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Mannose receptor independent uptake of transmembrane glycocluster immunostimulant TADM by macrophages

Dominik Eichin [a,b], Jani Rahkila [c], Xiang-Guo Li [d], Ramesh Ekambaram [c], Robert Lassfolk [c], Reko Leino [c], Johannes Savolainen [a,e,*]

[a] *MediCity Research Laboratory, University of Turku, 20520, Turku, Finland*
[b] *InFLAMES Flagship, University of Turku, Turku, Finland*
[c] *Laboratory of Molecular Science and Engineering, Åbo Akademi University, 20500, Turku, Finland*
[d] *Turku PET Centre and Department of Chemistry, University of Turku, 20520, Turku, Finland*
[e] *Department of Pulmonary Diseases and Clinical Allergology, University of Turku and Turku University Hospital, 20520, Turku, Finland*

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ABSTRACT

Triacedimannose (**TADM**) is a synthetic trivalent acetylated glycocluster comprising β-1,2–linked mannobioses that in humans induces TNF *in vitro* and *in vivo*. The purpose of this study was to analyze whether uptake of acetylated glycoclusters of such β-1,2–linked mannobioses by human macrophages is dependent on the mannose receptor (CD206) or if it is mediated by transmembrane activation. In mannose receptor blocking assays, monocyte-derived polarized macrophages were incubated with carbohydrate test-compounds and their binding to the mannose receptor was demonstrated as inhibition of FITC-Dextran binding. For 1H NMR spectroscopy, macrophages were incubated with **TADM**. The cells were collected at 6 and 24 h of incubation, centrifuged and washed twice with PBS. We found dose-dependent blocking of the mannose receptor in macrophage carbohydrate constructs containing free hydroxyl groups, but not by the trivalent acetylated glycocluster molecules. NMR spectroscopic analyses demonstrated that **TADM** was found in washed cellular pellets after 6-h co-culture, while after 24-h co-culture **TADM** was no more detectable, suggesting cleavage of the acetyl groups *in vitro*. The Type 1 immune response enhancing effects of **TADM** and other, stereochemically and structurally similar, trivalent acetylated glycoclusters may be due to transmembrane uptake of macrophages independent of the mannose receptor.

1. Introduction

Candida albicans mannans containing β-1,2-oligomannoside structures induce IFN-ɣ responses and lymphoproliferation in practically all human beings [1]. These responses are not only induced by intact mannan but also by short hydrolyzed β-1,2-oligomannoside fragments [2] that induce TNF responses in macrophages [3]. We have earlier prepared, by a synthetic organic chemistry approach, oligosaccharide molecules mimicking the natural β-1,2-mannoside structures [4,5]. In addition, we also enhanced their immunostimulatory properties by creating oligovalent glycocluster constructs resulting in novel synthetic immunostimulatory molecules. One such molecule is Triacedimannose (**TADM**, Compound **1**), a synthetic trivalent acetylated glycocluster comprising β-1,2–linked mannobioses. **TADM** has been shown to induce IFN-ɣ and TNF production and to suppress allergic inflammation in human *in vitro* PBMC models, as well as in *in vivo* murine models [4–7]. This is in accordance with previous findings on immune responses induced by β-1,2-oligomannoside structures, implying that macrophages are activated [1,3]. The present study was undertaken to investigate this further and analyze, whether uptake of acetylated glycoclusters of β-1,2–linked mannobioses, and in particular **TADM**, by human macrophages is dependent on the macrophage mannose receptor (CD206) or is mediated by transmembrane uptake. In addition to **TADM**, two of its close, fully acetylated trivalent analogues with elongated linker structures (Compounds **2** and **3**), were investigated.

* Corresponding author. Department of Pulmonary Diseases and Clinical Allergology, University of Turku and Turku University Hospital, Hämeentie 11, 20520, Turku, Finland.

E-mail addresses: dominik.eichin@utu.fi (D. Eichin), jani.rahkila@abo.fi (J. Rahkila), xiali@utu.fi (X.-G. Li), ramcheme@gmail.com (R. Ekambaram), robert.lassfolk@abo.fi (R. Lassfolk), reko.leino@abo.fi (R. Leino), johsav@utu.fi (J. Savolainen).

