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The Route of Infection Influences the Contribution of Key Immunity Genes to Antibacterial Defense in *Anopheles gambiae*

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Keywords

Anopheles gambiae · Mosquito innate immunity · Complement-like protein · C-type lectin · *Serratia marcescens* · Oral infections

Abstract

Insect systemic immune responses to bacterial infections have been mainly studied using microinjections, whereby the microbe is directly injected into the hemocoel. While this methodology has been instrumental in defining immune signaling pathways and enzymatic cascades in the hemolymph, it remains unclear whether and to what extent the contribution of systemic immune defenses to host microbial resistance varies if bacteria invade the hemolymph after crossing the midgut epithelium subsequent to an oral infection. Here, we address this question using the pathogenic *Serratia marcescens* (*Sm*) DB11 strain to establish systemic infections of the malaria vector *Anopheles gambiae*, either by septic *Sm* injections or by midgut crossing after feeding on *Sm*. Using functional genetic studies by RNAi, we report that the two humoral immune factors, thioester-containing protein 1 and C-type lectin 4, which play key roles in defense against Gram-negative bacterial infections, are essential for defense against systemic *Sm* infections established through injection, but they become dispensable when *Sm* infects the hemolymph following oral infection. Similar results were observed for the mosquito Rel2 pathway. Surprisingly, blocking phagocytosis by cytochalasin D treatment did not affect mosquito susceptibility to *Sm* infections established through either route. Transcriptomic analysis of mosquito midguts and abdomens by RNA-seq revealed that the transcriptional response in these tissues is more pronounced in response to feeding on *Sm*. Functional classification of differentially expressed transcripts identified metabolic genes as the most represented class in response to both routes of infection, while immune genes were poorly regulated in both routes. We also report that *Sm* oral infections are associated with significant downregulation of several immune genes belonging to different families, specifically the clip-domain serine protease family. In sum, our findings reveal that the route of infection not only alters the contribution of key immunity genes to host antimicrobial defense but is also associated with different transcriptional responses in midguts and abdomens, possibly reflecting different adaptive strategies of the host.

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Introduction

Insects deploy several humoral and cellular innate immune effector mechanisms to clear bacterial infections. While antimicrobial peptides (AMPs) [1, 2], melanization [3, 4], phagocytosis [5, 6], and complement-mediated attack [7, 8] are often described as the main players in different contexts, several knowledge gaps remain as to their regulation, specificity, and relative contribution to microbial clearance. This is further complicated by the fact that the vast majority of bacterial challenges in model insects have been established through an artificial route, by pricking the cuticle to introduce the microbes directly into the hemocoel [9–12]. While this route of infection has allowed the dissection of systemic antimicrobial immune responses at different levels, it is associated with 2 major pitfalls: first, microbes are often introduced at large numbers to trigger pathogenesis, which might blur the readouts from distinct effector programs due to saturation effects; low-dose infections are most likely the norm in field conditions. For instance, it was recently shown that when a low dose of *Staphylococcus aureus* is injected into *Drosophila*, the melanization response but not hemocytes or Toll effectors plays a significant role in resisting the infection, whereas at higher doses, the role of hemocytes becomes predominant over that of melanization [13]. Second, this route of infection may not allow sufficient priming of the systemic response whether humoral or cellular. For instance, in the malaria vector *Anopheles gambiae*, invasion of the midgut epithelium by *Plasmodium* ookinetes triggers the release of a hemocyte differentiation factor, constituted of a lipoxin/lipocalin complex, into the hemolymph, which induces immune priming, preparing the host for a subsequent challenge. Lipocalin is produced by the abdominal wall, possibly in response to unknown signals originating from the invaded midgut [14]. Also, *Plasmodium* midgut invasion triggers the nitration of the basal surface of the midgut epithelium, which upon contact with hemocytes induces the release of hemocyte-derived microvesicles that activate the complement-mediated attack against invading parasites, through unknown factors they deliver [15]. These studies inform that midgut invasion seems to trigger different forms of innate immune priming which might not occur if this route is bypassed.

A large number of functional genetic studies in *A. gambiae* identified several immunity genes with roles in systemic antibacterial defense [11, 16–23]. However, since bacterial challenges in these studies were performed by cuticle pricking, it remains unclear whether these genes significantly contribute to immune defense against systemic infections established through the oral route (i.e., after midgut invasion). This is particularly important since a previous study in *Drosophila* revealed that the virulent *Serratia marcescens* (*Sm*) Db11 strain is resistant to the Imd-mediated immune response during septic infections but is susceptible to the local Imd response in the gut after oral infections [24]. *Sm* is a Gram-negative bacterium with a broad host range including plants, vertebrates, and invertebrates [25], and an opportunistic pathogen to vertebrates [26, 27] and invertebrates [24, 28]. Its ability to efficiently colonize the midguts of insects [24, 29] and to invade the midgut epithelium reaching into the hemolymph [24] makes it an attractive microbe to address whether the route of infection alters the contribution of key immunity genes to systemic immune responses. Furthermore, *Sm* is one of the bacterial species identified frequently as a member of the microbiota in lab- and field-collected *A. gambiae* mosquitoes [30–32], which makes it more relevant to studying host-parasite interactions in this important malaria vector. Certain isolates of *Sm* compromised *Plasmodium* development when introduced into the midgut through a blood or sugar meal, most likely due to certain virulence factors released by the bacteria [30, 31]. However, the physiological relevance of *Serratia* symbiosis in insects remains poorly characterized. Here, we chose *A. gambiae* C-type lectin 4 (CTL4) and thioester-containing protein 1 (TEP1) which exhibit prominent roles in defense against systemic Gram-negative bacterial infections [11, 16, 19, 21] to determine whether the contribution of immune genes to mosquito resistance to *Sm* infections varies with the route of infection (oral vs. injection). TEP1 and CTL4 are required for the clearance of *E. coli* systemic infections [11, 16, 19, 21]; however, the fact that *E. coli* is not pathogenic to mosquitoes and that susceptibility studies require the injection of large numbers of bacteria (approximately 150,000 CFUs [21]) into the hemolymph raise legitimate questions concerning the significance of the immune contribution of these genes using this bacterial infection model and route of infection. To clarify this situation, we used the virulent *Sm* DB11 bacterial strain that kills mosquitoes at much lower CFUs than *E. coli*, to assess the true contribution of CTL4 and TEP1 to antibacterial defense. We also used RNA-seq analysis to determine whether the different routes of infection are associated with distinct transcriptional responses in the midguts and abdomens of infected mosquitoes.

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Materials and Methods

A. gambiae Rearing

All experiments were performed with adult female *A. gambiae* G3 strain mosquitoes. Mosquitoes were maintained at 27 (±1)°C and 75 (±5)% humidity with 12-h day-night cycle. Larvae were reared in 752 cm^2 plastic pans at a density of approximately 150 larvae per pan and given tropical fish food. Freshly emerged adult mosquitoes were collected from larval pans using a vacuum collector and maintained on 10% sucrose and given BALB/c mice blood (mice were anesthetized with ketamine) for egg laying.

Double-Stranded RNA Synthesis and Gene Silencing by RNA Interference

Double-stranded RNA (dsRNA) synthesis was performed using the T7 RiboMax Express Large Scale RNA production system (Promega) according to the manufacturer's instructions, and dsRNAs were purified as previously described [33]. Primers used for dsRNA production are listed in online suppl. Table 1; see www.karger.com/doi/10.1159/000511401 for all online suppl. material. In vivo gene silencing was performed as previously described [33]. In brief, mosquitoes were microinjected with 69 nL of 4 μg/μL solution of gene-specific dsRNA and allowed to recover for 3–4 days before proceeding with *Sm* infections. The efficiency of gene silencing by RNAi for TEP1 and CTL4 was quantified by Western blot in hemolymph extracts of naive mosquitoes at 3 days after dsRNA injection, as previously described [21], using the following dilution of primary antibodies: rabbit αTEP1 (1:1,000) and rabbit αCTL4 (1:1,000). Rabbit αSRPN3 (1:1,000) was used to control for loading. The silencing efficiency of *Rel1* and *Rel2* was determined by qRT-PCR in naïve mosquitoes at 3 days after dsRNA injection.

RNA Extraction and Real-Time PCR

Total RNA was isolated from whole mosquitoes at the indicated time points using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and contaminant genomic DNA was removed by DNase I treatment. First-strand cDNA was produced from 1 μg of total RNA using the iScript cDNA synthesis kit as described by the manufacturer (Bio-Rad). qRT-PCR was performed in a CFX96 real-time detection system (Bio-Rad) using the SYBR Green JumpStart™ Taq ReadyMix (Sigma-Aldrich) according to the manufacturer's instructions. Relative gene expression was normalized relative to the mosquito gene encoding the ribosomal protein S7 and calculated using the comparative CT method after checking for the efficiency of target amplification. The primers used in qRT-PCR are listed in online suppl. Table 1.

Mosquito Infections with Sm and Survival Assays

Mosquito oral infections with *Sm* were performed by allowing mosquitoes to feed continuously on a sugar solution containing *Sm* that was prepared as follows. DsRed-expressing, gentamycin-resistant *Sm* strain DB11 [24] cultured exponentially at 37°C was washed with PBS and then diluted in a sterile 3% sucrose solution to a final OD_{600} = 1. Mosquitoes that fed on *Sm*-containing sugar pads were sorted out at 24 h after feeding on *Sm* with the help of a food colorant added to the sugar solution and used in subsequent experiments. Mosquitoes were maintained on *Sm*-containing sugar pads for the duration of the experiment. To determine whether our mosquito colony naturally contains *Sm*, DNA was extracted from a pool of 10 midguts dissected from either sugar-fed adult female mosquitoes or mosquitoes that have been feeding on the *Sm* DB11 strain for 24 h, using DNeasy Blood and Tissue Kit (Qiagen). A 100 ng of extracted DNA per sample was used to amplify a 175-bp amplicon of the LuxS gene involved in quorum sensing, using *Sm* LuxS-specific primers, For, 5′-TGCCTGGAAAGCGGCGATGG-3′, and Rev, 5′-CGCCAGCTCGTCGTTGTGGT-3' [34], according to the following program (45 s at 95°C; 60 s at 66°C; 60 s at 72°C) for 33 cycles. As internal control, we PCR amplified a 298-bp amplicon of the gene encoding *A. gambiae* ribosomal protein S7 (*Ag*_S7) using primers, For, 5′-AGAACCAGCAGACCACCATC-3′, and Rev, 5′-GCTGCAAACTTCGGCTATTC-3′, according to the following program (45 s at 95°C; 60 s at 60°C; 60 s at 72°C) for 33 cycles. Amplicons were separated on a 1.2% agarose gel, stained with ethidium bromide, and analyzed on ChemiDoc MP (Bio-Rad).

