Genetic exchange in *Leishmania* is facilitated by IgM natural antibodies

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Abstract

Host factors mediating *Leishmania* genetic exchange are not well defined. Here, we demonstrate that IgM antibodies, but not IgG or IgA, facilitate parasite genetic hybridization in vitro and in vivo. IgM induces the gradual and transient formation of a structured parasite clump in a process essential for *L. major* and *L. tropica* hybridization in vitro. Parasite hybrids and 3-nucleated parasites were observed inside this structure, named the *Leishmania* mating clump. IgM was also required for or significantly increased *Leishmania* hybrid formation in vivo. At minimum, we observed a 12-fold increase in the proportion of hybrids recovered from sand flies provided a second blood meal containing IgM compared to controls. Notably, genetic backcross events in sand flies were only observed in the presence of IgM, and were reproducibly recovered, reinforcing the relevance of IgM for *Leishmania* genetic exchange in vivo. The in vitro and in vivo *Leishmania* crosses from these studies resulted in full genome hybrids. *Leishmania* co-option of a host antibody to facilitate mating in the insect vector establishes a new paradigm of parasite-host-vector coevolution that promotes parasite diversity and fitness through genetic exchange.

49 **Main text**

50 *Leishmania* parasites are vector-borne pathogens transmitted to mammalian hosts by
51 phlebotomine sand flies[1]. Associated with poverty, leishmaniasis is prevalent in developing
52 countries and results in self-limiting, severely disfiguring, or fatal diseases that cause substantial
53 morbidity and mortality worldwide[2]. More than a billion people are at risk of infection, millions
54 are asymptomatic carriers, and the annual incidence of fatal visceral and cutaneous leishmaniases
55 are estimated at 100,000 to 1 million cases, respectively[3]. These statistics place leishmaniasis at
56 the forefront of parasitic diseases of public health importance.

57 *Leishmania* parasites are considered mostly asexual, reproducing via clonal propagation[4-
58 6], though the presence of natural hybrids and demonstration of genetic exchange in the sand fly
59 vector[7-10] indicate that genetic exchange is more likely 'facultative' as seen in other eukaryotic
60 microbes[11]. Genetic exchange is a powerful evolutionary route enabling the development of
61 pathogenicity in microbes and offers an experimental tool for dissecting complex phenotypes
62 underlying virulence[12].

63 Host and parasite factors mediating this process are not well understood. In earlier studies,
64 passage in sand flies was required to undertake this experimentally[10,13,14], but recently, hybrid
65 formation was observed in culture for a few species including *L. tropica*, albeit following exposure
66 to harsh conditions[10,15,16]. Nevertheless, in vitro hybrid generation is yet to be accomplished for
67 most *Leishmania* species, including *L. major*[10,15,16]. Importantly, genetic exchange or the
68 generation of fertile hybrids through backcrosses has only been reported once[8].

69 In this study, we focused on elucidating host factors that mediate *Leishmania* genetic
70 exchange, asking whether host blood ingested by sand flies provides some element(s) absent in
71 culture media that promotes fusion and the formation of hybrid parasites. By adding freshly

72 isolated dog plasma to culture media we succeeded in generating in vitro crosses of two genetically
73 labeled *L. major* strains, WR-SSU-HYG and FV1-FKP40-BSD (Fig. 1a). Hybrid generation was
74 only observed when accompanied by the formation of a structured spherical 'clump' composed of
75 live aggregating parasites (Fig. 1b, top panel; Supplementary Videos 1-3). In standard culture
76 media, in the absence of dog plasma, the parasite clump did not form (Fig. 1b, bottom panel;
77 Supplementary Videos 6,7) and hybrids were not recovered. The parasite clump forms gradually
78 and is maintained for 24-36h (Supplementary Video 4) before it starts to dissociate releasing viable
79 parasites.

80 Purification and fractionation of dog plasma using a 2 step-HPLC followed by MS-MS and
81 in vitro incubation of parasites with the HPLC fractions revealed that IgM was the molecule
82 responsible for the formation of the parasite clump (Supplementary Fig. 1). Parasite clumping was
83 retained in HPLC-eluted fractions of a commercially available IgM (Fig. 1c) but was absent with
84 a commercial human IgA or IgG (Fig. 1d), emphasizing the unique role of IgM in parasite
85 clumping. Importantly, fetal bovine serum (FBS) does not induce clumping (Fig. 1d) and it does
86 not contain IgM (Fig. 1e). Pertinently, the process of IgM-induced clump formation (Fig. 1f,
87 Supplementary Fig. 2a) is distinct from other aggregation phenomena such as with peanut
88 agglutinin (PNA), where the parasites agglutinate immediately and irreversibly, forming a
89 disorganized flat mesh-like structure of immotile parasites (Fig. 1g; Supplementary Fig. 2b;
90 Supplementary Video 5). The composition and temporal formation and dissociation of the
91 *Leishmania* parasite clump is also different from other reported structures, including the
92 *Leishmania* rosettes[17]. Furthermore, pentameric IgM was required (Fig. 1h; Supplementary Fig.
93 3), as IgM monomers failed to clump the parasites (Fig. 1i, Supplementary Fig. 3). Of relevance,

94 IgM-induced parasite clumping in eight diverse *Leishmania* species (Fig. 1j) strongly suggesting
95 that parasite clumping upon contact with blood is a general response in *Leishmania*.

96 To directly establish the contribution of the IgM-induced clump to genetic exchange in
97 vitro, we used 3 genetically marked *L. major* parasite strains with independent selectable markers
98 (WR-SSU-HYG [parental line 1], FV1-FKP40-BSD [parental line 2], and FV1-FTL-SAT
99 [parental line 3]) in various crosses. Stationary phase promastigotes from two parental lines were
100 mixed at a 1:1 ratio and cultured for 3 days, with or without IgM, in the absence of selection drugs,
101 and then grown in the presence of selective drugs for up to 30 days (Fig. 1k, l). Hybrids were
102 observed for all parental combinations, but only when the culture was supplemented with IgM
103 (Fig. 1k, l).

104 Collectively, our data demonstrate that IgM is critical for the generation of *L. major* hybrids
105 in vitro through its induction of a structured and transient living parasite clump. Furthermore,
106 without any other manipulation to the culture, in vitro hybrids from *L. tropica* crosses were only
107 observed in the presence of IgM under our experimental conditions (Supplementary Fig. 4).
108 Another breakthrough of this study was to have accomplished something sought after for many
109 years, the formation of *L. major* hybrids in vitro.

110 Our studies addressed the hypothesis that formation of the IgM-dependent parasite clump
111 promotes a closer contact between parasites, and this, in turn, increases the probability of
112 *Leishmania* hybrid formation. Using confocal microscopy, we observed that IgM was abundantly
113 distributed throughout the parasite clump in close association with *Leishmania* parasites, forming
114 the distinct spherical structure observed in vitro (Fig. 2a). Transversal sections provided further
115 evidence that *Leishmania* parasites lie in close proximity to each other within the parasite clump
116 and revealed that IgM localized to distinct pockets between parasites (Fig. 2b) and coated their

117 surface (Fig. 2c), potentially contributing to interactions holding the structure together. We then
118 used scanning electron microscopy to establish the similarity in the organization of closely
119 interacting parasites within the *L. major* (Fig. 2d, upper panel) and *L. tropica* (Fig. 2d, lower panel)
120 clumps. Importantly, we located hybrid events inside a *L. tropica* parasite clump using two parental
121 lines each harboring either a green or red nucleotide analog, only detected in the nucleus (Fig. 2e,
122 left and middle panels). Double labeled nuclei (yellow), signifying a hybridization event, or a
123 *Leishmania* hybrid, were only detected inside the parasite clump by confocal microscopy (Fig. 2e,
124 right panel). Promastigotes with 3 nuclei (Fig. 2f, red arrows) indicative of fusion events were also
125 observed inside the parasite clump by transmission electron microscopy. Our data provide direct
126 evidence that IgM permeates the parasite clump and coats the parasites and establish that
127 *Leishmania* fusion and hybrid formation take place within the parasite clump. Based on these
128 findings and their relevance in parasite fusion and hybrid formation, we termed this structure the
129 *Leishmania* mating clump (LMC).

130 Eukaryotic class II fusogens such as HAP2, intrinsic molecules identified in
131 *Chlamydomonas* and *Plasmodium*, are involved in membrane fusion between gametes. In contrast,
132 IgM is an extrinsic molecule originating from the mammalian host that brings *Leishmania* parasites
133 together to promote fusion, exposing a novel mechanism co-opted by a eukaryote to facilitate the
134 early key steps of genetic exchange. We hypothesize that the LMC creates an environment,
135 nutritional, physical, and biochemical that promotes parasite fusion and hybrid formation. It will
136 be important to further investigate whether the interaction between IgM and the parasite is
137 activating a signaling pathway, or whether the tight parasite scaffolding within the LMC is creating
138 a physical or nutritional stress that facilitates genetic exchange. Nevertheless, it is important to
139 emphasize that the addition of IgM to *Leishmania* culture media mimics natural exposure of

140 *Leishmania* to IgM in sand fly blood meals. Every time a sand fly takes a blood meal, a *Leishmania*
141 parasite that resides in the sand fly gut will interact with IgM-present in the blood of all vertebrate
142 animals[18-20].

