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Short communication

Gliding motility protein LIMP promotes optimal mosquito midgut traversal and infection by *Plasmodium berghei*

Saskia Egarter [a,1], Jorge M. Santos [b,1,2], Jessica Kehrer [a], Julia Sattler [a], Friedrich Frischknecht [a], Gunnar R. Mair [a,b,c,*]

[a] Parasitology, Department of Infectious Diseases, Heidelberg University Medical School, Heidelberg, Germany
[b] Instituto de Medicina Molecular João Lobo Antunes, Faculdade de Medicina da Universidade de Lisboa, Edifício Egas Moniz, Av. Prof. Egas Moniz, Lisbon, Portugal
[c] Iowa State University, Biomedical Sciences, Ames, IA, United States

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ABSTRACT

Substrate-dependent gliding motility is key to malaria transmission. It mediates host cell traversal, invasion and infection by *Plasmodium* and related apicomplexan parasites. The 110 amino acid-long cell surface protein LIMP is essential for *P. berghei* sporozoites where it is required for the invasion of the mosquito's salivary glands and the liver cells of the rodent host. Here we define an additional role for LIMP during mosquito invasion by the ookinete. *limp* mRNA is provided as a translationally repressed mRNP (messenger ribonucleoprotein) by the female gametocyte and the protein translated in the ookinete. Parasites depleted of *limp* (Δ*limp*) develop ookinetes with apparent normal morphology and no defect during *in vitro* gliding motility, and yet display a pronounced reduction in oocyst numbers; compared to wildtype 82 % more Δ*limp* ookinetes remain within the mosquito blood meal explaining the decrease in oocysts. As in the sporozoite, LIMP exerts a profound role on ookinete infection of the mosquito.

Gliding motility of malaria parasites is required for mosquito and mammalian host infection. Despite their different morphologies, all invasive life cycle stages of the malaria parasite – merozoite, ookinete and sporozoite – rely on a conserved gliding motility machinery to traverse diverse host cell barriers in order to establish host cell infections and continue the life cycle [1]. Many of the motility factors are shared with related apicomplexan parasites such as *Toxoplasma gondii*. The pear-shaped malaria merozoite invades red blood cells. The ookinete is a banana-shaped life cycle stage that is formed from a fertilised female gamete (zygote) in the mosquito midgut and required to establish an infection in the mosquito; in order to do so, the ookinete crosses the blood meal sac as well as the mosquito peritrophic matrix and midgut epithelium to transform into a sessile oocyst. Sporozoites are finally formed in a period of 2 weeks and are distinctly slender-shaped; they move significantly faster than ookinetes and migrate from the hemocoel through the salivary gland epithelium into the gland lumen as well as through the skin and across the blood vessel endothelium to ultimately invade hepatocytes. The 110 amino acid-long cell surface protein LIMP is expressed in ookinetes and sporozoites, but not merozoites. In the sporozoite, LIMP defines the parasite's ability to populate the mosquito's salivary glands and is essential for the establishment of an infection of the rodent liver. In the ookinete, a deletion of the LIMP gene produces a robust, 50 %-reduction in oocyst numbers [2]. The cause for this defect is however unknown. Here we sought to address the role of LIMP at this crucial bottleneck in the malaria parasite's life cycle focusing on key aspects of ookinete biology: development, motility and infectivity.

The formation of the *P. berghei* ookinete from the fertilized female gamete (zygote) depends on maternally provided gene products (mRNA) that are produced in the female gametocyte, but are only translated after fertilisation. Hundreds of mRNAs – some 50 % of the detected transcriptome – are provided in translationally silent mRNPs (messenger ribonucleoproteins) [3]. When translated into protein they are instrumental in many developmental processes of the ookinete and can even affect the formation of oocyst-derived sporozoites [4–8]. Evaluating the protein expression profile through C-terminal GFP-tagging of LIMP in

* Corresponding author at: Iowa State University, Biomedical Sciences, Ames, IA, United States
E-mail address: mair@iastate.edu (G.R. Mair).
[1] Authors contributed equally.
[2] Present address: Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, United States.

