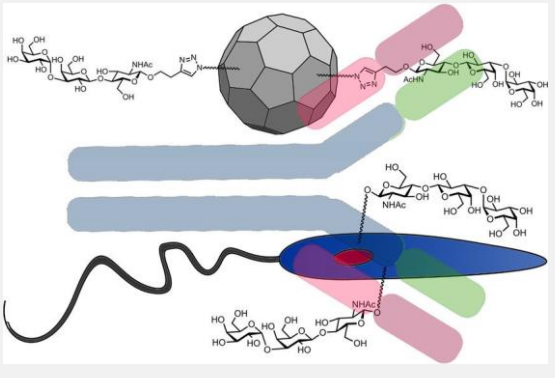
Leishmania vaccine design

Mohammad Nouri aliabadi and m Cheraghi and Jalili
Contact: m.nouri2000@yahoo.com
Iran _tehran Pharmaceutical and Biotechnology Technology Center

ABSTRACT: Secreted and surface-displayed carbohydrates are essential for virulence and viability of many parasites, including for immune system evasion. We have identified the α-Gal trisaccharide epitope on the surface of the protozoan parasites *Leishmania infantum* and *Leishmania amazonensis*, the etiological agents of visceral and cutaneous leishmaniasis, respectively, with the latter bearing larger amounts of α-Gal than the former. A polyvalent α-Gal conjugate on the immunogenic Qβ virus-like particle was tested as a vaccine against *Leishmania* infection in a C57BL/6 α-galactosyltransferase knockout mouse model, which mimics human hosts in producing high titers of anti-α-Gal antibodies. As expected, α-Gal-T knockout mice infected with promastigotes of both *Leishmania* species showed significantly lower parasite load in the liver and slightly decreased levels in the spleen, compared with wild-type mice. Vaccination with Qβ−α-Gal nanoparticles protected the knockout mice against *Leishmania* challenge, eliminating the infection and proliferation of parasites in the liver and spleen as probed by qPCR. The α-Gal epitope may therefore be considered as a vaccine candidate to block human cutaneous and visceral leishmaniasis.



sand flies, which regurgitate the parasite into the skin of their vertebrate hosts.[1] *Leishmania* species cause a spectrum of clinical symptoms[2,3] depending on the nature of the infecting strain and its interaction with the host immune system and other genetic factors.[4] In Mediterranean and South American countries, cutaneous leishmaniasis (CL) in humans is caused primarily by *L. mexicana*, *L. amazonensis*, and *L. braziliensis*, all of which induce ulcers localized to the skin. *L. amazonensis* is also responsible for diffuse cutaneous leishmaniasis, whereas *L. braziliensis*, in some circumstances, may cause lesions in mucosal surfaces. *Leishmania infantum* (in Europe, North Africa, and Latin America) and *Leishmania donovani* (in East Africa and the Indian subcontinent) are the causative agents of visceral leishmaniasis (VL),[5] in which the parasite spreads to internal organs and can be lethal if not treated.[6,7]

The carbohydrate-rich molecules found on the surface of *Leishmania* parasites include lipophosphoglycans, glycosylphosphatidylinositol-anchored proteins, glycosylphosphatidylinositol lipids, and proteophosphoglycans.[8] These glycoproteins are part of the glycocalyx of the promastigote infective form, playing an important role in infectivity[9,10] and sand fly interaction.[11] After transmission by blood-feeding sand flies of the genus *Lutzomyia* (in the New World), metacyclic *Leishmania* parasites interact primarily with phagocytic cells and then may be distributed to their resident tissues and organs, depending of the *Leishmania* species. While carbohydrates present on the parasite surface are likely to play an important role in mediating cell host adhesion,[12] the molecular mechanism of virulence of this parasitic disease is poorly understood.

