MGL orthologs that divalent glycan presentation increased the binding. To assess the utility of the glycopeptide binders of the MGL orthologs for MGL targeting, we performed uptake assays with fluorescein-MUC1 using murine dendritic cells. A diglycosylated MUC1 peptide was preferentially internalized in an MGL-dependent fashion, thus showing the utility for divalent MGL targeting. These findings may be relevant to a rational design of antitumor vaccines targeting dendritic cells via MGL.



INTRODUCTION

Over the past decade, it has become clear that the structural heterogeneity of glycans on proteins and lipids correlates with their essential functions in numerous biological processes.[1] An illustrative example is carbohydrate−lectin interactions governing the immune system. In innate immunity, the engagement of certain pattern recognition receptors (PRRs) by pathogen-derived ligands or self-ligands allows antigen-presenting cells (APCs) to recognize and discriminate between pathogens, tumor-associated antigens, or normal tissues. Interestingly, the majority of pathogen-derived ligands or autoantigens are glycoconjugates.[2] Of particular note is the natural and intrinsic immunosurveillance function of APCs that identify and eliminate tumor cells. Therefore, APCs are promising targets for advanced drug discovery on immune-mediated disorders such as cancer, autoimmune diseases, and chronic inflammation.[3]

In the immune system, glycan-binding proteins (GBPs) can be subdivided in several families with diverse and specialized functions. GBPs expressed by APCs are at the interface between the innate and adaptive immune system since they recognize endogenous glycoproteins as well as carbohydrates expressed by pathogens, thus contributing to the initiation of adaptive immune responses.[4] GBPs, also termed lectins, contain a highly conserved structural motif, the carbohydrate recognition domain (CRD), which facilitates selective carbohydrate recognition. Depending on their binding mode and affinity, cellular expression, or their biological functions, among other criteria, GBPs are conventionally classified in different groups.[1,5,6] In particular, myeloid C-type lectin receptors (CLRs) cover a large animal lectin family with a conserved structurally homologous CRD, which is characterized by Ca^{2+}-dependent binding sites as a common feature for lectin functionality.[2,7] In APCs, such as dendritic cells (DCs), CLRs often act as PRRs and promote the internalization and subsequent processing and presentation of antigens to trigger an efficient adaptive immune response.[3,8,9]

In the context of immunotherapy, the engagement of CLRs by glycan moieties has evidenced targeting of CLRs on APCs as a promising strategy to boost antigen presentation and, thereby, to direct strong and tailored $CD4^+$ and $CD8^+$ T cell

Received: August 22, 2017
Published: October 18, 2017

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

responses.[4,10,11] In this regard, herein we have confined our attention on the C-type lectin receptor macrophage galactose-type lectin (MGL, also referred to as Clec10A or CD301), a transmembrane type II galactose-specific C-type GBP exclusively expressed as an oligomer by DCs and macrophages. Distinctively, the conserved QPD (Gln-Pro-Asp) amino acid motif present in the CRD, which is coordinated with Ca^{2+} ions and the carbohydrate core, is the hallmark of MGL specificity for galactose (Gal) and terminal *N*-acetylgalactosamine (GalNAc) residues of N- or O-glycosylated proteins and lipids.[12,13] Physiologically, the expression of MGL by immature DCs is noteworthy, along with its resulting versatile key roles as initiator of adaptive immune responses.[14] As a receptor of self-ligands carrying GalNAc moieties, MGL can modulate T cell signaling to control inflammation and autoimmunity processes, and impede the migration of immature DCs.[14,15] Complementarily, MGL is also responsible for uptake of pathogens and tumor associated antigens.[16,17] Specifically, MGL engagement to tumor-associated mucin-1 (MUC1) glycoprotein containing GalNAc glycoforms not only enables the differentiation of primary colon carcinoma cells over normal epithelial cells[18] but also enhances activation of DCs.[17] Overall, as MGL is dynamic and versatile enough to contribute to both uptake and modulation of innate and adaptive immune responses, an accurate understanding of the molecular recognition process of tumor-associated antigens is crucial for designing novel anticancer vaccines with enhanced effectiveness.[19,20]

In a tumor environment, glycoproteins contain altered glycosylated patterns, such as under- and overexpressed, truncated or altered-branched glycans. Those glycan modifications in glycoproteins unmask abnormal carbohydrate cores that can be identified by lectins.[21] Strikingly, the overexpression of immature truncated O-linked glycans is found in most secreted and transmembrane glycoproteins, such as mucins, where this disorder is commonly associated with poor prognosis in several cancers and promoting the proliferation of cancer cells.[3,22] In particular, MUC1, a highly glycosylated transmembrane mucin protein playing protective roles as a mucous barrier for epithelial cells, represents one of the most promising targets for carcinoma vaccination and diagnosis. In fact, in cancer cells, the O-linked glycan density of its conserved 20 amino acid tandem repeat (GVTSAPDTRPAPGSTAPPAH) is drastically reduced, and hence, a different cellular pattern is expressed. The overexpression of the tumor-associated antigens Tn (O-linked GalNAcα) and T (O-linked Galβ1-3GalNAcα), and their sialylated counterparts in the five potential O-glycosylation sites that embody the tandem-repeats, unmasks tumor specific epitopes and triggers a tumor-specific immune response.[23,24] Accordingly, in the past decade, glycan and glycoprotein-based platforms[25] have led to advances in epitope mapping and antitumor vaccine design based on the MUC1 antigen.[26] Recently, we have developed a synthetic MUC1 glycopeptide microarray platform by which the epitope mapping of different anti-MUC1 mAbs was studied,[27,28] and the position and O-glycosylation state effects on the antigenicity of the immunogenic region were evaluated.[29] In addition, we showed the utility of this platform for exploring galectin specificities.[30]

In the present study, we employ a MUC1-based glycopeptide microarray to characterize the specificities of three MGL (Clec10a) orthologs, CD301 (human MGL, hMGL), CD301a (murine MGL1, mMGL1), and CD301b (murine MGL2, mMGL2). We believe that the findings reported here will facilitate the general use of evanescent-field fluorescence microarrays as a high-throughput screening approach to detect lectin ligands with high reproducibility. To transfer the microarray results to a relevant biological setting, we performed internalization studies using murine DCs. Our results demonstrate that GalNAc containing peptides are recognized and internalized by DCs in an MGL-dependent fashion. Collectively, this study may provide a basis for the rationale design of future cancer vaccines aiming at the targeting of lectins expressed by APCs.

RESULTS AND DISCUSSION

Synthetic MUC1 Screening Array Based on Evanescent-Field Fluorescence Principle. Glycan arrays represent a useful high-throughput platform to screen for glycan interactions with GBPs and antibodies.[31] However, due to the cross-reactivity of lectins with a broad spectrum of the glycome, an accurate glycan profiling of GBPs requires access to a large and defined collection of glycan structures. Thus, the Consortium for Functional Glycomics (CFG) established a global database, which compiles an exhaustive collection of glycan structures covering diverse protocol data of GBPs screening.[32] We have performed several trials using conventional microarray technique including consecutive washing to remove unbound proteins, but results were not conclusive. Although generally glycan arrays enable an accurate analysis of GBPs specificities in a single experiment, the prior washing steps to remove unbound fluorescent probes remain a challenge to avoid false negative results as a consequence of the general low affinity of the interactions of GBPs with glycans.[33,34] To address this, Hirabayashi et al. designed a new array platform based on evanescent-field fluorescence principle, in which no washing steps are required, and hence, even weak GBP−carbohydrate interactions under equilibrium conditions can be detected.[35] Briefly, the detection principle of an evanescent-field approach is based on the generation of an electromagnetic wave (evanescent field) within a limited distance from the surface, when a ray of light with a certain angle of incidence is totally reflected from the solid support material (high refractive index) into the solution phase (low refractive index). Overall, the lack of washing steps in this methodology permits monitoring of multiple lectin−carbohydrate interactions under equilibrium conditions.

