The Peptidoglycan Recognition Proteins PGRPLA and PGRPLB Regulate *Anopheles* Immunity to Bacteria and Affect Infection by *Plasmodium*

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Abstract

Peptidoglycan recognition proteins (PGRPs) form a family of immune regulators that is conserved from insects to mammals. In the malaria vector mosquito *Anopheles coluzzii*, the peptidoglycan receptor PGRPLC activates the immune-deficiency (Imd) pathway limiting both the microbiota load and *Plasmodium* infection. Here, we carried out an RNA interference screen to examine the role of all 7 *Anopheles* PGRPs in infections with *Plasmodium berghei* and *P. falciparum*. We show that, in addition to PGRPLC, PGRPLA and PGRPS2/PGRPS3 also participate in antiparasitic defenses, and that PGRPLB promotes mosquito permissiveness to *P. falciparum*. We also demonstrate that following a mosquito blood feeding, which promotes growth of the gut microbiota, PGRPLA and PGRPLB positively and negatively regulate the activation of the Imd pathway, respectively. Our data demonstrate that PGRPs are important regulators of the mosquito epithelial immunity and vector competence.

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Introduction

In sub-Saharan Africa, malaria parasites are mainly transmitted by 2 sibling species of mosquitoes, *Anopheles gambiae* and *Anopheles coluzzii*. Both species mostly bite humans and are permissive to *Plasmodium*, two characteristics that make them very efficient malaria vectors. A better knowledge on the interactions between parasites and mosquitoes is needed to identify targets for transmission-blocking strategies. After ingestion during blood feeding, *Plasmodium* parasites undergo sexual reproduction inside the midgut lumen of a female *Anopheles* mosquito and differentiate into motile ookinetes. Upon traversing the midgut epithelium, the ookinetes transform into oocysts, where parasites multiply and differentiate into sporozoites over a period of 1–2 weeks. Oocysts rupture and release thousands of sporozoites that invade the salivary glands and are transmitted to humans during subsequent bites. The ookinete-to-oocyst transition is the main bottleneck of the *Plasmodium* passage through the

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mosquito, largely due to mosquito immune reactions that drastically reduce the parasite numbers. These reactions include the NF-κB immune-deficiency (Imd) pathway that kills parasites in the midgut lumen and epithelium through an as yet uncharacterized mechanism [1, 2] and a complement-like pathway that eliminates ookinetes soon after crossing the epithelium and before transforming into oocysts [3].

In *Drosophila,* the Imd pathway is elicited by bacterial peptidoglycan via the peptidoglycan recognition protein (PGRP) LC [4, 5]. This pathway is highly regulated in a tissue-dependent fashion. In the gut, it is regulated both positively by the nonpeptidoglycan-binding protein PGRPLA and negatively notably by the amidases PGRPLB and PGRPSCs that cleave peptidoglycan into nonimmunogenic fragments [6–8]. PGRPLC and PGRPLA encode transmembrane proteins while PGRPLB and PGRPSCs are secreted [7, 9, 10]. In *Anopheles*, the Imd pathway is induced by the microbiota growth that follows blood feeding in a PGRPLC-dependent fashion [1]. Knocking down *PGRPLC* by RNA interference (RNAi) reduces antimicrobial gene induction and increases mosquito permissiveness to *Plasmodium* [1]. *Anopheles* PGRPLC and PGRPLA, but also PGRPLB, are all predicted transmembrane proteins.

The *Anopheles* genome encompasses 6 additional PGRP genes [11], encoding the predicted amidases PGRPLB, PGRPS2, and PGRPS3, and the noncatalytic PGRPLA, PGRPS1, and PGRPLD. The PGRPLA gene is reported to encode 2 isoforms referred to as PGRPLA1 and PGRPLA2 [11], which share an intracellular N terminus but differ in their C terminus that encompasses the PGRP domain [11]. Here, we investigated the role of PGRPs in regulating malaria infection in *A. coluzzii* mosquitoes (formerly known as *A. gambiae* M form). We identify PGRPLA and PGRPS2/S3 as new *Plasmodium* antagonists and reveal that PGRPLB enhances mosquito tolerance to *Plasmodium* infection. We also show that PGRPLA1 and PGRPLB positively and negatively regulate the *Anopheles* Imd pathway, respectively, similarly to their *Drosophila* orthologs.

Materials and Methods

Ethics Statement
Experiments performed in the UK were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Mosquito infections with *Plasmodium berghei* by blood feeding on parasitized mice were approved by the Imperial College Ethical Review Committee and carried out under the UK Home Office License PPL70/6347. All the procedures were of mild-to-moderate severity, and protocols were designed to minimize the numbers of animals used. Opportunities for reduction, refinement, and replacement of animal procedures were constantly reviewed and considered.

Experiments performed in Cameroon, including volunteer recruitment and mosquito maintenance, were carried out following protocols approved by the WHO and the Cameroon National Ethics Committee (agreement 039/CNE/MP/06). Screening of human volunteers was carried out on a voluntary basis after all participants and families were given extensive information on the program. Written informed consent was taken from each volunteer and/or their legal guardian prior to enrolment.

Mosquito Rearing
The Ngousso colony of *A. coluzzii* (formerly referred to as *A. gambiae* M form) was reared at 27°C and 80% humidity in a 12-h/12-h dark/light cycle. *Plasmodium falciparum* experiments in Cameroon were performed on a colony maintained on rabbit blood and 10% glucose solution. All other experiments were performed on a colony maintained on mouse and human blood and 10% fructose solution.

