Electronic supplementary information

Glycan-glycan interaction determines *Leishmania* attachment to the midgut of permissive sand fly vectors

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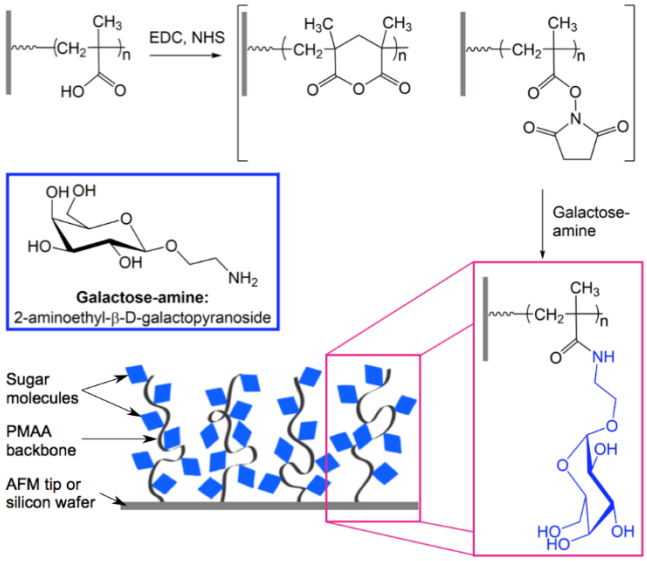
A Materials and methods

All chemicals, reagents and consumables were obtained from Sigma-Aldrich UK, unless stated otherwise.

***Leishmania* parasites and i*n vitro* culture:** *Leishmania mexicana* (MNYC/BZ/62/M379) and *Leishmania major* LV39 clone 5 (MRHO/SU/1959/Neal P) were originally obtained from cutaneous lesions from patients and have been routinely passaged through sand flies and mice since to maintain virulence. Lipophosphoglycan (LPG)-deficient and addback *L. mexicana* were a kind gift from Dr Thomas Ilg.[1, 2] The LPG-deficient mutant (*lpg1*$^{-/-}$) lacks the *LPG1* gene, which encodes a galactofuranosyltransferase required for synthesis of the LPG glycan core, rendering them deficient in LPG alone, and a corresponding addback line generated by stable transfection of an episomal copy of the *LPG1* gene (*lpg1*$^{-/-}$*+LPG1*). Parasites were maintained at 26°C on medium 199 supplemented with 20% fetal calf serum (Gibco), Basal Medium Eagles vitamins and gentamicin (50 μg ml^{-1}), pH 7.2. Nectomonad promastigotes were harvested from 2-3 day old transformed cultures and checked by microscopy.[3] Care was taken to use first passage promastigote cultures, initiated from lesion amastigotes, to ensure that the parasites recapitulated their sand fly program of development *in vitro*. Metacyclic promastigotes were generated by passaging a mid-log phase M199 promastigote culture into Graces medium (Gibco), supplemented with 20% fetal calf serum (Gibco), Basal Medium Eagles vitamins and gentamicin (50 μg ml^{-1}), pH 5.5 at 5 × 10^5 promastigotes ml^{-1} and harvested 5-7 days later. For the mutated *L. mexicana* lines, selection antibiotics were added to the culture medium: hygromycin (20 μg ml^{-1}) and phleomycin (2.5 μg ml^{-1}) for the *lpg1*$^{-/-}$ mutant; and hygromycin (20 μg ml^{-1}), phleomycin (2.5 μg ml^{-1}) and G418 (10 μg ml^{-1}) for the *lpg1*$^{-/-}$*+LPG1* addback line. Aliquots of nectomonad and metacyclic promastigotes were cryopreserved in 10% v/v dimethyl sulfoxide in fetal calf serum and stored in liquid nitrogen. Force spectroscopy maps of the glycopolymer binding were obtained from 12 *L. mexicana* WT nectomonads, 9 metacyclics, 12 LPG-deficient mutant nectomonads, 13 addbacks, and 9 *L. major* WT nectomonads.