In addition, advances in understanding the functions of glycosylation in mammalian proteins have also prompted the design of novel microarray platforms for mapping relevant disease epitopes and thus for vaccine design.[27,36,37] In view of these findings, we assume that a consistent microarray platform considering the natural MUC1 presentation will elucidate its molecular recognition by the CLR MGL and will provide insights into parameters such as glycan moiety, position, and multivalency effects. Hence, we adopted herein the evanescent-field fluorescence principle to screen the human and murine MGL orthologs using our previously reported microarray platform, based on the 20 amino acid tandem repeat within the MUC1 transmembrane domain. To this aim, we combined the standard solid-phase peptide synthesis (SPPS) assisted by microwave irradiation with a double activation protocol to incorporate the sugar amino acid derivatives.[38,39] By enzymatic sugar elongation, we obtained the highly O-glycosylated ligands,[38,40] and through the N-terminus functionalization with 5-oxohexanoic acid, we immobilized the library to the microarray support via the glycoblotting method.[27,41]

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

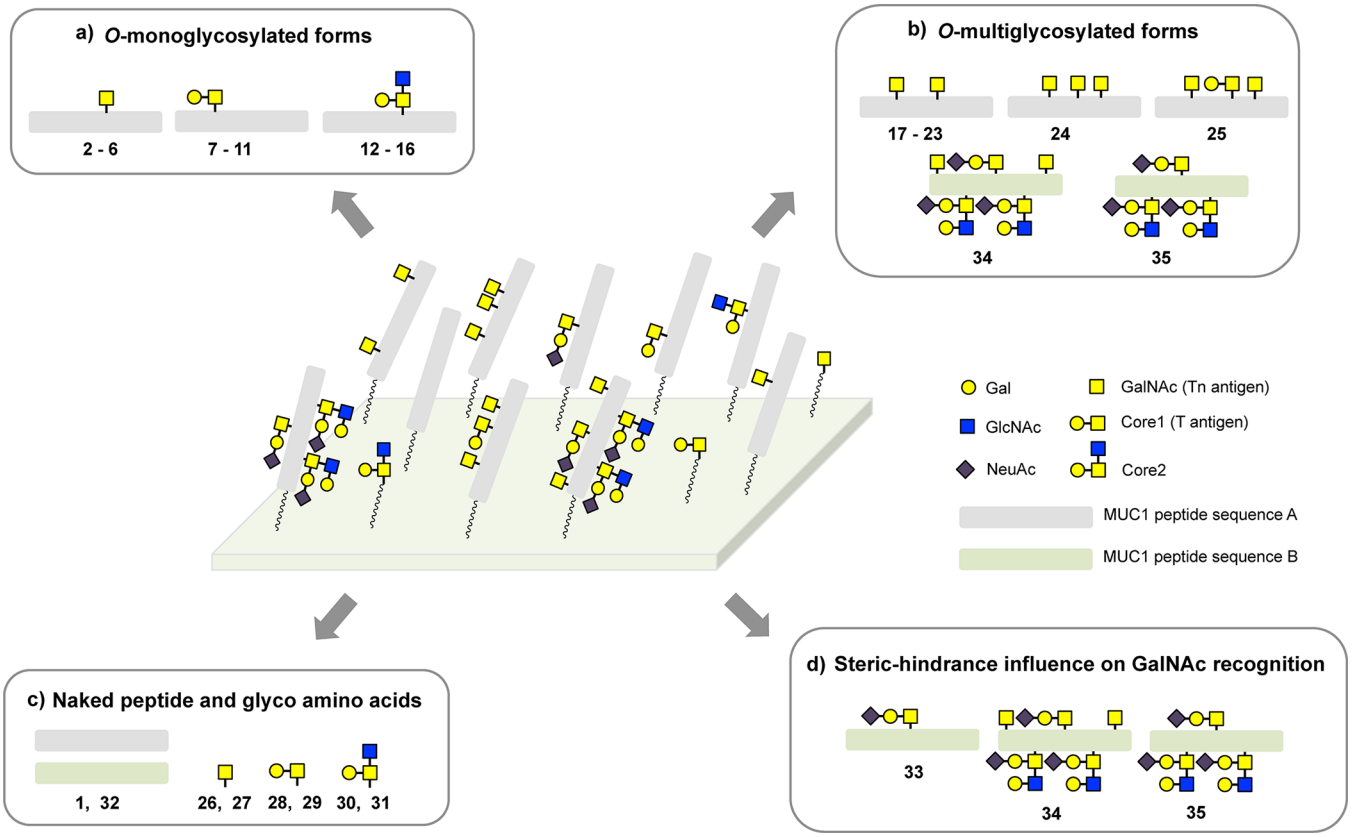


Figure 1. Versatile glycopeptide microarray platform based on the MUC1 tandem repeat. In this library, two different MUC1 sequences are contained: **1**–**25** represents a 23 amino acid fragment (gray box, GVT*S*APDT*RPAPGS*T*APPAHGVT), while **32**–**35** is a 20-mer (green box, PPAHGVT*S*APDT*RPAPGS*T*A). Asterisk shows the potential O-glycosylation sites. Compounds **24**–**30** correspond to O-glycosylated Ser and Thr amino acids with Tn antigen, T antigen (also named Core1), and Core2 glycan moieties, respectively (for further details, see Table S2 in Supporting Information).

Altogether, we are confident that the asset of our chemical library lies in its versatility to conduct different evaluations at once (Figure 1 and Supporting Information Tables S1 and S2 and Figure S1): (a) the glycan specificity and position impact of O-monoglycosylated forms containing *O*-glycan core structures representing cancer epitopes, commonly found in mucins (Tn antigen (GalNAcα1→) and T antigen or Core1 [Galβ(1→3)GalNAcα1→]), and the minimal trisaccharide core expressed in normal mucins (Core2 (Galβ(1→3)[GlcNAcβ(1→6)]-GalNAcα1→)[24] (**2**–**16**); (b) the multivalency effect on di-, tri-, and pentaglycopeptides (**17**–**25**, **34**, **35**); (c) the separate peptide and sugar contributions to the lectin binding (**1**, **32**, **26**–**31**); (d) the steric-hindrance influence on GalNAc specificity (**33**–**35**).

Epitope Mapping of Human and Murine MGL. MGL is expressed by both human and murine APCs. However, two distinct orthologs are found in mice (mMGL1 and mMGL2), while in humans only one is expressed (hMGL).[12,42,43] In the present work, we describe the interaction of human and murine MGL with a synthetic mucin-like microarray library (for further details, see Experimental Section and Supporting Information Table S2 and Figures S1 and S2). As a prior test, we first screened the antibreast tumor antibody SM3 with the tumor-associated antigens expressed in our mucin-like glycopeptide platform by evanescence microarray (see Supporting Information Figure S4). We deem the observation of the well-studied diagnostic specificities of SM3 with naked (**1** and **32**) and glycosylated MUC1 peptides in the immunodominant PDTR domain[44] (**4**, **9**, **14**, and **33**) and the use of Fc fragment and another mAb as negative control (data not shown) as a sufficient proof to validate the herein presented strategy.

First, we focused our analysis on the carbohydrate specificity of mMGL1 (see Figure S3 for representative array images), and results are shown in Figure 2A. As expected, lectin mMGL1 cannot recognize nonglycosylated peptides (compounds **1** and **32**). In contrast, mMGL1 recognizes monoglycosylated MUC1 carrying single GalNAc structures (**3** and **6**) and extended T antigen (**10**, **11**) and Core2 (**15**). Remarkably, this is the first reported evidence of epitope recognition in mMGL1 centered on glycan position, as the binding profile depends not only on the carbohydrate moiety but also on the glycosylated peptidic region. Regarding the glycan moiety, the preference of mMGL1 for GalNAc over terminal Gal is in agreement with previous studies on hMGL showing that GalNAc establishes additional interactions provided by the 2-acetamide group.[45,46] These previous structural findings might support the fact that mMGL1 showed higher affinity to single GalNAc than T antigen and Core2 structures. Consistently, the sialylated T antigen on region PDTR (peptide **33**) was not recognized by mMGL1.

As discussed previously, the peptide region may have an influence on mMGL1 recognition. We hypothesize that the primary sequence specificity for GalNAc structures at the Thr in the GSTA fragment might occur as a result of the conformation dependence of the GalNAc position on specific lectin engagement.[47,48] The contribution of vicinal residues is

