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Increased melanizing activity in *Anopheles gambiae* does not affect development of *Plasmodium falciparum*

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Serpins are central to the modulation of various innate immune responses in insects and are suspected to influence the outcome of malaria parasite infection in mosquito vectors. Three *Anopheles gambiae* serpins (SRPN1, -2, and -3) were tested for their ability to inhibit the prophenoloxidase cascade, a key regulatory process in the melanization response. Recombinant SRPN1 and -2 can bind and inhibit a heterologous phenoloxidase-activating protease and inhibit phenoloxidase activation *in vitro*. Using a reverse genetics approach, we studied the effect of SRPN2 on melanization in *An. gambiae* adult females *in vivo*. Depletion of SRPN2 from the mosquito hemolymph increases melanin deposition on foreign surfaces such as negatively charged Sephadex beads. As reported, the knockdown of SRPN2 adversely affects the ability of the rodent malaria parasite *Plasmodium berghei* to invade the midgut epithelium and develop into oocysts. Importantly, we tested whether the absence of SRPN2 from the hemolymph influences *Plasmodium falciparum* development. RNAi silencing of *SRPN2* in an *An. gambiae* strain originally established from local populations in Yaoundé, Cameroon, did not influence the development of autochthonous field isolates of *P. falciparum*. This study suggests immune evasion strategies of the human malaria parasite and emphasizes the need to study mosquito innate immune responses toward the pathogens they transmit in natural vector–parasite combinations.

innate immunity | malaria | mosquito | serpin

The concept that immune reactions of the mosquito vector limit the development of malaria parasites during the sporogonic cycle was proposed over seventy years ago (1). Recent detailed laboratory-based analysis of the population dynamics of avian, murine and human malaria parasites in different vector species has identified a strong developmental bottleneck during the transition of ookinetes to oocysts coinciding with the transition of the parasite through the midgut epithelium (2). This bottleneck was also observed by using field isolates of *Plasmodium falciparum* (3).

The mechanisms of parasite killing during midgut invasion have been under intense investigation recently, by using rodent and avian parasite model systems. Cell biological observations can separate two major events, ookinete lysis during the passage through the midgut epithelium and ookinete melanization in the basal labyrinth (for review see ref. 4). The mechanisms leading to lysis are poorly understood but appear to involve several hemocyte-derived molecules such as TEP1, LRIM1 and APL1 (5–7).

Parasite melanization occurs in specific genetic backgrounds of the mosquito vector (6, 8, 9). In contrast to lysis, the molecular events leading to melanization are better understood and involve activation of a prophenoloxidase (PPO) cascade. Recognition of a foreign object leads to the sequential activation of several serine proteases and results ultimately in the activation of a PPO-activating protease (PAP). As its name indicates, this enzyme in turn cleaves PPO into the active phenoloxidase form (10). Both biochemical (11) and genetic studies (12) have shown that this conversion of PPO to PO is a rate-limiting step in the production of eumelanin. The PPO cascade is controlled tightly by both positive and negative regulators. Although melanization is unlikely to be a major factor in natural resistance to *P. falciparum* (8, 13), it was recently reported at high frequencies in the field and its control was mapped to two quantitative trait loci (Pfmel1 and -2) in the *Anopheles gambiae* genome (7).

The recent increase of understanding vector–parasite interactions led to the proposal of novel malaria disease intervention strategies based on genetic or chemical targeting of the mosquito innate immune responses (14). Conceptually ideal targets of such immunomodulators would be negative regulators of parasite lysis or melanization: their absence or inactivation could enhance these responses, thereby reducing parasite numbers. One class of versatile inhibitors is serpins, serine protease inhibitors that act as suicide-like substrates, irreversibly inhibiting their targets, which are mainly serine proteases. Serpins control a wide variety of defense reactions in most animals, among them blood clotting, complement activation, and apoptosis (15). Insect serpins have been found to regulate the activation of the Toll pathway and the PPO cascade (for recent review see (16). The *An. gambiae* genome encodes eleven potentially inhibitory serpins (SRPNs) (17), of which at least three are linked to infection by malaria parasites (9, 18, 19). Based on phylogenetic analyses we proposed that SRPN2, and the closely related SRPN1, are negative regulators of the PPO cascade in *An. gambiae* (9). Importantly, wild-type expression of *An. gambiae* SRPN2 was found to be necessary for the rodent malaria parasite, *Plasmodium berghei* to survive midgut invasion (9).

This study elucidates further the biological function of SRPN2 by using biochemical techniques and addresses whether this immune factor also is necessary for *P. falciparum* development in autochthonous parasite/mosquito combinations in areas of human malaria transmission.