Double-Stranded RNA Preparation and Gene Silencing
PCR amplification of 120- to 600-bp-long regions of the PGRP genes was performed using T7-tagged primers (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000452797) and Ngousso mosquito cDNA or the control bacterial gene encoding β-galactosidase *(LacZ)* as templates. After purification with the QIAquick PCR Purification Kit (Qiagen), PCR products were used as templates for double-stranded RNA (dsRNA) synthesis using the T7 MEGAscript kit (Ambion). Remaining DNA was degraded with 1 μl TURBO DNase (Ambion) for 15 min at 37°C. dsRNAs were purified using the RNeasy Mini Kit (Qiagen). One- to 2-day-old mosquitoes were injected with 69 nL of 3 mg·mL^{-1} (Fig. 1–3, online suppl. Fig. S1–S3) or 6 mg·mL^{-1} (Fig. 5, online suppl. Fig. S4) dsRNA solution in the thorax using a Nano-injector (Drummond). For *PGRPS2/S3* RNAi, dsRNA targeting each gene was designed separately, and both dsRNA solutions were mixed prior to injection. For regular *Plasmodium* infections, an infectious blood meal was offered to mosquitoes 4 days after injection. For experiments involving antibiotic treatment, dsRNAs were injected 24 h after the start of antibiotic feeding, and *Plasmodium* infections were performed 48 h after injection. For experiments involving *Enterobacter cloacae* oral infections, dsRNAs were injected into freshly emerged mosquitoes 48 h before bacterial oral infections, and *Plasmodium* infections were carried out 48 h after bacterial infections. Quantification of the efficiency of PGRPLA dsRNA targeting was performed on midguts 24 h after injection and 24 h after a blood feed offered to the mosquitoes 30 h after injection.

Antibiotic Treatment
Freshly emerged adult mosquitoes were fed on sterile water supplemented with 25 μg·mL^{-1} gentamycin (Sigma), offered on a cotton pad that was changed every 12 h for 3 days [12]. Gentamycin was diluted in water rather than in sucrose to prevent its retention in the crop, the mosquito sugar storage organ. Sugar cubes were provided as an alternative source of sugar. Treatment efficacy was checked by plating homogenized mosquitoes on LB-agar

plates. No colony grew from the gentamycin-treated mosquitoes while many colonies were observed in the untreated control sample.

E. cloacae *Oral Infections*

E. cloacae cultures were grown overnight at 37°C in LB medium, washed twice in sterile PBS, and resuspended in sterile sucrose. Bacterium-containing sucrose solutions were offered to recently emerged mosquitoes on cotton pads for 48 h.

P. berghei *Infections*

GFP-expressing *P. berghei* parasites [13] were injected intramuscularly into Balb/C or CD1 mice. Two days later, parasitemia was assessed by Giemsa staining (typically 4–6%), and the presence of gametocytes was verified. Mosquitoes were allowed to feed for 10–15 min on anesthetized infected mice and maintained at 21°C. Unfed mosquitoes were discarded 48 h after the blood meal. Midguts were dissected 7–8 days after the blood meal, fixed in 4% paraformaldehyde for 45 min, washed 3 times in PBS for 20 min, and mounted on microscope slides using Vectashield medium (Vector Laboratories). Fluorescent parasites were counted using a fluorescence microscope. Experiments were included in the analysis if at least 1 mosquito was alive in each RNAi condition at the time of dissection (n = 9–36 per condition, average 22).

P. falciparum *Infections*

Infections with *P. falciparum* field isolates were carried out during the rainy seasons from May to June in 2007 and 2008. School children volunteers were enrolled at the village of Mfou, near Yaoundé, Cameroon. Gametocyte carriers were identified by Giemsa staining of thick blood smears. Venous blood was drawn from antecubital fossa by venipuncture, and the serum was replaced by malaria-naïve AB serum. Blood was maintained at 37°C in a membrane-feeding device covered with Parafilm M (Bemis) as a skin substitute, and mosquitoes were allowed to feed on infected blood for 30 min. Blood-fed mosquitoes were separated from unfed mosquitoes 5–6 h later and subsequently kept at 27 ± 2°C. Mosquitoes were dissected 8 days later and their midguts were stained with a solution of 0.4% Mercurochrome. Red-stained oocysts were counted by light microscopy. Experiments were included in the analysis if at least 15 mosquitoes were alive in each RNAi condition at the time of dissection (n = 17–60 per condition, average 34).

Gene Expression

Gene expression was examined in the midgut, the region of the gut located between the proventriculus (included) and the malpighian tubules (excluded), and the fat body, the external part of the abdomen including the cuticle and the underlying tissue. Tissues were homogenized in TRIzol (Invitrogen) using a Precellys 24 homogenizer (Bertin) with 0.5-mm-wide glass beads (Bertin). RNA was extracted in chloroform and precipitated with isopropanol, washed with 70% ethanol, and resuspended in molecular-biology grade water. cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara) and 700 ng RNA as a template. qPCR was carried out on an ABI 7700 real-time PCR machine (Applied Biosystems), using SYBR Premix Ex-Taq-Tli RNase H Plus (Takara). Primer sequences are indicated in online supplementary Table S2. The ribosomal transcript *S7* was used as normalization control.

