

# *Anopheles gambiae* immune responses to Sephadex beads: Involvement of anti-*Plasmodium* factors in regulating melanization <sup>☆</sup>

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## Abstract

We have performed a global genome expression analysis of mosquito responses to CM-25 Sephadex beads and identified 27 regulated immune genes, including several anti-*Plasmodium* factors and other components with likely roles in melanization. Silencing of two bead injection responsive genes, *TEPI* and *LRIM1*, which encode proteins known to mediate *Plasmodium* killing, significantly compromised the ability to melanize the beads. In contrast, silencing of two *Plasmodium* protective c-type lectins, *CTLA* and *CTLMA2*, did not affect bead melanization. This data suggest that the anti-*Plasmodium* factors have dual functions, as determinants of both *Plasmodium* killing and melanization of the parasite and other foreign bodies, while the *Plasmodium* protective factors are specifically utilized by the parasite for evasion of mosquito defense mechanisms.

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## 1. Introduction

Insects are continuously exposed to a diverse array of pathogenic microbial flora, viruses, and parasites, against which they defend themselves by means of structural barriers and innate immune responses. Activation of immune responses is initiated by the recognition of an invading pathogen via pattern recognition receptors that can interact with pathogen-associated molecular patterns,

such as bacterial lipopolysaccharide (LPS) and peptidoglycan. This immune response activation can be either direct, as in the case of phagocytosis, or indirect, through the mediation of serine protease signal amplification cascades and/or intracellular signaling pathways that control transcription of antimicrobial effector genes (Dimopoulos, 2003).

Melanization plays a key role in the invertebrate defense system through wound healing and pathogen encapsulation. This process involves the enzymatic oxidative conversion of tyrosine to eumelanin and is mediated by prophenoloxidases (PPOs) which, in turn, are activated by serine protease cascades (Soderhall and Cerenius, 1998; Christensen et al., 2006). One of the best-studied defense mechanisms against the malaria parasite *Plasmodium* in *Anopheles* mosquitoes is the melanotic encapsulation of ookinetes and early oocysts (Collins et al., 1986). This melanization is thought to occur through the deposition of melanin, which is derived from the hemolymph, on the

<sup>☆</sup>EW conducted the microarray assays, synthesis of dsRNAs, and the quantitative RT-PCR (validation of microarray expression data and efficiency of gene silencing) and wrote the manuscript. LL conducted the time-course analysis of bead melanization, melanization assays with gene-silenced mosquitoes, RNA preparation, and the statistical analysis.

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parasite surface (Gorman et al., 1998; Blandin et al., 2004). Genetic mapping experiments with a refractory *Anopheles gambiae* strain, that can encapsulate a variety of *Plasmodium* parasites, have identified three quantitative trait loci, *Pen1*, *Pen2*, and *Pen3*, that control the melanotic encapsulation of *Plasmodium cynomolgi* B (Zheng et al., 1997, 2003). Two of these loci, *Pen2* and *Pen3*, are also implicated in encapsulating a closely related species *P. cynomolgi* Ceylon while the third locus appears to be unique for encapsulation of this parasite. Furthermore, different QTLs were shown to differ in their contribution to parasite melanization between different refractory mosquito families. The L3-5 refractory strain has therefore been considered as genetically heterogeneous and melanization of different parasites appears to be controlled by somewhat different pathways (Zheng et al., 2003).

Recent studies have identified four mosquito genes (*TEPI*, *LRIMI*, *CTLA*, and *CTLMA2*) that encode proteins that can affect the ability of both melanization-capable refractory and susceptible mosquitoes to kill the *Plasmodium* parasites (Blandin et al., 2004; Osta et al., 2004a). The thioester-containing protein 1 (*TEPI*), a complement-like protein, has been shown to specifically bind to the surface of the ookinete stage of *Plasmodium* parasites in susceptible mosquitoes (Blandin et al., 2004). RNAi-mediated depletion of *TEPI* causes a five-fold increase in the number of oocysts developing in the midgut of susceptible mosquitoes, while in the melanizing refractory strain *TEPI* silencing abolishes parasite killing and oocysts develop normally (Blandin et al., 2004). *TEPI* has been localized between the ookinete surface and the melanin layer of the capsule but it has not been clear whether its presence is necessary for the melanization reaction in addition to the killing (Blandin et al., 2004). In susceptible mosquitoes, ookinetes that appear to be more strongly bound by *TEPI* are often smaller than normal and have condensed nuclei; findings which suggest that these parasites have been killed during the invasion process (Blandin et al., 2004). These observations suggest that *TEPI* can mediate plasmodiocidal activity independently of a melanization reaction (Blandin et al., 2004).

Silencing of another anti-*Plasmodium* factor, the leucine rich-repeat immune protein 1 (*LRIMI*), has been reported to increase oocyst loads by 3.6-fold in the midgut of susceptible mosquitoes, with no melanization of parasites (Osta et al., 2004a). *LRIMI* is expressed in both the carcass tissues and midgut of naïve mosquitoes and is specifically up-regulated upon *Plasmodium* invasion of the midgut epithelium (Osta et al., 2004a). Similarly to *TEPI* the implication of *LRIMI* in melanization has not been previously addressed. While *TEPI* has been shown to participate in the mosquito's anti-bacterial defense through its role in phagocytosis, it is not clear whether *LRIMI* has antimicrobial action or is specific for defense against *Plasmodium* parasites (Levashina et al., 2001; Osta et al., 2004a).