Results

Binding and Inhibition of the Moth *Manduca sexta* PAP3 by *Anopheles* SRPNs. The sequence similarity of SRPN1 and -2 with *M. sexta* serpin 3 (SPN3), particularly in the amino acid residues sur-

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The authors declare no conflict of interest.

Abbreviations: MR, melanization response; PAP, PPO-activating protease; PPO, prophenol oxidase; RCL, reactive center loop.

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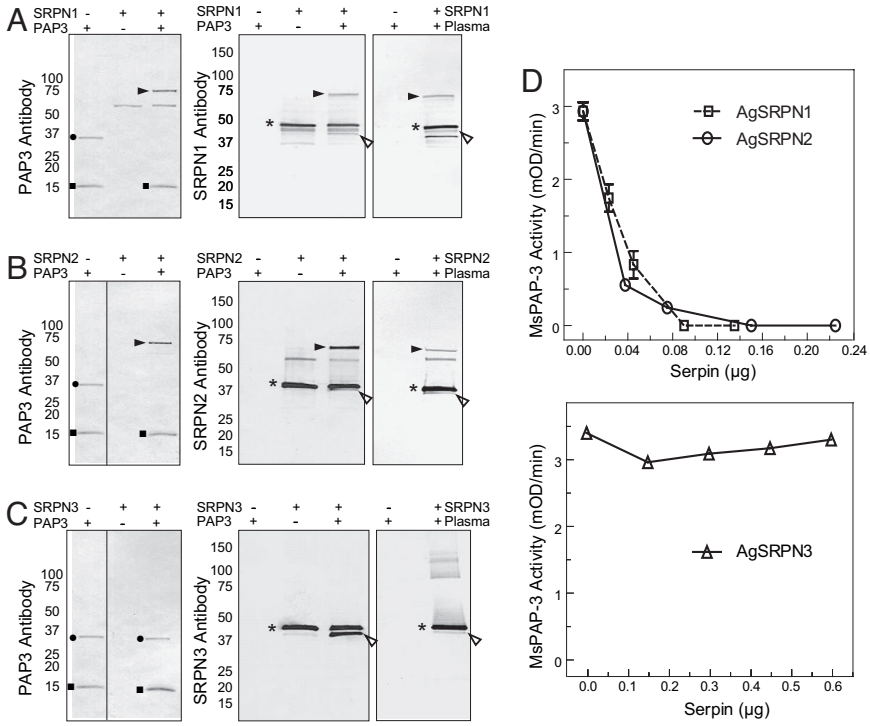

Fig. 1. SRPN1 and -2 bind and inhibit *M. sexta* PAP. (*A–C*) Immunoblot analysis of serpin–PAP3 complex formation. Purified PAP3 (*Left* and *Center*) or plasma from *M. sexta* plasma (which contains PAP3; *Right*) were incubated with recombinant AgSRPN1, -2 ,or -3 (*A*, *B*, and *C*, respectively). Samples were subjected to SDS/PAGE and immunoblotting. Potential complexes were visualized by using antibodies against PAP3 (*Left*) or against the indicated SRPN (*Center*). Symbols represent: SRPN-PAP3 complexes, filled arrowheads; catalytic domain of PAP3, circles; clip domain of PAP3, squares; noncomplexed intact form of SRPNs, asterisks; bands of slightly lower molecular weight probably representing cleaved forms of SRPNs, open arrowheads. Unlabeled polypeptides possibly represent *E. coli* proteins contaminating the SRPN preparations and recognized by the antisera. (*D*) Inhibition of PAP3 activity by recombinant SRPN1 and -2 but not -3. PAP3 and recombinant SRPN1, -2, or -3 were incubated at room temperature for 15 min before adding IEAR-pNA as a substrate. The activity was measured as the rate of increase in absorbance at 405 nm. Three replicates were performed, except in the case of AgSRPN3, where one replicate was tested. Error bars indicate SEM.

rounding the scissile bond in the reactive center loop (RCL), supported the prediction that *Anopheles* SRPN1 and SRPN2 might inhibit the *M. sexta* PAP; the mosquito SRPN3 differs significantly from SRPN1 and SRPN2 in the sequence near the putative scissile bond, and was therefore predicted to have different protease target specificities (9).