Septic infections with *Sm* were performed by the intrathoracic microinjection of dsRNA-treated mosquitoes with a suspension of DsRed-expressing gentamycin-resistant *Sm* strain DB11 in PBS (OD_{600} = 0.0005). Mosquitoes treated with dsRNA specific to the β-galactosidase gene (ds*LacZ*) served as control. Mosquito survival was scored over a period of 8–10 days after *Sm* septic or oral infections. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the log-rank test. Experiments were repeated at least 3 times using different mosquito and bacterial batches. At least 50 mosquitoes were utilized per sample per experiment.

Scoring Sm CFUs in Infected Mosquitoes

To determine *Sm* CFUs in whole mosquitoes following septic injections, batches of 8 mosquitoes each per genotype were grinded using a micropestle in 400-μL Luria Bertani (LB) broth at 24 h after *Sm* injections. The homogenate was serially diluted in the LB medium. After overnight culturing at 37°C on LB agar supplemented with gentamycin, CFUs were scored under a fluorescence stereomicroscope.

To determine *Sm* CFUs in the hemolymph, the hemolymph was collected 72 h after oral infections with *Sm* by perfusion as follows. In brief, mosquitoes were perfused with 5 μL of PBS injected into the thorax using a Nanoject II (Drumond Scientific) nanoinjector, and the perfused hemolymph was collected from a small incision made in the 3rd abdominal segment. Hemolymph was collected in sterile ice-cold PBS from batches of 4 or 5 mosquitoes each, serially diluted, and then plated on LB agar with the appropriate antibiotic. CFUs were scored after culturing overnight at 37°C on LB agar supplemented with gentamycin. Statistical significance was calculated using the Mann-Whitney test in GraphPad Prism software (version 6.0). Medians were considered significantly different if $p < 0.05$.

For the bacterial fitness experiment, ds*CTL4* and ds*LacZ* (control) mosquitoes were injected with *Sm* prepared from a fresh bacterial culture (OD_{600} = 0.0005) or *Sm* collected by hemolymph perfusion from wild-type mosquitoes that have been feeding on *Sm* for 24 h. Mosquitoes injected with hemolymph perfusate received 4, 207, and 331 *Sm* CFUs in the 3 independent biological experiments performed. *Sm* proliferation in injected mosquitoes was scored by homogenizing batches of 8 whole mosquitoes each in the LB medium at 24 h after *Sm* injection. The homogenate was serially diluted in the LB medium. CFUs were scored under a fluorescence stereomicroscope after culturing overnight at 37°C on LB agar supplemented with gentamycin.

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Mosquito Treatment with Cytochalasin D

Cytochalasin D was dissolved in DMSO to make a 1 mg/mL stock solution from which a 62.5 μg/mL (120 μM) working solution in PBS was prepared. Each mosquito was injected with 69 nL of the working solution. Control groups included mosquitoes injected with PBS only and those injected with 6.25% DMSO in PBS. Mosquitoes injected with PBS, DMSO, or cytochalasin D were allowed to recover for 6 h before feeding on *Sm* (OD_{600} = 1) and for 24 h before injection with *Sm* (OD_{600} = 0.0005).

RNA Extraction, Library Preparation, and Sequencing

RNA was extracted from midguts and abdomens dissected from untreated control female mosquitoes (fed on 3% sugar solution) and from female mosquitoes treated by oral *Sm* feeding, *Sm* injection, and sterile PBS injection using a hybrid modified Trizol/RNeasy protocol (Qiagen). Untreated mosquitoes served as control for mosquitoes fed on *Sm* and PBS-injected mosquitoes as control for *Sm*-injected mosquitoes. By including these respective controls, we would be assessing transcriptional responses that are *Sm* specific in each route, which allows us to focus on genes regulated by *Sm* itself and not secondary to the infection procedure. The abdomen specimen refers to the whole abdomen excluding the gut in addition to the malpighian tubules and ovaries which were pulled out with the gut during dissection. Among treated mosquitoes, RNA was extracted from the indicated tissues at 6, 12, and 24 h after treatment. RNA quantification was performed using Qubit RNA HS Assay and quality check procedures via AATI Fragment Analyzer. QuantSeq 3′ mRNA-Seq Library Prep Kit (Lexogen) was used for construction of 3′ end RNA-seq libraries. Libraries were checked with the Qubit DNA Assay kit and AATI Fragment Analyzer again before pooling and sequencing. Illumina NextSeq 500 platform with standard protocol for 75-bp single-end read sequencing was utilized to sequence libraries at the Cornell Life Sciences Sequencing core facility. Three to six million reads were obtained per sample, which is equivalent approximately to a 20× or more coverage of the transcriptome. Quality control of raw reads was performed with FastQC, followed by trimming of the reads by BBMap (https://sourceforge.net/projects/bbmap/) and then mapping to the *A. gambiae* transcriptome (AgamP4.12) using Salmon [35].

Differential Gene Expression and Gene Ontology

Differential expression was analyzed on the transcript level using Bioconductor package DESeq2 [36]. A model with 2 categorical variables was fitted, 1 variable for the replicate and a second variable that contained a separate level for each of the 18 combinations of tissue (abdomen or midgut), time (6, 12, and 24 h), and treatment (*Sm* oral infection, *Sm* injection, and PBS injection), plus a level for the untreated control (only sugar fed) at time zero. Differential expression was analyzed by fitting a generalized linear model and testing for a significant difference in coefficients for treatment and control. This analysis was performed within each combination of time and tissue by comparing read counts between *Sm* oral infection and the untreated control, between PBS injection and the untreated control, and between *Sm* injection and PBS injection of the same time point. The transcript-specific *p* values for differential expression were adjusted for a false discovery rate, and only transcripts with a false discovery rate below 0.05 were labeled as differentially expressed. Genes with at least one differentially expressed transcript (DET) were labeled differentially expressed. To identify transcripts whose expression changes significantly and uniquely in response to oral feeding of *Sm* relative to untreated control and those whose expression changes significantly and uniquely in response to *Sm* injection relative to PBS injection, we took all transcripts that are differentially expressed in each treatment of interest (*p* value adjusted for false discovery rate <0.05) and removed all transcripts that showed differential expression in the same direction in any of the other comparisons, either according to false discovery rate-adjusted *p* value (*p* value <0.05) or according to fold change (fold change >1.5). The fold change criterion was included to be confident that the remaining transcripts are actually treatment specific. Enrichment of differentially expressed genes was tested for each of treatment-control comparison according to 4 classifications, namely, gene ontology terms from the molecular function and biological process ontology, KEGG pathways, and gene families. The enrichment tests used Wallenius noncentral hypergeometric distribution to account for transcript-length-dependent bias for detecting differential expression as implemented in the R package *goseq* [37]. The false discovery rate was calculated by selecting all groups from all 4 classifications that contain >1 significantly differentially expressed gene in any of the comparisons of differential expression and applying the Benjamini-Hochberg correction [38] to the enrichment *p* values of all these groups. Only terms with a false discovery rate below 0.05 were reported.

Results

Sm *Invades the Hemolymph after Mosquito Oral Infection*

Sm efficiently colonizes the gut of *A. gambiae* mosquitoes after oral infection [29, 39]. It also colonizes the ovaries and is vertically transmitted to the progeny, which renders this bacterium an important tool for paratransgenic approaches that aim to generate mosquitoes resistant to *Plasmodium* infection [39]. Here, we monitored, during 3 consecutive days, *Sm* dynamics in the gut and hemolymph of mosquitoes feeding continuously on the *Sm* DB11 strain suspended in 3% sucrose solution. *Sm* DB11 is known to be virulent to insects, nematodes, and mice [24, 27, 28]. The results showed that *Sm* CFUs in the hemolymph are high on day 1 and then drop on days 2 and 3 after oral infection despite continuous feeding on *Sm* (Fig. 1a). Even though the highest numbers of *Sm* CFUs were detected on day 1, they were generally low, not exceeding 250 CFUs per mosquito. A similar trend was observed in the gut, whereby *Sm* CFUs dropped significantly by days 2 and 3 after oral infection; however, the guts generally contained much higher numbers (approximately 10,000-folds more) of *Sm* CFUs at all 3 days, relative to the hemolymph (Fig. 1b), suggesting that only few bacteria are present in the hemolymph at a given time despite the efficiency of *Sm* colonization of the gut. There

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were no significant differences in *Sm* CFUs in the sugar pads between all 3 days that could explain the significant drop observed in the gut CFUs at days 2 and 3 (Fig. 1c), indicating that *Sm* remains viable in the sugar solution for several days.

The Route of Hemolymph Invasion by Sm Alters the Contribution of CTL4 and TEP1 to Bacterial Clearance

The fact that *Sm* invades the mosquito hemolymph in low numbers after oral infection, mimicking natural bacterial infections in the field, renders it an attractive model to address whether the route of hemolymph invasion (thoracic injection vs. crossing of the midgut) influences the contribution of key humoral immune factors to systemic antibacterial defense. To that purpose, we selected CTL4 and TEP1 as candidates due to their essential role in the systemic immune response against septic (i.e., through thoracic injection) Gram-negative bacterial infections [11, 19, 21, 40], specifically CTL4, which forms a heterodimeric complex with the lectin CTLMA2 that protects mosquitoes from septic *E. coli*, *Pseudomonas*, and *Enterobacter cloacae* infections [21]. First, we assessed the contribution of these genes to the susceptibility of *A. gambiae* adult female mosquitoes to septic *Sm* infections established through thoracic injection. Mosquitoes treated with gene-specific dsRNA for CTL4 and TEP1 were injected with an *Sm* suspension in PBS (OD_{600} = 0.0005) at day 3 after dsRNA administration. At this OD, the CFUs injected per mosquito ranged between 19 and 113. Both *CTL4* and *TEP1* knockdown (kd) compromised mosquito survival to injected *Sm* (Fig. 2a, b; online suppl. Fig. 1a, b) compared to *LacZ* kd control. Also, *Sm* proliferation in these genotypes was significantly higher than that in the control group (Fig. 2c, d). The *Sm* DB11 strain used herein is gentamycin resistant and expresses DsRed [24], which allows accurate measurement of CFUs in whole mosquito lysates, without interference from natural *Sm* strains whose presence seems to be sporadic and minor in our mosquitoes (online suppl.

