

Acid-Triggered Degradable Reagents for Differentiation of Adaptive and Innate Immune Responses to *Leishmania*-Associated Sugars**

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Abstract: Lipopolysaccharides (LPS) of *Leishmania* spp are known to alter innate immune responses. However, the ability of these sugars to specifically alter adaptive T-cell responses is unclear. To study cap sugar–T-cell interactions, pathogen mimics (namely glycodendrimer-coated latex beads with acid-labile linkers) were synthesized. Upon lysosomal acidification, linker breakdown releases glycodendrimers for possible loading on antigen presenting molecules to induce T-cell growth. T-cell proliferation was indeed higher after macrophage exposure to mannobioside or -trioside-containing glycodendrimers than to non-functionalized beads. Yet, blocking phagolysosomal acidification only reduced T-cell proliferation with macrophages exposed to beads with an acid-labile-linker and not to covalently-linked beads. These sugar-modified reagents show that oligosaccharides alone can drive T-cell proliferation by acidification-requiring presentation, most significantly in NKT receptor (CD160)-restricted T cells.

Protozoan parasites of the genus *Leishmania* are intracellular pathogens capable of surviving inside macrophages, contributing to their unusual persistence even in the face of robust immune responses.^[1] This unique survival tactic employed by the parasite has also foiled attempts to design effective vaccines to prevent Leishmaniasis in the 98 countries worldwide where it is endemic.^[2] The role of the lipid portion of the lipophosphoglycan (LPG) in maintaining the virulence of the pathogen is well documented,^[3] but evidence for any specific role of the carbohydrates present in LPG was only recently discovered.^[4,5] Our studies showed that synthetic carbohydrates alone, in the appropriate context, are sufficient to modulate innate immune responses mediated by macrophages by TLR2-dependent pathways.^[4,5] Latex beads coated with pathogen-associated oligosaccharides served as “artificial pathogens” that altered the innate immune response based on the sugars present.^[4,5] However, the potential role of these sugars in influencing T-cell-based responses remains

unclear. Latex beads are a common platform for biologists to create multivalent cell-mimicking displays by covalent conjugation of the polymeric particles to specific molecules.^[4–7] However, these stable linkages are not amenable to release of the conjugated compounds from the bead for subsequent processing within the phagolysosomal compartment for presentation to T cells, including CD1d-restricted NKT cells. Herein we report the design and synthesis of glycodendrimer structures with acid-labile linkers as reagents to test for the possibility that upon acidification-based cleavage in macrophage phagolysosomes, oligosaccharides can trigger T-cell responses.

We set out to design a spacer between the sugars and the bead that would be stable under neutral conditions outside the cell, but would be hydrolyzed upon contact with an environment below pH 5.0, which is the pH of an activated phagolysosome.^[8,9] In this context, the challenge was creation of a sugar-containing structure sized similarly to the MHCII or CD1 binding pockets. Binding of molecules to these antigen-presenting receptors is crucial for a T-cell response and depends on size and polarity.^[10] To address this issue, a small dendrimeric core was designed that could bring together three separate oligosaccharides. With this design, we hypothesized that after macrophage-mediated phagocytosis of the beads and subsequent phagolysosomal acidification, glycodendrimers could be released and presented to T cells. The glycodendrimer-coated beads should then promote increased T-cell proliferation compared to uncoated beads (Scheme 1). If acidification is blocked, glycodendrimers are not antigen-presented to T cells, proliferation secondary to this presentation is ablated (Scheme 1). It is possible that T-cell proliferation can be driven by non-antigen presentation specific signals including the cytokine IL12.