Table 1. VectorBase accession numbers of the genes and transcripts mentioned in the paper

Gene or transcript	Accession No.
PGRPLA	AGAP005205
PGRPLA1	AGAP005205-RC = RF
PGRPLA2	AGAP005205-RA and RD
PGRPLB	AGAP001212
PGRPLC	AGAP005203
PGRPLD	AGAP005552
PGRPS1	AGAP000536
PGRPS2	AGAP006343
PGRPS3	AGAP006342
S7	AGAP010592
CEC1	AGAP000693

Statistical Analyses

Statistical analyses based on generalized linear mixed models (GLMM) were performed in R (version 3.2.3). Prevalence data were examined by ANOVA χ^2 test on a logistic regression (glmer). Intensity data are based on oocyst counts of all mosquitoes, including noninfected ones; they were analyzed by Wald Z test on a zero-inflated negative binomial regression (glmmADMB). qPCR data were analyzed by ANOVA χ^2 test on a common linear regression (lmer). GLMM analyses extract from the data the contribution of a fixed component (the covariates in question, here RNAi challenge) and a random component (here experiments). The odds ratios shown in the figures were calculated as the exponential of the fixed effect estimates. Sample size was determined according to standard protocols (oocyst counts and gene expression). Mosquitoes were excluded if they died during the experiment or did not blood feed (oocyst counts and expression data on blood-fed samples).

Accession Numbers

The accession numbers of the genes and transcript mentioned in this paper are reported in Table 1.

Results

PGRPs Influence Mosquito Permissiveness to Plasmodium

We investigated the roles of PGRPs in mosquito permissiveness to the rodent parasite *P. berghei* through an RNAi screen. We injected mosquitoes with dsRNA targeting each PGRP gene and challenged them with *P. berghei* 4 days later via blood feeding on infected mice. One week later, we monitored infection by counting the number of oocysts in each individual midgut and assessing the proportion of infected mosquitoes, referred to as prevalence, and the number of oocysts per midgut, referred to
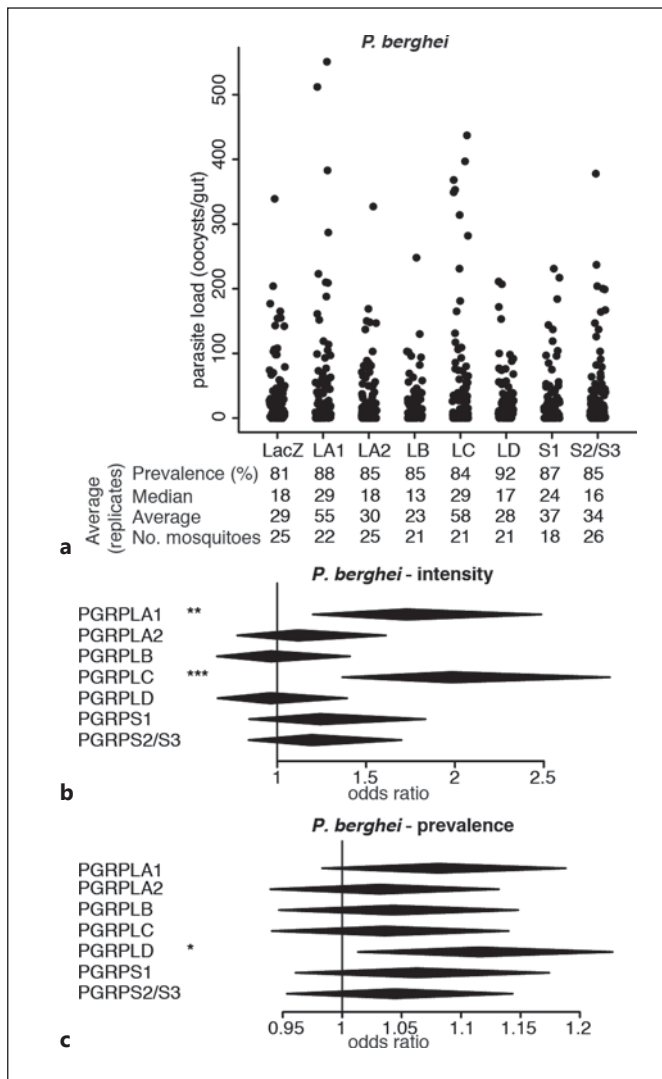
Fig. 1. Effect of RNAi on PGRP genes on *P. berghei* infections. **a** Strip-chart representation of the oocyst load of *P. berghei* upon RNAi on the genes encoding PGRPs. RNAi targeting the bacterial gene *LacZ* was used as a control. Each dot shows the oocyst load in an individual gut, and data show pools of 4 independent replicates. **b**, **c** Forest plots showing the effect of knocking down *Anopheles* PGRP genes on the intensity (**b**) and prevalence (**c**) of *P. berghei* infection, resulting from the same experiments as data shown in **a**. Statistical data show the result of a Wald *Z* test following zero-inflated negative binomial regression model fitting. The center and horizontal diagonal of each diamond show the odds ratio of the fixed effect and its confidence interval, respectively. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table S1). The prevalence of infection was not affected when silencing *PGRPLC* or *PGRPLA1* but increased when targeting *PGRPLD* (Fig. 1a, c).

Next, we screened for the effects of PGRPs on human malaria parasites *P. falciparum.* We chose to infect mosquitoes with the blood of gametocyte carriers in Cameroon as this experimental setup allows us to assess infections from a naturally diverse set of parasites having developed in vivo. We injected mosquitoes with dsRNA and offered them an infectious blood sample 4 days later. In addition to the strong negative impact of *PGRPLC* on infection intensity reported previously [1], we observed that silencing *PGRPLA2*, but not *PGRPLA1*, also increased mosquito permissiveness to *P. falciparum* (Fig. 2a, b; online suppl. Table S2). Silencing *PGRPS2* and *PGRPS3*, which we could only target together due to their high sequence similarity, increased *P. falciparum* infection intensity and prevalence, while silencing *PGRPLB* significantly decreased infection prevalence (Fig. 2; online suppl. Table S2).