The zymogen form of PAP3 has an apparent molecular weight of ≈50 kDa when analyzed by SDS/PAGE and is activated by cleavage at a single site, separating catalytic and clip domains as two fragments of 35 and 15 kDa, respectively (20). We have confirmed the report (21) that when *M. sexta* SPN3 and an activated preparation of PAP3 were mixed, the 35-kDa catalytic domain disappeared and a higher molecular weight band representing the covalently linked protease–serpin complex appeared (see arrowhead in Fig. 5, which is published as supporting information on the PNAS web site). Similar results were obtained when we mixed PAP3 with *Anopheles* SRPN1 or SRPN2 (filled arrowheads in Fig. 1 *A Left* and *B Left*, respectively). The identity of the higher-molecular-mass putative SRPN/PAP complexes was confirmed by their detection with specific antibodies against PAP3 or the respective mosquito serpins (Fig. 1 *A* and *B Left* and *Center*). The same antibodies labeled a band at a similar position when the respective serpin was mixed with *M. sexta* plasma (Fig. 1 *A* and *B Right*), indicating that these two mosquito serpins inhibit and covalently bind with the *M. sexta* protease also in the complex mixture of plasma proteins. In contrast, the absence of a similar band in Fig. 1*C Left* and *Center*, indicated absence of covalent binding between recombinant PAP3 and SRPN3. Instead, a serpin band of slightly reduced mass appeared, suggesting that SRPN3 is a substrate rather than an inhibitor of PAP3. When SRPN3 was incubated with *M. sexta* plasma, higher bands at ≈100 and 120 kDa recognized by SRPN3 antibody did appear (Fig. 1*C Right*), indicating that SRPN3 may inhibit and covalently bind *M. sexta* plasma proteases other than PAP3.

The inhibition of PAP3 by SRPN1 and -2 was confirmed by assays of PAP3 enzyme activity. These two SRPNs inhibited PAP3 in a concentration-dependent manner (Fig. 1*D Upper*). However, SRPN3 did not significantly affect PAP3 activity even at much higher serpin concentrations (Fig. 1*D Lower*).

Inhibition of Spontaneous Melanization and PPO Activation in Larval Plasma. Inhibition of PAP3 by SRPN1 and -2 would be expected to prevent melanization in *M. sexta* larval plasma, which undergoes spontaneous melanization at room temperature (changing color from light green to dark brown or black) after collection through a wound. The plasma sample used in this experiment became black within 40 min if untreated or treated with SRPN3; however, its color remained unchanged in the presence of SRPN1 or -2 (Fig. 2*A*), indicating that SRPN1 and SRPN2 indeed inhibit the PPO activation cascade.

The melanization cascade is known to be activated by infection. Indeed, as shown by comparison of the two left-most bars in Fig. 2*B*, addition of *Micrococcus luteus* to plasma substantially enhanced PPO activation. This microbial induction was prevented by preincubation of plasma with either SRPN1 or SRPN2, but was unaffected by SRPN3.

Increased Melanization in *An. gambiae* Hemolymph. We used melanization of negatively charged C-25 Sephadex beads to assess

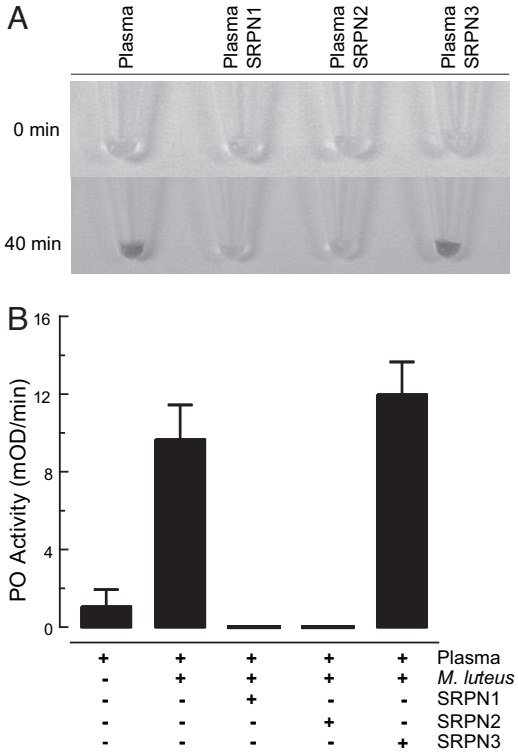

Fig. 2. SRPN1 and -2 inhibit PO activity and spontaneous melanization. (*A*) Inhibition of spontaneous melanization of freshly prepared *M. sexta* plasma. Five microliters of day-1 fifth-instar larval plasma were mixed with 0.3 μg of recombinant SRPN1, -2, or -3. Photographs taken at the indicated times after mixing demonstrate the inhibiting effect of SRPN1 and -2 but not -3 on melanin synthesis. (*B*) Day-1 fifth-instar *M. sexta* larval plasma was mixed with recombinant SRPN1, -2, or -3 as indicated. After 10 min at room temperature, *Micrococcus luteus* was added, and mixtures were incubated for 10 additional min. PO activity was measured by using dopamine as a substrate, as described in *Materials and Methods*; bars indicate Mean ± SE; $n = 3$.