143 The ubiquity of IgM in vertebrate blood[18-21] combined with the multiple and promiscuous
144 (Supplementary Table 1) blood-feeding behavior of the sand fly[22] led us to hypothesize that this
145 antibody is a major facilitator for the initiation of genetic exchange in vivo, in the sand fly gut.
146 Experiments were performed using conditions that mimicked the natural progression of sand fly
147 infections, including incorporation of a second blood meal that is needed to prevent loss of
148 naturally acquired parasites[22]. Having first established that IgM persists in the sand fly midgut for
149 at least 24 hours after each blood meal (Fig. 3a), we performed an experiment where we allowed
150 sand flies to feed on a cutaneous leishmaniasis lesion initiated by two *L. major* parental lines (Fig.
151 3b, BM1), to pick the parasites up as part of a natural blood meal [23,24]. We then followed the natural
152 sand fly feeding behavior by offering them a second uninfected blood meal six days after feeding
153 on cutaneous lesions (Fig. 3b, BM2). The second blood meal was either devoid of IgM or
154 supplemented with 500 µg/ml of IgM (Fig. 3b, BM2), a concentration at or below those reported
155 in the blood of different animals (Supplementary Table 2). Additionally, a control group was
156 maintained on a single infected blood meal (Fig. 3b, dotted arrow). Fourteen days after the initial
157 infection, sand fly guts from each group were dissected and plated individually to screen for
158 parasite hybrids (Fig. 3b). For the group initially infected with parental lines combination 1x2,
159 IgM increased the proportion of hybrid formation by up to 12-fold in sand flies getting a second
160 blood meal containing IgM compared to sand flies given a second blood meal devoid of IgM or
161 sand flies given a single infectious blood meal (Fig. 3c, d, Supplementary Fig. 5a). In these two
162 control groups, we observed a significantly lower proportion of hybrids as was previously

163 reported[13,14]. The similar proportion of hybrids observed in flies given a second blood meal devoid
164 of IgM or sand flies given a single infectious blood meal suggest that this may be a basal level of
165 chance mating within the confined space of the sand fly midgut. It is worth noting that both control
166 group scenarios would not occur in nature since an infected sand fly will always take a second
167 blood meal 5-6 days after infection[1,22]. Strikingly, control groups lacking IgM did not generate any
168 hybrids in parental lines combinations 1x3 and 2x3, further emphasizing the importance of IgM in
169 *Leishmania* hybrid formation in vivo (Fig. 3c, d, Supplementary Fig. 5a). Of note, the parasite
170 load, and percent metacyclics were similar in all experimental groups (Supplementary Fig. 5b-g).
171 The pronounced effect of IgM on parasite hybrid formation when provided to sand flies six days
172 after naturally acquired infections (BM2) suggests that proliferative stages are prone to genetic
173 exchange. We argue that hybrids may form soon after a sand fly takes a blood meal but can only
174 be detected later in the infection cycle after undergoing multiple cycles of division[1,23]. Collectively,
175 the above data establish that IgM facilitates *Leishmania* hybrid formation inside the sand fly gut
176 after naturally acquired infections, likely by bringing parasites closer together within a LMC that
177 promotes fusion and hybrid generation. This is supported by the observation of structures similar
178 to in vitro LMCs within the midgut (Supplementary Fig. 6; Supplementary Videos 7-12).

179 Corroborating previous findings[10], we observed hybrid formation only in sand flies and did
180 not find them in *Leishmania* skin lesions or draining lymph nodes containing 2 different parasite
181 lines (Supplementary Fig. 7). In the mammalian host, the *Leishmania* parasite is intracellular,
182 residing in a parasitophorous vacuole inside macrophages[25,26]. It may be that a lack of opportunity
183 for IgM to interact with amastigotes accounts for the absence of hybridization in the mammalian
184 host. Whole-genome sequencing of the double drug resistant hybrids (Supplementary Fig. 8)
185 showed that the crosses in sand flies and those generated in vitro were full genome hybrids (Fig.

186 3e; Supplementary Fig. 8-10). Moreover, aneuploidy commonly seen in cultured parasites[10,13,14]
187 were observed in this study at comparable levels (Supplementary Fig. 9, 10).

188 For use in positional cloning, generation of recombinant progeny by mating of hybrids to
189 each other or to parental lines is required. Previous studies showed that the frequency of this
190 progeny in *L. major* was considerably lower than it was for initial F1 hybrid formation, greatly
191 hindering its utility[8]. To test whether insights from IgM-mediated mating could improve F1 hybrid
192 recovery as well as confirm their fertility, we performed a backcross in the presence of IgM. This
193 involved crossing a HYG/BSD double resistant (1x2) F1 hybrid with two clones of *L. major*
194 parental line 2 bearing a SAT marker to allow identification of mating progeny. Sand flies were
195 allowed to feed on a cutaneous lesion initiated by mixed infections of the (1x2) F1 hybrid and
196 parental line 2' or 2" of *L. major* (Fig. 4a, BM1), and then given a second uninfected blood meal
197 six days later (BM2), either supplemented with 500 µg/ml of IgM or devoid of IgM (Fig. 4a, BM2).
198 We consistently observed hybrid formation of this backcross, but only in sand flies fed with blood
199 containing IgM (Fig. 4b, c; Supplementary Fig. 11a). Hybrid generation was not influenced by
200 parasite density or percent metacyclics that were similar in all experimental groups
201 (Supplementary Fig. 11b-g). A diagram outlining the approach to the generation of backcrosses is
202 provided in Supplementary Fig. 12.

203 Whole-genome sequencing of the triple drug resistant hybrids showed that the second-
204 generation backcross progeny in sand flies bore the expected patterns of recombination between
205 the F1, and 3rd parent genomes also observed in previously reported backcrosses[8]. Importantly,
206 no 'backcross progeny' were obtained with sand flies given a second blood meal devoid of IgM,
207 providing conclusive evidence of its importance in *Leishmania* genetic exchange in a natural
208 setting. Our data strongly suggest that IgM facilitates fertile hybrid formation and enhances their

209 frequency in sand flies consequently promoting *Leishmania* diversity. Importantly, the
210 reproducible and increased recovery of backcrosses opens the way for more in-depth genetic
211 analysis of *Leishmania* parasites.

212 Historically, aggregation of *L. donovani* by IgM in culture was reported once as a
213 standalone observation[27]. Our work provides direct experimental evidence of a critical role for
214 IgM in LMC formation that promotes the first key steps of *Leishmania* genetic exchange in vitro
215 and in vivo after a naturally acquired infection. Moreover, our data describe a new biological
216 function for an antibody by implicating IgM in genetic exchange in a parasite. The exploitation of
217 host IgM by *Leishmania* is of significant consequence. IgM is an evolutionarily conserved natural
218 antibody that is always present in the blood of all vertebrates after birth[19-21], independent of the
219 immune status of the animal[28-33]. *Leishmania* evolved to exploit the pentameric properties of host
220 IgM and the biological feeding pattern of sand flies to enhance the likelihood of mating. Sand flies
221 ingest blood every 5-6 days to produce eggs[1]. We hypothesize that when a *Leishmania*-infected
222 sand fly takes a subsequent blood meal, the periodic influx of IgM encounters parasites that have
223 evolved to be at a stage that provides the density required to initiate the formation of the LMC in
224 the confinement of the gut, a critical first step of genetic exchange that promotes fusion and hybrid
225 generation. As such, in addition to metacyclic promastigotes, this implicates the two major
226 proliferative stages, leptomonad and retroleptomonad promastigotes, as potential participants in
227 the process of genetic exchange.

228 The fact that the *Leishmania* parasite co-opted a natural antibody from its mammalian host
229 to advance its reproductive fitness uncovers a critical step in genetic exchange, providing an insight
230 into the intimate evolutionary relationship between an insect vector, a parasite, and a mammalian
231 host.

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346 **Main figures**

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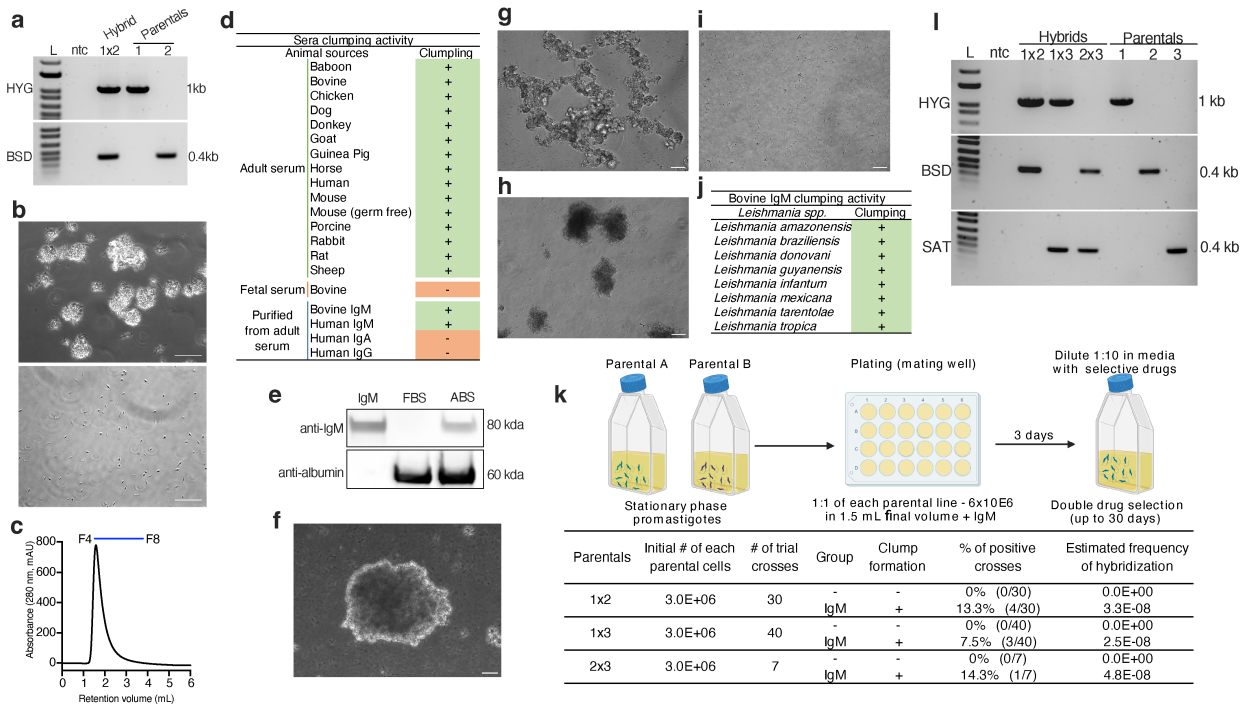