Together, these data indicate that beside PGRPLC, PGRPLA, PGRPLD, and PGRPS2/PGRPS3 also participate in the resistance to *Plasmodium*, while PGRPLB may be implicated in the tolerance to parasites. The antiparasitic function of PGRPLC is linked to the induction of the Imd pathway due to the microbial growth following a blood meal [1]. We then focused on PGRPLA and PGRPLB as their *Drosophila* orthologs are known to positively and negatively regulate the Imd pathway, respectively [7, 8]. We investigated whether their effect on parasite infections was mediated by similar regulatory roles on the *Anopheles* Imd pathway.

PGRPLA Is a Positive Regulator of the Imd Pathway in the Midgut

First, we investigated the role of PGRPLA in the induction of antimicrobial peptides in the midgut, where its *Drosophila* ortholog positively affects immune responses. We followed the expression of *Cecropin1 (CEC1)*, an antibacterial gene induced by the Imd pathway, in the midguts of sugar- and blood-fed mosquitoes 3 and 4 days after dsRNA injection, respectively, in order to follow the blood meal-induced Imd response [1]. We found that *CEC1* was induced 4.9-fold in the midgut of blood-fed versus sugar-fed mosquitoes. Simultaneous knockdown of both *PGRPLA1* and *PGRPLA2* reduced the blood meal-induced *CEC1* expression (Fig. 3a). The same effect was observed upon specific *PGRPLA1* knockdown, while knockdown of *PGRPLA2* had no effect on *CEC1* expression (Fig. 3a). These results suggested that PGRPLA1 is a

as intensity. We confirmed the previously reported increase in infection intensity upon *PGRPLC* knockdown [1] and observed that *PGRPLA1* silencing had a similar effect on *P. berghei* infection (Fig. 1a, b; online suppl.

positive regulator of the Imd pathway. When testing the efficiency of knockdown of *PGRPLA1* and *PGRPLA2*, we found that *PGRPLA1* was reduced by 44 and 37% in sugar- and blood-fed mosquitoes, respectively, while *PGRPLA2* was only reduced by 18 and 24%, respectively. In terms of isoform specificity, *PGRPLA2* dsRNA did not consistently affect *PGRPLA1*, while *PGRPLA1* dsRNA slightly decreased *PGRPLA2* expression by 7 and 15% prior to and after blood feeding, respectively. Thus, we cannot rule out that the specific effect found on *PGRPLA1* and not *PGRPLA2* is not due to the difference in efficiency and specificity of these dsRNAs.

VectorBase annotation of PGRPLA suggests that this gene is transcribed as 4 transcripts, 2 of them sharing the exact same sequence encoding for PGRPLA1, while PGRPLA2 would actually correspond to 2 isoforms sharing the same PGRP domain, 1 of which lacks the transmembrane and intracellular domains (Fig. 4a). According to RNA-sequencing data added to VectorBase, a whole additional region is also transcribed in PGRPLA [14–16]. As this region is ortholog to a PGRPLA region in *Anopheles merus* and *Anopheles quadriannulatus* [16], it might be an additional unpredicted exon of the gene. This suggests that the exact sequences of PGRPLA transcripts may need further investigation.

In *Drosophila*, the receptors PGRPLC and PGRLE induce the Imd pathway through a RIP Homotypic Interaction Motif (RHIM), which is also present in *Drosophila* PGRPLA [17]. Alignment of this RHIM to *Anopheles* PGRPLA revealed a high similarity in a sequence of 27 amino acids, in a region shared by both PGRPLA1 and PGRPLA2 (Fig. 4b). These data suggest that both isoforms of *Anopheles* PGRPLA carry a RHIM domain.