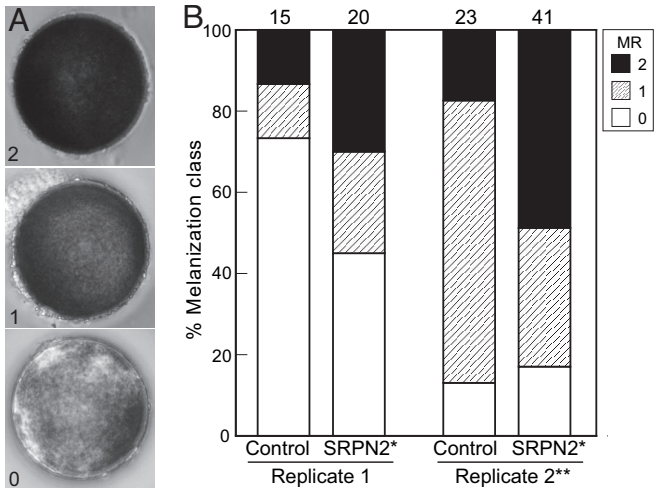

Fig. 3. Depletion of SRPN2 causes increased bead melanization. Negatively charged C-25 Sephadex beads were injected into the thoraces of Yaoundé mosquitoes 4 d after dsRNA injection. (*A*) Beads were recovered 24 h after injection, examined by microscopy, and scored for MR, according to percentage of bead surface coverage by melanin and the thickness of the melanin layer (see *Material and Methods*). (*B*) Quantitative analysis of bead melanization response. Each bar represents the proportion of mosquitoes that melanized beads to the extent of MR categories 0, 1, or 2. Number of mosquitoes used in each experiment is indicated above each bar. Likelihood-ratio χ^2 test was used to assess effects of *, treatment (d.f. = 1, $\chi^2 = 6.14$, $P = 0.013$) and **, replicate (d.f. = 1, $\chi^2 = 13.3$, $P = 0.0003$).

whether *SRPN2* influences the PPO cascade in adult female mosquitoes *in vivo* (Fig. 3). Two independent replicate experiments were performed in which single beads were injected in groups of control (*dsGFP*-injected) or experimental (*dsSRPN2*-injected) mosquitoes. The extent of bead melanization was scored quantitatively as explained in the legend of Fig. 3 and exemplified in Fig. 3*A* [melanization response (MR) 0, 1, and 2]. Interestingly, a measurable difference between the replicates was detected (likelihood-ratio χ^2 test; d.f. = 1, $\chi^2 = 13.3$, $P = 0.0003$). Melanization was increased overall in the second replicate (Fig. 3*B*), possibly because of differential exposure to environmental factors such as larval or adult nutrition in the experiments (22, 23). In addition, and despite this overall difference between replicates, prior treatment of adult female mosquitoes with *dsSRPN2* RNA significantly increased the level of bead melanization: in both replicates, the most prominent melanin capsules (MR2) were substantially more frequent in the *SRPN2* knockdown mosquitoes as compared with the controls (likelihood-ratio χ^2 test; d.f. = 1, $\chi^2 = 6.14$, $P = 0.013$).

***Plasmodium falciparum* Development in SRPN2-Depleted Mosquitoes.** It was shown that presence of SRPN2 in the hemolymph of *An. gambiae* facilitates development of a rodent malaria parasite (*P. berghei*) to the oocyst stage in the mosquito (9). Therefore we tested whether SRPN2 is also implicated in successful development of the human malaria parasite, *P. falciparum*. Experimental female *An. gambiae* (Yaoundé strain), were treated with *dsSRPN2*, and controls were treated with *dsGFP*. By using Western blot analysis, SRPN2 depletion from the hemolymph of experimental vs. control mosquitoes was monitored at several time points after injection (Fig. 4*A*), together with the level of another immunity factor, TEP1, that was used as loading control. Clear and specific SRPN2 depletion was visible by 2 days after injection and the serpin was virtually absent from the hemolymph of experimental mosquitoes 4–6 days after injection. As observed (9), in the standard G3 mongrel strain, silencing *SRPN2* in the Yaoundé mosquitoes caused the formation of melanotic pseudotumors throughout the body (data not shown) and had a strong effect on *P. berghei* development (Table 1 and see *Discussion*). Relative to the controls, the overall number of parasites that detectably crossed the midgut (oocysts plus melanized parasites) decreased by 45% and the mean oocyst number was reduced by 88%. Importantly, melanized parasites increased from 0–79% of parasites that crossed.

