Click multivalent neoglycoconjugates as synthetic activators in cell adhesion and stimulation of monocyte/machrophage cell lines

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The efficient synthesis of fluorescent and non-fluorescent multivalent neoglycoconjugates is described by means of the Cu(I) catalyzed azide–alkyne 1,3-dipolar cycloaddition ("click-chemistry"). A well-defined glycopolymer, glycocyclodextrin or glycocluster architecture displaying galactose or lactose epitopes has been chosen. Cellular assays using U-937 and RAW 264.7 monocyte/macrophage cells showed that these glycocompounds have the capability to act as synthetic activators mimicking the lipopolysaccharide (LPS) effects. Thus, the click compounds promote cell adhesion and stimulation of monocytes, measured as an increase in the amount of TNFα, facilitating their differentiation to macrophages.

Introduction

Cell-surface oligosaccharides play a vital role in biological processes such as cell adhesion, signal transduction and regulation as well as bacterial and viral infections.[1–3] Carbohydrates mediate specific multivalent interactions with soluble or membrane proteins called lectins.[4] Artificial multivalent carbohydrate models or glycomimics[5] have been prepared by conjugation of the ligand sugar part to a scaffold to ascertain the specific interactions involved in these carbohydrate–protein bindings.[5] Nowadays, a variety of these multivalent structures are available: linear polymers or glycopolymers, liposomes, dendrimers, beads or nanoparticles.[6] The utility of these glycomimics has been widely investigated for biotechnology, pharmaceutical and medical applications.[7]

Bacterial endotoxin (lipopolysaccharide, LPS), the major molecular component of the outer membrane of Gram-negative bacteria,[8–10] activates monocytes and macrophages to produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α).[11] These cytokines contribute to the efficient control of growth and dissemination of invading pathogens. All the LPSs share a common structural principle comprising three parts: (1) a hydrophobic glycolipid (lipid A), (2) a core oligosaccharide containing 2-keto-3-deoxy-octulonic acid (KDO) and other sugars including a heptose, and (3) a hydrophilic polysaccharide composed of a chain of highly variable repeating oligosaccharide subunits (O-antigenic side chain). There are ongoing efforts to search for synthetic LPS analogues for the activation of monocytes/macrophages.[12] In order to overcome the structural heterogeneity of endotoxin and in the search for new synthetic activators of monocytes/macrophages, multivalent neoglycoconjugates possessing a well-defined architecture were sought as optimal candidates. To optimize the preparation of such compounds, an easy, versatile and efficient synthetic methodology is required and, for this aim, it was decided to use "click-chemistry"[13] for their construction.

The efficiency and versatility of this methodology has been recently evidenced by the multiple applications that have been found[14–17] in the particular case of the Cu^{I} catalyzed 1,3-dipolar cycloaddition of alkynes and azides.[18,19] In the present work, this reaction has been applied as the synthetic strategy for the formation of the glycan–scaffold link by conjugation of complementary functionalized building-blocks considering the easy preparation of alkyne–azide scaffold and sugar derivatives. Furthermore, the modularity of this reaction has allowed the fluorescent labeling of these structures, enhancing their potential use in experiments where visualization is required. Cell adhesion of monocyte/macrophage cell lines and the production of the pro-inflammatory cytokine TNF-α were selected as the experimental proofs to evaluate the capabilities of these synthetic glycocompounds as activators.

Results and discussion

Synthesis of multivalent glycoconjugates

To achieve the pursued goals, three types of synthetic multivalent glycoconjugates possessing different architectures were selected: glycopolymers (Scheme 1), glycocyclodextrins (Scheme 2) and sugar-based glycoclusters (Scheme 3) considering that the required starting materials to be used as scaffolds (polystyrene, β-cyclodextrin and glucose) are inexpensive. These materials also allow easy access to multivalent glycoconjugates with different carbohydrate densities. In the present study, Gal and Lac were the selected carbohydrates to decorate the multivalent constructs considering that different receptors with specificity for Gal epitopes have been identified in macrophages.[20–22]

The required clickable functionalized scaffolds for the click grafting of carbohydrate appendages were prepared following

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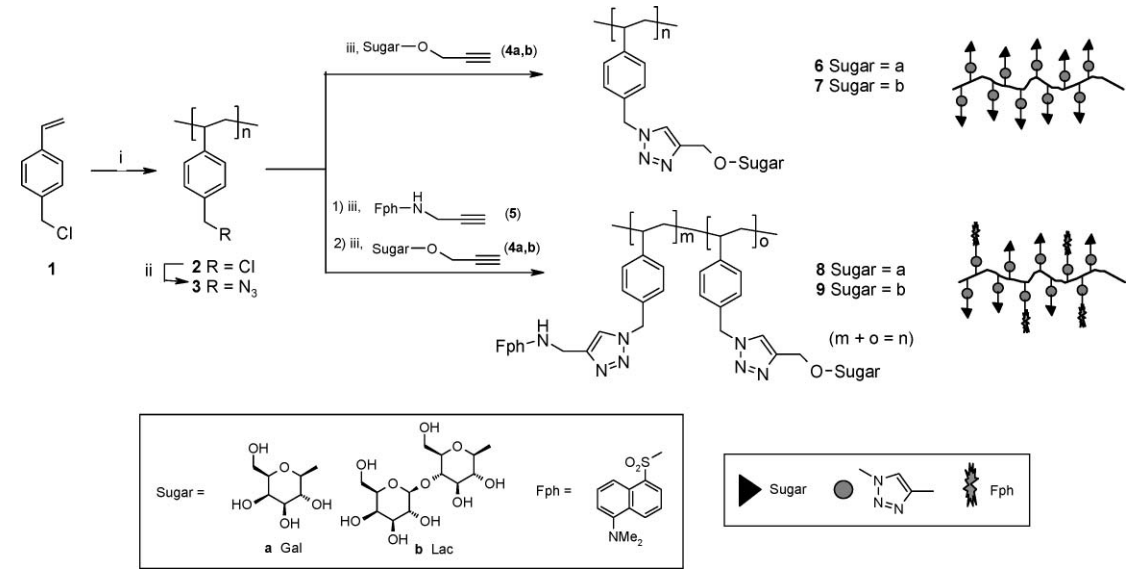
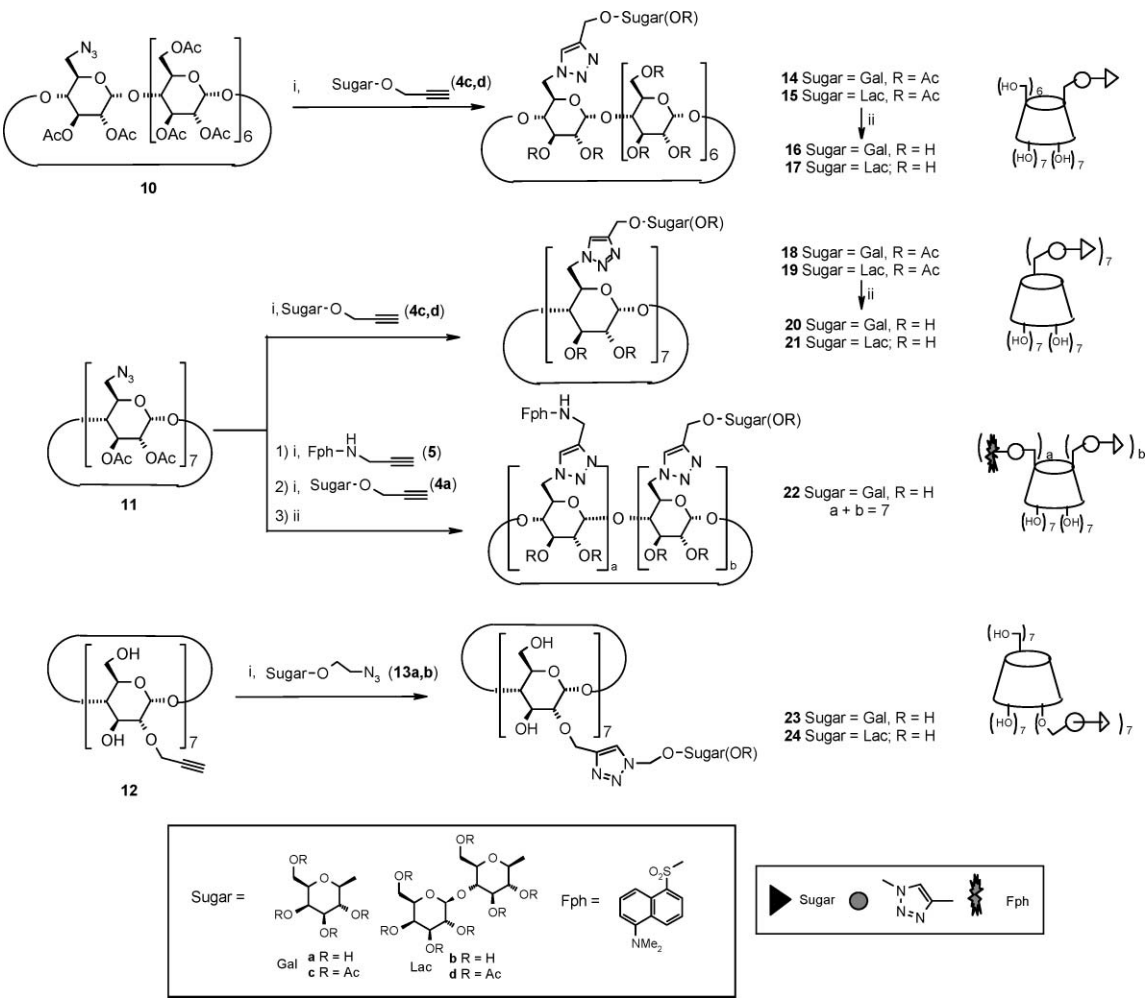
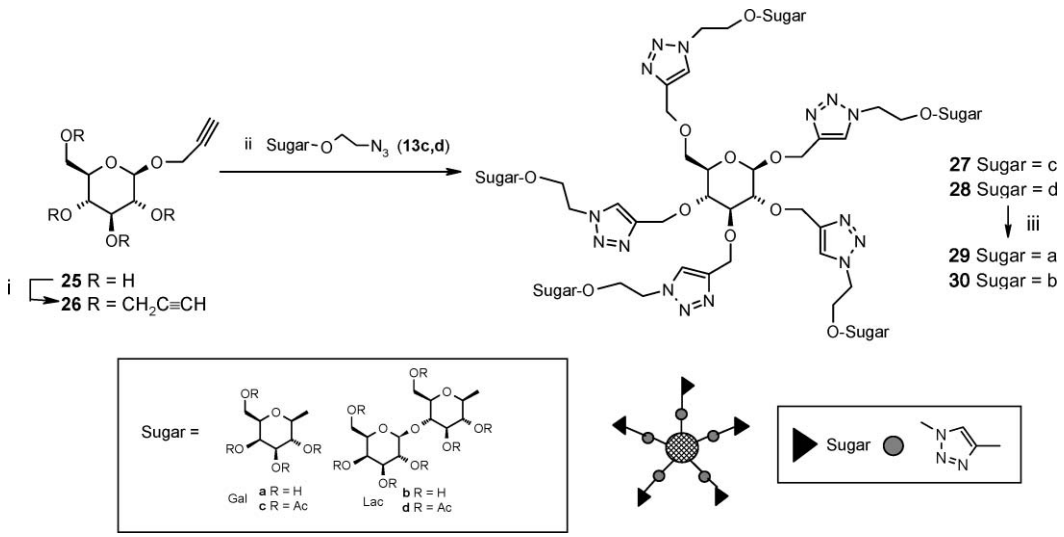