Among the most interesting pathogen-related carbohydrates is the α-Gal epitope, a name given to both the Gal-α1,3-Gal disaccharide[13] and the Gal-α1,3-Gal-β1,4-GlcNAc trisaccharide;[14] we use the latter designation here. Recently, high levels of anti-α-Gal antibodies were detected in cured patients with cutaneous leishmaniasis,[15] raising the possibility that this highly immunogenic[16,17] structure could be present on the parasite

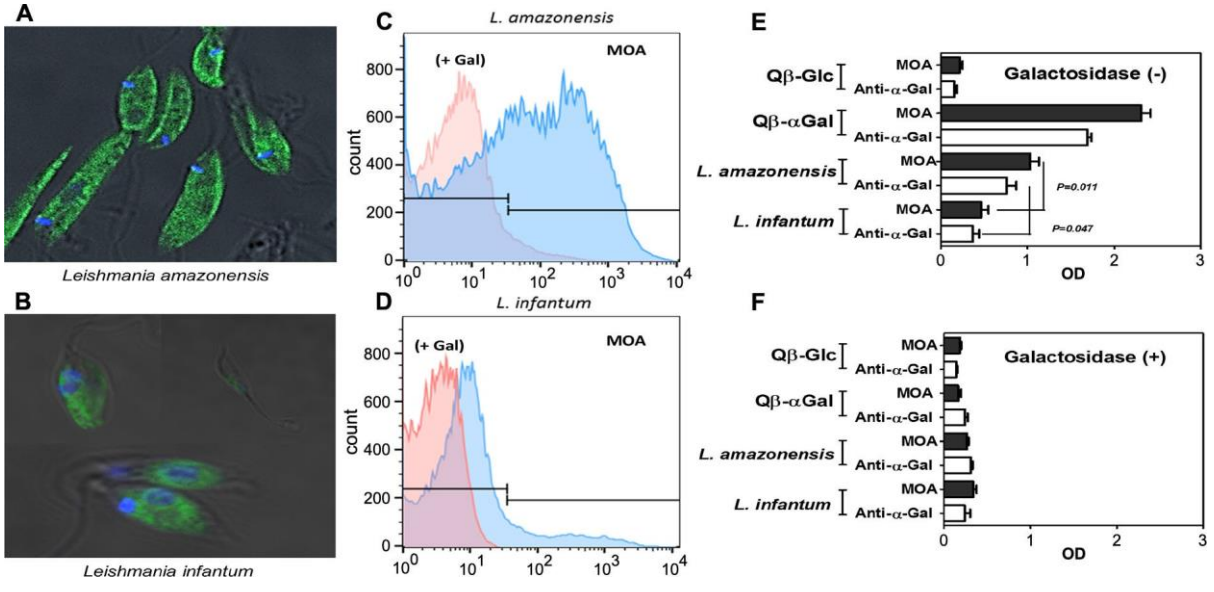


Figure 1. α-Gal epitope identification. (A, B) Fluorescence microscopy of *L. amazonensis* and *L. infantum* promastigote cells labeled with 3−5 μg/mL of FITC-labeled MOA (green = labeled MOA, blue = DAPI-stained nuclei, scale bar = 10 μm). (C−D) Flow cytometry (FACScan) of the cells treated as in panels A and B; *x*-axis = fluorescein fluorescence (488 nm excitation). "+ Gal" represents the same samples pretreated with 0.2 M galactose before addition of MOA-FITC. (E) ELISA performed with the indicated *Leishmania* extracts (10 μg/mL) as antigen, and biotinylated MOA (2 μg/mL) and anti-α-Gal antibody (3 μg/mL) as probe reagents. Qβ virus-like particles (2 μg/mL) bearing high density of either α-Gal or glucose were used as positive and negative controls, respectively. (F) ELISA as in panel E, with pretreatment of the antigens with green coffee bean α-galactosidase (overnight, 10 U/mL). All experiments were performed in triplicate.