Separate samples of *dsRNA*-injected experimental and control mosquitoes were infected in parallel with seven independent field isolates of *P. falciparum*. Six infections led to the development of *P. falciparum* oocysts in both *dsSRPN2*- and *dsGFP*-treated mosquitoes (Fig. 4*B* and Tables 1 and 2). In remarkable contrast with the *P. berghei* study, no melanized *P. falciparum* were found in the 312 midguts of experimental and control mosquitoes that were examined (Table 1). The SRPN2 depletion also did not alter significantly the prevalence of *P. falciparum* infection (Table 2; likelihood-ratio χ^2 test; d.f. = 1, $\chi^2 = 0.566$, $P = 0.452$), and an observed slight difference in oocyst load between the two treatment groups (Fig. 4*C*) was not statistically significant (Table 1, Mann–Whitney *U* test, $P = 0.208$).

Discussion

Melanization is a central and well-conserved innate immune response of virtually all arthropods and tightly regulated by inhibitors, which are themselves evolutionarily conserved. Based on the sequence similarities between the RCLs of SRPN1 and -2 with the PPO activation cleavage site, we had postulated that

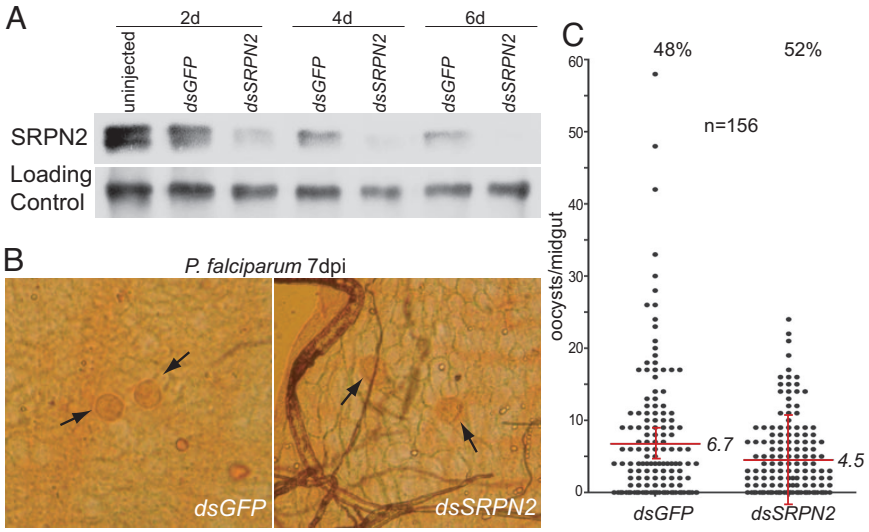

Fig. 4. SRPN2 knockdown does not affect *P. falciparum* oocyst development. Female Yaoundé mosquitoes were treated with *dsSRPN2* or control GFP dsRNA and infected 4 d after injection with field isolates of *P. falciparum*. Midguts were dissected 7 days after infection, and oocysts were counted after staining with mercurochrome. (*A*) SRPN2 knockdown by RNAi is efficient in *An. gambiae* Yaoundé strain mosquitoes. An immunoblot of 200 ng of hemolymph proteins isolated from mosquitoes treated with *dsSRPN2* or *dsGFP* as a control was probed with rabbit anti-SRPN2 antibody (1:1,000), stripped, and reprobed with rabbit anti-TEP1 antibody (1:500) for loading control. (*B*) Representative images of developing oocysts (black arrows) on midgut epithelia obtained from experimental and control-treated females. (*C*) Graph summarizing *P. falciparum* oocyst infection intensities after SRPN2-KD and control treatment. A total of 156 midguts were examined per treatment (each dot represents a single midgut). Arithmetic means of oocyst numbers are indicated by horizontal red bars, and the prevalence of infection is indicated above each data cloud. Vertical error bars indicate 1 ± SE. Mann–Whitney *U* test: $P = 0.208$.

SRPN1 and -2 can act as inhibitors of the melanization response by inhibiting the last step in the PPO cascade (9).