We then investigated whether PGRPLA1 promotes resistance to *P. berghei* in a bacterium-dependent fashion. Two days after dsRNA injection, mosquitoes were orally infected with *E. cloacae*, a Gram-negative bacterium previously found in the mosquito microbiota, and infected with *P. berghei* 48 h later. We observed that silencing *PGRPLA1* had a stronger effect on intensity and prevalence of *P. berghei* infection in *E. cloacae*-infected than noninfected mosquitoes, as shown by a significant increase in the *PGRPLA1*-to-*LacZ* odds ratios (Fig. 3b; online suppl. Fig. S3A). To examine whether the PGRPLA1-mediated response was induced by the midgut microbiota, we orally treated mosquitoes with the antibiotic gentamycin and infected them with *P. berghei* 3 days later. We observed that the antibiotic treatment significantly reduced but did not eliminate the effect of PGRPLA1 on *P. berghei* infection intensity, while the effect on prev-
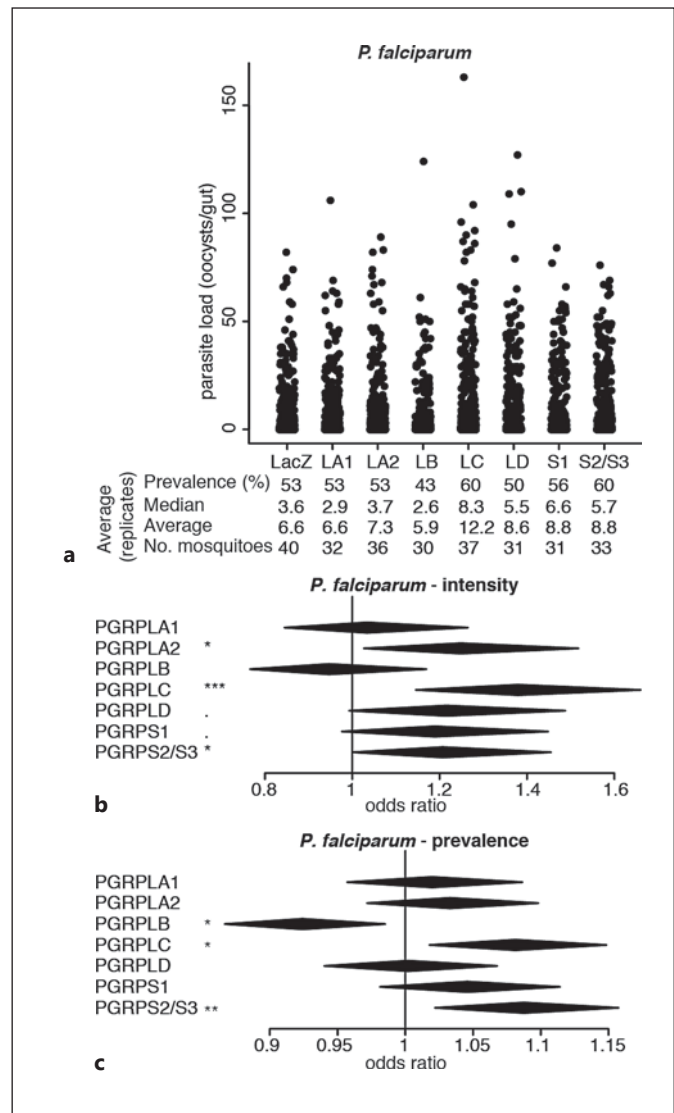

Fig. 2. Effect of RNAi on PGRP genes on *P. falciparum* infections. **a** Strip-chart representation of the oocyst load of *P. falciparum* upon RNAi on the genes encoding PGRPs. RNAi targeting the bacterial gene *LacZ* was used as a control. Each dot shows the oocyst load in an individual gut, and data show pools of 11 independent replicates. **b, c** Forest plots showing the effect of knocking down *Anopheles* PGRP genes on the intensity (**b**) and prevalence (**c**) of *P. falciparum* infection, resulting from the same experiments as data shown in **a**. Statistical data show the result of a Wald *Z* test following zero-inflated negative binomial regression model fitting (**b**) and of an ANOVA following a logistic regression model fitting (**c**). The center and horizontal diagonal of each diamond show the odds ratio of the fixed effect and its confidence interval, respectively. $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

alence was lost (Fig. 3c; online suppl. Fig. S3B). These results suggested that PGRPLA1 protection is partly microbiota independent. This prompted us to investigate whether the PGRP domain of PGRPLA1, as previously reported in *Drosophila* PGRPLA and *Anopheles* PGRPLA2, lacks essential features for peptidoglycan binding [8]. Indeed, sequence alignment revealed that 8/10 amino acids involved in binding peptidoglycan in PGRPLCx are absent from PGRPLA1 (Fig. 4c).