Scheme 1 Synthesis of click non-fluorescent and fluorescent multivalent glycopolymers. *Reagents and conditions*: (i) Ref. 33; (ii) NaN_3, DMF, 75 °C, 72 h; (iii) $(EtO)_3P{\cdot}CuI$, DMF, 90 °C, 15 min, MW (800 W).



Scheme 2 Synthesis of click non-fluorescent and fluorescent multivalent glycocyclodextrins. *Reagents and conditions*: (i) $(EtO)_3P{\cdot}CuI$, DMF, 90 °C, 15 min, MW (800 W); (ii) MeOH, Et_3N.



Scheme 3 Synthesis of click multivalent glycoclusters. *Reagents and conditions*: (i) NaH, $BrCH_2C{\equiv}CH$, THF (ii) $(EtO)_3P{\cdot}CuI$, toluene, reflux, 1 h; ii) MeOH, Et_3N.

already reported methods in the case of known compounds or by conventional chemistry for new compounds. Thus, for the synthesis of glycopolymers poly[*p*-(chloromethyl)styrene] **2**[23] allowed the easy introduction of the azide function by the nucleophilic substitution of the chlorine atom in the reaction with sodium azide giving the corresponding poly[*p*-(azidomethyl)styrene] **3** in high yield. In the case of glycocyclodextrins, the grafting of sugar moieties was planned to be performed at the primary face as well as at the secondary face to study the potential influence of such a difference in the topology of these compounds. The per-*O*-acetylated monoazide (**10**[24]) and heptaazide (**11**[25]) β-cyclodextrin derivatives were used as starting materials in the first case, whereas the per-2-*O*-propargyl derivative **12** was prepared to gain access to the secondary face. Finally, sugar-based glycoclusters were constructed using the per-*O*-propargylated glucose derivative **26** that was prepared by per-*O*-alkylation of propargyl β-D-glucopyranoside **25**.

The complementary functionalized glycosides derived from D-Galactose (Gal) and lactose (Lac) to be grafted to the scaffolds were easily prepared following already reported procedures. Thus, the propargyl glycosides **4a**,**b**[26] and the azidoethyl glycosides derived from Gal and Lac **13a**,**b**[27,28] were obtained starting from the corresponding per-*O*-acetylated sugars.

The click coupling reactions were performed using the conditions previously described by us: $(EtO)_3P{\cdot}CuI$ as organic-soluble catalyst, DMF as solvent and microwave irradiation or heating in refluxing toluene.[29] These conditions allowed the synthesis of the desired multivalent compounds in short reaction times and high yields (yields ranging 79–93%). To ensure the complete grafting of the "clickable" functions present in the scaffolds a 20% equivalent excess per function of the complementary monoazide or monoalkyne derivative was used.

As indicated above, fluorescent labeling of the neoglycoconjugates was also performed to enhance their utility, especially in biological assays where visualization could facilitate the quantification. The synthesis of the fluorescent multivalent counterparts was designed by the modular incorporation of the fluorophore and the sugar appendages using in both cases "click-chemistry" in order to attain the maximum synthetic economy. Conjugation of the alkynyl dansyl derivative **5**[30] was first carried out by reaction with 0.25 equiv. per azide group, under the same reaction conditions mentioned above, followed by *in situ* reaction with 1.2 equiv. per azide of the appropriate monoalkyne sugar derivatives **4a**,**b**

Previous to the development of "click-chemistry", the formation of the covalent ligand–scaffold link in multivalent neoglycoconjugates had been performed by a variety of strategies such as the formation of thiourea or amide bridge, by reaction of amines with isothiocyanates or carboxylic acids, respectively, by glycosylation or by nucleophilic substitution, as has been largely documented.[6,31] The "clicking" strategy offers advantages in relation to those methodologies since it enables the rapid and high yielding preparation of carbohydrate conjugates by using the heterocyclic triazole as a tether for the carbohydrate decoration. In addition, the flexibility of the this strategy is particularly notorious in the synthesis of the fluorescent counterparts. The sequential coupling of the alkynyl fluorophore derivative and the propargyl glycosides to the polyazide scaffolds allowed the efficient preparation of these labeled multivalent neoglycoconjugates.

All the cycloaddition compounds were easily isolated and purified and when necessary they were de-*O*-acetylated to obtain the corresponding hydroxylated derivatives to perform the biological assays.

Biological assays

To evaluate the ability of the neoglycoconjugates as synthetic activators, the adhesion of monocytes to cell culture plates was measured. The non-adherent human promonocytic U-937 cell line was selected, since it has been established as a useful model for monocyte adhesion studies.[32] U-937 cells usually grow in suspension in a medium supplemented with fetal bovine serum while after their stimulation with LPS or phorbol 12-myristate 13-acetate (PMA) and their differentiation to macrophages, they can grow as a monolayer attached to the tissue culture flask.[33]

U-937 cells were re-suspended in a serum free medium and added to wells coated with distilled water (control) or solutions containing 40 μg of each glycocompound (glycopolymers **6**, **7**, glycocyclodextrins **16**, **17**, **20**, **21**, **23** and **24**, and glycoclusters **29** and **30**). After three hours of incubation at 37 °C, plates were washed twice with phosphate buffered saline and cell adhesion was estimated by counting the attached cells per field by microscopy (Fig. 1A). All the compounds assayed have a positive effect promoting cell adhesion (cells per field values ranging from ≅ 40 to 75 *versus* 10 for control). U-937 adhesion was dose-mediated for the Lac-containing glycocyclodextrins **17** and **21** at 20 and 40 μg per well (Fig. 1B). Attached cells were maintained for an additional day in complete medium and then cells were counted. For each condition, the number of cells per field was approximately double, indicating that the cells attached to the plate through the neoglycoconjugates retain their capability to grow and divide.

