Article

Leishmania genetic exchange is mediated by IgM natural antibodies

https://doi.org/10.1038/s41586-023-06655-8

Received: 15 June 2022

Accepted: 19 September 2023

Published online: 25 October 2023

Check for updates

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Host factors that mediate *Leishmania* genetic exchange are not well defined. Here we demonstrate that natural IgM (IgMn)[1–4] antibodies mediate parasite genetic exchange by inducing the transient formation of a spherical parasite clump that promotes parasite fusion and hybrid formation. We establish that IgMn from *Leishmania*-free animals binds to the surface of *Leishmania* parasites to induce significant changes in the expression of parasite transcripts and proteins. *Leishmania* binding to IgMn is partially lost after glycosidase treatment, although parasite surface phosphoglycans, including lipophosphoglycan, are not required for IgMn-induced parasite clumping. Notably, the transient formation of parasite clumps is essential for *Leishmania* hybridization in vitro. In vivo, we observed a 12-fold increase in hybrid formation in sand flies provided a second blood meal containing IgMn compared with controls. Furthermore, the generation of recombinant progeny from mating hybrids and parental lines were only observed in sand flies provided with IgMn. Both in vitro and in vivo IgM-induced *Leishmania* crosses resulted in full genome hybrids that show equal patterns of biparental contribution. *Leishmania* co-option of a host natural antibody to facilitate mating in the insect vector establishes a new paradigm of parasite–host–vector interdependence that contributes to parasite diversity and fitness by promoting genetic exchange.

Leishmania parasites are vector-borne pathogens transmitted to mammalian hosts through phlebotomine sand flies[5]. Associated with poverty, leishmaniasis is prevalent in developing countries and results in self-limiting, severely disfiguring or fatal diseases that cause substantial morbidity and mortality worldwide[6]. More than 1 billion people are at risk of infection, millions are asymptomatic carriers and the annual incidences of fatal visceral and cutaneous leishmaniases are estimated at 100,000 to 1 million cases, respectively[7]. These statistics place leishmaniasis at the forefront of parasitic diseases of public health importance.

Leishmania parasites are considered mostly asexual, reproducing through clonal propagation[8–10]. However, the presence of natural hybrids and the demonstration of genetic exchange in the sand fly vector[11–14] indicate that genetic exchange occurs but is facultative, as seen in other eukaryotic microorganisms[15]. Genetic exchange is a powerful evolutionary route that facilitates the development of pathogenicity in microorganisms and offers an experimental tool for dissecting complex phenotypes that underlie virulence[16].

Host and parasite factors that mediate genetic exchange are not well understood in *Leishmania*. In earlier studies, passage in sand flies was required to undertake this experimentally[11,17,18]. However, hybrid formation has been observed in vitro for a few species, including *Leishmania tropica*, albeit following exposure to harsh non-physiological conditions, including DNA damage[15]. Nevertheless, in vitro hybrid generation has yet to be accomplished for most *Leishmania* species, including *Leishmania major*[11,19,20]. Notably, genetic exchange or the generation of fully fertile hybrids through backcrosses has only been reported once[13].

In this study, we focus on host factors that mediate *Leishmania* genetic exchange and explore whether host blood ingested by sand flies provides some element (or elements) absent in culture medium that promotes cell fusion and the formation of hybrid parasites.

IgM–*Leishmania* clumps generate hybrids

The addition of freshly isolated *Leishmania*-naive (referred to as naive henceforth) dog plasma to culture medium in vitro produced

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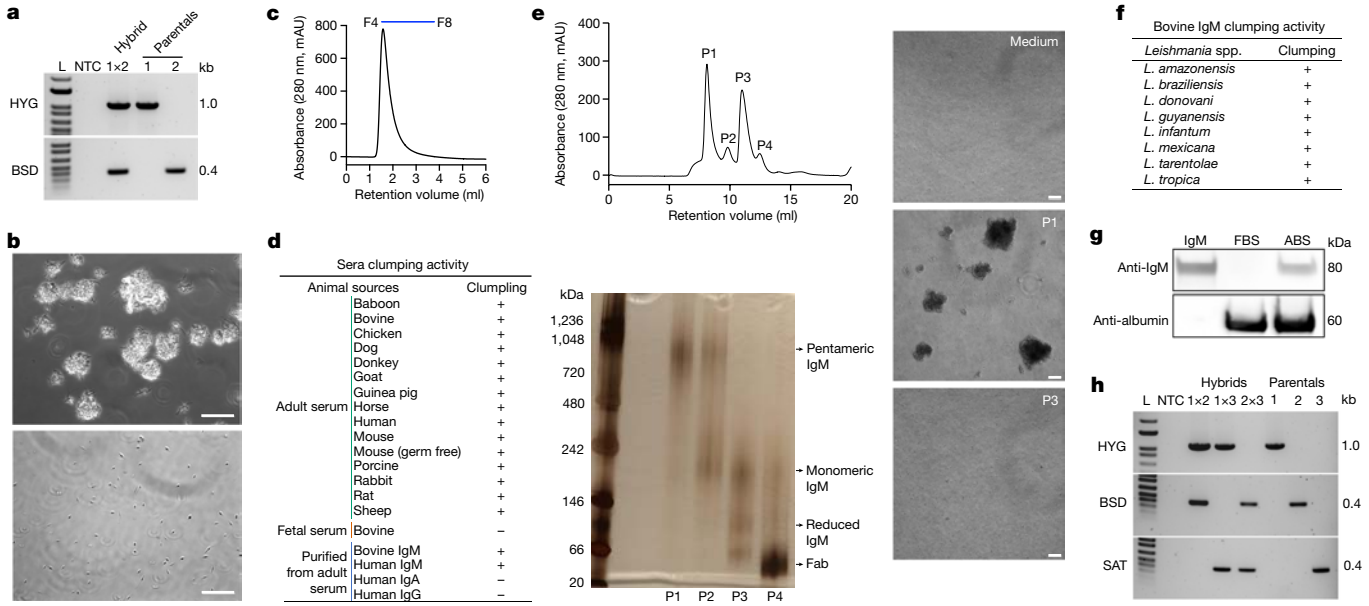


Fig. 1 | IgM facilitates *Leishmania* parasite clumping and promotes hybrid formation in vitro. a, Genotyping of *L. major* hybrids by PCR targeting parental-selectable drug markers HYG and BSD. **b**, Phase-contrast image of *L. major* in culture medium containing 20% FBS with (top) or without (bottom) 5% adult inactivated naive dog plasma. Images taken 30 min after cells were seeded in fresh medium. **c**, Human IgM eluted from a HiTrap IgM column. *Leishmania* clumping activity in IgM-eluted fractions F4–F8 (bar). **d**, Presence (+) or absence (–) of *L. major* clumping activity in inactivated naive adult sera from different vertebrate species, FBS or commercial immunoglobulins (IgM, IgG or IgA) purified from naive adult bovine serum (ABS) or naive adult human serum. **e**, Top left, high-resolution gel filtration chromatography of IgM on a Superdex-200 column after reduction and alkylation. Buffer used was PBS. Bottom left, native gel electrophoresis of HPLC peaks P1–P4 after silver staining. Molecular weights of bands representing pentameric, monomeric or reduced IgM, and the IgM Fab fraction are indicated. Right, phase-contrast images of *L. major* promastigotes in culture medium containing 20% FBS alone (medium) or 50 μg ml^{-1} of either pentameric (P1) or monomeric (P3) IgM fractions. $n = 3$ biological replicates. Images were taken 2 h after cells were seeded in fresh medium. **f**, Clumping activity of various *Leishmania* species in the presence of naive adult bovine IgM at 50 μg ml^{-1}. **g**, Western blot detecting IgM in FBS and ABS. Serum albumin was used as the loading control. $n = 2$ biological replicates. **h**, Genotyping of *L. major* hybrids by PCR targeting parental-selectable drug markers HYG, BSD and SAT. *n* detailed in Extended Data Fig. 2b. L, 1 kb-plus ladder; NTC, no template control. Scale bars, 50 μm (**b**,**e**).

a successful cross of two genetically labelled *L. major* strains: WR-SSU-HYG/GFP (resistant to hygromycin B (HYG)) and FV1-FKP40-BSD (resistant to blasticidin (BSD)) (Fig. 1a). Hybrid generation, as monitored through the recovery of double-drug resistant parasites, was only observed when accompanied by the formation of a spherical clump composed of live aggregating parasites (Fig. 1b, top, and Supplementary Videos 1–3). The parasite clumps gradually formed and were maintained for 24–36 h (Supplementary Video 4) before starting to spontaneously dissociate and release viable parasites. In standard culture medium, without naive dog plasma, the parasite clump did not form (Fig. 1b, bottom, and Supplementary Videos 5 and 6), and hybrids were not recovered.

Next, we purified and fractionated naive dog plasma using a two-step high-performance liquid chromatography followed by tandem mass spectrometry (HPLC–MS/MS) strategy. In vitro incubation of parasites with HPLC fractions revealed that IgM was the molecule responsible for parasite clump formation (Extended Data Fig. 1). Parasite clumping was retained in HPLC-eluted fractions of a commercially available IgM (Fig. 1c), but was absent with commercial human IgA or IgG (Fig. 1d). Notably, the pentameric structure of IgM was required for clumping activity because IgM monomers did not induce parasite clump formation (Fig. 1e). IgM-induced parasite clumping was observed in eight diverse species (Fig. 1f), which suggested that parasite clumping following contact with blood is a general response in *Leishmania*. Of note, fetal bovine serum (FBS) did not induce clumping (Fig. 1b, bottom) and does not contain IgM (Fig. 1g). Transient agglutination of *Leishmania donovani* by IgM has been previously reported[21], and this response, which we now refer to as parasite clumping, was not observed with FBS or human umbilical cord serum. These sera are both naturally deficient in IgM[22], thereby reinforcing our current observations.

To directly establish the contribution of the IgM-induced clump to genetic exchange in vitro, we used three genetically marked parasite strains with independent selectable markers in various crosses: *L. major* WR-SSU-HYG/GFP (parental line 1, resistant to HYG); *L. major* FV1-FKP40-BSD (parental line 2, resistant to BSD); and *L. major* FV1-FTL-SAT (parental line 3, resistant to nourseothricin (SAT)). Stationary-phase promastigotes (which contain a mixture of non-metacyclic and metacyclic forms) from two parental lines were mixed at a 1:1 ratio and cultured for 3 days, with or without IgM and without drug treatment, and then grown in the presence of selective drugs for up to 30 days (Extended Data Fig. 2a). Hybrids were observed for all parental combinations, but only when the culture was supplemented with IgM (Extended Data Fig. 2b and Fig. 1h). This result establishes a causal relationship between the IgM-mediated formation of a parasite clump and hybrid generation. Of note, IgM-mediated hybridization in vitro was independently verified by another laboratory. We emphasize that even when successful, not every mating experiment produced hybrid progeny, a result also commonly seen in sand fly crosses in vivo. Collectively, our data demonstrate that IgM is crucial for the generation of *L. major* hybrids in vitro by inducing the formation of a transient spherical clump of live parasites. Similar results with *L. tropica*, a different *Leishmania* species, were obtained without any manipulation to the culture medium other than addition of IgM (Extended Data Fig. 2a,c,d).

IgMn binds *Leishmania*

The observation of parasite clump formation in naive sera and in sera from germ-free animals (Fig. 1d) led us to propose that IgMn antibodies are responsible for parasite clumping by binding *Leishmania* parasites. IgMn antibodies, known as pre-immune antibodies, are generated

without known antigen exposure or T cell assistance[1]. They are typically characterized as oligonucleotide-specific low-affinity antibodies that bind foreign microbial antigens present in the cell wall of bacteria or fungi, and on the surface of viruses[1]. Known ligands of IgMn include carbohydrates, lipopolysaccharide, lipoteichoic acid, peptidoglycans and phosphorylcholine, among other microbial cell wall components[22]. To verify that parasite clumping is mediated by natural antibodies, we first confirmed the naive status of human, dog and mouse sera by comparing their IgG response with that of sera from *Leishmania*-infected animals. We screened the sera against both a lysate of *Leishmania* parasites and the recombinant kinesin protein 39 antigen (rK39), a parasite antigen routinely used for diagnosis of visceral leishmaniasis[23]. rK39 was used as a negative control as it is not a surface-associated protein, and IgMn mostly recognizes surface-associated antigens in microorganisms[1]. As expected, naive sera did not contain *Leishmania*-specific IgG antibodies, whereas significantly higher IgG titres were observed in *Leishmania*-infected sera (Extended Data Fig. 3a). By contrast, IgM antibodies in naive sera recognized native *Leishmania* antigens but did not react with rK39 (Extended Data Fig. 3b). This result supports the hypothesis that IgMn antibodies are binding to the parasites. Of note, both naive and *Leishmania*-infected sera contain IgMn, and as such mediate parasite clumping (Extended Data Fig. 3c), a finding of epidemiological significance.

Next, we demonstrated that purified IgMn binds to high-molecular-weight proteins in a *Leishmania* crude lysate or a *Leishmania* membrane preparation (Fig. 2a and Extended Data Fig. 3d). When O-linked and N-linked glycans were removed from the *Leishmania* lysate through treatment with PNGase F or a cocktail of O-linked and N-linked glycosidases, we only observed a 32.4% and 33.1% loss of IgMn binding, respectively (Fig. 2b). This partial loss of binding suggests that other ligands apart from glycans may be of significant relevance for IgMn binding to the *Leishmania* parasite. Recognition of *Leishmania* by IgMn is probably due to the polyreactive property of IgMn antibodies[1], which bound to many protein bands in the *Leishmania* crude lysate (Fig. 2a). We propose that IgMn binding may not be directed at a specific *Leishmania* molecule, but is likely to be broad and bind multiple ligands[1].

As the surface of *Leishmania* parasites is rich in the cell surface glycolipid lipophosphoglycan (LPG) and other *Leishmania* phosphoglycans, we asked whether these were potential targets for IgMn-mediated clumping. We tested *L. major* mutants devoid of LPG alone (*Δlpg1*$^{-}$) or deficient in all phosphoglycans through loss of the Golgi GDP-mannose transporter LPG2 (*Δlpg2*$^{-}$) or the UDP-Gal transporters LPG5A and LPG5B (*Δlpg5a*$^{-}$*/b*$^{-}$)[24]. All of the mutants formed IgM-dependent *Leishmania* clumps (Fig. 2c). This result establishes that *Leishmania* surface phosphoglycans are not required for *Leishmania* clumping formation.

These findings prompted the question that parasite clumping is a different process to parasite agglutination induced by lectins such as peanut agglutinin (PNA). PNA binds to terminal galactose residues in LPG and, therefore, does not agglutinate all *Leishmania* species or *L. major* metacyclics[25]. By contrast, IgMn induced clump formation in both metacyclic and non-metacyclic promastigotes and in all *Leishmania* parasite species tested (Fig. 1f). This result indicated that the mechanisms of action of IgMn and PNA binding to *Leishmania* are different and that the biological consequences are also distinct. In contrast to the transient and living clump formed by the addition of 50 μg ml^{-1} of IgMn, the same concentration of PNA caused parasites to agglutinate immediately and irreversibly (Fig. 2d), a process that was maintained when using low concentrations of PNA (Extended Data Fig. 3f). Following the addition of PNA, the parasites formed a disorganized, flat, mesh-like structure of irreversibly bound immotile parasites that did not mediate hybrid formation (Fig. 2d, Extended Data Fig. 3f,g and Supplementary Video 7). Of note, the composition and temporal formation and dissociation of the IgMn-induced *Leishmania* parasite clump was also different from other reported structures, including *Leishmania* rosettes[26].

We visualized IgMn binding to the surface of *Leishmania* parasites by confocal microscopy (Fig. 2e). We observed that IgMn was abundantly distributed throughout the parasite clump (Fig. 2f). Additionally, transverse sections revealed that IgMn localized in distinct pockets among parasites (Fig. 2g) and on the surface of parasites (Fig. 2h, inset), which potentially contributed to interactions holding the structure together.

IgMn alters parasite gene expression

To investigate whether IgMn binding to *Leishmania* alters the state of clumped parasites, we compared transcript and protein expression profiles of IgMn-treated and untreated *Leishmania*. Principal component analysis (PCA) showed that the mRNA expression profiles of six biological replicates from IgMn-clumped *Leishmania* parasites were distinct from those of control untreated parasites (Fig. 3a). Moreover, the transcription profile across the independent samples showed distinctive patterns of mRNA expression (Fig. 3b). Of 565 differentially expressed transcripts, 326 were upregulated and 239 were downregulated in IgMn-clumped *Leishmania* parasites compared with controls (Supplementary Table 1). IgMn-clumped *Leishmania* parasites showed upregulated transcripts coding for proteins such as serine/threonine protein kinase, CMGC/RCK protein kinase, cyclin-dependent kinase, histone methyltransferase, kinesin and DNA ligase (Supplementary Table 3).

The proteome expression profile of IgMn-clumped parasites was also distinct from that of controls (Fig. 3c), for which 15 proteins (8 upregulated and 7 downregulated) were differentially expressed (Fig. 3d and Supplementary Tables 2 and 4). The three most relevant upregulated proteins in IgMn-clumped parasites were orthologues of a polo-like kinase involved in cell division[27], a p25α protein implicated in cell fusion[28] and coronin, a protein in *Leishmania* that regulates microtubule remodelling during cytokinesis[29] (Supplementary Table 4).

Next, we set out to test the hypothesis that IgMn-induced upregulation of transcripts and proteins results in fusion and hybridization events inside the *Leishmania* parasite clump. Using scanning and transmission electron microscopy, we visualized the clump and identified promastigotes with three nuclei, indicative of fusion events inside the parasite clump (Extended Data Fig. 4a–c). A recent report demonstrating the presence of three nucleated promastigotes inside the sand fly gut[30] suggests that our in vitro findings mimic a natural parasite fusion event in vivo. To demonstrate hybridization events by confocal microscopy, we utilized two parental lines for which the nuclei were labelled with different modified nucleotide analogues that produced green or red fluorescence (Fig. 3e and Extended Data Fig. 4f, left and middle), which enabled the detection of nuclear fusion as yellow. Screening multiple confocal images of *xz* slices taken through the clump, we located yellow nuclei inside *L. major* (Fig. 3e, right) and *L. tropica* (Extended Data Fig. 4f, right) parasite clumps, indicative of hybridization events. Collectively, our data provide direct evidence that IgMn binds and activates transcriptional and translational machinery in clumped parasites that ultimately results in *Leishmania* fusion and hybrid formation inside, an event we name the *Leishmania* mating clump (LMC).