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

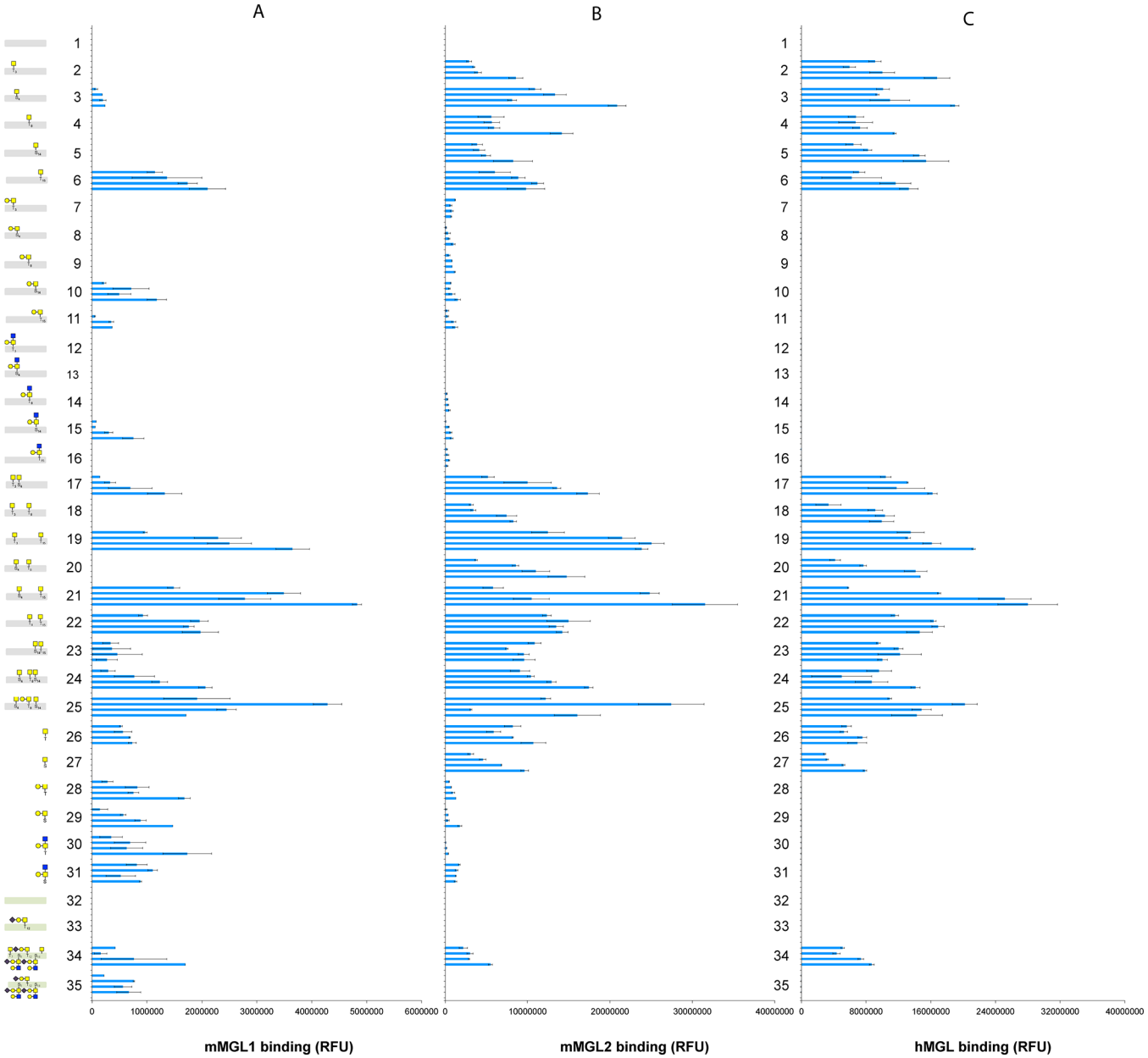


Figure 2. Glycopeptide profiling of commercially available poly-His-tagged recombinant mMGL1 (A), mMGL2 (B), and hMGL (C) with mucin-like glycopeptide by evanescent-field fluorescence microarray. Relative fluorescence units (RFU) are represented in a bar chart, in which every column represents the fluorescence average intensity with the corresponding standard deviation of four replicates per each spotted concentration (12.5, 25, 50, and 100 μM).

also demonstrated as the binding profile differs if the glycosylated amino acid is surrounded by the peptide or directly attached to the array (such as **3** vs **27** and **6** vs **26**). Interestingly, all single Thr and Ser amino acids with extended glycans are also generally recognized (**28**−**31**). In contrast, if we put the focus on the residue attached to the GalNAc (as **3** and **27** vs **6** and **26**), we suppose that particular structural features between the GalNAc moiety and the underlying Thr/Ser, such as the torsion angle of the glycosidic linkage, might affect the global conformation and thus influence mMGL1 recognition.[49,50] Analysis of the effect of multiglycosylation also provides essential information to understand the binding.[51] Our results show that the number of glycosylation positions and glycan attached site influences the binding affinity. Glycopeptides **17**−**23** contain two GalNAc moieties at different positions. To our view, there is an enhancement of the affinity of di-O-glycosylated forms displaying Tn antigen at both GVTS and GSTA regions (**19** and **21**), unlike those in which the PDTR motif is involved (**18**, **20**, and **22**). In relation to glycosylation in contiguous Ser and Thr residues, as GVTS (**17**) and GSTA (**23**) motifs, the binding is abrogated in comparison to longer distance separation glycans containing peptides (**17** and **23** vs **19** and **21**). Finally, the attachment of three or more glycans generally leads to a lower binding intensity of mMGL1 (peptides **24**, **34**, and **35**). A clear example is the comparison of peptides **19** and **34**, as the latter shows a minor intensity probably due to the steric hindrance effect generated by the presence of extended Core2 structures in the contiguous Ser residues. Interestingly, the general affinity of mMGL1 with terminal galactose units[42,43] seems to be partially

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

compensated by the lack of Tn antigen in **35**. Also, further studies may explain the observed higher intensity for **25** where a terminal galactose is included in the PDTR region (see below). In conclusion, the unique interaction profile of mMGL1 showing peptide-sequence specificity and different binding mechanisms for multiglycosylation requires further studies, such as a MUC1 library with diverse short *O*-glycan moieties on different positions.

Second, we also evaluated the carbohydrate specificity of the other murine ortholog (mMGL2) and hMGL (see Figure S3A for representative array images). Foremost, as shown in Figure 2B,C, our results confirm the previously reported preference of mMGL2 and hMGL to recognize terminal GalNAc structures (**2**−**6**, **17**−**27**, **34**).[42,52] Remarkably, unlike mMGL1, in both hMGL and mMGL2, a nonrestricted recognition MUC1 fragment has been appreciated for the O-monoglycosylated ligands (**2**−**6**). In addition, the binding interactions of mMGL2 with the extended GalNAc structures, Core1 (**7**−**11**) and Core2 (**12**−**15**), were also detected. It has been previously reported that monovalent units of Core1 and Core2 structures are also recognized by mMGL2.[43] Strikingly, our recently reported interaction analysis with MUC1 glycopeptides carrying Tn antigen in the immunodominant PDTR region has demonstrated a positive binding contribution of the amino acids adjacent to the sugar moiety to the hMGL engagement.[46] On this account, the similar binding intensities observed (**2**−**16**), in both hMGL and mMGL2, may suggest a weak and nonspecific MUC1 peptide-sequence dependence. In addition, we observed the same binding pattern of mMGL2 and hMGL for the carbohydrates directly attached to Ser and Thr amino acids (**26**−**31**), supporting a main role of the carbohydrate moiety in the recognition. Further, as observed for mMGL1, we correlate the presence of two glycan moieties with an increased binding interaction. Nonetheless, differently from the mono-glycosylated forms (**2**−**6**), in which all ligands act alike, the inclusion of a GalNAc core at both the GVTS and GSTA regions (**19** and **21**) seems to enhance recognition by hMGL and mMGL2, even more than tri-*O*-glycopeptides (**24** and **25**). Lastly, the presence of Core2 pentameric saccharide at neighboring position to GalNAc epitope (**34**) clearly disrupts the interaction of **19** with hMGL and mMGL2.

For the three MGL orthologs, there is a tendency to bind to diglycosylated peptides especially **19** and **21**. Noteworthy, both peptides have the glycan moiety on terminal positions in the sequence (**19**, **21** vs **17**, **18**, **20**, **22**, **23**), and intermediate multiclustering may affect the binding (**19**, **21** vs **24**, **25**, **34**). Future investigations may corroborate if this result correlates with the minimal distance required to accommodate two MGL lectins in one glycopeptide.

The so far unknown capacity of mMGL1 to interact with Tn-MUC1 in a sequence-specific manner prompted us to further confirm this novel binding profile. To this aim, we used the extracellular part of mMGL1 containing the CRD fused to the Fc fragment of human IgG1. The Fc fragment was selected as it can display lectins in a dimeric form (Figure S5) and can be detected by a secondary Ab with a conjugated fluorophore.[53] The resulting mMGL1-Fc chimeric protein was tested with our chemical library (Figure 3 and representative array images in Figure S3B). By using this chimeric protein, we observed a similar binding pattern to the His-tagged recombinant mMGL1, but we could get higher fluorescence average intensities and more homogeneous results. We can reconfirm the primary preference for the GSTA fragment bearing GalNAc