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2. Experimental

2.1. Synthesis of glycoclusters comprising β-1,2–linked dimannoses

Synthesis of the trivalent acetylated glycoclusters comprising β-1,2–linked dimannoses (compounds **1**–**3**), the deacetylated congener of **TADM** (**TDM**, compound **4**) and the other deacetylated mono- and oligovalent carbohydrate constructs (compounds **5**–**10**) have been described in our earlier work [4,5]. The structures studied here are depicted in Fig. 1.

2.2. Preparation of polarized macrophages

Human buffy coats were obtained from the Finnish Red Cross and PBMCs were extracted by Ficoll-Paque PLUS (17-1440-03, GE Healthcare, Helsinki, Finland). PBMCs were then frozen until needed. $CD14^{+}$ monocytes were extracted by positive selection according to the manufacturer's instructions (CD14 MicroBeads, human (130-050-201); MiltenyiBiotec, Lund, Sweden) and cultured into macrophages in HEC medium (RPMI 1640 with 10 % FCS, 4 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin) with 25 ng/mL M-CSF (300-25, Peprotech, London, United Kingdom). After 5 days, the macrophages were polarized towards an anti-inflammatory M2 (M(IL-4+IL-13)) or a pro-inflammatory M1 (M(LPS + IFN-ɣ)) phenotype for two days (IL-4 (200-04) and IL-13 (200-13) were obtained from Peprotech, London, United Kingdom; LPS was obtained from Sigma (L3024), Helsinki, Finland; IFN-ɣ (285-IF) was obtained from R&D Systems, Abingdon, United Kingdom). 10 ng/mL IL-4, 35 ng/mL IL-13, 35 ng/mL IFN-ɣ and 100 ng/mL LPS were used, respectively. Macrophages were verified with flow cytometry by their expression of CD68 and CD206 as M2 cells (Fig. S1). For this, anti-human CD68-AF647 (sc-20060 AF647, Santa Cruz Biotechnology. Inc., Heidelberg, Germany), CD206-APC (550 889, BD Biosciences, Helsinki, Finland) and their Mouse IgG1 Isotype control antibody (557 783 and 557 783, BD Biosciences, Helsinki, Finland) were used. Staining of CD68 was performed following permeabilization with BD's Cytofix/Cytoperm kit (554 714, BD Biosciences, Helsinki, Finland).

2.3. Mannose receptor blocking assays

For the mannan-binding assay purified, buffy coat derived monocytes were differentiated in the presence of M-CSF, IL-4 and IL-13 to mannose receptor positive M2 macrophages. These were incubated with three concentrations (10, 50 and 100 μM) of ten different synthetic carbohydrate constructs that are depicted in Fig. 1, including the acetylated glycocluster lead compounds **1**–**3**, and mannan from *Saccharomyces cerevisiae* (M7504, Sigma, Helsinki, Finland), from *Candida albicans* or hydrolyzed mannan from *Candida albicans* (strain CBS 5982, serotype A, both in-house, originally obtained from Centraalbureau Voor Schimmelculture, Delft, The Netherlands) as control substances. The lyophilized acetylated glycoclusters were first solubilized with vigorous vortexing at 37 °C to the highest achievable solubilized concentration of 0.5 mg/ml. In addition, some cells were differentiated with LPS and IFN-ɣ to M1 macrophages (that have a very low expression of mannose receptor) and exposed to mannan. To determine mannan binding, polarized macrophages were detached from their culture plate and replated to a 24-well plate at a density of 100 000 cells per well. Each well was incubated with 0–100 μg/mL mannan or 10–100 μM of the test-compounds, respectively, for 30 min at 37 °C. This was followed by incubation of the cells with 100 μg/mL FITC-Dextran (FD40S, Sigma, Helsinki, Finland) for 2 h at 37 °C. Cells were recorded on a flow cytometer (BD Fortessa, BD Biosciences, Helsinki, Finland) and analyzed with FlowJo (Treestar, Ashland, USA). All incubations were performed in HEC medium with 0.5 % DMSO. As a negative control, M2 cells that had not been incubated with FITC-Dextran, and as a positive control, cells only exposed to FITC-Dextran, were used.

