Virus-like particle display of the α-Gal carbohydrate for vaccination against *Leishmania* infection

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Supporting Information

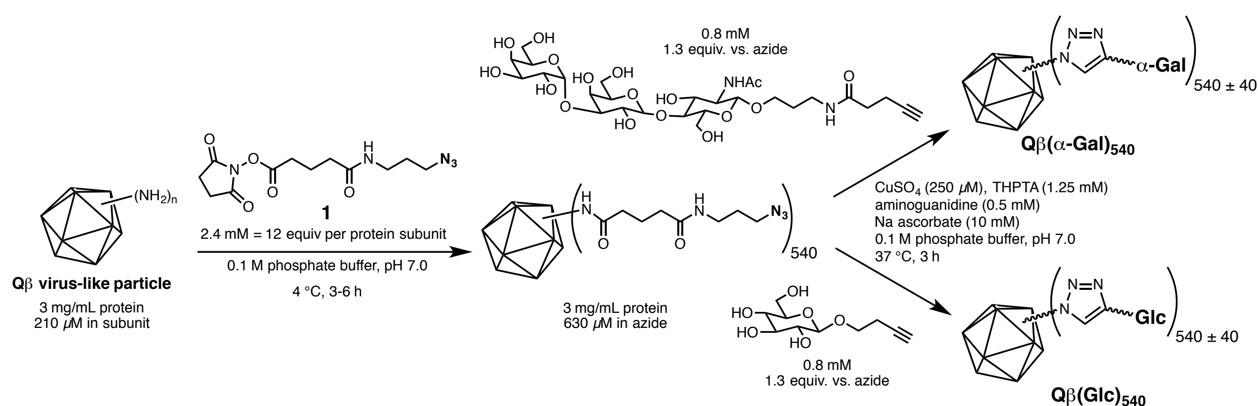
***Leishmania* parasites.** Promastigote forms of reference strains PH8 for *Leishmania amazonensis* (IFLA/BR/1967/PH8), and BH401 for *Leishmania infantum* (MCAN/BR/2002/BH401) were used. The parasites were initially isolated from hamsters, *Mesocricetus auratus*, that had been infected for two months. *L. amazonensis* promastigotes were isolated from injured lesion in the muzzle and *L. infantum* isolated from spleen fragments and grown in the NNN (Novy, McNeal and Nicolle) medium with Schneider's (SIGMA®), supplemented with 2% urine, 1% vitamin solution (BME Vitamins 100x-SIGMA®), 1% L-glutamine (200 mM), 10% bovine fetal serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco®). The cultures were maintained in BOD at a temperature of 23° C ± 1 °C. After expansion, the promastigote cells were preserved in a solution containing Middle Schneider's medium (SIGMA®), 10% Glycerol, and 20% of BSA, and the strains were kept in liquid nitrogen at "Laboratorio Fisiologia de Insetos Hematófagos – LFIH" Parasitology Department, Federal University of Minas Gerais, until use. Parasite species were confirmed by PCR/RFLP as described previously.[1]

Mice Vaccination and infection. Groups of 3-5 female αGalT-KO mice[2] were immunized with 10 µg Qβ-α-Gal once per week for three weeks; control groups were vaccinated with 10 µg of unmodified Qβ virus-like particles in PBS. All injections were subcutaneous, the first dose at the base of the tail (rump) and the following doses in the flank. One week later (week 4), the antibody levels were checked in serum by ELISA against Qβ-α-Gal (with minimal response against Qβ-Glu, showing that the protein response was blocked or not detected). All immunized mice were then subjected to infectious challenge. Parasites for infection of mice were prepared using parasites in stationary phase. Parasites from culture were washed 3X with PBS, centrifuged for 5 min to 3,000 g and then resuspended in 1 x sterile PBS with final volume required to inoculate 10 µL of *L. amazonensis*, and 200 µL *L. infantum* per animal, each delivering a dose of $1x10^7$ promastigote cells. Uninfected inoculation-control mice were administered the same volumes of sterile saline in the same manner.

Protein extracts. Total protein extracts were prepared from $1x10^8$ of promastigote form of *L. infantum* and *L. amazonensis* parasites. The parasites were centrifuged at 2000 g for 10 min, and washed twice with phosphate buffered saline (PBS) and suspended in 3 mL of Lysis buffer [20 mM of Hepes, 10 mM of KCl, 1.5 mM of MgCl2, 250 mM of sucrose, 1 mM of DTT, 0.1 mM PMSF and 300 µl protease inhibitor cocktail (GE Healthcare, Piscataway, USA)], submitted to 5 cycles of freezing (liquid N_2) and thawing (42° C). Then, the extracts were subjected to centrifugation at 8000 g for 20 min at 4°C. The supernatant was collected and stored at -70° C for upcoming experiments.[3] The protein samples were dosed by colorimetric method of bicinchoninic Acid (BCA), using the kit "BCA Protein Assay Reagent" (Thermo Scientific, Waltham, USA) according to the manufacturer's recommendations.