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349 **Fig 1. IgM facilitates *Leishmania* parasites clumping which promotes hybrid formation in**
350 **vitro**. (**a**) *Leishmania* hybrids genotyping by PCR targeting parental selectable drug markers HYG
351 (Hygromycin) and BSD (Blasticidin). (**b**) Phase contrast image of *L. major* in culture media
352 containing 20% fetal bovine serum (FBS) without (bottom panel) or with 5% adult inactivated dog
353 serum (top panel). Images taken 30 min after cells were seeded in fresh media. Scale bars = 20
354 µm. (**c**) Human IgM eluted from a HiTrap® IgM column. *Leishmania* clumping activity was
355 observed in fractions F4-F8 corresponding to elution of IgM. **(d)** Presence (+) or absence (-) of *L.*
356 *major* clumping activity in inactivated adult sera from different vertebrate species, FBS, or
357 immunoglobulins (IgM, IgG, IgA) purified from adult bovine or human sera. (**e**) Western blot to
358 detect IgM in FBS and adult bovine serum (ABS). Serum purified bovine IgM, positive control;
359 serum albumin, loading control. (**f**) Phase contrast image of *L. major* in culture media containing

360 20% FBS with bovine IgM at 50 µg/mL after 24h and (**g**) with peanut agglutinin (PNA) at 50
361 µg/mL after 24h. Scale bars = 50 µm. (**h**) Phase contrast image of *L. major* in culture media
362 containing 20% FBS with bovine IgM (pentameric form) at 50 µg/mL after 24h or (**i**) with bovine
363 IgM (monomeric form) at 50 µg/mL after 24h. (**j**) Clumping activity of various *Leishmania* species
364 in the presence of bovine IgM at 50 µg/mL. (**k**) (top) Workflow for in vitro crossing of *L. major*
365 parental lines. Mating wells were established with 50 µg/mL IgM in complete Schneider's media.
366 Double drug resistant hybrid parasites were cloned before genotyping. (bottom) Summary of data
367 from in vitro crossing of parental lines. 1x2 (n=30), 1x3 (n=40), 2x3 (n=7). (**l**) *Leishmania major*
368 hybrids genotyping by PCR targeting parental selectable drug markers HYG (Hygromycin), BSD
369 (Blasticidin) and SAT (Nourseothricin). Parental 1, WR-SSU-HYG; Parental 2, FVI-FKP40-
370 BSD; parental 3, FVI-FTL-SAT; ntc, no template control; L, 1kb plus ladder (Invitrogen).
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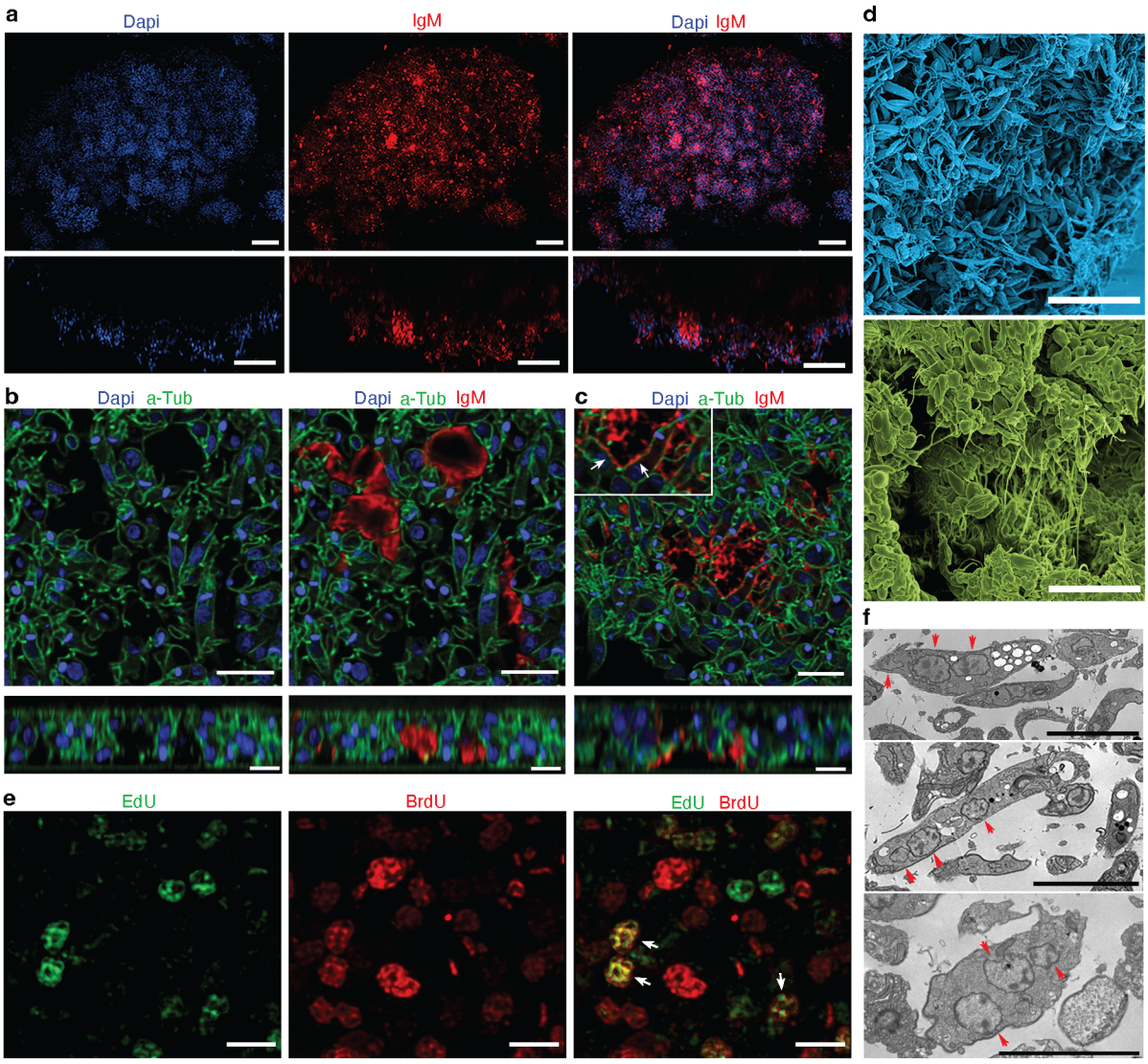

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378 **Fig 2. Characterization of the *Leishmania* mating clump. (a)** Confocal immunofluorescence
379 wide Z and tile images showing the association of IgM with the *L. major* mating clump after 24h
380 of culture. Dapi (nuclei, kinetoplast) blue; IgM, red. Scale bar = 40 µm. Bottom rectangles for each
381 panel represent XZ slices. Scale bar = 30 µm. **(b)** Confocal immunofluorescence of a 5 µm
382 transversal section of the *L. major* mating clump. Dapi (nuclei; kinetoplast), blue; alpha-tubulin,
383 green; IgM, red. IgM localizes between cells within the clump (right panel). Scale bars = 5 µm.
384 Bottom rectangles for each panel represent XZ slices. Scale bars = 3 µm. **(c)** IgM colocalizes on
385 the surface of parasites (arrows in inset). Scale bar = 5 µm. Bottom rectangles represent XZ slices.

386 Scale bar = 3 µm. **(d)** SEM of a *L. major* (upper panel) and *L. tropica* (lower panel) mating clump
387 showing the 3D organization of promastigote forms. Scale bar: 15 µm. **(e)** Confocal
388 immunofluorescence of a 5 µm transversal section of the *L. tropica* mating clump. *L. tropica*
389 promastigotes were grown to stationary phase in the presence of EdU or BrdU then extensively
390 washed and used at 1:1 ratio to assemble the LMC with IgM. EdU, green; BrdU, red. White arrows
391 point to yellow nuclei indicative of fusion and exchanged genetic material. Scale bars, 3 µm. (**f**)
392 TEM of a *L. tropica* mating clump showing cells with 3 nuclei. Arrow, individual nucleus within
393 a parasite cell body. Scale bar: 5 µm.
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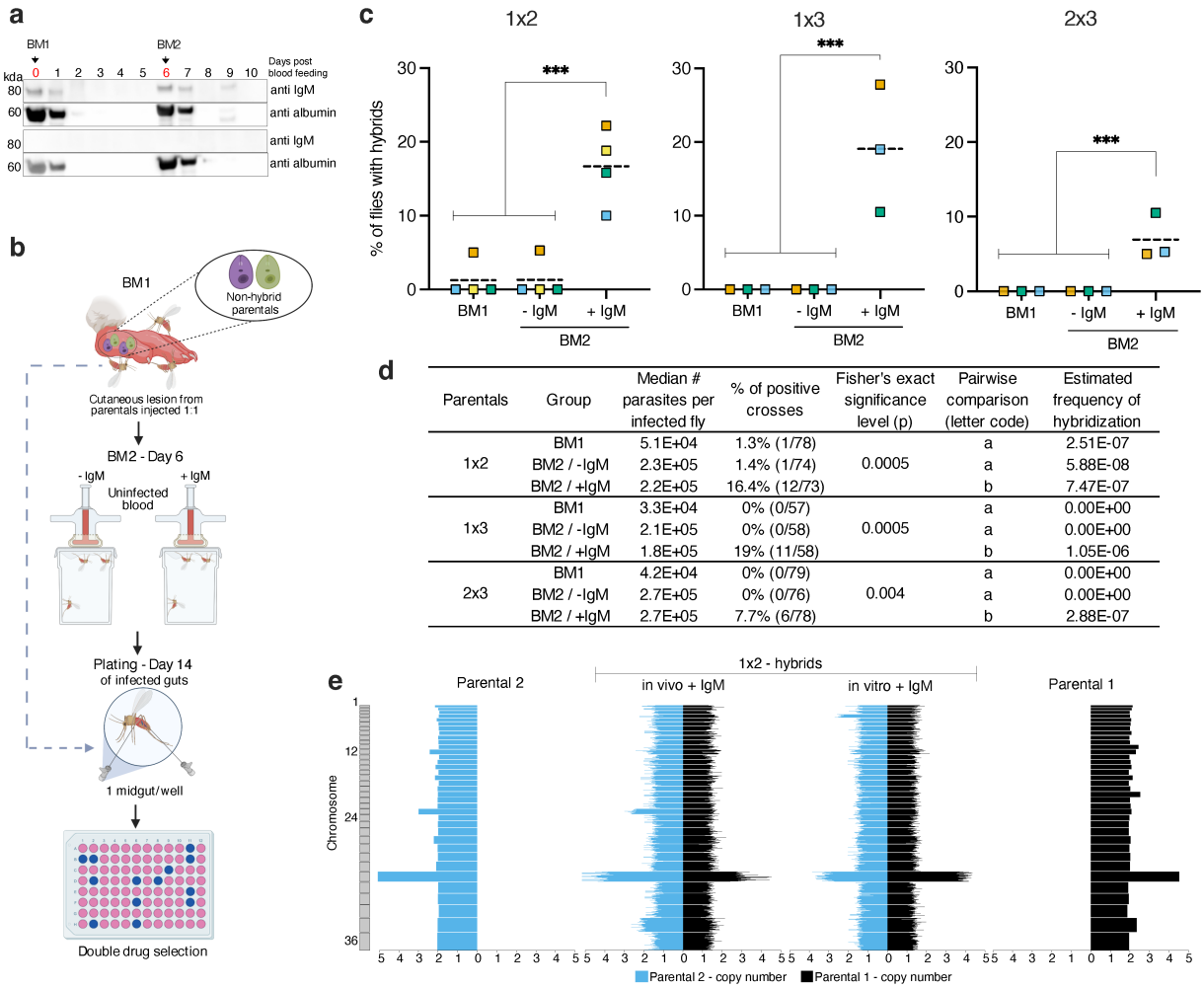