Two *Plasmodium* infection-responsive c-type lectins, *CTLA* and *CTLMA2*, have been shown to play a protective role in parasite development in the midgut; RNAi-

mediated depletion of these two lectins in a susceptible *A. gambiae* strain results in melanization of *Plasmodium* ookinetes (Osta et al., 2004a). These two mosquito factors appear to promote susceptibility of the mosquito to *Plasmodium* by inhibiting killing by *LRIMI*, and the subsequent melanization of the invading parasites (Osta et al., 2004a). It is interesting that melanization of parasites does not occur when *LRIMI* is silenced along with either of these two c-type lectins; this finding suggests that either the melanization occurs after *Plasmodium* killing by *LRIMI* or that the melanization requires *LRIMI* upon silencing of the c-type lectins (Osta et al., 2004a).

Both *Plasmodium*-refractory and -susceptible *A. gambiae* can also melanize Sephadex beads, with the melanization response being stronger in the refractory strain than in the susceptible strain (Gorman et al., 1998; Chun et al., 1995; Paskewitz and Riehle, 1994). In a melanization reaction similar to that seen for ookinetes in refractory *A. gambiae* midguts, beads are melanized through a humoral mechanism, rather than a cellular hemocyte-mediated process (Chun et al., 1995; Gorman et al., 1998). The strongest *P. cynomolgi* B encapsulation QTL, *Pen1*, has also been linked to the melanization of Sephadex beads, suggesting that the regulation of bead and *Plasmodium* involves at least some of the same pathways and components (Gorman et al., 1997). More recent studies have shown that the genetic basis of encapsulation mediated refractoriness can differ for different parasite species (discussed above) (Zheng et al., 2003; Niare et al., 2002).

Bead melanization can serve as a model for studies of processes that mediate parasite encapsulation (Gorman et al., 1997). In a natural population of *A. gambiae*, a positive genetic correlation between Sephadex bead melanization and bacterial clearing (not melanization) has been documented, suggesting that melanization and anti-microbial defense share common components and mechanisms (Lambrechts et al., 2004). The rate and intensity of bead melanization depend on its surface charge. Neutral Sephadex beads (carbohydrate matrix) are melanized more rapidly and strongly than are negatively charged Sephadex beads (with the same matrix but also containing a carboxymethyl functional group); glass beads, like other immunologically inert and non-charged bodies, are not melanized at all (Paskewitz and Riehle, 1994, 1998; Gorman et al., 1998). These differences suggest that bead melanization exhibits a certain degree of specificity and is not a spontaneous event occurring upon invasion by any entity recognized as non-self.

In the present study, we have characterized the *A. gambiae* molecular immune responses to implanted CM-25 Sephadex beads and made use of RNAi-mediated gene-silencing assays to analyze the role of selected bead inoculation-inducible immune genes in regulating the melanization process. These beads are known to be melanized by a genetically selected refractory strain, similarly to *Plasmodium*, but not by a selected susceptible 4arr strain (Paskewitz and Riehle, 1998).

## 2. Experimental procedures

### 2.1. Mosquitoes

All experiments were performed with the *A. gambiae* Yaoundé strain. Mosquitoes were reared as described in Lavazec et al., 2005. The Yaoundé *A. gambiae* strain, used for these studies, can transmit both *Plasmodium berghei* and *Plasmodium falciparum* parasites and does rarely melanize parasites (Bonnet et al., 2001; Tahar et al., 2002; Lambrechts, unpublished observations).

### 2.2. Melanization assays

CM-25 Sephadex beads (Sigma-Aldrich, Steinheim, Germany) range from 40 to 120  $\mu\text{m}$  in diameter, and visual inspection was used to select the smallest beads for inoculation. Beads were rehydrated in saline solution (1.3 mM NaCl, 0.5 mM KCl, 0.2 mM CaCl<sub>2</sub> [pH 6.8]) and stained with 0.001% methyl green to aid in visualization (Paskewitz and Riehle, 1994). Five-day-old adult female *A. gambiae* were immobilized briefly on ice, and one bead per mosquito was inoculated with <0.1  $\mu\text{L}$  of saline solution through the left side of the thorax into the hemolymph, using a heat-pulled capillary needle (Paskewitz and Riehle, 1994). Mosquitoes that were able to fly at 24 h post-inoculation (for gene silencing experiments) or at 30 min to 48 h post-inoculation (for the time-course experiment) were frozen and dissected in a mixture of saline solution and 0.01% methyl green. Beads were recovered, and melanization was scored according to three categories: no visible melanization, patchy melanization (dotted or partially melanized beads), and complete melanization. A nominal logistic analysis of the three melanization categories was used to determine statistical differences (Agresti, 1990; Hosmer and Lemeshow, 2000). In all analyses, replicate (considered a random factor) was included as a potential confounder.

