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Synthesis of multivalent tuberculosis and *Leishmania*-associated capping carbohydrates reveals structure-dependent responses allowing immune evasion

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Abstract

Mycobacterium tuberculosis and the protozoan parasites of the genus *Leishmania* are intracellular pathogens that can survive in macrophages—the very white blood cells of the immune system responsible for engulfing and ultimately clearing foreign invaders. The ability of these pathogens to hide within immune cells has made the design of effective therapies, including vaccines, to control tuberculosis and leishmaniasis particularly challenging. Herein we present the synthesis and development of carbohydrate-based probes to demonstrate that only changes in pathogen-associated surface oligosaccharides are sufficient to change cellular immune responses and thereby let a pathogen hide from immune surveillance.

Mycobacterium tuberculosis and the protozoan parasites of the genus *Leishmania* are intracellular pathogens that can survive in macrophages—the very white blood cells of the immune system responsible for engulfing and ultimately clearing foreign invaders. The ability of these pathogens to hide within immune cells has made the design of effective therapies, including vaccines, to control tuberculosis and leishmaniasis particularly challenging. Tuberculosis claims two million deaths annually worldwide¹; leishmaniasis is endemic over much of 88 countries in Africa, India, southern Europe, and Central and South America.² *Mycobacterial* glycolipid mannosylated lipoarabinomannan (ManLAM, Figure 1) is a major contributor to *M. tuberculosis* evasion and subversion of host immune responses.³ All pathogenic strains of *Mycobacterium* (*M. tuberculosis*, *M. leprae*), as well as the vaccine strain BCG, bear caps consisting of either a dimannoside or a trimannoside. This mannose cap is a major structural element engaged in receptor binding due to its prominent external location and is important in determining immunopathogenesis of *Mycobacteria* spp.³ The cytokine IL-12 is critical for production of a productive T helper type 1 immune response against either *M. tuberculosis* or *Leishmania* spp. Treatment of cells with ManLAM from either *M. tuberculosis* or BCG results in very limited IL-12 production leading to a non-polarized, mild immune response.⁴ ManLAM IL-12 inhibition was found to correlate with the presence of mannose caps or glycosylphosphatidylinositol (GPI) acyl residues.^{4b} Oligotriose and dipalmitoylphosphatidylethanolamine (Man 3-DPPE) treatment was found to induce IL-12 production in peritoneal macrophages.⁵ Although many groups have documented the role of the lipid portion of these glycolipids in pathogen virulence⁶ and

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Supporting Information Available: Synthetic protocols, assay conditions, and NMR spectra.

enzymatic cleavage of various mannan and glucan structures have been shown to alter various immune responses⁷, herein we present the first demonstration that a synthesized trimannose cap sugar of ManLAM *alone* is able to alter IL-12 production of macrophages via a Toll-like receptor (TLR)-2 dependent manner. We present the synthesis and development of new chemical tools to demonstrate that different surface carbohydrates are sufficient to change cellular immune responses and thereby let a pathogen hide from immune surveillance.

One of the most abundant molecules found on the cell surfaces of bacteria, parasites and viruses are carbohydrates.⁸ Therefore, we reasoned that carbohydrates could be at least partially responsible for differences in immune responses seen between virulent and non-virulent pathogens. We envisioned making “artificial pathogens” of approximately the same size (one micron) as *Leishmania* and *M. tuberculosis* that are coated only with the well-defined carbohydrate capping structure associated with these pathogens to compare the difference in cellular response when only the carbohydrate coat is altered. Such clean comparison experiments are impossible to do through genetic modification of the carbohydrate coat of the pathogens themselves because the gene products responsible for synthesis of the capped structures also have other functions.⁴

Alpha-linked trimannose is one of the major components both in the outer lipophosphoglycan (LPG) of *Leishmania* parasites¹⁰ and the lipoarabinomannan (LAM) of *M. tuberculosis* cell walls¹¹ and therefore makes an excellent synthetic target (Figure 1). To date, several synthetic approaches have been reported to construct *Leishmania* capping structures including oligomannose and branched oligosaccharides primarily for vaccine development.¹² We required a route to the trimannose structure that would allow a handle at the reducing end of the sugar to attach it to a polymeric bead for a multivalent display. The known trimannoside **2**^{12c} was then prepared using fluorous solid-phase extraction (FSPE)-based iterative synthesis from mannose trichloroacetimidate **1** (Figure 1). The fluorous-tag was cleaved by ozonolysis; oxidation with Jones reagent provided a carboxylate handle for further coupling with amine-functionalized beads. Global deprotection with sodium in ammonia at -78 °C furnished the desired fully deprotected trimannose **4** in good yield. As a control, lactose was prepared with the same linker by a similar sequence from the known allyl lactoside.¹³