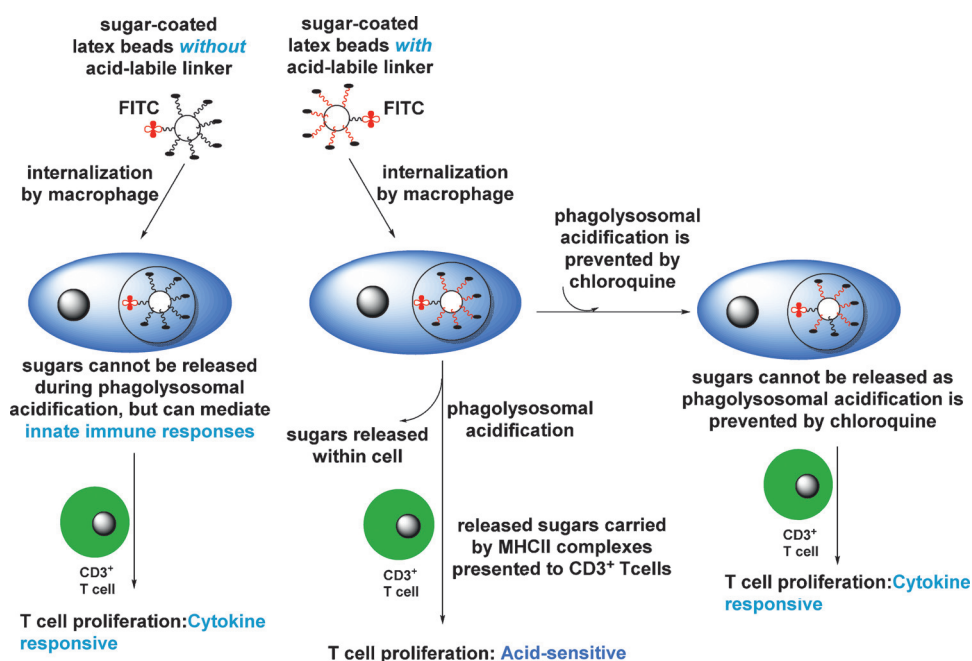
Our previous studies^[4,5] indicated that a specific oligosaccharide, 1,2-linked- α -mannotrioxide, altered the innate immune response to *Leishmania* infection. Additional studies looked at antibody production to this and other oligosaccharides^[11] Strong antibody responses were found to the trimeric 1,2-linked- α -mannopyranoside^[5] as well as a tetrasaccharide. This tetrasaccharide was shown to be a very minor component of most *Leishmania* surface coats^[12] and thus was left out from our studies. Along with alpha-linked mannotrioxides, alpha-i-mannobiosides are major components in the lipophosphoglycan of *Leishmania* parasite as well and therefore made obvious synthetic targets.^[13] Since little antigen presentation dependent response would be expected from an individual, covalently linked alpha-mannobioside or -trioside coating each bead, a dendrimeric linkage **3** (Figure 1) was placed between the acid-labile linker and the sugars. Upon presen-

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Scheme 1. Proposed T-cell proliferation pathway caused by latex beads linked to multiple mannobioside and -trioside dendrimers via an acid-labile linker.

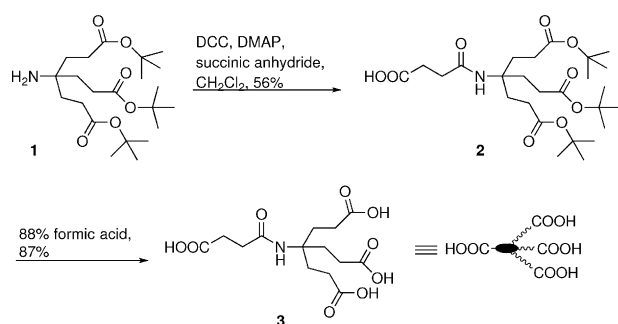


Figure 1. Synthesis of dendrimer **3**. DCC = Dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine.

tation to T cells, a significant immune response could be generated owing to the release of a glycodendrimer as an antigen to bind to MHCII or CD1d. Specific attention was given in the plan for this dendrimer to ensure its water solubility as the coupling reactions needed to be carried out in aqueous medium to preserve the integrity of the commercially available fluorescein isothiocyanate-associated latex beads. To this end, **2** was prepared using modified previously reported methods with the purification carried out by crystallization rather than the previously reported chromatographic separation procedure, starting from the commercially available amine **1** (Figure 1).^[14] In this way, a new water-soluble dendrimer **3** was obtained by acid hydrolysis of **2**.