### 2.3. RNA extractions

Total RNA was extracted from each whole mosquito sample using the Tri-Reagent<sup>®</sup> kit (M.R.C. Inc., Ont., Canada) according to the manufacturer's instructions. RNA was treated with the DNA-free<sup>®</sup> kit (Ambion, Austin, TX, USA).

### 2.4. Probe sequence design and microarray construction

Release 2a *A. gambiae* cDNA sequences were retrieved from Ensembl ([www.ensembl.org/Anopheles\\_gambiae](http://www.ensembl.org/Anopheles_gambiae)). These sequences were predicted using a combination of ab initio, EST, and protein similarity-based methods (Birney et al., 2004; Curwen et al., 2004; Stalker et al., 2004). The transcripts were annotated with the EnsMart utility (Hammond and Birney, 2004; Kasprzyk et al., 2004). Sixty-mer oligonucleotides for the 14,180 predicted

*A. gambiae* transcripts that corresponded to 13,118 genes were designed using Oligo Picky software according to the software developer's instructions (Chou et al., 2004). Oligonucleotide sequences were designed to be complementary to regions within 1 kb of the 3' UTR of transcripts and had a minimal sequence identity overlap with non-target transcript sequences. Microarrays were constructed through *in situ* synthesis of oligonucleotides on glass slides by Agilent Technologies ([www.agilent.com](http://www.agilent.com)).

### 2.5. Microarray analysis

Cy-3 and -5 fluorochrome labeled cRNA probes were synthesized from 2 to 3  $\mu\text{g}$  RNA using the Agilent Technologies low-input linear amplification RNA labeling kit according to the manufacturer's instructions. Probe quantity was determined with a Beckman DU640 spectrophotometer, and 16-h hybridizations were performed with the Agilent Technologies *in situ* hybridization kit according to the manufacturer's instructions. After the prescribed washes, microarrays were instantaneously dried with pressurized air. Microarrays were scanned with an Axon GenePix 4200AL scanner using a 10- $\mu\text{m}$  pixel size. Laser power was set to 60%, and the PMT was adjusted to maximize the effective dynamic range and minimize the pixel saturation (Axon Instruments, Union City, CA). The spot size, location, and quality were determined using GenePix software Pro 6.0 algorithms, and potential misidentifications of spot location and quality were corrected manually. Scan images were analyzed, and Cy-5 and -3 signal and ratio values were obtained using GenePix software. The minimum signal intensity was set to 100 fluorescent units, and the signal-to-background ratio cut-off was set to 2.0 for both Cy-5 and -3 channels. Three biological replicates were performed for each experimental set. The background-subtracted median fluorescent values for good spots (no bad, missing, absent, or not found flags) were normalized according to a LOWESS normalization method to eliminate dye-specific influences on expression ratios, and Cy-5/3 ratios from replicate assays were subjected to *t*-tests at a level of significance of  $P = 0.05$  using the TIGR MIDAS and MeV software (Dudoit et al., 2003). Expression data from all replicate assays were averaged with the GEPAS microarray preprocessing software prior to logarithm (base 2) transformation (Herrero et al., 2003). Previous self-hybridization assays with the same type of microarrays established a lower-limit cut-off value for the significance of gene regulation of 0.8 in log<sub>2</sub> scale, which corresponds to 1.7-fold regulation according to previously established methodology (Yang et al., 2002). Microarray-assayed gene expression of seven genes was further validated with quantitative real-time polymerase chain reaction (RT-PCR) (Pearson correlation coefficient  $P = 0.271$ ; best-fit linear-regression  $R^2 = 0.2343$ ; and the slope of the regression line  $m = 0.8962$ ) (Table 1).

## 2.6. RNAi gene silencing

Sense and antisense RNAs were synthesized from PCR-amplified gene fragments using the T7 Megascript kit according to the manufacturer's instructions (Ambion). The sequences of the primers used are listed in the supplementary materials section (Table S2). One-day-old adult females were injected with 101.2 nL of dsRNA (2 mg/mL, in RNase free water) on the right side of the thorax using a Nanoject apparatus (Drummond, Broomall, PA, USA) and allowed to recover for 4 days before the bead melanization assay (Blandin et al., 2004). A GFP dsRNA was injected in the control group mosquitoes for comparative assays to the gene-silenced mosquitoes.

## 2.7. Real Time quantitative (RTQ) PCR

The efficiency of gene silencing was established using RT quantitative PCR (RTQ-PCR) 4 days after dsRNA injection (i.e., at the time of bead inoculation) (Fig. 3B). Expression data were also validated by RTQ-PCR on the same samples used for microarray hybridizations. RNA was extracted from the female mosquitoes and reverse-transcribed using Superscript III (Invitrogen) with random hexamers. RTQ-PCR reactions were performed using the

QuantiTect SYBR Green PCR Kit (Qiagen) and ABI Prism 7300 Detection System. All PCR reactions were performed in triplicate. The *A. gambiae* ribosomal protein S7 gene was used for normalization of cDNA templates. Quantification was determined by the Pfaffl method (Pfaffl, 2001). The sequences of primers used are listed in the supplementary materials section (Table S2).