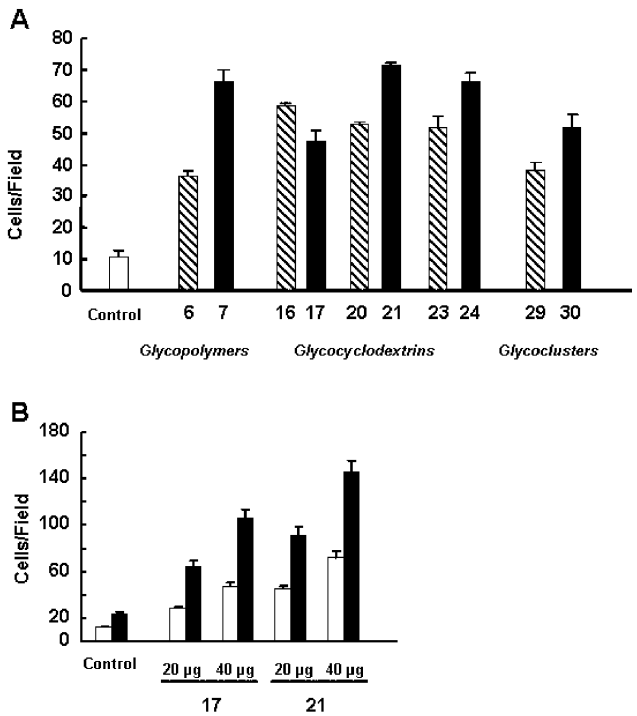


Fig. 1 Adhesion of U-937 cells to cell culture plates coated with neoglycoconjugates. **A**. U-937 cells were plated on tissue culture plates coated with distilled water (control) or neoglycoconjugates (40 μg). Adhesion was measured as number of cells per field. Epitope nature is indicated: Gal, dashed bars, Lac, solid bars, **B**. Effects of two different concentrations (20 and 40 μg) of compounds **17** and **21** on cell adhesion and growth. Number of cells was measured 3 h after the plating of the cells (open bars) and 24 h after incubation in a complete medium as described in the Experimental section. Results are the means ± S.E.M. from at least 10 different fields for each independent experiment ($n = 5$).

To facilitate the detection of the neoglycoconjugates, a fluorescent moiety was added. First, we investigated if the dansyl-containing neoglycoconjugates were able to mediate U-937 cell adhesion. The results obtained (Fig. 2) show that the labeled compounds **8**, **9** have a similar behaviour to their non-fluorescent homologues **6**, **7**, while the Gal fluorescent compound **22** has a slightly higher binding capacity than its non-fluorescent counterpart **20**.

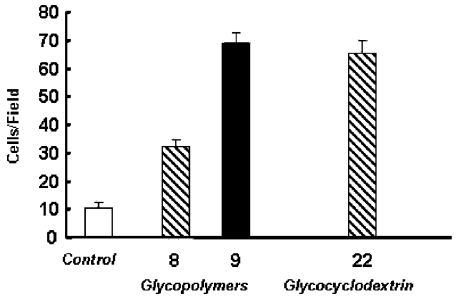


Fig. 2 Influence of fluorescent labelling on the adhesion of U-937 cells to cell culture plates. U-937 cells were plated on cell culture plates coated with dansyl-labeled glycopolymers **8** and **9**, and Gal-glycocyclodextrin **22** (40 μg). Control cells were plated on non-coated (distilled water) cell culture plates. The mediated adhesion was measured as number of cells per field. Epitope nature is indicated: Gal, dashed bars, Lac, solid bars. Results are the means ± S.E.M. from at least 10 different fields for each independent experiment ($n = 5$).

From the adhesion experiments, carried out with previously non-stimulated U-937 cells, it can be suggested that the neoglycoconjugates probably have the ability to stimulate monocyte differentiation and in response to this stimulation, facilitate their adhesion to the culture plates. This effect would probably mimic the action of LPS in cell differentiation and attachment.[33]

In addition, some conclusions regarding the influence of the architecture, topology and nature of the saccharide appendages present in the click glycocompounds can be extracted. First, the Lac epitope in the neoglycoconjugates (compounds **7**, **21**, **24** and **30**) determines higher levels of adhesion compared to the Gal-containing compounds (**6**, **20**, **23** and **29**) regardless of the chemical structure of the scaffold. The monovalent glycocyclodextrin derivatives are the only exception to this behaviour: the Gal-containing cyclodextrin **16** exhibits a slight higher cell binding efficiency that its Lac-containing homologous **17**.

Second, the data obtained from glycocyclodextrins indicate that multivalency does not significantly affect cell adhesion mediated by these compounds. In fact, the cells per field values are similar for the monovalent (compounds **16** and **17**) and the heptavalent glycocyclodextrins (compounds **20**, **21**, **23** and **24**). Also, the cell adhesion efficiency in glycocyclodextrins is independent of the face, primary or secondary, used for the presentation of the sugar epitopes: the Gal-containing (**20** and **23**) and the Lac-containing heptavalent glycocyclodextrins (**21** and **24**) have similar binding capabilities regardless of the face to which the sugar epitope has been attached.

Finally, regarding the nature of the scaffold, Gal-containing cyclodextrins display a higher capacity for adhesion than the corresponding glycoclusters or glycopolymers whereas the Lac heptavalent cyclodextrins and glycopolymers exhibit a comparable binding capacity. In general, glycocyclodextrins followed by glycopolymers appear to be the ligands of choice to mediate monocyte adhesion.

Since the adhesion experiment suggested the possibility that neoglycoconjugates were able to induce monocyte differentiation and therefore mediate cell adhesion, a series of assays were designed to evaluate their macrophage-stimulating capability. For this purpose the adherent RAW 264.7 cells were used. In this cell line, the binding of the fluorescent neoglycoconjugates to

the cell surface, its internalization and the synthesis of the pro-inflammatory cytokine TNF-α, a marker of the macrophage stimulation, were evaluated.

First, binding assays of the dansyl-labeled neoglycoconjugates were performed at 4 °C to preclude the phagocytosis of the neoglycoconjugates. The fluorescent-labeled compounds **8**, **9** and **22** were added to RAW 264.7 cells. The plasma membrane associated fluorescence was observed after an overnight incubation by fluorescence microscopy and quantified by measuring individual cells fluorescence from at least 10 different cellular fields (Fig. 3). The Gal-containing cyclodextrin **22** was significantly the glycoconjugate that produced the higher fluorescence signal associated with the plasma membrane while the binding by the Gal- and Lac-containing glycopolymers, **8** and **9** respectively, was lower. Binding assays with previously LPS-stimulated (1 μg ml^{-1}) RAW 264.7 cells were also carried out (Fig. 3). Interestingly, this stimulation produced a significant decrease in the fluorescence associated with the cells for the compounds containing either Gal (**8** and **22**) or Lac (**9**) epitopes.

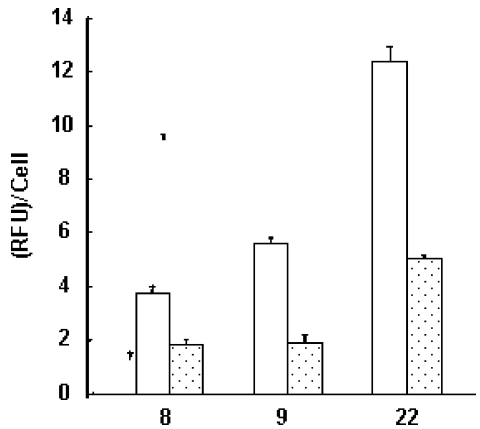


Fig. 3 Binding of fluorescent-labeled neoglycoconjugates to RAW 264.7 cells. RAW 264.7 cells were grown to 60% confluency in complete medium as described in the Experimental section, fasted for 24 h and incubated overnight with the different fluorescent compounds. Cells were pre-incubated (dotted bars) or not (open bars) with LPS for 4 h and then incubated at 4 °C with glycopolymers **8** and **9** and Gal-cyclodextrin **22** (400 μg ml^{-1}) in the presence of LPS. Fluorescence was quantified by fluorescence microscopy as described in the Experimental section. Results are the means ± S.E.M. from at least 50 different cells for each independent experiment ($n = 3$).