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limp::gfp mutants by live fluorescence microscopy found no translation in asexual stage parasites nor gametocytes; *limp::gfp* is translated in the ookinete stage and the protein is then mainly visible near or in the crystalloid, a transient and putative storage organelle of the ookinete [9] (Fig. 1**A** and previously shown in [2]). We observed no translation of *limp* in sexual precursor cells (gametocytes), despite the transcript being readily detected by RT-PCR in this life cycle stage (see *limp* input lanes labelled with red arrows in Fig. 1B). Using parasite clones expressing GFP-tagged translational repressors DOZI (a homolog of yeast DHH1 DEAD-box RNA helicase) and CITH (a homolog of yeast repressor of translation initiation protein SCD6) [3,10] we identified *limp* mRNA to be associated with both proteins by RNA-immunoprecipitation (RIP) followed by RT-PCR detection of the bound transcript. Monoclonal anti-GFP antibodies and a protein G sepharose affinity reagent were used to immunoprecipitate DOZI::GFP and CITH::GFP and any bound mRNA from enriched gametocyte populations (lanes IP-αGFP); controls included the use of an unrelated anti-cmyc IgG (lanes IP-αcmyc) or omission of IgG altogether (lanes IP beads). Like *p25* and *p28* – both are hallmark translationally repressed transcripts encoding ookinete surface proteins and thus important and promising antimalarial transmission-blocking vaccine targets [11–13] – *limp* mRNA is bound by DOZI and CITH (Fig. 1B). As expected, mRNA encoding the DNA/RNA-binding protein ALBA-3, which is translated in the gametocyte, does not co-IP with DOZI or CITH. The RIP data explain the lack of LIMP expression in the gametocyte; *limp* mRNA is a *bona fide* maternally provided mRNA that is kept translationally quiescent in a complex containing DOZI and CITH in gametocytes. Once translated, LIMP affects *in vivo* transmission from the infected experimental mouse host to *Anopheles* female mosquitoes; *limp* gene deletion mutants experience a 50 % reduction in oocyst numbers [2]. This decrease could result from defects in ookinete development, motility, adhesion or invasion, but the underlying cause is not clear. In order to test whether their motility was affected, we performed *in vitro* ookinete cultures from gametocyte-infected blood. Knock-out parasites produced apparently normal ookinetes as evaluated by differential interference contrast (DIC) microscopy (Fig. 1C); they showed no defect in gliding motility; a drop of purified ookinetes in Roswell Park Memorial Institute (RPMI) 1640 medium was placed on a glass slide and imaged using an inverted Zeiss Axiovert 200 M widefield microscope with an XBO75 xenon lamp, the AxioVision 4.7.2 software, a 63X objective and a CoolSNAP™ HQ2 high resolution CCD camera (Photometrics); images were captured every 20 s for 15 min; the moving ookinetes were finally analysed using the

