Supplemental Information

Mosquito Midgut Prostaglandin Release

Establishes Systemic Immune Priming

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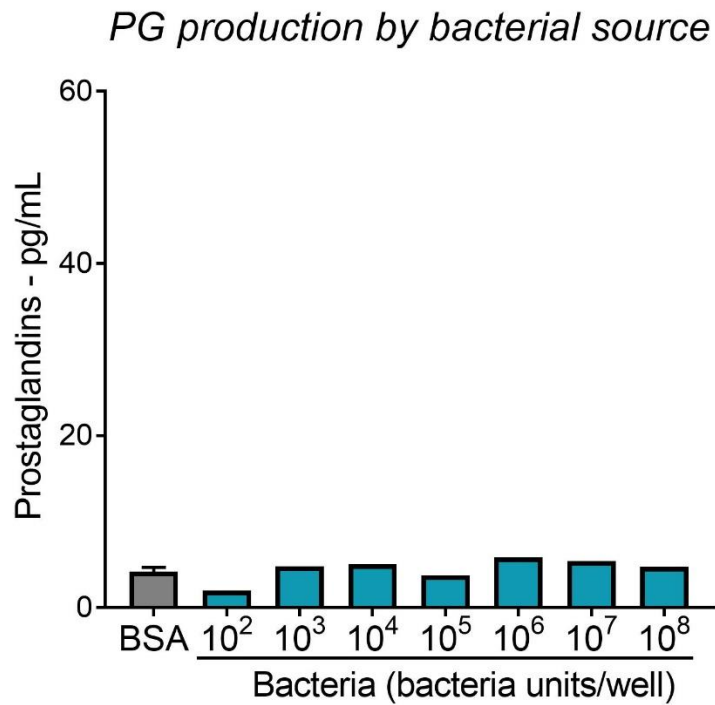
PG production by bacterial source

Figure S1. PG production by bacterial source, related to Figure 1. Prostaglandin levels were measured by ELISA in a BSA suspension containing crescent amounts of bacteria, ranging from 100 to 100.000.000 bacteria/ well. Levels were calculated in pg/mL and compared with BSA solution without bacteria.
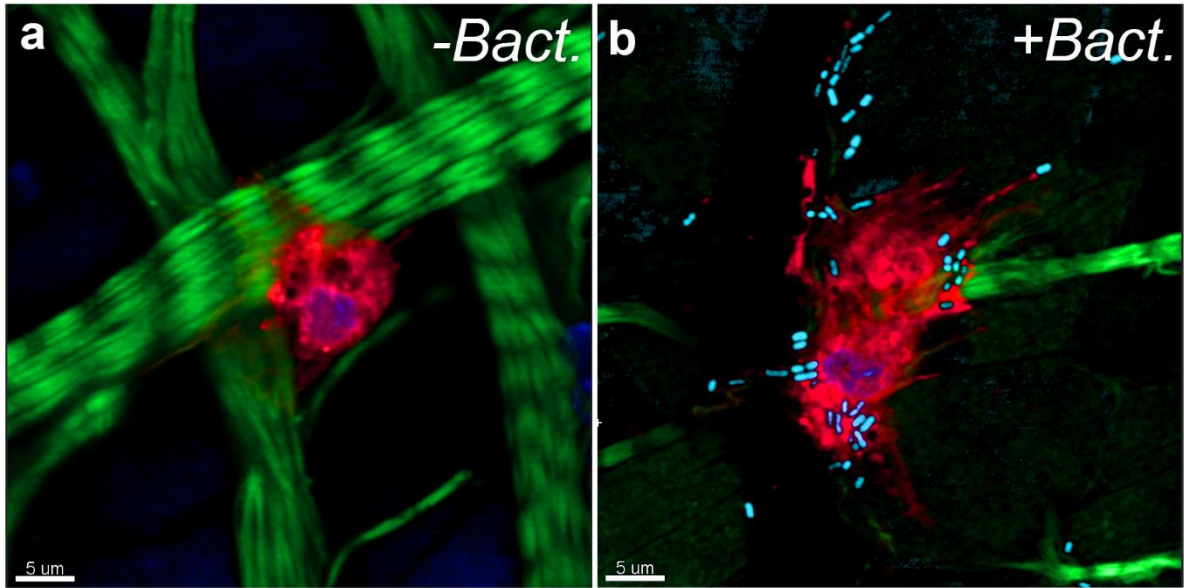
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Figure S2. Intracellular bacteria can be observed in hemocytes associated with midgut basal surface, related to Figure 1. (a) Hemocyte attached to the midgut basal surface in control mosquitoes fed with BSA 6 hours post feeding. **(b)** Hemocyte attached to the midgut basal surface in mosquitoes fed with bacteria 6 hours post feeding. Bacteria is observed inside and in close contact with hemocytes attached to basal lamina. Actin, green; Hemocytes, red; Nuclei, blue; Bacteria, cyan. Scale bar: 5μm.
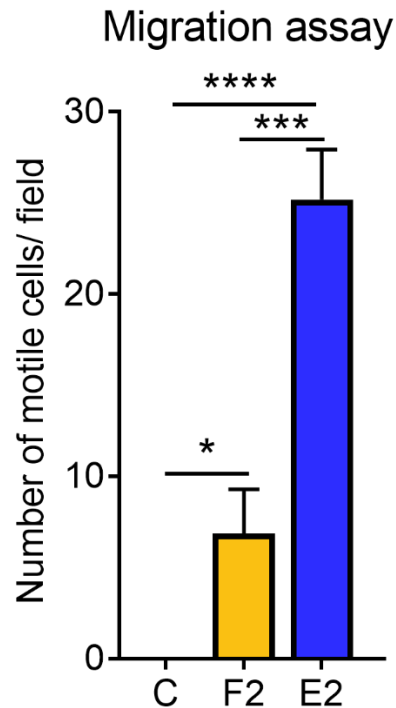
Figure S3. PGF2 chemotaxis, related to Figure 2. Quantification of motile Sua 5.1 *Anopheles* cells per field after exposure to PBS (Ctr) and 7 µg of PGF2 and PGE2 for 16 hours. Error bars represent mean ± SEM. ANOVA Dunn's multiple comparison test, *P≤0.05, ***P≤0.001, ****P≤0.0001. Quantification of migrating cells was performed in at least 2 independent experiments. In each experiment 10 fields per treatment were randomly chosen and counted. Detailed information of biological replicates and exact p values are listed in supplementary table 6 and 7.
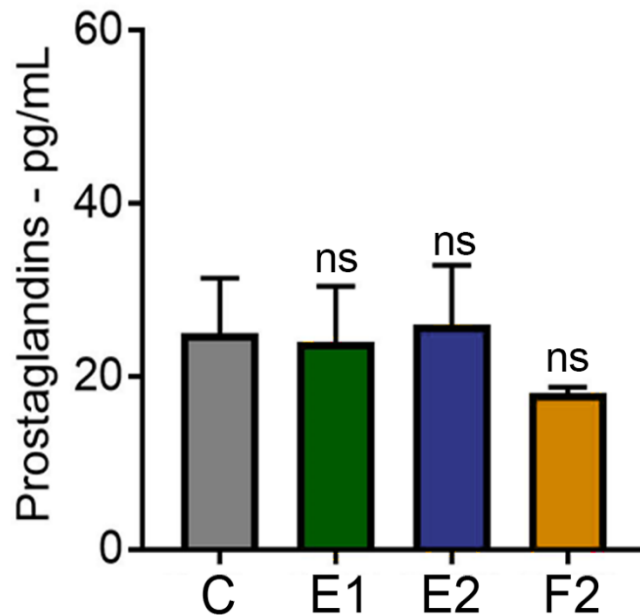
Figure S4. PG measurement on the hemolymph of PG treated mosquitoes 4 days post injection, related to Figure 3. Hemolymph prostaglandin levels four days after prostaglandin systemic injection. Mosquitoes were injected with 500nM of PGE1 (E1), PGE2 (E2) or PGF2 (F2). Error bars represent mean ± SEM. *ANOVA* Dunn's multiple comparison, NS, P>0.05. Each experimental group was compared with the control. The hemolymph of 10 mosquitoes was polled per biological replicate. At least 2 independent experiments were performed. Detailed information of biological replicates and exact p value number are listed in supplementary table 12.