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Parentals	Group	Median # parasites per infected fly	% of positive crosses	Fisher's exact significance level (p)	Pairwise comparison (letter code)	Estimated frequency of hybridization
1x2	BM1	5.1E+04	1.3% (1/78)	0.0005	a	2.51E-07
	BM2 / -IgM	2.3E+05	1.4% (1/74)		a	5.88E-08
	BM2 / +IgM	2.2E+05	16.4% (12/73)		b	7.47E-07
1x3	BM1	3.3E+04	0% (0/57)	0.0005	a	0.00E+00
	BM2 / -IgM	2.1E+05	0% (0/58)		a	0.00E+00
	BM2 / +IgM	1.8E+05	19% (11/58)		b	1.05E-06
2x3	BM1	4.2E+04	0% (0/79)	0.004	a	0.00E+00
	BM2 / -IgM	2.7E+05	0% (0/76)		a	0.00E+00
	BM2 / +IgM	2.7E+05	7.7% (6/78)		b	2.88E-07

Fig 3. The impact of IgM on *Leishmania* hybrid formation in the sand fly vector. **(a)** Immunoblot detecting IgM in the sand fly midgut after multiple blood meals. Sand flies were provided an artificial uninfected blood meal with or without IgM. Arrows indicate the timing of the first (BM1, 0 days) and second (BM2, 6 days) blood meals. Pools of 15 midguts were used. 7.5 µg of total protein was loaded per lane. **(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. Bovine IgM was used. **(c)** Percent of sand flies harboring hybrids for 3 different combinations of parental lines (1x2; 1x3; 2x3) in single fed sand flies or in sand flies fed twice in the presence or absence of IgM. Colors indicate independent experiments. **(d)** Cumulative data

416 from independent experiments for infection status and estimated frequency of hybridization per
417 fly. 1x2 (n=4), 1x3 (n=3), 2x3 (n=3). Per each biological replicate ~ 20 infected flies per group.
418 Fisher's exact test, $p < 0.05$ is considered significant. Pairwise comparison indicate source of group
419 significance: a, no difference; b, significant difference. **(e)** Whole-genome analysis of in vitro- and
420 in vivo-generated hybrids for parental line combination 1x2. Biparental ancestry was confirmed
421 across the whole genome for all crosses. Parental 1, WR-SSU-HYG; Parental 2, FVI-FKP40-BSD;
422 Parental 3, FVI-FTL-SAT. *$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

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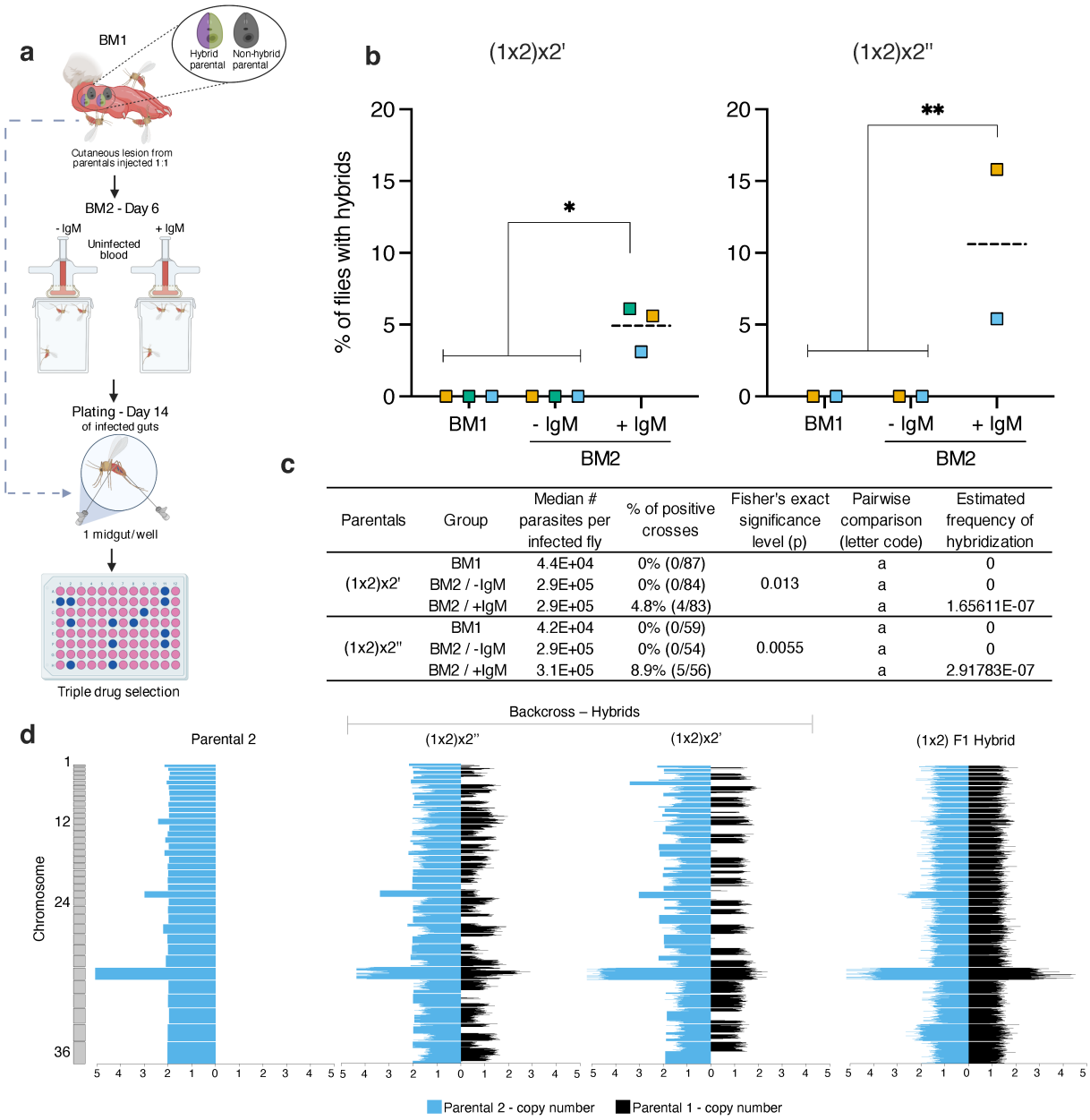

Parentals	Group	Median # parasites per infected fly	% of positive crosses	Fisher's exact significance level (p)	Pairwise comparison (letter code)	Estimated frequency of hybridization
(1x2)x2'	BM1	4.4E+04	0% (0/87)	0.013	a	0
	BM2 / -IgM	2.9E+05	0% (0/84)		a	0
	BM2 / +IgM	2.9E+05	4.8% (4/83)		a	1.65611E-07
(1x2)x2''	BM1	4.2E+04	0% (0/59)	0.0055	a	0
	BM2 / -IgM	2.9E+05	0% (0/54)		a	0
	BM2 / +IgM	3.1E+05	8.9% (5/56)		a	2.91783E-07

Fig 4. The impact of IgM on *Leishmania* genetic exchange 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 (1x2) F1 hybrid was injected at 1:1 proportion with the parental 2 harboring different resistance markers (parental 2'and parental 2''). Bovine IgM was used. **(b)** Percent of sand flies harboring hybrids in single fed sand flies or in sand flies fed twice in the presence or

451 absence of IgM. Colors indicate independent experiments. **(c)** Cumulative data from independent
452 experiments for infection status and estimated frequency of hybridization per fly. (1x2) x 2' [n=3];
453 (1x2) x 2'' (n=2). Per each biological replicate ~ 30 infected flies per group. Fisher's exact test, p
454 < 0.05 is considered significant. Pairwise comparison indicate source of group significance: a, no
455 difference; b, significant difference. **(d)** Whole-genome analysis of in vivo-generated backcross
456 hybrids for parental line combination 1x2. Where the biparental ancestry was consistent across
457 each chromosome, recombination events have broken this up in the backcrosses, making parental
458 copy number discontinuous always in the same direction through parental 2. Parental 1,WR-SSU-
459 HYG; Parental 2, FVI-FKP40-BSD; Parental 2', FVI-FKP40-SAT; Parental 2'', FVI-SSU-SAT.
460 *$P < 0.05$, **$P \leq 0.01$.