α-Gal antigen linked to Qβ-virus like particle and conjugate preparation. Qβ virus-like particles were prepared and purified as described previously;[4] details of VLP production and purification are given elsewhere.[5] All particles were characterized by size-exclusion chromatography, dynamic light scattering (Wyatt DynaPro), microfluidic gel electrophoresis (Agilent Bioanalyzer 2100, using Protein 80 chips), and electrospray ionization mass spectrometry on an accurate-mass time-of-flight instrument (Agilent G6230B); representative samples were further examined by transmission electron microscopy and multi-angle light scattering (Malvern Viscotec). In all cases, standard properties of size and composition were observed, with the particles showing narrow size distributions and high protein purity (less than 5% protein impurities detected). Protein concentrations in solution were measured with the BCA method (Protein Reagent kit, Pierce, USA), standardized with bovine serum albumin. For conjugate preparation, α-Gal trisaccharide (α-Gal-OH, Carbosynth US, LLC, San Diego, CA; this compound was also made in house as previously described[6]) and glucose were converted to their respective alkyne derivatives by Lewis acid-mediated glycosylation of 3-butyn-2-ol. Each alkyne was attached to Qβ virus-like particles by a two-step procedure in which the protein nanoparticle was first acylated with an azide-terminated N-hydroxysuccinimide ester and then addressed by copper-catalyzed azide-alkyne cycloaddition.[7]

For convenience, the synthetic procedures are summarized here:



All particles are approximately 30 nm in diameter (dynamic light scattering); the product particles are very similar in physical properties (effective charge as determined by native gel electrophoresis; size as determined by dynamic light scattering and size-exclusion chromatography)

ELISA for α-Gal epitope detection. High-binding ELISA plates (NUNC) were coated (overnight at 4° C, or 1 h at room temperature) with 10 µg/mL of *L. infantum* or *L. amazonensis* extracts (50 mM carbonate-bicarbonate buffer, pH 9.5). In place of the extracts, the Qβ-α-Gal antigen (2 µg/mL) was used as a positive α-Gal-displaying control; Qβ-Glc was used as negative control. After incubation and washing, free microplate binding sites were blocked with 2% bovine serum albumin (BSA, Sigma Aldrich) in phosphate buffered saline (PBS), pH 7.4. Each well was then incubated with one of two α-Gal binding reagents: (a) purified polyclonal mouse IgG anti-α-Gal antibody obtained as described elsewhere[3] (2 µg/mL), or (b) mushroom *Marasmius oreades* (MOA) lectin-HRP conjugate (EY Laboratories), which binds specifically to blood group B and terminal Gal-α1,3-Gal residues (5 µg/mL). After washing, each anti-α-Gal well (category a) was treated with 50 µL biotinylated anti-mouse IgG (1:2500 dilution in PBS with 2% BSA, Amersham, GE Healthcare Life Sciences, UK), washed, and then with 50 µL streptavidin-horseradish peroxidase conjugate (1:4000 dilution, Amersham, UK) in PBS-BSA. All incubation steps were performed at 37 °C for 1 h. All wells were developed with 100 µL of peroxidase substrate SigmaFast™ OPD (o-phenylenediamine dihydrochloride and urea hydrogen peroxide, Sigma-Aldrich) with quenching of the reaction by addition of 2N sulfuric acid. Absorbance measurements were performed in a Multiskan GO instrument, using SkanIt 3.2 software (Thermo Scientific). To determine antibody specificity, the VLP conjugates were placed on 96 wells plate and treated overnight at 28ºC with 0.1 U/well of α-galactosidase enzyme (from green coffee beans, Sigma-Aldrich G8507). After the incubation, the ELISA was performed as described.

ELISA for anti-α-Gal antibody detection. Serum samples from immunized mice were evaluated using the same ELISA procedure as above, coating the plates with Qβ-α-Gal (5 µg/mL). After incubation with serum samples (1 h at 37 °C) and washing, monoclonal goat anti-mouse IgG (Amersham) HRP conjugate was used as secondary antibody (1:4000 dilution), followed by peroxidase substrate and detection as above. To further validate that the observed response is specific to the α-Gal sugar, the Qβ-α-Gal reagent was plated and then treated overnight at 28ºC with 0.1 U/well of green coffee bean α-galactosidase (Sigma G8507). After the incubation, ELISA analysis gave little to no signal above background.

Source of samples for DNA extraction. Mouse tissue were collected 10 weeks post-infection from spleen and liver of C57BL6 KO or WT mice infected by *L. infantum* (MCAN/BR/2002/BH401) and *L. amazonensis* (IFLA/BR/1967/PH8). The infection was performed as described above and naive mice were used as controls. Tissues were removed by using different scissors or scalpels to avoid cross-contamination and were minced with Potter grinders and then carefully homogenized in 1.5-ml microtubes with single-use blue pellet pestles (Polylabo, Paris, France) in phosphate-buffered saline. Aliquots of the homogenates were stored at -80°C until DNA extraction. DNA extraction was performed using the "Genomic DNA from tissue" kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's recommendations. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Quantitative PCR. Extraction of DNA and evaluation of spleen parasite load by qPCR. DNA was extracted from liver and spleen samples using NucleoSpin®Tissue (Macherey-Nagel) according to the manufacturer's instructions. The parasite load was calculated by qPCR according to a method described

elsewhere with minor modifications.[8,9] The parasite burdens were estimated using the following primers: Forward, 5'-TGTCGCTTGCAGACCAGATG-3' and Reverse, 5'-GCATCGCAGGTGTGAGCAC-3'. These primers amplified a 90 bp fragment of a single-copy-number Leishmania DNA polymerase gene (GenBank: AF009147). The host's housekeeping β-actin gene was used as endogenous control in order to normalize initial DNA concentrations and to verify sample integrity. The primers used to amplify a 307-bp fragment of β-actin were as follows: Forward, 5′ CTTCTACAACGAGCTGCGCG 3′ and Reverse, 5′ TCATGAGGTAGTCGGTCAGG. PCR was carried out in a final volume of 10 μL containing 2 pmol of each DNA polymerase primers, SYBR®Green (Applied Biosystems), 4 μL of DNA with a concentration of 5 ng/μL and enough volume of ultrapure water. Reactions were processed and analyzed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The following steps were programmed: 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Parasite quantification for each spleen sample was calculated by interpolation from the standard curve included in the same run, performed in duplicate, and expressed as the number of parasites per 100,000 of host cells.

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