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

Tiago D. Serafim[1#*], Eva Iniguez[1#], Ana Beatriz F. Barletta [2], Johannes S.P. Doehl[1], Mara Short[1], Justin Lack[3], Pedro Cecilio[4] Vinod Nair[5], Maria Disotuar[1], Timothy Wilson[1], Iliano V. Coutinho-Abreu[1], Fabiano Oliveira[1], Claudio Meneses[1], Carolina Barillas-Mury[2], John Andersen[4], José M.C. Ribeiro[4], Stephen M. Beverley[6*], Shaden Kamhawi[1*], Jesus G. Valenzuela[1*]

Affiliations:

1. Vector Molecular Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA.
2. Mosquito Immunity and Vector Competence Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA.
3. NIAID Collaborative Bioinformatics Resource, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA.
4. Vector Biology Section, Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA.
5. Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA.
6. Department of Molecular Microbiology, School of Medicine, Washington University, St. Louis, MO, USA.

[*]Correspondence to:
jvalenzuela@niaid.nih.gov;stephen.beverley@wustl.edu;skamhawi@niaid.nih.gov;tiago.donatelliserafim@nih.gov

[#]Equally contributed

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 *Leishmania* clump that releases viable parasites 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 reported here resulted in full genome hybrids with comparable recombination structures that arose by fusion of the two parental lines. *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.

Main:

Leishmania parasites are vector-borne pathogens transmitted to mammalian hosts by phlebotomine sand flies[1]. 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[2]. More than a billion people are at risk of infection, millions are asymptomatic carriers, and the annual incidence of fatal visceral and cutaneous leishmaniases

are estimated at 100,000 to 1 million cases, respectively[3]. These statistics place leishmaniasis at the forefront of parasitic diseases of public health importance.

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

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

In this study, we focused on elucidating host factors that mediate *Leishmania* genetic exchange, asking whether host blood ingested by sand flies provides some element(s) absent in culture media that promotes fusion and the formation of hybrid parasites. By adding freshly isolated dog plasma to culture media we succeeded in generating in vitro crosses of two genetically labeled *L. major* strains, WR SSU-Hyg and V1 fkp40-Bsd (Fig. 1a). Hybrid generation was only observed when accompanied by the formation of a structured spherical 'clump' composed of live aggregating parasites (Fig. 1b, top panel; Supplementary Videos 1-3). In standard culture media, in the absence of dog plasma, the parasite clump did not form (Fig. 1b, bottom panel; Supplementary Videos 6,7) and hybrids were not recovered. The parasite clump forms gradually

and is maintained for 24-36h (Supplementary Video 4) before it starts to dissociate releasing viable parasites.

Purification and fractionation of dog plasma using a 2 step-HPLC followed by MS-MS and in vitro incubation of parasites with the HPLC fractions revealed that IgM was the molecule responsible for the formation of the parasite clump (Supplementary Fig. 1). Parasite clumping was retained in HPLC-eluted fractions of a commercially available IgM (Fig. 1c) but was absent with a commercial human IgA or IgG (Fig. 1d), emphasizing the unique role of IgM in parasite clumping. Importantly, fetal bovine serum (FBS) does not induce clumping (Fig. 1d) and it does not contain IgM (Fig. 1e). Pertinently, the process of IgM-induced clump formation (Fig. 1f, Supplementary Fig. 2a) is distinct from peanut agglutination of *Leishmania*. With peanut agglutinin (PNA), the parasites agglutinate immediately and irreversibly, forming a disorganized flat mesh-like structure of immotile parasites (Fig. 1g; Supplementary Fig. 2b; Supplementary Video 5). The composition and temporal formation and dissociation of the *Leishmania* parasite clump is also different from other reported structures, including the *Leishmania* rosettes[17]. Furthermore, the pentameric form of IgM was required for parasite clumping (Fig. 1h; Supplementary Fig. 3) that was lost when IgM monomers were added to parasite cultures (Fig. 1i, Supplementary Fig. 3). Of relevance, IgM-induced parasite clumping in eight diverse *Leishmania* species (Fig. 1j) strongly suggesting that parasite clumping upon contact with blood is a shared ancestral response in *Leishmania*. A transient agglutination of *L. donovani* by IgM, that we now refer to as parasite clumping, has been previously reported, and this response was not observed with FBS or human umbilical cord serum, both naturally deficient in IgM[18], reinforcing our current observations.