IgMn mediates in vivo hybridization

The ubiquity of IgMn in vertebrate blood[2–4,31] combined with the multiple[32] and promiscuous blood-feeding behaviour of the sand fly (Supplementary Table 5) led us to propose that IgMn is also a major facilitator for the initiation of genetic exchange in vivo in the sand fly gut. To address this hypothesis, experiments were performed using conditions that mimicked the natural progression of sand fly infections, including incorporation of a second blood meal that is needed to prevent the loss of naturally acquired parasites following defecation of the earlier digested meal[32]. First, we determined by western blotting that IgMn persists in the sand fly midgut for at least 24 h after each

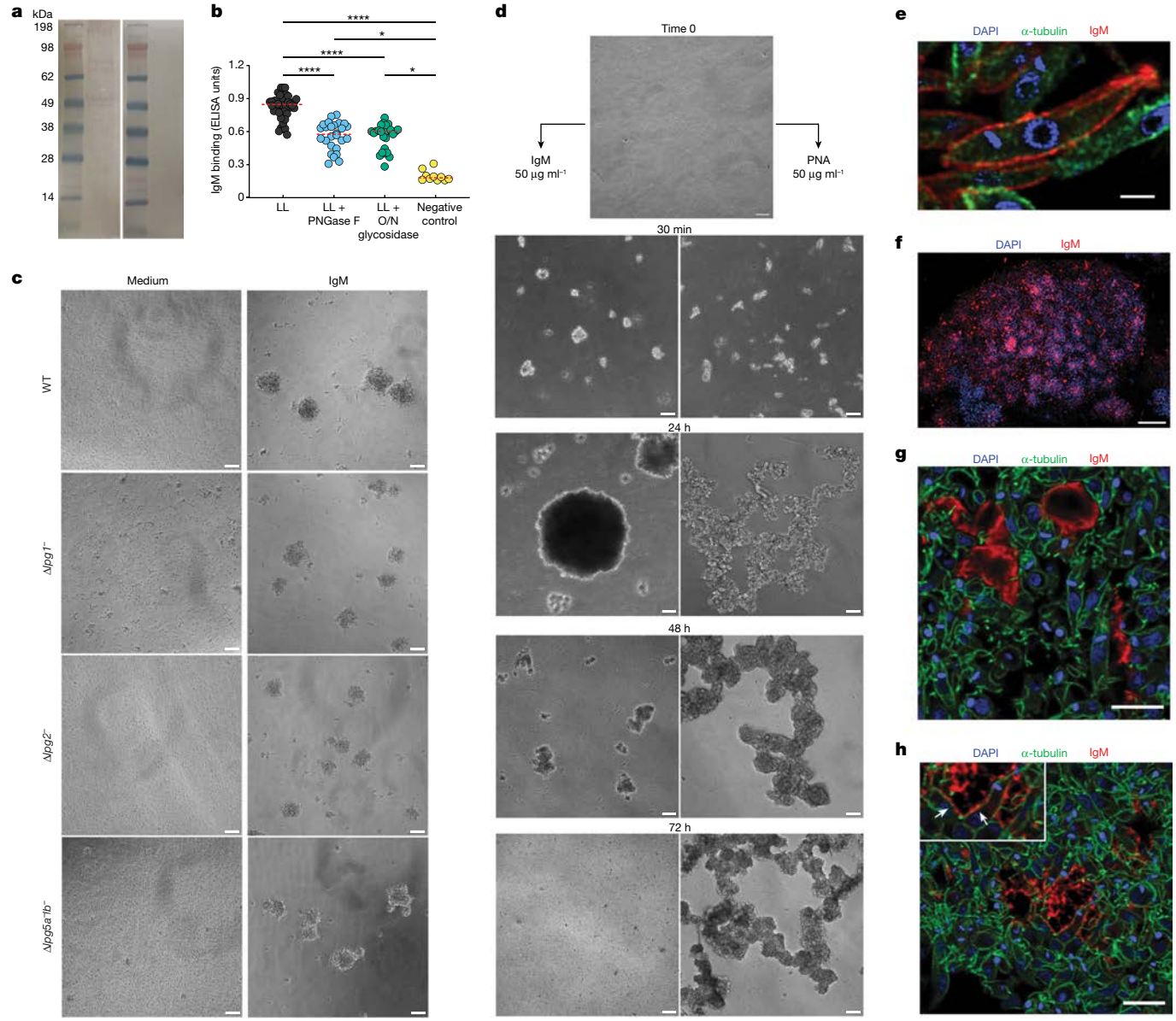


Fig. 2 | IgMn antibodies bind to *Leishmania* parasites. a, Left, western blot of *L. major* crude lysate detected by IgM purified from naive ABS. Right, *L. major* crude lysate was incubated with secondary antibody alone as a control. $n = 2$ biological replicates. **b**, ELISA of *L. major* lysate (LL) treated or not with PNGase F or an O-linked and N-linked glycosidase mix (O/N glycosidase) detected by purified naive IgM. Bovine serum albumin was used as a negative control protein. $^*P < 0.05$, $^{****}P \leq 0.0001$. Statistical analysis by Kruskal–Wallis with Dunns' multiple comparison test. Data represented as individual values by scatter plot. Dashed bar, median. $n = 3$ biological replicates. **c**, Promastigotes of *L. major* (LV39 strain) wild type (WT) or mutants devoid of LPG alone (Δ*lpg1*$^-$) or deficient in all phosphoglycans (Δ*lpg2*$^-$ or Δ*lpg5a*$^-$/*b*$^-$) were incubated in complete Schneider's medium without (left) or with 50 µg ml^{-1} purified bovine IgMn (right). $n = 3$ biological replicates. Images were taken after 2 h. **d**, IgMn-induced clump formation differs from PNA agglutination. *L. major* parasites were incubated with 50 µg ml^{-1} bovine IgMn (left) or PNA (right) and imaged over the course of 72 h. $n = 2$ biological replicates. **e**, Confocal immunofluorescence image of *L. major* after 5 min of incubation with IgMn. **f**, Confocal immunofluorescence wide *z* and tile image showing the association of IgM with an LMC after 24 h of culture. **g**,**h**, Confocal immunofluorescence images of a 5-µm transversal section of an LMC. Panels represent *xz* slices. **h**, Inset, IgMn colocalizes on the surface of parasites after 24 h (arrows). For **e**–**h**, $n = 3$ biological replicates. DAPI was used to stain nuclei or the kinetoplast. Scale bars, 2 µm (**e**), 5 µm (**h**, inset), 30 µm (**g**,**h**) or 50 µm (**c**,**d**,**f**).

blood meal (Fig. 4a). Next, we performed an experiment whereby we allowed sand flies to feed on a cutaneous lesion initiated by two *L. major* parental lines (Fig. 4b, BM1), thereby picking both parasites up as part of a natural blood meal[33,34]. We then waited 6 days before offering the sand flies a second uninfected blood meal (Fig. 4b, BM2), estimated as the time a sand fly needs to lay its eggs and take a second or subsequent blood meal in field conditions. The second blood meal was provided either through a feeder, supplemented with or without 500 µg ml^{-1} of IgMn, or by having sand flies feed on an uninfected mouse as a natural source of blood and IgMn (Fig. 4b, BM2). For membrane feedings, the use of 500 µg ml^{-1} of IgMn is at or below physiological concentrations reported in the blood of different animals (Supplementary Table 6). Additionally, a control group was maintained on a single infected blood meal (Fig. 4b, dotted arrow). Fourteen days after the initial infection (8 days after the second blood meal), sand fly guts from each group were dissected and plated individually to screen for parasite hybrids (Fig. 4b). For the group initially infected with the parental line combination 1×2, IgMn significantly increased the proportion of hybrid formation by up

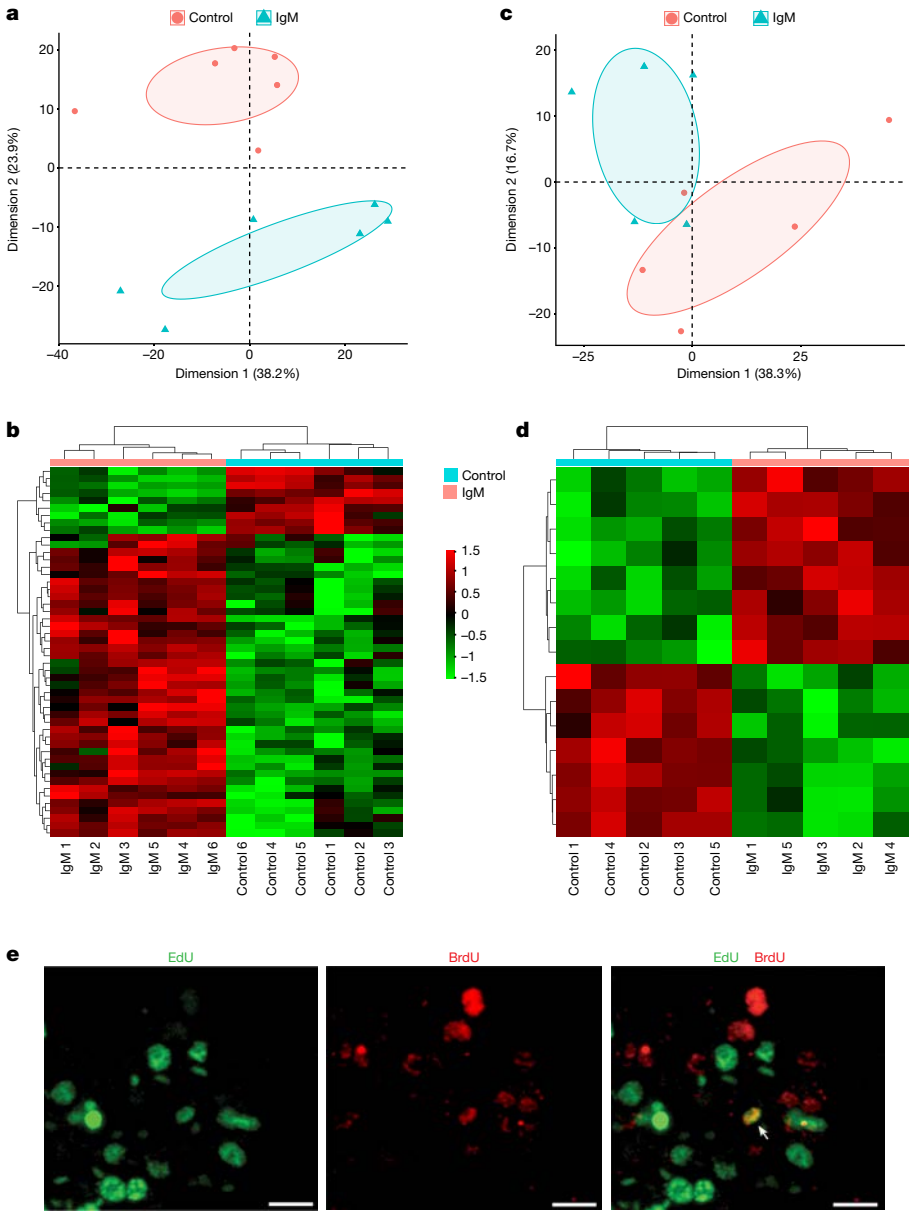
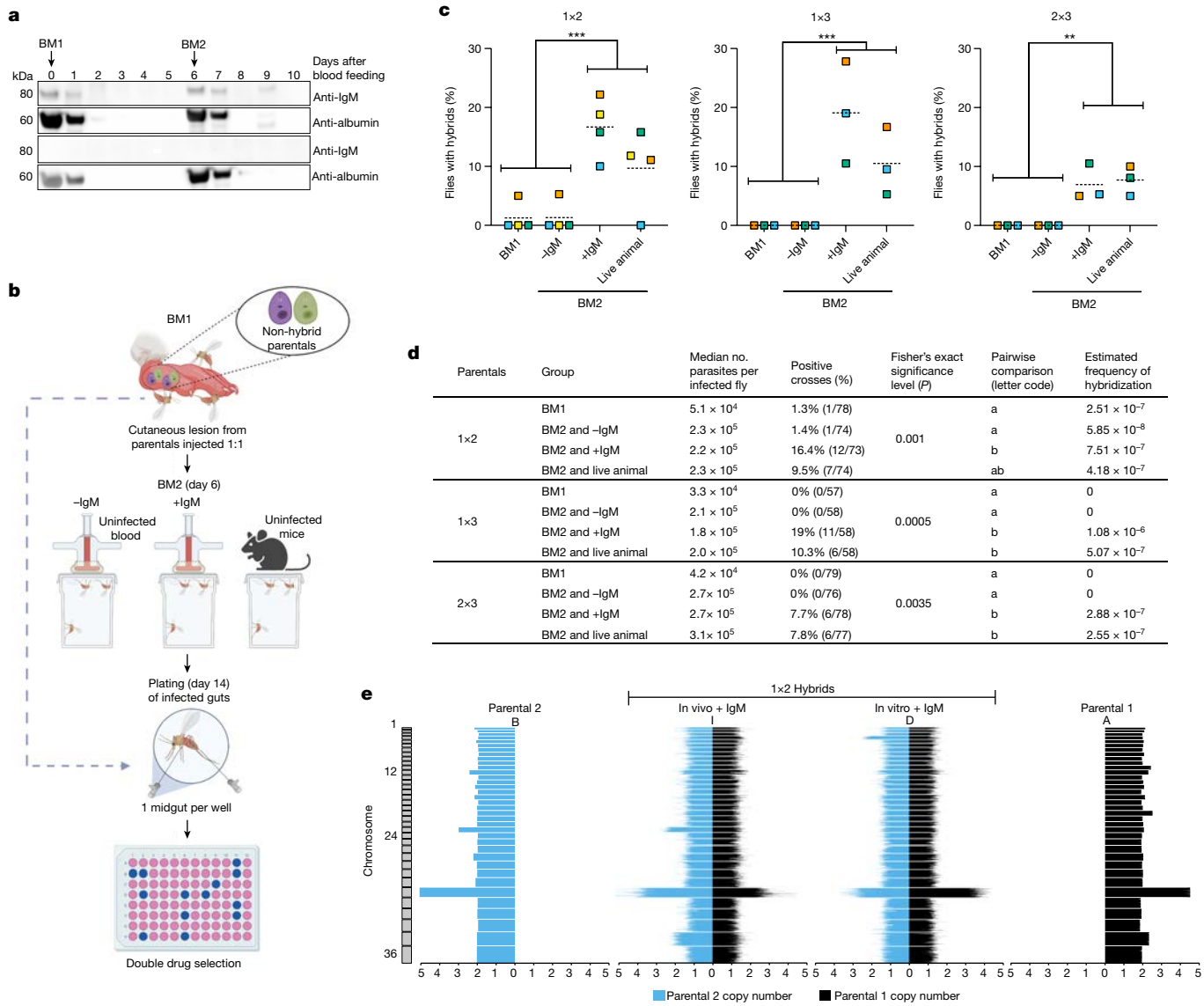


Fig. 3 | IgMn induces changes in *Leishmania* transcription and protein expression profiles and promotes *Leishmania* hybridization inside the parasite clump. a,**c**, PCA of differentially expressed transcripts (**a**) and proteins (**c**) from IgMn-clumped (IgM) and untreated (Control) parasites. Each point represents an independent experiment. **b**,**d**, Heatmaps of the 50 most significant differentially expressed transcripts (**b**) and of the differentially expressed proteins (**d**) in IgMn-clumped compared with untreated parasites. **e**, Confocal immunofluorescence image of a 7-µm transverse section from LMCs. Promastigotes were grown to stationary phase in the presence of 5-ethynyl-2′-deoxyuridine (EdU) or 5-bromo-2′-deoxyuridine (BrdU) then extensively washed and used at a 1:1 ratio to assemble LMCs with IgMn. The white arrow points to yellow nuclei indicative of fusion and exchanged genetic material. Scale bars, 3 µm.

to 12-fold in sand flies that received a second blood meal containing IgMn compared with sand flies given a second blood meal devoid of IgMn or sand flies given a single infectious blood meal (Fig. 4c,d and Extended Data Fig. 5a). In these two control groups, the observed proportion of hybrids was similar to what has been previously reported[17,18]. The similar proportion of hybrids observed in flies given a second blood meal devoid of IgMn or sand flies given a single infectious blood meal suggested that this may be a basal level of chance mating within the confined space of the sand fly midgut. Notably, control groups lacking IgMn did not generate any hybrids in parental line combinations 1×3 and 2×3, which further emphasized the importance of IgMn in *Leishmania* hybrid formation in vivo (Fig. 4c,d and Extended Data Fig. 5a). It is worth noting that both control group scenarios (a single blood meal and a second without IgM) would not occur in nature because an infected sand fly will always take a second IgM-containing blood meal 5–6 days after infection[5,32]. This is concordant with the observation that sand flies feeding on an uninfected mouse as a source of IgMn in the second blood meal also generated a significantly higher proportion of hybrids compared with control groups (Fig. 4c,d). Of note, the parasite load and per cent metacyclics were similar across all experimental groups (Extended Data Fig. 5b–g). The substantial effect of IgMn on parasite hybrid formation in sand flies provided a second blood meal 6 days after naturally acquired infections (BM2) suggested that proliferative stages are prone to genetic exchange. We argue that hybrids may form soon after a sand fly takes a blood meal but can only be detected later in the infection cycle after undergoing multiple cycles



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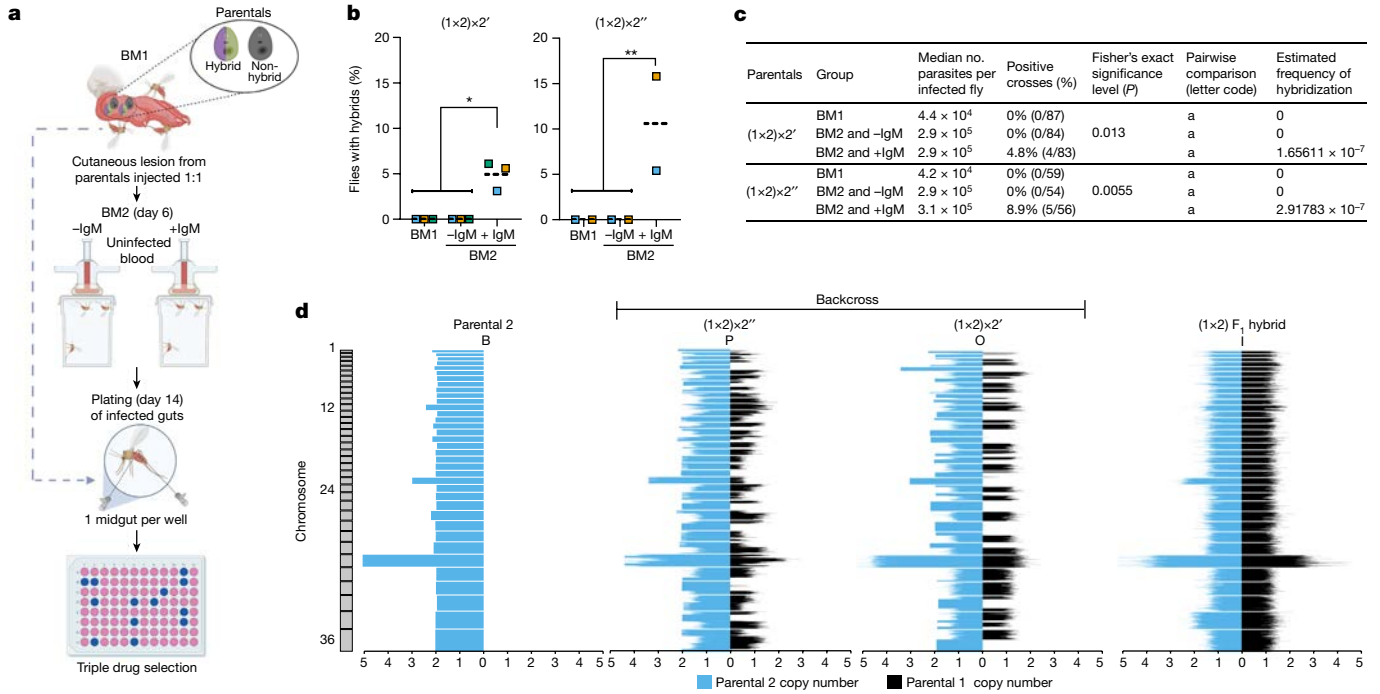
Parentals	Group	Median no. parasites per infected fly	Positive crosses (%)	Fisher's exact significance level (*P*)	Pairwise comparison (letter code)	Estimated frequency of hybridization
1×2	BM1	5.1×10^4	1.3% (1/78)	0.001	a	2.51×10^{-7}
	BM2 and –IgM	2.3×10^5	1.4% (1/74)		a	5.85×10^{-8}
	BM2 and +IgM	2.2×10^5	16.4% (12/73)		b	7.51×10^{-7}
	BM2 and live animal	2.3×10^5	9.5% (7/74)		ab	4.18×10^{-7}
1×3	BM1	3.3×10^4	0% (0/57)	0.0005	a	0
	BM2 and –IgM	2.1×10^5	0% (0/58)		a	0
	BM2 and +IgM	1.8×10^5	19% (11/58)		b	1.08×10^{-6}
	BM2 and live animal	2.0×10^5	10.3% (6/58)		b	5.07×10^{-7}
2×3	BM1	4.2×10^4	0% (0/79)	0.0035	a	0
	BM2 and –IgM	2.7×10^5	0% (0/76)		a	0
	BM2 and +IgM	2.7×10^5	7.7% (6/78)		b	2.88×10^{-7}
	BM2 and live animal	3.1×10^5	7.8% (6/77)		b	2.55×10^{-7}

Fig. 4 | The impact of IgMn on *Leishmania* hybrid formation in the sand fly vector. a, Immunoblot detecting IgMn in the sand fly midgut after multiple blood meals. Sand flies were provided an artificial uninfected blood meal with or without IgMn. Arrows indicate the timing of the first (BM1, 0 day) and second (BM2, 6 days) blood meals. Pools of 15 midguts were used and 7.5 µg of total protein was loaded per lane. $n = 2$ biological replicates. **b**, Experimental design for the generation of in vivo crosses after sand flies acquire parasites by feeding on a footpad lesion containing two *L. major* parental lines (BM1). For BM2, FBS was added to rabbit red blood cells with (+IgM) or without (–IgM) 500 µg ml^{-1} adult bovine IgMn and provided through a membrane feeder, and uninfected mice were used as a blood source for IgMn. **c**, Per cent of sand flies harbouring hybrids for three different combinations of parental lines (1×2, 1×3 and 2×3) in single-fed or twice-fed sand flies, in the presence or absence of IgMn, or in sand flies that fed on a live mouse. Each value (colour) indicates an independent experiment. Dashed bar, mean. **d**, Cumulative data for infection status and estimated frequency of hybridization per fly from independent experiments (1×2, $n = 4$; 1×3, $n = 3$; 2×3, $n = 3$). A total of 20 infected flies per group were used per biological replicate. Fisher's exact test, $P < 0.05$ is considered significant. Pairwise comparisons indicate the source of group significance: a, no difference; b, significant difference. **e**, Whole-genome analysis of in vitro-generated and in vivo-generated hybrids for the parental line combination 1×2. Upper case letters provide an identifier for each *Leishmania* parental or hybrid. Biparental ancestry was confirmed across the entire genome for all crosses. Parental 1, WR-SSU-HYG/GFP; parental 2, FVI-FKP40-BSD; parental 3, FVI-FTL-SAT. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Panel **b** was created using BioRender (https://biorender.com).

of division[5,32]. Collectively, the above data establish that IgMn facilitates *Leishmania* hybrid formation inside the sand fly gut after naturally acquired infections, probably by binding to and altering the phenotype of parasites within the LMC to promote fusion and hybrid generation. This hypothesis is supported by the observation of structures resembling in vitro LMCs within the sand fly midgut (Extended Data Fig. 4d,e and Supplementary Videos 8–12). Whole-genome sequencing of the double-drug-resistant hybrids showed that the crosses in sand flies and those generated in vitro were full genome hybrids (Fig. 4e and Extended Data Figs. 6 and 7). Moreover, patterns of biparental contribution were similar between hybrids generated in vitro and in vivo (Extended Data Fig. 7a,b).