Because of the limited amount of hemolymph that can be recovered from a mosquito, the PPO cascade in *An. gambiae* still awaits detailed biochemical analysis. Therefore, to test the hypothesis whether SRPN1 and/or 2 can inhibit spontaneous melanization by blocking PO activity, we used the detailed knowledge of the melanization system in *M. sexta* (11). Here we report that recombinant mosquito SRPN1 and -2 indeed inhibit spontaneous melanization of *Manduca* plasma and abolish its PO activity (Fig. 2). The sequence similarity of the PO activation cleavage site with the RCL of SRPN1 and -2 supports the conclusion that one or more PAPs are natural serine protease targets of these two serpins. Consistent with current models of serpin inhibition of serine proteases, we show that SRPN1 and -2 indeed form specific covalent complexes with recombinant *M. sexta* PAP3 (20), whereas a closely related serpin, SRPN3, which has a diverged RCL sequence, does not (Fig. 1). Consistent with the specificity of complex formation, only SRPN1 and -2, but not SRPN3, inhibit the enzymatic activity of recombinant PAP3.

Reverse genetic analysis supports further the biochemical evidence that SRPN2 modulates the activation of PPO by inhibiting an as yet unidentified mosquito PAP. In *An. gambiae*, SRPN2 is expressed throughout the life cycle and is expressed in fat bodies and ovaries of adult female mosquitoes (24). Knockdown of this gene in adult females leads to spontaneous melanization (9) and increases the melanin layer that is deposited on negatively charged Sephadex beads (Fig. 4). These results indicate that SRPN2 limits the activation of PPO systemically and may be used to prevent accidental triggering of the melanization response *in vivo*.

We reported (9) that SRPN2 function greatly facilitates the development of *P. berghei* in its mosquito vector to proceed beyond the ookinete stage and this was verified in the present study. In the absence of SRPN2 nearly 90% of the invading *P. berghei* ookinetes are killed; of those *ca.* nearly half are cleared by lysis, whereas the other half become melanized (Table 1). However, the association of *An. gambiae* and *P. berghei* is solely laboratory-based as this vector/parasite combination does not occur in the field. To test whether the knockdown of SRPN2 also changes the melanization response of *An. gambiae* toward a natural parasite, we repeated the experiments with field isolates of *P. falciparum*. In contrast to the effect observed with *P. berghei*, absence of SRPN2 from the hemolymph did not change *P. falciparum* oocyst load and no melanized human malaria parasites were observed. Although resistance to *P. falciparum*

Table 1. Effect of *SRPN2* KD on the development of *Plasmodium* spp. oocysts

Species	Gene KD	No. of Exp.	*n*	Prev., %	Oocysts per midgut*	Melanized ook. per midgut*	Fold reduction†	*P*‡
P. berghei	*SRPN2*	2	26	73	13 ± 3	48 ± 12	8.5	0.002
	Control		26	89	111 ± 32	0		
P. falciparum	*SRPN2*	6	156	67	6.7 ± 0.8	0	1.5	0.208
	Control		156	68	4.5 ± 0.4	0		

Control, *dsGFP* was used as control treatment; Exp., experiment; *n*, number of mosquitoes analyzed; Prev., prevalence; ook., ookinete.
*Arithmetic mean ± one standard error.
†Ratio of arithmetic means of developing oocysts per midgut.
‡7–8 days after infection, Mann–Whitney *U* test.

Table 2. No effect of *SRPN2* KD on *P. falciparum* prevalence

Parasite isolate	Percentage infected (*n*)*†		Gametocytes per μl of blood
	Control	*dsSRPN2*	
1	12.2 (41)	11.1 (27)	Not confirmed
2	48.6 (35)	38.5 (13)	15
3	50.0 (18)	81.8 (11)	12
4	56.3 (32)	65.0 (20)	10
5	90.9 (22)	73.1 (26)	Not confirmed
6	0.0 (30)	0.0 (20)	12
7	78.5 (93)	93.7 (63)	10

Control, *dsGFP* or *dsLacZ* were used as control treatment; *n*, number of mosquitoes analyzed.

*Percentage of midguts containing developing oocysts 7–8 days after infection.

†Likelihood-ratio χ^2 test (d.f. = 1, $\chi^2 = 0.566$, $P = 0.452$).

infection in field populations of *An. gambiae* has been observed (25), melanized parasites have been reported at low frequencies in autochthonous *P. falciparum*/*An. gambiae* combinations (13). Two possible explanations have been proposed for the rarity of observed melanization of *P. falciparum* in the field: negative selection driven by the high fitness cost of the melanization response (26), or active immune suppression by the malaria parasite (27). The first explanation is not pertinent to the present study, where high level of melanization was indeed achieved by experimental SRPN2 silencing (despite causing an observed reduction in longevity, (9). The second explanation is also inapplicable, as experiments of L.L. (unpublished work) with the same *P. falciparum* isolates as in this study did not detect melanization suppression.