2.4. Nuclear magnetic resonance spectroscopic analyses

For the nuclear magnetic resonance spectroscopic analyses, polarized macrophages were detached from their culture plate and replated to a 24-well plate at a density of 100 000 cells per well, in 400 μL media/well. Each well was incubated as such or 100 μM of **TADM**. The cells were collected at 6 and 24 h of incubation and centrifuged at 1600 rpm for 10 min. The cells were washed twice with PBS and centrifuged as above. Cell pellets were then stored at −70 °C for NMR spectroscopic analysis. The cell pellets were thawed and diluted with 100 μL D2O after which 1H NMR spectra were recorded on a Bruker AVANCE III HD NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 600.16 MHz (1H) equipped with a TCI Prodigy CryoProbe. A total of 4k transients were recorded on both **TADM** and the negative control using NOE presaturation to suppress the water signal. The spectra of the duplicate experiments were added together.

2.5. Deacetylation of TADM in human serum

Additionally, 1.5 mg **TADM** was incubated in 1 ml human serum (Sigma-Aldrich) at 37 °C to determine the stability of the acetyl groups outside of cells, and the deacetylation was followed by 1H NMR spectroscopy over approximately 4 days using a Bruker AVANCE III operating at 500.12 MHz (1H) equipped with a Smartprobe: BB/1H. NOE presaturation was used to suppress the water signal.

3. Results

3.1. Mannose receptor blocking assays

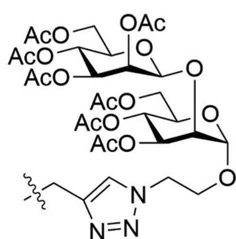
Binding and uptake of FITC-Dextran at +37 °C via mannose receptor was analyzed by flow cytometry and is depicted in Fig. 2. We could find dose-dependent blocking of mannose receptor in macrophages by glycocluster molecules with free hydroxyl groups, but not by the acetylated glycocluster molecules (**1**–**3**). The dose-dependent blocking was statistically significant with compounds **4**–**9** but not with compound **10**. In addition, experiments performed at 4 °C revealed no significant FITC-Dextran binding or uptake (Fig. S2).

3.2. Nuclear magnetic resonance spectroscopic analyses

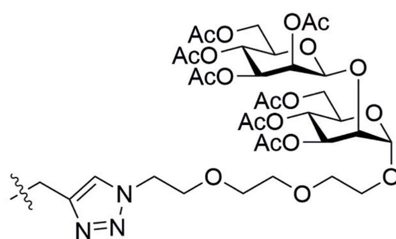
To investigate whether the acetylated glycocluster **TADM** (Compound **1**) penetrates through the macrophage cell wall, 1H NMR spectroscopic analysis of the twice washed M2 macrophage cellular pellets after 6- and 24-h incubation with **TADM** was performed. The data are presented in Fig. 3, together with the spectra of negative control (macrophages alone) and that of pure **TADM**. The carbohydrate region (3–5 ppm, not shown) of the incubated spectra is crowded and some signals close to the water peak are lost due to solvent suppression. For detecting the presence of **TADM,** the most suitable region is the one showing the strongest signals from the acetyl groups (1.70–2.15 ppm). In particular, the acetyl group resonance from the 2-position of the non-reducing end of the mannoside moiety (2.10 ppm) is well separated from the other signals (Fig. 3). In addition, seven other signals are seen in the region 1.88–1.97 ppm.

In the 6 h samples (A), the acetyl group region clearly contains peaks associated with **TADM** (D). In the 24 h sample (B), however, these peaks are significantly weaker and only the aforementioned well-separated peak (2.10 ppm) can be clearly identified. At this timepoint, the remaining part of the acetyl group region more closely resembles that of the non-treated cells (C). These results suggest at least partial deacetylation of **TADM** inside the cells over time. In the **TADM** spectra there is an increase of other acetylated compounds (1.97–2.07 ppm) in the 24 h sample (B) as compared to the 6 h sample (A) and macrophage control sample (C). Also increased acetate (1.75 ppm) is seen in the 24 h sample (B) as compared to the mere macrophage derived acetate in 6 h sample

Trivalent



TADM (1)



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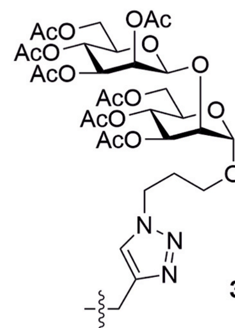