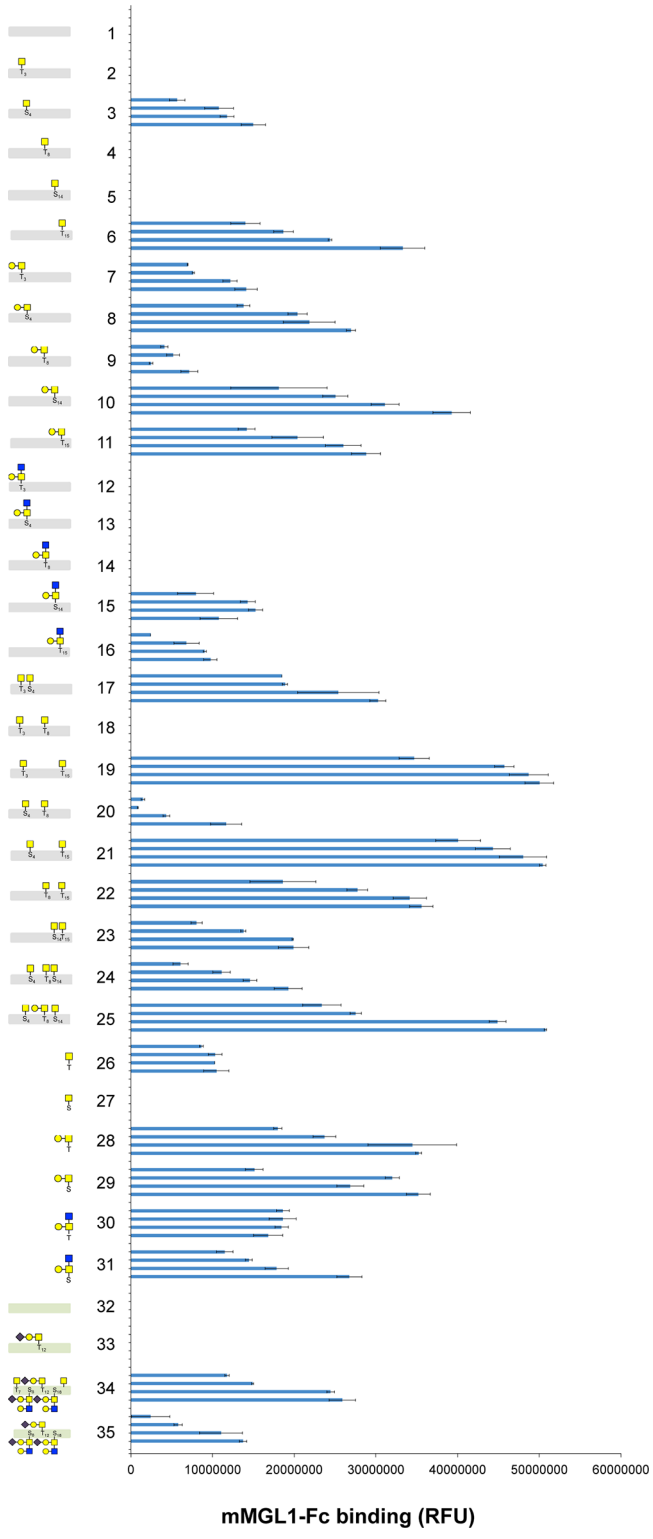


Figure 3. Glycopeptide profiling of mMGL1-Fc chimera with a mucin-like glycopeptide by evanescent-field fluorescence microarray. Relative fluorescence units (RFU) are represented in a bar chart, in which every column represents the fluorescence average intensity with the corresponding standard deviation of four replicates per each spotted concentration (12.5, 25, 50, and 100 μM).

structures as a novel mMGL1 epitope. In addition, owing to the enhanced binding avidity of the mMGL1-Fc chimera, the detection of ligands with less affinity was also possible in the array, such as monoglycosylated forms bearing Core1 and

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

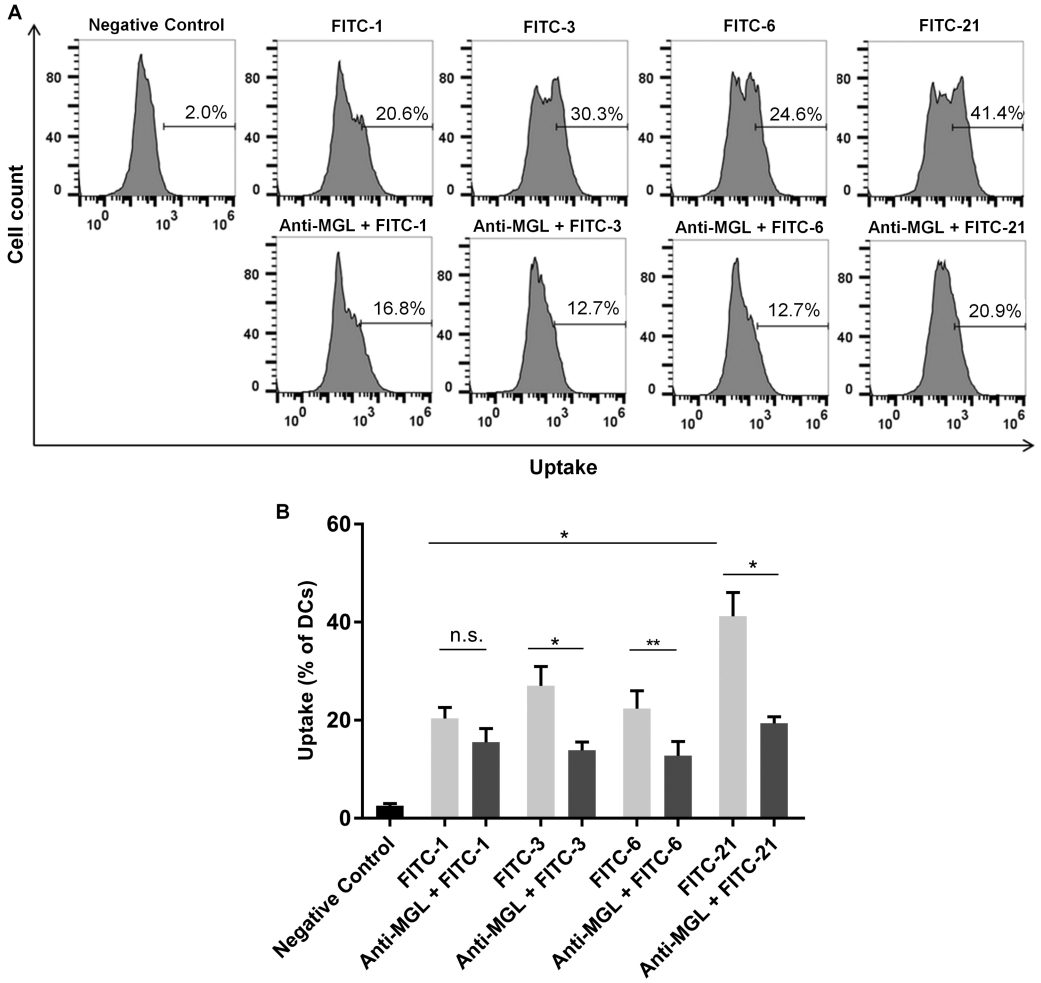


Figure 4. MGL-dependent uptake of FITC-labeled glycopeptides **1**, **3**, **6**, and **21** by murine CD11c$^+$ DCs. (A) Stained DCs (negative control) and stained DCs pulsed with the FITC-labeled glycopeptides were analyzed for internalization of the glycopeptides by flow cytometry (representative histoplots of one experiment are shown). (B) Percentage of CD11c$^+$ DCs that have internalized the glycopeptides. Data in (B) represent the summary of three independent experiments. Statistical analysis of the combined results was performed using the paired two-tailed Student's *t* test ((*) $p < 0.05$, (**) $p < 0.01$, $n = 3$).

Core2 glycans (**7**–**9** and **14** and **16**), and the diglycosylated ligand **20**. These novel findings may provide an explanation of the different binding intensities between triglycosylated peptides **24** and **25**. As this method based on mMGL1-Fc chimera was more sensitive, we could observe novel ligands as peptide **9** with Core1 at PDTR region. However, mMGL1 had no affinity for peptide **4** with GalNAc on the same site. Consequently, the higher binding of **25** in comparison to **24** might be due to the inclusion of Core1 at the PDTR region.

MGL-Dependent Uptake of Glycoconjugates into Murine DCs. It was shown that the glycan preference of murine CLRs may differ from their human orthologs.[2] However, the similar substrate selectivity of hMGL and mMGL, especially for the murine ortholog mMGL2, provides the opportunity to study glycopeptide-based MGL targeting using murine APCs.

To analyze the potential of glycopeptide-based MGL targeting, we preselected peptides from our library to be tested for uptake into murine DCs. For this purpose, we performed a larger scale synthesis of a smaller library of peptides containing fluorescein isothiocyanate (FITC) to monitor the uptake by DCs. Peptides were synthesized as previously described including β-alanine and 5,6-carboxyfluorescein at the N-terminus. A spacer between FITC and peptide was included to avoid the removal of the last amino acid during peptide–resin cleavage on acidic conditions.[54] To confirm the dependence of MGL for GalNAc as substrate, we used the naked MUC1 (FITC-**1**) as control. As monoglycosylated peptides we chose FITC-**3** and FITC-**6** with GalNAc on positions Ser4 and Thr15, respectively. To evaluate the influence of bivalency, we selected FITC-**21** with both Ser4 and Thr5 glycosylated. This glycopeptide manifested high affinity for murine MGL1/2 and human MGL.