***In vivo* binding assays of *Leishmania* promastigotes to sand fly midguts:** Phosphate buffered saline (PBS, pH 7.2)-washed parasites were suspended in M199 promastigote culture medium at a density of 5 × 10^8 ml^{-1} and fed to 5-day old flies (taken in an unfed and uninfected state from the sand fly colony) through a chick skin membrane with a Hemotek® artificial bloodfeeding system. Infected flies were maintained at 26°C for 1.0 h before dissection of the midgut into PBS. Dissected midguts were fully opened along their longitudinal axis in a fresh drop of PBS using insulin needles (30G) and transferred sequentially to 3 more drops, incubating for 30 s each time. Following this, guts were transferred to 30 μl PBS in 1.5 ml tubes and ground using a pestle. The homogenate was counted for the number of gut-attached parasites. As indicated, 50 mM sugars, amino-sugars or 10 mM EGTA were co-fed with parasites to the sand flies to test their ability to compete for parasite binding.

***Leishmania mexicana* LPG purification from enriched populations of either nectomonad or metacyclic promastigotes:** *L. mexicana* WT nectomonad and metacyclic promastigote pellets were washed three times in PBS. Pellets were kept at –80˚C until use. Prior to extraction, pellets of 4 × 10^9 nectomonads and 2 × 10^9 metacyclics were lyophilized under N_2 for 13 h using a Mini Lyotrap (LTE Scientific, UK) and fragmented with a spatula. Phospholipids were obtained from the cells with extraction solvent A (chloroform/methanol, 1:1) and sonication. Extracts were separated using an Eppendorf 5602R centrifuge at 4000 rpm (2600 g_n) for 10 min. Supernatants containing phospholipids were removed and 5 ml of solvent B (chloroform/methanol/water, 1:2:0.8) were added to the pellets, followed by sonication and centrifugation. LPG was extracted from the pellets using solvent C (9% butanol) before concentration using a speed vacuum. LPG concentration was determined by Schiff's stain for carbohydrates on a SDS-PAGE of separated material.



Scheme S1: Reaction scheme for glycopolymer synthesis, showing the orientation of the sugar on the PMAA backbone.

AFM tip functionalization: Poly(methacrylic acid) (PMAA) brushes were synthesized from initiator functionalized atomic force microscopy (AFM) cantilevers (MLCT-D, Bruker AFM Probes, USA with nominal spring constants of 30 pN nm^{-1}) and silicon control wafers (Prolog Semicor, Ukraine) as previously described.[4] After 20 min of polymerization (resulting in brushes typically between 8 and 15 nm thick as measured by ellipsometry in ambient conditions), the PMAA-coated cantilevers were stored in foil-wrapped glass Petri dishes for up to one month before use. PMAA brushes were converted to poly(*N*-2-(*β*-D-galactosyloxy)ethyl methacrylamide) glycopolymers by immersing the coated cantilever chips and control wafers in a solution of equal parts 0.1 M *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC) and 0.2 M *N*-hydroxysuccinimide (NHS) in deionized (DI) water. After 2 h the samples were rinsed with DI water and dried under a gentle nitrogen gas flow. 150 µl of a 10 mg ml^{-1} solution of 2-aminoethyl-*β*-D-galactopyranoside[5] was carefully added to each cantilever chip/wafer and left overnight before rinsing with DI water and drying under a gentle nitrogen gas flow. Both of these steps were undertaken at room temperature in 5 cm polystyrene Petri dishes. The glycosylation reaction is summarized in **Scheme S1**. A similar method was used to create glucose-coated AFM tips, using 2-aminoethyl-*β*-D-glucopyranoside.

Sample preparation for force spectroscopy: Lectin-coated surface preparation was based on a method described elsewhere.[6] Mica discs (Agar Scientific, UK) were attached to a specimen disc using UV-cured optical adhesive and the mica surface was cleaved with tape before functionalization. The surface was modification with silanized-amine groups using (3-aminopropyl)triethoxysilane (APTES, 1% v/v in toluene), and the subsequent APTES-coated substrate was annealed for 30 min at 120°C. The substrate was then immersed in a glutaraldehyde solution (12.5 % v/v in DI water) for 2 h, after which the substrate was rinsed with DI water and immersed in a 0.5 mg ml^{-1} solution of SBA in PBS for 14 h at 5°C, before being rinsed with PBS. A back-filling agent (1 M ethanolamine in PBS) was then added to the sample and left overnight at 5°C to passivate unreacted aldehyde groups. Samples were rinsed with PBS before use. Parasites were immobilized by physisorption to a positively charged surface. Glass microscope slides were sonicated in acetone, ethanol and DI water and dried under a nitrogen gas flow. A freshly made solution of 0.2% w/v polyethyleneimine (PEI) in DI water was added dropwise to the clean slides and left for 1.5 h. Parasites were used from cryopreserved stocks and washed extensively in PBS. A small volume of the parasite suspension (no more than 100 µl) was added to the centre of a PEI-coated slide and left for 1.5 h to settle. The slide was then gently rinsed with PBS and transferred into the AFM for analysis. For scanning force microscopy (SFM) images in air, parasites

were fixed with formalin, washed with PBS and resuspended in a 1:1 mixture of DI water and PBS. A drop of this solution was then added to a clean microscope slide and left to dry for 2 h before imaging.