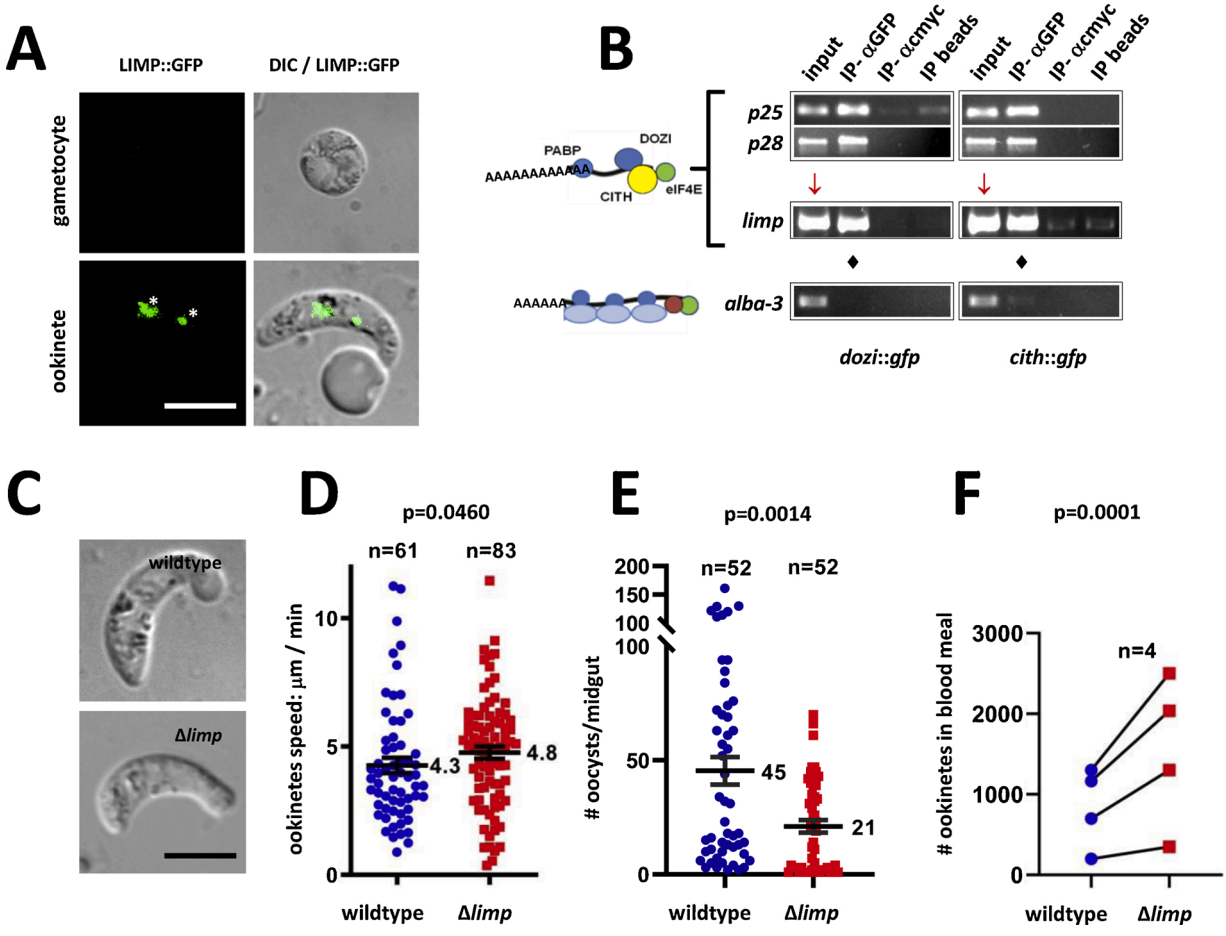

Fig. 1. LIMP is maternally provided to the ookinete where it ensures optimal mosquito infection. (A) Live fluorescence of *limp:gfp* blood stage gametocyte shows the absence of LIMP::GFP expression, while imaging of blood meal-retrieved ookinetes highlights LIMP::GFP localisation to discrete foci (*) at and near the crystalloid. Scale bar = 5 µm. (B) RT-PCR analyses of DOZI::GFP and CITH::GFP RNA immunoprecipitation (RIP) eluates demonstrate that *limp* co-precipitates with both translational repressors. Input: total gametocyte mRNA; IP-αGFP: IP with anti-GFP antibody; IP-αcmyc: IP with anti-c-myc antibody; IP beads: no antibody used for IP. (C) Representative images of wildtype and Δ*limp* ookinetes isolated from mosquito midguts. Scale bar = 5 µm. (D) Quantification of parasite motilities of wildtype and Δ*limp* ookinetes. Mann-Whitney test; means with SEM are indicated. 3 independent ookinete cultures.(E) Oocyst numbers from wildtype and Δ*limp*-infected mosquitoes. Mann-Whitney test; means with SEM are indicated. 2 independent membrane feeding assays. Midguts with no parasites were removed from the graph. Mean prevalence of infection was 74 % for both groups (Chi-square test, p = 0.8877). (F) Number of wildtype and Δ*limp* ookinetes retained in the mosquito blood meal bolus 24 h after infection. Ratio paired *t* test is indicated. 4 independent membrane feeding assays. Lines connect data points from the same replicate experiment.