Corroborating previous findings[11], we observed hybrid formation only in sand flies and did not find them in *Leishmania* skin lesions or draining lymph nodes containing two different parasite lines (Extended Data Fig. 8). In the mammalian host, the *L. major* parasite is intracellular, residing as a single parasite in a parasitophorous vacuole inside macrophages[35,36]. It may be that a lack of opportunity for IgMn to interact with amastigotes accounts for the absence of hybridization in the mammalian host.



Parentals	Group	Median no. parasites per infected fly	Positive crosses (%)	Fisher's exact significance level (*P*)	Pairwise comparison (letter code)	Estimated frequency of hybridization
(1×2)×2′	BM1	4.4×10^4	0% (0/87)		a	0
	BM2 and –IgM	2.9×10^5	0% (0/84)	0.013	a	0
	BM2 and +IgM	2.9×10^5	4.8% (4/83)		a	1.65611×10^{-7}
(1×2)×2″	BM1	4.2×10^4	0% (0/59)		a	0
	BM2 and –IgM	2.9×10^5	0% (0/54)	0.0055	a	0
	BM2 and +IgM	3.1×10^5	8.9% (5/56)		a	2.91783×10^{-7}

Fig. 5 | The impact of IgMn on the generation of recombinant progeny from hybrids in the sand fly vector. a, Experimental design for the generation of in vivo backcrosses after sand flies acquire parasites by feeding on a footpad lesion containing one non-hybrid *L. major* parental line and one hybrid *L. major* parental line. A (1×2) F_1 hybrid was injected at a 1:1 proportion with two clones of parental 2 bearing a different resistance marker (parental 2 and parental 2″). For BM2, FBS was added to rabbit red blood cells with (+IgM) or without (−IgM) 500 µg ml^{-1} adult bovine IgMn and provided through a membrane feeder. **b**, The per cent of sand flies harbouring hybrids in single-fed sand flies or in sand flies fed twice in the presence or absence of IgMn. Each value (colour) indicates an independent experiment. Dashed bar, mean. **c**, Cumulative data for infection status and estimated frequency of hybridization per fly from independent experiments. (1×2) × 2′, $n = 3$; (1×2) × 2″, $n = 2$. A total of 30 infected flies per group were investigated per biological replicate. Fisher's exact test, $P < 0.05$ is considered significant. Pairwise comparisons indicate source of group significance: a, no difference; b, significant difference. **d**, Whole-genome analysis of in vivo-generated backcross hybrids for parental line combination 2 × (1×2) F_1 hybrid. Upper case letters provide a unique identifier for each *Leishmania* parental or hybrid. For instances in which the biparental ancestry is consistent across each chromosome, recombination events have broken this up in the backcrosses, making the parental copy number discontinuous always in the same direction through parental 2. Parental 1, WR-SSU-HYG; parental 2, FVI-FKP40-BSD; parental 2′, FVI-FKP40-SAT; parental 2″, FVI-SSU-SAT. $^*P < 0.05$, $^{**}P \leq 0.01$. Panel **a** was created using BioRender (https://biorender.com).

IgMn promotes genetic exchange in vivo

For use in positional cloning, the generation of recombinant progeny from F_1 hybrids mated to each other or backcrossed to parental lines is required. Previous studies have shown that the frequency of these progenies in *L. major* is considerably lower than it is for initial F_1 hybrid formation, which greatly hinders its utility[13]. To test whether insights from IgMn-mediated mating could improve F_1 hybrid recovery and confirm their fertility, we performed a backcross in vivo in the presence of IgMn. This strategy involved crossing a HYG/BSD double-resistant (1×2) F_1 hybrid with one of two clones of *L. major* parental line 2 bearing a SAT marker to enable identification of mating progeny. Sand flies were allowed to feed on a cutaneous lesion initiated by mixed infections of the (1×2) F_1 hybrid with either parental line 2′ or 2″ (Fig. 5a, BM1), and were then given a second uninfected blood meal 6 days later (BM2), either supplemented with 500 µg ml^{-1} of IgMn or devoid of IgMn (Fig. 5a, BM2). A diagram outlining the approach for the generation of backcrosses is provided in Extended Data Fig. 9a. We consistently observed hybrid formation of backcrosses, but only in sand flies fed a second blood meal containing IgMn (Fig. 5b,c and Extended Data Fig. 9b). Hybrid generation was not influenced by the parasite density or by the per cent of metacyclics, which were similar across all BM2 experimental groups (Extended Data Fig. 9c–f). Whole-genome sequencing of the triple-drug-resistant hybrids showed that the backcrosses were full genome hybrids (Fig. 5d and Extended Data Figs. 6 and 7) and exhibited numerous recombination events between the F_1 parent (HYG/BSD) and the third parent (SAT) genomes, as seen previously in rare successful backcrosses[8]. These data establish that IgMn facilitates fertile hybrid formation and enhances their frequency in sand flies, ultimately promoting *Leishmania* diversity in nature. The reproducible and increased recovery of backcrosses also opens the way for a more in-depth genetic analysis of *Leishmania* parasites.

Aneuploidy commonly seen in cultured parasites[20,37,38] was observed in this study at comparable levels in our in vitro and sand fly hybrids (Extended Data Fig. 7). Furthermore, the DNA content of tested hybrids resulting from in vitro and in vivo experiments were 77% diploid and 23% triploid for *L. major*, whereas *L. tropica* hybrids were 100% tetraploid (Extended Data Fig. 10), similar to previously reported species differences[11,13,17,20].

Discussion

Our work provides direct experimental evidence of a crucial role for IgMn antibodies in binding to parasites to form the LMC, which promotes the first key steps of *Leishmania* genetic exchange in vitro and in vivo after a naturally acquired infection. Moreover, our data describe a new biological function for a natural antibody, implicating the binding of IgMn to *Leishmania* parasites in genetic exchange.

In reproduction and genetic exchange of eukaryotes, an organism usually produces all the molecules required for these processes[39]. In this work, we showed that *Leishmania* binds to IgMn, an extrinsic molecule originating from the mammalian host, triggering transcriptional and proteomic changes to promote fusion and hybridization, a new mechanism that facilitates early key steps in genetic exchange.

The dependence of *Leishmania* on host IgMn, an evolutionarily conserved antibody that is always present in the blood of all vertebrates after birth[28,30,31] independently of previous antigen exposure[40–45] is of significant consequence. Our data indicated that *Leishmania* takes advantage of the pentameric properties of host IgMn and the biological feeding pattern of sand flies, which need to ingest blood every 5–6 days to produce eggs[5], to enhance the likelihood of mating. We propose that when a *Leishmania*-infected sand fly takes a subsequent blood meal, the periodic influx of IgMn encounters parasites in the confinement of the gut at a developmental stage and density required to initiate the formation of the LMC, leading to activation of the transcriptional and translational machinery of the clumped parasites, a crucial first step of genetic exchange that promotes fusion and hybrid generation. As such, in addition to metacyclic promastigotes[11], this implicates the two major proliferative stages, leptomonad and retroleptomonad promastigotes, as potential participants in the process of genetic exchange.

In summary, this work demonstrates that the *Leishmania* parasite co-opts a natural antibody from its mammalian host to advance its reproductive fitness in the insect vector, uncovering a previously unappreciated critical first step in genetic exchange, and providing new insights into the intimate relationships that develop between a blood feeding insect vector, a parasite and the mammalian host.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-023-06655-8.

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Methods

Ethics statement

All animal experimental procedures were reviewed and approved by the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee under animal protocol LMVR4E. The NIAID DIR Animal Care and Use Program complies with the Guide for the Care and Use of Laboratory Animals and with the National Institutes of Health (NIH) Office of Animal Care and Use and Animal Research Advisory Committee guidelines. Detailed NIH Animal Research Guidelines can be accessed at https://oma1.od.nih.gov/manualchapters/intramural/3040-2/. *Leishmania*-infected human and dog serum samples were collected according to human and animal protocols approved by the institutional review boards of the Federal University of Sergipe, Brazil ("Interactions between pathogens and host in the pathogenesis of visceral leishmaniasis"; protocol number 1.353.887), and the University of Iowa (protocol number 2031721-002), respectively. Human naive serum samples were obtained from the NIH Blood Bank. Serum samples from naive beagle dogs, and immune and naive mice serum samples were provided by the Division of Veterinary Resources (DVR/NIH) under animal protocol LMVR4E.

Animals

Six-to-eight-week-old female BALB/c and Swiss Webster mice were obtained from Envigo and Charles River laboratories, respectively. Female sand flies (*Lutzomyia longipalpis*, Jacobina strain) were used at 4–6 days of age after emerging. Mice were housed on a 6:00–20:00 light cycle. Lights turned on at 6:00 and turned off at 20:00. The room temperature was held between 21 and 24 °C, and the humidity was held between 50 and 55%. Animals were housed under pathogen-free conditions at the NIAID Twinbrook animal facility, Rockville, USA.

Parasites

***Leishmania* parasites used in this study.** The following *Leishmania* parasites were used: *L. major* WR 2885 (isolated from a soldier deployed to Iraq[46]); *L. major* MHOM/IL/80/Friedlin; *L. tropica* MHOM/SU/74/K27 (American Type Culture Collection (ATCC), 50129); *Leishmania braziliensis* MHOM/BR/75/M2903 (ATCC, 50135); *Leishmania infantum* MCAN/BR/09/52 (isolated from a dog spleen in Natal, Brazil[47]); *L. donovani* MHOM/SD/62/1S; *Leishmania tarentolae* (Tar II; ATCC, 30143); *Leishmania mexicana* MNYC/BZ/62/m379; and *Leishmania amazonensis* IFLA/BR/67/PH8. For LPG mutants, *L. major* strain LV39 clone 5 (RHO/SU/59/P) was used and the following knockout lines generated as previously described: Δ*lpg1*$^-$ (ref. 48), Δ*lpg2*$^-$ (ref. 49) and Δ*lpg5a/b*$^-$ (ref. 50). Parasites were grown at 27 °C in Schneider's *Drosophila* insect medium (Lonza, 04-351Q; MilliporeSigma, S0146-1L) supplemented with 20% heat-inactivated FBS (Gibco, 16140071) (complete Schneider's medium (C-SM)) or C-SM plus 250 μM of adenosine (MilliporeSigma, A9251) (C-SMA). Parasites were maintained by serial passage in BALB/c mice or Golden Syrian hamsters depending on the dermotropic or viscerotropic characteristics of the parasite species. Promastigotes were kept in culture for a maximum of ten passages. *L. major* metacyclic promastigotes were purified by PNA (Vector Laboratories, L-1070) and used for in vitro experiments and infection of mice footpads as previously described[51].

Leishmania parental lines

The various *Leishmania* lines used in crossing experiments were constructed as detailed below. For parental line 1, *L. major* WR-SSU-HYG/GFP, *L. major* (WR 2885) was used as background for generation of a HYG parental line that also harbours the *EGFP* gene. The *EGFP* gene was amplified by PCR using the mEGFP-C1 plasmid as a template for the forward primer 5′-ACAGCGAGATCTATGGTGAGCAAGGGCGAGGA-3′, and reverse primer 5′-ACAGCGGGTACCCTTGTACAGCTCGTCCATGC-3′. The forward and reverse primers add the BglII or KpnI restriction sites to the amplicon, respectively. The PCR product was cloned into the Lexsy_hyg2 plasmid (Jena Bioscience, EGE-232). The linearized construct (2 μg) was used to transfect mid-log phase culture promastigotes on a 4D-NucleofectorTM X unit (Lonza) using pulse code FI-115 (T cell, Human, unistim. HE). Transfected parasites bearing rRNA locus-integrated EGFP were selected for growth in the presence of 100 μg ml^{-1} HYG (Goldbio, H270). Clones were selected by limiting dilution and assessed for chromosome integration. Parental line 2, *L. major* FVI-FKP40-BSD[52], and parental line 3, *L. major* FVI-FTL-SAT[53], were generated as previously described. For generation of parental line 2′, *L. major* FVI-FTL-SAT, we followed the procedures described for line 2, but used a heterozygous of the gene *FKP40* with a construct harbouring SAT. For parental line 2″, *L. major* FVI-SSU-SAT/RFP, *L. major* MHOM/IL/80/Friedlin/FV1 was used as background for the generation of a SAT parental line that also harbours the mRuby gene. The mRuby gene was amplified by PCR using the mRuby-C1 plasmid as template for the forward primer 5′-ACAGCGAGATCTATGAACAGCCTGATCAAAGA-3′, and reverse primer 5′-ACAGCGGCGGCCGCTCACAGATCCTCTTCAGAGATGAGTTTCTGCTCCCCTCCGCCCAGGCCGGC-3′. The forward and reverse primers add the BglII or NotI restriction sites to the amplicon, respectively. The PCR product was cloned into the Lexsy_sat2 plasmid (Jena Bioscience, EGE-234). The linearized construct (2 μg) was used to transfect mid-log phase culture promastigotes on a 4D-NucleofectorTM X unit (Lonza) using pulse code FI-115 (T cell, Human, unistim. HE). Transfected parasites bearing rRNA locus-integrated mRuby were selected for growth in the presence of 100 μg ml^{-1} SAT (Goldbio, N-500-100). Clones were selected by limiting dilution and assessed for chromosome integration. *L. tropica* (K27, ATCC, 50129) was used as background for the generation of parental line 4, *L. tropica* K27-SSU-HYG/GFP, and parental line 5, *L. tropica* K27-SSU-SAT, respectively. Parental line 4 was made following the same steps as described for parental line 1. Parental line 5 was made the same way as parental line 2″ but without the addition of any fluorescent gene. mEGFP-C1 and mRuby-C1 were a gift from the M. Davidson Laboratory (Addgene, plasmid 54759, http://n2t.net/addgene:54759, RRID:Addgene_54759; Addgene, plasmid 54552, http://n2t.net/addgene:54552, RRID: Addgene_54552).

Leishmania antigen preparation

L. major (WR 2885) parasites (1–2 × 10^8) were washed and pelleted down by centrifugation. To obtain a crude lysate, cell pellets were subject to lysis by the addition of 100 μl of 1% SDS in 10 mM Tris, pH 7.5, followed by vortexing for 1 min and a 10-min incubation at 37 °C. Protein concentration was quantified by BCA (ThermoFisher Scientific, 23227). To obtain the *Leishmania* parasite lysate, cell pellets were subjected to 5 cycles of sonication, 30 s per cycle, followed by the addition of 100 μl of 1× PBS. Samples were centrifuged at 8,000*g* for 30 min at 4 C, and the supernatant was collected. Protein concentration was quantified by BCA (ThermoFisher Scientific, 23227). rK39 was donated by S. Reed (HDT Bio).

IgM identification from plasma by HPLC

Canine plasma (1.5–2 ml) was inactivated at 56 °C for 30 min. Inactivated plasma was passed through a 0.22 μm PES membrane (Millipore MILLEX GP, SGLP033RB) and diluted to 7 ml in PBS containing 5 mM EDTA (buffer A). The inactivated, diluted plasma was then applied to a Sephacryl S200 (16/60, GE Healthcare) gel filtration column equilibrated with buffer A. Plasma components were eluted at 1 ml min^{-1}, and 2 ml fractions were collected and tested for *Leishmania* clumping activity. Active fractions were then combined, concentrated and diluted several times with 10 mM sodium phosphate (pH 6.0) buffer (buffer B) before application to a SP Sepharose (16/10, GE Healthcare) ion exchange column equilibrated with buffer B. Proteins were eluted with a gradient of 0–1 M NaCl in buffer B. Activity was distributed throughout the major peak, particularly at the leading edge. These fractions were pooled and chromatographed on a Superdex 200 (10/300, GE Healthcare) gel

filtration column equilibrated with buffer A, which resulted in further purification of the major component that produced bands on SDS–PAGE gels of approximately 70 and 25 kDa, consistent with the heavy and light chains of IgM, respectively. This sample was active in *Leishmania* clumping assays and was analysed after tryptic digest by electrospray ionization MS. As an additional means of purification, pooled fractions of IgM purified by gel filtration were diluted in 20 mM $NaPO_4$ pH 7.5 (buffer C) containing 0.8 M ammonium sulfate and applied to a 1 ml HiTrap IgM purification HP column (GE Healthcare) equilibrated with buffer C containing 0.8 M ammonium sulfate. The column was washed until the absorbance reached baseline levels. IgM was then eluted stepwise in buffer C and 0.5 ml fractions were collected. Activity in the *Leishmania* clumping assay closely aligned with the peak of absorbance.

Commercial IgM preparation for in vitro and in vivo experiments

Commercially available IgM antibodies used in this study were obtained from MilliporeSigma and contained preservatives such as sodium azide. To avoid any potential toxicity to the parasites, removal of preservatives was undertaken. IgM from human (MilliporeSigma, I8260) and bovine (MilliporeSigma, I8135) sources were purified by passing them through a Sephacryl-S-200 gel filtration column (Cytiva) equilibrated with either buffer A without EDTA or PBS. For IgM equilibrated with buffer A without EDTA, further purification steps using a HiTrap IgM purification HP column are given above. Protein-containing fractions eluted with PBS were pooled and the volume reduced to the desired concentration by ultrafiltration. Protein-containing fractions were then pooled together, and the volume reduced to the desired concentration by ultrafiltration. Concentration of the eluate if needed was performed using 100 kDa amicon filters. A concentration of 3–5 mg ml^{-1} was a good range of stock concentration that enabled the addition of small volumes to culture medium. The purified IgM solution was sterilized by filtration (0.22 µm membrane syringe filter), and aliquots were stored in low-binding-protein tubes at −20 °C. Cycles of freeze and thaw to the IgM solution were avoided, as this can break its pentameric structure. Once the aliquot was defrosted, it was maintained at 4 °C. IgM remained stable if not contaminated.