The observed discrepancy in survival of the two *Plasmodium* species in the SRPN2 knockdown may have two additional alternative explanations. It may be that the loss of *P. berghei* is caused by ookinete killing in the midgut epithelium because of overproduction and circulation of toxic byproducts of systemic melanin synthesis. *P. falciparum* might be less sensitive to the same toxins and therefore can survive. If melanization of ookinetes is the consequence rather than the cause of their killing (5), no *P. falciparum* melanization would occur. Alternatively, melanization of the parasite might require specific recognition of *P. falciparum* surface molecules, which are either absent from *P. falciparum* or have evolved not to be recognized by the *An. gambiae* surveillance system. The recent report that TEP1 limits *P. falciparum* survival in laboratory experiments (28) would argue against this hypothesis, as TEP1 is known to bind to the *P. berghei* surface and limit survival of that parasite in *An. gambiae* (5). Future experiments are required to discriminate robustly amongst these hypotheses. For now the key finding of the present study is that even artificial activation of an immune response to high level (here the melanization cascade) has different effects on the rodent vs. the human malaria parasite. If the modulation of immune responses is to be used for future control measures, it will be necessary to explore the underlying mechanism(s) that cause these observed differences; findings observed in model systems will need to be validated in the natural parasite/vector combinations that will be targeted for intervention.

Materials and Methods

Insect Rearing. All experiments were performed on *An. gambiae* mosquitoes of the Yaoundé strain (29), which is of Cameroonian origin and was maintained by standard procedures (30). *M. sexta* were originally obtained from Carolina Biological Supply (Burlington, NC). Larvae were reared as described (31).

***P. berghei* Maintenance and Infections.** The *P. berghei* GFP-CON transgenic 259cl2 strain (32) was passaged in CD1 or TO female mice and infections were performed according to (33). Parasite prevalence and mean intensity of infection was determined by fluorescent light microscopy in midguts dissected 7 or 8 days after infection.

Recombinant SRPN Production. *SRPN1*, *-2*, and *-3* gene fragments encoding the respective full-length mature proteins were amplified from cDNA from 2-d-old naïve females and cloned into pGEMTeasy vector. Primer sequences are published as *Supporting Materials and Methods* on the PNAS web site. An NcoI/SalI fragment of *SRPN1* and an NcoI/XhoI fragment of *SRPN3* were subcloned into the pETM11 expression vector. An NcoI/SalI fragment of *SRPN2* were subcloned into pETM50. The N-terminal His-tagged SRPN1 and 3 recombinant proteins and the recombinant DsbA-SRPN2 fusion protein were expressed and purified; the DsbA tag was removed subsequently by TEV cleavage as reported (34, 35).

αSRPN3 Polyclonal Antibody Production. A chimeric protein composed of GST (GST) fused to amino acids 226–418 of SRPN3, was produced from the pGEXSRPN3 expression vector. A *SRPN3* gene fragment was amplified by RT-PCR using cDNA from 2-d-old females and cloned into pGEMTeasy vector (*Supporting Materials and Methods*). Subsequently, a BamHI-XhoI fragment of *SRPN3* was subcloned into BamHI-SalI sites of the pGEX4T3 expression vector (Amersham Pharmacia). Recombinant GST-SRPN3 was expressed and used for immunizations of two rabbits as described (9).

Inhibition of Spontaneous Melanization of *Manduca sexta* Larval Plasma. Day-1 fifth-instar moth larvae were chilled on ice, the abdomen surface sterilized with ethanol, and hemolymph collected into a microcentrifuge tube from a cut in the dorsal horn. Hemocytes were removed by centrifugation at 2,000 × *g* for 10 min. Five microliters of the plasma was immediately mixed with 0.3 μg of recombinant serpin in a total volume of 10 μl. The mixtures were incubated at room temperature and photographed after 40 min.

Detection of Serpin–Protease Complexes by Immunoblot Analyses. Active recombinant *M. sexta* PAP3, previously separated from the zymogen by anion exchange chromatography, was kindly provided by Maureen Gorman (Kansas State University, Manhattan, KS). PAP3 (0.1 μg) was mixed with SRPN1, -2, or -3 (0.08 μg). A similar mixture of PAP3 with *M. sexta* serpin-3 was used as a positive control to confirm that the PAP3 was active and capable of forming a complex with the serpin (Fig. 5). After incubation for 15 min at room temperature, the mixtures were treated with SDS sample buffer at 95°C for 5 min and resolved by SDS/PAGE using 4–20% Tris·HCl Ready gels (Bio-Rad, Hercules, CA). Proteins were transferred onto a nitrocellulose membrane and subjected to immunoblot analysis using 1:1,500 diluted antiserum against PAP3 as the primary antibody (21). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) was used as the secondary antibody (1:3,000 dilution), and the alkaline phosphatase-conjugate substrate kit (Bio-Rad) was used to visualize the antibody binding. Antisera against mosquito serpins as the primary antibody (1:1,000 dilution) were used in the same fashion to analyze mixtures of PAP3 with SRPN1, -2, or -3. The same amounts of serpins were mixed with 1.5 μl of freshly prepared *M. sexta* larval plasma. The antisera against *Anopheles* serpins and *Manduca* PAP3 were absorbed against an *Escherichia coli* extract to reduce cross-reaction with bacterial proteins in the recombinant protein samples.