To directly establish the contribution of the IgM-induced clump to genetic exchange in vitro, we used 3 genetically marked parasite strains with independent selectable markers (*L. major* – WR/GFP/Hyg [parental-1], *L. major* – V1/Fkp40/Bsd [parental-2], and *L. major* – V1/Ftl/Sat [parental-3]) in various crosses. Stationary phase promastigotes from two parental lines were mixed at a 1:1 ratio and cultured for 3 days, with or without IgM, in the absence of selection drugs, and then grown in the presence of selective drugs for up to 30 days (Fig. 1k, l). Hybrids were observed for all parental combinations, but only when the culture was supplemented with IgM (Fig. 1k, l) establishing a causal relationship between IgM, formation of a parasite clump and hybrid generation. Collectively, our data demonstrate that IgM is critical for the generation of *L. major* hybrids in vitro through its induction of a structured and transient living parasite clump. Furthermore, without any other manipulation to the culture, in vitro hybrids from *L. tropica* crosses were only observed in the presence of IgM under our experimental conditions (Supplementary Fig. 4).

Another breakthrough of this study was to have accomplished something sought after for many years, the formation of *L. major* hybrids in vitro. We achieved it by adding IgM to *Leishmania* culture media that historically uses FBS that does not contain IgM (Fig. 1e).

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

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

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

Leishmania to IgM in sand fly blood meals. Every time a sand fly takes a blood meal, a *Leishmania* parasite that resides in the sand fly gut will interact with IgM-present in the blood of all vertebrate animals[19-21].

The ubiquity of IgM in vertebrate blood[19-22] combined with the multiple and promiscuous (Supplementary Table 1) blood-feeding behavior of the sand fly[23] led us to hypothesize that this natural antibody is a major facilitator for the initiation of genetic exchange in *Leishmania* parasites in vivo, in the sand fly gut. To address this hypothesis, we performed experiments using conditions that mimicked the natural progression of sand fly infections, including incorporation of a second blood meal that is needed to prevent loss of naturally acquired parasites[23]. Having first established that IgM persists in the sand fly midgut for at least 24 hours after each blood meal (Fig. 3a), we performed an experiment where we allowed sand flies to feed on a cutaneous leishmaniasis lesion initiated by two *L. major* parental lines (Fig. 3b, BM1), to pick the parasites up as part of a natural blood meal [24,25]. We then followed the natural sand fly feeding behavior by offering them a second uninfected blood meal six days after feeding on cutaneous lesions (Fig. 3b, BM2). The second blood meal was either devoid of IgM or supplemented with 500 µg/ml of IgM (Fig. 3b, BM2), a concentration at or below those reported in the blood of different animals (Supplementary Table 2). Additionally, a control group was maintained on a single infected blood meal (Fig. 3b, dotted arrow). Fourteen days after the initial infection, sand fly guts from each group were dissected and plated individually to screen for parasite hybrids (Fig. 3b). For the group initially infected with parental lines combination 1+2, IgM increased the proportion of hybrid formation by up to 12-fold in sand flies getting a second blood meal containing IgM compared to sand flies given a second blood meal devoid of IgM or sand flies given a single infectious blood meal (Fig. 3c, d, Supplementary Fig. 5a). In these two control groups, we observed a significantly lower proportion