## 3. Results and discussion

### 3.1. Bead melanization kinetics follow an acute-phase response pattern

Melanization response kinetics to inoculated beads were determined at 1- to 12-h intervals for up to 2 days after inoculation in order to determine an appropriate time point for assaying bead inoculation-inducible gene expression (Fig. 1A). Bead melanization was very rapid; the percentage of partially or completely melanized beads increased with time to 90% during the first 24 h after bead inoculation, after which a plateau was reached in this mosquito strain (Fig. 1A). Although the first melanin spots were observed after 30 min in only 10% of the mosquitoes, most (80%) had started melanizing the beads at 3 h post-inoculation (Fig. 1A). This rapid response suggests that

Table 1  
Induction of immune genes after inoculation of individual beads into female *A. gambiae*

ID	gene	Fold regulation	
		Array	RTQ-PCR
ENSANGT00000013041	Leucine rich-repeat immune protein 1 (LRIM1)	2.1	1.98
ENSANGT00000006849	Leucine-rich repeat (LRR)	2.9	
ENSANGT00000014371	Leucine-rich repeat (LRR)	1.7	
ENSANGT00000011338	Leucine-rich repeat (LRR)	2.3	
ENSANGT00000006045	Leucine-rich repeat (LRR)	2.2	
ENSANGT00000018394	Leucine-rich repeat (LRR)	2.1	
ENSANGT00000016857	Thioester-containing protein (TEP1)	1.6	1.93
ENSANGT00000006629	Thioester-containing protein (TEP13)	2.2	
ENSANGT00000018121	Thioester-containing protein (TEP9)	2	
ENSANGT00000008943	Gram-negative bacteria-binding protein (GNBPA2)	2.4	3.41
ENSANGT00000017694	Gram-negative bacteria-binding protein (GNBPB1)	1.9	
ENSANGT00000011564	Fibrinogen domain immunolectin (FBN34)	2.3	3.86
ENSANGT00000015683	Fibrinogen domain immunolectin (FBN)	2.1	
ENSANGT00000016439	Fibrinogen domain immunolectin (FBN)	2	
ENSANGT00000008282	Fibrinogen domain immunolectin (FBN)	1.8	
ENSANGT00000016460	Fibrinogen domain immunolectin (FBN)	1.7	
ENSANGT00000025439	PGRPLC1	1.9	3.33
ENSANGT00000010670	Galectin (GALE6)	1.8	
ENSANGT00000021903	Scavenger receptor SCRQB3	2.1	
ENSANGT00000015891	Scavenger receptor	1.8	
ENSANGT00000008141	C-type lectin (CTLSE2)	2	
ENSANGT00000010646	Serine protease	1.8	4.06
ENSANGT00000019907	Serine protease SNAKE	2.2	
ENSANGT00000002437	Prophenoloxidase (PPO3)	2.3	
ENSANGT00000022667	Prophenoloxidase (PPO6)	1.7	3.24
ENSANGT00000009751	Hemocyanin	2.1	
ENSANGT00000027038	Defensin	2.4	

The expression data for seven genes obtained by microarray analysis were validated using real-time quantitative polymerase chain reaction (RTQ-PCR) (Pearson correlation coefficient  $P = 0.271$ ; best-fit linear-regression  $R^2 = 0.2343$ ; and the slope of the regression line  $m = 0.8962$ ).

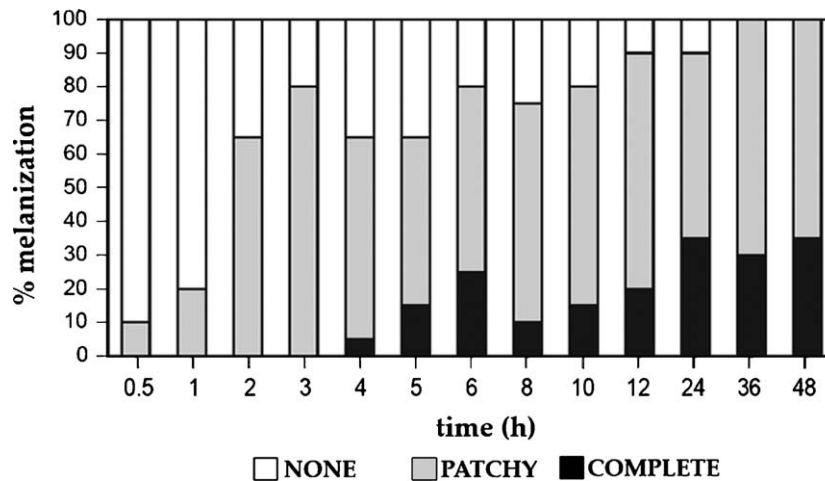


Fig. 1. Time-course analysis of bead melanization. The proportion of mosquitoes with no, patchy, or complete melanization of the bead is given for different times post-inoculation. Each bar corresponds to 20 mosquitoes from two independent replicate experiments. While there was no significant difference between the replicates (d.f. = 2;  $\chi^2 = 1.71 \times 10^{-13}$ ;  $P = 1.0$ ), even at particular time-points (d.f. = 24;  $\chi^2 = 20.2$ ;  $P = 0.687$ ), the increase in melanization over time was highly significant (d.f. = 24;  $\chi^2 = 123.7$ ;  $P < 0.0001$ ).

many of the proteins required for melanization were already present in the mosquito hemolymph before inoculation and may have been further enriched through bead inoculation-inducible gene expression. The relatively small proportion of completely melanized beads, even after 48 h of incubation, suggests the existence of melanization-inhibitory factors that either inhibit the melanization reaction in the vicinity of the bead or bind to and mask the bead. Soluble extracellular matrix proteins, for instance, could play a protective role of this kind (Gorman et al., 1998). The inflicted injury and the associated opportunistic infections upon bead injection is unlikely to be much greater than that caused by injection of PBS which is done through the same type of microcapillary needle. In fact, mortality of PBS injected control mosquitoes were quite similar to bead inoculated mosquitoes in these assays (data not shown).