Figure S5. Gene expression of HPX7 and HPX8 in the midgut 3 hours after bacterial feeding, related to Figure 4. (a) HPX7 relative mRNA expression in the midgut 3 hours post feeding (3hpf) with BSA (-Bact) or BSA containing bacteria (+Bact). Error bars in **a** and **b** represent mean ± SEM. Mann-Whitney U test, ***P≤0.001. Fifteen midguts were pooled for each biological replicate. Two independent experiments with at least 2 biological replicates for each treatment. Detailed information of biological replicates and exact p value number are listed in supplementary table 19.

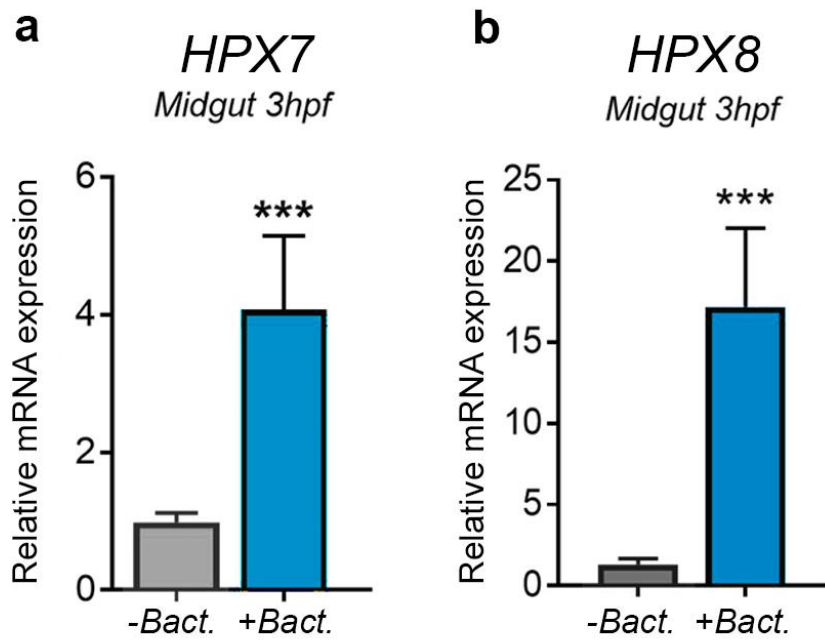**Video S1. Sua 5.1 migration activity in response to PBS. Related to Figure 2.**

Video S2. Sua 5.1 migration activity in response to PGE2 exposure. Related to Figure 2.

Video S3. Hemocyte patrolling activity in control mosquitoes. Related to Figure 2.

Video S4. Hemocyte patrolling activity in bacteria fed mosquitoes. Related to Figure 2.

Supplementary tables

Groups	Exp 1	Exp 2	Exp 3	Comparison	P value
Sugar fed (SF)	20.37 ± 1.53, n=4	7.87 ± 0.424, n=4	5.743 ± 0.6664, n=3	SF Vs C	0.7193
Blood fed (C)	19.8 ± 2.224, n=4	24.3 ± 3.136, n=4	26.93 ± 14.43, n=2	C Vs I	< 0.0001
P.berghei INF (I)	127.3 ± 53.65, n=2	113.7 ± 39.42, n=4	142.7 ± 19.21, n=3	SF Vs I	< 0.0001

Table S1 – Prostaglandin levels in the hemolymph in response to *P.berghei* infection 24 hours post feeding. Related to Figure 1. (SF) sugar fed. (C) Blood fed control. (I) *P.berghei* infected. *ANOVA Tukey's multiple comparison test*. **Each n is a biological replicate of a pool of 10 mosquitoes. Each independent experiment has at least 2 biological replicates (2 pools of 10 mosquitoes).**

Groups	Exp 1	Exp 2	Comparison	P value
Blood fed (C) + bacteria	24.99 ± 3.642, n=3	25.6 ± 1.823, n=3	C +Bact Vs I+Bact	0.0043
P.berghei INF (I) + bacteria	85.94 ± 10.75, n=3	89.54 ± 11.86, n=2		
Blood fed (C) + bacteria	35.22 ± 2.463, n=4	59.28 ± 4.619, n=4	C -Bact Vs I-Bact	0.6943
P.berghei INF (I) - bacteria	59.13 ± 12.24, n=4	42.78 ± 13.42, n=3		

Table S2 – Prostaglandin levels in the hemolymph in response to *P.berghei* infection 24 hours post feeding with (+ Bact) and without (-Bact) the presence of the microbiota. Related to Figure 1. (C) Blood Fed control. (I) *P.berghei* infected. Mann-Whitney U test. **Each n is a biological replicate of a pool of 10 mosquitoes. Each independent experiment has at least 2 biological replicates (2 pools of 10 mosquitoes).**

Treatment	Group	Exp 1	Exp 2	Comparison	P value
- Bact	Midgut	211.5 ± 11.72, n=3	264.3 ± 59.33, n=2	Midgut Control Vs Bacteria	0.0042
	Fat Body	28.71 ± 10.18, n=2	19.79 ± 7.551, n=2		
	Hemolymph	28.37 ± 2.472, n=2	-	Fat Body Control Vs Bacteria	0.5705
+Bact	Midgut	443.7 ± 102.8, n=2	521.7 ± 94.17, n=2		
	Fat Body	18.51 ± 2.601, n=2	22.88 ± 0.000, n=1	Hemolymph Control Vs Bacteria	0.0376
	Hemolymph	60.95 ± 6.018, n=2	-		