of hybrids as was previously reported[13,14]. The similar proportion of hybrids observed in flies given a second blood meal devoid of IgM or sand flies given a single infectious blood meal suggest that this may be a basal level of chance mating within the confined space of the sand fly midgut. It is worth noting that both control group scenarios would not occur in nature since an infected sand fly will always take a second blood meal 5-6 days after infection[1,23]. Strikingly, control groups lacking IgM did not generate any hybrids in parental lines combinations 1+3 and 2+3, further emphasizing the importance of IgM in *Leishmania* hybrid formation in vivo (Fig. 3c, d, Supplementary Fig. 5a). Of note, the parasite load, and percent metacyclics were similar in all experimental groups (Supplementary Fig. 5b-g). The pronounced effect of IgM on parasite hybrid formation when provided to sand flies six days after naturally acquired infections (BM2) suggests 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 of division[1,23]. Collectively, the above data establish that IgM facilitates *Leishmania* hybrid formation inside the sand fly gut after naturally acquired infections, likely by bringing parasites closer together within a LMC that promotes fusion and hybrid generation. This is supported by the observation of structures similar to in vitro LMCs within the midgut (Supplementary Fig. 6; Supplementary Videos 7-12).

Corroborating previous findings[10], we observed hybrid formation only in sand flies and did not find them in *Leishmania* skin lesions or draining lymph nodes containing 2 different parasite lines (Supplementary Fig. 7). In the mammalian host, the *Leishmania* parasite is intracellular, residing in a parasitophorous vacuole inside macrophages[26,27]. It may be that a lack of opportunity for IgM to interact with amastigotes accounts for the absence of hybridization in the mammalian host. Whole-genome sequencing of the double drug resistant hybrids (Supplementary Fig. 8)

showed that the crosses in sand flies and those generated in vitro were full genome hybrids (Fig. 3e; Supplementary Fig. 8-10) and establishes that they arose by fusion of the two parental lines. Moreover, the levels of aneuploidy commonly seen in cultured parasites[10,13,14] were observed in this study (Supplementary Fig. 9, 10).

To establish whether hybrids resulting from in vivo experiments are fertile, we performed a backcross between the (1+2) f1 hybrid and one of its original *L major* parental lines genetically modified to produce the 2' and 2" lines bearing a new marker each (Fig. 4). This is of relevance since only a single backcross has been reported for *Leishmania* hybrids to date[8]. Mimicking natural conditions, we let sand flies feed on a cutaneous leishmaniasis lesion initiated by the (1+2) f1 hybrid and parental line 2' or 2" of *L. major* (Fig. 4a, BM1). Sand flies were given a second uninfected blood meal six days after feeding on cutaneous lesions (BM2), either supplemented with 500 µg/ml of IgM or devoid of IgM (Fig. 4a, BM2). We consistently observed hybrid formation of this backcross, but only in sand flies fed with blood containing IgM (Fig. 4b, c; Supplementary Fig. 11a). Of note, the parasite load, and percent metacyclics were similar in all experimental groups (Supplementary Fig. 11b-g). Whole-genome sequencing of the triple drug resistant hybrids showed that the second-generation crosses (f2 hybrids) in sand flies are backcrosses to parental line 2 (Fig. 4d, blue plot) where recombination events have broken up (breakpoints) the consistent biparental contribution of the first generation cross (Fig. 4d; discontinuous black plots spaces on f2 hybrids). The absence of f2 hybrids in sand flies given a second blood meal devoid of IgM provides conclusive evidence of its importance in *Leishmania* genetic exchange in a natural setting. Our data strongly suggest that IgM facilitates fertile hybrid formation and enhances their frequency in sand flies consequently promoting *Leishmania*

diversity. Importantly, the reproducible generation of backcrosses opens the way for more in-depth genetic analysis of *Leishmania* parasites.