With the capping sugar and the control sugar lactose in hand, a suitable one micron-sized support was required for multivalent display of these sugars. Latex beads are commonly used in immunoassays due to their inertness and commercial availability. Moreover, beads with high concentrations of imbedded fluorophores enable various fluorescent assays.¹⁴ For example, such beads have been utilized for agglutination tests for the detection of antibodies or investigation of heparin-binding properties, for the analysis of blood cell populations, and for the identification of specific cell membrane markers.¹⁵ Commercially available latex beads **B0** (microspheres, 1 μ m diameter and yellow-green fluorescent) derivatized with carboxylate groups (3.5×10^{-4} mmol carboxylate groups per mL) were chosen because the size of these beads mimics the size of the *Leishmania* and *M. tuberculosis* pathogens and the FITC-label allows use of a common immunofluorescent assay for the observation of bead uptake by macrophages. In order to avoid spatial proximity of sugars on the surface of the beads and improve the accessibility of sugars to possible macrophage binding partners, an ethylenediamine spacer was attached to the carboxylated bead surface under standard peptide coupling conditions (Scheme 1). Although numerous coupling reagents¹⁶, including carbodiimides, phosphonium-based reagents, and aluminum-based reagents, have been utilized for amine coupling reactions, restrictions such as stability of generated intermediates, by-products, and cost determine the choice of reagent for solid-phase synthesis under aqueous conditions. Among the relatively inexpensive carbodiimide

coupling reagents, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) mediated coupling reactions with *N*-hydroxysuccinimide (NHS)¹⁷ have the advantage of formation of a relatively stable NHS-ester intermediate and also allow easy purification to eliminate urea by-products or excess EDC/NHS. A combination of EDC and NHS in deionized water proved successful for the synthesis of the necessary amide linkages (Scheme 1). The subsequent coupling reaction of carboxymethyl-trimannose and -lactose with bead-linked amine spacer gave the desired beads **B1** and **B2**. Each coupling step was repeated twice with 30 to 50 equivalents of the sugar to ensure complete reaction. The density of sugar was calculated by the given density of the carboxylates (1.296×10^{-14} mmol/bead). In addition, the Kaiser colorimetric test was used as an indicator of coupling reaction progress.¹⁸ Finally, a phenol-sulfuric acid assay was also used to confirm sugar loading densities on the bead surfaces.¹⁹

With the two desired beads in hand, we examined the effect of the sugar-coated beads on the inflammatory response mediated by macrophages. Murine macrophages were first stimulated by the known activational molecules, lipopolysaccharide (LPS) and interferon gamma (IFN γ). Lactose-coated beads **B1** and trimannose-coated beads **B2** were incubated with the stimulated macrophages and the levels of the proinflammatory cytokine interleukin-12 (IL-12) produced was measured 24 hours post exposure.²⁰ IL-12 plays a significant role in the link between innate and adaptive immunity²¹ (Figure 2). Comparable levels of IL-12-p40, the regulated portion of the bioactive IL-12p70, were produced when macrophages were exposed to lactose-coated beads **B1** and the sugar-free beads **B0**. In contrast, the presence of the trimannose-coated beads **B2** significantly diminished IL-12p40 production. *The innate immune response was significantly dampened to normally stimulatory signals only by the change of the carbohydrate structure presented on the bead.*

To test for the possible mechanism of this dampening effect, the macrophages were treated with antibodies against the extracellular lectin binding domain of Toll-like receptor 2 (TLR2). TLR2 is known to play a crucial role in pathogen recognition and activation of innate immune responses.²² Blocking TLR2 eliminated the differences in production of IL-12p40; there was no difference between the trimannose-coated or negative control coated beads. Isotype IgG1 antibodies were used as a control to demonstrate that the effect was specific to antibodies against TLR2. The possibility of recognition via mannose receptors^{4b} was also investigated via antibody inhibition and was not found to have a significant effect in leading to alteration of IL-12p40 production (data not shown). This data clearly implicates a TLR2-mediated pathway for this newly discovered trimannose-mediated immune suppression.

In conclusion, the synthesis of pathogen mimics with well-defined carbohydrate coats allowed the first demonstration that carbohydrates alone in the appropriate context are sufficient to dampen innate immune responses mediated by macrophages via TLR2-dependent pathways. The revelation that these carbohydrate moieties produce immune response alteration is critical to the production of the next generation immunomodulators and vaccines against complex pathogens such as *M. tuberculosis* and *Leishmania* and provide a good model system for dissecting and unveiling innate immune mechanisms induced by specific carbohydrate structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
 Capping structure in lipoarabinomannan (ManLAM) of *M. tuberculosis* and
 lipophosphoglycan (LPG) of *Leishmania*.

Figure 2.

Effect of cap sugar coated-latex beads (B0 = latex bead, B1 = lactose coated beads, and B2 = trimannose-coated beads) on the production of IL-12p40 by murine macrophages, J774 cells, stimulated by LPS and IFN γ . [Asterisks denote a significant change between the TLR2 blocking from control isotype and/or non-blocking-stimulation only treated cells ($p < 0.05$) via Student's t -test.] For comparison, no stimulation resulted in 200 ± 50 ng/mL IL-12p40 production. Stimulation alone had the same affect as stimulation with the latex bead (B0).

Scheme 1. Synthesis of multivalent tuberculosis and *Leishmania* capping structures on latex beads.