Adsorption of anti-LPG onto AFM tip: *Leishmania* LPG antibody (CA7AE) was obtained from Bio-Rad. The antibody was attached by physisorption to the tip using glutaraldehyde to aid adhesion. The antibody was stored in multiple aliquots at 2.5 mg/mL in 0.5 mL of DI water and frozen until use. MLCT-C cantilevers (Bruker, with nominal spring constants of 10 pN nm^{-1}) were cleaned in Piranha solution (H_2SO_4:H_2O_2, 1:1, 97%:30%) for 20 min before being rinsed in DI water. The cantilevers were then immersed in 10 mL of 1% APTES in toluene for 2 h. This was followed by immersing in 2.5% glutaraldehyde in PBS for 1 h, washing in PBS and storing in PBS overnight at 4°C. Glass slides were cleaned by sonication in acetone, ethanol, and DI water sequentially and dried under nitrogen and stored. On the day of experiments, fresh PEI (0.2% w/v) in DI water was added dropwise to the centre of the cleaned glass slides and left for 1.5 h. A frozen aliquot of antibody was thawed and 500 µL of PBS added. The cantilevers were incubated in this solution for 2 h. Parasites were extensively washed in PBS via centrifugation and 100 µL added to the PEI-coated slides and left for 1.5 h.

Force spectroscopy measurements: Measurements were undertaken using an MFP3D-BIO AFM (Asylum Research, USA). The spring constants of glycopolymer-coated probes were between 17 and 27 pN nm^{-1}, measured using the in-built thermal noise calibration in Igor Pro (Wavemetrics Inc., USA). For all force maps, the applied trigger force was 250 pN, the *z*-range was 1 µm and the *z*-velocity was 1.98 µm s^{-1}. All experiments were performed at ambient temperature in liquid. SFM images in air were obtained using a Dimension 3100 AFM (Veeco, USA). Parasite viability was confirmed by the observation of flagellum movement during and after adhesion experiments using the in-built optical microscope and bright field illumination.

Specificity of glycopolymer-coated AFM tips: The peak adhesive forces stated in the caption to **Fig. 2** comprised maps taken in different areas of the sample as follows: four 32 × 32 galactose-mica maps (4,096 data); nine 32 × 32 or 36 × 36 galactose-SBA maps (> 10,000 data), and six 32 × 32 or 36 × 36 galactose-SBA maps in the presence of free galactose-amine (~ 7,000 data).

Applying thresholds to adhesion data: It is common practice to reject force curves that do not contain any adhesive events when analysing force data from biological samples.[7-9] This can be achieved by applying force thresholds to the data, which are selected based on the observed noise levels within the force curve data. In this case, fluctuations in the approach or retraction force curves were ~20 pN, and therefore if only force events above 20 pN are included in analysis, these passive curves no longer influence the results. The effective areal force is a useful tool to compare relative adhesion strength between different parasite-glycopolymer combinations. It uses the mean force for data above the threshold, $F_{>t}$, and the number of events above the threshold, $n_{>t}$, to generate an effective force per unit area, F_{eff}, by

$$F_{\text{eff}} = \frac{n_{>t}}{n} n_{1\mu m} F_{>t},$$

where n is the number of events in the map, $n_{1\mu m}$ is the number of events in 1 µm and if A is the area of the force map in μm^2, then

$$n_{1\mu m} = n / A.$$

This approach takes the effective force as constituting an array of points with the area of one pixel in the force map, rather than an array of points the size of the tip-sample contact area. Since the number of pixels and map size were consistent between experiments, this is a reasonable means of comparison, assuming that all molecules within the pixel are available to bind to the AFM tip and contribute to the measured interaction. This method accounts for both the frequency and size of

forces between the cell and a probe, and gives a measure of the overall interaction strengths that could be expected if the probe had a large surface area with the same chemical characteristics as the AFM tip and the individual binding interactions added up cumulatively across the contact area between the cell and the probe.