Table S3 – Prostaglandin production by different tissues in response to bacterial feeding. Midgut, Fat Body and hemolymph from BSA fed (-Bact) and BSA + Bacteria fed (+Bact). Related to Figure 1. Unpaired t-test. **Each n is a biological replicate of a pool of 10 mosquito's tissues and hemolymph. Each independent experiment has at least 2 biological replicates (2 pools of 10 mosquitoes).**

Tissue	Group	Exp 1	Exp 2	Comparison	P value
Midgut	- Bact	3.553 ± 0.4916, n=3	2.444 ± 2.444, n=2	Midgut control Vs Midgut Bacteria	0.0079
	+ Bact	6.178 ± 0.5114, n=3	5.894 ± 0.3157, n=2		

Table S4 – PGE2 release by midguts in response to bacterial feeding. Related to Figure 1. Midguts from mosquitos fed with BSA (-Bact) or BSA + Bacteria (+Bact). Unpaired t-test. **Each n is a biological replicate of a pool of 10 midguts. Each independent experiment has at least 2 biological replicates (2 pools of 10 midguts).**

Treatment	Exp 1	Exp 2	Exp 3	Comparison	P value
-Bact	7, n=5	9, n=12	9, n=8	-	-
+Bact	20, n=6	17, n=11	17, n=10	Control Vs Bacteria	0.0028

Table S5 – Hemocytes attached to midgut in response to bacterial feeding. Hemocytes were visualized and counted on the midgut surface of mosquitoes fed with BSA (-Bact) or BSA + Bacteria (+Bact). Related to Figure 1. Mann-Whitney U test. **Each n represents an individual mosquito. Each independent experiment has at least 5 biological replicates (individual midguts).**

Treatment	Migration (fields)	Comparison	P value
Control	0 ± 0, n=10	-	-
E2	25.18 ± 2.753, n=11	Control Vs E2	<0.0001

Table S6 – Migration activity of SUA 5.1 after exposure to PGE2. Related to Figure 2. Agarose spot containing PBS (Control) or PGE2 was placed in contact with SUA 5.1 cells. Mann-Whitney U test. **N represents the number of fields counted for each condition.**

Treatment	Migration (fields)	Comparison	P value
Control	0 ± 0, n=15	-	-
F2	6.85 ± 2.832, n=20	Control Vs F2	0.0104

Table S7 – Migration activity of SUA 5.1 cells after exposure to PGF2. Related to Figure 2. Agarose spot containing PBS (Control) or PGF2 was placed in contact with SUA 5.1 cells. Mann-Whitney U test. **N represents the number of fields counted for each condition.**

Treatment	Distance traveled (total number of hemocytes)	Comparison	P value
-Bact	48.69, n=123	-	-
+Bact	66.95, n=156	Control Vs Bacteria	<0.0001

Table S8 – Distance traveled by hemocytes on the first hour of movie. Related to Figure 2. Total number of hemocytes from 4 midguts per treatment, from 4 independent experiments. Mann-Whitney U test. **N represents the total number of hemocytes recorded in 4 independent experiments.**

Treatment	Speed (total number of hemocytes)	Comparison	P value
-Bact	0.4224, n=123	-	-
+Bact	0.6493, n=156	Control Vs Bacteria	<0.0001

Table S9 – Hemocytes speed. Related to Figure 2. Total number of hemocytes from 4 midguts per treatment (-Bact and +Bact), from 4 independent experiments. Mann-Whitney U test. **N represents the total number of hemocytes recorded in 4 independent experiments.**

Treatment	Exp 1	Exp 2	Comparison	P value
Buffer	1.543 ± 0.1871, n=7	1.07 ± 0.1816, n=6	-	-
E1	2.344 ± 0.6015, n=6	2.969 ± 0.3665, n=8	Buffer Vs E1	0.0015
E2	4.349 ± 0.6917, n=7	6.559 ± 1.231, n=7	Buffer Vs E2	<0.0001
F2	3.176 ± 0.6019, n=7	4.077 ± 0.7272, n=8	Buffer Vs F2	<0.0001

Table S10 – Granulocyte percentage per mosquito four days after systemic prostaglandin injection. Related to Figure 3. Mann-Whitney U test. **N represents the number of individual mosquitoes used per experiment. At least 6 individual mosquitoes were used per condition for each of the 2 independent experiments.**

Treatment	Exp 1	Exp 2	Exp 3	Comparison	P value
Buffer	11.5, n=22	39, n=32	142, n=9	-	-
E1	5, n=15	9.5, n=30	137.5, n=6	Buffer Vs E1	0.1271
E2	2, n=33	14, n=21	34, n=11	Buffer Vs E2	<0.0001
F2	1, n=20	30, n=12	59, n=11	Buffer Vs F2	0.0247

Table S11 – Effect of prostaglandin systemic injection on antiplasmodial immunity. Related to Figure 3. Median number of oocysts per midgut dissected 10 days post infection. Three independent infections were performed and pooled together for analysis**.** *ANOVA Dunnet multiple comparison*. **Table represents non-randomized data and therefore treatments have different numbers of individual mosquitoes. Buffer (total n=63); PGE1 (total n=51); PGE2 (total n=65); PGF2 (total n=43).**

Treatment	Exp 1	Exp 2	Comparison	P value
Buffer	29.29, n=1	24.68 ± 6.218, n=2	-	-
E1	34.33, n=1	23.63 ± 6.333, n=2	Buffer Vs E1	0.9980
E2	27.16, n=1	25.63 ± 6.78, n=2	Buffer Vs E2	0.9986
F2	14.18, n=1	17.88 ± 0.6347, n=2	Buffer Vs F2	0.7398

Table S12 – Prostaglandin levels in the hemolymph four days after systemic injection of prostaglandin. Related to Figure 3. **Two independent experiments were conducted, and each biological replicate corresponded to a pool of 10 mosquitoes. First experiments had one biological replicate and experiment 2 has 2 pools of 10 mosquitoes.** *ANOVA* **Dunn's multiple comparison test.**

Treatment	Exp 1	Exp 2	Comparison	P value
Buffer	2.001 ± 0.3575, n=5	1.575 ± 0.4388, n=6	-	-
E1	3.989 ± 1.496, n=5	4.745 ± 0.6649, n=6	Buffer Vs E1	0.0025
E2	7.632 ± 2.227, n=5	6.166 ± 0.2753, n=4	Buffer Vs E2	<0.0001
F2	2.308 ± 0.7018, n=5	2.147 ± 0.5869, n=7	Buffer Vs F2	0.6075

Table S13 – Granulocytes percentage of naive mosquitoes injected with the hemolymph from prostaglandin pre-treated mosquitoes. Related to Figure 3. **Two independent experiments were performed, each one with a different batch of hemolymph. At least 4 individual mosquitoes were counted per condition in two independent experiments. Mann-Whitney U test. All individuals were considered for statistical analysis.**