### 3.2. Genome responses to inoculated Sephadex beads

We used a microarray-based approach to assess the global gene expression response of female mosquitoes to injected Sephadex beads, comparing the gene expression of bead-injected and PBS-injected mosquitoes. This experimental strategy was chosen since it was unclear to which degree the transcriptional induction of potential bead melanization factors was dependent on the specific bead surface characteristics or simply the presence of any foreign body or injury. Due to the rapid initiation of melanization, an early time point of 2 h after bead inoculation was chosen for the gene expression assay (Fig. 1A). The robustness of the microarray assays was validated by quantifying transcript abundance of selected genes using RTQ-PCR assays (Table 1). The global gene expression response was comprised of 267 up-regulated and 38 down-regulated

transcripts that were differentially regulated by at least 1.7-fold (Fig. 2, Supplemental Table 2). The major functional classes of bead-inoculation regulated genes encoded immune response-related (discussed in greater detail below), redox/stress-related, and cytoskeletal/structural proteins (Fig. 2). A large proportion of bead-induced transcripts encoded stress-related proteins (Fig. 2). Regulation of some of these genes may reflect the toxicity of intermediary compounds that are produced during the melanization reaction. Of the 12 redox/stress-related transcripts that were up regulated, three encode cytochrome P450s (ENSANGT00000008167, ENSANGT00000024354, ENSANGT00000016217), a family that plays an important role in detoxification. Only one redox-related transcript, encoding an aldo keto reductase (ENSANGT00000008370), was down regulated after bead inoculation (Fig. 2). Furthermore, 11 transcripts involved in cytoskeletal/structural functions were also up regulated; such genes are likely to play an important role in wound healing and hemocyte migration (Fig. 2). One of these transcripts, encoding a putative cuticle protein (ENSANGT00000026505), was up regulated by 2.3-fold 2 h after bead inoculation (Supplemental Table 2). Cuticle proteins are likely to be involved in wound healing and have also been shown to be expressed by hemocytes (Muñoz et al., 2002; Bartholomay et al., 2004). Several of the induced transcripts were related to housekeeping functions (transport, metabolism, proteolysis/digestion, replication/transcription/translation) (Fig. 2, Supplemental Table 2). It is likely that the experimental approach of assaying mRNA abundance in the whole mosquitoes failed to detect transcripts that were induced in specific cell types, such as the hemocytes, because of their low overall representation in the total RNA. Comparatively, mosquito global gene expression response 4 h after a low

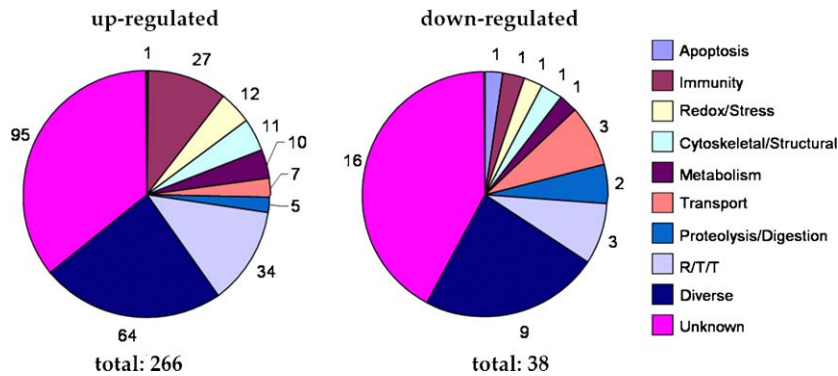


Fig. 2. Pie charts showing the functional class distribution of the 266 upregulated and the 38 down-regulated genes at 2h after bead inoculation into female *Anopheles gambiae*. The functional classes are indicated in the figure.

dose of heat-inactivated *Staphylococcus aureus*, *Salmonella typhimurium*, and *Beauveria bassiana* comprised 289 up-regulated transcripts, including genes of immune and stress function, and 54 down-regulated transcripts (Aguilar et al., 2005).