Multi-peak force curve analysis: A bespoke MATLAB (MathWorks, USA) routine was used to extract additional data from force–distance curves, reporting the extension and adhesive force for each binding event > 30 pN. This operation was performed for all relevant curves within the force map (i.e. those on the parasite).

Statistical methods: Differences between selected parameters were evaluated in Igor Pro (version 6.36) using a one-way analysis of variance (ANOVA) test[10] and p-values were subsequently calculated using the two-tailed Mann-Whitney t-test[11] with a significance level of 0.05. The following notation was used to indicate significance between different values: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

1. T. Ilg, *EMBO J.*, 2000, **19**, 1953-1962.
2. T. Ilg, M. Demar and D. Harbecke, *J. Biol. Chem.*, 2001, **276**, 4988-4997.
3. M. E. Rogers, M. L. Chance and P. A. Bates, *Parasitology*, 2002, **124**, 495-507.
4. P. Chapman, R. E. Ducker, C. R. Hurley, J. K. Hobbs and G. J. Leggett, *Langmuir*, 2015, **31**, 5935-5944.
5. A. Y. Chernyak, G. V. M. Sharma, L. O. Kononov, P. Radha Krishna, A. B. Levinsky, N. K. Kochetkov and A. V. Rama Rao, *Carbohydr. Res.*, 1992, **223**, 303-309.
6. M. Sletmoen, T. K. Dam, T. A. Gerken, B. T. Stokke and C. F. Brewer, *Biopolymers*, 2009, **91**, 719-728.
7. H. J. Busscher, W. Norde and H. C. van der Mei, *Appl. Environ. Microbiol.*, 2008, **74**, 2559-2564.
8. V. Dupres, F. D. Menozzi, C. Locht, B. H. Clare, N. L. Abbott, S. Cuenot, C. Bompard, D. Raze and Y. F. Dufrêne, *Nature Meth.*, 2005, **2**, 515-520.
9. G. Francius, S. Lebeer, D. Alsteens, L. Wildling, H. J. Gruber, P. Hols, S. De Keersmaecker, J. Vanderleyden and Y. F. Dufrêne, *ACS Nano*, 2008, **2**, 1921-1929.
10. B. R. Martin, *Statistics for Physical Science*, Academic, Boston, 2012.
11. R. J. Barlow, *Statistics: a guide to the use of statistical methods in the physical sciences*, Wiley, Chichester, 1989.

B Adhesive force histograms

The adhesive force of interactions between different AFM tip coatings and *Leishmania mexicana* was recorded. The distribution for interactions between *L. mexicana* and galactose AFM tips is shown in **Fig. 4a**. Here, the distributions for anti-LPG-coated AFM tips (**Figure S1**) and glucose-coated AFM tips (**Figure S2**) are given.

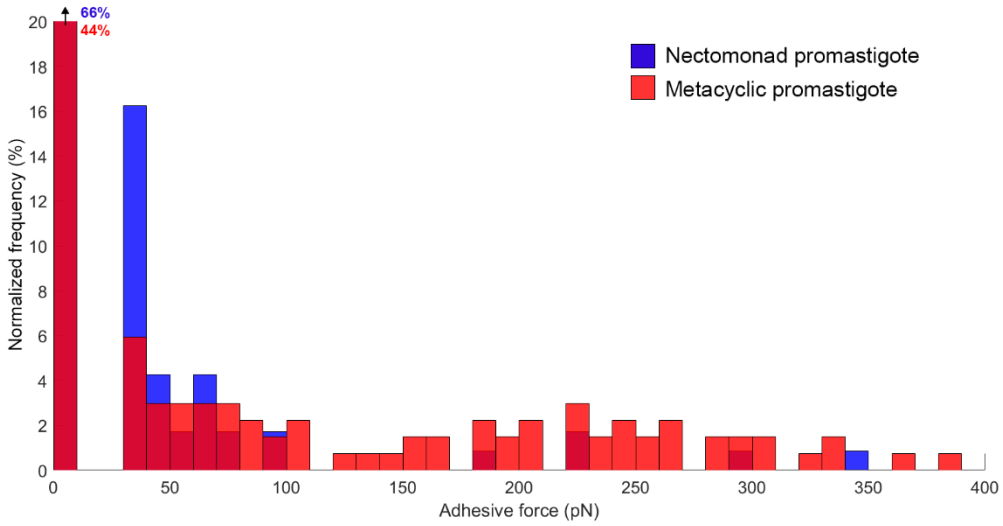


Figure S1. Typical adhesive force histograms for nectomonad (blue) and metacyclic (red) promastigotes interacting with an anti-LPG coated AFM tip.