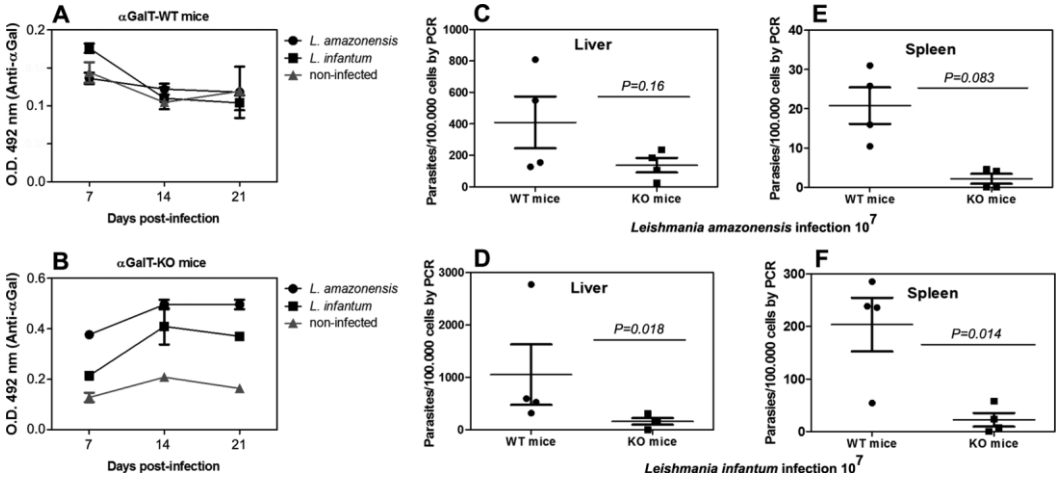


Figure 2. Infection with *Leishmania* parasites. (A) Anti-α-Gal serum IgG antibody levels in infected α-galactosyltransferase wild type mice (αGalT-WT, serum dilution 1/100). (B) Anti-α-Gal serum IgG antibody levels in infected α-galactosyltransferase knockout mice (αGalT-KO, serum dilution 1/100); same key as panel A. (C, E) Parasite load levels (qPCR) in liver and spleen samples of αGalT-WT and αGalT-KO mice infected with 10^7 *Leishmania amazonensis* cells. (D, F) Parasite load levels (qPCR) in liver and spleen samples of αGalT-WT and αGalT-KO infected mice with 10^7 *leishmania infantum* cells. Three or more independent experiments were performed in each group containing 3−7 mice.

surface, stimulating anti-α-Gal antibody production beyond the levels normally present in healthy individuals.[14,18] We describe here the identification of the α-Gal epitope on the promastigote form of *Leishmania amazonensis* and *Leishmania infantum*, the use of the α-galactosyltransferase knockout mouse (αGalT-KO) of Thall and colleagues[19] as a murine model for leishmaniasis, and a polyvalent α-Gal conjugate as an effective vaccine candidate against parasite infection in this model. Since only humans and higher apes among all mammals lack the α-galactosyltransferase gene,[20] the αGalT-KO mouse mimics the human immune system's ability to generate anti-α-Gal antibodies to a much greater degree than wild-type rodents. These mice have been used primarily in studies of xenotransplant rejection and fertilization,[21,22] but are also of great potential interest for studies of any pathogen that displays α- Gal.

To see if *Leishmania* falls into this category, we investigated the presence of the α-Gal epitope on the promastigote forms of *L. infantum* (MCAN/BR/2002/BH401) and *L. amazonensis* (IFLA/BR/1967/PH8). The *Marasmius oreades* agglutinin (MOA) lectin, which binds strongly to the α-Gal trisaccharide, the related type B branched trisaccharide, and the Gal-α1,3-Gal disaccharide (but not the isomeric α1,2-α1,4- and α1,6-disaccharides),[23,24] was used as the key reagent for these studies. Promastigote cells (10^8 per mL) of *L. infantum and L. amazonensis* were labeled with 3−5 μg/mL of fluorescein-