### 3.3. Sephadex beads elicit molecular immune responses

Twenty-seven of the up-regulated genes encoded putative proteins predicted to be implicated in the mosquito's innate immune system, based on their homology to known immune genes and previous studies (Table 1). Only one immune-related gene, cecropin (ENSANGT00000011963), was down regulated upon bead inoculation (Fig. 2). Of the 27 up-regulated putative immune genes, 20 (71%) encode putative pattern recognition receptors (Table 1) that are members of the TEP, Gram-negative bacteria-binding protein (GNBP), fibrinogen domain immunolectin (FBN), scavenger receptor, peptidoglycan recognition protein (PGRP), and lectin and leucine rich repeat (LRR) families. At least some of these pattern-recognition receptors may be involved in the bead melanization process, either directly by participating in capsule formation as proteins cross linked with melanin or indirectly by triggering the activation of serine protease cascades that activate PPOs. Among these putative pattern recognition receptor genes were the anti-*Plasmodium* *TEP1* and *LRIM1*, which were both up-regulated by approximately two-fold after bead inoculation (Table 1). In contrast, the two *Plasmodium* protective c-type lectins *CTLMA2* and *CTLA* did not show significant differential regulation (data not shown). *TEP1* is known to mediate the phagocytosis of bacteria and killing of *Plasmodium* in the midgut epithelium (Levashina et al., 2001; Blandin et al., 2004). A *Drosophila melanogaster* GNBP is implicated in the activation of the Toll pathway, together with a PGRP (Gobert et al., 2003), and some FBN immunolectins have been linked with anti-*Plasmodium* defense (Dimopoulos lab, unpublished observations). PGRPs have also been implicated in the activation of melanization reactions (Lee et al., 2004). In the present study, two serine proteases were

up-regulated after bead inoculation, a finding that may point to their involvement in proteolytic cascades that activate PPOs (Table 1) (Soderhall and Cerenius, 1998; Volz et al., 2005). Two PPOs and a hemocyanin gene were also induced by bead inoculation (Table 1). Hemocyanin is a phenoloxidase-like enzyme and may also play a role in the melanization reaction (Cerenius and Soderhall, 2004). These and previous data confirmed the immune-eliciting capacity, at the transcriptional level, of Sephadex beads in *A. gambiae* and suggests that putative pattern-recognition receptors, such as *TEP1* and *LRIM1*, can recognize these foreign bodies; the *A. gambiae* innate immune responses to challenge are to a significant degree regulated at the transcriptional level (Dimopoulos et al., 2002, 1998). Mosquito responses to a low dose of heat-inactivated *S. aureus*, *S. typhimurium*, and *B. bassiana* induced 38 putative immune genes at 4h after injection, suggesting a potentially lower immune-eliciting capacity of the bead surface, which lacks the complexity and variety of injected bacteria. The responses to bacteria challenge were also quite different at the qualitative level, with only one immune gene, *TEP1*, being up-regulated by both bead and bacteria injection (Aguilar et al., 2005).

### 3.4. Role of immune genes in bead melanization

An RNAi-mediated gene-silencing approach was used to assess the capacity of selected immune genes to influence the bead melanization reaction. In total, 10 genes were chosen for gene silencing (Fig. 3A); eight genes encoding *TEP1*, *LRIM1*, *PGRPLC1*, *GNBPA2*, *PPO6*, *SP10646*, *FBN9* and *FBN34* were selected from those that were transcriptionally induced by bead injection. Among the bead injection induced genes, *TEP1* and *LRIM1* were chosen because of their known implication in *Plasmodium* killing prior to melanization (Osta et al., 2004a; Blandin et al., 2004). GNBPs and PGRPs have been proposed to function as melanization promoting pattern-recognition receptors. Serine proteases are part of the prophenoloxidase activation system and phenoloxidases initiate the oxidative conversion of tyrosine to eumelanine (Soderhall