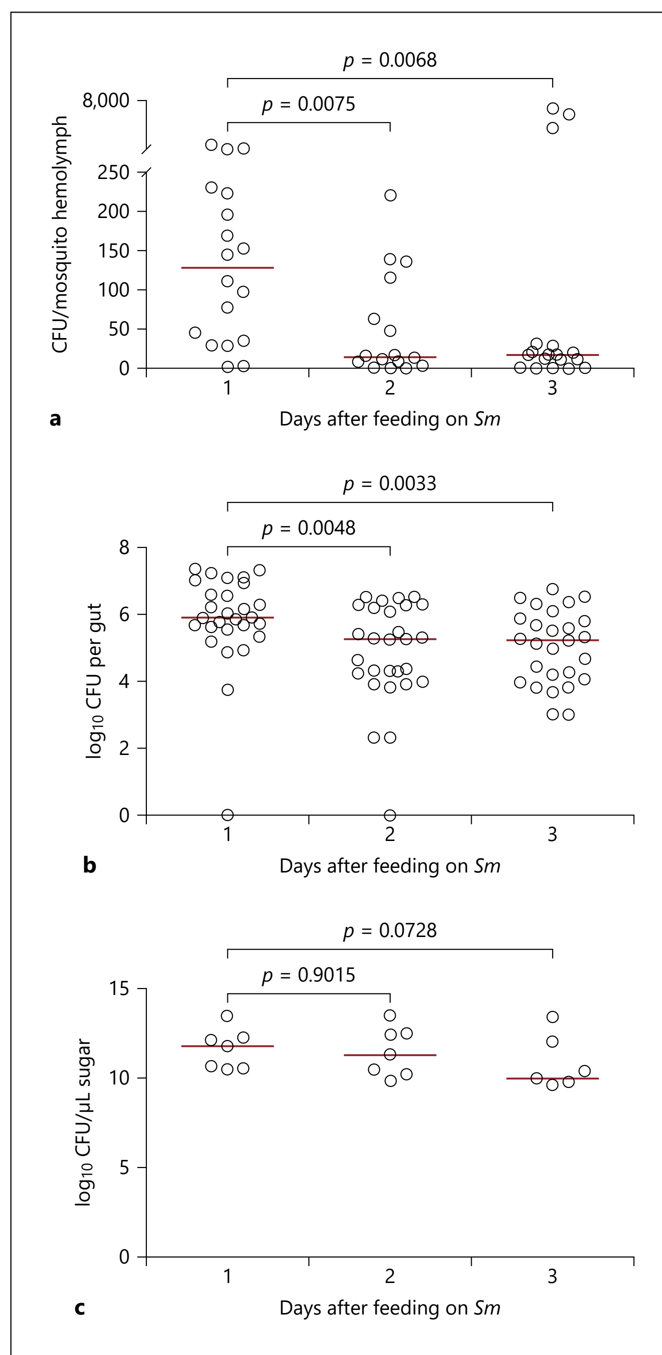

Fig. 1. *Serratia marcescens* acquired through the oral route crosses the midgut epithelium into the hemolymph. **a** Hemolymph was collected by perfusion from batches of 5 mosquitoes each, at the indicated time points after feeding on *Sm*, and plated on LB agar containing the appropriate antibiotic. Each point on the scatter plot represents the mean CFU per batch per mosquito. Data were pooled from 5 independent biological experiments. **b** Guts of individual mosquitoes were dissected at the indicated time points after feeding on *Sm*, homogenized, and plated on LB agar containing the appropriate antibiotic. Data were pooled from 3 independent biological experiments. Each point on the scatter plot represents 1 midgut. **c** Bacterial counts were monitored in sugar pads harboring *Sm* (OD_{600} = 1) over a period of 3 days. Data shown are from 7 independent biological experiments. Each point on the scatter plot corresponds to CFU/μL of sugar solution in the pad per experiment. Medians are represented by red lines. Statistical analysis was performed using the Mann-Whitney test, and medians were considered significantly different if $p < 0.05$. *Sm*, *Serratia marcescens*; CFU, colony-forming unit.

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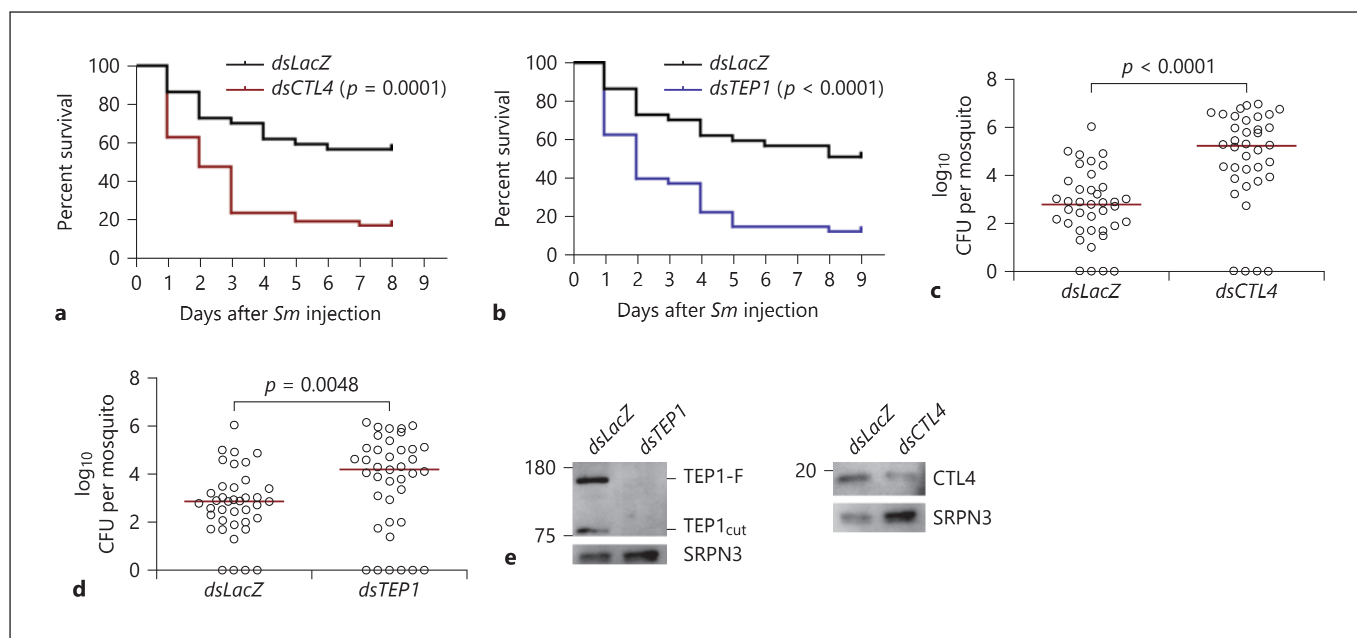

Fig. 2. TEP1 and CTL4 are required for defense against systemic *Sm* infections. **a**, **b** Survival assays of the indicated mosquito genotypes after injection with *Sm* (OD_{600} = 0.0005). One representative experiment is shown from at least 3 independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the log-rank test. **c**, **d** Bacterial proliferation assays conducted on the indicated mosquito genotypes injected with *Sm* (OD_{600} = 0.0005). Batches of 8 whole mosquitoes were grinded each in the LB medium at 24 h after infection, and CFUs were scored on LB plates supplemented with the appropriate antibiotic. Each circle on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test, and medians were considered significantly different if $p < 0.05$. Data shown are from 9 independent biological experiments. **e** Western blots showing the knockdown efficiencies of TEP1 and CTL4. αSRPN3 was used to control for loading. TEP1, thioester-containing protein 1; CTL4, C-type lectin 4; *Sm*, *Serratia marcescens*; CFU, colony-forming unit.

Fig. 2). This is further corroborated by a recent published work from our lab, in which the total OTUs belonging to the genus *Serratia* in the midguts of *A. gambiae* mosquitoes collected from our insectary over a 7-month period were about 12% [41]. Western blot analysis showed that both TEP1 and CTL4 were efficiently knocked down (Fig. 2e). Interestingly, when the same strain was used to establish systemic infections through oral feeding in *CTL4* and *TEP1* kd mosquitoes, no effect on survival was observed relative to the control group (Fig. 3a; online suppl. Fig. 3). We performed hemolymph perfusions at 72 h after oral infection to score the numbers of *Sm* that invaded the hemocoel in the different mosquito genotypes. Our data showed that *Sm* CFUs in the hemolymph of *CTL4* and *TEP1* kd mosquitoes were low and similar to those in the control (Fig. 3b); median values were 84.8, 43.6, and 42.2 for *LacZ*, *CTL4*, and *TEP1* kd mosquitoes, respectively. This indicates that the immune function of these proteins becomes nonessential when *Sm* invades the hemolymph through the oral route. The differential contribution of CTL4 and TEP1 to immune defense against *Sm* in the 2 routes of infection is not attributed to differences in the numbers of *Sm* introduced into the hemolymph between both routes; 19 to 113 CFUs of *Sm* were injected into the mosquito hemolymph during septic infections, while the numbers of *Sm* that reached the hemolymph of wild-type mosquitoes at 24 h after oral infection ranged from 2 to 280 CFUs (Fig. 1a). Despite both numbers being relatively low, *Sm* proliferated dramatically in *CTL4* and *TEP1* kd mosquitoes at 24 h after *Sm* injection (Fig. 2c, d), while in oral infections, *Sm* CFUs in the hemolymph remained low in these genotypes, even at 72 h after feeding (Fig. 3b). A similar profile was noted in the *LacZ* kd controls of both treatments (injection vs. oral), suggesting that bacteria invading the hemolymph from the gut exhibit a more controlled proliferation compared to those injected directly into the hemolymph.