Treatment	Group	Exp 1	Comparison	P value
dsLacZ	Control	1.214 ± 0.2907, n=10	dsLacZ control Vs dsLacZ E2	0.0015
	E2	3.705 ± 0.5343, n=11		
dsEvokin	Control	1.786 ± 0.3813, n=9	dsEvokin control Vs dsEvokin E2	0.5101
	E2	1.81 ± 0.2417, n=10		

Table S14 – Granulocyte percentage of *Evokin-silenced* mosquitoes injected with prostaglandin E2. Related to Figure 3. One experiment was performed and at least 9 individual mosquitoes were analyzed per condition. Mann-Whitney U test. **All individuals were considered for statistical analyses.**

Treatment	Exp 1	Comparison	P value
dsLacZ BF	18.3 ± 3.256, n=3	-	-
dsIMPer BF	49.24 ± 1.075, n=3	dsLacZ Vs dsIMPer	0.0008

Table S15 – Prostaglandin hemolymph levels in blood-fed *IMPer-silenced* mosquitoes. Related to Figure 3. Three biological replicates, containing a pool of 10 mosquitoes each, were used for dsLacZ and dsIMPer groups. Unpaired t-test. **All individuals were considered for statistical analysis.**

Treatment	Exp 1	Exp 2	Exp 3	Comparison	P value
dsLacZ	10, n=11	4, n=7	7, n=5	-	-
dsIMPer	21, n=9	14, n=5	26, n=9	dsLacZ Vs dsIMPer	0.0005

Table S16 – Number of hemocytes associated with the midgut basal surface in blood-fed *IMPer-silenced* mosquitoes. Related to Figure 3. Three independent experiments were performed and at least 5 individual mosquitoes were counted per group in each experiment. **All individuals were considered for statistical analysis. Mann-Whitney U test.**

Treatment	Exp 1	Exp 2	Comparison	P value
dsLacZ BF	1.917 ± 0.2524, n=9	1.451 ± 0.2583, n=8	-	-
dsIMPer BF	5.443 ± 1.098, n=9	7.125 ± 1.271, n=9	dsLacZ Vs dsIMPer	<0.0001

Table S7– Granulocyte percentage in blood-fed IMPer-silenced mosquitoes. Related to Figure 3. Two independent experiments were performed and at least 8 individual mosquitoes were counted per experimental group. Mann-Whitney U test. **All individuals were considered for statistical analysis.**

Hemolymph injection from	Exp 1	Exp 2	Comparison	P value
dsLacZ BF	0.9124 ± 0.2232, n=10	2.387 ± 0.5136, n=7	-	-
dsIMPer BF	2.554 ± 0.2817, n=10	4.09 ± 0.8691, n=12	dsLacZ Vs dsIMPer	0.0030

Table S18 – Granulocyte percentage in naïve mosquitoes injected with the hemolymph of blood-fed *IMPer-silenced* mosquitoes. Related to Figure 3. Two independent experiments were performed with different batches of hemolymph. At least 7 individual mosquitoes were counted in each experimental group. Mann-Whitney U test. **All individuals were considered for statistical analysis.**

Gene	Treatment	Exp 1	Exp 2	Exp 3	Comparison	P value
HPX7	-Bact	1.235 ± 0.4633, n=3	1.007 ± 0.08537, n=3	1.053 ± 0.3286, n=2	-	-
	+Bact	7.075 ± 2.47, n=3	2.894 ± 0.7942, n=3	2.26 ± 0.4295, n=3	Control Vs Bacteria	0.0003
HPX8	-Bact	1.48 ± 0.9318, n=3	1.005 ± 0.1001, n=2	1.307 ± 0.6975, n=3	-	-
	+Bact	21.48 ± 8.114, n=3	23 ± 16.36, n=2	8.999 ± 3.119, n=3	Control Vs Bacteria	0.0002

Table S19 – HPX7 and HPX8 gene expression in the midgut after bacterial feeding. Related to Figure 4. Three independent experiments were performed and at least 2 biological replicates were used per condition. Each biological replicate is a pool of 10 mosquito midguts. Mann-Whitney U test. **All pools obtained were considered for statistical analysis.**

Gene	Treatment	Exp 1	Exp 2	Comparison	P value
HPX7	dsLacZ BF	1.024 ± 0.1581, n=3	1.018 ± 0.133, n=3	-	-
	dsIMPer BF	3.597 ± 1.062, n=3	7.038 ± 3.784, n=3	dsLacZ Vs dsIMPer	0.0022
HPX8	dsLacZ BF	1.233 ± 0.3213, n=5	0.7897 ± 0.2643, n=4	-	-
	dsIMPer BF	5.198 ± 2.485, n=5	34.09 ± 16.29, n=4	dsLacZ Vs dsIMPer	0.0040

Table S20 – HPX7 and HPX8 gene expression in the midgut in blood-fed *IMPer-silenced* mosquitoes. Related to Figure 4. Two independent experiments were performed, at least 3 biological replicates were used per condition. Each biological replicate is a pool of 10 mosquito midguts. Mann-Whitney U test. **All pools were considered for statistical analysis.**

Treatment	Exp 1	Exp 2	Exp 3	Comparison	P value
dsLacZ BF	25.33 ± 5.634, n=3	21.66 ± 9.546, n=3	34.29 ± 9.41, n=3	-	-
dsIMPer BF	47.67 ± 4.847, n=3	64.34 ± 14.45, n=3	60.86 ± 1.719, n=2	LacZ Vs IMPer	0.0003
dsHPX7 +BF	31.68 ± 2.978, n=3	34.08 ± 6.472, n=3	48.02 ± 2.13, n=2	LacZ Vs HPX7	0.3598
dsIMPer + dsHPX7 BF	21.16 ± 3.203, n=3	37.91 ± 10.08, n=3	35.95 ± 7.495, n=4	LacZ Vs IMPer + HPX7	0.7701

Table S21 – Effect of IMPer and HPX7 co-silencing on prostaglandin levels after blood feeding. Related to Figure 4. Three independent experiments were performed and at least 2 biological replicates were used per condition. Each biological replicate is a pool of 10 mosquitoes (hemolymph). *ANOVA* Dunn's multiple comparison test. **All pools were considered for statistical analysis.**

Treatment	Exp 1	Exp 2	Exp 3	Comparison	P value
dsLacZ BF	42.16 ± 6.438, n=6	39.21 ± 3.172, n=6	23.94 ± 2.45, n=8	-	-
dsIMPer BF	84.02 ± 17.09, n=6	44.96 ± 3.579, n=6	47.12 ± 5.179, n=4	LacZ Vs IMPer	0.0002
dsHPX8 BF	34.55 ± 2.85, n=6	29.47 ± 3.629, n=6	17.69 ± 2.705, n=4	LacZ Vs HPX8	0.6972
dsIMPer + dsHPX8 BF	36.5 ± 6.015, n=6	26.97 ± 3.242, n=6	24.6 ± 4.957, n=4	LacZ Vs IMPer + HPX8	0.8520