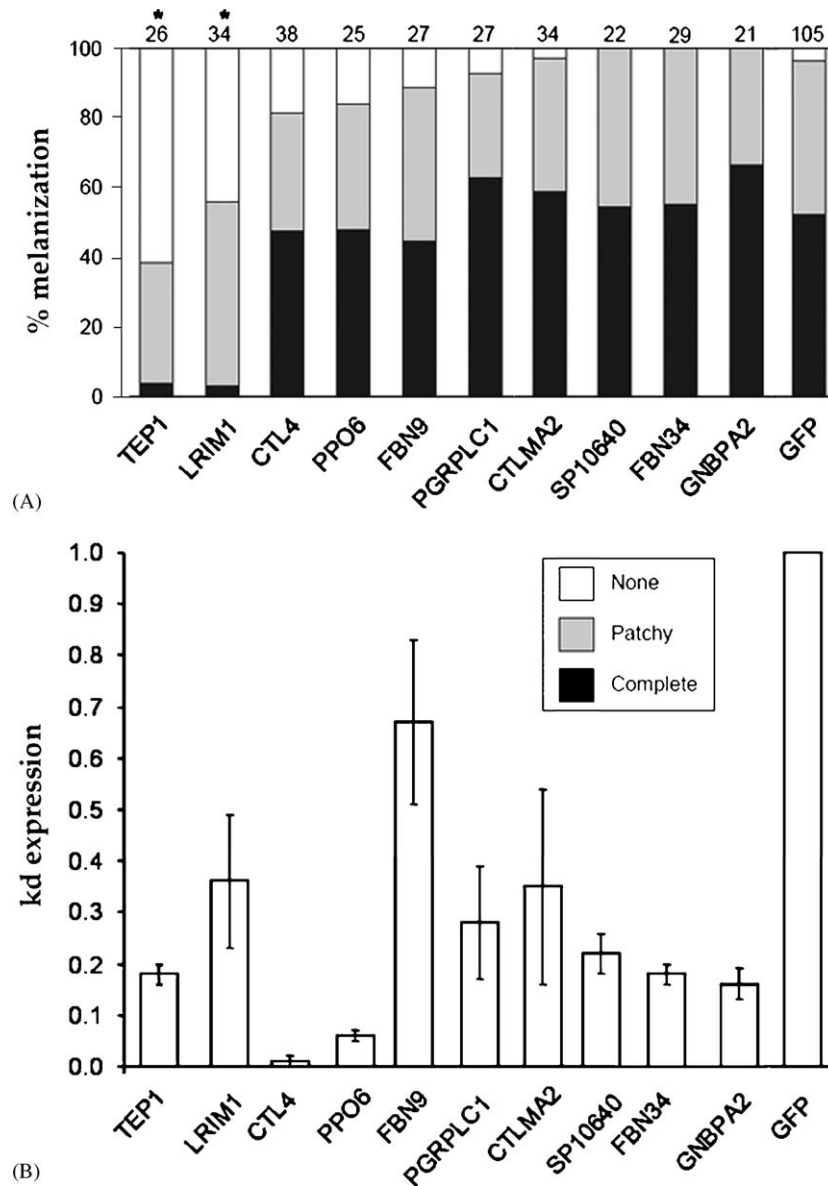


Fig. 3. (A) Effect of gene silencing (KD) on bead melanization in female *A. gambiae*. At 24 h post-inoculation, beads were recovered, and melanization was scored according to three categories: no visible melanization, patchy melanization (dotted or partially melanized beads), and complete melanization; according to nominal statistic analysis with  $P < 0.01^*$ . Mosquitoes injected with a GFP dsRNA served as controls. Assays were replicated three times and the numbers of mosquitoes inoculated are also indicated for each silenced gene. (B) Efficiency of specific gene-silencing (KD) was determined by real-time quantitative PCR as a measure of % transcript depletion compared to the GFP dsRNA injected control mosquitoes used as control. All assays were replicated three times and error bars represent standard error of the mean.

and Cerenius, 1998; Volz et al., 2005; Lee et al., 2004; Cerenius and Soderhall, 2004) (Fig. 3A). Of the two bead inoculation induced PPOs, *PPO6* was selected for silencing assays because of its high expression at the adult stages of female *A. gambiae*, in contrast to *PPO3* which is mostly expressed at the larval stages (Table 1) (Muller et al., 1999). Two novel immune factors, FBN9 and FBN34, which encode FBNs were included in the screen because of their implication in antimicrobial and anti-*Plasmodium* defense (Dimopoulos lab, unpublished data). Of the tested immune factors, only *TEP1* and *LRIM1* showed a strong influence on the melanization of beads ( $P < 0.001$ ); their

RNAi-mediated depletion prior to bead inoculation caused a significant decrease (~55% and 40%, respectively) in the percentage of beads that were partially or completely melanized (Fig. 3A). The other genes, whose silencing did not affect the melanization phenotype significantly, may still participate in the melanization reaction but not play a regulatory role (Table 1). Indeed, all the tested genes are members of larger gene families whose potential functional redundancy might mask the single-gene-silencing phenotype. The proteins produced by some of these genes may also be stable over time and therefore not efficiently depleted upon gene silencing, or the genes may be

expressed in tissues and cell types that are inaccessible to the injected dsRNA (Fig. 3B). The c-type lectins *CTLA* and *CTLMA2* were also included in the transient reverse genetic screen because of their known implication in protecting *Plasmodium* parasites from mosquito killing and melanization (Osta et al., 2004a) (Fig. 3A). It is interesting that these two factors had no effect on the melanization phenotype after RNAi depletion and are not differentially expressed upon bead inoculation (Fig. 3A, data not shown). This result suggests that these mosquito factors are specifically employed by *Plasmodium* parasites for protection against defense reactions, rather than providing general protection against pathogens or foreign bodies in the hemolymph.

#### 4. Conclusions

CM-25 Sephadex beads are readily melanized in mosquitoes by mechanisms that to some extent are controlled by some of the same genetic loci that are responsible for encapsulating certain *Plasmodium* species (Zheng et al., 2003; Gorman et al., 1997). Several genes that are involved in pattern recognition and serine protease cascade regulation have been shown to be capable of influencing the melanotic encapsulation of *Plasmodium* (Blandin et al., 2004; Osta et al., 2004a; Abraham et al., 2005; Michel et al., 2005; Volz et al., 2005). Four of these genes, *TEP1*, *LRIM1*, *CTLA*, and *CTLMA2*, are of particular interest because of their role in killing and protecting *Plasmodia* from both melanizing refractory and non-melanizing susceptible strains of *A. gambiae* (Blandin et al., 2004; Osta et al., 2004a).