To avoid future costly toxicology studies, a known and well-studied linker was sought to attach our glycodendrimer display. A water-soluble symmetric acid-labile linker with two amine groups at the terminal was ideal to allow robust amide coupling reactions to attach all fragments together. Inspired by examples of acid-labile linkers designed for target specific

delivery or pH-related studies,^[15] we chose compound **4** (Figure 2) as a linker. This structure is known to degrade completely at pH 5 within 16 h, the pH and timescale found in the phagolysosome when processing cargo.^[15b] Compound **4**^[15] and the requisite carboxylic acid-terminated mannobiosides and manno-triosides were synthesized as previously reported.^[4,16]

With all fragments in hand, including the commercial latex beads derivatized with carboxylic acid groups (microspheres with 1 μm diameter to mimic the size of the parasite and a fluorescent green FITC (fluorescein isothiocyanate) label to allow use of a common immunofluorescent assay to track bead uptake by macro-

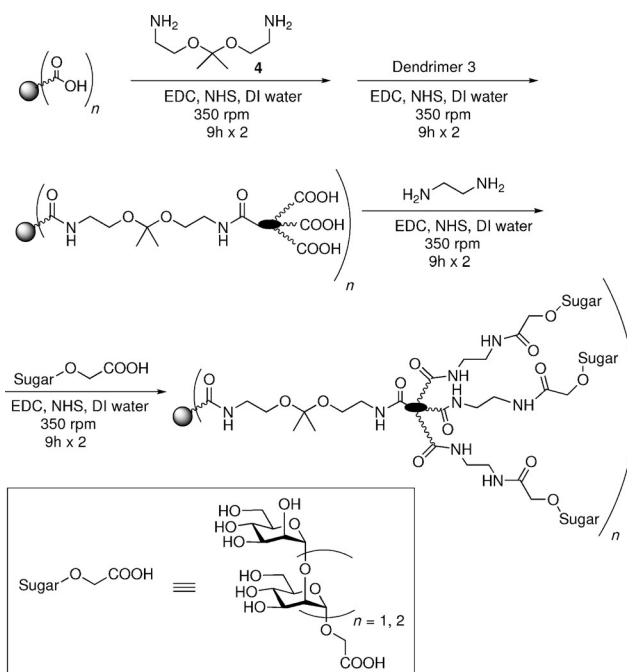


Figure 2. Synthesis of dendrimeric *Leishmania* capsular sugar structures on latex beads linked via an acid-cleavable linker. EDC = 1-ethyl-3-[3-dimethylamino-propyl]carbodiimide hydrochloride, NHS = N-Hydroxysuccinimide, DI = deionized.

phages), the final construction of the desired reagent commenced (Figure 2). All fragments were attached to latex beads sequentially using 1-ethyl-3-[3-dimethylamino-propyl]-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS)-mediated amide coupling reactions to enable by-products as well as excess reagents to be easily washed from

the beads with deionized water.^[17] The completion of amide couplings was monitored by a Kaiser test^[18] at each step and each coupling step was carried out twice to attain maximum coupling efficiency.

Once the acid-modifiable glycodendrimer bead reagents were generated, we set out to test their ability to mediate immune T-cell responses via measurement of T-cell proliferation. Activated macrophages derived from bone marrow (BMM) were incubated with uncoated (Un), acid-labile linked α -1-2-mannobioside dendrimer (BA) and mannotriose dendrimer (TA) coated beads and covalently linked di- and tri- α -1-2-mannobioside (BC) and mannotriose (TC) coated beads to evaluate bead uptake (Figure 3). Fluores-