Table S22 - Effect of IMPer and HPX8 co-silencing on prostaglandin levels after blood feeding. Related to Figure 4. Three independent experiments were performed and at least 2 biological replicates were used per condition. Each biological replicate is a pool of 10 mosquitoes (hemolymph). ANOVA Dunn's multiple comparison test. **All pools were considered for statistical analysis.**

Treatment	Exp 1	Exp 2	Comparison	P value
dsLacZ BF	2, n=29	6, n=29	-	-
dsIMPer BF	5, n=28	14, n=29	LacZ Vs IMPer	0.0001
dsHPX8 BF	3, n=21	8, n=29	LacZ Vs HPX8	0.6805
dsIMPer + dsHPX8 BF	3, n=21	8, n=29	LacZ Vs IMPer + HPX8	0.1289

Table S23 – Effect of IMPer and HPX8 co-silencing on the number of hemocytes attached to midgut surface. Related to Figure 4. Two independent experiments were performed and at least 20 individual mosquitoes were analyzed per condition per experiment. Hemocyte numbers from the 2 experiments were combined for statistical analysis. *ANOVA* Kruskal-Wallis non-parametric test. **All individuals were considered for statistical analysis.**

Hemolymph injection from	Exp	Comparison	P value
dsLacZ BF	1.869 ± 0.3943, n=12	-	-
dsLacZ INF	6.148 ± 1.16, n=4	dsLacZ BF Vs dsLacZ INF	0.0022
dsHPX7 BF	3.189 ± 0.71, n=10	dsHPX7 BF Vs dsHPX7 INF	0.3930
dsHPX7 INF	2.365 ± 0.4351, n=10	-	-

Table S24 – Effect of HPX7 silencing on HDF production and granulocyte proportion after hemolymph transfer from mosquitoes control and *P.berghei* infected. Related to Figure 4. Granulocyte proportion from individual mosquitoes were used for statistical analysis. Mann-Whitney U test. **All individuals were considered for statistical analysis.**

Hemolymph injection from	Exp	Comparison	P value
dsLacZ BF	1.883 ± 0.2944, n=12	-	-
dsLacZ INF	6.069 ± 2.02, n=11	dsLacZ BF Vs dsLacZ INF	0.0036
dsHPX8 BF	2.63 ± 0.3749, n=14	dsHPX8 BF Vs dsHPX8 INF	0.5795
dsHPX8 INF	2.481 ± 0.5752, n=14	-	-

Table S25 – Effect of HPX8 silencing on HDF production and granulocyte proportion after hemolymph transfer from blood-fed control and *P.berghei* infected mosquitoes. Related to Figure 4. Granulocyte proportion from individual mosquitoes were used for statistical analysis. At least 11 individual mosquitoes were used per treatment. Mann-Whitney U test. **All individuals were considered for statistical analysis.**

Treatment	Exp 1	Exp 2	Comparison	P value
Hemolymph dsLacZ BF	114.5, n=14	71, n=21	LacZ BF Vs LacZ INF	0.0009
Hemolymph dsLacZ INF	39, n=17	19, n=30	LacZ BF Vs HPX8 BF	0.3876
Hemolymph dsHPX8 BF	111, n=15	41, n=21	HPX8 BF Vs HPX8 INF	0.5154
Hemolymph dsHPX8 INF	59.5, n=20	25, n=22	LacZ INF Vs HPX8 INF	0.0770

Table S26– Effect of HPX8 silencing on HDF production and antiplasmodial immunity after hemolymph transfer from blood-fed control and *P.berghei* infected mosquitoes. Related to Figure 4. Median number of oocysts per midgut 10 days post infection. Two independent infections were performed, and the number of parasites was counted in the midguts of individual mosquitoes. The 2 experiments were combined for statistical analysis. Mann-Whitney U test. **All mosquitoes were considered for statistical analysis. Table represents non-randomized data and therefore treatments have different numbers of individual mosquitoes. DsLacZ BF (total n=35); dsLacZ INF (total n=47); HPX8 BF (total n=36); HPX8 INF(total n=42).**

Accession Number	Gene	Primer sequence
AGAP004036	HPX7 qPCR Forward	GTTCTACAGTCCGCACACCA
	HPX7 qPCR Reverse	GCGTGGGATTGAGAAAGGTA
AGAP004038	HPX8 qPCR Forward	ATGGAGCGTTTTTGATCCAG
	HPX8 qPCR Reverse	TGATCCATCAGCGAGAAGAG
AGAP013327	IMPer qPCR Forward	GGGTGCTGTGTGACAATACG
	IMPer qPCR Reverse	CCATCGCGTTAAATTCACCT
AGAP009281	Evokin qPCR Forward	CAGCCGAAAGTGAACAAACA
	Evokin qPCR Reverse	ATGGTGGCCATTGTATACGG
AGAP010592	RpS7 qPCR Forward	AGAACCAGCAGACCACCATC
	RpS7 qPCR Reverse	GCTGCAAACTTCGGCTATTC

Table S27 – Oligonucleotides used for qPCR analysis. Related to Figures 3 and 4.

Transparent Methods

Mosquitoes, cell line and mouse feeding

Anopheles gambiae mosquitoes (G3 strain – CDC) were reared at 28°C, 80% humidity under a 12h light/dark cycle and maintained with 10% Karo syrup solution during adult stages. Mosquito infections with *Plasmodium berghei* were performed using transgenic GFP *P.berghei* parasites (ANKA GFPcon 259cl2) maintained by serial passages into 3 to 4 week old female BALB/c mice (Charles River, Wilmington, MA, USA) from frozen stocks. Infectivity was measured by parasitemia levels and *in vitro* exflagellation counting as previously described (Billker et al., 1997). Four to five-day old mosquitoes were fed when mice reached 3-5% parasitemia and 1-2 exflagellation per field. Three to 4 weeks old uninfected mice were used to feed blood-fed control mosquitoes. After feeding, both control and infected mosquitoes were maintained at 19°C, 80% humidity and 12h light/dark cycle until the day of hemolymph collection or dissection.

Ethics statement

Public Health Service Animal Welfare Assurance #A4149-01 guidelines were followed according to the National Institutes of Health Animal (NIH) Office of Animal Care and Use (OACU). These studies were done according to the NIH animal study protocol (ASP) approved by the NIH Animal Care and User Committee (ACUC), with approval ID ASP-LMVR5.

Prostaglandin sample preparation

Midguts and fat body were dissected in Hanks Buffer Salt Solution (HBSS) without calcium and magnesium and placed in a 24-well containing 400μl of HBSS with Calcium and Magnesium. Ten tissues were used for each biological replicate and were incubated for one hour at 28°C for prostaglandin release. For total measurement of prostaglandins, the content of the well was homogenized with a pestle, frozen and thawed, and ultimately centrifuged at 12,000 rpm for 10

minutes at 4°C. The supernatant was stored at -80°C until assayed. To measure prostaglandin release by the midgut, ten midguts were placed in a 24-well plate and incubated for 1 hour at 28°C. Only the supernatant was collected and centrifuged before sample storage. For hemolymph samples, ten mosquitoes were perfused with 5μl each of HBSS with calcium and magnesium. Samples were centrifuged at 12,000 rpm for 10 minutes at 4^{0}C and stored at -80°C until assayed.