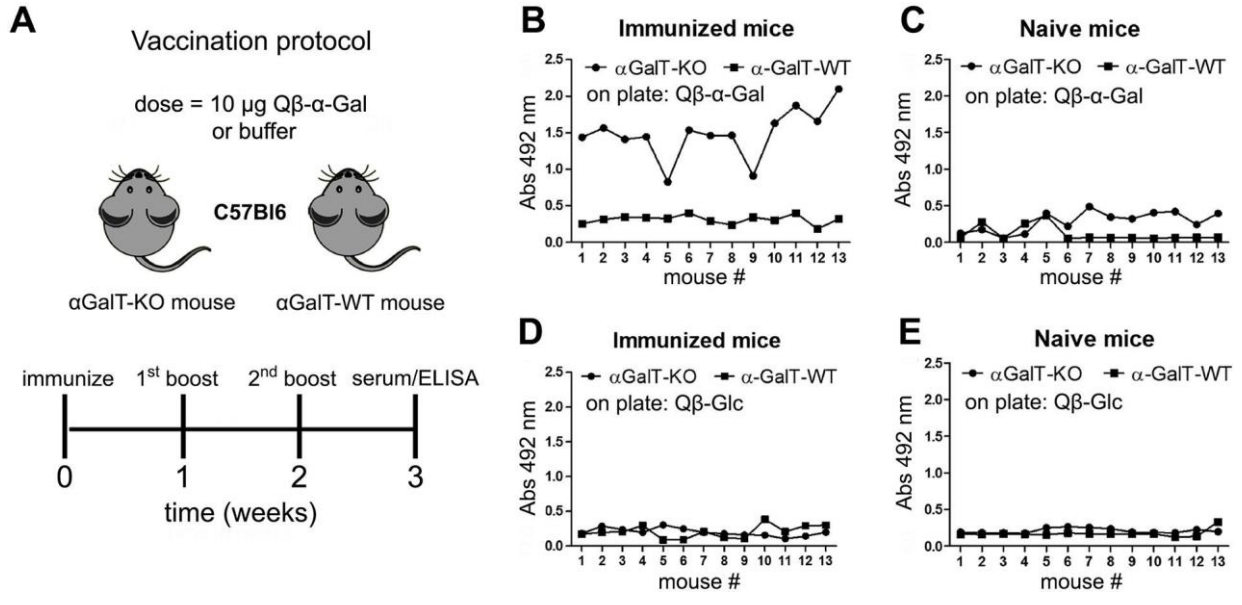


Figure 3. Anti-α-Gal IgG antibody production by mice vaccinated with Qβ–α-Gal antigen. (A) Protocol employing 13 mice per group. (B–E) ELISA for detection of serum antibodies (serum dilution 1/100); *x*-axis = each mouse in the group, arbitrarily numbered. (B) Serum from immunized mice, against Qβ–α-Gal immobilized on the ELISA plate; (C) serum from naive mice, against plated Qβ–α-Gal; (D) serum from immunized mice, against plated Qβ-Glc; (E) serum from naive mice, against plated Qβ-Glc.

labeled MOA and examined by fluorescence microscopy[25] and flow cytometry.[26] Strong staining of the *L. amazonensis* cell surface, compared to moderate staining of *L.* infantum, was observed (Figure 1A,B), which was confirmed by flow cytometry (Figure 1C,D). The addition of a large excess (0.2 M) of galactose largely abrogated binding, as expected. Lastly, *L. amazonensis* and *L. infantum* lysates were plated and incubated either with biotinylated MOA or purified anti-α-Gal antibody in an ELISA-type assay (Figure 1E).[27] As before, the presence of α-Gal was confirmed for both species, but to a greater degree for *L. amazonensis*. Preincubation with an α-galactosidase enzyme reduced binding by more than 90% (Figure 1F), showing that the terminal galactose residue is critical. Taken together, these results provide strong evidence that both *L. infantum* and *L. amazonensis* express the α-Gal epitope on their surfaces, the latter in greater amount or accessibility.