While a single GalNAc residue at position Ser4 (FITC-**3**) or at position Thr15 (FITC-**6**) did not significantly increase the uptake and internalization by differentiated bone-marrow-derived DCs (BMDCs), the FITC-**21** glycopeptide was markedly more internalized when compared to the remaining glycoconjugates (41.2% ± 2.8%, Figure 4). This finding indicates that the two GalNAc residues present at Ser4 and Thr15 of the peptide chain markedly enhanced DC uptake compared with the control peptide (20.3% ± 2.2%, Figure 4B).

To further evaluate if the uptake of the glycopeptides was dependent on MGL as indicated by the glycopeptide arrays, an anti-MGL blocking antibody was used. It is noteworthy that the selected anti-MGL blocking antibody was previously described

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012–9021

to block both murine orthologs of the human MGL, mMGL-1, and mMGL-2.[55,56] In the case of the nonglycosylated peptide FITC-**1**, some background uptake by DCs was observed but this unspecific endocytosis was MGL-independent as MGL blockade had no effect on internalization. Interestingly, a pronounced inhibitory effect of the MGL blockade on the internalization of FITC-**21** by BMDCs was observed (19.4% ± 2.8%, Figure 4B), which was even reduced to background level of uptake in the MUC1 control peptide. Moreover, the inhibitory effect of the MGL blockade was also visible after incubation with FITC-**3** and FITC-**6**, suggesting also MGL-dependent uptake of these FITC-conjugated peptides carrying one GalNAc residue. Thus, MGL-dependent recognition and DC uptake of the mucin-derived glycopeptides were shown, highlighting the utility of the rationally designed glycopeptides to target DCs through MGL.

MGL engagement by glycans present in MUC1 tumor antigens was shown to activate extracellular signal-regulated kinases 1 and 2 (ERK1/2) and nuclear factor κB (NF-κB) pathways, leading to DCs maturation and induction of $CD8^+$ T cell responses.[57] Clearly, a rational design of synthetic antitumor vaccines, particularly of multicomponent vaccines, is of crucial importance. In these vaccines, common denominators can be found: a glycosylated MUC1 tandem repeat, further T cell epitopes and immune stimulating epitopes (often Toll-like receptor agonists).[26] The work presented in this study highlights the critical role of the carbohydrate position in MUC1-derived glycopeptides for MGL binding and MGL-dependent uptake into DCs. This highly structure-dependent-manner of MGL targeting can address simultaneously two common denominators of multicomponent vaccines: the tumor antigen and an immune stimulating epitope, due to the role of MGL in antitumor immune responses. Therefore, the identified glycopeptides could be considered as building blocks toward rationally designed anticancer vaccines.

CONCLUSIONS

In the present work, we applied microarray technologies to analyze the interaction of murine and human orthologs of the CLR MGL with tumor-related carbohydrate structures present in mucin-like glycopeptides. Specifically, using our microarray platform based on the evanescent-field fluorescence principle, we were able to monitor, under equilibrium conditions, the similar recognition profile of human MGL and murine mMGL2 with natural mimetic MUC1 glycoforms. The structure−activity relationship showed for the first time the peptide sequence-specific recognition of mMGL1 to GalNAc moieties expressed in tumor-altered mucins. Therefore, the sugar site is important for MUC1-mMGL1 binding. In addition, we have confirmed the multivalency effect to recognize different O-glycosylated states at different positions, along with the peptide backbone contribution. To our knowledge, the overall specificity for tumor-related glycopeptides observed herein is in accordance with the well-known capacity of MGL to act as an endocytic receptor of tumor-related antigens. Thus, we are confident that the results of this study including the affinity of mMGL2 with MUC1-related T and Core2 structures, or the peptide-sequence dependence of mMGL1 might facilitate a better understanding of the specific functions of MGL in murine cancer. Finally, the MGL-dependent uptake of MUC1-derived glycopeptides into murine DCs highlights the potential of MGL-based DC targeting which may be used to design novel anticancer vaccines with an enhanced effectiveness.

EXPERIMENTAL SECTION

Materials. Poly-His-tagged recombinant MGL proteins (Clec10A or CD301, CD301a and CD301b) were purchased from R&D Systems, Inc. and were provided at 50 μg lyophilized from a 0.2 μm filtered solution in PBS. CD301a/b and CD301 were reconstituted at 100 μg/mL and 200 μg/mL in sterile PBS, respectively. The specified buffer used in the present study for microarray assay is a solution of TSM buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) containing 0.05% (v/v) Tween-20 and 1% (w/v) BSA. Chemicals for buffer preparation and albumin from bovine serum (Cohn fraction V, pH 7.0, 96%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 5,6-Carboxyfluorescein was purchased from Sigma-Aldrich Co. LLC (Milwaukee, USA). DY550-tagged 6-His epitope antibody was acquired from Novus Biologicals, LLC. Cy3-labeled streptavidin was obtained from Life Technologies (Thermo Fisher Scientific Corporation). Other solvents and chemicals used in this study were supplied by Wako Pure Chemical Industries (Osaka, Japan), Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan), and Sigma-Aldrich Co. LLC (Milwaukee, USA) and were of the highest purity available. Microarray slides (75 mm × 25 mm × 1 mm) were supplied by Sumitomo Bakelite Co., Ltd. (Tokyo, Japan). Micro cover glasses (18 mm × 18 mm) were purchased from Matsunami Glass Ind., Ltd. (Osaka, Japan). Silicon rubber sheets (60 mm × 24 mm × 0.1 mm) were purchased from Fuso Rubber Co., Ltd. (Hiroshima, Japan). Fluorescence images of microarray slides were measured on GlycoStation reader 1200 (GlycoTechnica Ltd., Yokohama, Japan) at 10 μm resolution on a Typhoon Trio plus variable mode imager (GE Healthcare) with a green laser (532 nm) and a 580 BP 30 filter at a PMT voltage of 600 V and normal sensitivity. The digital images of fluorescence responses were analyzed using ArrayVision software version 8.0 (GE Healthcare) The median value of relative fluorescence intensity was used; spot intensities were determined by subtracting the average pixel intensity from the median pixel intensity of the local background within the spots. Fluorescence of each spot is shown as the average of four replicate spots used to construct histograms showing the lectin-binding profile. Error bars are included showing the standard deviation for each (glyco)peptide−lectin interaction.

Preparation of MUC1 (Glyco)peptide Library. Synthesis was carried out by solid-phase peptide synthesis and incorporating protected Fmoc-Ser/Thr(glycan)-OH at the glycosylation site. All compounds were synthesized manually following published protocols.[39] For the microarray assay, a polyethylene glycol linker and oxohexanoic acid were incorporated at the N-terminus of the glycopeptides[58] (see also Table S2 and Figure S1). For the DC uptake assays, FITC-(glyco)peptides were synthesized following the same protocol and including a β-Ala and 5,6 carboxyfluorescein on N-terminal to monitor the internalization. All compounds were purified by preparative RP-HPLC and characterized by RP-HPLC or RP-UPLC and HR-MS. All compounds used in this work exist in a ≥95% purity (see details in Supporting Information)

Fabrication of MUC1 Glycopeptide Microarray. Following our reported protocol,[27] microarray AO/PC-copolymer slides were first deprotected by 2 N HCl treatment overnight at rt and rinsed with Milli-Q H_2O. Next, compounds **1**−**35** were robotically printed in quadruplicate at four concentrations (12.5, 25, 50, and 100 μM) in 25 mM AcOH-Pyr (pH 5.0), 0.0025% (w/v) Triton X-100 (see also Figure S2). To complete the oxime bond formation, printed slides were incubated for 1 h at 80 °C. Then, the remaining free aminoxy groups were capped by treating the slides with an aqueous solution of succinic anhydride (10 mg/mL) for 4 h at room temperature. Subsequently, slides were rinsed with Milli-Q H_2O and dried by centrifugation before use.

MGL Binding Assay. Printed slides were interrogated with recombinant tagged MGL proteins and MGL-Fc chimera using a microarray technology based on the evanescent-field fluorescence-

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

assisted detection principle.[35] First, a silicone rubber sheet with three chambers was attached to the slide surface. Then, slides were pretreated with TMS buffer containing 0.05% (v/v) Tween-20 for 5 min and dried by centrifugation. Subsequently, a cover glass was set in each well, and 30 μL of a premixed solution of His-tagged MGL (12.8 μg/mL) and anti-His-mIgG-DY550 (32 μg/mL) in TMS buffer containing 0.05% (v/v) Tween-20 and 1% (w/v) BSA was infused through the interstice of slide and cover.