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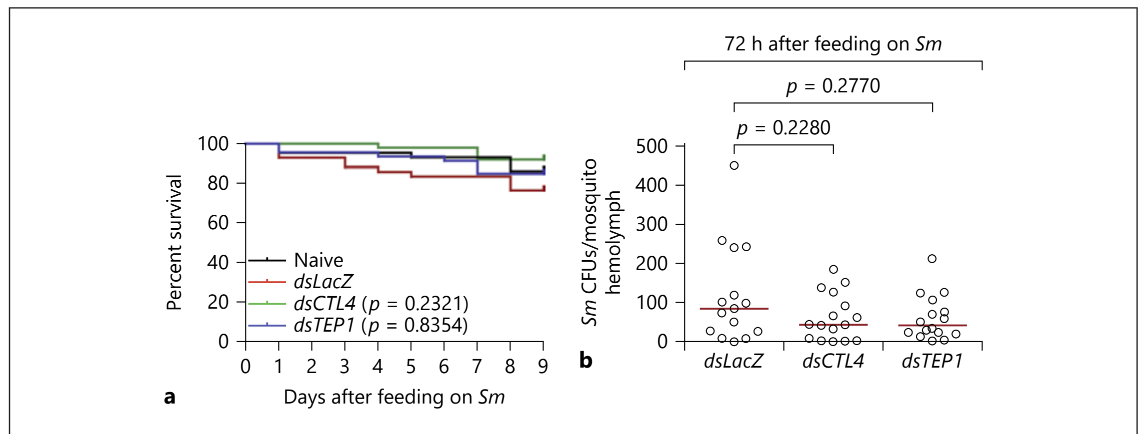

Fig. 3. CTL4 and Tep1 are not required for defense against *Sm* oral infections. **a** Survival assays of the indicated mosquito genotypes after oral infection with *Sm* (OD_{600} = 1). One representative experiment is shown from at least 3 independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the log-rank test. **b** Bacterial proliferation assays conducted on the indicated mosquito genotypes after oral infection with *Sm* (OD_{600} = 1). Hemolymph was perfused from batches of 5 mosquitoes at 72 h after feeding on *Sm*, and CFUs were scored on LB plates supplemented with the appropriate antibiotic. Each circle on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (black lines) were considered significantly different if $p < 0.05$. Data shown are from 3 independent biological experiments. TEP1, thioester-containing protein 1; CTL4, C-type lectin 4; *Sm*, *Serratia marcescens*; CFU, colony-forming unit.

To try to explain the controlled proliferation of *Sm* in the hemolymph after oral feeding, we hypothesized that the process of midgut invasion might influence the fitness of bacterial cells possibly due to exposure to oxidants (reactive oxygen and nitrogen species) generated as part of the local epithelial immune response, which are known to damage bacterial cells (reviewed in [42]). For instance, oxidants generated by dual oxidase in the *Drosophila* gut limit microbial proliferation [43], and nitric oxide produced in the *A. gambiae* midgut by heme peroxidase 2 and NADPH oxidase 5 enhances *Plasmodium* cytotoxicity [44]. To determine if bacteria exhibit an altered fitness after crossing the midgut, the hemolymph was collected by perfusion from wild-type mosquitoes that have fed on *Sm* during 24 h, and bacterial cells in the perfusate were pelleted by centrifugation, washed, and injected into *LacZ* and *CTL4* kd naïve mosquitoes. The same mosquito genotypes injected with *Sm* (OD_{600} = 0.0005) prepared from a fresh batch culture were used as control. Challenged mosquitoes were homogenized 24 h later to score *Sm* CFUs. The results show that bacteria prepared from hemolymph perfusates were able to proliferate to the same extent as those originating from a fresh culture, indicating that bacterial cells that cross the gut into the hemolymph do not seem to suffer from a reduced growth fitness (Fig. 4).

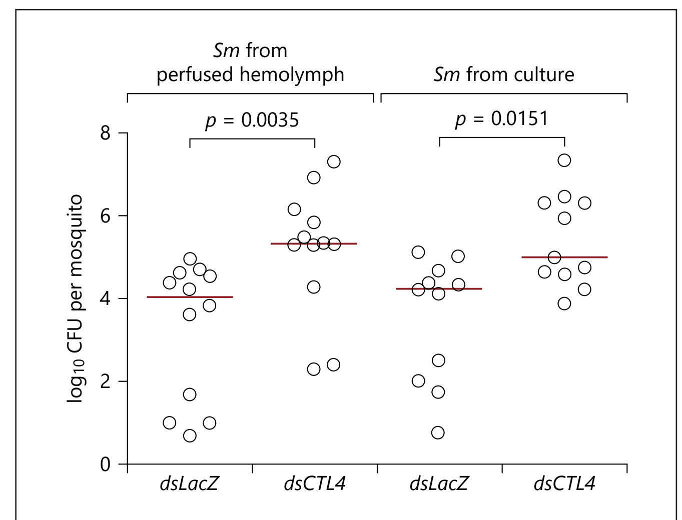

Fig. 4. Invasion of the midgut epithelium does not alter the fitness of *Sm*. Bacterial proliferation assays conducted on the indicated mosquito genotypes after injection with *Sm* prepared from a fresh bacterial culture (OD_{600} = 0.0005) or collected from hemolymph perfusions of mosquitoes that have fed on *Sm* for 24 h. Batches of 7 whole mosquitoes were grinded in the LB medium at 24 h after infection, and CFUs were scored on LB plates supplemented with the appropriate antibiotic. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using the Mann-Whitney test. Medians (red lines) were considered significantly different if $p < 0.05$. Data shown are from 3 independent biological experiments. *Sm*, *Serratia marcescens*; CFU, colony-forming unit.

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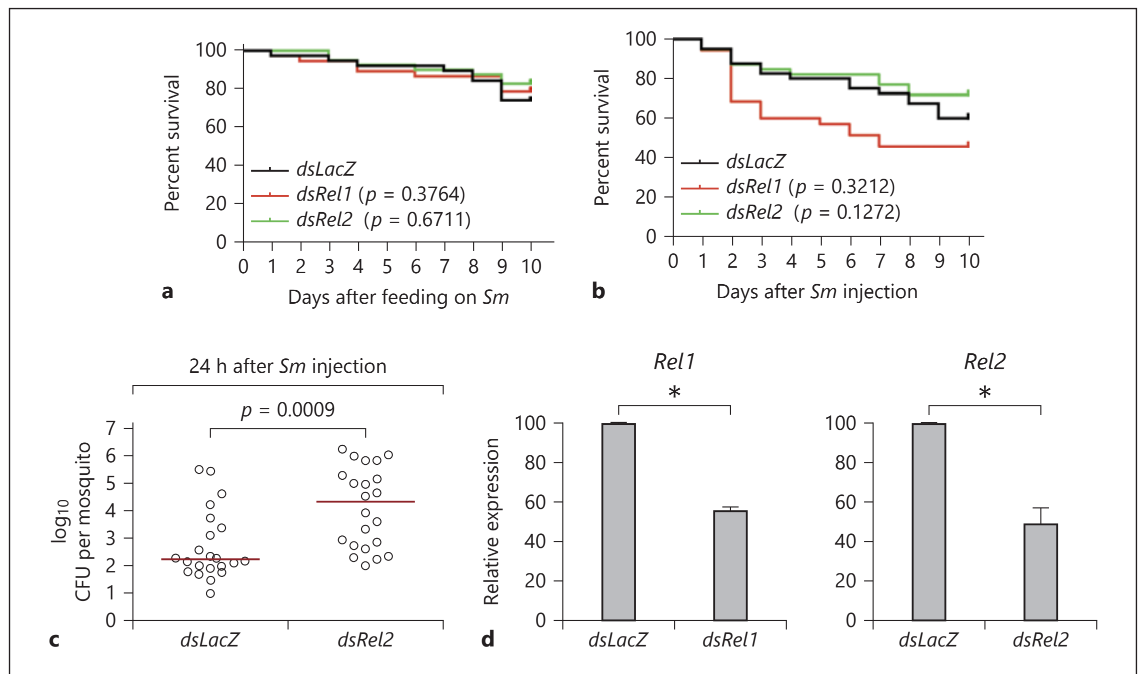

Fig. 5. The Rel2 signaling pathway plays a role in mosquito tolerance and resistance against systemic but not oral *Sm* infections. Survival assays following *Sm* oral (OD_{600} = 1) (**a**) and systemic (OD_{600} = 0.0005) (**b**) infections in mosquitoes silenced for either Rel1 or Rel2. One representative experiment is shown from at least 3 independent biological experiments. The Kaplan-Meier survival test was used to calculate percent survival. Statistical significance of the observed differences was calculated using the log-rank test. **c** Bacterial proliferation assays conducted on *Rel2* kd mosquitoes injected with *Sm* (OD_{600} = 0.0005). Batches of 8 whole mosquitoes were grinded in the LB medium at 24 h after infection, and CFUs were scored on LB plates supplemented with the appropriate antibiotic. Each point on the scatter plot represents the mean CFU per mosquito per batch. Statistical analysis was performed using Robust ANOVA in R. Data shown are from at least 3 independent biological experiments. **d** Transcript levels of Rel1 and Rel2 measured by qRT-PCR in whole female mosquitoes at 3 days following injection of their respective dsRNA. Error bars represent standard error of the mean of 2 biological repeats. Statistical analysis was done using Student's *t* test. *Sm*, *Serratia marcescens*; CFU, colony-forming unit.

The Rel2 Pathway and Phagocytosis Are Dispensable for Defense against Sm Systemic Infections Established after Gut Invasion

The mosquito Rel2 pathway is involved in defense against systemic infections with Gram-negative and Gram-positive bacteria [45, 46], and also protects against *P. falciparum* ookinetes [45, 47, 48]. To determine the contribution of Rel2 to systemic defense against *Sm* that invade the hemolymph following an oral infection, *LacZ* (control), *Rel2*, and *Rel1* kd mosquitoes were fed continuously on sugar pads containing a suspension of *Sm* at an OD_{600} = 1, and their survival was scored over 10 days after challenge. Neither Rel1 nor Rel2 silencing compromised mosquito survival to oral *Sm* infections (Fig. 5a; online suppl. Fig. 4a). In contrast, when systemic infection in these mosquito genotypes was established by injecting *Sm* into the hemocoel, *Rel2* kd compromised mosquito survival (Fig. 5b; online suppl. Fig. 4b) and resistance (Fig. 5c) as noted from the enhanced bacterial proliferation relative to control. Hence, the Rel2 pathway contributes to immune defense against *Sm* systemic infections established through septic injury but not through feeding. The efficiency of Rel1 and Rel2 silencing in our hands is 44 and 50% (Fig. 5d), which is similar to what was reported previously for these genes [46, 49].