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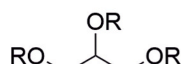

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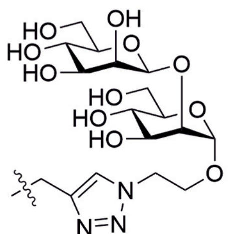
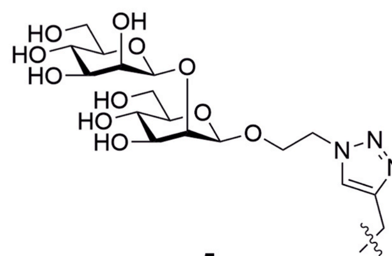


TDM (4)



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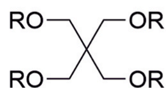
Tetravalent



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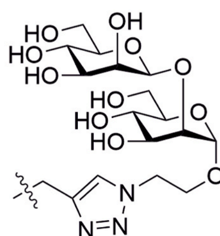

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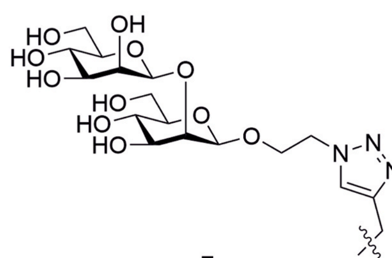

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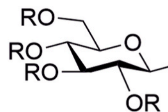
Pentavalent



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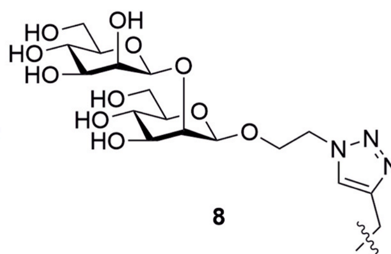

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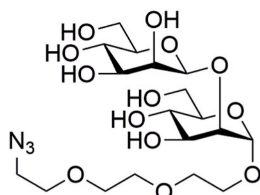
Monovalent



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Divalent

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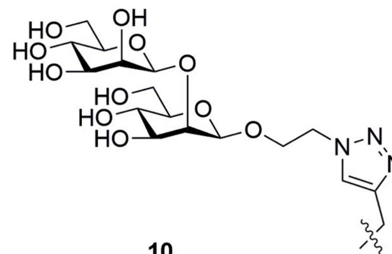

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Fig. 1. Structures of the carbohydrate compounds studied.