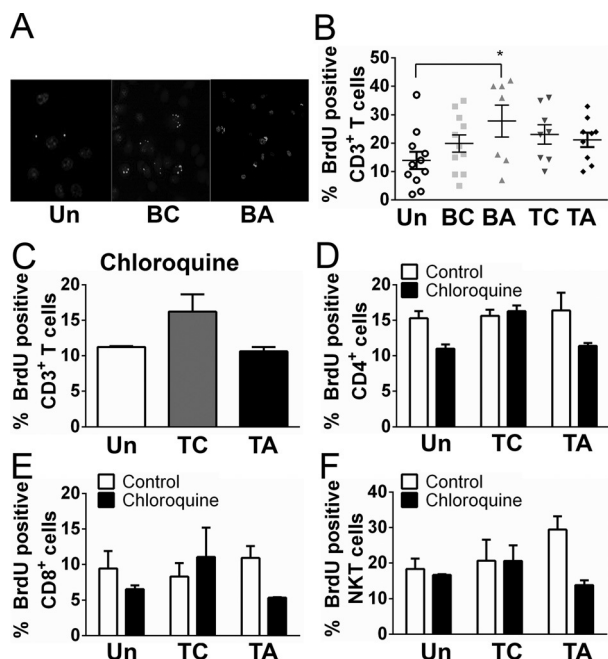


Figure 3. A) Fluorescence microscopy of bead uptake by activated bone marrow-derived macrophages (BMM). B) CD3⁺ T-cell proliferation after three-day co-culture with activated BMM and beads. $n=3$ experiments in duplicate or triplicate. C) CD3⁺ T-cell proliferation after chloroquine pre-treatment of activated BMM then co-cultured as in B. D) CD4⁺ T cell, E) CD8⁺ T cell, and F) NKT cell proliferation with cells treated as in C. Duplicate samples for C to F. Beads: Un = uncoated, BC = mannobioside covalent linkage, BA = mannobioside dendrimer acid-labile linkage, TC = mannotriose covalent linkage, TA = mannotriose dendrimer acid-labile linkage. Error bars denote $\pm$ SE, * $p \leq 0.05$ via Student's t -test.

cence microscopy revealed that differences in uptake occurred within the first 2–4 h of exposure (Figure 3A) but were equivalent by 48 h.

To evaluate T-cell proliferation in response to oligosaccharides, Un, BC, BA, TC, or TA beads were added to BMM. After 2 h, all non-internalized beads were removed and CD3⁺ T cells added. CD3⁺ T cells represent the collective CD4⁺, CD8⁺, NKT cell populations. T-cell proliferation was measured by BrdU incorporation after 3 days. Total CD3⁺ proliferation was increased in T cells co-cultured with macrophages exposed to both covalently linked mannotriose^[19] and mannotriose dendrimer beads with acid-labile linkers (Figure 3B). Macrophage exposure to uncoated beads pro-

moted limited basal co-cultured T-cell proliferation, indicating a role for multivalent sugars beads in promoting T-cell proliferation (Figure 3B). T-cell proliferation can be driven by specific antigen presentation, but also by non-antigen presentation specific signals including the cytokine IL12.^[20] Our previous studies^[4,5] demonstrated that IL12 was produced by macrophage interaction with oligosaccharides. To distinguish the importance of antigen presentation within an acidified phagolysosome vs. non-specific proliferation, we blocked lysosomal acidification with chloroquine (Scheme 1). Chloroquine-pre-treated macrophages induced decreased total CD3⁺ T-cell proliferation in response to TD (Figure 3C). Proliferation for the total CD3⁺ T-cell population was analyzed at the levels of CD4⁺, CD8⁺, and NKT cell populations (Figure 3D–F). A decrease in proliferation trending towards significance ($p=0.0585$) was observed in TD-presented to NKT cells (Figure 3F). CD1, the cognate receptor restricted to NKT cells, has previously been shown to present diverse glycolipid antigens.^[21] Our glycodendrimers mimic the size of CD1 glycolipid antigens demonstrating the immunostimulatory nature of *Leishmania* LPG sugars in the absence of lipids or proteins. These data conclusively show that oligosaccharides drive T-cell proliferation by acidification-requiring presentation, most significantly in NKT receptor (CD160)-restricted T cells (Figure 3).

Latex beads linked to glycodendrimers with an acid-labile linker showed efficient bead uptake by macrophages. Previous groups have indicated that *Leishmania* sugars conjugated to proteins could induce B cell responses, specifically antibody production.^[22] Zwitterionic polysaccharides have been shown to be processed as antigens and presented via MHC II to CD4⁺ T cells,^[23] but neutral carbohydrates are commonly thought to be poorly immunogenic. In this study, for the first time, we have successfully shown that carbohydrates alone, without any carrier proteins or lipids as seen with glycoconjugate vaccines,^[23e] were sufficient to induce T-cell proliferation after macrophage presentation. Further studies are underway to demonstrate the role of specific carbohydrate antigen-presentation during in vivo *Leishmania* infection.

Keywords: biological activity · carbohydrates · immunoassays · immunology · *leishmania*

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