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474 **Methods**

475 **Ethics statement**

476 All animal experimental procedures were reviewed and approved by the National Institute of
477 Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee under animal
478 protocol LMVR4E. The NIAID DIR Animal Care and Use Program complies with the Guide for
479 the Care and Use of Laboratory Animals and with the NIH Office of Animal Care and Use and
480 Animal Research Advisory Committee guidelines. Detailed NIH Animal Research Guidelines
481 can be accessed at https://oma1.od.nih.gov/manualchapters/intramural/3040-2/.

482 **Animals**

483 Six to eight weeks old female BALB/c and Swiss Webster mice were obtained from Envigo and
484 Charles River laboratories, respectively. Animals were housed under pathogen-free conditions at
485 the NIAID Twinbrook animal facility, Rockville, MD.

486 **Parasites**

487 *Leishmania* parasites used in this study: *Leishmania major* (WR 2885) isolated from a soldier
488 deployed to Iraq[34]; *Leishmania major* (MHOM/IL/80/Friedlin); *Leishmania tropica*
489 (MHOM/SU/74/K27 - ATCC 50129); *Leishmania braziliensis* (MHOM/BR/75/M2903 - ATCC
490 50135); *Leishmania infantum* (MCAN/BR/09/52) isolated from a dog spleen in Natal, Brazil[35];
491 *Leishmania donovani* (MHOM/SD/62/1S); *Leishmania tarentolae* (Tar II – ATCC 30143);
492 *Leishmania mexicana* (MNYC/BZ/62/m379); *Leishmania amazonensis* (IFLA/BR/67/PH8).
493 Parasites were grown at 27°C in Schneider's Drosophila Insect Medium (Lonza BioWhittaker, 04-
494 351Q) supplemented with 20% heat inactivated fetal bovine serum (FBS) (Gibco, 16140071) and
495 250 µM of adenosine (MilliporeSigma, A9251). Parasites were maintained by serial passage in
496 BALB/c mice or Golden Syrian hamsters, depending on dermotropic or viscerotropic parasite

497 species characteristics. Promastigotes were kept in culture for a maximum of 10 passages. *L. major*
498 metacyclic promastigotes were purified by peanut agglutinin (PNA) (Vector Laboratories, L-1070)
499 and used for in vitro experiments and infection of mice footpads as described[36].

500 ***Leishmania* parental lines**

501 The various *Leishmania* lines used in crossing experiments were constructed as detailed below.
502 For parental line 1, *L. major* – WR-SSU-HYG/GFP, *L. major* (WR 2885) was used as background
503 for generation of a hygromycin B (HYG) parental line that also harbors the EGFP gene. The EGFP
504 gene was amplified by PCR using the mEGFP-C1 plasmid as a template for the forward primer 5-
505 ACAGCGAGATCTATGGTGAGCAAGGGCGAGGA-3, and reverse primer 5-
506 ACAGCGGGTACCCTTGTACAGCTCGTCCATGC-3. The forward and reverse primers add the
507 BgIII or KpnI restriction sites to the amplicon, respectively. The PCR product was cloned into the
508 Lexsy_hyg2 plasmid (Jena Bioscience, EGE-232). The linearized construct (2 µg) was used to
509 transfect mid-log phase culture promastigotes on a 4D-NucleofectorTM X unit (Lonza) using pulse
510 code FI-115 (T cell, Human, unistim. HE). Transfected parasites bearing rRNA locus-integrated
511 EGFP were selected for growth in the presence of 100 µg/mL Hygromycin B (Goldbio, H270).
512 Clones were selected by limiting dilution and assessed for chromosome integration. Parental line
513 2, *L. major* – FVI-FKP40-BSD[37] and Parental line 3, *L. major* – FVI-FTL-SAT[38] were generated
514 as previously described. For generation of parental line 2', *L. major* – FVI-FTL-SAT, we followed
515 the procedures described for line 2, but used a heterozygous of the gene FKP40 with a construct
516 harboring Nourseothricin (SAT). For parental line 2'', *L. major* – FVI-SSU-SAT/RFP, *L. major*
517 (MHOM/IL/80/Friedlin/FV1) was used as background for the generation of a SAT parental line
518 that also harbors the mRuby gene. The mRuby gene was amplified by PCR using the mRuby-C1
519 plasmid as template for the forward primer 5-

520 ACAGCGAGATCTATGAACAGCCTGATCAAAGA-3, and reverse primer 5-
521 ACAGCGGCGGCCGCTCACAGATCCTCTTCAGAGATGAGTTTCTGCTCCCCTCCGCCC
522 AGGCCGGC-3. The forward and reverse primers add the BgIII or NotI restriction sites to the
523 amplicon, respectively. The PCR product was cloned into the Lexsy_sat2 plasmid (Jena
524 Bioscience, EGE-234). The linearized construct (2 µg) was used to transfect mid-log phase culture
525 promastigotes on a 4D-NucleofectorTM X unit (Lonza) using pulse code FI-115 (T cell, Human,
526 unistim. HE). Transfected parasites bearing rRNA locus-integrated mRuby were selected for
527 growth in the presence of 100 µg/mL Nourseothricin (Goldbio, N-500-100). Clones were selected
528 by limiting dilution and assessed for chromosome integration. *L. tropica* (K27 – ATCC 50129)
529 was used as background for the generation of parental line 4, *L. tropica* - K27-SSU-HYG/GFP and
530 parental line 5, *L. tropica* - K27-SSU-SAT, respectively. Parental line 4 was made following the
531 same steps as described for Parental line 1. Parental line 5 was made the same way as parental line
532 2'' but without the addition of any fluorescent gene. mEGFP-C1 and mRuby-C1 were a gift from
533 the Michael Davidson lab (Addgene plasmid # 54759, http://n2t.net/addgene:54759,
534 RRID:Addgene_54759; Addgene plasmid # 54552, http://n2t.net/addgene:54552,
535 RRID:Addgene_54552).
536

537 **In vitro *Leishmania* clumping activity**

538 Various Leishmania species were cultured in complete Schneider's medium in 25 cm^2 flasks.
539 $4x10^6$ /mL stationary phase promastigotes were dispensed into a well of a 24-well plate in the
540 presence of 50 µg/mL of bovine IgM to test clumping activity. For *L. major*, sera from different
541 animal sources were also tested for clumping activity in culture. Naïve beagle dog and germ-free
542 mouse blood were provided by the Division of Veterinary Resources (DVR/NIH) under animal

543 use protocol LMVR4E. Serum was obtained by centrifugation at 1000xg/10 min, inactivated at
544 56°C for 30 min, filtered sterilized through a 0.22 µm PES membrane (Millipore MILLEX GP,
545 SGLP033RB) and stored at -80 in aliquots and used without thaw/freeze cycles. Sera acquired
546 from commercial vendors are: Baboon (MilliporeSigma, S6884); Bovine -adult (MilliporeSigma,
547 B9433); Bovine -fetal (Gibco, 16140071. R&D, S12495, S11595); Chicken (MilliporeSigma,
548 C5405); Dog (BioChemed Services, TD092321NIH); Donkey (MilliporeSigma, D9663); Goat
549 (MilliporeSigma, G9026); Guinea Pig (MilliporeSigma, G9774); Horse (MilliporeSigma, H1270);
550 Human (MilliporeSigma, H4522, H6914, P2918, S7023, S1-M); Mouse (MilliporeSigma, S25-M,
551 S3509, S7273); Porcine (MilliporeSigma, P9783); Rabbit (MilliporeSigma, R4505); Rat
552 (MilliporeSigma, S7648); Sheep (MilliporeSigma, S2263). Sera were heat inactivated at 56ºC for
553 30 min and filtered sterilized before usage at 20% final concentration.
554

555 **In vitro *Leishmania* mating clump assembly for recovery of hybrids**

556 Parental lines of *L. major* were cultured in complete Schneider's medium in 25 cm^2 flasks. $3x10^6$
557 stationary phase promastigotes from each parental were mixed in a well of a 24-well plate with 1.5
558 mL of fresh complete Schneider's medium + 50 µg/mL of bovine IgM, empirically determined as
559 the optimal concentration that supports the formation and dissociation of the mating clump in vitro.
560 Mating wells were maintained at 27ºC for 3 days and then diluted 1:10 in fresh media in a 25 cm^2
561 flask containing selective drugs. Cultures were followed for up to 30 days to allow for double-drug
562 resistant cell lines to grow. Selective drugs were combined according to the parental lines used per
563 experiment. The concentration used for each selective drug was as follows: Hygromycin B - 100
564 µg/mL (Goldbio, H270); Blasticidin - 10 µg/mL (Goldbio, B-800-500); Nourseothricin - 100
565 µg/mL (Goldbio, N-500-100). Once resistant cells grew, the polyclonal culture was cloned by

566 plating 0.5 cell/well in a 96-well plate under double-drug pressure. After clone selection,
567 genotyping was carried out using primers as displayed in Supplementary Table 3. Clone cultures
568 were also a source of genomic DNA for deep sequencing. An illustration of the experimental
569 design for in vitro *Leishmania* genetic exchange is provided in Fig. 1g. Frequencies of
570 hybridization were estimated by: $\frac{\#\ positive\ crosses}{(Initial\ \#\ of\ one\ parental)*(\#\ trial\ crosses)}$

571

572 **Mice infection**

573 Metacyclic promastigotes were harvested from stationary phase cultures and purified using peanut
574 agglutinin (Vector Laboratories, L-1070) as previously described[36]. Purified *L. major* metacyclic
575 promastigotes ($1x10^5$) of each parental line were mixed at a 1:1 ratio and injected into a BALB/c
576 mouse footpad. Animals that developed a swollen non-ulcerated (<5 mm thickness) footpad lesion
577 at weeks 4 to 6 post-injection were used to feed sand flies. After sand fly feeding on infected
578 animals, amastigotes were harvested from the infected footpads and skin draining lymph nodes as
579 previously described[39].