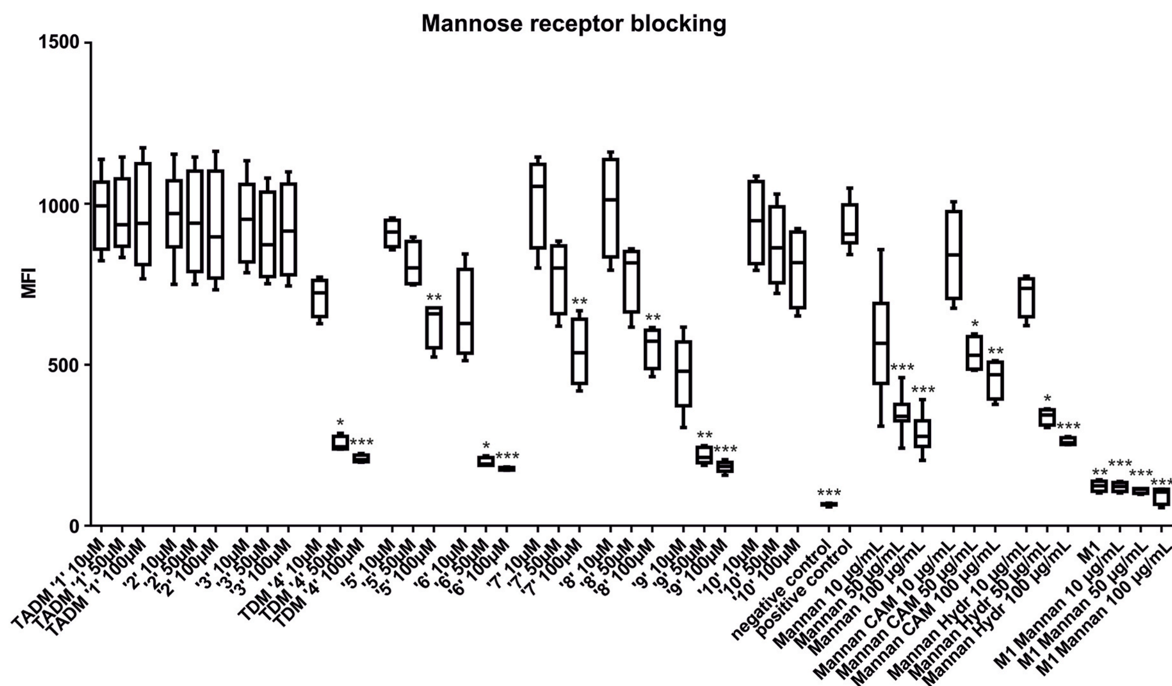

Fig. 2. Human macrophage mannose receptor blocking is dependent on free hydroxyl groups. Synthetic carbohydrate molecules were incubated with mannose receptor positive human macrophages and their receptor-blocking efficacy was determined by FITC-Dextran binding. No blocking could be observed with our lead compound **TADM** or other acetylated molecules (**1-3**), while blocking was observed with molecules that had free hydroxyl groups (**4-10**). Data was obtained by flow cytometry. Mannan stands for *S. cerevisiae* mannan, Mannan CAM stands for *C. albicans* mannan and Mannan Hydr stands for Hydrolyzed *C. albicans* mannan. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Kruskal Wallis test with post-hoc Dunn's comparison, compared to the positive control. N=4-15.