Our work provides direct experimental evidence of the critical role of IgM in promoting 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 an antibody by implicating IgM in genetic exchange in a parasite. The exploitation of host IgM by *Leishmania* is of significant consequence. IgM is an evolutionarily conserved natural antibody that is always present in the blood of all vertebrates after birth[19-21], independent of the immune status of the animal[28-33]. *Leishmania* evolved to exploit the pentameric properties of host IgM and the biological feeding pattern of sand flies to enhance the likelihood of mating. Sand flies ingest blood every 5-6 days to produce eggs[1]. We hypothesize that when a *Leishmania*-infected sand fly takes a subsequent blood meal, the periodic influx of IgM encounters parasites that have evolved to be at a stage that provides the density required to initiate the formation of the LMC in the confinement of the gut, a critical first step of genetic exchange that promotes fusion and hybrid generation. As such, in addition to metacyclic promastigotes, this implicates the two major proliferative stages, leptomonad and retroleptomonad promastigotes, as potential participants in the process of genetic exchange.

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

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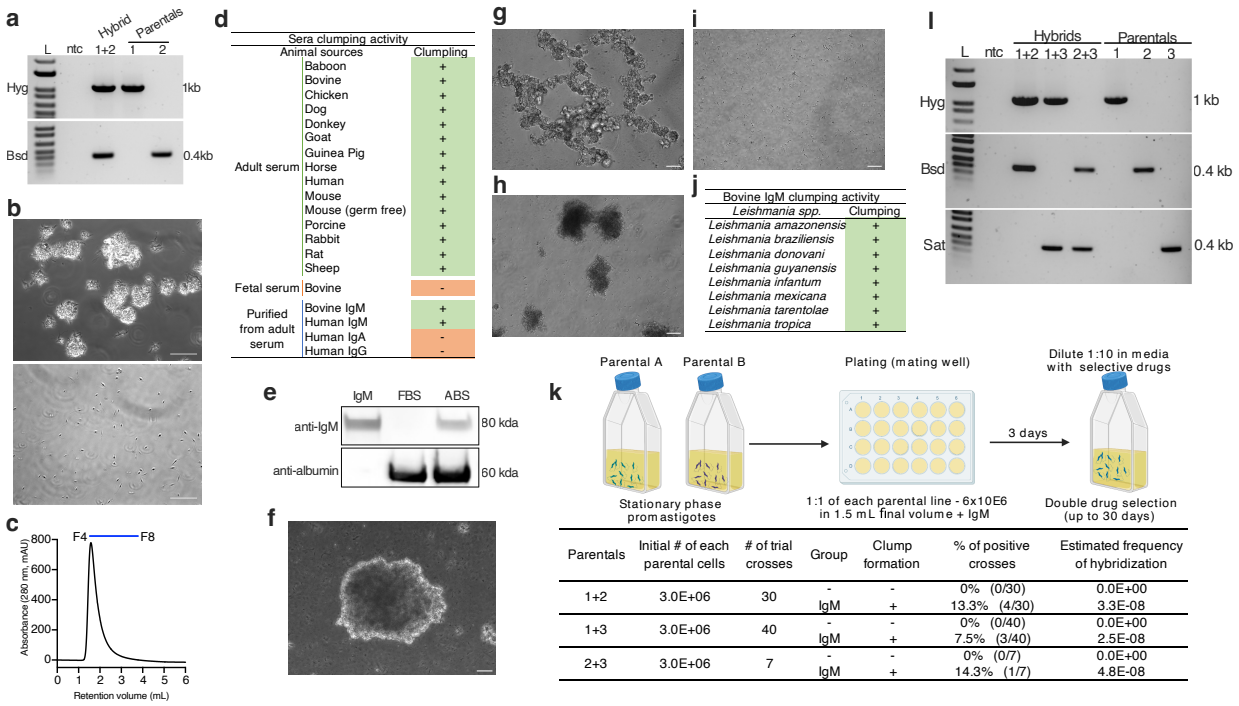
Data availability: All data is present in the main text or the supplementary materials. Codes generated on this work used for copy number bioinformatics analysis on https://github.com/jlac/Leishmania_allelic_copy_number. Whole genome sequencing raw reads sent to NCBI and waiting for approval and release.