Phagocytosis is an important innate immune response that was shown to control host susceptibility to septic bacterial infections in *A. gambiae* [11, 50] and *Drosophila* [51–54]. Additionally, blocking phagocytosis in *Drosophila* adults by cytochalasin D injection compromised the survival of the flies to oral *Sm* infections [24]. Based on these data, we hypothesized that the dispensable roles of CTL4, TEP1 and the Rel2 pathway in defense against *Sm* that gains access into the hemolymph after oral infection may be due to a primary role of phagocytosis in controlling host susceptibility through this route. To address this

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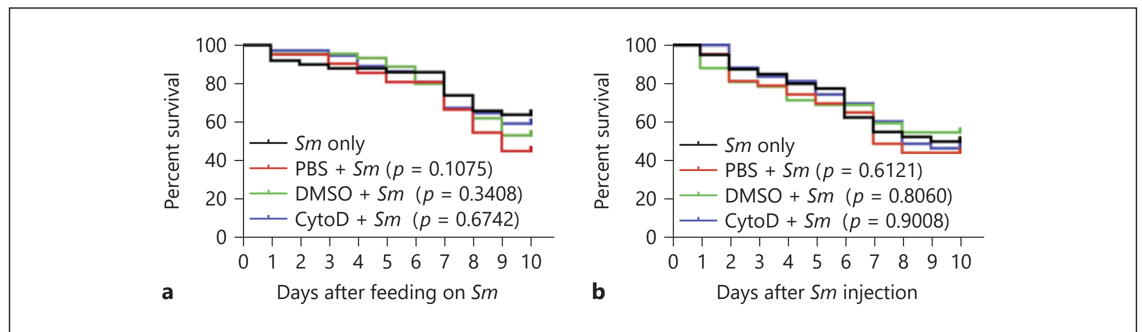

Fig. 6. Blocking phagocytosis by cytochalasin D does not seem to significantly impact mosquito susceptibility to oral or systemic *Sm* injections. Survival of noninjected mosquitoes or mosquitoes pre-injected with either PBS, DMSO, or cytochalasin D was monitored over a period of 10 days following oral (OD_{600} = 1) (**a**) or systemic (**b**) *Sm* infections. One representative experiment is shown from at least 3 independent biological experiments. The Kaplan-Meier survival test was used to calculate the percent survival. Statistical significance of the observed differences was calculated using the log-rank test. *Sm*, *Serratia marcescens*.

point, mosquitoes injected intrathoracically with 69 nL of 62.5 μg/mL solution (120 μM) of cytochalasin D in PBS were allowed to feed continuously on a sugar solution containing *Sm* at 6 h after cytochalasin D injection. Survival assays revealed that cytochalasin D treatment did not affect mosquito susceptibility to oral *Sm* infections (Fig. 6a; online suppl. Fig. 5a), despite the fact that the concentration of cytochalasin D used herein is higher than that which blocked phagocytosis in *Drosophila* adults [24] and *A. gambiae* cell lines [55]. Hence, our data suggest that, in the mosquito, phagocytosis may not play an essential role in controlling *Sm* that escapes into the hemolymph after oral infections. Also, cytochalasin D treatment did not compromise mosquito survival to *Sm* injection (Fig. 6b; online suppl. Fig. 5b), which was not surprising to us due to the primary immune defensive role of the humoral factors TEP1 and CTL4 in this infection route.

Abdomen and Midgut Transcriptional Responses after Sm Oral and Septic Infections

The fact that CTL4- and TEP1-mediated systemic immune responses did not provide resistance to *Sm* invading the hemolymph from the gut prompted us to monitor whether oral infection primes the tissue-specific expression of an immune gene repertoire in the fat body or midgut that could explain the dispensable roles of CTL4 and TEP1 in this route of infection. To that purpose, abdomens (excluding gut, malpighian tubules, and ovaries) and midguts (excluding hind- and foreguts) were dissected from wild-type mosquitoes at 6, 12, and 24 h after feeding on a 3% sucrose solution containing *Sm* Db11 (OD_{600} = 1) or after injection with an *Sm* suspension in PBS (OD_{600} = 0.0005), and transcriptional responses were monitored by RNA-seq. Mosquitoes fed on 3% sucrose solution or injected with sterile PBS were used as controls for *Sm* oral infection and *Sm* injection, respectively. Three independent biological experiments were performed. All DETs were determined according to a false discovery rate of 0.05 (online suppl. Table 2). In the abdomens, 70, 87, and 123 DETs were identified, respectively, at 6, 12, and 24 h after *Sm* oral infection with respect to untreated controls (i.e., only sugar fed), whereas 16, 11, and 47 DETs were identified at the respective time points after *Sm* injection with respect to PBS injection only (Fig. 7a, c), indicating that oral infection triggers more profound transcriptional changes in the abdomen than injection. In midguts, 406, 296, and 106 DETs were identified at 6, 12, and 24 h after *Sm* oral infection with respect to untreated controls, whereas 16, 12, and 12 DETs were identified at the respective time points after *Sm* injection (Fig. 7b, d), again indicating that oral infection had a greater influence on the midgut transcriptome than injection, which is rather expected since in the context of direct injection into the hemocoel, *Sm* is unlikely to invade the gut epithelium from the basal side. The low numbers of DETs in abdomens and midguts of *Sm*-injected mosquitoes are also likely due to the fact that PBS injection itself regulated a large number of transcripts in these tissues at all 3 time points (Fig. 7e, f). Of note, although the PBS solution used in these treatments is sterile, bacteria attached to the mosquito cuticle can still be introduced into the hemolymph due to the wounding process.

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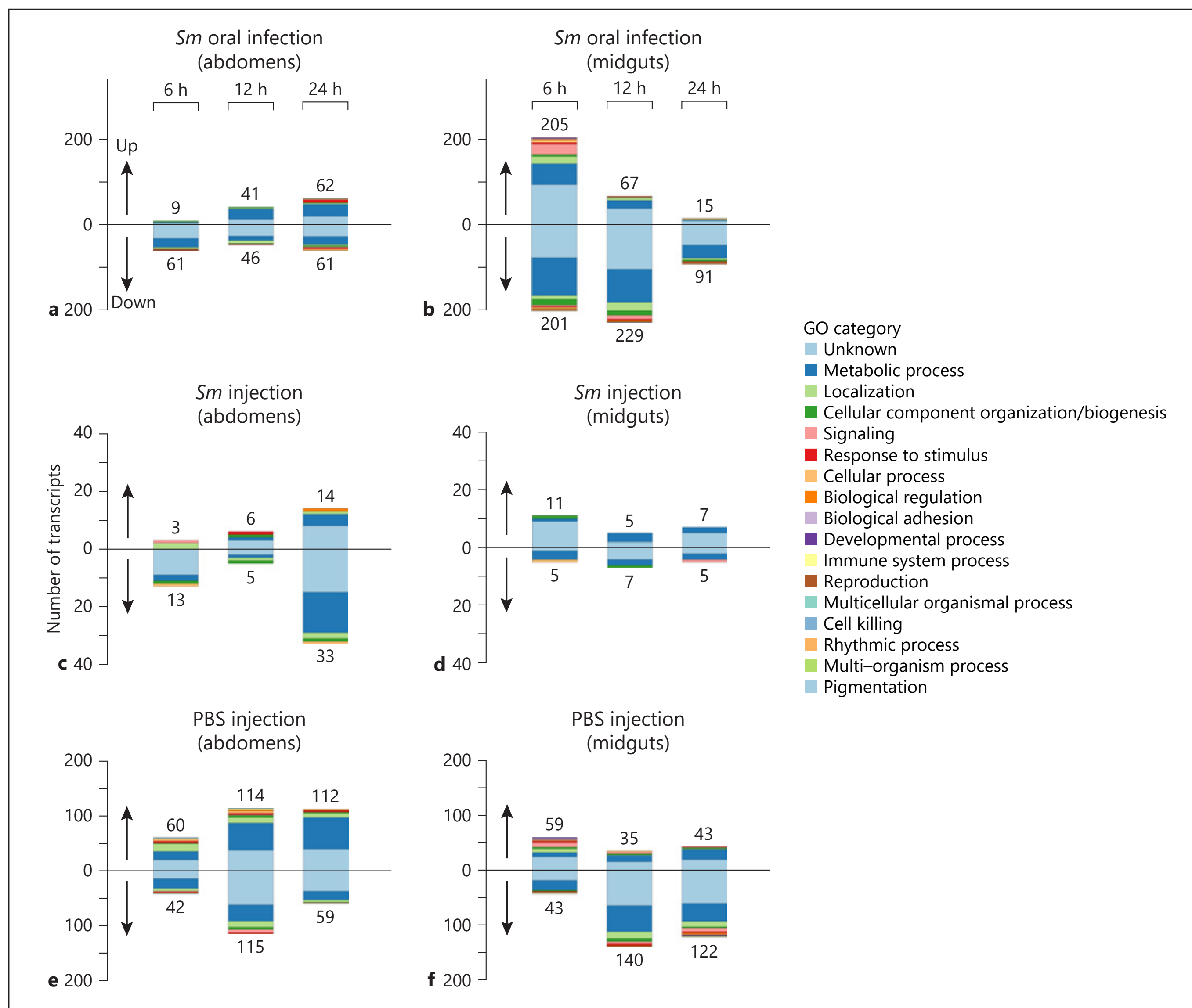

Fig. 7. Number of DETs per treatment, colored according to top-level gene ontology terms within the "biological process" ontology. **a**, **b** DETs in abdomens and midguts, respectively, of mosquitoes fed on *Sm*. **c**, **d** DETs in abdomens and midguts, respectively, of mosquitoes injected with *Sm*. **e**, **f** DETs in abdomens and midguts, respectively, of mosquitoes injected with PBS. For this plot, each transcript was assigned to its top-level gene ontology term within the "biological process" ontology. Whenever a transcript was associated with >1 top-level term, the least common term was chosen. DETs, differentially expressed transcripts; *Sm*, *Serratia marcescens*.