In vitro *Leishmania* clumping activity

Various *Leishmania* species were cultured in C-SM in 25 cm^2 flasks. In total, 4×10^6 per ml stationary-phase promastigotes were dispensed into a well of a 24-well plate in the presence of 50 µg ml^{-1} of bovine IgM to test clumping activity. For *L. major*, sera from different animal sources were also tested for clumping activity in culture. Serum was obtained by centrifugation at 1,000*g* for 10 min, inactivated at 56 °C for 30 min, filter sterilized through a 0.22 µm PES membrane (Millipore MILLEX GP, SGLP033RB) and stored at −80 °C in aliquots for single use. The following sera were acquired from commercial vendors: baboon (MilliporeSigma, S6884); bovine, adult (MilliporeSigma, B9433); bovine, fetal (Gibco, 16140071. R&D, S12495, S11595); chicken (MilliporeSigma, C5405); dog (BioChemed Services, TD092321NIH); donkey (MilliporeSigma, D9663); goat (MilliporeSigma, G9026); guinea pig (MilliporeSigma, G9774); horse (MilliporeSigma, H1270); human (MilliporeSigma, H4522, H6914, P2918, S7023, S1-M); mouse (MilliporeSigma, S25-M, S3509, S7273); porcine (MilliporeSigma, P9783); rabbit (MilliporeSigma, R4505); rat (MilliporeSigma, S7648); and sheep (MilliporeSigma, S2263). Sera were heat-inactivated at 56 °C for 30 min and filter sterilized before use at 20% final concentration.

In vitro LMC assembly for recovery of hybrids

Parental lines were grown in C-SM until reaching the stationary phase. Freshly isolated parasites after passage in the sand flies were routinely used, with no more than 15 passages in culture. The parasites were incubated at a temperature of 27 °C in a CO_2-free environment. To prepare the parental lines for the mating setup, the frozen stock was thawed, cultured in C-SM under drug pressure and allowed to grow for 3–4 days to late log phase. To obtain sufficient parasites, a new passage of the drug-resistant line was initiated without drug pressure. The parasites were cultured in 25 cm^2 upright culture flasks. Once the cultures reached the stationary phase, the cells were centrifuged and counted to determine the total number of parasites available for subsequent steps. For the assembly of mating wells, 24-well plates were used (96-well plates did not result in successful recovery of hybrids). Each well was seeded with an equal proportion of the two parental lines at a density of 4×10^6 parasites per ml (2×10^6 parasites per ml of each parental line) in a final volume of 1.5 ml per well. Parasite density and volume of medium were reproduced for recovery of hybrids at the reported frequency. C-SMA was used to provide sufficient nutrient support for the rare hybrid cells. Several wells were prepared as it was anticipated that many wells would not produce hybrids owing to the low frequency of double-resistant cells. Both parental lines were determined to be in good condition, exhibiting normal growth rates and healthy cell shape and movement, before initiating the crossing trial. Preservative-free IgM was added to each well to achieve a final concentration of 50 µg ml^{-1}. The contents of the wells were gently mixed by pipetting, and clumping formation was immediately observed. The plates were tightly sealed with Parafilm to prevent evaporation of the medium and were incubated at 27 °C. Careful monitoring was necessary to ensure that the Parafilm remained intact, as cracks could lead to evaporation. The clumps reached their peak sphere formation within 24 h, showing dense organization. Over time, the clumps began to dissociate, permitting parasite growth. After 3–4 days, a turbidity pattern was observed, indicating that the parasites are in late log/stationary phase. At this time, the clumps typically lost their spherical form, either partially or completely, depending on the parasite strains or species used. More specifically, after 3–4 days in mating wells, the parasites had usually become loosely associated or easily separated, with any remaining clump scaffolding settling at the bottom of the well. The entire contents of each well were then collected and transferred to separate selection flasks in a final volume of 15 ml. In brief, 1.5 ml was transferred to a new flask with 10 ml of fresh medium using 3.5 ml to wash and completely remove cells from the mating well into the flask. The selection flasks were prepared with C-SMA supplemented with the respective double-selective pressure drug. Flasks were maintained in an upright position at 27 °C. The cultures in the selection flasks were monitored weekly for up to 30 days. Non-hybrid parasites often started to slow down and die off within 2 days from transfer of the mating wells to the selection flasks. However, parasites under drug pressure sometimes took longer to die. In such cases, all parasite-positive flasks were pelleted after 1–2 weeks and resuspended in fresh medium (C-SMA with selective drugs). Selective drugs were combined according to the parental lines used per experiment. The concentration used for each selective drug was as follows: HYG, 100 µg ml^{-1} (Goldbio, H270); BSD, 10 µg ml^{-1} (Goldbio, B-800-500); SAT, 100 µg ml^{-1} (Goldbio, N-500-100). Once double-resistant cells grew, the polyclonal culture was cloned by plating 0.5 cell per well in a 96-well plate under double-drug pressure. After clone selection, genotyping was carried out using primers listed in Supplementary Table 7. Clone cultures were also a source of genomic DNA for deep sequencing. Only clones positive for double-resistance markers by PCR were considered and carried to whole genome sequencing. An illustration of the experimental design for in vitro *Leishmania* genetic exchange is provided in Fig. 1g. Frequencies of hybridization were estimated as: $\frac{\text{No. of positive crosses}}{(\text{Initial no. of one parental}) \times (\text{no. trial crosses})}$.

L. major hybrids were only recovered in the presence of IgM. However, owing to the low frequency of in vitro crossings compared to in vivo, some trials did not succeed.

A detailed step-by-step procedure to maximize IgM-induced *L. major* mating in vitro

An improved detailed step-by-step procedure to maximize IgM-induced *L. major* mating in vitro has been published[54]. This protocol was

developed to maximize the production of *Leishmania* hybrids in vitro. Our protocol was developed by mimicking as closely as possible the environment inside the gut of the sand fly, and we successfully and consistently produced hybrids in vivo. It is important to note that producing hybrids in vitro is less optimal than producing them in vivo. Following this step-by-step protocol faithfully and carefully will significantly improve the success of producing hybrids in vitro. Inside the sand fly gut, IgM is digested after 24 h, a crucial step that allows the parasite clump to form during the first 24 h and then dissociate to free hybrids for further development. Having an excess of IgM or always having IgM in culture (in vitro) will prevent the parasite clump from dissociating and leading to death of the parasites and absence of hybrid recovery in vitro. The use of controlled amounts of purified IgM in culture and the transfer of the parasite clump to a culture without IgM at 24 h after mating prevents the constant formation of the parasite clump and mimics in vivo conditions, mainly IgM digestion and parasite clump dissociation in the gut of the sand fly.

Mouse infection

Metacyclic promastigotes were collected from stationary-phase cultures and purified using PNA (Vector Laboratories, L-1070) as previously described[25]. Purified *L. major* metacyclic promastigotes (1×10^5) of each parental line were mixed at a 1:1 ratio and injected into a BALB/c mouse footpad. Animals that developed a swollen non-ulcerated (<5 mm thickness) footpad lesion at weeks 4–6 after injection were used to feed sand flies. After sand fly feeding on infected animals, amastigotes were collected from the infected footpads and skin draining lymph nodes as previously described[55].

Sand fly infection

Four-to-six-day-old female *L. longipalpis* (Jacobina strain) sand flies were infected by feeding on mice footpad lesions of BALB/c mice. For first (F_1) and second (backcross) generation crosses, culture purified metacyclic promastigotes of two parentals were injected in the mouse footpad at a 1:1 proportion. In F_1 cross experiments, mice were injected with parentals 1×2, 1×3 and 2×3. For backcross experiments, mice were injected with a (1×2 F_1 hybrid) $\times$ 2 harbouring a different resistance marker. Illustration of the experimental design for natural feeding on mice is provided in Figs. 4b and 5a. In brief, mice were anaesthetized by intraperitoneal injection of a mixture of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}). The infected footpad was then inserted into a cardboard pint containing the sand flies. Sand flies were allowed to feed for 2 h in the dark. After feeding, blood-fed female sand flies were sorted and maintained at 26 °C with 75% humidity and 30% sucrose solution ad libitum. *L. longipalpis* is not the natural vector of *L. major*, but it is a permissive sand fly species that has been shown to support the complete development of several *Leishmania* species from natural parasite acquisition to successful transmission by bite[56–58]. Importantly, midgut development related to parasites numbers and stages of differentiation in *L. longipalpis*–*L. major* infections were similar to those reported for *Phlebotomus papatasi*–*L. major* and *L. longipalpis*–*L. infantum* natural occurring combinations[32].

Preparation of the second blood meal provided to sand flies

For the second blood meal given 6 days after infection to a proportion of the sand flies, defibrinated rabbit blood (Spring Valley Laboratories) was centrifuged at 1,000*g* for 10 min and the normal serum containing IgM was discarded. Red blood cells were washed three times with 10 ml of PBS (Lonza BioWhittaker, BE17-516F) followed by centrifugation at 1,000*g* for 10 min. Red blood cells were washed an additional time with FBS (Gibco, 16140071). Blood was reconstituted by adding FBS at the same volume of the serum initially removed. Sand flies were allowed to feed for 2 h on a glass feeder containing reconstituted blood supplemented or not with bovine IgM (MilliporeSigma, I8135) at 500 µg ml^{-1}. Sand flies were also fed on anaesthetized Swiss Webster mice as a live animal source representative of a naturally imbibed second blood meal containing IgM. Unfed and blood-fed flies were sorted after feeding and kept on 30% sucrose solution ad libitum for a total of 14 days after the initial infected blood meal. Females infected by feeding on mice lesions only were used to represent sand flies that have taken a single infected blood meal.

Sand fly midgut counts

To assess maturity of the infection, *Leishmania*-infected sand fly midguts were individually transferred to a 1.7 ml microtube (Denville Scientific, C2172) filled with 30 µl of PBS. Midguts were then ground using a disposable pellet mixer and a cordless motor (Kimble, 7495400000). For counting, promastigotes were slowed down by a further 1:5 or 1:10 dilution in formalin/PBS at 0.005% final concentration. Then, 10 µl of the sample were loaded onto a Neubauer improved chamber (Incyto, DNC-NO1) and parasites were counted under a phase-contrast Axiostar plus microscope (Zeiss) at ×40 magnification. The number of parasites per midgut was back calculated on the basis of the total sample volume and subsequent dilution.

In vivo recovery of hybrids

Only midguts of sand flies that developed a mature infection, recognized by an opaque anterior midgut distended by the presence of a mass of parasites[32], were plated in the search for hybrids. Infected midguts were dissected in PBS on microscope slides using tweezers and fine needles. Each midgut was transferred to a 1.7 ml microtube (Denville Scientific, C2172) filled with 50 µl of C-SM and homogenized using a pestle with a cordless motor. One midgut was plated per well in a 96-well plate (Alkali Scientific, TP9096) at a final volume of 100 µl. An antibiotic cocktail (ABTs) that has no effect on parasite growth (Extended Data Fig. 5h) and viability was included in complete medium to control contamination with gut microbiota. ABTs consisted of penicillin–streptomycin (Gibco, 100 U ml^{-1}), gentamicin (Sigma, 50 µg ml^{-1}), caspofungin (Sigma, 15 µg ml^{-1}) and 5-fluorocytosine (Sigma, 30 µg ml^{-1}). For each independent experiment, a minimum of 20 infected midguts per group were collected. After plating, wells were allowed to stabilize and grow for 24 h before the addition of 100 µl per well of C-SM containing selective drugs (2× concentrated). Cultures were followed for up to 30 days for double-drug-resistant or triple-drug-resistant cell lines to grow. Contaminated wells not considered. The concentration of selective drugs, cloning and genotyping procedures were performed as described for in vitro recovery of hybrids. Frequencies of hybridization were estimated by: $\frac{\text{No. positive crosses}}{(\text{Median no. of parasites per fly}) \times (\text{no. of trial crosses})}$.

IgM stability in blood-fed sand flies and its absence in fetal serum

To estimate how long IgM is maintained in the midgut after blood feeding, *L. longipalpis* were provided two uninfected blood meals on days 0 and 6, with or without 500 µg ml^{-1} of bovine IgM. Midguts were dissected daily on days 0–10 ($n = 15$) after the initial blood meal and collected in 50 µl RIPA lysis buffer (ThermoFisher Scientific, 89901) supplemented with phenylmethylsulfonyl fluoride (MilliporeSigma, 93482) and Halt Protease Inhibitor cocktail (ThermoFisher Scientific, 87786). Samples were gently macerated with a disposable pellet mixer and a cordless motor, then vortexed and centrifuged at 12,000 r.p.m. for 10 min at 4 °C. The supernatant was collected, and its protein content was quantified by BCA (ThermoFisher Scientific, 23227). A total of 7.5 µg of protein was loaded in a 15-well NuPAGE 4–12% Bis-Tris protein precast gel (ThermoFisher Scientific, NP0335BOX), run under reducing conditions by the addition of NuPAGE Sample Reducing agent with DL-dithiothreitol (DTT) (ThermoFisher Scientific, NP0009) and then denaturation at 95 °C for 5 min. Proteins were transferred to a 0.45 mm nitrocellulose iBlot 2 transfer stack membrane using an iBlot 2 Gel Transfer device (ThermoFisher Scientific, IB23001). To check

for successful loading of antigens, No-Stain Protein Labeling reagent (Invitrogen, A44717) was used following the membrane manufacture's protocol. Next, membranes were blocked overnight at 4 °C in 5% non-fat milk diluted in Tris-buffered saline, 0.1% Tween-20 (TBST) followed by 3 h of incubation at room temperature with sheep anti-bovine IgM (Bio-Rad, AAI179) diluted at 1:1,000. Membranes were washed three times with TBST followed by incubation for 30 min with rabbit anti-sheep IgG H+L secondary antibody conjugated to horse radish peroxidase (HRP) (Abcam, Ab6747) at a 1:5,000 dilution. Membranes were washed three times with TBST and blots were developed by the addition of SuperSignal West Pico PLUS Chemiluminescence substrate (ThermoFisher Scientific, 34579). The signal was captured after 2 min using an Azure c600 chemiluminescence imager (Azure Biosystems). Membranes were stripped by adding 10 ml of Restore Western Blot Stripping buffer (ThermoFisher Scientific, 21059) for 15 min at 37 °C, followed by two washes with TBST. Blots were re-blocked overnight and incubated with recombinant rabbit anti-bovine serum albumin (BSA) antibody (Abcam, ab192603) at 1:1,000 dilution for 3 h, followed by a 30 min incubation with HRP-conjugated goat anti-rabbit IgG H+L secondary antibody (Cell Signaling Technology, 7074 S) at 1:1,000 dilution. Blots were re-imaged as detailed above. To assess the presence of IgM in fetal serum, NuPAGE 4–12% Bis-Tris protein precast gels (ThermoFisher Scientific, NP0335BOX) were loaded with 1 µg of bovine IgM (Bio-Rad, AAI179), FBS (Thermo-Fisher Scientific, 16140-071) or ABS (Millipore-Sigma, B9433) under reducing conditions followed by denaturation at 95 °C for 5 min. The pattern of the proteins was visualized by Coomassie blue staining using an eStain L1 protein staining system. For immunoblotting, proteins were transferred onto nitrocellulose membranes, blocked and probed with sheep anti-bovine IgM at 1:1,000 followed by a 30 min incubation with HRP-conjugated anti-sheep IgG H+L secondary antibody (Abcam, Ab6747) diluted at 1:5,000. Membranes were stripped and incubated with recombinant rabbit anti-BSA antibody at 1:1,000 followed by a 30 min incubation with HRP-conjugated goat anti-rabbit IgG H+L secondary antibody (Cell Signaling Technology, 7074S) at 1:1,000 and imaged after addition of the chemiluminescence substrate.

Binding of IgM to *Leishmania* by western blotting

A *Leishmania* crude lysate (10 µg) was incubated at 95 °C for 5 min, loaded into a Bolt 4–12% Bis-Tris gel Plus precast gel (ThermoFisher Scientific, NP0335BOX) and ran under non-reducing conditions. Proteins were transferred to a 0.45 mm nitrocellulose iBlot 2 transfer stack membrane using an iBlot 2 Gel Transfer device (ThermoFisher Scientific, IB23001). Membranes were stained with Ponceau S solution (Sigma, P7170) to check for successful loading of antigen. Western blots were run following the iBind Flex Western Device manufacture's protocol (ThermoFisher Scientific). Purified bovine IgMn (3 mg ml^{-1}) was diluted at 1:500, and sheep anti-bovine IgM-AKP (Bio-Rad, AAI19AB), used as the secondary antibody, was diluted at 1:1,000. The same protocol was followed for *Leishmania* membrane proteins (10 µg) extracted using a Mem-PER Plus Membrane Protein Extraction kit (ThermoFisher Scientific, 89842), according to the manufacture's protocol. After transfer of proteins to the nitrocellulose membrane, it was probed with a pool of human sera ($n = 5$) diluted to 1:25 followed by the secondary goat anti-human IgM-AP (Southern Biotech, 2020-04) diluted at 1:1,000. The reaction was developed by the addition of Western Blue stabilized substrate for alkaline phosphatase (Promega, S3841) and stopped with water. Controls with no primary antibody added were run in parallel. Protein bands were visualized by Coomassie blue staining using an eStain L1 protein staining system.

Binding of IgG and IgM to *Leishmania* by ELISA

Immuno 4HBx ELISA plates (ThermoFisher Scientific, 3455) were coated with *Leishmania* lysate (10 µg per well) or rK39 (25 ng per well). Antigens were diluted in carbonate bicarbonate buffer (pH 9.6, Sigma, C3041) and incubated overnight at 4 °C. Plates were blocked for 2 h at room temperature with 200 µl per well of 20% heat-inactivated FBS (Gibco, 16140071) diluted in buffered saline, 0.1% Tween-20 (TBST, Sigma, T9039). Plates were washed 6× with TBST followed by incubation with human, dog or mouse serum samples in a dilution range of 1:50 to 1:400. Secondary antibodies were diluted as follows: goat anti-human IgG-AP (Sigma, A1543) at 1:10,000; anti-dog IgG-AP (Sigma, A0793) at 1:5,000; goat anti-human IgM-AP (Southern Biotech, 2020-04) at 1:1,000; and goat anti-dog IgM-AP (Bethyl Laboratories, A40-116AP) at 1:1,000. Primary and secondary antibodies were diluted in TBST–5% FBS, and plates were washed 6× with TBST between each incubation using a 405 TS Microplate washer (Biotek). The optical density (OD) at 405 nm wavelength was recorded 30–60 min after the addition of the p-nitrophenyl phosphate liquid substrate system (Sigma, N7653).

Binging of IgM to *Leishmania* after in situ deglycosylation (ELISA)

For deglycosylation, ELISA plates were coated with *Leishmania* lysate (2.5 µg per well) and incubated overnight at 4 °C. Plates were blocked for 2 h at room temperature with TBST–20% FBS followed by 6× washes with TBST. In brief, each well was treated with 2 µl PNGase F (New England Biolabs, P0705L) and 5 µl of 10× Glycol-buffer for deglycosylation of N-linked glycans, or with 2 µl Protein Deglycosylation kit II (New England Biolabs, P6044S) and 5 µl of 10× Deglycosylation Mix Buffer 1, for deglycosylation of both N-linked and O-linked glycans. Each well was treated following the manufacturer's protocol for nondenaturing digestion in a final volume of 50 µl per well. Plates were sealed and incubated overnight (22–24 h) at 37 °C. Untreated wells with either *Leishmania* lysate (2.5 µg per well) or BSA (2.5 µg per well) were included in the plate. After enzyme treatment, plates were washed 6× with TBST followed by incubation with purified bovine IgM (3 mg ml^{-1}) diluted at 1:200. Sheep anti-bovine IgM-AKP (Bio-Rad, AAI19AB) was used as the secondary antibody at 1:2,000. Primary and secondary antibodies were diluted in TBST–5% FBS, and plates were washed 6× with TBST between each incubation using a 405 TS Microplate washer (Biotek). The OD at 405 nm wavelength was recorded 60–120 min after the addition of the p-nitrophenyl phosphate liquid substrate system (Sigma, N7653).

IgM depolymerization

To obtain monomeric IgM antibodies, 1 mg of bovine IgM (Millipore-Sigma, I8135) was subjected to a digestion buffer exchange (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% NaN_3; pH 8.0) to a final concentration of 1 mg ml^{-1}. Afterwards, 1 ml of IgM was incubated in the presence of 6 mg 2-mercaptoethylamine at 37 °C for 30 min in the dark. The reduced IgM was further alkylated in the presence of 16 mg iodoacetamide, followed by purification using a Superdex 200 Increase (10/300 GL; GE Healthcare) equilibrated with buffer A without EDTA. After size exclusion, a clear separation between the pentameric (peak 1, P1) and monomeric (peak 3, P3) IgM fractions were observed by native gel electrophoresis (NativePAGE Novex Bis-Tris Gel system; Life Technologies). All fractions corresponding to defined peaks were pooled together, adjusted to a suitable concentration and frozen at −20 °C until further use.