PGRPLB Downregulates Systemic Induction of the Imd Pathway

We also investigated whether PGRPLB is a negative regulator of the Imd pathway, similarly to its *Drosophila* ortholog [6, 7]. According to VectorBase annotations, PGRPLB encodes 2 isoforms, which share the same PGRP domain and differ in their transmembrane domains (Fig. 4a). We silenced *PGRPLB* by targeting a region shared by both predicted transcripts and monitored the expression of *CEC1* 6 h to 6 days after dsRNA injection in the mosquito midgut and fat body to examine local and systemic Imd responses, respectively. We found that *PGRPLB* silencing had no effect on *CEC1* expression in the midgut, but it significantly increased *CEC1* expression in the fat body 6 days later (Fig. 5a, b; online suppl. Fig. S4A). As PGRPLB is highly induced in the gut after blood feeding (Fig. 5c), we studied its role in the induction of the immune response after a blood meal provided 30 h after dsRNA injection. Our results indicate that *CEC1* is induced in the midgut, but not in the fat body, after blood feeding (online suppl. Fig. S4B). PGRPLB silencing did not affect *CEC1* expression in the gut but significantly induced *CEC1* in the fat body 72 h after the blood meal (Fig. 5d, e). These data suggested that, similarly to *Drosophila*, PGRPLB is involved in preventing activation of a systemic immune
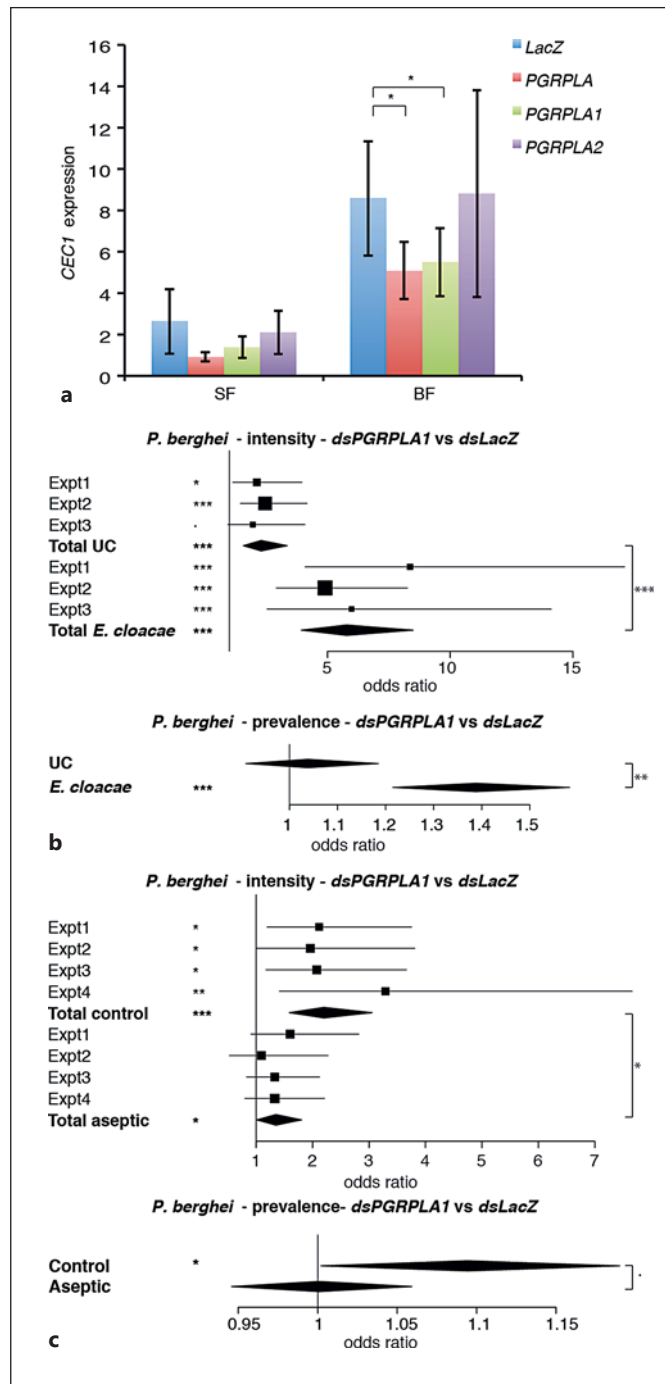

Color version available online

Fig. 3. Effect of *PGRPLA* RNAi on Imd pathway induction and on *P. berghei* infection. **a** *CEC1* expression in midguts of sugar-fed mosquitoes (SF) or 24 h after blood feeding (BF) when targeting the whole PGRPLA gene or only one of its isoforms (*PGRPLA1 or PGRPLA2*). RNAi targeting the bacterial gene *LacZ* was used as a control. **b** Effect of *PGRPLA1* RNAi on *P. berghei* live oocyst intensity (upper panel) and prevalence (lower panel) in unchallenged (UC) or *E. cloacae* orally infected mosquitoes. **c** Effect of *PGRPLA1* RNAi on *P. berghei* live oocyst intensity (upper panel) and prevalence (lower panel) in control or gentamycin-treated (aseptic) mosquitoes. Squares represent the odds ratio of the fixed effect for each gene in each independent replicate, and error bars represent 95% confidence intervals. In each chart, square sizes are proportional to mosquito numbers. Diamonds represent the summary of independent replicates. Statistical data show the result of ANOVA following a common linear regression model fitting (**a**), a Wald Z test following zero-inflated negative binomial regression model fitting (**b**, **c**; intensity data), and ANOVA following a logistic regression model fitting (**b**, **c**; prevalence data). ˙ $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

response. Interestingly, *PGRPLB* expression was efficiently knocked down in the midgut but not in the fat body (online suppl. Fig. S4C, S4D). This is probably due to its very low expression in the fat body, which is 41- and 15-fold lower than in the midgut of sugar- and blood-fed mosquitoes, respectively (online suppl. Fig. S4C, S4D). These data suggested that PGRPLB is required in the gut and/or potentially other tissues, but not in the fat body, to prevent the activation of a late systemic response.