After 2 h of incubation at rt in a humidified chamber, the slides were directly scanned and the fluorescence intensities were measured with a GlycoStation system (GlycoTechnica Ltd., Japan). Next, to analyze whether additional binding motifs could be detected at higher GBP concentrations, the solution was carefully removed and replaced by the premixed solution. As before, the slides were directly scanned without additional washing steps. In the case of mMGL1-Fc chimera analysis, no changes in binding were observed for successive slide incubations with a premixed solution of mMGL1-Fc (6.94 μg/mL) and anti-hIgG-Cy3 (8 μg/mL) (data not shown).

Fluorescence intensities were measured with a GlycoStation system and analyzed with the ArrayVision software. The net intensity value of each spot was obtained from the background value subtraction, and the average relative fluorescence unit (RFU) was plotted as a histogram by using Microsoft Excel software. From high and low RFU value, the MGL specificity was identified, the error bars being the standard deviation.

Mice. C57BL/6 mice (Charles River) were housed in the animal facility of the University of Veterinary Medicine Hannover under controlled temperature and humidity and pathogen-free conditions. Food and water were provided ad libitum. DCs in all experiments were generated from bone marrow of C57BL/6 mice.

Cell Culture. Bone marrow cells were isolated from tibia and femur of C57BL/6 mice by flushing the bones with complete IMDM medium (supplemented with 2 mM L-glutamine, 100U/mL of penicillin, 100 μg/mL of streptomycin, 10% fetal calf serum (PAN Biotech)). The collected cell suspension was kept cold, filtered through a 40 μm cell strainer, and centrifuged (300*g*, 5 min, 4 °C). After centrifugation, the cell pellet was suspended in erythrocyte lysis buffer (10 mM Tris, pH 7.5, and 144 mM NH_4Cl) and incubated at rt for 5 min. Cells were then washed twice in medium and stored at 1×10^8 cells/mL in 90% FCS with 10% DMSO at −150 °C. The stocks of bone marrow cells from C57BL/6 mice were cultured in a T75 cell culture flask with complete IMDM medium with 5% supernatant of GM-CSF producing X63 cells. Medium was exchanged every 48 h, and cells were cultured 10 days to ascertain differentiation into $CD11c^+$ DCs.

Uptake of FITC-Labeled Glycoconjugates by DCs. DCs were washed two times with IMDM medium (supplemented with 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin) and seeded in a 96-well round-bottom plate (5×10^5 cells/well in 100 μL). In some experiments, an anti-mouse MGL blocking antibody (clone ER-MP23, Bio Rad) was added at a concentration of 50 μg/mL to assess MGL-dependent uptake of the glycoconjugates. After incubation of the DCs for 1 h (37 °C, 5% CO_2), cells were pulsed for 10 min with FITC-**1**, FITC-**3**, FITC-**6**, and FITC-**21** at a concentration of 10 μg/mL. A negative control with nonpulsed DCs was also included. DCs were then washed two times with cold PBS and suspended in 100 μL of FACS buffer (PBS, 1% FCS, 1 mM EDTA) containing anti-CD16/32 (Fc-blocking antibody, dilution 1:100, clone 93, eBioscience) at 4 °C for 15 min. Afterward, cells were suspended in FACS buffer with APC-conjugated anti-CD11c antibody (1:200 dilution, clone N418, eBioscience) and incubated for 30 min at 4 °C in the dark. The cells were then washed two times with 200 μL of FACS buffer and further suspended in 200 μL of FACS buffer. Flow cytometry measurements were performed by placing the 96-well round-bottom plate in the autosampler of the Attune NxT flow cytometer (Thermo Fisher Scientific). Flow cytometry data were analyzed using the FlowJo version 10 software (Tree Star). In all flow cytometry assays, the same gating strategy was performed for all experimental conditions within one experiment.

Statistical Analysis. Statistical analysis was performed using the GraphPad Prism 7 software (GraphPad, La Jolla, CA). Combined data of all experiments were analyzed using the two-tailed paired Student's *t* test ($p < 0.05$ was considered statistically significant).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01242.

Additional tables, figures, and characterization data of new compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*B.L.: phone, +49 511 953 6135; e-mail, bernd.lepenies@tiho-hannover.de.

*F.G.-M.: phone, +81 80 706 9033; e-mail, faynagm@sci.hokudai.ac.jp.

ORCID

Shin-Ichiro Nishimura: 0000-0002-6608-8418

Fayna Garcia-Martin: 0000-0001-9118-3874

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Tomoko Nakayama for her great assistance on the synthesis and characterization of FITC-(glyco)peptides. This work was partly supported by a grant for "Innovation COE Project for Future Medicine and Medical Research" from the Ministry of Education, Culture, Science, and Technology, Japan, JSPS KAKENHI Grant 25220206 and JSPS Wakate B KAKENHI Grant 24710242. F.G.-M. acknowledges the Special Coordination Fund for Promotion of Science and Technology, through the Ministry of Education, Culture, Sports, Science & Technology's project for accelerated innovation of the fostering system for female scientists. G.A. acknowledges Canon Foundation in Europe for the postdoctoral fellowship between 2014 and 2015. J.T.M. and B.L. acknowledge funding from the European Union's Horizon 2020 research and innovation program (Marie Sklodowska-Curie Grant Agreement 642870, ETN-Immunoshape). Previously, funding from the Collaborative Research Center (SFB) 765 was crucial for the research program of B.L.

■ ABBREVIATIONS USED

Ab, antibody; ACN, acetonitrile; APC, antigen presenting cell; CLR, myeloid C-type lectin receptor; Core2, Galβ(1→3)[GlcNAcβ(1→6)]GalNAcα1→; CRD, carbohydrate recognition domain; DC, dendritic cell; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; Gal, galactose; GBP, glycan binding protein; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption/ionization; MGL, macrophage galactose lectin; MUC, mucin; MS, mass spectrometry; PEG, polyethylene glycol; PRR, pattern recognition receptor; RFU, relative fluorescence units; RP-HPLC, reversed-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis; sT, Neu5Acα(2→3)Galβ-(1→3)GalNAcα1→; T antigen or Core1, Gal β(1→3)GalNAc α1→; TFA, trifluoroacetic acid; Tn antigen, GalNAcα1→; TOF, time-of-flight

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

REFERENCES

(1) Varki, A.; Cummings, R. D.; Esko, J. D.; Freeze, H. H.; Stanley, P.; Bertozzi, C. R.; Hart, G. W.; Etzler, M. E. *Essentials of Glycobiology*, 2nd ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 2009.

(2) Rabinovich, G. A.; van Kooyk, Y.; Cobb, B. A. Glycobiology of Immune Responses. *Ann. N. Y. Acad. Sci.* **2012**, *1253* (1), 1−15.

(3) Pinho, S. S.; Reis, C. A. Glycosylation in Cancer: Mechanisms and Clinical Implications. *Nat. Rev. Cancer* **2015**, *15* (9), 540−555.

(4) Mayer, S.; Raulf, M.-K.; Lepenies, B. C-Type Lectins: Their Network and Roles in Pathogen Recognition and Immunity. *Histochem. Cell Biol.* **2017**, *147* (2), 223−237.

(5) Weis, W. I.; Taylor, M. E.; Drickamer, K. The C-Type Lectin Superfamily in the Immune System. *Immunol. Rev.* **1998**, *163*, 19−34.

(6) Cummings, R. D.; Pierce, J. M. The Challenge and Promise of Glycomics. *Chem. Biol.* **2014**, *21* (1), 1−15.

(7) Lepenies, B.; Lee, J.; Sonkaria, S. Targeting C-Type Lectin Receptors with Multivalent Carbohydrate Ligands. *Adv. Drug Delivery Rev.* **2013**, *65*, 1271−1281.

(8) Monzavi-Karbassi, B.; Pashov, A.; Kieber-Emmons, T. Tumor-Associated Glycans and Immune Surveillance. *Vaccines (Basel, Switz.)* **2013**, *1* (2), 174−203.

(9) Geijtenbeek, T. B. H.; Gringhuis, S. I. C-Type Lectin Receptors in the Control of T Helper Cell Differentiation. *Nat. Rev. Immunol.* **2016**, *16*, 433−448.

(10) van Vliet, S. J.; García-Vallejo, J. J.; van Kooyk, Y. Dendritic Cells and C-Type Lectin Receptors: Coupling Innate to Adaptive Immune Responses. *Immunol. Cell Biol.* **2008**, *86* (7), 580−587.

(11) Johannssen, T.; Lepenies, B. Glycan-Based Cell Targeting to Modulate Immune Responses. *Trends Biotechnol.* **2017**, *35* (4), 334−346.