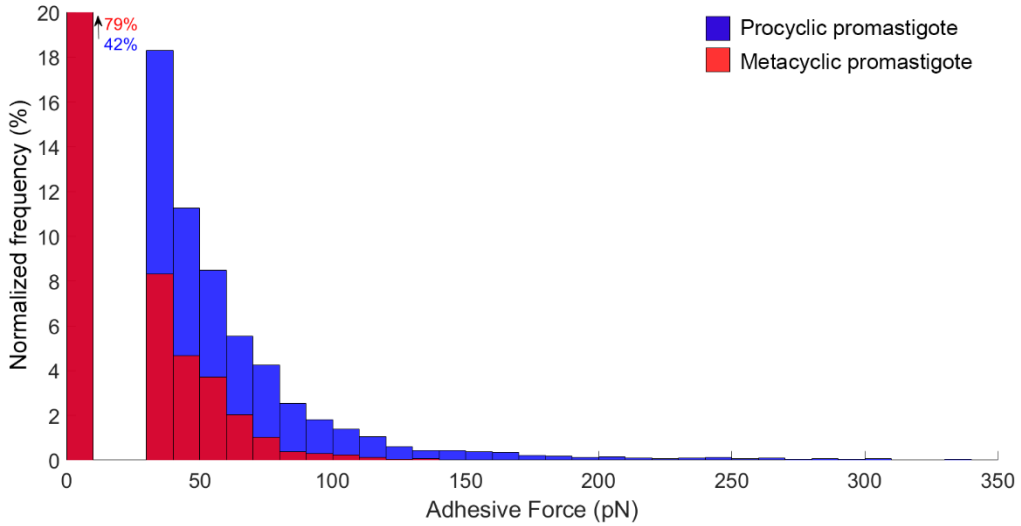


Figure S2. Typical adhesive force histograms for procyclic (blue) and metacyclic (red) promastigotes interacting with a glucose coated AFM tip. For the synthesis of the glucose-coated brushes, the chemical procedure was identical to that of the galactose brushes, but with the 2-aminoethyl-*β*-D-galactopyranoside replaced by 2-aminoethyl-*β*-D-glucopyranoside. In control experiments, glucose glycopolymer-coated tips displayed specificity for the *N*-acetyl-D-glucosamine-binding lectin concanavalin A; confirmed by loss of adhesion upon the addition of free, competing glucose-amine.

C Number of events per force curve

The distribution of interactions between *L. mexicana* and galactose AFM tips is shown in **Fig. 4b**. Here, the distributions for anti-LPG coated AFM tips (**Figure S3**) and glucose coated AFM tips (**Fig. S4**) are given.

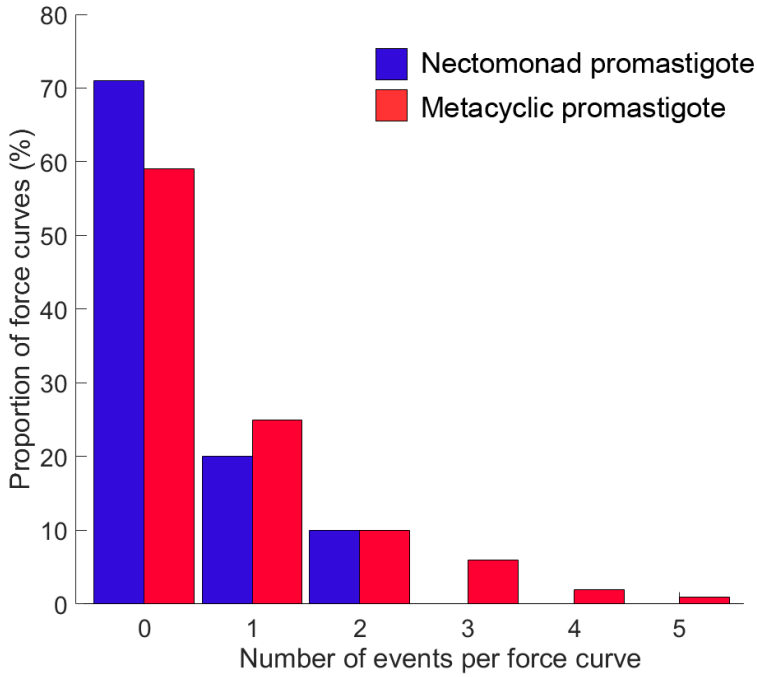


Figure S3. Typical adhesive force histograms for interactions between anti-LPG AFM tips and *L. mexicana* promastigotes.