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Functional classification of all DETs in abdomens and midguts from all treatments revealed that metabolic genes are the most represented class followed by those involved in cellular localization (Fig. 7; online suppl. Table 2). Surprisingly, immunity genes were underrepresented in both abdomens and midguts from all treatments suggesting that oral and septic infections with *Sm* have little effect on the transcriptome of immunity genes. When comparing the DETs in midguts of mosquitoes injected with *Sm* to those of mosquitoes fed on *Sm*, a small overlap was observed (online suppl. Fig. 6a). The same was noted for abdomens (online suppl. Fig. 6b), indicating that different physiological responses are triggered in response to the different routes of *Sm* infection. To determine whether hemolymph infection following *Sm* injection or midgut crossing triggers route-specific unique responses to

Sm in abdomens, we identified the transcripts whose expression changes significantly in response to *Sm* injection (*Sm*_inj) relative to PBS-injected (PBS_inj) control but not in response to any other treatment (i.e., *Sm* oral feeding [*Sm*_of] vs. untreated control [UC] or *Sm*_inj vs. UC or PBS vs. UC) and transcripts whose expression changes significantly in response to *Sm*_of relative to UC but not in response to any other treatment (i.e., *Sm*_inj vs. PBS_inj or *Sm*_inj vs. UC or PBS_inj vs. UC). In abdomens, where physiological responses are expected to be more relevant to hemolymph infection with *Sm* due to the presence of the fat body and sessile hemocytes, only one transcript, vacuolar protein sorting 60 (Vps 60; AGAP005100), showed significant change in expression unique to *Sm*_inj versus PBS_inj (online suppl. Table 3). Vps proteins are involved in the formation of multivesicular bodies which play important roles in the endocytic degradation of proteins and also in the formation of exosomes [56], which are small extracellular vesicles that mediate intercellular communication to regulate several biological processes including tissue repair [57]. The upregulation of Vps60 in abdomens may reflect enhanced investment in repair processes in response to *Sm* infection. Tissue repair and regeneration processes are crucial for host tolerance to infection [58]. Alternatively, this upregulation may indicate an increase in the protein secretory capacity of the fat body in response to immune activation and infection, which in *Drosophila* was associated with enhanced tolerance to infection [59]. On the other hand, 3 transcripts showed a downregulated expression profile in abdomens unique to *Sm*_of versus UC (online suppl. Table 4) including, CCR4-NOT (CNOT) transcription subunit complex 3 (AGAP009030), very long-chain enoyl-reductase (AGAP010714), and UPF0518 (AGAP011705). CNOT is a large multi-subunit RNA deadenylase, composed of catalytic and noncatalytic subunits that is conserved in eukaryotes and plays key roles in mRNA degradation and turnover, hence controlling the rate of protein expression [60]. It also plays an effector role in miRNA-mediated gene silencing [61]. As such, CNOT is involved in regulating several physiological processes in the cell including cell death, autophagy, immunity, inflammation, and differentiation to mention a few [60, 62]. It is tempting to speculate that the downregulation of CNOT3, a noncatalytic subunit essential for CNOT activity [63], in abdomens may increase the stability of mRNAs involved in immunity, tissue repair, or stress response which might favor host tolerance to systemic infection established after feeding on *Sm*. The very long-chain enoyl-reductase is involved in the synthesis of sphingolipids and glycerophospholipids [64], and its downregulation may indicate a shift in lipid metabolism, whereas UPF0518 has no known function. GO enrichment analysis of the route-unique transcripts listed in online suppl. Tables 3 and 4 suggests that the global physiological response associated with the oral route includes most of that associated with *Sm* injection (except 4 genes), in addition to other specific functions (online suppl. Tables 5, 6). These oral route-specific responses are mainly attributed to the midgut (33 out of 36 genes) and are enriched in biological processes related mainly to protein translation, protein folding, protein modification, DNA damage repair, and cell cycle regulation among others (online suppl. Table 5). These responses could reflect the pathology induced by *Sm* to the midgut epithelium.

A Wallenius noncentral hypergeometric distribution was used to test for the enrichment of GO terms, KEGG pathways, and gene families in the total set of differentially regulated genes in abdomens and midguts of mosquitoes fed on or injected with *Sm*, relative to UC and PBS-injected control, respectively. The results identified 13 unique terms (4 gene families, 5 KEGG pathways, and 4 GO terms) that were significantly overrepresented, the majority of which were associated with functions related to protein translation, processing, and export, followed by terms related to metabolic processes, in particular oxidative phosphorylation (OXPHOS), and 1 associated with immunity (online suppl. Table 7). Concerning immunity, only the clip-domain serine protease family (CLIPs) was significantly overrepresented in the midgut of mosquitoes at 12 h after *Sm* oral infection (Fig. 8; online suppl. Table 7). In total, 10 CLIPs were downregulated in this treatment including CLIPC4, CLIPB4, CLIPB1, CLIPC9, CLIPB13, CLIPA8, CLIPA4, CLIPA6, CLIPA1, and CLIPA7. CLIPs are key components of serine protease cascades that regulate important insect immune responses specifically melanization and Toll pathway activation [3, 65, 66]. Among the enriched CLIPs, CLIPB4 and CLIPC9, both catalytic clips, are involved in the melanization of *P. berghei* ookinetes in refractory mosquito backgrounds [67, 68], while CLIPA8 and CLIPA7 are noncatalytic CLIPs that act as positive and negative regulators of *Plasmodium* melanization, respectively [67]. The melanization response to fungal infections requires CLIPA8 [69], while both CLIPA8 and CLIPC9 play an essential role in the melanization response to bacterial infections [68, 70, 71]. CLIPA1, CLIPA4, CLIPA6, and CLIPB1 do not seem to be involved in *Plasmodium* melanization [67], whereas the roles of CLIPC4 and CLIPB13 in the melanization response remain to be elucidated. The

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downregulation of this significant number of CLIP genes suggests that *Sm* oral infection may suppress the melanization response regulated by several of these CLIPs. Of note, the differential regulation of CLIPs in the midgut is most likely attributed to hemocytes attached to the midgut surface and not to the midgut epithelium per se, as insect CLIPs are mainly expressed in hemocytes and fat body cells [65, 66]. Indeed, several of the overrepresented CLIPs in our study including CLIPA7, CLIPA8, CLIPB1, CLIPB4, CLIPB13, CLIPC4, and CLIPC9 were among the genes identified in transcriptomic studies of mosquito hemocytes [72, 73]. Also, PPO6 which is hemocyte specific [73, 74] was among the DETs identified in mosquito midguts in response to *Sm* oral infections (online suppl. Table 2), further indicating that some of the immunity genes identified in the midgut transcriptome are attributed to midgut-attached hemocytes rather than to the midgut epithelium. Indeed, there is evidence that contact between midgut epithelial cells and the gut microbiota which occurs during *Plasmodium* midgut invasion initiates systemic immune priming by triggering hemocyte differentiation and their attraction to the midgut surface where they present antimicrobial activities including complement activation [14, 15, 75]. Of note, the KEGG pathway enrichment analysis identified 12 genes involved in OXPHOS that are all downregulated in the midgut after feeding on *Sm*, suggesting that midgut infection may be triggering a shift in the gut metabolic program. As for the abdomens, only the FOXO signaling pathway is enriched after *Sm* injections but not feeding (online suppl. Table 7). In *Drosophila*, the FOXO transcription factor activates AMP production under nutritional stress independent of Toll and Imd pathways [76]. FOXO is also required for *Drosophila* to survive oral infections with *Sm*[77]. Whether FOXO signaling plays a similar role in mosquito immunity against bacterial septic injections and oral infections remains to be elucidated.

In addition to CLIPs, few other genes belonging to distinct immune gene families, though not overrepresented, were also downregulated after *Sm* oral but not septic infections (online suppl. Table 2). These include, Eiger, GNBPB1, the scavenger receptors SCRB5, SCRB7, and SCRB9, PPO6, TEP2, and CTLMA1. Eiger was downregulated in abdomens at 24 h after *Sm* oral infections. It is a TNF ortholog which, in *Drosophila*, is also expressed in the fat body [78] and plays an important role in regulating melanization, AMP expression, and immune defense against extracellular pathogens [78, 79]. GNBPB1 was also downregulated in abdomens at 24 h after *Sm* oral infections, and it was previously shown to contribute to anti-*Plasmodium* immunity [22]. Two members of the scavenger receptor gene family, SCRB5 and SCRB7, were downregulated in abdomens at 12 and 6 h after *Sm* oral infections, respectively, while SCRB9 was downregulated in the midgut at 6 h after infection. The role of these receptors in mosquito immunity has not been investigated, but members of this family are involved in the phagocytic uptake of bacteria in *Drosophila* [80–82]. PPO6, TEP2, and CTLMA1 were also downregulated in the midgut in response to *Sm* oral infection. PPO6, a phenoloxidase expressed in mosquito adults, is involved in the melanization reaction to *P. berghei* ookinetes, bacteria, and fungi [33, 69, 70]. While the roles of TEP2 and CTLMA1 in immune defense are unknown, certain members of the TEP and CTL families are key players in the mosquito antimicrobial defense [19, 21, 33, 83–85]. On the other hand, only 4 immunity genes were upregulated after *Sm* oral infections; galectin 5 and cecropin A were upregulated in abdomens, whereas CTL6 and lysozyme C7 (LYSC7) were upregulated in the midgut at the indicated time points (online suppl. Table 2). Whether these genes are involved in controlling *Sm* proliferation in the hemolymph following oral infection will require further investigations. Altogether, these results suggest that *Sm* invasion of the hemolymph following oral infection is seemingly associated with transcriptional suppression of several immune genes involved in different facets of the humoral and cellular immune response.