(12) van Vliet, S. J.; Saeland, E.; van Kooyk, Y. Sweet Preferences of MGL: Carbohydrate Specificity and Function. *Trends Immunol.* **2008**, *29*, 83−90.

(13) Gibadullin, R.; Farnsworth, D. W.; Barchi, J. J.; Gildersleeve, J. C. GalNAc-Tyrosine Is a Ligand of Plant Lectins, Antibodies, and Human and Murine Macrophage Galactose-Type Lectins. *ACS Chem. Biol.* **2017**, *12* (8), 2172−2182.

(14) van Kooyk, Y.; Ilarregui, J. M.; van Vliet, S. J. Novel Insights Into the Immunomodulatory Role of the Dendritic Cell and Macrophage-Expressed C-Type Lectin MGL. *Immunobiology* **2015**, *220* (2), 185−192.

(15) van Vliet, S. J.; Paessens, L. C.; Broks-van den Berg, V. C. M.; Geijtenbeek, T. B. H.; van Kooyk, Y. The C-Type Lectin Macrophage Galactose-Type Lectin Impedes Migration of Immature APCs. *J. Immunol.* **2008**, *181* (5), 3148−3155.

(16) van Sorge, N. M.; Bleumink, N. M. C.; van Vliet, S. J.; Saeland, E.; van der Pol, W.-L.; van Kooyk, Y.; van Putten, J. P. M. N-Glycosylated Proteins and Distinct Lipooligosaccharide Glycoforms of Campylobacter Jejuni Target the Human C-Type Lectin Receptor MGL. *Cell. Microbiol.* **2009**, *11* (12), 1768−1781.

(17) Napoletano, C.; Rughetti, A.; Agervig Tarp, M. P.; Coleman, J.; Bennett, E. P.; Picco, G.; Sale, P.; Denda-Nagai, K.; Irimura, T.; Mandel, U.; Clausen, H.; Frati, L.; Taylor-Papadimitriou, J.; Burchell, J.; Nuti, M. Tumor-Associated Tn-MUC1 Glycoform Is Internalized Through the Macrophage Galactose-Type C-Type Lectin and Delivered to the HLA Class I and II Compartments in Dendritic Cells. *Cancer Res.* **2007**, *67* (17), 8358−8367.

(18) Saeland, E.; van Vliet, S. J.; Bäckström, M.; van den Berg, V. C. M.; Geijtenbeek, T. B. H.; Meijer, G. A.; van Kooyk, Y. The C-Type Lectin MGL Expressed by Dendritic Cells Detects Glycan Changes on MUC1 in Colon Carcinoma. *Cancer Immunol. Immunother.* **2007**, *56* (8), 1225−1236.

(19) Serruto, D.; Rappuoli, R. Post-Genomic Vaccine Development. *FEBS Lett.* **2006**, *580* (12), 2985−2992.

(20) van der Bruggen, P.; Van den Eynde, B. J. Processing and Presentation of Tumor Antigens and Vaccination Strategies. *Curr. Opin. Immunol.* **2006**, *18*, 98−104.

(21) Fuster, M. M.; Esko, J. D. The Sweet and Sour of Cancer: Glycans as Novel Therapeutic Targets. *Nat. Rev. Cancer* **2005**, *5* (7), 526−542.

(22) Kufe, D. W. Mucins in Cancer: Function, Prognosis and Therapy. *Nat. Rev. Cancer* **2009**, *9* (12), 874−885.

(23) Hang, H. C.; Bertozzi, C. R. The Chemistry and Biology of Mucin-Type O-Linked Glycosylation. *Bioorg. Med. Chem.* **2005**, *13* (17), 5021−5034.

(24) Kudelka, M. R.; Ju, T.; Heimburg-Molinaro, J.; Cummings, R. D. Simple Sugars to Complex Disease—Mucin-Type O-Glycans in Cancer. *Adv. Cancer Res.* **2015**, *126*, 53−135.

(25) Rillahan, C. D.; Paulson, J. C. Glycan Microarrays for Decoding the Glycome. *Annu. Rev. Biochem.* **2011**, *80* (1), 797−823.

(26) Gaidzik, N.; Westerlind, U.; Kunz, H. The Development of Synthetic Antitumour Vaccines From Mucin Glycopeptide Antigens. *Chem. Soc. Rev.* **2013**, *42* (10), 4421−4442.

(27) Matsushita, T.; Takada, W.; Igarashi, K.; Naruchi, K.; Miyoshi, R.; García-Martín, F.; Amano, M.; Hinou, H.; Nishimura, S.-I. A Straightforward Protocol for the Preparation of High Performance Microarray Displaying Synthetic MUC1 Glycopeptides. *Biochim. Biophys. Acta, Gen. Subj.* **2014**, *1840* (3), 1105−1116.

(28) Coelho, H.; Matsushita, T.; Artigas, G.; Hinou, H.; Cañada, F. J.; Lo-Man, R.; Leclerc, C.; Cabrita, E. J.; Jimenez-Barbero, J.; Nishimura, S.-I.; García-Martín, F.; Marcelo, F. The Quest for Anticancer Vaccines: Deciphering the Fine-Epitope Specificity of Cancer-Related Monoclonal Antibodies by Combining Microarray Screening and Saturation Transfer Difference NMR. *J. Am. Chem. Soc.* **2015**, *137* (39), 12438−12441.

(29) Rangappa, S.; Artigas, G.; Miyoshi, R.; Yokoi, Y.; Hayakawa, S.; García-Martín, F.; Hinou, H.; Nishimura, S.-I. Effects of the Multiple O-Glycosylation States on Antibody Recognition of the Immunodominant Motif in MUC1 Extracellular Tandem Repeats. *MedChemComm* **2016**, *7*, 1102−1122.

(30) Artigas, G.; Hinou, H.; García-Martín, F.; Gabius, H.-J.; Nishimura, S.-I. Synthetic Mucin-Like Glycopeptides as Versatile Tools to Measure Effects of Glycan Structure/Density/Position on the Interaction with Adhesion/Growth-Regulatory Galectins in Arrays. *Chem. - Asian J.* **2017**, *12* (1), 159−167.

(31) Oyelaran, O.; Gildersleeve, J. C. Glycan Arrays: Recent Advances and Future Challenges. *Curr. Opin. Chem. Biol.* **2009**, *13* (4), 406−413.

(32) Blixt, O.; Head, S.; Mondala, T.; Scanlan, C.; Huflejt, M. E.; Alvarez, R.; Bryan, M. C.; Fazio, F.; Calarese, D.; Stevens, J.; Razi, N.; Stevens, D. J.; Skehel, J. J.; van Die, I.; Burton, D. R.; Wilson, I. A.; Cummings, R.; Bovin, N.; Wong, C.-H.; Paulson, J. C. Printed Covalent Glycan Array for Ligand Profiling of Diverse Glycan Binding Proteins. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101* (49), 17033−17038.

(33) Smith, D. F.; Song, X.; Cummings, R. D. Use of Glycan Microarrays to Explore Specificity of Glycan-Binding Proteins. *Methods Enzymol.* **2010**, *480*, 417−444.

(34) Hirabayashi, J.; Yamada, M.; Kuno, A.; Tateno, H. Lectin Microarrays: Concept, Principle and Applications. *Chem. Soc. Rev.* **2013**, *42* (10), 4443−4458.

(35) Kuno, A.; Uchiyama, N.; Koseki Kuno, S.; Ebe, Y.; Takashima, S.; Yamada, M.; Hirabayashi, J. Evanescent-Field Fluorescence-Assisted Lectin Microarray: a New Strategy for Glycan Profiling. *Nat. Methods* **2005**, *2* (11), 851−856.

(36) Blixt, O.; Cló, E.; Nudelman, A. S.; Sørensen, K. K.; Clausen, T.; Wandall, H. H.; Livingston, P. O.; Clausen, H.; Jensen, K. J. A High-Throughput O-Glycopeptide Discovery Platform for Seromic Profiling. *J. Proteome Res.* **2010**, *9* (10), 5250−5261.

(37) Westerlind, U.; Schröder, H.; Hobel, A.; Gaidzik, N.; Kaiser, A.; Niemeyer, C. M.; Schmitt, E.; Waldmann, H.; Kunz, H. Tumor-Associated MUC1 Tandem-Repeat Glycopeptide Microarrays to Evaluate Serum- and Monoclonal-Antibody Specificity. *Angew. Chem., Int. Ed.* **2009**, *48* (44), 8263−8267.