The global gene expression responses of female mosquitoes to bead inoculation showed an up-regulation of 27 immune genes of which at least two, *TEP1* and *LRIM1*, could regulate melanization (Fig. 3). Among the up-regulated genes were also several other factors with likely roles in melanotic encapsulation, including PPOs, serine proteases, and several types of pattern recognition receptors (Table 1). While the majority of the selected genes had no effect, gene silencing of the anti-*Plasmodium* factors *TEP1* and *LRIM1* significantly influenced the mosquito's capacity to melanize the Sephadex beads (Fig. 3), suggesting an essential role for these proteins in the mediation of melanization, as well as *Plasmodium* killing (Fig. 4). Both *TEP1* and *LRIM1* have adhesive properties, and *TEP1* has been shown to associate with the parasite (Fig. 4) (Blandin et al., 2004; Osta et al., 2004a, b). These factors are most likely associating directly with the bead surface, and from which they either recruit the melanization factors or serve as part of the proteinaceous capsule that is cross-linked and melanized. *Plasmodium* parasites are able to utilize the mosquito c-type lectins *CTLA* and *CTLMA2* to protect themselves from being killed and subsequently melanized (Fig. 4) (Osta et al., 2004a). The lack of an effect after silencing suggests that *CTLA* and *CTLMA2* are not involved in protecting foreign bodies from melanization

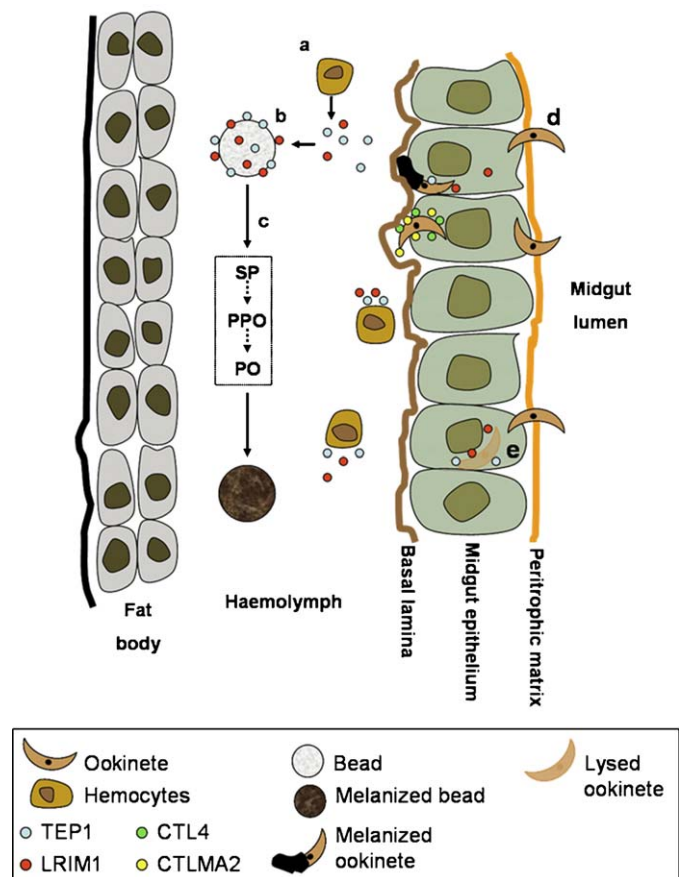


Fig. 4. Schematic hypothetical model of bead melanization and parasite killing in *A. gambiae*. (a and b) *TEP1/LRIM1* produced by the hemocytes and secreted into the hemolymph recognizes and binds to implanted beads within the hemolymph of the mosquito; (c) binding of *TEP1/LRIM1* to the bead activates the melanization machinery, via the prophenoloxidase (PPO) cascade. Binding of *TEP1/LRIM1* to the bead triggers a cascade of serine proteases, which leads to the proteolytic cleavage and activation of PPOs, the key enzymes for melanin production; (d) *TEP1*, *LRIM1*, *CTLA*, and *CTLMA2* are expressed in naïve mosquitoes and are induced during ookinete invasion. *TEP1* and *LRIM1* bind to the parasites and activate melanization. *CTLA* and *CTLMA2* protect the parasites from being killed by the mosquito factors *TEP1* and *LRIM1*; (e) some parasites undergo *TEP1*- or *LRIM1*-mediated lysis within the midgut epithelium.

but instead are specifically involved in protecting *Plasmodium* from being killed (Fig. 3). The lack of effect on melanization that we observed for some of the tested genes may also be a function of redundancy in protein function or stability of the proteins over time.

Our findings, together with those of previous studies, suggest that melanization in *A. gambiae* requires *TEP1* and *LRIM1*, which have also been implicated in *Plasmodium* killing in the absence of melanization; these two factors may play independent roles in the two processes. Although the role of melanization in limiting the vectorial capacity of malaria-transmitting mosquitoes in the field appears to be insignificant, melanization has served as a useful model for studying one of the mechanisms of *Plasmodium* killing because of its easily detected phenotype (Schwartz and Koella, 2002; Collins et al., 1986). A better understanding

of this reaction and other aspects of the anti-*Plasmodium* defense in mosquitoes can facilitate the development of novel control strategies for malaria.

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### Appendix A. Supplementary Materials

The online version of this article contains additional supplementary data. Please visit [doi:10.1016/j.ibmb.2006.07.006](https://doi.org/10.1016/j.ibmb.2006.07.006).

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