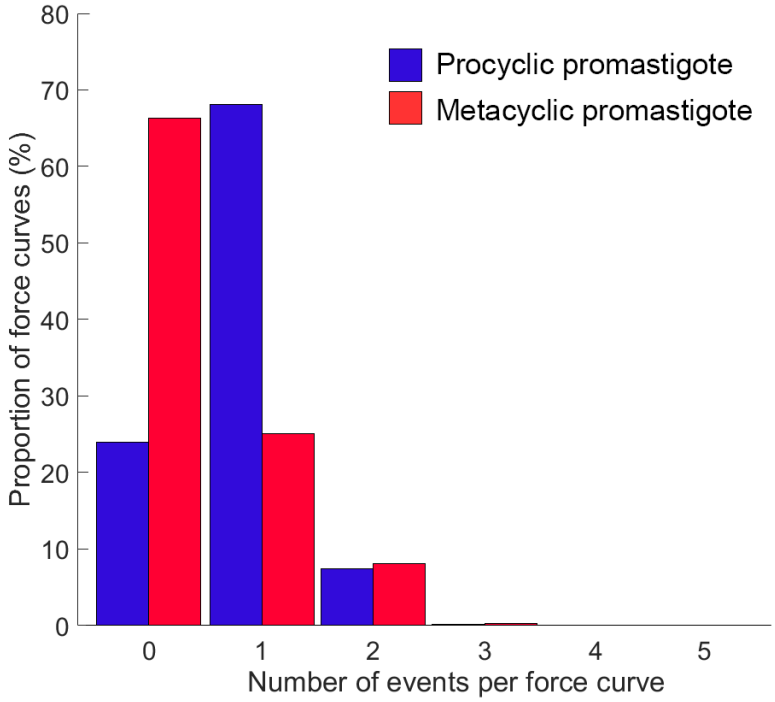


Figure S4. Typical adhesive force histograms for interactions between glucose-coated AFM tips and *L. mexicana* promastigotes.

D Force-distance curves

Examples of force-distance curves between anti-LPG coated AFM tips and metacyclic promastigotes are provided.

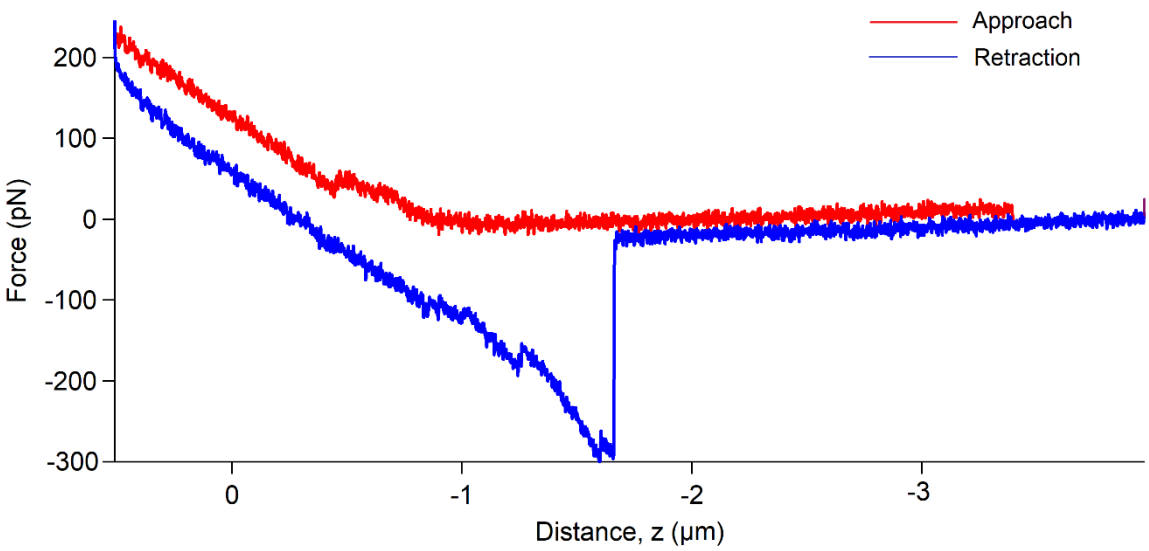


Figure S5. Forces measured as the anti-LPG-coated AFM tip moves towards (approach) and away from (retraction) a metacyclic parasite.