Prostaglandin ELISA assay

Hemolymph, midgut, and fat body prostaglandin levels were determined using Prostaglandin Screening ELISA and Prostaglandin E2 Monoclonal ELISA kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 50 μl of standard/ sample was pipetted in a pre-coated 96-well plate. Aliquots (50 μl) of polyclonal PG antibody or monoclonal PGE2 antibody and PG or PGE2 conjugated to acetylcholinesterase (AChE) were added to each well and allowed to incubate at room temperature (PG screening kit) or 4°C (PGE2 monoclonal kit) for 18 hours. After incubation, wells were washed five times with 400 μl of wash buffer provided, followed by addition of 200 μl of Ellman's reagent substrate for development. Absorbance was read at 412nm with a Spectro fluorimeter plate reader (Molecular Devices, Sunnyvale, CA, USA) 90 minutes after addition of the substrate.

Hemocyte collection and quantification

Hemocytes were collected as previously described (Rodrigues et al., 2010, Ramirez et al., 2015). Briefly, a fine incision (half a millimeter) was made in the mosquito between the last 2 abdominal segments using fine-tip forceps, and then mosquitoes were injected into the thorax with an anticoagulant solution (60% Schneider's Insect medium, 10% fetal bovine serum, and 30% citrate buffer). Anticoagulant buffer was injected until 10 μl was recovered from the incision made in the abdomen using a sterile siliconized pipette tip. The collected sample was applied to a sterile

disposable haemocytometer slide (10 µl capacity, Neubauer Improved, iNCYTO C-Chip DHC-N01) and the hemocytes were counted under a light microscope (40x objective). Hemocytes were counted and the proportion of granulocytes was calculated relatively to the rest (prohemocytes and oenocytoids) for each individual mosquito.

Hemolymph collection and transfer

Hemolymph collection was performed as previously described (Rodrigues et al., 2010, Ramirez et al., 2015). In short, hemolymph was collected by perfusion as described above. Depending on the purpose of the experiment mosquitoes were perfused with either a modified anticoagulant buffer for transfer experiments (95% Schneider media, 5% citrate buffer) or HBSS with calcium and magnesium for ELISA assays. Each mosquito was injected with enough buffer to recover 5µl of hemolymph. Pools of ten mosquitoes were used for each biological replicate, for a total hemolymph volume of 50 µl. After collection, the hemolymph was centrifuged at 4ºC, 9,300g for 10 minutes to remove hemocytes. Cell-free hemolymph was then transferred to a new microcentrifuge tube and stored at -80ºC until used. Transfer of cell-free hemolymph to sugar fed naïve mosquitoes was done by microinjection of a total of 138nl per mosquito. We transferred the equivalent of one-fiftieth of the hemolymph from the donors to the recipients. The effect of cell-free hemolymph transfer was evaluated 4 days post injection by hemocyte counting in the recipients to determine granulocyte proportion.

Eicosanoid injection

3 to 4-day-old *An. gambiae* mosquitos were anesthetized on ice and injected with Prostaglandin E1, E2 or F2 (approximately 500 nM) (Cayman Chemical, Ann Arbor, MI, USA). Prostaglandin stock solutions were stored at -80ºC in 100% Ethanol. To perform mosquito injections, prostaglandins were dried under a stream of nitrogen gas and resuspended in modified

anticoagulant solution (95% Schneider media, 5% citrate buffer). Three days post-injection mosquitoes were either fed with a *P. berghei*-infected mouse to assess its effect on *Plasmodium* infection or perfused 4 days post injection to determine granulocyte proportion and collect hemolymph for further transfer.

Artificial feeding of bacteria from the microbiota

To isolate the mixture of bacteria used for feeding, sugar-fed mosquitoes from our colony were cleaned in 70% Ethanol and sterile PBS and dissected in sterile PBS. Pools of 5 midguts were placed in sterile PBS, homogenized and inoculated in liquid LB media to grow at 28°C and 250rpm for at least 16 hours in a shaker incubator. The cultivable population was frozen and kept at -80°C as stocks. A pre-inoculum was set up in LB media from the frozen stocks containing the bacterial mixture and allowed to grow overnight at 28°C, 250rpm in a shaker incubator. On the day of the experiment, the pre-inoculum was diluted in fresh LB media and allowed to grow for 2 hours in the same condition described above. Briefly, after 2 hours of growth, bacteria were washed with sterile PBS to remove toxins and the concentration of the culture was estimated based on the Optical Density (OD) of the culture. At 600nm, 1OD was considered the equivalent of 10^9 bacteria/mL. 3 to 4-day-old mosquitos were treated with 10% sterile sucrose solution containing antibiotics Penicillin and Streptomycin (100U/mL and 100µg/mL, respectively) for 2 days prior the bacterial feeding. Control group was fed with a sterile 10% Bovine Serum Albumin (BSA) solution in HBSS without calcium and magnesium and the bacteria group was fed with the same solution containing 4×10^9 bacteria per feeder. Arachidonic acid 1µg/µl (Cayman Chemical, Ann Arbor, MI, USA) was supplemented in the 10% BSA solution to provide enough substrate for prostaglandin synthesis. Hemocyte biology and dynamics were analyzed 6 hours post feeding and prostaglandin production was evaluated 24 hours post feeding.

Visualizing hemocytes attached to the midgut basal lamina

To stain hemocytes, the day before any dsRNA treatment or feeding, 3 to 4-day-old mosquitoes were injected with 69nl of 100µM Vybrant CM-DiI cell labelling solution (ThermoFisher Scientific, Waltham, MA, USA) for a final concentration of approximately 3.5µM in the hemolymph. To preserve hemocyte-midgut attachment, tissues were quick fixed using a higher concentration of fixative injected straight into the hemolymph of the mosquito. Mosquitoes engorged from feeding either with 10% BSA solution or blood were ice-cold anesthetized, injected with 276nl of 16% paraformaldehyde, and rested for 40 seconds before midgut dissection in 4% paraformaldehyde solution. After dissection, midguts were placed in ice-cold PBS and opened longitudinally to remove the bolus. Cleaned, opened tissues were then fixed overnight at 4°C in 4% paraformaldehyde. The following day, midguts were washed twice with PBS, blocked for 40 minutes with PBS containing 1% BSA, and washed twice with the same solution. For actin and nuclei staining, midguts were incubated for 30 minutes at room temperature with 1U of phalloidin (Alexa Fluor 647, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) and 20µM Hoechst 33342 (405, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA), both diluted in PBS. Tissues were mounted in microscope slides using Prolong Gold Antifade Mountant (Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA). Hemocytes were visualized by confocal microscopy and the number of hemocytes per midgut in each biological condition was also analyzed.