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

Fig 1. IgM facilitates *Leishmania* parasites clumping which promotes hybrid formation *in vitro*. (**a**) *Leishmania* hybrids genotyping by PCR targeting parental selectable drug markers Hyg (Hygromycin) and Bsd (Blasticidin). (**b**) Phase contrast image of *L. major* in culture media containing 20% fetal bovine serum (FBS) without (bottom panel) or with 5% adult inactivated dog serum (top panel). Images taken 30 min after cells were seeded in fresh media. Scale bars = 20 µm. (**c**) Human IgM eluted from a HiTrap® IgM column. *Leishmania* clumping activity was observed in fractions F4-F8 corresponding to elution of IgM. **(d)** Presence (+) or absence (-) of *L. major* clumping activity in inactivated adult sera from different vertebrate species, FBS, or immunoglobulins (IgM, IgG, IgA) purified from adult bovine or human sera. (**e**) Western blot to detect IgM in FBS and adult bovine serum (ABS). Serum purified bovine IgM, positive control; serum albumin, loading control. (**f**) Phase contrast image of *L. major* in culture media containing 20% FBS with bovine IgM at 50 µg/mL after 24h and (**g**) with peanut agglutinin (PNA) at 50 µg/mL after 24h. Scale bars = 50 µm. (**h**) Phase contrast image of *L. major* in culture media

containing 20% FBS with bovine IgM (pentameric form) at 50 µg/mL after 24h or (**i**) with bovine IgM (monomeric form) at 50 µg/mL after 24h. (**j**) Clumping activity of various *Leishmania* species in the presence of bovine IgM at 50 µg/mL. (**k**) (top) Workflow for *in vitro* crossing of *L. major* parental lines. Mating wells were established with 50 µg/mL IgM in complete Schneider's media. Double drug resistant hybrid parasites were cloned before genotyping. (bottom) Summary of data from *in vitro* crossing of parental lines. 1+2 (n=30), 1+3 (n=40), 2+3 (n=7). (**l**) *Leishmania* hybrids genotyping by PCR targeting parental selectable drug markers Hyg (Hygromycin), Bsd (Blasticidin) and Sat (Nourseothricin). Parental 1, *L. major*/WR/SSU/GFP/Hyg; Parental 2, *L. major*/FVI/FKP40/Bsd; parental 3, *L. major*/FVI/FTL/Sat; ntc, no template control; L, 1kb plus ladder (Invitrogen).