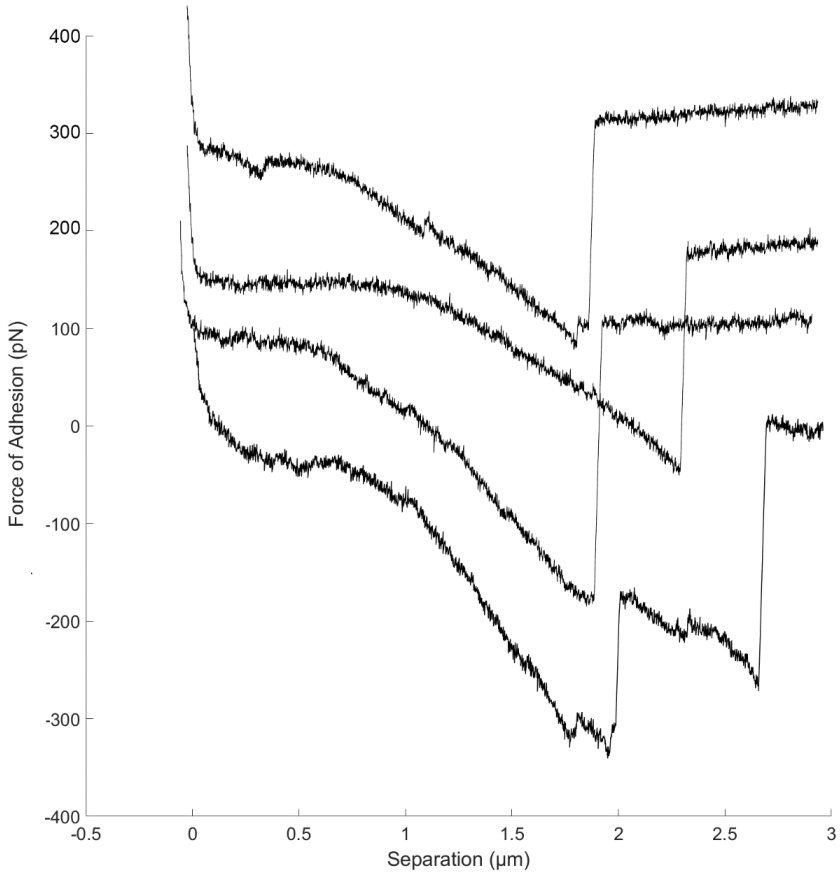


Figure S6. Four examples of retraction curves for an anti-LPG-coated AFM tip from a metacyclic parasite. These have been offset for clarity; the separation $z = 0$ corresponds to zero force on the cantilever.

E LPG western blot analysis

The absence of LPG in the *lpg1*$^{-/-}$ parasites was confirmed by Western Blot prior to use in AFM and gut binding experiments.

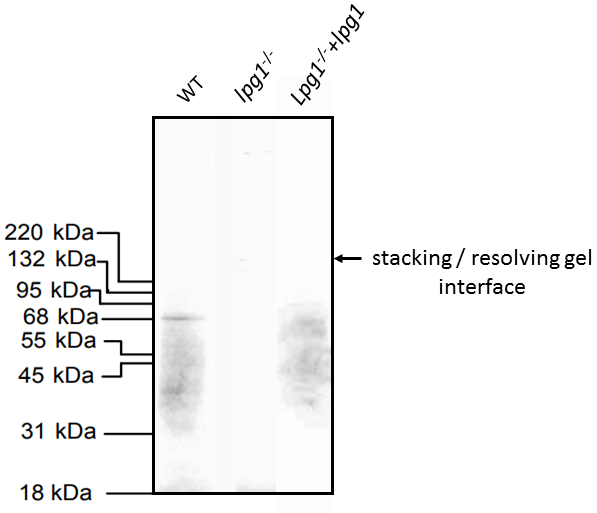


Figure S7. LPG from wild type (WT), LPG-deficient (*lpg1*$^{-/-}$), and LPG-restored (*lpg1*$^{-/-}$*+LPG1*) *Leishmania mexicana* parasites. LPG extracts were probed with the monoclonal antibody (CA7AE), which recognizes the galactose-mannose-phosphate disaccharide repeats of LPG.