Midgut PGE2 Immunostaining

3 to 4-day-old mosquitoes were fed with 10% BSA solution with or without bacteria, as previously described. Six hours post feeding, mosquitoes were dissected in PBS, cleaned to remove bolus, and fixed using 4% paraformaldehyde overnight at 4°C. Midguts were submitted to a mild

permeabilization with Tween 20 since prostaglandins are lipids and could be removed with this treatment. Then, midguts were blocked using PBS containing 1% BSA for 6 hours at room temperature and stained overnight using rabbit anti-PGE2 (1:300) (Anti-Prostaglandin E2 antibody, ab2318, Abcam, Cambridge, UK). Then midguts were washed with PBS containing 1% BSA and 0.1% gelatin and incubated with secondary antibodies goat anti-rabbit (Alexa Fluor 594, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) (1:500) for 2 hours at room temperature. Midguts were washed three times with the same buffer, washed twice with PBS, and then incubated with 20µM Hoechst 33342 (405, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) and 1U of phalloidin (Alexa Fluor 647, Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) as described above. Microscope slides were mounted using Prolong Gold Antifade Mountant (Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA).

Agarose-spot migration assay

A protocol was adapted from Wiggins & Rappoport, 2010 (Wiggins and Rappoport, 2010). Briefly, 1% low melting agarose solution in PBS was heated on a hot plate to facilitate the dissolution of the agarose. After complete dissolution, 1% low melting agarose solution was placed at 40°C to cool down. For the prostaglandins, the equivalent of 7.1 µg was dried under a nitrogen gas stream in a glass tube to remove the ethanol and resuspended in 5µl of sterile PBS. Five microliters of the cooled down 1% low melting agarose solution was mixed with 5µl of PBS with or without prostaglandins. A ten-microliter drop was placed in a 35mm glass-bottom dishes (Mat Tek corporation, Ashland, MA, USA). The chamber was then placed at 4°C for 5 minutes and the media containing 5 x 10^5 Sua 5.1 cells was pipetted in the well. Four hours after seeding the cells, the complete media was replaced by Schneider media 1% FBS and analyzed next morning by

counting the number of cells that entered the agarose per field. Imaging was performed on a Leica Epifluorescence (Leica Microsystems, Wetzlar, Germany) inverted microscope with a 40x objective using phase contrast and migration was recorded overnight every minute.

Hemocyte Live Imaging

3 to 4 days old mosquitoes were treated with sterile sucrose 10% solution containing antibiotics as described above. The next day mosquitoes were fed with saline solution with sodium bicarbonate (0.15M NaCl and 0.01M Na_2CO_3, pH 7.4) containing CFDA-SE (Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA) 1:500 for midgut staining to use as a reference. Then, only engorged mosquitoes were injected with a lipophilic dye, Vybrant DiD cell-labelling solution (Abs 644/ Em 665, Molecular probes, ThermoFisher Scientific, Waltham, MA, USA), for hemocyte identification and starved until the next day. Mosquitoes were fed with 10% BSA solution with or without bacteria as described above. After 4 hours post feeding, mosquitos were imaged between a coverslip and glass slide at 1-minute intervals using variable z-steps. To quantify hemocyte behavior, images from 2 hours of acquisition were analyzed using Imaris 8.3.1 (Bitplane, Concord, MA, USA). Hemocytes were first automatically identified and tracked. Next, tracks were manually edited and corrected for sample drift. Visualization of tracks was graphed using the Imaris MATLAB XTension "Plot all tracks with a common origin" (Peter Beemiller). Track statistics were exported and analyzed in Prism (GraphPad, San Diego, CA, USA) using the Mann-Whitney test with outliers removed.

Confocal Microscopy

Images were captured using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a 63x oil immersion objective (zoom factor of 4) equipped with a photomultiplier tube/hybrid detector. Samples were visualized with a 488-nm argon laser for CFDA-SE (midgut

staining for live imaging), a DPSS 561-nm laser for CM-DiI, a HeNe 594-nm laser for Alexa Fluor 594 (rabbit secondary antibody), and a HeNe 633-nm laser for Alexa Fluor 633 (phalloidin). Hoechst was excited using a 405-nm diode laser. Images were taken using sequential acquisition and variable z-steps. Image processing was performed using Imaris 8.3.1 (Bitplane, Concord, MA, USA) and Adobe Photoshop CC (Adobe Systems, San Jose, CA, USA).

dsRNA synthesis

The RNA interference (RNAi) silencing of heme-peroxidases (HPX7, HPX8 and IMPer) and Evokin was performed as previously described (Oliveira Gde et al., 2012, Ramirez et al., 2015, Kumar et al., 2010). In short, 3 to 4-day-old female *An. gambiae* were cold-anesthetized and injected with 69nl of a 3-µg/µl dsRNA solution specific for each gene. DsRNA was generated from a cDNA template from *A.gambiae* and the MEGAscript RNAi kit (ThermoFisher Scientific, Waltham, MA, USA). Primers were designed specifically for targeted genes and are listed as follows: for HPX7 (AGAP004036), a 689 bp fragment was amplified with external primers (F-AACTGTCCTCCACGCTGTTT and R- CACCCTGGGCAAGTATTCAC), the product of this reaction was amplified again using internal primers containing T7 promoters and generating a smaller fragment, 418 bp (F-TAATACGACTCACTATAGGGATCAAAACGAAGGCCAAATG and R-TAATACGACTCACTATAGGGCCAGCGAGATCAACTGGTTC). For HPX8 (AGAP004038), the same strategy was used, where a 718 fragment was amplified using external primers (F- AACCACCAACGCTATCGAAC and R- CGACATAAACTGGCCGAACT). Using the external PCR product as template a smaller fragment (450 bp) was amplified with T7 promoters (F- TAATACGACTCACTATAGGGTCACTGCCGTTTGAACTGTC and R-TAATACGACTCACTATAGGGCCACACACCGTCCTCGTAG). For IMPer (AGAP013327), a

463-bp fragment was amplified containing T7 promoters straight from cDNA (F-TAATACGACTCACTATAGGGTCGGTGCTGGAAAAGGATGG and R-TAATACGACTCACTATAGGGGTTGGCGCGAGCTAAACACG). For Evokin (AGAP009281), a 166-bp fragment was amplified with T7 promoters from cDNA (F-TAATACGACTCACTATAGGGTGGCGGTACTCCTGCTACTC and R-TAATACGACTCACTATAGGGGCCGTTATACACTTCCCACC). A 218-bp fragment was amplified from LacZ gene cloned into pCRII-TOPO vector using M13 primers to generate a dsRNA control as previously described (Molina-Cruz et al., 2012). Gene silencing efficiency obtained with this method was 81% for HPX7, 60% for HPX8, 97% for IMPer in the midgut and 80% for Evokin in the fat body.