580

581 **Sand fly infection**

582 Four to six days old female *Lutzomyia longipalpis* (Jacobina strain) sand flies were infected by
583 feeding on mice footpad lesions of BALB/c mice. For first (F1) and second (backcross) generation
584 crosses, culture purified metacyclic promastigotes of two parentals were injected in the mouse
585 footpad at a 1:1 proportion. In F1 crosses experiments, mice were injected with parentals 1x2, 1x3
586 and 2x3. For backcross experiments mice were injected with a (1x2 F1 hybrid) x 2 harboring a
587 different resistance marker. Illustration of the experimental design for natural feeding on mice is
588 provided in Fig. 3b & 4a. Briefly, mice were anesthetized by intraperitoneal injection of a mixture

589 of ketamine (100mg/kg) and xylazine (10mg/kg). The infected footpad was then inserted in a
590 cardboard pint containing the sand flies. Sand flies were allowed to feed for 2 hours in the dark.
591 After feeding, blood-fed female sand flies were sorted out and maintained at 26°C with 75%
592 humidity and 30% sucrose solution *ad libitum*. *Lutzomyia longipalpis* is not the natural vector of
593 *L. major* but it is a permissive sand fly species that has been shown to support the complete
594 development of several *Leishmania* species from natural parasite acquisition to successful
595 transmission by bite[40, 41,42]. Importantly, midgut development related to parasites numbers and
596 stages of differentiation in *Lu. longipalpis*/*L. major* infections was similar to those reported for
597 *Phlebotomus papatasi / L. major* and *Lu. longipalpis / L. infantum* natural occurring
598 combinations[43].
599

600 **Preparation of the second blood meal provided to sand flies**

601 For the 2nd blood meal given six days post-infection to a proportion of the sand flies, defibrinated
602 rabbit blood (Spring Valley Laboratories) was centrifuged at 1000xg for 10 min and the normal
603 serum containing IgM was discarded. Red blood cells (RBCs) were washed three times with 10mL
604 of PBS (Lonza BioWhittaker, BE17-516F) followed by centrifugation at 1000xg for 10 min. RBCs
605 were washed an additional time with FBS (Gibco, 16140071). Blood was reconstituted by adding
606 FBS at the same volume of the serum initially removed. Sand flies were allowed to feed for 2 hours
607 on a glass feeder containing reconstituted blood supplemented or not with bovine IgM
608 (MilliporeSigma, I8135) at 500 µg/mL. Unfed and blood-fed flies were sorted out after feeding
609 and kept on 30% sucrose solution *ad libitum* for a total of 14 days after the initial infected blood
610 meal. Females infected by feeding on mice lesions only were used to represent sand flies that have
611 taken a single infected blood meal.

612 **Sand fly midgut counts**

613 To assess maturity of the infection, *Leishmania*-infected sand fly midguts were individually
614 transferred to a 1.7mL microtube (Denville Scientific, C2172) filled with 30µL of PBS. Midguts
615 were then ground using a disposable pellet mixer and a cordless motor (Kimble, 7495400000). For
616 counting, promastigotes were slowed down by a further 1:5 or 1:10 dilution in formalin/PBS at
617 0.005% final concentration. Then, 10 µL of the sample were loaded onto a Neubauer improved
618 chamber (Incyto, DNC-NO1) and parasites were counted under a phase contrast Axiostar plus
619 microscope (Zeiss) at 40X magnification. The number of parasites per midgut was back-calculated
620 based on the total sample volume and subsequent dilution.

621

622 **In vivo recovery of hybrids**

623 Only midguts of sand flies that developed a mature infection, recognized by an opaque anterior
624 midgut distended by the presence of a mass of parasites[43], were plated in search for hybrids.
625 Infected midguts were dissected in PBS on microscope slides using tweezers and fine needles.
626 Each midgut was transferred to a 1.7mL microtube (Denville Scientific, C2172) filled with 50 µL
627 of complete Schneider's medium and homogenized using a pestle with a cordless motor. One
628 midgut was plated per well in a 96-well plate (Alkali Scientific, TP9096) at a final volume of 100
629 µL. An antibiotic cocktail (ABTs) that has no effect on parasite growth (Supplementary Fig. 13)
630 and viability was included in complete media to control contamination with gut microbiota. ABTs:
631 Penicillin-Streptomycin (Gibco, 100 U/mL); Gentamicin (Sigma, 50 µg/mL); Caspofungin
632 (Sigma, 15 µg/mL); 5-fluorocytosine (Sigma, 30 µg/mL). For each independent experiment, a
633 minimum of 20 infected midguts per group were collected. After plating, wells were allowed to
634 stabilize and grow for 24h prior to the addition of 100 µL/well of complete Schneider's media

635 containing selective drugs (2x concentrated). Cultures were followed for up to 30 days for double
636 or triple-drug resistant cell lines to grow. The concentration of selective drugs, cloning, and
637 genotyping procedures were performed as described for in vitro recovery of hybrids. Frequencies
638 of hybridization were estimated by: $\frac{\#\ positive\ crosses}{(Median\ \#\ of\ parasites\ per\ fly)*(\#\ trial\ crosses)}$.

639

640 **IgM identification from plasma by HPLC**

641 Canine plasma (1.5-2 mL) was inactivated at 56°C for 30 min. Inactivated plasma was passed
642 through a 0.22 µm PES membrane (Millipore MILLEX GP, SGLP033RB) and diluted to 7 mL in
643 PBS containing 5 mM EDTA (buffer A). The inactivated, diluted plasma was then applied to a
644 Sephacryl S200 (16/60, GE Healthcare) gel filtration column equilibrated with buffer A. Plasma
645 components were eluted at 1 mL/min, and 2 mL fractions were collected and tested for *Leishmania*
646 clumping activity. Active fractions were then combined, concentrated, and diluted several times
647 with 10 mM sodium phosphate (pH 6.0) buffer (buffer B) before application to an SP Sepharose
648 (16/10, GE Healthcare) ion exchange column equilibrated with buffer B. Proteins were eluted with
649 a gradient of 0-1 M NaCl in buffer B. Activity was distributed throughout the major peak,
650 particularly at the leading edge. These fractions were pooled and chromatographed on a Superdex
651 200 (10/300, GE Healthcare) gel filtration column equilibrated with buffer A, resulting in further
652 purification of the major component that produced bands on SDS-PAGE gels of approximately 70
653 and 25 kDa consistent with the heavy and light chains of IgM. This sample was active in
654 *Leishmania* clumping assays and was analyzed after tryptic digest by electrospray ionization mass
655 spectrometry. As an additional means of purification, pooled fractions of IgM purified by gel
656 filtration were diluted in 20 mM NaPO4 pH 7.5 (buffer C) containing 0.8 M ammonium sulfate
657 and applied to a 1 mL HiTrap IgM purification HP column (GE healthcare) equilibrated with buffer

658 C containing 0.8 M ammonium sulfate. The column was washed until the absorbance reached
659 baseline levels. IgM was then eluted stepwise in buffer C and 0.5 mL fractions were collected.
660 Activity in the *Leishmania* clumping assay closely aligned with the peak of absorbance.
661

662 **Commercial IgM preparation for in vitro and in vivo experiments.**

663 Commercially available IgM antibodies used in this study were obtained from MilliporeSigma and
664 contained preservatives such as sodium azide. To avoid any potential toxicity to the parasites,
665 removal of preservatives was undertaken. IgM from human (MilliporeSigma, I8260) and bovine
666 (MilliporeSigma, I8135) sources were purified by passing them through a Sephacryl-S-200 gel
667 filtration column (Cytiva) equilibrated with either buffer A minus EDTA or PBS. For IgM
668 equilibrated with buffer A minus EDTA, further purification steps using a HiTrap IgM purification
669 HP column are given above. Protein-containing fractions eluted with PBS were pooled and the
670 volume reduced to the desired concentration by ultrafiltration.
671

672 **IgM stability in blood fed sand flies and its absence in fetal serum.**

673 To estimate how long IgM is maintained in the midgut after blood feeding, *Lu. longipalpis* were
674 provided two uninfected blood meals on days 0 and 6, with or without 500 µg/mL of bovine IgM.
675 Midguts were dissected daily on days 0 to 10 (n= 15) post initial blood meal and collected in 50µL
676 of RIPA lysis buffer (Thermo-Fisher Scientific, 89901) supplemented with phenylmethylsulfonyl
677 fluoride (PMSF) (MilliporeSigma, 93482) and Halt Protease Inhibitor Cocktail (Thermo-Fisher
678 Scientific, 87786). Samples were gently macerated with a disposable pellet mixer and a cordless
679 motor, then vortexed and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was
680 collected, and its protein content was quantified by BCA (Thermo-Fisher Scientific, 23227). A