F Supporting data overview

The supporting data comprises three files and one zipped folder. These are described in turn.

1) PeakAdhesiveForcesOnParasites.csv

This file contains the peak adhesive force output from the force maps generated using the Asylum Research software in Igor Pro. Each numerical value in any given column is the peak adhesive force in Newtons of a pixel in the force map that was within the masked area i.e. on the top of the parasite body. This file contains the data for all of the different parasite types which were probed with galactose, according to the following key:

A = *Leishmania mexicana* WT nectomonads
B = *Leishmania mexicana* WT metacyclics
C = *Leishmania mexicana* (*lpg1*$^{-/-}$) nectomonads
D = *Leishmania mexicana* (*lpg1*$^{-/-}$*+LPG1*) nectomonads
E = *Leishmania major* WT nectomonads

The initial row contains the description of the numerical data, with the parasite type letter followed by an integer number to indicate a different parasite: e.g. B2 would be the second WT *Leishmania Mexicana* metacyclic parasite included in the study.

2) LmexNectomonadMultiPeak.csv

This file contains the force event data from the multi-peak analysis of category A parasites (*Leishmania mexicana* WT nectomonads). In this sheet, the parasite is indicated in the same way, with parasite A1 in this file corresponding to parasite A1 in the peak adhesive force file. In this case, both the magnitude of the force (N) and the extension from the cell surface (m) have been recorded. Each row represents a single force event from the force curves in the masked area i.e. those on top of the parasite body, calculated from the same force – distance curves as those included in the peak adhesive force file. The

number of items in the column differs because only force events are included, so more will be added for curves with multiple measurable events, and no entry is included for curves without a measurable binding event in the curve. On the right-hand side of the columns containing the force event data is a separate table containing the proportion of curves containing *x* number of adhesive events. This has been normalised such that *x* = 2 with a value in the column for A2 of 0.18 would be equivalent to 18% of curves on parasite A2 containing 2 adhesive events.

3) LmexMetacyclicMultiPeak.csv

This file is the equivalent of LmexNectomonadMultiPeak.csv but for parasite category B (*Leishmania mexicana* WT metacyclics). These data show the variation in the parasite population, which was tested in this work, and enable the reader to verify the conclusions of the work based on the full details of the measured adhesive interactions.

4) ForceMaps.zip

This file contains five spreadsheets produced in Microsoft Excel following the naming convention "X_IndividualParasite_ForceMaps.xlsx". These files contain the peak force value obtained per force curve at each point measured on the parasite body. Each value in a cell represents the force value demonstrated in the force maps in **Figure 4a,b,d,e,f**. These files are labelled with X = A, B, C, D, E, with each letter representing the same group as in 1) above. Within each file are multiple tabs. Each tab represents a different parasite, with the tab naming convention "X expI pJ", where X represents the category of parasite that is being probed with galactose, I represents the experiment the parasite was mapped in and J represents the number of the parasite within the experiment. For example, "A exp 2 p3" would be the third parasite mapped during the second experiment using *Leishmania mexicana* WT nectomonads. Any tabs which state "FLAGELLUM END" denote a second map on one of the parasites, purely examining the end of the parasite as opposed to the usual map position towards the centre of the parasite body. These maps were not used in general analysis but are included for completeness.

The data in these maps has been colour-coded using conditional formatting. Yellow corresponds to no measurement; green to forces less than 100 pN; and red to those 100 pN or greater. The 100 pN threshold can readily be altered by the user.

Force spectroscopy data from the article are available:

- **Figure 3d, f** can be found in B_IndividualParasite_ForceMaps.xlsx
- **Figure 3e** can be found in A_IndividualParasite_ForceMaps.xlsx
- Data for **Figure 4a** can be found in PeakAdhesiveForcesOnParasites.csv (blue and red including data from a single parasite of each type)
- Data for **Figure 4b** can be found in LmexNectomonadMultiPeak.csv and LmexMetacyclicMultiPeak.csv