Discussion

In this study, we identify 2 new *A. coluzzii* PGRPs involved in the vector-parasite interactions. We reveal that PGRPLA has an antiparasitic effect and positively regulates the Imd pathway, similar to what was shown previously for PGRPLC [1]. Although our data suggest that PGRPLA1 and PGRPLA2 affect negatively *P. berghei* and *P. falciparum*, respectively, previous experiments performed in our laboratory using the G3 mosquito strain
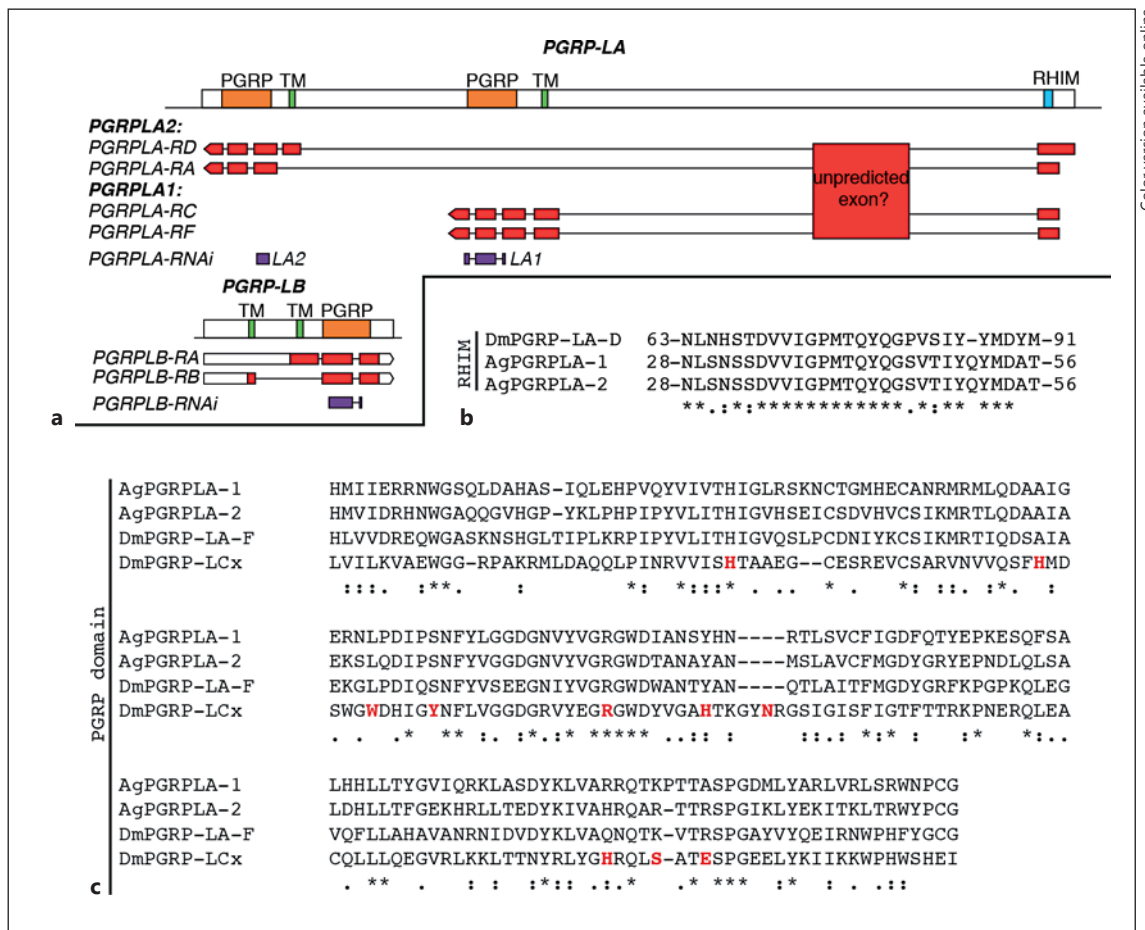

Fig. 4. Map of PGRPLA and PGRPLB and sequence features of PGRPLA. **a** Map of the PGRPLA and PGRPLB gene loci. Predicted PGRP domains are shown in orange, predicted transmembrane domains (TM) in green and the predicted RHIM in turquoise (see online version for colors). The map of each VectorBase-predicted transcript is shown below each gene, including untranslated regions in white and coding DNA sequences in red. According to RNA-Seq data [14–16], a transcribed region that may participate in the sequence of PGRPLA transcripts is also indicated as "unpredicted exon." dsRNA-targeted regions are shown in purple. **b**, **c** Sequence alignments of PGRPLA RHIM (**b**) and PGRP domain (**c**) with their homologous sequences in *Drosophila* PGRPLA (**b**, **c**) and PGRPLCx (**c**). In *Drosophila*, there is no single isoform encompassing both an RHIM and a PGRP domain, hence the sequence of PGRPLA$_D$ isoform is shown for the RHIM and the sequence of PGRPLA$_F$ isoform for the PGRP domain. **c** Amino acids shown in red are in direct contact with the peptidoglycan monomer "TCT" (tracheal cytotoxin) [18]. Sequence alignments were performed with ClustalW [19] and manually curated. *, positions with fully conserved residues; :, positions with conservation of "strong" groups (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW); ·, positions with conservation of "weaker" groups (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY).