In visceral leishmaniasis, caused by *L. infantum,* parasites are able to escape from the host immune system and reach internal organs such as liver or spleen, whereas in cutaneous leishmaniasis *L. amazonensis* usually stay at the site of the infection. C57BL/6 mice have been used to study pathogenesis of experimental cutaneous and visceral leishmaniasis.[28] However, in spite of intense effort with this model, greater consistency is needed, particularly over multiple *Leishmania* strains and numbers of parasites used for infection. The presence of α-Gal on the parasite makes these experiments even less reliable as an indicator of the human response since α-Gal is a self sugar in these wild-type C57BL/6 mice.

We therefore compared the response of α-GalT-KO vs α-GalT-WT mice to intraperitoneal infection with 10^7 promastigotes of *L. infantum* and subcutaneously with the same number of *L. amazonensis*. Blood samples for ELISA were collected at days 7, 14, and 21; liver and spleen samples were collected for quantitative PCR (qPCR) 10 weeks postinfection. The *Leishmania*-infected α-GalT-WT produced levels of anti-α-Gal antibodies identical to uninfected controls, representing a negligible response against the α-Gal epitope (Figure 2A). In contrast, α-GalT-KO infected mice produced anti-α-Gal antibodies to a moderate extent, significantly greater than noninfected controls (Figure 2B). Infection with *L. amazonensis* induced a more intense anti-α-Gal response, consistent with its higher level of α-Gal display. Parasite loads in liver and spleen were assessed by qPCR, with significantly greater variability for α-GalT-WT mice than α-GalT-KO mice. Differences in liver infection at 10 weeks were not significant for either *Leishmania* strain between the two mouse models ($p > 0.05$, Figure 2C,E), but both parasites were found in the spleens of α-GalT-WT mice to a significantly greater extent than α-GalT-KO mice (Figure 2D,F).

These findings suggested that α-GalT-KO mice were more resistant than α-GalT-WT toward *Leishmania* infection, perhaps because of the anti-α-Gal antibodies produced by the knockout animals. The highly variable nature of the response of WT mice (panels C, D, E, F) is to be expected given the many factors that contribute to the extent of infection in the absence of a controlling factor, including parasite dissemination from the site of injection and the draining lymph nodes to other organs.[29−32] To further test the proposed role of an induced anti-α-Gal immune response, we immunized both mouse strains with the previously reported[33,34] Qβ virus-like particle bearing approximately 540 copies of the α-Gal trisaccharide, designated Qβ–α-Gal. We hoped that high anti-α-Gal antibody levels would be generated against the glycoconjugate, and that this response would protect these mice more effectively from *Leishmania* infection. The immunization protocol is shown in Figure 3A, in which groups of mice were immunized with 10 µg Qβ–α-Gal particles (containing approximately 1 µg of attached α-Gal) and boosted twice (at one-week intervals) with the same dose.

The immune response of these animals compared to nonimmunized control mice was assessed by ELISA against two reagents: the Qβ–α-Gal immunogen and an analogous particle (Qβ-Glc) bearing glucose moieties (approximately 500