The fluorescence detected in these experiments clearly indicates that the neoglycoconjugates bind to the plasma membrane of RAW cells. In addition, the assays provide insight into some structure–function relationship regarding the nature of the neoglycoconjugates: scaffold architecture and the saccharide appendages. The relative fluorescence values obtained in the case of glycopolymers (**9** > **8**) indicate that Lac is preferred over Gal to mediate the specific binding of glycopolymers to the cell surface of monocyte cell lines and are in agreement with the previous findings in the adhesion of U-937 to the cell plates. Complementary to this, the fact that the Gal-containing cyclodextrin **22** produces the higher fluorescence can be explained by the concurrence in the same construct of a cyclodextrin scaffold and a Gal epitope, in line again with the adhesion experiments performed with U-937. These data support the idea that the nature of the scaffold and the saccharide moiety may determine the specificity of the binding in these cells. In addition, the assays performed with LPS-stimulated cells at 4 °C are indicative of a competition for the same membrane receptors between LPS and the Gal- and Lac- derivatives.

The effect of calcium on the binding of the neoglycoconjugates was assessed by removing this cation from the media by adding EDTA. For this purpose, fasted cells were washed extensively with Krebs–Henseleit Hepes buffer pH 7.4 without Ca^{2+} and supplemented with EDTA 5 mM. Compound **22** (400 μg ml^{-1}) was then added to cells in the presence or absence of calcium and incubated at 4 °C overnight. The cells incubated in a medium containing Ca^{2+} showed a higher fluorescence (18.5 ± 0,8 RFU per cell) than those incubated in the absence of this cation (3,3 ± 0,3 RFU per cell) (Fig. 4), indicating that the presence of Ca^{2+} highly facilitates the binding of this glycocompound to the cells. This result suggests the involvement of C-type lectins in the binding to RAW cells of the neoglycocompounds.[34,35]

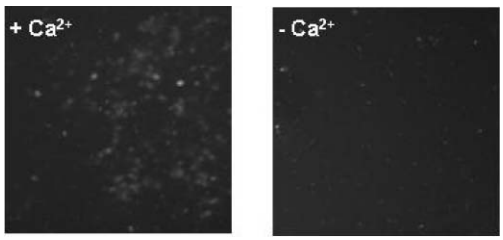


Fig. 4 Fluorescence microscopy of RAW 264.7 cells incubated at 4 °C with Gal-glycocyclodextrin **22** in the presence or absence of Ca^{2+}. Fluorescent photographs using an Olympus IX70 microscope equipped with a WU excitation filter are shown.

Second, the total uptake of the neoglycoconjugates by RAW 264.7 cells at 37 °C was measured. Cells were incubated overnight with the fluorescent-labeled compounds **8**, **9** and **22**. After extensive washing, the uptake was visualized by fluorescence microscopy. Fig 5A shows that RAW cells incubated with the Gal-branched glycocyclodextrin **22** mainly accumulate this compound in the cytosol. A lower fluorescence incorporation was observed when cells were incubated with the dansyl-glycopolymers (data not shown).

To quantify the uptake of the neoglycoconjugates, incubated cells were scraped and the total amount of fluorescence per mg protein in the cell extracts was measured (Fig. 5B). The uptake of the compounds was again significantly higher for the neoglycocompound containing Gal in a cyclodextrin scaffold. In all cases, the fluorescence per mg protein of the cells incubated with the different compounds increased when the cells were previously pre-incubated with LPS. This increase is especially significant in the case of glycocyclodextrin **22**. The LPS-stimulated uptake results point to the idea that macrophage differentiation is a requirement for the efficient uptake of the neoglycoconjugates.

Third, to confirm the hypothesized stimulating-effect of the Gal-cyclodextrin in cultured monocytes RAW 264.7 cells were incubated for 18 h with glycocyclodextrin **22** (100 and 400 μg ml^{-1}) and mRNA levels for TNF-α were measured by real time PCR. Cells incubated with LPS for 18 h were included as a positive control for cell stimulation. The results (Fig. 6) show that TNF-α mRNA production depends on the concentration of compound

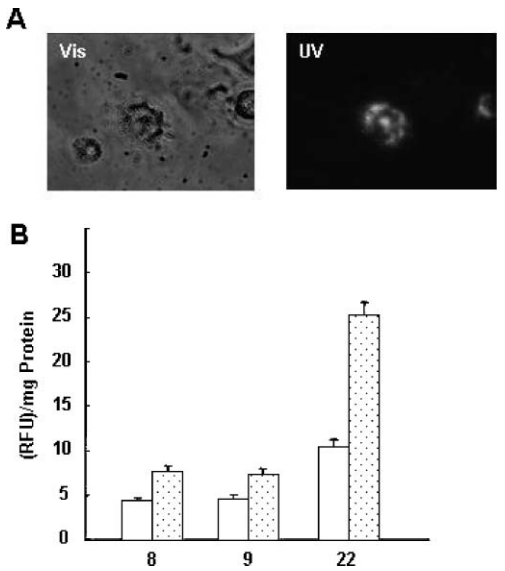


Fig. 5 Uptake of fluorescent-labeled neoglycoconjugates into RAW 264.7 cells. **A**. Fluorescence microscopy of RAW 264.7 cells incubated at 37 °C with Gal-glycocyclodextrin **22**. Visible (Vis) and fluorescent (UV) photographs are shown. **B**. Fluorescence of RAW 264.7 cells that were pre-incubated (dotted bars) or not (open bars) with LPS for 4 h and then incubated overnight with glycopolymers **8** and **9** and Gal-cyclodextrin **22** (400 μg ml^{-1}) at 37 °C. Fluorescence was quantified by spectrofluorometry as described in the Experimental section. Results were normalized to the amount of protein of each sample. Results are the means ± S.E.M. from each independent experiment ($n = 3$).

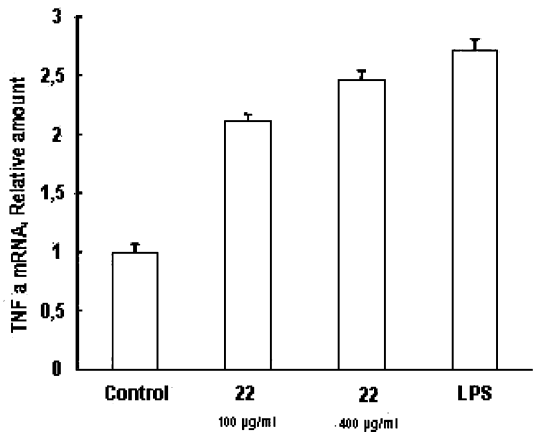


Fig. 6 Stimulation of RAW 264.7 by glycocyclodextrin **22**. Relative amount of TNF-α mRNA RAW 264.7 cells were incubated for 18 h in the absence (control) or the presence of **22** (100 and 400 μg ml^{-1}) or LPS (1 μg ml^{-1}). Relative amounts of TNF-α mRNA were measured by a real time PCR.

22. The amount of TNF-α mRNA at concentrations of 400 μg ml^{-1} was similar to the production observed in LPS-stimulated cells, indicating clearly the stimulating potential of this glycocompound.

Since the results show that LPS-differentiated cells can uptake higher amounts of the glycocompounds (see Fig. 5), we evaluated the intrinsic capacity of these glycocompounds to mediate differentiation and uptake of their fluorescent counterparts. For this purpose, RAW cells were pre-incubated with nothing (control cells), LPS (1 μg ml^{-1}) and the non-fluorescent compound **20** (100 or 400 μg ml^{-1}) at 37 °C for 6 h. After this time, the media was changed and compound **22** alone was added and the cells incubated overnight. Cells were detached using 0.1% SDS and fluorescence was measured by spectrofluorometry. Fig. 7A shows that pre-incubated cells with glycocyclodextrin **20**, at both concentrations have an uptake of glycocyclodextrin **22** comparable to those pre-incubated with LPS. These results confirm that Gal-containing glycocyclodextrins stimulate the differentiation of monocytes in a similar manner to LPS.