(38) Matsushita, T.; Hinou, H.; Fumoto, M.; Kurogochi, M.; Fujitani, N.; Shimizu, H.; Nishimura, S.-I. Construction of Highly Glycosylated Mucin-Type Glycopeptides Based on Microwave-Assisted Solid-Phase

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021

Syntheses and Enzymatic Modifications. *J. Org. Chem.* **2006**, *71* (8), 3051−3063.
(39) García-Martín, F.; Hinou, H.; Matsushita, T.; Hayakawa, S.; Nishimura, S.-I. An Efficient Protocol for the Solid-Phase Synthesis of Glycopeptides Under Microwave Irradiation. *Org. Biomol. Chem.* **2012**, *10*, 1612−1617.
(40) Fumoto, M.; Hinou, H.; Ohta, T.; Ito, T.; Yamada, K.; Takimoto, A.; Kondo, H.; Shimizu, H.; Inazu, T.; Nakahara, Y.; Nishimura, S.-I. Combinatorial Synthesis of MUC1 Glycopeptides: Polymer Blotting Facilitates Chemical and Enzymatic Synthesis of Highly Complicated Mucin Glycopeptides. *J. Am. Chem. Soc.* **2005**, *127* (33), 11804−11818.
(41) Nishimura, S.-I.; Niikura, K.; Kurogochi, M.; Matsushita, T.; Fumoto, M.; Hinou, H.; Kamitani, R.; Nakagawa, H.; Deguchi, K.; Miura, N.; Monde, K.; Kondo, H. High-Throughput Protein Glycomics: Combined Use of Chemoselective Glycoblotting and MALDI-TOF/TOF Mass Spectrometry. *Angew. Chem., Int. Ed.* **2005**, *44* (1), 91−96.
(42) Tsuiji, M.; Fujimori, M.; Ohashi, Y.; Higashi, N.; Onami, T. M.; Hedrick, S. M.; Irimura, T. Molecular Cloning and Characterization of a Novel Mouse Macrophage C-Type Lectin, mMGL2, Which Has a Distinct Carbohydrate Specificity From mMGL1. *J. Biol. Chem.* **2002**, *277* (32), 28892−28901.
(43) Singh, S. K.; Streng-Ouwehand, I.; Litjens, M.; Weelij, D. R.; García-Vallejo, J. J.; van Vliet, S. J.; Saeland, E.; van Kooyk, Y. Characterization of Murine MGL1 and MGL2 C-Type Lectins: Distinct Glycan Specificities and Tumor Binding Properties. *Mol. Immunol.* **2009**, *46* (6), 1240−1249.
(44) Schol, D. J.; Meulenbroek, M. F.; Snijdewint, F. G.; vonMensdorff-Pouilly, S.; Verstraeten, R. A.; Murakami, F.; Kenemans, P.; Hilgers, J. "Epitope Fingerprinting" Using Overlapping 20-Mer Peptides of the MUC1 Tandem Repeat Sequence. *Tumor Biol.* **1998**, *19* (Suppl. 1), 35−45.
(45) Jégouzo, S. A. F.; Quintero-Martínez, A.; Ouyang, X.; dos Santos, Á.; Taylor, M. E.; Drickamer, K. Organization of the Extracellular Portion of the Macrophage Galactose Receptor: a Trimeric Cluster of Simple Binding Sites for N-Acetylgalactosamine. *Glycobiology* **2013**, *23* (7), 853−864.
(46) Marcelo, F.; García-Martín, F.; Matsushita, T.; Sardinha, J.; Coelho, H.; Oude-Vrielink, A.; Koller, C.; André, S.; Cabrita, E. J.; Gabius, H.-J.; Nishimura, S.-I.; Jimenez-Barbero, J.; Cañada, F. J. Delineating Binding Modes of Gal/GalNAc and Structural Elements of the Molecular Recognition of Tumor-Associated Mucin Glycopeptides by the Human Macrophage Galactose-Type Lectin. *Chem. - Eur. J.* **2014**, *20* (49), 16147−16155.
(47) Madariaga, D.; Martínez-Sáez, N.; Somovilla, V. J.; García-García, L.; Berbis, M. Á.; Valero-Gonzalez, J.; Martín-Santamaría, S.; Hurtado-Guerrero, R.; Asensio, J. L.; Jimenez-Barbero, J.; Avenoza, A.; Busto, J. H.; Corzana, F.; Peregrina, J. M. Serine Versus Threonine Glycosylation with A-O-GalNAc: Unexpected Selectivity in Their Molecular Recognition with Lectins. *Chem. - Eur. J.* **2014**, *20* (39), 12616−12627.
(48) Madariaga, D.; Martínez-Sáez, N.; Somovilla, V. J.; Coelho, H.; Valero-Gonzalez, J.; Castro-López, J.; Asensio, J. L.; Jimenez-Barbero, J.; Busto, J. H.; Avenoza, A.; Marcelo, F.; Hurtado-Guerrero, R.; Corzana, F.; Peregrina, J. M. Detection of Tumor-Associated Glycopeptides by Lectins: the Peptide Context Modulates Carbohydrate Recognition. *ACS Chem. Biol.* **2015**, *10* (3), 747−756.
(49) Corzana, F.; Busto, J. H.; Jiménez-Osés, G.; Asensio, J. L.; Jimenez-Barbero, J.; Peregrina, J. M.; Avenoza, A. New Insights Into Alpha-GalNAc-Ser Motif: Influence of Hydrogen Bonding Versus Solvent Interactions on the Preferred Conformation. *J. Am. Chem. Soc.* **2006**, *128* (45), 14640−14648.
(50) Corzana, F.; Busto, J. H.; Jiménez-Osés, G.; García de Luis, M.; Asensio, J. L.; Jimenez-Barbero, J.; Peregrina, J. M.; Avenoza, A. Serine Versus Threonine Glycosylation: the Methyl Group Causes a Drastic Alteration on the Carbohydrate Orientation and on the Surrounding Water Shell. *J. Am. Chem. Soc.* **2007**, *129* (30), 9458−9467.
(51) Godula, K.; Bertozzi, C. R. Density Variant Glycan Microarray for Evaluating Cross-Linking of Mucin-Like Glycoconjugates by Lectins. *J. Am. Chem. Soc.* **2012**, *134* (38), 15732−15742.
(52) van Vliet, S. J.; van Liempt, E.; Saeland, E.; Aarnoudse, C. A.; Appelmelk, B.; Irimura, T.; Geijtenbeek, T. B. H.; Blixt, O.; Alvarez, R.; van Die, I.; van Kooyk, Y. Carbohydrate Profiling Reveals a Distinctive Role for the C-Type Lectin MGL in the Recognition of Helminth Parasites and Tumor Antigens by Dendritic Cells. *Int. Immunol.* **2005**, *17* (5), 661−669.
(53) Maglinao, M.; Eriksson, M.; Schlegel, M. K.; Zimmermann, S.; Johannssen, T.; Götze, S.; Seeberger, P. H.; Lepenies, B. A Platform to Screen for C-Type Lectin Receptor-Binding Carbohydrates and Their Potential for Cell-Specific Targeting and Immune Modulation. *J. Controlled Release* **2014**, *175*, 36−42.
(54) Jullian, M.; Hernandez, A.; Maurras, A.; Puget, K.; Amblard, M.; Martinez, J.; Subra, G. N-Terminus FITC Labeling of Peptides on Solid Support: the Truth Behind the Spacer. *Tetrahedron Lett.* **2009**, *50* (3), 260−263.
(55) Dupasquier, M.; Stoitzner, P.; Wan, H.; Cerqueira, D.; van Oudenaren, A.; Voerman, J. S. A.; Denda-Nagai, K.; Irimura, T.; Raes, G.; Romani, N.; Leenen, P. J. M. The Dermal Microenvironment Induces the Expression of the Alternative Activation Marker CD301/mMGL in Mononuclear Phagocytes, Independent of IL-4/IL-13 Signaling. *J. Leukocyte Biol.* **2006**, *80* (4), 838−849.
(56) Vukman, K. V.; Ravidà, A.; Aldridge, A. M.; O'Neill, S. M. Mannose Receptor and Macrophage Galactose-Type Lectin Are Involved in Bordetella Pertussis Mast Cell Interaction. *J. Leukocyte Biol.* **2013**, *94* (3), 439−448.
(57) Napoletano, C.; Zizzari, I. G.; Rughetti, A.; Rahimi, H.; Irimura, T.; Clausen, H.; Wandall, H. H.; Belleudi, F.; Bellati, F.; Pierelli, L.; Frati, L.; Nuti, M. Targeting macrophage galactose type C-type lectin (MGL) induces DC signaling and activation. *Eur. J. Immunol.* **2012**, *42* (4), 936−945.
(58) García-Martín, F.; Matsushita, T.; Hinou, H.; Nishimura, S.-I. Fast Epitope Mapping for the Anti-MUC1Monoclonal Antibody by Combining a One-Bead-One-Glycopeptide Library and a Microarray Platform. *Chem. - Eur. J.* **2014**, *20* (48), 15891−15902.

DOI: 10.1021/acs.jmedchem.7b01242
J. Med. Chem. 2017, 60, 9012−9021