RNA extraction, cDNA synthesis, and qPCR analysis

RNA samples from midguts were obtained using TRIzol (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer instructions. Briefly, pools of 15 midguts were dissected in PBS, placed in TRIzol, and homogenized with a motorized pestle. Two hundred microliters of chloroform were added (1/5 of TRIzol volume) to 1mL of TRIzol for the aqueous phase separation. Total RNA was precipitated with 2-propanol (1:1) added to the aqueous phase and resuspended in 30μl of nuclease free water. RNA concentration was measured at 260nm using a Nanodrop (ThermoFisher Scientific, Waltham, MA, USA). One microgram of total RNA was used for complementary DNA (cDNA) synthesis using Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Gene expression was assessed by quantitative PCR (qPCR) using the resulting cDNA as a template. We used the DyNamo SYBR green qPCR kit (ThermoFisher Scientific, Waltham, MA, USA) with target specific primers listed on table S27 and the assay ran on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules,

CA, USA). Relative quantitation results were normalized against *An. gambiae* ribosomal protein

S7 (RpS7) as internal standard and analyzed using the ΔΔCt method (Livak and Schmittgen, 2001,

Pfaffl, 2001). Statistical differences of fold change values between the conditions was determined

using the Mann-Whitney test (GraphPad, San Diego, CA, USA). Each independent experiment

was done with three biological replicates (three pools of 15 tissues) for each condition tested, and

each one was analyzed by qPCR in duplicates (technical replicates). At least three independent

experiments were performed.

<u>Statistical analysis</u>

All statistical analyses were performed using GraphPad Prism 5 (GraphPad, San Diego, CA, USA).

All analyses where pertinent were conducted using either the student Unpaired t-test, Mann-

Whitney Test, ANOVA Dunnett multiple comparison and ANOVA Kruskal-Wallis test. The test

choice was determined based on the variation of the sample, if parametric or non-parametric, and

the number of treatments in each experiment. Significance was assessed at $p < 0.05$. The error bars

represent the Standard Error of the Mean (SEM).

<u>Key resources table</u>

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Polyclonal anti-PGE2	Abcam	Ab2318
Anti-Rabbit IgG Alexa Fluor 594	Molecular Probes/ Invitrogen/ ThermoFisher Scientific	A-11037
Cell lines		
Anopheles gambiae Sua 5.1*	BEI Resources	MRA-922
Organisms strains		
Anopheles gambiae G3 strain (CDC)	BEI Resources	MRA-112
GFP - *Plasmodium berghei* (ANKA GFPcon 259cl2)	BEI resources	MRA-865
BALB/c mice	Charles River	N/A
Chemicals		

Hanks Buffer Salt Solution (HBSS) with Calcium and Magnesium	Sigma	H8264
Hanks Buffer Salt Solution (HBSS) without Calcium and Magnesium	Sigma	H6648
Prostaglandin Screening ELISA kit	Cayman Chemical	514012
Prostaglandin E2 Monoclonal ELISA kit	Cayman Chemical	514010
Schneider's Insect Medium	Sigma	S0146
Prostaglandin E2	Cayman Chemical	14010
Prostaglandin F2α	Cayman Chemical	16010
Prostaglandin E1	Cayman Chemical	13010
Bovine serum albumin, fatty acid free	Sigma/ Roche	10775835001
Penicillin-Streptomycin	Sigma	P4333
Arachidonic Acid	Cayman Chemical	90010
Vybrant CM-DiI cell labeling solution	Molecular Probes/ Invitrogen/ ThermoFisher Scientific	V22888
Alexa Fluor 647 Phalloidin	Molecular Probes/ Invitrogen/ ThermoFisher Scientific	A22287
Hoechst 33342 solution (20 mM)	Molecular Probes/ Invitrogen/ ThermoFisher Scientific	62249
Prolong Gold Antifade Mountant	Molecular Probes/ Invitrogen/ ThermoFisher Scientific	P36934
16% Paraformaldehyde	Electron Microscopy Sciences	15710
Vybrant CFDA-SE	Molecular Probes/ Invitrogen/ ThermoFisher Scientific	V12883
Vybrant DiD cell labelling solution	Molecular Probes/ Invitrogen/ ThermoFisher Scientific	V22887
MEGAscript RNAi kit	ThermoFisher Scientific/ Invitrogen	AM1626
TRIzol reagent	ThermoFisher Scientific/ Invitrogen	15596018
QuantiTect Reverse Transcription Kit	Qiagen	205314
DyNAmo HS SYBR Green qPCR Kit	ThermoFisher Scientific	F410L
Oligonucleotides		
Primers for cloning, dsRNA synthesis and qPCR	This paper/ Eurofins	N/A
Software		
Graphpad Prism	GraphPad	N/A

IMARIS 8.3.1	Bitplane	N/A
Adobe Photoshop CC	Adobe Systems	N/A
Specific Tools used in this manuscript		
SKC, Inc. C-Chip™ Disposable Hemacytometers (Neubauer Improved)	Fisher Scientific	SKC, Inc. DHCN015
35mm Dish/ High tolerance 1.5 Coverslip/ 14mm Glass Diameter	Mat Tek corporation	P35G-0.170-14-C

Supplemental References

BILLKER, O., SHAW, M. K., MARGOS, G. & SINDEN, R. E. 1997. The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of Plasmodium berghei in vitro. Parasitology, 115 (Pt 1), 1-7.

RODRIGUES, J., BRAYNER, F. A., ALVES, L. C., DIXIT, R. & BARILLAS-MURY, C. 2010. Hemocyte differentiation mediates innate immune memory in Anopheles gambiae mosquitoes. Science, 329, 1353-5.

RAMIREZ, J. L., DE ALMEIDA OLIVEIRA, G., CALVO, E., DALLI, J., COLAS, R. A., SERHAN, C. N., RIBEIRO, J. M. & BARILLAS-MURY, C. 2015. A mosquito lipoxin/lipocalin complex mediates innate immune priming in Anopheles gambiae. Nat Commun, 6, 7403.

WIGGINS, H. & RAPPOPORT, J. 2010. An agarose spot assay for chemotactic invasion. Biotechniques, 48, 121-4.

OLIVEIRA GDE, A., LIEBERMAN, J. & BARILLAS-MURY, C. 2012. Epithelial nitration by a peroxidase/NOX5 system mediates mosquito antiplasmodial immunity. Science, 335, 856-9.

KUMAR, S., MOLINA-CRUZ, A., GUPTA, L., RODRIGUES, J. & BARILLAS-MURY, C. 2010. A peroxidase/dual oxidase system modulates midgut epithelial immunity in Anopheles gambiae. Science, 327, 1644-8.

MOLINA-CRUZ, A., DEJONG, R. J., ORTEGA, C., HAILE, A., ABBAN, E., RODRIGUES, J., JARAMILLO-GUTIERREZ, G. & BARILLAS-MURY, C. 2012. Some strains of Plasmodium falciparum, a human malaria parasite, evade the complement-like system of Anopheles gambiae mosquitoes. Proc Natl Acad Sci U S A, 109, E1957-62.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25, 402-8.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res, 29, e45.