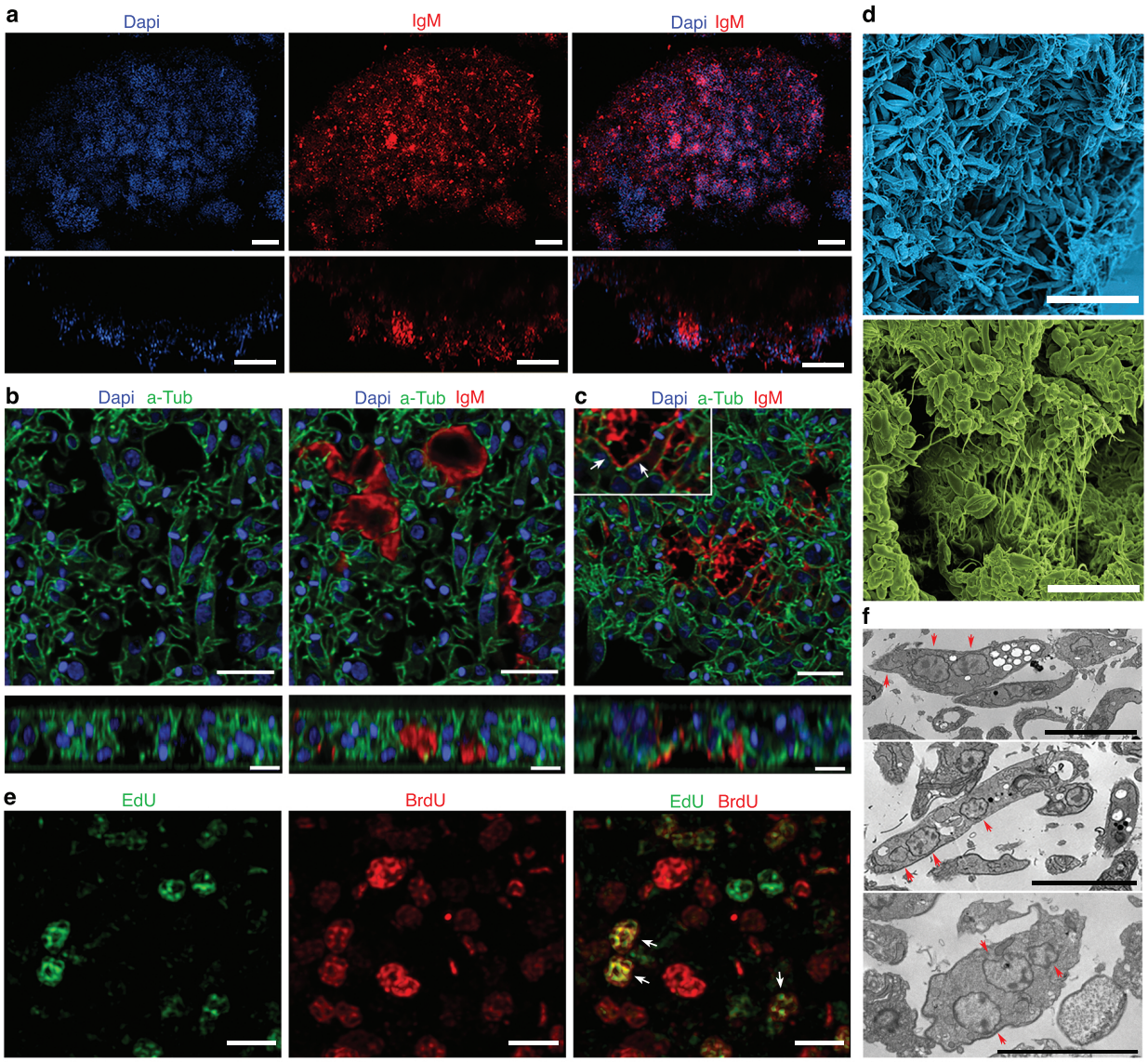