manual tracking plug-in of ImageJ [14] revealing a speed of just below 5 μm × min^{-1} for mutant and wildtype parasites (Fig. 1D). Knock-out ookinetes appeared to show a slight increase in gliding speed. However, this increase is only marginal ($p = 0.0460$) and we believe it does not bear any biological relevance. We next tested the ability of *in vitro*-cultured ookinetes to infect the mosquito vector in a controlled standard membrane feeding assay (SMFA). To this end, wildtype and mutant ookinetes were first purified, then mixed with 500 μl naïve mouse blood and finally offered to female *Anopheles stephensi* mosquitoes for 30 min in two independent feeding experiments (either offering 1.6 or 6 million ookinetes at a concentration of 3200 or 12,000 ookinetes × μl^{-1}). On day 10 post-infection, oocyst numbers were quantified following mercurochrome staining. The gene deletion mutant reproduced the 50 % reduction in oocyst numbers observed in our previous publication using direct mosquito feeds on infected mice [2] (Fig. 1E). Prevalence of infection was as follows: replicate 1–58% wildtype *vs* 68 % knock-out (Chi-square test, $p = 0.4272$) – and replicate 2–89% wildtype *vs* 79 % knock-out (Chi-square test, $p = 0.2898$). Mean prevalence of infection calculated by combining both replicates was 74 % for both wildtype and knock-out parasites (Chi-square test, $p = 0.8877$). By bypassing the *in vivo* differentiation of ookinetes, our results show that the lower number of oocysts produced by knock-out parasites cannot be attributed to an *in vivo* defect in zygote-to-ookinete transformation. To characterize the knock-out's failure to produce high oocyst numbers in more detail, we finally quantified ookinetes in the blood meal after 24 h of four independent SMFA. Therefore, midguts from infected mosquitoes were collected in RPMI and the blood meal isolated; the number of retained ookinetes was quantified via haemocytometer. We found the reduction in oocyst numbers to correlate with an accumulation of ookinetes in the mosquito blood meal bolus. On average, we observed an 82% increase in retained knock-out ookinetes when compared to the wildtype controls (Table 1 and Fig. 1F), indicating a role for LIMP in attachment/-traversal/invasion of the mosquito midgut by the ookinete. Given the observed 50 % reduction in knock-out oocyst numbers, one could expect to find twice as many ookinetes retained in the blood meal bolus (*i.e.*, 200 % of wildtype numbers, or a 100 % increase). Instead, we observe, on average, an 82 % increase from wildtype in the number of retained ookinetes. This is slightly lower than the predicted 100 % increase. However, it is conceivable that some stochastic events might have resulted in an underestimation of retained ookinetes (e.g., loss of ookinetes during sample processing or partial excretion of the blood bolus prior to gut collection). Therefore, it is plausible to anticipate that, with further replication of this experiment, the average percent increase would progressively get closer to the theoretical value of 100 %.In summary, our data show that LIMP protein is produced from translationally repressed, maternally provided mRNA in the ookinete where it helps to optimize malaria parasite infection of the mosquito; *limp*-depleted zygotes develop into motile ookinetes with apparent normal morphology, and yet display a defect in escaping the mosquito blood meal and reaching the midgut basal lamina where they can transform into the sporozoite-producing oocyst stage and continue the life cycle. The accumulation of Δ*limp* mutants in the midgut lumen is reproducible and strong, but not absolute, allowing the establishment of oocysts in a laboratory environment where *P. berghei* typically can produce hundreds of oocysts in an infected mosquito. In the field on the other hand, absence of the conserved *P. falciparum* LIMP protein might well cause a complete transmission blockade of human malaria parasites. The much smaller number of *P. falciparum* gametocytes present in malaria-positive individuals – 450 rather than 10,000 found in the laboratory for the rodent model *P. berghei* – translates into field mosquitoes typically hosting, on average, around 6 ookinetes, and just 2 oocysts per infected mosquito; in addition, only $^1/_3$ of mosquitoes actually do become infected [15]. The gametocytemia numbers under laboratory conditions can reach 10 % of the total parasite population in the rodent model; it is thus that we observe large numbers of oocysts produced in wildtype conditions. The absence of LIMP causes a 50 % reduction in oocyst numbers. In the context of the high baseline infection this is not enough to block the life cycle; this occurs ultimately during salivary gland and liver cell infection by the sporozoite [2]. The accumulation of morphologically normal ookinetes in the mosquito blood meal is also documented for mutants depleted of the perforin-like proteins PPLP3/MAOP, PPLP4 and PPLP5 [16–19], or the cell-traversal-protein-for-ookinetes-and-sporozoites (CelTOS) [20,21]; here, the block in *P. berghei* transmission is almost absolute. Ookinete traversal of the mosquito midgut epithelium is not completely understood and it has not been proven unequivocally whether the parasite predominantly penetrates the cells of the epithelial tissue of the midgut – perhaps with the help of pore-forming PPLPs and CelTOS – and/or moves between these cells in order to reach the basal lamina of the distal side of the midgut where they establish oocysts that go on to produce sporozoites [22]. The midgut invasion defect of Δ*limp* parasites could result from a defect in adhesion to the peritrophic matrix, the luminal side of the midgut epithelium or a failure to traverse the epithelial cell layer. These scenarios would be consistent with our previous findings for the sporozoite, where both adhesion and traversal processes rely on LIMP [2]. Perhaps this dual role for LIMP could be used to interfere with both malaria parasite transmission stages: ookinete and sporozoite.

Table 1
Data summary for ookinete retention experiments. Number of retained ookinetes is plotted in Fig. 1F.

Experiment #	Total # ookinetes used (x10^6)	Ookinetes/μL of blood at feed (x10^3)	# retained ookinetes		% increase from wildtype
			wildtype	**Δ*limp***	
1	1.6	3.2	1163	2038	75
2	6.0	12.0	1300	2500	92
3	4.0	8.0	200	350	75
4	2.5	5.0	700	1300	86
average	***3.525***	***7.05***	***841***	1547	***82***

Declarations of Competing Interest

None.

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