Discussion

In all organisms, the vast majority of microbial infections are established through initial interactions between microbes and host at barrier epithelia. There is growing evidence, in both invertebrates and vertebrates, that the route of infection determines the adaptive strategies of the host in terms of the nature of immune responses engaged to deal with the insult [86, 87]. In *Drosophila*, oral

Fig. 8. Heatmap of estimated log-fold changes of all CLIP genes. Log-fold changes were estimated for each comparison indicated by column labels. The column labels start with symbol for control (UC, unchallenged; iPBS, PBS injection), followed by a symbol for the tissue (a, abdomen; g, gut). The symbols for the treatments are OF (oral feeding) and iSm (injection of *Serratia marcescens*). The timing of treatment is indicated in hours (h). The row dendrogram shows a hierarchical clustering of the dissimilarities between CLIP genes in their log-fold change patterns among all comparisons shown in the plot. CLIP, clip-domain serine proteases.

(For figure see next page.)

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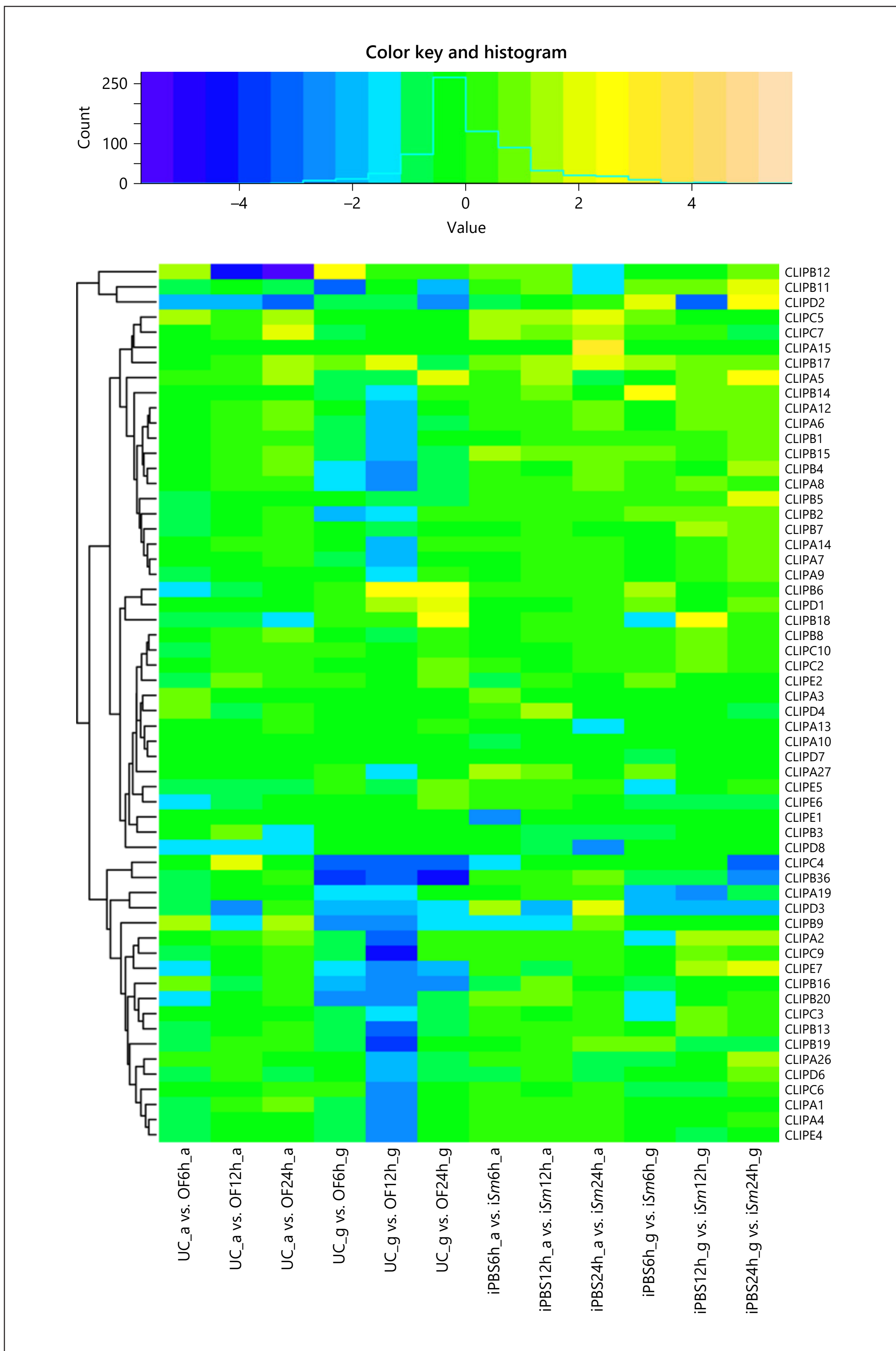

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and systemic infections with *Pseudomonas entomophila* triggered the evolution of resistance in fly populations that was infection-route specific [86]. Also, oral infection of *Drosophila* with different RNA viruses revealed different patterns of virus clearance and immune priming compared to systemic injections [87]. In a similar context, *Anopheles coluzzii* oral infections with O'nyong nyong arbovirus shared little overlap in transcriptional responses with intrathoracic injections [88]. Route-specific immune responses have been also described in mammals. For instance, the intranasal administration of the vaccinia virus to mice triggered a stronger adaptive response in magnitude and diversity compared to local intradermal injections [89]. In another study, infection of mice with *Brucella melitensis* through 3 different routes, intradermally, intraperitoneally, and intranasally, revealed route-specific contributions of the 3 lymphoid populations, $CD4^+$ T cells, B cells, and $\gamma\delta^+$ T cells [90]. Interestingly, the authors also showed that the type IV secretion system which is required for *Brucella* persistence in the lungs after intranasal infections does not seem to promote persistence in the skin after intradermal infections, suggesting that the route of infection influences not only the physiology of the immune response but also the contribution of certain virulence factors to microbial persistence. In this work, we used the *Sm* DB11 strain as a model mosquito pathogen to score the contribution of 2 key humoral antibacterial factors, TEP1 and CTL4, to immune defense against hemolymph infections established either through injection or midgut invasion after oral feeding, in adult *A. gambiae* female mosquitoes.

Sm is detected in the mosquito hemolymph 1 day after oral infection, but the numbers drop significantly during the following 2 days concomitant with a reduction in *Sm* CFUs in the midguts. This reduction is not due to increased bacterial death in the sugar pads used to feed mosquitoes since bacterial CFUs in the pads did not change significantly during this period. The reduction in *Sm* hemolymph CFUs in days 2 and 3 could be explained by immune defenses active at the level of the midgut epithelium that may restrict the numbers of bacteria that successfully invade the midgut into the hemolymph [24, 29]. It may also reflect a reduction in mosquito feeding due to chronic infection of the gut by *Sm*. In fact, the gustatory receptor gene *Gr9* was shown to be associated with *Sm* infection phenotype of *A. gambiae* midguts, and silencing this gene increased *Sm* colonization of the midgut, indicating that *Sm* infection may trigger a behavioral feeding response [29]. The composition of the gut microbiota in *Drosophila* also influences its foraging behavior [91]. We did not address whether blood feeding would influence the dynamics of *Sm* invasion of the hemolymph; however, we expect that it would be more difficult for *Sm* to cross the midgut during blood feeding since the peritrophic matrix [92] and the dityrosine network produced by a peroxidase dual oxidase system [93] will likely restrict midgut permeability to microbes.

We found that CTL4 and TEP1 are required for mosquito resistance to *Sm* infections of the hemolymph established following injection but not oral infection. The latter route may trigger systemic immune priming by gut epithelia creating redundancy in bacterial defense among different arms of the immune response. The numbers of *Sm* that gain access to the hemolymph after oral infection are likely to be small as inferred from our hemolymph perfusion assays. In *Drosophila*, melanization was shown to be essential for immune defense against septic infections with a small dose of *S. aureus*[13]. Our attempts to measure PO activity following oral *Sm* infections were not conclusive, as some trials showed activation while others did not (data not shown). There are 2 plausible explanations for this inconsistency: first, hemolymph invasion after oral infection is likely to occur in waves and not at one single time point which makes it difficult to pinpoint the optimal time point for measuring hemolymph PO activity. Second, small numbers of bacteria are most likely reaching the hemolymph in each wave, as inferred from the small numbers of *Sm* CFUs scored in the hemolymph after oral infections (Fig. 1), which might not trigger a measurable PO activity. This is in contrast to bacterial injections where the time of hemolymph infection and the dose of introduced bacteria can be optimized to trigger a measurable PO response [70, 94]. However, the fact that silencing TEP1, a key upstream regulator of the mosquito melanization response [69, 70, 95], did not alter mosquito susceptibility to *Sm* oral infection and that PPO6 and several CLIPs were downregulated after feeding on *Sm* suggest that melanization may not play an essential role in this route of infection. We observed that bacteria invading the hemolymph from the gut remained at low numbers, as compared to those injected directly into the hemolymph, even in control (ds*LacZ*) mosquitoes. It remains unclear whether this is attributed to a longer generation time (i.e., reduced cell division rate) associated with bacteria that cross the midgut into the hemolymph but not with those directly injected into the hemolymph. Reduced proliferation is expected to benefit bacterial persistence since the release of cell wall components, such as peptidoglycan, during bacterial cell division would activate PGRPs leading to Imd pathway acti-

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vation in fat body cells [20, 96–98]. However, it is worth noting that the mosquito complement-like system, which plays a key role in antibacterial immunity, may not be sensitive to bacterial proliferation since TEP1 was shown to efficiently bind *E. coli* bioparticles [40, 95]. Another possibility is that exposure to the midgut triggers some alterations of the outer membrane of bacteria, resulting in changes in susceptibility to immune effectors [99]. We showed that bacteria that invade the hemolymph from the midgut proliferate efficiently after extraction and injection into the hemolymph of control or ds*CTL4* mosquitoes, indicating that they have not lost fitness. These results suggest that the combination of the midgut invasion process and the exposure to the hostile hemolymph environment may impose a certain stress on the bacteria associated with a reduced proliferation rate. This stress may have been relieved through the process of extraction and washing before the cells are injected into another mosquito.