Inhibition of PAP3 Activity. Recombinant PAP3 (0.1 μg) was preincubated with recombinant mosquito serpins at various concentrations adjusted to a total volume of 10 μl with buffer A (0.1 M Tris·HCl, pH 8.0/0.1 M NaCl). After incubation at room temperature for 15 min, 190 μl of 50 μM *N*-acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR-*p*NA) in buffer A was added, and the protease activity was measured by monitoring at 405 nm in a PowerWave340 microplate reader (Bio-Tek, Winooski, VT). Three replicates were performed with SRPN1 and SRPN2, and one replicate at higher concentrations was tested with SRPN3.

Inhibition of Prophenoloxidase (proPO) Activation in *Manduca* Larval Plasma. Plasma from day-1 fifth instar *M. sexta* larvae was prepared as described (36). Recombinant mosquito serpins SRPN1, -2, or -3 (0.5, 1, and 1 μg, respectively) were incubated with 1 μl of larval plasma. After incubation at room temperature for 10 min, 1 μl of 1 μg/μl *M. luteus* suspension was added to the mixtures and further incubated for 10 min. Phenoloxidase substrate solution (20 mM dopamine in 50 mM sodium phosphate, pH 6.5; 200 μl) was added, and PO activity was measured by monitoring absorbance at 470 nm in a microplate reader. Three replicates with three different larval plasma samples were examined.

SRPN2 Knockdown by RNAi. DsSRPN2 was produced and injected, and its knockdown was monitored as described (9).

Bead Melanization Assays. Assays were performed essentially as described in ref. 37. Negatively charged CM-25 Sephadex beads (Sigma-Aldrich, St. Louis, MO) were rehydrated in 1.3 mM NaCl, 0.5 mM KCl, and 0.2 mM CaCl2 buffer (pH6.8) with 0.001% methyl green. Single beads with diameters ranging from 40 to 80 μm were injected on ice into the thoraces of individual mosquito 4 days after dsRNA treatment. Beads were recovered 24 h after inoculation, examined by microscopy for level of melanization, and grouped according to (*i*) the percentage of the bead surface that was covered by melanin and (*ii*) the thickness of the melanin layer. Three broad classes of MR were identified: MR0 (<100% melanin coverage); MR1 (100% melanin coverage, thickness = 0); MR2 (100% melanin coverage, thickness = 1). Bead melanization in control and treatment groups was compared with an ordinal logistic analysis of the three classes including the replicate as a random confounding factor.

***Plasmodium falciparum* Carrier Recruitment.** *P. falciparum* gametocyte carriers were recruited from five to eleven-year old children in the School district of Mfou, 30 km from Yaoundé, Cameroon. Surveys were carried out in collaboration with the medical team in charge of malaria treatment at the local hospital. Thick blood smears were taken from each volunteer, air-dried, Giemsa-stained, and examined by microscopy for the presence of *P. falciparum*. Children with asexual parasitemia (>1,000 parasites per microliter) were treated in accordance with national guidelines. Asymptomatic gametocyte-positive children were recruited for the study. This procedure was approved by the Cameroonian and World Health Organization ethical review committees.

Experimental Mosquito Infections. Mosquito infections were performed by artificial membrane feeding. Gametocyte carrier blood was collected by venipuncture into heparinized tubes. To avoid transmission blocking immune factors from the gametocyte carrier (30), the blood was centrifuged at 2,000 $\times$ *g*, and the serum was replaced with nonimmune AB serum, adjusting the hematocrit to 50%. Female mosquitoes, 4 d after dsRNA injection, were allowed to feed on this blood mixture ad libitum through Parafilm membrane. Nonfed or partially fed females were removed after 24 h. Oocysts were counted 7 days after feeding by mercurochrome staining of midguts. *P. falciparum* gametocytes were counted against 500 leukocytes, and the estimate of gametocyte density was obtained assuming a standard number of 8,000 leukocytes per microliter of blood.

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