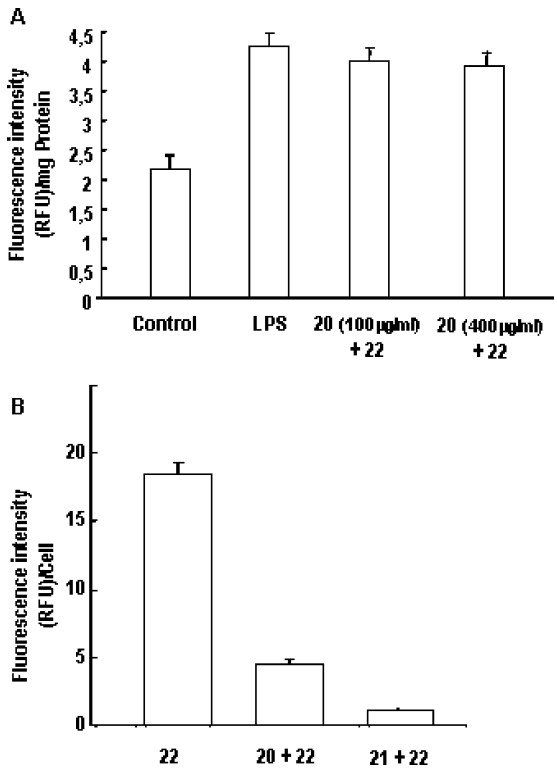


Fig. 7 Glycocyclodextrin **22** binding and uptake in RAW 264.7 cells **A**. Fluorescence of RAW 264.7 cells that were pre-incubated in the absence (control) or in the presence of LPS and 100 or 400 μg ml^{-1} of unlabeled glycocompound **20** for 6 h and then incubated with Gal-glycocyclodextrin **22** (400 μg ml^{-1}) at 37 °C. Results were normalized to the amount of protein of each sample. **B**. Fluorescence per cell of RAW 264.7 cells pre-incubated or not with 400 μg ml^{-1} of the unlabeled glycocompounds **20** (Gal) or **21** (Lac) for 4 h and then incubated at 4 °C with the labeled Gal-glycocyclodextrin **22** (100 μg ml^{-1}). Fluorescence was quantified by fluorescence microscopy as described in the Experimental section. Results are the means ± S.E.M. from at least 50 different cells for each independent experiment ($n = 3$).

Once the stimulating-effect of the multivalent neoglycoconjugates and their capability to act as synthetic activators in monocyte/macrophage cells was demonstrated, a competition assay was performed to probe the specificity of the surface receptor of monocytes/macrophages for glycocompounds bearing Gal- or Lac-appendages. RAW-264.7 cells were pre-incubated with the non-fluorescent glycocompounds containing Gal (**20**) or Lac (**21**) during 4 h prior the supplementation overnight with fluorescent compound **22** at 4 °C. The cell-associated fluorescence was quantified as above (Fig. 7B). The significant decrease of the fluorescence observed when the cells were pre-incubated with the Gal- or Lac-compounds is indicative of the specificity of the

surface receptors for these epitopes. This result corroborates the rational design of neoglycoconjugates containing these epitopes as hypothesized.

Conclusions

From the results described herein, it can be concluded that click multivalent neoglycoconjugates with a well-defined structure have the capability to act as synthetic activators since they promote cell adhesion and stimulation of monocytes. These processes depend on the architecture of the neoglycoconjugates as well as on the nature of the saccharide epitope grafted to those neoglycoconjugates but it is independent of the multivalency degree. Gal and Lac-containing glycocyclodextrins were demonstrated to be the neoglycoconjugates with the highest adhesion and stimulation capabilities. The fluorescent labeling of the multivalent neoglycoconjugates enhances their utility, without modification of their binding and stimulating properties. This allows their application in experiments where visualization was required. Experiments with dansyl-labeled neoglycoconjugates have demonstrated their capability to affect the cytokine production by stimulating the TNF-α expression in RAW 264.7 cells. This stimulation is dose-dependent and mediates similar production levels of TNF-α to those observed in LPS stimulated cells. Therefore, these synthetic compounds act as LPS surrogates that can provide, without the heterogenicity of natural endotoxins, a repetitive stimulation and adhesion of monocytes in cell cultures. Considering the flexibility and efficiency of "click-chemistry", these well-defined and custom-made click multivalent neoglycoconjugates appear to be valuable compounds with applications not limited to the activation of monocytes/macrophages but also with potential for the development of therapeutics.

Experimental

General synthetic methods

Unless otherwise noted, commercially available reagents and solvents were used as purchased without further purification. TLC was performed on Merck Silica Gel 60 F_{254} aluminium sheets. Reagents used for developing plates include ceric sulfate (1% w/v) and ammonium sulfate (2.5% w/v) in 10% (v/v) aqueous sulfuric acid, iodine, ethanolic sulfuric acid (10% v/v) and by UV light when applicable. Flash column chromatography was performed on silica gel Merck (230–400 mesh, ASTM). Melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. IR spectra were recorded on a Satellite Mattson FTIR. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker (300–400 MHz) spectrometer. *J* values are given in Hz. FAB mass spectra were recorded on a Fisons VG Autospec-Q spectrometer, using *m*-nitrobenzyl alcohol or thioglycerol as matrix. Matrix-assisted laser desorption/ionization and time-of-flight mass spectrometry (MALDI-TOF MS) were recorded on a Bruker Daltonics (AUTOFLEX) spectrometer using DGB as matrix

Synthesis of polymer 3

A solution of polymer **2**[23] (1.0 g, MW = 3700), sodium azide (2.13 g, 5 equiv.) and Bu_4NI (20 mg) in DMF (10 mL) was heated at 75 °C for 72 h. After this time, CH_2Cl_2 (100 mL) was added and the organic solution washed with water (5 × 15 mL). The organic phase was dried (Na_2SO_4) and evaporated. Addition of MeOH (75 mL) gave a solid that was filtered and dried under vacuum to give **3** as a solid (88%).

Synthesis of heptakis(2-*O*-propargyl)-β-cyclodextrin 12

Compound **12** was prepared starting from heptakis(2-*O*-propargyl-3,6-di-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin[36] and following the procedure described in the literature[37] for the de-*O*-silylation. mp >325 °C; $[\alpha]_D = +101$ ($c = 0.25$ in methanol); IR (KBr): $\nu = 3430, 3288, 2923, 2117, 1636, 1363, 1159, 1049\ cm^{-1}$; ^{1}H NMR (DMSO-$d_6$, 300 MHz) (selected signals): $\delta = 4.98$ (d, $J =$ 3.3 Hz, 7 H, H-1), 2.49 (br s, 7 H, C≡CH); ^{13}C NMR (DMSO-d_6, 75 MHz): $\delta = 99.8, 81.7, 79.7, 78.4, 77.7, 72.5, 71.3, 59.6, 58.5$; MS (MALDITOF): *m*/*z*: calcd for: $C_{63}H_{84}O_{35}Na$ 1423.5 $[M + Na]^+$; found: 1423.5.

Synthesis of propargyl 2,3,4,6-tetra-*O*-propargyl-β-D-glucopyranoside 26

Propargyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside[38] (0.8 g, 2.3 mmol) was de-*O*-acetylated by the standard Zemplen procedure using NaOMe–MeOH. The crude product, which showed identical physical and spectroscopic data to those reported in literature,[39] was dissolved in anhydrous THF (20 mL) and NaH (0.7 g) was added under an argon atmosphere. After standing at room temperature for 30 min, propargyl bromide (6.8 mL) was dropwise added and the reaction mixture magnetically stirred for 24 h. The reaction mixture was cooled in an ice bath and MeOH (5 mL) was added dropwise. After 15 min, the solvent was evaporated and the resulting crude was purified by column chromatography (AcOEt–hexane 1 : 2) to give **26** as a syrup (580 mg, 68.4% yield): $[\alpha]_D = -25.0$ ($c = 1$ in chloroform); IR (film): $\nu = 3289, 2117, 1445, 1354, 1075\ cm^{-1}$; ^{1}H NMR ($Cl_3CD$, 300 MHz) $\delta = 4.60$–4.30 (several m, 9 H; H-1,2,3, 3 × CH_2C≡CH), 4.25 (dd, $J = 15.9$, 2.3 Hz, 1 H; CH_2CCH), 4.18 (dd, $J = 15.9$, 2.4 Hz, 1 H; CH_2C≡CH), 3.84 (dd, $J = 10.9$, 1.7 Hz, 1 H; H-6), 3.76 (dd. $J = 10.9$, 4.2 Hz, 1 H; H-6′), 3.28 (t, $J = 8.5$ Hz, 1 H; H-4), 3.50–3.35 (m, 3 H; H-5, CH_2C≡CH), 2.48 (m, 5 H; 5 × C≡CH); ^{13}C NMR (Cl_3CD, 75 MHz): $\delta = 100.7, 83.2, 80.9, 80.1, 80.0, 79.8, 79.5, 78.7, 75.9, 75.2, 74.8, 74.5, 74.1, 68.3, 60.2, 60.0, 59.2, 58.6, 55.9$; HRMS (FAB+): *m*/*z*: calcd for $C_{11}H_{22}O_6Na$: 393.1314 $[M + Na]^+$; found: 393.1312.