Rel2 silencing did not affect mosquito survival to oral *Sm* infections, suggesting that the Rel2/Imd pathway may be either nonessential for defense against *Sm* invasion of the hemolymph following an oral infection or that the pathway is not activated through this route. In *Drosophila*, *Sm* was sensitive to the local Imd response in the gut but failed to activate the systemic response in the fat body after crossing the gut epithelium into the hemolymph [24]. Currently, it is not possible to accurately score the activation of the mosquito Rel2 pathway due to the absence of a specific gene expression signature associated with this pathway. However, in our RNA-seq analysis, several immunity genes that are known to be at least partially regulated by Rel2, such as TEP1, APL1, several CLIPs, and FBNs among others [45, 47], were not upregulated neither in the midgut nor in the abdomens after oral *Sm* infection, suggesting that the Imd pathway may not be activated through this route. Phagocytosis is an essential determinant of *Drosophila* susceptibility to *Sm* oral infection [24]. However, this does not seem to be the case in *A. gambiae* since the treatment of mosquitoes with cytochalasin D did not alter their susceptibility to oral *Sm* infection. This result may suggest either the existence of functional redundancy among different branches of the immune response in this route of infection or that phagocytosis may not be essential when small numbers of bacteria are present in the hemolymph. Indeed, a recent study in *Drosophila* revealed that hemocyte-deficient flies did not succumb to a low-dose *S. aureus* infection, but a high dose did compromise their survival [13].

Our RNA-seq analysis identified a limited number of DETs in the midguts after *Sm* oral infection, specifically at the late 24-h time point. This agrees with a previous microarray-based study that compared the transcriptional responses in the guts of antibiotic-treated mosquitoes at 3 days after *Sm* oral infection with those of antibiotic-treated uninfected mosquitoes [29]. Another microarray-based study in *A. gambiae* that compared the gut transcriptomes of antibiotic-treated and untreated mosquitoes also identified a limited set of differentially expressed genes [100]. On the other hand, oral infections in *Drosophila* trigger dramatic changes in the gut transcriptome [101, 102]. This discrepancy can be explained by the different nature of the food source of both species; while *Drosophila* feeds mainly on fermented and rotten fruits rich in yeast and bacteria [103, 104], *A. gambiae* mosquitoes feed mainly on human blood which is sterile. It should also be noted that in oral infections, the number of DETs increased in the abdomens with time, whereas the inverse was observed in midguts. This DETs pattern in abdomens may be due to the continuous crossing of *Sm* from the gut into the hemolymph triggering physiological responses in the fat body and/or hemocytes attached to it or due to signaling between the gut epithelium on the one hand and the fat body and hemocytes on the other. Interorgan communication has been mainly studied in *Drosophila* whereby pathogen-infected intestinal cells signal to hemocytes, which in turn regulate intestinal regeneration [105, 106]. There is also evidence for signaling between the gut and fat body in *Drosophila* to regulate energy homeostasis [107, 108]. Our RNA-seq analysis also revealed that the response to *Sm* oral infections is more pronounced in the midgut, and it becomes even more pronounced when the analysis is restricted to transcripts that uniquely respond to feeding on *Sm*. Most of these transcripts are associated with biological processes related to protein translation, cell cycle, and DNA repair, which may not be surprising since *Sm* infection of the *Drosophila* gut was shown to trigger significant damage to the gut epithelium that alters cell morphology and physiology [109]. Epithelial damage of the gut and enhancement of gut physiological responses associated with stress, cell renewal, and proliferation have been also observed in *Drosophila* intestinal infections with *Erwinia carotovora* [101]. Only 4 genes showed an expression pattern unique to *Sm* injection suggesting that most of the transcriptional response is triggered by the wounding process per se. This was not surprising since a previous study by Dimopoulos et al. [110] showed that the predominant transcriptional responses triggered by septic

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and sterile injury in the refractory L3–5 mosquito strain were shared, suggesting that most of these responses are attributed to injury and/or wound healing; injury-specific transcriptional responses were dominated by functional groups pertaining to carbohydrate metabolism, whereas septic infection was dominated by immunity genes. Interestingly, a separate study showed that wounding of *A. gambiae* mosquitoes by the injection of water or dsRNA triggers the killing of *P. falciparum* parasites in a TEP1-dependent manner [111]. To better understand the relationship between wounding and immune defense to *Plasmodium*, the authors performed a genome-wide analysis of the transcriptional response to wounding in adult *A. gambiae* mosquitoes and identified 53 genes with statistically significant regulation that were enriched mainly in genes involved in proteolysis-related processes including several CLIP genes. Wounding also triggered the expression of several immunity genes with known anti-*Plasmodium* roles such as TEP1, LRIM1, APL1C, and FBN9 among others [112]. The fact that wounding triggers the expression of several mosquito immunity genes explains most likely why no immunity genes showed an expression pattern unique to *Sm*_inj in our study. The complex physiological responses triggered by wounding in other insects (reviewed in [113]) lend further support to our conclusion. In *Drosophila*, for instance, where the wound healing process is best characterized, cellular responses mediated by hemocytes, epithelial cells, and fat body cells act in concert with humoral factors including hemolectin and fondue to seal the wound, clear tissues debris, and initiate soft clot formation that becomes eventually melanized by the action of crystal cell-derived phenoloxidase [114–122]. Interestingly, fat body cells were also shown to secrete antimicrobial peptides locally to protect from wound infection [115].

Our transcriptomic analysis revealed that metabolic genes are the most represented functional class of all DETs in abdomens and midguts from all treatments. Knowing that metabolism is at the core of all biological processes, this result comes as no surprise. There is currently ample evidence in mammals, specifically from studies in mice, that cellular metabolism shapes the activation and differentiation of myeloid and lymphoid immune cells during infection [123, 124]. This relation has been particularly addressed in macrophages, whereby proinflammatory macrophages of the M1 type exhibit a metabolic shift to aerobic glycolysis associated with the production of nitric oxide, reactive oxygen species, and prostaglandins, whereas M2 macrophages exhibit a shift towards OXPHOS and increased dependency on a complete Kreb's cycle fueled by glucose, fatty acids, and glutamine (reviewed in [125]). Our knowledge of immunometabolism in mosquitoes is fragmented, with emerging evidence pointing toward a cross-talk between metabolic genes or metabolic signaling pathways and immune defense processes. In *A. stephensi*, *P. falciparum* infection was shown to induce the expression of insulin-like peptides that favor parasite development by suppressing the NF-κB signaling pathway in the midgut and by triggering metabolic shifts in this tissue independent of NF-κB [126]. Human insulin ingested by mosquitoes during blood feeding was also shown to enhance *Plasmodium* development by inhibiting NF-κB-dependent immune responses [127]. This reciprocal effect between immunity and insulin signaling has been also reported in *Drosophila* [128]. Another example of cross-talk between immunity and metabolism in mosquitoes is the finding that Lipophorin, a multifunctional carrier involved in lipid transport and metabolism, and its receptor are upregulated in *Aedes aegypti* mosquitoes following infection with Gram-positive bacteria and fungi in a Toll/Rel1-dependent manner [129]. In *A. gambiae*, *apolipophorin-II/I* was shown to control *TEP1* expression during systemic infections with *E. coli* and *Beauveria bassiana* [130]. Metabolic decisions may also influence the outcome of mosquito infection with microbes independent of immunity, and this has been mainly studied in the context of *Plasmodium* infections. For instance, the susceptibility of refractory and susceptible strains of *A. gambiae* to infection with *P. berghei* was shown to be influenced by broad metabolic differences between these strains, whereby the refractory strain exhibits rapid utilization of lipids, impaired mitochondrial respiration, and increased glycolytic activity leading to higher ROS production that is toxic to malaria parasites [131]. In a more recent study, Lampe et al. [132] elegantly showed that the timely expression of blood-meal-inducible miR-276 finely regulates the rate of amino acid catabolism, terminating the investment in reproductive processes and providing excess resources for the sporogonic development of *P. falciparum*. In a similar context, it would be interesting to determine whether metabolic shifts induced by blood feeding would influence mosquito resistance to bacterial and fungal infections and through what mechanisms. It was interesting to note that genes involved in OXPHOS were overrepresented in our KEGG pathway enrichment analysis, and all were downregulated in the midgut after feeding on *Sm*. This metabolic shift away from OXPHOS (a catabolic process) may reflect increased dependency on anabolic processes such as aerobic glycolysis that would be re-

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quired to promote midgut tissue repair in response to the damage triggered by *Sm* intestinal infection [24]. Tissue repair processes are known to be anabolic in nature and contribute to host tolerance to infection [124, 133]. Our transcriptomic analysis also revealed that the abdomen transcriptome was substantially larger in *Sm* oral infections relative to injections, at all 3 time points (compare Fig. 7a, c), despite the fact that injections resulted eventually in higher loads of *Sm* in the hemolymph. While these results may reflect different adaptive strategies of the host in response to different routes of infection with the same microbe, they possibly pinpoint also to a potential key role of the midgut epithelium in priming immune and non-immune physiological responses in the fat body and hemocytes that should act in concert to control hemolymph infections.

In conclusion, we provide evidence using gene silencing and transcriptomic analysis that the dynamics of immune defense to bacterial hemolymph infections through the midgut are different from those of hemolymph infections established by septic injections. The key difference between both routes is that the first involves the gut as a natural route towards establishing systemic infection while the second utilizes the more artificial or "naturally less common" wounding process to do so. Being at the front line of microbial defense, it is not surprising that the midgut epithelium, in addition to its classical evolutionary conserved role in local immune defense through its physical impermeability and chemical defenses, also plays an important role in priming physiological responses in distant organs that provide the host with better resistance and tolerance in case the microbe succeeds in crossing this barrier to establish a systemic infection. The nature of these protective physiological responses and how they are primed by the midgut epithelium remain largely unknown.

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Statement of Ethics

This study was carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, USA). Animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (permit number 17-10-451). The IACUC functions in compliance with the Public Health Service Policy on the Humane Care and Use of Laboratory Animals (USA) and adopts the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

M.A.O. and A.S.D. conceived and designed the study. A.S.D. performed all functional genetic studies. M.A.O. and N.B. designed the transcriptomic analysis. N.B. and X.Y. performed the RNA-seq library construction and sequencing. M.A.O., H.D., N.B., and X.Y. analyzed the RNA-seq data. M.A.O. wrote the manuscript. All authors critically reviewed and edited the manuscript.

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