681 total of 7.5 µg of protein was loaded in a 15-well NuPAGE 4%–12% Bis-Tris protein precast gel
682 (Thermo-Fisher Scientific, NP0335BOX), run under reducing conditions by the addition of the
683 NuPAGE Sample Reducing Agent with DL-Dithiothreitol (DTT) (Thermo-Fisher Scientific,
684 NP0009), and then denaturation at 95°C for 5 min. Proteins were transferred to a 0.45mm
685 nitrocellulose iBlot 2 Transfer Stack membrane using the iBlot 2 Gel Transfer device (Thermo-
686 Fisher Scientific, IB23001). To check for successful loading of antigens, No-Stain Protein
687 Labeling Reagent (Invitrogen, A44717) was used following the membrane manufacture's
688 protocol. Next, membranes were blocked overnight at 4°C in 5% non-fat milk diluted in Tris-
689 Buffered Saline, 0.1% TWEEN 20 (TBST) followed by 3 hours of incubation at RT with sheep
690 anti-bovine IgM (Bio-Rad, AAI179) diluted at 1:1000. Membranes were washed three times with
691 TBST followed by incubation for 30 min with rabbit anti-sheep IgG H+L secondary antibody
692 conjugated to horse radish peroxidase (HRP) (Abcam, Ab6747) at a 1:5000 dilution. Membranes
693 were washed three times with TBST and blots were developed by the addition of SuperSignal West
694 Pico PLUS Chemiluminescence substrate (Thermo-Fisher Scientific, 34579). The signal was
695 captured after 2 min using the Azure c600 chemiluminescence imager (Azure Biosystems).
696 Membranes were stripped by adding 10 ml of Restore Western Blot Stripping Buffer (Thermo-
697 Fisher Scientific, 21059) for 15 minutes at 37°C, followed by two washes with TBST. Blots were
698 re-blocked overnight and incubated with recombinant rabbit anti-bovine serum albumin antibody
699 (Abcam, ab192603) at 1:1000 dilution for 3 hours, followed by a 30 min incubation with HRP-
700 conjugated goat anti-rabbit IgG H+L secondary antibody (Cell Signaling Technology, 7074S) at
701 1:1000 dilution. Blots were re-imaged as detailed above. To access the presence of IgM in fetal
702 serum, NuPAGE 4%–12% Bis-Tris protein precast gels (Thermo-Fisher Scientific, NP0335BOX)
703 were loaded with 1 µg of either Bovine IgM (Bio-Rad, AAI179), fetal bovine serum (Thermo-

704 Fisher Scientific, 16140-071) or adult bovine serum (Millipore-Sigma, B9433) under reducing
705 conditions followed by denaturation at 95°C for 5 min. The pattern of the proteins was visualized
706 by Coomassie blue staining using the eStain™ L1 protein staining system. For immunoblotting,
707 proteins were transferred onto nitrocellulose membranes, blocked, and probed with sheep anti-
708 bovine IgM at 1:1000 followed by a 30 min incubation with HRP-conjugated anti-sheep IgG H+L
709 secondary antibody (Abcam, Ab6747) diluted at 1:5000. Membranes were stripped and incubated
710 with recombinant rabbit anti-bovine serum albumin antibody at 1:1000 followed by a 30 min
711 incubation with HRP-conjugated goat anti-rabbit IgG H+L secondary antibody (Cell Signaling
712 Technology, 7074S) at 1:1000 and imaged after addition of the chemiluminescence substrate.
713

714 **IgM depolymerization**

715 To obtain monomeric IgM antibodies, 1 mg of bovine IgM (MilliporeSigma, I8135) was subjected
716 to a digestion buffer exchange (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% NaN_3; pH 8.0)
717 - final concentration of 1 mg/ml. Afterward, 1 ml of IgM was incubated in the presence of 6 mg
718 2-mercaptoethylamine at 37 °C for 30 minutes in the dark. The reduced IgM was further alkylated
719 in the presence of 16 mg iodoacetamide, followed by purification using a Superdex 200 Increase
720 (10/300 GL; GE Healthcare) equilibrated with Buffer A minus EDTA. After size exclusion, a clear
721 separation between the pentameric (peak 1 – P1) and monomeric (peak 3 – P3) IgM fractions were
722 observed via native gel electrophoresis (NativePAGE Novex Bis-Tris Gel system; Life
723 Technologies, Carlsbad, CA, USA). All fractions corresponding to defined peaks were pooled
724 together, adjusted to a suitable concentration and frozen at -20 °C until further use.
725
726

727 **Mass spectrometry**

728 Protein identification was carried out with a standard bottom-up method. The sample was reduced
729 with DTT, alkylated with iodoacetamide, then digested using Lys-C followed by trypsin. The
730 digested sample was cleaned using C18 tips (Agilent, OMIX10) and subjected to the LC-MS
731 (EASY nLC 1200 and Orbitrap Fusion Lumos mass spectrometer, Thermo Scientific). The
732 peptides were separated using a PepMap 100 C18 reversed phase column, and a standard data-
733 dependent acquisition was performed. The survey MS1 (Top Speed mode, 3 seconds) was done
734 using the Orbitrap mass analyzer, and the ddMS2 was done using the Linear Ion Trap where the
735 fragmentation was done with CID. Dynamic exclusion was set for the duration of 20 seconds. The
736 data analysis was done using PEAKS Studio software (Bioinformatcs Solutions Inc.). The database
737 used is the UniProt proteome UP000002254 (Dog, taxonomy id 9615). The number of identified
738 peptides, and protein abundance in the sample were confirmed by the PEAKS scores.

739

740 **Video recording and stereomicroscopic imaging**

741 Midgut videos were taken using an iPhone 10 camera connected to the Stemi 508 stereomicroscope
742 (Zeiss) ocular by a microscope mount (iDu Optics, LabCam iPhone 10 microscope adapter with
743 built-in 30mm 10X WF lens). Phase contrast images and videos were recorded using a K5 camera
744 coupled to a Thunder Imager microscope (Leica). Raw files were opened using Fiji ImageJ
745 software[44] and exported at 25 to 90 frames per second.

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750 **Immunofluorescence of in vitro *Leishmania* mating clumps**

751 After 24 hours of formation in vitro, whole Leishmania mating clumps were washed twice with
752 PBS before fixation with 4% Paraformaldehyde (PFA) overnight at 4ºC. Fixation was followed by
753 permeabilization with 0.1% PBS Triton for 10 minutes, then 0.1 M glycine in PBS solution for 10
754 minutes, and a final blocking step with 0.1% Triton/0.1% BSA/ PBS for an hour at room
755 temperature. Mating clumps were then incubated at 4ºC overnight with mouse anti-alpha-tubulin
756 monoclonal antibody (MilliporeSigma, T6199) conjugated to Alexa 555 (1:1000), and sheep anti-
757 bovine IgM (Bio-Rad, AAI179) conjugated to Alexa 647 (1:1000) diluted in blocking buffer. Anti-
758 Bovine IgM antibody was conjugated to an Alexa 647 fluorophore using the READILINK™
759 647/674 Antibody Labeling Kit (Bio-Rad, 1351006) following the manufacturer's instructions.
760 The next day, mating clumps were washed three times with blocking buffer, ten minutes each, and
761 then washed two times with permeabilization buffer, both at room temperature. Clumps were
762 counterstained with 2 µM of Hoechst 33342 (Thermo Fisher Scientific, 62249) in permeabilization
763 buffer for 30 minutes at room temperature, followed by a quick rinse of 0.1% Triton/PBS. Clumps
764 were placed in a u-slide angiogenesis (IBIDI, 81506) well in Prolong gold antifade medium
765 (Thermo Fisher Scientific, P10144) for imaging. For the sections, mating clumps were fixed in 4%
766 paraformaldehyde overnight at 4ºC, processed on the Leica ASP 6025, and then embedded in
767 histology grade paraffin. All samples were sectioned at 3 µm. Mating clump section pretreatment
768 was performed on the Leica Bond RX. Sections were baked for 30 minutes at 60ºC and then
769 dewaxed for 30 seconds in Bond Dewax solution (Leica, AR9222) heated to 72ºC. Sections were
770 rehydrated with absolute ethanol washes followed by 1x ImmunoWash (StatLab, ACR-015), then
771 subjected to a 20-minute treatment with Epitope Retrieval Solution 1 (Leica, AR9961) heated to
772 100ºC. Slides were then washed with PBS and stained. Steps for immunofluorescence of sections

773 were based on Zaqqout et al. 2020[45]. Briefly, slides were permeabilized with PBS/gelatin/Triton
774 0.25%, twice for 10 minutes each at room temperature, followed by blocking with 5% BSA/PBS
775 for one hour at room temperature in a humid box. The primary antibodies listed above were
776 incubated in a blocking solution and kept overnight in a humid box at 4ºC. The next day, slides
777 were washed three times with PBS and then with permeabilization solution for 10 minutes at room
778 temperature, and counterstained with 2 µM of Hoechst 33342 in the same buffer for 30 minutes at
779 room temperature followed by a quick rinse with 0.1% Triton/PBS. Another final rinsing step with
780 10mM $CuSO_4$/ 50mM NH_4Cl solution for 10 minutes and a final short rinse with water was
781 performed. Slides were closed using Prolong gold antifade mounting media and a coverslip.
782

783 **Confocal Microscopy**

784 Images were captured using a Leica TCS SP8 DMi8 inverted fluorescence confocal microscope
785 (Leica Microsystems) equipped with a photomultiplier tube/hybrid detector and a Leica DFC345
786 Monochrome camera. Images were taken using sequential acquisition, variable z-steps, mosaic
787 size, and integration. Images were viewed with the 40x, 63x, and 100x oil immersion objective
788 (zoom factor of 2, 3, or 4), and data were collected using the Leica Application Suite X software
789 platform. White light laser and specific emission and excitation range were applied depending on
790 the fluorophore used. The following spectra for excitation/emission were used: 488/520 for EdU
791 and BrdU; 555/580 for alpha-tubulin; 594/620 for BrdU and EdU; and 647/665 for IgM. DAPI
792 was excited using a 405-nm diode laser. Images were taken using sequential acquisition and
793 variable z-steps. Whole mating clumps were imaged in the Z-wide mode (8-bit) and tile mode to
794 capture the entire clump. Individual fields were merged and stitched together to mount the
795 composite of the whole clump. Image processing was performed using Imaris 9.2.1 (Bitplane,