IgM identification by MS

Protein identification was carried out with a standard bottom-up method. The sample was reduced using DTT, alkylated with iodoacetamide, then digested using Lys-C followed by trypsin. The digested sample was cleaned using C18 tips (Agilent, OMIX10) and subjected to the LC–MS (EASY nLC 1200 and Orbitrap Fusion Lumos mass spectrometer, Thermo Scientific). The peptides were separated using a PepMap 100 C18 reversed phase column, and a standard data-dependent acquisition was performed. The survey MS1 (top speed mode, 3 s) was done using an Orbitrap mass analyzer, and the ddMS2 was done using the Linear Ion Trap, for which the fragmentation was done with CID. Dynamic

exclusion was set for the duration of 20 s. Data analysis was done using PEAKS Studio software (Bioinformatcs Solutions). The database used was the UniProt proteome UP000002254 (Dog, taxonomy ID 9615). The number of identified peptides and protein abundance in the sample were confirmed by the PEAKS scores.

Video recording and stereomicroscopy imaging

Midgut videos were taken using an iPhone 10 camera connected to a Stemi 508 stereomicroscope (Zeiss) ocular using a microscope mount (iDu Optics, LabCam iPhone 10 microscope adapter with built-in 30 mm ×10 WF lens). Phase-contrast images and videos were recorded using a K5 camera coupled to a Thunder Imager microscope (Leica). Raw files were opened using Fiji ImageJ software[59] and exported at 25–90 frames per second.

Immunofluorescence of in vitro LMCs

After 24 h of formation in vitro, whole LMCs were washed twice with PBS before fixation with 4% paraformaldehyde overnight at 4 °C. Fixation was followed by permeabilization with 0.1% PBS Triton for 10 min, then 0.1 M glycine in PBS solution for 10 min, and a final blocking step with 0.1% Triton, 0.1% BSA–PBS for 1 h at room temperature. Mating clumps were then incubated at 4 °C overnight with mouse anti-α-tubulin monoclonal antibody (MilliporeSigma, T6199) conjugated to Alexa 555 (1:1,000), and sheep anti-bovine IgM (Bio-Rad, AAI179) conjugated to Alexa 647 (1:1,000) diluted in blocking buffer. Anti-bovine IgM antibody was conjugated to an Alexa 647 fluorophore using a READILINK 647/674 Antibody Labeling kit (Bio-Rad, 1351006) following the manufacturer's instructions. The next day, mating clumps were washed three times with blocking buffer, 10 min each, and then washed two times with permeabilization buffer, both at room temperature. Clumps were counterstained with 2 µM of Hoechst 33342 (ThermoFisher Scientific, 62249) in permeabilization buffer for 30 min at room temperature, followed by a quick rinse of 0.1% Triton–PBS. Clumps were placed in a u-slide angiogenesis (IBIDI, 81506) well in Prolong gold antifade medium (ThermoFisher Scientific, P10144) for imaging. For the sections, mating clumps were fixed in 4% paraformaldehyde overnight at 4 °C, processed on a Leica ASP 6025, and then embedded in histology-grade paraffin. All samples were sectioned at 3 µm. Mating clump section pretreatment was performed on a Leica Bond RX. Sections were baked for 30 min at 60 °C and then dewaxed for 30 s in Bond Dewax solution (Leica, AR9222) heated to 72 °C. Sections were rehydrated with absolute ethanol washes followed by 1× Immuno-Wash (StatLab, ACR-015), then subjected to a 20-min treatment with Epitope Retrieval Solution 1 (Leica, AR9961) heated to 100 °C. Slides were then washed with PBS and stained. Steps for immunofluorescence of sections were based on a previous method[60]. In brief, slides were permeabilized with PBS, gelatin and Triton 0.25%, twice for 10 min each at room temperature, followed by blocking with 5% BSA–PBS for 1 h at room temperature in a humid box. The primary antibodies listed above were incubated in a blocking solution and kept overnight in a humid box at 4 °C. The next day, slides were washed three times with PBS and then with permeabilization solution for 10 min at room temperature, and counterstained with 2 µM of Hoechst 33342 in the same buffer for 30 min at room temperature followed by a quick rinse with 0.1% Triton–PBS. Another final rinsing step with 10 mM $CuSO_4$ and 50 mM NH_4Cl solution for 10 min and a final short rinse with water was performed. Slides were sealed using Prolong gold antifade mounting medium and a coverslip.

Confocal microscopy

Images were captured using a Leica TCS SP8 DMi8 inverted fluorescence confocal microscope (Leica Microsystems) equipped with a photomultiplier tube/hybrid detector and a Leica DFC345 monochrome camera. Images were taken using sequential acquisition, variable *z*-steps, mosaic size and integration. Images were viewed with using ×40, ×63 and ×100 oil-immersion objectives (zoom factor of 2, 3 or 4), and data were collected using the Leica Application Suite X software platform. White light laser and specific emission and excitation ranges were applied depending on the fluorophore used. The following spectra for excitation/emission were used: 488/520 nm for EdU and BrdU; 555/580 nm for α-tubulin; 594/620 nm for BrdU and EdU; and 647/665 nm for IgM. DAPI was excited using a 405-nm diode laser. Images were taken using sequential acquisition and variable *z*-steps. Whole mating clumps were imaged in the *z*-wide mode (8-bit) and tile mode to capture the entire clump. Individual fields were merged and stitched together to mount the composite of the whole clump. Image processing was performed using Imaris 9.2.1 (Bitplane, Oxford Instruments). Maximum intensity projection is represented in *xy* pictures, and cross-sections of between 2 and 3 µm were obtained with the ortho-slicer feature from Imaris. Images were acquired using 8-bit resolution, size 1,024 by 1,024 and with a 400–600Hz speed. Gaussian filter was applied to all the pictures using the default settings during processing in Imaris. Images were pseudocoloured as confocal camera is black and white for increased sensitivity. No gamma changes were performed. All raw images are available on request.

BrdU and EdU staining

Leishmania promastigotes were incubated with 10 µM EdU (Abcam, ab146186) or BrdU (Abcam, ab142567) for 4 days to reach the stationary growth phase. Parasites were then washed twice with PBS, and an equal proportion of each of the labelled promastigotes was used for mating clump assembly. Mating clumps were fixed and processed for sections as described above. Five micrometre (*L. tropica*) and 7-µm (*L. major*) sections were washed with PBS, permeabilized with 0.2% Triton–PBS for 10 min, then incubated with 0.1 M glycine for 10 min at room temperature. Coarser sections were needed to find less frequent hybridization events in the *L. major* clumps compared with the *L. tropica* clumps. Samples were then blocked with 0.5% BSA–PBS for 10 min and then briefly washed with PBS before the Click reaction was performed. We used a Click-it EdU Cell Proliferation kit to image incorporated Edu with Alexa Fluor 488 dye (Invitrogen, C10337). The reaction was prepared following the manufacturer's instructions and incubated with the samples for 30 min at room temperature in a humid and dark chamber. After the Click reaction, sections were washed three times with PBS and blocked with 0.5% BSA–PBS for 1 h at room temperature. Primary mouse monoclonal anti-BrdU (MOBU-1) (Invitrogen, B35128) (1:100) was incubated in blocking buffer overnight at 4 °C. The next day, sections were washed with blocking buffer three times, 10 min each, and incubated with goat anti-mouse Alexa 594 secondary antibody (Invitrogen, A-11005) at 1:1,000 in 0.1% Triton–PBS for 2 h at room temperature. Slides were washed three times with 0.1%Triton–PBS for 10 min each and then counterstained with 2 µM Hoechst 33342 in 0.1% Triton–PBS for 30 min at room temperature. A final rinse was done with 10 mM $CuSO_4$ and 50 mM NH_4Cl solution for 10 min and followed by a quick rinse with water before the slides were sealed with Prolong gold antifade mounting medium and a coverslip.

Genome sequencing and analysis

Genomic DNA was extracted from cloned parasite culture pellets using an E.Z.N.A. DNA/RNA Isolation kit (Omega Biotek, R6731) and following the manufacturer's recommendations for cultured cells. Sample concentration was measured using a DS-11+ spectrophotometer (DeNovix). A minimum of 3 µg of DNA was sent for sequencing (Novogene). Genomic DNA was randomly fragmented by sonication, then DNA fragments were end polished, A-tailed and ligated with full-length adapters for Illumina sequencing followed by further PCR amplification with P5 and indexed P7 oligonucleotides. The PCR products were purified with the AMPure XP system and libraries were checked for size distribution using an Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified by real-time PCR (to meet the criteria of

3 nM). Qualified libraries were fed into Illumina sequencers after pooling to obtain effective concentrations and the expected data volume.

For Figs. 4e and 5d and Extended Data Fig. 7a,b, raw sequencing reads were trimmed using Trimmomatic (v.0.39)[61] and mapped using BWA-MEM (v.07.17)[62] to the TriTrypDB-52_LmajoprFriedlin reference genome with the resistant gene sequences inserted as additional contigs. PCR duplicates were marked using Samblaster (v.0.1.2.5)[63], and germline variants were called jointly across all samples using HaplotypeCaller/GATK (v.4.1.9.0) and using default settings. Resulting variants were then filtered down to only biallelic sites with no missing data across all samples. The genomic insertion locations for each resistance gene were validated by examining split reads from each sample and identifying the location of reads with one pair member mapped to the construct and the other pair member mapped to the human genome. Any locations with fewer than three supporting split read pairs were excluded from consideration. To quantify the allelic copy number of each contributing parent in F_1 offspring, resulting BAM files were first used as input to the PAINT software suite (release 1.0)[64] to generate chromosome-level somy levels (Extended Data Fig. 7c). A custom perl script was then used to execute the following steps with the multisample VCF and chromosome somy estimates as input: (1) identify homozygous SNP differences between parents for each cross, ignoring sites at <20× depth in both parental strains; (2) for each SNP identified in step 1, calculate the frequency of each allele based on read counts supporting each allele (for example, allele 1 proportion = (no. reads supporting allele 1 at position 1)/(total sequencing depth at position 1); (3) for a given F_1, multiply the proportion of each allele by the chromosome-specific somy to get the copy number estimate for each parent. For the backcross hybrids analysis, the same procedure outlined above was used, and recombination breakpoints were identified as regions for which the parental copy number along a chromosome shifted by ≥0.8 (to allow for 20% variance in absolute copy number estimation) across ≥10 kilobases. All resulting copy number breakpoint calls were visually confirmed (for example, Fig. 5d). The script used to execute the steps above is available at https://zenodo.org/record/8231973 or https://github.com/jlac/Leishmania_allelic_copy_number. For Extended Data Fig. 6, raw sequencing reads were assessed for quality using FastQC software. Fastq files were imported and processed using Geneious prime software (v.2021.2.2; https://www.geneious.com). Clean reads were mapped to the reference genome TriTrypDB-52_LmajorFriedlin using bowtie 2 plugin. BAM files with aligned reads were analysed using qualimap (v.2.2.1)[65]. The minimum genomic coverage among all samples was 22.2×. Using bowtie 2 (medium sensitivity), reads were aligned to an artificial chromosome with selective markers (Supplementary Data 1). The artificial chromosome was created by concatenating the resistance genes integrated into the parental lines, HYG, BSD and SAT, buffered by a 1.2 kb (150 bp repeated 8 times) sequence from mouse (*Mus musculus*) chromosome 1.

Preparation of *Leishmania* pellets for transcriptomics and proteomics analyses

L. major parasite mating wells were prepared as described in the section 'In vitro LMC assembly for recovery of hybrids'. At 24 h after incubation with IgM, IgM-treated parasites or untreated controls were pelleted and washed once with PBS. Of note, at this time point, most parasites treated with IgM will be in clumping formations. Pellets of control and IgM-treated groups were stored at −80 °C.

Transcriptome sequencing and analysis

Total RNA was extracted from parasite pellets using a Qiagen RNeasy plus mini kit (Qiagen, 74134) and following the manufacturer's recommendations for cultured cells. Sample concentration was measured using a DS-11+ spectrophotometer (DeNovix). A minimum of 5 µg of total RNA was sent for library preparation and sequencing (Novogene). In brief, mRNA was purified from total RNA using poly-T oligonucleotide-attached magnetic beads. After fragmentation, the first-strand cDNA was synthesized using random hexamer primers followed by synthesis of the second-strand cDNA. The library was ready after end repair, A-tailing, adapter ligation, size selection, amplification and purification. The library was checked with Qubit and real-time PCR for quantification, and a bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on an Illumina NovaSeq 6000 platform according to an effective library concentration and data amount. A minimum of 50 million paired reads per sample were obtained.

Raw RNA sequences were prepared and analysed using the CLC genomics workbench (v.22; Qiagen). Sequences were trimmed (prepare raw data workflow) to remove poor-quality sequences and adaptors. Trimmed reads were mapped, and the differential expression of genes for IgM compared with control groups was calculated for six biologically independent sample pairs (RNA-seq and differential gene expression analysis workflow). TriTrypDB-59_LmajorFriedlin was used as a reference genome. As previously described[66], filtering was performed on all mapped gene counts to exclude genes where the sum of counts in all conditions was inferior to ten. Significant associations were considered when a P adjusted was smaller than 5% ($P < 0.05$) and $\log_2$ fold change larger than 0.5 (±). PCA was performed using $\log_2$ transcript per million reads. Heatmaps were generated using $\log_2$ transcript per million read z-scores. Heatmaps and PCA plots were generated using the GraphBio web app (v.2.2.7)[67].

Proteome preparation and analysis by MS

Cell pellets were thawed on ice and immediately lysed in 26 µl of 50 mM HEPES, pH 7.4, 5% SDS with vigorous mixing by vortexing for 5 min. The lysates were sonicated five times in a water bath at 30 °C with 5-min intervals, freeze–thawed on dry ice twice and sonicated three times more as mentioned above. The lysates were cleared by centrifugation at 12,500 r.p.m. at 8 °C for 10 min, and the protein concentration was estimated by BCA (Thermo Fisher Scientific) using BSA as a standard. A total of 134 µg of proteins from each sample was reduced in 5 mM DTT in 50 µl of lysis buffer for 40 min at 37 °C. Samples were cooled and alkylated in 15 mM iodoacetamide for 20 min. The samples were adjusted to 1.2% H_3PO_4, and 350 µl of 50 mM HEPES, pH 7.1, in 90% methanol was added. The samples were processed with an S-Trap Plate (Protifi) using 5 µg trypsin in 100 mM TEABC per well. The eluted peptides were dried under vacuum, dissolved in water and quantitated by fluorescence assay (Pierce). A total of 50 µg of each sample was labelled using TMT-10 plex reagents in 50% AcCN in 75 µl of 100 mM TEABC at room temperature for 1.5 h. After quenching, the samples were consolidated and reduced in volume by 80% under vacuum. 420 µl of 0.1% TFA was added, and the final pH adjusted to 2.0 with 10% TFA. The peptides were desalted using an Oasis HLB, a 10 cc vacuum cartridge, dried, dissolved in 100 µl of 50 mM TEABC and fractionated on a 3 × 150 mm C_{18} Extend HPLC column (Agilent) using an AcCN gradient to 50% in 5 mM TEABC. A total of 60 fractions were collected and consolidated into 12 fraction pools which were lyophilized. The samples were dissolved in 25 µl of 0.1% formic acid, 3% acetonitrile and quantitated by a micro-colorimetric peptide assay (Pierce). The proteomic relative quantification was performed using ten samples (five non-treatment, five IgM-treated). LC–MS data acquisition was performed using an Orbitrap Fusion Lumos (Thermo Fisher Scientific) connected to an EASY-nLC 1200. Each TMT-labelled peptide sample was loaded onto a 2-cm PepMap trap column (ThermoFisher Scientific, no. 164946) and separated using a 25-cm PepMap column (ThermoFisher Scientific, no. ES903). The mobile phase solvent was water with 0.1% formic acid, and peptide elution was achieved using acetonitrile in 0.1% formic acid: 0–24% for the first 120 min, then 24–48% for the next 60 min. The TMT reporter ions were monitored as follows: survey MS1 was acquired with the 120,000-resolution setting every 3 s. Data-dependent MS2 was acquired with Linear Ion Trap after CID. Synchronous precursor

selection was carried out, and MS3 was acquired with Orbitrap at the 50,000-resolution setting after HCD at 65% NCE.

Raw MS files were processed in MaxQuant (v.2.1.1.0)[68]. Quantification of the sample was performed using TMT, and the results are detailed in the file proteinGroups.txt. The identification of protein fractions, reporter ions and protein groups is detailed in Supplementary Table 8. The Andromeda search engine was used for spectral identification based on a database search against the *L. major* strain Friedlin (TrytripDB-59). For identification, a false discovery rate of 1% was used as threshold for both the peptide and the protein levels. For modifications: acetyl (protein N-term) and oxidation (M) were set as variable modifications, and carbamidomethyl (C) as a fixed modification. MaxQuant default settings were used for all other parameters. Type was set to Reporter in MS3 and 10plex TMT. The output table proteinGroups.txt from MaxQuant was loaded into Perseus (v.2.0.7.0)[69] for downstream analyses. Data were filtered to remove contaminants and reverse peptides that match a decoy database and proteins identified only by site. For unambiguous identification, proteins with at least one unique peptide were used for analyses. The corrected intensities were $\log_2$-transformed. Data were normalized by subtracting the median to centre the distribution at zero. Annotation was imported from the Tritryp database. For quantification, only proteins that were detected in seven out of the ten biological replicates were considered. Missing values were imputed using a random normal distribution of values with the mean set at the mean of the real distribution of the values minus 1.8 standard deviation and a standard deviation of 0.3 times the standard deviation of the distribution of the measured intensities. The probability of differential expression between groups was calculated using the Student's *t*-test, using the permutation based false discovery rate of 250 randomizations. Heatmaps and PCA plots were generated using the GraphBio web app[67].

Scanning electron microscopy

LMCs were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, 15960). After fixation, the clumps were rinsed in 0.1 M sodium cacodylate buffer and fixed with 1% OsO_4 reduced with potassium ferrocyanide in buffer. After a couple of washes in water, the clumps were dehydrated in 100% ethanol and dried using multiple exchanges of hexamethyldisilazane. On the following day, clumps were mounted on SEM pins using carbon adhesive tabs. Gentle pressure was applied to split open clumps to reveal their content, sputter coated with 5 nm of Iridium using an EMS 300TD Quorum sputter coater and imaged on a SU 8000 scanning electron microscope. Scanning electron micrographs were converted to RGB colour in Adobe Photoshop CS6 from greyscale. The embedded scanning electron microscopy image details were masked using the Fill-Content Aware function. The *L. major* picture mask was modified with a hue/saturation layer (hue: 28, saturation: 13, lightness: −34). The background was selected as a mask and the contrast was modified with the levels adjustment layer. The background mask was coloured cyan (#0eb7f6) with a colour fill adjustment layer set at an opacity of 85% and fill of 90% using an overlay transfer mode. For *L. tropica*, the picture mask was modified with a hue/saturation layer (hue: 28, saturation: 13, lightness: −34). The background was selected as a mask and the contrast was modified with the levels adjustment layer. The background mask was coloured green (#9acd32) with a colour fill adjustment layer set at an opacity of 85% and fill of 85% using an overlay transfer mode.

Transmission electron microscopy

LMCs were fixed as per the scanning electron microscopy protocol. After fixing the organoids with 1% reduced OsO_4 and dehydrating with 100% ethanol, the clumps were infiltrated and embedded with 100% Spurr's resin in a flat mould. The flat mould with clumps were excised and mounted on BEEM capsules for trimming. Ultrathin sections were collected using a Leica UC7 ultramicrotome and imaged on an 80 kV Hitachi 7800 TEM using an AMT XR81B bottom mount camera. Image capture engine software amt (v.7.0.0.149).

DNA content analysis by flow cytometry

DNA content analysis was performed as previously described[70] with modifications. In brief, exponentially growing cells were collected by centrifugation (2,500*g*, 15 min) and washed in PBS supplemented with 5 mM EDTA. Cells were fixed at 4 °C in a mixture of methanol and PBS (7:3) for 1 h. Cells were then collected by centrifugation and washed once in PBS supplemented with 5 mM EDTA and passed through a 30 µm Filcon (BD Biosciences, 3406000). Around 2×10^7 cells were re-suspended in 1 ml of FxCycle PI/RNase staining solution (ThermoFisher, F10797) and kept in the dark at room temperature for 1 h. Cells were then collected by centrifugation and re-suspended in 1 ml of PBS supplemented with 5 mM EDTA. Flow cytometry events, 50,000 cells or more, were collected using MACSQuant analyzer 16 (Miltenyi Biotec) and analysis was performed using FlowJo (v.10.8; BD Bioscience).