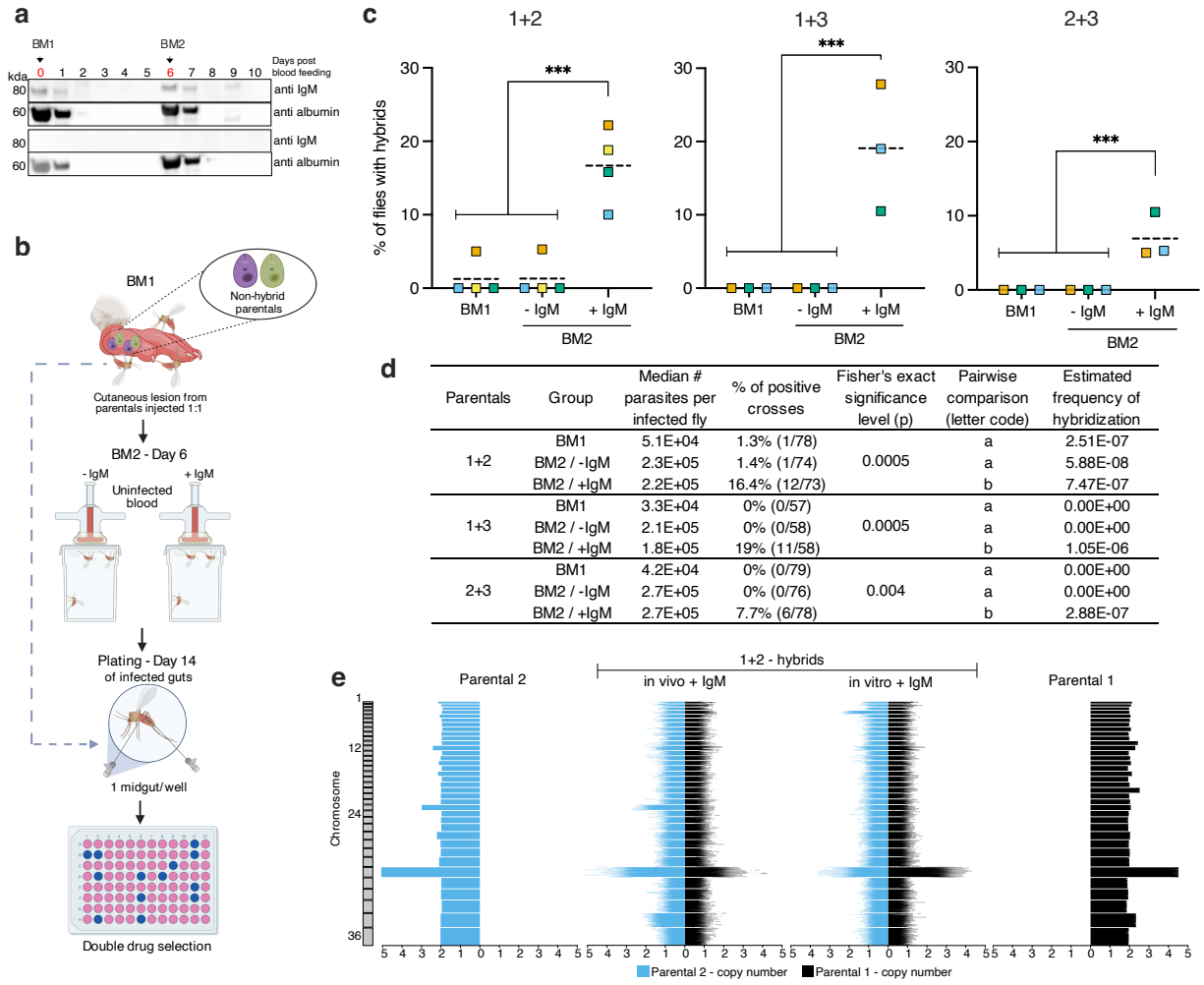