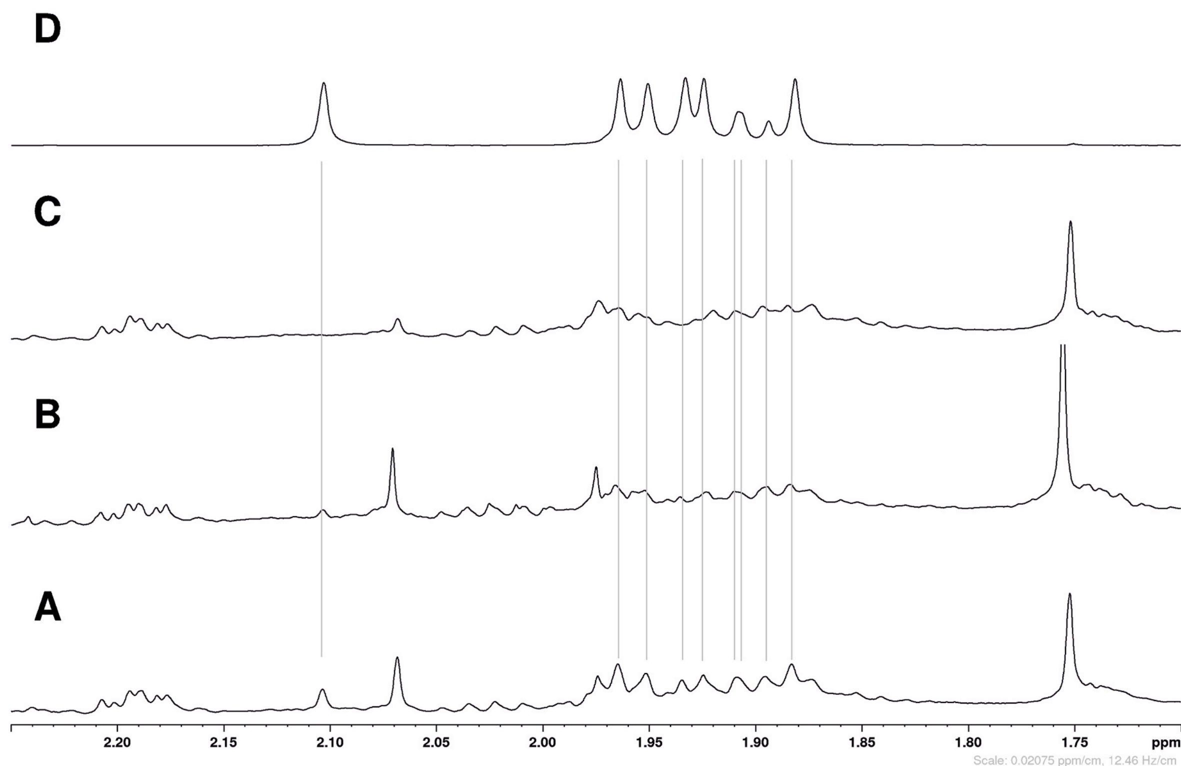

Fig. 3. ^{1}H NMR analysis (acetyl peak region) of twice washed M2 macrophage cellular pellets after 6-hour incubation with **TADM** (A), after 24 h (B), negative control, macrophage cellular pellets without **TADM** (C) and pure **TADM** (D). **TADM** acetyl peaks are indicated with vertical bars (1.89-1.97 and 2.10 ppm). Acetate peak at 1.75 ppm.

(A) and the macrophage control sample (C) (Fig. 3).

3.3. Deacetylation of TADM in human serum

When **TADM** was incubated with human serum at 37 °C, approximately 1–1.5 equivalents of acetate were formed over 24 h. This is consistent with 1–1.5 acetyl groups per molecule being hydrolyzed, indicating a significantly slower deacetylation than that taking place inside the treated cells (Fig. 4, Figs. S3 and S4).

4. Discussion

In earlier work, we have prepared oligosaccharide molecules mimicking the natural β-1,2-mannoside structures which also have enhanced immunostimulatory properties. This was done by creating oligovalent glycocluster constructs. This work, carried out jointly at the Organic Chemistry Laboratory of Åbo Akademi University and the MediCity Research Laboratory of the University of Turku, has resulted in novel synthetic immunostimulatory molecules [4,5]. Mannan induced lymphoproliferation can be inhibited by both α-methyl mannoside and anti-HLA DR antibodies, indicating that the immune response to mannan is mediated by both mannose receptor (CD206) and MHC II [8]. We therefore became interested to test selected, representative synthetic β-1,2-manno-oligosaccharides and their potential interaction with the mannose receptor. To test this we used buffy coat derived M2 type macrophages because they are rich in cell surface CD206 expression.

To visualize the binding to the mannose receptor, FITC-Dextran with a molecular weight of 40 000 was used. This molecule has been routinely used to study the mannose-receptor, as dextrans are able to bind to it and block it [9,10]. Furthermore, antibodies specific for the mannose receptor dose-dependently inhibited the uptake of FITC-Dextran into tumor associated macrophages or monocyte-derived dendritic cells, a cell type closely related to our monocyte-derived macrophages [11,12]. Although it is obvious that peracetylated dimannoside hardly will interact directly with the mannose receptor, we found it important to demonstrate it here. It is known that the macrophage scavenger receptor SR-A has the ability to bind and mediate endocytosis of acetylated low-density lipoprotein [13]. In the present study we showed that uptake of acetylated glycoclusters of β-1,2–linked mannobioses, especially **TADM,** by human macrophages, is independent of the macrophage mannose receptor (CD206) and is mediated either by a different receptor or transmembrane uptake. While in our earlier work we could rule out TLRs 2, 3, 4, 5, 7, 8 and 9 as potential binding partners for **TADM** by using in ligation screening, mannose containing ligands have been reported to interact with various receptors on the surface of macrophages [5]. Some of these receptors such as Dectin-2 and Mincle can likely be excluded, as despite their higher expression in M1

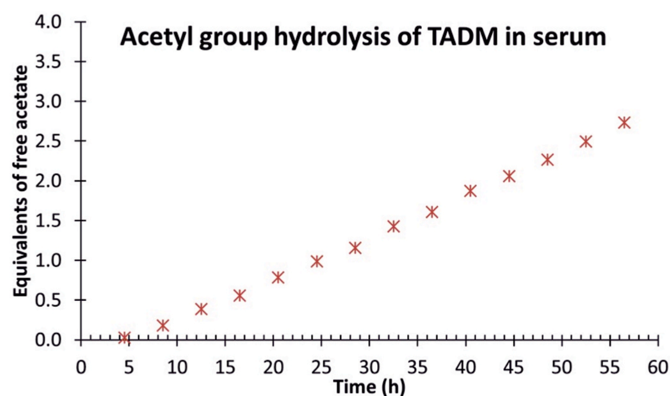

Fig. 4. TADM incubated in human serum at 37°C to determine the stability of the acetyl groups. Deacetylation followed by water suppressed ^{1}H NMR spectroscopy at 37°C.

macrophages, these cells only showed negligible FITC-Dextran uptake in our hands [14]. Others though, such as scavenging receptors or DC-SIGN, cannot be ruled out and might require additional studies.