Statistical analysis

Data manipulation and statistical analysis were performed in R (v.4.1.1) with custom code written in Rstudio (v.1.4.1717). Functions from the following packages were used in our scripts: abind[71], chron[72], data.table[73], e1071[74], emmeans[75], FedData[76], gsubfn[77], gt[78], ijtiff[79], janitor[80], lubridate[81], MASS[82], plyr[83], rcompanion[84], readxl[85], reshape[86], R.utils[87], rlist[88], Rmisc[89], rstatix[90], stats[91], stringr[92], tidyverse[93] (contains: dplyr, ggplot2, forcats, tibble, readr, stringr, tidyr, purr), utils[91], WRS2[94] and xlsx[95]. To compare hybrid formation success between the different experimental conditions (BM1, BM2 and −IgM, BM2 and +IgM) with the different parental line combinations (1×2, 1×3 and 2×3) as detailed in Figs. 4d and 5c, we applied an expanded Fisher's exact test that could cope with $r \times n$ matrices over a Chi-square test owing to the occurrence of low expected values (<5). As our data matrices exceed the standard 2×2 format, we followed the Fisher's exact test with a pairwise test with the pairwsieNominalIndependence function from the R package rcompanion for two-dimensional matrices, in which at least one dimension exceeded two levels. The output of this test was converted into a letter code using the cldList function from the R package rcompanion for improved test output representation; equal letters indicate no significant difference, whereas different letters indicate a significant difference ($P < 0.05$). Kruskal–Wallis with Dunns' multiple comparison test was applied to compare between the groups (*Leishmania* lysate (LL), LL with PNGaseF, LL with *O*/*N* glycosidase, and negative control), detailed in Fig. 2b. Graph shows individual values with median of cumulative data from three biological replicates. $^*P < 0.05$, $^{****}P \leq 0.0001$. A negative control group was run in one plate of the three biological replicates. Similar results for the negative control group are shown in Extended Data Fig. 3e. For the statistical analysis of ELISA assays performed using rK39 protein or *Leishmania* lysate as target antigens between the groups, detailed in Extended Data Fig. 3a,b, we applied a Mann–Whitney test. Graphs show the individual values and median of four human, dog or mouse serum samples. $^*P < 0.05$, $^{**}P \leq 0.01$. Mann–Whitney test was performed when measuring IgM binding to *Leishmania* lysate compared with BSA (negative control protein) by ELISA. Cumulative data from three biological replicates. $^{****}P \leq 0.0001$, detailed in Extended Data Fig. 3e.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data are present in the main text or the supplementary materials. Whole-genome and transcriptome sequencing raw reads files have

been deposited into the NCBI BioProject database with the identifier PRJNA988832. Proteome sequencing raw files have been deposited into the PRIDE database with the project identifier PXD038652. Source data are provided with this paper.

Code availability

Codes generated on this work used for copy number bioinformatics analysis are available at https://zenodo.org/record/8231973 and https://github.com/jlac/Leishmania_allelic_copy_number. Codes generated for this work used for statistical analysis are available at https://github.com/joedoehl/IgM-promotes-genetic-exchange-of-Leishmania.

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Acknowledgements We would like to thank Y. Rangel Gonzalez, R. Salas Carrillo and B. Mills from VMBS, NIAID for technical support; S. Doe and D. Akueson from VMBS, NIAID for sand fly insectary support; C. Percopo from LMVR, NIAID for flow cytometry support; A. de Souza from the Department of Medicine, Division of Nephrology and Hypertension, Georgetown University for critical reading of the manuscript; G. A. Nardone, M. Suzuki and L. R. Olano of the Research Technologies Branch, NIAID, for MS analysis; C. Brodskin from Fiocruz, Bahia, Brazil, and P. Lima dos santos, Programa de Pós-graduação em Ciencias da Saúde (Graduate Program in Health Science), Federal University of Sergipe for *Leishmania*-infected human serum; C. Petersen from the University of Iowa, USA, for *Leishmania*-infected canine serum; and S. Kuhn of the NCBR Research Technologies Branch, NIAID, for support with RNA sequencing analysis. This research was supported by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases, and NIH grant R01-AI29646 to S.M.B. The schematics in Figs. 4b and 5a and Extended Data Figs. 2a, 8a,c and 9a were created using BioRender (https://biorender.com).

Author contributions T.D.S. developed the hypothesis. T.D.S., S.K., J.G.V., S.M.B., I.V.C.-A., C.B.-M., J.A. and F.O. contributed to experimental design. E.I. planned the experiments. T.D.S., E.I., J.S.P.D., A.B.F.B., J.A., M.D., V.N., M.S., P.C., T.W. and T.L.A.S. performed the experiments. T.D.S., J.M.C.R., J.S.P.D., J.L., A.B.F.B., J.G.V., S.K., S.M.B., E.I., T.L.A.S. and J.V.-R. analysed the data. C.M. performed sand fly insectary work. J.G.V., S.K. and S.M.B. supervised the project. All authors wrote the manuscript. P.C. performed the in vitro IgM experiment with two different *Leishmania* species, *L. major* and *L. tropica*, and was able to replicate the IgM-mediated hybridization process five times, independently. A.B.F.B. and P.C. contributed equally.

Competing interests The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41586-023-06655-8.

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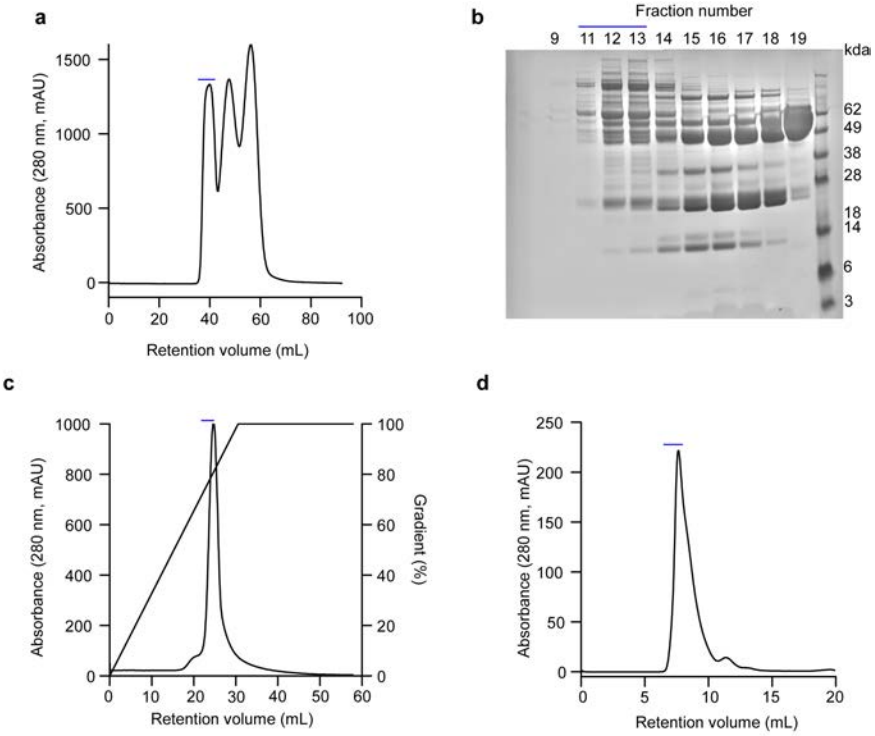
Peer review information *Nature* thanks Álvaro Acosta-Serrano, Jeremy Mottram and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.



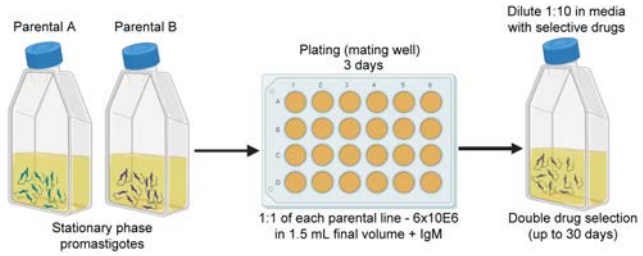
e

Mass Spec. top hits

Uniprot	Annotation	Area
J9NVC6	Immunoglobulin heavy constant mu	1.11E+10
J9P9J6	Immunoglobulin heavy constant mu	6.05E+09
J9JHH5	Joining chain IgM, IgA	3.00E+09
H9GWR8	Ig-like domain-containing	2.89E+09
F1PG16	CD5-like	2.22E+09
J9NWX7	Ig-like domain-containing	6.19E+08
P01874	Ig mu chain C region	3.12E+08
F6UME0	Alpha-2-macrogoblulin	3.05E+08

Extended Data Fig. 1 | Identification of IgM in blood serum as the *Leishmania* clumping factor. (**a**) Gel filtration chromatography of inactivated dog plasma on Sephacryl S-200. Buffer: 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 5 mM EDTA. Bar represents HPLC fractions corresponding to *Leishmania* clumping activity. (**b**) SDS-PAGE of fractions obtained from gel filtration chromatography shown in panel A. Bar represents fractions containing clumping activity that were pooled for ion exchange chromatography. (**c**) Ion exchange chromatography of pooled fractions 11–13 from B. HPLC gradient, a sodium chloride gradient of 0 – 1 M in 20 mM sodium phosphate, pH 6.0. Bar represents fractions pooled for high resolution gel filtration chromatography. (**d**) High-resolution gel filtration chromatography on Superdex-200. Buffer: 20 mM sodium phosphate (pH 7.4), 150 mM NaCl. Bar represents fractions pooled for protein identification. (**e**) Mass spectrum analysis of the most abundant proteins in pooled fractions from (**d**).

a



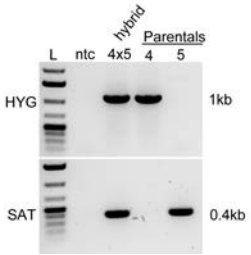
b

L. major parentals	Initial # of each parental cells	# of trial crosses	Group	Clump formation	% of positive crosses	Estimated frequency of hybridization
1x2	3.0E+06	30	-	-	0% (0/30)	0.0E+00
			IgM	+	13.3% (4/30)	3.3E-08
1x3	3.0E+06	40	-	-	0% (0/40)	0.0E+00
			IgM	+	7.5% (3/40)	2.5E-08
2x3	3.0E+06	7	-	-	0% (0/7)	0.0E+00
			IgM	+	14.3% (1/7)	4.8E-08

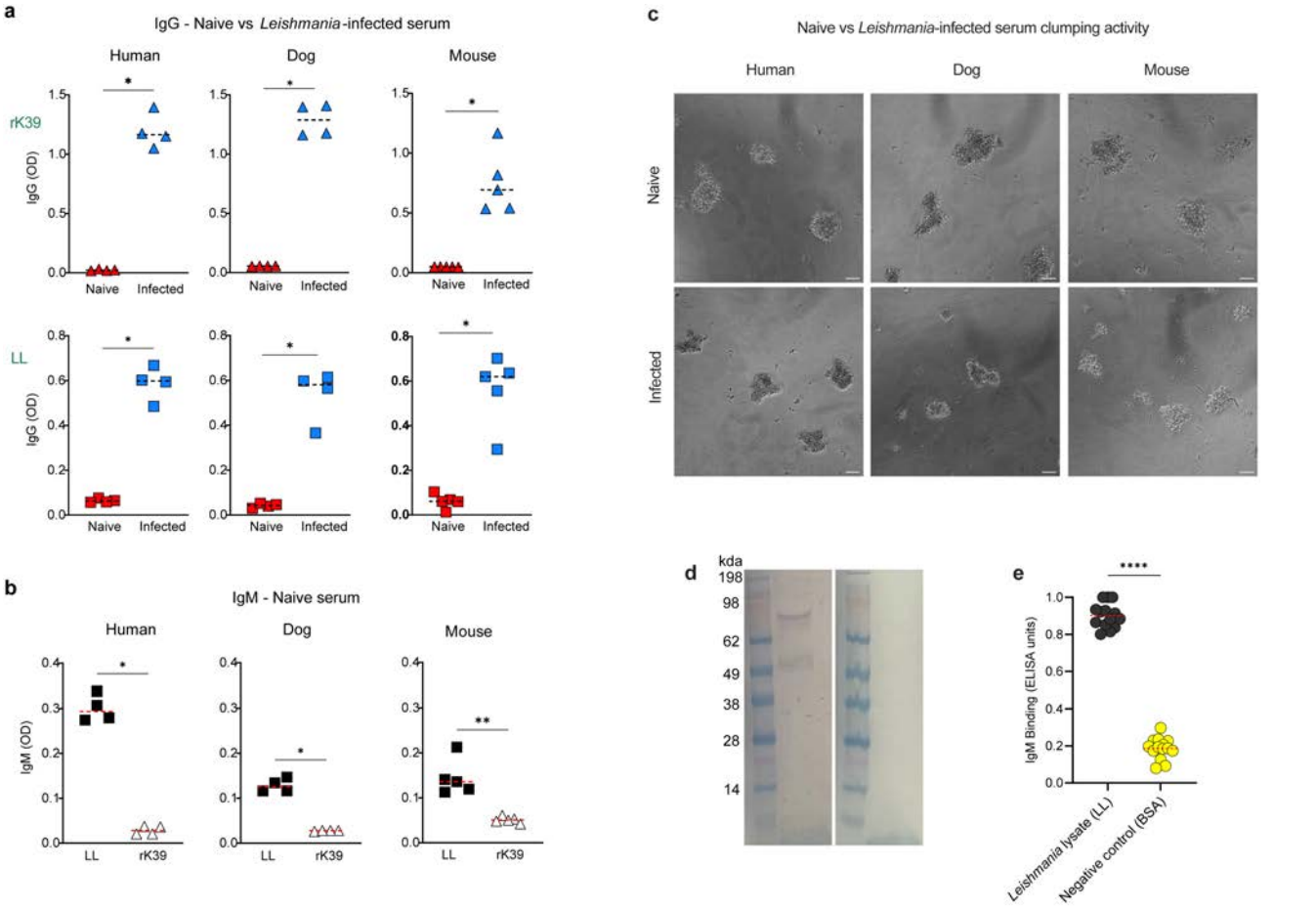
c

L. tropica parentals	Initial # of each parental cells	# of trial crosses	Group	Clump formation	% of positive crosses	Estimated frequency of hybridization
4x5	3.0E+06	12	-	-	0% (0/12)	0.0E+00
			IgM	+	33.3% (4/12)	1.1E-07

d



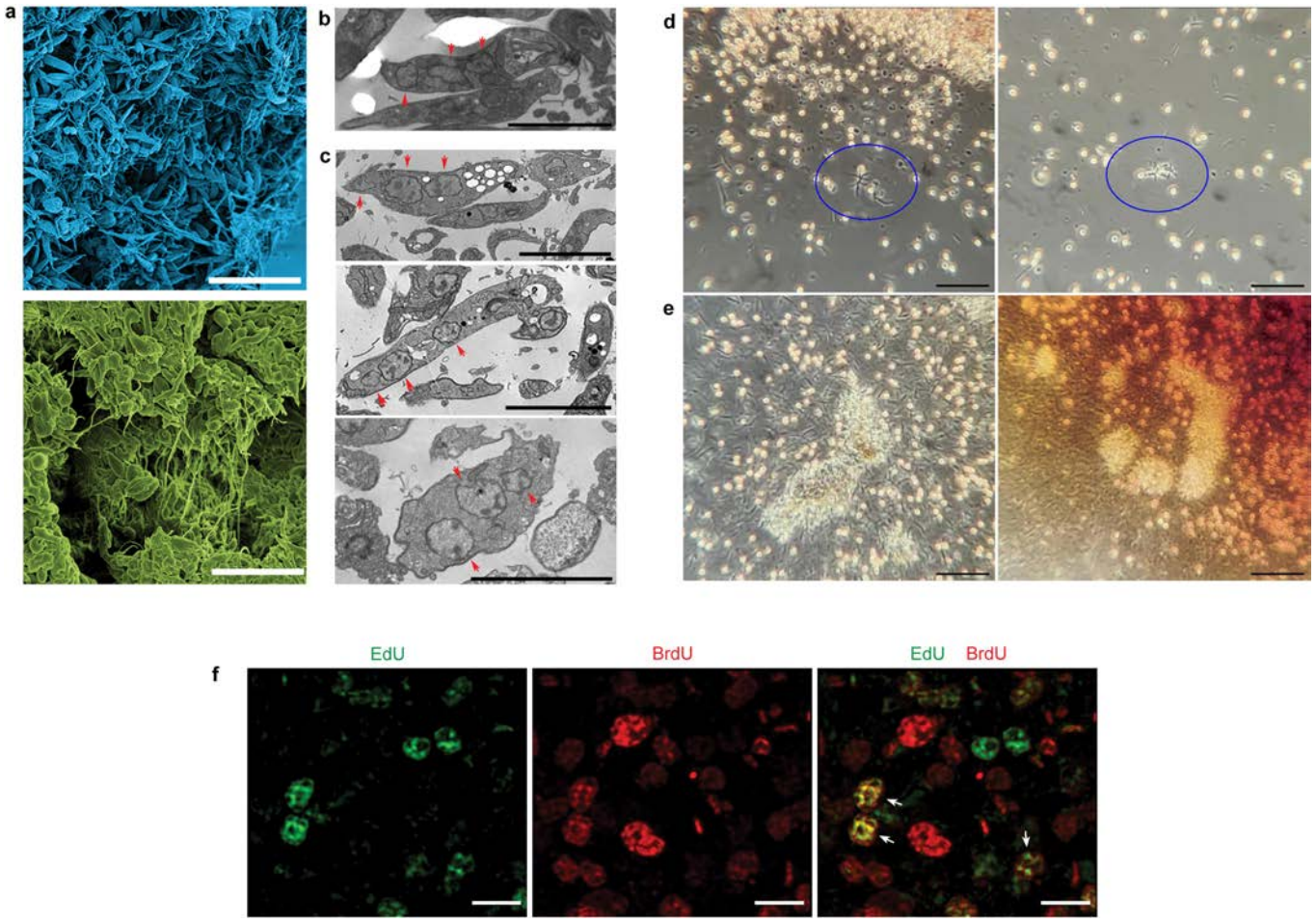
Extended Data Fig. 2 | IgM promotes *Leishmania* hybrid formation in vitro. (**a**) Workflow for in vitro crossing of *Leishmania* parental lines (*L. major* or *L. tropica*). Mating wells were prepared with 50 µg/mL IgM in complete Schneider's media. An improved detailed step-by-step procedure to maximize IgM-induced *Leishmania major* mating in vitro is available at https://doi.org/10.21203/rs.3.pex-2345/v1. (**b**) Data summary of hybrid formation from in vitro crossing of *L. major* parental lines: Parental 1, WR-SSU-HYG; Parental 2, FVI-FKP40-BSD; parental 3, FVI-FTL-SAT. *L. major* hybrids genotyping by PCR targeting parental selectable drug markers available at Fig. 1h. (**c**) Data summary of hybrid formation from in vitro crossing of *L. tropica* parental lines: Parental 4, K27-SSU-HYG; Parental 5, K27-SSU-SAT. (**d**) *L. tropica* hybrids genotyping by PCR targeting parental selectable drug markers HYG, and SAT. Parental 4, K27-SSU-HYG; Parental 5, K27-SSU-SAT; ntc, no template control; L, 1kb-plus ladder.