General procedure for the synthesis of non-fluorescent glycopolymers 6 and 7

A solution of the polystyrene derivative **3** (0.100 g), the corresponding propargyl sugar derivative **4a, b**[26] (0.76 mmol, 1.2 equiv. per azide group) and the copper catalyst $[(EtO)_3P{\cdot}CuI]$ (0.063 mmol, 0.1 equiv. per azide group) in DMF (10 mL) was irradiated at 800 W and 90 °C in a Milestone Star Microwave Labstation until the IR spectra of the reaction mixture showed complete disappearance of the starting material (15 min). The reaction mixture was added dropwise to MeOH (100 mL). The white precipitate that appears was filtered and washed with MeOH, dichloromethane and ethyl ether. The resulting solid was dissolved in water (10 mL) and freeze dried to get a white powder.

Gal-glycopolymer 6

Obtained in 79% yield: IR (KBr): ν = 3484, 1663, 1498, 1390, 1256, 1100 cm^{-1}; ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 144.5, 133.6, 128.0, 125.0, 103.2, 75.6, 73.8, 71.0, 68.6, 61.9, 61.0, 53.0.

Lac-glycopolymer 7

Obtained in 82% yield: IR (KBr): ν = 3430, 1663, 1498, 1439, 1389, 1256, 1097 cm^{-1}; ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 144.0, 127.8, 124.7, 103.9, 101.9, 80.8, 75.5, 74.9, 73.3, 70.6, 68.2, 61.7, 60.5, 53.0.

General procedure for the synthesis of fluorescent glycopolymers 8 and 9

A solution of the polystyrene derivative **3** (0.100 g), the propargyl dansyl derivative **5**[30] (0.03 mmol, 0.05 equiv. per azide group) and the copper catalyst [$(EtO)_3P{\cdot}CuI$] (0.063 mmol, 0.1 equiv. per alkyne group) in DMF (10 mL) was irradiated at 800 W and 90 °C in a Milestone Star Microwave Labstation until the IR spectra of the reaction mixture showed complete disappearance of the starting material (15 min). At this moment, the corresponding propargyl sugar derivative **4a**,**b** (0.76 mmol, 1.2 equiv. per alkyne group) was added and the reaction mixture irradiated for additional 15 min. The work-up procedure was identical as that indicated for the synthesis of non-fluorescent glycopolymers **6**, **7**.

Fluorescent Gal-glycopolymer 8

Obtained 0.179 g. IR (KBr): ν = 3386, 1644, 1513, 1226, 1141, 1067 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) (selected signals): δ = 8.12 (br s; H-triazole), 6.90. 6.35 (2 br s; Ar); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 144.7, 144.0, 127.5, 127.3, 127.1, 124.4, 124.2, 102.6, 75.1, 73.2, 70.4, 68.0, 61.3, 60.3, 52.5.

Fluorescent Lac-glycopolymer

Obtained 0.313 g. IR (KBr): ν = 3384, 2921, 1644, 1054 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) (selected signals): δ = 8.15 (br s; H-triazole), 6.91, 6.33 (2 br s; Ar), 5.47 (br s; $ArCH_2N$); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 143.8, 132.8, 127.3, 124.5, 103.98, 101.8, 80.7, 75.4, 74.9, 73.2, 70.5, 68.1, 61.6, 60.4, 52.4.

General procedure for the synthesis of non-fluorescent glycocyclodextrins functionalized at the primary face 14, 15, 18 and 19

A solution of the 6-azido cyclodextrin derivative **10**[24] or **11**[25] (0.100 mmol), the corresponding propargyl sugar derivative **4a**,**b** (1.3 equiv. per azide group) and the copper catalyst [$(EtO)_3P{\cdot}CuI$] (0.1 equiv. per azide group) in toluene (15 mL) was refluxed for 1.5 h. The reaction mixture was evaporated and the crude purified by column chromatography to yield the corresponding glycocyclodextrin.

Monovalent Gal(OAc)-glycocyclodextrin 14

Column chromatography (AcOEt–hexane 10 : 1 to AcOEt) gave **14** (79%) as an amorphous solid: $[\alpha]_D$ = +97 (c = 1 in chloroform); IR (KBr): ν = 1750, 1372, 1235, 1047 cm^{-1}; ^1H NMR (Cl_3CD, 300 MHz) (selected signals): δ = 7.63 (s; H-triazole), 5.60 (d, J = 4.0 Hz, 1 H); ^{13}C NMR (Cl_3CD, 75 MHz): δ = 170.9–169.30 (19 peaks), 143.7, 125.9, 100.3, 97.15, 97.1, 96.8, 96.8, 96.7, 96.6, 96.5, 77.5, 77.3, 77.1, 76.9, 76.7, 76.5, 75.9, 71.5, 71.3, 71.1, 70.9, 70.8, 70.5, 70.5, 70.3, 70.2, 70.0, 69.8, 69.7, 69.5, 69.4, 68.8, 67.2, 62.8, 62.7, 62.5, 62.3, 61.3, 49.4, 20.8, 20.8, 20.7, 20.6; MS (MALDITOF): m/z: calcd for $C_{99}H_{131}N_3O_{64}Na$: 2408.7 [M + Na]$^+$; found 2408.8.

Monovalent Lac(OAc)-glycocyclodextrin 15

Column chromatography (AcOEt) gave **15** (75%) as a solid: mp 156–158 °C; $[\alpha]_D$ = +80 (c = 1 in chloroform); IR (KBr): ν = 1750, 1371, 1232, 1046 cm^{-1}; ^1H NMR (Cl_3CD, 300 MHz) (selected signals): δ = 7.63 (s, H-triazole); ^{13}C NMR (Cl_3CD, 75 MHz): δ = 170.9–169.0 (17 peaks), 143.8, 126.0, 101.0, 100.8, 99.7, 97.1, 97.0, 96.8, 96.6, 96.5, 83.2, 80.9, 77.2, 77.0, 76.2, 76.0, 75.8, 75.1, 74.7, 74.4, 74.3, 74.1, 72.7, 71.6, 71.5, 71.3, 71.0, 70.7, 70.5, 70.4, 70.2, 70.1, 70.0, 69.7, 69.4, 69.1, 68.4, 66.7, 62.6, 62.3, 62.1, 60.8, 60.2, 60.0, 59.3, 58.7, 55.9, 49.4, 20.8, 20.6, 20.5; MS (MALDITOF): m/z: calcd for $C_{119}H_{189}N_{21}O_{77}Na$: 3167.1 [M + Na]$^+$; found: 3167.1.

Heptavalent Gal(OAc)-glycocyclodextrin 18

Column chromatography (CH_2Cl_2–MeOH 20 : 1) gave **18** (88%) as a solid: mp 184–186 °C; $[\alpha]_D$ = +7.5 (c = 1 in chloroform); $[\alpha]_{436}$ = 10.0 (c 1, chloroform); IR (KBr): ν = 1752, 1372, 1228, 1048 cm^{-1}; ^1H NMR (Cl_3CD, 300 MHz) (selected signals): δ = 7.76 (s; H-triazole); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 170.4, 170.3, 169.5, 144.2, 125.8, 100.8, 96.4, 84.5, 70.8, 69.8, 68.7, 67.1, 65.5, 61.1, 20.8, 20.6; MS (MALDITOF): m/z: calcd for $C_{184}H_{245}N_{21}O_{112}Na$: 4623.8 [M + Na]$^+$; found: 4624.0.