Fig 2. Characterization of the *Leishmania* mating clump. (a) Confocal immunofluorescence wide Z and tile images showing the association of IgM with the *L. major* mating clump after 24h of culture. Dapi (nuclei, kinetoplast) blue; IgM, red. Scale bar = 40 µm. Bottom rectangles for each panel represent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

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

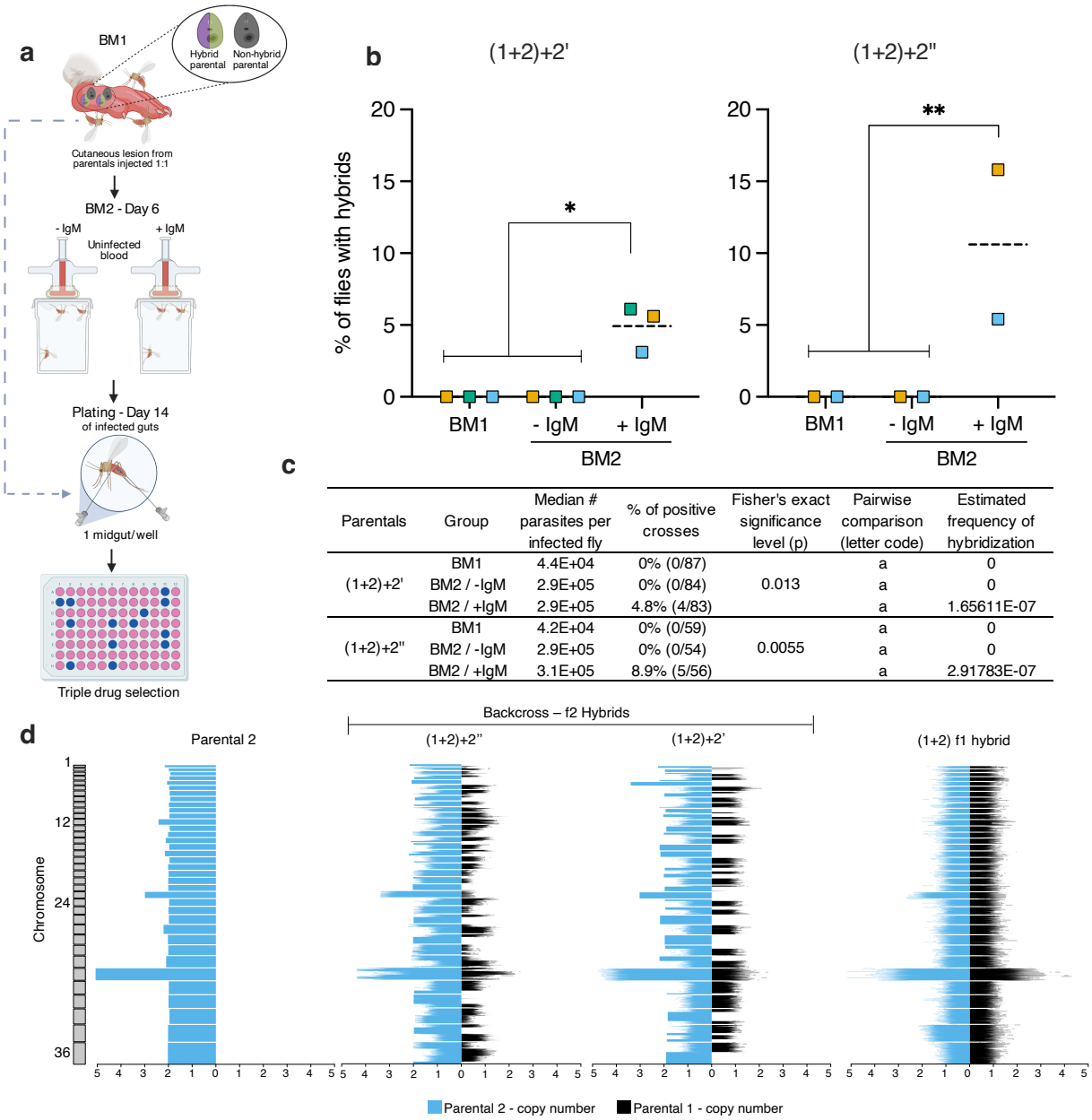


d

Parentals	Group	Median # parasites per infected fly	% of positive crosses	Fisher's exact significance level (p)	Pairwise comparison (letter code)	Estimated frequency of hybridization
1+2	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
1+3	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
2+3	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 (1+2; 1+3; 2+3) 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

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



Parentals	Group	Median # parasites per infected fly	% of positive crosses	Fisher's exact significance level (p)	Pairwise comparison (letter code)	Estimated frequency of hybridization
(1+2)+2'	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
(1+2)+2"	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 f2 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) 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

absence of IgM. Colors indicate independent experiments. **(c)** Cumulative data from independent experiments for infection status and estimated frequency of hybridization per fly. (1+2) + 2' [n=3]; (1+2) + 2'' (n=2). Per each biological replicate ~ 30 infected flies per group. Fisher's exact test, $p < 0.05$ is considered significant. Pairwise comparison indicate source of group significance: a, no difference; b, significant difference. **(d)** Whole-genome analysis of in vivo-generated f2 backcross hybrids for parental line combination 1+2. Where the biparental ancestry was consistent across each chromosome, recombination events have broken this up in the f2s, making parental copy number discontinuous always in the same direction through parental 2. Parental 1, *L. major*/WR/SSU/GFP/Hyg; Parental 2, *L. major*/FVI/FKP40/Bsd; Parental 2', *L. major*/FVI/FKP40/Sat; Parental 2'', *L. major*/FVI/SSU/Ruby/Sat. *$P < 0.05$, **$P \leq 0.01$.