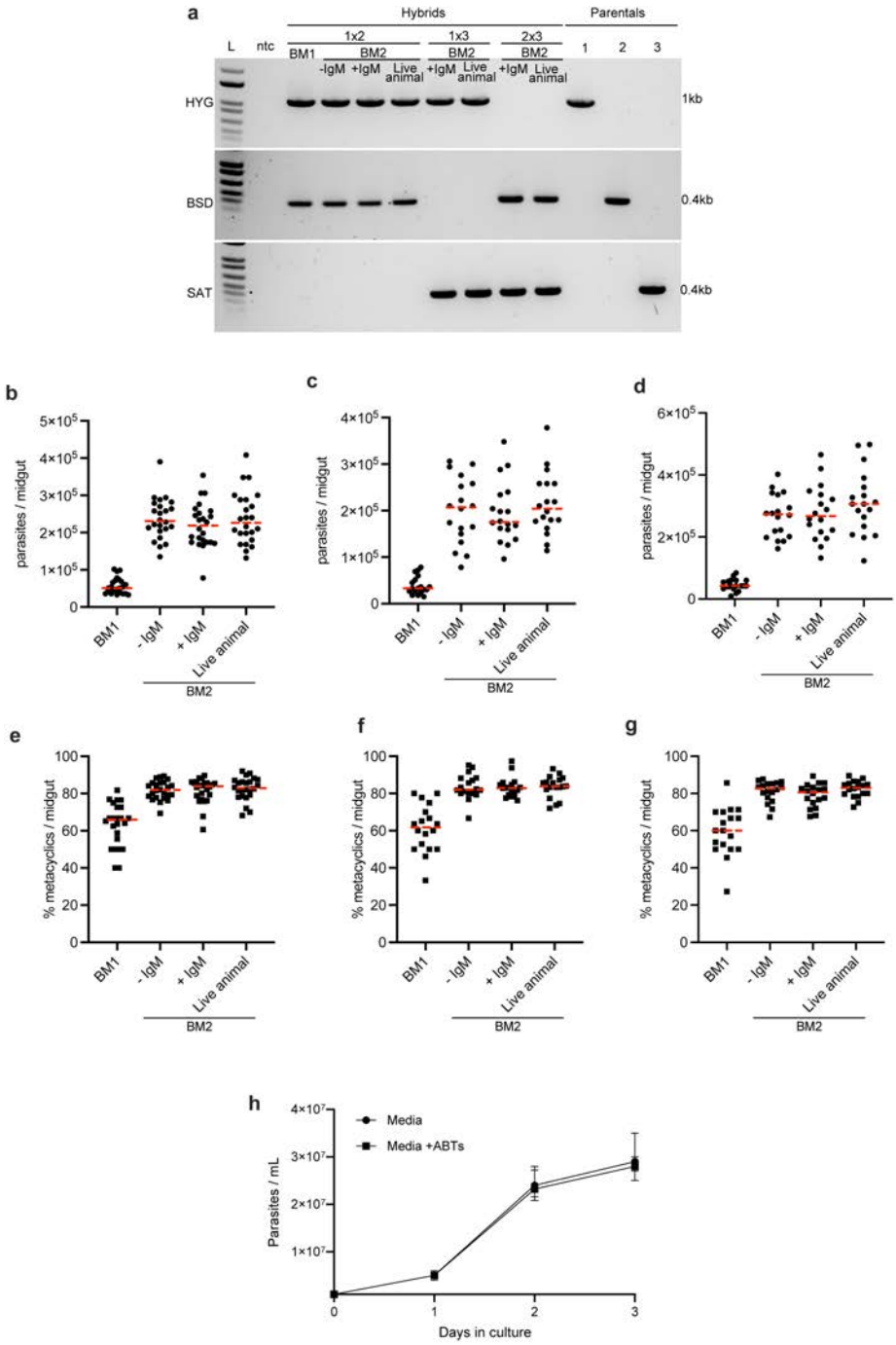
g

Parentals	Initial # of each parental cells	# of trial crosses	Group	Clump formation	Aglutination	% of positive crosses	Estimated frequency of hybridization
1x2	3.0E+06	12	-	-	-	0% (0/12)	0.0E+00
			IgM	+	-	8.3% (1/12)	2.8E-08
			PNA 50	-	+	0% (0/12)	0.0E+00
			PNA 12.5	-	+	0% (0/12)	0.0E+00

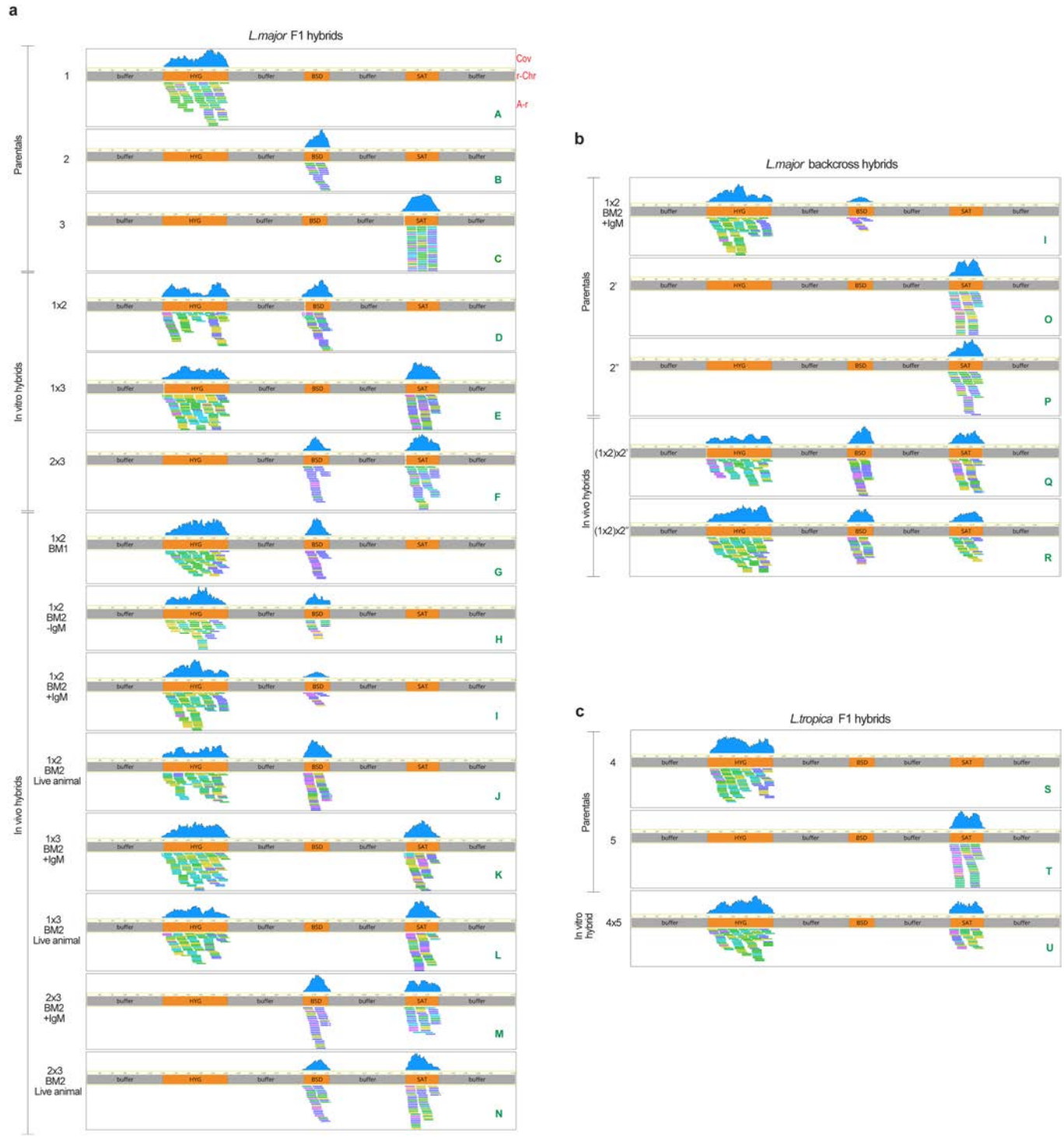
Extended Data Fig. 3 | IgM natural antibodies and sera from naive animals bind to *Leishmania* parasites. (**a**,**b**) Sera from naive or *Leishmania*-infected adult mammals were used as indicated. IgG (**a**) or IgM from naïve serum (**b**) ELISA performed using recombinant K39 (rK39) protein or *Leishmania* lysate (LL) as target antigens. Statistical analysis by Mann-Whitney test between groups, *P < 0.05, **P ≤ 0.01. n = 4. (**c**) *Leishmania* clumping pattern 2 h after incubation with naïve or *Leishmania*-infected sera. *L. major* parasites were incubated in complete Schneider's supplemented with 5% naïve or *Leishmania*-infected sera from adult mammals as indicated (n = 4-5). Scale bars = 50 µm. (**d**) Western blot of *L. major* enriched cell wall membrane preparation incubated with naïve adult human sera detected by anti-IgM (left panel). Right panel, *L. major* enriched cell wall membrane preparation incubated with only secondary antibody as control. n = 2. (**e**) Purified naïve IgM (IgMn) binding to *Leishmania* performed ELISA using LL or a negative control protein (bovine serum albumin) as target antigens. Statistical analysis by Mann-Whitney test between groups, ****P ≤ 0.0001. n = 3. (**f**) PNA agglutination does not promote *Leishmania* hybrid formation. Images taken 12 h after incubating parasites with varying concentrations of PNA. Scale bars = 50 µm. (**g**) Data summary of hybrid formation from in vitro crossing of *L. major* parental lines 1 and 2. Mating wells were prepared with 50 µg/mL IgM or with 6.25, 12.5, 25 and 50 µg/mL PNA in complete Schneider's media. ELISAs data represented by individual values scatter plots. Dashed bars, medians.



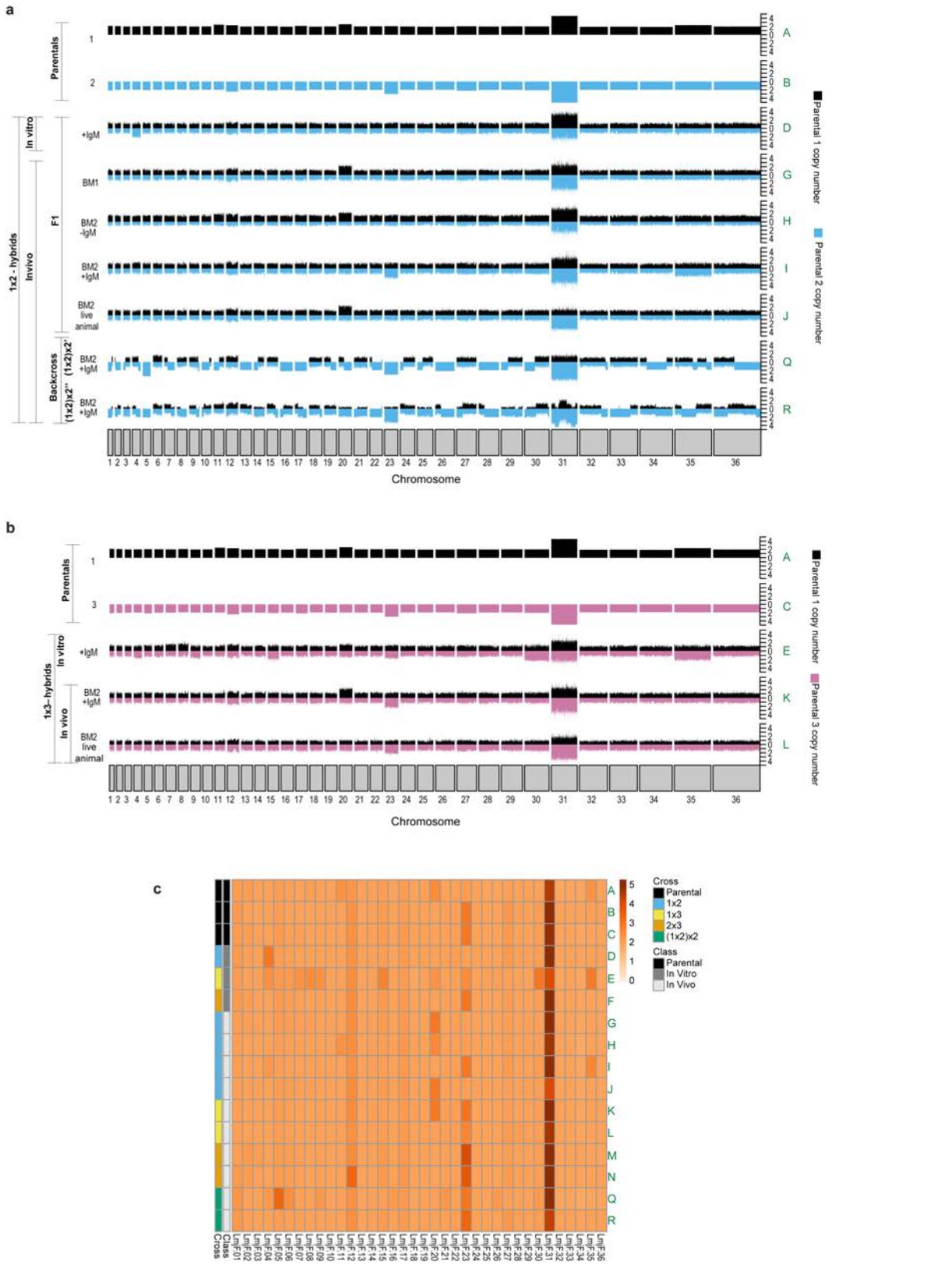
Extended Data Fig. 4 | IgM promotes *Leishmania* mating clump formation and hybridization inside the mating clump. (**a**) SEM of *L. major* (upper panel) and *L. tropica* (lower panel) mating clump showing the 3D organization of promastigote forms. Scale bar, 15 µm. (**b**) TEM of a *L. major* mating clump showing a cell with 3 nuclei. Scale bar, 3 µm. (**c**) TEM images of a *L. tropica* mating clump showing cells with 3 nuclei. Scale bars: 5 µm. Arrows point to individual nuclei within a parasite cell body. Three-nucleated cells are suggestive of an earlier step that precedes the merging of nuclei. The cell cycle progression follows a strict pattern were Nucleus, Kinetoplast, Flagellum (NKF) division is always constant and goes from 1N1K1F to 2N2K2F culminating with cytokinesis. Note the presence of 3 nuclei without changes to either the kinetoplast or flagellum suggestive of a potential early fusion event inside the LMC. (**d**, **e**) *Leishmania* mating clump formation within the sand fly midgut. Twelve days post-infection with *L. major*; *Lu. longipalpis* females were provided with a second reconstituted blood meal containing IgM (500 µg/mL). (**d**) Initiation of *Leishmania* mating clump (LMC) formation 30 min after imbibing a second blood meal (blue circles). (**e**) Parasites merge to form larger LMCs within 24 h after imbibing a second blood meal. Scale bars = 50 µm. LMCs formation in sand flies can also be observed in Supplementary Videos 11 and 12. (**f**) Confocal immunofluorescence of a 5 µm transversal section from *L. tropica* mating clump. Promastigotes were grown to stationary phase in the presence of EdU or BrdU then extensively washed and used at a 1:1 ratio to assemble the LMC with IgMn. EdU, green; BrdU, red. White arrows point to yellow nuclei indicative of fusion and exchanged genetic material. Scale bars, 3 µm.



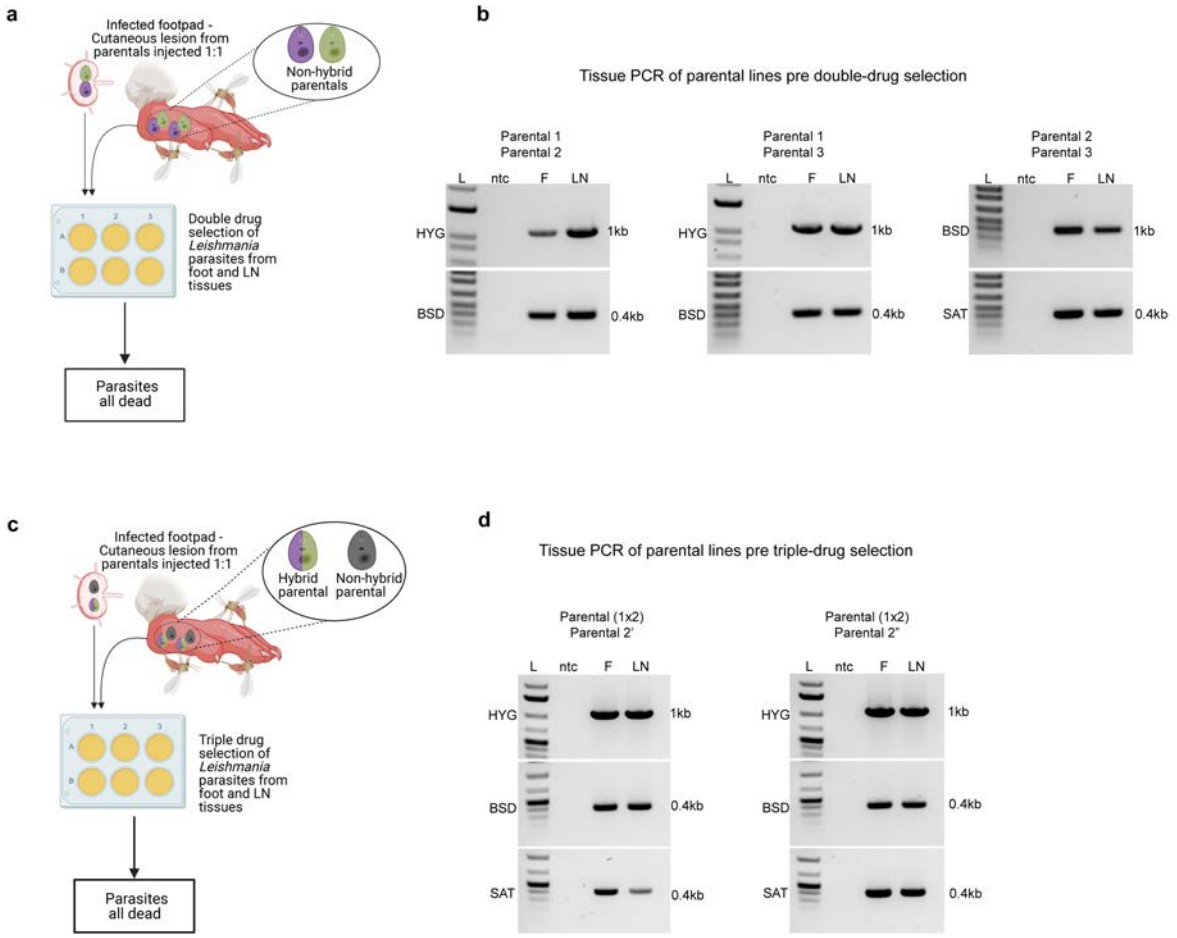
Extended Data Fig. 5 | Hybrid genotyping and infection status in sand flies after a naturally acquired *Leishmania* infection from mice lesions composed of two parental lines. (**a**) *Leishmania major* hybrids formed in s and flies given one infectious blood meal (BM1) or provided 6 days later with an additional uninfected bloodmeal (BM2) in the presence (+IgM) or absence (−IgM) of IgM were genotyped by PCR targeting parental selectable drug markers HYG (Hygromycin), BSD (Blasticidin) and SAT (Nourseothricin). Parental 1, WR-SSU-HYG; Parental 2, FVI-FKP40-BSD; Parental 3, FVI-FTL-SAT; ntc, no template control; L, 1kb-plus ladder. Double drug resistant hybrid lines were cloned before genotyping. A single hybrid exemplar is shown for positive events from each group. "n" detailed on Fig. 4d. Parasite number (**b**,**c**,**d**) and percentage of metacyclic promastigotes (**e**,**f**,**g**) in *L. major*-infected *Lu. longipalpis*. At 6 days post-infection, a proportion of the sand flies were provided a second uninfected blood meal via a membrane feeder composed of rabbit red blood cells reconstituted with fetal bovine serum with (+IgM) or without (−IgM) 500 µg/ml adult bovine IgM or allowed to feed on uninfected mice as a blood source for natural IgM. Infection status of individual sand flies was assessed at 14 days after the first blood meal (8 days after the second bloodmeal). Parental line combination 1×2 (b,e) n = 4, 1×3 (c,f) n = 3, 2×3 (d,g) n = 3. data represented by individual values scatter plots. Dashed bars, medians. (**h**) An antibiotic cocktail (ABTs) was used to control contamination with sand fly gut microbiota when isolating parasites from sand fly midguts. ABTs has no effect on parasite growth and viability. ABTs: Penicillin-Streptomycin (100 U/mL); Gentamicin (50 µg/mL); Caspofungin (15 µg/mL); 5-fluorocytosine (30 µg/mL). Growth curve represented by median ± interquartile range. n = 3.