Heptavalent Lac(OAc)-glycocyclodextrin 19

Column chromatography (AcOEt then AcOEt–MeOH 30 : 1) gave **19** (87%) as a solid: mp 194–196 °C; $[\alpha]_D$ = +14.5 (c = 1 in chloroform); IR (KBr): ν = 1750, 1370, 1225, 1047 cm^{-1}; ^1H NMR (Cl_3CD, 300 MHz) (selected signals): δ = 7.76 (br s, 7 H; H-triazole), 5.36 (br s, 7 H; H-4′ lactose), 5.25–3.50 (several m), 2.15, 2.05, 2.03, 1.97, 1.93 (5 s, 189 H; 63 × Ac); ^{13}C NMR (Cl_3CD, 75 MHz): δ = 170.4, 170.2, 170.1, 169.8, 169.6, 169.2, 144.0, 126.0, 100.9, 100.2, 96.4, 76.1, 72.7, 71.5, 71.0, 70.5, 69.7, 69.0, 66.6, 63.0, 62.0, 60.7, 60.4, 50.1, 21.2, 20.9, 20.8, 20.7, 20.5; MS (MALDITOF): m/z: calcd for $C_{184}H_{245}N_{21}O_{112}Na$: 4623.8 [M + Na]$^+$; found: 4624.0.

Synthesis of fluorescent glycocyclodextrin functionalized at the primary face (22)

A solution of the 6-azido cyclodextrin derivative **11** (0.100 g), the propargyl dansyl derivative **5** (0.15 mmol, 0.25 equiv. per azide group) and the copper catalyst [$(EtO)_3P{\cdot}CuI$] (0.063 mmol, 0.1 equiv. per azide group) in DMF (5 mL) was irradiated at 800 W and 90 °C in a Milestone Star Microwave Labstation for 6 h. At this moment, the corresponding propargyl sugar derivative **4a** (0.76 mmol, 1.2 equiv. per alkyne group) was added and the reaction mixture irradiated for an additional 2 h. The work-up procedure was identical to that indicated for the synthesis of non fluorescent glycocyclodextrins **14**, **15**, **18** and

19. The crude product was de-*O*-acetylated by NaOMe–MeOH. Evaporation of the solvent was followed by purification by column chromatography (acetonitrile–water 3 : 1) giving **22**. ^{1}H NMR, ^{13}C NMR and MS (MALDITOF) showed a mixture of different compounds with **22** as the general formula.

General procedure for the synthesis of glycocyclodextrins functionalized at the secondary face 23 and 24

A solution of the propargylated cyclodextrin **12** (0.100 mmol), the corresponding azido sugar derivative **13a**,**b**[27,28] (1.5 equiv. per alkyne group) and the copper catalyst [$(EtO)_3$P·CuI] (0.1 equiv. per azide group) in DMF (15 mL) was irradiated at 500 W and 90 °C in a Milestone Star Microwave Labstation for 40 min. Evaporation gave a crude that was purified by column chromatography (acetonitrile–water 2 : 1 to 1 : 1).

Heptavalent Gal(OAc)-glycocyclodextrin 23

Isolated as a solid in 92% yield: mp > 250 °C (decomp.); $[\alpha]_D$ = +34 (c = 0.5 in water); $[\alpha]_{436}$ = +160 (c = 0.5 in water); IR (KBr): ν = 3381, 2991, 1636, 1152, 1080, 1037 cm^{-1}; ^{13}C NMR (DMSO-d_6, 200 MHz): δ = 143.4, 125.0, 103.4, 99.9, 82.0, 78.9, 75.3, 73.3, 73.0, 71.5, 70.4, 68.1, 67.1, 64.2, 60.4, 54.8, 49.6. MS (MALDITOF): m/z: calcd for $C_{161}H_{259}N_{21}O_{112}Na$: 4303.8 [M + Na]$^+$; found: 4303.4.

Heptavalent Lac(OAc)-glycocyclodextrin 24

Isolated as a solid in 86% yield: mp > 250 °C (decomp.); $[\alpha]_D$ +96 (c = 0.5 in water); $[\alpha]_{436}$ = +188 (c = 0.5 in water); IR (KBr): ν = 3996, 1645, 1373, 1157, 1035 cm^{-1}; ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 143.5, 125.4, 103.8, 102.5, 100.0, 81.7, 80.6, 78.5, 75.1, 74.8, 73.2, 72.9, 71.5, 70.5, 68.1, 67.3, 64.9, 63.8, 60.4, 59.6, 49.8; MS (MALDITOF): m/z: calcd for $C_{161}H_{259}N_{21}O_{112}Na$ 4303.8 [M + Na]$^+$; found: 4303.4; $C_{161}H_{259}N_{21}O_{112}K$ 4319.9 [M + K]$^+$; found: 4319.3.

General procedure for the synthesis of hydroxylated glycocyclodextrins 16, 17, 20 and 21

A solution of the corresponding per-*O*-acetylated derivatives **14**, **15**, **18** or **19** (0.05 mmol) in MeOH–DMF–Et_3N (8 : 2 : 1 ml) was refluxed for 60 h. Evaporation gave a crude that was crystallized to yield the corresponding hydroxylated derivatives **16**, **17**, **20**, or **21**, respectively.

Monovalent Gal(OH)-glycocyclodextrin 16

Crystallization in AcOEt–MeOH gave **16** in 90% yield as a solid: mp > 250 °C (decomp.); $[\alpha]_D$ +110 (c = 1 in water); ^{1}H NMR (D_2O, 300 MHz) (selected signals): δ = 8.16 (s; H-triazole); ^{13}C NMR (D_2O, 75 MHz): δ = 143.6, 126.9, 102.0, 101.9, 101.5, 83.0, 81.3, 81.1, 80.7, 75.2, 73.1, 72.8, 72.7, 72.0, 71.8, 71.7, 71.4, 70.6, 68.6, 61.7, 61.0, 60.2, 59.1, 51.1; MS (MALDITOF): m/z: calcd for $C_{51}H_{83}N_3O_{40}Na$: 1400.5 [M + Na]$^+$; found: 1440.5.

Monovalent Lac(OH)-glycocyclodextrin 17

Crystallization in MeOH gave 64% yield as a solid: mp > 165–166 °C; ^{1}H NMR (D_2O, 400 MHz) (selected signals): δ 8.13 (s; H-triazole); ^{13}C NMR (D_2O, 100 MHz): δ = 143.5, 127.0, 103.0, 102.0, 101.9, 101.5, 101.3, 83.0, 81.3, 81.1, 80.7, 78.3, 75.3, 74.8, 74.3, 73.0, 72.8, 72.7, 72.5, 72.0, 71.8, 71.4, 70.9, 70.5, 68.5, 61.8, 61.0, 60.2, 60.1, 59.1, 51.2; MS (MALDITOF): m/z: calcd for $C_{57}H_{93}N_3O_{45}Na$: 1562.50 [M + Na]$^+$; found 1562.60.

Monovalent Gal(OH)-glycocyclodextrin 20

Crystallization in MeOH gave **20** in 80% yield as a solid: mp > 250 °C (decomp.); $[\alpha]_D$ = +10 (c = 0.5 in water); $[\alpha]_{436}$ = +20 (c = 0.5 in water); IR (KBr): 3400, 1638, 1156, 1076, 1048 cm^{-1}; ^{1}H NMR (D_2O, 300 MHz): δ = 8.1 (s, H-triazole); ^{13}C NMR (D_2O, 75 MHz): δ = 140.9, 124.5, 99.4, 98.3, 79.6, 72.4, 70.0, 69.7, 68.9, 68.0, 67.3, 65.8, 58.3; MS (MALDITOF): m/z: calcd for $C_{105}H_{161}N_{21}O_{70}Na$: 2858.0 [M + Na]$^+$; found: 2858.3.

Heptavalent Lac(OH)-glycocyclodextrin 21

Crystallization in MeOH gave 95% yield: ^{1}H NMR (D_2O, 300 MHz) (selected signal): δ = 8.11 (br s, H-triazole); MS (MALDITOF): m/z: calcd for $C_{147}H_{231}N_{21}O_{105}Na$: 3995.5 [M + Na]$^+$; found: 3995.6.

General procedure for the synthesis of glucose-based glycoclusters 27–30

A solution of the propargylated glucose derivative **26** (0.100 mmol), the corresponding azide sugar derivative **13c**,**d** (1.3 equiv. per alkyne group) and the copper catalyst [$(EtO)_3$P·CuI] (0.1 equiv. per alkyne group) in toluene (15 mL) was refluxed for 1 h. The reaction mixture was evaporated and the crude purified by column chromatography to yield the corresponding glycocluster.