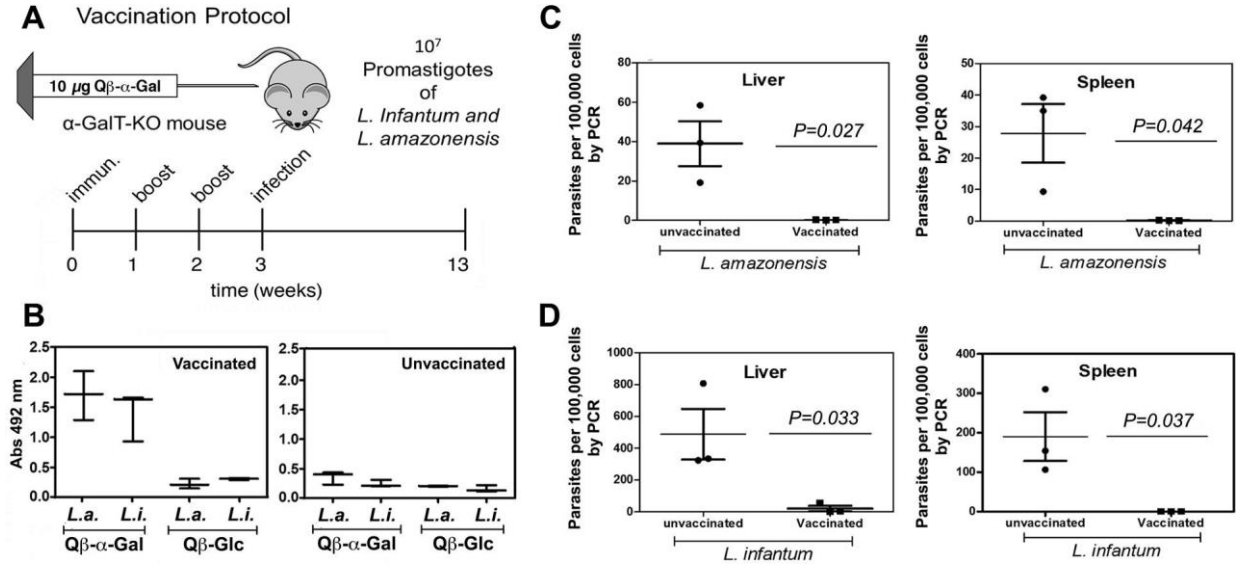


Figure 4. Protection from *Leishmania* infection in α-GalT-KO mice. (A) Vaccination and infection protocol. (B) Detection of anti-α-Gal IgG antibody response by ELISA at week 2.5, for animals immunized with Qβ−α-Gal vs Qβ-Glc. "L.a." = mice later infected with *L. amazonensis*; "L.i." = mice later infected with *L. infantum*. (C) qPCR determination of parasite load from liver and spleen of vaccinated and unvaccinated αGalT-KO mice infected subcutaneously with 10^7 promastigotes of *Leishmania amazonensis*. (D) qPCR determination of parasite load from liver and spleen of vaccinated and unvaccinated αGalT-KO mice infected intraperitoneally with 10^7 promastigotes of *Leishmania infantum*. Three or more independent experiments were performed in groups containing from 3 to 7 mice.

per particle) in place of α-Gal. As shown in Figure 3B, the immunized α-GalT-KO mice produced a strong IgG anti-α-Gal response, whereas α-GalT-WT gave no such response above background. The immune response against the carrier Qβ protein was not independently assessed, but is unlikely to have contributed significantly to the results, as evidenced by the modest intensities of ELISA signals against Qβ-Glc (Figure 3D). As expected, nonimmunized mice showed no IgG antibodies against Qβ−α-Gal or Qβ-Glc particles (Figure 3C,E).

Following vaccination as above with Qβ−α-Gal (immunization + two boosts), groups of 3−5 mice were assessed for anti-α-Gal antibody production (Figure 4B) and then were infected with 10^7 *L. amazonensis* or *L. infantum* by the appropriate route. Ten weeks later, the animals were sacrificed and qPCR was performed on samples collected from liver and spleen from all groups. Parasite loads in both organs were found to be undetectable, or nearly so, for all vaccinated and infected α-GalT-KO mice, whereas the unvaccinated mice all harbored the parasite, in widely varying amounts (Figure 4C−D). Preliminary tests showed no difference between unvaccinated mice and mice immunized with Qβ-Glc in *Leishmania* challenge (data not shown).

CONCLUSIONS

Carbohydrates present on the surface of the *Leishmania* parasite have been proposed to accomplish several functions, many of which contribute to immune system evasion and pathogenesis.[9,10] Since the α-Gal epitope has been found on many pathogens including organisms related to *Leishmania*, and since an α-Gal immune response had recently been found in *Leishmania* patients, we looked for and found evidence of α-Gal on two representative *Leishmania* species.