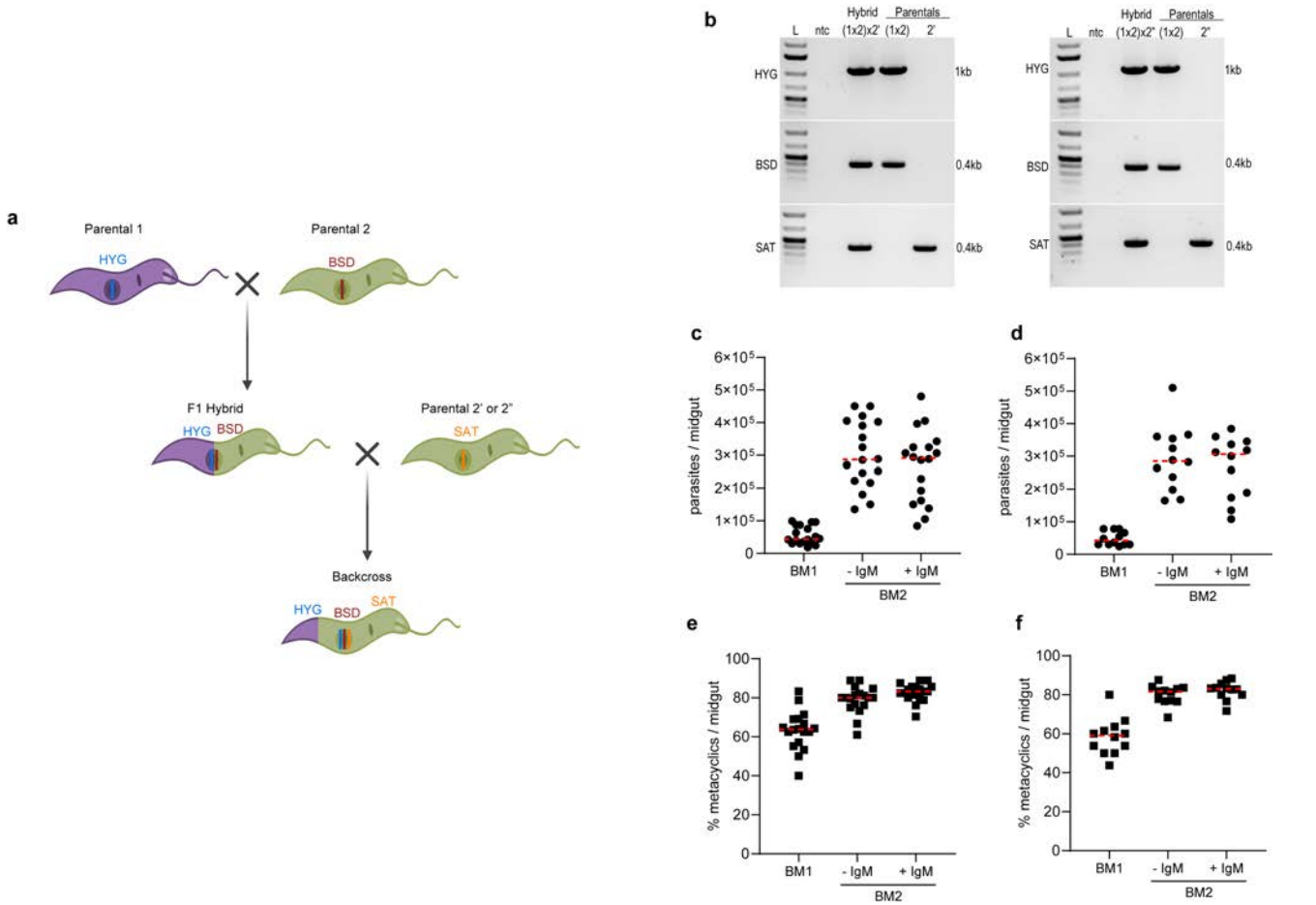
Extended Data Fig. 6 | Resistance markers whole-genome analysis of parental and hybrid lines. Schematic display of an artificial chromosome containing arbitrary loci of the three antibiotic resistance genes (Hygromycin – HYG, Blasticidin – BSD, Nourseothricin – SAT) used as parental markers in this study. (**a**) *Leishmania major* F1 hybrids. (**b**) *L. major* backcross hybrids. (**c**) *L. tropica* F1 hybrids. Raw reads were processed and displayed using Geneious prime software v2021.2.2. Upper case letters in green provide a unique identifier for each *Leishmania* parental or hybrid. Red abbreviations present on first panel of (a) are applicable to all panels in the figure: Cov, coverage; r-Chr, artificial resistance chromosome; A-r, aligned reads. Panel I was repeated from (a) to (b) for illustrative purposes as this was the selected F1 hybrid for backcrosses.



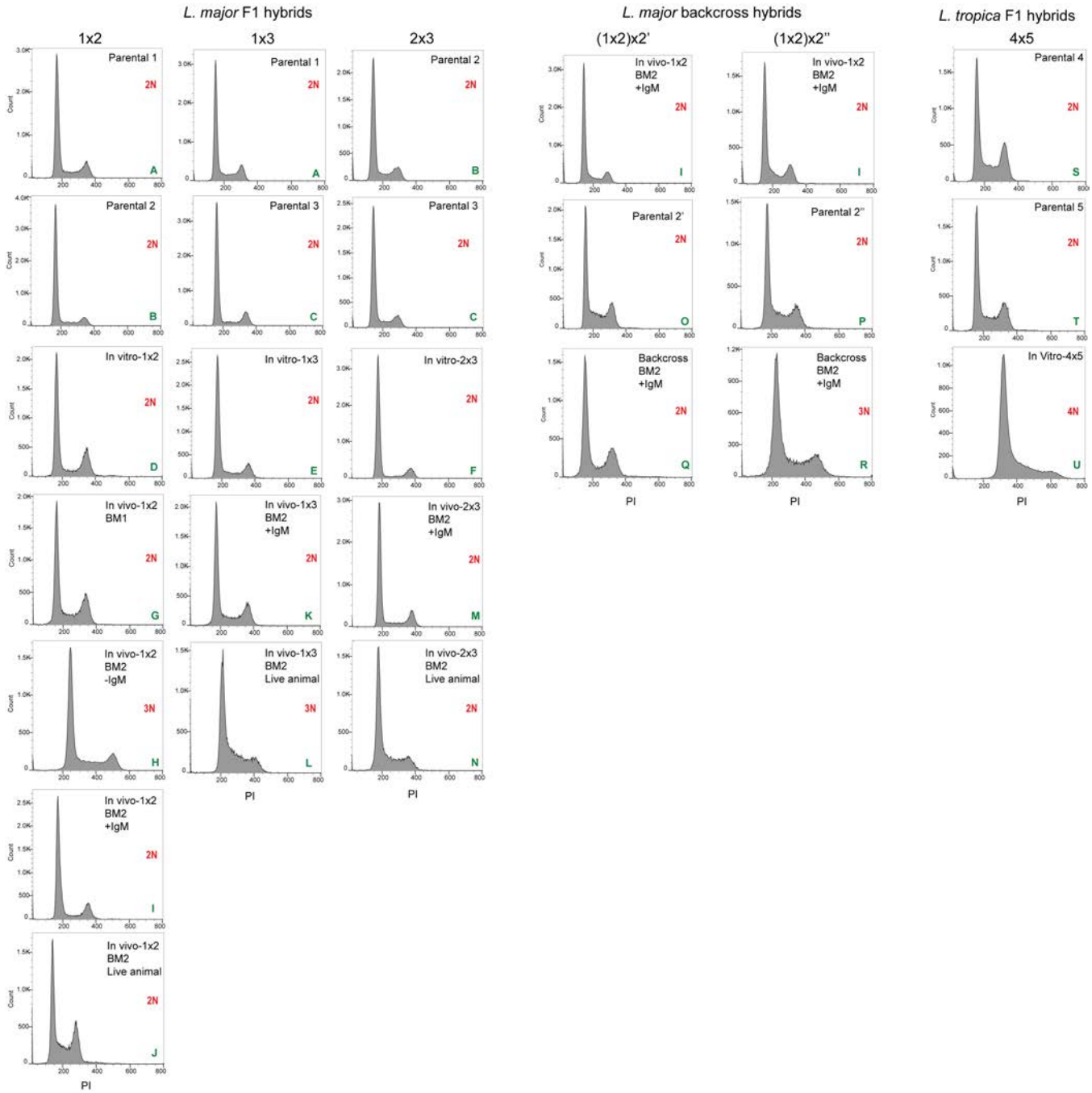
Extended Data Fig. 7 | Whole-genome analysis of *Leishmania major* hybrids. (**a**, **b**) Allele-specific chromosomal copy number as inferred by read depth and allelic proportions. Biparental ancestry was confirmed across the whole genome for crosses between parentals 1×2 and backcrosses of (1×2) F1 hybrid with parental 2′ or parental 2″ (**a**) and a parental 1×3 cross (**b**). Biparental inheritance of backcrosses for 1×2 crosses are also shown in Fig. 5d. Upper case letters provide a unique identifier for each *Leishmania* parental or hybrid. (2×3) crosses not present because within strains there were very few fixed differences between the parents, and therefore not enough informative sites to call ancestry. Parental 1 plot was repeated from (a) to (b) for illustrative purposes as this parental composed both F1 crosses setup. (**c**) Somy heatmap of sequenced *Leishmania major* parentals and hybrids. Whole-genome analysis shows characteristic *Leishmania major* aneuploidy chromosome distribution in all samples. (**a**-**c**) Parental 1, WR-SSU-HYG; Parental 2, FVI-FKP40-BSD; Parental 3, FVI-FTL-SAT. Parental 2′, FV1-FKP40-SAT; Parental 2″, FV1-SSU-SAT. Upper case letters provide a unique identifier of each *Leishmania* parental or hybrid.



Extended Data Fig. 8 | Evaluation of parental lines and hybrids in infected mouse tissue. (**a**) Experimental design. Mouse footpads were injected with a 1:1 mixture of two *L. major* parental line combinations 1 and 2, 1 and 3, or 2 and 3. After lesion development, 3 to 5 weeks post injection, infected footpads were exposed to sand flies for their first blood meal. Tissue from the infected footpad (F) and draining lymph node (LN) were then disrupted and seeded in complete Schneider's media (6 mL) for 3 days to allow for amastigote differentiation into promastigotes. Half of the material was then cultured in double drug pressure for selection of hybrids. (**b**) The second half of the tissue from (a) was used for DNA extraction to confirm the presence of both parental lines by genotyping. n = 12 (1×2), 9 (1×3 and 2×3). (**c**) Experimental design. Mouse footpads ware injected with a 1:1 mixture of (1×2) F1 hybrid and parental 2 harboring a different resistance marker (SAT) at the same (2′) or different (2″) chromosome locus. Sand fly feeding on mouse lesions and tissue processing were carried out as outlined above. Half of the tissue from the footpad and draining lymph node was then cultured in triple drug pressure for selection of hybrids. (**d**) The second half of the tissue from (c) was assessed for the presence of both parental lines by genotyping. n = 9 [(1×2)x2′], 6 [(1×2)×2″]. Parental 1, WR-SSU-HYG; Parental 2, FV1-FKP40-BSD; Parental 3 = FV1-FTL-SAT; Parental 2′, FV1-FKP40-SAT; Parental 2″, FV1-SSU-SAT.



Extended Data Fig. 9 | IgM promotes *Leishmania* backcross hybrids in the gut of sand flies. (**a**) Diagram outlining the generation of backcrosses. Sand flies were fed on mice lesions composed of a parental and a hybrid line. *L. major* Parental 1 (WR-SSU-HYG) and Parental 2 (FVI-FKP40-BSD) resistant to hygromycin (HYG) or blasticidin (BSD), respectively, were crossed to produce F1 hybrids. HYG/BSD double resistant F1 hybrids were backcrossed to *L. major* Parental 2′ (FV1-FKP40-SAT) or 2″ (FV1-SSU-SAT), both resistant to Nourseothricin (SAT) inserted at loci in chromosome 16 and 27, respectively. This resulted in the recovery of backcross hybrids resistant to HYG/BSD/SAT. (**b**) *Leishmania major* backcross hybrid genotyping by PCR targeting parental selectable drug markers HYG, BSD and SAT. (1×2) F1 hybrid (a cross between *L. major* Parental 1 and Parental 2); Parental 2′, FV1-FKP40-SAT; Parental 2″, FV1-SSU-SAT.; ntc, no template control; L, 1kb-plus ladder. Triple drug resistant hybrid lines were cloned before genotyping. Only sand flies provided a second bloodmeal containing IgM produced backcross hybrids. A single backcross hybrid exemplar is shown for positive events from each group. "n" detailed on Fig. 5c. Parasite number (**c**,**d**) and percentage of metacyclic promastigotes (**e**,**f**) in *L. major*-infected *Lu. longipalpis*. At 6 days post-infection, a proportion of the sand flies were provided a second uninfected blood meal via a membrane feeder composed of rabbit red blood cells reconstituted with fetal bovine serum with (+IgM) or without (−IgM) 500 µg/ml adult bovine IgM. Infection status of individual sand flies was assessed at 14 days after the first blood meal (8 days after the second bloodmeal). Parental line combination (1×2)×2′ (**c**,**e**) n = 3; (1×2)x2′ (**d**,**f**) n = 2.



Extended Data Fig. 10 | Ploidy histograms of sequenced *Leishmania* parentals and hybrids. Cloned parasite lines were evaluated by FACS to determine DNA content. Parental 1, WR-SSU-HYG; Parental 2, FVI-FKP40-BSD; Parental 3, FVI-FTL-SAT. Parental 2', FV1-FKP40-SAT; Parental 2", FV1-SSU-SAT; Parental 4, K27-SSU-HYG; Parental 5, K27-SSU-SAT. Ploidy indicated in red uppercase number/letter. Upper case green letters on lower right corner provide a unique identifier of each *Leishmania* parental or hybrid.

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Last updated by author(s): Aug 21, 2023

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Leica Application Suite X v3.7.4; Azure cSeries Capture v2.1; Illumina NovaSeq Control v1.7.5; SoftMax Pro v5.2; Xcalibur v4.4; GE Unicorn v5.31; MACSQuantify v2.13.3.
Data analysis	GraphPad Prism v9; PEAKS Studio v10.6; Imaris v9.2.1; Geneious prime v2021.2.2; CLC Genomics Workbench v22; MaxQuant v2.1.1; Perseus v.2.0.7; FlowJo v10.9; FIJI-ImageJ v1.54; Trimmomatic v0.39; BWA-MEM v07.17; Samblaster v0.1.2.5; HaplotypeCaller/GATK v4.1.9.0 GraphBio http://www.graphbio1.com/en/ v2.2.7; PAINT https://ageless.sourceforge.net/ Release 1.0; Codes generated on this work used for copy number bioinformatics analysis on https://zenodo.org/record/8231973 or https://github.com/jlac/Leishmania_allelic_copy_number; Codes generated on this work used for statistical analysis on https://github.com/joedoehl/lgM-promotes-genetic-exchange-of-Leishmania. R v4.1; Rstudio v1.4; Packages: abind, chron, data.table, e1071, emmeans, FedData, gsubfn, gt, ijtiff, janitor, lubridate, MASS, plyr, rcompanion, readxl, reshape, R.utils, rlist, Rmisc, rstatix, stats, stringr, tidyverse (contains: dplyr, ggplot2, forcats, tibble, readr, stringr, tidyr, purr), utils, WRS2, xlsx. Version numbers of these packages are all shown in the references.

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- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All data are available in the main text or the supplementary materials.
Databases used on this study: TrytripDB; UniProt proteome UP000002254 (Dog, taxonomy id 9615).
Whole-genome and transcriptome sequencing raw reads files: NCBI BioProject ID PRJNA988832. Proteome sequencing raw files: PRIDE project PXD038652.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

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Recruitment	*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The number of sand flies per group was not determined based on sample size calculation. Instead, we opted for the maximum number of sand flies per group that our sand fly colony could provide. We employed a substantial quantity of sand flies to account for the inherent variability of sand fly natural infection. In each independent experiment, we utilized a range of 2000 to 3000 flies, considering the typical mortality rates observed in laboratory conditions. To ensure comparability among groups at all time points, matched samples were employed. Additionally, for ex vivo and in vitro experiments, we conducted a minimum of 3 independent biological repetitions. This is the first time where Leishmania hybrids were consistently isolated in vitro. No prior observations were available, and no statistical calculations of sample size was undertaken. Instead, we based our sample sizes on standard protocols for identification of hybrid organisms. All experiments had at least three independent biological replicates.
Data exclusions	No data were excluded
Replication	Statistical significance was found in all independent experiments reported. We conducted a minimum of three independent biological

Replication	replicates. IgM-mediated hybridization in vitro was independently verified by another laboratory with two different Leishmania species, L. major and L. tropica. They were able to replicate the IgM-mediated hybridization process five times, independently.
Randomization	Sand flies were randomly assigned to the different groups. During the establishment of hybrids, randomization of parental lines is not possible since Leishmania cultures are grown at specific antibiotic pressure, matched at age, and mixed to be cultured, fed to sand flies or injected in animals in defined quantities. In this study with Leishmania parasites and female sand flies, all tested at the same age, and conditions, no covariates can impact the study results.
Blinding	Outcomes from sequencing and PCR of potential hybrids were blinded, since samples were coded during sequencing and preliminary bioinformatic analysis. No blinding applied to other experiments. Blinding was not relevant as experimental outcomes do not reflect subjective scoring

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

Unconjugated bovine IgM (Millipore Sigma, cat. I8135): Amini et al. Immunochemical properties of a 60 kDa cell surface-associated heat shock-protein (Hsp60) from Helicobacter pylori. FEMS immunology and medical microbiology (1996-12-31)

Unconjugated human IgM (Millipore Sigma, cat. I8260): Abdullah M, et al. Killing of dsrA mutants of Haemophilus ducreyi by normal human serum occurs via the classical complement pathway and is initiated by immunoglobulin M binding.
Abdullah M, et al. Infection and Immunity, 73(6), 3431-3439 (2005)

Sheep anti-bovine IgM (polyclonal, Bio-Rad, cat. AAI179): Nebl, T. et al. (2002) Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. J Biol Chem. 277 (45): 43399-409.

HRP rabbit anti-sheep IgG H+L (Abcam, cat. Ab6747): Li J et al. Overexpression of PFKFB3 promotes cell glycolysis and proliferation in renal cell carcinoma. BMC Cancer 22:83 (2022).

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AKP Rabbit anti-dog IgG (whole molecule) (polyclonal, Sigma, cat. A0793):Barsoum SC, et al. Delivery of recombinant gene product to canine brain with the use of microencapsulation. The Journal of Laboratory and Clinical Medicine, 142(6), 399-413 (2003).

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Validation

All antibodies utilized in this study have been acquired commercially and all references of their use are noted in the "Antibodies use" section.

Animals and other research organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research, and Sex and Gender in Research

Laboratory animals	Six to eight weeks old female BALB/c and Swiss Webster mice. Sand flies Lutzomyia longipalpis, jacobina strain, females, were used with 4 to 6 days of age after emerging. Mice were held on a 6AM-8PM light cycle. Lights come on at 6AM and go off at 8PM. Room temperature was held between 69-75 Fahrenheit, while humidity was held between 50-55%.
Wild animals	No wild animals were used in the study
Reporting on sex	Insect females are the vectors for Leishmania parasites. Only female mice were used.
Field-collected samples	No field collected samples were used in the study
Ethics oversight	All animal experimental procedures were reviewed and approved by the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee under animal protocol LMVR4E. The NIAID DIR Animal Care and Use Program complies with the Guide for the Care and Use of Laboratory Animals and with the NIH Office of Animal Care and Use and Animal Research Advisory Committee guidelines. Detailed NIH Animal Research Guidelines can be accessed at https://oma1.od.nih.gov/manualchapters/intramural/3040-2/.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Parasite cultured cells were collected by centrifugation and washed in PBS supplemented with 5 mM EDTA. Cells were fixed at 4°C in a mixture of methanol and PBS (7:3) for 1h. Cells were then collected by centrifugation and washed once in PBS supplemented with 5 mM EDTA and passed through a 30 µm Filcon. 2x10E7 cells were resuspended in 1ml of FxCycle PI/RNase staining solution and kept in the dark at room temperature for 1h.
Instrument	Miltenyi Biotec MACSQuant Analyzer 16
Software	Data acquired with MACSQuantify v2.13.3. Data analyzed with FlowJo v10.9.
Cell population abundance	Each sample analyzed events of a single clone parasite. Population abundance is 100%.
Gating strategy	All single cells were counted for the DNA content. No gating strategy necessary as only one color was acquired.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.