796 Oxford Instruments). Maximum intensity projection (MIP) is represented in xy pictures, and cross-
797 sections of between 2-3 µm were obtained with the ortho-slicer feature from Imaris.
798
799 **BrdU and EdU staining**
800 *Leishmania* promastigotes were incubated with 10 µM of EdU - 5-Ethynyl-2'-deoxyuridine
801 (Abcam, ab146186) or BrDu - 5-bromo-2'-deoxyuridine (Abcam, ab142567) for 4 days to reach
802 the stationary growth phase. Parasites were then washed twice with PBS and an equal proportion
803 of each of the labeled promastigotes was used for mating clump assembly. Mating clumps were
804 fixed and processed for sections as described above. Three-micrometer sections were washed with
805 PBS, permeabilized with 0.2% Triton/PBS for 10 minutes, then incubated with 0.1M glycine for
806 10 minutes at room temperature. Samples were then blocked with 0.5% BSA/PBS for 10 minutes
807 and then briefly washed with PBS before the Click reaction was performed. We used the Click-it
808 EdU Cell Proliferation Kit to image incorporated Edu with Alexa Fluor 488 dye (Invitrogen,
809 C10337). The reaction was prepared following the manufacturer's instructions and incubated with
810 the samples for 30 minutes at room temperature in a humid and dark chamber. After the click
811 reaction, sections were washed three times with PBS and blocked with 0.5% BSA/PBS for one
812 hour at room temperature. Primary mouse monoclonal anti-BrdU (MOBU-1) (Invitrogen, B35128)
813 (1:100) was incubated in blocking buffer overnight at 4ºC. The next day, sections were washed
814 with blocking buffer three times, 10 minutes each, and incubated with goat anti-mouse Alexa 594
815 secondary antibody (Invitrogen, A-11005) at 1:1000 in 0.1% Triton/PBS for 2 hours at room
816 temperature. Slides were washed three times with 0.1%Triton/PBS for 10 minutes each and then
817 counterstained with 2 µM of Hoechst 33342 in 0.1% Triton/ PBS for 30 minutes at room
818 temperature. A final rinse was done with 10mM $CuSO_4$/ 50mM NH_4Cl solution for 10 minutes

819 and followed by a quick rinse with water before the slides were closed with Prolong gold antifade
820 mounting media and a coverslip.
821
822 **Genome sequencing**
823 Genomic DNA was extracted from cloned parasite culture pellets using the E.Z.N.A.® DNA/RNA
824 Isolation Kit (Omega Biotek, R6731) and following the manufacturer's recommendations for
825 cultured cells. Sample concentration was measured by a DS-11+ spectrophotometer (DeNovix
826 Inc.). A minimum of 3 µg of DNA was sent for sequencing (Novogene Co.). Genomic DNA was
827 randomly fragmented by sonication, then DNA fragments were end polished, A-tailed, and ligated
828 with full-length adapters for Illumina sequencing followed by further PCR amplification with P5
829 and indexed P7 oligos. The PCR products were purified with the AMPure XP system and libraries
830 were checked for size distribution using an Agilent 2100 Bioanalyzer (Agilent Technologies) and
831 quantified by real-time PCR (to meet the criteria of 3 nM). Qualified libraries were fed into
832 Illumina sequencers after pooling to obtain effective concentrations and the expected data volume.
833
834 **Genomic analysis**
835 For Supplementary Fig. 8, raw sequencing reads were assessed for quality using the FastQC
836 software. Fastq files were imported and processed using Geneious prime software v2021.2.2
837 (https://www.geneious.com). Low quality reads were trimmed from fastq files (< 20) and
838 contaminating adapter primer sequences were removed using BBDuk trimmer plugin. Trimmed
839 reads were mapped to the reference genome TriTrypDB-52_LmajorFriedlin using bowtie 2 plugin.
840 BAM files with aligned reads were analyzed with qualimap v2.2.1[46]. The median genomic
841 coverage of all samples was 33.1X. Using bowtie 2, unmapped reads were aligned to an artificial

842 chromosome with selective markers. The artificial chromosome was created by concatenating the
843 resistance genes integrated into the parental lines, HYG (Hygromycin), BSD (Blasticidin) and
844 SAT (Nourseothricin), buffered by a 1.2kb (150bp repeated 8 times) sequence from mouse (*Mus*
845 *musculus*) chromosome 1. For Fig. 3e, 4d and Supplementary Fig. 9, raw sequencing reads were
846 trimmed using Trimmomatic v0.39[47] and mapped using BWA-MEM v07.17[48] to the TriTrypDB-
847 52_LmajoprFriedlin reference genome with the resistant gene sequences inserted as additional
848 contigs. PCR duplicates were marked using Samblaster v0.1.2.5[49] and germline variants were
849 called jointly across all samples using the HaplotypeCaller tool from GATK v4.1.9.0 and using
850 default settings. Resulting variants were then filtered down to only biallelic sites with no missing
851 data across all samples. The genomic insertion locations for each resistance gene were validated
852 by examining split reads from each sample, and identifying the location of reads with one pair
853 member mapped to the construct and the other pair member mapped to the human genome. Any
854 locations with less than 3 supporting split read pairs were excluded from consideration. To quantify
855 the allelic copy number of each contributing parent in F1 offspring, resulting BAM files were first
856 used as input to the PAINT software suite[50] to generate chromosome-level somy levels
857 (Supplementary Fig. 10). A custom perl script was then used to execute the following steps with
858 the multisample VCF and chromosome somy estimates as input: 1) identify homozygous SNP
859 differences between parents for each cross, ignoring sites at <20X depth in both parental strains;
860 2) for each SNP identified in step 1, calculate the frequency of each allele based on read counts
861 supporting each allele (e.g., allele1 proportion = (# reads supporting allele 1 at position1)/(total
862 sequencing depth at position 1); 3) for a given F1, multiply the proportion of each allele by the
863 chromosome-specific somy to get the copy number estimate for each parent. For the backcross
864 hybrids analysis, the same procedure outlined above was used, and recombination breakpoints

865 were identified as regions where parental copy number along a chromosome shifted by >=0.8 (to
866 allow for 20% variance in absolute copy number estimation) across >=10 kilobases. All resulting
867 copy number breakpoint calls were visually confirmed (e.g., Fig. 4d). The script used to execute
868 the steps above is available here: https://github.com/jlac/Leishmania_allelic_copy_number .
869

870 **Scanning Electron Microscopy (SEM)**

871 Leishmania mating clumps were fixed in 2.5% Glutaraldehyde in 0.1M Sodium Cacodylate Buffer,
872 pH 7.4 (Electron Microscopy Sciences, 15960). Post fixation, the clumps were rinsed in 0.1M
873 sodium cacodylate buffer and fixed with 1% OsO4 reduced with potassium ferrocyanide in buffer.
874 After a couple of washes in water, the clumps were dehydrated in 100% ethanol and dried using
875 multiple exchanges of hexamethyldisilazane (HMDS). On the following day, clumps were
876 mounted on SEM pins using carbon adhesive tabs. Gentle pressure was applied to split open
877 clumps to reveal their content, sputter coated with 5nm of Iridium using an EMS 300TD Quorum
878 sputter coater, and imaged on a SU 8000 SEM.
879

880 **Transmission Electron Microscopy (TEM)**

881 *Leishmania* mating clumps were fixed as per SEM protocol. After postfixing the organoids with
882 1% reduced OsO_4 and dehydrating with 100% ethanol, the clumps were infiltrated and embedded
883 with 100% Spurr's resin in a flat mold. The flat mold with clumps were excised and mounted on
884 BEEM™ capsules for trimming. Ultrathin sections were collected using a Leica UC7
885 ultramicrotome and imaged on an 80kV Hitachi 7800 TEM using an AMT™ bottom mount
886 camera.
887

888 **Statistical analysis**

889 All data manipulation and statistical analysis was performed in R (v. 4.1.1) with custom code
890 written in Rstudio (v. 1.4.1717). Functions from the following packages were used in our scripts:
891 abind[51], chron[52], data.table[53], e1071[54], emmeans[55], FedData[56], gsubfn[57], gt[58], ijtiff[59], janitor[60],
892 lubridate[61], MASS[62], plyr[63], rcompanion[64], readxl[65], reshape[66], R.utils[67], rlist[68], Rmisc[69], rstatix[70],
893 stats[71], stringr[72], tidyverse[73] (contains: dplyr, ggplot2, forcats, tibble, readr, stringr, tidyr, purr),
894 utils[71], WRS2[74], xlsx[75]. To compare hybrid formation success between the different experimental
895 conditions (BM1, BM2/-IgM, BM2/+IgM) with the different parental line combinations (1x2, 1x3
896 & 2x3) as detailed in figures 3d and 4c, we applied an expanded Fisher's exact test that could cope
897 with r x n matrices over a chi-square test due to the occurrence of low expected values (<5). Since
898 our data matrices exceed the standard 2x2 format, we followed the Fisher's exact test with a
899 pairwise test with the "pairwsieNominalIndependence" function from the R package rcompanion[64]
900 for 2-dimensional matrices, in which at least one dimension exceeded 2 levels. The output of this
901 test was converted into a letter code using the "cldList" function from the R package rcompanion[64]
902 for improved test output representation; equal letters indicate no statistically significant difference,
903 while different letters indicate a statistically significant difference (<0.05).

904

905 **Methods references**

906

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End notes:

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Data availability: All data is present in the main text or the supplementary materials. Codes generated on this work used for copy number bioinformatics analysis on https://github.com/jlac/Leishmania_allelic_copy_number. Whole-genome sequencing raw reads files are deposited on SRA database under BioProject PRJNA848835. https://dataview.ncbi.nlm.nih.gov/object/PRJNA848835?reviewer=g3jklmr2bpu6ehm01aagjr68jv.