Gal(OAc)-glycocluster 27

Column chromatography (AcOEt–MeOH 15 : 1) gave **27** (93%) as a solid: mp 112–114 °C; $[\alpha]_D$ = −17.7 (c = 1 in chloroform); $[\alpha]_{436}$ = −34.3 (c = 1 in chloroform); IR (KBr): ν = 2937, 1750, 1371, 1225, 1051 cm^{-1}; ^{1}H NMR (Cl_3CD, 300 MHz) (selected signals): δ 7.99, 7.90, 7.88, 7.85, 7.74 (5 s, 5 H; H-triazole); ^{13}C NMR (Cl_3CD, 75 MHz): δ = 170.3, 170.0, 169.4, 169.1, 144.7, 144.6, 144.5, 144.1, 124.7, 124.6, 124.3, 102.3, 100.9, 83.9, 81.6, 74.7, 70.8, 10.6, 68.9, 68.4, 67.5, 67.4, 66.9, 66.3, 66.6, 65.4, 64.5, 62.6, 61.1, 49.9, 20.6, 20.6, 20.4; MS (MALDITOF): m/z: calcd for $C_{101}H_{137}N_{15}O_{56}Na$: 2478.8 [M + Na]$^+$; found: 2478.5.

Lac(OAc)-glycocluster 28

Column chromatography (AcOEt–MeOH 15 : 1) gave **28** (92%) as a solid: mp 149–151 °C; $[\alpha]_D$ = −13 (c = 1 in chloroform); IR (KBr): ν = 2958, 1753, 1371, 1230, 1053 cm^{-1}; ^{1}H NMR (Cl_3CD, 300 MHz) (selected signals): δ = 7.99, 7.91, 7.89, 7.85, 7.73 (5 s, 5 H; H-triazole), 5.35 (d, J = 1.3 Hz, 5 H; H-4 Gal); ^{13}C NMR (Cl_3CD, 75 MHz): δ = 170.3, 170.1, 169.9, 169.6, 169.5, 169.1, 144.7, 144.5, 144.2, 124.6, 124.5, 124.2, 101.0, 100.4, 76.1, 76.0, 72.8, 72.5, 71.3, 70.9, 70.6, 69.1, 67.7, 66.6, 61.9, 61.8, 60.7, 49.8, 49.7; MS (MALDITOF): m/z: calcd for $C_{161}H_{217}N_{15}O_{96}Na$: 3921.5 [M + Na]$^+$; found: 3921.8.

General Procedure for the synthesis of hydroxylated glycoclusters 29 and 30

A solution of the per-*O*-acetylated derivatives **27** or **28** (0.05 mmol) in MeOH–Et_3N (8 : 2 ml) was refluxed for 3 h. Evaporation gave a crude that was purified by column chromatography to give the corresponding glycoclusters **29** or **30**.

Gal(OH)-glycocluster 29

Column chromatography (AcOEt–MeOH 15 : 1) gave **29** (94%) as a solid: mp >250 °C (decomp.); $[\alpha]_{436} = +3$ ($c = 0.5$ in water); IR (KBr): $\nu = 3408, 2925, 1118, 1076$ cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) (selected signals): $\delta = 8.20, 8.16, 8.13, 8.10$ (4 s, 5 H; H-triazole); ^{13}C NMR (DMSO-d_6, 75 MHz): $\delta = 143.8, 143.6, 143.2, 143.1, 143.0, 124.7, 124.6, 103.4, 101.1, 83.2, 83.1, 76.9, 75.3, 73.7, 73.3, 70.4, 68.1, 67.0, 65.5, 65.0, 64.8, 63.8, 61.8, 60.4, 49.5$; MS (MALDITOF): *m/z*: calcd for $C_{61}H_{97}N_{15}O_{36}Na$: 1638.6 $[M + Na]^+$; found, 1638.7.

Lac(OH)-glycocluster 30

Column chromatography gave **30** (94%) as a solid: mp > 280 °C (decomp.); $[\alpha]_D = +10.4$ ($c = 0.5$ in water), $[\alpha]_{436} = +20.6$ ($c = 0.5$ in water); IR (KBr): $\nu = 3392, 2922, 1146, 1070$ cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) (selected signals): $\delta = 8.17, 8.13, 8.12, 8.08, 8.08$ (s, 5 H; H-triazole); ^{13}C NMR (Cl_3CD, 75 MHz): $\delta = 143.5, 124.5, 103.7, 102.3, 80.5, 75.3, 74.8, 74.7, 73.1, 72.8, 70.5, 68.0, 67.2, 60.3, 60.2, 49.5$; MS (MALDITOF): *m/z*: calcd for $C_{91}H_{147}N_{15}O_{61}Na$ 2448.9: $[M + Na]^+$; found: 2448.9.

Cell culture

U-937 human histiocytic lymphoma cells (ECACC No. 85011440) were grown in suspension in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Cultek, Madrid, Spain), 2 mM glutamine, plus 100 units per ml penicillin and 0.1 mg ml^{-1} streptomycin (Sigma, St. Louis, MO, USA). Mouse monocyte macrophages, RAW 264.7 (ECACC No. 91062702) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal bovine serum, plus the same supplements as for U-937 cells. All cell lines were maintained at 37 °C in a humidified incubator containing CO_2 (5%) and air (95%).

Monocyte adhesion assays

U-937 cells were collected, washed free of serum and placed in serum free RPMI medium. The neoglycoconjugates tested were dissolved at 1 mg ml^{-1} in distilled water and then were added to a 24 well plate to achieve a concentration of 20 or 40 μg ml^{-1} in a final volume of 500 μl of water. The water in the wells was allowed to completely evaporate overnight. Next 4×10^4 U-937 cells in RPMI 1640 medium without serum were plated in the wells and were incubated at 37 °C for 3 hours. After the attachment period, the medium and the non-adhered cells were aspirated and the plates were washed gently twice with 1 ml of cold of phosphate buffered saline. Adhesion was measured by counting the adhered cells by microscopy. At least 10 different cellular fields were examined in each plate at 100× magnification.

Labelling studies

For labelling studies, RAW 264.7 cells were used at 60–70% confluence. Cells were incubated in medium without FCS for 8 h and then treated in the absence or presence of 1 μg ml^{-1} lipopolysaccharide 055:B5 (Sigma, St. Louis, MO) for 4 hours. Neoglycoconjugates bearing Glc, Gal and Lac appendages (**9–11** and **24**) marked with a dansyl group were dissolved in water and added to the growth media in a concentration of 100 or 400 μg ml^{-1} of each labelled neoglycoconjugate. After incubation of 24 hours at 4 °C and 37 °C the cells were washed extensively with cold PBS and analyzed by fluorescence microscopy using an Olympus IX70 microscope equipped with a WU excitation filter. Image acquisition was performed under constant conditions in every field examined. Digital image acquisition and processing were performed with an Olympus DP10 camera and the NIH image software package, respectively. At least 10 different cellular fields were examined in each plate at 100× or 200× magnification. Alternatively, fluorescence intensity was assayed using a Shimadzu RF-5301PC spectrofluorophotometer. Cells were scraped and the total amount of fluorescence in the cell extracts was measured at λ_{Ex} 335 and λ_{EM} 518 nm. Results were normalized using the total amount of protein of the cell extracts.[40]

Quantitative RT-PCR

Specific RNA concentrations were quantified by RT-real time PCR. Total RNA was isolated from the RAW 264.7 cells using a guanidinium thiocyanate method.[41] Three μg of total RNA was used as a template to generate the cDNA by reverse transcription (RT) with the first-strand cDNA synthesis kit (GE Health Care Life Sciences, Uppsala, Sweden) using a oligodT-*Not*I primer as per manufacturer's protocol. Real time PCR was performed using double-stranded DNA-specific dye SYBR Green I. The RT products were amplified using the following primers: TNFα 5′-CCTGTAGCCCACGTCG TAGC-3′ and 5′-TTGACC-TCAGCGCTGAGTTG-3′[42] and β-actin 5′-GGCCAACCGTG-AAAAGATG-3′ and 5′-GGATCTTCATGAGGTAGTCTGTC-3′. Amplification, data acquisition and data analysis were carried out using a LightCycler 480 system (Roche Applied Science, Barcelona, Spain). In each run, standard curves were generated for a primer set by serial dilution of plasmid DNA encoding the relevant cDNA. Melting curves were generated after each run to verify melting temperatures of the amplicon, and the purity of RT-PCR was additionally verified by running the PCR products on agarose gel (data not shown). TNF-α mRNA amounts were calculated using the real time PCR standard curve method.[43]

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