In this study, binding and uptake via the mannose receptor could only be observed at +37 °C, but not at +4 °C. This temperature sensitivity has been observed before and is likely due to the low affinity of the receptor [15]. At +37 °C, we could find dose-dependent blocking of mannose receptor in macrophages by the mono- and oligovalent carbohydrate constructs with free hydroxyl groups, but not with the fully acetylated glycoclusters. The dose-dependent blocking was weaker and not statistically significant with compound **10**. Notably, already in our earlier work [4,16,17] it became evident that clear correlations between the degree of valency and stereochemical configuration vs. the biological response of the mannoside-derived glycoassemblies are difficult to deduce. While in the present work the divalent compound **10** stereochemically is analogous to the trivalent, tetravalent and pentavalent glycoclusters **5**, **7** and **8**, respectively, its divalent structure is, however, likely to result in significant differences in conformational behavior and solubility. Consequently, the underlying reasons for its different response may be due to a multitude of factors which due to the limited scope of the present study are impossible to address in detail.

Interestingly, a dose-response stereochemical discrimination (not only for compound **10**) can be observed between compounds with stereochemistry beta-alfa (**4**, **6**, and **9**) and those with stereochemistry beta-beta (**5**, **7**, **8** and **10**). As shown in our earlier work [4], stereochemical configuration of the carbohydrate derived assemblies significantly influences their biological properties. This can at least partially be related to their minimum energy conformations (influenced by the alfa vs. beta-linkages in their carbohydrate units) consequently also influencing their binding to biological targets [5]. While the beta-alfa linked compound **1** (**TADM**) has been proven as a potential biologically active lead molecule *in vitro* and *in vivo* [6,7], the corresponding beta-beta analogue has shown to be inactive [4]. In the absence of thorough structure-activity relationship studies on larger compound libraries (combined with advanced molecular modeling) the exact biological binding mechanisms can, however, not be deduced.

As the mannose receptor was not involved, we performed 1H NMR spectroscopic analyses. These demonstrated, that **TADM** was found in washed cellular pellets after 6-h co-culture of macrophages with the glycocluster molecule. Interestingly, however, after 24-h co-culture the signals from **TADM** were significantly weaker compared to the 6-h timepoint, suggesting hydrolysis of the acetyl groups over time. Nevertheless, this hydrolysis seems only to take part once **TADM** has been taken up by the cells, as it was relatively stable under biological conditions when exposed to human serum alone. This hints, that **TADM** may penetrate the cell walls of macrophages directly and independently of the mannose receptor and before the hydrolysis of its acetyl group becomes too prevalent to prevent it. However, it is also possible that **TADM** could loosely co-precipitate with the cell walls over time, leading to its detection in NMR after 6 h due to detachment in D2O, but not after 24 h when it would be more firmly attached. Nevertheless, it is more likely for **TADM** to penetrate the cell wall, because also other acetylated compounds as well as acetate are increased in the 24 h sample. This hypothesis is in line with other research on acetylated carbohydrates that shows that they can penetrate the cell membrane and be hydrolyzed inside the cells by intracellular esterases [18]. However, further studies are required to determine the specific binding partner of **TADM** and shed more light into the immunological processes that are induced by it.

To conclude, we have shown in earlier studies that **TADM** can act as a Type 1 immunoresponse enhancing adjuvant in *in vivo* acute and chronic allergy mouse models [6,7]. In the current study, we demonstrate that the observed effects of **TADM** are mannose receptor independent.

CRediT authorship contribution statement

Dominik Eichin: Writing – review & editing, Writing – original

draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Jani Rahkila:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xiang-Guo Li:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Ramesh Ekambaram:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Robert Lassfolk:** Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Reko Leino:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Johannes Savolainen:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2024.109166.

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