Fig. 5. Effect of *PGRPLB* RNAi on the local and systemic activation of the Imd pathway. **a**, **b**, **d**, **e** *CEC1* expression in the midguts (**a**, **d**) and fat bodies (**b**, **e**) of mosquitoes after injection of *PGRPLB* or *LacZ* control dsRNA in sugar-fed (SF; **a**, **b**) or blood-fed (BF; **d**, **e**) conditions. **c** *PGRPLB* expression in sugar-fed mosquitoes or 24 h after blood feeding. Statistical data show the results of ANOVA following a common linear regression model fitting. ˙ $p < 0.1$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
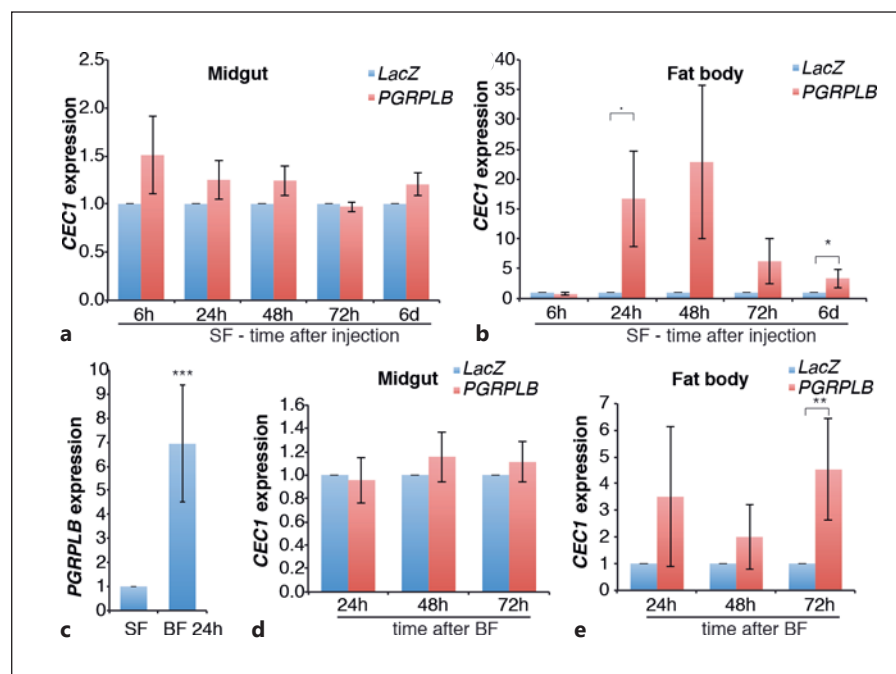
infected with *P. berghei* revealed an antiparasitic function for PGRPLA2 [20]. As both isoforms share the exons encoding the intracellular domain, including the RHIM motif, and are predicted not to bind peptidoglycan, we hypothesize that their differential effects on Imd pathway activation and anti-*Plasmodium* responses depend on their expression levels rather than functional differences. Indeed, *P. falciparum* and *P. berghei* infection of the mosquito midgut occur with different timing and at different temperatures. In that respect, published data suggest that *PGRPLA1* and *PGRPLA2* expression differs between mosquito strains and *Plasmodium* infection stages [21–24], and we found that *PGRPLA2* expression is 14 and 10 times lower than that of *PGRPLA1* in the midguts of sugar- and blood-fed mosquitoes of our colony, respectively. Moreover, as mentioned in the results, the observed phenotypic difference may arise from *PGRPLA1* dsRNA having a higher silencing efficiency and being less isoform specific than *PGRPLA2* dsRNA. However, we cannot rule out that the differences found between PGRPLA1 and PGRPLA2 are not biologically relevant, they may notably be linked to the absence of a transmembrane domain in one of the predicted PGRPLA2 isoforms.

Oral bacterial challenge and antibiotic treatments revealed that the effect of PGRPLA1 is only partly bacteria dependent. Therefore, the effect of PGRPLA1 on *Plasmodium* in aseptic mosquitoes may be due to basal induction

of the Imd pathway by PGRPLA1 dimerization. The RHIM domain of PGRPLA1 is highly conserved with that of *Drosophila* PGRPLA, which is shown to induce the Imd pathway [8]. The observed increase in the antiparasitic effect of *PGRPLA1* silencing upon bacterial challenge suggests that PGRPLA1 also participates in a bacterium-mediated induction of the Imd pathway, perhaps through heterodimerization with PGRPLC. Induction of the Imd pathway by heterodimerization has been reported in *Drosophila* between PGRPLCx, which binds peptidoglycan, and PGRPLCa, which does not [25].

Our results also identify PGRPLB as a negative regulator of the Imd pathway, promoting tolerance to *P. falciparum*. More specifically, our data suggest that PGRPLB is needed in the gut and perhaps other tissues, but not in the fat body, to prevent induction of a systemic immune response. This is in contrast to what is known in *Drosophila*, where PGRPLB is required both in the gut and in the fat body to control systemic responses [7]. This difference between *Anopheles* and *Drosophila* PGRPLB may relate to the fact that the former is a predicted transmembrane protein, potentially scavenging peptidoglycan fragments as they traverse the gut epithelium, while the latter is a secreted protein [7], likely scavenging peptidoglycan monomers in the gut lumen and potentially in the hemolymph. Indeed, peptidoglycan fragments are known to diffuse through the gut and other epithelia into the hemolymph, where they re-

motely induce a systemic response [26]. *Anopheles* PGRPLB expression is enriched in the midgut and the salivary glands [27], 2 organs harboring microbial communities, further implicating this protein in epithelial tolerance to microbiota. Alternatively, the effect that we report on the systemic response may also happen via an antibacterial effect of PGRPLB, which may prevent bacteria from entering the mosquito hemolymph. In the tsetse fly, PGRPLB is shown to be a negative regulator of the Imd pathway, participating in tolerance to symbionts [28], and also to have a direct antiparasitic effect against trypanosomes [29]. Our results do not point to such an antiparasitic effect of *Anopheles* PGRPLB against *Plasmodium*. In our experimental setting, the role of PGRPLB on *Plasmodium* infection is probably reduced due to the fact that *PGRPLB* is itself a target of the Imd pathway [30]; hence, its silencing may induce Imd pathway activation leading to its overexpression. A deletion of the gene is required to determine the full effect of PGRPLB in *Plasmodium* infections.

Taken together, our data demonstrate that PGRPs are key regulators of the mosquito immune system and permissiveness to *Plasmodium*. We reveal that PGRPLA is a positive regulator of the Imd pathway and that its antiparasitic activity is partly bacteria dependent, while PGRPLB negatively regulates the same pathway, participating in the tolerance to the gut microbiota.

Author Contributions

M.G., F.T., F.H.R., and G.K.C. designed the experiments. M.G., F.T. and F.H.R. performed the experiments. A.C. and I.M. organized gametocyte carrier recruitment in Cameroon and provided infrastructure and intellectual input for work on *P. falciparum*. M.G., F.T., and G.K.C wrote this paper. All the authors commented on the paper and approved the final version.

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