Antibodies against α-Gal have been reported to protect against experimental malaria infection,[35] assist in the healing of burn wounds,[36] improve immunogenicity in HIV vaccination,[37] show lytic action against *Trypanosoma cruzi* parasites,[38] and enhance immunogenicity in anticancer vaccine development.[16] These diverse functions show that the anti-α-Gal immune response, while strong in all healthy humans, is also more complex than may be commonly appreciated. To study this for *Leishmania*, the α-galactosyltransferase knockout mouse can be a useful tool. Indeed, we showed here that these animals are susceptible to immunization against causative organisms of cutaneous and visceral leishmaniasis with a conjugate vaccine that presents only the α-Gal carbohydrate in common with the parasites.

METHODS

Details of the handling of *Leishmania* samples, extract preparation, ELISA, Qβ virus-like particle preparation, qPCR, and immunization are provided in Supporting Information.

Virus-like Particles. Qβ virus-like particles (of "wild type" sequence) were prepared, purified,[39] chemically derivatized,[34] and characterized as previously described.

Mice. All animals and experiments were handled in strict accordance with the guidelines of the Research Ethics Committee of the UFMG, approved under the protocol

having disrupted alleles of the α1,3-GalT gene (α1,3GalT-

number 137/2011. Female C57BL/6 mice (6−8 weeks old) [19]

KO) were used. These mice have the H-2b genetic background and are bred and maintained at the animal facility of Federal University of Minas Gerais, Belo Horizonte, Brazil.

Fluorescence Microscopy and Flow Cytometry. Cells were fixed for 40 min in 2% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.4), and washed three times in phosphate buffer at 4 °C for 15 min. Cells were then labeled with 3−5 μg/mL of MOA lectin-fluorescein isothiocyanate (FITC) for 60 min, washed, mounted on slides with Mowiol reagent (DABCO, Sigma a 2,3%), and observed using a fluorescent Nikon Eclipse Ti, (USA), with fluorescence illumination and a FITC filter-barrier system. Pictures were

taken with Kodak Tri-X pan 400 ASA film. Lectins conjugated with FITC were obtained from EY laboratories, San Mateo, CA.

Fluorescent signals were quantified using a Becton Dickinson FACScan (Becton Dickinson), excitation 488 nm, emission 530 nm (±15 nm), adjusted to a fixed channel using standard Brite Beads (Coulter) prior to determining fluorescence. Samples were briefly vortexed before introduction to sheath fluid. Data acquisition and manipulation were performed with CellQuest and FlowJo version X.0.7 (Tree Star Inc.). Lectin binding was blocked by the addition of 0.2 M galactose to verify specificity.

Mice Immunization for Antibody Detection. We initially verified the competence of α1,3-GalT-KO mice, previously immunized, to produce antibodies (primarily IgG) against α-Gal epitopes. Immunization was performed in groups of 13 mice by subcutaneous injection of 10 μg doses of Qβ−α-Gal or unmodified Qβ particle at the base of the tail (not injection into the tail vein), followed by subcutaneous boost injections of the same dose at 1 week intervals in the flank. A control group of 13 mice was similarly treated with unmodified Qβ virus-like particle.

Only α-GalT-KO mice were used for vaccination and parasite challenge given the tolerance of α-GalT-WT mice to α-Gal epitopes.[40] Groups of 3−5 female α-GalT-KO mice were immunized (initial and two boost injections) as described above; control groups received PBS only. One week after the third injection, serum antibody levels were checked by ELISA. Each each animal was then challenged with 10^7 *Leishmania* parasites prepared as described in Supporting Information: *L. amazonensis*, MCAN/BR/2002/BH401, causing cutaneous leishmaniasis, so injected into the footpad in 10 μL volume and *L. infantum*, MCAN/BR/2002/BH401, causing visceral leishmaniasis, so injected i.p. in 200 μL volume.

Notes

The authors declare no competing financial interest.

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